Extraction, bioactivity, and stability of wheat bran alkylresorcinols

by

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Abstract

Whole grain intake may be linked to a lowered risk of chronic diseases and such positive effects might be attributed to phenolic lipids, alkylresorcinols (ARs), found in cereal bran. This study aimed to characterize ARs in wheat bran (WB), investigate the influence of environmental factors on AR composition, measure AR bioactivity including antioxidant activity (AA) and investigate AR stability during baking. Moreover, the prebiotic potential of WB-soluble dietary fibre (SDF) was explored using yogurt models. Specific objectives were achieved by the following projects.

The effects of cultivar, and region on the ARs content in 24 wheat cultivars grown in Ontario were studied, by GC-MS and their AA were evaluated. TPC (3.0 to 58.0 mg FAE/g), DPPH (5 to 68%), ORAC (6.0 to 94.0 μmol TE/g) and ARs (21.0 to 1522.0 μg/g) of WB extracts were significantly affected by location and cultivar ($P < 0.05$).

The stability of WB-ARs was studied using different bread formulations. Bread ARs (1.1 to 82.9 mg/100 g) were heat stable during baking at 255 °C for 12 min. A positive correlation was observed between TPC and ORAC ($R^2 = 0.90$).

Two extraction methods were used to compare the AR contents of WB. The % ARs per extract using acetone versus SC-CO_2 extraction of both WB samples studied were in the range of 10.9% -15.6% and 5.1% - 6.6%, respectively.

The prebiotic potential of WB-SDF to enhance lactic acid bacteria (LAB) survival in yogurt models was explored. Total DF (53%) was counted as a sum of SDF (6%) and IDF (47%). HPLC analysis of DF fractions treated with alkaline hydrolysis showed that IDF (84.2%) had a higher phenolic acid (PA) content than SDF (15.8%). There was a significant difference in total bacterial count (9.1 log CFU/mL), pH (4.8) and TTA

(1.4%) in yogurt samples containing 4% WB compared to controls during the four week cold storage period (4 °C).

These results could lead to practical applications of WB-ARs and SDF in the functional foods and nutraceutical industry. Furthermore, increased WB utilization will reduce Agri-Food leftovers, thus improving environmental sustainability.

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Finally, I dedicate this thesis to my family with special thanks to my dear husband, Metin Gunenc, and my beloved sons, Bilal and Cemal Gunenc. Thank you for your understanding, giving me the strength to hold on to my ideas, encouraging me to pursue my dreams, and for always giving me support when I needed it. I am so proud of my family and wish you all the best.

List of Publications

This thesis is based on the following original publications.

1. Gunenc, A., HadiNezhad, M., Tamburic-Ilincic, L., Mayer, P.M., Hosseinian, F. Effects of region and cultivar on alkylresorcinols content and composition in wheat bran and their antioxidant activity, *Journal of Cereal Science,* **2013,** 57:405-410.
2. Gunenc, A., Tavakoli, H., Seetharaman, K., Mayer, P.M., Fairbanks, D., and Hosseinian, F. Stability and antioxidant capacity of alkylresorcinols in Canadian hard and soft red wheat bran during baking, *Food Research International,* **2013**, 51:571-578.
3. Gunenc, A., and Hosseinian, F. Comparision of traditional and super critical carbon dioxide extraction of alkylresorcinols and their homolog profile and bioactivity, *Manuscript.*
4. Gunenc, A., and Hosseinian, F. Wheat bran enhances lactic acid bacterial survival and growth in yogurt and hold high antioxidant capacity. *Manuscript.*

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List of Abbreviations

AAPH	2, 2'-azobis (2-amidino-propane) dihydrochloride
AA	Antioxidant activity
AH	Antioxidants
ARs	Alkylresorcinols
AXOS	Arabionoxylan oligosaccharides
CFI	Canadian Foundation for Innovation
CFU	Colony forming unit
DPPH	2,2-diphenyl-1-picrylhydrazyl
DF	Dietary fiber
E	Environment
F	Fluorescein
FE	Ferulic acid equivalent
FOS	Fructooligossacharides
G	Genotype
GC-MS	Gas chromatography/mass spectrometry
HPLC	High performance liquid chromatography
HRWB	Hard red wheat bran
IDF	Insoluble DF
LDL	Low density lipoproteins
m/m	mass/mass
MRS	Man Rogosa Sharpe

OMAFRA	Ontario Ministry of Agriculture, Food and Rural Affairs
ON	Ontario
ORAC	Oxygen radical absorbance capacity
ORAS	2'-oxoalkylresorcyclic acid synthase
PDA	Photodiode array detector
PP	Polyphenols
RNO	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention time
SC-CO_2	Supercritical carbon dioxide
SDF	Soluble DF
SRWB	Soft red wheat bran
TE	Trolox equivalent
TLC	Thin layer chromatography
TPC	Total phenolic content
TTA	Total titratable acidity
WB	Wheat bran
WEP	Water extractable polysaccharide

Chapter 1 General Introduction

1.1 Significance of wheat

Wheat, *Tricticum aestivum,* is one of the oldest and most widely grown grain crops, followed by corn (maize), and rice. It was first cultivated as a food crop about 10,000 BCE somewhere along the Fertile Crescent in the Near East.[1,2] Wheat agriculture was considered to be in a small region of southeast Turkey and northeast Syria around the Middle Euphrates (the longest river of Eastern Asia, average coordinates 37°00' N, 38°60' E).[1,3] The genus name for wheat, *Triticum,* comes from the Latin word tero (I thresh), within the grass family *Poaceae.* The current binomial name, *Tricticum aestivum,* refers to bread wheat, distinguishing it from tetraloid macaroni wheat (*Triticum durum*). Most of the grown wheat in the world is the *aestivum* species.[4]

In Canada, wheat was first cultivated around 1605 near Annapolis Royal, Nova Scotia.[5] It is believed that Selkirk settlers from Scotland were the first to successfully harvest wheat in Western Canada in the early 1800's.[6] Currently wheat is grown all over Canada, mainly in the Canadian Prairies with Alberta, Manitoba, and Saskatchewan being the three major growing provinces.[7] In Canada for the last three years (2010-2012), wheat has been cultivated on 8.2-9.3 million hectares producing about 23.1-26.2 million metric tons.[8] Canada is one of the largest wheat producers and exporters in the world, with almost 70% of the produced wheat being exported. Two thirds of the world's wheat production is used for human consumption and about one-sixth is used for livestock feed. The remaining portion is used to produce starch, paste, malt, dextrose, gluten, alcohol and other products.[5]

Wheat exists in more than 30,000 varieties, and is grouped into six major classes

according to the planting and harvesting dates, as well as the hardness, colour, and shape of the kernels. These classes are as follows: a) hard red spring, b) hard red winter, c) soft red winter, d) hard white wheat, e) soft white wheat, and f) durum.[4] Hard wheat contains high levels of protein and gluten and is generally used for leavened bread. Common white wheat and soft red winter wheat have low protein content and are used for pastries, cookies, cakes. All-purpose flour is made by combining both hard and soft wheat. Durum wheat is used for pasta, semolina, couscous and Arabic flat breads. These above mentioned properties are influenced by a combination of genetic and environmental factors (genotype (G) and environment (E) and the interaction GxE). For instance, grain hardness is largely determined by the genotype (variety), but the more significant contributors to aspects of quality, such as protein content, are the result of growth conditions such as soil fertility, rainfall, and temperature throughout the growing season, at harvest time, and during storage and transport.[4,5]

1.2 Wheat structure

Wheat kernels contain three layers; the endosperm, bran, and germ (Figure 1.1). The endosperm makes up approximately 83% of the kernel weight and contains 70-75% of the protein present in the wheat. Also, it is the center of the kernel, rich in cellulose, starch, and gluten, and is surrounded by a single cell aleurone layer.[9]

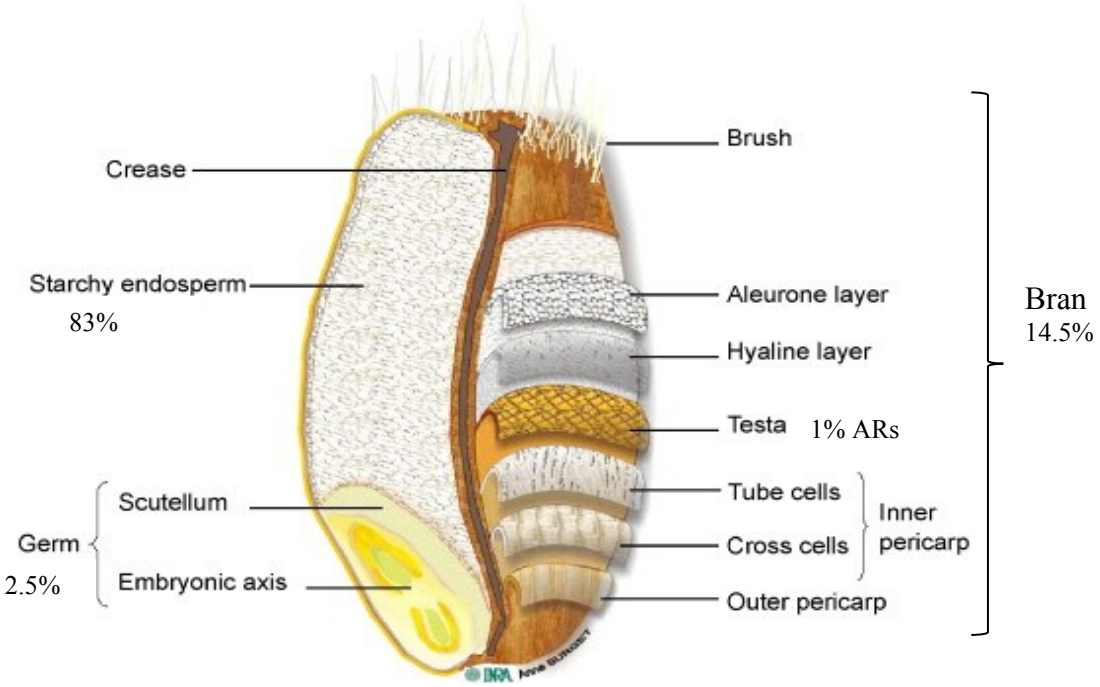


Figure 1-1 The three wheat fractions: bran, germ, and endosperm, adopted from Surget, and Barron[10]

The bran layers enclose the endosperm and are abundant in vitamins, minerals protein, and large amounts of insoluble dietary fiber.[9] The bran layer is about 14.5% of the kernel weight. The germ, the embryo of the kernel, accounts for about 2.5% of the wheat kernel. This layer contains vitamins B, and E, protein (8%), fat, and minerals.[11,12]

In the late 1800s, the pericarp, testa, aleurone, and nucellus (hyaline) bran layers/fractions were described in one of the first research report papers that was published on wheat bran.[13] Bran layers are generally regarded as the epidermis, hypodermis, cross cells, tube cells, testa, hyaline epidermis, and aleurone. Bran is also referred to by region of the grain such as outer, immediate, or inner layers.[14]

1.3 Wheat bran production

Wheat bran is a by-product of conventional flour milling and has little value compared with wheat.[15,16] Generally wheat bran is produced by a roller milling process of the wheat kernels.[15] This method offers a relatively clean separation of the bran and germ from the endosperm. Moreover, by using this method, the bran fractions are removed with almost all of the bran layers intact.[17,18]

Commercial wheat bran consists of the pericarp, seed coats, and aleurone layers, along with bits and pieces of the endosperm. Wheat bran from conventional milling consists of the outer portions of the kernel with approximately 10-20% of the endosperm still present.[19] More detailed information about the modern flour milling process and milling conditions[16] is available.[15] In brief, water is added for cleaning, tempering the wheat and toughening the bran. After this process, the endosperm is separated from the bran by milling. Wheat bran yield is about 21.1-36.5% of the kernel weight.[20] Generally millers try to produce large (>2000 μm) bran pieces and shorts in order to optimize the flour yield.[15] However, bran thickness is not the only variable determining the post-milling particle size.[18] Grinding speed, equipment, and wear during milling also influence the geometry of the bran pieces.[21] The milling efficiency can affect the bran particle size as well as the biological differences in the thickness of the aleurone layer which can determine the amount of clean endosperm that is removed from the bran.[22]

Total global wheat crop production is approximately 600–700 million tonnes of wheat bran annually (~1.4×10^{12} lb/~2.4×10^{10} bushels).[23] Global wheat bran consumption increases annually by about 5%.[24] Wheat bran is commonly used today for many things, such as food, livestock feed, and in cosmetic and biomass products.[25]

1.4 Wheat nutrition

Wheat is an excellent source of carbohydrates (59.6%), proteins (11.7%), lipids (2.2%), minerals (1.5%) and fibre (13.3%).[26] During the milling process, the removed layers of wheat bran (14.5%) and germ (2.5%) represent 75% of the phytonutrients in the wheat kernel (Figure 1.1).[27,28]

The term 'phytochemical' refers to every naturally occurring chemical substance present in plants, especially those that are biologically active.[29] The known phytochemicals in whole grains and wheat include dietary fiber, vitamins, minerals, lignans, phytoestrogens, phenolic compounds, phytic acid, benzoic, cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavonones, amino phenolic compounds, flavonoids, coumarin derivatives, polyphenols, phytosterines, saponins, catechins, tocotrienls and tocopherol, tannin, caretenoids, ferulic acid and diferulates. [27,29] The major bioactive compounds and their composition and content in whole wheat grain, wheat bran, and germ fractions are shown in the Table 1.1.

Table 1-1 Average content (g/100g) of the major bioactive compounds in whole-grain wheat, wheat bran, and germ fractions (%)*, adapted from Fardet[30]

Bioactive compounds	whole-grain wheat[a]	Wheat bran[a]	Wheat germ[a]
α-Linolenic acid	NA[b]	0.16	0.53
Sulfur compounds	0.5	0.7	1.2
Total free glutathione	0.007	0.038	0.27
Dietary fiber[d]	13.2	44.6	17.7
Lignins	1.9	5.6	1.5
Oligosaccharides[e]	1.9	3.7	10.1
Phytic acid	0.9	4.2	1.8
Minerals and trace elements	1.12	3.39	2.51
Vitamins	0.0138	0.0398	0.0394
B-vitamins	0.0091	0.0303	0.0123
Vitamin E	0.0047	0.0095	0.0271
Carotenoids	0.00034	0.00072	NA
Polyphenols	0.15	1.1	>0.37
Phenolic acids	0.11	1.07	>0.07
Flavonoids	0.037	0.028	0.3
Lignans	0.0004	0.005	0.0005
Alkylresorcinols	0.07	0.27	NA
Betaine	0.16	0.87	0.85
Total choline	0.12	0.17	0.24
Total free inositols	0.022	0.025	>0.011
Phytosterols	0.08	0.16	0.43
Policosanol+melatonin+*para*-aminopbenzoic acid	0.00341	0.0029	>0.00186
Total	>15.4	51.5	>23.9
Subtotal (without dietary fibre)	2.2	6.9	>6.2

*Mean % of bioactive compounds found in wheat bran, whole-grain wheat and wheat germ are calculated as follows: % = (minimum+maximum) /2

[a] Expressed as g/100g food

[b] No data found

[c] Total free glutathione is given as glutathione equivalents=reduced glutathione + (oxidized glutathione x 2)

[d] Dietary fibre content is measured according to the AOAC method

[e] Oligosaccharides include fructons, raffinose and stachyose

The contents of certain bioactive components present in wheat, rye, oat and barley cereal grains are given in Table 1.2.[31] Cultivar and growing region may affect the content of the bioactive components that are present. The components presented in Table 1.2 have been reported having physiological functions in the human body, with dietary fiber appearing to act as a carrier in some cases.[32]

Table 1-2 Major bioactive components (μg/g dry matter) contained in whole grain wheat, rye, oats, and barley, adopted from Frolich et al.[31]

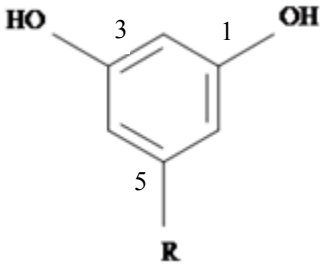
Component	Wheat	Rye	Oats[a]	Barley[a]
Phytic acid	390-1,350	540-1,460	420-1,160	380-1,116
Tocols	28-80	44-67	16-36	46-69
Phenolic acids	326-1,171	491-1,082	351-873	254-675
Phytosterols	670-960	1,098-1,420	618-682	899-1,153
Alkylresorcinols	220-650	797-1,231	Not present	32-103
Avenantramides	Not present	Not present	42-91	Not present

[a] Husked and naked cultivar

1.5 Alkylresorcinols structure and sources

ARs belong to a large group of phenolic lipids that differ mainly by the odd-numbered hydrocarbon chain attached to position 5 on the benzene ring.[33] Structurally, ARs consist of 1, 3-dihydroxy-5-alkylbenzene (5-alkylresorcinol) which is alkylated at position 5 by a saturated, odd numbered hydrocarbon side-chain containing 13-27 carbon atoms as shown in Figure 1.2. Depending on the source of the ARs, the structure can vary according to the degree of unsaturation, chain length, ring or chain substituted functional groups on the alkyl chain or aromatic ring, and position of the alkyl chain.[33,34] These isoprenoid phenolic lipids are present as mixtures of several homologues and derivatives.

The 5-alkyresorcinols are commonly given abbreviations which describe the alkyl chain length and the degree of unsaturation. For instance, C19:0, C19:1, and C19:2 indicate a saturated chain of 19 carbon atoms, an unsaturated chain of 19 carbons with one double bond, and an unsaturated chain of 19 carbons with two double bonds, respectively. These abbreviations do not carry any indication of the double bond-location. Generally, saturated 5-*n*-alkylresorcinol homologues ranging in chain length from C15:0-C25:0 are most common in cereal grains. ARs with wide structural variety are found in various plant families and include 5-*n*-alkyl-, 5-alkenyl-, 5-oxoalkyl-, 5-oxoalkenyl and 5-hydroxyalkylresorcinols (Table 1.3).[35,36]



R= $(CH_2)_n (CH_3)$

n =14, 16, 18, 20, 22, 24

1,3-dihydroxy-5-alkylbenzene (5-alkylresorcinol)

Figure 1-2 The 5-n-alkylresorcinol structure, modified from Ross et al.[34]

Table 1-3 Various 5-*n*-alkyl, 5-alkenyl-, 5-(oxoalkyl)-, 5-(oxoalkenyl)-, and 5-(hydroxylalkenyl)-resorcinols isolated from wheat and rye, adopted from Ross et al.[34]

Alkylresorinol derivative (R)	Structure	Molecular weight (Da)
5-*n*-Alkylresorcinols		
C17:0	5-*n*-Heptadecylresorcinol	348
C19:0	5-*n*-Nonadecanylresorcinol	376
C21:0	5-*n*-Heneicosylresorcinol	404
C23:0	5-*n*-Tricosylresorcinol	432
C25:0	5-*n*-Pentacosylresorcinol	480
5-*n*-Alkenylresorcinols		
C17:1	5-(Heptadecenyl)-resorcinol	346
C19:1	5-(Nonadecenyl)-resorcinol	374
C21:1	5-(Heneicosenyl)-resorcinol	402
C23:1	5-(Triocosenyl)-resorcinol	430
C25:1	5-(Pentacosenyl)-resorcinol	458
C19:2	5-(Nona-10*Z*, 13*Z*-decadienyl)-resorcinol	372
C21:2	5-(Henei-12*Z*, 15*Z*-cosadienyl)-resorcinol	400
C23:2	5-(Tri-14*Z*, 17*Z*-cosadienyl)-resorcinol	428
C25:2	5-(Penta-16*Z*, 19*Z*-cosadienyl)-resorcinol	456
5-Oxoalkylresorcinols		
C19:Oxo	5-(2-Oxononadecanyl)-resorcinol	390
C21:Oxo	5-(2-Oxoheneicosanyl)-resorcinol	418
C23:Oxo	5-(2-Oxotriocosanyl)-resorcinol	446
C25:Oxo	5-(2-Oxopentacosanyl)-resorcinol	474
5-Oxoalkenylresorcinols		
C19:1, Oxo	5-(2-Oxononadecenyl)-resorcinol	388
C21:1, Oxo	5-(2-Oxoheneicosenyl)-resorcinol	416
C23:1, Oxo	5-(2-Oxotriocosenyl)-resorcinol	444
C25:1, Oxo	5-(2-Oxopentacosenyl)-resorcinol	472
C19:2, Oxo	5-(2-Oxo,-10*Z*, 13*Z*-nonadecadienyl)-resorcinol	386
C21:2, Oxo	5-(2-Oxo,12*Z*,15*Z*-heneicosenyl)-resorcinol	414
C23:2, Oxo	5-(2-Oxo,14*Z*,17*Z*-tricosadienyl)-resorcinol	442
C25:2, Oxo	5-(2-Oxo,16*Z*,19*Z*-pentacosadienyl)-resorcinol	470
5-Hydroxyalkenylresorcinols		
C19:1,Hydroxy	5-(2-Hydroxynonadecenyl)-resorcinol	390
C21:1,Hydroxy	5-(2-Hydroxyheneicosenyl)-resorcinol	418
C21:1,Hydroxy	5-(4-Hydroxyheneicosenyl)-resorcinol	418
C23:1,Hydroxy	5-(4-Hydroxytricosenyl)-resorcinol	446
C25:1,Hydroxy	5-(4-Hydroxypentacosenyl)-resorcinol	474
C21:2,Hydroxy	5-(4-Hydroxy,12*Z*,15*Z*-heneicosenyl)-resorcinol	416
C23:2,Hydroxy	5-(4-Hydroxy,14*Z*,17*Z*-tricosadienyl)-resorcinol	444

It has been reported that ARs have been found in eleven plant families including *Anacardiaceae, Ginkgoaceae, Proteaceae, Myrsinaceae, Primulaceae, Myristicaceae, Iridaceae, Compositae, Leguminosae and Gramineae*.[37] The amount of ARs present varies largely depending on the plant source. Some plants contain ARs that vary from 0.005% (m/m, mass/mass) for *Hordeum vulgare* (barley) grains to 0.3% (m/m) for *Secale cereale* (rye) grains.[37,38] The highest concentration of ARs has been reported in the oil extract of cashew nut shells containing up to 20% (m/m) of ARs.[33]

The existence of both a water soluble polar 'head' (dihydroxybenzene group) and a water insoluble non-polar alkyl 'tail' gives ARs their amphiphilic nature. This is of great significance with regards to their analysis, absorption, metabolism and potential bioactivity.[33] The octanol/water partition coefficients (log $P_{o/w}$) for ARs are between 7.0 and 13.4, and the hydrophobicity increases with increasing alkyl chain length.[34,39]

Although they can be isolated from a wide variety of sources, ARs in edible food sources provide the greatest dietary advantage with the essential dietary intake of ARs coming from foods containing whole-grain rye or wheat kernels.[34,38] There has been a large amount of research reporting the potential positive effects of ARs on humans.[40] However, the initial interest in ARs started decades ago from concerning findings that suggested ARs in cattle feed caused inhibition of growth and decrease of appetite in animals. This led to the need and development of a rapid method for determining the presence of ARs.[40,41]

ARs are detected during kernel development in cereals, and the amount has been found to decrease as the grain matures.[42] This decrease might be due to dilution effect caused by the increase in proportion of starch and protein in the maturing grain kernel.

ARs have the ability to protect plants from mold and other harmful organisms such as bacteria, which attack from the outside. The location of ARs in the outer layers (in the bran of cereal grains) enhances these protective effects. [43] [44] The exact location of ARs in a cereal kernel has been determined to be in the intermediate layer between the testa and pericarp, with this layer containing 99% of the ARs.[45]

1.6 Alkylresorcinols in cereals

The highest levels of ARs are found in rye[46], wheat[47], and triticale[46], with the lowest levels being reported in barley.[46,48] Although trace amount of ARs have been reported in rice, millet, oats, corn, and sorghum[34-36,45,46,48,49], other studies found no ARs present in these grains.[36,46,50] The AR contents of whole cereal grains, the bran fraction, and the flour is shown in Table 1.4.

Table 1-4 Alkylresorcinol content of whole cereal grains, bran and flour[35,45,46]

Cereal	Fractions	Range (μg/g, dry matter)
Rye	Whole grain	560-1444
	Bran	2400-4108
	Flour	69-79
Wheat	Whole grain	264-943
	Bran	2210-3225
	Flour	29-45
Triticale	Whole grain	430-700
	Bran	2780-3080
Barley	Whole grain	41-74
	Bran	210

The AR content is reported to vary widely within and between species due to environmental, agricultural and genetic factors.[51-53] Normally, AR contents in the bran

fractions of a given cereal are two to five times greater than its respective whole grain ARs content.[36,46] The ratio of AR homologue C17:0/C21:0 is unique for each of the cereal grains, and is approximately 0.2 in triticale, 0.1 in wheat, and 1.0 in rye bran. ARs are of interest as biomarkers of whole grain cereal intake, and as such are an aid in understanding the link between whole grain consumption and health.[54]

The Figure 1.3 shows the varying amounts of each of the AR homologues in the different cereal grains: rye, common wheat, durum wheat, triticale, and barley.[34,49,55] Each grain has predominant homologues specific to that grain type. The most abundant AR homologues in rye, common wheat, durum wheat, triticale, and barley are C19:0, C21:0, and C25:0.

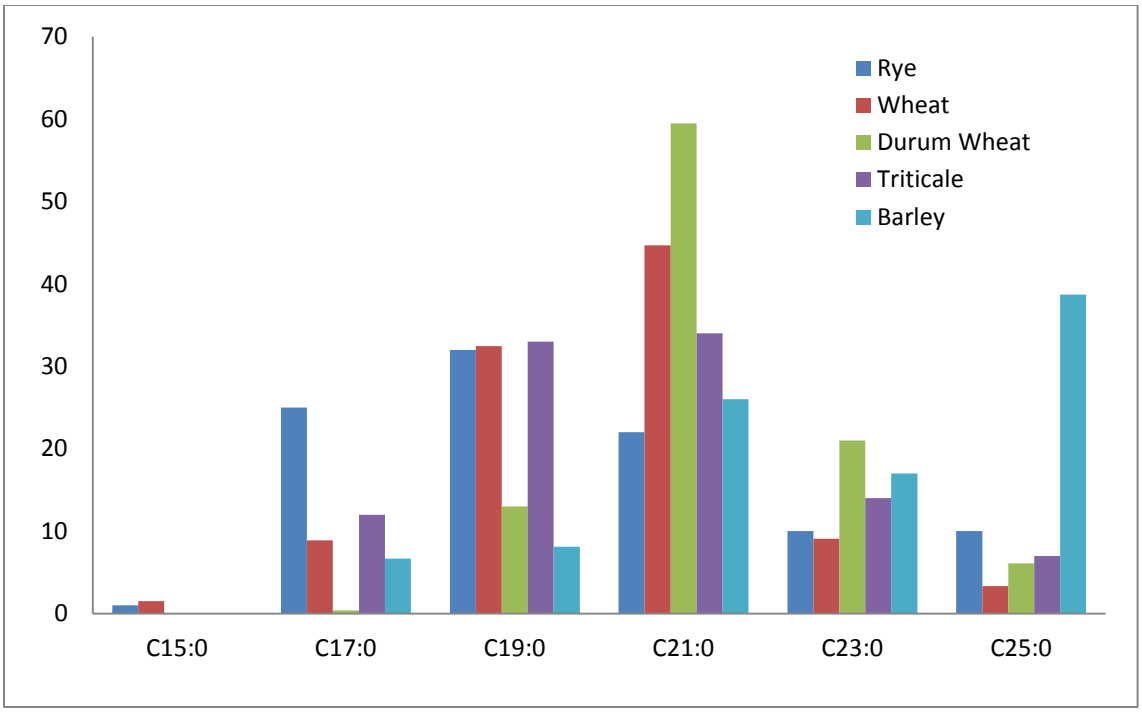


Figure 1-3 Typical percent distribution of the alkylresorcinol homologues C15:0-C25:0 found in rye,[46] common wheat,[47] durum wheat,[55,56] triticale,[46] and barley,[46,48] modified from Söderholm[57]

1.7 Biosynthesis of alkylresorcinols

Plant phenolic compounds are synthesized from two main pathways: the shikimate pathway and the polyketide (acetate) pathway. The shikimate pathway forms phenylpropanoids such as the hydroxycinnamic acids and coumarins, and the polyketide pathway produces simple phenols which lead to the formation of quinones.[33] The largest groups of phenolics (flavonoids) are formed through a combination of both pathways.

The biosynthesis of resorcinolic lipids (a form of phenolic lipids) are believed to be derived from the polyketide (acetate) pathway by 2`-oxoalkylresorcylic acid synthase (ORAS), a type III polyketide synthase.[58] Polyketide biosynthesis begins with polyketomethylenic chains, $-(CH_2\text{-}C)_m-$, derived from acetic acid and the activated forms

of acetyl-S-CoA and malonyl-S-CoA.[59] The biosynthesis pathway for polyketides resembles the pathway for fatty acids. For both of them, the formation of linear chains occurs by the addition of C_2 units. A fatty acyl-coA reacts with malonyl coenzyme A three times to form a tetraketide intermediate by an aldol condensation-type reaction.[60] The alkyl side chain (the odd numbered side chain) of ARs is the direct result of the fatty acid precursor.[61] The intermediate compound undergoes an aldol condensation-type ring closure and hydrolysis to release the coeznyme A to form alkylresorcylic acid. The next step is non-enzymatic decarboxylation to yield 5-n-alkylresorcinols. [43] ORAS synthesizes triketide and tetraketide pyrones as well as pentaketide resorcylic acids and resorcinols from the fatty acyl-CoA starter unit (Figure 1.4).[58]

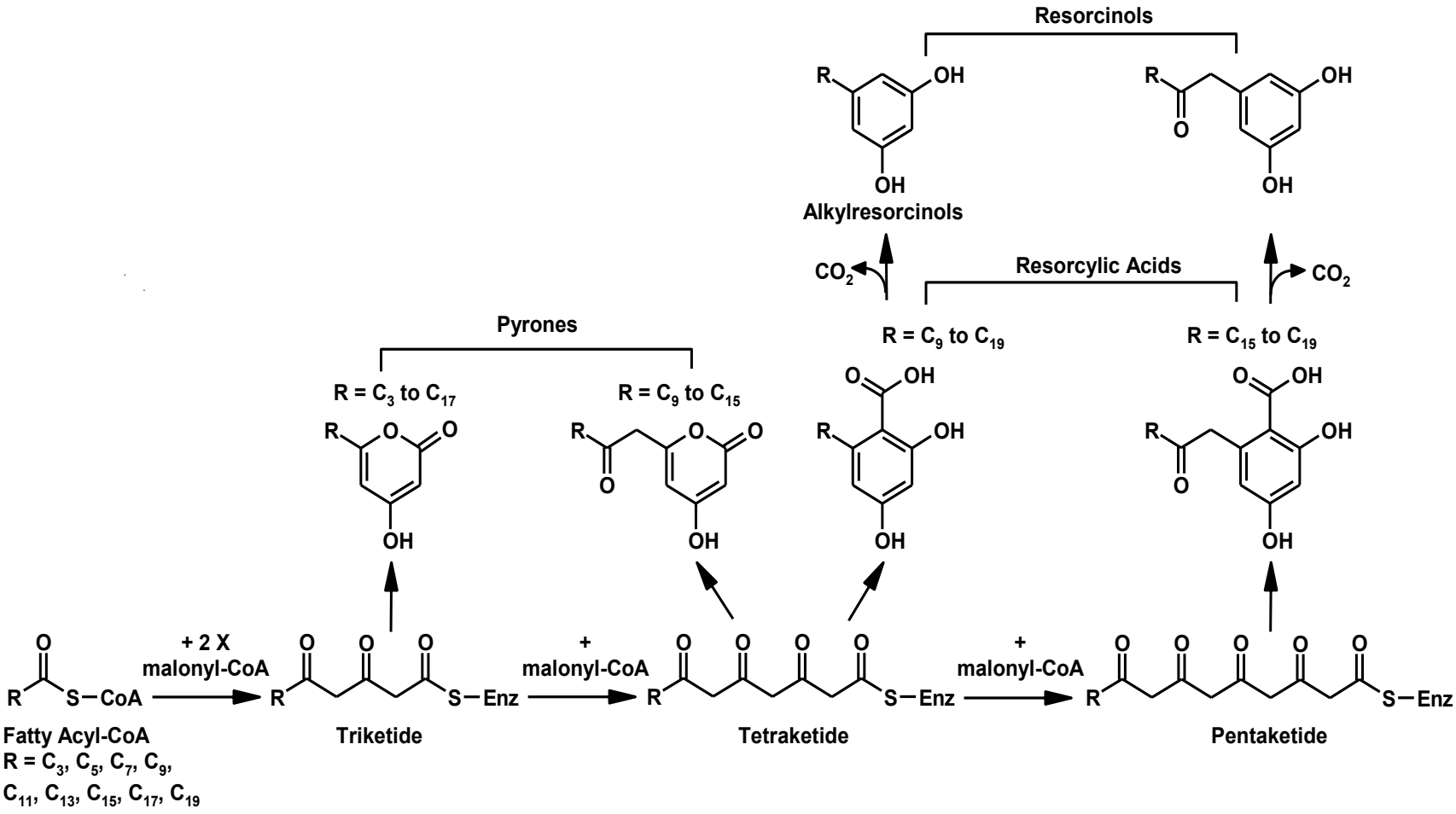


Figure 1-4 The biosynthesis of alkylresorcinols and various polyketide (triketide, tetraketide and pentaketide) resorcylic acids, adopted from Yu et al.[58]

1.8 Extraction of alkylresorcinols

Different solvent extraction methods are commonly used for the extraction of ARs from cereals using organic solvents such as acetone, ethyl acetate, and methanol. However, using acetone and ethyl acetate are not suitable for the extraction of ARs from bread.[46] For this purpose, a hot-1-propanol: water (3:1, v/v) solution is used for the extraction and complete recovery of ARs from baked products.[46]

It was believed that baking destroyed or reduced the ARs content in bread, since only low amounts of ARs were detected in bread.[50,62] Later experiments showed that ARs remain stable during baking and food processing.[38,56,63]

ARs are amphiphillic compounds that become more water insoluble with increasing alkyl chain length.[33] For this reason, cereal grain ARs have been traditionally extracted using organic solvents, especially acetone, and ethyl acetate, at room temperature for 16-24 h with a sample to organic solvent ratio between 1:40 and 1:50 (w/v). [34] There are several disadvantages to using the organic solvent extraction methods, including long extraction time, toxic waste generation, and lack of selectivity. Additionally, there is concern for food security, safety, and quality when organic solvents are used.[64] Supercritical carbon dioxide (SC-CO_2) extraction can be used as an alternative to the traditional solvent extraction methods as explained in the following section.

