AN ABSTRACT OF THE THESIS OF

Shi Feng for the degree of Master of Science in Food Science and Technology presented on June 4, 2014.

Title: Aroma-Active Compounds in 'Centennial', 'Citra' and 'Nelson Sauvin' Hop Varieties and Their Aroma Contribution to Dry-Hopped Beer

Abstract approved:

Michael C. Qian

The objectives of this study were to 1) measure and compare the compositional differences of essential oil among three hop cultivars ('Centennial', 'Citra' and 'Nelson Sauvin'), 2) identify the odor-active aroma compounds in three varieties of hops and 3) investigate the behavior of hop-derived aroma compounds in beers prepared by dry-hopping approach with three hop varieties.

The major compositions of essential oil of three hop varieties ('Centennial', 'Citra' and 'Nelson Sauvin') were determined. Myrcene, β-caryophyllene and α-humulene were dominant compositions in all cultivars while certain esters as well as linalool and geraniol were relatively abundant in 'Centennial' and 'Citra' hops. The odor-active compounds in these three hop varieties were identified using gas chromatography-mass spectrometry/olfactometry (GC-MS/O) and aroma extract dilution analysis (AEDA). Application of AEDA revealed myrcene (celery, balsamic notes), isovaleric acid (smelly,

rancid and cheese notes) and geraniol (citrus note) as the most important aroma components in all hop cultivars by presenting the highest flavor dilution (FD) factors, followed by S-methyl methanethiosulfonate (radish, cabbage notes), linalool (floral, sweet notes) and vanillin (vanilla note). S-methyl methanethiosulfonate, having unique radish, cabbage notes, was detected in the hops for the first time and showed high FD factors in ‘Centennial’ and ‘Citra’ hops. Several sulfur-containing compounds were also identified as important contributors to hop aroma. Dry-hopped beer with ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’ revealed an increase of myrcene and α-humulene whereas the increase of β-caryophyllene was only in ‘Citra’ variety. Linalool and geraniol increased significantly in hopped beer. Due to the importance of linalool and aroma contribution differences of stereoisomers, the chiral isomers in both hops and beers were further studied. The results demonstrated the prevalence of R-linalool in hops and a conversion of R-linalool to S-linalool in beer was observed in control beer when hops were added at beginning of wort boiling.

Aroma-Active Compounds in ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’ Hop Varieties and Their Aroma Contribution to Dry-Hopped Beer

by

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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 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.

Shi Feng, Author

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CHAPTER 1 GENERAL INTRODUCTION

The hop plant, *Humulus lupulus L.*, belongs to the family *Cannabinaceae* (Neve 1991). The cultivated hop, *H. lupulus*, is a perennial climbing plant native to much of the Northern hemisphere between 35° and 55° N. Germany and America are the two biggest contributors to hop production around the world in the Northern hemisphere. Hops are also widely cultivated in countries located in Southern hemisphere, such as Australia, New Zealand and South Africa (Lewis and Young 2002, Briggs et al. 2004).

Hops were first cultivated for their herbal and medicinal properties (Neve 1991). The earliest record of the use of hops in beer brewing can be dated back to 1079 (Moir 2000). Originally, hops were added to prolong the shelf life of beer, but their continued use is due to the bitterness and pleasant flavor they contribute to beer. Hop resins are related to the bitterness of beer while hop essential oils are responsible for the hoppy aroma. Resins are major components of lupulin glands, which are found most extensively at the base of hop bracteoles, and can be divided as soft resins and hard resins (Neve 1991). Soft resins largely comprise α-acids and β-acids, and the former ones are the precursors of iso-α-acids, which play important role as the bitter principles of beer. Essential oils are also produced in lupulin glands and usually make up about 0.5-1.5%, in some case up to 3%, of the weight of the whole hops (dried hop cones) (Neve 1991, Lewis and Young 2002). Essential oil constituents introduce characteristic hoppy aroma to beer and can be generally classified into three groups: 1) hydrocarbon components; 2) oxygenated components; and 3) sulfur-containing components.

Hop cones are typically dried in kiln to a final moisture level of about 10% w/w or less. The dried cones, packed as conventional bales or pockets, are usually too bulky and expensive to transport and store. Moreover, the brewing value of hops loses gradually during storage. Therefore, various products have been developed to improve the hop utilization. The main hop products include: 1) hop pellets; 2) hop extract using organic solvent; 3) hop extract by CO_2 extraction 4) hop oil by steam distillation (Neve 1991, Lewis and Young 2002). Hop pellets are compressed powders obtained from whole hops by hammer milling. They are more stable than the whole hops and have higher extraction efficiency. Organic solvent and liquid/supercritical CO_2 are both employed to extract hop oil in addition to steam distillation.

Hops can be added at certain points of beer brewing based on specific brewing purposes. Hops or hop preparations are added at the beginning of the wort boiling to give bitterness. Late hop additions at the last five to ten minutes of the boil or in the whirlpool are mainly applied to replace the aroma loss during steam evaporation in wort boiling process (Harris 1976). Alternatively, dry hops can be added to fermented beer during conditioning or even directly to finished beer in cask, which is known as dry hopping, to give pure hop aroma (Briggs et al. 2004). During the brewing process, the key flavor composition (essential oils) of hops are alternated in various ways, therefore, hop addition performed at different points of brewing may have a quite diverse influence on the final beer flavor (Sharpe and Laws 1981, Kaltner and Mitter 2003) (**Figure 1**).

There are over 100 hop varieties in commercial use throughout the world. In this

study, three hop varieties (‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’) were investigated, among which the first two cultivars are American-grown and the third one is from New Zealand (https://www.hopunion.com/hop-varieties/). ‘Centennial’ has strong citrus aroma and is used for both aroma and bitterness enhancements. ‘Citra’ hop, known for its intense aroma characteristics, is added to beer as aroma variety since it imparts strong citrus and tropical fruit characters. ‘Nelson Sauvin’, possessing intense passionfruit and white wine-like flavors, is also effective for bittering to make it an excellent dual-purpose hop.

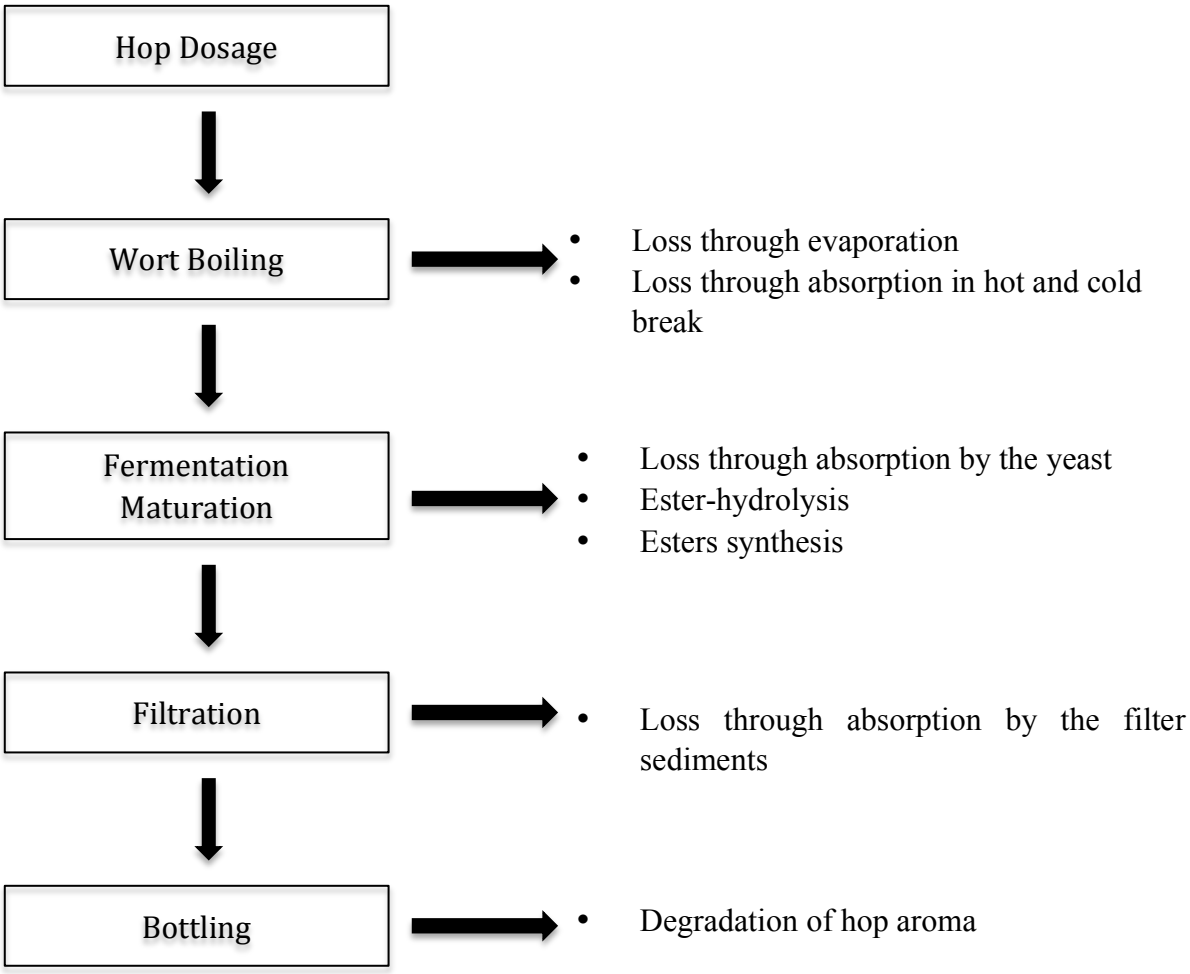


Figure 1. Schematic description of the possible loss and conversion of the compositions of hop oil during the brewing process (Kaltner and Mitter 2003).

CHAPTER 2 LITERATURE REVIEW

2.1 Hop Aroma Study

Considered as the 'spirit of beer', hops have been widely investigated by flavor chemists and brewers during last decades. Investigators are eager to decipher the mystery of hops and especially that of hop aroma so that more measures could be taken to control as well as develop beer flavor. Following the first extraction and investigation of hop oil fractions in the early 19th century, extensive studies have been conducted to explore the aroma profile of hops. The earliest systematic description of hop essential oil was made by Chapman, who revealed the presence of myrcene, humulene, caryophyllene as well as oxygenated components, linalool, geraniol, "luparone," "luparenol," "luparol," and linalyl isononoate in hops (Chapman 1895a, Chapman 1895b, Chapman 1903, Chapman 1928a, Chapman 1928b, Chapman 1929). About two decades later, the presence of myrcene, humulene, and caryophyllene in hop materials was confirmed by the investigation of Zatec hop oil (Stevens 1967). Meanwhile, from the same study, farnesene was isolated and, undecan-2-one was extracted and believed to be identical as "luparone" in Chapman's study. The invention of gas chromatography (GC) (James and Martin 1952) was a milestone in the history of analytical chemistry and its application to the exploration of hop oil has taken the understanding of hop chemistry to the next level. The first application of GC to hop oil analysis was conducted by Howard and his colleagues (Howard 1956, Howard 1957, Rigby and Bethune 1957). In 1963, a research of Bullion hop oil revealed the presence of approximately 200 components and tentatively identified

that the oxygenated fraction containing a complex mixture of alcohols (2-methylbutanol, linalool, nerol, geraniol, nerolidol, etc.), carbonyls (methylnonyl ketone and numerous other methyl ketones, citral and other aldehydes), and esters of carboxylic acids with straight chains, branched chains and unsaturated straight and branched chains (Jahnsen 1963). In the same year, the presence of 2-methylbutanol, linalool, methylnonyl ketone, methylundecyl ketone, geraniol, and the mono epoxides of caryophyllene and humulene were found to be major constituents in the non-saponifiable constituents of the oxygenated fraction of Brewer's gold hop oil (Roberts 1963). Afterwards, Buttery and Ling reported 76 components in five American hops and proposed that the peak patterns and area percentages of certain compounds could be used to distinguish between the varieties (Buttery and Ling 1967). In 1975, GC coupled with flame photometric detection was documented to specifically analyze the sulfur-containing compounds in hop oil and at least thirty components were detected (Pickett et al. 1975). At the end of 20th century, according to a comprehensive list compiled from 75 papers, about 500 compounds have been currently identified in hop essential oils (Nijssen et al. 1996). Nowadays, the chemical composition of hop oil is conventionally described as three classes: hydrocarbons, oxygenated components and sulfur-containing components (Sharpe and Laws 1981, Neve 1991).

So far, as the development of the analytical technique, it is becoming clear that the major components of essential oil fraction are not necessarily responsible for the characteristic hoppy aroma (Howard and Stevens 1959). In order to determine the

importance and influence of individual volatiles in hops, the relevant sensory thresholds of the odorants must be considered. This suggests that dominant constitutes in hops may not be the most flavor-impact compounds if they have high sensory thresholds, and on the other hand, some aromatic components with low concentration even trace amount can really contribute to the aroma character of hops due to their low thresholds in the sample matrix. Therefore, increasing number of flavor scientists has paid more attention to aroma-active compounds in hops instead of only looking at the overall chemical composition. By using gas chromatography-olfactometry (GC-O), *trans*-4,5-epoxy-(E)-2-decenal, linalool and myrcene were identified as the key aroma contributors in fresh and dried hop cones (Spalter Select variety), while (E,Z)-1,3,5-undecatriene, 1,3(E),5(Z),9-undecatetraene, (Z)-1,5-octadien-3-one, and ethyl 2-methylpropanoate and methyl 2-methylbutanoate were identified as important hop odorants in Spalter Select hop variety (Steinhaus and Schieberle 2000). Using aroma extract dilution analysis (AEDA), linalool and myrcene were found to present the highest flavor dilution (FD) factors in five different hop varieties (Hallertau Perle, Hallertau Hersbrucker Spat, Slowenian Golding, Hallertau Smaragd, US Cascade), followed by 2-isopropyl-3-methoxypyrazine, 3-methylbutanoic acid and geraniol (Steinhaus et al. 2007). Almost at the same time, a study of odor-active compounds in the spicy fraction of hop essential oil from four different varieties tentatively identified 14-hydroxy-β-caryophyllene as the responsible compound for herbal/spicy note of hop oils, and geraniol, linalool, β-ionone and eugenol were also proved to be important (Eyres

et al. 2007). Kishimoto and his colleagues discovered 4-mercapto-4-methylpentan-2-one (4MMP) as the contributor to the fruity, black currant-like aromas in Simcoe, Summit, Apollo, Topaz and Cascade cultivars (Kishimoto et al. 2008). Similarly, 'Nelson Sauvin', grown in New Zealand, was reported to contain 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), which contribute to the exotic fruity-like, white wine-like flavor to finished beer (Takoi et al. 2009). More recently, Tomahawk hop, a recently developed super alpha cultivar (containing high content of α-acids), was proved to contain a wide variety of odorant polyfunctional thiols, which included β-sulfanyl acetate, 3-sulfanyl-2- ethylpropyl acetate, 3-sulfanylhexan-1-ol, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) (Gros et al. 2011).

These discoveries indicate that hop oil is a rather complex mixture of volatiles. Furthermore, different hop cultivars may present a unique aroma profile although containing similar chemical compositions. Being abundant in the hop oil, hydrocarbons however contribute far less to the overall aroma than do the oxygenated components and sulfur-containing components.

2.2 Hop Aroma Compounds in Essential Oil and Their Formations

The chemical composition of hop oil is conventionally described as three classes: hydrocarbons, oxygenated components and sulfur-containing components. In the following sections, the major aroma compounds in hops and their formation are summarized based on chemical classes.

2.2.1 Hydrocarbons

Hydrocarbon fraction, which is extremely volatile, usually makes up about 40-80% of the hop essential oil (Moir 1994). It can be classified into two major groups: monoterpenes and sesquiterpenes (**Figure 2**).

The monoterpenes are organic compounds consist of two isoprene units and have the molecular formula $C_{10}H_{16}$. They can be subdivided into three groups, the acyclic, the monocyclic and bicyclic. The most abundant monoterpene is the acyclic terpene myrcene (may account for 30% of the whole oil), which is very labile and impart pungent balsamic smell to fresh hops (Howard and Slater 1957). The sensory threshold of myrcene was found as low as 13 ppb, which suggests myrcene as an important odorant in hydrocarbon fraction. β-ocimene, the structure of which was also characterized as acyclic terpene (Sutherland 1952), was identified in hop oil (Stevens 1967). The monocyclic terpenes that have been identified are limonene, ρ-cymene, α-phellandrene, β-phellandrene, and for bicyclic monoterpenes, α-pinene, β-pinene and sabinene have been reported (Buttery et al. 1963, Buttery et al. 1964, Sharpe and Laws 1981).

