Characterization of Local Canadian Beer and Brewers' Spent Grain through the Identification of Phenolics and Evaluation of Their Antioxidant Activities

by

Md. Jiaur Rahman

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University of Manitoba

Winnipeg

DEDICATED TO MY RESPECTED FATHER AND MOTHER

ABSTRACT

Phenolic compounds are derived in beer from malts, hops and other ingredients during brewing process. The first objective of the research work was to examine the profiles of major hydroxycinnamic acids (HCAs) and their derivatives of a laboratory-made, selected Canadian and foreign commercial beer extracts as well as evaluation of their antioxidant activities. Using the Folin Ciocalteu assay, the total phenolic content (TPC) of the beer extracts ranged from 3.72 ± 0.23 to 13.73 ± 0.49 mg GAE/ 100 mL beer. The total flavonoid content (TFC) was also found to vary from 0.82 ± 0.02 to 5.28 ± 0.04 mg CE/ 100 mL in the beer extracts. Major HCAs such as ferulic acid, *p*-Coumaric acid, sinapic acid and a key derivative of ferulic acid, 4-vinyl guaiacol (4-VG) were identified and quantified using high performance liquid chromatography with diode array detection (HPLC-DAD). A significant variation of TPC, TFC, HCAs and 4-VG content were observed across different beer extracts tested. Antioxidant activities of beer extracts, determined by DPPH radical and ABTS radical cation scavenging as well as reducing power assays, significantly correlated ($p < 0.05$; $p < 0.01$) with TPC and TFC. 4-vinyl guaiacol showed its significant correlation with ABTS radical cation scavenging and reducing power assays. These findings suggest that beer phenolics and 4-VG, the key derivative of the ferulic acid, may have a protective role in the beer as an antioxidant.

Phenolics in food grains may exist as bound form with cell wall carbohydrates especially with lignin and arabinoxylan. Effective processing e.g. heat treatment can breakdown cell walls by disrupting cell membranes and cleaving covalent bonds to release low molecular phenolics. The second objective of the research work was to examine the effect of various oven heat

treatments (100, 140 & 160^{0}C) on the extractability of bound phenolics from the brewers' spent grains (BSG) and analyse their profiles by chemical and instrumental methods. BSG treated at 160^{o}C showed a two-fold higher TPC (172.98 ± 7.3 mg GAE/100 g defatted meal) and TFC (16.15 ± 2.22 CE/100 g defatted meal), when compared to the untreated BSG extracts. Heating BSG at 160^{o}C showed two-fold higher DRSC (22.67 ± 6.93 to 46.26 ± 2.17 µmole TE/g defatted meal) and FRAP (8.30 ± 0.49 to 17.27 ± 1.15 µmole/g defatted meal) values than the corresponding untreated BSG extracts. Eleven phenolic acids were identified and quantified by Ultra Performance Liquid Chromatography with Photodiode Array (UPLC-PDA) with their amounts varying significantly ($p < 0.05$) at 160°C. Chlorogenic acid was the predominant phenolic acid present in all fractions, however, its amount decreased from 107.18 ± 2.29 to 71.28 ± 4.74 µg/g in the defatted meal. This was attributed to its conversion to caffeic acid that was identified at 140°C and 160°C.This supports the ability of the higher temperature to cleave the esterified bonds. Therefore, the observed results indicate that heat processing releases bioactive phenolic acids except the chlorogenic acid, from their bound forms.

Keywords: Beer, Hydroxycinnamic Acids, 4-Viny Guaiacol, Antioxidant Properties, Brewers' Spent Grain, Heat- Treatments, Valorization, Bioactive Phenolics.

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AAPH	2,2'-azobis (2-aminopropane) dihydrochloride
DPPH	1, 1-diphenyl-2-picrylhydrazyl
UPLC	Ultra performance liquid chromatography
PDA	Photo diode array
HPLC	High performance liquid chromatography
DAD	Diode array detector
PBS	Phosphate buffer solution
ROS	Reactive oxygen species
PUFA	Polyunsaturated fatty acids
TBA	Thiobarbituric acid
TE	Trolox equivalents
CVD	Cardiovascular disease
ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonate)
GAE	Gallic acid equivalents
4-VG	4-Vinyl Guaiacol
4-VP	4-Vinyl Phenol
4-VS	4- Vinyl Syringol
HCAs; HBAs	Hydroxycinnamic Acids; Hydroxybenzoic Acids
FA, SA, *p*-CA	Ferulic Acid, Sinapic Acid, *p*-Coumaric acid

Lists of Manuscripts

1. Rahman, M.J., Liang, X., Eskin, N.A. M., Eck, P., Thiyam-Holländer, U. (2019). Identification of Hydroxycinnamic Acids and Derivatives of Selected Canadian and Foreign Commercial Beer Extracts and Determination of their Antioxidant Properties. *LWT- Food Science and Technology, Minor* Revision for Acceptance in Process.
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CHAPTER 1

1.1 General Introduction

Beer is the most popular alcoholic beverage, which is commonly consumed worldwide including Canada. The history of beer making is over 5000 years and starting with ancient brewers, the German purity law from 1516 to today's modern industrial brewing (Wang et al., 2016; Williams, 1996). Among quality characteristics of beer, flavor is the most key attribute (Wannenmacher et al., 2018). Different beers have distinct flavor that popularizes a specific brand, while shelf life is the main factor for maintaining flavor stability and nutritional value. Maintaining flavor stability and longer shelf life of commercial beers remains a main challenge to brewers worldwide because of longer transportation and storage times (Wannenmacher et al., 2018; Leitao et al., 2011; Zhao et al., 2010). In addition, excess oxygen picked up during the brewing process, bottling and canning process causes oxidation leading to flavor instability of commercial beer. The main ingredients of commercial beers are malt (barley), hops, water and yeast. The main ingredients, malt (barley) and hops are good sources of endogenous phenolic antioxidant (Gerhäuser & Becker, 2009). Phenolic substances, which are derived from beer making ingredients may play a key role in beer flavor stability and enhance shelf life by delaying, retarding, or preventing oxidation processes because of their anti-oxidative properties (Zhao et al., 2010; Guido et al., 2005). In addition, barley phenolics especially hydroxycinnamics acids (HCAs), undergo changes to their various derivatives of vinyl and ethyl phenols via decarboxylation during malting, wort boiling and fermentation (Callemien & Collin, 2009; Vanbeneden et al., 2006; Vanbeneden et al., 2008). The HCAs and their derivatives appear to act as remarkable

radical scavenging molecules and not extensively studied as the phenolics from hops (Gerhäuser & Becker, 2009; Vanbeneden et al., 2006; Guido et al., 2005).

Brewer's spent grain (BSG) is a by-product of the beer industry. Generally, it consists of the external layers of malted barley, which are separated from the mash before fermentation. It is produced in big amounts worldwide, estimated at 39 million tons on average. It is a good source of fiber (30-50% w/w), protein (19-30% w/w), lipid (8-10%), ash (2-5%), essential amino acids and a number of bioactives such as arabinoxylans, protein hydrolysates and phenolic compounds, which are staple components of human diet (Lynch et al., 2016; Steiner et al., 2015; Mussatto, 2014; Mussatto et al., 2006). However, currently the application of BGS is limited and they are used as low-grade animal feeds or discarded (Lynch et al., 2016; Steiner et al., 2015). Because of its high nutritional composition, BSG could be very attractive source of food ingredients, due to its low cost and availability. Phenolics in BSG mostly exist as bound form with complex carbohydrates especially lignin and arabinoxylan like other cereal grains. Because of these complexes, a negligible amount of bound phenolics is released during digestion. Recently, several studies reported that heat treated processing may liberate low molecular weight phenolics from its bound form and hence increase the antioxidant capacity of extracts of interest (Li et al., 2013; Terpinc et al., 2011; Xu et al., 2007). However, this processing technique has not been applied to the phenolic composition of BSG yet. The aim of this study was to investigate the effect of heat treatments on the release of bound phenolics and also to investigate their contributions to the antioxidant activities of BSG extracts.

1.2 Major Objectives

The short-term objective of this research was to investigate the profile of major hydroxycinnamic acid derivatives of a laboratory-made, local Canadian and foreign commercial beers and evaluate the antioxidant activities of beer extracts. Another objective was to investigate the effect of heat-treatments on the release of bound phenolics and antioxidant activity of the BSG meals. The specific objectives are briefly discussed below:

1.2.1 Objective I

1.2.1.1 To investigate the profiles of major HCAs and their derivatives, in a laboratory-made, selected Canadian and foreign beer consumed in Canada using HPLC-DAD.

1.2.1.2 To determine the antioxidant properties of the beer extracts using *in vitro* screening assays.

1.2.1.3 A Pearson correlation statistical analysis was conducted between antioxidant screening assays and TPC, TFC, HCAs and 4-VG to establish the antioxidant properties of beer extracts, and individual HCAs and their derivatives.

1.2.2 Objectives II

1.2.2.1 To investigate the effect of oven heat treatments on the extractability of bound phenolics of BSG and analyse their profiles by chemical and UPLC- PDA method, respectively.

1.2.2.2 To characterize the free radical scavenging activities of BSG extracts using *in vitro* screening methods.

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CHAPTER 2

2 Review of literature

2.1 Phenolic compounds and their synthesis in plants

Phenolic compounds are derivatives of plants' secondary metabolism known as secondary metabolites. They are the well-studied group of compounds for their antioxidant properties and other mechanisms of interest (Scalbert & Williamson, 2000). In response to stress conditions, phenolics are synthesized by plants (Naczk & Shahidi, 2004) through the Shikimic acid and acetic acid pathways from two aromatic amino acids, namely phenylalanine and tyrosine (Saltveit, 2010; Santos-Sánchez et al., 2019; Kulbat, 2016; Giada, 2013; Hollman, 2001). Most plant phenolic acids are synthesized from phenylpropanoids, which is the main product of the shikimic acid pathway (Santos-Sánchez et al., 2019; Giada, 2013). In contrast, simple phenols are the main products of the acetic acid pathway. Flavonoids, the most abundant group of polyphenols in nature, are synthesized by combination both of these pathways (Giada, 2013; Sánchez-Moreno, 2002). Furthermore, condensed tannins, a group of polymer polyphenols are synthesized through condensation and polymerization phases during the synthesis of flavonoids (Giada, 2013).

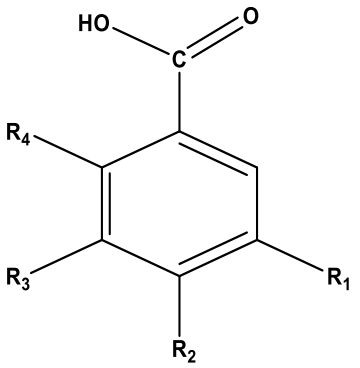
2.2 Classification, structure, and chemistry of phenolics

Phenolics are the most studied group of secondary metabolites with more than 8000 identified in cereals, legumes, fruits, vegetables, various conventional and non-conventional oilseeds, alcoholic and non-alcoholic beverages and its related by-products (Cheynier, 2012; Bravo, 1998). Phenolics are known to reflect a diverse structure. The main diversity of structure of phenolic is "heterogenicity" that range from simple to highly complex

compounds (e.g. polymer) characterized by several aromatic rings consisting of one or more hydroxyl groups (Cheynier, 2012). In addition, these aromatic rings may bear other functional groups such as esters, methyl ethers, and glycosides, and thus show big diversity in their structures. According to the diversity of structures, plant phenolics can be categorized into three major classes. Firstly, simple phenolics which include various simple phenols, volatile phenols, hydroquinone, resorcinol and pyrocatechol which are derived from benzoic acids, hydroxybenzoic and hydroxycinnamic acids (Giada, 2013; Vermerris & Nicholson, 2008). Secondly, phenolic acids that are major components of cell wall structures of whole grains (Stuper-Szablewska & Perkowski, 2019; Shahidi & Ambigaipalan, 2015). Thirdly, polyphenolics which carry large group of phenolic compounds with simple structure to complex such as flavonoids and their derivatives, anthocyanins, proanthocyanins, stilbenes, coumarins, lignans, including the highly polymerized complements, e.g. tannins and lignin (Shahidi & Ambigaipalan, 2015; Vermerris & Nicholson, 2008). According to the distribution and localization in plant's tissues, plant phenolics can also be categorized as free, soluble conjugates (e.g. esterified fraction, etc.) and insoluble bound phenolics (Stuper-Szablewska & Perkowski, 2019; Shahidi & Yeo, 2016; Acosta-Estrada et al., 2014; Wong, 2006). From a nutritional point of view, this classification of phenolics is essential because the metabolism and absorption in the small intestine and colon as well as the physiological effects of phenolics depend on their solubility characteristics (Acosta-Estrada et al., 2014). Insoluble phenolics are not bioaccessible in the small intestine, however, may be partially released in the colon by microbiota fermentation. Soluble phenolics both free and conjugates can cross

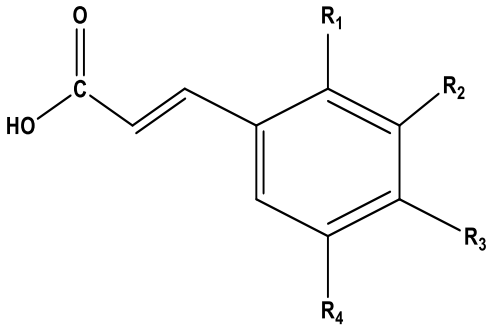
the intestinal barrier into the blood, unchanged or as metabolites (Acosta-Estrada et al., 2014; Pérez-Jiménez et al., 2013; Giada, 2013; Sánchez-Moreno, 2002).

2.2.1 Phenolic acids and its derivatives



Names	R_1	R_2	R_3	R_4
Gallic acid	OH	OH	OH	H
Protocatechuic acid	OH	OH	H	H
P-Hydroxybenzoic acid	H	OH	H	H
Vanillic acid	OCH_3	OH	H	H
Syringic acid	OCH_3	H	OCH_3	H
Salicylic acid	H	H	H	OH
Gentisic acid	OH	H	OH	H

Figure 2.1. Basic structure and lists of the main hydroxybenzoic acids (Source: Giada, 2013)



Names	R_1	R_2	R_3	R_4
Ferulic acid	H	OCH_3	OH	H
p-Coumaric acid	H	H	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H
o-Coumaric acid	OH	H	H	H
m-Coumaric acid	H	OH	OH	H

Figure 2. 2. Basic structure and lists of major hydroxycinnamic acids (Source: Giada, 2013)

Phenolic acids (PAs) are the most abundant group of phenolics found in the plant kingdom. Most of them and their derivatives are an integral part of human diet (Yang et al., 2001). They can be categorized as a) hydroxybenzoic acids (HBAs) and b) hydroxycinnamic acids

(HCAs). Hydroxybenzoic acids are the simplest form of PAs found in plants having seven carbon atoms (C_6-C_1) in their structures (Stuper-Szablewska & Perkowski, 2019; Kulbat, 2016; Shahidi & Ambigaipalan, 2015; Giada, 2013). On the other hand, HCAs are the most plentiful PAs in plants and having nine carbon atoms (C_6-C_3) in their respective structures. The major hydroxybenzoic and hydroxycinnamic acids and their basic chemical formulas are drawn **in figures 2.1 and 2.2,** respectively. The major HBAs found in various foods and beverages are, gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, gentisic acid, syringic acid, salicylic acid and vanillic acid (Shahidi & Ambigaipalan, 2015; Sánchez-Moreno 2002) while major HCAs are ferulic, caffeic, *p*-coumaric, and sinapic acid, which are most plentiful in nature (Shahidi & Ambigaipalan, 2015; Young et al. 2001). A great amount of these compounds usually can be found in the outer coat, the aleurone layer and in embryos of cereal grains and seeds, while starchy endosperm of kernels contains trace amounts (Stuper-Szablewska & Perkowski, 2019; Deng et al., 2012; McKeehen et al., 1999). The main hydroxycinnamic acid found in whole grains is ferulic acid (Balashubashini et al., 2003). It has been stated that HCAs and their esters show higher antioxidant activities such as chlorogenic acid etc. However, the arrangement and the number of hydroxyl groups present in the structures of a molecule involved could be attributed to the antioxidant properties of phenolic acids and their esters, including many other characteristics.

2.2.2 Flavonoids and its derivatives

Flavonoids are the most commonly studied polyphenols and important ingredients of the human diet (Bravo, 1998). They are potent natural antioxidants from plants and their antioxidant potential depends on the hydroxyl groups which are present in positions 3' and 4' of the B ring (Shahidi & Ambigaipalan, 2015; Giada, 2013). In plant-based foods, a different class of flavonoids can be found; however, their structure depends on a C_2-C_3 double bond that is found in the heterocyclic pyrone ring and the degree of hydroxylation. The major classes of flavonoids include flavanols, flavones, flavonols, flavanones isoflavones, flavan-3-ol, anthocyanins, and anthocyanidins, which are structurally different from each other (Shahidi & Ambigaipalan, 2015; Giada, 2013; Vermerris & Nicholson, 2008).

