AN ABSTRACT OF THE THESIS OF

Victor Alexander Algazzali for the degree of Master of Science in Food Science and Technology presented on August 8, 2014.

Title: The Bitterness Intensity of Oxidized Hop Acids: Humulinones and Hulupones

Abstract approved:

______________________________________________________

Thomas H. Shellhammer

The goal of this research was to investigate the bitterness intensity of known hop acid oxidation products, humulinones and hulupones. This was carried out by first creating suitable extracts of humulinones and hulupones of high purity for sensory testing. Using previously established oxidation methods and the addition of preparative liquid chromatography, high purity extracts of humulinones and hulupones were prepared; 93% and 92% purity respectively as measured by High Pressure Liquid Chromatography (HPLC).

The isolated humulinone and hulupone extracts were then spiked into unhopped lager, which was prepared at the Oregon State University pilot brewery. The beer samples were spiked in concentration intervals, ranging from 8 mg/L to 40 mg/L for the humulinones and hulupones. Additionally, a set of beers were prepared with commercially available iso-α-acid extract in the concentration range of 6mg/L to 30mg/L. The humulinone, hulupone, and iso-α-acid beer samples were evaluated by a trained panel in Quantitative Descriptive Analysis (QDA) sensory testing.

The trained QDA panel on beer bitterness found humulinones to be 66% as bitter as iso-α-acid (+/-13%), and hulupones to be 84% as bitter as iso-α-acids (+/-10%). The
sensory results were substantially higher than previous estimates of 35% for humulinones and 50% for hulupones (26, 29). Additionally, the trained panel was able to detect the bitterness intensity of humulinones at a concentration as low as 8 mg/L, which is the first estimation of the bitterness detection threshold of humulinones. The conclusions of this work demonstrated that the bitterness intensity of hulupones and humulinones is greater than what was previously suspected and that their influence on beer flavor should not be ignored.
The Bitterness Intensity of Oxidized Hop Acids: Humulinones and Hulupones

by
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APPROVED:

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Head of the Department of Food Science and Technology

Dean of the Graduate School

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

______________________________
Victor Alexander Algazzali, Author
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I would like to thank Dr. Thomas Shellhammer for allowing me the opportunity to work in his laboratory for nearly three years. During this time, Tom provided a learning environment, where I was often allowed to follow my curiosities in chemistry and brewing science, and ultimately acquire a great education. The aura of the Shellhammer lab is that of a family, a family of professional beer nerds, and it was an unforgettable experience working with my friends and colleagues: Jeff Clawson, Daniel Sharp, Peter Wolfe, Daniel Vollmer, Rachel Hotchko, Ellen Parkin, Meghan Peltz, and many others.

Time for a beer.
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The Bitterness Intensity of oxidized Hop acids: Humulinones and Hulupones.

Chapter I: Hop composition, products, and storage

1.1 Introduction to hops

Hops and beer share a long history, thought to begin more than a thousand years ago. In 822 AD, the first use of hops in beer was documented in the monasteries of northern France, where Abbot Aldahard of Corbie decreed that his abbey porter receive a fraction of hops, as written in his Consuetudines Corbeiens (1). Today, more than a century later, hops are still used as a main ingredient in beer because of tradition and other beneficial reasons. Hops have been anecdotally and empirically proven to contain antibacterial properties and provide microbial stability to beer (2,3). Hops are thought to increase the flavor stability of beer by infusing the beverage with many antioxidant compounds capable of reducing oxidative species (4,5); although some evidence has shown that this point is controversial, as hops do contain pro-oxidant compounds as well (6,7). Most interestingly, a component of the hop resin, xanthohumol, has been shown to have anti-cancerous properties, and can potentially be used as a chemopreventive supplement (8,9). Unfortunately, imbibing numerous pints of ale for anti-cancerous effects is not practical because the concentration of anti-cancerous compounds is too low in beer; one would fall from alcohol poisoning before achieving any health benefits.
Above all, the best reason hops are still an essential ingredient in beer is because of the sensory properties they contribute. In other words, people like the flavor of hops in beer. For a brewer, hops serve as the spice cabinet, capable of imparting beautiful aromas ranging from floral to woody to fruity, etc. The aroma contribution of hops is of great importance, however brewers also select hops for their contribution to taste, namely bitterness. Hops contain bitter tasting compounds which are used to balance the natural sweetness of wort made from malted grains. Ales and lagers fermented with brewer’s yeast, *Saccharomyces cerevisiae* and *Saccharomyces pastorianus* respectively, generally finish fermentation with 1% to 5% residual sugar. The bitter tasting compounds of hops are married with the innate sweetness of beer, transforming it into an aesthetically pleasing beverage.

It goes unsaid amongst conversations with brewers that the word “hop” or “hops” refers to the processed hop cones of the female *Humulus Lupulus* plant or a hop product made from the female hop cones. Male plants are useful in breeding and the development of new hop varieties, but otherwise are segregated from female plants as the act of pollination will result in lower hop cone yield and quality \(10\). Hop cones are flowers layered in bracteoles that grow on the vines of the perennial *Humulus Lupulus* plant. These hardy plants are grown on trellises that can reach heights upwards of 20 feet, making harvesting the tall plant somewhat of a challenge. The use of specialized trucks to remove tall hop vines and the use of mechanized hop cone separators has made cultivation of hop plants less laborious \(10\). In the northern
hemisphere, the *Humulus Lupulus* plants typically begin growth in early spring, March to April, and are harvested in the fall, August to September. The harvested vines are then stripped of their cones by a mechanical separator. The cones are dried from roughly 75% to 9% moisture, cooled, and packaged in bails or other specialty packaging before leaving the hop cultivar’s facility (II).

1.2 Hop additions to beer: bitterness and aroma

Hops can be added at various time points in the brewing process, generally with the intended purpose to add hop bitterness, to add hop aroma, or perhaps to add both. To add primarily bitterness to a beer, a brewer would add hops early in the kettle boil. The conventional wisdom is that hop α-acids will be thermally isomerized to form bitter iso-α-acids, while the aroma compounds of hop oil will be volatilized or boiled off. It is evident that kettle hopping imparts some aroma to beer, however more importantly kettle hopping adds bitterness in a predictable and calculated manner. To add primarily hop aroma to a beer, a brewer would add hops towards the end of the boil or even after the boil during the cold side of production. A common method is to add hops into the fermenter, a technique known as “dry hopping”. The conventional wisdom is that dry hopping only adds the aroma compounds of hop oil to beer and absolutely no bitterness; because the bitter iso-α-acids will not form without the presence of heat. While it is true that iso-α-acids do not form without heat in a brewery setting, the underlying assumption is that iso-α-acids are the only bitter
compounds in hops. It has been shown that other hop acids, including oxidized hop acids are bitter, and perhaps even other components of the hop such as polyphenols can add significant bitterness (12). The notion that hops can contribute bitterness to beer in a ways other than α-acid isomerization due to heat, is challenging the conventional wisdom. The hopping techniques used by brewers to add exclusively hop aroma or hop bitterness are perhaps not so black and white.

