THESIS

CHARACTERIZING THE IMPACT OF PACKAGE TYPE ON DIFFERENT BEER STYLES USING ADVANCED ANALYTICAL TOOLS

Submitted by

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ABSTRACT

CHARACTERIZING THE IMPACT OF PACKAGE TYPE ON DIFFERENT BEER STYLES USING ADVANCED ANALYTICAL TOOLS

In 2020 there was over 9,000 breweries in the US, increasing the beer market competition and driving the importance of product stability under variable storage conditions. More breweries, specifically craft breweries, than ever before are choosing to package in cans due to ongoing effects of the current pandemic, the growing availability of smaller can line systems, and increased mobile canning options. Foundational beer stability research has focused on light lager styles packaged in bottles. Limited research has been conducted studying flavor stability in styles relevant to the American craft brewing industry, nor any comparisons of how package type (i.e., cans and bottles) affects flavor stability. Industry utilizes trained sensory panels to evaluate flavor stability; a resource that is both time consuming and expensive. Thus, this is a tool that is often inaccessible or inadequate for providing relevant and timely stability data.

This research project, a collaboration between New Belgium Brewing and Colorado State University, aims to address the package-type knowledge gap and sensory panel restrictions by utilizing advanced analytical tools to characterize the changes in metabolite profiles over time between cans and bottles. A low-hopped amber ale (AA) and high-hopped India Pale Ale (IPA) were chosen for their distinct style and relevance to the American craft brewing industry. One batch of an IPA and AA was packaged into cans and bottles, then aged for a six-month period. The samples were stored under cold temperatures (4°C) for the first 30 days, and then at room temperature (20°C) for the subsequent time. Aliquots were collected biweekly for a total of 13

timepoints throughout the six-month aging period and stored at -80°C until chemical analysis. Chemical analysis was conducted by gas chromatography coupled to a mass spectrometer detector (GC-MS) and direct analysis in real time mass spectrometry (DART-MS) to address the research questions. Multivariate (MVA) and univariate (UVA) statistical analysis of the GC-MS data allowed for the characterization of the impacts of package container on the chemical profiles of AA and IPA over time. MVA of the DART-MS data explored the predictive power of the tool for streamlining beer flavor stability analysis.

Partial least squares discriminant (PLS-DA) and Multiple Analysis of the Variance (MANOVA) statistical analyses were used to explore data produced by GC-MS and helped define a group of 17 detected metabolites important to explaining the data variation. PLS-DA models of AA samples demonstrated good model fit and package type predictability ($R^2 = 0.981$, $Q^2 = 0.964$). This was not observed in IPA which indicates package effects are styles dependent. Differences in AA samples are due, in part, to can and bottle baseline differences in the detected amino acid and ester metabolites. Differences in the physical packaging process of cans, oxidations, and low hop polyphenol concentrations are proposed mechanisms for explaining the observed baseline differences. Analysis of variance (ANOVA) found ten metabolites in AA cans significantly ($P \geq 0.05$) changing over time as compared to four metabolites in AA bottles. This indicates higher instability in cans for AA samples. Four detected hop volatiles (humulene, **β**-myrcene, **α**-calacorene, pinocarvone), identified by estimated marginal mean of linear models (95% confidence interval) had exhibited significant changes over time that were dependent on package type interactions, but to varying magnitudes and directions depending on the metabolite's polarity and susceptibility to packaging material interactions (e.g., scalping). PLS-DA models of data produced by DART-MS indicated a poor model fit and lack of beer storage

time predictability in AA samples (R^2 = 0.554, Q^2 = -0.151) and IPA samples (R^2 = 0.622, Q^2 = -0.079). These results lack the evidence that DART-MS is a useful tool for streamlining beer stability analysis. However, results for package type predictability matched GC-MS analysis conclusions in that package type predictability is style dependent. The overall study results demonstrate there is much nuance in the effects of package type on beer flavor stability, and those effects depend on style, packaging material, and the individual metabolite. Targeted analysis is needed to fully understand the mechanisms driving the effects of package type on beer stability.

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CHAPTER 1 – INTRODUCTION

1.1 What is beer?

Beer is an alcoholic beverage that is made from the fermentation of four main raw ingredients; malted barley, hops, yeast, and water. A liquid called wort is brewed from water, malt, and hops to form fermentable sugars for yeast to metabolize the carbohydrate energy source through alcoholic fermentation. During fermentation the sugar substrate is transformed into ethanol, carbon dioxide, and other flavor active chemical compounds. There are many examples of alcoholic beverages that require the fermentation of various sources of fermentable sugars. Examples of other sugar sources include fruits (grape, apple, pear), cereals (barley, wheat, millet, sorghum, rice, corn), and tubers (potato, sweet potato, cassava, sugar beet)[3, 4]. What makes beer the beverage that has been ingrained in our civilization for thousands of years is that the sugar source is a malted cereal grain[5].

1.2 The Four Main Raw Ingredients Necessary to Produce Beer

1.2.1 Hordeum vulgare - Barley

Barley (*Hordeum vulgare*) is a cereal grain in the Poaceae, or grass, family. It grown mainly for its use in brewing and animal feed[6]. The United Stated Department of Agriculture (USDA) reported 165 million acres of barley were planted in the United States in 2020, and of that, 6.5 million acres were grown in Colorado[7]. For use within the brewing industry, the barley seeds must first undergo a process known as malting. The malting process consists of three phases: steeping, germination, and kilning. During steeping the barley seeds are placed in water between 13-16°C with forced air running through the mixture. This environment initiates

plant growth. Once growth is initiated, the grain is transferred to a germination vessel. During the germination phase various enzymes are produced in the endosperm, the energy storage site of the barley seed. Examples of such enzymes include **β**-glucanases responsible for breaking down cell walls, lipases responsible for lipid oxidation, and proteases which break down the protein matrix that surround small and large starch granules[3]. Additional examples include **α**-amylase and **β**-amylase, the two enzymes responsible for the further breakdown of the starch granules during the brewing process.

Before the seed can grow into a plant, growth is quenched by applying heat during the kilning phase. Successful malting will result in preserved sugars, enzymes, and amino acids important to the brewing process. Additionally, time, temperature, and airflow can be adjusted during malting to produce a variety of flavors through Maillard and caramelization reactions. The various flavors and aromas that are created during malting are then used by brewers to produce various styles of beer[8].

1.2.2 Humulus lupis - Hops

Hops (*Humulus lupis)* are a vinous plant in the Cannabaceae family. The cones are the flowering body of the plant and represent the part of the plant used in brewing. In 2020, 104 million pounds of hops were produced in the US corresponding to a an economic value of $619 million[9]. Initially hops were used to add flavor and stabilize beer shipped long distances from European colonies around the world. Today, hops are used to impart bitterness, flavor, and more recently turbidity[10]. Hops are mostly made up of cellulose, water, proteins, tannins, hop resins, and essential oils. The latter two constituents, housed within the lupulin glans, are what give the plant its brewing value. The hop resins contain **α**-acids and **β**-acids which are important for

providing a bitter flavor. The **β**-acids have antimicrobial properties, preventing the growth of some beer spoiling microorganisms. The essential oil fraction contains various terpenes, sulfur containing compounds, and other flavor active compounds that provide pleasant, as well as unpleasant, flavors and aroma[11].

There are a wide range of hop cultivars that are used in the brewing industry. Both genetic (i.e., cultivar) and environmental (i.e., growing region) conditions influence the size, yield, hop acid content, and essential oil profiles of hops. The **α**-acid and hop oil content can be leveraged by brewers to create unique flavor profiles in beer. Additionally, the scope of cultivars available to brewers is not static. Hop breeding programs around the nation and world work to create new varieties that can compete with environmental and disease pressures, produce a high yielding crop, and contribute novel flavors for brewers[12]. Hops are commercially processed into many forms such as whole cone, pellets, and extracts. Like malts, brewers adjust hops within recipes to create specific beer styles.

1.2.3 Saccharomyces species - Yeast

Brewer's yeast (*Saccharomyces sp.*) is the ovoid single-celled fungi responsible for alcoholic fermentation in beer production. It is a eukaryotic organism and has the capacity to undergo aerobic metabolism and fermentation. Yeast metabolize glucose, sucrose, fructose, maltose, maltotriose, and galactose for sources of energy[13]. Specifically, in an oxygen rich environment yeast will undergo aerobic respiration resulting in the conversion of sugars into carbon dioxide and water. Under environments of high sugar concentration, metabolic pathways of respiration are repressed under what is called catabolic repression, or the Crabtree Effect. This forces the cellular functioning of the yeast to undergo fermentation as the mechanism for energy

production. The byproducts of fermentation include ATP, ethanol, carbon dioxide, and other flavor active compounds[14].

There are hundreds of strains of *Saccharomyces sp.* that behave uniquely. For example, some yeast strains are capable of fermenting at warmer temperatures, some prefer to ferment at cooler temperatures, others will ferment quickly, and some are high producers of specific flavor active compounds such as diacetyl and esters. Yeast can be grown under natural and biotechnical means to provide new and novel strains. Additionally, as this is the transformative component of the brewing process, the products of fermentation are controlled by the substrates at the start of the process[14]. Thus, the choice of malt, hops, and yeast will contribute to the overall flavor profile of the final beer product.

1.2.4 Water

Water, known as liquor in brewing, is the largest component of beer by volume. Water is used throughout the brewing process, described in *Section 1.3*. Water should be essentially pure; however, it is common for brewers to adjust the salts in their liquor to control flavor attributes. The ratios of calcium, chloride, and sulfates can be adjusted to achieve specific flavor profiles resulting from salt interactions with malt and hops. Target ratios have been determined from historical beer producing locations; Burton-in-Trent for pale ales, Dublin for stouts, and Pilsen for pale lagers are such examples. Thus, water quality represents yet another way to manage and manipulate flavor profiles to produce a complex beer matrix[15].

1.3 Overview of the Brewing Process

1.3.1 The brewhouse

The brewing process first begins in the brewhouse where milled malts and water are combined in a vessel called the mashtun to create the "mash" (Figure 1). Typically, the water is preheated to around 65°C before combining with the malt to optimize activity of malt enzymes important for the conversion of large sugar molecules (e.g., amylose and amylopectin) into small fermentable sugars. The enzymes responsible for sugar conversion are **α**-amylase and **β**-amylase. Mashes can range from thirty minutes to two and a half hours in length to allow time for enzymatic activity. At the end of the mash, the resulting liquid is called "wort". The main fermentable carbohydrates that make up wort are glucose, maltose, sucrose, and maltotriose[16].

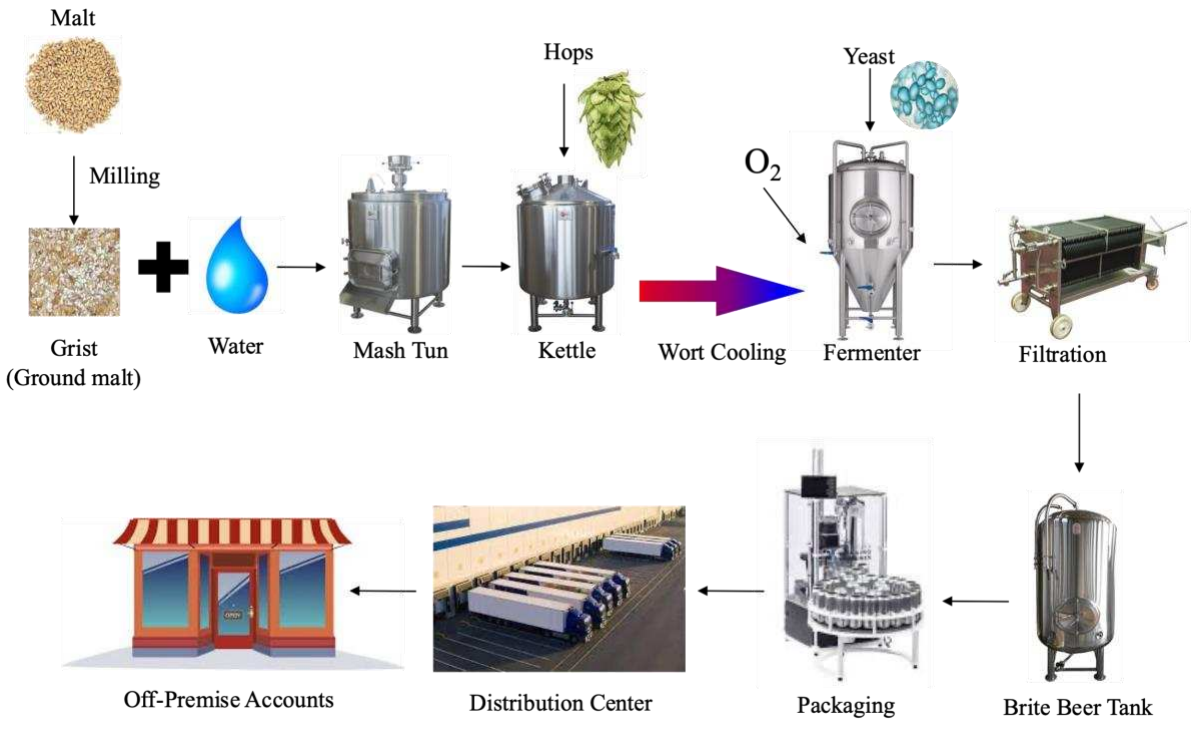

Figure 1. A generalized process flow diagram representing the brewing process.

After mashing, the liquid wort is separated from the grain solids during a process called lautering to recover residual sugars and clarify the wort. Then, the wort is transferred to a vessel called the kettle where it is boiled for one to two hours (Figure 1). Hops are now added at distinct times to achieve desired sensory outcomes. Hop **α**-acids undergo isomerization transformation when heated resulting in iso-**α**-acids, the compound that cause bitterness in beer. The longer hops are boiled, the more isomerization occurs resulting in a higher degree of bitterness in the beer. The closer hops are added to the end of the boil, the less essential oil is volatized, increasing the concentration of terpenes and other flavor compounds in the beer. Brewer's may also choose to use less hops to produce a malt forward beer. Additional outcomes from the boil are microbial stabilization, sugar concentration from water evaporation, and the formation of protein-polyphenol complexes which settle out in the form of trub which is then removed before fermentation[17].

1.3.2 Fermentation and maturation

At the end of the boil the hopped wort is cooled through a heat exchanger before moving into a fermenter (Figure 1). The cooled wort is oxygenated to promote cellular growth of the yeast. This is the only point in the brewing process when oxygen is desirable. Small carbohydrate molecules generated in the brewhouse are now available to yeast to metabolize through fermentation. The end products of fermentation are carbon dioxide, ethanol, heat, and other flavor active compounds[14]. Because heat is produced during fermentation, temperature control provides an additional method for specification of desired sensory profiles. For example, fermentations carried out at higher temperatures will result in higher ester production[18, 19]. Typical ale fermentations are carried out at 18-25°C for 2-3 days, whereas lager fermentations

are performed at 5-15°C for much longer periods of time. Fermentation is complete when all the fermentable sugars have been metabolized by the yeast. This is tracked by measuring sugar concentration in units of specific gravity or degrees Plato.

After fermentation is complete the beer enters maturation where yeast settle out and unwanted flavor active compounds dissipate. The beer is then chilled and yeast are removed to clarify the beer. Clarification can be achieved through time, food safe fining agents, filtration, or centrifugation (Figure 1). Lastly, the bright beer is carbonated with carbon dioxide.

1.3.3 Packaging

Once a beer is matured it is ready for packaging so that it may reach the consumer. There are two main types of packaging options available to the brewer; “large volume” in the form of kegs and casks, and bottles and cans referred to as “small-pack”, or single use single serving. Small-packed beers make up most of the packaged beer, representing 91% of the sales market in 2000[20]. Packaging is essential to both the economy and quality of beer. Among kegs, bottles, and cans there are numerous sizes, materials, and shapes in which a beer can be presented to the consumer. It is important to appeal to the consumer and provide a high-quality and consistent product; thus, packaging quality must be a high priority.

Kegs are found at restaurants, bars, and other off-premises accounts where the beer is served through a draught line system. Cans and glass bottles are the most common form in which a consumer will bring beer home for consumption. Although similar in function and size offerings, cans and bottles are inherently different. In the United States, cans are made of aluminum coated on the inside with a protective food grade polymer, and come in two pieces (i.e., can body and the lid). Bottles are made from brown, green, or clear glass and come in two

pieces (i.e., bottle and the crown) as well. A packaging line fills the container with beer and attaches the lids of the can, or crown of the bottle. The can lid is hermetically sealed, or airtight, while the seal of the crown is permeable and allows air ingress over time[21]. The opaque material of the aluminum blocks ultraviolet (UV) light from encountering the beer, preventing known light induced reactions that can results in undesirable flavor changes known as "skunking". Transparent glass bottles are permeable to UV light. Brown glass is therefore more often used due to its ability to block more UV light than clear and green glass[20, 22].

Packaging lines for bottles and cans are intricate manufacturing machines with various moving parts and pieces with the sole purpose of getting beer to the consumer. For the sake of quality, packaging lines must be designed to reduce oxygen pickup, keep the product cold, and be easily cleanable. As will be discussed later, oxygen will cause beer flavor instability and should be below 200ppb total O_2 in the final packaged product. Cans are more susceptible to oxygen pick up during the packaging process due to the large surface area of the can opening before the lid is attached and the inability to completely purge the can of air via vacuum due to its weak structure prior to filling. Glass bottles can undergo air evacuation via vacuum, resulting in less oxygen pick up during packing. Maintaining temperatures below 0°C during packaging will keep carbon dioxide in solution at levels of 2.1 to 2.7 volumes and aid in keeping oxygen out. Optimal cleaning of the packaging line will also ensure microbial stability and therefore flavor stability of the final product[20].

1.4 Beer Styles

1.4.1 Overview of beer styles

Beer comes in many flavors, or styles, depending on the raw ingredients and the way they are used during the brewing process, as explained in the previous sections. As raw ingredient options evolve and new technologies become available to the brewer, new styles are innovated. With enough market and industry acceptance, these new flavors become a new style. The Beer Judge Certification Program (BJCP) is the accepted organization overseeing the beer style guidelines that outline the characteristics a beer must meet to be considered a certain style. The guidelines consist of a standard format for the style description including overall impression, appearance, aroma, flavor, mouthfeel, history, and characteristic ingredients[8, 23]. As of 2020, there were over 75 beer styles, each with its own flavor, aroma, and color to distinguish it from other styles[24].

1.4.2 Dichotomy of beers 1: Lagers and ales

Beer styles can be categorized into two simplified groups, lager or ale[21]. The primary difference between a lager and an ale is the species of *Saccharomyces* yeast used for fermentation. Specifically, lager beers are produced using the bottom fermenting strain, *Saccharomyces pastorianus,* and ferment at cooler temperatures (5-15°C). Lagers have a longer production time because of the colder fermentation temperature, and are crisp and refreshing in flavor[8]. They are typically lower in alcohol content, color, and fruity aromas. Examples of lager styles are pilsner and the American lager. Conversely, an ale is produced using the top fermenting yeast species, *Saccharomyces cerevisiae,* and ferment typically around 18-25°C.

They are characteristically fruitier due to higher ester concentrations and are also higher in alcohol[18, 21].

Ales have been brewed for thousands of years, whereas lagers are relatively newer, increasing in popularity in the late 1800's with the German migration to North America. Now, lagers are the highest produced beer style in the world[8, 18]. The modern-day breweries producing most of these lagers are large breweries that have internal research and development laboratories. This has resulted in a bias of the foundational beer research to a focus on lagers. As the brewing industry continues to evolve and invent new styles with the onset of the craft beer revolution, it is apparent that the research performed for lager styles often does not translate to other beer styles.

