THESIS

METABOLITE FINGERPRINTING OF HOPS (*HUMULUS LUPULUS*) TO TRACK CHEMICAL VARIATIONS

Submitted by

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ABSTRACT

METABOLITE FINGERPRINTING OF HOPS

(*HUMULUS LUPULUS*) TO TRACK CHEMICAL VARIATIONS

In the brewing industry, identification of quality crops that provide unique organoleptic properties to beer flavor (aroma, taste) are of critical importance. Hops represent a key ingredient in beer and are utilized to impart specific flavors. India Pale Ales (IPAs) are a popular style of "hoppy beers" in the U.S. and customer expectations for consistency, quality, and unique organoleptic properties of hops are growing. While the contribution of chemical compounds in hops (Humulus lupulus) such as alpha-acids (e.g. humulone) is well-understood, the influence of the hop metabolome (e.g. composition of hop chemical compounds) is still in the early stages of discovery. There is a gap in the knowledge regarding our understanding of chemistry variations in hops among cultivars and growing locations that impact the sensory quality. Traditional sensory evaluation relies on the ability to organize a group of unbiased and trained panelists, who are also subject to sensory fatigue, which can add to the challenge of this method. An alternative approach, ambient mass spectrometry (AMS) is an objective, intuitive, analytical tool capable of rapid chemical fingerprinting. The overall goal of this research is to develop a robust, high-throughput assay using AMS technology to evaluate hop quality that is reflective of

both cultivar and environmental variations impacting sensory. To address this goal, twelve hop samples were sourced from three different suppliers across four different farms located in Washington and Oregon over two growing seasons. The samples included three commercial cultivars, Cascade, Centennial, and Strata. The hop samples were extracted using an 80% ethanol solution and fingerprints were acquired by Direct Analysis in Real Time Mass Spectrometry (DART-MS). The resulting data were used to train predictive models and validation was performed to evaluate classification accuracy. Additionally, authentic standards of important hop compounds (hop alpha-acids, terpenes) were used to putatively annotate DART-MS signals reflective of sensory attributes. This study demonstrates the potential of this approach for rapid evaluation of hops quality and lays groundwork for further method optimization. Ultimately, implementation of this tool could have applications for quality assurance programs and for phenotyping of hops for producers and craft brewers.

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Chapter 1 Literature Review

1.1 Introduction

With the growing craft beer industry in the United States, hops (*Humulus lupulus* L.) have reached a new record of 113 million pounds produced in 2019. Hops are one of the four main ingredients of beer, and their use has greatly increased as craft brewers produce beer with enhanced aroma and taste (*e.g.,* bittering). Importantly, hops are a chemically diverse ingredient, and over 100 hop compounds have been demonstrated to influence beer flavor[1]. Specifically, hops contribute to (i) aromatics of the beer (~90 compounds) and (ii) bitterness (~10 compounds), and this chemistry can be influenced by both genetics (hop cultivar) and the environment (agricultural system)[2,3]. Because of this chemical variation, the brewing industry requires "hop quality" reports to inform brewers on the expected bitter and aroma properties of the material. However, comprehensive chemical profiling of hops is costly and low-throughput, and therefore most evaluations are performed by integrating sensory analysis. Further, sensory analysis is also low-throughput and subjective. Therefore, there is a critical need to develop rapid, high-throughput analytical chemical assays that can inform on the overall chemistry and quality of the hops.

1.2 Botanical Features of Hops and Agronomic Background

Hops are the flowering part of the hop plant (*Humulus lupulus*), a perennial vine climbing plant species that is part of the Cannabaceae family[4]. The common hop plant is dioecious with separate male and female plants, where the female plants are sought after for the flowers (*e.g.,* hop cones) that they produce[5]. The hop cone flower secretes a sticky resinous precipitate from lupulin glands that contains aromatic organoleptic flavonoids, phenols, and other compounds of

interest. Hop cones have historically been used for their medicinal properties to treat ailments such as anxiety and insomnia, but more recently they are prominently used in commercial beer production[5,6].

Female plants are propagated from cuttings and are almost exclusively produced for the commercial brewing industry while male plants are kept separate and are produced mainly to generate genetic material for breeding new varieties[5,7–9]. Hop plants can grow as tall as 20 feet (6 meters) in a single season and the plant consists of perennial roots (*e.g.,* rhizomes), vines (*e.g.,* bines), green leaves and the flowering hop cones as seen in figure 1.1[10,11]. Hop cones from the female plants grow from small buddings (*e.g.,* burrs) on the bine and into pinecone-shaped flowers. The outer green, papery, scale-like petals (*e.g.,* bracts) of the flower protect the inner scales (*e.g.,* bractoles) and glandular trichomes (*e.g.,* lupulin glands) at the base of the petals (Figure 1.1)[7]. Lupulin glands secrete a yellowish resin, otherwise known as lupulin, which contains essential oils and polyphenol. Lupulin has antimicrobial, preservative qualities as well as organoleptic characteristics of interest for use in beer[6,8,12].

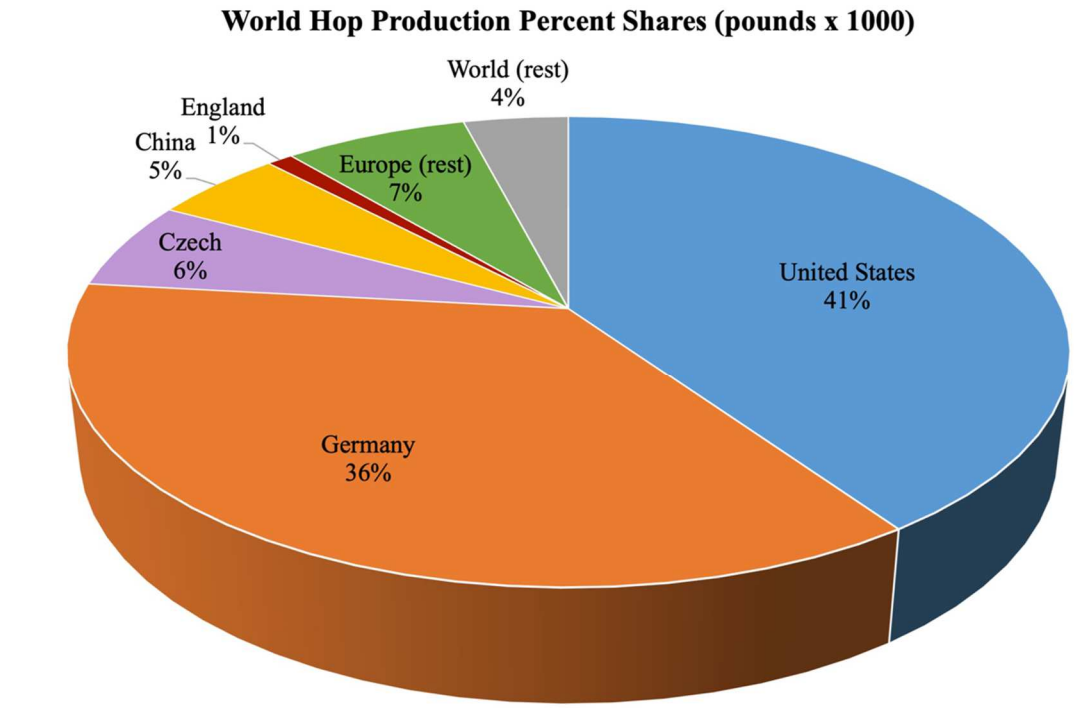
Figure 1.1. Female hop plant and hop cone. A: The bine of the plant supports tri-lobed green leaves and flowering hop cones. B: The outer green petal-like bracts protect the inner flower where the yellow lupulin resin is secreted by glands at the base of the bracts.

1.3 Current Hop Production

Historical records suggest that the hop plant originated in temperate regions of Asia, possibly China, then migrated outwards east and west to Europe and North America[6,12]. As a result of this migration, five different taxonomic varieties of hops emerged from *H. lupulus L.* and are classified based on their respective geographical location and morphological characteristics[6,12]. Over the last century there has been a dramatic shift in where hops are produced relative to where they originated. For example, hop production in European countries (such as Germany) has significantly decreased while at the same time hop production has increased in the United States (Figure 1.2)[13,14].

The cultivation of hops has resulted in genetic variation that is characterized by a wide range of uniquely different phenotypes[12]. Today, roughly 97% of all hops produced are utilized

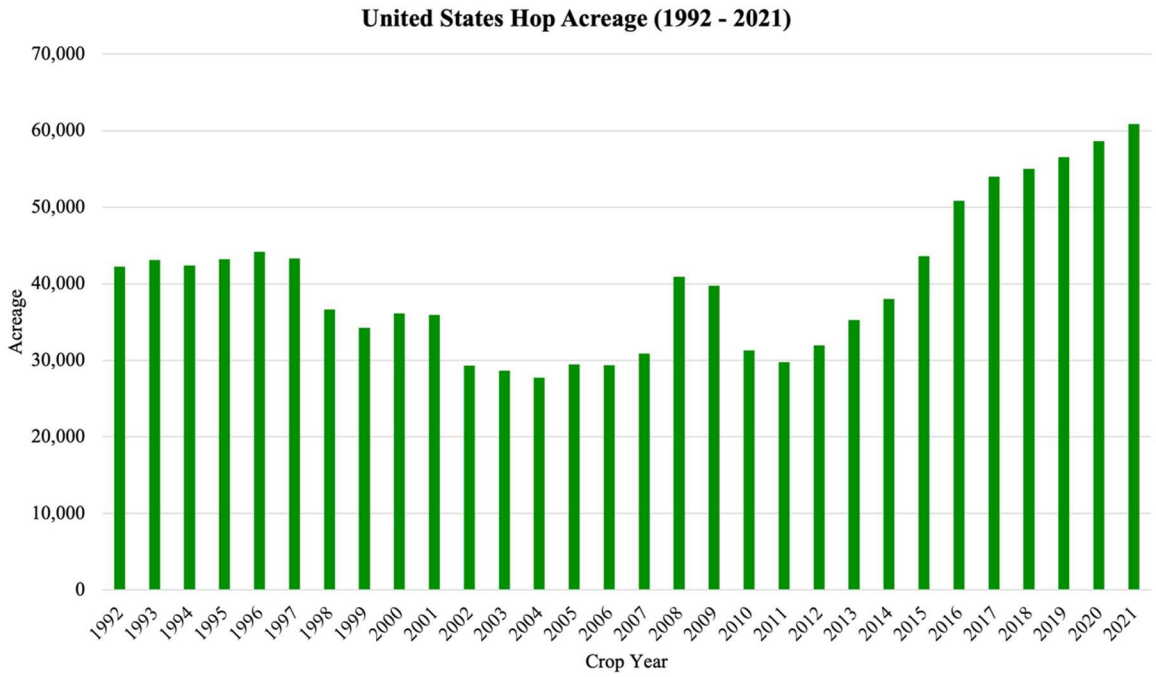
Figure 1.2. World Hop Production. The United States and Germany are lead producers in the global hop industry. Europe (rest) includes Austria, Belgium, France, Poland, Romania, Serbia, Slovakia, Slovenia, Spain, and Ukraine. World (rest) includes Australia, New Zealand, Russia, South Africa, Argentina, Canada, and Japan.



Source: IHGC Economic Commission November 2020 Report. Prepared by HGA

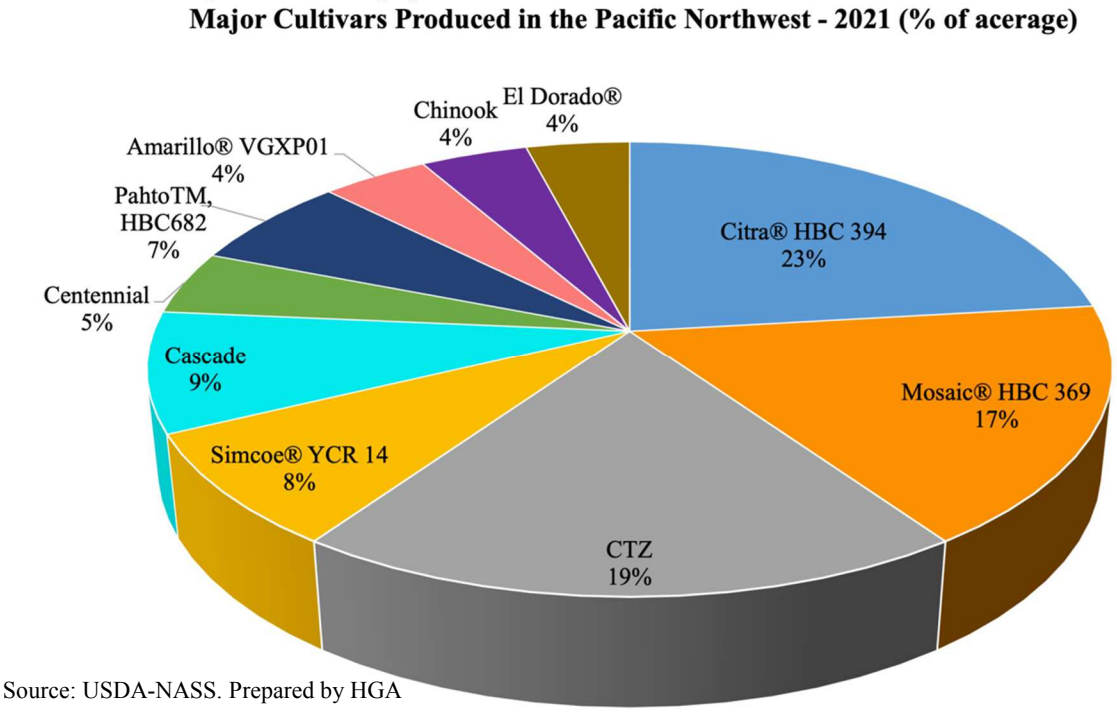
by the brewing industry; thus growers are specifically catering to the sensory phenotypes relevant for beer production[9]. The United States is one of the leading hop producers in the world, combined with Germany it accounts for 75-80% of the worlds produced hops[5,9,15]. In the United States, hop production continues to increase (Figure 1.3) and has reached a record of 113 million pounds in 2019[16]. Additionally, there is vast variation in different hop cultivars currently available from United States producers with the aromatic Citra® cultivar topping the list (in terms of most acreage) in 2021 (Figure 1.4) [17]. In the United States, the major region for hop production is the Pacific Northwest (PNW) including specifically the states Washington, Oregon and Idaho[13].

Figure 1.3. Annual United States Hop Acreage (1992-2021). Acreage dedicated to hop production in the United States over the last 10 years has steadily grown.



Source: USDA-NASS and HGA Hop Acreage Reports

Figure 1.4. Major Hop Varieties. Leading hop cultivars produced in the United States, Citra® currently is the most popular.



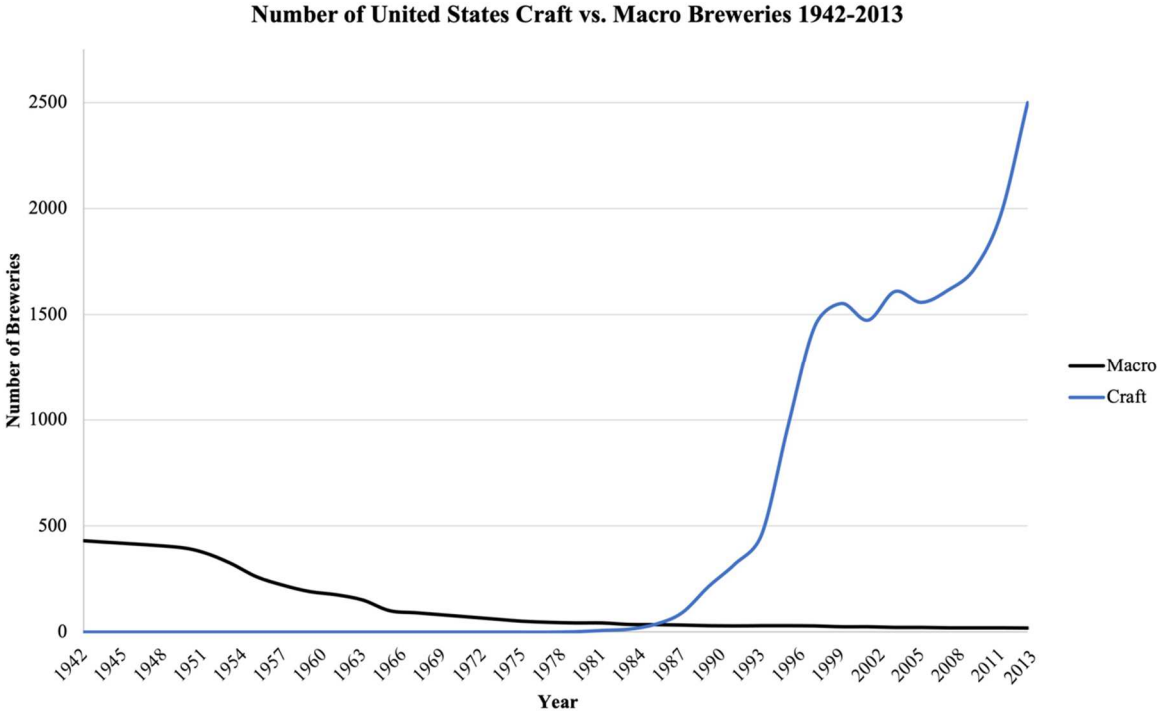
Source: USDA-NASS. Prepared by HGA

1.4 The Evolution of American Beer

A major consideration when growing and selecting hops is the intended usage in the brewing process. Historically, prior to its extensive use in beer production, hops and hop shoots were first considered as a medicinal and nutritional plant as cited by Plinius the Elder[18]. Germany was the first region to notably expand on the usage of hops in beer production and incorporate the ingredient in a more systemic way, leading to hopped beers dominating the Northern-European market in 1500 and the development of *Reinheitsgebot*, a law stipulating that hops are one of the four main ingredients in beer[11]. Exploration overseas led to the use of hops not only as a flavoring agent to beer, but also for the added benefit of its antimicrobial preservative properties allowing for beer to be stored for longer periods thus the India Pale Ale (IPA) was born[6,9,11].

The development of the American beer industry wasn't straightforward, enduring interruptions in the nineteenth and twentieth centuries from two World Wars and Prohibition leading to a significant decrease in number of breweries (Figure 1.5)[19–22]. In fact there was a decrease in the number of breweries from 2300 to only 60 between 1880 and 1960[4]. Coming out of World War II, the rebirth of all sectors of society and marketplace globalization changed the landscape of American beer. The focus changed to optimize on low-cost mass production of

Figure 1.5. Number of United States Craft and Macro Breweries. In the United States from 1947 to 2013 craft breweries exploded in number versus the few macro brewers that remained.



Source: Elizinga-Tremblay-Tremblay; Brewers Association National Sales and Production Data

simply flavored beers designed to appeal to all drinkers, "a one-size-fits-all" Standard American Lager[23]. The combined effect of a reduced number of breweries with the rebirth of beer production post World War II led to the dominance of the United States beer industry by a few large breweries that are now defined as macro breweries[24].

The most notable attribute of the United States beer industry is its continual change in concentration and landscape[24]. Specifically, the United States has seen a steady decrease in the number of macro breweries coupled with a drastic increase in craft breweries starting in the mid-1980s (Figure 1.5). Craft breweries are defined as "small and independent, producing 6 million barrels of beer or less" by the Brewers Association[25]. Furthermore, the craft brewery segment, otherwise known as microbreweries, are distinct from their macro brewery brethren in the fact that they brew a variety of styles – ales, stouts, even lagers. Additionally, craft brewers adhere to brewing traditions that do not include the use of adjuncts or artificial ingredients and instead focus on an artisanal craftsmanship with an emphasis on quality raw materials that are locally sourced and supportive of their local community[21,23].

1.5 Utilization of Hops in Beer

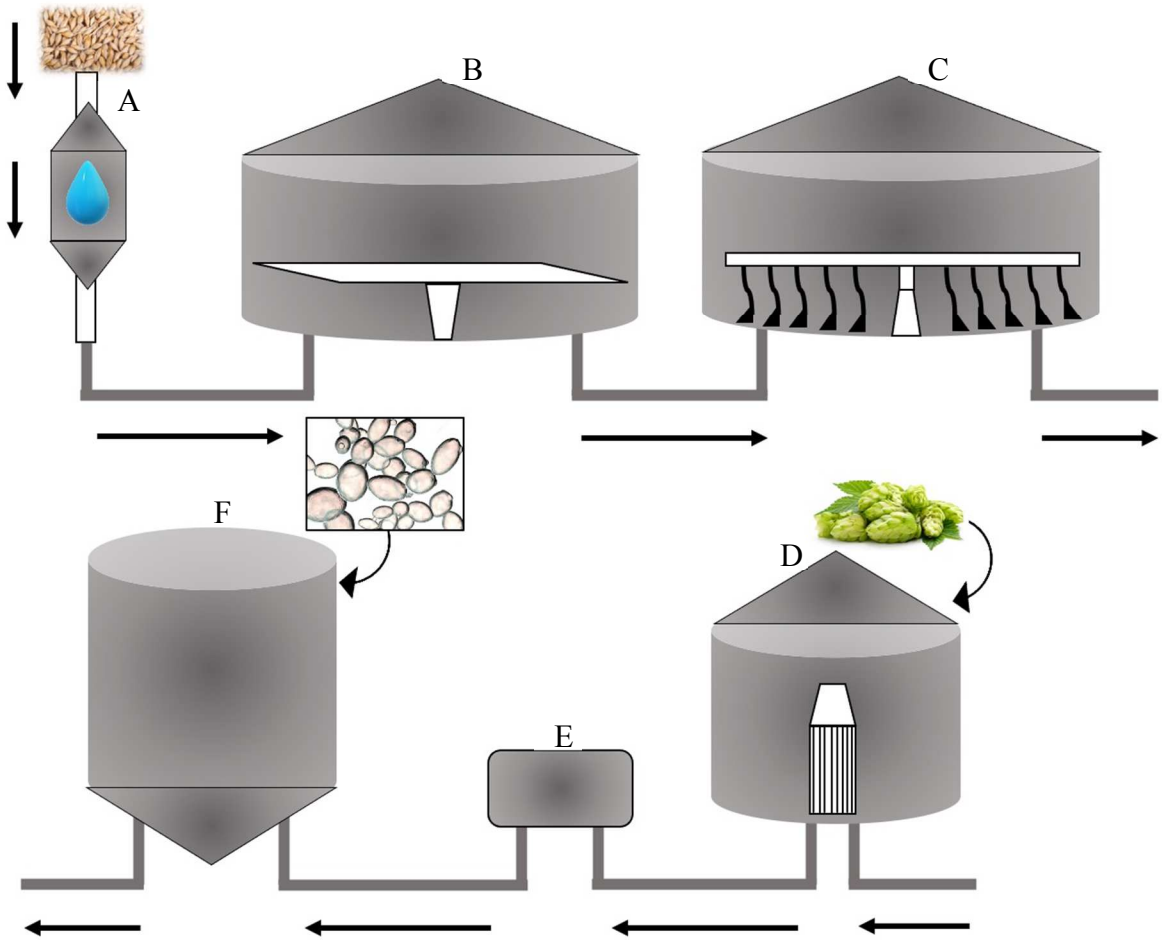
During the reign of the sessionable lagers made by macro breweries, hop usage dropped from 0.56 to 0.49 pounds in 1943 with mixed reactions from consumers throughout the years until reaching an all-time low in 2004[23]. The microbrewery movement starting the "craft beer revolution" began in 1965 with Anchor Steam Brewing on the west coast[21,23,24]. Starting from the initial introduction of the IPA style of beer when it came to the United States with the first settlers to the revival of the style in the 21st century, hop utilization has become a critical ingredient of the brewing industry.

Historically, hops were utilized for imparting bittering characteristics to balance the malt sweetness, however, craft brewers in modern times have shifted their focus to highly aromatic and flavorful hops[8,26]. With the emergence of consumer demand for a variety of beer styles, including highly hopped ales, there has been a significant increase in hop usage which has driven increases in hop production observed within the last decade (Figure 1.3). Additionally, breeding

of new hop cultivars has evolved to stay relevant to the ever changing beer industry trends and we now see classifications of hops as alpha, for bittering characteristics, aromatic, and dual-purpose[8]. The combined effect of a rise in hop usage and the development of novel hop cultivars for various flavor phenotypes is that brewers now have the tools to meet customer demand for complex, highly aromatic, hoppy beers (Figure 1.4)[27]. For example, depending on the intent of the beer style, a brewer may utilize different hops; alpha hops for high bitterness styles such as Imperial IPA, or aromatic hops for lower bitterness Pale Ale styles with high hop flavors of citrus and tropical fruity[28].

With consideration of the different flavor impacts of hops, brewers can also manipulate how and where hops are used in the brewing process. A general overview of the brewing process is visualized in Figure 1.6. As previously mentioned, there are four primary ingredients of beer; water, malted barley, hops and yeast[29]. Hydrolyzed sugar is extracted from malted barley creating wort, the base ingredient for alcohol fermentation. The wort is boiled and flavoring additions, such as hops, are added. Following the boil, the wort is cooled through a heat exchanger prior to introducing yeast, the microorganism responsible for alcohol production. Alcohol production by yeast through consumption of the hydrolyzed sugars, otherwise known as fermentation, takes from 7 days to multiple weeks depending on the strain of yeast used. When fermentation reaches terminal, the beer is clarified and then packaged into bottles, cans, and kegs. Hops are introduced into the brewing process at various stages, the boiling step to impart bitterness or later during fermentation as a cooled hop addition to extract more aromatics (*e.g.,* dry-hopping)[4,30].

Figure 1.6. The Brewing Process. A: Crushed malted barley is hydrolyzed with water. B: The mash tun homogenized with mixer. C: The wort is separated from the grain and separation is facilitated with steel rakes that cut deep channels in the grain bed. D: Wort is boiled in the kettle where hops are added. E: A heat exchanger cools down the hot wort. F: Cool wort and yeast are added to the fermenter and in 7-14 days depending on yeast strain the sugar in the wort is converted to alcohol.



