AN ABSTRACT OF THE DISSERTATION OF

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Title: Factors that Influence the Aroma and Monoterpene Alcohol Profile of Hopped Beer

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Thomas H. Shellhammer

Hop aroma in beer is related to the unique compositional chemistry of the hops used in the brewing process. While the range of these compositions is quite diverse and primarily dependent on hop cultivar[1], other studies have also shown that cultivation, seasonality, harvesting[2], processing[3,4], and storage practices[5,6] contribute to differences in hop composition. However, it should be noted that the aroma and composition of fresh and processed hops[7] is different than the subsequent finished beer. This irreconcilable difference that exists between hops and the finished product has been a confounding variable for brewing scientists, in large part due to the complexity and diversity of the compounds that are transferred from hops to beer, but also due to an incomplete understanding of the interactions between these compounds and the aromas they elicit. Of the many compounds found in hops, those belonging to the class known as monoterpene alcohols have consistently been useful indicators of changes in hop aroma due to different brewing practices.

Notable differences exist between American and European hops in terms of the types of flavor they contribute to beer. Brewers tend to describe the former as contributing citrusy, fruity and in some instances floral aromas to beer, while the latter

are often described as contributing herbal, tobacco, woody, and spicy notes. Single-hop brewing trials were carried out using either American hops (Cascade, Chinook, Centennial, Citra, or Simcoe) or European hops (East Kent Goldings, Hallertau Mittlefrueh HHA or Saaz) to identify hop-derived volatiles that contribute to American hop aroma in beer. The eight resultant beers were evaluated using both sensory and instrumental analyses. The sensory analysis identified Centennial as having the highest piney and green hop aromas, while Citra and Simcoe were characterized as being very fruity, citrusy, and tropical (especially Citra). The Hallertau Mittlefrueh (HHA) beers were similar to the East Kent Goldings, and these two were more floral and rose-like than the Saaz sample with more melon character than the American cultivars. Volatile analysis of the beer samples was performed using a stir-bar sorptive extraction (SBSE) of the beer samples followed by quantification by gas chromatography mass spectrometry (GC-MS). In general, the beers brewed with the American hop varieties were higher in aroma and in monoterpene alcohols.

In addition to hop oil-derived aroma, previous studies have demonstrated that non-volatile hop-derived precursors, specifically glycosides, survive the boil process and can be hydrolyzed to release volatile aglycones capable of contributing to aroma. To investigate this, twelve single hopped pilot scale beers were brewed using pellet, supercritical extract, and spent hop fractions of Citra, Simcoe, Centennial, or Cascade cultivars in order to investigate the contribution of these different hop fractions to the aroma of kettle hopped beers. The spent hop treatments produced beers that had

noticeable, albeit low, hop aroma which suggest that the water-soluble components left behind in the spent hops may contribute to hop aroma. The intensity and nature of the hop aroma in the Spent treatments was hop variety. However, contributions of water soluble components from spent hops to hop aroma in beer was very subtle, especially compared to the pellet and extract treatments.

Aqueous extracts of the spent material from pilot scale supercritical CO_2 fluid extraction (SFE) of hop pellets were treated to investigate the impact of different hydrolysis treatments and on the aroma and volatile profile. Aroma profiles were evaluated using descriptive analysis by a trained panel. Volatiles arising from hydrolysis treatments of aqueous extracts of the spent materials were measured using SBSE and GC-MS. The intensity and nature of the hop aroma was treatment specific. Acidic hydrolysis of water soluble extracts produced the most intense *Overall* and *Pine* aroma. Differences in the aroma intensities due to the hydrolysis from the addition of different enzyme preparations were present but subtle. Aromas liberated by ale yeast produced different profiles than the lager yeast. All treatments showed increases in aglycone content and changes in aroma profile when treated with hydrolytic enzymes preparations.

However, fundamental studies that examine the extraction of glycosides during brewing and their subsequent hydrolysis by yeast have not been fully investigated. Furthermore, extraction of other hop-derived compounds into beer show a strong dependency on the hop cultivar being used and the point at which it is added. Therefore, the extent of glycoside extraction due to hopping regime, cultivar, and their

hydrolysis due to yeast β-glucosidase activity was investigated. The glycoside concentration of worts made with three different hopping regimes and three cultivars was measured. Additionally, β-glucosidase activities for 80 different yeast strains and their effect on aglycone concentration in wort was determined. Glycoside content was measured by the difference in volatile aglycone concentrations between samples treated with purified β-glucosidase and untreated samples. Aglycone concentration was measured by SPME GC-MS. Results showed that yeast have a wide range of abilities to hydrolyze glycosides with a maximum hydrolysis occurring after three days of fermentation regardless of yeast activity. Although it was shown that yeast are capable of glycoside hydrolysis, glycoside concentrations in wort are low and have small contributions to hop aroma. These results help explain the extent to which different brewing yeasts and hopping regimes contribute to hoppy beer aroma through the hydrolysis of non-volatile hop-derived compounds.

Finally, in order to investigate the effect of hopping regime on the monoterpene alcohol content and sensory attributes of beer, 6 single hop beers were made using different hop additions and evaluated by sensory and instrumental analysis. Beers were brewed while varying two factors: hop cultivar (Simcoe and HHA) and timing of hop addition (60 min. boil, 25 min. whirlpool, or 48-hour dry-hopping). Additionally, the impact of yeast strain on treatment was investigated. Each treatment was compared to an unhopped control using SBSE GC-MS and descriptive sensory analysis. Multivariate statistical analysis were used to described the between relationships between instrumental and sensory results. Whirlpool additions produced

beers with the highest concentrations of geraniol, linalool, and β-citronellol; beers brewed with highly aromatic Simcoe hops produced more intense and individually distinct aromas for each hopping regime compared to the HHA hopped beers. Conversely, beers brewed with HHA hops showed less intense aromas with less distinction between hopping regimes, except for the dry-hopped treatment, which was characterized by a more floral type aroma than the other HHA. This research shows that despite the popularity of dry-hopping as an aroma hopping method, whirlpool additions can also produce intensely aromatic beers.

Factors that Influence the Aroma and Monoterpene Alcohol Profile of Hopped Beer

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Daniel C. Sharp

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APPROVED:

_____

Major Professor, representing Food Science and Technology

_____

Head of the Department of Food Science and Technology

_____

Dean of the Graduate School

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

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Daniel C. Sharp, Author

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CONTRIBUTION OF AUTHORS

Dr. YanPing Qian assisted with analytical measurements using Stir-Bar-Sorptive-Extraction Gas Chromatography Mass Spectrometry for chapters 2, 3, 4 and 6. Gina Shellhammer assisted with data analysis and experimental design for chapter 6. Jeff Clawson assisted with sensory analysis and beer production for chapters 2 and 3. Jan Steensles provided preliminary yeast strain selection for chapter 6.

Dedicated to my girls, Amelia and Ada.

"It's personal freedom, not hundred dollar bills that lights the soul's cigar."
Tom Robbins, *B is for Beer*

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Chapter 1 - Introduction

THE HOP PLANT

The hop plant belongs to the genus *Humulus* of the Cannabaceae family and includes the species *H. japonicas, H. yannanensis, and H. lupulus*[8], the latter of which has been used as an ingredient in the production of beer since at least 1079[9], if not earlier. Of the three hop species, only *H. lupulus* contains components of value to brewing beer and, with the exception of its limited use in pharmacology[10] or as ornamentals, is almost exclusively cultivated for brewing purposes. As a dioecious perennial, the hop plant is a climbing bine capable of heights ranging from 2 – 6 meters on trellised structures and are grown primarily in temperate climates where a considerable amount of the growing season has greater than 13 hours of daylight and a steady supply of water. Since most hop plants require special growing conditions, their cultivation is generally limited to between the 35th and 55th parallels north and south of the equator[8]. While the bulk of hop cultivation occurs in the Pacific Northwest Region of the United States and in the Hallertau Region of Bavaria in southeast Germany[11], other growing regions, such as the U.K., Czech Republic, Australia, and New Zealand, also produce style-defining hop cultivars. A breakdown of the global hop acreage and production by country is shown in Table 1.

Table 1:Global hop acreage and production from 2015 year[11]. *=estimates. Discrepancies in totals due to rounding.

Country	Acreage (ha)	production (mt)
Europe (total)	29050	41748
Germany	17855	28337
Czech Republic	4622	4843
Poland	1444	2242
Slovenia	1406	1678
England	895	1357
Spain	543	1029
France	440	555
Romania	270	195
Austria	249	298
Belgium	143	208
Slovakia	137	94
Bulgaria	14	26
Portugal	12	23
Netherlands	4	3.2
Ukraine	380*	380*
Turkey	320	212
Russia	242	194*
Belarus	58	54
Switzerland	16	21
American (total)	18729	26728
USA	18478	36389
Argentina	146	220
Canada	105*	120*
Asia (total)	2461	6230
China	2320	5954
Japan	141	276
Africa total	395	769
South Africa	395	769
Australia/New Zealand (total)	877	1940
Australia	488	1201
New Zealand	389	740
World	51512	87415

The inflorescence of mature female hop plants, called strobiles or hop cones, contain glandular trichomes, often called lupulin glands, located at the base of bracteoles[12]. These lupulin glands contain the bulk of the components of interest to brewers, although other components of value are located within the vegetative material of the hop cone as well. A general summary of the chemical composition of dried hop cones is shown in Table 2. Of principle importance to brewers are the α-acids and the essential oil fraction. Alpha-acid content indicates the bittering potential for a given hop cultivar and in depth studies and reviews regarding the role and chemistry of hop-derived bitterness are available[13–15]. While the α-acids are indeed an important fraction of hops as they pertain to bitterness, the fractions associated with aroma will be the focus of the discussion herein.

Table 2: Typical composition of dried hop cones [16–18]

Principle Components	Concentration (%w/w)
Cellulose-lignins	40.0 - 50.0
Proteins	15.0
Alpha acids	0.6 - 24.0
Beta acids	2.0 - 13.2
Water	8.0 - 12.0
Minerals	8.0
Polyphenols and tannins	3.0 - 6.0
Lipids and fatty acids	1.0 - 5.0
Hop oil	0.5 – 4.8
Monosaccharides	2.0
Pectins	2.0
Amino acids	0.1

The essential oil fraction has been attributed as the primary source of hop-derived aroma in beer[19], however, it is likely that the compositional chemistry of hop essential oil is more important to the aroma profile than the total overall oil content[20]. The composition of essential oil is extremely complex; there are over 450 identified chemical compounds and suggestions that the total number of existing compounds exceeds 1000[21]. Furthermore, its composition is quite diverse and is different for each hop cultivar[1], although studies have also shown that cultivation, seasonality, location, harvesting[2], processing[3,4], and storage practices[5,6] contribute to differences in hop composition and overall quality. For these reasons, hop chemists have not been able to identify a single compound that either describes or indicates the aroma contributions of a given hop cultivar. That being said, researchers have been able to identify volatiles in hops that exist in sufficient quantities relative to their aroma thresholds and that are likely contributors to overall aroma[7,22,23]. However, many of the compounds found in hop oil exist in quantities well below sensory detection thresholds and therefore may not contribute to the aroma profile of hops, particularly after being selectively extracted and diluted into beer, unless in the presence of other compounds which augment their sensory detection.

Of the many classes of compounds found in hop oil, the majority belong to the class of terpenes or terpenoids[24]. Terpenes are a diverse class of lipids with more the 20,000 species[25] and make up the majority of hop oil, although not in its entirety. Much of the compositional chemistry of hop oil is well studied and in-depth reviews

are available[13,24,26,19,26]. The majority of aromatic compounds in hop oil are derived from a few key parent terpenes and it is thought that they are biosynthesized by the plant as a defense against insects[27], while the oxygen-containing terpenes, known as terpenoids, function as membrane constituents, photosynthetic pigments, electron transport carriers, growth substances, and plant hormones. Terpenes contain carbon atoms in multiples of 5 ranging from 10-40 carbon atoms and are composed of isoprene units (C_5H_8) formed through biosynthetic pathways within the plant[28,29]. While a single isoprene unit is the only hemiterpene, oxygen-containing hemiterpenes or hemiterpenoids, such as isovaleric acid and 3-methyl-2-buten-1-ol, are more a diverse class and can contribute to hop aroma[30]. Monoterpenes (C_{10}) are the product of two isoprene units and include α-pinene, β-pinene, β -myrcene, ρ-cymene, and limonene among others, while the monoterpenoids include linalool, geraniol, nerol and geranyl acetate. Similarly, sesquiterpenes and the oxygen-containing sesquiterpenoids are comprised of 3 isoprene units and include caryophyllene, E, β –farnesene, humulene, farnesol and humulene epoxides. Terpenes or terpenoids larger than C_{15} backbones are either not generally found in hop oil or are not considered to be volatile enough to contribute directly to aroma due to higher molecular weight. Although it is conceivable that they could degrade into more volatile products. Other classes of compounds found in hop oil include aldehydes, ketones, methyl esters, and sulfur compounds[31]. Of particular note is the impact of sulfur containing compounds such as 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) which are found in trace levels but also have very low

odor thresholds[32] and are commonly found in newer hop cultivars that exhibit intense grapefruit, tropical fruit, guava, and black-currant like aromas[33].

The distribution of essential oils in hop cones is not uniform and is dependent on the specific tissue. For example, β-myrcene is found exclusively in lupulin glands while the monoterpene, linalool, is found mainly in the floral tissue of the hop plant but only in trace amounts. The sesquiterpenes, humulene and caryophyllene, are not specific to lupulin and are found in almost identical ratios in lupulin, leaves and flowers[28]. Therefore, the amount of vegetative material relative to lupulin glands obtained during hop processing will affect the quality of the final product.

In addition to the essential oil fraction, non-volatile metabolites in hops have been implicated as precursors to aroma in beer. One example of these metabolites are glycosides, which are thought to exist in plants as a way to increase the water solubility and thus facilitate transport of otherwise polar compounds throughout the cell. Chemically speaking, glycosides represent a large class of compounds defined as having a sugar moiety linked at its anomeric carbon to another functional group[34]. Polysaccharides technically fall under this definition, although in practice the definition is often refined to only linkages between a sugar moiety (glycone) and a non-sugar moiety (aglycone). The glycosides are further classified by their glycone, the configuration (α or β) of the glycosidic linkage, and its aglycone. Within hops, the range of glycosides is quite diverse, although studies have shown that the majority contain β-D-glucose as a glycone linked to broader range of aglycones[35]. Due to their increased molecular weight and polarity relative to their respective aglycone,

glycosides are less volatile and more water soluble thus increasing their extraction from the vegetative matter into the aqueous wort matrix and able to survive heating during wort boiling. Upon their hydrolysis during fermentation or aging, it is possible that liberated aglycones may exist in sufficient quantities to contribute to hop aroma. The glycoside content and parameters affecting the extent of their hydrolysis is investigated in this study.

There are over 100 available commercial hop cultivars, each with its own set of agronomics for a given growing region and a unique chemical composition that in turn yields unique characteristics to beer. Historically speaking, beer styles reflected the characteristics of the hops grown in that region. However, development of cultivars with higher yields and better storage stability coupled with improved processing and storage capabilities have resulted in a global hop market. A summary

of the major hop cultivars in the U.S. as a percentage of total production is showed in

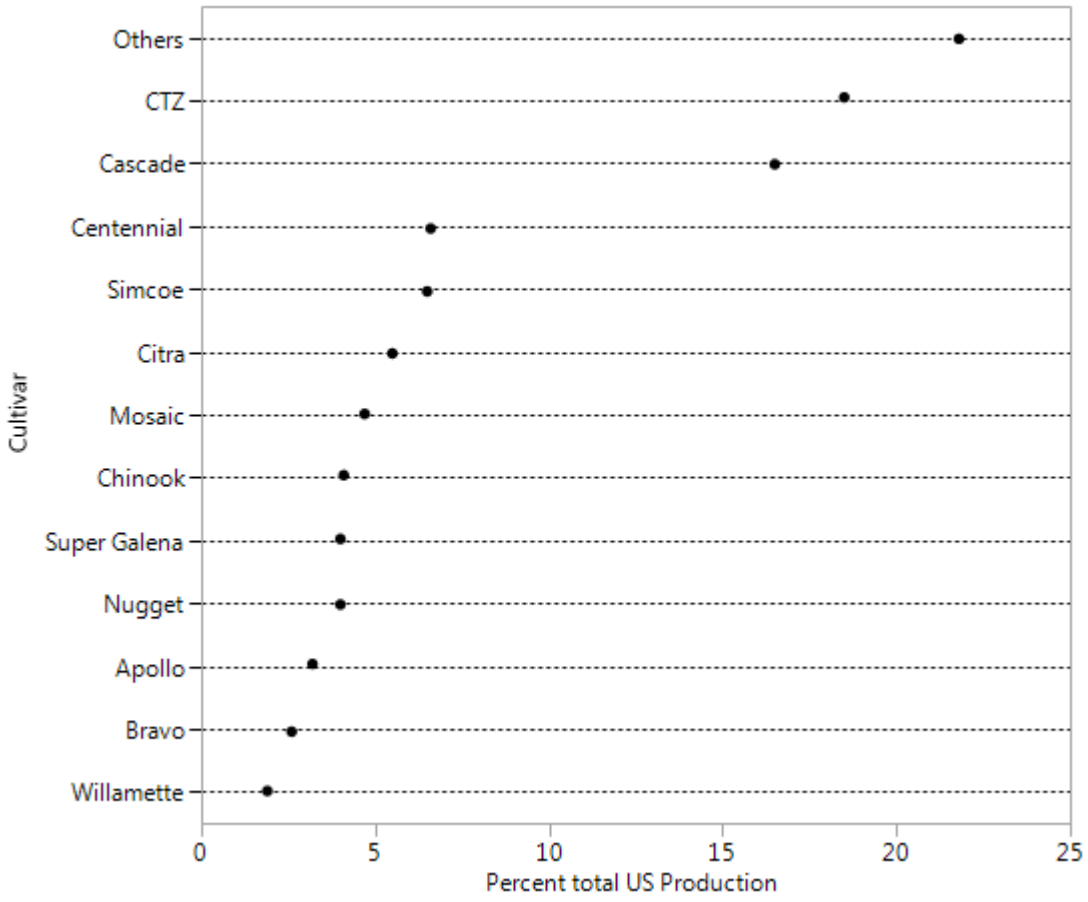

Figure 1. Since the chemistry of each hop cultivar is unique, the role of different hop cultivars in the volatile and aroma profile of beer was investigated in the work presented here, with efforts to select hop cultivars of industrial and historical relevance from diverse growing regions and lineages.

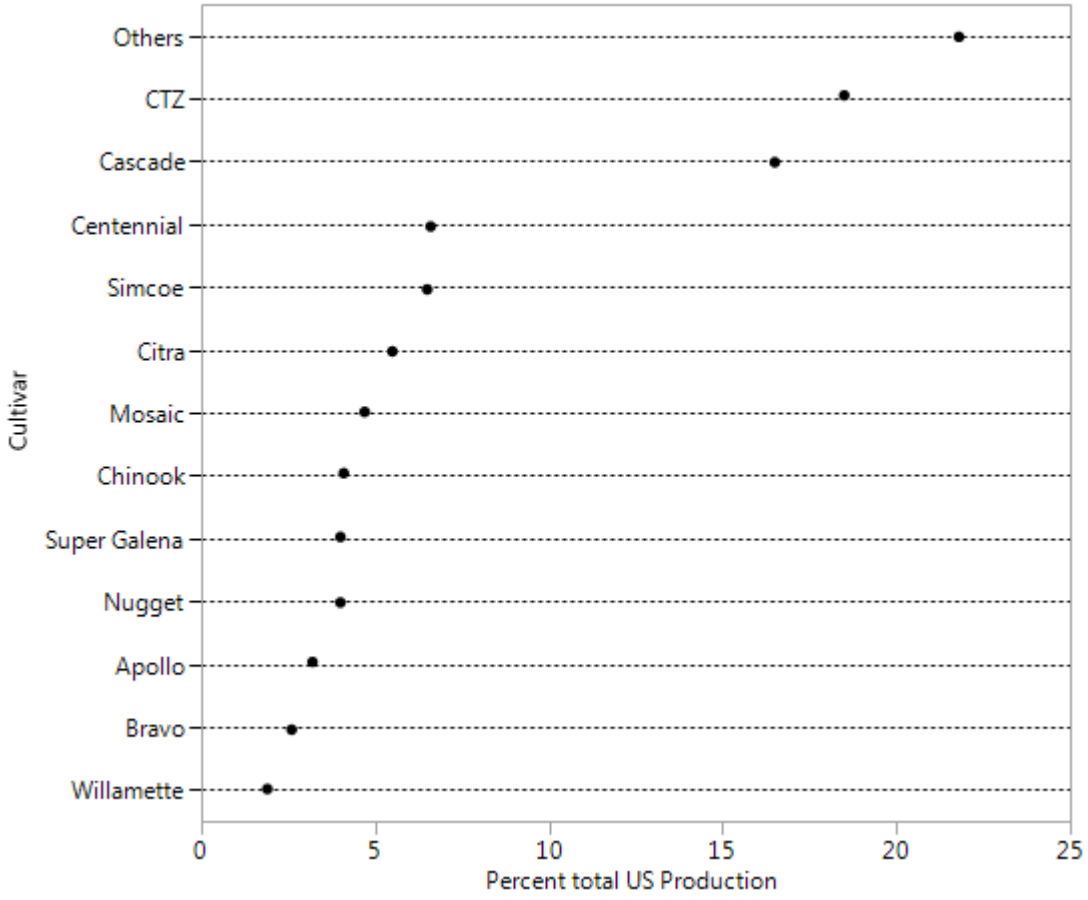

Figure 1: Major U.S. Hop Varieties expressed as percentage of total U.S. production of 36388.6 metric tons in 2015.[36]

Since hops are susceptible to a range of pests and diseases, hop breeders have focused intensively on improving disease resistance and overall plant vigor in new hop cultivars. However, in addition to breeding for healthy hops, breeders select for traits of agronomic and industrial relevance as well. For most of the 20th century, hop breeding efforts were focused on disease resistance and increasing α-acid yield. Most breeding efforts towards hop aroma were aimed at creating local substitutes of established cultivars from other growing regions[37]. While breeders often developed lines of hops with pleasant aromas, it wasn't until the recent paradigm shift of brewing styles, spurred by the renaissance of craft beer in the United States, that breeders began developing hops with novel and intense aroma profiles. However, it would be unfair to discredit the role of the hop breeder in transforming the status quo of hop aroma, as both the hop breeder and brewer both play a significant role. Nevertheless, hop acreage dedicated to aroma type hops has increased dramatically in the last 10 years[38], a trend that will likely continue but with an increasingly diverse composition of cultivars.

HOP PROCESSING

Except for the occasional seasonal practice of using undried fresh hop cones, most brewers add hop material derived from dried hop cones. Once harvested and picked, hops are dried immediately (~8-12% moisture w/w) and compressed into bales for longer term storage. After being baled, they can be used as-is or processed into a number of other hop products ranging from a pelletized version of hop cones to

purified and concentrated pre-isomerized α-acid extract. There are numerous resources that detail many of the possible products derived from hops[12,17,39]. However, in regard to the research presented here, three hop products are considered: Type 95 hop pellets, supercritical CO_2 extract (SFE), and spent hop material. Previous studies have focused on the impact of pelletizing conditions[40,41] and extraction conditions[17,42,43] on the quality of pellets and their extracts respectively.

While many brewers use whole cone hops, there are many advantages to using pelletized hops such as increase storage stability[40], handling, and extraction during brewing. The most common hop pellet used is the Type 95 hop pellet named for containing 95% of the original vegetative matter of the whole hop cone. In order to further increase these advantages, pellets can also be extracted using SFE. The remaining vegetative matter from the extraction process, called spent hops, contains ~25% w/w of water soluble substances which can be further extracted and concentrated to be used in brewing[44]. One of the principle goals of the research presented here is to investigate the role and extent to which this material is capable of contributing to hop aroma in beer in comparison to hop pellets and their extracts.

HOPS AND BREWING

Hops are primarily used in brewing to provide bitterness and aroma, in addition to mouthfeel and microbial stability, to finished beer. Depending on their point of addition during brewing, different process related phenomena can affect the utilization and extraction of different hop components and their subsequent contributions to beer. As such, the aroma of raw, dried hops often differs greatly from

the aroma they produce in beers. This phenomenon is primarily due to the extent of extraction, volatilization, and changes of hop-derived compounds during the brewing process. These effects differ greatly depending on when hops are added. Hops may be added anytime during the brewing process but are typically added sometime between the start of wort boiling and up to final beer filtration, although creative brewers have been known to add hops at every possible stage of a beer's production. Generally, hop additions are divided by process points during brewing: kettle additions (early or late), whirlpool hopping, hot wort or hop back hopping, and dry-hopping. It is generally accepted that as hops are added later in the brewing process the volatilization of hop-derived volatiles decreases, thus retaining more aroma. While this may be a useful guide, it falls woefully short of addressing the quality or nature of the diverse aromas hops can lend to beer.

Hops are often added early on in a kettle boil primarily to isomerize α-acids and provide bitterness to beer. As such, the amount of hops added to the kettle is dictated by the level of bitterness desired for a given style with common hopping rates ranging from 0.10 g/L to ~5g/L. Hops added at the beginning of wort boiling are used primarily for adding bitterness because of the greater extent of α-acid isomerization and hop oil volatilization. Nevertheless, despite the intense volatilization effects of boiling wort, a noticeable aroma persists in kettle hopped beers that makes them noticeably different from unhopped beers. Some studies suggest that sesquiterpene oxidation products may be formed during wort boiling and are responsible for subtle spicy aromas[45–48]. Hops may be added at any point throughout the boil to yield

different aromas although they are commonly added within the last 15 minutes of wort boiling (late hopping) when more intense aromas are desired..

At the end of a kettle boil, the hot wort is circulated within the vessel to create a whirlpool effect as a means to consolidate solids and precipitates. This provides the brewer with another opportunity to add hops to hot (not boiling) wort. The effect of this addition on hop aroma in beer was examined in this study. Although, the intent of most late-hopping and whirlpool-hopping additions are to increase aroma in beer, these additions are often calculated based on the α-acid, or bittering potential, of the hops rather than oil composition, in order to account for the bitterness contributions of those additions. In 1992, the hop aroma unit (HAU) was proposed[49] as a way to calculate hop dosing but it has yet to be adopted by brewers. Late hop and whirlpool dosages can often reach well above 5 grams of hops per liter of wort or beer. A slight modification of the whirlpool hop addition is the addition of hops to a vessel placed inline between the whirlpool and the wort chiller, known as a hop back, through which hot wort passes and extracts hop volatiles.

Hops may also be added once wort is cooled in a practice called dry-hopping. Dry-hopping can take place anytime during or after fermentation prior to clarification. Due to the lower temperatures of fermenting vessels (12-25°C) and conditioning vessels (1-15°C) relative to the kettle, hop-derived volatiles are less likely to be lost due to temperature effects. However, when hops are added prior to or during active fermentations, studies have shown significant losses of hop volatiles, likely due to the stripping effects of CO_2 production during fermentation, adsorption of hydrophobic

compounds to yeast cells, or partitioning into foam[50]. In addition to volatile losses due to fermentation, hop-derived compounds are also transformed by yeast[50,51], which may help explain the transfer rates in excess of 100% as observed by some researchers[52–54]. A thorough discussion of yeast and hop biotransformations is provided by Praet et al.[51] of hop-derived. A short summary of these biotransformation is shown below.

- Carbonyls reduced to hydroxyls[55]
- Ester hydrolysis and trans-esterification [56]
- Hop degradation products to fruity esters [57,58]
- Monoterpene alcohols are isomerized [50,59]
- Cysteine conjugates are transformed into thiols [60]
- Glycosidically bound aroma precursors are hydrolyzed[61]

One explanation of this increase in monoterpene alcohols may be due to the liberation of aglycones from glycosides either by acid or enzymatic hydrolysis[62] during fermentation or aging. Although acid hydrolysis in most beers (pH=4.2-4.7) would not likely occur rapidly, it may occur over the course of lagering or extended aging. This is particularly true for more acidic beers with pH < 4.0, which are also often aged for 6 months to many years. However, even in a study of aged wine (pH~3-3.4), monoterpene alcohol conjugated glycosides were still present after 2 years[63] suggesting that complete hydrolysis of glycosides due to acidic conditions is long process under normal beer storage conditions. However, yeasts have also been shown to exhibit hydrolase activity toward glycosides[64] with an optimal functionality at pH 4.5-5.2[65]. Enzymatic hydrolysis of glycosides is dependent on the specificity of a given enzyme for the substrate. The class of enzymes for the hydrolysis of β-D-

glucose linkages, known as β-glucosidases (E.C. 3.2.1.21)[66] displays different substrate specificity and tolerances to glucose inhibition depending on its source[67]. Nevertheless, yeast play a significant role in hop aroma in beer so long as hops are added prior to yeast removal.

In short, brewing process, raw ingredients, and fermentation heavily influence the hop aroma of finished beer. Fundamental studies focusing on these factors and how they relate to specific volatile and nonvolatile markers will help brewers better utilize hops in order to obtain the sensory characteristics they desire in beer and help guide hop breeders during new cultivar development. The research presented in the following chapters investigates these issues by focusing on the influence of hop cultivar, hop products, hopping regime, and yeast biotransformations on the analytical and sensory profiles of dry-hopped beer.

Chapter 2 - An exploratory study toward describing American hop aroma in beer

Daniel C. Sharp, Yanping Qian, Jeff Clawson, and Thomas H. Shellhammer

ABSTRACT

Notable differences exist between American and traditional European hops in terms of the types of flavor they contribute to beer. Brewers tend to describe the former as contributing citrusy, fruity and in some instances floral aromas, while the latter are often described as contributing herbal, tobacco, woody, and spicy notes. Single-hop brewing trials were carried out with Cascade, Chinook, Centennial, Citra, Simcoe, East Kent Goldings, Hallertau Mittlefrueh (HHA) and Saaz to identify hop-derived volatiles characteristic of American hop aroma in beer. The eight resultant beers were evaluated using both sensory and instrumental analyses. The sensory analysis identified Centennial as having the highest piney and green hop aromas, while Citra and Simcoe were characterized as being very fruity, citrusy, and tropical (especially Citra). The HHA was similar to the East Kent Goldings, and these two were more floral and rose-like than the Saaz sample with more melon and DMS than the American cultivars. Volatile analysis of the beer samples was performed using a stir-bar sorptive extraction (SBSE) of the beer samples followed by quantification by gas chromatography mass spectrometry (GC-MS). Principal components analysis of the instrumental data identified distinct differences between the citrusy American cultivars (Centennial, Chinook and Citra) and the non-citrusy European cultivars. Mapping the sensory data with the instrumental data via Generalized Procrustean

Analysis revealed interrelationships between the aromatic descriptors and the individual volatile compounds that were separated by the GC.

INTRODUCTION

Much of the aroma quality in beer contributed by hops (*Humulus lupulus)* can be attributed to the essential oil fraction produced in glandular trichomes, called lupulin glands, of hops. The composition of the hop essential oil found in the lupulin is extremely complex; over 450 chemical compounds have been identified, and research suggests the total number may exceed 1000[21]. For current in-depth reviews on the aroma chemistry of essential oil from hops and in beer see Sharpe and Laws[19], Schönberger and Kostelecky[24] and Briggs et al.[12] to name a few. Indeed, as suggested by the sheer number of possible chemical combinations due to the diversity of hop cultivars, it has been difficult for hop analysts to provide a short list of chemicals that can predict the aroma impact of hops in a finished beer. In addition, low sensory detection thresholds in the parts per trillion range, synergistic effects of compounds[68] and varying brewing techniques for imparting aroma can influence the composition of hop aroma in the finished beer which further confounds the complexity of hop aroma analysis. While it is true that extrinsic harvest and post-harvest conditions and handling of hops impact hop aroma[31,69], perhaps the biggest factor affecting hop aroma in beer, all else being equal, is the cultivar(s) used in beer production. Previous work by Peacock et. al[70] and results from an industry survey of brewing professionals (n=201) conducted in the Oregon State University (OSU) Brewing Laboratory[71] regarding opinions of how specific hop cultivars contribute to the flavor and aroma in

beer show a clear distinction between beers made with either American hop cultivars or European hop cultivars. It is the intention of the work presented here to investigate the differences between beers brewed with American and European hops using chemical and sensory analysis with the goal of advancing the understanding of what characterizes American hoppy beer aroma in relation to beers made with traditional European hop cultivars..

MATERIALS AND METHODS

Cultivar selection

Data from an industry survey of 201 brewing professionals' opinions of how specific hop cultivars contribute to the flavor and aroma in beer were used to select specific hop cultivars to include in a study of citrus/fruity aromas in hops[71]. This survey was aimed at understanding brewers' expectations about hop flavor in beer that originates from specific hop cultivars. A clear difference was observed among American and European hop cultivars whereby the top five American hop cultivars were rated as citrusy compared to three prominent European hop cultivars which brewers felt were not citrusy. The European hops were expected to deliver herbal, floral, spicy and woody aromas. Using input from brewing scientists working for commercial breweries in conjunction with the OSU hop survey, eight hop cultivars were selected for investigation in this study. Cascade, Chinook, Centennial, Citra (all four courtesy of John I. Haas, Yakima, WA) and Simcoe (courtesy of the Craft Brewers Alliance, Portland, OR) were selected based on their flavor profile and current demand by craft brewers seeking American hop aroma. Hallertau Mittlefrueh

and Czech Saaz (courtesy of John Barth & Sohn GmbH, Nürnberg, Germany) were selected as representatives of continental-noble hop cultivars while UK East Kent Goldings (courtesy of Boston Beer Company, Boston, MA) was chosen because of its mild aroma and its historic significance to the British hop pedigree. Hops (2009 harvest) were donated to OSU and stored at -20°C until brewing in 2010. Bittering acid content and total essential oil content for the hops used in this study are shown in Table 4.

Beer Production

Eight single hop beers were brewed in the OSU pilot brewery and hopped using a constant mass approach of three hop additions and fermented using ale yeast. Each single hop beer was brewed in the OSU pilot brewery using a grist comprised of 70% pale lager malt and 30% liquid adjunct (Clearbrew 60/44 IX, Cargill). Hop pellets were added to each 2 hL brew using a constant mass approach: 0.6 g/L at 5 minutes into a 60-minute boil, 1.13 g/L at 5 minutes before kettle knock out and 0.45 g/L in the hop back post whirlpool (2.18 g/L total). Dosage using a constant mass minimized the variation in hop aroma intensity from sample to sample rather than adjusting hopping levels based on alpha acids. Beers were fermented and conditioned at 18°C with an ale yeast (Wyeast 1056, Wyeast Laboratories, Wyeast, OR), and then ramped down to 1 °C over four days. Beers where then filtered, carbonated to 2.8 volumes CO_2 and packaged into brown 350 ml glass bottles. Finished, packaged beers were stored at 1 °C until analysis. The maximum iso-alpha acid concentration as measured by HPLC was 25 mg/L, and finished beers had approximately 5% ethanol

by volume. The eight resultant beers were evaluated using sensory and instrumental analyses.

Sensory Analysis

A quantitative descriptive analysis technique was used for describing and quantifying sensory attributes of single hopped beers. The sensory panel consisted of twelve trained panelists, many of whom had been extensively involved with previous sensory work regarding beer evaluations. Samples of beer (60 ml) were presented to the panelists in 300 ml glasses capped with clear- plastic, odorless lids. Samples were evaluated within two hours of serving and were evaluated at ambient temperature (20˚C). The final descriptive ballot was based on 18 descriptive terms for beer aroma with a focus on hop-derived aromas. The descriptive terms were developed during the training exercises and each term was accompanied by an aroma reference standard in beer to aid panelists in identification and agreement of aroma and descriptors. The descriptive ballot included (in order as they appeared on the ballot): Fruit Cocktail, Guava, Passion Fruit, Papaya, Banana, Melon, Grapefruit, Lemon, Estery, Green Apple, Rose, Floral, Green Hop, Piney, Onion/garlic, Soy Sauce, Buttery, DMS (Dimethyl Sulfide). All descriptors were rated on a 16-point intensity scale (0=none, 15=extreme intensity). Panelists trained six times over a two week period prior to data collection. On each day the panel came together, all 8 beers were presented individually to each panelist in a panelist-specific random order.

During testing, panelists evaluated 4 beers, took a brief rest and then evaluated another 4 beers. Each beer was evaluated 5 independent times on 5 separate

days/sessions. ANOVA and multiple comparisons by Tukey's Least Square mean was performed using XLStat 2009. Principle component analysis (PCA) of panelist data, averaged over all replications, was performed using the covariance matrix and a Varimax rotation. Sensory panel analysis was performed using the XLStat sensory package.