1.4.3. Dichotomy of beers 2: Malty and hoppy

Another way in which beers are simplified into a dichotomous classification is based on the sensory characteristics of "hoppy" and "malty". This distinction is not based on the yeast strain, as is the case for lagers and ales, but instead on the specific usage of hops and malt as an ingredient to impart a specific dominant flavor characteristic[21, 25]. Importantly, this dichotomy is independent of the lagers and ale style classification, meaning an ale can be hoppy or malty depending on the style, as is true for lagers[21]. A malty beer is one that uses a variety of malts and minimal hops to create a complex malt-forward sensory profile. They also tend to be darker in color from the browning effect of Maillard reactions. Maillard reactions are a chemical transformation between a reducing sugar and amino acid. Styles that are considered malty include amber ales, stouts, porters, and Vienna lagers. A hoppy beer is one that uses one or more hops to create a dominant hoppy sensory profile. A hoppy profile can be in the form of

bitterness, flavor or aroma. Bitterness comes from higher levels of iso-α-acids, formed from the isomerization of α-acids during the kettle boil. A high α-acid hop cultivar is therefore typically used to create a highly bitter beer. Flavor and aroma come from the preservation of hop oils through the late addition of hops to the boil, or as a "dry hop" in the fermenter. Styles that are considered hoppy include pale ales, India pale ales (IPA), and New England IPA[21].

1.4.4 Beer is a complex chemical matrix

In the most basic sense, beer is a water-ethanol solution that is the product of fermentation. However, beer should be considered not as a simple matrix, but one that is vastly diverse. During the fermentation process, the four main ingredients are transformed into a complex matrix containing thousands of unique chemical compounds[18, 26]. The concentrations and ratios of these compounds depend on the starting substrates and the microorganism performing the transformation. After packaging, these compounds continue to undergo chemical transformations as the beer ages. Beer is not a static matrix and is constantly changing.

The composition of this infinitely complex matrix includes both volatile and non-volatile chemicals[1]. Volatile compounds are those with a higher vapor pressure making them more responsible for the aroma of beer. They are broken down into common subcategories that include carbonyls, cyclic acetals, heterocyclic compounds, esters, and sulfur compounds sugars[1]. Non-volatile compounds are those that stay within the beer matrix and will contribute to a beer's mouthfeel. Common non-volatiles found in beer include hop acids, polyphenols, amino acids, sugars, and inorganic salts[1, 27].

1.5 The Brewing Industry and Economics

1.5.1 The brewing industry can be divided into two groups: macro-breweries and craft beer

Breweries in the U.S. are divided into two general groups based on production size and ownership, macro-breweries and craft breweries[28]. These distinctions formed around the 1970's when homebrewing was popular and microbreweries first appeared in the market[29]. In the 1990's, the popularity of "craft" beer exploded and with it the formation of the Brewers Association (BA) in 2005, a 501(c)(6) non-profit trade organization. The BA created official definitions for the various groups of beer manufacturers. According to them, a macro-brewery is defined as a large national or international non-craft brewery, often with multiple brewing facilities around the world that produce more than six million barrels annually (e.g., AB InBev)[30]. A common beer style produced by a macro-brewery is the American light lager. The BA defines a craft brewery as one that is small, producing less than six million barrels annually, and independent with no more than 25% cross ownership. Another key aspect of craft beer is innovation, specifically within the variety of beer styles they produce. Craft beer is further divided into six market segments: microbreweries, brewpubs, taproom breweries, regional breweries, contract breweries, and alternating proprietors[30].

1.5.2 The brewing industry is a large contributor to the national and state economy

The BA produce and publish frequent economic and production reports on the brewing industry at the national and state level. In their 2020 report, the overall national retail dollar sales for macro- and craft breweries was $94.1 billion, with craft contributing $22.2 billion of that total. Approximately 150 billion barrels of beer was produced in the U.S., and craft accounted for 12.3% of that production. There were 8,884 breweries and of those, 8,764 were craft

breweries and 120 were macro-breweries[31]. The Beer Institute, another brewing industry advocacy group, reported in their 2020 document the brewing industry produced $333.8 billion in economic impact based on the jobs and wages of the brewing industry, representing 1.6% of the U.S. Gross Domestic Product. They also report roughly 2 million jobs were created across the various sectors of the industry[32]. Although the Beer Institute does not differentiate craft in their statistics, the BA reports craft beer provided more than 400,000 jobs across breweries, retailers, and wholesalers[31].

In Colorado, as of 2019 there were 433 craft breweries making a $2 billion impact on the U.S. economy. Craft beer employs 15,752 Coloradoans and produced just under one million barrels of beer[33]. There are two macro-breweries not included in these statistics, AB InBev (Fort Collins, CO) and Molson Coors (Golden, CO), and therefore the values reported by the BA are not a complete representation of the beer industry in Colorado.

1.5.3 American craft beer trends

1.5.3.1 Evolution of craft beer styles away from light lagers

According to Elzinga et.al, the craft beer revolution started in 1977 with Anchor Brewing Company and New Albion Brewing opening their doors in California[34, 35]. Prior to 1977, beer was a homogenous beverage produced by what is defined today as macro-breweries. The main style in production was the light lager which are light in body, pale in color, and often produced with adjuncts such as corn and rice[34, 35]. With the onset of craft breweries came the introduction of different styles with unique flavor profiles, filling gaps in the macro-brewery dominated market. Craft brewers began to introduce styles that were popular and common in Europe, such as dark lagers and ales, which at that point were not typical in the U.S. beer

market[34, 35]. While macro-breweries marketed to a large audience, craft brewers took lessons from the wine industry and marketed to consumers interested in pairing their beers with food, and to those who enjoyed flavor idiosyncrasies.

The success of the craft beer approach has been recognized and with this shift in consumer preferences macro-breweries are now branching out from the light lager styles and producing a wider range of styles. Therefore, it is no longer just craft brewers that are producing beers with more unique flavor and chemical profiles. Nevertheless, the majority of beer produced and sold in the U.S. today is still the light lager[36].

1.5.3.2 Introduction of the heavily hopped India Pale Ale style

Another important trend started by the craft beer industry is the increased usage of hops. Anchor Brewing Company was the first craft brewery in the U.S. to produce the now popular India Pale Ale (IPA) style[34]. Today, hoppy ales, specifically IPA, continue to be the biggest selling and most important styles in craft beer[30]. The development of new beer styles is one of the defining attributes of the craft beer industry[30]. With almost 45 years since the introduction of craft beer, and now over 9000 craft breweries in the U.S., beer has become significantly more diverse relative to the peak of light lager style.

1.5.3.3 Package type preferences in craft beer

Over the last decade, craft brewing has driven a shift in single use package type usage. Packaging in single use and single serving containers, mainly glass bottles and aluminum cans, is a critical method for craft brewers to get their products to the customer outside of kegged and draft beers at retail locations, such as bars, restaurants, and liquor stores. In the beginning of the craft revolution, glass bottles were the preferred method of packaging[37]. In the early 2000's, that began to shift with the availability of small can lines to the market creating a steady increase

in the percentage of craft beer packaged in aluminum cans[38]. Benefits of using cans include reduced costs to the brewer with a lighter and more compact package and improvements in beer quality. Cans provide an air-tight closure preventing oxygen ingress and lead to flavor changing oxidation reactions. They are also opaque and keep out UV light which will induce additional flavor changing reactions in the beer[37]. In 2015, the usage of cans and bottles in craft beer packaging was about 20% and 80%, respectively. By 2018, the usage had shifted to 40% cans and 60% bottles. By 2020, the usage had shifted drastically to 80% cans and 20% bottles, exhibiting a complete swing in package type usage over five years (Figure 2)[37, 39].

A major cause of the drastic shift seen in package type usage in 2020 can be attributed to the COVID-19 pandemic [2, 40]. Restaurants and bars were shut down for significant periods of time, causing a decrease in draught beer sales. Therefore, breweries had to shift their strategy for

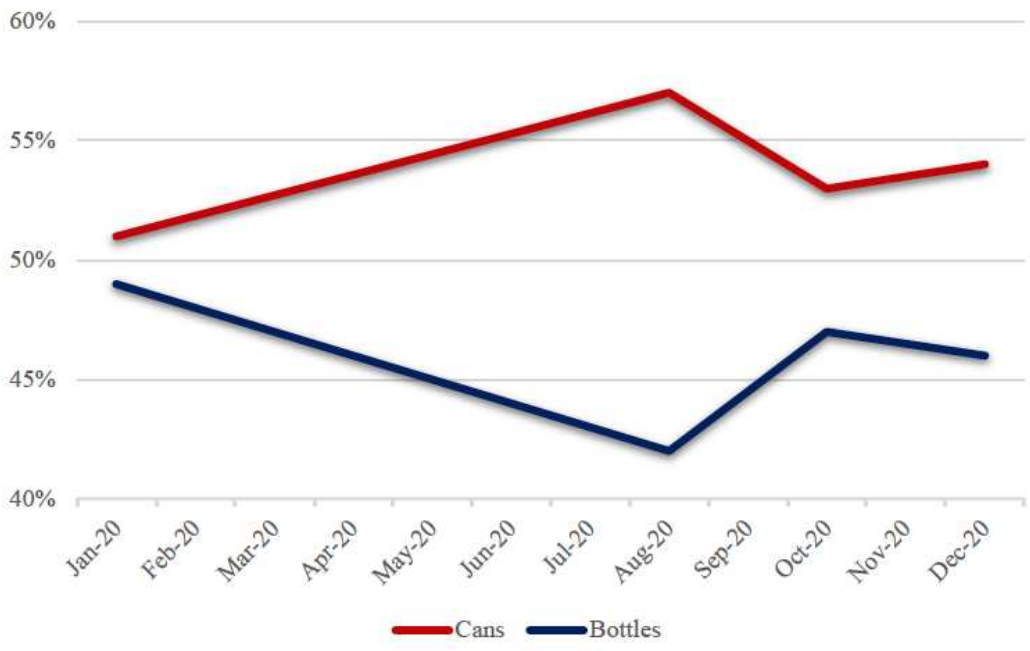

Figure 2. 2020 craft beer package use percentages.
A line graph demonstrating the effects of the COVID-19 pandemic on the proportion of craft beer packaged in cans (red) and bottles (blue) from January through December 2020. *Adapted from Whatson, 2021[2]*

getting product to the consumer. Many craft breweries that were not canning before the pandemic purchased canning lines or used mobile canning services to package beer for on-premise to-go sales[40]. Can lines were preferred over bottling lines due to consumer preference, costs, and advancements in small craft canning line technology and accessibility[37]. The long-term effects of the pandemic on packaging trends remain to be seen, but it is likely that with the increased investment in canning technology across craft brewers cans will remain the preferred package type for the foreseeable future.

1.5.4 Competition in the U.S. beer market

The beer industry is a global market. Products from the U.S. are sent around the world while at the same time beers from around the world are imported for American consumers to enjoy. According to the BA, in 2020 19.5% of the beer sales (by volume) in the U.S. was imported beer, representing a 0.6% increase from the previous year. At the same time, craft beer decreased 9.3%. As a whole, the U.S. 2020 beer sales volume decreased 2.9% from the previous year, potentially indicating increased competition from alternative alcoholic beverages (e.g., wine, spirits) as well as the impacts of the COVID-19 pandemic[39]. However, while the volume of beer sales decreased, the number of overall U.S. breweries increased 4.5% (8,502 to 8,884) in 2020.

Regardless of the brewing industry segment (i.e., international, U.S. macro-brewery, or U.S craft brewery) the industry is highly competitive. To compete, breweries must use various means to win the loyalty of the consumer including preference of style, packaging presentation and, arguably the most important, flavor integrity and stability.

1.6 Beer Stability

1.6.1 What makes a quality beer?

The designation of beer "quality" is independent of style and brewery size. The consumer, who is the final judge, expects their beer to meet a certain "degree of excellence"[41], that is maintained across batches and over time for a specific beer brand. In other words, a consumer wants consistency in the sensory characteristics (i.e., aroma, taste, and mouthfeel) they are familiar with for a particular product, or what is often referred to as "true to brand". This is essential for building brand loyalty [42]. Although this is defined specifically for craft beer, this definition is applied across all breweries and beer styles.

In addition to brand consistency, beer should be free from off flavors to be considered a quality product. Off flavors are chemical compounds that impart flavor and aroma notes that are undesirable in the sensory profile of a beer. These chemical compounds could originate from raw materials, improper brewing processes, microbial contamination, aging, amongst many others. Some off flavors should never be present in beer, including diacetyl which imparts a buttery aroma and flavor, acetaldehyde which imparts a green apple flavor and aroma, or trans-2-nonenal (T2N) which imparts a papery flavor[42]. Additionally, a flavor can be considered "off" because it is inappropriate for a specific style or brand. For example, an amber ale should not have lactic acid notes present however, is appropriate in sour ales such as Berliner Weisse. In summary, all brewers should strive to meet the consumer's expectations, produce beer free from off flavors, and maintain a product's sensory characteristics, or flavor stability.

1.6.2 There are three types of beer stability: physical, biological, flavor

Beer is assessed on a range of metrics which utilize all the senses: color, clarity, foam, aroma, carbonation, mouthfeel, and taste. The brewer aims to preserve the original state of these metrics for as long as possible because they do not know when their products will reach the consumer. To maintain customer loyalty, a key aspect to a successful business operation, it is imperative that beers taste fresh and are a true representation of the brand, or "true to brand". This maintenance of the original quality of beer over time is called "beer stability". There are three categories when considering beer stability. The first is the physical stability which mainly refer to as turbidity or haze. The second is biological stability, or the absence of contamination and spoilage effects. These first two, in general, are easily controlled by the brewer. The final category is flavor stability, which is less easily controlled and becomes an important aspect of beer stability once the beer is packaged and out in the market.

1.6.2.1 Physical Stability

Physical stability, also referred to as colloidal stability, mainly encompasses haze produced by interactions between proteins and polyphenols. These chemical species originate from malt and hops. It is worth noting that haze can also manifest from biological contamination, starches, pentosans, and oxalic acid[43]. When beer temperatures reach below 0°C, the polyphenol-protein complexes break out of solution into what is called chill haze. Chill haze is reabsorbed back into solution once the beer is warmed, however, chill haze will become permanent haze after multiple cycles of chilling and warming[44]. To control haze formation beer should be stored at a constant cool (+0°C) temperature, and temperature fluctuations should be minimized. Haze is also controlled by removing the main haze causing substrates (i.e., proteins and polyphenols) during raw material selection and the brewing process, preventing

agitation that will speed the chemical reactions between proteins and polyphenols, decreasing oxidation potential, preventing UV light contact, removal of heavy metals such of iron, and usage of beer stabilizer products such as polyvinyl polypyrolidone (PVPP)[44]. In general, the customer prefers bright clear beer, except for newer "hazy" styles such as hazy India Pale Ales. Physical stability should be an important consideration for the brewer.

1.6.2.2 Biological stability

Biological instability is caused by microbiological contamination of beer. Microbiological contamination can be caused by bacteria, yeast, or molds, and have a range of deleterious effects on a beer's quality including off-flavor production, haze formations, over carbonation and attenuation, and gushing. Due to beer's low pH, alcohol content, limited nutrient availability, and hop acids' bactericidal effects, pathogenic contamination is not a concern when considering biological stability[44, 45]. Good cleaning practices should be observed throughout the brewing process to prevent contamination. Contaminating microorganisms can come from raw materials (e.g., *Fusarium* in malt), wild yeasts present in the environment, cross-contamination from yeast and bacteria used in other brand production, and improperly cleaned equipment[44]. At accounts with draught line systems, care should be taken to properly clean the equipment to prevent the buildup of biofilms and microorganism growth than can cause biological instability and poor beer quality post-packaging[45].

1.6.2.3 Flavor Stability

Beer flavor is the combination of taste, aroma, and mouthfeel of a beer in which thousands of flavor active compounds are responsible[45]. Beer flavor is not static and begins to change as soon as it enters the final package. Flavor active compounds attributed to a beer's sensory profile undergo various chemical reactions resulting in the formation and degradation of

compounds. These chemical compounds that are not part of a beer's flavor profile are considered off flavors and contribute to the staling of beer flavor. When off flavors are detected through aroma, taste, or mouthfeel, they have passed the flavor threshold and a beer is no longer "true to brand". The degradation of desirable flavor compounds results in the lessening or absence of favorable flavor attributes and contributes to beer staling. Therefore, brewer's want their beers to have good flavor stability in which flavor changes are minimized. This goal remains one of the more difficult aspects of beer to control[43].

There are many factors that affect beer stability throughout the brewing process however, the biggest impacts occur during packaging, transportation, and distribution[45]. The three major impacts on flavor stability are time, temperature, and oxygen, although there are other sources of beer staling[43]. The longer beer is stored, the more time is allowed for chemical reactions to proceed and affect flavor compound concentrations, therefore beer should be consumed as close to packaging date as possible. Higher temperatures increase reaction rates of chemical reactions that lead to beer staling. Reactions rates differ between reaction types depending on temperature and substrate; thus the formation and degradation of beer aging compounds is not constant nor linear. Still, reaction rates increase with increased temperatures making refrigeration during transport, distribution, and storage essential to ensure flavor stability[43, 45]. Finally, oxygen is a highly reactive species and responsible for many chemical reactions leading to beer staling[46]. Oxygen is picked up during the packaging process as well as through bottle crowns during storage, a phenomenon that is not a concern with canned beer [43]. Brewers therefore aim to minimize oxygen levels in the beer prior to packaging [46] and oxygen pickup during the packaging process (<100ug/L)[47]. Total packaged oxygen (TPO), dissolved oxygen plus

oxygen in the package headspace, is often monitored to ensure machinery and protocols are optimized.

Shelf life, or best by date, is the length of time in which a beer product can be expected to look, taste, smell, and feel as expected. Shelf life is not an indication of food safety, rather beer stability. Through monitoring of a product under potential market conditions, it is up to the brewer to determine the shelf life of their products. A beer is most stable when stored under refrigeration. Unfortunately, this is not guaranteed once the product leaves the production facility and should be a consideration when assigning shelf life and when establishing protocols with distributors and accounts. Since beer styles age differently depending on raw materials and alcohol content, a unique shelf life is applied to each brand[48].

Beer staling comes in the form of many off flavors and aromas depending on the starting material, chemical reaction, temperature, and time. Beer is inherently unstable and is therefore always changing. Figure 3 represents the general trends in beer aging over time. The pleasant bitterness intensity steadily decreases due to the oxidation of iso-α-acids from hops[47], while sweeter flavors and aromas such as honey, sherry, caramel and toffee increase steadily. Aroma notes of ribes, or black currant leaves, tomato leaves, and catty will increase initially, reach a maximum intensity, and then decrease. Perhaps the most notable beer staling attribute, in part due to its low flavor threshold (0.1ug/L) and the extensive number of studies focusing on its link to beer staling [44], is the papery or wet cardboard flavor. This is a simplified schema of the beer aging process and will shift and change depending on beer style and the starting substrates within a beer[1]. Other general trends across varying styles may include a decrease in favorable esters, alcohol, and dimethyl sulfide (DMS), harsh bitterness and astringent finish, and notes of cheese,

soy sauce, leathery and wine/sherry, especially in severely aged beers [1, 45]. Table 1 outlines common staling taints and their sensory impacts as well as their causes.

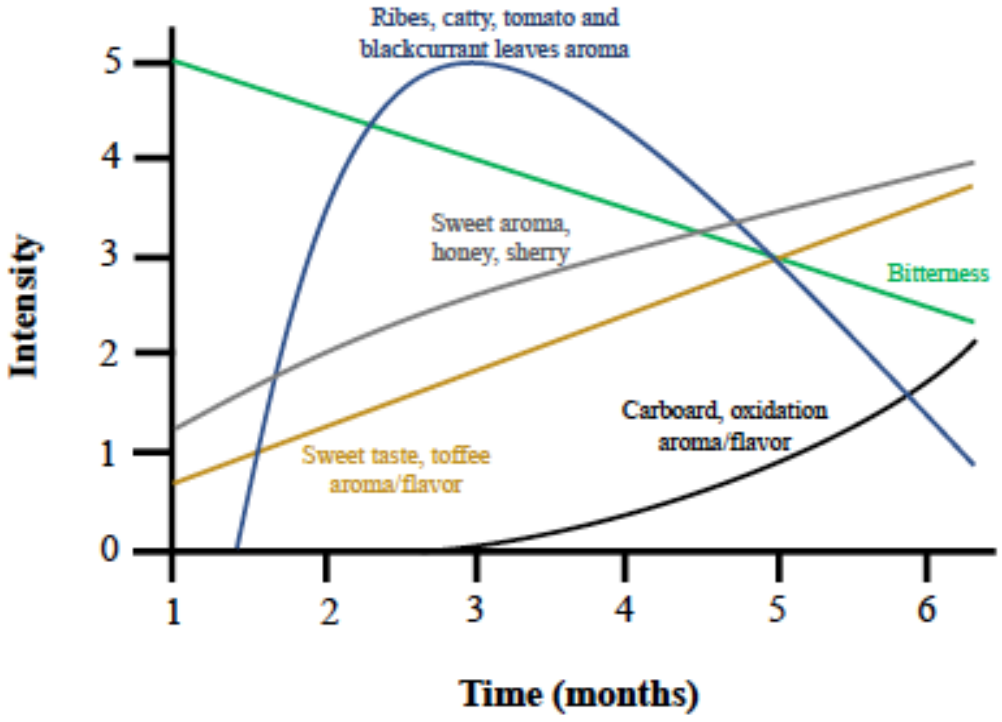

Figure 3. Generalized beer flavor and aroma changes over time.
Over a six-month aging period bitterness (green) will decrease, and various sensory attributes such as toffee (yellow), carboard (black), sherry (gray), and ribes (blue) will increase. The rates of change in these sensory characteristics depend on time, temperature, and total packaged oxygen. Adapted from Vanderhaegen et al., 2006 [1].

