DISSERTATION

VARIATION IN MALT METABOLITE CHEMISTRY AND IMPACT ON MALT AND BEER FLAVOR

Submitted by

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ABSTRACT

VARIATION IN MALT METABOLITE CHEMISTRY IN GENETICALLY DIVERSE POPULATIONS, THE CONNECTION OF MALT SENSORY ANALYSIS, AND IMPACT ON BEER FLAVOR

Growing and malting barley are processes that are imperative to the production of beer and distilled spirits. The rise of craft malting and brewing have provided many opportunities for the "science and art" of manipulating and bending barley to new limits to create new alternatives for the industry in terms of style and flavor. Understanding the mechanisms of how raw ingredients and their interactions influence product quality is critical for any industry. The organoleptic traits of food and beverages are highly discernable by consumers based on flavor (taste and aroma), appearance, and mouthfeel. These organoleptic attributes are variable and define style, drive consumer trends, and therefore influence the decisions regarding traits that are desirable to breed for in crops. There is a gap in the knowledge regarding how genotype, environment, and processing conditions affect the quality and flavor of beer. Understanding the sources of chemical variation among barley varieties can inform on breeding, malting, and production techniques for novel attributes of the final product. The goal of this research is to understand 1) the chemical variation in non-volatiles observed among heirloom barley malts in order to determine if they offer novel chemical traits; 2) the chemistry of malt hot steep extracts and the links between specific metabolites of the hot steep extracts and their resulting sensory attributes; and 3) the chemical and sensorial basis for differences in beer flavor attributed to experimental barley varieties under controlled conditions. Broadly, a comprehensive non-

targeted metabolomics approach was utilized for all studies. Study 1: ultra-performance liquid chromatography mass spectrometry (UPLC-MS) for non-volatiles detection, chemical characterization, and multivariate statistical analyses. In Study 2: three metabolomics platforms: UPLC-MS, Gas chromatography mass spectrometry (GC-MS) for detection of volatiles, and headspace solid phase microextraction (HS/SPME) GC-MS, as well as sensory testing, and multivariate statistical analyses. Study 3: HS/SPME-GC-MS for aromatic volatiles detection, sensory analysis (including check-all-that-apply [CATA], qualitative descriptive analysis [QDA], and projective mapping/napping [PM/N]), and both univariate and multivariate statistical analyses. The results of these studies supported the role of barley genetics, environment, and processing in both quality and flavor differences and provided new information on the types of volatile and non-volatile metabolites that can vary due to these factors.

Study 1: we concluded that the metabolites detected in this study (e.g. lipids, organic acids) have demonstrated effects on malt quality and flavor and have potential to contribute to novel chemistry to heirloom barley varieties. The widening of genetic diversity through breeding and introduction of the novel metabolite chemistry traits can allow maltsters and brewers access to more varieties with unique and improved traits.

Study 2: the data highlight the utility of the hot steep extract to differentiate malt for flavor and chemistry and indicate specific compounds that drive the most dominant flavors observed in a population of pale malts. These research findings support the value of sensory assessments of malt hot steeps when assessing the quality of malt, defect elimination, and potential for flavor development for craft malts.

Study 3: the data supported the role of barley genetics in beer flavor and provided new information on the types of volatile metabolites that can vary in controlled systems. This

research could be useful in prediction of flavor based on variety, malting, and brewing analytics. Predictions regarding variation in metabolites could be useful in variety development, selection of varieties for specific beer styles, and malting or brewing protocol design.

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PREFACE

Contribution of Authors

All authors played a role in reviewing the manuscript of their respective chapters. Harmonie Bettenhausen was primarily responsible for all data collection, analysis, interpretation, and writing of the manuscripts. Dr. Adam Heuberger was responsible for coordination, design, ideas, and funding-procurement for these projects. Dr. Linxing Yao was integral in coordination and implementation of metabolomics in all cases.

Chapter 2: Dr. Jamie Sherman and her lab (Liz Elmore and Hannah Turner) were responsible for the collection of germplasm (heirlooms), agronomy, and malting of barley for this project. Heather Omerigic was key in assisting in the chemical extractions for this large project.

Chapter 3: Lindsay Barr conducted the sensory for this project with the team at New Belgium Brewing (Fort Collins, CO, USA). Heather Omerigic was key in assisting with sensory and preparation of samples.

Chapter 4: Dr. Patrick Hayes (and the whole of Barley World) was responsible for the development of projects and compilation of the germplasm, coordination of labs, maltings, brewing, packaging, and sensory of this project. Dr. Scott Fisk was responsible for the growing, loving, and malting of these germplasms. Dr. Javier Hernandez contributed to the statistical analysis on sensory. Amanda Benson and Veronica Vega from Deschutes Brewing (Bend, Oregon, USA) were responsible for leading the pilot brewing, brewing quality analytics, and sensory at those breweries. Drs. Juyun Lim and Sue Queisser were responsible for the consumer

sensory panel at OSU. Dr. Thomas Shellhammer and his lab were responsible for the laboratory panel conducted at OSU. All authors contributed heavily to this manuscript.

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CHAPTER 1 – INTRODUCTION

The role of barley in society

Globally, cereal crops (plant family Poaceae) are the world's richest food source. According to the 2017 report by the Food and Agriculture Organization of the United Nations, barley (*Hordeum vulgare* L.*)* accounted for 21 million metric tons of grain that were produced for human foodstuffs, beverages, and livestock feed. Currently, barley is the fourth most important cereal crop in the world and is grown in over 100 countries [1].

Barley is one of the world's oldest domesticated crops, having been cultivated in the Nile River Valley of Egypt over 17,000 years ago. For nearly as long, humans have sought to improve the quality and quantity of the food, beverages, and animal feed derived from this crop [2]. Studies of barley, as well as other cereal grasses such as wheat (*Triticum aestevum*) have enhanced our understanding of grain breeding, agronomics, production, and consumption habits immensely [3]. Barley is highly adaptable and very tolerant of unfavorable environments, such as cold, drought, and unamenable soils [1]. A grass of the family Poaceae, the tribe Triticeae, and the genus *Hordeum,* barley is an annual, self-pollinating, diploid species with 2n = 14 chromosomes and a genome size of ~5.1 Gigabases with approximately 30,000 genes [1]. The relative simplicity of its genome, the potential genetic diversity of the species, and the availability of a whole genome sequence make it an ideal organism for genetic studies and breeding efforts [4].

Traditionally, breeding efforts have been focused primarily on agronomic traits, such as improvements in yield, disease resistance, harvest index, and protein content. Barley is an early maturing crop with a relatively short season. It is drought-tolerant and more tolerant to adverse soils such as those that are alkaline or saline. The crop experiences one of two basic seasons–

types: Winter or Spring barley. Winter cultivars undergo a vernalization period before they can carry out their development and produce flowers in the spring. Both winter and spring cultivars can germinate in under five days, provided temperatures are conducive to plant growth and development (12- 24˚C) [2]. After budding, spring barley matures within 60-90 days, making it a desirable crop in regard to water and nutrient use efficiency [5]. Barley exists as 2-row or 6-row types (nearly subspecies in their genetic differences), the terminology of which is derived from the physical morphology. The trait is determined by multiple genes that lead to the development of either two or six kernels consecutively along the barley head. Both types have alternating sets of three spikelets, however, it is only in two-row barley that the central spikelets are fertile and develop seed creating a flat-shaped head, as opposed to the development of all six rounded kernels that develop in six-row types [6]. Six-row barley tends to yield smaller kernels due to the tightly packed nature of those kernels, thereby yielding less starch and therefore less overall extract for brewing [6, 7]. Two- and six-row barleys can both exist as winter or spring varieties. While this is variation is not uncommon for cereal crops, the barley model is interesting in that distinct sub-types have been bred for very different processing conditions. 'Food barley' tends to be high in protein (nutrients for people and animals) while high protein content is negatively associated in malting barley. Six-row barley tends to have higher protein, whereas two-row is lower in protein, a more desirable characteristic for North American brewers. Malting barley is bred for chemistry associated with desirable malting characteristics (rapid germination due to hormone-associated inhibition, glycosidases, resistance to kilning) and fermentation (high in saccharides and free amino acids, low in beta-glucans and protein) [8]. Malting barley is a major raw material for the brewing industry. For beer production, breeding optimized varieties that

contain distinct beverage fermentation and flavor (e.g. taste, aroma) properties is an active area of research.

Utility of malted barley grain in the beer industry: the brewing process

The brewing process and the creation of beer is a complex process with many important steps, several raw ingredients (water, hops, malt, yeast, etc.) that are either processed or remain alive. As this has been a widely-studied and written-about topic, this section aims to be brief and focused on the subject of barley and malt. Every step in this process, like the malting process, is closely regulated in order to create the "big picture" product at the end. However, there are also many variations on the process that lead to alterations in raw ingredients, how they interact, and how we perceive them.

After barley grain is malted (discussed below), the finished malt is milled, (similarly to flour) so that it increases the surface area, allowing it to dissolve well in water, and allowing more access for enzymatic activity during the mashing step. This process serves to separate the husk from the endosperm and to expose interior starch. Once milled, it is known as "grist". The grist (which still contains endogenous amylases) is mixed with water (this mixture is known as "the mash") and heated for several hours to encourage a process known as "saccharification" during which the remaining barley starches are converted to simple sugars. The step during which saccharification occurs is known as "mashing" and immediately following it, the liquids are separated from the solids. Solids are discarded or repurposed, while the liquid, known as "wort" will undergo further processing to become beer. Wort is loosely defined as "an aqueous medium comprised of mainly fermentable sugars derived usually from starch-rich cereals but also assimilable nitrogen, oxygen, sources of sulfur and phosphates, the vitamin biotin, calcium, and magnesium ions, together with trace elements such as copper and zinc" [9, 10]. The

proportions of these components will depend on the composition of the raw ingredients. Malt is rich in these necessary components for yeast health and growth. Normally, wort contains 70-75% fermentable sugars, comprised of glucose, fructose, sucrose, maltose, and maltotriose. The remaining carbohydrates are considered "unfermentable," meaning they are longer-chain saccharides and branched polymers, unable to be broken down and made available for yeast [9, 10]. Free Amino Nitrogen (FAN), discussed in detail below, is influenced by the variation in raw materials, specifically malt, which is the primary source of FAN for yeast. Understanding malt chemistry and composition is imperative to understanding and manipulating how carbohydrates and proteins are utilized in the process.

The wort is collected from the spent grains and continues to the next step: boiling. Boiling the wort is an essential part of the process, and the way it is performed affects the final beer quality and flavor. Of principal importance in the boil is isomerization and subsequent solubilization of the bitter substance alpha acid from added hops. Boiling the wort also pasteurizes it, freeing it from bacterial contamination. Enzyme activity is halted and therefore, the remaining saccharide composition of the wort and hence the dextrin content of the final beer is set. Proteins which become insoluble through destruction of structure form a solid precipitate called the "trub," which is removed from the wort prior to fermentation. The wort is then clarified and chilled. After cooling, brewers add yeast which will ferment the solubilized simple sugars into alcohol and carbon dioxide.

Utility of malted barley grain in the beer industry: macro-level malt chemistry

To deem barley acceptable for the malting process, certain quality parameters are assessed, quality parameters for malting barley are distinct from food barley. Malting is a process by which cereal grains are germinated and partially broken down ("modification") in such a way

that their starch becomes available for conversion to simple sugars. These simple sugars are the substrate for fermentation of beer and distilled spirits; although many grains can be used, barley is the most common raw material for the brewing and distilling industry due to chemical properties that make it a highly efficient and uniform malting grain. The primary function of a barley seed is to produce a mature barley plant. Malting takes advantage of the chemical signals associated with this function to release simple sugars as described above. The first step in malting is to assess and utilize only prime barley that not only has excelled in the field but can create quality malt with novel or consistent attributes, depending on the end use. There are a few grain quality parameters the barley should fulfill regardless of malting style, such as moisture. Moisture in raw barley grain should not exceed 12% [11]. This prevents the growth of mold and decreases the likelihood of mycotoxin build up within the stored grain. Uniform germination is key so that grain modification, which refers to enzymatic degradation of cell walls (by enzymes released from the aleurone layer/scutellum) to convert starches to sugars in the endosperm, is consistent throughout the batch. Nitrogen and protein content are important attributes in assessing grain quality [12]. Acceptable nitrogen and protein levels in two-row malting barley (most common in North America) are less than 2% nitrogen and 11.0-13.0% protein. Low nitrogen content does not fulfill the growth and maturation needs of yeast during fermentation, whereas high protein content decreases the volume of starch within the endosperm cavity, therefore decreasing potential extract [5]. Protein content of the barley grain affects the metabolomic composition and enzyme levels of malt, which is important for the brewer to be aware of, as high protein limits starch degradation, affects mouthfeel, foam stability, and decreases extract available to the brewer. Low protein limits enzymatic activity and modification is difficult. Hot and dry environments (such as in Colorado) tend to result in higher protein [13].

The barley must not show signs of disease, pre-harvest sprout damage, damage from insects or from fertilizers or pesticides. It is only when the raw barley is deemed acceptable upon inspection and analysis that the stages of malting can begin. Barley must also have a low prevalence of the mycotoxin deoxynivalenol (DON), which is the result of fusarium head blight (FHB) in barley. Fusarium head blight (FHB), characterized by premature bleaching on the "heads" of grains, is caused by the Fusarium fungus that grows on cereal grains such as wheat, barley, oats, and corn. This fungus is encouraged by wet and humid weather and is therefore more common in temperate climates. It produces the mycotoxin deoxynivalenol (DON), or vomitoxin, which causes nausea and vomiting if consumed at high enough levels in humans and livestock and is problematic to the maltster and brewer, leading to potential problems in malting and brewing. Both barley and malt from the same lot should undergo testing because barley DON levels are not correlated to malt DON levels. The FDA has set "advisory limits" of DON on finished wheat products to be 1ppm, but no limits are set for malt or beer.

Malting: how macro-level chemistry is achieved from barley grain

The phenotype of the raw barley (i.e. protein, moisture content) determines the process and outcome of malting, and it has a major impact on flavor and flavor stability during brewing. The metabolites in malted barley, such as nitrogenous compounds (amines), carbohydrates, amino acids, and lipids (fatty acids and their esters) and the interactions they have with hops, water, and yeast under the conditions of brewing, influence the flavor in beer and subsequent stability. Steeping, the first step in malting barley, involves partially immersing dried barley grain in water (at 14-16°C) to increase moisture content (to a final moisture content of 42-48%), which stimulates germination. This stage takes 24-48 hours and requires turning and venting of the steeping grains (for oxygen replenishment and carbon dioxide release) [12, 14]. Depending

on the maltster, time of year, and barley genotype, the specifics and timing of these steps often varies. Once the "chit" (coleorhiza) has emerged from the grain, the barley is moved to germination beds. These beds maintain a specific temperature (between 16-20°C) and aeration level (usually by auger) with the goal of stimulating enzymes that initiate protein degradation and hydrolyzation of starch, also known as endosperm 'modification'. There are several enzymes released into action during germination. For example, one suite of enzymes degrades β-glucan (β-glucanase). Excess β-glucan increases viscosity in wort (hot water mixed with ground malt) and is an undesirable characteristic in beer. During the starch modification that occurs during germination, α- and β-amylase are both released. These are essential enzymes which break up the native barley starches amylopectin and amylose. This starch breakdown results in glucose chains of varying length, including glucose, maltose, maltotriose, and other saccharides for downstream consumption by yeast during fermentation. Kilning is the last stage in malting and is highly variable. Over the course of 24-30 hours, the now "green" malt is dried and cured to affect a roasted flavor and reduce grain moisture level. Kilning temperature is incrementally increased over the course of several hours to achieve they style of malt desired [12, 14].

Over-or under-modification can result in beer with too much or too little protein (resulting in underdeveloped yeast or off-flavors in aged beer) or too much or too little residual dextrin (which results in an undesirable result of sweeter-than-expected beer) [15]. Kilning also influences the rate of lipid oxidation in malt. Lipid oxidation causes the formation of detrimental characteristics in barley and beer. For example, the development of trans-2-nonenal (cardboard off-flavor/aroma) is due to lipid oxidation in malt [16, 17]. For this reason, it is imperative to know the composition of barley when malting.

The current goal of barley breeding is to optimize both agronomic performance and malting quality

Malted barley (malt) varieties are bred specifically for characteristics that promote good malting and brewing performance. In beer, malt is the primary source of the starch and enzymes necessary to produce the fermentable sugars which yeast convert to ethanol and carbon dioxide [14]. Malt provides not only food for yeast, but also color (e.g. melanoidins) and flavor compounds (e.g. esters and aldehydes) which contribute to the final product. There are several parameters that affect the acceptability of malted barley and it is imperative that malting quality parameters be understood as to their limitations and utilization. These criteria (explained below) and the preservation of quality control of malt is necessary to ensure consistency, processing efficiency, and quality of the final product in the malthouse, brewery, and distillery [7, 11, 18]. The malt Certificate of Analysis (COA) lists the results of these standardized tests that serve to indicate how malt will perform. The COA includes parameters required by the maltster as well as parameters that are useful to brewers to manage and alter the brewing process. These analyses are performed using a standard laboratory based mashing regime for the wort. The methods for measuring wort are governed by the three main brewing institutions: The Institute of Brewing and Distilling (IoB), the European Brewing Convention (EBC), and the American Society of Brewing Chemists (ASBC). Each institution suggests its own methods. The IoB analysis involves homogenizing and holding the temperature of the mix at 65.5°C, which reflects the use of infusion mashing of ales. The EBC utilizes the Congress mash, found predominantly in the United States, which involves mashing at 45°C and increasing the temperature to 70°C, mimicking the process used in lager brewing [12, 19]; this process is similar to the ASBC regime. The primary limitation of the Congress mash is that it is not reflective of true brewery conditions. For example, the Congress mash method requires milling grains very fine (0.2mm) in

the laboratory for analysis; this is very unlike conditions found in a typical brewhouse, in which grain diameter is often contingent upon the mill and brewhouse- a parameter that can affect predictability of performance. The COA includes many useful features and parameters:

1. Barley variety and crop year indicators. Differences in barley variety (genetics) and crop year or location (environment) affect malting and brewing processes. Barley varieties exhibit highly different malting quality and flavor characteristics which will influence other quality parameters, such as enzyme levels, protein levels, and flavor. Crop year or location changes affect malting due to differences in dormancy and hydration [20].
2. Malt Sieve Analysis, which serves to measure the size and any damage of kernels. Kernels should be plump, homogenous, and should consist of over 90% of the measured kernels. Kernels and husks should be free of damage, to provide a sufficient filter bed during the mashing portion of the brewing process [2].
3. Friability, which is the ability to be crushed/mealiness of a malt. This serves as an indicator of the level of modification and, when used in tandem with β-glucan, can be used to indicate cytolytic modification [2].
4. Malt moisture, which is impacted by steeping, germination, and kilning. Brewers desire malt with values between 4 and 6% due to storage issues [2].
5. Malt extract measures the amounts of fermentable sugars. It determines the amount of alcohol that can be made from one ton of grain. The higher the extract level the more alcohol that can be made from a given amount of grain. Malt extract is measured by malting the grain and measuring the amount of soluble sugar (like glucose and

maltose) in wort. The measure is presented as a percentage (dry basis). The desired standard for malt is a minimum malt extract level of 80% [2].

6. Protein (soluble) is the primary indicator of proteolytic action, the breakdown of proteins into peptides and amino acids, during the malting process, as well as the level of modification [2].
7. Color, which is produced by the same mechanism as Maillard Reaction Products, is the interaction of reducing sugars and amino acids.
8. Protein (total) is inversely proportional to extract, so brewers generally desire a lower protein malt, being cognizant, however, that protein-derived amino acids (called free amino nitrogen or "FAN", discussed below) are essential to yeast health. Protein, which is barley variety-dependent, is also related to foam stability and haze [21].
9. β-glucans, polymers of which are found in the cell walls of the starchy endosperm of barley, are highly viscous and can cause a number of problems in brewing, such as reduced rates of wort separation and beer filtration and also the formation of hazes, gels, and precipitates [22]. β-glucan can also be used as an indication of cytolytic modification – how much of the cellular structure of the barley endosperm has been degraded during the malting process to unlock access to the starch granules inside [22]. The germination of the barley grain during malting results in the activation of many enzymes that convert the starch in barley into simple sugars. Beta-glucans persist into finished beer if β-glucanases are not activated during malting (temperatures over 60 °C deactivate β-glucanase) or if the malt is poorly modified during the germination portion of malting [23]. Starch and protein degradation are

essential for a clear wort and a resulting beer that is free of off-flavors or textures created by β-glucans.

10. Diastatic Power (DP) is the combination of starch degrading enzymes such as α-amylase, β-amylase, limit dextrinase and α-glucosidase. DP impacts fermentability and can be influenced by adjusting the mash temperature. Higher levels of enzymes support increased attenuation (reduced residual saccharides/dextrins) and higher levels of adjunct ingredient addition.
11. α-Amylase is one of the most important enzymes to the mash in the brewing process. It progressively breaks opens chains of amylose and amylopectin, which are large polymers, into smaller, more bite-sized units, exposing it further to digestion by β-amylase. Through fermentation, most saccharides are taken up by yeast, with the exception of residual dextrins that are integral to the character of beer in terms of sweetness, body, and mouthfeel.
12. Free amino nitrogen (FAN) is an essential part of brewing and can greatly influence the intended flavor of fresh beer and the flavor stability of the beer over time. During aging, remaining nitrogenous compounds tend to form undesirable flavors in the beer. For example, amino acids, such as L-lysine and L-proline, which are absorbed during fermentation at different rates, (L-lysine is absorbed rapidly and L-proline has little to no absorption) also influence the speed of fermentation. A normal fermentation time is about 72-100 hours. A wort that is supplemented with L-lysine can complete fermentation in approximately 48 hours with complete absorption of the L-lysine but can lead to an increase in vicinal diketone levels (VDK) in wort, leading to higher 2,3-butanedione and diacetyl (butterscotch, buttered popcorn) off-flavors over time.

This is due to the rapid fermentation time that does not allow the VDK to be reabsorbed by yeast [24]. FAN is provided by the malt, and yeast utilize nitrogen-containing compounds to form enzyme and growth proteins. Wort with high FAN tends to produce excess higher alcohols (fusel) and esters that lead to undesirable flavor. After fermentation, excess nitrogen sources can produce off-flavors in the beer due to increased esters, aldehydes, or fusel alcohol. FAN levels are regulated by monitoring changes in the grist (grain that has been milled in preparation for the mash step of brewing) composition and seasonal variations of raw materials (*i.e.* barley), to control yeast cultures and overall growth. Most brewing yeast requires approximately 100 mg of FAN per liter extract for adjunct brewing, and 200 mg/L for all-malt brewing to successfully ferment the wort [12, 15]. Traditionally, large-scale adjunct brewers have sought out barley that is higher in FAN. FAN is therefore important for yeast nutrition – a wort that is lacking in amines can result in a "stuck fermentation" (fermentation that has halted) whereupon the yeast have run out of a nitrogen source and cannot continue to ferment. The resulting beer suffers raised VDK levels (described earlier) leading to increased production of diacetyl, and the unutilized amines that remain in the beer result in a chill haze (undesirable in most beers). Excess FAN can produce off-flavors such as meaty, hot-dog, umami flavors, as well as reduce the stability of the flavor over time [24].

New outlook into malt for distinct sensory traits

In the brewing industry, there is a relentless pursuit for quality control, brand development, and customer satisfaction. The quality of all raw materials used in brewing (malt, water, yeast, and hops) and their impact on final product flavor is becoming increasingly

important. Simple sensory evaluation of raw materials gives an immediate indication of quality and/or the presence of any off-flavors, disease, or mishandling. Current sensory analysis of malt is not, however, a requirement, nor is it normalized or standardized. Brewers have historically viewed “malt quality” as a perception of personal views which encompass specifications which they believe protect the integrity of their flagship or brand. However, the "malt spec" is by no means as reliable as many brewers would admit. Congress mash has historically been utilized to evaluate malt quality, but has recently been subject to scrutiny and change [25]. The analyses which are rendered by the congress mash are odor, turbidity, pH, color (including after boiling), viscosity, soluble nitrogen content, free amino nitrogen, and final degree of attenuation [26]. There has recently been an incentive to standardize the malt sensory process and lexicon. The malt hot steep sensory method, validated by ASBC in 2017 was designed for the industry and by the industry with the intent of doing just this – standardizing the method and the way malt is talked about as it is evaluated [27]. The hot steep method is, briefly, a hot water extraction of malt, whereupon malt is not treated as if it is to be brewed (as in the Congress Mash), but simply for a sensory assessment of malt quality [28, 29]. The data generated by the hot steep method provides information regarding sample quality via the aromas and flavors present; in other words, it generates a "fingerprint" for each malt type in terms of aroma and taste characteristics and based on the variety of barley utilized, the environment it was grown in, and how it was malted. One outcome of this method is that undesirable traits or traits that are directly connected to malt or barley imperfections are easily identified. As of the time of this study, it was not possible to correlate malt hot steep sensory with beer flavor. This is considered one of the main product attributes that influences food choice, such as in breads or baked goods. Healthier or alternative food options, such as gluten-free or sugar-free, are often lacking in flavor quality,

which obstructs consumption and thus has a measurable health impact. Historically, flavor research has focused on improving flavor by decreasing or masking undesirable attributes or increasing levels of desirable attributes or attributes able to mask other undesirable attributes. The scientific approach to this has been to define the flavor profile, by using a trained sensory panel, then analytically identify the chemistry which drives those attributes. This methodology involves separating out the chemical components by chromatography and tasting or smelling them individually and in isolation to determine which compounds evoke the attributes of interest. While these methods are unquestionably effective at identifying sources of off-flavors, or for finding characteristic flavor compounds, they have limited ability to define sensory interactions or complexity. To advance our understanding of flavor perception, driven by many chemical factors, a holistic approach which considers the sum and the interplay of those factors and their relationship to the sensory attributes of interest is needed.

Malt Metabolomics: metabolite chemistry of malt, metabolite variation, and the potential impact on beer quality

Given the recent development of high-throughput sensory techniques and the numerous chemical and genetic factors involved in crop selection, metabolomics is a useful and relevant technique for investigating cereal crop data. Metabolomics allows for the application of large-scale chemo-sensory data collection to the analysis of different crop varieties. This method was applied to barley either alone or in combination with supporting sensory data to make determinations about crop quality. In each case, the barley was tested in the context of its most relevant agricultural product: wheat was tested in the form of bran (since most wheat is consumed in the form of bread) and barley was tested as brewing malt (since most barley intended for human consumption in the United States is used for brewing malt, and a smaller

portion as bran). This demonstrates the efficacy of metabolomics techniques as part of the decision tree of quality control for cereal grains; despite their different products and uses, each species benefit from metabolomics analysis.

Foodomics, a novel branch of metabolomics, works to characterize and profile food metabolites and is able to describe assessments of quality, authenticity, safety, and traceability [30]. The metabolome of food represents the collection of small molecules (molecular weight < 2 kDa) called metabolites, that are present in foods and are derived from animals, plants, and microorganisms. Metabolites in foods such as barley, malt, and beer are subject to changes during processing (such as malting, brewing, fermentation), storage, aging, microbial decomposition, and dynamic chemical reactions or contamination. Each variety of barley, batch of malt, and glass of beer has its own characteristics depending upon the presence and abundance of certain metabolites and the combination thereof [31]. Herein lies the usefulness of metabolomics in understanding the connections between genotype, environment, and phenotype (via sensory or quality assessments). The chemistry of barley influences brewing parameters and the final beer quality. Recent studies have demonstrated that small molecules in barley grain are highly variable among barley genotypes, and that interactions between barley genetics and environment (GxE) further influence the chemistry of the malt [32, 33]. Malting quality is based on both differences in protein structures of barley (which tells us there is variation in enzymatic action) and the expression of the genes involved [13]. The metabolomics approach allows us to understand the mechanisms and indicators of certain biochemical processes, such as determining the compounds responsible for certain flavor traits in beer and the routes that these compounds take to arrive at the point where a staling/off-flavor is created [34-36].

Metabolomics studies have recently been utilized in investigations to identify new markers of quality traits to breed superior plant lines. [13, 37]. The results of this study suggest that metabolism and quality traits are co-influenced by barley GxE factors and demonstrate the usefulness of metabolites as efficient markers of quality traits, suggesting the need for further research into the metabolites and biochemical processes which may contribute to beer flavor and flavor stability. Given the breadth of variation in malt metabolites due to barley genotype described, there is the potential that malt genotype may also influence the flavor stability of beer. Malt plays a key role as the keeper and transporter of precursors for many of the flavor compounds in beer. Previous research demonstrates that barley genetics can influence beer flavor. However, the chemical basis for differences in beer flavor attributed to barley variety is only in the beginning stages of definition [29, 31, 34, 38-40]. Broadly, there are several chemical classes of interest regarding malting and brewing which continue to be investigated by studies involving metabolomics.