Instrumental analysis of single hop beer

Hop aroma compounds were analyzed using a stir bar sorptive extraction-gas chromatography-mass spectrometry (SBSE-GC-MS) method described previously[72–74]. A 10 ml beer sample was diluted with 10 ml of water in a 20 ml vial, in which 20 µL of octyl propionate internal standard solution was added. A stir bar (Twister) coated with poly(dimethylsiloxane) (PDMS) phase (1 cm length, 0.5 mm thickness, Gerstel Inc., Baltimore, MD) was used for extraction. The sample was extracted at room temperature with the stir bar for 3 h at a speed of 1000 rpm. After extraction, the stir bar was rinsed with distilled water, dried carefully with paper and placed into a sample holder for GC-MS analysis.

GC-MS analyses were performed using an Agilent 7890 gas chromatograph with a 5975 mass selective detector (Agilent, Santa Clara, CA). Samples were loaded into a thermal desorption unit (TDU) by a multi-purpose autosampler (Gerstel). A cooled injection system (CIS4, Gerstel) was used at the sample inlet. The TDU had an initial temperature of 25 ºC. After the sample was loaded, the TDU was heated at a rate of 300 ºC/min to a final temperature of 250 ºC and held for 2 min. The TDU inlet was in split-less mode during thermal desorption, while the CIS4 was in a solvent vent

mode with a venting flow of 50 ml/min for 4.0 min, at a venting pressure of 5 psi. After the solvent vent, the CIS4 was switched to split-less mode for 3.0 min, then changed to split mode with a venting flow of 50 ml/min. The initial temperature of the CIS4 was kept at -80 °C then ramped at a rate of 10 ºC/s to a final temperature of 250 ºC and held for 10 min.

Compounds were separated with a DB-WAX column (30 m length, 0.25 mm i. d., 0.5 μm film thickness, Phenomenex, Torrance, CA). The oven temperature was programmed at 40 °C for a 2 minute hold, then to 230 ºC at a rate of 4 ºC min^{-1} with a 5 minute hold. A constant helium column flow of 2.5 ml/min was used. A column splitter was used at the end of the column, 1 ml min^{-1} column flow was introduced to the MS, and the other 1.5 ml min^{-1} column flow was vented out. The MS transfer line and ion source temperature were 280 and 230 ºC, with an ionization voltage of 70 eV. Analytical standard solutions in 5% ethanol were used to build a standard curve using response factors from the selected mass ions. Triplicate analysis was performed and the average values were reported. Terpene and terpenoid concentration data were converted to flavor units by dividing the measured concentrations by sensory threshold estimates obtained from literature. Principle components analysis of the peak area data identified those compounds that correlated with differences among the eight hops. Similarly, Generalized Procrustean Analysis was used to visualize the instrumental data with the sensory data and thereby find correlations between specific volatile components and perceived aromas.

RESULTS

Sensory Analysis of Single Hop Beers

Panel Analysis

Panelist analysis was performed using mixed model analysis of variance. Panelists performance scores were based on their ability to repeatedly score treatments, the number of attributes they were able to use to discriminate between treatments, and lack of contribution to interaction effects (panelist by treatment or panelist by rep). Of the 12 panelists three were removed from subsequent analysis due to poor performance scores (data not shown). Results from mixed model analysis of variance post-panelist removal is shown in Table 3.

The sensory analysis identified large differences in the aromatic profile and intensities among the eight different cultivars (Figure 2). ANOVA of sensory results for all sensory descriptors across all treatments showed no significant difference between treatments in the mean sensory scores for Fruit Cocktail, Melon, Estery, and Soy Sauce descriptors (Table 5). In general, the American cultivars, with the exception of Chinook, were more intensely aromatic as compared to the European cultivars, with Citra, Simcoe and Centennial hops rated higher in Grapefruit, Passion Fruit, Piney and Green hop (Table 5). One should note the presence of buttery and banana descriptors and the unexpectedly low levels of piney and grapefruit descriptors in the Cascade sample. Cascade had a significantly higher mean score for Buttery than all the treatments, which may indicate insufficient diacetyl reduction. This sample may not be representative of the characteristic Cascade aroma and as such was viewed as an

outlier. Consequently, it was removed from subsequent sensory multivariate statistical analyses.

Mean sensory scores of each attribute for all treatments were used to cluster groups by Agglomerative Hierarchical Clustering analysis (AHC) based on dissimilarity of the treatments (Figure 3). PCA of the sensory data showed interrelationships among the sensory descriptors and the hop cultivars (Figure 4 and Figure 5). The first dimension (accounting for 51% of the variation) was anchored in the positive direction by Piney and Green hop descriptors and in the negative direction by DMS and Melon terms. The second dimension (accounting for 23% of the variation) was anchored in the positive direction by guava. The y-axis serves as a separation between the American (positive dimension) and European (negative dimension) hop cultivars. Centennial (sitting in the lower right quadrant of Figure 4) had the highest Piney and Green hop scores but was low in the fruity and tropical fruit aromas. Citra was located in the upper right quadrant of the same figure and was characterized as being very fruity, citrusy, and tropical. Simcoe was positioned along D3, which was anchored by Onion/Garlic. Interestingly, Chinook sat near the middle of the PC space with notes of Floral, Rose and Green apple. The HHA was similar to the East Kent Goldings, and these two were more floral and rose-like than the Saaz sample and more melon and noticeable levels of DMS than beers made with the American cultivars. It should be noted that DMS is not typically associated with hop character is more likely a derived from malt derived constituents[39].

Instrumental Analysis

The GC-MS analysis resulted in the detection of a broad range of over 300 peaks. Some of the initial peak identifications were esters, which were likely fermentation related and not hop related. Concentrations of these esters are shown in Linalool, myrcene, α-pinene, β-pinene, limonene, trans-β-caryophyllene, and α-terpineol were identified as hop-derived aroma compounds in the single hopped beers. Relative contributions of a compound to a particular matrix can be gauged by their flavor units, which is a function of the measured concentration of a compound in the analytical matrix divided by the accepted sensory threshold in the same matrix. Flavor unit results for the hop-derived compounds investigated here are shown in Table 6. It should be noted that flavor units are often disputed due to the variability of sensory detection threshold values reported in the literature and therefore one should avoid placing too much emphasis on them. Furthermore, the character and intensity of a given compound often changes when in the presence of other compounds[75]. Nevertheless, flavor units provide a helpful indicator of the approximate contributions of a compound to aroma.

The principal components analysis of the GC data yielded distinct separations of the hop cultivars (Figure 7 and Figure 8). Centennial and Simcoe anchored the positive and negative ends of Dimension 1 respectively, while Citra and Saaz anchored the positive and negative ends of Dimension 2, respectively. Citra also anchored the positive end of Dimension 3. In much the same manner as in the sensory PCA, the American hop cultivars were positioned on right hand side of the biplot and

the European cultivars on the left hand side. The use of Generalized Procrustean Analysis (GPA) as outlined by Noble and Ebeler[76] allowed for the comparison of the GC and sensory data and how they relate to different hop cultivars (Figure 9).

DISCUSSION

The sensory analysis data identified prominent differences among the 8 different hop cultivars. The 8 treatments were clustered in 4 groups (Figure 3) using AHC. When compared to genetic groupings[77] treatments reflected the differences between hops of European descent (Hallertau Mittlefrueh, Saaz, EK Goldings) and American descent (Simcoe, Citra, Chinook, Centennial), with the exception of Chinook being grouped with hops of European descent. In particular, Citra and Simcoe were clustered together, and while their full pedigree is not known they are of a newer breed of post millennium North American hops compared to Cascade, Centennial and Chinook. When averaged across all descriptive terms, Citra and Centennial were rated the highest in aroma while Saaz and East Kent Goldings were rated the lowest (Figure 2). The American cultivars had the highest scores for grapefruit, passion fruit, piney, and green hop whereas the European cultivars were rated low in all of these categories. Within the American cultivars there exists a range of aromatic differences. Centennial had the highest piney and green hop aromas, but was low in the fruity and tropical fruit aromas. Citra and Simcoe were characterized as being very fruity, citrusy, and tropical (especially Citra), while Chinook had notes of floral, rose and green apple. In contrast, the European cultivars were rated higher than the American counterparts in terms of melon and DMS. The HHA had the highest

floral and melon aromas of all the hops. While Saaz and East Kent Goldings had the highest DMS scores of the set, they were often rated low in aromatic intensity across nearly all of the attributes. Lack of any appreciable variation in the esters and the higher alcohol phenylethyl alcohol, with the possible exception of Simcoe, indicates that the fermentations were consistent across the 8 hop treatments (Figure 6). Furthermore, all of these compounds were detected well below sensory detection threshold levels. The Estery descriptor was not significant for any of the treatments. It is interesting to note that phenylethyl alcohol has a rose-like aroma[78], and despite the only subtle variation in this compound across the hop treatments, there were differences in the level of the rose descriptor that was hop cultivar dependent (Table 5). This may be due to other hop-derived compounds that have floral like aromas such as β-citronellol or geraniol[79].

When mean hop scores are plotted against a total oil content (Figure 10) most do not correlate with increasing hop aroma with the exception of Guava (R^2=0.8), Passionfruit(R^2=0.6), and Grapefruit (R^2=0.76) suggesting that these descriptors are correlated with increasing oil content. This is not to say that more oil means more aroma, only that for the cultivars and hopping regimes used in this study, increased oil content was associated with increased higher Grapefruit, Guava, and Passion Fruit aromas. Additionally, the high oil contents of Citra and Simcoe hops may be introducing significant leveraging effects on the correlation coefficients.

Principal components analysis of the GC data yielded distinct separations between the American cultivars Centennial, Chinook, Citra, and the European

cultivars such as HHA thereby identifying a clear separation between the citrusy hops and the European (non-citrusy) hop.

The PCA procedure is both a data reduction procedure and a means of studying interrelationships within a complex data set. In terms of data reduction, we used it to find those factors (compounds and their flavor units, in this case) that have the greatest influence on differences in the sample treatments (aromatic components from beer made from individual hop cultivars). For instance, α-pinene and limonene are aligned closely with Chinook and opposite HHA. We can infer that compounds associated with these hops may be distinguishing features of Chinook hop aroma in beer and relatively less so for HHA. The close proximity of these peaks in the PCA space indicates they were highly correlated with each other in this study. Finally, since these compounds lie on Dimension 1, which describes the greatest amount of variation in the data set, these compounds may be very important for assessing differences among the set of eight hop cultivars.

Mapping the sensory data to the instrumental data via Generalized Procrustean Analysis revealed interrelationships between the aromatic descriptors and the individual volatile compounds that were separated by the GC. Using the GPA, we can see that Citra, and to a lesser extent Simcoe, are highly correlated with guava, fruit cocktail, and onion/garlic notes and that these aromas are associated with α-pinene, β-pinene, and limonene. The clear separation of Citra and Simcoe from the other hop cultivars reflects the strong pungent aromas characteristic of Citra and Simcoe hops. These descriptors are likely derived from very odor active thiols found in many

cultivars of North American descent[32,33]. Centennial, and to a lesser extent Chinook, were correlated with more rose and green apple like aromas that could not be related to the aroma compounds studied here. A clear separation in the PC space is shown between the European hops and the modern American hop cultivars Citra and Simcoe Cascade was characterized by mostly buttery, banana, and melon notes. These aromas, especially buttery and banana, are associated with common beer defects, diacetyl and isoamyl acetate respectively. These defects indicate possible problems during or after fermentation and therefore this particular hop treatment was viewed as an outlier in terms of the sensory analyses. However, this is surprising since Cascade did not have higher ester concentrations than the other treatments, specifically isoamyl acetate, which would indicate inconsistent fermentations. Also, Cascade hops are well known for having a very citrus and floral like quality, yet these attributes were not described in the study. This demonstrates that despite the moderate hopping rates used in this study, strong non-hop-derived aromas can overwhelm and mask hop aroma in beer. Also, previous research has shown that a number of other factors, such as harvest timing and location[2,80], processing[3,41], and storage[40] can influence the aroma characteristics for a given sample of hops. Hops used in the study presented here were from a single lot of hops and were not controlled for the factors mentioned above and it is possible that within cultivar variation of hop quality may be a factor in determining the hop aroma in beer. As such, further investigations into the within-cultivar variation effects on hop aroma are warranted.

In this study, hops were added at three different points throughout the brewing process in order to mimic common industry practices. However, this eliminated the possibility to investigate changes in hop aroma due to different hopping regimes. Future studies would benefit from single hop additions and allow for the investigation of differences in hop aroma due to cultivar and addition effects. Furthermore, one should bear in mind that even though the specific hop compounds identified in this study are associated with certain aromas and cultivars, they may not necessarily be responsible for those aromas. Considering the large number of volatile compounds found in hops and beer, it is likely that the compounds examined do not fully explain the sensory differences. For example, sulfur containing hop compounds have been associated in previous studies as contributors to American hop aroma[81], particularly to the guava and tropical aromas in hops such as Simcoe, Citra and Cascade. Although these compounds have been found in trace levels, their contribution to aroma is substantial due to their extremely low flavor thresholds. The compounds investigated here were those primarily reported by hop suppliers on hop specification sheets: Myrcene, Linalool, Humulene, and Caryophyllene. However, the purpose of the specifications are primarily for cultivar identification purposes and not for recipe formulation or to describe the flavor and aroma profile of a given hop. With the popularity of aroma hops increasing, it would be helpful for hop suppliers to provide a more detailed analysis of hop volatiles to aid the brewer in recipe formulation. Nevertheless, while only a handful of compounds were quantified in this study of the

hundreds of volatile compounds found in beer, clear differences between the aromas produced by the American and European hop cultivars were found.

ACKNOWLEDGEMENTS

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Table 3: F-values from mixed model analysis of variance of descriptive attributes. **Bold =** significant at $p<0.05$.

Source	DF	Fruit Cocktail	Guava	Passion Fruit	Papaya	Banana	Melon	Grapefruit	Lemon	Estery	Green Apple	Rose	Floral	Green Hop	Piney	Onion/ Garlic	Soy Sauce	Buttery	DMS
Products	7	1.5	**4.5**	**4.8**	2.2	**2.8**	1.0	**4.8**	1.7	0.7	2.0	1.5	**2.3**	**5.0**	**5.4**	**4.3**	0.7	**8.7**	**7.3**
Assessors	8	**17.2**	**2.9**	**4.9**	1.3	**3.9**	**3.5**	**4.5**	**7.6**	**9.2**	**15.6**	**2.7**	**7.6**	**6.3**	**2.3**	2.0	**7.0**	**6.3**	**5.5**
Sessions	4	1.3	1.2	1.3	0.6	2.8	1.3	0.6	0.6	2.1	2.8	0.6	0.5	0.8	0.1	0.5	0.2	0.7	2.4
Products*Assessors	56	**1.8**	**2.3**	**1.6**	**1.5**	**1.8**	1.0	**2.2**	**1.6**	**2.9**	1.2	**1.9**	**2.2**	**2.1**	**2.0**	**2.8**	1.6	**2.7**	1.3
Products*Sessions	28	0.9	1.5	1.0	0.9	0.9	1.2	1.3	1.1	0.9	1.1	1.0	0.8	1.0	1.3	1.4	1.2	0.9	0.8
Assessors*Sessions	32	1.0	0.7	1.1	2.9	0.8	0.7	1.3	0.9	1.2	0.6	**1.9**	0.9	0.9	**1.6**	1.2	1.2	1.0	0.9

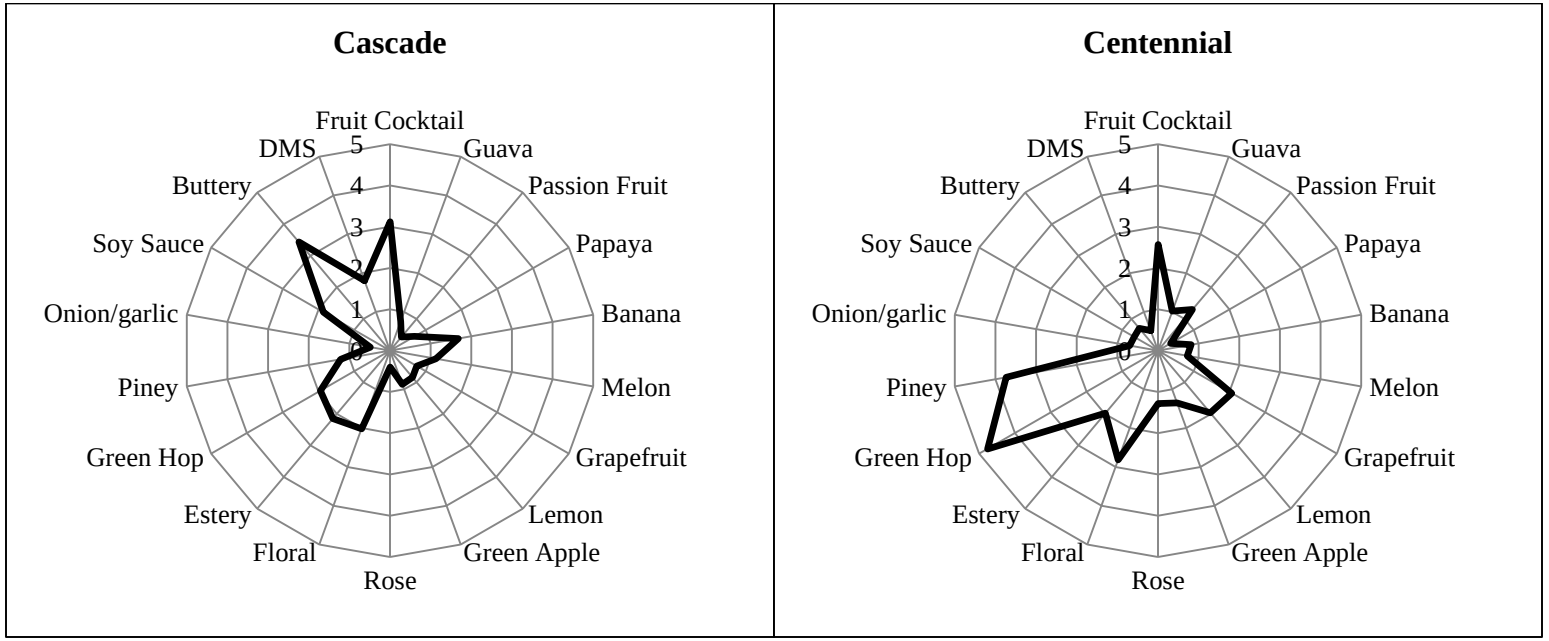
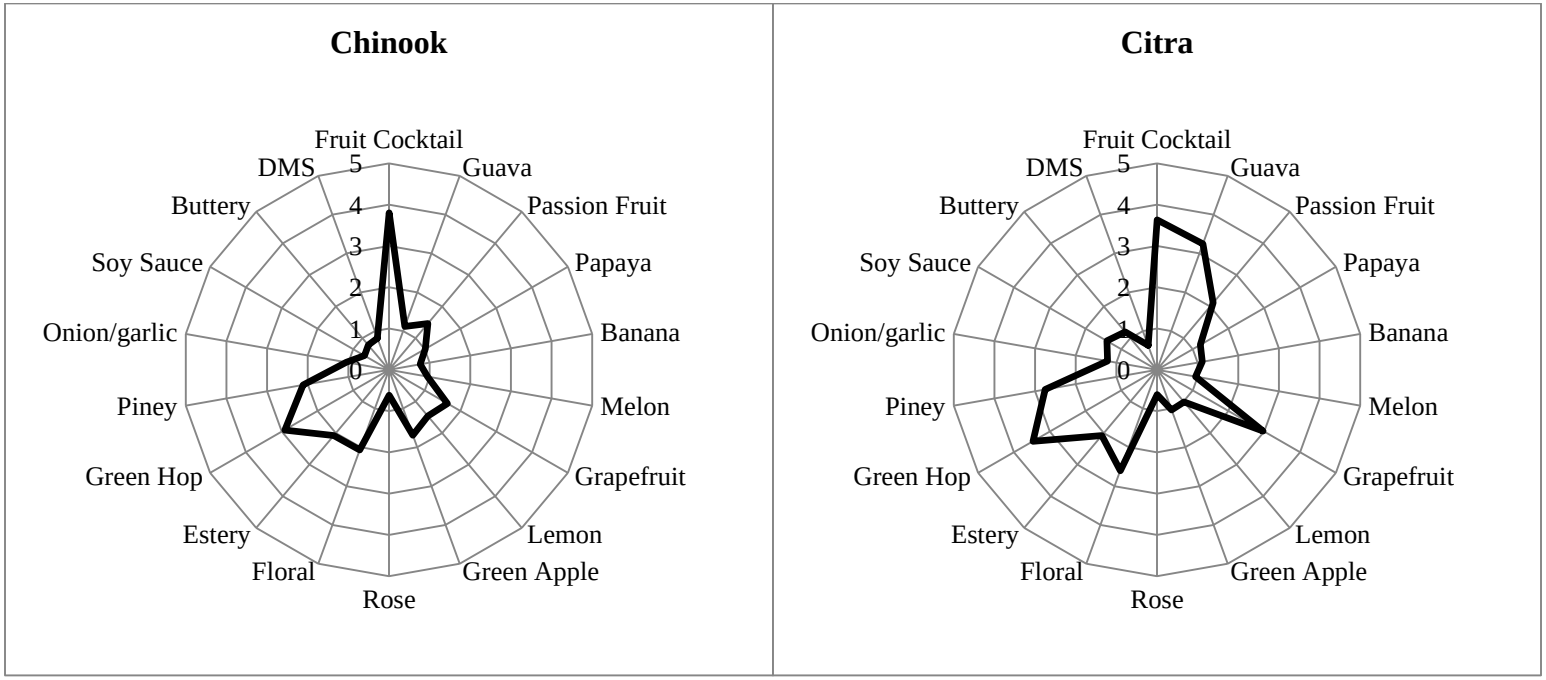


Figure 2: Spider diagrams of aromatic descriptors for each of the single hop beers. Scale = 0-16 (0-5 shown for detail).



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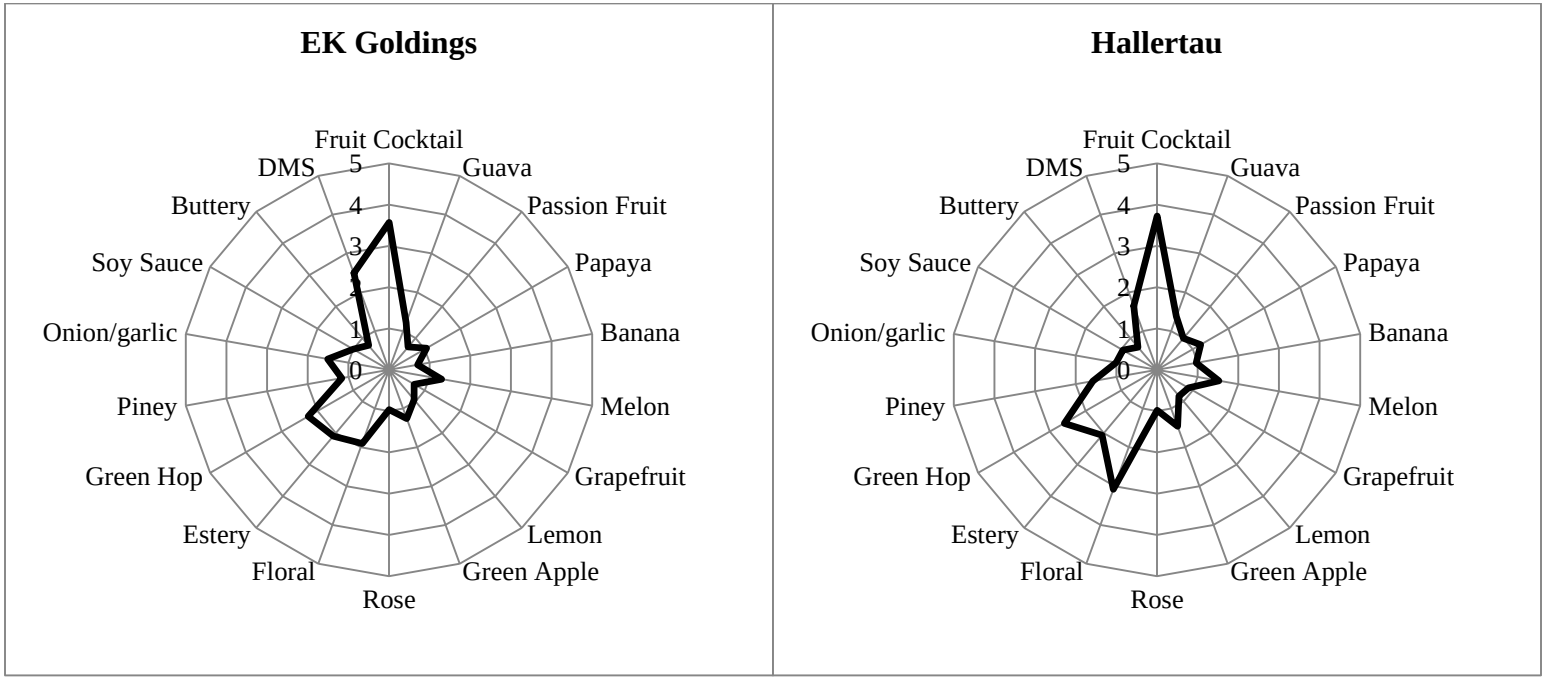

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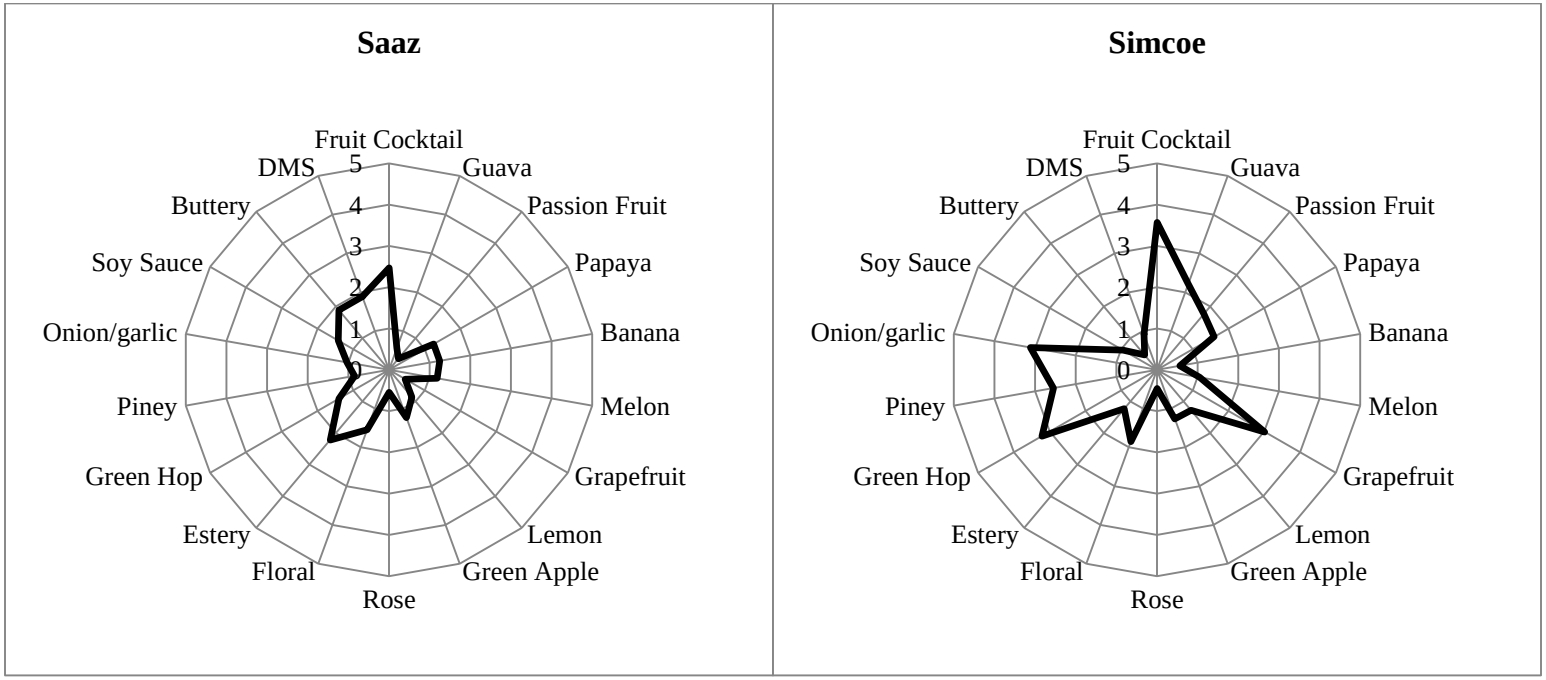

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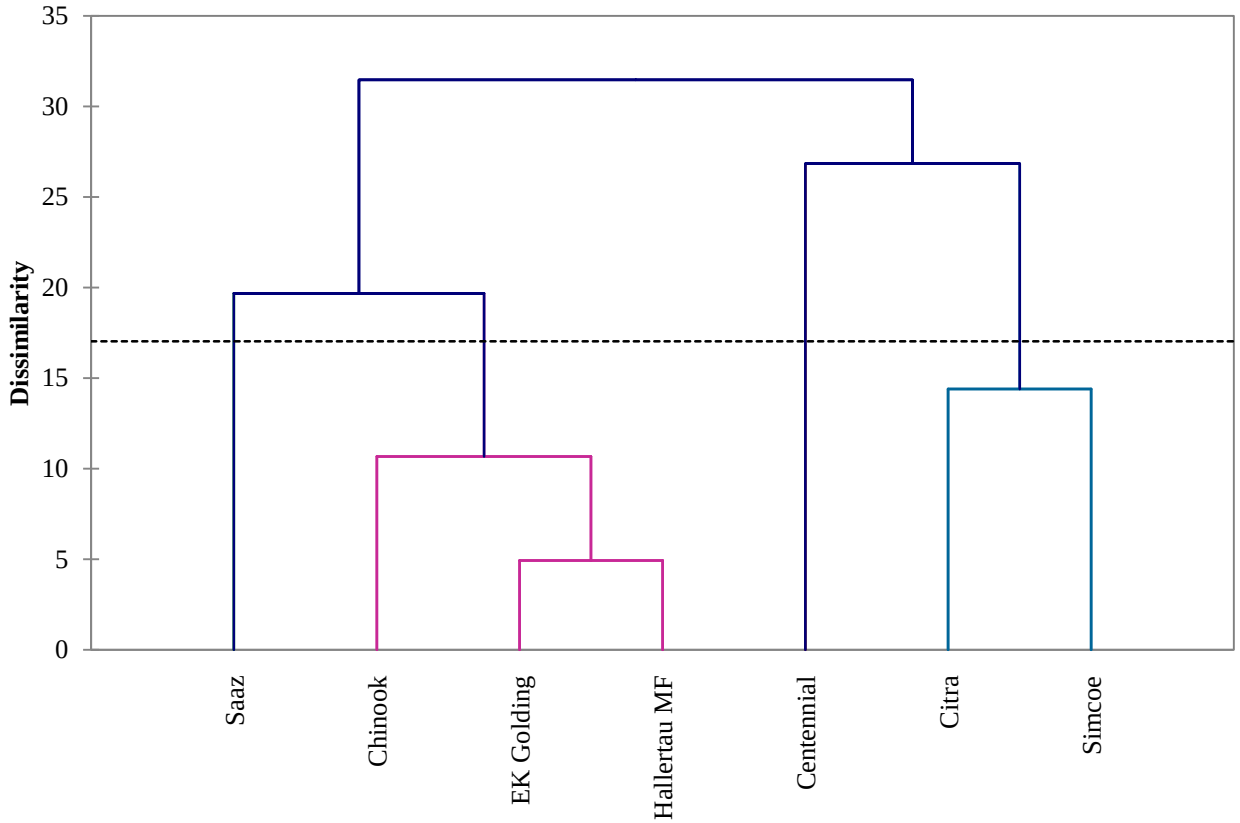


Figure 3: Agglomerative Hierarchical Clustering on Sensory Data

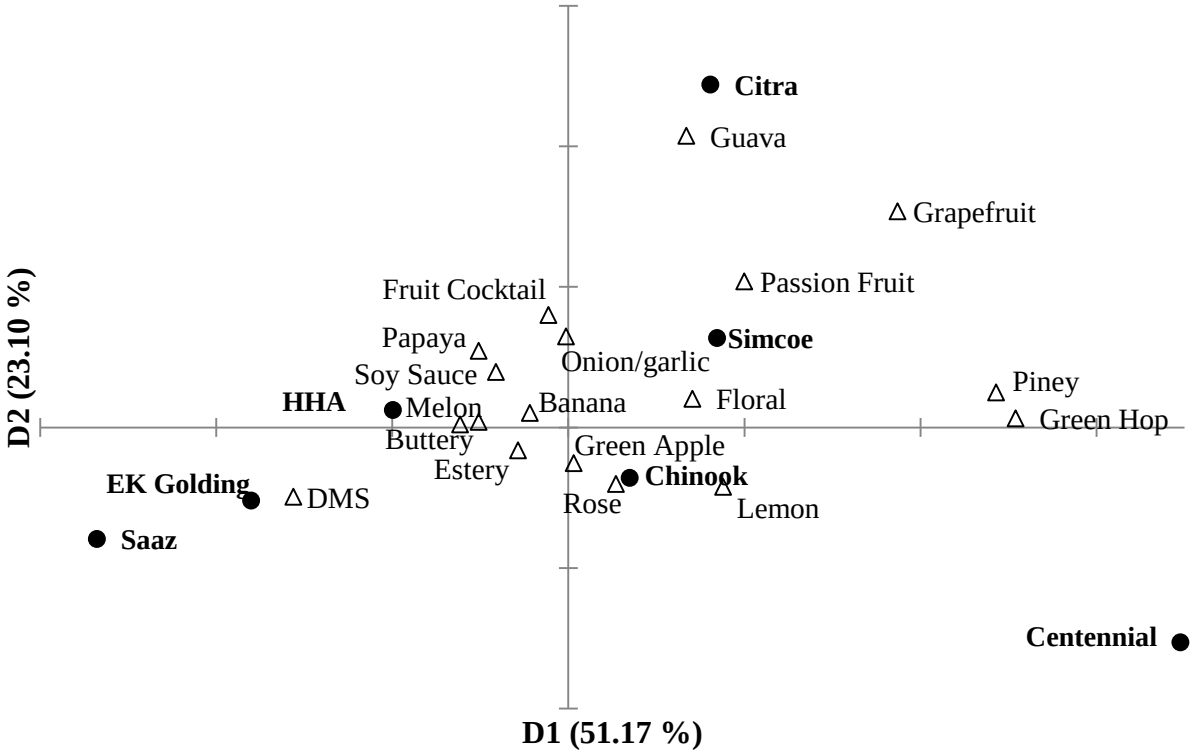


Figure 4: Principle components analysis (D1 and D2) of sensory descriptive data from single hop beer evaluation. Dimensions 1, 2 and 3 account for 88.5% of the total variation

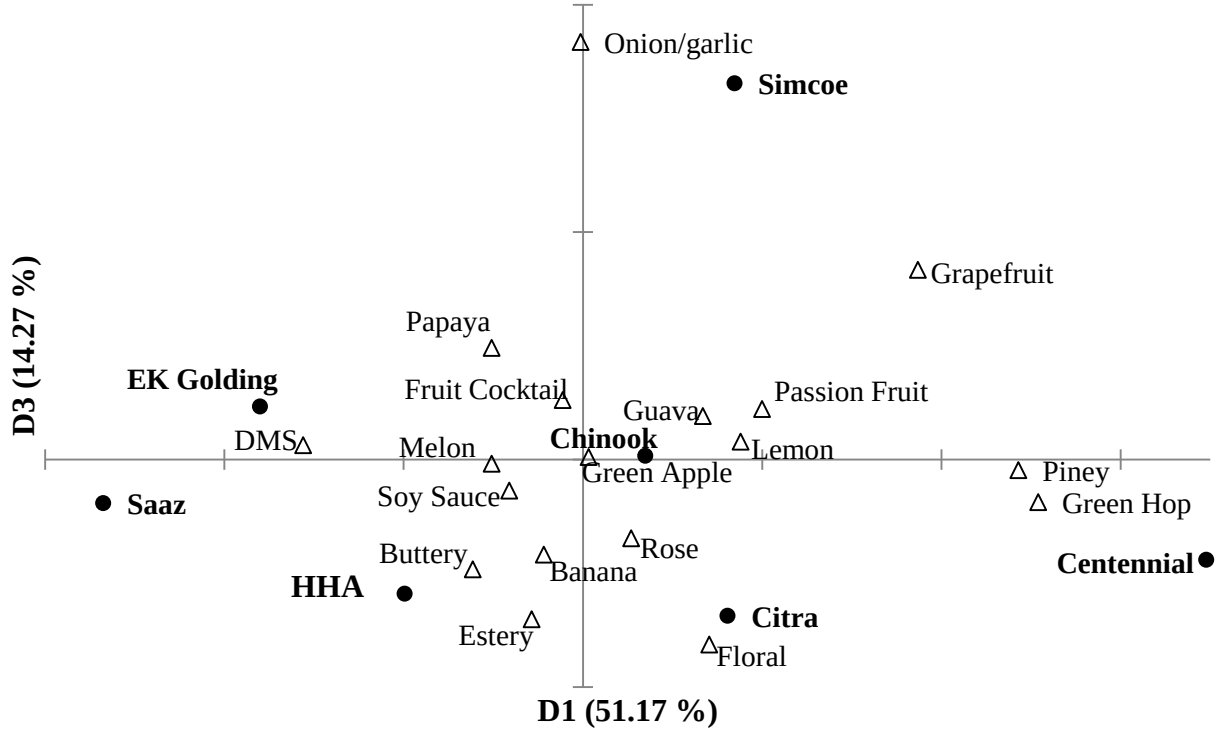


Figure 5: Principle components analysis (D1 and D3) of sensory descriptive data from single hop beer evaluation. Dimensions 1, 2 and 3 account for 88.5% of the total variation

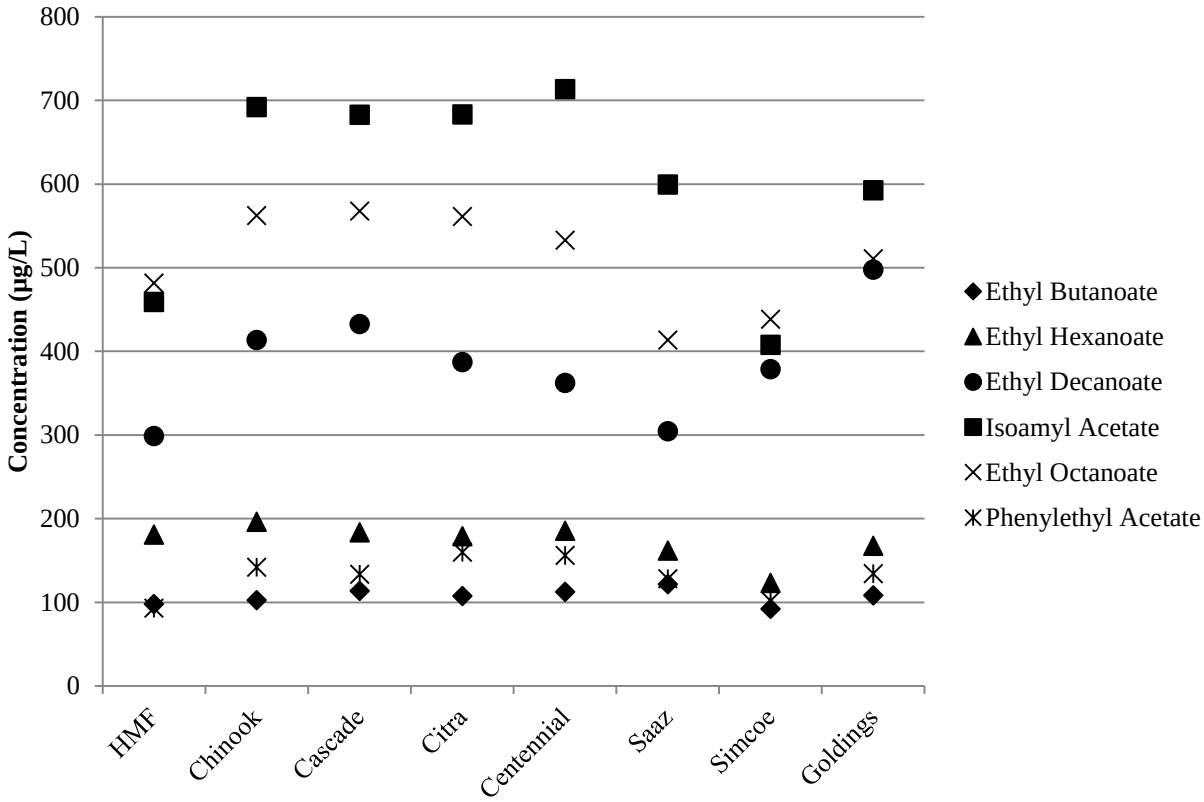


Figure 6: Esters and high alcohol comparisons among the 8 separate hop treatments.