Table 1.Typical beer staling flavors and aromas and their causes
Adapted from Stewart, 2004 [44]

Taint Descriptor	Flavor/Aroma	Cause
Oxidized	Papery, cardboard, “dull”, toffee	Storage, oxygen
Catty/*Ribes*	Tomcats, black currant leaves, tomato plants	High in package oxygen
Aldehyde	Rotting apples	Storage, high oxygen
“Cooked”	Over pasteurization, grainy, “dull”, toffee	Storage, high oxygen, high temperatures

1.6.3 Important flavor stability compounds and reaction types

Although flavor stability research has been conducted over many decades, it is still difficult for the brewer to control. For many years the scope of flavor research was limited to lagers and staling aldehydes[43, 48]. There has been a recent increase in research with an expanded focus on additional beer styles and the subsequent relevant aging compounds[48], however, our knowledge of beer aging mechanisms and flavor active compounds still present many gaps. The chemical reactions occurring during beer storage are both oxidative and non-oxidative, affecting the volatile and non-volatile chemical fractions.

Beer aging mechanism are complex where intermediates and products of one reaction pathway become the substrates for another. It is the formation and deteriorated of compounds that lead to stale beer. Table 2 reviews volatile compounds known to increase in beer during storage.

Table 2. Compounds in beer grouped by chemical ontology that have been shown to change in abundance during storage, and their sensory impacts
Adapted from Vanderhaegen et al., 2006[1]

Chemical Ontology	Compound	Known Flavor Impacts
Aldehydes (carbonyl)	acetaldehyde	Green apple
	E-2-nonenal (T2N)	Paper, cardboard
	E-2-octenal	Bitter, cardboard, stale
	E,E-2,4-decadienal	Oily, rancid, deep-fried, papery
	E,E-2,6-nonadienal	
	2-methylbutanal	Malty, alcoholic, vinous, banana, solvent
	3-methylbutanal benzaldehyde	
	2-phenylacetaldehyde	Malty, cherry, almost, cheese
	3-(methylthio) propionaldehyde	Floral, honey, sweet
Ketones (carbonyl)	E-beta-damascenone	Berries, stale hopped beer
	Diacetyl	Butterscotch, butter, rancid
	3-methyl-2-butanone	
	4-methyl-2-butanone	
	4-methyl-2-pentanone	
	2,3-pentanedione	Fruity, butterscotch, honey, woody
Cyclic acetals	2,4,5-trimethyl-1,3-dioxolane	

	2-isopropyl-4,5-dimethyl-1,3-dioxolane 2-isobutyryl-4,5-dimethyl-1,3-dioxolane 2-sec butyl-4,5-dimethyl-1,3-dioxolane	
Heterocyclic compounds	furfural 5-hydroxymethylfurfural 5-methylfurfural 2-acetylfuran 2-acetyl-5-methylfuran 2-propionylfuran furan furfuryl alcohol furfuryl ethyl ether 2-ethoxymethyl-5-furfural 2-ethyoxy-2,5-dihydrofuran maltol dihydro-5,5-diemethyl-2(3H)-furanone 5,5-dimethyl-2(5H)-furanone 2-acetylpyrazine 2-methoxypyrazine 2,6-dimethylpyrazine trimethylpyrazine tetramethylpyrazine	Caramel, bready, cooked meat, papery, husk Almond, spicy, caramel, burnt, phenolic Sugar cane, woody Caramel, malty, sweet, toasted, roasted Sweet, candy floss, caramel
Ethyl esters	ethyl-3-methylbutyrate ethyl-2-methylbutyrate ethyl-2-methylpropionate ethylnicotinate diethyl succinate ethyl lactate ethyl phenylacetate ethyl formate ethyl cinnamate	 Honey, sweet, mead, sherry Fruity, solvent Fruity, sweet, cinnamon, cider, strawberry
Lactones	gamma-nonalactone gamma-hexalactone	Coconut, vanilla, glue, rancid, peach Peach, fruity
S-compounds	dimethyl trisulfide 3-methyl-3-mercaptobutyl formate	Onion, garlic, cabbage, sulfur

1.6.3.1 Chemical compound groups relevant to beer aging

Compounds can be grouped into two broad classifications: volatiles and non-volatiles. Beer is made up of both and within each the compounds are further sub-divided based on chemical ontology. Chemical ontologies may overlap and it is possible for a chemical compound to have multiple chemical ontology classes. Vanderhaegen, et. al., classifies the volatile compounds associated with beer aging into the following major chemical ontologies; carbonyls (aldehydes, Strecker aldehydes, ketones), esters, and sulfur containing compounds, although cyclic acetals, lactones, and heterocyclic compounds are described as well [1].

Structurally, carbonyls have a carbon/oxygen double bond making this a large group of compounds. They've received the most attention and interest in beer staling studies since they are known to cause staling in other foods. Initial research found T2N and acetaldehyde as two important carbonyls which increase over time[1, 45, 48]. T2N and acetaldehyde impart a cardboard/papery and green apple flavor, respectively. The increase in T2N is due to the oxidation of unsaturated fatty acids, however its formation is not universal in all beer. Some researchers have not characterized T2N as present or increasing in concentration during beer storage, leading to the conclusion that additional aldehydes and pathways must also be responsible for staling off flavors[1, 49]. Research has shown that under heat acidified conditions (40°C), or forced aging, T2N will increase more rapidly than at room temperature (20°C) and brings into question the appropriateness of forced aging practices.

Carbonyl concentrations in beer are low yet have a strong flavor impact due to their low flavor threshold. Unfortunately, this proves problems in chromatographic detection of these compounds without proper extraction methods due to the more abundant compound peaks masking the small carbonyl peaks[1]. The absence of T2N or other carbonyls could also be a

result of carbonyl scavengers within the beer matrix[46]. Apparent from the previous research, carbonyls play an important role in beer aging and lead to flavor active beer staling compounds, however, detecting these compounds can be difficult.

Another important group of carbonyls are **α**-dicarbonyls. They are products of Maillard reactions, interactions between reducing sugars and amino acids under heated conditions, which occur in the brewhouse and during the malting process. **α**-dicarbonyls are the substrates for many staling compounds found in beer, most notably Strecker aldehydes. These compounds are a result of Strecker degradation of amino acids, a reaction type explained later in *Section 1.6.3.2*.

An ester is the product of a reaction between an organic acid and an alcohol. During fermentation, yeast synthesize esters to produce favorable fruity characters in beer[14]. During being aging, these favorable esters deteriorate and result in a dulling effect to beer flavor profiles. Additionally, volatile ethyl esters are formed during aging and are known to contribute to beer aging flavors such as wine-like, sweet, peachy, and fruity, although these tend to be uncharacteristic and unfavorable [1].

Sulfur compounds have very low flavor thresholds and therefore small changes in their concentrations will have a great effect on beer flavor. During beer aging, sulfur compounds can be formed. One of the most notable sulfur compounds in beer is referred to as catty or ribes, as in the black currant genus. The responsible compound was found to be 3-methyl-3-mercaptobutyl. Other sulfur compounds can impart onion and drain-like off flavors.

Beer aging research has largely focused on volatile compounds as they correlate to beer aroma, while non-volatiles have been less explored in relation to beer stability. Non-volatiles tend to affect a beer's bitterness and mouthfeel, which are equally important to beer flavor. The *trans* isomers of beer iso-**α**-acids are most sensitive to deterioration during storage, leading to the

trans/cis isomer ratio as a potential beer aging marker. Other non-volatile aging markers have been described [50, 51], however they have not been identified to effect flavor. Finally, polyphenols are readily oxidized and can lead to harsh bitterness and astringency in beer[1].

1.6.3.2. Reaction types relevant to beer aging

The formation and degradation of the chemical compounds reviewed in the previous section are the results various chemical reactions and pathways that can also be grouped by their type. Research in beer aging has been able to elucidate and characterize some pathways leading to beer staling compounds. The known relevant reaction types from these studies and their associated substrates and products are listed in Table 3.

Table 3. Known beer aging reactions and their associated substrates and products

Reaction Type	Substrates	Product
Formation of reactive oxygen species (ROS)	Oxygen, ground state ($^3O_2$)	Singlet ($^1O_2$), superoxide ($O_2^{\bullet-}$), hydroperoxyl radical ($^{\bullet}OOH$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2)
Oxidation of higher alcohols	Ethanol, 2-methyl-propanol, 2-methyl-butanol, 3-methyl-butanol, 2-phenyl-ethanol, ROS	1-hydroxyethyl radical, acetaldehyde, long chain carbonyls
Oxidation of fatty acids	Hydroxy and hydroperoxyl fatty acids	Trans-2-nonenal, aldehydes
Maillard reaction	Reducing sugars and amino acid	Melanoidins, alpha-dicarbonyls, heterocyclic compounds, furfuryl
Strecker reactions	Alpha-dicarbonyl and amino acid	Strecker aldehydes
Aldol condensation	Carbonyl and amino acid catalyst	Low flavor threshold carbonyls
Degradation of hop bitter acids	Alpha acids, beta acids, iso-alpha-acids	Alkanones, alkenals, alkadienals
Breakdown of glycosides	Allene triols, acetylene diols, glycosylated beta-demascenone precursors	Beta-demascenone
Acetalization of aldehydes	2,3-butanediol and aldehyde	Cyclic acetals
Ester formation	Organic acids from oxidized hop acids, Strecker degradation, yeast metabolism	Ethyl esters

Reactive oxygen species (ROS) come in various forms and are highly reactive with other components of beer leading to the formation of beer staling compounds. ROS are formed in beer during storage from light, energy (e.g., increased temperatures, agitation), and catalytic activity by metal ions. The formation of ROS is catalyzed by iron (Fe) and copper (Cu) through various pathways, however, the most notable is hydroxyl radical (•OH) formation through the Fenton and Haber Weiss reactions. Other ROS formation is due to pro-oxidants, compounds that increase hydrogen peroxide, such as Fe, Cu, and Maillard reaction products. Alternatively, antioxidants quench ROS and inactivate pro-oxidative metal ions. The ROS of most concern is the hydroxyl radical. Its formation is lagged during beer storage, and its reactivity is unspecific, reacting with many beer compounds and therefore alludes the antioxidants present in beer. To avoid ROS formation, it is best to keep metal ions and oxygen in beer as low as possible[1].

ROS can cause the oxidation of alcohols. The most abundant alcohol in beer is ethanol, however there are others present in lower concentrations. Hydroxyl radicals react with ethanol to form the radical 1-hydroxyethyl, which then degrades to acetaldehyde. It has been proposed that other alcohols form radical species once oxidized which then lead to longer chain carbonyls and staling aldehydes[49].

The oxidation of fatty acids is understood to be the cause of staling and rancidity in other foods[46], which is why this pathway has received much attention when studying beer staling. Although the mechanisms are still not totally understood, most evidence shows fatty acid oxidation occurring mainly prior to beer storage. The two main fatty acids present in wort, linoleic and linolenic acid, come from barley. During mashing, lipases and lipoxygenases oxidate linoleic and linolenic acid into hydroxy and hydroperoxyl fatty acids. During the boil, although

the enzymes are deactivated, ROS are formed from heating and the presence of oxygen resulting in the formation of additional hydroxy and hydroperoxyl fatty acids [49, 52]. These intermediates are non-enzymatically converted to T2N and other aldehydes. T2N forms adducts of amino acids and proteins in the brewhouse which protect it from release by yeast during fermentation. During storage T2N is released from its adduct form through acid hydrolysis due to the beer's low pH, explaining the increase in T2N during storage[52]. Since T2N and other oxidized fatty acid compounds form during the mashing and boiling steps of brewing, they can be controlled through optimization of protocols in the brewhouse.

Maillard reactions occur during the boiling of wort and the kilning step of malt production (Figure 4). With each of these processes, high temperatures facilitate reactions between reducing sugars and amino acids. This reaction occurs in all processed food production, where the end results are color and flavor active compound formation. Maillard reaction mechanisms are complex and its relation to beer staling has been minimally researched. The

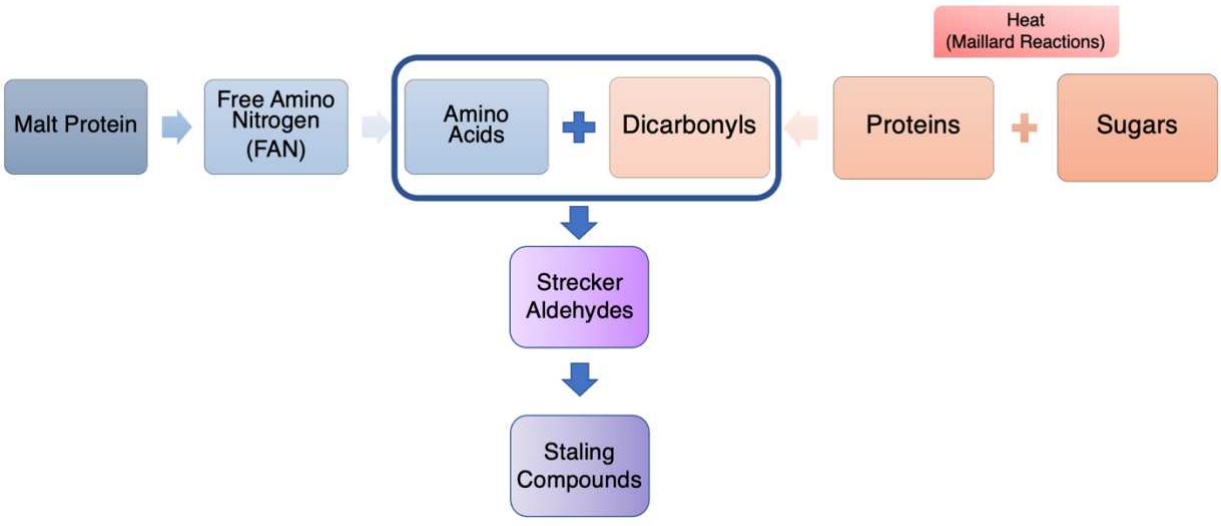

Figure 4. Schema depicting the formation of staling compounds from Strecker degradation reactions.
Amino acids from malt and dicarbonyls, a product of Maillard reactions from thermal stress in the brewhouse, undergo Strecker degradation reactions and result in products that cause beer staling off flavors and aromas.

products of Maillard reactions are called melanoidins which have been found to be both pro-oxidants and antioxidants in beer. These reaction products result in bready, sweet, and wine-like off flavors in aged beer. Furfuryl is another melanoidin that leads to 2-furfuryl ethyl ester which is well known for imparting a harsh solvent off flavor. **α**-dicarbonyls are another melanoidin which play an important role in Strecker reactions and aldol condensation (Figure 4).

Strecker degradations occur between an amino acid and an alpha-dicarbonyl, where a cascade of reactions result in the formation Strecker aldehyde (SA), which is an amino acid with one less carbon, and an alpha-aminoketone[52]. SAs increase during beer storage, especially at higher temperatures, and may lead to flavor impacts in beer[53]. The resulting flavor attributes of SAs can be generalized as caramel, toffee, nutty, fruity, meaty, and brothy notes. Researchers have correlated some amino acids to their resulting SA and the resulting sensory attributes. For example, the amino acid valine and its corresponding SA will impart a fruity green apple aroma, the proline SA results in bready characters, and phenylalanine SA results in pungent floral rose aromas[53].

One study found that beer stored for 20 days at 50°C in the presence of proline, acetaldehyde and heptanal will undergo an aldol condensation reaction to form T2N, where proline is suspected to be the catalyst[54]. Proline is an amino acid found in wort and is not assimilated by yeast; therefore, it will always be present in beer. While this pathway is plausible for the formation of flavor active carbonyls, it is not yet fully understood if these reactions occur during normal storage conditions[52].

Beer bitterness is a result of iso-**α**-acids. There are three iso-**α**-acids depending on the functional side group (-R) that exist in both *trans* and *cis* forms; isohumulone, isocohumulone, and isoadhumulone. *Trans*-iso-**α**-acids are more susceptible to ROS degradation than the *cis*

form. Carbon-centered radicals have also been shown to cause degradation of iso-α-acids, providing evidence that oxidative degradation may occur with and without the presence of oxygen[1]. Oxidative degradation leads to a decrease in the intensity and freshness of the bitterness profile in beer during storage. Today, hop products called tetrahydroiso-α-acids are used due to their resistance to oxidative degradation. Isohumulone is also suspectable to UV light degradation and may results in the formation of 3-methyl-2-butene-1-thiol (MBT) which imparts a skunky flavor[52].

β-demascenone, an important norisoprenoid, has a very low flavor threshold and causes berry-like and stale hop flavors in highly hopped beers. The formation may be caused by acid hydrolysis of precursors or the hydrolysis of glycosylated β-demascenone, through either non-enzymatic or enzymatic yeast breakdown [49, 52]. The formation of this highly flavor active compounds may indicate the formation of additional flavor active compounds through similar pathways, however, this remains to be fully elucidated in the research.

Flavor active cyclic acetals are the products of a condensation reaction between 2,3-butanediol, the by-product of diacetyl reduction by yeast during fermentation, and an aldehyde such as acetaldehyde. Cyclic acetals may be considered off flavors in beer.

Positive ester concentrations are reduced during storage by enzymatic and chemical hydrolysis into acetate esters and ethyl esters. Esterases are released into solution from yeast after fermentation or during bottle conditioning. Without a pasteurization step, the enzymes remain active and will continue enzymatic hydrolysis. Chemical hydrolysis depends on pH and storage temperature. Formation of undesirable ethyl esters rise from the esterification between ethanol and organic acids, which impart wine and brandy-like off flavors. Acid precursors may

come from yeast fermentation, oxidation products of hop acids, or derived from the Strecker degradation pathway[52].

In addition to the chemical reactions involved with beer aging described in this section, it is important to recognize the physical interactions of flavor active compounds with the packaging materials and the effects they have on flavor stability. Scalping is the term used to describe the movement of flavor active compounds from a food or beverage on or into the packaging material [55]. Beer can coatings and bottle liners are constructed from a variety of polymers, mainly epoxys and acrylics. Although they should be inert, it has been demonstrated that flavor active compounds from beer (i.e., hop volatiles, esters, alcohol) absorb into the can coating and bottle liner polymer at various rates depending on polar interactions and will affect flavor stability [56, 57]. Scalping can result in an overall decrease of compounds in the beer liquid and a muting of the organoleptic properties of the original product.

1.6.4 What we know about flavor stability mechanisms is limited

While more mechanisms of beer aging are being studied with continuing research, there is still much about beer flavor stability that is not understood. For example, the compounds that lead to leathery and sweet flavors are not known[46]. Additionally, most research published on beer stability has been conducted on lagers, and within that research only a few compounds are being observed, most notably T2N[43]. This is a narrow scope in the entirety of beer aging and leaves gaps in the knowledge. More work needs to be conducted in the areas of various beer styles and their aging properties [48], identification of additional compound markers of beer aging [43], how packaging materials interact with beer flavor compounds, non-targeted approaches to studying beer aging, and investigations into new methods of flavor preservation.

