Functional properties of triticale bran bioactives using *in vitro* and *in vivo* model systems

by

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Abstract

Part of the solution to reducing the prevalence of chronic diseases is the employment of food as medicine. Whole grain consumption has been associated with a variety of health benefits owing to their bioactive constituents, namely fibre and phytochemicals. The overall aim of this study was to discover the underlying mechanisms behind the positive physiological effects of whole grains and further elucidate their nutritional value. In particular, the bioactive properties of alkylresorcinols (ARs), phenolic lipids present almost exclusively in cereal grains, and soluble dietary fibre, potential prebiotics, were investigated using triticale bran (TB), an underestimated cereal crop.

Specific objectives were met through the following projects. Conditions for large-scale extraction of ARs from cereal grains were optimized using response surface methodology (RSM). Isolation of ARs for 16-24 h at a solid-to-solvent ratio of 1:40 (weight per volume) and temperature of 24 °C produced the highest yield with the least amount of co-extractives/unknowns. The antioxidant and anti-inflammatory activities of ARs were evidenced by *in vitro* model systems, ORAC, DPPH, and COX assay whereby results were in agreement with *ex vivo* model systems, RAW 264.7 macrophage cell cultures induced by AAPH or LPS. Subsequently, the *in vivo* effects of ARs were evaluated and oxidative stress markers in liver and heart tissues of mice supplemented 0.5% ARs and mice fed 10% TB demonstrated antioxidant protection. Additionally, water extractable polysaccharides (WEP) from cereal samples were isolated by varying treatments; the highest yield with the least amount of impurities was achieved by boiling water extraction followed by successive enzyme treatments, dialysis and successive ethanol fractionations. Using yogurt as fermentation model, probiotic yogurt in the presence of TB produced significantly higher TTA values, lower pH levels, and improved microbial viability compared to controls. In addition to the prebiotic potential of TB, likely owing to its high fibre content, isolates of WEP also demonstrated antioxidant activity as measured by ORAC assay.

Findings of this study will contribute to a better understanding of the underlying mechanisms behind the health benefitting properties of whole grains.

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List of Publications

This thesis is based on the following original publications:

1. Agil R, Oomah DB, Mazza G, and Hosseinian F, Optimization of alkylresorcinols extraction from triticale bran using response surface methodology. Food and Bioprocessing Technology. 5:2655-2664 (2012).
2. Agil R and Hosseinian F, Dual functionality of triticale as a novel dietary source of prebiotics with antioxidant activity in fermented dairy products. Plant Foods for Human Nutrition. 67: 88-93 (2012).
3. Agil R, Hosseinian F, Determination of water-extractable polysaccharides in triticale bran. Journal of Food Composition and Analysis. 34: 12-17 (2014).
4. Agil R, Patterson ZR, Mackay H, Abizaid A, and Hosseinian F, Triticale bran alkylresorcinols enhance resistance to oxidative stress in mice fed a high-fat diet. Foods. 5(1): 5 (2016).
5. Agil R, Gliwa, J, Willmore WG, and Hosseinian F, Protective effects of triticale bran alkylresorcinols against inflammation and oxidative stress measured by *in vitro* and *ex vivo* model systems. *Manuscript submitted to Journal of Functional Foods*

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List of Abbreviations

AAPH	2, 2'-azobis (2-amidino-propane) dihydrochloride
AA	Ascorbic acid
ARs	Alkylresorcinols
ATCC	American type culture collection
AUC	Area under the curve
BSA	Bovine serum albumin
CFU	Colony forming unit
COX	Cycloxgenase
DF	Dietary fiber
DHBA	3,5-Dihydroxybenzoic acid
DHPPA	3-(3,5-Dihydroxyphenyl)-propanoic acid
DM	Dry matter
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTNB	5,5`-Dithiobis-(2-nitrobenzoic acid)
DuP-697	5-Bromo-2-(4-fluorophenyl)-3-(4-(methylsulfonyl)phenyl)-thiophene
EDTA	Ethylenediaminetetraacetic acid
FA	Ferulic acid
Fe	Iron
FOS	Fructooligossacharides

GC-MS	Gas chromatography/mass spectrometry
GR	Glutathione reductase
GSH	Glutathione (reduced)
GSSG	Glutathione disulfide (oxidized glutathione)
GTT	Glucose tolerance test
HPLC	High performance liquid chromatography
HF	High fat
IDF	Insoluble dietary fibre
LDL	Low density lipoproteins
LPS	Lipopolysaccharide
MeSH	β-Mercaptoethanol
Mn	Mn
MRS	Man Rogosa Sharpe
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide phosphate
NED	N-(1-Naphthyl)ethylenediamine dihydrochloride
NO	Nitric oxide
iNOS	Inducible nitric oxide synthase
OMAFRA	Ontario Ministry of Agriculture, Food and Rural Affairs
ORAC	Oxygen radical absorbance capacity
PDA	Photodiode array detector
PBS	Phosphate buffered saline
PP	Polyphenols

PVDF	Polyvinylidene fluoride
RNO	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Retention time
SC-CO_2	Supercritical carbon dioxide
SDF	Soluble DF
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
TBST	Tris-buffered saline-Tween
TE	Trolox equivalent
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TNB	Tris-buffered saline-Tween
TPC	Total phenolic content
TTA	Total titratable acidity
WEP	Water extractable polysaccharide

Chapter 1 Literature Review

1.1 Background

With more than 50, 000 edible plants in the world, only 15 plant species provide 90% of the world's food energy intake, of which cereal grains contribute to two-thirds of this supply (*1*). Due to a growing human population, an increased demand exists for nutritionally valuable, high quality cereals with low costs attached. With the limited availability of non-renewable resources, cereal crops are no longer produced exclusively for food applications but for non-food applications such as biofuels and biomass. Whole grains are staple foods due to their rich concentration of carbohydrates, dietary fibre, protein and micronutrients (*2*). Cereal grains are the edible plant seeds belonging to the grass family, *Poaceae* (or *Gramineae*); major grain types include wheat, rice and maize, as well as minor grains, rye, triticale, oat, barley and millet. A large percentage of the global cereal production is imported to developing countries that depend on these grains as their primary source of food and animal feed (*3*). To overcome the gap in supply and demand for cereal crop production, particularly in these developing countries, an alternative solution might be to provide farmers with an additional crop with a higher yield potential and better tolerance to poor environmental conditions such as drought.

1.2 Wholegrain Structure and Nutrition

The three principal components of the whole grain/kernel are the bran, germ and endosperm which respectively account for 10-15%, 3%, and 80-85% of the total grain weight (Figure 1.1). The bran is the outer coating of the seed that protects it against environmental threats such as insects, microorganisms, and poor weather conditions. The germ contains the

plant embryo and the endosperm supplies the food that allows it to germinate and grow (*4*).

According to the US Food and Drug Administration (FDA), for a food to be considered a good or an excellent source of nutrients, it must provide a minimum of 10% or 20% respectively, of the recommended daily value from a standard serving size. Thus, whole grains are an 'excellent' source of nutrients rich in carbohydrates (29%), fibre/complex carbohydrates (12-14%), minerals (0.8-1.5%), as well as B vitamins (thiamin & niacin) and vitamin E (α-tocopherol). Whole grains are also a 'good' source of protein (10-16%) and low in fat (1-3%) (*5, 6*).

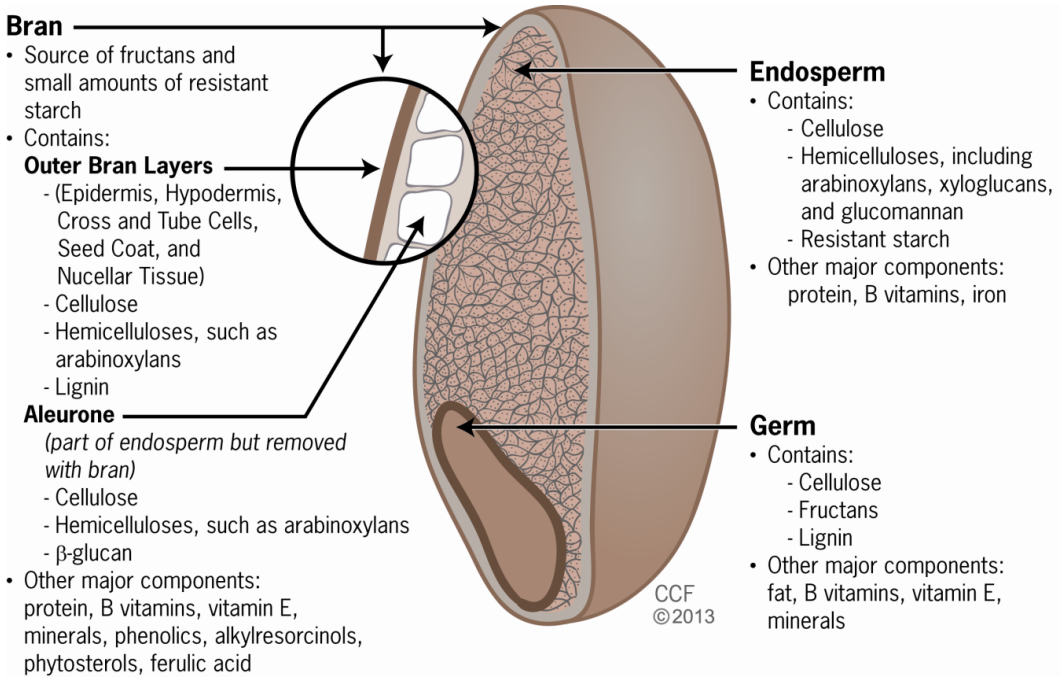


Figure 1.1 - Average wholegrain composition using wheat as an example, adopted from Bernstein et al. (*7*).

Additionally, cereal grains carry an abundance of bioactive components which are predominantly found in the bran fraction and germ fraction of cereal grains and include compounds such as phenolic acids, flavonoids, lignans, alkylresorcinols, phytosterols, tocols, and avenantramides. Consumption of whole grains protects against chronic diseases such as cancer, diabetes, cardiovascular disease, and obesity as evidenced by numerous epidemiological studies; these health benefits are generally linked to their bioactive components in situ with fibre, a proposed carrier of these bioactives in the human body (*8*). Increased satiety and transit time, reduced glycemic response, increased nutrient absorption and improved gut microflora are some of the mechanisms responsible for the disease preventative role of dietary fibre in synergy with the antioxidant, anti-inflammatory and anticarcinogenic actions of whole grain bioactives (vitamins, minerals and phytochemicals) (*9*).

1.3 Triticale

1.3.1 History

Triticale is a hybrid that was first developed in Scotland in 1875, in an effort to merge the positive attributes of parent plants wheat and rye using conventional plant breeding methods (*10*). Over a century later, research on triticale was initiated in Canada by the University of Manitoba in 1953. Their research proved to be successful when the first triticale variety, Rosner, was released in North America in 1969 followed by other varieties, Welsh and Carman (*11, 12*). However, these new triticale varieties were not without their faults, including such issues as late maturation, weak-straw, shriveled grain characteristics, low test weight, and limited yield potential due to a high incidence of sterile florets.

Thereafter, research in Manitoba was replaced by agronomy programs in Lancombe, Alberta and Swiftcurrent, Saskatchewan by Agriculture and Agri-Food Canada (*13*). The future of triticale was once again promising by the late 1980's when agronomic and grain quality limitations were overcome by genetic solutions found internationally. These new findings were applied to the development of new Canadian varieties available to farmers for commercial crop production. Triticale had become 'the crop for all seasons' due to its advantageous qualities of high yield potential and tolerance to non-optimal growing environments, attributes originating from parent plants, wheat and rye respectively (*14, 15*).

1.3.2 Varieties and Production

Cereal grain varieties of a particular species are generally categorized by their adaptability to planting at different times of the year and are either spring or winter types. Winter varieties are sown in the fall, germinate and undergo vegetative growth, become dormant during the winter season, then convert to reproductive growth in the spring once they have flowered, and mature by early summer (*16*). Winter varieties are advantageous as they are high-yielding, can be harvested early in the growing season to free up land for other crops, and require less irrigation since they can utilize snow for moisture and growth. There are currently only two varieties of winter triticale in Canada, Pika and Bobcat, which are grown in Southern Alberta and the prairies (*15, 17*). In areas where winters are too warm or too severe, spring varieties are grown; they are sown in early spring and mature by late summer.

Spring triticales are advantageous over other spring cereals due to their drought tolerance under dryland conditions. Canadian spring triticale varieties are as follows: Pronghorn, AC Alta, AC Certa, AC Copia, and AC Ultima grown in Alberta, Manitoba and Saskatchewan, as well as Banjo grown only in Manitoba (*18*). The good disease resistance of profile of spring and winter

triticale varieties aid in breaking disease cycles in cereal crop rotations which ultimately improves yield. Although research on genetic modifications of triticale varieties have been conducted in the US and Europe, there are currently no GM varieties of triticale as they have all been developed through selective breeding processes(*14, 15*).

World production of triticale has grown considerably within the past decade by 75%, due predominantly to the considerable growth of and research on triticale in Europe. The leading producer of triticale on a global scale is Poland with just over 4.2 million metric tonnes produced in 2013 alone. Canada contributed only a small fraction to the world triticale production with less than 30, 000 metric tonnes produced in 2013 (*19*). Nonetheless, there has been a steady increase in the production of triticale in Canada from 17, 000 hectares in 1996 to 300, 000 hectares in 2003(*15*). In order for this growth to continue, the potential for this crop and its diversity of applications must be realised (*18*).

1.3.3 Applications

Although the main use of triticale has always been as a source of animal feed, current applications include that of food products, and industrial uses (*14, 15, 20-29*). The use of triticale grains for animal feed among other cereal grain types can be associated with its high energy content, and stable compositional quality. Triticale grains have a rich and desirable content of minerals and vitamins with generally higher levels of fibre and protein than wheat and rye respectively. The high protein content of triticale, as well as its generally higher levels of the amino acid lysine than that of barley and wheat, one of the limiting amino acids in cereal grains, provides the advantage of a decreased need for protein supplementation in the diet of ruminant animals (*6, 15, 26, 28, 30*). Although triticale starch content and fermentation is generally equal to that of other cereal grains(*31*), its digestibility is higher than barley and oats which is

important for maximizing the availability of nutrients for growth (*32*).

With the limited availability of non-renewable resources, cereal grains are no longer produced exclusively for food and feed applications, but for their non-food applications such as biofuel, particularly bioethanol. Sugars derived from the starchy cereal grains of wheat or triticale are what allow for the production of bioethanol through the process of fermentation. While the high crop yield and low cost of triticale make it an ideal grain choice for bioethanol production, it has also demonstrated high alcohol yield likely due to its high starch content and protein composition (*23*). In addition to the grain, triticale straws, which are the dry stalks that remain after the grain and chaff have been removed, are also used for industrial applications as biomass for power generation (*33*).

While the human food market for triticale and its incorporation in multi-grain breads is very low, the high energy and lysine content of triticale make it a very suitable grain for human diets. Triticale has comparable levels of protein and gluten strength to that of wheat while also adding a nutty flavour to food products. In addition to the potential application of triticale in traditional bread making, it has also been used to make breakfast cereals, health-food bars, and international foods such as the middle-eastern sweet, halwa, and Ethiopian bread, injera (*29*). The poor bread making quality of rye and the digestibility problems of barley in cattle increase the need for exploring other avenues of grain sources such as triticale. Although triticale is an excellent candidate for animal feed, it has yet to be well recognized for human food applications due to the insufficient demand for its significant utilization in food products and lack of efforts invested in triticale breeding for food use. Factors which may be attributed to the limited research data available on the composition and functional characteristics of triticale composites, particularly in the bran (*26, 34*).

With more than 50, 000 edible plants in the world, only 15 plant species provide 90% of the world's food energy intake, of which cereal grains contribute to two-thirds of this supply (*1*). Due to a growing human population, an increased demand exists for nutritionally valuable, high quality cereals with low costs attached. With the limited availability of non-renewable resources, cereal crops are no longer produced exclusively for food applications but for non-food applications such as biofuels and biomass.

Whole grains are staple foods due to their rich concentration of carbohydrates, dietary fibre, protein and micronutrients (*2*). Cereal grains are the edible plant seeds belonging to the grass family, *Poaceae* (or *Gramineae*); major grain types include wheat, rice and maize, as well as minor grains, rye, triticale, oat, barley and millet. A large percentage of the global cereal production is imported to developing countries that depend on these grains as their primary source of food and animal feed (*3*). To overcome the gap in supply and demand for cereal crop production, particularly in these developing countries, an alternative solution might be to provide farmers with an additional crop with a higher yield potential and better tolerance to poor environmental conditions such as drought.

1.4 Alkylresorcinols

1.4.1 Sources and Structure

Alkylresorcinols are phenolic lipids that were first isolated in 1989 from *Ginkgo biloba* (Ginkgoaceae) and have been detected in numerous other sources thereafter. The wide array of AR sources in nature includes families of higher plants, fungi, algae, mosses, bacteria as well as the marine sponge, *Haliclona sp.*, belonging to the animal family (*35*). The amount of ARs is incredibly variable across different families and species. Cereal grains of the Gramineae family

possess the highest consumable levels of ARs (*36*). The highest levels of ARs are found in rye, followed by wheat and triticale, with low levels reported in barley (*36-43*).

According to past studies, trace amounts of ARs have been detected in rice, millet, oat, corn and sorghum (*44-49*), however, other studies found no ARs to be present in these grains (*36, 39, 50*). There is also a large variation in AR content of within and between species due to environmental, agricultural and genetic factors (*51-53*). As well, levels are impacted by grain maturity such that AR content is highest during the early stages of grain development and decreases as the grain matures (*45*). However, during baking and processing, ARs are stable and levels remain relatively unchanged throughout (*54*).

ARs are 1, 3-dihydroxy-alkylbenzene derivatives with an aromatic ring and an alkyl chain generally at the C-5 position (Figure 1.2). Depending on the source, the structure of these resorcinolic lipids can vary in degrees of unsaturation, chain length, ring or chain substituted functional groups on the alkyl chain or aromatic ring, and the position of the alkyl chain (*35, 55*). ARs generally occur as mixtures of several homologues and derivatives. The most common homologues in cereal grains are saturated 5-*n*-alkylresorcinols ranging in chain length from C15-C25. As seen in Figure 1.3, substituted (5-(2-oxoalkylresorcinol) and 5-(2-hydroxyalkylresorcinols) and mono-unsaturated (5-alkenylresorcinols) derivatives have also been detected, although to a much lesser extent (*37, 39, 56-58*).

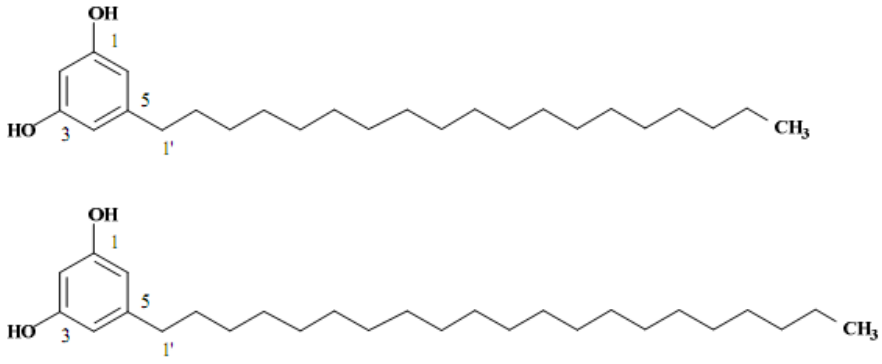


Figure 1.2 - The major 5-n-alkylresorcinols in triticale, C:19 and C:21 homologues (*59*).

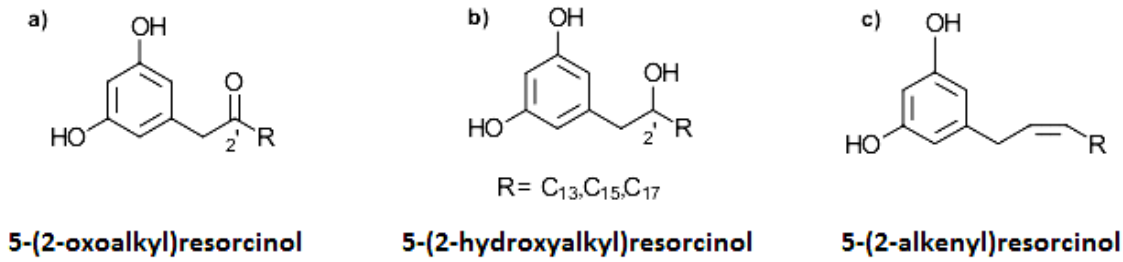


Figure 1.3 – Examples of **(a)** -oxo and **(b)** –hydroxy substituted 5-n-alkylresorcinols and **(c)** monounsaturated 5-n-alkenylresorcinols (*35*).

Found almost exclusively in the bran, particularly the seed coat/testa, ARs contribute to 0.01-0.15% of the whole kernel dry weight (*41*) with only trace levels identified in the endosperm and germ layers (*36, 39, 60, 61*). The AR content in the bran fractions of a given cereal is often two to five times higher than its respective whole grain content (*36, 39*). A valuable property of ARs is the ratio of homologues C17 to C21 which is unique for each cereal

grain types, thereby allowing for a method of distinction amongst consumed grains (Table 1.1). The ratio of homologues C17:0/C21:0 is approximately 0.2 in triticale, 0.1 in wheat and 1.0 in rye bran (*38, 40, 55*).

Table 1.1 - Ranges of alkylresorcinol content in whole, bran, and flour fractions of cereal grains (μg/g DM), their predominant homologues, and C17:0/C21:0 ratios, adopted from Agil and Hosseinan (*62*).

Cereal	Cereal Fraction	Range (μg/g DM)	Predominant Homologues	C17:0/C21:0
Rye	Whole Grain	560-1444	C19, C21	0.8-1.3
	Bran	2400-4108		
	Flour (endosperm)	69-79		
Common Wheat	Whole Grain	264-943	C21, C19	0.09-0.24
	Bran	2210-3225		
	Flour (endosperm)	29-45		
Triticale	Whole Grain	430-700	C21, 19	0.18-0.55
	Bran	2780-3080		
Barley	Whole Grain	41-74	C25, C21	0.05-0.46
	Bran	210		

1.4.2 Biosynthesis

Alkylresorcinols are just one of numerous types of plant secondary metabolites containing a phenol group and are collectively referred to as phenolics, compounds containing one or more aromatic rings bearing a hydroxyl functional group. Unlike primary metabolites, secondary metabolites are not essential for growth and development; however, they aid in plant growth and survival by serving various roles such as attractants for pollinators (anthocyanins),

structural roles (lignins), protectants against UV rays (carotenoids), and defence against insects and fungi (cyanogenic glucosides)(*63*).

Although phenolics have a wide range of chemical structures and biological activities, they are derived from one of two main routes of biosynthesis the acetate/malonate pathway and the shikimate pathway. Primary metabolites, erythrose-4-phosphate and phosphoenolpyruvate, products of glycolysis and the pentose phosphate cycle, enter the shikimic acid pathway which goes on to branches out and form two major classes of phenolics, phenylpropanoids and hydrolysable tannins.

The acetate/malonate pathway utilizes primary metabolite, acetyl-CoA, a product of glycolysis, as a precursor for the synthesis of phlorotannins and polyketides. Polyketides are synthesized in a manner similar to that of fatty acids such that acetyl-CoA is converted to malonyl –CoA by acetyl-CoA carboxylase, followed by a series of condensation reactions with several malonyl-CoA derived extender units via polyketide synthases (PKSs). Three types of PKS exist, type I, II, and III, the latter of which is responsible for the synthesis of resorcinolic lipids (*64*). In particular, alkylresorcinol synthases (ARSs) are type III PKS which produce 5-n-alkylresorcinols (*65*). Unlike fatty acid synthases, polyketide synthases add the next C_2 unit to the growing chain before reduction of the previous unit to a methylene group is complete. This slight variation is what leads to the incorporation of double bonds and other functional groups such as hydroxyls and keto groups in the growing chain, resulting in the formation of poly-β –ketoacids. These linear reactive poly-β –ketoacids favor an intramolecular condensation reaction leading to the formation of a ring structure likely due to the nucleophilic and electrophilic potential of the methylene and carbonyl groups within the poly-β –ketoacids chain (*35*).

The biosynthesis of 5-alkylresorcinols begins with a condensation reaction of a fatty

acyl-CoA starter unit with three malonyl-CoA extender units forming a tetraketide intermediate. Following ring closure and dehydration to form a benzene ring, coenzyme A is released by hydrolysis, resulting in 6-alkylresorcinolic acid which undergoes nonenzymatic decarboxylation to form 5-alkylresorcinol (*64*). As seen in Figure 1.4, the 5-alkylresorcinol chain length is dependent on the acyl-CoA precursor such that C15 homologue, 5-pentadecylresorcinol would likely involve palmitoyl-CoA (16:0) as the starter unit (*65*). In the case of stilbenes, chalcones, and flavonoids, the latter of which is the largest category of phenolics, they are synthesized through a combination of both the shikimic acid and acetate/malonate pathways (*66*).

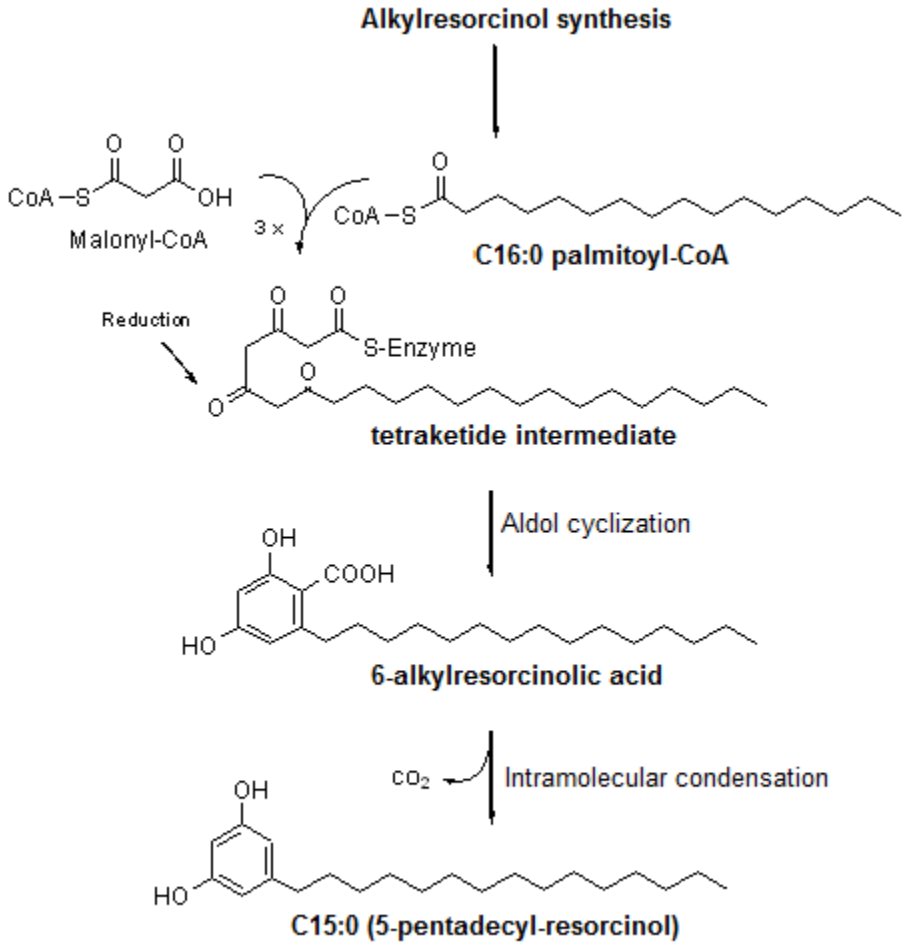


Figure 1.4 - Predicted mechanism of 5-n-alkylresorcinol synthesis by example of C15:0, adapted from Baerson(*65*) and Dayan(*67*).

1.4.3 Alkylresorcinols as biomarkers of wholegrain consumption

Cereal grains are the only source of ARs for feeding purposes, making them an excellent candidate for biomarkers of wholegrain intake. The role of ARs as biomarkers is extremely important for the validation of disease preventative properties and health benefits resulting from the inclusion of whole grains cereals in the diet (*68, 69*). Numerous studies on the pharmacokinetics of ARs *in vivo* and in vitro have determined that levels of whole grains consumed in the diet correlate with levels of intact ARs measured in the faeces as well as AR metabolites extracted from urine samples of these individuals (*60, 69-72*). Recently, Jansson et al. (2010) suggested that ARs can also be used as long-term biomarkers of whole grain intake because a small percentage of ARs consumed from whole grains often reside in the adipose tissue and accumulated proportions correlate with long term intake (*73*).

1.4.4 Pharmacokinetics

1.4.4.1 Digestion and Absorption

Depending on the dose and source (e.g. pericarp/testa vs. aleurone layer), AR digestibility and absorption varies between 60-79%, wherein AR uptake decreases with increasing levels consumed (*74-76*). AR digestibility may also be affected by other foods administered, as suggested by a study demonstrating AR absorption increased when subjects were served an AR free meal 4h after AR intake (*77*). The effect of nibbling (7 small meals/day) and ordinary (3 large meals/day) meal frequencies on AR digestibility has also been studied with no variations

detected amongst them (*75*).

In a study where rats were fed C21:0 radiolabelled ARs that were traced in their blood, urine, feces and body tissues, it was determined that AR levels never fully reach zero (*74*); these results were in agreement with other studies measuring AR concentrations in plasma (*60, 77-81*). It is proposed that this occurrence may be due to enterohaptic circulation, slow turnover of ARs liberated from storage in body tissues, or the individual and specied dependent differences in the mechanism by which ARs are metabolised, stored, or released after intake (*60, 77, 82, 83*).

The absorption pathway of ARs is theoretically similar to that of structurally related fat soluble compounds such as tocopherols. Bile acids in the gut allow for the solubilization of such hydrophobic molecules as tocopherols and ARs into micelles (*84*). Through passive diffusion, micelles cross the unstirred layer and release their contents into the enterocyte membrane where ARs are packaged into chylomicrons and secreted into the lymphatic system where they are broken down by lipoprotein lipase (*74, 77, 85*). Chylomicron remnants carrying residual compounds such as ARs are then taken up by the liver where they are packaged in VLDL and carried to various tissues of the body such as skeletal muscle or adipose tissue (*41, 85*). Some of the ARs from VLDL may also be transferred to HDL or end up in LDL as a result of VLDL metabolism (Figure 1.5).

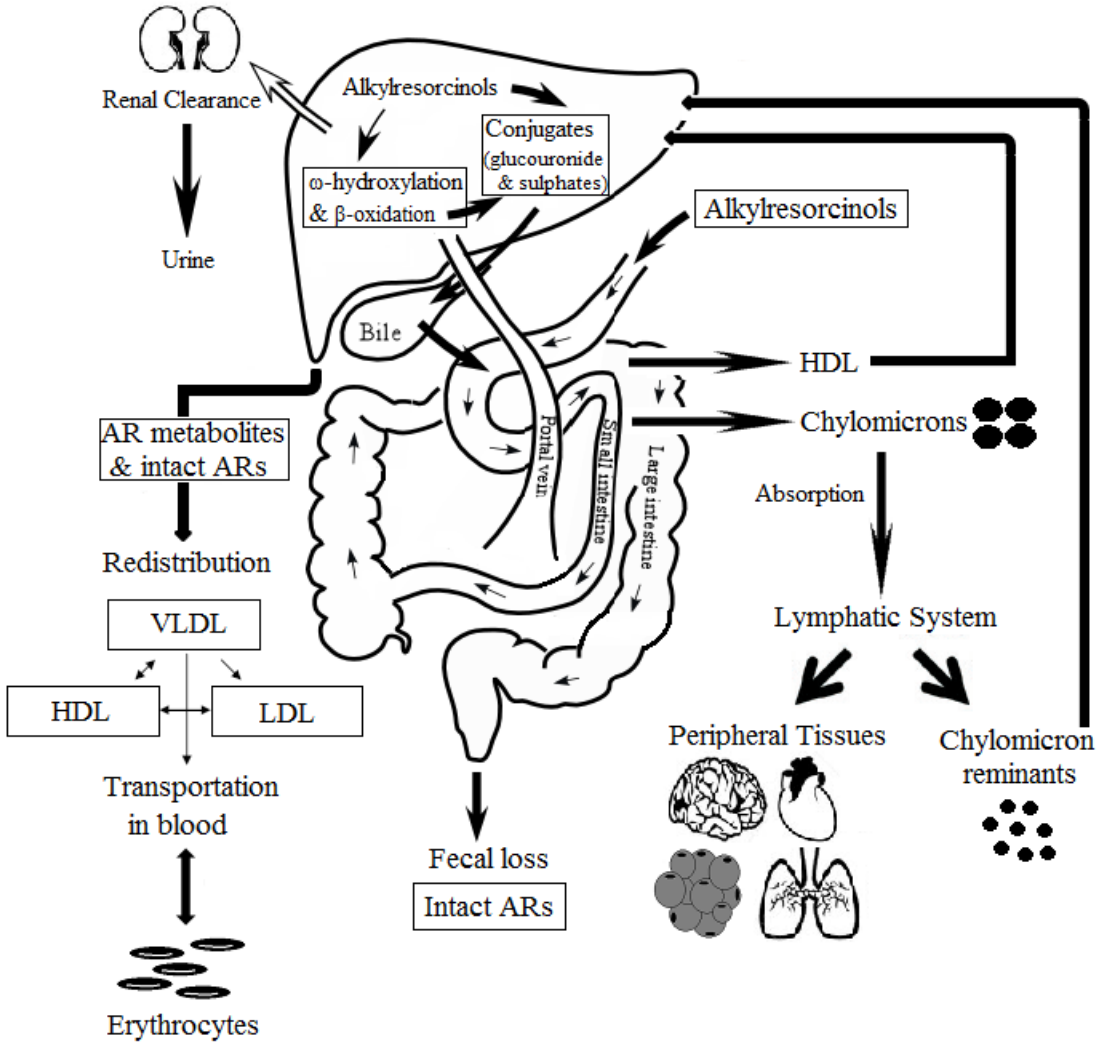


Figure 1.5 - Proposed pathways for alkylresorcinol (AR) absorption, distribution, metabolism and elimination adapted from Hosseinian(*86*).

Due to the amphiphillic nature of ARs, they are easily incorporated into biological membranes as seen in both *in vitro* (*35, 87*) as well as *in vivo* studies (*78, 85*) whereby longer-chain AR homologues (C17:0 and C19:0) exhibited a greater ease of integration into membranes than their shorter-chain counterparts. Erythrocyte AR membrane levels increase with wholegrain consumption and remain stable in fasting blood samples; this correlation suggests that ARs are

incorporated into and potentially stored in erythrocyte membranes (*78*). Variation in uptake patterns of short and long chain AR homologues could not be distinguished in studies on pig plasma kinetics and ileal recovery results in both humans and pigs (*74, 75, 82*). However, studies on the human plasma kinetics of ARs found there to be a variation in the absorption of patterns of AR homologues such that relative bioavailability increased with increasing chain length (*77*).

1.4.4.2 Metabolism and Elimination

The proposed mechanism of ARs metabolism is based on the metabolic stages of structurally similar tocopherols and is summarized in Figure 1.5. ARs metabolism is initiated by a cytochrome P450 enzyme whereby hydroxyl groups on the phenolic ring are conjugated then undergo ω-oxidation, followed by shortening of the alkyl chain and conversion of the ω-hydroxyl group to a carboxylic acid. Lastly, a series of stages of β-oxidation follow resulting in the formation of metabolites, DHPPA (3-(3,5-Dihydroxyphenyl)-1-propanoic acid) and DHBA (3,5-dihydroxybenzoic acid). A proportion of these metabolites may undergo a second phase of metabolism prior to urinary excretion, where they are conjugated to glucuronides and sulfates to increase their polarity and water solubility (*41*).