Malt metabolite chemistry: role of sulfur compounds

Sulfur compounds in malting and brewing are of utmost importance. These compounds are volatile, with very low organoleptic thresholds (detectable in parts per billion). Trained sensory panels can recognize sulfur attributes even when the compounds are undetectable by analytical instruments. Sulfur compounds, to complicate the issue, are sensitive to changes in pH and temperature, and thereby readily convert into other compounds in response to those changes. Sulfur-compounds which react with ketones create a catty or barnyard goat-like aroma in beer [43]. Sulfur flavor is desirable in some styles of beer (e.g. Saison Farmhouse style), but is generally associated with aging and poor flavor stability. For example, 3-methyl-2-butene-1-thiol (associated with ‘lightstruck’ flavor), which is often found in beer exposed to excessive light or

aging and is caused by a reaction between hop α-acids and riboflavin in beer, is easily controlled by proper quality control and monitoring of the compounds. Sulfitic attributes, derived from sulfuric acid or sulfate salts (SO_4^-), include sulfur dioxide (lit match) and hydrogen sulfide (rotten eggs). Sulfidic (hydrogen sulfide, H_2S) attributes comprise mercaptans (garbage in methyl mercaptans, while higher mercaptans give off burnt rubber), *garlic, light struck* (*skunky*), autolyzed yeast (*hot dog water*), and butyl mercaptans (*shrimp-like*). Vegetative sulfur attributes include dimethyl sulfide (DMS) and dialkyl sulfides which give off cooked/canned vegetable properties. Whereas sulfitic attributes are rare and regulated (legally restricted to 10ppm) in the U.S., sulfidic flavors, produced naturally, are not regulated, but they are considered very undesirable in beer. Hydrogen sulfide, thiols, and mercaptans are being widely studied regarding their control, origin, and biotransformation during malting and brewing [41-44].

Malt metabolite chemistry: role of lipids

Although not a major component of beer, free fatty acids (FFA) are considered undesirable in the finished product. FFAs are a "foam-negative" compound, relating to their surface absorption tendencies upon interaction with foam-positive proteins [12, 36]. Medium chain fatty acids such as hexanoic, octanoic, and decanoic acid can result in off-flavors as *rancid, vomit, goat-like, cheese-like*. These volatile off-flavors are formed by the yeast during fermentation [45-47]. However, long-chain unsaturated fatty acids, such as linoleic and linolenic acids, are more often derived from malt and lead to the formation of staling off-flavors (lipid oxidation) in beer. Saturated fatty acids (*e.g.* palmitic and stearic) from malt are also related to gushing (spontaneous foaming over when beer is opened). Trans-2-nonenal, a common staling compound in beer is formed from oxidized lipid components. Lipid oxidation in malt has also been reported to cause lautering (filtering) problems during the brewing process [17]. In recent

years, an effort to create low-lipoxygenase malt (LOX-less) has been made. The kilning process affects the lipoxygenase in malt and its ability to oxidize lipids. Fatty acids in barley and malt also play a role in the amount of extract obtained from brewing and the attenuation (the percentage of sugars converted into alcohol and carbon dioxide by fermentation) [48].

Malt metabolite chemistry: role of amino acids and other nitrogenous compounds

Amino acids, from a malting and brewing perspective, are of utmost importance to understand. They are the most readily available source of nitrogen for yeast, derived from malt and wort, they are critical to fermentation performance and beer quality. Amino acids partially make up FAN, which is defined as the sum of individual amino acids, ammonium ions, and small peptides in wort [49]. All-malt wort typically contains ~19 amino acids which are taken up preferentially by yeast [31, 42]. The uptake of amino acids and creation of by-products of yeast create changes in wort composition which influence organoleptic properties of the finished product. Changes in the concentrations of amino acids in wort tend to influence and change nitrogen metabolism because the yeast amino acids are derived from the amino acids in the wort [42, 50]. The most important amino acids in wort are: isoleucine, valine, phenylalanine, glycine, alanine, tyrosine, lysine, histidine, arginine and leucine. These amino acids are responsible for the "regulation of biosynthesis of flavor-active compounds formed by yeast" [42].

Malt metabolite chemistry: Maillard Reaction Products

Malt is the most reliable source for Maillard Reaction Products (MRPs), but beer is complex, and the exact origin of MRPs created in beer is still under investigation [51]. Applying metabolomics methods, such as high-pressure liquid chromatography (HPLC) combined with ultraviolet detection (UV) has proved to be a successful venture into identifying the etiology and

pathway of MRPs in brewing. It has been shown that MRPs which are created in malt do not correlate with what is in beer [28], and so it is important to understand the transformation during brewing. Maillard reactions occur more commonly at higher temperatures, with low moisture levels, and under basic conditions with pentose sugars (e.g. arabinose, xylose) rather than with glucose and maltose (hexose, disaccharides) and with amino acids that have more propensity to react, such as lysine and glycine. Melanoidins, a result of the Maillard Reaction, lends to color in malt and beer. Maillard Reactions occur both during the kilning stage of malting and in the kettle during the wort boiling stage of brewing [52]. There are three stages in the creation of MRPs. The first is the creation of Amadori rearrangement products (ARP) through the reaction of amino and imino groups in free amino acids, peptides, and proteins with reducing sugars. The most common ARPs are N-ε-fructosyllysine (FL) and N-ε-maltulosyllysine (ML). Both compounds have been identified in malt and beer. The second stage is the degradation of ARPs into vicinal dicarbonyl compounds (e.g. glyoxal, 3-deoxyglucosone) and the formation of dicarbonyl compounds in a reaction with polysaccharides – this is what is known as "caramelization" and many compounds are formed as a result of this, such as furfural [51] from the dehydration of the aforementioned ARPs. The third stage involves formation of glycated amino acids and Strecker degradation, which forms aldehydes present in beer during fermentation. There are many MRPs yet to be investigated in malt and beer, as well as understanding how processing, barley variety, malt type, and the interaction with other raw ingredients (hops, yeast) may affect quality and flavor of malt and beer regarding those products.

Broadly, chemical variation can result in diversified breeding

The understanding of chemical variation among malting barley varieties will allow for more diversity in breeding programs for flavor development/improvement. Fermentation and

processing studies (for beer and distilled spirits) have advanced our knowledge of quality control for the raw ingredients in these products. Although the available body of knowledge seems vast, there is much to be learned about the complex nature of fermentation and production of wares such as beer, distilled spirits, breads, and other value-added manufactured goods. These products have multiple raw ingredients, processing steps, and complex chemistry; the result is that multiple factors influence their sensory quality. As such, an equally complex method of analysis is required to examine the relationship between quality and chemosensory properties of barley. Similarly, although barley is one of the world's essential cereal crops, its quality as a foodstuff is not well-matched with known organoleptic properties. The value of metabolomics to assist in the selection of varieties for malting (brewing) is still being assessed as malting barley is a chemically complex crop and the same compounds that affect its functionality for food/beverage preparation also have bearing on its flavor. Breeding for malting quality barley is an extended time- and effort-involved process. Breeding programs have excelled at choosing potential malting cultivars for yield, disease resistance, and to a lesser degree, malting quality. However, this selection has also led to a narrowing of the genetic pool resulting in a decrease of genetic diversity and flavor traits (e.g. as described in tomato) [4, 53]. In the world of brewing, hops have variety names (e.g. Cascade, Centennial), whereas malt is more commonly referred to the stylized product of the malting process (e.g. 2-row, base, Munich), and not by the barley from which it originated. This disparity has developed within the brewing industry because barley is utilized not as a plant but as a processed seed (malt). These grains have been domesticated and utilized for thousands of years, and humans have created thousands of varieties of barley. However, over time and through much culling of agriculturally undesirable barley (according to "big" beer), brewers currently rely on a short list of acceptable malting and brewing varieties

[54] governed by the aforementioned AMBA. This to "help ensure an adequate supply of high quality malting barley for the malting, brewing, distilling, and food industries" [54]. This practice, however, has narrowed the genetic diversity of barley, creating a genetic bottleneck of varieties able to perform agronomically, but unable to provide novel malting quality and flavor traits. The governance of barley research and limited variety releases has resulted in barleys bred for large adjunct brewers (such as Coors and Anheuser Busch-InBev). Traditionally, these large-scale adjunct and high-gravity brewers have required barleys with traits such as high diastatic power levels, capable of extraordinary enzymatic effort to convert adjunct sugars. What is needed, in regard to diversity, is barleys bred for all-malt beers brewed in the U.S. Large maltsters require barley that is consistent and efficient, diversity is admonished due to the technical challenges it presents for automation and throughput. Heirloom barley varieties may offer the U.S. market the opportunity for craft to build a new market for novel varieties which lend agronomic, quality, and flavor diversity and a new tool for craft brewers to use in the creation of unique products. Because malting chemistry so specific, there is correspondingly narrow genetic diversity within cultivated malting barley varieties. Only 31 2-row and 7 6-row malting varieties were approved to be grown in the 2020 season in North America by AMBA. This is a conundrum which has imposed unintentional restrictions on flavor and product differentiation in beer, as recent work shows that barley genetics influence beer flavor. As such, breeding programs have begun to investigate the introduction of heirloom cultivars for the purpose of exposing domesticated varieties to new genetics. Metabolomics is well-positioned to predict how these varieties may influence both the chemistry of the malt and the flavor of the resulting beer.

Metabolomics approaches combine well with supporting sensory methodology when appropriate.

Over the past few decades, evaluating wheat and barley, malt, and beer using untargeted methodologies has successfully identified hundreds of chemical compounds responsible for organoleptic properties such as sensation (mouthfeel, such as tingling from carbonation) and aroma. Several compounds identified in the literature have exhibited good correlation with specific sensory descriptors and have been used as chemical markers for attributes. Still, evaluation of barley malt based on taste or aroma perception separately is rather limiting. Malt and beer should be evaluated in their original matrix, where all human senses can integrate to achieve a perception of the product as a whole. In terms of malt flavor quality, there is lack of chemical and sensorial agreement which has created inconsistency and ambiguity in the term "quality" in literature. Despite many extensive studies, the key to highly desirable or undesirable characteristics of malt and beer are still not fully understood. The difficulty in capturing a holistic sense of the word "quality" can be attributed to the complexity of malt and beer matrices in the analytical and sensory aspect. Many papers lack experimental design that includes comprehensive chemical and sensory evaluation. The causative relationship between the chemistry and the quality of both wheat and malt/beer has only recently been studied.

The study on relationships between malt and beer sensory and chemistry has just recently begun. Malt's contribution to the final profile of beer is complemented by hops and yeast fermentation by-products, creating a unique organoleptic experience. However, much of the flavor contributed by barley has been attributed to the malting process and thus breeding programs have focused on malt quality outcomes, rather than positive or unique flavor attributes. The current range of malts available to brewers, which are pale or base malts, have not been considered major contributors to a beer's overall "malt flavor" profile. Craft brewers have

recently shifted focus, asking barley breeders and maltsters to provide malting quality specifications better suited for all-malt brewing, including lower protein, DP, and FAN [55, 56]. Craft brewers have also continued to demand commercial "heirloom" varieties (e.g. Barke®, Golden Promise®, and Maris Otter®) based on reported, anecdotal contributions to beer flavor, despite the fact that these varieties do not adhere to traditional agronomic and malting quality standards [29]. Developing a deeper understanding of the contributions of barley genotype to malt and beer flavor is an area of active research [29, 34, 40, 57]. Bettenhausen et al. (2018) demonstrated that commercially available malts of similar type - but made from different genotypes and malted at different locations - had different metabolite profiles and led to different beer sensory outcomes. Metabolomics is a powerful tool for the evaluation of malt and beer flavor because it is able to identify volatile compounds and precursor compounds using various types of chromatography and mass spectrometry [13, 31, 34, 58].

The long-term goal of this/my research

The long-term goal of my research seeks to build upon these findings to identify genetic, environmental, and processing factors that regulate the chemical composition of food, and to use these findings to improve crop varieties, increase sustainability for the industry, and develop new, chemical targets to evaluate (barley) malt for quality and flavor improvement for health.

First, this research sought to **characterize novel malt flavor from a population of heirloom barley varieties.** Currently, commercial malts are derived from narrow genetic diversity, and the brewing industry would benefit from new germplasm with novel flavor. A population of heirloom malt was profiled using metabolomics methods to understand chemical variation to facilitate breeding for flavor. I *hypothesized* that malt derived from heirloom barley contains a unique profile of metabolites that influences beer flavor, specifically via phenolics,

fatty acids, and amino acids. To test this hypothesis, metabolomics was performed on malt from 160 heirloom barley varieties. The population was also chemo-typed and a subset of malt will be brewed for sensory evaluation of the beer.

Secondly, I sought to **understand chemical variation in hot steep malt extracts and how these influences sensory evaluation of malt.** A hot steep malt extract method has been recently validated to evaluate malt quality for brewing, and previous studies have demonstrated that sensory evaluation of the extract can differentiate malts. I *hypothesized* that hot steep extract metabolites vary among different malts, and that this metabolite variation influences sensory traits (*i.e.* the influence of butyric acid in the creation of the fruit punch/sour vomit trait). To test this hypothesis, metabolomics was performed on hot steep extracts from 10 commercial malts, and the data was integrated with sensory evaluation of the extracts. This Aim resulted in the discovery of new metabolite markers for high-throughput screening of malt to predict sensory outcomes of the extract, and this can facilitate quality control/assurance in a brewing program.

Finally, efforts were made to **understand variation in sensory attributes and volatile compounds in lager brewed from genetically distinct malts using an integrated sensory and non-targeted metabolomics approach.** As sensory data from nano-brews prepared from micro-malts was used to document significant contribution and genotype and/or environment to specific beer flavor descriptors [7]. Herein, I investigated the effects of barley genotypes on sensory attributes and metabolite profiles of finished beer were made using larger-scale malts and brews. The malting quality data, beer analytical data, sensory data, and beer volatile metabolite profiles of the three doubled-haploid (DH) lines were compared to those for the well-established cultivar CDC Copeland. CDC Copeland is widely grown and used in the North American malting and brewing industries. This research tested the *hypothesis* that barley genetic variation can, through

malt, affect desirable sensory attributes of lager beer, and that these phenotypes are associated with differences in the volatile profiles.

Hypothesis and Goals

In this dissertation, I test the ***broader hypothesis*** that variation in malt metabolite chemistry can be broadened and deepened in the industry by utilizing genetically diverse populations (Chapter 2), that this variation can be detected using sensory analysis of the malt (Chapter 3), and that this variation can impact beer flavor (Chapter 4). My specific hypotheses were that:

1. There will be metabolomic differences among heirloom barley genotypes.
2. The differences in barley chemistry will be reflected in the chemistry of the final product (beer).
3. Certain barley and/or malt metabolites can be markers for beer flavor and/or flavor stability.
4. Differences in the beer chemistry will impact sensory attributes of beer through flavor and flavor stability.

Goals/Objectives:

1. To use metabolomics platforms to evaluate flavor in malt and beer.
2. To evaluate flavor and flavor stability based on sensory and metabolite analysis.
3. To determine beer volatile and/or non-volatile metabolite markers for flavor and flavor stability.
4. To determine the co-varying metabolites regarding how malt genotype affects beer flavor and the associations among metabolites that influence beer flavor and flavor stability.

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CHAPTER 2 – METABOLOMICS OF HEIRLOOM BARLEY SUPPORTS INTROGRESSION OF NOVEL CHEMICAL TRAITS FOR MALTING AND BREWING[1]

Overview

Barley (*Hordeum vulgare* L.) is an important global food crop, and malted barley is a major ingredient in the brewing industry. Currently, only a small number of varieties meet quality standards for brewing, and there is interest in developing varieties with new chemical traits that affect flavor. Heirloom varieties have the potential to improve chemical diversity in barley. To describe metabolite variation in a population of heirloom malting barley, determine structure of this variation within the population and metabolites that influence this structure, and to determine the extent of variation compared to modern varieties. Non-targeted reverse phase UHPLC-TOF-MS metabolomics was performed on extracts from 130 heirloom varieties and two commercial varieties, and metabolites were annotated based on spectral interpretation and matching of accurate mass data. Principal component and hierarchical clustering analyses (PCA-HCA) determined population structure and metabolites most associated with variation in the system. The analysis detected 2,669 compounds, of which 202 were annotated and included metabolites related to fermentation and flavor properties of malt including amino acids, lipids, carbohydrates, organic acids, and Maillard Reaction Products. PCA-HCA characterized sub-populations within the heirloom based on metabolite chemistry and formed seven chemogroups. Fermentation and flavor related metabolites that trended among chemogroups included leucine, proline, epicatechin, maltol, and maltotriose.

[1] This chapter is a modified form of a manuscript in preparation for the Journal Metabolites: Harmonie M. Bettenhausen, Liz Elmore, Heather Omerigic, Hannah Turner, Linxing Yao, Jamie Sherman & Adam L. Heuberger

This analysis of metabolite variation in malting barley supports the potential to utilize heirloom to breed for new varieties with novel flavor traits. Metabolomics-based chemogrouping of can develop population sub-structures to facilitate selection during breeding.

Introduction

Barley (*Hordeum vulgare* L.) is a major cereal crop used in the malting and brewing industries. Through plant breeding, barley varieties have been developed and optimized for very distinct processing conditions. 'Malting' barley is bred for a chemical profile achieved thought malting, a process where the seed is germinated and then kilned to produce a food product. The malt chemical profile consists of enzymes (glycosidases, proteases, amylases), saccharides, and other metabolites that facilitate fermentation in brewing. The malt chemical properties that facilitate fermentation define the quality of the malt, for example extract (saccharides), protein, and free amino nitrogen (FAN; amino acids and other nitrogenous metabolites). In contrast, food barley has been bred for distinct chemical properties of the unmalted grain, such as high-protein kernels [1].

In addition to facilitating fermentation, malt metabolites also contribute to flavor of the finished food. As barley grain is processed into malt, flavor is largely dictated by the specific malting regime and resulting malt type (Caramel, Crystal, Roasted) [2]. The metabolite changes that occur during malting affect both primary and specialized metabolite production [3]. The malting regime consists of three sequential, essential steps. In the first step of steeping, primary metabolites are physiologically released, and protein, saccharides, and lipids are utilized as energy substrates for the growing embryo, leading to germination. Next, during germination, several specialized metabolites are produced, such as phenolics released from the breakdown of hemicelluloses [4]. Last, upon kilning, metabolite-metabolite interactions can occur to generate

new classes of metabolites, for example, esters (created from lipids) [5], Maillard Reaction Products, and Strecker aldehydes, (amino acid-saccharide interactions) that result in malt flavor and color [6]. Amino acids are particularly important for their role in both the developing embryo, generation of secondary metabolite food products, and the dynamics of fermentation during brewing [7].

The need for the very specific quality-chemistry of malting barley results in a very narrow genetic diversity within cultivated varieties. The major genetic sub-groups of barley are the 2-row or 6-row types, as well as Spring or Winter growth habits. But there is only minor genetic variation beyond these major differences due to the strict requirements for malt quality. For example, the main regulatory group in the U.S. has approved only 31 2-row and 7 6-row varieties to be grown in the 2020 season in North America for malting purposes [8]. The strict regulation macro-level chemistry, combined with the basic need to breed for good agronomics and yields, is a conundrum in a brewing industry that is defined by product differentiation and flavor.

Recent studies demonstrate that barley genetics and varieties can indeed influence flavor. As such, barley breeders have recently begun to introgress new genetics into crossing blocks, specifically making use of landrace or heirloom cultivars for novel nutrition, flavor, and adaptation to environments [9, 10]. In several modern breeding programs, breeders hypothesize that new chemical traits can be incorporated from less-domesticated genetics to influence flavor, independent of quality-level chemistry that is necessary for adequate fermentation in brewing. Over the past decade, several studies confirm that barley genotype does impact beer flavor, shown extensively in laboratory/controlled environment brewed beers (non-commercial studies) [11-13]. The flavor differences originating from the malts were somewhat attributed to variation

in malt modification, which is the extent to which the endosperm breaks down during the malting process. [14]. A companion study demonstrated that that barley genotype, as well as process and environment, contribute to beer flavor [14]. Variety-based flavor differences were also shown in a study that incorporated metabolomics of the malt [15]. Bettenhausen et al. (2020) utilized metabolomics and sensory to profile three varieties which were also used by Herb et al. (2017), all grown and malted in the same environment, again demonstrating the power of metabolomics to better understand contributions to malt and beer flavor [15, 16].

Importantly, a major limitation of these previous studies is that they evaluated a small number of varieties due to limitations in malting, brewing, and sensory. And this presents a major challenge for breeding new varieties, which involves screening a large pool of genetics, and the corresponding malting, brewing, and sensory evaluation of hundreds to thousands of barley varieties is not feasible for flavor analysis. As a high-throughput method, metabolomics offers an approach to pre-screen varieties for novel metabolite traits that are predicted to impact flavor. Here, we evaluate and demonstrate the application of metabolomics to facilitate developing new malting barley varieties. Malt metabolites were profiled within a large population of 'heirloom' barley, which is a source of genetics to introgress new flavor traits into commercial varieties. Heirloom barley varieties were developed approximately 50-100 years ago, and while not yet optimized for quality, they are considered an important tool for generating new malt flavor. We hypothesize that metabolite variation in heirloom malt is distinct from high-quality commercial malt. Further, by comparing metabolite profiles of heirlooms to control varieties, we can estimate the potential for creating novel metabolite phenotypes. Further, the procedure of using metabolomics to form chemical sub-groups, hereby referred to as "chemogroups" is demonstrated. Chemogrouping was used to reduce dimensions in the

metabolite data from this large, diverse population, with the goal of establishing chemogroups to focus on the introduction/introgression of new chemical diversity. The metabolite variation among chemogroups in this population is described herein.

Materials and Methods

Plant materials and description of the population

A total of 130 barley varieties were selected for metabolomics analysis (Table S.1). The 130 varieties were derived from a larger heirloom barley population of 233 varieties established between 50 to 100 years ago. The 233 varieties were grown and tested in 2016-2017 and the subset of 130 was selected for having the 2-row trait, adequate grain yield, agronomic performance, and protein.

Barley and malting

For metabolomics analysis, barley lines were grown under irrigation in 2017 at the Montana State University Post Research Farm, Bozeman, MT, U.S.A. (latitude 45.41° N, longitude 111.00° W, elevation 1455 m) on Amsterdam silt loam soil with plots consisting of 3 m rows seeded at 90 seeds m^{-1}. Under normal irrigation. The lines were planted in an augmented randomized complete block design [17] with 7 blocks and 5 check plots per block. The check varieties, Hockett and, Odyssey were randomly assigned plots within each block and the heirloom lines were randomly assigned to the remaining plots.

Malting

Malting was performed with the use of Custom Laboratory Products (Milton Keynes, U.K.) steep/germ tanks and a kiln. Samples of barley (120 g), plumped over a 5.5/64” sieve, were

loaded into round steeping cages (19.05 cm diam. x 12.7 cm tall) with four quadrants. Each steep tank accommodated four cages, allowing 16 samples to be malted simultaneously. The modern cultivar Odyssey was used as a malt quality control and was placed in each steep run to ensure uniformity of steeping among runs. Using the CLP's Combi Micro Malter (Buckingham, UK, https://www.curiomalting.com/micromaltings-range) The steeping regime consisted of 48 h, in which grain was continually maintained at 15 °C and underwent a multi-steep program with a steep/rest pattern of 10 h steep, 18 h rest, 6 h steep, 10 h rest, and 4 h steep, with an average target moisture of 45%. Germination consisted of 96 h at a constant 15 °C. Throughout steeping and germination, humidity was maintained at >98% and agitation consisted of 5 min of cage turning at 0.61 RPM in every 30 min period. Aeration with moist air through the grain occurred for 1 out of every 10 min. After germination, samples were kilned via forced air in the CLP kiln over a 24 h period consisting of 12 h at 60 °C, 6 h at 65 °C, 2 h at 75 °C, and 4 h at 85 °C. Upon completion, samples contained on average 4.0% moisture and were manually de-culmed [18].

Malt quality analysis

Testing followed the guidelines set forth by the American Society of Brewing Chemists (ASBC) with only minor modification, as detailed below. Enzyme extraction was performed on 12.5 g of grist mixed with 250 mL 0.5% NaCl dissolved in ultra-pure water. Samples were mixed continually for 30 m at 20 °C followed by 30 m of filtration (with samples turned over at 25mL). Validated analysis of α-amylase was performed in accordance with ASBC Malt-7 and the Gallery operating protocol "Alpha-Amylase in Malt, version 1 – 06/2016 (Thermo Fisher Scientific Vantaa, Finland). Validated analysis of Diastatic Power was performed in accordance with ASBC Malt-6 and the Gallery operating protocol "Diastatic Power", version 4-5/2016 and the Thermo Scientific D-Glucose reagent kit, product code: 984304. Wort extraction was

performed on 40 g of grist mixed with 320 mL of ultra-purified water. Samples were extracted with a congress mash in a 16-cup, benchtop mash bath (IEC, Australia). Samples were filtered to 80 mL before being turned back into the filter and a final filtration performed to 160 mL. Filtration time was recorded as the time from initial pour to accumulation of 160 mL. The 1-mL samples were taken at the one-hour mark after filtration started and FAN, β-glucan, and soluble protein were all measured with a Gallery analyzer. Validated FAN analysis was performed as described in D101313_08_Insert_Alpha-Amino_Nitrogen (Thermo Fisher Scientific) and described by [10]. Validated β-glucan analysis was performed as described in D10951_07_insert_B-Glucan_(High_MW) (Thermo Fisher Scientific) and described by [19]. Validated soluble protein analysis was performed as described in D10415_02_Insert_Total_Protein_Biuret (Thermo Fisher Scientific).

Malt metabolite extraction

To extract grain non-volatile metabolites, malted barley was first powderized using a coffee grinder (Bodum, Inc., Triengen, Switzerland). Next, 100 mg of powderized grain (from each variety) was transferred to a VWR 2 Dram glass vial with phenolic cap. Analysis of non-volatile metabolites was conducted with a methyl-tert-butyl-ether (MTBE):methanol: water (6:3:1) biphasic extraction of the malt as reported in [20]. A portion of the aqueous fraction was combined with the organic fraction as a stacked injection [20], and analyzed using reverse phase ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) using methods as previously described for detection of semi-polar and nonpolar metabolites including flavonoids (e.g. catechins), terpenoids (phytosterols), alkaloids (hordatines, hordatines), pigments (carotenoids), and lipids (free fatty acids, phosphatidylcholines), and malt flavor/Maillard reaction products including maltol and maltoxazine [21].

Replicate injections (n=2) of each sample were used as quality control to account for analytical variation. Coefficient of variance (CV) percentages were calculated for all metabolites between the replicate samples to evaluate variation in the system and were reported as < 25%, considered acceptable variation for these metabolomics platforms [22]. Mass spectra from each of three metabolomics platforms were converted to the .cdf file format and processed and annotated using the workflows described in [21]. Metabolite quantities were normalized to total ion current and relative abundance for each molecular feature was determined by the mean area of the chromatographic peaks among replicate injections (n = 2). Non-volatile metabolites were identified and annotated by spectral matching in RamSearch software [23] to an in-house database of ~1,500 compounds, MSFinder software (as a validation tool for LC annotations (v3.26, RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa, Japan)) [24, 25], and to external and theoretical databases including NIST v14 (http://www.nist.gov), Metlin [26], Human Metabolome Database (HMDB) [27], and FooDB [28]. Chemical ontologies were established using HMDB and the ClassyFire package in R [29].

Statistics

Agronomic and malt data were corrected using replicated controls and the Augmented RCB design in Agrobase (https://www.agronomix.com/). Statistical models were used on the annotated malt metabolites and quality traits. Principal Component Analysis (PCA) and Hierarchical Clustering Analysis (HCA), to create chemogroups, was conducted on mean-centering and Pareto-scaled metabolite and quality data using SIMCA software v.14.1/15 (Sartorius Stedim Biotech, Umea, Sweden) [30]. Metabolite abundances were compared using two-way ANOVA, via the aov function in the R statistical environment v. 3.2.4 for malt variety with a p threshold of 0.05. False discovery rate adjustment was performed on the ANOVA p-

values using the Benjamini-Hochberg algorithm [31]. O2PLS models were conducted in SIMCA v. 14.1 on the scaled and centered data sets for malting quality traits (y) and annotated metabolites (x).

