AN ABSTRACT OF THE DISSERTATION OF

Patricia M. Aron for the degree of Doctor of Philosophy in Food Science and Technology, presented on March 8, 2011.

Title: The Effect of Hopping Technology on Lager Beer Flavor and Flavor Stability and the Impact of Polyphenols on Lager Beer Flavor and Physical Stability.

Abstract approved: _____
Thomas H. Shellhammer

Beer is one of the most extensively consumed beverages world-wide and it is almost always brewed with hops (*Humulus lupulus, L.*). Hops provide beer with bitterness, aroma, flavor and texture and also enhance specific beer properties such as foam stability, clarity (colloidal stability), color, flavor stability and microbial stability. Hops are a dioecious species, with female plants producing the hop strobilus (cone). The cone is an inflorescence, which is the entire part of the plant that holds the flowers. Hop cones contain lupulin glands (the source of the hop bittering resins), essential oils, and polyphenols (prenylflavonoids).

Beer prenylflavonoids such as the flavan-3-ols and their condensed products, the proanthocyanidins, represent a class of readily oxidizable compounds capable of hindering or preventing the oxidation of other molecules present in beer. Flavan-3-ols and proanthocyanidins have recently gained significant consideration as potential beer flavor modifiers and/or stabilizers. However their roles in beer flavor stability have not yet been fully realized.

In this study polyphenols were extracted from spent hop (*Humulus lupulus* L. cv Galena) solid materials and dosed into commercial lager beers. Chemical analysis of the fresh and aged beers confirmed an anti-staling effect of the dosed polyphenol extract as measured by antioxidant capacity assays: FRAP, DPPH• and ESR. The polyphenol rich extract was subjected to phloroglucinolysis and analyzed via reverse-phased chromatography/mass spectroscopy-electrospray ionization (RP-HPLC/MS-ESI) to determine flavonoid content. C-18 RP-HPLC analysis of the extract revealed that it was

99% phenolic in nature, with a procyanidin mean degree of polymerization (mDP) of 2.72.

Based on these findings beers were subsequently brewed with and without hop products (*Humulus lupulus* L. cv Galena) to target the effect of the complete hop (pellets), hop bittering acids only (CO_2 extract), hop polyphenols only (spent hop solids) and no hop components (Control) on beer flavor and flavor stability. Spent Hop and Pellet Hop beers scored highest in antioxidant potential as measured by the FRAP assay, howeer ESR results were contradictory. Even after force-aging, Pellet hopped beers were lowest in total aldehydes and Control beers were highest in total aldehydes, indicating a protective effect for whole hop products on staling aldehyde formation. Sensorially, the Spent Hop and Pellet Hop beers were characterized by high Piney and Tropical fruit notes, with significant increases occurring after force-aging. The Control beers were rated as being higher in Overall Aroma Intensity, and were judged as being high in Cardboard aroma after force-aging.

Preliminary findings from the brewing trials indicated that significant changes in polyphenol levels occur during accelerated aging. The brewing trial was therefore repeated and beers were profiled for phenolic content and investigated for changes in phenolic content during aging. Beer polyphenols were extracted with Sephadex LH20 resin and subjected to phloroglucinolysis to reveal subunit composition and proanthocyanidin mDP. Although the sephadex extracts were phenolic in nature, proanthocyanidins only accounted for up to 2% of the total phenolic material. Total flavanoid and proanthocyanidin content of the beers increased initially during storage, with eventual decreases occurring after 6 weeks of storage at 30°C. Beers high in hop polyphenols did not suppress the loss of *iso*-alpha acids during aging and were once again assessed as least flavor stable of the beers by ESR T150.

The Effect of Hopping Technology on Lager Beer Flavor and Flavor Stability and the Impact of Polyphenols on Lager Beer Flavor and Physical Stability.

by
Patricia M. Aron

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APPROVED:

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I understand that my dissertation will become a part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Patricia M. Aron, Author

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Robert T. Foster, Cindy Lederer and Patrick L. Ting assisted with the data collection, analysis, and writing for Chapter 2.

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Introduction

HOPS

Hops (*Humulus lupulus* L.) are dioecious, perennial vines within the family Cannabinaceae that grow in nearly all temperate regions of the world. Hops are grown as a niche crop almost exclusively for the purpose of brewing beer. Major agricultural centers for hop production include the Hallertau region of Germany and the Pacific Northwest states of the U.S. (Idaho, Oregon and Washington), with lesser agricultural centers in England, China, Slovenia, Czech Republic, Australia, New Zealand and South Africa. Although hops contribute a mere fraction of the beer raw ingredient bill, the use of hops immensely impacts the flavor and quality of finished beer. Hops can provide beer with bitterness, aroma, flavor and texture and also enhance specific beer properties such as foam stability, clarity (colloidal stability), color, flavor stability and microbial stability.

Hop Cultivation. Hops are grown in yards on trellis systems (low 6 ft or high 12 ft) that allow for the vines to climb. Although wild hops can be quite hardy, commercial varieties are susceptible to a diversity of pests and diseases. Female plants produce the hop strobilus (cone). The cone is an inflorescence, which is the entire part of the plant that holds the flowers. The flowers of hops are very small and reduced; they do not have all the flower parts, like petals, sepals, etc. There is one flower on each bracteole of the cone, sterile bracts alternate with flower holding bracteoles around the strig (cone stem). The flowers are actually an encased ovule with two stigmas coming from it, with about 20 - 40 flowers per cone. In the United States male plants are typically not kept in commercial yards. Fertilization of female plants results in production of seeds which are believed to impart undesired flavors (seed fatty acids) and complicate the brewing process (seed removal). Therefore, U.S. brewers typically prefer seedless hops.

Types of hops. There are two species of hops: *Humulus lupulus* and *Humulus japonicas*. However *H. Japonicas* do not produce the necessary resins used for beer

bittering and so are used purely for ornamental purposes. Within *H. lupulus* hops are classified into two general categories based on their bittering acid content: aroma hops and bittering hops. However some bittering hops also provide substantial aroma and can be considered as all-purpose hops.

Hop cones. Several components comprise the whole of a hop cone (cellulose, lignin, proteins, lipids, waxes, resins, essential oils and polyphenols)[1], however brewers mainly concern themselves with just two components: the resins and essential oils. Hop resins are secreted by the lupulin glands and contain the alpha acids, which once isomerized (*iso*-alpha acids) are responsible for the bitter quality of beer (**Figure 1.1**). The essential oils contain a complex mixture of several hundred compounds. Because the resins and essential oils sum to less than 20% of the entire hop cone by mass [1], the use of whole hops is not the most efficient practice.

Hop products. A number of hop products are now commercially available and can be utilized throughout the brewing process to provide beer with sensorial bitterness and added-value (antimicrobial activity, foam stability, flavor enhancement and flavor stability)[2-6]. Products such as pelletized hops, pre-isomerized pelletized hops, resin concentrated pelletized hops and resinous extracts (prepared by critical or liquid CO_2 extraction) are commonly used in place of whole hop cones today due to their increased efficiency and utilization. More refined, advanced hop products such as pre-isomerized extracts and reduced extracts (tetrahydro-*iso*-alpha-aicds, hexahydro-*iso*-alpha acids, and rho-*iso*-alpha acids) are also available for trimming and special applications (foam enhancement and light stability). Consequently, the use of hop pellets is also in decline, leaving a large portion of the hop cone as a waste stream of spent hop solids/powder. This spent hop solid material is generally rich in polyphenols[7, 8].

Hop and beer polyphenols. Polyphenolic prenylflavonoids (flavonol glycosides, flavanols, and proanthocyanidins) comprise another class of secondary plant metabolites produced by hops [9] (**Figure 1.2**). Several polyphenols have been found in hops, however the majority of polyphenols found in beer are said to derive from malt (up to 80%)[10, 11]. Polyphenol concentrations in lager beers can range anywhere from 50 to 150 mg/L [12]. Several classes of phenolic compounds have been found in beer, including

simple phenols, benzoic acid derivatives, cinnamic acids, coumarins [13-17], chalcones, flavanones, flavones, flavan-3-ols, proanthocyanidins, several types of flavonoid-glucosylated compounds [11, 15, 18], alpha acids, *iso*-alpha acids and other miscellaneous compounds [15, 19]. The final polyphenol content of beer depends largely on brewing practice and raw materials. Flavan-3-ol and proanthocyanidin capacity to improve food oxidative stability has been well established in several food systems[20] and recently, these antioxidants have been considered for their potential to improve beer flavor stability [17, 21-25].

BEER FLAVOR STABILITY

While it is preferred that flavor improves during the maturation process, formation of undesirable flavors inevitably occurs during beer storage. More problematic is that occurrence of aged-flavors varies from one beer style to another, with lager beer seeming especially sensitive [4, 26, 27]. Of the many chemicals involved in beer flavor modification, a few key groups have been identified: diketones, sulfur compounds, aldehydes and volatile fatty acids [4, 26, 28, 29]. In general, beer aging results in decreased bitter taste, increased sweet taste and increased caramel, ribes (black currant), and toffee-like aromas. Carbonyl compounds such as trans-2-nonenal (cardboard aroma) form during beer storage from the oxidation of fatty acids and have been attributed to aged-beer flavor due to their very low flavor thresholds [30, 31]. To date more than 700 compounds have been reported in various beer types [32] with specific volatiles resulting from a multitude of aging reactions: the Maillard reaction, the formation of linear aldehydes and esters, ester degradation, acetal formation, etherification, degradation of hop bittering acids and presence of phenolic compounds [3, 21, 24, 33]. The occurrence of each reaction depends on beer type, storage temperature, and dissolved oxygen content [34, 35].

Oxygen in beer. Limiting dissolved oxygen levels in finished beers to $\leq$ 50 ug/L should prevent most undesirable effects on flavor and haze stability [4]. Quality control criteria recommends 0.2 mg/L or less of dissolved oxygen for packaged beer [32, 36] and modern filling equipment is capable of achieving < 0.1 mg/L total package oxygen.

Reactive oxygen species and metal catalysts. It is generally thought that aged-beer flavor depends heavily on the oxidative degradation of beer compounds by reactive oxygen species (ROS) [4, 34, 37-40]. ROS can be either oxygen or nitrogen radicals, or

even non-radicals with the potential to oxidize or convert to oxidizing radicals. Flavor deterioration and related oxidative changes in beer molecules do not correlate directly with absolute molecular oxygen content [38]; molecular oxygen (O_2) itself does not react directly with compounds such as SO_2, sulfite ions or polyphenols. Reactions involving oxygen are thought to proceed in one-electron steps via the formation of free radicals [41], a process that can be catalyzed by transition metals [42]. In the presence of a metal catalyst such as Fe(II) or Cu(I), oxygen can capture an electron to form superoxide anion (O_2^-). Upon protonation, superoxide forms the perhydroxyl radical ($OOH^\bullet$). Generally most of the superoxide (pKa 4.8) originating in beer (pH ~ 4.5) exists in this protonated and more reactive state [26, 32]. Superoxide may also undergo reduction to form peroxide anion (O_2^{2-}). Peroxide ion can in turn become protonated to form hydrogen peroxide (H_2O_2) [34]. Furthermore, iron can catalyze the generation of hydroxyl ($OH^\bullet$) and peroxyl radicals ($OOH^\bullet$) from H_2O_2 via the Haber-Weiss and Fenton reactions.

The protective role of polyphenols - antioxidants. Polyphenolic flavanoids are capable of scavenging free radicals [43], which allows them to protect other molecules, such as flavor compounds, from oxidation. The electron configuration of flavan-3-ols allows for easy release of electrons to free radical species ($R^\bullet$). Release of an electron then transfers the radical character to the flavan-3-ol ($F^\bullet$), a radical that is generally more stable and less harmful than the initial radical species. The oxidation of flavan-3-ols predominantly produces semiquinone radicals. Semiquinone radicals can ultimately couple through nucleophilic addition to produce oligomers that posssess the same number of reactive catechol/pyrogallol structures as the parent molecule. The flavanoid oligomers produced via this process may then behave as antioxidants themselves [44].

The protective role of polyphenols – metal chelators. Flavan-3-ols and proanthocyanidins may act as indirect anti-oxidants by binding and effectively reducing concentrations of divalent transition metals from solution [45, 46]. (+)-Catechin strongly complexes iron and copper cations in preferred stoichiometric binding ratios of Fe(II)/procyanidin (2:1) and Cu^{2+}/procyanidin (4:1) [47]. Flavan-3-ol/proanthocyanidin potential to chelate metals depends on hydroxylation pattern and degree of polymerization; ortho-dihydroxy configurations on the B-ring and higher degree of

polymerization lead to increased metal-flavanoid complex formation and stability [48]. Flavones chelate metal ions, specifically Cu(II), between the 5-hydroxyl and 4-oxo group[49]. At increased pH, the flavonols myricetin and quercetin as well as (+)-catechin can chelate Cu(II) at the ortho-catechol group of the B-ring. Flavonols myricetin and quercetin also bind Fe (III) between the 5-hydroxyl and the 4-oxo groups (tested at pH 5.5).

Polyphenol pro-oxidant potential. Despite their antioxidant nature, polyphenols also have the potential to act as pro-oxidants. Flavan-3-ols, proanthocyanidins and flavonols may potentially promote ROS formation and behave in a pro-oxidant manner. Redox cycling of phenolics can be catalyzed by cations such as iron and copper to result in ROS that are capable of altering lipids, proteins, enzymes, and other biological molecules [50, 51]. Flavanoids that contain a gallic acid moiety show greatest pro-oxidant potential. Although galloylated flavanoids have rarely been reported in beer (5-20 mg/L) [52], and gallic acid is usually present only at ug/L concentrations [15], the 3'4'5' trihydroxyflavans (prodelphinidins) are believed to have similar chemical functionality to gallic acid[34]. Prodelphinidins can function as coupled reducing agents in a pro-oxidant manner. Prodelphinidins [11, 19] reported in beer to date include the following: (-)-gallocatechin,(-) -epigallocatechin, (-)-galloatechin-(+)-catechin dimer, and ent-(-)-epigallocatechin-(+)-catechin dimer [19, 53]. Flavonols also display pro-oxidant activity due to their propensity to form quinones that are highly prone to redox-cycle [44] . Flavonols can reduce Fe(III) to Fe(II) and Cu(II) to Cu(I), metals that are responsible for promoting oxidation via Fenton and Haber-Weiss reactions.

Role of polyphenols in beer haze. Despite having a reputation for improving food oxidative stability, beer polyphenols can negatively impact beer colloidal stability by instigating haze formation; protein-polyphenol complexes are the most frequent cause for haze production [4, 23]. Barley hordein proteins are the haze active (HA) proteins found in beer. Because HA-proteins have on the order of 20 mol% proline they display a high affinity for polyphenols. Polyphenol- protein complexation most likely involves hydrogen bonding and hydrophobic stacking of proline and polyphenol rings. Formation of protein-PP haze depends on beer pH, alcohol content, ionic strength, as well as

phenolic composition [54-57]. Flavanoids are known constituents of permanent beer haze. The flavan-3-ol monomers (-)-epicatechin and (+)-catechin and (+)-gallocatechin bind, but do not cross link HA proteins. However, proanthocyanidin oligomers possess two or more binding sites within the same molecule, allowing them to crosslink HA proteins [58]. Haze formation varies with proanthocyanidin molecular weight, subunit composition, interflavanoid bond orientation, number and placement of the hydroxyls on the heterocyclic C and aromatic B rings [39, 59-61]. Because trimer, tetramer and higher proanthocyanidin oligomers less readily survive the brewing process, the proanthocyanidin dimers are thought to play the most significant role in beer haze [56]. However, oxidized flavanols instigate chill haze and once condensed (polymerized) into proanthocyanidins can partake in the formation of permanent haze [62].

Beer stabilization. Several methods have been employed for beer colloidal stabilization including: prolonged cold storage, cold filtration, fining with gelatin, isinglass, or tannic acid, addition of proteolytic enzymes and treatments with adsorbents [4, 63]. One of the more commonly used adsorbent resins, polyvinylpyrrolidine (PVPP) was commercially introduced in 1961[64] to specifically target and remove beer PPs. While other approaches may be used to target PPs, fining with PVPP is practiced commonly due to its relative ease of use and low cost. PVPP is a neutral polyamide that has an affinity for beer-PPs because it is structurally similar to polyproline, a known HA-peptide [4, 28, 63].

Polyphenol removal, addition and beer flavor. Although the aim of PVPP use is to eliminate the PPs involved in haze formation, the reality is that several PP classes are affected: simple phenolic acids, flavonol glycosides, procyanidins, prodelphinidins, proanthocyanidins and complexes of PPs and proteins [58, 63]. Model experiments indicate that PVPP may preferentially adsorb the potentially prooxidant prodelphinidins, while maintaining the antioxidant pool of procyanidins, yet this phenomenon has not been sufficiently substantiated in beer. According to O'Reilly, effective PVPP dosing rates differ by beer type [65]. If flavanoid dimers and oligomers are the target, lower doses (15-20 g/hL for single use) of PVPP may be applied, whereas extremely high doses

on the order of 100 g/L are needed to remove excessive quantities of monomers [66]. This was corroborated by Mitchell and coworkers [23].

As antioxidants, polyphenols have the potential to influence the oxidative mechanisms responsible for aged beer flavors. However, very little is understood regarding the impact of polyphenols, or their exclusion (via fining with PVPP or use of CO_2 extracts), on aged beer flavor development. Despite their antioxidant potential, some polyphenols can show pro-oxidant behavior and thus both attractive [21, 24, 34, 35, 39, 67] and unattractive [34, 40, 68-70] flavor properties have been attributed to beer polyphenols. Polyphenols likely undergo changes during malting and brewing [16, 19] and seem to have greatest potential on flavor stability during the mashing and wort boiling steps [21, 71]. Polyphenols also contribute significant reducing power to beer [35, 39], and have been ascribed to nonenal reduction during wort boiling . Sensory experiments also allude to positive effects of hop-PPs on beer flavor stability [21, 24]. Foster [72] reports that hop polyphenol rich extracts can improve the oxidative stability (ESR lagtime) of light and dark beer and fruit juices, however other ESR lagphase studies fail to show that polyphenols (catechin, phenolic acids, and dimeric proanthocyanidins) significantly diminish free radical formation in beer during storage or in wort during brewing [40, 69, 73, 74]. Moreover several reports claim that the antioxidative properties of hop products are unrelated to their polyphenol content and that it is the hop bittering acids that contribute the strongest source of antioxidants in the beer [75-77].

Regardless, over the last decade, brewing scientists and hop chemists have given substantial attention to this polyphenol rich spent hop material. Brewing trials conducted with pellets, CO_2 extract and spent hops [78] indicate that pellet hopped beers age slightly better and have more pleasant aroma than extract beers, and that spent hops contribute pleasant, hoppy, slightly fruity aromas and tastes as judged by panelists, even after accelerated storage. To date at least five patents have been filed in reference to the advantages of brewing with hop polyphenols and spent hop material [6, 7, 79-81].

RESEARCH JUSTIFICATION AND OBJECTIVES

Flavor instability resulting from beer storage remains one of the most important quality problems in the brewing industry. Although research has focused on aged beer

flavor stability via a multitude of analytical methods, it remains very difficult to comprehensively and accurately evaluate the aging flavor of beer; no single compound or measurement exists to adequately address the multifaceted course of aging. Moreover, pale lager beers are especially sensitive to flavor degeneration [26, 33, 82-84].

Beer aging is caused primarily by oxidative reactions that transform into products associated with compromised product quality. Flavanoid polyphenols represent a class of readily oxidized compounds. As beer constituents they are directly involved in haze formation and can be removed by PVPP. Both attractive and unattractive flavor properties have been attributed to beer polyphenols; some report antioxidative roles for polyphenols in beer flavor [24, 34, 35, 39, 85] while others report pro-oxidative roles [34, 40, 69, 86]. Sensory experiments also allude to positive effects of hop-polyphenols on beer flavor stability [21]. Although the use of whole hops and whole hop pellets seems to be in decline, and thus the total contribution of polyphenols to beer is in decline, evidence exists to suggest that whole hop or spent hop material has something special to offer the brewer in terms of flavor stability.

Regardless, the debate over the impact of polyphenols on beer flavor remains unresolved. The goal of these projects was thus to provide a better understanding of how hop polyphenols affect beer beer flavor and flavor stability and how polyphenol composition changes with regard to storage time to ultimately affect beer quality parameters. The information ultimately achieved from this research could help brewers to better understand the extent to which polyphenols play a role in beer flavor stability.

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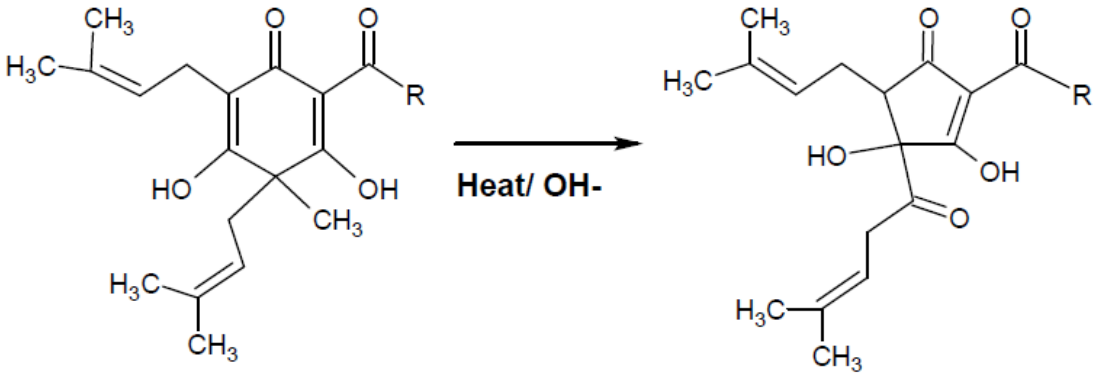

Figure 1.1. Hop alpha acids isomerize during wort boiling to produce *iso*-alpha acids, the main bittering acids in beer.

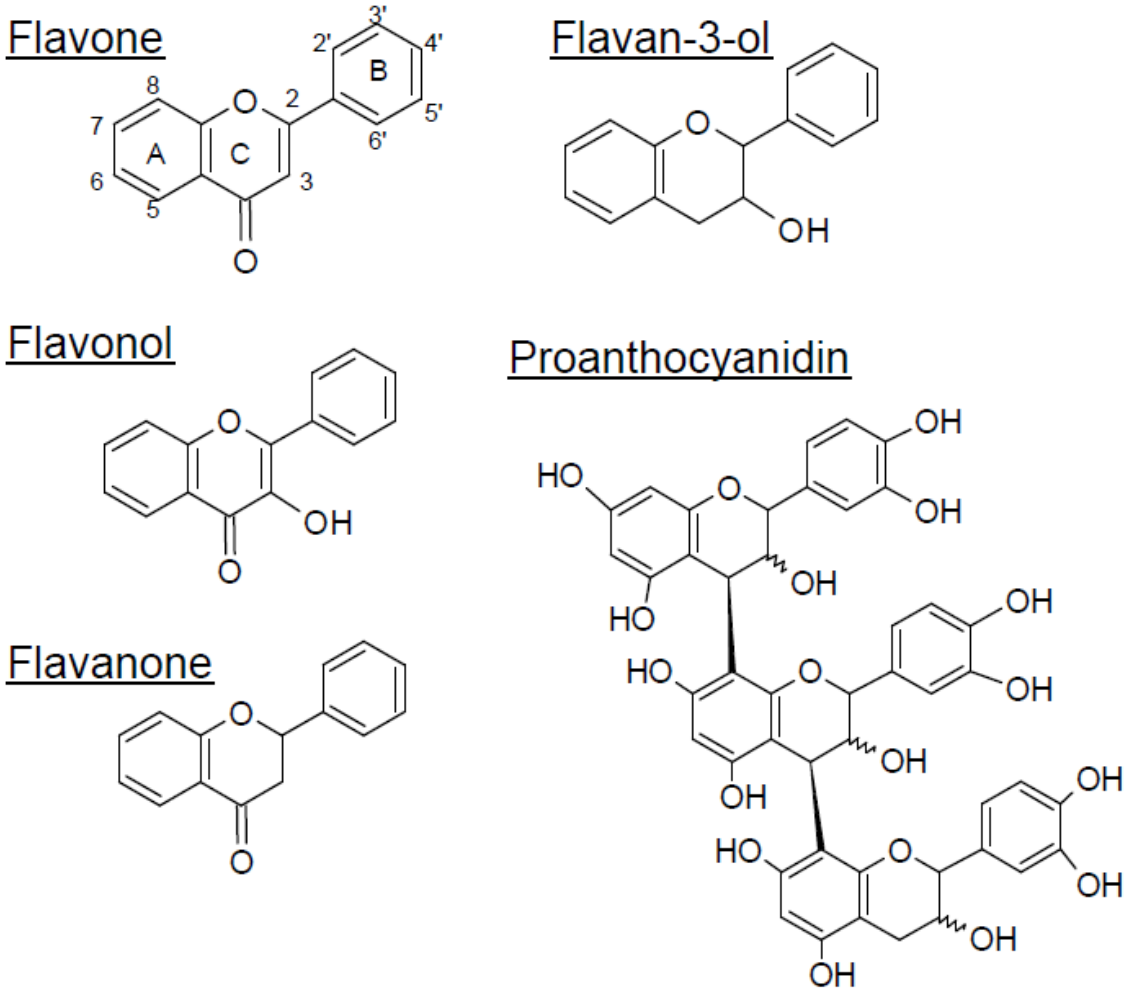

Figure 1.2. Prenylflavonoid phenolic and proanthocyanidin (condensed tannin) structures.

Chapter 2.

Hop derived polyphenols contribute antioxidant capacity and flavor potential to lager beer.

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ABSTRACT

A polyphenol rich extract was isolated from spent hop solids (*Humulus lupulus* L. cv Galena) and dosed into a commercial lager beer at 100 ppm. Beer with (PP) and without (No-PP) added polyphenols was bottled, pasteurized and force-aged in the dark at 21ºC and 29ºC. The polyphenol rich extract was also subjected to phloroglucinolysis and analyzed via reverse-phased chromatography-mass spectroscopy - electrospray ionization (RP-HPLC-MS-ESI) to determine flavonoid content. RP-chromatography of the extract revealed that it was 99% phenolic in nature, however phloroglucinolysis indicated that (+)-catechin and (-)-epicatechin as well as other flavan-3-ols represented only 2% of the material by weight (w/w%) with a procyanidin mean degree of polymerization (mDP) of 2.72. The extract also contained caffeic acid, methylated xanthohumols, flavan-3-ol-glycosides, flavanonol glycosides, flavonol glycosides and other unknown compounds. Chemical analysis of the fresh and aged beers confirmed an anti-staling effect of the dosed polyphenol extract as measured by antioxidant capacity assays: FRAP, DPPH• and ESR. Metal analysis (ICP-AES) demonstrated that the polyphenol extract also preferentially reduced Cu levels (~10 ppb over No-PP beers) in the fresh and aged beers. Sensorially, beers treated with polyphenols were statistically different from beers which did not receive polyphenols. More importantly a significant temperature effect for (decreased) cardboard aroma formation was seen in the PP treated beer stored over 6 weeks at higher temperature.

KEYWORDS: hops; polyphenols; flavan-3-ols; proanthocyanidins; flavonols; beer flavor; antioxidants

INTRODUCTION

Hops (*Humulus lupulus*) provide beer with bitterness, flavor, aroma and microbial stability. Presently, commercial brewers tend to favor the use of processed or specialized hop-derived bittering products [1-7, 80, 87, 88] due to their increased utilization, cost effectiveness, and utility for special applications (i.e. providing light stability). Products such as resin concentrated pelletized hops (prepared via sieving) and resinous extracts (prepared by critical, supercritical or liquid CO_2 extraction or ethanolic extraction) have

largely taken the place of whole or pelletized hop cones, leaving a considerable amount of spent hop solid material as a waste stream. Hop solid material may account for up to 80% of the original hop cone by mass and in terms of flavor, may have a lot more to offer the brewer than has been assumed.

Hop solids are a rich source of polyphenols [6, 80], with hops providing upwards of 20% of a light lager beer's phenolic profile [89, 90]. Of the polyphenols found in beer, the flavanols, their condensed products the proanthocyanidins and flavonols **(Figure 2.1)** receive considerable attention because of their roles as antioxidants in many foods and beverages [8, 20, 91-93]. Flavor changes that occur in beer during storage or under temperature abuse are generally credited to the degradation of beer compounds by reactive oxygen species (ROS) [34, 91, 94, 95]. As antioxidants (**Figure 2.2**), flavanols and proanthocyanidins may act protectively against ROS initiated formation of aged beer flavors. Flavanols, flavonols and proanthocyanidins can also act as natural metal chelators [45, 46] (**Figure 2.3**) that are capable of influencing beer flavor modifying reactions promoted by transition metals, and also play roles in the formation of beer flavor [6, 78, 96-98] and enhancement of beer flavor stability [6, 7, 99, 100]. Depending on degree of polymerization, flavanols also impart both bitterness and astringency: with monomers said to provide bitterness [101, 102] and polymers denoting greater astringency [101-103]. Several studies have considered the impact of polyphenols on beer flavor and flavor stability, yet the results remain somewhat conflicting.

As antioxidants, polyphenols have the potential to influence the oxidative mechanisms responsible for aged beer flavors. Despite their antioxidant potential, some polyphenols can show pro-oxidant behavior and thus both attractive [34, 91-93, 100, 104] and unattractive [34, 68, 70, 105, 106] flavor properties have been attributed to beer polyphenols. Dadic and Belleau [96] dosed purified phenols and polyphenols, as well as their oxidized counterparts, to water and beer to examine their impact on beer flavor. Several of the compounds increased beer bitterness, some denoted a harsh bitterness and others provided increased astringency. Delcour and colleagues [107-109] brewed beers with varying levels of polyphenols by using modified raw materials (proanthocyanidin free malt and tannin-free hop extracts) and commercial materials (regular malt and whole

hops) to test the influence of dimeric and polymeric proanthocyanidins on beer flavor and flavor stability. Differences of 225mg/L in polyphenols failed to test significant in triangle tests and paired-comparison tests indicated that panelists could only detect slight organoleptic differences between the beers. Moreover, panelists judged the no-polyphenol beers as more bitter and more astringent than the control beers. Mikyska and coworkers [100] treated wort with polyvinylpyrrolidine (PVPP) to reduce the apparent polyphenol content of finished beers. Their work concluded that hop and malt derived polyphenols contribute to harsh bitterness. Forster conducted brewing trials that included treatments with spent hop solids [78]. According to Forster spent hop solids contribute pleasant, hoppy, slightly fruity aromas and tastes to beer, even after high temperature storage. Previously published work done at Oregon State University [98] corroborates these results. A polyphenol rich extract was prepared from spent hop solids and dosed into a commercial light lager. Panelists informally described beers to which polyphenol rich extracts were added as having intense 'fig and fruit-like' aromas and a 'more rounded and fuller flavor than the base beer', with considerable hop aroma. However, when dosed into beers at high levels (200 mg/L) the extract imparted beers with an undesirable bitterness.

Antioxidant studies indicate that hop polyphenols ((+)-catechin and ferulic acid) affect beer reducing activity (chemical analysis), formation of stale carbonyls, formation of UV-active compounds and degradation of the *iso*-α-acids [93, 104, 110]. McMurrough and colleagues treated beer with PVPP at 100g/hL to find that removal of polyphenols via PVPP treatment decreases beer reducing capacity by 9-38% as measured by DPPH• analysis. Despite this, the authors could not determine any marked differences in flavor stability of forced aged-lager beer following PVPP treatment [91]. Antioxidant studies conducted by Foster [111] indicate that hop-solid derived extracts can improve the oxidative stability of light and dark beer and fruit juices as measured by Electron Spin Resonance (ESR). However other ESR lagphase studies are conflicting, demonstrating that polyphenols (catechin, phenolic acids, and dimeric proanthocyanidins) may not significantly diminish free radical formation in beer during storage or in wort during brewing [74, 105, 106, 112]. Additionally, other findings indicate that it is the hop

bittering acids (humulones and lupulones) that provide the largest antioxidative protection to beer (as measured by chemical analyses), not the polyphenols [75-77]. Despite these reports, some hop scientists and brewing researchers regard spent hop solids with admiration, insisting that they have something special to offer the brewer in terms of sensorially perceived flavor stability; to date several patents have been filed regarding the use of hop polyphenols or spent hop solids in brewing [6, 7, 79-81].

The objective of this study was to examine the potential for a spent hop-derived polyphenol extract to impact the flavor stability of a commercial pale lager beer. Under the hypothesis that the addition of hop polyphenols would increase the potential staling resistance of lager beer, pale lager beer was dosed with a polyphenol rich hop extract. A domestic pale lager beer produced by a U.S. brewing company was chosen as the testing matrix because of its relative low level of hop derived polyphenols. The spent hop solids were obtained from a commercial hop supplier from the same lot of Galena hops. Galena hops were chosen because they are used to produce CO_2 extracts utilized for bittering commercial lager beers and therefore spent Galena hop material is readily available. Flavor stability was assessed by sensory and chemical analyses.

MATERIALS AND METHODS

Materials. Spent Galena hop powder following CO_2 extraction was generously donated from John I. Haas. Wheat gliadin was obtained from MGP Ingredients Inc. (Atchison, KS). Ferric ammonium citrate (green) was purchased from Fisher Chemicals. Ethylenediaminetetraacetic acid disodium salt, dihydrate (EDTA), *p*-dimethylamino-cinnamaldehyde, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), acetonitrile, pectin and phenolic standards (rutin, quercetin, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO). Flavanoid procyanidin dimers B1, B2 and B3 were graciously prepared as previously described [113] by Hui-Jing Li and Max L. Deinzer of Oregon State University. Grape seed extract was generously supplied by Patrick Ting of MillerCoors brewing company. Low-viscosity carboxymethylcellulose was supplied by Hercules Inc. ICP metal standards (Iron and Copper), and ICP grade HNO_3 and Sodium Acetate Trihydrate were purchased from VWR International, BDH (West Chester, PA, USA). Divergan RS PVPP

was obtained from BASF (Ludwigshafen, Germany). Ferric chloride anhydrous and phosphoric acid were obtained from EMD (Gibbstown, NJ), Trolox was obtained from Calbiochem® (La Jolla, CA). HPLC- solvents, Methanol and 96-well plates were obtained from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid was purchased from Merck kGaA (Darmstadt, Germany). Hydrochloric acid and 2,2,4-trimethylpentane from JT Baker (Phillipsburg, NJ), ammonium hydroxide obtained from Ashland (Columbus, OH). All solvents were HPLC grade. Water was purified to HPLC grade with a Millipore MilliQu apparatus (Bedford, MA).

Total polyphenol (TP) and total flavanoid (TF) content of polyphenol rich extracts and beers. TP and TF were measured according to the EBC Analytica methods (9.11 and 9.12) [114] using a Shimadzu PharmaSpec UV-1700 spectrophotometer, Shimadzu Corporation (Columbia, MD).

Analyses pertinent to antioxidant properties of the beer. The antioxidative activity of the hop treated beers was evaluated by three assays. This first assay, the 'ferric reducing antioxidant power' or 'ferric reducing antioxidant activity of plasma' (FRAP) assay is a simple colorimetric method that measures the ferric reducing (Fe(III) to Fe(II)) ability of the beer sample. FRAP was performed using 96 well microplates according to the methods of Benzie and Strain [115] and Firuzi and coworkers[116]. FRAP results are reported as Trolox Equivalents (ppm TE). The second assay, DPPH• activity, measures the samples ability to quench the stable radical radical DPPH• (2,2 diphenyl-1-picrylhydrazyl radical) and was performed according to modified of the methods of Sanchez-Moreno et al. [117]. A 125 uL sample of beer was pipette into a cuvette (1 cm pathlength) containing 2.3 mL of DPPH• reagent (6.1×10^{-5}M DPPH• in MeOH) and the change in absorbance was monitored at 515nm. The third assay Electron Spin Resonance (ESR) was performed as per the methods of Uchida and Ono [118, 119] with a Bruker EMX 6/1 Electron Paramagnetic Resonance Spectrometer controlled by Bruker WinAcquisition 3.04 Software with AquaX sample cell and 48rpm Peristaltic Pump: Attenuation – 9dB; Power – 25.26 nW; Center Hall Field – 3472.0 G; Sweep Width – 10.0 G, Spin-trap agent: alpha-phenyl-t-butylnitrone (PBN) (Sigma Aldrich), Internal Standard: 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-^{15}N-oxyl (4-OH Tempol)

aqueous solution and External Standard: commercial lager beer (courtesy of MillerCoors Brewing Company).

Metal ions by inductively coupled plasma atomic emission spectroscopy (ICP-AES). ICP-AES for copper and iron was performed according to the ASBC proposed method [120].

Haze. Haze was measured using a Hach 2100 AN Turbidimeter (Loveland, CO). Samples were degassed, tempered to room temperature and haze was recorded in NTUs.

Total Package air. Total package air was conducted according to ASBC Standard Methods (Beer-13).

Benchtop extraction of spent hop-derived polyphenol extract. Spent hop-derived polyphenol rich extracts were first produced at lab scale using two different resins: high-capacity C-18 resin (Alltech Chromatography Co., Deerfield, IL) or Amberlite^{TM} FPX66 Food Grade adsorbent resin (Rohm and Haas, Philadelphia, PA). To 2 liters of water, 100 g (50g/L) of spent Galena hops were added and acidified with acid (3N HCl) to pH 3.0. Acidification was done in effort to minimize solubilization of residual alpha acids. The mixture was heated to 40°C and held there for 30 min. Wheat gliadin (suspended in 95% ethanol) was added to the mixture at 0.5% w/w in order to mimic action of wort proteins in the kettle and boiled for 30 min. Following a coarse filtration with cheese cloth, the extract was refrigerated overnight (4°C), centrifuged (16,000 g, 15 min. at 10°C), alkalized (pH 7, 5N NaOH), treated with EDTA (10g/L) to reduce pro-oxidative metals (Cu and Fe), filtered through a Whatman No. 1 and re-acidified (pH 3.0, 3N HCl). EDTA treated-extract (100mL) was applied to a 6 mL (0.5 g) solid phase extraction cartridge containing either a high-capacity C-18 or Amberlite^{TM} FPX66 Food Grade adsorbent resin, rinsed with 20 mL of MQ water, and the polyphenols of interest were eluted with 10 ml of 95% EtOH. The eluted fraction was kept at -4°C overnight, centrifuged (16,000 g ,15 min., 3°C) and decanted. Extracts were concentrated under vacuum (80% by volume) and dosed at a target rate of 115 ppm (+)-catechin equivalents (TPP method) into 40 mL of lager beer in an equivalent volume of ethanol (up to 2mL). Control beers were prepared by dosing 40 mL of lager beer with 2 mL 95%

ethanol. These samples were frozen and subsequently analyzed for metals by ICP-AES and anti-radical activity by ESR.

Pilot scale extraction method optimization. After the preliminary trials, spent-hop derived polyphenol rich extracts were produced in a pilot facility and polyphenols were isolated using a 4.5×15cm Chromaflex (Kontes, Vineland, NJ) preparative column containing either a high-capacity C-18 or Amberlite^{TM} FPX66 adsorbent resin. Deionized brewhouse water was heated to 40°C in a steam kettle and acidified to pH 3.0, spent hop material (45g /L) was added and mixed while maintaining pH 3.0 (3N HCl) for 30 minutes (40°C), coarse filtered through cheese cloth to remove large particles, then transferred back to a steam kettle. Wheat gliadin was added at 0.5 %w/w or 0.1%w/w. The solution was gently boiled (up to 97°C, 30 min.), transferred hot into a clean, sanitized 15 gallon keg and held at 1°C overnight. The following day, the hop extract was decanted and centrifuged at 8,300 g using a pilot-scale centrifuge (Westfalia Separator, Northvale, NJ, model KA 2-06-075). This aqueous spent hop extract was then concentrated using a Centritherm (Alfa Laval, Lund, Switzerland, 0.8kg/cm^2, 64°C, 59 RPM or 0.7L/min) to 1 L and stored under nitrogen at 1°C overnight. The concentrated extract was further centrifuged 2 x 10,000 RPM (5C), alkalized to pH 7.0 (5N NaOH), treated with EDTA (10g/L), filtered (whatman no. 1) then reacidified to pH 3 (3N HCl). 250 mL of concentrated extract was applied to either the C18 or Amberlite resin, washed with 2 volumes (500 mL) of MQ water and eluted with 95% ethanol. The polyphenol rich extracts were kept chilled, concentrated under vacuum, reconstituted in ethanol (up to 2 mL) and dosed into an unhopped pale lager (100 ppm TPP). Dosed beers and control beers were frozen and subsequently analyzed by ESR and ICP. Because the 0.5% gliadian led to increased undesireable precipitation issues in the final extract, the lower dosing of 0.1%w/w (100g/L Gliadin in 95% EtOH, 0.1% by weight) was chosen for the final pilot scale process.

Pilot scale production of spent hop-derived polyphenol rich extract. The optimized pilot scale extraction was implemented for production of the polyphenol rich extract to be used in the final staling trial. Eight liters of EDTA treated-extract was applied to a 4.5×15cm Chromaflex (Kontes, Vineland, NJ) preparative column

containing a high-capacity Amberlite™ FPX66 Food Grade adsorbent resin, rinsed with 1.0 L of MQ water, and the polyphenols of interest were eluted with 500 ml of 95% EtOH. The eluted fraction was then concentrated under vacuum to an approximate volume of 100 ml, kept at -4°C overnight, centrifuged (16,000 g ,15 min., 3°C) and decanted.

Analysis of polyphenol rich extract by HPLC and HPLC-ESI-MS. Two methods were used to analyze the polyphenol content of the Amberlite extract used in the final staling trial. Total polyphenols were analyzed by RP-HPLC-DAD (Agilent Technologies1100) via the methods of Callemien and Collin [121], using C18 Prevail (Grace Davison Discovery Sciences, Deerfield, IL), 5 um, 250 mm x 4.6 mm-i.d. and accompanying guard column of the same material. Gradient elution was accomplished using water containing 1% acetonitrile and 0.1% formic acid (A) and acetonitrile (B): 97-91% A, 0-5 min, 91—84% A, 5-15 min; 84-50% A, 15-45 min; 50-10%A, 45-48 min; 48-51 min isocratic; initial conditions for 15 min. For the ESI source, the following conditions were applied; negative mode, dry temperature 350°C; dry gas 10.0 L/min; nebulizer 50.0 psi, trap drive 47.5, skim 1 -38.3 volt, skim 2 -6.0 volt, octopole RF amplitude 120.0Vpp, capillary exit -113.0 volt, scan begin 50m/z, scan end 1800 m/z. The Amberlite extract was also subjected to acid-catalysis in the presence of phloroglucinol according to the methods of Kennedy and Jones [122] and subsequently analyzed by HPLC-DAD-ESI-MS (Agilent Technologies) according to modified methods of Taylor et al [123]. The reversed-phase method consisted of two Chromolith RP-18e (100- 4.6 mm) columns connected in series with accompanying guard column (Chromolith RP-18e, 5-2.6mm) all purchased from EMD chemicals (Gibbstown, NJ). The procedure utilized a binary gradient of 1%v/v aqueous acetic acid (A) and acetonitrile containing 1%v/v acetic acid (B). Eluting peaks were monitored at 280 nm: 1.0mL/min; 5% B at 0 min, linear gradient from 5- 10% B, 0-10 min; 10- 30% B 10-20 min; 30-55% B, 20-40 min.; 55- 90%B 40-41min.; 90%B, 41-51 min. The column was washed with 5% B for 5 minutes prior to the next injection. This method was used in place of the previously published one in order to achieve separation necessary for subsequent sample analysis via ESI-MS. Acid catalysis was performed in the presence of

excess phloroglucinol to determine subunit composition of phloroglucinolysis products [122]. (+)-Catechin was used as a quantitative standard and grape seed extract was used to confirm elution times of subunits. Mean degree of polymerization (mDP) of the hop solid Amberlite extract and grape seed extract were determined by summing all subunits (in moles, extension and terminal) and then dividing by the sum of all terminal units (in moles). In this manner a conversion yield (%flavanoids) could also be calculated: sum of the mass of all subunits (minus the mass of phloroglucinol from adducts) divided by the original mass of the material that underwent phloroglucinolysis.

Addition of hop polyphenol rich extract to lager beer for staling trial. Forty eight liters of fresh commercial full strength lager (kegged) was transferred to a clean, sanitized, degassed (CO_2), 15 gallon keg, dosed with 320 ml of hop polyphenol extract (15,000 ppm) to achieve 100 ppm hop polyphenols, shaken, and then transferred to another clean, sanitized keg. This treatment was referred to as the polyphenol-treated beer (PP). A control beer, without polyphenols (No-PP), was prepared in the same fashion substituting 320 ml of 95% ethanol for the polyphenol extract. The PP and No-PP beers were packaged using 355 ml bottles (total package air $\leq$ 0.60 ml) and batch pasteurized at 60°C for approximately 10 minutes (to achieve 25 PU).

Beer Staling Experiment. Bottles of PP and No-PP were force-aged in the dark at 21°C and 29°C and real-time aged under cold storage at1°C for up to six weeks. Samples (6 bottles) were pulled every week (weeks 1 through 6) from the experiment start date and held at 1°C until analyzed. All chemical and sensory analyses were performed on samples from real-time aged and force-aged beers after 3 and 6 weeks to assess impact of temperature abuse and polyphenol dosing on beer staling character.

Analytical Statistical Analysis. An ANOVA was conducted per analytical method for polyphenol addition, temperature, and time effects as well as for temperature × time interaction. Correlation matrixes were constructed and used to compare results of analytical assays to each other as well as to sensory results. Statistical analysis was performed using S-plus software (TIBCO Software, Palo Alto, CA).

Sensory Procedure: Descriptive Analysis. The sensory panel consisted of seven trained panelists (ages 21-54, four males, and three females) two of which were

professional panelists, three OSU staff or faculty, one undergraduate student, and one graduate student. Six of the seven panelists had been extensively involved with previous sensory work regarding beer evaluations. Thirty mL (~one-ounce) samples were presented to the panelists in 12-ounce, red glasses that were capped with clear- plastic, odorless lids. The glass color was successful in masking the beer differences due to haziness present in the polyphenol treated samples which was not present in the samples without polyphenol additions. Samples were poured within one hour of serving and were evaluated at ambient temperature (68˚F/20˚C). Panelists rinsed with spring water after each sample and waited until their palates were free from taste or mouth drying sensations before proceeding to the next sample. Training took place over two weeks (six one-hour training sessions). There were 9 testing sessions which took place over four days with a maximum of 3 test sessions per day. Panelists were required to wait at least 30 minutes between sessions.

The final descriptive ballot was based on consensus terms including six aroma descriptors and three basic taste/mouth feel descriptors: Overall Aroma, Cardboard/Papery, Apple, Tropical Fruit, Caramelized, Sulfide, Bitter Taste, Sweet Taste, and Astringent Mouth Feel. All descriptors were rated on a 16-point intensity scale (0 = none, 15 = extreme intensity) and panelists were provided with aroma and basic taste references to help them identify and agree upon the aroma or taste characteristics of the beer samples.

Sensory Experimental Design. The design was set up to investigate if there were any perceivable sensory changes due to time and temperature applied to beer dosed with polyphenols (PP) and beers without polyphenols (No-PP). Age parameters were 0 wks, 3 wks, and 6 wks and temperature parameters were 1˚C, 21˚C, and 29˚C. For testing, panelists evaluated treated and untreated samples in triplicate three times (3 replications), over 9 testing sessions, each panelist received 6 samples per session resulting in a total evaluation of 54 samples (3 samples of No-PP and 3 samples of PP for each storage temperature x 3 storage temperatures x 2 treatments x 3 replications = 54 samples in total). Panelists evaluated the samples monadically (one at a time).

This was a partially randomized block design which means that within each testing session, panelists received three No-PP samples followed by three PP samples or they received three PP samples followed by three No-PP samples. The serving order of PP first or No-PP first was randomized *across* testing sessions and also *across* panelists so that PP and No-PP samples were seen in the first and second positions an approximate equal number of times.

In addition to blocking on PP or No-PP, randomization took place within replication. This means that the samples in the first rep (PP and No-PP) were evaluated, followed by the samples in the second rep (PP and No-PP), and finally by the samples in the third rep. In addition, samples *within* PP or *within* No-PP were randomized so that each of those three samples were seen in first, second, and third serving order positions an approximate equal number of times.

Sensory Statistical Analyses. An ANOVA was conducted per descriptor for polyphenol additionl, temperature, and time effects as well as for temperature × time interaction. For statistical testing, panelist effect was random and polyphenol, temperature, and time effects were fixed. Statistical analysis was performed using PC.SAS 9.1 (SAS Institute, Inc., Cary, N.C.). Statistically significant sample effects were further analyzed to see where mean differences existed using Tukey's HSD test at the 95% confidence interval ($p \leq 0.05$). For principle component analysis (PCA), panelist data, averaged over replications, were analyzed by factor analysis using the varimax rotation and the covariance matrix (SPSS® v 15.0 (Chicago, IL)).

RESULTS

Extraction method optimization. Initially the spent hop solid material was found to be high in copper, likely a carryover pesticide residue used in the hop fields. Copper is a transition metal that could interfere with the antiradical power of the extracts by ESR and increase pro-oxidative flavor reactions. The polyphenol extraction method was therefore optimized to reduce pro-oxidant metal content by alkalizing the aqueous extract to pH 7- 8 and chelating metals with EDTA. ESR analysis of chelated extracts yielded ESR lag times and T-150 values that were not significantly different than the base beer, which was an improvement over previous extraction conditions. Preliminary bench

top experiments were conducted to compare C-18 resin and Amberlite™ FPX66 resin effect on yield and anti-oxidative quality of the hop polyphenol isolates. Results indicated that the use of Amberlite™ FPX66 resin in conjuction with EDTA alkalization produced isolates equal to or lower in contaminant metals (Cu and Fe) than the control beer and also produced ESR lag times and T-150 values that were significantly better than the base beer **(Figures 2.4 and 2.5)**.

Once extraction conditions were modified to produce extracts with very low residual metal ions and significantly strong antioxidant properties (via FRAP and ESR indices), the extraction procedure was scaled up to pilot scale in preparation for the staling trial. Results of the pilot scale extraction and subsequent preparative chromatography using the two adsorbent resins indicated that extracts prepared using Amberlite resin were higher in total polyphenols, total flavanoids, lower in pro-oxidant metals (Cu and Fe) and had improved lagtimes and T150 values as measured by ESR than extracts produced with C18 adsorbent resin. Therefore, the Amberlite™ FPX66 resin was used to produce the extracts used in the accelerated beer staling trial (**Figures 2.6 and 2.7)**.

Polyphenol characterization of Amberlite Extract. Two HPLC methods were used to gain insight on the polyphenol content and character of the Amberlite polyphenol rich hop extract. The first method allowed for quantification of compounds with a maximum absorbance at 280nm while the second method allowed for identification of subunit composition. Amberlite™ FPX66 resin was used to isolate 300 mL of an ethanolic polyphenol rich extract from 450 mL of a spent hop solid hot water extract (prepared as described above by extracting 45.25 g of spent material in 1 Liter of MQ water). The 300 mL ethanolic polyphenol rich extract was concentrated by rotoevaporation and freeze dried to yield 0.94 g of a light yellow fluffy powder. 3.9 mg of the PP rich powder was dissolved in 1 mL of MeOH and analyzed via RP-HPLC [121]. The PP rich powder contained minor amounts of the flavanol monomers epicatechin, catechin, epigallocaetchin and gallocatechin, as well as small amounts of the dimers B1, B2 and B3 (deduced by elution times of standards) and was determined to be 98.8% phenolic in nature (expressed as (+)-catechin equivalents absorbing at 280nm).

The PP rich powder was also subjected to acid-catalyzed degradation in the presence of phloroglucinol [122]. During acid-catalysis proanthocyanidin interflavanoid C-C linkages are cleaved in a mild acidic solution to form C-4 carbocations that react with a strong nucleophile (phloroglucinol) to form monomer-nucleophile adducts (**Figure 2.8**). In this manner proanthocyanidin dimers and oligomers are broken down into monomeric subunits that can be identified as terminal or extension units (extension-phloroglucinol adducts). A proanthocyanidin rich grape seed extract (determined to be 61% procyanidin w/w) and monomeric standards ((+)-catechin, (-)-epicatechin (-)gallocatechin, (-)-epigallocatechin, rutin, quercetin and caffeic acid) were used in addition to MS-ESI in order to identify subunits and clarify elution times of monomeric subunits.

Consistent with the total phenolics measurement by RP-HPLC, results of phloroglucinolysis indicate that only 2% of the Amberlite extract made from spent hop solids was comprised of procyanidins (flavan-3-ols) by mass (w/w). Analysis of subunit composition results allowed for the determination of the Amberlite extract apparent polymeric polyphenol mDP (2.72). Ten major phloroglucinolysis products were observed that could further be categorized into either extension or terminal proanthocyanidin subunits. Extension subunits consisted of (-)-epigallocatechin, (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate and one unknown flavan-3-ol that was analyzed by ESI-MS (RT = 7.7 min, MW 414), possibly an isomer of (+)-catechin (varying in hydroxylation pattern), likely produced due to heating and alkalization[124] that took place during the extraction and isolation process. Terminal subunits consisted of (+)-gallocatechin, (-)-epigallocatechin, (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate. The predominant subunits by mass were extension units (-)-epigallocatechin and (-)-epicatechin and terminal unit (-)-epigallocatechin. Molar proportions of the subunits are expressed in **Table 2.1**. The predominant subunits by molar ratio were (-)-epigallocatechin-phloroglucinol (16%), (+)-catechin-phloroglucinol (31%), and (-)-epigallocatechin (19%). The ratio of (-)-epicatechin to (+)-catechin was 0.37 (1.7 x more (+)-catechin) and only 5% of the flavanoid content consisted of galloylated monomers.