AR metabolites, DHPPA and DHBA, have been detected in both conjugated and free form in rat urine (*74*), human urine (*81, 88-93*) and human plasma (*94-97*). Metabolism and conjugation of ARs was first evidenced when recovery of radiolabelled AR in urinary samples of rats was greatly increased through application of enzymatic hydrolysis. This suggested that metabolites of AR were present in urine, while this was not the case in feces (*74*). It was later determined that the more hydrophilic metabolite DHBA is mainly unconjugated in urine in comparison with DHPPA (*93*). The extent of this variation was found mainly in glucuronide conjugates where 67% of total DHBA were present in urine as free aglycones versus only 46%

of total DHPPA existing as such. In the case of sulfate conjugates of DHPPA and DHBA, no apparent variations in the extent of conjugation amongst the two metabolite forms were found (*90*).

Absorbed ARs are likely secreted into bile where they are metabolized to more water soluble forms due to the highly insoluble nature of ARs in water, DHPPA and DHBA, to be carried by the bloodstream to the kidney and then excreted as conjugated and free forms in urinary waste (*41, 93*) as seen in Figure 1.5. A small proportion of absorbed ARs may also be stored and accumulated in body tissues (adipose tissue and/or biological membranes); this is mainly evident in cases of prolonged feeding of AR-rich diets, as opposed to a single dose feeding (*41, 74, 78, 93, 98*). ARs not absorbed by the small intestine pass through the digestive tract and are eliminated mainly as unchanged ARs in fecal waste (*74*).

1.4.5 Health Benefits

1.4.5.1 Antioxidant Activity

Reactive oxygen species (ROS) are standard products of aerobic metabolism generated as a host cell defense mechanism against foreign invaders. Oxidative stress is the result of an imbalance of ROS in which the level of oxidants exceeds that of antioxidants and leads to damaging effects on cellular components such as DNA, protein, and lipids. Such damaging effects to crucial cellular components can contribute to an increased risk or prevalence of degenerative diseases such as cancer (*99, 100*). Compounds capable of preventing or delaying oxidative degradation at concentrations much lower than those of an oxidizable substrate are classified as antioxidants. The degree of antioxidant activity of a compound is dependent on its structural properties which determine whether it can donate hydrogen atoms or electrons to free radicals to produce a stable chain breaking product (*101*).

Tocopherols (Vitamin E) are well established natural antioxidants in living systems due their structural components, particularly the hydroxy group that serves as the main source of their radical scavenging and hydrogen donation abilities as well as their hydrophobic phytyl chain that allows for their solubility in cell membrane (Figure 1.6a) (*102*). The structural resemblance of ARs to tocopherols has been cause for speculation that they too hold antioxidant potential *in vitro* due to their hydroxyl groups attached to the aromatic ring at C1 and C3, as well as *in vivo* antioxidant membrane protection due to their lipophilic alkyl tail that eases incorporation into cell membranes (Figure 1.6b) (*8, 103*). Although they are considered weak antioxidants in comparison to tocopherols, they do possess antioxidant properties which are more evident *in vivo* than *in vitro* (*103-112*).

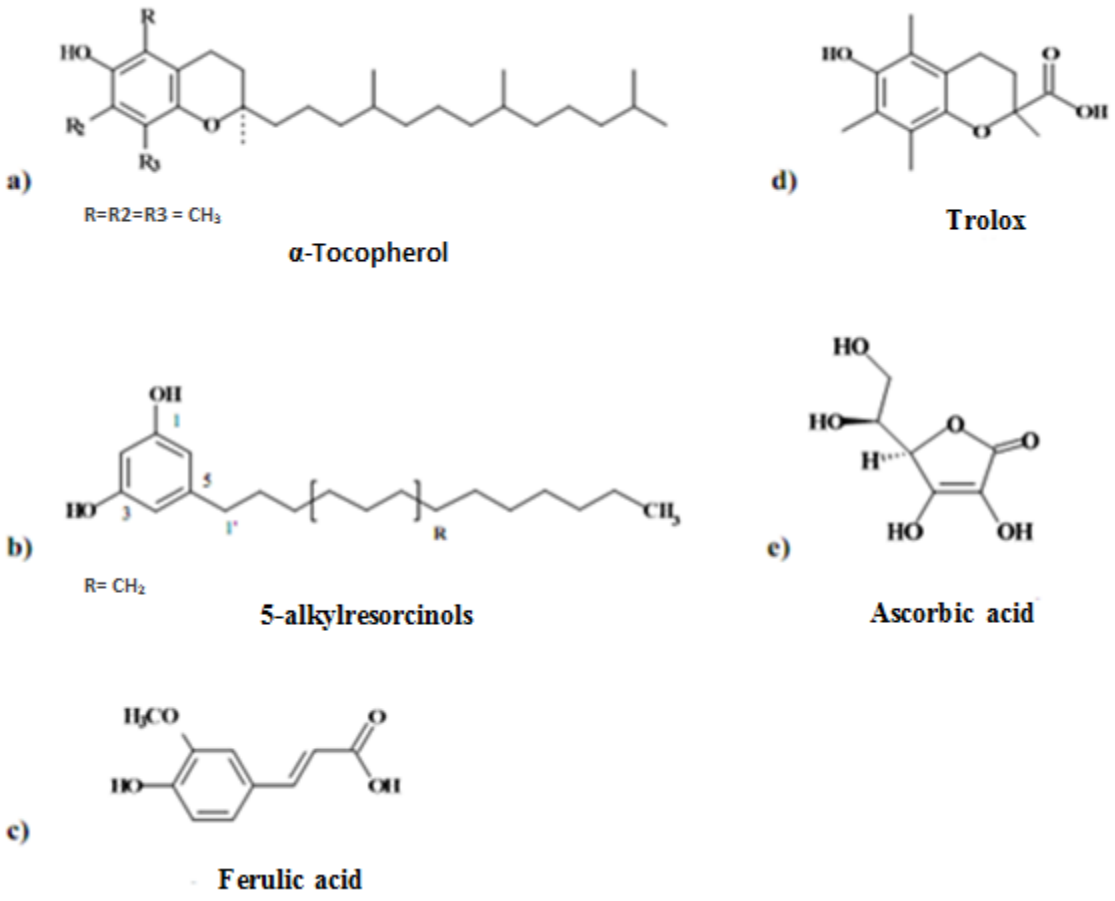


Figure 1.6 - Chemical structures of standard antioxidants compared to that of 5-alkylresorcinols (*62*).

Lipid peroxidation (Figure 1.7) involves hydrogen abstraction to form a lipid radical ($L^{\bullet}$) (equation 1), which can react with oxygen to form a lipid peroxyl radical (equation 2). The peroxyl radical propagates the chain reaction by abstracting hydrogen from another lipid (equation 3), usually the rate-limiting step in lipid peroxidation (*113*). Cell membrane constituents, phospholipids and proteins, are the direct targets of lipid oxidation (*114, 115*). As lipid oxidation of cell membranes progresses, the polarity of the lipid-phase and oligomer protein formation increases. However, the molecular mobility of lipids, number of sulfhydryl (SH)

groups, and resistance to thermo-denaturation decrease (*115*). Increased levels of lipid oxidation products are associated with diabetes and atherosclerosis (*114, 115*). Oxidation of low-density LDL has been reported to be involved in the development of atherosclerosis and cardiovascular disease (*114, 115*). Oxidized cholesterol or fatty acid moieties in plasma LDL can lead to the development of atherosclerosis (*114*). Ideally, antioxidants work by blocking the propagation step through hydrogen atom donation. Antioxidants can also interfere with the oxidation process by chelating catalytic metals and acting as free radical scavengers (*116*).

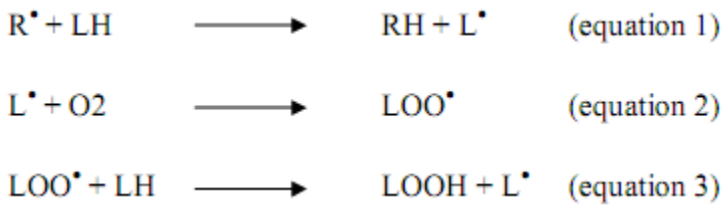


Figure 1.7 - Lipid peroxidation reaction sequences (*62*).

In suspensions of erythrocyte injected with hydrogen peroxide and homologs of 5-n-alkylresorcinols, ARs were found to decrease peroxidation products induced by hydrogen peroxide at micromolar concentrations. Homolog C15:0 had the greatest antioxidant effect followed by C19:0, C23:0 while C5:0 (olivetol) had minimal effects. In liposomal emulsions of phosphatidyl choline, AR homologues extracted from rye protected lipids and fatty acids of the membrane bilayer against Fe^{2+} induced peroxidation at micromolar concentrations; the level of protection by ARs positively correlated with AR chain length (*106*). These results suggested that the antioxidant potential of AR homologs is dependent on their incorporation into the lipid bilayer (Figure 1.8) and their ease of incorporation is determined by their chain length (*104*).

Winata and Lorenz (*105*) determined that AR homologue, pentadecylresorcinol (C15:0), effectively slowed the rate of oxidative rancidity in oils as well as lipid oxidation in cereal products at AR concentrations of 0.050-0.075%. A recent study also identified that AR derivative, 1,3-dihydroxy-5-(tridec-4′,7′-dienyl), effectively inhibited lipid peroxidation of linoleic acid measured by an FTC (ferric thiocyanate) method (*117*). These findings suggest that ARs may prove to be useful as natural antioxidants in the food industry (*118*).

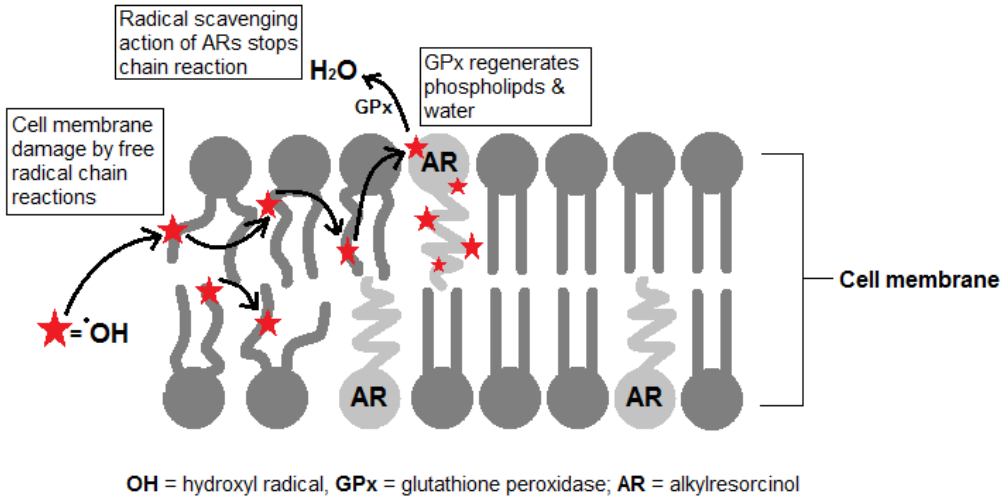


Figure 1.8 - Theoretical illustration of a hydroxyl radical (•OH) scavenged by ARs incorporated in cell membrane.

DPPH is a stable free radical commonly used in studies to measure the kinetics and hydrogen donating potential of AR homologues in comparison to well-known antioxidants, vitamin E tocopherols, trolox, ferulic acid (FA), and ascorbic acid (AA) (Figure 1.6b-e) (*103, 107, 108, 117, 119*) . Through the employment of a DPPH assay, ARs (C15:0-C25:0) decreased the initial DPPH concentration by 50% at EC_{50} concentrations of 157-195 µM in comparison to tocopherols with an EC_{50} range of 16-27 µM (*108*). This study and others employing such

methods as the AAPH induced chemiluminescence method, FRAP (ferric reduction ability of plasma) and autoxidation of triacylglycerol/PUFA substrates, found ARs were only effective at higher concentrations that were double that of known antioxidants, ferulic acid and ascorbic acid (*103, 117*) triple that of trolox (*108, 119*) and over ten times that of tocopherols (*107*), making them comparatively weak antioxidants. Nonetheless, ARs possess sufficient antioxidant strength for use as natural antioxidants in food industry applications (*108*).

The antioxidative potential of ARs in preventing oxidation of lipoproteins induced by copper (II) was measured in human plasma LDL by measuring conjugated diene complex formation over time. Results showed that ARs, particularly C15:0, effectively delayed LDL oxidation by 65 min at concentrations of 25 μM; thus AR antioxidant activity is more prevalent in lipophilic interactions within biological systems (*120*).

1.4.5.2 Antiinflammatory Activity

Arachidonic acid is an unsaturated fatty acid present in cell membranes and is freed from attachment to phospholipids of the bilayer by Ca^{2+} stimulation of phospholipase enzymes. This release initiates a cascade of metabolic events resulting in the production of various pro-inflammatory eicosanoids such as thromboxane (TX) which induce platelet aggregation (*121*). ARs can inhibit human platelet TX production by blocking phospholipase activity or enhance TX synthesis by increasing membrane permeability at higher AR concentrations. This increase in membrane permeability allows cations such as Ca^{2+} to pass through; thereby stimulating phospholipase activity that leads to an increase in TX levels *in vivo* (*122*).

1.4.5.3 Anticancer Activity

Several studies, although in vitro, have demonstrated the effectiveness of ARs and related resorcinolic lipids as cytotoxic agents against various cancer cell lines (*117, 119, 123-129*). In one particular study, DNA damage acquired by HT29 human colon cancer cells due to exposure to genotoxic substances: hydrogen peroxide (H_2O_2) and fecal water, was effectively inhibited by AR homologues by 40% at concentrations of 100 μM and 50 μM against H_2O_2 and fecal water respectively (*103*).

As suggested earlier for the antioxidant activity of ARs, the hydroxylation at C-1 and C-3 and chain length at C-5 are also significant structural properties responsible for the level of cytotoxic protection by ARs (*125*). In a series of studies conducted by Gasiorowski et al. (*130*), it was determined that ARs possess antimutagenic activity in lymphocyte cultures against direct acting genotoxic compounds (Mitomycin C, MMS and DRC) and indirect promutagens (benzo[a]pyrene and 2-AF) by stimulating apoptosis of highly damaged cells (*130-132*). Later studies confirmed these results, such that ARs exhibited cytotoxic effects against human colon tumour, promyeloid leukemia, hepatocellular carcinoma cell lines and squamous carcinoma cells by inducing programmed cell death (*128, 133, 134*).

In addition to increasing apoptotic elimination of cells damaged by mutagens, ARs also employ another mechanism of cytotoxic action known as DNA cleavage. Cancer cells often develop resistance mechanisms to anticarcinogenic agents by adopting the ability to repair DNA damaged by such agents, thereby lessening their effects. Therefore, the solution to this particular predicament is to target the proteins involved in the DNA repair pathways such as base excision repair (BER), nucleotide excision repair (NER), and DNA mismatch repair (*135*). In this context, ARs have been reported as mediators of DNA damage by strand scission in the presence of Cu

(II), and in some cases O_2, as well as inhibiting DNA polymerase β from repairing the damaged strands (*135-137*). Once again, chain length was found to have an impact whereby the success of DNA cleavage by ARs was greater with increasing chain length of up to 17 carbons (*135, 137*). Therefore, findings from the aforementioned studies on the anticarcinogenic effects of ARs suggest the optimal chain length for cytotoxic action to be that of 15 to17 carbons in the alkyl chain (*35, 41*).

1.4.5.4 Anti-obesity Activity

It has been speculated that ARs cause appetite suppression in animals by observation of farm animals on a rye-rich feed, therefore a feed rich in ARs, showed poor growth in comparison to those consuming other cereals such as wheat or maize. Although several animal studies have been conducted on this basis, results remain inconclusive due to contradicting findings. Some studies have found a direct correlation between diets rich in rye with antinutritional effects whereas others have found no such effect on growth by ARs. It has been proposed that the effect of ARs on reducing animal growth has been attributed to soluble fibers in rye as the causative factor, particularly arabinoxylans (*41*).

As a result of the interaction of ARs with various enzymes, a range of metabolic effects have been documented. Glycerol-3-phosphate dehydrogenase (GPDH) is a membrane enzyme essential for lipid and carbohydrate metabolism in addition to sustaining the membrane potential across the inner mitochondrial membrane during glycolysis (*138*). Triglyceride synthesis and storage in adipose tissue is dependent on the generation of its precursor glycerol-3-phosphate which is controlled by the activity of the dehydrogenase enzyme, GPDH. The activity of this enzyme is most pronounced during cell differentiation into adipocytes. NAD is a substrate which

binds to the coenzyme domain of GPDH (glycerol-3-phosphate dehydrogenase) causing changes in its conformation resulting catalyzed enzyme activity. *In vitro* studies suggest that ARs inhibit triglyceride synthesis by competing with NAD for the GPDH binding site, thereby inhibiting enzyme activity. This in turn prevents triglyceride synthesis as seen *in vivo* by the reduced accumulation of triglycerides during 3T3-L1 cell differentiation (*139-141*).

Leptin is an adipose derived hormone that regulates energy intake and metabolism; It acts to stimulate glycogen synthesis by inhibition of phosphorylase *a.* In the presence of AR related compound, resorcinol (1,3-Dihydroxybenzene), the inhibitory effect of leptin has been found to be enhanced (*142*). ARs and their relative analogues have also shown to reduce insulin absorption in intestinal loops isolated from anesthetized dogs (*143*).

1.4.5.5 Antimicrobial Activity

Due to their strong antibacterial and antifungal activity, ARs are biosynthesized specifically during the seedling stage to protect the plant against predators (*35, 43*). Various sources of ARs from cereal grains (*43, 144*), to the waxy epicuticular layer of barley (*145*), and the peel of mango fruits (*146, 147*) have been isolated and exhibited antifungal activity against a number of types and strains of pathogenic fungi such as *Aspergillus niger, Penicillium chrysogenum, Fusarium culmorum* and *Alternaria alternata*. The inhibitory capacity of ARs against microorganisms is more pronounced in resorcinolic lipids with a greater degree of unsaturation, chain length, and hydroxyl groups and a greater antimicrobial effect can be observed against gram positive bacteria versus gram negative (*148*). Alkylresorcinol homologues with longer and more saturated chains also exhibit antiparasitic activity against *Briomphalaria glabrata* (mollusks) and *Filaria* (worms) (*35, 123, 149*).

1.4.5.6 Membrane Interactions

Similarly to phospholipids, resorcinolic lipids consist of a hydrophilic head (polar aromatic ring) and a hydrophobic tail (alkyl chain at C-5). This amphiphillic nature of ARs is what allows them to easily incorporate into cell membranes, resulting in changes in membrane structure and properties such as phospholipid mobility, membrane stability, fluidity and permeability (*35, 41, 85, 120, 150*). In an effort to mimic biological membranes, liposomes have been constructed from various mixtures of phospholipids and compared to similar systems incorporating ARs. The resulting effect of utilising ARs for liposomal membranes was enhanced stabilization of the bilayer as well as effective entrapment and retention of aqueous solutions. These effects of ARs were further enhanced by the presence of other lipophilic compounds (i.e. cholesterol, fatty acids) in the membrane and when ARs were incorporated prior to bilayer formation (*150-153*). In a recent study investigating methods to improve sphingomyelin–cholesterol liposomes for drug delivery purposes, it was found that the incorporation of ARs enhanced solute encapsulation *in vitro* and reduced the elimination rate of the liposome from circulation *in vivo* (*154*). Due to the enhancing effects of ARs on liposome stability, the application of ARs in liposomal drug delivery has been proposed (*155*).

1.4.6 Methods of Extraction

The methods for extraction of alkylresorcinols from cereal grains have been a research topic of interest for over half a century now, and have been progressively evolving over the years. To date, there are three main techniques that can be used to extract alkylresorcinols from grains; traditional solvent extraction, soxhlet extraction and super critical carbon dioxide extraction. The afore-mentioned techniques will be discussed in further detail in the following sections.

1.4.6.1 Traditional Solvent Extraction

Resorcinolic lipids are generally amphiphillic compounds and with increasing chain length (>10 carbon alkyl chain) they are rendered insoluble in water (*35*). For this reason, ARs from cereal grains are commonly extracted using hydrophobic organic solvents such as acetone, ethyl acetate, methanol, ethanol, diethyl ether and hexane (*55*). Although most ARs are soluble in methanol and ethanol, the longer chain homologues are not extractable to the same extent as they are in acetone and ethyl acetate. Additionally, the use of methanol as a solvent choice is discouraged due to its toxicity, it's poor ability to extract longer chain homologues C: 23 and C: 25, and it's tendency to extract other undesirable co-extractives (*156*).

The amount of solvent used per weight of grain product can often vary but the commonly used ratio of sample weight (g) to solvent volume (ml) is between 1:40 and 1:50 (w/v; g/mL). A large volume of solvent is used to completely immerse the grains and thereby allow maximum surface area coverage of extractable ARs thereby increasing yield. In some cases, where lesser solvent volumes are used, multiple extraction steps are often required (*55*).

Since ARs are highly concentrated in the outer layers of cereal grains (pericarp/testa and aleurone layer), it would be apparent that separation of the different layers by milling should be carried out in order to reduce extraction time as well as sample amount used (*41*). However, milling can also have negative effects such as increasing the level of undesirable co-extractives and making further analysis more difficult (*156*). Studies have shown that an extraction time of 16-24hr is ideal in extracting a high yield of AR homologues (*41, 157*).

1.4.6.2 Soxhlet Extraction

In 2004, Zarnowski and Suzuki conducted a study that successfully established an

alternative to previously published procedures (*158*). This new method involved the application of a Soxhlet apparatus whereby lipids were isolated and yielded extracts differed in their resorcinolic lipid contents, homologue compositions and presence of contaminating co-extractives. This variation was dependent on the polarity of the solvent used for extraction with Soxhlet apparatus, and it was found that the ideal solvent for this form of procedure was cyclohexane. Although acetone and ethyl acetate had the highest yields of total resorcinolic lipids, cyclohexane had the lowest content of undesirable lipids and other co-extractives, making it more suitable for direct qualitative and quantitative determination of alkylresorcinols using this procedure. Also, this procedure only requires an extraction time of 2-6hr making it much faster than the traditional 16-24hr extraction time.

1.4.6.3 Super critical carbon dioxide Extraction

In 2005, Francisco et al. developed a new method for effectively extracting ARs by using supercritical CO2 (SCCO2) whereby conditions of pressure, temperature, and co-solvent type could be set. When carbon dioxide gas is both heated and compressed above its critical point in a synchronized fashion, SC-CO2 is the result (*159*).These super-critical conditions can act as an extraction solvent and the effectiveness of SCCO2 as a solvent is dependent on the afore-mentioned conditions. This method is beneficial in comparison to past procedures followed due to its non-toxic, non-flammable, inexpensive and environmentally friendly properties (*37, 160, 161*). It is also of greater advantage as it is much faster, allows for the separation of high and low molecular weight AR components, and yields qualitatively cleaner bran fractions due to the utilization of co-solvents ethanol & methanol, rather than the traditional organic solvents acetone and ethyl acetate (*37, 161*). With future optimization of this method, the settings and conditions

for this procedure were found to be ideal at temperature 70°C, pressure 35MPa, with a flow rate of 25g/min for 4h (1° extraction step), and 5-g/min for 4h (2° fractionation step) using co-solvent ethanol (*37*). While past studies by Francisco et al. have found SCCO2 to yield 8-80% more ARs when compared with traditional extraction using acetone, a recent study in 2007, by Landberg et al., found no significant difference in total AR content amongst the two methods when comparing against traditional extraction with ethyl acetate (*160*).

1.4.7 Methods of Analysis

1.4.7.1 Colorimetry

A colorimetric method of analysis has been used in several studies to quantify total AR concentrations by measuring the intensity of electromagnetic radiation in the visible spectrum transmitted through AR extracts (*61, 159, 162-168*). This method of analysis was first developed by Musehold in 1973 and has been adapted since to produce more accurate results that are in agreement with other methods. This method is based on a diazonium salt which reacts with phenolic compounds to form an azo-complex that develops a reddish violet colour with a maximum absorption peak (λ=520nm) distinct for 5-alk(en)ylresorcinols (*165, 168*). Various diazotized compounds have been used as reagents for this method, such as sulfonic (*53, 162, 169*), *p*-nitroaniline (*170*), Fast Blue B BF4 (*61, 163*), Fast Blue B Zn (*165, 167*), and Fast Blue RR salt (*167*). Current methods employ either one of the three Fast Blue salts; however, the methods employed vary depending on the type of salt used. It is important to note that colorimetry does not distinguish amongst the various AR homologues present in the extract as it only quantifies based on the sum of aromatic rings reacting with the diazonium salt (*61*). Therefore, this method is ideal for rapid, simple, cost-effective detection and quantification of

total ARs (*165-167*). Further analysis by chromatography is recommended for confirmation of the results obtained by colorimetry and to differentiate between the different AR homologues (*55, 171*).

1.4.7.2 Thin Layer Chromatography (TLC)

TLC has been used a qualitative method for the identification and separation of ARs (*42, 157, 160, 171, 172*). In this method, a plate coated with a layer of silica gel or aluminum oxide, acts as the stationary phase. Once the sample is applied on the plate, a solvent mixture is drawn up the plate causing different analytes to separate at different rates, thereby allowing for separation of the compound of interest. The plate is sprayed with a reagent such as Fast Blue that reacts with the separated bands of ARs to form colors that range in intensity; bands vary from pink to deep crimson depending on the chain length (*173*). TLC is an ideal preliminary method for detection, isolation and purification of AR homologues, as it is a rapid and simple procedure. However, further analysis by HPLC or GC is essential for the quantitative determination of ARs (*35, 120, 171, 173*).

1.4.7.3 High Performance Liquid Chromatography (HPLC)

For a more in depth analysis of the AR homologue composition of extracts, either HPLC (*39, 110, 157, 172-177*) and/or GC have been employed (*38, 42, 90, 111, 112, 156, 157, 167, 168, 174, 178-183*). HPLC separates, identifies, and quantifies AR homologues based on their characteristic retention times that are dependent on AR-chain length and degree of unsaturation. Given that the majority of AR homologues extracted from cereal grains are non-polar, a reverse-phase HPLC with a non-polar C18 column as the stationary phase and a polar solvent mobile

phase is generally used (*50, 161, 172, 175, 177*). Use of a gradient system produces optimal results with clear peak separation points and reduced background noise in the resultant chromatogram; a combination of methanol and water (v/v) or a step-wise gradient of 70-99% MeOH have been commonly used (*94, 172*). The characteristic retention times of the AR homologues are made available by a UV detector set at 280 nm (*157, 161, 172, 175*) , a photodiode array (PDA) detector (*61, 174*) or a coulometric electrode array detector (CEAD) (*91, 176*) set at a scanning range of 250-350 nm. Other parameters such as the flow-rate of the mobile phase, column temperature, pump pressure, and the exact ratio of solvent gradient used varies depending on the specific system used. HPLC coupled to an atmospheric pressure chemical ionization multistage mass spectrometer (DAD-APcI-MSn) effectively characterizes substituted AR homologues with saturated, monoenoic, dienoic, and/or oxygenated side-chains (*56*) and HPLC-CEAD allows for sensitive analysis and maximum recovery of 98-107% AR homologues (*176*).

1.4.7.4 Gas Chromatography (GC)

Gas chromatography separates and quantifies the relative amounts of AR homologues based on their individual boiling points, thus their retention times, which are visualized by a flame ionization detector (FID) or mass spectrometer (MS) (*184*). While GC alone does not positively identify all components of an AR extract, GC-MS identifies AR components with high accuracy through their characteristic ionized fragments of mass to charge ratio (m/z) and retention times (*37, 156*). The characteristic ion peaks are generally 320, 348, 376, 404, 432 and 460 m/z for respective AR homologues C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0 (*185*). Alternative methods such as gas chromatography/tandem mass spectrometry (GC/MS/MS) and

normal-phase liquid chromatography/tandem mass spectrometry have been used for rapid analysis of ARs in human plasma and red blood cells (RBC) and exhibited lower limits of detection and quantification in comparison to that of GC-MS (*179, 186*).

1.5 Phenolics as Antioxidants

Antioxidants can be classified under two major categories, enzymatic (e.g. superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic antioxidants (e.g. vitamin C and E). The ability of natural antioxidants to make a significant contribution to our body's natural defense system against the damages of excess ROS production is dependent on a variety of aspects including its structural nature (e.g. position & number of hydroxy groups on the benzene ring), mechanism of action (e.g. hydrogen atom or single electron transfer), bioavailability (e.g. level absorbed through the small intestine), and interaction with other antioxidants (e.g. synergistic or antagonistic) (*101, 187*). In order to protect against oxidative damage, antioxidants may carry out their protective actions through one or more of the following mechanisms: oxygen depletion, quenching of singlet oxygen, chelation of metal ions, catalyzers of ROS production, ROS scavenging or chain termination of radical formation, and oxidative damage repair (*101*). To be effective antioxidants, phenolics must intercept and react with ROS at a faster rate than the substrate which may be a lipid, fat or protein (Figure 1.7). As such, the strength of the OH bond of phenolic compounds is a determining factor of their antioxidant potential since weaker OH bonds will react faster with free radicals. Additionally, phenolics must form relatively stable free radicals that react at a much slower rate with the substrate (LH) but rapidly with the radical ($LOO^{\bullet}$), thereby terminating the chain reaction (*188*). Thus suitability of phenolic compounds and other molecules for employment as food additives or

antioxidant drugs depends on the aforementioned factors (*187*).

Phytochemicals are chemical compounds naturally occurring in plants that are not considered essential nutrients (secondary metabolites), however they possess bioactive properties that elicit positive physiological effects and have been associated with a reduced risk of major chronic diseases. While phytochemicals vary individually in their bioactivities, studies have determined there to be a greater protective benefit from the complex combination of phytochemicals in foods than as isolated components due to their combined synergistic effects (*189*). To date, over 5000 phytochemicals have been identified and can be classified as carotenoids, phenolics, alkaloids, nitrogen-containing compounds, and organosulfur compounds (*190*). The occurrence, content and type of phytochemicals vary among plant species. Grains contain a wide variety of phytochemicals including phenolic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, amino phenolic compounds, tocotrienols and tocopherol (*191*).

Phenolic compounds present in grains have antioxidant properties associated with the health benefits of grains and grain products. The most prevalent phenolic compounds in cereal grains are flavonoids and phenolic acids. A significant role of phenolics in plants is that of a primary defense system against herbivores, pathogens and free radical stress due to ultraviolet light. In the body, phenolics are said to have a similar protective role as they have been shown to scavenge reactive oxygen species (ROS), especially under conditions of oxidative stress, and impede their damaging effects on cells and their components (*192*). Thus, foods containing high levels of antioxidants, such as fruits, vegetables, and grains, are often recommended.

Phenolics are compounds with at least one aromatic ring and hydroxyl group. Depending on their structure, they can be further classified as phenolic acids, flavonoids, stilbenes,

coumarins or tannins (*193*). There are two major categories of phenolic acids depending on whether they are derivatives of hydroxybenzoic acid or hydroxycinnamic acid. These phenolic acids may exist in free form, as conjugates esterified to sugars, proteins and other low molecular weight components in the plant matrix, or in bound form where they are associated with cell wall polysaccharides. The predominant phenolic acid in whole grains and cereal bran is ferulic acid, followed by p-coumaric acid, both of which are hydrohydroxycinnamic acids (*194*).

1.6 Phenolics as Anti-inflammatory Agents

As a part of the body's response to stressful stimuli such as infection or tissue injury, a signaling cascade of pro-inflammatory mediators such as cytokines, chemokines, and eicosanoids, are activated to induce an inflammatory response. Part of this physiological response involves the release of ROS and RNS to attack and foreign invaders through oxidative damage (*195*). Although these reactive species are released as a defense mechanism, they do not discriminate among microbial and host cell targets. Therefore, if this physiological response is dysregulated, as in the case of chronic inflammation, host cell damage is inevitable and the effects can be detrimental (*196*).

Phenolic compounds may impart their anti-inflammatory effects through a variety of proposed mechanisms such as the down-regulation of pro-inflammatory transcription factors or enzyme systems involved, inhibition of pro-inflammatory enzyme activity, or through their antioxidant effects resulting in an increase in endogenous antioxidant system activity (*197, 198*).

1.7 Dietary Fibers as Prebiotics

Dietary fibers (DF) are the complex carbohydrates that make up the plant cell wall and are

resistant to hydrolysis by human digestive enzymes of the small intestine. Dietary fibre can be further classified as soluble (SDF) or insoluble dietary fibre (IDF) depending on their ability to dissolve in water and their degree of fermentation in the colon (*199*). Unlike oilseeds which are predominantly made up of SDF, the bran of cereal grains mostly consists of IDF. While the physiological effects of DF can vary depending on the fiber type, the health benefits of DF are well-recognized, principally in the prevention of various chronic diseases and in the amelioration of gut health (*200*).

Insoluble fibre absorbs water helping to increase bulk, soften stools and shorten transit time through the intestinal tract, an effective means of preventing constipation and promoting regularity. Soluble fibre readily dissolves in water forming a viscous gel that delays gastric emptying and induces satiety (*201*). Insoluble fibers are resistant to fermentation in the large bowel whereas soluble fibres are readily fermentable and act as prebiotics promoting the growth of colonic microflora (probiotics) that elicit health benefitting effects in the gut (*202*). Soluble fibers inhibit micelle formation by binding bile acids, thereby increasing fecal excretion of bile acids and lowering total cholesterol levels (*203*). Incorporation of fibre-rich byproducts (eg. bran) can enhance the nutritional value of foods by increasing their fibre content, resulting in reduced cholesterol, fat and improved gut health (*204-206*).

Oftentimes, the total phenolic content of plants is underestimated due to a large proportion of phenolic compounds (PC) being linked to cell wall polysaccharides (dietary fibre) (*207*). Unlike free or unbound phenolics, dietary fibre-phenolic compounds (DF-PC) are not bioaccessible in the small intestine. Low molecular weight polyphenols, carotenoids and vitamins are dietary antioxidants which are bioaccessible and at least, partially absorbed through the small intestine because they can be effectively solubilized by digestive enzymes and

intestinal fluids. However, conjugation of phenolics to DF makes them inaccessible to digestive enzymes, allowing them to continue intact through to the colon where they can then be fermented by the action of bacterial enzymes (*208*). Of the total dietary antioxidants travelling through the small intestine, 50% are linked to dietary fiber, the majority of which are polyphenolics. When dietary antioxidants arrive in the colon, they are released from the fiber matrix and metabolized by bacterial microbiota, consequently producing an antioxidant environment (*209*). Thus dietary fibers in wholegrains may possess dual functionality through their antioxidant and prebiotic health benefits.

1.8 Thesis Objectives

The principle aim of this study was to investigate the chemical composition and functional properties of bioactive components in triticale bran, an underutilized grain type with high yield potential and nutritional value. To achieve this aim, the following objectives were carried out:

1. Extraction, quantification and characterization of triticale bran ARs
    a. Optimization of AR extraction using response surface methodology (RSM) for large-scale processes.
    b. Quantification of total AR content by colorimetric determination
    c. Characterization & quantification of AR homologues using HPLC-PDA and confirmation by GC-MS and FTIR

2. Discovery of the anti-inflammatory and antioxidant potential of ARs
    a. Antioxidant capacity measurement using *in-vitro* and *ex-vivo* model systems
    b. Anti-inflammatory measurement using *in-vitro* and *ex-vivo* model systems

3. Understanding the *in vivo* effects of ARs on oxidative stress induced by a high fat diet
    a. To measure the impact of AR incorporation in a high fat diet on the body weight, food intake and glucose tolerance of mice
    b. To determine the impact of AR incorporation in a high fat diet on oxidative stress markers in mice heart and liver tissues

4. Determination of water-extractable polysaccharides (WEP) in triticale bran
    a. Characterization and quantification of WEP composition in TB

b. Measuring the effects of enzyme treatment and ethanol fractionation on the yield potential of WEP
c. Measuring the molecular weight distribution (MWD) profile of extracted WEP components

5. Evaluation of the prebiotic potential of TB using an *in-vitro* fermentation model system and the antioxidant potential of WEP extracted from TB
    a. To examine the impact of TB on the growth of probiotic bacteria in yogurt by measuring TTA, pH, and total bacteria counts.
    b. Evaluation of the antioxidant capacity of WEP extracted from TB.

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

Chapter 1 provides background information on whole grains, particularly triticale bran, and the nutritional value and bioactive properties of their constituents. Information on AR bioactivity, metabolism and methods of extraction and analysis are also covered.