2.3 Major sources of phenolic compounds

The major sources of phenolics include fruits, vegetables, legumes, cereals, oilseeds, nuts, herbs, spices, and alcoholic and non-alcoholic beverages as reviewed by many recent studies (Stuper-Szablewska & Perkowski, 2019; Shahidi & Ambigaipalan, 2015; Giada, 2013; Vermerris & Nicholson, 2008). Cereals are recognized as a good source of phenolics with high amounts of phenolic acids, flavonoids, and proanthocyanidins that are present in their aleurone layer (Stuper-Szablewska & Perkowski, 2019). Wheat is a major cultivated cereal crop of Canada. Phenolics in wheat exist as free, insoluble-bound and soluble conjugated forms (Stuper-Szablewska & Perkowski, 2019; Liyana-Pathirana & Shahidi, 2007; Onyeneho & Hettiarachchy, 1992). The major phenolics in wheat are ferulic, *p*-coumaric, and vanillic acid, all of which exist as free forms. However, these compounds are also found together with other phenolic compounds, for example chlorogenic, gentisic, caffeic, syringic, and *p*-

hydroxybenzoic acid (Stuper-Szablewska & Perkowski, 2019; Liyana-Pathirana & Shahidi 2007; Onyeneho & Hettiarachchy, 1992). Barley, the second major cereal crop of Canada, is a good source of bioactive phenolics. Barley is the main ingredient of brewing industries. In Canada, barley is normally used to make malt, for animal feed and human consumption. Like wheat, phenolics in barley grains exist as free, esterified and in bound form (Andersson et al., 2008; Bonoli et al., 2004). Many recent studies have been reported on the phenolic profiles of barley extracts, including evaluation of their antioxidant activities (Carvalho, Curto, & Guido, 2015; Meneses, Martins, Teixeira, & Mussatto, 2013; Gamel & Abdel-Aal, 2012; Hajji et al., 2018; Harnanz et al., 2001; Yu, Vasanthan, & Temelli, 2001). These studies reviewed the TPC, free radical scavenging activities, and metal chelation activities of barley extracts. Several phenolic acids such as sinapic acid, ferulic acid, *p*-coumaric acid, caffeic acid, syringic acid, 4-hydroxybenzoic acid, chlorogenic acid and protocatechuic acid and flavonoids such as catechin and epicatechin etc. (**Figure 2.3**) were identified and quantified by many recent studies (Carvalho et al., 2015; Gamel & Abdel-Aal, 2012; Hajji et al., 2018; Harnanz et al., 2001; Meneses et al., 2013; Yu et al., 2001). Also, oats, rye, and corn are also good sources of bioactive phenolics, and their profiles reviewed by several recent studies (Stuper-Szablewska & Perkowski, 2019; Shahidi & Ambigaipalan, 2015). Legumes are rich sources of phenolics. The hulls of legumes contain higher phenolics than the endosperm. For example, a higher amount of TPC was found in hulls fraction of beans (6.7-27.0 mg catechin equivalents/g extracts) than whole seeds (4.9-9.36 mg/g extracts) (Madhujith & Shahidi, 2005). The authors identified major phenolic acids of the bean hulls extracts includes ferulic, *p*-coumaric, vanillic, caffeic, and sinapic acid, etc.

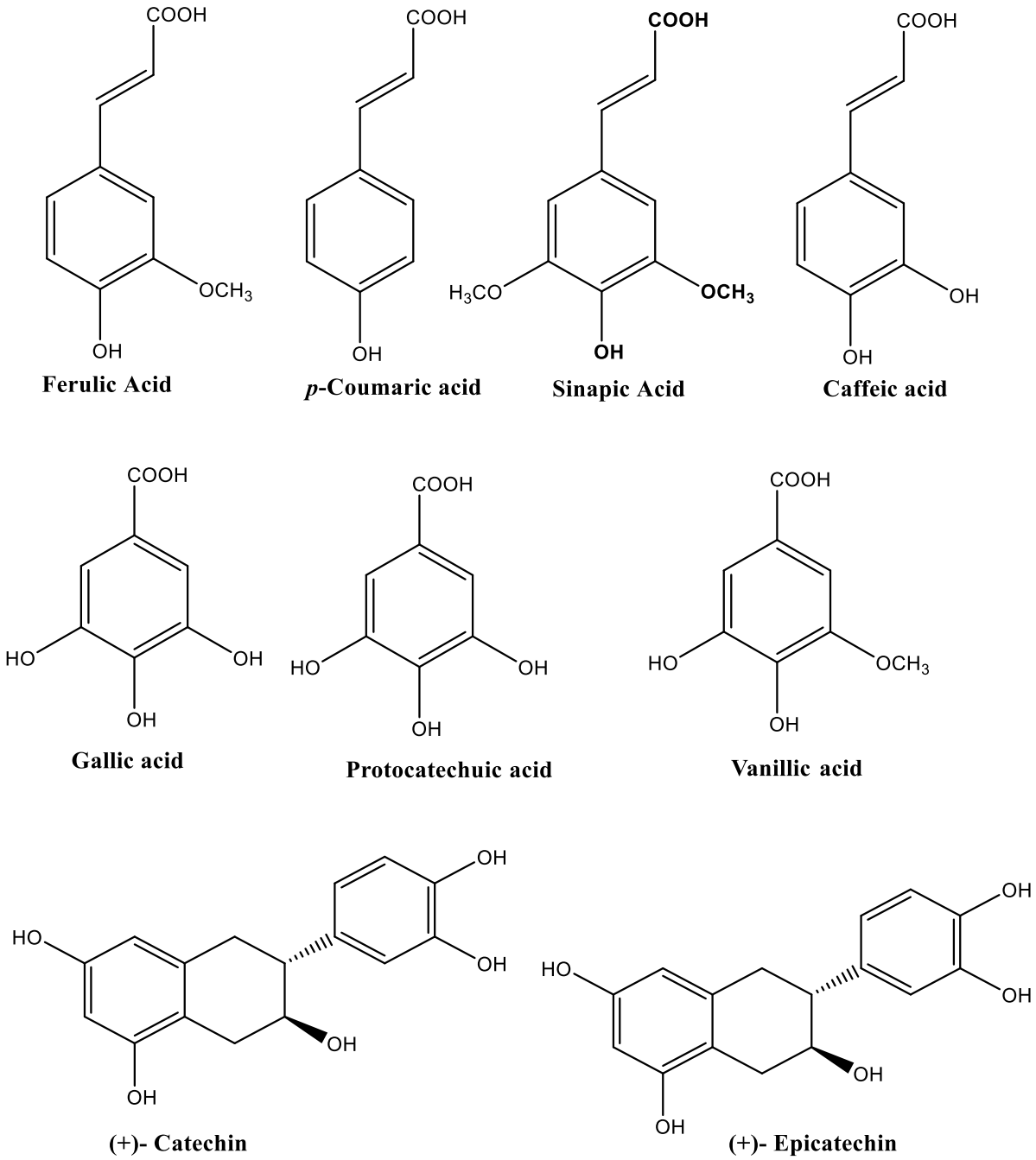


Figure 2.3. Chemical structures of identified phenolics in barley (Name of the compound adopted from Carvalho et al., 2015; Gamel & Abdel-Aal, 2012; Yu et al., 2001).

The oilseeds are also good sources of different phenolic acids, coumarins, flavonoids, tannins, etc. as reported by a number of recent studies (Khattab et al., 2014, 2010; Rahman et al., 2017, 2018b; Terpinc et al., 2011). Sinapic acid is one of the major phenolic acids found in brassica oilseeds (Rahman et al., 2017, 2018b; HadiNezhad, Rowland, & Hosseinian, 2015; Terpinc et al., 2011). Fruits are rich sources of bioactive phenolics and their antioxidant and bioactivities in both *in vitro* and i*n vivo* systems have been well studied and documented (Ambigaipalan et al., 2017; Sangiovanni et al., 2013; Jean-Gilles et al., 2011; González-Barrio et al., 2010; Gođevac et al.,2009; Hosseinian et al., 2007; Beekwilder, 2005a, b). Berries, grapes, apples, citrus, and pomegranates are known as super fruits worldwide. They are good sources of polyphenols, especially flavonols (e.g. quercetin, kaempferol, myricetin, etc.), proanthocyanidins (e.g. procyanidins and prodelphinidins) and esterified phenolic acids, for example, ferulic, sinapic, *p*-coumaric, caffeic, gallic, and chlorogenic acid (Ambigaipalan et al., 2017; Furuuchi et al., 2011; Beekwilder, 2005b; Sánchez-Rabaneda et al., 2004). Vegetables are a rich source of bioactive polyphenols. The profiles and the content of bioactive polyphenols in various groups of vegetables have been reviewed by many studies (Grosso, 2018; Shahidi & Ambigaipalan, 2015; Lima et al., 2014; Gaida, 2013, Shahidi et al., 2010). Green leafy vegetables, for example, lettuce, kale and spinach, contain high level of flavonoids at 0.80 - 2.241 mg/g fresh weight (Howard et al., 2002).

2.4 Extraction and characterization of phenolics

The extraction techniques of phenolics can be categorized as I) Conventional methods; II) Modern methods. The conventional methods are generally used in a laboratory for small-scale extraction (Kartsova & Alekseeva, 2008) which includes solid-liquid extraction (SLE),

and liquid-liquid extraction, among others. However, these methods have several drawbacks, for example, low selectivity, higher solvent volume, lower efficacy of extraction, longer time of extraction, solvent residue, lower yields, and environmental pollution, etc. (Brglez Mojzer et al., 2016). In contrast, most common novel methods for the extraction of phenolics includes I) ultrasound-assisted extraction (UAE), II) enzyme-assisted extraction (EAE), III) microwave-assisted extraction (MAE), IV) pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE). The major advantages of these techniques are I) higher yield, II) shorter time and III) solvent-free extracts (Brglez Mojzer et al., 2016; Michalak & Chojnacka, 2015; Kadam et al., 2013; Ibanez et al., 2012; Jeon et al., 2012). Among them, SFE is mostly preferred method by a food and pharmaceutical industry due to minimal or no use of organic solvents, and high yield without loss of compound of interest (Brglez Mojzer et al., 2016; Michalak & Chojnacka, 2015; Kadam et al., 2013; Ibanez et al., 2012). Despite the extraction of phenolics from natural sources, the high-value phenolic compounds also can be manufactured through chemical or enzymatic synthesis and/or microbial biosynthesis. Various modern techniques such as HPLC, LC-MS, LC-MS/MS, and NMR are generally used to identify and characterize the phenolics obtained from plant extracts and biosynthesis (Zhong, 2010; Kartsova & Alekseeva, 2008).

2.5 Phenolics and their role as protective antioxidants

2.5.1 Lipid oxidation and its mechanism

Lipid oxidation is a key cause of deterioration of foods and its by-products. It also has negative effects on biological systems such as tissue damage, etc. The oxidation of foods may occur from harvesting to storage conditions including processing time. The major consequences of lipid oxidation of foods and their products include development of off-odors and off-flavors, breakdown of nutritional ingredients such as essential fatty acids, fat-soluble vitamins, and other bioactives, which lead to change in taste, texture, color and even formation of potentially toxic metabolites (Błaszczyk et al., 2013, Smet et al., 2008; Zhong, 2010), thus decreasing the shelf-life and nutritional value of foods and feeds. On the other hand, *in vivo* biological systems, peroxidation of polyunsaturated fatty acids of cell membranes has adverse cellular effects that may cause several diseases, for example, inflammation, atherosclerosis, cancer, and aging, etc. (Kruidenier & Verspaget, 2002; Floyd & Hensley, 2002; Davies, 2000). Several oxidations may occur in food systems namely autoxidation, photooxidation, thermal and enzymatic oxidation (Vercellotti et al., 1992). The main factors facilitating lipid oxidation are the temperature and water activity of the materials (Lingnert, 1992) and lipid degradation (Lingnert, 1992). In addition, a number of factors that may catalyze lipid oxidation include light, heat, transition metal ions ($Cu^{2+,}$ Fe^{2+}, etc.), hemoproteins, metalloproteins and cellular enzymes such as lipoxygenase (Shahidi, 2000; Vercellotti et al., 1992).

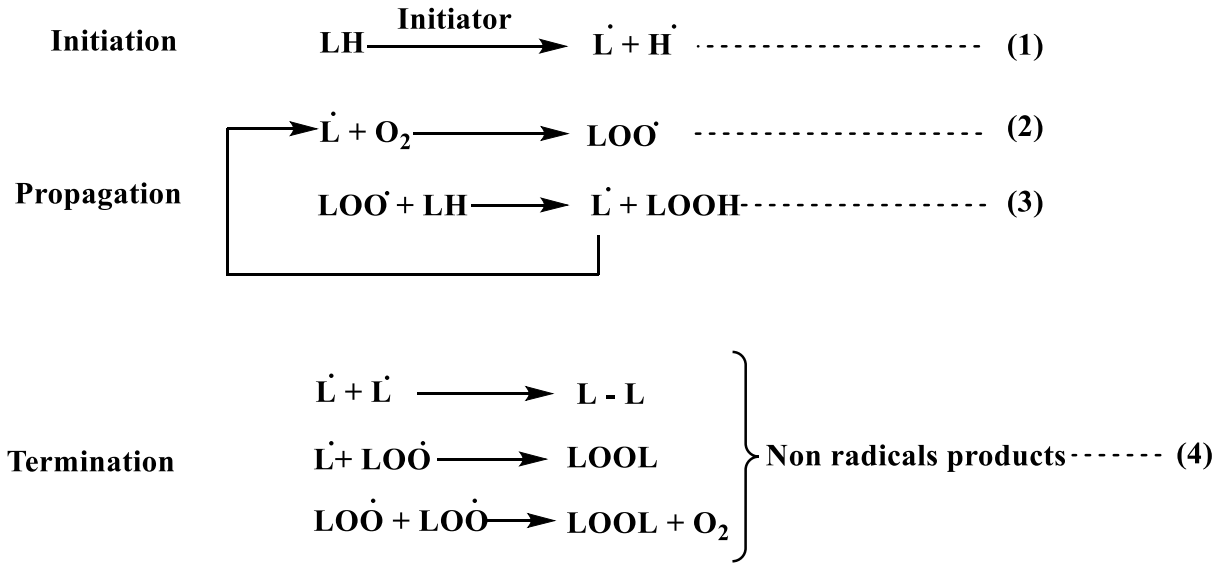


Figure 2.4. Simple schematic pathways of lipid autoxidation and its mechanism of reactions (Source: Shahidi & Ambigaipalan, 2015)

Autoxidation is one of the primary pathway that degrade lipids in foods. It occurs via a free radical mechanism in which atmospheric oxygen is added to the unsaturated fatty acid chains of lipid molecules. The reaction can be catalyzed by various initiators as mentioned above. Autoxidation with the three aforementioned steps of a) initiation, b) propagation, and c) termination, all of these leads to a series of complex chemical changes of foods (Shahidi & Zhong, 2005; Shahidi & Wanasundara 1992). A simplified scheme autoxidation and its mechanism are given in **Figure 2.4.**

Oxidation of foods and by-products take place slowly at the initial stage. However, it increases suddenly after crossing the induction period. **Figure 2.4,** the initiation process (Reaction 1) of oxidation is a quite complex, which involves removing an electron and/or hydrogen atom from a lipid compound (LH) and form a lipid radical ($L^{\cdot}$). Because of losing a

hydrogen atom from a polyunsaturated fatty acid (PUFA), the rearrangement of the methylene-interrupted double bonds occurs, as a result, conjugated dienes (CD) and trienes formed. These CD and trienes are primary indicators of lipid oxidation (Shahidi & Zhong, 2005; Shahidi & Wanasundara, 1992). During the propagation process (**Figure 2.4,** Reaction 2), lipid-derived acryl radical ($L^{\cdot}$) reacts with O_2 and forms peroxyl radicals ($LOO^{\cdot}$) or abstracts a hydrogen atom from another lipid compound (**Figure 2.4,** Reaction, 3) to form hydro - peroxides (LOOH) which are known as the principal products of a lipid oxidation. Hydro-peroxides are very reactive molecules that easily break down to several secondary products of oxidation, such as ketones, aldehydes, alcohols, volatile organic acids, hydrocarbons, and epoxy molecules etc. Some of these compounds have undesirable odors, however, threshold values of their off flavor are very low. Meanwhile, peroxyl (LOO·), hydroxyl (·OH), alkoxyl (LO·), and new lipid radical (L·) are produced due to breakdown of hydro-peroxides and promote the chain reaction of free radicals (Shahidi & Wanasundara 1992). During the termination phase (**Figure 2.4, reaction 4**), radicals deactivation happens by forming stable non-radical derivatives, including a number of polymeric molecules through radical-radical disproportionation or radical-radical coupling (Zhong, 2010; Erickson, 2002).

2.5.2 Mechanism of antioxidant action of phenolic compounds

Antioxidant is a chemical molecule which can delay oxidation of lipid-enriched foods or other compounds by stopping the reactions of oxidation such as initiation and /or propagation of oxidizing chain reactions (Sang et al., 2002; Velioglu et al., 1997). Phenolic compounds and their derivatives can act as antioxidants because of their redox properties. Because of these properties, they can quench singlet and triplet oxygen, neutralize free radicals, decompose

peroxides, etc. (Sang et al., 2002; Shahidi, 2000; Osawa et al., 1994). In addition, they are metal ion chelators, decomposer of secondary products, and inhibitor of a number of prooxidative enzymes, among others (Sang et al., 2002; Osawa et al., 1995). However, the antioxidant properties of phenolics depend on the number of hydroxyl groups and their location in the structures of interest (Sang et al., 2002; Cao et al., 1997). For example, a phenolic compound (AH) can donate a single electron and/or hydrogen atom to lipid-derived radicals (L·/ L0·/L00·) and convert them into non-radical lipid derivatives (**figure 2.5, Reaction 1**). After donating an electron and/or a hydrogen atom, the phenolic compound also produces its own antioxidant free radical (A*), which is more stable and does not participate in promoting the autoxidation (Kiokias et al., 2008). However, chain-propagation reaction of oxidation can be interfered by the antioxidant free radicals (**figure. 2.5, Reactions 2 and 3**) and converted radical derivatives to non-radical substances.

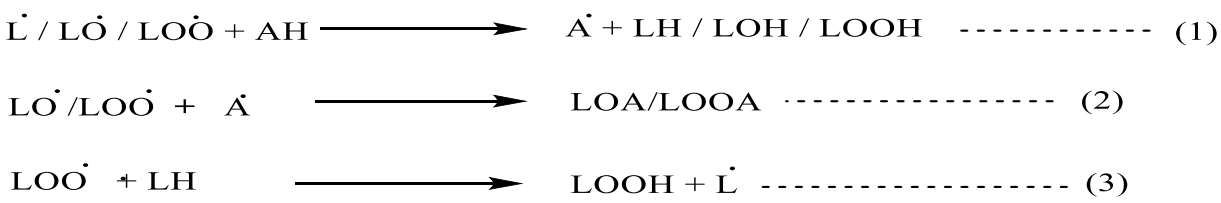


Figure 2.5. Antioxidant action of phenolic compounds

2.6 Phenolics and polyphenolics in various commercial beer

Beer is a source of endogenous antioxidants namely phenolic acids, flavonoids, stilbenes, products of Maillard reaction, and sulfites (Zhao et al., 2010). These endogenous antioxidants especially phenolic substances have appeared to prevent oxidation, which is the key reason of flavor stability and deterioration of commercial beer quality. Phenolic acids

especially hydroxycinnamic acids and their decarboxylated derivatives can impart very strong flavor and can enhance the shelf life of beer. Flavonoid and stilbenes, which are mainly derived from hops may have several health benefits as reviewed by several most research studies and reviews (Działo et al., 2016; Gutiérrez-Grijalva et al., 2016; Kumar et al., 2014; Rasouli et al., 2017; Tresserra-Rimbau et al., 2017) as well as also act as an antioxidant like phenolic acids in beer. Recently, many studies have been published on beer phenolics and their antioxidant activities. Zhao and his co-workers conducted a study (Zhao et al., 2010) on the thirty-four commercial beers including twenty-seven Chinese and seven internationals to analyze their TPC and antioxidant activities, as well as several major PAs, which were identified and quantified by an HPLC method. The TPC significantly varied from 152.01 to 339.33 mg GAE/L sample among the beer extracts, depending on the beer type. Gallic and ferulic acid were the predominant phenolic acids compared to other phenolics identified (**Figure 2.6**).