Given that the Amberlite extract was determined to be nearly 99% phenolic in nature yet proved to be low in proanthocyanidin content, the Amberlite extract was examined post phloroglucinolysis for content of other flavonoid compounds. The proposed identity of some of the unknown compounds is reported in **Table 2.2** (MS-ESI), these include: caffeic acid, methylated xanthohumols, flavan-3-ol-glucosides, flavanonol glucosides (hesperidin), flavonol glucosides (rutin as well as quercetin and kaempferol derivatives) (**Figure 2.9**). The majority of the peaks eluting after 18 minutes were suspected to be flavonol or flavanonol-glucosides. However, we were unable to confidently determine the identity of any peaks eluting past 20 minutes, the time at which flavonol monomers such as quercetin (RT = 24. 5, MW = 302) would elute.

Accelerated beer staling trial. The performance of the hop polyphenols in a beer medium was tested using an accelerated storage protocol. Commercial lager beer was dosed with the hop polyphenol extract, bottled and pasteurized. The bottled beer was stored at 1, 21 and 29°C and samples were pulled weekly and frozen or stored at 1°C until analysis.

Polyphenols and Total Flavanoids content of the beers. Hop polyphenol extracts were dosed into a commercial full strength lager at a target of 100 ppm of added hop polyphenols (PP). A control beer was also prepared by dosing commercial lager with an equivalent volume of 95% EtOH (No-PP). Beers were monitored at weeks 0 (fresh), 4 (3 weeks storage) and 7 (6 weeks of storage) of the staling trial to assess whether or not polyphenol levels change during storage. Two spectrophotometric analyses were used to assess polyphenol level of the beers. The first assay, the total polyphenols (TPP) assay is a general assay that measures the beers reducing capacity. Overall the dosed (PP) and undosed (No-PP) beers were significantly different in levels of total TPP ($p < 0.01$) (**Figure 2.10**). Beers dosed with polyphenols resulted in 144 ppm total polyphenols. Un-dosed beers (No-PP) resulted in 81.2 ppm total polyphenols. After three weeks of storage TPP increased in the dosed and the undosed beers, regardless of treatment, with samples stored at 29°C scoring significantly higher in TPP ($p < 0.01$ for both). PP dosed beers saw a significant increase in levels of TPP up to week 6, regardless of storage temperature. Inversely, undosed force-aged beers (No-PP)

decreased in TPP content slightly from week 3 to week 6. Untreated beers stored at 29°C were also significantly higher in TPP than those stored at 21°C ($p < 0.001$).

The second spectrophotometric analysis used to assess polyphenol levels is the total flavanoids (TF) assay. The TF assay is specific to flavan-3-ols in that it relies on a condensation reaction with the flavanol C-ring and results are reported in (+)-catechin equivalents (CE). Beers dosed with PP were significantly higher in TF than undosed beers: (PP = 35.6 CE, No-PP =19.8 CE, $p < 0.001$). When considering the PP dosed beers separately TF decreased slightly after three weeks of storage, regardless of temperature ($p < 0.01$), but increased back to starting levels after 6 weeks of storage. The No-PP beers did not experience any significant changes in TF due to storage time or temperature, however beers stored at 21°C increased slightly in TF by week three and then returned to starting levels by week six. No significant changes in TF levels occurred for No-PP beers stored at 29°C.

Metals by ICP. Metal analysis (Cu and Fe) for all samples was performed by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES). Commercial retail samples of fresh and aged (29°C, > 6 weeks) lager beers were also tested for metal composition. Results indicate that PP treatment of the commercial lager significantly reduced ($p < 0.01$) total metals (Cu and Fe, ppb) from about 60 ppb (No-PP) to 50 ppb (PP) at week 0. Furthermore, the PP beers also contained lower total metals than both the fresh and aged retail commercial lager samples. The decrease in metals was driven by significant reduction in Cu levels ($p < 0.001$). Fe levels were not significantly different in the treated and untreated beers ($p = 0.14$). Aging of both treatments (PP and No-PP), as well as the retail lager, did not significantly affect total metals at either storage temperature (**Figure 2.11**). The undosed beers stored at 29°C experienced a slight increase in total metals after six weeks, which was driven by an increase of about 10 ppb Cu.

Haze by Nephelometry. Beer haze (NTU) was monitored for both treatments (PP and No-PP) at 0 weeks, and after 3 and 6 weeks of storage under forced aging at 21°C and 29°C. Beer samples were poured into clean beakers, sonicated to remove gas and tempered to room temperature before analysis. Forced aging had no effect on the haze

levels of the No-PP control beer at either storage temperature (**Figure 2.12**). The PP treatment produced roughly a five-fold increase in haze in the fresh beers as compared to the No-PP treated beers (PP = 9.6 NTU, No-PP = 1.71 NTU). By three weeks storage the PP treated beer showed a slight haze increase from 9.6 NTU to 12.4 NTU for both temperature treatments, which in turn nearly doubled by the end of the 6 week study.

Chemical antioxidant measurements. Three analytical assays were employed to determine the chemical antioxidant capacity of the beers: Ferric Reducing Antioxidant Power (FRAP) and 2,2 diphenyl-1-picrylhydrazyl radical (DPPH•) bleaching assay, and Electron Spin Resonance (ESR) spectroscopy. A high FRAP reading is indicative of a higher anti-oxidant content of beer. As observed in the laboratory scale extraction optimization experiments, the addition of 100 ppm of hop derived polyphenols dramatically increased ($P<<0.001$) the antioxidant power of the final beer as measured using the FRAP assay (**Figure 2.13**) from 996 TE for the No-PP beer to 1742 TE for the PP beer. FRAP values remained constant throughout storage for the PP treated samples and increased during storage for the No-PP beers ($p = 0.006$), with negligible differences seen between the two storage temperatures.

The DPPH• bleaching assay is a complimentary chemical analysis to the FRAP assay. Two different DPPH• indices were used to gauge antioxidant power: (1) integrated area of the decay in absorbance (bleaching) during the first 10 minutes of the assay and (2) the bleaching half life (sec) of the absorbance decay curve. Larger integrated area represents lower bleaching power while longer half life indicates a slower bleaching rate – both represent lower antioxidant power relative to larger areas and longer half lives. The addition of polyphenols to the commercial lager resulted in significant improvement in antioxidant power as measured by the DPPH• bleaching assay (**Figure 2.14**), decreasing the half life significantly ($p = 0.05$) from 157.50 to 123.0 seconds for un-abused beer. Unlike the FRAP results which remained constant with time, the DPPH• area and half life in the PP treatment rose significantly ($p < 0.01$) over the 6 weeks of storage to meet that of the No-PP control, especially under high temperature storage (PP = 158.0 sec, No-PP = 160 sec, 6 weeks, 29°C). The No-PP treatments did

not change significantly in anti-oxidant power as measured by the DPPH• assay during storage at either storage temperature.

Electron Spin Resonance (ESR) spectroscopy is used to detect radicals in beer. Beers with greater endogenous antiradical concentration are thought to have delayed radical formation during the ESR analysis. ESR is commonly used by larger commercial breweries to detect radicals involved in beer flavor degradation reactions, as such ESR has become an important tool for evaluating the flavor stability of beer and predicting beer shelf life [118, 119, 125]. Beer samples were pulled at week 0, and after 1, 3 and 6 weeks of accelerated storage at 21°C and 29°C, frozen immediately in 50 mL test tubes and kept frozen until analysis. Beers were also pulled from force-aging during week 4 and then kept at 1°C for analysis at week 7 (temperature abused for 3 weeks and then stored at 1°C for 3 weeks). A common index in ESR measurement is the lagtime (delay or lag phase in radical formation) and the T150 value (ESR signal at 150 minutes into the run). The anti-radical capacity (expressed as T150 and lagtime) of the beers is reported in **Figure 2.15.** A low T150 (radical concentration after 150 minutes of analysis) and a long lagtime are indicative of increased shelf stability or flavor stability. Polyphenol dosed beers showed improved flavor stability potential over the control (No-PP) beers at week 0. The No-PP beers scored 31% higher in T150 values and 16.1% lower in lagtime than the PP-dosed beers. Interestingly trends for T150 values increased slightly (decreased flavor stability) during storage after 1 and 3 weeks, and then T150 values decreased (increased flavor stability) again after 6 weeks of storage. This was true for both the No-PP and PP-Dosed beers, regardless of storage temperature, with the effect more extreme in the PP-Dosed beers. For example PP dosed beers showed substantially reduced anti-radical capacity/flavor stability (expressed as T150 values) through the initial weeks of aging, then between the 3rd and 6^{th} week of storage the anti-radical capacity increased drastically again. The exception to this was for the No-PP beers stored at 21°C which actually decreased slightly in T150 after 1 week of storage with no noticeable changes after 6 weeks of storage. The same but reverse trend was true for lagtime values. Something of interest to note was that No-PP beers showed slightly improved anti-radical capacity after six weeks of storage at 1°C. Another noteworthy

observation was the anti-radical capacity improved radically for beers that were removed from accelerated storage after 3 weeks of temperature abuse and placed back into cold storage for 3 weeks at 1°C. Returning the abused beers to cold storage actually improved the shelf life/flavor stability scores of the beers over their original scores recorded before any temperature abuse took place (T150 values), regardless of the abused storage temperature.

Sensory Results. Sensory data were analyzed to answer these two questions: 1) Did differences exist due to polyphenol treatments versus beers not treated with polyphenols, and 2) within each of the PP and No-PP treatments, did differences exist due to temperature and time of storage? Results of ANOVA are reported in **Table 2.3** and spider plots showing overall mean scores and changes with time for each treatment are reported in **Figures 2.16 and 2.17**.

Across PP and No-PP (PP Effect). Beers treated with polyphenols (PP) were statistically different from beers which did not receive polyphenols (No-PP) ($p < 0.01$) for all descriptors except Tropical Fruit aroma; however, for each descriptor tested, there was also a significant interaction (polyphenol-by-panelist) effect ($p < 0.01$). Therefore the relevance of this significant main effect must be interpreted cautiously on a descriptor-by-descriptor basis.

Across all attributes there was a separation of PP and No-PP as seen in the PCA Map (**Figure 2.18**). A PCA takes into account all the descriptors at once and then allows you to see the relationship of the descriptors to each other and how these descriptors combine (on the positive or negative end of a principle component) to characterize the samples. PP samples fell on the positive end of PC1 and were characterized as having higher overall aroma, caramelized aroma, bitter taste, and to a lesser degree cardboard aroma, whereas, No-PP samples fell primarily on the negative end of PC1 and these samples can be described as having sulfide aroma, apple aroma, and sweet taste (**2.18**). PC1 accounts for 40% of the total variability in the samples. The general conclusion from the PCA is that the addition of polyphenols created a beer that was discernibly different than the untreated control in terms of aroma and flavor. While the addition of the polyphenols appears to have resulted in stale/aged flavors relative to the No-PP control, within the PP

treatment there appears to be some suppression of staling flavors, namely cardboard aroma, with temperature and/or time.

Examining Temperature and Time Effects within PP and No-PP separately. We predicted from the outset (based on previous work with hop polyphenols) that the addition of the polyphenols would likely change the flavor of the base beer. Yet, our interest in this study was to learn whether the addition of the polyphenols retarded staling, thus we were looking for time, temperature and/or time×temperature interactions within each of the two treatments. There were significant time and temperature effects for several different attributes within beers treated with polyphenols as well as with the No-PP controls (**Table 2.3**).

Aroma and Flavor attributes. For both PP and No-PP, there were significant time effects for the Overall Aroma scores ($p < 0.05$). The intensities of overall aroma increased across time regardless of the polyphenol treatment. Overall, the Apple attribute was higher for the No-PP control than the PP treated beers. Within each of the treatments there was no significant change with time or temperature. Tropical Fruit scores for the No-PP indicated that there was a significant temperature effect ($p < 0.01$); 21˚C samples (mean 2.4; 3 = ”slight”) were rated significantly higher than 29˚C (mean 1.9) and 1˚C were not significantly different from 21˚C or 29˚C samples. Within the PP treated there was no effect of time or temperature. The Cardboard/papery descriptor was higher overall in the PP treatment relative to the No-PP treatment ($p<0.01$) albeit just slightly higher. However, within the PP treatment, there appeared to be some evidence of staling reduction in that the Cardboard descriptor was lower in the 21˚C storage compared to the 1˚C control. Conflicting with this was the fact that the 29˚C treatment was similar to the 1˚C control. Interesting results were observed for the Caramelized descriptor. While it was higher overall for the PP treatment, there was a significant suppression on the increase in this aroma over time in that treatment. In contrast, the No-PP control had low levels of Caramelized to begin with and a significant rise with time and temperatures ($p \leq 0.01$). This result offers some preliminary evidence that the polyphenols may retard the formation of Caramelized aromas with storage time. For Sulfide there was not a significant temperature or time effect but there a significant temperature ×time interaction

($p < 0.05$) for the No-PP treatment. Overall, the PP treatment resulted in significantly lower Sulfide aroma. The PP treatment contributed higher bitterness ($p < 0.01$) relative to the No-PP control. For No-PP, there were significant temperature and time effects ($p < 0.05$), in that the beers stored at 21˚C and 29˚C increased slightly in bitterness during storage, while No-PP beers kept at 1˚C did not increase in bitterness. Although the PP treated beers were higher in bitterness at week 0, there were no significant changes due to storage, regardless of storage temperature. Conversely the No-PP samples were characterized as having sweeter taste than the PP treated samples. This may have been due in part to the increase in bitterness in the PP treatment. Panelists gauged the PP treatment to be higher in astringency compared to the No-PP control. However panelists did not find significant changes in beer astringency due storage time or temperature.

DISCUSSION

Preliminary extraction of spent hop solids indicated that the material can be a significant source of copper. Because copper and other transition metals are well known to increase aged flavor formation by participating in ROS promoting reactions such as the Fenton and Haber Weiss reactions and transition metals may negatively interfere with ESR anti-radical potential measurement, it was necessary to direct modification of the extraction procedure toward limiting the copper content of the final ethanolic extract. The final hop extraction procedure using EDTA at pH 7-8 and Amberlite™ FPX66 resin produced a hop polyphenol extract with very low levels of residual transition metals and higher levels of total polyphenols. The Amberlite™ FPX66 resin extract resulted in dramatically improved ESR and FRAP readings. The extraction of spent hop solids on a pilot scale yielded a larger quantity of this extract which was then added to a commercial lager beer for an accelerated staling trial.

Analysis by RP-HPLC indicated that the polyphenol rich powder was nearly 99% phenolic in nature (absorbance at 280nm, expressed as (+)-catechin equivalents), and contained relatively low amounts of flavan-3-ol monomers, small amounts of procyanidin B-type dimers, and a larger quantity of later eluting compounds (likely flavonol and flavanonol glycoside species). Because Amberlite™ FPX66 is a food grade resin used by the commercial juice industry to extract phenolics, it was not surprising that the

lyophilized extract was predominantly phenolic in nature, as assessed by HPLC. Acid-catalysis in the presence of phloroglucionol [122] allowed for the quantification of flavan-3-ol monomeric subunits and estimation of mDP. Although other authors report finding much higher yields of flavan-3-ols and procyanidins from hops [123], it is not surprising that the Amberlite extract was comprised of only 2% flavanoid material by mass (w/w) with a mDP of 2.72. The initial aqueous extraction of the spent hop solids was designed to mimic the extraction of hop phenolics during the kettle boil, at green wort pH, and in the presence of barley proteins, in this experiment gliadin. During kettle boiling proteins, polyphenols and carbohydrates interact and complex to form hot trub or hot break [32]. In this manner, a fair amount protein-reactive polyphenols (mostly dimers and oligomers) [58] may be removed from the hot wort. Thus it is not surprising that the aqueous extraction of the spent hop solids in a hot, acidic kettle produced a final ethanolic extract low in oligomeric flavanoid species and potentially higher in glycosidic flavonol (rutin as well as quercetin and kaempferol derivatives) and flavanonol species (hesperidin) that less favorably react to crosslink and precipitate barley proteins in the kettle. Flavonols of these species have been determined in beer and hops previously [15, 52, 126-128].

The performance of hop polyphenols in a commercial lager beer was tested using accelerated storage (force-aging) where bottled beer was stored at 1, 21 and 29°C. This resulted in PP-dosed beers being higher in TPP (33 ppm) and TF (15.8 CE) than the No-PP dosed beers. TPP increased in the beers after three weeks of storage, regardless of treatment, with samples stored at 29°C scoring significantly higher in TPP ($p < 0.01$ for both). PP dosed beers saw a significant increase in levels of TPP up to week 6 at both storage temperatures, however No-PP beers decreased slightly in TPP from week 3 to week 6. Untreated beers stored at 29°C were also significantly higher in TPP than those stored at 21°C ($p < 0.001$). Slight decreases in levels of TF were seen after 3 weeks of storage for both the PP and No-PP beers, with levels rising back to original values after 6 weeks (although slightly lower). Changes in phenolic content due to processing and storage in foods and beverages has been reported previously [129, 130]. Flavonoids have been known to undergo de-polymerization, epimerization and other chemical

modifications during storage [130, 131] and temperature abuse which could cause apparent levels of TPP measured by spectrophotometry to change. It is interesting to note that the levels of TF did not change significantly over time. This could partially be explained by the fact that the flavanoids contribution of the phenolic extract was determined to be lower than anticipated as measured by HPLC analysis.

Phenolic compounds of the flavonoid family are well known contributors to beer haze via protein-polyphenol complexation [4, 23] and are also recognized as transition metal chelators [45, 46]. Thus increased haze formation and decreased metal content seen in the PP dosed beers were not unexpected. Haze formation and metal reduction correlated well with increased TPP levels ($r = 0.93$, $r = 0.83$). Yet equivalent correlations were not seen with TF content, potentially suggesting that haze formation depends more on concentration of another species of flavonoid (not the flavan3-ols) and that metal chelating ability of the PP dosed beer is only somewhat, but not heavily, dependent on flavanoid content. A noteworthy observation was that the hop polyphenol extract preferentially chelated and effectively lowered the measureable content of copper ions (fresh = 7 ppb, aged = 15 ppb) in the commercial lager beer, but the same was not true for iron. This could be due to flavanol/flavonol/flavanonol preference for metal chelation of specific species of Cu and Fe. Flavones chelate Cu (II) (pH 7.4 and 5.5) between the 5-hydroxyl and 4-oxo group. At increased pH, the flavonols myricetin and quercetin and the flavan-3-ol (+)-catechin chelate Cu(II) at the ortho-catechol group of the B-ring. It is important to note that at beer pH, (+)-catechin would not effectively chelate copper, but flavonols would [49]. Although metal speciation (Fe II vs. Fe III and Cu I vs. Cu II) was not investigated in this study, some authors have investigated metal speciation in beer [132, 133] . The apparent ability of hop polyphenols to chelate and effectively reduce Cu and Fe ions in beer is noteworthy due to the fact Cu and Fe are free radical catalyzing transition metals with potential to act as pro-oxidants [26, 32, 134, 135]. Thus their effective reduction in the beer matrix during storage could increase beer shelf stability, even during high temperature storage as seen in this experiment. Further investigation of the relationship between metal speciation and polyphenol-chelation could lead to insights

on how these beer attributes affect mechanisms responsible for aged beer flavor formation.

Because the FRAP assay is a reduction-capacity assay similar in nature to the TPP assay, it was not unexpected that FRAP results correlated well with TPP results (r = 0.88). TF results were less correlated with FRAP results (r = 0.71), indicating that the FRAP assay may be more reactive to other classes of polyphenols besides flavan-3-ols and their oligomers. Conversely, TPP and TF results did not correlate as well with both DPPH• metrics. Although fresh PP-dosed lager beers showed increasing anti-oxidant capacity via DPPH• analysis, the DPPH• area and half life in the PP treated beers rose significantly over 6 weeks of storage, indicating a loss of anti-oxidant power due to storage. The No-PP beers did not experience any loss in anti-oxidant power as measured by the DPPH• assay. FRAP results correlated well with a decrease in DPPH• area (r = -0.77) but not for DPPH• half life. On the other hand, TF results correlated better with decreased DPPH• half life (r = -0.64) and not with DPPH• area. Neither metric for phenolics correlated well with ESR metrics for T150 and lagtime, yet ESR results indicate that dosing PP into lager beer may increase beer shelf stability. The No-PP beers scored 31% higher in T150 values and 16.1% lower in lagtime than the PP-dosed beers. Some interesting trends were observed with changes in ESR metrics during aging: trends for T150 values increased slightly (decreased flavor stability) during storage after 1 and 3 weeks, and then T150 values decreased (increased flavor stability) again after 6 weeks of storage in both the No-PP and PP- dosed beers, regardless of storage temperature. The same but reverse trend was true for lagtime values. Something of interest to note was that No-PP beers showed slightly improved anti-radical capacity after six weeks of storage at 1°C and beers that experienced force-aging that were returned to cold storage for three weeks showed improved flavor stability (T150). Reasoning behind this remains unresolved at this time. Further investigation of this phenomenom is warranted.

Sensory analysis of the aged beers indicated a significant effect due to the addition of hop polyphenols across nearly all descriptor attributes, excluding Tropical aroma. PP samples were characterized as having higher overall aroma, caramelized aroma, bitter taste, increased astringency and a lesser degree of aging induced cardboard

aroma. Increased aroma derived from spent hop solid addition to beer has been previously described [6, 78, 79, 97], with some authors purporting that these observations could be related to glycoside flavor precursors found in the spent hop solid material[98] The increased characterization by attributes such as astringency and bitterness are not surprising; PPs ranging from low to high molecular weight may elicit an astringent response [103] and flavan-3-ol monomers such as (+)-catechin and (-)-epicatechin are known to impart bitterness to beer [98, 101, 102]. No-PP beers were described as having sulfide aroma, apple aroma, and sweet taste.

CONCLUSION

A polyphenol rich extract was isolated from spent hop solids using Amberlite™ FPX66 resin. Dosing this extract into a commercial lager beer produced a beer with increased antioxidant potential that was judged by tasters to be different than the untreated control lager beer in terms of aroma and flavor. Moreover treatment of a commercial lager with polyphenol extracts of this nature provided beers with a suppression mechanism for formation of some staling flavors with time and/or temperature, namely in the case of Cardboard aroma.

AKNOWLEDGEMENTS

The authors would like to acknowledge Jeff Clawson of Oregon State University, Scott Garden of John I. Haas, Roman Ortiz and Dana Sedin of MillerCoors Brewing Company, and the Hop Research Council for providing financial support for this work.

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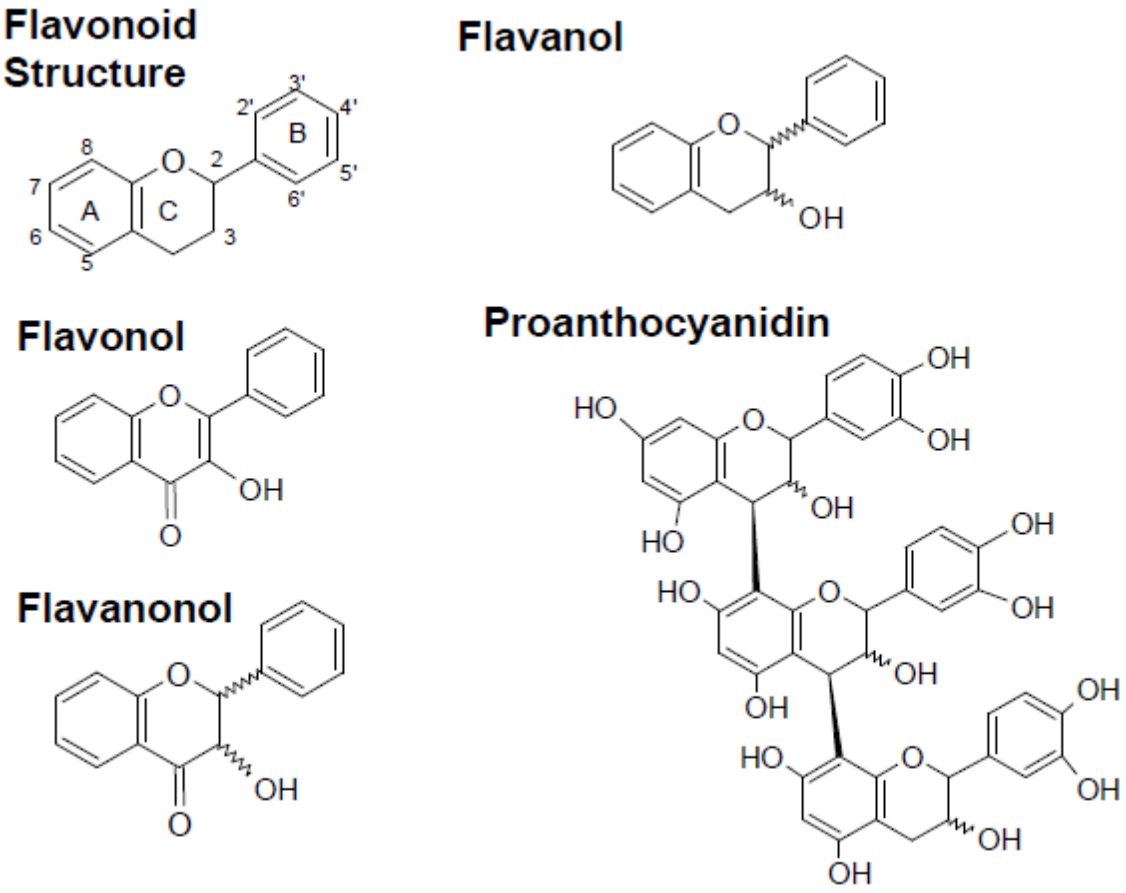

Figure 2.1. Flavonoid structures: flavanols, flavonols, flavanonols and proanthocyanidins

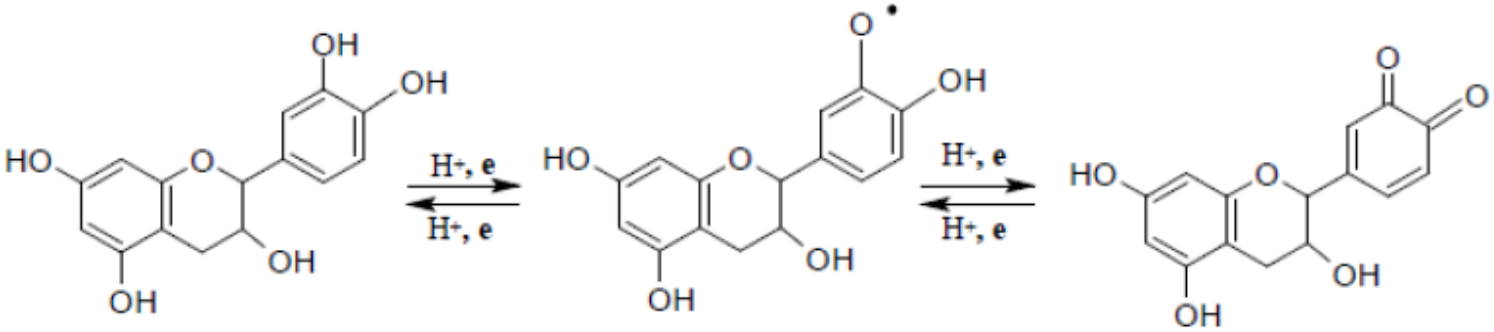

Figure 2.2. Antioxidantive mechanism of proanthocyanidins and flavonoid monomers; oxidation leads to formation of semiquinone radicals.

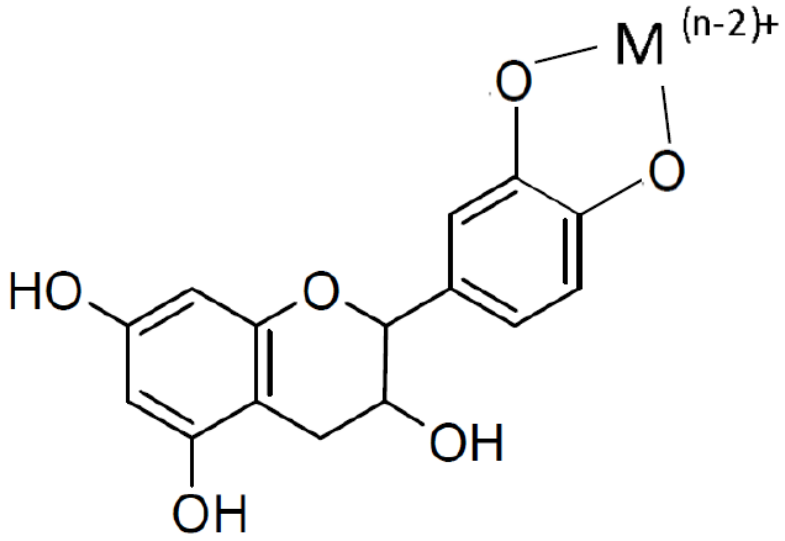

Figure 2.3. Flavan-3-ol mechanism of metal chelation between the ortho-hydroxyls (2’ and 3’) of the B-ring.

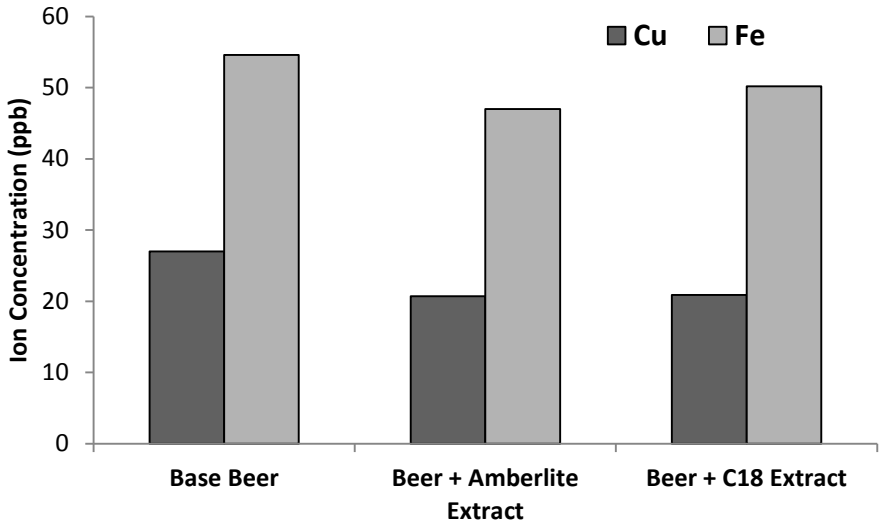

Figure 2.4. Copper and ion content in commercial lager beer due to polyphenol addition: Effect of resin type

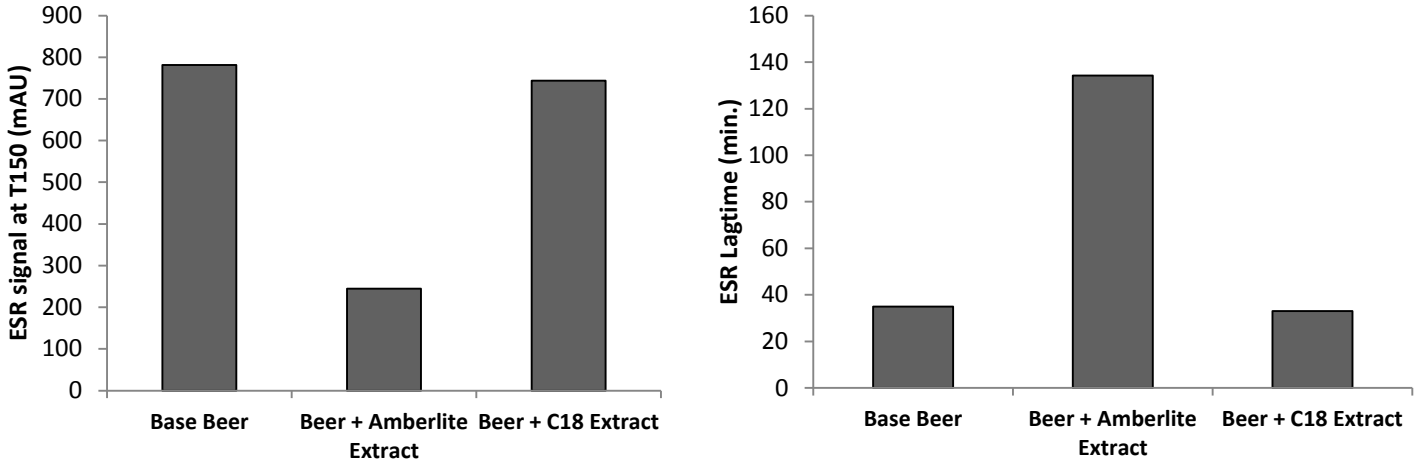

Figure 2.5. ESR, T-150 and lag times in polyphenol dosed commercial lager beer (Base beer = 65 ppm total polyphenols, Beer + Extracts = 200 ppm total polyphenols).

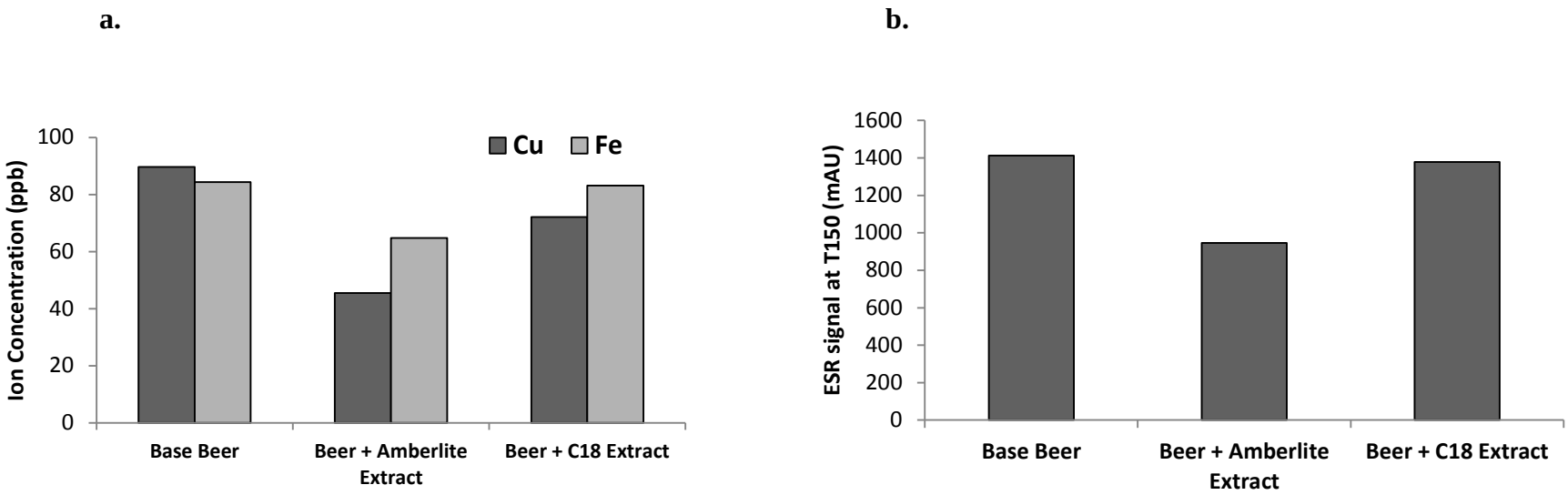

Figure 2.6. Pilot scale *filtered all-malt lager **beers: Effect of resin type **a.** Copper and ion content effect of resin type **b.** ESR T150 *Filtration with diatomaceous Earth **Produced at Oregon State University.

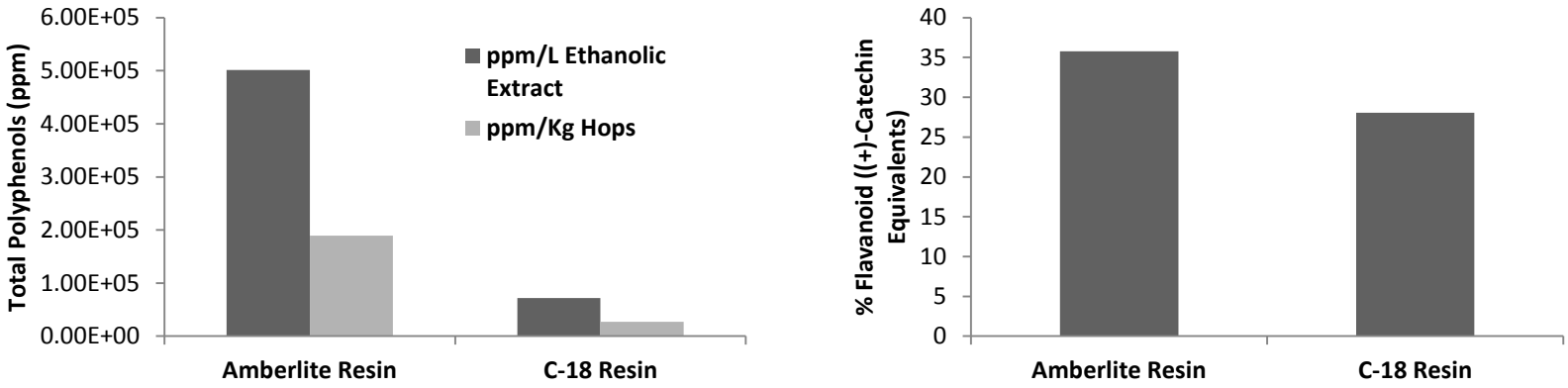

Figure 2.7. Effect of resin type on polyphenol content: a)Total polyphenols (ppm) by volume of extract and by mass of original spent hop solid material (Kg). b) Percent of polyphenols that are flavanoid (+)-catechin equivalents.

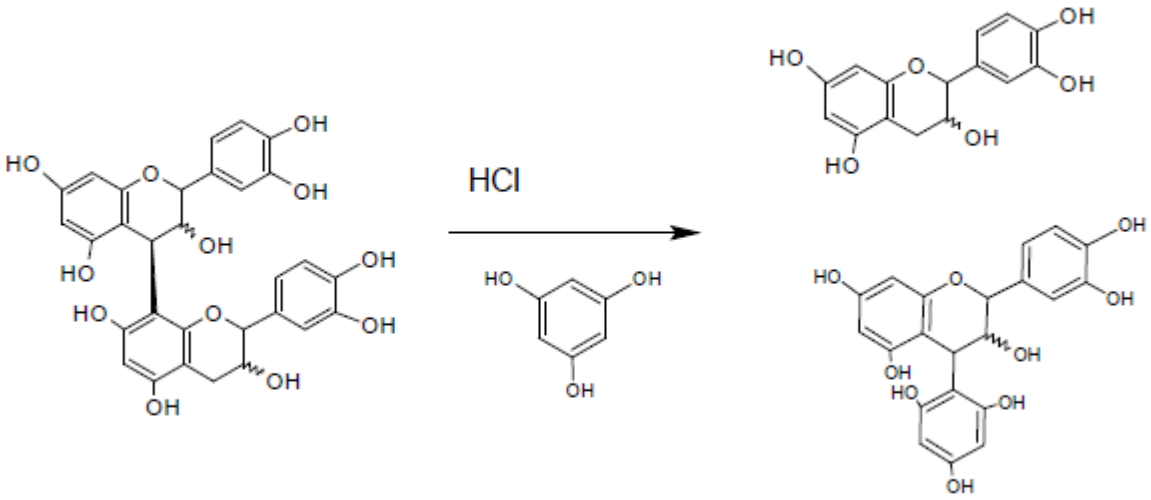

Figure 2.8. Acid catalysis degradation of proanthocyanidins in the presence of phlorglucinol produces monomeric subunits and subunits with phloroglucinol adducts.

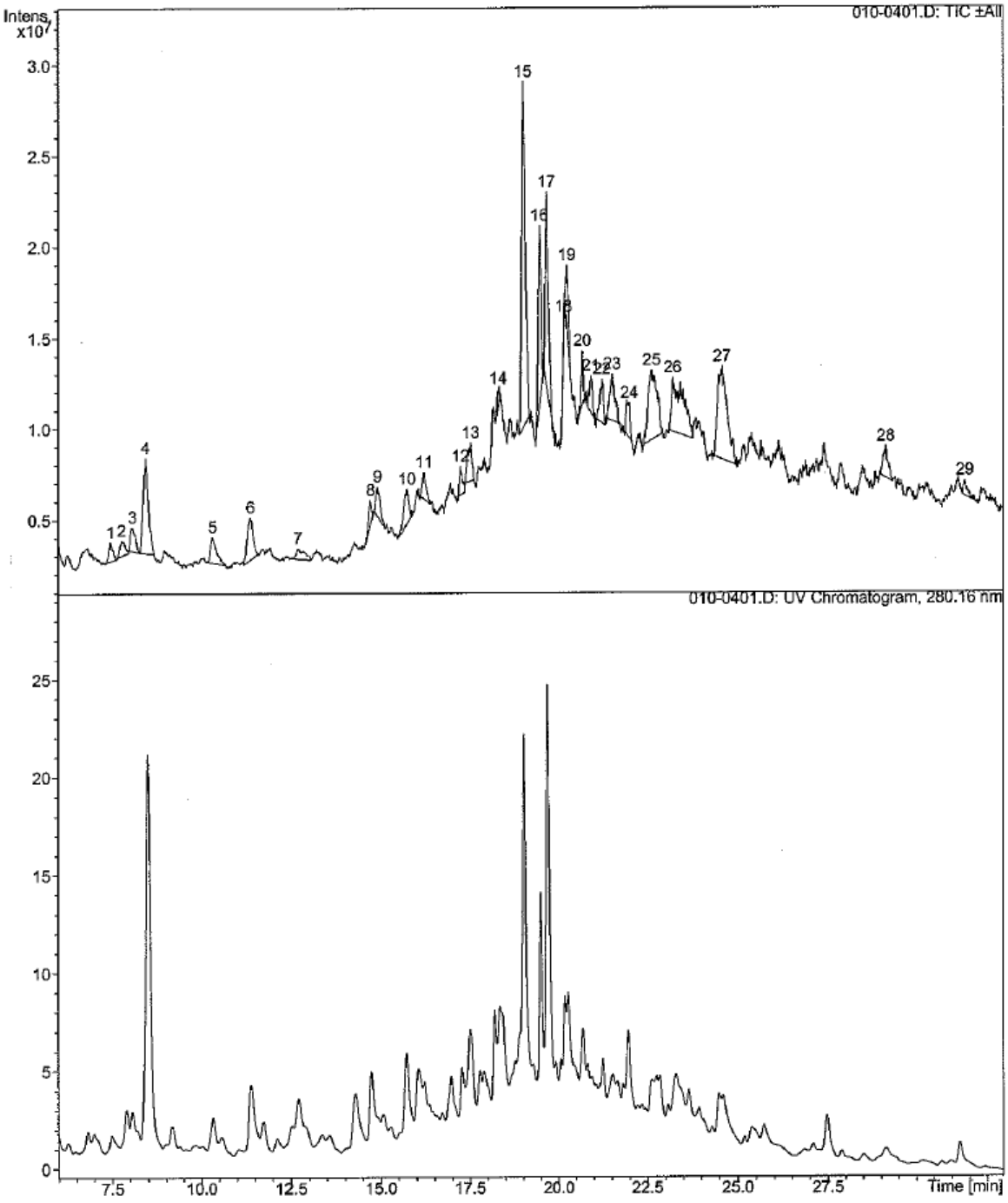

Figure 2.9. RP-HPLC-MS-ESI Total ion chromatogram (top) and UV chromatogram showing peaks absorbing at 280 nm (bottom) following phloroglucinolysis. Peaks are identified in Table 2.2.

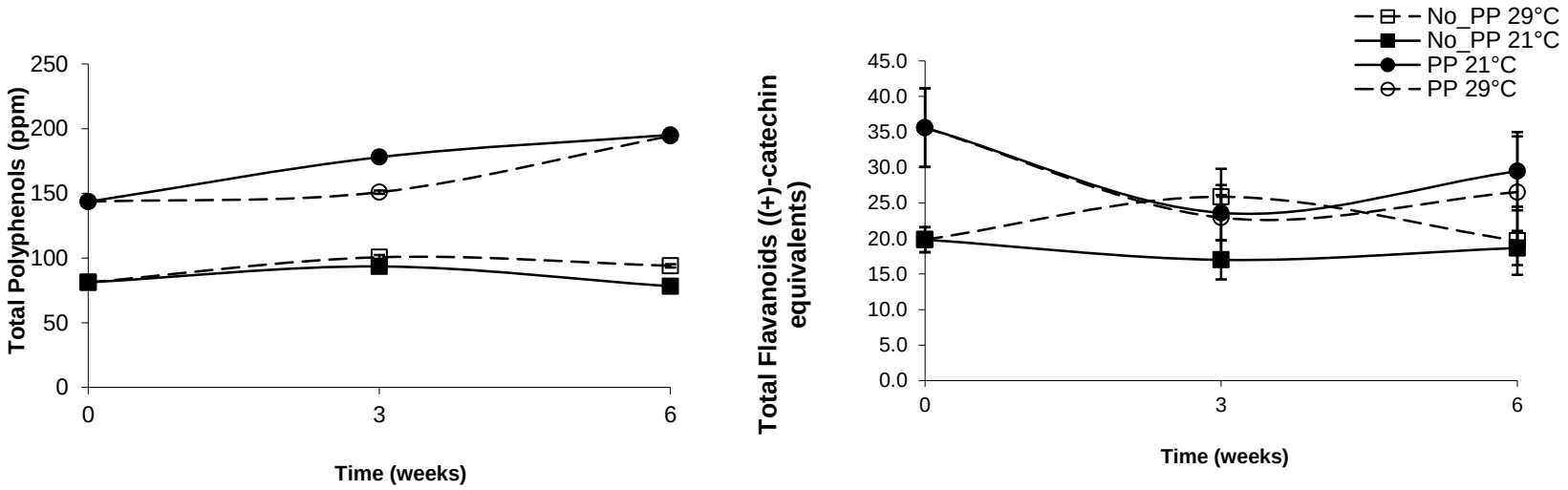

Figure 2.10. Total polyphenols and flavanoids polyphenol treated (PP) and non treated (No-PP) commercial lager beer.

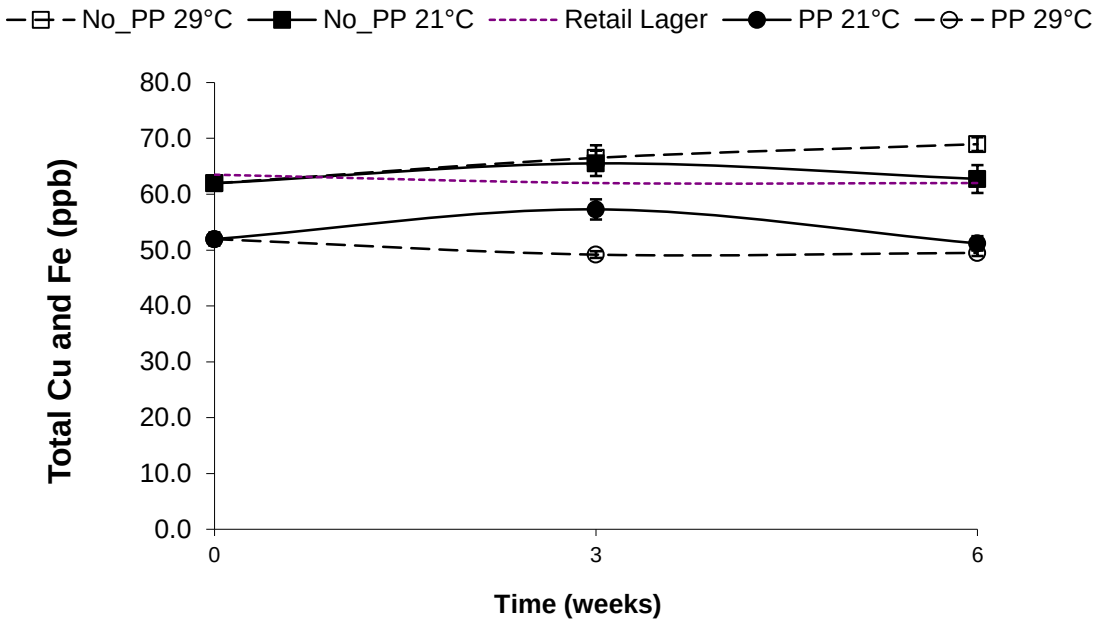

Figure 2.11. Copper and iron levels in polyphenol treated (PP) and non treated (No-PP) commercial lager beer.

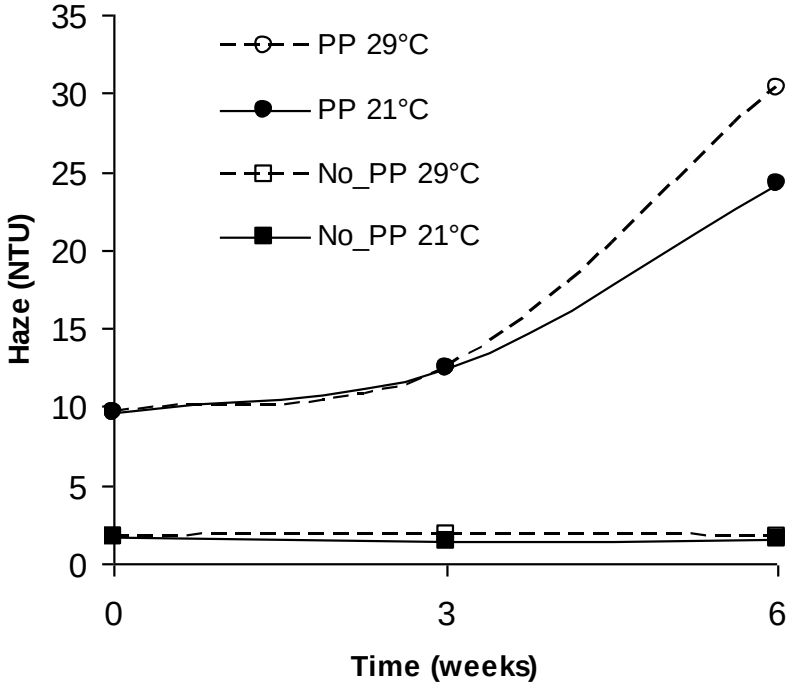

Figure 2.12. Beer haze as a function of polyphenol treatment (polyphenol treated (PP) and non treated (No-PP)) during storage at varying storage temperatures.

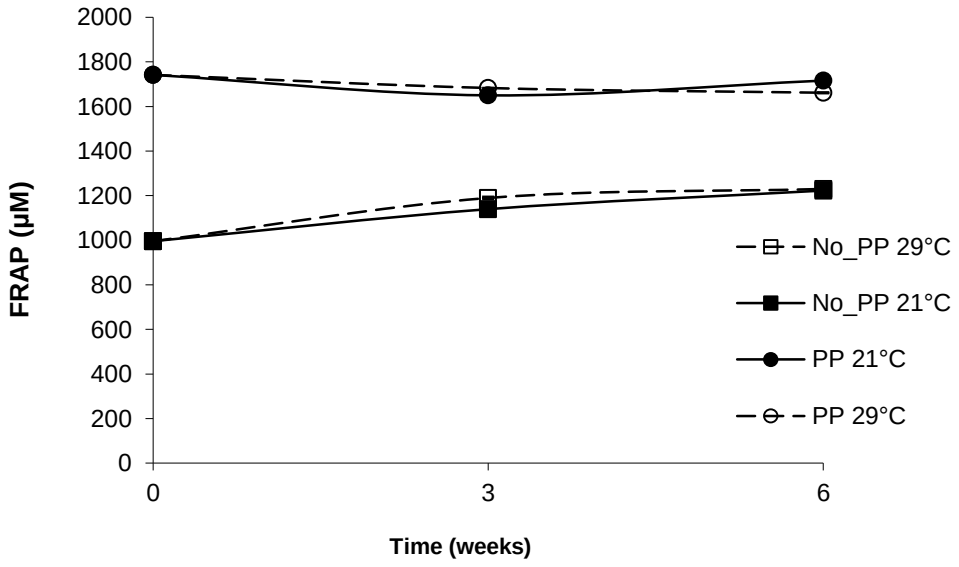

Figure 2.13. FRAP results for polyphenol treated (PP) and non treated (No-PP) commercial lager beers during storage at varying storage temperatures.

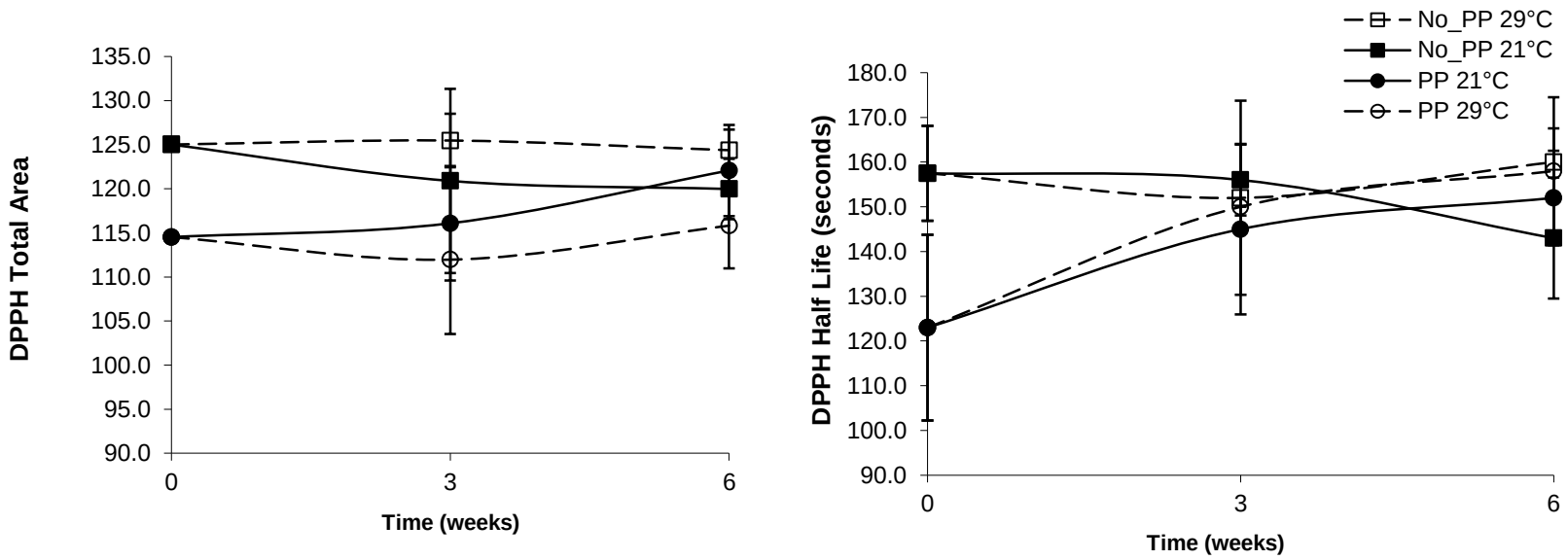

Figure 2.14. DPPH bleaching indices: integrated area (absorption over 10 minutes) and bleaching half life (sec).