Data were z-transformed and resulting z-scores were used to create heatmaps utilizing hierarchical clustering and Spearman's rank correlation (rs) methods. Heatmaps with hierarchical clustering were generated in R using the gplots, ggplots2, Reshape2, and stats packages with the heatmap.2, melt, and hclust functions [32-34]. Chemogrouping, as defined above, although determined by chemical composition, was used to highlight chemically similar groups throughout this research. Prior to heat-mapping, metabolite data were normalized within each of the experimental varieties via z-transformation (normalized peak area – mean peak area/standard deviation of total peak area of each metabolite). The resulting z-scores were converted into colors and grouped using hierarchical clustering. Coefficient of Variation (CV) was calculated as: $CV = \sigma/\mu * 100$, where σ is the standard deviation or the metabolite for each individual cultivar within a treatment and μ is the mean abundance for the metabolite for each individual cultivar within a treatment. The CV was calculated for each the heirloom population and controls, and for chemogroups, averaged across chemogroups, and represented as a heat map. Fold change (FC), a measure describing the degree of quantity change between final and original metabolite abundances.was calculated as the ratio of the average mean of that chemogroup divided by the control chemogroup for each metabolite. The FC was calculated for chemogroups for each metabolite and represented as a heat map. Boxplots and calculation of means of individual metabolites were created using GraphPad Prism v. 9.1.1 (Graphpad Software, San Diego, CA, USA).

Results

Assessment and patterns of metabolite variation in heirloom barley

LC-MS metabolomics analysis of the 130 2-row heirlooms was performed to study variation in small molecules compared to two modern, control varieties: Hockett and Odyssey. The LC-MS method resulted in the detection of 2,568 compounds, of which 202 were annotated as metabolites (Figure 2.1, Table S.2). The annotated metabolome was comprised of lipids (139), organic acids (12), phenolics and benzenoids (21), nitrogenous compounds (11), and others (19).

Metabolite variation was first modeled in this system using multivariate approaches. Principal Component Analysis (PCA) was applied to the 202 metabolites and resulted in 12 components that explained 83% of the variation (Figure 2.2a). The variation explained was skewed towards PC1 (53.3%), for example PC2 and the remaining components were all less than 6.4%. The two control varieties (Hockett and Odyssey) had near identical chemical profiles as indicated by the PC1-PC2 scores plot, and they co-localized near the center of the PCA.
The PCA indicated clusters of varieties based on chemical composition, as opposed to an equal and normal distribution of observations within the scores plot. Therefore, hierarchical cluster analysis (HCA) was applied to the PCA to establish clusters of varieties defined by their metabolite profile. The PCA-HCA analysis resulted in seven groupings, hereby termed "chemogroups" that were assigned to each variety (I-VII) for further analysis (Figure 2.2b, Figure S.1).

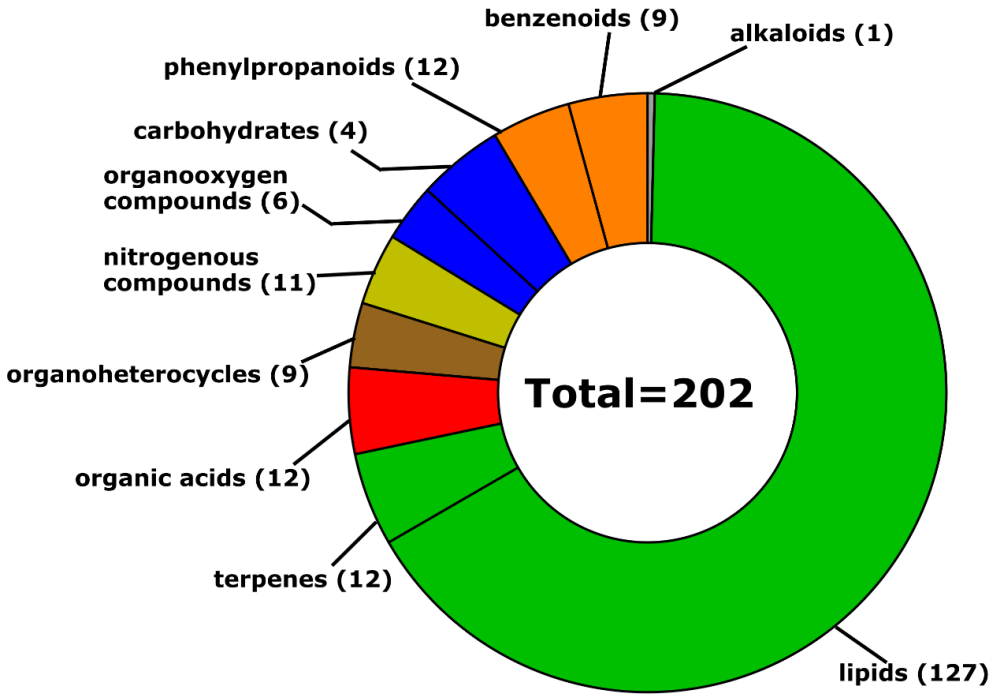

Figure 2.1 Pie chart of annotated malt metabolites and corresponding chemical class. A total of 202 metabolites were annotated from the heirloom malt. The pie chart displays metabolites by broad chemical class (black text) and the total number of metabolites in that class (in parenthesis).

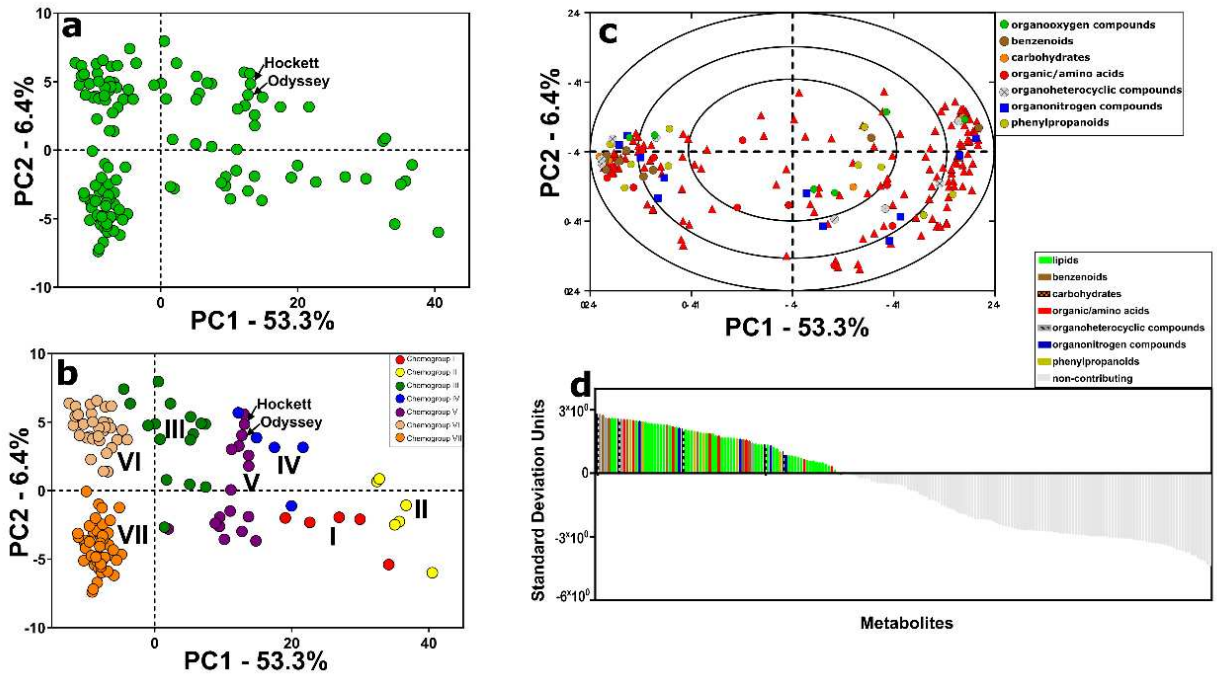

Figure 2.2 Principal component analysis (PCA) and chemogrouping of heirloom malt. (a) PCA scores plot for analysis of malt metabolite variation (202 metabolites) from 128 heirloom varieties and 2 controls (Hockett, Odyssey). (b) Hierarchical clustering was performed on the scores data and formed seven chemo-groups, labeled I-VII and colored accordingly. (c) PCA loadings plot performed on 128 two-row heirloom malted barley varieties. The PCA loadings and scores plot of PC1 and PC2 were correlation scaled and ellipses denote 0.5, 0.75, and 1.0 correlation values and colored by chemical class, (d) Example contribution plot of Chemogroup I. The contribution plot shows why metabolite deviates from the average. Metabolites (x-axis) are colored by chemical class and shown in terms of standard deviations from the average (y-axis).

An analysis of the PCA loadings indicated several compounds as major contributors to an assignment within a chemogroup (Figure 2.2c). PC1, which separated all chemogroups except for VI and VII, was largely driven by several lipids (e.g. glycerolipids), maltotriose (a saccharide, **12**), amino acids valine (**647**) and biocytin (**170**), and several benzenoid compounds (e.g. 2-aminobenzoic acid, **1141**) and phenylpropanoids (e.g. epicatechin, **2232**). Analysis of a metabolite's importance in defining each chemogroup was conducted using the 'distance to model' function applied to the PCA-HCA data in SIMCA software. The result of this analysis is

a contribution plot that ranks metabolites in order of relevance to defining the metabolome of a set of varieties (chemogroups), and an example contribution plot for Chemogroup I is shown in Figure 2.2d. The distance to model data for all chemogroups is reported in Table S.3, and the five most important metabolites that define each chemogroup is shown in Table 2.1.

Analysis of metabolite variation among and within chemogroups

The influence of a metabolite or set of metabolites to defining a chemogroup, and relationship among chemogroups was further evaluated via z analysis. In this method, a z score is assigned to each metabolite that indicates its 'uniqueness' to that chemogroup, and then HCA is applied to the metabolite and chemogroup data to form clusters that indicate broad trends Non-lipids (organic acids, amino acids, benzenoids) and lipids were separated in this analysis for improved visualization of the data (Figure 2.3, Figure S.2, Table S.7). For non-lipids, Chemogroups IV, VI, and VII had similar profiles, yet Chemogroups I, II, and III were very distinct, with Chemogroup V being non-descript. Proline (**178**) was more abundant in Chemogroup IV and was the least abundant in Chemogroup 1. Leucine (**1313**) was least abundant in Chemogroups IV-VII and most abundant in Chemogroups I-III, whereas maltol (**264)** displayed the opposite trend (Figure 2.3a). For lipids, Chemogroup I and VI had the most unique lipid profiles. Other chemogroups exhibited an influence of lipids but to a smaller extent (Figure S.2). Next, the metabolite data was evaluated to quantify the extent of variation within and among chemogroups using coefficient of variation (CV). A CV can indicate the breadth of variation within a group, and therefore comparisons indicate the potential to develop varieties that are high in a specific metabolite. Here, the CV for each metabolite was evaluated in the heirlooms compared to the high-quality controls, as well as the chemogroup of the controls.

The CV was therefore evaluated in two ways: the heirloom population CVs relative to the CVs of the two control varieties, Odyssey and Hockett; and among CVs calculated within chemogroups and then compared (Figure 2.3b-c). The CV was also evaluated among varieties within the heirloom population (n=128) (Table S.2). For the comparison of control and heirloom groups (Figure 2.3b), the controls had moderately high variation in an organonitrogen amide (**236**). N1,N10-diferuloylspermidine, a phenylpropanoid (**638**), and a glycerophosopholipid (**1295**), but most metabolites were consistent (CV < 75%). Among the metabolites with the lowest CV in the control group were several lipids, the saccharides maltotriose and β-D-galactose, amino acids valine (**647**) and proline (**178**), and maltol (**264).** The CV data reveal that metabolite variation was much lower within the control group compared to the heirlooms, and most metabolites in the heirlooms had a CV of greater than 75%, with some CVs exceeding 120% such as several triglycerides (**233**, **361**,**495, 581, 693**) and an amide (**236**).

Table 2.1 Distance to model – contribution scores for chemogroups

Chemogroup	Classification	Subclass	Metabolite	DTM-I	DTM-II	DTM-III	DTM-IV	DTM-V	DTM-VI	DTM-VII
Chemogroup I	organoheterocycles	pteridines	2,4-diamino-6,7-diisopropylpteridine	1.72	1.28	1.3	0.06	0.56	-0.86	-0.85
	organoheterocyclic compounds	quinolines	4-benzyl-3H-pyrrolo[2,3-c]quinoline	1.72	1.2	1.28	0.02	0.58	-0.81	-0.87
	carbohydrates	saccharides	maltotriose	1.71	1.22	1.19	0.01	0.61	-0.74	-0.92
	organic acids	amino acids	biocytin	1.66	1.6	1.25	0.24	0.75	-0.84	-0.86
	lipids	lysophospholipids	LysoPC(16:0)	1.66	1.19	1.22	0.1	0.57	-0.71	-0.93
Chemogroup II	lipids	phosphatidylcholines	PC(18:2(9Z,12Z)/18:2(9Z,12Z))	0.67	5.35	-0.19	1.02	1.76	-0.94	-0.23
	lipids	phosphatidylcholines	PC(20:4(5Z,8Z,11Z,14Z)/18:4(6Z,9Z,12Z,15Z))	0.05	5.24	-1.79	1.05	1.91	-0.73	0.02
	organic acids	amino acids	2-aminoheptanoic acid	-0.04	4.64	-2.56	2.55	1.88	-0.31	0.04
	lipids	glycerophospholipids	PA(22:4(7Z,10Z,13Z,16Z)/15:0)	0.7	4.45	-0.24	3	2.45	-0.65	-0.44
	lipids	lysophospholipids	PE(20:1(11Z)/15:0)	-0.07	4.39	-2.03	2.79	1.2	-0.05	0.01
Chemogroup III	phenylpropanoids	flavonoids	epicatechnin	1.55	0.75	1.45	0.09	0.42	-0.49	-1
	phenylpropanoids	flavonoid glycosides	apiin	1.5	0.85	1.37	0.03	0.35	-0.69	-0.76
	organic acids	amino acids	L-Valine	1.65	1.12	1.32	0.02	0.45	-0.77	-0.85
	organoheterocycles	pteridines	2,4-diamino-6,7-diisopropylpteridine	1.72	1.28	1.3	0.06	0.56	-0.86	-0.85
	organoheterocyclic compounds	imidazopyrimidines	adenine	1.37	0.86	1.29	-0.05	0.18	-0.58	-0.72
Chemogroup IV	lipids	lysophospholipids	PE(16:0/18:1(9Z))	-0.14	3.71	-2.42	3.58	1.76	-0.11	0.06

Chemogroup	Classification	Subclass	Metabolite	DTM-I	DTM-II	DTM-III	DTM-IV	DTM-V	DTM-VI	DTM-VII
	lipids	phosphatidylcholines	PC(16:0/18:0)	-0.13	3.09	-2.03	3.57	1.06	-0.35	0.1
	lipids	lysophospholipids	LysoPE(18:2(9Z,12Z)/0:0)	-0.17	1.17	-0.89	3.32	-0.16	-0.14	0.09
	lipids	glycerolipids	DG(18:3(9Z,12Z,15Z)/18:2(9Z,12Z)/0:0)	-0.06	3.83	-2.16	3.25	1.9	-0.26	0.03
	lipids	glycerophospholipids	PA(22:4(7Z,10Z,13Z,16Z)/15:0)	0.7	4.45	-0.24	3	2.45	-0.65	-0.44
Chemogroup V	lipids	glycerophospholipids	PA(22:4(7Z,10Z,13Z,16Z)/15:0)	0.7	4.45	-0.24	3	2.45	-0.65	-0.44
	lipids	phosphatidylcholines	PC(20:4(5Z,8Z,11Z,14Z)/18:4(6Z,9Z,12Z,15Z))	0.05	5.24	-1.79	1.05	1.91	-0.73	0.02
	lipids	glycerolipids	DG(18:3(9Z,12Z,15Z)/18:2(9Z,12Z)/0:0)	-0.06	3.83	-2.16	3.25	1.9	-0.26	0.03
	organic acids	amino acids	2-aminoheptanoic acid	-0.04	4.64	-2.56	2.55	1.88	-0.31	0.04
	lipids	lysophospholipids	PE(16:0/18:1(9Z))	-0.14	3.71	-2.42	3.58	1.76	-0.11	0.06
Chemogroup VI	lipids	monoacylglycerides	MG(18:4(6Z,9Z,12Z,15Z)/0:0/0:0)	-1.21	-2.45	-0.56	0.27	-0.64	1.09	0.27
	lipids	triglycerides	TG(18:2(9Z,12Z)/16:0/20:5(5Z,8Z,11Z,14Z,17Z))	-1.14	-2.5	-0.25	0.15	-0.53	1.06	0.23
	lipids	sphingolipids	N-(2R-Hydroxytricosanoyl)-2S-amino-1,3S,4R-octadecanetriol	-1.23	-2.22	-0.48	0.09	-0.28	1.05	0.3
	lipids	phosphatidylcholines	PC(15:0/20:3(8Z,11Z,14Z))	-1.23	-2.55	-0.43	0.06	-0.55	1.05	0.34
	carbohydrates	hexoses	beta-D-galactose	-1.07	-2.38	-0.19	0.22	-0.19	1	0.19
Chemogroup VII	lipids	diradylglycerols	DG(18:2(9Z,12Z)/18:2(9Z,12Z)/0:0)	-1.2	-1.17	-0.5	-0.04	-0.41	0.05	1.01
	lipids	diradylglycerols	DG(16:1(9Z)/20:3(5Z,8Z,11Z)/0:0)	-1.42	-1.41	-0.55	0.02	-0.51	0.29	1
	organoheterocycles	indoles	n-methyltryptamine	-1.2	-1.5	-0.41	-0.36	-0.8	0.29	0.9
	lipids	diradylglycerols	DG(18:1(9Z)/18:1(9Z)/0:0)	-1.17	-1.11	-0.55	0.04	-0.24	0.14	0.87

Chemogroup	Classification	Subclass	Metabolite	DTM-I	DTM-II	DTM-III	DTM-IV	DTM-V	DTM-VI	DTM-VII
	lipids	glycerolipids	DG(20:4(8Z,11Z,14Z,17Z)/16:0/0:0)	-1.27	-1.49	-0.58	-0.01	-0.35	0.34	0.86

Analysis of CVs among chemogroups (Figure 2.3c) indicate that most metabolites had a similar variation among chemogroups. However, three sets of metabolites were shown to have CVs that varied among chemogroups, denoted in Figure 2.4c. This contrasts with the much higher CVs of the rest of the heirloom population, which were 4- to 5-fold higher for each metabolite. Looking at a comparison among the chemogroups, II and IV had the highest number of high CV scores for metabolites spanning across all classes, whereas chemogroups VI and VII contained the lowest number of high CV scores, indicating that these had the least amount of unique variation across all chemical classes.

Abundance and fold change trends among chemogroups

In addition to describing the formation of chemogroups, and the metabolite variation among and within these groups, it is also important to describe differences in abundances. Abundances were compared for each chemogroup relative to the chemogroup V, which contained the two controls (V) using ANVOA and fold change analysis. ANOVA of the 202 metabolites supports that 202 of the 202 metabolites varied among chemogroups (Table S.6). Heatmaps (Figures S.3 and b, Table S.5) displaying fold change (FC) indicate large abundance differences the control chemogroup. Chemogroups I, II, and III, for example, exhibited greater variation among some lipid metabolites indicating less abundance of these metabolites (Figure S.3). Notably, chemogroups I, II, and III were more abundant in proline (**178**) whereas chemogroups IV, VI, and VII were less abundant (compared to the controls Odyssey and Hockett and Chemogroup V). Several lipids (glycerolypids, phosphatidylcholines) were more abundant in Chemogroups I and II, compared to the other chemogroups. In Chemogroups II and VII, there were several phenylpropanoids and phenolics (such as epicatechin, **2232** and cinnamic acid, **1099)** that were more abundant than in other chemogroups.

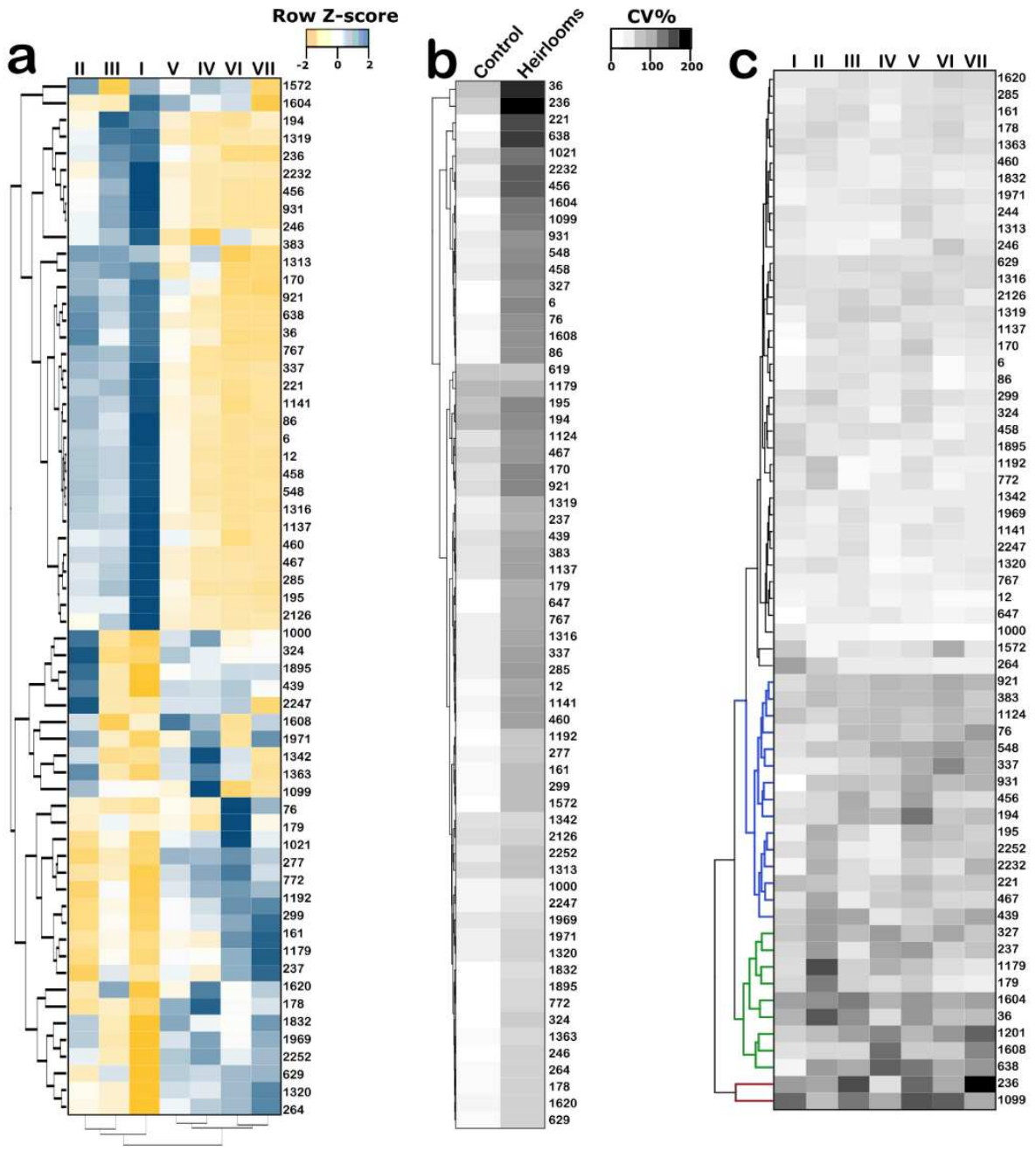

Figure 2.3 Heatmaps of Z-scored abundances and coefficient of variation (CV). Prior to heatmapping, metabolite data were normalized within each variety via z-transformation normalized peak area - mean/standard deviation of total peak area of each metabolite). The resulting z-scores were converted into colors and grouped using hierarchical clustering. Heat maps with hierarchical clustering were built within chemicals classes, as described (a) 62 non-lipids. The color in each cell represents the z-transformed abundances of the averaged replicates (n = 2) per malt sample. Z-transformation was based on the mean abundance and standard

deviation of the metabolite across all samples. Heat map of the relative standard deviation of metabolites identified in the heirloom barley chemogroups represented as coefficient of variation. Gray squares represent the mean coefficient of variation (CV) for each of the non-lipid metabolites (b) between the control varieties and the population and (c) among chemogroups (n=7).

Further analysis was conducted on select flavor and fermentation-relevant metabolites to evaluate trends within and among chemogroups, as a measure to display variation in this population using bar charts (Figure 2.4). Leucine (**1313**), an amino acid important to the creation of flavor in beer, was highest in Chemogroup I (mean = 37,678 AU) and lowest in group VI (mean = 24,441 AU) (Figure 2.4a). Maltol (**264**), a major malt flavor metabolite, was lowest in group I (mean = 14,389 AU) and highest in group VII (mean = 60,085 AU) (Figure 2.4b). A similar trend was observed for proline (**178**), an amino acid that important to yeast nutrition. Proline was highest in group IV (mean = 70,066 AU) and lowest in group I (mean = 33,144 AU) (Figure 2.4c).

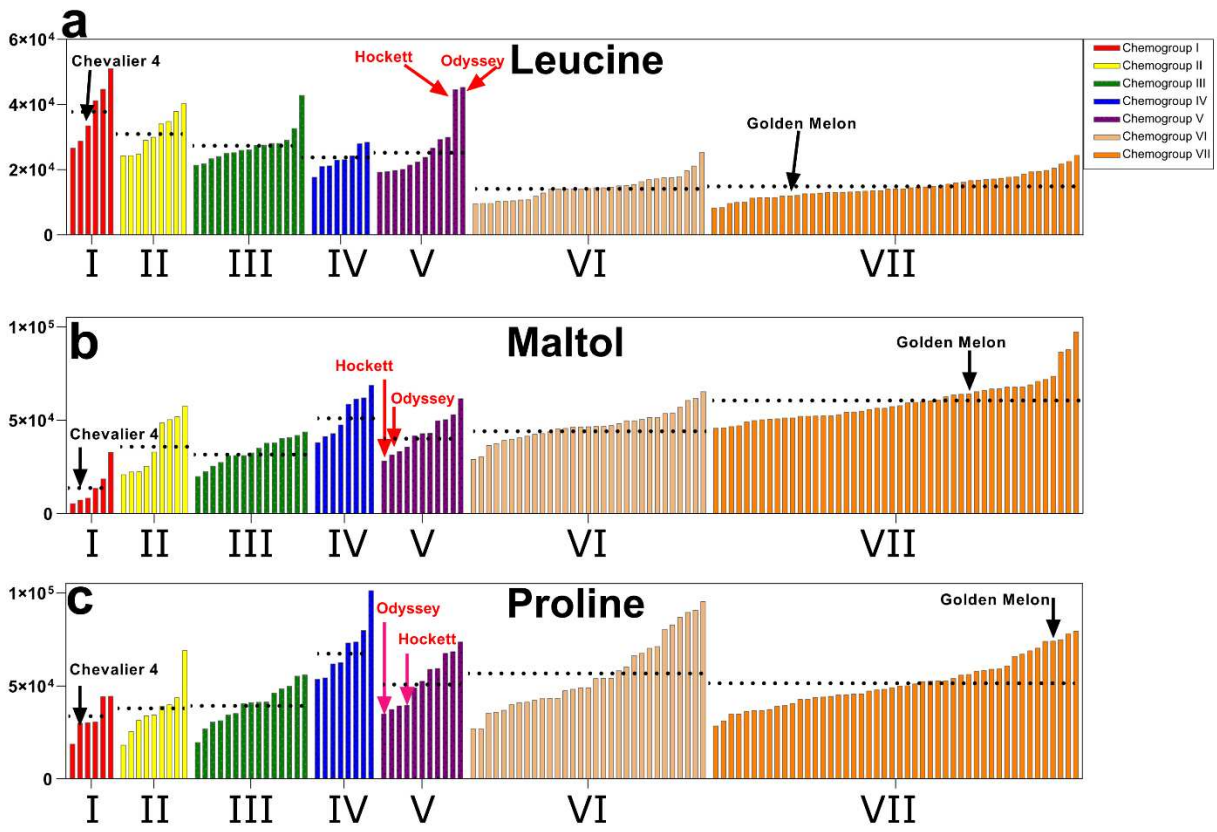

Figure 2.4 Boxplots exemplifying HCA groups and varying levels of flavor precursors among chemogroups and varieties. (a) leucine (b) maltol and (c) proline. Two check varieties are called out in pink text with pink arrows. Dotted lines represent the mean of each chemogroup.