1.7 Quality Testing for Craft Breweries

1.7.1 Laboratory analyses – quality control and quality assurance

For reasons outlined in previous sections, beer quality is important to breweries. Brewers can monitor beer quality through various laboratory tests which are divided into two groups of testing based on the ultimate purpose: quality control (QC) and quality assurance (QA). According to Mary Pellettieri, "QC is controlling the process and product output by conducting a measurement, usually as close to the operation as possible. QA is controlling the measurement output by assuring the data via calibration or checking a measurement tool with a standard [58]." Often QC tests are run by production personnel in real time so that process changes can be made in a timely manner. QA analyses ensure that the QC data collected during production is accurate and precise. Both QC and QA analyses are necessary to ensure a high-quality product. A robust quality program requires a tailored suite of QC and QA analytical, microbiological, and sensory tests performed throughout the brewing process from raw materials to finished product. The testing suite is unique to each brewery because of the different processes and raw materials used, styles of beer produced, and quality risk factors found across brewery productions.

In general, analytical tests detect and quantify a particular analyte. Those values are monitored to ensure they are within an acceptable range to a specific brand. For example, color, pH, bitterness units (BU), vicinal diketones (VDK), sugar concentration, and alcohol content are commonly measured in quality programs, however, this is not an exhaustive list.

Microbiological tests aim to detect contaminating microbes, as well as ensure the health of essential microbes. This is performed using various microbiological tools such as cell counting, viability staining, plating, sterile filtration, Gram's staining, catalase tests, and in more advanced labs, polymerase chain reactions (PCR) analysis.

In addition to these laboratory tests, breweries are monitoring quality through sensory assessments. This often entails a trained sensory panel to assess beer throughout the process to detect any deviations from a brand's pre-determine sensory profile. Panelists are looking for off flavors as well as describing a beer's attributes in its appearance, mouthfeel, aroma, and taste. Although not a requirement, it is best practice to have a sensory panel that is trained to produce statistically significant results. Training a panel requires the use of often expensive sensory standards to calibrate the members so they can accurately detect and identify off flavors and aromas. Panelists are also trained to know each brand's sensory profile.

1.7.2 Current flavor stability testing practices within the craft industry

Flavor stability is directly and indirectly tested within the three quality divisions described above: microbiological, analytical, and sensory. By testing a finished product for contaminating microorganisms and determining microbial stability, breweries can confirm that unwanted microbes are not present and do not have the potential for altering the flavor of a beer.

Using advanced analytical tools, such as liquid and gas chromatography coupled with various detectors (e.g., mass spectrometry, electron capture, flame ionization), known aging compounds and markers outlined in *Section 1.6* are detected and quantified. If aging compounds are above the threshold concentration, which is the concentration at which point an average person is capable of detecting, there is evidence that the beer is no longer true to brand. These tests are performed on beers that have been naturally aged in the market, beers aged in house under cold storage (4°C), beers aged in house under warm storage at room temperature (20°C), and after forced aging conditions. Forced aging is a process in which packaged beer is placed

under higher temperatures (≤ 37°C) to expedite the aging process from many months and years to one or two weeks[51].

Additional analytical testing aims to identify aging potential. This can be done through measuring the total pacakged oxygen (TPO) in a can or bottle. TPO measures the oxygen that is dissolved in solution plus the oxygen contained within the headspace of the package. There are many instruments available for this type of analysis. Unfortunately, the technology is expensive and often cost prohibitive for smaller brewing operations. Due to oxygen's reactive nature, this must be performed at the time of packaging. As this is a destructive test, multiple tests are taken throughout the packaging run and averaged. Measuring the thiobarbituric acid index (TBI) in malt and wort, and quantification of 2-thiobarbituric acid (TBA) in beer are additional analyses that reflect heat stress during the brewing process [59]. As described in *Section 1.6*, heat stress in the brewhouse causes the formation of Maillard reaction products, which are substrates leading to staling compounds downstream in the process. In knowing the degree of heat stress, brewers can better understand a beer's staling potential and adjust brewhouse operations to decrease a beer's aging potential.

The most direct, and arguably most important, method of assessing flavor stability is through sensory analysis by a trained panel. Depending on the questions being asked, there are many tests used in a brewery sensory program. Someone with the knowledge of which test to use, how to administer the panel and conduct the statistical analysis once a sensory panel provides feedback is necessary to run a successful sensory program. The validated sensory methods used include the paired comparison, triangular, duo-trio, threshold of added substances, descriptive analysis, tetrad, difference from control, and ranking test [60].

The most common test performed is the descriptive analysis test [61]. This test is used with trained tasters to systematically determine the description of a beer's attribute profile using an agreed upon lexicon. The descriptive analysis test is used to evaluate test brews, in quality control, to train tasters, and to profile competitor's brands[60]. Once a brand's descriptive analysis is conducted and panelists are trained on the attributes that make a brand true to type, the other sensory tests can be administered to detect statistical differences in flavor over time, or flavor stability.

1.7.3 Sensory panels are powerful tools but are limiting

Sensory panels are limiting for many brewing facilities because of the expertise, financial investment, and time needed to run a successful sensory program. Although not a requirement, it is sometimes the case that a brewery will hire at least one individual trained in sensory science who understands the needs around test choice, statistical analysis, training, panel selection, equipment (e.g., glassware, sensory standards), and facility design.

The cost to employ someone with this specific skill set is significant, and there are additional costs associated with constructing an effective facility to run sensory panels and train panelists. Appropriate glassware and off-flavor spikes must be purchased, which are often expensive if ordering from a certified spike manufacturer. The purpose of training is to convert a subjective human panelist into an objective sensory instrument. Like the needs of a laboratory instrument, a panelist needs calibration and qualification, therefore multiple training sessions are needed and the costs quickly become cumbersome.

Sensory panels also require significant time investment. Extensive hours of training are necessary to ensure a reliable sensory instrument[62]. Additionally, to assess flavor stability

enough time must elapse for a beer to naturally age before a sensory panel can check if a brand is true to type at a given timepoint. This aging period is typically 16-weeks [50], a timeframe that does not allow for a brewer to make quick and educated decisions about flavor stability in their products, or understand how process changes have or have not improved stability quickly. Research showing the applicability of forced aging to speed up the aging process is variable. In the non-volatile fraction of beer, it was shown that forced aging is a valid approach to speed up the aging process and reduced the timeline from 16 weeks to 5 days[51]. Alternatively, T2N increased to levels above threshold within a few days when stored at 40°C, whereas the concentration of T2N was not detected in beers stored at 20°C after 4 months of storage [1]. Researchers continue to explore new stability methods, and while some demonstrate potential as complimentary tools for beer sensory analysis they have not succeeded in decreasing the time and training requirements of a sensory panel [61, 62].

1.7.4 Advanced and novel analytical tools are needed for assessing flavor stability

There will always be a need for sensory assessment of beer, still, this method can be inaccessible nor timely. There is a need for advanced analytical tools that can quickly assess beer freshness and stability [46]. In additional to expediting the process, another potential benefit of using an analytical instrument is a reduction in the noise of the results. Although sensory panelists are calibrated, a low number of panelists, which is often the case, can lead to potentially erroneous results.

Analytical analyses can be either targeted or non-targeted, where the prior looks at specific known compounds of interest and the later looks at all detectable compounds. Targeted analysis would allow a brewer to look at known staling compounds and markers as well as

compounds that are important to a beer flavor's profile. This approach is useful in many cases but is in essence limiting due to the remaining knowledge gaps in our understanding of staling compounds as described in *Section 1.6.4*.

Non-targeted analysis captures all the detectable compounds in a beer and turns it into a picture in the form of a chemical profile or fingerprint. Using statistical tools, the ideal profile is compared to unknowns to assess if the two match. In the context of flavor stability, the ideal profile would be a fresh beer and the unknown would be that beer at various ages. The non-targeted approach enables a holistic view of a beer that is more representative of the assessments of a taste panelists.

Currently flavor stability is assessed using sensory evaluations, and could benefit from the coupling with quicker, high precision analytical tools. In the following section, two analytical tools will be discussed and their capabilities within beer flavor stability assessment will be explored.

1.8 Advanced and novel analytical tools are needed for assessing flavor stability

1.8.1 Non-targeted mass spectrometry is a powerful approach for assessing beer flavor stability

As described in the previous section, a non-targeted approach using analytical instrumentation looks at all the chemical compounds (i.e., metabolites) that are detectable by the instrument being used. This approach is referred to as metabolomics and can be applied to various analytical platforms. Liquid chromatography mass spectrometry (LC-MS) and gas chromatography mass spectrometry (GC-MS) are two commonly employed instrument platforms in the field of beer metabolomics which can be used in both targeted and non-targeted analysis. In both methods, chromatography is utilized to separate metabolites over time with detection by

mass spectrometry. The data output of both platforms is a list of molecular features that each have a corresponding mass spectrum which is then used to generate compound annotations by comparing the mass spectra of known compounds in spectral libraries.

As described in its name, LC-MS analysis involves separation in the liquid phase and is ideal for the detection of non-volatile metabolites. Examples of non-volatile metabolites found in beer include iso-**α**-acids from hops, and flavonoids which are responsible for flavors and mouthfeel in beer[50]. Alternatively, GC-MS involves separation in the gas phase and is thus ideal for the detection of metabolites that are volatile, or readily enter a gaseous state. Examples of volatile metabolites found in beer include esters, hop oils, and aldehydes, and contribute to beer aroma. Some metabolites, such as amino acids and smaller sugars (e.g., mono-, di-, and trisaccharides), are semi-volatile and can be detected by GC-MS after a chemical derivatization step to increase their volatility and thermal stability.

Metabolomics is a relatively new tool with utility for a wide range of applications. Recently, brewing research has begun to utilize this tool to look at various questions including the characterization of different beer styles [18, 63], investigating the profiles of raw materials and their effects on finished beer [64, 65] and, to a limited degree, evaluation of beer flavor stability [50, 51, 66-68]. Demonstrated by the research previously conducted, metabolomics is a powerful tool for exploring beer flavor stability research.

While there is a large benefit of looking at samples holistically with a non-target approach, detecting a larger number of metabolites pose challenges to data interpretation. Often the ability to interpret data and assign spectral data as a specific metabolite (i.e., annotation) is limited by the spectral databases, or libraries, available. This is especially challenging for complex matrices such as beer which contain metabolites originating from the raw ingredients, water chemistry,

and microbial (e.g., yeast and bacteria) metabolism and biotransformation products. Currently, an open-source beer metabolite spectral library has not been created. There is an ongoing need for the generation of a public spectral database of known beer metabolites.

1.8.2 Ambient ionization mass spectrometry as a novel approach for expediting flavor stability assessment

Ambient ionization mass spectrometry was introduced in 2004 and can be defined as ""the ionization of unprocessed or minimally modified samples in their native environment, and it typically refers to the ionization of condensed phase samples in air [69]." Analysis in a sample's natural state minimizes, or eliminates, the need and time requirements of sample preparation, rendering this tool a quick, easy, high throughput alternative approach to food analysis. Ambient ionization mass spectrometry techniques are often categorized into three groups based on their desorption method: liquid extraction, plasma desorption, and laser ablation. The technology is used in various fields including biomedical, forensics, environmental, and food and agriculture.

While there are many techniques that fall under the broad umbrella of ambient ionization, one method that has shown potential for food and agriculture applications is Direct Analysis in Real Time (DART). For example, DART coupled with mass spectrometry (DART-MS) has been used to detect contaminant pesticides in wine [70] and bisphenol A (BPA) in food [69], in food fraud (e.g., fish and spices)[71, 72], in product differentiation [69, 73, 74], and to quantify analytes of interest (e.g., caffeine in coffee) [75]. DART uses a heated plasma, usually helium, to desorb and ionize molecules in a sample prior to mass spectrometry detection[69]. DART-MS can ionize compounds in either positive or negative mode and can often detect a combination of polar, non-polar, volatile, and non-volatile molecules. Because DART-MS can analyze liquids

that are absorbed to a solid surface directly and detect a wide range of metabolites, it is particularly suitable for high throughput analysis of beer.

While GC-MS and LC-MS result in a chemical profile consisting of a list of assigned spectra to a specific metabolite, DART-MS provides a single chemical fingerprint of the analyzed sample. When used in combination with predictive modeling and machine learning computational tools, this analytical platform enables high throughput screening for quality control. The power of this tool can be demonstrated in looking at an application of food fraud assessments. For example, an unknown fish sample is analyzed and a chemical fingerprint is created. The fingerprint is entered into a predetermined statistical model and its likeness to known fish samples are assessed. From this data, an analyst can rapidly determine if a food sample is true to its labeling. Similarly, previous research has demonstrated the use of DART-MS, in combination with predictive modeling, to differentiate specific beer brands and their places of origin [73, 74]. This indicates DART-MS as a potential analytical tool for further beer analysis, such as beer flavor stability.

1.8.3 GC-MS and DART-MS as powerful analytical tools for assessing beer flavor stability

The brewing industry provides significant financial impact to the U.S. economy. It is a global market where product travels long distances to reach the consumer. Additionally, it is an industry that continues to change with time. With the recent growth of the craft beer industry, preferences in style and packaging choices are shifting. Beer continues to develop as a complex matrix, and the fundamental research on beer flavor and stability, often focusing on bottled light lagers, proves to contain many gaps in the knowledge base. With the increased competition in the beverage market, flavor stability continues to be important for brewers to maintain, however,

the current methods of assessing flavor stability are limiting due to the time and financial investments required. Technological advances have resulted in various analytical tools (e.g., GC-MS and DART-MS), that can be used in combination with predictive modeling to improve our understanding of the changing beer matrix of today and its flavor stability.

CHAPTER 2 – CHARACTERIZING THE IMPACT OF PACKAGE TYPE ON BEER STABILITY THROUGH A METABOLOMICS APPROACH UTILIZING GAS CHROMATOGRAPHY MASS SPECTROMETRY TOOLS

2.1 Introduction

The beer industry is a competitive global industry regardless of market segment (i.e., international, U.S. macro-brewery, or U.S. craft brewery). In 2020, there were 8,884 breweries in the U.S. (8,764 craft breweries, 120 macro-breweries) that produced 150 billion barrels of beer [76, 77]. Still, the 2020 U.S. beer sales by volume decreased 2.9%, and 19.5% of the total sales was from imported beer, signifying increased competition in the alcohol beverage market [76, 77]. To compete, breweries must use various means to win consumer loyalty including matching product to consumer preference trends, packaging presentation through container type and label design, and maintaining high quality and flavor stability. A quality beer that builds brand loyalty is one that is free from off flavors and meets a certain degree of excellence across a complex set of sensory characteristics that are maintained across batches and over time [41].

Beer is a complex matrix composed of water, ethanol, and thousands of volatile and non-volatile flavor active compounds. Beer flavor is the combination of taste, aroma, and mouthfeel[45]. A beer's chemical, and thus flavor, profile begins to change as soon as it enters the final package. During storage, flavor active compounds undergo chemical reactions that lead to both compound degradation and formation. This process can result in a reduction of favorable sensory attributes and the formation of undesirable ones, contributing to the aging, or staling, of beer[1]. The rate at which these changes occur depends on factors such as dissolved oxygen, temperature, agitation, and starting substrates. At a certain point, the flavor profile of a beer is no longer considered "true to brand". The ability for a beer to maintain its original flavor profile is

known as beer stability and can be determined through chemical and sensory evaluations. In a competitive global market where the time it takes for product to reach the consumer is increasing, maintaining the original flavor and chemical profile is an important goal of the brewer yet, beer stability remains one of the more difficult aspects to control[43, 78].

Many mechanisms of beer aging have been studied and include oxidation reactions[1, 79], Strecker aldehyde formation[80], hop volatile and acid degradations[1, 56], and ultraviolet (UV) light effects[46, 81]. However, much of the published research on beer stability has been conducted on light lagers and a limited group of compounds (e.g., volatiles and carbonyls)[82]. The most studied and notable aging marker is trans-2-nonenal, which imparts a paper-like flavor and drying mouthfeel[1, 43, 83]. This is a narrow and dated scope when considering the innovations of new beer styles coming from the craft beer industry, the emergence of new hop and barley cultivars, and technological process advancements. Along with aging chemical reactions, compounds in beer during storage interact with the packaging material where metabolites migrate into the packaging material through a phenomenon known as flavor scalping [55]. This effect has been shown to impact hop volatiles in both canned and bottled beer [56, 57]. There remains much about beer flavor stability, particularly in new craft beer styles, that is not well understood [43]. More research on understanding the mechanisms of beer stability in a comprehensive range of beer styles [48] and identifying additional markers of beer aging [43] is needed.

Beer aging and the chemical transformations referenced previously occur in the final package container. Bottles and cans, referred to as “small-pack”, are the most common ways for breweries to package and sell their products [20]. Over the last decade there has been a complete shift in small pack container usage in the craft brewing sector. In 2015, the breakdown was 20%

cans and 80% bottles. In 2020 the percentages flipped to 80% cans and 20% bottles[2, 37]. Causes of this dramatic shift can be attributed to the recent availability of small canning lines and the COVID-19 pandemic [2, 40]. While the long-term effects of the pandemic on packaging trends remain to be seen, it is likely that with the increased investment in canning technology across craft brewers, cans will remain the preferred package type for the foreseeable future.

Although similar in function, cans and bottles are inherently different. Cans are made of aluminum and are coated on the inside with a polymer, often epoxy and acrylic, to protect the liquid from metal taints and the aluminum from corrosion. The can lid is hermetically sealed to the can body, making it an airtight closure. Cans are more susceptible to oxygen pick up during the packaging process due to the large surface area of the can opening before the lid is attached, and the inability to completely purge the can of air via vacuum due to its weak structure [20]. The opaque aluminum material blocks UV light, preventing light induced reactions that result in an undesirable "skunking" effect. In contrast, bottles are made from brown, green, or clear glass, although brown is most often used due to its ability to block more UV light [20, 22]. Glass bottles undergo air evacuation via vacuum, often resulting in less oxygen pick up during packing. The cap, or crown, is also lined with a polymer similar to the coating of cans and is permeable at the glass-liner interface, allowing air to ingress over time[21]. The differences between cans and bottles affect the risk potential for different types of aging reactions, adding container type to the list of variables that affect flavor stability. The differences in aging mechanisms and degree of impact between cans and bottles has not previously been studied.

In this study, a non-targeted metabolomics approach was used to explore packaging effects on beer chemical stability in two craft-relevant, non-light lager styles. The use of a non-targeted analytical approach allowed for novel discoveries of potential metabolites important to beer

stability. Our focus on styles beyond light lagers and including various packaging types extends the knowledge of flavor stability mechanisms. Results from the study are relevant to modern breweries and beer styles and provide valuable information to make scientifically backed decisions around packaging and package type best practices.

2.2 Materials and Methods

2.2.1 Brewing parameters and storage

Sample beer was brewed at New Belgium Brewery Company (Fort Collins, CO). One batch of an amber ale (AA) (5.2% ABV, 22 IBU) and India pale ale (IPA) (7.0% ABV, 50 IBU), from the same brite tank was packaged into bottles and cans. Packaging of the two container types occurred on the same day for each batch and packaging of the two batches occurred within the same week. Both canned and bottled samples were flash pasteurized as a final stabilization step in the packaging process. The IPA was packaged into 12 oz. cans and bottles, and the AA was packaged into 12 oz. bottles and 16 oz. cans. Post-packaging, the batches were screened by a trained sensory panel at New Belgium Brewing Company and passed quality checks. Total package oxygen (TPO) was measured by Pentair Haffmans Automatic Inpack TPO/CO2 meter during the packaging run. Measurements were taken throughout the run and then average. The averages passed brewery quality control specifications and were as follows for each of the four treatments: AA cans (41.3 ppb TPO), AA bottles (85.4 ppb TPO), IPA cans (76.5 ppb TPO), IPA bottles (104 ppb TPO).