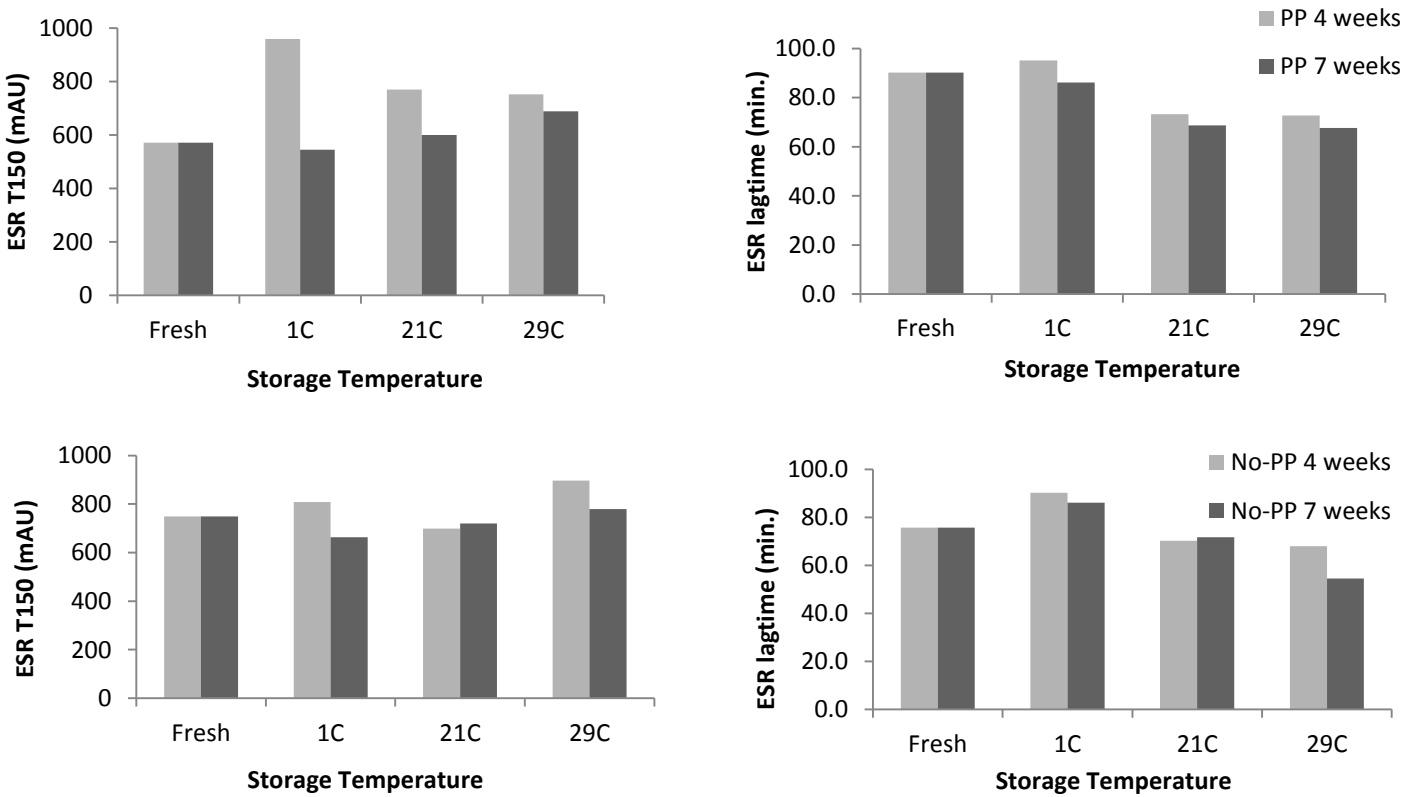

Figure 2.15. ESR indices: T150 represents the ESR signal (mAU) at time = 150 minutes. Lagtime represents radical formation during the lagphase, expressed in minutes, for fresh and aged (4 and 7 weeks) commercial lager dosed with (PP) and without (No-PP) polyphenols.

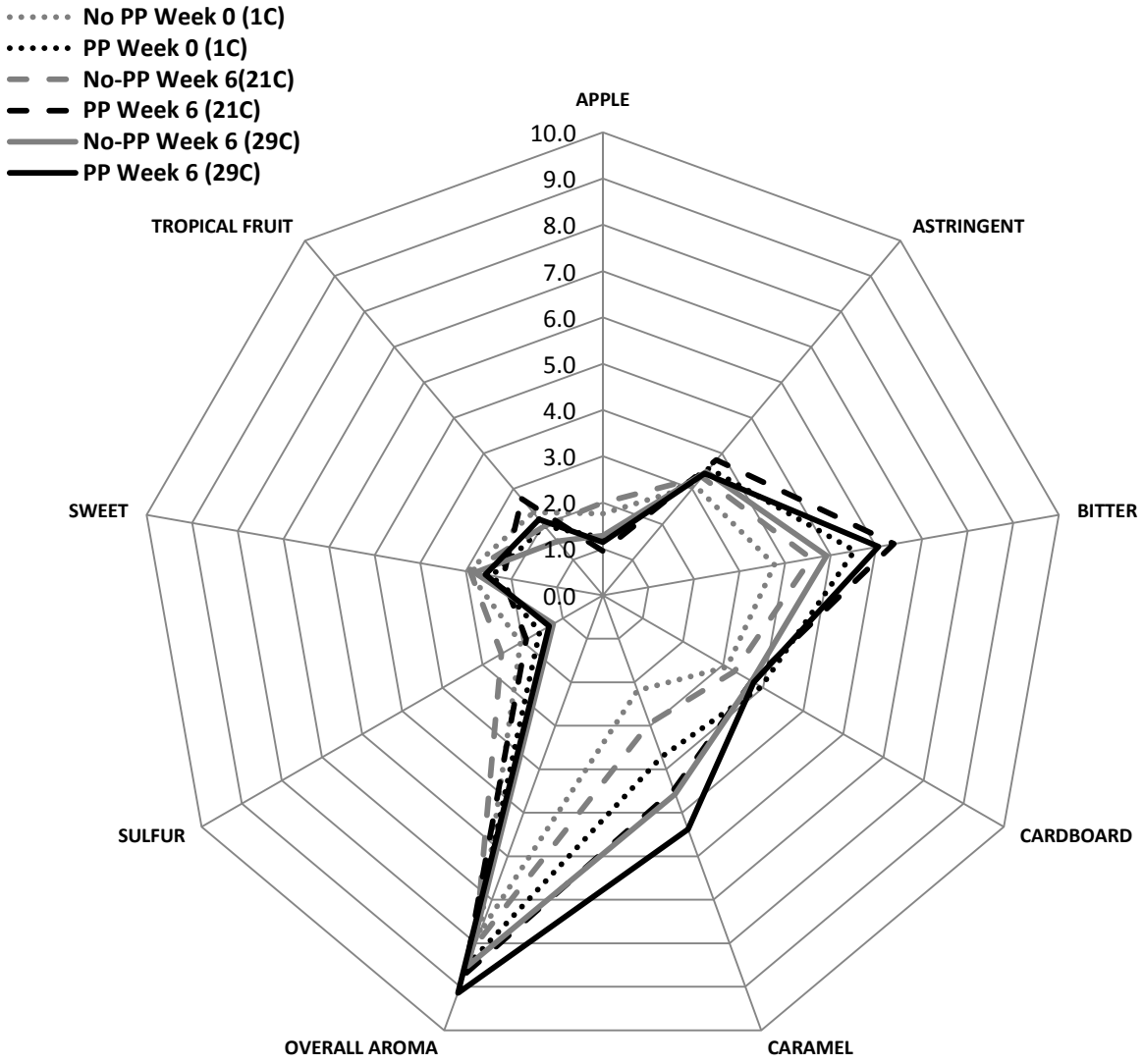

Figure 2.16 Sensory Scores of polyphenol treated (PP) and untreated (No-PP) commercial lager: fresh beers (Week 0, 1C) compared to beers aged over 6 weeks at 21 and 29˚C.

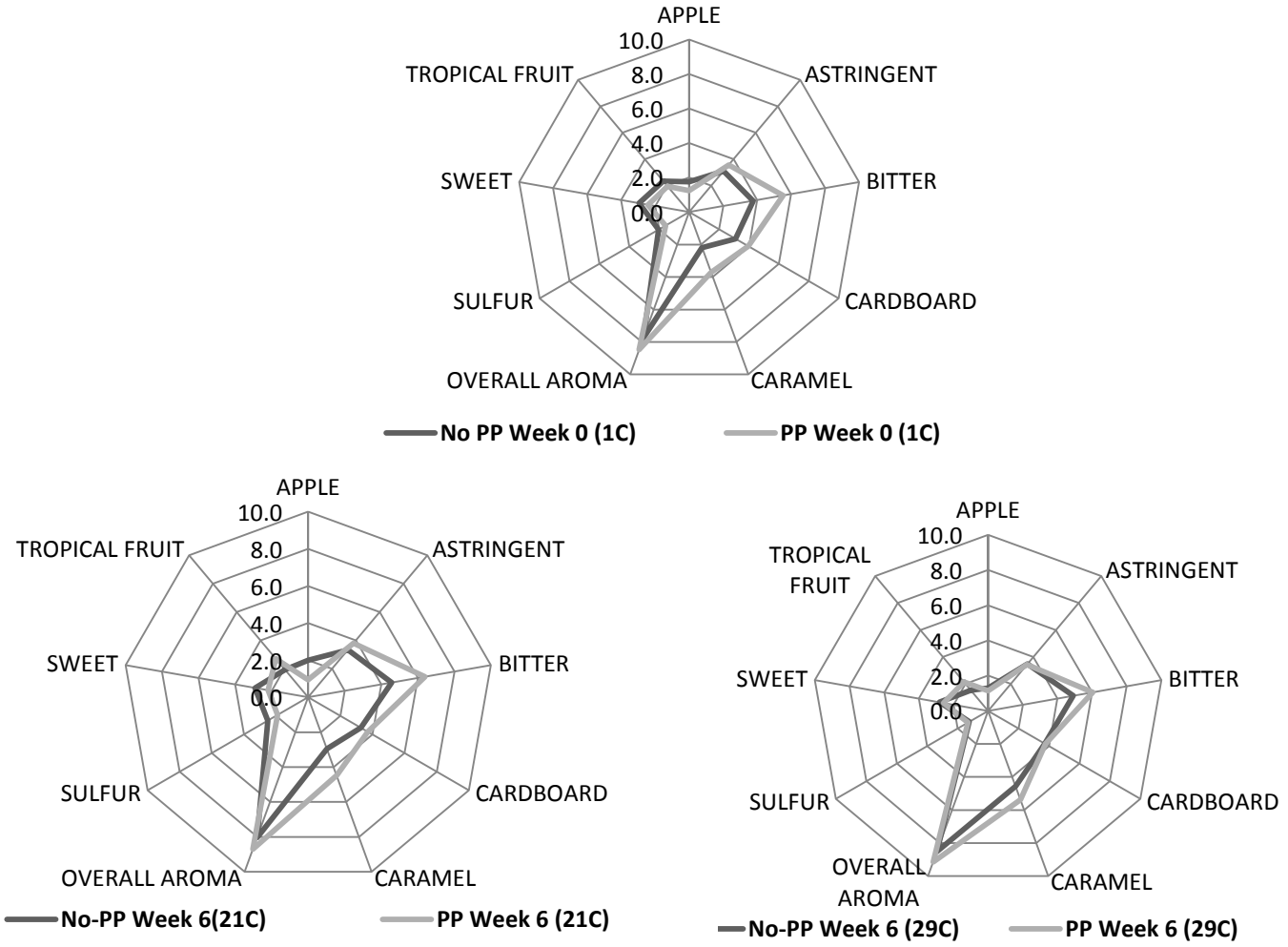

Figure 2.17. Comparison of Sensory Scores for Polyphenol Treated (PP) and Untreated (No-PP) for Fresh and Aged (6 weeks) Commercial Lager after at 1, 21 and 29˚C

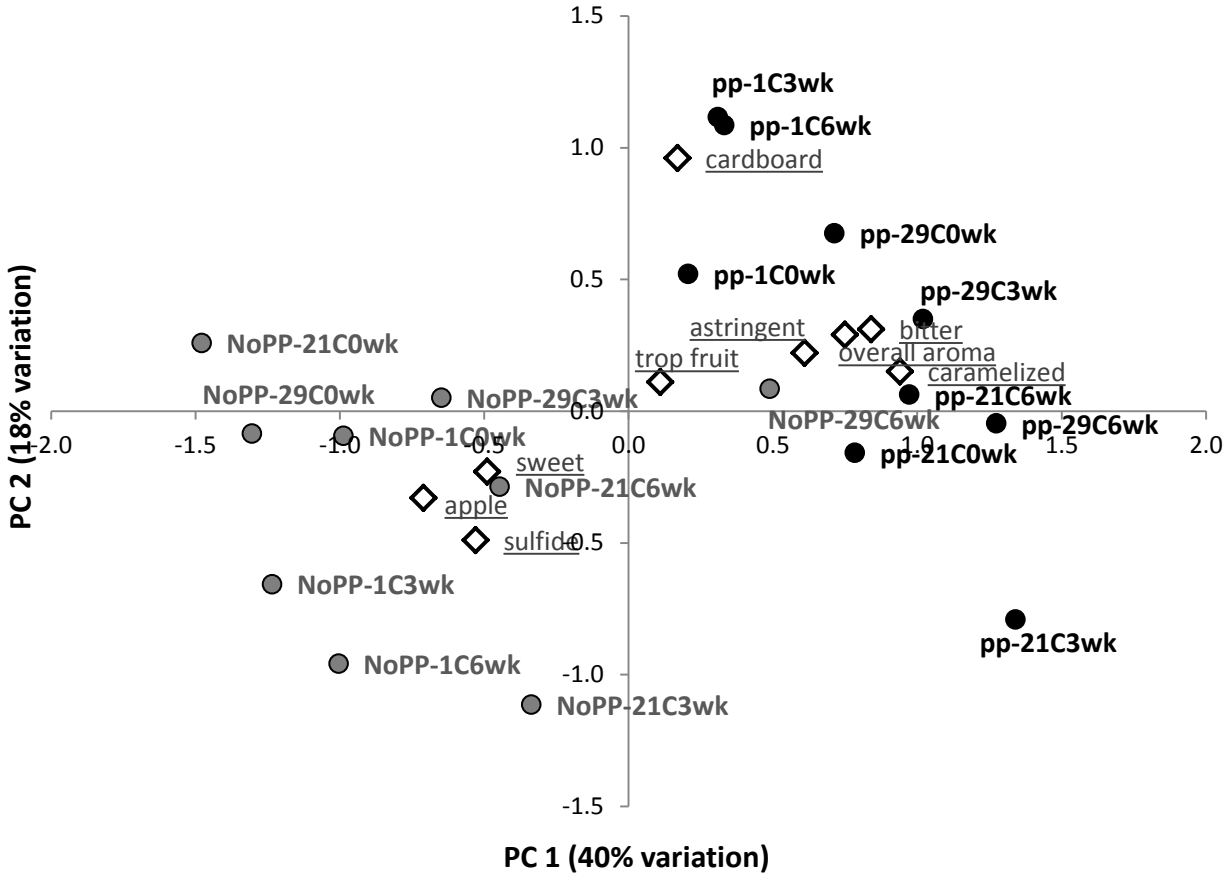

Figure 2.18. Principal component analysis (PCA) map based on aroma, basic taste, and mouth feel descriptors

List of Tables.

Table 2.1 Amberlite extract proanthocyanidin composition by phloroglucinolysis

Extension[a]	**EGC-P**	**C?-P**	**C-P**	**EC-P**	**ECG-P**
	111.75	29.36	217.76	60.80	20.08
Terminal[a]	**GC**	**EGC**	**C**	**EC**	**ECG**
	33.29	109.36	58.38	41.16	13.02

[a]Proportional composition of proanthocyanidin subunits in moles, and with the following subunit abbreviations: (-P), phloroglucinol adduct of extension subunit; EGC, (-)-epigallocatechin; C?, (-)-catechin*; C, (+)-catechin; EC, (-)-epicatechin; ECG, (-)-epicatechin-*O*-3-gallate. *proposed identity by LC-MS-ESI is an isomer of (+)-catechin.

Table 2.2. Proposed Identity of flavonoid compounds comprising the Amberlite hop solid extract

Compound #[a]	RT	m/z	Proposed Identity	Confirmed Via[b]
*	5.1	584.0	(-)-epigallocatechin-gallate-phloroglucinol	gse
*	6.3	306.0	(+)-gallocatechin	std
1.0	7.4	414.0	(?)-catechin isomer-phloroglucinol	
2.0	7.7	448.0	Unknown	
3.0	8.0	414.0	(+)-catechin-phloroglucinol	gse
4.0	8.4	414.0	(-)-epicatechin-phloroglucinol	gse
*	10.3	306.0	(-)-epigallocatechin	std
5.0	10.3	444.0	(+)-catechin-gallate	std
6.0	11.3	290.0	(+)-catechin	std
7.0	12.7	414.0	Unknown	
*	13.6	136.0	Caffeic acid	std
*	14.5	584.0	(-)-epicatechin-gallate-phloroglucinol	gse
8.0	14.7	368.0	Methylated xanthohumol	
9.0	17.5	206.0	Unknown-phloroglucinol	
10.0	15.7	290.0	(-)-epicatechin	std
11.0	16.2	520.0	Unknown	
12.0	17.2	368.0	Methylated xanthohumol	
13.0	17.5	626.0	(-)-epigallocatechin-3-glucorhamnoside	
14.0	18.3	610.0	Hesperidin	
15.0	19.0	610.0	Rutin	std
*	19.2	444.0	(-)-epicatechin-gallate	
16.0	19.4	464.0	Quercetin-3-glucoside	
17.0	19.6	358.0	Unknown	
18.0	20.1	594.0	Kaemferol-gluco-rhamnoside	
19.0	20.2	534.0	Kaemferol-3-O-C6"-O-malyonyl-glucoside	
20.0	20.7	448.0	Kaemferol-glycoside	
*	20.7	580.0	Naringin	std
21.0	20.9	468.0	Unknown-phloroglucinol	
22.0	21.2	520.0	Unknown	
23.0	21.5	394.0	Unknown	
24.0	22.2	372.0	Unknown	
25.0	22.6	364.0	Unknown	
26.0	23.2	364.0	Unknown	
*	24.5	302.0	Quercetin	std
27.0	24.6	378.0	Unknown	
28.0	29.1	352.0	Unknown	
29.0	31.4	378.0	Unknown	

[a]Retention Times are based on RP-HPLC according to modified methods of Taylor et al.[123] and compounds marked with an asterix (*) that are not numbered are in order of retention time. [b]Compound identification was confirmed via use of grape seed extract (gse) or standards (std).

Table 2.3. ANOVA Significance levels for differences in trained beer panel descriptors across and within polyphenol treatments

	PP[1]	Within PP			Within No-PP		
Descriptor	**PP**	**Temp**	**Time**	**Temp×Time**	**Temp**	**Time**	**Temp×Time**
AROMA							
Overall Aroma	**	NS	**	NS	NS	*	NS
Cardboard/Papery	**	**	NS	NS	NS	NS	NS
Apple	**	NS	NS	NS	NS	NS	NS
Tropical Fruit	NS	NS	NS (0.06)	NS	*	NS	NS
Caramelized	**	**	NS	NS	**	**	**
Sulfide	**	NS	NS	NS	NS	NS	*
BASIC TASTES & MOUTH FEEL							
Bitter	**	NS	NS	NS (0.1)	*	*	NS
Sweet	**	NS	NS	NS	NS	NS	NS
Astringent	**	NS	NS	NS	NS	NS	NS

*,** Significant at $p<0.05$ and $p<0.01$, respectively. NS = Not significant

[1]The significance for the main effect of polyphenols (referred to as PP) is presented but it should be noted that there was a significant "PP by Panelist" interaction for each of the nine descriptors listed. Therefore the results need to be interpreted with caution and on a descriptor-by-descriptor basis.

Chapter 3.

Polyphenol rich hop products impact lager beer flavor and flavor stability

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ABSTRACT

Beers were prepared with and without hop products (Pellet Hops, Hop CO_2 Extract, Spent Hops, *Iso*-alpha acid Extracts and no Hops) to target the effect of hop components (*iso*-alpha-acids and polyphenols) on beer flavor stability. All kettle hopping regimes reduced the levels of beer soluble Fe and Cu, with the greatest effect observed with Pellet hopping. Hopping with Spent Hop material increased beer bitterness potential (BUs), despite low levels of *iso*-alpha-acids. Hopping regime also had a significant effect on initial %Trans and on the decline in total *iso*-alpha acids and *trans*-*iso*-alpha acids over storage. Spent Hop and Pellet Hop beers scored highest in antioxidant potential as measured by the FRAP assay. ESR results contradicted FRAP results; beers high in polyphenols were assessed as least flavor stable by ESR and the least haze stable by nephelometry. Pellet hopped beers were lowest in total aldehydes, while the unhopped Control beers were highest in total aldehydes. The Extract and Spent Hop treated beers endured larger increases in total aldehydes than the Pellet treated beers, indicating a protective effect for whole hop products on staling aldehyde formation. Sensorially the Spent Hop and Pellet Hop beers were characterized by high Piney and Tropical fruit notes, with significant increases occurring after force-aging. The Control beers were rated as being higher in Overall Aroma Intensity, and were judged as being high in Cardboard aroma after force-aging. Specific aldehydes did not correlate well with sensory scores, indicating that the predominant aromas characterizing the Pellet and Spent Hop beers were likely due to another class of compounds that were not investigated in this study.

KEY WORDS: ESR, FRAP, lager beer flavor, hops, polyphenols, aldehydes.

INTRODUCTION

Hop cones (*Humulus lupulus*) have long been used to impart special flavors and preservative qualities to beer. Several components comprise the whole of a hop cone (cellulose, lignin, proteins, lipids, waxes, resins, essential oils and polyphenols)[1],

however brewers mainly concern themselves with just two components: the resins and essential oils. The resins contain the alpha acids, which once isomerized are responsible for the bitter quality of beer. The essential oils contain a complex mixture of several hundred compounds. Because the resins and essential oils sum to less than 20% of the entire hop cone by mass [1], the use of whole hops is not the most efficient practice. A number of hop products are now commercially available and can be utilized throughout the brewing process to provide beer with sensorial bitterness and added-value (antimicrobial activity, foam stability, flavor enhancement and flavor stability) [136-139]. Products such as pelletized hops, pre-isomerized pelletized hops, resin concentrated pelletized hops and resinous extracts (prepared by critical or liquid CO_2 extraction) are commonly used in place of whole hop cones today due to their increased efficiency and utilization. More refined, advanced hop products such as pre-isomerized extracts and reduced extracts (tetrahydro-*iso*-alpha-aicds, hexahydro-*iso*-alpha acids, and rho-*iso*-alpha acids) are also available for trimming bitterness and special applications (foam enhancement and light stability). Consequently, the use of hop pellets is also declining, leaving a large portion of the hop cone as a waste stream of spent hop solids/powder.

This spent hop material is rich in polyphenols (PPs)[6, 80]. Lager beers typically contain anywhere from 50 to 150 mg/L PPs [140] and depending on beer style and hopping technology 20% or more of these PPs can originate from hops [89, 90]. PPs represent a broad class of compounds that contain at least one phenol unit and are further classified by the type and number of subcomponents. PPs of the Flavonoid family (**Figure 3.1**.) receive measurable attention because of their capacity to improve food and beverage oxidative stability [141]. One class in particular, the flavan-3-ols and their condensed products, the proanthocyanidins, are effective antioxidants [39, 92, 93] and natural metal chelators that influence beer flavor [6, 96, 97, 142, 143] and flavor stability [6, 7, 99, 100] as well as other quality parameters (foam, color and colloidal stability) [141].

Flavor changes that occur during beer storage can be attributed to the degradation of beer compounds by reactive oxygen species (ROS) [39, 94, 144, 145] as well as non-oxidative reactions [146-148]. Aged flavor formation varies from one beer style to

another, with remarkable changes often noted in paler lager beers [149-151]. Aged light lager beers are generally characterized as having decreased bitterness and increased sweet, caramel, and toffee-like flavors and aromas [150, 152]. Several hundred compounds may undergo a multitude of aging reactions to ultimately play a role in aged beer flavor [32, 93, 100, 136, 148]. The occurrence of each reaction depends on beer type, storage temperature, dissolved oxygen content, pH and concentrations of other beer analytes such as sulfites, ascorbic acid, reductones, enzymes, transition metals and polyphenols [87, 92, 144, 148, 150, 153, 154].

Numerous compounds have been targeted as staling indicators in beer. The loss of *iso*-alpha acids can be monitored and correlated to bitterness deterioration during aging. The *trans*-stereoisomers of the *iso*-alpha acid analogues are more prone to oxidation than the cis-steroisomers during aging, especially under temperature abuse, and thus the ratio of *trans* to cis *iso*-alpha acids can be used as a staling indicator [155, 156]. Other compounds identified as key players in beer flavor modification include, but are not limited to, sulfur compounds, esters, maillard reaction products, straight chain aldehydes, strecker aldehydes, lactones, and volatile fatty acids [28, 149]. Oxidation of fatty acids in particular leads to formation of staling aldehydes during beer aging. Aldehydes such as *trans*-2-nonenal (cardboard/papery) can impact beer flavor even at sub-ppb levels due to their very low flavor thresholds [157]. Despite not being present at threshold concentrations, other straight chain carbonyls are also monitored as beer oxidative flavor indicators because their concentrations increase alongside increases in oxidative flavors during beer aging [154, 158-160].

As antioxidants, polyphenols have the potential to influence the oxidative mechanisms responsible for aged beer flavors. However, very little is understood regarding the impact of polyphenols, or their exclusion (via use of CO_2 extracts), on aged beer flavor development. Despite their antioxidant potential, some polyphenols can show pro-oxidant behavior and thus both attractive [39, 92, 93, 100, 110, 144] and unattractive [40, 68-70] flavor properties have been attributed to beer polyphenols. Foster [72] reports that spent-hop derived extracts can improve the oxidative stability (ESR lagtime) of light and dark beer and fruit juices, however other ESR lagphase studies fail to show that

polyphenols (catechin, phenolic acids, and dimeric proanthocyanidins) significantly diminish free radical formation in beer during storage or in wort during brewing [40, 69, 74, 112]. Moreover several reports claim that the antioxidative properties of hop products are unrelated to their polyphenol content and that it is the hop bittering acids, the humulones (alpha-acids) and lupulones (beta-acids) that contribute the strongest source of antioxidants in the beer [75, 76, 137].

Regardless, over the last decade, brewing scientists and hop chemists have given substantial attention to this polyphenol rich spent hop material. Brewing trials conducted with pellets, CO_2 extract and spent hops [143] indicate that pellet hopped beers age slightly better and have more pleasant aroma than extract beers, and that spent hops contribute pleasant, hoppy, slightly fruity aromas and tastes as judged by panelists, even after accelerated storage. To date at least five patents have been filed in reference to the advantages of brewing with hop polyphenols and spent hop material [6, 7, 79-81].

The objective of this study was to examine the potential for hop-derived polyphenols to impact the flavor stability of lager beer. Beers were brewed with three hopping regimes to target the effect of the complete hop (pellets), hop bittering acids only (CO_2 extract), and hop polyphenols only (spent hop solids). Unhopped control beer was brewed and fermented to provide a blank beer against which comparisons could be made. A post-fermentation treatment of pre-isomerized alpha-acid extract was also incorporated into the experiment to determine if adding *iso*-alpha acids downstream provides any protective effect against beer staling. The hop materials were obtained from a commercial hop supplier from the same lot of Galena hops. Galena hops were chosen because they are used to produce CO_2 extracts utilized for bittering commercial lager beers. Flavor stability was assessed by sensory and chemical analyses.

MATERIALS AND METHODS

Hop products. Spent Galena hop powder (post-CO_2 extraction), hop pellets, CO_2 hop extract and pre-isomerized hop extract were obtained from John I. Haas, Inc. (Yakima, WA, USA). All hop products were of the same lot.

Reagents and materials. Ferric ammonium citrate (green) was purchased from Fisher Chemicals. Ethylenediaminetetraacetic acid disodium salt, dihydrate (EDTA), *p*-

dimethylamino-cinnamaldehyde, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), acetonitrile and pectin were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO) . Low-viscosity carboxymethylcellulose was supplied by Hercules Inc. (Wilmington, DE). C-8 SPE columns were purchased from Supelco (Bellefonte, PA). Acrodisc® LC 13 mm Syringe Filters (0.45 um PVDF) were purchased from Pall Corporation (East Hills, NY). Divergan RS PVPP was obtained from BASF (Ludwigshafen, Germany). ICP metal standards (Iron and Copper), and ICP grade HNO_3 and Sodium Acetate Trihydrate were purchased from VWR International, BDH (West Chester, PA, USA). Divergan RS PVPP was obtained from BASF (Ludwigshafen, Germany). Ferric chloride anhydrous and phosphoric acid were obtained from EMD (Gibbstown, NJ), Trolox was obtained from Calbiochem® (La Jolla, CA). HPLC- solvents, methanol and 96-well plates were obtained from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid was purchased from Merck kGaA (Darmstadt, Germany). Hydrochloric acid and 2,2,4-trimethylpentane from JT Baker (Phillipsburg, NJ), ammonium hydroxide obtained from Ashland (Columbus, OH). DCHA-*Iso* ICS-I2 standard was obtained from ASBC. All solvents were HPLC grade. All aldehydes (2-methylpropanal, 2-methylbutanal, 3-methylbutanal, 2-furaldehyde, benzaldehyde, phenylacetaldehyde, *trans*-2-nonenal, and cis-11-hexadecenal) and o-(2,3,4,5,6-Pentafluorobenzyl) hydroxylamine hydrochloride (PFBOA) were purchased from Sigma-Aldrich (St. Louis, MO).

Lab scale experiments. Benchtop experiments were conducted to calculate approximate polyphenol yield per gram of both spent hop and pellet hop material. Five (5) g spent hop material or 0.63 g of pellets were dosed into 1 L of pale all-malt wort, boiled for 60 minutes and analyzed for total polyphenol content as the per EBC analytica method 9.11. Pale all-malt wort was also treated with Divergan RS PVPP at 50g/hL to determine potential for PVPP to reduce apparent barley-polyphenol content of the wort. Finished Control beer was also treated with Divergan RS PVPP at 50 g/hL to determine its potential to remove polyphenols chelated to pro-oxidative metals Fe and Cu.

Production of pale lager beer treated with various hop products. Following benchtop and preliminary experiments, an experimental design was optimized to include four kettle hopping regimes and one post-filtration hopping regime: *Control* (no hop

product), *Pellet Hop* (complete hop), *Spent Hop* (hop polyphenol rich), *Extract* (*iso*-alpha-acid rich) and *Iso-Dosed* (pre-isomerized alpha acid, post fermentation and filtration). Two-85 gallon batches of lager wort (100% Pale 2-Row malt, 18°P O.G.) were produced in the OSU pilot brewery, blended and treated with Divergan RS PVPP at 50g/hL to remove barley polyphenols. The PVPP was allowed to settle overnight and removed by coarse filtration (Pall, HS 2000). Unhopped lager wort was used as a Control: 138 L of wort and 81 L of sterile brewhouse water were transferred to the brewhouse kettle and boiled for 60 minutes (final OG=11.8°P). Following whirlpool, 185 L of hot wort was cooled, transferred into a sanitized brewhouse fermentor containing 1.5 kg of yeast, fermented (13°C) and lagered cold (2 weeks, 1°C). The Control treatment was not replicated in the kettle. For the kettle hop treatments 75 L batches of wort and 26.0 L of sterile brewhouse water were transferred to the kettle, treated with a hop-product and boiled for 60 minutes (12°P). All treatments were performed in triplicate beginning with a new batch of unhopped wort. For the *Pellet Hop* treatment, pellet hops were added at a target yield of 15 ppm *iso*-α-acid, assuming 40% utilization based on preliminary results (28.5g at beginning of a 60 min. boil and 28.5 g at 5 min. to end of boil). For the *Spent Hop* treatment, spent hop material was added to exceed projected polyphenol contribution of the pellet hop treatment, also based on preliminary results (200 g at beginning of a 60 min. boil and 100 g. at 5 min. to end of boil). For the *Extract* treatment, the CO_2 extract was added at a target 15 ppm *iso*-α-acid yield, assuming 35% utilization based on preliminary work (7.5 g extract at 60 min. to end of boil). Following whirlpool, 40.0 kg of the kettle-treated wort (OG = 11.3 to 12.0°P) was cooled and transferred into a sanitized 100 L (26.4 gallons) fermentor containing 0.4 kg yeast. The wort was fermented (12.8 - 15.6 °C), racked into sterile- 5 gallon (18.9 L) Cornelius kegs and lagered (2 weeks, 1°C), then coarse and sterile filtered into sterile 3 gallon (11.3 L) or 5 gallon (18.9 L) Cornelius kegs. Following filtration the finished beers were analyzed for *iso*-α-acid content via HPLC-DAD. Pre-isomerized α-acid extract was then dosed (0.7 mL extract) in 10 kg of Control beer post filtration to attain a level of *iso*-α-acids consistent with that of the finished Extract and Pellet hop treated beers. This treatment

was called *Iso Dosed.* Two 5-gallon kegs of *Iso* Dosed beer were prepared for each storage temperature of the trial (2 at 1°C and 2 at 30°C).

Accelerated staling trial. Finished beers (10 kg of beer into 3 gallon, 11.3 L kegs) were not carbonated but were kept under nitrogen at 1°C and 30°C for up to eight or twelve weeks during which samples were pulled weekly. Chemical analyses were performed to assess the impact of hop treatment and storage temperature on beer flavor stability from 0 to 8 weeks.

Total polyphenols (TP) and total flavanoids (TF). TP and TF were measured according to the EBC Analytica methods (9.11 and 9.12) [114] using a Shimadzu PharmaSpec UV-1700 spectrophotometer, Shimadzu Corporation (Columbia, MD).

Antioxidant properties. The antioxidative activity of the hop treated beers was evaluated by two assays. This first assay, the 'ferric reducing antioxidant power' or 'ferric reducing antioxidant activity of plasma' (FRAP) assay is a simple colorimetric method that measures the ferric reducing (Fe^{3+} to $Fe^{2+)}$ ability of the beer sample. FRAP was performed using 96 well microplates according to the methods of Benzie and Strain [115] and Firuzi and coworkers[116]. The second assay Electron Spin Resonance (ESR) was performed as per the methods of Uchida and Ono [118, 119] with a Bruker EMX 6/1 Electron Paramagnetic Resonance Spectrometer controlled by Bruker WinAcquisition 3.04 Software with AquaX sample cell and 48rpm Peristaltic Pump: preliminary work courtesy of Coors Brewing (now MillerCoors) and final staling trial courtesy of Sierra Nevada Brewing Company.

Metal ions by inductively coupled plasma atomic emission spectroscopy (ICP-AES). ICP-AES for Copper and Iron was performed according to the ASBC proposed method [120].

Haze. Haze was measured using a Hach 2100 AN Turbidimeter (Loveland, CO). Samples were degassed, tempered to room temperature and haze was recorded in NTUs.

Bitterness Units. Bitterness units (BUs) were measured according to ASBC methods of analysis [161].

***Iso*-α-acids.** *Iso*-α-acids were measured by HPLC-DAD (Agilent 1200, Hewlett Packard, Palo Alto, CA) according to the methods of Donley, J.R. [162] and the ASBC standard method for HPLC analysis of hop components in beer. A 250 x 4.6 mm, 5-µm column (Supelco Discovery C-18, Sigma Aldrich) was used. Mobile phase A consisted of 75:24:1 MeOH:H_2O:H_3PO_4 %v/v and mobile phase B consisted of 100% MeOH. Eluting peaks were monitored at 270 nm. Elution conditions were as follows: 1.3 mL/min (30 °C); 7 µL injection; 100% A for 17 minutes, a linear gradient from 0 to 60% B for 1 min, a linear gradient from 60% B to 25%B for 1 min, a linear gradient from 25% to 0% B for 1 minute and then isocratic at 100% A for 6 minutes.

Ratio of *trans* to *cis* (%TRANS) by HPLC. %Trans *iso*- α-acids was measured by HPLC-DAD at 270 nm according to the methods of Araki et al. [156] and DeCoomen et. al [155]. The method utilized the same column as for the measurement of *Iso*-α-acids. Mobile phase A consisted of MilliQ water adjusted to pH of 2.81 with H_3PO_4 and mobile phase B consisted of 100% Acetonitrile. An isocratic method was used: 48% A and 52% B at 1.4 mL/min, 30°C, 5 µL injection, for a total of 30 min per sample. %Trans was calculated as the sum of *trans*-*iso*-cohumulone and *trans*-*iso*-humulone divided by the sum of cis-*iso*-cohumulone and cis-*iso*-humulone then converted into percent.

Volatile aldehyde analysis. Volatile aldehyde analysis of the beers by solid phase microextraction-gas chromatography/mass spectroscopy (SPME-GC/MS) was carried out according to modified methods of Saison et al (2008) [163] and Vesely et al (2003) [164]. A 65µm PDMS-DVB fiber (23 guage, Supelco) was used for the extraction of staling aldehydes. 10mL of degassed beer was added to a 20mL vial, followed by 10µL of internal standard solution (*cis*-11-hexadecenal) and 3.5 g of sodium chloride (NaCl). The vial was capped, vortexed for 1 minute and placed in the autosampler for immediate analysis. Capillary GC-MS was performed using an Agilent 6890N GC with a 5973 mass selective detector (Agilent Technology, Palo Alto, CA). Samples were analyzed on a DB-5 column (30m x 0.25mm i.d., 0.5µm film thickness, J&W Scientific of Agilent Technologies). The carrier gas was helium at a constant flow rate of 1.1mL/min. The oven temperature was initially at 40°C and then increased to 140°C at 10°C /min, and again increased to 250°C at 7°C/min at final time = 14.00 minutes. The total run time

was 39.71 minutes. The injector temperature was 250°C. The splitless mode was used. The SPME fiber was first pre-extracted for 10.0 min in the derivatization vial and then extracted for 60.0 minutes in the sample vial (incubated at 50°C for 5.0 min at 250 rpm). The ion source temperature was set at 230°C and the MS quad temperature was set to 150°C . The mass spectra were obtained in SIM mode for ion selection at m/z 181.

Preparation of internal standard for SPME-GC-MS. To a 50 mL volumetric flask half filled with ethanol, 30µL of cis-11-hexadecenal was added and then brought to 50mL with ethanol. A 4 mL aliquot of this solution was then transferred to another 50mL volumetric flask and brought to volume with ethanol.

Derivative solution preparation. A stock solution was prepared by adding 0.150 g o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBOA) to a 25mL volumetric flask which was brought to volume with MQ water. The stock solution was kept refrigerated at 4°C until ready for use. For each analysis a separate derivative solution was prepared by adding 100µL of the PFBOA derivative stock solution to 10mL of water in a 20mL vial.

Sensory descriptive analysis. The sensory panel consisted of thirteen trained panelists (ages 21 – 58, 7 males, 6 females), many of the panelists had been extensively involved with previous sensory work regarding beer evaluations. Thirty (30) mL (~one-ounce) samples were presented to the panelists in 8-ounce, clear glasses that were capped with clear- plastic, odorless lids. Samples were poured within one hour of serving, and kept on ice until evaluation at ambient temperature (68˚F/20˚C).

The initial descriptive ballot was based on six consensus aroma descriptors and three taste descriptors: overall aroma, cardboard honey, tropical fruit, caramel, and apple aromas and bitter, astringent and metallic tastes. All descriptors were rated on a 16-point intensity scale (0=none, 15=extreme intensity) and panelists were provided with aroma references to help them identify and agree upon the aroma characteristics of the beer samples. The final descriptive ballot (final staling trial) was based on eight consensus aroma descriptor terms: Overall Aroma Intensity, Cardboard/Papery, Piney, Honey, Tropical Fruit, Caramelized, Apple and Sulfury/Skunky. All descriptors were rated on a 16-point intensity scale (0=none, 15=extreme intensity) and panelists were provided with

aroma references to help them identify and agree upon the aroma characteristics of the beer samples.

Sensory experimental design. The complete randomized block design was set up to investigate if there were any perceivable sensory changes due to storage time and temperature applied to beer treated with various hop products. The twelve samples tested represented two storage replicates for the Control and Iso Dosed treatments (these treatments were not replicated in the kettle), three kettle replicates for Pellet and Extract kettle hopped treatments and only two replicates for the Spent Hop kettle hopped treatment. Spent Hop treatment #1 was not used in the sensory trial (1°C or 30°C) because of inadequate sample volume. For testing, panelists evaluated each of the twelve samples qualitatively in duplicate. Panelists evaluated the samples monadically (one at a time). Testing took place over four separate sessions, 12 samples per session.

Sensory Statistical analyses.

ANOVA. An analysis of variance (ANOVA) was conducted per descriptor. Statistical analysis was performed using PC SAS 9.1 (SAS Institute, Inc., Cary, N.C.). Statistically significant sample effects were further analyzed to see where mean differences existed using Tukey's HSD test at the 95% confidence interval ($p \leq 0.05$).

PCA. For principle component analysis (PCA), panelist data, averaged over replications, were analyzed by factor analysis using the varimax rotation and the covariance matrix (SPSS® v 15.0 (Chicago, IL).

RESULTS AND DISCUSSION

Lab scale experiments. Benchtop experiments were conducted to calculate approximate polyphenol (PP) yield per gram of spent hop and pellet hop material added to the wort. Five grams (5 g) spent hop material added to 1 L of pale all-malt wort, boiled for 60 minutes, resulted in 59.2 mg PP per g of Spent Hop, 0.63 g of pellets dosed into 1 L of pale all-malt wort, boiled for 60 minutes resulted in 124.9 mg PP/g Pellet hop. The malt itself was calculated to contribute 0.70 g of PP/g malt. The treatment of Pale all-malt wort with Divergan RS PVPP at 50g/hl reduced the apparent barley-polyphenol content of the wort by 36.7%. The treatment of finished Control beer with Divergan RS PVPP at

50g/hL reduced the total concentration of the pro-oxidant metal Fe by 19.4%, however no significant reduction was seen in the concentration of another pro-oxidant metal, Cu.

Preliminary beer staling trial. The performance of the various kettle hop treatments on lager beer flavor stability was initially tested using an accelerated storage protocol. The kegged beer was stored at 1°C and 30°C and samples were pulled weekly and frozen for future analysis. Results indicated that significant loss of polyphenols resulted from fermentation and filtration. As such, hopping regimes used in the final staling trial were adjusted to account for this. A sensory evaluation was also conducted following the preliminary staling trial. Sensory data were analyzed to answer these two questions: 1) Did differences exist due to hop kettle treatments, and 2) within each of the treatments, did differences exist due to storage temperature over 8 weeks? The experimental design for the preliminary staling trial was similar to that used for the final staling trial, with fourteen trained panelists (ages 21 – 58, 8 males, 6 females). Each sample was evaluated in duplicate for overall flavor and aroma. Excluding any effect of storage temperature, statistical analysis using ANOVA failed to provide any evidence that beers produced from various kettle hopping regimes were statistically different for any of the aroma descriptors: overall aroma, cardboard/papery aroma, tropical fruit aroma, caramelized aroma and honey aroma. However, samples were found to differ significantly in bitter taste ($p < 0.05$), regardless of storage temperature; the pellet beer being most bitter (mean score = 6.1), the control treatment the least bitter (mean = 4.0) and Spent Hop and Extract treatments being equi-bitter (4.7 and 4.6 respectively). As expected there was a storage temperature effect with beers stored at 30°C scoring significantly higher than those stored at 1°C in Overall Aroma ($p < 0.001$), Cardboard/Papery ($p < 0.001$), and Metallic taste ($p < 0.01$). More importantly, the hop pellet treatment stored at 30°C resulted in significantly lower cardboard aroma than the control and other hop treatments. Interestingly, panelists also scored beers stored at 30°C significantly lower in honey aroma than those stored at 1°C ($p < 0.05$). Samples stored at 30°C were also rated higher in caramelized and tropical fruit, aromas as well as higher in astringent taste. Samples stored at 1°C were characterized as more bitter.

Final Staling Trial

Wort parameters. Wort parameters are reported in **Table 3.1**. The Control treatment was kettle boiled in bulk and thus no standard error is reported for Control wort specifications. The Iso Dosed treatment was prepared post-fermentation from the Control beer so data is not shown in **Table 3.1**.

Treatment of wort with PVPP. In order to reduce the polyphenol contribution of the malted barley and the potential for beer to form haze, the wort was treated with Divergan RS PVPP. The unhopped wort contained 248 ppm polyphenols by the total polyphenols assay. Treatment of the wort with 50g/hL PVPP resulted in a 31.5 % reduction in barley polphenols (170 ppm PP in PVPP treated wort) and 41.2% reduction in barley flavanoids (from 51.8 ppm to 30.5 ppm in PVPP treated wort).

Finished beer parameters. Finished beer specifications are reported in **Table 3.2**. As stated in the methods, pre-isomerized α-acids were added to 10 kg of unhopped lager beer to attain a level of *iso*-α-acids consistent with that of the finished Pellet and Extract hop treated beers (13 ppm). As stated previously the Iso Dosed beers were not produced in replicate form in the kettle, however 2 - 5 gallon kegs were stored at each storage temperature to observe the effects of downstream hopping on lager beer flavor stability.

Evaluation of polyphenol content. While it has been established that polyphenol losses occur due to trub formation during boiling, adherence to yeast cells during fermentation and removal by filtration/fining, the fate of PPs on packaged beer remains uncertain [128]. Wort, fresh beers, cold-aged and force-aged beers were analyzed for their polyphenol content via two assays. The first assay, the total polyphenols assay relies on the use of ammonium iron citrate as a reducing reagent. It is a non-specific assay that can also be used to determine the relative reductive capacity of the beer. An advantage of the assay is that it is simple, highly reproduceable, relatively quick and can be performed with a spectrophotometer. A disadvantage of the assay is that haziness complicates the results. Results may be more consistent if beers are clarified to brightness, however clarification means that some of the precipitated (polyphenol-protein complexes) polyphenols are removed. Therefore, older, hazy beers have a tendency to produce lower total polyphenol readings [165]. A second disadvantage of the assay lies in the fact that

several classes of compounds containing hydroxyl groups may elicit a response, and reactivity will depend on the subset of chemical compounds, as well as their stereochemistry, present in the beer. It must also be taken into consideration that this phenolic assay's basic mechanism is an oxidation/reduction reaction in which the ferric reagent oxidizes phenols, thus the presence of oxidized flavanols or proanthocyanidins in the beer would result in the beers containing less observed 'total phenolic' material.

Consistent with the preliminary staling trial, significant loss of total polyphenols (PPs) resulted from the fermentation and filtration processes. The Spent Hop treatments lost an average of 26% PPs during fermentation and filtration, while the other treatments lost significantly less: Extract (13%), Pellet (7%) and Control (14%). Comparison of sample means in the fresh beers indicates that Spent Hop kettle treatment produced beers significantly higher in PP than the Control, Extract, Iso Dosed and Pellet treatments ($p < 0.05$), however no other significant differences were seen in PP levels of the finished fresh Pellet, Extract, Iso Dosed or Control beers. PPs were monitored from week 0 through week 8 over both storage temperatures. No significant changes in total polyphenol concentrations due to 8 weeks of storage at either 1 (Cold-Aged) or 30°C (Force-Aged) were measured via this assay. However, PP levels did decrease slightly in some treatments, most notably during force-aging and in the Control beers (**Figure 3.2**). This is consistent with the literature stating that beer aging results in decreased PP content, most likely due to chemical degradation, oxidation, polymerization, etc [151] that results in the formation of other species whose chemical reactivity remains largely unspecified.

The second assay used to determine polyphenol content of the wort, finished fresh, cold-aged and force-aged beers is the total flavanoids assay. The total flavanoids assay is specific for flavan-3-ols in that it relies on a condensation reaction to occur between the flavanol A-ring and chromagen reagent. The resultant condensation product absorbs light at 335 nm and results are reported as ppm (+)-catechin. Disadvantages of the assay are that it cannot be used to discern relative content of monomeric, dimeric or oligomeric flavanoids in beer and that the assay's color yield varies by degree of polymerization as well as C ring configuration. For example (-)-

epicatechin (2,3-*cis*) yields a greater color than its stereo-isomer (+)-catechin (2,3-*trans*)[166]. Comparison of total flavanoids in the fresh beers indicates that the Spent Hop kettle treatment produced a beer highest in flavanoids ($p < 0.05$), which was anticipated. The Spent Hop beer was significantly higher in flavanoids than the Control, Iso Dosed, Extract and Pellet Hop treatments ($p < 0.05$). Storage for 8 weeks at both 1 and 30 °C did not significantly change the levels of total flavanoids present in the beers. However, as seen in the levels of PPs, total flavanoids did decrease after 8 weeks of storage at both storage temperatures, with greater losses seen due to force-aging at 30°C. This is consistent with the scientific literature on flavan-3-ol content and the effects of food processing and storage in a broad range of food and beverage systems [167-170]. Lower flavan-3-ol levels generally appear in cooked or stored foods due to epimerization, chemical modification, degradation and de-polymerization of oligomers and polymers [129, 130, 170, 171]. In beer it is known that flavanol monomer and dimer concentrations ((+)-catechin, (-)-epicatechin, prodelphinidins and procyanidin dimers) decrease [12, 39, 52, 151] during storage or aging, with dimeric flavanoids diminishing more quickly than their monomeric counterparts. However, it is also reported that after several weeks of storage, relative levels of beer PPs increase [172]. This could partially be explained by occurrence of PP polymerization and chemical alterations due to uptake of oxygen [173, 174]. Evidence consistent with oxidation of proanthocyanidins in other food systems suggests that oxidation may occur during storage [175-177], with phenyl ring oxidation dependent on proanthocyanidin configuration and conformation [176, 178-181], system pH and presence of metal catalysts [175, 177]. An interesting observation seen in all of the beers during this study is that levels of flavanoids decreased sharply initially and then once again gradually began to increase again at five or six weeks of storage, and this was regardless of hopping regime or storage temperature. As expected there was a high correlation between polyphenols and flavanoid levels for the fresh ($r^2=0.888$), cold-aged ($r^2=0.953$) and force-aged ($r^2=0.998$) beers.

Evaluation of haze. Largely a function of its polyphenol and protein content, beer is fundamentally prone to colloid formation. Thus without proper treatment, both temporary and permanent hazes may develop during beer storage. The most frequent

cause for haze production involves formation of protein-polyphenol complexes within the beer matrix [28, 149, 182-186]. Formation of protein-polyphenol haze depends on beer pH, alcohol content, ionic strength, as well as phenolic composition [184-187]. However not all polyphenols are involved in colloidal stability. Haze active (HA) polyphenols must be able to effectively crosslink HA-proline-rich proteins into a stable network to result in precipitation. Flavanoids are known constituents of permanent beer haze. The flavan-3-ol monomers may bind, but do not cross link HA proteins. However, proanthocyanidin oligomers do crosslink HA proteins [61, 183, 184, 188, 189]. Therefore it was not unexpected that beers higher in phenolic and flavanoid content would have increased haze potential. Haze potential of the finished fresh lager beer varied by hopping treatment. Comparing the fresh finished beers, there was a statistically significant treatment effect ($p < 0.001$) with the Spent Hop (4.8 ± 0.26 NTUs) treatment significantly higher ($p < 0.05$) than the Extract (3.0 ± 0.46 NTUs), Iso Dosed (1.75 NTUs) and Control (1.75 NTU). The Spent Hop and Pellet hop (3.7 ± 0.34 NTUs) kettle treated fresh beers were not statistically different. The Pellet hopped beers were hazier than the Control and Iso Dosed beers ($p < 0.05$), but the Control and Iso Dosed beers were not different in levels of haze. Comparing beers stored for 4 weeks at 1 or 30°C, there was a significant treatment effect ($p < 0.001$) for beers stored at high temperature (30°C, force-aged) but not for cold-aged beers. Spent Hop kettle treated beers were haziest (18.5 ± 1.9 NTUs), significantly hazier ($p < 0.05$) than all other force-aged beers (Pellet (11.4 ± 1.0 NTUs), Extract (8.9 ± 1.2 NTUs), Control (6.74 NTUs) and Iso Dosed (2.51 NTUs)). The Pellet beers stored at 30°C were hazier ($p < 0.05$) than the Iso Dosed beers, but no significant differences between other samples were seen after storage 4 weeks of force-aging. When comparing 4 week force-aged beers to fresh beer, there was a significant treatment effect ($p < 0.001$), however trends for haze production in 4 week force-aged beers was similar to trends seen in fresh beers. The Control and Iso Dosed treatments did not increase significantly in haze after 4 weeks at 30°C, while the Extract, Pellet and Spent Hop treated beers became much hazier ($p < 0.05$). After 8 weeks of force-aging, haze levels seen in the fresh beers increased ($p < 0.05$) dramatically for all

beers, but aged beers did not differ from each other in the levels of haze ($p = 0.27$) (**Figure 3.3**).