1.3 Hop cone composition and anatomy

Hops contain thousands of important compounds known as ‘secondary metabolites’. Secondary metabolites are substances that do not participate in vital metabolic functions but help the plant interact with the surrounding environment (13). For example, secondary metabolites may fend off predators or pathogens, or they may attract animals to aid in pollination and seed dispersal; they are passed on through hop generations as evolutionary traits. The majority of secondary metabolites can be categorized as hop acids, hop oils, or polyphenols, and they are most concentrated in the hop cone.

The exact composition of hop cones can vary greatly between hop varieties, although generally they follow a common profile. Hops contain two major groups of hop acids called α-acids and β-acids ranging from 2.0 – 17.0% and 2.0-10.0% respectively on a dry weight basis of the kilned hop cone (Table 1)(14). The hop acid content of the hop cone, especially the α-acid content, is arguably the most important component to
brewers and growers. Another important feature is the hop oil content, ranging from 0.5 – 3.0% of the total hop cone weight. The majority of the remaining weight of the hop cone is attributed to water and plant macromolecules, like polyphenols, cellulose, lignin, and protein.

Table 1. Hop cone components by weight

<table>
<thead>
<tr>
<th>Hop Components</th>
<th>Concentration (%w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose + lignin</td>
<td>40.0 - 50.0</td>
</tr>
<tr>
<td>Protein</td>
<td>15.0</td>
</tr>
<tr>
<td>Alpha acids</td>
<td>2.0 - 17.0</td>
</tr>
<tr>
<td>Beta acids</td>
<td>2.0 - 10.0</td>
</tr>
<tr>
<td>Water</td>
<td>8.0 - 12.0</td>
</tr>
<tr>
<td>Minerals</td>
<td>8.0</td>
</tr>
<tr>
<td>Polyphenols and tannins</td>
<td>3.0 - 6.0</td>
</tr>
<tr>
<td>Lipids and fatty acids</td>
<td>1.0 - 5.0</td>
</tr>
<tr>
<td>Hop oil</td>
<td>0.5 - 3.0</td>
</tr>
<tr>
<td>Monosaccharides</td>
<td>2.0</td>
</tr>
<tr>
<td>Pectin</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Hough et al. (14)

The hop cone anatomy is that of a stabile, containing bracts and smaller bracteoles that cover that contain lupulin glands and may contain seeds if pollinated (Figure 1). The lupulin gland is home to the hops resins: hop oils, hop acids, and other compounds, and it can be considered the pearl of the hop cone. The lupulin gland is especially valuable in brewing because it is filled with strong sensory impacting molecules. There are at least 250 aroma compounds above aroma threshold concentration in hop oil and numerous hop acids that can provide prominent bitter taste (15).
Figure 1. Anatomy of hop cones

Hough et al. (14)
1.4  Hop Acids

1.4.1  Humulones, α-acids

The humulones or α-acids are a group of organic acids with a cyclohexane backbone, which contain at least three known different R-groups (Figure 2). The R-groups are short alkane structures seen in Figure 3 (co, hu, ad), and they denote the α-acid “analog”. Humulones or α-acids have three analogs known as cohumulone, humulone, and adhumulone, which are only different by one carbon and three hydrogens; 347.1 m/z (cohumulone) and 361.1 (humulone/adhumulone) (16). Other hop acids have the same R-groups (co, hu, ad) and the same nomenclature system, however they have a different backbone structure.
1.4.2 Lupulones, β-acids

Figure 4. Structure of Lupulones, β-acids

Lupulones or β-acids (Figure 4) have a similar chemical structure to α-acids. They have the same cyclohexane backbone, and five of the six carbons in the ring have the same constituents bound to them. The β-acids have an additional 5-carbon prenyl group (3-methyl-but-2-en-1-yl), in place of an alcohol group, bound to one of the carbons in the alkane ring. This is a distinctive feature of β-acids, and β-acid degradation products. The additional 5-carbon group gives β-acids the molecular weights 399.2 (co) and 413.2 (hu/ad) (16).

1.4.3 α and β acids do not contribute bitterness to beer

The structure of α acids makes them slightly soluble in beer, with a maximum solubility of roughly 14 ppm (17). It has been shown through sensory testing that α-acids at maximum concentration in beer did not produce perceivable bitterness (17).
The low solubility of \( \alpha \)-acids combined with their seemingly nonexistent bitterness makes their impact on beer flavor negligible. Similarly, \( \beta \)-acids have no role in beer flavor. \( \beta \)-acids contain an additional 5 carbon group, which makes them more hydrophobic and essentially insoluble in beer. Although \( \alpha \)-acids and \( \beta \)-acids themselves have negligible importance in beer flavor, they have the special ability to transform into other compounds that are extremely bitter.
1.5 The bitter isohumulones, iso-α-acids

![Figure 5. Structure of isohumulones, iso-α-acids](image)

The most well-known product of α-acids is the isomerized α-acids, known as the isohumulones or iso-α-acids. Iso-α-acids do not naturally exist in hop cones, but are a product of α-acids after processing. To form iso-α-acids, α-acids needs to do undergo a structural rearrangement, going from a 6-membered carbon ring to a 5-membered carbon ring, in an event brewers term isomerization; although the product is not an isomer by the strict chemistry definition. The molecular weights of iso-α-acids are nearly identical α-acids, measuring at 347.0 m/z (co) and 361.0 m/z (hu/ad) (16). (Intellman, Hoffman 2011). Although their molecular weights may be similar, the 5-carbon ring and oxidation state of iso-α-acids cause them to behave chemically differently. The additional keytone group and the reduction from cyclohexadiene to cyclopentene makes iso-α-acids more polar and soluble in beer; making them most importantly, very bitter (16).
Iso-α-acids can be synthesized from α-acids using metal catalysts like calcium or magnesium in laboratory setting (18). Also, iso-α-acids can be synthesized through thermal isomerization. As alluded to earlier in the text, this is the process occurs when hops are placed in the kettle with boiling wort. It has been shown that during wort boiling the reaction rate of α-acid isomerization is dictated by only two factors, temperature and time (19). Surprisingly other factors in wort such as calcium concentration, saccharide quality, and pH surprisingly do not effect the isomerization rate. For this reason, brewers add hops to boiling kettle for typically an hour to 90 minutes to impart bitterness, maximizing the isomerization of α-acids to iso-α-acids.

In a similar light to minimize the amount of hop materials used, hop growers over the years have selectively hybridized hops to maximize their α-acid content. “High-α” varieties such as Summit or CTZ may contain up to 20% α-acids by weight and are very efficient at bittering beer (19). These varieties are used to bitter beer but also used to create further concentrated iso-α-acid extracts and other advanced hop acid extracts. Hop raw materials are so densely concentrated in hop acids that they are added to beer in substantially small amounts, magnitudes less than 1% by weight of a recipe; however they greatly influence beer flavor (14).

1.6 Ideal storage conditions for hop products

It is of upmost importance to brewers that hop products are of high quality and that they maintain their quality through storage. Storage times can be very long, as hops
are harvested once a year but beers are brewed year round. For breweries to produce consistent beers, the quality and flavor of raw ingredients like hops need to stay consistent throughout the year.