Chapter 2 defines the optimum AR extraction parameters for large-scale processes using RSM. This chapter examines the influence of temperature and solid-solvent ratio on the yield of saturated, unsaturated and total ARs.

Chapter 3 determines the anti-inflammatory and antioxidant potential of ARs. This chapter identifies the potential of ARs to impede the activity of pro-inflammatory markers and free radical generators using both *in vitro* and *ex vivo* model system.

Chapter 4 assesses the *in vivo* effects of AR incorporation in the diet of high fat fed mice

on weight, food intake, and glucose tolerance. Additionally, the ability of ARs to provide antioxidant protection *in vivo* was elucidated by measuring the level of oxidative stress markers in the liver and heart tissues of mice post-mortem.

Chapter 5 identifies the WEP composition in TB while also determining the effects of enzyme treatment and ethanol fractionation on WEP content, composition, and MWD profiles.

Chapter 6 identifies the prebiotic and antioxidant potential of TB dietary fibre, particularly soluble dietary fibre, through the incorporation of TB and probiotics in yogurt as a fermentation model, as well as the extraction and antioxidant capacity measurement of WEP from TB.

Finally, in Chapter 7, the outcomes of each chapter are summarized with an overall conclusion addressing the contributions of this thesis to current knowledge and suggest future directions for these findings.

Outcomes of this research may lead to greater utilization and demand for triticale and its bioactive components as a functional food or as nutraceutical ingredients. Current uses of triticale are predominantly for animal feed purposes and biofuel production, whereas food applications of triticale are limited due to a lack of research on triticale composition and bioactivity. The increase in food applications for triticale, a cost-effective hybrid cereal grain with high yield potential, will greatly benefit and improve the economy of Ontario grain farmers and the Canadian Agri-food industry overall.

Chapters 2-6 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:

- Ms. Rania Agil, 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. David B. Oomah, a research scientist at Agriculture and Agri-Food Canada, provided technical expertise for the experimental design of ARs extraction optimization using RSM.
- Dr. Giuseppe Mazza, a research scientist at Agriculture and Agri-Food Canada, provided the facility and instruments needed to carry out the experiments for the optimization of ARs extraction.
- Dr. Alfonso Abizaid, an associate professor from the department of neuroscience at Carleton University, provided the facility, animals and research expertise for the assessment of AR incorporation in the diets of high fat fed mice.
- Dr. William G. Willmore, an associate professor from the institute of biochemistry at Carleton University, provided the macrophage cells as well as the facility to culture and store them. Dr. Willmore also provided technical expertise and training.

1.9 Connecting Statement to Chapter 2

In Chapter 1, the health benefits and nutritional importance of whole grains and their bioactive constituents, which reside predominantly in the bran fraction, have been discussed. More specifically, the bioactive constituents of TB including dietary fibre and phenolics have been presented, with the bioactivity, bioavailability, extractability, and analytical methodology of ARs discussed at greater length.

In Chapter 2, optimization conditions for ARs extraction from triticale bran are determined using response surface methodology.

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

- Agil, R.*, Gunenc, A., and Hosseinian, F.S. Biologically Effective Phenolic Lipids in Cereal Bran: Alkylresorcinols Extraction and Analysis. Ottawa-Carleton Chemistry Institute (OCCI) Day. Ottawa, Ontario. May 21, 2010. Institutional; Poster Presentation.
- Agil, R.*, Hosseinian, F.S., Oomah, B.D., and Mazza, G. Optimization of Alkylresorcinols Extraction from Triticale Bran. Canadian Institute of Food Science & Technology (CIFST)/ Agriculture and Agri-Food Canada (AAFC) Conference. Winnipeg, Manitoba. May 30- June 1, 2010.
- Agil R, Oomah DB, Mazza G, and Hosseinian F, Optimization of alkylresorcinols extraction from triticale bran using response surface methodology. Food and Bioprocessing Technology. 5:2655-2664 (2012).

Chapter 2 Optimization of alkylresorcinols extraction from triticale bran using response surface methodology

2.1 Abstract

Optimization conditions for alkylresorcinols (ARs) extraction from triticale bran were determined using response surface methodology. A central composite design was used to determine the effects of extraction temperature (degrees Celsius) and solid-to-solvent ratio (weight per volume) on yield of saturated, unsaturated and total ARs. Extraction of ARs was affected significantly (p value≤0.05) by temperature and solid-to-solvent ratio on the yield of saturated, unsaturated and total ARs. The highest quantity of total ARs from triticale bran was extracted at 24 °C between 16 and 24 h at a solid-to solvent ratio (weight per volume) of 1:40 and ranged from 278 to 308 mg/100 g, while saturated and unsaturated ARs were 163 to 225 mg/100 g and 22 to 29 mg/100 g, respectively. The ARs identified by high-performance liquid chromatography included: C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0. Also some unsaturated analogues (12.4% to 14.0%) were found including C17:1, C19:1, C21:1 and C23:1. Analysis of variance (p value=0.05) indicated that the response surface methodology (RSM) developed for saturated, unsaturated and total ARs were adequate and explained most of the variability (85% to 89%) with high coefficient of multiple determination (R^2=0.89). The main effect of the response variable was quadratic rather than a linear function. Results showed that RSM is a tool that is useful for optimizing the experimental conditions of ARs extraction as it clearly defined the solid to solvent ratio and temperature parameters that release the highest yield of ARs from the cereal sample matrix.

2.2 Introduction

Studies have shown that consumption of whole grains have beneficial health effects, including reduced risk of diabetes (*9, 210*), obesity, and coronary heart diseases (CHD) (*73, 154*), in addition to their antioxidant and anticarcinogenic properties (*95, 103, 211*). ARs have been shown to have health benefits against risk factors of coronary vascular disease (CVD) such as type II diabetes, obesity and coronary heart disease (CHD). Some of these health benefits may be attributed to the presence of alkylresorcinols (*73, 95, 211*). However, currently triticale has limited use in the human diet, and thus it is (*212*) desirable to evaluate bioactive compounds with potential health benefits such as alkylresorcinols. Cereal 5-n-alkylresorcinols (AR) are lipophilic 1,3-dihydroxybenzene derivatives that differ mainly with their odd-numbered hydrocarbon chains attached to position 5 of the benzene ring (Figure 2.1) (*37, 186*). ARs are named in a similar way as fatty acids. The chemical formula of AR is C (n+6) H(2n+6) O(2), where n represents the number of carbons in the alkyl chain. For example, the chemical formula of AR C17:0 would be C (17+6) H(2(17)+6) O(2) which makes it $C_{23}H_{40}O_2$. As an example, AR C19:0 and C19:1 with a hydrocarbon chain with 19 carbon atoms is shown in Figure 2.1. The alkyl chains are mainly in saturated forms and length varying from C15 to C27 (*37, 178, 186*). Names based on the resorcinol structure (e.g. 5nonadecylresorcinol, **1**) are more commonly used than IUPAC nomenclature (5nonadecylbenzene1,3diol).

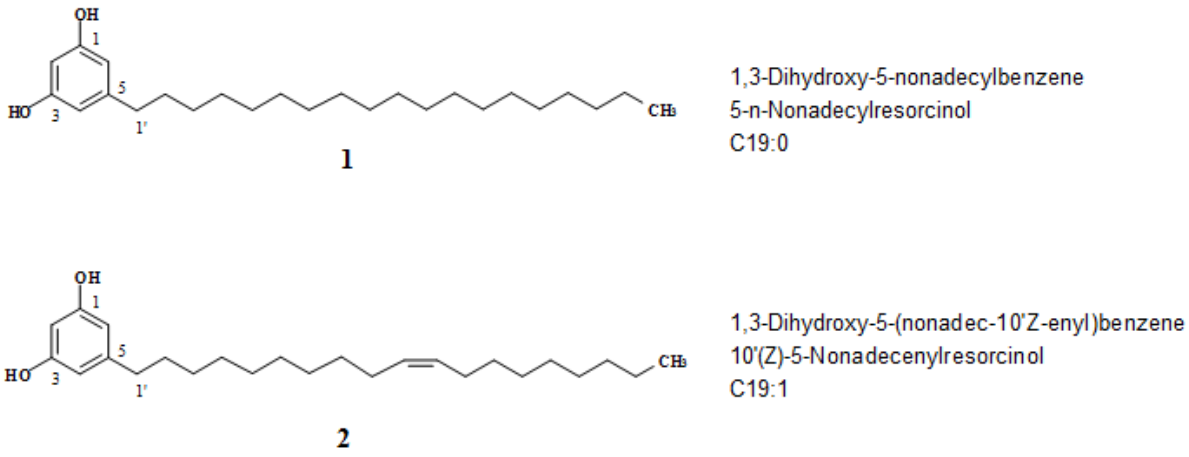


Figure 2.1 - Chemical structure of 5-alkylresorcinol C19:0 and C19:1

ARs are found mainly in the bran fraction of cereal grains, particularly in wheat and rye (*36, 69*). Wheat and rye brans contain 221-323 mg/100 g and 276-411 mg/100 g of alkylresorcinol, respectively (*38*). The alkylresorcinol content ranges between 48.9-61.8 mg/100 g in wheat,43.9-64.7 mg/100 g in triticale,56.8-76.1 mg/100 g in rye, and 0.4-0.5mg/100 g in barley on dry weight basis (*36, 38, 39, 43*). Trace amounts of ARs have also been found in oat, corn and sorghum (*50*). The content of alkylresorcinols is reported to vary widely due to environmental, agricultural and genetic factors (*43, 52*). The alkylresorcinol content and composition of triticale bran has not been reported.

ARs from cereal grains are commonly extracted using organic solvents such as acetone, ethyl acetate, methanol, and ethanol (*50*). Although most ARs are soluble in methanol and ethanol, the longer chain homologues are not extractable to the same extent as in acetone and ethyl acetate. Factors such as extraction temperature and solid to solvent ratio are important for developing, improving, and optimization of extraction processes (*213, 214*). Also, different variables and their interactions on dependent variables can affect the extraction process;

therefore, the use of statistical and mathematical analysis models to optimize the extraction of bioactives in plants is often helpful (*215, 216*). Central composite experimental design using response surface methodology is commonly used to evaluate the effects of independent variables and their interactions on a dependent variable (*213, 214, 217, 218*). The objective of this study was to use response surface methodology (RSM) to optimize the solid to solvent ratio and temperature for extraction of alkylresorcinols from triticale bran for large scale processes.

2.3 Materials and methods

2.3.1 Sample preparation

Triticale (spring triticale, cultivar Ultima) bran grown in Edmonton region in Alberta was received in three bags containing 5 Kg bran by a collaborator from Alberta Agriculture, Food and Rural Development (Edmonton, Alberta, Canada). Samples were mixed properly in each bag and sampling in triplicates was done randomly from top, middle and bottom parts of the bags. Prior to extraction, the bran was ground to the recommended 2 mm size (*36, 50*) using a Thomas Wiley Mill (model ED-5, Arthur H. Thomas Co., Philadelphia, Pennsylvania, USA). Samples were stored in sealed plastic bags and kept in the freezer (-30°C) prior to analysis.

2.3.2 Materials

Acetone and methanol were HPLC grade (Fisher Scientific Co., Ottawa, ON). The alkylresorcinol standards including C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0 were purchased from ReseaChem GmbH (Burgdorf, Switzerland).

2.3.3 Experimental design

Response surface methodology (RSM) was used to determine the influence of two independent variables, extraction temperature (X1) and solid to solvent ratio (X2), considered the most important variables affecting alkylresorcinol extraction. The experimental design was a modification of Box's central composite design for two factors each at five levels, as described by (*215*). The end and central values were chosen from the results of preliminary tests. The experimental design consisted of 12 points, including four replications at the central point, and was carried out in a random order. The response variables were yield (Y_1-Y_{14}) of saturated, unsaturated, and total alkylresorcinols (Table 2.1).

Regression models were evaluated for each response variable. Models tested possible linear, quadratic and linear cross-product relationships, analysed through the centre point. A model was considered an adequate approximation of the true surface if the error due to lack-of-fit was not significant ($P>0.05$) and the variation due to regression was significant ($P\leq0.05$). Regression parameters were used to interpret significant treatment effects at P-value$\leq$0.05. Goodness-of-fit tests were performed on the model using the backward elimination procedure. Response surface graphs were plotted, and optimum responses were identified within feasible treatments for the different response-dependent variables. The response surface regression (RSREG) procedure uses the method of least squares to fit quadratic response surface regression models. Response surface model analysis by the RSREG procedure was performed according to the Statistical Analysis System SAS (version 9, SAS Institute Inc, Cary, NC).

The second order equation used in the response surface analysis was:

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

Where $beta_0$, $beta_i$ and $beta_{ii}$ are the regression coefficients for intercept, linear, quadratic and

interaction terms, respectively, and X_i, and X_j are the independent variables. Response surface plots were obtained using predicted values from the fitted model, by keeping the least effective independent variable fixed at a constant value while modifying the other variables.

The extraction temperature at 24 ºC and solid to solvent ratio of 1:40 as the zero point in the model was selected based on available literature (Table 2.1) (*36, 50*). The lower (-1), middle (0) and upper levels (+1) of two independent variables were then determined using RSM (*213, 215*). For this experimental design, acetone was the solvent selected for ARs extraction due to its suitability and effectivity in isolating ARs from cereal bran samples (*55*).

2.3.4 Alkylresorcinol extraction

ARs from triticale bran were extracted with acetone at the solid to solvent ratios (w/v, g/mL) and temperatures (ºC) indicated in (Table 2.1). The extraction was carried out in a 4 L glass beaker with a solvent volume of 2.5 L and an axial agitator (63.5 mm diameter, 1250 rpm) as reported previously (*213, 217*). Temperature was controlled through the use of a large water bath (PolyScience refrigerated/heating circulating bath, Model 1187) with a 28 L reservoir and a working access of 26 x 26 x 26 cm. The location of the impeller was 40 mm from the bottom, which was also one third of the liquid depth above the vessel bottom. The impeller was 41 mm from the wall with 15º angle to ensure constant mixing and to maximize diffusion of particles in solvents as described by (*213*). During extraction, 2 mL of extract was taken periodically to optimize the extraction time and determine the equilibrium in terms of mass transfer (*213, 217*). Sampling was performed at 2 h intervals up to 12 h and then every 4 h up to 24 h and 12 h until the end of the 48 h study. All values are reported on a dry matter basis.

2.3.5 High-performance liquid chromatography (HPLC) analysis

An Agilent 1100 HPLC system equipped with a photodiode array detector (PDA), autosampler, and Agilent ChemStation Plus software (Agilent Technologies Inc., Palo Alto, CA) was used for the analyses. The separation of alkylresorcinols was accomplished on a reversed-phase Zorbax SB-C_{18} column (5 μm, 250 mm × 3.0 mm). The gradient conditions were in accordance with the method by Mullin & Emery (1992) (*50*). The mobile phases were A (50 mM phosphoric acid) and B (methanol) at a flow rate of 0.4 mL/min, a running time of 52 min, and an injection volume of 10 µL for each sample. The gradient system used was solvent B: 0-9 min, 90%; 10-44 min, 100%; 45-52 min, 90%. The elution of compounds of interest was monitored at 280 nm. Spectral data (220-600 nm) were also examined for all samples for the presence of other phenolics. Alkylresorcinol standards including C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0 were prepared in ethyl acetate. A calibration curve (0.1 to 10 mg/mL) was prepared for each of the alkylresorcinol standards. Due to unavailability of standards, unsaturated alkylresorcinols were identified according to their UV profile and molecular mass values. The unsaturated alkylresorcinols in samples were expressed as C19:0 equivalents.

2.3.6 Gas chromatography–mass spectrometry (GC-MS) analysis

GC/MS was conducted according to the method by Athukorala, Hosseinian & Mazza, (2010). The extracts (10 µL) were trimethylsilylated (TMS) with 200 µL of *bis*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (TMCS) and 100 µL of ethyl acetate at 75 °C for 30 min. The reaction mixture was allowed to cool, evaporated under continuous N_2 flush, and re-dissolved in ethyl acetate for analysis. The GC/MS analysis was conducted with an Agilent 6890/5973 GC-MS network gas system (Agilent Technologies, Wilmington, Delaware, USA)

equipped with a splitless injector, a network mass selective detector, and a 7683 series Agilent autosampler. A DB-17HT high-temperature capillary column was used (30 mm x 0.25 mm I.D., 0.1 μm film thicknesses, J & W Scientific, Folsom, California, USA). Helium was used as the carrier gas. The temperature of the injector was 300 °C and the flow rate was 1.3 mL/min. The initial oven temperature of 50 °C (1 min) was ramped at 10 °C/min to 280 °C, increased to 350 °C at 5 °C/min and then held constant for 10 min. The Agilent 5973 quadrupole mass spectra was operated in the electron ionization (EI) mode at 70 eV, source temperature of 280 °C and temperature of 150 °C, in the scan range of 35 to 350 *m/z*. Data were collected with Agilent enhanced ChemStation software (standard MSD version) and compared against the NIST (v.02) and Wiley (v. 138) libraries (Palisade Corp., Newfield, New York, USA). Compounds were identified by comparing their spectra with those in the library and with their relevant standards.

2.3.7 Fourier transform infrared (FTIR) Spectroscopy

FTIR of ARs in all samples (in triplicate) was determined according to the method of Hamed and Allam (2006), using a Nicolet™ 380 FTIR (Thermo Electron Corp. Madison, Wisconsin, USA) equipped with OMNIC Spectra™ software. Samples in dried pelletized form (1 mg) were placed on a diamond attenuated total reflectance (ATR) crystal then pressed using a pressure tower as recommended by Thermo Electron Corp. Spectra were recorded in duplicates between 400 and 4000 cm-1 at 4 cm-1 resolution, applying 32 scans. Background spectra were taken in the empty chamber.

2.4 Results and discussion

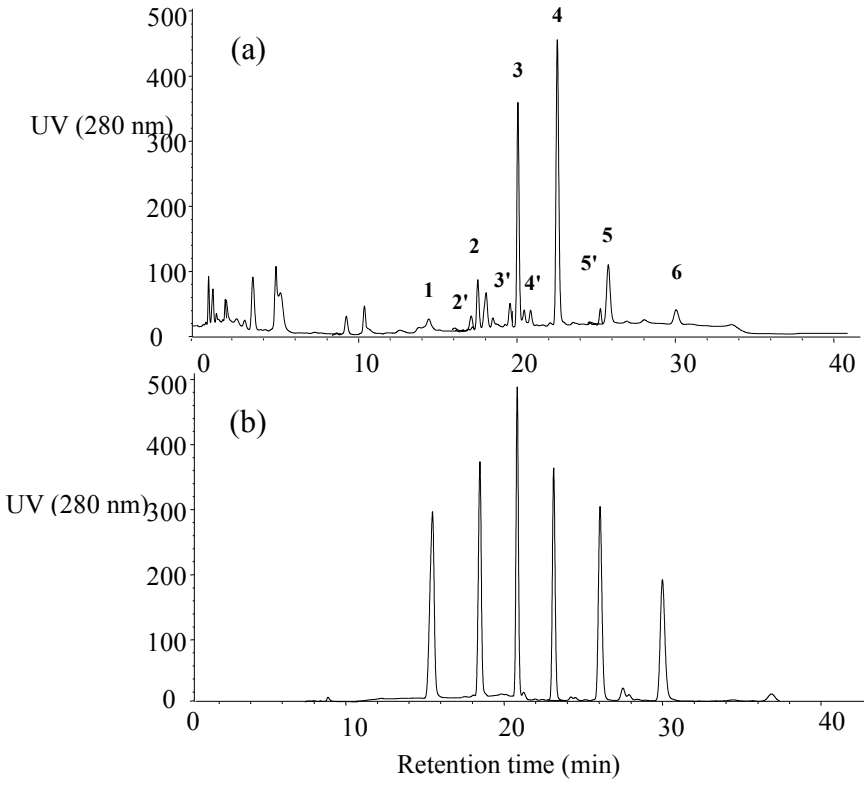
2.4.1 Alkylresorcinol content and composition

HPLC and GC/MS were used for the separation, quantification and identification of the alkylresorcinols. HPLC chromatograms for alkylresorcinol standards C15:0, C17:0, C19:0, C21:0 C23:0 and C25:0 and ARs extracted with acetone from triticale bran are shown in (Figure 2.2). Alkylresorcinols (mg/100g) distribution in triticale bran under different conditions is shown in Table 2.1. Saturated alkylresorcinols were the major ARs in all samples constituting 86 to 88%, while unsaturated ARs represented only 12.4 to 14% of the total ARs. Comparable results were reported by Chen et al. (2004) (*38*) for saturated and unsaturated ARs in cereal brans. Unsaturated alkylresorcinols were separated from the saturated analogues based on their molecular mass (Figure 2.2). Under these conditions, the total ARs content ranged from 277.5 to 308.3 mg/100g while saturated and unsaturated ARs were between 163.4 to 224.9 mg/100g and 22.0 to 28.5 mg/100g, respectively (Table 2.1). The alkylresorcinol content in wheat bran (221.1-322.5 mg/100g) and in rye bran (275.8-410.8 mg/100g) reported earlier (*38, 39*) support results obtained from this study. The ARs content in triticale bran was up to six times higher (277.5 to 308.3 mg/100g) than reported for triticale grains (43.9-64.7 mg/100g) (*36, 38, 39*). This indicates that triticale bran is a valuable fraction of triticale with elevated levels of ARs.

Table 2.1 Process variables and surface response for alkylresorcinols (mg/100 g, dry matter) solid-liquid extraction with acetone from triticale bran, using HPLC.

Run	(X1) Extraction temperature (ºC)	(X2) Solvent to solid ratio (v/w)	Surface Response														
			Yield%*	C15:0**	C17:0**	C17:1**	C19:0**	C19:1**	C21:0**	C21:1**	C23:0**	C23:1**	C25:0**	Saturated**	Unsaturated**	Unknown**	Overall**
1	12 (-1)[a]	20 (-1)	89.3	0.9±0.02	3.6±0.2	0.8±0.1	18.6±0.2	3.6±0.5	22.6±0.3	0.6±0.4	9.3±0.2	0.6±0.4	3.9±0.1	58.8±0.6	4.9±0.4	12.6±0.3	76.3±0.6
2	36 (+1)	20 (-1)	90.7	1.5±0.3	13.6±0.5	6.5±0.3	30.2±0.1	5.2±0.8	35.3±0.9	1.0±0.5	13.8±0.4	1.0±0.2	5.8±0.6	100.2±0.9	13.7±0.3	154.5±0.8	268.4±0.5
3	12 (-1)	60 (+1)	89.8	0.8±0.7	7.6±0.5	3.7±0.3	18.1±0.3	3.6±0.1	21.6±0.6	0.7±0.1	8.6±0.6	0.8±0.2	3.9±0.9	60.6±0.7	3.7±0.9	22.2±0.5	86.5±0.8
4	36 (+1)	60 (+1)	93.4	1.4±0.03	16.3±0.1	7.5±0.3	28.4±0.5	5.0±0.4	34.9±0.09	1.4±0.6	15.1±0.5	1.6±0.8	6.9±0.7	102.9±0.7	15.5±0.5	176.0±0.5	294.4±0.6
5	24 (0)	10 (-1.41)	95.2	0.1±0.5	0.9±0.07	0.5±0.01	2.2±0.06	0.5±0.02	3.1±0.05	0.2±0.06	1.6±0.04	0.1±0.9	0.9±0.1	11.1±0.2	1.2±0.8	0.3±0.3	12.5±0.7
6	24 (0)	70 (+1.41)	87.8	0.9±0.03	15.6±0.6	7.7±0.5	36.2±0.1	6.3±0.1	44.6±0.2	1.1±0.7	18.9±0.3	1.8±0.05	7.7±0.4	125.6±0.4	17.0±0.6	28.9±0.7	171.5±0.6
7	7.1 (-1.41)	40 (0)	88.3	0.7±0.2	6.7±0.03	3.1±0.06	17.5±0.06	2.9±0.04	22.8±0.02	0.8±0.2	9.6±0.2	1.1±0.4	4.1±0.6	61.5±0.6	7.9±0.7	14.9±0.8	84.3±0.6
8	40.9 (+1.41)	40 (0)	91.0	0.7±0.04	6.7±0.2	3.2±0.4	16.3±0.5	3.4±0.6	19.5±0.3	0.6±0.1	8.2±0.8	1.2±0.03	3.5±0.1	54.8±0.1	8.4±0.2	13.1±0.3	76.3±0.1
9	24 (0)	40 (0)	91.8	2.3±0.3	24.3±0.2	12.0±0.5	54.8±0.2	9.8±0.3	68.8±0.2	1.9±0. 5	30.4±0.3	2.6±0.3	11.6±0.5	224.9±0.7	28.1±0.1	55.3±0.5	308.3±0.5

[a] Coefficient of multiple determination; **significant at 0.05 level ; *significant at 0.1 level; [NS] insignificant



Peak	R	Names	MW*
1	C15:0	5-n-pentadecylresorcinol	320
2'	C17:1	5-n-(heptadecenyl)-resorcinol	346
2	C17:0	5-n-heptadecylresorcinol	348
3'	C19:1	5-(nonadecenyl)-resorcinol	374
3	C19:0	5-n-nonadecylresorcinol	376
4'	C21:1	5-(heneicosenyl)-resorcinol	402
4	C21:0	5-n-heneicosylresorcinol	404
5'	C23:1	5-(trieicosenyl)-resorcinol	430
5	C23:0	5-n-tricosylresorcinol	432
6	C25:0	5-n-pentacosylresocinol	460

Figure 2.2 - HPLC chromatograms (at 280 nm) for alkylresorcinols of (a) triticale bran and (b) standards. *Molecular weight obtained from GC-MS.

While, C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0 were the major saturated ARs in triticale bran, C17:1, C19:1 C21:1 and C23:1 were the major unsaturated analogues (Figure 2.2). This is in agreement with results reported previously (*36, 38, 50*). Also the unsaturated ARs had

a shorter retention time compared to the corresponding saturated analogues. Five unknown minor peaks (RT= 5.6, 9.8, 10.3, and 18.1min) were included in the total ARs because they had UV spectra similar to ARs (Figure 2.2). In addition, these samples were analyzed for the presence of other phenolics such as phenolic acids (280 nm), proanthocyanidins/catechins (280 and 320 nm) and lignans (280 nm). The presence of other phenolics was negligible, indicating that the extraction method was selective for ARs. The results of HPLC analyses confirmed an increase of ARs in the range of 286.4 to 308.3 mg/100g at 24 °C to 36 °C (Table 2.1). Some unknown peaks were detected at higher temperatures than 24 °C. These compounds have not been identified at this time. The standard calibration curve of the assay showed acceptable linearity in the range of 0.001-1.00 mg/mL of homologues C15:0-C25:0 with a correlation coefficient of 0.99. GC/MS was used to validate the results obtained from HPLC. GC/MS analysis showed the distinct molecular ions for each of the ARs. The presence of AR homologues was confirmed by the 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). The most abundant molecular ion peak was 268 m/z for all TMS derivatives of ARs (Figure 2.2). These results agree with published results (*55*). For unsaturated ARs, C17:1 at 346 m/z, C19:1 at 374 m/z, and C21:1 at 402 m/z, it was not possible to verify the location of the double bonds (*55*).

Alkylresorcinols in triticale and triticale by-products can act as biomarkers of grain intake since 5-n-alkylresorcinols are almost exclusively present in cereal grains and are not present in appreciable amounts in other foods (*39, 50*). Future studies are needed to investigate the effect of alkylresorcinols in triticale by-products in animals and humans.

2.4.2 Time-course study

The time course study on the extraction yield of ARs (Figure 2.3) indicated that the highest

amount of ARs from triticale bran was obtained between 16 and 24 h; thereafter extraction efficiency declined. Acetone is one of the most common solvents used for extracting ARs from grains at room temperature for 16–24 h with a solid to solvent ratio of 1:40 to 1:50 (g/mL), and the results obtained from this study agree with published results (*36, 50, 69*). Previous studies also show that solid to solvent ratio and temperature play critical roles in the extraction of bioactives in plants (*213, 214, 219*). Our results also showed that saturated ARs increased up to 20 h extraction (Figure 2.3) and then declined. Unsaturated ARs continued to increase after 20 h. The increase in unsaturated ARs may be due to the presence of oxygen resulting in the oxidation of the unsaturated double bonds. Oxidation of fatty acids is known to proceed in the presence of oxygen that is incorporated into the formation of more unsaturated fatty acids (*116, 220*). Another possibility is the greater solubility of unsaturated ARs in the solvent system allows for a greater release of these homologues from the matrix overtime (*221*).

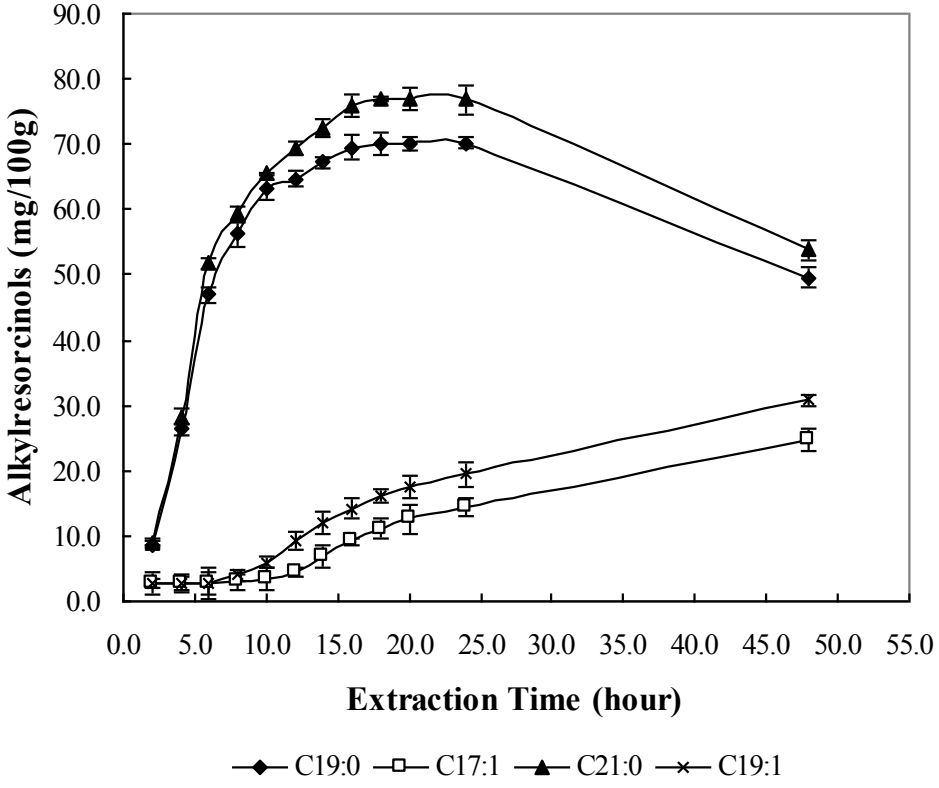


Figure 2.3 - Extraction of saturated and unsaturated alkylresorcinols in triticale bran with acetone at 24 °C and solid to solvent ratio of 1:40 (w/v).

2.4.3 Response surface model

Analysis of variance for the two process variables (Table 2.2) revealed that temperature was the major factor affecting the yield of saturated and unsaturated ARs. However, temperature had no significant effect ($P>0.05$) on the yield of total ARs, probably due to the significant amount (15-20%) of unknown ARs in the samples (Table 2.3). The solid: solvent ratio had significant effect ($P\leq0.05$) on the yield of saturated and unsaturated ARs (Table 2.5). The cross product (Table 2.3) between process variables (temperature and solid: solvent ratio) was not significant, indicating the process variables were not dependent on each other; hence response

surface diagrams could not be drawn for the interaction. Analysis of variance for the three major response variables (Table 2.4) indicated that the response surface models developed for saturated, unsaturated, and total ARs were adequate, possessing no significant lack of fit ($P>0.05$), and explained most of the variability with high coefficient of multiple determination ($R^2=0.89$). The main effect of the response variable (Table 2.3) was quadratic ($P\leq0.05$); i.e., second order. Cacace & Mazza (2003) reported optimization conditions for the extraction of anthocyanins from black currants with aqueous ethanol to be a quadratic rather than linear function. Cui et al. (1994) also reported similar results for aqueous extraction process for flaxseed gum. Our results showed that RSM is a tool that is useful to optimize experimental conditions for extraction of ARs.

Table 2.2 - Regression coefficient of predicted quadratic polynomial models for the response for alkylresorcinols in triticale bran.

Coefficient	**Saturated**	**Unsaturated**	**Total**
βo	-196.56	-29.80	-269.67
Model			
Linear			
β_1	22.22	3.09	+27.94
B_2	2.14	0.29	+3.08
Quadratic			
β_{11}	-0.45	-0.05	-0.23
B_{22}	-0.07	-0.01	-0.07
Interaction			
β_{12}	0.00	0.00	0.01
R^{2a}	83.72	82.93	89.21
F Value	4.60	3.81	3.81
Pr > F	**	**	*

[a] Coefficient of multiple determination; **significant at 0.05 level ; *significant at 0.1 level; [NS] insignificant

Table 2.3 - Analysis of variance of the regression parameters of the predicted response surface quadratic models for alkylresorcinols in triticale bran.

Regression	***df***	**Sum of Squares**	***R*-value[a]**	***F* Value**	**Pr > *F***
Saturated alkylresorcinols					
Linear	2	16347	0.2374	2.71	NS
Quadratic	2	34409	0.8998	5.71	_[b]
Cross product	1	0.25	0.0010	0.00	NS
Total Model	5	50757	0.8372	3.37	_[c]
Unsaturated alkylresorcinols					
Linear	2	346.67	0.2594	2.88	NS
Quadratic	2	624.44	0.8681	5.19	_[c]
Cross product	1	2.30	0.0017	0.04	NS
Total Model	5	972.80	0.8293	3.23	_[c]
Total alkylresorcinols					
Linear	2	51297	0.35	2.89	NS
Quadratic	2	40073	0.87	2.26	_[c]
Cross product	1	62.41	0.01	0.01	NS
Total Model	5	91432	0.89	2.06	_[c]

df degrees of freedom; *NS* insignificant; [a] Coefficient of multiple determination; [b] Significant at 0.05 level; [c] Significant at 0.1 level

Table 2.4 - Analysis of variance for the response surface quadratic model for alkylresorcinols in triticale bran.

Residual	***df***	**Sum of Squares**	**Mean Square**	***F* Value**	**Pr > *F***
Saturated alkylresorcinols					
Lack of Fit	3	14857	4952.42	4.59	NS
Pure Error	3	3236.57	1078.86		
Total Error	6	18094	3015.64		
Unsaturated alkylresorcinols					
Lack of Fit	3	327.28	109.09	9.68	NS
Pure Error	3	33.79	11.26		
Total Error	6	361.07	60.18		
Total alkylresorcinols					
Lack of Fit	3	47170	15723	7.80	NS
Pure Error	3	6044.45	2014.82		
Total Error	6	53214	8869.06		

df degrees of freedom; *NS* insignificant.

Table 2.5 - Analysis of variance for the overall effect of the process variables on responses.

Factor	***df***	**Sum of Squares**	**Mean Square**	***F* Value**	**Pr > *F***	**Critical Value**	
						Coded	**Uncoded**
Saturated alkylresorcinols							
Temperature (X1, °C)	3	33043	11014	3.65	_[a]	0.05	24.79
Solid:Solvent ratio (X2, w/v)	3	22827	7609	2.52	_[b]	0.47	54.06
Unsaturated alkylresorcinols							
Temperature (X1, °C)	3	642	214	3.56	_[a]	0.12	30.01
Solid:Solvent ratio (X2, w/v)	3	429	143	2.37	_[b]	0.47	54.13
Total alkylresorcinols							
Temperature (X1, °C)	3	57795	19265	2.17	_[b]	0.24	28.01
Solid:Solvent ratio (X2, w/v)	3	38713	12904	1.45	_[b]	0.73	61.99

df degrees of freedom; *NS* insignificant; [a] Coefficient of multiple determination; [b] Significant at 0.05 level; [c] Significant at 0.1 level.