Evaluation of pro-oxidant metals by ICP. Although several species of transition metals have been reported in beer [132], two metals in particular have been identified as instigators of unfavorable beer aging reactions. Iron (III) and copper (II) predominate in beer and have relatively lower reduction potentials which makes them more inclined to promote reactions that instigate flavor instability [144]. Due to their ability to expedite oxidative mechanisms via the Fenton and Haber-weiss reactions, Cu and Fe levels were monitored in the wort, the finished beer and at weeks 4 and 8 of both cold and force-aging (**Table 3.3**). Kettle treatment with all three hopping regimes produced worts significantly lower in both Cu than the Control unhopped wort: Pellet = 12 ppb, Spent Hop = 11 ppb, Extract = 17 ppb and Control = 72 ppb. A significant treatment effect was thus seen in the finished beers for levels of Cu. The Control (29 ppb) and Iso dosed (34 ppb) treatment resulted in the highest ppb Cu, followed by the Extract treated fresh beers (28 ppb), then the Pellet (20 ppb) and finally the Spent Hop treatment (19 ppb). After 4 and 8 weeks of storage, treatment effects on copper levels persisted regardless of storage temperature, however there were no significant changes in levels of Cu due to storage.

Kettle treatment with all three hopping regimes produced worts significantly lower in Fe than the Control unhopped wort: Pellet = 55 ppb, Spent Hop = 88 ppb, Extract = 118 ppb and Control = 307 ppb. A significant treatment effect for Fe levels was also seen in the finished fresh beers: Pellet = 80 ppb, Spent Hop = 103 ppb, Extract = 109 ppb, Iso Dosed = 100 ppb and Control = 148 ppb. The treatment effect for Fe remained throughout the 8 weeks of storage, regardless of storage temperature and no changes in Fe levels were seen due to aging in the kettle hopped beers, except in the case of the force-aged Pellet treatments which increased from 80 ppb (fresh beer) to 109 ppb (4 weeks) to 116 ppb (8 weeks).

Sources of transition metals in beer have been explored and their fate during the brewing, fermentation, and clarification process has been examined by several investigators. Reportedly, raw material derived iron and copper only minutely influence

the flavor stability of the final beer. Worts are typically higher in transition metals than beer due to the presence of several classes of compounds (proteins, amino acids and polyphenols) capable of chelating metals [132, 134, 190, 191] during wort boiling. Yeast may also absorb and effectively reduce metal content during fermentation [192]. This is especially true regarding copper and iron [132, 190, 191]. Iron and copper levels were monitored in the wort and after fermentation (post-DE filtration) in this experiment. While significant reductions in both Fe and Cu were effected due to the addition of hopping products in the kettles, the levels of iron in the finished beer were on the high side (see below) of recommended levels. It was suspected that the levels of iron seen in the finished beers resulted from the malt and investigation confirmed this suspicion. However, filtration (coarse and sterile) via DE plate and frame filtration augmented the Fe levels of the beers, which could potentially confound the results in consideration of flavor stability.

Irwin and colleagues [144] attest that the rate of beer flavor deterioration is significantly accelerated by trace amounts (<100 ppb) of Cu(II). Bamforth and Parsons[145] go even further to suggest that even lower concentrations of transition metals (Fe, Cu, Zn, Co and Mn) may impair beer flavor stability (< 50 ppb for Cu specifically) [36]. Beers containing higher levels of copper II ions (40 to 95 ug/L) in particular are more prone to stale flavor formation. Iron appears to be a bit less reactive than copper, however excess endogenous iron can result in a metallic off-taste in beer [132]. According to the recommendations of these reports the beers in this experiment were within acceptable range for Cu, but above recommended levels for Fe.

Evaluation of beer bitterness

Aged light beers are generally characterized as having decreased bitterness after prolonged or high temperature storage [150, 152]. Beer bitterness is typically reported as Bitterness Units (BUs). As an analytical method it is frequently used in the brewing industry because of its ease of use and because it generally correlates well with sensory bitterness, especially in lighter beers. However, the bitterness unit analysis is not a direct measure of *iso*-alpha-acids because it accounts for other bitter and non-bitter compounds that absorb at 275 nm. This

accounts for the fact that despite being equivalent in ppm *iso*-alpha acid content (see below), the Pellet Hop, Extract and Iso Dosed finished fresh beers varied significantly in their BU levels ($p < 0.01$). The Pellet and Spent Hop treatments resulted in the highest BU values (21.2 and 20.9 respectively) followed by the Extract (17.1), the Iso Dosed (15.4) and finally the Control, unhopped lager with 4.7 BUs. This result indicates that lager beer high in polyphenols can produce considerable levels of BUs, despite being low in *iso*-α acids. The Spent Hop beers were dosed with 200 g at 60 min. and 100 g. at 5 min. to end of boil for a total of 300g/101L hop solids (2970 mg/L, 12°P wort) to produce 16.2 BUs or 183 ppm hop solids/BU. Changes in BUs were not monitored during storage because it is generally thought that changes in BU levels correlate to the degradation of *iso*-alpha acids during beer storage [193].

The loss of *iso*-alpha acids during storage was monitored via use of solid phase extraction in conjuction with HPLC via two methods. The first method measures the total *iso*-alpha-acids present in the wort and beer samples, but does not allow for the identification of specific analogue stereo-isomers. The advantage of the method is that it is more cost effective in that it uses methanol as a mobile phase solvent over acetonitrile and that it is relatively rapid. Total *iso*-α-acids of the finished beer are reported in **Table 3.4**. The Pellet Hop, Extract and Iso Dosed beers were not significantly different in *iso*-alpha-acids ($p < 0.05$) concentration. Consistent with our intention to produce a beer low in *iso*-alpha-acids and high in polyphenols, the Spent Hop treated fresh beer was significantly lower in *iso*-alpha-acids ($p < 0.05$).

As reported in several studies [104, 110, 136, 147, 155, 193, 194], significant loss of *iso*-alpha-acids resulted from beer storage. Storage at 1°C for 8 weeks resulted in a significant degradation of *iso*-alpha-acids ($p < 0.001$). A significant storage temperature effect was also seen; samples stored at 30°C for 8 weeks were lower in *iso*-α-acids than those stored at 1°C for 8 weeks ($p < 0.05$). The mean *iso*-alpha-acid content of the beers at 0, 1, 3, 6 and 8 weeks of storage is reported in **Table 3.4** and changes due to storage are reported in **Figure 3.4**.

The Extract treated beers lost the most *iso*-alpha-acids by ppm after 8 weeks at both storage temperatures (ΔCold-Aged = 4.62, ΔForce-Aged = 5.57), followed by the Pellet (ΔCold-Aged = 3.82, ΔForce-Aged = 5.07), Iso Dosed (ΔCold-Aged = 3.13, ΔForce-Aged = 4.35), and Spent Hop (ΔCold-Aged = 2.10, ΔForce-Aged = 2.30) treated beers. When the loss of *iso*-alpha-acids was calculated as a percentage loss of the initial *iso*-alpha-acid present in the beer, the Spent Hop treated beers lost the greatest percentage (ΔCold-Aged = 38.4%, ΔForce-Aged = 42.0%), followed by the Extract (ΔCold-Aged = 33.4%, ΔForce-Aged = 40.31%), then Pellet (ΔCold-Aged = 28.9%, ΔForce-Aged = 38.3%), then Iso Dosed (ΔCold-Aged = 23.1%, ΔForce-Aged = 32.2%). Reports indicate that beers high in reductive capacity (high polyphenol content) have a stabilizing effect on the degradation of *iso*-alpha-acids [8, 195-197]. Unfortunately, the results seen here do not adequately support the idea that hop polyphenols contribute to a beer's resistance against *iso*-alpha-acid degradation. This could in part be because of the nature of the analytical assay that does not allow for quantification of specific *iso*-alpha-acid stereo-isomers.

The *trans*-stereoisomers of the *iso*-alpha acid analogues are more prone to oxidation than the cis-stereo-isomers during aging, especially under temperature abuse, and thus the ratio of *trans* to cis *iso*-alpha-acids can be used as a staling indicator [155, 156]. To determine the effect of increased hop polyphenol content on the ratio of *trans* to cis *iso*-alpha-acids, a second HPLC method was used that allows for quantification of the *trans* and cis stereoisomers of the three major *iso*-alpha-acid analogues. When comparing the fresh beers, sufficient evidence was provided to indicate that hopping regime affects the initial ratio of *trans* to cis isomers, referred to %Trans (reported in Table V). Specifically, the Spent Hop fresh beers were highest in the *trans* stereo-isomers (%Trans = 53.9), followed by the Extract (%Trans = 49.2), Pellet (%Trans = 47.0%) and then the Iso Dosed (%Trans = 21.0%). There is no 'Control treatment' for this analysis because the control had no *iso*-α-acids.

As anticipated, hopping regime significantly affected the decline of %Trans over 8 weeks of storage ($p<0.05$). A significant temperature effect was also seen with temperature abused samples having a greater decline in %Trans after 8 weeks of storage ($p < 0.001$). When comparing decline of %Trans for cold-aged samples, the Pellet, Extract, Iso dosed treatments were not significantly different in their rate of %Trans decline (3.04%, 2.68%, and 1.44% respectively). The Spent Hop treatment experienced the greatest decline in %Trans over time ($p < 0.05$). Interestingly after 8 weeks of force-aging, only the Iso-dosed treatment showed significantly less decline than the Extract, Pellet and Spent Hop beers (5.26%, 12.1%, 12.3% and 13.5% respectively). This result might indicate that beers lowest in polyphenols allow for greatest protection against *trans*-*iso*-alpha-acid degradation, however the results are conflicted by the fact that the fresh beers were significantly different in %Trans initially. When the loss of *trans*- *iso*-alpha-acids was calculated as a percentage loss of the initial *trans*-alpha-acid present in the beer (**Figure 3.5**) the results changed. For cold-aged beers, only the Spent Hop treated beers lost significantly more *trans* by percentage (ΔCold-Aged = 13.09%), than the other beers:Extract (ΔCold-Aged = 5.45%), Pellet(ΔCold-Aged = 6.46%) , Iso Dosed(ΔCold-Aged = 6.85%). After force-aging for 8 weeks there were no significant differences in the percentage of initial *trans* lost (Spent Hop = 25.14%, Extract = 24.66%, Pellet= 26.15%, and Iso Dosed= 25.05%), providing insufficient evidence that polyphenol content prohibits the rate of decline in %Trans under cold-storage conditions or that polyphenol content exacerbates a decline in %Trans in heat abused beer.

Assesment of beer antioxidant potential. Polyphenol capacity to improve food oxidative stability has been well established [141], and thus these antioxidants have been considered for their potential to improve beer flavor stability [93, 100, 182, 198-200]. Flavan-3-ols and proanthocyanidins behave as antioxidants that are capable of scavenging free radicals [43] in food systems due to their electron configuration. Flavan-3-ols readily donate electrons to free radical species ($R^{\bullet}$) to assume the radical character ($F^{\bullet}$) themselves, and effectively result in a radical that is generally more stable and less harmful than the initial radical species. As stated previously, flavan-3-ol oxidation predominantly produces semiquinone radicals, that couple through nucleophilic addition

to produce proanthocyanidin oligomers. These oligomers may retain reactive anti-radical configuration [201], with varying antioxidant potential determined by substitution patterns, stereochemistry and interflavanoid bond orientation [178, 180, 202-204].

The relative antioxidant or anti-radical capacity of the finished fresh and aged beers was assessed via two methods. The first method, the ferric reducing antioxidant power (FRAP) assay is a relatively simple colorimetric method that measures the beer's reducing capacity or antioxidant capacity by its propensity to reduce iron (III) to iron (II). In that regard it is similar to the method used to determine total polyphenols, and thus it may also be used to determine general polyphenol content of a sample. Comparison of the fresh beers indicates that hopping regime significantly affects the antioxidant capacity of lager beer as per the FRAP assay. FRAP values are reported in **Table 3.6** and **Figure 3.5** as Trolox Equivalents (ppm). Of the fresh beers those brewed with Spent Hops were highest in FRAP value, significantly higher than all other beers ($p < 0.001$). This was expected due to their increased polyphenol content. Accordingly a high linear correlation between FRAP antioxidant value and both phenolic assays resulted for fresh beers: total polyphenol ($r^2 = 0.913$) and total flavanoid content ($r^2 = 0.916$). Cold and Force-Aged beer samples were analyzed at three time points each over a twelve week period. Cold-Aged beers were not significantly different in their FRAP antioxidant capacities than fresh beers, even after 12 weeks of storage, and results correlated well with total flavanoid content (cold-aged beer $r^2=0.956$ and force-aged beer $r^2 = 0.974$). Force-aging for 12 weeks caused a decline in the antioxidant capacity for all of the beers, but the FRAP response was not significantly different for force-aged beers than for fresh beers. The Pellet and Spent Hop beers had similar losses of antioxidant capacity by ppm Trolox equivalents, however when calculated as a percent loss of initial antioxidant capacity, the Pellet treatment maintained its antioxidant potential better than the other beers, especially better than the Extract and Iso dosed beers ($p < 0.05$). After 12 weeks of aging at 30°C, the Pellet beers lost a mean of 1.6 % antioxidant capacity, the Spent Hop beers lost about 7.6% antioxidant capacity, the Control 10.2%, the Iso Dosed 11.8% and the Extract fared the worst with 12.3% loss in antioxidant capacity. It is interesting that the Pellet hopped beers fared so well after force-aging. The Pellet and Spent Hop beers saw a similar

decline in %PP, yet the Pellet beers maintained antioxidant capacity throughout aging. Whether or not this was due to a synergistic effect due to the presence of both polyphenols and *iso*-, alpha-, and beta- acid derivatives in the Pellet beers as suggested by some[75, 137], or to variations in the chemical nature of the polyphenols present in the Pellet beers remains to be resolved.

To investigate the antiradical potential of the beers, a second method, Electron Spin Resonance (ESR) was utilized. ESR is used in the brewing industry to measure the formation of radicals relevant to beer oxidation, in essence to predict beer shelf life [125, 205]. Samples are exposed to atmospheric oxygen and high temperature (60°C) while the relative radical concentration is measured. The longer a beer suppresses radical formation (lagtime), the better the predicted shelf life of the beer. Likewise a measurement of the radical population (ESR signal intensity) may be taken at a specific time, usually at t= 150 minutes (T150). A lower T150 correlates to increased anti-radical potential. A shelf stable beer would be expected to have a relatively low T150 and a relatively long lagtime [125].

ESR was performed on the fresh beers and on the aged beers after 12 weeks of storage. Hopping regime and storage time significantly affected anti-radical potential of the lager beer, reported as T-150 (**Table 3.7 and Figure 3.6**) regardless of storage time or storage temperature ($p < 0.05$). Comparison of the fresh beers indicated that all treatments, except the Iso-dosed treatment, had significantly higher T150s than the Control. The Iso-dosed (T150 = 125) treatment had the lowest T150 followed by the Control (T150 = 131), Pellet (T150 = 162), Extract (T150 = 164), then Spent Hop (T150 = 203) beers. A significant treatment effect was seen following 12 weeks of storage, regardless of cold or force-aging, with all T150 values improving slightly after storage. The Spent Hop ($p < 0.05$) and the Extract ($p < 0.10$) treatments differed from the Control beers, both having higher T150 values and thus decreased anti-radical capacity. Consistent with the fresh beers, force-aged Spent Hop beers had the highest mean T150 value while the force-aged Iso Dosed beers had the lowest mean T150 after 12 weeks of temperature abuse, followed by Control, Pellet, then Extract beers.

Transition metals such as Fe and Cu are integral players in the catalysis of free radical chain reactions and thus are known to increase ESR signal intensity of beer and other fermented beverages [206-209]. Likely due to the high concentration of iron in all of the beers vs. commercial lager beers, lagtimes were not attainable in the beer samples. However a comparison of T150 values to iron or copper did not indicate that there was any correlation between ESR T150 and metal content of the beers, and nor did the sum of iron and copper. In fact a slight negative correlation existed between metal content and ESR T150; T150 value increased (less anti-radical potential) as the metal content of the beers decreased. For example the Spent Hop beers were significantly lower in Fe than the Control beers, yet the Control beers had a significantly lower mean T150 ($p < 0.05$). The only variance in the beers was the way in which they were kettle boiled, the Control beers being kettle boiled in bulk with the hopped beers kettle boiled in smaller volume in a smaller kettle. Yet, the same trend was seen when comparing only the three kettle hop treatments. It is likely that because all of the finished beers had iron levels above 80 ppb, well above the recommended levels for flavor stability, that variation above 80 ppb may not have as much of an effect on the ESR signal. A positive correlation between sulfur dioxide [69, 125, 207] content and beer ESR lagtimes has been reported by several authors. Sulfur dioxide levels in the finished beers were not assessed based on the assumption that the kettle hopped beers were produced according to an experimental design that would eliminate this variation. However, a comparison of total polyphenols (TPP) indicated that ESR T150 values correlated well with polyphenol content of the beer. As Total polyphenol content increased, ESR T150 increased, especially after storage at high temperature: fresh beer ($r^2 = 0.781$), cold-aged beer ($r^2 = 0.794$), force-aged beer ($r^2 = 0.969$). A similar effect was observed with total flavanoids: fresh beer ($r^2 = 0.971$), cold-aged beer ($r^2 = 0.793$), force-aged beer ($r^2 = 0.980$).

While several ESR lagphase studies indicate that polyphenol levels do not positively influence the formation of free radicals either during the production of or in finished alcohol based beverages [40, 69, 75, 137, 209, 210], this study indicates that polyphenol levels may have a detrimental effect on free radical formation, and thus beer flavor stability, as measured by ESR. One plausible explanation is that some polyphenols

are capable of behaving in a pro-oxidant nature, as has been seen with ascorbic acid [69]. Flavan-3-ols, proanthocyanidins and flavonols (which were not directly measured) may in fact promote the formation of reactive oxygen species because of their propensity to reduce cations such as iron and copper [51, 211] into more active states. Specifically, flavonoids such as prodelphinidins (3'4'5'trihydroxyflavans) show strong pro-oxidant potential [144] because they can function as coupled reducing agents(i.e. Fe (III) → Fe(II)). Reported prodelphinidin [90, 212] concentrations in beer (mg/L) [213] should adequately promote oxidative reactions leading to flavor instability. Flavonols found in beer [213] can also display prooxidant activity due to their propensity to reduce iron and copper to the active forms necessary to prompt Fenton and Haber-weiss reactions [1, 213]. Although it has not yet been substantiated with sufficient evidence, it is plausible to assume that the presence of certain species of flavonoids in beer could act to reduce iron and copper to their more reactive states during the ESR analysis, and thereby affect a beer's estimated shelf stability via this method. Because this is the same mechanism by which the total polyphenol and the FRAP assays work, it is not surprising that both assays are so highly correlated with high T150 values.

An interesting paradox presents itself because flavan-3-ols, proanthocyanidins and flavonols are known to act as anti-oxidants by effectively binding and reducing apparent concentrations of divalent transition metals [214-216]. However, flavonoid potential to chelate metals depends on hydroxylation pattern, degree of polymerization [217] and several other factors such as media pH. At beer pH (5.5) flavan-3-ols such as (+)-catechin would not be expected to effectively chelate copper, however hop-derived flavonols would [218]. In fact, flavonols purportedly reduce Fe (III) to their preferred binding species Fe(II) before association, and thus could potentially promote radical formation (via instigation of Fenton's reaction) during the ESR analysis. Other flavonoids such as kaempherol, luteolin and (+)-catechin more readily complex Fe (III). This is of significance because in order for transition metals to be effective catalysts in radical reactions during natural beer aging they must be present in, or transitioned to, their free (unbound) or ionic forms (Fe(II) and Cu(I)) [219, 220]. Chelated transition metals are potentially less active in the promotion of free-radical related reactions, but at

beer pH which compounds are chelating which metals? And, is it possible that the true anti-oxidant or anti-radical potential of some polyphenols may not be measureable by ESR/PBN spin trapping due to this phenomenon? More investigation is necessary in order to begin unraveling the intricacies of this paradox.

Assessment of Beer Flavor Stability

In practical application beers hopped with spent hops or polyphenol rich products have proven to have enhanced flavor stability and thus improved shelf-life [6, 7, 79-81, 111, 143]. In the present study beer flavor stability was assessed via two methods: instrumentally by monitoring aldehydes pertinent to aged-beer flavor and sensorially by trained panel.

Although numerous compounds have been targeted as staling indicators in beer [28, 149], much attention has been given to the presence of carbonyl compounds. Aldehydes in particular are thought to play an important role in the deterioration of beer flavor and aroma during storage. Oxidation of amino acids via strecker degradation is one plausible mechanism by which aged flavor aldehydes may be produced: an amino acid reacts with an alpha di-carbonyl compound to be converted into an aldehyde with one less carbon atom. Some examples are the conversion of leucine to 3-methylbutanal, alanine to acetaldehyde, valine to 2-methyl-propanal, methionine to methional and phenylalanine to phenylacetaldehyde. Strecker degradation may take place during wort boiling and during beer storage. Aldehydes pertinent to aging can also result from other chemical pathways related to lipid oxidation[147, 221], maillard reactions[222, 223], and the degradation of proteins[224]. Aldol condensation reactions between aldehydes and ketones can also occur to produce flavor important compounds such as *trans*-2-nonenal[1]. The very low flavor threshold of some aldehydes makes them key players in aged beer flavor, even if present at sub-ppb levels in beer [157]. Furaldehyde, a Maillard product, has been established as an indicator of temperature abuse despite its very high flavor threshold. While other aldehydes may be equally less-flavor-active they are also used as aged beer flavor indicators because their concentrations increase in conjunction with increases in other oxidative flavors during aging [154, 158-160, 164].

Fresh and aged beers were analyzed for content of twelve (12) aldehydes via SPME-GC-MS with on fiber derivatization (PFBHA): isobutyraldehyde, 2-methylbutyraldehyde, isovaleraldehyde, valeraldehyde, hexanal, furaldehdye, heptanal, methional, octanal, benzaldehyde, phenylacetaldehyde, and *trans*-2-nonenal. A standard curve was produced for each aldehyde by incremental dosing of known concentrations into the fresh un-hopped control beer (Control). Concentrations of aldehydes were back-calculated to these standard curves. Levels of aldehydes for the Fresh, Cold-Aged and Force-Aged beers are reported in **Tables 3.7, 3.8 and 3.9**. As stated previously, the Iso Dosed and Control treatments were not produced as kettle replicates, they were created in bulk and stored in duplicate vessels, thus discussion of statistical analyses for aldehydes were limited to the comparison of means between treatments that were truly replicated in the kettle: Pellet, Extract and Spent Hop. Comparisons were then drawn back to the Control and Iso Dosed treatments, but not to a statistically significant level. The concentrations of aldehydes in the beers were regarded in several different ways. Comparisons were made between levels (ppb) of each individual aldehyde, and also between the sum (total aldehydes) and percent composition of aldehydes for the fresh, cold-aged and force-aged beers. Changes in levels of each aldehyde, total aldehydes and percent composition due to storage were also evaluated.

Aldehydes in fresh beers. As seen in **Table 3.7**, fresh beers varied in levels of total aldehydes. The Control, Iso Dosed and Spent Hop beers were highest in total aldehydes, while fresh beers produced from Pellet and Extract kettle treatments resulted in lower total aldehydes. When looking at percent composition, phenylacetaldehyde (31.4% mean for all treatments pooled) was the predominant aldehyde by percentage in all fresh beers (including Control and Iso Dosed) except for the Pellet treatment which was also characterized with high levels of methional. Levels of phenylacetaldehyde were as high as 18.4 ppb (Control) and as low as 3.32 ppb (Pellet). Six aldehydes represented roughly 90% of the mean ppb total aldehydes in the fresh beers: phenylacetaldehyde (31.44%), methional (18.73%), isobutyraldehyde (13.70%), furaldehyde (11.34%), isovaleraldehdye (9.44%), 2-methylbutyraldehyde (5.16%). The aldehydes *trans*-2-nonenal, valeraldehyde, hexanal, heptanal and octanal were present at less than 1 ppb in all fresh beers and thus comprised respectively low percentages of total aldehydes in the beers.

Of the kettle hopped treatments, the fresh beers produced from Spent Hop kettle treatment were significantly higher in levels of isobutyraldehyde ($p < 0.01$), 2-methylbutyraldehyde ($p < 0.01$), isovaleraldehyde ($p < 0.01$), valeraldehyde ($p < 0.05$), furaldehyde ($p < 0.01$), benzaldehyde ($p < 0.01$) and phenylacetaldehyde ($p < 0.01$). However the Pellet and Extract kettle treated fresh beers did not differ in levels of these seven aldehydes. Interestingly, levels of methional were significantly different in all three of the fresh kettle treated beers ($p < 0.01$). No significant differences in levels of hexanal ($p = 0.23$), heptanal ($p = 0.23$), octanal ($p = 0.28$), or *trans*-2-nonenal ($p = 0.42$) resulted in the finished fresh kettle treated beers.

Aldehydes in cold- aged beers. After cold-aging, all treatments showed an increase in total aldehydes. Consistent with the fresh beers, Pellet kettle treated beers were lowest in total aldehydes, followed by the Extract kettle treatment, the Spent Hop kettle treatment, the unhopped Control and the Iso Dosed beers. Again, phenacetaldehyde was the predominant aldehyde by percent composition (34.6%) in the cold-aged beers. Driven by all treatments, levels of phenylacetaldehyde were highest in the Control, Iso Dosed and Spent Hop treatments. Isobutyraldehyde (34.6%) and methional (16.0%) were the next most abundant aldehydes by percent when means for all treatments were considered. Phenylacetaldehyde and methional have been recognized as a key indicators of aged beer flavor [225], and thus increases in these aldehydes from aging is consistent with the literature.

Of the kettle hopped treatments, cold-aged beers produced from the Spent Hop treatment were highest in total aldehydes, comparable to levels seen in the control beers. Of the kettle hopped beers, the Spent Hop cold-aged beers were significantly higher than both the Pellet and Extract hopped cold-aged beers for two aldehydes: isobutyraldehyde ($p < 0.01$) and valeraldehyde ($p < 0.01$). The Spent Hop kettle treated beers were higher than the Pellet kettle hopped cold-aged beers, yet not significantly higher than the Extract kettle hopped cold-aged beers for four aldehydes: 2-methylbutyraldehyde ($p < 0.05$), isovaleraldehyde ($p < 0.05$), methional ($p < 0.05$) and phenylacetaldehyde ($p < 0.01$). All three kettle hopped cold-aged beers were significantly different in levels of furaldehyde ($p < 0.01$), with Spent hop characterized by the highest levels, then Extract, then the Pellet hopped beers. Extract hopped cold-aged beers were significantly higher than the Pellet

hopped cold-aged beers in hexanal ($p < 0.05$) and *trans*-2-nonenal ($p<0.05$), but not significantly higher than the levels seen in Spent Hop treated beers. No significant differences were seen in the levels of *trans*-2-nonenal and hexanal between the Spent Hop or Pellet treated beers. No significant differences were measured in the levels of heptanal ($p = 0.7$), octanal ($p = 0.39$) or benzaldehyde ($p = 0.12$) for the kettle treated cold-aged beers.

Aldehydes in force-aged beers. Although reactions that take place at extreme conditions may not appropriately represent reactions that might take place under real time storage conditions, force-aging beers at 30°C allowed us to investigate the effects of hopping regime on beer aging under high temperature (force-aging) and over a shorter period of time. Even after force-aging, the beer produced with a Pellet kettle treatment was lowest in total aldehydes, followed by the Extract kettle treatment, the Spent Hop kettle treatment, and the Iso Dosed post-fermentation treatment. The beer produced from the unhopped Control treatment was highest in total aldehydes after forced aging. Isobutyraldehyde (33.3%) was the predominant aldehyde by percent composition when all treatments were pooled. This was driven by the Control and the Spent Hop treatments. Levels of phenylacetaldehyde were highest for all other treatments and comprised 31.6% of the total aldehydes (mean of pooled treatments). Of the kettle hopped treatments, force-aged beers produced from the Spent Hop material were significantly higher in levels of isobutyraldehyde ($p << 0.01$) and methional ($p < 0.01$) than either the Pellet or Extract treated force-aged beers. The Spent Hop kettle treated force-aged beers were significantly higher in furaldehyde ($p < 0.05$) than the Pellet treated beers, yet not significantly different than the Extract treated beers.

Changes in aldehydes due to storage. Changes in aldehyde levels due to force and cold-aging were investigated. The levels of total aldehydes in the fresh and aged beers as well as differences due to aging are reported in **Table 3.10**. Total aldehydes (ppb) increased significantly during storage over 8 weeks for all kettle treatments, with greater increases seen when beers were stored at 30°C (**Figure 3.7**, force-aged $p << 0.001$ and cold-aged $p < 0.05$). All kettle hopped beers were different in levels of total aldehydes after cold storage and force-aging, with the Spent Hop beers highest in total

aldehydes ($p < 0.05$). There was a treatment effect for changes in total aldehydes for cold-aged beers ($p < 0.05$), but not for force-aged beers. The Extract treated beers endured the largest increase in total aldehydes, followed by the Spent Hop beers and then the Pellet treated beers. Pellet and Spent Hop treated beers did not differ significantly in increases in total aldehyde levels after cold storage.

Again, phenylacetaldehyde was a predominant aldehyde by percent composition, increasing in percentage of total aldehydes during storage at both temperatures. Phenylacetaldehyde (estery, floral, flowery, roses) has been indicated as an important flavor contributor to beer because of its potentially low flavor threshold (<1 ppb[225] to 105 ppb[226]). If the 1 ppb threshold level is accurate, levels of phenylacetaldehyde in the fresh, cold-aged and force-aged beers would be expected to impact overall aroma of the beers. Higher increases were seen in the force-aged beers, ($\text{Extract}\Delta = 8.21$ ppb, $\text{Pellet}\Delta = 9.90$, and $\text{Spent Hop}\Delta = 2.14$), bringing the levels of phenylacetaldehdye in the Extract and Pellet beers to equivalent levels seen in the fresh and aged Spent-hopped beers.

The percent contribution of isobutyraldehyde to total aldehydes increased significantly due to storage (fresh =13.9%, cold-aged =16.4%, force-aged =33.6%). Storage at either temperature resulted in a large increase in ppb isobutyraldehyde for all treatments, with greater increases seen due to force-aging. Force-aging of Spent Hop kettle treated beers resulted in the greatest increase in isobutyraldehyde (fresh = 5.8 ppb, force-aged = 26.4 ppb). Isobutyraldehyde is a strecker aldehyde produced from valine with a reported flavor threshold of 1ppm. It has been described as extremely diffusive, pungeant, green, grassy and straw-like, however at extreme dilution may take on an almost ‘pleasant fruity, banana-like’ aroma. It has been postulated that polyphenols may have a catalytic role in strecker degradation [1]. The degradation of valine to isobutyraldehyde is reported to occur more rapidly in the presence of dehydro-ascorbic acid, an oxidized derivative of ascorbic acid having three keto-groups on the pyran ring. Once oxidized from their di- or tri-hydroxy forms to quinonic forms, perhaps polyphenols might behave in a similar manner. Further investigation should be conducted to confirm this phenomenom. Isobutyraldehyde is also a product of humulone

hydrolysis via the isomerization of humulone to allo-isohumulone[227]. However, this is likely not the explanation for the levels of isobutyraldehyde seen in the Spent Hop beers, because similar levels were recorded in the fresh and force-aged Control beers which did not contain any humulones.

After storage, methional was still a key player in total aldehyde composition for all treatments, however its contribution to total aldehydes declined due to cold and force-aging; when treatments were pooled, both storage temperatures resulted in significant losses of the % of methional to the total aldehyde content (fresh =18.9%, cold-aged =16.2% and force-aged = 6.6%). This was due to the fact that the Spent Hop and Pellet beers showed a decline of methional (ppb) after force-aging, while the Extract treated beers increased in methional after aging (ΔSpent Hop = -1.66, ΔPellet = -1.84 and ΔExtract = 1.1 ppb). These results are inconsistent with the literature. Methional (cooked potato, worty) has been linked to aged beer flavor previously, with substantial increases seen in beers during normal storage and accelerated storage[225]. Thresholds for methional have been reported as less than 0.5 ppb by Soares da costa et al.[225] to 4.2 ppb by Saison et al.[226], thus levels seen in the force-aged beers could be expected to impact the overall aroma profile of the beers.

Furaldehyde is a product of the Maillard reaction and used as an indicator of temperature abuse despite its very high odor threshold (15,157 ppb or greater[226]). The Spent Hop and Control beers were highest in furaldehdye in the fresh beers, however aging at either storage temperature did not affect the levels of furaldehyde in these beers. The levels of furaldehyde in the Extract beers increased due to storage, with significant increases resultant from force-aging at 30°C (fresh beer = 1.51 ppb, cold-aged = 2.82 ppb, force-aged = 3.58 ppb) to match the levels seen in the fresh Control and Spent Hop beers. This insinuates that beers produced with Extract (without polyphenols) are more susceptible to heat oxidation than beers hopped with Pellets (with polyphenols) and perhaps there is a protective effect of polyphenols against temperature abuse during aging.

Isovaleraldehyde (3-methylbutyraldehyde), a strecker degradation product formed from leucine, is considered a tertiary flavor constituent by Meilgaard[154]. Characterized

as having malty, cherry, apple, almond and cocoa like aromas it is thought to have a relatively subtle effect on beer flavor and aroma, with a flavor threshold of 46 ppb or higher [226]. Although present below threshold levels, the fresh Spent Hop and Control beers were highest in isovaleraldehyde and the levels were not significantly augmented by cold or force-aging in these beers. Signficant increases were seen after cold-aging in the Extract beers and after force-aging in both the Extract and Pellet beers to match levels seen in the Control beers.

2-Methylbutyraldehyde is characterized by almond, apple-like and malty aromas. By percentage it was the 6th most predominant compound of total aldehydes in the fresh beers. Levels of 2-methylbutyraldehyde were highest in the fresh Spent Hop and Control beers, but after 8 weeks of force-aging, the beers did not differ significantly in levels of this aldehyde. 2-methylbutyraldehyde has a flavor/odor threshold of 46/56 ppb, and thus the levels resulting in these beers would not be expected to greatly impact their overall aroma.

Although benzaldehyde (almond, cherry), valeraldehyde (fruity, nutty) and hexanal (winey) were well below flavor or odor thresholds, similar increases resulted due to storage with the Extract beers suffering the largest increases. Levels of heptanal and octanal did not different between treatments and no changes were observed to occur due to storage. However, it is important to consider that some compounds behave differently in the presence of other compounds to elicit odor and flavor, even when they exist at sub-threshold concentrations[226].

Trans-2-nonenal (cardboard/papery) has been recognized as an important contributor to aged beer flavor due to its very low (<1 ppb[226]) flavor threshold. *Trans*-2-nonenal is a product of lipid oxidation of poly-unsaturated linoleic acid isomers, and is thought to also be released during aging from imine adducts[228]. Surprisingly *trans*-2-nonenal levels did not increase dramatically during aging, and its contribution to the total aldehyde content decreased overall during storage (fresh = 11.4%, cold-aged =9.9% and force-aged = 7.0%). The Iso Dosed treatments had the highest increase in *trans*-2-nonenal at both storage temperatures, followed by the Extract treatment. Increases for *trans*-2-nonenal were greatest for beers aged at 30°C: Extract (Δ0.12 ppb) > Pellet (Δ0.09 ppb) >

Spent Hop (Δ0.04 ppb). Because of its potency (cardboard aroma at 0.035 ppb), even small changes in the levels of *trans*-2-nonenal resultant from aging could impact the aroma profile of lager beers.

Assessment of beers by sensory analysis.

Despite the analytical data indicating increases in flavor compounds pertinent to aging, analytically determined chemical compounds do not always explain observed sensory changes. This may be due to synergies, masking effects, or panelist inability to detect certain compounds or to discern differences between the effects of compounds with similar aroma profiles. The aged-beers were therefore subjected to evaluation by a trained sensory panel. Due to the large number of beers that needed to be evaluated, panelists only evaluated the beers for aroma profiles using 8 descriptors: Overall Aroma Intensity, Piney, Cardboard, Tropical Fruit, Caramel, Honey, Apple and Skunky/Sulfury.

When all treatments and both aging temperatures were considered hopped beers were discerned as being different than unhopped beers (**Figure 3.8**). The Spent Hop and Pellet beers were characterized by high Piney and Tropical fruit notes, with significant increases occurring after force-aging. As seen in the principle component plot, scores for Piney and Tropical fruit dominated the plot, masking differences due to other aroma attributes. **Figure 3.9** shows the principle component plot with Piney and Tropical Fruit descriptors removed, allowing for differences in other descriptors to pull out on the plot. The Control beers were rated as being higher in Overall Aroma Intensity, with changes in intensity resulting from storage. When treatments were pooled by temperature there was no significant effect of storage temperature for Overall Aroma, Cardboard, Tropical Fruit or Skunky/Sulfury. There was a significant storage temperature effect for Piney and Apple with force-aged beers significantly higher in Piney and Apple aroma than cold-aged beers. Cold-aged beers scored significantly higher in Caramel and Honey aromas. When all treatments were considered based on individual aroma descriptors differences pulled out (see below). All reported differences were significant to $p = 0.05$ or less. Mean scores for each aroma attribute are reported in **Table 3.11**.

Overall Aroma Intensity. Panelist scores for Overall Aroma Intensity ranged from 6.8 (several treatments) to 8.0 (Control, cold-aged). The cold-aged unhopped Control and Spent Hop treatments ranked highest in overall aroma intensity. Of the force-aged beers, panelists scored the Spent Hop beers highest in overall aroma, followed by the Pellet and Control beers. For force-aged beers, the Iso Dosed and Extract were lowest in overall aroma, significantly lower than the Spent Hop beers.

Piney. Mean panelist scores for Piney ranged from a low of 2.0 (Iso Dosed, force-aged) to a high of 4.4 (Pellet, force-aged). Panelists rated the force-aged Pellet and Spent Hop treatments as most Piney. The Pellet hop and Spent Hop force-aged beers were significantly more Piney than all other beers, regardless of storage temperature, with the exception of the Extract force-aged beers, which were significantly lower than the Pellet but not significantly lower than the Spent Hop force-aged beers. There were no significant differences amongst the cold-aged beers in Piney aroma.

Cardboard. Panelists ratings for Cardboard aroma were as low as 1.8 (Spent Hop, cold-aged and Pellet , hot-aged) and as high as 3.3 (Control, cold-aged). Panelists rated the Control beers highest in Cardboard aroma, regardless of storage temperature. The cold-aged Control beers were significantly higher than Pellet or Spent Hop cold-aged beers. Although the Control force-aged beers were higher in Cardboard aroma than all other force-aged beers, there were no significant differences between Cardboard scores between treatments for the force-aged beers.

Tropical Fruit. Mean panelist scores for Tropical Fruit aroma ranged from 2.1 (Iso Dosed, force-aged) to a high of 4.0 (Pellet, force-aged). Pellet and Spent Hop beers were significantly higher in tropical fruit aromas than all other beers, regardless of storage temperature. There were no significant differences in Tropical Fruit aroma for other beer treatments.

Caramel. Mean panelist scores for Caramel aroma ranged from 1.8 (Pellet, force-aged) to 4.6 (Spent Hop, cold-aged). The Spent Hop cold-aged beers were significantly higher in overall caramel aroma than all other beers, regardless of storage temperature. The Pellet hopped beer force-aged beers were ranked lowest in Caramel aroma,

significantly lower than the Spent Hop and Pellet cold-aged beers, but not significantly different than the other force-aged beers.

Honey. Mean panelist scores for Honey aroma ranged from 1.3 (Control and Pellet, force-aged) to 2.4 (Spent Hop, cold-aged). Panelists ranked the Spent Hop cold-aged beers highest in Honey, followed by the Control cold-aged beers.

Apple. Scores for Apple aroma spanned from 2.3 (Spent Hop, cold-aged) to 3.2 (Iso Dosed, force-aged). Panelists did not discern any significant differences for Apple aroma for specific treatments at either storage temperature, despite the temperature effect seen when pooling treatments.

Sulfur/Skunky. Sulfur/Skunky scores were as low as 0.5 (Pellet, force-aged) and as high as 2.2 (Control, cold-aged). Panelists rated the control treatments highest in Sulfur/skunky, with the Control cold-aged beers significantly higher than the Extract, Pellet and Spent Hop treated cold-aged beers. Force-aged beers were not significantly different from each other in sulfur/skunky aroma.

Relationship between sensory and analytical results.

A PCA plot was constructed to compare all analytical results from the aged-beers and to summarize the analytical data (**Figure 3.10**). Overall the force-aged beers separated slightly from cold-aged beers due to increased Haze and higher total aldehyde content. The Spent Hop treated beers became distinguished from the other beers, regardless of temperature, ranking high in Total Polyphenols, Total Flavanoids, FRAP (high antioxidant potential) and T150 (low anti-radical potential). This was not surprising given the high correlations existing for the beers between theses analytical markers. The Pellet and Extract beers were characterized as high in *iso*-alpha-acids. The Control and Iso Dosed beers were distinguished by their high metal content.

Several PCAs were constructed to answer the question of whether the sensory results correlate with the analytical markers. Because the aldehyde analytical results were expected to have higher correlations with the sensory results, several PCAs were constructed to compare treatments at storage time and temperature (fresh, cold-aged,

force-aged beers). Separate PCAs were constructed to assess correlations between the sensory results because aldehydes such as phenylacetaldehyde, isobutyraldehyde and methional were predominant, comprising a high percentage of total aldehydes. PCAs were constructed to assess correlations between the sensory results and aldehydes that were present above 1 ppb (methional, benzaldehyde, phenylacetaldehyde, furaldehyde, isovaleraldehyde and isobutyraldehyde) and below 1ppb (hexanal, valeraldehyde, octanal, 2-methylbutyraldehyde, heptanal and *trans*-2-nonenal) for cold and force-aged beers (**Figures 3.11 and 3.12**).

Cold-aged Control beers were characterized by the most aroma descriptors(Apple, Cardboard/Papery, Sulfur/Skunky, Tropical Fruit) with these attributes grouped with hexanal, *trans*-2-nonenal and valeraldehyde. The cold-aged Iso Dosed and Spent Hop beers were characterized by the most aldehydes with phenylacetaldehyde and isovaleraldehdye being grouped with with Overall Aroma Intensity, and Honey and Piney being grouped with methional, benzaldehyde, furaldehyde and isobutyraldehdye, octanal and heptanal. The cold-aged Extract and Pellet beers were not characterized by many of the aroma attributes and did not group well with any of the aldehydes.

Force-aging the beer allowed differences to emerge on the PCA plots with the Control still characterized by Skunky/Sulfury, and Cardboard/Papery aroma descriptors that were grouped with phenylacetaldehdye, isovaleraldehyde and isobutyraldehyde. The force-aged Iso Dosed beers were characterized as being high in *trans*-2-nonenal, hexanal, 2-methylbutyraldehyde, phenylacetaldehyde, isovaleraldehyde, isobutyraldehyde, methional, with these aldehydes grouped with aroma attributes of Cardboard/Papery, Caramel and Honey. The force-aged Extract beers were characterized as high in Caramel and Cardboard and Sulfur/Skunky, yet were not grouped closely to any aldehydes. The Spent Hop force-aged beers were high in Apple, Tropical Fruit, Overall Aroma, Piney and Honey with the attributes grouped to furaldehyde, methional, valeraldehyde and heptanal. Pellet force-aged beers were high in Tropical Fruit, Overall Aroma Intensity, Piney, Apple and only one aldehyde: benzaldehyde.

Based on the PCA results, comparisons between aroma attributes and aldehydes were made to identify if any correlations existed. Very few correlations were significant. Positive linear correlations were found between Apple aroma and 2-methylbutyraldehyde after force aging (r^2= 0.876), despite the aldehyde being present at well below published thresholds. Honey correlated to furaldehyde levels after force-aging (r^2=0.724), most likely due to the effects of high temperature abuse on other compounds present in the beer because furaldehyde was also not present at levels near stated threshold levels. No correlations existed for Overall Aroma Intensity and any of the predominate aldehydes or between *trans*-2-nonenal and Cardboard/Papery aroma. However, the aroma attributes for Piney and Tropical Fruit were highly correlated (force-aging r^2 = 0.905). The polyphenol rich beers (Pellet and Spent Hop) aged at high temperature were assessed as high in Piney and Tropical Fruit, yet these aroma attributes did not correlate well with levels of Total Polyphenols or Total Flavanoids. Nor were correlations found between these aroma attributes or any of the assessed aldehydes, indicating that these aromas are likely due to another class of compounds that were not investigated in this study. The aroma attributes credited to the Pellet and Spent Hop beers are consistent with other investigations that ascribe fruity fig-like [142], pleasant tropical fruit and hoppy aromas to hop pellet and spent hop products[143]. Goldstein and colleagues[97] attribute these aroma attributes to water soluble glycoside flavor precursors in the hops and spent hops that have undergone chemical or enzymatic hydrolysis .

CONCLUSION

Beers were produced with different hop products to represent beers varying in levels of hop derived phenolics as well as iso-alpha-acid content. As expected Pellet and Spent hop treated beers were highest in phenolic content. Phenolic levels declined slightly throughout force-ageing, while levels of flavanoids decreased initially and then gradually increased again at five or six weeks of storage, regardless of hopping regime. Not unexpectedly increases in beer phenolic content caused increases in haze, especially after force-aging. All kettle hopping regimes reduced the levels of beer soluble Fe and Cu over the Control treatment, with the greatest effect observed with Pellet hopping. Hopping with Spent Hop material augmented beer bitterness potential as measured by

BUs, despite low levels of *iso*-alpha-acids. Hopping regime also had a significant effect on initial %Trans and on the decline in *iso*-alpha-acids and *trans*-*iso*-alpha acids over storage. Spent Hop and Pellet Hop beers scored highest in antioxidant potential as measured by the FRAP assay. Howeer ESR results contradicted these results, indicating that increasing polyphenol levels may have a detrimental effect on free radical formation in beer, and thus beer flavor stability, as measured by ESR. Analysis of aldehyde levels in the fresh and aged beers indicates that hopping regime affects initial aldehyde composition. Total aldehydes (ppb) increased significantly during storage over 8 weeks for all kettle treatments, with greater increases seen when beers were stored at 30°C. Even after force-aging, Pellet hopped beers were lowest in total aldehydes and the unhopped Control beers were highest in total aldehydes. The Extract treated beers endured the largest increase in total aldehydes, followed by the Spent Hop beers and then the Pellet treated beers indicating a protective effect for whole hop products on staling aldehyde formation. Sensorially the Spent Hop and Pellet beers were characterized by high Piney and Tropical fruit notes, with significant increases occurring after force-aging. The Control beers were rated as being higher in Overall Aroma Intensity, with changes in intensity resulting from storage. Control beers were also judged as being higher in Cardboard aroma than other beers after force-aging.

Although some positive linear correlations were found between aroma attributes and aldehydes (Apple aroma correlated well with 2-methylbutyraldehyde and Honey correlated to furaldehyde), in general specific aldehydes did not correlate well with sensory scores, indicating that the predominant aromas characterizing the Pellet and Spent Hop beers were likely due to another class of compounds that were not investigated in this study. We hypothesize that these aromas could result from chemical or enzymatic hydrolysis of glycoside flavor precursors found in the hop pellets and spent hop pellets.

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List of Figures.

a). **b).**

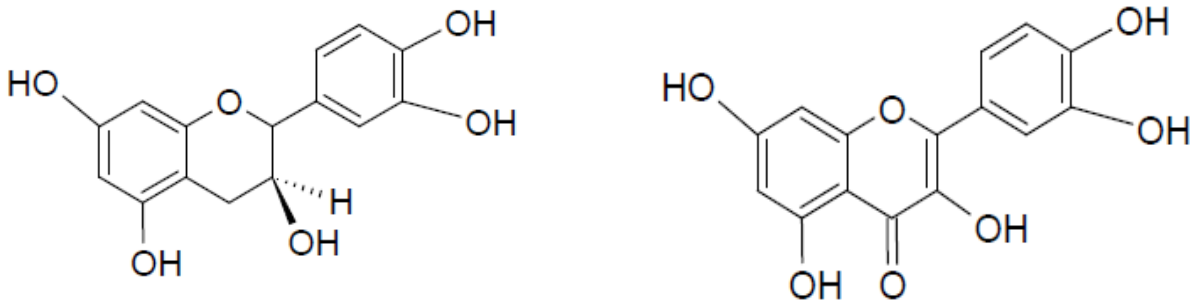

Figure 3.1. Structures of flavonoid polyphenols a). flavan-3-ol b). flavonol

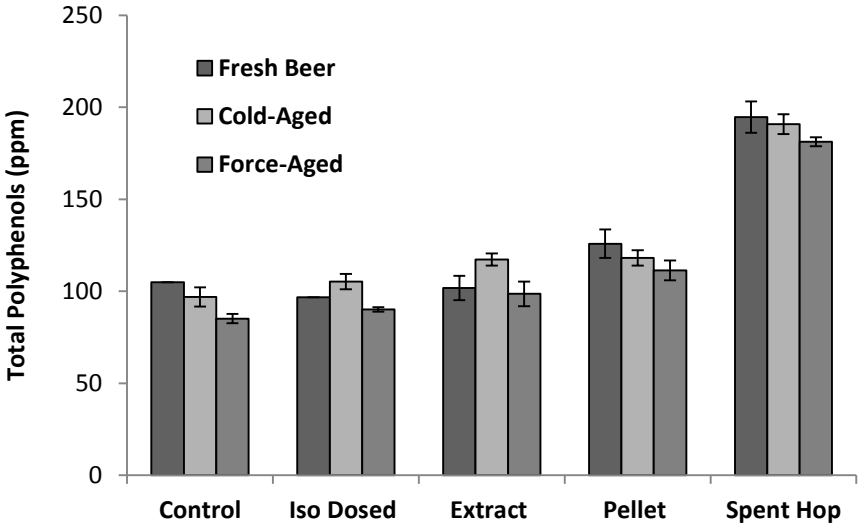

Figure 3.2. Change in total polyphenols during aging reported as mean values ± standard error.

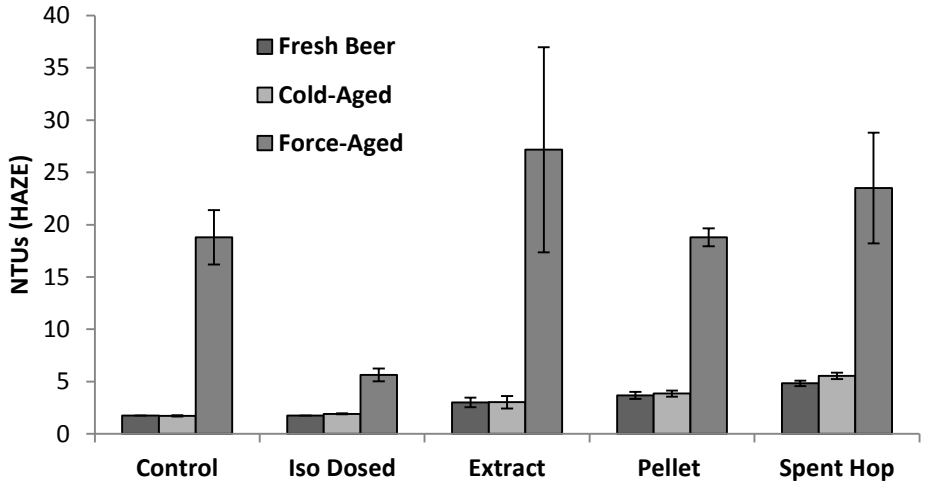

Figure 3.3. Changes in haze during aging reported as mean values ± standard error.

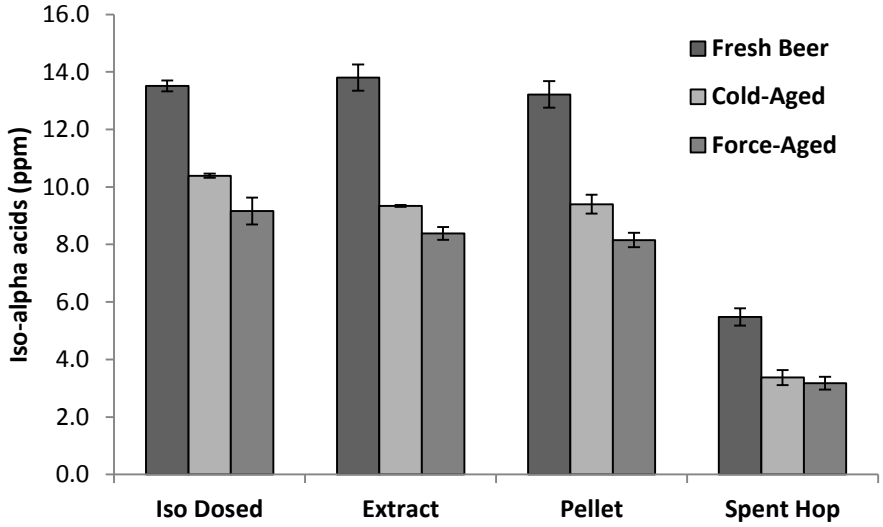

Figure 3.4. Changes in *iso*-alpha-acid content during aging reported as mean values ± standard error.

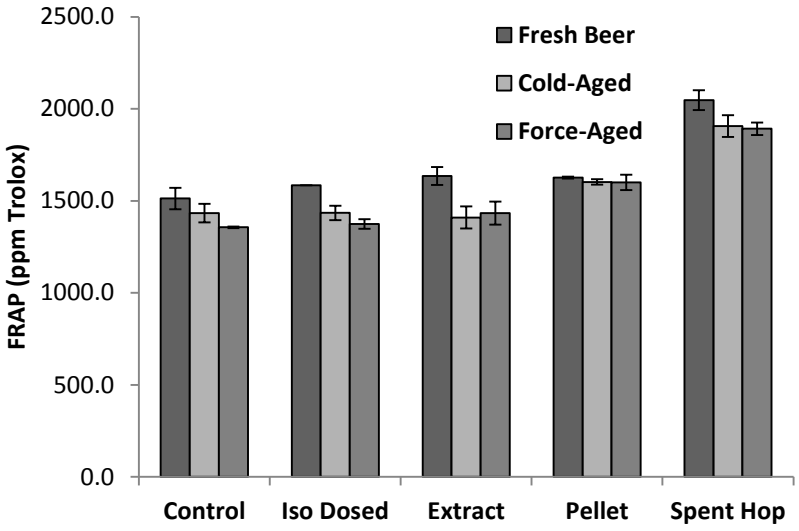

Figure 3.5. Antioxidant Capacity as per the FRAP Assay: high FRAP = high antioxidant activity.