1.9 Supercritical carbon dioxide (SC-CO_2) extraction of ARs

Supercritical fluid technology has gained increased interest as a green technology, especially for use in the food industry. Fluids are put into the supercritical phase by increasing the pressure and temperature above the critical point. Once in the supercritical

phase, fluids have liquid-like solvent power and gas-like diffusivity.[65] Carbon dioxide (CO_2) is the most widely used supercritical fluid, and when heated and compressed above its critical point, SC-CO_2 is produced. Figure 4.1 in Chapter 4 shows the SC-CO_2 instrument in our research laboratory at Carleton University

SC-CO_2 can be used as an extraction solvent to extract the ARs, and the efficiency of this extraction method depends on the pressure, temperature, and co-solvent used.[66] The SC-CO_2 extraction technique is very attractive in comparison to traditional organic solvent procedures because of its non-toxic, non-flammable, low-cost, and environmentally friendly properties.[35,66] Additionally, it has a faster extraction time than the traditional techniques and the CO_2 is safe and easy to remove after the extraction is complete. The use of SC-CO_2 technology has been well established for the extraction of many different food products, including essential oils.[67] It has also been used to extract ARs from wheat and rye bran at the optimum conditions of 70 ºC and 35 MPa with a flow rate of 25 g/min for 4 h followed by using 10% ethanol as a co-solvent/modifier.[35] Because of its non-polar nature, pure CO_2 does not solubilize the polar ARs. For that reason, ethanol has been suggested as a co-solvent when using SC-CO_2 for the removal of ARs from wheat and rye bran. Previous studies have found SC-CO_2 to yield 8-80% more ARs than when traditional extraction was done using acetone.[66] However, some recent studies have found there are no significant differences in the total AR yield between the two methods.[35,68]

1.10 ARs analysis methods

In literature, many studies have described the isolation of ARs from different plant matrices and the analysis of extracts by different techniques. These studies and the

chromatographic techniques used in AR analysis have been comprehensively reviewed.[33,34]

ARs in rye were reported in 1967 as a mixture of homologues C15:0-C25:0. Since then, the analysis and isolation of ARs in cereal grains have been performed using a variety of techniques.[69] A number of analytical methods have been employed to quantify AR concentration in plasma such as gas chromatography/mass spectrometry (GC-MS),[70] gas chromatography/tandem mass spectrometry),[71] and liquid chromatography/tandem mass spectrometry.[72] The following sections include detailed information about determination techniques.

1.10.1 Colorimetric determination of ARs

ARs were first thought to be growth inhibitors for the livestock that were fed with the rye, causing lower weight gain compared to animals fed with other cereal grains. This initiated screening of the rye cultivar and lines using colorimetric studies to determine which had the lowest possible concentrations of ARs.[40,69] A colorimetric analysis method has been used in several studies to quantify the total ARs content by measuring the intensity of the electromagnetic radiation in the visible spectrum that is transmitted through an AR extract.[53,73-75] This method is based on a diazonium salt reacting with phenolic compounds to form an azo-complex which develops a reddish violet colour with a maximum absorption peak at 520 nm distinct for ARs.[75,76] There have been various diazotized compounds used as reagents for the method, including Fast Blue B BF4,[47,74] Fast Blue B Zn[70,75] and Fast Blue RR salt.[70] However, the colorimetric method does not differentiate between the various AR homologues present in the extract as it only quantifies based on the number of aromatic rings that react with the diazonium salt.[47] For

this reason, the colorimetric method is only good for rapid and cost-effective detection and quantification of the total ARs content.[77] Further chromatographic analysis is needed for confirmation and differentiation between the different AR homologues.[51,78]

1.10.2 Thin layer and paper chromatography

Thin layer chromatography (TLC) and paper chromatography have been used for the identification and separation of ARs qualitatively.[45,79-81] Cereal grains have been analyzed by TLC using silica gel or alumina on the plates.[82-84] After running the TLC plate it is stained with a staining reagent such as Fast Blue B to indicate the presence of any ARs. TLC is relatively simple and easy to perform. However, for quantitative determinations, accurate chromatographic techniques must be used.

1.10.3 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) with reverse phase (RP) columns has been used in many studies to analyze the composition of various AR extracts.[36,50] Identification of AR homologues is based on their characteristic retention times, with these being dependent on the AR-chain length and the degree of unsaturation.[37,50,85] It has been found that using a gradient elution system[36,86] gives a faster and better separation than when using an isocratic system. [34 ,84] RP-HPLC with a non-polar C18 column as the stationary phase and a polar solvent (e.g. methanol) as the mobile phase is generally used to separate ARs.[37,50,86] Gradient systems produce optimal results with clear peak separation and reduced background noise in the resulting chromatogram. A photodiode array detector (PDA) [87,88] at 280 nm has been used for the detection of ARs from cereal bran.[80,89]

1.10.4 GC-MS

Gas chromatography combined with mass spectrometry (GC-MS) separates and quantifies the relative amounts of the different AR homologues based on their individual retention times which are visualized by the MS detector. It seems to be an efficient method for the determination of ARs content from different matrices.[46,86,88] Generally a non-polar stationary phase is used in the GC column, with a flame ionization detector (FID) and electron impact (EI) for ionization in the MS stage of the procedure. GC alone is not enough to identify all of the AR components of the extracts. Using GC-MS identifies the AR components with high precision through their characteristic ionized fragments of specific mass to charge (m/z) ratios and retention times.[35,90] In GC-MS studies, the ion with m/z ratio of 268 is a typical peak for di-trimethylsilylated (TMS) AR[46]. The characteristic m/z ion peaks for each AR homologues starting from C15:0 to C25:0 are 320, 348, 376, 404, 432 and 460 respectively.[91]

1.11 Bioactivity of alkylresorcinols

ARs have been reported to have various biological activities. Most of the activities of ARs are thought to be based on their ability to interact with biological membranes. For instance, ARs are capable of binding to the hydrophobic regions of some proteins and modulating their enzymatic activity. [92] The AR homologues C15:0 and C19:0 have been reported to inhibit the activity of glycerol 3-phosphate dehydrogenase at micromolar concentrations.[93,94] Data concerning other homologs (AR-C21:0-C23:0 and C24:0) are missing. In the following topics some aspects of bioactivity such as antioxidant activity and the measuring methods used to determine this is covered.

1.11.1 Free radicals and antioxidant activity

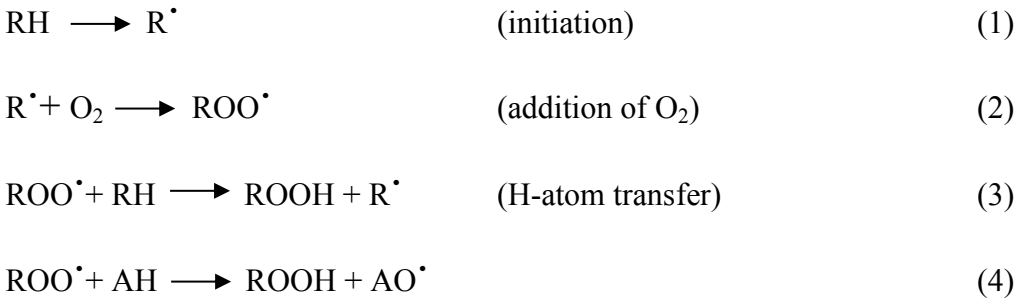
Oxidative damage is caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS). They are classified as "free radicals" and produced as a byproduct of aerobic metabolism. Cell damage can be caused by an imbalance of free radicals, in which the level of oxidants exceeds that of antioxidants.[95] Free radicals are believed to contribute to several inflammatory and degenerative diseases.[96] Some dietary antioxidants include radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors.[97] There is a link between increased levels of lipid oxidation products and diseases such as diabetes and atherosclerosis.[98,99] For example, oxidized cholesterol of fatty acid moieties in plasma LDL can lead to the development of atherosclerosis.[98]

Phenolic antioxidants are an important class of compounds for inhibiting the oxidation of materials for both commercial and biological importance.[100] An antioxidant is defined as a substance in foods that when present at low concentrations compared to those of an oxidizable substrate significantly decreases or prevents the adverse effects of reactive species, such as ROS and RNS, in the normal physiological function of humans.[97,101] However, not all reductants involved in a chemical reaction are antioxidants. Only those compounds capable of protecting the biological target from oxidants meet this principle.[102]

There are several mechanisms of actions for antioxidants such as **a)** serving as physical barriers to prevent ROS generation such as a UV filter or cell membranes, **b)** chemical traps that quench ROS by absorbing energy and electrons, such as carotenoids, and anthocyanins, **c)** catalytic systems that neutralize ROS, for instance, the antioxidant enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase[103], **d)**

binding/inactivation of metal ions to prevent ROS generation by ferritin, catechins, and by chain-breaking antioxidants such as ascorbic acid, tocopherols, and flavonoids.[104]

There are two pathways for oxidation in which antioxidants can play a preventive role including H-atom transfer (HAT) and single electron transfer (SET). The HAT pathway of lipid peroxidation is illustrated below: [100]



Once a free radical ($R^{\bullet}$) has been generated, a chain reaction is created by the reactions 2 and 3. Through these chain cycles, many lipid molecules (R-H) are converted into lipid hydroperoxide (ROOH), resulting in oxidation and rancidity of fats. The role of an antioxidant such as phenolic antioxidants (AH), is to interrupt the chain reaction.[100]

An effective AH must be a relatively stable free radical and react slowly with substrate (RH) but quickly with hydroxyperoxide ($ROO^{\bullet}$). The most effective lipid-soluble chain-breaking antioxidant in human blood plasma is known as α-tocopherol, the most active component of Vitamin E.[105]

The rate of reaction of substrate RH with peroxyl radicals depends on the barrier height for transfer of an H-atom from AH. The bond dissociation enthalpy (BDE) [106] in phenolic antioxidants is an important factor in determining the efficacy of an antioxidant,

since the weaker the OH bond the faster will be the reaction with free radicals.[100] The BDE of phenol provides a reference value for all phenolic antioxidants, and Wright et al. (2001) has studied the procedures for estimating the O-H BDE based on group additivity rules and tested on several classes of phenolic antioxidants including commercial ones used as food additives. On the basis of their study results, they were able to interpret relative rates for the reaction of antioxidants with free radicals, including a comparison of both HAT and SET mechanism in which HAT was dominant in most cases. [100]

In the work [92] by Kozubek and Nienartowicz, it was found that ARs decreased peroxidation products at micromolar levels in suspensions of erythrocytes injected with hydrogen peroxide and homologs of ARs. The homolog AR-C15:0 had the highest antioxidant effect followed by C19:0, and C23:0. From this study it was suggested that the antioxidant potential of AR homologs is dependent on their chain length, with the ARs with shorter alkyl chain lengths having higher antioxidant activity. [92] In another study [107], Winota and Lorenz showed that AR homolog-C15:0 slowed the rate of oxidative rancidity in oil as well as lipid oxidation in cereal products at AR concentrations of 0.050-0.075%. These findings suggest that ARs might be useful as natural antioxidants in the food industry.[108]

In conclusion, many studies shown that ARs from cereal bran isolates serve as antioxidants in food and biological systems. However, detailed information of about how ARs act in the membranes, in different emulsion or food systems, the potential of ARs to act as preventive antioxidants, and if any synergistic effect of ARs exist, are only a few of the many questions that remain to be answered in regards to the antioxidant activity of ARs.[109]

1.11.2 Oxygen radical absorbance capacity (ORAC)

The peroxyl radical is an important physiological radical. It is involved in the propagating steps in lipid peroxidation chain reactions. They can be formed *in vivo* through reactions of carbon centered radicals with other reactive oxygen species like peroxyl radicals. The ORAC assay measures the inhibition of peroxyl-radical-induced oxidants and reflects classical radical chain breaking antioxidant activity by H-atom transfer.[110] Basically, peroxyl radicals are produced from thermal decomposition of AAPH (2, 2'-azobis (2-amidino-propane) dihydrochloride) and react with a fluorescent probe (oxidizable protein substrate) to form a nonfluorescent product that can be quantitated by fluorescence.[111]

The ORAC value for an antioxidant is calculated based on the areas under the reaction kinetic curves for the antioxidant sample, antioxidant standard, and the blank. Trolox is generally used as the antioxidant standard, and results are expressed as in micromoles of trolox equivalents per unit of the sample (μmole TE/g).[112]

The ORAC assay has several advantages compared to other antioxidant scavenging capacity assays such as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. For instance, it measures scavenging activity against a physiological relevant radical which is peroxyl radical, and it is well known that it plays a role in the oxidation of lipids in human bodies and food systems.[97] Also, the assay working conditions are conducted under physiological pH and for high throughput analysis. Lastly, the assay takes into consideration both kinetic and thermodynamic properties of antioxidant-radical reactions.[112]

1.11.3 Diphenyl-picryhydrazyl (DPPH)

This assay has found extensive use in screening the antioxidant properties of pure compounds and botanical extracts. It is technically simple and can be performed using a UV-Vis spectrophotometer.[113] The 2, 2-diphenyl-1-picryhydrazyl (DPPH) radical is a long-lived organic nitrogen radical and has a deep purple color. The purple chromogen radical is reduced by antioxidants to the corresponding pale yellow hydrazine and the reducing power of the antioxidants is measured by monitoring the absorbance decrease at 515-528 nm. This method was first reported by Brand-Williams et al.[114] The main advantage of this assay over other antioxidant capacity measurement assays is its broad solvent compatibility with aqueous, polar, and non-organic solvents.[113] The calculated percent DPPH remaining is proportional to the antioxidant concentration, and the EC_{50} is defined as the effective concentration that causes a 50% decrease in the initial DPPH amount.[114]

1.12 Prebiotic activity

The definition of dietary fiber (DF) by AACC (the American Association of Cereals Chemist) includes "cell wall polysaccharides, lignin and associated substances resistant to hydrolysis by the digestive enzymes of humans". Most of those mentioned "conjugated compounds" are related dietary antioxidants such as phenolic acids. The most abundant phenolic compound in wheat is ferulic acid (Figure 5) followed by sinapic acid, p-coumaric acid, and caffeic acid.[115,116] Most grain polyphenolic compounds (~95%) are linked to cell wall polysaccharides. Those dietary fiber-phenolic compounds are covalently bound to polysaccharides through ester bonds. For instance, ferulic acid (Figure 5) is bound to the arabinoxylans via the acid group acylating the primary

hydroxyl at the C5 position of α-arabinofuronasyl residues (Figure 6).[117] They might be hydrolyzed through wheat related endoxylanases or specific intestinal bacteria possessing arabinoxylan-degrading enzymes. This hydrolysis leads to the formation of arabinoxylan oligosaccharides (AXOS), representing a new class of candidate prebiotics. Little information is currently available on the prebiotic potency of AXOS *in vivo*.[118]

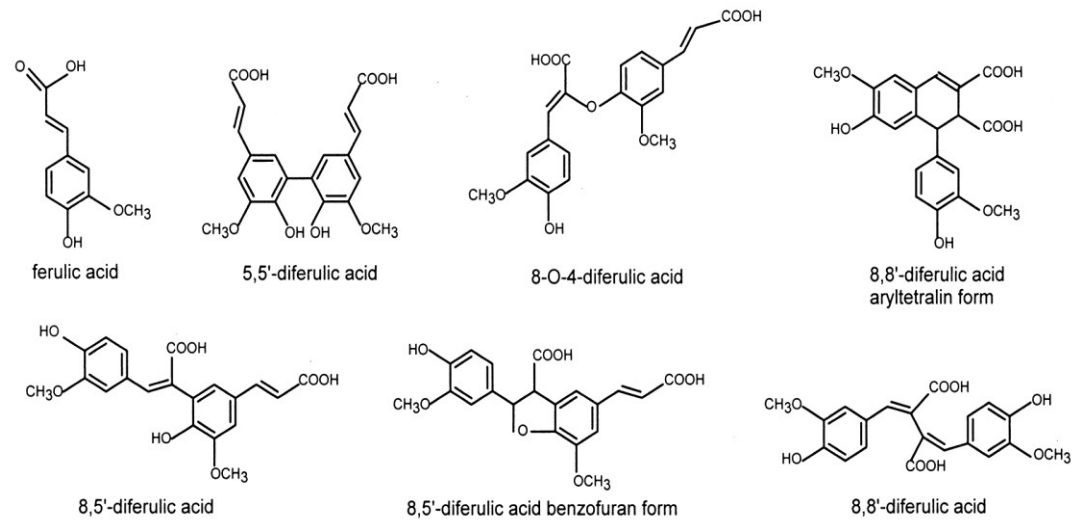


Figure 1-5 Chemical structures of ferulic acid and diferulic acid identified in plant cell walls, adopted from de Vries et al.[119]

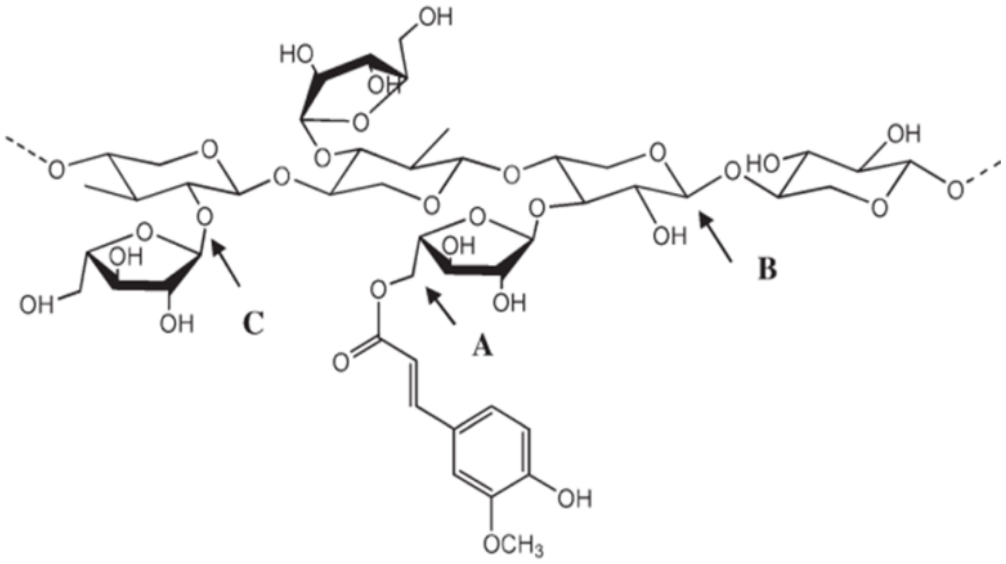


Figure 1-6 The structure of ferulic acid esterified to units of arabinoxylan. A: ferulic acid linked to 0-5 arabinose chain of arabinoxylan; B: β-1,4-linked xylan backbone; C: α-1,2-linked L-arabinose.[117]

DF can also be used as a source of prebiotics. Prebiotics are defined as non-digestible food ingredients such as fructooligosaccharides (FOS), inulin, galactooligosaccharides and other related carbohydrates that beneficially affect the host by stimulating the growth, or activity of one or a limited number of bacteria such as Lactobacilli and Bifidobacteria.[120]

It has been reported that only the intestinal microflora is able to disrupt the DF matrix and release the associated AOX in soft physiological conditions (neutral pH, 37 ºC). Also, the human microflora can hydrolyze, reduce, decarboxylate, demethylate, and dehydroxylate polyphenols (PP), and produce several metabolites.[121] The colon is a complex ecosystem with more than 400 different species of bacteria (>95% concentrated in the colon). PP are metabolized by gut microbiota and produce metabolites such as hydroxyphenylacetic, phenylpropionic and phenylbutric acids with potential health benefits to the host.[122] As a result, the bacterial species in the human gut have important metabolic and immune functions. For instance, short chain fatty acids, which are the metabolites from DF, can act as prebiotics and increase the population of beneficial colonic bacteria. DF polyphenols and their metabolites can also inhibit the growth of pathogenic bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. Current findings on these intestinal health effects of polyphenols is based on *in vitro* and animal studies, however, confirmation in humans is needed.[123,124]

1.13 Thesis objectives

This thesis was undertaken as a project within the Ontario Ministry of Agriculture, Food, and Rural Affairs (OMAFRA). OMAFRA works as a catalyst for transforming agriculture, food sectors, and rural communities for a healthier Ontario and is committed to protecting the Ontario environment.

This study aimed to characterize ARs in wheat bran, investigate the influence of environmental factors on AR composition, measure AR bioactivity including antioxidant activity and investigate AR stability during baking. Moreover, the prebiotic potential of WB-soluble dietary fibre (SDF) was explored using yogurt models. Specific objectives were achieved by the following:

1. To extract and characterize wheat bran ARs
    - Extractions by both traditional and SC-CO_2 methods
    - Characterizations of ARs by GC-MS and HPLC-PDA
2. To investigate environmental factors on wheat bran-ARs content and composition
    - Effects of region and cultivar on ARs
    - Effects of region and cultivar on antioxidant activity
3. To determine the stability of ARs at high temperatures using baking as a food model
    - AR content and composition before and after baking
    - Antioxidant activity before and after baking
4. To evaluate prebiotic activity of wheat bran in yogurt as a fermented food model
    - Effects of wheat bran addition on the growth/survival of lactic acid bacteria
5. To characterize phenolic composition of wheat bran by HPLC-PDA

- Alkaline hydrolysis of phenolics and characterization

This thesis is publication based, and each chapter covers the manuscripts already published or soon to be published.

Chapter 1 provides background information on wheat, as well as on the extraction, quantification, and determination of ARs, and on the bioactivity, stability, and prebiotic activity of wheat bran extracts.

Chapter 2 evaluates the effects of cultivar and region on the composition of ARs in wheat grown in Ontario (ON), using GC-MS. The effects of region and cultivar on the ARs in WB are closely examined and the results can be used for screening and breeding purposes.

Chapter 3 determines whether ARs in bread are stable during baking. This chapter gives detailed information about how different amounts of AR affect the homologue composition during baking.

Chapter 4 compares the traditional AR extraction method with the SC-CO_2 extraction method, the characterization of ARs by HPLC, as well as comparing different solvent systems in measuring antioxidant activity of WB extracts.

Chapter 5 investigates the capability and efficiency of WB soluble dietary fibre to enhance bacterial survival and growth in yogurt as well as to measure the bioactivity of the polysaccharides.

Finally, in Chapter 6, the overall conclusion is presented along with the contributions this thesis has made to current knowledge. It summurizes all findings and provides direction for future studies.

Outcomes of this research might lead to practical applications of wheat bran ARs and soluble dietary fiber in food, and nutraceutical applications for Agri-Food companies. Furthermore, enhanced WB utilization will reduce Agri-Food by product/leftovers and thus improve environmental sustainability.

1.14 Contribution of the authors to the manuscript based chapters

Chapters 2-5 are manuscript-based, and have been either presented in their edited form, or are soon to be published. Selected results from these chapters were also used to prepare conference presentations. The titles of the manuscripts and conference presentations are specified in the connecting statements for each chapter.

The contributions of the authors to the research presented in this thesis are as follows:

- Mrs. Aynur Gunenc, in her capacity as the author of this thesis and first author of the manuscripts and the conference presentations enumerated in the connecting statements to each manuscript-based chapter, conducted the reviews of literature, designed the experiments, the practical experimental work in the laboratory, and interpretation of results, the writing of this thesis, and the preparation of the manuscripts for publication.
- Dr. Farah Hosseinian, in her capacity as PhD Thesis supervisor, provided her technical expertise, guided the author of this thesis throughout all the stages of planning and experimental work, analyzing and interpreting the results, writing, and correcting this thesis, and preparing the manuscripts for publications.
- Dr. Mehri Hadinezhad, the Research Associate of Dr. Hosseinian Lab, provided technical expertise during the revision of the manuscript and submission process.
- Dr. Koushik Seetharaman from Guelph University provided the wheat bran samples.
- Dr. Paul Mayer from the University of Ottawa granted the use of his GC-MS instrument for analysis and characterization of the AR extracts.

- Dr. Lily Tamburic-Ilincic from Guelph University provided wheat bran samples grown in different location of Ontario and years for screening purposes.
- David Fairbanks from Algonquin College aided in making the bread used experimentally as reported in Chapter 3, and allowed the use of their culinary facilities during the research concerning the stability of ARs during baking.

1.15 Connecting Statement to Chapter 2

In Chapter 1, the economic importance of the wheat crop, the composition of WB, the health benefits of WB, and extraction methods, and the methods to analyze ARs content have been presented and discussed.

In Chapter 2, the contributions that the cultivar and region have on the ARs content and antioxidant activity of the wheat grown in Ontario, Canada are investigated.

The work presented in Chapter 2 has been used to prepare one conference presentation (poster) and one publication, as follows:

- Gunenc, A. Tamburic-Ilincic, L., and Hosseinian, F. New Approach for food safety assessment of Ontario wheat, OMAFRA-the 9th Annual Food Safety Research Forum, Guelph, ON, Canada, May 5th, **2011**.

- Gunenc, A. Tamburic-Ilincic, L., and Hosseinian, F. New Approach for food safety assessment of Ontario wheat, OCCI (Ottawa-Carleton Chemical Institute) Poster Day, University of Ottawa, Ottawa, ON, Canada, **2011**.

- Gunenc, A., Hadinezhad, M., Tamburic-Ilincic, L., Mayer, P. and Hosseinian, F. Effects of region and cultivar on alkylresorcinols content and composition in wheat bran and their antioxidant activity, *Journal of Cereal Science*, **2013,** 57: 405-410.

Chapter 2 Effects of region and cultivar on alkylresorcinols content and composition in wheat bran and their antioxidant activity

2.1 Abstract

This study evaluated the effects of cultivar and region on the composition of alkylresorcinols (ARs) of 24 wheat bran samples from 6 cultivars grown in four locations (Bath, Nairn, Palmerstone and Ridgetown) in Ontario, Canada, using gas chromatography/mass spectrometry (GC-MS). Total phenolic content (TPC) of wheat bran extracts was determined by Folin-Ciocalteau method and the antioxidant activity of wheat bran extracts was measured by 2,2-diphenyl-1-picryhydrazyl radical (DPPH) scavenging activity and oxygen radical absorbance capacity (ORAC) assays. The highest AR contents (μg/g) were found in cultivars Emmit (1522), Harvard (1305), Warthog (1170), and Superior (853) grown in Ridgetown. The relative percentages of saturated and unsaturated ARs were 89% and 11%, respectively. Total AR content and composition, TPC, and the antioxidant activity of wheat bran extracts were significantly affected ($P < 0.05$) by location and cultivar. TPC, % DPPH, and ORAC values for different wheat bran sample extracts ranged from 3 - 58 mg FAE/g, 5 - 68 (%), and 6 - 94 (μmol TE/g), respectively. This work provides a detailed examination of region and cultivar effects on the potential of ARs in wheat bran and results from which can be used for screening and breeding purposes.

2.2 Introduction

2.2.1 Climatic conditions affect wheat production

Wheat, maize and rice are the three most important cereal crops, representing approximately 2 billion tons of the total global production. Amongst these three crops, wheat is the most widely grown and consumed cereal by humans.[125] Wheat production needs suitable growing season temperatures and precipitation to obtain commercial yields.

Table 2.1 shows the ten year average Canadian wheat production and yield (1,000 tonnes) by province. The majority of Canadian wheat is produced in Saskatchewan (46% of total production), Alberta (30%), Manitoba (14%), Ontario (9%), Quebec (1%) and the Atlantic region produces less than 1% of total wheat production.

The climatic range for wheat production along with the variability in genotypes for each wheat class means that yields and quality vary significantly by location. Also the climatic values represent long term mean conditions and there is considerable variation from year to year.[126]

Table 2-1 Average 1999-2008 Canadian wheat production and yield by province[127]

Province	Wheat Production ('000 tonnes)			Wheat Yield (Kg/ha)		
	Spring Wheat	Durum	Winter Wheat	Spring Wheat	Durum	Winter Wheat
British Columbia	59			2,590		
Alberta	6,064	841	145	2,740	2,410	3,000
Saskatchewan	7,674	3613	227	2,060	2,020	2,560
Maniotoba	3,184	44	421	2,730	2,314	3,690
Ontario	183		1,636	3,310		5,020
Quebec	134		6	3,090		2,990
New Brunswick	9		2	3,070		3,370
Nova Scotia	3		8	3,020		3,930
Prince Edward Island	28		7	2,990		3,020
Canada	17,338	4,485	2,452	2,370	2,080	4,310

2.2.2 Wheat bran-phytochemicals affected by environmental factors

Phytochemicals present in grains have the potential to reduce human diseases such as cardiovascular diseases[128], diabetes[129] and cancer.[130] Some of the mentioned health benefits may be attributed to antioxidant activity of phenolic compounds such as ferulic acid, other polyphenols (lignans, anthocyanins and alkylresorcinols (ARs)), caretonoids and vitamin E.[30]

Wheat has significant levels of antioxidants, and among these phenolic compounds may elicit the highest health benefit. They exist in free, bound and soluble conjugated forms. The most dominant free phenolic compounds are ferulic, *p*-coumaric and vanillic acids. They are found with other phenolics including caffeic, chorogenic, gentisic, syringic, and *p*-hydroxybenzoic acids.[131] Previous studies have shown that bioactive

compounds (tocopherols, sterols, ARs, folates, phenolic acids, and fiber components) can be affected by different environmental factors.[52,125,132] The interactions between wheat genotype and the environment in which wheat is grown, and possibly genotype-environment interactions can likely strongly influence the levels of grain antioxidants.[132] Shewry et al. (2010) showed significant correlations between the contents of bioactive components (ARs, sterols, tocols, folates, phenolic acids and fiber compounds) and environmental factors (precipitation and temperature) differing in amount between grain samples grown in different years or different sites.[125]

Quantification of phytochemicals in whole grains and their products is crucial for marketing purposes. Unfortunately, studies mainly select for new cultivars having high yield. In some countries such as Canada and Australia, end use quality is an another purpose for breeding.[133]

2.2.3 Alkylresorcinols in wheat bran

ARs are phenolic lipids, also called resorcinolic lipids, predominantly present in the bran fraction of rye grains, closely followed by triticale and wheat.[35,134] ARs are located in the intermediate layers of pericarp and testa in the grain.[45] The structure of ARs is 1, 3-dihydroxy-5-alkylbenzene derivatives with odd-numbered, mostly saturated hydrocarbon side chains in the range of 15-25 carbon atoms. Also, ARs have been suggested as markers for whole grain, rye products and a biomarker for human intake of whole grain wheat and rye even though the total ARs in wheat or rye differs in cereal species, the relative homologue composition of ARs remains almost constant within the species.[49]

ARs affect physicochemical properties of biological membranes due to their

amphiphilic character. They have their antibacterial and antifungal activity.[30] They have potential to modulate the activity of some enzymes, and improve membrane phospholipid bilayer properties.[135] For instance, long chain ARs mixtures have been reported to prevent the peroxidation of fatty acids and phospholipids in liposomal membranes as well as autoxidative processes in triglycerides and fatty acids.[136]

ARs in cereal grains and cereal grain products have been quantified using gas chromatography (GC)[137] and high performance liquid chromatography (HPLC)-Coularray-Based Electrochemical Detection.[134] Also, AR metabolites in human urine have been determined by gas chromatography/mass spectrometry (GC-MS).[138]

2.2.4 Objectives

The primary objective of this study was to determine the effects of cultivar and region on AR content and composition of 24 wheat bran samples from 6 wheat cultivars grown in 4 different regions in Ontario using GC-MS. Also, another objective was to evaluate the effect of AR content and total phenolic content (TPC) on the antioxidant activity of wheat bran extracts by measuring oxygen radical absorbance capacity (ORAC) and 2, 2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity.

2.3 Materials and methods

2.3.1 Samples

The wheat bran samples were provided by the Plant Agriculture Department at Guelph University, Ridgetown, Ontario (ON), Canada. The samples were from six winter wheat cultivars including three hard red wheats (AC Morley, Harvard and Warthog) and three soft red wheat (Emmit, Superior and FT Wonder). The samples were grown in 4

different regions of Ontario in 2008; Bath, Nairn, Palmerstone and Rigdetown. Each region is illustrated in the map below of Ontario (Figure 2.1). The average precipitation, maximum and minimum temperatures from May 1st to June 31st in 2008 in the aforementioned regions were 265 mm, 21.5 °C and 9.5 °C for Bath; 340 mm, 21.0 °C and 10.1 °C for Nairn; 394 mm, 19.1 °C and 8.5 °C for Palmerstone; 340 mm, 20.9 °C and 9.9 °C for Ridgetown, respectively (Table 2.2).