Sesquiterpenes have one more isoprene unit than monoterpenes. Therefore, except for three forms (the acyclic, the monocyclic and bicyclic) monoterpenes have, sesquiterpenes also present as tricyclic form. Farnesene is the only acyclic sesquiterpene that has been found in hop oil, and its presence could only be proved in some varieties such as Saaz (Zatec) hops (Sharpe and Laws 1981). Later plant breeding studies suggest that the presence of farnesene is a sex-linked character controlled by a single pair of genes with presence dominant to absence (Briggs et al. 2004). Germacrene B and germacrene D

were found as monocyclic sesquiterpenes, in which, germacrene D was present at very low concentrations in hop varieties studied probably related to the role it plays as a precursor for several other sesquiterpene compounds (Naya and Kotake 1971, Naya and Kotake 1972). α-humulene and β-caryophyllene are two of the most prominent sesquiterpenes in hop oil, which are responsible for 8-33% and 4-22% of the whole oil, respectively (Stevens 1967). It was reported that α-humulene and β-caryophyllene could be perceived at a relatively higher concentration of 120 ppb and 64 ppb respectively (Guadagni et al. 1966). α-humulene was one of the first volatiles that identified in hop oil and β-caryophyllene, was originally characterized in clove oil and then identified in hop oil (Sharpe and Laws 1981). The first identification of β-selinene was in oil from hop variety OW 153 in 1964 and its isomer α-selinene was reported in the same year from Hersbruck hops (Buttery et al. 1964, Stevens 1964). Moreover, other sesquiterpenes, such as α-ylangene, α-copaene, β-copaene, α-muurolene, γ-muurolene, α-cubebene, calamenene, γ-calacorene, α-calacorene and β-elemene, were identified in hop oils (Sharpe and Laws 1981).

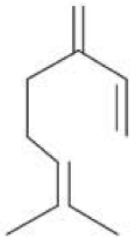
From the current biogenetic theory, terpene hydrocarbons are formed from oxygenated intermediates (Neve 1991). During the hop ripening, trace of oxygenated compounds of the essential oil emerges firstly then followed by the formation of cyclic sesquiterpenes (e.g., α-humulene and β-caryophyllene), and finally the monoterpene myrcene is formed (Briggs et al. 2004). As the hop ripens, the synthesis of myrcene becomes the dominant pathway so that the percentage of myrcene could represent the

ripeness of the cones.

Terpenes, as the largest class of plant secondary metabolites, are assembled biosynthetically from five carbon units of isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP) (Lichtenthaler et al. 1997, Eisenreich et al. 1998). So far, two pathways have been discovered for the biosynthesis of IPP, the classic mevalonate (MVA) pathway, which is active in the cytosol and a relatively novel route, the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway bounded to the plastidic compartment (**Figure 3**). During the MVA pathway, three units acetyl-CoA condense to form 3-hydroxy-3-methylglutary-coenzyme A (HMG-CoA) which, after reduction by 2 NADPH, yields mevalonate and, IPP is gained by the transformation of MVA under consumption of 3ATP and loss of CO_2 (Lichtenthaler et al. 1997). Through the new DOXP pathway, IPP forms from the DOXP, which is the product of a head-to-head condensation of glyceraldehyde-3-phosphate (GA-3-P) and 'activated acetaldehyde' generated from pyruvate (Lichtenthaler et al. 1997, Eisenreich et al. 1998, Lichtenthaler 1998, Lichtenthaler 1999). IPP can be converted into DMAPP by collaborating with an isomerase, and then DMAPP condense with a molecule of IPP to form the parent of the monoterpenes, geranyl pyrophosphate (GPP), which can further react with IPP to give the parent of sesquiterpenes, farnesyl pyrophosphate (FPP) (**Figure 4**) (Dudareva et al. 2004, Schwab et al. 2008). For instance, the major monoterpene myrcene is formed from GPP by eliminating of pyrophosphoric acid, and α-humulene and β-caryophyllene are thought to be formed from trans,trans-farnesyl pyrophosphate and trans,cis-farnesyl cation

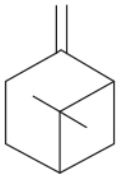
inspectively (Briggs et al. 2004). Formations of other terpenes are based on the similar principles.

Monoterpenes:

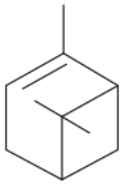


myrcene

limonene

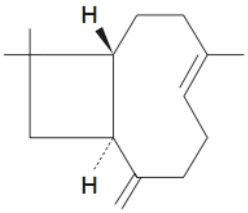


β-pinene

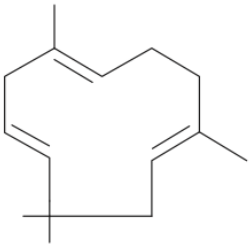


α-pinene

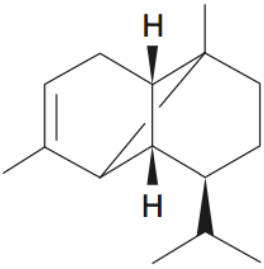
Sesquiterpenes:



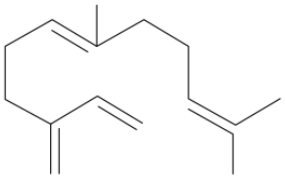
β-caryophyllene



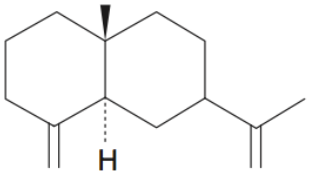
α-humulene



copaene



β-farnesene



β-selinene

Figure 2. Terpenes in hop oil.

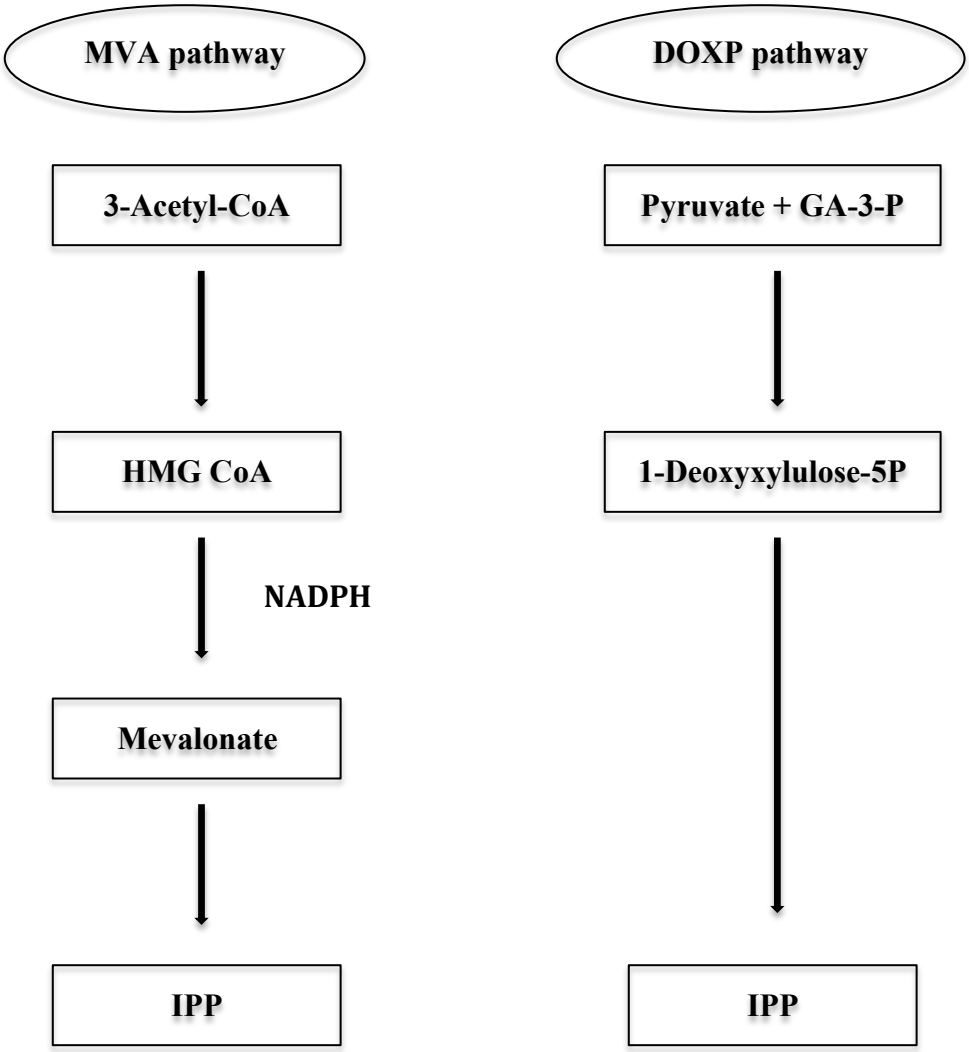


Figure 3. Isopentenyl pyrophosphate (IPP) biosynthesis: (A) via the MVA pathway, (B) via the DOXP pathway.

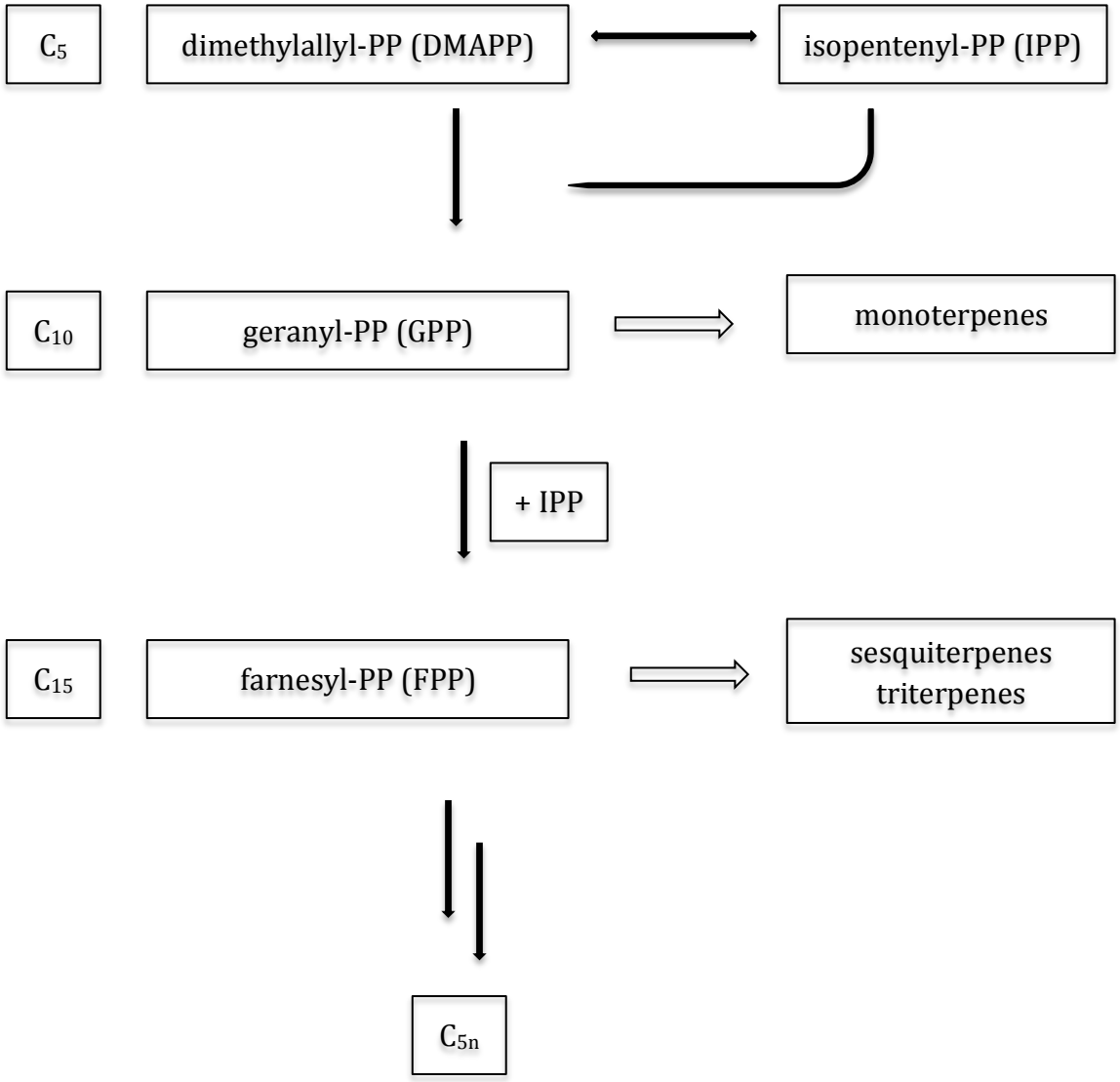


Figure 4. Biosynthesis of higher terpenes from IPP and DMAPP.
(IPP: isopentenyl pyrophosphate, DMAPP: dimethylallyl pyrophosphate, GPP: geranyl pyrophosphate, FPP: farnesyl pyrophosphate.)

2.2.2 Oxygenated Components

The oxygenated fraction of hop oil, which represents approximately 30% of the total oil, is even more complex than hydrocarbon components. In an early review of essential oil of hops, the authors reported 60 aldehydes or ketones, 70 esters, 50 alcohols, 25 acids and 30 oxygen heterocyclic compounds under the category of oxygenated components (Sharpe and Laws 1981). However, oxygenated compounds can be described as two simple classes: terpenoids and non-terpenoid components. Terpenoids (**Figure 5**), appearing as oxygenated forms of the terpenes, are derived compounds of terpene modifications and rearrangements. Most studied terpenoids in hops, such as terpene alcohols (e.g., linalool, geraniol, nerol, α-terpineol) and terpene aldehyde (e.g., geranial, neral and citronellal) are usually described as floral, citrus or lemon notes and considered to contribute positively and largely to hop aroma. Except for few terpene-derived compounds, the rest of oxygenated compounds are non-terpenoid constituents, some of which may also cause great impact on hop aroma profile. Aliphatic and aromatic alcohols in hop oil such as 2-methyl-1-propanol and 3-methyl-1-butanol have been identified and reported to contribute to hop character (Sharpe and Laws 1981). Important aldehydes are always perceived as green and grassy notes (e.g., hexanal). Esters and ketones can partly responsible for the floral and fruity aromas in hops (e.g., 2-methylbutyl acetate, isoamyl propionate and methyl octanoate). Acids present in hop oil usually give rancid, ink and cheese smells (e.g., isovaleric acid). In addition, the importance of aroma compounds

depends on the hop varieties and the sensory thresholds of specific compounds within a certain sample matrix.

The formation of terpenoids shares the same biosynthesis pathway with terpenes. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), formed by the MVA pathway and the DOXP pathway, condense and produce geranyl pyrophosphate (GPP). Transesterification of geranyl pyrophosphate is likely to be the source of a series of geranyl esters such as geranyl acetate, propionate and isobutyrate. Mild (enzymatic) hydrolysis of these esters gives the geraniol while linalool is formed under acid condition hydrolysis (Briggs et al. 2004). *cis, trans*-isomerization of geraniol could lead to nerol, and further oxidation of geraniol and nerol gives the mixture of the aldehydes geranial and neral. Citronellol is formed by the reduction of the 2,3-double bond in either geraniol or nerol, and nerol readily cyclizes to give rise of α-terpineol. Except for the terpenoid pathway, flavor compounds can also derive from the metabolism of fatty acids and amino acids.

Volatile fatty acid derivatives, such as straight-chain alcohols, aldehydes, ketones, acids, esters and lactones, constitute another large class of plant volatiles and are found ubiquitously in the plant kingdom at high concentrations (Dudareva et al. 2006, Schwab et al. 2008). Basically, there are two major processes to form these aromatics: β-oxidation and the lipoxygenase (LOX) pathway. β-Oxidation involves successive removal of C2 units (acetyl CoA) from the parent fatty acid (Goepfert and Poirier 2007, Schwab et al. 2008). In many essential oils isolated from different plants, short- and medium-chain

linear carboxylic acids, which are formed by repeated β-oxidative cycles followed by the action of an acyl CoA hydrolase, have been found. Aliphatic short- and medium-chain aldehydes and alcohols are probably formed by enzymatic reduction of the parent acyl CoAs (Flamini et al. 2007). Specifically, aroma ester production depends on the supply of acyl CoAs formed during β-oxidation and alcohols. The synthesis of various kinds of esters can be regulated by alcohol acyl transferases (AAT), which combines various alcohols and acyl CoAs (Schwab et al. 2008). Volatile fatty acid derivatives such as hexanal, cis-3-hexenol and 3,6-nonadienal, contributing 'fresh green' odor to fruits, vegetables and green leaves, are derived from C_{18} unsaturated fatty acids (e.g., linoleic acid or linolenic acid) which undergo dioxygenation in a reaction catalyzed by lipoxygenases (LOX) (Feussner and Wasternack 2002, Schwab et al. 2008).

Meanwhile, the formation of a wide range of plant volatiles including aldehydes, alcohols, esters and acids can be traced back to the amino acid precursors, such as alanine, valine, leucine, isoleucine, and methionine (Dudareva et al. 2006). For instance, branch-chain volatile namely 2-methylbutyl acetate, which derived from branched-chain amino acid, contributes strong fruity note and has been associated with the aroma of apples and melons (Schwab et al. 2008). Meanwhile, isovaleric acid, which formed from leucine, is also important aromatic component in various plants. During the amino acid metabolism, amino acids could undergo an initial deamination or transamination resulting in the formation of the corresponding α-keto acid. After that, subsequent decarboxylation followed by reductions, oxidations and/or esterifications leads to aldehydes, acids,

alcohols and esters (Reineccius 2006).