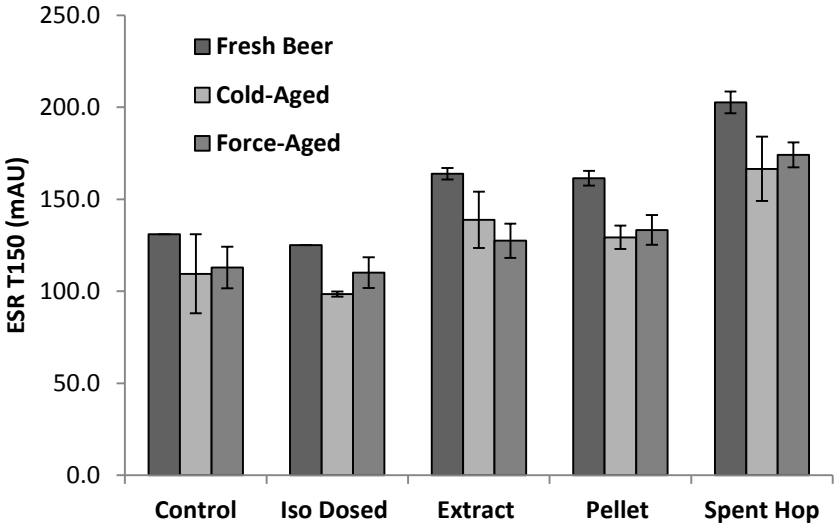

Figure 3.6. Antiradical Capacity as per ESR T150, the ESR signal (mAU) at time = 150 minutes: high T150 = low anti-radical potential. Mean values are expressed ± standard error.

Figure 3.7. Changes in total aldehydes in the hopped treatments due to aging, displayed as mean values (n = 3) ± standard error.

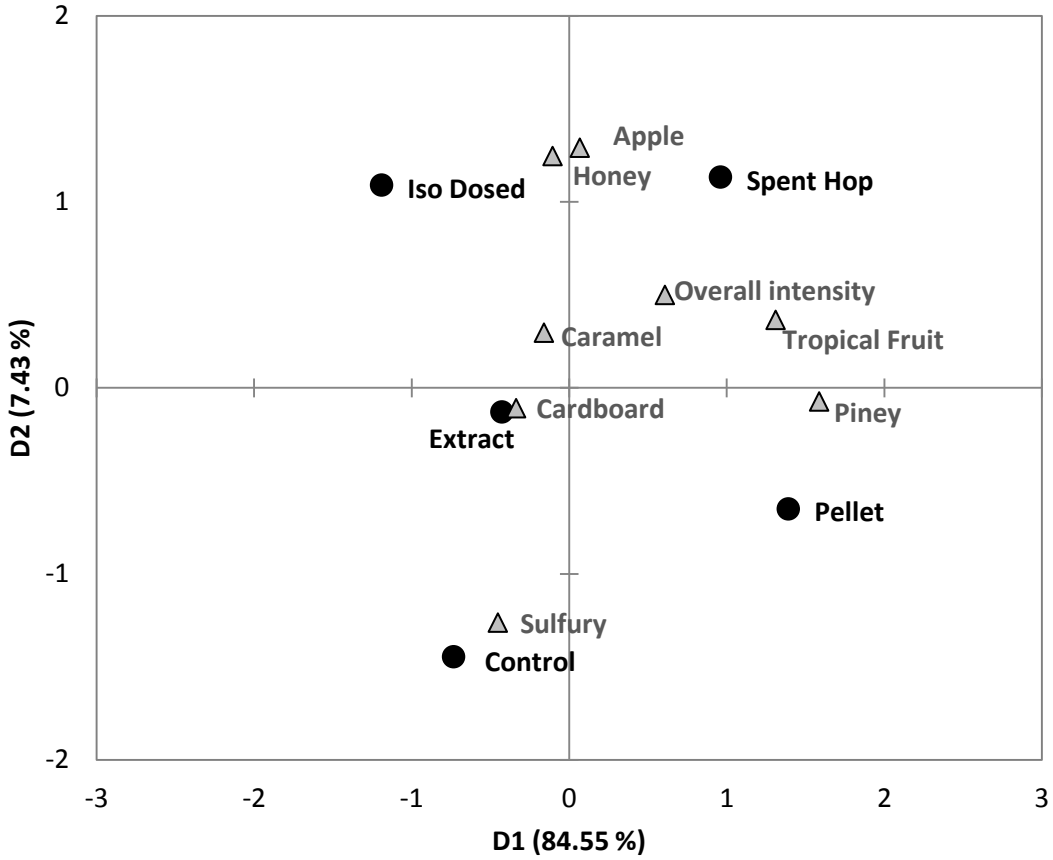

Figure 3.8. Principle Component Analysis for force-aged beers (8 weeks) by treatment with all sensory descriptors.

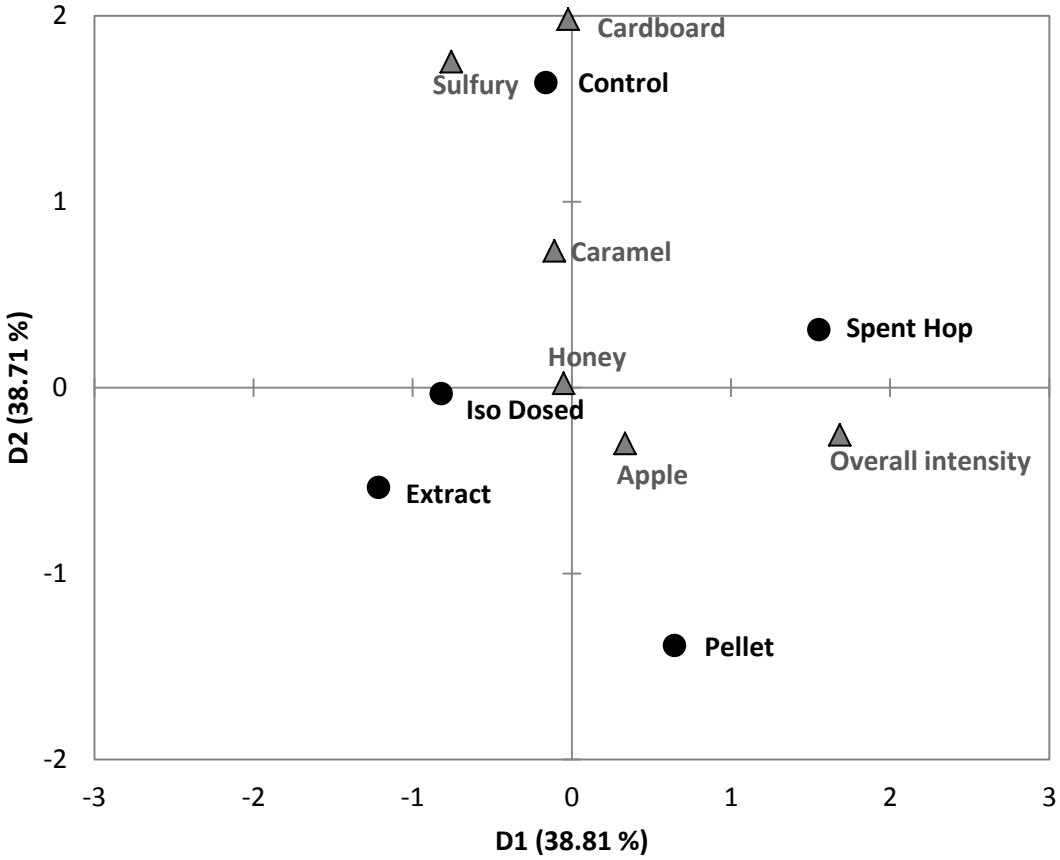

Figure 3.9. Principle component analysis for force-aged beers showing sensory aroma descriptors with the dominant attributes of 'Piney' and 'Tropical Fruit' removed.

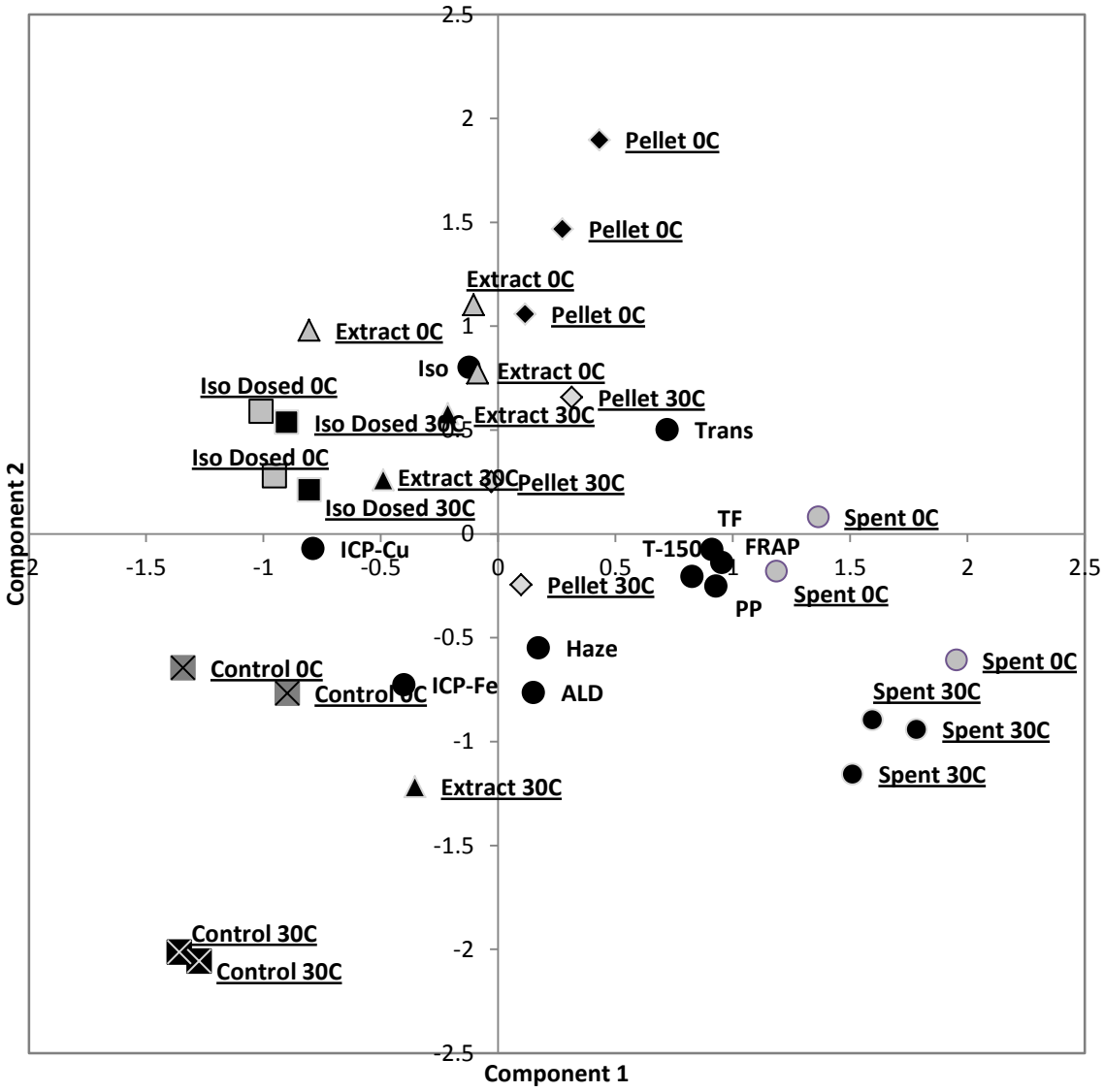

Figure 3.10. PCA showing all analytical values, treatments (replicates) and total aldehydes (ALD) for aged beers (30°C, 8 weeks).

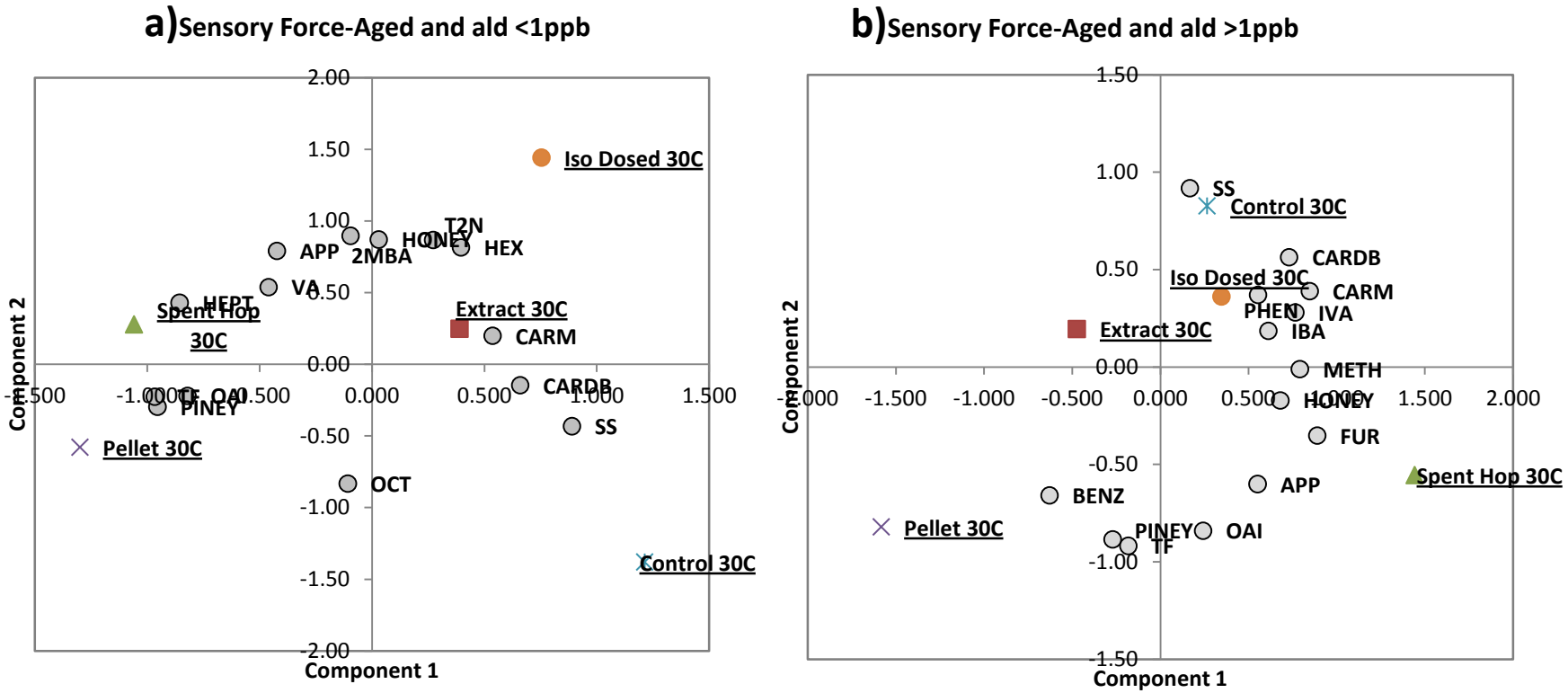

Figure 3.11. PCAs for force-aged sensory analysis and aldehydes: a) aldehydes present below 1 ppb b)aldehydes present above 1 ppb in force-aged beer (30°C, 8 weeks)

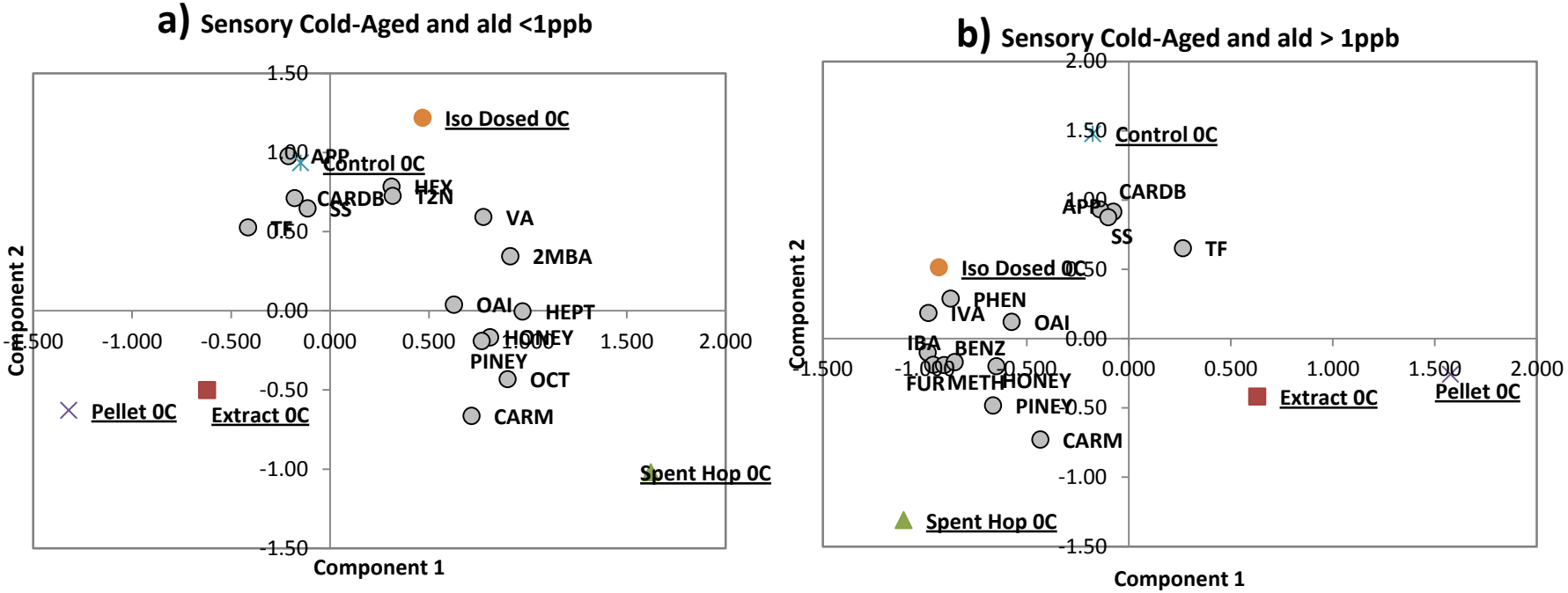

Figure 3.12. PCAs for cold-aged sensory analysis and aldehydes a) aldehydes present below 1 ppb b) aldehydes present above 1 ppb in cold-aged beers (8 weeks, 1°C).

List of Tables.

Table 3.1. Wort Parameters

Treatment	Specific Gravity	Degree Plato	pH	Total Polyphenols (ppm)	Total Flavanoids (ppm)
†Control	1.047	11.6	4.92	123	18
Extract	1.051 ± 6.9E-04	12.4 ± 0.0	5.22 ± 0.01	117 ± 3.0	19 ± 0.4
Pellet	1.050 ± 5.8E-04	12.4 ± 0.1	5.26 ± 0.02	136 ± 3.8	22 ± 0.1
Spent Hop	1.050 ± 7.3E-04	12.2 ± 0.1	5.34 ± 0.01	262 ± 7.6	36 ± 1.3

Wort results are reported as mean values for each treatment (n=3) ± standard error. †n=1 , Control beer was kettle boiled in bulk, no true replicates, thus no standard error is reported. No Iso Dosed data is reported because Iso Dosed was a post-fermentation treatment.

Table 3.2. Finished Beer Parameters

Treatment	Specific Gravity	pH	*Iso*-alpha acids	BU	Total Polyphenols (ppm)	Total Flavanoids (ppm)
†Control	1.009	4.2	--	4.7	105	24
†Iso Dosed	1.008	4.2	13.3	15.4	97	23
Extract	1.010 ± 0.003	4.3 ± 0.00	13.8 ± 0.3	17.1 ± 0.2	102 ± 1.1	28 ± 0.5
Pellet	1.010 ± 0.006	4.3 ± 0.03	13.2 ± 0.3	21.3 ± 0.3	126 ± 1.2	28 ± 0.3
Spent Hop	1.010 ± 0.005	4.5 ± 0.05	5.48 ± 0.2	21.0 ± 0.4	195 ± 1.3	37 ± 0.6

Finished beer results are reported as mean values for each treatment (n=3) ± standard error. † n=1 , Control beer was kettle boiled in bulk and Iso Dosed beer was a post-fermentation treatment with no true kettle replicates, thus no standard error is reported.

Table 3.3. Pro-oxidant metal content during beer processing, finishing and aging.

	Wort		Fresh Beer		Cold-Aged		Force-Aged	
Treatment	**Cu**	**Fe**	**Cu**	**Fe**	**Cu**	**Fe**	**Cu**	**Fe**
†Control	71.6	307.1	29.0	147.4	32.3	148.6	34.9	171.0
†Iso Dosed	--	--	34.1	99.6	35.4	108.9	32.5	99.8
Extract	16.7 ± 2.8	118.0 ± 6.4	28.3 ± 1.1	108.9 ± 1.8	33.0 ± 0.9	117.1 ± 3.8	30.7 ± 2.3	129.8 ± 18.8
Spent Hop	11.2 ± 0.5	87.6 ± 12.2	20.4 ± 2.9	102.6 ± 6.2	26.0 ± 4.0	109.5 ± 7.7	21.3 ± 1.7	107.8 ± 4.0
Pellet	11.9 ± 0.7	54.7 ± 0.9	19.4 ± 1.3	79.1 ± 11.5	25.1 ± 1.8	86.8 ± 11.0	24.5 ± 0.6	115.5 ± 24.1

Copper (Cu) and Iron (Fe) results are reported as ppb mean values for each treatment (n=3) ± standard error. † n=1 , Control beer was kettle boiled in bulk, no true replicates, thus no standard error is reported. Iso Dosed was a post-fermentation treatment to the Control, thus no wort values and no standard error are reported.

Table 3.4. *Iso*-alpha-acid levels during aging.

	Week	††Iso Dosed	†Extract	†Pellet	†Spent Hop
Fresh Beer	0	13.51	13.95 ± 0.32	13.22 ± 0.46	5.48 ± 0.30
Cold-Aged (1°C)	1	12.94 ± 0.03	13.57 ± 0.10	12.29 ± 0.17	4.47 ± 0.31
	3	15.62 ± 0.07	14.32 ± 0.13	14.11 ± 0.19	4.92 ± 0.36
	6	12.14 ± 0.01	10.62 ± 0.28	10.76 ± 0.13	3.76 ± 0.25
	8	10.39 ± 0.07	9.34 ± 0.04	9.40 ± 0.33	3.37 ± 0.26
Force-Aged (30°C)	1	13.52 ± 0.11	13.12 ± 0.25	12.68 ± 0.16	4.71 ± 0.49
	3	14.08 ± 0.09	13.60 ± 0.05	13.20 ± 0.10	4.73 ± 0.39
	6	10.97 ± 0.31	10.22 ± 0.07	10.57 ± 0.18	3.66 ± 0.27
	8	9.16 ± 0.46	8.39 ± 0.22	8.15 ± 0.25	3.18 ± 0.22

Mean ppm *Iso*-alpha-acid over storage at 1°C and 30°C. Mean values ± standard error for each treatment. † n=3 means for true kettle replicates. †† n=2 means (not true kettle replicates). No Control data is displayed because the Control Beers had no *iso*-alpha-acids.

Table 3.5. Decline in %Trans due to storage.

Treatment	Fresh	Cold-Aged	Force-Aged	ΔCold-Aged	ΔForce-Aged
†Iso Dosed	21.0 ± 0.00	19.6 ± 0.10	15.7 ± 0.10	1.44 ± 0.10	5.26 ± 0.10
†† Extract	49.2 ± 0.22	46.5 ± 0.30	37.1 ± 0.33	2.68 ± 0.37	12.1 ± 0.28
†† Pellet	47.0 ± 0.25	43.9 ± 0.31	34.7 ± 0.48	3.04 ± 0.30	12.3 ± 0.54
†† Spent Hop	53.8 ± 1.10	46.8 ± 0.50	40.3 ± 0.96	7.05 ± 1.2	13.5 ± 0.92

Mean decline in %Trans over storage at 1°C and 30°C. Mean values ± standard error for each treatment. † n=3 means for true kettle replicates. †† n=2 means (not true kettle replicate). No Control data is displayed because the Control Beers had no *iso*-alpha-acids.

Table 3.6. Ferric Reducing Activity of Plasma (FRAP) results

Treatment	Fresh Beer	Cold-Aged	Force-Aged	Δ%Cold-Aged	Δ%Force-Aged
Control	1513.0	1433.9 ± 50.3	1356.3 ± 3.8	5.21 ± 0.3	10.24 ± 3.2
Iso Dosed	1584.9	1434.3 ± 38.7	1374.8 ± 25.9	7.36 ± 2.4	11.75 ± 1.6
Extract	1635.0 ± 48.8	1409.5 ± 60.2	1433.8 ± 62.4	13.79 ± 1.1	12.31 ± 1.1
Pellet	1626.0 ± 4.9	1602.5 ±14.9	1599.7 ± 41.9	2.36 ± 0.26	1.63 ± 2.3
Spent Hop	2047.5 ± 53.7	1905.7 ± 59.0	1891.7 ± 33.3	9.50 ± 1.8	7.57 ± 0.92

Mean values of FRAP (Trolox Equivalents, ppm) are displayed with standard error (n=3), Control and Iso Dosed were not replicated in the kettle, only during storage (n=2) .

Table 3.7. Electron Spin Resonance (ESR) Results.

Treatment	Fresh Beer	Cold-Aged	Force-Aged	Δ%Cold-Aged	Δ%Force-Aged
Control	131.00	109.5 ± 21.5	112.9 ± 11.31	21.5 ± 21.5	18.0 ± 11.3
Iso Dosed	125.00	98.5 ± 1.33	110.2 ± 8.38	26.5 ± 1.33	14.8 ± 8.4
Extract	163.8 ± 3.1	138.8 ± 15.3	127.5 ± 9.34	25.0 ± 13.7	36.4 ± 6.5
Pellet	161.5 ± 4.0	129.3 ± 6.37	133.3 ± 8.06	32.2 ± 7.2	28.2 ± 6.3
Spent Hop	202.7 ± 5.9	166.5 ± 17.5	174.1 ± 6.79	36.2 ± 11.9	28.6 ± 12.5

Mean values of ESR (T150) values are displayed with standard error (n=3), Control and Iso Dosed were not replicated in the kettle, only during storage (n=2) .

Table 3.7. Aldehyde levels in the fresh beers

Aldehyde	†Control	†IsoDosed	Extract	Pellet	Spent Hop
IBA**	5.85	4.28	2.26 ± 0.48^{a}	1.96 ± 0.11^{a}	5.75 ± 0.51^{b}
2MBA**	1.86	1.33	0.81 ± 0.23^{a}	0.94 ± 0.15^{a}	1.93 ± 0.12^{b}
IVA**	3.83	2.20	1.60 ± 0.28^{a}	1.54 ± 0.36^{a}	3.70 ± 0.19^{b}
VA*	0.31	0.59	0.25 ± 0.05^{a}	0.27 ± 0.02^{a}	0.43 ± 0.04^{b}
HEX^{NS}	0.59	0.79	0.42 ± 0.06^{a}	0.38 ± 0.03^{a}	0.49 ± 0.02^{a}
FUR**	3.92	3.58	1.51 ± 0.47^{a}	1.98 ± 0.01^{a}	4.77 ± 0.73^{b}
HEPT^{NS}	0.31	0.32	0.30 ± 0.01^{a}	0.30 ± 0.01^{a}	0.31 ± 0.00^{a}
METH**	6.83	8.42	2.47 ± 0.11^{a}	3.66 ± 0.33^{b}	5.83 ± 0.40^{c}
OCT^{NS}	0.31	0.33	0.33 ± 0.01^{a}	0.35 ± 0.01^{a}	0.35 ± 0.01^{a}
BENZ**	1.53	1.55	0.40 ± 0.11^{a}	0.60 ± 0.03^{a}	1.00 ± 0.10^{b}
PHEN**	18.4	16.3	5.73 ± 0.92^{a}	3.32 ± 0.36^{a}	12.7 ± 1.13^{b}
T2N^{NS}	0.10	0.13	0.06 ± 0.01^{a}	0.05 ± 0.01^{a}	0.07 ± 0.00^{a}

Mean aldehyde levels in fresh beer ± standard error. Mean is based on 3 replicates for Extract, Pellet and Spent Hop treatments. †No true replicates for Control and Iso Dosed (no standard error reported). ^{a,b,c} means within a row with different letters are significantly different from one another at Tukey's HSD at the 5% level and ANOVA at *$p < 0.05$ or **$p < 0.01$, NS = no significant difference. Abbreviations: IBA, isobutyraldehdye; 2MBA, 2-methylbutyraldehdye; IVA, isovaleraldehyde; FUR, furaldehyde; METH, methional; BENZ, benzaldehyde; PHEN, phenylacetaldehyde; VAL, valeraldehyde; HEX, hexanal; HEPT, heptanal; OCT, octanal: T2N, *trans*-2-nonenal.

Table 3.8. Aldehyde levels in cold-aged beers

Aldehyde	†Control	†IsoDosed	Extract	Pellet	Spent Hop
IBA**	32.6	10.6	10.4 ± 0.46^{a}	10.3 ± 1.67^{a}	26.38 ± 1.35^{b}
2MBA ^{NS}	2.06	2.65	2.24 ± 0.20^{a}	2.15 ± 0.13^{a}	2.55 ± 0.25^{a}
IVA^{NS}	4.86	4.44	2.98 ± 1.10^{a}	3.16 ± 0.35^{a}	4.51 ± 0.59^{a}
VA^{NS}	0.36	0.48	0.60 ± 0.06^{a}	0.45 ± 0.04^{a}	0.62 ± 0.07^{a}
HEX^{NS}	0.59	1.16	0.81 ± 0.09^{a}	0.67 ± 0.08^{a}	0.58 ± 0.01^{a}
FUR*	3.23	3.84	3.58 ± 0.33^{ab}	2.80 ± 0.07^{a}	4.83 ± 0.67^{b}
HEPT^{NS}	0.34	0.36	0.35 ± 0.01^{a}	0.36 ± 0.02^{a}	0.36 ± 0.01^{a}
METH**	3.73	2.10	3.57 ± 1.33^{a}	1.82 ± 0.38^{a}	4.17 ± 0.25^{a}
OCT^{NS}	0.40	0.33	0.34 ± 0.02^{a}	0.36 ± 0.01^{a}	0.38 ± 0.02^{a}
BENZ^{NS}	1.10	1.19	1.12 ± 0.14^{a}	1.40 ± 0.18^{a}	1.18 ± 0.08^{a}
PHEN^{NS}	14.8	18.1	13.9 ± 2.60^{a}	12.7 ± 1.19^{a}	14.9 ± 0.89^{a}
T2N^{NS}	0.09	0.31	0.18 ± 0.02^{a}	0.14 ± 0.02^{a}	0.11 ± 0.01^{a}

Mean aldehyde levels in force-aged (30^{0}C) beer ± standard error. Mean is based on 3 replicates for Extract, Pellet and Spent Hop treatments. †No true replicates for Control and Iso Dosed (no standard error reported). ^{a,b,c} means within a row with different letters are significantly different from one another at Tukey's HSD at the 5% level and ANOVA at *$p<0.05$ or **$p<0.01$, NS = no significant difference. Abbreviations: IBA, isobutyraldehdye; 2MBA, 2-methylbutyraldehdye; IVA, isovaleraldehyde; FUR, furaldehyde; METH, methional; BENZ, benzaldehyde; PHEN, phenylacetaldehyde; VAL, valeraldehyde; HEX, hexanal; HEPT, heptanal; OCT, octanal: T2N, *trans*-2-nonenal.

Table 3.9. Aldehyde levels in the force-aged beers

Aldehyde	†Control	†IsoDosed	Extract	Pellet	Spent Hop
IBA**	5.73	7.49	4.48 ± 0.69^{a}	3.50 ± 0.16^{a}	7.91 ± 0.62^{b}
2MBA*	1.60	2.11	1.43 ± 0.22^{a}	0.94 ± 0.26^{a}	2.07 ± 0.10^{b}
IVA*	3.48	3.92	2.45 ± 0.28^{ab}	1.65 ± 0.61^{a}	3.79 ± 0.27^{b}
VA**	0.46	0.49	0.34 ± 0.01^{a}	0.34 ± 0.03^{a}	0.46 ± 0.00^{b}
HEX*	0.58	0.97	0.57 ± 0.04^{a}	0.38 ± 0.05^{b}	0.49 ± 0.0^{ab}
FUR**	2.71	4.12	2.82 ± 0.52^{a}	1.07 ± 0.12^{b}	4.39 ± 0.31^{c}
$HEPT^{NS}$	0.32	0.35	0.32 ± 0.01^{a}	0.30 ± 0.01^{a}	0.36 ± 0.05^{a}
METH*	4.37	7.13	4.26 ± 0.16^{ab}	3.56 ± 0.55^{a}	6.64 ± 0.78^{b}
OCT^{NS}	0.35	0.37	0.35 ± 0.00^{a}	0.33 ± 0.00^{a}	0.53 ± 0.19^{a}
$BENZ^{NS}$	1.03	2.01	1.37 ± 0.46^{a}	0.62 ± 0.24^{a}	1.70 ± 0.16^{a}
PHEN**	13.3	21.2	9.76 ± 1.43^{ab}	5.07 ± 1.11^{a}	13.8 ± 0.73^{b}
T2N*	0.07	0.20	0.08 ± 0.01^{a}	0.04 ± 0.01^{b}	0.07 ± 0.00^{ab}

Mean aldehyde levels in cold-aged (0^{0}C) beer ± standard error. Mean is based on 3 replicates for Extract, Pellet and Spent Hop treatments. †No true replicates for Control and Iso Dosed (no standard error reported). ^{a,b,c} means within a row with different letters are significantly different from one another at Tukey's HSD at the 5% level and ANOVA at *$p<0.05$ or **$p<0.01$, NS = no significant difference. Abbreviations: IBA, isobutyraldehdye; 2MBA, 2-methylbutyraldehdye; IVA, isovaleraldehyde; FUR, furaldehyde; METH, methional; BENZ, benzaldehyde; PHEN, phenylacetaldehyde; VAL, valeraldehyde; HEX, hexanal; HEPT, heptanal; OCT, octanal: T2N, trans-2-nonenal.

Table 3.10. Total aldehydes and changes during aging.

Treatment	Fresh	Cold-Aged	Force-Aged	ΔCold-Aged	ΔForce-Aged
Extract	16.2 ± 2.34^{a}	28.2 ± 3.83^{a}	40.1 ± 4.75^{a}	12.1 ± 2.21^{a}	23.9 ± 5.91^{a}
Pellet	15.4 ± 1.01^{a}	17.8 ± 2.82^{b}	36.3 ± 2.68^{a}	2.45 ± 1.90^{b}	20.9 ± 3.70^{a}
Spent Hop	37.4 ± 2.29^{b}	42.3 ± 2.61^{c}	60.6 ± 1.86^{b}	4.88 ± 2.51^{ab}	23.2 ± 3.98^{a}

Total aldehydes (ppb) for fresh, cold-aged and force-aged beers, represented as the treatment mean ± standard error for twelve aldehydes. Changes in cold-aged and hot-aged beers were calculated by subtractimg the fresh mean value from the aged-mean values. ^{a,b,c} means within a column with different letters are significantly different from one another at Tukey's HSD at the 5% level ($p<0.05$)

Table 3.11. Sensory aroma descriptor mean values.

Treatment	Storage Temp.	Overall Aroma***	Piney***	Cardboard***	Tropical Fruit***	Caramel***	Honey**	Apple^{NS}	Sulfur/ Skunky***
Control	0	8.0 ± 1.8^{a}	2.2 ± 2.1^{c}	3.3 ± 2.4^{a}	2.8 ± 1.9^{b}	2.8 ± 2.4^{bc}	2.2 ± 2.1^{ab}	2.8 ± 1.8^{a}	2.2 ± 2.9^{a}
Iso Dosed	0	6.9 ± 1.4^{c}	2.3 ± 1.8^{c}	2.3 ± 1.5^{ab}	2.7 ± 1.7^{b}	2.4 ± 1.7^{bc}	1.8 ± 1.4^{ab}	2.8 ± 1.9^{a}	1.1 ± 1.7^{ab}
Extract	0	6.9 ± 1.2^{c}	2.1 ± 1.9^{c}	2.2 ± 1.7^{ab}	2.4 ± 1.8^{b}	3.0 ± 1.9^{b}	2.0 ± 1.4^{ab}	2.5 ± 1.9^{a}	0.9 ± 1.6^{b}
Pellet	0	6.8 ± 1.2^{c}	2.3 ± 1.8^{c}	1.9 ± 1.8^{b}	2.8 ± 2.0^{b}	2.7 ± 2.1^{bc}	1.7 ± 1.5^{ab}	2.5 ±1.7^{a}	0.9 ± 1.6^{b}
Spent Hop	0	8.0 ± 1.6^{ab}	2.5 ± 2.0^{c}	1.8 ± 1.5^{b}	2.5 ± 1.7^{b}	4.6 ± 2.1^{a}	2.4 ± 1.7^{ab}	2.3 ± 1.9^{a}	0.8 ± 1.5^{b}
Control	30	7.0 ± 1.4^{bc}	2.4 ± 1.9^{c}	2.7 ± 2.0^{ab}	2.4 ± 1.6^{b}	2.2 ± 2.1^{bc}	1.3 ± 1.3^{b}	2.6 ± 1.9^{a}	1.6 ± 2.2^{ab}
Iso Dosed	30	6.8 ± 1.5^{c}	2.0 ± 1.8^{c}	2.4 ± 1.8^{ab}	2.1 ± 1.6^{b}	2.1 ± 1.6^{bc}	1.8 ± 1.7^{ab}	3.2 ± 2.0^{a}	0.9 ± 1.5^{b}
Extract	30	6.8 ± 1.3^{c}	2.7 ± 1.9b^{c}	2.0 ± 1.7^{b}	2.8 ± 1.6^{b}	2.2 ± 2.1^{bc}	1.7 ± 1.5^{ab}	2.9 ± 1.7a	1.0 ± 1.8a^{b}
Pellet	30	7.5 ± 1.5^{abc}	4.4 ± 2.3^{a}	1.8 ± 1.5^{b}	4.0 ± 1.6^{a}	1.8 ± 1.8^{c}	1.3 ± 1.2^{b}	2.9 ± 1.3^{a}	0.5 ± 1.0^{b}
Spent Hop	30	7.8 ± 1.6^{ab}	4.0 ± 2.2^{ab}	2.4 ± 1.9^{ab}	3.9 ± 1.7^{a}	2.2 ± 2.0^{bc}	1.8 ± 1.6^{ab}	3.3 ± 1.9^{a}	0.6 ± 1.2^{b}

Sensory treatment means ± standard deviation. Mean is based on 13 panelists, 4 reps, and 2 temperatures (n=104). ^{a,b,c} means within a column with different letters are significantly different from one another at Tukey's HSD at the 5% level ($p<0.05$). Mean values are based on a 16 point intensity scale where 0 = none, 1 = just detectable, 3 = slight, 5 = slight to moderate, 7 = moderate, 9 = moderate to large, 11 = large, 13 = large to extreme, 15 = extreme. *,**, *** significant at $p<0.05$, $p<0.01$, and $p<0.001$, respectively. ^{NS} = not significant ($p>0.05$).

Chapter 4.

Phenolic profiling of lager beer during aging in relation to hopping technology

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ABSTRACT

Lagers beers were produced with varying hopping regimes to study the effect of different hop products on the polyphenol content of finish lager beers: Spent Hop Solid hopped, Pellet hopped, Extract hopped and Unhopped beers. Finished beers were then subjected to a staling trial to investigate the effects of force-aging on changes in polyphenolic profiles. Polyphenol and total flavanoid content of the beers were directly measured via EBC standard methods. Polyphenol rich extracts were produced from the beers using Sephadex LH20 resin. Beers produced with Spent Hop solids were higher in total polyphenols, flavanoids and proanthyocyanidins than beers hopped with other products. Phloroglucinolysis was used in conjunction with RP-HPLC-ESI-MS to reveal subunit composition and proanthocyanidin mDP. Six major phloroglucinolysis products were observed, however galloylated flavanols were not detected in any of the beers. The predominant subunits by molar ratio were (+)-catechin followed by (-)-epigallocatechin, except for beers brewed solely with Spent Hops, which also high in (-)-epicatechin by molar ratio. The major extension subunit was (+)-catechin for all treatments. Although the Sephadex extracts were phenolic in nature, proanthocyanidins only accounted for up to 2% of the total phenolic material. Total flavanoid and proanthocyanidin content of the beers increased initially during storage, with eventual decreases occurring after 6 weeks of storage at 30ºC. Beers high in hop polyphenols did not suppress the loss of *iso*-alpha acids during aging and were also assessed as least flavor stable of the beers by ESR T150. However the presence of hop polyphenols suppressed the formation of staling aldehydes during aging as measured by SPME-GC-MS.

KEYWORDS: hops; polyphenols; flavan-3-ols; proanthocyanidins; beer aging; beer flavor; antioxidants

INTRODUCTION

Beer is one of the most extensively consumed beverages world-wide. Its production dates back to the Sumerians and ancient Egyptians and has become a source of national pride for more modern nations. Today, beer is almost always brewed with hops (*Humulus lupulus, L.*). Hops provide beer with bitterness, aroma, flavor and texture and

also enhance specific beer properties such as foam stability, clarity (colloidal stability), color, flavor stability and microbial stability. Hops are a dioecious species, with female plants producing the hop strobilus (cone). The cone is an inflorescence, which is the entire part of the plant that holds the flowers. Hop cones contain lupulin glands (the source of the hop bittering resins), essential oils, and polyphenols.

Polyphenols represent a class of secondary plant metabolites widely distributed throughout the plant kingdom and include many classes of compounds from simple phenolic acids, to more complex polymers such as proanthocyanidins (also referred to as tannins) [229, 230]. The most important class of polyphenols for consideration in beer and related products is that of the 2-phenylbenzopyrans (**Figure 4.1**). This class contains several groups of phenolic compounds that may or may not be glucosylated or galloylated, such as the flavans, flavanones, flavones, flavonols, flavan-3-ols, etc. The flavan-3-ols and their condensed (polymerized) products, the proanthocyanidins account for astringent and bitter properties of many commonly consumed plant based food products [101-103]. The proanthocyanidins also receive appreciable attention due to their propensity to behave as antioxidants. However in beer they are known causative agents of undesirable temporary and permanent haze and thus are not always regarded as desirable contributors to finished beer.

Proanthocyanidin content in beers varies depending on style and hopping technology [10, 25, 231]. To date several flavan-3-ol monomers (including glycosides and gallates), dimers (5 B-type, 2 A-type) and trimers (C-type, 1 procyanidin, 2 prodelphinidin) have been reported in beer [18, 113, 128]. However not all are involved in colloidal instability [18, 232]: flavan-3-ol monomers may bind, but do not cross-link with haze active proteins. Proanthocyanidin oligomers on the other hand possess several binding sites within the same molecule that allow for protein-polypheol haze formation [58]. Proanthocyanidin propensity to form haze is dependent on molecular weight, subunit composition, interflavanoid bond orientation as well as placement and degree of hydroxylation [39, 59, 61, 233]. Higher oligomer proanthocyanidins (trimer, tetramer and beyond) less readily survive the brewing process. Therefore dimers are thought to play a sizeable role in beer haze formation [56]. However, oxidized flavanols may also instigate

chill haze and it is plausible that monomers polymerize during beer production and aging to eventually participate in permanent haze formation [62].

Regardless, proanthocyanidins are known antioxidants and are thought to play a role in improving beer flavor stability [17, 21-25]. Flavan-3-ols behave as antioxidants via several mechanisms: scavenging of free radicals, chelation of transition metals, and mediation and inhibition of enzymes [43]. Proanthocyanidins and flavanoids behave as free radical scavengers due to their electron configuration that allows for release of electrons to free radical species ($R^{\bullet}$). The radical character thus can be transferred to the flavanoid, ($F^{\bullet}$), considered a relatively stable and potentially less harmful species than the initial radical. Flavanoid oxidation is generally thought to procede through formation of semiquinone radicals which may further undergo nucleophilic addition to produce oligomers, thereby maintaining the reactive catechol structures and scavenging ability of the parent molecule (**Figure 4.2**) [44]. Flavan-3-ols may also act as indirect antioxidants by strongly chelating iron and copper cations, as well as other transition metals, from solution [45, 46]; chelated transition metals are potentially less active in the promotion of free-radical related reactions [29]. Flavan-3-ols/proanthocyanidin potential to chelate metals depends on hydroxylation pattern and degree of polymerization; ortho-dihydroxy configurations on the b-ring and higher degree of polymerization lead to increased metal-flavanoid complex formation and stability [48]. Flavanols such as (+)-catechin chelate at the ortho-catechol (ortho-hydroxy) group on the B-ring, with metal speciation and beer pH affecting chelation [218].

Despite the promising antioxidant potential of flavanoids, a comprehensive understanding of flavanoid or proanthocyanidin involvement in beer flavor stability does not exist. In contrast to the roles of other hop derived ingredients, such as the isomerized alpha acids and their reduced products (bittering acids), the absolute value of hop derived flavonoids is not well realized. Several studies indicate that brewing with hop derived polyphenols favorably impacts beer flavor and flavor stability [96, 98, 107-109, 143]. However chemical analyses pertinent to oxidation evidence conflicting results. Some studies indicate that hop polyphenols markedly improve beer shelf stability by increasing

beer reducing activity [24, 104, 110, 111] while others do not clearly show that polyphenols effect differences in beer flavor stability [39, 40, 69, 73, 74].

To add to this confusion, very little is understood regarding the fate of polyphenols during the brewing and aging processes [128]. Polyphenol losses occur due to trub formation, adherence to yeast cells during fermentation and removal by filtration/fining media. Polyphenols also likely undergo degradative reactions by oxidative mechanisms [151], forming other species whose chemical reactivity remains largely unknown. Furthermore, flavanoids may take part in maillard reactions and endure structural rearrangements that cause color changes in beer during storage [121]. Studies indicate that flavanol monomer and dimer concentrations ((+)-catechin, (-)-epicatechin, prodelphinidins and procyanidin dimers) decrease [39, 52, 151], with dimeric flavanoids receding more rapidly than their monomeric counterparts. However, after several weeks of storage, relative levels of PPs may once again increase [121, 172]. This could partially be explained by occurrence of PP polymerization and chemical alterations due to uptake of oxygen [173, 174]. During a recent evaluation investigating the effect of high temperature storage on *iso*-alpha-acid levels in research lager beers we noticed substantial and relatively rapid changes in the uncharacterized fraction of our RP-C18 HPLC chromatograms, specifically in the early eluting fraction. The extraction method for *iso*-alpha acids utilizes solid phase extraction (SPE) with C-8 resin, a hydrophobic resin that can be used to extract phenolic material. Based on the elution patterns and spectral data we hypothesized that a majority of these early eluting peaks represent barley and hop polyphenols, with the latter comprising the majority of hop treated samples.

In order to understand the polyphenolic profile and effect of aging on the polyphenolic profile of these lager beers, the previous experiment was repeated and beers were brewed with varying hopping technology: using whole hop (pellets), hop bittering acids only (CO_2 extract), hop polyphenols only (spent hop solids) and no hops (control). In this manner the effect of hopping technology on phenolic profiling and changes during aging could also be investigated. Beer polyphenol content was investigated using EBC methods of beer analysis for total polyphenols and total flavanoids by spectrophotomry. Beers were also extracted using sephadex LH20 resin and analyzed for total polyphenols

by RP-C18-HPLC. Proanthocyanidin composition was investigated using phloroglucinolysis in conjunction with RP-HPLC-ESI-MS. Flavor stability was also assessed by chemical analyses to assess oxidative, aging-related changes in the beers.

MATERIALS AND METHODS

Materials. Spent hop pellets (*Humulus Lulupulus* cv. Chelan, post-CO_2 extraction), hop pellets, CO_2 hop extract and pre-isomerized hop extract were generously donated from John I. Haas. Ferric ammonium citrate (green) was purchased from Fisher Chemicals. Ethylenediaminetetraacetic acid disodium salt, dihydrate (EDTA), *p*-dimethylamino-cinnamaldehyde, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), acetonitrile, pectin and phenolic standards (rutin, quercetin, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin) were obtained from Sigma-Aldrich Chemical Co (St. Louis, MO). Flavanoid procyanidin dimers B1, B2 and B3 were graciously prepared as previously described [113] by Hui-Jing Li and Max L. Deinzer of Oregon State University. Grape seed extract was generously supplied by Patrick Ting of MillerCoors. Low-viscosity carboxymethylcellulose was supplied by Hercules Inc. (Wilmington, DE). C-8 SPE columns were purchased from Supelco (Bellefonte, PA). Divergan HM PVPP was generously donated by BASF (Ludwigshafen, Germany). All solvents were HPLC grade, and all other reagents were of analytical reagent grade. ICP metal standards (Iron and Copper), and ICP grade HNO_3 and sodium acetate trihydrate were purchased from VWR International, BDH (West Chester, PA, USA). Ferric chloride anhydrous and phosphoric acid were obtained from EMD (Gibbstown, NJ). HPLC- solvents, Methanol and 96-well plates were obtained from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid was purchased from Merck kGaA (Darmstadt, Germany). Hydrochloric acid and 2,2,4-trimethylpentane from JT Baker (Phillipsburg, NJ), ammonium hydroxide obtained from Ashland (Columbus, OH). All solvents were HPLC grade. Water was purified to HPLC grade with a Millipore MilliQ apparatus (Bedford, MA). Clearsweet, a high glucose liquid adjunct was generously donated by Cargill (Minneapolus, Minnesota).

Production of pale lager beer treated with various hop products. A base wort (75% Pale GW 2-Row malt, 25% Clearsweet 95%, 321 L 12°P) was produced in the OSU pilot plant. Unhopped lager wort was treated with Divergan HM PVPP-PVI at

80g/hL to remove barley polyphenols and reduce transition metals concentration. The PVPP-PVI was allowed to settle overnight and removed by coarse filtration (Pall, HS 2000). The filtered wort was stored chilled at 1°C until hop treatment and boiling. Unhopped lager wort was used as a control: 12 L of wort were transferred to the kettle and boiled for 60 minutes (Target O.G. = 12.00°P). This treatment was named *Control*. For the hop treatments 12 L batches of wort were transferred to the kettle, treated with a hop-product and boiled for 60 minutes. All treatments were performed in quadruplicate beginning with a new batch of unhopped wort. For the *Pellet Hop* treatment, pellet hops were added at a target yield of 11 ppm *iso*-α-acid, assuming 35% utilization based on results of previous brewing trials (4.3g at 60 min. and 4.3 g at 5 min. to end of boil). For the *Spent Hop* treatment, spent hop material was added to yield a high polyphenol content, based on results of previous brewing trials (30 g at 60 min. and 30 g. at 5 min. to end of boil). For the *Extract* treatment, alpha-acid extract was added at a target 11 ppm *iso*-α-acid yield, assuming 35% utilization based on previous results (1.13 g extract at 60 min. to end of boil). Following whirlpool, the kettle-treated wort was cooled and transferred into a sanitized 18.9L (5 gallons) fermentor to which approximately 140 mL of lager yeast (Wyeast 2007, 7.8 x 10^8 cells/ml) was added. The wort was fermented (13°C) for approximately one week, racked into sterile- 5 gallon (18.9 L) Cornelius kegs and lagered (2 weeks, 1°C). Lagered beer was sterile filtered into 3 (11.3 L) gallon Cornelius kegs (Pall, HS 400).

Staling Trial. Finished beers (8 kg in 3 gallon kegs) were stored at 30°C for six weeks during which samples were pulled bi-weekly and analyzed immediately or frozen (-80°C) for future analysis. Chemical analyses were performed to assess the impact of hop treatment on changes in polyphenol composition and staling potential during aging.

Polyphenol of the beers by spectrophotometry. Total polyphenols and total flavanoids were measured according to the EBC Analytica methods (9.11 and 9.12) [114] using a Shimadzu PharmaSpec UV-1700 spectrophotometer, Shimadzu Corporation (Columbia, MD).

Beer flavanoids solid-phase extractions (Sephadex LH20). Extraction of the beer for each treatment was done in duplicate according to the methods of Callemien and

Collin [121]. Beer was applied to a 12 mL SPE column containing 3 g Sephadex LH-20 resin that had been preconditioned for 4 hours with methanol/water (30:70v/v). 60 mL of beer (degassed) was applied to the column, rinsed with 40 mL of methanol/water (30:70v/v), and the compounds of interest were eluted using 70 mL of acetone/water, concentrated to dryness (30ºC) and reconstituted in 2 mL of HPLC grade methanol. Samples were stored in the freezer until analysis).

Total polyphenols by RP-HPLC-DAD. Phenolic Sephadex beer isolates were analyzed for total polyphenols by RP-HPLC-DAD (Agilent Technologies1100) via the methods of Callemien and Collin [121], using C18 Prevail (Grace Davison Discovery Sciences, Deerfield, IL), 5 µm, 250 mm x 4.6 mm-i.d. and accompanying guard column of the same material. Gradient elution was accomplished using water containing 1% acetonitrile and 0.1% formic acid (A) and acetonitrile (B): 97-91% A, 0-5 min, 91—84% A, 5-15 min; 84-50% A, 15-45 min; 50-10%A, 45-48 min; 48-51 min isocratic; initial conditions for 15 min.

Acid Catalysis in the presence of excess phloroglucinol (Phloroglucinolysis). Phenolic Sephadex beer isolates underwent phloroglucinolysis as per modified methods of Kennedy and Jones[122] and Taylor et al.[123] 200uL of extract was combined with 200µL of liquid phloroglucinol reagent (0.2N HCl, 2.5 g phlor./25 mL MeOH, 0.5 g ascorbic acid), heated in a water bath (50ºC) for 20 minutes, and the reaction was quenched with an equivalent volume (400 µL) of 40mM sodium acetate. The 1:1 volume of phloroglucinol-sample:sodium acetate was used over the previously published ratio to increase the concentration of phenolics in the final sample mixture for HPLC analysis. Acid catalysis was done in the presence of excess phloroglucinol to determine subunit composition of phloroglucinolysis products [122]. The decreased dilution was sufficient to stop phloroglucinolysis and did not affect retention times or elution order of the investigated compounds.