Hop products are best stored in freezing, inert conditions to prevent hop acid and hop oil degradation. It was discovered in the 1950s that hop acid degradation during storage is due to oxidation reactions (20). To control oxidation reactions, evidence has shown that inert packaging is the best way to minimize hop acid degradation due to minimal exposure to oxygen, followed by cold temperatures during storage, although a combination of both is ideal (21,22).

Research has shown that hop pellets can be stored refrigeration temperatures +2 °C for 12 months and exhibit no significant change in hop acid composition, if the pellets are stored in inert (N₂) atmosphere (21). However, hop cones that were stored in large bales (not inert) at -5°C for just 3 months, suffered greater α-acid degradation than the same hops stored -20°C (23). The conclusion from these two studies is that keeping oxygen away from hops is the single best storage technique, otherwise extremely cold conditions, like -20°C, will reduce α-acid degradation of hops. This is a noteworthy realization, because often large volumes of hop cones are not sold in inert packages.

Overall, the best possible storage conditions for hops is an inert atmospheric and freezing temperatures to reduce hop acid oxidation.
1.7 Hop acid oxidation

Oxygen containing species in hops and atmospheric oxygen are the culprits in hop acid oxidation. The conventional understanding is that oxygen directly interacts with hop acids in an event called “autooxidation”. However, some research supports the theory that oxygen indirectly oxidizes hop acids by first reacting with hop oil compounds, like β-pinene, turning them into pro-oxidants, which in turn oxidize hop acids (24). Preventing the interactions between hop oils and hop acids in hop cones is unavoidable because of their proximity within the lupulin gland.

1.8 Humulinones

1.8.1 α-acid oxidation

Scientists began investigating α-acid oxidation and its products in the 1950s (25). Palamand and Aldenhoff in 1973 reported that there are four or more oxidation products from α-acid oxidation reactions performed in a laboratory setting (26). Few researchers have examined if these discovered compounds are of actual significance in beer and in hops. Tanguchi et al state in their recent manuscript on hop aging, that the most important group of oxidized α-acids formed during hop aging is the humulinones (27). Tanguchi and others also reported a newly discovered compound named, 4’-hydroxy-allohumulinone, which appeared in similar concentrations in hops as the
humulinones. Other than the mention of this novel compound, humulinones are recognized as the primary oxidation product of α-acids.

1.8.2 Structure

![Structure of oxidized α-acids, humulinones](image)

Figure 6. Structure of oxidized α-acids, humulinones

Humulinones are structurally identical to iso-α-acids with the exception of one additional oxygen on a member of the 5-carbon ring. This discrepancy is reflected in the increase in molecular weight of humulinones by 16 (m/z): 363.1 (co) and 377.1 (hu/ad) versus iso-α-acids 347.0 (co) and 361.0 (hu/ad) (16). The structural similarity of humulinones to iso-α-acids and their proven proliferation in hops are reasons enough to inspire scientists to examine their bitterness.

1.8.3 Bitterness

In 1955 Cook et al wrote in their manuscript focused on the creation of oxidized α-acids that “humulinones are bitter” (28). In 1964, Whitear and Hudson published estimates of humulinones bitterness, stating that humulinones are 35% as bitter as iso-
α-acids, however “…very little is known of their importance in beer flavor” (29). These findings are the extent of published knowledge on the bitterness of humulinones.

1.9  Hulupones

1.9.1  β-acid oxidation

There has historically been more interest in the oxidation of β-acids than the oxidation of α-acids because the possibility of non-bitter β-acids becoming oxidized to bitter oxidized β-acids is an intriguing idea. It is often mentioned in the topic of hop aging that old hops maintain their bitterness over time because potentially bitter α-acids are lost however potentially bitter oxidized β-acids which are gained. There is no direct evidence to support this claim, although many bitter oxidized beta acids have been identified.

Haseleu et al reported 12 oxidized β-acids that can be found in beer with bitter recognition thresholds of less than 100 mg/L (30). The most bitter of the reported oxidized β-acids was hulupones at about 8 mg/L. Tanguichi et al. measured the concentration of hop acid oxidation products during storage, and reported hulupones as being the major product of β-acid degradation (27). The research of Krofta et al. confirmed that hulupones are the most bitter and abundant hop acids formed during forced aging of β extracts, and noted that the oxidized β-acid extract tasted about 35-
40% as bitter as iso-α-acid extract in beer (31). While there have been numerous oxidized β-acids reported there seems to be agreement amongst researchers that hulupones are the most abundant product.

1.9.2 Structure

![Figure 7. Structure of oxidized β-acids, hulupones](image)

Hulupones are structurally similar to their parent compound in that they contain two 5-carbon prenyl groups bound to a single carbon of the carbon ring; the distinct feature of β-acids. However one major change between hulupones and lupulones (β-acids) is the shrinking of the cyclic carbon backbone from a 6-carbon to a 5-carbon ring. The mechanism of this reaction is not well understood, but the structural change makes hulupones more similar to iso-α-acids and humulinones, as they both have 5-carbon rings as well. The new structure allows hulupones to be very soluble in beer and perhaps substantially bitter. The new molecular weight of hulupones is 317.0 m/z (co) and 331.1 m/z (hu/ad) (32).
1.9.3 Bitterness

In 1960 Spetsig et al was the first to comment on the bitterness of hulupones, simply stating that “hulupones are bitter” (33). In 1973 Palamand et al published an article examining the bitterness intensity of many hop acids, and noted that hulupones are about 50% as bitter as iso-α-acids (26). Two decades later, Briggs et al cites the complete opposite finding, stating that hulupones are twice as bitter as iso-α-acids (34). There is much disagreement between the estimations of previous researchers likely due to the variability of sensory methods at the time. None of the former publications mention any details of their testing procedure, sample preparation, or statistics used in determining their estimated values. The goal of this study is to use modern sensory methods and clear reporting to help elucidate the bitterness intensity of hulupones and humulinones and their potential impact on the flavor of beer.

1.10 Transmission of humulinones and hulupones to beer

1.10.1 Whirlpool hopping and dry hopping

Researchers have shown that the formation of humulinones and hulupones can occur during hop storage and hop aging (28, 31). Other researchers have shown that the creation of humulinones and hulupones occurs when hops are added to boiling wort (29, 30, 31). Lastly, one source has shown that emergence of humulinones and hulupones can occur during beer storage and beer aging (27). It is likely that
humulinones and hulupones form in each of these ways, but regardless of how they are formed their presence in beer is directly connected the amount of hops used. Common craft beer styles like Pale Ales and India Pale Ales use very large amounts of hops in the later stages of the brewing process as compared to conventional kettle hopping. To achieve a prominent hop aroma it is common to add hops at the rate of 2 g/L to 20 g/L during the whirlpool and dry hop stages. On the other hand, to achieve a prominent bitter taste it is common to add hops at a rate of 0.5 g/L to 2 g/L during the wort boiling stage. The former techniques, whirlpool hopping and dry hopping, use about tenfold the amount of hops by weight for a given beer. Because whirlpool hopping and dry hopping opportunity to transmit substantial amounts of humulinones and hulupones to beer is much likelier in beers made with whirlpool hop and dry hop additions. The transmission of humulinones and hulupones to beer during hop aroma additions is an interesting notion for future research.