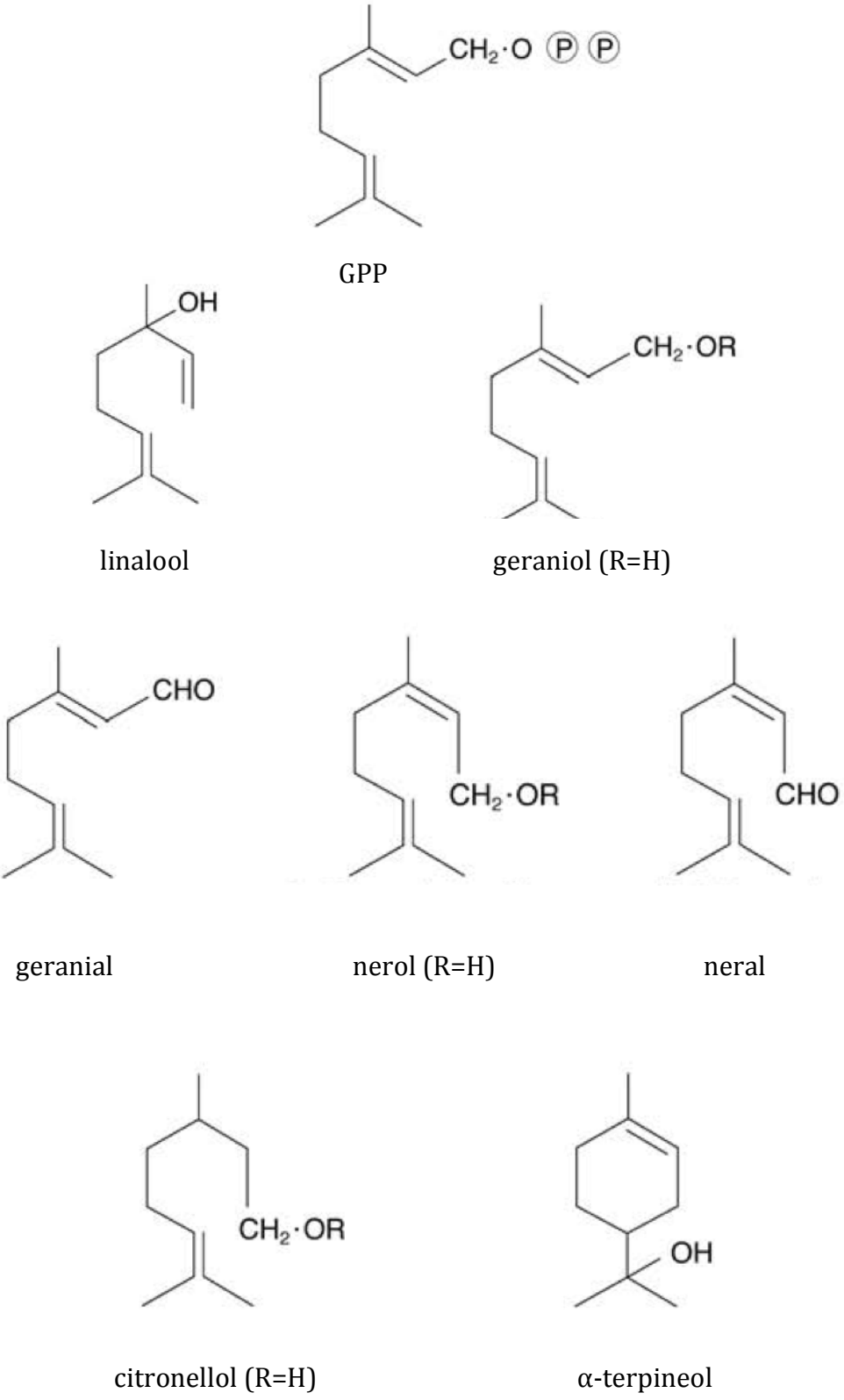


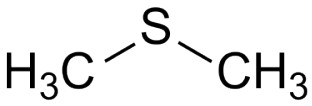
Figure 5. Terpenoids (derived from GPP) in hop oils**.**

2.2.3 Sulfur-Containing Components

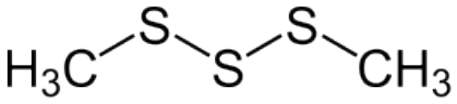
Only trace levels of sulfur-containing components (generally<1%) are detected in hop oil, their influence on the whole aroma spectrum however should not be underestimated due to the low sensory thresholds they present. A large proportion of hops grown in the world are exposed to sulfur dioxide during kilning to achieve uniform appearance. Meanwhile, hop plants may be treated with elemental sulfur to prevent fungal disease (Neve 1991). Both treatments are considered to affect the profile of sulfur-containing compounds in hop oil. For instance, it was reported that sulfuring caused reduction even elimination of some sulfur-containing compounds in hops (Pickett et al. 1976). Besides, it was recorded that the occurrence of 3-allkythiophenes in certain hop oil could be attributed to abnormally high residual sulfur levels (Collin 2003).

A number of sulfur-containing constitutes, which are always described as undesirable sulfury, cooked vegetable, garlic, and onion-like notes with low thresholds, have been identified in hops including thioesters, thiophenes, straight-chain sulphides, episulfides and miscellaneous sulfur compounds (**Figure 6**) (Sharpe and Laws 1981, Collin 2003). Being most readily detected in hop oil, thioesters, to be more accurate S-methyl thioesters (e.g.，S-methyl-4-methylpentanethioate, S-methyl-2-methylbutanethioate and S-methyl hexanethioate), are highly flavor active and many of them possess flavor thresholds ranging from 0.3 ppb to 50 ppb (Peppard 1981). Another group of thioesters, namely S-methylthiomethyl thioesters, was also identified in hop oils. It was concluded that thioesters occur naturally during hop ripening,

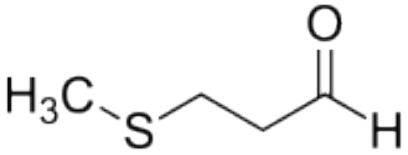
nevertheless, great portion of them could be formed from heating process (Peppard 1981). A possible route for the thioester biogenesis in hops was demonstrated as thiolysis of acyl coenzyme A (CoA) by methanethiol coming from methionine degradation (Collin 2003). An alternative pathway was suggested as the reaction of methanethiol with straight- and branched-chain fatty acids and/or the alkanoyl side chains of α- and β- acids in hops (Peppard 1981). Straight-chain sulphides, which can be further divided into methylsulfides and polysulfides, also play an active role in hop aroma profile. The polysulphide, dimethyl trisulphide, considered to be formed from S-methylcysteine sulphoxide during steam distillation, has a sulfury, cooked vegetable and onion-like aroma with a low sensory threshold at 0.1 ppb (Sharpe and Laws 1981, Briggs et al. 2004). Moreover, other methylsulfides have also been identified, such as 3,3-dimethylallyl methyl sulfide, the level of which is enhanced in hop oil by heat treatment (Collin 2003). Similarly, Episulphides can be formed from the reaction of sesquiterpenes caryophyllene and humulene with elemental sulfur. However, since the thresholds of these episulphides are in the ppm range, they are not as aroma active as thioester and those straight chain sulphides (Peppard et al. 1980). Meanwhile, myrcene can also react with sulfur however a suitable activator is needed. Besides, sulfur compounds such as dimethyl disulfide (garlic note), methional (boiled potato note) and methanethiol (cooked cabbage note) found in hop oils are revealed to be formed from methionine and cysteine through amino acid metabolism (Jones et al. 2004).



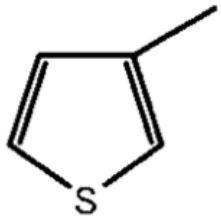
dimethylsulfide (DMS)



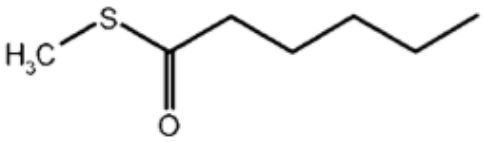
dimethyltrisulfide (DMTS)



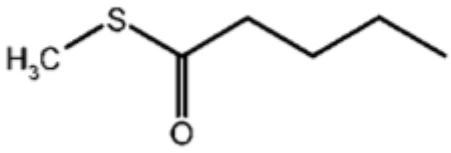
methional



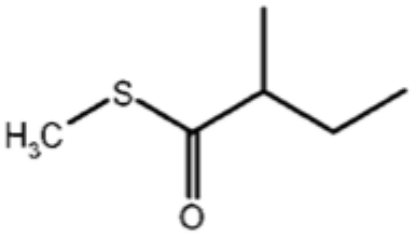
3-methylthiophene



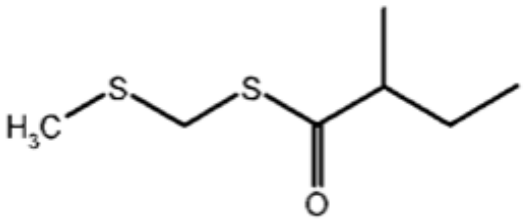
S-methylthiohexanoate



S-methylthiopentanoate



S-methyl-2-methylthiobutanoate



S-methylthiomethyl-2-methylthiopropionate

Figure 6. Sulfur-containing compounds in hop oils.

2.3 Flavor Analysis of Hops

2.3.1 Aroma Extraction

Due to the possible low concentrations they may present in the hop matrix, aroma components need to be isolated from hop sources and concentrated before being introduced to the analytical instruments. In order to obtain the target volatile constituents from hop materials, other compositions of hops/hop products such as cellulose, resins, protein, water and ash should be removed or avoided during the extraction without causing loss of volatile components. Since 1900s, various methods have been developed to fulfill the isolation purpose of hop oils but beyond doubt, no approach can accurately reflect the aroma profile actually present in hops. The aroma extraction methods can be generally classified as solvent associated approach and solventless method.

Steam Distillation (SD)

Steam distillation (SD) is the earliest method used in the extraction of hop aroma, and the essential oil obtained from this approach has been extensively investigated over hundred years. Since the late stage of 19^{th} century, SD has become a popular and universal approach used to obtain the aroma isolates, which can largely facilitate the investigation of hop aroma spectrum (Chapman 1895, Howard and Slater 1957, Kovačevič and Kač 2002). Nevertheless, the conventional steam distillation method has several drawbacks that should be carefully considered. For the original steam distillation, three-hour heating at 100°C is a common process to complement the separation of the bulk of essential oil during which, degradation of some compounds may happen and

potential artifacts could be formed from thermal reaction (Pickett et al. 1975). Besides, some water-soluble constituents could be washed out during the steam distillation since they prefer to stay in aqueous phase instead of organic phase (Briggs et al. 2004). Simultaneous distillation-extraction method (SDE) (also known as Likens-Nickerson method) was developed in 1964 for the purpose of hop oil analysis (Likens and Nickerson 1964, Chaintreau 2001) and since then, has been repeatedly used and reported in research papers related to hop investigation (Perpète et al. 1998, Eri et al. 2000, Lermusieau et al. 2001). The SDE starts with the distillation of both sample and solvent and then, is actualized by the extraction between sample vapors and solvent vapors on the condenser surface. Due to the utilization of both solubility and volatility during the sample isolation, SDE has been proved highly efficient and therefore largely applied to various areas such as analysis of flavors and fragrances, or pollutants (Perpète et al. 1998, Eri et al. 2000). SDE method itself can also be employed under different conditions, for instance, atmospheric pressure SDE (A-SDE), vacuum-SDE (V-SDE) and SDE for large-scale operations, according to specific research purposes. Working as a one-step isolation-concentration approach, SDE is very efficiently to obtain a volatile extract with limited amount of organic solvent. The weakness here is that, although aroma extracts prepared by SDE can present nearly all the volatiles in a sample, their proportions may only poorly reflect the true profile in the sample (Reineccius 2006). Furthermore, SDE method was evidenced to discriminate some important food aroma compound such as furaneol (Pickenhagen et al. 1981), which is also concerned by some flavor chemists.

Solvent Extraction

Organic solvents are most commonly used in aroma isolation of hops (Steinhaus and Schieberle 2000, Steinhaus et al. 2007). Solvent extraction takes the advantage of the solubility of target compounds in applied solvents. It is a quite easy and widely used way to isolate aroma components from hop matrix without high temperature heating process, during which artifact could occur from the thermal reaction. One drawback of solvent extraction is that the affinity of the aroma compounds to selected solvent may lead to varying extent extraction.

The solvent extract is always further processed by special distillation approach such as solvent assisted flavor evaporation (SAFE) since non-volatile materials need to be removed from the volatiles. SAFE is a compact and versatile distillation method which can offer fast and reliable isolation of volatiles from complex matrices (Engel et al. 1999). In connection with a high vacuum pump, SAFE system could implement the separation of volatiles under low temperature distillation, which reduces the risk of altering the aroma profile. Although various solvents with different polarities could be adopted in the extraction (Weurman 1969), from a general view, dichloromethane was claimed to be the best solvent for hop aroma extraction (Chaintreau 2001) (**Figure 7**). In spite of the convenience, the alliance of solvent extraction involving organic solvents with further distillation is time-consuming to some extent and involves multi-step operations. These extra procedures may introduce non-ignorable bias and errors into the description of aroma profile due to the loss or conversion of volatiles. In addition, the solvent disposal,

safety issues and environmental concern also need to be taken into account when using solvent extraction method.

Furthermore, due to the complexity of hop aroma profile, pre-fractionation of the aroma isolate (essential oil) collected by solvent extraction method needs to be considered before introducing the sample into analytical instruments, in order to simplify the further identification and quantification analyses.

In the early days, investigators fractionated the essential oil of hops by performing distillation under different temperature ranges (Chapman 1895). Subsequently, column chromatography with silica gel was introduced to prepare the essential oil by united with diverse organic solvents that having different polarities (Kirchner and Miller 1952, Howard 1957) and since then, has become a conventional and widely adopted method for hop oil fractionation. Basically, in the fractionation, the aroma concentrate is loaded onto a silica gel column and then eluted with different organic solvents in a certain order. The principle of this approach is to fractionate the aroma extract by the polarity of various compounds. Based on the different affinities of volatile components to the polar stationary phase and the mobile phase with gradient polarities, the volatiles can be separated into individual fractions. It was found that the hydrocarbon component of hop oil was quantitatively eluted from silica gel by light petroleum, whereas the oxygenated compounds were not eluted until ether was passed through the column (Howard 1957). This fractionation method was also known as adsorption chromatography and extensively applied in hop analysis (Howard and Slater 1957, Howard and Stevens 1959). However,

the silica-gel fractionation technique still has its inherent limitations (Marsili 2010): 1) the presence of water can decrease the silica gel activity; 2) some solutes or solvents can remain fixed onto the column which leads to irreversible adsorption; 3) degradation of labile compounds could be catalyzed by silica and artifact formation could happen.

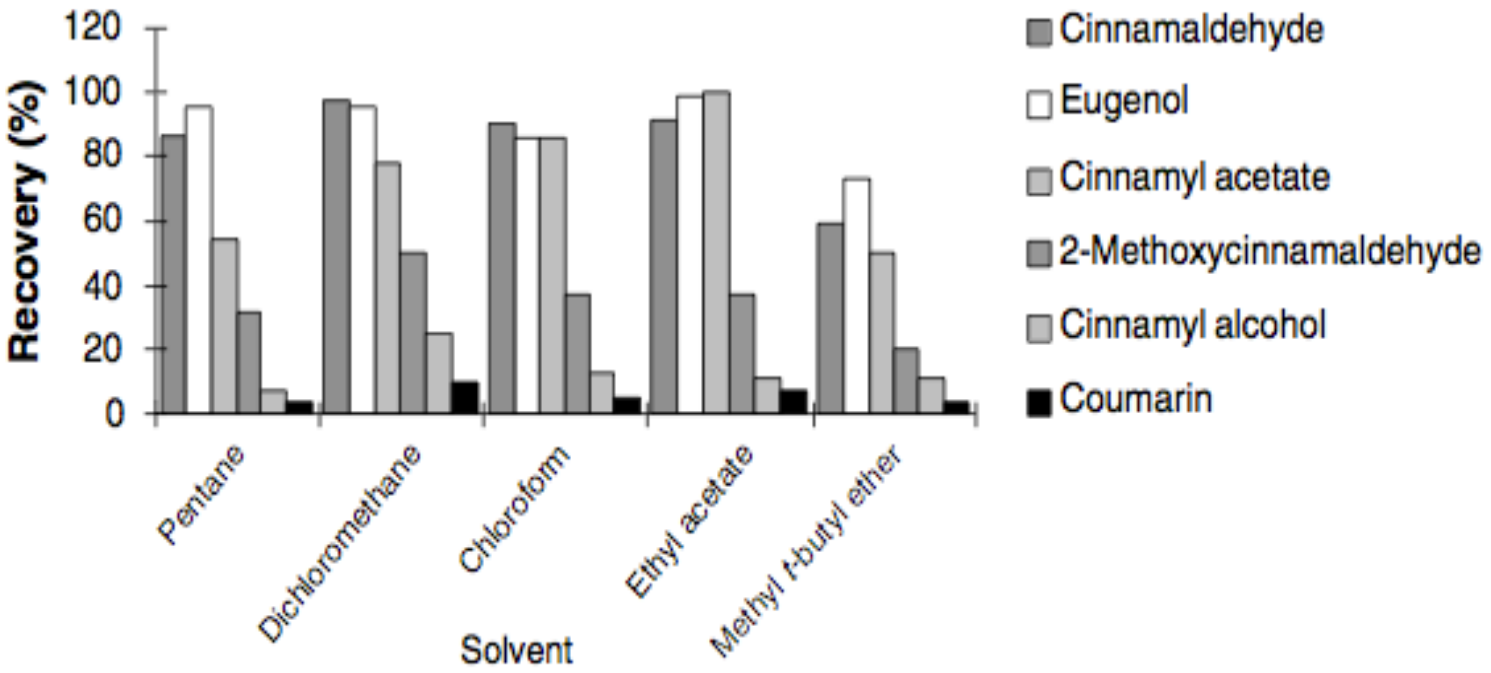


Figure 7. Comparison of solvent efficiency (Chaintreau 2001). Average recoveries are 47%, 59%, 53%, 58% and 36% for pentane, dichloromethane, chloroform, ethyl acetate, and methyl t-butyl ether, respectively.