Proanthocyanidin content by RP-HPLC-ESI-MS. The Sephadex extracts were also subjected to acid-catalysis in the presence of phloroglucinol and subsequently analyzed by HPLC-DAD-ESI-MS according to modified methods of Taylor et al [123]. The reversed-phase method consisted of two Chromolith RP-18e (100- 4.6 mm) columns

connected in series with accompanying guard column (Chromolith RP-18e, 5-2.6mm) all purchased from EMD chemicals (Gibbstown, NJ). The procedure utilized a binary gradient of 1%v/v aqueous acetic acid (A) and acetonitrile containing 1%v/v acetic acid (B). Eluting peaks were monitored at 280 nm: 1.0mL/min; 5% B at 0 min, linear gradient from 5- 10% B, 0-10 min; 10- 30% B 10-20 min; 30-55% B, 20-40 min.; 55- 90%B 40-41min.; 90%B, 41-51 min. The column was washed with 5% B for 5 minutes prior to the next injection. For the ESI source, the following conditions were applied; negative mode, dry temperature 350°C; dry gas 10.0 L/min; nebulizer 50.0 psi, trap drive 47.5, skim 1 -38.3 volt, skim 2 -6.0 volt, octopole RF amplitude 120.0Vpp, capillary exit -113.0 volt, scan begin 50m/z, scan end 1800 m/z. (+)-Catechin was used as a quantitative standard, and grape seed extract was used to confirm elution times of subunits. ESI-MS was used to confirm identity of the flavanoid compounds of interest. Mean degree of polymerization (mDP) of the Sephadex extracts were determined by summing all subunits (in moles, extension and terminal) and then dividing by the sum of all terminal units (in moles). In this manner a conversion yield (%flavanoids) could also be calculated: sum of the mass of all subunits (minus the mass of phloroglucinol from adducts) divided by the original mass of the material that underwent phloroglucinolysis.

ESR. Electron Spin Resonance (ESR) was performed as per the methods of Uchida and Ono [118, 119] with a Bruker EMX 6/1 Electron Paramagnetic Resonance Spectrometer controlled by Bruker WinAcquisition 3.04 Software with AquaX sample cell and 48rpm Peristaltic Pump: Attenuation – 9dB; Power – 25.26 nW; Center Hall Field – 3472.0 G; Sweep Width – 10.0 G, Spin-trap agent: alpha-phenyl-t-butylnitrone (PBN) (Sigma Aldrich), Internal Standard: 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-^{15}N-oxyl (4-OH Tempol) aqueous solution and External Standard: commercial lager beer (courtesy of MillerCoors Brewing Company) and was conducted courtesy of Nick Kaiser, Applied Brewing Technology, MillerCoors, Milwaukee, WI, USA.

Metal Ions. Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) according to the ASBC proposed method [120].

***Iso*-α-acids.** *Iso*-α-acids were measured by HPLC-DAD (Agilent 1200, Hewlett Packard, Palo Alto, CA) according to the methods of Donley, J.R. [162] and the ASBC

standard method for HPLC analysis of hop components in beer. A 250 x 4.6 mm, 5-µm column (Supelco Discovery C-18, Sigma Aldrich) was used. Mobile phase A consisted of 75:24:1 MeOH:H_2O:H_3PO_4 %v/v and mobile phase B consisted of 100% MeOH. Eluting peaks were monitored at 270 nm. Elution conditions were as follows: 1.3 mL/min (30 °C); 7 µL injection; 100% A for 17 minutes, a linear gradient from 0 to 60% B for 1 min, a linear gradient from 60% B to 25%B for 1 min, a linear gradient from 25% to 0% B for 1 minute and then isocratic at 100% A for 6 minutes.

Ratio of *trans* to *cis* (%Trans) by HPLC. %Trans *iso*-α-acids was measured by HPLC-DAD at 270 nm according to the methods of Araki et al. [156] and DeCoomen et. al [155]. The method utilized the same column as for the measurement of *Iso*-α-acids. Mobile phase A consisted of MilliQ water adjusted to pH of 2.81 with H_3PO_4 and mobile phase B consisted of 100% Acetonitrile. An isocratic method was used: 48% A and 52% B at 1.4 mL/min, 30°C, 5 µL injection, for a total of 30 min per sample. %Trans was calculated as the sum of *trans-iso*cohumulone and *trans-iso*humulone divided by the sum of cis-*iso*cohumulone and *cis-iso*humulone then converted into percent.

%Trans = ([*trans-iso*cohumulone] + [*trans-iso*humulone] / [cis-*iso*cohumulone] + [cis-*iso*humulone]) x 100%

Volatile aldehyde analysis. GC- MS volatile aldehyde analysis of the beers by solid phase microextraction-gas chromatography/mass spectroscopy (SPME-GC/MS) was carried out according to modified methods of Saison et al (2008) [163] and Vesely et al (2003) [164]. A 65um PDMS-DVB fiber (23 guage, Supelco) was used for the extraction of staling aldehydes. 10mL of degassed beer was added to a 20mL vial, followed by 10uL of internal standard solution (*cis*-11-hexadecenal) and 3.5 g of sodium chloride (NaCl). The vial was capped, vortexed for 1 minute and placed in the autosampler for immediate analysis. Capillary GC-MS was performed using an Agilent 6890N GC with a 5973 mass selective detector (Agilent Technology, Palo Alto, CA). Samples were analyzed on a DB-5 column (30m x 0.25mm i.d., 0.5µm film thickness, J&W Scientific of Agilent Technologies). The carrier gas was helium at a constant flow rate of 1.1mL/min.

The oven temperature was initially at 40°C and then increased to 140°C at 10°C /min, and again increased to 250°C at 7C/min at final time = 14.00 minutes. The total run time was 39.71 minutes. The injector temperature was 250°C . The splitless mode was used. The SPME fiber was first pre-extracted for 10.0 min in the derivatization vial and then extracted for 60.0 minutes in the sample vial (incubated at 50°C for 5.0 min at 250 rpm). The ion source temperature was set at 230°C and the MS quad temperature was set to 150°C . The mass spectra were obtained in SIM mode for ion selection at m/z 181.

Preparation of internal standard for SPME-GC-MS. To a 50 mL volumetric flask half filled with ethanol, 30 µL of *cis*-11-hexadecenal was added and then brought to 50 mL with ethanol. A 4 mL aliquot of this solution was then transferred to another 50 mL volumetric flask and brought to volume with ethanol.

Derivative solution preparation. A stock solution was prepared by adding 0.150 g o-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBOA) to a 25 mL volumetric flask which was brought to volume with MQ water. The stock solution was kept refrigerated at 4°C until ready for use. For each analysis a separate derivative solution was prepared by adding 100µL of the PFBOA derivative stock solution to 10mL of water in a 20mL vial.

Evaluation of beer parameters. Fresh and aged beers were analyzed for color (EBC method [234]) and BUs [161] by spectrophotometer (Perkin Elmer Lamda 20), pH, specific gravity, original gravity, residual extract, alcohol (%w/w, %v/v), calories, apparent extract and real degree of fermentation (Anton Paar Beer Alcolyzer Plus) by Quynh Le and Mark Swokowski courtesy of MillerCoors brewing company.

Analytical Statistical Analysis. ANOVAs were conducted to assess significance of analytical results and correlation matrixes were constructed compare results of different analytical measurements. Statistical analysis was performed using S-plus software (TIBCO Software, Palo Alto, CA).

RESULTS AND DISCUSSION

Finished Beer Parameters. Finished lager beers were produced in quadruplicate for each of the following four hopping regimes: Pellet, Extract, Spent Hop and Control (no hop product). Beers in this study were brewed with 25% Clearsweet adjuct and 75% 2-Row Pale Malt. The formulation for these beers was based on preliminary brewing trials that implicated malt as a source of pro-oxidative transition metals, especially iron (Fe). Replacing the malt bill with 25% adjuct therefore decreased the malt Fe contribution of the beers (**Figure 4.3**). Treatment of the wort with Divergan HM PVPP-PVI at 80g/hL also reduced transition metal content: 63% for Fe and 58% for Cu (**Figure 4.4**). Beers were analyzed at week 0 (Fresh Beers) and after 6 weeks of storage at 30°C (Aged Beers). Finished beer parameters for fresh and aged beers are reported in **Table 4.1**. SO_2 values were also measured in the fresh beers; all treatments scored ≤ 0.1 ppm SO_2. Hopping regime did not significantly affect fresh beer parameters (Color, pH, Specific Gravity, Original Gravity, alcohol, residual extract, apparent extract, real degree of fermentation or Calorie content (Kilojoules per 100mL, 1KJ = 4.2 Calories). Most beer parameters did not change significantly due to aging. However trends were seen for some parameters such as color, where all treatments experienced an increase in color after 6 weeks of storage, with the unhopped control treatment enduring the greatest increase in color (ΔColor = Control, 1.06; Pellet, 0.72; Extract, 0.68; Spent Hop, 0.64). Color changes due to high temperature storage have been reported previously and could be explained by changes in phenolic material or maillard product formation [39, 121, 151, 235]. The Control unhopped beers also experienced a decrease in pH from 4.35 to 3.85 pH units, while the other treatments did not change in pH after six weeks of storage.

Evaluation of Polyphenol Content. While it has been established that polyphenol losses occur during beer production, the fate of polyphenols in packaged beer remains uncertain [128]. Fresh and Aged beers were analyzed for their polyphenol content via several methods. Firstly beers were evaluated directly by spectrophotometric methods for total polyphenols and total flavanoids. Both methods of analysis are relatively simple and rapid. The total polyphenols assay is non-specific and will react to

many classes of compounds that are phenolic in nature, while the second assay the total flavanoids assay is more specific for flavanoids; it relies on a condensation reaction to occur between the flavanol A-ring (that contains meta-oriented di- or tri-hydroxy substituted benzene rings) and a chromagen reagent. Secondly beers were extracted using Sephadex LH20 resin and analyzed for total polyphenols (absorbance at 280 nm) and for proanthocyanidin character (phloroglucinolysis) by RP-HPLC and ESI-MS.

Total Polyphenol Content. Polyphenols were monitored directly for the fresh beer (**Figure 4.5**). As anticipated, beers brewed with Spent Hop material were significantly higher than all other treatments in total polyphenols. However the other treatments did not differ in levels of total polyphenols as measured by spectrophotometry. The extraction of beers with Sephadex yielded methanolic extracts that could be directly injected into the HPLC for analysis. Sephadex LH 20 resin works via both adsorption and size exclusion mechanisms and allows for the extraction of hydrophobic material such as flavanoids and proanthocyanidins (monomers to oligomers). It was thus not surprising that the sephadex extracts were high in phenolic material. (+)-Catechin was used as a standard and results are reported in ppm (+)-catechin. The Spent Hop treated beers proved highest in total phenolics for the fresh beers ($p<0.05$), followed by the Pellet treated beers. However the Control and Extract beers were not significantly different in levels of total polyphenols.

Changes in Total Polyphenols due to force-aging. The polyphenol content of aged beers was measured directly (after 2, 4 and 6 weeks of aging by spectrophotometry) and after sephadex extraction (after 3 and 6 weeks via RP-HPLC). Measurement of total polyphenols by spectrophotometry did not indicate that any changes in levels of total polyphenols occurred due to aging, even after six weeks at 30C. However the results from the analysis of the polyphenol rich sephadex extracts told another story. Beers produced from all treatments saw an increase in total polyphenol levels by the third week of aging. Increases were significant for the Pellet, Extract and Spent Hop beers, yet not for the Control beers. After six weeks of storage all beers saw a decline in total polyphenol content. By the sixth week of storage the Spent Hop kettle treated beers suffered significant losses in total polyphenols (Δ520ppm). However, despite the downward trend in polyphenol levels seen from week 3 to week 6, the other beers did not change significantly from levels seen in

the fresh beers. A loss of phenolic character due to beer aging has been previously reported. Beer aging, especially at high temperature, may result in decreased polyphenol content, most likely due to chemical degradation, oxidation, and polymerization reactions [39, 121] , all processes that may result in the formation of other species whose chemical reactivity remains largely unspecified.

Total Flavanoids and proanthocyanidin content. Fresh beers were also analyzed for flavanoid and proanthocyanidin content via two methods. The first method, the total flavanoids assay is specific for flavonoids that possess a single bond between the 2,3 position of the C-ring. The resultant condensation product absorbs light at 335 nm and results are reported as ppm (+)-catechin or (+)-catechin equivalents (CE). Total flavanoids of the fresh beers are reported in **Figure 4.6**. The Spent Hop kettle treated fresh beers were significantly highest in total flavanoids (28.3 CE), followed by the Control (19.6 CE) and Pellet (19.5 CE) treatments ($p < 0.05$). The Extract hopped fresh beers were lowest in mean total flavanoids (15.9 CE). The high levels of flavanoids in the Spent Hop treated beers was anticipated, however it was surprising that the Pellet hopped beers were not different in flavanoid content than the Control beers. While it was not surprising that Extract treated beers were low in total flavanoids, it was interesting that kettle boiling with Extract (alpha-acid-rich kettle extract) reduced levels of flavanoids in the wort over levels resulting in the Control beers.

Disadvantages of the total flavanoids assay are that it cannot be used to discern relative content of monomeric, dimeric or oligomeric flavanoids in beer and that the assay's color yield varies due to stereochemistry ((-)-epicatechin (*2,3-cis*) >> color than its stereoisomer (+)-catechin (*2,3-trans*)), by degree of polymerization and configurations of the C-ring [166]. Therefore a second method was also employed to assess relative flavanoid and proanthocyanidin content of the beers. The methanolic sephadex extracts were subjected to phloroglucinolysis- acid catalyzed degradation in the presence of phloroglucinol, which is a very strong nucleophile. The resultant products yield monomeric flavanol units and monomeric-flavanol-phloroglucinol adducts (**Figure 4.6**). During phloroglucinolysis proanthocyanidin dimers and oligomers are depolymerizd into monomeric subunits which can be recognized as either terminal subunits (monomeric

flavanols) or extension subunits (extension-phloroglucinol adducts). The combination of these flavanol subunits and phloroglucinol adducts can be used to assess relative proanthocyanidin content as well as an estimated mean degree of polymerization (mDP). Moreover it also provides information on subunit molar composition. A proanthocyanidin rich grape seed extract (determined to be 61% procyanidin w/w) and monomeric standards ((+)-catechin, (-)-epicatechin (-)gallocatechin, (-)-epigallocatechin) were used in addition to MS-ESI in order to identify subunits and clarify elution times of monomeric flavan-3-ols. Results of phloroglucinolysis are reported in **Figure 4.6 and Table 4.2.** Six major phloroglucinolysis products were observed that could further be categorized into either extension or terminal proanthocyanidin subunits. Extension subunits consisted of, (+)-catechin, (-)-epicatechin. Terminal subunits consisted of (+)-gallocatechin, (-)-epigallocatechin, (+)-catechin, and (-)-epicatechin. Although (-)-epicatechin-3-O-gallate was present in the grape seed extract, no measureable quantities of galloylated flavanols were detected in any of the beers. Molar equivalents of the subunits were calculated based on individual UV response relative to (+)-catechin and are expressed in **Table 4.2**. The predominant subunits by molar ratio were (+)-catechin (Spent Hop = 38%, Pellet = 38%, Extract = 37% and Control = 33%), followed by (-)-epigallocatechin (Spent Hop = 12%, Pellet = 21%, Extract = 23% and Control = 28%) for all but the Spent Hop treatment, which had (-)-epicatechin as its second highest subunit by molar ratio. The major extension subunit was (+)-catechin-phloroglucinol for all treatments (Spent Hop = 14%, Pellet= 11%, Extract = 10% and Control = 8%). Estimated yields for beers produced from each hop treatment are also reported in **Table 4.2**. The Spent Hop beers had the highest yield of proanthocyanidins (1.58%), followed by the Control and Pellet beers (0.95 and 0.93%, respectively) and then the Extract, which was significantly lower than all other treatments at 0.74% yield. This indicates that although the Sephadex extracts were phenolic in nature, proanthocyanidins made up less than 2% of that phenolic material. Moreover, kettle hopping with Pellet hops did not augment the proanthocyanidin content of the beers over that already contributed by the malt and kettle hopping with Extracts may reduce the ultimate proanthocyanidin content of lager beers. Estimated mDPs are also reported in **Table 4.2**. Not unexpectedly, beers treated with Spent Hop material had the

highest overall mDP (1.35), followed by the Pellet (1.20), Control (1.14) and Extract beers (1.10). Results of phloroglucinolysis are consistent with those reported by Callemien and Collin, with (+)-catechin making up the majority of both the terminal and extension subunits. Differences were however seen, in that in this study gallocatechin was not detected as a terminal subunit and the Spent Hop beers had relatively higher amounts of (-)-epicatechin in the form of both extension and terminal subunits than other beers. Unfortunately very few studies have attempted to extract and quantify beer polyphenols so comparisons for proanthocyanidin yield data could not be found.

Changes in Total Flavanoids and proanthocyanidins due to ageing. The effect of aging on flavanoid levels was also investigated (**Figure 4.7**). Total flavnoids by spectrophotometer were measured after weeks 2, 4 and 6 of high temperature storage. Total flavanoid content increased initially for all treatments and then decreased after six weeks of force-aging at 30°C. The Spent Hop beers lost the most flavanoids by percent (percent of initial content lost) after six weeks of aging (23%) followed by the Control beers (20%), Pellet beers (15%) and finally the Extract beers (12%). Results of phloroglucinolysis corroborated these results, however losses of known flavan-3-ol content were greater for all treatments, except in the case of the Spent Hop beer which actually showed minimal loss of flavanoids after 6 weeks of aging at 30C. As seen in **Table 4.2**, all beers showed a significant decrease in proanthocyanidin yield due to storage time, except for the Spent Hop treatment. The Control beers lost 36%, the extract beers lost 27%, the Pellet beers lost 15% and Spent Hop beers lost only 3.6%. Six weeks of aging at 30°C caused a slight decline in mDP for all treatments, but not to a significant extent. Predominant losses for known subunits were from losses in (+)-catechin-phloroglucinol-adducts (extension subunit), (+)-catechin and (-)-epicatechin. Surprisingly levels of these subunits did not decline as dramatically in the Spent Hop treated beers. Moreover, (-)-epigallocatechin and (+)-gallocatechin monomeric subunits actually increased after six weeks of aging. Although relatively few investigations have focused on flavanoid changes in beers during aging, several investigations on flavan-3-ol content in other food systems tell a similar story [167-170]. Lower flavan-3-ol content generally results due to food processing and heating due to epimerization, chemical modifications,

degradation and polymerization [129, 130, 170, 171]. In beer it is known that flavanol monomer and dimer concentrations ((+)-catechin, (-)-epicatechin, prodelphinidins and procyanidin dimers) decrease [39, 52, 121, 151] during storage or aging, with dimeric flavanoids diminishing more quickly than their monomeric counterparts. Moreover, increases in polyphenol levels have been reported to occur after several weeks of storage [172]. Evidence consistent with oxidation of proanthocyanidins in other food systems and in beer systems suggests that oxidation may occur during storage [173-177], with phenyl ring oxidation dependent on proanthocyanidin configuration and conformation [176, 179, 236-238], system pH and presence of metal catalysts [175, 177]. An interesting observation seen in all of the beers during this study is that levels of flavanoids increased initially and then hit a sharp decline after six weeks of storage (except in the case of the Spent Hop beers as per HPLC). This could plausibly be due to proanthocyanidin depolymerization and subsequent repolymerization. However, further investigation is warrented to confirm this.

Evaluation of pro-oxidant metals by ICP. Although several species of transition metals have been reported in beer [132], iron and copper are predominant transition metals found in beer that act as pro-oxidant agents by partaking in the formation of reactive oxygen species (ROS) via the Fenton and Haber-weiss reactions [144]. Cu and Fe levels were monitored in the fresh beer (**Figure 4.8**). No significant treatment effects resulted for either Fe or Cu levels in the finished beers. Pellet hopping produced beers lower in Fe content (49 ppb), followed by the Control (59 ppb), Spent Hop (63 ppb) and then the Extract beers (78 ppb). Cu levels did not vary between treatments, ranging from 26 to 29 ppb. Several investigators report that maintaining low levels of Cu and Fe in finished beers is imperative to beer flavor stability [38, 132, 144]. To avoid acceleration of beer flavor deterioration, levels of Cu should be below 50 ppb, while Fe levels may be less detrimental due to lower reactivity, levels of Fe should also be kept low. The levels of metals in these beers are in specification with recommendations. Because there were no significant differences in total levels of metals due to treatment, there is no evidence that hopping regime or polyphenol level significantly affects metal content of beers that have previously been stripped of a heavy

portion of malt derived metals via treatment of wort with PVPP-PVI. However previous experimentation in our lab indicates that the use of hop products, Pellets especially can effectively lower the Fe and Cu content of unclarified (PVPP-treated) wort and beer.

Evaluation of beer bitterness and changes due to aging. Fresh and aged beers were evaluated for beer bitterness via three analytical methods: Bitterness Units (BUs), ppm iso-alpha acids by HPLC and ratio of *trans* to *cis* isomers of the *iso*-alpha acid analogues by HPLC. Analytical bitterness scores for the Fresh and aged beers are reported in **Table 4.3**. There is no 'Control treatment' for this analysis because the control had no *iso*-α-acids. The measurement of BUs is standard in industry because of its relative ease of use. However, the bitterness unit analysis is somewhat general in its measurement because it accounts for several chemical species that absorb at 275 nm. Fresh beers were significantly different in levels of BUs ($p<0.05$), with the Pellet hopped beers scoring highest (14.1 BUs), followed by the Spent Hop (13.0), Extract (10.8 BUs) and finally the unhopped Control beers (1.5 BUs). Although the Spent Hop beers were relatively high in BUs, they were lowest in *iso*-alpha acids as measured by HPLC, with the exception of the Control Beers, which had no *iso*-alpha-acids. This result indicates that lager beer high in polyphenols can produce considerable levels of BUs, despite being low in *iso*-α acids. Consistent with our intention to produce a beer low in *iso*-alpha-acids and high in polyphenols, brewing with spent hop solids produced beers significantly lower in *iso*-alpha-acids ($p < 0.05$), with fresh beers at 3.5 *iso*-alpha acids. The Extract beers finished lower than the target 11 ppm *iso*-alpha acid levels due to unexpectedly low utilization in the kettle (9.5 ppm), while the Pellet beers were on target with 11.2 ppm *iso*-alpha acids.

Aged beers are generally characterized as having decreased bitterness after prolonged or high temperature storage [150, 152]. Changes in *iso*-alpha acid content were monitored after six weeks of storage (**Figure 4.9).** The Extract and Pellet hopped beers lost 1.15 and 1.45 ppm *iso*-alpha acids respectively, while the Spent Hop beer lost 0.36 ppm *iso*-alpha acids. Changes in BUs were not monitored during storage because it is generally thought that changes in BU levels correlate to the degradation of *iso*-alpha acids during beer storage [193]. Although other investigators report that beers high in

reductive capacity (high polyphenol content) have a stabilizing effect on the degradation of iso-alpha-acids during aging [8, 195-197], the results seen here do not adequately support this idea. This could in part be because of the nature of the analytical assay that does not allow for quantification of specific *iso*-alpha-acid stereo-isomers.

The *trans*-stereoisomers of the iso-alpha acid analogues are more prone to oxidation than the cis-stereo-isomers during aging, especially under temperature abuse, and thus some investigators have used the ratio of trans to cis *iso*-alpha-acids as a staling indicator [155, 156]. To determine the effect of increased hop polyphenol content on the ratio of trans to cis *iso*-alpha-acids, a second HPLC method was used that allows for quantification of the *trans* and *cis* stereoisomers of the three major *iso*-alpha-acid analogues. **Figure 4.10** depicts overlayed HPLC chromatograms for finished beers representative of all four treatments at week 0. The figure displays differences in eluting peaks which correlate well with total polyphenol levels (described previously) as well as six peaks that represent the cis and trans isomers of the three main *iso*-alpha-acid analogues. Fresh and aged beers did not differ significantly in their %Trans for any of the hopped treatments. Although the Pellet hopped treatments lost about 13% of their initial %Trans, it was not significantly higher than the losses seen by the Extract (11.6% loss) and Spent Hop (11.4% loss) treated beers after six weeks of aging at 30°C **(Figure 4.9).** This is in contrast to data previously collected in our lab, that indicates that hopping regime may affect initial ratio of *trans* to *cis iso*-alpha acids as well as the %Trans loss due to storage. Regardless, the data do not provide sufficient evidence that beer polyphenol content suppresses the rate of decline in %Trans experienced during high temperature storage.

Assessment of Beer Flavor Stability.

There exists evidence that brewing beers with Spent Hop solids or polyphenol enriched products enhances flavor stability and thus beer shelf life [6, 79, 81, 111, 143, 239]. Beer flavor stability was assessed via two chemical methods: the first utilized Electron Spin Resonance to monitor the effect of hopping technology on beer anti-radical (shelf life) potential monitoring and the second utilized SPME-GC-MS to measure staling aldehydes pertinent to aged-beer flavor.

Anti-radical potential by Electron Spin Resonance. Electron Spin Resonance (ESR) was utilized to investigate the antiradical potential of the fresh and aged beers. ESR is used in the brewing industry to measure the formation of radicals relevant to beer oxidation, in essence to predict beer shelf life [125, 205]. Samples are exposed to atmospheric oxygen and high temperature (60ºC) while the relative radical concentration is measured. The longer a beer suppresses radical formation (lagtime), the better the predicted shelf life of the beer. Likewise a measurement of the radical population (ESR signal intensity) may be taken at a specific time, usually at t= 150 minutes (T150). A lower T150 correlates to increased anti-radical potential. A shelf stable beer would be expected to have a relatively low T150 [125].

While hopping regime significantly affected the anti-radical potential of the lager beers ($p < 0.05$), reported as T-150 (**Figure 4.11**), storage at 30C for 6 weeks had no effect. Comparison of the fresh beers indicated that all treatments had significantly higher T150s than the Control. The Control treatment had the lowest T150 followed by the Pellet and Extract beers (which were not significantly different from each other), and then the Spent Hop beers, which were significantly highest in T150 values. These results suggest that beers without any hops (i.e. no *iso*-alpha acids, no hop derived polyphenols) have improved shelf stability as measured by ESR. This is contradictory to reports that claim that the antioxidative/anti-radical properties of hop products correlate well with hop bittering acid content, the humulones (alpha-acids) and lupulones (beta-acids) [75, 76, 137]. These results also suggest that the presence of hop derived polyphenols may negatively impact the flavor stability of beer as assessed by ESR; high T150 (low flavor stability) correlated positively with proanthocyanidin content in these beers ($r = 0.80$). Moreover, the beers in this study, with the exception of the Spent Hop hopped beers, were not drastically different in total polyphenol levels and in fact the Control beers were slightly higher in polyphenol levels than the Extract beers. A number of ESR lagphase studies indicate that polyphenol levels may not positively influence the formation of free radicals during processing in alcohol based beverages [40, 69, 75, 137, 209, 210]. It is possible that some polyphenols may in fact promote the formation of reactive oxygen species because of their propensity to reduce cations such as iron and copper [51, 211]

into more active states. Specifically, flavonoids such as prodelphinidins (3'4'5'trihydroxyflavans) show strong pro-oxidant potential [144] because they can function as coupled reducing agents(i.e. Fe (III) → Fe(II)). Reported prodelphinidin [212, 231] concentrations in beer (mg/L) should adequately promote oxidative reactions leading to flavor instability. However, results of phloroglucinolysis indicate that the Control beers in fact had the highest molar concentrations of the prodelphinidins (-)-epigallocatechin and (+)-gallocatechin. It is plausible therefore that another class of flovonoids, such as the flavonols, which were not directly measured in this study could be the causative agents. Flavonols, also found in beer , have the propensity to reduce iron and copper to the active forms necessary to prompt Fenton and Haber-weiss reactions [1, 232]. However, recently we investigated the phenolic content of a polyphenol rich extract that when dosed into beer improved ESR T150 values, and this hop derived polyphenol rich extract was actually high in total flavanoids and determined to be rich in flavonols and flavanonols. Moreover other antioxidant capacity assays and sensory experiments allude to positive effects of polyphenols on beer flavor stability [8, 111, 143, 239]. This presents a rather interesting paradox: flavonoid compounds are known antioxidants yet their anti-oxidant character does not appear to be measurable via ESR-PBN spin trapping. Further investigation into this paradox is warranted.

Resistance to aldehyde formation during force-aging. A wide range of compounds have been designated as potential staling indicators in beer [28, 149], however the very low flavor threshold of some aldehydes makes them key players in aged beer flavor, even if present at sub-ppb levels in beer [157, 240]. Furthermore, other aldehydes, although definitively less flavor-active, can be reliable aged beer flavor indicators because their concentrations increase in conjunction with the appearance of other oxidative flavor changes during aging [158-160, 164].Although aldehydes can be produced via other chemical pathways (lipid oxidation[147, 221], maillard reactions[222, 223], and the degradation of proteins[224]), the primary cause of aldehyde formation in beer is thought due to the oxidation of amino acids via strecker degradation that cause the conversion of leucine to 3-methylbutanal, alanine to acetaldehyde, valine to 2-methyl-propanal, methionine to methional and phenylalanine to phenylacetaldehyde, etc.

Strecker degradation may take place during wort boiling and during beer storage. To date very little has been reported regarding the effect of polpyhenol content on aldehyde formation in aged beers.

Fresh and aged beers were analyzed for content of twelve (12) aldehydes via SPME-GC-MS with on fiber derivatization (PFBHA): isobutyraldehyde, 2-methylbutyraldehyde, isovaleraldehyde, valeraldehyde, hexanal, furaldehdye, heptanal, methional, octanal, benzaldehyde, phenylacetaldehyde, and *trans*-2-nonenal. A standard curve was produced for each aldehyde by incremental dosing of known concentrations into the fresh un-hopped control beer (Control). Concentrations of aldehydes were back-calculated to these standard curves. Levels of aldehydes for the fresh and aged beers are reported in **Table 4.4**.

Total aldehydes. There was a significant effect of hopping regime on total aldehydes. The fresh Extract beers, which were lowest in total polyphenols, scored highest in total aldehydes (82 ppb), followed by the Spent Hop beers (72 ppb) (highest in total polyphenols), then the Control beers (62 ppb) and lastly the Pellet hopped beers with 46 ppb total aldehydes. The predominant aldehyde in the fresh beers, regardless of hopping regime or polyphenol content was methional (ranging from 32-33% of total aldehydes). The next highest aldehyde by percent was furaldehyde, which was significantly higher in the Spent Hop beers, but not significantly different between the other hopping treatments (Spent Hop = 25%, Extract, Control and Pellet = 19%). Other key players by percentage were phenylacetaldehyde and Isobutyraldehyde. The aldehydes valeraldehyde, hexanal, heptanal, octanal and trans-2-nonenal were all present below 1 ppb, this was consistent for all treatments.

Effect of aging on Aldehyde content. There was a significant storage effect ($p<0.01$) on total aldehydes levels for all treatments (**Figure 4.12**). After aging at 30°C for six weeks the Extract beers remained highest, suffering the largest increase in total aldehydes from 82 ppb to 183 ppb The Pellet hopped beers also suffered a large increase in total aldehydes, increasing from 46 ppb to 144 ppb. The aged Control beers increased from 63 ppb to 139 ppb and the Spent Hop treated beers experienced the smallest increase in total aldehydes, increasing just 56 ppb from 72 ppb in the fresh beers to 128

ppb in the aged beers. The predominant aldehyde by percent in the aged beers was still methional (24 to 29%), however aldehydes such as furaldehyde, a maillard product that is an indicator of heat abuse increased to make up 24 to 40% of the total aldehdyes. The Spent Hop, Extract and Pellet beers endured the largest increases in furaldehyde (32, 29 and 28 ppb respectively), indicating that neither polyphenol levels nor presence of iso-alpha acids offer resistance to increases in maillard products such as furaldehyde. Interestingly there was a high correlation between high furaldehyde levels and high T150 values (low anti-radical potential) by ESR (r= 0.89). Levels of isobutyraldehyde also increased dramatically for the Control (Δ20 ppb) and Extract beers (Δ20 ppb), while Pellet and Spent hop beers did not increase as dramatically (Δ6ppb and 7 ppb). Phenylacetaldehyde increased for all treatments by 18 to 21 ppb, except for the Spent Hop beers which only increased by 2 ppb. In fact low phenylacetaldehyde levels correlated well with high levels of polyphenols by HPLC (r = 0.914), total polyphenols by spectrophotometry (r = 0.90), proanthocyanidins by HPLC (r = 0.965) and total flavanoids by spectrophotometry (r = 0.99), indicating that there could be a protective effect of high polyphenols against formation of phenylacetaldehyde in aging beers. Levels of the lesser abundant aldehydes did not change as drastically, with heptanal and octanol not increasing significantly during aging. Aged beers were not significantly different in levels of hexanal or trans-2-nonenal, although all beers contained above threshold levels in trans-2-nonenal (Pellet = 0.20 ppb, Extract = 0.24ppb, Spent Hop =0.17 ppb and Control beers = 0.16 ppb).

CONCLUSION

In summary, we have produced lagers beers according to four hopping regimes in order to better understand the effect of hopping technology on polyphenol profiles and the consequence of aging at high temperature (30C) for six weeks on changes in polyphenolic profiles of the lager beers. The four hopping regimes were chosen to represent beers constituting varying levels of polyphenols and *iso*-alpha-acids. Beers were brewed with 25% liquid adjunct and the wort was treated with Divergan HM PVPP-PVI to produce pale lager beers low in transition metals (Fe and Cu) with reduced barley polyphenol content. Hopping regime was not found to significantly affect fresh or aged

beer parameters. However increases in color were seen after six weeks of accelerated storage, with the largest increases seen in the Control beers.

The fresh and aged beers were analyzed for phenolic content via spectrophotometric and HPLC analysis. Results indicated that beers brewed with Spent Hop solids were significantly higher in total polyphenols, total flavanoids and proanthocyanidins, with all methods of analysis correlating well with proanthocyanidin content ($r = 0.97$ and $r = 0.99$). The extraction of beers with Sephadex LH20 resin produced methanolic extracts that could be diretly injected into the HPLC for phenolic analysis. Phloroglucinolysis allowed for the quantification of flavanol subunits as well as proanthocyanidin mDP. Six major phloroglucinolysis products were observed: extension subunits consisted of, (+)-catechin, (-)-epicatechin and terminal subunits consisted of (+)-gallocatechin, (-)-epigallocatechin, (+)-catechin, and (-)-epicatechin. However galloylated flavanols were not detected in any of the beers. The predominant subunits by molar ratio were (+)-catechin (Spent Hop = 38%, Pellet = 38%, Extract = 37% and Control = 33%), followed by (-)-epigallocatechin (Spent Hop = 12%, Pellet = 21%, Extract = 23% and Control = 28%) for all but the Spent Hop treatment, which had (-)-epicatechin as its second highest subunit by molar ratio. The major extension subunit was (+)-catechin-phloroglucinol for all treatments (Spent Hop = 14%, Pellet= 11%, Extract = 10% and Control = 8%). Although the Sephadex extracts were phenolic in nature, proanthocyanidins made up less than 2% of that phenolic material. The Spent Hop beers had the highest yield of proanthocyanidins (1.58%), however, kettle hopping with Pellet hops did not produce beers that were different in proanthocyanidin content from the unhopped Control beers. Not unexpectedly, beers treated with Spent Hop material had the highest overall mDP (1.35). The total polyphenol content of the beers did not appear to change during storage as measured by the EBC standard method, however analysis of the Sephadex extracts indicated that increases did decline over six weeks of storage for the Spent Hop treated beers. Total flavanoid and proanthocyanidin content of the beers changed during storage, with increases seen initially and eventual decreases after 6 weeks of storage at 30C. The Spent hop beers lost 23% of flavanoids due to aging as measured by the total flavanoids assay, however phloroglucinolysis revealed that only 4% of the flavanoid material was

lost in the Spent Hop treatments due to storage. Bitterness analysis indicated that lager beer high in polyphenols can produce considerable levels of BUs, despite being low in *iso*-α acids. However we could not provide sufficient evidence that beer polyphenol content suppresses loss of *iso*-alpha acids during aging. The Spent Hop beers also showed the least flavor stability as assessed by ESR T150, suggesting that beers with augmented levels of hop derived polyphenols may negatively impact flavor stability as assessed by ESR. After aging at 30C for six weeks Spent Hop treated beers experienced the smallest increase in total aldehydes, increasing just 56 ppb from 72 ppb in the fresh beers to 128 ppb in the aged beers, indicating that presence of hop polyphenols may infact suppress formation of staling aldehydes in beers during aging.

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List of figures.

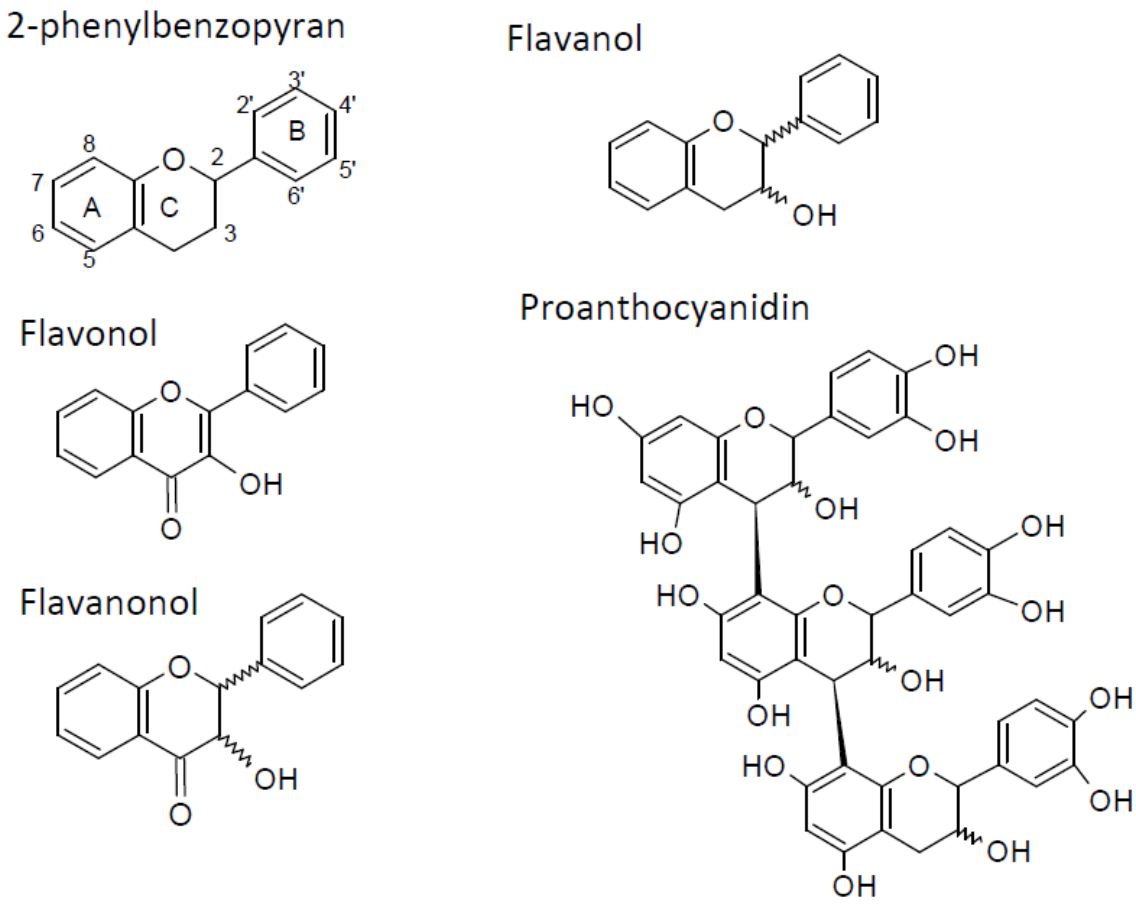

Figure 4.1. Flavonoid subclass phenylbenzypyran structures: flavan-3-ol, flavonol, flavanonol, and condensed flavan-3-ols (proanthocyanidin).

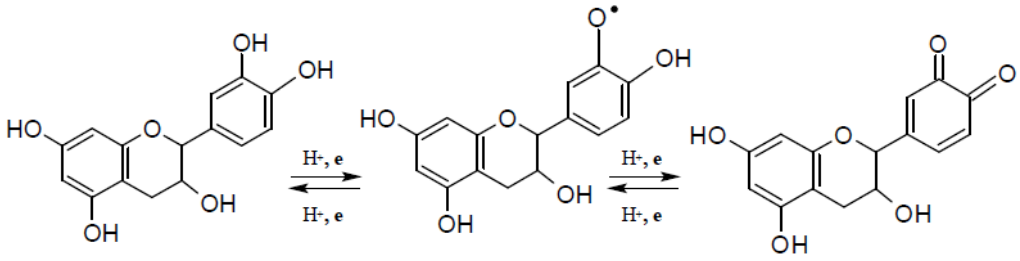

Figure 4.2. Phenolic oxidation and radical formation procedes through formation of a semiquinone intermediate.

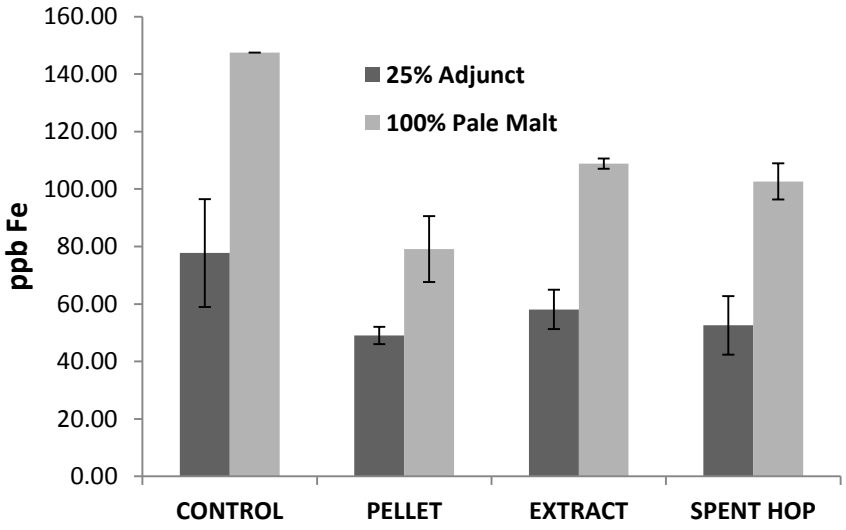

Figure 4.3. The effect of brewing with 25% adjunct (Clearsweet) vs. 100% all pale malt on mean values of wort iron content ± standard error.

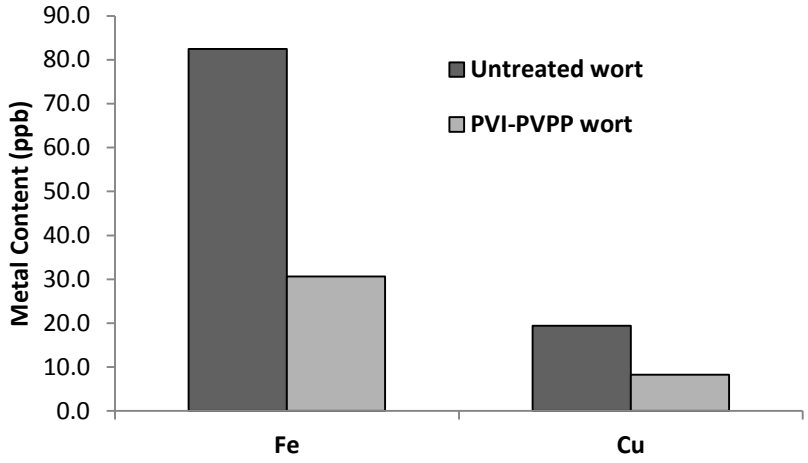

Figure 4.4. Treating wort with HM PVPP-PVI reduces apparent copper (ppb) and iron (ppb) concentrations.

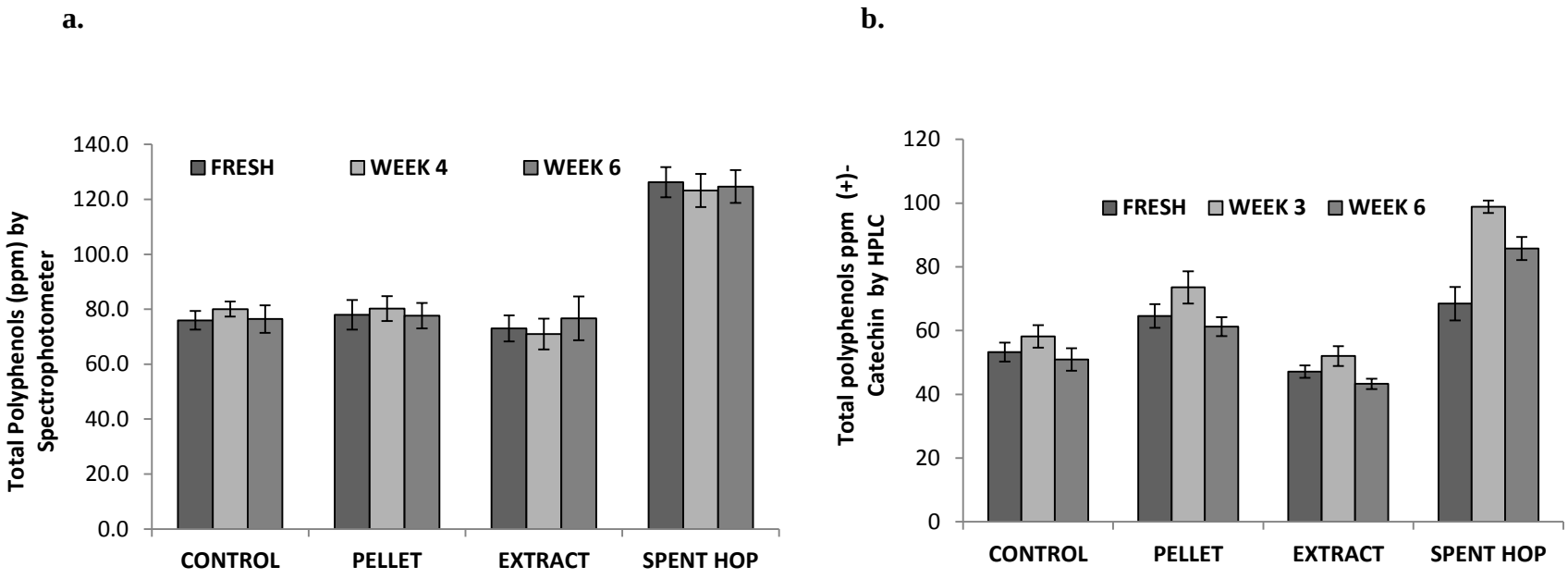

Figure 4.5. Total polyphenols mean values (n = 4) ± standard error in lager beers during aging at 30°C: a. Total polyphenols reported as (+)-catechin by the EBC standard method using a spectrophotometer b. Total polyphenols reported as (+)-catechin (ppm) by RP-HPLC.

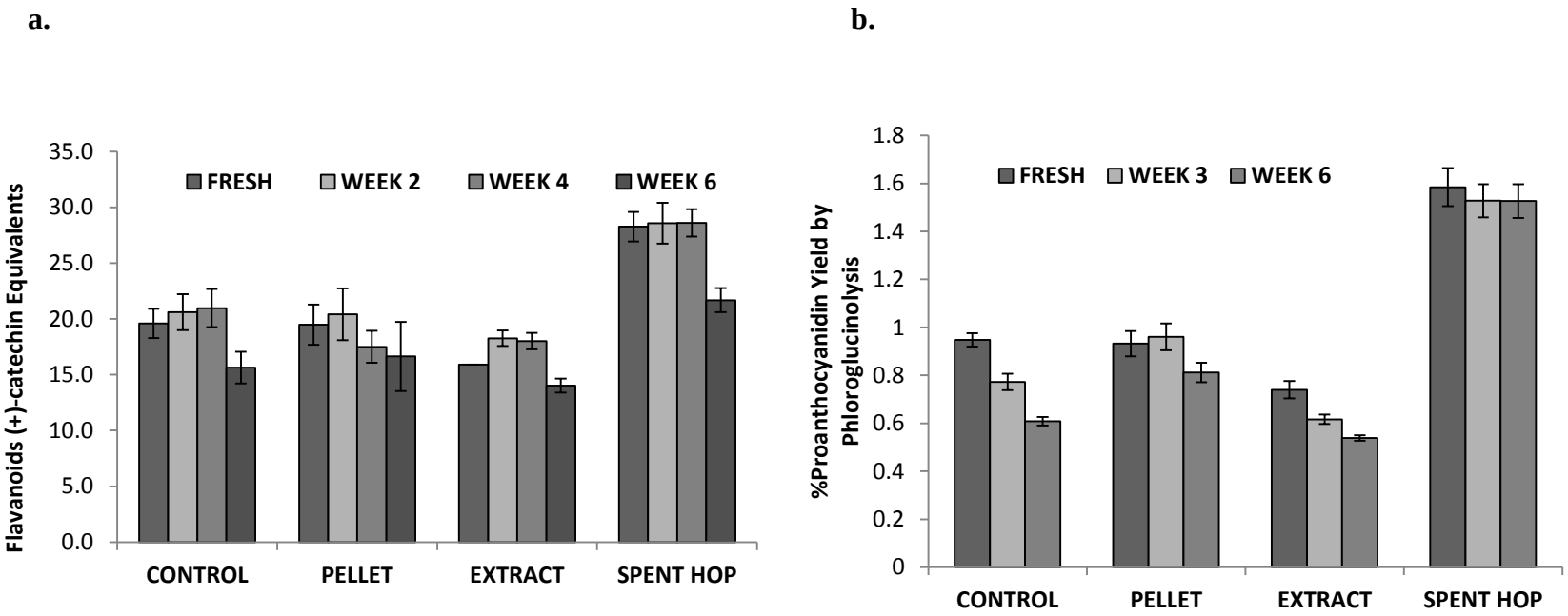

Figure 4.6. Total flavanoid mean values ± standard error in lager beers during aging at 30ºC : a. Total flavanoids reported as (+)-catechin equivalents (CE) by the EBC standard method using a spectrophotometer. b. % Proanthocyanidin yield resultant from phloroglucinolysis as measured by RP-HPLC-ESI-MS.

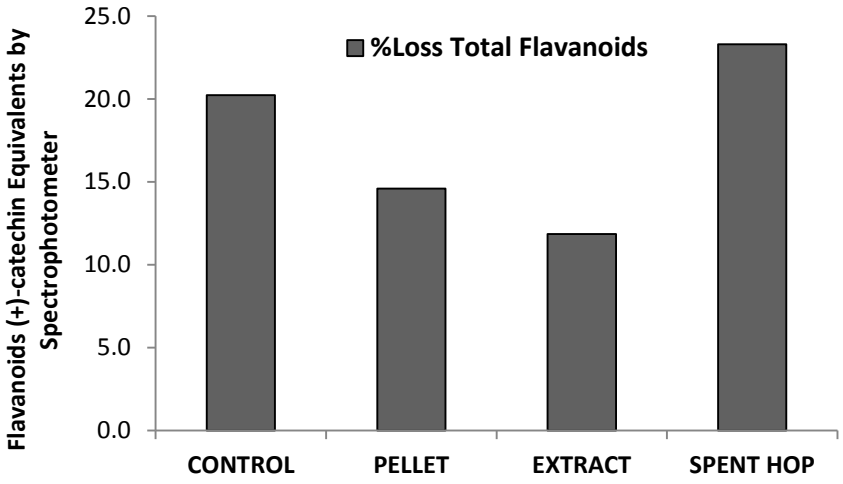

Figure 4.7. %Loss in total flavanoids due to six weeks of aging at 30ºC reported as (+)-catechin equivalents as measured by the EBC standard method using a spectrophotometer.

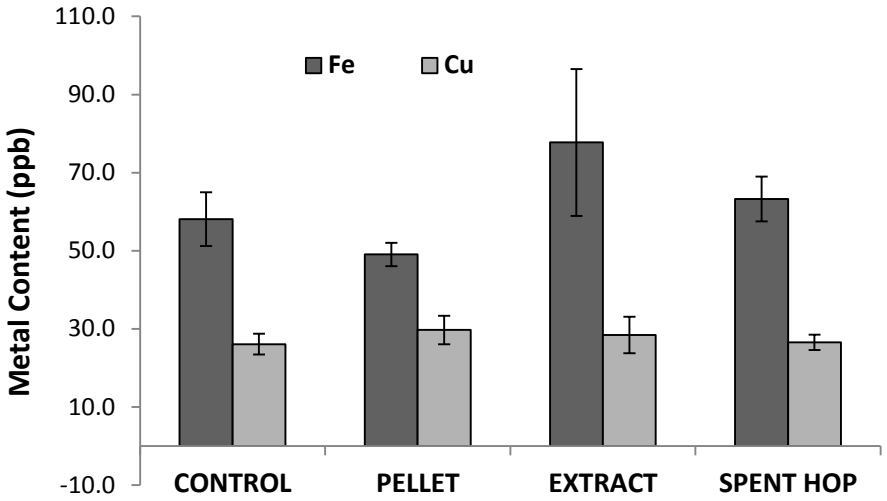

Figure 4.8. Metal content of the fresh lager beers reported as mean values of Fe (ppb) and Cu (ppb) ± standard error.