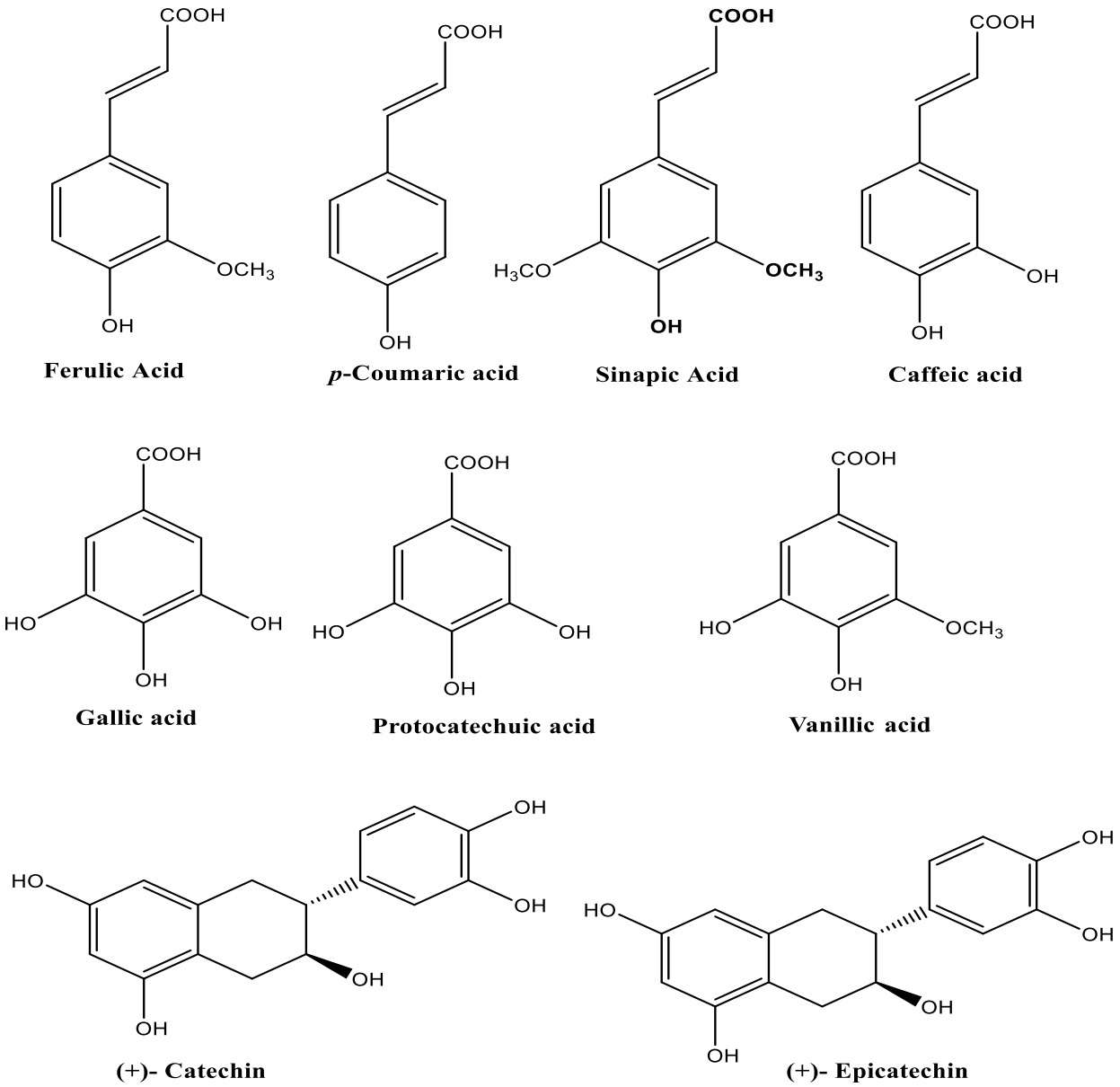


Figure 2.6. Chemical structures of phenolic compounds identified in commercial beer extracts (Names of the compound adopted from Leitao et al., 2011; Zhao et al., 2010; Piazzon et al., 2010).

Piazzon et al. (2010) conducted a study on seven different types of beer samples such as abbey, bock, ale, pilsner, lager, wheat, and dealcoholized beers and analysed the phenolics and antioxidant activities of these beer extracts. They reported that bock beer showed the highest TPC (875 ± 168 mg GAE/L sample) while the lowest amount of TPC (366 ± 73 mg GAE/L sample) was observed in dealcoholized beer extract. FA was found to be the main PA in the seven beer extracts tested followed by *p*-coumaric, caffeic, sinapic, vanillic, syringic, and 4-hydroxyphenyl acetic acid (**Figure 2.6**) as identified by HPLC-ECD method. The antioxidant activity of seven beer extracts was determined using the FRAP assay. The FRAP activity increased among seven beer extracts in the order abbey > ale > wheat > pilsner > lager > dealcoholized. Quifer-Rada and his co-workers (Quifer-Rada et al., 2015) identified 47 phenolics including phenolic acids, flavones, flavonols, flavanols, hydroxycinnamoylquinics, alkylmethoxyphenols, alpha- & iso-alpha-acids, prenylflavonoids, hydroxyphenyl acetic acids, in four beer samples such as lager, Pilsen, Märzenbier and non-alcoholic beer using HPLC-MS/MS. Seven of these compounds have been recognized in beer for the first time: caffeic acid-O-hexoside, feruloyl quinic acid, sinapic acid-O-hexoside, coumaric acid-O-hexoside, kaempferol-O-hexoside, catechin-O-dihexoside, and apigenin-C-hexoside-pentoxide. Leitao et al. (2011) conducted a study on the various beer extracts obtained from different steps of beer processing and used to analyze their individual phenolic compounds and antioxidant activities. The beer extract showed significantly lower antioxidant activities compared to other beer processing materials. However, the antioxidant activities of different beer processing extracts remained unaffected during the processing steps, as opposed to the TPC, that showed a 3-fold increase (Leitao et al., 2011). The authors

identified ten phenolic compounds using the HPLC method in the different beer processing extracts. Ferulic acid was the major compound followed by sinapic acid, caffeic acid, *p*-coumaric acid, protocatechuic acid, vanillic acid, catechin, chlorogenic acid, caffeic acid and epicatechin (**Figure 2.6**) while boiled wort extract showed higher ferulic acid content (133 ± 23 µM) compared to other samples tested in the study.

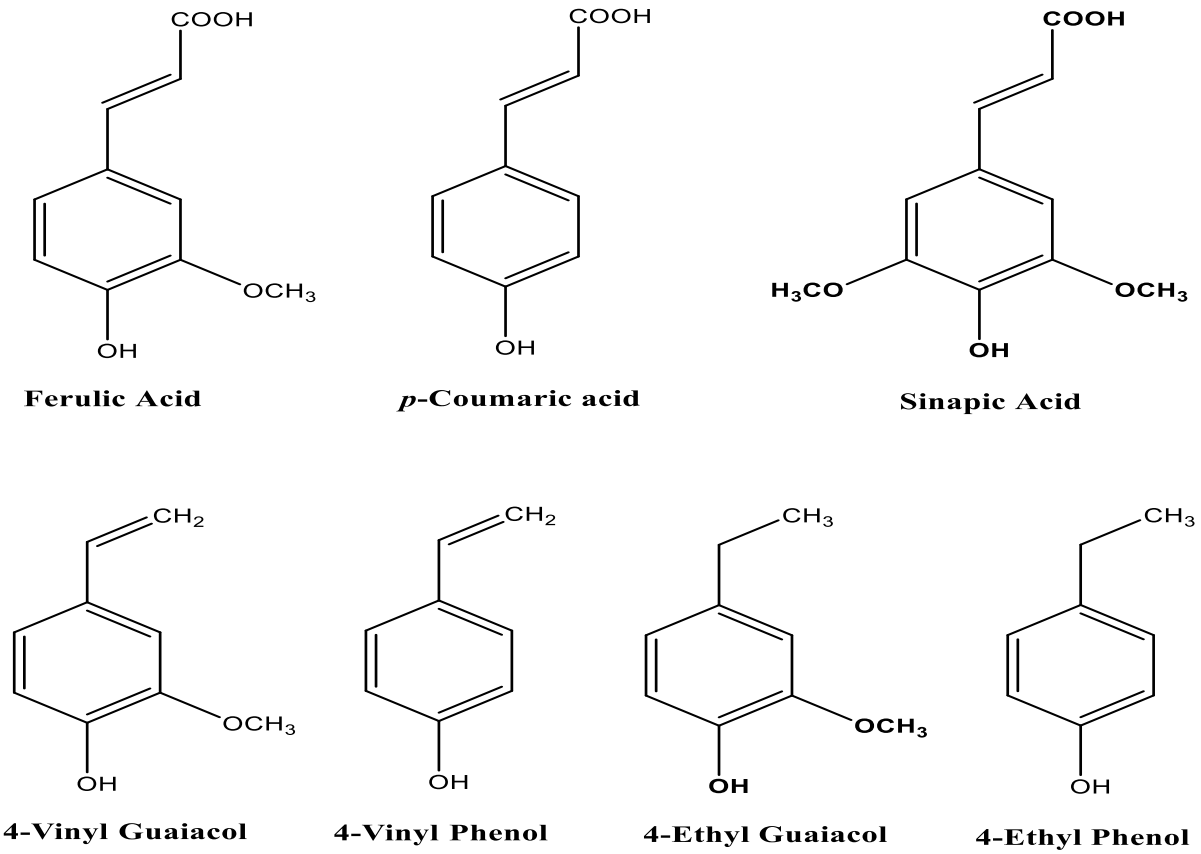


Figure 2.7. Chemical structures of major hydroxycinnamic acids and their derivative phenols identified in commercial beers (Name of the compounds adopted from Vanbeneden et al., 2006).

Vanbeneden and his co-worker conducted a study (Vanbeneden et al., 2006) using three beer samples such as wheat beer, dark and pilsner beer including one wort sample to determine the major HCAs and their derivatives by an isocratic HPLC-ECD method. HCAs, for example, sinapic acid, ferulic acid, and *p*-coumaric acid and their derivative flavor active volatile phenols such as 4-VG and 4-VP were identified (**Figure 2.7**). The study reported that ferulic acid showed higher content (0.3896 ± 0.0043 - 2.6267 ± 0.0085 ppm) compared to *p*-coumaric acid (0.2394 ± 0.0033 - 1.4794 ± 0.0064 ppm) and sinapic acid (0.1281 ± 0.0031 - 0.5035 ± 0.0046 ppm), among the samples tested while wort extract showed highest hydrocinnamic acids content. The TPC, individual phenolics and antioxidant activities of various commercial beers varied significantly probably because of their style, origin, and raw materials used during processing & production. Ferulic acid and its derivative 4-Vinyl guaiacol was a predominant phenolic compound found in beer extract. These studies focused on the relationship between phenolic content and their antioxidant activities. However, very limited findings were observed on the hydroxycinnamic acids and their contributions in increasing flavor active volatile phenols and their identification as well as antioxidant activities of Canadian beers.

2.7 Phenolics and polyphenolics of brewers spent grains

Brewers' spent grains (BSG) is a good source of bioactives, for example, phenolics, bioactive fibers (e.g. arabinoxylan and β-glucan, etc.) and protein hydrolysates, etc. In this study, the main interest was in the phenolics of BSG and their characterization through identification and antioxidant activities affected by various oven heat treatments. There have been a

number of studies on the phenolic extracts from BSG meal and evaluation of their antioxidant activities (Moreira et al., 2013; McCarthy et al., 2012; da Rosa Almeida et al., 2017; Szwajgier et al. 2010). Moreira et al. (2013) conducted a study on four-light BSG samples (e.g. Pilsen, melano, melano 80, carared), and two dark BSG samples (e.g. chocolate, black, etc.) to analyze their phenolic profiles and antioxidant activities. Microwave assisted extraction technique was used to extract phenolics of BSG and a number of *in vitro* assays such as DPPH, ABTS and deoxyribose assays were used to measure the antioxidant activities of BSG extracts. HPLC-MS/MS technique was used to identify phenolic compounds of each BSG extract. The study reported that both light and dark BSG extracts were a good source of phenolic compounds where light BSG extracts showed higher TPC compared to dark BSG extracts. The BSG extract showed significant free radical scavenging effects in several *in vitro* assays (Moreira et al., 2013). Pilsen BSG extract (light BSG) significantly showed higher DPPH, ABTS and hydroxyl radical scavenging activities when compared to other BSG extracts tested in the same study.

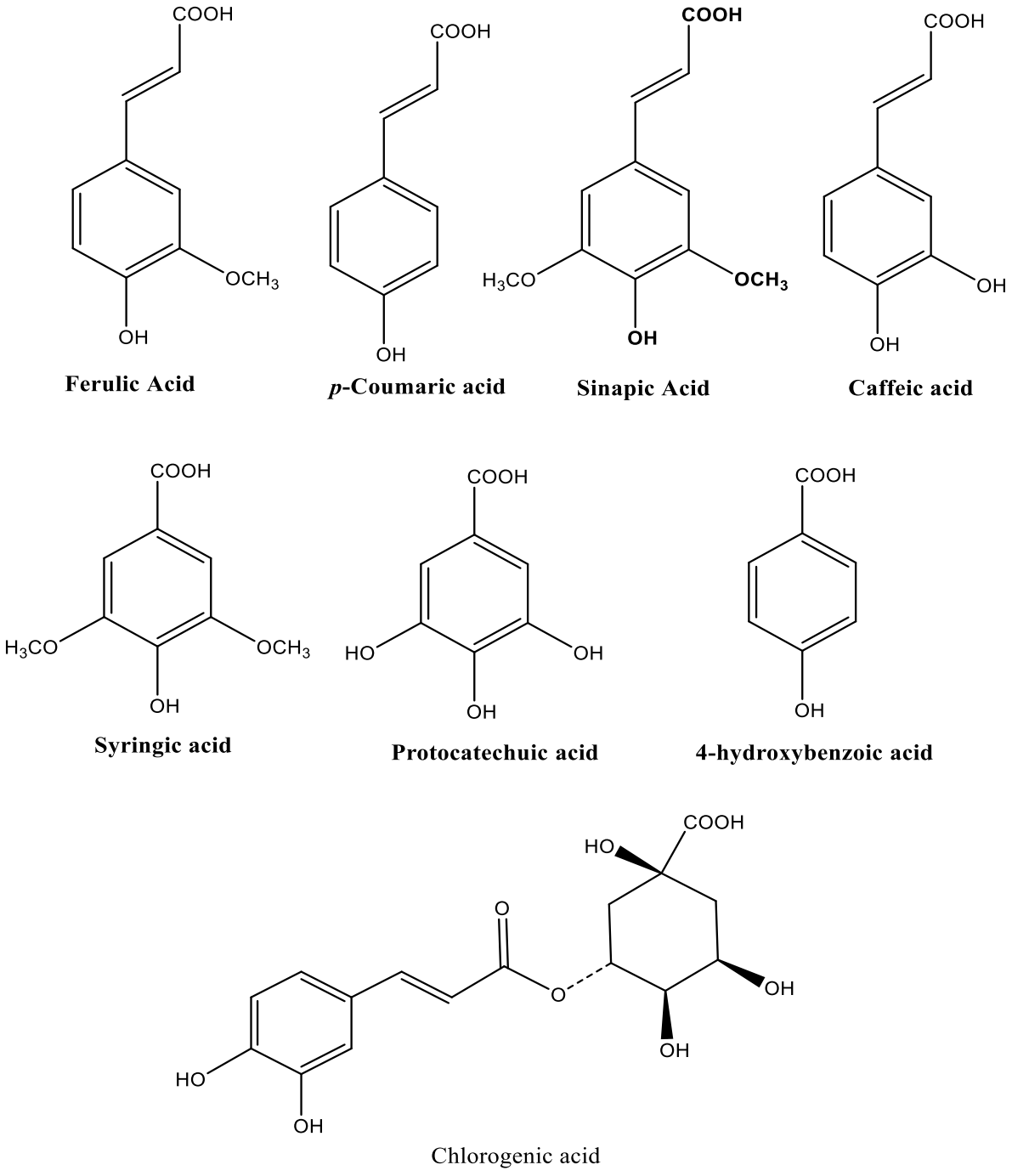


Figure 2.8. Chemical structures of identified phenolic acids in BSG by HPLC analysis (Name of the compounds adopted from Szwajgier et al., 2010; Moreira et al., 2013).