Solid-Phase Microextraction (SPME)

Solid-phase microextraction (SPME) is a simple and efficient sample preparation technique favored by investigators in various research areas such as food analysis, biomedical analysis, environmental analysis and pesticide residual analysis (Beltran et al. 2000, de Fátima Alpendurada 2000, Kataoka et al. 2000, Ulrich 2000). It is also undoubtedly approved by hop chemists and therefore widely performed in hop studies (Field et al. 1996, Kovačevič and Kač 2001). Since invented in 1989, SPME has been widely used and introduced for direct coupling with gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), which proved to be a successful combination for the analysis of volatile and semi-volatile organic compounds (Kataoka et al. 2000).

Working without solvent, SPME integrates sampling, extraction, concentration and sample introduction into a single step by using a fused-silica fiber which is coated on the outside with an appropriate stationary phase (Vas and Vékey 2004). Several types of coating fibers are currently available and the selection of fibers is based on the affinity of the fiber for a specific analyte. For example, non-polar polydimethylsiloxane (PDMS) fiber and more-polar polyacrylate (PA) fiber are preferred for the extraction of non-polar and more-polar analytes respectively. In headspace (HS)-SPME, the fiber is exposed in the vapor phase above an analytical sample in solid of liquid form, meanwhile, in direct immersion (DI)-SPME, the fiber is directly immersed in a liquid sample (Kataoka et al. 2000). Analytes are extracted and concentrated on the coating fiber by absorption/adsorption process. SPME is an equilibrium method and the extraction is dependent upon partitioning among the coated fiber, headspace and the sample matrix for

HS-SPME, and for DI-SPME, on partitioning between coated fiber and liquid sample phase.

Although with impressive advantages, SPME could not deny its shortcomings at the same time. In general, SPME has relatively high detection limit due to the non-exhaustive extraction, and its application range is narrowed down by the limitation of fiber coating materials available in the market. Also, the SPME fiber is easy to have carry-over, which may lead to the poor reproducibility (Prosen and Zupančič-Kralj 1999). Furthermore, considering about the limited surface of the SPME fiber, competition effects between volatile compounds can cause biases in the quantitative determination of compounds (Roberts et al. 2000).

Stir Bar Sorptive Extraction (SBSE)

Being a solventless sample preparation method, SBSE is environmentally friendly and commonly used for aqueous matrices. A stir bar is a magnetic stirring rod coated with a sorbent phase. Derived from SPME and sharing a similar extraction mechanism to that of SPME, SBSE relies on trapping of the target analytes from the sample by an adsorbent material, and the extraction of solutes from the aqueous solution into the extraction medium is determined by the partitioning coefficient of the solutes between the sorbent phase and the aqueous phase (Baltussen et al. 2002, David and Sandra 2007). During the extraction process, a stir bar is added into the sample solution and the sample is stirred typically for 30-240 min. After completion of the stirring time, the stir bar is removed by a stainless steel wire, dried with tissue paper and transferred into the thermodesorption system for solutes release (Baltussen et al. 1999). The main advantage of SBSE over SPME is that SBSE uses an extraction phase volume about 50-250 times

larger than SPME, which consequently presents extremely high sensitivities (He et al. 2013). However, the limited applicable and commercially available coating (most common one is PDMS-coated stir bar) may restrict the further improvement and application of SBSE (Prieto et al. 2010).

SBSE has been widely applied in trace analysis in environmental, food and biomedical fields (David and Sandra 2007). For example, it has been largely used for the analysis of hop aroma compounds in beer by different researchers (David et al. 2001, Kishimoto et al. 2005, Kishimoto et al. 2006).

In summary, no method is perfect and can accurately reflect the aroma compounds actually present in a sample matrix. Thus an isolation method should be carefully chosen based on the specific analytical objective.

2.3.2 Instrumental Analysis of Aroma Extract

At present, the bulk of hop aroma research is accomplished mainly by reasonable combination and application of gas chromatography (GC), olfactometry (O) and mass spectrometry (MS) (Steinhaus and Schieberle 2000, Roberts et al. 2004, Eyres et al. 2007, Steinhaus et al. 2007, Kishimoto et al. 2008, Shellie et al. 2009).

Gas Chromatography-Mass Spectrometry (GC-MS)

As the provider of excellent separation powers and extreme sensitivity, gas chromatographic technologies are ideally suited to aroma studies. Coupling with a wide range of detectors, gas chromatography (GC) has been extensively applied to research of hops/hop products to serve different purposes of aroma investigations (Field et al. 1996, Steinhaus and Schieberle 2000, Marriott et al. 2001, Kovačevič and Kač 2002). Samples injected into GC need to be thermally labile and with relatively high volatility, or the

degradation of certain compounds may happen and high boiling point components could stay in the GC system so that lead to contamination. One major limitation of GC is the sample capacity due to the thickness of the packing materials for capillary column used in advanced GC technique, which limit the separation efficiency of one-dimensional GC. The separation ability of gas chromatography could be further increased by the application of multidimensional gas chromatography (MDGC), in which a sample is first separated by one column, and the simplified subsamples are then applied onto a following column for further separation (Nielsen 2010). Generally, MDGC techniques can be divided into two classes: 1) conventional two-dimensional GC (only small portion of the effluent from the first column will be transferred to the second column) and 2) comprehensive two-dimensional GC (entire effluent from the pre-separation column will be transferred to the second column).

Mass spectrometry (MS) is a detection method. The working process of MS can be generally concluded as three steps: 1) convert a molecule to an ion by placing a charge on it; 2) resolve the generated ions according to their mass-to charge ratio (*m/z*) in the mass analyzer; 3) detect the ion fragments (Nielsen 2010). The key of an MS is the mass analyzer and nowadays four types of MS are commonly used: quadrupole-MS, ion trap (IT)-MS, time-of-flight (TOF)-MS and Fourier-transform (FT)-based MS. Since MS possesses outstanding advantages in qualitative and quantitative analysis, it is routinely coupled to gas chromatography (GC) to realize the identification and quantification of aroma compounds. Similar, multidimensional gas chromatography (MDGC) has been worked with mass spectrometry (MS) and then applied to various scientific fields, for

instance essential oils study, to achieve high-resolution analysis (Roberts et al. 2004, Eyres et al. 2007).

Gas Chromatography-Olfactometry (GC-O)

In 1971, the first GC-olfactometry (GC-O) was reported (Leland et al. 2001). GC-O refers to a unique and valuable analytical method, which combines the resolution power of capillary GC with sensitive and selective human assessor as a detector. Usually, the effluent coming out of GC column is split into two portions, one portion is transferred to a GC detector while the other portion is introduced to a sniffing port and perceived by humans. This technique is used to determine the odor activity and relative importance of volatiles in a sample extract. In fact, it is generally most ideal to apply the combination of GC-MS with olfactometry to the inspection of aroma profile since it could greatly facilitate the identification of odorants by getting an odor profile and a MS chromatogram simultaneously.

The concept of odor activity values (OAV), which is defined as concentration/threshold where the threshold is lowest concentration detectable by humans in the sample, was derived in 1962 (Leland et al. 2001). Generally, chemicals that are at OAV levels higher than one should be detectable by humans. Nevertheless, threshold values in a particular sample matrix are seldom available, which limits the utilization of conventional quantitative method to estimate the aroma impact of certain odorant in a complex sample matrix.

Aroma extract dilution analysis (AEDA) is one of the GC-O dilution techniques used to effectively estimate the OAV of volatiles in a sample matrix (Ullrich and Grosch 1987). It is a quantitative GC-O procedure based on successive dilutions of an aroma

isolate until no odor could be perceived by olfactory panelists. The impact of an odor-active compound is indicated by its flavor dilution (FD) factor since the FD value is proportional to the OAV of the compound in air. The FD factor is the number of times, expressed as fold, that a sample can be diluted before reaching its detectable limitation (Leland et al. 2001, Delahunty et al. 2006, d'Acampora Zellner, Dugo et al. 2008). The disadvantage of AEDA method is that it is a time-consuming process. Moreover, large variation in individual's sensitivities may make it a subjective method. However, the use of reasonable size of experienced panelists could be helpful to minimize the human bias of this method.

2.4 Water-Soluble Hop Flavor Precursors

Although the investigation of hop aroma in this study only focused on the essential oil of hops, the attention to the occurrence of glycosidically bound flavor compounds in hop materials has been paid recent years and a new group of aromatic contributors has been discussed. Glycosides are defined as odorless compounds that contain a sugar component (usually β-D-glucose) in its cyclic form and another component, which is attached to the sugar at its glycosidic carbon. When this second components are non-sugar moieties, they are referred to as aglycons. Generally, under acid or enzyme catalysis, the glycosides can be hydrolyzed to sugars and free aglycons. In 1999, the study of remaining hop solids after liquid carbon dioxide extraction indicated the potential aroma contribution of water-soluble fraction to the flavor of beer (Goldstein et al. 1999). The water-soluble fraction of hops, which makes up 20 to 25% of the hop solids (remainder after the isolation of hop resins and oils), contains proteins, polyphenols, carbohydrates and inorganics. And in this fraction, hop glycosides were the

class of compounds that proved to be potential to produce flavor. At the same year, a method of making kettle hop flavorants were built as a utilization of extracted hops after CO_2 extraction (Goldstein et al. 1999). In fact, in the wine industry, the glycosides in grapes were investigated for decades because of their potency to affect the flavor in wine (Francis et al. 1992). Meanwhile, in the beer industry, it was reported that the hydrolysis of glycosides might be responsible for the increase of linalool at the end of boiling or during the whirlpool rest (Mitter et al. 2001). More recently, it was considered to be possible that linalool and β-damascenone, liberated from hop glycosides, could contribute to hop aroma of beer (Kollmannsberger et al. 2006). The mystery of water-soluble hop flavor precursors is still under investigation and waiting for a more comprehensive disclosure.

2.5 Hop Aging

The composition of essential oil experiences tremendous change during hop storage. In an early study of hop aging, the concentration of individual aroma constituents was compared between the essential oils of two-month stored hops and three-year stored hops (Tressl et al. 1978). The total oil content was decreased after three years and presented as only half of the content of original hops. The amount of esters kept nearly constant, however, most of the ketones increased considerably, which could be derived from both hop resins and hydrocarbons in hop oil. Most aldehydes were detected only in three-year stored hops. Fatty acids such as 2-methylpropionic, 2-methylbutyric, and 3-ethylbutyric acids were increased significantly, which might be derived from degradation of hop resin components. The terpenes, decomposed by oxidation and polymerization, decreased from 88 to 7% in the hop oil. It has been proposed that the autoxidation of myrcene might lead

to the increase of linalool, geraniol, nerol, citral together with the cyclic limonene, while the formation of sesquiterpene epoxides of caryophyllene and humulene, such as caryophyllene epoxide, humulene epoxide Ⅰ and humulene epoxide Ⅱ, were also observed (Lam et al. 1986, Briggs et al. 2004). The formation of extra terpene alcohols, sesquinterpene epoxides and fatty acids could contribute floral/citrus note, herbal/spicy note and rancid/cheesy note respectively, therefore aging of hops may change the hop aroma profile largely and introduce stronger hoppy aroma to hopped beer, (Lam et al. 1986, Goiris et al. 2002).

CHAPTER 3 IDENTIFICATION OF ODOR-ACTIVE VOLATILES IN 'CENTENNIAL', 'CITRA' AND 'NELSON SAUVIN' HOP VARIETIES BY GC/OLFACTOMETRY-MS

3.1 Abstract

Essential oils of three different hop varieties ('Centennial', 'Citra' and 'Nelson Sauvin') were obtained by solvent extraction followed with solvent assisted flavor evaporation (SAFE). Pre-fractionation on silica gel column further separated them into a polar fraction in dichloromethane and a non-polar fraction in pentane. The odor-active compounds in both fractions were identified using gas chromatography-mass spectrometry/olfactometry (GC-MS/O) and aroma extract dilution analysis (AEDA). It was determined that myrcene, isovaleric acid and geraniol were the most potent aroma components in all three hop cultivars based on the highest flavor dilution (FD) factors, followed by S-methyl methanethiosulfonate, linalool and vanillin. S-methyl methanethiosulfonate, having unique radish, cabbage notes, was detected in hops for the first time with high FD factors in 'Centennial' and 'Citra' hops. Several sulfur-containing compounds, perceived as garlic, sulfury notes, were also identified to be important contributors to hop aroma.

Keywords: SAFE; GC-MS/O; AEDA; Flavor dilution factor; S-methyl methanethiosulfonate.

3.2 Introduction

Hops (*Humulus luplus* L.) are considered as an indispensible brewery ingredient since they contribute bitterness and characteristic aroma to beer. So far, hop resin, which is responsible for the bitter taste of beer, has been well studied. Nevertheless, the key of the hop aroma, hop essential oil, is far from fully understood due to the chemical complexity and varietal dependency.

Steam distillation is one of the earliest and most common methods to obtain hop oil for further analysis (Chapman 1928a, Howard and Slater 1957), however the possible artifacts formation during steam distillation may change the oil composition and consequently impart undesired error in the flavor evaluation (Pickett et al. 1975). Organic solvent extraction combined with solvent assisted flavor evaporation (SAFE) is an ideal and efficient alternative to conventional steam distillation (Engel et al. 1999, Steinhaus and Schieberle 2000).

The invention of gas chromatography (GC) (James and Martin 1952) was a milestone in the history of analytical chemistry and its application to the exploration of hop oil has taken the understanding of hop chemistry to the next level. The first application of GC to hop oil analysis was conducted by Howard and his colleagues (Howard 1956, Howard 1957, Rigby and Bethune 1957). In 1963, a research of Bullion hop oil revealed the presence of approximately 200 components and tentatively identified that the oxygenated fraction containing a complex mixture of alcohols (2-methylbutanol, linalool, nerol, geraniol, nerolidol, etc.), carbonyls (methylnonyl ketone and numerous other methyl ketones, citral and other aldehydes), and esters of carboxylic acids with straight chains, branched chains and unsaturated straight and branched chains (Jahnsen

1963). In the same year, the presence of 2-methylbutanol, linalool, methylnonyl ketone, methylundecyl ketone, geraniol, and the mono epoxides of caryophyllene and humulene were found to be major constituents in the non-saponifiable constituents of the oxygenated fraction of Brewer's gold hop oil (Roberts 1963). Afterwards, Buttery and Ling (1967) reported 76 components in five American hops and proposed that the peak patterns and area percentages of certain compounds could be used to distinguish between the varieties (Buttery and Ling 1967).

Gas chromatography-olfactometry (GC-O) combining the resolution power of capillary GC with sensitive and selective human nose as a detector has been used to study aromatic compounds in a complicated sample matrix such as hop oil (Mayol and Acree 2001, Delahunty et al. 2006, Eyres et al. 2007, d'Acampora Zellner et al. 2008, Takoi et al. 2009). Aroma extract dilution analysis (AEDA) is a GC-O based method, which is based on successive dilution of an aroma isolate (Ullrich and Grosch 1987). This method can identify the relative importance of odor-active compounds of an aroma extract (Grosch 1993, Iraqi et al. 2005, Fan and Qian 2006). By using gas chromatography-olfactometry (GC-O), *trans*-4,5-epoxy-(E)-2-decenal, linalool and myrcene were identified as the key aroma contributors in fresh and dried hop cones (Spalter Select variety), while (E,Z)-1,3,5-undecatriene, 1,3(E),5(Z),9-undec-atetraene, (Z)-1,5-octadien-3-one, and ethyl 2-methylpropanoate and methyl 2-methylbutanoate were identified as important hop odorants in Spalter Select hop variety (Steinhaus and Schieberle 2000). Using aroma extract dilution analysis (AEDA), linalool and myrcene were found to present the highest flavor dilution (FD) factors in five different hop varieties (Hallertau Perle, Hallertau Hersbrucker Spat, Slowenian Golding, Hallertau

Smaragd, US Cascade), followed by 2-isopropyl-3-methoxypyrazine, 3-methylbutanoic acid and geraniol (Steinhaus et al. 2007). Almost at the same time, a study of odor-active compounds in the spicy fraction of hop essential oil from four different varieties tentatively identified 14-hydroxy-β-caryophyllene as the responsible compound for herbal/spicy note of hop oils, and geraniol, linalool, β-ionone and eugenol were also proved to be important (Eyres et al. 2007). Kishimoto and his colleagues discovered 4-mercapto-4-methylpentan-2-one (4MMP) as the contributor to the fruity, black currant-like aromas in Simcoe, Summit, Apollo, Topaz and Cascade cultivars (Kishimoto et al. 2008). Similarly, 'Nelson Sauvin', grown in New Zealand, was reported to contain 3-sulfanyl-4-methylpentan-1-ol (3S4MP) and 3-sulfanyl-4-methylpentyl acetate (3S4MPA), which contribute to the exotic fruity-like, white wine-like flavor to finished beer (Takoi et al. 2009). More recently, Tomahawk hop, a recently developed super alpha cultivar (containing high content of α-acids), was proved to contain a wide variety of odorant polyfunctional thiols, which included β-sulfanyl acetate, 3-sulfanyl-2-ethylpropyl acetate, 3-sulfanylhexan-1-ol, 3-sulfanyl-4-methylpentan-1-ol (3S4MP) (Gros et al. 2011).