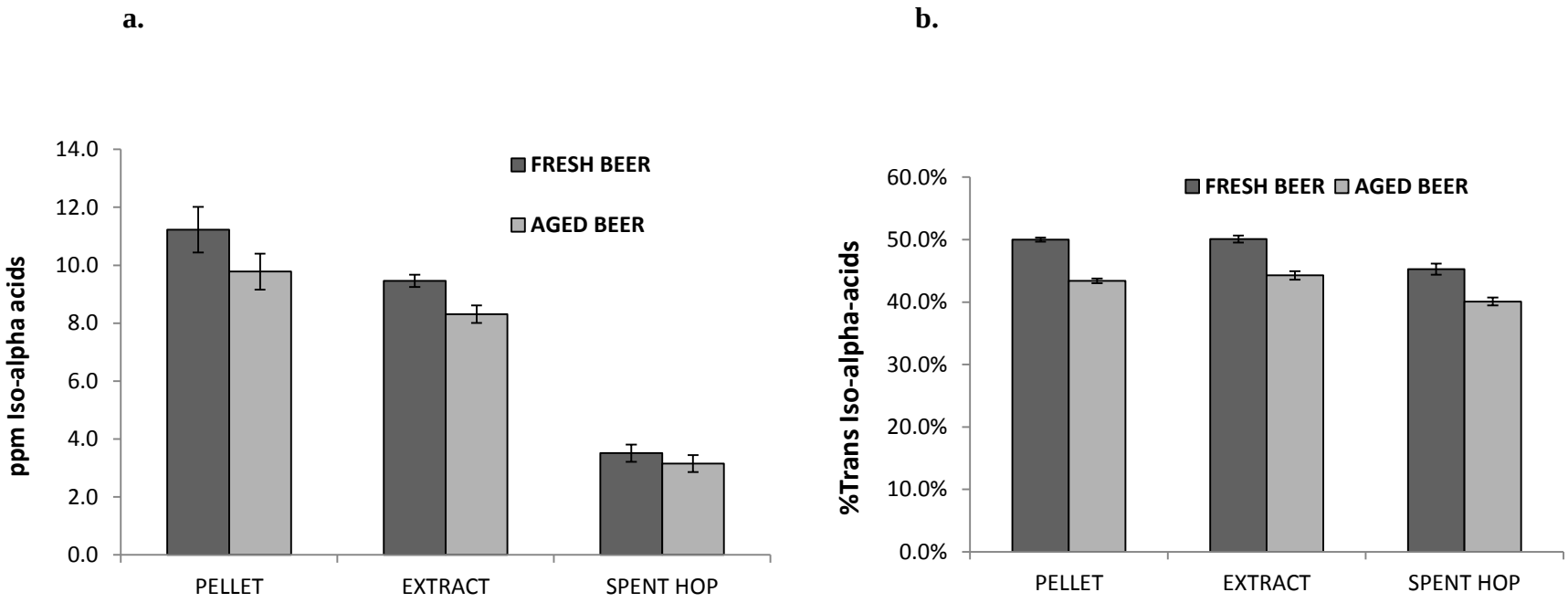

Figure 4.9. Bitterness values for fresh and aged beers reported as mean values ± standard error: a. *Iso* alpha acid content b. Ratio of *trans* to *cis* isomers of alpha acid analogues (%Trans).

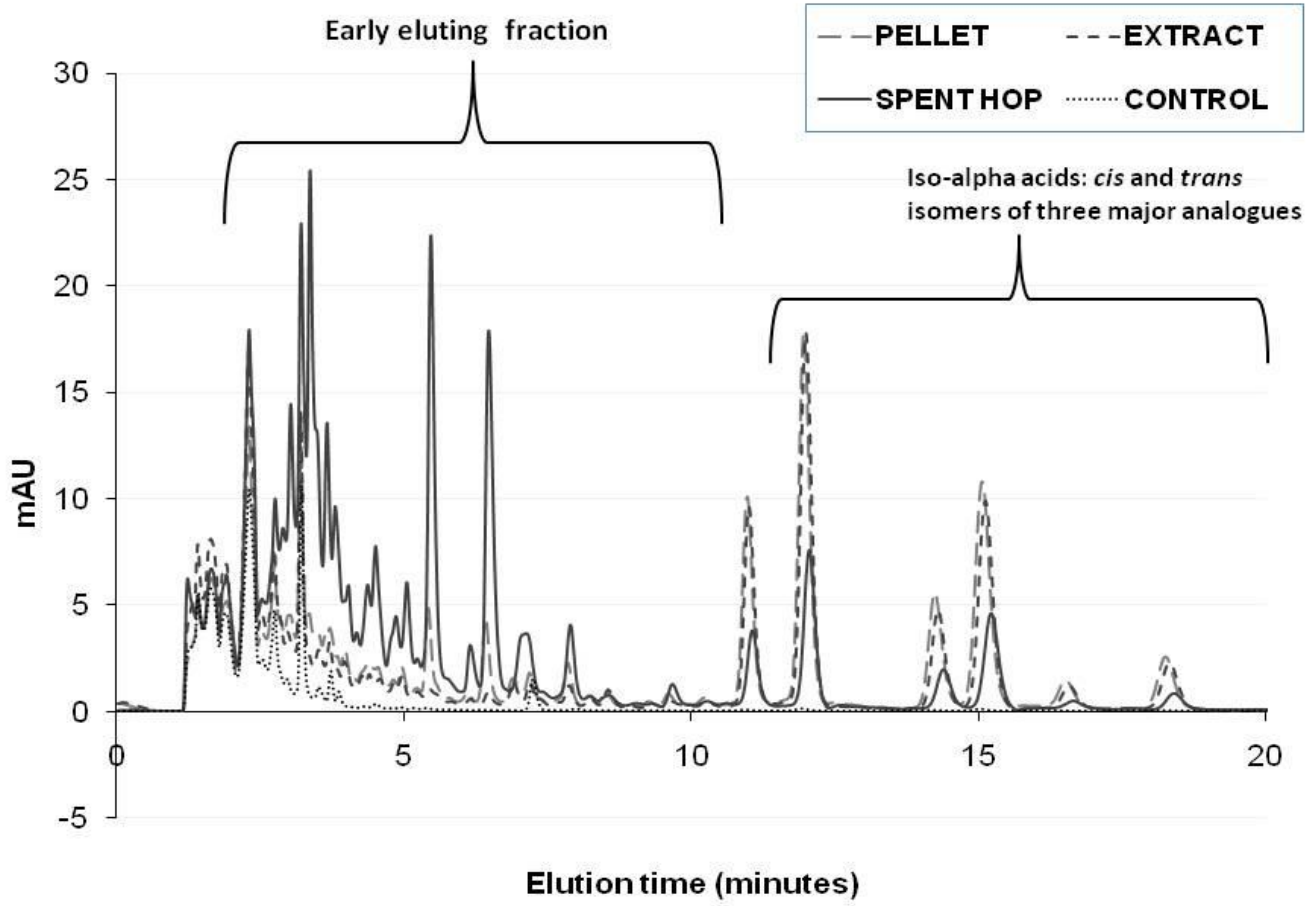

Figure 4.10. HPLC chromatogram of C-8 extractions of fresh beers showing early eluting fraction and six peaks representing the *cis* and *trans* isomers of three major *iso*-alpha-acid analogues.

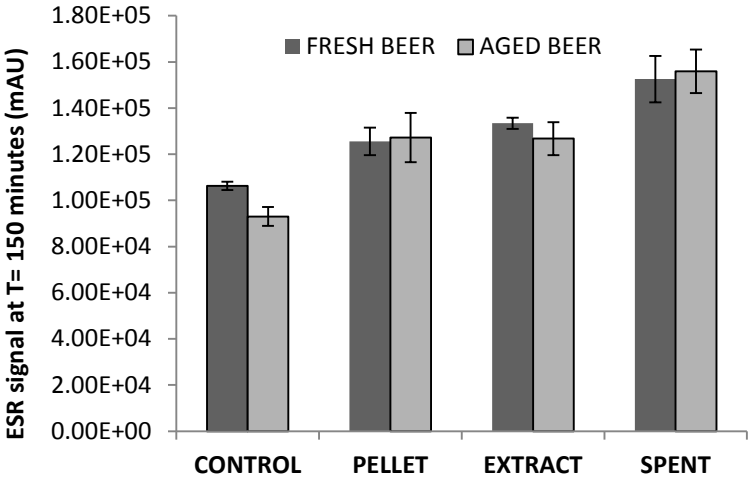

Figure 4.11. Beer antiradical capacity or flavor stability as measured by Electron Spin Resonance. Values reported as T150 values, representing the signal (mAU) of each sample at time = 150 minutes.

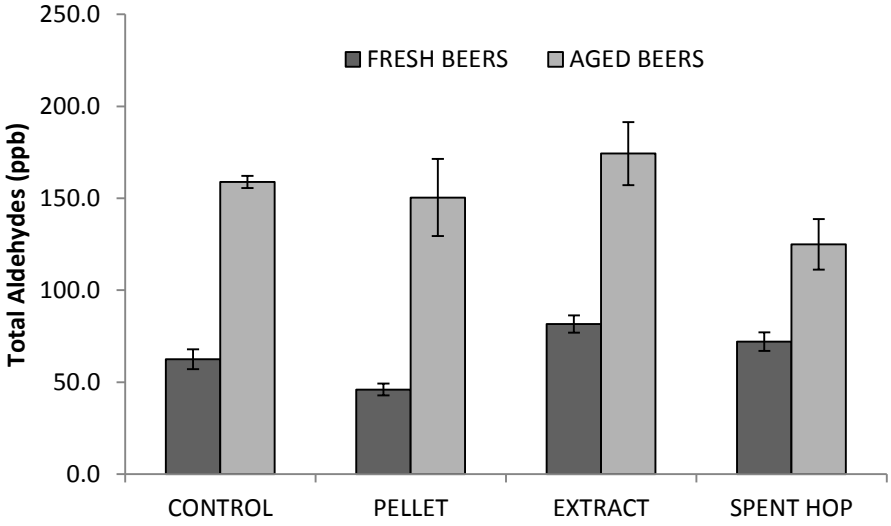

Figure 4.12. Total aldehydes (ppb) representing the sum of 12 aldehydes in the fresh and aged beers (6 weeks of storage at 30°C) are reported as mean values ± standard error.

List of tables.

Table 4.1. Finished beer parameters for the fresh and aged (6 weeks, 30°C) beers

	Color[a] (EBC)	pH[a]	Specific Gravity [a]	Original Gravity [a] (°P)	Alcohol[a] % w/w	Alcohol[a] % v/v	Residual Extract[a] %w/w	Apparent Extract[a] % w/w	†RDF % w/w	††KJ/100 mL
FRESH BEERS										
CONTROL	6.77±0.41	4.35±0.02	1.0051±0.0006	10.91±0.72	3.92±0.28	4.98±0.35	3.28±0.21	1.46±0.10	71.6±0.4	168 ±12
PELLET	6.28±0.19	4.46±0.02	1.0052±0.0003	11.55±0.05	4.23±0.03	5.38±0.04	3.34±0.03	1.38±0.04	72.2±0.3	177±1
EXTRACT	6.71±0.71	4.44±0.02	1.0055±0.0003	11.28±0.41	4.10±0.16	5.22±0.20	3.30±0.12	1.40±0.07	71.9±0.4	173±6
SPENT	7.09±0.21	4.47±0.02	1.0057±0.0001	11.11±0.23	4.00±0.09	5.09±0.12	3.31±0.06	1.46±0.03	71.4±0.2	170±4
AGED BEERS										
CONTROL	7.83±0.25	3.85±0.02	1.0052±0.0003	11.53±0.16	4.24±0.03	5.39±0.05	3.30±0.10	1.34±0.08	72.8±0.3	177±3
PELLET	7.00±0.23	4.42±0.01	1.0050±0.0001	11.64±0.11	4.31±0.04	5.47±0.05	3.27±0.05	1.29±0.03	72.8±0.3	179±2
EXTRACT	7.39±0.47	4.44±0.02	1.0057±0.0001	11.97±0.06	4.38±0.03	5.57±0.04	3.48±0.03	1.46±0.03	72.2±0.2	184±1
SPENT	7.73±0.15	4.47±0.03	1.0056±0.0001	11.44±0.17	4.16±0.08	5.29±0.10	3.35±0.02	1.43±0.02	71.9±0.3	176±3

[a]All values expressed as mean values (n=4) ± standard error. †Real degree of fermentation %. ††Kilojoules per 100 mL of beer.

Table 4.2. Phloroglucinol results for fresh (0 Weeks) and aged (3 weeks and 6 weeks) beers

Sample Name	WEEK[a]	GC[a]	C-P[a]	EC-P[a]	EGC[a]	C[a]	EC[a]	mDP[b]	YIELD[c]
CONTROL	**0**	44.34±1.36	24.73±0.93	11.61±0.76	71.15±3.23	96.18±4.05	30.34±1.16	1.14±0.01	0.95±0.03
CONTROL	**3**	32.28±2.63	21.42±1.54	10.43±0.54	59.77±2.73	84.51±5.05	26.19±1.42	1.15±0.00	0.77±0.03
CONTROL	**6**	44.46±4.83	7.34±0.40	12.52±0.48	49.27±2.12	44.19±2.34	16.74±0.54	1.13±0.01	0.61±0.02
PELLET	**0**	35.46±2.65	31.26±1.38	17.41±0.49	60.14±2.14	108.9±3.46	31.67±2.31	1.20±0.00	0.93±0.05
PELLET	**3**	40.05±1.97	27.64±2.38	16.09±1.40	71.70±1.57	94.44±8.34	35.29±2.81	1.18±0.01	0.96±0.06
PELLET	**6**	35.76±1.74	19.52±1.86	12.84±0.83	56.83±2.57	89.01±3.65	29.41±0.97	1.16±0.01	0.81±0.04
EXTRACT	**0**	29.67±1.72	21.10±1.65	9.53±1.02	48.63±2.70	80.50±4.47	25.15±2.06	1.10±0.01	0.74±0.04
EXTRACT	**3**	41.38±2.87	9.33±0.57	15.39±0.99	51.52±1.09	43.28±1.66	16.11±0.80	1.16±0.01	0.62±0.02
EXTRACT	**6**	43.57±4.08	5.93±0.11	11.31±0.73	41.09±2.70	37.12±1.51	13.36±0.65	1.13±0.01	0.54±0.01
SPENT	**0**	35.64±3.43	62.14±4.96	53.96±5.51	52.51±6.28	173.7±9.17	67.77±3.96	1.35±0.01	1.58±0.08
SPENT	**3**	28.46±4.57	52.38±3.52	48.60±3.99	82.19±4.92	174.7±8.60	68.24±4.17	1.27±0.01	1.53±0.07
SPENT	**6**	48.21±2.04	51.42±1.73	48.72±3.46	73.84±3.73	172.9±4.42	65.12±2.91	1.27±0.01	1.53±0.07

[a]Phloroglucinol results for fresh (0 Weeks) and aged (3 weeks and 6 weeks) beers expressed as mean values (n=4) of molar equivalents ± standard error from individual UV response to (+)-catechin with the following subunit abbreviations: (-P)-phloroglucinol adduct of extension subunit; GC, (-)-gallocatechin; C, (+)-catechin; EC, (-)-epicatechin; EGC, (-)-epigallocatechin. [b]Apparent mean degree of polymerization based on known phloroglucinol subunit composition. [c]Percent conversion yield resultant from conversion of phenolic Sephadex extracts to known proanthocyanidin subunits.

Table 4.3 Bitterness parameters of the fresh and aged (6 weeks, 30ºC) lager beers

	BUs[a]	FRESH *Iso* (ppm)[a]	AGED *Iso* (ppm)[a]	FRESH †%Trans[a]	AGED †%Trans[a]	DECLINE[c] *Iso* (ppm)	DECLINE[c] †%Trans	††%DECLINE †%Trans
PELLET	14.13±0.66	11.23±0.79	9.78±0.62	50.0±0.3	43.4±0.4	1.45	6.6	13.2
EXTRACT	10.75±0.40	9.46±0.21	8.31±0.30	50.1±0.5	44.3±0.7	1.15	5.8	11.6
SPENT HOP	12.99±1.03	3.51±0.30	3.16±0.29	45.3±0.9	40.1±0.6	0.36	5.2	11.4
CONTROL[b]	1.51±0.07	--	--	--	--	--	--	--

[a]Values reported as mean values (n=4) ± standard error of the mean. [b]No *iso*-alpha acids or %Trans data is displayed for the Control beers because the Control beers were unhopped. [c] 'Decline' is equivalent to loss due to storage (Fresh – Aged values). †%Trans = ([*trans*-*iso*cohumulone] + [*trans*-*iso*humulone]/[*cis*-*iso*cohumulone] + [*cis*-*iso*humulone]) x 100%. ††%Decline in Trans =([%Trans of fresh beer - %Trans of Aged beer]/%Trans Fresh beer) *100%.

Table 4.4. Mean aldehyde levels in fresh beer

FRESH	IBA**	2MBA*	IVA^{NS}	FUR^{NS}	METH^{NS}	BENZ^{NS}	PHEN^{NS}	VAL^{NS}	HEX^{NS}	HEPT^{NS}	OCT^{NS}	T2N*
CONTROL	6.45±0.49^{ab}	5.00±0.22^{ab}	5.76±0.48	12.4±1.91	21.2±2.36	1.91±0.35	8.11±0.96	0.49±0.07	0.41±0.04	0.23±0.02	0.46±0.01	0.08±0.01^{ab}
PELLET	4.70±0.55^{a}	3.24±0.33^{a}	4.13±0.45	8.90±1.81	15.8±1.42	1.85±0.38	5.89±0.32	0.29±0.01	0.46±0.01	0.24±0.05	0.39±0.01	0.06±0.01^{a}
EXTRACT	9.38±0.46^{b}	6.16±0.58^{b}	6.99±0.74	15.9±1.58	27.0±3.32	2.51±0.22	11.7±1.38	0.51±0.04	0.63±0.04	0.20±0.01	0.43±0.01	0.12±0.01^{b}
SPENT HOP	5.45±1.02^{a}	4.03±0.71^{a}	6.05±1.01	18.6±1.60	23.4±0.07	2.68±0.39	9.88±1.55	0.46±0.07	0.66±0.07	0.29±0.02	0.43±0.02	0.09±0.00^{ab}
AGED	**IBA^{NS}**	**2MBA^{NS}**	**IVA****	**FUR^{NS}**	**METH^{NS}**	**BENZ^{NS}**	**PHEN^{NS}**	**VAL^{NS}**	**HEX^{NS}**	**HEPT****	**OCT^{NS}**	**T2N^{NS}**
CONTROL	26.9±6.39	4.02±0.44	6.19±0.72^{a}	33.2±3.53	34.9±7.02	4.1±0.86	26.7±6.17	0.96±0.17	0.72±0.10	0.20±0.02^{a}	0.49±0.05	0.16±0.03
PELLET	10.8±2.50	8.65±2.03	11.9±0.75^{bc}	44.1±8.54	41.4±3.60	7.8±2.91	23.7±12.48	0.92±0.27	1.07±0.35	0.23±0.04^{b}	0.43±0.05	0.20±0.08
EXTRACT	21.4±5.58	9.28±1.24	14.4±1.95^{b}	45.2±8.74	43.8±3.66	3.5±0.68	33.2±2.73	0.93±0.24	1.63±0.63	0.23±0.03^{a}	0.51±0.10	0.24±0.06
SPENT HOP	12.3±0.82	4.70±1.15	7.77±0.91^{ac}	50.3±13.46	34.3±2.62	3.9±0.53	12.0±1.00	0.92±0.16	0.88±0.08	0.22±0.02^{a}	0.56±0.03	0.17±0.02

Mean is based on 4 replicates ± standard error for Extract, Pellet , Spent Hop and Control treatments. ^{a,b,c} means within a column for Fresh or Aged subgroups with different letters are significantly different from one another at Tukey's HSD at the 5% level and ANOVA at *$p < 0.05$ or **$p < 0.01$, NS = no significant difference. Abbreviations: IBA, isobutyraldehdye; 2MBA, 2-methylbutyraldehdye; IVA, isovaleraldehyde; FUR, furaldehyde; METH, methional; BENZ, benzaldehyde; PHEN, phenylacetaldehyde; VAL, valeraldehyde; HEX, hexanal; HEPT, heptanal; OCT, octanal: T2N, trans-2-nonenal.

Chapter 5.

A Discussion of Polyphenols in Beer Physical and Flavor Stability

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ABSTRACT

Generally referred to as polyphenols (PPs), beer flavonoids such as the flavan-3-ols and their condensed products, the proanthocyanidins, represent a class of readily oxidizable compounds capable of hindering or preventing the oxidation of other molecules present in beer. Flavan-3-ol and proanthocyanidin capacity to improve oxidative stability has been well established in other food systems, and thus these antioxidants have recently gained significant consideration as potential beer flavor modifiers and/or stabilizers. The duality of their presence in beer is that PPs complex with proteins in the beer matrix to form temporary and permanent hazes. Undesirable physical instability caused by PP-protein interactions can be resolved via use of adsorptive resins such as polyvinylpyrrolidine. While there is no doubt that polyphenol removal increases beer shelf stability in terms of haze formation, the impact of polyphenol removal on beer flavor remains unresolved. This review discusses the sources, content and impact of polyphenol presence and removal on beer physical and flavor stability.

KEY WORDS: beer, haze, flavan-3-ols, flavor, polyphenols, proanthocyanidins.

INTRODUCTION

Maintaining beer quality through the various stages of maturation, distribution and shelf storage remains an extensive challenge. While several attributes are used to establish overall beer quality, two aspects in particular have received considerable attention: colloidal and flavor stability. The establishment of colloidal stability in beer renders a beer ‘bright’, or haze free. A commonly used commercial stabilization treatment involves the addition of polyvinylpyrrolidine (PVPP) to finished beer. PVPP effectively removes polyphenols (haze precursors) and haze from beer. While stabilization treatments have resolved a majority of beer colloidal stability issues, the issue of flavor stability remains a challenge, especially for pale lager beers that are more sensitive to flavor deterioration during aging. Most aged-beer flavors have been attributed to oxidative mechanisms. As potential antioxidants and natural metal

chelators, beer polyphenols such as the flavan-3-ols and their condensed products, the proanthocyanidins, may influence the oxidative mechanisms responsible for aged beer flavors. However, very little is understood regarding the impact of their presence on aged beer flavor. This review presents information on the occurrence of flavan-3-ols and proanthocyanidins in beer and the effect of their presence and removal on the species and mechanisms involved in beer physical and flavor stability.

Beer Flavor Stability: Species and Mechanisms

Beer flavor stability. Flavor changes that occur during beer maturation play a critical role in the product quality and brand identity of beer. Successful brands that strive for maximum flavor stability are generally well received and recognized for such by consumers. While it is preferred that flavor improves during the maturation process, formation of undesirable flavors inevitably occurs during beer storage. More problematic is that occurrence of aged-flavors varies from one beer style to another, with lager beer seeming especially sensitive [149-151]. Of the many chemicals involved in beer flavor modification, a few key groups have been identified: diketones, sulfur compounds, aldehydes and volatile fatty acids [28, 149, 150, 241]. In general, beer aging results in decreased bitter taste, increased sweet taste and increased caramel, ribes (black currant), and toffee-like aromas. Carbonyl compounds such as trans-2-nonenal (cardboard aroma) form during beer storage from the oxidation of fatty acids and have been attributed to aged-beer flavor due to their very low flavor thresholds [154, 242]. Other carbonyls have also been used as chemical indicators of beer oxidative flavor development even though they typically exist at concentrations below the human detection threshold in beer. Compounds such as acetaldehyde, 2-furaldehyde (furfural), 5-hydroxymethyl-2-furaldehyde (5-hydroxymethyl furfural or 5 –HMF) and β-damascenone are considered useful chemical staling-indicators because their concentrations increase alongside increases in oxidative flavors during beer aging [243-245]. To date more than 700 compounds have been reported in various beer types [32] with specific volatiles resulting from a multitude of aging reactions: the Maillard reaction, the formation of linear aldehydes and esters, ester degradation, acetal formation, etherification, degradation of hop bittering acids and presence of phenolic compounds [24, 100, 136, 148]. The

occurrence of each reaction depends on beer type, storage temperature, and dissolved oxygen content [144, 246].

Oxygen in beer. Limiting dissolved oxygen levels in finished beers to ≤ 50 ug/L should prevent most undesirable effects on flavor and haze stability [149]. The ingress of oxygen during all stages of brewing and maturation should be limited to ensure that beer maintains maximum flavor shelf life (up to 52 weeks). Quality control criteria recommends 0.2 mg/L or less of dissolved oxygen for packaged beer[32, 138] and modern filling equipment is capable of achieving < 0.1 mg/L total package oxygen. However, even under reduced oxygen conditions, non-oxidative flavor modification reactions such as esterifications, etherifications, Maillard reactions and glycoside and ester hydrolysis may still occur in bottled beer [147, 148] due to production of $OH^{\bullet}$ via the Fenton reaction or during thermally or photochemically induced homolysis of some weak bonds of organic beer molecules [247]. Regardless, it is generally thought that aged-beer flavor depends heavily on the oxidative degradation of beer compounds by reactive oxygen species (ROS) [39, 40, 144, 145, 149, 248].

Reactive oxygen species and metal catalysts. ROS can be either oxygen or nitrogen radicals, or even non-radicals with the potential to oxidize or convert to oxidizing radicals. Flavor deterioration and related oxidative changes in beer molecules do not correlate directly with absolute molecular oxygen content ; molecular oxygen (O_2) itself does not react directly with compounds such as SO_2, sulfite ions or polyphenols. The non-radical molecular oxygen ($^3O_2$) in its ground state is slow to react with most organic compounds in their singlet ground states. This is due to the nature of oxygen's outermost, bonding-available electrons, the electrons available to form covalent bonds [41, 249, 250]. In oxygen these electrons exist in separate molecular orbitals, with spins aligned, in a triplet state ($^3O_2$). According to Danilewicz [249], oxygen in its triplet state resembles a di-radical. Under normal conditions, molecular oxygen in its triplet ground state cannot directly react with molecules that possess paired electrons with anti-parallel spins, molecules such as polyphenols that exist in their singlet state. This would violate Pauli's exclusion principle, and thus the reaction could only take place if spin inversion were to occur, a process that would require a large and unlikely energy input. The

activation energy required for oxygen to react with a lipid is also relatively large, between 35 and 65 kcal/mol [251]. Reactions involving oxygen are thus thought to proceed in one-electron steps via the formation of free radicals [41], a process that can be catalyzed by transition metals [205]. In the presence of a metal catalyst such as Fe^{2+} or Cu^{+}, oxygen can capture an electron to form superoxide anion (O_2^{-}). Upon protonation, superoxide forms the perhydroxyl radical ($OOH^{\bullet}$). Generally most of the superoxide (pKa 4.8) originating in beer (pH ~ 4.5) exists in this protonated and more reactive state [32, 150]. Superoxide may also undergo reduction to form peroxide anion (O_2^{2-}). Peroxide ion can in turn become protonated to form hydrogen peroxide (H_2O_2) [144]. Furthermore, iron can catalyze the generation of hydroxyl ($OH^{\bullet}$) and peroxyl radicals ($OOH^{\bullet}$) from H_2O_2 via the Haber-Weiss and Fenton reactions. Bamforth and colleagues provide a comprehensive review of oxygen and oxygen radical chemistry pertaining to malting and brewing[138]. The involvement of other metals in radical generation has not been as thoroughly investigated, but d-block elements such as manganese are capable of catalyzing reactions that produce ROS and may act synergistically along with iron and copper to catalyze oxidative staling reactions [134, 252]. **Figure 5.1** details reactions leading to the formation of ROS.

Occurrence and effect of transition metals in beer. Although a broad range of transition metals have been reported in beer, iron (III) and copper (II) exist at higher concentrations in beer and have relatively lower reduction potentials which makes them more prone to participate in reduction reactions in beer [144]. Sources of transition metals in beer have been explored and their fate during the brewing, fermentation, and clarification processes has been examined by several investigators. A concise summary of references pertaining to transition metal content in beer can be found in a 2008 article by Zufall and Tyrelll[132]. Bamforth and Parsons [145] suggest that traces of transition metals (Fe, Cu, Zn, Co and Mn) may cause detriment to beer flavor stability, even when present in bound forms, and should be eliminated at all stages of the brewing process (<50 ppb for Cu specifically) [138]. This suggestion has been re-iterated by Irwin and colleagues [144], who attest that the rate of beer flavor deterioration is significantly accelerated by trace amounts (<100 ppb) of Cu (II). Specifically, beers containing

higher levels of copper II ions (40 to 95 ug/L) exhibit increased stale flavor intensity. This effect is seen to a lesser extent with iron, however excess endogenous iron can result in a metallic off-taste in beer [132]. High manganese concentration has been linked to the production of a sherry like off aroma during beer aging. Zufall and Tyrell report that Fe, Cu, and Mn exist in malt and wort in an approximate ratio of 6:1:2, respectively[132]. However iron and copper from the raw materials side have relatively little influence on flavor stability of the final beer. Wort is typically higher in transition metals than beer. Wort boiling can reduce metal content of finished beers; chelating, nitrogenous (proteins and amino acids), and polyphenolic compounds originating from raw materials (malt and hops) act to bind and effectively remove a portion of the total metals [132, 134, 190, 191]. During fermentation, yeast absorbs and intracellularly distributes transition metals to effectively diminish the metal content of finished beer [192]. This is especially true regarding copper, iron and zinc [132, 190, 191]. However, evidence exists to suggest otherwise in regard to manganese. Cellular uptake of manganese by yeast is lower, and thus significant losses of manganese may not be seen during fermentation. Recently Pohl and Sergiel [133] investigated Cu speciation and the rate of staling in beer. The authors defined three groupings of Cu species, differing in hydrophobicity and charge: hydrophobic, cationic and residual Cu. The majority (74-82%) of the Cu found in beer exists in the residual fraction. Hydrophobic species accounted for 10-14% of the total copper found in beer, likely present in beer as polyphenolic-bound species, and the cationic species or free Cu contributed 12-13% of the total Cu. Results of these findings corroborated data compiled by Svendsen and Lund [253], who found that ~72% of total Cu in beer exists in the non-cationic form. Characterization of transition metal speciation in beer could be of use to brewers; transition metals must be in their free or ionic forms in order to effectively catalyze radical reactions [219, 220].

Brewers should also keep in mind that, beyond playing significant roles in beer off- flavor formation, trace metals have also been associated with beer colloidal instability in the forms of haze and gushing [190, 254-257].

Beer Physical Stability

Gushing. A beer may exhibit gushing, explosive release of carbon dioxide gas, upon opening when the pressure is reduced from approximately 2 ½ atmospheres to atmospheric pressure. Several factors are known to cause this physical phenomenon: presence of trace metals, oxidation, and prolonged low temperature storage [255]. Transition metals of the fourth or higher periods are known causative agents of gushing [257] . While copper may not be as active as iron or other metals tested, copper may accelerate oxidative reactions that cause gushing. The addition of chelating agents such as EDTA can be used to prevent metal-induced gushing. Beer itself possesses endogenous metal chelators that may provide a natural resistance against gushing [217, 254].

Beer haze. Consumers expect lager and filtered beer to be 'bright' or haze free, a quality resulting from colloidal stability. A beer is considered 'bright' if no haze forms when chilled to 4°C (40°F) or below [28, 32]. However, because beer is intrinsically colloidally-unstable, without proper treatment, chill haze (non-permanent) may develop that can lead to sedimentation and precipitation (permanent haze). Beer haze results from the interaction of beer constituents that aggregate to form visible particles in solution that reflect light [254]. Constituents known to play a role in haze formation include protein, tannin (polyphenol), carbohydrate, oxygen and metal ions. Several metals have been found in haze: aluminium, barium, calcium, chromium, copper, iron, lead, magnesium, manganese, molybdenum, nickel, phosphorus, silicon, silver, strontium, tin, vanadium and zinc [256] . Metals such as copper, iron and aluminium can exist in the haze at several thousand fold higher concentrations than in the parent beer. Other metals such as lead, nickel, tin, vanadium and molybdenum are less concentrated in the haze, with manganese, calcium and magnesium even less concentrated. Such high presence of iron and copper in beer haze is not unexpected as these metals are easily chelated by phenolic, amino and carboxyl groups. It is therefore also not surprising that haze concentrates metals such as iron and copper; protein-polyphenol complexes are the most frequent cause for haze production [149, 258].

The haze active (HA) protein found in beer derives from barley hordein. Because HA-proteins have on the order of 20 mol% proline [46] they display a high affinity for polyphenols (PPs). Siebert et al. [58] have described a model (**Figure 5.2**) in which PPs crosslink HA-proteins, in a similar fashion to when beer PPs react with parotid-derived proline-rich lingual proteins to elicit the organoleptic sensation of astringency [28, 58, 259, 260]. In this model protein-PP complexes form a large network when the number of HA-PP binding sites equal the number of available HA-protein binding sites in the beer matrix. The nature of haze formation in this manner likely involves hydrogen bonding and hydrophobic stacking of proline and PP rings associated with π-bonding (**Figure 5.3**) [261]. Formation of protein-PP haze depends on beer pH, alcohol content, ionic strength, as well as phenolic composition [259-262].

Beer stabilization. Protein-PP complex formation has received extensive attention and thus precursors to this complex have become the target for beer haze treatments. Several methods have been employed for beer colloidal stabilization including: prolonged cold storage, cold filtration, fining with gelatin, isinglass, or tannic acid, addition of proteolytic enzymes and treatments with adsorbents [63, 149]. One of the more commonly used adsorbent resins, polyvinylpyrrolidine (PVPP) was commercially introduced in 1961[64] to specifically target and remove beer PPs. While other approaches may be used to target PPs, fining with PVPP is practiced commonly due to its relative ease of use and low cost. PVPP is a neutral polyamide that has an affinity for beer-PPs because it is structurally similar to polyproline, a known HA-peptide (both possess a five-membered nitrogen containing ring with hydrogen bonding sites) (**Figure 5.4**)[28, 63, 149]. PVPP-PP binding involves hydrogen bonding and hydrophobic stacking, roughly the same mechanism as protein-PP binding [260, 261].

Beer Polyphenols.

Phenolic compounds represent a group of chemical substances characterized by the presence of at least one phenol unit. Classified by the type and number of phenolic subcomponents present, PPs are generally divided into categories: the hydrolysable tannins and the flavonoid elagitannins or phenylpropanoids: flavones, flavonols,

flavanonals, flavanones, flavan-3-ols and condensed flavan-3-ols (proanthocyanidins) (**Figure 5.5**) [229, 230].

PP concentrations reportedly range from 50 to 150 mg/L in lager beers [121]. Depending on beer type, up to 80% of beer PPs are said to derive from malt, with the remainder originating from hops [231, 263]. Several classes of phenolic compounds have been found in beer, including simple phenols, benzoic acid derivatives, cinnamic acids, coumarins [14, 16, 232, 264, 265], chalcones, flavanones, flavones, flavan-3-ols, proanthocyanidins [231, 232, 266], alpha acids, *iso*-alpha acids and other miscellaneous compounds [232, 267]. The final PP content of beer depends largely on brewing practice and raw materials. The flavan-3-ol monomers (**Figure 5.6**) and proanthocyanidins oligomers receive measurable attention based on their roles in flavor, foam, colour, and colloidal beer quality parameters [268]. To date at least 8 flavan-3-ol monomers (including glycosides and gallates), 7 dimers (5 B-type, 2 A-type) (**Figure 5.7**) and 3 trimers (C-type, 1 procyanidin, 2 prodelphinidin) have been reported in beer [266, 269]. Another class of flavonoids, the flavonols also receive measurable attention due to their roles in metal chelation and potential to promote ROS formation. The following flavonols have been determined in beer: kaempferol, kaempferol-rhamnoside [126], quercetin [127], quercitrin, isoquercitrin [52], and rutin [128, 232]. Callemien and Collin [128] recently reviewed the structures and properties of all phenolic compounds found in malt, hops and beer.

Haze-active beer polyphenols. Although roughly 80 known phenolic compounds have been determined in beer [232, 266], not all are involved in colloidal instability. HA-PPs must be able to effectively crosslink HA-proline-rich proteins into a stable network to result in precipitation. Flavanoids are known constituents of permanent beer haze. The flavan-3-ol monomers (-)-epicatechin and (+)-catechin and (+)-gallocatechin bind, but do not cross link HA proteins. However, proanthocyanidin oligomers possess two or more binding sites within the same molecule, allowing them to crosslink HA proteins [58]. Haze formation varies with proanthocyanidin molecular weight, subunit composition, interflavanoid bond orientation, number and placement of the hydroxyls on the heterocyclic C and aromatic B rings [39, 61, 189, 233]. Specifically, tri-hydroxy

flavanoids bind more readily than di-hydroxy flavanoids, vicinal or ortho oriented hydroxyls bind better than meta-oriented hydroxyls, and (-)-epicatechin oligomers bind slightly better than (+)-catechin oligomers. Because trimer, tetramer and higher proanthocyanidin oligomers less readily survive the brewing process, the proanthocyanidin dimers are thought to play the most significant role in beer haze [259]. However, oxidized flavanols instigate chill haze and once condensed (polymerized) into proanthocyanidins participate in the formation of permanent haze [62].

Polyphenols as antioxidants. Despite having a negative association with beer haze formation, flavan-3-ol and proanthocyanidin capacity to improve food oxidative stability has been well established [141], and thus these antioxidants have been considered for their potential to improve beer flavor stability [24, 100, 258, 265, 268, 270]. Flavan-3-ols behave as antioxidants via several mechanisms: scavenging of free radicals, chelation of transition metals (**Figure 5.8**), and mediation and inhibition of enzymes [43]. The electron configuration of flavan-3-ols allows for easy release of electrons to free radical species ($R^{\bullet}$). Release of an electron transfers the radical character to the flavan-3-ol ($F^{\bullet}$), a radical that is generally more stable and less harmful than the initial radical species. The oxidation of flavan-3-ols predominantly produces semiquinone radicals (**Figure 5.9**). Semiquinone radicals couple through nucleophilic addition to produce oligomers that retain the number of reactive catechol/pyrogallol structures, in effect preserving their scavenging ability . Relative ease of flavanol oxidation and free radical scavenging activity correlates to structure and stereochemistry: (-)-epicatechin is more easily oxidized than (+)-catechin; C-4→C-8 dimers oxidize more readily than C-4→C-6 dimers [271]; antioxidant activity increases from monomer to trimer then decreases from trimer to tetramer [180, 272]. Presence of galloyl groups, number and position of hydroxyl groups enhance activity, whereas methoxylations and glycosylations inhibit activity [178, 181].

Polyphenols as metal chelators. Flavan-3-ols and proanthocyanidins may act as indirect anti-oxidants by binding and effectively reducing concentrations of divalent transition metals from solution [214, 215]. Procyanidins (3', 4'-dihydroxyflavans) such as (+)-catechin strongly complex iron and copper cations (stability constants from 9 to

9.35 (log K)) in preferred stoichiometric binding ratios of Fe^{2+}/procyanidin (2:1) and Cu^{2+}/procyanidin (4:1) [216]. Flavan-3-ols/proanthocyanidin potential to chelate metals depends on hydroxylation pattern and degree of polymerization; ortho-dihydroxy configurations on the b-ring and higher degree of polymerization lead to increased metal-flavanoid complex formation and stability [217]. Formation constants for catechin-copper chelate complexes have been assessed spectrophotometrically: (+)-catechin($K_{Cu/CuL}$ = 14.45). Mira et al. [218] investigated the flavonoid chelation capacity of Cu (II) and Fe (III) using UV spectroscopy and electrospray ionization mass spectrometry. Flavones (flavonols and flavanonols) and the flavanol catechin readily chelate metal ions. Specifically, flavones chelate Cu (II) (pH 7.4 and 5.5) between the 5-hydroxyl and 4-oxo group. At increased pH, flavonols such as myricetin and quercetin and the flavan-3-ol (+)-catechin chelate Cu (II) at the ortho-catechol group of the B-ring. It is important to note that at beer pH, (+)-catechin would not effectively chelate copper, but flavonols on the other hand, which can be sourced from hops, effectively chelate copper at beer pH. Flavonols myricetin and quercetin also bind Fe (III) between the 5-hydroxyl and the 4-oxo groups (tested at pH 5.5). The authors remark that at pH 5.5 the flavones studied chelate Fe (II), suggesting that flavonoids chelate iron more effectively when iron is in its bivalent form and thus at beer pH, and flavonols (myricetin and quericetin) would reduce Fe (III) to Fe (II) before association. However, other flavonoids such as kaempherol, luteolin and (+)-catechin more readily complex Fe (III). This is important because, as stated previously, in order for transition metals to be effective catalysts in radical reactions, they must be present in their free or ionic forms.

Polyphenols as pro-oxidants. Regardless of reports that indicate an overall protective role for beer PPs, flavan-3-ols, proanthocyanidins and flavonols may potentially promote ROS formation and behave in a pro-oxidant manner. Redox cycling of phenolics can be catalyzed by cations such as iron and copper to result in ROS that are capable of altering lipids, proteins, enzymes, and other biological molecules [51, 273]. Of the major flavan-3-ols and their condensation products, those that contain a gallic acid moiety show greatest pro-oxidant potential. Gallic acid moieties can antioxidatively bind with Fe (III) to form ROS scavenging complexes, however when Fe (III) concentration is

more than twice the gallic acid concentration, the complex becomes pro-oxidant by forming H_2O_2 derived hydroxyl radicals via a Fenton type reaction. Proanthocyanidins may also reduce Cu (II) to Cu (I), with Cu (I) capable of auto-oxidation to form more ROS. Although galloylated flavanoids have rarely been reported in beer (5-20 mg/L) [52], and gallic acid is usually present only at ug/L concentrations [232], the 3'4'5' trihydroxyflavans (prodelphinidins) are believed to have similar chemical functionality to gallic acid [144]. Specifically, prodelphinidins can function as coupled reducing agents (prooxidants). Moreover, reported prodelphinidin concentrations in beer (mg/L) [232] should be sufficient to drive oxidative reactions, including the oxidation of primary alcohols to aldehydes [144]. Prodelphinidins [231, 267] reported in beer to date include the following: (-)-gallocatechin, (-)-epigallocatechin, (-)-galloatechin-(+)-catechin dimer, and ent-(-)-epigallocatechin-(+)-catechin dimer [53, 267]. Although not contributors to haze, another species of flavonoids, the flavonols also display pro-oxidant activity due to their propensity to form quinones that are highly prone to redox-cycle [274] . Flavonols can reduce Fe(III) to Fe(II) and Cu(II) to Cu(I), metals that are responsible for promoting oxidation via Fenton and Haber-Weiss reactions. Flavonol ability to reduce Fe(III) is likely due to the 2,3 double bond on the C-ring that increases molecular planarity and provides increased rigidity[218]. When the A and C rings are aligned with more planarity, the 3-hydroxyl/4-oxo groups and 5-hydroxyl/4-oxo groups align more closely. Flavonols myricetin and quercetin readily reduce Fe(III). Flavonoid-copper reduction activity seems to depend more on number of hydroxyl groups present, and not as much on the presence of a 2,3 double bond on the C-ring.

Cu^{2+} reduction study shows that a large number of copper ions are reduced per flavonoid. In fact, all flavonoids studied by Mira et al.[218] showed an increased reducing capacity for copper ions rather than for iron ions, most likely due to the standard redox potentials of the metals: $Cu^{2+/}Cu^{+}$ (+0.15V) and $Fe^{3+/}Fe^{2+}$ couple (+0.77 V).

However, even the flavonols may behave antioxidatively via their capacity to chelate iron and copper; chelated transition metals are potentially less active in the promotion of free-radical related reactions.

Polyphenols and Beer Flavor

PPs undeniably behave as antioxidants (procyanidins and their condensed products) in food systems and hence improve food and beverage functionality in terms of foamability, oxidative stability and heat stability[141]. However, epimerization, degradation and de-polymerization of flavanol oligomers and polymers have been known to occur during food and beverage processing and storage [129, 130, 170]. The fate of PPs during brewing, processing and aging must also therefore be regarded. PP losses due to trub formation, adherence to yeast cells during fermentation and removal by filtration/fining media are inherent, but what fate is bestowed upon PPs during aging? Unfortunately, the fate of PPs on packaged beer remains unresolved [128]. During beer aging PPs are gradually degraded by oxidative mechanisms[151] into other species whose chemical reactivity remains largely unknown. Flavanol monomer and dimer concentrations ((+)-catechin, (-)-epicatechin, prodelphinidins and procyanidin dimers) reportedly decrease [39, 52, 121, 151], with dimeric flavanoids receding more rapidly than their monomeric counterparts. However, after several weeks of storage, relative levels of PPs may once again increase [172]. This could partially be explained by occurrence of PP polymerization and chemical alterations due to uptake of oxygen [173, 275].

Polyphenol contribution to bitterness and astringency. PPs of the flavonoid family provide beer with astringency [101, 102, 276], perceived organoleptically as a drying or puckering sensation on the tongue. PPs of both low and high molecular mass may elicit an astringent response [103]. Flavan-3-ol monomers such as (+)-catechin and (-)-epicatechin can also impart bitterness to beer [101, 102, 142]. Dadic and Belleau[96] added purified PPs and oxidized counterparts to both water and beer. Sensory analysis confirmed that addition of PPs to beer added a harsh bitterness and increased astringency. McLaughlin, Lederer and Shellhammer[142] substantiated these findings. The authors added varying levels (0,100, 200 mg/L) of PPs extracted from a Galena spent-hop (material remaining post-critical CO_2 extraction), along with *iso*-alpha acids to a commercial lager beer. The beer samples with *iso*-alpha acids and 100 or 200 mg/L of spent hop-derived PPs were assessed as being more bitter by sensory panellists. The

samples that included known monomers of (+)-catechin, (-)-epicatechin, (-)-epigallocatechin and (-)-epicatechin gallate, also had a longer bitterness duration and gave higher intensities for 'harsh', 'medicinal' and 'metallic'. Surprisingly, the spent-hop polyphenolic extract itself was characterized by intense fig and fruit-like aromas and when dosed into the base beer, provided remarkable hoppy aroma and flavor. Goldstein and colleagues[97] report that these observations could be related to glycoside flavor precursors found in the spent hop material. According to the authors, water soluble glycosidic hop flavor precursors may undergo chemical or enzymatic hydrolysis to create a variety of flavor-active compounds that ultimately impact the overall hop aroma and flavor of the beer.

Polyphenol removal, addition and beer flavor. While the benefits of PPs removal by PVPP on increased shelf stability have been well established, questions arise about the potential for PVPP treatment to impact beer organoleptic quality and flavor stability [70, 100, 144, 147]. Malt and hop derived PPs of the flavonoid family are considered the main natural antioxidants in wort and beer. PPs reportedly provide up to 60% of the endogenous reducing capacity [39] to wort and beer [246]. Moreover, PP ability to scavenge free radicals [145] [206], interact with aldehydes [173] and chelate pro-oxidant transition metals can provide protection against formation and degradation of important beer flavor components: the formation of stale carbonyls, protect degradation of UV-active compounds, guard *iso*-humolunes from decomposition and shield sulfites from oxidation [24, 275, 277].

Although the aim of PVPP use is to eliminate the PPs involved in haze formation, the reality is that several PP classes are affected: simple phenolic acids, flavonol glycosides, procyanidins, prodelphinidins, proanthocyanidins and complexes of PPs and proteins [58, 63]. Model experiments indicate that PVPP may preferentially adsorb the potentially prooxidant prodelphinidins, while maintaining the antioxidant pool of procyanidins, yet this phenomenon has not been sufficiently substantiated in beer.

McMurrough and colleagues [39] reported that the treatment of beer with PVPP at 100 g/hL effectively decreases beer reducing capacity by 9-38% as measured by $DPPH^{\bullet}$ analysis. Despite this, the authors could not determine any marked differences in flavor

stability of forced aged-lager beer following PVPP treatment. Moreover the addition of exogenous PPs to PVPP treated beer (prodelphinidin B3, procyanidin B3 and (+)-catechin), resulted in rapid flavanol oxidation as well as increased chill haze. However, the PP additions did not significantly affect development of staling indicators [39, 64]. Mikyska and co-authors [100] investigated the effect of a modified content of malt and hops in the brewhouse in conjunction with the effect of PVPP treatment of beer on beer haze and flavor stability. While the addition of malt and hop PPs in the course of wort boiling improved reducing activity and carbonyl content in fresh and stored beers, both types of PPs influenced 'harsh taste'. PVPP treatment did not negatively affect stale flavor formation, but did have a positive effect on the flavor stability of heat-aged beers. Decreased staling of force-aged beers correlated well with PP content in the brewhouse; both hop and malt PPs slowed down flavor depreciation during a nine month storage period, with the primary effect seen during the first four months. Recently, Bushnell et al. [70] reported that partial removal of PPs by PVPP did not significantly affect flavor stability of the beers studied by a sensory panel. However, in contrast to the 9 months of Mikyska's study, beers in this study were only force-aged for 30 days (30°C) and exposed to one PVPP stabilization regime (0.18 g/L) [70]. According to O'Reilly, effective PVPP dosing rates differ by beer type [65]. If flavanoid dimers and oligomers are the target, lower doses (15-20 g/hL for single use) of PVPP may be applied, whereas extremely high doses on the order of 100 g/L are needed to remove excessive quantities of monomers [66]. This was corroborated by Mitchell and coworkers [258].

Gerhauser et al. [232, 267] attempted to identify which PPs are specifically absorbed by PVPP. The authors used ultrafiltration to isolate adsorbed PVPP components. Initial results were not very encouraging as the PVPP residue consisted largely of degradation and rearrangement products of beer PPs, including five structural classes of compounds, 28 compounds in total that displayed little antioxidative potential in biological assays.

As another approach for PP removal, Andersen and Skibsted [112] added hexamethylenetetramine (HMT) during mashing to reduce endogenous PP content of wort. HMT addition reduced the concentration of (+)-catechin, prodelphinidin B-3, and

procyanidin B-3 in wort and beer. The oxidative stability of the wort and beer was then tested via Electron Spin Resonance (ESR). No differences were seen in the treated vs. untreated wort by ESR. Likewise, other ESR lag phase studies indicate that PPs (catechin, phenolic acids, and dimeric proanthocyanidins) may not significantly affect the formation of free radicals in beer during storage or in wort during brewing [40, 69]. Moreover, beers produced with and without HMT treatment gave similar flavor acceptance scores by sensory panellists. The authors conclude that PPs have little to no effect on the oxidative stability of beer, results that corroborate those of previous studies [69, 278]. The authors attest that this is because PPs are present only in very low concentrations in beer, thus reducing their potential to act anti-oxidatively. However, if the PPs are sufficiently present during earlier stages of brewing, they may provide more of a protective effect against oxidation. Andersen and Skibsted also hopped beer with and without hop PPs to conclude that added PPs have no effect on the oxidative stability of the beer, work that supports that of Takaoka et al. [74]. Work by Foster and researchers at Oregon State University contradict this [72].

Foster investigated the potential of a spent-hop derived PP extract (prepared at Oregon State University) to improve the oxidative stability of light and dark beer and fruit juices. Addition of the PPs to beer demonstrated enhanced antioxidative potential as measured by ESR[72]. Recent work conducted at Oregon State University (unpublished) supports this. A PP-rich extract, isolated from spent Galena hop material was dosed into a commercial lager (100 ppm). Chemical analysis confirmed an anti-staling effect of dosed spent hop material as measured via several antioxidant capacity assays (FRAP, DPPH, and ESR). Sensorially, beers treated with PPs were statistically different from control beers due to the presence of increased tropical and fruity aromas, however a significant temperature effect of reduced staling (cardboard aroma) was seen in the PP dosed beer after 6 weeks at high temperature storage. In a separate experiment, beer was brewed with varying kettle hop treatments, with and without hop PPs. Analysis of the beer by FRAP indicated that using pellets and spent hop material improved the antioxidant capacity of fresh finished lager beer, and that this capacity persisted through aging at high temperature for up to eight weeks. After eight weeks storage, significant

increases in overall and cardboard aromas were seen in lager beers aged at 30°C ($p < 0.01$), with pellet hop treatment scoring lower in cardboard aroma than the control and other hop treatments.

Forster [143] has conducted several brewing trials that corroborate these effects observed with pellets and spent hops on beer flavor stability. Forster brewed beer with pellets (PP rich) and CO_2 extract (PP free). Results indicate that pellet hopped beers had a more pleasant aroma than beers hopped with CO_2 extract and that pellet hopped beers aged slightly better than extract hopped beers. In a second experiment Forster brewed with PP rich spent hops (derived from processing of type-45 pellets) at the beginning and at the end of boil. Colour and foam were not influenced, but the beer brewed with hop PPs did affect physical stability, especially when boiled for 90 minutes. The beers containing spent hops could be described as pleasant, hoppy, slightly fruity in aroma and taste. Beers that were dosed at 600 g/hL at 90 minutes did possess a marked bitterness, however no harsh bitterness occurred with shorter boiling times. The majority of tasters judged PP rich beers positively, even after 4 weeks of storage at 27°C. Beer without the hop PP addition was deemed undrinkable and aged after 4 weeks of storage at 27°C.

Hop polyphenols, beer flavor and patents. To date at least five patents have been filed and published regarding the use of hop PPs in fermented beverages. U.S. Patent 578325, entitled 'Method of preparing a full hop flavored beverage of low bitterness' by inventors Ting and coworkers was granted in 1998[6]. This patent entails a method of preparing light stable, hop flavored, fermented beverages that possess less bitterness, yet have comparable hop flavor to a fermented beverage that is prepared with whole hops. The high PP hop flavoring residue originates from a solid spent-hop material resultant from super-critical CO_2 extraction of whole hops. The patent is assigned to Miller Brewing Company. A second patent held by Ting and other coworkers, U.S. Patent 0161491 A1, published in 2004 [7], entails the methods and compositions for reduction of staling of fermented malt beverages, beverages, foodstuffs and cosmetics. Hop solids, rich in PPs were derived via a number of extraction methods from spent hop material and are intended to improve the flavor stability of malt beverages. U.S. Patent 7258887 B2, also held by Ting and colleagues [79] and assigned

to Miller Brewing Company, was published in August 2007. The patent relates to the treatment of beer with PP rich hop solids (from liquid CO_2 extraction) that provide light stable malt beverages with hop flavor and mouthfeel that is 'indistinguishable' from whole hops. Application for a United States Patent (0254063 A1) was made by Aerts and co-workers [80], of Chemisch en Biochemisch Onderzoekscentrum in (Ghent, Belgium) in 2007, entitled 'Use of hop PPs in beer'. The patent relates to a method of brewing with PP rich extracts. The extracts, prepared from hops are said to contribute mouthfeel, reducing power and stability to beer. In 2007, inventors Collin and Jerkovic applied for a patent with the World Intellectual Property Organization (#WO 068344 A2) [81]. The patent application outlines the use of spent hop material, rich in stilbenes and flavanoids, as an antioxidant for addition to comestibles such as beer.