2.4.4 Effect of process variables on yield of alkylresorcinols

Extraction of ARs was affected significantly ($P \leq 0.05$) by temperature and solid to solvent ratio. Temperature and solid to solvent ratio influenced the content of saturated, unsaturated (Figure 2.4A and 2.4B), and total alkylresorcinols (Figure 2.5). The extraction of ARs increased to its maximum levels at 24 ºC and then declined with a further increase of temperature. This clearly shows that temperature is an important process variable for the extraction of ARs. These results also show that temperatures above the optimum range of 24 ºC can lead to breakdown of these compounds (Figure 2.5). At temperatures below the optimum range (7-12 ºC), the extraction of ARs was almost one third of maximum values (Figure 2.5) and the peaks corresponding to C15:0, C17:1, C21:1 were very small in these extracts (Table 2.1). Solubility is likely an important factor influencing the increase of ARs at elevated temperature. This agrees with previous data obtained for extraction of other bioactive phenolics in plants (*213*). ARs yields increased almost linearly with the increase of solid to solvent ratio and this increase occurred at the temperature range used (Figure 2.5). The total ARs yields at 36 ºC was lower than that obtained at 24 ºC (Figure 2.5 and Table 2.1) and thus temperatures above 24 ºC are not recommend for the extraction of these compounds.

The selected settings of mixer and agitation speed (1250 rpm) helped the extraction of ARs with low variability in their content (R^2=0.89). These factors were reported to be critical for rapid mixing and suspension of particles in the solvent and reproducibility of results (*213*). This study suggests that the highest quantity of ARs can be obtained at 24 ºC between 16 and 24 h with a solid to solvent ratio of 1:40. This is consistent with previous results for the extraction of ARs from plants (*38, 178*).

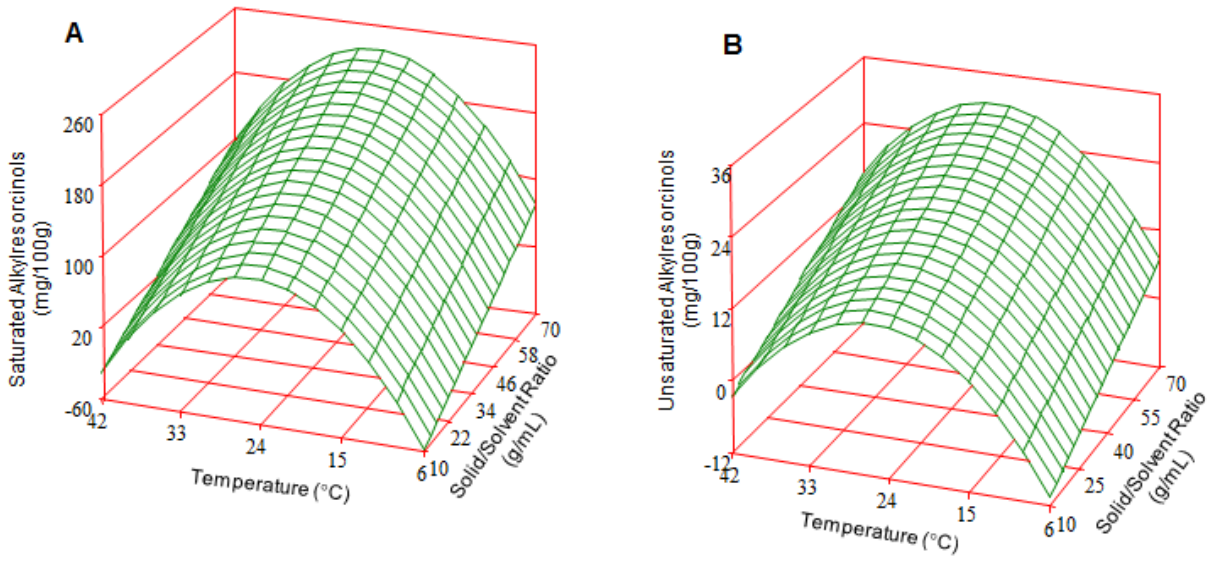


Figure 2.4 - Response surface for the effect of temperature (°C) and solid to solvent ratio (triticale bran to acetone) on A) saturated, and B) unsaturated alkylresorcinols in triticale bran.

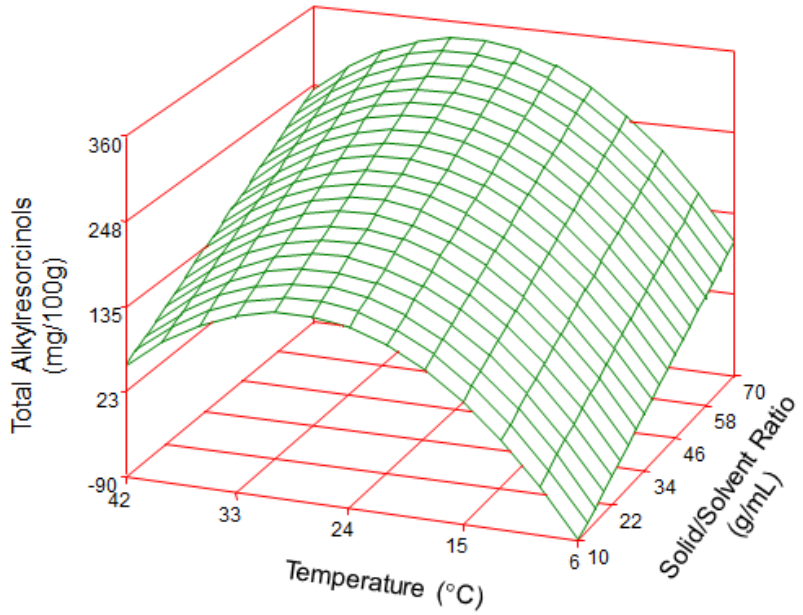


Figure 2.5 - Response surface for the effect of temperature (°C) and solid to solvent ratio (triticale bran to acetone) on total alkylresorcinols in triticale bran.

2.4.5 FTIR profiles

The FTIR profiles of samples were used to support the results obtained from HPLC and GC-MS. The FTIR spectra of ARs extracts (at 24 ºC, solid to solvent ratio of 1:40 after 24 h) along with one of the alkylresorcinol standards (C21:0) are shown in (Figure 2.6A and 2.6B). The FTIR profiles of the extracts obtained from triticale bran (Figure 2.6B) were similar to the ARs standards from C21:0 (Figure 2.6A), indicating that these samples consisted of bioactive alkylresorcinols. The broad band with high intensity ~3290 cm^{-1} corresponded to the stretching vibrations of the OH groups on the phenol rings (Figure 2.6B) (*222*). Absorption at 2900 and 3000 cm^{-1} are attributed to the C-H stretching vibrations of the CH_2 and CH_3 aliphatic groups of the alkyl tail. The 1500–1600 cm^{-1} absorption band was attributed to aromatic ring stretching vibrations. The nearly identical bending vibrations in the fingerprint region of 400-1500 cm^{-1} further establish the presence of ARs in sample extracts and indicate minimal impurities being present that may interfere with spectral absorption patterns.

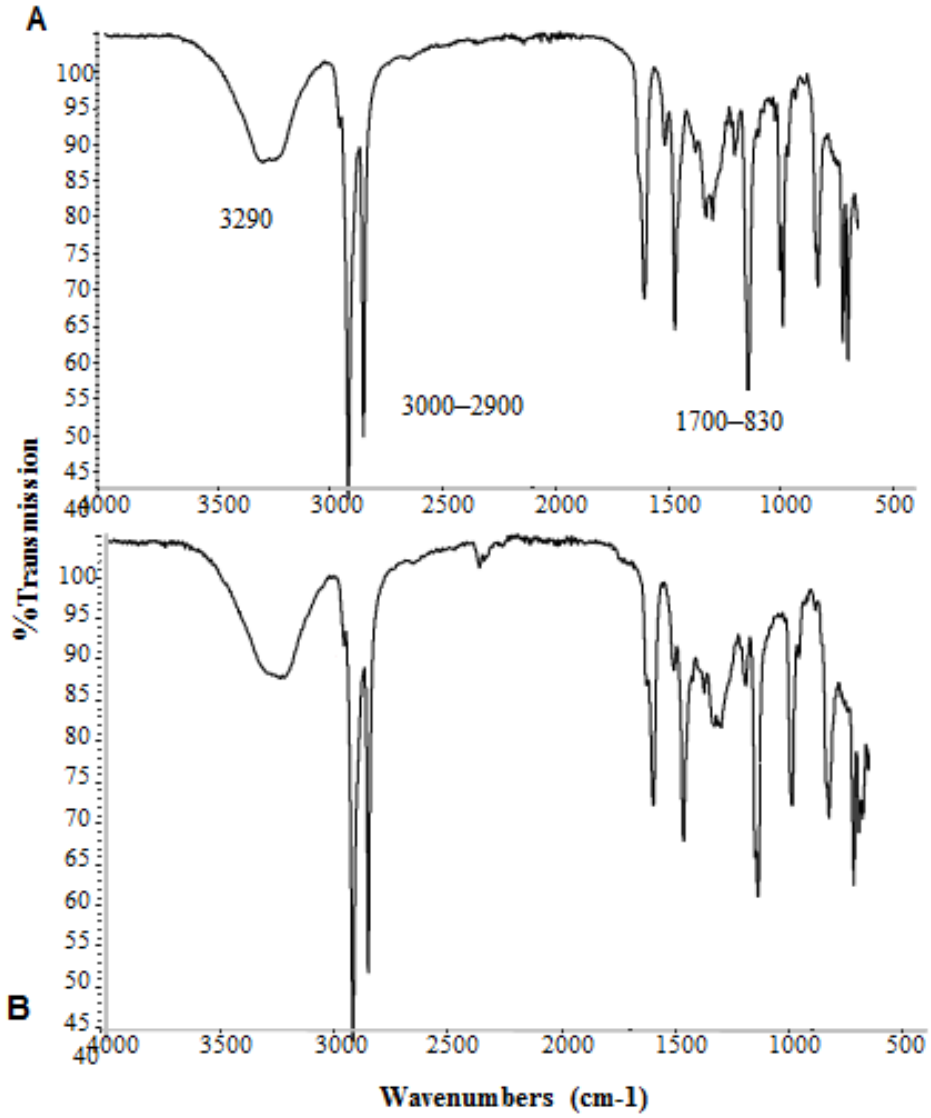


Figure 2.6 - FTIR spectra of **A** alkylresorcinol standard (C21:0), and **B** alkylresorcinols extracted with acetone from triticale bran.

2.5 Conclusion

RSM can be used to optimize extraction of alkylresorcinols from triticale bran. The high correlation of the model (r=0.93) indicated that it accurately expressed the influence of independent variables on the response that was measured. The model showed that the highest amount of ARs from triticale bran can be extracted at 24 ºC for 16 to 24h at a solid to solvent ratio (w/v) of 1:40. Triticale bran contained high levels of alkylresorcinols (277.5 to 308.3 mg/100g) and thus can be used as a source of these dietary bioactives. Saturated ARs were the major ARs in all samples and ranged from 86 to 88% of total ARs content. C17:0, C19:0 C21:0 and C23:0 were the major ARs in all samples. The presence of high levels of ARs in triticale bran may be a significant factor to take into account with respect to its potential benefits for human nutrition and health.

2.6 Connecting Statement to Chapter 3

Chapter 2 provided an in depth evaluation of the effects of solid-to-solvent ratio, temperature and time on the extractability of total ARs as well as saturated and unsaturated AR homologue contents from triticale bran thereby allowing for the determination of optimum parameters for ARs extraction.

In Chapter 3, the extent to which ARs can impede the activity of biologically relevant oxidative stressors such as the DPPH radical and peroxyl radicals generated by AAPH are evaluated through *in vitro* and *ex vivo* model systems. In addition, the ability of ARs to inhibit the activity of inflammatory stressors such as nitrite production induced by lipopolysaccharides and inhibition of pro-inflammatory enzyme, cyclooxygenase-2, is determined using *in vitro* and *ex vivo* model systems.

The work presented in Chapter 3 has been used to prepare one publication as follows:

- Agil R, Gliwa, J, Willmore WG, and Hosseinian F, Protective effects of triticale bran alkylresorcinols against inflammation and oxidative stress measured by *in vitro* and *ex vivo* model systems. Manuscript submitted to Journal of Functional Foods. (2016).

Chapter 3 Protective effects of triticale bran alkylresorcinols against pro-oxidative stressors and pro-inflammatory agents

3.1 Abstract

Modern medicine aims to find preventative means against increasingly prevalent chronic diseases by investigating the health benefits of various foods in the diet and bioactive compounds responsible. The main objectives of this study were to determine the antioxidant and anti-inflammatory capacity of alkylresorcinols (ARs) extracted from triticale bran (TB). Antioxidant activity assays exhibited an ORAC value of 183.7 μmol trolox equivalent (TE)/g and 20.1% DPPH radical inhibition by TB-ARs. Furthermore, RAW 264.7 macrophage-like cells exposed to peroxyl radical generator, AAPH, and endotoxin, LPS, increased in cell viability by 32% and cell production of pro-inflammatory marker, nitrite, decreased by 35% in the presence of 80.0 μg/mL TB-ARs. Additionally, COX-2 activity induced by arachidonic acid was significantly inhibited by 49% in the presence of 83.34 μg/mL TB-ARs ($P < 0.05$). These results suggest a strong future potential for cereal bran ARs as novel functional food ingredients due to their health benefitting properties.

3.2 Introduction

Oxidative stress and systemic inflammation are major contributing factors to the onset of various chronic diseases such as cancer, cardiovascular disease (CVD), obesity and diabetes (*223-226*). Free radicals are highly reactive chemical species due to their unpaired electron. As a by-product of aerobic respiration, mitochondria produce reactive oxygen species (ROS) such as superoxide $O_2^{\cdot -}$ and hydrogen peroxide, H_2O_2. During inflammation, macrophages and endothelial cells produce reactive oxygen species as a protective mechanism against tissue damage by harmful stimuli. Additionally, the reactive nitrogen species (RNS), nitric oxide (NO), is an important cell signalling molecule and powerful vasodilator. In addition to these endogenous sources, exogenous sources of ROS include ultraviolet light, ionizing radiation and other environmental stressors (*227*). Overproduction of ROS can lead to cellular damage and necrosis, thus cells produce antioxidant enzymes which reduce and quench these ROS in addition to antioxidant molecules such as glutathione that allow for the regeneration of these enzymes (*228*). When the cell's ability to detoxify ROS is compromised and antioxidant molecules have been depleted, oxidative stress results (*229*). Both acute inflammation and reactive oxygen species are a part of the body's natural order of things; however, aging, continued exposure to environmental stressors and unhealthy lifestyle habits such as overeating, lead to oxidative stress and chronic inflammation. The toxic and damaging effects of prolonged oxidative stress and inflammation include cellular dysfunction, tissue destruction and fibrosis, predecessors for the onset of chronic diseases (*230*).

In today's society, measures to reduce the risk of chronic disease are sought after through the investigation of foods that present beneficial health effects to biological systems. Fruits, vegetables, legumes and grains are well established sources of protein, carbohydrates, nutrients

and bioactives with health promoting properties (*231*). In particular, whole grains are well established in literature as rich sources of phytochemicals such as alkylresorcinols (AR) which are predominantly found in the bran layer (*9, 191*). These phenolic lipids owe their amphiphilic nature to their polar aromatic ring and non-polar alkyl chain at the C5 position. It has been speculated that this property of ARs allows for its ease of incorporation into cell membranes where it can carry out its antioxidant effects by ring stabilized oxidation of its hydroxyl groups (*62*).

While there have been some studies on the antioxidant activity of ARs from wheat and rye grains, none have investigated the bioactive potential of ARs from triticale (*108, 109, 111*) . Triticale is a hybrid of wheat and rye that possesses both a high yield potential and environmental tolerance to disease and soil conditions. As a part of our on-going research on the development of functional food ingredients from bioactive components of cereal grains, the aims of the present study were to: 1) Verify the total AR content of TB by a rapid screening method employing Fast Blue B salt, 2) Investigate the antioxidant activity of TB-ARs by *in vitro* measures, ORAC and DPPH radical scavenging activity assays, 3) Investigate the *ex vivo* antioxidant capacity of TB-ARs by measuring the cell viability of RAW 264.7 macrophage-like cells exposed to AAPH peroxyl radical scavenger in the presence of AR extract, 4) Evaluate the anti-inflammatory potential of TB-ARs by *in vitro* measures, COX-2 inhibition assay, and 5) Evaluate the *ex vivo* anti-inflammatory potential of TB-ARs by measuring the level of nitrite produced by cells exposed to LPS in the presence of ARs.

3.3 Materials and methods

3.3.1 Materials

Acetone, methanol, ethanol, 1-propanol, phosphoric acid and acetic acid were obtained from Caledon Laboratories (Georgetown, ON, Canada). Mono-and dibasic potassium phosphate, fluorescein, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), rutin, 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2, 2-dipheny-1-picryhydrazyl radical (DPPH), Fast Blue B salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS from *Escherichia coli*, N-(1-Naphthyl)ethylenediamine dihydrochloride (NED), sulfaniliamide, phosphate buffered saline (PBS), were acquired from Sigma (Oakville, ON, Canada). Alkylresorcinol standard C15:0 (1,3-Dihydroxy-5-pentadecyl-benzene) was obtained from ReseaChem GmbH (Burgdorf, Switzerland).Dimethyl sulfoxide (DMSO) and bovine serum albumin (BSA) were obtained from Bioshop (Burlington, ON, Canada). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were acquired from Life Technologies (Grand Island, NY, USA. Triticale bran (AC Ultima, spring triticale) was provided by Agriculture and Agri-Food Canada (Lethbridge, AB, Canada). Oat bran (OB) and wheat bran (WB) samples were purchased from a commercial source (Bulk Bran) in Ottawa, ON, Canada. $NaNO_2$ crystals (sodium nitrite) were obtained from Anachemia Chemicals LTD (Montreal, QC, Canada).

3.3.2 Extraction procedure

In accordance with the optimized procedure outlined in the previous chapter, the extraction of ARs was carried out as follows. Alkylresorcinols were extracted from bran samples with acetone at a ratio of 1:40 (w/v; g/mL) for 24 h under continuous agitation at room temperature. After vaccum filtration using Whatman #1 filter paper, the supernatant was collected and stored at 4 ºC prior to analysis.

3.3.3 Colorimetric determination of total ARs

Fast Blue B (FBB) stock solutions was prepared by dissolving FBB salt in 5% acetic acid at a solid-to-solvent ratio of 1:1000 (w/v; g/mL). An FBB working solution was prepared by the addition of 40 mL of 1-propanol to 10 mL of stock. A concentration of 250 µg/mL of AR homologue standard, C15:0, was prepared in acetone. Sample volumes of 150 µL AR extract, and standard volumes of 0, 5, 20, 30, 50 and 100 µL were evaporated to dryness by a stream of N_2 gas and re-dissolved in 4 mL of FBB working solution. Following a 5 h incubation period at 4 ºC under dark conditions, 200 µL of sample, standard, and blank (FBB working solution) were plated on a 96-well quartz plate and the absorbance read at 520 nm using an Epoch microplate spectrophotometer with Gen5 software (BioTek Instruments,Winooski, VT, USA). Using the standard calibration curve prepared, the total content of ARs in cereal bran extracts was calculated based on the formula established by (*168*).

3.3.4 *In vitro* evaluation of antioxidant and anti-inflammatory activity

3.3.4.1 ORAC assay

The ORAC values of AR extracts from TB, WB, and OB were determined in accordance

with procedures previously described in literature (*232*). Using an automated plate reader (FLx800 with Gen5 software, BioTek Instruments, Winooski, VT, USA), the extent to which ARs (65 µg/mL) can delay fluorescence decay caused by peroxyl radical generator, AAPH over a period of 1h was determined by comparing the net area under the curve for each bran sample against that of a trolox standard curve. Final results were calculated as mg Trolox equivalent (TE)/g of bran sample and rutin was used a positive control sample.

3.3.4.2 DPPH assay

The % DPPH radical scavenging activity of AR extracts from TB, WB, and OB were determined by addition of 60 µM DPPH in 100% methanol to sample extracts (65 µg/mL) at a ratio of 30:2 (v/v) for a total volume of 200 µL in a 96-well plate. Using an Epoch microplate spectrophotometer with Gen5 software (BioTek Instruments, Winooski, VT, USA), the absorbance was read at 515 nm for a period of 1 h at 5 min intervals. To determine the DPPH scavenging effect of AR extracts, the following formula was used to calculate percentage inhibition:

$$\% \text{ DPPH inhibition} = \left(1 - \frac{Abs_{sample\ t0}}{Abs_{control\ t}}\right) \times 100\%$$

where *Abs control* was the absorbance of DPPH at time zero t_0 and *Abs sample* was the absorbance of sample extracts in DPPH solution at time *t*. The aforementioned procedure was followed with some minor modifications from literature (*233, 234*).

3.3.4.3 COX assay

Using a COX fluorescent inhibitor screening assay kit purchased from Cayman Chemical (Ann Arbor, MI, USA), the anti-inflammatory potential of ARs was evaluated. Concentrations of 20.83, 41.67, 83.34, 166.67, and 333.34 µg/mL of TB-AR extracts were prepared in DMSO and

10 µL volumes were plated in combination with 150 µL of assay buffer (100 mM Tris-HCl, pH 8.0), 10 µL heme, 10 µL fluorogenic substrate (10-acetyl-3,7- dihydroxyphenoxazine), and 10 µL human recombinant COX-2 enzyme. A 25 nM (0.01µg/mL) solution of 5-bromo-2[4-fluorophenyl]-3-[4-methylsulfonylphenyl (DuP-697), a potent inhibitor of COX-2, was used a positive control and 100% DMSO was used as a blank. Immediately after adding 10 µL of arachidonic acid to initiate the reaction, the plate was read at excitation and emission wavelengths of 544 and 590 nm respectively, on a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany). Percentage inhibition of COX-2 enzyme activity was calculated as follows:

% COX-2 Inhibition = ($\frac{Initial\ Acvtivity - Sample\ Activity}{Initial\ Activity}$) x 100%

The aforementioned procedure was closely followed in accordance with guidelines set out by Cayman Chemical Company.

3.3.5 *Ex vivo* evaluation of antioxidant and anti-inflammatory activity

3.3.5.1 Cell culture treatment

The murine macrophage cell line (RAW 264.7) was supplied by the American Type Culture Collection (ATCC; Manassas,VA, USA) and cultured in DMEM supplemented with 10% FBS. The cells were maintained in an atmosphere of 5% CO2 at 37 °C and sub-cultured every other day.

To evaluate the cytoxicity of ARs, cells were seeded in 96-well plates at a density of (2 x 10^4 cells/100 µL), incubated for a 24 h period, washed and fresh media was applied. Cytotoxicity was analysed using an MTT assay after 24 h of continuous exposure to 2 µL of TB-ARs extract

in 95% EtOH (1.0-800.0 μg/mL). Prior to measuring the absorbance at 570 nm against a reference wavelength of 630nm (SpectraMax 340PC384 microplate reader, Molecular Devices, Sunnyvale, CA, USA), 10 μL of MTT (5 mg/mL in PBS) was added, incubated for 1 h at 37 °C, and the media replaced with DMSO (*235*).

3.3.5.2 AAPH-stimulated cytotoxicity assay

Cells (2 x 10^4 cells/100 μL) were seeded in 96-well plates, incubated for 24h, and replaced with fresh media. Cells were pre-treated with TB-ARs extract (80 μg/ mL) for 24h and replaced with fresh media prior to AAPH (3 mM) exposure for incubation periods of 10, 30, 60, and 120. The absorbance of viable cells was measured spectrophotometrically at 570nm against a background wavelength of 630nm by MTT assay. Results were compared to that of control cells exposed to AAPH in the absence of AR extract.

3.3.5.3 Nitrite assay

The following procedure for the measurement of nitrite accumulation in LPS-stimulated macrophage-like cells was modified from (*236*). Nitrite production was stimulated in RAW 264.7 macrophage cells (2 x 10^4 cells/100 μL) by introduction of 1 μg/mL of LPS for 24 h in the presence or absence of TB-ARs extract (10.0, 20.0, 40.0, and 80.0 μg/ mL). Griess reagent (1:1 mixture of 0.1% NED in H_2O and 1% sulfanilamide in 5% H_3PO_4) was added to the cells at a ratio of 1:1, incubated in the dark for 10 min and read at 540 nm by UV-Vis spectrophotometry (SpectraMax 340PC384 microplate reader, Molecular Devices, Sunnyvale, CA, USA). A standard curve was generated by known concentrations of $NaNO_2$ and the derived linear equation was used to calculate the concentration in samples.

3.3.6 Statistical analysis

Extractions were carried out in triplicate, *in vitro* assays were analysed in triplicate and *ex vivo* assays were analysed in replicates of six. Data are expressed as means values ± SEM. Analysis of variance was performed by one-way analysis of variance (ANOVA) with significant differences ($P < 0.05$) between means determined by the Student's t-test (SAS version 9.4, SAS Institute Inc., Cary, NC, USA).

3.4 Results and discussion

3.4.1 Quantitative determination of total AR content

In this study, the total AR contents of TB, RB, WB, and OB extracts were measured and compared by a colorimetric method involving Fast Blue B. This diazotized salt is selective for 1,3-dihydroxy-benzene rings, forming a reddish-violet coloured azo-complex is detected at 520 nm (*237*). This method is a reliable and effective means of comparing the total AR contents in cereal grains as it has been optimized and used for several years (*108, 163-165, 167, 238*). While HPLC is important for identifying the AR homolog composition of cereal samples, the method of colorimetric determination using FBB is fast and valid means of conducting preliminary screening for the presence of in a sample and generating comparative results of total ARs among multiple samples of varying cultivars, regions or varieties.

From this method, the total AR content in TB was measured at 192.8 mg C15:0 equivalent/100 g of bran sample (Figure 3.1). By comparison, the AR content of TB was significantly greater than that of WB (174.4 mg/100 g) and 14% less than that of RB at 218.6 mg/100g ($P < 0.05$). As anticipated, OB contained a substantially lower level of ARs (16.7 mg/100g) with less than 10% of the amount measured in TB. These results are in agreement with

literature findings as rye grains, on average, have been found to have the highest level of ARs amongst cereal grains, whereas OB typically has near negligible levels of ARs by comparison (*77, 239, 240*). Although the AR content in wheat grains varies considerably among classes, common/commercial wheat has generally exhibited a lower AR content than that of triticale species (*37, 167*) .

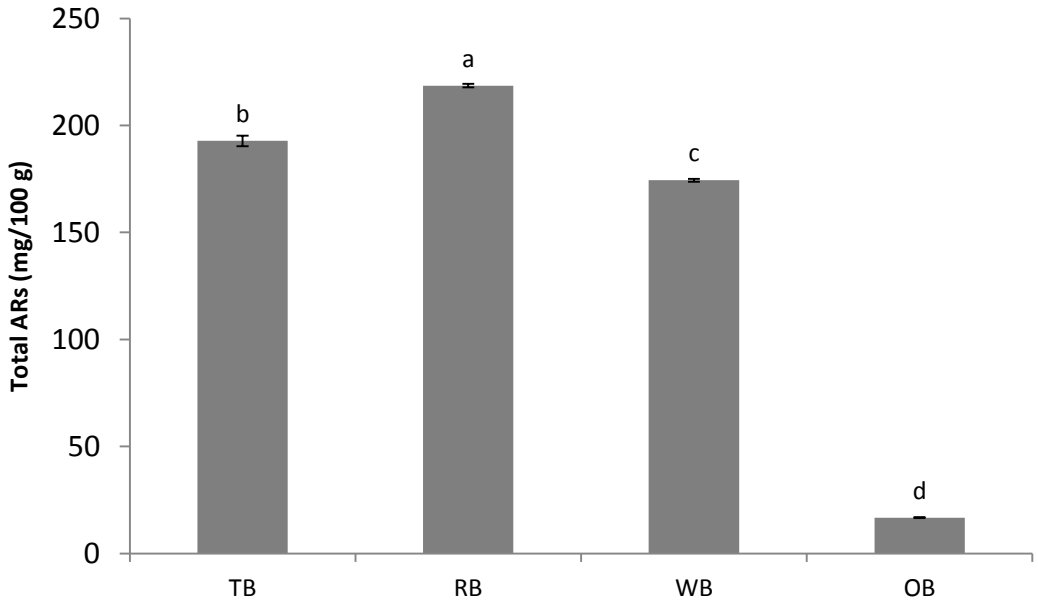


Figure 3.1 - Total AR contents of extracts from cereal bran samples by colorimetric determination using Fast Blue B reagent. RB: rye bran; WB: wheat bran; OB: oat bran. Results are expressed as mean values ± SEM. Values with different letters are significantly different ($P < 0.05$).

3.4.2 *In vitro* Antioxidant activity analysis

Molecules capable of inhibiting or delaying oxidative degradation at much lower concentrations than the oxidizable substrate are considered antioxidants. To determine the

antioxidant strength of these molecules, model systems such as ORAC, DPPH and other model systems reflective of antioxidant reactions *in vivo* are often employed.

3.4.2.1 Peroxyl radical scavenging effect of ARs

The ORAC assay is considered one of the more biologically relevant model systems of antioxidant reactions due to the peroxyl radicals used and their constant generation within a reasonable timeframe (*241*). Through this assay, the radical chain breaking activity of antioxidants by hydrogen atom transfer is reflected. The antioxidant strength of a compound is relative to its structural characteristics as this dictates whether it can readily transfer hydrogen atoms or electrons to free radicals and form a stable end product (*242*). From measured ORAC values, TB-ARs demonstrated a similar trend reflective of the AR contents found in bran samples (Figure 3.2). Although the ORAC value of TB-ARs (183.7 μM TE/mg) was greater than that of WB-ARs (174.6 μM TE/mg), it did not significantly differ in peroxyl radical scavenging capacity ($P > 0.05$). . This outcome may be explained by the difference in AR homologue profiles among cereal bran samples. Alkyresorcinol homologues present in cereal grains vary in chain length from C15:0 to C25:0, however, each species of grains has been found to have its own unique distribution profile (*212*). The predominant AR homologues in wheat, rye and triticale are C19:0 and C21:0, however, wheat typically has a much higher ratio of C21:0 to C19:0 in contrast to rye where the opposite is true, and triticale contains a fairly equal proportion of these two homologues (*62, 243*). Longer chain alkylresorcinols have been reported to possess a higher radical scavenging potential (*103, 106*), thus a possible contributing factor to the lack of significant difference in ORAC between TB and WB.

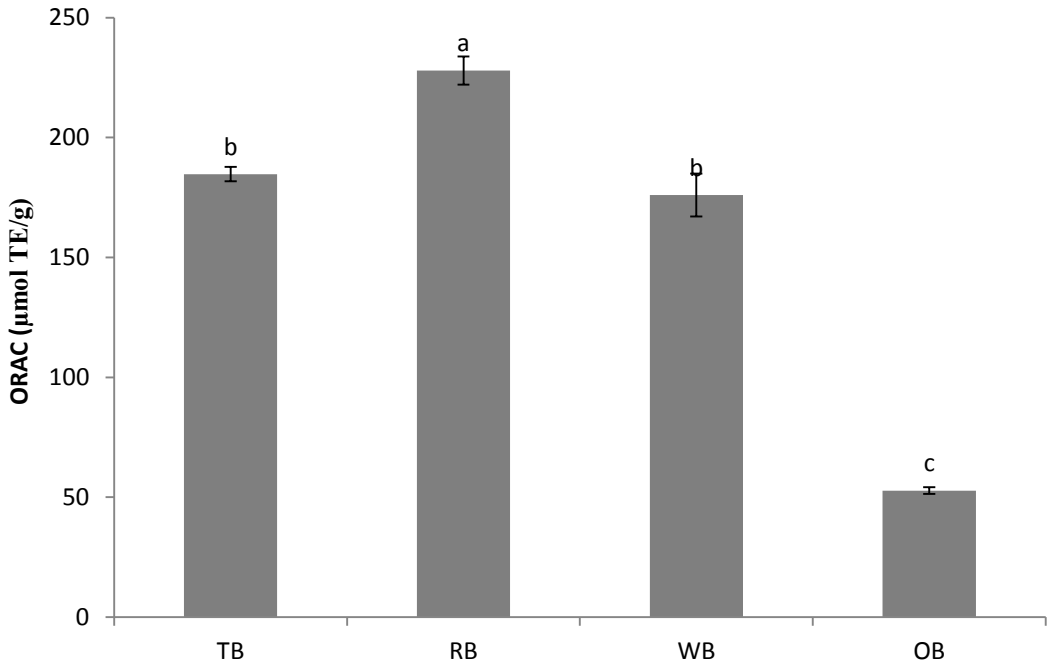


Figure 3.2 - Antioxidant activity of AR extracts from cereal bran samples determined by ORAC in µmol TE/g. RB: rye bran; WB: wheat bran; OB: oat bran. Results are expressed as mean values ± SEM. Values with different letters are significantly different ($P < 0.05$).

3.4.2.2 DPPH radical inhibition effect of ARs

The organic nitrogen radical, DPPH, is stable and does not have to be generated thus allowing for a controlled environment in measuring the reducing capacity of antioxidants by electron transfer. When the DPPH radical is quenched, it loses its deep purple color that absorbs at 515nm, thus the scavenging effect of antioxidants is determined by the loss of absorbance over time reported as %DPPH inhibition.

Results of this assay demonstrated a consistent pattern with that of total AR contents and ORAC values such that TB-ARs maintained a radical scavenging capacity greater than WB and OB, yet remained less than that of RB with 20.1% DPPH inhibition (Figure 3.3). The unexpectedly higher scavenging capacity of OB-ARs may be due to the likely presence of non-

alkylresorcinol components that may have been extracted due to the lack of ARs present in oat cereal grains. If these molecules are smaller in size than ARs they have the advantage of easier access to the DPPH radical, resulting in an overestimation of antioxidant potential in this sample (*244*). Nonetheless, these findings were comparable to literature findings which have reported the %DPPH radical scavenging capacity of multiple cultivars of wheat to be in the range of 21-54% and 43% for rye (*108, 111*).

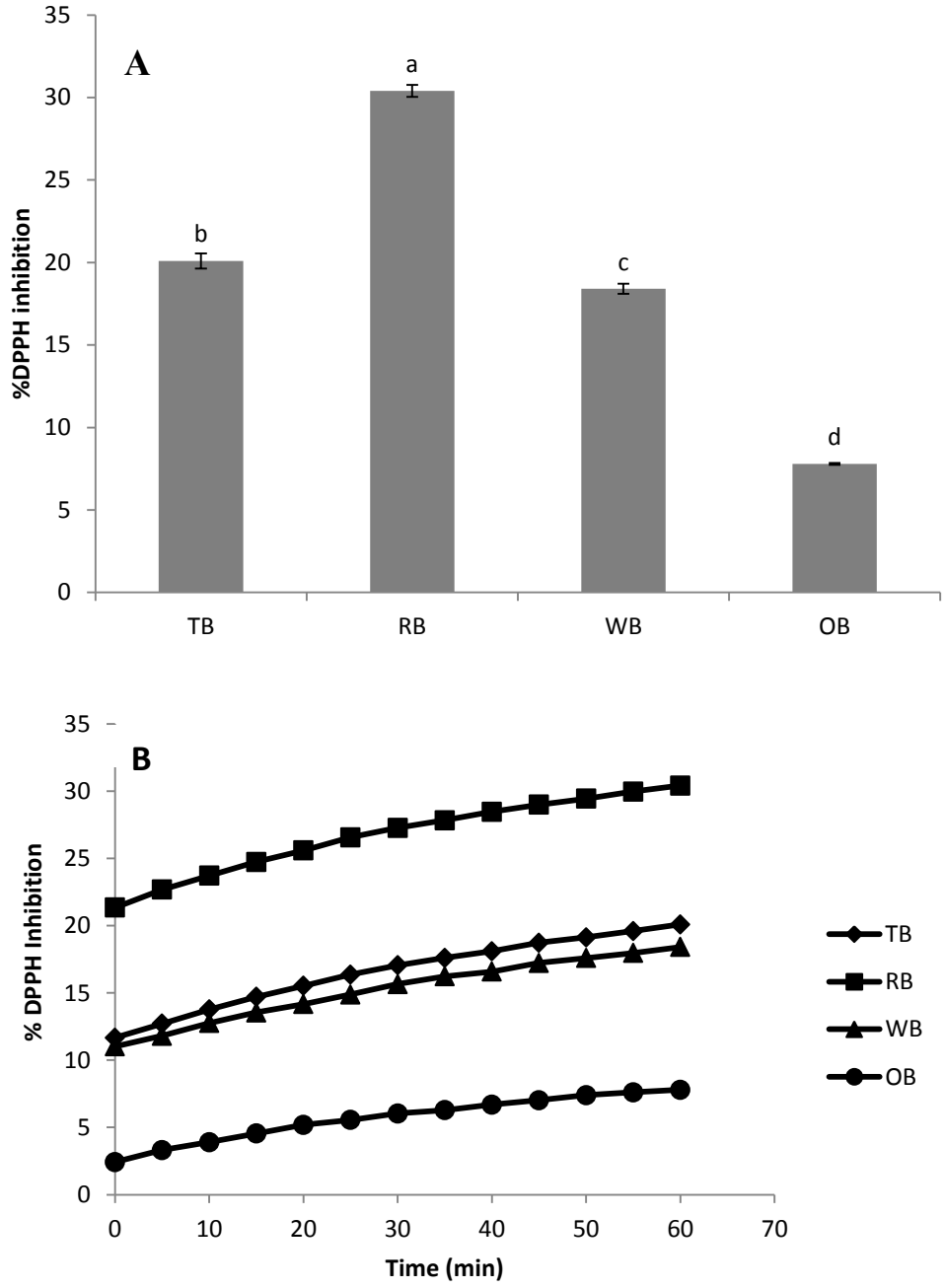


Figure 3.3 - Inhibition % (A) and kinetics (B) of antioxidant activity of AR extracts from cereal bran samples using the DPPH radical. TB: triticale bran; RB: rye bran; WB: wheat bran; OB: oat bran. Results are expressed as mean values ± SEM. Values with different letters are significantly different ($P < 0.05$).