1.6 Hops Chemistry

The yellow lupulin oils from the center of the hop cones contains flavoring compounds important to the brewing industry, including hydrophobic bittering resins and essential oils[4,26,31]. The phytochemical composition of lupulin is very complex. It has been suggested to contain over 1000 different compounds, although currently only 450 have been identified through analytical chemical assessment[4,27,32]. There are many different combinations of hop chemical compounds

that result in the unique flavor phenotype of a given cultivar. This unique hop chemical fingerprint, is determined by cultivar (genetic) and environmental differences (*e.g.,* farming practices, climate, soil, harvest maturity, age of plant, etc.), although there is a gap in our understanding of the full extent of environmental impacts[26]. Characterization of the chemical composition of hops is important to enable prediction of how it will perform in beer production[33]. The chemical composition of hops broadly includes bittering acids, volatile aromatic compounds, and polyphenols[34,35]. The lupulin fraction of hops can be broken into two categories; hop resins and hop oils[36]. Additionally, hop polyphenols contained in the green plant matter (*e.g.,* bracts) are important to consider since they can impact the final flavor and appearance of beer depending on hop variety and utilization[37].

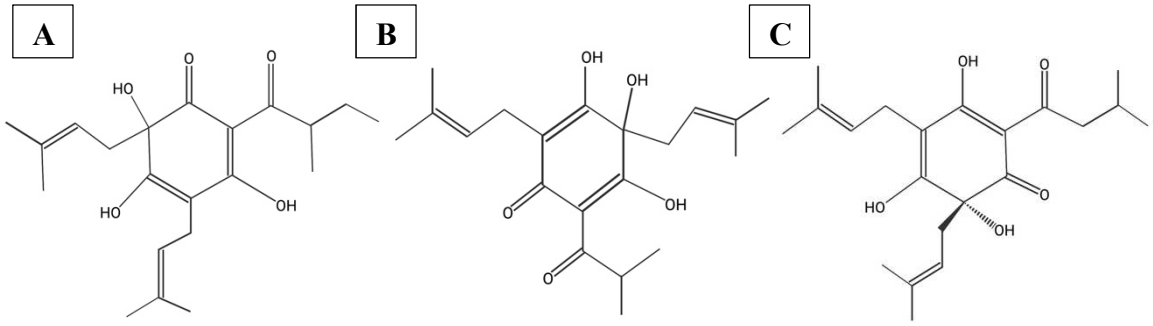
1.6.1 Hop Resins and Bittering Acids

A well-known flavor characteristic of hops is bitterness, a gastronomic balance to the sweetness of the malted barley in beer[38]. Total hop resins include both hard and soft resins; hard resins are comprised of prenylflavonoids and xanthohumol[26]. Bittering acids, the main component for contributing bitter flavors, include both alpha- and beta- acids that are found in the soft resin fraction of lupulin[36].

Alpha-acids found in hop soft resins are direct precursors to the primary bittering compounds perceived in beer, iso-alpha-acids[39]. The precursor alpha-acids are thermally converted into isomerized alpha-acids during the boiling of the wort in the brewing process (Figure 1.6D); this key step is where the brewer can manipulate alpha-acid hop content for the intended bitterness amount in the final beer product[29,39]. Alpha-acid content is dependent on hop variety and is, on average, 10 wt% although some specifically bred high alpha hops can contain as much as 19 wt%. Thus, the percentage of alpha-acid composition is an important part of the

commercial hop value[36]. There are three main alpha-acid metabolites (Figure 1.7); humulone, cohumulone, and adhumulone[39]. Since overall alpha-acid content varies depending on hop cultivar, so does the different concentrations and ratios of the alpha-acid metabolites, all of which influences the overall final bitterness perceived in the beer[36,39]. Variations in alpha-acid metabolite concentrations and total percentage are a crucial part of the overall hop chemical fingerprint.

Figure 1.7. Alpha-acids humulone, cohumulone and adhumulone. Important alpha-acids that primarily contribute to the bittering flavoring in beer: A: humulone, B: cohumulone, and C: adhumulone



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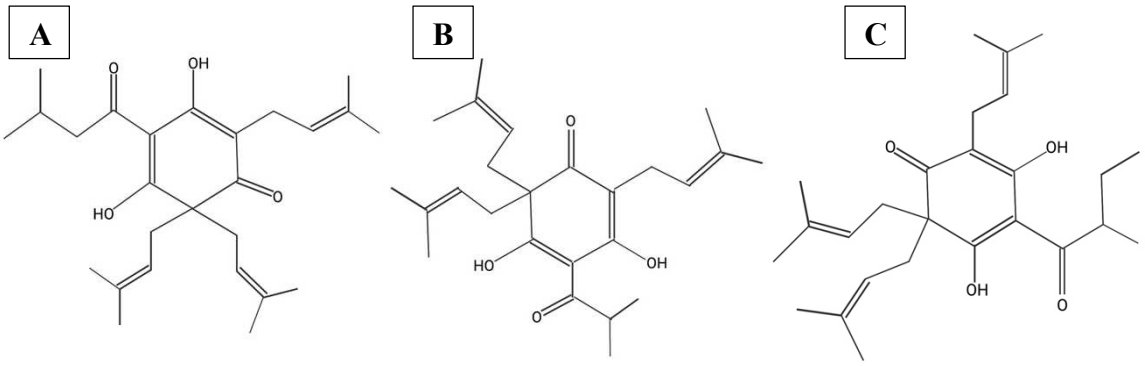
Hop beta-acids are secondary in terms of importance to bitterness flavors. Due to the more basic and hydrophobic nature of beta-acids, they are typically less abundant as they are less soluble in aqueous conditions even at boiling temperatures compared to alpha-acids[36,39]. Figure 1.8 shows the beta-acid analougues; lupulone, colupulone, and adlupulone[39].

Figure 1.8. Beta-acids lupulone, colupulone, and adlupulone. Beta-acids are secondary metabolites that impart bittering flavors: A: lupulone, B: colupulone, and C: adlupulone.



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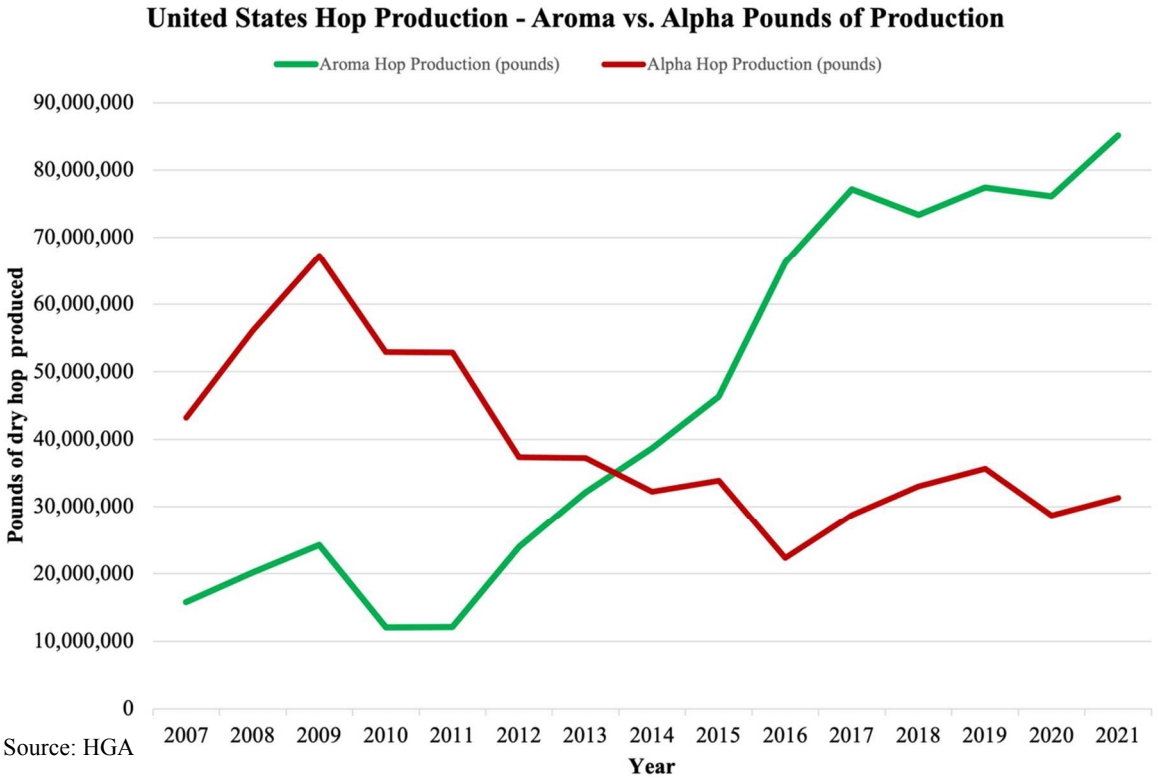
1.6.2 Volatile Aromatic Compounds

Volatile aromatic compounds of hops found in the essential oil fraction of lupulin are important to the brewing industry and include terpenes, terpenoids, oxygenated compounds, and thiols[26]. Depending on the compounds present, concentrations will vary due to their chemical nature (*e.g.,* solubilities, interacting side chains, acidity, etc.). The resulting composition of compounds will have synergistic effects that are crucial in beer products to produce harmonious "hoppy" aromas and flavors[4]. With the increase in global hop production over the last two decades, the ratio of aromatic to alpha hop varieties has shifted from 44:56 to 61:39 respectively (Figure 1.9)[17,26]. This represents an important swing in the types of hops being produced that has been driven by craft brewers shifting their focus to more aromatic and flavorful hop attributes. Additionally, as brewers focus on more aromatic and flavorful beers, hopping rates have increase two- to three-fold[26].

Essential oils comprise 0.5 to 3.0% (v/w) of the whole hop cone and within this fraction there are estimated to be over 1000 terpenes, creating a very heterogeneous and complex mixture where terpenes and their oxygenated analogues are the most abundant of the hop

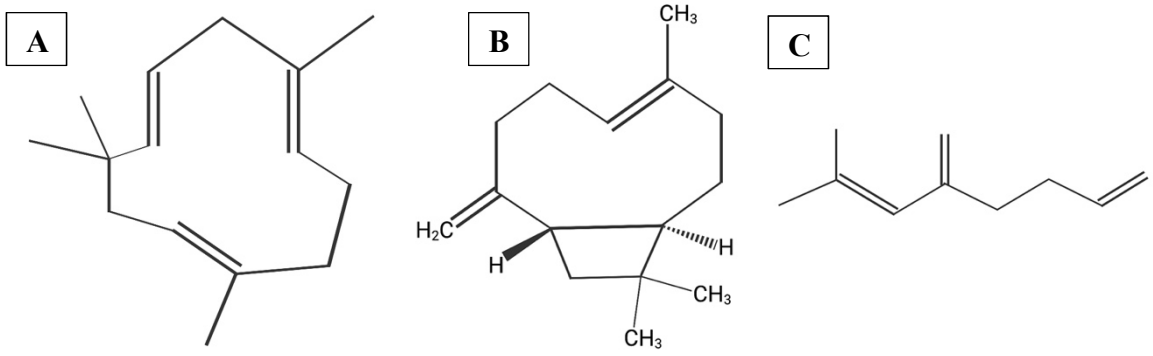
Figure 1.9. United States Hop Production – Aroma versus Alpha Pounds of Dry Hops Produced 2007 to 2021. Before 2014 alpha hops dominated the market. Then as IPAs began to lead in sales as the leading beer style, aroma hops became more popular. Today, more aroma hops are produced than alpha hops.



Source: HGA

phytochemicals[3,40]. Terpenes are hydrocarbons and include monoterpenes (50-70% of total essential oil composition) and sesquiterpenes (30-50% of total essential oil composition)[9,26,40]. The monoterpene β-myrcene and the sesquiterpenes α-humulene and β-caryophyllene (Figure 1.10) impart hop aromatics and flavors of *woody* and *herbal* and are the primary terpenes (by abundance) in most hop cultivars[31,41]. Although most of these compounds are lost during the boiling step of the brewing process due to their high volatility, they may act as precursor metabolites to aroma-active oxygenated analogues that play a significant role in the final beer[36,39,42,43]. Characterization of these terpene compounds helps hop growers and breeders to determine the complexity of the terpene profile as this has been shown to differ between

Figure 1.10. Terpenes α-humulene, β-caryophyllene, and β-myrcene. Terpenes are primary and secondary metabolites important to hop sensory: A: α-humulene, B: β-caryophyllene, and C: β-myrcene.



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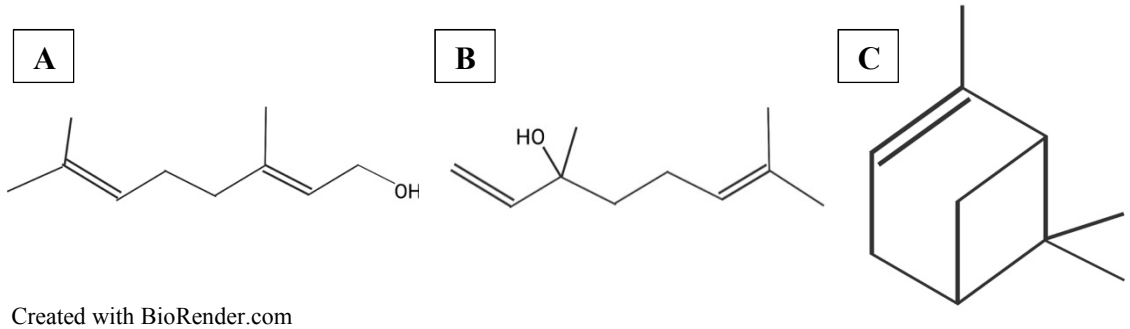
cultivars[27]. For example, bittering hops have higher β-myrcene content while more aromatic varieties showcase a higher α-humulene content[43].

Additionally, terpene profile composition can be monitored during crucial hop cone development to signal ripeness[2,39,42]. The specific terpene compounds that are important markers of cone development to predict the hops performance in beer is still widely debated among hop growers and breeders. For example, recent research by Lafontaine at Oregon State University suggests that geraniol is an important monoterpene alcohol to monitor due to its positive correlation with approaching harvest date and its relation to Cascade, a known late-picking hop variety[2]. Other potentially important terpenes as markers for harvest include linalool, limonene, α- and β-pinene, and citronellol (Figure 1.11). These compounds range from *floral* and *citrus* to *pine* characteristics, especially if utilized in late hopping and cold extraction additions[4,26].

Other volatile compounds include terpenoids, esters, aldehydes, ketones, and alcohols which can impart a variety of aromatics. Specifically, terpenoids, esters, alcohols and ketones impart *sweet fruity* and *floral* characteristics while aldehydes provide more *green* and *grassy* attributes[4,40]. These other volatile compounds are attributed to the synergistic overall "hoppy"

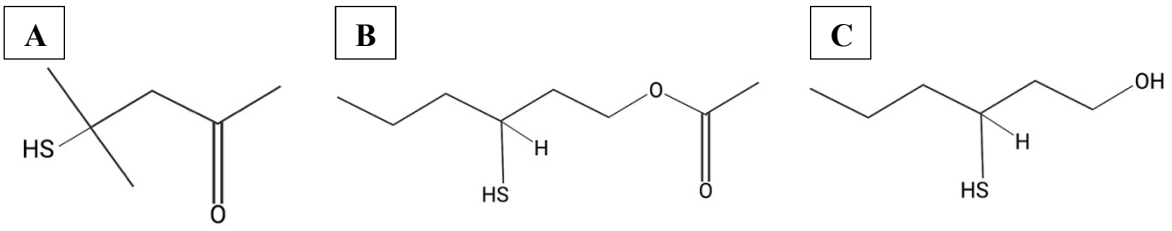
characters desired and highlighted in most IPA and Pale Ale style beers[39]. Although more soluble compared to the precursor terpenes, these other compounds are still loss due to their volatile nature and thus brewers will optimize extraction of these characteristics by utilizing late hop or cold extraction additions[26,39]. Other important volatile compounds seen in Figure 1.11 include terpenoids, terpenoid alcohols and other terpenes that impart a range of flavors from *floral* geraniol to *fruity* linalool and *piney* α-pinene to name a few[9,27].

Figure 1.11. Examples of other important volatile compounds including terpenoids, terpenoid alcohols, and other terpenes such as geraniol, linalool, and α-pinene. A: geraniol and B: linalool imparts *floral* and *fruity* characteristics. C: α-pinene contributes *pine* aromas and flavors. These compounds may be metabolite indicators for when hops are ready for harvest.



Polyfunctional thiols are sulfur containing compounds that comprise <1% of the total essential oil fraction. Despite their low presence in both beer and hops compared to other compounds, they have low sensory thresholds thus imparting intense aromatics and flavors even at low concentrations[26,27]. The main thiol compounds of interest in hops and hopped beers as depicted in Figure 1.12 are 4-methyl-4-mercaptopentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA), and 3-mercapto-1-hexanol (3MH)[2]. Previous research by Gros et. al. and Chenot et. al. demonstrated that these polyfunctional thiols exist in both a free and bound form. In hops, thiols are typically present in a bound form and are thought to be released by yeast through biotransformation during fermentation[44,45]. Other research by Lafontaine et. al suggests that

Figure 1.12. Thiol compounds 4-methyl-4-mercaptopentan-2-one (4MMP), 3-mercaptohexyl acetate (3MHA), and 3-mercapto-1-hexanol (3MH). A: 4-methyl-4-mercaptopentan-2-one (4MMP), B: 3-mercaptohexyl acetate (3MHA), and C: 3-mercapto-1-hexanol (3MH) produce tropical and fruity flavors including *passion fruit*, *black current*, and *guava*.



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perhaps hop-derived enzymes also play a role in freeing bound thiols[2,46]. They showed that free thiols and their bound precursors help brewers determine how to utilize the hops in the brewing process and that understanding their composition can help to predict hop cone maturity since environment (*e.g.,* agronomic practices) can impact thiol development. More research is needed on thiol compounds and related precursors, their importance in relation to hop cone development as well as the role yeast and hop-derived enzymes plays in converting thiols from bound precursors to free in hopped beer. In addition to thiol precursors, other compound precursors may be present in a bound form found in both lupulin and the bract green plant matter of hop cones[26,46].

1.6.3 Polyphenols

Polyphenols are mostly present in the green plant matter of the hop cones (bracts) although they may also be present in low quantities in the lupulin[47]. Hop polyphenols can impact beer flavor, appearance, astringency (a mouthfeel that is drying and puckering in characteristic) and stability over time[37]. Polyphenols additionally provide the antimicrobial preservative quality important to hops[6,9]. While less considered for their contribution to flavors and aromatics of

hopped beers, polyphenols are an important consideration, especially when utilizing cold hop extraction methods during the brewing process in order to avoid their negative impacts.

1.7 Environmental Impact on Hops

It is well-known that hop cultivar (*e.g.,* genetics) will greatly influence the resulting chemical phenotype, however it is speculated that agronomic factors (*e.g.,* environment) may also be influential[32,42]. This is supported by previous studies in wine grapes (a relatable perennial crop) that demonstrates the crucial role of environment in the development of distinct flavors. This concept, known as terrior, is capitalized on in the marketing of wine from different regions[48]. However, there remains a gap in knowledge regarding how the environment, or terrior, impacts hops. Recent research of terpene biosynthesis and secondary reactions rendering oxidative metabolites suggests that both genetics (cultivar) and agronomic factors (*e.g.,* growing environment, geography, agronomic practices, etc.) are important in determining hop essential oil composition[2,3]. For example, hexyl glucosides (known aroma compounds that imparts a *grassy* note) have been shown to be increased in hops growing in environments with higher incidence of herbivore insects[49]. Hop breeding programs may take advantage of hop parent plants (male and female hop germplasms) for new variety crosses that have demonstrated responses (or resistance) to environmental influences that impact the resulting hop phenotype like the above example.

The hop community is just beginning to understand the environmental factors contributing to desired sensory and quality attributes. There remains a need for further investigation to better understand the synergistic combination of agronomy with biotransformation pathways that are impacting hop chemistry. However, this goal is further complicated by the continued impacts of climate change that are resulting in increasing global

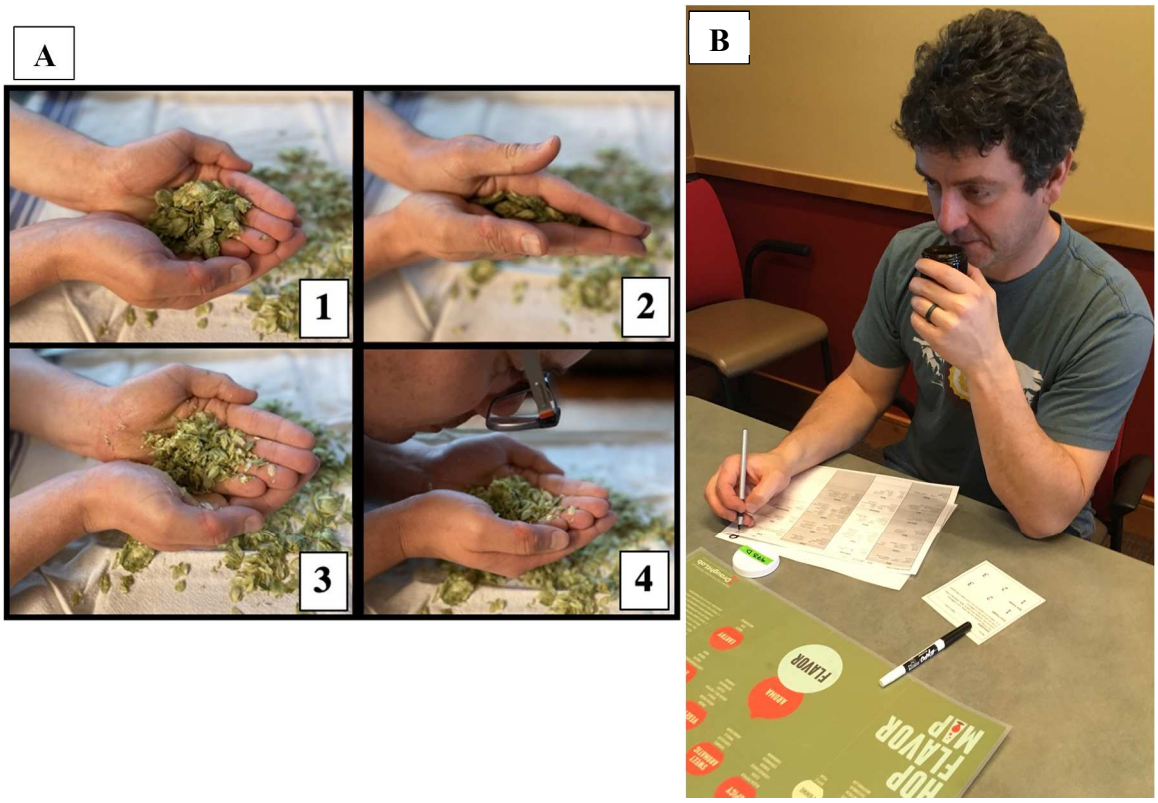
temperatures and unpredictable weather. Since 1990, the National Oceanic and Atmospheric Administration (NOAA) has been a part of monitoring the United States National Climate Assessment every four years as mandated by the Global Change Research Act of 1990[50]. In March 2019, NOAA fire weather research showed that increased temperatures and drought is directly correlated with an increased risk and extent (*e.g.,* duration, acreage) of wildfires in the United States[51]. One of the impacts of increased wildfire activities is smoke exposure to nearby crops which can lead to "smoke taint" – a phenomenon that has been well documented in wine grapes. A recent report indicated that smoke taint resulted in a $400 million loss in the Australian wine industry due to unsellable product. [52,53]. Researchers are just beginning to explore the impact of smoke on hops, starting with the development of validated methods for the detection of known smoke metabolites[54]. There remains an extensive gap in our understanding of how smoke manifests in hop aromas and flavors as well as processes to mitigate the effects. The example of smoke taint illustrates the importance of environment on hop chemistry and highlights the need for continued research efforts to better understand all aspects of agronomic impact on hop phenotype.

1.8 Hop Sensory Analysis

Many brewers typically select their hops as part of an annual contract with a producer. The hop selection is performed during harvest using manual sensory evaluation[9]. There are some standardized methods for hop sensory analysis including the Hop Tea and Hop Grind methods that were developed by the Association of Brewing Chemists (ASBC) and can be found online at the ASBC Methods of Analysis[55,56]. These methods are designed to be performed by trained panelists in a controlled environment and represent an extension of the traditional hand rub method typically utilized during hop selection.[57] The hand rub method involves breaking up

whole flower or pelletized hops between the palms, exposing the aromatic hop essential oil compounds found in the lupulin glands (Figure 1.13A). The Hop Grind and Hop Tea methods include a mechanical homogenization of the hops followed by sensory of either the dry ground hop material (Hop Grind) or of a cold-water extraction (Hop Tea). While the Hop Grind method

Figure 1.13. Hand rub VS Hop Grind method for sensory analysis of hops. A: Hand rub method: Hops are rubbed between the palms to break up lupulin glands and expose aromatics. B: Hop Grind method: Hops are previously ground and presented to panelists typically in blind settings (coded).



is a more standardized alternative to the hand rub method (Figure 1.13 B), the Hop Tea method is intended to simulate the effect of dry hopping. However, despite the development of these new standardized methods, the hand rub method remains the method of choice during hop selection (Figure 1.13A), a crucial moment in determining which hop cultivars and products from which hop growers are purchased.

While the quality of sensory is the primary output of interest, sensory analysis of hops and highly hopped beers can be fatiguing, limiting the number of samples analyzed by panelists at a given time[55,56]. Additionally, ensuring a non-biased, objective sensory panel can be difficult and requires training[58,59]. Not only is it important to train the panel on both desirable hop attributes and off-flavors, but it is also important to create a language or lexicon with associated aroma scaling that is agreed on by all participating panelists[60]. Finally, predicting how hops will perform especially considering variations in chemical compounds that may interact (*e.g.,* the synergistic effect *floral*, *fruity* linalool has on other aromatic compounds such as *woody*, *spicy* β-myrcene) to choosing to brew a single hop versus different combinations of multiple varieties remains a challenge[61]. High quality aroma and flavor is the primary end goal for products produced using hops. Therefore, while sensory analysis will likely remain an important part of quality assurance of hops and associated products, it's use is limited by time and labor while also being constrained to processing small sample sets at one time and by ensuring objective data collection.