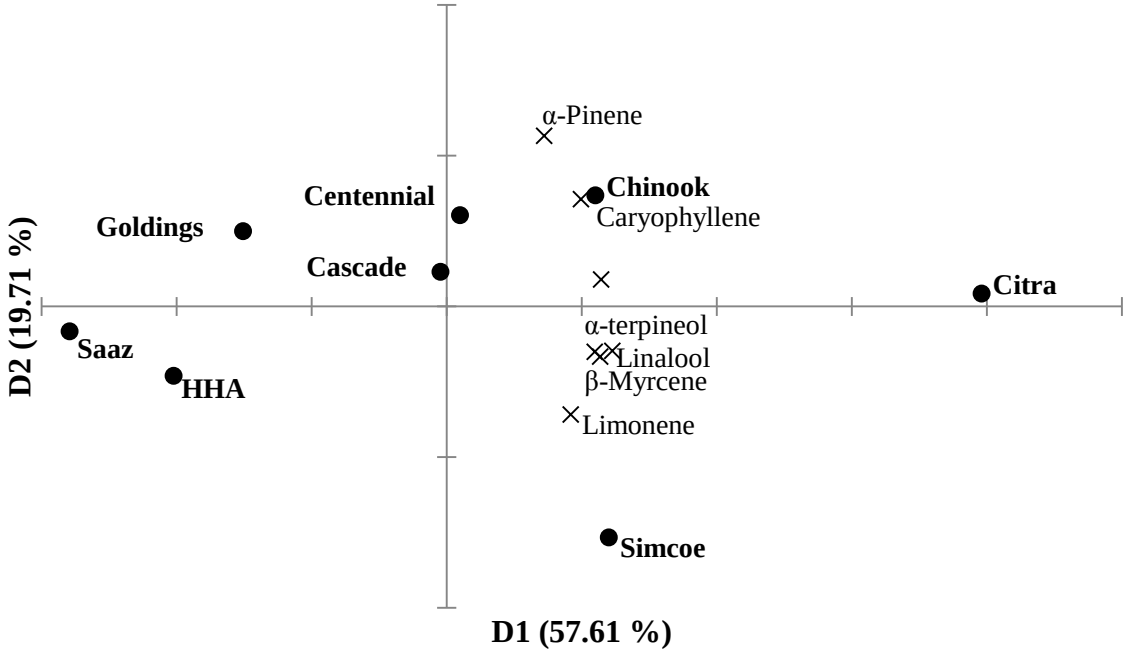


Figure 7: Principle component analysis of flavor unit data of hop compounds found in beer from GC-MS instrumental analysis. Dimension 1, 2 and 3 account for 89.4 % of total variation.

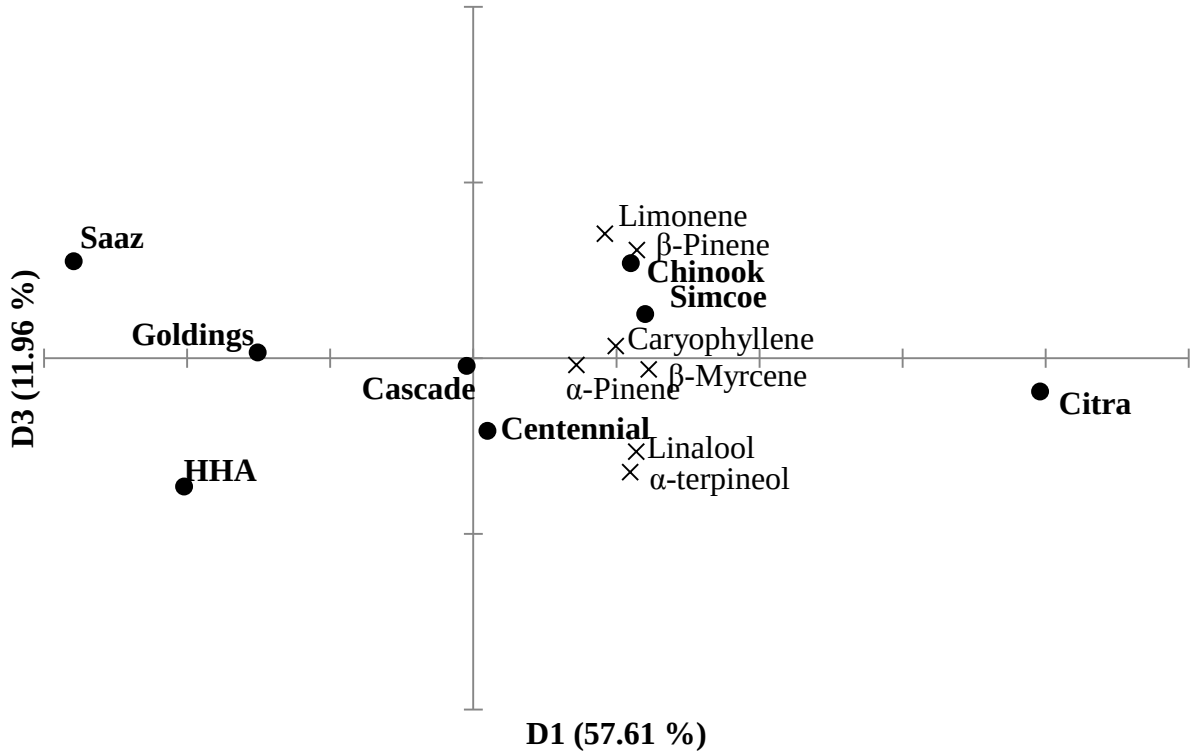


Figure 8: Principle component analysis of flavor unit data of hop compounds found in beer from GC-MS instrumental analysis. Dimension 1, 2 and 3 account for 89.4 % of total variation.

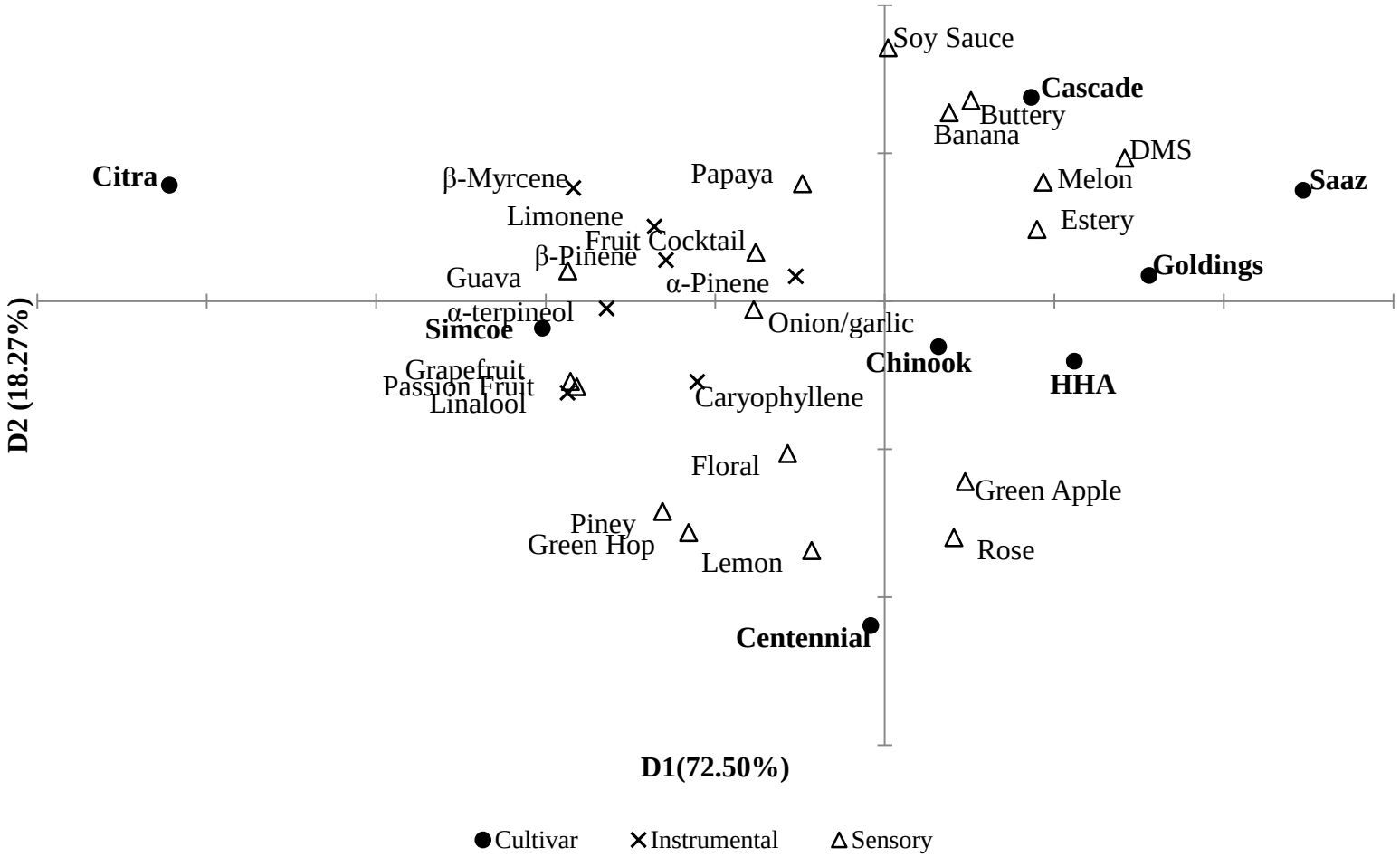


Figure 9: Generalized Procrustean analysis of combined sensory and GC-MS data from single hop beer evaluation. Dimensions 1 and 2 account for 90.78% of the total variation.

Table 4: Hop pellet specifications by cultivar

Cultivar	% alpha acids	Oil content (ml/100g)
Citra	12.3	1.68
Simcoe	12.2	1.64
Hallertau Mittlefrueh	3.8	0.67
Saaz	3.4	0.60
Cascade	5.8	0.82
Centennial	10.9	0.73
Chinook	11.8	0.69
East Kent Goldings	6.9	0.60

Table 5: Means and Tukey's multiple comparisons of descriptive analysis results.

	American					European		
Descriptor	Citra	Cascade*	Centennial	Simcoe	Chinook	EK Golding	Hallertau	Saaz
Fruit Cocktail^{ns}	3.6^{a}	3.2^{a}	2.4^{a}	3.8^{a}	3.4^{a}	3.1^{a}	3.2^{a}	2.6^{a}
Guava	4.0^{a}	0.9^{c}	1.2^{bc}	2.5^{b}	1.3^{bc}	1.5^{bc}	1.3^{bc}	0.6^{c}
Passion Fruit	2.5^{a}	0.5^{c}	1.3^{abc}	2.0^{ab}	1.6^{abc}	0.7^{c}	0.9^{bc}	0.3^{c}
Papaya	1.2^{a}	0.6^{ab}	0.3^{b}	1.2^{a}	0.6^{ab}	0.7^{ab}	0.8^{ab}	0.8^{ab}
Banana	1.0^{ab}	1.5^{a}	0.7^{ab}	0.4^{b}	0.4^{b}	0.5^{b}	0.8^{ab}	1.2^{ab}
Melon^{ns}	0.6^{a}	1.1^{a}	0.4^{a}	0.7^{a}	0.7^{a}	0.9^{a}	1.0^{a}	1.0^{a}
Grapefruit	2.7^{a}	0.7^{bc}	2.0^{ab}	3.0^{a}	1.7^{abc}	0.6^{bc}	1.0^{bc}	0.4^{c}
Lemon	1.1^{ab}	1.0^{ab}	2.1^{a}	1.6^{ab}	1.4^{ab}	1.1^{ab}	1.1^{ab}	0.9^{b}
Estery^{ns}	2.4^{a}	1.9^{a}	2.0^{a}	1.2^{a}	2.1^{a}	2.1^{a}	1.9^{a}	2.1^{a}
Green Apple	0.9^{a}	0.7^{a}	1.1^{a}	1.0^{a}	1.4^{a}	0.9^{a}	1.6^{a}	1.2^{a}
Rose	0.8^{ab}	0.2^{b}	0.9^{ab}	0.5^{ab}	0.6^{ab}	1.2^{a}	1.2^{a}	0.6^{ab}
Floral	2.9^{ab}	2.2^{ab}	3.3^{ab}	1.9^{b}	2.0^{ab}	2.2^{ab}	3.4^{a}	1.8^{b}
Green Hop	3.5^{ab}	2.0^{bc}	5.2^{a}	3.2^{b}	3.3^{b}	2.8^{bc}	3.0^{bc}	1.4^{c}
Piney	2.5^{b}	1.1^{bc}	4.2^{a}	2.2^{bc}	2.4^{b}	1.2^{bc}	1.7^{bc}	0.7^{c}
Onion/garlic	1.5^{b}	0.6^{b}	0.8^{b}	3.8^{a}	1.3^{b}	1.6^{b}	1.3^{b}	1.0^{b}
Soy Sauce^{ns}	1.2^{a}	1.4^{a}	0.7^{a}	1.0^{a}	0.8^{a}	1.2^{a}	0.9^{a}	1.2^{a}
Buttery	1.2^{bc}	3.8^{a}	0.9^{bc}	0.6^{c}	0.9^{bc}	0.9^{bc}	0.8^{c}	2.0^{b}
DMS	0.7^{b}	2.1^{a}	0.6^{b}	0.8^{b}	0.8^{b}	2.6^{a}	1.7^{ab}	2.2^{a}

^{ns}= no significant difference between all treatments for that descriptor; Means with same letter superscript are not significantly different for that attribute. *Treatment removed from multivariate testing due to defects (buttery).

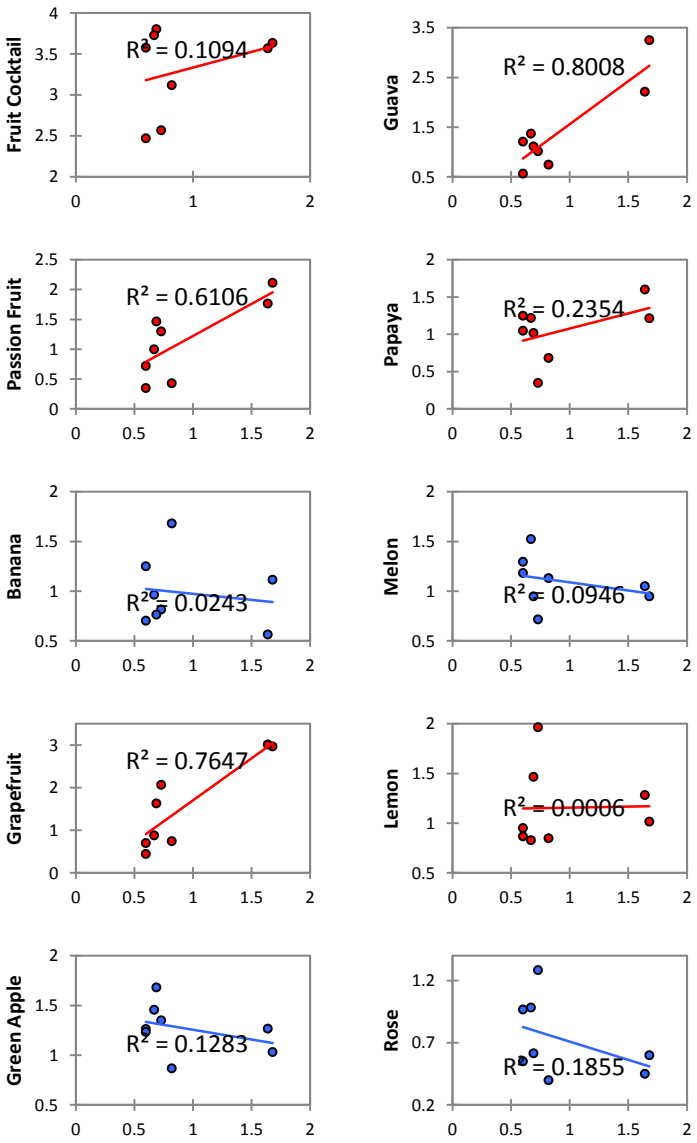
Table 6: Concentration (µg/L) and flavor unit data of hop-derived aroma compounds found in single hopped beers.

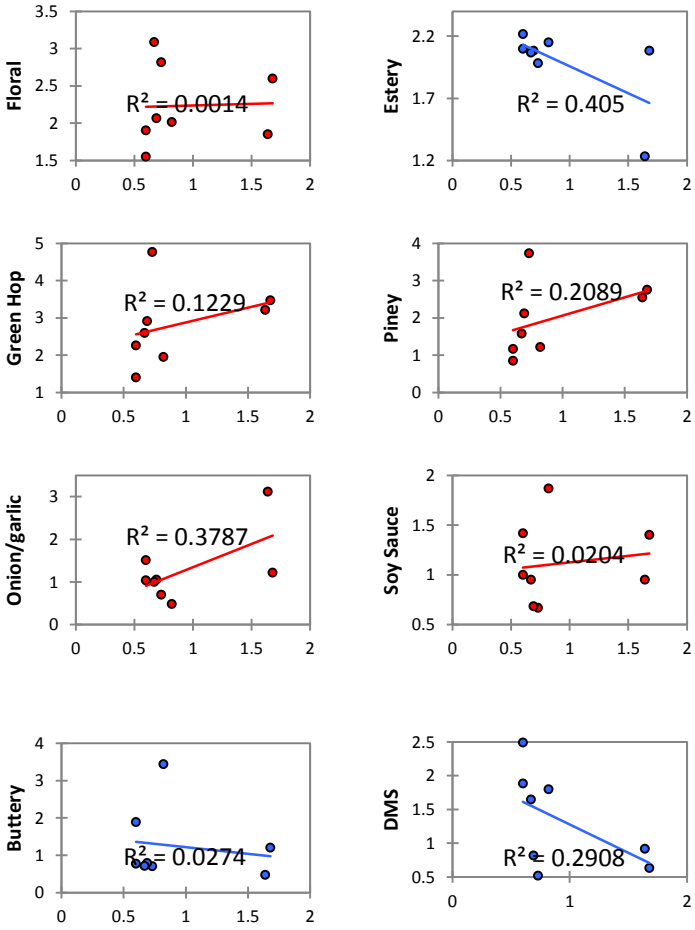
Compound	HHA	Chinook	Cascade	Citra	Centennial	Saaz	Simcoe	Goldings	Sensory Threshold (µg/L)
α-Pinene	13.1 2.2	13.7 2.3	13.6 2.3	13.7 2.3	13.5 2.3	13.2 2.2	13.0 2.2	13.5 2.3	6.0[82]
β-Pinene	21.3 0.2	25.1 0.2	24.3 0.2	25.2 0.2	23.8 0.2	22.7 0.2	24.8 0.2	23.6 0.2	140[82]
Myrcene	33.2 2.6	46.0 3.5	61.2 4.7	126.8 9.8	33.5 2.6	27.5 2.1	74.2 5.7	35.6 2.7	13.0[82]
Limonene	22.1 2.2	23.0 2.3	22.4 2.2	23.2 2.3	22.1 2.2	22.6 2.3	23.6 2.4	22.1 2.2	10.0[82]
Linalool	36.8 1.4	31.0 1.2	25.3 0.9	91.4 3.4	66.3 2.5	3.4 0.1	69.7 2.6	19.2 0.7	27.0[83]
E, β-caryophyllene	15.1 0.2	16.0 0.3	15.1 0.2	16.0 0.3	15.7 0.2	15.1 0.2	15.1 0.2	15.1 0.2	64.0[82]

Bold values represent concentration data. Flavor units are shown below concentrations.
FU < 1 Little to no flavor contribution, FU = 1-2 Moderate flavor contribution, FU > 2 Significant flavor contribution
[82]water; [83] beer

APPENDIX

Figure 10: Scatterplot matrix and Correlation Coefficients of Sensory Descriptors vs. Essential Oil content (ml/100g hops).




Floral
R² = 0.0014
Estery
R² = 0.405
Green Hop
R² = 0.1229
Piney
R² = 0.2089
Onion/garlic
R² = 0.3787
Soy Sauce
R² = 0.0204
Buttery
R² = 0.0274
DMS
R² = 0.2908

Chapter 3: Comparison of the contributions of hop pellets, super critical fluid hop extracts, and extracted hop material on the aroma and terpenoid content of lager beers.

Daniel C. Sharp, YanPing Qian, Jeff Clawson and Thomas H. Shellhammer

ABSTRACT

Brewers who create hop-forward styles such as American style India Pale Ales typically add hops toward the end of or after wort boiling to avoid aroma volatilization and thereby impart strong hop-derived aromas. However, previous studies have demonstrated that despite the volatilization effects of boiling wort, hops that are added early in kettle boil can contribute to hop aroma[46]. Non-volatile hop-derived precursors, specifically glycosides, survive the boil process and can be hydrolyzed to release volatile aglycones capable of contributing to aroma. Twelve single-hopped pilot scale (3hL) beers were brewed using pellet, supercritical CO_2 extract, and spent hop fractions of Citra, Simcoe, Centennial, or Cascade cultivars in order to investigate the contribution of these different hop fractions to the aroma of kettle hopped beers. Pellet, extract and spent additions consisted of a single hop addition 5 minutes into a 60-minute boil. Volatile analysis of beers was performed using stir bar sorptive extraction (SBSE) of beer samples and quantified using gas-chromatography mass spectrometry (GC-MS). Beers were analyzed for the common terpenoid compounds: α-pinene, β-pinene, β-myrcene, limonene, linalool, E, β-caryophyllene, α-humulene, and α-terpineol. In addition, beers were evaluated using descriptive sensory analysis. The descriptive sensory data identified significant differences among the cultivar and hop product treatments. The spent hop treatments produced beers that had noticeable

hop aroma which suggest that the water-soluble components left behind in the spent hops may contribute to hop aroma in beer. The intensity and nature of the hop aroma in the Spent treatments was hop variety specific. However, contributions of water soluble components found in spent hops to hop aroma in beer were very subtle, especially compared to the pellet and extract treatments.

INTRODUCTION

Hops are primarily used in brewing to impart flavor and aroma to finished beer. Much of the hop aroma in beer is derived from volatile compounds found in the essential oil fraction of hops[26]. In order to add hop aroma to beer, brewer's will typically add hops (pelletized or whole cones) later in the brewing process in order to extract the essential oil fraction. Alternatively, brewers may add hop extracts which have been produced via supercritical fluid extraction (SFE) using carbon dioxide or via ethanolic extracts. CO_2 extracts contain mostly the nonpolar compounds found in hops including the essential oil fraction while ethanol extracts will include some of the polar components, such as some polyphenols (xanthohumol, for instance) and chlorophyll. The former tend to be amber in color while the latter are green[84]. The chemical profile of the essential oil fraction varies by cultivar[85], but consists primarily of volatile hydrocarbons, oxygenated components and sulfur containing components[19]. For the most part, these compounds are relatively volatile and minimally soluble in an aqueous matrix such as wort. While it has been shown the some of these compounds may be converted to more water soluble, less volatile compounds, such as humulene epoxides[86,87], capable of surviving the kettle and ultimately contributing to kettle

aroma[46], most do not survive the entire brewing process[88]. Interestingly, anecdotal reports from brewers indicate that all else being equal, clear differences in flavor are observed between beers made with pellets and those made with extracts. Based on these reports it is reasonable to hypothesize that the extraction process does not fully extract all the components responsible for contributing hop aroma and flavor to beer. Therefore, examination of the left over, "spent" material after SFE could lead to greater insight into these differences.

After hops are extracted by supercritical CO_2, the remaining solid material, often referred to as "spent hops", contains a water soluble fraction that makes up approximately 25% (w/w) of the spent hop material[84]. In addition, this material contains polyphenols, nitrates, free amino acids, and protein but only minor amounts of residual essential oil and alpha acids[89]. Researchers have shown that the water soluble fraction of hops contain compounds, specifically non-volatile aroma precursors known as glycosides[35]. Glycosides are water-soluble compounds in which a sugar, most commonly β-D-glucose, is bound to a functional group via glycosidic bond [35]. Due to the presence of a sugar moiety and increased molecular weight, these compounds are more water soluble and less volatile than their aglycones, which may lead to greater retention throughout wort boiling. Once extracted into the wort/beer matrix, glycoside hydrolysis can occur due to acidic conditions or enzymatic action[90], thus releasing volatile aglycones and creating aroma. Once aglycones are hydrolyzed from their associated sugar moiety in significant quantities they may become an active contributor to hop aroma in beer[91].

It was the purpose of this study to examine the contribution of pellets, their extracts obtained from SFE, and the left over spent hop material to the hop aroma profile of single-cultivar kettle-hopped beers made using 4 different hop cultivars: Simcoe, Citra, Cascade, or Centennial. These cultivars were selected to investigate which fractions of newer American aroma type hops contribute to kettle type aroma.

MATERIAL AND METHODS

Hop Products

During the winter/spring of 2011, Yakima Chief performed small scale supercritical fluid CO_2 extractions of Simcoe, Centennial, Citra, and Cascade hops from the 2010 North American Harvest. For each variety, a sample of pellets, the CO_2 extracts and resulting spent material were used to brew single cultivar kettle hopped beers. For each variety, a 1200 g sample of pelletized hops was extracted using supercritical carbon dioxide. Each extraction run consisted of 400 g of raw pellets and 3 runs were performed for each variety. The extracted material, the residual spent material and the pelletized starting material were used in the studies presented herein. Hop pellets and spent material were vacuum packaged in UV/gas/vapor barrier bags and stored at -20°C until use. The total essential oil of pellets and spent material were measured by steam distillation according the American Society of Brewing Chemists standard method [92].

Hop acids analysis by spectrophotometry and high performance liquid chromatography were performed by Yakima Chief Hops (Yakima, Washington). Hop

acid reduction from pellets by SFE was determined to be greater than 95% for each variety; the reduction in hop oils was assumed to be similar.

Beer Production

Twelve single-hopped beers were brewed in the OSU pilot brewery using wort (12°P original gravity) made from a grist of 98.5% 2-row malt and 1.5% acidulated malt and fermented with a lager yeast (Wyeast 2007) pitched at 24.0 x 10^6 cells/ml. Beers were fermented at 14 °C for 3 weeks, cooled to 8 °C over 4 days and then lagered for 1 week. Beers where then filtered (Pall HS 2000), carbonated to 2.7 volumes CO_2 and packaged into 355 ml amber glass bottles. Finished packaged beers were stored at 1°C until analysis.

Hop Additions

Hop pellets, extract and spent additions consisted of a single dosage to 170 L of boiling wort 5 minutes into a 60-minute boil. The mass of each hop dosage for each brew is shown in Table 7. The pellets were dosed at 1 g/L while the extract and spent hop dosages were calculated based on the analytical specifications of the hop pellet (Table 7). Dosage calculations (equations 1 & 2) are discussed in further detail below. In general, hop dosage determinations were calculated to be representative of their relative compositions in hop pellets for each hop cultivar and the upper limit of hops dosage was built around the variety with the highest hop acid (Simcoe – 13.3% alpha acids) necessary to achieve a beer with less than 35 BU. Hop Dosage Calculations: Hop dosages were calculated individually for each treatment.

Table 7: Mass of hop pellet, extract, and spent material added to 170 L of wort. * Cascade pellets treatment was removed from analysis due to contamination.

Variety	Pellets	Extract	Spent Material
Simcoe	170	50	139 g
Citra	170	41.9	140 g
Cascade	170*	44.4	150
Centennial	170	37.5	148

Pellets

Because aroma characteristics and not bittering characteristics were investigated, a constant mass (170 g) approach was used for pellet dosing of all 4 varieties. This maintained a consistent and comparable contribution of aroma from pellets on a per mass basis. The mass determination was calculated so as to not exceed ~35 BU in the finished beer. In order to maintain a 35 BU limit, pellet dosing was calculated using the sample with the highest α content (Simcoe=13.3% α-acid; Table 8). This resulted in a 1 g/L hop additions to the boiling wort.

Extracts

Concentration of the nonpolar hop components is a direct result of the extraction process, therefore hop extracts contain higher concentrations of α-acids, β-acids, and essential oil than their pellet starting material. Consequently, dosing at the same level as for pellet treatments (1 g/L) would be unconventional and without practical merit. Since hop extracts contained >96% of α-acids found in the pellets, dosing was calculated based on the percent contribution of α-acids equivalent to 170 g of pellets for each cultivar (equation 1). This method ensured that pellet samples and extract samples had approximately the same amount of bittering acids and oils dosed into each beer.

$$m_{extract} = \frac{m_{pellet}(\alpha_{pellets})}{\alpha_{extract}} \quad (1)$$

where m = mass and α=α-acid %.

Spent material

Spent hop doses were calculated to represent the mass of hop material and chemical components that were not extracted by SFE relative to the amount of pellets used. The percent mass of hop material not accounted for by hop acids and essential oils was assumed to be the non-extracted material. The mass of spent material additions was then calculated from the non-extracted material fraction of the pellets used (equation 2).

$$m_{spent} = m_{pellets}(\delta) \quad (2)$$

$$\text{where } \delta = 1 - (\alpha_{pellets} + \beta_{pellets} + oil_{pellets})$$

$\equiv$ approximate fraction of material not extracted during SFE;

m = mass and α=α-acid %, β= β-acid%, oil= Oil (%v/w).

Examination of Table 7 shows that the combined mass of extract and spent hop material for each cultivar is greater than the mass of pellets used. This incomplete mass balance is an artifact of the approximate compositional analysis of extracts and spent hops.

Instrumental analysis of beers

Stir-Bar Sorptive Extraction and GC-MS Analysis

The concentrations of common terpenes, terpene alcohols and sesquiterpene alcohols were quantified using an Agilent 7890A gas chromatograph on a ZB wax column (30m x 0.25 mm ID x 0.25 µm; Zebron) with helium as the carrier gas at a

flow rate of 1.0 ml/min. Compounds were identified using an Agilent 5975C single quadrupole mass spectrometer with electron impact ionization at 70 eV operating in scan mode (m/z 35-350).

Stir bar sorptive extractions (SBSE) were performed using a polydimethylsiloxane (PDMS) coated magnetic stir bar (10mm x 0.5 mm; Gerstel). Samples were diluted 1:1 with deionized water and stirred at 1000 RPM in 40 ml amber screw top vials for three hours at 20°C. 4-octanol (Sigma-Aldrich) was added to final concentration 150 ppb to each vial as an internal standard. After extraction, stir bars were removed, rinsed with distilled water, and gently dried by blotting with lint free tissue (Kim-wipes, Kimberly-Clark) before being desorbed via a thermal desorption unit (TDU; Gerstel). Samples were desorbed according to the instrumental parameters described below for gas chromatographic separation and detection by mass spectrometry (GC-MS). All instrumental measurements were performed in duplicate.

Instrumental parameters

Stir bars were placed into a Thermal Desorption Unit (TDU; Gerstel) for temperature-programmed thermal desorption. The temperature program began at 25°C and increased at a rate of 120°C /min to a final temperature of 250°C and held for 2 minutes. After desorption, analytes were cryofocused with liquid nitrogen (-80°C) in a CIS4 programmed temperature vaporizing (PTV) injector (Gerstel). Once cryofocusing was complete, the injector inlet was programmed at a ramp rate of 10°C/s from -80°C to 250°C with a 54-minute hold at the final temperature

Standards for the following target analytes were purchased from Sigma-Aldrich: α-pinene, β-pinene, β-myrcene, limonene, linalool, β-caryophyllene, α-humulene, α-terpineol. The purity of each standard used for quantitation was determined and used to correct concentrations for calibration curves. A standard stock solution was made in dichloromethane and added to a 5% (v/v) ethanol/water solution to obtain the following concentrations for a calibration curve: 1 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, 100 ppb, 250 ppb, and 500 ppb. All calibration solutions were analyzed according to the SBSE sample preparation and analysis methodology previously described.

Hop Acid Analysis in Beer

Hop acids in the beers were analyzed by American Society of Brewing Chemists Standard Method of Analysis for iso-α-acids in beer by HPLC[93]. Iso-α- acids were observed at 270 nm and non-isomerized acids at 314 nm. A Kinetex C-18 100x 4.6 mm column operated at 40°C was used for reverse phase analyte separation.

Sensory Analysis of Beers

Descriptive sensory analysis consisted of 12 trained panelists, most of whom had been extensively involved with previous sensory work regarding beer evaluations. Each panelist was presented with 60 ml samples in ~ 200ml clear glasses capped with clear-plastic, odorless lids. Samples were kept cold on ice for no more than 2 hours prior to evaluation. Ambient temperature during evaluation was 18- 20°C. The descriptive ballot was based on 11 descriptive terms for beer aroma with a focus on hop-derived aromas; *overall aroma intensity (OAI), cedar, citrus, tobacco/earthy,*

floral, *grassy/herbal*, *onion/sweaty*, *pine*, *spicy*, *stone fruit*, and *tropical fruit*. The aromatic attributes were scaled using a 16-point scale with 0 anchoring "none" and 15 anchoring "extreme". The descriptive terms were developed during the training sessions, which met 6 times over the course of 2 weeks prior to data collection. On each testing day, 11 beers were presented individually to each panelist in a panelist-specific random order. Although 12 beers were included in the overall design of the study (4 cultivars x 3 hop products), a noticeable and distracting defect was noticed in the Cascade Pellet sample early in sensory analysis trainings and was removed from all future sensory testing. During testing, panelists evaluated 6 beers, given a 2-hour break, and then evaluated the remaining 5 beers. Each beer was evaluated independently and in a random fashion on 5 separate days/sessions. Summary statistics and mixed model analysis of variance of panelist performance was evaluated evaluated using XLSTAT 2009. Principle component analysis of the panelist data averaged over all replications was performed using the Varimax rotation and the covariance matrix.