3.4.3 *In vitro* anti-inflammatory activity analysis

3.4.3.1 Effect of ARs on COX-2 activity

Part of the initial stages in the inflammatory response of tissues to a stimulus is the activation of phospholipase A2 which acts on membrane phospholipids to release the polyunsaturated fatty acid, arachidonic acid. Subsequently, cyclooxygenase enzymes catalyze the formation of prostaglandins (PGs) G_2 and PGH_2, as well as thromboxanes (*245*). Two isoforms of cyclooxygenase exit, COX-1, which is continuously expressed as a part of normal cellular homeostasis, and COX-2 which is mainly expressed under acute inflammatory conditions (*246*). Due to the key role of COX enzymes in the inflammatory pathway, it has become an important target of clinical studies for the identification of non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit prostaglandin and thromboxane synthesis typically by binding their active sites (*247*). In this study, inhibition of COX-2 activity by TB-ARs is measured by the absence of resofurin, a highly fluorescent reaction product of PGG_2 with ADHP (10-acetyl-3,7-dihydroxyphenoazine).

Initial COX-2 activity was inhibited by 19.2% in the presence of 20.83 µg/mL of AR extract from TB (Figure 3.4). Concentration dependant inhibitory effects were witnessed at TB-AR concentrations of 20.83, 41.67, 83.34 and 166.67 µg/mL, beyond which the degree of inhibition did not change significantly. The inhibitory concentration of TB-ARs in which almost 50% of the initial COX-2 activity was inhibited (IC_{50}) was close to 83.34 µg/mL which is a substantially higher concentration than potent COX-2 inhibitor, DuP-697 at 0.01 µg/mL. Nevertheless, the IC_{50} of TB-ARs is comparable to that of common NSAIDs, acetaminophen, aspirin, and sodium salicylate with $IC5_{50}$ values for COX-2 at 20, 50 and 100 µg/mL respectively (*248*).

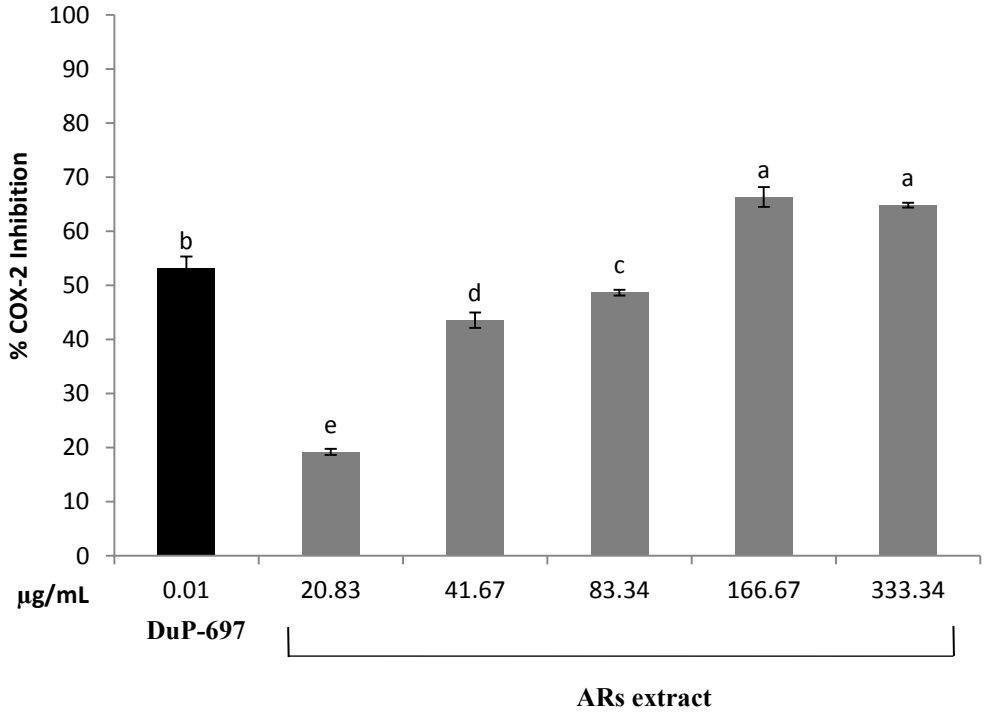


Figure 3.4 - Inhibition % of COX-2 activity by AR extracts from triticale bran. Results are expressed as % of control and are the mean values ± SEM. Values with different letters are significantly different ($P < 0.05$).

3.4.4 *Ex vivo* antioxidant activity analysis

3.4.4.1 Effect of ARs on cell viability of AAPH-stimulated RAW 264.7 cells

To test the cytotoxicity of ARs, the ability of cells to convert MTT which is yellowish in solution, to formazan, a bluish purple product that is measured spectrophotometrically. Since formazan can only be produced by active mitochondrial dehydrogenases of live cells, cytotoxicity in RAW 264.7 cells is determined based on the absorbance intensity of converted dye.

As such, the incubation of cells with extracts (1.0–800.0 µg/mL) resulted in moderate cytotoxic action for the tested extracts (Figure 3.5). Concentrations at or above 200.0 µg/mL proved to be too high a concentration as cell viability was drastically reduced to a very low range of 2-10%. TB-AR extracts did not cause cell death or exhibit significant cytotoxicity in RAW 264.7 cells (83%-95% cell viability) at concentrations of 10, 20, 40, and 80 µg/ml; thus, these concentrations were used for further studies.

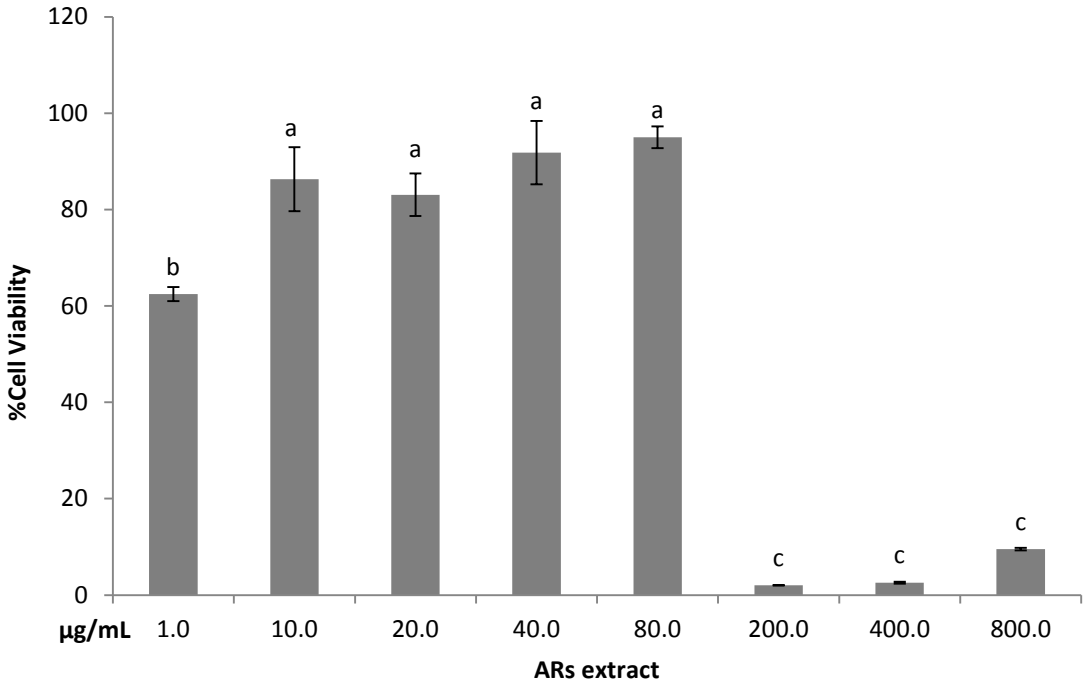


Figure 3.5 - Effects of increasing concentrations of AR extracts from triticale bran on the viability of RAW 264.7 cells. Cell viability was evaluated by MTT assay. Results are expressed as % of control and are the mean values ± SEM. Values with different letters are significantly different ($P < 0.05$).

At physiological temperature (37°C), AAPH decomposes to tert-amidinopropyl radicals ($ROO^{\bullet}$) which rapidly convert to two dioxyl (hydroperoxyl) radicals in the presence of oxygen. These radicals go on to oxidize proteins and polyunsaturated lipids of cell membranes to protein and lipid peroxides that damage cells, implicate cellular function, and eventually lead to necrosis (*249*). As seen in Figure 3.6, intracellular oxidative stress induced by AAPH (3mM) was substantially reduced in the presence of TB-ARs (80 μg/ml). Based on the significantly higher absorbance values ($P < 0.05$) maintained throughout the 2h treatment with AAPH in the presence of extract, ARs seem to increase the metabolic of activity of cells in an effort to return them to a normal homeostatic state (Figure 3.6). Studies have suggested that ARs may have a more pronounced antioxidant effect *in vivo* due to their amphiphilic nature that allows them to more easily incorporate into the lipid bilayer and impart their radical scavenging, antioxidant effects(*104, 153*). Our recent findings demonstrated that ARs may also possess significant antioxidant effects *in vivo* through the evaluation of ORAC, SOD activity, and oxidized glutathione levels measured in heart and liver tissues of mice fed a high fat diet in the presence and absence of TB-ARs (*250*). The cytoprotective effect of ARs against AAPH induced damage in RAW 264.7 cells further confirms their antioxidant properties elucidated in literature (*62, 108, 109, 111*).

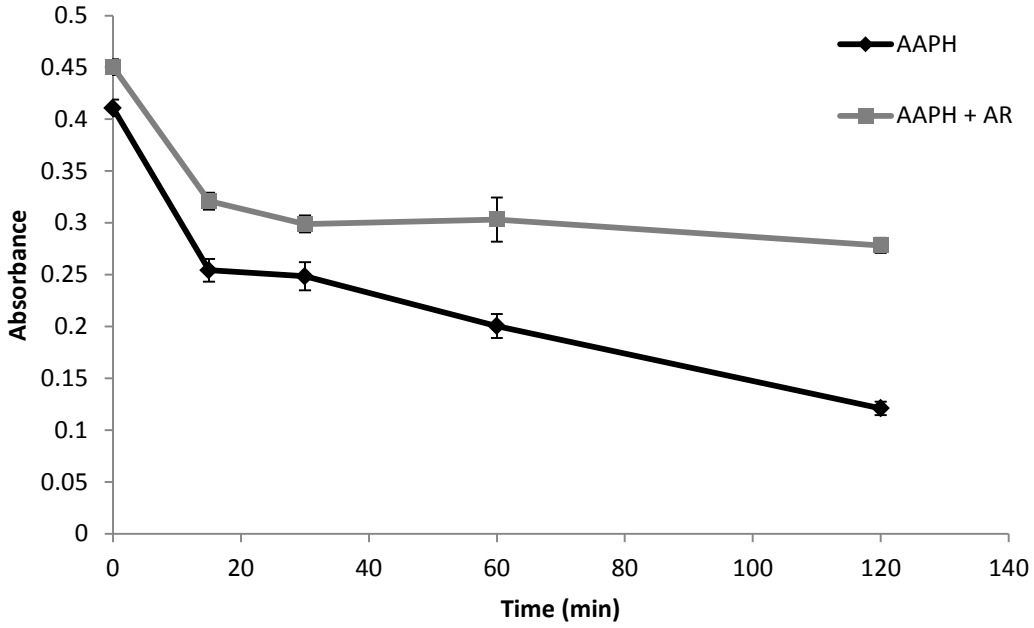


Figure 3.6 - Effects of TB-ARs on the viability of AAPH-stimulated RAW 264.7 cells determined by MTT assay. Cytotoxicity was stimulated by AAPH (3mM) in cells (2 x 104 cells/100 µL) for 0, 15, 30, 60 and 120 min in the presence of 80 µg/ml of AR extract from triticale bran. Results are expressed as absorbance of viable cells at 570 nm against a reference of 630 nm and are the mean values ± SEM. Values were significantly different among groups at each time point ($P < 0.05$).

3.4.5 *Ex vivo* anti-inflammatory activity analysis

3.4.5.1 Effect of ARs on nitrite production of LPS-induced RAW 264.7 cells

The bacterial endotoxin LPS elicits a strong immune response in macrophages resulting in the activation numerous pro-inflammatory enzymes including inducible nitric oxide synthase (iNOS) which produces RNS such as NO. Since NO has a short half-life, it rapidly oxidizes to nitrite/nitrate by oxygen. Thus the production of NO released by LPS stimulated macrophage-like cells was estimated by supernatant nitrite.

Co-treatment of cells with TB-ARs significantly reduced nitrite accumulation in a dose-dependent manner ($P < 0.05$). As such, the lowest tested dose of 10 µg/mL AR extract decreased nitrite production by 25% and a near 3-fold decrease at the highest tested dose of 80 µg/mL (Figure 3.7). The inhibition potential of ARs against COX-2 activity and LPS-stimulated NO production by macrophages is representative of the significant anti-inflammatory capacity of ARs. Results of this study are also evidenced by findings of significantly reduced COX-2 and iNOS protein and mRNA expression in LPS stimulated RAW 264.7 cells treated with AR extracts from RB (*183*). Studies on the inhibition of soybean lipoxygenases by resorscinolic lipids from cereal bran suggest the mechanism behind their bioactive properties may be partially due to their ability to bind the active sites of the enzyme and outcompete its substrate (*251*). The ability of ARs to easily incorporate into cell membranes may suggest their accessibility and interaction with membrane bound proteins such as the membrane receptor protein, phospholipase A2, by blocking its active site and preventing the release of arachidonic acid, a pro-inflammatory mediator and substrate.

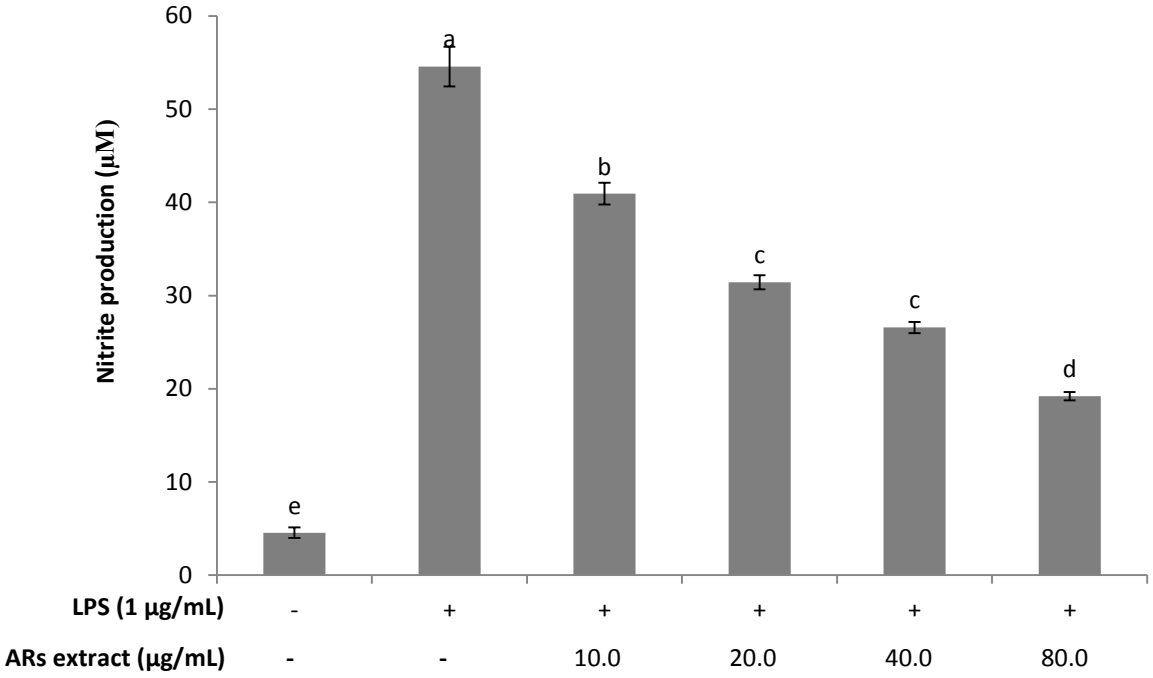


Figure 3.7 - Effects of TB-ARs on nitrite production by LPS-stimulated RAW 264.7 cells determined using Griess reagent. Cells (2 x 104 cells/100 µL) were stimulated by LPS (1 µg/mL) for 0, 15, 30, 60 and 120 min in the presence of AR extract from triticale bran (10, 20, 40, 80 µg/mL). Results are expressed as mean values ± SEM. Values with different letters are significantly different ($P < 0.05$).

3.5 Conclusion

Through the reaction of extracts from bran samples with Fast Blue B, the total of AR content of TB Ultima was determined and compared to that of positive controls, RB and WB, as the negative control OB. Total AR content in bran samples was highest in RB, closely followed by TB, and WB, whereas OB contained extreme low amounts by comparison. While the antioxidant activity of ARs has been previously reported, this study introduces new findings on the antioxidant activity of ARs extracted from TB using DPPH and ORAC assays. Additionally, the significant antioxidant effect of TB-ARs against cytotoxic damage induced by AAPH was determined. In measuring the inhibition of COX-2 activity by TB-ARs, their IC_{50} was within the range of notable NSAIDs, aspirin, ibuprofen, and acetaminophen, suggesting that ARs possess anti-inflammatory properties in addition to their antioxidant effects. The high degree to which ARs reduced the accumulation of nitrite stimulated by LPS in macrophages suggests a dual functionality of ARs to not only scavenge RNS such as nitrite and nitric oxide, but to potentially inhibit pro-inflammatory mediators such as iNOS by interacting with and consequently blocking its active site from its corresponding substrate.

Chronic inflammation has been implicated in many human diseases through the up-regulation pro-inflammatory transcription factors and proteins. These proteins include enzymes such as iNOS and COX-2 which play central roles in the production of pro-inflammatory cytokines, prostaglandins, ROS and RNS. Continuous production of these compounds can lead to the development of cancer caused by the inflammatory microenvironment created that aids in the progression and growth of tumors. Thus, the free radical scavenging and anti-inflammatory activity of ARs may contribute to reducing the risk of these chronic diseases through their application as functional food ingredient and neutraceticals, or through the increased

consumption of cereal bran in the diet.

Future studies aim to investigate the structure function relationship of ARs and their bioactive properties as well as the mechanisms behind their free radical scavenging and anti-inflammatory activity revealed in this study. Through future evaluation of individual AR homologue activity using both *in vitro* and *ex vivo* methods, we can better understand the relation of side chain length to the strength, functionality of ARs as well as their ability to interact and potentially bind enzyme active sites or their ease of incorporation into lipid bilayers. Furthermore, additional studies evaluating the *in vivo* activity and effects of ARs through animal studies and clinical trials need to be carried out before ARs can be considered functional food ingredients for the risk reduction of various pathologies linked to oxidative stress and chronic inflammation.

3.6 Connecting Statement to Chapter 4

In Chapter 3, the antioxidant and anti-inflammatory capacity of ARs was determined using *in vitro* and *ex vivo* model systems. Through this study, the effective concentration of ARs could be determined as well as the maximum cytotoxic concentration of ARs in RAW 264.7 macrophage cells. In addition novel findings evidencing the anti-inflammatory nature of ARs, results of this chapter further confirm previous studies reporting their antioxidant properties.

In Chapter 4, effects of ARs on obesity development and oxidative stress are assessed. The impact of AR incorporation in the diet of high fat fed mice on weight, food intake, and glucose tolerance is monitored over the course of 12 weeks. Additionally, the extent to which ARs in the diet aid in the prevention of oxidative stress is evaluated through ex-vivo analysis of oxidative stress markers in the mice liver and heart tissues.

In Chapter 2, optimization conditions for ARs extraction from triticale bran are determined using response surface methodology.

The work presented in Chapter 4 has been used to prepare two conference presentations and one publication, as follows:

- Agil, R.* and Patterson, Z.R., Mackay, H., Abizaid, A., and Hosseinian, F. Triticale bran alkylresorcinols enhanceresistance to oxidative stress in obesity-induced mice. 7th International Conference and Exhibition on Nutraceuticals and Functional Foods in Istanbul, Turkey (October 14-17, 2014). International; Poster Presentation.
- Agil, R.* and Patterson, Z.R., Mackay, H., Abizaid, A., and Hosseinian, F. Triticale bran alkylresorcinols enhanceresistance to oxidative stress in obesity-induced mice. Canadian Oxidative Stress Consortium in Ottawa, ON (June 11-13, 2014). National; Oral and Poster Presentation.

- Agil R, Patterson ZR, Mackay H, Abizaid A, and Hosseinian F, Triticale bran alkylresorcinols enhance resistance to oxidative stress in mice fed a high-fat diet. Foods. 5(1): 5 (2016).

Chapter 4 Triticale bran alkylresorcinols enhance resistance to oxidative stress in obesity-induced mice

4.1 Abstract

Triticale (× *Triticosecale* Whitm.) is a cereal grain with high levels of alkyresorcinols (AR) which are concentrated in the bran. These phenolic lipids have been shown to reduce or inhibit triglyceride accumulation and protect against oxidation, however, their biological effects have yet to be evaluated *in vivo*. The objective of this study was to determine the effects of ARs extracted from triticale bran (TB) added to a high a fat-diet on the development of obesity and oxidative stress. CF-1 mice were fed a standard low fat (LF) diet, 60 % high-fat diet (HF) and HF diets containing either 0.5% AR extract (HF-AR), 10% TB (HF-TB) or 0.5% vitamin E (HF-VE). Energy intake, weight gain, glucose tolerance, fasting blood glucose (FBG) levels and body composition were determined. Oxygen radical absorbance capacity (ORAC), superoxide dismutase (SOD) activity, and glutathione (GSH) assays were performed on mice liver and heart tissues. Liver and heart tissues of HF with AR extract diet groups possessed significantly higher ($P < 0.05$) peroxyl radical scavenging activity (0.53 & 0.54 μM TE/mg of protein), glutathione levels (0.68 & 0.41 μM GSH/mg of protein) and lower oxidized glutathione/reduced glutathione (GSSG/GSH) ratios (0.14 & 0.18) than HF diet groups (0.31 & 0.33 μM TE/mg of protein, 0.47 and 0.20 μM GSH/mg of protein, 0.41 & 0.51 GSSG/GSH ratios). Although other parameters did not show significant differences, mice supplemented ARs did exhibit improved FBG levels, glucose tolerance, as well as increased SOD activity in the liver in comparison to control HF mice. Findings of this study suggest that ARs may serve as a preventative measure against oxidative damage through their application as functional foods and neutraceuticals.

4.2 Introduction

The rising incidence rate of obesity has become a serious public health concern, resulting in a growing consumer demand for a healthy diet by means of natural health promoting products (*252, 253*). Obesity is a disorder associated with an increased risk of numerous diseases including type II diabetes and cardiovascular disease (CVD). Studies implicate the co-morbidities in obesity to be a result of oxidative stress as a chief underlying source (*254*). Oxidative stress is an imbalance in reactive oxygen species (ROS) production that overwhelms the body's antioxidant defense system. This defense system includes both enzymatic and nonenzymatic mechanisms to scavenge and detoxify ROS and reactive intermediates (*255, 256*). Some of the major contributors to this system are superoxide dismutase (SOD), which catalyzes the conversion of highly reactive superoxide (O_2^-) molecules into less reactive product, hydrogen peroxide (H_2O_2). Enzymes, glutathione peroxidase (GPx) and catalase can further reduce H_2O_2 by decomposition into water and oxygen. Antioxidant enzymes rely on endogenous antioxidants such as glutathione (GSH), for hydrogen and electron donations necessary for the reduction and detoxification of ROS (*256*). Fat accumulation correlates with oxidative stress as evidenced by increases in ROS production, ratio of oxidized glutathione to reduced glutathione (GSSG/GSH) and NADPH oxidase expression, as well as a decrease in antioxidant enzyme expression in the adipose tissue of obese mice (*223*).

Whole grain consumption is associated with a variety of health benefits including a reduced risk of obesity and related diseases. Whole grains are rich in nutrients and phytochemicals which have been linked to the improvements seen in the antioxidant protection, weight loss, BMI, and blood-lipid metabolism of subjects fed a diet rich in whole-grains (*68, 257, 258*). The health-related functionality of whole grains is principally due to the presence of

bioactive compounds in the bran. Some of these health benefits may be attributed to alkylresorcinols (ARs), bioactive compounds present almost exclusively in the bran. These phenolic lipids possess a polar aromatic ring and a hydrophobic alkyl chain generally at the C5 position. Hydroxyl groups are attached to the aromatic ring at C1 & C3 and may serve as sources of ARs' radical scavenging activity by hydrogen donation (*62*). Due to similarities in structural properties, ARs are often compared to vitamin E (tocopherols) which is a well-established antioxidant (*108*), and studies have reported similarities in their metabolism after ingestion (*41, 62, 88*).

ARs have also been shown to reduce or inhibit triglyceride accumulation (*259*), in addition to their anti-inflammatory properties (*62*) & antioxidant protection (*109*). In a recent study by Oishi et al., mice fed a 28% fat and 20% sucrose diet showed positive physiological effects when also supplanted with ARs (0.4% wheat bran ARs) in comparison to mice fed the same diet lacking ARs (*260*). Researchers found the incorporation of ARs in the diet suppressed the risks of obesity and glucose intolerance generally associated with a high fat, high sucrose diet by increasing insulin sensitivity and cholesterol excretion. This is one of a very few and limited number of studies that has investigated the physiological effects of ARs. Nonetheless, the antioxidant potential of ARs *in vivo* is a question that remains inconclusive and has yet to be evaluated Thus the objective of this study was to determine the effects of AR extracts from triticale bran (TB) added to a high a fat-diet on the development of obesity and oxidative stress.

4.3 Materials and methods

4.3.1 Materials

Acetone was purchased from Caledon Laboratories Ltd. (Georgetown, ON).

Alkylresorcinol standards were ordered from ReseaChem GmbH (Burgdorf, Switzerland). Potassium phosphate buffer (KPi) was purchased from Mallinckrodt (Paris, Kentucky). Fluorescein, Trolox, 2,2'-azobis(2-methylpropionamidine)dihydrochloride (AAPH), super oxide dismutase, manganese(II) chloride ($MnCl_2$), 5,5`-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione reductase (GR), and 2-vinylpyridine (VP) were purchased from Sigma-Aldrich (Oakville, ON). Ethylenediaminetetraacetic acid (EDTA), sulfosalicylic acid (SA), glutathione (GSH), bovine serum albumin (BSA), nicotinamide adenine dinucleotide (NADH), 2-mercaptoethanol (MeSH), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from BioShop (Burlington, Ontario). Oxidized glutathione (GSSG) was purchased from Santa Cruz Biotechnology (Dallas, Texas).

4.3.2 Sample preparation and analysis

Triticale bran (TB) was provided by Agriculture and Agri-Food Canada (Lethbridge, Alberta). Bran was milled to a 2 mm particle size prior to extraction using a Thomas Wiley Mill (model ED-5, Arthur H. Thomas Co., Philadelphia, PA, USA). Complete methods of extraction, characterization, and quantification of ARs were followed in accordance with our previous studies (*261*). Alkylresorcinols were extracted from TB for 24 hours with acetone, at a 1:40 solid-to-solvent ratio (w/v; g/mL). The extracted solution was removed of all acetone by rotary evaporation using a BüchiRotavapor R-215 (New Castle, Delaware). The dried product was weighed and stored at -20 °C prior to use.

An Alliance HPLC system e2695 Separation Module with a photodiode array detector from Waters (Milford, Massachusetts) equipped with a reverse-phase C18 column (150 x 4.6 mm, 5μm) and Empower 3 software was used for analysis. AR sample extracts and standards

(C15:0, C17:0, C19:0, C21:0, C23:0 and C25:0) were prepared in methanol and concentrations were determined according to the calibration curve (0.125 to 2 mg/mL) of each standard. The method of HPLC analysis was modified from Gunenc et al. (*110*) to separate and quantify total AR content and homolog composition present in our extract. A gradient system using 4% acetic acid in water (Solvent A) and 1% acetic acid in methanol (Solvent B) was applied at a flow rate of 1 mL/min, with a column temperature of 35 ºC and an injection volume of 10 uL. The gradient system used initiated at 90% B for 10 min, followed by 100% B for 30 min, and returned to 90% B for a final 10 min.

4.3.3 Animals and diets

The study protocol was approved by the Local Ethics Committee at Carleton University and all experimental procedures complied with the Canadian Council on Animal Care (CCAC) guidelines. Male CF-1 mice (~6 weeks old; n = 40) were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). CF-1 mice were selected due to their suitability as general multipurpose models that are safe and effective for testing. Throughout the entirety of the study, mice were maintained on a standard 12 hour light dark cycle (lights on at 08:00). Mice were allowed to acclimatize to vivarium conditions for 1 week, during which time they were left undisturbed with ad libitum access to water supply and conventional chow (2014 Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories, Mississauga, Ontario). Following this habituation period, a two week baseline period was carried out in which mice continued to be fed the same conventional chow with their food intake and body weight measures recorded daily. Subsequently, animals were randomly assigned to one of five groups (n = 8 per group), each of which were fed different diets for a period of 10 experimental weeks.

Groups were categorised by diet exposure type as follows: A) low fat conventional chow for maintenance, B) high-fat TD.06414 from Harlan Laboratories, C) high-fat containing 0.5% Vitamin E (5 g/kg feed), D) high-fat containing 0.5% AR extract (5 g/kg feed), and E) high-fat containing 10% TB (100 g/kg feed) with groups designated LF, HF, HF-VE, HF-AR, and HF-TB respectively. The compositions of the control LF and HF diets are shown in Table 4.1.

Body weight and food intake were recorded daily. The total duration of the study was 12 weeks and at the end of this period, mice were fasted for 12 hours prior to sacrifice by decapitation. Blood glucose levels were measured at time of death using a Contour® next EZ Blood Glucose Monitoring System (Bayer Inc., Toronto, Ontario) and organs were stored at -80 °C immediately after being removed.

Table 4.1 - Composition of control diets.

Ingredient	Low-fat diet	High-fat diet
Carbohydrate (%)	48.0	27.3
Protein (%)	14.3	23.5
Fat (%)	4.0	34.3
Saturated (%)	0.7	12.7
Monounsaturated (%)	0.8	16.1
Polyunsaturated (%)	2.5	5.5
Fibre (%)	22.1	6.5
Soluble (%)	4.1	--
Insoluble (%)	18.0	6.5
Energy density (kcal/g)	2.9	5.1
Energy from carbohydrate (%)	67.3	21.3

4.3.1 Glucose tolerance test (GTT)

During the experimental period, a glucose tolerance test was carried for all groups by intraperitoneal (IP) injection of 20% D-glucose solution to overnight- fasted animals (12 hour fast). Blood samples were collected from the tail vein and glucose levels measured by Contour Blood Glucose Monitor at time 0 (prior to injection), 15, 30, 60 and 120 min after injection to determine the rate of reduction of glucose level. The dose of glucose solution administered was 0.5% (0.5 mL/100 g) of the mouse's total body weight.

4.3.2 Carcass analysis

Magnetic resonance imaging (MRI) was carried out courtesy of Health Canada using an EchoMRI4in1TM mouse composition analyzer (EchoMRI, Houston, Texas) to measure the % body fat and % lean fat of the decapitated animal carcasses.

4.3.3 Tissue preparation

Dissected liver and heart tissues were fixed in solution at a ratio of 1:5 (w/v; g/mL), homogenized using a Tissuemiser 130 V 50/60 HZ, 125 W (Fisher Scientific, Ottawa, Ontario), centrifuged at 12 000 g for 15 min at 4 °C, and the supernatant was collected and stored at -80 °C until analysis. Tissue samples used for GSH assays were homogenized in ice cold 5% SA that had been briefly bubbled in nitrogen gas. Samples used for SOD and ORAC assays were homogenized in a solution of 100 mM potassium phosphate buffer solution (pH 7.5) with 1 mM EDTA.

4.3.4 Protein determination

Protein levels were determined based on the method proposed by Bradford M.M. (*262*), using bovine serum albumin as a standard. Absorbance was read at 595 nm using a SpectraMax 340PC384 microplate reader (Molecular Devices, Sunnyvale, California).

4.3.5 Antioxidant activity assays

4.3.5.1 Oxygen radical absorbance capacity (ORAC) assay

ORAC assays were carried out with minor modifications of procedures previously described by us (*261*). All solutions were prepared in 100 mM potassium phosphate buffer (pH 7.5) which was also used as the blank. Varying concentrations of Trolox (a water-soluble vitamin E analog) were used as standard to create a reference range. Fluorescence was measured using an

automated plate reader (FLx800 with Gen5 software, BioTek Instruments, Winooski, VT) for a total runtime of 90 min, at excitation and emission wavelengths of 485 and 525 nm. Final results were calculated as Trolox equivalents per mg of protein.

4.3.5.2 Super oxide dismutase (SOD) activity

The activity of SOD in tissue samples was measured indirectly by monitoring the inhibition of superoxide-induced NADH oxidation, seen as a decrease in absorbance at 340 nm over a period of 10 minutes (*263*). The following solutions (10 µL each) were added sequentially to a 96-well plate: 50 mM EDTA, 25 mM MnCl2 , 2.7 mM NADH, and 50 µL of supernatant (6-8 different concentrations), blank (100 mM KPi), or standard (1 µg/mL SOD). The reaction was initiated by the final addition of 39 mM MeSH. One unit of SOD activity represents the amount of enzyme in the sample that inhibits NADH oxidation reaction by 50%. Results are expressed in U of SOD/mg of protein.