1.9 Hop Chemical Analysis

The analysis of hop chemistry has advanced over the past two decades and instrumentation such as liquid (LC) and gas chromatography (GC) coupled with mass spectrometry (MS) offer new opportunities for understanding this complex chemical matrix[3]. While sensory analysis continues to be a critical method used by hop growers, breeders, and brewers; analytical analysis is becoming an important tool contributing to the assessment of hop quality. Additionally, as mentioned previously, our continued climate uncertainty with increased global temperatures and unpredictable weather events (*e.g.,* wildfires) further motivates the need

to understand the chemical profile of hops and the impact of environmental and agronomic practices on the phenotype.

The primary focus for hop chemistry analysis is first establishing validated methods for detection of small molecules that contribute to the hops flavor and aroma profiles. Metabolomics is defined as the study of small molecules (small volatile/non-volatile molecules < 1500 da) in a biological system[62,63]. There are two approaches to this type of chemical analysis: metabolite profiling and metabolite fingerprinting. Metabolite profiling is slow and comprehensive, separating out each individual compound with chromatography (*e.g.,* GC or LC) prior to detection, annotation, and relative quantification (Figure 1.14B and 1.15). Metabolite fingerprinting is rapid and non-specific, foregoing the separation step (*e.g.,* high sensitivity) to enable detection of a selection of compounds (likely the most abundant) that is representative of the sample (*e.g.,* fingerprint) (Figure 1.14A). Metabolite profiling can be used to understand how specific small molecules impact and contribute to specific biochemical pathways and is often referred to as "discovery". The metabolite fingerprint is focused less on the identification of any specific compound and instead on the generation of a chemical pattern or fingerprint that can be correlated with a sample phenotype.

Figure 1.14. Metabolite fingerprinting VS metabolite profiling. A: Metabolite fingerprinting does not use separation while B: metabolite profiling relies on separation of metabolites prior to detection and quantification.

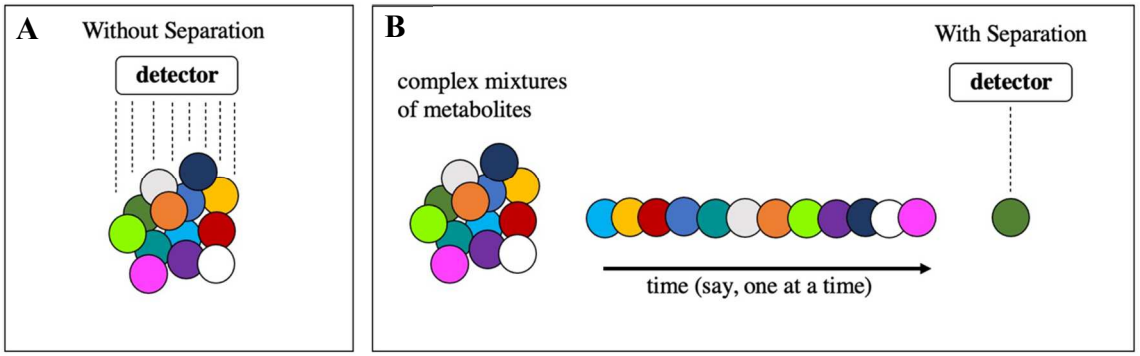


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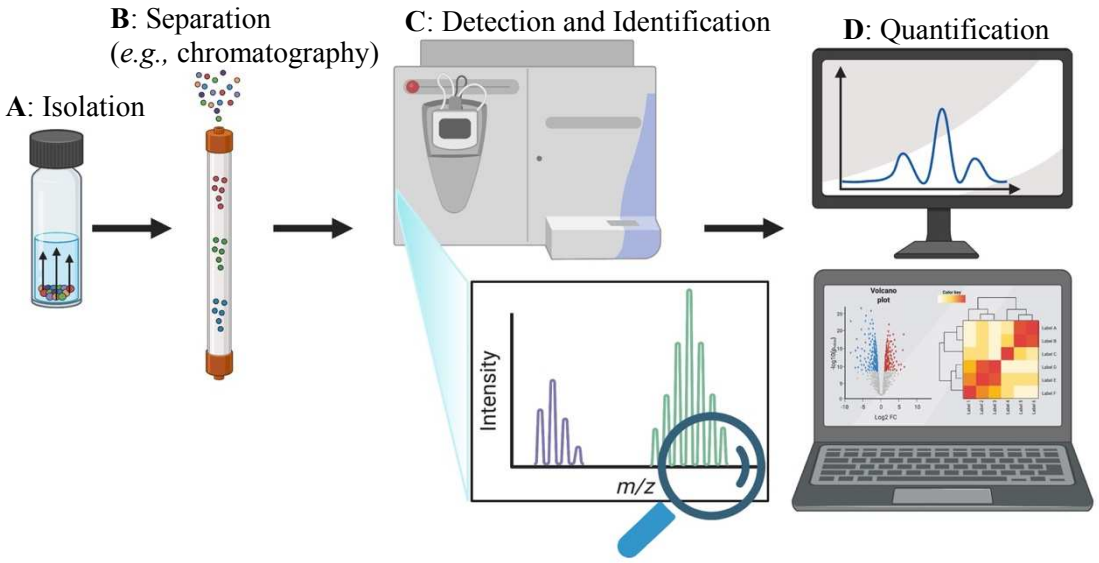
Figure 1.15. Workflow for metabolite profiling. Metabolite profiling begins with A: isolation of metabolites of interest, B: separation utilizing chromatography, followed by C: detection, identification and finally D: quantification of metabolites[3].



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1.9.1. Sample Preparation Methods

Prior to analytical chemical analysis it is often necessary to separate non-volatile and volatile components to improve detection. Sample preparation can occur for both hops and hopped beer, however liquid hopped beers often require less to no sample preparation. For non-volatile analysis, typically extraction with a solvent like methanol or iso-octane is performed followed by a minor pH adjustment to isolate hydrophobic compounds[47,64]. Important non-volatile compounds in hops include bittering acids, polyphenols, and other prenylated flavonoids. For both hops and hopped beer analysis, isolation of these non-volatile compounds of interest from other compounds found in beer (*e.g.,* yeast, malt metabolites, etc.) is helpful for detection and quantification.

For volatile compounds, the most utilized method for metabolite extraction from hops is steam distillation. While this method offers a relatively simple approach where ground hops are

boiled for several hours (up to 3 hours or longer), this can take time and the thermal activity of boiling can produce artifacts[2,3]. Other extraction methods for both non-volatiles (bittering acids) and volatiles (terpenes, thiols, etc.) include Stir Bar Sorptive Extraction (SBSE)[65,66]. Stir bars coated with polydimethyl siloxane (PDMS) are subjected to thermal desorption in capped vials allowing trapped metabolites to be released to detection instrumentation such as GC-MS [67]. It is important to note that SBSE methods are limited to the analysis of liquids. Other extraction methods for volatile compounds include utilizing solvents of varying polarities such as dichloromethane (DCM) and hexane (HEX) to extract compounds from the hop essential oils [43]. Using different solvents can be useful in isolating hop compounds of interest depending on the chemical nature of those metabolites (*e.g.,* DCM is efficient in isolating β-myrcene). Other nonpolar solvents that have been previously used include ethanol and methanol[32,68]. One final extraction method utilizes super critical carbon dioxide to extract hop essential oils including terpenes and terpenoids[69,70]. Similar to the steam distillation method, temperature should be considered as a possible factor that can lead to metabolite artifacts. These other extraction methods may be ideal for isolating volatile compounds; however some extraction methods require more time and labor for sample preparation and extraction[69,71]. Additionally, it is important to consider what solvents are used for extraction as some solvents may be more hazardous than others.

For most of the chemical analysis methods involving chromatography, a general workflow is followed involving isolation of metabolites of interest, separation (chromatography), detection and identification (mass spectrometry), and finally quantification (Figure 1.15)[3]. It is important to keep in mind that analysis of hop metabolites in beer has additional challenges due to dilution and biochemical transformations from both the inclusion of malt metabolites and the

biotransformation yeast contributes, thus creating multiple analytical targets[3]. Regardless, if the metabolites are isolated from hops alone or hopped beers, they are hidden in complex matrices that can make detection and thus identification and quantification difficult.

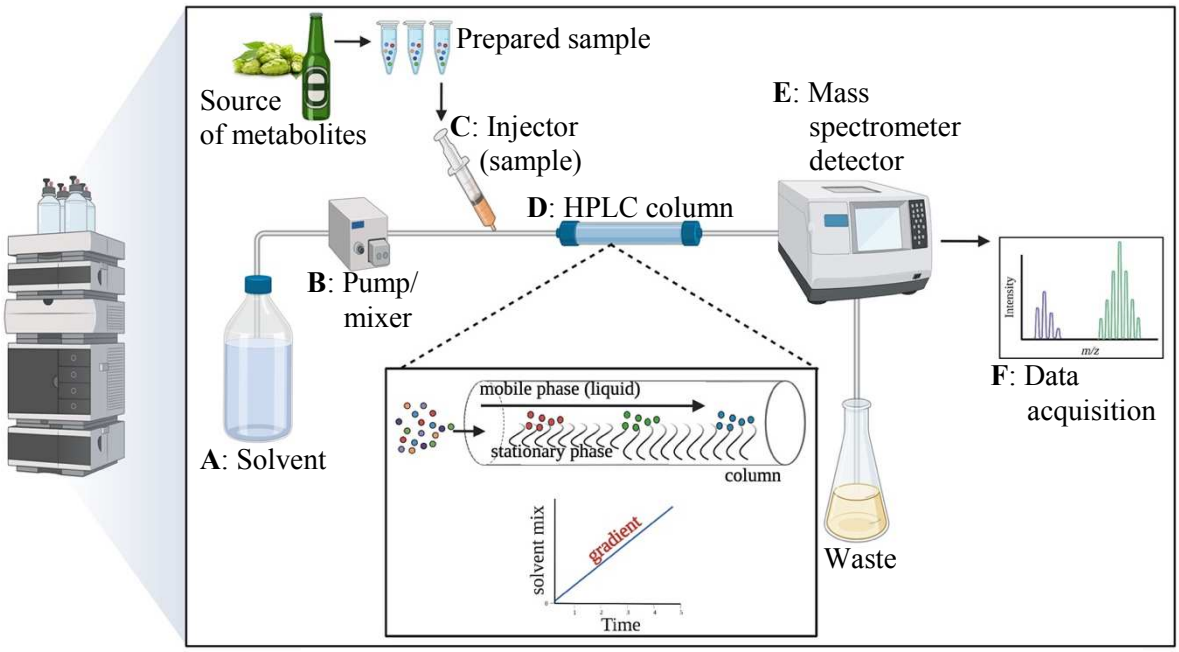
1.9.2. Metabolite Profiling of Non-volatiles

The analysis of non-volatiles can be achieved through more than one method. The most commonly utilized method for hop analysis following suggested ASBC methods of analysis is focused on the quantitation of isomerized bittering metabolites in beer (terpenes, alpha-acids, beta-acids, phenols, etc.) using ultraviolet-visible spectrophotometry (UV-VIS-SPEC) monitoring absorbance at 275nm[72]. Craft breweries typically advertise not only the percent alcohol content of the beer, but also the bitterness units (BU) that result from this assay. The results of this analysis represent a total of all compounds that absorb at 275nm, including some aromatic volatile compounds like terpenes. This method has multiple advantages including being relatively low cost, rapid (requiring only an hour of labor), and high-throughput[40,72]. However, in order to isolate and detect individual bittering metabolites, more advanced methods involving separation are required.

High-Performance Liquid Chromatography (HPLC) is an ideal method for analyzing non-volatile hop metabolites (Figure 1.16) such as iso-alpha (isomerized alpha-acids), alpha- and beta- bittering acids (Figures 1.7 and 1.8)[64]. ASBC has developed standardized HPLC methods for hop producers to analyze hops, hop pellets, and hop extracts[73,74]. Other important compounds that can be analyzed using this technique include phenols and prenylflavonoids that impact attributes in beer such as foam stability and medicinal or preservative properties[6,9,75,76]. Separation by HPLC is based on compound polarity manipulated using differing solvent gradients, enabling detection and quantification of individual metabolites (Figure 1.16D, E and

F). HPLC methods are convenient for measuring non-volatile compounds especially if already solubilized in aqueous form, such as in the beer matrix. Additionally, the selectivity of HPLC methods (*e.g.,* temperature control, solvent composition, and light exposure) is beneficial. Bittering acids can oxidize quicker with increased temperatures and isomerized compounds are light sensitive making HPLC methods advantageous. HPLC however is limited to requiring sample preparation and time for testing and data analysis[64].

Figure 1.16. HPLC-MS for analysis of non-volatile metabolites. A: Different solvents are mixed B: with a pump or mixer. C: The sample is injected and as metabolites go through D: the column they are separated based on chemical nature such as polarity (as solvent mixture gradients change over time). E: Separated metabolites then are detected by the mass spectrometer collecting F: data in the form of chemical profiles[3].



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1.9.3. Metabolite Profiling of Volatiles

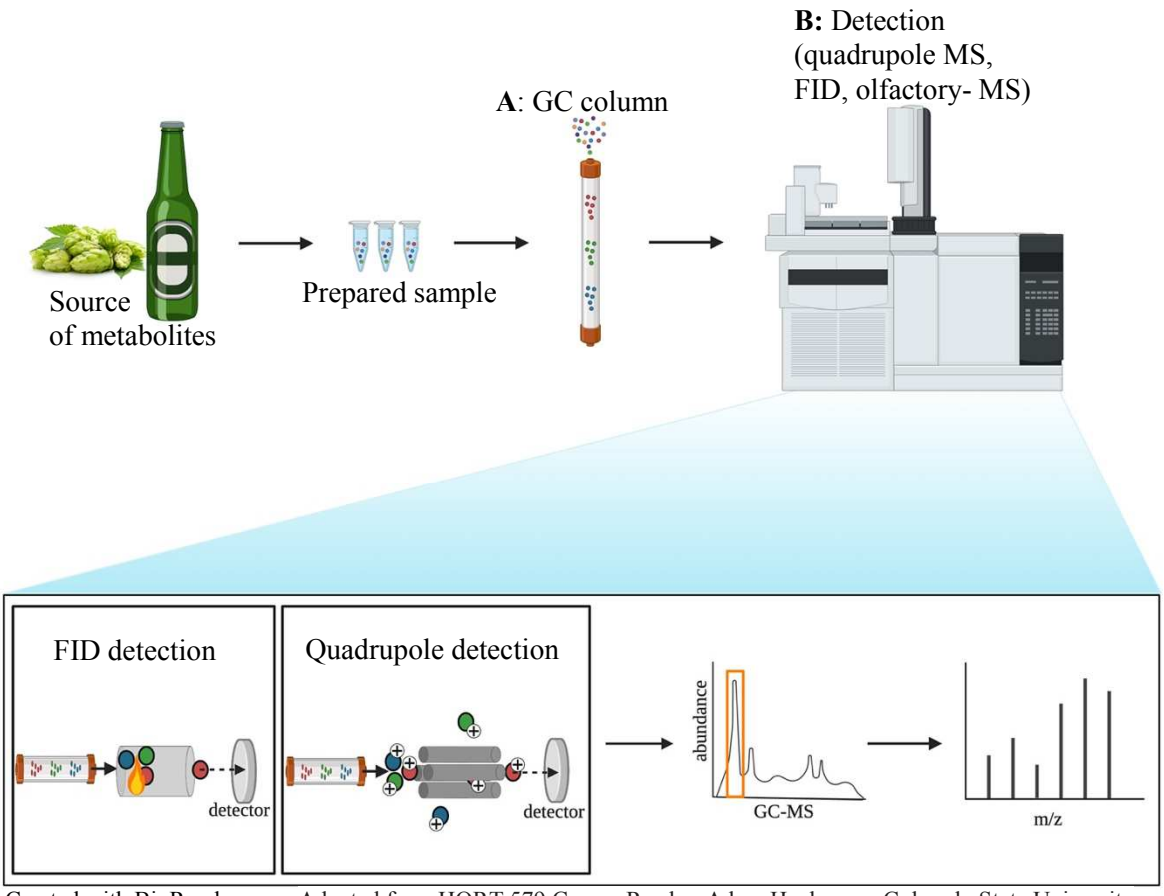
Chemical characterization of essential oils, mainly focusing on the volatile compounds, by GC is well documented[2,3,31,45,77]. Separation by GC is an important step prior to detection, such as by MS, to separate out hop metabolites that either are structurally similar or have the same mass weight. While the utilization of GC coupled with analytical detector instrumentation

platforms (*e.g.,* MS) have proven capable of identifying and quantifying major hop metabolites (terpenes, terpenoids, etc.), validated detection and quantification of minor chemical compounds (thiols, aldehydes, fatty acids) remains a challenge[3].

GC in general is one of the most common methods of separation prior to detection and analysis of volatile hop essential oil compounds (Figure 1.17), [40]. Analysis of hop aromatic compounds (terpenes and terpenoids, including β-myrcene, β-caryophyllene and α-humulene) by GC began in the 1960-1970s when the instrument was first introduced[78]. Various detection platforms can be coupled to GC including quadrupole mass spectrometer detector (GC-MS), flame ionization detection (GC-FID), and olfactory coupled mass spectrometer (GC-O-MS)[2,31,32,40]. ASBC standardized methods for analyzing hop essential oils via GC-FID is widely used by hop producers[79]. GC-O-MS studies on hops currently is expanding since it offers additional sensory analysis with the olfactory adaptor[69]. The inclusion of olfactory, used by several researchers, allows for the determining which metabolites impart which sensory attributes (such as monoterpenes linalool and geraniol imparting *floral* and *fruity* aromas)[80,81]. Connecting important sensory attributes to compounds in hops is important for hop growers, breeders, and brewers to optimize agronomy, processing, and brewing practices. While all these platforms vary in how the metabolites are detected, the analysis process follows the same general workflow (Figure 1.15 and Figure 1.17); 1) metabolite isolation, 2) separation via chromatography, 3) detection, 4) identification by MS or FID, and 5) quantification.

Another important platform emerging in volatile metabolite profiling is headspace solid phase microextraction gas chromatography mass spectrometry (HS/SPME-GS-MS). This method offers a gentle process (requiring no sample preparation) that harnesses thermodynamic equilibrium in a closed system (*e.g.,* vial) to isolate volatile compounds[3]. HS/SPME-GS-MS is

Figure 1.17. GC separation for analysis of volatile metabolites. Prepared samples are injected into A: a GC column that separates metabolites based on volatility. B: Separated metabolites then flow into the detector unit (quadrupole MS, FID, olfactory-MS)[3].



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beneficial due to its increased sensitivity allowing for detection of metabolites at low concentrations (compounds that GC-MS struggles to detect), but the analysis is slow and requires longer run times. Although some of these odorant compounds are at low concentrations compared to other hop essential oil metabolites, they have an intense impact on the sensory output and were detected for the first time using HS/SPME-GC-MS (*e.g.,* thiol compounds - 4MMP, 3MHA, 3MH)[32].

Although GC is ideal for separating compounds prior to detection (via MS or FID) of aromatic hop compounds, there remains limitations to the methods. The cost for sample

preparation, instrument run time, and materials can be expensive, especially when coupling specialized technology platforms[3]. Run times can be up to 30 minutes or longer, in particular when increasing sensitivity such as for HS/SPME-GC-MS[32]. Additionally, there can be false identifications, misinterpretations, and incorrect quantifications when metabolites co-elute or if there are artifacts created by the method or instrumentation (*e.g.,* heat, temperature)[3]. Finally, GC is not amendable to separating large non-polar compounds that also have the potential to contaminate the column if samples are not properly prepared[40].

1.9.4. Methods of Metabolite Fingerprinting

Metabolite fingerprinting methods sometimes utilize sample preparation as described above, however some chemical fingerprinting technologies, specifically ambient mass spectrometry (AMS), can analyze "real world" biological samples with little to no sample preparation[82,83]. Recall that metabolite fingerprinting differs from metabolite profiling creating a whole pattern that is detected at once (*e.g.,* rapid) instead of utilizing a separation method (*e.g.,* chromatography) to detect compounds one at a time (Figure 1.14). Unlike conventional mass spectrometry (MS) technologies which typically requires more time for labor intensive sample preparation and run time, AMS is much quicker with rapid ionization technologies and operate under ambient conditions[84,85].

AMS technologies have recently seen rapid growth over the past decade and ~30 different AMS techniques are commercially available, some of which have been integrated into food industries to meet demands for regulations, certification programs, and fraudulent countermeasures[83,86]. Previous research has demonstrated the utility of AMS in applications for analysis of animal products (*e.g.,* meat, beef lard, beef tallow), herbs (*e.g.,* mulberry leaves, oregano, herbal medicines), beer, coffee, and honey to name a few[83,87–90]. These methods have

the potential to be tools for authentication and rapid screening to be used by hop breeders, hop growers, and craft brewers.

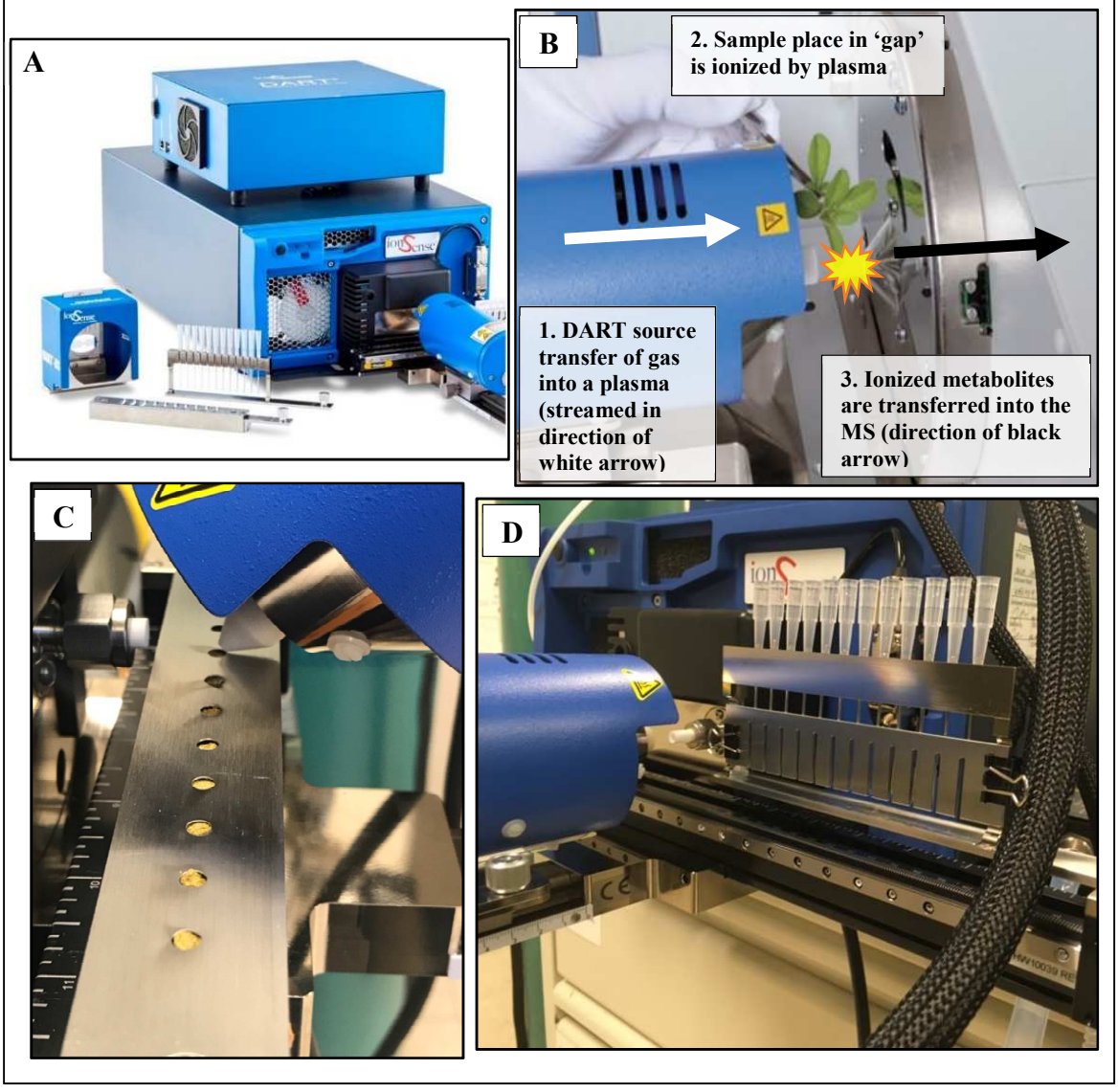
Direct Analysis in Real Time Mass Spectrometry Metabolite Fingerprinting

Direct analysis in Real Time Mass Spectrometry (DART-MS) is an AMS technology that is capable of rapid chemical fingerprinting of biological samples[86]. Additionally, DART-MS requires little to no sample preparation and in some instances the biological sample can be analyzed as is (Figure 1.18B and C). DART-MS has been applied in the agriculture, pharmaceutical, food and beverage industries for a variety of applications such as fast screening, authentication, detection of contaminations and fingerprinting both volatiles and nonvolatile metabolites of interest[89,91–95].

DART-MS (Figure 1.18A) is a benchtop mass spectrometer that operates under ambient conditions[96]. Both solid and liquid samples can be analyzed by DART-MS (Figure 1.18C and D). For example it has been used for the analysis of olive oil, honey, and beer and solid samples such as coffee beans, plant material, and solid medicines[88,89,92,93,97]. The sample is exposed to ionizing plasma and ionized metabolites then flow into the mass spectrometer (Figure 1.18B)[98]. DART-MS then produces an instantaneous chemical fingerprint of the sample. This technology is ideal for its rapid analysis, ability to operate in open air conditions for ease of use, requiring little to no prior sample preparation, and its versatility to analyze both solid and liquid samples.