1.11 Measuring hop acid oxidation

1.11.1 HSI

Little method development has been published in means of measuring oxidized hop acid products. The current standardized method to measure hop acid oxidation is known as the Hop Storage Index [HSI], which is an indicator of hop acid quality, rather than quantification or identification measurement (36). The hop storage index
method was developed in 1970 by Gail Nickerson and Sam Likens at Oregon State University with the goal to measure the deterioration of hop α-acids and β-acids (36). The strategy of the method is to simply extract hop acids from a hop sample using alkaline methanol, and then to measure an absorbance ratio of 275nm to 325nm. The absorbance ratio correlates well with the ratio of oxidized α-acids and β acids to unadulterated α-acids and β-acids in the hop sample: HSI = \frac{A_{275\,nm}}{A_{325\,nm}}. According to the ASBC method hops of good keeping quality exhibit HSI values of less than 0.32 after 6 months of storage (36). This method may help determine the extent of hop acid oxidation and hop quality, however, it does not help identify or quantify the hop acid oxidation products.

1.11.2 HPLC

The best modern technique to characterize hop acids is High Performance Liquid Chromatography [HPLC]. HPLC allows researchers to separate compounds within a complex mixture, to identify compounds interest, and to measure the concentration of those compounds. Although no method has yet been standardized by the ASBC to measure oxidized hop acids in beer via HPLC, hop scientist have used existing methods or adapted methods. Published methods in literature use a C18 reverse phase column with an acidic mobile phase of methanol or acetonitrile with water (27, 30, 31, 32). In the following work, a similar approach was used, adapting the ASBC Beer-23 HPLC method to measure oxidized hop acids.
Chapter II: The Bitterness Intensity of Oxidized Hop acids: Humulinones and Humulinones

Manuscript to be reviewed

The following is a manuscript to be submitted to and reviewed in the Journal of the American Society of Brewing Chemists (ASBC) pages 21-48.
The Bitterness Intensity of Oxidized Hop acids: Humulinones and Hulupones

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ABSTRACT: Humulinone and hulupone extracts were prepared for evaluating their bitterness intensity in beer. Previously established methods for oxidizing alpha and beta acids followed by preparative liquid chromatography resulted in humulinone and hulupone extracts of purity; 93% and 92% respectively as measured by HPLC. The humulinone and hulupone extracts were dosed into unhopped lager over a range of concentrations from 8 mg/L to 40mg/L. Similarly, purified iso-α-acids were dosed into unhopped beer at concentrations ranging from 6mg/L to 30mg/L. A 9 member trained panel scaled the bitterness intensity of all samples in 5 replicated testing sessions. Humulinones were found to be 66% as bitter as iso-α-acid (+/-13%), and hulupones were found to 84% as bitter as iso-α-acids (+/-10%). The results of this study suggest that humulinones and hulupones are more bitter than previously suspected.

KEYWORDS: humulinones, hulupones, bitterness, sensory, oxidation, hops.
INTRODUCTION

Interest in the oxidization products of hop acids began in the 1950s when Cook and Harris published their article titled “Humulinone, a new constituent of hops” (25). These researchers identified a group of oxidized α-acids, humulinones, in hops at a concentration of 1 to 2% by weight. Two years later Verzele and Govaert published their article “Humulinone: its Alleged Occurrence in Hops”. As alluded to in their title, Verzele and Govaert were skeptical of the existence of humulinones in hops, and declared that humulinones were oxidation artifacts created during the hop acid extraction process (37). This was a worthy observation as it has been shown that peroxides in ether will oxidize α-acids to humulinones (28); nonetheless the existence of humulinones in hops remained debatable.

The discovery of a group of oxidized β-acids, named hulupones, brought about more disagreement amongst researchers. In 1960, Spetsig et al stated that dried hop cones contain up to 3% hulupones by weight (33). One year later in 1961, Stevens et al measured hop cones to contain only 0 - 0.5% hulupones by weight of the dried hop cone in a survey he conducted on many hop varieties (38). Before researchers could agree on the abundance of humulinones and hulupones and the best methods to quantify them, research began which sought to determine the potential bitterness of these oxidized hop compounds.
Cook et al. 1955 was the first to comment on the flavor of humulinones, simply stating that they were “bitter” with no further description (25). A similar observation was made about hulupones in 1960 by Spetsig et al. stating “…hulupones are bitter.” (33). It became accepted that oxidized hop acids were bitter, and in the following decade researchers began to estimate the bitterness intensity of humulinones and hulupones.

In 1964, Whitear et al. found that a beer dosed with 70mg/L of humulinones reached approximately the same bitterness of a beer with added 25mg/L of iso-α-acids, inferring that humulinones are 35% as bitter as iso-α-acids (29). This conclusion was made with little consideration to sensory technique, as there was no mention of how the beer samples were prepared, how the concentrations were measured, or how the sensory testing protocol was executed. There is no mention of the number of panelists, the replications, or the statistical analysis used. Additionally, no further work has been carried out since then to confirm the bitter intensity of humulinones. For the bitterness intensity of hulupones, in 1973 Palamand et al. stated that hulupones are about 50% as bitter as iso-α-acids (26). Conversely, Briggs et al. cites the complete opposite finding, stating that hulupones are reported to be twice as bitter as the iso-α-acids (34). The bitterness intensity of humulinones and hulupones is evidently still not agreed upon.

It is likely that some of the variation in previous findings was attributed to the lack of standardized testing methodology. A group of recent publications have revisited the topic of humulinones and hulupones, using modern chemistry separation techniques like High Performance Liquid Chromatography (HPLC) (27, 30, 31, 32). These works
have shown that humulinones and hulupones are in fact the most abundant oxidized hop acids in aged hops (27, 31), and that they can be found in beer at substantial concentrations (30, 32). The purpose of this research is to conclusively determine the bitter intensity of humulinones and hulupones in comparison to iso-\(\alpha\)-acids, using standardized sensory methods and modern chemistry techniques.

Figure 8. Bitter compounds of interest and their origin\(^a\)

\(^a\)**1**abc (co,n,ad) humulone, **2**abc (co,n,ad) isohumulone, **3**abc (co,n,ad) humulinone, **4**abc (co,n,ad) lupulone, **5**abc (co,n,ad) hulupone**
EXPERIMENTAL

**Humulinone preparation.** The oxidation procedure of Cook *et al* in 1955 was followed to produce humulinones 3\textit{a/b/c} from α-acids 1\textit{a/b/c} (3). A solution of α-acid in ether and cumene hydroperoxide was suspended over a saturated sodium bicarbonate layer for 4 days at 20°C. The starting material was 200 grams of a commercial α-acid extract 50%, composed of α-acid analogs 1\textit{a/b/c}. 58 grams of crude humulinones 3\textit{a/b/c}, as measured by HPLC-MS-TOF, was collected having the appearance of a yellow crystalline residue (yield ~50%). 15 grams of the humulinones were washed with 100 mls of ether and dried followed by successive washes with cold distilled water until the yellow color dissipated. The resulting white residue was dried and prepared for freeze-drying. After freeze-drying 7.8 grams humulinones 3\textit{a/b/c} in the form of a fine white powder was obtained.