Immediately after packaging, samples were stored under cold conditions (3°C) at the brewery. Due to logistical constraints, the time between package day and week 0 sample day was 14 days for AA and 6 days for IPA. Once all samples were package, they were transferred to

Colorado State University (CSU) where they were stored under cold conditions for the first 30 days, then stored at room temperature (20°C) for 150 days, for a total of 6 months storage time. Storage conditions were established to mimic typical packaged beer storage conditions. Shelf life of both brands were previously determined by a trained sensory panel to be 5 months.

2.2.2 Sampling and sample preparation

Throughout the 6-month aging period, samples were collected bi-weekly, starting with the day samples were transferred to CSU (i.e., week 0 or baseline), resulting in a total of 13 time points. At each sampling time, three unique packaged samples (n=3) in each treatment were randomly selected from storage resulting in a total of 156 samples over the course of the aging period. For each sample, multiple aliquots were collected in 2-mL glass vials and stored at -80°C until chemical analysis. At the time of chemical analysis, samples were randomized, thawed, and conditioned to room temperature (20°C) over a 1-hour period, at which point the samples were degassed by sonication.

2.2.3 Analysis of small non-volatile molecules in beer by Gas Chromatography Mass Spectrometry (GC-MS)

2.2.3.1 Polysaccharide cleanup protocol

A sample clean-up procedure was performed prior to derivatization. This process precipitated out large polysaccharides to prevent interference with metabolite detection. 200µL degassed beer, 400µL methanol, and 400µL acetonitrile was added to a glass vial and agitated for 1 hour at 20°C, followed by a 20-hour incubation at 3°C then centrifuged. A pooled QC sample comprised of 22µL from each sample was created with the supernatant from this step.

75µL of the sample supernatant and pooled QC from this step was transferred to a new glass vial and dried down for 25 minutes using nitrogen gas. Vials were capped and then stored at -80°C until derivatization.

2.2.3.2 Sample derivatization protocol

Samples were derivatized by methoxyamine HCl (25mg/mL pyridine) and MSTFA+1% TMCS. The glass vials containing the dried down supernatant was brought to room temperature and 50µL methoxyamine HCl (25mg/mL pyridine) was added to resuspend the contents. Vials were incubated at 60°C for 45 minutes then sonicated for 10 minutes before a second incubation at 60°C for 45 minutes. 50µL MSTFA+1% TMCS was added and incubated for a final round at 60°C for 40 minutes. Vials were brought to room temperature over 10 minutes.

2.2.3.3 GC-MS analysis

A Clarus 690 gas chromatography system (PerkinElmer Waltham, MA, USA) coupled to a PerkinElmer Clarus SQ 8T mass detector was used to detect small semi-volatile and non-volatile molecules. Separation was conducted with a TG-5MS column (Thermo Scientific, 30m x 0.25mm x 0.25mm). One microliter of derivatized sample was injected at a 1:12 split ratio and 1.0 mL/min helium gas flow. Samples were injected in randomized order with a pooled QC injected between every six samples. The oven profile consisted of an 80°C hold for 30 seconds, ramping 15°C/min to 330°C, with an 8-minute hold at the end of the run. Masses between 50-620 m/z were scanned at 4 scans/second after electron impact ionization operating at 70eV. The injector temperature was held at 285°C, the transfer line was held at 280°C, and the source was held at 260°C.

2.2.4 Analysis of small volatile molecules in beer by Headspace Gas Chromatography Mass Spectrometry (HS-GC-MS)

For all samples, 2mL of degassed beer was pipetted into a 20mL headspace (HS) vial and immediately capped with a crimper. A pooled QC sample comprised of 500μL of each sample was prepared using the degassed room temperature samples. Samples were analyzed in a randomized order and a pooled QC was injected every six samples. Samples were stored at 3°C until loaded in the autosampler.

A Clarus 690 gas chromatography system coupled to a PerkinElmer (Waltham, MA, USA) Clarus SQ 8T mass detector was used to detect small volatile and semi-volatile. Separation was conducted with an Elite-624Sil MS column (PerkinElmer, 30m x 0.25mm x 1.4 mm). The TurboMatrix 40 Trap headspace sampler (PerkinElmer) was used as an HS sampler. Prior to injection samples were heated to 80°C for 20 min followed by preconcentration for 1 cycle in a TurboMatrix air monitoring trap (PerkinElmer). Absorption of compounds to the trap was performed at 25°C at a vial pressure of 40 psi. Vaporization was performed at 20 psi and 260°C for 0.5 min. The HS needle was held at 120°C and the HS transfer line was held at 140°C. Samples were injected at a 1:10 split ratio and 1.0-mL/min helium gas flow with the column pressure set to 23 psi. The oven profile consisted of a 35°C hold for 5 min, ramping 6°C/min to 245°C. Masses between 35-350 m/z were scanned at 4 scans/second after electron impact ionization operating at 70eV. The injector temperature was held at 180°C, the transfer line was held at 260°C, and the source was held at 240°C.

2.2.5 Statistical analysis

The open source XCMS software was used to define a matrix of molecular features as previously described[84, 85]. The *RAMClustR* package operating within the R Programming

(version 4.0.3) environment was used to cluster co-varying and co-eluting features[86, 87]. Metabolite annotations were performed by spectral searching against the NIST, GOLM and an in-house proprietary database in the program RamSearch (Colorado State University, CO, USA)[88]. Data from each analysis was processed independently.

The resulting datasets were then z-score transformed and combined into a final data matrix and used for multivariate (MVA) statistical analyses. The SIMCA software (Umetrics, Version 17) was used to perform principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA), data scaled by unit variance (UV). Variable importance factors (VIP) from model biplots were used to identify metabolites of interest. Those metabolites with a VIP score ≥ 2 were flagged for further UVA testing. Multiple analysis of variance (MANOVA), performed in R Programming using the *manova()* function of the *mvnormtest* package, was also used to screen and identify metabolites of interest ($P \leq 0.05$)[89].

Univariate (UVA) statistical analysis was then used to explore the final group of metabolites of interest. Assumptions of normality were checked, and log or square root transformations were performed if necessary. A linear model was created and model fitness assessed by the multiple R^2 value. Metabolites with a linear model fit of $R^2 \geq 0.900$ were considered adequate. The change over time and the estimated marginal means at specific time points were explored using the *emmeans()* and *emtrends()* function of the *emmeans* package for R Programming[90], where significance was determined at the 95% confidence interval and $P \leq 0.05$.

2.3 Results and Discussion

2.3.1 Broad analysis of the effects of package type on beer stability indicate style influence

Non-targeted metabolite profiling is a powerful tool that can be used to detect metabolite variations in a large dataset. In the present study, this approach was used for all sample treatments (i.e., AA can, AA bottle, IPA can, and IPA bottles) across 13 timepoints (n=156) resulting in the detection of 351 molecular features across both analytical platforms. Of those molecular features, 76 total metabolites were annotated, 46 by GC-MS detection and 27 by HS-GC-MS detection (Table 6). The methods use, described in *Section 2.2*, detected volatile and small non-volatile polar compounds including amino acids, terpenes, esters, alcohols, carbohydrates, and carbonyls. The metabolites and their relative abundances from both detection platforms were combined into one data matrix and normalized about the mean (i.e., z-score) prior to multivariate analysis (MVA). An unsupervised principal components analysis (PCA) was performed on the 76 annotated metabolites and all samples after outliers were removed (n = 144) to determine the source of variation across the entire dataset (Figure 5). Outliers were

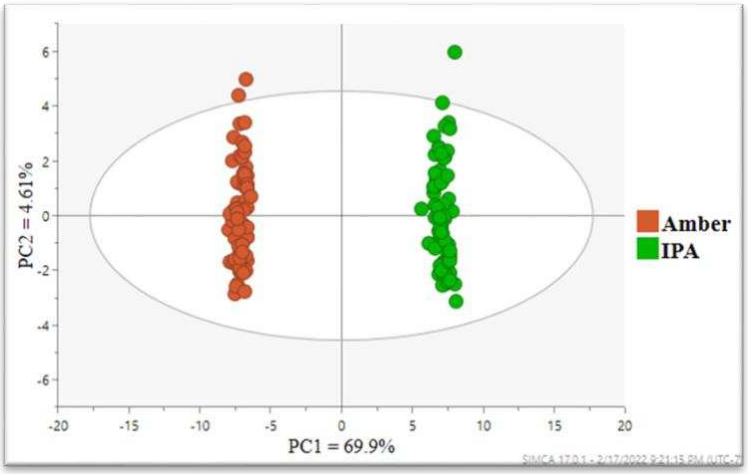

Figure 5. Principal components analysis (PCA) of combined GC-MS and HS-GC-MS datasets.
The combined data matrix used in the formation of this model was normalized about the mean (z-score). The model is separating by style across PC1, with orange circles representing AA samples and green circles representing IPA samples.

defined as datapoints with a Hotelling's T^2 Crit > 95%. The PCA model explains 74.5% of the total variation across the first two components, where PC1 explains 69.9% of the variation and PC2 explains 4.6% of the variation. The model is well fit ($R^2 = 0.867$) and clearly separates. AA and IPA across PC1, indicating style as the main source of variation across all samples. This is to be expected with AA and IPA being unique and vastly different beer styles. AA are a malt forward style made with a low to medium level of hops utilized in the recipe. IPA's have a prominent hop profile due to the higher hopping rates in its recipe formulation. Thus, it is expected that metabolite profiles of different styles should be the greatest source of variation regardless of storage time or package type. The PCA model supports this expectation and thus serves as a positive check on our dataset.

To further investigate package type differences, a supervised partial least squares discriminant analysis (PLS-DA) was applied to the AA samples (Figure 6). The PLS-DA model for AA samples explains 30.2% of the total variation across the first two components, where PC1 explains 18.8% of the variation and PC2 explains 11.4% of the variation (Figure 6a). The model fit is good and predictive ($R^2 = 0.981$, $Q^2 = 0.964$). The canned and bottled samples are distinctly separating across PC1 indicating packaging type as the main source of variation across all AA samples. This provides evidence there are metabolite differences between AA cans and AA bottles. From the loadings plots (Figure 6b) we see which metabolites are driving the variation in the data. The metabolites on the furthest left in the biplot are strongly associated with bottled AA (e.g., 2-methylbutyl butyrate, isobutyl isobutyrate) and the metabolites furthest right in the biplot are strongly associated with canned AA (e.g., myo-inositol, humulene).

The PLS-DA model was applied to IPA samples separately (Figure 7). The model explains 15.0% of the total variation across the first two components, where PC1 explains 7.53%

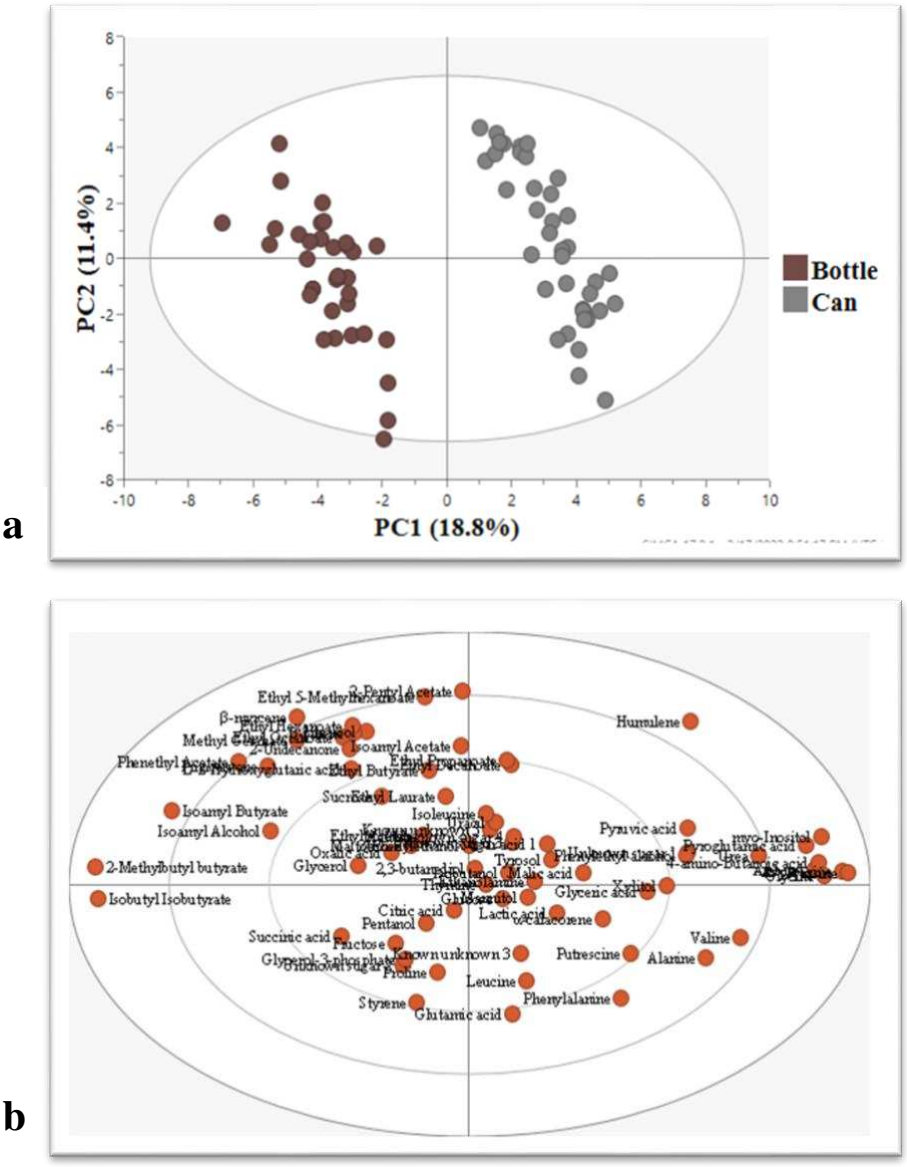

Figure 6. Partial least squares discriminant analysis (PLS-DA) models for AA colored by package type, and the associated loadings plot.
a) PLS-DA model for AA with brown circles representing bottled samples and gray circles representing canned samples. Model is separating data by package type across PC1, exhibiting package type predictability. (*b*) The associated loadings of the AA model showing metabolites driving the observed separation in both directions.

of the variation and PC2 explains 7.48% of the variation (Figure 7a). The model fit and predictive power ($R^2 = 0.667$, $Q^2 = 0.115$, respectively) are weak, indicating a lack of package type predictability for IPA samples and metabolite differences between IPA cans and IPA bottles. Still, the bottles are generally grouping on the left side of the model and the cans are

generally grouping on the right side, similar to the trend seen in the AA model. Again, the loadings plot (Figure 7b) indicates metabolites that are driving that trend. Because there is strong predictability in only one of the two styles represented in this study, the metabolite variations between cans and bottles appears to be style dependent.

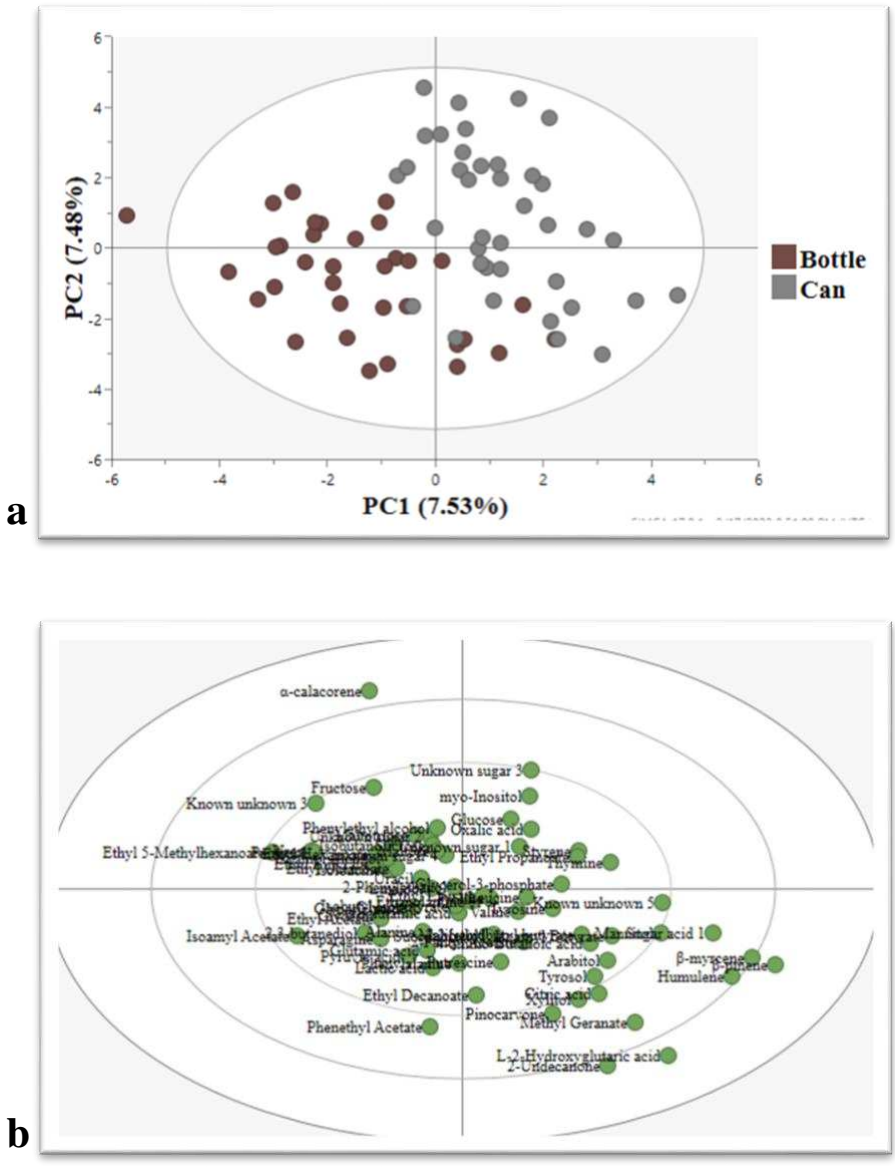

Figure 7. Partial least squares discriminant analysis (PLS-DA) models for IPA colored by package type, and the associated loadings plot.
(*a*) PLS-DA model for IPA with brown circles representing bottled samples and gray circles representing canned samples. Model shows a lack of separation and predictability by packaging type. (*b*) The associated loadings of the IPA model.

The variable importance of the projection (VIP) value for each metabolite in the model represents their importance in driving the observed variation. For both PLS-DA models, metabolites with a VIP ≥ 2 were flagged as metabolites of interest for further statistical investigation. A MANOVA test was also conducted, and the metabolites with significance ($P \leq 0.05$) in the interaction between storage week number and package type were flagged as additional metabolites of interest. In total, 17 metabolites consisting of compounds from amino acid, ester, terpene, alcohol, and carbonyl chemical classes were flagged between the two methods. A complete list of the metabolites of interest and the methods used to identify them can be found in Table 4.

Figure 8 displays a heatmap with hierarchal clustering to visualize important relationships within the data. The scales are based on z-score normalization where the intensity

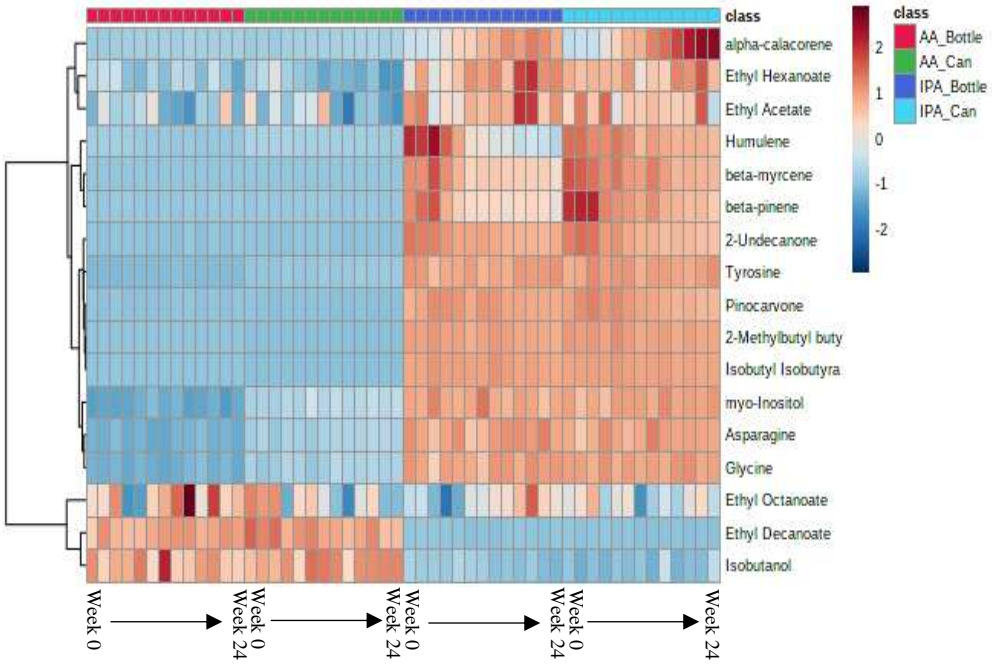

Figure 8. Heatmap visualization of relationships between treatment, metabolite, and time.
The figure was created with the averaged z-score normalized relative abundances of each sample treatment, labeled as "class", at the 13 bi-weekly sample time points.