Previous research has demonstrated that DART-MS has the potential for rapid metabolite fingerprinting that can be used to train a predictive model to discriminate samples and to accurately predict the classifications of unknowns[93,99]. For example, one study demonstrated the use of DART-MS to discriminate samples of monofloral honey based on geographical origin with high prediction ability ranging from 89.2% to 98.4% depending on the model[94]. Another

Figure 1.18. Overview of DART-MS. A: DART-MS is a portable, benchtop system[94]. B: The DART source (blue cylinder) emits a plasma into the MS, the sample is placed in the gap between the DART and the MS, and ionized metabolites are transferred into the MS[94]. DART is amendable to both liquid and solid samples, C: solid hop T90 pellet samples and D: liquid hop extracts on stainless-steel needles.



study demonstrated the use of DART-MS to classify olive oil based on the detection of specific polyphenols [91]. DART-MS is an ideal technology for the analysis of hops, hop extract, and beer because it is capable of training a predictive model to differentiate samples based on

classifications (*e.g.,* cultivar, growing environment, etc.) and authentic standards can be used to validate metabolites important to hops.

Previous use of DART-MS with beer and hops is limited but has been attempted. For example, DART-MS analysis of beer was successful at discriminating different beer brand samples (95% accuracy) and was able to detect beta-acids and their aging analogues[92,100].. Additionally, when compared to HPLC technologies, DART-MS was validated for detecting beta-acids and their associated oxidized beta-acids and could detect decomposition of beta acids overtime when hops were stored at ambient temperatures[101]. Further evaluation of DART-MS for the analysis of hops is important because this rapid, objective technology could be an ideal addition to quality assurance and control (QA/QC) programs for hop growers and brewers, as well as being a selection screening tool for hop breeders.

Fourier Transform Infrared Spectroscopy Metabolite Fingerprinting

Another high-throughput, rapid technology that has been considered to support food quality assurance programs, as a tool to countermeasure food adulteration, and to discriminate geographical and botanical origin is Fourier Transform Infrared Spectroscopy (FTIR). Similar to DART-MS, FTIR is capable of instantaneously producing chemical "fingerprints" that represent spectrochemical patterns of the sample being tested[102]. The non-invasive, non-destructive technology of FTIR allows for sample discrimination even though the sensitivity and specificity are less as compared to MS [103]. Previous research on olive oil and honey demonstrated that FTIR was capable of training a model to discriminate samples based on botanical origin as successfully as other platforms (*e.g.,* GC-MS)[104].

FTIR has been utilized in applications for QA/QC measures and to combat food fraud for animal products, oregano, olives, and beer[105–108]. Additionally, previous research in criminal

forensics (illegal drug use), pharmaceuticals, and food industries (*e.g.,* oil adulteration) has demonstrated the use of FTIR to train a predictive models for discrimination of samples and classification of unknowns[103,109]. Limited research has been completed on in-process monitoring of beer by FTIR for sugar analysis, but to my knowledge FTIR has not been evaluated for the analysis of hops or hopped beers[105]. FTIR offers unique capabilities and advantages for the analysis of hops that may offer this as an alternative rapid method of analysis compared to conventional MS technologies (*e.g.,* GC-MS, HPLC, etc.).

In the following work I evaluated the potential of DART-MS and FTIR as tools for characterization of hop metabolites with the goal of enabling an objective, high-throughput screening method. I hypothesized that chemical fingerprints of hops detected by DART-MS and FTIR can be used to train a predictive model to predict hop quality. I tested this hypothesis through the following objectives: 1) determined optimal extraction and analytical settings for DART-MS, 2) evaluated collected chemical fingerprints from DART-MS and FTIR to train predictive models to classify hops, and 3) validated that the chemical fingerprints were reflective of known quality and sensory attributes import to hops.

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Chapter 2 DART-MS Method Development

2. Introduction

According to the Brewers Association (BA), the Craft Brewing industry contributed approximately $76.2 billion to the United States economy in 2017 and more than 500,000 jobs[1]. Hops (*Humulus lupulus)* are a huge part of the industry and a key flavor and aroma ingredient in many styles made today. Craft brewers now use 40-50% of all hops produced domestically[2]. Hops are a global commodity and production in the United States has significantly increased over the last decade[3,4]. However, the increasing hop usage per barrel of beer is driving up prices for consumers and also resulting in higher expectations for consistent quality and organoleptic properties. India pale ale (IPA), which is a broad category of the many styles of "hoppy" beers created to elicit "high hop aroma…with a moderate to assertive hop bitterness", is the leading style in the craft brewing industry[5,6]. Hop growers and craft brewers are tasked with maintaining consistent, intensely "hoppy" and flavorful hop products and hop flavored fermented beverages using established quality assurance and control (QA/QC) programs often depending heavily on sensory analysis.

Characterization of hop metabolites (small volatile/non-volatile molecules < 1500 da) such as hydrocarbons (*e.g.,* essential oils such as linalool, geraniol, and others), terpenes (*e.g.,* monoterpenes, β-myrcene, and others), and organic acids (*e.g.,* alpha-, beta-, and isomerized acids) will lead to a better understanding of the roles these compounds play in important brewing chemistry reactions[7,8]. Examples of areas in the hop growing and brewing industries that would directly benefit from rapid chemical analysis tools include existing in-process QA/QC methods (*e.g.,* hop selection tools, rapid in-field hop testing methods) and process developments (*e.g.,*

new cultivars, hop products and beer styles) to improve quality of hop breeding, growing, harvesting, processing (*e.g.,* drying) programs, and utilization in the brewing process.

Recently there has been increased development and application of ambient mass spectrometry systems (AMS), novel technologies that are capable of rapid, objective chemical characterization of various mediums. AMS can generate results faster (*e.g.,* instantaneous), a distinct advantage as compared to conventional mass spectrometry (MS) platforms that are typically coupled with time intensive liquid or gas chromatography (LC and GC)[9] separations. AMS has been utilized in various food industries to meet demands for regulations, certification programs, and fraudulent countermeasures[10]. Direct Analysis in Real-Time Mass Spectrometry (DART-MS) is a specific type of AMS that has been used for analysis of foods such as animal products (*e.g.,* meat, beef lard, beef tallow), herbs (*e.g.,* mulberry leaves, oregano, herbal medicines), beer, coffee, and honey to name a few, for authentication against food fraud and origin recognition[11–14].

One of the benefits of DART-MS is its ability to test a variety of mediums ranging from solids to liquids with minimal to no sample preparation. The versatility of DART-MS as an analytical tool makes it an ideal candidate for most food industries. In this research I have focused on the evaluation of DART-MS as a tool for high-throughput characterization of hops including both hydrolyzed hop compounds in liquid extract samples and solid processed hops as solid pellets. Prior to evaluation of this tool to answer our research questions it was necessary to perform method development and optimization to ensure high quality and reproducible results.

2.1 Hop Extractions and Analytical Settings Method Development

DART-MS is capable of testing solid hop samples in the form of pellets (such as T-90 pellets); however, it was observed that the dry whole flower plant material would easily clog the

inlet port of the MS during analysis of these samples (Figure 2.1A). For the following work, whole flower hops were sourced and therefore, phase one of the method development process focused on the use of extractions. I hypothesized that: 1) extractions would provide a physically homogenous sample and 2) different types of solvents would extract different types of compounds from the hops, thus allowing the discrimination to focus on chemicals that are important to hop sensory and quality. Overall, 6 extraction methods were tested with two hop varieties for analysis on DART-MS (Figure 2.1B):

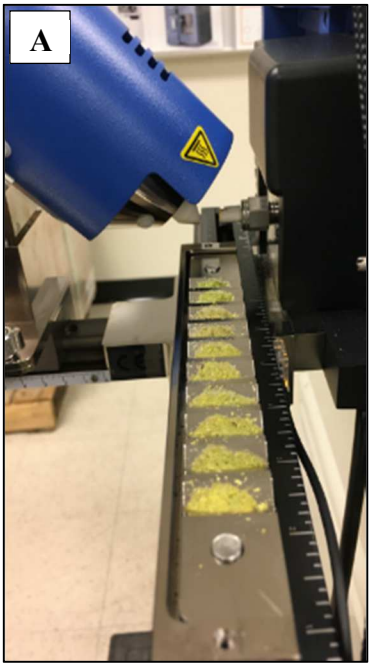
- Method A – 80/20 methanol/water
- Method B – 95/5 methanol/water
- Method C – 80/20 ethanol/water
- Method D – 95/5 ethanol/water
- Method E – 3/2/1 MTBE/methanol/water
- Method F – 3/2/1 MTBE/methanol/water

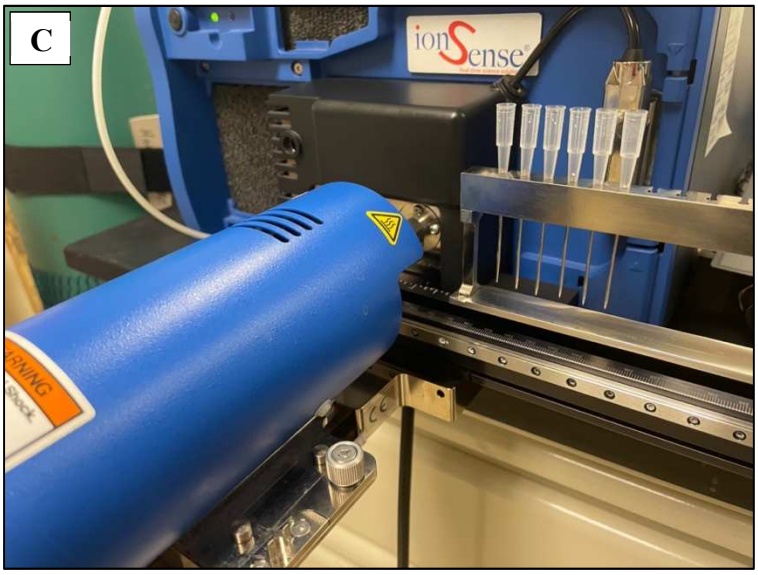
A simple, quick hop extraction utilizing solvents that are easier to handle, cheaper, and accessible (such as methanol and ethanol) would be most ideal for quality programs in the hop and brewing industries. The above simple extractions were primarily based on previous literature from the Research Center for Brewing and Food Quality in Munich Germany that has applied simple extractions for headspace-GC-MS[15]. Other studies also support simple ethanol and methanol extractions for hops analysis[16]. In addition to hop extraction method development and validation, DART-MS also required optimization of specific analytical settings such as railway and autosampler speed, temperature, and ionization mode settings.

2.1.1 Railway and Autosampler Settings

The DART-MS operates using a stainless steel autosampler to hold a certain number of samples (up to 12 samples per test run depending on the autosampler holder). The autosampler moves right to left as each sample is "scanned", being exposed to the ionizing plasma flowing into the mass spectrometer (MS). Different adapters allow for samples of different physical state (*e.g.,* solid or liquid) to be tested. Examples of adapters include a stainless-steel grooved metal plate for small particulate samples (such as ground hops) and a stainless-steel 12-Dip-IT® adapter that holds glass or stainless-steel needles that have been dipped in liquid samples (such as hop extraction samples) (Figure 2.1A and C). For each autosampler holder, additional

Figure 2.1. Example extractions for DART-MS method. A: DART sampling of ground whole flower hops quickly clogged the inlet. B: Example of extractions of hop samples display varying color properties. C: Custom autosampler to enable sampling of up to 12 extracts in a single run. Pictured is a half run comprised of 6 stainless steel needles.

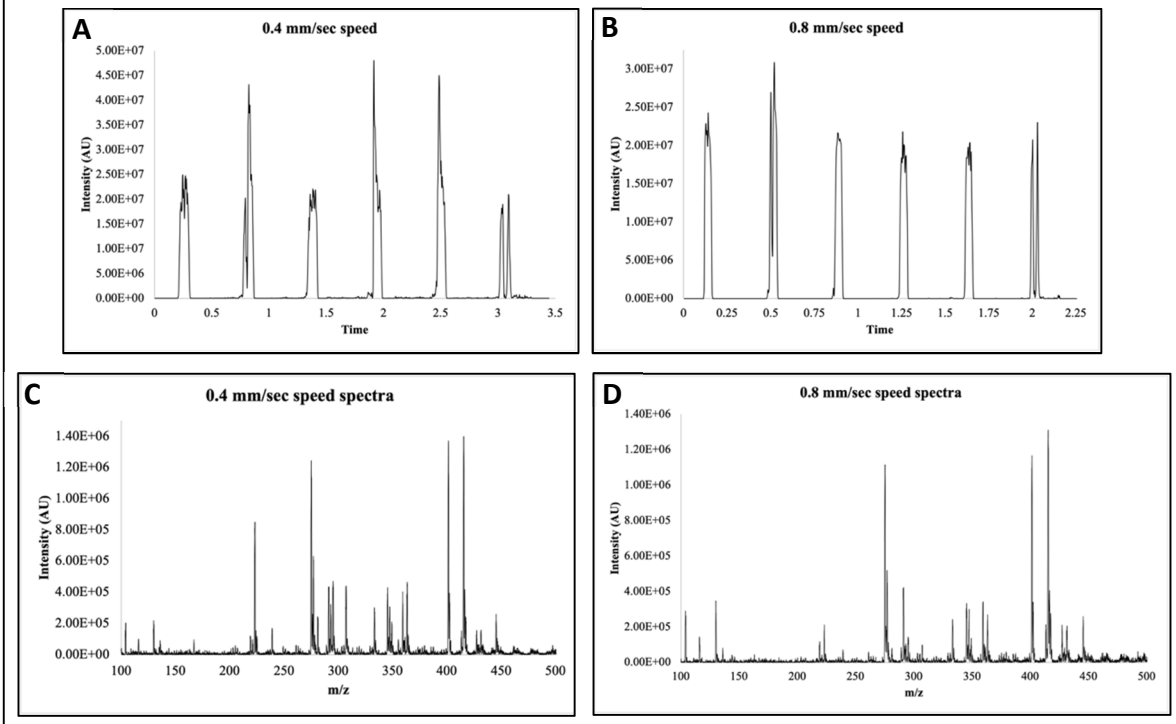




analytical settings are determined such as the speed of the autosampler and exposure contact time with the ionization source for each sample. Optimization of the rail settings is necessary to maximize data collection. The optimized railway settings ensure the sample has enough exposure to the ionization plasma to collect a full scan without collecting excessive background noise.

Railway settings specifically for the 12-Dip-IT® adapter were assessed following an iterative testing plan trialing different combinations of railway speeds and sample exposure times. The "sample speed" controls the speed of the railway as it moves perpendicular to the adapter holding the samples in front of the ionization plasma and the MS detector inlet. The "contact closure delay" determines how long each sample is exposed to the ionization source. For each test, spectra collected from different settings for railway speeds (mm/sec) and sample contact exposure delays (sec) were compared. Spectra were evaluated for (i) acquisition of high abundant peaks of interest (peaks of specific mass charges putatively annotated as important hop compounds) and (ii) low relative intensity of background noise. Referencing the DART-MS scans and spectra of different railway speeds in Figure 2.2 demonstrates that different speeds change both the DART-MS scans and the hop chemical fingerprint spectra pattern. Based on this evaluation, a railway speed setting of 0.8 mm/sec was determined to be optimal based on spectral richness and minimization of background noise (Figure 2.2D). Additionally, the 0.8 mm/sec speed enabled collection of 6 sample scans in a shorter time, 2.1 minutes (Figure 2.2B) compared to 3.2 minutes when using a speed setting of 0.4mm/sec (Figure 2.2A).

Figure 2.2. DART-MS railway speed settings. A: 0.4 mm/sec railway speed showing 6 samples scanned over 3.20 minutes. B: 0.8 mm/sec railway speed showing 6 samples scanned over 2.1 minutes. C: 0.4 mm/sec railway speed spectra. D: 0.8 mm/sec railway speed spectra, a richer chemical fingerprint while maintaining low background noise.



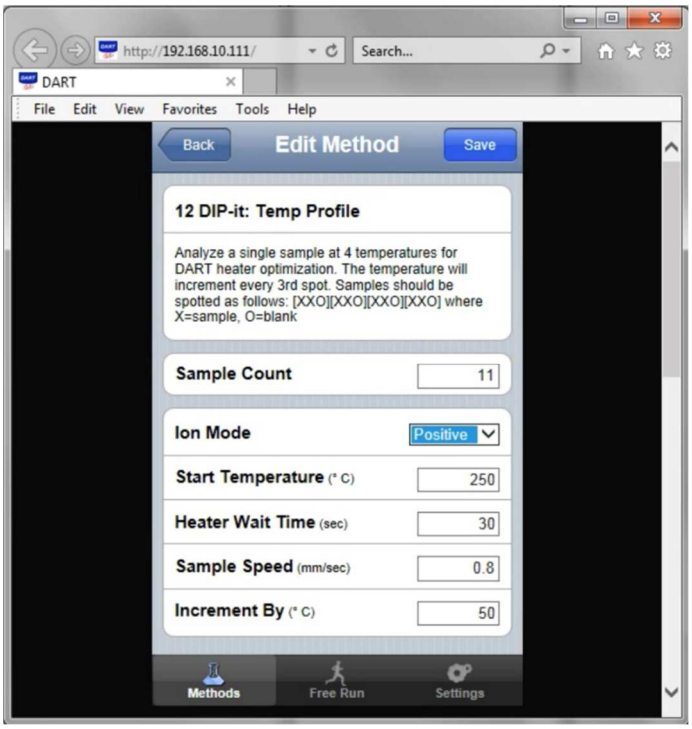
After trialing many different speeds and times, the optimal railway and autosampler settings were determined to be a "sample speed" of 0.8mm/sec and "contact exposure delay" of 10 seconds. These analytical settings were entered into the DART-MS railway software as depicted in Figure 2.3. Settings are easily adjusted using the user-friendly software interface to edit each method and can be optimized depending on the specific specimen and medium being tested. The method type selected to edit is determined based on the autosampler used. Other settings beyond "sample speed" and "contact closure delay" such as temperature settings, including "run temperature", and "ion mode" settings were optimized as described in later sections.

Figure 2.3. DART-MS railway speed software settings. 12-Dip-IT® method DART-MS software settings with "Sample Speed" of 0.8 mm/sec and "Contact Closure Delay" of 10 seconds

2.1.2 Temperature Settings

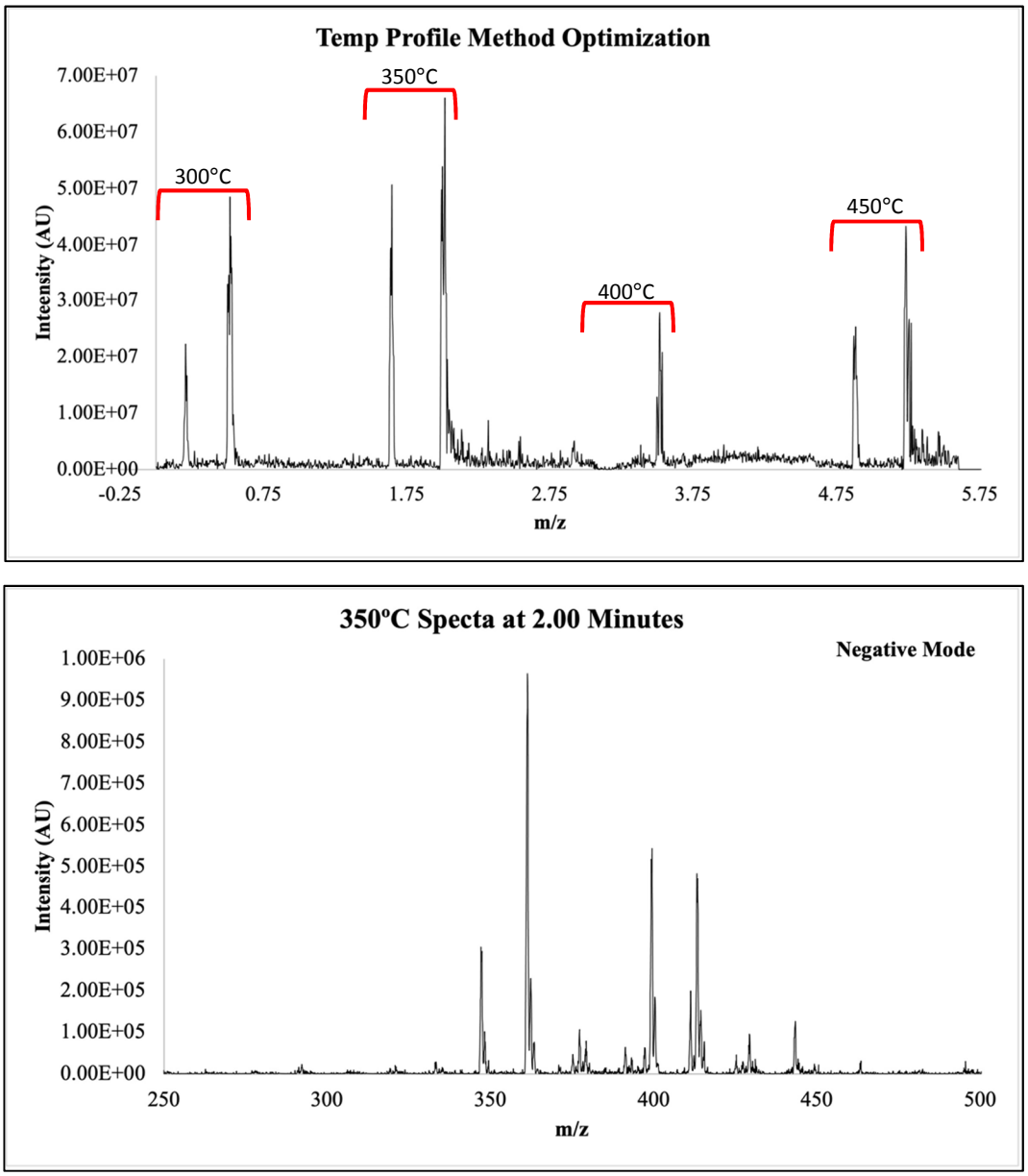
Temperature can also define the ionization efficiency of the sample. Multiple different temperatures can be trialed in a single scan using the "Temp Profile" method template, in this case the 12-Dip-IT® method for analyzing liquids. The "Starting Temperature (°C)" is chosen by the user (<150°C - 500°C) and increases by intervals as defined by "Increment By (°C)" as depicted in Figure 2.4. This was performed three times in a pattern of samples and blanks such that there are two samples followed by one blank for a total of twelve test points. Thus, if X=sample and O=blank then the order was [XXO][XXO][XXO][XXO] testing four different temperature settings in a single test run.

Figure 2.4. DART-MS temperature optimization software settings. 12-Dip-IT® "Temp Profile" temperature optimization method where the "Start Temperature" and the intervals of temperature increase defined as "Increment By" were entered into the user defined fields. Sample spaces and blank spaces (stainless-steel needles dipped in sample and clean needles respectively) to see differences in the resulting scan and spectrum that are produced.



Similar criteria used for optimizing railway movement settings was also used in selecting optimal temperature settings; spectra chemical fingerprints of hop extract samples were reviewed iteratively to select the best temperature setting that produces the richest fingerprint, acquiring peaks with the highest relative abundance and the least amount of background noise as visualized in Figure 2.5. Based on this evaluation it was determined that 350°C was the optimal temperature for ionization of the hop extract samples.

Figure 2.5. Temp Profile method testing four different ionization temperatures in negative mode. A: Four different ionization temperatures were tested starting at 300°C increasing at a temperature interval of 50°C. The scans at 350°C had the highest relative abundancy proving to be the ideal ionization temperature. B: Spectra from the most abundant scan at 2.00 minutes ionized at a temperate of 350°C resulting in a rich chemical fingerprint with low background signal.



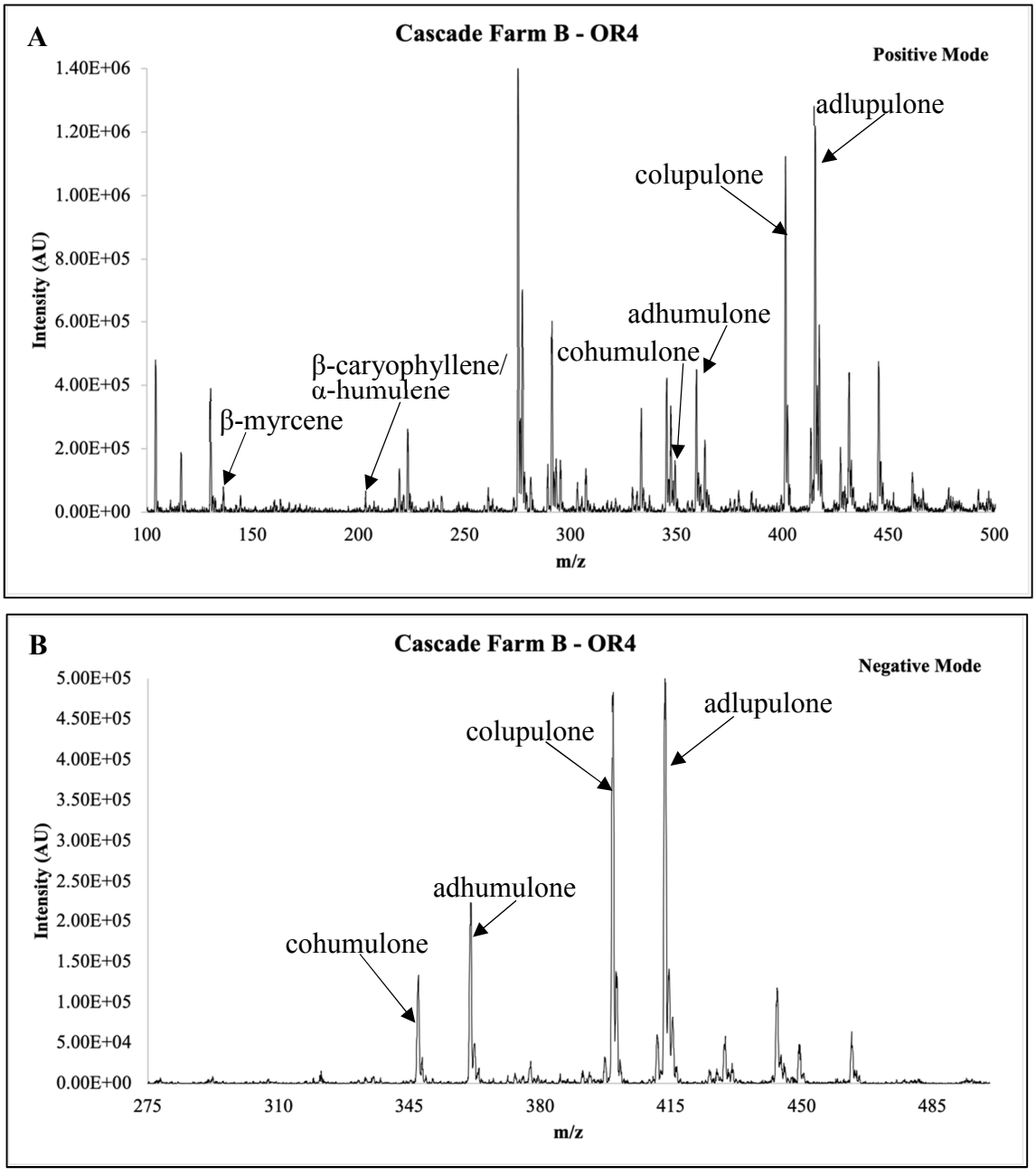
2.1.3 Ionization Mode

DART-MS can be performed in 'positive' or 'negative' ionization mode, which forms positive/negative ions from the hop sample extracts. As seen in Figure 2.6, some hop compounds are more amenable to positive ionization, and others to negative, and so the ionization mode setting can further focus on the types of hop chemicals ultimately detected. Positive mode acquisitions enabled the detection of a wider variety of compounds, including both terpenes and alpha-acids resulting in richer chemical fingerprints, whereas negative mode detected only alpha-acids (Figure 2.6). Additionally, positive mode ionization enabled the detection of both volatile compounds (terpenes) and non-volatile compounds (alpha- and beta- acids), an advantage over traditional metabolomics approaches. For example, historically, terpene analysis of hop oils is performed using GC coupled with a flame ionization detector (GC-FID) while alpha- and beta-acids are analyzed utilizing high pressure liquid chromatography mass spectrometry (HPLC-MS) methods[17,18]. While positive mode ionization did result in richer spectra, it was also considered that the "cleaner" spectra obtained with negative mode ionization may lead to more predictive models. Thus, it was decided that all samples would be analyzed by both positive and negative ionization modes to evaluate through the data analysis pipeline.