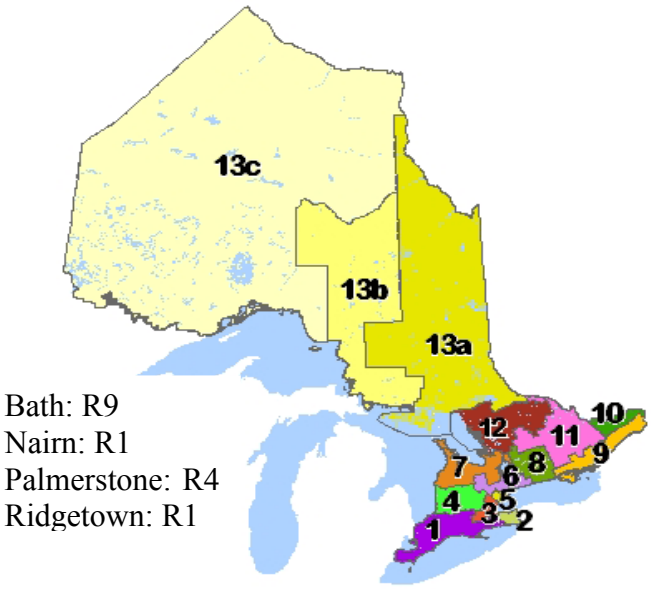


Figure 2-1 The map of Ontario regions, adopted from Ontario Ministry of tourism, culture and sport [139]

Table 2-2 The average precipitation (mm), and temperatures (°C) for each region

Region	Precipitation	T-min	T-max	ΔT
Bath	265	9.5	21.5	12.0
Nairn	340	10.1	21.0	10.9
Palmerstone	394	8.5	19.1	10.6
Ridgetown	340	9.9	20.9	11.0

Fine wheat (hard and soft) bran was prepared by abrading outer layers of grain in a Satake TM 05 laboratory scale pearler (Satake Co, Japan) to remove 10% (by weight) of bran. Prior to extraction, the bran was ground to the recommended 2 mm size[140] using a Thomas Wiley Mill (model ED-5, Arthur H. Thomas Co., Philadelphia, Pennsylvania, USA). Samples (24 wheat bran) were stored in sealed plastic bags and kept in the freezer (-20 °C) prior to analysis.

2.3.2 Materials

This section contains all detailed information about materials used not only for Chapter 2 but also for the following Chapters including 3, 4 and 5.

N-O-bis-(trimethylsilyl)-tri-fluoroacetamide with 1% trimethylchlorosilane was purchased from VWR International (Ottawa, Canada) for derivatization of samples before GC analysis. Solvents, acetone, ethanol, HCl, 1-propanol, and ethyl acetate were analytical grade and purchased from Caledon Laboratories LTC (Georgetown, ON, Canada). The AR standards (C15:0, C17:0, C19:0. C21:0, C23:0 and C25:0) in addition to C22:0 (Internal standard) were over 98% pure and purchased from ReseaChem GmbH (Burgdorf, Switzerland). Mono-and dibasic potassium phosphate, fluorescein, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), rutin, 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), Folin-Ciocalteau reagent, α-tocopherol, and 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), NaOH and phenolphthalein indicators were over 98% pure and obtained from Sigma (Oakville, ON, Canada). Ferulic acid standard (99% purity) was purchased from Fluka Analytical (Sigma) (Oakville, ON, Canada). Sodium carbonate was obtained from Church and Dwight Canada Corp. (Mississauga, ON, Canada).

The enzymes, α-amylase from *Bacillus licheniformis* (Type XII-A, saline solution ≥500 U/mg protein, EC 232-752-2) and protease from *Bacillus licheniform* (saline solution ≥2.4 U/g protein, EC 232-560-9) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The starter cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* (B-548; USDA) and *Streptococcus salivarius* ssp. *thermophilus* (14485; ATCC) were employed. The probiotics used were *Lactobacillus acidophilus* (B-4495, USDA) *and Bifidobacterium lactis* (41405, USDA), Man Rogosa Sharpe (MRS) broth liquid and MRS agar media were purchased from Oxoid Ltd. (Basingstoke, United Kingdom).

The phenolic acid standards (over 97% purity); gallic, proto-catechic, p-OH-benzoic, chlorogenic, caffeic, vanillic, syringic, p-coumaric, sinapic, ferulic, o-coumaric, the flavanoid standards; pyrogallol, catechin, epicatechin, rutin, quercetin-3-beta-glucoside, epicatechin gallate, myricetin, quercetin, apigenin and kaempherol were purchased from Sigma-Aldrich (St. Louis, Missouri, USA)

2.3.3 Extraction of alkylresorcinols (ARs)

ARs in wheat bran samples (1g) were extracted with acetone in a 1:40 (w/v) ratio for 24 h, by continuous stirring (Stirrer-VWR, Corning®, VMS-C4) at room temperature[140] and filtered using Whatman double filter paper (number 1). The precipitate was discarded and the acetone was evaporated to dryness from the supernatant using a Rotavapor (Buchi-Brinkman, R110, Switzerland). The extract was stored in the dark at -20°C until further analysis of TPC, DPPH, ORAC and GC-MS. All measurements were conducted in triplicate.

2.3.4 GC-MS analysis

GC-MS analysis was performed according to the method of Athukorala et al.[35] Extracts (200 µl) were spiked with 200 µl of internal standard AR C22:0 (0.5 mg/mL). The solvent was evaporated under nitrogen, and were derivatized with trimethylsilyl (TMS) using 200 µL of bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimetylchlorosilane (TMSC) and 100 µl of ethyl acetate. The reaction mixture was heated at 75 °C for 30 min and excess reagent was then evaporated under nitrogen and re-dissolved in ethyl acetate for GC-MS analysis. The GC-MS analysis was conducted with a Hewlett Packard HP 6890 Plus (GC) and Model G1540A equipped with a network mass selective detector. A DB-17HT high-temperature capillary column was used (30 m x 0.25 mm I.D., 0.1 µm film thicknesses, J & W Scientific, Folsom, California). Helium was used as the carrier gas with a flow rate of 1.3-mL/min and split injection by 10:1 ratio. The initial oven temperature of 150°C was held for 2 min and the temperature was increased by 10 °C/min to 320°C. Upon reaching 320°C, it was held for 7 min and the temperature was increased to 325°C for 1 min. A Hewlett Packard HP 5973 (MS) was operated in the electron ionization (EI) mode at 70 eV, a source temperature of 280°C and temperature of 150°C, in the scan range of *m/z* 35 to 350. Data were collected by ChemStation software (Shawnee, KS, USA). Identification of components was done by comparing their spectral data with ARs-standards (C15:0 to C25:0). The MS was used to confirm the presence of AR homologues by their molecular ion peaks at m/z 320 (C15:0), 348 (C17:0), 376 (C19:0), 404 (C21:0), 432 (C23:0) and 460 (C25:0). Data were compared against the NIST (v.02) and Wiley (v.138) libraries (Palisade Corp., Newfield, New York).[35]

2.3.5 Measuring total phenolic content (TPC) of wheat bran extracts

TPC was determined by using modified procedures of the Folin-Ciocalteu method of Gao et al.[141] Folin-Ciocalteu reagent was diluted 10 times with deionized water. The extract (200 µL) was added to of freshly diluted Folin-Ciocalteu reagent (1.9 mL). Then 1.9 mL of sodium carbonate solution (60 g/L) was added to the mixture. It was left for 2 h incubation at ambient temperature and the absorbance of the mixture was read at 725 nm against a blank of distilled water by using the Cary 50Bio UV-Visible Spectrophotometer (Varian Inc., Australia). Ferulic acid was used as standard.[142]

2.3.6 Measuring antioxidant activity by DPPH scavenging activity assay of wheat bran extracts

DPPH scavenging activity was performed according to a modified method of Brand-Williams et al.[142] 200 µL of extracts were reacted with 3.8 mL of DPPH solution (60 µm DPPH solution freshly prepared in 95% ethanol solution). The absorbance (A) values of the mixture at 515 nm were read against a blank of pure 95% ethanol at 0 and 30 minutes. Moreover, DPPH assay was performed on each standard AR homologue and a mixture of standard AR homologues at equal concentrations (2 mg/ml ethanol solution). Antioxidant activity was calculated as percent discoloration as shown in the following equation.[142]

$$\%DPPH = \left[1 - \left(\frac{A_{Sample,t=30\,\min}}{A_{Control\,t=o\,\min}}\right)\right] \times 100$$

2.3.7 Oxygen radical absorbance capacity (ORAC) assay of wheat bran extracts

The ORAC of each wheat bran extract as well as AR standards was measured according to the procedure described by Ou et al.[143] The ORAC assay was carried out on a fluorometric micro plate reader (FLx800™ Multi-Detection Microplate Reader with Gen5™ software, BioTek Instruments, Ottawa, Canada). Peroxyl radicals were generated by AAPH, and fluorescence microplate reader was used at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Trolox was used as standard (100, 50, 25, 12.5, 6.25 μM) and rutin was used as control (10 and 20 μM). Proper dilutions of bran extracts were made with ORAC buffer (potassium phosphate buffer, pH 7.4). For each ORAC run, a micro plate was prepared containing 20 μL of Trolox standards, Rutin control, and sample dilutions, as well as 120 μL of fluorescein (FL) solution. All ORAC analyses were performed at 37 °C with a 20 min incubation and 60 min run time. After the incubation, 60 μL of 153 mM AAPH was added to each well for a final volume of 200 μL. The results were calculated using the differences of areas under the FL decay curves between the blank and a sample and were expressed as micromole Trolox Equivalents per gram of sample (μmol TE/g).[143]

2.3.8 Statistical analyses

All statistical analyses were performed using a two-way-analysis of variance (ANOVA) (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). Significant treatments were further analyzed with the Duncan's multiple range test ($P < 0.05$). Significant correlations ($P < 0.05$) between ARs content and other measured parameters were performed using Microsoft Excel (Version 2010).

2.4 Results and Discussion

2.4.1 ARs analysis of wheat bran using GC-MS

2.4.1.1 Total ARs content and related composition

The total AR contents (μg/g) as well as the relative % saturated and unsaturated AR homologue contents of wheat bran samples from 6 cultivars grown in 4 different regions of ON in 2008 are shown in Table 2.3. Also, the GC-MS chromatograms for AR-standards and AR extract from the Emmit cultivar grown in Ridgetown is shown in Figure.2.2.

The saturated ARs were confirmed by their molecular ion as follows, at m/z 464 (C15:0), 492 (C17:0), 520 (C19:0), 548 (C21:0), 576 (C23:0), 604 (C25:0) and the base peak at m/z 268 for all ARs. The peaks with this characteristic m/z value were considered resorcinolic derivatives. The same observations were reported in literature.[34,35] The unsaturated peaks were assigned according to the MS data obtained from each GC-MS run and compared with literature data. [34] Among 24 wheat bran samples, ARs were found in 23 samples (Table 2.3). The cultivar Emmit had the highest amount of total ARs (1522 μg/g wheat bran sample) compared to all other cultivars. Athukorala et al.(2010), also reported 680 μg/g as total ARs contents in wheat bran sample from a commercial source.[35] The cultivar AC Morley grown in Palmerstone region was found to be free of ARs. It is not clear why this cultivar did not show any detectable quantity of ARs. More studies on another sample from the same cultivar and region are needed to confirm this result.

ARs in wheat are mostly present in the outer layers of bran.[90] Landberg et al. (2007) analyzed total ARs in different wheat grain fractions and reported 576, 2923 and

1024 μg/g of total ARs in ethyl acetate extracts of whole grain wheat, wheat bran and wheat aleurone, respectively.[68] Therefore, the bran removal technique used is an important factor in determining the total AR content. During grain milling, contamination of bran with refined flour could result in much lower ARs detection in wheat bran.

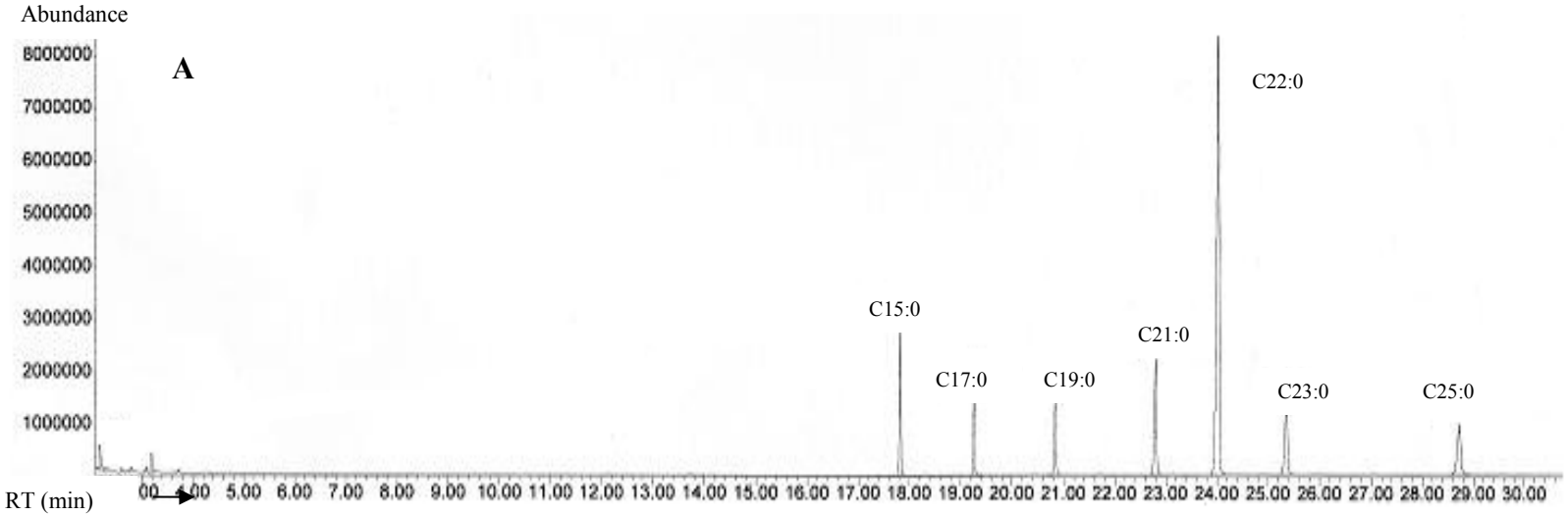
Although the total ARs content shown as in Table 2.3 was different for each cultivar, the relative saturated: unsaturated ratio of ARs remained close to 89:11and our findings were in agreement into the values reported in literature.[137]

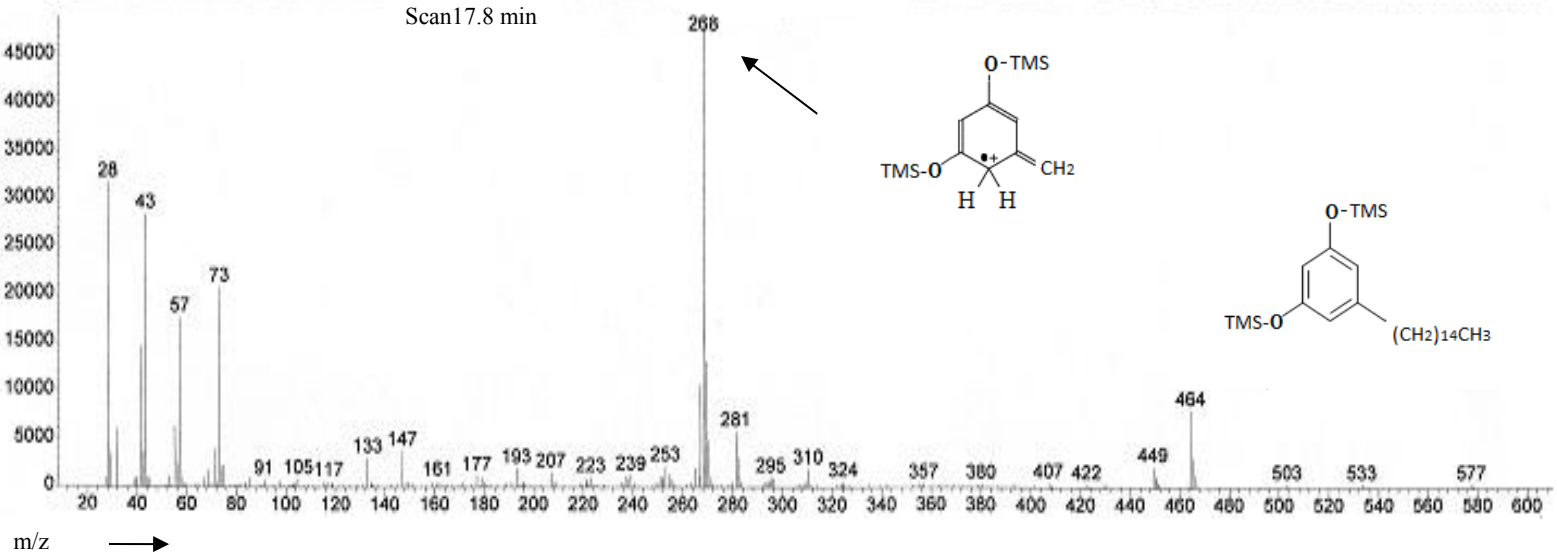
Table 2-3 Total ARs content in μg/g, and related % saturated (Sat), unsaturated (Unsat) and homologue

Region	Composition	AC Morley	Emmit	FT Wonder	Harvard	Superior	Warthog
Bath	Total- ARs	338 [jk]	138 [lm]	137 [lm]	367 [jk]	175 [l]	216 [l]
	Sat.ARs	88 [h]	100 [a]	81 [j]	90 [defg]	84 [i]	88 [h]
	Unsat. ARs	12 [c]	nd	19 [a]	10 [def]	16 [b]	12 [c]
	C17:0	4	3	nd	4	3	3
	C19:0	31	24	28	31	22	25
	C21:0	42	51	53	43	41	41
	C23:0	11	14	nd	8	12	13
	C25:0	nd	8	nd	4	6	6
Nairn	Total- ARs	458 [ij]	664 [gf]	509 [hi]	60 [gh]	801 [de]	259 [kl]
	Sat.ARs	90 [fgh]	91 [cd]	92 [cd]	89 [fgh]	89 [fgh]	88 [fgh]
	Unsat. ARs	10 [cde]	9 [efg]	8 [g]	11 [c]	11 [cde]	12 [c]
	C17:0	2	2	2	2	2	2
	C19:0	25	22	23	27	19	25
	C21:0	47	51	54	46	51	42
	C23:0	11	11	8	9	11	13
	C25:0	5	5	5	5	6	6
Palmerstone	Total- ARs	nd[b]	779 [def]	845 [de]	21 [m]	137 [lm]	363 [jk]
	Sat.ARs	nd	93 [c]	92 [cd]	100 [a]	100 [a]	90 [def]
	Unsat. ARs	nd	7 [g]	8 [gf]	nd	nd	10 [def]
	C17:0	nd	3	2	nd	nd	3
	C19:0	nd	28	16	43	24	28
	C21:0	nd	45	38	57	76	45
	C23:0	nd	11	33	nd	nd	12
	C25:0	nd	6	3	nd	nd	22
Ridgetown	Total- ARs	366 [jk*]	1522 [a]	725 [ef]	1306 [b]	853 [d]	1170 [c]
	Sat.ARs	83 [ij]	92 [cd]	97 [b]	91 [cde]	90 [efgh]	90 [def]
	Unsat. ARs	17 [ab]	8 [gf]	3 [h]	9 [fg]	10 [cde]	10 [def]
	C17:0	3	3	3	2	2	3
	C19:0	24	26	30	30	19	26
	C21:0	41	49	48	48	50	46
	C23:0	11	10	11	8	13	10
	C25:0	4	4	5	3	6	5

[a] Values are means of triplicates with different letters in rows for each characteristic are significantly different ($P < 0.05$) in Duncan's multiple range tests

[b] nd = not detected


Abundance
A
C22:0
C15:0
C21:0
C17:0
C19:0
C23:0
C25:0
RT (min)


Scan17.8 min
268
O-TMS
TMS-O
CH2
H H
O-TMS
TMS-O
(CH2)14CH3
m/z

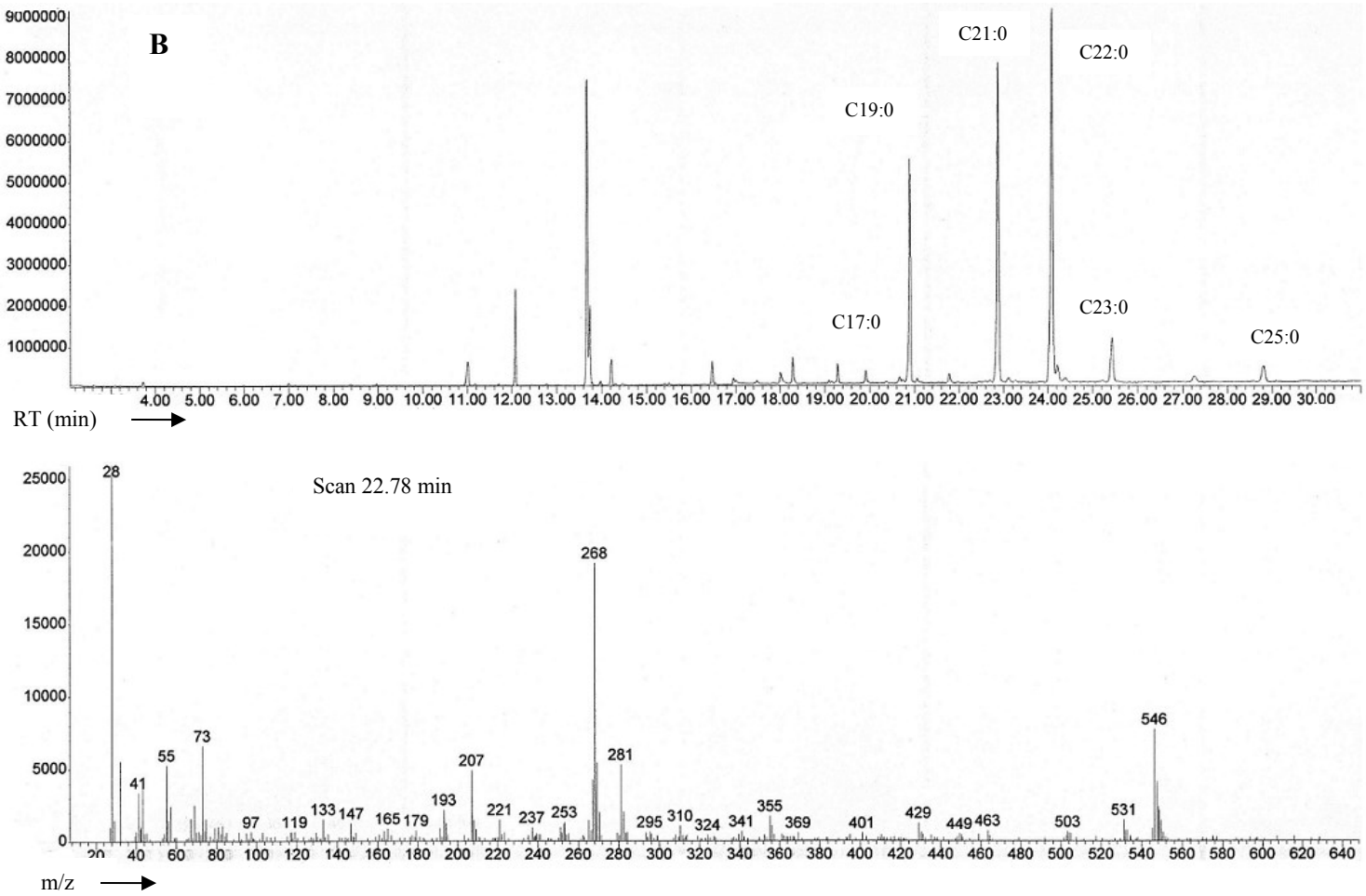


Figure 2-2 A) GC-MS of derivitized ARs standards (C15-C25) and the fragmentation pattern, B) GC-MS of cultivar Emmit grown in Ridgetown region.

2.4.1.2 Effects of region and cultivar

Two-way ANOVA demonstrated that the region, cultivar and interaction of the two showed significant ($P < 0.001$) effects on all measured parameters (Table 2.4).

Average total AR contents for different wheat varieties were highest in Ridgetown and lowest in Bath regions. On the other hand, for each region, the cultivar Emmit had the highest overall total AR contents with AC Morley having the lowest. The ANOVA also revealed that for each cultivar; the lower the total ARs, the higher the relative %unsaturated ARs. Genetic factors, climate, season, grain maturity, cereal type and soil conditions have been reported to affect ARs content.[38]

Overall, average of total AR contents (607 μg/g) for soft wheat (Emmit, Superior, and FT Wonder) was higher than than that of hard red wheat (488 μg/g; AC Morley, Harvard, and Warthog). This finding was in agreement with the study of Hengtrakul et al.(1990), where hard wheat had lower AR content than soft wheat.[144]

Table 2-4 Alkylresorcinols (ARs) analysis results for cultivar and region effects

Cultivar[e]	Total ARs[a]	Sat.ARs	Unsat.ARs	C17:0	C19:0	C21:0	C23:0	C25:0	C17/C21	TPC[b]	DPPH[c]	ORAC[d]
Morley	387 [e]	86 [e]	14 [a]	2.8 [a]	26.9 [b]	43.2 [c]	10.2 [bc]	2.9 [c]	0.07 [a]	29 [d]	41 [bc]	28 [d]
Emmit	776 [a]	95 [a]	5 [e]	2.5 [b]	24.9 [bc]	50.1 [b]	11.7 [ba]	5.8 [ab]	0.05 [c]	36 [b]	39 [c]	47 [a]
FT Wonder	554 [cb]	91 [c]	9 [c]	1.7 [c]	24.4 [bc]	47.7 [b]	13.4 [a]	3.8 [bc]	0.03 [d]	26 [e]	48 [a]	48 [a]
Harvard	574 [b]	93 [b]	7 [d]	2.2 [ab]	34.9 [a]	44.9 [c]	7.9 [c]	3.1 [c]	0.05 [b]	29 [d]	44 [b]	44 [ab]
Superior	491 [d]	91 [c]	9 [c]	1.7 [c]	21.3 [c]	54.7 [a]	9.1 [abc]	4.2 [bc]	0.03 [d]	32 [c]	37 [cd]	40 [bc]
Warthog	502 [cd]	89 [d]	11 [b]	2.6 [a]	26.2 [b]	41.2 [c]	11.2 [ab]	7.8 [a]	0.06 [b]	40 [a]	35 [d]	38 [c]
Region												
Bath	229 [d]	89 [b]	11 [a]	2.6 [a]	26.9 [a]	45.7 [b]	9.9 [a]	3.9 [a]	0.07 [a]	33 [b]	21 [d]	26 [d]
Nairn	549 [b]	90 [b]	10 [b]	2.3 [b]	23.7 [a]	48.3 [a]	10.8 [a]	4.9 [a]	0.04 [c]	34 [b]	50 [b]	38 [b]
Palmerstone	358 [c]	92 [a]	8 [d]	2.8 [c]	26.9 [a]	47.7 [c]	10.3 [a]	4.8 [a]	0.05 [b]	19 [c]	37 [c]	31 [c]
Ridgetown	990 [a]	91 [b]	9 [c]	2.6 [a]	26.6 [a]	46.7 [ab]	10.3 [a]	4.8 [a]	0.05 [b]	42 [a]	54 [a]	68 [a]

[a] Total ARs = µg/g of sample, Sat. ARs= %Saturated ARs, Unsat ARs = %Unsaturated ARs, all ARs homologue (C17:0-C25:0) are in (%).

[b] TPC = Total Phenolic Content as mg Ferulic Acid Equivalent (FAE) /g

[c] DPPH = 1, 1-diphenyl-2-picrylhydrazyl as %DPPH inhibition after 30 min

[d] ORAC = Oxygen Radical Absorbance Capacity as TE µmole/g (Trolox Equivalent)

[e] Values are means of triplicates with different letters in columns are significantly different ($P < 0.0001$) in Duncan's multiple range tests

2.4.1.3 The relative % of homologue composition

All assignments of homologues C15:0-C25:0 were made by comparison of retention times of the respective reference standards. Also, each peak assignment was confirmed by fragmentation pattern in the MS data. A homologous series of saturated C17:0-C25:0 were detected in wheat bran extracts which was in agreement with literature reports.[145] In all wheat bran samples, C21:0 and C19:0 homologues were the most abundant. Wheat bran was also reported to have some AR derivatives including ARs with unsaturated alkyl chains, keto groups, or a combination of keto groups and unsaturated alkyl chains.[78] It has been suggested that these minor groups of AR derivatives exhibit various biological activities related to their amphiphilic characteristics.

Only 1% of the AR homologue, C15:0 was detected in cultivar Emmit in both Ridgetown and Nairn regions, and cultivar Superior in Bath region (data not shown). The highest to lowest AR homologue order was C21 > C19 > C23 > C25 > C17 for all samples. These findings are in agreement with results reported in the works of Ross et al. and Knodler et al.[34,145] The relative homologue composition of ARs in wheat samples has been reported to be 3-6% C17:0, 29-42% C19:0, 46-55% C21:0, 4-12% C23:0, and 1-4% C25:0. The ratio of C17:0 to C21:0 for durum wheat, common wheat, and rye is 0.01, 0.1, and 1.0, respectively. [38] The ratio of C17:0/C21:0 ranges from 0.03 to 0.07 and these ratios are suggested to be an index for determining the cereal grain source. The ratio of 0.01, 0.1 and 1.0 was reported for durum wheat, common wheat and rye respectively.[130] Our study demonstrated that these ratios could be used to differentiate common wheat cultivars.

2.4.2 TPC of wheat bran extracts

The TPC of all wheat bran samples were analyzed by Folin-Ciocalteu method. Results were significantly different ($P < 0.05$) among wheat bran-acetone extracts (Table 2.5). Wheat bran from cultivar Emmit grown in Ridgetown had the highest TPC (58 mg FAE/g) and it was lowest for Harvard cultivar from Palmerston (5 mg FAE/g). This wide range reflects the TPC dependency on the wheat cultivars. In wheat, several major phenolic compounds are present including phenolic acids, ARs, flavonoids, and phenolic acid diacyl glycerols, phenolic aldehydes and ferulates.[30] There was a small but significant correlation between total ARs and TPC of wheat brans ($R^2 = 0.20$, $P < 0.05$).

Table 2-5 TPC (mg FAE /g), % DPPH, and ORAC (μmol TE/g) values* of WB extracts

Region	Analysis	AC Morley	Emmit	FT Wonder	Harvard	Superior	Warthog
Bath	TPC[a]	33 ^{fged}	43 ^{b}	13 ^{j}	31 ^{fgeh}	35 ^{ced}	41 ^{cb}
	% DPPH[b]	43 ^{fg}	11 ^{ml}	15 ^{kl}	18 ^{jkl}	19 ^{jkl}	20 ^{jk}
	ORAC[c]	41 ^{egf}	21 ^{i}	20 ^{i}	29 ^{ihg}	20 ^{i}	25 ^{ih}
Nairn	TPC	41 ^{cb}	28 ^{gih}	29 ^{fgih}	38 ^{cbd}	30 ^{fgeh}	38 ^{cbd}
	% $DPPH_c$	68 ^{a}	26 ^{ji}	60 ^{bac}	63 ^{ba}	52 ^{edc}	33 ^{hi}
	ORAC	33 ^{ihgf}	36 ^{ehgf}	56 ^{d}	33 ^{ihgf}	47 ^{ed}	24 ^{ih}
Palmerstone	TPC	3 ^{k}	15 ^{j}	27 ^{ih}	5 ^{k}	24 ^{i}	38 ^{cbd}
	% DPPHc	5 ^{m}	60 ^{bac}	63 ^{ba}	43 ^{fg}	18 ^{kl}	36 ^{hg}
	ORAC	6 ^{i}	45 ^{edf}	68 ^{c}	20 ^{i}	20 ^{i}	28 ^{ihg}
Ridgetown	TPC	39 ^{cbd}	58 ^{a}	34 ^{fed}	43 ^{b}	37 ^{cbd}	42 ^{b}
	% DPPHc	47 ^{fe*}	57 ^{bdc}	56 ^{bedc}	52 ^{edc}	61 ^{bac}	50 ^{fed}
	ORAC	29 ^{ihg}	85 ^{ba}	48 ^{ed}	94 ^{a}	74 ^{bc}	75 ^{bc}

[a] TPC = Total Phenolic Content as mg Ferulic Acid Equivalent (FAE) /g
[b] DPPH = 1, 1-diphenyl-2-picrylhydrazyl as %DPPH inhibition after 30 min
[c] ORAC = Oxygen Radical Absorbance Capacity as μmole TE (Trolox Equivalent) /g
[*] Values are means of triplicates with different letters in rows for each analysis are significantly different ($P < 0.05$) in Duncan's multiple range tests

Two-way ANOVA showed that region, cultivar and their interactions were significantly different ($P < 0.001$) in TPC (Table 2.5). Average TPC was highest for Ridgetown and lowest for Palmerstone regions. Differences among the cultivars at each location were also significant ($P < 0.001$); Warthog had the highest TPC with FT Wonder having the lowest.