RESULTS AND DISCUSSION

Hop Products

Hop acid specifications (Table 8) for each variety and material were provided by Yakima Chief. Oil content of the extracts were estimated using the percent reduction of acids after extraction as an assumed percent extraction of oil. The amount of residual hop oil in the spent hop material was measured using steam distillation. All of the spent material yielded less than 0.1 ml/100 g spent hop material. These data

confirm that spent material did not have appreciable amounts of hop oil to contribute to aroma in beer when added to boiling wort during the brewing process.

Beer Production

Routine analytical data for finished beers is shown in Table 9. One can observe that the high α-hops (Simcoe and Citra) resulted in beers with more bitterness than the other two. These two hops produced beers with the target ~35 BU while hops with the lower α-acid contents resulted in beers with lower BU's, however beers made with spent hops (α-acid ≤0.5%) had 12 – 16 BUs and 6 – 9 ppm iso-α acids. While some BUs were expected in spent hop treatments due to the extraction of polyphenols, the residual iso-α acids were surprisingly high given that the starting hop material was so low in measureable α-acids. As to be expected, the residual α acids levels in the spent hop beers were very low. The alcohol and residual extract was consistent across all 12 brews averaging 4.1% w/w and 3.6% w/w, respectively.

Instrumental analysis of pellet, extract, and spent material single hopped beers

Instrumental analysis data identified statistically significant differences in target analytes among the 12 beers. However, it should be noted that although statistical differences exist, the level at which compounds were detected by GC-MS was at the lower end of the sensitivity level for the analysis method and therefore concentrations of < 1 ppb are estimates (Table 10), which suggests that most of these target analytes are likely volatized during kettle boiling or not present to any significant degree in the starting material. In addition, the concentrations of the target analytes were considerably lower than their sensory aroma detection thresholds in beer

or water. With the exception of β-pinene, all target analytes were found at concentrations 800 times below their aroma thresholds and it is likely that the contribution of each target analyte to the overall aroma for each treatment is quite low.

Table 8: Hop Product Specifications

	Simcoe			Citra			Centennial			Cascade		
	Pellets	Extract	Spent	Pellets	Extract	Spent	Pellets	Extract	Spent	Pellets	Extract	Spent
UV												
α-Acid(%)	13.3	45.5	0.5	12.2	49.5	0.1	8.2	37.1	0	5.4	20.6	0.1
β-Acid(%)	3.9	15.5	0.3	3.3	14.5	0.1	3.2	19.2	0.2	5.7	33.6	0.2
HSI	0.30	0.25	1.18	0.30	0.27	1.25	0.30	0.28	1.99	0.27	0.23	1.41
HPLC												
[α-Acid] ppm	12.5	41.8	0.6	10.8	44.4	0.3	8.0	35.4	0.3	5.2	20.9	0.3
[β-Acid] ppm	3.3	13.9	0.3	3.2	14.7	ND	2.9	17.4	ND	4.9	28.8	0.1
Cohumulone %	18.7	18.5	18.7	23.9	23.2	25.8	25	24.4	28.1	31.8	32.3	34.9
CoLupulone %	41.6	40.6	45.1	56.8	55.7	ND	48.3	47.5	ND	48.5	48.2	54.7
Oil (ml/100g)	1.50		<0.1	2.00		<0.1	1.00		<0.1	1.05		<0.1

Table 9: Chemical analyses of beers

	Simcoe			Citra			Centennial			Cascade		
	Pellets	Extract	Spent	Pellets	Extract	Spent	Pellets	Extract	Spent	Pellets	Extract	Spent
BU	37.3	25.5	15.8	36.7	31.1	12.2	25.9	21.2	13.8	17.8	18.0	12.5
[α] ppm	4.2	1.0	0.5	2.3	5.1	0.9	2.7	2.1	0.2	2.1	2.1	0.6
[iso- α] ppm	27.5	22.3	9.1	32.4	26.6	6.8	21.5	17.9	9.3	13.5	14.4	9.7
pH	4.7	4.71	4.7	4.7	4.7	4.7	4.8	4.8	4.7	4.8	4.7	4.7
Alcohol % w/w	4.3	4.0	4.1	4.1	4.0	4.2	4.2	4.1	4.0	4.2	4.1	3.9
RE % w/w	3.4	3.8	3.8	3.6	3.8	3.3	3.4	3.4	3.4	3.5	3.4	3.8

Table 10: Concentration (µg/L) of hop aroma compounds found in beer determined by SBSE GC-MS.

	Odor Threshold	Simcoe			Citra			Centennial			Cascade		
		Pellets	Extract	Spent	Pellets	Extract	Spent	Pellets	Extract	Spent	Pellets	Extract	Spent
Compound													
α-Pinene	140[c]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-Pinene	6[c]	0.6	0.2	0.1	0.3	0.6	0.2	0.3	0.3	0.1	0.3	0.3	0.1
β -myrcene	195[a]	0.8	1.2	0.6	0.9	6.6	0.3	0.5	1.1	0.4	0.6	1.2	0.2
Limonene	100-1400[b]	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
R/S Linalool	83[a]	ND	0.1	ND	0.1	0.6	ND	0.1	0.1	ND	0.1	0.1	ND
E,β-caryophyllene	770[a]	0.7	0.7	1.1	0.4	0.4	0.8	0.8	0.7	0.5	0.4	0.4	ND
α-Humulene	310[a]	0.2	0.1	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
α -Terpineol	330[b]	1.0	1.8	0.5	3.3	5.3	0.6	1.7	2.5	0.7	1.7	1.6	0.7

Values are means of duplicate injections. [a]5% ABV beer[94], [b]beer[95], [c]water[95]. ND indicates not detected.

Sensory analysis of beers

The relatively low hopping rate and early hop addition during beer production resulted in beers with low levels of hop aroma regardless of treatment type. The sub-detection threshold concentrations of the target analytes are reflected in the sensory results. Despite low target analyte concentrations and sensory scores, the descriptive sensory data identified statistically significant differences among the 11 different hop treatments for all sensory attributes with the exception of *Cedar* and *Spicy* (Table 11). Mixed model ANOVA results (Table 11) from descriptive panel analysis show a significant treatment by panelist interaction for all attributes except cedar and spicy which indicates that each panelist used the scale differently for each treatment. The lack of other significant interaction effects (treatment*rep, panelist*rep) indicates that panelists were repeatable from one session to another. These differences can be visualized in the spider diagrams (Figure 11) of the sensory scores averaged across all panelists and replications and also in the PCA biplots (Figure 12).

Spent treatments resulted in beers that were lower in hop aroma than their Pellet or Extract counterparts (Table 12). And, spent treatments did not result in higher aroma than Pellet or Extract treatments for a given cultivar. Nevertheless, the Spent treatments produced beers with perceptible hop aroma, and in one instance, the Simcoe Spent hop treatment resulted in beers that had higher overall aroma than Extract and Pellet treatments from other varieties. This suggests that the water-soluble components left behind in the spent hops can contribute hop aroma in some cases.

When examined across all treatments (Pellet, Extract and Spent) there were significant differences in hop aroma based on variety. When looking at hop aroma intensity, Citra produced the most intense aroma followed by Simcoe, Centennial and Cascade (Table 12). Conversely, the Cascade hop had subtle to negligible hop aroma, particularly in the Spent treatment.

Table 11: Summary of p-values associated with F-values generated from ANOVA of sensory evaluation data.

Treatment	DF	OAI	Cedar	Citrus	Tobacco Earthy	Floral	Grassy Herbal	Onion Sweaty	Pine	Spicy	Stone Fruit	Tropical Fruit
Treatment	11	< 0.001	0.603	< 0.001	0.014	< 0.001	0.001	< 0.001	< 0.001	0.771	0.062	< 0.001
Panelist	11	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Rep	4	0.294	0.623	0.245	0.637	0.972	0.404	0.041	0.373	0.471	0.112	0.926
Treatment*Panelist	121	< 0.001	0.191	< 0.001	0.056	0.006	< 0.001	< 0.001	0.000	0.190	< 0.001	< 0.001
Treatment*Rep	44	0.164	0.283	0.949	0.644	0.490	0.434	0.209	0.676	0.507	0.523	0.779
Panelist*Rep	44	0.803	0.693	0.431	0.983	0.857	0.896	0.223	0.996	0.398	0.019	0.861

OAI = Overall Aroma Intensity

Table 12: Means comparisons of Overall Hop Aroma Intensity (OAI) via Tukey's HSD.

Treatment	Mean*	Groups				
Citra Extract	6.24	A				
Citra Pellet	4.88		B			
Simcoe Extract	4.47		B	C		
Simcoe Pellet	4.30		B	C		
Simcoe Spent	4.21		B	C		
Centennial Extract	4.14		B	C	D	
Centennial Pellet	3.98			C	D	E
Citra Spent	3.88			C	D	E
Centennial Spent	3.81			C	D	E
Cascade Extract	3.44				D	E
Cascade Spent	3.28					E

*Means from 16-point scaling data where 0 = none, 3 = slight, 7 = moderate and 15 = extreme. Means with the same letter are not significantly different at the 0.05 confidence level with Tukey adjustment. Note: Cascade pellet treatment removed due to contamination.

Citra Extract was ranked significantly higher than all other treatments for *Overall intensity*, *Citrus*, *Pine*, *Stone fruit*, *Floral* and *Tropical fruit* descriptors. The extent of the differences in the hop aroma intensity coming from the Citra Extract treatment resulted in it behaving somewhat like an outlier relative to all other hop treatments. This is particularly obvious when examining the PCA biplots. It sits in the far right region of the PC space in Figure 4 and drives the differences along PC 1. Why this particular hop yields such an intense aroma is not clear. Of all the hop cultivars used Citra pellets had the highest oil content (Table 8) and it is likely the Citra extract had the highest oil of the four cultivars extracts. Brewers often associate greater oil content with increased aroma, and while the extract oil content was not measured it was estimated to be the highest of the four extracts. Clearly, this hop contains a set of hop aromatics that are very potent.

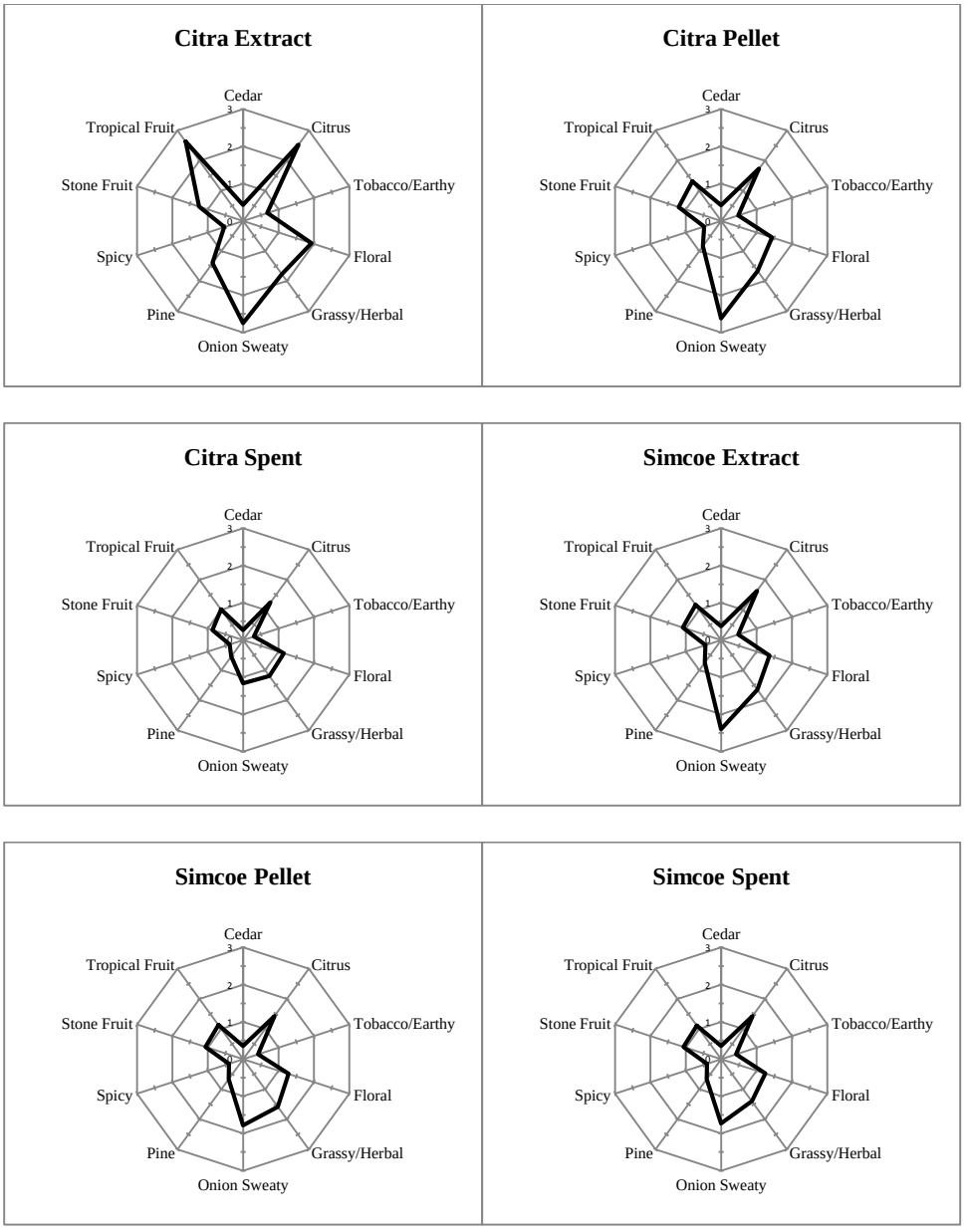
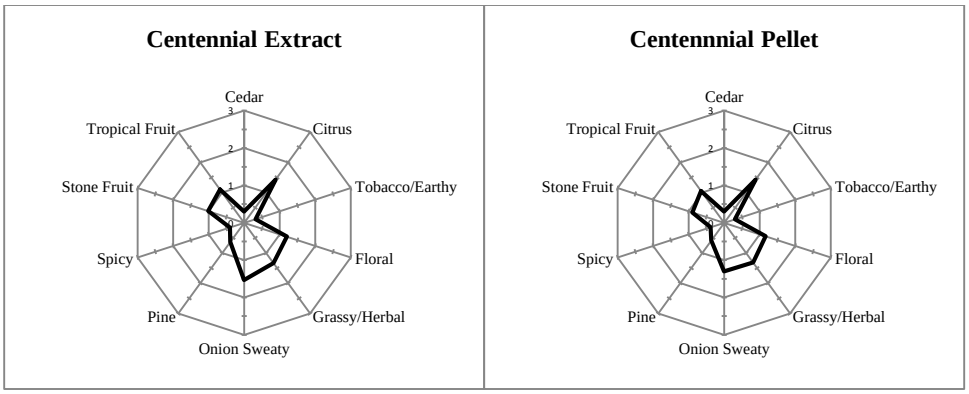
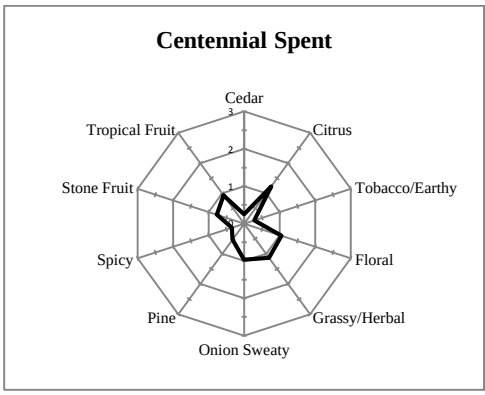
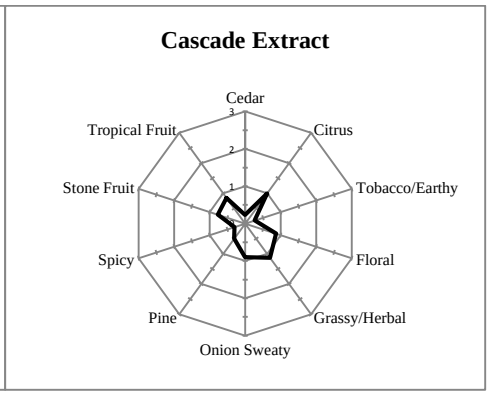


Figure 11: Spider diagrams of aromatic descriptors for each of the treatments. Scale 0-7 (0-3 displayed only). Cascade pellet treatment removed due to sensory defects.







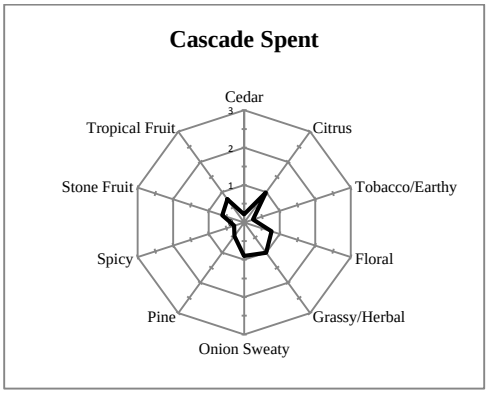


Figure 11: (continued)

Removing the Citra Extract treatment from the PCA allows one to better understand the differences among the remaining group of hops without the significant leveraging effect of the high aroma Citra Extract sample (Figure 13). In its absence, one can see that the spent hop treatments lie in the left side of the PC space indicating that they are lower in overall intensity (*OAI*) and specifically *Onion/Sweaty* and *Pine* aromas. That being said, the Centennial Spent treatment was aligned with and highest on PC2. It separated itself from the others by being higher in *Tobacco/Earthy*, *Spicy* and *Cedar* notes. Separately, Citra Spent was somewhat aligned with and highest on PC3. It was associated with *Floral*, *Citrus* and *Tropical Fruit* aromas.

The interactions among mixtures of aromatic compounds can result in unexpected aromas and one should keep in mind that the instrumental data does not provide a complete picture of the aroma characteristics of each treatment. For instance, potent thiols such as 4-mercapto-4-methylpentan-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA) were not analyzed in this study, but have been shown to contribute to tropical, passionfruit, and box tree aromas in wines such as Sauvignon Blanc[96]. They have also been shown to contribute to citrus and tropical aromas in hops and resulting beers [32,81]. Additionally, previous research has identified oxidation products of humulene and caryophyllene as contributors to kettle hop aroma[46,86,87,97]

Previous research has mentioned the possibility of contributions from hop-derived glycosides to beer and patents demonstrate that these glycosides are present in spent hop material[44,98]. The results presented here indicate that for the hopping rates

(1/gL), the yeast strain, and lagering conditions used in this study, spent hops have very minimal contribution to the aroma of finished beer. However, work by Daenon et al.[99] shows that the ability of yeast to hydrolyze glycosides and allow the release of volatile aglycones varies between yeast strains. Furthermore, the hydrolysis or biotransformation of any hop-derived products to yield volatile compounds are likely dependent on a number of factors such as extraction efficiency, contact time and timing between substrates and yeast, and temperature and matrix effects such as pH[65]. Previous research has shown that the presence of 10% glucose has an inhibitory effect on glycoside hydrolysis and therefore may not occur until later in the fermentation/aging once glucose concentrations are decreased[100]. Therefore, it is conceivable that the conditions in this study were not suitable for significant biotransformation of the compounds found in spent hop material.

Many of the components of brewing value (e.g. α-acids, essential oil content and profile) are cultivar dependent and it is likely that contributions from spent hop material are no different. Furthermore, the sensory results indicate a possible cultivar effect (Table 12). In this case, Simcoe spent hop treatments produced beers with a slightly higher Overall Aroma Intensity score than beer made with Cascade Spent hops. While the instrumental results (Table 10) of beers revealed little to no difference between cultivars for any of the target analytes, there may be other analytes found in spent hop material not investigated in this study that contribute to hop aroma in beer. Glycosides are a diverse class of compounds requiring only a free hydroxyl group on aglycones for glycoconjugation. In this study, α-terpineol and linalool were the only

target analytes to contain free hydroxyl groups and therefore the only potential hydrolysis products from glycosides. Inclusion of more alcohol containing compounds commonly found in hops, such as geraniol, nerol, cis-3-hexenol, or β-citronellol may help describe the subtle increases in aroma related to spent hop treatments.

It is clear that for the conditions used in this study, contributions from spent hop material to hop-derived aroma in beer are minimal, especially when compared to pellets or hop extracts. Further investigations into factors such as yeast type, hydrolysis kinetics, cultivar and point of hop addition would yield more detailed information on the extent to which spent hop material, and specifically glycosides, can contribute to hop aroma in beer.

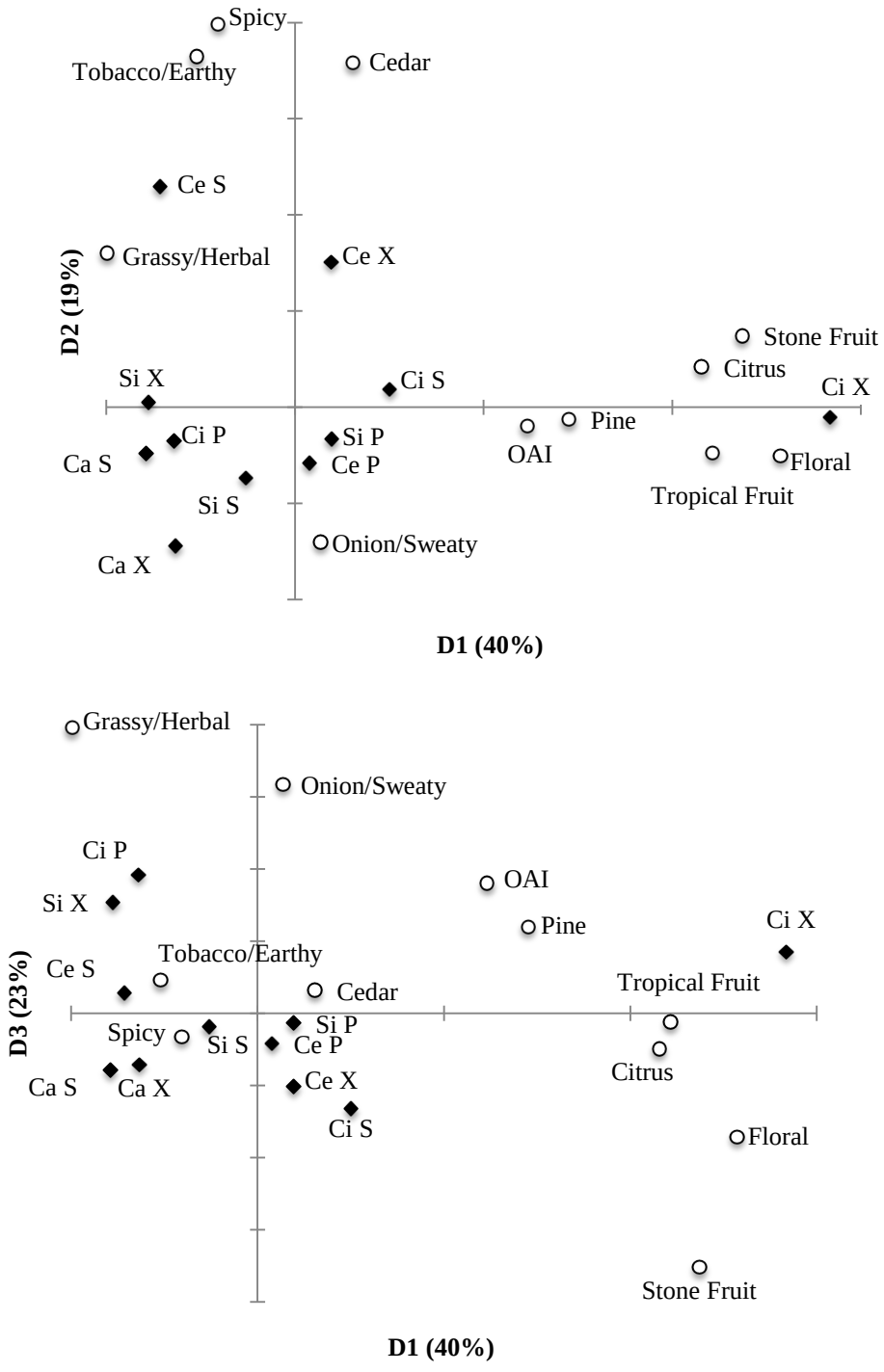


Figure 12: Principle component analysis of sensory descriptive data. Dimensions 1, 2 and 3 account for 82% of the total variation. ♦ = treatments, ○= descriptors; Ce = Centennial, Ci = Citra, Ca = Cascade, Si = Simcoe; P = pellet, X = extract, S = spent

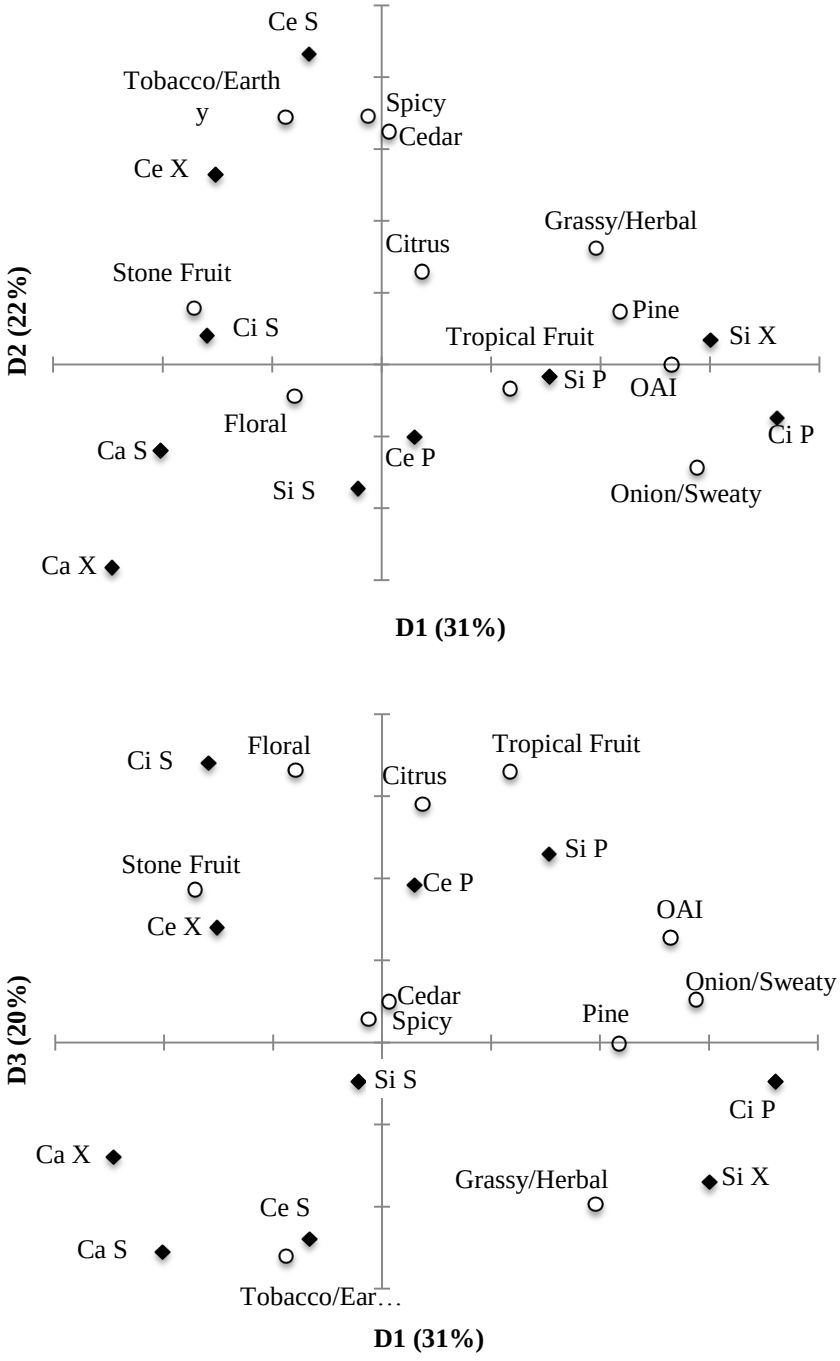


Figure 13: Principle component analysis of sensory descriptive data with Citra extract treatment removed. Dimensions 1, 2 and 3 account for 73% of the total variation. ♦ = treatments, ○= descriptors; Ce = Centennial, Ci = Citra, Ca = Cascade, Si = Simcoe; P = pellet, X = extract, S = spent

CONCLUSIONS

Instrumental results from SBSE showed little to no difference among treatments for all the target analytes. All target analytes were detected at concentrations below 5 ppb and did not survive the kettle boil. Cultivar by product interactions were observed for treatments, notably, Citra extract producing significantly higher aroma than all the other cultivars and products. Also, Simcoe spent hop treatments produced beers noticeably higher in aroma than beers made using spent hops from Cascade, Citra, or Centennial hops. However, contributions of water soluble components found in spent hops to hop aroma in beer was very subtle, especially compared to the pellet and extract treatments. Therefore, for the conditions used in this study where the hopping rate for the spent material was less than 1g/L, the majority of kettle hop aroma came from the more nonpolar components extracted during SFE and very little from spent hop material.

ACKNOWLEDGEMENTS

The authors would like to thank the Hop Research Council for funding this research. In addition, many thanks to Karl Vanevehoven at Yakima Chief Hops for performing supercritical fluid extraction of hop material.

Chapter 4: Examination of hydrolysis methods for the liberation of glycosidically bound terpene alcohols from aqueous Simcoe spent hop extracts.

Daniel C. Sharp, YanPing Qian, Thomas H. Shellhammer

ABSTRACT

Brewers rely on the extraction of essential oil and the non-polar fractions from hops to impart flavor and aroma to beer. This extraction traditionally takes place during the brewing process, but can also occur outside the brewery at extraction facilities, often using supercritical CO_2 fluid extraction (SFE). Extraction of hops by SFE produces a non-polar fraction, sold as hop extract, and a more polar and water soluble fraction of the remaining spent hop material that may also contain residual compounds or precursors capable of contributing to hop aroma in beer.

The central goal of the project presented here was to understand the origin of the aroma contributed by the water-soluble components of hops. Aqueous extracts of the left over spent material from pilot scale SFE of hop pellets were used to investigate the impact of different hydrolysis treatments and different hop cultivars on the aroma and volatile profile. Aroma profiles were evaluated using descriptive analysis by a trained panel. Volatiles arising from hydrolysis treatments of aqueous extracts of the spent materials were measured using a stir bar sorptive extraction (SBSE) and gas-chromatography mass spectrometry (GC-MS).

Results of descriptive sensory analysis identified slight but unique differences among the different treatments. This suggests that the water-soluble components left behind in the spent hops may contribute to hop aroma in beer. The intensity and nature

of the hop aroma was treatment specific. Acidic hydrolysis of water soluble extracts produced the most intense *Overall* and *Pine* aroma. Aroma intensities due to the hydrolysis from the addition of enzyme preparations depended on the specific enzyme used. Aromas liberated by ale yeast produced greater intensities than the lager yeast. Additionally, all treatments showed increases in aglycone content and changes in aroma profile when treated with hydrolytic enzymes preparations.

INTRODUCTION

Brewers have traditionally added hops, in either pellet or cone form, to beer as a way to provided flavor and aroma. However, the use of pelletized and cone hops in the brewery presents certain disadvantages, such as poor extraction efficiencies, storage instability, and substantial waste handling to name a few. As an alternative, various hop extracts provide a cost effective way to mitigate many of the challenges associated with traditional hop products. After the initial picking and kilning process, hops can be extracted using supercritical carbon dioxide to remove the bittering acids and oil fraction[17] and depending on the parameters used during SFE, different non-polar fractions may be removed[43].

SFE extracts the non-polar compounds (i.e. oils and acids) from hops while leaving the polar compounds in the "spent" material. Previous research suggests that this spent hop material contains residual compounds that play an important role in the flavor and aroma of beer[35] and as a result several patents have been issued for the preparation and use of additional extracts prepared from the spent hop material[101,102]. Many plant systems contain non-volatile, water soluble glycosides[103,104] that vary due

to cultivar[105,106] and may contribute to aroma after undergoing hydrolysis. These compounds, such as glycosides, have been shown to exist in hops and hop spent material[101,107].

Glycosides consist of a carbohydrate moiety, bound at its anomeric carbon to the hydroxyl group of a non-sugar moiety, called an aglycone. The sugar moiety can be a monosaccharide, disaccharide, or polysaccharide. The type of glycosidic bond and sugar moiety defines the specificity of enzymes capable of hydrolyzing the bond and releasing the aglycone. The resulting metabolites of enzymatic hydrolysis fall into three distinct categories: 1) simple alcohols 2) terpene alcohols 3) and carbonyl compounds[107]. Investigations of different commercial enzyme treatments have been investigated as a means to liberate aglycones in hops[108], however, additional investigations into novel preparations were carried out in the study presented here. Aqueous extracts of the left over, spent material from pilot scale supercritical fluid CO_2 extractions (SFE) of hop pellets were used to investigate the impact of different hydrolysis treatments on the aroma and volatile profile of the water-soluble fraction of hops. Aroma profiles were evaluated using descriptive analysis by a trained panel. Volatiles arising from hydrolysis treatments of aqueous extracts of the spent materials were measured using stir bar sorptive extraction (SBSE) and gas chromatography-mass spectrometry (GC-MS).

MATERIALS AND METHODS

Experimental design

Figure 14 represents the experimental flow of various extraction methods, treatments, and analysis methods. Extraction methods and treatments are described in the respective methodology sections. Because SFE requires special equipment, hexane extractions were performed on Simcoe pellets for comparison in an attempt to establish a more readily accessible extraction method. All spent hop samples were hydro-distilled to remove residual unextracted volatiles (essential oil) and to extract the water soluble fraction. The aqueous spent hop slurry was then filtered to separate the aqueous extract from the water extracted spent hop material (residue). A screening of different glycoside hydrolysis treatments (enzymatic, acid, yeast) was performed using aqueous spent hop extracts from Simcoe hops. All treatments were compared to an untreated control using SBSE GC-MS and descriptive sensory analysis.

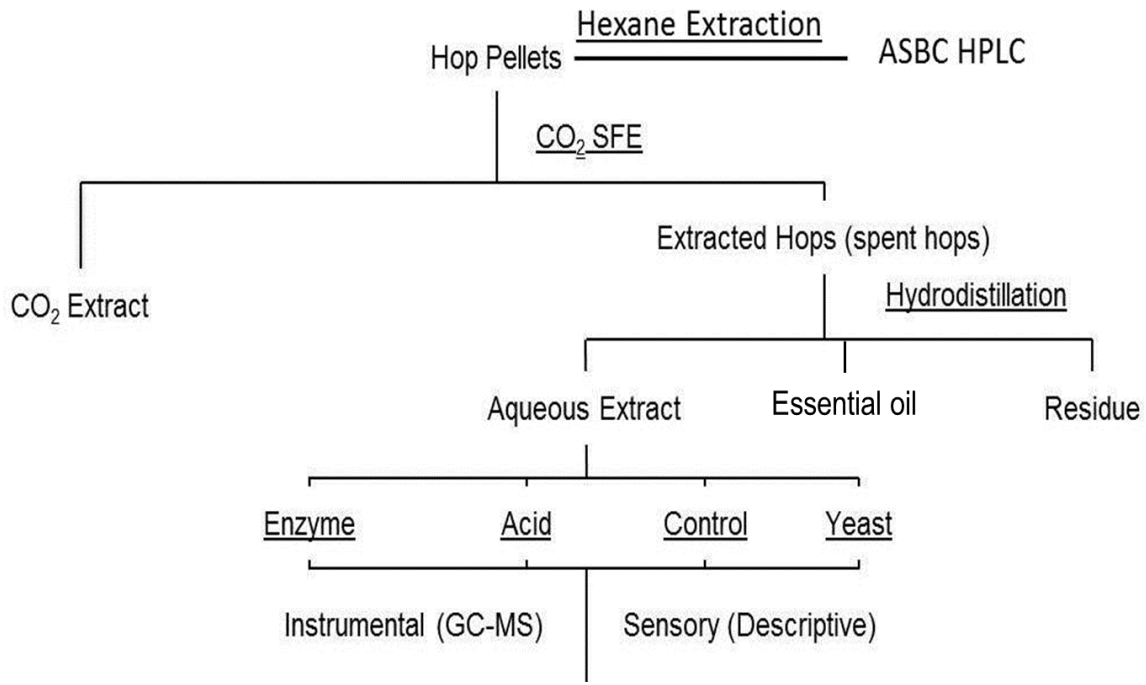


Figure 14: Extraction/treatment flow chart of materials.