2.1.4 Optimal Hop Extraction Method

To compare all extraction methods and ionization mode settings, two hop varieties were extracted with each method, and then the extracts were pooled together to represent a range of metabolites that would be expected in a trial setting. DART-MS was conducted on each of the pooled extracts in positive and negative mode, and spectra were evaluated for the following: (i) number of peaks observed in each scan (ii) reproducibility of the data when sampled multiple times and (iii) for the presence of known quality compounds.

Figure 2.6. Positive and Negative Ionization modes. A: DART-MS spectra of hop extraction sample in positive (+) mode; marked m/z that are predicted to correspond to hops metabolites. B: DART-MS spectrum of hop extraction sample in negative (-) mode; marked m/z that are predicted to correspond to hops metabolites.



The OPLS-DA modeling is reported in Table 1 and Figure 2.7, with Pareto scaling applied to the data scaled all metabolites, rendering low abundant compounds equally as important as high abundant compounds in the analysis. Overall, all methods resulted in good

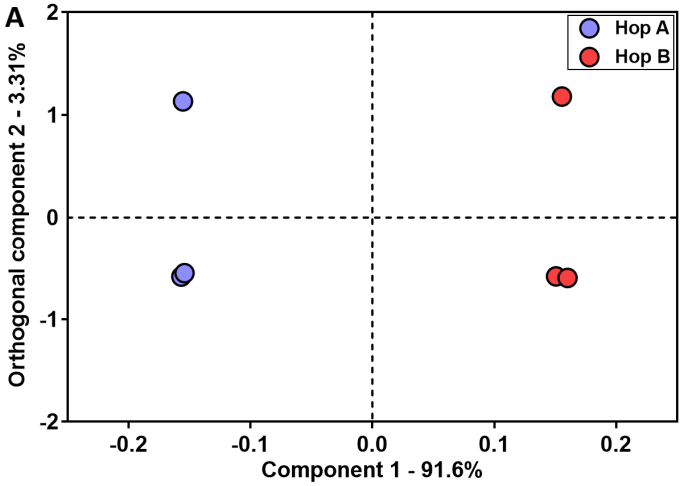
model fits with R^2 greater than 90%. After analyzing all extraction methods, Methods B, C, and D in both positive and negative mode were determined to be ideal for hops analysis (Table 1) with high Q^2 predictability greater than 87%. Method A had low predictability attributed to user error, so this method wasn't considered further. These data support that the extraction and DART-MS system can be optimized to detect distinct classes of compounds for hop quality analysis.

Table 1. Model Fit (R^2) and Predictive Power (Q^2)
positive (+) and negative (-) modes

Method	R^2	Q^2
Method A+	0.951	0.32
Method B+	0.943	0.947
Method C+	0.990	0.974
Method D+	0.985	0.936
Method A-	0.951	0.32
Method B-	0.943	0.947
Method C-	0.930	0.990
Method D-	0.943	0.878

Taken together results from Figures 2.7 and Table 1, the OPLS-DA supports choosing Method C (80/20 ethanol/water) as the optimal extraction method to carry out for the hop analysis study in both positive and negative ionization modes. Table 2 below summarizes all analytical settings and extraction methods attempted highlighting which settings and extractions are the best for whole flower hop analysis using DART-MS.

Figure 2.7. OPLS-DA modeling. A: Example OPLS-DA scores plot showing ability for DART-MS data to be used to discriminate samples. B: Graphical representation of model fit and predictive power of the method.



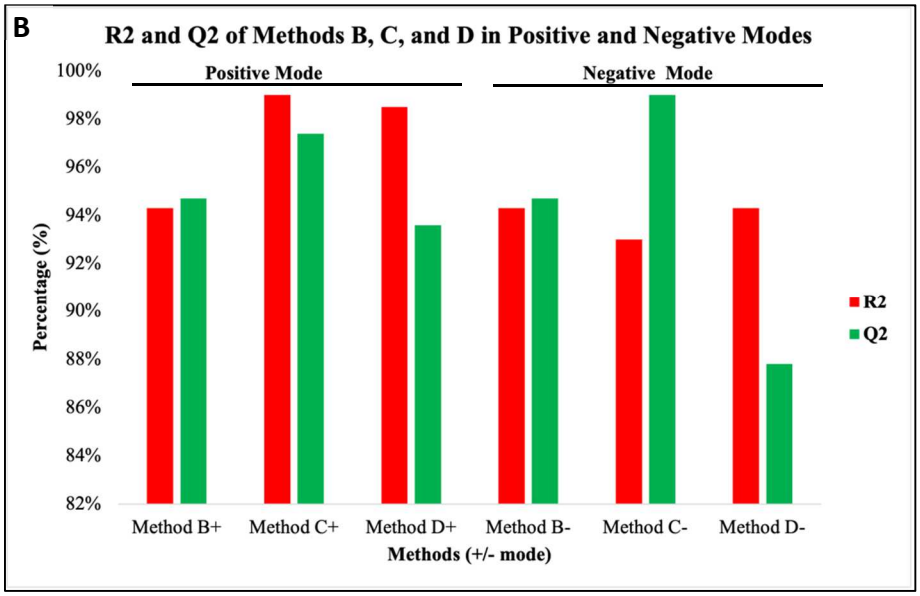


Table 2. Summary of Method Development trials for DART-MS analysis of hops.

Method (mode)	Extraction Solvent (v/v)	Comments
Method A (+/-)	80/20 methanol/water	simple method, easy to replicate, solvents are generally safe to use
Method B (+/-)	95/5 methanol/water	same as Method A; expected to extract more terpenes than Method A
Method C (+/-)	80/20 ethanol/water	same as Method A; ethanol is more easily accessible, but expected to be slightly worse for terpenes
Method D (+/-)	95/5 ethanol/water	same as Method C; but expected to be slightly better for terpenes
Method E (+/-)	3/2/1 MTBE/methanol/water	Same as Method F, but acetonitrile was added to further reduce potential interference of sugars
Method F (+/-)	3/2/1 MTBE/methanol/water	a biphasic method producing two samples: an **aqueous** and **organic** extract from hops; the organic was expected to focus the method on terpenes (oils) by removing highly water-soluble compounds; overall a difficult method with many steps, and only worthwhile if other methods failed
Other Methods	**Method Parameter**	**Comments**
Method G	Raw hops (no extraction)	Could not run whole flower, as the flowers are very dry post kilning and they would blow up into the DART via the He ion source and could cause significant damage to the instrument; decided to try to run raw ground hops gently mixed with high purity lab grade water; difficult to keep the ground hops separated as the hops easily floated away from static electricity during weighing process; difficult to keep hops moist, and the water evaporated with differing amounts of evaporation between the 12 samples as it took time to prepare each; ion source evaporated all the water upon contact as it is high temperature at 400 °C
Method H	Autosampler	We created a custom method for autosampling hops extracts (Section X.X.X below); this was used for all our analyses, and enabled sampling of 6-12 extracts in a single 'run' (approximately 1 min per run)
Method I	DART-MS temperatures	Used 12 Dip IT™ temp profile methods; looked into different temperatures by starting at 300 °C and having it increase in temperature in 50c increments; 350 °C chosen as it had the cleanest/less noise for spectras/chromatograms (see Figure XX below)
Method J	Ionization Mode	Different ionization modes (positive/negative) is selective for different compounds depending on each compounds potential for certain ionization modes being positive or negative

2.2 Custom Dip-IT™ Autosampler and Reducing Background Noise

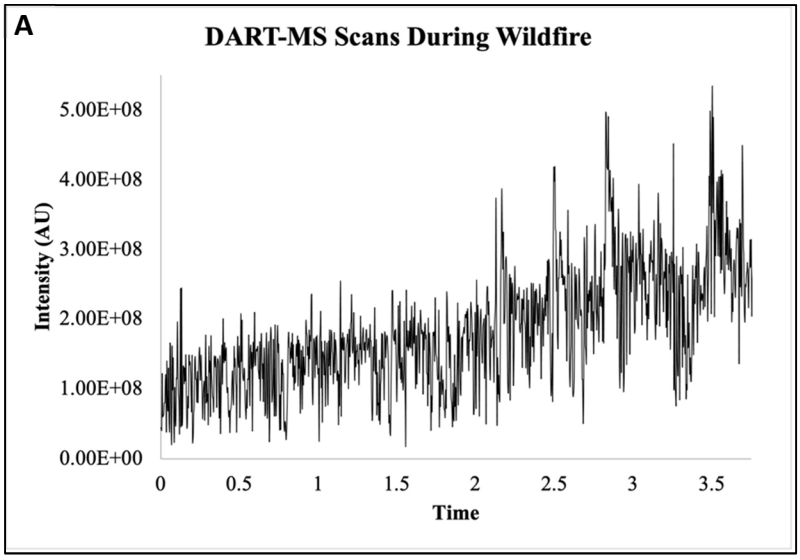
At the time of DART-MS testing during the fall of 2020, Fort Collins, Colorado and surrounding areas were greatly impacted by smoke from the Cameron Peak Fire, the largest fire in Colorado history to date[19]. From August until the fire was contained two months later, smoke was heavy in the city of Fort Collins, being directly west from the fire, and the smoke easily carried into buildings (Figure 2.8)[20]. Smoke easily permeated most buildings even though local

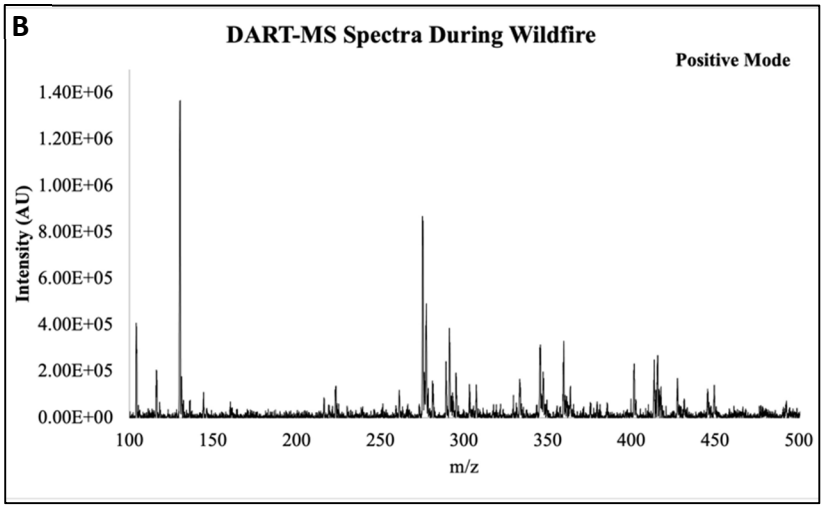
Figure 2.8. Smoke Column north of Boyd Lake Loveland, Colorado from the Cameron Peak Fire. Daily thick smoke plumes covered cities like Loveland and Fort Collins directly west of the fire.

authorities advised civilians to keep doors and windows closed during high smoke exposure.

With smoke easily recognized by smell alone, DART-MS was also detecting the surrounding smokey ambient air. This resulted in a significant increase in background signal (*e.g.,* noise) to the point that it was impossible to discern between background and sample signal (based on total ion counts – TIC) (Figure 2.9). Technical experts from both IonSense™ and

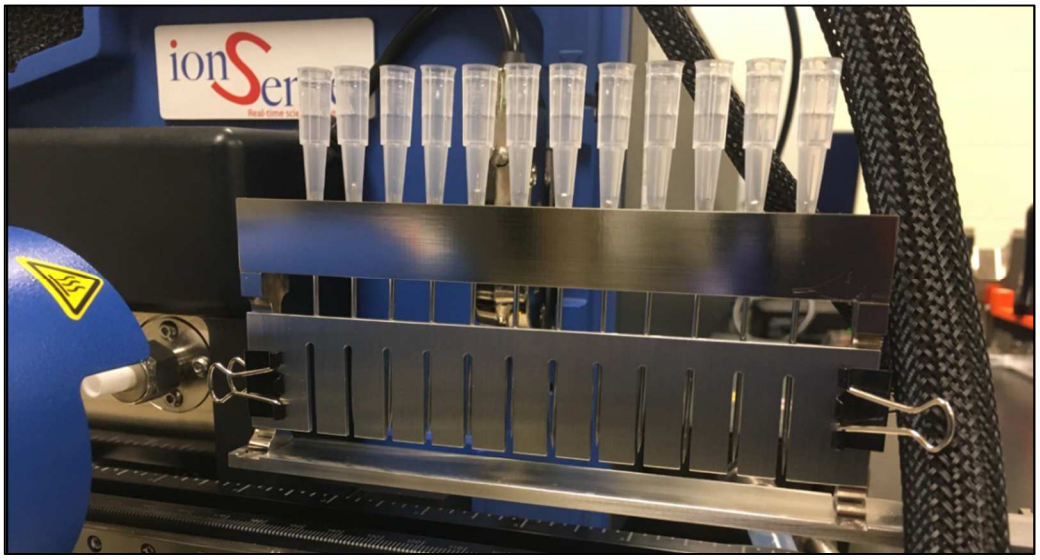
Figure 2.9. Detection of smoke in the surrounding ambient air impacts DART-MS scans and spectra. A: The pickup of smoke from the surrounding air disrupted the scan making it unclear where the scans of the actual hop extraction samples occurred at any point during the analysis where several samples were being tested during the run (up to twelve samples). B: An example spectrum (positive mode), the spectra is missing key peaks since it is difficult to discern which scans are of hop samples.





Waters™ confirmed that this challenge was systemic and a common issue with the platform. In fact, this was such a known issue that IonSense™ was in the process of developing systems upgrades to create a software update known as JumpShot technology. This update would solve ambient air interference by modifying how the ionization of the sample was executed from a constant flow of ionizing helium gas to a pulsated flow[21].

Figure 2.10 DART-MS scans and spectrum with addition of stainless-steel plate physical barrier. 12-Dip-IT® autosampler with attached fabricated stainless-steel plate with slots for exposing the sample needles loaded with hop extract.



While waiting for the software upgrade, an alternative solution to DART-MS sample testing was developed by the Prenni lab at Colorado State University through a collaborative effort with Wilson Machining, a local Fort Collins metal fabricator. A slotted stainless-steel plate physical barrier was designed that would block the ionizing helium from flowing into the mass spectrometer detector except for when sample was present (Figure 2.10). Blocking the flow of the ionizing plasma between the samples produced cleaner scans and spectra. Creating clean scans and spectra by incorporating the stainless-steel plate as a physical barrier was key to reducing excessive background noise pick-up and made it clear when hop samples were being analyzed in the scan, improving data analysis post testing. After successfully validating the stainless-steel plate by comparing scans and spectrum before and after applying the stainless-steel plate barrier, the incorporation of this adapter was included in the method for the experiment moving forward.

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Chapter 3 DART-MS and FTIR Metabolite Fingerprinting of Hops (*Humulus lupulus* L.) to Track Variation in Chemistry

3.1 Introduction

According to the Brewers Association (BA), the Craft Brewing industry contributed approximately $76.2 billion to the United States economy in 2017 and more than 500,000 jobs[1]. Hops (*Humulus lupulus)* are a huge part of the industry and a key flavor and aroma ingredient in many beer styles made today. Hops are a global commodity and with the increasing demand comes not only increased prices for consumers, but also higher expectations for consistent quality and organoleptic properties. India pale ale (IPA), which is a broad category of the many styles of "hoppy" beers, was created to elicit "high hop aroma…with a moderate to assertive hop bitterness" and is currently the leading style in the craft brewing industry[2,3].

The United States is one of the leading hop producers in the world, combined with Germany it accounts for 75-80% of the worlds produced hops[4–6]. Craft brewers now use 40-50% of all the hops produced domestically[7]. Importantly, hops are a chemically diverse ingredient, and over 100 hop compounds have been demonstrated to influence beer flavor[8]. Specifically, hops contribute to (i) aromatics of the beer (~90 compounds) and (ii) bitterness (~10 compounds), and this chemistry can be influenced by both genetics (hop cultivar) and the environment (agricultural system)[9,10]. Identification of compounds as metabolites (small volatile/non-volatile molecules < 1500 da) such as hydrocarbons (*e.g.,* essential oils such as linalool, geraniol, and others), terpenes (*e.g.,* monoterpenes, terpenoids, β-myrcene, and others), and organic acids (*e.g.,* beta-, alpha-, and isomerized acids) will lead to better understanding of the roles they play in important brewing chemistry reactions[11,12].

Previous research by Oregon State University (OSU) demonstrated genetic (cultivar) variation in the hop metabolome and that this variation was correlated with sensory attributes[9]. Hop cultivars were shown to display differences in metabolite expression (*e.g.,* essential oils) which affected the sensory perception[9,13]. Another study demonstrated an impact of processing treatment on the hop metabolome and sensory measured in both the raw hops and beer[13].

Characterization of the hop metabolome is an important step towards understanding potential quality markers of hops, future technologies (*e.g.,* hop selection tools, rapid in-field hop testing methods, etc.), and process developments to improve the quality of hop breeding, growing, harvesting, processing (*e.g.,* drying) programs, and utilization in the brewing process. Currently the most common method of analysis for hops used in quality assurance and control (QA/QC) programs for hop growers and craft brewers is sensory analysis. American Society of Brewing Chemists (ASBC) have created robust, standardized sensory methods for hops and hopped beers. While the quality of sensory is the primary output of interest, sensory analysis of hops and highly hopped beers can be fatiguing, limiting the number of samples analyzed by panelists at a given time[14,15]. Additionally, ensuring a non-biased, objective sensory panel can be difficult and requires training[16,17]. Better understanding the mechanisms of chemistry and the impact genetics, growing environment, and processing factors have on the overall sensory perception could help to answer questions of why certain hop cultivars or hops grown in certain locations yields beer with improved consumer acceptance.

Ambient Mass Spectrometry (AMS) is an analytical technology that has seen rapid growth over the past decade. Unlike conventional mass spectrometry (MS) technologies which typically require labor intensive sample preparation, AMS utilizes rapid ionization technologies operating under ambient conditions and require little to no sample preparation[18,19]. The

simplified design of AMS technologies allows for analysis of samples in their "real world" state[20]. Currently ~30 different AMS techniques are commercially available, some of which have been integrated into food industries to meet demands for regulations, certification programs, and fraudulent countermeasures[21,22]. Previous research has demonstrated AMS application for animal products (*e.g.,* meat, beef lard, beef tallow), herbs (*e.g.,* mulberry leaves, oregano, herbal medicines), beer, coffee, and honey to name a few[21,23–26]. Direct Analysis in Real-Time Mass Spectrometry (DART-MS) is a specific type of AMS that can test a variety of mediums ranging from solids to liquids with minimal to no sample preparation.

Another high-throughput, rapid technology that additionally has been considered to support food quality assurance is Fourier Transform Infrared Spectroscopy (FTIR). Similar to DART-MS, FTIR is capable of instantaneously producing "fingerprints" that represent spectrochemical patterns of the sample being tested[27]. FTIR has also been utilized in food applications for quality assurance measures and to combat food fraud for animal products, oregano, olives, and beer[28–31]. In this study I evaluated the potential of DART-MS and FTIR as tools for characterization of hop metabolites with the goal of enabling high throughput screening of hops for quality assurance as liquid extractions. I hypothesized that chemical fingerprints of hops detected by DART-MS and FTIR can train a predictive model to predict hop quality. I tested this hypothesis through the following objectives: 1) evaluated collected chemical fingerprints and generated accurate predictive models to classify hops by genetic and environmental differences and 2) validated that the collected fingerprints were reflective of known quality and sensory attributes important to hops.

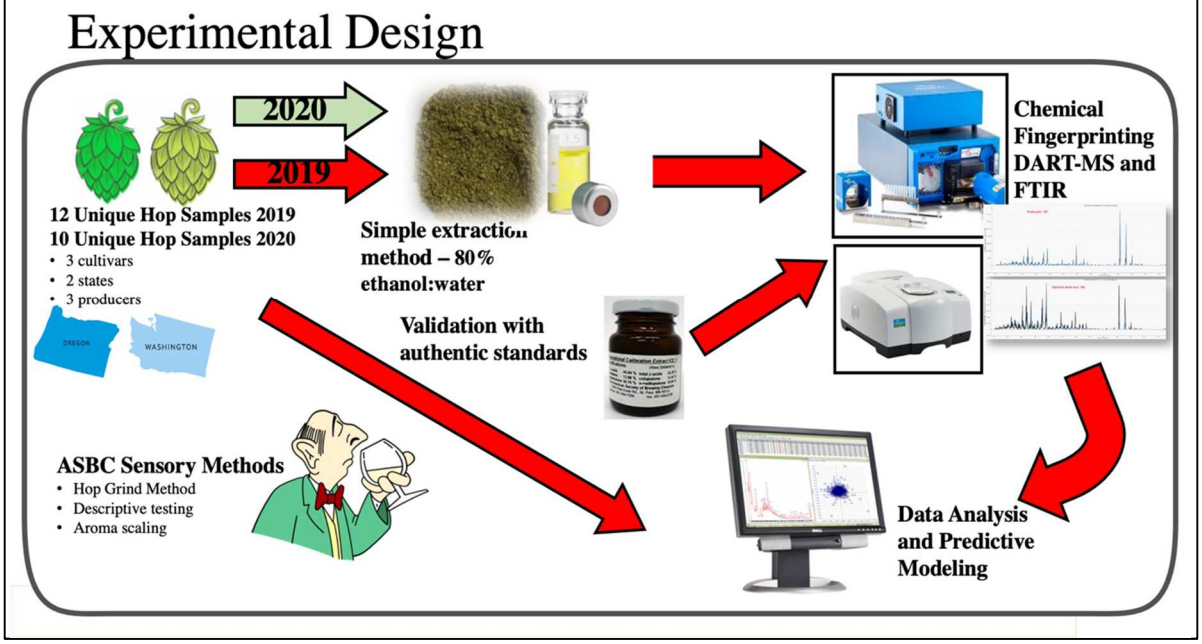
3.2 Methods

3.2.1 Experimental Design

Hop samples were sourced from three commercial hop suppliers in the Pacific Northwest region, specifically in Oregon and Washington. One of the hop suppliers had two different growing locations, resulting in a total of four unique growing locations. Three of the hop suppliers were in Oregon with locations in the cities Gervais, Woodburn, and Silverton, and the fourth was in Yakima Valley Washington. Both Oregon and Washington are recognized as two of the primary areas for hop production in the United States[32]. Three hop cultivars: Cascade, Centennial, and Strata, were sourced from each growing location, resulting in 12 unique hop samples for the 2019 harvest season. A second year of sampling was performed during the 2020 harvest season; the same three cultivars were sourced from the same three hop growers with the same four growing locations. Two of the hop lots sampled during the 2019 harvest season were discontinued by the hop grower and thus a total of 10 unique hop samples were acquired in 2020.

The experimental design is summarized in Figure 3.1[33,34]. Briefly, hop samples were homogenized and extracted with 80% ethanol/water. Subsequently, the hops were analyzed by both DART-MS and FTIR and the data was used to train and test multivariate predictive models. Simultaneously, descriptive sensory analysis following ASBC standardized methods was also performed on all samples.

Figure 3.1. Experimental Design. Hops were sampled two years; harvest season 2019 and 2020. Three different cultivars were sampled from two states (Oregon and Washington), three different hop suppliers, and four distinct growing locations. Hop fingerprints then were collected from DART-MS and FTIR[29,30]. Authentic standards were used to support detection confidence of known hop metabolites. In parallel, sensory analysis was performed on all samples. Predictive modeling determined the ability of DART-MS and FTIR to discriminate samples and detect hop compounds important to sensory and quality.