It should be mentioned that the present study analyzed acetone extracts of all wheat bran samples for TPC, ORAC and DPPH assays. As reported by Winata and Lorenz, our preliminary experiments also demonstrated that acetone extracts were more selective for AR extractions in comparison with ethanol, ethyl acetate, and propanol.[146]

However, it co-extracts other compounds which affect the measured TPC and antioxidant activity. Liyana-Pathirana and Shahidi reported TPC of 67 and 59 mg FAE/g in soft and hard wheat bran, respectively, using 80% ethanolic crude extracts.[147]

2.4.3 Antioxidant activity of wheat bran extracts by DPPH scavenging activity assay

The antioxidant activity of all wheat bran-acetone extracts was measured using a DPPH assay (Table 2.5). This assay measures the ability of antioxidants to reduce the DPPH radical which can be calculated by the percentage of discolorization; the higher this percentage, the stronger the antioxidant.[114] There were significant differences ($P < 0.05$) in % DPPH-discoloration of all assayed samples. All % DPPH inhibition results ranged between 5 and 68. It was interesting the same cultivar, AC Morley, from different regions, possessed the highest and lowest antioxidant potentials based on this assay. There was a small but significant correlation between total ARs of wheat bran samples and their % DPPH ($R^2 = 0.36$, $P < 0.05$).

Two-way ANOVA showed that region, cultivar and their interactions, were significantly different ($P < 0.001$) for antioxidant activity (Table 2.5). Mpofu et al. (2006) also reported that antioxidant properties of wheat and wheat products are affected by genotype and environment.[132] Wheat brans from Ridgetown and Bath regions had the highest and lowest antioxidant activity, respectively. The antioxidant activity of different cultivars as %DPPH-discoloration ranged from 35% (Warthog cultivar) to 48% (FT Wonder cultivar) and the antioxidant activity of different regions ranged from 21% (Bath region) to 54% (Ridgetown region). These findings suggest that region has a greater effect than cultivar type on antioxidant activity.

Overall, the %DPPH inhibition of soft wheat (41%) was slightly higher than that of hard wheat (40%). Using a similar protocol and DPPH method, Liyana-Pathirana and Shahidi (2006) obtained % DPPH inhibition values of 58% for soft wheat and 55% for hard wheat brans extracted with 80% ethanol.

Table 2.6 shows the antioxidant activity of individual AR homologue standards and their mixture. At a ratio of 4-5 mol AR/mol DPPH the antioxidant activity ranged from 20 to 30% discoloration of DPPH. The highest observed antioxidant activity (31.5 ± 1.8%) belonged to the mixture of AR standards with a ratio of 4.3 mol/mol DPPH, indicating some synergistic effects of the mixture. Kamal-Eldin et al.(2000) also reported a weak hydrogen donation activity for 5-n-pentadecylresorcinol (C15:0); 20% at 7.7 mol phenol/mol DPPH after 60 min.[148]

Although ARs do not possess a hydroxyl group in the ortho-position, another factor that can affect the efficiency of an antioxidant for fat systems is the lipophilicity, which allows better penetration in the lipids micelles. A comparison between the relative antioxidant activities (RAA) of different phenolic compounds in a test system including linoleic acid micelles stabilized with Na dodecyl sulfate (pH 7.4, Temp. 50 ^{o}C) showed ferulic acid and caffeic acid (with OH-group in ortho-position) cannot be considered as an effective antioxidant under these conditions since they cannot penetrate through lipid micelles.[26]

Our results showed that the AR molar ratio in wheat bran extract used for the DPPH assay ranged from 0.02-3.6 mol/mol DPPH, with the lowest ratio belonging to Harvard cultivar from Palmerstone region and the highest Emmit cultivar from Ridgetown region. Comparing these ratios with the AR standards and their antioxidant

activity, it can be concluded that other phenolic compounds in the extract such as ferulic acid, have more pronounced effects on the overall antioxidant capacity of wheat bran when compared to ARs.

2.4.4 ORAC assay of wheat bran extracts

ORAC measures antioxidant inhibition of peroxyl radicals and reflects classical radical chain-breaking antioxidant activity by H-atom transfer.[110] The peroxyl radical generated from thermal decomposition of AAPH in aqueous buffer reacts with a fluorescent probe to form a non-fluorescent product. Higher ORAC values indicate less fluorescent decay over a certain period of time.

ORAC values of all wheat bran-acetone extracts ranged between 6 and 94 μmole TE/g (trolox equivalent) as shown in Table 2.5. ORAC values were significantly different ($P < 0.001$) for region and cultivar and their interaction (Table 2.4). The wheat bran cultivars grown in Ridgetown and Bath locations showed the highest and lowest respective antioxidant capacities. Although differences in ORAC value among cultivars were not significant, AC Morley had the lowest ORAC value. The correlation between total AR contents and ORAC values were significantly related (R^2 =0.78, $P < 0.05$). Also a small but significant correlation (R^2= 0.41, $P < 0.05$) was found between two employed antioxidant assays.

Overall, ORAC values of soft wheat (50 μmole TE/g) were higher than hard wheat (37 μmole TE/g). Moore et al. reported the ORAC of eight Maryland-grown soft wheat samples and ORAC values ranged from 33 to 47 μmole TE/g.[149] Another study also reported the ORAC values of six different whole wheat varieties to be between 20 to 37 μmole TE/g.[150]

Table 2-6 Antioxidant assays of individual and mixture of ARs standard

AR Standards	Concentration in mole (phenol/ DPPH)	%DPPH*	ORAC (μmoleTE/g)*
C15:0	5.2	19.2 ± 1.0	199.4 ± 3.8
C17:0	4.8	23.0 ± 0.6	202.1 ± 5.3
C19:0	4.4	20.3 ± 2.0	228.1 ± 7.6
C21:0	4.1	20.7 ± 1.3	142.8 ± 4.3
C23:0	3.8	20.9 ± 0.0	178.2 ± 5.6
C25:0	3.6	20.1 ± 1.7	215.0 ± 9.8
ARs-mix[a]	4.3	31.5 ± 1.8	245.9 ± 7.6

[a] ARs-mix comprises of all individual AR in gram ratio similar to their ratio in wheat bran extract and the mol presented in the Table is the sum of their individual calculated mole

* Values are means of triplicates ± standard deviations (SD), %DPPH = 1, 1-diphenyl-2-picrylhydrazyl as % inhibition after 30 min, ORAC = Oxygen Radical Absorbance Capacity as μmole TE (Trolox Equivalent) / g

The ORAC values for AR standards are presented in Table 2.6. There were only minor differences between AR standards, and a mixture of ARs standards showed higher antioxidant activity than individual ARs as was found with DPPH assay results.

2.5 Conclusion

The current study investigated the effects of region and cultivar on total AR content, relative composition analysis, as well as total phenolic count and antioxidant activity of wheat bran extracts. The results showed that the Ridgetown region has greater potential for varieties rich in AR content. The highest amount of ARs was found in cultivar Emmit and the most common AR homologues in wheat brans were C21:0, followed by C19:0, C23:0, C25:0 and C17:0. TPC and antioxidant activity of wheat bran extracts were significantly affected by location and cultivar and their interaction. Our work provides a detailed examination of region and cultivar effects on AR content in

wheat bran and presents a prospective for producing wheats rich in ARs. These results can be used for screening, breeding and genetic modification purposes. Thoroughly designed animal and pilot human studies are needed to investigate the bioavailability of wheat ARs from different wheat-based food products.

2.6 Connecting Statement to Chapter 3

Chapter 2 provided a detailed examination of region and cultivar effects on potential of ARs in wheat bran and the results can be used for screening and breeding purposes.

In Chapter 3, the stability of natural levels of ARs in baked breads was investigated by analyzing and comparing total and homologue AR levels using GC-MS. Also, the study aimed to determine how the baking process affects the TPC and antioxidant activity of ARs by measuring ORAC and DPPH inhibition.

The work presented in Chapter 3 has been used to prepare two conference presentations (oral) and one publication, as follows.

- Gunenc, A. and Hosseinian, F., 2011. Potential of alkylresorcinols in Canadian red hard and red soft wheat bran and their stability during baking, American Association of Cereal Chemists (AACC)-International Conference, Palm Springs, CA, USA, October 16-19.

- Gunenc, A., Seetharaman, K., Mayer, P. Fairbanks, D., and Hosseinian, F., 2011. Potential of alkylresorcinols in Canadian red hard and red soft wheat bran and their stability during baking, Canadian Section of American Oil Chemists'Society (CAOCS), 24th Conference on Fats and Oils, Edmonton, AB, Canada, September 26-27.

- Gunenc, A., Tavakoli, H., Seetharaman, K., Mayer, P.M., Fairbanks, D. and Hosseinian, F., 2013. Stability and antioxidant capacity of alkylresorcinols in Canadian hard and soft red wheat bran during baking, Food Research International, 51:571-578.

Chapter 3 Stability and antioxidant activity of alkyresorcinols in breads enriched with hard and soft wheat bran

3.1 ABSTRACT

Alkylresorcinols (ARs) are phenolic lipids that are mostly found in rye and wheat bran. In this study, the heat stability of ARs and the AR homologue composition of hard red wheat bran (HRWB) and soft red wheat bran (SRWB) were studied using four different bread formulations. ARs in wheat bran samples were extracted with four different solvents whereas ARs were extracted from the breads using hot 1-propanol. Gas chromatography-mass spectrometry (GC-MS) was used to quantify AR contents of wheat bran and bread samples. The different breads used in each trial were: A) 100% white flour (control breads), B) 30% wheat bran, C) 30% residue bran after AR extraction, and D) 30% wheat bran plus 2% crude ARs extract. AR content in breads varied from 1.1 to 82.9 mg/100 g, demonstrating that they were heat stable during baking. After 1 h of cooling, all breads showed significantly different ($P < 0.05$) inner temperatures, heights, and weights from that of the control bread. Furthermore, bread D displayed an improved height than breads B and C ($P < 0.05$). The effects of baking on total phenolic content (TPC) and antioxidant activity were also determined. A positive correlation was observed between oxygen radical absorbance capacity (ORAC) and TPC ($R^2 = 0.90$).

3.2 Introduction

3.2.1 Bread, the major staple food

Many food products such as bread, pasta, noodles, and breakfast cereal products are made from cereal grains.[151] One of the most important and widely consumed foods in the world is cereal products. Among the cereals, wheat and rye are the most suitable basic ingredients for bread making.[152,153] Bread consumption provides 50% carbohydrate, 30% protein, and 50-60% vitamin B of the daily requirements for people of industrialized nations.[152] The main antioxidant components found in cereals are phenolic compounds such as phenolic acids, ARs, tannins, and anthyocyanins.[154] Some of these phytochemical compounds may reduce the risk of many diseases such as cancer, cardiovascular disease, obesity, type 2 diabetes, cataracts, osteoporosis, and urinary tract infections.[30, 33, 135, 155] It is becoming more obvious that these health benefits are related to the consumption of whole grain cereal products due to their composition of micronutrients, phytochemicals, and dietary fiber.[30]

3.2.2 ARs in bread

ARs are phenolic compounds predominantly present in the bran fractions of wheat, rye, and triticale.[52] AR content has been reported to be present in cereal brans at levels of 36-320 mg/100g in rye, 32-101 mg/100g in wheat, and 58-163 mg/100g in triticale.[46] Even though the total amount of ARs in wheat or rye varies between cereal species, the relative homologue composition of ARs remains almost the same within species.[145] For instance, the relative homologue composition of ARs in wheat samples has been reported as 3-6 % C17:0, 29-42 % C19:0, 46-55 % C21:0, 4-12 % C23:0, and 1-4 % C25:0. ARs are only found in the inner pericarp, hyaline layer, and testa of cereal grains. Thus a high

amount of ARs is present in bran and very minimal levels in the endosperm. The ratio of C17:0/C21:0 can be used to differentiate between three main cereals containing ARs such as 0.1 for wheat, 0.01 for durum wheat and 1.0 for rye.[45]

If wheat bran or whole grain flour is added in adequate amounts to specific food products, it can enhance potential health benefits. A substantial amount of bran addition may cause dough to get too hydrated and thus non-elastic, consequently leading to a decrease in leavening volume and tougher more firm dough.[153] Some earlier studies have indicated that fermentation and baking could decrease the amount of ARs in bread; however, later studies have shown that the problem lies in the poor extraction of ARs rather than being caused by baking. Ross et al.(2003) used different solvents to extract ARs from breads containing rye bran and showed that a mixture of hot propanol and water was the best solvent to extract ARs completely with high yield.[38] The extraction conditions were hot-propanol/water (3:1, v/v) with three fold extractions at 100 ºC (2 times x 2h followed by 1 time x 1h) using fresh solvent each time. Currently, there is a lack of research studies on the determination of AR content and quality in Canadian wheat breads and how that is influenced during baking. Hirawan et al.(2010) studied how the effects of cooking could alter the antioxidant properties of commercial, regular and whole-wheat spaghetti.[156] They found that whole wheat spaghetti brands exhibited significantly higher contents of phenolic compounds and ferulic acid in comparison to regular spaghetti brands.

3.2.3 Objectives

The objective of this study was to investigate the stability of natural levels of ARs in baked breads by analyzing and comparing total and individual AR homologue quantity

using gas chromatography/mass spectrometry (GC-MS). This was accomplished by producing wheat breads of differing formulations and measuring the level of ARs in these breads after baking. Moreover, the study aimed to determine how the process of baking affects the total phenolic count (TPC) and the antioxidant activity of ARs; the antioxidant activity was measured by oxygen radical absorbance capacity (ORAC) and 2, 2-diphenyl-1-picryhydrazyl radical (DPPH) radical scavenging activity assays.

3.3 Materials and methods

3.3.1 Materials

Hard red wheat bran (HRWB) and soft red wheat bran (SRWB) were obtained from Kraft-Canada. All the other ingredients needed for bread making were supplied by the Culinary Department of Algonquin College, Ottawa, Ontario, Canada. Detailed information of all chemical materials used for this study can be found in the section 2.3.2.

3.3.2 Analysis of ARs

3.3.2.1 Extraction of ARs in wheat bran samples

ARs from two WB samples were extracted by ethanol in a ratio of 1 to 40 (w/v) according to the methods of Mullin et al. [87] After 24 h of stirring at room temperature, the samples were filtered using double Whatman filter paper. The precipitate was discarded and the solvents were evaporated from the supernatant using a Rotavapor (Buchi-Brinkman, R110, Switzerland) and the dried product was weighed and stored at -20 °C until it was ready to be used in bread trial D. The residues of HRWB and SRWB after ethanol extraction were dried at room temperature and used for bread trial C which was based on 30% extracted wheat bran (EWB). Also, the same extraction method was

repeated using acetone, ethyl acetate and 1-propanol to compare ARs content and composition. All analyses were performed in triplicates.

3.3.2.2 Extraction of ARs in bread samples

The freeze-dried bread samples were crushed and milled to a particle size of less than 0.5 mm. The bread samples were extracted with 1-propanol and water in accordance with Ross et al. [38] The milled samples (0.5 g for breads B and D and 1 g for breads A and C) were placed in 30 ml glass tubes and capped tightly. Later, 200 µl of internal standard (0.5mg/ml, C22:0) and 10 ml of 1-propanol: water (3:1, v/v) were added to the tubes and placed in a boiling water bath for 2 hours while being mixed by vortexer every 30 min. After allowing tubes to cool to room temperature, they were centrifuged at 2300 x g for 10 min; supernatants were then transferred to 50 ml tubes and capped tightly. The same extraction procedure was repeated two more times.[38] Eventually, all the supernatants from the repeated extractions were collected and combined in one tube. Later, 4 ml portions of each sample extract were dried under nitrogen stream. All analyses were performed in triplicate.

3.3.2.3 GC-MS analysis

ARs in all extracted samples were analyzed by GC-MS and all detailed information about analyzing conditions is in section 2.3.4.

3.3.3 Bread making procedure

3.3.3.1 Bread preparation

In this study, seven different bread trials have been prepared using white flour (as a negative control) and wheat brans (HRWB and SRWB) according to a modified method[52]

of Andersson et al.(2010). The four different trials of bread were baked with: A: 100% white flour (WF or Control Bread); B: 30% non-extracted wheat bran (NEWB); C: 30% extracted wheat bran (EWB); and D: 30% (NEWB) plus crude 2% crude AR extract. David Fairbanks who is the chef from Algonquin College baked the breads according to the recipe shown in the Table 3.1. All analyses were performed in triplicate.

Table 3-1 All bread trials and recipes for bran samples: HRWB (hard red wheat bran) and SRWB (soft red wheat bran)

Bread types	Bread trials [a]	Bread recipes
A	70% WF + 30% WF	140 g WF + 60 g WF + 144 g water + 1.9 g salt + 2.5 g yeast
B	70% WF + 30% NEWB	140 g WF + 60 g NEWB + 144 g water + 1.9 g salt + 2.5 g yeast
C	70% WF + 30% EWB	140 g WF + 60 g EWB + 144 g water + 1.9 g salt + 2.5 g yeast
D	70% WF + 30% NEWB + 2% crude ARs extract	140 g WF + 60 g NEWB + 4 g crude ARs extract + 144 g water + 1.9 g salt + 2.5 g yeast

[a] EWB = Extracted wheat bran, NEWB = Non-extracted wheat bran, WB = Wheat Bran, WF =White Flour

3.3.3.2 Baking procedure

The bread making process included 5 steps: 1) mixing, 2) fermentation, 3) makeup, 4) proofing and 5) baking (Figure.3.1). The baking dough was prepared on a flour weight basis with 200 g flour mixture, 2.5 g dry yeast (Saf-Instant, lesouffre yeast corporation, Milwaukie, Wisconsin, U.S.A.), 1.9 g sodium chloride (Windsor Table Salt, The Canadian Salt Company Limited, Point-Claire, Quebec, Canada), and tap water (48°C). The ingredients were mixed as a straight dough method for 10 minutes in a countertop mixer (Hobart Legacy, Hobart Corporation, Troy, OH) at 2nd speed and the doughs were

left in a leavening cupboard (Metro proofer, Inter-metro industries corporation, California, USA) at 34°C at 60% Relative Humidity (RH) for 60 min. The doughs were then divided into 3 pieces (3x100 g), molded, and placed in baking loaf pans laced in non-grazed molds, then placed in a second leavening cupboard at 39°C at 85% RH for 60 min. The fermented dough was baked for 12 min at 255 °C in a rotation oven (Garland-Master 200, USA). The inner temperature, as well as the weight and height of the breads were measured after 60 minutes of cooling. The breads were put in sealed plastic bags, frozen and freeze-dried for further analysis.

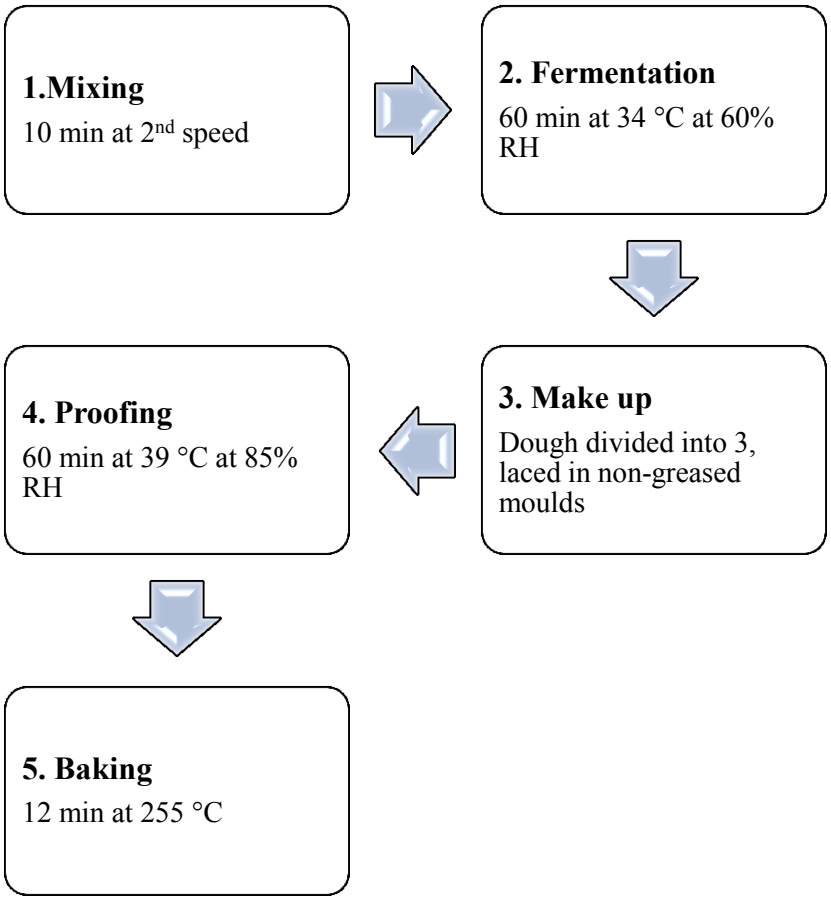


Figure 3-1 The bread making procedure

3.3.4 Antioxidant activity

3.3.4.1 Sample extraction

For the extraction procedure, 15 mL of ethanol (95%):1 N HCl (85:15, v/v) was mixed with 1.0 g of milled sample in 50 mL brown bottles and was continuously shaken for 6 h at 300 rpm and at an ambient temperature. The mixture was then centrifuged at 7,800 x g (Thermo Scientific, Sorval Legend XTR) at 5°C for 15 min. The resulting supernatant fluid was then stored at -20°C in the dark, and kept till further analysis of TPC, DPPH, and ORAC[142]. All analyses were performed in triplicates.

TPC, DPPH and ORAC measurement of WB and bread samples were performed by following sections 2.3.5, 2.3.6 and 2.3.7, respectively.

3.3.5 Statistical Analysis

All statistical analyses were performed, using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) using Fisher t-test, LSD was used to determine if there was a significant difference ($P < 0.05$) among triplicates of total and homologue of ARs before and after baking. Also the same analysis was performed to see if there was significant difference ($P < 0.05$) between ORAC, DPPH and TPC data sets.

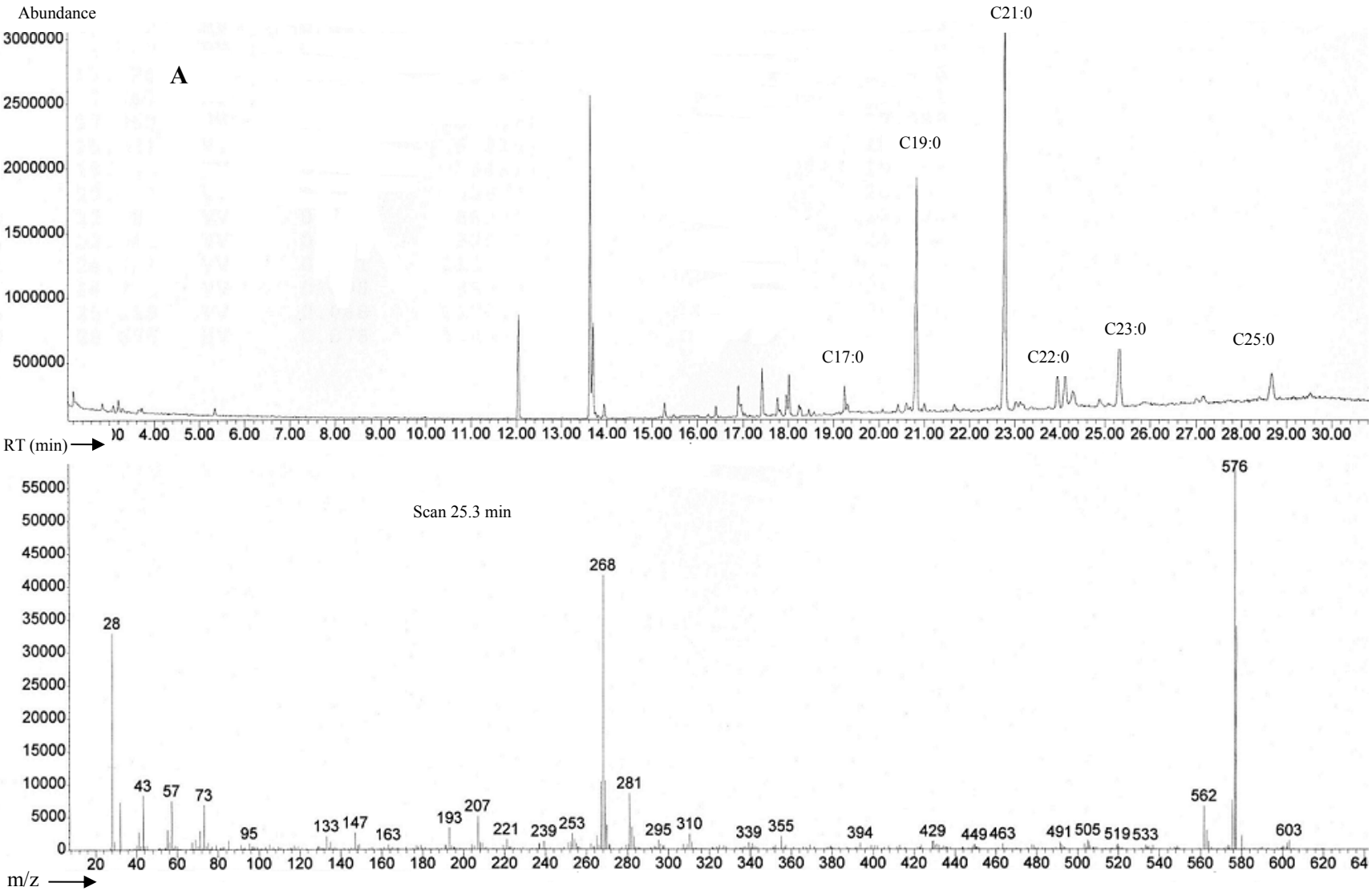
3.4 Results and Discussion

3.4.1 AR content and homolog composition in wheat bran

GC-MS analysis was used to measure the total content of saturated, unsaturated ARs (mg/100g), and level of individual homologues (%) in wheat bran samples. The saturated ARs were confirmed by comparison of retention times of AR standards (C15:0-C25:0) and their molecular ion mass to charge ratio (m/z) as follows, 464 (C15:0), 492

(C17:0), 520 (C19:0), 548 (C21:0), 576 (C23:0), and 604 (C25:0). The main fragmentation ions were observed at m/z 268, 267, and 281; this is most likely due to McLafferty rearrangement, β cleavage and γ cleavage, respectively. The peaks with these characteristic m/z values were considered resorcinolic derivatives and this is in agreement with results reported in literature.[34,35,140]

Table 3.2 shows the AR content and composition in bran samples. The wheat bran samples were HRWB, SRWB, HRWB-R, SRWB-R and WF (control). Four different solvents including acetone, ethanol, ethyl acetate and 1-propanol were used to extract the above WB samples. The effect of various extraction solvents on AR content (mg/100g) in WB samples is shown in Table 3.2.


Abundance
A
C21:0
C19:0
C23:0
C25:0
C17:0
C22:0
RT (min)
Scan 25.3 min
576
268
28
281
562
m/z

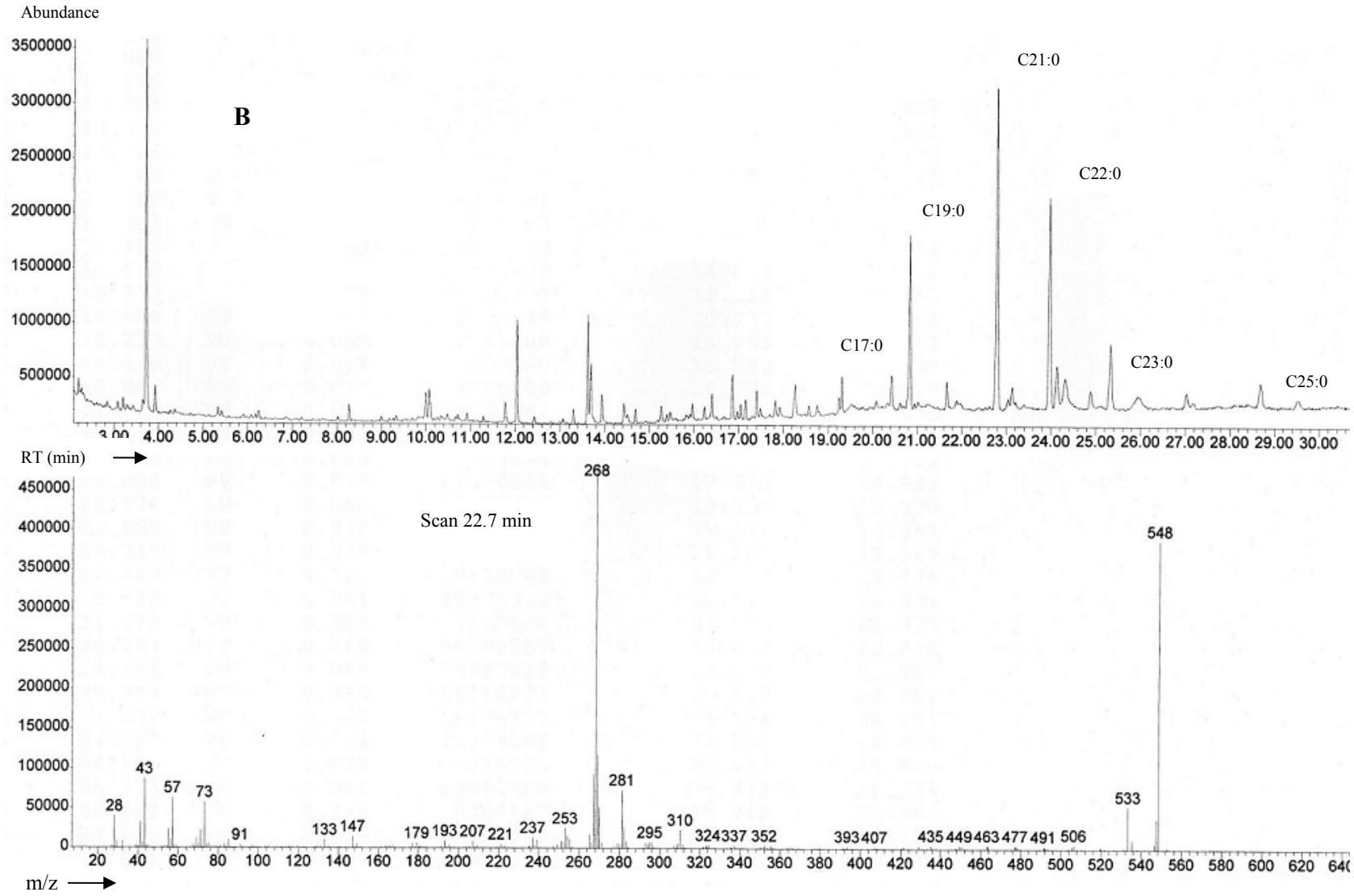


Figure 3-2 GC-MS results of ARs from HRWB before baking (A), and in bread 1D (30%wheat bran plus 2% crude ARs extract) (B)

Table 3-2 Total AR content (mg/100 g), % saturated (Sat), unsaturated (Unsat) and homologue (C15-C25) ARs in wheat bran, bran-residue and white flour.