Since different hop cultivars could have a different aroma profile and therefore, contribute differently to the beer aroma, the goal of the current study was to achieve the characterization of odor-active components in three varieties of hops by AEDA.

3.3 Materials and Methods

Chemicals and Equipment

Strata Si-1 Silica cartridges (200mg/3ml) were obtained from Phenomenex (Torrance, CA, USA). Pentane (Nanograde, Mallinckrodt Baker, Phillipsburg, NJ) and

dichloromethane (HPLC grade, Burdick & Jackson, Muskegon, MI) were freshly distilled. Methanol was obtained from Fisher Scientific (HPLC grade, Pittsburgh, PA). Anhydrous sodium sulfate (99.9%, ACS certified) was supplied by Mallinckrodt (Mallinckrodt Baker, Phillipsburg, NJ). All the chemical standards used in volatile compound identification were obtained from Sigma-Aldrich (St. Louis, MO) with the highest commercial purity (>95%).

Hop Samples

'Centennial' hop cones were supplied by Indie Hops, Portland, Oregon. Whole cones of 'Citra' hops and 'Nelson Sauvin' hops were obtained from Jonhn I. Haas, Yakima, Washington. All the hop samples were packaged in airtight bags, stored at -20°C prior to analysis.

Isolation and Pre-Fractionation of Hop Volatiles for Aroma Analysis

Thirty grams of dried hop cones of each variety were ground in a by blender. The blended hop cones were extracted twice (200 ml for each) with freshly distilled dichloromethane (total volume = 400 ml) in a 500 ml Mason jar. Hop materials were extracted for in total 32 hours at room temperature. The mixture was filtrated and the extract was further isolated by solvent assisted flavor evaporation (SAFE) (Glasbläserei Bahr, Manching, Germany) at 50 °C. Non-volatile material was removed and hop oil was achieved after SAFE. The essential oil was dried over anhydrous sodium sulfate and concentrated to 1 ml. 100μL of essential oil of each hop variety were loaded to the silica cartridge. Nonpolar fraction was eluted with distilled pentane (3 ml) while polar fraction of hop oil was washed out by distilled dichloromethane (5 ml). Both fractions of hop oil were concentrated to 200 μL.

Gas Chromatography Coupled Simultaneously with Mass Spectrometry and Olfactometry (GC-MS/O)

GC-MS/O analysis were carried out on an Agilent 6890 GC equipped with an Agilent 5973 mass selective detector and an olfactometer (Agilent, Santa Clara, CA). The samples were analyzed on a ZB-Wax column (30 m×0.25 mm i.d., 0.5 μm film thickness; Phenomenex, Torrance, CA). The column carrier gas was helium at a constant flow rate of 2.5ml/min. The column effluent was split 1:1 into MS and olfactometer. Sample (1 μl) was injected into the GC injector at a 1:5 split ratio. The GC injector temperature was set at 250 °C. The oven temperature was programmed at 40 °C for 2 min hold and then to 230 °C at a rate of 3 °C/min, with a 5.0 min hold at the final temperature. The MS transfer line and ion source temperature were 280 °C and 230 °C, respectively. The electron impact energy was 70 eV, and the mass range was from 35 to 350 amu. Mass spectra of unknown compounds were compared with those in the Wiley 275.L database (Agilent Technologies Inc.). A series of alkanes (C_5-C_{25}) was analyzed using MS to establish the Kovats retention indices (RIS).

Four panelists participated in the olfactory analysis. All of them were experienced in GC-O analysis and were trained for describing aroma compounds in professional language. The odor intensities were relatively scaled as “very weak”, “weak”, “moderate”, “strong” and “extreme”. The retention time, odor descriptor, and intensity value were recorded. Each fraction was replicated two times by each panelist. The odor intensity was the averaged from the results of all the panelists when an aroma was registered.

Aroma Extract Dilution Analysis (AEDA)

AEDA analysis was performed using a Hewlett-Packard 5890 series II gas chromatography (Agilent Technologies, Palo Alto, CA) coupled with a flame ionization

detector (FID) and a sniffing port. Samples were separated using a ZB-Wax capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Torrance, CA). The column carrier gas was nitrogen at a constant flow rate of 2ml/min. The column effluent was split 1;1 into FID and olfactometer. Sample (1 μl) was injected into the GC injector at a splitless model. The GC injector and detector temperatures were 250°C. The oven temperature was programmed at 40 °C for a 1 min hold and then up to 230 °C at a rate of 4 °C/min, with a 5.0 min hold at the final temperature.

FD factor of volatiles in nonpolar and polar fractions was determined by AEDA. The original odor extract of each fraction was stepwise diluted with pentane (nonpolar fraction) or dichloromethane (polar fraction) using a series of 1:1 dilution. 100 μl of the concentrate (FD=1) and each diluted sample (FD=2, 4, 8, etc.) were separated on GC and in parallel evaluated by GC-O. Three panelists were selected for the AEDA study. All of the samples were replicated twice by each panelist. The corresponding retention times and aroma descriptors were recorded.

Aroma Compound Identification

Identification of aroma compounds was based on the following criteria: odor description, mass spectra, and retention indices (RIs) relative to those of pure reference compounds. Retention indices were determined using a series of standard linear alkanes C_5-C_{25} under the same chromatographic conditions.

Identification of S-methyl methanethiosulfonate by Heart-Cut Multidimensional Gas Chromatography-Olfactometry (MDGC-O)

The identification of S-methyl methanethiosulfonate was carried out on an Agilent 6890 equipped with an Agilent 5973 mass selective detector (Agilent, Santa Clara, CA). The first column was a DB-Wax cappillary column (30 m×0.25 mm i.d., 0.5 μm film

thickness; Agilent Technologies, Palo Alto, CA) and the second column was a DB-5 capillary column (30 m×0.25 mm i.d., 0.5 μm film thickness; Agilent Technologies, Palo Alto, CA). Helium was used as carrier gas at a flow rate of 4.6ml/min in the first column and 0.6ml/min in the second column. The effluent from the column one was directed to either 1) FID or 2) column two. Sample (1 μl) was injected into the GC injector at a splitless model. The GC injector and FID temperatures were 250°C. The oven was programmed at 80 °C for a 1 min hold and then up to 230 °C at a rate of 3 °C/min, with a 15.0 min hold at the final temperature for column one. For column two, the oven was programmed at 60 °C for a 2 min hold and then up to 230 °C at a rate of 3 °C/min, with a 5 min hold at the final temperature. The effluent from the column two was directed to both of 1) MS and 2) sniffing port. The MS transfer line and ion source temperature were 280°C and 230°C, respectively. The electron impact energy was 70 eV, and the mass range was from 35 to 350 amu. The sniffing port was heated at 230°C.

3.4 Results and Discussions

Although the oil content of a specific hop variety from different sources can be quite diverse (https://www.hopunion.com/'Centennial'/), hops were reported to express good varietal uniformity of composition under different environmental conditions (Likens and Nickerson 1967). Under this condition, 'Centennial' is always associated with a strong citrus-like impression, while 'Citra' and 'Nelson Sauvin' possesses a tropic fruit/ exotic fruit-like character besides the citrus note (Takoi et al. 2009, Takoi et al. 2010).

Aroma Fractionation and GC-MS/O of Hop Oils

To simplify the sample matrix and facilitate the identification of volatiles, hop oils of three hop varieties were pre-fractionated into two fractions: nonpolar fraction and

polar fraction. The simultaneous analysis of GC-MS/O facilitated the effective association of odorants and corresponding volatile compounds and therefore, provided useful and solid information for MS identification.

From GC-MS, in total, 28 compounds were identified in the nonpolar fractions (**Table 1** and **Figure 8**)，and 62 components were identified or tentatively identified from the polar fractions (**Table 2** and **Figure 9**). Only four odorants were detected by olfactometry in the nonpolar fractions, while in the polar fractions, 36 odorants were perceived but six of them could not be identified and listed as unknown compounds (**Table 3**)**.**

In the nonpolar fractions, terpenes were responsible for the majority. Terpenes are typically associated with balsamic, turpentine, woody and spice notes and in this case, can be positive contributors to the hop aroma. Nevertheless, among detected terpenes, only four of them could be perceived by GC-O due to the high sensory thresholds most terpenes possessed. From the odor assessment, myrcene was recorded as balsamic, celery notes with the extreme intensity, which could imply its importance to the aroma profile of hop oils. In addition, α-pinene, β-phellandrene and β-caryophyllene were detected by GC-O panelists. α-pinene was described as orange peel note with a moderate intensity. β-phellandrene was perceived as mint, turpentine notes while β-caryophyllene displayed a woody smell. However, the perception of these two compounds was weak.

The polar fractions mainly consisted of oxygenated compounds. In the polar fractions, 2-methylbutyl acetate, isoamyl propionate, 2-methyl-1-butanol, methyl octanoate, acetic acid, linalool, methyl (Z)-4-decenoate, isovaleric acid, (2E, 4E)-nona-2,4-dienal, geraniol, S-methyl methanethiosulfonate and vanillin showed strong

intensities in all hop varieties. Among these aromatics, esters were contributors to the fruity or citrus note. Acids such as acetic acid gave vinegar smell while isovaleric acid imparted strong rancid perception. Both linalool and geraniol presented extreme intensity and were reported as pleasant floral, citrus notes respectively. S-methyl methanethiosulfonate was identified in the hop oil for the first time. It imparted a radish/cabbage smell and presented with extreme intensity in all varieties. Vanillin was also recorded as extreme intensity and smelled like vanilla. 2-methyl-1-butanol was described as wine-like note while (2E, 4E)-nona-2,4-dienal displayed a special oily, steamed grain smell. In addition, one unknown compound, which imparted pine aroma, was detected with at least moderate intensity in all the hop varieties. Furthermore, three aromatics, all of which were described as garlic, preserved vegetable, sulfury notes, were also indicated as potent aroma contributors with at least moderate intensities to all the examined hops. Two of them were tentatively identified as S-methylthiomethyl 2-methylbutanethioate and S-methyl methanethiosulfonate, and one is an unknown volatile.

Volatiles including diacetyl, hexanal, S-methyl 4-methylpentanethioate, S-methylthiohexanoate, cis-3-hexenol, methional, methyl nonanoate, octanoic acid and nonanoic acid contributed to a different extent to the hop aroma based on the hop cultivars. Some aroma components, however, contributed positively only in certain varieties, for instance, 2-methylbutyl isobutyrate, geranial and nerol in ‘Centennial’, methyl heptanoate and isobutyric aicd in ‘Centennial’ and ‘Citra’, and nonanal in ‘Centennial’ and ‘Nelson Sauvin’.

Although GC-O revealed useful information about the aroma contribution of single volatiles to the whole hop aroma spectrum, it mainly served as a technique to identify aroma compounds by collaborating with MS detector and the more detailed description of odor-active compounds in hop oils had been made by the flavor dilution (FD) factor obtained from AEDA.

Table 1. Hop oil composition in nonpolar fractions.

No.	RI[a]	Compounds	No.	RI[a]	Compounds
1	1032	α-pinene	15	1495	α-copaene
2	1100	β-pinene	16	1528	β-cubenene
3	1152	myrcene	17	1621	β-caryophyllene
4	1212	limonene	18	1659	(E)-β-farnesene
5	1213	β-phellandrene	19	1686	α-humulene
6	1244	cis-ocimene	20	1703	α-amorphene
7	1253	γ-terpinene	21	1727	valencene
8	1260	trans-β-ocimene	22	1728	β-selinene
9	1275	p-cymene	23	1734	α-selinene
10	1286	1,2,4-trimethylbenzene	24	1759	α-farnesene
11	1290	δ-terpinene	25	1771	δ - cadinene
12	1399	1,3,5-undecatriene	26	1789	cadina-1,4-diene
13	1461	α-cubebene	27	1800	α-cadinene
14	1485	α-ylangene	28	1917	α-calacorene

[a]Retention Index.

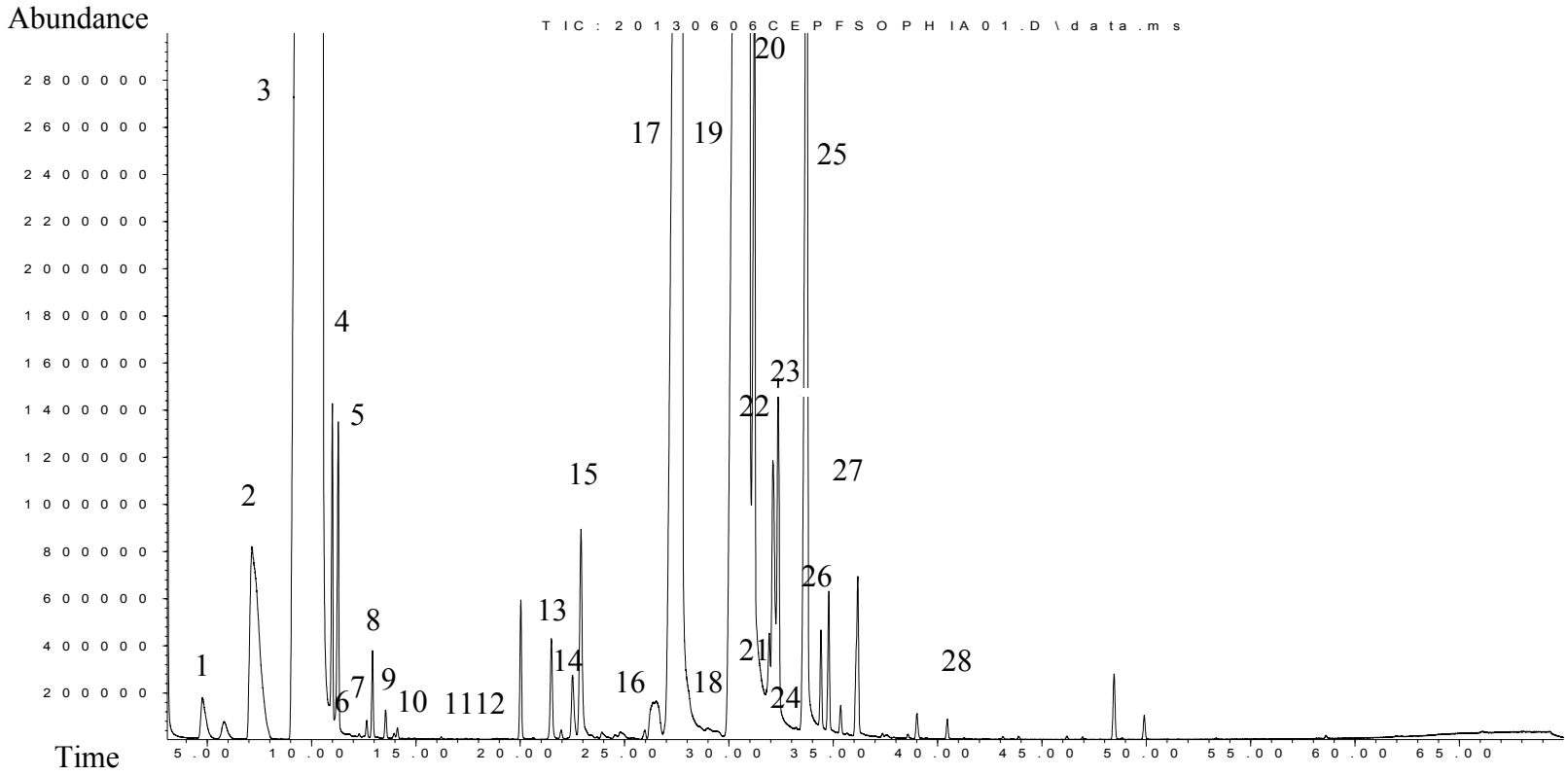


Figure 8. Compounds identified in nonpolar fractions (numbers refer to the compounds identified in **Table 1**).

Table 2. Hop oil composition in polar fractions.

No.	RI[a]	Compounds	No.	RI[a]	Compounds
1	987	diacetyl	26	1404	S-methylthiohexanoate
2	1012	4-methyl-2-pentanone	27	1409	cis-3-hexenol
3	1014	2-methyl-3-buten-2-ol	28	1433	acetic acid
4	1064	isobutyl propanoate	29	1447	heptyl isobutyrate
5	1081	isobutyl isobutyrate	30	1461	methional
6	1101	hexanal	31	1464	isoamyl hexanoate
7	1130	2-methylbutyl acetate	32	1493	methyl nonanoate
8	1142	butyl isobutyrate	33	1518	benzaldehyde
9	1174	isobutyl 2-methylbutyrate	34	1531	methyl 4-nonenoate
10	1179	2-heptanone	35	1547	isobutyric acid
11	1181	isoamyl propionate	36	1549	octyl isobutyrate
12	1195	2-methylbutyl isobutyrate	37	1550	linalool
13	1199	2-methyl-1-butanol	38	1603	2-undecanone
14	1247	methyl 5- methyl hexanoate	39	1606	methyl decanoate
15	1269	isoamyl butyrate	40	1609	methyl Z-4-decenoate
16	1271	methyl heptanoate	41	1659	isovalerica acid
17	1285	acetoin	42	1684	neral
18	1331	prenol	43	1704	geranial
19	1341	6-methyl-5-hepten-2-one	44	1743	pentanoic acid
20	1348	methyl 6-methylheptanoate	45	1756	nerol
21	1354	S-methyl 4-methylpentanethioate	46	1763	geranyl acetate
22	1358	isobutyl hexanoate	47	1778	citronellol
23	1364	1-hexanol	48	1783	geranyl propionate
24	1380	methyl octanoate	49	1785	neryl isobutyrate
25	1398	nonanal	50	1800	3-methyl-2-butenoic acid

Table 2 (Continued). Hop oil composition in polar fractions.