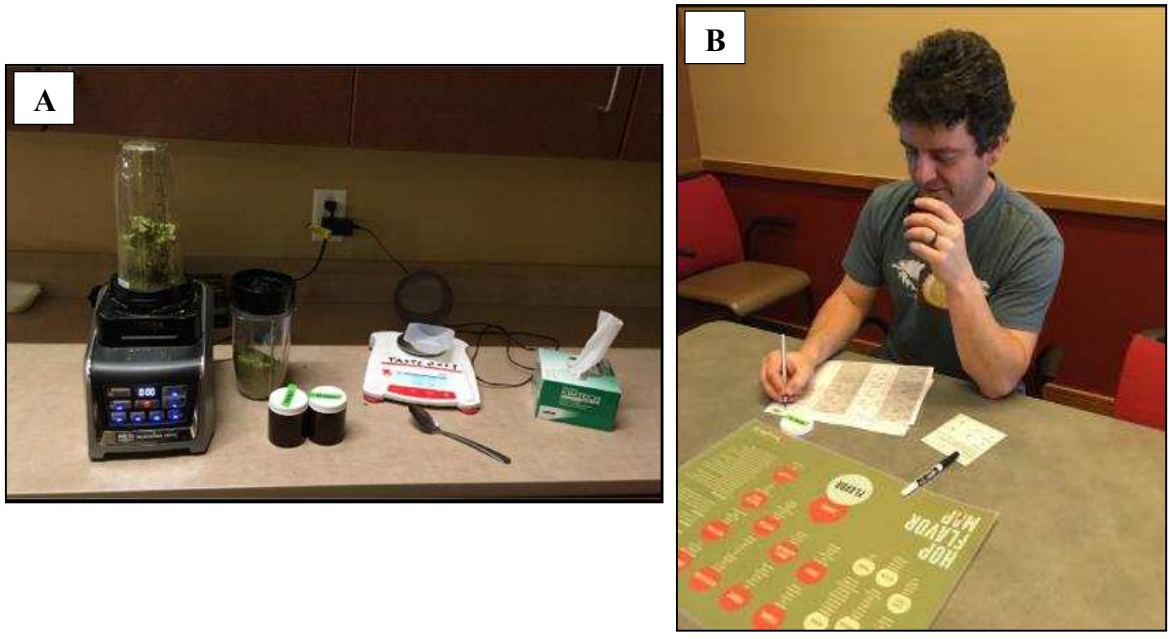
3.2.2 Hop Sampling

Whole flower hops were harvested during the optimal time of hop cone maturity, which was highly dependent on cultivar and environmental factors such as weather or geographic location. The growers followed best agronomic practices, utilizing data from previous years and sensory analysis to determine the optimal harvest time for each hop cultivar. Samples of whole flower hops were dried and cured by the suppliers following Hop Sampling guidelines provided by the USDA[35]. One pound of each hop sample was packaged and shipped overnight to Fort Collins, CO on ice. Hop samples were stored at 4°C in a dark and dry area prior to DART-MS, FTIR, or sensory analysis.

3.2.3 Sensory Analysis

Hop samples stored at 4°C were prepared for sensory analysis following the ASBC Hop Grind Sensory Evaluation Method[14]. Using a Ninja Blender with blending cups and blade caps, hop samples were ground at a medium to high setting for 4 sec, shaken for 2 sec, and then ground for an additional 4 sec (Figure 3.2A). Hop samples were weighed out into 3-5 g samples and aliquoted into amber glass jars with caps. Sensory samples were stored at 4°C until ready for analysis.

Figure 3.2. Sensory Analysis. A: Hop samples were prepared for panelists following the ASBC Hop Grind Method, grinding samples using a ninja blender. B: Equal amounts of samples were presented to panelists for descriptive analysis.



The experimental design for processing hop samples for sensory analysis was dictated by a randomized block design. Samples were presented in a blind setting to 10 trained panelists for aroma analysis as depicted in Figure 3.2B. Panelists analyzed the samples using descriptive and aroma intensity analysis[36]. All hop samples were tested three separate times over a three-month time span.

3.1.4 Hop Extraction

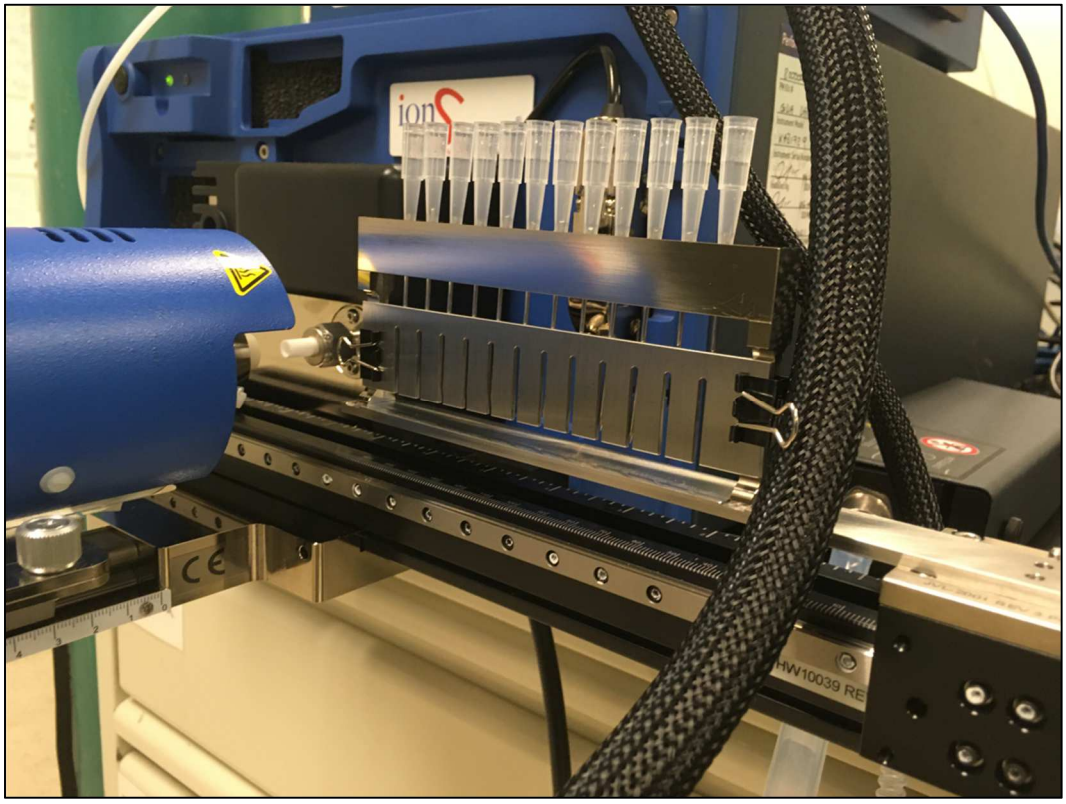
Hop samples stored at 4°C were prepared following the method development in Chapter 2, with the 80/20 ethanol/water extraction method for all hop samples harvested in the 2019 and 2020 seasons. This optimal method was based primarily on a report from the Research Center for Brewing and Food Quality, Munich Germany[37]. Other literature also supports the use of solvent extraction with ethanol and methanol and such methods have been demonstrated for the detection of volatile compounds such as terpenes and nonvolatile compounds such as bittering acids[37,38]. Furthermore, a simple solvent extraction method is ideal for hop growers, breeders, and brewers that require rapid, high-throughput analysis[39]. Hop samples were homogenized using a mortar and pestle with liquid nitrogen to quench metabolism. Samples were then stored at -80°C until ready for extraction. Each hop sample (100 mg) was extracted with 1 mL of an 80% ethanol solution (200 proof ethanol and LCMS grade water). Samples were vortexed for 2 hours at 4°C and then centrifuged at 4°C, whereupon liquid extract (supernatant) was separated from the plant material (pellet). The plant material was discarded. For each of the unique hop samples (12 hop samples for harvest year 2019, 10 hop samples for harvest year 2020), three technical replicates were produced. Aliquots of 80 µl from each replicate was used for analysis by DART-MS. Extracted hop samples were stored at -80°C until ready for analysis. A pooled QC sample was prepared by combining the equal amounts of each hop sample extract (50 µl each). Separate pooled QCs were generated for samples from 2019 and 2020.

3.1.5 DART-MS

DART-MS Workflow

Hop extracts were analyzed using the DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Inc., Saugus, MA) coupled with a single quadrupole mass spectrometer (ACQUITY QDa; Waters Corporation, Manchester, UK) via a Vapour interface (IonSense, Inc., Saugus, MA). The DART-SVP was equipped with a motorized rail system where the 12-Dip-IT® adapter with sampling needles held at the optimal position would move perpendicular to the flow of the Helium ionizing gas (Figure 3.3).

Figure 3.3. Example extractions for DART-MS method. Custom autosampler to enable sampling of up to 12 extracts in a single run. Pictured is a half run comprised of 6 stainless steel needles.



The extracted hop samples were analyzed in a randomized order. The relevant pooled QC was analyzed after every seventh sample. Hop extracts were introduced to the DART-MS by

dipping a custom cut stainless steel needle into each extract. The analysis of each sample consisted of 6 needles dipped into the same sample extract. The needles were placed onto the 12-Dip-IT® adapter for the sample introduction rail system (IonSense, Inc., Saugus, MA) for analysis.

Settings for the DART-SVP were modified from a previous study[40]. The helium gas flow was set to 3LPM and heated at 350°C for both positive and negative ionization modes. The cone voltage was set to 15V. Spectra were collected in both positive and negative ionization modes separately over the mass range of 100-600 m/z. The speed of the motorized rail system holding the 12-Dip-IT® adapter with 6 stainless steel sampling needles was set to 0.8 mm/sec with a contact closure delay of 10 seconds and standby temperature of 300°C. This method ensures one scan per each sample needle on the 12-Dip-IT® adapter for up to 12 scans (12 needles) per each run.

Authentic Standards

Authentic analytical hop standards for β-myrcene, β-pinene, geraniol, linalool, β-carophyllene, citral, α-humulene, (R)-(+)-Limonene, α-pinene, nerol, cohumulone, N+adhumulone, colupulone, N+adlupulone, alpha-acids and beta-acids were analyzed using the same instrument and testing conditions as described for the extracted hops. Standards were sourced from Sigma Alderich at ≥ 98% purity (Sigma-Aldrich, St. Louis, MO) and SPEX CertiPrep at concentrations of 1000 µg/mL concentration (SPEX CertiPrep, Omaha, NE). The ICE-4 international calibration extract was sourced from the American Society of Brewing Chemists (ASBC) contained a mixture of hop standards at the following concentrations: Cohumulone (10.98%), N+adhumulone (31.60%), Colupulone (13.02%), N+adlupulone (13.52%), Total alpha-acids (42.58%), and Total beta-acids (beta-acids; 26.54%) (ASBC, St.

Paul, MN). Six replicates of each standard were analyzed by DART-MS at the concentrations as described above.

Data Analysis

DART-MS data was processed using a beta version of WRC Abstract Model Builder (Waters Corporation, Manchester, UK). Sampling replicates were selected using a threshold requiring 20% or greater total ion current as compared to the most abundant replicate for that sample. This manual filtering resulted in selection of ~ 3-6 sampling replicates for each sample. Spectra from the selected sampling replicates were then averaged to generate a single representative spectrum for each sample. The resulting spectra were normalized to the total ion current for each sample followed by peak binning at an interval of 1.0 m/z, resulting in 500 total m/z bins.

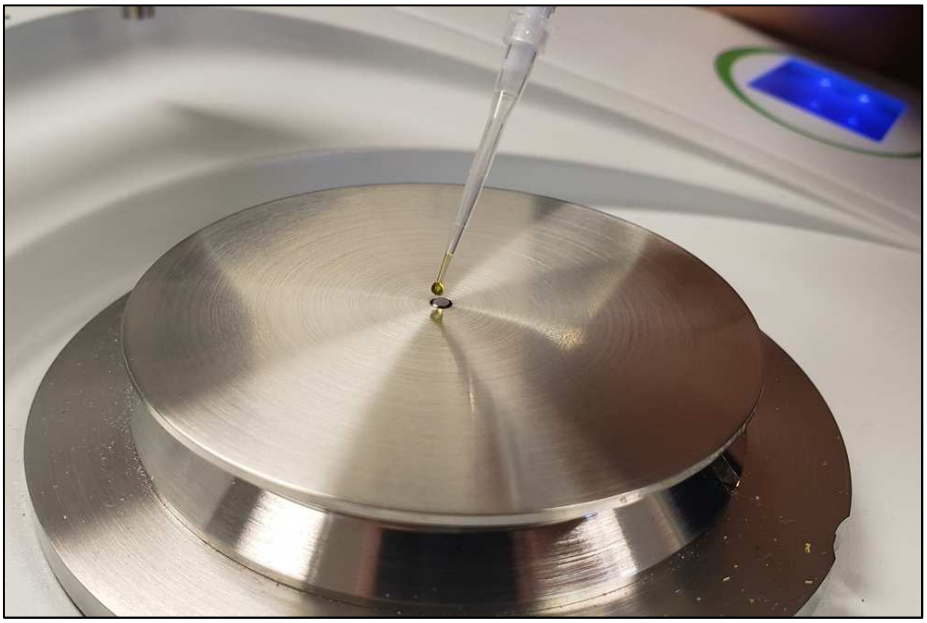
3.2.6 FTIR Methods

FTIR Workflow

Hop extracts were detected within the wavenumber region of 4000 to 650 cm^{-1} using a Spectrum 400 series FTIR (Perkin Elmer) with Universal Attenuated Total Reflectance (ATR) Sampling Accessory equipped with a Diamond crystal sampling surface (Figure 3.4). Spectra were collected at a resolution of 4 cm^{-1}, collecting 4 scans per sample. For each sample, 3 μL of hop extract was pipetted onto the crystal detector and allowed to dry for 30 seconds prior to detection (Figure 3.4). The crystal was cleaned in between each sample with 100% LCMS grade methanol, allowing the crystal to visibly fully dry before analyzing the next hop extract. Hop extract samples were analyzed in a randomized order. After every seventh sample, a pooled QC sample was tested. Prior to taking any sample measurements and after every eighth sample

analyzed, a background scan was performed to scan the environment and "zero out" the instrument.

Figure 3.4. Example extractions for FTIR method. A 3μL amount of sample is pipetted on to the FTIR crystal and allowed to dry for 30 seconds prior to spectral acquisition.



Data Analysis

FTIR data was processed using PerkinElmer Spectrum IR software (PerkinElmer Ltd, Beaconsfield, UK). Data pre-processing included (1) normalization and (2) calculation of the first and second derivatives to increase data quality. The FTIR data was first pre-processed using the Perkin Elmer Spectrum IR software which was also used to collect the fingerprints. Pre-processing steps of FTIR data are helpful prior to multivariate modeling to eliminate background noise and to highlight differentiating features[41]. Using the "Macros" function, different data processing procedure profiles were created. The different data processing step options "normalization" and "derivatization" were chosen. For the derivatization processing step, 1st and 2nd degree derivatives were compared to each other as well as comparing the interval range of points the derivative step covers; 25-, 37-, 49- and 149-point intervals. Each individual spectra

data file per hop extract sample then was combined into one data matrix using R Studio software and an in-house built program. This produced robust models where the derivative degree and the point interval coverage with the best fit and predictability power was selected from the comparisons of the pre-processing steps.

Chemometric Modeling for DART-MS and FTIR

Supervised multivariate statistical modeling using orthogonal projections to latent square discriminant analysis (OPLS-DA) was applied to evaluate differentiation of hops (3 cultivars x 4 environments) using DART-MS (collected in both positive and negative ionization modes) and FTIR spectral fingerprints. All data was pareto scaled except for QC analysis that was UV scaled. This type of predictive modeling is class based (in this case, cultivar). The quality of the OPLS-DA is described by two metrics: R^2 (indicating the overall model fit) and Q^2 (indicating the predictive power determined after cross-validation). A Q^2 of greater than 50% supports that the model can predict a class (cultivar) greater than by chance, and a value of more than 90% indicates good predictive power in a true-to-type setting.

3.3 Results and Discussion

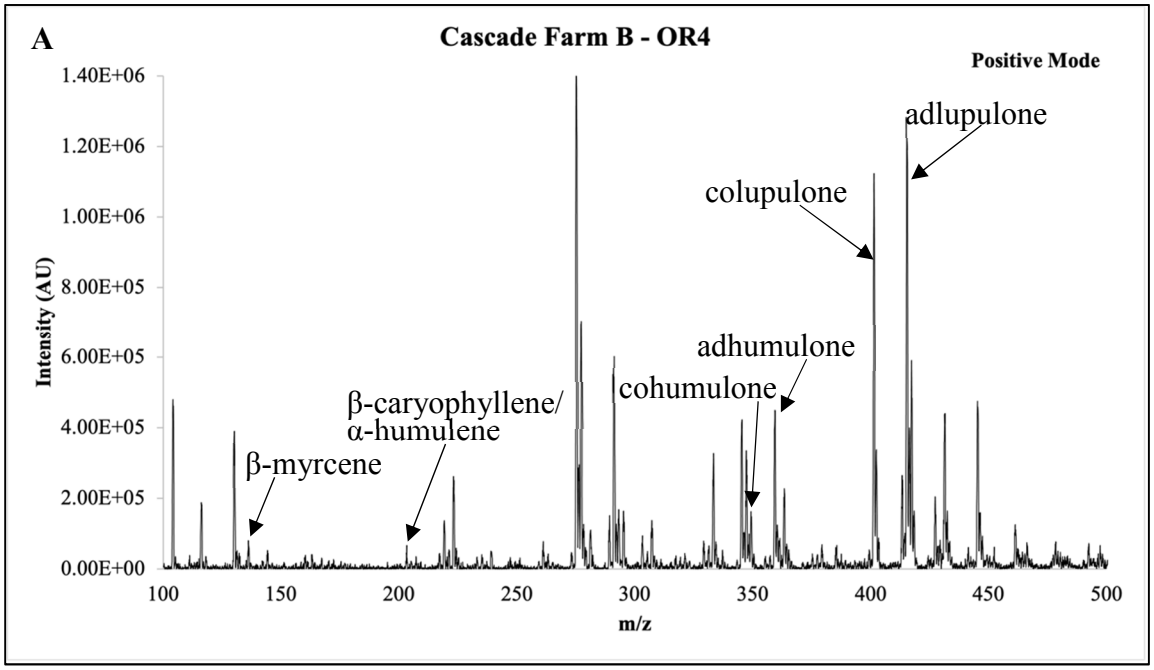
3.3.1 DART-MS analysis enables detection of non-volatile and volatile hop compounds.

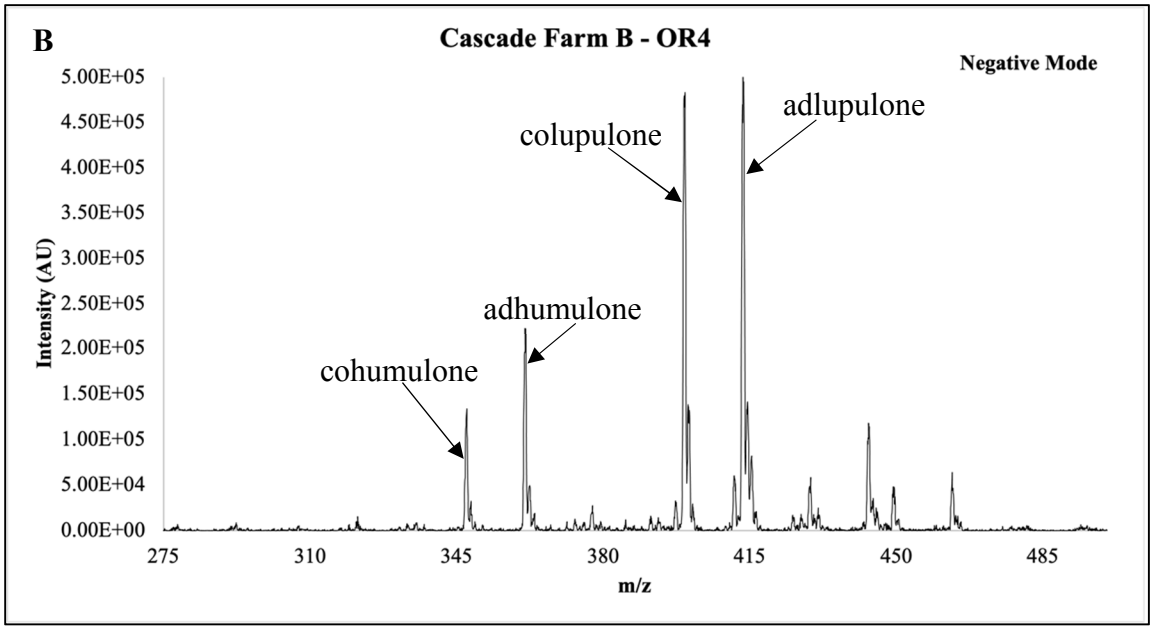
Hop Extracts

Analysis with DART-MS generated near instantaneous chemical fingerprints from the 80% ethanol hop extracts (Figure 3.5). All samples were analyzed using both positive and negative ionization modes. As expected, negative ionization was ideal for detection of bittering acids as seen in the spectrum of a Cascade hop extract (Figure 3.5B). However, positive ionization resulted in a much richer (in terms of the number of compounds detected) chemical

fingerprint (Figure 3.5A). For example, positive ionization enabled detection of both non-volatile bittering acids and volatile terpenes.

Figure 3.5. Example spectra of Cascade from one location in positive mode and negative mode. Selected ranges of m/z are shown for illustration purposes. A: Cascade Oregon (OR) Farm 4 positive mode, B: Cascade OR Farm 4 negative mode. Labeled peaks correspond to putative compound annotations supported by detection of authentic standards listed in Table 1.





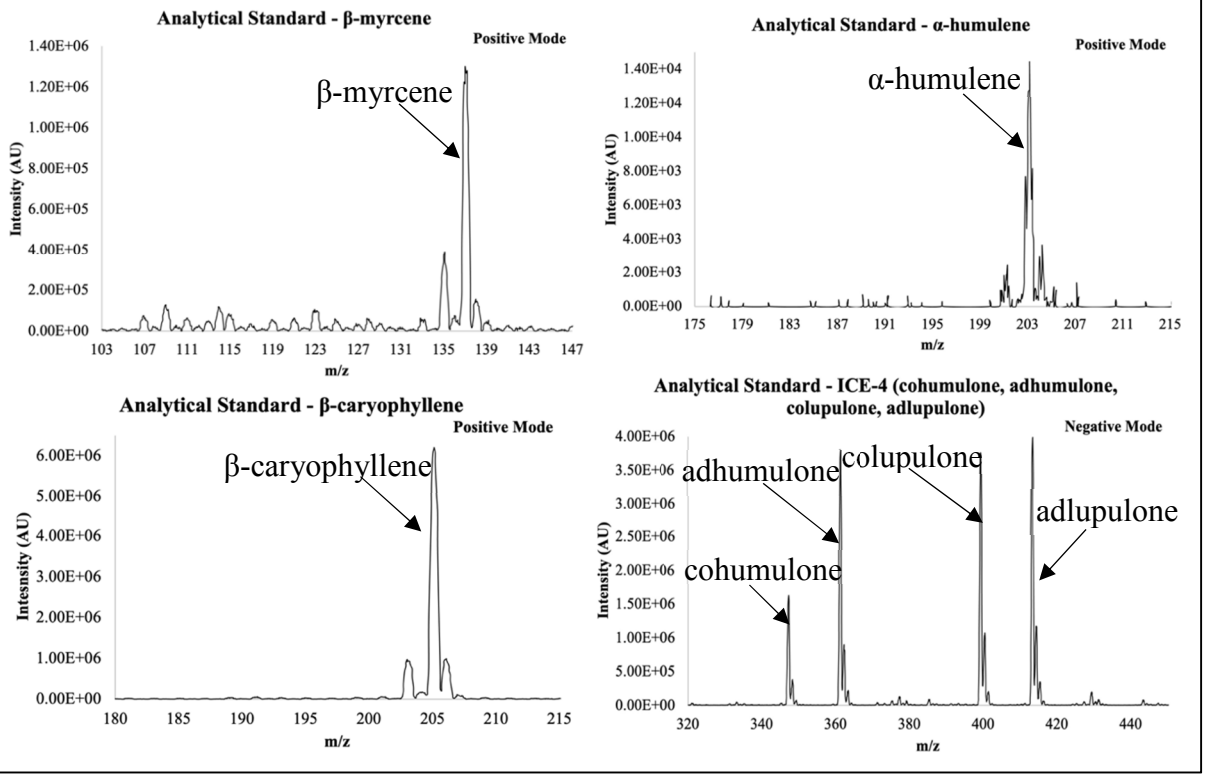
To support the annotation of specific peaks in the experimental spectra, a number of authentic standards representing known hop compounds were analyzed by DART-MS. Table 1 lists all 14 compounds tested from 11 different authentic standards (ICE-4 standard contains four compounds: cohumulone, adhumulone, colupulone, and adlupulone). In total, annotation of 7 experimental peaks was supported by detection of the corresponding authentic standards: β-myrcene, α-humulene, β-caryophyllene, cohumulone, colupulone, adlupulone and adhumulone (Figure 3.5 and Figure 3.6).

Table 1. Authentic standards evaluated using DART-MS.