Table 4. List of metabolites considered important to explaining data variation and the justification as indicated shading.

Class	Subclass	Metabolite	Sensory Attribute	Detection	VIP_a	VIP_b	*P*-value$_c$
Carboxylic acid	Amino acids	Glycine	NA$_d$	GC-MS	2.00	0.76	0.81
		Tyrosine	NA	GC-MS	2.21	0.50	0.68
		Asparagine	NA	GC-MS	2.10	0.76	0.34
	Carboxylic acid ester	Ethyl Acetate	nail polish remover, solvent, fruity, sweet	HS-GC-MS	0.22	1.02	0.02
		Isobutyl isobutyrate	grape skin, pineapple, tropical	HS-GC-MS	2.18	0.12	0.71
Lipid	Fatty acid ester	Ethyl decanoate	caprylic, soapy, estery	HS-GC-MS	0.59	1.42	<0.001
		Ethyl octanoate	apple, sweet, fruity, sour apple	HS-GC-MS	0.59	0.43	<0.001
		Ethyl hexanoate	apple, anise seed, citrus, solvent	HS-GC-MS	0.56	0.59	0.02
		2-Methylbutyl butyrate	fruity, pear, apricot, tropical, spicy, apple	HS-GC-MS	2.13	0.87	0.40
	Monoterpene	Pinocarvone	minty	HS-GC-MS	0.97	0.78	0.001
		β-myrcene	spicy, citrus, resinous, piney, lemon, woody	HS-GC-MS	0.79	2.46	0.74
		β-pinene	woody, green, resinous, dry	HS-GC-MS	0.59	2.45	0.25
	Sesquiterpene	Humulene	spicy, herbal, grassy, woody, clove	HS-GC-MS	1.71	1.76	<0.001
		α-calacorene	citrus, spicy, woody	HS-GC-MS	0.66	2.09	<0.001
Organooxygen	Alcohol	Isobutanol	malty, solvent	HS-GC-MS	0.22	0.54	0.01
		myo-Inositol	NA	GC-MS	2.17	0.74	0.03
	Carbonyl	2-Undecanone	varnish, bitter, green plants, geranium, fruity, citrus	HS-GC-MS	0.58	1.59	0.005

(*a*) VIP scores from PLS-DA model of amber samples
(*b*) VIP scores from PLS-DA model of IPA samples
(*c*) *P*-values from ANOVA and the interaction between package type and week number
(*d*) NA (not applicable) due to a lack of sensory information in the literature

of the red squares represent a positive magnitude relationship, and the intensity of the blue squares represent a negative magnitude relationship. Figure 8 represents the relationships between sample treatment and the 17 selected metabolites of interest over time, indicated by the averaged sample replicates (n = 3) at each timepoint along the bottom axis of the graph from left to right within each sample treatment. We see most metabolites are lower in relative abundance in the AA samples than in the IPA samples. There are two metabolites that follow an opposite relationship and are higher in relative abundance in AA than IPA. Those metabolites are ethyl decanoate and isobutanol. Ethyl octanoate is grouped with these metabolites within the hierarchal clustering, however it appears to display a more sporadic trend than the rest of the metabolites of interest. This is shown by the two main groups in the hierarchal clustering. Furthermore, the heatmap displays a group within the AA samples that are lower in AA bottles than in AA cans. Those metabolites are myo-inositol, asparagine, and glycine. Within the IPA treatment, the hop acids humulene, **β**-myrcene, and **β**-pinene display an obvious decrease overtime as the intensity of the color moves from a dark red to light blue over week 0 to week 24. Interestingly, the hop acid **α**-calacorene increases over time. Additionally, within this group there are differences between cans and bottles. The relationships displayed in Figure 8 will be explored further in *Section 2.3.2* and *Section 2.3.3*.

2.3.2 Baseline differences in metabolite abundance in cans and bottles appear to be a source of package type differences in amber ale

Once a broad analysis of the variation in the data was determined, focused univariate analyses (UVA) were applied to explore the observed MVA results (Table 5). First evaluated was the data to identify significant differences in metabolite abundance at baseline (e.g., week 0) between package type in both beer styles. Of the 17 metabolites of interest, eight had baseline

significant ($P \geq 0.05$) differences between cans and bottles in the AA samples, and six in the IPA samples (Table 5, Figure 9). Six out of eight of the metabolites with significant baseline

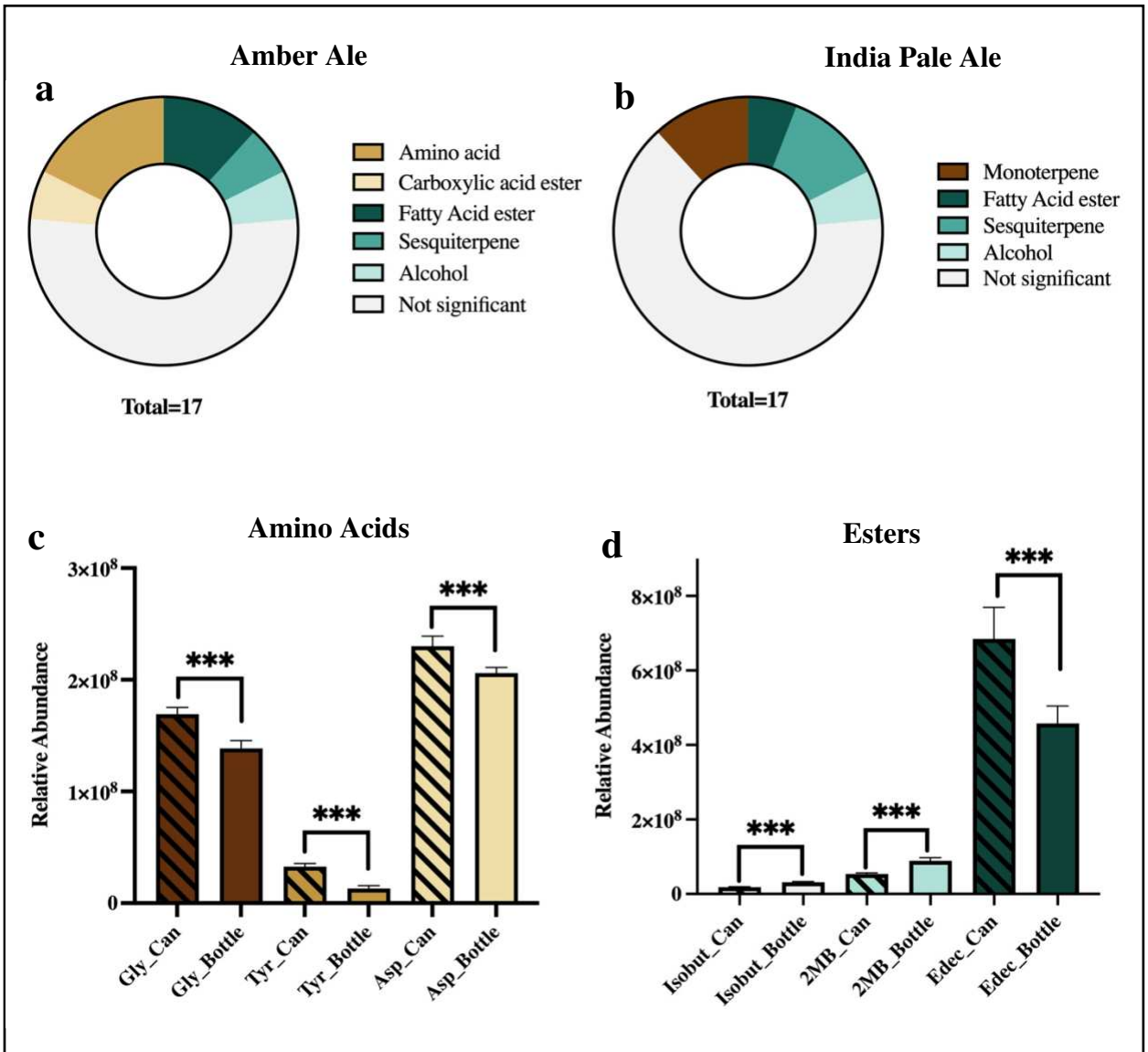

Figure 9. Generalized baseline differences in AA samples by chemical class.
(*a*) Proportion graph of chemical classes with baseline (week 0) differences in AA samples, (*b*) Proportion graph of chemical classes with baseline (week 0) differences in IPA samples, (*c*) Amino acids (Gly = glycine, Try = tyrosine, Asp = asparagine) with significant (*** indicates P <0.001) baseline differences between cans and bottles in AA samples, (*d*) Esters (Isobut = isobutyl isobutyrate, 2MB = 2-methylbutyl butyrate, Edec = ethyl decanoate) with significant (*** indicates P <0.001) baseline differences between cans and bottles in AA samples.

differences in AA were also flagged from the VIP scores of the PLS-DA model. This indicates the baseline differences explain, in part, the separation observed between the cans and bottles in the AA PLS-DA model. Two main chemical class groups arise from the list of eight metabolites including esters and amino acids. By exploring the chemical properties of these compounds and the literature proposed, potential mechanisms driving the baseline differences in AA begin to form.

Isobutyl isobutyrate and 2-methylbutyl butyrate are two esters for which the baseline abundance was observed to be significantly higher in bottles than in cans. Esters readily move from beer to the atmosphere and are susceptible to oxidation[1]. One potential mechanism explaining the baseline difference in these compounds is the packaging process and the physical parameters of the package. A can has a larger surface area exposed to the air before the can lid is sealed compared to a bottle and its cap. This could lead to more volatilization of esters from beer in an open can during packaging, which would result in a lower baseline relative abundance. The difference in baseline abundance may also be a result of a greater oxidation reduction in cans than bottles by the initial total packaged oxygen (TPO). It has been shown that esters are readily oxidized and that these reactions occur after packaging once the ground state oxygen that makes up the TPO becomes reactive oxygen species (ROS) [1, 52]. There is a lag phase from when TPO becomes ROS and begins oxidative deterioration. The length of the lag phase varies with the different beer matrixes and its endogenous antioxidant properties[91]. It appears the lag phase occurred within the 14-day time gap between when AA samples were packaged and when the baseline samples were collected in the lab. The difference in antioxidant properties may explain the lack of baseline differences in IPA. Hops contain hundreds of polyphenols that have antioxidative properties [78, 92]. Because IPA styles utilize high hopping rates, the endogenous

antioxidant activity of the IPA matrix protects against oxidation compared to the lower hopped AA samples. In contradiction to what is seen in isobutyl isobutyrate and 2-methylbutyl butyrate, ethyl decanoate starts higher in cans. The mechanisms driving this difference could result from ester formation which was previously observed, however, the cause is not fully understood [1].

Esters provide important flavor and aroma characteristics in beer. Each style or brand has its own unique group of esters that make up the flavor profile, along with other flavor active compounds. A reduction in esters will result in an overall dampening of the flavor, where the formation of esters will impart unintended aromas. In both scenarios the overall balance of the beer is affected[48, 49]. Ultimately, it is up to the brewer to decide which esters are important to each of their brands, and if a change in a specific ester's abundance will negatively affect the organoleptic properties of that brand.

The amino acid group, consisting of tyrosine, glycine, and asparagine, are a second chemical class important to the baseline difference discussion. The relative abundance of the three detected amino acids were observed to be significantly lower in bottles than cans in AA. No significant difference in these metabolites was observed in the IPA samples. It is well understood that amino acids will bind to silica glass and this is often a source of analytical error when preparing samples for amino acid analysis in a lab setting[93, 94]. Although not previously documented, it is possible that amino acids in beer adsorb to the inside of a glass bottle, accounting for the baseline differences between package type observed in the present data.

To understand the style-dependent effect of baseline differences of amino acids, consider the differences in hop usage between AA and IPA. The antioxidant properties of hops are the result of hundreds of polyphenol compounds[92]. It has been documented extensively that these polyphenols will bind to proteins, peptides, and therefore amino acids[78, 95]. The higher

hopping rates used in the production of IPAs results in increased levels of polyphenols in solution that could bind the amino acids in the beer matrix. Furthermore, the amino acids in beer originate from malt and thus higher in IPAs because of its higher finishing gravity. If amino acids are bound to polyphenols, the adsorption effect would be impeded which could reflect the lack of baseline difference observed between package types in the IPA style.

Recent work has shown that left over free amino acids in beer will react with Maillard compounds formed during mashing and undergo Strecker degradation reaction. The resulting Strecker aldehyde (SA) products are associated with negative staling flavors and aromas[1, 52]. Each amino acid will result in a unique SA [53] and their sensory impacts continue to be explored. In beers with lower hopping rates (e.g., amber ale) where amino acid – polyphenol binding is minimal, canned product could be at a higher risk for SA formation due to the higher abundance of amino acids at baseline. This could ultimately lead to sensory differences of the same batch of beer packaged into both bottles and cans over time.

Taken together, these results demonstrate that baseline differences are a major variable explaining the predictability of package type in AA samples and therefore, the discussion has focused on the metabolites with significant baseline differences in this style. Targeted investigations on the mechanisms driving baseline differences needs continued exploration.

2.3.3 Metabolite changes over time are dependent on package type

In addition to investigating the unexpected differences observed at baseline, we next evaluated how the abundance of the metabolites of interest changed over time, as this relates to beer chemical stability and how package type influences those changes. The *P*-values reported from the ANOVA analysis (Table 4) indicate significant changes in a metabolite over time that

are dependent on package type. The estimated marginal means of linear trends was performed by the *emtrends()* function in R Programming once assumptions of normality were met and a linear model was fitted. The analysis produced a 95% confidence interval, indicating a significant change over time for a metabolite in each sample treatment (i.e., AA bottle, AA cans, IPA bottles, IPA cans) and is reported (Table 5).

Of the 17 metabolites of interest, ten were found to be significant from the estimated marginal means of linear trends analysis. There were six metabolites that significantly changed over the 24-week aging period in IPA bottles, five in IPA cans, four in AA bottles, and ten in AA cans (Figure 10a). All four of the metabolites identified in AA bottles were also identified in AA cans. The additional six metabolites in AA cans were tyrosine, 2-methylbutyl butyrate, myo-inositol, ethyl acetate, ethyl octanoate, and ethyl hexanoate. It is important to note the later three metabolites did not have an acceptable model fit and therefore conclusions regarding their influence on the variation in the data is limited. Still, there are six additional metabolites significantly changing over time in AA cans as compared to bottles, implying a general reduction in chemical stability in AA cans as compared to AA bottles. Whether these compounds have an impact on the organoleptic properties of the beer is outside the scope of this study but warrants further investigation.

Visualization of the linear models and ANOVA *P*-values reveals hop terpenes, volatile beer aroma compounds, as an interesting chemical class to evaluate. The focus of this discussion will be around the important detected terpenes in this study; humulene, **β**-myrcene, **α**-calacorene, pinocarvone (Figure 10). The terpenes exhibit a decrease in relative abundance except for **α**-calacorene, which increases over time. **β**-pinene will not be included because it lacks significance determined from the ANOVA or the estimated marginal means.

Table 5. Univariate analysis of the seventeen metabolites of interest.

Metabolite	R^2[a]	IPA			AA		
		Baseline[b]	OT[c] Bottle	OT Can	Baseline	OT Bottle	OT Can
Glycine	0.950	0.58	No	No	<0.001	No	No
Tyrosine	0.990	0.24	No	No	<0.001	No	Yes
Asparagine	0.940	0.74	No	No	<0.001	No	No
Ethyl acetate	0.428[d]	0.56	No	No	0.21	No	Yes
Isobutyl isobutyrate	0.998	0.96	No	No	<0.001	No	No
Ethyl decanoate	0.977	0.88	No	No	<0.001	Yes	Yes
Ethyl octanoate	0.170	0.04	Yes	No	0.06	No	Yes
Ethyl hexanoate	0.687	0.20	Yes	No	0.74	No	Yes
2-Methylbutyl butyrate	0.997	0.62	No	No	<0.001	No	Yes
Pinocarvone	0.994	0.001	No	Yes	0.33	No	No
β-myrcene	0.994	0.05	Yes	Yes	0.76	Yes	Yes
β-pinene	0.966	0.06	No	No	0.41	No	No
Humulene	0.980	<0.001	Yes	Yes	<0.001	Yes	Yes
α-calacorene	0.964	0.02	Yes	Yes	0.47	Yes	Yes
Isobutanol	0.794	0.02	No	No	0.43	No	No
myo-Inositol	0.954	0.14	No	No	<0.001	No	Yes
2-Undecanone	0.991	0.16	Yes	Yes	0.50	No	No

(*a*) Demonstrates fitness of a linear model after necessary transformations to meet assumptions of normality

(*b*) *P*-values produced from the *emmeans()* function in R Programming to determine significance in relative abundance in week 0 samples between cans and bottles within each style

(*c*) Over Time (OT) demonstrates if a linear model is significantly changing over the 24-week aging period with a 95% confidence interval in each style and package type. Analysis performed by the *emtrends()* function in R Programming

(*d*) Metabolites with R^2 values < 0.900 are highlighted in red and considered as an unacceptable model fit

No general trends are seen across the terpenes due to the varying chemical properties of this large group of compounds, a result consistent with the literature[56, 96]. Temperature, alcohol content, and pH of beer, and the terpene's water solubility, or polarity, can all affect the propensity for scalping into can and crown liners[56, 57]. Humulene, a hop volatile with sensory descriptors of earthy and herbal, appears to exhibit liner scalping to the greatest extent of all the terpenes. Humulene significantly changes over time in both AA and IPA and is dependent on package type ($P < 0.001$). The linear models for both styles show humulene decreases at a greater magnitude in bottles than in cans. Previous work has shown bottle cap liners scalped hop volatiles to a greater extent than can liners and could be reflected in the present data[56]. When considering the surface area of a bottle cap liner to the liner of a can, the greater scalping impact of the bottle cap is not an intuitive result. A possible mechanism for this observed effect could be explained by an equilibrium of metabolites within the can liner and beer matrix from the beer's constant contact with the liner during storage. Whereas in bottled beer volatile metabolites are trapped into the headspace and do not have the ability to reenter the liquid, thus the bottle cap liner maintains all the metabolites within. Furthermore, the bottled samples had a higher average TPO than cans, and oxygen ingress through bottle crowns is generally accepted. This could increase the oxidation reactions occurring in IPA bottles, exacerbating the decrease of humulene.