Hop Products

Simcoe hops from the 2011 harvest were used to examine the effect of hydrolysis method on the aglycone contents and sensory profile of spent hop extract. Hops were pelletized and extracted using SFE by Yakima Chief Hops (Yakima, WA). Each run consisted of 400 g of raw pellets and 3 runs were performed. Pellets and the respective spent material were individually vacuum packaged into UV/vapor barrier bags and stored at -25 °C until use. Hop oil specifications for pellets and spent material were measured by hydrodistillation[92]. Hop acid specifications were supplied by the processer except for the hexane extraction study described below.

Hexane Extraction

Hexane extractions of Simcoe pellets were carried out to determine the number of hexane extractions needed to reduce residual alpha acid content to below the residual alpha acid content obtained by SFE (0.3%). Prior to extraction, two 25 g samples of Simcoe hop pellets were ground using a Magic Bullet grinder for 30 seconds and then combined. Next, 5.0 g of ground pellets were extracted with 50 ml hexane per extraction step and shaken at 300 rpm on a shaker table for 1 hour. Seven extraction steps were performed with hexane. After extraction, hops were filtered using a 9.0 cm Buchner funnel with vacuum filtration using Whatman #541 filter paper. Extracted hop powder was then washed with an additional 20 ml hexane. The hop powder was then removed and allowed to dry in a watch glass in the hood for 30 minutes. After drying, 2.5 g of extracted hop powder was weighed into a glass sample

jar and the bittering acids content was determined by HPLC using the American Society of Brewing Chemists standard method[109].

Total Essential Oil determination

Total essential oil content of hop pellets and spent hop material were determined by hydrodistillation according to the American Society of Chemists standard for oil measurements[92].

Extraction of water soluble components

Table 13: Buffer composition used for hop extraction

Brewing Salt	Concentration
KH_2PO_4	1.03 g/L
$CaSO_4$	0.31 g/L
$MgCl_2$	1.41 g/L
NaCl	0.11 g/L
$(NH_4)SO_4$	1.79 g/L

An aqueous pH 4.2 buffer solution[98] (Table 13) composed of typical brewing salts was used to extract spent hops. Preliminary hop dosing levels typically found in commercially prepared (hopped) wort (max 1 g/L) did not produce noticeable aroma attributes. For this reason, a higher dosing rate (50 g/L) was used.

Spent hops were boiled in 3 L of buffer solution for 3 hours using a large (5 L) round bottom flask and mantle at a dosing rate of 50 g spent hops per liter of buffer. This aqueous extraction was not unlike the hydrodistillation procedure for removing and quantifying total essential oils in hops. After 3 hours of boiling and subsequent cooling, the aqueous mixture was coarsely filtered through cheesecloth. The filtered

extract was then boiled briefly in a large round bottom flask for 2 minutes as a sanitation step. The extract was then cooled overnight before being divided into aliquots for sensory and instrumental analyses. Untreated samples were frozen and stored in glass vials with foiled lined lids at -25°C. The treated samples were incubated accordingly as described in the *Hydrolysis treatments* section, frozen, and stored at -25°C until analysis.

Hydrolysis treatments

Samples of the water-soluble extract were treated with either a lager yeast, an ale yeast, an acid addition, or 1 of 4 enzyme treatments. A control (not treatment) of aqueous hop extract was also prepared. Prior to treatment, samples were thawed at room temperature. An internal standard of octyl β-D-glucopyranoside equivalent to 100 µg/L 1-octanol was added to aqueous hop extract samples in order to monitor the extent of glycoside hydrolysis. Controls were prepared using the buffer solution with yeast (Ale + Buffer, Lager + Buffer) with the internal standard mentioned above and aqueous extract only (Extract) with the internal standard. In a similar fashion negative controls were prepared but with no internal standards (Ale + Buffer NoIS, Lager + Buffer NoIS). Treatment details are outlined in Table 14. Yeast control samples consisted of a yeast addition to the buffer solution only. For yeast treatments Saflager-55 dried lager yeast (Lager) or Lallemand dried West Coast Ale yeast (Ale) was dosed into the aqueous hop extract at a rate of 1.75×10^6 cells/ml. Enzymatic hydrolysis was accomplished using either purified β-glucosidase (Sigma; Sigma-Aldrich), NovArom Blanc (NB; Novozymes), Vino Taste Pro (VTP; Novozymes), or Rapidase AR2000

(Rapidase; DSM Food Specialties). Each was dosed per manufacturer's instructions. For the acid hydrolyses treatment (pH 2.7), the pH of the aqueous hop extract was reduced to 2.7 with 6 M HCl and incubated for 1 hour 90°C according to methodology by Ibarz et al. [110]. Samples were centrifuged and then frozen until analysis. All treatments plus the control were performed in triplicate. Yeast treatments in buffer were not replicated (n=1) and therefore not included in Tukey HSD tests of instrumental data.

Table 14: Aqueous Hop Extract Treatment Details

Treatment	Treatment Specs	Source	Dosage	Conditions
Saflager-55 dried lager yeast (Lager)			1.75×10^6 cells/ml	18°C for 72 hours, shaken
Lallemand dried West Coast Ale yeast (Ale)			1.75×10^6 cells/ml	18°C for 72 hours, shaken
Sigma Aldrich β-glucosidase (Sigma)	β-glucosidase ≥ 750 BDGU/g	*Aspergillus niger*	500 mg/L	30°C for 48 hours
Novozymes Novarom Blanc (NB)	β-glucosidase ≥ 200 BDGU/g	*Aspergillus niger*	1000 mg/L	30°C for 48 hours
Novozymes Vino Taste Pro (VTP)	Polygalacturonase ≥ 2500 PGNU/g, β -glucanase (exo-1,3-) ≥ 75 BGXU/g	*Trichderma harzianum, Aspergillus niger*	500 mg/L	30°C for 48 hours
Rapidase AR2000 (Rapidase)	Polygalacturonase ≥ 25,000 AVJP/g, β-glucosidase ≥ 4,000 BDGU/g	*Aspergillus niger*	1000 mg/L	30°C for 48 hours
pH 2.7 (pH 2.7)			Adjusted w/ 6 M HCl	90°C for 1 hour

BDGU= β-D-glucosidase units, PGNU = polygalacturonase units, BGXU=exo-β-glucanase units, AVJP= viscosimetric activity on apple juice. All activity units reported by suppliers.

Instrumental Analysis

Fermentable sugars in the aqueous extracts

Samples of the aqueous extracts from Simcoe spent hops were sent to the Anheuser-Busch InBev Technical Center in St. Louis, Missouri for fermentable sugar analysis by HPLC. Analysis was performed on a Perkin-Elmer model # 1020 LC Plus chromatograph. Instrument parameters are shown below. Aqueous hop extract was not diluted prior to injection while the samples of a typical wort were diluted 1:10. Quantification and identification of fermentable sugars maltotriose, maltose, fructose, and glucose were determined using external standards and calibration.

Table 15: HPLC Analysis Instrumental Conditions

Column:	Supelco apHera NH2 polymer column, 25 cm x 4.6 mm, 5 um
Colum Temperature:	80°C
Column Eluent:	Deionized, Millipore-filtered, degassed water
Flow Rate:	0.4 ml per minute
Sample Size:	10 μL
Detector:	Refractive Index
Detector Temperature:	Ambient

Stir-Bar-Sorptive-Extraction and GC-MS Analysis

Volatiles were quantified and identified using an Agilent 7890A gas chromatograph equipped with a 60m x 0.25 mm ID x 0.5 µm capillary ZB-Wax column (Zebron) using helium as the carrier gas at a flow rate of 2.5 ml/min. Compounds were identified using an Agilent 5975C single quadrupole mass spectrometer with electron impact ionization at 70 eV operating in scan mode (m/z 35-350).

Stir bar sorptive extractions (SBSE) were performed using a polydimethylsiloxane (PDMS) coated magnetic stir bar (10mm x 0.5 mm; Gerstel). Samples were diluted 1:1 with saturated NaCl solution and stirred at 1000 RPM in 40 ml amber screw top vials for three hours at 20°C. 4-octanol (Sigma-Aldrich) was added to final concentration 150 ppb to each vial as an internal standard. After extraction, stir bars were removed, rinsed with distilled water, and gently dried by blotting with lint free tissue (Kim-wipes , Kimberly-Clark) before being desorbed via a thermal desorption unit (TDU; Gerstel). Samples were desorbed according to the instrumental parameters described below for gas chromatographic separation and detection by mass spectrometry (GC-MS). All instrumental measurements were performed in duplicate.

Stir bars were placed into a Thermal Desorption Unit (TDU; Gerstel) for temperature-programmed thermal desorption. The temperature program began at 25°C and increased at a rate of 120°C /min to a final temperature of 250°C and held for 2 minutes. After desorption, analytes were cryofocused with liquid nitrogen (-80°C) in a

CIS4 programmed temperature vaporizing (PTV) injector (Gerstel). Once cryofocusing was complete, the injector inlet was programmed at a ramp rate of 10°C/s from -80°C to 250°C with a 54-minute hold at the final temperature

Standards for the following target analytes (Table 16) were purchased from Sigma-Aldrich. The purity of each standard used for quantitation was determined and used to correct concentrations for calibration curves. A standard stock solution was made in dichloromethane and added to a 5% (v/v) ethanol/water solution to obtain the following concentrations for a calibration curve: 1 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, 100 ppb, 250 ppb, and 500 ppb. All calibration solutions were analyzed according to the SBSE sample preparation and analysis methodology previously described.

ANOVA and Tukey's HSD were used to identify differences among treatments for each compound of interest. Principle component analysis of the hop volatiles identified the compounds that correlated with differences among the treatments using XLStat software.

Table 16: Target hop aroma compounds quantified by GC-MS

α Pinene	ρ-Cymene	α-Humulene	Geranyl-acetate	β-Ionone
β Pinene	Citronellal	E-β-Farnesene	β-citronellol	Caryophyllene Oxide
3-Carene	Linalool	Z-Citral	Nerol	Eugenol
Myrcene	β-Caryophyllene	α -Terpineol	β-Damascenone	α-Eudesmol
Limonene	Terpinen-4-ol	E-Citral	Geraniol	β-Eudesmol

Descriptive Sensory Analysis

A trained panel was used to describe the aroma profiles of samples due to hydrolysis method and cultivar. The panel consisted of panelists with previous experience evaluating hop aroma in beer. Frozen samples were thawed and allowed to come to room temperature 1 hour prior to evaluation. Each panelist was presented with 5 ml samples in ~ 50 ml clear glass vials capped with a polypropylene screw cap. Ambient temperature during evaluation was 20°C. The intensities of the attributes were scaled using an 8-point scale with 0 anchoring "none" and 7 anchoring "very high".

Descriptive sensory analysis of the hydrolysis treatments was performed using a panel of 9 trained panelists. Panelists evaluated the aroma of Lager, Ale, Sigma, VTP, Control, and pH 2.7 treatments. The descriptive ballot was based on 7 descriptive terms with a focus on hop-derived aromas; *Overall aroma intensity, Herbal/Iced Tea, Vegetative/Grassy, Floral, Fruity/Citrus, Pine, and Honey.* The descriptive terms were developed during the training sessions, which met 4 times over the course of 2 weeks prior to data collection. On each testing days, 6 samples were presented individually to each panelist in a panelist-specific random order. Although 10 samples were included in the overall design of the study, the yeast in buffer solution treatments (Ale + Buffer, Lager + Buffer) were determined to have no noticeable aroma early in training sessions and not included in the testing sessions to save time. Likewise, the commercial enzyme treatments were narrowed to 1 commercial enzyme and the purified β-glucosidase from Sigma Aldrich to reduce

panelist fatigue. Each sample was evaluated independently and presented in a random fashion on 4 separate days. Data were evaluated using XLSTAT 2012. Principle component analysis of the panelist data, averaged over all replications, was performed using the Varimax rotation and the covariance matrix.

RESULTS

Instrumental Analysis

Hop Products

Hop acid specifications (Table 17) were provided by Yakima Chief. Oil quantitation of the spent material, carried out at OSU, yielded less than 0.1 ml/100 g spent hops. These data confirm that spent material did not have appreciable amounts of hop oil to contribute to aroma. Furthermore, the aqueous extraction procedure used in this study was very similar to the hydrodistillation method used to remove and measure the essential oil content in hop material[92], thus we could observe whether any residual oil remained in the samples. Furthermore, any oil in the spent hops was collected in the distillate receiver during hot water extraction/hydrodistillation and not returned to the extraction mixture.

Hexane Extractions

The α and β acid percentages shown in Figure 15 are the result of successive extractions of ground pellets with hexane. Even after 7 extractions, α acid concentration was above the 0.3% level obtained from supercritical CO_2 extraction. The intent of this phase of the study was to determine the suitability of hexane extraction for separating the non-water soluble components from hops compared to

supercritical CO_2 extraction. When using bittering acids as an indicator for non-water soluble component separation it is clear that hexane extractions would be too solvent- and time-intensive as a means for resin and oil removal compared to CO_2 extraction. Therefore, SFE is recommended as way to separate non-water soluble fractions from hop pellets.

Table 17: Simcoe hop product specifications

Specification	Pellets	Extract	Spent
UV			
α-Acid(%)	11.9	47.8	0.3
β-Acid(%)	3.7	17.0	0.3
HSI	0.317	0.250	1.167
HPLC			
α-Acid(%)	11.6	41.8	0.6
β-Acid(%)	3.1	13.9	0.3
Cohumulone %	16.8	18.5	18.7
CoLupulone %	40.6	40.6	45.1
Oil (ml/100g)	1.80	Not measured	<0.1

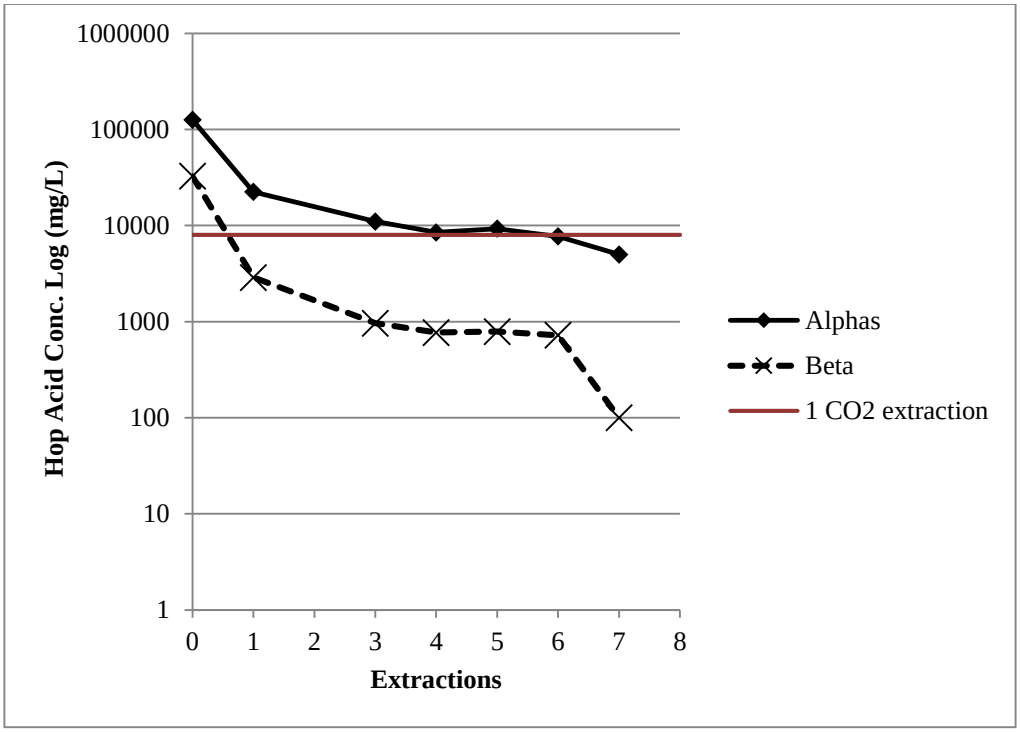


Figure 15: Hop acids concentration after successive hexane extractions in relation to concentration after one extraction by CO_2 SFE.

Fermentable Sugar Analysis

Analysis of fermentable sugars by HPLC was performed courtesy of Anheuser-Busch InBev Technical Center in St. Louis, Missouri. Figure 16 shows the chromatogram of fermentable sugars in aqueous spent hop extract and typical wort. Note, that the chromatogram of the typical wort was diluted 1:10 with water while the extract sample was not diluted. Although, the response from the spent hop extract was below the lowest calibrated concentration point for the calibration curve (1g/L), the fermentable sugar concentration in the hop extract was estimated to be lower than 2 g/L. Future investigation with more sensitive carbohydrate analysis is necessary to determine conclusively if the water soluble fraction of hops contributes significant amounts of fermentable carbohydrates. However, considering the high levels of spent hops used in this study it is likely that any contribution from hops to carbohydrates capable of being fermented by brewing yeast is negligible.

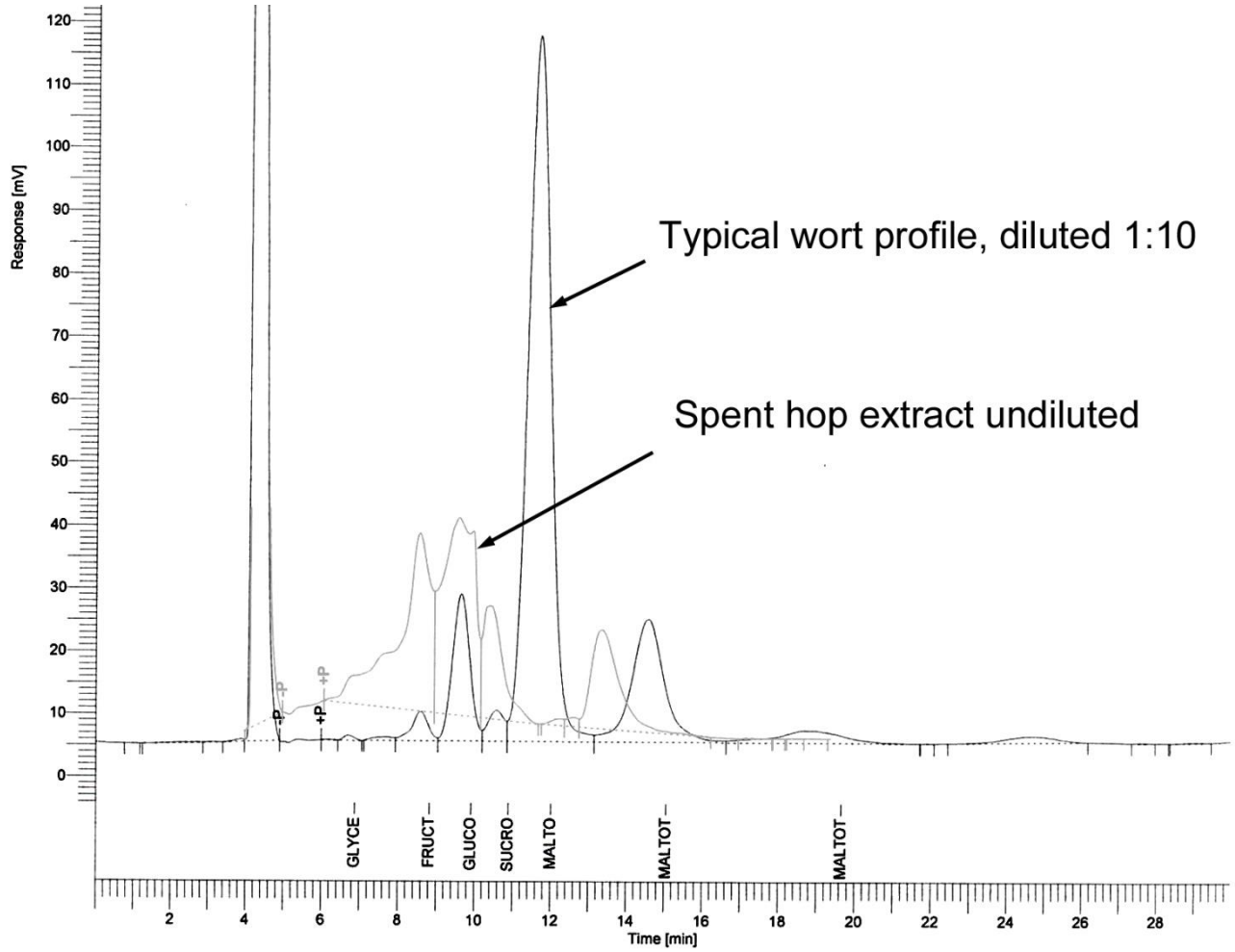


Figure 16: HPLC chromatogram of fermentable sugars in aqueous extract (no dilution) and typical wort (1:10 dilution).

Internal Standard Analysis

The extent of glycoside hydrolysis was estimated by examining the hydrolysis of the internal standard octyl β-D-glucopyranoside relative to its aglycone 1-octanol (Figure 17). From a statistical standpoint (Table 18), no difference in the extent of hydrolysis (i.e. amount of 1-octanol following treatment) was found among the Ale, Sigma, VTP or NovoBlanc treatments. Rapidase was found to have significantly less 1-octanol than Ale and Sigma treatments, but not NB or VTP treatments. Lager yeast treatments had significantly lower amounts of 1-octanol than all the enzyme treatments and the Ale yeast but more than the Extract (control) and pH 2.7 treatments. It is clear that the lager and ale yeasts had lower activities relative to their positive controls (Ale in Buffer/Lager in Buffer). However, contradictory results were observed for the pH 2.7 sample, which had very little 1-octanol yet showed high amounts of other aglycones indicative of glycoside hydrolysis (Table 19).

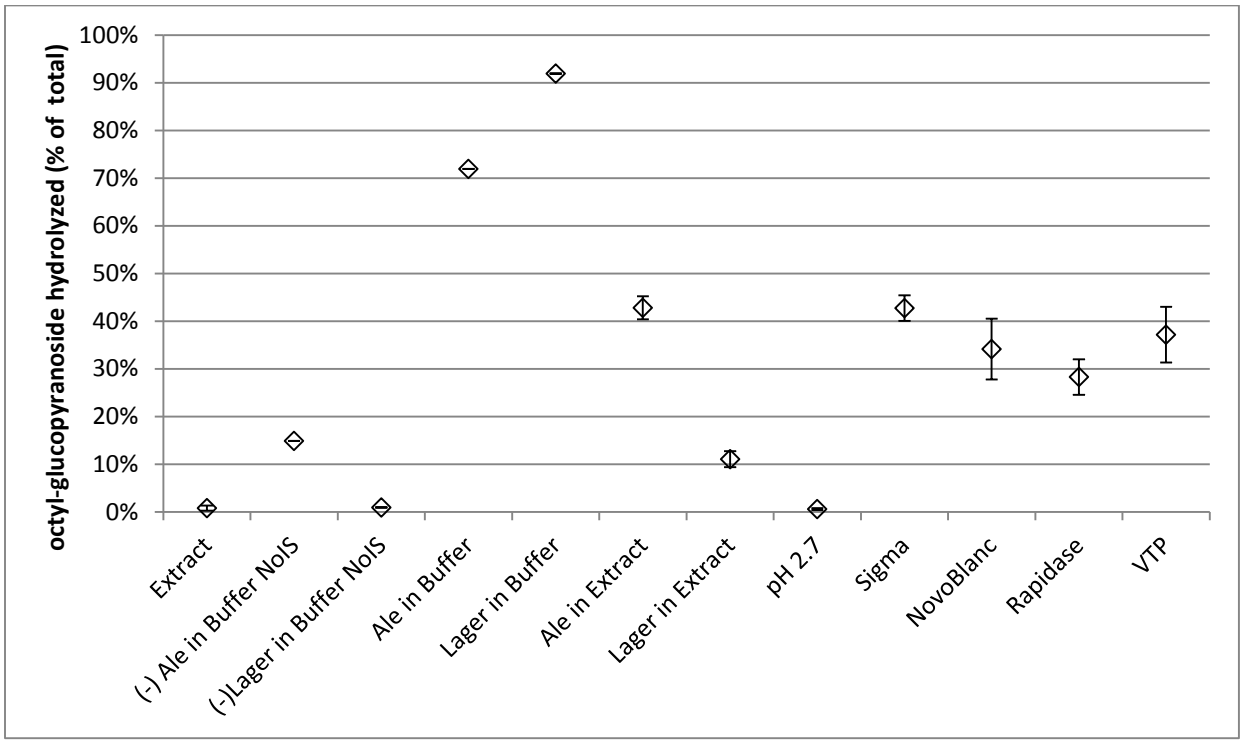


Figure 17: Percentage of 1-octanol remaining after treatments as measured by SBSE GC-MS, n=3. Yeast in buffer treatments n=1. Error bars = standard deviation.

Table 18: Tukey's pairwise comparisons of extent of hydrolysis treatments

Treatment	Mean (%)	Groups			
Ale	42.8	A			
Sigma	42.7	A			
VTP	37.2	A	B		
NovoBlanc	34.1	A	B		
Rapidase	28.3		B		
Lager	11.0			C	
Extract	0.8				D
pH 2.7	0.6				D

Aglycone Analysis

Concentrations of hop aroma compounds determined by SBSE GC-MS are summarized in Table 19. α-pinene, β-pinene, 3-carene, myrcene, limonene, ρ-cymene, citronellal, E-β-farnesene, geranyl-acetate, and β-ionone were not detected in any treatment. Rows shaded in gray highlight those compounds that displayed significant and practical differences among treatments. The control sample had low levels of linalool, β-caryophyllene, α-humulene, Z-citral, α –terpineol, E-citral, β-citronellol, geraniol, caryophyllene oxide, eugenol, and α-eudesmol. This indicates that low levels of aroma compounds can still be found in the spent hop material despite having been extracted first by supercritical CO_2 and then hydrodistillation.

In terms of treatment effects, no difference was observed for α-humulene, α-eudesmol, or β-eudesmol for all treatments. Additionally, although differences were observed among treatments for β-caryophyllene, Z-citral, and E-citral, it is unlikely that a perceivable sensory difference would be detected among samples with less than a 2 ppb difference in concentrations, with the exception of β-damascenone which has an extremely low odor threshold. Interestingly, the Rapidase, Vino Taste Pro, Sigma Aldrich enzyme treatments and the acid treatment had the highest levels of linalool while the yeast treatments were significantly lower. Furthermore, the lager yeast treatment had significantly less linalool than the original control base, which indicates that the fermentation process actually decreased linalool concentration. A significantly higher concentration of β-citronellol was observed in the both the lager and yeast treatments than in the control and all other treatments. However, no significant

difference in concentrations of other compounds was found between either of the yeast treatments and the control. This decrease in linalool and increases in β-citronellol could be attributed to hydrophobic adsorption to the yeast membrane or by further biotransformations into β-citronellol or nerol by yeast as observed by Takoi et al.[111] and King et al.[112]

In general, enzyme treatments had significantly higher levels of geraniol, linalool, and eugenol, while the pH 2.7 treatments had significantly higher levels of β-damascenone, terpinen-4-ol and α –terpineol. In fact, over a 15-fold increase in α –terpineol was observed for the acid treated sample over the control sample. Increased concentrations of α –terpineol in acidic conditions have been reported elsewhere[113] which may partially explain the high level of α –terpineol found in the acid hydrolyzed treatments. Enzymatic treatments all produced significantly higher concentrations of geraniol than the acid treatments and aqueous extract.

Table 19: Concentration (µg/L) of aroma compounds in Simcoe aqueous spent hop extracts.

Compound	Control (Base)	Ale Yeast	Lager Yeast	Rapidase	Vino Taste Pro	Sigma Aldrich	NovoBlanc	pH 2.7	Lager + Buffer	Ale + Buffer
Linalool*	9.45^{c}	$8.14^{c,d}$	1.99^{d}	$24.48^{a,b}$	27.99^{a}	$27.15^{a,b}$	12.66^{c}	20.52^{b}	ND	ND
β-Caryophyllene*	5.13^{c}	5.13^{c}	5.05^{c}	5.25^{c}	5.17^{c}	6.63^{b}	5.12^{c}	7.54^{a}	ND	ND
Terpinen-4-ol*	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	0.38^{b}	ND^{b}	3.84^{a}	ND	ND
α-Humulene^{NS}	0.07	0.06	0.04	0.10	0.05	0.14	0.05	0.06	ND	0.07
Z-Citral*	0.35^{c}	$0.57^{b,c}$	0.31^{c}	$0.88^{b,c}$	$1.61^{a,b}$	$0.96^{b,c}$	2.66^{a}	$0.68^{b,c}$	0.15	0.37
α –Terpineol*	8.82^{d}	7.10^{d}	6.49^{d}	24.90^{b}	$11.68^{c,d}$	$21.08^{b,c}$	8.45^{d}	155.01^{a}	ND	ND
E-Citral*	$0.85^{a,b,c}$	$0.60^{b,c}$	0.56^{c}	$1.01^{a,b}$	$0.68^{b,c}$	1.27^{a}	$0.74^{b,c}$	0.47^{c}	0.26	0.24
β -citronellol*	0.31^{b}	3.88^{a}	2.67^{a}	1.06^{b}	1.01^{b}	1.26^{b}	0.68^{b}	1.07^{b}	0.09	1.15
Nerol*	ND^{c}	0.94^{c}	0.06^{c}	4.82^{a}	3.03^{b}	2.99^{b}	3.17^{b}	ND^{c}	ND	ND
β-Damascenone*	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	ND^{b}	2.89^{a}	ND	ND
Geraniol*	24.71^{c}	21.30^{c}	$17.02^{c,d}$	48.05^{a}	39.54^{b}	$42.97^{a,b}$	50.32^{a}	10.03^{d}	ND	ND
Caryophyllene Oxide*	2.60^{b}	2.47^{b}	2.46^{b}	2.58^{b}	2.54^{b}	5.09^{a}	2.41^{b}	2.70^{b}	ND	2.11
Eugenol*	3.21^{c}	3.23^{c}	3.14^{c}	10.16^{a}	9.46^{a}	10.30^{a}	6.02^{b}	3.20^{c}	1.30	1.49
α-Eudesmol^{NS}	0.26	0.23	0.18	0.15	0.09	0.23	0.16	0.46	0.09	0.76
β-Eudesmol^{NS}	ND	0.11	0.01	ND	ND	0.01	ND	ND	ND	0.43

α-Pinene, β-Pinene, 3-Carene, Myrcene, Limonene, ρ-Cymene, Citronellal, E-β-Farnesene, Geranyl-acetate, and β-Ionone were not detected.
*****attributes are significant $p < 0.001$. ^{NS} compounds are not significant ($p > 0.05$). Sample means with different superscripts within a row are significantly different from one another at $p < 0.05$ by Tukey's HSD test. ND=not detected.

Principle Components Analysis

Investigation of the PCA of the instrumental data reveals that PC 1 is anchored in the positive direction by Z-citral, linalool, eugenol, and to a lesser extent E-citral. The commercial enzyme treatments (VTP, NovoBlanc, and Rapidase) were all associated with these compounds. The negative PC 1 axis was anchored by β-citronellol and β-eudesmol and associated with Ale and to a lesser extent the Lager treatments. The positive axis of dimension 2 was anchored by α-terpineol, terpinen-4-ol, caryophyllene, and and β-damescenone. These compounds were associated with the pH 2.7 treatment. Finally, the Sigma treatment was associated with caryophyllene oxide, and α-humulene each of which anchored dimension 3 in the primary axis.

The PCA identified four groups of treatments. Three of the enzyme treatments (Rapidase, Vino Taste Pro, and NovoBlanc) group together and were in a quadrant opposite to the yeast group (ale and lager). The Sigma Aldrich enzyme sat by itself along PC3 and the pH 2.7 treatment sat by itself along PC2. The control was positioned at the origin of the PCA axes. Interestingly, the instrumental data from the hop extract hydrolysis contradicts (to some extent) the hydrolysis of the internal standard. For instance, the pH 2.7 treatment had very high levels of α-terpineol and β-damescenone while at the same time had the lowest internal standard hydrolysis activity. It should be noted that the internal standard hydrolysis behavior does not represent the differences seen in the hop volatiles.

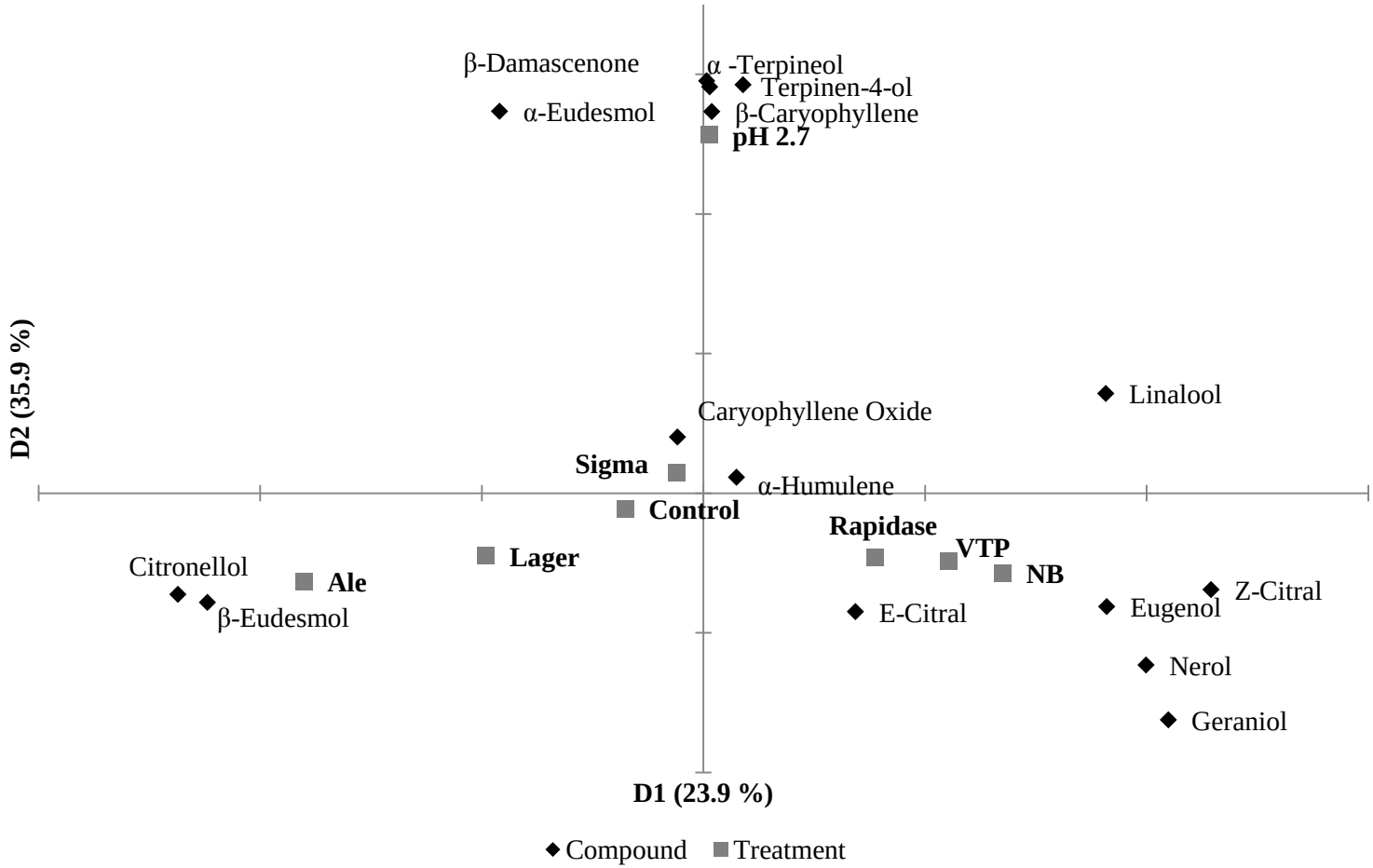


Figure 18: Principle component analysis of instrumental data. Dimension 1 and 2 account for 59.8% of the variation. Dimensions 1, 2 and 3 account for 86.2 % of the total variation.