Class	Compound	Detected (mode)	signal (m/z)
Terpenes	β-myrcene	yes (positive)	136
	α-humulene	yes (positive)	204
	β-caryophyllene	yes (positive)	204
	α-pinene	no	
	β-pinene	no	
	limonene	no	
	nerol	no	
	geraniol	no	
	linalool	no	
	citral	no	
Bitter acids	cohumulone	yes (negative)	348
	colupulone	yes (negative)	400
	adlupulone	yes (negative)	414
	adhumulone	yes (negative)	362

*Note that depending on ionization mode the m/z will deviate +1 m/z in positive mode and -1 m/z in negative mode

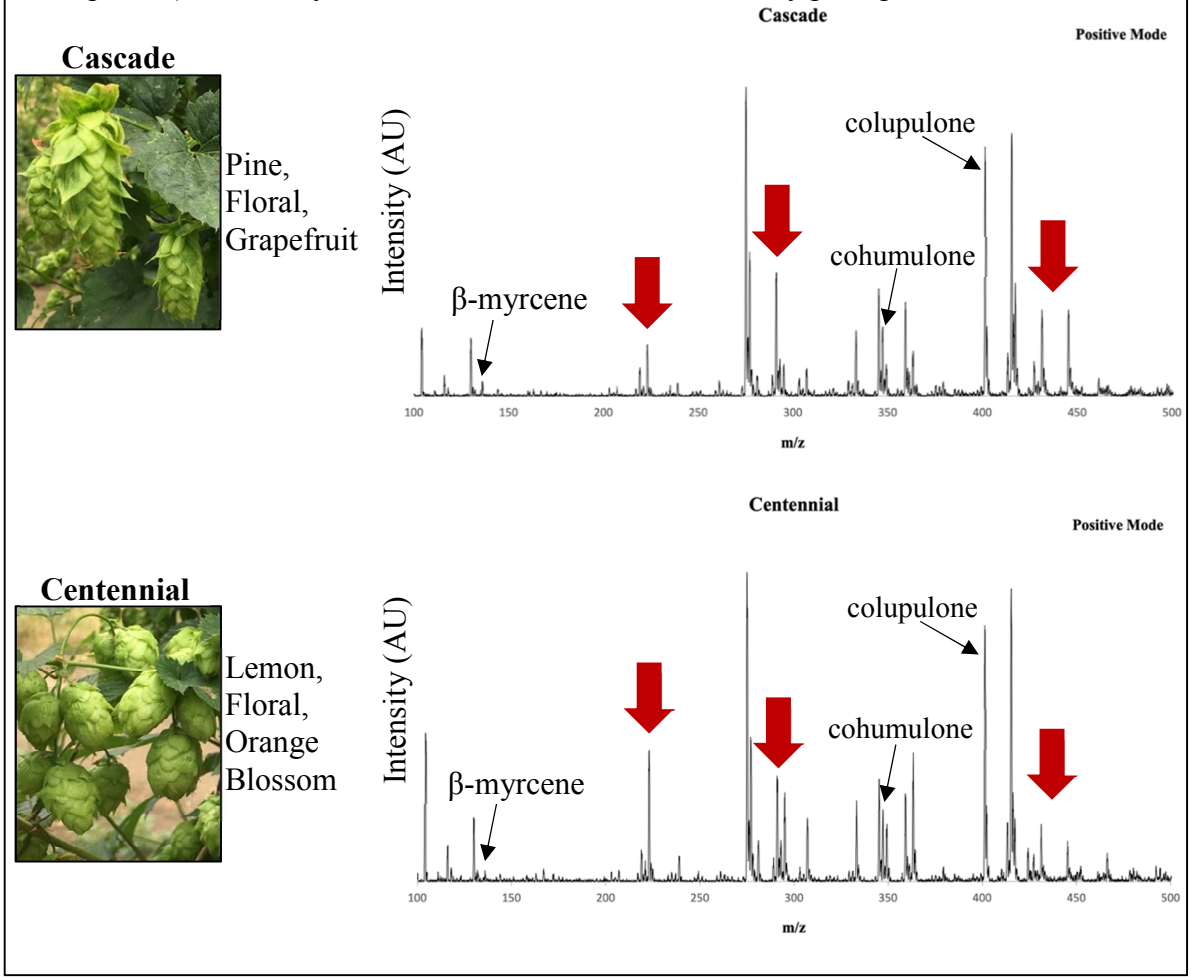
Figure 3.6. Authentic hop quality compounds detected using DART-MS. A: β-myrcene, B: α-humulene C: β-caryophyllene, and D: ICE-4 containing four compounds, cohumulone, adhumulone, colupulone, and adlupulone.



The analysis of authentic standards supports that we are detecting compounds of known importance to hop quality. However, we are specifically interested not in the presence or absence of a specific peak, but in the pattern or "fingerprint" of all the peaks detected in the hop sample. For illustration, if we compare the positive ionization DART-MS spectra for two different hop cultivars, Cascade (Washington Farm 3) and Centennial (Washington Farm 3) we can see that there are distinct differences in the overall peak pattern as indicated by the red arrows in Figure 3.7. Specifically, we see a difference in the ratios of terpenes, such as β-myrcene (*green*, *spicy*, and *hoppy*), and two important bittering acids cohumulone and colupulone[42]. Variations in concentrations of β-myrcene may affect the perception of *herbaceous* or *hoppy* and *resinous* aromas and flavors[43]. Additionally, cohumulone has the potential to

impart more bittering flavors in beer compared to colupulone[44,45]. Other variations may be more subtle and hard to discern by visual comparison of the chemical fingerprint. This overall variation in the chemical fingerprint is also supported by the known sensory differences among the cultivars; *pine*, *grapefruit*, and *floral* for Cascade and *lemon, floral,* and *orange blossom* for Centennial.

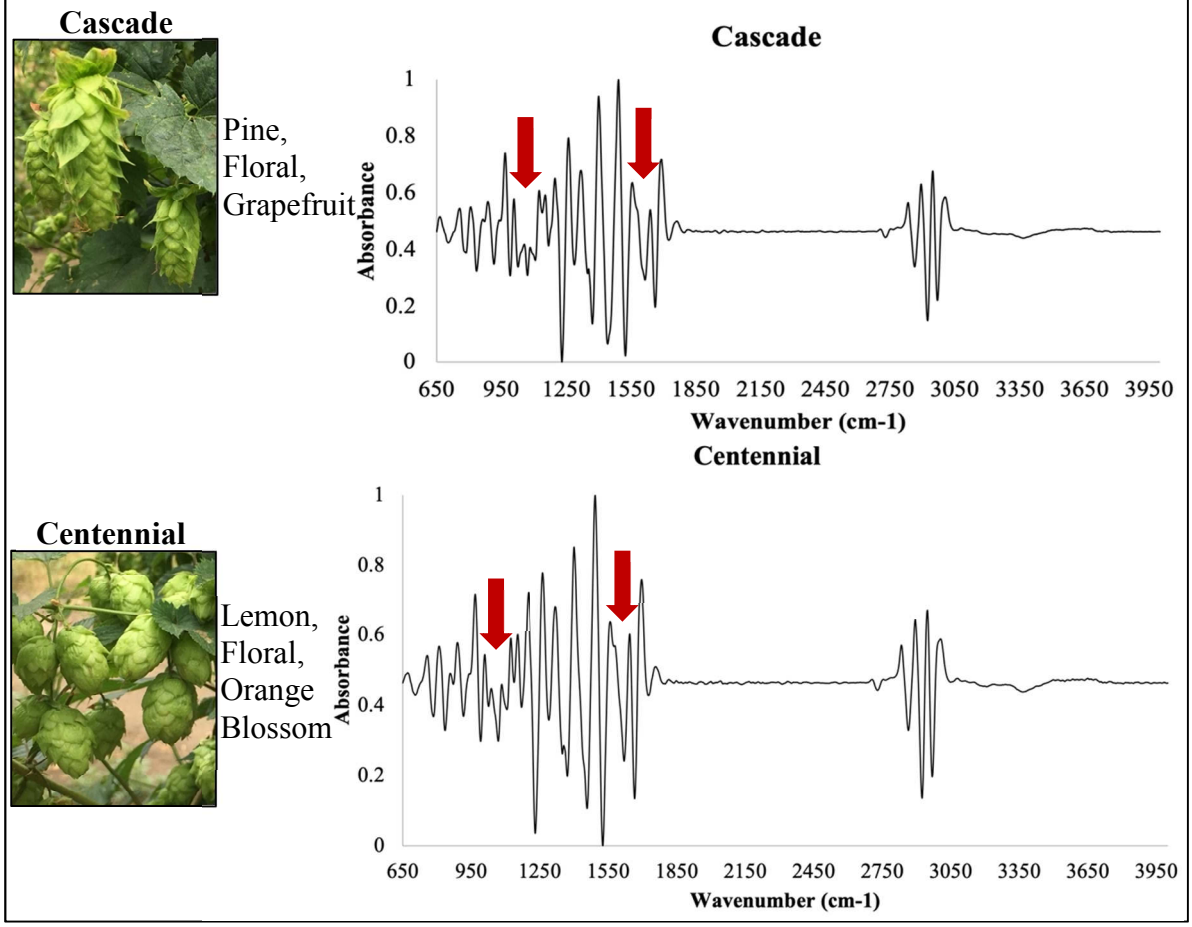
Figure 3.7 Example spectra of two different cultivars Cascade and Centennial in positive mode. Differences in presence and absence of metabolites (red arrows) with varying relative intensities (Intensity AU), comparing Cascade and Centennial spectra. The differences in terpene (β-myrcene) and bittering acid (cohumulone and colupulone) ratios may be reflective of variations in sensory perceptions.



3.3.2 FTIR enables detection of spectral hop fingerprints.

Similar to the results obtained by DART-MS, the FTIR fingerprints demonstrated qualitative differences in the spectra patterns when comparing between cultivars. For illustration, a visual comparison of the spectrochemical fingerprints for Cascade and Centennial again from Washington Farm 3 (Figure 3.8) illustrates differences in the wavenumber regions around 950 to 1250 cm-1 and 1600 to 1750 cm-1. These wavenumber regions may indicate differences in oxygenated compounds such as alcohols (1100 cm-1) or ester compounds (1200 and 1740 cm-1).

Figure 3.8. FTIR Spectrum. Comparison of hop extracts Cascade and Centennial from WA, some differences are easy to discern, but others in the spectrophotochemical fingerprints are less noticeable. The red arrows show where some differences are easy to see comparing the two hops.

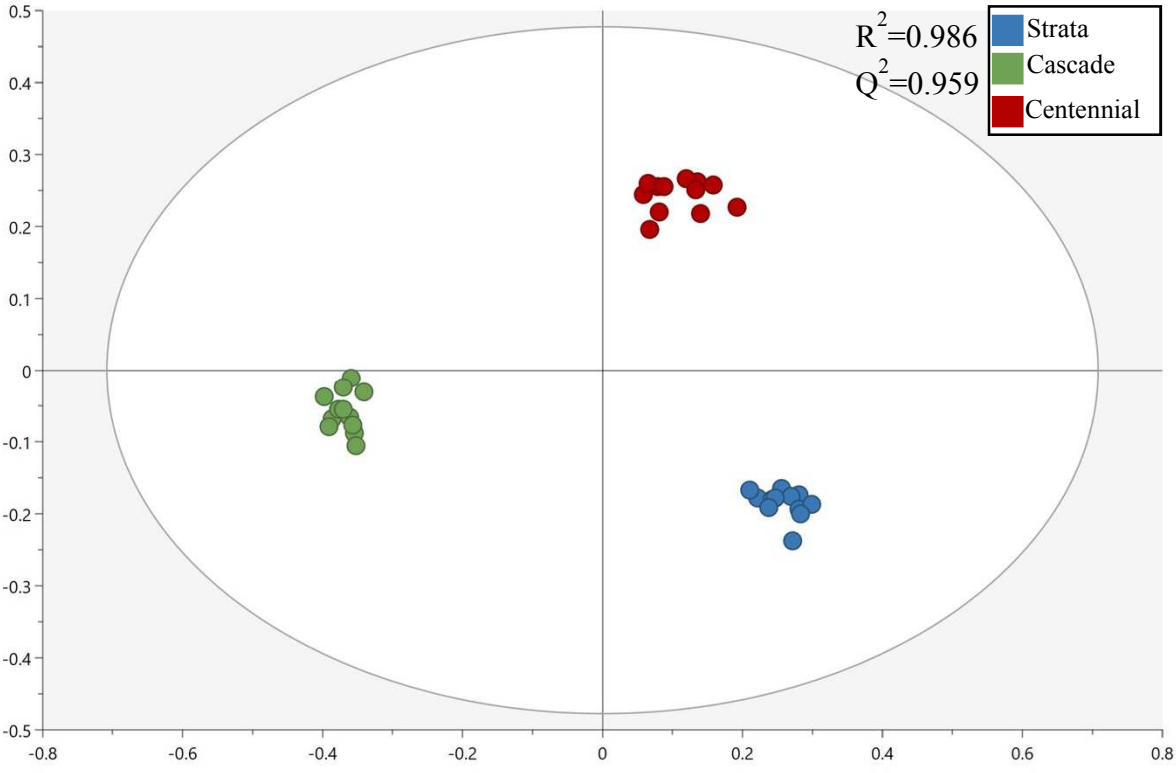


Differences in other areas of the fingerprint are subtle and difficult to discern by visual inspection.

3.3.3 DART-MS fingerprints can Discriminate Hop Samples Based on Cultivar

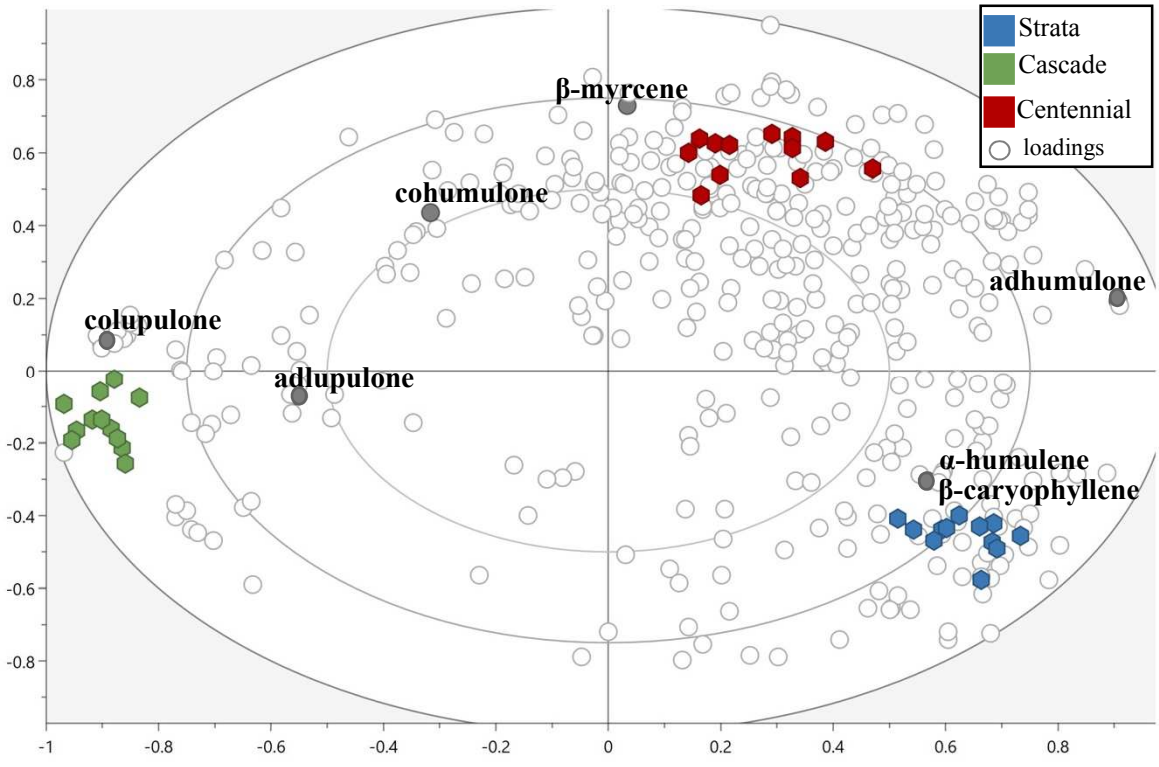
OPLS-DA models were successful in classifying samples by cultivar using spectral data from both positive and negative ionization modes. Visualization of the OPLS-DA model for positive ionization DART-MS data (Figure 3.9) demonstrates clear differentiation of the three cultivars Centennial, Cascade and Strata. The R^2 and Q^2 metrics were 0.986 and 0.959, respectively, indicating that the model is robust with a good model fit and high predictability. Further investigation of the positive ionization OPLS-DA model from crop year 2019 revealed the influence of specific compounds on the separation observed in the model. This can be

Figure 3.9. OPLS-DA model of positive ionization DART-MS spectra from the 2019 harvest season. Clear separation is observed between cultivars Centennial (green), Cascade (blue), and Strata (red). R^2 reflects the model fit and Q^2 reflects the predictive power. Values close to 1 indicate a strong and highly predictive model.



visualized as a biplot which represents both the scores (samples) and the loadings (compounds) (Figure 3.10). The compound annotations that were supported by detection of authentic standards were highlighted in the analysis (Figure 3.10, solid gray circles) and can be interpreted as higher in abundance if co-localized to the scores (solid hexagons) for a given cultivar. For example, we can see that α-humulene and β-caryophyllene both strongly co-localized with Strata samples

Figure 3.10. OPLS-DA cultivar scores (colored hexagons) and loadings (open and gray circles) of positive ionization DART-MS data. Cultivars include Strata (blue hexagons), Cascade (green hexagons), and Centennial (red hexagons) with all loadings detected (open circles) including the seven annotated compounds (solid gray circles) α-humulene, β-caryophyllene, β-myrcene, adhumulone, cohumulone, adlupulone, and



(blue hexagons). Likewise, β-myrcene co-localized with Centennial (red hexagons) and colupulone and adlupulone co-localize with Cascade (green hexagons). The localization of cohumulone indicates that it is more abundant in both Centennial and Cascade than in Strata.

Similarly, adhumulone appears to be higher in abundance in Centennial and Strata when compared to Cascade.

Previous literature has demonstrated the value of α-humulene, β-caryophyllene, and β-myrcene variation for differentiation of hop cultivars and hop application types (*e.g.,* aromatic, bittering, and dual-purpose)[46]. Aromatic hops tend to show a higher α-humulene content, whereas bittering hops are higher in β-myrcene and dual-purpose have complex to intermediate terpene profiles. This trend is also reflected our data where the aromatic cultivar Strata is most associated with α-humulene compared to Cascade and Centennial. Our results also agree with alpha and beta acid composition based on essential oil analysis (Table 2) as provided by the hop growers following ASBC methods of analysis [47–51]. Specifically, this data indicates that the

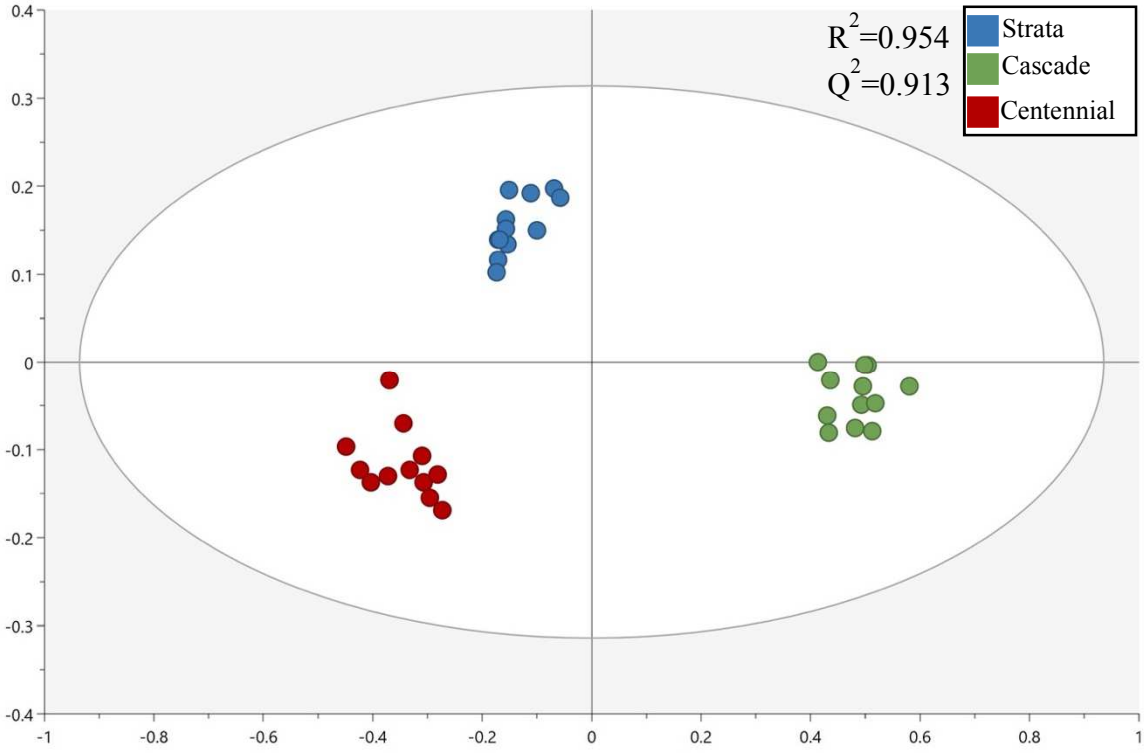
Table 2. Alpha Acid, Beta Acid, and Essential Oil Analysis (Percent of Total Essential Oil)

	Cascade	Centennial	Strata
alpha acids (adhumulone)	5-8%	9.5-11.5%	12-15%
beta acids (colupulone, adlupulone)	4-7%	3.5-4.5%	5-6%
cohumulone	40%	29-30%	21%
β-myrcene	45-60%	45-55%	52-65%
α-humulene	10-16%	10-18%	22-30%
β-caryophyllene	3-6%	5-8%	5-12%

Strata cultivar has higher α-humulene and β-caryophyllene content compared to Cascade and Centennial which is also reflected in our data.

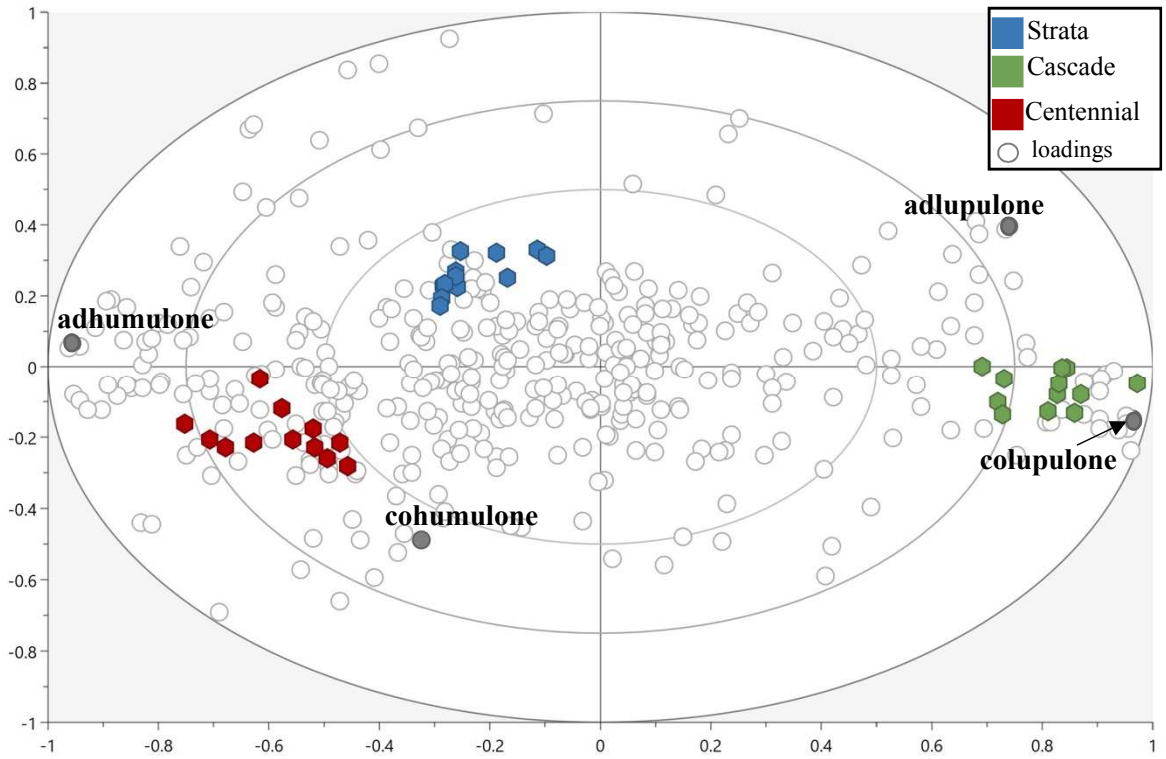
Briefly, while the spectral data generated using negative ionization mode was less rich and resulted in the detection of almost exclusively the non-volatile bittering acids (Figure 3.9), successful OPLS-DA models were also generated with this data. Similar separation and grouping among the different cultivars Centennial, Cascade and Strata was observed in the model visualization (Figure 3.11), with R^2 and Q^2 values of 0.954 and 0.913 for, respectively, indicating a well fit and predictive model.

Figure 3.11. OPLS-DA scores plot of negative ionization DART-MS spectra from the 2019 harvest season. Clear separation is observed between cultivars Strata (blue), Cascade (green), and Centennial (red). R^2 reflects the model fit and Q^2 reflects the predictive power. Values close to 1 indicate a strong and highly predictive model.



Four of the seven annotated compounds were detected in negative mode and are represented as solid gray circles in the biplot (Figure 3.12). Similar to what was observed in the models generated with the positive ionization mode data, colupulone and adlupulone are co-localized with Cascade, cohumulone is co-localized with Centennial, and adhumulone is co-localized with Centennial and Strata.

Figure 3.12. OPLS-DA cultivar scores (colored hexagons) and loadings (open and gray circles) of negative ionization DART-MS data. Cultivars include Strata (blue hexagons), Cascade (green hexagons), and Centennial (red hexagons) with all loadings detected (open circles) including the four annotated compounds (solid gray circles) adhumulone, cohumulone, adlupulone, and colupulone.



3.3.4 DART-MS Pooled QC fingerprints indicates method performance reproducibility

Using pooled QC's is important during normal analysis to determine if the method of collecting data is reproducible. OPLS-DA model of positive ionization mode 2019 harvest year data including pooled QC fingerprints in Figure 3.13 visualizes the QC scores and cultivars as filled circles (QC (yellow), Strata (blue), Cascade (green), and Centennial (red)), where the QC scores are clustering together in the center of the model. This is supportive of a reproducible method. Further, investigating the coefficient of variation (CV) of the pooled QC data in Figure 3.14 as a histogram reveals that 90% of the mass bins representing the spectral data of the QC fingerprints has a CV of 20% or less. This is indicative of a good method performance.

Figure 3.13. OPLS-DA QC Scores. The QC scores (yellow) are clustering in the center, separating from the cultivars Strata (blue), Cascade (green), and Centennial (red).