Major phenolic acids (PAs), for examples ferulic acid (FA), *p*-coumaric acid (*p*- CA), syringic acid, and sinapic acid (SA), as well as one dehydrotrimer and numerous isomeric ferulate dehydrodimers (**Figure 2.8**) were identified and quantified by HPLC- MS/MS analysis. Chocolate and black extracts displayed the lowest volume of FA and *p*-CA content than other phenolic acids identified. Szwajgier et al. (2010) conducted a study to investigate the release of PAs from the meal of BSG using a novel ferulic acid esterase, which derived from *Lactobacillus acidophilus* K1. The author identified and quantified a number of PAs such as FA, SA, *p*-CA, caffeic acid, syringic acid, 4-hydroxybenzoic acid, chlorogenic acid and protocatechuic acid (**Figure 2.8**) by HPLC-MS/MS analysis where FA was found as predominant compound (336.3 ± 16.0 mg/100 g of dry matter) compared to other PAs identified. Da Rosa Almeida et al. (2017) characterized BSG extracts by determining the TPC, TFC, and antioxidant activities of the extracts using four different assays. A number of phenolic acids and flavonoids, for example, syringic acid, gallic acid, kaempferol, and catechin, respectively, were identified by HPLC. Syringic acid showed the highest concentration (122.2 mg/kg dry weight) followed by catechin (84.4 mg/kg dry weight), gallic acid (32.2 mg/kg dry weight) and kaempferol (31.2 mg/kg dry weight). Also, several *in vitro* chemical assays, for instance, DPPH, ABTS, β-carotene/linoleic acid and metal chelation ability were used to evaluate the antioxidant activity of the extracts obtained from the BSG sample. McCarthy et al. (2012) conducted a study to examine the ability of BSG extracts obtained from four pale (P_1-P_4) and black (B1-B4) BSG meals against the genotoxic effects of oxidants, for example, 4-nitroquinoline 1-oxide (4-NQO), hydrogen peroxide (H_2O_2), 3-morpholinosydnonimine hydrochloride (SIN-1), and tert-butylhydroperoxide (t-BOOH) in

U937 cells. The authors found that extracts (B_1-B_4) from black BSG significantly exhibited protective activity against H_2O_2-induced DNA damage while extracts of P_2, B_2, B_3, and B_4 from BSG significantly displayed defence against SIN-1-induced DNA damage, SIN-1, a potent vasodilator, which releases nitric oxide and forms superoxide naturally under physiological conditions. However, none of the extracts showed a protective effect on DNA damage induced by t-BOOH and 4-NQO. The TPC, individual phenolics and antioxidant and biological activities of various spent grains varied significantly, which might be because of genotype, origin, and harvesting time of barley used in beer processing and production. Ferulic acid was a predominant phenolic compound found in BSG extracts.

2.8 Research Gap

Though some beer phenolic compounds and their antioxidant activities had been investigated, the profiles of hydroxycinnamic acids and their derivatives of commercial beers are poorly characterised. According to the best of our knowledge, no study has demonstrated contents or the in vitro antioxidant properties of decarboxylated derivatives of hydroxycinanamic acids in beer.

For brewers' spent grains (BSG), few reports have addressed the phenolic compounds and their contribution to antioxidant activities. However, no study describes the impact of heat treatments (toasting) on the release of bound phenolics and antioxidant activities of BSG meal.

Therefore, after reviewing most recent studies on the phenolic compounds and antioxidant activities of beer, and BSG extracts, following research gaps are concluded:

2.8.1 Limited data are available on the profiling of major hydroxycinnamic acids and derivatives and their identification as well as antioxidant activities of laboratory-made, selected Canadian and commercial beer extracts

2.8.2 Very limited studies have been done on the impact of heat treatment (toasting) on the release of bound phenolics of BSG and their role in antioxidant activities

2.9 Hypothesis

2.9.1 Laboratory-made, selected Canadian and foreign commercial beers will be good sources of hydroxycinnamic acids and their derivatives, which will contribute to the antioxidant capacity of the drinks.

2.9.2 Oven heat treatment (toasting) will have an impact on the release of bound phenolics as well as on the antioxidant activities of BSG extracts.

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CHAPTER 3

3. Manuscript 1: Identification of Hydroxycinnamic Acids Derivatives of Selected Canadian and Foreign Commercial Beer Extracts and Determination of their Antioxidant Properties

3.1 Abstract

Phenolic compounds are derived in beer from grains and hops during processing and production. The phenolics from beer samples were extracted by liquid-liquid technique using ethyl acetate (1:1). Thus, the total phenolic content (TPC) of beer extracts, from selected Canadian and foreign commercial, and a laboratory-made beer were examined and ranged from 3.72 ± 0.23 to 13.73 ± 0.49 mg GAE/100 mL beer. The total flavonoid content (TFC) of the same beer extracts varied from 0.82 ± 0.02 to 5.28 ± 0.04 mg CE/100 mL beer. Of particular interest were the hydroxycinnamic acids (HCAs) and derivatives in beer extracts because of their antioxidant properties. Main HCAs and a key derivative of ferulic acid, 4-vinylguaiacol (4-VG), were identified in beer extracts by high performance liquid chromatography with diode array detector (HPLC-DAD). Significant variations in TPC, TFC, HCAs and 4-VG content were observed among the beer extracts. Antioxidant activities of beer extracts, determined by radical scavenging and reducing power assays, were positively correlated ($p < 0.05$) with TPC, TFC**,** and 4-VG but were negatively correlated with individual HCAs ($p > 0.05$). The positive correlation of 4-VG with ABTS radical scavenging and reducing power assays is reported first time. These findings suggest that TPC and a key derivative of the ferulic acid, 4-VG, may have the protective role in the beer as an antioxidant.

Keywords: Beer, hydroxycinnamic acids, derivative phenols, antioxidant potential and flavor stability

3.2 Introduction

Beer is the most commonly consumed alcoholic beverage worldwide (Stewart, 2013). Canadians drink more beer (51%) than wine (28%) and spirits (27%) (WHO, 2016). The flavor stability and shelf life of beer still remain a big challenge to brewers (Leitao et al., 2011; Zhao et al., 2010). Different beers have distinct flavors that popularizes a specific brand while their shelf life is important for preserving flavor quality and nutritional value. Oxygen content and oxidation are the main causes of flavor and quality deterioration of commercial beers (Zhao et al., 2010). A number of natural antioxidants, for example, phenolics, sulfite, and Maillard reaction products, are present in beer (Zhao et al., 2010; Guido et al., 2005). Of these, phenolics are of specific interest to brewers for the reason that they can delay, retard or could prevent oxidation during brewing process (Guido et al., 2005).

Phenolics are secondary metabolites, which are extensively distributed in plant foods. They display a wide range of antioxidant and biological activities in food and biological systems, which is mostly dependent on their structures and the number of functional groups (Rahman et al., 2017, 2018 a, b). The major phenolics found in beer are phenolic acids, flavonoids and stilbenes (Wannenmacher et al., 2018; Callemien & Collin, 2009). Many of these phenolics act as flavor precursors and antioxidants. For example, HCAs and their decarboxylated derivatives can impart a very strong flavor to beer (Callemien & Collin, 2009; Vanbeneden et al., 2006; Vanbeneden et al., 2008). These derivatives are produced in beer during wort

boiling and fermentation by decarboxylation (Vanbeneden et al., 2006; Vanbeneden et al., 2008).

Many studies have been conducted on beer phenolics and their antioxidant activities (Leitao et al., 2011; Zhao et al., 2010; Piazzon et al., 2010; Li et al., 2007; Lugasi, 2003). These studies are mainly focused on the relationship between total phenolic content and antioxidant activities of beer extracts with identification of individual phenolic compounds. Very limited data are available, however, on the profiles of hydroxycinnamic acids (HCAs) and their derivatives in commercial beers and their identification (Vanbeneden et al., 2006; Vanbeneden et al., 2008; Lyuke et al., 2008). In fact, the profiles of phenolics, individual HCAs, and their derivative phenols in local Canadian beer, have not been extensively studied. These compounds could be important to enhance the shelf life of beer, because of their antioxidant properties (Richard- Forget et al., 1995), which still remains to be examined. Therefore, the aim of this study was to investigate the major HCAs and their derivatives, in a laboratory produced beer and selected Canadian and foreign beers consumed in Canada using HPLC-DAD. In addition, the antioxidant activities of all beer extracts were studied using *in vitro* screening assays. A Pearson correlation statistical analysis was conducted between antioxidant screening assays and TPC, TFC, HCAs and 4-VG to establish the antioxidant properties of beer extracts and individual HCAs and their derivatives.

3.3 Materials and Method

3.3.1 Samples

Thirteen beer samples (Table 1), one laboratory, six local Canadian and six foreign commercial beers consumed in Canada were used in this study. The selection of beers used

in this study was based on what was available in the market place. Those chosen represented well-recognized and established brands regularly sold commercially. With the exception of the one Home Brew, all of the commercial beers (foreign and local) selected have been consumed regularly and form part of the standard beer products purchased by consumers for quite a long time. Local Canadian beers were kindly supplied by the Canadian Malting Barley Technical Centre (CMBTC) (Winnipeg, MB, Canada); Trans Canada Brewing Co (Winnipeg, MB, Canada), Half Pints Brewing Co. (Winnipeg, MB, Canada). Foreign commercial beer samples were purchased from the local Liquor Mart (Winnipeg, MB, Canada) and laboratory beer were prepared in the lab using a standard method.

Table 3. 1. Characteristics of collected beers

No.	Beer Brand	Beer Type	Alcohol content (%, v/v)	Ingredients	Country of origin
1.	*Stir Stick Stout	Dark	5.6	Barley Malt and hop	Canada
2.	*Lamp Lighter	Amber ale	5.2	Barley Malt and hop	Canada
3.	*Arrow Strong	IPA	6.8	Barley Malt and hop	Canada
4.	*Laboratory Beer	Dark	-	Barley Malt	Canada
5.	*CMBTC beer-I	Blonde Ale	5.53	Barley Malt and hop	Canada
6.	*CMBTC beer-II	Blonde Ale	5.53	Barley Malt and hop	Canada
7.	*CMBTC beer-III	Blonde Ale	5.56	Barley Malt and hop	Canada
8.	Erdinger Weissbier	lager	5.3	Barley Malt, Wheat malt and hops	Germany
9.	Holsten Weizen	lager	5.4	Barley Malt and hop	Romania
10.	Guinness Draught	Dark	4.2	Barley Malt and hop	Ireland
11.	Coors Banquet	lager	5.0	Barley Malt and hop	USA
12.	Miller Genuine	lager	4.7	Barley Malt and hop	USA
13.	Faxe Royal Strong	Dark	8.0	Barley Malt and hop	Denmark

*Indicate fresh beer tested; #CMBTC=Canadian Malting Barley Technical Centre

3.3.2 Chemicals

Various organic solvents such as ethyl acetate, methanol, and reagents, for example, anhydrous sodium sulfate, sodium hydroxide, potassium ferricyanide, sodium nitrite, mono and dibasic sodium and potassium phosphates, sodium chloride, hydrochloric acid, ferrous chloride, ferric chloride, Folin-Ciocalteu reagent, and sodium carbonate were purchased from Sigma-Aldrich (Oakville, ON, Canada) and Fisher Scientific (Ottawa, ON, Canada). Standard sinapic acid, ferulic acid, Gallic acid, *p*-coumaric acid, 4-vinyl guaiacol, catechin, 2, 2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), and Trolox, were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

3.3.3 Extraction of Beer Phenolics

Beer phenolics were extracted using liquid-liquid technique as stated by the method of Leitao et al. (2011) and Zhao et al. (2010) with some modifications. Beer samples were degassed by sonication for 10 min using an ultrasonic bath (VWR International, West Chester, PA 19380l). The degassed beer solution (50 mL) were acidified to pH 2 with 6 M HCl. Ethyl acetate (1:1 v/v) was used as solvent for the extraction of beer phenolics (four times) from the acidified slurries. The combined organic solvents were evaporated at 35^{o}C with a Buchi Rotavapor R-205 (Buchi Labortechnik AG, CH-9230 Flawil, and Switzerland). After evaporation, the remaining residue was dissolved in 5 mL methanol and stored at -20°C for further analysis.

3.3.4 Determination of Total Phenolics Content (TPC)

The TPC of beer extracts was determined by the Folin Ciacalteu assay described by Singleton & Rossi, (1965) and Chandrasekara & Shahidi, (2010). Briefly, each beer extract (0.5 mL) and Folin-Ciocalteu phenol reagent (0.5 mL) was taken in the test tubes and mixed properly. After 3 min, 1 mL of $NaCO_3$ (7.5 %) and 8 mL of d.H_2O were added to each tube and incubated at room temperature for 35 min in the dark. After that, the tubes were centrifuged at 3000 g for 10 min and the absorbance read at 725 nm using a spectrophotometer (DU 800 Series, Beckman Coulter, Inc., Fullerton, California, USA). TPC was calculated as gallic acid equivalent (GAE) milligram per 100 mL beer.

3.3.5 Determination of Total Flavonoid Content (TFC)

The TFC of beer extracts was measured by $AlCl_3$ method as stated by Chandrasekara & Shahidi (2010). Each beer extract (0.25 mL), 1 mL of d.H_2O and 75 µL of $NaNO_2$ solution (5%) were mixed in a test tube. After 5 min, 10% $AlCl_3$ solution (75 µL) and then, after 1 min, 0.5 mL of NaOH (1 M), 0.6 mL of d.H_2O were added. After mixing instantly, tubes were incubated for 15 min at room temperature in the dark. Then, the absorbance of each tube was read at 510 nm by the same spectrophotometer against a blank without beer extract. The TFC was expressed as mg catechin equivalents (mg CE) per 100 mL of beer.

3.3.6 HPLC-DAD Analysis of Beer Extracts

Identification and quantification of HCAs and their derivatives were measured by an HPLC method described by Harbaum-Piayda et al. (2010) with major modifications. Analysis was carried out using HPLC-DAD instrumentation (Ultimate 3000; Dionex, Sunnyvale, CA, USA) equipped with on-line degasser, autosampler, binary pump, column heater and diode array

detector (DAD). Separation of individual phenolics was accomplished on a Kinetex 2.6 µ m XB-C18 LC Column (2.6 mm, 150 x 4.6 mm) (Phenomenex Inc., Torrance, CA, USA) maintained at flow rate of 0.6 mL/min and injection volume of 10 µL at 25 ^{o}C . The gradient elution was accomplished by 0.1 % (v/v) formic acid in HPLC grade water as solvent A and 0.1 % (v/v) formic acid in HPLC grade methanol as solvent B. The gradient elution system was operated as follows 0-4 min, 35 - 42 % B; 4-15 min, 46 - 60 % B; 16-18 min, 100-100 % B and 19-22 min, 35 -35 %. The chromatograms were acquired at 280 and 320 nm and data were analyzed with the Chromeleon software (Version 7.2 SR4). Identification of the HCAs and their derivatives was carried out with authentic standards by comparison of their retention times and UV spectra. Quantification of each phenolic was carried out by plotting HPLC peak areas against concentrations with standard curves of authentic standards. For the external standard calibration curves *trans*-ferulic acid, *p*-coumaric acid, sinapic acid, and 4-vinylguaiacol, were used.

3.3.7 DPPH Radical Scavenging Assay

DPPH radical scavenging capacities (DRSC) of the beer extracts were measured as described by Thiyam et al. (2006). To each beer extract (50 µL), 2.95 mL of a DPPH solution (0.1 mM) was placed in a spectrophotometric cuvette (n = 3). A control was also prepared. The cuvettes were covered and incubated for 10 min at room temperature in the dark. Absorbance was then read at 516 nm using the same spectrophotometer. The DRSC for each beer extract was calculated as micromoles Trolox equivalent (µmole TE) per 100 mL of beer.

3.3.8 ABTS Radical Cation Scavenging Assay

The ABTS radical cation scavenging capacities (ARSC) of beer extracts were measured as stated by the procedure of Van den Berg et al. (1999) and Chandrasekara & Shahidi (2010). The ABTS solution was made by mixing of 2.5 mM AAPH and 2.0 mM ABTS (1:1, v/v) in saline phosphate buffer solution (0.1 M, pH 7.4, 0.15 M NaCl). The mixed solution was heated up for 20 min at 60^{o}C using a water bath in the dark. After cooling at room temperature, the solution was filtered with P8 filter paper. A 40 µL aliquot of each beer extract and 1960 µL of ABTS solution was mixed in the test tubes. After 6 min, the absorbance was read at 734 nm using the same spectrophotometer. ARSC values were calculated as micromoles of Trolox equivalents per 100 mL beer.

3.2.8 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP of beer extract was determined following the method of Oyaizu (1986) and Chandrasekara & Shahidi (2010). Beer extract (0.5 mL), 2.5 mL of a phosphate buffer solution (0.2M, pH 6.6) and potassium ferricyanide (1%, w/v) were added in a centrifuge tube and mixed properly. After incubating for 20 min at 50^{o}C, 2.5 mL of 10% TCA was added to each tube and centrifuged at 1750 g for 10 min. After, 2.5 mL of the supernatant, 2.5 mL of d.H_2O, and 0.5 mL of 0.1% (w/v) Iron (III) chloride were added in a test tube. Then, the absorbance of the well mixed solution was read at 700 nm using the spectrophotometer. The results were calculated as micromole of Trolox equivalents (TE) per 100 mL of beer.

3.3 Statistical Analysis

All experiments used in the study were conducted in triplicate. Data were reported as means ± standard deviation (SD). One way analysis of variance (ANOVA) and Tukey HSD test

identified differences among means using IBM SPSS Statistics version 22 (Armonk, New York, USA). Correlation coefficients between antioxidant screening assays and TPC, TFC, HCAs and 4-VG were then established by the Pearson product moment correlation at $p > 0.05$ and $p > 0.01$.