Sample [*]	Extraction solvent	Total-ARs [**]	Sat.AR	Unsat.AR	C17	C19	C21	C23	C25
HRWB	Acetone	282.6 ± 3.9 [a]	94.1 ± 0.7 [ab]	5.9 ± 0.7 [b]	2.0 ± 0.1 [de]	25.3 ± 1.6 [b]	53.2 ± 2.1 [bc]	9.6 ± 0.24 [def]	3.9 ± 0.1 [ef]
HRWB	Ethyl acetate	241.8 ± 0.7 [b]	91.8 ± 1.5 [ab]	8.2 ± 1.5 [b]	1.8 ± 0.2 [ef]	24.4 ± 0.4 [b]	51.5 ± 0.8 [bcd]	9.6 ± 0.3 [def]	4.4 ± 0.7 [def]
HRWB	1-propanol	195.8 ± 0.9 [d]	93.1 ± 0.8 [ab]	6.9 ± 0.8 [b]	2.1 ± 0.1 [d]	26.1 ± 0.5 [b]	49.0 ± 0.6 [cdef]	10.7 ± 0.1 [def]	5.0 ± 0.47 [de]
HRWB	Ethanol	109.5 ± 4.0 [f]	95.6 ± 0.2 [ab]	4.4 ± 0.2 [b]	1.8 ± 0.1 [ef]	23.3 ± 0.0 [b]	56.1 ± 0.6 [a]	6.8 ± 3.6 [f]	7.5 ± 3.4 [b]
SRWB	Acetone	241.0 ± 2.1 [b]	93.4 ± 0.6 [ab]	6.6 ± 0.6 [b]	2.2 ± 0.2 [d]	25.0 ± 0.9 [b]	52.3 ± 1.4 [bcd]	9.8 ± 0.6 [def]	4.0 ± 0.3 [def]
SRWB	Ethyl acetate	218.5 ± 4.6 [c]	92.2 ± 0.3 [b]	7.8 ± 0.3 [b]	1.7 ± 0.1 [ef]	23.3 ± 0.0 [b]	53.1 ± 0.2 [bc]	9.8 ± 0.0 [def]	4.2 ± 0.1 [def]
SRWB	1-propanol	187.5 ±10.4 [e]	92.7 ± 0.1 [ab]	7.3 ± 0.1 [b]	1.8 ± 0.1 [ef]	24.7 ± 0.9 [b]	48.9 ± 1.2 [cdef]	11.7 ± 0.1 [def]	5.5 ± 0.3 [cd]
SRWB	Ethanol	68.6 ± 2.3 [g]	92.2 ± 0.2 [b]	7.8 ± 0.2 [b]	3.2 ± 0.1 [b]	31.4 ± 0.4 [a]	45.5 ± 0.4 [ef]	8.5 ± 0.1 [ef]	3.5 ± 0.1 [f]
HRWB-R	Acetone	46.8 ± 3.8 [h]	100 [a]	nd	3.0 ± 0.1 [b]	25.8 ± 0.0 [b]	50.3 ± 0.9 [cde]	14.3 ± 0.2 [cd]	6.6 ± 0.5 [bc]
HRWB-R	Ethyl acetate	36.9 ± 1.1 [i]	100 [a]	nd	2.2 ± 0.4 [d]	24.9 ± 0.3 [b]	50.5 ± 1.3 [cde]	15.0 ± 1.1 [cd]	7.4 ± 0.2 [b]
HRWB-R	1-propanol	34.5 ± 1.5 [ij]	100 [a]	nd	nd	nd	27.4 ± 0.9 [h]	54.9 ± 1.3 [b]	17.7 ± 0.4 [a]
HRWB-R	Ethanol	14.2 ± 0.5 [l]	100 [a]	nd	2.6 ± 0.2 [c]	25.4 ± 0.2 [b]	50.4 ± 1.1 [cde]	14.6 ± 0.6 [cd]	6.9 ± 0.2 [b]
SRWB-R	Acetone	20.1 ± 0.5 [k]	82.6 ± 1.8 [c]	17.4± 0.0 [a]	nd	23.2 ± 5.3 [b]	44.9 ± 9.3 [f]	14.3 ± 2.8 [cd]	nd
SRWB-R	Ethyl acetate	14.2 ± 0.3 [l]	100 [a]	nd	nd	26.4 ± 6.8 [b]	54.8 ± 5.8 [ab]	18.7 ± 1.4 [cd]	nd
SRWB-R	1-propanol	18.9 ± 1.9 [kl]	100 [a]	nd	nd	nd	33.6 ± 1.1 [g]	66.3 ± 1.1 [a]	nd
SRWB-R	Ethanol	4.1 ± 0.1 [mn]	100 [a]	nd	nd	24.8 ± 1.6 [b]	55.9 ± 1.1 [ab]	19.2 ± 0.5 [c]	nd
WF	Acetone	8.6 ± 0.4 [m]	78.1 ± 1.1 [c]	21.9 ± 1.1 [a]	nd	30.4 ± 1.1 [a]	47.5 ± 1.0 [def]	nd	nd
WF	Ethyl acetate	nd [***]	nd	nd	nd	nd	nd	nd	nd
WF	1-propanol	nd	nd	nd	nd	nd	nd	nd	nd
WF	Ethanol	30.2 ± 0.5 [j]	100 [a]	nd	4.8 ± 0.2 [a]	31.1 ± 0.3 [a]	47.4 ± 0.0 [def]	12.0 ± 0.0 [def]	4.6 ± 0.2 [def]

[*] HRWB = hard red wheat bran, SRWB = soft red wheat bran, HRWB-R= hard red wheat bran-residue, SRWB-R = soft red wheat bran-residue, WF= white flour.

[**]Values are means of triplicates ± standard deviations (SD). Values with different letters in columns are significantly different from each other at $P < 0.05$. Correlation of variation (CV %) range is 3.54. [***]nd = not detected.

There was a significant difference ($P < 0.05$) in the total AR content extracted by the four different solvents used in each trial. The highest yield was recorded for acetone, and in addition it was found to be a more selective solvent for AR extracts compared to other solvent systems.[155] For the WB samples, the order of the solvents giving the highest to lowest AR yield was acetone > ethyl acetate > 1-propanol >ethanol.

Although the total AR content was different for each WB sample, the relative % saturated and unsaturated ARs for HRWB remained close to that of SRWB and our findings is comparable to the values reported in literature.[34,35,140]

It has been suggested that ARs may have some inhibitory effects on metabolic enzymes. The length of the alkyl chain affects the extend of inhibition, with C17:0 and C19:0 being the most effective inhibitors.[94] ARs were also able to prevent triglyceride accumulation in cultured 3T3-L1 cells, indicating that they may inhibit triglyceride synthesis *in vivo*.[94] It has been reported by Ross et al.(2004) that wheat bran has minor AR derivatives including ARs with unsaturated alkyl chains, keto groups, or a combination of both keto groups and unsaturated alkyl chains.[34] These minor AR derivatives are claimed to have many specific biological activities that are related to their amphiphilic characteristics.[157,158] Unsaturated ARs may affect cell membrane permeability and prevent LDL oxidation in humans, similar functionality to polyunsaturated fatty acids.[159] A thorough explanation of the chemistry, nutritional effects, and bioactivities of ARs is discussed in more detail in reviews by Kozubek and Tyman[33] and Ross et al.[34] The unsaturated peak values were assigned according to the MS data obtained from each GC-MS run and then compared with literature data.[34] Further studies need to be carried out on whether ARs have any bioactivity in higher

mammals.

Acetone extracts showed significantly higher ($P < 0.05$) AR content and both of the wheat bran samples had very close AR homologue composition. The order of individual AR homologues from highest to lowest was C21:0 > C19:0 > C23:0 > C25:0 > C17:0. In this study, the relative AR homologue composition for both WB samples was determined to be 1.8-3.2 % for C17:0, 23.3-31.4% for C19:0, 45.5-56.1 % for C21:0, 6.8-10.7 % for C23:0, and 3.5-7.5 % for C25:0. Our findings were close to those ranges and C21:0 and C19:0 were the dominant AR homologues in the WB samples which is in agreement with other reports in literature such as those of Knodler et al.[145] Meanwhile, for the residue brans, the homologue composition was not similar before and after extractions. Some AR homologues such as C17:0 and C25:0 were not found in the extraction of the residue brans. Both of these homologues were also found in the lowest amounts in regular bran. The difference in AR content between extracted and regular bran was high and our findings were parallel to the work of Hedkvist.[160] In the study, they also found ARs in the acetone extract of residue rye bran (41-57 mg/100g).[160]

The WF samples yielded only a small amount of ARs, 8.6 mg/100g in acetone and 30.2 mg/100g in ethanol extracts (Table 3.2) that was consistent with the report by Hengtrakul et al.[144] The low residual amounts might due to improper separation of the bran, resulting in having ARs in the white flour sample.[144]

Table 3-3 Total content (mg/100 g), saturated (Sat), unsaturated (Unsat) and homologue composition (%) of ARs in all bread trials

Bread Coding*	Total AR **	Sat.AR	Unsat.AR	C17:0	C19:0	C21:0	C23:0	C25:0
A	1.1 ± 0.6 ^{f}	100 ^{a}	nd***	nd	nd	100^{a}	nd	nd
1B	51.4 ± 0.1 ^{c}	90.6 ± 0.2 ^{c}	9.4 ± 0.2 ^{a}	2.1 ± 0.8 ^{a}	23.1 ± 0.5 ^{c}	45.4 ± 1.7 ^{f}	13.3 ± 0.3 ^{b}	6.8 ± 0.3 ^{b}
1C	7.7 ± 1.2 ^{e}	100 ^{a}	nd	nd	27.6 ± 0.3 ^{b}	72.4 ± 0.3 ^{b}	nd	nd
1D	82.9 ± 2.0 ^{a}	91.3 ± 0.4 ^{b}	8.6 ± 0.4 ^{b}	1.6 ± 0.1 ^{a}	22.2 ± 0.8 ^{d}	49.3 ± 2.3 ^{d}	12.7 ± 0.2 ^{c}	5.6 ± 0.6 ^{c}
2B	39.6 ± 6.3 ^{d}	90.6 ± 0.2 ^{c}	9.4 ± 0.1 ^{a}	0.8 ± 0.3 ^{b}	22.2 ± 0.1 ^{d}	46.4 ± 0.3 ^{ef}	13. 8 ± 0.7 ^{a}	7.3 ± 0.3 ^{a}
2C	9.6 ± 0.5 ^{e}	100 ^{a}	nd	nd	32.5 ± 0.8 ^{a}	67.7 ± 0.6 ^{c}	nd	nd
2D	76.9 ± 0.9 ^{b}	90.8 ± 0.1 ^{c}	9.2 ± 0.1 ^{a}	2.0 ± 0.2 ^{a}	21.7 ± 0.1 ^{d}	47.7 ± 0.1 ^{de}	12.8 ± 0.3^{c}	6.4 ± 0.2 ^{b}

*1 = hard red wheat bran (HRWB), 2 = soft red wheat bran (SRWB), A = control, B =30% non-extracted bran added, C = 30% extracted bran added, D = 30% non-extracted bran + 2% crude ARs extract added.

**Values are means of triplicates ± SD. Values with different letters in columns are significantly different from each other at ($P < 0.05$). CV % is 7.15.

**nd= not detected

3.4.2 AR content and homologue composition in bread samples

In this study, four different solvents including acetone, ethanol, ethyl acetate and 1-propanol, were used to extract ARs from wheat bran samples but in order to extract ARs from wheat bran and add to bread samples, ethanol was used since it is the only food grade solvent that commonly applies in food industries. On the other hand, a mixture of hot propanol and water was used to extract ARs from bread samples. Ross et al.(2003) used different solvents to extract ARs from breads containing rye bran and showed that a mixture of hot propanol and water was the best solvent to extract ARs completely with high yield and release the ARs bound from starch-lipid complexes. [38] The total AR and related homologue content of all breads trials were analyzed using GC-MS (Figure.3.2B) and the results are shown in Table 3.3. All breads tested in this study, contained ARs and it was shown that AR was not degraded during baking which is in agreement with literature [38,52] as was expected.[34 42]

A significant difference ($P < 0.05$) was seen in the total amount of ARs yielded from the WB samples. The highest amount of ARs yielded from the trials was from bread D, followed by B, C, and A which was the control that was expected to have the lowest yield.

Meanwhile, when it came to the homologue AR composition in breads, B and D had very similar values before the baking was carried out. Interestingly, after the baking process, the homologue composition of breads B and D had hardly been affected as there was only a slight decrease in C21:0 and C19:0 while there was an actual increase in C23:0 and C25:0 in bread B. However, the AR homologue content of breads A and C did change after baking.

Overall, the homologue ARs content had not been significantly altered for both WB samples during baking and only a slight decrease was observed in C21:0 and C25:0 content of bread D, as opposed to an increase in its C23:0 homologue content.

3.4.3 Effects of ARs on bread physical quality

After baking, the breads were cooled to room temperature under a baking cloth for around 1 h. Then the inner temperatures, heights, and weights of breads were recorded. All treatments were significantly different ($P < 0.05$) in temperature, height and weight from control bread (Table 3.4). The height differences of bread B (containing WB) and C (containing residue bran) were not significant ($P > 0.05$). Meanwhile bread D (containing highest amount of ARs) showed a significant difference ($P < 0.05$) in height in comparison with B and C breads (Table 3.4). This study suggests that ARs may have potential in bread manufacturing by improving the quality of bread. The control bread (A) had the lowest inner temperature (35.6 ± 1.3 °C) and greatest height (6.8 ± 0.1 cm) amongst all trials. This was expected since the control bread didn't include any bran as an ingredient. Apart from the control bread, breads 1D and 2D had the greatest heights of 5.80 and 5.40 cm, respectively. Upon an overall comparison, it was acknowledged that the breads with the highest amount of ARs were also the most elevated and raised trial breads; the ranking from highest to lowest for both HRWB and SRWB is D > B = C. There was a positive correlation between bread heights and AR content ($R^2 = 0.90$). The images of each of the bread slices are shown in Figure 3.3.

Table 3-4 Effects of ARs on bread physical quality: The inner temperature, height and weight of bread samples after 1 h cooling.

Bread trials*	Inner temperature (°C)	Height (cm)	Weight (g)
A	35.6 ± 1.3**d	6.8 ± 0.1 a	89.3 ± 0.1 c
1B	43.9 ± 0.3 ba	5.1 ± 0.2 d	96.7 ± 0.1 a
1C	44.9 ± 0.9 a	4.8 ± 0.1 d	96.7 ± 0.1 a
1D	43.7 ± 0.9 ba	5.8 ± 0.2 b	96.7 ± 0.1 a
2B	40.4 ± 0.2 c	4.9 ± 0.2 d	94.7 ± 0.1 b
2C	42.4 ± 0.4 b	4.8 ± 0.1 d	96.7 ± 0.0 a
2D	42.8 ± 0.3 b	5.4 ± 0.1 c	96.7 ± 0.0 a

*1 = hard red wheat bran (HRWB), 2 = soft red wheat bran (SRWB), A =control, B=30% non-extracted bran added, C = 30% extracted bran added, D= 30% non-extracted bran + 2% crude ARs extract added.

**Values are means of triplicates ± SD. Values with different letters in columns are significantly different from each other at ($P < 0.05$). CV% for temperature, height and weights are 1.93, 0.98, and 50 respectively.

A 1B 1C 1D

A 2B 2C 2D

Figure 3-3 Breads baked with 100% white flour (A), 30% non-extracted wheat bran (1B, 2B), 30% extracted wheat bran (1C, 2C) and 30% non-extracted wheat bran and 2% crude ARs extract added (1D, 2D)

1= hard red wheat bran (HRWB) and 2 = soft red wheat bran (SRWB)

Thus this specific method of AR extraction and its addition to the dough, made a significant difference perhaps due to the chemical behavior of these lipids that were added to the dough. These results showed that bread height did increase upon changing the amount of AR content. ARs are amphiphilic phenolic lipids due to the polarity of the dihydroxybenzene group and the hydrophobic alkyl carbon chain. ARs with amphiphilic properties, like emulsifiers, can act as dough softeners, changing the gelatinization behavior of starch by making complexes with amylose and as a result delay water penetration which has also been suggested to occur during baking.[46] They also have the ability to travel to interfaces between two physical phases and thereby lower surface tension and form dispersions. Moreover, ARs have been suggested to form amylose-lipid complexes during baking as mentioned by Ross et al. [38] This might explain the effects ARs have on the baking quality of yeast-leavened wheat breads.

3.4.4 TPC

TPC was expressed as milligrams of ferulic acid equivalents per gram of sample (mg FAE/g) and the results of these measurements are shown in Table 3.5. The aim was to compare whether the phenolic content of the wheat bran had been affected during baking. For this reason, all bread ingredients, HRWB, SRWB, HRWB-R, SRWB-R and WF were studied in addition to seven types of bread trials. Before the baking trials, TPC values of both wheat bran samples (HRWB and SRWB) were significantly different ($P < 0.05$) among residue brans and WF. Statistical analysis showed that TPC values of HRWB was 1D > 1B = 1C and SRWB was 2D = 2B = 2C.

Table 3-5 ORAC, % DPPH, and TPC values of wheat bran, bran-residue, white flour and bread samples.

Sample*	ORAC	% DPPH	TPC
Bran			
HRWB	104.5 ± 0.8 b**	29.8 ± 3.2 a	4.9 ± 0.3 a
SRWB	112.5 ± 7.4 a	26.8 ± 1.0 a	4.5 ± 0.1 a
HRWB-R	33.2 ± 1.6 d	21.0 ± 2.1 b	2.0 ± 0.0 c
SRWB-R	33.4 ± 1.0 d	28.5 ± 0.7 a	2.2 ± 0.8 bc
WF	30.6 ± 7.9 ed	7.1 ± 2.21 d	1.2 ± 0.9 c
Bread			
A	13.5 ± 1.1 g	15.0 ± 0.1 c	1.4 ± 0.3 c
1B	28.2 ± 1.1 ed	20.2 ± 1.7 b	2.0 ± 0.4 c
1C	34.8 ± 4.4 d	19.6 ± 1.0 b	1.5 ± 0.3 c
1D	114.6 ± 5.1 a	29.9 ± 0.7 a	4.8 ± 0.1 ba
2B	18.9 ± 2.0 gf	20.7 ± 3.7 b	2.2 ±0.2 c
2C	24.8 ± 0.4 ef	22.8 ± 1.9 b	1.7 ± 0.2 c
2D	71.9 ± 1.7 c	27.3 ± 0.9 a	3.2 ± 1.6 bc

ORAC = Oxygen Radical Absorbance Capacity as μmole TE (Trolox Equivalent)/g

DPPH= 1, 1-diphenyl-2-picrylhydrazyl as %DPPH inhibition

TPC=Total Phenolic Content as mg Ferulic Acid Equivalent (FAE) /g

*1 = HRWB (hard red wheat bran), 2 = SRWB (soft red wheat bran), R = residue. A = control, B = 30% non-extracted bran added, C= 30% extracted bran added, D = 30% non-extracted bran + 2% crude ARs extract added

**Values are means of triplicates ± SD. Values with different letters in columns are significantly different from each other at $P < 0.05$. CV % ranged between 9.63% and 16.92%.

The results also indicated that the effects of heat treatment on TPC of two WB samples was limited and less than 1 mg/g which is in agreement with the work of Li et al.[161] In wheat bran, phenolic compounds are the key contributors to the total in *vitro* antioxidant capacity and thus a high TPC reflects a high total antioxidant activity. It is noteworthy to mention that during the extraction process, it is possible to have slight hydrolysis of polyphenolic compounds due to acidic extracting conditions thus the higher antioxidant activity.[161]

3.4.5 Antioxidant activity by ORAC

An ORAC assay is used to measure the relative potential of antioxidants which prevent biological molecules from free radical attacks.[143,161] High ORAC values indicate high antioxidant capacity in a sample extract. It has been reported by Li et al[161] that the ORAC values of extracts from bran and bran-containing muffins ranged from 3.0 to 25.3 μmole TE/g. In addition, another study[150] determined that the ORAC values of the free fractions of six diverse whole wheat ranged from 19.5 to 37.4 μmole TE/g. Meanwhile, the ORAC values obtained in our study were higher than both of the studies mentioned above. Furthermore, a significant correlation ($P < 0.05$) was found between TPC content and the ORAC values ($R^2 = 0.90$). Thus perhaps, the phytochemicals found in whole grains or specifically in wheat bran could be the major factors and main role players responsible for the health benefit of whole grain consumption.

The ORAC values of all samples in this study ranged between 13.5 to 112.5 μmole TE/g (Trolox equivalent) as shown in Table 3.5. There was significant difference ($P < 0.05$) among sample values as SRWB had higher ORAC values than HRWB. Bread D had the highest ORAC values in all of the studied bread trials. Therefore, the breads

which contained ARs had higher ORAC values than the control breads with bread D having significantly higher values than breads B and C.

3.4.6 Antioxidant activity by %DPPH

The %DPPH is based on the measurement of the reducing ability of antioxidants and is calculated as a percentage discoloration with a higher percentage suggesting a higher antioxidant capacity.[114] The %DPPH scavenging activity of all samples studied during baking is shown in Table 3.4 and ranged from 7.1 to 29.9. Korycinska et al[136] reported that the %DPPH of whole grain products should be between 7% to 43% indicating those values were in agreement with findings from this study. The control breads had the lowest %DPPH compared to all other bread trials. Bread D contained the highest amount of ARs which also had the highest antioxidant activity compared to the other bread types. The ranking order of %DPPH inhibition values for WB samples was D > B = C. The antioxidant activity of WB samples was significantly different from WF ($P < 0.05$). Interestingly, there were no significant differences between the antioxidant activity of WB and SRWB containing residue ($P < 0.05$). This suggests that the presence of other components in bran might affect the antioxidant activity of the above samples. Additionally, the total phenolic components in WB are approximately 3g/100g[30] which is almost 50 to 60 times higher than its corresponding total ARs (32 to 100 mg/100g).[46] This is likely why the antioxidant activity of WB and SRWB containing residue remained the same. The ORAC assay is a method that can mimic antioxidant activity of phenols in biological systems, making it a more ideal method for antioxidant measurement[143]. Unlike DPPH results, ORAC showed a difference between the antioxidant activity of WB and SRWB containing residue ($P < 0.05$) (Table 3.5).

3.5 Conclusion

In this study, seven bread trials were conducted with the same baking conditions, using two different wheat bran samples to determine whether AR contents and antioxidant capacity changed. The results showed that naturally occurring wheat bran ARs were heat stable during baking. The breads with the highest amount of ARs were also the most elevated and raised trial breads. Furthermore, TPC results showed a positive correlation with AR content (R^2 = 0.75, P < 0.05). Additional studies are necessary to determine the optimum amount of ARs that should be added in bread or other food model systems.

3.6 Connecting Statement to Chapter 4

In Chapter 3, the stability of natural levels of ARs in baked breads have been investigated by analyzing and comparing total and homologue AR levels using GC-MS. Also, the study aimed to determine how the baking process affects the TPC and the antioxidant activity of ARs by measuring ORAC and DPPH.

In Chapter 4, the characterization of wheat bran ARs by HPLC was studied for different wheat cultivars. The extraction process included both of traditional and supercritical fluid extraction. Also, this chapter gives comparison of antioxidant measurement of wheat bran extracts by different solvent systems.

The work presented in Chapter 4 will be submitted for a publication.

Chapter 4 Comparison of traditional and supercritical carbon dioxide extractions of ARs, their homolog profile, and bioactivity

4.1 Abstract

Supercritical carbon dioxide (SC-CO_2) extraction is a non-toxic, non-flammable, low-cost, and environmentally friendly technology which has gained increased interest in the food industry. The SC-CO_2 and traditional organic solvent methods were used to extract wheat bran (WB) ARs from different cultivars. The AR content and profiles were obtained using RP-HPLC and PDA detection. Alkaline hydrolysis and liquid-liquid partitioning steps were carried out to examine ester linked phenolics in WB fractions with soluble free, soluble conjugated and bound forms. Also, the effects of extraction solvents on TPC, DPPH and ORAC were investigated by using three different solvents containing acidified ethanol, 50% and 100% acetone. Results showed that the AR content was higher in acetone extracts compared to SC-CO_2 extracts. The most dominant AR homologue was C21:0, followed by C19:0, C23:0, C17:0 and C25:0. HPLC analysis of phenolics from different WB fraction identified ten phenolic acids of which ferulic acid was the most predominant phenolic acid and mostly found in bound fractions. The same fractions were analyzed for flavonoid contents and six flavonoids were identified with rutin being the predominant one. Two-way ANOVA showed that cultivar, solvent type and interactions of the two, had significantly different effects on TPC, DPPH, and ORAC values ($P < 0.05$). The mean ORAC and DPPH values were highest in the acidified ethanol extracts, whereas the highest TPC values were obtained from the 50% acetone extracts.

4.2 Introduction

Consumption of cereal grain and the risk of chronic diseases are inversely related.[162] These health benefits might be attributed to the antioxidant capacity of phenolic compounds in cereals such as ARs and phenolic acids. [163] Traditionally, ARs have been extracted by different organic solvents including acetone, ethanol, and ethyl acetate.[36,79,164] The SC-CO_2 extraction technique is very attractive in comparison to traditional organic solvent procedures because of its non-toxic, non-flammable, and environmentally friendly properties.[35] SC-CO_2 can be used as an extraction solvent to extract ARs, and the efficiency of this extraction method depends on the pressure, temperature, and co-solvents used.[66] Additionally, it has a faster extraction time than traditional techniques and the CO_2 is safe and easy to remove after the extraction is complete. The use of SC-CO_2 technology has been well established for the extraction of many different food products, including essential oils.[67] It has also been used to extract ARs from wheat and rye bran at optimum conditions of 70 ºC and 35 MPa with a flow rate of 25 g/min for 4 h followed by using 10% ethanol as a co-solvent/modifier.[35] Due to its non-polar nature, pure CO_2 does not solubilize the polar ARs. For this reason, ethanol has been suggested as a co-solvent when using SC-CO_2 for the removal of ARs from wheat and rye bran. Previous studies have found SC-CO_2 to yield 8-80% more ARs than the traditional extraction using acetone.[66]

Phenolic acids are a predominant group of phenolic antioxidants and can be classified into hydroxybenzoic acids and hydroxycinnamic acids. Ferulic acid is the major phenolic acid of hydroxycinnamic acid derivatives and found esterified or etherified to cereal cell wall components.[165] Polyphenolic (PP) compounds may exist in

free or esterified/etherified soluble and insoluble form bound to cell wall constituents like polysaccharides, protein, lignin, cutin or suberin. Insoluble polyphenolic compounds are considered the major contributors to the total antioxidant capacity of cereals. [115] Most literature data on food, and related biological, nutritional, and epidemiological studies address extractable PP antioxidants.[115,166,167] However, the analytical methods do not determine the amount of PP remaining in the residues of aqueous organic extraction, named as non-extractable polyphenols, and there is a lack of information in literature on this group of antioxidants. Analytical methodology for cereal PP consists of an extraction with aqueous-organic solvents to obtain soluble polyphenols, followed by a hydrolysis treatment to liberate the bound form. Since acidic hydrolysis might degrade hydroxycinammic and benzoic acids, alkali hydrolysis is the main procedure usually performed in the aqueous-organic extracts for ester bond cleavage.[115] The major non-extractable PP in DF in the diet are ferulic acid, caffeic acid, hesperidin, naringin, catechin, epicatechin, ellagic acid, gallic acid derivatives, protocatechin, and *p*-hyrdoxybenzoic acid.[168] Many study reported the influence of different extraction solvents on the content of natural antioxidants in extract.[169] Solvents such as acetone, methanol, ethanol, propanol and ethyl acetate have been commonly used for the extraction of phenolics from plants.[170]

The objectives of the present work were; I) to investigate feasibility of SC-CO_2 for extracting ARs from wheat bran and compare the SC-CO_2 method with the traditional solvent extraction method of ARs; II) to evaluate the effects of extraction solvents on the antioxidant potential of WB samples by measuring TPC, ORAC and DPPH values; and III) to characterize phenolic fractions as soluble free, soluble conjugated and bound form

in WB.

4.3 Materials and methods

4.3.1 Materials

Seven different WB samples were used for this study, including two winter and five spring cultivars (Table 4.1). The winter wheats, hard red wheat bran (HRWB) and soft red wheat bran (SRWB) were obtained from Kraft-Canada. The rest were from Agriculture and Agri-Food Canada (AAFC) breeding programs and were hard spring wheat. The main difference in the samples from the breeding program was the bran color. Win B and Win A were sister lines and they had white bran color as Snowbird, a registered cultivar. The other two, AC Brio and AC Tahoe were also registered but they had red bran color and usually AC Brio was harder than AC Tahoe. The two genetic lines (Win A and Win B) and AC Brio were from eastern Canada while Snowbird and AC Tahoe was from the Winnipeg program. All chemicals used for extractions and analyses were as described in the section 2.3.2.

Table 4-1 Detailed information about all WB samples used for this study

Sample	Source*	Season	Hardness	Colour
SRWB	Kraft Canada	winter	soft	red
HRWB	Kraft Canada	winter	hard	red
AC Brio	AAFC-eastern Canada	spring	hard	red
AC Tahoe	AAFC-Winnipeg	spring	hard	red
Snowbird	AAFC-Winnipeg	spring	hard	white
Win A	AAFC-eastern Canada	spring	hard	white
Win B	AAFC-eastern Canada	spring	hard	white

* AAFC = Agriculture and Agri-Food Canada

4.3.2 Extraction of ARs

ARs were extracted using a traditional solvent extraction method, and the green/environmentally friendly SC-CO_2 method. The traditional solvent extraction of ARs has been already described in section 2.3.3.

The SC-CO_2 extraction was performed in a SFE-1000F-2-FMC50 system, from Thar Technology Inc., Pittsburg, USA. It consists of two pumps, one for CO_2 and the other for a co-solvent, an automated back pressure regulator (ABPR), an extraction vessel and two collector vessels (Figure 4.1). The SC-CO_2 extraction parameters were chosen based on published studies as summarized in Table 4.2. Ground wheat bran samples (HRWB and SRWB; 150 g) were placed in the extraction vessel and CO_2 was pumped at 25 g/min (flow rate). The pressures of extraction, collector-1 and -2 vessels were set at 350, 120, and 60 bars respectively. The temperatures of extraction, collector-1 and -2 vessels were set at 70, 45 and 35 ˚C respectively. The extractions were performed for 6 h, including 2 h with 100% SC-CO_2 and 4 h with a co-solvent added (10% ethanol). The extracts obtained with co-solvent were further evaporated and stored at -20 ˚C for further analysis. The AR extraction was made in triplicates.

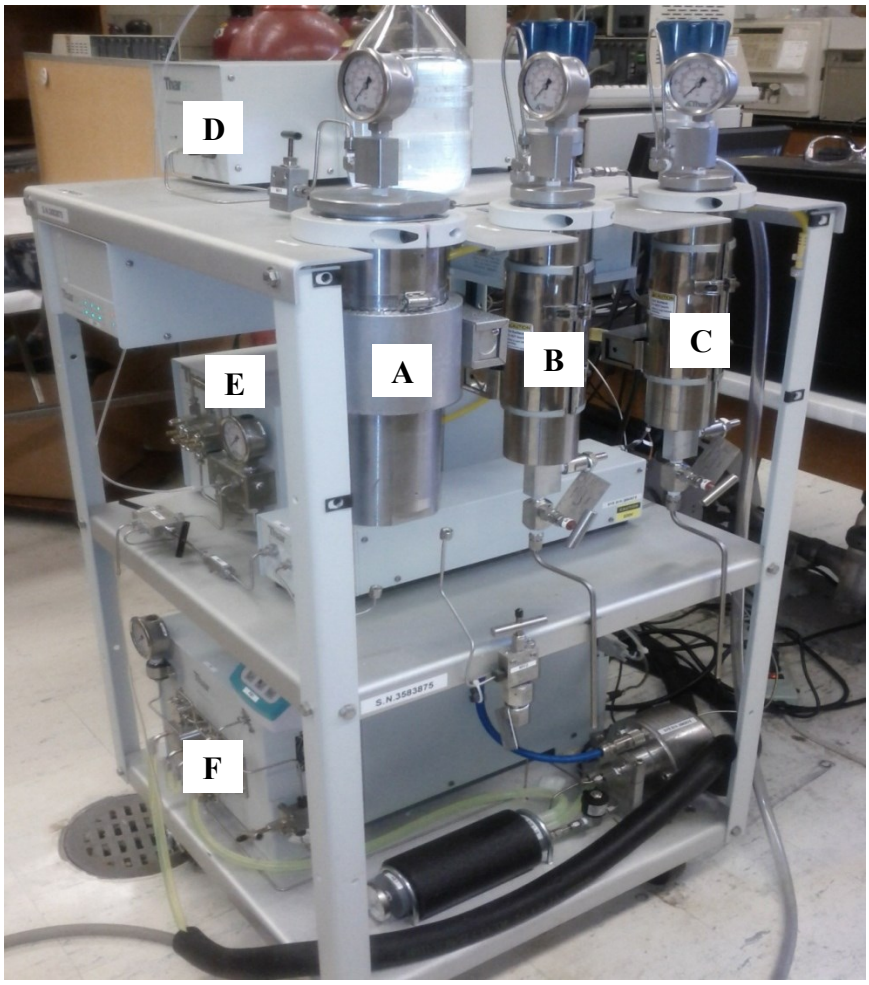


Figure 4-1 SC-CO_2 extraction instrument in Dr. Hosseinian's research lab at Carleton University. The letters A, B, C, D, E and F represent the extraction vessel, collector-1, collector-2, automated back pressure regulator (ABPR), co-solvent pump, and CO_2 pump, respectively.

Table 4-2 Comparison of SC-CO2 parameters for ARs extraction of cereal bran

Parameters	Landberg et al.[68]	Francisco et al.[66]	Athukorala et al.[35]
Sample weight(g)	20	25	100
Extraction temperature (ºC)	70	40, and 55	70
Extraction pressure (bar)	350	80, 150, 300 and 350	350
Extraction time (h)	6	2	2, 4, 6, 8, 10
CO2 flow (g/min)	5	5	25
% Co-solvent (ethanol)	10	10	10

4.3.3 Different solvent extractions for antioxidant activity measurements

Three different solvents were used to extract phenolic compounds from WB samples and compare solvent effects on the antioxidant activity measurements. The extraction solvents were 100% acetone, 50% acetone (in water), and acidified ethanol (95% ethanol: 1N HCl (85:15, v/v)). The first two extractions were performed with a 1:4 ratio (w/v) for 24 h,[140] and the last one was performed with a 1:15 ratio (w/v) for 6 h (section 3.3.4.1).[171] The resulting supernatant fluid was then stored at -20°C for further analysis (TPC, DPPH and ORAC). All analyses were performed in triplicates.

4.3.4 Fractional extraction (soluble free, soluble conjugated, and bound phenolics)

HRWB and SRWB phenolic compounds (phenolic acids and flavonoids) were extracted according to the modified method of Kim et al.[172] Soluble free, soluble conjugated and bound phenolics were individually extracted in three separate fractions as shown in Figure 4.2. Soluble free phenolics were extracted twice from defatted bran samples by mixing with 80% methanol (1/10, w/v ratio) for 1 h at room temperature (RT)

and centrifuged at 3900 g for 15 min. The combined supernatants were driedthen re-dissolved in water and acidified to pH 2 by 12 M HCl. The soluble free phenolics fraction was extracted twice by ethyl ether (1/1, v/v), dried then re-dissolved in methanol for HPLC analysis. The second extract fraction was assigned as soluble conjugated phenolics. The extraction was started the same way for free fraction however after drying the supernatant, samples were re-dissolved in 2 M NaOH and agitated at RT for 4 h to hydrolyze and release the conjugated phenolics. After hydrolysis, the acidification and ethyl acetate extraction steps were followed the same as in the extraction of soluble free phenolics steps. Finally, the bound phenolics fraction was obtained by re-dissolving the dried residue from the first extraction (crude phenolics extraction) in 2 M NaOH (1:40, w/v) and agitating for 4 hour at RT followed by acidification and ethyl acetate extraction. The dried extracts were re-dissolved in MeOH for the determination of phenolic acid and flavanoid content. Extracts were filtered with a 0.45 μm PTFE filter before HPLC analysis.