No.	RI[a]	Compounds	No.	RI[a]	Compounds
51	1815	2-tridecanone	57	1961	heptanoic acid
52	1821	geranyl isobutyrate	58	1970	caryophyllene oxide
53	1837	geraniol	59	1973	S-methyl methanethiosulfonate
54	1852	hexanoic acid	60	2068	octanoic acid
55	1882	benzyl alcohol	61	2206	nonanoic acid
56	1913	5-methylhexanoic acid	62	2546	vanillin

[a]Retention Index.

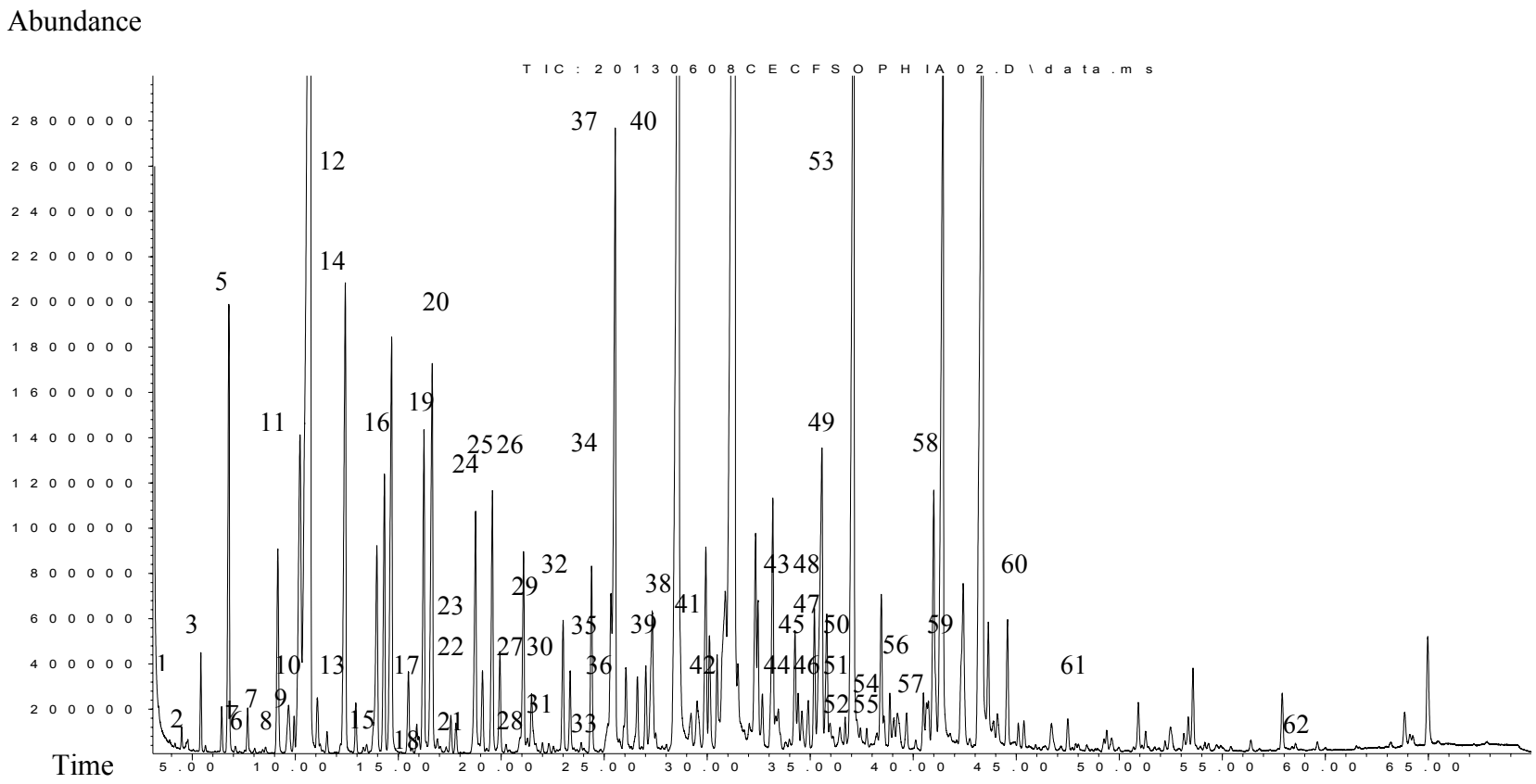


Figure 9. Compounds identified in polar fractions (numbers refer to the compounds identified in **Table 2**).

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Table 3. Aroma compounds detected in three different hop varieties ('Centennial', 'Citra' and 'Nelson Sauvin') by GC-MS/O.

No.	RI[a]	Aroma compounds	Descriptor[b]	Fraction	Basis of identification[c]
1	987	diacetyl	cheesy, buttery	P	MS, RI
2	1032	α-pinene	orange peel, pine	NP	MS, RI
3	1101	hexanal	grassy	P	MS, RI
4	1130	2-methylbutyl acetate	fruity, nail polish	P	MS, RI
5	1152	myrcene	celery, balsamic	NP	MS, RI
6	1173	Unknown	pine	P	
7	1181	isoamyl propionate	fruity	P	MS, RI
8	1195	2-methylbutyl isobutyrate	fruity, soda	P	MS, RI
9	1199	2-methyl-1-butanol	wine, fruity, sweet	P	MS, RI
10	1213	β-phellandrene	mint, turpentine	NP	MS, RI
11	1228	Unknown	pine tree, almond	P	
12	1271	methyl heptanoate	fruity, sweet	P	MS, RI
13	1354	S-methyl 4-methylpentanethioate	preserved vegetable	P	MS, RI
14	1380	methyl octanoate	citrus, soapy, fatty	P	MS, RI
15	1398	nonanal	citrus, floral	P	MS, RI
16	1404	S-methylthiohexanoate	cabbage	P	MS, RI
17	1409	cis-3-hexenol	green, grassy	P	MS, RI
18	1433	acetic acid	vinegar, sour	P	MS, RI
19	1461	methional	potato, soy sauce	P	MS, RI
20	1493	methyl nonanoate	floral, cooked rice	P	MS, RI
21	1547	isobutyric acid	cheese, rancid	P	MS, RI
22	1550	linalool	floral	P	MS, RI
23	1609	methyl (Z)-4-decenoate	milky, green	P	MS, RI
24	1621	β-caryophyllene	woody, spice	NP	MS, RI
25	1659	isovaleric acid	smelly, rancid	P	MS, RI

Table 3 (Continued). Aroma compounds detected in three different hop varieties: 'Centennial' (CE), 'Citra' (CI) and 'Nelson Sauvin' (NS).

No.	RI[a]	Aroma compounds	Descriptor[b]	Fraction	Basis of identification[c]
26	1701	(2E,4E)-nona-2,4-dienal[d]	steamed grain, oily	P	RI
27	1704	geranial	citrus, soapy	P	MS, RI
28	1730	S-methylthiomethyl 2-methylbutanethioate[e]	garlic, preserved vegetable	P	RI
29	1756	nerol	sweet, floral, citrus	P	MS, RI
30	1837	geraniol	citrus, lemon	P	MS, RI
31	1845	Unknown	fatty, nutty	P	
32	1897	S-methylthiomethyl 4-methylpentanethioate[e]	garlic, gross, fatty	P	RI
33	1970	caryophyllene oxide	woody, incense	P	MS, RI
34	1973	S-methyl methanethiosulfonate	radish, cabbage	P	MS, RI
35	2000	Unknown	preserved vegetable	P	
36	2041	octanoic acid	sweaty, rancid	P	MS, RI
37	2133	Unknown	ink, rancid	P	
38	2206	nonanoic acid	sweaty	P	MS, RI
39	2227	Unknown	rancid, cheese	P	
40	2546	vanillin	vanilla	P	MS, RI

[a]Retention Index.
[b]Descriptor perceived at the sniffing port.
[c] MS, compounds identified by MS spectra; RI, comparison to pure standard, and with the RI from the literature.
[d]Compound was identified by comparison to pure standard.
[e]Compounds were tentatively identified by aroma note and RI form the literature.

AEDA of Three Hop Varieties

The essential oils of three hop varieties were pre-fractionated into nonpolar and polar fractions for AEDA. Each fraction was diluted stepwise with corresponding solvent (1+1 by volume) and all dilutes were analyzed by GC-O in duplicate. Serial dilutions were evaluated and flavor dilution (FD) factor of each odorant was calculated as an estimate for the contribution of single volatiles to the overall hop aroma profile.

Aroma extract dilution analysis (AEDA) showed that myrcene possessed the highest FD factor (FD=512) followed by α-pinene (FD=32), in the nonpolar fractions of three hop varieties. Other hydrocarbons such as β-phellandrene (FD≤8) and β-caryophyllene (FD≤4) were also detected by GC-O however did not display high FD factors. In the polar fraction, AEDA revealed isovaleric acid and geraniol with the highest FD values of all varieties, followed by S-methyl methanethiosulfonate, vanillin and linalool (**Table 4**). Isovaleric acid was one of the most import aroma compounds among the oxygenated components based on its high FD factor especially in 'Citra' hop (FD=256). Geraniol presented the highest FD factor (FD=256) in 'Centennial', and in 'Citra' and 'Nelson Sauvin', the FD value was 128. S-methyl methanethiosulfonate, which was detected in hops for the first time, presented a high FD factor (FD=128) in 'Centennial' cultivar and a relatively high FD factor (FD=64) in 'Citra', however a moderate FD fractor (FD=16) in 'Nelson Sauvin'. Vanillin was detected with a relatively high FD factor (FD=64) in all three cultivars. Linalool showed a relatively high FD value (FD=64) in 'Centennial' and 'Citra' hops. In addition, an unknown volatile 6 showed a relatively high FD factor (FD=64) in both 'Citra' and 'Nelson Sauvin' varieties. It was perceived with strong pine, turpentine aroma notes but could not be identified from GC-MS.

Some odorants, however, showed high FD values only in ‘Centennial’ and ‘Citra’ varieties. For instance, diacetyl strongly contributed a buttery aroma in ‘Centennial’ (FD=64) while (2E,4E)-Nona-2,4-dienal gave a strong oily, steamed grain notes to ‘Citra’ (FD=64). An unknown compound 35 added preserved vegetable aroma note to ‘Citra’ with a FD factor 64, compared to FD factor 16 in ‘Centennial’ and ‘Nelson Sauvin’. At the same time, sulfur-containing aromatic, S-methylthiomethyl 2-methylbutanethioate (tentatively identified) contributed the most in ‘Citra’ hops (FD=32) compared with the other two cultivars. In addition, methyl (Z)-4-decenoate largely contributed milky, green notes to ‘Centennial’ (FD=16) and ‘Citra’ (FD=16) instead of ‘Nelson Sauvin’ (FD=4). Geranial and nerol were only detected by GC-O in ‘Centennial’ hop oil, they contributed citrus, lemon, soapy notes which may play a role due to the dominant citrus aroma of ‘Centennial’.

Based on the AEDA, it was clear that although plenty of monoterpenes and sesquiterpenes could be identified by GC-MS in the nonpolar fractions, many of them could not be perceived by GC-O due to their high sensory threshold, which suggested their limited influence on the overall aroma profile of hops. On the contrary, oxygenated compounds and sulfur-containing components in the polar fractions were more potent aromatic contributor to hop aroma. It was reported that free fatty acids (e.g., isovaleric acid) presented 0.8-3% in fresh hops, and were proposed to be developed during storage (Tressl et al. 1978, Hanke et al. 2010) due to the degradation of hop resins. These acids impart intense rancid, cheesy flavor to beer when late hopping or dry hopping is applied. Linalool was widely confirmed as a significant contributor to the hoppy aroma in beer (Sakuma et al. 1991, Hanke et al. 2010). Geraniol, however, was believed to be more

cultivar-specific than linalool (Steinhaus et al. 2007). Moreover, the initial high concentration of geraniol in hop was proved to increase the concentration of geraniol and β-citronellol in the finished beer (Takoi et al. 2010). Vanillin was revealed to be important odorant in hop oil and it was also reported as a flavor contributor to beer. However, vanillin is not only derived from hops since it was detected in unhopped beer (Kishimoto et al. 2006). Thioester, such as S-methylthiomethyl 2-methylbutanethioate (tentatively identified), were proved to importantly contribute to the garlic, preserved cabbage flavor in hops, which have also been studied and discussed in plenty of hop investigations focusing on volatile sulfur compounds (Peppard 1981, Collin 2003). Although sulfur compounds were quite odor-active in certain hop varieties, they were not considered to be very desirable in beer products and their influence on beer flavor based on different utilizations of hops during brewing.

Identification of S-methyl methanethiosulfonate by 2D GC

Due to the capacity limit of one-dimensional GC, coeluting of effluents happened and precluded the identification of aroma compounds. 2D GC further separated the coeluting compounds by introducing a small portion of the target peaks from the first separation column to a second column. And based on the polarity difference of the packing materials of two columns, coeluting compounds were separated. S-methyl methanethiosulfonate was coeluted with caryophyllene oxide from one-dimensional GC. After cut from the first column, the coeluting peak of S-methyl methanethiosulfonate and caryophyllene oxide was directed to the second column. Two compounds were separated and S-methyl methanethiosulfonate was identified by MS and perceived by Olfactometry.

S-methyl methanethiosulfonate was identified as a potent aroma compound in hops, especially in ‘Centennial’ and ‘Citra’ varieties, for the first time. Although not reported in the hop before, S-methyl methanethiosulfonate is believed to work as phytoalexin in vegetables such as cabbage, broccoli and cauliflower (Kyung and Fleming 1997). And it was proposed to derive from the degradation of S-methyl-L-cysteine sulfoxide catalyzed by C-S lyase, which presumedly induced by wounding the vegetable tissues. S-methyl methanethiosulfonate was described as sauerkraut-like flavor and its detection threshold was estimated as 5 ppm (Chin and Lindsay 1994). However, a study of sauerkraut revealed that sauerkraut sulfur flavor correlated linearly with dimethyl trisulfide and S-methyl methanethiosulfonate. Moreover, the sauerkraut sample with the highest concentration of S-methyl methanethiosulfonate presented the most sauerkraut sulfur flavor although the concentration of S-methyl methanethiosulfonate was measured below the reported threshold value (Johanningsmeier et al. 2005). And in the current research, the high odor activity of S-methyl methanethiosulfonate in hops was proved.

In conclusion, although about 100 compounds were identified in hops, only small portion of them were demonstrated to contribute to hop aroma. Moreover, oxygenated volatiles and sulfur-containing compounds, compared with terpenes, were revealed to be more important to the overall hop aroma profile.

Table 4. Odor-active volatiles detected in three different hop varieties: 'Centennial' (CE), 'Citra' (CI) and 'Nelson Sauvin' (NS).

No.	Aroma compounds	Descriptor[a]	RI[b]	Fraction	FD-Factor[c]		
					CE	CI	NS
1	diacetyl	cheesy, buttery	987	P	64	8	16
2	α-pinene	orange peel, pine	1032	NP	32	32	32
3	hexanal	grassy	1101	P	4	8	1
4	2-methylbutyl acetate	fruity, nail polish	1130	P	16	16	8
5	myrcene	celery, balsamic	1152	NP	512	512	512
6	Unknown	pine, turpentine	1173	P	32	64	64
7	β-phellandrene	mint, turpentine	1177	NP	8	4	4
8	isoamyl propionate	fruity	1181	P	4	2	4
9	2-methylbutyl isobutyrate	fruity, soda	1195	P	2	<1	<1
10	2-methyl-1-butanol	pear, fruity, sweet	1199	P	8	4	8
11	Unknown	pine tree, almond	1228	P	8	4	1
12	methyl heptanoate	fruity, sweet	1271	P	2	2	<1
13	S-methyl 4-methylpentanethioate	preserved vegetable	1354	P	8	2	2
14	methyl octanoate	citrus, soapy, fatty	1380	P	8	8	4
15	nonanal	citrus, floral	1398	P	8	<1	4
16	S-methylthiohexanoate	Preserved vegetable, cabbage	1404	P	8	8	2
17	cis-3-hexenol	green, grassy	1409	P	8	2	4
18	acetic acid	vinegar, sour	1433	P	2	2	2
19	methional	potato, soy sauce	1461	P	16	8	4
20	methyl nonanoate	floral, cooked rice	1493	P	8	4	8
21	isobutyric acid	cheese, rancid	1547	P	8	8	<1
22	linalool	floral	1550	P	64	64	16
23	methyl (Z)-4-decenoate	milky, green	1609	P	16	16	4

Table 4 (continued). Odor-active volatiles detected in three different hop varieties: 'Centennial' (CE), 'Citra' (CI) and 'Nelson Sauvin' (NS).