Discussion

Here, a non-targeted metabolomics approach was utilized to describe chemical diversity in malt derived from a diverse set of heirloom varieties, and compared variation in this population to to optimized modern varieties as controls. A biphasic extraction protocol was utilized to optimize the extraction of a wide-range of chemical compounds for detection using UHPLC-MS and to include analysis of lipids, which are an important but understudied component of malt chemistry [35, 36].

It is well known that the abundance and composition of lipids in malt and into the brewing process have an impact on yeast performance during fermentation and the production of

flavor-active lipid esters [36]. However, it is a challenge for maltsters and brewers to understand the influence of barley genetics and malt variety on the amount and composition of lipids which impact fermentation and brewing efficiency [21, 36, 37]. It has been suggested that lipids also have an effect on gelatinization temperature, seed water uptake in barley during the steeping step in malting, foam stability, and enzymatic activity throughout the brewing process [38].

This analysis allowed for the detection of 202 compounds present in the heirloom barley malt variety metabolome. In our study, 202 compounds were identified and placed into 10 chemical classes that varied within and among chemogroups, supporting the potential for new breeding targets for flavor and quality in heirlooms. Predicting phenotype from genetic, environmental, and processing information is a great challenge in diverse and intricate biological systems such as malting barley [39]. The potential to apply metabolomics to plant breeding has been extensively discussed [40-43] and the major value is that metabolite profiles are directly related to important quality phenotypes of food crops. While non-targeted metabolomics can also provide fingerprints and, the fact that many metabolites directly affect quality makes these datasets a major challenge to decipher. Therefore, a limitation is applying metabolomics to plant breeding is the size and complexity of the data, and uncharacterized links to quality phenotypes.

One method to reduce the complexity of omics data in plant breeding is to apply a form of dimension reduction. In genomic selection, this is often done via PCA or kinship analysis, and result in sub-populations based on genetic patterns. Here, we applied this same principle with chemical data: structure within the population of heirlooms was determined based on similarities and differences of metabolite profiles. This PCA-HCA analysis reduced the data from 130 varieties to 7 groups. The major value of this grouping to breeding is to simplify the selection process – instead of breeding with and evaluating130 varieties, sub-sets could be tested as

representatives within the chemogroup. The two control varieties, Hockett and Odyssey, had similar profiles and were within the same Chemogroup V, and this group was located centrally within the PC scores plot (Figure 2.2), supporting these varieties and this chemogroup has an 'ordinary' malt metabolite profile. For breeding, this indicates that Chemogroup V is a less ideal source of varieties for novel malt traits. The chemogroups that were most different from the control-containing Chemogroup V were Chemogroups I-V, and metabolite analysis indicated these were defined by increased abundances in important amino acids such as valine (**647**), proline (**178**), leucine (**1313**), phenylalanine (**931**), tryptophan (**2252**), and glutamine (**548**), as well as maltol (**264**). Further, some chemogroups were more heavily defined by lipids (Chemogroups I, VI, and VII), and others by known flavor compounds [Chemogroups I-III], and these classes of compounds have different ways of contributing to flavor [15, 21, 36, 41]. The flavor-active precursor compounds detected in this study have demonstrated effects on beer flavor (Table S.2). Many of the metabolites are precursors to Maillard Reaction Products in beer (examples and references). The compounds detected in this study (e.g. lipids, organic acids) have demonstrated effects on malt quality and flavor, and have potential to be precursors to beer flavor and aging. However, there was not an influence of simply one class of metabolites that may influence flavor and quality. Differences in malting flavor has to do with varietal practices combined with malting practices.

To assess the extent of variation within and among chemogroups, CVs were evaluated, and a CV can indicate the plasticity of a metabolite trait to be enhanced via breeding. First, the CVs of the heirloom population (n=128) were compared to the controls (n=2). The heirloom population was much more diverse, understandably, since the control varieties are bred to be consistent, whereas the heirlooms are not. The higher and more inconsistent CVs among the

heirlooms indicate that these are unique varieties that may offer novel traits to broaden the pool of genetic and chemical diversity that exists (Figure 2.3b). CVs among the chemogroups showed three broad trends, including some much higher CVs (>100%). Notably, the amino acids valine (**647**), proline (**178**), leucine (**1313**) had low CVs, indicating stability among and within chemogroups, but phenylalanine (**931**), tryptophan (**2252**), and glutamine (**548**) had higher CVs, indicating more variation and ability to be manipulated through breeding. These amino acids are important for many biochemical processes in the malting and brewing processes, and the ability to have control over their abundances is desirable [44, 45]. Ultimately, metabolites with higher CVs will be strong targets for screening and breeding for genotypes with flavor and quality attributes amenable to the industry.

The influence of metabolites which define a chemogroup and explain relationships among chemogroups was further evaluated via z-analysis. The z-scores (Table S.7, Figure 2.3a, Figure S.2) indicate similar chemical trends among Chemogroups I-III. As Chemogroup I seems to be the most extreme in trend (with very high and very low z-scores, as indicated by the intensity in color), implying variation that is unlike the other chemogroups. Chemogroups II and III share this metabolite variation following the same trend as Chemogroup I, yet less intensely. Chemogroups IV-VII also share similar trends in both lipid and non-lipid classes, but again, lacking in intensity of variation. These profiles are important to note when considering the barley variety, how it can be optimized, and how it is to be utilized in the brewing process. An increased lipid profile, as in Chemogroup I, and to a lesser extent, Chemogroups VI and VII, could be a liability, as it may lead to increased lipid oxidation [46, 47]. The abundance of lipids, as well as the composition of lipids in malt, may play a role and indicate that they should be considered together with other metabolites when considering genotype selection and

optimization of varieties [15]. The most abundant compounds in Chemogroup I, the most diverse, were two phenylpropanoids: epicatechin (**2232**) and apiin (**456**), both of which are sources of natural antioxidants for disease prevention, health promotion, and are known for their influence in the formation of haze, color, taste, and foam stability in barley [48]. Also notably abundant in Chemogroup I was phenylalanine (**931**), which, is significantly correlated with haze formation in beer [49].

In addition to describing the metabolite variation among and within the chemogroups, abundances were compared via fold change (FC) for each chemogroup relative to the chemogroup V, which contained the two controls (Figure S-3). Among both lipid and non-lipid metabolites, Chemogroups I-III demonstrated very different metabolomes compared to the other chemogroups with the amino acids and lipids contributing significantly to this distinction. Chemogroups I-III displayed the highest FC compared to Chemogroup V spanning many chemical classes. Those with a FC > 2 included phenylalanine (**931**), epicatechin (**2232**), adenine (**1124**), apiin (**456**), and several lipids (Table S.5, Figure S.3). This supports previous research regarding the concentration and the quality of lipids in beer, which play a role in foam stability and staling, depend on the composition in the lipid content of finished malt [50].

The potential to breed for a specific metabolite profile is an important future endeavor. In addition, a malting regimen for each variety can be optimized to enhance metabolite expression. In the future, specific chemical compounds and genetic markers for quality and flavor must be identified for the benefit of breeders. Taken together, the results of this study support the potential to breed for a more unique barley varieties with novel traits utilizing the heirloom genetic pool.

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CHAPTER 3 – MASS SPECTROMETRY METABOLOMICS OF HOT STEEP MALT EXTRACTS AND ASSOCIATION TO SENSORY TRAITS[2]

Overview

In the brewing industry, there is value in defining sensory attributes of malt, and recent protocols have been developed that enable analysis of aroma and taste. One method, the 'hot steep', is a hot water extract that is highly reproducible and able to distinguish malt flavor. However, the chemistry of the hot steep extracts has not been fully defined, and the links between specific metabolites of the hot steep and their resulting sensory attributes remains largely unknown. Here, a study was designed to describe the metabolite chemistry of hot steep extracts, and to characterize variation in this chemistry and corresponding sensory by comparative analysis of 12 commercial pale malts. Metabolomics was performed on the 12 malt hot steep extracts using three mass spectrometry platforms to detect volatiles (HS/SPME-GC-MS) and non-volatiles (UHPLC-TOF-MS and GC-MS). The analysis detected a total of 1,026 compounds including lipids, organic acids, esters, and Maillard Reaction Products (MRPs), of which 162 compounds (15.7%) varied among the 12 hot steep extracts. Sensory of the 12 hot steep extracts was performed using an integrated Check All That Apply and quantitative analysis method for 14 traits, and the data revealed *cereal, grassy,* and *dough* aromas were the attributes that varied. The metabolomics and sensory data were integrated using OPLS analysis. The analysis revealed 64 compounds strongly associated with *cereal* aroma and included MRPs. A total of 23 compounds were strongly associated with *grassy* aroma including alkane/alkenes,

[2] This chapter is a modified form of the following article published in the Journal of the American Society of Brewing Chemists: Harmonie M. Bettenhausen, Lindsay Barr, Heather Omerigic, Linxing Yao & Adam L. Heuberger (2021) Mass Spectrometry Metabolomics of Hot Steep Malt Extracts and Association to Sensory Traits, Journal of the American Society of Brewing Chemists, DOI: 10.1080/03610470.2020.1869499

benzenoids, organic acids, lipids, and fatty acid esters. Taken together, these data highlight the utility of the hot steep extract to differentiate malt for flavor and chemistry and indicate specific compounds that drive the most dominant flavors observed in this population of pale malts.

Introduction

In 2014, the Brewers Association published a "Malting Barley Characteristics for Craft Brewers" white paper that reported malt flavor as a major gap in industry knowledge [1]. The industry responded by establishing several new tools that enable the description of malt flavor. One method, known as the "hot steep", is a rapid and affordable measure of malt flavor for its wide accessibility, low-cost of materials, and reproducibility across laboratories [2]. The hot steep was developed as an alternative to the Congress Mash, a procedure that was designed to estimate a maximum theoretical extract of wort and is therefore focused on extracting and measuring saccharides. While similar, the hot steep utilizes lower extraction temperatures and shorter incubation times than the Congress Mash, essentially reducing the saccharide content of the extract for a better evaluation of malt aroma (excess saccharides can mask or otherwise impact sensory of aroma). This hot steep protocol was developed and validated by the American Society of Brewing Chemists in 2017 [2]. While this protocol is still new, it is thought that the overall value of analyzing the hot steep can include quality control of malt and brewing recipes, differentiation products for marketing, and potentially understanding a contribution of malt flavor metabolites to beer flavor.

Malt flavor, an integration of taste, mouthfeel, and aroma, is complex as chemicals in malt can contribute to one or all of these categories. For example, malt polyphenols can lead to astringency and tartness [3, 4]. Malt flavor is largely determined by malting regimen, which affects to the final content (variation) in non-volatile and volatile chemicals through physical

processes such as moisture, airflow, and pH [5, 6]. A major class of malt flavor metabolites are the Maillard Reaction Products (MRPs, e.g. pyrans, furans, pyrazines) that contribute to *toasted, roasted, malty, dough, caramel,* and *cracker* aromas as well as color [7, 8]. Further, aliphatic alcohols, aldehydes, ketones, organic acids, and lipids contribute to malt flavor [4, 9] . In previous work by Beal and Mottram, it was demonstrated that methylbutanals (aldehydes) such as 3-methylbutanal (also known as isovaleraldehyde), also affect so-called "malty" aromas (e.g. *chocolate*, *malty*, *roasted*, *biscuit*) of the lighter, pale malts [10].

Variation in malt chemistry and flavor can be determined using various sensory analyses of the malt and/or malt extracts. Methods for quality assurance often involve visual inspection for color and defects, smelling for off or mold-like aromas, and then chewing the malt for a broad view of its acceptability. However, chewing is considered ineffective to fully describe malt flavor, as some flavor active compounds are only detected after extracting in hot water, as would occur with brewing [11]. Both hot water extracts and cold water pastes had been attempted for sensory research on malt, by which Quantitative Descriptive Analysis (QDA) was then used develop and evaluate a lexicon [12, 13]. Recently, new sensory methods have been developed for analysis of the malt hot steep. In 2017, DraughtLab, LLC published the Base Malt Flavor Map as a lexicon, and designed a corresponding Check All That Apply and Quantitative Descriptive Analysis (CATA/QDA) tool to evaluate malt flavor [14]. The lexicon uses common flavor descriptors (e.g. *cereal, bready*) and divides flavor within a hierarchy of three color-coded categories: taste, aroma, mouthfeel. The combination of the hot steep method, DraughtLab lexicon, CATA (to develop the lexicon utilized in this study), and subsequent QDA data scoring enables the ability to describe malt, quantify variation in sensory, and to potentially differentiate malts based on this variation.

Here, we report the metabolite chemistry of hot steep extracts, and characterize variation in this chemistry and sensory by comparative analysis of 12 commercial malts. The 12 malts were all pale, within a narrow specification window (base malts), and so variation in metabolite chemistry and sensory traits was designed to be more subtle among major classes of malt (*i.e.* obvious sensory differences among pale *vs.* crystal *vs.* chocolate malt). A non-targeted metabolomics approach was utilized to characterize metabolites extracted using the hot steep method. The hot steep data was integrated by first deciphering sensory trends using Principal Component Analysis, and associating those trends to metabolites using Orthogonal Projection to Latent Structures (OPLS), a regression analysis often employed in metabolomics studies to associate metabolite data to phenotypes [15]. In addition to reporting the metabolite chemistry of hot steep malt extracts, we specifically tested the hypotheses that, when sourced from different commercial operations: (*i*) the metabolite chemistry of pale malt will vary; (*ii*) that pale malts can be discriminated based on sensory attributes; and (*iii*) flavor metabolites vary in the hot steep and can be associated to sensory traits.

Experimental Procedures

Plant materials, malting, and malt quality

The 12 malts used in this study were provided by 5 commercial maltsters (denoted A-E) as representatives of pale/base malts within a specification window of: low color (below 2° Lovibond), 3-5% moisture, 10-11% protein, 80% Fine Grind (FG) minimum. Malt specifications for the samples used in this study are provided in Table 3.1. The malts utilized for this study all adhered to American Malting Barley Association malting specifications [16]. Malt quality analyses were performed either in-house or at the Hartwick College Center for Craft Food &

Beverage (Oneonta, New York, U.S.A.) using methods of analysis set by the American Society for Brewing Chemists for α-amylase, β-glucan, color, diastatic power (DP), extract, FAN, soluble protein, total protein, and viscosity.

Hot steep extraction of the malt

For sensory analysis, hot steep extracts of the malts were performed at New Belgium Brewing Company (Fort Collins, CO, USA) using the American Society of Brewing Chemists Methods of Analysis Malt-14 [2]. The same protocol was performed on the malts at Colorado State University for metabolomics analysis, for which the extracts were flash frozen in liquid nitrogen and stored at -80°C until chemical analysis was performed.

Sensory analysis of the hot steep extracts

Sensory analysis of the 12 hot steep extracts was performed by a trained panel at New Belgium Brewing Company in collaboration with DraughtLab, LLC. The first session was solely focused on lexicon development, and a panel consisted of 11 participants (6 female, 5 male, ages 25-55) and was tasked to describe aroma using smelling and sipping (not taste, mouthfeel, or appearance). This training also included foods that exhibited the sensory attributes predicted to act as key flavors in evaluating malt. The panel used a Check All That Apply (CATA) method in the DraughtLab Pro software environment (v.1, New York, New York, USA) using the ASBC Base Malt Flavor Map (v.1) as a reference.

Each participant was instructed to choose the best descriptors for each of four randomly selected malt hot steep extracts. After data collection, the panel reviewed the results and formed a consensus on 14 sensory attributes that can define malt flavor: *bread dough, corn/DMS, dried grass, grainy, Grape Nuts™, green grass, green tea, hay, honey, nutty, papery/cardboard, pasta*

water, Play-Doh™, and woody. A second training was performed with the same panel to introduce scaling for each of the 14 traits.

Table 3.1 Sources of malt used in this study

Maltster	Barley Variety	Malt Type	Location Grown	Moisture (%)	Extract FGDB (%)	Color (°L)	β-glucan (mg/L)	Soluble Protein (%)	Total Protein (%)	S/T (%)	FAN (mg/L)	Alpha Amylase (D.U.)	Diastatic Power (°L)
A	Merit 57	Brewers Malt	Wyoming/Montana	4.2	81.0	1.9	NR	NR	11.5	42.0	NR	65	140
	Pinnacle	Pale	North Dakota	4.0	80.0	3.1	NR	NR	11.7	42.0	NR	45	85
	Synergy	Pilsen	Wyoming/Montana	4.5	81.0	1.6	NR	NR	11.3	37.0	NR	65	170
B	NR	Pale	British Columbia	4.5	80.0	1.9	NR	4.5	10.9	44.9	NR	NR	NR
	NR	Pilsner	NR	4.5	82.4	1.9	55	4.1	9.0	45.9	NR	NR	NR
C	NR	Base	NR	4.5	80.5	2.0	140	5.0	10.5	NR	NR	55	150
D	Genie	Base	San Luis Valley, CO	4.5	81.5	2.0	NR	4.8	11.5	42.0	NR	55	140
	Genie	Pale		3.5	81.5	3.1	NR	4.9	11.5	43.0	NR	40	90
	Genie	Pilsner		3.8	81.0	1.9	132	1.6	11.5	38.4	185	53	117
E	Genie	Munich	Northern Colorado	3.3	81.0	2.7	70	5.0	12.1	41.9	192	57	130
	AC Metcalfe	Pale		3.9	80.4	2.1	88	4.8	12.2	39.9	185	56	137
	Genie	Vienna		3.3	80.3	5.0	91	4.9	12.1	40.7	150	47	86

NR=Not Reported

Subsequent sensory sessions then evaluated the 12 malt hot steep extracts on two separate days, with each hot steep extract being presented in duplicate (randomized and blind-coded). During each testing session, panelists assessed the orthonasal aroma and flavor by mouth of the hot steep extracts with new randomized blind codes for the samples, using Quantitative Descriptive Analysis (QDA) [17, 18]. The sensory analysis was performed the same day hot steep extracts were prepared, within 1 hr of preparation. Sensory attributes measured on a line scale from 0-10, where 0 was 'not detected' and 10 was the most the panelist had ever experienced in the malt extracts.

Mass spectrometry metabolomics of hot steep extracts

Volatile and non-volatile metabolites in the hot steeps were detected using a non-targeted metabolomics approach. Analysis of volatiles was performed using headspace solid-phase microextraction gas chromatography-mass spectrometry (HS/SPME-GC-MS) with methods as

previously described [7]. Analysis of non-volatiles was conducted with a methyl-tert-butyl-ether (MTBE):methanol:water (6:3:1) biphasic extraction of the hot steep, as reported in [19]. The aqueous layer was dried under a stream of nitrogen gas, derivatized via methoximation and silylation [20], and then injected into a GC-MS using methods as previously described [21]. A portion of the aqueous fraction was also combined with the organic fraction and analyzed using reverse phase ultra-high-performance liquid chromatography MS (UHPLC-MS) using methods as previously described [22]. Replicate injections (n=3) of each sample were used as quality control to account for analytical variation. Coefficient of variance (CV) percentages were calculated for all metabolites between the duplicate samples to evaluate variation in the system and were reported as < 10%, considered acceptable variation for these metabolomics platforms [23]. Mass spectra from each of three metabolomics platforms were converted to the .cdf file format and processed and annotated using the workflows described in [7] (GC) and [22] (LC). Metabolite quantities were normalized to total ion current and relative abundance for each molecular feature was determined by the mean area of the chromatographic peaks among replicate injections (n = 2). Volatile metabolites were identified by spectral matching in RamSearch software [24] to an in-house database of ~1,500 compounds, MSFinder software (as a validation tool for LC annotations (v3.26, RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa, Japan) [25, 26], and to external and theoretical databases including NIST v14 (http://www.nist.gov), Metlin [27], Golm Metabolome Database [28], Human Metabolome Database (HMDB) [29], and FooDB [30]. Chemical ontologies were established using HMDB and the ClassyFire package in R [31].

Statistical analyses

Sensory

In order to analyze data from the sensory panel, Principal Component Analysis (PCA) was conducted on the quantitative scores for each of the 14 sensory traits using unit variance (UV) scaling (SIMCA software v15, Sartorius Stedim Biotech, Umea, Sweden) [32]. The quantitative scores were the mean of n=11 participants were collected for each sensory attribute. Quality control on the sensory panel was performed by compared PCA distributions of the UV-scaled raw quantitative scores (within attribute) to participant-scaled scores (UV-scaled within participant), but no differences were observed and therefore raw (non-normalized) values were used for the remainder of the study. Quality control was also performed on each panelist using PCA to identify outlier observations, and none were identified nor removed.

Metabolomics

Volatile and non-volatile metabolite abundances were compared using analysis of variance (ANOVA) via the *aov* function in the R statistical environment v4.0 [33], with "malt source" as the major factor, and false discovery rate adjustment was performed on the ANOVA *p*-values using the Benjamini-Hochberg algorithm [34], for n=2 injections per malt source. Principal Components Analysis (PCA) and Orthogonal Projection to Latent Structures (OPLS), performed in SIMCA software, was used to assess and separate systematic variation based on linearity and orthogonality. For metabolite analysis, PCA was conducted on 1,026 annotated metabolites following unit variance (UV) scaling. OPLS was conducted by regressing the sensory PC score (*y*; PC1 for *grassy/cereal* analysis; PC2 for *dough* analysis) against UV-scaled metabolite data (*x*). Predictive power (Q^2) of the two OPLS models was determined via cross-validation, by which the data was divided into seven parts and 1/7th of the data was removed,

and the model was built on the remaining 6/7th of data remaining, and the removed 1/7th of data are predicted from the model.

Results

Sensory analysis revealed variation among the hot steep samples, a normal distribution of attributes, and a trend among grassy/cereal traits

A sensory analysis was performed on the 12 hot steeps to understand if the pale malt extracts vary for aroma, and if so, which attributes were the largest contributors to the variation. The analysis was comprised of forming a custom lexicon (resulting in 14 aroma traits), training a panel, and quantifying each of the 14 traits. PCA was conducted on the aroma scores and supports differences in the overall sensory profiles of the 12 pale malts (Figure 3.1) [35]. The first two PCs explained 70.2% of the variation and separated each of the 12 malt hot steeps without any clear trend or groupings, for example by malt type (e.g. pale, pilsner) (Figure 3.1A). Neither PC1 nor PC2 exhibited large separation based on maltster (A-D), although Maltster E did cluster along PC1. The PCA loadings plot did display two major trends of sensory: *cereal* and *grassy* traits (PC1) and *dough* traits (PC2) (Figure 3.1B). Importantly, the *cereal* and *grassy* traits can be considered as negatively correlated to each other based on the loadings data for PC1, and non-correlated to *dough* traits.

Metabolomics revealed chemical variation among pale malt hot steeps

Metabolite variation of the hot steeps was determined using a non-targeted metabolomics workflow, and profiles were established using three MS platforms: non-volatile metabolites via UHPLC-MS and GC-MS, volatile metabolites via HS/SPME-GC-MS. The HS/SPME-GC-MS, UHLPC-MS, and GC-MS detected 868, 1,771, and 1,020, compounds,

respectively, of which were categorized and annotated as 452 volatile and 574 non-volatile metabolites [Table S.8]. The metabolome of the hot steep was comprised of 9 different chemical classes, including organooxygen compounds (aldehydes, ketones, carbohydrates), organonitrogen compounds (amines, amides), organoheterocyclic compounds (MRPs, indoles), organic acids (amino acids, carboxylic acids), lipids (terpenoid lipids, fatty acid esters), alkaloids, alkanes/alkenes, benzenoid compounds (phenolics, phenylpropanoids), and organosulfur compounds. (Figure 3.2A, 3.2B).

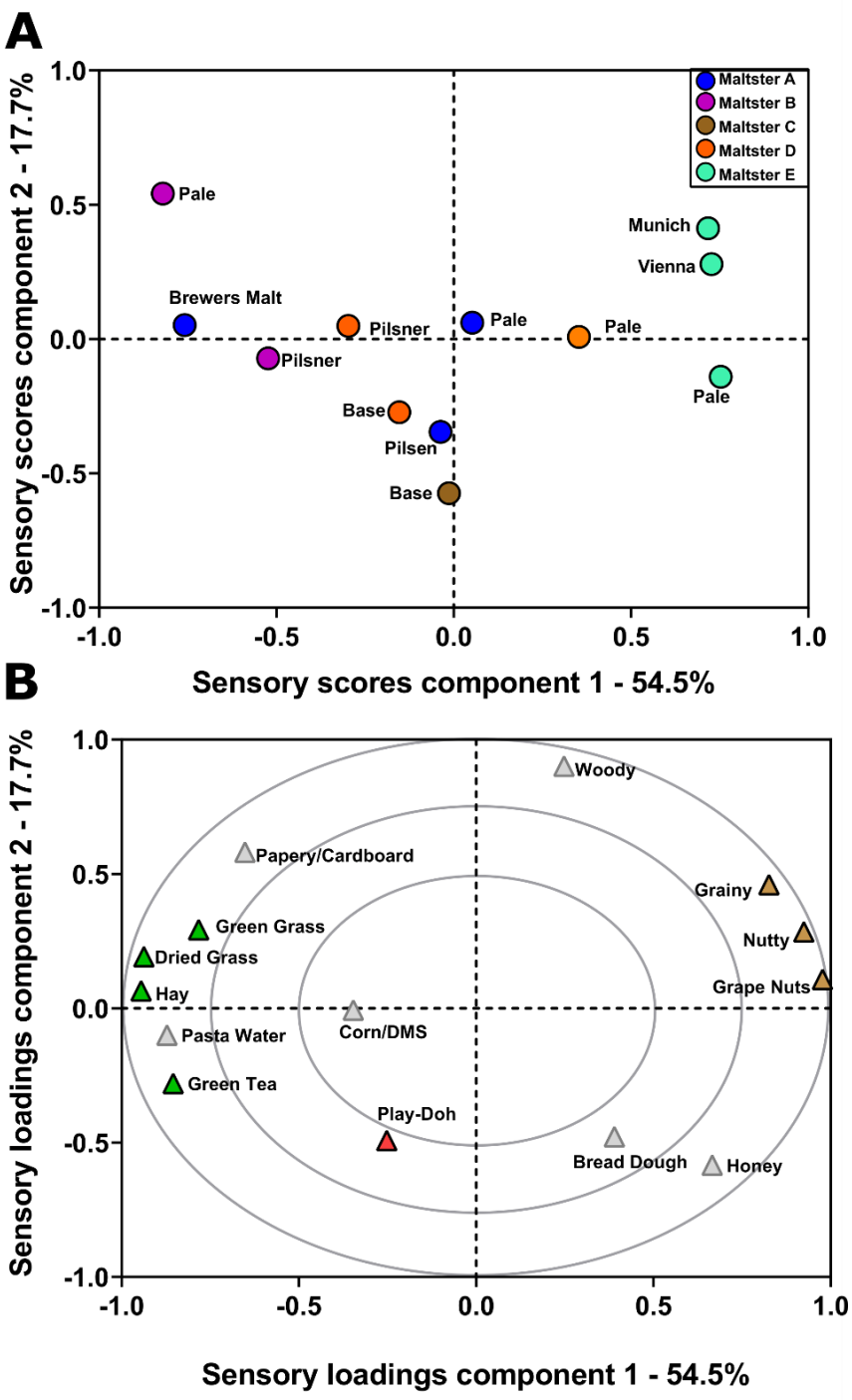

Figure 3.1 Principal component analysis (PCA) of sensory data from the 12 hot steeps. PCA was performed on data for 14 sensory traits quantified for the 12 malt hot steeps. (A) Correlation-scaled PC scores plot based on panel consensus data with (B) corresponding correlation-scaled loadings plot. Loadings were colored according to broad sensory trait as grassy (green), cereal (brown), dough (red), and all others (gray). Both scores and loadings were correlation scaled.