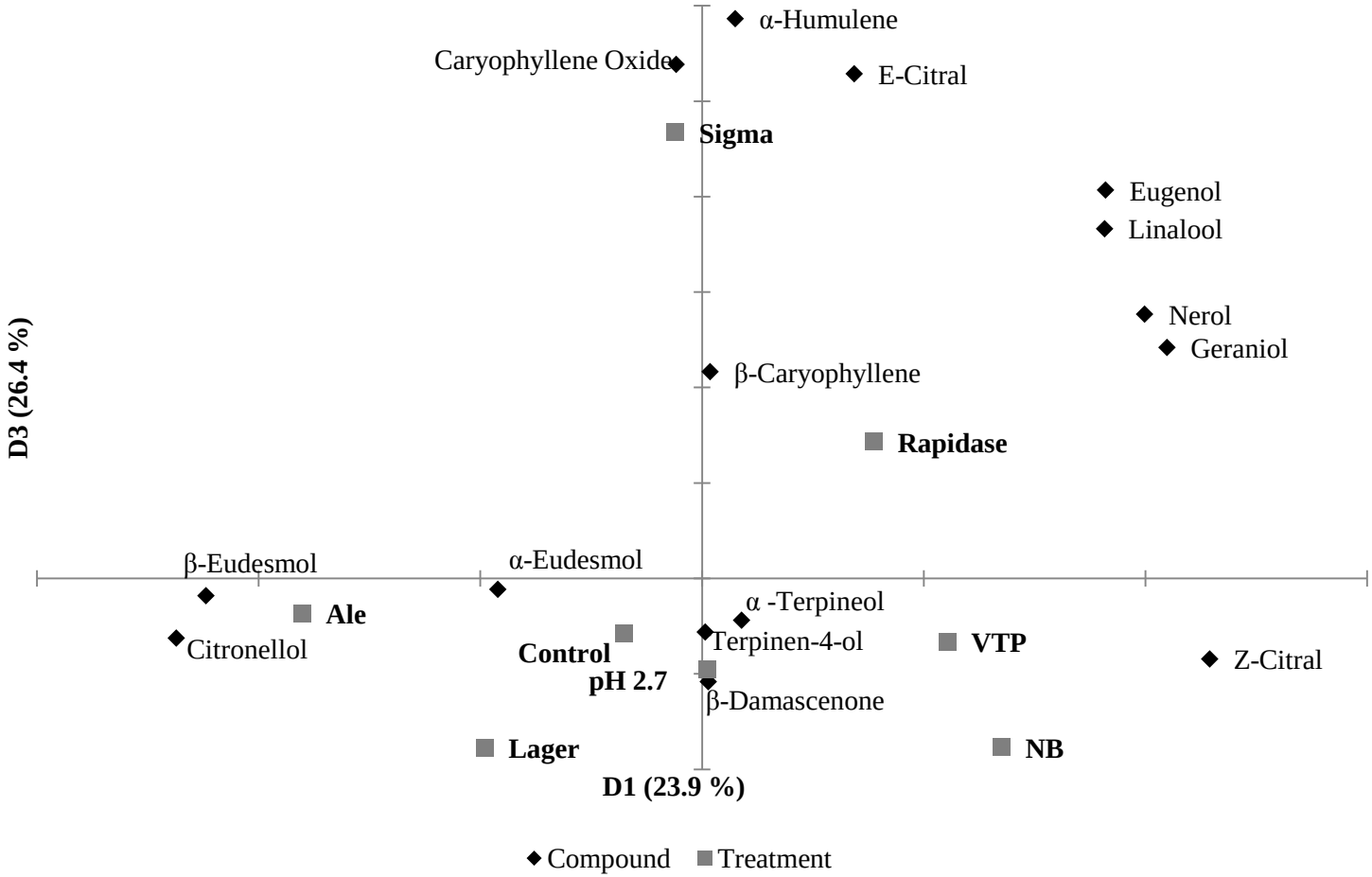


Figure 19: Principle component analysis of instrumental data. Dimension 1 and 3 account for 50.3% of the variation. Dimensions 1, 2 and 3 account for 86.2 % of the total variation.

Sensory analysis of Spent Hop Extracts

Panel Analysis

Mixed model analysis of variance examining panel performance revealed significant repetition x treatment, repetition x panelist, and panelist x treatment interactions in addition to significant main effects for many of the descriptors (data not shown). Closer examination revealed the first testing session (rep 1) was significantly different than other testing sessions for each treatment and panelist, an indication that the panel was unfamiliar with the testing protocol and format. For this reason, results from the first testing session were removed prior to further statistical analysis. Mixed model analysis of variance using testing sessions 2 through 4 of panelist, treatment, and repetition effects and interactions are shown in Table 20. A significant sample by panelist interaction effect was observed for Overall, Floral, Fruity/Citrus, and Pine descriptors. However, this common in sensory testing due to panelists perceiving the samples differently and utilizing the scales differently from one another depending on the treatment.

Enzyme treatments

Despite the low levels of aroma compounds measured instrumentally, the samples were not devoid of aroma and many had a perceptibly unique aroma. Mean rating, ANOVA and Tukey-Kramer multiple comparison test results for descriptive analysis of the treatments are shown in Table 21. For all treatments, no significant difference was found between ratings for treatments for Vegetative/Grassy, Floral, Citrus, and Honey descriptors. The pH 2.7 treatment scored highest and was rated

significantly higher than all the other treatments for the *Pine* descriptor and was one of the highest in *Overall* aroma. This agrees with the relatively high levels of α-terpineol in the pH 2.7 treatments, a compound with a pine like characteristic[95]. In terms of *Overall* aroma intensity, no significant difference was found between the control, VTP, lager, or ale treatment.

Table 20: F-values of mixed model analysis of variance of descriptive attributes. Panelist factor = random. **Bold =** significant at p-value < 0.05.

	DF	Overall	Herbal/ Iced Tea	Vegetative/ Grassy	Floral	Fruity/Citrus	Pine	Honey
Panelist	8	**12.5**	**21.9**	**7.1**	**12.3**	**10.1**	**15.7**	**9.2**
Sample	5	**7.5**	**3.7**	1.6	1.1	2.1	**3.2**	1.1
Rep	3	1.0	1.0	0.3	0.4	0.1	1.3	0.6
Rep*Sample	15	1.8	1.5	0.6	0.8	0.9	0.9	1.0
Rep*Panelist	24	0.7	1.5	1.5	0.8	1.1	0.9	0.7
Sample*Panelist	40	**1.7**	1.3	0.9	**1.6**	**2.0**	**1.6**	0.9

Table 21: Summary results of descriptive sensory analysis.

	Treatment					
Attribute	pH 2.7	Sigma	Ale	Lager	VTP	Control
Overall Aroma Intensity*	5.1[a]	4.6[b]	4.4[b]	4.2[b]	4.1[b]	4.1[b]
Herbal/Tea*	4.1[a]	3.8[a,b]	3.6[a,b]	3.3[b]	3.9[a,b]	3.3[b]
Vegetative/Grassy[NS]	3.4	3.1	2.4	2.8	3.1	3.1
Floral[NS]	2.2	2.4	2.3	2.5	2.3	1.9
Fruity/Citrus[NS]	2.7	2.6	2.6	2.6	2.0	2.0
Pine*	1.9[a]	1.4[a,b]	1.3[a,b]	1.0[b]	1.3[**a,b**]	1.3[a,b]
Honey[NS]	2.4	2.3	2.4	2.0	2.0	2.0

***** attributes are significant at $p < 0.001$. [NS] attributes are not significant ($p > 0.05$). Sample means with different superscripts within a row are significantly different at $p < 0.05$ by Tukey's HSD test.

The principle components analysis of sensory data displayed interrelationships among the target compounds and the treatments. The first dimension was anchored primarily in the the postive direction by the *Pine* descriptor. The second dimension was anchored primarily by the *Fruity/Citrus* descriptor. The pH 2.7 treatment was rated significantly higher than all other treatments for *Pine*, and at least as intense or higher than all other treatments for *Overall* aroma. The extent of the differences in the hop aroma intensity coming from the pH 2.7 treatment resulted in it behaving somewhat like an outlier relative to all other treatments. This is particularly obvious when examining the PCA biplots. It sits in the far right region of the PC space in Figure 20 and drives the differences along PC 1, much as it did in the instrumental results. Clearly, acid hydrolysis of glycosides produces a set of hop aromatics that are relatively more potent, particularly for *Pine* like notes. The overall aroma was most likely driven by the higher pine notes in the pH 2.7 treatment relative to the other treatments. To overcome the strong influence of the pH 2.7 treatment in the PCA, it was removed and the analysis repeated (Figure 21).

With pH 2.7 treatment removed, the PC 1 was anchored in the positive direction by honey and herbal/tea descriptors. The Sigma treatment, and to a lesser extent the Ale treatment, was correlated with *honey, herbal/tea*, and *floral* descriptors. Within the enzyme treatments, the Sigma product (purified β glucosidase) yielded greater intensity of hop aroma than the VTP (blend of β glucosidase and pectinase). The ale yeast yielded greater aroma than the lager yeast.

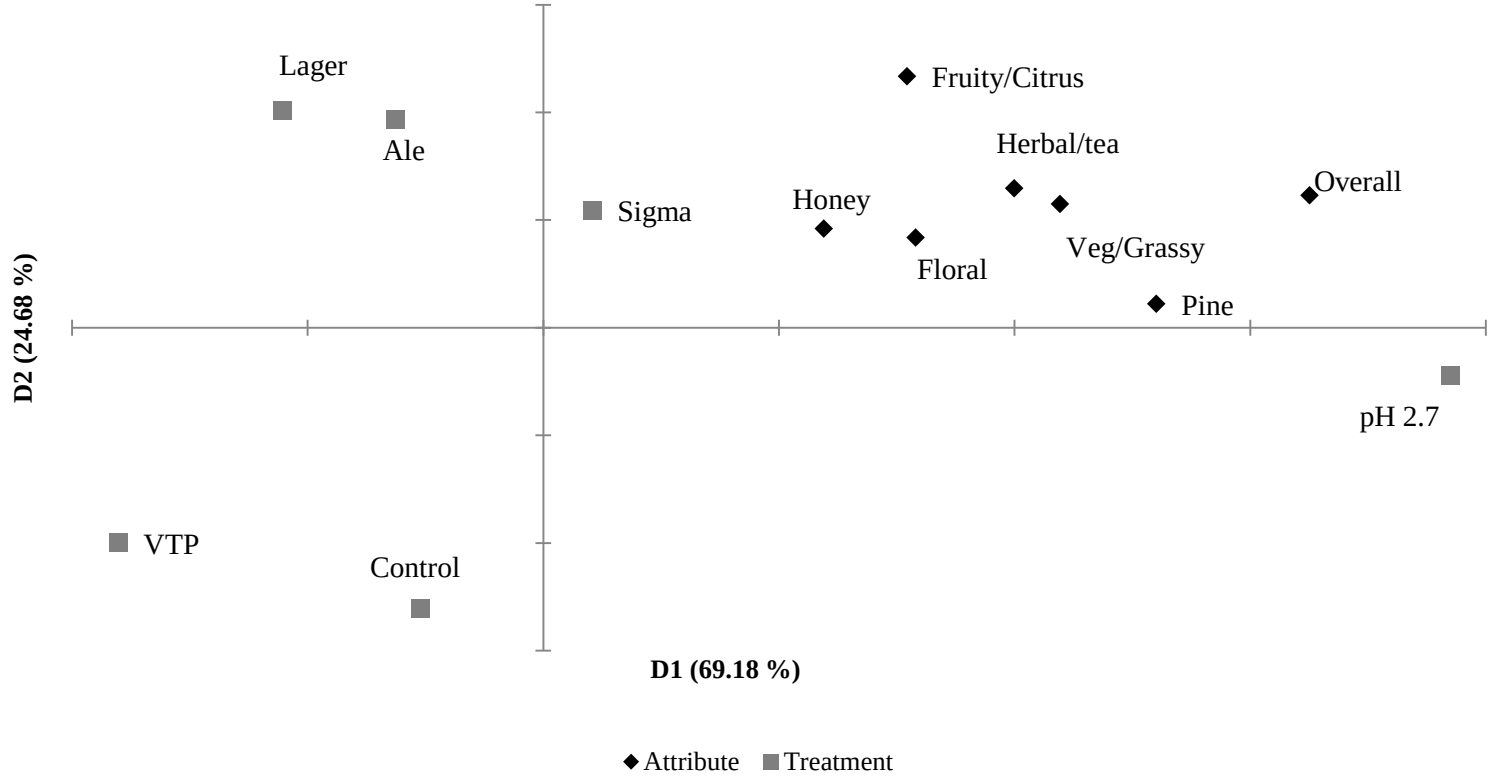


Figure 20: Principle component analysis of sensory descriptive data with pH 2.7 treatment. Dimensions 1 and 2 account for 94% of the total variation (w/ pH 2.7 treatment).

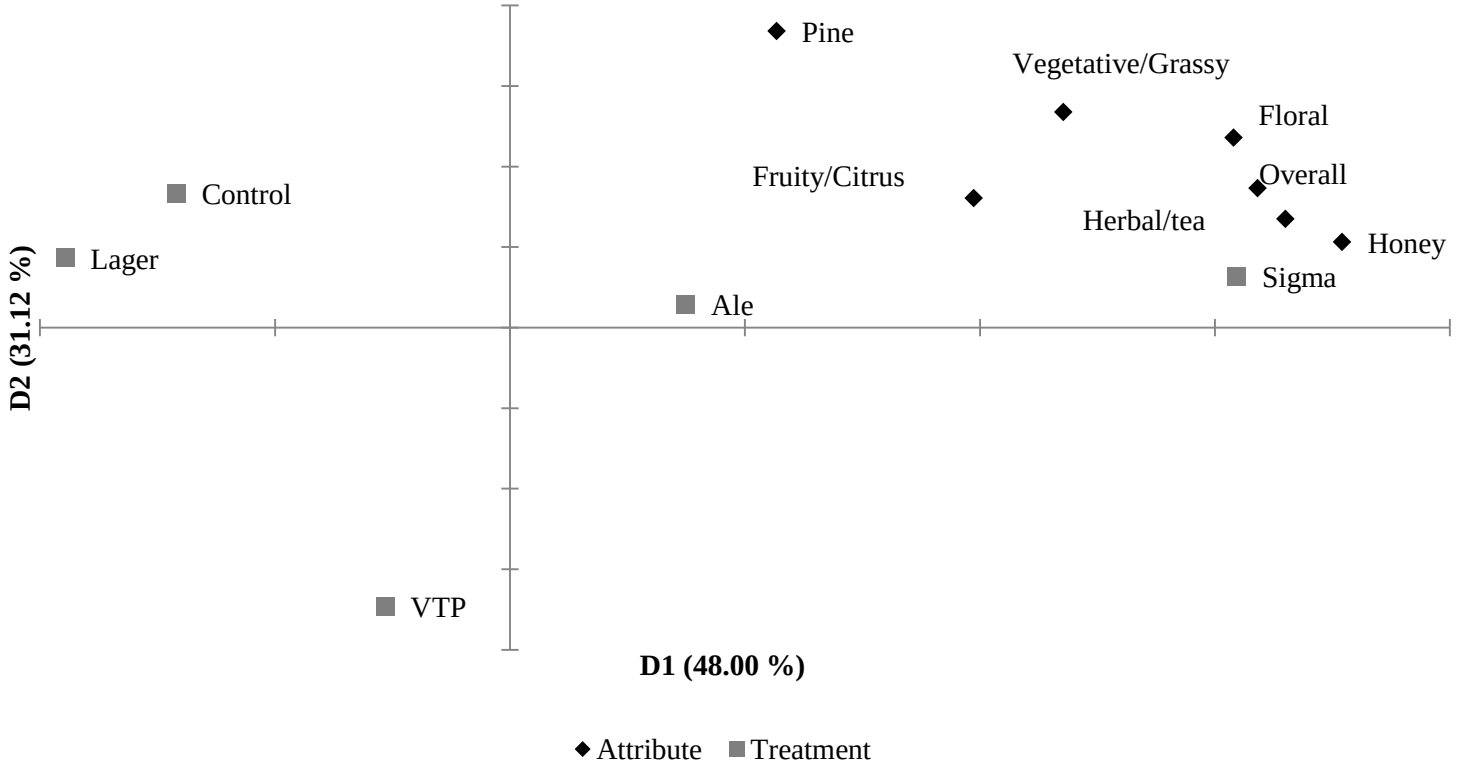


Figure 21: Principle component analysis of sensory descriptive data without pH 2.7 treatment. Dimensions 1 and 2 account for 79% of the total variation (w/o pH 2.7).

DISCUSSION

Spent hop material yielded detectable amounts of hoppy aroma to an aqueous buffer system that had not been hopped with traditional (oil-containing) hop material. However, the hop dosing rates evaluated in this study (50g/L) far exceeds normal hopping regimes in traditional brewing practices (1 g/L -5 g/L)[38]. Yet even at these high dosages, relatively low levels of volatile aroma compounds were measured using instrumental techniques (all compounds in the low µg/L range). Although statistical differences among treatments were found for caryophyllene, caryophyllene oxide, E- and Z-citral, and α-humulene, the differences were quite small and not likely to have a have significant contribution to the sensory profile. However, this is to be expected since these compounds are not derived from glycoside hydrolysis and found primarily in the essential oil fraction of hops. The relatively low concentrations of terpenes, sesquiterpenes, and sesquiterpenoids found in the spent hop extracts for all treatments and the control indicates that most of these compounds were removed during SFE and aqueous extraction. In comparison, significant increases in the concentrations of terpene alcohols was present among hydrolysis treatments and relative to the untreated control. The differences among the hydrolysis methods investigated in this study provide different options for determining the content and profile of glycosidically bound terpene alcohols in hops.

From this study, it is quite apparent that the extent of hydrolysis/release of aroma aglycones is yeast strain dependent as shown by the differences between lager and ale treatments. However, it is also clear that aroma profiles produced by

enzymatic hydrolysis, and especially acid hydrolysis, are not comparable to yeast derived hydrolysis. On the one hand, the use of purified β-glucosidase from Sigma Aldrich, may be a useful indicator of the aroma potential derived from β-D-glucosides only. While on the other hand, the use of a multi-enzyme preparation, such as Rapidase, may be useful for investigating the contributions broader range of glycosides. In contrast, fast acid hydrolysis offers a relatively quick gauge of the total content of glycosidically bound terpene alcohols. Though results from this study and others[108,114] shows increases in the terpene alcohol content of aqueous spent hop extracts treated with commercial enzymes, there is no consensus on the actual mechanism responsible for glycoside hydrolysis and the role of their contribution to hop-derived aroma in beer. While, many wine yeasts are capable of hydrolyzing glycosides in grape must[115], the contributions from brewing yeasts are not clear. Daenen et al.[99] screened 58 yeasts that are commonly found in brewing environments to characterize their ability to hydrolyze glycosidically bound substrates. The results of the screening showed a strain dependent activity for *Saccharomyces* strains from the enzyme exo-1,3- β -glucanase. Only some of the *Saccharomyces* strains screened showed specific 1,4 β-glucosidase activity. Alternatively, *Brettanomyces* yeasts showed increased glucosidase activity compared to the *Saccharomyces* strains, in particular *Br. Custerii*. However, among the enzyme treatments used in this study, aglycone concentrations were lowest for the preparation containing exo-1,3 β -glucanase which suggests that although yeasts may contain the ability to hydrolyze glycosides using exo-1,3 β -glucanases, their specificity for substrates found in hops

that contain terpene alcohols is lower compared to β-glucosidase specific activity (VTP; Table 14).

Kanauchi and Bamforth[65] showed that brewing yeasts do have the ability to hydrolyze the β-linkage between the parent sugar molecule and the aglycones and that the enzyme responsible for glycoside hydrolysis is located primarily intracellularly. Since the extent of hydrolysis is dictated by the presence of the enzyme found primarily with the yeast cell, then its ability to hydrolyze aroma compounds would be relatively low. Therefore, hydrolytic activity could be attributed to cellular leakage, or the result of autolysis during fermentation that would effectively leak enzymes into the medium. Furthermore, the extent of hydrolysis as a function of yeast strain also dictates the contribution to overall hop flavor, as shown by the differences in terpene alcohol concentrations between the ale and lager treatments used in this study. Therefore, contributions to hop aroma from glycosides hydrolyzed by different yeast strains must be examined on a per yeast basis. Additionally, previous research examining differences among German cultivars has shown that while the same aglycones were found in all of the observed cultivars, the concentrations of the specific aglycones differed among them[114]. Finally, the presence of β-damascenone in the acid treated samples supports findings comparing fresh and aged beers[116] and may be an indicator of the gradual acid hydrolysis of glycosides or other precursors over time.

CONCLUSIONS

Removal of non-polar components from hop pellets by hexane extraction proved too solvent intensive to be practical compared to SFE. Aqueous extracts of spent hop material (50g/L) contained less than 2 g/L of fermentable carbohydrates. Results from this project suggest that hop aroma is slightly influenced by the non-oil components present in hops. Based on sensory and instrumental analysis, it is clear that aroma compounds are indeed liberated upon treatment with exogenous enzymes. The sensory data pointed to the pH 2.7 treatment as being a standout relative to the other treatments. It yielded the greatest overall and piney hop aroma intensity. The presence of β-damascenone and increases in α-terpineol produced by acid hydrolysis of aqueous hop extracts supports previous research suggesting that beer storage may lead to hydrolysis of glycosides. Enzymatic treatments produced different terpene alcohol profiles depending on the enzyme used. Finally, differences in terpene alcohol content of the different yeast strains indicates variability among yeast strains to hydrolyze glycoside from hops. While increases in hop-derived volatiles in spent hop extracts appears to be a combination of both acid and enzymatic hydrolysis, the respective kinetics, time scale and contributions of these hydrolysis phenomenon are unclear. Additionally, the overall contribution of glycosides to conventionally hopped beers should not be overstated. Dosage rates used in this study were many times higher than those used in brewing yet modest increases to terpene alcohol concentrations were observed. In this light it likely that the impact of non-oil

components to hop aroma is small compared to the contributions from oil derived aromatic compounds that predominate total hop aroma.

ACKNOWLEDGEMENTS

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Chapter 5 - The effect of hopping regime, cultivar and β-glucosidase activity on monoterpene alcohol concentrations in beer.

Daniel C. Sharp, Jan Steensels and Thomas H. Shellhammer

ABSTRACT

Previous studies show that the complexity of hop aroma in beer can be partly attributed to the hydrolysis of glycosidically bound monoterpene alcohols extracted from hops during the brewing process to release volatile aglycones. However, fundamental studies that examine the extraction of glycosides during brewing and their subsequent hydrolysis by yeast have not been fully investigated. Furthermore, extraction of other hop-derived compounds into beer show a strong dependency on the hop cultivar being used and the point at which it is added. Therefore, this study focused on the extent of glycoside extraction due to hopping regime, cultivar, and their hydrolysis due to yeast β-glucosidase activity. Glycoside concentration of worts made with three different hopping regimes and three cultivars was measured. Additionally, β-glucosidase activities for 80 different yeast strains and their effect on aglycone concentration in wort was determined. Glycoside content was measured by the difference in volatile aglycone concentrations between samples treated with purified β-glucosidase and untreated samples. Aglycone concentration was measured by SPME GC-MS. Results showed that yeast have a wide range of abilities to hydrolyze glycosides with a maximum hydrolysis occurring after three days of fermentation regardless of yeast activity. Although it was shown that yeast are capable of glycoside hydrolysis, glycoside concentrations in wort are low and have small contributions to

hop aroma. These results help explain the extent to which different brewing yeasts and hopping regimes contribute to hoppy beer aroma through the hydrolysis of non-volatile hop-derived compounds.

INTRODUCTION

The complex flavor profiles of beer can be attributed to the diverse range of ingredients available to brewers. Of the 4 basic ingredients used in beer, water, malted barley, hops and yeast, the latter two are used in relatively small amounts compared to the former, yet their contributions to aroma and flavor are quite substantial and beers made without them would hardly be recognizable. With regard to hops, the majority of aroma is derived from the hop essential oil fraction[12] while aromas generated by yeast are mostly byproducts of aerobic respiration and anaerobic fermentation. However, previous researchers have shown that interactions between hops and yeast also increase or modify the chemical profile responsible for the aroma properties of beer[51].

A diverse class of compounds, called glycosides, is present in beer, the bulk of which are derived from the hydrolysis of β-glucans found in barley[117], however hops have been shown to contain glycosides[35,105] capable of being hydrolyzed into volatile aglycones. By definition, a glycoside contains at least one monosaccharide moiety (glycone) linked at its anomeric carbon (α or β configuration) to the oxygen of a hemiacetal hydroxyl group of another moiety (aglycone). Due to their inter-glycone linkages, polysaccharides also fall into the broadest definition of a glycoside, however for the purpose of this study, a glycoside is defined as a linkage between a sugar moiety and non-sugar moiety. Furthermore, the range of glycosidically associated

aglycones found in hops appears to be quite diverse[35] including polyphenols, however those that are classified as monoterpene alcohols are of particular importance due to their connection to hop aroma[12,49].

Glycosides can be hydrolyzed by acid induced hydrolysis[62,118] or by enzyme catalyzed hydrolysis. The latter case first requires the hydrolysis of any inter-saccharide bonds of oligosaccharides, if present, to yield a monosaccharide, commonly β-D-glucose, linked to an aglycone followed by subsequent hydrolysis of the glucosidic linkage[62]. β-glucosidase is capable of hydrolyzing β-D-glucosides, however these enzymes exhibit a range of substrate specificity depending on their origin[119]. Once hydrolyzed, the aglycone is released and, if volatile, contributes to aroma. An example of this is the glycoside S-linalyl-β-D-glucopyranoside, which is not aromatic, but when the glucose is cleaved it releases linalool, which is very aromatic.

Many interactions between hops and yeast have been investigated[59], however the contribution of hop-derived glycosides to aroma in finished beer upon yeast hydrolysis remains unclear. In contrast, significant research on the topic in other fermented beverages, such as wine[64,104,120–122], shows clear contributions from hydrolyzed glycosides to aroma. Besides work by Daenen et al.[99] and Kanauchi and Bamforth[65] little work has been done investigating the β-glucosidase activity of different brewing related yeast strains in relation to hop-derived aroma in beer.

In this study, we report the β-glucosidase activities of a diverse range of brewing yeasts compared to the activities of non-brewing related yeasts, the timing of

glycoside hydrolysis during common ale fermentation conditions, and the role of hopping regime and hop cultivar on the extraction of terpene-alcohol glucosides

MATERIAL AND METHODS

Yeast Screening of β-Glucoside Hydrolysis Activity

Yeast selection

Eighty different yeast strains were obtained from both commercial and research yeast suppliers in order to measure their β-glucoside hydrolysis activity. Yeast strains included brewing related strains, such as ale, lager, and *Brettanomyces spp.*, as well as wine yeasts and other non-brewing related yeasts. Yeast isolates were supplied by Lallemand (Canada) and Wyeast (Hood River, OR) and the Verstrepen VIB Systems Biology lab group (VIB; Katholieke Universiteit Leuven, Belgium). Yeast type and origin was reported by the supplier (Table 22). Yeast isolates were stored on Yeast Peptone Dextrose (YPD) slants at 3° C until use.

Table 22: Information of yeast used for β-glucosidase screening

Supplier	Species as reported by supplier	Origin/ID	Quantity
Lallemand	*Saccharomyces cerevisiae*	Ale	1
Lallemand	*Saccharomyces cerevisiae*	Ale	17
Lallemand	*Saccharomyces cerevisiae*	Baking	1
Lallemand	*Saccharomyces cerevisiae var bayanus*	Champagne	2
Lallemand	*Saccharomyces cerevisiae*	Kölsch	1
Lallemand	*Saccharomyces cerevisiae*	Lager	5
Lallemand	*Saccharomyces boulardii*	Probiotic	1
Lallemand	*Saccharomyces cerevisiae*	Wheat beer	2
Lallemand	*Saccharomyces cerevisiae*	Wine	24
Lallemand	*Saccharomyces cerevisiae S.uvarum*	Wine	1
Lallemand	*Saccharomyces cerevisiae var bayanus*	Wine	8
VIB	*Saccharomyces cerevisiae*	Ale	7
VIB	*Dekkera anomala*	Brett(ale)	2
VIB	*Dekkera bruxellensis*	Brett(ale)	1
VIB	*Candida versatilis*	Cucumber Brine	1
VIB	*Kluyveromyces marxianus*	Figs	1
VIB	*Scheffersomyces stipitis*	Insect	1
VIB	*Saccharomyces pastorianus*	Lager	1
VIB	*Debaryomyces nepalensis*	Sake	1
VIB	*Saccharomyces cerevisiae*	Wine	2

Sample Prep

Yeast isolates were assayed for β-glucosidase activity according to the method described by Daenen *et al*. (2007)[99] but modified for fluorometric measurement as outlined by Fia *et al* [115]. Single colonies were inoculated in 10 mL of Wickerman's MYGP medium (3 g/L malt extract, 3 g/L yeast extract, 10 g/L glucose, 5 g/L peptone, pH 5.5) and incubated 24 hours at 25 °C. Following incubation, 1.5 ml of yeast suspensions was transferred to microcentrifuge tubes and centrifuged for 5 minutes (4650 g at 4 °C). Supernatant was decanted for extracellular measurements (EC) while the remaining yeast pellet was rinsed (2x) with cold sterile saline (0.9% NaCl) and resuspended in 1.5 ml sterile McIlvaine Buffer (pH 5.0) for cell associated measurements (CA). 100 µl of EC and CA samples were transferred each to 4 replicate wells of a black 96 well plate for fluorescence measurements. Additionally, 100 µl of suspended yeast in McIlvaine buffer was added to clear 96 well plates for yeast density measurements.

Activity measurement by fluorescence

After yeast samples were prepared and loaded into 96 well plates 100 µl of the fluorophore containing substrate 4-methylumbelliferone (1 mM, 4-MUG; Sigma Aldrich) was added to each well and the fluorescent emission at 445nm was immediately measured using excitation wavelength of 365 nm with fluorescence microplate reader (Molecular Devices Gemini XPS). All measurements were conducted at 30°C. Fluorescence readings were taken at 0 and 20 min with shaking at five minute intervals. Each plate was read against blanks and a calibration reading of

100 U/L of β-glucosidase. A calibration curve was constructed in McIlvaine buffer at 0, 5, 10, 25, 50, 100, and 250 U/L of β-glucosidase. Cell density was measured at 605 nm with a 96-well plate spectrophotometer (Molecular Devices SpectraMax 190) to normalize effects due to yeast cell density.

Wort production

Wort for bench top trials was brewed in the OSU pilot brewery using a grist of 98.5% 2-row malt and 1.5% acidulated malt and boiled for 60 minutes prior knockout into 20-liter plastic buckets. Yeast extract (Yeastex, Brewers Supply Group) was added at a rate of 10 mg/L 15 minutes prior to the end of wort boiling. Iso-hop extract was added to the kettle prior to whirlpool separation to a final concentration of ~ 25 mg/L iso-α-acids for microbial stability. Buckets were hot filled, covered with locking lids and frozen (-20°C) until use. Prior to use for bench top trials, buckets were thawed in boiling water, dispensed (1 L) into 2-liter media bottles and autoclaved for 20 minutes (121°C, 124 kPa). Starting gravity of the base wort used for all the investigations was 12°P.

Hydrolysis timing

Two ale yeast strains were randomly selected from the upper (high activity) and lower (low activity) quartiles of the ale yeast β-glucosidase activities obtained from the yeast screening described above. 1-liter lab scale fermentations were carried out in 2-liter autoclave sterilized bottles fitted with an airlock. Yeast were pitched into the wort at a rate of 18.0×10^6 cells/ml, aerated by shaking on a shaker table for 5 minutes at 300 rpm and then spiked with octyl-glucopyranoside (Sigma Aldrich) in pH

5 McIlvaine buffer to final concentration of 224 µg/L octylglucoside (100 µg/L 1-octanol). In addition, purified β-glucosidase dissolved in sterile pH 5 McIlvaine buffer was dosed into the model wort solution to final activity of 250 U/L (calculated). An untreated control of model wort solution was prepared for comparison. Treatments and the control were incubated for 15 days at 18°C. Samples of each treatment and control were taken at 1 day intervals starting with day 0 (prior to enzyme/yeast addition) and analyzed using headspace solid-phase-microextraction and GC-MS as described below.

Instrumental analysis

Quantification by headspace-solid phase micro extraction gas chromatography-mass spectrometry

The concentrations of target analytes for all volatiles were quantified using an Agilent 6890 gas chromatograph with a DB-wax column (30m x 0.25 mm ID x 0.25 µm; Agilent) using helium as the carrier gas at a flow rate of 1.0 ml/min. Compounds were identified using an Agilent 5972A single quadrupole mass spectrometer with electron impact ionization at 70 eV. Target analytes were quantified using selective ion monitoring (SIM) using the following ions for each analyte: m/z 69 (1-octanol and 4-octanol) and m/z 93 (α-terpineol, nerol, and geraniol), and m/z 109 (linalool and β-citronellol)

Headspace-Solid Phase Micro Extractions (HS-SPME) were performed using a polydimethylsiloxane (PDMS) coated fiber (100µm film thickness x 1 cm long; Supelco). 8 ml of each sample was placed into a nitrogen purged 20 ml screw top

amber vial with 3 g sodium chloride. After the addition of 4-octanol (Sigma-Aldrich) to a final concentration 150 ppb each vial was sealed with screw on magnetic caps and PTFE septa. Pre-incubation, stirring, extraction, and injection were all performed using a Multipurpose Auto Sampler2 (MPS2; Gerstel). Samples were pre-incubated for 15 min at 30°C and adsorbed by piercing the vial septa and exposing the fiber to the headspace for 45 minutes with agitation. After adsorption, the fiber was desorbed into the GC sample inlet (splitless mode, 250 °C) for 10 minutes. A thermal temperature program for all samples was as follows: 50°C initial temperature with a 1-minute hold followed by a 4°C/min ramp to 90°C with a 5 min hold, 5°C/min ramp to 185°C with a 6.5 min hold, 3°C/min to 230°C with 10 min hold.

Analytical grade standards for the following target analytes were purchased from Sigma-Aldrich: linalool, β-citronellol, nerol, geraniol, 1-octanol, 4-octanol, and α-terpineol. The purity of each standard used for quantitation was used to correct concentrations for calibration curves. For unfermented (non-yeast treated samples, calibration curves were made in a model wort solution (pH 5.0 McIlvaine buffer). For fermented (yeast treated) samples, calibration curves were made in a model beer solution (5% v/v ethanol in pH 4.2 citrate buffer). Calibration curves were made using the following concentrations: 1 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, 100 ppb, 250 ppb, and 500 ppb. All calibration solutions were analyzed according to the HS-SPME GC-MS sample preparation and analysis methodology previously described.

Effect of hopping regime on glycoside extraction

2-liter lab scale boils were performed to investigate the role of a possible cultivar by hopping regime interaction on glycoside extraction from hops during brewing. A 60-minute boil, 25 min whirlpool rest at ~100°C (not boiling), and 72-hour dry-hop addition (18°C) hopping regimes were investigated individually in addition to an unhopped control. Samples of Simcoe, Hallertau Mittlefrueh and Columbus hops from the 2014 harvest were used individually for each hopping regime to examine the differences in extraction for different cultivars. Hops were dosed at a constant rate of 2g/L for all cultivars to examine their relative contributions. Each hop extraction was performed independently in triplicate using wort as described above. Boils were performed in 5000 ml round bottom borosilicate flasks and electric (20 amp) round bottom mantle. For all treatments, wort was boiled for 60 minutes and adjusted to its pre-boil mass with 95°C deionized water to account for water loss due to evaporation. Each flask was swirled vigorously for 30 seconds in order to create a whirlpool effect. In the case of whirlpool hopping treatments, hops were added prior to swirling. After swirling, flasks were allowed to sit for 25 minutes. Two, 1 liter aliquots of each lab scale boil was decanted into a 2-liter sterilized media bottle, capped and allowed to cool overnight in an 18°C chamber. Once cooled, dry-hops were added where applicable and β-glucosidase was added (250U/L) to 1 of the bottles for each treatment and incubated for 72 hours at 18°C along with the non-enzyme treated bottle. Octyl glucoside was added to each bottle to a final concentration of 224 µg/L.

Volatile analysis was performed as described in the HS-SPME GC-MS methodology section.

RESULTS AND DISCUSSION

Yeast Screening of β-Glucoside Hydrolysis Activity

The ability of 80 different brewing yeast strains to hydrolyze a β-glucoside linkage was measured using a fluorometric enzymatic assay (Figure 22). Generally speaking, yeast type (lager, ale, brett) was not an indicator of hydrolase activity. That is, not all ale yeast had low activity while all brett yeast had high activity. This is in contrast to work by Daenen et al.[99] who were not able to find significantly high levels of β-glucosidase activity for ale and lager yeasts. These differences may be due to the specific range of yeast studied or differences in the timing of substrate additions relative to yeast metabolic activity. Interestingly, 65% of the yeast in the present study exhibited higher EC hydrolase activity than CA activity. This is in contrast to results found by Kanauchi and Bamforth[65] which showed very little EC hydrolase activity in brewing yeasts. However, these differences are likely associated to the differences in the preparation methodology of CA samples used in each study. In their study, Kanauchi and Bamforth[65] disrupted yeast cells in order to extract the cellular contents of the yeast whereas CA hydrolases were not extracted from yeast in the study presented here.