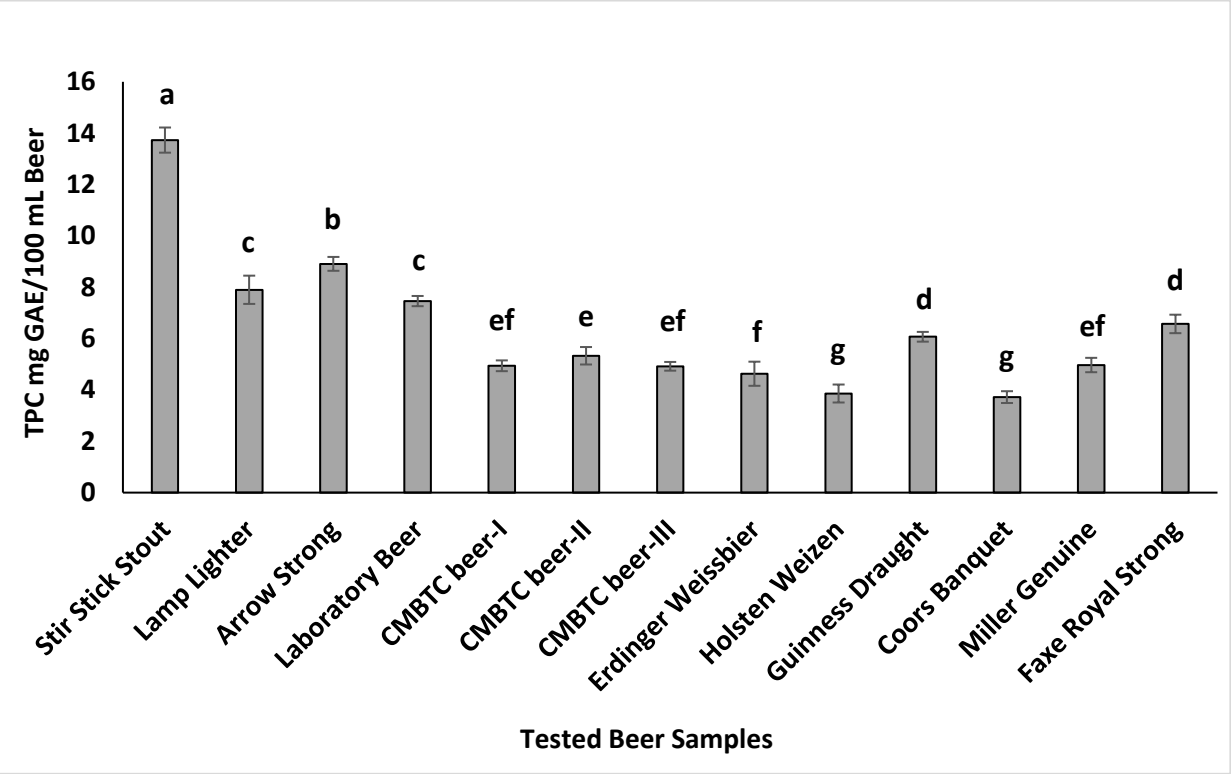

Figure 3.1. Total phenolic content (TPC) of a laboratory-made, local Canadian and foreign commercial beer extracts. Data represents as mean ± SD (N = 3). Mean for the samples followed by different letters are significantly different at $p < 0.05$. *SD= Standard Deviation; *CMBTC = Canadian Malting Barley Technical Centre; *GAE = Gallic Acid Equivalent.

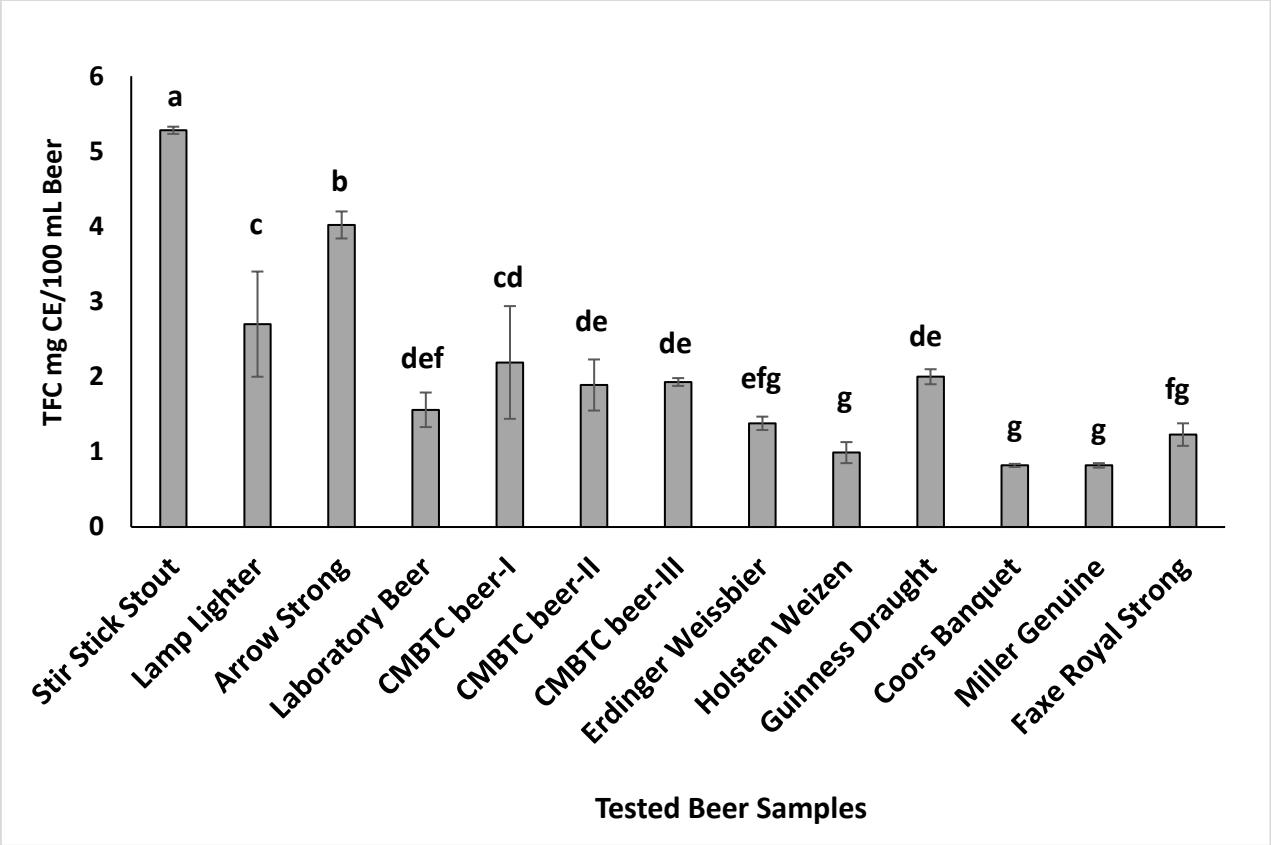

Figure 3.2. Total flavonoids content (TFC) of a laboratory-made, local Canadian and foreign commercial beer extracts. Data represents as mean ± SD (N = 3). Mean for the samples followed by the different letters are significantly different at $p < 0.05$.

*CE = Catechin Equivalent.

3.4. Results and Discussion

3.4.1 Total Phenolic Content (TPC) and Total Flavonoids (TFC) of Beer Extracts

The TPC in the laboratory, local Canadian and foreign commercial beer crude extracts varied significantly from 3.72 ± 0.23 to 13.73 ± 0.49 mg GAE/100 mL beer as shown in **Figure 3.1.** These values are lower than the results published by Oladokun et al. (2016) and Dvorakova et al. (2007) who reported that TPC ranged from 7.4- 25.6 and 7.0 - 24.0 mg GAE/100 mL

beer in their respective commercial beer extracts. The differences in TPC values between our findings and the literature could be attributed to beer storage conditions (e.g. fresh and /or stored), and polyvinylpyrrolidine (PVPP) treatments. During storage, polyphenols are gradually degraded by oxidative mechanisms as result of beer aging (Vanderhaegen et al., 2003) while PVPP treatment is occasionally used by brewers to remove phenolics involved in haze formation in the finished beer (Siebert & Lynn, 1998). The variability in TPC may be due to beer type, raw ingredients, brewing techniques, as well as the fermentation time employed by the industry (Moura-Nunes et al., 2016; Leitao et al., 2011). The Stir Stick Stout, a local beer, showed significantly higher TPC (13.73 ± 0.49 mg /100 mL beer) compared to the other extracts while the lowest amount of TPC was recorded for Coors Banquet and Holsten Weizen beer extracts, respectively (3.72 ± 0.23 and 3.86 ± 0.19 mg /100 mL beer, respectively). The laboratory beer extract, however, had significantly higher TPC than the CMBTC produced and the commercial beer extracts tested (**Figure 3.1**).

The TFC of thirteen beer extracts are presented in **Figure 3.2.** These beer extracts exhibited significant variation in their TFC values, ranging from 0.82 ± 0.02 - 5.28 ± 0.04 mg CE/100 mL beer. The Stir Stick Stout extract also exhibited the highest amount of TFC (5.28 ± 0.04 mg / 100 mL) while the lowest TFC was found in Coors Banquet (0.82 ± 0.02 mg/mL beer). The Coors Banquet extract, however, showed a statistically similar TFC value with Miller Genuine, Erdinger Weissbier, and Faxe Royal Strong extracts, respectively. The TFC of the crude beer extracts in this study were slightly lower than that reported by Obruča et al. (2009) in their beer extracts (5.34-7.68 mg QE/100 mL beer). Such differences could be due to the beer

type, brewing technique, raw ingredient, fermentation as well as storage conditions discussed previously for TPC (Moura-Nunes et al., 2016; Leitao et al., 2011).

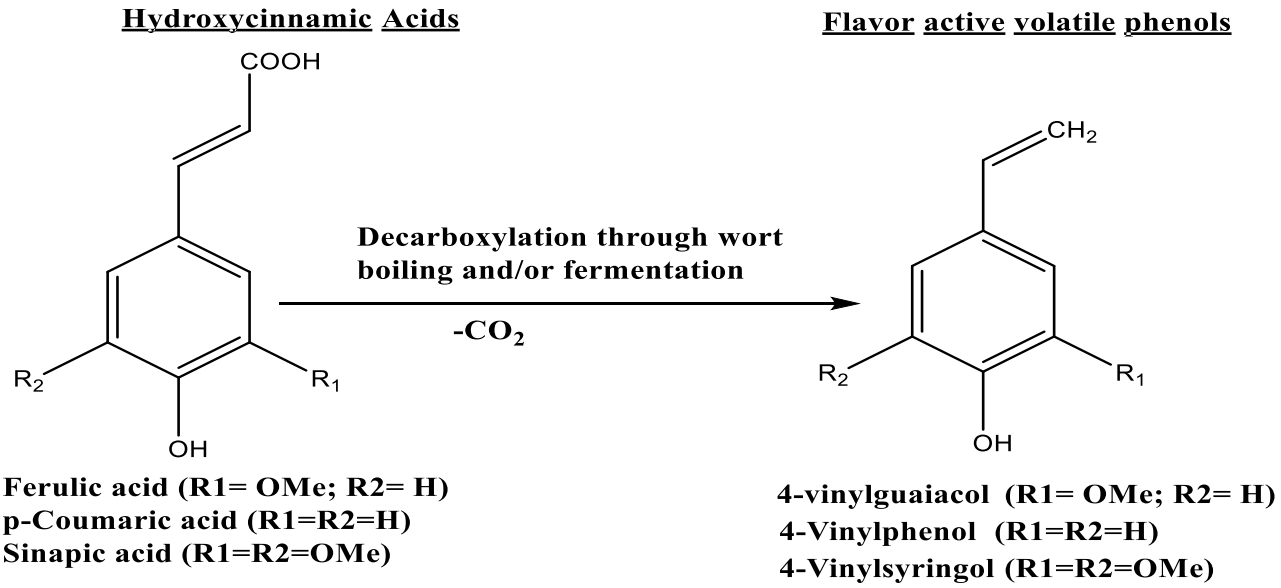

Figure 3.3. Formation of flavor active volatile phenols from hydroxycinnamic acids. Source: Vanbeneden et al. (2006).

Table 3.2. Retention time and UV spectra of Identified hydroxycinnamic acids and 4-vinylguaiacol of various beers extracts by HPLC-DAD.

Phenolic compounds	Retention time, t_R (min)	Absorption UV spectra (nm)
p- Coumaric acid	8.867	209.60, 220.98, 222.82, 309.31
Sinapic acid	9.110	235.96, 236.08, 322.86, 323.26
Ferulic acid	9.287	196.40, 216.90, 218.04 233.86, 322.97
4- Vinylguaiacol	17.49	201.05, 202.68, 211.69, 213.42, 214.15

3.4.2 Identification and Quantification of Hydroxycinnamic Acids and their Derivative Phenols by HPLC-DAD

Table 3.3. Major hydroxycinnamic acids and 4-vinylguaiacol content (µg/100 mL beer) of laboratory, local Canadian and foreign commercial beer extracts.

Beer Samples	Hydroxycinnamic acids and their derivatives			
	Ferulic acid	*p*- Coumaric acid	Sinapic acid	4-Vinylguaiacol
Stir Stick Stout	83.90 ± 0.35^{h}	79.79 ± 0.61^{def}	58.07 ± 0.27^{f}	289.39 ± 73.08^{a}
Lamp Lighter	123.52 ± 0.98^{g}	124.58 ± 43.5^{cde}	74.68 ± 0.42^{ef}	288.42 ± 17.64^{a}
Arrow Strong	132.38 ± 0.26^{e}	187.93 ± 0.76^{a}	81.52 ± 0.01^{e}	274.93 ± 30.86^{a}
Laboratory Beer	177.26 ± 0.25^{d}	172.14 ± 0.37^{ab}	88.96 ± 0.68^{cde}	128.29 ± 21.35^{b}
CMBTC beer-I	157.61 ± 1.79^{f}	170.02 ± 2.45^{ab}	106.78 ± 1.40^{bc}	117.73 ± 1.75^{b}
CMBTC beer-II	160.93 ± 0.43^{e}	170.20 ± 1.22^{ab}	108.94 ± 0.28^{bc}	105.78 ± 1.31^{b}
CMBTC beer-III	177.35 ± 0.39^{d}	201.06 ± 1.98^{a}	107.54 ± 1.26^{bc}	98.67 ± 2.99^{b}
Erdinger Weissbier	296.02 ± 4.88^{a}	132.95 ± 0.51^{cd}	86.38 ± 0.03^{cde}	145.52 ± 2.78^{b}
Holsten Weizen	179.55 ± 0.83^{d}	115.71 ± 0.30^{cdef}	244.55± 20.64^{cde}	53.74 ± 0.85^{b}
Guinness Draught	120.03 ± 0.06^{g}	166.2 ± 18.57^{abc}	111.93 ± 0.58^{b}	109.02 ± 0.64^{b}
Coors Banquet	182.06 ± 1.17^{c}	80.95 ± 0.18^{f}	84.00 ± 0.35^{bcde}	92.64 ± 0.21^{b}
Miller Genuine	210.51 ± 3.5^{d}	63.80 ± 1.12^{ef}	93.72 ± 0.87^{de}	133.76 ± 27.28^{b}
Faxe Royal Strong	223.06 ± 0.42^{b}	159.91 ± 2.93^{abc}	105.06 ± 0.14^{bc}	90.36 ± 1.62^{b}

Data represents as mean ± SD (N = 3). Mean for the samples followed by the different letters are significantly different at $p < 0.05$. *SD= Standard Deviation; *CMBTC= Canadian Malting Barley Technical Centre.

Hydroxycinnamic acids (HCAs), the major phenolics in wort and beer (Lentz, 2018), are derived from the main ingredients of beer making such as malt, wheat, rice, maize, etc. They can be converted into vinyl derivatives by decarboxylation during wort boiling and/or

fermentation (**Figure 3.3**). These derivatives have very low organoleptic thresholds (McMurrough et al., 1996), but may contribute significantly to the aroma and flavor of the finished beer (Lentz, 2018). In this study, three major HCAs, *p*-coumaric acid (*p*-CA, t_R = 8.86 min), sinapic acid (SA, t_R = 9.11 min), ferulic acid (FA, t_R = 9.28 min), and a key derivative of FA, 4-vinylguiacol (4-VG, t_R = 17.49 min), were identified using HPLC-DAD by comparing their retention times and UV absorption spectra with authentic standards (Table. 3.2). However, an unknown compound (t_R = 3.09 min) was found to be a major compound in the tested beer samples but has not been discussed further as it does not belong to the hydroxycinnamic acids group.

4-Vinylguaiacol (4-VG), a flavor active phenol, is the decarboxylated derivative of ferulic acid, which enhances the desirable clove smoky flavour in the finished beer (Callemien & Collin, 2009). It is generally produced in beer *via* decarboxylation (Vanbeneden et al., 2006; Vanbeneden et al., 2008) (**Figure 3.3**). The 4-VG content of beer extracts shown in **Table 3.3** ranges from 53.74 ± 0.85 to 289.39 ± 73.08 µg /100 mL sample. Local Canadian beers such as Stir Stick Stout, Lamp Lighter, and Arrow Strong extracts exhibited significantly ($p < 0.05$) higher 4-VG content (274.93 ± 30.86, 288.43 ± 17.64 and 289.39 ± 73.08 µg /100 mL sample, respectively) than other extracts. However, no significant differences were found in 4-VG content between laboratory, CMBTC-I, II, III and foreign commercial beer extracts. Vanbeneden and co-workers, (2006) reported that the 4-VG contents in a pilsner, wheat, and dark specialty beer were 13.90 ± 0.20, 111.19 ± 1.67 and 58.70 ± 0.40 08 µg /100 mL sample, respectively. Zhu and Cui, (2013) found the contents of 4-VG and 4-vinyl phenol (4-VP) in the

top-fermented wheat beers were 261.33 and 100.95 µg/100 mL, respectively while 4-VP was not found in any of the 13 beer extracts included in this study.

Ferulic acid (FA), a major HCAs in cereal grains and beer wort (Lentz, 2018), is the precursor of 4-VG in beer (Callemien & Collin, 2009). The FA content of various beer extracts is shown in **Table 3.3**. There was a considerable variation of FA content between the laboratory-made, local Canadian and foreign commercial beer extracts, which ranged from 83.9 ± 0.35-296.02 ± 4.88 µg/100 mL sample. Erdinger Weissbier extract showed significantly higher FA content (296.02 ± 4.88 µg/100 mL beer sample) compared to other beer extracts. These results are consistent with a study by Zhao et al. (2010) who reported FA was the predominant phenolic acid in 34 commercial beers examined ranging from 51.0 -313.0 µg / 100 mL sample. Piazzon et al. (2010) also found FA was the major phenolic acid in seven different beers examined with concentrations ranging from 34.0 ± 23.0 to 200.0 ± 47.0 µg / 100 mL beer sample. Stir Stick Stout extract had the lowest amount of FA (83.9 ± 0.35 µg / 100 mL sample) but the highest amount of 4-VG, which could be attributed to its conversion during malting, kilning, mashing and fermentation (**Figure 3.3**).

p-Coumaric acid (*p*-CA)**,** the precursor of 4-VP, is present in various beer extracts as shown in **Table 3.3**. Arrow Strong and CMBTC beer-III extracts had the highest *p*-CA content compared to other beer extracts tested (**Table 3.3**). The *p*-CA content in Laboratory beer, CMBTC-I & II, Erdinger Weissbier and Coors Banquet were not significantly different. The total *p*-CA content ranged from 63.80 ± 1.12 - 201.06 ± 1.98 µg/100 mL. These results are consistent with Vanbeneden et al. (2006) who reported *p*-CA content in Wort, Pilsner, Wheat and Dark beer, 147.94 ± 0.64, 141.95 ± 2.08, and 23.94 ± 0.34 µg / 100 mL, respectively but are higher

than the values reported by Zhao et al. (2010) who found the *p*-CA content among commercial beers ranged from 1.0-112 µg / 100 mL.