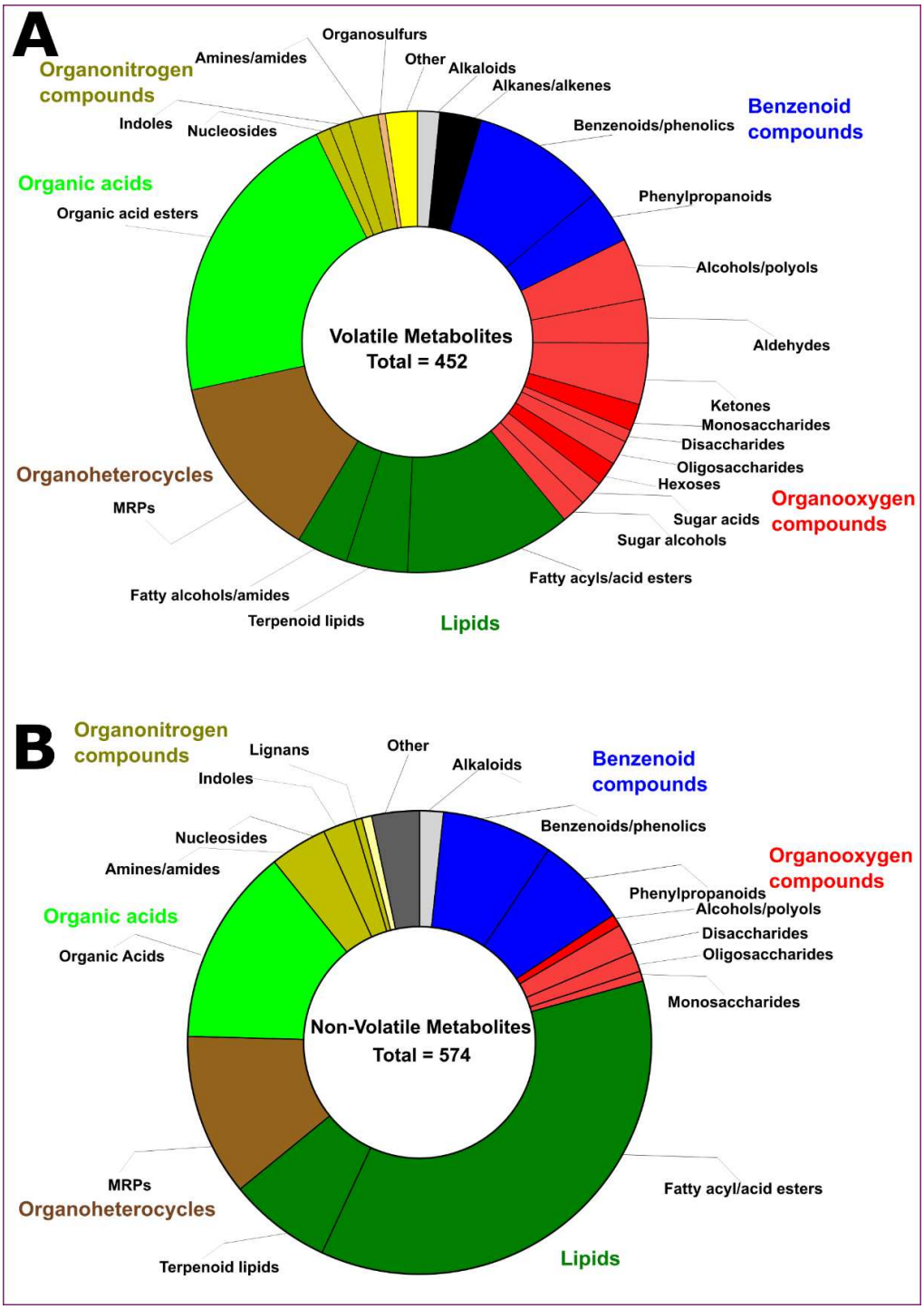

Figure 3.2 Annotated hot steep metabolites and corresponding chemical class. A total of 1,026 metabolites were annotated in hot steep extracts and pie charts were developed for (A) 452 volatile compounds and (B) 574 non-volatile compounds, respectively. Pie charts display metabolites by broad chemical class (black text).

Analysis of variance (ANOVA) revealed 162 of the 1,026 metabolites (15.7%) varied among the five malt sources (Table S.8; FDR adjusted $p \leq 0.05$).

Principal Component Analysis (PCA) was performed to evaluate metabolite variation. Among the 12 hot steep samples, PCA of the 1,026 annotated hot steep metabolites generated 4 PCs that explained 71% of the variation (Figures 3.3A, 3.3B). PC1, PC2 and PC3 explained 28.0%, 20.8%, and 16.2% of the variation (respectively). Further, the PCA was evaluated for trend attributed to "maltster." The data revealed that for the 5 maltsters, there was only a slight "maltster effect," whereupon Maltster E and Maltster A were more tightly clustered, indicating similarity within the two maltsters (Figure 3.3B). However, it should be noted that the malts of Maltster E and the Pilsner of Maltster D are driving much of the variation in the system. The PC1 and PC2 loadings (Figure 3.3C) indicated some trends in metabolite classes associated with variation among the hot steeps, again with Maltster E and the Pilsner of Maltster D responsible for most of the variation seen in the system. Specifically, lipids (non-volatile fatty acids/acyls) were generally more abundant in the pale malts of Maltsters A, B, and D, as well as in the base malt of Maltster D and the pale malt of Maltster A (Figure 3.3C, purple squares), but notably lower in the malts of Maltster E. Organic acids were more abundant in the base malt of Maltster C, the malts of Maltster E, and the pilsner malts of Maltsters B and D (Figure 3.3C, green circles). Other metabolite classes, volatile and non-volatile, were generally equally distributed in the loadings plot, indicating that none of the malts exhibited a major trend in being higher or lower in any one chemical class.

Several hot steep metabolites co-varied with cereal, grassy, and dough aroma

To investigate relationships between the hot steep metabolites and each of the 14 sensory attributes, an OPLS model was developed for two sensory principal components (correlation-

scaled PC1 scores for the *cereal/grassy trend;* correlation-scaled PC2 scores for *dough)*. The OPLS algorithms for *cereal/grassy,* and *dough* resulted in predictive and orthogonal components that explained 89.6% ($Q^2 = 0.68$) and 44.2% ($Q^2 = 0.24$) of the variation, respectively. (Table 3.2, Figure 3.4).

OPLS correlation-scaled loadings thresholds were used to determine if a metabolite was associated with the *cereal* or *grassy* sensory attribute. Metabolites were associated to the *cereal* attribute if the Component 1 (predictive) loadings thresholds were greater than 0.75, and less than 0.25 for the orthogonal component. Metabolites associated with *grassy* were given loadings thresholds of (< 0.50 / < 0.25; predictive/orthogonal). In this case, g*rassy* metabolites *were* allowed a more relaxed threshold based on interpretation of the biplot, which was skewed towards *cereal* traits and with the potential to mask the *grassy* trend (Figure 3.4, as uneven loadings distribution across the predictive component). A total of 70 volatile and 17 non-volatile metabolites were associated with cereal/grassy (Tables 3.2-3.4 and Figure 3.4); Associated with PC2 (dough), were 20 volatile compounds and 48 non-volatile compounds (Tables 3.3 and 3.4,). The OPLS data support a relationship between the metabolites detected in this study and the grassy/cereal trend, however *dough* model fit was poor and no clear associations between metabolites and dough aroma could be established.

The OPLS scores plot displays the same trends seen in PCA (Figure 3.4), indicating the three malts of Maltster E are abundant in compounds associated with MRPs and conversely, the two malts of Maltster B are abundant in esters which lead to the formation of *grassy* attributes. Importantly, when overlaying chemical class information with sensory traits, flavor-related trends were observed. The cereal aromas of *grainy, nutty, grape nuts,* and *honey* co-varied with

many MRPs (Figure 3.4). The *grassy* (*green grass, hay, green tea, dried grass*) co-varied with a set of lipids, various organic acids, and phenolic/benzenoid compounds (Figure 3.4).

Table 3.2 R^2 and Q^2 of principal components relating to dominant sensory attributes

Component	Observations	Threshold Used	R^2	Q^2	Volatile metabolites	Non-Volatile metabolites	Metabolites below the threshold
Component 1 (*Cereal; Grassy*)	12	corr >0.75;orthogonal corr < 0.25	0.89	0.68	50;5	14;2	963;1,1019
Component 2 (*Play-doh/Dough*)	12	>0.75	0.44	0.24	20	48	958

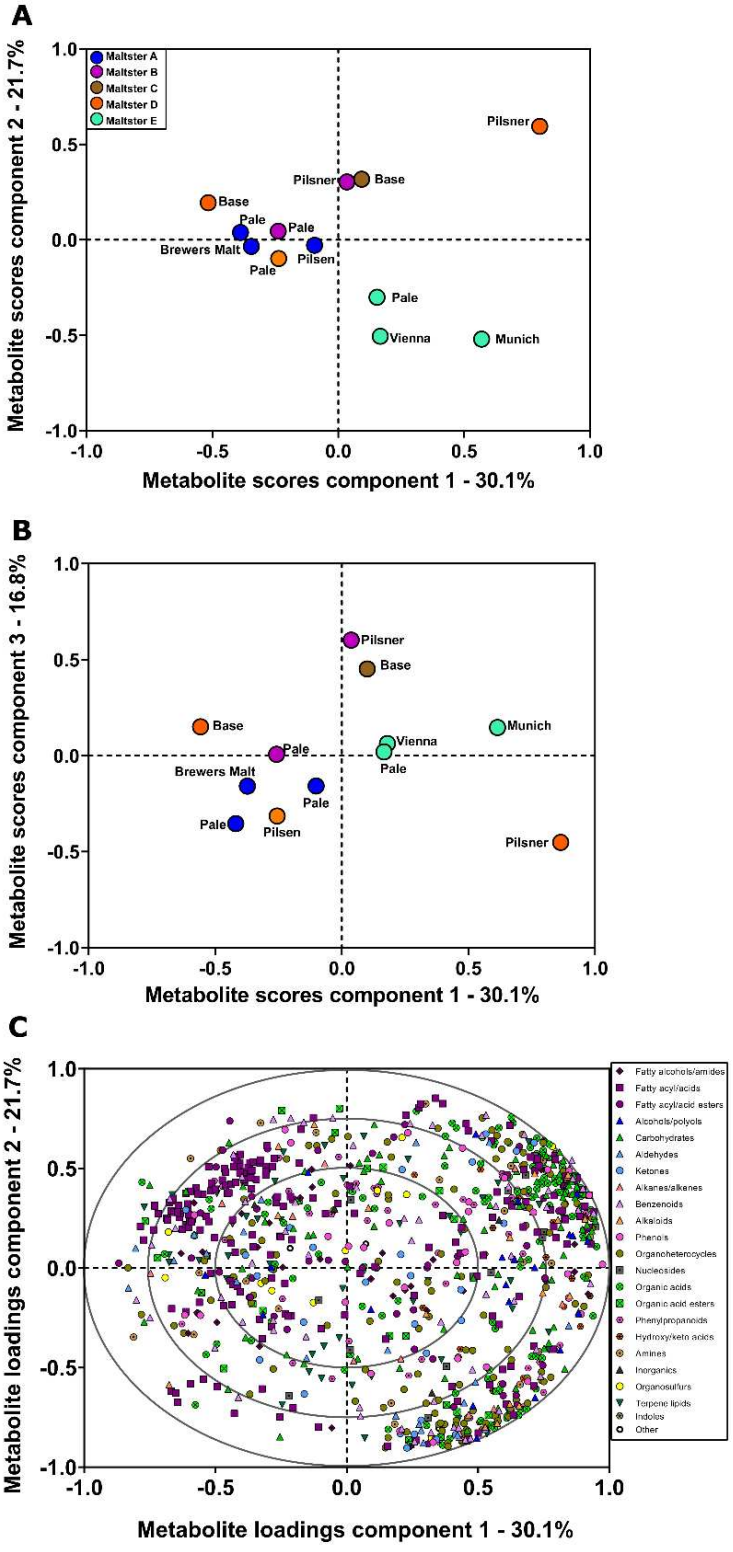
A
Maltster A
Maltster B
Maltster C
Maltster D
Maltster E
Pilsner
Pilsner
Base
Base
Pale
Pale
Brewers Malt
Pilsen
Pale
Pale
Vienna
Munich
Metabolite scores component 2 - 21.7%
Metabolite scores component 1 - 30.1%
B
Pilsner
Base
Base
Munich
Vienna
Pale
Pale
Brewers Malt
Pale
Pale
Pilsen
Pilsner
Metabolite scores component 3 - 16.8%
Metabolite scores component 1 - 30.1%
C
Fatty alcohols/amides
Fatty acyl/acids
Fatty acyl/acid esters
Alcohols/polyols
Carbohydrates
Aldehydes
Ketones
Alkanes/alkenes
Benzenoids
Alkaloids
Phenols
Organoheterocycles
Nucleosides
Organic acids
Organic acid esters
Phenylpropanoids
Hydroxy/keto acids
Amines
Inorganics
Organosulfurs
Terpene lipids
Indoles
Other
Metabolite loadings component 2 - 21.7%
Metabolite loadings component 1 - 30.1%

Figure 3.3 Principal component analysis (PCA) of metabolites data from the 12 hot steeps. PCA was performed on the 1,026 annotated metabolites from the 12 hot steeps. PCAs were generated for (A) PC1 and PC2 scores plot, (B) PC1 and PC3 scores plot, and (C) correlation scaled loadings. Loadings were colored according to chemical class. Analyses were based on n =2 extraction replicates per genotype that were pooled via mean averaging prior to PCA.

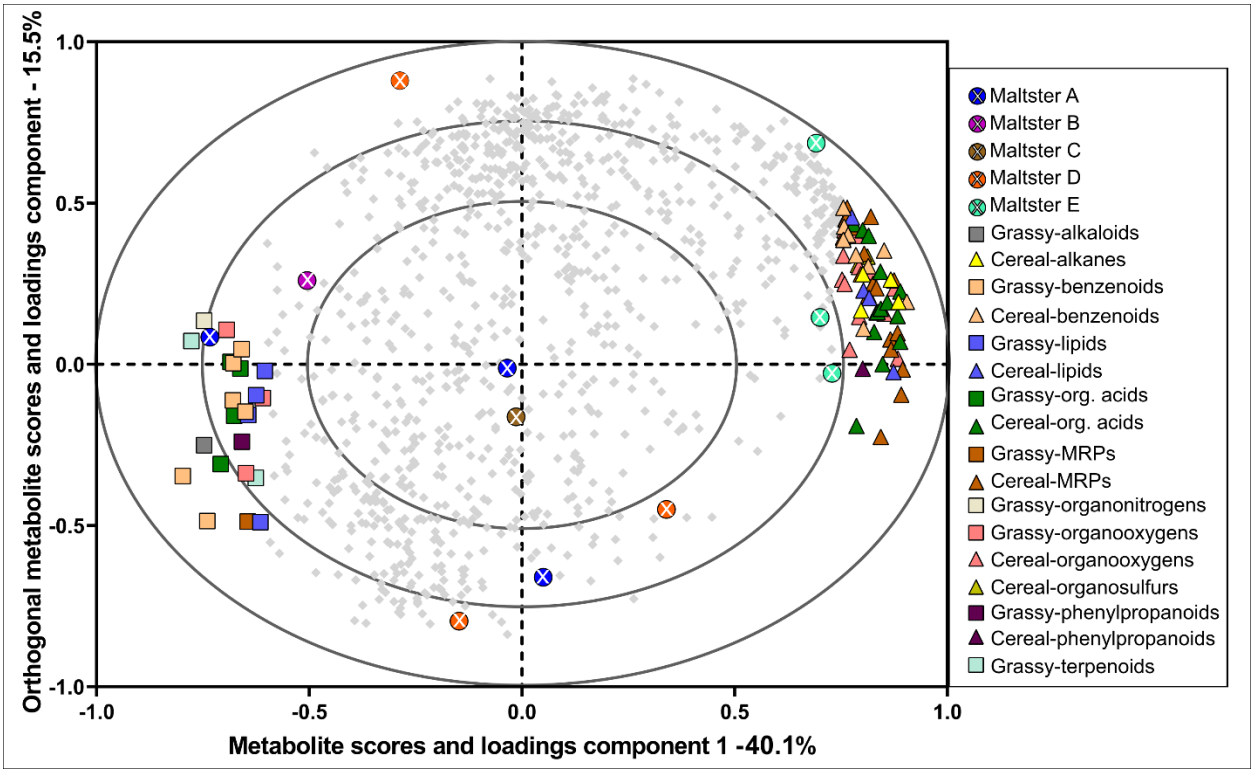

Figure 3.4 Orthogonal projection to latent structures analysis (OPLS) of metabolites and the grassy/cereal sensory trend. OPLS was conducted on the 12 malts for x = 1,026 metabolites and y = correlation-scaled PC1 scores from sensory analysis. Data is plotted as a biplot for correlation scaled OPLS scores (circles colored as per maltster; samples) and loadings (squares for grassy loadings; squares for cereal loadings; grey diamonds for metabolites which did not meet the threshold of loading corr). Loadings were colored according to chemical class if they met the threshold of loading corr > 0.75 and orthogonal corr < 0.50 and orthogonal corr < 0.25.

The *grassy* traits were also associated with decreased abundance of the MRPs. Organic acids, generally, were equally distributed among the malts and therefore were not generally associated with a type or specific sensory trait.

The data was evaluated to determine if trends of metabolite classes could distinguish each sensory trend. Across chemical classes, MRPs were associated with *cereal* descriptors (Table

3.3) and malt lipids and benzenoids were associated with *grassy* descriptors (Table 3.4), whereas more organic acids were associated with *cereal* than with *grassy.* Among sensory trends, *cereal* aroma was associated with maltoxazine (LC-354; likely no associated aroma, but marker for/has a role in beer aging), pyrazines, such as 3,5-diethyl-2-methylpyrazine (SPME-157; *meaty, nutty)*, 2-furoic acid (LC-170; *caramellic)*, methional (SPME-202; *umami, brothy*), and 2-hydroxy-5-methyl-3-hexanone (SPME-212; *chocolate*). *Grassy* aroma was associated with many lipids including esters, glycerolipids, and lipid amides, 5-ethyl-4-octanone (SPME-94; *fresh, herbal, sweet, vegetable*), menthone (SPME-242; *mint)*, and 2-ethylhexanal (SPME-189; *grass, green, herbal, fruity*) (Table 3.4, Table S.8). *Dough* aroma was associated with many lipids including glycerolipids, 2-oxo isovaleric acid *(*GC-110; *sweat, cheese, pungent),* 3-methylbutanal (SPME-197; *aldehydic, malty, peach, sour),* 3-nonen-1-ol *(*SPME-89*; fresh, wax, grease, cilantro)*, 2-methylpentanoic acid-like *(*SPME-75*; cheese, sour)*, 2-acetylthiazole *(*SPME-208*; nut, toasted corn chip, bread)*, and 1-pyrroline-2-carboxylate *(*GC-337; *shrimp, seafood).*

Discussion

This study found that in a population of 12 base malts from 5 commercial maltsters, (all with similarly requested malting quality parameters), variation in the hot steep sensory and hot steep metabolites was observed. While metabolite variation was seemingly minor (17.8% differed by ANOVA) when compared to previous malt metabolomics reports [22, 36, 37] , with ANOVA variations of >50%, this still conferred variation in sensory attributes of the hot steep. The relatively small variation in the metabolome also validates the overall experimental design of this study; all malts were adequate for use in a commercial setting.

The malt metabolomics data reported herein describes 452 volatile and 574 non-volatile metabolites detected in the hot steep extracts, many with known effects on fermentation and flavor including lipids, fatty acid esters, amines, phenolics, and organic acids.

Prior to the development of the hot steep malt sensory method, the "Congress mash" was used for sensory evaluation of malt samples [29]. The hot steep malt sensory evaluation method is now utilized as a method slated to improve quality of malt, malt sensory, and beer sensory as a result of utilizing these malts [5,6]. The predictive ability of this method, though much more rapid than brewing entire batches of beer, has yet to be fully analyzed and understood. In this study, we were able to identify relationships of hot steep malt sensory with chemistry. However, determining if relationships are predictive will require further experiments. The hot steep method, applied to assessing malt quality, could be a highly effective tool, as some sensory attributes are known to be correlated with imperfections in barley or in malting process [38].

In this study, we binned individual sensory attributes (e.g. *green tea, green grass*) into a single sensory trend characterized as c*ereal/grassy and* quantified this trend among malts using PCA. The reason for this stems from the issue of multicollinearity (correlation between variables, in this case, between the sensory attributes) and the confounding nature of the highly-correlated sensory attributes. Using the OPLS model with each individual sensory attribute would have resulted in a poor estimation of each individual sensory attribute.

Table 3.3 Metabolites highly associated with the *cereal* attributes in the malt hot steep.

Study Identifier	Class	Subclass	Metabolite	Flavor[a]	Correlation-scaled loadings[b]	Pvalue[c]
GC-7	Alkanes	alkanes	heneicosan-1-ol, n-	NR	0.78	< 0.01
GC-10			tetradecane	NR	0.83	< 0.01
GC-12			tricosane	NR	0.76	0.02
SPME-7			3-methoxy-pentane	alkane	0.83	0.05
SPME-15	benzenoids	benzaldehydes	benzaldehyde	almond, bitter, burnt sugar, cherry, sweet	0.79	0.10
SPME-40		phenols	2-methoxyphenol isomer 1	medicine, phenolic, smoke, spicy, sweet, vanilla, woody	0.81	0.02
SPME-16		phenylacetaldehydes	2-phenyl-4-pentenal	floral, fruity	0.87	0.02
SPME-18			3-methyl-2-phenylbutanal	bitter, fruity, green	0.89	0.02
SPME-17			4-methyl-2-phenyl-2-pentenal	cocoa, nutty, rose, sweet	0.82	0.03
SPME-24			5-methyl-2-phenyl-2-hexenal-like	aldehydic, bitter, butyric, chocolate, cocoa, fruity, green, nutty, sweet	0.89	0.02
SPME-23			5-methyl-2-phenyl-2-hexenal-like	aldehydic, bitter, butyric, chocolate, cocoa, fruity, green, nutty, sweet	0.85	0.03
LC-122	carbohydrates	cyanogenic glycosides	lotaustralin	NR	0.75	0.14
GC-104	Lipids	fatty acyl/acid esters	nonadecanoic acid methyl ester	NR	0.80	0.02
GC-105			octadecatrienoic acid methylester, 9,12,15-	NR	0.82	0.01
SPME-88		fatty alcohols	3-hexen-1-ol	fresh, green, leafy, moss	0.87	0.04
LC-226		long chain fatty acids	(-)-aerocyanidin	NR	0.77	0.03
SPME-122	organic acids	carboxylic acid esters	2-aminocaprylic acid	NR	0.79	0.00
SPME-104			2-mercaptopropanoic acid	meaty, roasted, sulfurous	0.85	0.02
SPME-107			acetic acid, 1,1-dimethylethyl ester	sour, tart, vinegar	0.83	0.05
SPME-111			acetic acid, phenyl ester	sour, tart, vinegar	0.86	0.03
SPME-113			prenyl isobutyrate	berry, butter, fruity	0.79	0.05
LC-187		carboxylic acids	2-[7]acetic acid	NR	0.80	0.04
LC-74			3-(1h-imidazol-5-ylmethyl)-6-(1h-indol-3-ylmethyl)piperazine-2,5-quinone	NR	0.76	0.01
LC-76			azidamfenicol	NR	0.78	0.02

Study Identifier	Class	Subclass	Metabolite	Flavor[a]	Correlation-scaled loadings[b]	Pvalue[c]
GC-207			glycylglycine	NR	0.76	0.01
GC-213			homoserine lactone, n-octanoyl-	NR	0.76	0.01
LC-437			l-tyrosine	NR	0.80	0.07
LC-304			n-acetyl-l-phenylalanine	NR	0.79	0.05
GC-255		keto acids	butanoic acid, 2-oxo-	NR	0.81	0.02
GC-260			pyruvic acid, 4-hydroxyphenyl-	NR	0.80	0.02
SPME-128	Organoheterocycles	benzodiazines	3-methylcinnoline	NR	0.87	0.02
GC-269		diazines	cytosine, 5-methyl-	NR	0.87	0.02
SPME-140		furans	2,5-diethyltetrahydrofuran	NR	0.90	0.02
SPME-139		furans	2-furanmethanol	alcoholic, bitter, bread, burnt, caramel, coffee, musty, sweet	0.84	0.02
LC-170		furans	2-furoic acid	NR	0.80	0.01
LC-268		piperazines	2-(benzenesulfonyl)-3-[2-(4-methyl-1-piperazinyl)-4-oxo-3-pyrido[1,2-a]pyrimidinyl]-2-propenenitrile	NR	0.79	0.03
SPME-147		piperazines	piperazine	ammonia	0.80	0.03
SPME-155		pyrazines	2-isopentyl-3,6-dimethyl pyrazine	fruity	0.82	0.05
SPME-154			2-methoxy-5-methylpyrazine	NR	0.76	0.05
SPME-157			3,5-diethyl-2-methylpyrazine	baked, meaty, nutty, vegetable	0.77	0.05
SPME-158			3-ethyl-2,5-dimethylpyrazine	cocoa, nutty, potato, roasted	0.80	0.07
SPME-12		pyridines	4-vinylpyridine	tea	0.88	0.01
LC-347			ciclopirox	NR	0.75	0.02
GC-290			pyridine, 4-hydroxy-	NR	0.81	0.02
GC-294		pyrroles	pyrrole-2-carboxylic acid	NR	0.78	0.01
LC-354		pyrrolidines	maltoxazine	NR	0.76	0.03
LC-388		tetrahydroisoquinolines	salsoline-1-carboxylate	NR	0.76	0.02
SPME-171	organooxygen compounds	acetals	3-ethyl-2,5-dimethyl-3-hexene	earthy, green, mushroom, violet	0.84	0.01
SPME-202			methional diethyl acetal	cabbage	0.84	0.04
GC-308		alcohols	pantothenic acid	NR	0.90	0.01

Study Identifier	Class	Subclass	Metabolite	Flavor[a]	Correlation-scaled loadings[b]	Pvalue[c]
SPME-196		aldehydes	2-methylbutanal	almond, cocoa, coffee, musty, nutty	0.88	0.02
SPME-197			3-methylbutanal	aldehydic, chocolate, ethereal, malty, peach, sour	0.89	0.02
GC-88			5-hydroxymethylfurfural	NR	0.89	0.03
SPME-198			fur-2-aldehyde	NR	0.88	0.03
LC-68		alkyl-phenylketones	hydroxykynurenine	NR	0.76	0.02
LC-295		benzoquinones	quinone-like	NR	0.76	0.03
SPME-97		enals	(e)-2-methyl-2-butenal	fruity, green	0.79	0.01
SPME-209		ketones	2-butanone	ether	0.88	0.01
SPME-216			3-nonanone	fresh, fruity, herbal, jasmine, leafy, spicy, sweet	0.80	0.05
SPME-207			5-methyl-2-hexanone	NR	0.85	0.02
SPME-225			pyrrol-2-methylketone	bread, nut, walnut	0.87	0.02
SPME-183	organosulfur compounds	thioethers	2-(Methylthio)ethanol	meaty, sulfurous	0.84	0.04
SPME-232			dimethyl trisulfide	sulfur	0.81	< 0.01
LC-160	Phenylpropanoids	flavanones	flavone-like	NR	0.83	0.02

a=Predicted flavor attribute based on information in FooDb; NR=No flavor information reported. [30]
b= Correlation-scaled loadings examine the strength and direction of the relationship between the metabolite(s) and the sensory component (*cereal*), metabolites shown are those which met the threshold for this analysis, |< 0.75|.
c= From ANOVA supporting variation among the n = 12 malts

Table 3.4 Metabolites correlated with the *grassy* attributes in the malt hot steep (orthogonal corr < 0.50)

Study Identifier	Class	Subclass	Metabolite	Flavor[a]	Correlation-scaled loadings[b]	Pvalue[c]
SPME-2	alkaloids	alkaloids	quinine-like	bitter	-0.62	0.13
SPME-19	benzenoids	benzenoic acid esters	4-ethylbenzoic acid, cyclopentyl ester	NR	-0.68	0.13
SPME-29			styrene	NR	-0.68	< 0.01
SPME-31			vanillic acid	vanilla, sweet	-0.66	0.14

Study Identifier	Class	Subclass	Metabolite	Flavor[a]	Correlation-scaled loadings[b]	Pvalue[c]
SPME-32			salicylic acid	NR	-0.60	0.30
SPME-42			3,4-dimethoxyphenol	NR	-0.63	0.02
SPME-37		phenols	3-hydroxymandelic acid, ethyl ester	NR	-0.65	0.08
SPME-59	Lipids	fatty acid esters	eicosanoic acid	NR	-0.71	0.14
SPME-64			methyl hexadecanoate	fatty, oily, waxy	-0.75	0.02
LC-224		fatty acyls	gamma-linolenic acid	NR	-0.78	0.11
SPME-55	organic acids	carboxylic acid esters	2-aminobutanoic acid	NR	-0.65	0.05
SPME-123			guanidinoglutaric acid	NR	-0.64	0.15
SPME-120			phosphonoacetic acid	NR	-0.66	0.14
SPME-126		keto acid esters	butyl levulinate	bitter, caramel, fruity, herbal	-0.66	0.01
SPME-150	organoheterocycles	purines	hypoxanthine	tea	-0.69	0.16
LC-263	organonitrogen compounds	amines	palmitoleoyl ethanolamide	NR	-0.61	0.12
SPME-181	organooxygen compounds	alcohols	diethyl carbitol	NR	-0.75	0.35
SPME-189		aldehydes	2-ethylhexanal	aldehydic, fatty, fresh, fruity, grass, green, leafy, sweaty	-0.64	< 0.01
SPME-94		carbohydrates	(±)-5-hydroxy-4-octanone	NR	-0.80	0.04
SPME-46		phenolic glycosides	phlorin	fruity, lemon, orange	-0.68	0.09
SPME-234	phenylpropanoids	chalcones	2-hydroxychalcone	NR	-0.68	0.02
SPME-242	prenol lipids	monoterpenoids	l-menthone	bitter, camphor, herbal, menthol, peppermint, raspberry	-0.74	0.02

a=Predicted flavor attribute based on information in FooDb; NR=No flavor information reported. [30]
b= Correlation-scaled loadings examine the strength and direction of the relationship between the metabolite(s) and the sensory component (*cereal*), metabolites shown are those which met the thresholds for this analysis, corr $|< 0.50|$ and $|< 0.25$.
c= From ANOVA supporting variation among the n = 12 malts

After it was determined that the attributes did, in fact, relate and display a pattern through PCA (e.g. *grassy, hay, green tea* were all determined to be a part of *grassy*), these attributes were reduced from fourteen to three. This was an effective method to reduce the data and find associations to the trends instead of attempting to determine which sensory attribute (among the other highly-correlated attributes) was responsible for inference or prediction [39]. It is in this way that two distinct flavor attributes/classes were evaluated in detail for associations between malt (hot steep) chemistry and effects or relation to flavor, *cereal* and *grassy.*

The malts, themselves, as was noted in the relatively low number of significant compounds (ANOVA; Table S.8), are similar enough (as they all meet specific AMBA malting requirements) to produce desirable and consistent results, yet different enough to elicit sensory responses which allowed us to see variation through sensory and chemistry. The two chemically and sensorially diverse attributes (*cereal* and *grassy*) were assessed to encompass desirable traits of malt. The hot steep data included many metabolites with known effects on fermentation and flavor, including fatty acid esters, amines, phenols, and MRPs which are known contributors to flavor.