No.	Aroma compounds	Descriptor[a]	RI[b]	Fraction	FD-Factor[c]		
					CE	CI	NS
24	β-caryophyllene	woody, spice	1628	NP	4	2	4
25	isovaleric acid	smelly, rancid	1659	P	128	256	64
26	(2E,4E)-nona-2,4-dienal[d]	steamed grain, oily	1701	P	16	64	16
27	geranial	citrus, soapy	1704	P	4	<1	<1
28	S-methylthiomethyl 2-methylbutanethioate[e]	garlic, preserved vegetable	1730	P	16	32	8
29	nerol	sweet, floral, citrus	1756	P	8	<1	<1
30	geraniol	citrus, lemon	1837	P	256	128	128
31	Unknown	fatty, nutty	1845	P	8	16	1
32	S-methylthiomethyl 4-methylpentanethioate[e]	garlic, gross, fatty	1897	P	16	16	16
33	caryophyllene oxide	woody, incense	1970	P	8	16	1
34	S-methyl methanethiosulfonate	radish, cabbage	1973	P	128	64	16
35	Unknown	preserved vegetable	2000	P	16	64	16
36	octanoic acid	sweaty, rancid	2041	P	16	8	2
37	Unknown	ink, rancid	2133	P	8	16	<1
38	nonanoic acid	sweaty	2206	P	2	4	8
39	Unknown	rancid, cheese	2227	P	<1	16	<1
40	vanillin	vanilla	2546	P	64	64	64

[a] Descriptor perceived at the sniffing port. [b] Retention Index. [c]Flavor Dilution factor.
[d]Compound was identified by comparison to pure standard.
[e]Compounds were tentatively identified by aroma note and RI form the literature.

CHAPTER 4 EVALUATION OF THE BEHAVIOR OF HOP-DERIVED AROMA COMPOUNDS IN DRY-HOPPED BEER

4.1 Abstract

The major compositions of essential oil of three hop varieties (‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’) were determined. It showed that myrcene, β-caryophyllene and α-humulene were dominant compositions in all hop cultivars while esters as well as linalool and geraniol were relatively abundant in ‘Centennial’ and ‘Citra’ varieties. A further study of hop aroma characters in beers dry-hopped with ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’ was performed with stir bar-soptive extraction (SBSE) technique. Compared with control beer, dry-hopped beers prepared with ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’ varieties revealed an increase of myrcene and α-humulene whereas the increase of β-caryophyllene was only in ‘Citra’ variety. Linalool and geraniol increased significantly in hopped beer. Due to the importance of linalool and aroma contribution differences of stereoisomers, the chiral isomers in both hops and beers were further studied by solid phase microextraction (SPME) and a chiral column was used. The results demonstrated the prevalence of R-linalool in hops and a conversion of R-linalool to S-linalool in beer was observed in control beer when hop was added at beginning of wort boiling.

Keywords: Hop oil composition; dry-hopping; SBSE; linalool isomer; chiral.

4.2 Introduction

The fate of hop aromatic volatiles during brewing process are important and consequently, widely investigated. Hops can be added at various stages to achieve different flavor and aroma profile of beer. Hops can be added at the early stage of wort boiling process to give bitterness but meanwhile, late-hopping can be applied to introduce more hoppy aroma to the final beer by reducing the evaporation of hop volatiles during the boiling progress. Nevertheless, it has been reported that most of the hop oil constitutes can be lost even at the last five-minute boiling when hop oil is added at late-hopping stage (Haley and Peppard 1983). Furthermore, in the application of hopping methods involving hop additions before fermentation, hop volatiles may be altered and expended under several situation: 1) carrying out by carbon dioxide during fermentation; 2) hydrophobic or high molecular weight components absorption by yeast; 3) metabolization and esterification by yeast (King and Dickinson 2003, Kishimoto et al. 2006). Dry-hopping is an alternative hopping technique, during which pure hop aroma could be efficiently impart into beer. It involves adding hops or hop products during the beer conditioning or even directly to the beer cask, which is a cold extraction of hop material into an alcoholic solution. The role of hops in brewing has been widely investigated. Tressl et al. reported more than 110 volatiles of a German beer, forty-seven of which had been found in Spalter hops (Tressl et al. 1978). Humulenol Ⅱ was considered as a contributor to hoppy aroma in American beers, while geraniol and linalool were demonstrated to be responsible for the floral note of beer brewed with Cascade hops (Peacock et al. 1980, Peacock et al. 1981). Comparision of dry-hopped beer and late-hopped beers indicated that dry-hopping method kept more representative volatiles from original hop oil than late-hopping (Haley and Peppard 1983). Investigators have attempted to build a clear theoretical

connection between hop characteristics and final beer flavor based on which, a better control of hop-derived beer flavor could be achieved.

Linalool is widely found in essential oils. It occurs in nature in both dextrorotatory (S-linalool) and laevorotatory (R-linalool) forms. The investigation of linalool enantiomeric distribution in essential oils has been largely conducted (Gaydou and Randriamiharisoa 1987, Bernreuther and Schreierc 1991, Schubert and Mosandl 1991) and nowadays, cyclodextrin derivatives (CDs) are largely applied as chiral selector for direct enantimer GC separation of volatile optically active components (Bicchi et al. 1999). In a chiral evaluation of linalool in 42 esential oils, R-linalool was revealed to be more common than S-linalool (Özek et al. 2010). Linalool is indicated as one of the most important aroma-impact volatiles in beer (Schönberger and Kostelecky 2011). Although both linalool enantiomers are descriped as floral-like note, the threshold of R-linalool in beer is 2.2 ppb, which is much lower than the threshold of S-linalool (180 ppb) (Kaltner and Mitter 2009). Since hops are the direct source of linalool in hopped beer, the understanding of the ratio of R-linalool in hops as well as beers is of great importance. A recent study of the enantiomeric distribution of linalool revealed the ratio of R-linalool between the range of 92% to 94% in nine different hop varieties and regardless of hop processing (Kaltner and Mitter 2009).

Stir bar-sorptive extraction (SBSE) and solid-phase microextraction (SPME) have been commonly combined with GC-MS to perform quantitative analysis (Baltussen et al. 1999, Prosen and Zupančič-Kralj 1999). SBSE has been successfully employed in the study of alcoholic beverage analysis such as beer, wine and whisky (Demyttenaere et al. 2003, Ochiai et al. 2003, Hayasaka et al. 2003, Kishimoto et al. 2005). Meanwhile, SPME has been commonly utilized in the hop studies such as hop variety verification and essential oil determination (Field et al. 1996,

Kovačevič and Kač 2001). In the current study, major oil compositions of three hop varieties were determined. The evaluation of the behavior of hop-derived aroma compounds in beers was achieved by SBSE method and gas chromatography (GC) coupled with chiral column and SPME preparative method was employed to investigate the enantiomeric composition of linalool in both hops and beers.

4.3 Materials and Methods

Chemicals and Equipment

Pentane (Nanograde, Mallinckrodt Baker, Phillipsburg, NJ) was freshly distilled. Citric acid, monohydrate granular (500g) was from J.T. Baker (Phillipsburg, NJ). Sodium chloride was supplied by EMD (Philadelphia, PA). All the chemical standards used in volatile compound identification were obtained from Sigma-Aldrich (St. Louis, MO) with the highest commercial purity (>95%).

Hop Samples

'Centennial' and 'Citra' hop cones were supplied by Freshops, Philomath, Oregon. 'Nelson Sauvin' hop pellets were obtained from BSG Handcraft, Shakopee, Minnesota. All the commercial hop samples were packaged in airtight bags, stored at -20°C prior to analysis.

Hop Essential Oils

Total oil content of hops was determined by steam distillation based on ASBC method (Hops-13 2009). For 'Centennial' and 'Citra', 100 grams of whole hops were mixed with 1.5 liters of distilled water and then blended for 30 seconds using a 3.8 liter stainless steel blender (Waring CB15). The blended mixture was transferred to a 5000 ml round bottom boiling flask and another 1.5 liters distilled water was used to rinse blending jar and ensure the complete transfer of hop content to the flash. For 'Nelson Sauvin', 100 grams of pellets were added

directly to the boiling flask with 3 liters of distilled water. The distillation lasted for 4 hours and volume of the oil was measured after the system was cooled down. The collected oils were stored in 4 ml brown glass vials capped with foil lined screw-top caps at 5°C under nitrogen.

Determination of Major Oil Compositions

Major oil compositions of three hop varieties were determined by ASBC method (Hops-17 2009). The determination was performed on an Agilent 6890 gas chromatography (GC) coulpled with an flame ionization detector (FID) and an Agilent 5973 mass selective detector (MSD). The samples were analyzed on a ZB-Wax column (30 m×0.25 mm i.d., 0.5 μm film thickness, Phenomenex). The column carrier gas was helium at a constant flow rate of 2 ml/min. The column effluent was split 1:1 into FID and MSD. Sample (1μl) was injected into the GC injector at a 1:20 split ratio. The injector temperature and FID temperature were 250°C. The oven temperature was programmed at 50°C for a 1 minute hold and then to 120°C at a rate of 2°C /min, and to 230°C at a rate of 3°C /min, with a 5 min hold at the final temperature. The MS transfer line and ion source temperature were 280 °C and 230 °C, respectively. The electron impact energy was 70 eV, and the mass range was from 35 to 350 amu. Quantification of compounds was determined by using an internal standard method with 2-octanol (Hops-17 2009). The identification of oil components were fulfilled by GC-MS, unknown compounds were compared with those in the Wiley 275.L database (Agilent Technologies Inc.) and confirmed using in house standards.

Pilot-Scale Brewing

Cascade, ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’ were used in the brewing process and all the beers were prepared in the pilot plant (Food Science & Technology Department, Oregon State University). The base beer was brewed with Cascade hops, which were added at the beginning of

the wort boiling process. When appropriate, wort was pitched with yeast, fermented at 20°C for 14 days and then, held at 1°C for 33 days. After the cold stabilization, the experimental beer was filtered through 3-micron (nominal) cellulose pads. The finished beer was evenly divided and stored into four kegs. One keg of beer was saved as control beer and the other three portions of beer were dry-hopped independently at a 10-L volume scale with different hop varieties by adding the hop materials directly into the base beer at a one lb/Bbl hopping rate. Beers were dry-hopped for three days.

Hop Volatile Components Analysis in Beer by Stir Bar Sorptive Extraction-Gas Chromatography-Mass Spectrometry (SBSE-GC-MS)

A sample of 10 ml of beer was diluted with Milli-Q water at 1:1 ratio (by volume) in a 40 ml glass vial, and 25 µl of internal standard (4-octanol solution, 150ppb) was added to the vial. A stir bar (length=10mm, thickness=0.5mm) coated with poly(dimethylsiloxane) (PDMS) phase (Gerstel, Baltimore, MD) was used to extract the volatile constitutes from beer samples. The extraction was performed for 3 h at a speed of 1000rpm. After extraction, the stir bar was then removed from the vial and washed with Milli-Q water, dried with a Kim wipe (Kimberly-Clark Profesionalmc, Roswel, GA) tissue paper, and placed into the glass sample holder of sampler tray (Gerstel,Inc.).

Thermal desorption of the volatile compounds from the PDMS-coated stir bar was performed in the TDU autosampler (Gerstel,Inc.), which was programmed from 25°C to 250°C (held for 2 min) at a rate of 120°C/min in a splitless mode. The desorbed analytes from the TDU were cryofocused in a programmed temperature vaporizing (PTV) injector (CIS4, Gerstel,Inc.) at -80 °C using liquid nitrogen. After desorption, the CIS4 inlet was programmed from -80 °C to 250°C at a rate of 10 °C/s and held for 54 min. An Agilent 7890A gas chromatograph coupled with 5975C mass spectrometry detector (Agilent Technologies, Little

Falls, DE) was used for the analysis. A ZB-Wax column (60m, 0.25mm i.d., 0.5 μm film thickness; Phenomenex Inc., Torrance, CA) was used to fulfill the separation. Helium at a constant flow rate of 2.5ml/min was used as the carrier gas. The initial oven temperature was set at 60°C for one min, raised to 140°C at a rate of 5°C/min, then to 200°C at a rate of 2°C/min, and finally to 230°C/min at a rate of 4°C/min and held for 10 min. The MS was set up to scan ions with a mass-to-charge ratio (m/z) of from 35 to 350 and in the electron-impact mode at 70 eV. The MS source temperature was set at 230°C.

Standard Calibration Curve

In total, 13 hop-derived terpenes and terpenoids were chosen as target compounds for quantification and the stock solution was prepared as mixture of all the volatiles. An eight-point (1, 5, 10, 25, 50, 100, 250 and 500ppb) standard curve was built and y-axis and x-axis were plotted as peak area ratio and concentration ratio for each point respectively.

$$y = mx + b$$

$$\left(\frac{\text{Std}peakarea}{ISpeakarea}\right) = m\left(\frac{\text{Std}conc}{ISconc}\right) + b$$

The regression coefficients were calculated using the Chemistation data analysis software. If the calculated concentration falls below the detection limit, then the concentration was stated as 'not detected'.

Linalool Chiral Isomer Analysis by SPME Method

A three-phase (DVB/Carboxen/PDMS) solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA) was used to extract and concentrate volatiles from the headspace of samples. For hop sample, one gram of each variety was added into a 20 ml glass vial. For beer sample, 2 ml of beer was diluted with 8 ml citric acid solution (saturated with sodium chloride, PH=3) in a 20 ml glass vial, and 20 μl internal standard (4-octanol, 20ppm) was added to the vial. The glass vial

was sealed with Teflon septum lid. The sample was incubated at 50°C for 5 min, and extracted by the SPME fiber at the same temperature for 50 min. The volatiles were desorbed from SPME fiber at 250°C for 5 min in splitless mode.

The chiral analysis was carried out on an Agilent 6890-5973 mass selective detector (Agilent, Santa Clara, CA). Linalool isomers were separated using a CycloSil-B column (30 m×0.25 mm i.d., 0.25 μm film thickness; Agilent). The column carrier gas was helium at a constant flow rate of 2.5ml/min. The GC injector temperature was set at 250 °C. The oven temperature was programmed at 60 °C for a 1 min hold and then to 220 °C at a rate of 3 °C/min, with a 10 min hold. The MS transfer line and ion source temperature were 280°C and 230°C, respectively. The electron impact energy was 70 eV, and the mass range was from 45 to 350 amu. Linalool enantiomers were identified on MS by comparing to standard compounds. The percentage of R-linalool was calculated by total mass ion abundance.

Statistic Analysis

The major compositions in essential oils of three hop varieties and target compounds in four different beers were compared respectively using analysis of variance (One-way ANOVA) with the Duncan's test ($P<0.05$). SPSS 17.0 software (SPSS Inc., IL, USA) was employed to conduct the One-way ANOVA.

4.4 Results and Discussions

Different hop cultivars could have a different aroma profile and therefore, contribute differently to the beer flavor. In spite of the varietal difference, hops or hop preparations influence the beer flavor to different extent due to abundant variables during the brewing process. It has been observed that the hop aroma characters of beers depend on the time of hop addition (Kishimoto et al. 2005). Compared with other hop addition techniques, dry-hopping method is

the most straightforward way to introduce pure hop aroma to the final beer without any possible interfere or effect from wort boiling and fermentation. However, it was stated that only a small portion of hop oil could be transferred to the beer by dry-hopping procedure and different components might behave differently when partitioning between hops and beer (Pickett et al. 1975).

Hop Oil Composition

Oil content of three hop cultivars was determined by steam distillation and was compared with the data from commercial source (**Table 5**). From 100 grams of hop materials, 1.30 ml of 'Centennial' oil and 1.05 ml of 'Nelson Sauvin' oil were extracted respectively by steam distillation. Compared with the other two cultivars, a much higher yield of essential oil was presented for 'Citra' as 2.65 ml. The oil contents of 'Citra' and 'Nelson Sauvin' measured in this study were consistent with commercial values, while the data of 'Centennial' was slightly lower than reference.

Major hop oil components of three hop varieties were listed in **Table 6**. The content of identified compounds was expressed as mg/g of hop materials. In all varieties, terpene compounds namely myrcene, β-caryophellene and α-humulene were measured to be dominant compositions, followed by 2-methylbutyl isobutyrate and δ-cadinene. The varietal differences among hop cultivars were quite apparent. 'Citra' hop was rich in myrcene, the content of which was four times of that in 'Centennial' and 'Nelson Sauvin'. 'Citra' also possessed the highest amount of β-caryophellene while α-humulene was most abundant in 'Nelson Sauvin'. β-pinene, linalool, methyl z-4-decenoate, methyl geranate and geraniol were relatively high in 'Centennial' and 'Citra', but only a small amount showed in 'Nelson Sauvin'. Compared with 'Centennial' and 'Nelson Sauvin', 'Citra' hop had a higher content of isoamyl propionate, β-phellandrene,

methyl octanoate, 2-undecanone, methyl decanoate, β-selinene and α-selinene. Furthermore, 'Centennial' presented a relatively higher amount of esters such as isobutyl 2-methylbutyrate and geranyl isobutyrate in relation to the other two varieties. The content of other detected components in the hop oils were lower than 0.1mg/g.

Table 5. Oil contents of 'Centennial', 'Citra' and 'Nelson Sauvin' hops (ml/100g hop).

Hop variety	Oil Content	Oil Content from Commercial Data *
'Centennial'	1.30	1.5-2.5
'Citra'	2.65	2.2-2.8
'Nelson Sauvin'	1.05	~1.1

*https://www.hopunion.com/hop-varieties/

Table 6. Major compositions of essential oils of 'Centennial' (CE), 'Citra' (CI) and 'Nelson Sauvin' (NS) hops (mg/g hop).