Sinapic acid (SA), the precursor of 4-vinylsyringol (4-VS), is a potent antioxidant. Its content in the various beer extracts is shown in **Table 3.3**. SA content in various beer extract varied from 58.07 ± 0.27 to 244.05 ± 20.64 µg/100 mL. Holsten Weizen beer extract had the significantly highest amount (244.05 ± 20.64 µg/100 mL) of SA with the lowest level observed in the Stir Stick Stout extracts (58.07 ± 0.27 µg/100 mL). These values are higher than those of Socha et al., 2017 who reported a significant variation in SA content ranging from 21.0 to 99.0 µg/100 mL in their beer samples. Vanbeneden et al. (2006) reported SA content in Wort, Pilsner, Wheat and Dark beer, 12.81 ± 0.31, 24.46 ± 0.31, 50.35 ± 0.46, and 31.35 ± 0.41 µg/100 mL, respectively. The variation of HCAs such as FA, SA, *p*-CA and 4-VG content in beer extracts could be influenced by the same factors affecting both TPC and TFC discussed previously.

3.4.3 Antioxidant activities of beer extracts

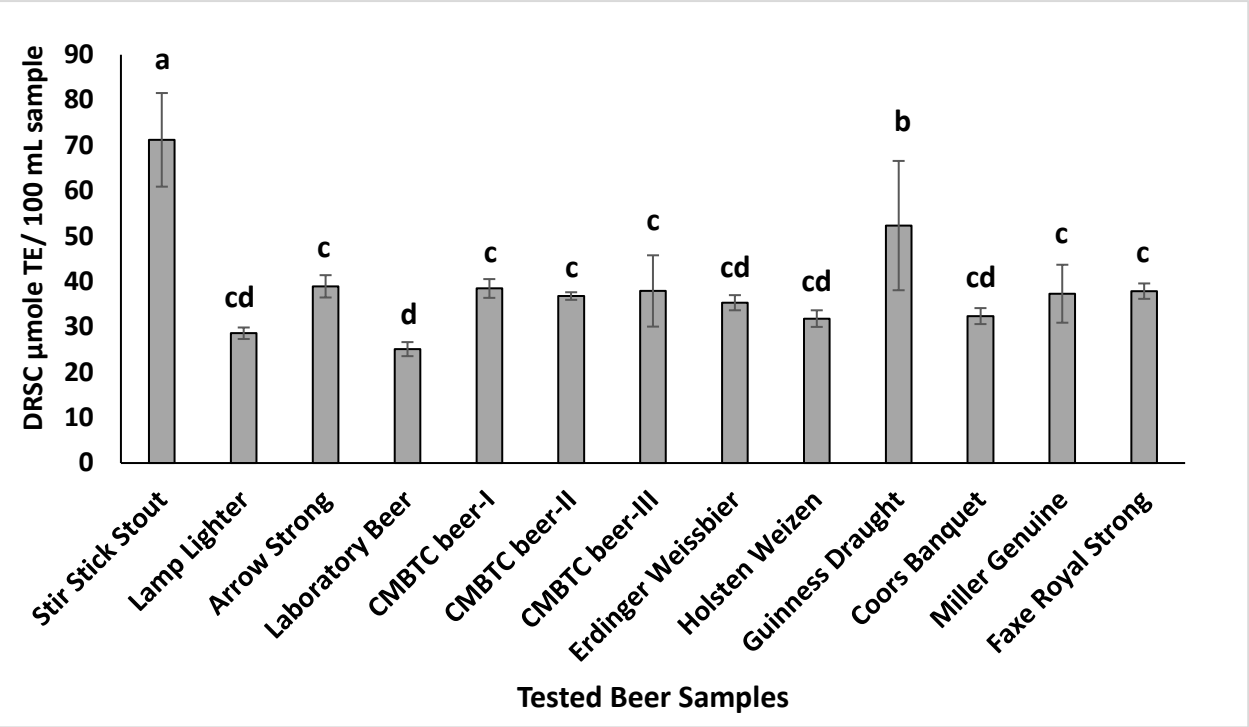

Figure 3.4 DPPH radical scavenging capacity of laboratory, local Canadian and foreign commercial beer extracts. Data represents as mean ± SD (N = 3). Mean followed by different letters are significantly different at $p < 0.05$. TE* = Trolox Equivalent.

Phenolic compounds can act as a potent antioxidant by scavenging free radicals in both food and biological systems. The DPPH radical scavenging capacity (DSSC) of crude beer extracts is shown in **Figure 3.4** with values ranging significantly ($p < 0.05$) from 25.11 ± 1.55 to 71.24 ± 10.32 µmole TE/100 mL beer. The Star Stick Stout extract exhibited significantly ($p < 0.05$) higher DPPH radical inhibition (71.24 ± 10.32 µmole/100 mL) compared to the other beer extracts tested while the laboratory beer extracts had the lowest DRSC (25.11 ± 1.55 /100 mL). With the exception of Star Stick Stout, Arrow Strong, and Laboratory extracts, the

remaining crude beer extracts had statistically similar DRSC values. The higher DRSC reported in the Stir Stick Stout extract could be due to its higher TPC and TFC. The reported DRSC values agreed with earlier data reported by Zhao et al. (2010), Leitao et al. (2011), and Socha et al. (2017), respectively. The DRSC values reported by Zhao et al. (2010) ranged from 24 to 135 µmole TE/100 mL and by Socha et al. (2017) varied from 23.0 ± 3.0 to 67.0 ± 1.55 TE/100 mL in various dark beer extracts.

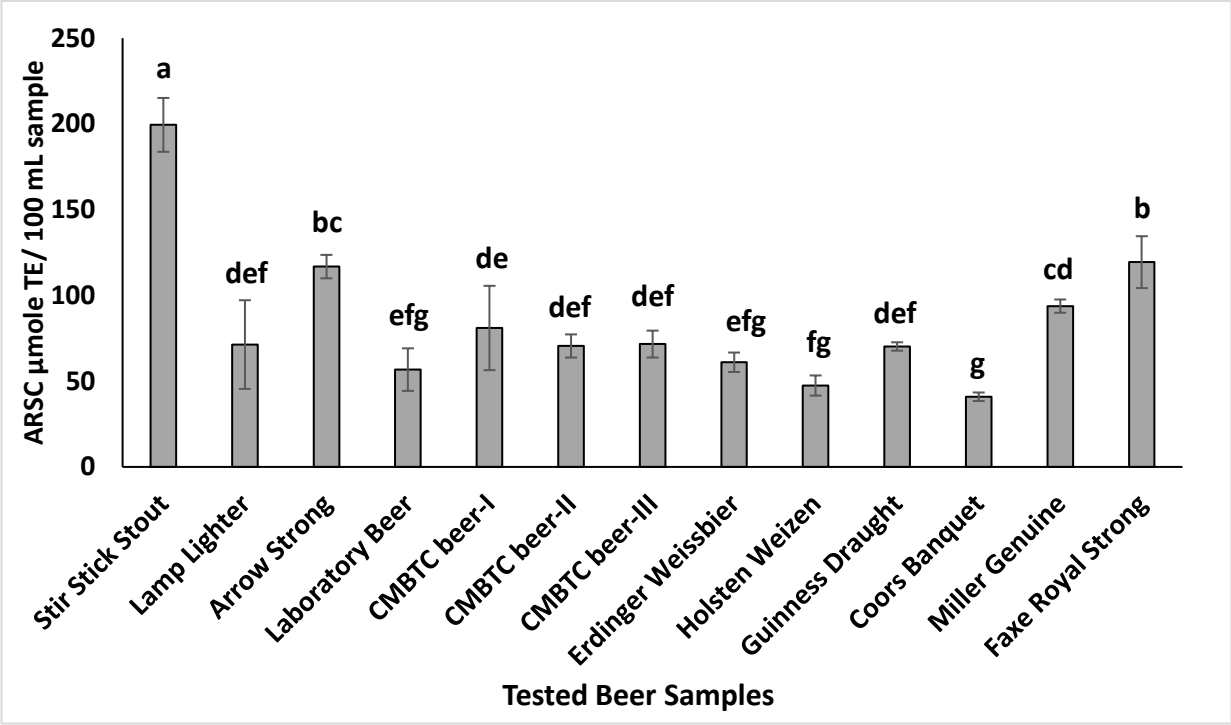

Figure 3.5. ABTS radical cation scavenging capacity (ARSC) of laboratory-made, local Canadian and foreign commercial beer extracts. Data represents as mean ± SD (N = 3). Mean for the samples followed different letters are significantly different at $p < 0.05$. *TE = Trolox Equivalent.

The ARSC assay is used to measure free radical scavenging potential of plant and biological extracts. **Figure 3.5** shows the ARSC values of various crude beer extracts, which ranged from 40.92 ± 2.49 to 199.42 ± 15.70 µmole TE/100 mL beer. These results are consistent with data by Zhao et al. (2010) who reported that the ARSC of 34 commercial beer extracts varied from 55.0 ± 4.0 to 195 ± 5.0 µmole TE/100 mL beer. However, ARSC values are higher than those of Socha et al., 2017 who reported a significant variation in ARSC ranging from 25.0 ± 3.0 to 72.0 ± 0.00 TE/100 mL in various dark beer extracts. The Stir Stick Stout extract had highest ARSC (199.42 ± 15.70 µmole TE/100 mL beer) while the Coors Banquet extract had lowest ARSC value (40.92 ± 2.49 µmole TE/ 100 mL sample). Statistically similar ARSC values were observed between Lamp Lighter, CMBTC beer-II, CMBTC-III, Erdinger Weissbier, Guinness Draught, and Laboratory beer extracts.

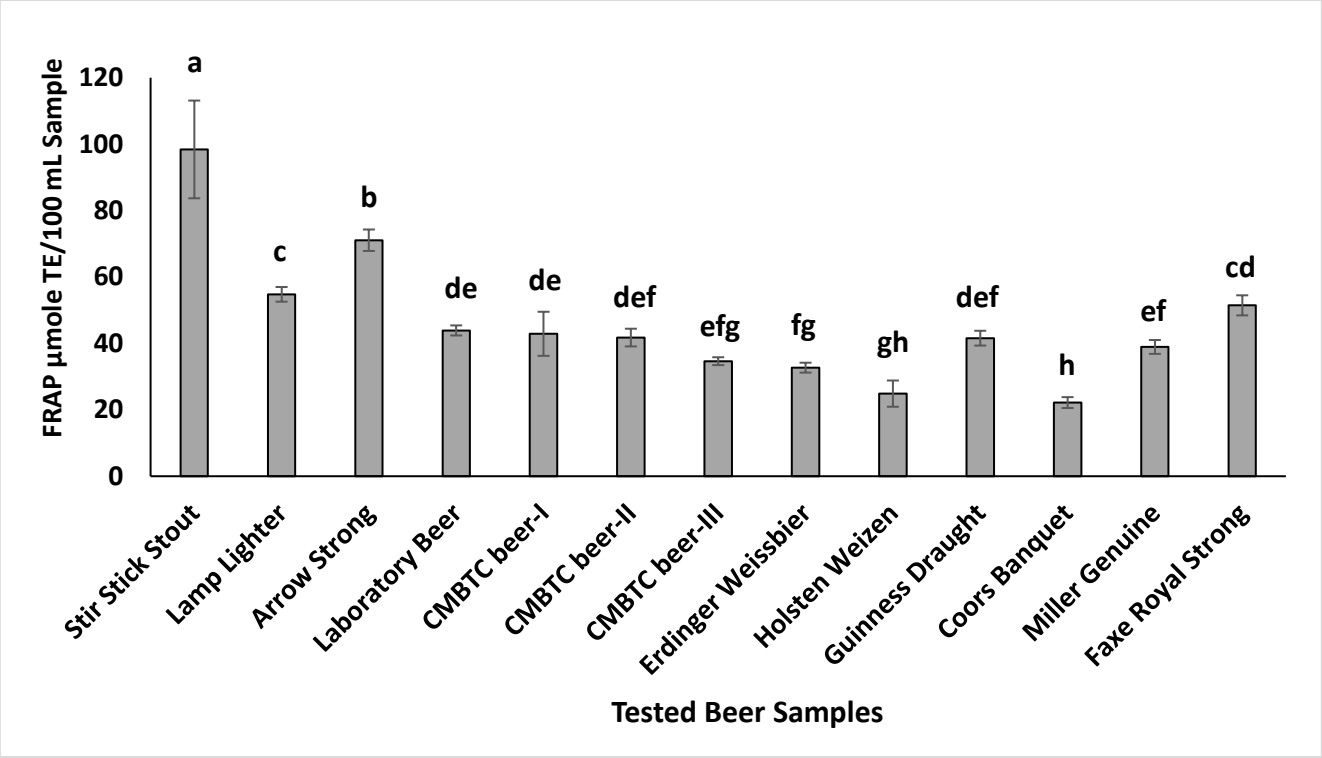

Figure 3.6. Ferric reducing antioxidant power (FRAP) capacity of laboratory-made, local Canadian and foreign commercial beer extracts. Data represents as mean ± SD (N = 3). Mean for the samples followed different letters are significantly different at $p < 0.05$. *CMBTC = Canadian Malting Barley Technical Centre; *TE = Trolox Equivalent.

As shown in **Figure 3. 6,** FRAP values also varied significantly among various beer crude extracts with values ranging from 22.17 ± 1.47 to 98.41 ± 14.71 µmole TE/100 mL beer. The Stir Stick Stout beer extract exhibited significantly ($p < 0.05$) higher FRAP value compared to other beer extracts tested. A comparison of the results obtained for both DPPH and ABTS assays showed similar results for the Stir Stick Stout beer extract. The lowest FRAP value were observed in the Coors Banquet extract while no significant differences were found for

FRAP between CMBTC beer-I, CMBTC beer-II, CMBTC-III, Guinness Draught, Miller Genuene, and Laboratory beer extracts. The observed FRAP values are lower than data reported by Piazzon et al. (2010) for seven commercial beer samples, which ranged from 101-284 µmole TE/100 mL, consistent with the variation in TPC. In this study, the increase in DRSC, ARSC and FRAP values also followed the increase in TPC. Many of the factors discussed earlier influence the latter (Moura-Nunes et al., 2016; Leitao et al., 2011).

Table 3.4. Correlations among the total phenolic and flavonoid content, HCAs, 4-vinylguaiacol and antioxidant activities evaluation assays

	TPC	TFC	FA	*p*-CA	SA	4-VG	DRSC	ARSC	FRAP
TPC	1	.901**	-.492	-.305	-.444	.836**	.661*	.859**	.971**
TFC	-	1	-.551	-.021	-.354	.849**	.686**	.787**	.925**
FA	-	-	1	.069	.298	-.533	-.104	-.432	-.512
p-CA	-	-	-	1	.339	-.105	-.357	-.497	-.310
SA	-	-	-	-	1	-.261	-.194	-.384	-.434
4-VG	-	-	-	-	-	1	.368	.639*	.844**
DPPH	-	-	-	-	-	-	1	.798**	.690**
ABTS	-	-	-	-	-	-	-	1	.920**
FRAP	-	-	-	-	-	-	-	-	1

TPC, total phenolic content, TFC, total flavonoid content, FA, ferulic acid, FA, flavonoid content, *p*- CA, *p*-coumaric acid, SA, sinapic acid, 4-VG, 4-vinyl guaiacol, DRSC, DPPH radical scavenging capacities, ARSC, $ABTS^{\bullet+}$ radical scavenging capacities, FRAP, ferric reducing antioxidant power

**. Correlation is significant at the 0.01 level.

*. Correlation is significant at the 0.05 level.

3.4.4 Correlation between TPC, TFC, individual HCAs content, 4-VG content and beer antioxidant activity examined by Pearson correlation analysis

A Pearson correlation analysis was carried out to further examine the antioxidant properties of beer phenolics, particularly the relationship between TPC, TFC, the major HCAs, and 4-VG content with antioxidant activity (**Table 3.4**). Both TPC and TFC showed a strong correlation with DPPH radical scavenging capacity (r = 0.661, $p < 0.05$ and r = 0.686, $p < 0.01$, respectively), ABTS radical cation scavenging capacity (r = 0.859, $p < 0.01$ and r = 0.787, $p < 0.01$, respectively) and ferric reducing antioxidant power (r = 0.971, $p < 0.01$ and r = 0.925, $p < 0.01$, respectively). These results strongly suggest that phenolics make important contributions to the antioxidant properties of beer extracts. This is in agreement with data presented by Zhao et al. (2010) who also found a strong correlation between TPC and DRSC, ARSC, FRAP assays. The single HCAs, however, did not show any correlation with antioxidant evaluation assays (**Table 3.4**). This might be because of their lower amount observed (**Table 4.2**) that may be caused by transformation to its vinyl derivatives during malting, wort boiling and fermentation via decarboxylation (Vanbeneden et al., 2006; Vanbeneden et al., 2008). 4-VG, a derivative of FA showed a positive correlation with ARSC and FRAP (r = 0.639, $p < 0.05$ and r = 0.844, $p < 0.01$, respectively), respectively. However, it showed a moderate correlation (r = 0.368, $p > 0.05$) with DRSC assay but was insignificant. Thus, 4VG may exert a greater contribution to the antioxidant activities of beer. For all these assays, DRSC, ARSC, and FRAP, significantly ($p < 0.01$) and positively correlated with each other (r = 0.690-0.920). These correlations indicate that not only the TPC, but also a key derivative of FA, 4-VG could contribute significantly to the antioxidant activities of finished beers.