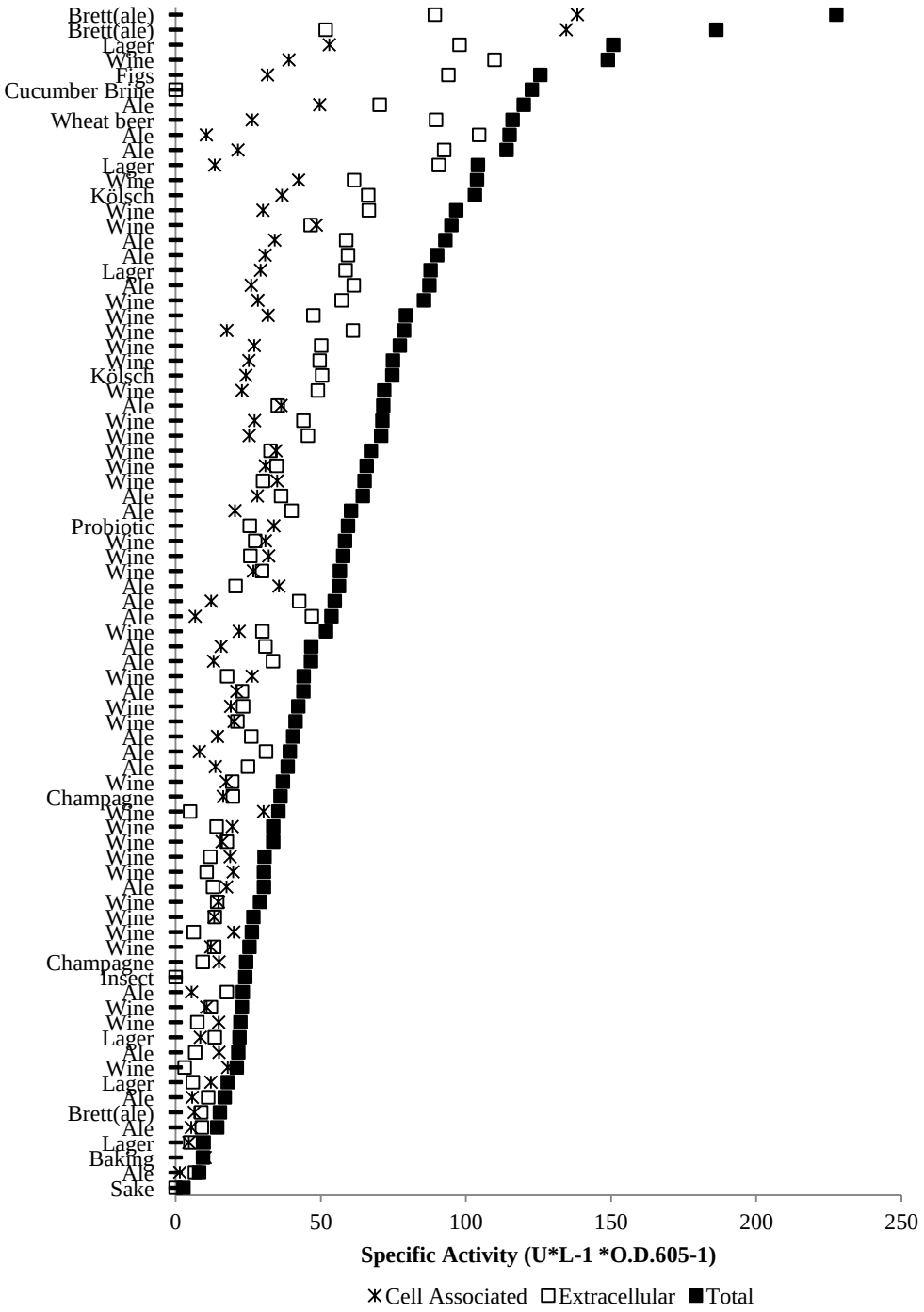

Figure 22: Specific β-glucosidase activity of yeast (n=80) by 4-MUG fluorometric assay. Yeast are sorted in descending total activity. One unit of enzyme is able to hydrolyze 1µmole of substrate per min at pH 5. Data are normalized to cell density at λ=605nm.

Hydrolysis timing

The extent of glucoside hydrolysis in 12°P wort by purified β-glucosidase (250U/L)) and two ale yeasts with high (120 U/L) and low (16 U/L) hydrolysis activities was monitored over time. Extent of hydrolysis was determined by measurement of the hydrolysis product of octyl glucoside, 1-octanol, by HS-SPME GC-MS (Figure 23). After 96 hours, all treatments had reached their maximum hydrolysis. The purified enzyme treatment obtained 98% hydrolysis of the octyl glucoside substrate after 96 hours while both yeast samples, regardless of hydrolysis activity, were able to hydrolyze only ~11% of the substrate over the course of 10 days, both reaching a maximum after 72 hours of fermentation. The control sample produced no measurable amount of the aglycone 1-octanol, indicating that hydrolysis was not attributed to the wort matrix. The results here suggest that although yeast may exhibit different glucoside hydrolysis activities, they are not necessarily indicative of the extent of hydrolysis over the time scale of a normal brewing fermentation (4-15 days). Furthermore, the 87% decrease in the extent of hydrolysis between purified β-glucosidase suggests that yeast expression of enzyme hydrolysis activity appears to be inhibited by the conditions used in this study. Previous researchers have shown that glucose levels and anaerobic conditions may inhibit the expression of β-glucosidase by *Saccharomyces cerevisiae* and thereby limit the extent of glycoside hydrolysis. Since wort was initially aerated to encourage yeast growth, it is possible that aerobic conditions were sufficient for glucosidase expression by the yeast until oxygen levels were depleted. Ting et al. also observed low glycoside hydrolysis by yeast in the

presence of glucose[100] and suggest that hydrolysis may not occur until after primary fermentation and during condition and aging in the presence of yeast, although results here did not see any increase due to the extended contact with yeast (10 days) in anaerobic conditions.

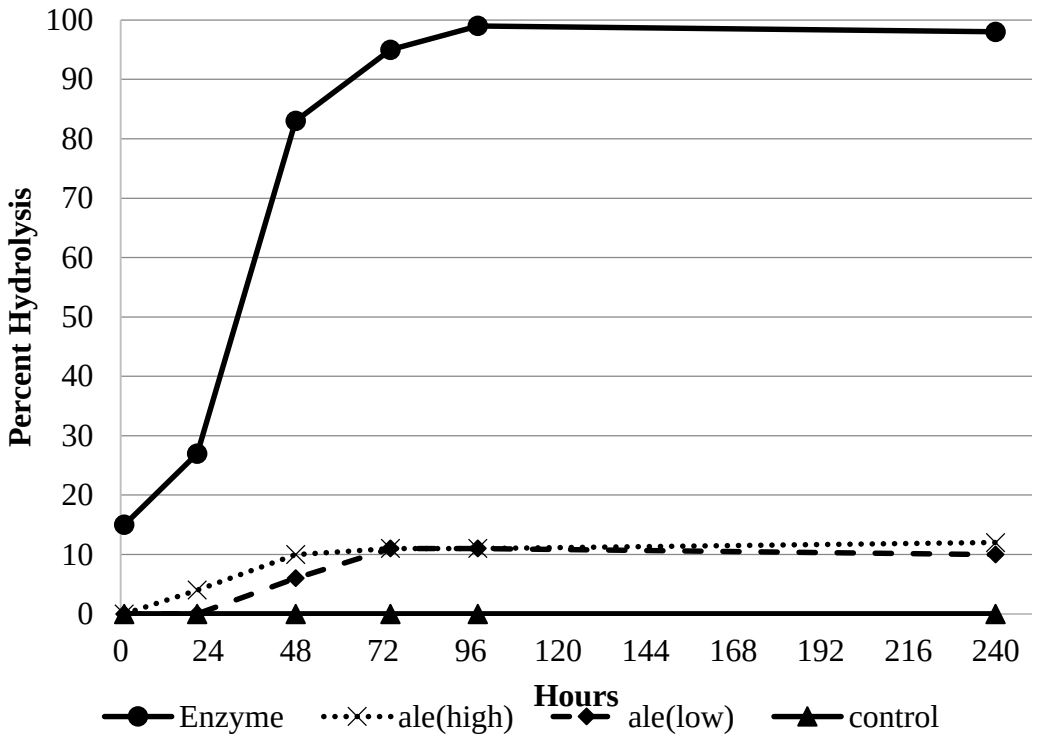

Figure 23: Percent hydrolysis of octyl-glucoside in wort by purified β-glucosidase (enzyme; 250 U/L) and ale yeasts. n=1 for each time point.

Effect of hopping regime on glycoside extraction

Volatile analysis of terpene alcohol concentrations found in wort made with different hopping regimes and cultivars are shown in Table 23 and in unhopped controls (Table 24). Results are the average of three experimental replication for each treatment combination. With the exception of β-citronellol, which is not typically found in hops and likely an isomerization product of geraniol by yeast[59], the concentrations of monoterpene alcohols for Simcoe and Hallertau treatments are likely higher than what would be found in unfermented beers hopped at the same rate, likely due to the lack of terpenoid loss associated with fermentation[50]. ANOVA results show significant cultivar x addition effects for all target analytes with the exception of the surrogate glycoside hydrolysis product 1-octanol (Table 25). This is to be expected since it is generally accepted that monoterpene alcohol content in beer depends on the hop cultivar and how it is used in the brewing process. In the situations were enzyme x cultivar or enzyme x addition interactions were significant, examination of the mean concentrations in Table 23 actually show a decreasing trend in target analytes as a result of the enzyme treatment rather than an increase. These same results are also reflected to some degree in paired t-tests between enzyme and non-enzyme treated wort (Table 26). A significant difference was found between enzyme treatment and non-enzyme treatments for 1-octanol concentrations with the average difference in concentration of 1-octanol between enzyme treated wort and no-enzyme treated wort being 88.8 µg/L. This indicates that hydrolysis of octyl glucoside to 1-octanol was occurring in the enzyme treatments. Partial hydrolysis was observed in the untreated

samples as well (max=15.8µg/L), however 1-octanol was not detected in unhopped/untreated control samples. This partial hydrolysis of octyl-glucoside in some of the hopped non-enzyme treated samples, but not in the unhopped control (Table 24) could be due to native glucosidase found in the hops. Previous reports have found β-glucosidase activity in other plant systems such as grapes[123], however the glycoside hydrolysis activity in hops has yet to be published.

For all other target analytes, no significant difference was found between the concentrations of each monoterpene alcohol in enzyme treated and non-enzyme-treated worts. These results are in stark contrast to previous research on hop-derived glycosides, which generally show slight increases in terpene alcohol contents due to hydrolysis of hop-derived glycosides. However, up until now, previous research has focused primarily the hydrolysis of concentrated extracts from hops[105,124,125]. In the instances where slight increases in hydrolysis products were observed in beers hopped at common hopping rates (1-5g/L), either no account was given to the relevancy of these increases in light of between sample variation[59], or increases were not fully quantified[35]. In studies where increases in monoterpene alcohol concentrations were quantified, they were generally small in relation to contributions from the essential oil containing fraction and odor detection thresholds. For examples, increases in linalool concentrations due to glycoside hydrolysis ranged from 0.2 µg/L to 16.5 µg/L depending on the hopping rate[59,105].

Results from the study presented here show that if hops contain glycosides capable of increasing the concentration of monoterpene alcohols in wort or beer upon

hydrolysis, then their concentrations are not significant enough to overcome the between sample variation of compounds found in the lab scale trials used in this study. Furthermore, the results of this study support previous research presented in chapters 3 and 4 that the contribution of hop-derived glycosides to hop aroma in wort or beer is minimal. However, it should be pointed out that studies by other researchers have focused primarily on the extended storage of beers over the course of at least 10 days of storage in beer. In this study, treatments were subjected to shorter hydrolysis times (72 hours) and carried out in wort with a measured pH=5.1, compared to finished beer with, which typically has a pH range of 4.2-4.7. Nevertheless, results from this study show that different hopping regimes and cultivars do not extract significantly different amounts of monoterpene alcohol glycosides capable of being hydrolyzed by purified β-glucosidase. This is not to say that glycosides are not present in hops. In fact, results from (chapter 4) show that hops do indeed contain glycosides and this result confirms similar findings published elsewhere. However, their concentration in beer is quite low unless hops are used in high amounts (>10g/L), and even then their contribution to aroma is relatively small in regard to their aroma threshold in beer, aroma contributions from the essential oil fractions of hops, and process variability.

Table 23: Concentrations (µg/L) of terpene alcohol in wort treated with β-glucosidase compared to untreated wort for different hop cultivars and hopping additions. (n=3)

	Kettle Hopped											
	Columbus				Hallertau Mittlefrueh				Simcoe			
	β-glucosidase		No β-glucosidase		β-glucosidase		No β-glucosidase		β-glucosidase		No β-glucosidase	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1-octanol	93.0	3.0	6.9	1.6	99.1	1.1	0.0	0.0	102.7	4.6	9.3	8.3
Linalool	6.5	5.6	29.8	25.6	0.0	0.0	0.3	0.5	0.0	0.0	3.4	5.8
α-terpineol	0.0	0.0	2.0	1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-citronellol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Nerol	8.2	2.7	2.5	0.5	0.0	0.0	0.0	0.0	0.0	0.0	6.0	8.0
Geraniol	19.6	4.4	20.9	9.3	0.0	0.0	0.0	0.0	18.9	13.2	15.6	15.1

	Whirlpool hopped											
	Columbus				Hallertau Mittlefrueh				Simcoe			
	β-glucosidase		No β-glucosidase		β-glucosidase		No β-glucosidase		β-glucosidase		No β-glucosidase	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1-octanol	93.7	3.3	11.6	2.5	89.4	8.0	2.9	5.0	97.4	10.4	14.3	1.2
Linalool	125.6	3.6	143.1	6.0	75.1	4.5	78.5	6.7	94.9	15.9	111.8	21.5
α-terpineol	0.0	0.0	6.4	0.6	2.7	4.6	4.4	2.6	0.0	0.0	0.0	0.0
β-citronellol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.8	1.9	2.5	3.4
Nerol	0.0	0.0	8.6	5.9	0.0	0.0	0.3	0.6	15.2	2.6	21.5	0.5
Geraniol	165.5	10.1	139.6	26.2	7.8	0.9	7.0	3.0	240.4	49.5	316.0	28.4

Table 19 (continued)

	Dry-Hopped											
	Columbus				Hallertau Mittlefrueh				Simcoe			
	β-glucosidase		No β-glucosidase		β-glucosidase		No β-glucosidase		β-glucosidase		No β-glucosidase	
	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev	Mean	Std Dev
1-octanol	97.0	1.2	12.6	2.6	98.0	7.4	2.8	4.8	101.8	0.7	12.9	2.2
Linalool	169.8	14.9	182.7	13.8	114.2	12.8	99.3	18.9	139.1	8.4	129.2	11.1
α-terpineol	2.7	3.1	8.9	2.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
β-citronellol	0.4	0.5	0.6	0.6	0.0	0.0	0.0	0.0	1.0	1.1	1.4	1.2
Nerol	16.1	2.0	9.8	5.4	0.0	0.0	0.0	0.0	23.7	4.0	25.4	1.7
Geraniol	420.4	73.1	436.9	79.0	11.6	2.3	5.8	2.8	253.2	27.7	263.9	36.6

Table 24: Concentrations (μg/L) of terpene alcohol in wort treated with β-glucosidase compared to untreated wort for unhopped wort. (n=1)

	Unhopped	
	β-glucosidase	No β-glucosidase
1-octanol	88.82	ND
Linalool	ND	ND
α-terpineol	ND	ND
β-citronellol	ND	ND
Nerol	ND	ND
Geraniol	ND	ND

Table 25:ANOVA F-statistics of HS-SPME GC-MS results for each target analyte. **Bold** = significant at $p<0.05$.

		Linalool	1-octanol	α-terpineol	β-citronellol	Nerol	Geraniol
addition	**2**	**609.7**	1.7	**7.3**	2.7	**37.0**	**206.7**
cultivar	**2**	**76.8**	12.5	20.1	6.8	**91.0**	**198.5**
enzyme	1	3.4	**5001.9**	**17.5**	0.2	1.7	0.7
addition*cultivar	**4**	**7.9**	0.8	**7.9**	**2.6**	**19.4**	**78.5**
addition*enzyme	2	2.5	**4.3**	1.90	0.1	4.7	0.3
cultivar*enzyme	**2**	**3.9**	**4.7**	**12.5**	0.2	**3.7**	1.3

Table 26: Summary results from one-sided paired t-test of enzyme treated beers vs non-enzyme treated beers. (Ha: D1 >D2), n=3, DF=26, alpha=0.05.

	1-octanol	Linalool	α-terpineol	β-citronellol	nerol	geraniol
Difference	88.8	-5.9	-1.8	-0.1	-1.2	-7.6
t (Observed value)	55.485	-1.787	-3.059	-0.879	-1.060	-1.116
|t| (Critical value)	1.7	1.7	1.7	1.7	1.7	1.7
p-value (one-tailed)	< **0.0001**	0.957	0.997	0.806	0.851	0.863

CONCLUSIONS

Brewing yeasts (Saccharomyces spp.) exhibit a broader range of abilities to hydrolyze glycosides than previously thought and there was no indication that either lager or ale yeasts exhibited higher activities than the other. In bench scale fermentations, hydrolysis activity of ale yeasts appeared to be inhibited by high glucose concentrations (12 °P) and anaerobic conditions, regardless of having a high or low β-glucosidase activity, however, purified β-glucosidase from almonds was not. Finally, different hopping regimes or cultivars did not extract significantly different amounts of monoterpene glycosides from hops in lab scale brewing trials.

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Chapter 6 - Contributions of select hopping regimes to the terpenoid content and hop aroma profile of ale and lager beers

Daniel C. Sharp, YanPing Qian, Gina Shellhammer, Thomas H. Shellhammer

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3340 Pilot Knob Road
St. Paul, MN 55121

ABSTRACT

Hops have long been used to impart aroma and flavor to beer. Recently, brewers have dramatically increased the complexity and intensity of aromas in hop-forward beers by using diverse hopping regimes. For this study, the terpenoid content and sensory attributes of beers made using different hop additions was measured. Beers were brewed while varying two factors: hop cultivar (Simcoe and Hallertau Mittlefrueh) and timing of hop addition (60 min. boil, 25 min. whirlpool, or 48-hour dry-hopping). Additionally, the impact of yeast strain on treatment was investigated. Each treatment was compared to an unhopped control using stir bar sorptive extraction (SBSE) GC-MS and descriptive sensory analysis. Multivariate statistical analysis showed relationships between instrumental and sensory techniques. Whirlpool additions produced beers with the highest concentrations of geraniol, linalool, and β-citronellol; beers brewed with highly aromatic Simcoe hops produced more intense and individually distinct aromas for each hopping regime compared to the Hallertau Mittlefrueh hopped beers. Conversely, beers brewed with Hallertau Mittlefrueh hops showed less intense aromas with less distinction between hopping regimes, except for the dry-hopped treatment, which was characterized by a more floral type aroma than the other Hallertau treatments. This research shows that despite the popularity of dry-hopping as an aroma hopping method, whirlpool additions can produce more intensely aromatic beers.

INTRODUCTION

Hop aroma in beer is related to the unique compositional chemistry of the hops used in the brewing process. While the range of these compositions is quite diverse and primarily dependent on hop cultivar[1], other studies have also shown that cultivation, seasonality, harvesting[2], processing[3,4], and storage practices[5,6] contribute to differences in hop composition. However, it should be noted that the aroma and composition of fresh and processed hops[7] is different than the subsequent finished beer. This irreconcilable difference that exists between hops and the finished product has been a confounding variable for brewing scientists, in large part due to the complexity and diversity of the compounds that are transferred from hops to beer, but also due to an incomplete understanding of the synergy between these compounds and the aromas they elicit.

As stated by Weitstock *et al*.[126], the selective transfer of hop compounds to beer or wort during brewing is thermodynamically seen as an extraction process: a function of temperature and time[127]. Manipulation of these factors with different hop cultivars defines the hopping regime of a recipe and has been used by brewers for centuries to impart style defining aromas that range from subtle and nuanced[47,48] to intense and complex[49,70]. However, despite the success of imparting unique aromas, the scientific approaches for predicting and controlling the consistency of hop aroma in beer has been outpaced by the creativity of brewers. In response to a growing interest for new and unique aromas, hop breeders continue to release new aroma type cultivars with novel aromas such as tropical fruit, stone fruit, melon and berry[128].

There are many challenges with tracking hop aroma throughout the brewing process. The complex compositional chemistry of hops[12,30,129–131] is not fully known. It is not surprising that a simple list of target analytes has yet to be produced since over 450 compounds have been identified in the aroma-rich essential oil fraction of hops alone and that over 1000 may exist[21]. This challenge is compounded by inadequate methodology for the routine analysis of hops[132] and an incomplete understanding of the changes that occur during brewing and fermentation.

Numerous studies have focused on the fate of hop-derived compounds in beer[88,133] as a function of cultivar[81,134,135] and hopping regime [81,136,137]. However, they do not fully account for the fermentation effects that alter some of the hop-derived compounds in beer. These fermentation effects include volatile stripping, aroma masking, solubility changes, and a number of direct metabolic biotransformations of hop compounds by yeast[51]. In particular, yeast exhibit β-glucosidase[124,138] activity capable of hydrolyzing non-volatile glycosides from hops[35,105]. This hydrolysis liberates volatile aglycones which may contribute to hop aroma to beer[98]. Since non-volatile glycosides are not routinely targeted for analysis in hops and are not detected in volatile analyses, their contribution is often overlooked. Additionally, researchers have shown that geraniol can also be reduced into other terpenoids such as β-citronellol, a compound not typically found in hops. Both of the biotransformation phenomena mentioned above have been shown to be yeast strain dependent[50,59,99]. Therefore, yeast strain in addition to brewing practices and hop cultivar, is an important consideration when investigating hop aroma in beer. With these challenges

in mind, investigations into the relationships between brewing processes, ingredients, and the aromas they produce are of primary interest to brewing scientists with regard to recipe formulation and improving product consistency. Additionally, as more acreage is dedicated to aroma type hops[139] and brewers continue to pay a high premium, there exists need to identify factors that lead to improved yields and consistency from hops by determining the relationship between common brewing practices and the aromas they elicit.

Of the many classes of volatile compounds found in hops, the class of terpenoids, which includes terpenes, sesquiterpenes and their related alcohols, makes up the largest percentage of hop essential oil[140]. The aroma profile of complex products, such as beer, is the result of not just one compound or class, but rather the interactions between them[75]. Nonetheless, terpenoids represent an important class of compounds that contribute diverse aromas to beer ranging from floral, citrusy, fruity, to woody, green, herbal, and pine[49,56,141].

Many methods have been proposed for the analysis of volatiles in beer due to its complex matrix and diverse composition. However, many of these analytical methods rely on techniques that suffer from long workups, poor analyte recovery, or decreased sensitivity. Stir-bar sorptive extraction (SBSE) and gas chromatography mass spectrometry analysis (GC-MS) is a reliable analysis technique[28] for volatile analysis in beer[143] that offers high sensitivity, high analyte capacity, ease of preparation with high sample throughput and low artifact formation[142]. SBSE has also been shown to overcome many of the challenges presented by the analysis of the beer

matrix, especially when compared liquid-liquid extractions, and it works quite well for hop aroma analysis in beer[81,143]. However, improved chemical analysis capabilities have far outpaced our knowledge of how chemical data relate to aroma[144]. Therefore, the greatest challenge lies not in the ability to detect and measure hop-derived volatiles, but in how volatiles relate to brewing process factors and the aromas they produce.

The purpose of this paper is to determine the aroma sensory profile of single hopped beers made using different hopping regimes, cultivars, and yeasts. Sensory results were used to guide the targeted analysis of 23 hop-derived volatiles in the beers using SBSE. Additionally, the β-glucosidase activity of each yeast strain was measured to investigate the effect of yeast hydrolase activity on hop aroma and hop volatiles. This study provides fundamental research on the contribution of common hopping regimes to the compositional chemistry of finished beers and its relationship to aroma.

MATERIALS AND METHODS

Experimental design

Beers were brewed by varying 3 factors: hop cultivar (Simcoe and Hallertau Mittlefrueh), the timing of hop addition (60 min. boil, 20 min. whirlpool, or 48-hour dry-hopping), and yeast type (ale yeast, lager yeast, wine yeast). Unhopped control beers were also made for comparison to treatment beers. A partially-replicated design (also referred to as an augmented design) was used in order to monitor process variability (within-treatment variability) while maximizing the number experimental factors. In contrast to a fully-replicated design, only certain levels of each factor were replicated and then used as an indication of process variability for all levels of that factor. Replicated treatments were those that included ale yeast treatments and either Simcoe hops or control samples; i.e., all ale fermentations of kettle hopped Simcoe, whirlpool Simcoe, dry-hopped Simcoe treatments and an unhopped control were replicated. Hallertau Mittlefrueh treatments and lager treatments were not replicated during beer production.

Pilot Scale Brewing

Single-hop beers were made at the Oregon State University Pilot Research Brewery (4 hL) using a single infusion mash (68°C) with a grist of 98% 2-row Pale Ale malt (Great Western Malting, Vancouver, WA) and 2% acidulated malt (Weyermann Malting, Bamberg, Germany). The final mineral content of brewing water contained 50 mg/L Ca^{2+} from $CaCl_2$ and 50 mg/L Ca^{2+} from $CaSO_4$ with a final mash pH of 5.3. To avoid non-hop related variation in the sample matrix, a common

high-gravity wort (HGW) was produced and diluted from 20°P to 12°P for all treatments and controls. The wort used for all treatments and controls was boiled for 60 minutes in the kettle, followed by a 25-minute whirlpool rest, cooled to fermentation temperature, and oxygenated with sterile oxygen to ~15 mg/L prior to yeast pitching.

Hop Treatments

Commercial hop suppliers and regional breweries supplied hop pellets from the 2012 harvest and these were stored at -10°C until brewing trials in the summer of 2013. Hop cultivars were chosen based their differences in hop aroma character, chemical profile, and geographic origin. For all single-hop beer treatments, hop pellets were dosed at a fixed rate of 1.5 g/L. Each hop addition was performed independently of other additions for each brew (i.e., one hop addition per treatment). A summary of hopping treatments is shown in Table 27. Unhopped controls were brewed for comparison to treatment samples. Isohop® extract (John I. Haas, Yakima, WA) was added at the beginning of kettle boiling for all treatments and controls to achieve a concentration of 25 mg/L iso-alpha acid (IAA). For kettle additions, pellets were added at the beginning of a 60-minute boil. Whirlpool hop treatments were added after boiling and held for 25 minutes at ~100°C (not boiling) during the whirlpool rest before cooling. Dry-hop treatments were placed in nylon mesh bags and added to fermentation vessels just prior to terminal gravity during diacetyl rest (hence in the presence of yeast). After 48 hours of contact time at 18°C mesh bags with dry-hops were removed from the fermentation vessels.

Yeast and Fermentation

All hop treatments, including controls, were fermented with ale yeast (American Ale™ 1056, Wyeast, Hood River, Oregon), lager yeast (Bohemian lager™ Wyeast 2124), or wine yeast (OSU2, Oregon State University). OSU2 wine yeast (*Saccharomyces cerevisiae*) was obtained from the wine yeast culture collection at Oregon State University and chosen because of its high β-glucoside hydrolase activity. The ale and lager yeast strains were chosen based on high industry relevance due to common usage throughout the brewing industry and low contribution of fermentation-derived aroma to beer. Ale and lager yeast were bottom cropped from healthy fermentations in the normal production of a local commercial brewery and pitched at a rate of 18.0 x 10^6 cells/ml for ale yeast and 24.0 x 10^6 cells/ml for lager. OSU2 wine yeast colonies isolated on YPD agar (yeast, peptone, dextrose) were used to inoculate sterilized wort for stepped propagation. Colonies were inoculated into 10 ml of 8°P sterilized wort and grown at 25°C. Subsequent lab scale aerobic propagations were performed at three 10-fold increases in propagation volume up to 1000 ml (8°P, 25°C, 24 hours each). Afterwards, a 10-liter and 40-liter propagation was carried out in larger propagation vessels (White Labs Ferm-Flask, SABCO). Brewery scale aerobic propagations were carried out at 20°C in 10°P wort for 48 hours.

Beer fermentations were carried out at 18°C for treatments using ale or wine yeasts and at 13°C for lager treatments. After fermentation and diacetyl reduction, all beers were conditioned at 7°C for eight days and then 0°C for three days before being

filtered (Pall HS 2000 filter pads) and carbonated to 2.7 volumes of CO_2 prior to packaging in 355 ml amber bottles.

Yeast β-Glucosidase Quantification

Since some yeasts express β-glucosidase enzyme activity capable of hydrolyzing hop-derived glycosides that could potentially contribute to hop aroma in beer, the β-glucosidase activity of the yeast cultures used in this study was measured. Yeast isolates were assayed for β-glucosidase activity using a method adapted from Daenon *et al*. 2007[99]. Yeast samples obtained from each pitching culture of lager, ale, and wine, were isolated on yeast peptone dextrose (YPD) agar plates and then incubated at 25°C for 48 hours. Single colonies were inoculated into 20 mL of Wickerman's malt yeast glucose peptone (MYPG) medium (3 g/L malt extract, 3 g/L yeast extract, 10 g/L glucose, 5 g/L peptone, pH 5.5) and incubated at 25°C for 24-48 hours or until yeast growth reached stationary phase as determined by spectrophotometric absorbance measurements (λ=605 nm). Following incubation, each yeast suspension was centrifuged for 10 minutes (4650 g at 4°C). The supernatant was decanted and retained for the extracellular measurements (EC). The yeast pellet was re-suspended and rinsed twice with cold (4°C) sterile saline (0.9% w/v) and re-suspended in 10 ml of sterile McIlvaine Buffer (pH 5.0) for cell associated measurements (CA). 200 µL each of the supernatant and suspended yeast were separately inoculated into 10 mL of filter-sterilized 5 mM ρ-NPG (ρ-nitrophenyl glucopyranoside) medium in pH 5 McIlvaine buffer. The cultures were incubated for one hour at 30°C. Following incubation, cultures were centrifuged at 4650 G for five

minutes. The supernatant (1.0 mL) was mixed with 2.0 mL sodium carbonate (0.2 M, pH 10.2) and the absorbance of the solution was measured at 405 nm on a UV/Vis spectrophotometer (Shimadzu). β-glucosidase activity reported as µmole ρ-nitrophenol released per g of dry cells per mL of supernatant (U/L). Each sample was read against blanks and a calibration. An external calibration curve was constructed in McIlvaine buffer with 0, 5, 10, 25, 50, 100, and 250 U/L of purified β-glucosidase (Sigma-Aldrich).

To determine dry cell mass, 5.0 mL of the culture was removed prior to enzyme analysis and filtered using pre-weighed 0.45-micron cellulose filters. The pre-weighed filters with yeast were then dried for 24 hours in a 60°C oven and weighed after cooling.

Sensory Analysis

For all sensory tests, 60 ml beer samples were served in 300 ml clear glasses loosely covered with clear polyethylene terephthalate (PET) lids. Prior to evaluation, all samples were allowed to equilibrate to room temperature (~20°C) for ~20 minutes to maximize aroma and to minimize temperature changes during evaluation. Panelists were asked to assess the orthonasal aroma for each beer. For selected treatments, difference testing was performed prior to descriptive analysis.

Triangle Tests

Discrimination testing was performed prior to descriptive analysis to determine if detectable differences were present between the experimental replicates and between kettle hopped and unhopped controls for each yeast strain. Discrimination

tests were carried out according to the American Society of Brewing Chemists methodology for triangle tests[145]. Panelists (n=18) were recruited from brewing science courses at Oregon State University and asked to identify which sample was different from the other two. Presentation order was randomized and balanced across panelists. Panelists were allowed to re-assess samples, but only in the assigned presentation order.

Descriptive Analysis

A twelve-member panel performed descriptive analysis of the aroma for all 18 hop treatments plus unhopped controls. Due to a large sample size (n=21), each sensory session was blocked by yeast type. During each session, every panelist was presented with the six treatments for each yeast strain plus the unhopped control. Presentation order was uniquely randomized for each panelist, and each session was repeated six times for a total of 18 sessions.

Sensory descriptors were selected by group consensus of attributes that best described the hop aroma of preliminary pilot beers made with higher hopping rates and with comparisons to commercial beers. The following attributes were agreed upon by the panel: Overall Hop Aroma Intensity (OHAI), Pine/Resinous, Grassy/Hay, Herbal, Floral, Citrus, Stone Fruit, Tropical Fruit, Cooked Cabbage/Vegetable, and Clove/Phenolic. Panelists used the above attributes to evaluate the aroma of treatment and control beers using the following interval scale: 0= none, 1-very low, 2=low, 3 low-medium, 4=medium, 5=medium high, 6 = high, 7 = very high.

Instrumental Analysis

Stir-Bar Sorptive Extraction and GC-MS Analysis

Volatiles for each beer were quantified and identified using an Agilent 7890A gas chromatograph equipped with a 60m x 0.25 mm ID x 0.5 µm capillary ZB-Wax column (Zebron) using helium as the carrier gas at a flow rate of 2.5 ml/min. Compounds were identified using an Agilent 5975C single quadrupole mass spectrometer with electron impact ionization at 70 eV operating in scan mode (m/z 35-350).

Stir bar sorptive extractions (SBSE) were performed using a polydimethylsiloxane (PDMS) coated magnetic stir bar (10mm x 0.5 mm; Gerstel). Samples were diluted 1:1 with saturated NaCl solution and stirred at 1000 RPM in 40 ml amber screw top vials for three hours at 20°C. 4-octanol (Sigma-Aldrich) was added to final concentration 150 ppb to each vial as an internal standard. After extraction, stir bars were removed, rinsed with distilled water, and gently dried by blotting with lint free Kim-wipes (Kimberly-Clark) before being desorbed via a thermal desorption unit (TDU; Gerstel). Samples were desorbed according to the instrumental parameters described below for gas chromatographic separation and detection by mass spectrometry (GC-MS). All instrumental measurements were performed in duplicate.

Instrumental parameters

Stir bars were placed into a Thermal Desorption Unit (TDU; Gerstel) for temperature-programmed thermal desorption. The temperature program began at 25°C

and increased at a rate of 120°C /min to a final temperature of 250°C and held for 2 minutes. After desorption, analytes were cryofocused with liquid nitrogen (-80°C) in a CIS4 programmed temperature vaporizing (PTV) injector (Gerstel). Once cryofocusing was complete, the injector inlet was programmed at a ramp rate of 10°C/s from -80°C to 250°C with a 54-minute hold at the final temperature

Standards for the following target analytes were purchased from Sigma-Aldrich: α-pinene, β-pinene, 3-carene, β-myrcene, limonene, β-citronellal, linalool, β-caryophyllene, E-β-farnesene, α-humulene, Z-citral (neral), α-terpineol, E-citral (geranial), geranyl acetate, nerol, β-damascenone, geraniol, β-ionone, caryophyllene oxide, eugenol, α-eudesmol, β-eudesmol, and terpine-4-ol. The purity of each standard used for quantitation was determined and used to correct concentrations for calibration curves. A standard stock solution was made in dichloromethane and added to a 5% (v/v) ethanol/water solution to obtain the following concentrations for a calibration curve: 1 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, 100 ppb, 250 ppb, and 500 ppb. All calibration solutions were analyzed according to the SBSE sample preparation and analysis methodology previously described and produced a linear response over the concentration range (R^2>0.97).

Data Analysis

Analysis of variance was carried out on the instrumental analytes and sensory descriptors using a linear mixed model. Tukey-Kramer adjustment to the standard errors allowed posthoc multiple comparisons of all hop treatments. When sensory responses data were skewed toward zero, a common occurrence with descriptive

analysis, models were fitted using Poisson regression. Before conducting best approximate inference of the models, Akaike and Bayesian Information criteria (AIC/BIC) was used to determine the model of best fit. All summary statistics, analysis of variance, and posthoc multiple comparisons tests for instrumental and sensory data were generated using SAS/STAT software, Version 9.2 of the SAS system software for Windows (Copyright 2002-2008 by SAS Institute Inc., Cary, NC, USA). Multivariate analysis was performed using XLStat (Copyright © 2015 by Addinsoft, New York, NY, USA) and consisted of principle component analysis (PCA) using a covariance of n-1 of the observations and dissimilarity grouping using agglomerative hierarchal clustering analysis (AHC).

RESULTS AND DISCUSSSION

Process variation

A partially replicated design was used as a measure of within-treatment variation while still maximizing the number of factors and levels of the study design. Discrimination testing was used to determine if significant sensory differences could be detected between replicates. Results of the triangle test comparisons (Table 28) show that panelists were not able to distinguish between replicate beers for all hop regimes made with ale yeasts (p-value > 0.05). This strongly suggests that no detectable sensory differences existed between the replicated beers for a given treatment and that any differences found among treatments were attributable to factor effects and not due to within-treatment process variation. Since sensory differences were not found between any of the replicated treatments both replicates for a given treatment were treated as the same unit in subsequent analyses.

Yeast effects

The effect of yeast on hop aroma was investigated by fermenting treatments with three yeast types: ale, lager, and wine yeast. The wine yeast used in this study was selected due to its high β-glucosidase activity, and previous research has shown that yeast may contribute to hop aroma by hydrolyzing hop-derived glycosides[99]. For this reason, the β-glucosidase activity of each yeast was determined (Figure 24) to examine its relationship to hop aroma in beer. The wine and ale yeast had statistically higher cell-associated and extracellular activities (p-value<0.001) than the lager yeast. Statistical differences were not found between ale and wine yeast (p-value>0.05).

Despite statistically different hydrolysis activities between the ale and lager yeasts, the descriptive panel was not able to describe any differences in hop aroma between ale and lager treatments using any of the sensory attributes. This means that any increases in aglycones due to the higher β-glucosidase activity of ale yeast or any other yeast strain dependent biotransformation of hop-derived compounds was not significant enough for panelists describe.