Myrcene, a hop volatile with sensory descriptors of "hoppy" and freshly herbaceous, is also significantly decreasing over time in all sample treatments. Unlike humulene, package type is not a determining variable ($P = 0.74$). The decrease in relative abundance is likely due to oxidation reactions. The lack of package effect and the difference in results observed for humulene may be due to the higher water solubility of myrcene (0.077g/L). In contrast, humulene has a lower

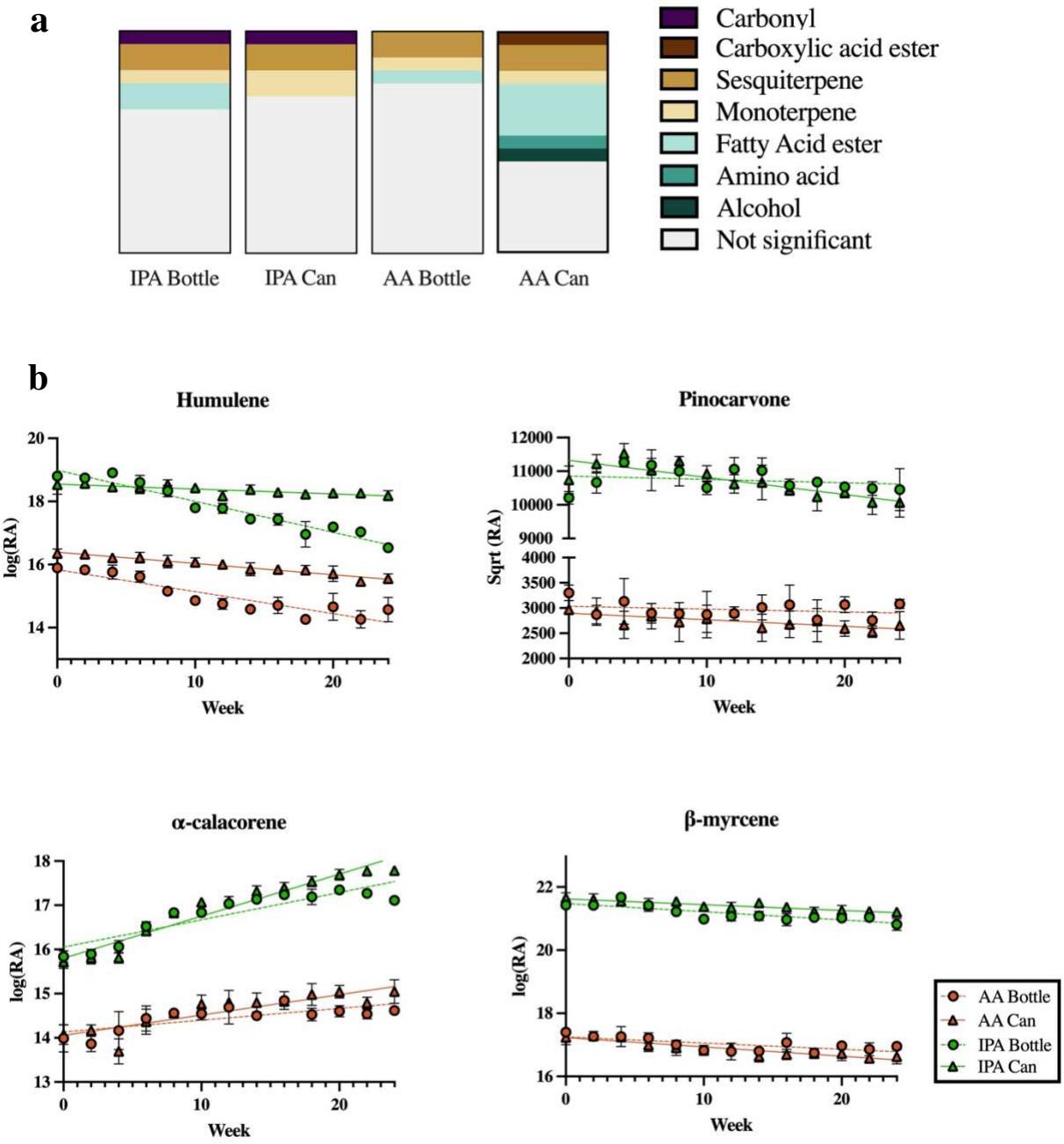

Figure 10. Generalize significant changes over time by chemical class and liner modeling of important terpene metabolites.

(*a*) Proportion charts for each sample treatment and the chemical classes significantly (95% confidence interval) changing over time of the seventeen identified metabolites of interest. (*b*) Linear modeling of relative abundance (RA) of four important terpenes (humulene, **β**-myrcene, **α**-calacorene, pinocarvone) significantly ($P \leq 0.05$) changing over time. Each terpene linear model graphs the four sample treatments, with orange representing AA, green IPA, dashed line/circle symbol bottle, and solid line/triangle symbol as cans. Each time point symbol represents the mean (n=3) with standard error bars.

water solubility (0.011g/L), is more non-polar, and therefore is readily scalped by the liner polymers. This result is consistent with previous studies on hop volatile scalping[56, 57].

Pinocarvone, described as minty, follows a decreasing trend over time that is different from humulene and myrcene. In IPA cans, pinocarvone is significant in its interaction between storage week and package type ($P = 0.001$). Pinocarvone has the highest water solubility (0.62g/L) of all the detected terpenes and is the only metabolite with an oxygen molecule in its chemical structure. These chemical property differences may explain why pinocarvone behaves differently, and exhibits a greater scalping effect by the can liner than the bottle cap. There would have to be a difference in the polymer material of the can and crown liner to explain pinocarvone's affinity for the can liner material however, the polymer make up is unknown in this study and is also often unknown to brewers. This information is necessary to fully understand the interaction of these hop terpenes with its packaging[55]. The muted scalping effect in the AA samples could be due to the lower relative abundance as compared to IPA.

The only terpene with an increase over time is **α**-calacorene, a compound known to impart spicy and herbal aromas[97]. The effects seen over time are dependent on package type ($P < 0.001$) and is observed in both AA and IPA. The increase in **α**-calacorene could be explained by formation through oxidation or enzymatic release, which has been previously demonstrated for other terpenes[96, 98]. The rate of **α**-calacorene is greater in cans for both AA and IPA suggesting that package type has a greater impact than style on the observed changes in this compound.

It is not surprising that the terpenes detected in this study exhibit varying behaviors during aging with their unique chemical properties resulting in different interactions based on style and package type. The implications for flavor impact are greater for IPAs due to the

importance of hop volatiles in IPA styles, however, regardless of style any change in abundance of flavor active compounds could affect the chemical balance and impact the original sensory profile. A targeted analysis could elucidate in more detail how package type and style impact important hop volatiles.

2.4 Conclusion

A non-targeted metabolomics approach was used to explore style and packaging effects on beer chemical stability. The two styles chosen were alternatives to light lagers, which have historically dominated the field of beer stability research, to broaden the knowledge of flavor stability mechanisms. The use of a non-targeted analytical approach allowed for novel discoveries of potential metabolites and mechanisms important to beer stability. Predictability of package type was dependent on style. AA demonstrated differences between cans and bottles while IPA did not through multivariate analysis. Additional univariate analysis showed the baseline differences between cans and bottles mainly drove the variation in AA. The presumed mechanisms are oxidation, volatilization during packaging, and glass material adsorption. Although the predictability of package type in IPA was weak, changes over time in the detected terpenes proved important to understanding aging mechanisms in IPA. Aligning with previous work, the changes in hop volatiles depend on the metabolite's chemical properties and their interaction with the packaging material, specifically the can and bottle crown liners.

The results of this study do not conclude there is a general best package type for all styles, rather it demonstrates there is much nuance in the effects of package type depending on style and each individual metabolite. A quantitative targeted analysis is needed to fully understand the mechanisms driving the effects of package type on beer stability. Although

outside the scope of this study, pairing targeted or non-targeted analytical analysis with sensory outcomes would provide additional important information to the brewer since the sensory experience is equally important to the subject of beer stability. Ultimately, this type of work should provide relevant knowledge so that brewers may make scientifically backed decisions around packaging, package type best practices, and shelf-life determinations.

CHAPTER 3 - INVESTIGATING THE USE OF DART-MS AS AN ADVANCED ANALYTICAL TOOL TO EXPEDITE BEER FLAVOR STABILITY ANALYSIS

3.1 Introduction

Beer flavor is the combination of taste, aroma, and mouthfeel of a beer for which thousands of flavor active compounds are responsible[45]. Beer flavor is not static and begins to change as soon as it enters the final package. Flavor active compounds attributed to a beer's sensory profile undergo various chemical reactions resulting in the formation and degradation of compounds. The chemical compounds that are not part of a beer's flavor profile are considered off flavors and contribute to the staling of beer flavor. When off flavors are detected through aroma, taste, or mouthfeel, they have passed the flavor threshold and a beer is no longer "true to brand". The degradation of desirable flavor compounds results in the lessening of a beer's favorable flavor attributes, which also contributes to beer staling. Therefore, brewer's want their beers to have good stability in which flavor changes are minimized. This goal remains one of the more difficult aspects of beer to control[43].

The most direct method of assessing flavor stability is through sensory analysis by a trained panel. Someone with the knowledge of which test to use, how to administer the panel, and conduct statistical analysis is necessary to run a successful sensory program. The most common test performed is the descriptive analysis test[61]. This test is used to systematically determine the description of a beer's profile of flavor attributes using an agreed upon lexicon. Once a brand's descriptive analysis is conducted and panelists are trained on the attributes that make a brand true to type flavor stability can be tested.

Although using a sensory panel for determining flavor stability, and in turn shelf life of a product, is the most common method it come with some challenges and limitations. Sensory

panels can be unobtainable for breweries because of the expertise, financial investment, and time needed to run a successful sensory program. Although not a requirement, it is often the case that a brewery will hire at least one individual trained in sensory science. The cost to employ someone with this technical skill set is significant. Additionally, there are costs for acquiring the appropriate glassware and off-flavor spikes. Sensory panels also require significant time investment. Extensive hours of training are necessary to ensure a reliable panelist[62]. Furthermore, enough time must elapse to naturally age beer before a sensory panel can check if a brand is true to type at a given timepoint. This aging period is typically 16-weeks [50], a timeframe that does not allow for a brewer to make quick and educated decisions about flavor stability in their products.

There will always be a need for sensory assessment of beer, but this method of assessing beer quality is not always accessible or timely. There is need for advanced analytical tools that can quickly assess beer freshness and stability[46]. Research showing the applicability of forced aging to speed up the aging process is variable [1, 51] and remains a debated means to reducing aging time. Although methods are being explored[61, 62], a reliable protocol for expediting beer flavor stability analysis has yet to be identified.

Ambient ionization mass spectrometry is an advanced analytical tool currently used in various capacities to streamline sample analysis. It is defined as ""the ionization of unprocessed or minimally modified samples in their native environment, and it typically refers to the ionization of condensed phase samples in air[69]." Analysis in a sample's natural state minimizes or eliminates the need and time requirements of sample preparation, rendering this tool a quick, easy, high throughput alternative approach for food analysis.

While there are many techniques that fall under this broad umbrella of ambient ionization, one method that has shown potential for food and agriculture applications is Direct Analysis in Real Time (DART). DART coupled with mass spectrometry (DART-MS) has been used to detect contaminant pesticides in wine [70], bisphenol A (BPA) in food [69], in food fraud (e.g., fish and spices) [71, 72], in product differentiation [69, 73, 74], and to quantify analytes of interest (e.g., caffeine in coffee)[75]. DART uses a heated plasma, usually helium, to desorb and ionize molecules in a sample prior to mass spectrometry detection[69]. DART-MS can ionize compounds in either positive or negative mode and can often detect a combination of polar, non-polar, volatile, and non-volatile molecules. Because DART-MS can analyze liquids that are adsorbed to a solid surface directly it can detect a wide range of metabolites.

While gas and liquid chromatography mass spectrometry result in a chemical profile consisting of a list of assigned spectra to a specific metabolite, DART-MS provides a single chemical fingerprint of the analyzed sample. When used in combination with predictive modeling and machine learning computational tools, this analytical platform enables high throughput screening, suggesting it is particularly suitable for high throughput analysis of beer. Previous research has demonstrated the use of DART-MS combined with predictive modeling to differentiate specific beer brands and their places of origin[73, 74]. This indicates DART-MS as a potential analytical tool for beer analysis, such as beer flavor stability.

In this study, a non-targeted and predictive metabolomics approach was used to explore DART-MS as a high throughput tool for streamlining beer flavor stability analysis. The use of DART-MS allowed for minimal to no sample preparation and rapid instrument analysis. Coupling the expedited analysis flow to successful predictive modeling would greatly decrease time and costs associated with flavor stability analysis. Focusing on styles beyond light lagers

and including various packaging types extends the knowledge of DART-MS capabilities in beer analysis. Results from the study are relevant to modern breweries and beer styles and could provide valuable information for making scientifically backed decisions around product shelf life and variables that impact flavor stability.

3.2 Materials and Methods

3.2.1 Brewing parameters and storage

See Chapter 2, *Section 2.2.1 Brewing parameters and storage*.

3.2.2 Sampling and sample preparation

See Chapter 2, *Section 2.2.2 Sampling and sample preparation.*

3.2.3 Chemical fingerprint generation by Direct Analysis in Real Time Mass Spectrometry (DART-MS)

3.2.3.1 Method optimization

Method parameters for analysis by Direct Analysis in Real Time Mass Spectrometry (DART-MS) instrumentation were optimized prior to sample analysis. Specifically, the method ionization mode and plasma temperature were optimized to obtain the highest total ion signal and to maximize spectral richness (i.e., number of peaks). Final optimization parameters were determined to be ionization under positive mode using a helium flow rate of 3L/min at a temperature of 350°C. The motorized linear rail system was set to a speed of 0.4mm/second and such that the distance between the source exit cap and the mass spectrometer inlet was 2.8cm. The source exit cap diameter was 2.5mm and the cone voltage was set to 15V. The final total run time was six minutes.

To minimize ionization of ambient air between samples, a steel plate manifold was eventually designed, fabricated, and attached to the motorized linear rail system outfitted with the 12 DIP-it® Holder. Sample introduction was performed using custom steel needles. The steel needles and plate were cleaned by sonication in deionized water then in methanol and dried with nitrogen gas to prevent sample carry over.

3.2.3.2 Sample analysis

The DART-MS analysis was conducted using the DART-Standardized Voltage and Pressure (DART-SVP) model ion source (IonSense, Inc., Saugus, MA) coupled by a Vapour interface (IonSense, Inc., Saugus, MA) to a single quadrupole mass spectrometer (ACQUITY QDa; Waters Corporation, Manchester, UK). Masses were scanned from 50-600 m/z in positive ionization mode to acquire spectra.

Quality control samples were made using a 50:50 mix of IPA and AA sample and were inserted into the randomized run order every ten samples. To determine the effectiveness of the custom steel plate attachment, samples were analyzed over two runs. The first analysis occurred over four days without the custom steel plate attachment and utilizing the Ionsense® DIP-it® Tips glass applicators. The second analysis occurred over two days with the custom steel plate and utilizing custom steel needles. For each sample run, six steel needles, either glass or steel, were dipped into the degassed liquid sample resulting in six replicate chromatograms per sample collected.

3.2.4 Statistical analysis

Data processing was performed with the beta version of the software WRC Abstract Model Builder (Waters Corporation, Manchester, UK). Six scans corresponding to each sample

were selected, spectra were background subtracted, normalized to total ion current, and averaged to generate one spectrum per sample. Peaks were binned at 0.5 m/z intervals to create a data matrix of molecular masses. SIMCA software (Umetrics, Version 17) was used to perform multivariate statistical analysis (MVA) on the data matrix utilizing principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA) on unit variance scaled data.

3.3 Results and Discussion

3.3.1 Instrument analysis was susceptible to ambient conditions and carryover contamination

Samples from the 13 bi-weekly time points were analyzed in the first analysis run over four days. The vendor-supplied glass Dip-It sticks were used for sample introduction. MVA was performed on the data matrix produced by the WRC Abstract Model Builder, and an unsupervised PCA model was created. The PCA model explained 64.5% of the total variation across the first two components, where PC1 explained 46.7% of the variation and PC2 explained 17.8% of the variation (Figure 11a). The model fit is acceptable ($R^2 = 0.880$) and clearly separates AA and IPA samples, indicating style as the main source of variation across all samples. This result matches previous work conducted looking at DART-MA capabilities to predict specific beer brands and their place of origin[73, 74].

The overall goal was to evaluate DART-MS capabilities to streamline beer stability assessments, thus a supervised PLS-DA model was created for each style based on time by week number. The AA model indicates a poor model fit ($R^2 = 0.66$) and a lack of beer age predictability ($Q^2 = 0.042$). Similarly, the IPA model had a poor model fit ($R^2 = 0.629$) and lacked beer age predictability ($Q^2 = 0.048$) (Figure 11b,c). The results suggest that DART-MS is

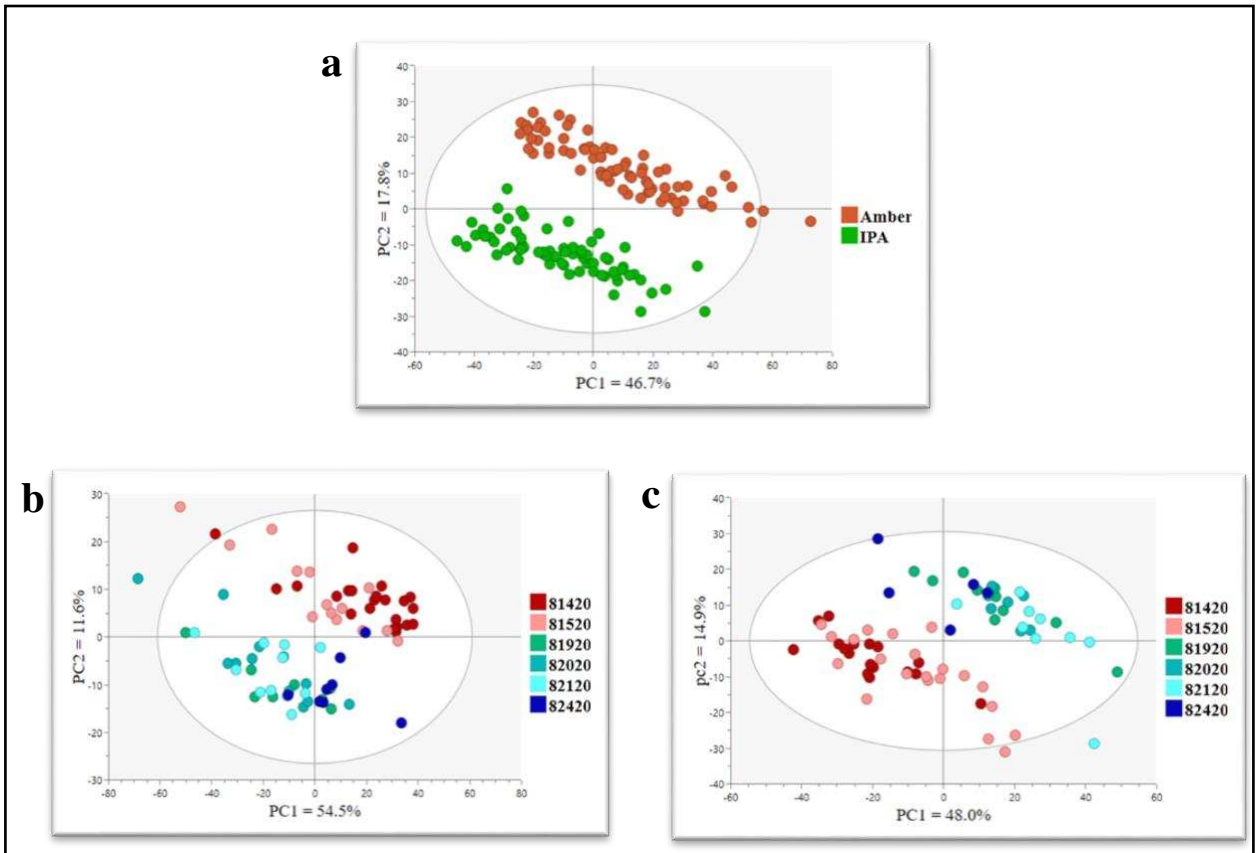

Figure 11. Multivariate analysis suggests style as biggest contributor to sample variation and instrument sensitivity to ambient conditions.
(*a*) PCA model is separating data by style indicating style is biggest contributor to sample variation, (*b*) PLS-DA model of AA colored by sample run date demonstrating run date effect, (*c*) PLS-DA model of IPA colored by sample run date also demonstrating run date effects strengthens the evidence that varying ambient conditions due to wildfire smoke affected sample analysis.

not suitable for predicting beer age and therefore is not a useful tool to streamline the process of determining beer flavor stability.