4.3.5.3 Gluatathione (GSH/GSSG) Assay

The assay for glutathione followed procedures similar to those previously described by Griffith O.W. (*264*). It is based on the reaction of GSH with DTNB to produce a yellow coloured end product that absorbs at 412 nm. Thus the concentration of GSH in the sample is dependent on the rate of TNB production. To measure total GSH, the following solutions were sequentially added to a 96-well plate: 0.55 mM NADPH (100 µL), 1.32 mM DTNB (10 µL), sample, standard or blank (10 µL), and 11 U/mL GR (10 µL). Ice cold 5% SA was used as a blank and to prepare GSH standard solutions (1.25, 2.5, 5, 10, and 20 µM). To determine the amount of GSSG within the samples, samples (50 µL) and a stock solution of GSSG standard (50 µL) were

individually treated with 60 μL of a 1:90 solution containing VP and 500 mM KPi (v/v). This process derivatizes GSH in order to quantify GSSG levels exclusive of GSH within the sample. In a separate 96-well plate, pre-treated samples were run against a GSSG standard curve (0.1, 0.2, 0.4, 0.7, 1, 1.5 and 2.0 μM) following the aforementioned procedure for total GSH. In determining total GSH, and GSSG, the actual concentration of GSH can be deduced using the following formula:

Total = GSH + 2GSSG

4.3.6 Statistical analysis

Three of the mice, one from group LF (n = 7) and two from HF (n = 6), died prior to the end of the study period, thus all data obtained from these mice have been excluded from the final results and statistical analyses. All experiments and analyses were performed at least in triplicate. Results are presented as means ± SEM. Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Means were considered significantly different at $P < 0.05$.

4.4 Results and discussion

4.4.1 Characterization of ARs extract from TB

The compositions of total ARs and individual homologues in triticale bran are shown in Table 4.2. The total amount of ARs found in triticale bran was 143.29 mg/100 g of which saturated ARs make up 82.0% and homologue C21:0 was the most prevalent at a concentration of 39.76 mg/100 g. These results are in agreement with literature findings and our previous studies (*37, 59*).

Table 4.2 - Total alkylresorcinol (AR) content and composition of homologues (mg/100 g) in extract from triticale bran (TB) determined by HPLC.

AR Homologue (mg/100 g)	
Saturated	
5-n-heptadecylresorcinol C 15:0	1.06 ± 0.2
5-n-heptadecylresorcinol C 17:0	12.44 ± 0.5
5-n-nonadecanylresorcinol C 19:0	28.08 ± 0.3
5-n-heneicosylresorcinol C 21:0	39.76 ± 0.3
5-n-tricosylresorcinol C 23:0	20.33 ± 0.2
5-n-pentacosylresorcinol C 25:0	15.86 ± 0.1
Unsaturated	12.12 ± 0.2
Unknown	13.61 ± 0.6
Total	143.29 ± 0.3

Analyses were performed in triplicate and results expressed in mean ± SD.

4.4.2 Body composition, weight, and intake parameters

During the 2-week baseline period in which all 5 groups were fed the same chow diet, groups showed similar means ($P > 0.05$) in weight (33.6-34.4 g), daily weight gain (0.15-0.22 g/day) and energy intake (17.3-18.4 Kcal/day). After the 10 week treatment period, animals fed high-fat diets were 21% heavier and exhibited a higher mean of daily weight gain ($P < 0.05$) compared to the standard control group LF. No statistical differences were found in cumulative

weight gain or body weight amongst HF treatment groups (HF-AR, HF-TB, HF-VE and HF) as seen in Figure 4.1A and 4.1B. Comparable results were found for the difference in mean energy intake ($P < 0.05$) between control groups HF and LF. On the other hand, the mean energy intake of groups, HF and HF-TB, were found to be significantly higher than that of HF-AR and HF-VE groups (Figure 4.2A). To estimate differences in the ability of ingested energy to be metabolized (*265*), metabolic efficiency (ME) across the 10-week treatment period was calculated as follows:

$$\text{ME} = \text{energy intake/body weight gain}$$

While a statistically significant difference cannot be seen, the HF-TB group exhibits a greater mean metabolic efficiency of 99 Kcal/g of body weight 227 when compared to the 83 Kcal/g for control HF animals (Figure 4.2B). Overall, the standard control group LF demonstrated the lowest mean values in weight (44.3 g), daily weight gain (0.17 g), energy intake (16.8 Kcal), and greatest metabolic efficiency (120.4 Kcal/g).

The values obtained for carcass analysis at the end of the experimental period are summarized in Table 4.3. No statistical difference was found in % fat amongst the HF groups irrespective of treatment received. However, HF-TB had the least % fat (24.1%) and statistically highest % lean muscle (72.9%) when compared with the remaining HF groups. These results depict a similar trend to that of metabolic efficiency findings. These findings suggest that incorporation of 0.5% AR extract or 0.5% vitamin E in a 60% high-fat diet does not prevent fat accumulation; however, incorporation of 10% TB may improve metabolic efficiency and promote an increase in %lean muscle.

An increasing number of studies have reported the beneficial effects of phenolic

compounds and bioactives in cereal grains on obesity. Phenolics such as anthocyanins, rutin, gallic acid, ferulic acid, genestein, and tea catechins can induce apoptosis of adipocytes, inhibit trigycleride accumulation, or regulate plasma lipids and cholesterol levels; these are some of the mechanisms that have been identified in which phenolics can act against the prevalent disease (*266, 267*). In this study, neither AR nor VE supplementation resulted in significant changes in body weight, composition or metabolic efficiency. Ross et al. (*98*) found coinciding results in which rats fed a basal diet supplemented with 4 g AR/kg feed for 4 weeks did not affect final body or organ weights. Nonetheless, the higher energy intake yet greater metabolic efficiency found for animals fed HF-TB compared to HF alone indicates that consumption of TB did attenuate weight gain and enhance metabolism in some respect, which is in agreement with existing literature (*268, 269*).

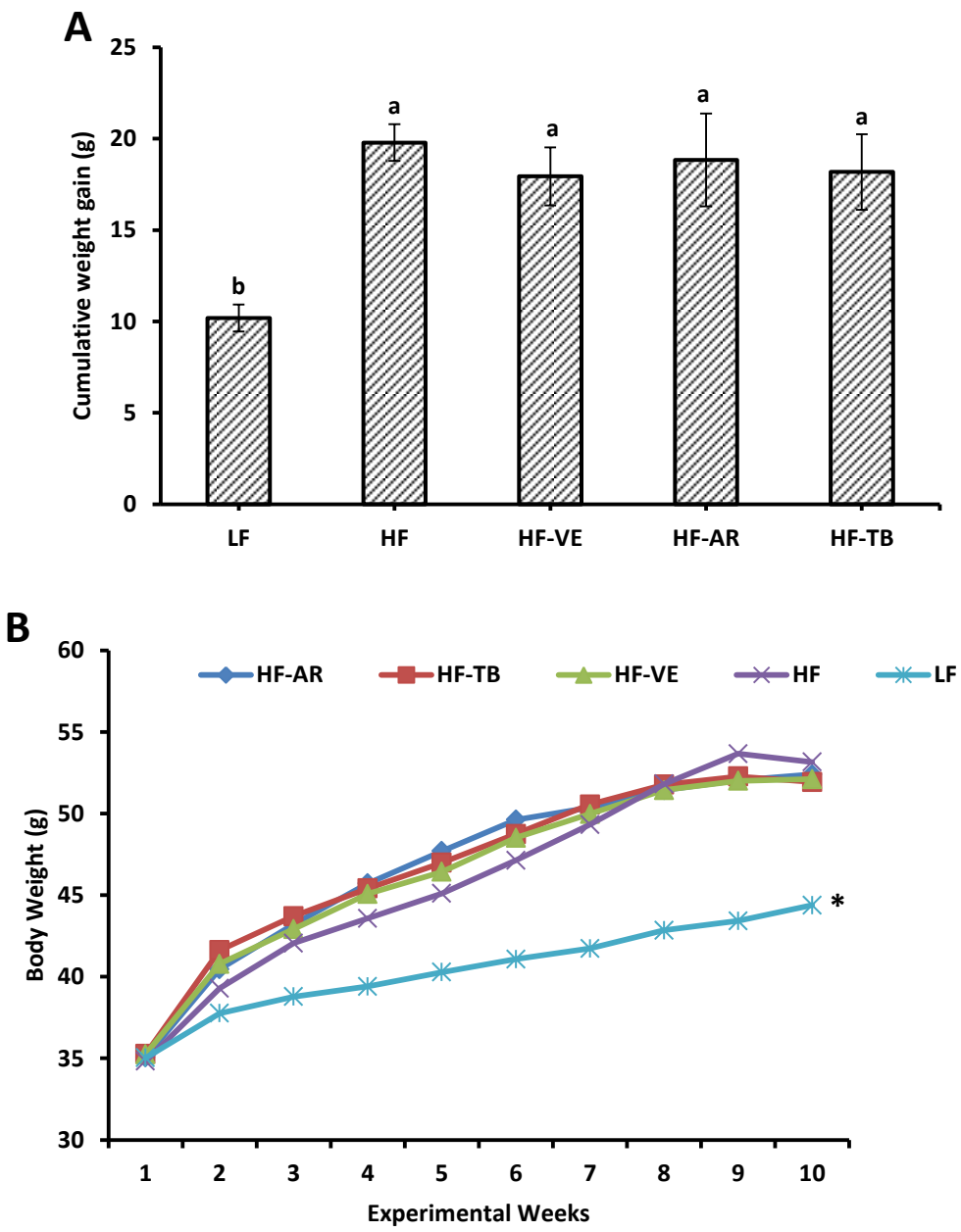


Figure 4.1 - Cumulative weight gain (A) and body weight after 10 experimental weeks (B). LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 0.5% VE added; HF-AR: group fed HF diet with 0.5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences ($P < 0.05$) are represented by different letters and * indicates statistical difference from HF group.

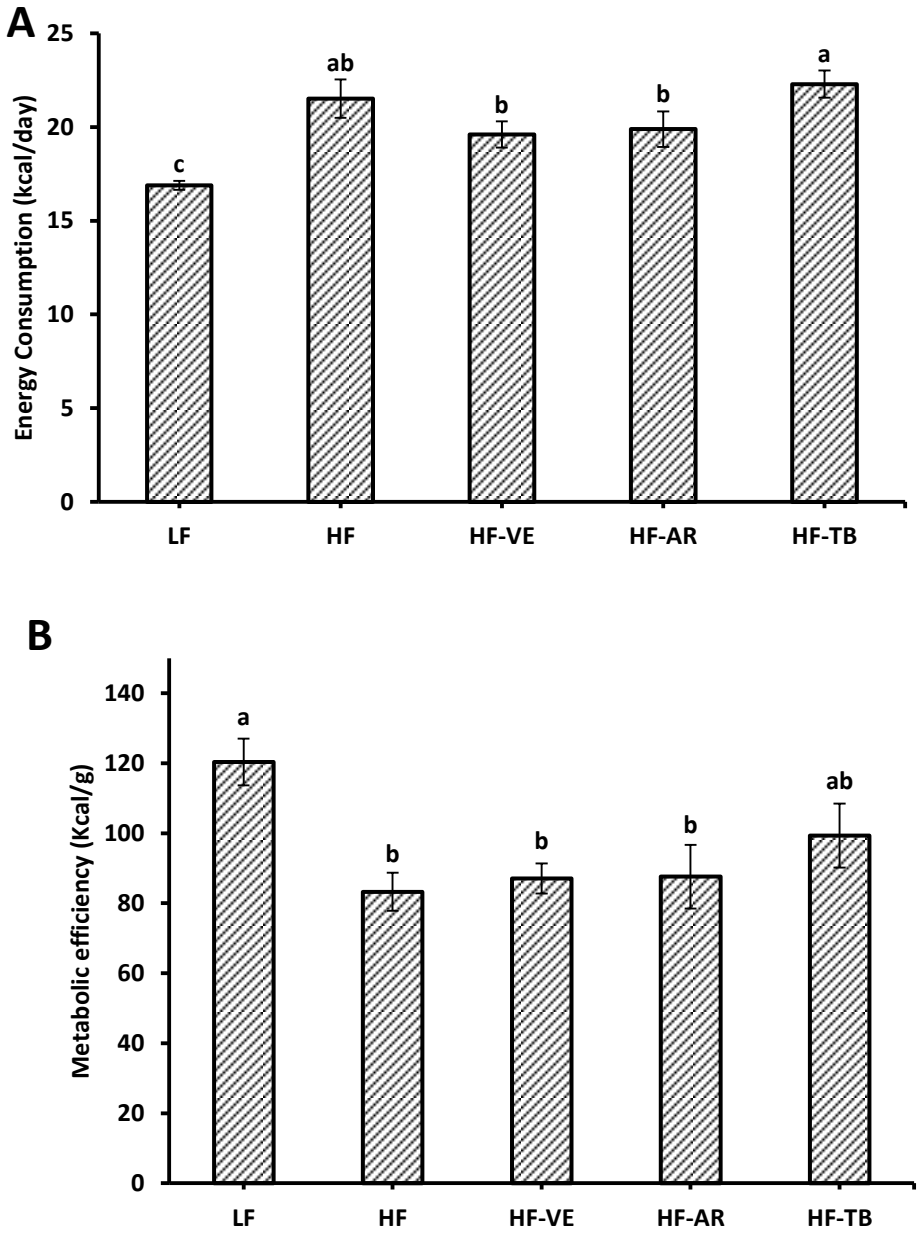


Figure 4.2 - Energy consumption (A) and metabolic efficiency after 10 experimental weeks (B). LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 0.5% VE added; HF-AR: group fed HF diet with 0.5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences ($P < 0.05$) are represented by different letters.

Table 4.3 - Body composition of mice carcasses.

Treatment[1]	Weight (g)	Fat (%)	Lean muscle (%)
LF	36.60 ± 1.35^{b}	18.02 ± 1.00^{b}	75.90 ± 0.89^{a}
HF	46.66 ± 1.06^{a}	27.00 ± 1.03^{a}	69.89 ± 1.35^{c}
HF-VE	45.17 ± 2.18^{a}	26.09 ± 0.63^{a}	70.87 ± 0.62^{bc}
HF-AR	45.83 ± 3.15^{a}	28.10 ± 1.92^{a}	67.79 ± 1.89^{c}
HF-TB	45.06 ± 2.32^{a}	24.14 ± 1.35^{a}	72.87 ± 1.43^{ab}

Body composition data obtained by EchoMRI4in1TM and expressed as % of decapitated mouse carcass body weight. [1] LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 0.5% VE added; HF-AR: group fed HF diet with 0.5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Data are presented as mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences among groups ($P < 0.05$) are represented by different letters.

4.4.3 Glucose Tolerance and Fasting Blood Glucose

Although differencs were not statistically significant, lower fasting blood glucose levels of animals on the HF-AR diet (6.1 mM) than the remaining HF treatment groups (7.2-8.0 mM) and control LF group (7.1 mM) are apparent in Figure 4.3. The results of the GTT seen in Figure 4.4A indicated no significant difference among all 5 groups at time 15, 30, and 120 min. At time 0 min blood glucose levels were significantly higher in the HF group with no marked differences found between the remaining groups. After 60 min, control LF mice exhibited a significantly lower drop in blood glucose than HF control and treatment groups with the exception of HF-AR. At each of the time points throughout the GTT, HF-AR mice had the lowest blood glucose levels amongst HF groups. Results of the GTT at different time points were used to calculate the area under the curve (AUC) in the glucose versus time graph. Based on results of AUC (Figure 4.4B), mice fed HF alone had a significantly higher mean AUC (2146.5) than that of mice fed the standard LF (1585.1). Among the HF groups, HF-AR had the lowest AUC (1810.8) followed by HF-TB (1941.5), HF-VE (1976.0) and HF.

An impaired glucose tolerance test and persistently high FBG levels are indicators of insulin resistance and hyperglycemia respectively; these conditions are often linked to obesity and diabetes. Insulin resistance is a failure of cells to respond to insulin hormone signalling, leading to reduced glucose uptake and hyperglycemia (*265, 270*). Although not statistically significant (P = 0.06), the addition of ARs did improve glucose tolerance as evidenced by a lower mean of area under the curve. Additionally, fasting blood glucose levels were significantly lower in mice fed HF-AR rather than HF alone. A recent study by Magnusdottir et al. (*71*) discovered an association between the plasma C17:0/C21:0 AR homolog ratio and increased insulin sensitivity in subjects with metabolic syndrome fed a diet rich in whole grain rye. In

examining the intestinal loops of anesthetized dogs administered a solution of alkyresorcinol and insulin, Sealock et al. (*143*) found alkylresorcinols and related compounds promoted the absorption of insulin from the gastrointestinal tract and into the circulating bloodstream, thereby significantly dropping blood glucose levels. Results of these studies suggest that ARs perhaps interact with insulin in a synergistic manner, potentially enhancing the functionality of insulin and its effectiveness in blood glucose clearance. Although further investigation is necessary to confirm this prospective and understand their mechanism of action, ARs show promising results in their potential to ameliorate glucose intolerance and insulin sensitivity commonly associated with obesity and diabetes (*260*).

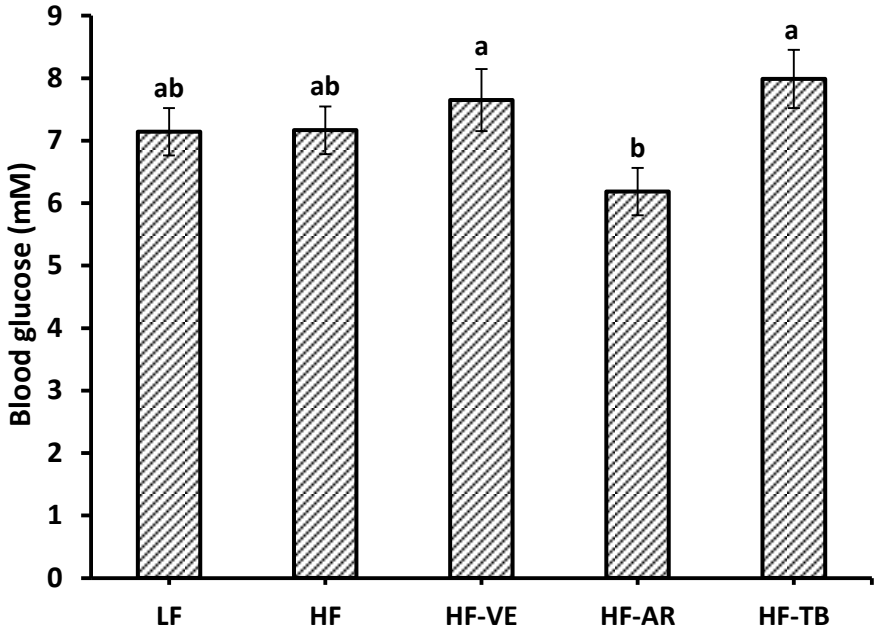


Figure 4.3 - Fasting blood glucose levels after 10 experimental weeks. LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 0.5% VE added; HF-AR: group fed HF diet with 0.5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences (P <0.05) are represented by different letters.

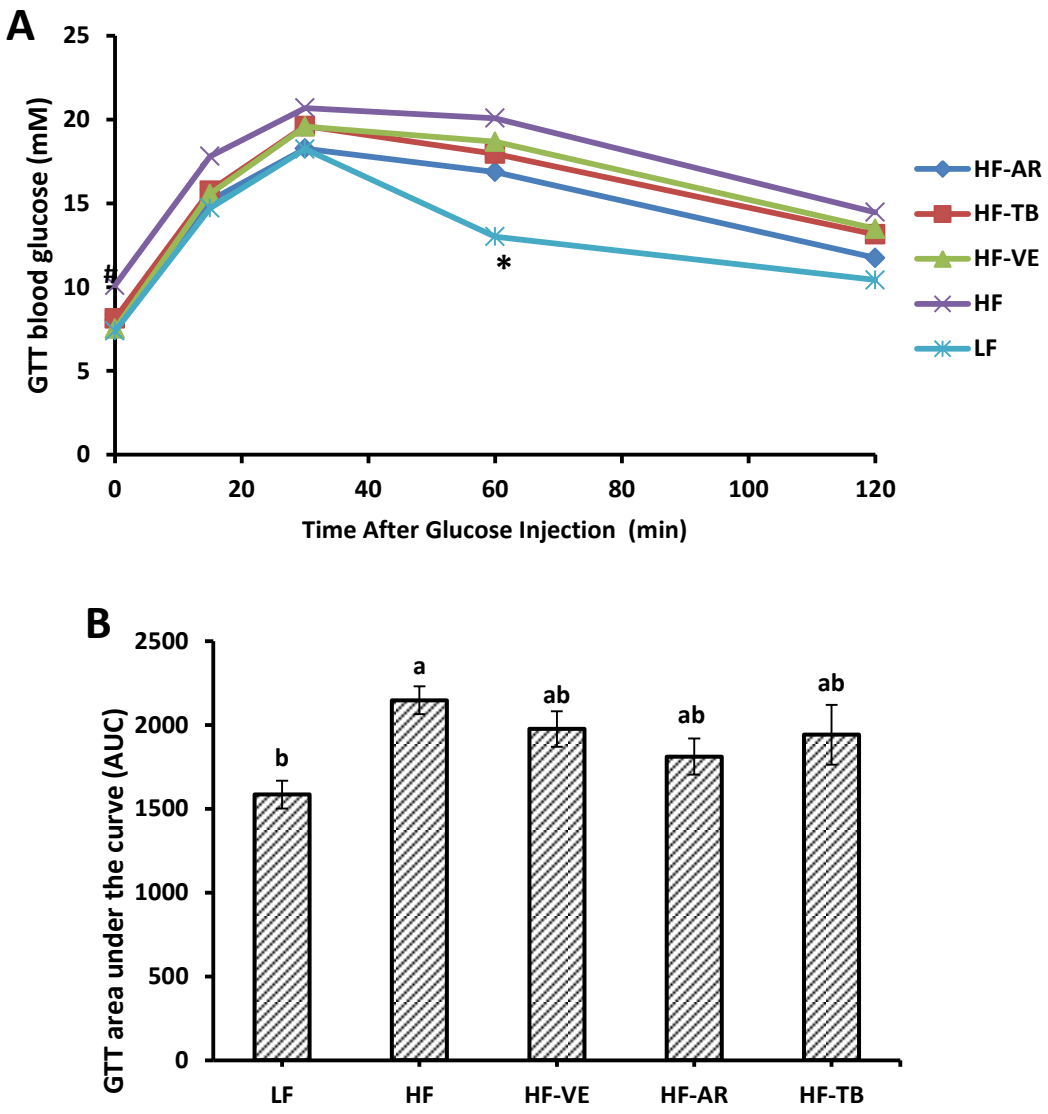


Figure 4.4 - Results of glucose tolerance test (GTT) mean blood glucose levels at 0, 30, 60, 90 and 120 min after intraperitoneal injection of glucose solution (A), and mean area under the curve of GTT graph. LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 0.5% VE added; HF-AR: group fed HF diet with 0.5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences ($P < 0.05$) are represented by different letters. # indicates statistical difference from the control group and * indicates statistical difference from HF group.

4.4.4 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay showed that mice fed a high-fat diet demonstrated poor antioxidant protection of the fluorescent probe from peroxyl radicals generated by APPH. Liver and heart tissues of HF control mice were found to have the lowest ORAC values of 0.31 and 0.33 μmol TE/g of protein respectively amongst all the groups (Figure 4.5). Supplementation of the high fat diet with 0.5% AR extract significantly improved antioxidant status ($P < 0.05$) as evidenced by much higher mean ORAC values of 0.53 and 0.54 μmol TE/g of protein respectively. Addition of 5% VE and 10% TB also showed marked improvements of 25-35% in the liver and 55-65% greater in the heart compared to the tissues of non-supplemented HF mice.

The ORAC assay best represents antioxidant reactions within biological systems by providing a controllable source of peroxyl radicals that react with a florescent probe, resulting in a non-fluorescent product; antioxidant capacity is quantified by comparison of net AUC (relative fluorescence units vs. time) to that of known antioxidant, trolox (*244*). The significantly higher ORAC levels in liver and heart tissues of mice a fed high fat diet with 5% ARs confirms literature results reporting the *in vitro* antioxidant potential of these dynamic phenolic lipids (*103, 108, 109, 111, 112, 117*). It is speculated that the amphiphillic nature of ARs carry out their antioxidant actions in a similar manner to that of tocopherols by easily incorporating in cell lipid bilayers and providing protection against lipid peroxidation by hydrogen atom transfer from hydroxyl groups of the phenol ring to peroxyl radicals thereby forming stable products (*62*).

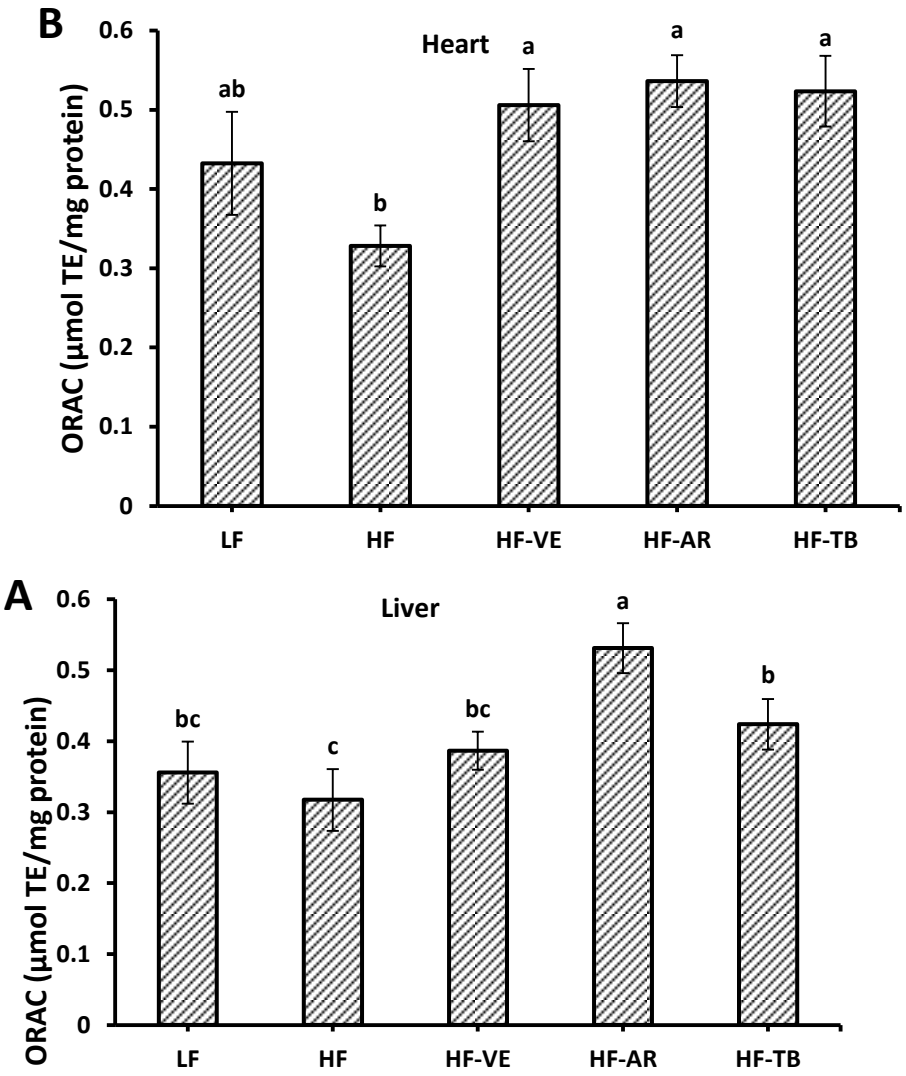


Figure 4.5 - Total antioxidant activity in liver (A) and heart tissue of mice as evaluated by the ORAC method (B). LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 0.5% VE added; HF-AR: group fed HF diet with 0.5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences among groups ($P < 0.05$) are represented by different letters.

4.4.5 Superoxide dismutase (SOD) Activity

Although ARs seemed to improve SOD activity levels by 30% when compared to control HF liver tissues, this difference was not statistically significant ($P > 0.05$). The same was true for the remainder of the groups such that the activity of the SOD enzyme increased by approximately 20% and 5% for VE and TB supplemented mice, respectively (Figure 4.6). In order to quantify units of SOD per mg of protein, differences in %inhibition of NADH oxidation must be observed at varying concentrations of tissue assayed. For this reason, no quantifiable differences were found for the SOD activity in mice heart tissues.

Superoxide dismutase is an enzyme that catalyzes the dismutation of superoxide ($O_2^{\cdot -}$) radicals into less reactive forms, hydrogen peroxide and oxygen. It is proposed that the superoxide anion can initiate and terminate lipid peroxidation, thus SOD concentrations in mammalian cells are not easily susceptible to change as enough SOD are expressed to predominantly suppress ROS imbalances linked to superoxide (*271*). Also, it is known that SOD is a strong antioxidant that out-competes the reactions of $O_2^{\cdot -}$ to protect the cell from its toxicity (*272*). The supplementation of 0.5% antioxidants (ARs or VE) with a high-fat diet did not significantly enhance SOD activity. However, the expected negative impact on SOD activity associated with increased levels of metabolic stressors such as ROS due to a high fat diet (*254*) were also not statistically pronounced. The lack of pronounced effects on SOD activity, whether they are negative or positive, is potentially due to the strength and self-sufficient nature of the SOD enzyme in successfully suppressing superoxide linked chain reactions. Thus, is it theorized that for significant changes in SOD activity to be apparent, the initiating source of oxidative stress must be substantially stronger and present for a more prolonged period of time than that experienced by the high-fat fed mice in this study.

In the future, other antioxidant enzymes such as glutathione peroxidase and catalase may be a more reliable measure of antioxidant effects in less extreme conditions of oxidative stress.

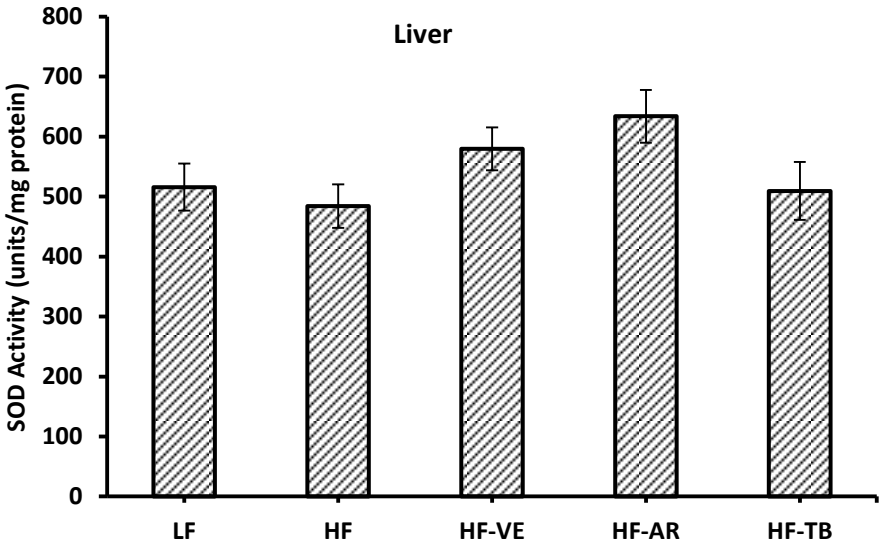


Figure 4.6 - Superoxide dismutase activity in liver tissue of mice. LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 5% VE added; HF-AR: group fed HF diet with 5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where no statistical differences among groups were found ($P > 0.05$).

4.4.6 Reduced glutathione (GSH) contents

Liver and heart tissues of the control HF group exhibited decreased levels of reduced GSH (0.47 μM and 0.20 GSH/mg of protein) and increased ratios of GSSG/GSH (0.41 and 0.51) respectively, in comparison to control LF mice (0.69 and 0.32 μM GSH/mg of protein; 0.27 and 0.32 GSSG/GSH). These results were expected since a high-fat diet is often correlated with increasing levels of ROS leading to oxidative stress (*273*). Overall, liver and heart tissues from

HF-TB and HF-AR mice had significantly higher GSH levels and significantly lower ratios of GSSG/GSH ($P > 0.05$) in comparison to mice fed high fat alone (Figure 4.7).

A chief contributor to the biological defense system against oxidative damage is the antioxidant molecule, glutathione, which is naturally synthesized in the body. In its reduced form, it directly interacts with reactive oxygen and nitrogen species or acts as a cofactor for enzymes. In healthy cells, glutathione is mainly in the reduced form and a rising ratio of oxidized to reduced glutathione (GSSG/GSH) is a significant marker of oxidative stress (*274*). The signify cant increase in GSH levels and decrease in the ratio of GSSG/GSH in HF-AR liver and heart tissues suggest that a diet supplemented with ARs may play an impacting role in the recycling of GSSG back to its reduced form as GSH. Although the mechanism of action requires further investigation, it would be presumed that ARs help increase GSH levels by direct reduction of GSSG through hydrogen atom transfer/donation or by free radical quenching that indirectly helps maintain higher GSH levels. Since GSH has multiple roles in the endogenous antioxidant defense system, it is likely that GSH cooperates with SOD in the removal of free radicals (*272*). This may explain the significant effects demonstrated by ARs on GSH, yet less pronounced or undetectable effects on SOD activity in the liver and heart respectively, as ARs may only impact SOD activity indirectly through GSH.

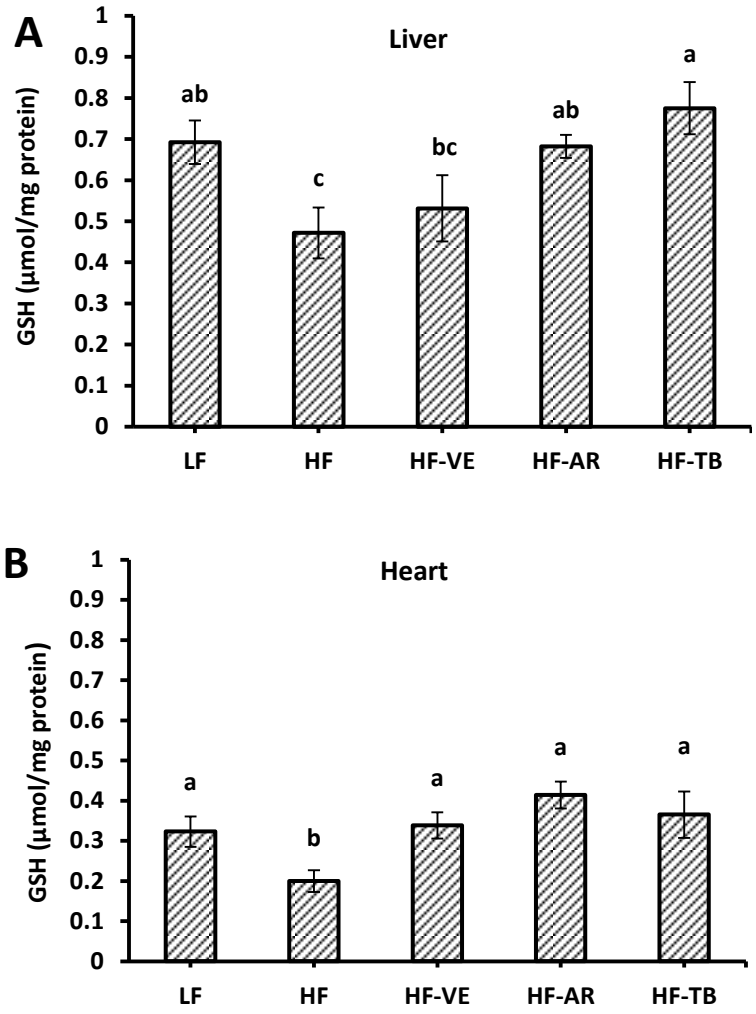


Figure 4.7 - Reduced glutathione levels (A & C) and ratio of oxidized glutathione to reduced glutathione (GSSG/GSH) in mice liver and heart tissues (B & D). LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 5% VE added; HF-AR: group fed HF diet with 5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences among groups ($P < 0.05$) are represented by different letters.