Initial results (data not shown) from the descriptive analysis showed that wine yeast contributed high amounts of clove, medicinal and phenolic aromas. Initial ANOVA and Tukey's post-hoc multiple comparison analysis of the descriptive analysis results showed that only the wine yeast contributed to the main factor effect of yeast for the clove/phenolic attribute. Additionally, no difference was found between the control beer and kettle hopped treatment for wine yeast (Table 28), and the clove/phenolic like aromas ultimately overwhelmed all other aromas contributed by hop treatments or any potential increases in volatile aglycones derived from glycoside hydrolysis. Because the wine yeast skewed the sensory data towards clove/phenolic, an attribute determined to be not of hop origin, the entire wine yeast treatment was removed from subsequent data analysis.

Despite the significant impact of the wine yeast on beer aroma, once it was removed from statistical analysis the comparison of descriptive analysis data for yeast type showed that panelists were not able to describe statistically significant differences between beers made using ale and lager yeasts treatments for a given for hop treatment. While more robust discrimination testing was not performed between ale

and lager beers for each hop treatment due to concerns of panelist fatigue, the inability of panelists to describe differences between ale and lager treatments suggests that hydrolysis of hop-derived compounds does not contribute to noticeable differences in hop aroma in beer.

Despite the fact that raw hops do not contain β-citronellol, SBSE results (Table 30) show that all the Simcoe hop additions increased β-citronellol concentrations in beer with the highest concentrations coming from Simcoe whirlpool treatments followed by Simcoe kettle hopped treatments. This increase is thought to be due to the biotransformation of geraniol into β-citronellol and supports previous reports by Takoi et. al[59] and King et. al[50]. Both dry-hop treatments show little increase in β-citronellol. This was expected for the Hallertau Mittlefrueh hop treatments, since they are not considered to be a geraniol rich hop[146] cultivar and thus lack the precursor for β-citronellol isomerization. Interestingly though, the Simcoe kettle hopped treatment and the Simcoe dry-hopped treatment had similar concentrations of geraniol (Table 30), yet the kettle hop treatment resulted in a higher concentration of β-citronellol. Furthermore, the dry-hop treatment had the lowest level of β-citronellol, suggesting that isomerization of geraniol into β-citronellol may have occurred during primary fermentation and not during dry-hopping or subsequent conditioning of the beers. This is in contrast to work by Takoi et. al 2014[59,146], which showed an increase in β-citronellol concentrations post primary fermentation during maturation as well as during primary fermentation. These differences suggest that the dry-hopping conditions used in this study (e.g. timing of dry-hop additions, conditioning

temperatures, and yeast contact time) were not conducive for the biotransformation of geraniol into β-citronellol by yeast.

Effects of hop addition

Triangle tests were performed between kettle hopped treatments and controls for both hop varieties and yeast strains to determine if the subtle aromas of kettle hopping treatments produced detectable differences. Panelists were able to detect differences between control beers and kettle hopped beers for both cultivars (Table 28), supporting previous work by Praet *et al*. 2015[46] that early kettle hop additions can indeed contribute to hop aroma, specifically, spicy and herbal type aromas. Descriptive analysis results (Table 29) show that Simcoe whirlpool hopped treatments produced the most intensely aromatic beers compared to Hallertau Mittlefrueh treatments. The Simcoe whirlpool treatments in particular were scored as having the highest OHAI, Pine/Resinous, Citrus, -Stone fruit and Tropical fruit aromas. Simcoe dry-hopped treatments were also described as having high OHAI, Stone fruit and Tropical fruit aromas. Simcoe kettle hopped treatments were described as having a predominantly Grassy/Vegetal aroma.

Aroma differences between Hallertau Mittlefrueh hop treatments and controls were subtler compared to the effect of Simcoe hop treatments. Panelists were not able to describe differences between the Hallertau Mittlefrueh treatments and the unhopped controls, with the exception of the Hallertau kettle hop treatment producing significantly higher Herbal/Spicy aroma and Hallertau dry-hop and whirlpool treatments producing significantly higher floral aromas (Table 29).

Multivariate analysis

Using cluster analysis based on dissimilarity, the treatments fell into three main groupings. Group 1 consisted of the control group, Hallertau Mittlefrueh dry-hopped and whirlpool hopped beers. This group was associated with the least amount of aroma as shown by its location along the negative 1st dimension of the PCA (F1) (Figure 26) and relatively low concentrations of target analytes detected by SBSE (Table 30). Additionally, all target analytes, except for, linalool, geraniol, and terpinen-4-ol, were below 10 ppb. Dry-hopped Hallertau Mittlefrueh and whirlpool Hallertau Mittlefrueh treatments were associated with low aroma and were grouped with unhopped controls (Table 28) while kettle hopped Hallertau treatments were associated with herbal/spicy aromas (Figure 26). The association of kettle hopped Hallertau Mittlefrueh treatments with more aroma than respective dry-hop and whirlpool hop treatments was unexpected since dry-hopping and whirlpool hopping are generally thought to produce more intense aroma. The fact that the kettle hop treatment had higher herbal/spicy aroma supports the work of Praet et al.[45] who found that kettle hopping leads to an increase in sesquiterpene oxidation products which are herbal, woodsy, and spicy in character.

Principle component analysis (Figure 26) of the descriptive sensory data showed that over 96% of the variation among samples was explained by the descriptive attributes used by the sensory panel. Driving variation on the first dimension (F1) and describing over 75% of the variation was Overall Hop Aroma Intensity, Tropical Fruit, Citrus and Pine/Resinous. Generally speaking, all the Simcoe

treatments were located on the positive end of the 1st dimension, whereas Hallertau Mittlefrueh treatments anchored the negative 1st dimension. Furthermore, all the kettle hopped treatments were located along the negative 2nd dimension (F2) while dry-hopped and whirlpool hopped treatments were aligned in the positive 2nd dimension.

Interestingly, both kettle hop treatments of Hallertau and Simcoe hops were grouped together in the 2nd group and located along the negative 2nd dimension that was described by cooked vegetable, grassy, and herbal aromas. It should be noted that the panel described the Cooked Cabbage/Vegetable descriptor as specifically *not* similar to the aroma of dimethyl sulfide (DMS), which is a common non-hopped derived defect in beer. Results from SBSE analysis showed sub-sensory detection threshold concentrations of all target analytes for both kettle treatments, even those attributed to herbal and grassy aromas such as α and β-eudesmol, β-caryophyllene, α-humulene, caryophyllene oxide, and eugenol. This suggests that other compounds not measured in the present study may be responsible for the herbal, spicy, grassy, and vegetative aromas associated with kettle hop treatments. Research by Praet *et al*. shows these aromas are partially due to the oxygenated derivatives of α-humulene and β-caryophyllene formed during the pro-oxidative environment of the brewing kettle[45].

Group 3 consisted of whirlpool and dry-hopped Simcoe treatments located along the positive 1st dimension. This group represented the most intensely aromatic of the three groups with the Simcoe whirlpool treatments associated with the highest overall hop aroma intensity attribute followed by the Simcoe dry-hopped treatment. The Simcoe whirlpool treatment was also described as having the highest

Pine/Resinous, Tropical fruit, and Citrus aromas, while dry-hopped Simcoe treatments were associated with the highest Stone fruit and Floral aromas. Simcoe hops have been shown to contain polyfunctional thiols, such as 4-mercapto-4-methyl-pentan-2-one (4MMP), 3-mercaptohexylacetate (3-MHA), and 3-mercaptohexanol (3-MA), and are a source of distinct and intense aromas in beer[81]. These sulfur-containing compounds have been described as catty, boxwood, currant, tropical fruit, sweaty, grapefruit, and passionfruit[32,33,147]. These compounds were not investigated in the present study, although they are likely key odor-active compounds that contribute the higher tropical fruit like aromas found in the Simcoe whirlpool treatments.

When assessing the SBSE results, it is worth noting that both cultivars produced similar concentrations of linalool for their respective hopping regimes. However, both of these cultivars lie at opposite ends of the PCA space when it comes to floral and fruity aromas as well as overall hop aroma intensity. This supports the idea that at these concentrations, linalool is not a likely driver of differences in overall hop aroma intensity[148] and that contributions from other compounds such as geraniol and β-citronellol, to name but a few, are also important considerations[68]. Table 30 shows that both dry-hop treatments produced similar levels of terpinen-4-ol regardless cultivar, but no association with each other in the first two principle components of the PCA (Figure 26). This means that terpinen-4-ol can be as used an indicator of dry-hopped beers for both of these cultivars, but does not indicate which cultivar was used. β-myrcene and geraniol on the other hand were higher for Simcoe dry-hopped

treatments compared and Hallertau dry-hop treatments and could therefore be used as potential indicators of Simcoe dry-hop treatments.

Brewer's often turn to dry-hopping to add intense aromas to beer. As outlined by Wolfe *et al*.[41], the low temperatures used during dry-hopping retains more hop-derived aromas by minimizing their volatilization due to the elevated temperatures (~100°C) used during brewhouse hop additions. However, despite its increasing use as a means to impart hop aroma to beer, dry-hopping presents significant disadvantages. First, since lower extraction temperatures are used to maximize volatile retention, dry-hopped beers require significantly longer extraction times (48 hours to 2 weeks) compared to hot-side brewhouse hop additions (~30 min to a few hours). Furthermore, dry-hopped beers often result in considerable product loss due to poor sedimentation and separation of hop material from beer. Also, the addition and removal of dry-hops to fermenting or conditioning vessels present considerable process and safety hazards due to the dangers associated with enclosed spaces and a high carbon dioxide environment. Finally, the addition of dry-hops adds the potential introduction of dissolved oxygen thereby decreasing flavor stability and increasing the formation of staling aromas. In this study, whirlpool Simcoe treatments generally produced a more intense hop aroma than the dry-hopped Simcoe treatments. These findings challenge the idea that dry-hopping is the most efficient way to impart aromas characteristic of terpenes and terpene alcohols and suggest that whirlpool hop additions may be an efficient alternative particularly if conditions are optimized for efficient extraction.

Hopping rates were based on a fixed rate for all treatments in order to investigate the contributions of each cultivar to hop aroma for each hopping regime. Ideally, a high hopping rate (>4 g/L) would have been used in order to amplify aroma. However, the hopping rate was constrained by the attempt to achieve industry relevant levels of hop-derived bitterness and the high alpha acid content of Simcoe hops (12.1% α-acid). As a result, a compromise in hopping rates was required. The hopping rates used in this study (1.5g/L) were modest compared to the average hopping rate used among US craft brewers (5.4 g/L) in 2014[38]. As such, the interaction effect between hop addition and hopping rate for a giving cultivar would be of great interest and practical use to brewers.

CONCLUSIONS

Cultivar had the least impact on kettle hop aroma, while differences in aromas for whirlpool and dry-hopping treatments were mostly dependent on cultivar. This suggests that cultivar has more of an influence on hop aroma in dry-hopped and whirlpool hopped beers than in early kettle hopped beers. The use of a yeast that produces high levels of phenolic off-flavor and aroma overwhelmed the hop aroma character and therefore should not be used in beers meant to highlight hop aroma. For the hopping rates used in this study, statistically significant increases in hop aroma were not observed due to increases in the glycoside hydrolysis activities of yeasts. In general, Simcoe whirlpool treatments were associated with the highest overall hop aroma intensity and tropical fruity aromas compared to dry-hop Simcoe treatments. Simcoe hop additions in the whirlpool resulted in the highest concentrations of β-citronellol when added to the whirlpool, presumably from the isomerization of geraniol by yeast. However, the addition of hops during the last 48 hours of fermentation was not sufficient for the geraniol biotransformation into β-citronellol.

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TABLES AND FIGURES

Table 27: Summary of hopping treatment conditions and dosages

Hop Treatment	Isohop Extract addition	Pellet addition
Unhopped	15.5 ml/hl, 60 min. boil	None
Kettle hopped	none	1.5 g pellets/L wort, 60 min. boil
Whirlpool hopped	15.5 ml/hl, 60 min. boil	1.5 g pellets/L wort, 25 min. whirlpool rest
Dry-hopped	15.5 ml/hl, 60 min. boil	1.5 g pellets/L wort, 48 hours during diacetyl rest (18°C)

Table 28: Triangle test comparisons between

Sample 1	Sample 2
DH Simcoe ale rep. 1	DH Simcoe ale rep. 2
KH Simcoe ale rep. 1	KH Simcoe ale rep. 2
WH Simcoe ale rep. 1	WH Simcoe ale rep. 2
Control ale	**KH Simcoe ale**
Control ale	**KH HHA ale**
Control lager	**KH Simcoe lager**
Control lager	**KH HHA lager**
Control wine	KH Simcoe wine
Control wine	KH HHA wine

Bold indicates a statistically significant difference between samples ($p>0.05$). HHA= Hallertau Mittlefrueh, KH = kettle hopped; WP = whirlpool hopped; DH = dry-hopped.

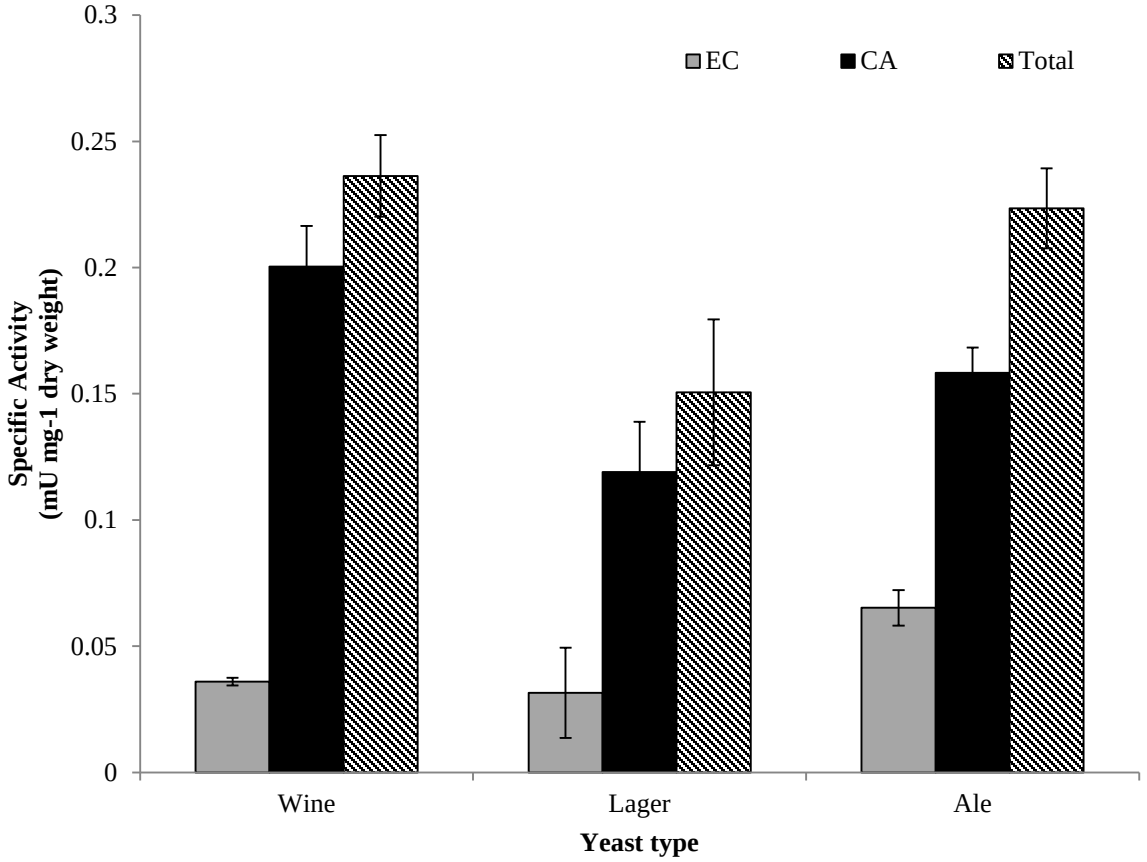

Figure 24: Specific activity of yeast-derived β-glucosidase enzyme activity on ρ-NPG of wine, lager, and ale yeasts. Extracellular (EC), Cell-Associated (CA) and the sum of EC and CA (total) activities are shown. Error bars show standard deviation.

Table 29: Mean descriptive analysis sensory scores and results from Tukey's HSD analysis. Values represent means of lager and ale treatments only.

Treatment	OHAI	Pine/ Resinous	Grassy/ Vegetal	Herbal/ Spicy	Floral	Citrus	Stone fruit	Tropical fruit	Cooked cabbage/ Vegetable	Clove/ Phenolic
HHA-DH	$3.8^{c,d}$	0.6^{d}	$1.5^{a,b}$	$0.8^{a,b}$	$1.8^{a,b}$	$1.0^{b,c}$	$1.8^{a,b}$	1.5^{b}	$1.1^{b,c}$	0.1^{a}
HHA-KH	$3.9^{b,c,d}$	$0.7^{b,c,d}$	$1.8^{a,b}$	0.9^{a}	$1.4^{a,b,c}$	0.7^{c}	$1.4^{b,c}$	1.5^{b}	1.6^{a}	0.1^{a}
HHA-WP	$3.8^{c,d}$	$0.5^{b,c}$	$1.5^{a,b}$	0.6^{b}	$1.6^{a,b}$	$0.9^{b,c}$	$*1.8^{a,b}$	1.6^{b}	$1.1^{b,c}$	0.1^{a}
Simcoe-DH	$4.3^{b,a}$	0.9^{b}	$1.5^{a,b}$	$0.8^{a,b}$	$1.9^{a,c}$	$1.2^{a,b}$	$2.1^{a,b}$	$2.1^{a,b}$	1.0^{c}	0.1^{a}
Simcoe-KH	$4.0^{b,c}$	0.9^{b}	2.0^{a}	1.0^{a}	1.4^{c}	$0.8^{b,c}$	1.3^{c}	1.7^{b}	1.8^{a}	0.2^{a}
Simcoe-WP	4.7^{a}	1.5^{a}	1.5^{b}	$0.7^{a,b}$	2.0^{a}	1.8^{a}	2.2^{a}	2.7^{a}	$1.3^{a,b,c}$	0.1^{a}
iso-control	3.5^{d}	$0.5^{c,d}$	1.4^{b}	0.6^{b}	1.3^{c}	$0.7^{b,c}$	$1.6^{a,b}$	1.4^{b}	$1.4^{a,b}$	0.1^{a}

Means with the same letter are not significantly different.
***HHA-KH and HHA-WH are significantly different. (α=0.05); HHA=Hallertau Mittlefrueh, KH=kettle hopped, WP=whirlpool hopped, DH=dry-hopped.**

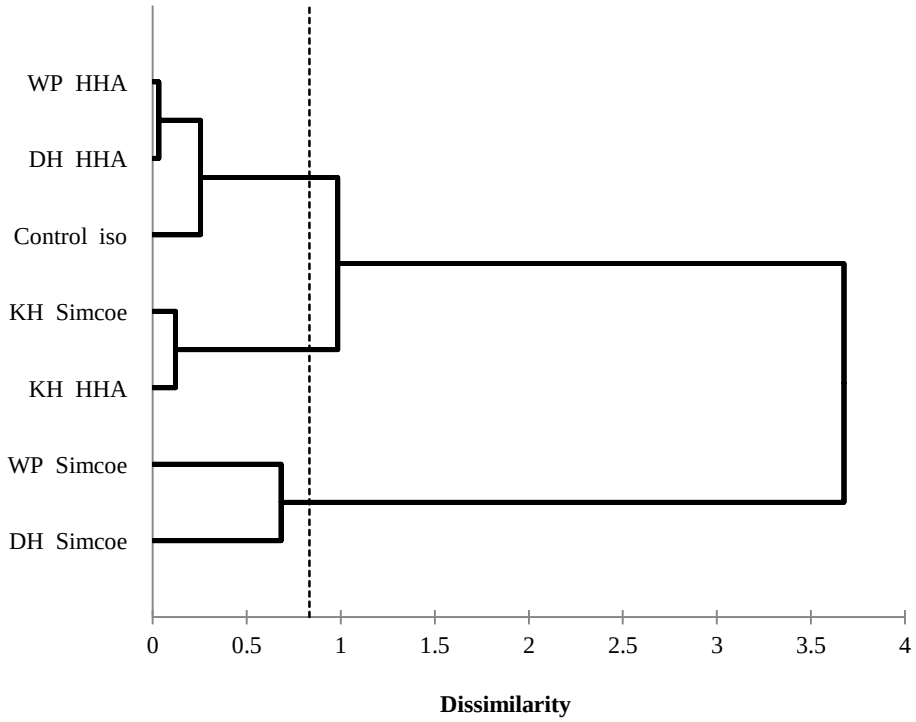

Figure 25: Agglomerative Hierarchical Clustering Analysis of descriptive analysis data. HHA=Hallertau Mittlefrueh, KH=kettle hopped, WP=whirlpool hopped, DH=dry-hopped.

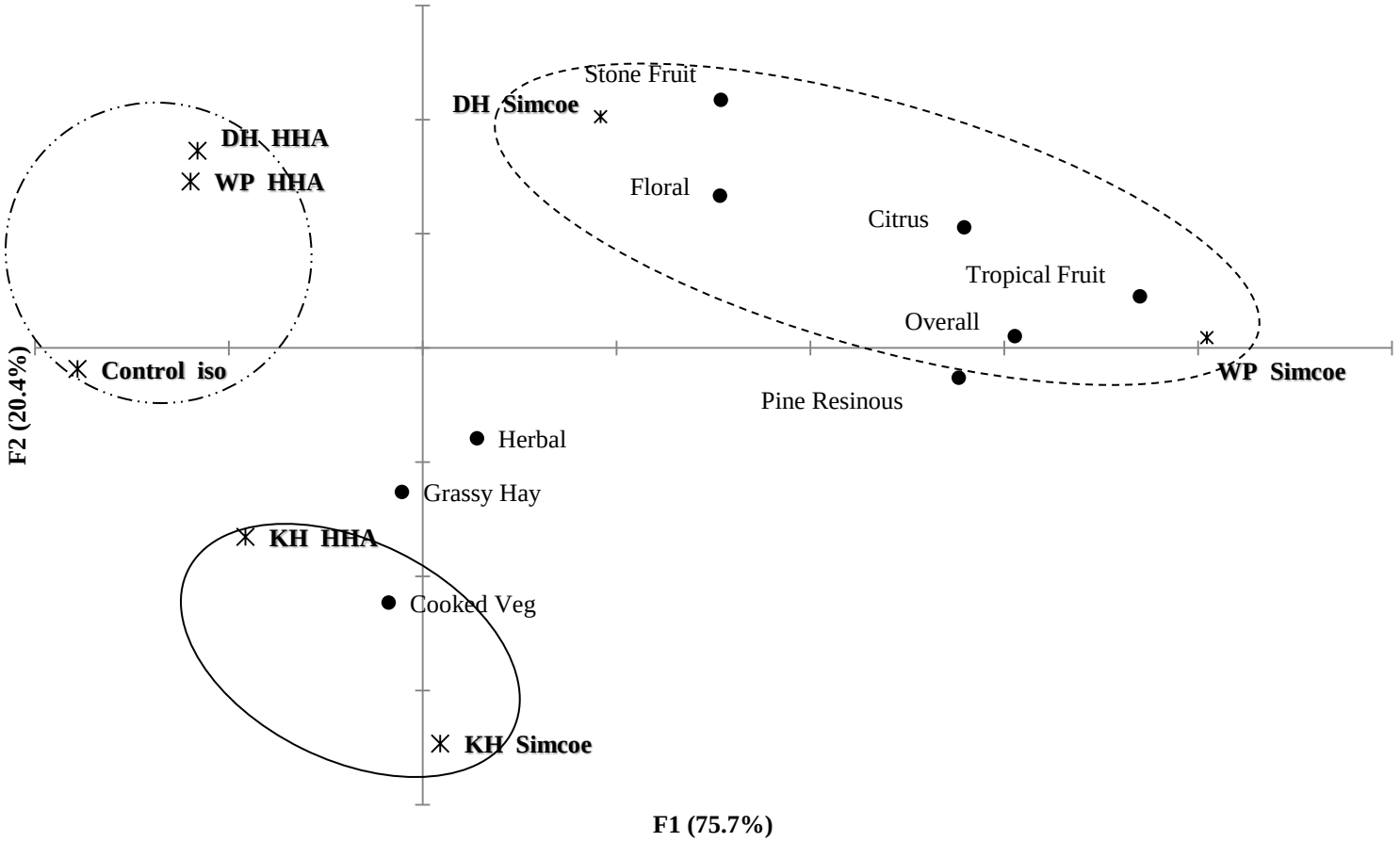

Figure 26: Principle Component Analysis biplot of descriptive sensory analysis data. HHA=Hallertau Mittlefrueh, KH=kettle hopped, WP=whirlpool hopped, DH=dry-hopped. Groupings from cluster analysis (Figure 26) are represented by ellipses. Group 1 = double dashed line, Group 2= solid line, Group 3= single dashed line.

Table 30: Concentration and aroma thresholds (µg/L) of hop-derived volatiles in beers brewed using different hopping regimes analyzed by SBSE GC-MS.

compound	odor threshold	unhopped	Hallertau Mittlefrueh			Simcoe		
		control	kettle	whirlpool	dry-hop	kettle	whirlpool	dry-hop
β-myrcene	195[a]	2.1±0.2	2.1±0.1	1.9±0.1	3.6±0.6	2.0±0.1	3.0±0.3	11.0±3.5
limonene	100-1400[b]	1.6±1.0	2.5±1.6	<1	<1	1.7±1.2	2.0±1.3	1.9±0.9
citronellal	n/a	1.2±0.3	1.7±0.8	1.2±0.5	1.7±0.7	1.3±0.5	1.8±0.3	1.6±0.6
(R/S)-linalool	83[a]	2.7±0.1	11.3±0.5	64.0±3.7	21.7±0.7	12.3±0.3	74.3±1.4	18.7±2.2
e-β-farnesene	550[b]	1.3±0.1	1.7±0.3	2.7±0.3	1.3±0.3	1.7±0.1	3.8±0.6	1.8±0.1
α-humulene	310[a]	<1	1.1±0.0	1.0±0.1	1.9±0.1	1.3±0.1	<1	1.9±0.1
α-terpineol	330[b]	1.4±0.5	3.6±0.3	4.8±0.1	2.2±0.3	6.2±0.2	11.3±0.5	3.1±0.3
geranial	n/a	n.d.	<1	<1	n.d.	<1	1.4±0.5	n.d.
geranyl acetate	449[a]	<1	<1	<1	<1	1.1±0.1	2.9±0.2	<1
β-citronellol	53[a]	4.1±0.2	6.2±0.4	5.8±0.2	3.6±0.5	15.9±1.1	40.5±0.8	4.6±0.3
nerol	632-975[a]	1.1±0.1	1.5±0.3	1.1±0.2	1.2±0.1	2.7±0.2	6.9±0.8	2.0±0.3
β-damascenone	177[a]	<1	<1	<1	<1	<1	<1	<1
geraniol	53[a]	4.0±0.4	8.5±2.0	7.4±1.1	6.4±1.7	24.6±2.4	66.0±10.4	26.3±3.8
caryophyllene-oxide	n/a	n.d.	<1	<1	<1	<1	1.9±0.3	<1
α-eudesmol	n/a	n.d.	4.2±0.5	5.3±0.5	3.2±0.4	n.d.	n.d.	n.d.
β-eudesmol	10000[b]	n.d.	4.4±0.2	3.4±0.3	2.0±0.1	1.8±0.	2.4±0.1	<1
terpinen-4-ol	n/a	n.d.	n.d.	8.1±2.3	39.2±0.7	1.4±0.9	9.0±1.0	41.7±5.7

Threshold values were determined in: [a]5% ABV beer[94], beer[145]. Concentration values are +/- standard error, <1 indicates below 1µg/L calibration limit, n.d.= not detected.

Concluding Remarks

The primary goal of the work presented in this thesis was to investigate several key brewing and hop factors during the brewing process to understand how they relate to the chemical and sensory profiles of beer. These primary factors included the investigation of hop cultivar, hop fraction, and hopping regime, in addition to the interactive effect of yeast hop and volatiles.

Regarding the effect of cultivar, we found that beers brewed with the classic American hops, Cascade and Centennial, along with the newer American hop cultivars, Citra and Simcoe, were separated from classic European aroma hops, East Kent Goldings, Hallertau Mittlefrueh (HHA) and Saaz using cluster analysis of descriptive sensory data. Interestingly, the American cultivar Chinook had a more similar aroma profile to the European hops than the American hops. Additionally, the contribution of Simcoe and Citra SFE hop extracts had the greatest impact on the nature of kettle hopped beers compared to the other cultivars examined.

While kettle hopped beers were not devoid of aroma and were noticeably different compared to unhopped beers, they produced beers with low aroma regardless of the cultivar or hop product used. Instrumental results from SBSE showed similar results for the target analytes which were detected at concentrations below 5 ppb and most likely volatilized during the kettle boil. Based on descriptive analysis results of beers brewed using different hop products (pellets, SFE extracts and spent material) we observed that SFE hop extracts, which were devoid of water soluble components, produced beers with a more similar kettle hop character to pellet hopped beers than

those hopped with spent hop material. This further supports the findings of other researchers that kettle hopped aroma is derived primarily from the hop oil fraction despite its volatility and not from the water soluble fraction. Furthermore, this supports centuries-old brewing observations that although kettle hop additions provided slight contributions to aroma, they are minimal compared to whirlpool or dry-hop additions.

Cultivar by product interactions were observed for hop aroma. Notably, Citra extract produced beers with significantly higher aroma than all the other cultivars and products. Also, Simcoe spent hop treatments produced beers with noticeably higher aroma than beers made using spent hops from Cascade, Citra, or Centennial hops. However, contributions of water soluble components found in spent hops to the hop aroma in beer were very subtle, especially compared to the pellet and extract treatments. Therefore, for the conditions used in this study where the hopping rate for the spent material was less than 1g/L, the majority of kettle hop aroma came from the more nonpolar components extracted during SFE and very little from spent hop material. With regard to hop addition, hop cultivar had the least impact on kettle hop aroma, while differences in aromas for whirlpool and dry-hopping treatments were mostly cultivar dependent. This suggests that cultivar has more of an influence on hop aroma in dry-hopped and whirlpool hopped beers than in early kettle hopped beers.

In general, Simcoe whirlpool treatments were associated with the highest overall hop aroma intensity and tropical fruity aromas compared to dry-hop Simcoe treatments. Simcoe hop additions in the whirlpool resulted in the highest

concentrations of β-citronellol, presumably from the isomerization of geraniol by yeast. In terms of impacts of yeast on aroma or monoterpenes alcohols, it is likely that the isomerization of free monoterpene alcohols plays a bigger role than transformations of glycosidically bound monoterpene alcohols. When hops were added to wort prior to fermentation (kettle hopped or whirlpool hopped) decreases in geraniol in and increases in β-citronellol were observed. This is in contrast to the addition of hops during the last 48 hours of fermentation, which did not produce beers with β-citronellol. While more work is necessary to identify conditions that optimized the isomerization of geraniol, it nevertheless gives brewers a process tool for manipulating hop aroma. However, we have seen here too, that yeast also play a significant role in masking hop aroma due to aromas produced as byproducts of fermentation. In our case we observed that the use of a yeast that produces high levels of phenolic off-flavor and aroma overwhelmed the hop aroma character and therefore should not be used in beers meant to highlight hop aroma. Similar masking effects were observed in beers with noticeable levels of dimethyl sulfide (DMS) and diacetyl aroma. Finally, different hopping regimes or cultivars did not extract significantly different amount of monoterpene glycosides from hops in lab scale brewing trials.

The work presented here found a negligible contribution of the water soluble fraction of hops, specifically glycosides, to beer aroma, especially when compared to contributions from essential oil fraction or variation in aroma due to brewing practices. Results from this thesis confirm work by previous authors that glucosides are present in hops but challenges the idea that they exist in sufficient enough quantities to have a

meaningful impact on the aroma of finished beer when typical hopping rates are used. Differences were observed in the monoterpene alcohol profiles of aqueous spent hop extracts treated with ale and lager yeasts (<2g/L glucose). However, while brewing yeast (Saccharomyces spp.) exhibited a broader range of β-glucosidase activities than previously thought, these activities appeared to be inhibited by the glucose concentrations found in wort and also by the anaerobic conditions of fermentation but not hop aqueous spent hop extracts. This presents unfavorable enzymatic hydrolysis conditions for yeast during the brewing process which, in combination with the low concentrations of glycosides found in hops, suggests that aroma contributions from the hydrolysis of monoterpene alcohol glycosides are minimal unless a concentrated glycoside extract and exogenous enzyme preparations are used. This approach may become more attractive to brewers as demand for hops continues to rise and brewers look for more efficient ways to utilize and extract the aroma potential of hops. In the bench scale trials used in this study, purified β-glucosidase extracted from almonds did not show glucose inhibition and may be useful for glycoside hydrolysis if concentrated glycoside extracts are used. In this light we observed that different enzymatic treatments produced different monoterpene alcohol profiles depending on the enzyme used which supports work by other researchers that the specificity of β-glucosidase or other glycosidases toward primary and tertiary alcohols. While increases in hop-derived volatiles in spent hop extracts appears to be a combination of both acid and enzymatic hydrolysis, the respective kinetics, time scale and contributions of these hydrolysis phenomena are unclear. What is clear from this study

is that while cultivar and hopping regime play an obvious role in the hop aroma of beer, the interaction effect between yeast and hop volatiles cannot be ignored. Fundamental studies examining these effects would help brewers to better control hop aroma and analysts better account for the differences between the volatile profile of hops and the profile of hop-derived volatiles in beer. Attempts to determine the inter relationships between hop chemistry, beer chemistry, and hop related aroma profiles have been ongoing for at over a century. Researchers and brewers have contributed substantially to this cause and their role cannot be understated. However the complex and diverse chemistry of hops, brewing practices, and wort/beer matrices compound the challenge of a comprehensive understanding of these relationships and despite the substantial scientific contributions to decoding hop-derived aroma in beer, it appears that there is substantially more work left to do compared to what has already been done.

Future Work

With regard to the analysis of hop volatiles in beer, much remains to be done. Analytical capabilities have increased dramatically over the years and many tools are available to the analyst for determining important chemical markers of hop aroma. However, despite increasing analytical techniques, it is impossible to analyze a sample *in toto* without employing sensory techniques. In this regard, sensory directed aroma analysis techniques provide the most comprehensive strategy to defining and tracking aroma throughout brewing. While identification of new compounds in hops and beer are novel and provide a more thorough understanding of the diversity of hop chemistry, what is needed is the identification of chemical markers in hops that relate to the aroma changes in beer due to different chemistries. This is not to say that these markers must be responsible for these aromas, only that they relate to sensory changes. In this way analysts may build statistical models that connect the raw ingredient profiles to hops and track changes in beer due to process developments.

However, of primary importance with regard to hop aroma in beer is improving the extraction of hop-derived volatiles while minimizing their loss, or more simply put, improving yield. Brewers are continually looking for ways to increase the aromas of hop forward beers. As the demand for hops continues to rise, brewers will need to become more efficient with the ways they use hops for aroma. Substantial research has been performed to maximize the utilization of bittering compounds in beers. However, a parallel equivalent of hop aroma utilization cannot be made. Recently, brewers have been experimenting with novel processing techniques that

improve the extraction of desired aromas, and while significant advancements have been made, particularly in dry-hopping, fundamental work remains. As it stands now, the most common practice for adding more hop aroma to beer, is to simply add more hops. Unfortunately, addition of more hops generally has an inverse effect on yield or extraction efficiency, not to mention beer losses. Therefore, investigations towards optimal extraction conditions of hop aroma at all stages of the brewing process would be beneficial. In terms of whirlpool and late hopping, investigations towards the optimal temperatures that maximize extraction and minimize volatilization would provide brewers with practical tools for increasing the utilization of hop material.

In terms of extraction efficiencies, brewers would be hard pressed to improve upon the yields found with SFE hop extracts. Ironically, those who would benefit the most from using extracts have generally rejected them in favor of hop products that provide the lowest utilization and extraction yields (whole cones, pellets). With regard to production of hop extracts, additional studies looking at SFE conditions that preferentially extract desired volatiles from new hop cultivars may provide enticing new hopping options to otherwise reluctant adopters.

With regard to hopping addition, it is unclear as to when to add hops relative to fermentation in order to obtain a certain aroma. For that matter, when, and to what extent do different yeast strains modify hop-derived volatiles during fermentation? Or, what are the differences in aroma when hops are dry-hopped in the presence of yeast and their required fermentation conditions compared to cold dry-hopping in the absence of yeast? These are but a few avenues for possible future research.

Significant strides have been made in both analytical techniques and the understanding of how hops influences aroma in beer. However, much work remains, particularly as the chemical profiles of hops continue to increase in diversity and brewers strives for better ways to utilize their hops. Indeed, hop aroma is complex - a function of its chemical complexity, despite advancements in analytical techniques that have allowed for the identification of many of the important aroma compounds in hoppy beers. The real challenge is interpreting the relationship between these compounds, their matrix, and their aromas. To what extent this is truly possible remains to be seen.

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