**Hulupone preparation.** The oxidation procedure of Wright 1963 was followed to produce hulupones 5\textit{a/b/c} from β-acids 4\textit{ab/c} (39). A solution of β-acids in alkaline ethanol and sodium sulfite was mixed under oxygen atmosphere at room temperature for 12 hours. The starting material was 100 grams of 75.8% β-acid extract, composed of β-acid analogs 4\textit{a/b/c}. The resulting product was a low-yielding slurry of hulupones 5\textit{a/b/c}, unidentified oxidized compounds, and unreacted β-acids as measured by
HPLC-MS-TOF. Lower than expected yields have been observed by others using this method (40).

The crude mixture of hulupones was dried by rotary evaporation, and dissolved in 1 liter of aqueous 0.2M NaOH. The solution was titrated with a 3.0M HCL solution to ~ pH 5.5 to precipitate of any remaining β-acids and other hydrophobic compounds. The precipitated compounds were discarded and the aqueous solution was acidified, extracted into ether, and prepared for preparative liquid chromatography.

**Reverse phase liquid chromatography [RP-LC].** Preparative RP-LC was used to further purify the crude mixture of hulupones. A 250ml cylindrical separatory column was packed with 100 grams of C18 silica gel base packing material (50μm particle size, 70Å pore size, 480 m²/g surface area, Supelco Discovery). A series of eluents in increasing ethanol concentration were used to stratify the mixture; 40%, 50%, 55%, 60%, 65%, 70%, and 90% ethanol. The 60%, 65%, and 70% ethanol eluates contained predominantly hulupones 5a/b/c (Figure 9) and these solutions were collected, combined, concentrated, and rerun a second time through the same column to further purify them. The collective purified eluate was extracted into ether, rotary evaporated, and freeze dried, resulting in 3.2 grams of purified hulupones 5a/b/c in the form of viscous, yellow solution.
Figure 9. Chromatographic results of preparative RP-LC eluates.
Identification of oxidized hop acids: High Performance Liquid Chromatography.

Humulinones 3a/b/c and hulupones 5a/b/c were identified at the Oregon State University Mass Spectrometry Laboratory with a Shimadzu Nexera HPLC coupled to a AB Sciex 5600 MS-MS-TOF. The column was a 4 x 100 mm i.d., 2.6µm, C18 (Phenomenex, Kinetex) column maintained at 40°C. The mobile phase was 50% acetonitrile, 49.9% water, and 0.1% formic acid, set to linearly increase to 70% acetonitrile, 29.9% water, and 0.1% formic acid over a 20 minute run. Compounds were detected at 275nm and 330nm, and masses (m/z) measured in negative ion mode (-[M-H]).

Subsequent HPLC work to quantify these compounds in beer samples was performed in the OSU Brewing Science Laboratory on a 1200 series Agilent instrument with a photo diode array detector. The concentrations of hop acids in beer samples were assayed using ASBC method Beer-23 (36) on the same C18 (Phenomenex, Kinetex) column measuring absorbance at 275nm for the iso-α-acids and humulinones and 330nm for the hulupones. These wavelengths were chosen considering the absorbance spectrum of each hop acid (Figure 10). The method was capable of separating all analogs (co/n/ad) of each hop acid: iso-α-acid 2a/b/c, humulinones 3a/b/c, and hulupones 5a/b/c (Figure 11).
Figure 10. Absorbance spectrum of A isocohumulone (2a), B cohumulinone (3a), and C cohulpone (5a) measured in 100% methanol.
Figure 11. HPLC chromatograms of standards: humulinones 3a/b/c, hulupones 5a/b/c, and iso-α-acids 2a/b/c.
Unhopped Lager Preparation. 300 Liters (~80 gallons) of unhopped lager beer was brewed in the Oregon State University research pilot brewery as the base for each treatment. The beer was brewed using 70% 2-row pilsner malt and 30% high glucose adjunct. The wort was fermented with lager yeast (strain 2124, Wyeast) at 55°C, and the final beer specifications were 5% ABV and 2.5% real extract.

Beer Treatment Preparation. The unhopped lager was individually dosed with each extract (humulinones, hulupones, and iso-α-acids) into 3 separate 1/6th barrel (~20L) kegs (Figure 12). The kegs were cold stored (~1°C) for 24 hours with intermittent agitation to allow for full solubilization of the added hop acids. The dosed beer treatments were sterile filtered with a 0.45µm PVDF cartridge filter (Pall Inc.) to remove any insoluble hop material and then assayed via HPLC to determine the exact hop acids concentration of each treatment. The dosed beers were then diluted with unhopped beer to achieve the desired concentrations: 6, 12, 18, 24, 30 (mg/L) for iso-α-acid, and 8, 16, 24, 32, 40 (mg/L) for humulinone and hulupones. All treatments were assayed in triplicate via HPLC to confirm final concentrations. The resulting treatments resided separately in 15 individual 12L (3gallon) kegs. The final beer was carbonated to approximately 2 volumes of carbon dioxide prior to sensory evaluation.

Safety Testing. Humulinone and hulupone extracts were dosed into beer at 200 mg/L (5 times the concentration of the highest level sensory sample) and sent to an external, certified commercial lab for safety testing. Both beers had undetectable levels of residual solvent (n-Hexane, detection level (DL) - 15.0 µg/L) and heavy metals
(Arsenic, Lead, and Mercury, detection levels of 10.0, 2.5, and 0.23 µg/L, respectively).

Figure 12. Sample preparation flow chart
**Sensory Testing and Experimental Design.** The OSU Bitterness Panel consisted of 9 members, 5 males and 4 females, ranging from ages 25 to 53. All 9 panelists had previous training on bitterness quality and descriptive analysis. A short training regime of 3 one-hour sessions was carried out over a period of 1 week. During the training sessions panelists practiced rating bitterness intensity on a scale of 0 to 15, and were allowed to experience all 15 beer samples prior to official testing.

The testing sessions were carried out the week after training in a conference room in Wiegand Hall at Oregon State University. Panelists were seated at a table and received a large tray (Appendix II) with the following items: 5 beer samples, 1 unhopped beer, a glass of 0.1% pectin rinse solution, a glass of water, 3 unsalted crackers, and 3 warm-up beer samples labeled “low” (6mg/L iso-α-acid), “medium” (18 mg/L iso-α-acid), and “high”(35 mg/L iso-α-acid). Panelists were instructed to first taste the warm-up samples to acclimate to the bitterness intensity of the following testing samples. Panelists then evaluated each beer sample in the order listed on their personal ballot. Between samples panelists were instructed to wait 1 minute, and to use pectin rinse and unsalted crackers as needed to alleviate sensory fatigue and bitterness carryover.

Testing samples were presented blind coded with 3 digit numbers. Each sample was served as a 60ml (~2oz) sample in a 240ml (~8oz) clear serving glass and served cold at approximately 4C. Panelists only rated one attribute, bitterness intensity, on a scale of 0 to 15. A total of 5 independent replications were performed on each sample, and
testing sessions were blocked by hop acid, meaning only beers of one hop acid type (iso-α-acid, humulinone, or hulupone) were evaluated together. Paper ballots were used to record panelist’s observations (Appendix I); the data was collated in Microsoft’s Excel and statistical analyses were performed using XLSTAT (Addinsoft version 2013.5.02).
RESULTS AND DISCUSSION

Instrumental Results

Creation of humulinone and hulupone extracts was a critical step before further research could be carried out since no commercial extracts of oxidized hop acid are currently available. For research purpose hulupone and humulinone extracts must be of high purity to ensure results are accurate and reproducible and to minimize the possible impact of impurities on flavor perception.