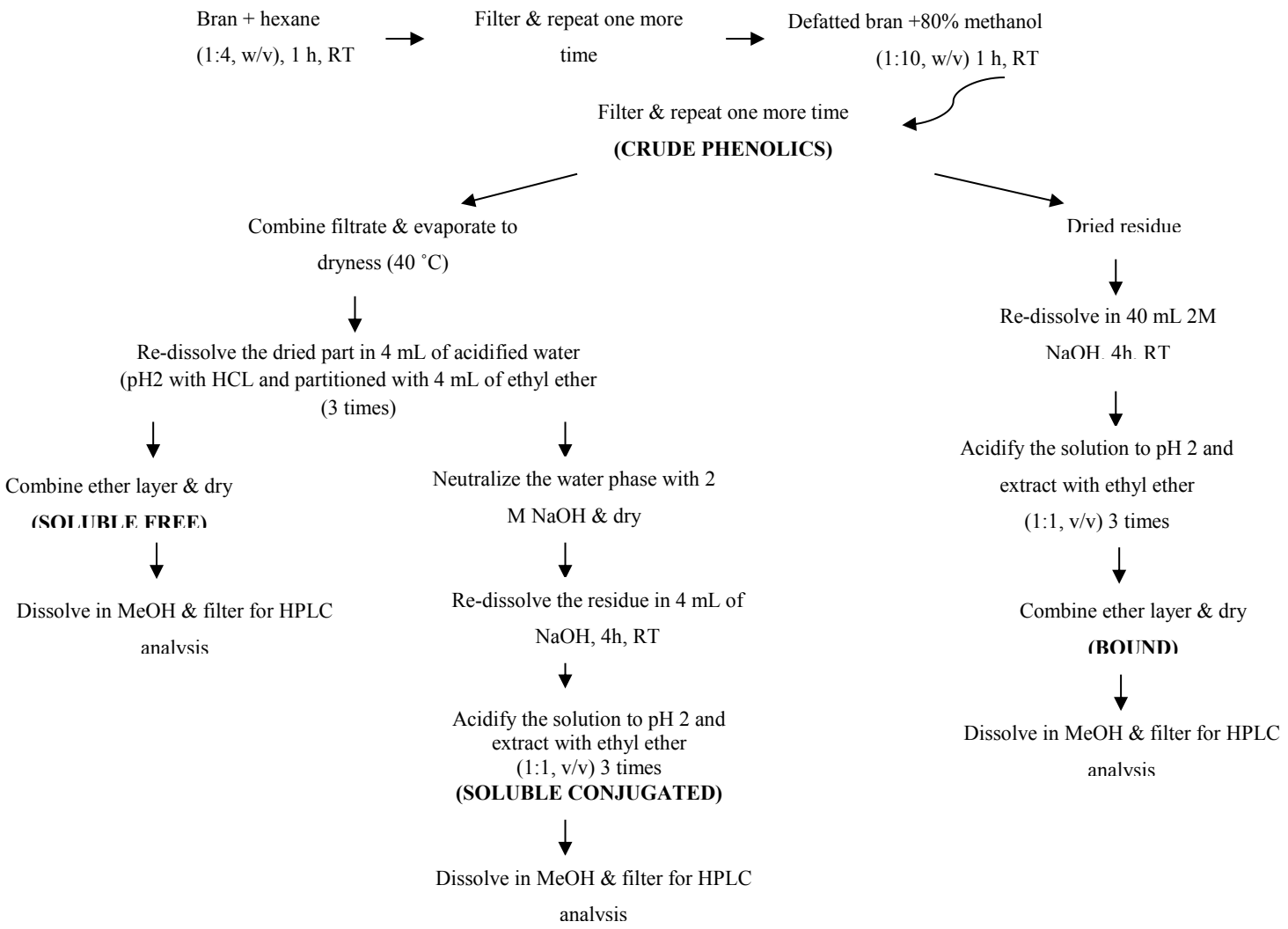


Figure 4-2 Soluble free, soluble conjugated and bound phenolic acid extractions procedures (modified from Kim et.al).[172]

4.3.5 HPLC analysis

Both traditional and SC-CO_2 crude extracts of ARs, and fractional extracts of phenolics were analyzed via a reverse phase (RP)-HPLC on the Alliance® HPLC system e2695 Separation Module with the 2998 Photodiode Array Dedector (PDA) from Waters (Mildford, Massacchusetts, USA). The software used was Empower 3. The details about HPLC run parameters are explained in the following sections.

4.3.5.1 HPLC analysis of ARs

The standards of ARs as a mixture of AR homologues C15:0-C25:0 and all sample crude extracts of ARs were prepared in methanol. A gradient program followed to separate the different homologues at a flow rate 1 mL/min and UV detection at 280 nm with a RP column (Synergi-Max-RP, 250 x 4.6 mm, 5 μm) a temperature set at 35 ˚C. Solvent A was 1% acetic acid in MeOH and solvent B was 2% acetic acid in water. The gradient program was summarized in Table 4.3.

Table 4-3 The gradient program for HPLC analysis of ARs

	Time	Flow	% A	%B
1	00.00	1.00	90.0	10.0
2	10.00	1.00	100.0	00.0
3	25.00	1.00	100.0	00.0
4	35.00	1.00	90.0	10.0
5	50.00	1.00	90.0	10.0

4.3.5.2 HPLC analysis of phenolics

Phenolic acid and flavonoid standards, samples, and fractional extracts were prepared in methanol. A gradient program was developed to separate phenolic acids and flavonoids in a single run with a Synergi-Max-RP column, at 35 ˚C. Solvent A was 0.01% formic acid/water and solvent B was 100% acetonitrile at a flow rate of 1.0 mL/min and a gradient program as shown in Table 4.4. The wavelengths used for qualitative and quantitative analysis for phenolic acid was 280 nm, and for flavonoids was 280 and 320 nm. Also, spectral data at 200-700 nm were recorded for all sample and standard. For all 11 phenolic acid and 10 flavonoid standards (Table 4.8), five different concentrations were prepared as a mixture of standards. Standard curves of each phenolic acid and flavonoid mixture were plotted using the corresponding peak areas. For all standard curves, the regression value ranged between 0.9956 and 1.0000. All samples were analyzed in duplicate and concentration of each phenolic acid and flavonoids was calculated using a standard curve equation and expressed in mg/g of wheat bran samples.

Table 4-4 The gradient program for HPLC analysis of phenolic acid and flavonoids

	Time	Flow	% A	%B
1	00.00	1.00	90.0	10.0
2	35.00	1.00	50.0	50.0
3	40.00	1.00	90.0	10.0
4	50.00	1.00	90.0	10.0

4.3.6 Antioxidant activity measurements

All wheat bran extracts from three different solvents (section 4.3.3) were analyzed for measuring TPC, DPPH, and ORAC values. The TPC, DPPH and ORAC measurements were performed by following the methods described in the sections 2.3.5, 2.3.6, and 2.3.7, respectively.

4.3.7 Statistical analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used to determine if there was a significant difference ($P < 0.05$) among triplicates of total and homologue of ARs for both traditional and SC-CO_2 extractions. Significant treatments were further analyzed with the Duncan's multiple range test ($P < 0.05$). Also a two-way analysis of ANOVA was performed to see if there was significant difference ($P < 0.05$) between ORAC, DPPH and TPC data sets of different solvents.

4.4 Results and discussion

4.4.1 AR content and homolog composition by traditional method

The total ARs and homologue contents (mg/100g) of different wheat cultivars extracted by acetone are presented in Table 4.5. The saturated ARs were confirmed in comparison by the retention times of AR standards (C15:0-C25:0) as shown in the Figure 4.3.

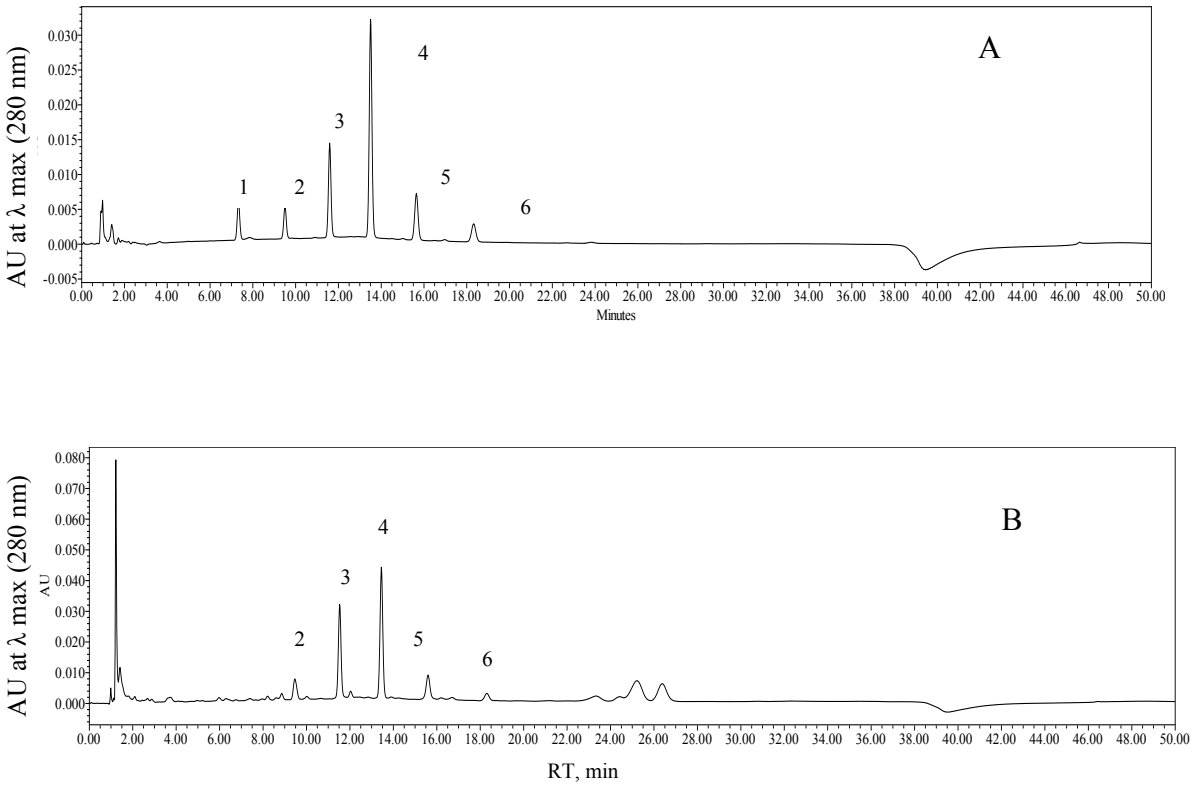


Figure 4-3 HPLC chromatograms of ARs standard mixture (A) and HRWB sample (B).The numbers represent each ARs homologue including C15:0 (1), C17:0 (2), C19:0 (3), C21:0 (4), C23:0 (5), and C25:0 (6)

Table 4-5 Total ARs and homolog contents (mg/100g WB) of different wheat cultivars extracted by acetone

Composition	HRWB	SRWB	AC Brio	AC Tahoe	Snowbird	Win A	Win B
Total- ARs	447.8 ± 10.1 [a]	305.8 ± 8.2 [b]	285.0 ± 12.3 [b]	258 ± 11.8 [c]	245.9 ± 9.0 [c]	192.3 ± 7.7 [d]	144.0 ± 2.0 [e]
C15:0	nd	nd	2.3 ± 0.1[b]	nd	10.7 ± 0.4 [a]	nd	nd
C17:0	44.0 ± 0.4 [a]	19.5 ± 1.5[ed]	34.3 ± 1.9 [b]	28.8 ± 2.8 [cb]	24.2 ± 0.1 [cd]	12.9 ± 0.9 [ef]	10.7 ± 3.8 [f]
C19:0	138.1 ± 5.1 [a]	92.9 ± 6.6 [b]	80.5 ± 1.0 [cb]	76.0 ± 4.5 [c]	79.2 ± 4.5 [cb]	59.9 ± 6.4 [d]	44.6 ± 3.2 [e]
C21:0	193.6 ± 2.5 [a]	136.4 ± 4.1 [b]	113.5 ± 6.3 [c]	92.2 ± 6.0 [d]	85.6 ± 5.9 [ed]	80.1 ± 3.5 [e]	58.9 ± 4.4 [f]
C23:0	53.0 ± 1.2 [a]	44.6 ± 1.7 [ba]	37.1 ± 2.2 [bc]	13.8 ± 4.9 [d]	26.5 ± 5.0 [dc]	28.4 ± 3.4 [dc]	23.5 ± 1.7 [dc]
C25:0	19.1 ± 0.9 [a]	12.9 ± 2.7 [b]	17.1 ± 2.9 [b]	47.4 ± 4.0 [a]	19.8 ± 0.3 [b]	11.0 ± 1.9 [b]	6.3 ± 0.1 [b]

[*] Values are means of triplicates ± standard deviations (SD). Different letters in rows for each characteristic are significantly different ($P < 0.05$) in Duncan's multiple range tests, nd = not detected

There was a significant difference ($P < 0.05$) in the total AR and homolog contents of all the samples extracted by acetone. The order of total AR content from highest to lowest of all WB samples was recorded as:

HRWB > SRWB=Brio > Tahoe=Snowbird > Win A > Win B

Due to unavailability of standards, unsaturated ARs were identified according to their retention time ranges, eluting prior to or after each saturated AR homologue (C15-C25).[173] Although the total AR content was different for each WB sample, the relative saturated (80.4%) and unsaturated ARs (19.6%) for HRWB remained in close range and our findings were in agreement with values reported in literature.[140,173] The ANOVA revealed that for each cultivar, the mean saturated AR content (74.4%) in winter wheats was higher than that of spring wheats (60.5%). Genetic factors, climate, season, grain filling period, cereal type and soil conditions have been reported to affect AR content.[46]

It has been suggested that ARs may have protective antioxidant effects in biological membranes and also have stimulant or inhibitory effects on some metabolic enzymes. The length of the alkyl chain affects the extent of inhibition, with C17:0 and C19:1 being the most effective inhibitors.[94] Moreover, resorcinolic lipids from cereal bran have shown inhibitory activity of soybean lipoxygenases[91] and prevent triglyceride accumulation in cultured 3T3-L1 cells, indicating that they may inhibit triglyceride synthesis *in vivo*.[93]

The order of individual AR homologue content from highest to lowest was C21:0 > C19:0 > C23:0 > C17:0 > C25:0. In this study, the relative AR homologue composition for all WB samples examined was determined to be in the ranges of 0-4.5% for C15:0, 6.2-11.9% for C17:0, 28.4-32.1% for C19:0, 31.3-44.6% for C21:0, 5.4-14.4% for C23:0,

and 4.2-10.5% for C25:0. The most abundant AR homologues were C21:0, and C19:0 in WB samples which is in agreement with other reports; Knodler et al,[145] and Gunenc et al.[173] The C15:0 homologue was only detected in Brio (2.3 ± 0.1mg/100g) and Snowbird (10.7 ± 0.4mg/100g) WB samples and that was expected due to the fact that C15:0 does not generally occur in WB compared to the other homologues (C17:0-C25:0). The ratio of C17:0 to C21:0 for durum wheat, common wheat, triticale, and rye are approximately 0.01, 0.1, 0.2 and 1.0 respectively.[46] For this study, the ratio of C17:0 / C21:0 ranges from 0.07 to 0.30 and these ratios are suggested to be an index for determining the origin of a cereal product. Our study showed that these ratios could be used to differentiate common wheat cultivars.

4.4.2 AR content and homolog composition by SC-CO_2 method

Both HRWB and SRWB were extracted with SC-CO_2. The total AR and homolog contents (mg/100 g WB) were presented in Table 4.6. The total AR content of HRWB and SRWB was calculated as 74.8, and 64.2 mg/100 g respectively, findings which were in agreement with that of Athukorala et al.[35] In their study, total AR contents for triticale Ultima, triticale Pronghorn, and wheat were recorded as 70, 36, and 68 mg/100g respectively; similar conditions were used in this study (70 ˚C, 25 g/min, 350 bar).

When both extraction methods were compared, the SC-CO_2 extracts had 35% (HRWB) and 38% (SRWB) lower total yields than acetone extracts. At the same time, the total AR content in SC-CO_2 extracts was 79% (HRWB) and 83% (SRWB) less than that of acetone extracts. In the traditional method, WB samples were extracted with 100% acetone for 24 h whereas in SC-CO_2 extraction, only 10% ethanol was used as a co-solvent for 4 h. When the extraction solvents and conditions were taken into

consideration, differences in AR content can be better explained. Also, HPLC analysis of the first extraction step (100% CO_2) confirmed that none of the ARs homologues were detected, indicating 100% SC-CO_2 solvent is not appropriate to extract polar lipids such as ARs. Fransciso et al.(2005) also reported that none of the AR homologues were detected in the extract when 100% CO_2 was used under the same extraction conditions (350 bar, 55-70 ˚C).[66] Therefore in our study, the first extraction step with 100% SC-CO2 was performed to remove the free-ARs lipid fraction. The combination of a polar co-solvent with a non-polar fluid (CO_2) increased the extractability of ARs. In the second extraction step of SC-CO_2 which incorporated a co-solvent, the sum of 1st and 2nd collectors had 61% (HRWB) and 55% (SRWB) fewer yields compared to the acetone extract.

In earlier studies, for acetone extractions of ARs from cereal bran, the most abundant AR-homologues have been reported to be C17:0-C21:0 (up to 85%, w/w).[66,90] However, in this study these homologues accounted for only 70% of ARs. Several factors such as a difference in sample source, sample nature, extraction method and protocol might suggest some reasons for these variations.[66] Although total yields and solvents used were different in both extraction methods, the homologue distribution pattern remained similar. The order of individual AR homologue contents from the highest to lowest percentage matched that of acetone extracts, C21:0 > C19:0 > C23:0 > C17:0 > C25:0.For SC-CO_2 extracts, the relative AR homologue content for both WB (HRWB and SRWB) samples were determined to be 5.3-7.8% for C17:0, 30.7- 43.8% for C19:0, 50.7-53.1% for C21:0, 8-11% for C23:0, and 2.7-3.1% for C25:0. The dominant AR homologues were C21:0 (52%) and C19:0 (37%), results that are in agreement with

Rebolleda et al.[174] Several factors such as genetic factors, climate, season, grain filling and soil conditions might affect the AR content,[35] similar %AR profiles were also found by Landberg et al.(2007) in the extraction of wheat bran using ethyl acetate and SC-CO_2.[68] Although similar AR profiles were obtained by both extraction methods, it must be emphasized that the highest AR/extract ratio was obtained when using a polar organic solvents such as acetone. The ratios of HRWB and SRWB for total %AR extracted with acetone were 15.6% and 10.9%, respectively. These ratios for SC-CO_2 extraction for HRWB and SRWB were 6.6% and 5.1% respectively. These findings are in agreement with literature.[68,174] Even though the higher AR yield obtained by traditional extraction method compared to SC-CO_2, solvent extractions present some disadvantages like long extraction times, toxic waste generation and a more lengthy final purification procedure.[66]

Table 4-6 Total ARs and homolog contents (mg/100g WB) of HRWB and SRWB samples extracted by SC-CO_2

Sample	Collector	C 17:0	C19:0	C21:0	C23:0	C25:0	ARs	Total ARs (1^{st} + 2^{nd})
HRWB	1^{st}	1.1 ± 0.5^{a}	5.2 ± 2.9^{b}	8.1 ± 4.1^{c}	1.5 ± 0.7^{b}	1.1 ± 0.8^{a}	17.0 ± 9.1^{b}	74.8 ± 17.3^{a}
	2^{nd}	2.6 ± 0.8^{a}	18.7 ± 2.9^{a}	29.9 ± 2.5^{a}	4.9 ± 0.5^{a}	1.7 ± 0.2^{a}	57.8 ± 7.8^{a}	
SRWB	1^{st}	1.9 ± 0.3^{a}	7.5 ± 0.1^{b}	13.8 ± 0.1^{cb}	2.6 ± 0.6^{ba}	0.6 ± 0.5^{a}	26.4 ± 1.8^{b}	64.2 ± 12.8^{a}
	2^{nd}	2.7 ± 1.6^{a}	11.2 ± 3.3^{ba}	19.6 ± 4.3^{b}	3.5 ± 1.2^{ba}	0.8 ± 0.3^{a}	37.8 ± 11.0^{ba}	

* Values are means of triplicates ± standard deviations (SD). Different letters in columns for each characteristic are significantly different ($P < 0.05$) in Duncan's multiple range tests.

4.4.3 Phenolic acids and flavonoids content and composition analysis of fractional extraction of phenolics

In the present study, alkaline hydrolysis and liquid-liquid partitioning steps were carried out to extract ester linked phenolics from both WB (HRWB & SRWB) and their fractions, assigned as soluble free, soluble conjugated and bound forms. The separation of phenolic acids in the WB samples and its alkaline extracts were carried out on a RP-HPLC. The phenolic acid content and composition (mg/g of sample) of HRWB and SRWB fractions including soluble free (A), soluble conjugated (B), and bound fractions (C) are shown in Table 4.7.

Table 4-7 Phenolic acid (PA) content (mg/g WB) and composition of HRWB and SRWB fractions including soluble free (A), soluble conjugated (B), and bound (C)

Sample	Fraction	Total (PA)	1*	2	3	4	5	6	7	8	9	10
HRWB	A	0.20±0.14	nd	nd	0.02±0.01	0.15±0.11	nd	nd	nd	0.02±0.01	nd	0.01±0.01
	B	0.44±0.04	0.08 ± 0.00	nd	0.05±0.04	0.22±0.16	0.10±0.09	nd	0.03±0.02	0.05±0.04	0.02±0.01	0.05±0.05
	C	3.01± 0.57	0.60 ± 0.75	nd	0.07±0.05	0.40±0.28	nd	0.06±0.04	0.23±0.26	0.33±0.29	0.08±0.02	1.52±1.49
SRWB	A	0.10±0.04	nd	nd	0.01±0.01	0.07±0.05	nd	nd	nd	nd	nd	nd
	B	1.82±2.02	0.47± 0.58	0.27±0.35	0.27±0.07	0.18±0.13	0.03±0.02	0.19±0.13	0.13±0.17	0.16±0.21	0.02±0.02	0.30±0.38
	C	2.09±1.66	0.44±0.31	nd	0.07±0.05	0.40±0.28	nd	0.03	0.04±0.03	0.15±0.03	nd	1.43±1.66

*1= gallic, 2 = proto-catechuic, 3 = p-OH-benzoic, 4 = chlorogenic, 5 = caffeic, 6 = vanillic, 7 = syringic, 8 = p-coumaric, 9 = sinapic, 10 = ferulic,
nd = not detected

Values are means of triplicates ± standard deviations (SD)

Fraction C (bound form) for both WB samples had the highest total phenolic acid content (52% for SRWB and 82% for HRWB) compared to the other fractions, A (soluble free) and B (soluble conjugated). Total phenolic acid contents ranged from 12-45% for B fractions, and 3-6% for A fractions. These findings were expected since most of the phenolic acids were found in bound form.[30]

With HPLC analysis, a total of ten phenolic acid peaks identified in the fractions (A, B and C) were respectively assigned as gallic, proto-catechmic, p-OH-benzoic, chlorgenic, vanillic, syringic, p-coumaric, sinapic, and ferulic acids with their retention times compared with standards (Figure 4.4). There were no significant differences ($P < 0.05$) in the content of each phenolic acid per fraction. The WB samples tested were winter red wheats. Further studies are needed to compare phenolic acid compositions indifferent cultivars and regions. Comparing the present study with published data was difficult because of differences in wheat cultivars used, pre-treatment of the flour or bran, and solvents used to extract free phenolics,[116] as well as differences in acidic and/or alkaline hydrolysis.[175]

Ferulic acid (FA) was detected as the predominant phenolic acid and mostly in bound form in C fractions (82-96%).Mean FA content ranged from 0.842-1.523 mg/g. High levels of FA in the C fractions showed that esterified FA was concentrated in those fractions and this finding is in agreement with literature.[176]

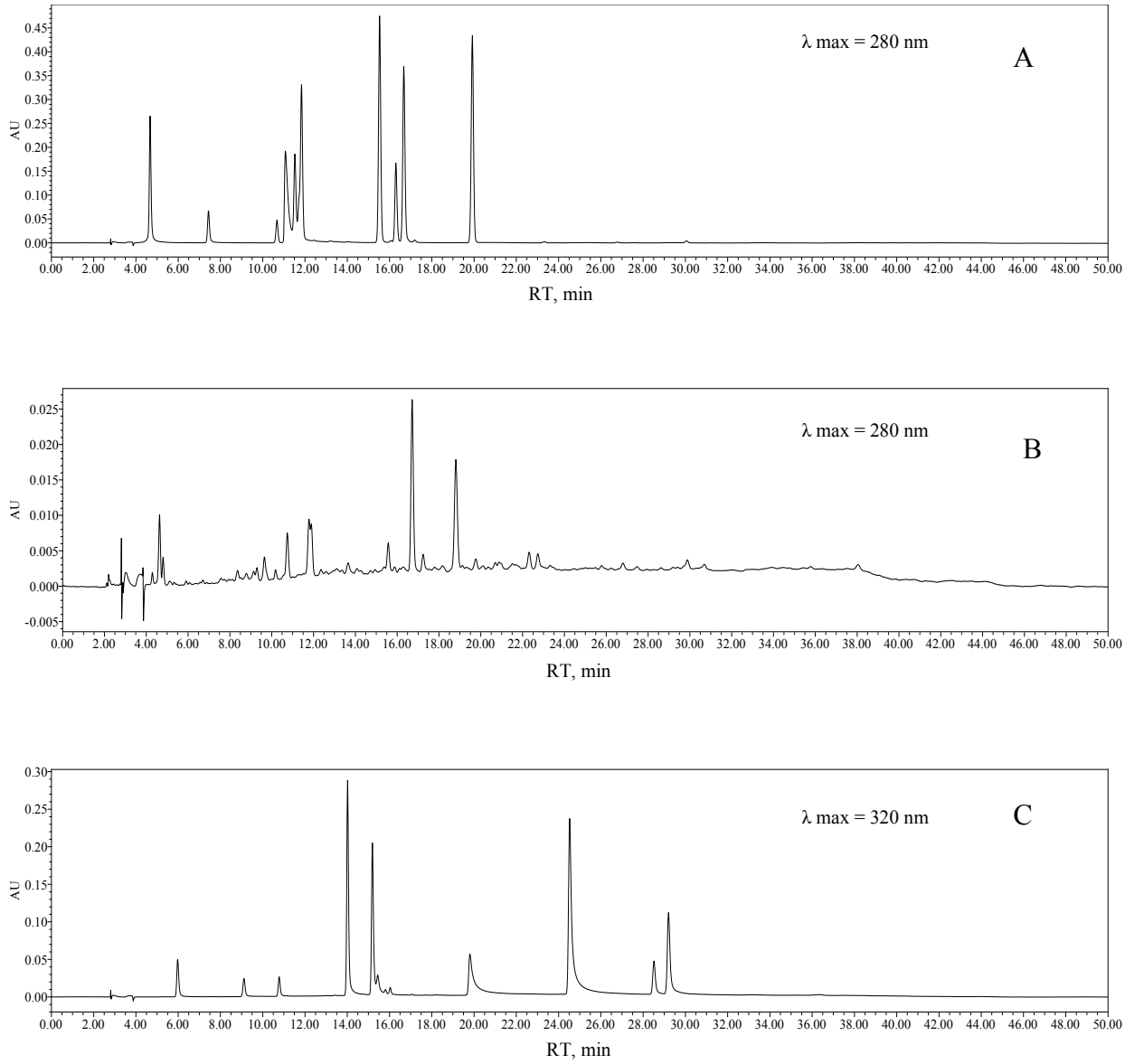


Figure 4-4 HPLC chromatograms of phenolic acid standard (A), SRWB soluble conjugated phenolic acids (B), flavonoid standards (C) (Look at Table 4.8 for each standard and corresponding retention times)

Table 4-8 Phenolic acid and flavonoids standards used for HPLC analysis and their retention times (RT) in minute

Phenolic acids	RT	Flavonoids	RT
Gallic acid	4.73	Pyrogallol	5.97
Proto-catechic acid	7.71	Catechin	9.13
p-OH-benzoic acid	10.76	Epicatechin	10.76
Chlorogenic acid	11.10	Rutin	14.00
Caffeic acid	11.56	Quercetin 3 beta glucoside	15.17
Vanillic acid	11.76	Epicatechin gallate	15.40
Syringic acid	11.89	Myricetin	19.71
p-coumaric acid	15.60	Quercetin	24.44
Sinapic acid	16.38	Apigenin	28.45
Ferulic acid	16.75	Kaempherol	29.14
o-coumaric	19.99		

The total phenolic content obtained in this study (3.703-4.036 mg/g) is in the range reported by Verme et al. (2009), whereby similar hydrolysis conditions were used. Their total phenolic acid content ranged from 2.784 for AC Andrew wheat bran to 4.043 mg/g for Kathew wheat bran.[175]

Moreover, the same fractions were analyzed for flavonoid content by HPLC and the results are presented in Table 4.9. This Table shows flavonoid content and composition (mg/g of sample) of HRWB and SRWB fractions containing the three fractions A, B and C. Six major flavonoid peaks in the fractions were pyrogallol, catechin, epicatechin, rutin, quercetin-3-beta glucoside and epicatechin gallate by comparison of their retention times with standards. The phytochemical profiles,

especially the flavonoid profiles of wheat, have only been studied in recent years.[116] Moreover, little is known about the inherent differences in phytochemical profiles. Those biologically active components have not received as much attention as the phytochemicals in fruits and vegetables although the increased consumption of whole grains and whole grain products has been linked to a reduced risk of chronic disease development. [163] Our findings for flavonoid content were also comparable to that of Feng et al.[177] They characterized flavonoids in the bran of four classes of wheat, including three hard red winter classes and one durum class; the mean flavonoid content found in this study was 0.30 mg/g compared to that of our findings of 0.64 mg/g.

Table 4-9 Flavonoids contents (FC, mg/g of WB) and composition of HRWB and SRWB fractionals including soluble free (A), soluble conjugated (B), and bound (C)

Sample	Fraction	Total (FC)	1*	2	3	4	5	6
HRWB	A	0.10 ± 0.02	0.07 ± 0.01	nd	0.01 ± 0.00	0.02 ± 0.02	nd	nd
	B	0.14 ± 0.10	nd	0.05 ± 0.04	nd	nd	0.10 ± 0.05	nd
	C	0.26 ± 0.19	0.11 ± 0.02	nd	nd	0.30 ± 0.22	nd	nd
SRWB	A	0.11 ± 0.06	0.04 ± 0.03	0.01 ± 0.00	0.14 ± 0.01	0.01 ± 0.01	nd	nd
	B	0.38 ± 0.28	0.02 ± 0.02	0.23 ± 0.17	nd	0.25 ± 0.33	nd	nd
	C	0.27 ± 0.03	0.05 ± 0.04	0.10 ± 0.07	nd	0.10 ± 0.07	nd	0.15 ± 0.01

*1 = pyrogallol, 2 = catechin, 3 = epicatechin, 4 = rutin, 5 = quercetin-3-beta glucoside, 6 = epicatechin gallate,

nd = not detected

Values are means of triplicates ± standard deviations (SD)

4.4.4 Antioxidant activity

Wheat contains many classes of antioxidant compounds including phenolic acids, carotenoids, tocopherols, and tocotrienols. Most of these compounds such as phenolic acids are present in free, esterified or glycosylated, and additionally as complexes with proteins and other plant materials. [115,116,149] Also, the concentration of each antioxidant compound in wheat grains depends on the genotype, growing conditions, and interactions between genotype and environment. For these aforementioned reasons, many extraction conditions have been used by different research groups making inter-laboratory comparisons of wheat antioxidant assays difficult.[112]

The purpose of this section was to compare three different solvent systems for extracting antioxidants from different wheat cultivars and their influence on antioxidant activity estimation by comparing TPC, ORAC and DPPH assays of those crude WB extracts.

The WB samples were extracted with acidified ethanol, 50% acetone and 100% acetone. Solvent effects on TPC, ORAC and DPPH were investigated. The results are presented below.

4.4.4.1 TPC

Solvent effects on TPC measurement was investigated using five hard spring wheats (Brio, Tahoe, Snowbird, Win A and Win B) as well as one hard (HRWB) and one soft (SRWB) winter wheat (Table 4.1). Extraction solvent types had significant effects ($P < 0.05$) on TPC as seen in Table 4.10.

Regardless of wheat variety, the WB samples extracted with 50% acetone displayed the highest TPC values followed by acidified ethanol and 100% acetone extracted samples. Use of 50% acetone for winter wheat (HRWB and SRWB) exhibited significantly higher TPC values than spring wheat, suggesting that 50% acetone might be a preferred solvent for extracting WB rather than acidified ethanol or 100% acetone.

Table 4-10 TPC (mg FAE/g WB) values of different wheat cultivars extracted by three different solvent systems

Sample	Acidified ethanol	50% acetone	100% acetone
HRWB	9.8 ± 0.7 [c]	26.3 ± 0.7 [a]	14.6 ± 1.2 [a]
SRWB	12.6 ± 0.6 [a]	16.8 ± 2.1 [b]	9.3 ±0.6 [b]
AC Brio	10.4 ± 0.4 [cb]	14.5 ± 1.6 [cb]	7.3 ±0.1[c]
AC Tahoe	11.1 ± 0.5 [b]	14.4 ± 2.6 [cb]	8.4 ± 0.9 [cb]
Snowbird	9.9 ± 0.4 [c]	13.3 ±0.6 [c]	6.9 ± 0.1[c]
Win A	8.5 ± 0.3 [d]	13.4 ± 1.6 [c]	7.5 ± 0.7 [cb]
Win B	8.7 ± 0.1[d]	12.3 ± 0.6 [c]	8.1 ± 2.2 [cb]

[*] Different letters in column for each solvent are significantly different ($P < 0.05$) in Duncan's multiple range and values are means of triplicates ± standard deviations (SD)
TPC = Total Phenolic Content as mg Ferulic Acid Equivalent (FAE) /g

4.4.4.2 DPPH

The free radical scavenging activity of different WB extracts with three different solvent systems was studied and the results are presented in Table 4.11.