MRPs play a major role in malt and beer flavor, and form during malting, brewing, fermentation, and during beer storage and the effect of the malting process on barley cannot be understated [40-42]. MRPs create flavor combinations, can mask lighter aromas (such as *fresh, clean, citrus* flavors), can potentially have a negative effect on yeast growth, and have the potential to inhibit enzymatic pathways involved in flavor compounds (aldehyde and pyruvate) [8]. Therefore, understanding of the chemical composition (saccharides and amino acids) of barley during malting regime in order to create or inhibit flavors is critical in understanding the composition of malt. As fructose and sucrose (monosaccharides) are abundant in barley, glucose

maltose, and maltotriose (di-, tri-saccharides) are only released by amylolytic activity during mashing (brewing) [8]. Understanding the total composition of the nitrogen and amino acid profile is essential in the creation of consistent or novel flavors and flavor combinations when malting or brewing. It is also important when considering free amino nitrogen (FAN), of which 70% is produced during malting [43]. Amino acids and other nitrogenous compounds are present in both malt and barley at varying abundances, and the subsequent stages of the Maillard Reaction involve the interaction of oxygenation compounds with reactive components (amines, amino acids), leading to flavor compounds such as furans, pyrazines, pyrroles, oxazoles, thiophenes, thiazoles, and other heterocycles [7]. The present study supports an association of MRPs to three hot steeps associated with the *cereal* sensory attributes (Table 3.3). These different malts had a diverse MRP profile, including maltoxazine, a marker in beer aging, pyrazines, hexanone, 2-furoic acid, and others which are attributed to the typical *roasted, toasted, coffee, nutty, caramel* flavors and aromas of MRPs. Considering the MRP pathways utilized to create the MRPs, it is, again, important to note that the total composition of amino acids in both barley and in malt is necessary to understand. Yeast, during fermentation, show preference for uptake of amino acids or FAN (which is the measure of the nitrogen compounds metabolized by yeast during fermentation) and this does not include proline [43, 44]. Proline, however, still contributes to the formation of MRPs, creating proline-specific products, which are potent odorants and bitter compounds, such as pyrrolidine (LC-309) and 1-pyrroline (GC-337), and 2-hydroxypyridine (GC-289) [45]. The “type” of amino acid involved in the reaction may play a larger role in flavor production, masking of lighter flavors, and potential for off-flavors during aging.

As stated in the results, two thresholds (< 0.25 and < 0.50) were utilized for *grassy* in order to express metabolites which did not meet the strict threshold of > 0.25. As the *cereal* threshold (> 0.75) explains that malts with higher amounts of metabolites (MRPs) are related to *cereal,* low amounts of these metabolites suggest that the malt trends toward *grassy.* For example, if the malt metabolites are reduced and particular metabolites are either low in abundance or in prevalence in a sample (MRPs), then the metabolites which are left would render the malt as more *grassy* due to the fact that those dominant compounds are lower (in abundance or prevalence). The *grassy* compounds in the model were responsible for driving the *grassy* trend if the dominant *cereal* traits are reduced (Table 3.4). Lipid and fatty acid profiles of genetically different barley varieties, as well as the relationship between barley quality and malt have been investigated recently [46]. There has been a negative correlation reported between total lipid content and quality of malt [47] which was also affected by climate and type of barley (winter, spring) grown. It was noted that the variety with lower polyunsaturated fats may be important in improving shelf life and subsequent beer quality. Longer-chain fatty acids (such as palmitic, stearic, oleic, linoleic, and linolenic) which are present in malt at higher levels will be present in wort, also affecting beer quality and stability [48]. Unsaturated linolenic and linoleic acids have been shown to contribute to beer oxidation and facilitate staling flavors during storage [49, 50].

In this study, six different malts were associated with *grassy* traits and had a profile of fatty acids/fatty acid esters, which is an important factor to consider when determining malt flavor and quality and its effect on beer flavor and quality (Figure 3.4). The abundance of free fatty acids and fatty acid esters, as well as the composition of free fatty acids in hot steeps play a definitive role in these factors and should be considered together with other metabolites when

considering and choosing different malts. The most abundant compounds in these different malts were long-chain fatty acids (such as linoleic and linolenic), 5-ethyl-4-octanone, hexadecenoic acid ester, menthone (a terpene lipid), and other glycerolipids, lipid amides, and esters (Table 3.4, Table S.8). The presence of these compounds in conjunction with the a low MRP/nitrogenous profile and many organic acids which contribute to *fresh, herbal,* and *grassy* flavors and aromas suggests not only that these compounds contribute to *grassy* attributes, but that these lipids and fatty acid esters (known for undesirable aromas such as *goaty* and *cheesy*) may also contribute to off flavors in aged malt and in beer. Butanoic acid (SPME-54) and ethyl hexanoate (*pineapple*), pentanoic acid (*red apple*, *aniseed*), and ethyl acetate (*fruity*, *tropical*, *pineapple*) are all associated with typically tropical or fruity aromas and flavors These profiles are important to note when considering the "type" of beer to be created, as these esters combined with the organic acid and benzenoid profile could lead to more competitive flavors in malt flavor. Lipids and fatty acid esters are important to malt (hot steeps) as it pertains to beer flavor, as they do contribute to *fruity*, *tropical fruit,* and *perfume-like* aroma character of beer, commonly reported to be produced by yeast during fermentation [51]. However, lipids and fatty acid esters, although only a portion of the non-volatile and volatile profile, can impart staling traits such as *cardboard, garbage,* and *papery.*

Regarding the *dough* attributes (Table 3.4), many lipids which lead to these staling traits, were present, including glycerolipids, 2-oxo isovaleric acid, and dodecanol (Table 3.4). When considering the hot steep as a method to evaluate malt flavor and consistency, these data suggest that the chemical composition of barley and malt are important when considering malting regime and how the malt is to be utilized, as novel flavors (*fruity, molasses, grassy*), or consistency are desired in the product, as beer is a highly complex beverage driven by chemical and mechanical

interactions (driven by malting and brewing processes) prone to leaving a brewer with undesirable results. A third sensory trait, *dough*, was characterized in the sensory analysis. However, while dough was clearly detected by panelists, no clear associations could be made for metabolites and dough. This may be because metabolites driving this attribute were not detected in our experiment, and therefore is an area for future work.

This study aimed to develop new, chemical targets to evaluate malt for brewing quality and flavor. An important outcome of this research was the goal of identifying target chemicals from the hot steeps that could be used to *predict malt quality* and *potential for flavor* using analytical chemistry techniques. These target chemicals were studied for their relationship (covariance) with sensory attributes and determined to answer the question "*Does what we perceive relate to the chemistry? Are these chemicals predictors of consistency, quality, and flavor of malt*?" The target chemicals that were evaluated for effect on flavor, specifically those that related to the broader categories of *cereal* and *grassy* are the result of Maillard Reactions and lipid oxidation reactions, respectively. Each different malt (from each different cultivar of barley) begins with differing amounts of lipids, amino acids, and saccharides, and what is created (malt) depends on the maltster's ability to bring those compounds to fruition through the process of malting, if they are able, given their initial abundances [6, 38, 52]. The association of lipids/fatty acid esters to *grassy* traits supports that these different malts may contain a larger lipid profile from the beginning, therefore prompting the abundance of fatty acid esters created in those malts (Figure 3.3B). The abundance of lipids associated with these different malts (of barley and subsequently malt) could be predictors of the quality of the malt, as lipid oxidation occurs rapidly [53] and the presence of certain types of lipids in malt influence yeast metabolism and beer quality [46].

Conclusions

The current results of this study support that differences in flavor profiles of malt hot steeps, assessed through rigorous sensory testing and metabolomics, are heavily influenced by maltster (which may relate to barley variety or region grown, since many of the barley varieties were the same). A total of 12 malts, malted by 5 different maltsters, were found to have distinct differences which contributed to the nuanced flavor profiles of each malt hot steep. Metabolomics characterized 1,026 compounds in hot steeps using three MS platforms. A trained sensory panel was able to describe and differentiate malts, mostly based on *cereal, grassy,* and *dough* attributes. The association analyses identified target compounds which were likely to be involved in the *cereal, grassy,* and *dough* malt flavor categories. These research findings support the value of sensory assessments of malt hot steeps when assessing the quality of malt, defect elimination, and potential for flavor development for craft malts. The measurements and procedures used in this research study have value in guiding decisions regarding malt selection but were not directly predictive of another. For example, *grassy* attributes in a hot steep are more likely indicative of fewer MRPs and not predictive of *grassy* flavor in a beer. Additionally, metabolomics can provide insights into the chemical basis of specific sensory descriptors. Distinct metabolomic profiles were detected within and between hot steeps, which were attributable to maltster. The new knowledge generated by this research can be capitalized upon by extending it to additional barley genotypes, differently crafted malts, different maltsters, malting regimens, and different beer styles.

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CHAPTER 4 – VARIATION IN SENSORY ATTRIBUTES AND VOLATILE COMPOUNDS IN LAGER BREWED FROM GENETICALLY DISTINCT MALTS: AN INTEGRATED SENSORY AND NON-TARGETED METABOLOMICS APPROACH[3]

Overview

Previous research demonstrates that barley genetics can influence beer flavor. However, the chemical basis for differences in beer flavor attributed to barley is not well defined. Here, the associations between beer volatile compounds and sensory descriptors were investigated in a controlled experiment, whereby barley genotype was the main driver of variation in the system. Beer was brewed from three advanced barley breeding lines and compared to a CDC Copeland control. Sensory studies were performed via three independent panels (a Consumer, Brewery, and Laboratory panel). The results suggest that the four beer samples have distinct flavor profiles that could be discriminated by the three sensory panels. Volatile compounds for the four beers were characterized using HS/SPME-GC-MS; quantitation and annotation were performed using a non-targeted metabolomics approach on 397 detected compounds. O2PLS data analysis supports that alkane/alkenes, benzenoids, amides/amines, and fatty acid esters were associated with the most desirable lager traits, compared to Maillard Reaction Products that were more abundant in the beers with "non-ideal" lager traits. Taken together, these data further support the role of barley genetics in beer flavor and provide new information on the types of volatile metabolites that can vary in controlled systems.

[3] This chapter is a modified form of the following article published in the Journal of the American Society of Brewing Chemists: Harmonie M. Bettenhausen, Amanda Benson, Scott Fisk, Dustin Herb, Javier Hernandez, Juyun Lim, Sue H. Queisser, Thomas H. Shellhammer, Veronica Vega, Linxing Yao, Adam L. Heuberger & Patrick M. Hayes (2020) Variation in Sensory Attributes and Volatile Compounds in Beers Brewed from Genetically Distinct Malts: An Integrated Sensory and Non-Targeted Metabolomics Approach, Journal of the American Society of Brewing Chemists, 78:2, 136-152, DOI: 10.1080/03610470.2019.1706037

Introduction

The potential for barley breeding to facilitate distinct beer flavor is a novel area of research. Considerable effort has been devoted to elucidating the genetic basis of malt chemistry traits [1]. Breeding schemes include making use of genetic variation that impacts the finished malt chemistry and quality, and this can be observed as quantitative trait loci (QTL) described for quantities of extract and protein [2, 3], protein composition (e.g. haze-related protein [4], glycosidases and sugar transporters [5]), and many other malt chemistry traits [6]. The relationship between barley genetic variation and malt chemistry may be through malt modification, another factor that has been demonstrated to affect beer flavor [7]. Importantly, a genetics approach was conducted whereby the barley cultivars Golden Promise and Full Pint were crossed, and their progeny evaluated for variation in beer sensory attributes, while malt modification was controlled. The study demonstrated that the variation in barley genetics can impact the flavor of beer [8].

The effects of barley genetics on beer flavor may be attributed to differences in the small molecule (*i.e.* metabolite) content of the malt. This can occur through two mechanisms: (*i*) differences in the genetics-mediated potential for biochemical/metabolic reactions to form metabolites during germination (*i.e.* seed metabolism and maturation of the embryo), and (*ii*) subsequent metabolite/metabolite interactions that occur during kilning, for example the heat-induced interactions of amino acids and saccharides that provide for Maillard reactions. A metabolomics study of the cultivar Maltasia showed how factors of the malting process influence malt amino acids, organic acids, and lipid metabolites [9]. Further, a comparative metabolomics study of 72 barley genotypes found a contribution of the barley genetics to quantities of malt amines, amino acids, alkaloids, phenolics, lipids [10]. While many of the flavor active metabolites in malt may be lost during wort boiling, several malt compounds are known to

directly transfer to the finished beer, for example maltols (sweet aromatic compounds) [11, 12] and hordatines (astringency compounds [13]). The effect of malt metabolite variation on beer chemical variation was described in a study of five malts and demonstrated effects on beer sensory through flavor stability pathways [14].

Several components of beer flavor can be attributed to malt metabolite chemistry. Defect elimination in beer flavor and enhanced beer flavor stability have been widely-studied in using the tools of malt metabolites research. Malt free amino nitrogen (FAN), for example, is comprised of amine and amino acids metabolites, and the total quantity of FAN is important for fermentation (via yeast nutrition) and flavor stability [15, 16]. Wort with high FAN tends to produce excess higher alcohols (fusel), esters, and aldehydes in beer, and these compounds usually are reported as undesirable flavors. Excess malt FAN can affect flavor stability through differential utilization of amino acids by yeast. Lysine is very rapidly utilized, and excess quantities affect fermentation rates such that excess vicinal-diketone (VDK) is produced in the beer and forms 2,3-butanedione and/or diacetyl (butterscotch, buttered popcorn) off-flavors [16]. Malt lipids and lipoxygenase enzymes may also impact paper/cardboard flavor in aged beer [17].

Desirable beer flavor can also be sourced from malt metabolites. The kilning step of malting produces Maillard Reaction Products (MRPs) resulting from the interaction of amino acids and saccharides in the germinated barley grain [18]. High molecular weight MRPs, which usually occur as complex heterocyclic compounds, provide color [19-21]. Small molecules, such as pyrazines, pyrroles, and furans, provide aromas such as roasted, nutty, and caramel [19, 22-24]. The type of MRP produced is affected by the composition of precursor amino acids and saccharides, and therefore it is hypothesized that the genetic and corresponding metabolite variation in the germinating grain will influence the flavor profile that develops after kilning.

Further, MRPs are known to affect the anti/pro-oxidant potential of the beer and are therefore likely to affect flavor stability during storage [25].

Malt may also affect beer flavor through indirect effects on ester synthesis during yeast fermentation. Beer ester types are classified as 'acetate' or 'ethyl'. Acetate esters are low molecular weight and highly volatile. Examples are ethyl acetate, isoamyl acetate, and phenyl ethyl acetate which are characterized by solvent, banana, and roses/honey aromas, respectively [26, 27]. Ethyl esters are medium molecular weight and contain a notable medium-chain fatty acid group, for example ethyl hexanoate, ethyl octanoate, and ethyl decanoate with anise/apple, sour apple, and floral aromas, respectively [27]). During brewing, the resulting alcohols can act as substrates to produce the many esters found in beer; approximately 60 have been detected, although not all are known to have organoleptic properties [28-32]. The effects of barley genetic variation, and the corresponding malt variation on the alcohol and subsequent aromatic ester production, are largely unknown.

The current study builds on the foundation established by Herb et al. (2017), in which sensory data from nano-brews prepared from micro-malts was used to document significant contribution and genotype and/or environment to specific beer flavor descriptors [7]. Herein, we investigated the effects of barley genotypes on sensory attributes and metabolite profiles of finished beer made using larger-scale malts and brews. Three doubled haploid (DH) barley breeding lines from the cross of Full Pint x Golden Promise were selected (based on agronomic performance, malting quality profile, and beer sensory attributes) from the larger set evaluated by Herb et al. (2017). These are referred to as "M341", "M285", and "M058" in this report. The malting quality data, beer analytical data, sensory data, and beer volatile metabolite profiles of the three DH lines were compared to those for the cultivar CDC Copeland. Copeland is a cultivar

that is widely-grown and used in the North American malting and brewing industries. This research tested the hypothesis that barley genetic variation can, through malt, affect desirable sensory attributes of lager beer, and that these phenotypes are associated with differences in the volatile profiles.

Experimental Procedures

Plant materials, malting, and malt quality

The three experimental breeding lines used in this study (AD120341, DH120058, and DH120285) were selected from the 200 doubled haploid progeny generated from the cross of the spring growth habit, 2-row, cultivars Golden Promise and Full Pint. For ease of reference, in this study the three lines are referred to as "M341", "M058", "M285", respectively. The spring growth habit, 2-row cultivar CDC Copeland was used in this study as a control. Grain of the three experimental selections was grown at Lebanon, Oregon USA and harvested in 2017. Grain of Copeland was sourced from Great Western Malting. All barley grain was malted at Oregon State University. The steeping regimes were adjusted for each of the four barleys to achieve a target steep-out moisture of 47%. Malt quality analysis was performed at the Hartwick College Center for Craft Food & Beverage (Oneonta, New York, U.S.A.) using methods of analysis set by the American Society for Brewing Chemists for α-amylase, β-glucan, color, diastatic power (DP), extract, FAN, soluble protein, total protein, and viscosity. The malt analyses are shown in Table 4.1.

Pilot scale brewing was performed at the Deschutes Brewery pilot plant (Bend, Oregon, U.S.A.) in January of 2018. Two batches (1.2 hL) of each beer were produced from the three DH lines and CDC Copeland. The research recipe, which was developed based on a preliminary

brew using Copeland malt, was intended to feature the malt, yet be a “drinkable” lager-type beer (not for sale purposes).

Table 4.1 Malt quality for the sources used in this study.

Line	Moisture	Friability	Extract	Color	β-glucan	SP	TP	S/T	FAN	DP	AA	Filtration	Clarity	pH
	%	%	%	°SRM	mg/L	%	%	%	mg/L	°L	DU	Time		
Copeland	4.5	90.3	82.1	1.54	74	4.84	10.8	44.8	195	146	58.6	normal	clear	5.86
M058	5.3	82.3	80.1	1.46	173	4.47	10.6	42.2	167	150	56.5	normal	clear	6.02
M341	5.7	68.7	81.4	1.55	727	4.11	10.6	38.8	154	124	46.5	normal	clear	5.98
M285	4.7	85	80.4	1.84	58	4.99	10.5	47.5	205	168	64.2	normal	clear	5.95

Color – based on Standard Reference Method (SRM); DP – diastatic power, based on Lintner units; AA – α- amylase, based on diastatic units (DU), 30 or above is required for proper conversion; TP – total protein, target of <14%; SP – soluble protein, based on dry basis; S/T – soluble/total ratio, a minimum of 30 is required to prevent lautering issues; FAN – free amino nitrogen, standard value is 180 ppm and above; Viscosity – typically 1.45 – 160 centipoise units (CPU); β-glucan – <180 indicates good lauterability, but this test only indicates the number of molecules found, not the molecular weight; Friability – indicator of lautering performance, >90% indicates good friability, <90% indicates under-modification (high viscosity polysaccharides such as β-glucan, leading to lautering difficulties); Moisture – optimum range for base malt is 3-6%; Extract – (as-is) high % is optimal; Clarity and Filtration Time are dependent upon multiple factors; pH – normal range between 5.0-5.7.

The final brewing parameters are provided in Supporting Information Section 1. Brewing quality analyses were performed at Deschutes Brewery (Table 4.2).

Sensory analysis of beer

Three sensory studies were performed by independent panels: herein referred to as (1) Consumer Panel, (2) Brewery Panel, and (3) Laboratory Panel. The complete methods and results of the three panels are described in detail in the Supporting Information Section 2. Briefly, the Consumer Panel – managed by the Center for Sensory and Consumer Behavior Research at Oregon State University - consisted of 101 participants asked to (*i*) rate "Overall Liking" of beer samples, (*ii*) choose appropriate characteristics from a list of 18 descriptors for a particular beer using the Check All That Apply (CATA) method, (iii) imagine an "ideal lager" beer and choose the appropriate descriptors utilizing the CATA method, and (i*v*) rank the top 3 qualities of an "ideal lager" beer. The Brewery Panel consisted of members of the Bells Brewery, Deschutes Brewery, Firestone-Walker Brewery, New Glarus Brewery, and Rahr Malting sensory panel (60 panelists), and participants were trained using foods that emulated the sensory attributes identified as important to evaluating malt flavor in beer.

Table 4.2 Beer quality.

Line	ABV	°P	SG	RE	AE	Beer pH	Color	RDF	IBU
Copeland	5.93 ± 0.16	11.55 ± 0.26	11.55 ± 0.26	2.46 ± 0.02	0.31 ± 0.03	4.94 ± 0.02	2.95 ± 0.15	79.73 ± 0.29	16.5 ± 0
M058	5.37 ± 0.04	10.91 ± 0.08	1 ± 0.08	2.65 ± 0.01	0.69 ± 0	4.59 ± 0.05	2.5 ± 0.3	76.74 ± 0.05	13.25 ± 0.75
M285	5.68 ± 0.19	11.01 ± 0.33	11.01 ± 0.33	2.26 ± 0.04	0.19 ± 0.03	4.63 ± 0.01	2.85 ± 0.15	80.4 ± 0.25	14.75 ± 1.25
M341	5.34 ± 0.14	11.13 ± 0.25	11.13 ± 0.25	2.94 ± 0.06	1 ± 0.02	4.8 ± 0.04	2.75 ± 0.05	74.71 ± 0.11	17.5 ± 1

From n = 2 batches of beer produced from each malt; each ABV – alcohol by volume; RDF – real degree of fermentation; IBU – international bittering units; °P – Degrees Plato, concentration of dissolved solids in wort to quantify the concentration of extract; SG – specific gravity; RE – real extract, based on attenuation of wort; AE – apparent extract, based on dissolved solids

The assessments of the beers were based on 16 sensory attributes (*e.g.* mouthfeel, aromas, described in Supporting Information Section 2). Each sample was compared with commercially available reference beers (Budweiser, Bud Light, Coors Banquet, Miller High Life) to characterize flavors and estimate variability within samples. The sensory assessments were coordinated by Deschutes Brewery. The Laboratory Panel consisted of 9 participants at Oregon State University, all of whom were trained on and performed the Directed Projective Mapping (or Napping®) sensory method [33, 34].

Detection of volatile metabolomics in beer and metabolomics data processing

Volatile metabolites in beer were detected using a non-targeted metabolomics approach via headspace solid-phase microextraction gas chromatography-mass spectrometry (HS/SPME-GC-MS). The workflow was performed as previously reported in [14], and the HS/SPME-GC-MS settings are described in the Supporting Information Section 3. Mass spectra from the HS/SPME-GC-MS analysis were converted to the .cdf file format, and peak detection, grouping, retention time alignment, and peak filling was performed using XCMS algorithms in the R statistical environment (v 3.5.1) [35]. The molecular features were deconvoluted into spectral clusters using the RAMclustR package in R [36]. The outputs of this data processing workflow resulted in a data set consisting of metabolites defined by (*i*) mass spectra, (*ii*) retention time, and

(*iii*) relative abundance and normalized to the total ion current as reported previously [36]. Metabolites were annotated by spectral matching in NIST MS Search software to an in-house database of ~1,500 compounds and to external and theoretical databases including NIST v14 (http://www.nist.gov) and the Human Metabolome Database [37].

Statistical analyses

Data from the three independent sensory panels were evaluated separately due to the differences in the procedures and scales employed. For the Consumer Panel CATA data, Cochran's Q test were conducted followed by McNemar's test. The scaled data (i.e. overall liking from Consumer Panel, intensity ratings from both the Brewery and Laboratory Panel) were evaluated by using analysis of variance (ANOVA). Data from the Brewery Panel were compared using two-way analysis of variance (ANOVA) via the *aov* function in the R statistical environment v. 3.5.1 [38]. Specifically, ANOVA was performed on the panel datasets (for each beer, independently) and it was determined there was variance among sensory attributes for each panel. A Two-Way ANOVA was performed on the Brewery Panel sensory data, using R via the *car* and *agricolae* packages [39, 40], for each brewery. The mean of the 16 sensory descriptors from the Brewery Panel were used as the response variables, beer, panelist and beer-panelist interactions as main effects and batches as replicates. To identify pairs of means which are statistically different, a Least Significant Difference test (LSD) was performed. The partitioning sources of variation, df, SS, and MS from ANOVA are provided in Table S.11. Principal Component Analysis (PCA) was conducted on each independent sensory dataset using SIMCA software v.15 (Sartorius Stedim Biotech, Umea, Sweden) [41]. The beer sensory attributes for the Consumer Panel (representing the hedonic "*overall liking*" score and unscaled sensory data) are presented in bar charts created in R using ggplots2 [42].

Volatile metabolite abundances were compared using ANOVA via the *aov* function in the R statistical environment v. 3.5.1 [38], and false discovery rate (FDR) adjustment was performed on the ANOVA *p*-values using the Benjamini-Hochberg algorithm [43]. Principal Components Analysis (PCA), and Two-way Orthogonal Partial Least Squares Analysis (O2PLS) were conducted on 397 annotated metabolites and sensory traits from each panel with SIMCA software v. 15 (Sartorius Stedim Biotech, Umea, Sweden) [41]. Respective sensory attributes of each independent sensory panel were integrated with the volatile metabolites for further multivariate analysis. Biplots of scores and loadings values from O2PLS models were conducted in SIMCA software on z-transformed data for sensory attributes (y) and annotated, unit variance scaled volatile metabolites (x). Predictive power (Q^2) was determined via cross-validation, by which the data was divided into seven parts and 1/7th of the data was removed, and the model was built on the remaining 6/7th of data remaining, and the removed 1/7th of data are predicted from the model.

Prior to heat-mapping, volatile metabolite data were normalized within each of the experimental varieties via z-transformation (normalized peak area – mean peak area/standard deviation of total peak area of each metabolite). The resulting z-scores were converted into colors and grouped using hierarchical clustering on the Spearman's rank correlation (r_s) between metabolite and sensory trait values [44]. Correlation plots were generated in R using the gplots, ggplots2, Reshape2, and stats packages via the *heatmap.2*, *melt,* and *hclust* functions [45-47].

Results

Malting and beer quality among the four malts and subsequent beers were within specifications

As shown in Table 4.1, the Copeland check was well-modified and achieved a balanced malt profile that met the adjunct malt specifications as defined by the American Malting Barley Association (AMBA) and was within the all-malt specifications except for FAN, which was higher than considered within-spec, according to AMBA [48]. The experimental malts had total protein levels, filtration times, and clarities comparable to Copeland. M285 came the closest to meeting the Copeland malt profile. The malt extract was lower than Copeland and the S/T, FAN, DP were all slightly higher than AMBA guidelines for all-malt two-row brewing. M285 had the lowest wort β-glucan, indicating that perhaps intentional under-modification could be manipulated to bring other specifications closer to guidelines. M058 was not fully modified, as evidenced by the high β-glucan and low malt extract. Other malt characteristics were within, or approaching, AMBA specifications. Additional modification might improve all parameters. M341 was under-modified, as evidenced by low friability and high β- glucan. Malt extract, however, was good and the S/T and measures of enzyme activity were suitable for the all malt brewer.