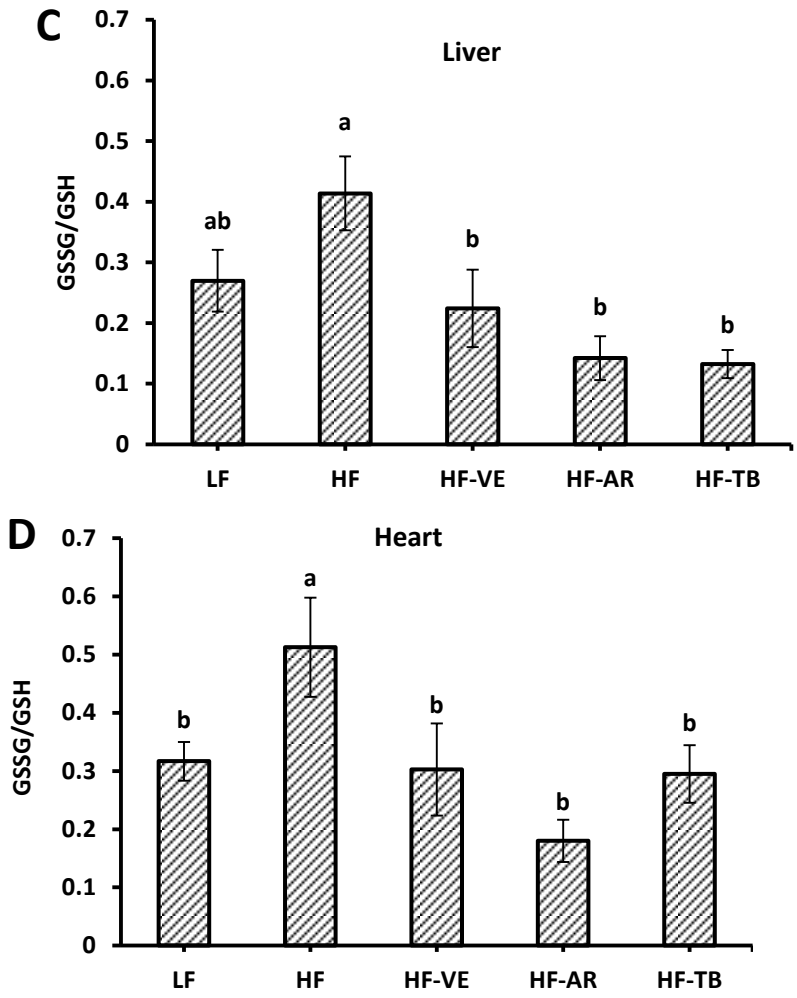


Figure 4.7 - Reduced glutathione levels (A & C) and ratio of oxidized glutathione to reduced glutathione (GSSG/GSH) in mice liver and heart tissues (B & D). LF: group fed standard low fat diet; HF: group fed high-fat diet; HF-VE: group fed HF diet with 5% VE added; HF-AR: group fed HF diet with 5% ARs extract added; HF-TB: group fed HF diet with 10% TB. Bars represent mean ± SE. Data were statistically analyzed using one-way ANOVA and DMRT where statistical differences among groups ($P < 0.05$) are represented by different letters.

4.5 Conclusion

Cereal grains are rich in phenolics, predominantly in the bran fraction, which also contains an abundance of other bioactive components that have been shown to protect against chronic diseases including obesity and diabetes (*9, 275*). Although ARs did not impact weight gain and body composition, results were promising in their ability to improve glucose tolerance and fasting blood glucose levels. Additionally, ARs proved to have antioxidant potential *in vivo* through their enhanced effects on oxidative stress markers, results which confirmed literature findings for the antioxidant capacity of ARs demonstrated *in vitro*. Alkylresorcinols are known to be weaker antioxidants than that of tocopherols based on *in vitro* studies (*108*); however results of this study demonstrate that several other physiological factors are involved *in vivo* as ARs had an overall greater effect than VE in improving oxidative stress as well as glucose tolerance. It has also been shown that AR chain length has an effect on their *in vivo* elimination kinetics such that the half-life of ARs in rats positively correlated with alkyl chain length (*276*). However, Korycinska et al. (*108*) found that chain length did not significantly impact their antioxidant status. Thus, future studies aim to measure and compare the effects of individual AR homologs *in vivo*. Also, the complex nature of the mechanisms involved in the body's antioxidant system requires further exploration to better understand the effects of antioxidant therapy and apply these findings to preventative measures against oxidative stress and chronic diseases.

4.6 Connecting Statement to Chapter 5

In Chapter 4, the impact of AR incorporation in a high-fat diet on food intake, body composition and weight, as well as glucose tolerance and obesity development in mice was elucidated. Additionally, this chapter provided insight on the antioxidant potential of ARs *in vivo* through *ex vivo* analysis comparing levels of oxidative stress markers such as GSH and SOD in the liver and heart tissues of mice fed AR incorporated high fat diets versus control diets lacking ARs.

In Chapter 5, the content and composition of water extractable polysaccharides in TB was determined in addition to investigating the effects of variations in extraction parameters on WEP yield and composition.

The work presented in Chapter 5 has been used to prepare one publication as follows:

- Agil, R. and Hosseinian, F., 2014. Determination of water-extractable polysaccharides in triticale bran. *Journal of Food Composition and Analysis*, *34*(1), pp.12-17.

Chapter 5 Determination of water-extractable polysaccharides in triticale bran

5.1 Abstract

The content, composition, and molecular weight distribution (MWD) profile of water-extractable polysaccharides (WEP) in triticale (*Triticosecale*) bran were determined. Results were compared against wheat (*Triticum* sp.) and rye (*Secale* sp.) bran, as well as triticale straw and flakes. The effects of the following conditions on the extractability of sugars were compared and respective fractions obtained: 1) boiling water extraction, WEP-I; 2) successive enzyme treatment and dialysis, WEP-II; 3) successive ethanol fractionation, WEP-III. Although the highest yield of WEP was generally found in fraction II, fraction III had the least level of simple sugars as unwanted co-extractives. The extractability of xylose (Xyl) and arabinose (Ara) in all bran samples significantly increased ($P < 0.05$) by up to 23.3% and 3.2% respectively, after precipitation with 80% ethanol. Amongst the cereal brans, wheat bran possessed a significantly higher ($P < 0.05$) molar % of Ara (19.5%) and Xyl (29.6%), followed by triticale bran (14.5% Ara, 17.2% Xyl), and lowest in rye bran (7.2% Ara, 13.4% Xyl). Triticale bran (TB) had higher molecular weight (MW) polysaccharides and the widest MW distribution range (4400-401000 Da) of WEP, suggesting greater bioactive potential and high grain hardiness; prospects to be further investigated in future studies.

5.2 Introduction

Triticale (*Triticosecale*) is a hybrid of wheat (*Triticum sp.*) & rye (*Secale sp.*), containing high levels of nutritionally beneficial compounds (*30*). Modern triticales (*Triticosecale AC Ultima*) are mostly hexaploid which originated from Triticum turgidum subsp. durum X rye (Secale cereale) cross. Triticale is rich in phenolics and dietary fibres consisting of both soluble and insoluble fibres (*28, 277*). Triticale possesses a combined advantage of the high yield potential of wheat, and the disease and environmental tolerance of rye. Animal studies have shown the digestibility of triticale to be generally higher than barley and relatively equal to rye (*22, 32*). The poor bread making quality of rye and the digestibility problems of rye in cattle increase the need for exploring other avenues of grain sources such as triticale .Although triticale is an excellent candidate for animal feed due to high protein, amino acid, polysaccharide and B vitamin content, it has yet to be well recognized for human food applications (*26*). The insufficient demand for its significant utilization in food products can be attributed to the limited research data available on the characterization of triticale composites, particularly in the bran.

Consumption of whole grains has been associated with a variety of health benefits such as a reduced risk of obesity, type II diabetes, cardiovascular disease and cancer. Although the exact mechanisms have yet to be discovered, it is understood that the physiological functions of their protective bioactive compounds reside in the bran fraction of cereal grains (*278*). Cereal bran such as wheat bran is a by-product of milling and usually accounts for 14–19% of the grains' weight (*279*). On average, WEP such as pentosans/arabinoxylans are part of soluble dietary fibre, making up to 30% of the total sugar content of cereal bran (*280*). Whole wheat and rye grain are common ingredients of the human diet, serving as essential sources of dietary fiber. The dietary fibre content in triticale (13-16%) is less than that of rye (15-21%) but generally

higher than wheat (11-14%) (*30, 280*). This can be attributed to the higher amount of cell wall polysaccharides being an essential part of the dietary fibre composite.

Recent studies have shown that water soluble pentosans (e.g. arabinoxylans), the major cell wall polysaccharides, may have potential health benefits, thus acting as quality markers of cereal grains (*261, 281*). The objectives of this study were to: i) characterize and quantify the WEP composition in triticale bran, ii) measure the effect of ethanol fractionation on the yield potential of WEP, and iii) measure the molecular weight distribution of these WEP components in triticale bran and all results were compared with that of triticale straw and flakes, as well as wheat and rye bran. In this study, the term water-extractable polysaccharide mainly refers to that of pentosans or arabinoxylans.

5.3 Materials and method

5.3.1 Sample preparation

Triticale bran, straw, and flakes (AC Ultima, spring triticale) were kindly provided by Agriculture and Agri-Food Canada (Lethbridge, AB). The bran is the hard outer layer of the cereal grain, while flakes are rolled and pressed wholegrain and straw are the dry stalks of the cereal plant that remain after the grain and chaff have been removed. Wheat and rye bran samples were purchased from a commercial source (Bulk Barn) in Kelowna, BC, Canada. All samples were ground to approximately 1.5 mm particle size using a Thomas Wiley Mill (Philadelphia, PA, USA); this was within the recommend range of a 1.0-2.0 mm particle size (*59*). The samples were dried in an oven at 105 ºC overnight, cooled in desiccators, and weighed. All results are reported relative to the oven-dried weight (ODW) of the sample and calculated according to the following equations (*282*). Samples were stored in sealed plastic bags and kept

in a freezer (-20 °C) prior to further analyses.

Calculations:

% Moisture (w/w) = 100-[(weight $_{dry\ sample\ plus\ dry\ pan}$ – weight $_{dry\ pan}$ / weight $_{sample\ as\ received}$) × 100]

% Total solids (w/w) = weight $_{dry\ sample\ plus\ dry\ pan}$ – weight $_{dry\ pan}$ / weight $_{sample\ as\ received}$ × 100

ODW=weight $_{air\ dry\ sample}$ × % Total solids/100

5.3.2 Scanning Electron Microscopy

Scanning electron microscopy (SEM) was employed to confirm the particle size of bran after grinding. Triticale bran was used as the representative sample whereby the sample was mounted on aluminum stubs, coated with a layer of gold (30 nm), and examined with a scanning electron microscope (SEM) (JSM-840A; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV (*283*).

5.3.3 Isolation of WEP

The WEP were sequentially extracted in triplicate from each grain sample, according to the procedures of (*261, 284*)). To remove fats, the fine powder (100 g), was extracted twice using hexane, at a 5:1 (v/w) solvent/solid ratio, for 1 h at room temperature (*285*). The mixtures were filtered through a Whatman No. 4 (15 cm) filter paper, and the defatted residue was dried and kept at -20 ºC until further analyses.

Defatted ground sample (10 g) was extracted with distilled water (1:100 w/v), stirred for 4 h at 65 °C, cooled, and centrifuged at 4000 rpm for 20 min using a Sorvall RC 5B Plus centrifuge (Mandel Scientific Company, Guelph, ON, Canada). The supernatant was kept, the extraction repeated, and supernatants from the first and second extraction combined. This extract

portion was designated WEP-I, representing the primary fraction of extract that has not been enzyme or ethanol treated.

To eliminate starch molecules and proteins from the sample, 1 mL of α-amylase solution (pH 6.9, 0.5-1.0 units/g) and 1 mL protease (pH 7.5, 2.4 units/g) from heat resistant *Bacillus licheniformis* as well as 1mL of amyloglucosidase (pH 4.5, 300 units/ml) from *Aspergillus niger* (Sigma-Aldrich Canada Ltd., Oakville, ON) were added to the supernatant solution and stirred overnight at 40 °C. To inactivate the enzymes, the solution was heated at 95 ºC for 5 min, then cooled and centrifuged at 4000 rpm for 20 min at room temperature. The supernatant solution was dialysed against deionized water (4 ºC for 72 h) using membrane tubing (3500 molecular weight cut off, Spectrum Laboratories, INC., CA, USA). This extract portion was designated WEP-II, representing enzyme treated extract.

This latter fraction of extract, WEP-II, was further fractionated by incremental increases of ethanol concentration including 20, 40, 60 and 80% (*279*). The mixture was stored at 4 °C overnight (16 h), centrifuged at 10,000 rpm for 30 min and the precipitate was collected. This resulted in four fractions WE-20, WE-40, WE-60 and WE-80, respectively. The last two digits refer to the ethanol concentration at which the precipitate was collected. Since the highest proportions of water-extractable polysaccharides were obtained by precipitation with 80% ethanol, this fraction was designated WEP-III representing enzyme and ethanol treated extract. All extract solutions (WEP-I,-II, and -III) were freeze-dried and stored in a -20 °C freezer until further analysis.

5.3.4 Molecular Weight Distribution

5.3.4.1 High Performance Liquid Chromatography (HPLC)

The molecular weights of polymeric carbohydrates was estimated using high performance size-exclusion chromatography on an Agilent 1100 HPLC system with an RI detector, autosampler, and Agilent ChemStation Plus software (Agilent Technologies Inc., Palo Alto, CA). The column comprised of triple Ultrahydrogel (2000, 250, and 120, 7.8 X 300 mm, Waters, Milford, MA, USA) equipped with a guard column (TSK PWH, TosoHaas GmbH, Stuttgart, Germany). Samples of WEP-III fractions, were dissolved in water, filtered through a 0.2 μm PVDF filter (BD, Franklin Lakes, NJ, USA) and injected (20 μL). Separation ensued for 90 min at 0.4 mL/min, with an isocratic condition using 0.1 M $NaNO_3$, as a mobile phase at 25 ºC. Dextran standards (1 mg/mL) of varying molecular weights (4,400, 9,900, 21,400, 43,500, 124,000, 196,000, 277,000 and 401,000 Da) were used as molecular weight markers. Processing and analysis of molecular weight distribution data were performed in triplicate using GPC software (Agilent Technologies Inc., Palo Alto, CA, USA).

5.3.4.2 Fourier transform infrared (FTIR) Spectroscopy

To confirm and compare results obtained by HPLC for molecular weight determination, FTIR was also employed in accordance with the method of Hamed and Allam (2006), using a Nicolet™ 380 FTIR (Thermo Electron Corp. Madison, Wisconsin, USA) equipped with OMNIC Spectra™ software. Samples in dried form (1 mg) were placed on a Diamond and ZnSe crystal plate as recommended by Thermo Electron Corp. Spectra were recorded in duplicates between 400 and 4000 cm-1 at 4 cm-1 resolution, applying 32 scans. Background spectra were taken in the empty chamber. Compounds were identified by comparing their spectral patterns with that of dextran standards of different molecular weights. All analyses were carried out in triplicate.

5.3.5 Simple sugar analysis

High performance gel chromatography was used for analysis of simple sugars using a Biorad Aminex HPX-87H column and the refractometer index (RI) detector. Prior to analysis, samples (WEP-I, -II, & -III) were converted from polymeric carbohydrates into the monomeric forms by hydrolysing in 72% H_2SO_4 for 1 h at 30 °C, followed by 4% H_2SO_4 (by diluting with water) for 1 h at 121 °C. Once the hydrolyzates cooled to room temperature, the pH was neutralized with calcium carbonate, and the solution was filtered through a 0.2 μm PVDF filter (BD, Franklin Lakes, NJ, USA). HPLC conditions were as follows: isocratic with 0.1 M $NaNO_3$ as a mobile phase, 75 ºC column temperature, 5 μL injection volume, and a 0.5 mL/minute flow rate a run time of 60 minutes. Sugar standards (0.1-1.0 mg/mL) purchased from Sigma, St. Louis, MO were used to determine the sugar composition of each sample and include D-(+)glucose, D-(+)xylose, D-(+)galactose, -L(+)arabinose, and D-(+)mannose. All analyses were carried out in triplicate.

5.3.6 Proximate Analysis

Moisture and total solids in the sample (10 g), were determined according to Laboratory Analytical Procedures (LAP) (*282*) measured as a mass difference after dehydration in a 105 ± 3 ºC conventional drying oven for 24 h and calculated using the following equations. Crude fat and crude protein were measured according to AOAC official methods (*286, 287*) by the automated LECO FP-528 machine (LECO Corp., Michigan, USA).

Klason lignin (lignin, cutin, silica) was obtained by mixing the residue after protein analysis with 72% H_2SO_4 for 4 h at room temperature. Acid was then removed by washing samples twice with 100 mL distilled water and samples dried in a 105 °C oven. Klason lignin

was measured as the difference in weight between oven dried samples before and after acid hydrolysis.

The crucibles containing residues from Klason lignin analysis were placed in the muffle furnace at 575 ± 25 °C for 8 h for ashing, transferred into a desiccator and cooled for 1 h. The ash content was measured by the difference between the weight of crucible before and after ashing. The cellulose content was calculated from the difference between ash and lignin.

Mineral analysis was performed using an inductively coupled plasma mass spectrometer (ICP-MS) method by a ThermoElemental X-7, ICP-MS unit coupled with a CETAC ASX 500 autosampler. ICP-MS calibration used 3 separate multi-element solutions (Inorganic Ventures Ltd., Christiansburg, VA, USA). Dilution blanks were measured from the internal standard spiked with 2% HNO3 (*288*). All analyses were carried out in triplicate.

5.3.7 Statistical analysis

Analysis of variance (ANOVA) was determined using JMP 5.1 software by Statistical Analysis System SAS (SAS Institute Inc., Cary, NC, USA) and when significant ($P < 0.05$) mean comparison was performed using Duncan's multiple range test. All analyses were carried out in replicates of 3 using a randomized design.

5.4 Results and discussion

5.4.1 Chemical composition

The chemical compositions (g/100g) of triticale bran, straw and flakes along with wheat and rye bran are shown in Table 5.1. Total sugars ranged from 22.2 g/100g in triticale straw to 43.0 g/100g in rye bran. This is in agreement with results reported in literature indicating that rye

has much higher total sugar content than wheat and triticale (*30, 289*). It was noteworthy that triticale straw contained a much higher content of lignin (18.4 g/100g) and cellulose (38.1 g/100g) (Table 5.1) compared to all remaining samples which were much closer in range (7.0 to 11.9 g/100g lignin and 18.9 to 24.1 g/100g cellulose). We were interested to look at triticale straw as a comparative for the purposes of broadening the knowledge base for the chemical composition and WEP content of different parts of the triticale plant.

Triticale straw was particularly of interest due to its recognized potential as a source of biomass for renewable energy such as biofuel; it is an environmentally friendly and cost-effective alternative. The mineral compositions (%) of triticale bran, straw and flakes along with wheat and rye bran are shown in Table 5.2. Twenty-six of the naturally occurring elements are known to be essential for life (*290*). A wide range of these nutritionally valuable elements can be obtained through the consumption of cereal grains/bran possessing more than 1/3rd of these essential minerals. Wheat bran (4.26%) contained the highest total mineral content, followed by triticale bran (3.61%), straw (1.8%), rye bran (1.71%) and triticale flakes (1.21%).

Table 5.1 - Chemical composition (g/100g) of grain samples.

	TB [a]	RB	WB	TF	TS
Sugars (non-cellulosic)[b]	31.2 ± 0.7	43.0 ± 1.4	33.6 ± 1.1	40.0 ± 2.1	22.2 ± 0.7
Klason lignin	10.0 ± 0.2	9.4 ± 0.1	11.9 ± 0.2	7.0 ± 0.2	18.4 ± 0.5
Cellulose	24.1 ± 1.0	18.9 ± 0.9	21.7 ± 1.1	19.0 ± 0.3	38.1 ± 0.8
Ash	6.2 ± 0.3	7.2 ± 0.1	6.5 ± 0.1	8.3 ± 0.2	8.1 ± 0.5
Fat	6.4 ± 0.3	6.6 ± 0.2	7.8 ± 0.6	7.2 ± 0.4	0.0 ± 0.0
Protein	6.3 ± 0.1	5.7 ± 0.2	5.3 ± 0.2	4.6 ± 0.3	8.8 ± 0.4
Moisture	8.6 ± 0.7	6.9 ± 0.3	8.5 ± 0.5	7.8 ± 0.1	7.2 ± 0.3

[a]TB: triticale bran; RB: rye bran; WB: wheat bran; TF: triticale flakes; TS: triticale straw.

[b]Total non-cellulosic sugars: 0.88X(%Ara + %Xyl) + 0.99 X (%Man + %Gal + %Glu) (*279*). Values are means (n = 3).

Table 5.2 - Mineral composition (%) of grain samples.

Sample	Ca	Mg	K	P	B	Zn	Fe	Mn	Cu	Total
Triticale Bran	0.10	0.47	1.51	1.27	0.03	0.06	0.07	0.09	0.01	3.61
Rye Bran	0.05	0.21	0.75	0.56	0.03	0.04	0.03	0.03	0.01	1.71
Wheat Bran	0.10	0.70	1.56	1.52	0.03	0.09	0.11	0.14	0.01	4.26
Triticale Flakes	0.05	0.14	0.52	0.36	0.03	0.03	0.03	0.04	0.01	1.21
Triticale Straw	0.25	0.11	1.07	0.16	0.03	0.02	0.17	0.04	0.01	1.86

[a]Ca: calcium; Mg: magnesium; K: potassium; P: phosphorous; B: boron; Zn: zinc; Fe: iron; Mn: manganese; Cu: copper. Values are means (n = 3) with standard error (SE) below 5%.

5.4.2 Scanning electron microscopy

Sample particle size has been shown to effect the composition and yield potential of extractives. Particle size may create inconsistencies in the analytical and chemical properties of the sample in a non-uniform manner such that an increased particle size can reduce levels of certain composites while increasing others (*291, 292*). Thus particle size is essential for optimal yield conditions and was verified by SEM prior to extraction and analyses using TB as the representative sample. As seen in Figure 5.1, a particle size of 1.5 mm, which is within the optimal range of 1.0-2.0 mm, was successfully achieved.

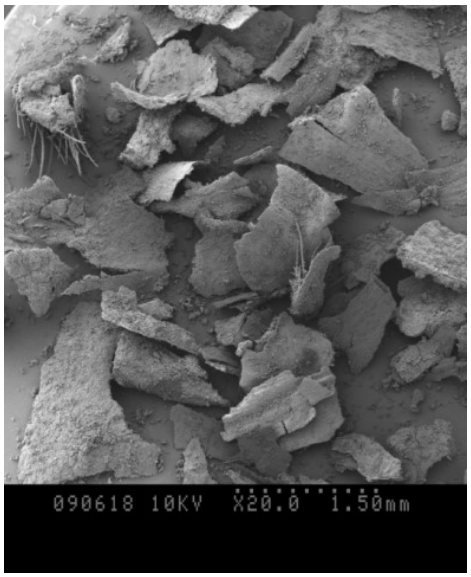


Figure 5.1 - Scanning electron micrograph of the intact cell wall/bran of triticale.

5.4.3 Fractionation of WEP

Ethanol fractionation showed that the distribution of the extractable polysaccharides ranged from 14.8 to 27.7 g/100g of extract fraction, WEP-II (Table 5.3). Studies in literature have reported comparable findings of WEP levels in cereal bran (12.7 to 22.1 g/100g) and flour

(1.35 to 2.75 g/100g) (*279, 293, 294*). Treating samples with 80% ethanol resulted in isolating WEP without the presence of simple sugars as undesirable co-extractives. Additionally, 80% ethanol can aid in the extraction of water-soluble pentosans with only ≤2% protein as an impurity (*294*). It is evident that the extractability of WEP is dependent on the concentration of ethanol used during fractionation. In this respect, yields of WEP increased with increasing ethanol concentrations that were above 20%; Yields almost tripled in fraction 80 (10.6 g/100 g) compared to that of fraction 20 (3.6 g/100 g) of rye bran. . Thus, the maximal level of WEP can be obtained at a concentration of 80% ethanol, a finding that is also in agreement with literature (*279*).

Table 5.3 - Yields of water extractable (WE) pentosans (g/100 g) derived by graded ethanol precipitation of extract fraction, WEP-II.

Sample[a]	Frac 20	Frac 40	Frac 60	Frac 80	Total WE pentosans
TB	3.2 ± 0.1	5.8 ± 0.1	5.6 ± 0.2	5.2 ± 0.1	19.8
RB	3.6 ± 0.0	8.2 ± 0.5	4.4 ± 0.2	10.6 ± 0.5	26.8
WB	6.4 ± 0.2	3.1 ± 0.1	9.1 ± 0.4	9.1 ± 0.3	27.7
TF	4.0 ± 0.2	4.6 ± 0.1	4.8 ± 0.1	4.6 ± 0.0	18.0
TS	3.4 ± 0.2	2.4 ± 0.1	2.4 ± 0.0	6.6 ± 0.3	14.8

[a]TB: triticale bran; RB: rye bran; WB: wheat bran; TF: triticale flakes; TS: triticale straw. Values are means (n = 3).

5.4.4 Molecular weight determination of WEP

The molecular weight values of water soluble pentosans were classified as follows using extract fraction, WEP-III: very low (≤4400 Da), low (4400-9900 Da), medium (21400-43500 Da), high (124000-401000 Da), and very high (≥401000 Da). Triticale bran exhibited the widest distribution of polysaccharides ranging from very low to very high (Table 5.4). Wheat and rye shared similar molecular weight distribution profiles, whereby medium to high molecular weight polymers ranging from 43500-196000 Da were lacking. High molecular weight polymers have been associated with grain hardiness due to their ability to form strong gel networks thereby adding to the rigidity of endosperm cell walls (*284, 295*). Triticale combines the high quality parameters of wheat with the high yield and tolerance to biotic and abiotic stresses of the rye parent. Additionally, it has been speculated that higher MW polysaccharides, which were most pronounced in TB, may possess greater antioxidant potential (*296*).

All dextran standards (4400-297000 Da) had the same FTIR profile as the spectra of samples, thereby confirming the presence of these polysaccharides in WEP-III fractions (Figure 5.2). The FTIR spectra displayed major absorption at 3400 cm^{-1} corresponding to the hydroxyl functional groups, and around 3010 cm^{-1} from C=O stretching. Absorption at around 830 cm^{-1} was caused by the C-H stretching from CH_2 groups.

Table 5.4 - Molecular weight distributions (MWD) screening of grain samples.

MWD of Standards (Da)	TB[a]	RB	WB	TF	TS
401000	(+)	(+)	(+)	(+)	(+)
277000	(-)	(+)	(+)	(+)	(+)
196000	(+)	(-)	(-)	(-)	(+)
124000	(+)	(-)	(-)	(-)	(+)
43500	(+)	(-)	(-)	(+)	(-)
21400	(+)	(+)	(+)	(+)	(-)
9900	(+)	(+)	(+)	(+)	(-)
4400	(+)	(+)	(+)	(+)	(-)

[a]TB: triticale bran; RB: rye bran; WB: wheat bran; TF: triticale flakes; TS: triticale straw.

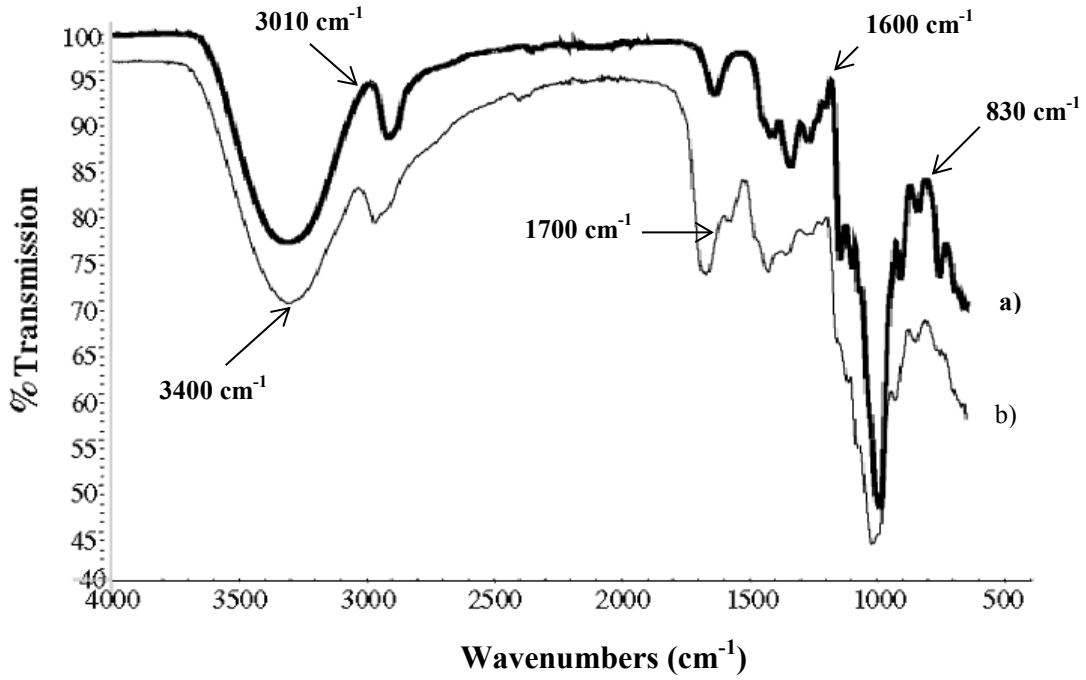


Figure 5.2 - The FTIR spectra of a) molecular weight standard dextran (196000 Da), and b) WEP-III fraction extracted from triticale bran.

5.4.5 Characterization of simple sugars

In this study, hot water (65 ºC, 4 h) was used to extract water-extractable materials. This temperature was also useful for the inactivation of endogenous enzymes and protein denaturing (*281*). The results of this study indicated that the majority of water soluble pentosans in the cell wall could not be extracted with water alone, thus employment of enzymes and 80% ethanol aided in the complete extraction of WEP. The WEP in cereal cell wall tissues can be separated from the insoluble residues by means of aqueous extraction. Recovery % ranged from 86.5 to 93.9, thus a small quantity of materials was lost during extraction or fractionation.

The sugar composition (g/100g) of WEP before and after enzyme treatments and after

precipitation with 80% ethanol in triticale bran, straw and flakes, as well as wheat and rye bran are shown in Table 5.5. Typical amylolytic enzymes including α-amylase and amyloglucosidase were used to hydrolyse α-1–4 and α-1–6 branch linkages in the starch polymer. Protease was used to remove any protein residue. In all samples, extractability of sugars differed significantly ($P < 0.05$) after treatment with enzymes, and after precipitation with 80% ethanol. In the case of xylose and arabinose a significant increase ($P < 0.05$) was generally found after each additional treatment, however, the same could not be said for glucose (non-cellulosic) and galactose. Differences in the extractability of individual sugars such as arabinose and xylose may be attributed to variances in their degree of binding or strength as polymers within the matrix of the cell wall (*297, 298*).

Overall, wheat bran possessed a significantly higher ($P < 0.05$) molar % of Ara (19.5%) and Xyl (29.6%) in comparison to triticale (14.5% Ara and 17.2% Xyl) and rye bran (7.2% Ara and 13.4% Xyl) as seen in Figure 5.3. All samples contained a higher proportion of xylose than arabinose, which is in parallel with literature findings (*30, 297, 299*). It was notable that the ratio of arabinose/xylose (calculated from the molar % values of Figure 5.3) was unique for each sample and were determined to be as follows: RB=0.25, TS=0.30, TF=0.47, WB=0.57 and TB=0.62. This suggests that the ratio of arabinose/xylose can be used as a distinguishing feature amongst samples.

Table 5.5 - Sugar composition (g/100g) of water-extractable pentosans in triticale bran, straw and flakes along with wheat and rye bran.

Sample[a]	Glc[b]	Xyl	Gal	Ara	Man	Total-experimental[f]	Total-calculated[g]
TB: WEP-I[c]	61.6 a ± 1.7	16.4 c ± 0.8	2.6 a ± 0.1	13.8 c ± 1.0	0.7 a ± 0.2	95.1 b	90.8 b
TB: WEP-II[d]	60.1 b ± 0.1	19.2 b ± 0.9	2.5 a ± 0.0	15.9 b ± 0.3	0.6 a ± 0.1	98.4 a	93.5 a
TB: WEP-III[e]	43.0 c ± 0.6	27.0 a ± 1.2	2.6 a ± 0.1	16.7 a ± 0.3	0.6 a ± 0.1	90.0 c	84.3 c
RB: WEP-I	69.4 b ± 1.5	12.2 b ± 0.2	1.7 a ± 0.1	6.5 b ± 0.1	1.0 a ± 0.0	90.8 c	87.8 b
RB: WEP-II	76.3 a ± 0.4	12.1 ± 0.1 b	1.7 a ± 0.0	6.6 b ± 0.1	0.9 a ± 0.0	97.5 a	94.5 a
RB: WEP-III	44.8 c ± 1.1	35.4 a ± 1.3	1.7 a ± 0.0	8.9 a ± 0.1	1.0 a ± 0.0	91.7 b	85.9 c
WB: WEP-I	43.8 a ± 0.6	27.5 b ± 1.1	2.8 a ± 0.9	18.1 b ± 1.3	0.7 ab ± 0.1	92.9 b	86.9 b
WB: WEP-II	43.8 a ± 0.7	27.7 b ± 0.1	2.9 a ± 0.0	18.2 b ± 0.1	1.0 a ± 0.0	93.5 a	87.5 a
WB: WEP-III	29.2 b ± 0.4	37.5 a 0.8	2.8 a 0.1	21.4 a ± 0.7	0.0 c ± 0.0	93.5 a	83.5 c
TF: WEP-I	72.6 a ± 0.4	9.6 c ± 0.5	1.7 a ± 0.1	4.1 c ± 0.2	0.9 ab ± 0.0	89.0 a	86.6 a
TF: WEP-II	68.3 b ± 0.8	11.1 b ± 0.3	1.8 a ± 0.0	5.8 b ± 0.0	1.1 a ± 0.0	88.0 b	85.3 b
TF: WEP-III	58.6 c ± 0.8	15.9 a ± 0.7	1.7 a ± 0.1	7.5 a ± 0.5	1.2 a ± 0.1	85.1 c	81.6 c
TS: WEP-I	5.8 a ± 0.3	25.2 c ± 1.6	1.2 a ± 0.3	4.1 c ± 0.9	ND	36.2 a	52.4 a
TS: WEP-II	5.7 b ± 0.3	29.8 b ± 1.1	1.2 a ± 0.0	8.0 ab ± 0.6	ND	44.7 b	50.0 b
TS: WEP-III	4.5 c ± 0.2	30.6 a ± 1.1	1.0 a ± 0.0	8.6 a ± 0.7	ND	44.6 b	49.7 c

[a]TB: triticale bran; RB: rye bran; WB: wheat bran; TF: triticale flakes; TS: triticale straw.

[b] Glc: glucose; Xyl: xylose; Gal: galactose; Ara: arabinose; Man: mannose. Different letters indicate differences in total lignan contents in samples at $P < 0.05$.

[c]WEP-I: After water extraction and prior to enzyme treatment.

[d]WEP-II: After α-amylase and protease treatment.

[e]WEP-III: After α-amylase and protease treatment and precipitation with 80% ethanol

[f] Total non-cellulosic sugars by HPLC.

[g]Total non-cellulosic sugars, calculated: 0.88 x (%Ara + %Xyl) + 0.99 x (%Man + %Gal + %Glc) (*279*)

Values are means (n = 3); Values followed by different letters are significantly different for the same grain sample under the different extraction conditions ($P < 0.05$).

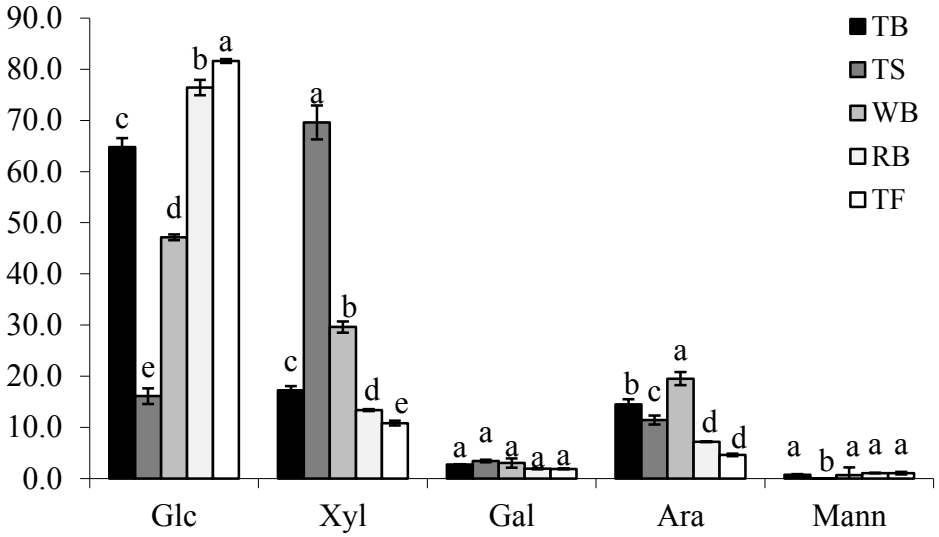


Figure 5.3 Molar % of sugars in triticale bran (TB), straw (TS) and flakes (TF) along with wheat (WB) and rye bran (RB). Values are means (n = 3); Values with different superscript letters for the same sugar are significantly different *($P < 0.05$).*

5.5 Conclusion

The optimal extraction conditions for water soluble polysaccharides/pentosans from cereal brans rendering the least amount of impurities is that of boiling water extraction, followed by enzyme treatment, dialysis and 80% ethanol treatment; Findings which are in agreement with comparable studies by Maes and Delcour (2002). Overall molar % of sugars indicates that triticale is a rich source of arabinose and xylose, sugars that are well recognized for their bioactive potential. Future studies need to investigate the relationship between the chemical structure and bioactivity of polysaccharides in TB.