Table 4-11 DPPH (%) of wheat bran extracts with different solvent systems

Sample	Acidified ethanol	50% acetone	100% acetone
WB	43.7 ± 1.2^{d}	28.3 ± 1.2^{a}	22.3 ± 3.6^{a}
SRWB	45.5 ± 2.1^{dc}	20.8 ± 1.6^{b}	17.6 ± 1.9^{a}
AC Brio	48.3 ± 0.3^{a}	17.6 ± 1.5^{cb}	21.6 ± 3.3^{a}
AC Tahoe	48.6 ± 0.1^{a}	13.7 ± 2.2^{cd}	20.9 ± 1.7^{a}
Snowbird	47.4 ± 0.3^{ba}	20.7 ± 6.5^{b}	20.6 ± 4.7^{a}
Win A	47.0 ± 1.1^{bac}	7.0 ± 1.6^{e}	20.8 ± 4.0^{a}
Win B	46.1 ± 0.7^{bc}	10.3 ± 0.8^{ed}	19.6 ± 3.4^{a}

* Different letters in column for each solvent are significantly different ($P < 0.05$) in Duncan's multiple range and values are means of triplicates ± standard deviations (SD)
DPPH = 1, 1-diphenyl-2-picrylhydrazyl as %DPPH inhibition after 30 min

Solvents used for antioxidant extractions had significant effects ($P < 0.05$) on DPPH scavenging activity. Regardless of wheat variety, the acidified ethanol extracts exhibited the greatest %DPPH inhibition values, followed by 100% acetone and 50% acetone extracted WB samples. Antioxidant activity ranged from 6.97% to 48.62% for all WB extracts. Free radical chain reaction is a chemical mechanism which is similar to lipid oxidation in food products. Also free radical scavengers are a group of potential antioxidants to be used as food additives and nutraceuticals.[178] By measuring DPPH, it provides an idea about the total free radical scavenging capacity of each WB extract.[179]

In 1999, Mageed and Fadel extracted WB antioxidants with hexane, chloroform, and ethanol, and compared their capacities by suppressing lipid peroxidation in cooked beef kept at 4 ˚C for 7 days. Their results showed ethanol extracts to be more effective than other solvents in the inhibition of lipid oxidation during 7 days of storage.[180] Their results indicate that ethanol may be more effective than less polar solvents for extracting

lipohilic antioxidants capable of inhibiting the formation of aldehyde compounds generated from lipid peroxidation; compounds which are detectable with a TBA (thiobarbituric acid) test. Further analysis of WB extract composition is needed to explain the different observations and correlations between TPC and radical scavenging activities.

Our finding shows that WB contains significant free radical scavenging activities against DPPH. It is reported that each solvent system extracts different antioxidant components depending on their polarities, and each antioxidant compound is likely to exhibit different free radical scavenging properties depending on the nature and mechanism of the free radicals used for the antioxidant measurements assays.[112] Therefore, more research is needed to investigate the chemical components involving total antioxidant activity

4.4.4.3 ORAC

ORAC measures antioxidant inhibition of peroxyl radical and represents radical chain-breaking antioxidant activity by H-atom transfer.[110] Acidified ethanol, 50% acetone, and 100% acetone extracts of the same WB samples showed different ORAC values as shown in Table 4.12, suggesting that extraction solvents may affect the overall estimation of ORAC values of a selected WB sample. The greatest ORAC values was observed in the acidified ethanol extracts for all WB samples, followed by 50% acetone and 100% acetone extracts. Our findings indicated that acidified ethanol was a suitable solvent for extracting WB antioxidants with high ORAC values and it is parallel with literature findings.[181]

It has been reported each solvent extracts different antioxidant components depending on their polarities, and each type of antioxidant compound is likely to exhibit different free radical scavenging properties depending on the nature and mechanism of the free radical used. [112] Also, it has been emphasized that the extraction solvents need to be optimized for each type of wheat material used.[181]

Table 4-12 ORAC (μmole of TE/g WB) values of different wheat cultivars extracted with different solvent systems

Sample	Acidified ethanol	50% acetone	100% acetone
HRWB	104.5 ± 0.8 [ba]	97.3 ± 5.4 [a]	19.3 ± 2.0 [b]
SRWB	112.5 ± 7.4 [a]	102.3 ±4.0 [a]	23.6 ± 1.0 [b]
AC Brio	89.4 ± 2.4 [c]	52.1 ± 4.7 [cb]	14.02 ± 0.5 [c]
AC Tahoe	91.1 ± 4.9 [c]	62.2 ± 7.8 [b]	21.4 ± 0.2 [b]
Snowbird	89.1 ± 5.9 [c]	42.53 ± 7.8 [c]	12.63 ± 0.7 [dc]
Win A	70.5 ± 4.8 [d]	46.4 ± 0.5 [c]	19.3 ± 1.0 [b]
Win B	101.6 ± 0.5 [b]	42.8 ±1.5 [c]	33.1 ± 6.3 [a]

[*] Different letters in column for each solvent are significant different ($P < 0.05$) by Duncan's multiple range test and values are means of triplicates ± standard deviations (SD)
ORAC = Oxygen Radical Absorbance Capacity as μmole TE (Trolox Equivalent) /g

Two-way ANOVA showed that cultivar, solvent and their interactions were significantly different ($P < 0.0001$) for TPC, DPPH and ORAC values as shown in Table 4.13. Average TPC, DPPH and ORAC values were the highest in winter wheats and lowest in spring wheats. For both DPPH and ORAC results, the two-way analysis showed that acidified ethanol extracts gave the highest results, whereas for TPC analysis, 50% acetone gave the highest values compared to the other extraction solvents.

Table 4-13 The two way ANOVA for cultivar and solvent effects on antioxidant activity measurements; TPC, DPPH and ORAC

CULTIVAR	TPC[a]	DPPH[b]	ORAC[c]
HRWB	16.90 [a]	31.47 [a]	73.72 [a]
SRWB	12.92 [b]	27.95 [bc]	79.49 [a]
AC Brio	10.73 [dc]	29.15 [ba]	51.85 [c]
AC Tahoe	11.30 [c]	27.75 [bc]	58.22 [b]
Snowbird	10.02 [d]	29.58 [ba]	48.09 [dc]
Win A	9.80 [d]	24.94 [d]	45.40 [d]
Win B	9.70 [d]	25.33[dc]	59.16 [b]
SOLVENTS			
acidified ethanol	10.13 [b]	46.67 [a]	94.12 [a]
50% acetone	15.86 [a]	16.90 [c]	63.67 [b]
100% acetone	8.89 [c]	20.50 [b]	20.47 [c]

[*] Different letters in column for each analysis are significantly different ($P < 0.0001$) in Duncan's multiple range tests and values are means of triplicates
[a] TPC = Total Phenolic Content as mg Ferulic Acid Equivalent (FAE) /g
[b] DPPH = 1, 1-diphenyl-2-picrylhydrazyl as %DPPH inhibition after 30 min
[c] ORAC = Oxygen Radical Absorbance Capacity as μmole TE (Trolox Equivalent) /g

4.5 Conclusion

The current study investigated and compared total AR contents, and their relative homologue composition in WB samples using traditional and SC-CO_2 extraction methods. The results showed that winter wheats had higher levels of ARs than spring wheats. The highest amount of ARs was found in HRWB for both extraction methods and the most common AR homologues were C21:0, followed by C19:0, C23:0, C17:0 and C25:0. Due to the amphiphilic nature of ARs, the extraction yield was higher for the traditional extraction using a polar organic solvent than for SC-CO_2 extraction. Even

though a higher AR yield was obtained by traditional solvent extraction, a valuable extract rich in ARs was been obtained by SC-CO_2 from WB. Further studies need to be conducted to determine the optimum extraction conditions of SC-CO_2, such as particle size, soaking before extraction, and temperature. Alkaline hydrolysis and liquid-liquid partitioning steps were carried out to extract ester linked phenolics from both winter wheats and their fractions, including soluble free, soluble conjugated and bound forms. HPLC analysis allowed identification of ten major phenolic acid peaks; FA was detected as the predominant phenolic acid found mostly in bound fractions. Also, the same fractions were analyzed for flavonoid content by HPLC and six major flavonoid peaks were identified. Further studies are needed for the antioxidant activity of each fraction and different cultivars from different regions need to be analyzed to compare soluble free, soluble conjugated and bound phenolic content. For the final part of the study, the effects of extraction solvent on TPC, DPPH and ORAC were investigated. This study reports that different extraction solvents have effects on the antioxidant activity measurements for WB samples. Findings from this study suggest that 50% acetone might be the recommended solvent for the preparation of WB extracts for TPC measurements and acidified ethanol could be used to extract WB bioactive components for both DPPH and ORAC measurements.

4.6 Connecting Statement to Chapter 5

In Chapter 4, the characterization of wheat bran ARs by HPLC was studied for different wheat cultivars. The extraction process included both traditional and supercritical fluid extraction methods. Also, this chapter gave us comparison of antioxidant measurement of different wheat varieties extracted with three different solvent systems.

In Chapter 5, the potential of WB soluble polysaccharides on the growth of bacterial survival and growth in yogurt as well as the antioxidant activity of the polysaccharides was investigated.

The work presented in Chapter 5 has been used to prepare one conference presentation (poster). Also, one publication will be submitted, as follows.

- Gunenc, A. and Hosseinian, F., 2012. Canadian Institute of Food Science and Technology (CIFST)-50th National Conference, Potential of adding red hard wheat bran on yogurt production and antioxidant activity measurements: total phenolics, DPPH, and ORAC, Niagara Falls, ON, Canada.

- Gunenc, A., and Hosseinian, F., 2013. Wheat bran enhances lactic acid bacterial survival and growth in yogurt and hold high antioxidant capacity

Chapter 5 Wheat bran enhances lactic acid bacterial survival and growth in yogurt and hold high antioxidant capacity

5.1 ABSTRACT

This study investigated the potential of hard red wheat bran (HRWB) polysaccharides to enhance bacterial survival and growth in yogurt as well as the antioxidant activity of the polysaccharides. Initially, the highest amount of bran that could be added to milk was determined, and 4% bran addition was found to be the optimum concentration of HRWB in yogurt for the entirety of the study. The microbial count, pH and total titratable acidity (TTA) were measured weekly in yogurt samples supplemented with probiotic bacteria (*Lactobacillus acidophilus* and *Bifidobacterium lactis*) in combination with 4% HRWB for 28 days stored at 4 ˚C. Results showed that there were significant differences ($P < 0.05$) in total bacterial count (9.1 log CFU/mL), pH (4.8) and TTA (1.4%) in the presence of 4% HRWB in yogurts compared to controls at the end of the cold storage period. The water extractable polysaccharides (WEP) from HRWB were enzymatically (α-amylase and protease) treated and dialyzed to obtain pure extract. The WEP exhibited strong antioxidant activity with an ORAC value of 52.48 μmol trolox equivalent/g, DPPH inhibition of 31.64%, and TPC value of 4.22 mg FAE/g. Also, the total dietary fiber (DF) content of HRWB was determined as 53% of the bran; 88.6% of DF as insoluble dietary fiber (IDF) and 11.4% of DF soluble dietary fiber (SDF). HPLC analysis of the alkaline hydrolyzed DF fractions showed that IDF had the highest phenolic acid and flavonoid content compared to SDF. This study suggests that wheat bran polysaccharides act as a good source of prebiotic, enhancing lactic acid bacteria growth in a yogurt model.

5.2 Introduction

Epidemiological studies have shown that whole grain consumption has been related to a decreased risk of chronic diseases[30], Particulary obesity[182], type II diabetes,[183] cardiovascular disease,[184] and cancer.[185] In addition to risk reduction, whole grain consumption has been reported to improve gut health.[186] Wheat is one of the main whole-grain cereals consumed in the world, containing about 13% dietary fibre and at least 2% bioactive compounds apart from fibre. Wheat bran (WB) on the other hand, is made up of 45% dietary fibre and 7% bioactive compounds.[187] WB can be used in food products due to its high fibre content and antioxidant properties.[188]

Prebiotics may be defined as "a nondigestible food ingredient or soluble dietary fibre". Cereal grain oligosaccharides acts as prebiotics and increase levels of beneficial bacteria in the large bowel, thereby improving gut health.[189] It is a nutritional substrate for probiotics in the colon that have the potential to improve host health.[190] Prebiotic oligosaccharides can be manufactured by extraction from plant materials through microbial/enzymatic synthesis and enzymatic hydrolysis of polysaccharides.[191] In addition to the prebiotic potential of cereal oligosaccharides, polyphenolic compounds such as ferulic, or gallic acids are related to prevention of diseases through possible mechanisms such free radical quenching, chelation of transition metals, reducing peroxides and stimulation of the antioxidant enzyme system.[192] Potential protective effects of phenolic acids and of oligosaccharides as prebiotics deserve to be studied in more depth.[30] Probiotics and prebiotics are mostly used in fermented dairy products that are the number one products of functional foods worldwide.[193] Therefore, research is essential for the discovery of new high-value bio-products and their potential use in

functional foods or nutraceuticals. The objectives of this study were to; 1) determine acceptable concentration of red hard wheat bran (HRWB) in yogurt production, 2) calculate the prebiotic effects of HRWB addition on microbial counts (CFU), pH and total titratable acidity (TTA) in yogurt samples containing starter cultures with or without probiotic bacteria, 3) measure antioxidant activity of water-extractable polysaccharides by using oxygen radical absorbance capacity (ORAC) and 2, 2-diphenyl-1-picryhydrazyl radical (DPPH) radical scavenging activity assays as well as total phenolic count (TPC), and 4) determine total dietary fiber content (soluble and insoluble dietary fiber) and analyze the phenolic acid and flavonoid composition of each fiber fraction.

5.3 Materials and method

5.3.1 Materials

All detailed information about the materials used for this study is included in section 2.3.2.

5.3.2 Sample preparation

Hard red wheat bran (HRWB) was provided by Kraft Canada. Samples were grounded to a 0.5 mm particular size by using a cyclone sample mill (UDY Corporation, CO, USA) from Agriculture-Canada (Ottawa, ON, Canada), freeze dried and stored in sealed plastic bags at -20 $^{\circ}$C until further use.

5.3.3 Probiotic activity

5.3.3.1 Milk Preparation and Sample Concentration

Pasteurized whole milk (homogenized 3.25%) was purchased at the local market. The milk was heated at 85°C for 15 minutes, cooled down to 42°C in a water bath and

transferred into 50 ml-sterile test tubes. The starter cultures, probiotics, and different HRWB concentrations were added and incubated at 42°C until the yogurt reached approximately pH 5.0.[194]

For determining optimum concentration of HRWB in yogurt without disrupting the fermentation, concentrations of 0, 2, 4, 6, and 8% (w/v) bran were added to 50 ml pasteurized milk and incubated at 42 ˚C until the completion of fermentation. All yogurt treatments of varying HRWB concentrations were carried out in triplicates.

5.3.3.2 Microbial cultures

For yogurt preparation, the starter cultures, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus,* were employed. *Lactobacillus acidophilus* and *Bifidobacterium lactis* were used as probiotic 1 and 2 respectively. The lyophilized bacteria were rehydrated and stored at -80°C in 32% glycerol before use. For each strain, 10 mL sterile aliquots of Man Rogosa Sharpe (MRS) broth liquid media was used to grow microorganisms and incubated at 42°C for 24 hours. For preparation of the stock culture, the activated tubes were used after three successive rinses with sterilized distilled water. Then the cultures were diluted with sterilized milk (121°C for 15 min in an autoclave) to obtain a concentration of 6.5 log bacteria cells/ml. Once the dilutions were made for from the stock culture 0.5 mL of each yogurt starter culture and 1.0 mL of required probiotics (pro 1 and 2) were added to the tubes depending on the treatments. The probiotics were added to yogurt samples as shown in Table 5.1 and the initial pH recorded. There were a total eight yogurt trials; four contained HRWB and four served as controls lacking HRWB. Samples with HRWB were compared against samples lacking HRWB as a probiotic substrate. All tubes were incubated at 42 ˚C for fermentation and

pH was measured after 4 h and 1 h thereafter. When the pH reached approximately 5, samples were stored at 4 °C.[194]

Table 5-1 The experimental design used to evaluate the effect of addition of HRWB sample on probiotic viability in different yogurt trials.

Yogurt trials*	Sample coding
Y	Y
Y + Pro 1	Y+1
Y + Pro 2	Y+2
Y + Pro 1 + Pro 2	Y+1+2
Y + HRWB	YB
Y + HRWB + Pro 1	YB+1
Y + HRWB + Pro 2	YB+2
Y + HRWB + Pro 1 + Pro 2	YB+1+2

*Y = control yogurt containing only starter cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophiles.* Pro 1 = *Lactobacillus acidophilus,* Pro 2 = *Bifidobacterium lactis,* HRWB = hard red wheat bran (4 %)

5.3.3.3 Microbial count

Total microbial counts were carried out on days 1, 7, 14, 21 and 28 in triplicate for each batch at different dilutions; four serial dilutions of 1 to 10. A 5 µl portion from each dilution was plated on MRS agar dishes in triplicate by using a spread plate technique. Colonies were counted after incubation at 42 °C for 24 hours.[194] Counted colonies were converted to log CFU (Colony Forming Unit) / mL usingthe following formula:

$$\log \frac{CFU}{mL} = \frac{1000\ \mu L \times \frac{CFU}{plate}}{5\ \mu L} \times dilution\ factor$$

5.3.3.4 pH and total titratable acidity (TTA)

Post-acidification was read at days 1, 7, 14, 21 and 28, using a Denver Instrument UB-5 pH meter (Denver Instrument, Bohemia, New York, USA). For the same days, TTA was also performed by titrating a mixture of yogurt and sterile water (1:9, v/v) with 0.1 N NaOH using 0.1 % phenolphthalein as an indicator.[195] The following equation was used to calculate TTA %:

$$TTA\ \% = Dilution\ factor\ (10) \times V_{NaOH} \times 0.1N \times 0.009 \times 100\%$$

V_{NaOH}: Volume of NaOH (mL) added to neutralize the acid and generate a colour change.

5.3.4 Extraction of prebiotics (water extractable polysaccharide, WEP)

Prebiotic or WEP extraction was carried out in triplicate by following the method of Escarnot et al.[196] Ground HRWB was extracted with distilled water (1:100, w/v) stirred for 4 h at 70 ºC, cooled, centrifuged at 6000 x g for 20 min in a Thermo Sorval centrifuge (Legend XT Series, Fisher Scientific, Nepean, ON, Canada) and the supernatant was kept. For the purpose of removing starch and proteins/peptides, the enzymes of α-amylase and protease from *Bacillus lichenformisis* were added to the supernatant solution (20 µL/100 mL) and stirred at 37 ºC for 24 h. The supernatant was then heated at 95 ºC for 5 min to inactivate the enzymes, cooled to room temperature and re-centrifuged at 6000 x g for 20 min. The supernatant solution was dialyzed against double distilled water for 48 h and replaced with fresh distilled water every 6 h to separate polysaccharides and other materials with a molecular weight cut-off of 3500 D (Spectra/Por dialysis

membrane, Rancho Dominguez, CA, USA) (Figure 4.1A). The extract solutions were kept at -20 ºC until further analysis.

5.3.5 Antioxidant activity

TPC, DPPH and ORAC measurement of WEP were performed by following sections 2.3.5, 2.3.6 and 2.3.7, respectively.

5.3.6 Total dietary fiber (DF): soluble (SDF) and insoluble (IDF) extraction

Insoluble dietary fibre (IDF) and soluble dietary fiber (SDF) of the bran sample (HRWB) were prepared based on AOAC Official Method 991.43 and the study of Guo & Beta.[176] Briefly, 5 g of HRWB was subjected to enzymatic digestions; firstly, heat stable α-amylase (250 µL, boiling water bath for 30 min), then alcalase protease (50 mg/mL, 500 µL, pH 7.5, 60 ˚C water bath for 30 min) and lastly, amyloglucosidase (1500 µL, pH 4.5, 60 ˚C water bath for 30 min) to remove starch and protein. After centrifugation at 10, 000 rpm, the residue was washed with hot water, ethanol (95%), and acetone (95%); it was then vacuum filtered and referred to as IDF. The combined supernatants from the washings was precipitated in ethanol (80%, preheated to 60 ˚C, 4 volumes) overnight and referred to as SDF. Both of the fractions (IDF and SDF) were placed in a fume hood and dried at 35-40 ˚C overnight to remove organic solvent.

5.3.7 Phenolic acid extraction from SDF and IDF

HRWB (100 mg), IDF(100 mg) and SDF (50 mg) were subjected to alkaline hydrolysis and liquid-liquid partitioning steps for releasing ester linked phenolics[176] as described in the section 4.3.4 (bound fraction). The dried alkaline extracts were re-

dissolved in MeOH for the determination of phenolic acid and flavanoid content. Extracts were filtered with a 0.45 μm PTFE filter before HPLC analysis.

5.3.8 Statistical analysis

The experiment was conducted according to a completely randomized design with triplicates. Analysis of variance (ANOVA) was determined using the GLM procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC) and when significant ($P < 0.05$) mean comparison was performed using Duncan's Multiple Range test.

5.4 Results and discussion

5.4.1 Optimizing HRWB concentration

The first objective of this study was to investigate an acceptable concentration of HRWB in yogurt. As a preliminary study, five different concentrations of HRWB including 0, 2, 4, 6, and 8% (w/v) were employed to determine the optimum concentration. All employed concentrations resulted in fermented yogurt products with an even texture as shown in Figure 5.1B. Microbial viability in standard yogurt (containing only starter cultures) with different concentrations of HRWB was observed over 28 days of cold storage and log CFU/mL data has been shown in Figure 5.1C. This graph gave detailed information about how different concentrations of HRWB affected starter cultures during the storage period and helped in determining the optimum concentration of HRWB.

There were significant differences ($P < 0.05$) in all HRWB concentrations (2-8%) compared to the control yogurt (having 0% HRWB). The yogurts that included 4 and 6% HRWB showed significantly higher CFU values compared to the yogurts containing 2 and 8% HRWB.. Meanwhile, the yogurts containing 6 and 8% of HRWB absorbed more liquid compared to other concentrations as shown in Figure 5.1B. Therefore, addition of 4% HRWB in yogurt as in the work [197] of Agil, et al. was determined to be the optimum concentration that could be added to milk for all yogurt trials as shown in Table 5.1.

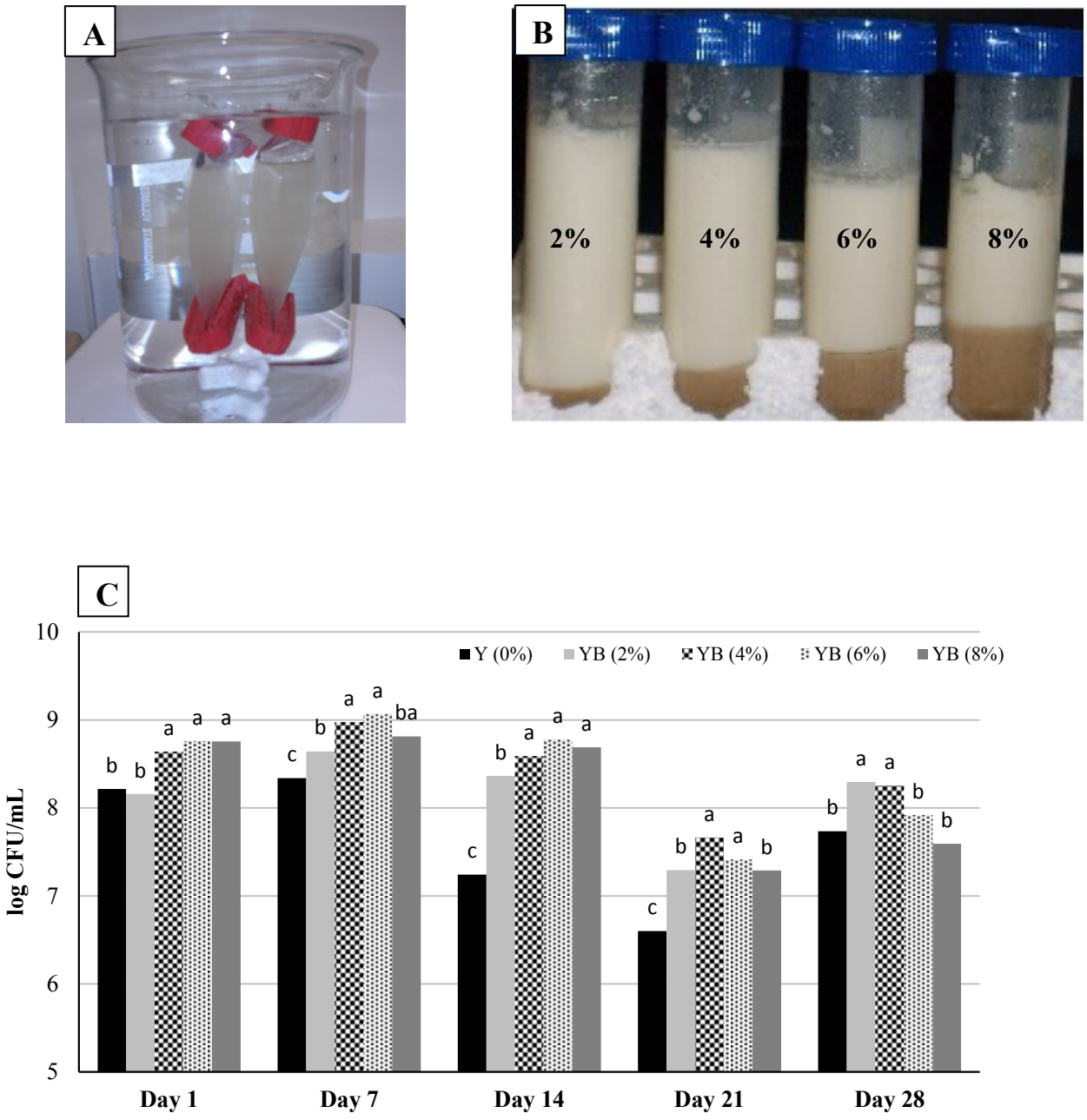


Figure 5-1 A) Dialysis of water extractable polysaccharides, B) Resultant yogurt products with various concentrations of hard red wheat bran (HRWB), C) Total microbial count (log CFU /mL) of lactic acid bacteria in yogurt containing starter cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophiles* with different concentrations of HRWB including 0, 2, 4, 6, and 8% over 28 days cold storage at 4 ˚C.

5.4.2 Prebiotic activity of HRWB addition on microbial count (log CFU/mL)

The total microbial counts at days 1, 7, 14, 21 and 28 in yogurt samples were shown in Figure 5.2. By day 1, microbial counts increased from an initial bacteria count of 6.5 to a range of 8.19-8.31 log CFU/mL in control yogurts, and 8.41-8.65 log CFU/mL in yogurts containing HRWB. There was a significant difference ($P < 0.05$) in the number of bacterial colonies between all yogurt samples containing and lacking HRWB. The yogurt trial, YB+1 (containing HRWB and probiotic 1), had the highest microbial count compared to all other treatments.

By day 7, the number of bacteria in control yogurts ranged from 7.94 to 8.34 log CFU/mL whereas the yogurt samples containing HRWB had between 8.87 and 9.15 log CFU/mL. The bacterial growth increased significantly ($P < 0.05$) in yogurts containing each or both probiotic bacteria in the presence of HRWB compared to their corresponding controls.

By days 14 and 21, the CFU values in yogurt samples with and without HRWB continued to be significantly different yet stable. By the last week of cold storage (day 28), yogurt samples lacking HRWB had markedly lower total bacteria counts of 7.03-7.65 log CFU/mL in comparison to 8.31-8.74 log CFU/mL viable bacteria found in yogurt samples containing HRWB. It has been suggested that the level of viable bacteria remaining in yogurt after 4 weeks of cold storage should be in the range of 6 to 8 log CFU/mL.[198] The control yogurts (Y, Y+1, Y+2, Y+1+2) have remained within range, but the yogurts containing HRWB (YB, YB+1, YB+2, YB+1+2) had higher bacteria counts in the range of 8.31 and 8.74 log CFU/ml. Especially the samples containing both pro 1 and 2 had the highest CFU count compared to the other treatment trials on day 28.

Overall, the yogurt samples containing HRWB demonstrated significantly higher bacteria counts ($P < 0.05$) throughout the 4 weeks of cold storage in comparison to control samples. These results may be attributed to the high levels of carbohydrates and micronutrient content of wheat and this finding was in agreement with Agil and Hosseinian. [199] From Figure 5.2, it can be elucidated that in the presence of HRWB, there was an increase in microbial viability in sample treatments consisting of probiotics. It may be a synergistic effect of HRWB on probiotics by comparison to its respective control samples lacking HRWB. These results showed that wheat bran might have a selective effect on increasing probiotic bacteria counts and starter cultures during cold storage from day 0 to day 14, and to a lesser extent on day 21. A decreasing or stable trend in CFU numbers may be a result of nutrient depletion over time.[200] For all microbes, certain nutrients such as iron and manganese are necessary to promote viability and growth. At the same time, probiotic bacteria have the ability to bind these elements and decrease accessibility by pathogenic bacteria.[201] Wheat bran consists of high quantities of manganese, and iron, with levels of 4-14 and 2.5-19 g/100g.[30] So, the presence of wheat bran in yogurt cultures containing probiotics may supply a source of micronutrients and oligosaccharides, selectively stimulating microbial growth or viability of these particular bacterial strains.[202]

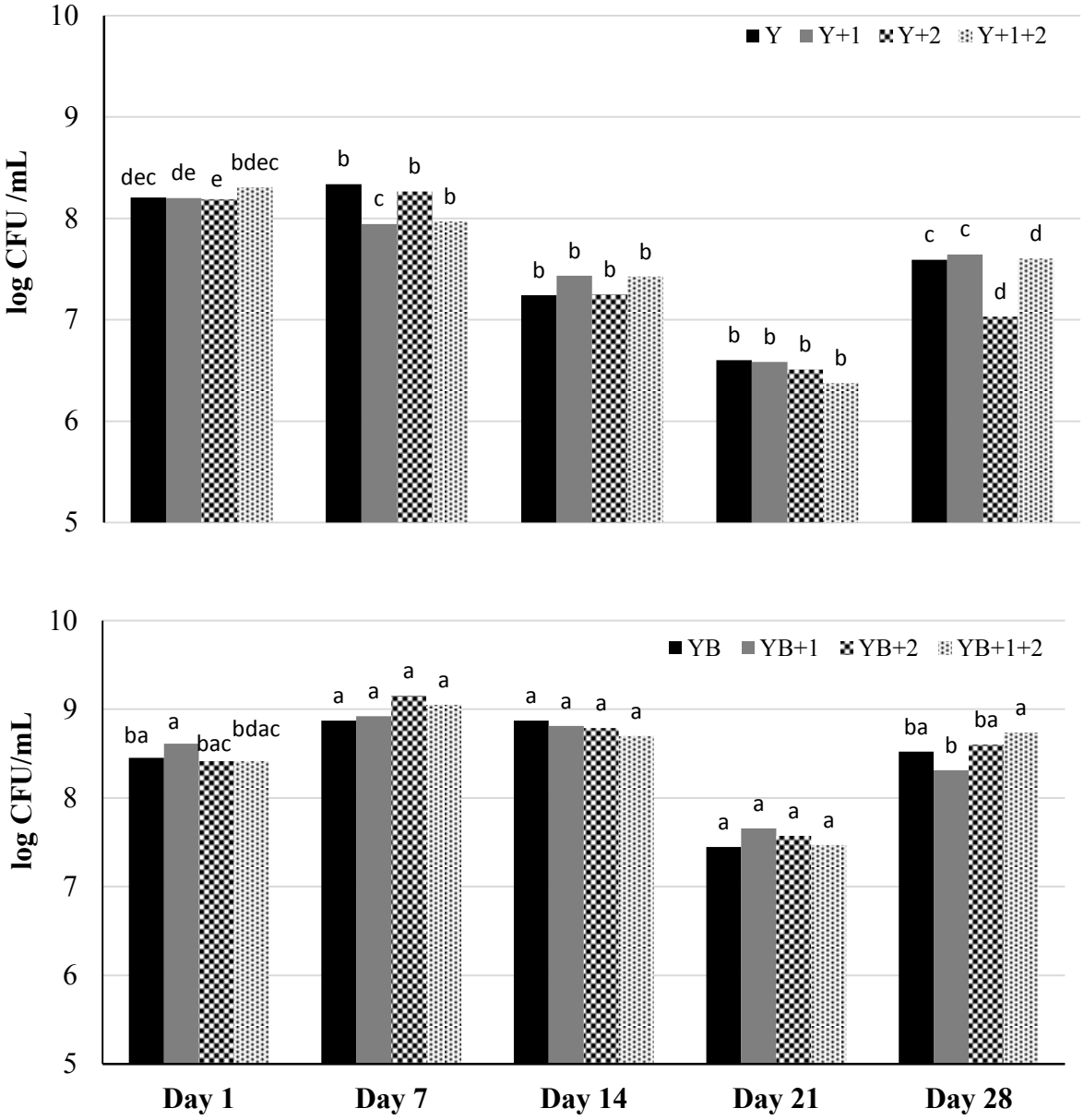


Figure 5-2 Total microbial count (log CFU /mL) in control yogurt (Y, Y+1, Y+2, Y+1+2), and yogurt with 4% wheat bran (YB, YB+1, YB+2, YB+1+2). Number 1, and 2 represent probiotic1 and 2. Different letters in columns in the same day are significantly different ($P < 0.05$) in Duncan's multiple range tests

5.4.3 Prebiotic activity of HRWB addition on pH and TTA

After fermentation, the pH gradually decreased to 5 in all yogurt samples. After day 1, yogurts containing HRWB were significantly more acidic ($P < 0.05$) than yogurts lacking HRWB by 0.35-0.87 pH units as shown in Figure 5.3. The pH of plain yogurt samples decreased steadily in the range of 0.32 to 0.85 units over the 28 day period, whereas the pH of HRWB yogurts dropped quickly by 0.75 to 0.88 units. The bacteria in yogurt samples with HRWB (YB, YB+1, YB+2, YB+1+2 samples) were more active and producing more lactic acid, causing the pH to drop from day 1 to day 28. With the exception of a significant 0.1 pH unit increase in control yogurts lacking HRWB on day 21, there was a significant difference ($P < 0.5$) between the yogurt treatments lacking versus those containing wheat bran, findings which are parallel to the work of Agil et al.[164] Also the control yogurts (Y+1 and Y+1+2) containing probiotic 1 and both probiotics had significantly lower pH values when compared to control yogurts (Y and Y+2) containing only starter cultures and probiotic 2 during the 4 week study period.