3.4 Conclusion

The study showed that the crude beer extracts exhibited strong antioxidant activities as determined by a number of different assays. A significant variation of major HCAs, namely FA, *p*-CA, and SA content, were identified and quantified by HPLC-DAD. The main derivative of FA identified, 4-VG, showed a positive ($p < 0.05$ and $p < 0.001$) correlation with $ABTS^{\bullet+}$ and FRAP assays. No other derivatives, neither 4-VP nor 4-VS, were detected in any of the crude beer extracts although these compounds could be present at levels too low to be detected. Furthermore, a positive correlation between TPC, TFC and antioxidant assays was found, although no significant correlations were evident between the major individual HCAs and antioxidant activity evaluation assays. The results suggest that in addition to the total phenolics, 4 -VG may have antioxidant action in finished beer. However, antioxidant properties of individual HCAs were not observed. Further research is recommended to examine the efficacy of the beer extracts, and 4-VG, on beer shelf life.

3.5 Acknowledgment

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3.6 Conflicts of Interest: The authors declare no conflict of interest.

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CHAPTER 4

4 Manuscript 2: Valorization of heat-treated brewers spent grain through identification of bioactive phenolics by UPLC-PDA and evaluation of their antioxidant activities.

4.1 Abstract

Thermal processing not only disrupts cell membranes and cell walls, but also cleaves covalent bonds to release low molecular weight phenolics. This study examined the impact of various oven heat treatments (100,140 & 160^{o}C) on the composition of phenolic acids and antioxidant activities in extracts obtained from defatted brewers' spent grain (BSG) meal. Heating BSG at 160^{o}C resulted in a two-fold increase of total phenolic content (TPC, 172.98 ± 7.3 mg gallic acid equivalent (GAE)/100 g defatted meal) and total flavonoid content (TFC, 16.15 ± 2.22 catechin equivalents (CE) /100 g defatted meal), respectively compared to the untreated BSG extracts. The antioxidant activities of treated BSG extracts, determined by radical scavenging and ferric reducing antioxidant power (FRAP) were significantly ($p < 0.5$) higher than the corresponding untreated BSG extracts. Eleven phenolic acids were identified and quantified by Ultra Performance Liquid Chromatography with Photodiode Array (UPLC-PDA) with their amounts varying significantly ($p < 0.05$) depending on the degree of toasting that the BSG was subjected to. Chlorogenic acid, an ester of caffeic and quinic acid was the predominant phenolic acid present in all fractions. This study found significant increases in TPC, TFC and individual phenolic acids as well as antioxidant activities in BSG extracts exposed to increasing oven temperatures. The results indicate that heat processing releases

bioactive phenolics from their bound forms due to breakdown of the covalent bonds, which may enhance the phenolic profiles and digestibility of BSG meal in the intestinal tract.

Keywords: Brewers Spent Grain, Heat Treated Processing, Bioactive Phenolics, UPLC-PDA and Antioxidant Properties

4.2 Introduction

Brewers' spent grain (BSG), a by-product of the brewing industry, is produced from barley malt during production of wort. According to literature, about 20 kg (Wb) of BSG are produced during the production of 100 L beer which represents about 31% of original malted barley weight (Lynch et al., 2016; Mussatto et al., 2006; Kunze, 2010). BSG is an inexpensive by-product available in large amounts throughout the year (Lynch et al., 2016; Mussatto et al., 2006). It is good source of protein (30-50% w/w), fibre (19-30% w/w) and essential amino acids, which are staple components in the human diet (Lynch et al., 2016; Mussatto, 2014; Mussatto et al., 2006). The current application of BSG, however, is limited to use as an animal feed. Nevertheless, due to its high nutritional composition, BSG could be very attractive for enhancing the nutritional value of foods through fortification, as it is both cheap and readily available (Lynch et al., 2016; Mussatto, 2014). The prospective of BSG as a functional ingredient and good source of health-promoting bioactives is slowly being recognised. A number of bioactives in BSG including phenolics, arabinoxylans (bioactive fibres), and hydrolysates forms of proteins, are receiving increasing attention for their potential health benefits (Steiner, Procopio, and Becker, 2015; Lynch et al., 2016). Phenolics are widely found in plant foods, and occur as soluble and insoluble bound forms in

food grains especially in the outer layer (the bran). Soluble phenolics include both free and conjugated phenolics; the conjugated phenolics can be esterified and etherified fractions. The conjugated phenolics are generally found in cell vacuoles where they form soluble complex with molecules such as sugars, lipids, proteins, amino acids and other phenolic compounds through ester and ether linkages. On the other hand, insoluble bound phenolics exist in the cell wall where they are covalently bound with cell wall components such as polysaccharides (e.g. cellulose, hemicellulose, beta glucan, arabinoxylans etc.), lignin, protein and pectin forming insoluble complexes with each other through ester and ether linkages (Shahidi & Yeo, 2016). Both conjugated and insoluble bound phenolics are known as bound phenolics because they are not soluble in organic solvents, which are commonly used for the extraction of phenolics and they cannot be released in the small intestine (Pérez-Jiménez et al., 2013). However, the bound phenolics pass into the colon where they are digested by microbiota during fermentation and can be released in the colon. These phenolics can be essential for gastrointestinal health reviewed by Pérez-Jiménez et al. (2013).

Several studies have examined BSG phenolics and their antioxidant activities (Moreira et al., 2012; McCarthy et al., 2012; da Rosa Almeida et al., 2017; Szwajgier et al., 2010). However, no data is available on the impact of heat treatments (toasting) on BSG phenolics and their antioxidant activities. The aim of this study was to investigate the effect of oven heat treatments on the extractability of bound phenolics from BSG and to analyse their profiles using both chemical and instrumental methods. Analysis of individual phenolic acid from the BSG extracts was performed by UPLC-PDA and their antioxidant activities were also monitored.

4.3 Materials and methods

4.3.1 Chemicals

Major organic solvents used in this study include acetone, hexane, methanol, ethyl acetate, diethyl ether etc. On the other hand, major reagents used to conduct the experiments are anhydrous sodium sulfate, sodium hydroxide, sodium chloride, dibasic and monobasic sodium phosphates, sodium nitrite, HCl, $AlCl_3$, potassium ferricyanide, ferrous chloride, Folin-Ciocalteu's reagent, ferric chloride, and sodium carbonate. These solvents and reagents were bought from Fisher Scientific Ltd. (Ottawa, ON, Canada) and Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Standards of gallic acid, catechin, Trolox, DPPH radical, and a number of phenolic acid standards were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

4.3.2 Sample collection and preparation

BSG samples used in the study were kindly supplied by Canadian Malting Barley Technical Centre (CMBTC), 303 Main St #1365, Winnipeg, MB R3C 3G7. Before heat treatments, BSG was dried at 55^{o}C for 24 hr using an electric oven (Thelco laboratory oven, Fisher Scientific, Oakville, ON Canada) and the final moisture content was below 10%.

4.3.3 Oven heat treatments of BSG

Before extraction of phenolics, the BSG samples were pretreated by an oven (Thelco laboratory oven, Fisher Scientific, Oakville, ON Canada) at 100, 140 and 160^{o}C for 30 min. The toasted BSGs were then powdered in a coffee bean grinder (model CBG5 series, Black & Decker, Canada Inc., Brockville, ON, Canada); a fine powder was finally obtained by passing

through a 0.5 mm sieve. The fine ground samples were defatted by the AOCS Soxhlet extraction method with hexane at 150^{o}C. Defatted BSG meal were air-dried and then used immediately for extraction of phenolics.

4.3.4 Extraction of phenolics fraction from defatted BSG meals

The phenolics from the defatted BSG meal were extracted by ultrasound assisted extraction (UAE) technique as stated by the method of Chandrasekara & Shahidi (2011c) with minor modifications. Both untreated and treated defatted BSG meal (3 g) were mixed with 60 mL of 70% acetone (N = 2) and sonicated for 20 min in an ultrasonic bath (250 HT, 120 volt, 6 Amp, 50-6 Hz, VWR International, and West Chester, PA 19380). After which, the samples were centrifuged for 5 min, 4000 g at 20 °C. The supernatants were collected, and the same step was repeated one more time. The residue was mixed again with 60 mL of 70% methanol (N= 1) following the same procedure as the acetone extraction. Both acetone and methanolic organic supernatants were combined and evaporated to dryness using rotary evaporator (V-800, 100-240V, 210W, 50-60HZ, Buchi Labortechnik AG, CH-9230 Flawil, and Switzerland) at the temperature of 40^{o}C. After evaporation, the remaining water phase of the extract was acidified to pH 2 using 6 M HCl solution. The phenolics were extracted five times from the acidified solution using diethyl ether and ethyl acetate (1:1 v/v). The combined organic solvent extract was evaporated under vacuum using a Buchi Rotavapor R-205 (V-800, 100-240V, 210W, 50-60HZ, Buchi Labortechnik AG, CH-9230 Flawil, and Switzerland) at 40^{o}C. Five milliliters of HPLC grade methanol were added to the residue and stored at - 20^{o}C until further analysis.

4.3.5 Determination of Total Phenolic Content (TPC)

A Folin-Ciacalteu reagent method as described by Singleton & Rossi, (1965) and Chandrasekara & Shahidi, (2010) was used to determine total phenolic content (TPC) of BSG extracts. Briefly, 0.5 mL of BSG extract and Folin-Ciocalteu phenol reagent (0.5 mL) was taken in a test tube and mixed properly. After 3 min of incubation, 1 mL of 7.5 % Na_2CO_3 and 8 mL of d.H_2O was added, the tubes were incubated for 35 min in the dark. The absorbance was read at 725 nm using. The total phenolic content (TPC) in BSG extracts was calculated as gallic acid equivalent (GAE) milligram per 100 gram of dry weight (DW).

4.3.6 Determination of Total Flavonoid Content (TFC)

The total flavonoid content (TFC) of BSG extracts was determined according to method described by Chandrasekara and Shahidi (2010). A 1 mL of BSG extract, 4 mL of d.H_20 and 0.3 mL of $NaNO_2$ solution (5%) were mixed in the test tubes. After 5 min, 0.3 mL of $AlCl_3$ solution (10%) was added to each tube. After 1 min, 2 mL of NaOH (1 M), 2.4 mL of d.H_20 were added and incubated for 15 min in the dark. The absorbance was read at 510 nm using a spectrophotometer. The TFC was calculated from a standard curve for catechin and calculated as mg catechin equivalents (mg CE) per 100 grams of defatted BSG meal.

4.3.7 Identification and quantification of phenolic acids by UPLC-PDA analysis

Identification and quantitative analysis of the phenolic acids of BSG extracts from treated and non-treated defatted meals were carried out using an UPLC-PDA method as described by Malunga and Beta (2015) with major modifications. The filtrated BSG extracts (0.20 μm) were analysed using a reverse-phased Ultra High Performance Liquid Chromatography (Acuity H class instrument (Waters, Milford, MA)) equipped with a Waters 2996 Photodiode

Array Detector. Separation was carried out on a 30 mm*2.1 mm, 1.8 µm C18 column (Waters Acuity UPLC) with the sample and column temperatures maintained at 15^{o}C and 30^{o}C, respectively with a 0.7 mL/min flow rate and one microliter injection volume. The mobile phase was 0.1 % (v/v) acetic acid in UPLC grade water as solvent A and 0.1 % (v/v) acetic acid in methanol as solvent B with a flow rate of 8 mL/min. A gradient elution system operated as follows; for 7.93 min: 9 - 15 % B (0-2 min); 15 - 16.5 % B (2 - 2.72 min), 16.5-19 % B (2.72-3.17 min), 19-25 % B (3.17-3.4 min), 25-26 % B (3.4-4.08 min), 26 -28 % (4.08-4.31 min), 28-35 % B (4.31 - 4.65 min), 35 -40 % (4.65 - 5.21 min), 40-48 % (5.21-5.44 min); 48-53% B (5.44-6.01 min); 53-70% B (6.01-6.8 min); 70-9% B (6.8-7.37 min); 9-9% B (7.37-7.93 min).
Phenolic acids such as hydroxybenzoic acids were monitored at a wavelength of 280 nm using UV-DAD while hydroxycinnamic acids were monitored at 320 nm wavelength. Identification of each phenolic acid was carried out by comparing their retention times and UV spectral characteristics of the UPLC peak with standard solutions of each type of phenolic acid. Quantitative determination was performed using an external calibration curve. For this purpose, various phenolic acid standards, for example, protocatechuic acid, chlorogenic acid, 4-hydroxybenzoic acid, gallic acid, caffeic acid, syringic acid, vanillic acid, *p*-coumaric acid, ferulic acid, and sinapic acid. A series dilution of these compounds were prepared using methanol: water (50:50) and used to obtain a respective calibration curve for each standard.

4.3.8 DPPH radical scavenging assay

DPPH radical scavenging activities of BSG extracts were measured according to the method of Thiyam et al. (2006). Fifty µL of BSG extracts were mixed with 2.95 mL of DPPH radical solution (0.1 mM) in the test tube and vortexed well. A control was prepared identically but

devoid of BSG extracts. All tubes were allowed to stand for 10 min at room temperature in the dark. The absorbance of each tube was then read at 516 nm using a Spectrophotometer. The DPPH radical scavenging effect of BSG extracts were expressed as micromoles Trolox equivalent (TE) per gram of defatted BSG meal.

4.3.9 Ferric-reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power (FRAP) of BSG extracts was determined as described by Oyaiza, (1986) with some modifications. A 0.5 mL aliquot of the BSG extract, 2.5 mL of a phosphate buffer solution (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v) were placed in the test tube and incubated for 20 min at 50^{o}C. Then, 2.5 mL of TCA solution (10%) was added to each tube and centrifuged for 10 min at 1750 g. A 2.5 mL of each supernatant was mixed with 2.5 mL of deionized water in separate test tubes. After that, 0.5 mL of $FeCl_3$ solution (0.1%; w/v) was added to each mixture and the absorbance read at 700 nm using a UV Spectrophotometer (DU 800 Series, Beckman Coulter, Inc., Fullerton, California, USA). A standard curve was prepared using Trolox. The results were expressed as micromole of trolox equivalents (TE) per gram of defatted BSG sample.

4.3.10 Statistical analysis

All experiments were conducted in triplicate. Data are reported as means ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey HSD test identified differences among means using IBM SPSS Statistics version 22 (Armonk, New York, USA).

4.4 Results and Discussion

4.4.1 Impact of oven heat treatments on the total phenolic and flavonoid content of BSG defatted meal

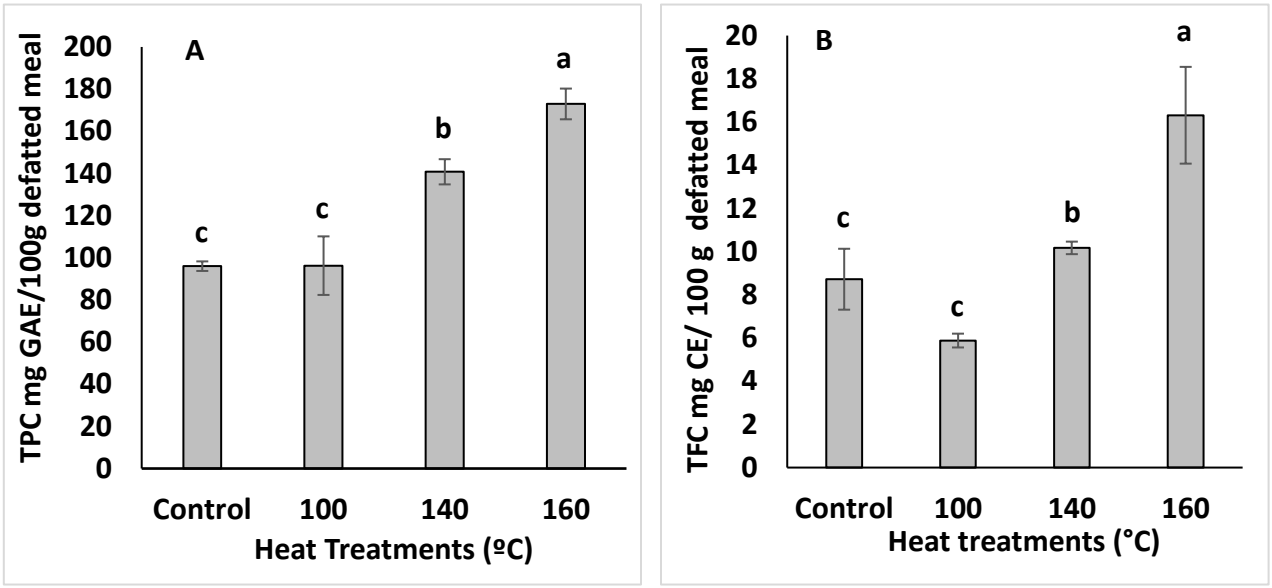


Figure 4.1. Total Phenolic content (TPC) (A) and Total flavonoids content (TFC) (B) of BSG extracts from defatted meal affected by oven heat treatments. Data represents as mean ± SD (N = 3). Mean followed by different letters are significantly different at $p < 0.05$. GAE*= Gallic Acid Equivalent, CE*= Catechin Equivalent.

The impact of heat treatments on the TPC of defatted BSG meal is shown in **Figure 4.1A**. The TPC value of untreated BSG extract was 96.0 ± 2.27 mg GAE/100 g defatted meal. This value significantly (*p < 0.05*) increased at temperature above 100°C as determined by Folin Ciocalteu assay (**Figure 4.1A**). The BSG extract heated at 160°C showed highest amount of TPC compared to the extracts heated at 100°C and 140°C. An almost two-fold increase in TPC (from 96.0 ± 2.27 to 172.97 ± 7.29 mg GAE/100 g defatted meal) was observed at 160°C while BSG treated at 140°C showed 1.5 fold increase in TPC (from 96.0 ± 2.27 to 140.78 ±

5.98 mg GAE/100 g defatted meal) compared to the sample heated at 100°C or the control. These findings are in agreement with previous studies by Jeong et al. (2004), Xu et al., (2007), Terpinc et al., (2011), and Li and Shah (2013), which reported that TPC increased significantly in citrus peel, camelina meal, and *pleurotus eryngii* when heated at different temperatures.