		Content (mg/g)					Content (mg/g)		
RI	Compound	CE	CI	NS	RI	Compound	CE	CI	NS
1012	4-methyl-2-pentanone	0.04±0.00c	0.01±0.00a	0.01±0.00a	1493	methyl nonanoate	0.02±0.00b	0.10±0.00c	<0.01a
1014	2-methyl-3-buten-2-ol	0.08±0.00c	0.02±0.00b	0.01±0.00a	1495	α-copaene	0.03±0.00a	0.03±0.00a	0.05±0.00b
1032	α-pinene	0.04±0.00b	0.04±0.00b	0.02±0.00a	1550	linalool	0.13±0.00b	0.22±0.00c	0.04±0.00a
1081	isobutyl isobutyrate	0.07±0.00b	0.02±0.00a	0.02±0.00a	1603	2-undecanone	0.02±0.00a	0.16±0.00c	0.09±0.00b
1100	β-pinene	0.22±0.00b	0.27±0.00c	0.03±0.00a	1606	methyl decanoate	<0.01a	0.26±0.00b	<0.01a
1130	2-methylbutyl acetate	<0.01a	0.01±0.00b	<0.01a	1609	methyl z-4-decenoate	0.17±0.00b	0.38±0.00c	0.06±0.00a
1152	myrcene	5.91±0.00b	19.38±0.06c	4.99±0.00a	1620	methyl geranate	0.49±0.00c	0.27±0.00b	0.02±0.00a
1174	isobutyl 2-methylbutyrate	0.17±0.00b	<0.01a	<0.01a	1621	β-caryophellene	0.80±0.01a	1.41±0.00c	0.88±0.01b
1181	isoamyl propionate	0.08±0.00b	0.15±0.00c	0.04±0.00a	1684	neral	0.03±0.00b	<0.01a	<0.01a
1195	2-methylbutyl isobutyrate	0.48±0.00c	0.33±0.00b	0.25±0.00a	1686	α-humulene	1.88±0.02a	2.07±0.01b	3.30±0.03c
1199	2-methyl-1-butanol	0.05±0.00c	<0.01a	0.02±0.00b	1704	geranial	0.02±0.00c	0.01±0.00b	<0.01a
1213	β-phellandrene	0.04±0.00b	0.18±0.00c	0.03±0.00a	1728	β-selinene	0.06±0.01b	0.23±0.00c	<0.01a
1260	tran-β-ocimene	0.01±0.00a	0.05±0.00b	0.01±0.00a	1734	α-selinene	0.04±0.00a	0.21±0.00c	0.05±0.00b
1271	methyl heptanoate	0.03±0.00b	0.04±0.00c	<0.01a	1756	nerol	0.02±0.00b	<0.01a	<0.01a
1275	para-cymene	0.01±0.00b	<0.01a	<0.01a	1763	geranyl acetate	0.01±0.00b	0.02±0.00c	<0.01a
1331	prenol	0.01±0.00b	<0.01a	<0.01a	1771	δ-cadinene	0.26±0.00a	0.31±0.00c	0.28±0.01b
1348	methyl 6-methyl heptanoate	0.04±0.00c	0.03±0.00b	0.02±0.00a	1783	geranyl propionate	0.02±0.01c	0.01±0.00b	<0.01a
1380	methyl octanoate	0.02±0.00b	0.14±0.00171c	0.01±0.00a	1815	2-tridecanone	0.02±0.00a	0.04±0.00b	0.06±0.00c
1398	nonanal	0.01±0.00b	<0.01a	<0.01a	1821	geranyl isobutyrate	0.13±0.00c	0.05±0.00a	0.07±0.00b
1423	trans-linalool oxide	0.01±0.00b	<0.01a	<0.01a	1837	geraniol	0.19±0.00c	0.12±0.00b	0.02±0.00a
1485	α-Ylangene	0.02±0.00b	0.01±0.00a	0.02±0.00b	1970	caryophyllene oxide	0.08±0.00c	0.02±0.00b	<0.01a

Mean±SE presented (n=2).
Duplicate analyses were performed and content of the volatiles was calculated for each replicate based on the area integration, and the average was determined**.** Concentration below calculation was recorded as '<0.01'.
a-c Different letters indicate significant differences.

SBSE Analysis of Terpenoids in Beers

The quantitative analysis of beers (a control beer and samples dry-hopped with 'Centennial', 'Citra' and 'Nelson Sauvin' hops respectively) was listed in **Table 7**. The transfer rate of selected hop-derived aroma compounds from hops to dry-hopped beers was calculated (**Table 8**). Theses compounds were previously identified in essential oil of three hop varieties ('Centennial', 'Citra' and 'Nelson Sauvin') and selected for the quantitative analysis based on their odor activity and aroma potential. Seven of them, which were α-pinene, myrcene, linalool, β-caryophyllene, geranial, nerol, geraniol and caryophyllene oxide were proved to be aroma-impact in three hop cultivars by previous aroma extract dilution analysis (AEDA). The rest were studied as potential odorants or marker compounds in hops or beers.

Six terpene compounds were examined in beers (**Figure 10**). As one of the most abundant components in hop essential oil, myrcene showed a remarkable increase in beers dry-hopped with 'Citra' and 'Nelson Sauvin', and some increase in 'Centennial' beer demonstrating that myrcene can be effectively transferred from hop to beer. With a low sensory threshold, myrcene could impart a characteristic and unique hoppy flavor to dry-hopped beer compared with beer prepared by early hopping methods, during which myrcene could be largely lost. However, high myrcene beer is not necessarily the most preferred beer. A balanced flavor profile is more important to consumers. β-Caryophyllene was only detected in beer hopped with 'Citra' variety, which had the highest amount of β-caryophyllene in hop oils among the three cultivars. The concentration of α-humulene was below detection limit in control beer and showed significant increase in 'Citra' beer and 'Nelson Sauvin' beer, the change of concentration in 'Centennial' beer was not significant. β-Caryophyllene and α-humulene also increased dramatically in hopped beers, the high sensory detection thresholds they possess might limit their odor contribution and therefore

their influence on hopped beers. α-Pinene, although indicated to be odor-active in hops, was below detection limit and sensory threshold in both control beer and hopped beers. Similarly, β-pinene was unlikely to impart to the flavor of finished beers by this cold dry-hopping process. Consequently, both α-pinene and β-pinene could impart few, if any, flavor to beer via dry-hopping regardless of variety. Compared with control beer, there was no significant change of limonene content in hopped beers of 'Citra' and 'Nelson Sauvin'. This was consistent with the lack of presence of limonene in three studied hop varieties (**Table 6**). In general, most terpenes possessed inefficient transfer from hop to beers (**Table 8**), and the result was consistent with that reported in previous study (Pickett et al. 1975).

Representatives of terpenoids, namely linalool, neral, geranial, geranyl acetate, nerol, geraniol and caryophyllene oxide, were investigated in the current study (**Figure 10**). Compared with control beer, significant increase of geraniol and linalool were found in all dry-hopped beers. Moreover, the highest concentration of geraniol showed up in 'Centennial' beer and the concentration of linalool was measured with highest value in 'Citra' beer, which was consistent with the varietal character of oil composition of three hop varieties (**Table 6**). Geraniol and linalool were revealed to be two of the most odor-active terpenoids in AEDA of hop study. Presenting a citrus-like pleasant aroma, geraniol could contribute positively to the hoppy aroma in beer (Peacock et al. 1981, Lam et al. 1986). Similarly, linalool, contributing a pleasant floral note, has been highly correlated with the presence of hop flavor in beer. The increases of nerol and caryophyllene oxide were significant in beers prepared with 'Centennial' and 'Citra', but not in 'Nelson Sauvin'. Geranial, neral and geranyl acetate were presented in the hop oils of 'Centennial' and 'Citra', but their concentrations were below the quantification limits in this study.

Although the determination of hop oil component concentration and understanding of their thresholds can give useful information for the aroma evaluation of beer, the influence of individual hop compounds to the overall aroma profile of beer is more difficult to determine. The aromatic contribution of some volatiles could be underestimated since additive effect of aroma compounds even at sub-threshold concentration (Guadagni et al. 1963, Hanke et al. 2010). It has been reported that some compounds could be perceived with a concentrations much lower than, even 10% of, their thresholds.

Table 7. Concentration (ppb) of hop-derived aroma compounds in beers.

Target compound	Control	Centennial	Citra	Nelson Sauvin	Threshold(ppb)
α-pinene*	<1a	<1a	<1a	<1a	2.5-62[e]
β-pinene	<1a	<1a	<1a	<1a	140[e]
myrcene*	54.9±3.8a	96.2±9.4a	262±7.1c	220±7.8b	13[d]
limonene	218±13.0b	114±6.3a	236±28.0b	210±12.0b	4-229[e]
linalool*	93.7±1.5a	157±9.7b	288±5.4c	140±0.1b	4-10[e]
β-caryophyllene*	<1a	<1a	11.3±1.0b	<1a	64-90[e]
α-humulene	<1a	2.1±0.5a	18.8±1.2b	17.2±0.6b	120[e]
neral	<1a	<1a	<1a	<1a	28-120[e]
geranial*	<1a	<1a	<1a	<1a	n/a
geranyl acetate	<1a	<1a	<1a	<1a	9-460[e]
nerol*	<1a	23.4±0.7c	15.1±0.5b	<1a	680-2200[e]
geraniol*	80.0±2.8a	194±1.8d	173±0.3c	98.6±3.8b	4-75[e]
caryophyllene oxide*	18.5±1.0ab	25.0±2.6bc	29.5±1.0c	16.1±0.1a	n/a

Mean±SE presented (n=2).
*Aroma compounds have been proved odor active from previous AEDA study.
a-c Different letters indicate significant differences.
[d] The values are shown relative to the threshold in water (Guadagni, Buttery et al. 1966).
[e] The values are shown relative to the threshold in different sources (Burdock 2001).
n/a means no available data.

Table 8. % Transfer of aroma compounds from hops to beer.

	% Transfer		
Compound	Centennial	Citra	Nelson Sauvin
α-pinene	0	0	0
β-pinene	0	0	0
myrcene	0.18%	0.28%	0.86%
limonene	0	0	0
β-caryophyllene	12.57%	22.89%	30.28%
α-humulene	0	0.21%	0
neral	0.03%	0.23%	0.14%
geranial	0	0	0
geranyl acetate	0	0	0
linalool	0	0	0
nerol	30.35%	n/a	0
geraniol	15.54%	20.07%	24.11%
caryophyllene oxide	2.11%	14.21%	0

n/a means data not available.

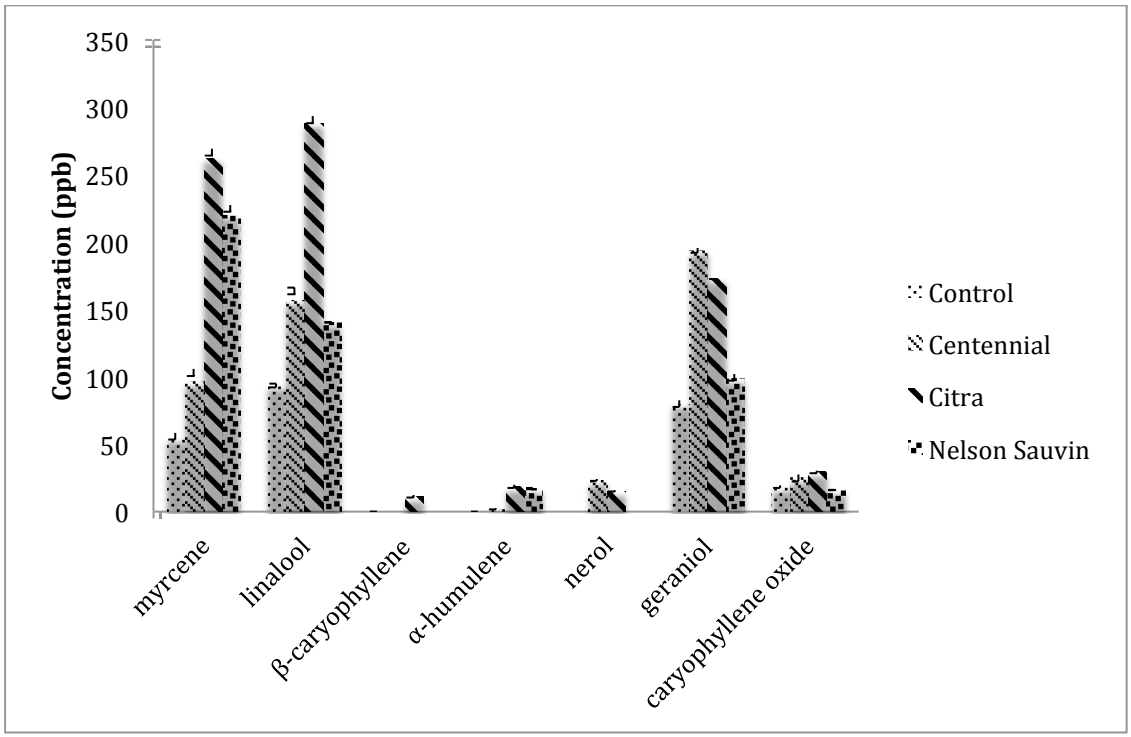

Figure 10. The composition of hop-derived aroma compounds in beers.

Chiral Analysis of Linalool

Linalool, as an important character-impact volatile in both hop and beer, has two stereoisomers, R-linalool and S-linalool. Both isomers give a floral-like aroma, however, the threshold of R-linalool in beer is much lower (2.2 ppb) compared to S-linalool (180ppb) (Kaltner and Mitter 2009). This suggests that the ratio of R/S-linalool in hops and beer is of great importance. Therefore, a further investigation of chiral distribution of linalool in hops and dry-hopped beers was conducted.

Table 9 shows an R-linalool ratio of 92-96% in diverse hop varieties, which indicates the absolute dominancy of R-linalool in hop samples. It was documented that linalool content of different hop cultivars may demonstrate a wide difference, and the R-/S-linalool ratio was depended on hop variety (Kaltner and Mitter 2009).

Compared with hops, the chiral distribution of R-linalool showed a much lower ratio in beers (**Table 9**). In the control beer, the percentage of R -linalool was 70%. However, in the 'Cascade' hop, R-linalool made up 92% of linalool. The low ratio of R-linalool in control beer was caused by the isomerization of R-linalool to S-linalool during wort boiling. The isomerization of R-linalool, to some extent, weakens the odor activity of linalool as an entirety in beer. In **Table 10**, the theoretical value of the concentration of R-linalool in beers, which was under the hypothesis of no isomerization during dry-hopping, was calculated. Compared with the theoretical value of R-linalool, the actual value of R-linalool in dry-hopped beers showed no significant difference. This indicated the negative isomerization from R-linalool to S-linalool during dry hopping process. The possible isomerization of R-linalool during hop aging and its stability in beer are of great interests and under further investigation.

Table 9. The percentage of R-linalool in hops and beers.

	Ratio of R-linalool			
Hops	Cascade	Centennial	Citra	Nelson Sauvin
	92%	93%	95%	97%
Beers	Control	Centennial	Citra	Nelson Sauvin
	70%	77%	80%	77%

Table 10. The concentration of R-linalool in beers.

	R-linalool concentration (ppb)			
	Control (Cascade)	Centennial	Citra	Nelson Sauvin
Theoretical Value	86	124	250	110
Actual Value	65	120	231	108

CHAPTER 5 CONCLUSION

Three hop varieties, ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’, were investigated in the current research. Major oil compositions of essential oils were determined and the identification of odor-active aroma compounds in three hop varieties by aroma extract dilution analysis (AEDA) was carried out. Moreover, behavior of hop-derived aroma compounds in beers dry-hopped with ‘Centennial’, ‘Citra’ and ‘Nelson Sauvin’ was investigated to further study the application of different hop cultivars in beers.

Hop oils from different varieties were demonstrated to have characteristic compositions with a similar pattern, following which the terpenes (such as myrcene, β-caryophyllene and α humulene) were responsible for majority of the essential oils. The rest were mainly terpenoids and aliphatic esters. The oil profile of diverse hop cultivars was distinct and the varietal uniqueness showed to be obvious.

Organic solvent extraction combined with solvent assisted flavor evaporation (SAFE) was proved to be an efficient approach for the extraction of volatile components (essential oil) from hop materials. Pre-fractionation by normal phase chromatography could largely simplify the hop oil and facilitate the further separation and analysis on gas chromatography (GC). Although GC-Olfactometry (GC-O) was useful for scanning the aroma spectrum of hops, AEDA was confirmed to give more detailed description of aroma compounds and their contributions to the overall aroma profile. According to AEDA, myrcene, geraniol and isovaleric acid were key aroma compounds in all of the three hop varieties, followed by S-methyl methanethiosulfonate, linalool and vanillin., S-methyl methanethiosulfonate was first identified in hops and importantly contributed to the hop aroma especially in ‘Centennial’ and ‘Citra’ hops. Besides, sulfur-containing

compounds were also proved to have an important impact on the whole aroma spectrum of hops.

Compared with control beer, dry-hopped beers revealed an increase of myrcene and α-humulene whereas the increase of β-caryophyllene was only in 'Citra' variety. Meanwhile, linalool and geraniol increased significantly in hopped beer. The investigation of the chiral distribution of linalool in hops and beers using gas chromatography coupled with chiral column indicated the prevalence of R-linalool in hops and a conversion of R-linalool to S-linalool in beer was observed in control beer when hop was added at beginning of wort boiling.

The useful information collected from this study will help the understanding of the aroma character of three varieties ('Centennial', 'Citra' and 'Nelson Sauvin') of hops and the selection of hops for dry-hopping purpose. In the future work, more effort will be made to characterize the unknown volatile components and meanwhile, the possible isomerization of R-linalool during beer aging and its stability in beer are of great interests and under further investigation.

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