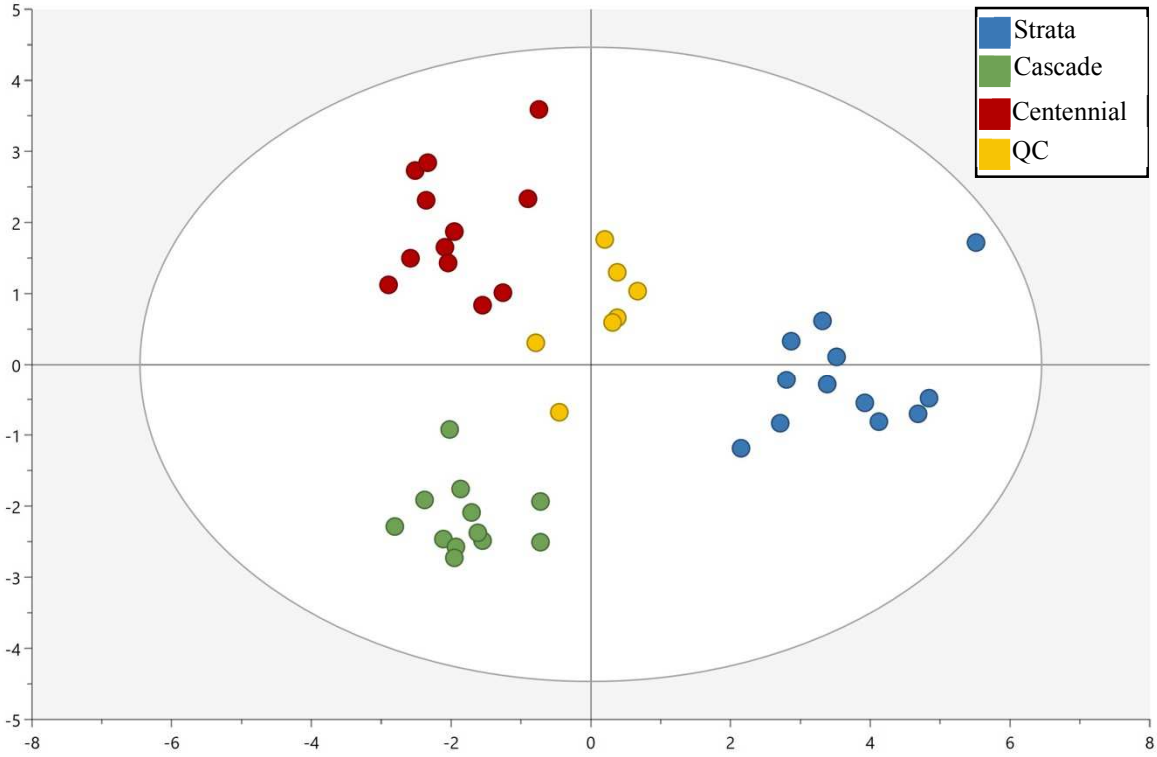
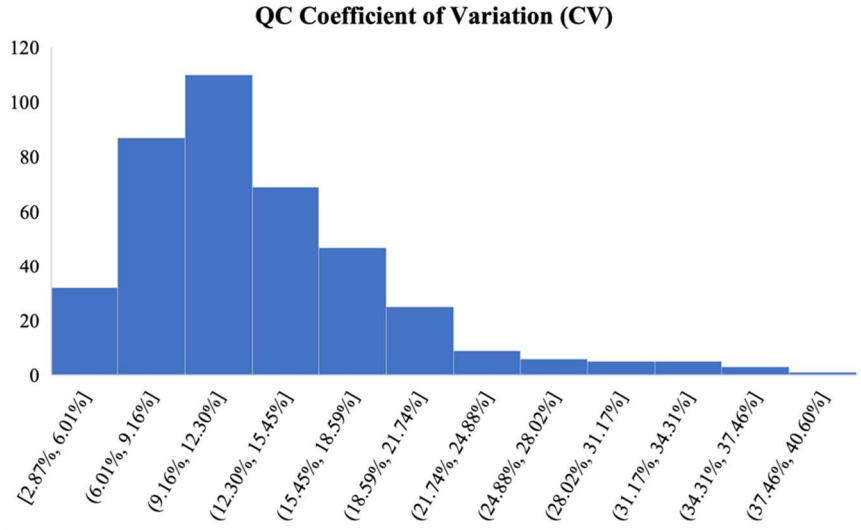


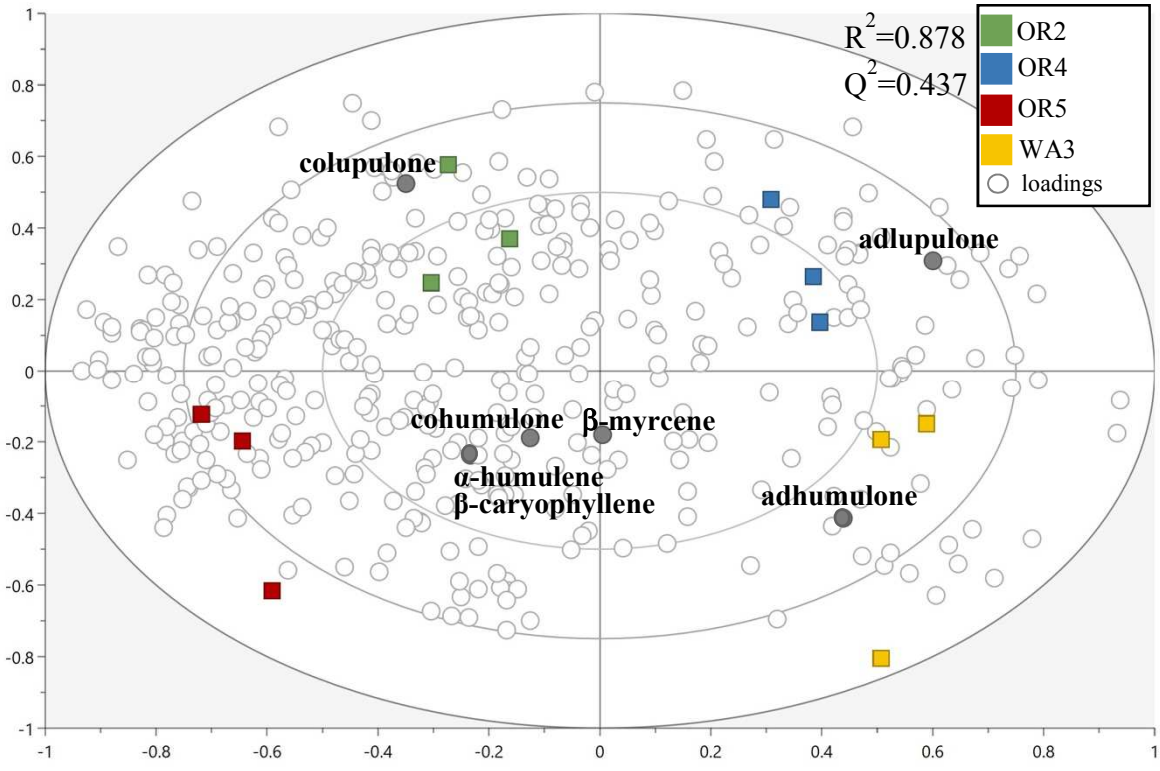
Figure 3.14. Coefficient of Variation (CV) of Pooled QCs. 90% of the mass bins representing the spectral data of the pooled QCs have a CV of 20% or less demonstrating a reproducible method of analysis.



3.3.5 DART-MS fingerprints are less effective at Discriminating Hop Samples Based on Environment

The DART-MS data (positive ionization) from the Centennial cultivar in 2019 was used to evaluate the potential for classification based on growing environment. The OPLS-DA model for environment is much less robust than what was observed for cultivar differences, R^2 and Q^2 metrics of 0.878 and 0.437, respectively (Figure 3.15). Although the model metrics indicate a less robust model fit and lower predictive power, there still appears to be some separation among the different growing locations. Visualizing the OPLS-DA model as a biplot with the metabolite loadings (gray circles), there are stronger associations of certain metabolites with different

Figure 3.15. OPLS-DA environment scores (colored hexagons) and loadings (open and gray circles) of positive ionization DART-MS data for cultivar Centennial. Three of the environment locations were in OR, OR1(green), OR4 (blue) and OR5(red), and one was in WA (yellow). The loadings include the seven annotated compounds (solid gray circles) α-humulene, β-caryophyllene, β-myrcene, adhumulone, cohumulone, adlupulone, and colupulone.



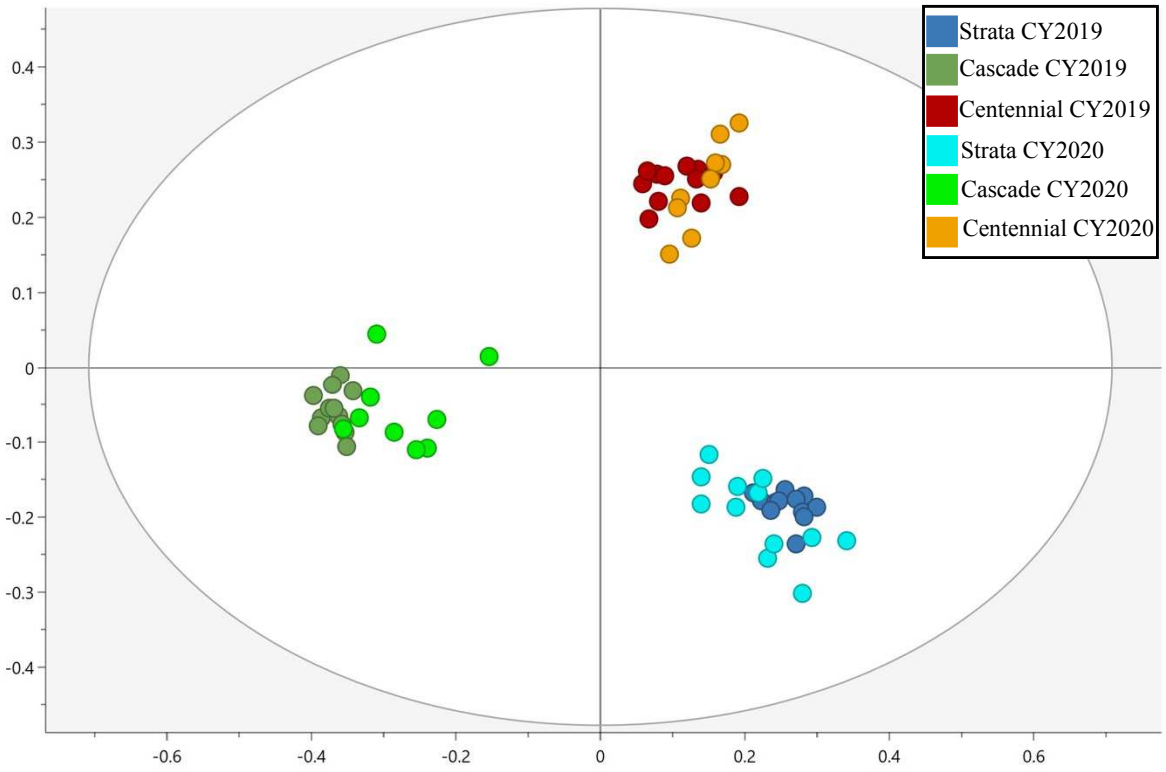
locations (colored squares; Oregon Farm 2 (green), Oregon Farm 4 (blue), Oregon Farm 5 (red) and Washington Farm 3 (yellow)). For example, the alpha-acid adhumulone is co-localizing with the Washington location while the beta-acid colupulone is co-localizing with the Oregon Farm 2.

These results demonstrate potential for this approach to be utilized for evaluation of environmental impact within the same cultivar. It is likely that differences between environments are more subtle than that between cultivars and thus in order to capture this variability, future work should include collection of a much larger number of hops samples and replicates for each growing location. Previous literature has demonstrated that the same cultivar, Cascade, from different geographical growing locations in Italy had varying hop cone chemical compositions[52]. Ultimately, the successful application of a method such as DART-MS that could quickly and easily differentiate hop samples based on environment could be hugely important for hop producers and brewers. DART-MS technology as a tool for QA/QC programs could help ensure consistency in hop products year over year. Additionally, as a screening tool, DART-MS could be used by hop breeders and growers selecting plants to advance in experimental trial lines or determining when hops are at maturity and ready for harvest. Utilizing DART-MS for plant selection in these scenarios could support sensory programs to reduce the time it takes to make these important decisions of which experimental plants to advance and when hops are ripe for picking.

Validation of predictive models across two crop years

The positive ionization mode DART-MS data collected from harvest year 2020 was used as an independent test set to validate the OPLS-DA models trained with DART-MS data collected from the 2019 harvest year. Figure 3.16 visualizes this validation showing the model based on the 2019 data (filled colored circles for each cultivar Strata (blue), Cascade (green), and

Figure 3.16. Testing the DART-MS positive mode OPLS-DA models. Scores plot of the OPLS-DA model trained using 2019 harvest year samples (positive ionization) shown in solid circles Strata (blue), Cascade (green), and Centennial (red). Predicted scores of 2020 harvest year samples shown solid circles Strata (light blue), Cascade (light green), and Centennial (light orange).



Centennial (red)) and the corresponding predicted scores using this model for the 2020 data (filled colored circles for each cultivar Strata (light blue), Cascade (light green), and Centennial (light orange)). The model was able to predict the cultivar of the 2020 harvest year hop samples based on the positive ionization DART-MS data with 100% accuracy.

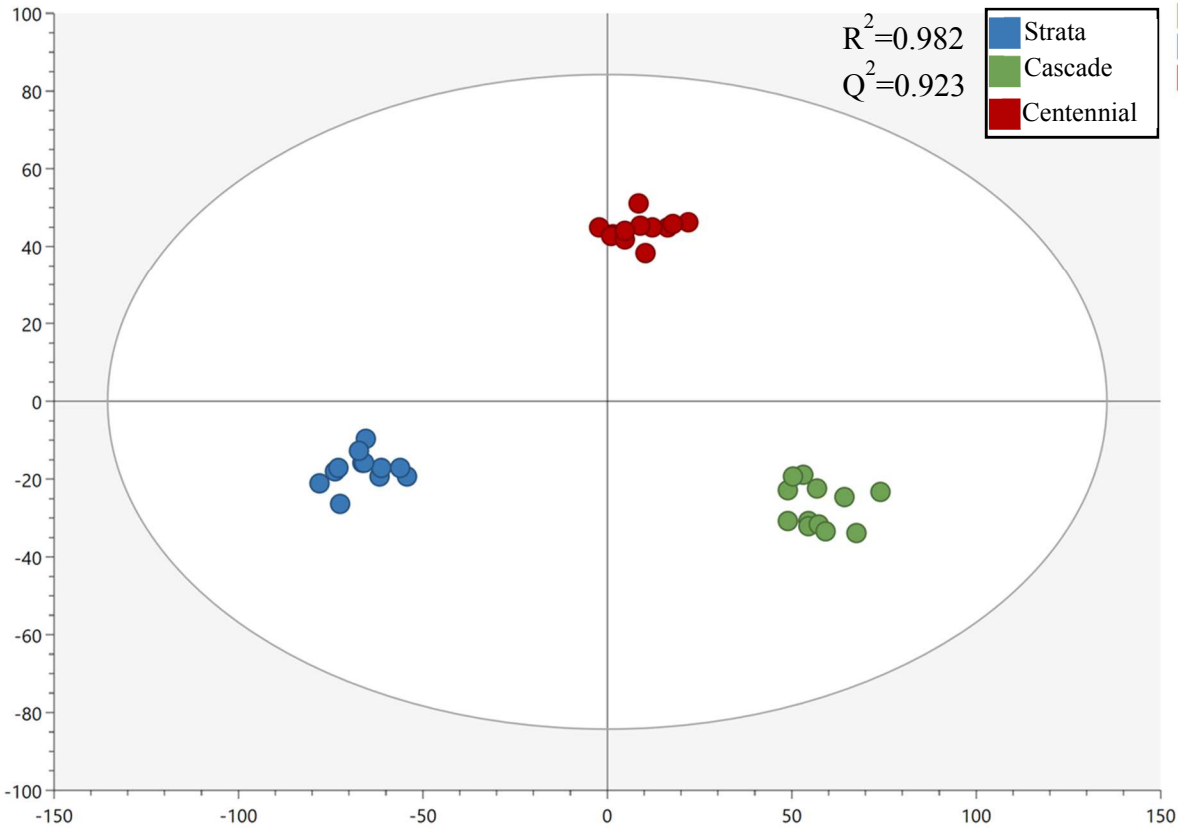
3.3.6 FTIR as an alternative analytical tool to Discriminate Hop Samples Based on Cultivar and Environment

FTIR was also evaluated as a high throughput analytical tool for characterization of hops. FTIR is less specific than DART-MS technology detecting broad chemical classes based on differences in bond energies. The many benefits of FTIR includes lower costs, the system is

intuitive and easy to use, and little to no sample preparation. FTIR and other spectroscopy approaches (*e.g.,* NIR) are commonly used as QA/QC tools in food industries and thus there may be lower barriers to adoption for this technology.

OPLS-DA models were successful in classifying samples by cultivar using spectral data from FTIR (Figure 3.17). Various data pre-processing approaches were evaluated, and it was determined that using the 2nd derivative covering 49-point intervals yielded the highest R^2 (overall model fit) and Q^2 (predictability power) of 0.982 and 0.923, respectively (Figure 3.17). This result demonstrates that FTIR is also a robust analytical method for classification of hop samples by cultivar with comparable predictive quality to DART-MS.

Figure. 3.17. OPLS-DA scores plot of FTIR data from the 2019 harvest season. Clear separation is observed between cultivars Strata (blue), Cascade (green), and Centennial (red). R^2 reflects the model fit and Q^2 reflects the predictive power. Values close to 1 indicate a strong and highly predictive model.



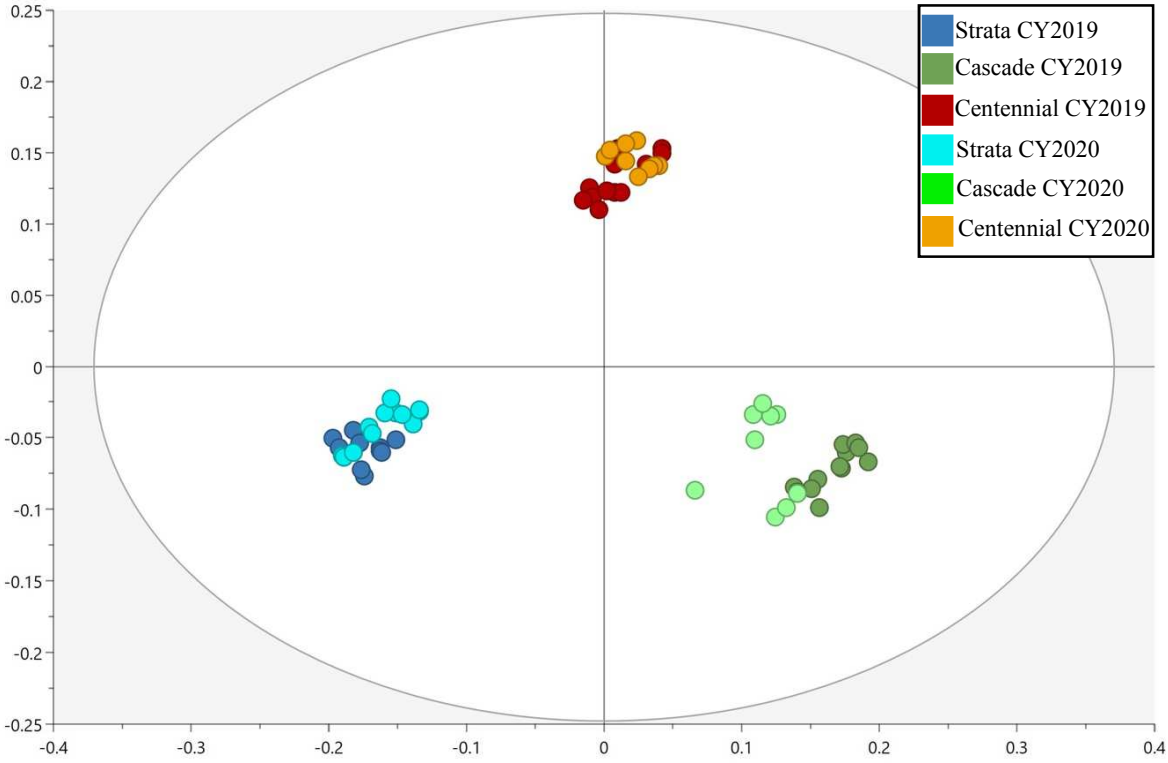
Differentiation by Environment was not achieved based on FTIR data

Training of a predictive OPLS-DA model to differentiate hop samples by growing location (*e.g.*, environment) using FTIR data was not successful (data not shown). As discussed above for DART-MS, it is likely that larger sample numbers are required to adequately capture the variation between environments.

Validation of predictive models across two crop years

The hop samples collected during the 2020 harvest were used as an independent test set to validate the FTIR OPLS-DA model trained by the previous year's 2019 hop samples. The 2020 hop samples were treated as unknowns to see if the trained model can correctly differentiate the samples based off the spectral FTIR data. Figure 3.18 visualizes the 2019 model

Figure 3.18. Testing the FTIR OPLS-DA models. Scores plot of the OPLS-DA model trained using 2019 harvest year samples shown in solid circles Strata (blue), Cascade (green), and Centennial (red). Predicted scores of 2020 harvest year samples shown solid circles Strata (light blue), Cascade (light green), and Centennial (light orange).



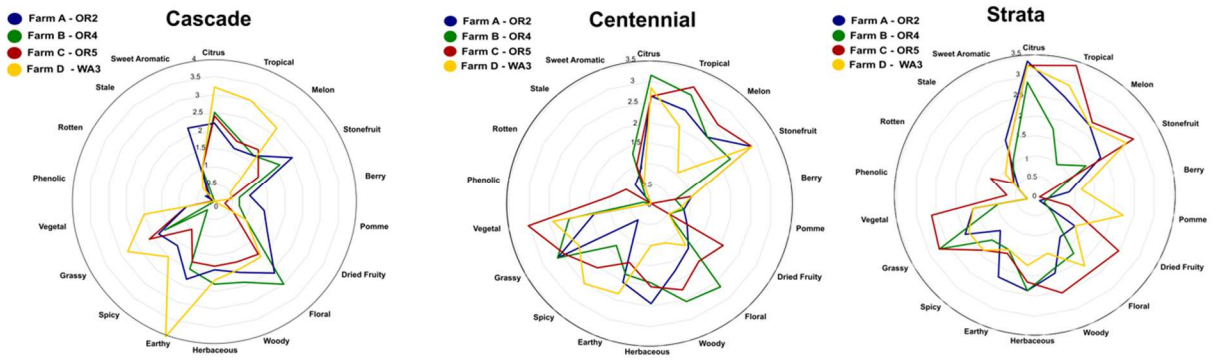
(filled colored circles for each cultivar Strata (blue), Cascade (green), and Centennial (red)) and then tested with the 2020 data (filled colored circles for each cultivar Strata (light blue), Cascade (light green), and Centennial (light orange)). The model was able to predict the cultivar of the 2020 harvest year hop samples based on the FTIR spectral data with 100% accuracy.

3.3.7 Sensory Analysis

Sensory analysis panelists were able to discriminate among cultivar and environment based on aroma. Spider plots for each individual cultivar demonstrate the differences that were detected based on cultivar and growing location (environment). These sensory analyses support the identified compounds detected by DART-MS. Cascade was determined to have less dominant aromatic attributes, with the exception of the WA farm cultivar, perceived as *earthy, citrus,* and *grassy* (Figure 3.19). Strata (Figure 3.19) was overall the most complex of the hops and determined to have the most *herbaceous* characteristics as well as being perceived as *citrus and tropical.* This result supports the DART-MS results in which Strata was observed to be associated with detected compounds α-humulene and β-caryophyllene (Figure 3.10) that contribute to more *herbal*, *woody*, and *spicy* aromas[53,54]. Centennial (Figure 3.19) was the most variable among location, hitting broad categories similarly (broadly *earthy*, *grassy*, *tropical*, *citrus*, and *stonefruit*), but nuanced depending on location. Again referencing Figure 3.10, we see Centennial covarying with β-myrcene, which according to literature can be perceived as *earthy* and *herbaceous*, but even more importantly is a precursor to other important odorants such as linalool and geraniol that contributes *citrus*, *fruity*, and *floral*[53]. Cascade and Centennial demonstrate more dual-purpose hop characteristics being more associated with intermediate terpene profiles, β-myrcene, and with alpha-acids and beta-acids. The dual-purpose potential is

also reflected in the sensory of Cascade and Centennial demonstrating less dominant characteristics and having a more broad "hoppy" profile.

Figure. 3.19. Sensory, spider plots



3.4 Conclusions and Future Directions

In this study I evaluated two rapid, high-throughput analytical technologies coupled with chemometric analysis for the characterization of hops based on cultivar (*e.g.,* genetics) and growing environment. Both technologies were assessed for the potential for objective analyses and true-to-type quality screening of hops samples.

My analysis supports that data generated by DART-MS can be used to train a predictive model to classify hops samples based on cultivar and that these cultivar differences may be linked to quality (*e.g.,* sensory). The data generated by DART-MS also shows strong potential for classification of hops based on environment, but more research with a larger number of hop samples is necessary to validate this capability. Additionally, the identification of specific compounds detected by DART-MS was supported by the analysis of authentic standards for compounds of known importance to hop sensory and quality; α-humulene, β-myrcene, β-caryophyllene, alpha-acids (cohumulone, adhumulone) and beta-acids (colupulone, adlupulone).

Spectroscopic data generated by FTIR was also successfully utilized to train predictive models for classification of hop samples by cultivar. Both DART-MS and FTIR have the benefits of being relatively low-cost, require few consumables (*e.g.,* solvents), have intuitive software, require minimal to no sample preparation, and are high-throughput with real time data acquisition. Both technologies have the potential for future applications as a tool for hop breeders, growers, and craft brewers. Future research could evaluate the potential of these technology to guide on breeding and agronomic management. Currently, both breeding and hop growing programs rely heavily on sensory analysis, where plants are either selected for breeding trials or major agronomic practice decisions depend on the sensory results (*e.g.,* harvest time). Analytical technologies like DART-MS and FTIR would be beneficial tools to quality assurance programs from hop breeders, growers, craft brewers to ensure consistency in hop products.

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