The beers brewed from the experimental malts produced beers that were fairly similar but were nonetheless expressive of the degree of modification during malting. Copeland had the highest alcohol (5.9% ABV) and the second highest fermentability (79.7%) while M341, which was undermodified, had the lowest alcohol (5.3% ABV), highest apparent extract (1%) and lowest fermentability (74.7%) (Table 4.2). As with the malt, beer made from M285 came closest to meeting the Copeland beer profile while the beer made from M058 was intermediate between Copeland and M341. Color, pH and bitterness was comparable across all beers.

The three experimental beers varied in their sensory profiles

Three sensory panels were performed to evaluate the experimental beers: a Consumer Panel, a Brewery Panel, and a Laboratory Panel (described in detail in the Supporting Information Section 2). The Consumer Panel *overall liking* (hedonics) data showed that M285 was liked significantly more than M341 ($p < 0.05$, Tukey's HSD test), with M058 and Copeland being statistically indifferent from others (Figure 4.1A, Table S10a).

The results show that the four beers could be differentiated among consumers. In particular, the attributes *bitter*, *citrus*, *crisp* ($p<0.05$), and potentially *floral*, *refreshing*, and *thin/watery* ($p<0.1$) were the driving forces of differentiating the four beers (Table S.10b); M285 was significantly less *bitter* than M341 and M058, significantly more *citrus* than M341 and Copeland, and significantly more *crisp* than the other 3 beers (Table S.10b). Next, data from the Consumer Panel was evaluated to determine which of the 18 traits represented "ideal lager" traits in beer. *Refreshing* and *crisp* were identified as the most desirable in lager beer, followed by *citrus, sweet,* and *light* (Figure 4.1B).

The Brewery Panel data showed (Table S.11a) that breweries differed in their identification of significant differences between beers for specific descriptors. Brewery 1 found only differences for *DMS*, Brewery 2 found differences for *floral* and *toffee*; Brewery 3 found differences for *astringent, honey, mouthcoating* and *roasted marshmallow*; Brewery 4 found differences for *earthy* and *grainy*; and Brewery 5 for *biscuit* and *roasted marshmallow*. The data indicate that there was little agreement across breweries (Table S.11a). Only *roasted marshmallow* was identified by Brewery 3 and Brewery 5. Brewery 3 identified the most differences between beers (4 descriptors). The other breweries each identified 2 unique descriptors, except Brewery 1, which identified only 1 difference (*DMS*). Panelists were a

significant source of variation at all breweries and for all descriptors. Within each brewery they have different ratings for each beer, but these ratings are consistent. There were significant differences between genotypes for specific attributes between breweries.

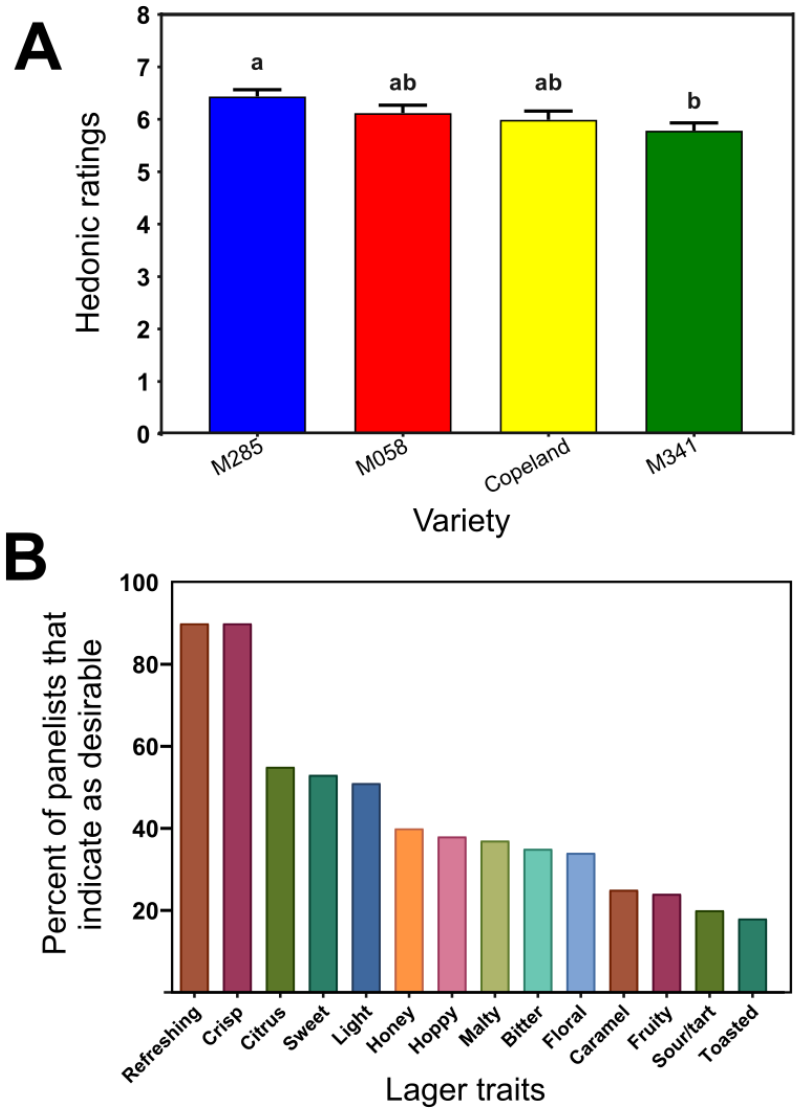

Figure 4.1 Sensory trait variation among the four beers. **(A)** Mean overall liking scores ± SEMs for M341, M285, M058, and Copeland obtained from the Consumer Panel (n = 101). Different letters indicate significant differences between beer samples (Tukey's honest significant difference test, $p<0.05$). **(B)** Percent of Consumers(n = 101) identified a sensory trait as a desirable trait in lager beer.

These are shown in Table S.11b as: M285, the variety favored for lager style beers by the consumer panel was not different from other beers for *DMS, toffee, honey,* or *biscuit*. M285 was the most different for *roasted marshmallow* compared to Copeland and M341 and the most *grassy* compared to Copeland. M285 was the least *floral, astringent, mouthcoating,* and *earthy* compared to Copeland.

The X and Y coordinate similarity/dissimilarity data from the Projective Mapping carried out by the Laboratory Panel, as described in Supplementary File 2 was analyzed using a Multi Factor Analysis (MFA). The analysis identified all four beers as being distinct from one another (data not shown). The Correspondence Analysis (CA) of the descriptor frequency data supported the MFA results however it resulted in three distinct samples where Copeland and M285 beers were grouped together (Supplementary Figure 4.4). These two beers were described as *sweet, graham cracker, bready, malty, fruity, and floral* and were more similar to each other. M341 was described as *floral, earthy, Play-doh, milk chocolate, cereal, hay, and bubblegum* and was distinct from the rest. M058 was described as g*rainy, doughy, toffee, nutty, and green tea* and was distinct from the rest. The MFA biplot supported the differences observed in the basic beer chemistry (i.e. ABV, RE/AE and RDF) whereby Copeland and M285 were similar to each other but different from M341 and M058. These differences in basic beer chemistry may present confounding effects on the perceived beer flavor.

To investigate relationships between the malt analytics, beer analytics, and sensory attributes among panels, a two-way orthogonal projection to latent structures (O2PLS) model was developed with the sensory and analytics data. O2PLS is an extension of PLS and was used to classify co-variation between variables among the two multivariate datasets. For the malt and beer analytics, the O2PLS algorithm resulted in three predictive and one orthogonal component

that explained 65.3% of the variation, with a predictive power of Q^2 = 99% to support that the model was not over-fit. Analysis of the scores (beer variety) and loadings (malt and beer analytics) indicate many of the analytics can be linked to beer profiles within the first two O2PLS components, as indicated by correlation values of greater than |0.50| (Supplementary Figure 3A). A similar model was created for the brewery panel (91.7% variation, Q^2 =83%) (Supplementary Figure 3B), the consumer panel (87.7% variation, Q^2 =83%), and the laboratory panel (84.7% variation, Q^2 =83%).

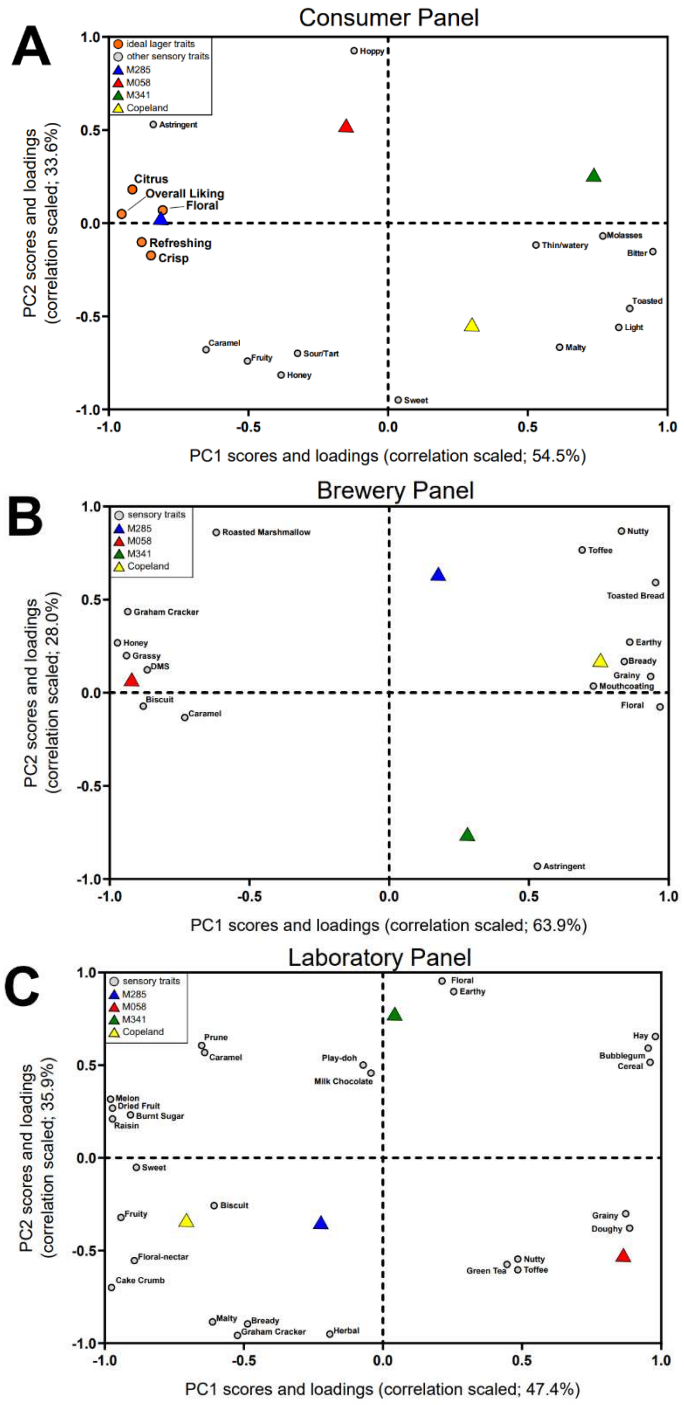

Figure 4.2 Principal component analysis (PCA). (A) PCA scores (triangles, beers) and loadings (circles, 18 Consumer Panel sensory attributes and a score or *overall liking* biplot.. The sensory attributes that co-varied with *overall liking* and were highest in M285 are denoted in orange. **(B)** PCA scores (triangles, beers) and loadings (circles, 16 Brewery Panel sensory attributes) biplot. **(C)** PCA scores (triangles, beers) and loadings (circles, 27 Laboratory Panel sensory attributes) biplot.

All four beers had distinct profiles of volatile metabolites

While the *crisp/refreshing* traits may be driven by non-volatile beer chemistry (for example, mouthfeel), the *citrus/floral* traits are expected to be driven by aromatic properties. To investigate if the beers varied in their volatile profiles, and if this variation was associated to the desirable lager traits *citrus* and *floral*, a non-targeted metabolomics experiment was performed on the four beers using HS/SPME GC-MS. The MS detection and subsequent chemoinformatics method resulted the characterization of 397 compounds, which were annotated using spectral matching and classified according to ontological information from public chemical databases (Table S.9). Analysis of variance (ANOVA) on the 397 compounds characterized 30 volatile metabolites (7.6%) that varied among the four beers (FDR adjusted $p < 0.05$, Table 4.3). PCA was conducted of the 397 compounds and this demonstrates that variation was attributed to the beer-type and resulted in three principal components (Figure 4.3). PC1 (29.4% of the variation) was attributed to a distinct volatile profile of M058, PC2 (18.3%) was associated with differences in M341, and PC3 (7.5%) demonstrated separation between M285 and Copeland.

O2PLS modeling characterized beer volatiles that co-varied with desirable lager sensory attributes and overall liking in M285

To investigate relationships between the beer volatiles and each of the beer descriptors from each independent panel, an O2PLS model was developed with the sensory and volatile metabolite data. The O2PLS algorithm resulted in four predictive and two orthogonal components that explained 94.4% of the variation, with a predictive power of $Q^2 = 68\%$ to support that the model was not over-fit.

Analysis of the scores and loadings indicate most of the 18 Consumer Panel sensory attributes can be linked to beer volatile metabolites within the first two O2PLS components, as

indicated by correlation values of greater than |0.50| (Figure 4.4A). A SIMCA ‘distance to model’ function was applied to characterize the metabolites with the largest contribution to explaining the variation in desirable lager traits (Figure 4.4B). The data indicate associations with fatty acid esters and benzoic acids, which are known classes of aroma compounds.

Table 4.3 Volatile metabolites that varied among the four beers.

Class	Subclass	Metabolite	Flavor Attribute[a]	p-values[b]	Compound ID[c]
lipids	fatty acid esters	ethyl decanoate ester-like	alkane, fatty	0.038	10
		ethyl decanoate ester-like	bitter, fruity	0.038	9
		methyl decenoate ester isomer	waxy, fruity, flowery	0.015	20
		methyl butanoate ester-like	sharp, acetic, fruity, cheesy, buttery	0.032	160
		methyl butanoate ester-like	spicy, warm, balsamic	0.012	92
		methyl decanoate ester	fatty, oily	0.034	64
		ethyl 3-butenoate ester	vegetal	0.001	326
		octadecenoic acid ester isomer	waxy	0.021	177
	MCFA esters	ethyl dodecanoate ester	alkane, floral	0.045	11
		n-decanoic acid ester	fatty, rancid	0.011	8
		octanoic acid ester	cheese, sweat	0.023	1
	sesquiterpenoids	terpene-like		0.021	206
		nerolidol	grape, fruity, apple, sweet, melon	0.001	27
benzenoids/heterocyclic	carboxylic acid esters	2-phenylethyl acetate ester	fruity, honey, possible sour	0.001	31
	benzenoids	benzene-like	phenolic, floral, grapefruit, citrus, spicy, fruity	0.031	281
		phenyl isobutyrate	vanilla	0.003	439
	benzoic acids	3-methoxybenzylamine	phenolic, floral, grapefruit, citrus, spicy, fruity	0.003	349
	benzodiazines	7-methyl-6-oxo-5-phenyl-1,3-diazaadamantane	na	0.032	15
	hydrazines	hydrazine-like	na	0.031	536
	pyridazines	3-methylpyridazine	fried potato, roasted barley, cocoa, coffee, tea, roasted filbert, roasted pecan, peanut, soy products, rum, whisky	0.004	587
	pyrroles	2-ethyl 1H-pyrrole		0.032	376
	quinoxalines	8H-pyrido[1,2-a]quinoxalin-8-one	coffee, nutty, roasted	0.001	328
	thioethers	1,2-dithiane	na	0.001	76
		4-methylpentanethioamide		0.001	597

nitrogenous	monoalkylamines	2-ethanamine		0.034	399
	carbonyl compounds	2-propyn-1-amine	geranium	0.038	615
organosulfurs	dialkyldisulfides	disulfide	sulfurous	0.042	562
	sulfoxides	methyl phenethylsulfoxide	sulfurous	0.014	397
misc	isoflavans	chromanone	herbal, spicy	0.002	280
	alkanes	methane isomer 01	alkane	0.038	621

a: previously reported flavor attributes
b: ANOVA p, FDR adjusted for n=3 injection replicates per beer
c: metabolite identifier for this manuscript

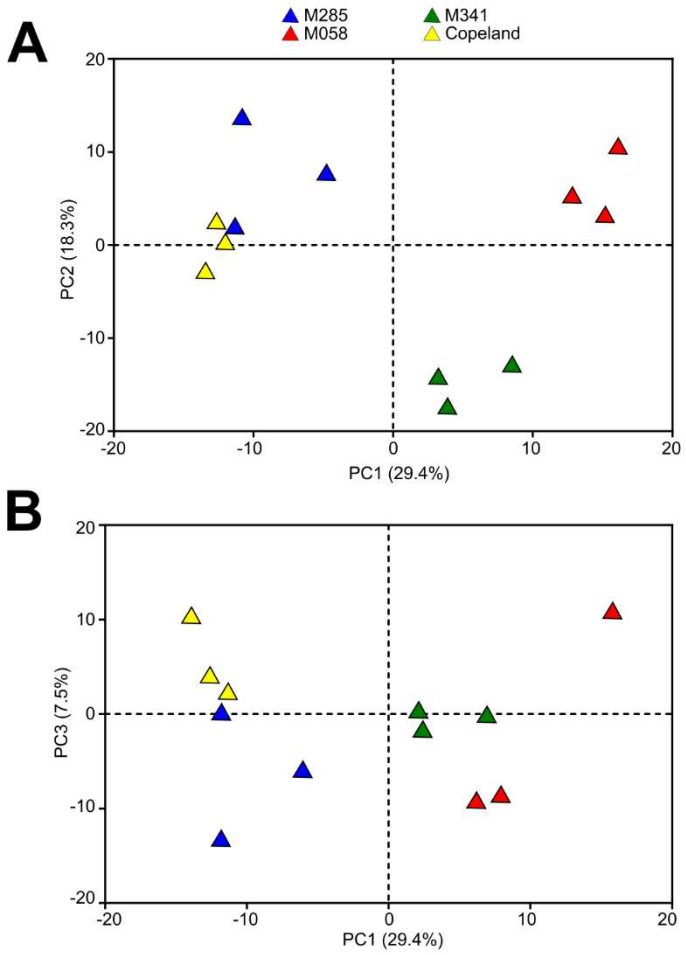

Figure 4.3 Volatile metabolite variation among the four beers. Principal component analysis (PCA) scores plots for the 397 annotated metabolites. **(A)** Top panel is PC1 vs. PC2 and **(B)** PC1 vs. PC3. Triangles represent volatile metabolite profiles for n = 3 HS/SPME-GC-MS extraction replicates for each beer type.

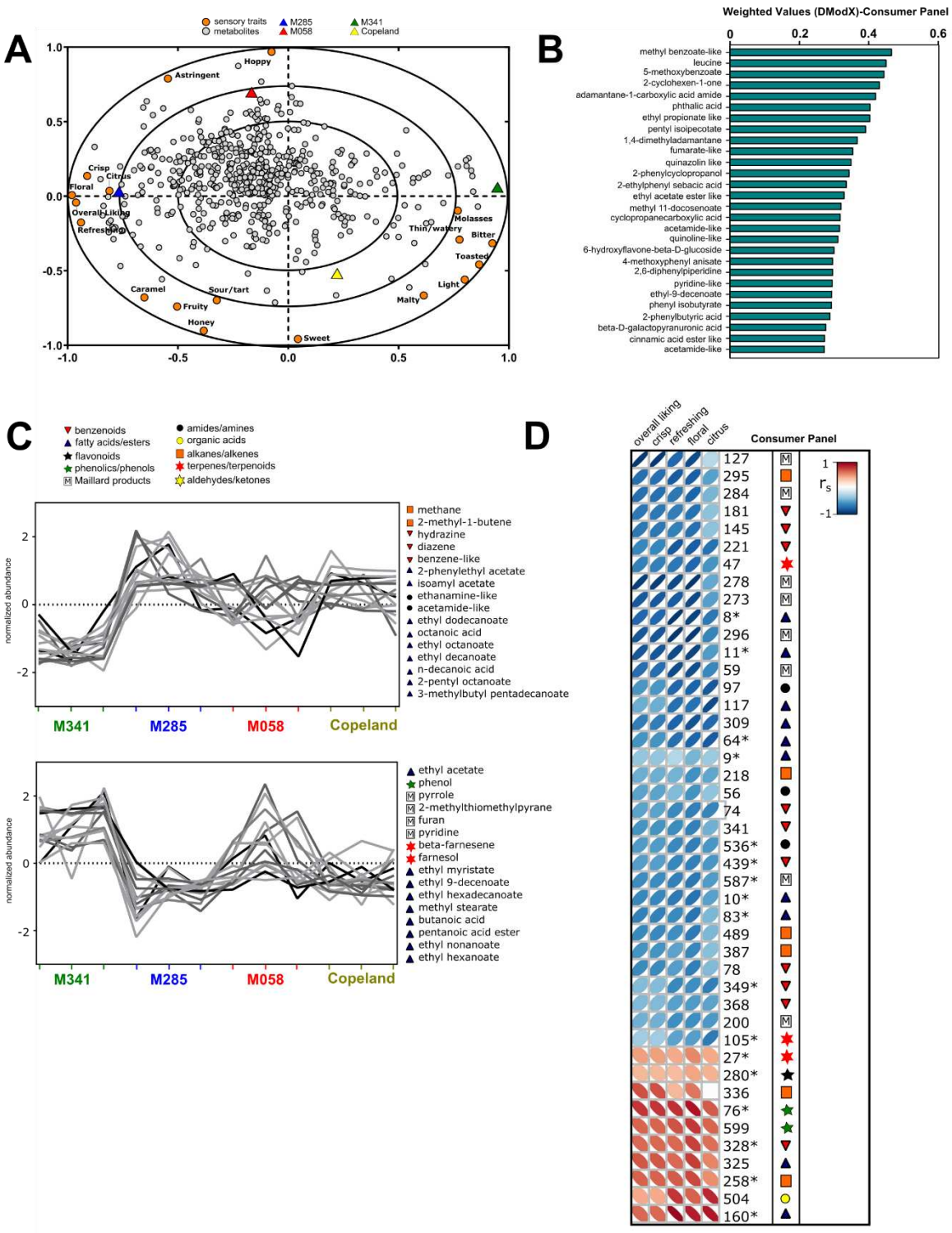

Figure 4.4 Co-variation analysis of sensory and chemical traits among the four beers. O2PLS analysis was conducted on the 18 Consumer Panel sensory attributes and *overall liking* (y variables) and 397 volatile beer compounds (x variables). **(A)** O2PLS scores (beers) and loadings (sensory attributes, metabolites) plot provides an overview of the model for the first two

of the four calculated explanatory components. Axes are correlation scaled scores and loadings, -1.0 to 1.0 **(B)** Distance to model plots are scores indicating a metabolite's cotribution to the full OP2LS model. **(C)** Normalized abundance plots of metabolites contributing to O2PLS Component 1 (x-axis of Panel A), generally with correlation values greater than 0.50 for Component 1, and less than 0.50 for Component 2. **(D)** Heat map of Spearman's rank correlation values for metabolites and the sensory data. Colors and the eccentricity of the ellipse indicate strength and direction of the correlation, and the data were arranged using hierarchical clustering.

Table 4.4 Volatile metabolites that were most distinct M285 beer

Class	Subclass	Metabolite	Flavor Attribute[a]	p-values[b]	M285 contribution scores[c]	r_s M285[d]	FC[e]	CV[f]	Compound ID[g]
benzenoids	benzenoids	methyl 3-benzoate	phenolic, floral, grapefruit, citrus, spicy, fruity	0.424	1.13		1.49	27	194
		benzene-like	astringent, phenolic	0.375	1.19		1.46	19	619
	benzamides	benzaldehyde	phenolic, floral, grapefruit, citrus, spicy, fruity	0.831	1.69		1.40	3	554
		benzamide-like	phenolic, floral, grapefruit, citrus, spicy, fruity	0.424	0.58		1.16	16	90
		benzamide-like	phenolic, floral, grapefruit, citrus, spicy, fruity	0.506	1.36		1.22	30	256
	benzeacetic acids	benzeacetic acid ester	na	0.506	1.10		0.99	14	585
		hydrazide-like	na	0.435	1.59		1.55	2	403
	benzenes	1-ethyl-4-methoxybenzene	phenolic, floral, grapefruit, citrus, spicy, fruity	0.404	0.75		1.03	5	534
	benzoic acid esters	4-methoxyphenyl anisate ester	putrid, sweet, cadaverous	0.409	1.40		1.09	27	449
		benzoic acid ester isomer 08	phenolic, floral, grapefruit, citrus, spicy, fruity	0.653	0.58		1.13	33	150
		benzoic acid ester isomer 10	phenolic, floral, grapefruit, citrus, spicy, fruity	0.445	1.68		2.96	5	575
		salicylic acid ester	solvent, astringent, mint	0.288	1.20		1.73	58	37
	benzoic acids	2-hydroxybenzoic acid	phenolic, floral, grapefruit, citrus, spicy, fruity	0.06	0.83		1.34	30	463
		butylbenzoate-like	phenolic, floral, grapefruit, citrus, spicy, fruity	0.528	1.35		1.96	50	539
		ethyl benzoate isomer 02	phenolic, floral, grapefruit, citrus, spicy, fruity	0.906	0.64		1.04	11	372
		ethyl benzoate-like	viscous	0.374	1.12		1.11	23	262
	benzophenones	2H-pyrazol-3-methanone	balsamic, rose, metallic, powdery, geranium, herbal	0.419	1.43		1.22	30	545
hydrocarbons	cycloalkane esters	cyclopentane methanol	herbal, camphor, minty	0.814	1.65		1.47	51	420
	cycloalkanes	cyclohexyl carbamate-like	na	0.318	1.74		1.36	17	560
lipids	acetate esters	acetate ester	sweat, urine, musty, flowery, musky, perfume, grass	0.978	0.76		1.48	39	110
	fatty acid esters	ethyl 2,4-pentadienoate	na	0.445	0.93		0.97	5	573

		ethyl pentanoate ester	vomit, barnyard	0.424	1.12		1.00	30	55
		fumarate	herbal, green, floral, fresh, plastic	0.345	2.18		2.39	44	522
		fumaric acid ester	herbal, green, floral, fresh, plastic	0.831	0.71		1.29	51	437
		glutaric acid ester isomer 02	na	0.473	1.49		1.42	58	382
		methyl butanoate ester-like	spicy, warm, balsamic	0.012	0.83	r_s > 0.50	1.27	5	92
		methyl decanoate ester	fatty, oily	0.034	1.91	r_s > 0.50	1.67	46	64
		succinate ester isomer 01	musty, apple, fruity	0.445	1.72		1.39	8	530
	fatty alcohol esters	(+/-)-dihydrofarnesol	flower, musky, oily	0.507	0.55		0.85	19	39
		decanol ester	alcoholic	0.943	2.27		1.60	31	355
	fatty amide esters	butyramide ester	na	0.424	0.59		1.47	68	592