Preparative Reverse Phase Liquid Chromatography (RP-LC). The process of iterative preparative RP-LC was satisfactory in purifying crude oxidized hop acid extracts (Figure 9). The crude mixture of hulupones (approximately 10%) was washed of undesired compounds, isolating a mixture of hulupones 5a/b/c with 92% chromatographic purity (Figure 11). The RP-LC procedure was not needed in the production of humulinones; the aforementioned peroxidation reaction of humulones 1a/b/c led to a humulinone mixture 3a/b/c of 93% chromatographic purity (Figure 11). Chromatographic purity was estimated by measuring the peak areas of desired compounds in comparison to the total peak areas of all compounds in the chromatogram, examined over the UV wavelengths of 200-400 nm. The impurities of the humulinones and hulupones extracts accounted for less than 7% of the extract composition. It was assumed the potential flavor impact was negligible; in sensory testing no unexpected flavors were reported.
**Mass Spectrometry.** To confirm the identity of humulinones and hulupones, mass spectrometry time of flight was used to measure the molecular weights and the corresponding chemical formulas. The results were consistent with previously proposed structures (Table 2) (27, 32). The humulinone and hulupone samples were also examined by scientists at a commercial hop processor (S.S. Steiner Inc.). The absorbance spectra of corresponding compounds were measured and found to be consistent with previous findings (Figure 10) (40). The absorbance spectrum pattern can be used as a tentative identification tool on an HPLC equipped with a photo diode array detector.

<table>
<thead>
<tr>
<th>Compound Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mass (m/z)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chemical Formula&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>363.1813</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;28&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>3b/c</td>
<td>377.1970</td>
<td>C&lt;sub&gt;21&lt;/sub&gt;H&lt;sub&gt;29&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
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<tr>
<td>5a</td>
<td>317.1758</td>
<td>C&lt;sub&gt;19&lt;/sub&gt;H&lt;sub&gt;26&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>5b/c</td>
<td>331.1915</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;26&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Compound numbers refer to chemical structures in Figure 1.

<sup>b</sup> Mass results (m/z) measured in negative ion mode [M-H]-.

<sup>c</sup> Chemical formulas determined from mass (m/z).
**Beer treatments.** The range of hop acid concentration levels were purposefully chosen to cover a range of sensory bitterness of just above detection to a strong bitter sensation, hence an iso-α-acid range of 6 to 30 mg/L was selected. The humulinone and hulupone concentration range of 8 to 40 mg/L was chosen based on bench trials to achieve a similar sensory bitterness range of iso-α-acids. The concentrations of beer treatments were verified in triplicate on HPLC (Table 3). The measured averages were within 15% of the target concentration level for each treatment and variation within replicate injections was no greater than 8% of the mean. These average values (mg/L) were used in statistical analysis comparing instrumental and sensory data.

Table 3. Concentration of hop acids in beer treatments

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>2a/b/c iso-α-acid (mg/L)b</th>
<th>Treatmenta</th>
<th>3a/b/c humulinone (mg/L)b</th>
<th>Treatmenta</th>
<th>4a/b/c hulupone (mg/L)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso 0</td>
<td>0.0 +/- 0.0</td>
<td>Hum 0</td>
<td>0.0 +/- 0.0</td>
<td>Hul 0</td>
<td>0.0 +/- 0.0</td>
</tr>
<tr>
<td>Iso 6</td>
<td>6.9 +/- 0.3</td>
<td>Hum 8</td>
<td>7.8 +/- 0.6</td>
<td>Hul 8</td>
<td>8.3 +/- 0.2</td>
</tr>
<tr>
<td>Iso 12</td>
<td>12.1 +/- 0.5</td>
<td>Hum 16</td>
<td>16.1 +/- 1.0</td>
<td>Hul 16</td>
<td>16.0 +/- 0.5</td>
</tr>
<tr>
<td>Iso 18</td>
<td>18.6 +/- 1.1</td>
<td>Hum 24</td>
<td>24.1 +/- 1.2</td>
<td>Hul 24</td>
<td>23.6 +/- 0.6</td>
</tr>
<tr>
<td>Iso 24</td>
<td>24.5 +/- 0.4</td>
<td>Hum 32</td>
<td>33.0 +/- 0.8</td>
<td>Hul 32</td>
<td>31.4 +/- 0.7</td>
</tr>
<tr>
<td>Iso 30</td>
<td>30.4 +/- 1.2</td>
<td>Hum 40</td>
<td>40.6 +/- 1.2</td>
<td>Hul 40</td>
<td>39.8 +/- 1.5</td>
</tr>
</tbody>
</table>

a Treatments are abbreviated names of the hop acids followed by the target concentration level.

b Concentrations of beer treatments in averages +/- 1 standard deviation.
Sensory Results

**Unhopped control and bitterness detection threshold.** An unhopped control was blind coded and placed amongst samples in each testing, labeled as Iso 0, Hum 0, and Hul 0 (Tables 3 & 4). The purpose of these samples was to determine if panelists could distinguish the unhopped beer from the lowest hop acid treatment. In all cases, the unhopped control (Iso 0, Hum 0, and Hul 0) was scored significantly lower than the lowest hop acid treatments (Iso 6, Hum 8, Hul 8) (Table 4). Thus all hop dosages at their lowest concentrations were above sensory detection thresholds. These findings for iso-α-acids and hulupones support the work of others who determined the threshold of iso-α-acids to be 5-6 mg/L (41), and the threshold of hulupones to be 7 to 8 mg/L (30). For humulinones, no research has been published on the bitterness threshold. While the focus of this research was not specifically to identify detection thresholds; humulinones were perceived to be bitter at 8 mg/L or less by a trained panel. The bitterness detection threshold of humulinones at 8mg/L is a new finding.
Table 4. Sensory bitterness scores of beer treatments averaged over all panelists

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bitterness Score</th>
<th>Treatment</th>
<th>Bitterness Score</th>
<th>Treatment</th>
<th>Bitterness Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iso 0</td>
<td>1.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Hum 0</td>
<td>2.2&lt;sup&gt;A&lt;/sup&gt;</td>
<td>Hul 0</td>
<td>2.0&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iso 6</td>
<td>3.9&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Hum 8</td>
<td>4.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>Hul 8</td>
<td>4.2&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iso 12</td>
<td>6.6&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Hum 16</td>
<td>6.6&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Hul 16</td>
<td>6.3&lt;sup&gt;C&lt;/sup&gt;</td>
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<tr>
<td>Iso 18</td>
<td>9.1&lt;sup&gt;D&lt;/sup&gt;</td>
<td>Hum 24</td>
<td>7.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Hul 24</td>
<td>8.7&lt;sup&gt;D&lt;/sup&gt;</td>
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<tr>
<td>Iso 24</td>
<td>11.7&lt;sup&gt;E&lt;/sup&gt;</td>
<td>Hum 32</td>
<td>10.4&lt;sup&gt;E&lt;/sup&gt;</td>
<td>Hul 32</td>
<td>11.5&lt;sup&gt;F&lt;/sup&gt;</td>
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<tr>
<td>Iso 30</td>
<td>12.8&lt;sup&gt;G&lt;/sup&gt;</td>
<td>Hum 40</td>
<td>12.7&lt;sup&gt;GH&lt;/sup&gt;</td>
<td>Hul 40</td>
<td>13.7&lt;sup&gt;H&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Treatments with the different superscripts are significantly different using Fischer’s LSD at α <0.05, compared across all 18 treatments.