5.6 Connecting Statement to Chapter 6

In Chapter 5, the optimal extraction conditions for WEP extraction from cereals and the content, composition and MWD profile of WEPs in triticale bran were determined.

In Chapter 6, the prebiotic potential of TB was evaluated using yogurt a fermentation model system for the detection of microbial viability by TTA and pH measurements. Additionally, the antioxidant properties of WEP extract from TB was determined by ORAC assay.

The work presented in Chapter 6 has been used to prepare one conference presentation and one publication:

- Agil, R.* and Hosseinian, F.S. Triticale bran: A novel dietary source of prebiotics and antioxidants in fermented dairy products. American Association of Cereal Chemists (AACC) International Annual Meeting. Palm Springs, California, Oct. 16-19, 2011. International; Oral Presentation.
- Agil, R. and Hosseinian, F., Dual functionality of triticale as a novel dietary source of prebiotics with antioxidant activity in fermented dairy products. Plant Foods for Human Nutrition. 67: 88-93 (2012).

Chapter 6 Dual functionality of triticale as a novel dietary source of prebiotics with antioxidant activity in fermented dairy products

6.1 Abstract

The objectives of this study were to: i) define the optimum concentration of triticale bran (TB) that can be incorporated in yogurt; ii) evaluate the prebiotic effects of TB on microbial viability, pH and total titratable acidity (TTA) in yogurt across 28 days of cold storage; and iii) measure the Oxygen Radical Absorbance Capacity (ORAC) of water-extractable polysaccharides (WEP) in TB. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* were used as starter cultures. *Lactobacillus acidophilus* and *Bifidobacterium lactis* were used as probiotics. A concentration of 4 % TB in yogurt was determined to be the maximum amount that could be added without causing synersis. By day 7, the number of bacteria greatly increased in yogurt samples containing TB and maintained higher viable bacteria counts at the end of the cold storage period, in comparison to controls ($P \leq 0.05$). Confirming this data was the lower pH levels and higher TTA values of TB yogurt samples exhibited throughout 28 days ($P \leq 0.05$). Polysaccharide extracts of TB exhibited strong antioxidant activity with an ORAC value of 33.86 ± 2.30 μmol trolox equivalents (TE)/g of bran. Results of this study suggest that TB may serve as a new prebiotic and antioxidant source for functional foods and nutraceutical applications.

6.2 Introduction

Studies have shown that consumption of whole grains have beneficial health effects including a reduced risk of cardiovascular disease (CVD), obesity, cancers, and type II diabetes in addition to a variety of other health benefits including improved gut health (*300*). Triticale (Triticosecale) is a hybrid of wheat (Triticum sp.) and rye (Secale sp.), containing high levels of nutritionally beneficial compounds. Triticale bran (TB) is rich in phenolics and dietary fibres consisting of both soluble and insoluble fibres (*277, 289*).

Cereal grain oligosaccharides function as prebiotics by increasing levels of beneficial bacteria in the large bowel thereby improving gut health (*301*). Prebiotics are typically non digestible food ingredients (i.e. soluble dietary fibres) that are resistant to human digestive enzymes but serve as food for probiotics to promote their growth and activity. Current sources of prebiotics include cereals such as wheat and barley, soybeans, chicory and some fruits and vegetables (*302*). In addition to the prebiotic potential of cereal oligosaccharides, they have also been shown to possess antioxidant activity owing to their bound phenolic acids (*303*).

Currently, there is considerable research in promising areas for the discovery of new high-value bioproducts and their use as functional foods and nutraceuticals. However, few are engaged in exploiting the value of triticale bran. Although studies have been conducted on the prebiotic effects of whole grain and wheat bran cereal on gut microbiota (*206*), as well as the antioxidant activity of oligosaccharides such as arabinoxylans isolated from wheat bran (*303*) such effects have yet to be explored in TB. The objectives of this study were: i) to determine the optimum concentration of TB that can be incorporated in yogurt, ii) to evaluate the prebiotic effects of TB on microbial viability, pH and titratable acidity in yogurt, iii) to measure the antioxidant capacity of WEP from TB, using an Oxygen Radical Absorbance Capacity (ORAC)

method.

6.3 Materials and method

6.3.1 Sample Preparation

Triticale bran, cultivar Ultima, was provided by Agriculture and Agri-Food Canada (Lethbridge, AB). Samples were ground to a 2.0 mm particle size, freeze dried, stored in sealed plastic bags, and kept in the freezer (-20°C) prior to any analyses.

6.3.2 Prebiotic and Probiotic Activity

Prebiotic and probiotic activity were measured according to a method by Espirito Santo et al. (*304*) and Behrad et al. (*305*) and explained as follows.

6.3.2.1 Selecting Prebiotic Concentration

To determine the ideal amount of bran that could be added without causing synersis, concentrations of 0.1, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.0, and 3.0 g of bran were added to 50 mL pasteurized milk and incubated at 42 ºC until completion of fermentation (pH 4.5).

6.3.2.2 Milk and TB Yogurt Preparation

Pasteurized, 3.25 % homogenized milk (commercial source in Ottawa, ON) was stirred at 85 °C for 15 min., portioned into sterile test-tubes (50 ml), and cooled to 42 °C (*305*). The starter cultures consisted of *Lactobacillus delbrueckii* subsp. *bulgaricus* (B-548; USDA) and *Streptococcus salivarius subsp.thermophilus* (14485; ATCC) were employed for the sole purpose of fermenting milk and are not considered probiotics. Meanwhile, *Lactobacillus acidophilus* (B-4495; USDA) and *Bifidobacterium lactis* (41405; USDA) were used as probiotic 1 and probiotic 2, respectively. Microbial cultures grown in liquid Man Rogosa Sharpe (MRS)

broth were rinsed with sterile water and diluted to a concentration of ~6.5 log colony forming units (CFU) per ml with sterilized milk. All yogurt samples were treated with the same conditions wherein equal volumes of each bacteria culture with equal concentrations (6.5 log CFU/ml) were added such that the total volume of bacteria present constituted 2 ml of the 50 ml final sample volume. The probiotics were added to the yogurt samples as seen in Table 6.1 and the initial pH recorded. Four treatments contained TB and the remaining four served as controls lacking TB. Thus, all samples consisted of equal levels of substrate. Samples where extra substrate was added were compared against samples lacking extra substrate thereby serving as controls. Tubes were incubated at 42 °C to allow for fermentation and the pH was measured after 4h and every 1h thereafter. Once a ~pH of 4.5 was reached, samples were stored at 4 °C.

Table 6.1 - Experimental design employed for the evaluation of TB prebiotic activity.

Microorganisms	Triticale bran	Sample coding
Y1 : *Streptococcus thermophilus* + *Lactobacillus bulgaricus*	-	1
Y1 + *Lactobacillus acidophilus* (Pro1)	-	2
Y1 + *Bifidobacterium lactis* (Pro2)	-	3
Y1 + Pro1&2	-	4
Y1	+	1S
Y1 + *Lactobacillus acidophilus* (Pro1)	+	2S
Y1 + *Bifidobacterium lactis* (Pro2)	+	3S
Y1 + Pro1&2	+	4S

Abbreviations: without TB (-); with TB (+); standard yogurt containing starter cultures *S. thermophilus* + *L. bulgaricus* (Y); probiotic *Lactobacillus acidophilus* (Pro1); probiotic *Bifidobacterium lactis* (Pro2).

6.3.2.3 pH and Total Titratable Acidity (TTA)

Using Denver Instrument UB-5 pH meter, the pH of yogurt samples was measured post-fermentation on Day 1, 7, 14, 21 and 28. To determine TTA, a mixture of yogurt and sterile water (1:9 v/v) was titrated with 0.1N NaOH using 0.1% phenylphthalein indicator.

6.3.2.4 Microbiological Analyses

Cell counts were taken at days 1, 7, 14, 21 and 28 in triplicate for each batch at different dilutions (four serial dilutions of 1/10). From each dilution, a 5 µl portion was plated on MRS agar dishes in triplicate using the spread plate method (*304*).

6.3.3 Extraction of Prebiotics (Water Extractable Polysaccharides/WEP)

Unless otherwise indicated, all chemicals for WEP extraction and antioxidant activity analyses were purchased from Sigma-Aldrich Canada Ltd., Oakville, Ontario. Triticale bran WEP were extracted as follows and carried out in duplicate (*297*). Ground TB was extracted with distilled water (1:100 w/v), stirred for 4h at 70 °C, cooled, centrifuged at 4000 rpm for 20 min and the supernatant kept. To eliminate starch molecules and proteins from the sample, 20 µl of α-amylase solution and 20 µl proteases from *Bacillus lichenformisis* (Type XII-A) was added, stirred overnight at 37 °C, cooled, centrifuged at 4000 rpm for 20 min at room temperature and the supernatant kept. The supernatant solution was placed in a dialysis tube (Spectra/Por® Dialysis Membrane wet in 0.1% Sodium azide, Molecular Weight Cut-Off: 3,500) against deionized water for 48 hours. Extract solutions were stored in a -20 °C freezer until further analysis.

6.3.4 Chemical Analyses

Moisture, ash, total sugars (non-cellulosic) and total cellulose in the sample were determined according to Laboratory Analytical Procedures (LAP) (*306*). Total nitrogen was measured according to AOAC official method (*287*) by the automated LECO FP-528 machine (LECO Corp., Michigan, USA). The crude fat content was extracted by Soxhlet extraction using hexane (*306*).

6.3.5 ORAC (Oxygen radical absorbance capacity) Assay

The ORAC assay using fluorescein as fluorescent probe, was carried out on a FLx800^{TM} Multi-Detection Microplate Reader with Gen5^{TM} software (BioTek Instruments, Ottawa, Ontario, Canada) as described by Georgiev et al. (*307*). The assays were prepared with 20 µl of either a trolox standard (100, 50, 25, 12.5, and 6.25 µM), rutin control (10 and 20 µM) or sample extract per well, in combination with 120 µl of fluorescein solution. After 20 min of incubation at 37ºC, 60 µl of 153 mM AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) was added for a total well volume of 200 µl. All ORAC analyses were performed at 37 ºC with a runtime of 60 min, an excitation wavelength of 485 nm and emission wavelength of 525 nm. Sample extract was analysed in triplicate and final ORAC values expressed as µmol Trolox equivalent per g of dried bran (µmol TE/g).

6.3.6 Statistical analysis

Analysis of variance (ANOVA) was determined using JMP 5.1 software by Statistical Analysis System SAS (version 9, SAS Institute Inc, Cary, NC). Values with statistically significant differences ($p \leq 0.05$) between the eight varying treatments were compared using Duncan's Multiple Range test and designated by different letters.

6.4 Results and discussion

6.4.1 Optimal bran concentration

The optimal concentration of TB employed was identified as the highest amount of bran that could be added to milk and maintain a stable yogurt product with a texture that would be acceptable to consumers as palatable yogurt. Therefore, 4% TB in yogurt; 2.0 g/50 ml of milk

was determined to be the ideal concentration to be employed for succeeding yogurt sample preparations as the concentration above that (6% TB) resulted in a yogurt product of uneven texture resembling that of spoiled yogurt (Figure 6.1).

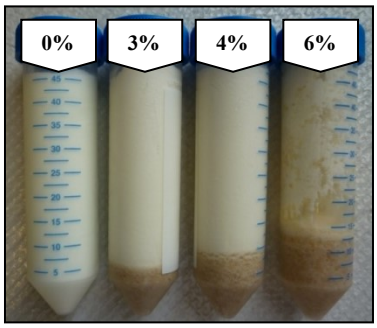


Figure 6.1 - Resultant yogurt products with varying concentrations of TB.

6.4.2 Microbial Viability

Microbial viability in all yogurt samples increased from an initial bacteria count of ~6.5 log CFU/ml at day 0, to a range of 8.2-8.5 log CFU/ml in control samples (1-4) and 8.7-8.9 log CFU/ml in yogurts containing TB (Figure 6.2). By day 7, the number of bacteria in TB yogurt samples remained steady in comparison to blank counterparts (1-4) which decreased to a range of 7.7-7.9 log CFU/ml ($P \leq 0.05$). The greater CFU counts in samples 1S-4S, in comparison to control samples, may be explained by the high levels of carbohydrates and micronutrients present in TB that are likely responsible for the increased probiotic viability.

Fermentation of carbohydrates by gut microflora is a selective process, as is the case with oligosaccharides which are fermented only by particular strains of bifidobacteria and lactobacilli. The typical endproducts of carbohydrate metabolism are short-chain fatty acids which confer a positive effect on bacteria by serving as an energy source (*308*). Additionally, certain elements such as manganese and iron (Mn and Fe) serve as essential nutrients for all microbes as they

promote viability and growth. However, probiotics bifidobacteria and lactobacilli are able to competitively bind these elements and decrease accessibility by pathogenic bacteria (*309*). Triticale bran consists of high quantities of these elements, Mn and Fe, with levels of 55.4 and 51.5 ug/g DM respectively (*310*). Therefore, the presence of TB in yogurt cultures containing probiotics, acts as a source of micronutrients (Mn and Fe) and prebiotic substrates (oligosaccharides) selectively stimulating microbial growth and viability of bacteria strains bifidobacteria and lactobacilli. From Figure 6.2 we can speculate that the increased microbial viability evident in sample treatments consisting of probiotics in the presence of TB indicate that there is a synergistic effect of TB on probiotics by comparison to its respective control sample also consisting of probiotics but lacking TB.

In all yogurt samples, bacteria counts began to drop by day 14 and continued to decrease steadily until day 28. TB yogurt samples demonstrated significantly higher bacteria counts ($P \leq 0.05$) throughout day 21 to 28 in comparison to control samples. The decrease in bacteria growth is a result of the reduced amount of sugars remaining in the yogurt, leaving bacteria with far less nutrients to consume and promote growth. By day 28, the end of the cold storage period, all yogurt samples lacking triticale (1-4) had markedly inferior total bacteria counts of 6.1-6.3 log CFU/ml in comparison to 7.5-8.3 log CFU/ml viable bacteria found in yogurt samples containing TB (1S-4S). It has been suggested that a range of 6 to 8 log CFU/ml is the recommended level of viable probiotic bacteria that should remain at the end of the cold storage period (*311*). Although plain yogurt samples 1-4 had bacteria counts near the lower limit, all yogurt samples demonstrated satisfactory counts within the acceptable range.

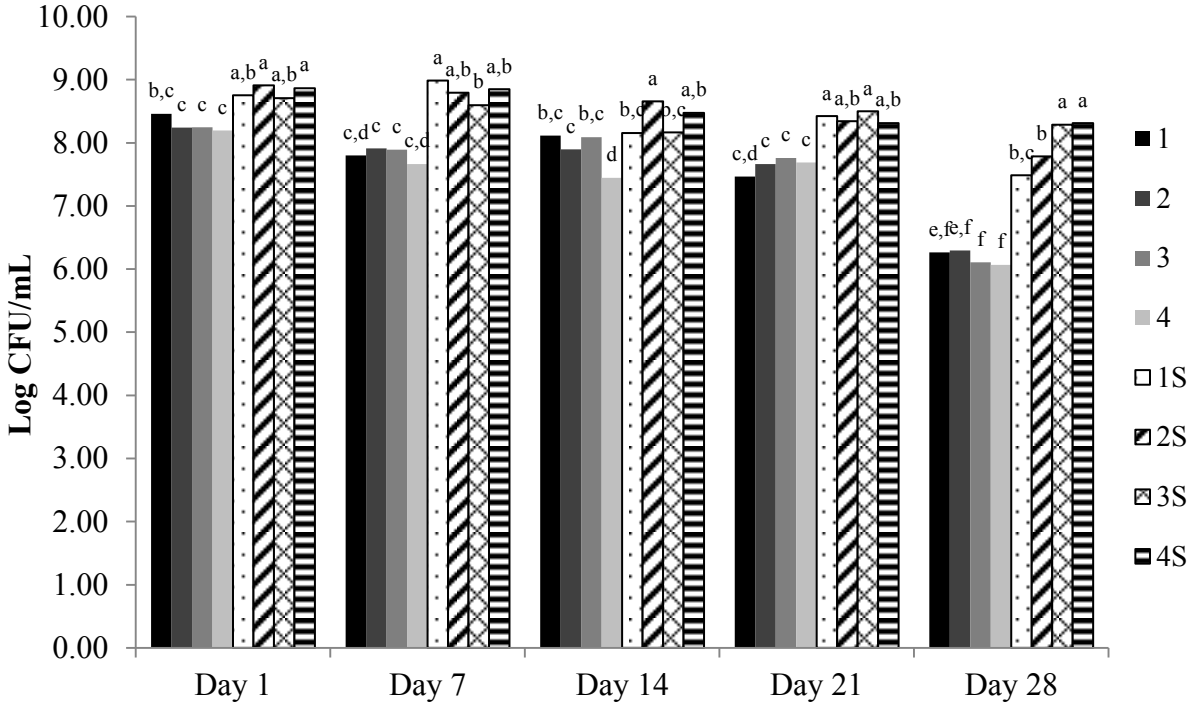


Figure 6.2 - Enumeration of lactic acid bacteria in yogurts without (1, 2, 3, 4) and with TB (1S, 2S, 3S, 4S). Values are means (N = 3); Values with different superscript letters for the same day are significantly different ($P < 0.05$).

6.4.3 pH and Total Titratable Acidity (TTA)

The milk initially had a pH of 7.0, once inoculated, the pH gradually decreased to a pH of 4.5, signifying that fermentation was complete and thereby representing day 0. Across day 1 to 28, the pH of plain yogurt samples remained stable, fluctuating only 0.05 to 0.10 units, whereas the pH of triticale yogurts dropped ~0.4 units by day 21. In the presence of triticale, bacteria are potentially more active and producing more lactic acid, thereby increasing yogurt acidity as seen in Figure 6.3 by the lower pH levels of these samples (1S-4S) across day 1 to 28 ($P < 0.05$).

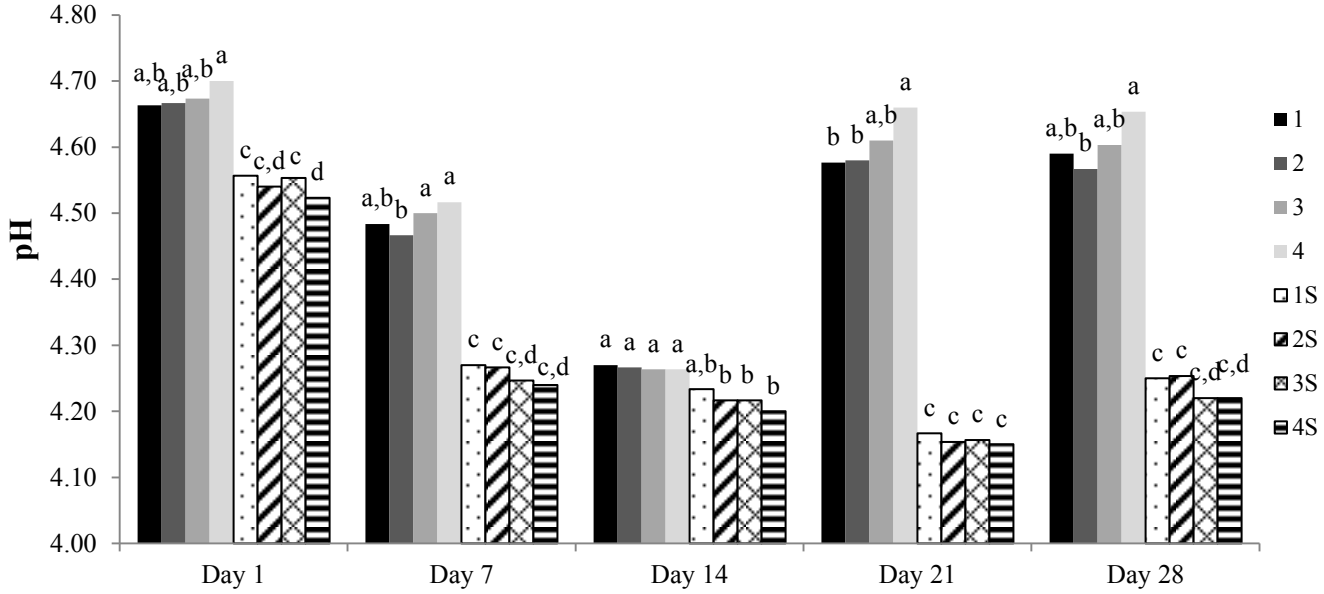


Figure 6.3 - pH values in yogurts without (1,2,3,4) and with (1S,2S,3S,4S) triticale bran. Values are means (N = 3); Values with different superscript letters on the same day are significantly different ($P < 0.05$).

Secondary metabolites such as lactate and ethanol from pyruvate and acid aldehyde pathways are glucose fermentation products that do not affect pH but may have a positive influence on cell viability (*312*). Additionally, the presences of volatile aroma components such as carbonyl compounds enhance cell viability without impacting pH levels (*313*). For these reasons, the significant drop in pH on day 21 (indicating strong acid formation capacity) did not appear to affect cell viability. Related studies have also reported similar findings of increased probiotic viability in yogurt samples with pH levels as low as 4.1 in the presence of a prebiotic source (*304, 314*).

The TTA test was employed as a method of quantifying the amount of lactic acid in

yogurt samples. Although other acids such as butyric and propionic acid are produced by probiotic bacteria, lactic acid is the most prevalent and was therefore chosen as the representative acid for quantification by TTA. All yogurt samples showed a general trend of rising TTA levels (% lactic acid) as seen in Figure 6.4, suggesting that lactic acid is being produced as a result of the growing number of bacteria. With the exception of sample 1S on day 7, TTA levels of TB yogurt samples were significantly greater ($P \leq 0.05$) than controls throughout the 28 days of cold storage. These results suggest that bacteria produce more lactic acid when TB is made available, thus further confirming findings obtained from pH monitoring.

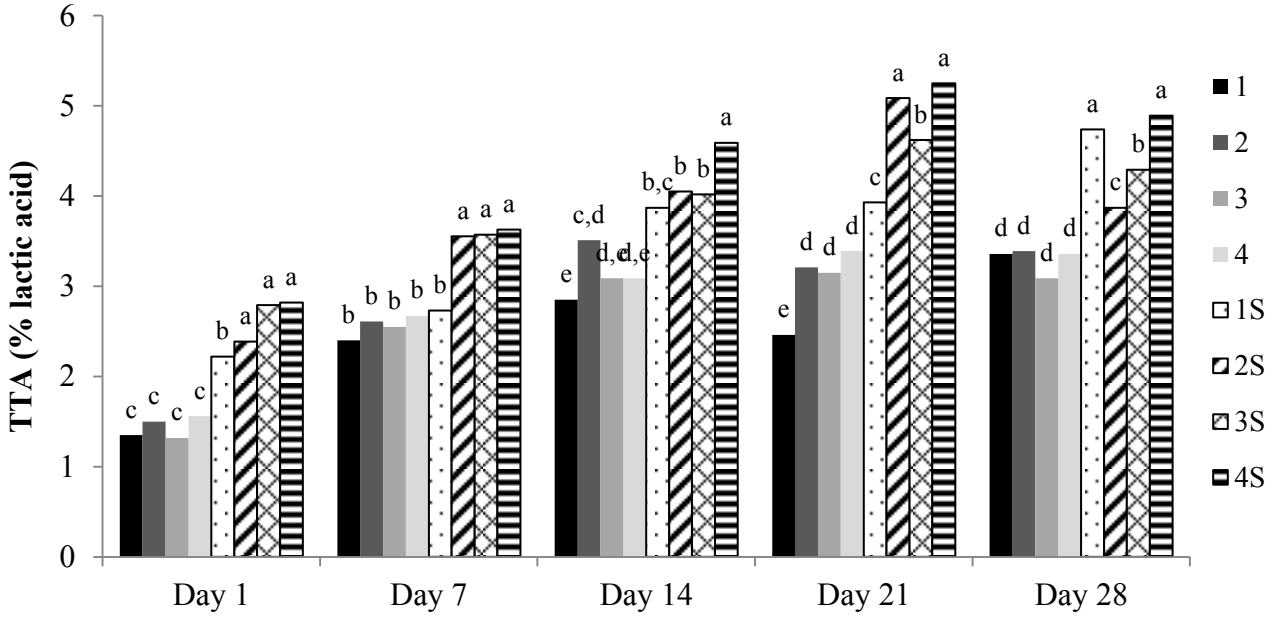


Figure 6.4 - TTA (after acidification) in yogurt with TB (1S, 2S, 3S, 4S) and without (1,2,3,4). Values are means (N = 3); Values with different superscript letters for the same day are significantly different ($P < 0.05$).

6.4.4 TB Composition and Yield of WEP

Chemical composition (g/100 g) of triticale bran is shown in Table 6.2. Total sugar was 55.3 g/100 g, in which 31.2 and 24.1 g/100 g constituted total non-cellulosic and cellulosic sugars respectively. The total amount of WEP extracted from TB was determined to be 19.8 g/100 g. From the difference between weight of dry sample before and after extraction, yield % was determined to be 93.9%. Results were obtained from triplicates and expressed on a dry matter basis.

Table 6.2 - Chemical Composition (Grams per 100 g of Dry Matter) of TB.

Sample	Sugars (non-cellulosic)	Cellulose	Ash	Fat	Protein	Moisture
TB	31.2	24.1	6.2	6.4	12.6	8.6

6.4.5 Antioxidant activity

The protective effect of an antioxidant was measured by comparing the area under the curve (AUC) of TB polysaccharide extract with that of a known antioxidant, Trolox, a water-soluble analogue of Vitamin E. ORAC is a widely accepted method for measuring the radical scavenging activity of natural products as it accurately measures both the degree of inhibition and inhibition time per assay. In utilizing this method, WEP extracts (33.86 ± 2.30 μmol TE/g) demonstrated strong antioxidant activity likely owing to the presence of bound phenolic acids. The antioxidant potential of WEP from TB is ~1/4th that of bound phenolic (128.6 ± 5.2 μmol TE/g) and above that of free phenolic (25.4 ± 1.43 μmol TE/g) isolates of TB (*277*). In addition to the presence of bound phenolic acids, such as ferulic acid, the presence of sugars with acetyl

groups (-C=O) and/or glycan-polymerization have been shown to have a large contribution to the antioxidant activity of polysaccharides (*303*).

Thus, soluble dietary fibres in TB may play a dual role in the gastrointestinal tract by acting as prebiotics enhancing the viability of gut microbiota and functioning as antioxidants released by the cereal matrix, especially after colonic fermentation (*315*). Therefore, average daily consumption of one yogurt per day (~125 g serving size) containing 4% TB (5 g) can potentially possess 0.99 g of WEP as a prebiotic source with an antioxidant level of 3386 ORAC units (μmol TE/100 g).

6.5 Conclusions

From this present study, we can conclude that Triticale bran shows promise as a source of prebiotics with antioxidant activity for future functional foods and neutraceutical applications, ultimately improving Canadian health and economy by adding value to waste. Future studies aim to separately evaluate the behaviour of probiotic bacteria in the presence of TB by employment of selective media for the enumeration of individual bacterial strains rather than total bacteria counts. Also, it is recognized that the perhaps excessive acid formation exhibited in TB yogurt samples near the latter half of the cold storage period may be undesirable since the product may affect sensory attributes. Therefore, sensory evaluation of yogurt samples using electronic sensors such as e-nose, e-tongue, as well as actual sensory panels will be employed in prospective studies. It would be interesting for prospective studies, to determine the relationship between the potential prebiotic effect of TB and characterization of comprising WEP such as the degree of purity, monosaccharide composition, degree of polymerization, and molecular weight distribution.

Chapter 7 General Discussion

Oxidative stress and chronic inflammation are known precursors to numerous illnesses, inflammatory disorders, and neurodegenerative diseases (*316-318*). Due to the production of ROS as a common byproduct of respiratory metabolism and inflammatory immune response, free radical damage and oxidative stress is a common phenomenon that occurs with the aging process (*319*). Nonetheless, other factors such as exposure to environmental stressors (e.g. UV radiation) and poor eating habits (e.g. high caloric intake) can contribute to the accelerated accumulation of ROS and early onset of oxidative stress related damages (*320, 321*).

While antioxidants cannot reverse the aging process or ROS accumulation that unequivocally occurs over-time, they have been shown to aid in the reduction of oxidative damage through the inhibition or delay of oxidation (*228, 255*). Additionally, inflammation is linked to oxidative stress such that an excess of free radicals attracts inflammatory mediators (*224*). Thus, researchers have re-introduced the concept of food as medicine by investigating the health benefitting properties of foods such as whole grains and the bioactive constituents responsible (*189, 275*). Additionally, limited food sources in today's economy have led to the exploration of non-traditional food sources otherwise considered waste byproducts of food processing (*322*). Therefore, the overall aim of this study was to evaluate the nutritional value and functional properties of triticale bran, the milling byproduct of an underestimated cereal crop. In particular, the bioactivities of cereal grain constituents, ARs and WEP, were measured using various model systems include *in vitro* and *in vivo* analysis.

Optimization conditions for alkylresorcinols (ARs) extraction from triticale bran were determined using response surface methodology. A central composite design was used to determine the effects of extraction temperature (degrees Celsius) and solid-to-solvent ratio

(weight per volume) on yield of saturated, unsaturated and total ARs. Extraction of ARs was affected significantly (p value≤0.05) by temperature and solid-to-solvent ratio on the yield of saturated, unsaturated and total ARs. The highest quantity of total ARs from triticale bran was extracted at 24 °C between 16 and 24 h at a solid-to solvent ratio (weight per volume) of 1:40. Triticale bran contained high levels of alkylresorcinols (277.5 to 308.3 mg/100g) and thus can be used as a source of these dietary bioactives. Saturated ARs were the major ARs in all samples and ranged from 86 to 88% of total ARs content. C17:0, C19:0 C21:0 and C23:0 were the predominant AR homologues in all samples. Results showed that RSM is a tool that is useful for optimizing the experimental conditions of ARs extraction.

The antioxidant and anti-inflammatory capacity of alkylresorcinols (ARs) extracted from triticale bran (TB) were determined by *in vitro* and *ex vivo* model systems. Antioxidant activity assays exhibited an ORAC value of 183.7 μmol trolox equivalent (TE)/g and 20.1% DPPH radical inhibition by TB-ARs. Furthermore, RAW 264.7 macrophage-like cells exposed to peroxyl radical generator, AAPH, and endotoxin, LPS, increased in cell viability by 32% and cell production of pro-inflammatory marker, nitrite, decreased by 35% in the presence of 80.0 μg/mL TB-ARs. Additionally, COX-2 activity induced by arachidonic acid was significantly inhibited by 49% in the presence of 83.34 μg/mL TB-ARs ($P < 0.05$). Future studies evaluating the *in vivo* activity and effects of ARs through animal studies and clinical trials need to be carried out before ARs can be considered functional food ingredients or natural therapeutic agents for reducing the risk of various pathologies linked to oxidative stress and chronic inflammation.

The *in vivo* effects of ARs, particularly their effects on oxidative stress induced by a high-fat diet, were determined by the incorporation of ARs in the diets of CF-1 mice. CF-1 mice were fed a standard low fat (LF) diet, 60 % high-fat diet (HF) and HF diets containing either 0.5% AR

extract (HF-AR), 10% TB (HF-TB) or 0.5% vitamin E (HF-VE). Liver and heart tissues of HF with AR extract diet groups possessed significantly higher ($P < 0.05$) peroxyl radical scavenging activity (0.53 & 0.54 μM TE/mg of protein), glutathione levels (0.68 & 0.41 μM GSH/mg of protein) and lower oxidized glutathione/reduced glutathione (GSSG/GSH) ratios (0.14 & 0.18) than HF diet groups (0.31 & 0.33 μM TE/mg of protein, 0.47 and 0.20 μM GSH/mg of protein, 0.41 & 0.51 GSSG/GSH ratios). Findings of this study suggest that ARs may serve as a preventative measure against risks of oxidative damage associated with high-fat diets and obesity through their application as functional foods and neutraceuticals. Future studies aim to investigate the structure-function relationship of ARs and their bioactive properties as well as the mechanisms behind their favourable biological effects. Through future evaluation of individual AR homologue activity using *in vitro* and *ex vivo* methods, we can better understand the relation of side chain length to the strength, and functionality of ARs.

The content, composition, and molecular weight distribution (MWD) profile of water-extractable polysaccharides (WEP) in triticale (*Triticosecale*) bran were determined. Results were compared against wheat (*Triticum* sp.) and rye (*Secale* sp.) bran, as well as triticale straw and flakes. The effects of the following conditions on the extractability of sugars were compared and respective fractions obtained: 1) boiling water extraction, WEP-I; 2) successive enzyme treatment and dialysis, WEP-II; 3) successive ethanol fractionation, WEP-III. Although the highest yield of WEP was generally found in fraction II, fraction III had the least level of simple sugars as unwanted co-extractives. Triticale bran (TB) had higher molecular weight (MW) polysaccharides and the widest MW distribution range (4400-401000 Da) of WEP, suggesting greater bioactive potential and high grain hardiness; prospects to be further investigated in future studies. Overall molar % of sugars indicates that triticale is also a rich source of arabinose and

xylose, sugars that are well recognized for their bioactive properties. Future studies need to investigate the relationship between the chemical structure and bioactivity of WEP in TB.

The objectives of this study were to: i) define the optimum concentration of triticale bran (TB) that can be incorporated in yogurt; ii) evaluate the prebiotic effects of TB on microbial viability, pH and total titratable acidity (TTA) in yogurt across 28 days of cold storage; and iii) measure the Oxygen Radical Absorbance Capacity (ORAC) of water-extractable polysaccharides (WEP) in TB. By day 7, the number of bacteria greatly increased in yogurt samples containing TB and maintained higher viable bacteria counts at the end of the cold storage period, in comparison to controls ($P \leq 0.05$). Confirming this data was the lower pH levels and higher TTA values of TB yogurt samples exhibited throughout 28 days ($P \leq 0.05$). Polysaccharide extracts of TB exhibited strong antioxidant activity with an ORAC value of 33.86 ± 2.30 μmol trolox equivalents (TE)/g of bran. These findings suggest the utilization of TB as a source of prebiotics with antioxidant activity for food applications, ultimately improving Canadian health and economy by adding value to waste. Future studies aim to separately evaluate the behaviour of probiotic bacteria in the presence of TB by employment of selective media for the enumeration of individual bacterial strains rather than total bacteria counts. Additionally, sensory evaluation of yogurt samples using electronic sensors such as e-nose, e-tongue, as well as actual sensory panels should be employed in prospective studies.

In summary, the antioxidant and anti-inflammatory activities exhibited by ARs, as well as the antioxidant and prebiotic potential of WEP isolates from TB, suggest advantageous properties for their applications as functional food ingredients and non-artificial preservatives. While the results of this study are promising, therapeutic and medicinal applications necessitate further studies and results based on clinical trials. Moreover, the optimum conditions established

in this study for the isolation of ARs and WEP from cereal bran may prove to be a valuable starting point for the development of large-scale extraction procedures in the food industry. Lastly, information from this study can add value to triticale, an otherwise undervalued crop, and help reduce Agri-food waste by promoting increased utilisation of bran.

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