CONCLUSIONS

Flavor instability resulting from beer storage remains one of the most important quality problems in the brewing industry. Although research has focused on aged beer flavor stability via a multitude of analytical methods, it remains very difficult to comprehensively and accurately evaluate the aging flavor of beer; no single compound or measurement exists to adequately address the multifaceted course of aging. Moreover, pale lager beers are especially sensitive to flavor degeneration [147, 148, 150, 195, 245]. Beer aging is caused primarily by oxidative reactions that transform into products associated with compromised product quality. Flavanoid PPs represent a class of readily oxidized compounds. As beer constituents they are directly involved in haze formation and can be removed by PVPP. Both attractive and unattractive flavor properties have been attributed to beer PPs; some report antioxidative roles for PPs in beer flavor [24, 39, 144, 246, 277] while others pro-oxidative roles [40, 69, 86, 144]. PPs likely undergo changes during malting and brewing [16, 39, 267] and seem to have greatest potential on flavor stability during the mashing and wort boiling steps [100, 279]. PPs also contribute significant reducing power to beer [246, 280], and have been ascribed to nonenal reduction during wort boiling [24]. Sensory experiments also allude to positive effects of hop-PPs on beer flavor stability [100]. Although the use of whole hops and whole hop pellets seems to be in decline, and thus the total contribution of PPs to beer is in decline,

evidence exists to suggest that whole hop or spent hop material has something special to offer the brewer in terms of flavor stability. Regardless, the debate over the impact of PPs on beer flavor remains unresolved.

List of Figures

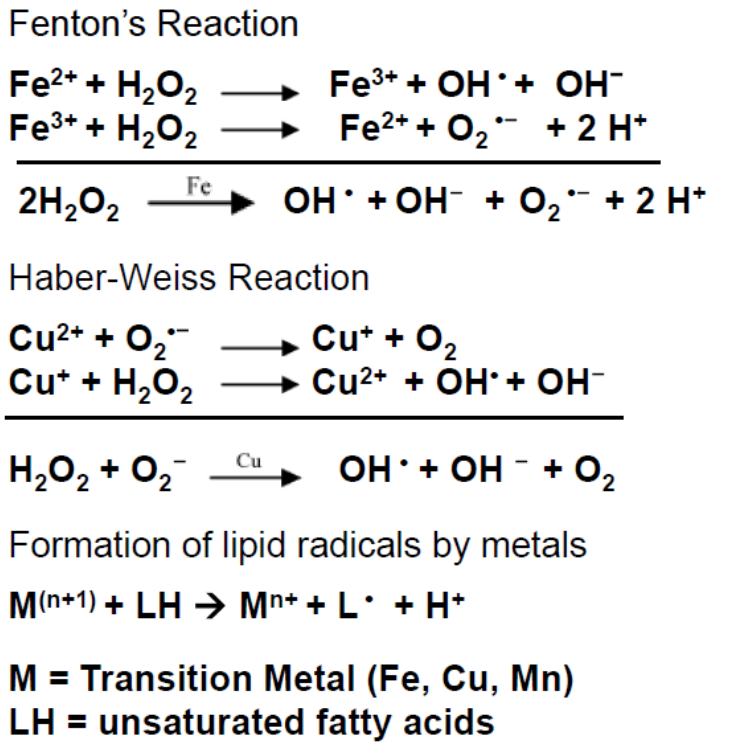


Figure 5.1. Reactions leading to the formation of reactive oxygen species (ROS).

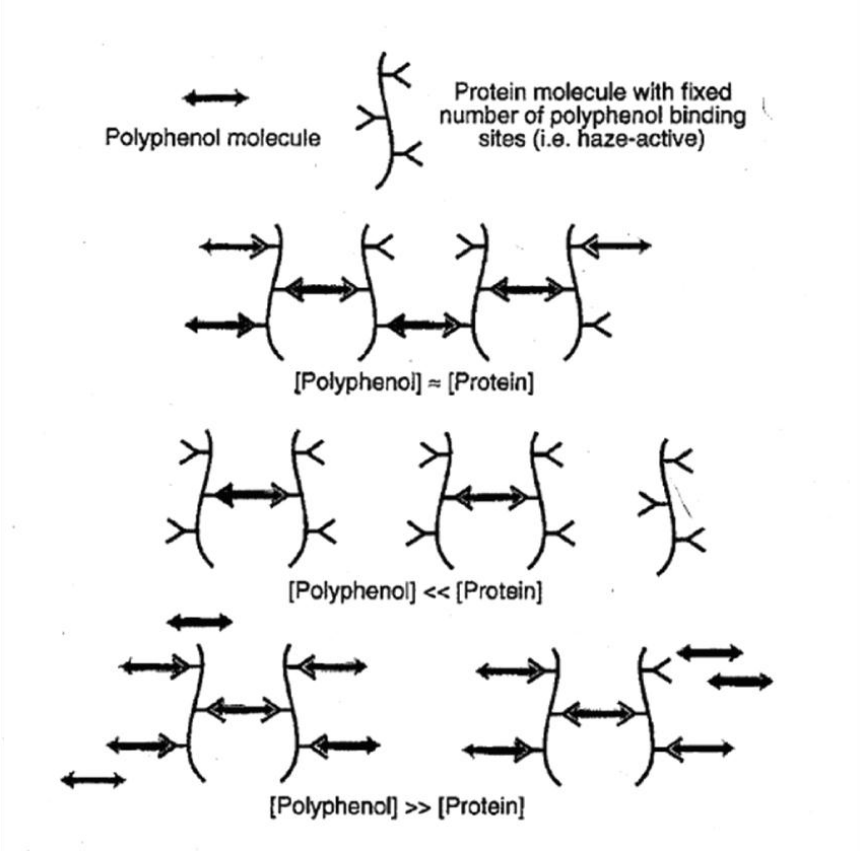


Figure 5.2. Concept of protein polyphenol interactions leading to haze; originally proposed by Siebert and Lynn [58]. (Reprinted with permission of J. Am. Soc. Brew. Chem.)

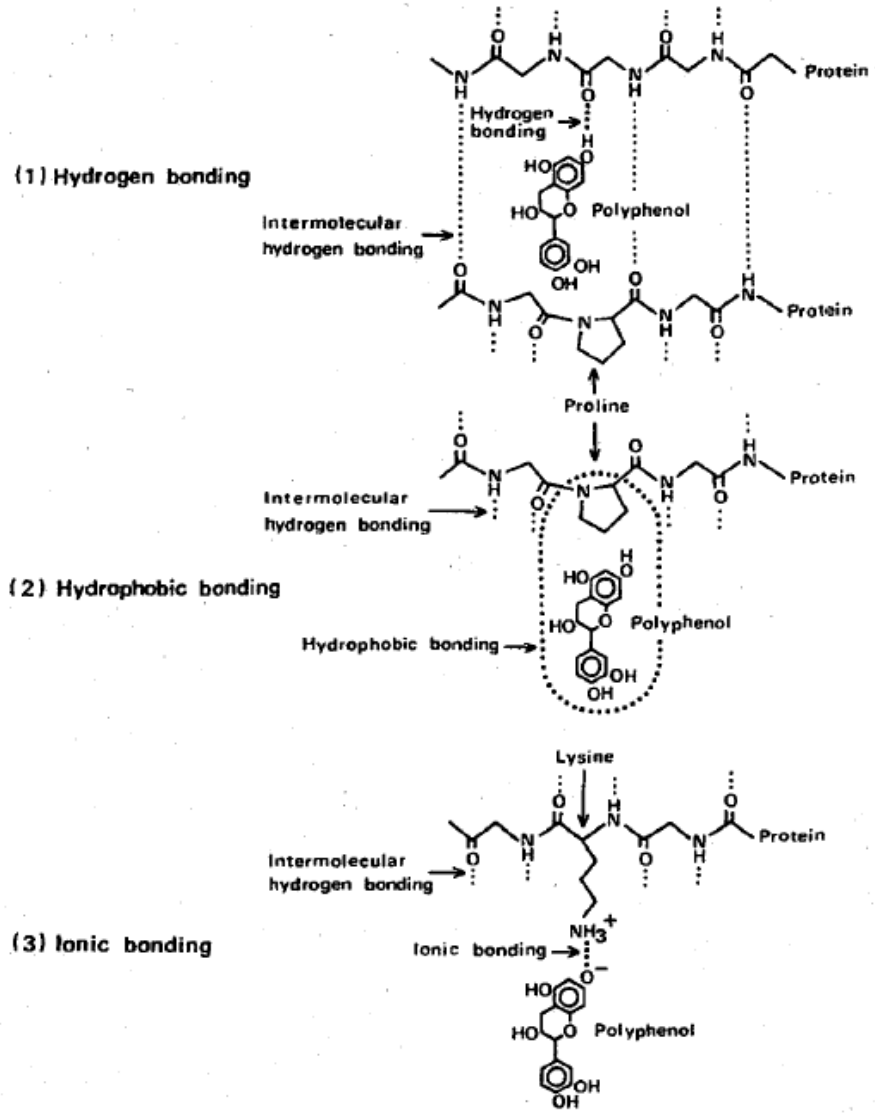


Figure 5.3. Mode of combination of proteins with polyphenols; originally proposed by Asano et al [54]. (Reprinted with permission of J. Am. Soc. Brew. Chem.)

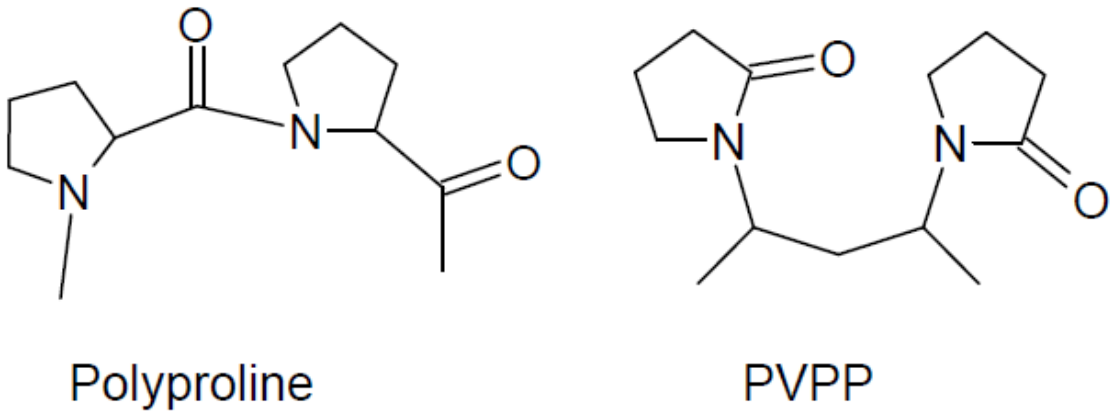


Figure 5.4. Similarity of polyproline and polyvinylpyrrolidine (PVPP); both possess a five-membered nitrogen containing ring capable of hydrogen bonding.

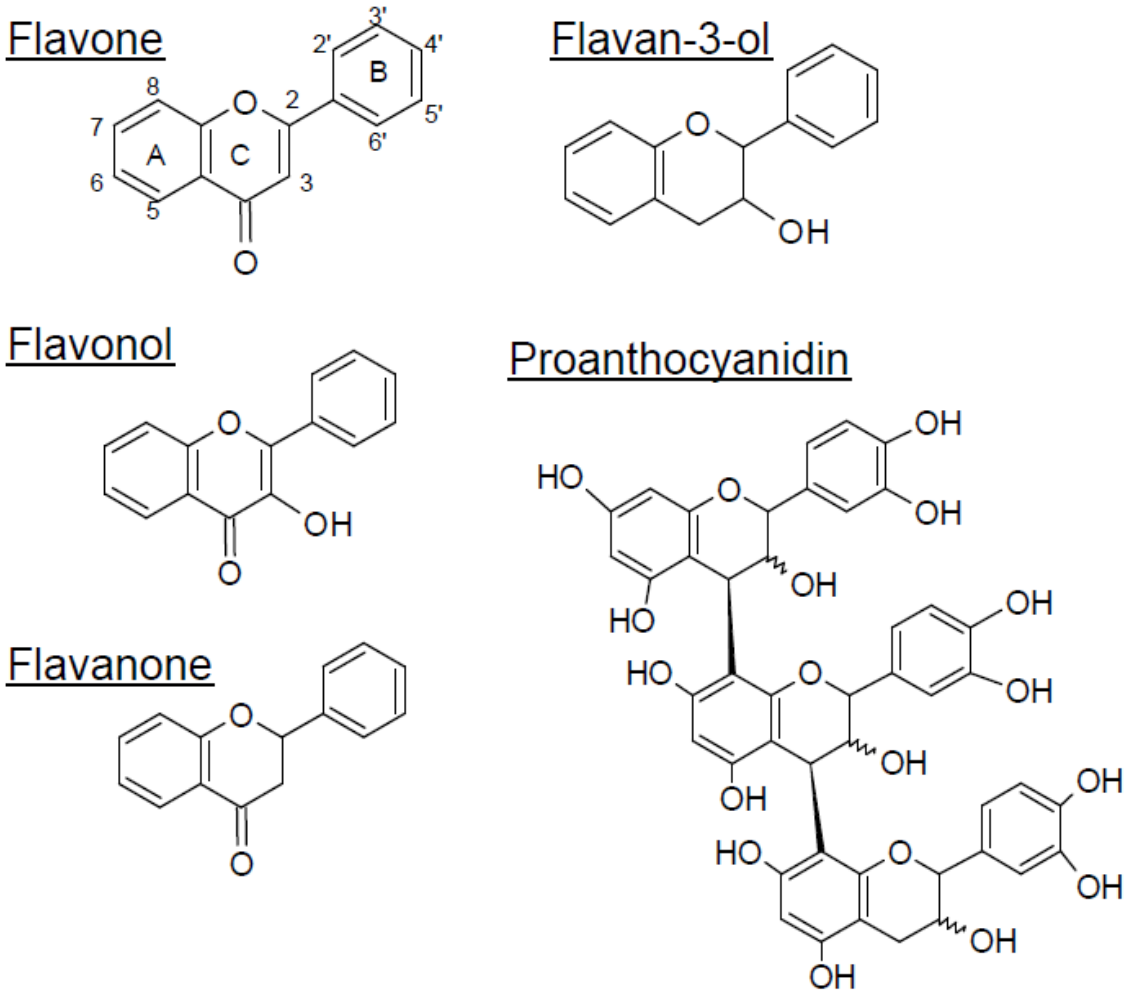
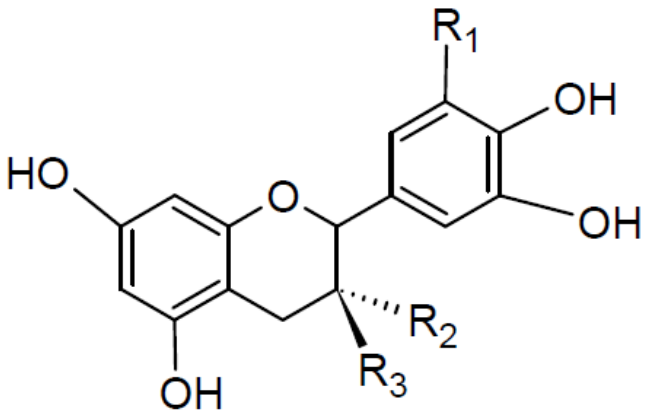


Figure 5.5. Flavonoid phenolic and proanthocyanidin (condensed tannin) structures.



Flavan-3-ol	R_1	R_2	R_3	
(+)-catechin	H	H	OH	Malt, Hops, Beer
(-)-epicatechin	H	OH	H	Hops, Beer
(-)-epigallocatechin	OH	OH	H	Beer
(-)-epicatechin-3-O-gallate	H	G*	H	Beer

Figure 5.6. Flavan-3-ol monomers found in malt, hops and beer. G* = gallate.

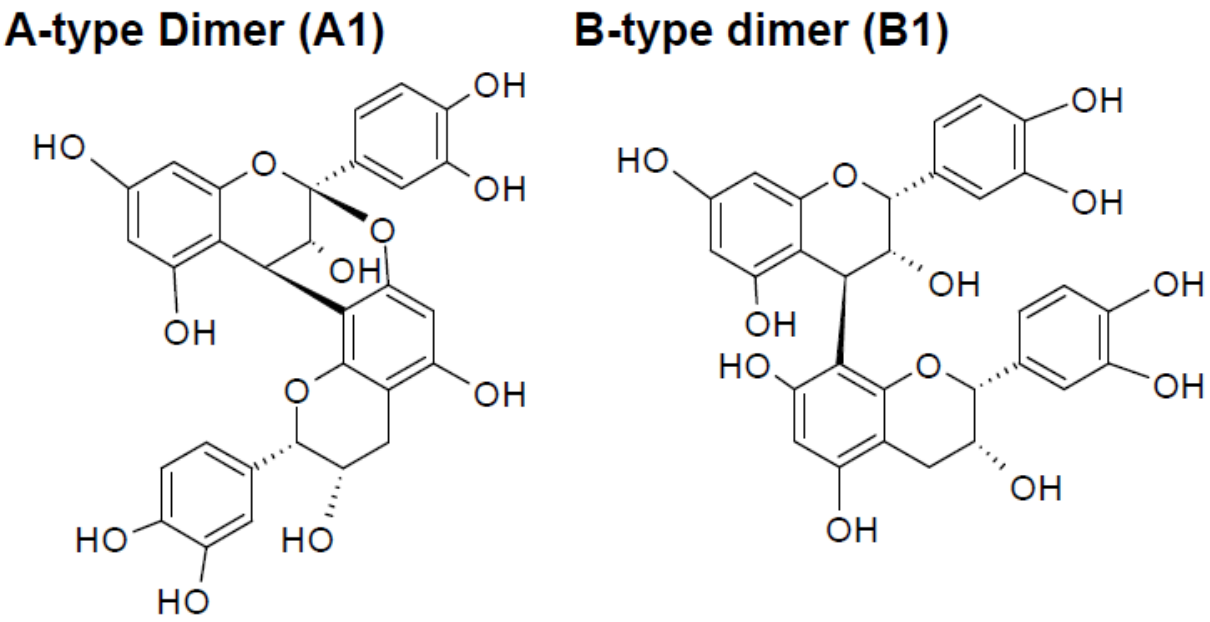


Figure 5.7. A-type and B-type proanthocyanidin dimers

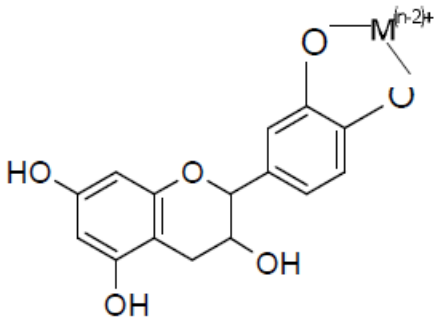


Figure 5.8. Flavanol 3’-4’ ortho-hydroxy chelation of metals.

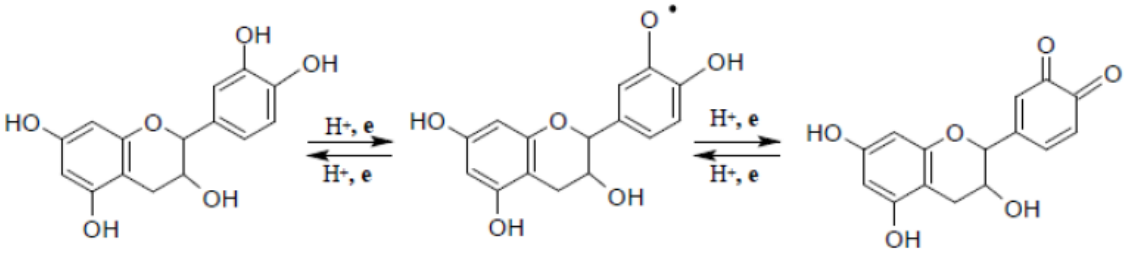


Figure 5.9. The oxidation of flavan-3-ols predominantly produces semiquinone radicals

Chapter 6

Identification of hop-derived antioxidative polyphenols – A note

Patricia M. Aron, Patrick L. Ting and Thomas H. Shellhammer

INTRODUCTION AND JUSTIFICATION

Hops (*Humulus Lupulus* L., Cannabinaceae) are an essential raw ingredient used in the brewing industry to bitter beer. Hops also provide beer with several other quality attributes such as microbial stability, foam stability, mouthfeel , color, flavor and flavor stability)[5, 87, 136, 149, 239, 281-283]. Some of these parameters are suspected to depend heavily on hop polyphenol contribution to finished beer. As a result, hop polyphenols have become the interest of many investigations focusing on beer stability. The results of these studies are conflicting: some indicate that polyphenols of the flavonoid family have a protective effect on beer flavor stability [93, 104, 110, 111, 284], and others imply that polyphenols either have no effect or negatively affect beer flavor stability [74, 91, 105, 106, 112].

The large number of different polyphenols found in hop plant materials complicates the identification of individual compounds, and hop solids – although having already been extracted and stripped of bittering resins by soft critical or super-critical CO_2 extraction- are no exception. Comparison of retention times and U.V. spectra of compounds in question to a known reference does not always provide adequate information to allow for unambiguous identification of individual compounds. However, HPLC-UV-DAD and MS/MS can assist in the partial structural elucidation and identification of polyphenols.

Research conducted at Oregon State University and at MillerCoors suggests that hop polyphenols have something of interest to offer the brewer in terms of flavor and flavor stability [8, 239, 283]. A polyphenol rich extract prepared from spent hop solid materials (*Humulus lupulus* L. cv Galena) was produced using Amberlite FPX adsorption resin. The extract was dosed at a rate of 100 ppm total polyphenols into lager beer and the beer was aged for eight weeks under cold and accelerated storage. The added polyphenols were found to improve the antioxidant capacity of a commercial lager beer as measured by antioxidant (FRAP and DPPH• •) and anti-radical (ESR) assays. Reverse-phase (C18)- HPLC-ESI-MS chromatography in conjunction with phloroglucinolysis revealed that the extract was nearly 99% phenolic in nature, with low levels of proanthocyanidins (2% by mass), traces of procyanidin monomers, B-type

dimers and a plethora of other compounds that are suspected to be xanthohumols, flavonols, flavanonols and their glycosylated counterparts. However it was unclear as to which of these compounds, if any alone or in synergy, were responsible for the improved anti-oxidative/anti-radical response elicited by the dosed extract.

Assessing an individual compound's, or even a class of compounds', anti-oxidative effect(s) on food systems or in living systems can be a complicated affair. Not all systems are alike and limitations of solubility and bioavailability further complicate matters. In this study the goal was to assess the potential for hop derived compounds to affect beer flavor stability. Therefore, our plan of attack involved combining several methodologies in hopes of determining which compounds found in the polyphenolic extract were responsible for improving lager beer flavor stability.

MATERIALS AND METHODS

Preparation of hop solids. A polyphenol isolate was produce via the methods published in Chapter 2. Briefly, 450 mL of a spent hop solid (*Humulus lupulus* L. cv Galena) aqueous extract was prepared by extracting 45.25 g of spent hop material in 1 L of water under simulated kettle boiling conditions. The aqueous extract was treated with EDTA under alkaline conditions (pH 7) to remove any contaminant copper from residual pesticides used in the hop fields. Amberlite^{TM} FPX66 resin was used to isolate polyphenols of interest and produce 300 mL of an ethanolic polyphenol rich extract was diluted with 50 mL of water, further concentrated by roto-evaporation (30^0 C), then freeze dried to yield 0.94 g of a light yellow fluffy powder (polyphenol isolate).

Preparation of hop solutions. To 5 mL of MQ water, 0.11 g of the polyphenol isolate was added and sonicated until solubilized. The entirety of the 5 mL aqueous solution was applied to a preconditioned (95% ethanol, followed by MQ water) C-18 solid phase extraction cartridge (60 mL, 10 g, Supelco, Bellefonte, PA). The compounds of interest were eluted with 60mL effluent in the order of solvent polarity or ethanol and water. Eight fractions were collected: 0%, 10%, 20%, 30%, 40%, 50%, 70% and 100% of 95% ethanol/water. The fractions were concentrated under roto-evaporation (30C) to a constant volume of 5 mL (hop solutions). The polyphenol isolate was added and extracted using C-18 separately for each fraction, i.e. a 5 mL aqueous solution was

prepared using the polyphenol isolate 8 times, applied to a new column each time and eluted with 60 mL of effluent ranging in polarity from 0-100% ethanol (95%) (polyphenol fractions).

Total polyphenols and total flavanoids were measured according to the EBC Analytica methods (9.1and 9.12) [114] using a Shimadzu PharmaSpec UV-1700 spectrophotometer, Shimadzu Corporation (Columbia, MD).

DPPH• radical bleaching assay. To a 10 mL test tube, 2 mL DPPH• stock reagent (2.9 mg/50 L Methanol) was added. 50 µL of hop solution was added, vortexed for 20 seconds, incubated for 10 minutes at room temperature and the absorbance was read at 518 nm. %DPPH• reduction = [(Absorbance 518 nm DPPH• – Absorbance of the test sample)/ Absorbance DPPH•] x 100%

HPLC/ESI-MS. The reversed-phase method consisted of two Chromolith RP-18e (100- 4.6 mm) columns connected in series with accompanying guard column (Chromolith RP-18e, 5-2.6mm) all purchased from EMD chemicals (Gibbstown, NJ). The procedure utilized a binary gradient of 1%v/v aqueous acetic acid (A) and acetonitrile containing 1%v/v acetic acid (B). Eluting peaks were monitored at 280 nm: 1.0mL/min; 5% B at 0 min, linear gradient from 5- 10% B, 0-10 min; 10- 30% B 10-20 min; 30-55% B, 20-40 min.; 55- 90%B 40-41min.; 90%B, 41-51 min. The column was washed with 5% B for 5 minutes prior to the next injection. For the ESI source, the following conditions were applied; negative mode, dry temperature 350°C; dry gas 10.0 L/min; nebulizer 50.0 psi, trap drive 47.5, skim 1 -38.3 volt, skim 2 -6.0 volt, octopole RF amplitude 120.0Vpp, capillary exit -113.0 volt, scan begin 50m/z, scan end 1800 m/z.

RESULTS

Analytical results are summarized in **Table 6.1** and depicted in **Figure 6.1**. As seen in **Figure 6.1** three fractions, 10%, 20% and 30% exhibited the greatest quenching effects as assessed by DPPH• radical quenching. Fractions of 10% and 20% ethanol were highest in total polyphenols and flavanoids.

Structural analysis of the polyphenolic components of fractions 2-8 was conducted by RP-C18 HPLC-ESI-MS (negative mode). Fraction 1 was not analyzed via HPLC-ESI-MS. The identification of the polyphenols in each fraction was complicated; hundreds of compounds were detected. Currently, we have proposed the identity of many compound, however many remain unidentified. Further analysis by MS/MS may assist to elucidate the identity of the compounds we were not able to propose identities for at this time. Structures of some of the known flavonoid polyphenols found in this study are presented in **Figure 6.2**.

Fraction 2 (10% ethanol) was high in total polyphenols and total flavanoids and also contributed the greatest antioxidant potential as measured by the DPPH• radical capacity assay. HPLC-ESI-MS results indicate that most of the components eluted between 0 – 10 minutes (retention time). A wide variety of compounds were found, which are tentatively characterized in Table 6.2. (+)-Catechin dimers and trimers, prodelphinidin dimers, hop bittering related compounds such as desoxy-alpha-acids, lupulone, and tetrahydrolupulone were tentatively identified.

Fraction 3 (20% ethanol) components eluted between 10 and 25 minutes retention time. This fraction was found to contain xanthohumol derivatives and humulinones (oxidized humulones).

Fraction 4 (30% ethanol) was found to contain several glucosides and rutinosides of quercetin and kaempferol which eluted between 15 and 25 minutes retention times. Fraction 4 was found to be low in total polyphenols, total flavanoids as measured by the spectrophotometric methods. However the DPPH• • capacity assay indicated that fraction 4 had substantial anti-oxidant capacity, equivalent to fractions 2 and 3.

Fraction 5 (40% ethanol) was lower in total polpyhenols and total flavanoids and also weak DPPH• antiradical capacity. Four peaks dominated fraction 5 which were characterized as quercetin-3-0-rutinoside, quercetin-3-O-glucoside, kaempferol-3-O-rutinoside, and kaempferol-3-O-glucoside.

Fractions 6, 7 and 8 consisted of low total polyphenols and total flavanoids and also had week DPPH• antiradical capacity. Multiple minor components eluted in these fractions : 25-40 minutes., 15-40 minutes, and 45-55 minutes respectively. These

fractions were found to contain dihydrocohumulone, oxidized-alpha-acids (cohumulinone and humulinone), humulone, colupulone, and xanthohumol. Interestingly, many unknowns found in fraction 7 show a pattern of $[M-1]^{-1}$ + CH_3COO-Na (82) adducts: m/z: 427.2, 509.2; 471.3, 553.3; 515.3, 597.3; 603.4, 685.4; 647.4, 729.3; 691.4, and 773.4.

CONCLUSION

While this work is still preliminary and many compounds in the extract remain unresolved, the findings shed some light as to which compounds elicit higher antioxidant responses via the DPPH• radical quenching assay. Fractions 2 and 3 scored equally as high in antioxidant potential (78% and 81% respectively) and were high in total polyphenols and flavanoids. Given the general conviction that polyphenols and flavanoids are strong antioxidants, this was not surprising. However, it is interesting that despite measuring low in total polyphenols and low in flavanoids, fraction 4 was classified as being equally as high in antioxidant potential (79%) by the DPPH• radical quenching assay. Moreover it is interesting that fractions 6, 7 and 8, which were found to be high in hop bittering acids and their derivatives, showed relatively weak DPPH• antiradical activity. This finding conflicts with results from past experiments that implied hop bittering acids contribute significant antioxidant/antiradical potential to lager beer [137]. It is our hope that continued investigation into the antioxidant nature of hop derived polyhenols in this manner will provide more information that will enable us to better understand hop polphenol roles in beer flavor stability.

Figures and Tables.

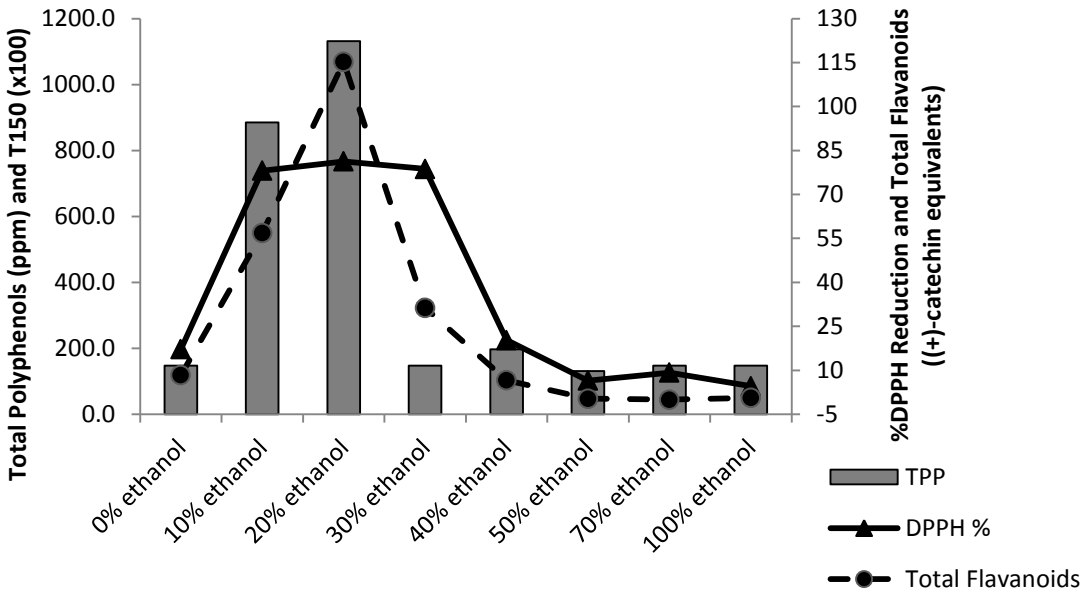


Figure 6.1. Fraction impact on total polyphenols, total flavanoids and DPPH• activity.

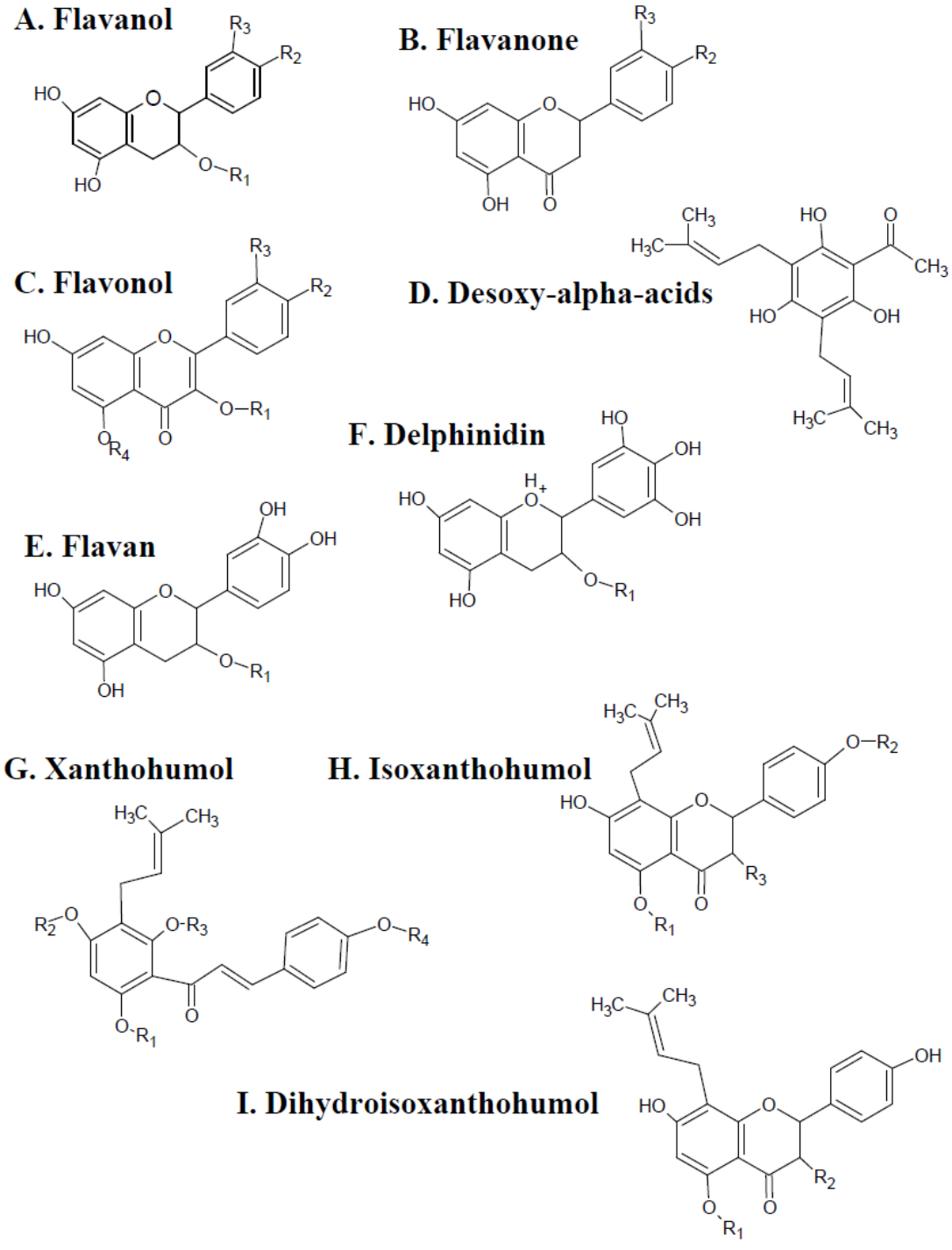


Figure 6.2. Structures of proposed compounds found in Table 6.2

Table 6.1. Polpyhenol fraction analytical data.

Fraction	Eluent (Ethanol/H2O)	Total Polyphenols ppm)	% DPPH Depression	Total Flavanoids (+)-catechin equivalents
1	0%	147.6	17.2%	8.4
2	10%	885.6	78.1%	56.9
3	20%	1131.6	81.3%	115.2
4	30%	147.6	78.7%	31.3
5	40%	196.8	20.3%	6.6
6	50%	131.2	6.5%	0.3
7	70%	147.6	9.2%	0.0
8	100%	147.6	4.6%	0.6

Name/Formula	$[M-H]^-$	Major Ions (m/z)	R1	R2	R3	R4
Fraction 2						
$C_{17}H_{16}O_6$ (A), (B), (C)	314.8	288.8, 271.8, 270.8, 110.8	H	OCH_3	OCH_3	
				OCH_3	OCH_3	
			H	OH	OCH_3	H
Desoxycohumulone (D)	330.9	242.8, 199.7, 167.7				
Desoxyhumulone (D)	344.8					
$C_{21}H_{14}O_9$ (E)	410.9	345.9, 344.8, 304.6, 290.7, 289.7, 288.7, 272.6, 260.7, 240.7, 180.8, 174.5, 164.7, 149.8	$C_6H_5O_2$ (dihydroxybenzene)			
Unknown	765.1	763.1, 737.1				
Unknown	564.9	476.7, 283.8, 282.9, 281.9, 149.8				
Unknown	345.9	327.9, 311.8, 210.7, 133.8				
Unknown	508.9	486.8, 294.9, 292.8, 259.7, 243.8, 242.8199.8, 109.9				
Unknown	827.1	664.9, 484.9, 325.0, 182.7				
Unknown	324.9	254.8, 211.8, 210.8, 166.8				
Unknown	327.9	269.9, 210.7, 133.8				
Unknown	422.9	345.9, 260.7, 210.7				
Unknown	647.0	370.9, 359.9, 326.9, 139.8				
Unknown	462.9	328.9, 213.8, 141.8				
Lupulone	412.9	338.0, 290.0, 280.9, 254.7, 161.8				
Unknown	395.0	380.0, 360.9,				

		179.8				
Unknown	430.9	395.9, 395.0, 371.9, 370.9, 208.7, 136.8				
Unknown	613.0	546.9, 413.0, 382.9, 381.9, 352.9, 205.8, 190.7, 115.9				
$C_{21}H_{20}O_{11}$ (C)	446.9	395.0, 323.0, 194.7, 151.8	glucoside	H		H
Unknown	352.9	190.7, 178.7, 134.7				
Catechin dimer (2E)	577.0	443.0, 336.9, 288.8, 190.8, 162.8	H			
tetrahydrolupulone	416.9	356.9, 354.8, 336.8, 322.9, 194.8, 162.8				
Unknown	336.9	321.0, 222.7, 208.8				
Catechin trimer (3E)	865.0	426.9, 409.1, 368.9, 288.8, 222.7, 204.7, 192.8				
Unknown	366.9	352.9, 192.8, 133.8				
Delphinidin dimer (F)	604.1	378.9, 192.8	delphinidin			
Unknown	409.2	351.2				
Unknown	379.2	367.0, 283.1				

Name/Formula	**$[M-H]^-$**	**Major Ions (m/z)**	**R1**	**R2**	**R3**	**R4**
Fraction 3						
Unknown	443.2	297.2, 245.1				
Methylated Xanthohumol (G)	395.0	360.9, 350.8, 313.1, 296.8	CH_3	CH_3	CH_3	CH_3
Unknown	427.0	374.9, 352.9, 178.7				
$C_{22}H_{18}O_8$ (E)	409.0	395.0, 350.9, 336.9, 284.9	$C_7H_5O_2$ hydroxybenzoate			
Unknown	425.0	378.9, 354.8, 307.8, 208.7, 190.7, 162.7				
*Iso*xanthohumol+	707.0	645.9, 353.9,	CH_3	H	I	

Dihydro*iso*xanthohumol (H+I)		352.9, 291.9, 190.7				
Xanthohumol (G)	352.9	266.8, 192.8, 178.8, 172.8, 134.8	CH_3	H	H	H
unknown	595.0	430.9, 400.9, 361.0, 268.9, 253.0, 192.8				
I	518.9	393.0, 356.9, 335.1, 307.9, 194.7, 192.7	glucoside			
Unknown	379.0	336.9, 288.8, 172.7				
Unknown	379.0	370.9, 300.9, 192.7, 176.8				
Unknown	395.2	381.2, 377.2, 361.2, 333.2, 311.1				
Unknown	578.9	393.0, 244.8, 202.7				
Unknown	693.0	670.9, 356.9, 290.9, 248.9, 194.8				
Unknown	381.0	363.0, 334.5, 280.8, 279.1, 262.7, 190.8				
Unknown	693.0	393.0, 357.9, 356.9, 290.9, 194.8				
Dihydrohumulone /cohumulinone	363.0	331.9				
Humulinone	377.0	364.1, 362.9, 236.8, 190.7				

Name/Formula	$[M-H]^-$	Major Ions (m/z)	R1	R2	R3	R4
Fraction 4						
Quercetin-5-O-glucoside-3-O-rutinoside (C)	771.3	427.2, 300.0, 299.0191.0	rutinoside	OH	OH	glucoside
Kaempferol-5-O-glucoside-3-O-rutinoside (C)	755.1	625.2, 463.1	rutinoside	OH	H	glucoside
E	611.3	479.1, 534.2, 480.1, 479.1431.1, 316.0, 166.9	rutinoside			
Unknown	739.1	667.2, 609.0, 394.0, 393.0, 334.0, 333.0, 263.9				
Quercetin-3-O-rutinoside (C)	609.0	597.0, 394.0, 333.0, 289.1, 254.0	rutinoside	OH	OH	H
Qucertin-3-O-rutinoside (C)	463.1	300.0	glucoside	OH	OH	H
Unknown	597.2	463.1, 300.0				
Unknown	715.0	693.0, 405.0, 357.9, 356.9, 194.8				
Kaempferol-3-O-(6”-O-malonylglucoside) (C)	533.0	464.8, 463.9, 462.9, 299.8	malonylglucoside	OH	H	H
Qucertin-3-O-(6”-O-malonylglucoside) (C)	548.9	506.9, 505.9, 504.9, 296.9	malonylglucoside	OH	OH	H
Kaempferol-3-O-rutinoside (C)	593.0	566.9, 394.0, 393.0, 362.9	rutinoside	OH	H	H
Kaempferol-3-O-(6”-O-oxalylglucoside) (C)	519.0	502.9, 392.7, 332.9, 286.9, 208.8	oxalylglucoside	OH	H	H
Unknown ($715^{+2}CH_2$)	743.1	620.9, 417.0, 373.0, 371.9, 370.9, 363.0, 209.8, 208.8				
Unknown	371.0	279.1, 210.0, 209.0, 165.0				
Unknown	393.2	379.2, 371.1, 363.4, 349.2, 335.2, 209.9, 209.1				
Humulinone	377.0	333.2, 223.0, 195.0				

Name/Formula	$[M-H]^-$	Major Ions (m/z)	R1	R2	R3	R4
Fraction 5						
Unknown	295.1	216.9				
Unknown	234.0	216.8, 162.9, 145.0				
8-prenylnarigenin (H)	339.2	265.0, 264.1, 250.0, 249.0, 216.9	H	H	H	
Kaempferol-3-O-isohexenoyl (C)	383.2	285.1, 216.9	isohexenoyl	OH	H	H
Unknown	427.2	395.1, 351.1, 337.2, 285.1				
Unknown	471.2	462.5, 380.2, 331.2, 216.8				
Unknown	381.2	363.2, 341.2, 321.1				
Unknown	515.3	457.2, 425.3, 395.1, 389.1, 379.2, 342.3				
Quercetin-3-O-rutinoside	609.0	573.3	rutinoside	OH	OH	H
Quercetin-3-O-glucoside (C)	463.1	301.0, 300.0	glucoside	OH	OH	H
Kaempferol-3-O-rutinoside (C)	593.0	533.2, 413.1, 285.0	rutinoside	OH	H	H
kaempferol-3-O-glucoside (C)	447.1	285.0, 284.0	glucoside	OH	H	H
Unknown	371.2	281.0, 265.0, 251.1, 243.0, 210.0, 209.0				
Unknown	381.2	364.2, 363.2, 306.1, 305.1, 275.2, 190.9				
Kaempferol-3-O-(6”-O-malonylglucoside) (C)	533.2	385.1, 384.1, 383.1, 312.1, 297.1280.1, 220.9	malonylglucoside	OH	H	H
Unknown	369.1	143.0				
Unknown	395.2	379.1, 378.4, 377.2, 319.1, 265.2				
Cohumulinone	363.2	249.0, 209.0, 141.0				
Unknown	317.2	248.0, 209.1, 205.0				
Humulinone	377.2	365.3, 293.1, 263.1, 223.0				
Colupulone	399.3	330.1, 305.0, 287.1, 141.0				

Name/Formula	$[M-H]^-$	Major Ions (m/z)	R1	R2	R3	R4
Fraction 6						
Unknown	327.2	239.0, 229.0, 211.0				
Unknown	603.3	489.3, 465.2				
Unknown	329.2	249.0, 229.0, 211.0				
Dihydrohumulone	363.2	294.1				
Cohumulinone	363.2	353.1, 345.2, 249.0, 209.0, 140.9				
Humulinone	377.2	309.1, 308.1,				
Unknown (377+H_2O)	395.2	331.1, 317.2, 263.0, 248.0				
Humulone	361.2	347.2, 297.2, 265.1, 263.0				
Unknown	365.3	321.2, 285.2, 284.3, 283.2				
Colupulone	399.3	330.2,				

Name/Formula	$[M-H]^-$	Major Ions (m/z)	R1	R2	R3	R4
Fraction 7						
8-prenylnarigenin (H)	339.2	261.8, 210.8	H	H	H	
Unknown	383.2	218.8				
Unknown	427.2	509.2, 275.0, 232.9				
Unknown	471.3	553.3, 283.1, 232.9, 166.8				
Unknown	515.3	597.3, 379.2,				
Unknown	559.3	641.3,				
Unknown	603.4	685.4, 574.6, 440.9, 411.2, 393.3, 269.2, 252.7				
Unknown	647.4	729.3, 550.0, 535.1, 232.9, 216.8				
Unknown	691.4	773.4, 675.2, 593.1, 561.1, 401.1, 396.2, 381.2, 232.9				
Dihydrohumulone/ cohumulinone	363.2	294.1, 201.9				
Dihydrohumulone/ cohumulinoe	363.2	249.1, 209.4				
Humulinone	377.2	309.1, 308.1, 248.9				
Xanthohumol	353.2	233.0				

Humulone	361.2	347.2, 297.2, 263.0				
Colupulone	399.3	330.2				

Name/Formula	**$[M-H]^-$**	**Major Ions (m/z)**	**R1**	**R2**	**R3**	**R4**
Fraction 8						
Unknown	367.0	232.8, 176.8				
Unknown (367+O)	383.0	348.9				
Unknown	397.1	277.9				
xanthohumol	353.1	299.0, 162.9				
Unknown	427.1	293.9				
Unknown	339.1	162.8				
Unknown (377+H2O)						
Humulone	361.2	347.2, 297.2, 265.1, 263.0				

Chapter 7

The future of hop chemistry research

The future of hop research remains bright. Although plentiful research has been conducted on elucidating the structures and roles of the hop bittering acids and reduced hop bittering acids on beer flavor and flavor stability, much less is known about hop polyphenols and their roles in beer flavor stability. Over the last couple of decades brewing scientists have begun to unravel the mysteries of hop polyphenols. However, there are still many stones to over-turn.

Firstly, the data on hop polyphenol content of hops by specific compounds or classes of compounds is lacking. Secondly, very few studies have investigated the fate of hop polyphenols during the brewing process. Relatively little is known about the extraction of polyphenols from hops into beer during processing and what chemical reactions polyphenols undergo throughout fermentation and finishing. There is some evidence to suggest that the glycosides of hop polyphenols have a lot to offer the brewer in terms of flavor potential. Several years ago researchers at Miller Brewing Company began looking into the effects of glycosides on beer flavor [285], however since then very little work has focused on hop glycosides or their impact on beer flavor. Recently Callemien and Collin reviewed [9] known phenolic compounds found in beer. The paper provides a great review of polyphenols in beer but what it lacks is data pertaining to quantification of specific compounds. To date most research done on hop polyphenols and beer polyphenols has focused on structural elucidation and compound identification. It is clear that there is a lot of work left to do in this area.

Projects that would naturally follow the work we have done over the past four years are suggested here:

1. Investigate the mechanisms of polyphenol metal chelation. In order to do this it is necessary to understand polyphenol affinity for metal speciation. Experiments could be done by dosing known phenolic compounds as well as fractions derived from hop material into model solutions that contain a known concentration of transition metals (suggestions: Fe(II), Fe (III), Cu(I) and Cu (II).

2. A follow up to this would be to look at the effects of dosing specific polyphenols or classes of polyphenols into model beer with and without transition metals that are known to interfere with ESR analysis, then subject the model solutions to ESR analysis. Questions of interest: Do polyphenols impact metal speciation in beer in a way that can decrease apparent flavor stability as measured by the ESR analysis? If polyphenols are actively chelating transition metals in solution, are they less available to behave as protective antioxidants?
3. Results of ESR analysis conflict with results from other antioxidant capacity analyses done in these studies. Interestingly investigators in Belgium and Germany have witnessed the same phenomenon, but no one quite understands why- or they are not willing to share what they do know about why. We theorize that some classes of compounds found in hops that are phenolic in nature interfere with the ESR analysis. It would be of interest to continue investigating this phenomenon.
4. In this study we monitored aldehyde formation and the results were somewhat mixed. It would be of interest to continue studying the effects of hopping technology on aldehyde formation in beer. Questions that arise include: Does hopping technology influence levels of amino acids (precursors to staling aldehydes) in the wort and finished beer? Are hop products a source of compounds that can either inhibit or increase rates of staling aldehyde formation in aging beer?
5. Glycosides- several studies indicate that brewing with spent hop material contributes hoppy aromas and pleasant character to beer, and that brewing with the material promotes balance and denotes body. The number of patents that have been granted or applied for pertaining to brewing with spent hop material and beer flavor is a bit alarming. Major breweries have trended toward the use of downstream products and extracts which are devoid of this material, yet they have researchers applying for patents that say brewing with what they've left out is good for beer flavor and flavor stability. The analytics in the case of ESR don't measure up, but sensory work seems to confirm that this effect is valid. It would

be interesting to conduct trials where beers are brewed with spent hop materials and fermented with different yeast strains to understand the effect of fermentation (glycoside release by yeast). Beers could also be brewed with spent hop material from different hop varieties and fermented with the same yeast strain.

6. Follow up work for that would be to look at the effect of using enzymes (enzymatic hydrolysis of the glycosidic linkages) on hop material and how that affects beer flavor potential.
7. Results of this work indicate that the phenolic material extracted from beer using Sephadex is very low in proanthocyanidin material- yet so many studies focus on (+)-catechin and other flavanoid monomers as key players in beer antioxidant capacity. It would be of interest to **a.** continue investigations to clarify what the other 98% of the phenolic material is on a mass basis and **b.** scan commercial beers for phenolic content and collect metrics on beer proanthocyanidin (including procyandin and prodelphinidin monomers) content.

The Effect of Hopping Technology on Lager Beer Flavor and Flavor Stability and the Impact of Polyphenols on Lager Beer Flavor and Physical Stability.

Conclusion

Beer was either dosed or brewed with hop or hop derived materials and investigated for the effect of hopping technology on beer flavor potential and beer flavor stability. When dosed into a commercial lager beer at 100 ppm a polyphenol rich hop extract prepared from Spent Hop material significantly impacted the flavor of the lager beers and also improved the flavor stability of the lager beer as measured by antioxidant and antiradical capacity assays. Analysis of the polyphenol rich extract indicated that although the material was phenolic in nature, it contained relatively low levels of proanthocyanidins and flavanoid mononers. Subsequent HPLC-ESI-MS analysis and fractionation with C18 resin indicated that several classes of compounds including flavonols, flavonol glycosides, xanthohumols, humulones and other hop bittering acid derivatives contributed to the phenolic character of extract.

Based on the positive assessment of dosed hop polyphenols on beer flavor stability, brewing trials using a spectrum of hopping products were conducted to target the effects of hop polyphenols and hop bittering acids on beer flavor potential and flavor stability. Sensorially beers that were hopped with polyphenol rich hop products (Spent Hop solids and Pellet Hops) were discerned as being different by panelists because they were characterized by Piney and Tropical fruit notes. Antioxidant assessment of the beers produced conflicting results. Beers with hop polyphenols scored high in antioxidant capacity as per the FRAP assay. However beers high in hop polyphenols were least flavor stable as measured by the ESR assay.

Preliminary findings from the first set of brewing trials indicated that beer polyphenol profiles change during aging. A subsequent brewing trial was conducted to produce beers with the same hop products in order to assess the effect of hopping technology on beer polyphenol profiles. The beers were found to be relatively low in proanthocyanidins and related monomers. Moreover, only the beers hopped with Spent Hop solids contained proanthocyanidins above levels seen in the Control, unhopped beers, suggesting that very few of the hop proanthocyanidins were either extracted into the wort or were able to survive through the brewing process. The second brewing trial produced the same conflicting results between polyphenol content and ESR anti-radical potential, indicating that ESR may not be the best analytical tool for assessing beer flavor stability. Beers from both brewing trials were investigated for aldehyde content. Total aldehyde levels increased due to aging in both trials, but it was difficult to determine the effect of hopping technology on aldehyde production in aging beers because results from the trials were inconsistent.

Further investigation is necessary in order to better ascertain the roles of specific polyphenols or classes of polyphenols in beer flavor stability.

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