The TFC of treated BSG extracts also increased significantly ($p < 0.05$) at the higher oven temperatures (**Figure 1B**). The TFC of BSG extracts increased from 21.8 ± 3.54 mg GAE/100 defatted meal to 40.79 ± 5.61 mg GAE/100 defatted meal in samples heated at 160°C for 30 min. The BSG extract at 160°C was significantly ($p < 0.05$) higher in TFC compared to extracts from 100°C, 140°C and the control (**Figure 4.1B**). Overall, these results suggest that the highest heat treatment was effective in cleaving the covalent bonds binding the phenolics to the plant components. This facilitated release of the bound phenolic compounds from their esterified and insoluble bound forms located in cell vacuoles and walls. However, TPC and TFC of BSG extract remained unaffected when treated at a temperature of 100°C. The lower temperature appeared to be unable to break down the covalent bonds between the phenolic compounds and other plant molecules. A linear increase was evident for TPC and TFC with the rise in oven temperature except for treatment at 100°C. These results indicate that the higher temperatures are able to modify the phenolic composition of BSG by releasing more bound forms.

4.4.2 Impact of heat treatment on phenolic acids composition of defatted BSG meal analysed by UPLC-PDA

Table 4.1. Impact of heat treatments on individual phenolic acid content (µg/ g) of defatted BSG meal analysed by UPLC-PDA

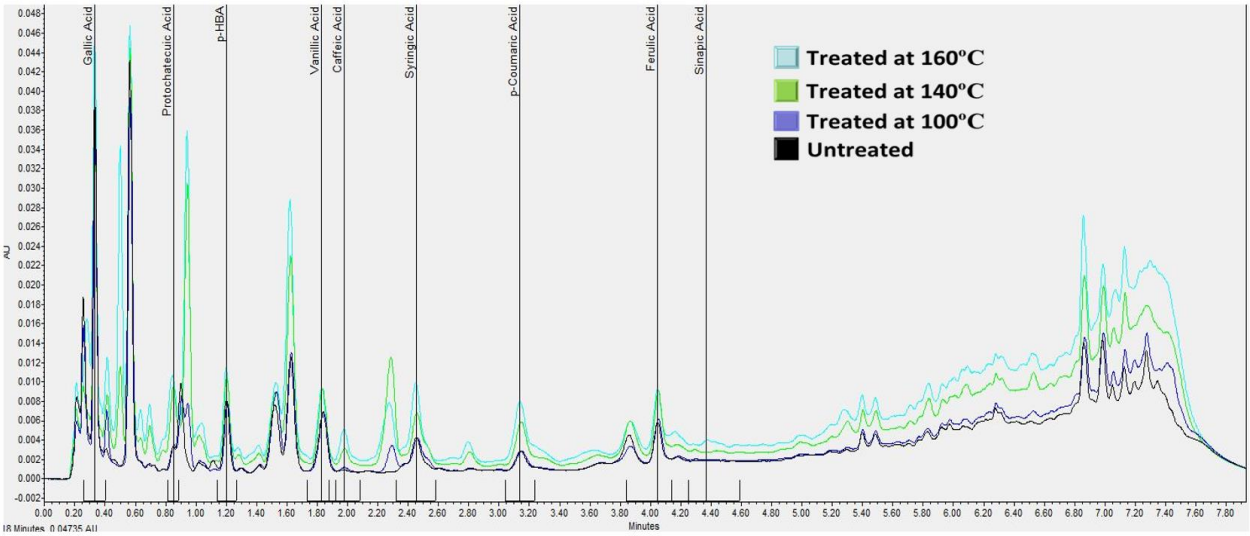
	Oven Heat Treatments			
Phenolic acids	Untreated/ Control	Temperature at 100°C	Temperature at 140°C	Temperature at 160°C
Gallic acid	57.36 ± 7.81^{b}	48.81 ± 7.34^{b}	53.90 ± 13.59^{b}	82.79 ± 9.30^{a}
Unknown 1	76.05 ± 0.92^{b}	68.34 ± 4.29^{ab}	72.57 ± 9.81^{ab}	83.62 ± 5.27^{a}
Protocatechuic acid	22.39 ± 1.95^{b}	18.39 ± 3.75^{b}	25.94 ± 4.16^{b}	37.41 ± 4.28^{a}
4-hydroxybenzoic acid	10.77 ± 0.90^{b}	9.95 ± 0.83^{b}	13.64 ± 1.70^{ab}	16.59 ± 3.36^{a}
Vanillic acid	20.84 ± 0.84^{b}	19.03 ± 1.25^{b}	23.48 ± 2.36^{ab}	26.59 ± 5.29^{a}
Syringic acid	13.72 ± 0.97^{c}	15.29 ± 0.75^{c}	23.42 ± 2.82 ^{b}	35.65 ± 3.85^{a}
Chlorogenic acid	107.18 ± 2.29^{a}	102.01 ± 5.36^{a}	81.92 ± 10.34^{b}	71.28 ± 4.74^{b}
Caffeic acid	-	-	3.79 ± 0.49	7.88 ± 0.73
p-Coumaric acid	3.71 ± 0.25^{c}	3.41 ± 0.22^{c}	7.68 ± 0.96^{b}	11.42 ± 0.57^{a}
Ferulic acid	9.5 ± 0.27^{b}	10.2 ± 0.62^{b}	15.58 ± 1.13^{a}	15.39 ± 1.29^{a}
Sinapic acid	-	-	-	3.64 ± 0.025
Unknown 2	23.91 ± 3.17^{c}	24.15 ± 2.72^{c}	31.73 ± 2.95^{b}	41.99 ± 2.04^{a}
Unknown 3	17.02 ± 1.92^{b}	14.88 ± 0.69^{b}	20.68 ± 1.40^{a}	23.31 ± 0.98^{a}

Data represents mean ± SD (N = 3). Mean for the samples followed by different letters are significantly different at $p < 0.05$.

The impact of heat treatment on the composition of phenolic acids in the defatted BSG meal extracts is shown in the **Table 4.1.** A positive impact of the oven heat treatments was observed by the increasing yield of individual phenolic acids. BSG thermally treated at 160^{o}C showed a significantly ($p < 0.05$) higher amount of hydroxybenzoic and hydroxycinnamic acids than the untreated BSG extract. Of all the phenolic acids, only chlorogenic acid

decreased significantly ($p < 0.05$). More specifically, after BSG treatment at 160°C, the major hydroxybenzoic acids such as gallic acid increased from 57.36 ± 7.81 to 82.79 ± 9.30 μg/g; protocatechuic acid from 22.39 ± 1.95 to 37.41 ± 4.28 μg/g; 4-hydroxybenzoic acid increased from 10.77 ± 0.90 to 16.59 ± 3.36 μg/g; vanilic acid increased from 20.84 ± 0.84 to 26.59 ± 5.29 μg/g ; syringic acid increased from 13.72 ± 0.97 to 35.65 ± 3.85 and unknown 1 (peak 1) increased from 76.05 ± 0.92 to 83.62 ± 5.27 μg/g. Changes in the major hydroxycinnamic acids at 160°C were, an increase in ferulic acid from 9.5 ± 0.27 to 15.39 ± 1.29 μg/g; an increase in *p*-coumaric acid from 3.71 ± 0.25 to 11.42 ± 0.5 μg/g; an increase in an unidentified compound 2 (peak 2) from 23.91 ± 3.17 to 41.99 ± 2.04 μg/g; and an increase in an unidentified compound 3 (peak 3) from 17.02 ± 1.92 to 23.31 ± 0.98 μg/g. In contrast at 160°C, chlorogenic acid, an ester of caffeic acid and quinic acid, decreased from 107.18 ± 2.29 to 71.28 ± 4.74 μg/g defatted meal. This was attributed to its conversion to caffeic acid when heated at both 140°C and 160°C. This further supports the ability of the higher temperatures to cleave the esterified and glycosylated bonds (Xu et al., 2007). A smaller sinapic acid peak appeared at 160°C with the amount of 3.64 ± 0.025 μg/g defatted meal (DW) indicating that sinapic acid was present in BSG as bound form. Xu and his co-workers reported that TPC and individual phenolic compound in the esterified, glycoside and bound

fractions from citrus meal decreased with increase in temperature while at the same time there was an increase in the free fraction.



(A)

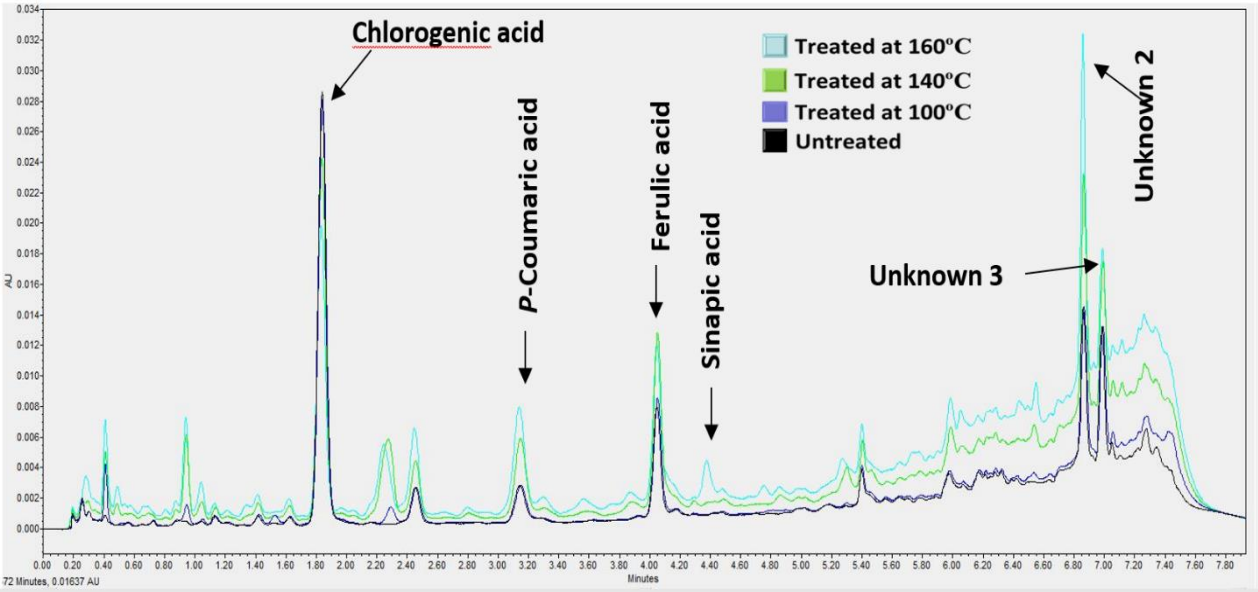


Figure 4.2. A Chromatogram showing the impact of heat treatments on the composition of hydroxybenzoic acids (at 280 nm, (A)) and hydroxycinnamic acids (at 320 nm, (B)) of defatted BSG meal analysed by UPLC-PDA.

4.4.3 Impact of heat treatment on the antioxidant activities of extracts from BSG defatted meal

The antioxidant activities of BSG phenolic extracts were investigated using two well-known assays such as DPPH radical scavenging and FRAP assay.

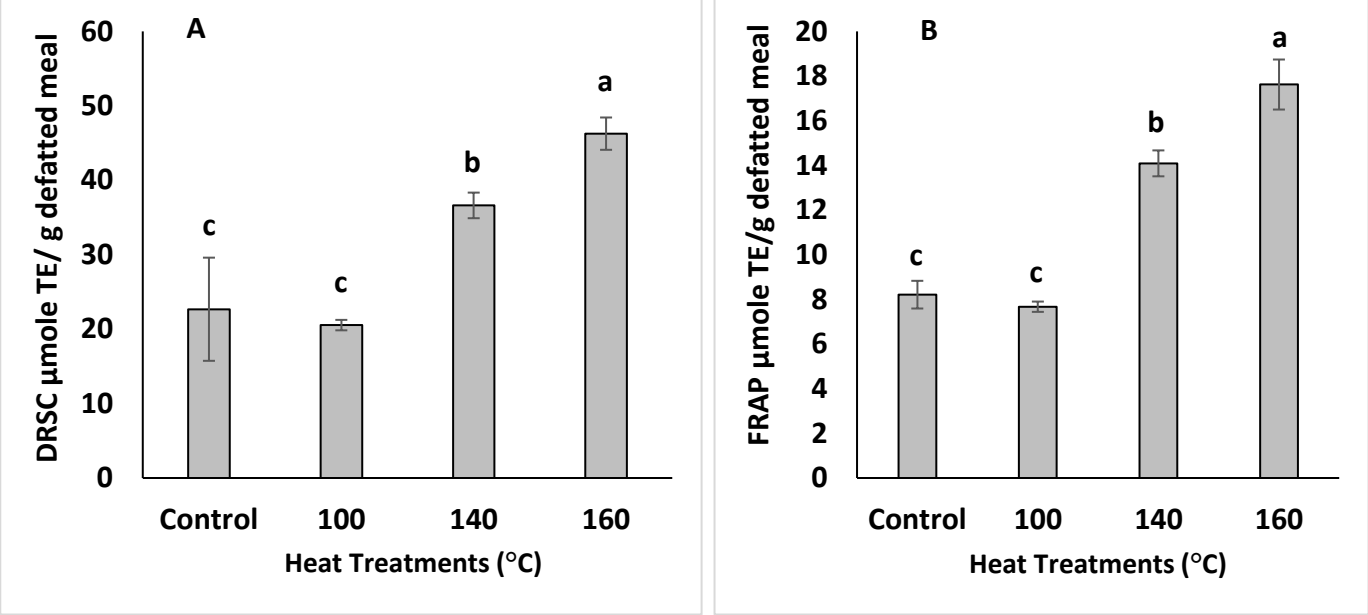


Figure 4.3. DPPH radical scavenging capacity (DRSC, A) and ferric-reducing antioxidant power (FRAP, B) of extracts from defatted BSG meal affected by various oven heat treatments. Means followed by the same letters are not significantly different at $p > 0.05$.

DPPH is an artificial free radical generally used to measure antioxidant activities of plant and biological samples of interest. The DPPH radical scavenging capacity (DRSC) of the BSG extracts increased with heating at different temperatures as shown in the **Figure 4.3 A.** The highest DPPH radical scavenging capacity was observed when treated at 160^{o}C, which also generated the largest amount of TPC and TFC. The DRSC values increased from 22.67 ± 6.93

to 46.26 ± 2.17 µmole/g defatted meal (DW) following treatment at 160^{o}C and was two-fold higher than control. The DRSC value also increased after being heated at 140^{o}C. The ferric-reducing antioxidant power (FRAP) of BSG extracts also increased significantly ($p < 0.05$) with oven heat treatments (**Figure 4.3 B**). For example, after being treated at 140^{o}C, FRAP values increased significantly ($p < 0.05$) from 8.30 ± 0.49 to 13.83 ± 0.77 µmole/g defatted meal (DW). The highest FRAP value was recorded at 160^{o}C increasing significantly ($p < 0.05$) to 17.27 ± 1.15 µmole/ g defatted meal (DW). Heating BSG at 100^{o}C, however, did not increase DRSC and FRAP values significantly, which is consistent with the level of TPC that was not significantly different from control at that temperature. These findings are in agreement with DRSC and FRAP values published by Jeong et al. (2004) and Xu et al., (2007) where antioxidant activities significantly increased in the extracts from citrus peel after being heated at different temperatures. Our results indicate that TPC of defatted BSG meal significantly ($p < 0.05$) increased at the higher oven temperatures, which paralleled the corresponding increase in DPPH radical scavenging capacities, and reducing power.

4.5 Conclusion

The study demonstrated a positive impact of heat treatments on the levels of TPC and TFC and their contribution to the antioxidant activities of BSG extracts. Several phenolic acids were identified and quantified by UPLC-PDA in the treated and untreated BSG extracts with chlorogenic acid being the predominant compound present. The study found that the heat treatments released hydroxylbenzoic and hydroxycinnamic acids from the cell structural materials. Heating the BSG extracts at 160^{o}C resulted in highest levels of TPC and TFC, as well as individual phenolic acids and free radical scavenging activities. These results indicate that

oven heat treatments facilitated the release of bioactive phenolics from the cell structure materials, where they are normally bound by covalent bonds, which may enhance the digestibility of BSG meal in the intestinal tract.

4.6 Acknowledgement

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CHAPTER 5

5 Summary, Conclusion and Future directions

The study showed significant variations in TPC and TFC between laboratory, local and foreign commercial beer extracts that exhibited strong antioxidant activities as determined by different assays. A significant variation of major HCAs, namely ferulic acid, *p*-coumaric acid, and sinapic acid content was observed among the beer extracts, identified and quantified by HPLC-DAD. The main derivative of ferulic acid identified, 4-VG, and its contents were not significantly ($p > 0.05$) different between laboratory-made, CMBTC produced and foreign commercial beer extracts, but showed a significant ($p < 0.05$ and $p < 0.001$) positive correlation with ABTS and FRAP assays. No other derivatives, neither 4-vinylphenol, a derivative of *p*- coumaric acid nor 4-vinylsyrinsol, a derivative of sinapic acid, were detected in any of the beer extracts although these compounds could be present at levels too low to be detected. Furthermore, a significant positive correlation between TPC, TFC and antioxidant activity evaluation assays was found, whereas no significant correlations were evident between the major individual HCAs and the results of antioxidant activity assays. Therefore, the results and correlation studies suggest that not only the beer phenolics but also the derivative of ferulic acid, 4-VG could contribute significantly to the antioxidant activities of beer. However, future research is recommended to investigate the impact of beer phenolics and 4-VG on the shelf life and flavor stability of the beer in a shelf life study.

The study showed a significant effect of oven heat treatment on the TPC and TFC and their contributions to antioxidant activities of BSG extracts. TPC and TFC of BSG extracts,

determined by Folin Ciocalteu's and $AlCl_3$ method, respectively and their DPPH radical scavenging capacities (DRSC) and ferric reducing antioxidant power (FRAP) increased significantly ($p < 0.05$) by increasing treatment temperature. UPLC-PDA analysis was used to identify and quantify eleven major phenolic acids from treated and untreated BSG extracts where chlorogenic acid was the major compound in both fractions. The study found significant increases of hydroxylbenzoic and hydroxycinnamic acids contents after being treated at 140°C and 160°C, respectively. However, after being treated at 160°C, the BSG extracts showed higher TPC, TFC, and individual phenolic acid content as well as DPPH radical scavenging capacities and reducing power. These results indicate that oven heat treatments caused release of bioactive phenolics from the cell structure materials where they exist as bound forms through covalent bonds, which may enhance the phenolic yield and digestibility of BSG meal in the intestinal tract. However, future research should investigate bio-accessibility and bioavailability of BSG phenolics in the small intestine and colon through preclinical and clinical studies.