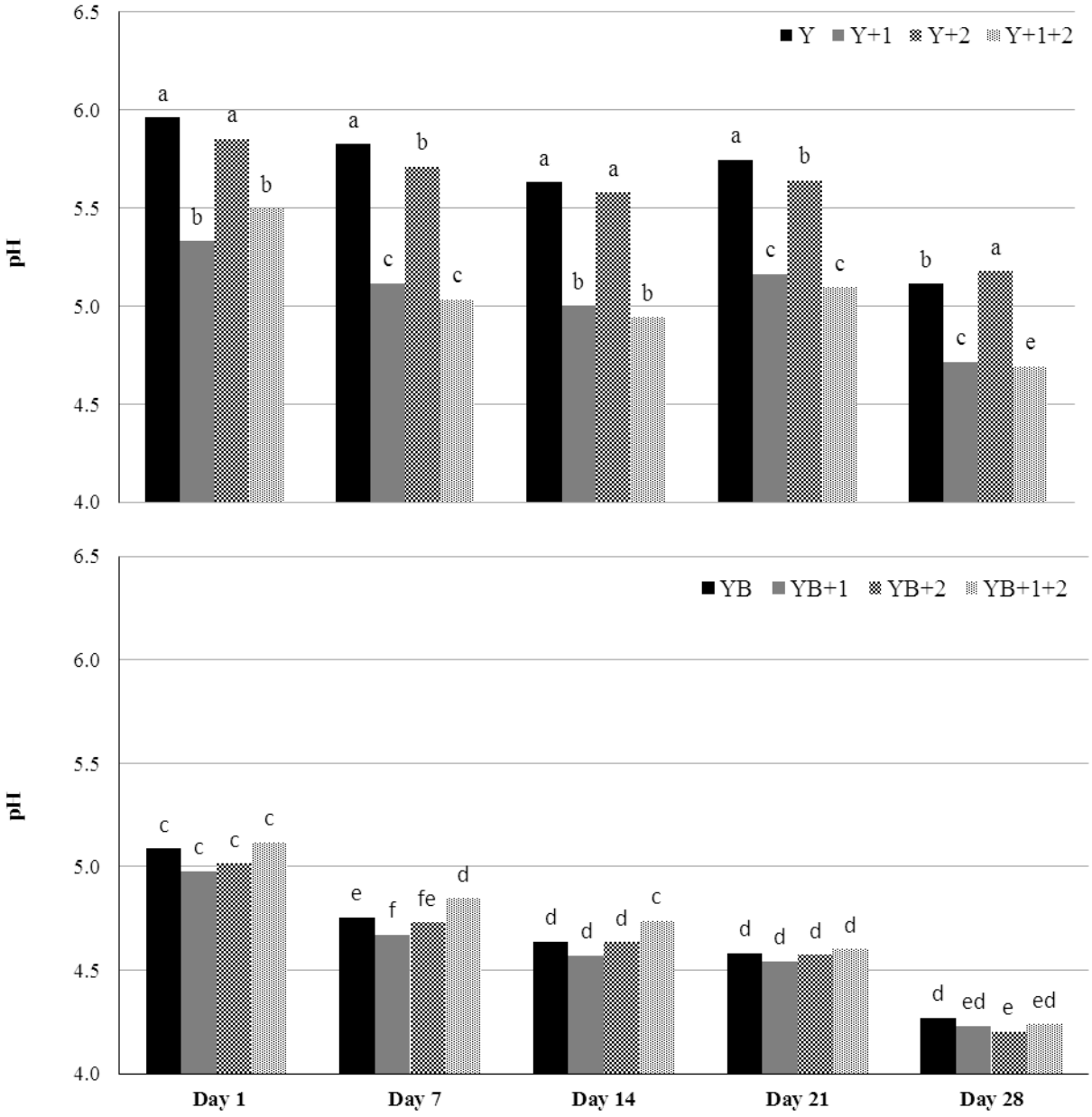


Figure 5-3 The pH values in control yogurt (Y, Y+1, Y+2, Y+1+2) and yogurt with 4% wheat bran (YB, YB+1, YB+2, YB+1+2). Number 1 and 2 represent probiotic1 and 2. Different letters in columns in the same day are significantly different ($P < 0.05$) in Duncan's multiple range tests.

Figure 5.4 illustrates the % total titratable acidity (TTA) of yogurt samples during the cold storage period. The TTA test was performed to quantify the amount of lactic acid in all yogurt samples. All yogurt samples showed increasing TTA levels as % lactic acid ($P < 0.05$) as seen in Figure 5.4, indicating that lactic acid is being produced as a result of the growing number of bacteria. The control yogurts had significantly lower TTA levels than corresponding yogurts consisting of HRWB. The reduction in microbial count in all yogurt trials can be attributed to the organic acid accumulation as a result of growth and fermentation. Interestingly, on day 28, both total microbial count and TTA values for yogurts containing HRWB and both probiotics (YB+1+2) had the highest values and were significantly different ($P < 0.05$) from their corresponding controls. These results suggest that the addition of wheat bran to yogurt trials caused bacteria to produce more lactic acid, confirming findings obtained from pH monitoring.

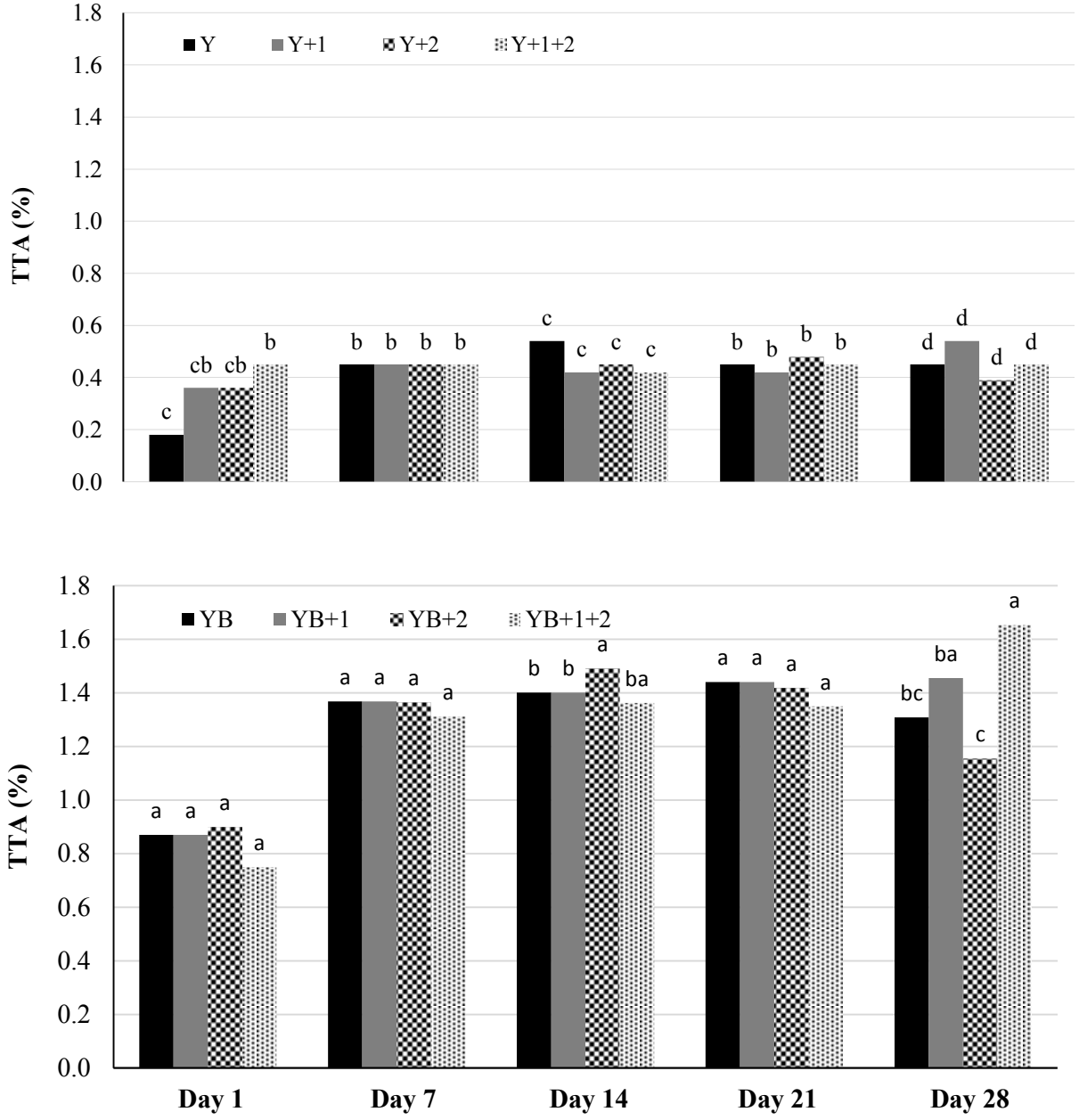


Figure 5-4 % Total titratable acidity (TTA) values for control yogurts (Y, Y+1, Y+2, Y+1+2) and yogurt with 4% wheat bran (YB, YB+1, YB+2, YB+1+2). Number 1 and 2 represent probiotic1 and 2. Different letters in columns in the same day are significantly different ($P < 0.05$) in Duncan's multiple range tests

5.4.4 Total dietary fiber determination

The total DF content was constituted 53% of the HRWB. Of this total, 88.6% was determined to be IDF and 11.4% SDF. The content and proportion of IDF and SDF are diverse among different types of cereals. This may be due to differences in seed morphology and most cereal grains including wheat, maize and barley have much higher contents of IDF than SDF. Our findings are in agreement with the study of Fardet.[30]

5.4.5 Phenolic acids and flavonoids content and composition analysis of fractional extraction of phenolics

In the present study, alkaline hydrolysis and liquid-liquid partitioning steps were carried out to extract ester linked phenolics from IDF, SDF, and HRWB. The separation of phenolic acids in its alkaline extracts was carried out on a RP-HPLC (section 4.3.5.2). The Table 5.2 shows phenolic acid content and composition (mg/g of sample) of IDF, SDF and HRWB.

Table 5-2 Phenolic acid (PA) content and composition (mg/g of sample) of SDF, IDF and HRWB

Sample	Total PA*	1	2	3	4	5	6	7	8	9
SDF	0.23 ± 0.02	0.10	nd	0.03	nd	0.01	0.01	0.02	nd	0.06
IDF	1.23 ± 0.01	0.15	0.06	0.08	nd	nd	0.03	0.07	nd	0.84
HRWB	2.64 ± 0.25	0.24	nd	0.17	0.89	0.04	0.05	nd	0.11	1.13

*1= gallic, 2= proto-catechuic, 3= p-OH-benzoic, 4= chlorogenic, 5= vanillic, 6= syringic, 7= p-coumaric, 8= sinapic, 9= ferulic, nd = not detected

Values are means of triplicates ± standard deviations (SD)

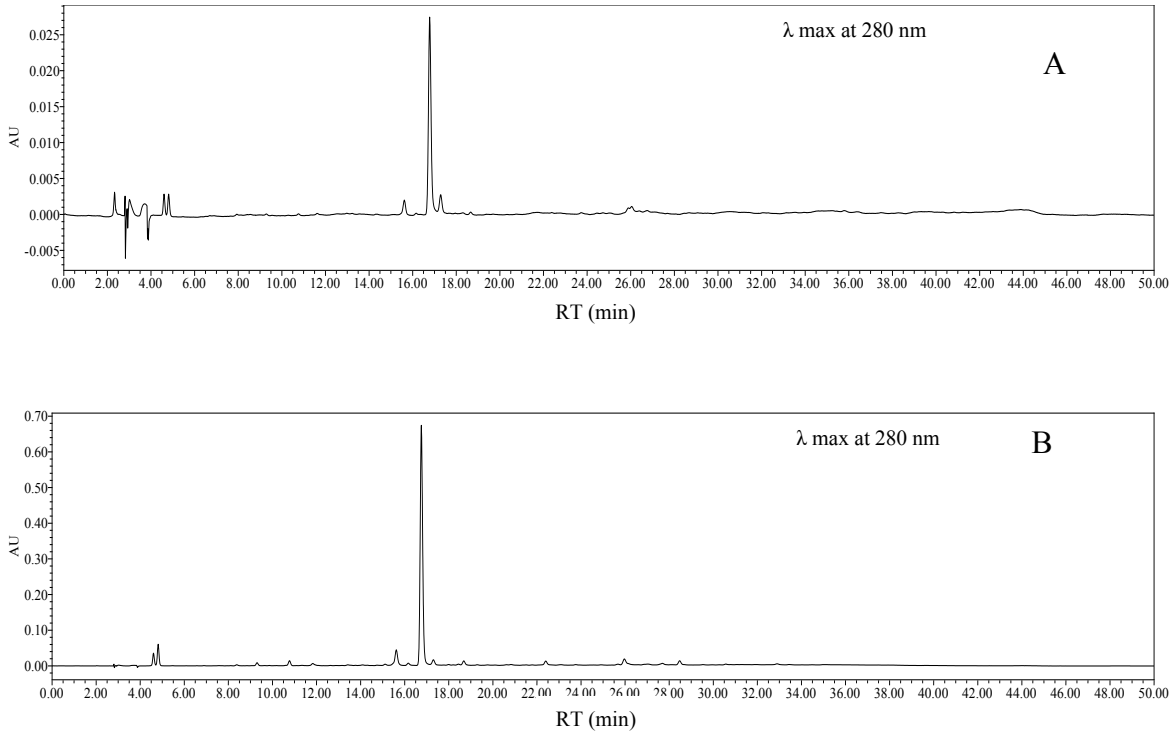


Figure 5-5 HPLC chromatograms of phenolic acid profile of soluble dietary fiber (A), and insoluble dietary fiber (B) of HRWB. Ferulic acid is the most abundant phenolic acid in both fractions. (Table 4.8 descries each standard and corresponding retention times)

IDF had the higher total phenolic acid content compared to SDF phenolic contents. All three samples, including IDF, SDF and HRWB, had alkaline hydrolysis first, followed by liquid-liquid extraction of bound phenolic acids. When HRWB phenolic acid content was compared to that of DF (IDF+SDF), the HRWB had a greater phenolic acid content of 2.64 mg/g of sample as opposed to 1.46 mg/g of sample. This was an expected each step of fractional extraction resulted in some loss of phenolic acids.[176]

With HPLC analysis of the samples, a total of eight phenolic acid peaks were identified in IDF, SDF and HRWB samples (Figure 5.5). They were assigned as gallic, proto-catechmic, p-OH-benzoic, chlorogenic, syringic, p-coumaric, sinapic and ferulic acids. Ferulic acid (FA) was detected as the predominant phenolic acid, and most of FA was found in IDF (40-42%). The mean FA content ranged from 0.06-1.13 mg/g. High levels of FA in the IDF showed that bound FA was concentrated in those fractions and this finding is in agreement with literature.[176]

Also, the same fractions were analyzed for flavonoid content by HPLC and the results were presented in Table 5.3. With HPLC analysis of the samples, four flavonoid peaks were identified and they respectively assigned as catechin, rutin, quercetin-3-beta glucoside, and epicatechin gallate. The IDF fraction had more flavonoids (0.22 mg/g) than the SDF fraction (0.07 mg/g). The phytochemical profiles, especially the flavonoid profiles of wheat, have only been studied in recent years.[116] Moreover, little is known about the inherent differences in phytochemical profiles. Those biologically active components have not received much attention as the phytochemicals in fruits and vegetables although the increased consumption of whole grains and whole grain products has been linked to a reduced risk of developing chronic diseases. [163] Our findings of

flavonoid content were also in close range with Feng et al.[177] In their study, flavonoids in the bran of four classes of wheat were characterized; three hard red winter classes and one durum class. They reported the mean flavonoid content to be 0.29 mg/g,and our corresponding flavonoid content of total DF and HRWB were 0.29 and 0.35 mg/g respectively and shown in the Table 5.3.

Table 5-3 Flavonoids content (FC in mg/g of sample) of SDF, IDF and HRWB

Sample	Total FC*	1*	2	3	4
SDF	0.07 ± 0.01	0.01	0.03	0.03	-
IDF	0.22 ± 0.09	0.07	0.07	0.08	-
HRWB	0.35 ± 0.12	0.10	0.13	-	0.12

*1 = catechin, 2 = rutin, 3 = quercetin-3-beta glucoside, 4 = epicatechin gallate, nd = not detected
Values are means of triplicates ± standard deviations (SD)

5.4.6 ORAC

Table 5.3 illustrates ORAC, DPPH and TPC findings of WEP. The antioxidant activity of WEP was measured using ORAC and DPPH assays. An ORAC assay is used to measure a sample's ability to prevent the oxidation of fluorescein generated by a peroxyl radical (AAPH) and compared with that of known antioxidant, Trolox (a water soluble analogue of vitamin E), at different concentrations [161] The ORAC value of WEP was found to be 52.48 μmol TE/g in this study. Within the same experimental conditions, our result showed a higher antioxidant activity than triticale bran, 33.9 μmol TE/g,[199] and the green and red lentil, 46.1 and 43.1 μmol TE/g, respectively.[197] In the study of Moore et al.(2005), they have reported the ORAC values of eight Maryland-grown soft wheat

samples to be in the range of 32.9 to 47.4 μmole TE/g.[149] Also in another study, the ORAC of the free fraction of six diverse whole wheats ranged from 19.5 to 37.4 μmole TE/g. [150] Our ORAC results are in agreement with both above mentioned studies. This antioxidant activity could be attributed to the presence of bound phenolic acids such as ferulic and p-coumaric acids.[203] Also, the presence of sugars with acyl groups and/or glycan-polymerization has been reported to have effects on the antioxidant activity of polysaccharides.[204]

Table 5-4 ORAC[1], DPPH[2] and TPC[3] values of WEP[4]

Anitoxidant assays	WEP
ORAC	52.48 ± 2.05
%DPPH	9.32 ± 0.18
TPC	2.96 ± 0.10

[1] ORAC = Oxygen radical absorbance capacity values was calculated as μmole Trolox Equivalent (TE)/g of sample.
[2]DPPH (%) = 2, 2-diphenyl-1-picryhydrazyl radical (DPPH) radical scavenging activity assay.
[3]TPC = Total phenolic count of crude extract was calculated as mg ferulic acid equivalent (FAE)/g of sample.
[4] WEP = Water extractable polysaccharides and values are means of triplicates ± standard deviations (SD)

5.4.7 DPPH assay

The antioxidant activity of WEP was also measured using DPPH assay. This assay measures the capacity of antioxidants to directly scavenge (react with) DPPH radicals by monitoring its absorbance at 517 nm and is calculated based on thepercentage discoloration/inhibition of DPPH.[114] The %DPPH inhibition of WEP was recorded as 9.32 ± 0.18% (Table 5.3), a values similar to to that of Mpofu et al.(2006); they have reported the %DPPH inhibition of six wheat cultivars to be in the range of 13.21 to

15.06%.[132]

5.4.8 TPC

The TPC of HRWB polysaccharide extract was analyzed by Folin-Ciocalteau method and was determined to be 2.96 ± 0.10 mg FAE/g of bran. In the study of Mpofu et al.(2006), they have reported the TPC of six wheat acidified methanol (HCl/methanol/water, 1:80:10, v/v) extracts to be in the range of 1.70 to 1.99 mg FAE /g of wheat.[132] In wheat, several major phenolic compounds are present including phenolic acids, ARs, flavonoids, phenolic acid diacyl glycerols, phenolic aldehydes and ferulates.[30] The most abundant phenolic acid compounds belong to the chemical class of hydroxycinnamic acids. The main one is ferulic acid (FA) followed by diferulic acids and by sinapic acid, p-coumaric acid, caffeic acid and benzoic acid derivatives. [115] About 95% of phenolic compounds in cereal grains are linked to cell wall polysaccharides. They are covalently bound to polysaccharides through ester bonds and classified as dietary fiber-phenolic compounds.[205]

Consequently, the addition of wheat bran in yogurt might affect the gastrointestinal tract by acting as potential prebiotics improving probiotic viability and functioning as antioxidants, especially after colonic fermentation.[30]

5.5 Conclusions

The current study investigated the potential of HRWB addition to enhance bacterial survival and growth in yogurt, and the antioxidant activity of WEP by using ORAC and DPPH assays. The outcomes of this study showed that wheat bran enhanced bacterial survival and growth in yogurt over a four week cold storage period. The overall decrease in pH and increase in TTA in yogurts containing HRWB during the storage period suggest that starter cultures and probiotics could consume wheat bran. Furthermore, WEP showed strong antioxidants activity and this activity is most probably related to its polyphenolic content. This antioxidant activity of WEP confirmed additional beneficiary effects of incorporating these natural bioactive compounds in a yogurt model system. It can be concluded that WB has potential as a source of prebiotics with antioxidant activity for future functional food and neutraceutical applications. Further investigations are needed for the evaluation of probiotic bacterial behavior in the presence of WB by employment of selective media for the enumeration of individual bacterial strains rather than total bacteria counts. Also, it would be interesting to study the sensory and rheological characteristics of yogurt containing wheat bran soluble polysaccharides and useful to know their impacts on yogurt sensorial and textural properties.

Chapter 6 General Conclusion and Future Directions

Whole grain consumption has been linked to a lowered risk of chronic diseases such as coronary heart diseases, type II diabetes, and some cancers. These health benefits may be attributed to bioactive components such as phenolics in cereal bran.[30] This study aimed to extract, characterize, and investigate the bioactivity of ARs in WB. Additionally, incorporation of bioactive phenolics and prebiotics (soluble dietary fiber) were investigated in fermented food products including bread and yogurt.

Many studies have shown that the bioactive compounds produced can be affected by different environmental factors. The effects of genotype, environment and genotype-environment interactions may affect the levels of grain antioxidants. The ***first project*** of this study aimed to determine the effects of cultivar and region on AR content and homolog composition of 24 WB samples from 4 different regions in Ontario. Moreover, the antioxidant activity of all WB samples was determined by measuring TPC, ORAC and DPPH methods. The cultivar Emmit had the highest amount of total ARs (1522 μg/g) compared to all other cultivars. Although the total AR content was different for each cultivar, the relative saturated (89%) and unsaturated (11%) ARs remained in close range. The C21:0 and C19:0 homologues were the most abundant in all WB samples. Only 1% C15:0 homologue was detected in cultivar Emmit in both Ridgetown and Nairn regions and cultivar Superior in Bath region. Also, the ratios of AR homologues C17:0 /C21:0 has been suggested to be an index for determining the source of a cereal product;[137] our study showed that these ratios could be used to differentiate between wheat cultivars. The two-way ANOVA analysis has shown that the region, cultivar and the interactions of

both variables were significant ($P < 0.001$) on all measured parameters. Mean total AR content for different wheat varieties were the highest and lowest in Ridgetown and Bath regions respectively. On the other hand, for each region, cultivars Emmit and AC Morley showed the highest and lowest mean total AR content respectively. Overall, the ANOVA revealed that for each cultivar, the lower the total ARs, the higher the relative % unsaturated ARs.

A two-way ANOVA showed that region, cultivar and their interactions were significantly different ($P < 0.05$) for TPC, DPPH and ORAC values. Cultivar Emmit grown in Ridgetown had the highest mean TPC content (58 mg FAE/g) and cultivar Harward from Palmerstone region had the lowest mean TPC content (5 mg FAE/g). This wide range shows the dependency of TPC on the type of wheat cultivar analysed. Wheat has major phenolic compounds including phenolic acids, ARs, flavonoids, and phenolic acid diacyl glycerols, phenolic aldehydes and ferulates.[30] For DPPH results, WB from Ridgetown and Bath regions had the highest and lowest antioxidant activity respectively. The overall %DPPH values of 41 soft wheats were higher than that of the 40 hard wheats measured. Also, ORAC results revealed that wheat grown in Rigdetown and Bath locations demonstrated the highest and lowest antioxidant capacities respectively. The correlation between total AR content and ORAC results was significantly related ($R^2 = 0.80$, $P < 0.05$). Overall ORAC values of soft wheat (50 μmole TE/g) were higher than hard wheat (37 μmole TE/g).

From this first project, it was discovered that the Ridgetown region has a greater potential for selected wheat cultivars to have high AR content. In the same region, the highest content of ARs has been found in cultivar Emmit, and all analyzed WB samples

had C21:0 as the most common AR homologue. Our results can be used for screening, breeding and genetic modification purposes. For future directions, the same cultivars and some others might be followed up for the following years by recording all data related to environmental conditions (climate, season, degree of maturity, soil conditions, and harvesting periods) to gain a better understanding of the relationships between cultivars rich in ARs and bioactive components. Moreover, thoroughly designed animal and human pilot studies are needed to investigate the bioavailability of wheat ARs from different wheat-based food ingredients and food products.

Cereal products are of the most important and widely consumed foods in the world. Among cereal types, wheat and rye are the most suitable basic ingredients for bread making. ARs are one of the main antioxidative phenolic compounds found in cereals. Thus, the objective of the ***second project*** was to investigate the stability of natural levels of ARs in baked breads by analyzing and comparing total and homologue AR levels before and after baking. Moreover, the effects of different solvents (acetone, ethanol, ethyl acetate, and 1-propanol) on the extractability of ARs were investigated. This study also aimed to determine how the baking process affects TPC levels as well as ORAC and DPPH potential.

Seven different bread trials have been prepared for this project, and GC-MS analysis was used to determine total content and homologue composition of ARs in each bread trial. There was a significant difference ($P < 0.05$) in the total AR content extracted by the four different solvents. Acetone gave the highest AR content compared to the other solvents (ethanol, ethyl acetate and 1-propanol). Although the total AR content in each solvent system was different, the relative saturated (92%) and unsaturated (8%) ARs

for WB samples remained in close range and the dominant AR homologues were C21:0 and C19:0 as was found in the first project. For the bread samples, a mixture of hot proponal and water was used to extract the ARs as suggested in literature.[46] This mixture of hot propanol and water was the best solvent recommended to extract ARs with high yield (82.9 mg AR/100g bread D) and release the ARs bound from starch-lipid complexes in bread. All breads tested in this study contained ARs (1.1-82.9 mg/100g bread) and it was shown that AR was did degrade during baking as was expected. Additionally, it was approved that the breads with the highest amount of ARs were also the most elevated and rised of the trial breads, and there was a positive correlation between bread heights and AR content (R^2= 0.90).

For TPC analysis, our results indicated that before baking TPC values of both WB samples were significantly different ($P < 0.05$). For ORAC analysis, our results showed that there was significant difference ($P < 0.05$) among sample values as SRWB had higher ORAC values than HRWB. Bread D (containing the highest amount of ARs) had the highest ORAC values in all of the studied bread trials. Overall, the breads high in AR content had higher ORAC values compared to the control bread. Meanwhile, DPPH analysis revealed bread D to possess the highest antioxidant activity compared to the other bread types. Our study showed that naturally occurring wheat bran ARs were heat stable during baking. ARs with amphiphilic properties, like emulsifiers, can act as dough softeners changing the gelatinization behavior of starch by making complexes with amylose and as a result delay water penetration which has also been suggested to occur during baking.[46] They may also travel to interfaces between two physical phases and thereby lower surface tension and form dispersions. Further investigations need to be

done for determining the optimum amount of ARs that should be added in bread or other food model systems.

Traditionally, ARs have been extracted by organic solvents, including acetone, ethanol and ethyl acetate.[36,79,164] Alternatively, SC-CO_2 can be used for ARs extraction which is non-toxic, non-flammable, inexpensive and faster than traditional methods.[66] Therefore, the ***third project*** aimed to test the potential of using SC-CO_2 for extracting ARs from WB and compare this green/environmentally friendly extraction method with the traditional solvent extraction method. At the same time, this project aimed to evaluate the effects of extraction solvents on the antioxidant potential of WB samples by measuring TPC, ORAC and DPPH values. Lastly, the phenolic profile of WB extract fractions was studied.

Our results showed that there was a significant difference ($P < 0.05$) in the total AR content of all the samples extracted by acetone. Although the total AR was different for each WB sample, the relative saturated and unsaturated ARs for HRWB remained in close range. The AR homologue compositions for all samples were also in close range, with C21:0 and C19:0 being the most. The total AR content of HRWB and SRWB was calculated as 75 and 64 mg/100g respectively by SC-CO_2 method. It was significantly lower ($P < 0.05$) than the traditional method. When the extraction parameters of both methods were considered, the lower AR content in SC-CO_2 extracts was expected due to solvent polarity differences (acetone versus SC-CO_2 with 10% ethanol) and extraction times (24 h versus 4 h). Although the total AR content differed between extraction methods, the homologue distribution remained analogous. The dominant AR homologues were AR C21:0 and C19:0, results similar to that of the first two projects. Overall, it must

be emphasized that the highest AR/extract ratio was obtained when a more polar organic solvents such as acetone was used. In this project, the ratio of ARs/extracts for acetone and SC-CO_2 extraction were recorded to be in the range of 15.6-10.9% to 6.6-5.1% for HRWB and SRWB respectively. Moreover, the alkaline hydrolysis and liquid-liquid partitioning steps were carried out to extract bound phenolics from WB. With HPLC analysis, ten major phenolic acid peaks were identified in the fractions which were respectively assigned as gallic, proto-catechmic, p-OH-benzoic, chlorgenic, vanillic, syringic, p-coumaric, sinapic, ferulic and o-coumaric acids. Overall, the total phenolic contents obtained in this study were in the range of 3.70-4.04 mg/g). Also, the same fractions were analyzed for flavonoid content by HPLC and six major peaks identified in the fractions were assigned as pyrogallol, catechin, epicatechin, rutin, quercetin and epicatechin gallate. The phytochemical profiles, especially the flavonoid profiles, have only been studied in recent years. For future directions, the effects of region and cultivar on phytochemical profiles need to be investigated.

Additionally, three solvent systems including acidified ethanol, 50% acetone and 100% acetone were used to extract crude WB antioxidants to investigate the solvent effects on antioxidant activity measurements. Regardless of wheat variety, the 50% acetone extracts showed the highest TPC values and all winter wheats exhibited significantly higher values ($P < 0.05$) than spring wheats. For DPPH analysis, acidified ethanol gave the highest values compared to the two solvents. Further composition analysis of WB extracts is needed to explain the different observation of the correlations between TPC and DPPH. More research is needed to investigate the chemical components involving total antioxidant activity. Also, acidified ethanol was the solvent

which gave the highest values for ORAC analysis. Two-way ANOVA revealed that cultivar, solvent, and their interactions were significantly different ($P < 0.0001$) for TPC, DPPH and ORAC values. Our study indicated that mean TPC, DPPH and ORAC values were highest in winter wheats and lowest in spring wheats. For both DPPH and ORAC analysis, acidified ethanol resulted in the highest values, whereas for TPC analysis, 50% acetone gave the highest values compared to the other extraction solvents used.

Furthermore, whole grain consumption has been reported to improve gut health.[186] Cereal grain oligosaccharides acts as prebiotics (a nondigestible food ingredients or soluble fibre) and increase the levels of beneficial bacteria in the large bowel thereby improving gut health.[189] WB might be used in food products due to its high fibre content and antioxidant properties.[188] The ***fourth project*** aimed to investigate the potential of WB soluble dietary fiber to enhance bacterial survival and growth in yogurt as well as the antioxidant activity of soluble dietary fiber from WB. Our study showed that 4% WB addition in yogurt was determined to be the optimum concentration of WB for all yogurt trials. This project outcome showed that WB improved the growth of microbial count over a four week cold storage (4 °C) period. The overall decrease in pH and increase in TTA in yogurts containing HRWB suggest that LAB (lactic acid bacteria) would consume wheat bran during the storage period. It can be concluded that WB has potential as source of prebiotics with antioxidant activity for future functional food and neutraceutical applications. Further *in vivo* investigations need to be carried out to evaluate the behavior of probiotic bacteria in the presence of wheat bran by employment of selective media for the enumeration of individual bacterial strains rather than total bacteria counts.

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