**Panelist performance.** The 5 replicated tests of each panelist were graphed, comparing bitterness scores (0 to 15) to the concentration values (mg/L) of each beer (Figure 13, A & C). For each panelist, the 5 observations per beer treatment were averaged and the average values were used to linearly regress hop acid concentration against bitterness score (Figure 13, B & D). The coefficients of determination (R<sup>2</sup> values) from each regression were used as a metric to measure panelist performance.
Figure 13. Responses and averaged responses of a “good” assessor and a “poor” assessor

\(^a\) A & B are the graphs of the “good” assessor and C&D are the graphs of the “poor” assessor

\(^b\) Graphs B & D were created by averaging data in graphs A & C and performing a linear regression on the data pertaining to each hop compound.
R² value is an indication of how well a panelist data fits a linear relationship. Although the relationship between iso-α-acid concentration and sensory bitterness is not completely linear (42), an increase in hop acid concentration should result in an increase in bitterness perception, making the R² coefficient an adequate predictor of panelist performance. Observing the R² results in Table 5, it is apparent that there were two groups of panelists. Those that could distinguish beer treatments in bitterness intensity yielded high R² values (R² > 0.82) while those who could not distinguish beer treatments had low R² values (R² < 0.49). For example, the data of panelist 4 is graphed in Figure 13 A & B illustrate this panelist’s ability to rate the beer treatments differently with low variation, resulting in a high average R² value of 0.97 (Table 5). In contrast, the data of panelist 2 is graphed in (Figure 13 C & D) and illustrate the large variation in the panelist’s results and the panelist’s inability to discriminate samples, resulting in a low average R² value of 0.46 (Table 5). Overall, two of the nine panelists (panelist 2 & 7) were removed from subsequent analyses (Table 6) because of their inability to discriminate beer treatments.
Relative bitterness intensity of hop acids calculated on a per panelist basis.

It was shown by Fritsch and Shellhammer 2008 that trained panelists differ when scoring the relative bitterness perception of various hop acids (42). In other words, the relationship of bitterness intensity between hop acids significantly varies from panelist to panelist. Taking into consideration this finding, a linear regression analysis approach was used on the individual data set within each panelist. The goal was to estimate the relative bitterness intensity of humulinones and hulupones to iso-α-acids on a per panelist basis.

A linear relationship existed between the sensory scores and hop acid concentrations. This relationship can be described as:

\[ Y_{ij} = M_{ij} X_{ij} + B_{ij} \]
where $Y_{ij} =$ the sensory bitterness score for panelist $i$ on hop acid $j$

$X_{ij} =$ the concentration level (mg/L) for panelist $i$ on hop acid $j$

$M_{ij} =$ the increase in sensory bitterness score for a unit increase in concentration (mg/L) for panelist $i$ on hop acid $j$.

$B_{ij} =$ the bitterness score at concentration 0 mg/L ($X_{ij} = 0$) for panelist $i$ on hop acid $j$.

This model was fitted to each panelist’s data, by adjusting $M_{ij}$ and $B_{ij}$ to form a best-fit line that minimized the residual sum of squares between predicted and observed results. The $M_{ij}$ values defined the increase in sensory bitterness score of an oxidized hop acid $j$ for a unit increase in iso-$\alpha$-acid concentration for panelist $i$. The panelist-specific $M_{i\text{Iso}}, M_{i\text{Hum}}, M_{i\text{Hul}}$ values are presented in Table 5.

To determine the concentration ratio for which iso-$\alpha$-acids ($X_{i\text{Iso}}$) are equally bitter to humulinones ($X_{i\text{Hum}}$) we can set the equation $Y_{i\text{Iso}}$ equal to $Y_{i\text{Hum}}$ and solve for $X_{i\text{Iso}}$:

$$Y_{i\text{Iso}} = Y_{i\text{Hum}}$$

$$M_{i\text{Iso}} X_{i\text{Iso}} + B_{i\text{Iso}} = M_{i\text{Hum}} X_{i\text{Hum}} + B_{i\text{Hum}}$$

Assuming all $B_{ij}$s to be equal for all panelist $i$ and all hop acids $j$, this equation reduces to:

$$M_{i\text{Iso}} X_{i\text{Iso}} = M_{i\text{Hum}} X_{i\text{Hum}}$$

and rearranges to:

$$X_{i\text{Iso}} = \left( \frac{M_{i\text{Hum}}}{M_{i\text{Iso}}} \right) X_{i\text{Hum}}$$
where $M_{i\text{iso}}$ and $M_{i\text{Hum}}$ are constants representing the concentration dependency of bitterness intensity for each hop acid, iso-\(\alpha\)-acid and humulinone, respectively.

The $B_{ij}$ values represent the bitterness intensity response of the non-dosed control sample for each panelist and each hop acid. These were similar in all cases thereby validating our assumption and confirming they could be eliminated in the process of simplifying the equibitter concentration relationships presented above. Thus, the concentration relationship between humulinones and iso-\(\alpha\)-acids at equal bitterness intensity is simply reduced to $(\frac{M_{i\text{Hum}}}{M_{i\text{iso}}})$. Using the values of $M_{i\text{iso}}$, $M_{i\text{Hum}}$, and $M_{i\text{Hul}}$ for each panelist (Table 5), we can determine the concentration ratio at which humulinones are equally bitter to iso-\(\alpha\)-acids $(\frac{M_{i\text{Hum}}}{M_{i\text{iso}}})$, and the concentration ratio at which hulupones are equally bitter to iso-\(\alpha\)-acids $(\frac{M_{i\text{Hul}}}{M_{i\text{iso}}})$. The relationships were calculated for each panelist and averaged (Table 6).
Table 6. Summary of panelist’s equi-bitter concentration ratios

<table>
<thead>
<tr>
<th>Panelist</th>
<th>$\frac{M_{Hul}}{M_{Iso}}$</th>
<th>$\frac{M_{Hum}}{M_{Iso}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.02</td>
<td>0.85</td>
</tr>
<tr>
<td>3</td>
<td>0.77</td>
<td>0.56</td>
</tr>
<tr>
<td>4</td>
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<td>0.87</td>
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<tr>
<td>5</td>
<td>0.80</td>
<td>0.55</td>
</tr>
<tr>
<td>6</td>
<td>0.85</td>
<td>0.58</td>
</tr>
<tr>
<td>8</td>
<td>0.80</td>
<td>0.64</td>
</tr>
<tr>
<td>9</td>
<td>0.68</td>
<td>0.61</td>
</tr>
</tbody>
</table>