At the time of sample analysis, the Cameron Peak fire was burning west of Fort Collins causing severely poor outdoor air quality. As a result, the ambient air conditions inside the lab fluctuated with the outside smoke particulate levels. This in turn created variable ambient conditions during instrument ionization of the samples and contributed to high background noise levels and poor baseline recovery in the chromatography. Upon further discussions with the instrument vendor, it was also determined that the ionizing helium gas stream could cause

sample carry over between consecutive glass Dip-It stick applicators, exacerbating the observed poor baseline recovery. Without sufficient baseline recovery, data processing proved difficult when determining the beginning and end of each sample in the chromatography. It was therefore determined analysis must be completed without smoke contamination in the lab, justified by the sample run day effect exhibited in the PLS-MA models (Figure 11b,c). Additionally, a means to block the ionizing helium stream between sample applicators was deemed necessary to achieve quality data and effective downstream analysis. Once these two conditions were met, the samples were analyzed in a second run.

3.3.2 DART-MS analysis lacks the capability of predicting beer age

Results from our first experiment demonstrated that analysis by DART-MS was unable to predict beer age at the bi-weekly level. Therefore, during the second experiment, only samples from weeks 0, 4, 8, 12, 16, 20, and 24 were analyzed to assess monthly predictability. In this experiment, three additional optimized conditions were met prior to sample analysis; the absence of contaminating smoke in the lab's ambient air, the fabrication of a steel plate blockade that prevented helium gas to flow between sample applicators adjacent on the sample rail, and the use of steel needle sample applicators, which were cleaned more effectively than the glass Dip-It applicators.

Samples from the seven time points were analyzed over two days. MVA was performed on the data matrix produced by the WRC Abstract Model Builder, and an unsupervised PCA model was created. The PCA model explained 63.0% of the total variation across the first two components, where PC1 explained 45.8% of the variation and PC2 explained 17.2% of the variation (Figure 12a). The model fit was acceptable ($R^2 = 0.862$), and clearly separates AA and

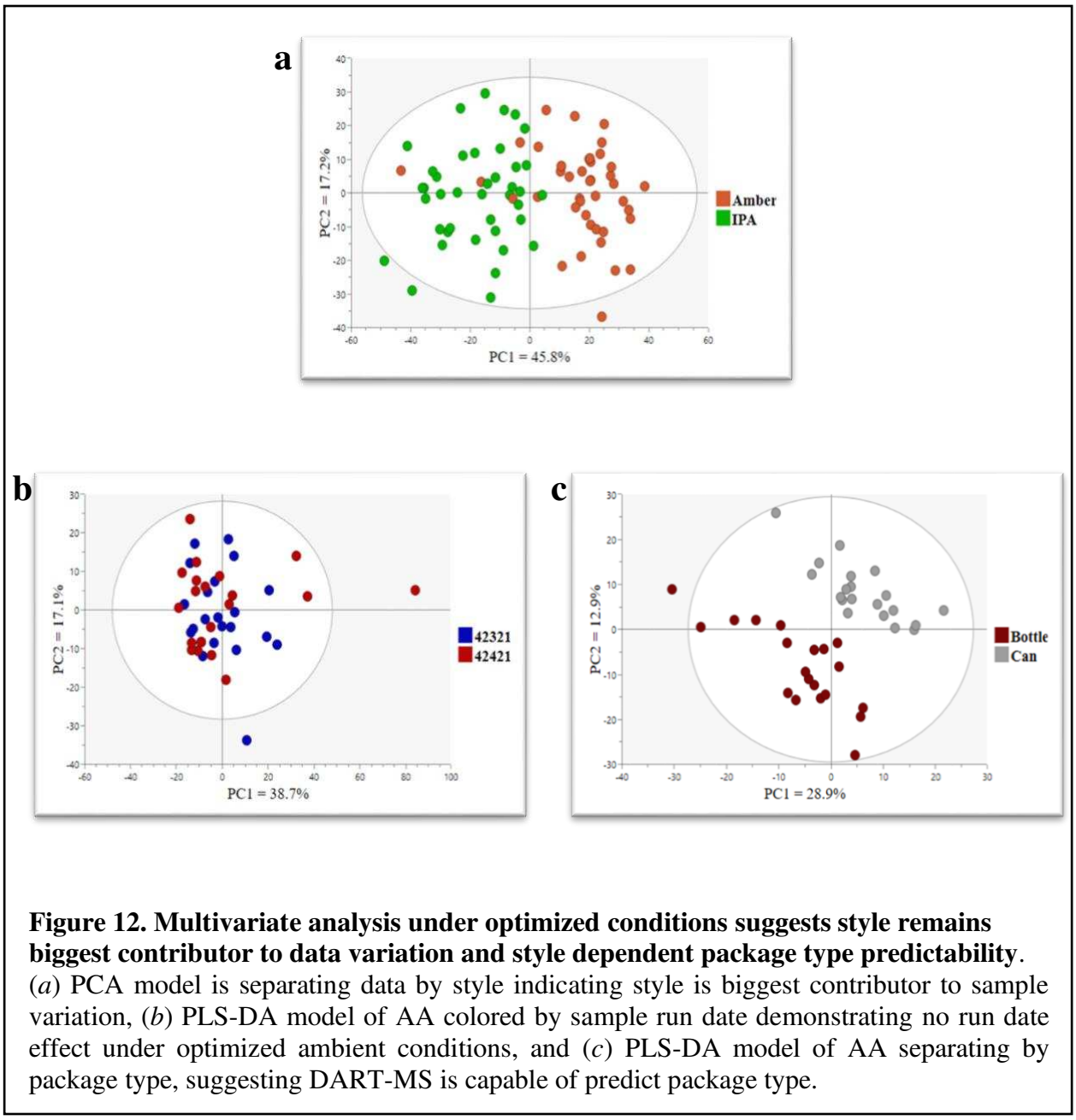

Figure 12. Multivariate analysis under optimized conditions suggests style remains biggest contributor to data variation and style dependent package type predictability. (*a*) PCA model is separating data by style indicating style is biggest contributor to sample variation, (*b*) PLS-DA model of AA colored by sample run date demonstrating no run date effect under optimized ambient conditions, and (*c*) PLS-DA model of AA separating by package type, suggesting DART-MS is capable of predict package type.

IPA samples, replicating the results from the first experiment that style was the main source of variation across all samples. The optimized conditions were successful in achieving improved baseline resolution in the chromatography (Figure 13) and eliminating the run date effect seen during the first analysis (Figure 12b). The model statistics between experiments one and two closely align suggesting that while the data quality improved, the optimized conditions did not affect which compounds were ionized.

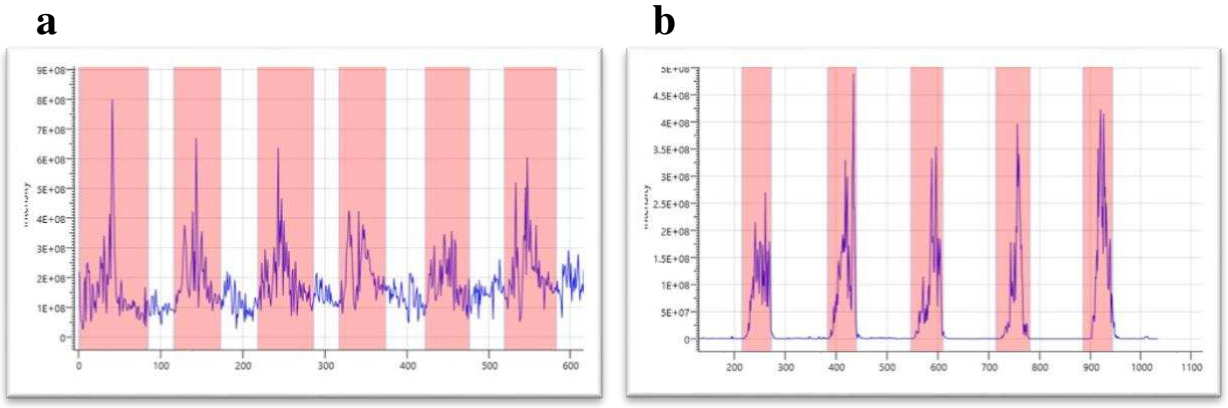

Figure 13. Chromatography from DART-MS analysis before and after optimization. (*a*) Chromatography of consecutive samples from experiment one during high smoke particulate ambient conditions in the lab resulting in poor baseline resolution and increased background noise, (*b*) Chromatography of consecutive samples from experiment two utilizing steel plate rail attachment, steel needles, and under clean air ambient environment resulting in improved baseline resolution and reduction in background noise.

Supervised PLS-DA models were created for each style based on time by week number. The AA model indicated a poor model fit ($R^2 = 0.554$) and a lack of beer age predictability ($Q^2 = -0.151$). Similarly, the IPA model had a poor model fit ($R^2 = 0.622$) and lack of beer age predictability ($Q^2 = -0.079$). This result also replicates what was observed in experiment one and suggests that DART-MS is not a suitable method for predicting beer age and therefore is not a useful tool to streamline the process of determining beer flavor stability.

A second set of PLS-DA models for each style was created to determine if analysis by DART-MS could predict package type, as was explored in Chapter 2. The AA model had an acceptable model fit ($R^2 = 0.788$) and exhibited package type predictability ($Q^2 = 0.846$) (Figure 12c). The IPA model had a poor model fit ($R^2 = 0.549$) and lack of package type predictability ($Q^2 = 0.039$). These results align with the results from Chapter 2, indicating that DART-MS is ionizing and detecting similar metabolites as those detected by GC-MS. Although there is a lack of evidence that DART-MS is a useful tool for assessing beer stability, it may be useful for

screening packaging type differences in different beer styles and could lead to important information around best packaging practices for brewers.

3.4 Conclusions

Breweries need methods to streamline and reduce costs of beer stability assessments. The current methods of utilizing a trained sensory panel are timely and financially inaccessible for many. Analysis by DART-MS paired with predictive modeling was explored as a potential high-throughput alternative to sensory evaluation. It was determined that the instrument was sensitive to ambient conditions (e.g., smoke), and required a physical barrier to prevent sample carry over between sample applicators to ensure quality data processing and accurate results. The data generated by DART-MS did not enable prediction of beer age at the bi-weekly or monthly time intervals. However, it did enable predication of package type and style type, suggesting that this analytical tool may be useful for other beer screening analyses outside of brand recognition, such as package type effects based on style and packaging materials. DART-MS is only one type of ambient ionization. Evaluation of other approaches, which enable detection of different compounds, is warranted to address the remaining need for a high throughput analytical tool to assess beer stability for the brewing industry.

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APPENDIX

Table 6. Annotated metabolites detected from GC-MS and HS-GC-MS analysis

Chemical Class	Chemical Subclass	Metabolite Name	Chemical Formula	Detection Method	RT (sec)	Average MW	Reported Sensory
Organooxygen	Alcohol and polyols	2,3-Butanediol	C4H10O2	GC-MS	213.38	90.121	butter, cream, fruity
Keto acid	Alpha-keto acid	Pyruvic acid	C3H4O3	GC-MS	220.62	88.0621	na
Hydroxy acid	Alpha hydroxy acid	Lactic acid	C3H6O3	GC-MS	226.7	90.0779	sour
Carboxylic acid	Amino acid	Alanine	C3H7NO2	GC-MS	248.32	89.0932	na
Carboxylic acid	Dicarboxylic acid	Oxalic acid	C2H2O4	GC-MS	268.31	90.0349	tart
Carboxylic acid	Amino acid	Isoleucine	C6H13NO2	GC-MS	275.56	131.1729	bitter
Carboxylic acid	Amino acid	Valine	C5H11NO2	GC-MS	308.31	117.1463	mildly bitter
Benzene	na	Phenylethyl alcohol	C8H10O	GC-MS	314.67	122.1644	rose, honey
Organic carbonic acid	Ureas	Urea	CH4N2O	GC-MS	316.13	60.0553	na
Organonitrogen	Amines	Ethanolamine	C2H7NO	GC-MS	335.79	61.0831	na
Organooxygen	Carbohydrate	Glycerol	C3H8O3	GC-MS	338.64	92.0938	sweet
Carboxylic acid	Amino acid	Leucine	C6H13NO2	GC-MS	349.56	131.1729	bitter
Carboxylic acid	Amino acid	Proline	C5H9NO2	GC-MS	352.35	115.1305	na
Carboxylic acid	Dicarboxylic acid	Succinic acid	C4H6O4	GC-MS	356.68	118.088	sour, umami
Carboxylic acid	Amino acid	Glycine	C2H5NO2	GC-MS	356.96	75.0666	sweet

Organooxygen	Carbohydrate	Glyceric acid	C3H6O4	GC-MS	368.01	106.0773	Na
Diazines	Pyrimidines	Uracil	C4H4N2O2	GC-MS	372.29	112.0868	na
Diazines	Pyrimidines	Thymine	C5H6N2O2	GC-MS	404.34	126.1133	na
Hydroxy acid	Beta hydroxy acid	Malic acid	C4H6O5	GC-MS	446.17	134.0874	tart, sour
Carboxylic acid	Amino acid	Pyroglutamic acid	C5H7NO3	GC-MS	464.1	129.114	umami
Carboxylic acid	Amino acid	4-amino-Butanoic acid	C4H9NO2	GC-MS	467.2	103.1198	na
Phenol	Tyrosols	Tyrosol	C8H10O2	GC-MS	485.22	138.1638	mild, sweet, floral
Hydroxy acid	Short-chain hydroxy acid	L-2-Hydroxyglutaric acid	C5H8O5	GC-MS	486.02	148.114	na
Carboxylic acid	Amino acid	Glutamic acid	C5H9NO4	GC-MS	505.87	147.1293	sour, mild umami
Carboxylic acid	Amino acid	Phenylalanine	C9H11NO2	GC-MS	513.14	165.1891	na
Carboxylic acid	Amino acid	Asparagine	C4H8N2O3	GC-MS	531.13	132.1179	na
Organooxygen	Carbohydrate	Unknown sugar 1	na	GC-MS	537.98	na	na
Organooxygen	Carbohydrate	Xylitol	C5H12O5	GC-MS	551.75	152.1458	sweet
Organooxygen	Carbohydrate	Arabitol	C5H12O5	GC-MS	556.91	152.1458	sweet
Organonitrogen	Amines	Putrescine	C4H12N2	GC-MS	562.21	88.1515	foul odor, rotting
Glycerophospholipid	Glycerophosphate	Glycerol-3-phosphate	C3H9O6P	GC-MS	571.95	172.0737	na
na	na	Known unknown 3	na	GC-MS	575.86	na	na
na	na	Sugar acid 1	na	GC-MS	584.18	na	na
Carboxylic acid	Tricarboxylic acid	Citric acid	C6H8O7	GC-MS	595.87	192.1235	tart, sour

na	na	Known unknown 5	na	GC-MS	614.17	na	na
Organooxygen	Carbohydrate	Fructose	C6H12O6	GC-MS	621.28	180.1559	sweet, fruity aroma
Organooxygen	Carbohydrate	Unknown sugar 2	na	GC-MS	625.24	na	na
Organooxygen	Carbohydrate	Glucose	C6H12O6	GC-MS	632.28	180.1559	sweet
Organooxygen	Carbohydrate	Unknown sugar 3	na	GC-MS	639.83	na	na
Carboxylic acid	Amino acid	Tyrosine	C9H11NO3	GC-MS	642.55	181.1885	bitter
Organooxygen	Carbohydrate	Mannitol	C6H14O6	GC-MS	645.49	182.1718	sweet
Organooxygen	Alcohol	myo-Inositol	C6H12O6	GC-MS	706.02	180.1559	neutral
Organooxygen	Carbohydrate	Sucrose	C12H22O11	GC-MS	917.23	342.1162	sweet
Organooxygen	Carbohydrate	Maltose	C12H22O11	GC-MS	925.45	342.2965	sweet
Organooxygen	Carbohydrate	Unknown sugar 4	na	GC-MS	936.9	na	na
Organooxygen	Carbohydrate	Maltotriose	C18H32O16	GC-MS	1138.5	504.4371	sweet
Carboxylic acid	Carboxylic acid ester	Ethyl Acetate	C4H8O2	HS-GC-MS	462.74	88.1051	nail polish remover, solvent, fruity, sweet
Organooxygen	Alcohol	Isobutanol	C4H10O	HS-GC-MS	559.57	74.1216	malty, solvent
Carboxylic acid	Carboxylic acid derivative	Ethyl Propanoate	C5H10O2	HS-GC-MS	675.42	102.1317	fruity, estery
Organooxygen	Alcohol and polyols	Isoamyl Alcohol	C5H12O	HS-GC-MS	710.43	88.1482	alcohol, banana, sweet, fusel
Organooxygen	Alcohol	Pentanol	C5H12O	HS-GC-MS	797.43	88.1482	sweet, balsamic, fusel
Lipid	Fatty acid ester	Ethyl Butyrate	C6H12O2	HS-GC-MS	869.63	116.1583	pineapple, mango, papaya, butter, sweet

Carboxylic acid	Carboxylic acid derivative	2-Pentyl Acetate	C7H14O2	HS-GC-MS	999.81	130.1849	citrus, spicy, ripe fruit, apple
Carboxylic acid	Carboxylic acid derivative	Isoamyl Acetate	C7H14O2	HS-GC-MS	1027.46	130.1849	banana
Benzene	Styene	Styrene	C8H8	HS-GC-MS	1066.59	104.1491	plastic, sweet, floral, balsamic
Carboxylic acid	Carboxylic acid ester	Isobutyl Isobutyrate	C8H16O2	HS-GC-MS	1098.6	144.2114	grape skin, pineapple, tropical
Lipid	Monoterpenoid	β-pinene	C10H16	HS-GC-MS	1196.12	136.234	woody, green, resinous, dry
Lipid	Monoterpenoid	β-myrcene	C10H16	HS-GC-MS	1208.84	136.238	peppery, spicy, citrus, resinous, piney, lemon, woody
Lipid	Fatty acid ester	Ethyl Hexanoate	C8H16O2	HS-GC-MS	1237.21	144.2114	apple, anise seed, citrus, solvent
Lipid	Fatty acid ester	Ethyl 5-methylhexanoate	C9H18O2	HS-GC-MS	1245.62	158.238	na
Lipid	Fatty acid ester	Isoamyl Butyrate	C9H18O2	HS-GC-MS	1270.74	158.238	fruity, green, apricot, pear, banana
Lipid	Fatty acid ester	2-Methylbutyl butyrate	C9H18O2	HS-GC-MS	1282.7	158.238	fruity, pear, apricot, tropical, spicy, apple
Lipid	Monoterpenoid	Pinocarvone	C10H14O	HS-GC-MS	1423.59	150.2176	minty
Lipid	Monoterpenoid	Linalool	C10H18O	HS-GC-MS	1447.21	154.253	citrus, floral, rose, woody
Benzene	na	2-Phenylethanol	C8H10O	HS-GC-MS	1522.74	122.1644	alcohol, floral, honey, sweet rose
Lipid	Fatty acid ester	Ethyl Octanoate	C10H20O2	HS-GC-MS	1570.01	172.2646	apple, sweet, fruity, sour apple
Benzene	na	Phenethyl Acetate	C10H12O2	HS-GC-MS	1707.9	164.2011	honey, floral, cabbage, fruity, rose, apply, sweet
Organooxygen	Carbonyl	2-Undecanone	C11H22O	HS-GC-MS	1743.71	170.2918	varnish, bitter, green plants, geranium, fruity, citrus
Lipid	Monoterpenoid	Methyl Geranate	C11H18O2	HS-GC-MS	1765.54	182.263	waxy, green, fruity, floral, citrus

Lipid	Fatty acid ester	Ethyl Decanoate	C12H24O2	HS-GC-MS	1849.09	200.3178	caprylic, soapy, estery
Lipid	Sesquiterpenoid	Humulene	C15H24	HS-GC-MS	1961.06	204.357	spicy, herbal, grassy, woody, clove
Lipid	Sesquiterpenoid	α-calacorene	C15H20	HS-GC-MS	2068.97	200.3196	citrus, spicy, woody
Lipid	Fatty acid ester	Ethyl Dodecanoate	C14H28O2	HS-GC-MS	2102.07	228.3709	caprylic, soapy, estery