**Average** | **0.84** | **0.66** |
**STDV**     | **0.10** | **0.13** |

Humulinones were found to be on average 66% as bitter as iso-α-acid (+/-13%) and hulupones 84% as bitter as iso-α-acids (+/-10%). The panelist-dependent nature of the relative bitterness of these compounds is evident in Table 6 in that these ratios vary across the panel. For instance with hulupones, panelist 1 found them to be equal in bitterness with iso-α-acids while panelist 9 found them to be only 68% as bitter. For humulinones, panelist 1 was again more sensitive to the oxidized hop acid finding the humulinones to be 85% as bitter as iso-α-acids while panelist 5 found them to be only half as bitter. So the reader must be cautioned that the grand averages presented in Table 6 have variation associated with them. Regardless, it should be apparent that iso-α-acids are more bitter than both hulupones and humulinones. As an example, the bitterness achieved by 15 mg/L of iso-α-acids is estimated to be equivalent in intensity
as 18 mg/L hulupones and 22.7 mg/L humulinones. This is represented graphically in Figure 14 where the concentrations for which humulinones and hulupones are equally bitter to iso-α-acids can be visualized.

![Graph showing concentrations of humulinones and hulupones estimated to achieve equal bitterness intensity to iso-α-acids.](image-url)

Figure 14. Concentrations of humulinones and hulupones estimated to achieve equal bitterness intensity to iso-α-acids.
This study examined the bitterness of three hop acids separately in beer, in order to gauge their relative bitterness intensities. However in commercial beers these hop acids will likely be found as mixtures at varying concentrations. It is not clear whether any synergy exists among these compounds or whether the bitterness contribution is simply additive. The impact of humulinones and hulupones on beer bitterness may be better understood with further investigation into their abundance in beer. It has been observed in hops that humulinones and hulupones are the major products formed during oxidation and storage (27, 31), however the extent to which these compounds are transmitted from hops into beer has not been examined. The possible oxidation of $\alpha$-acids and $\beta$-acids to form humulinones and hulupones during the brewing process is also largely not understood (30). Beers processed with large quantities of hops, such as dry hopped beers, may achieve high concentrations of humulinones and hulupones. An understanding of the abundance of humulinones and hulupones in commercial beers along with the bitterness intensity results of this study may help elucidate the importance of oxidized hop acids in beer bitterness and beer quality.

**Conclusion.** Using previously established synthesis methods and preparative liquid chromatography, high purity extracts of humulinones and hulupones were prepared for sensory testing (93% and 92% purity respectively). Humulinones and Hulupones were evaluated in unhopped lager and compared to iso-\(\alpha\)-acid. A trained descriptive analysis panel found humulinones to be 66% as bitter as iso-\(\alpha\)-acid (+/-13%), and hulupones to be 84% as bitter as iso-\(\alpha\)-acids (+/-10%). This study found the bitterness
intensity of humulinones and hulupones to be substantially higher than previous estimates of 35% for humulinones and 50% for hulupones (26, 29). While iso-α-acids were confirmed to be more bitter than oxidized α-acid and β-acids, both hulupones and humulinones were bitter enough to have a potentially significant impact on beer bitterness. Additionally, a trained panel was able to detect the bitterness of humulinones at a concentration as low as 8 mg/L; the first mention of humulinone detection threshold. The bitterness intensity of hulupones and humulinones is substantial and their influence on beer flavor should not be dismissed.
Chapter III: Future Work

In Verzele’s chapter titled, “The Future of Hop Chemistry” he proposes research ideas that he describes as interesting, short, and tailor-made for a student research project (16). The chapter advises researchers to take full advantage of modern chemistry separation instruments, to isolate hop compounds of interest, and to determine their importance in beer by performing simple sensory-like studies. The research done in this thesis essentially mimics this research approach, and has left many avenues of research regarding humulinones and hulupones.

As alluded to earlier in the text, determining the abundance of humulinones and hulupones in commercial beers would clarify their importance as flavor active compounds in beer. Unpublished data from this work has shown that these compounds may exist in commercial beers well above their threshold of detection, 8 mg/L. It was witnessed that concentrations of humulinones and hulupones were larger in beers brewed with larger quantities of hops, although further investigation should be done. This raises questions into the possible transmission and formation of humulinones and hulupones.

Anecdotally, it has been witnessed that adding hops during the cold side of production may increase the perceived bitterness of the beer. Although, this is a perplexing idea as iso-α-acids cannot form during these processing steps. This anecdote probes brewers to reconsider the notion that iso-α-acids are the only hop compound worth
measuring. It is hypothesized from this research and similar works that hopping protocols such as whirlpool hopping and dry hopping, incorporate oxidized hop acids, (humulinones and hulupones) and perhaps other bitter substances in to the beer. This is a research area that could be investigated relatively easily.

Beyond humulinones and hulupones, more than a dozen other oxidized α-acids and β-acids have been identified (27, 30, 31, 32). The research design of this study could easily be applied to test these newly discovered compounds. The best approach may be to investigate these novel hop compounds by first surveying commercial beers to determine which compounds exist in meaningful concentrations.

It is hoped that the findings of this work and future works will ultimately help improve the understanding of beer flavor and beer quality.
REFERENCES


40. Tynan, P., Murrough, T., and Byrne, J. Preparation, purification and separation by high performance liquid chromatography of humulinic acids, dehydrohumulinic acids, and hulupones. 96 (3):137–141 1990.

APPENDICES

Appendix A. Sample Sensory Ballot

Testing Day 1 PM 4.28.2014

Name: Victor A.

Taste the warm-up samples. Low: 1-2 Med: 7-8 High: 15-16

Rate the Set 1 samples on peak Bitterness intensity using a scale of 0 to 15. You may revisit samples.

Set 1

<table>
<thead>
<tr>
<th>649</th>
<th>323</th>
<th>968</th>
<th>444</th>
<th>720</th>
<th>359</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>9</td>
<td>7</td>
<td>13</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>

Please remove labels and return your glasses. Keep your warm-up samples as anchors. Take a break. Eat some of the provided crackers before starting set 2.

Rate the Set 2 samples on peak Bitterness intensity using a scale of 0 to 15. You may revisit samples.

Set 2

<table>
<thead>
<tr>
<th>398</th>
<th>766</th>
<th>557</th>
<th>512</th>
<th>881</th>
<th>473</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>5</td>
<td>12</td>
<td>2</td>
<td>15</td>
<td>8</td>
</tr>
</tbody>
</table>
Appendix C. Beer Decarbonation Method for HPLC

100 mls of cold carbonated beer was dispensed into a 250ml glass beaker. The beaker was placed in a water bath sonicator and set to sonicate for 15min at 20C. Large volumes of beer foam were produced during sonication; using a vessel with excess headspaces for foaming is recommended. After sonication the beer foam was allowed to collapse. The residual foam cling around the glass was collected back into solution by swirling the beer in the beaker. 1.5mls of the uncarbonated, room-temperature beer was transferred to a 1.8ml HPLC vial with a pre-slit PTFE screw cap lids.
Appendix D. Visual Depiction of Humulinone and Hulupone extracts

**Humulinone**  
A fine white powder

**Hulupone**  
A viscous yellow liquid