Class	Subclass	Metabolite	Flavor Attribute[a]	p-values[b]	M285 contribution scores[c]	r_s M285[d]	FC[e]	CV[f]	Compound ID[g]
		2-phenylbutyric acid	vomity, fruity, cheesy	0.312	0.55		1.46	62	515
		methyl butanoate ester-like	spicy, warm, balsamic	0.288	1.29	r_s > 0.50	1.63	46	117
	MCFA esters	caproleic acid ester	waxy, green, fatty, soapy, creamy, cheesy	0.133	1.73		1.27	12	45
		octanoic acid ester	cheese, sweat	0.023	0.74		0.99	5	1
	sesquiterpenoids	β--ionone	floral	0.586	1.40		1.94	79	276
		farnesol	anise, floral, grapefruit, wax-taste, lily	0.532	0.83		0.88	7	19
		nerolidol	grape, fruity, apple, sweet, melon	0.001	0.61		1.46	10	27
		terpene-like	na	0.021	0.56		1.47	38	206
organic acids	organic acid esters	ethyl acetate ester isomer 02	fruity, floral, apple, apricot, jasmine, blueberry	0.439	1.42		1.38	39	252
	amino acid esters	L-proline ester	na	0.694	1.19		3.40	130	311
	dicarboxylic acid esters	2,2,4-trimethyl-1,3-pentanediol diisobutyrate ester	ether	0.614	1.56		1.42	37	67

		dimethyl propanedioate	na	0.437	1.04		1.21	21	268
		ethane-like	ether	0.666	0.81		1.59	48	467
		methyl carbamate	na	0.485	1.30		1.37	37	561
	phthalic acids	1,2-benzenedicarboxylic acid ester	phenolic, floral, grapefruit, citrus, spicy, fruity	0.336	2.04		1.53	30	371
organoheterocyclic compounds	ferrocenes	1-hydroxypentylferrocene	na	0.607	0.80		1.15	23	310
		3-methylbutylidene	na	0.638	1.13		1.61	82	391
	furans	2-methylpropyl 2-(furan-2-carbonylamino)propanoate	sweet	0.375	1.56		1.43	16	63
		furazanamine	meaty, roasted, cooked, nutty	0.375	0.62		1.11	2	471
		furfural	wet dog	0.696	0.51	$r_s > 0.50$	1.41	71	56
	halobenzenes	imidazole-like	na	0.507	0.69		1.12	19	167
	indoles	3,3-dimethyl-3H-indole	floral, poop	0.345	1.48		1.41	37	614
	isophthalic acid esters	isophthalic acid, neopentyl octyl ester	phenolic	0.931	0.93		1.31	53	138
	ketones	ketone-like	na	0.453	0.76		2.28	77	203
	lactones	jasmine lactone	powerful, creamy, jasmine, peachy, coconut	0.375	2.49		1.62	10	82
	phenols	phenol-like	phenolic	0.424	1.37		1.28	19	604
		phenol-like	phenolic	0.472	2.13		1.45	7	599
		phenol-like	phenolic	0.48	1.28		1.05	13	321
	pyrans	4H-pyran-4-one	na	0.569	1.19		1.14	37	249
		benzopyran-like		0.417	1.07	$r_s > 0.50$	1.44	31	273
	pyridines	2-heptadecylpyridine	na	0.156	0.93		1.12	37	209
		pyridine-like	fruity, bitter, corn, cornchip, popcorn, fatty, tobacco, musty, nutty	0.375	0.80		2.69	136	365

Class	Subclass	Metabolite	Flavor Attribute[a]	p-values[b]	M285 contribution scores[c]	r_s M285[d]	FC[e]	CV[f]	Compound ID[g]
		pyridine-like	fruity, bitter, corn, cornchip, popcorn, fatty, tobacco, musty, nutty	0.532	0.63		2.24	53	581
	pyrimidines	pyridinone-like	na	0.526	1.25		1.111	30	136
	pyrroles	1H-pyrrole-2-carboxamide	na	0.848	1.55		1.48	23	73
		3,4-dihydro-5-methyl-4-[(7Z)-pentadecenyl]-2H-pyrrole	green, metallic, woody, herbal, smoke	0.387	0.65		1.01	40	201
		pyrrole-like	na	0.478	1.13		1.20	27	269
	quinazolines	quinazolin-like	green, metallic, earthy, leafy	0.548	2.14		1.41	10	495
organonitrogen compounds	dialkylamines	diethylamine	unpleasant	0.559	1.04		1.34	12	457
organooxygen compounds	alcohols	ethyl 1,3-propanediol	alcoholic	0.54	1.02		1.46	21	593
	aryl alkyl ketones	ethanone-like	woody, leathery, musk, cedar	0.057	1.08		1.13	29	531
		ethanone-like	woody, leathery, musk, cedar	0.822	0.54		1.36	30	405
	carbonyl compounds	2-propyn-1-amine	geranium	0.038	1.25		3.15	24	615
	dialdehydes	succindialdehyde	fruity	0.747	0.99		1.96	32	459
organosulfur compounds	dialkyldisulfides	disulfide	sulfurous	0.042	1.98		1.93	19	562
	sulfoxides	methyl phenethylsulfoxide	sulfurous	0.014	1.47		1.35	12	397
phenylpropanoids	alkyldienes	3-methylheptadiene	herbal, spicy	0.609	0.72	$r_s > 0.50$	1.05	18	218
	ketones	phenyl ketone	na	0.898	0.51		1.30	21	367

a: previously reported flavor attributes
b: ANOVA p, FDR adjusted for n=3 injection replicates per beer
c: VIP, variables important in projection scores
d: r_s: Spearman's rho, $r_s > 0.5$ indicates high correlation
e: FC, fold change from control (Copeland)
f: CV, coefficient of variance score, CV > 10 indicates increased variability
g: metabolite identifier for this manuscript

The sensory/chemistry cluster along O2PLS Component 1 demonstrates co-variation of M285 volatiles, desirable lager traits (*crisp, overall liking, refreshing, citrus, floral)*, and a negative association with M341 volatiles and Maillard-like sensory attributes (*molasses, bitter, toasted, malty*). The metabolites that are associated with this trend (correlations greater than 0.5 for Component 1, and less than 0.5 for Component 2) are shown in Figure 4.4C. Metabolites that were positively associated with desirable lager traits included alkane/alkenes (2), benzenoids (3), amides/amines (2), and fatty acid esters (9). Negative associations included compounds of the same classes, but also four metabolites that are putatively Maillard Reaction Products and two terpenes. These relationships of metabolites to sensory were validated using Spearman's rank correlation performed on this subset (and others) (Figure 4.4D).

Broad trends among chemical classes did not explain the differences among beers

The data was evaluated to determine if broad trends of metabolite classes could distinguish each of the four beers: specifically, for lipids (to include fatty acid ester formation), nitrogenous compounds, organic acids, and phenolics. Metabolite abundances were z-transformed to express the data as a profile within a variety, therefore a range in color denotes range in variation of a compound class within a variety, with very blue (high) or very yellow (low) indicate proportions of a metabolite's contribution to the profile. For lipids, M341 and Copeland had similar profiles, and M285 and M058 were more distinct. (Figure 4.5A). The lipids that were common between M341 and Copeland were a notably higher variation of fatty acid esters.

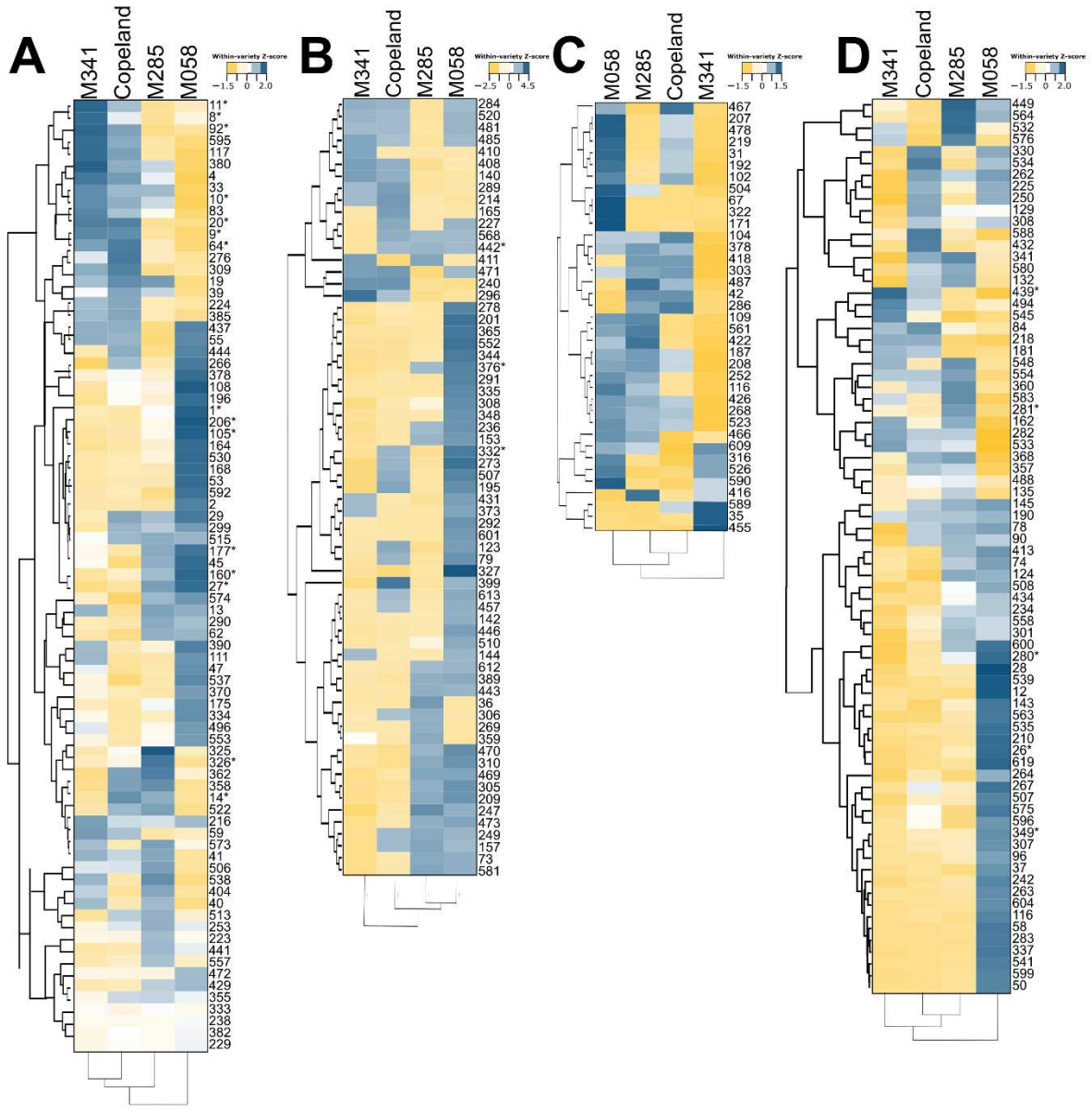

Figure 4.5 Analysis of volatile metabolite variation among the four beers within chemical classes. Prior to heatmapping, volatile metabolite data were normalized within each variety via z-transformation normalized peak area - mean/standard deviation of total peak area of each metabolite).The resulting z-scores were converted into colors and grouped using hierarchical clustering on the Spearman's rank correlation (r_s) between metabolite and sensory trait values. Heat maps with hierarchical clustering were built within chemicals classes, as described in Figure 4.4C for **(A)** 94 lipids **(B)** 65 nitrogenous compounds **(C)** 122 phenolic/benzenoid compounds and **(D)** 34 organic acids. The color in each cell represents the z- transformed abundances of the averaged replicates (n=3-5) per beer sample. Z-transformation was based on the mean abundance and standard deviation of the metabolite across all samples.

For example, the lipid profile of M341 and Copeland has higher proportions of dodecanoic acid (11) and n-decanoic acid (8). M058 lipids were higher in several terpenes and short-chain fatty acid esters including octanoic acid (ester) (14) and butanoic acid (ester) (83), and a sesquiterpenoid (206). The M285 lipid profile contained a lower abundance of many lipid metabolites, except for ethyl hexanoate (29), succinic acid ester (299), 17-octadecenoic acid ester (177), caproleic acid ester (45), nerolidol (19), ethyl 9-decenoate (13) and others. A similar pattern was observed for nitrogenous compounds (Figure 4.5B), whereby M058 had higher abundances of most compounds, M341 and Copeland were similar, and M285 has overall reduced abundance of most compounds in this class. The nitrogenous compounds most distinct in M285 included 4-methyl-3-nitropyridine (568), pyridine-like compounds, 3,5-methylpyrazolidinedione (236), ethenone-like compounds.

Organic acids and phenolics have differing trends, and all four beers had more distinct profiles from each other compared to the lipids and nitrogenous compounds (Figures 4.5B, 4.5C). For organic acids, M341 was the most lacking in variation of organic acids, while M058 was the most abundant. The organic acids most unique to M285 included ethyl propionate (208), ethyl-2-propionate (426), diethylmalonic acid (187), and methyl propionate (487). The phenolics most unique to M285 included 4-methoxyphenyl anisate (449), 5-methoxybenzoate (532), methyl benzoate (576), and other benzene compounds.

The O2PLS model supports fatty acid esters and MRPs association with other sensory attributes not identified as "ideal" in lager beer

The O2PLS analysis workflow was also used to characterize relationships between the chemistry and sensory of M341, M058, and Copeland beers and is summarized in the Supporting Information. Copeland (*grainy, bready, earthy, floral* from the Brewery Panel; *biscuit, sweet,*

fruity from the Laboratory Panel) was more abundant in 3-methylpyridazine, a compound that has been related to sensory attributes such as bread and toast [49] and ethyl 3-butenoate ester, related to vegetal or tropical aromas. The *biscuit, astringent,* and *hoppy* attributes (from the Consumer and Brewery Panels) and the *grainy, doughy,* and *DMS* (Laboratory Panel) associated with M058 displayed positive associations with amines and sulfur compounds, and negative associations with the fatty acid ester classes, whereas M341 (*floral, earthy* from the Laboratory Panel; no correlation from Brewery Panel; *molasses, bitter, toasted, light, malty* from the Consumer Panel) showed highly negative associations with the amines and sulfur compounds, but more positive associations were seen with the MRPs (Supplementary Figures 1, 2).

Discussion

This study characterized sensory and chemical variations in pilot beers that was hypothesized to be different due to barley genotypes. Support for accepting the hypothesis comes from (*i*) significantly different and distinct flavor profiles that could be discriminated by three independent, different types of sensory panels, (*ii*) distinct flavor chemistry mediated by volatile metabolites, and (*iii*) associations between volatile metabolites and flavor hedonics. These variations in beer sensory and volatile metabolites may be mediated through metabolite chemistry of the malt and/or the differences in malting and beer quality metrics. Several studies have reported the contribution of barley genotype (cultivar or variety) to malt metabolite variation and subsequent variation in beer [7, 8, 14, 50, 51]. Ultimately, variation in metabolites can be tracked back to variation at the genetic level and these polymorphisms can be used as a basis for selection in barley breeding programs.

Malt and beer analytics: predictors of beer flavor?

This study aimed to normalize malting specifications by adjusting for steep-out moisture. Steeping times were adjusted in process to account for differences in moisture. Standardizing

malt analytics for different barley genotypes in order to assess their potential flavor contributions is an ideal experimental framework but one that is a challenge to achieve. Herb et al (2017) addressed this issue in a large sample of malts that differed greatly for all specifications by focusing on different degrees of modification and its effects on beer flavor [7]. The conclusion was that while degree of malt modification did affect flavor, there was still a significant difference in the genetic component. In this study, only the control, Copeland, met malting quality specifications. Nonetheless, the brewer (V. Vega, Deschutes Brewing) did not express concern regarding the specifications of M285 and M058. M285, from which the preferred beer was made, approached malt specifications, but was lower in extract and higher in FAN, DP, and AA than the control. The other experimental line (M341) was notably deviant in one or more analytical properties. These differences in malt specifications are attributable to the genetic architecture of the barleys, and malts produced from these barleys produced beers with different flavor profiles.

This prompts the question "are the differences in beer flavor a direct outcome of differences in one or more malt specifications?" If this were the case, then specific malt attributes would be predictors of beer flavors. Copeland was judged suitable, by the brewer, for ales or lagers and likely to produce a crisp beer. The higher enzyme levels in M285 indicate the potential for a drier/crisper beer while the M058 might be expected to have green/grassy flavors due to higher moisture [52]. The poor modification of M341 could produce beers with higher body and *sweet*/cloying flavors and perhaps contribute to *grainy, malty* flavors. Malt analytical specifications are designed to allow brewers to predict brewing performance and are the principal determinant of whether or not a barley variety is recommend for malting and brewing [48]. The utility of malt analytical profiles for variety selection and predicting brewing

performance has, however, been questioned [53]. In this regard, it is noteworthy that the differences in beer analytics between the four genotypes were not as great as the difference in malt analytics. In other words, similar beers can be made from dis-similar malts. The current research – within the overall framework of "no red flags" malts and research beers approaching commercial specifications - suggests that malt and beer analytical attributes could potentially be useful for predicting the contributions of a barley variety to beer flavor. For example, the higher AA, DP and RDF in M285 (Tables 4.1, 4.2) may have a role in the *citrus, crisp, refreshing, and overall liking* sensory attributes of due to lower levels of monosaccharides in the finished beer. In contrast, M341 was less modified and had a higher °P, which would result in increased saccharides in the finished beer and would be consistent with the *caramel* and *molasses* attributes. RDF levels suggest that flavor differences are also a function of both malt modification and attenuation. Although the four beers are considered well-attenuated (>70%), the slight differences in leftover unfermented extract may contribute to the organoleptic properties perceived and may have been initially affected by the enzymes present in malt and their ability to perform (Table 4.1) [54]. The higher moistures associated with M058 and M341 may influence the graininess, grassiness, and astringency associated with these genotypes [48, 55]. It is fair to say that phenotype (through sensory) can be influenced by malt and beer analytics (through malting and brewing); slight process differences, combined with genetic variation, may have an impact on sensory, providing indicators of potential flavor, but cannot be relied upon as predictors alone.

Elucidating the chemical basis of desirable sensory traits in M285 lager

Through integrated sensory and metabolomic analysis, it has been determined that M285 produced a beer which had a higher *overall liking*, encompassing the five most desirable traits,

refreshing, crisp, citrus, sweet, and light. To understand flavor variation in beer attributed to malt, an integrated chemical and sensory analysis was performed with HS-SPME/GC-MS in combination with three sensory testing methods. The beer data included many metabolites with known effects on fermentation and flavor, including fatty acid esters, amines, phenols, and MRPs. Here, the chemical profile of the most "ideal lager" (M285) was further evaluated in detail for associations between malt quality, beer quality, beer chemistry, and affects on flavor, specifically through reduced MRPs and a unique profile of fatty acid esters.

Role of Maillard Reaction Products in desireable lager flavor

Maillard Reaction Products (MRPs) are produced during malting, during fermentation, and beer storage, [18, 19, 56], and have a major role in beer flavor. One MRP sub-class important to beer flavor are the furfurals, which are members of the furan and aldehyde families. Furfurals are abundant in dark, specialty malts, contributing to color and flavor development during wort boiling, persist through fermentation [57], and can affect the organoleptic properties of beer -- especially as it is exposed to light and heat during aging, creating staling (cardboard, metallic) flavors [58]. Evidence suggests furfurals provide *nutty, maple,* and *bready* aromas in darker beers, but the presence of furfural in lager beers results in a more sharp, crisp, lingering bitterness and increase in astringency [59], as seen in M285; while the abundance of furfural (compound 56) did not vary greatly among the beers, it was slightly more abundant in M285.

MRPs also create flavor combinations, whether by masking a lighter aroma or creation of a new attribute, as subsequent stages of the Maillard Reaction involve the interaction of oxygenated compounds with reactive components, such as amines, aldehydes, amino acids, etc. which lead to flavor compounds including furans, pyrazines, pyrroles, oxazoles, thiophenes, thiazoles, and other heterocycles. MRPs produce specific aromas depending on which amino

acid by which they were originally produced [60]. According to Wong, et. al., examples of this include proline and alanine, which resulted in flowery and fragrant aromas; phenylalanine and tyrosine produced dried roses aroma; aspartic acid and serine produced fruity aromas; glycine, lysine, threonine, and valine produced a caramel-like aroma; isoleucine and leucine produced a burnt caramel aroma; methionine developed a fried potato aroma; cysteine and methionine produced umami, meaty, soy sauce-like aromas and flavors [24, 60]. A combination of these amino acids would result in different types of aroma, with the stronger note dominating the aromas. In the case of M285, an abundance of amino acids which produced lighter, more sweet or citrusy aromas (e.g. proline ester, compound 311) may have been dominant over less abundant meaty-aroma-producing amino acids. This may have resulted in less predominance of furans and pyrroles (Figure 4.4) and increased ability of *crisp*, *light*, *citrus* esters from lipids and astringency from benzenoids to direct the flavor profile indicated by the Consumer Panel.

Role of fatty acids esters in desirable lager flavor

In this study, M285, associated with the five most desirable traits, *refreshing, crisp, citrus, sweet, and light*, had a profile that was less abundant in MRPs (Figure 4.4). The profile of MRPs in M285 is important factor to consider when breeding for barley that will be malted specifically to be brewed into an "ideal lager". The decreased amino acids and sugars involved in production of MRPs could influence the antioxidant properties of the beer, decreasing foam stability and promoting rapid aging (due to the lack of antioxidants) leading to more staling flavors over time.

The present study of four varieties further supports an association of fatty acids and their esters to specific sensory attributes is an important factor to consider when determining beer flavor and flavor stability (aging). The abundance of free fatty acids and fatty acid esters, as well

as the composition of free fatty acids in barley, malt, and beer may play a role and indicate that they should be considered together with other metabolites when considering genotype. The most abundant compounds in M285 were ethyl octanoate, ethyl dodecanoate, ethyl 9-decenoate, 2-pentyl octanoate, and n-decanoic acid (Figure 4.4). The presence of these compounds in conjunction with the low MRP profile suggests not only that these compounds contribute to the "ideal lager" attributes associated with M285, but that these fatty acid esters (known for undesirable aromas such as *goaty* and cheesy) may also contribute to off flavors as aging progresses, due to the less abundant MRP antioxidant profile in M285. In contrast, M341 and M058 had profiles consisting many fatty acid esters known to impart traditionally fruitier flavors in beer (Figure 4.5). Butanoic acid (pineapple), ethyl hexanoate (pineapple), pentanoic acid (red apple, aniseed), and ethyl acetate (fruity, tropical, pineapple) are all associated with tropical or fruity aromas and flavors [32, 61]. These profiles are important to note when considering the "type" of beer to be created, as these esters combined with an increased MRP profile could lead to heavier, more competitive flavors in beer as it ages (*e.g.* syrupy molasses combined with ripe tropical fruits) [62]. Therefore, the lack of a MRP antioxidant profile in M285 could be a liability.

Fatty acid esters are important to beer flavor, as they do contribute to *fruity*, *candy-sweet*, and *perfume-like* aroma character of beer, commonly reported to be produced by yeast during fermentation [63]. However, two sub-classes characterized to be higher in M285 include the acetate esters and medium chain fatty acids, which are largely uncharacterized, and may be attributed more to malt metabolite chemistry than yeast metabolism. As an example, lipids and fatty acids represent a small fraction of barley grain weight (about 2% [9]), but they do influence malting and brewing for example *sweet* and *fruity* sensory attributes [64] and staling traits such

as cardboard, old vegetables, and papery [65, 66]. Some malt fatty acids have well characterized negative effects on beer quality such as foam instability, hazy appearance, and flavor stability [65]. Unsaturated linolenic and linoleic acids have been shown to contribute to beer oxidation and facilitate staling flavors during storage [28, 65]. The *sweet, graham cracker, bready, malty, fruity,* and *floral* traits in M285 described by the Laboratory Panel may be attributed to this metabolite profile, as could the *grassy* described by the Brewery Panel. These data suggest that the chemical composition of barley, malt, and beer when malting and brewing is important and should be taken into consideration if specific profiles (i.e. "ideal lager"), novel flavors (tropical molasses), or consistency are desired in the product, as beer is a highly complex beverage driven by chemical and mechanical interactions prone to leaving a brewer with undesirable results otherwise [67].

A potential for secondary/specialized metabolites effects on desirable lager flavor

In addition to MRPs and lipids, several secondary (specialized) metabolites were characterized in M285. These included alkaloids, quinolines, terpenoids, other heterocyclic aromatic compounds which, in conjunction with other metabolites may also contribute to its "ideal lager" status. Beer made with M285 contained many phenolic compounds (Figure 4.4D), which contribute many different organoleptic properties to beer, with about 70-80% of those coming from malt and 20-30% from hops [68]. Benzoic acid derivatives, including hydroxybenzoic acid and gallic acid, contribute a balsamic-like astringency, urine-like and/or fruity flavor attributes to beer [69]. These attributes were noted by the Laboratory Panel in M285 and M341, to some extent, by the Consumer Panel. These two beers also had very different analytical profiles, as described in Tables 4.1 and 4.2, possibly contributing to the amplification of phenotype through metabolite expression. Cinnamic acid derivatives, such as ferulic acid,

found in barley, malt, and beer, contributes to bready or vanilla flavors, depending on circumstance, large variablility has been observed in the levels of these compounds [70, 71]. If not prevented, these esterified compounds undergo thermal or metabolic decarboxylation to more undesirable aromas [68].

Conclusions

This study investigated the effect of barley variety on beer flavor, as it relates to experimental lines, by profiling volatile metabolites using a non-targeted metabolomics approach, integrated with sensory data, malting analytics, and brewing analytics. Based on our results and analyses, we accept our hypothesis that barley genetic variation can, through malt, affect desirable sensory attributes of lager beer, and that these phenotypes are associated with differences in the volatile profiles. Using larger malts and beer volumes, we confirm prior reports, based on nano-beers, that barley genotypes derived from the cross of Full Pint x Golden Promise can make different, positive contributions to lager beer flavor. Further research is necessary to confirm these findings in other barley germplasm and with other beer styles. Differences in sensory attributes and metabolite abundances observed among the four barley genotypes are functions of genetic differences that lead to differential responses to the malting and brewing processes. Ultimately, metabolic compounds associated with specific sensory attributes can be tracked back to determinant genes by referring to the barley genome sequence. The chemical compositions of malts made from different barley genotypes, and beers made from those malts, are critical considerations for targeting specific beer profiles (i.e. “ideal lager”), novel flavors (tropical molasses), and ensuring product consistency: beer is a highly complex beverage driven by chemical and mechanical interactions prone to leaving a brewer with undesirable results. Taken together, these data further elucidate the chemical basis by which malt

genetics can influence beer flavor. Differences in sensory attributes and metabolite abundances among varieties are functions of malting and brewing processes, therefore this research could be useful in prediction of flavor based on variety, malting, and brewing analytics. Previous research describes the characterization of metabolic variation as related to phenotypic distribution of multiple malting and agronomic quality traits, as well as to sensory attributes. Predictions regarding variation in metabolites could be useful in variety development, selection of varieties for specific beer styles, and malting or brewing protocol design. For this research, each of the pieces fit into the puzzle, providing unique bits of information regarding analytics, sensory, and chemistry. The path forward in this research would involve standardizing the sensory lexicon and the training of the panels on attributes, creating cohesive templates for each descriptor, regardless of the type of sensory methods one uses. This would allow future "omics" to be further aligned with the appropriate sensory descriptors and analytics, giving each piece a place in the puzzle of an already complex system.

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CHAPTER 5 – GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I tested the ***broader hypothesis*** that variation in malt metabolite chemistry can be expanded on in the industry by utilizing genetically diverse populations (Chapter 2), that this variation can be detected using sensory analysis of the malt (Chapter 3), and that this variation can impact beer flavor (Chapter 4). My specific hypotheses were:

5. There will be metabolomic differences among heirloom barley genotypes.
6. The differences in barley chemistry will be reflected in the chemistry of the final product (beer).
7. Differences in the beer chemistry will impact sensory attributes of beer through flavor and flavor stability.
8. Certain barley and/or malt metabolites can be markers for beer flavor and/or flavor stability.

The purpose of these studies was to identify genetic, environmental, and processing factors that regulate the chemical composition of food, and to use these findings to improve crop varieties, increase sustainability for the industry, and develop new, chemical targets to evaluate (barley) malt for quality and flavor improvement for health.

The first study in this research attempts to characterize novel malt flavor from a population of heirloom barley varieties**.** A population of heirloom malt was profiled using metabolomics methods to understand chemical variation to facilitate breeding for flavor. The hypothesis was determined to be true, that malt derived from heirloom barley contained a unique profile of metabolites that can influence beer flavor, specifically via non-volatile precursor metabolites, such as fatty acids, organic acids, phenols, and amino acids. The population of

heirlooms was also chemogrouped via HCA, in order to allow analysis of trends by grouping chemically similar varieties together.

The second study sought to understand chemical variation in hot steep malt extracts and how these influences sensory evaluation of malt. As per the hypothesis, it was determined that hot steep extract metabolites did vary among different malts, and that this metabolite variation did influence sensory traits (*i.e.* the influence of butyric acid in the creation of the fruit punch/sour vomit trait). Metabolomics was performed on hot steep extracts from 10 commercial malts, and the data was integrated with sensory evaluation of the extracts. This new information will provide potential metabolite markers for high-throughput screening of malt to predict sensory outcomes of the extract, and this can facilitate quality control/assurance in a brewing program.

Finally, the third study proposed to understand variation in sensory attributes and volatile compounds in lager brewed from genetically distinct malts using an integrated sensory and non-targeted metabolomics approach. Here, we investigated the effects of barley genotypes on sensory attributes and metabolite profiles of finished beer made using larger-scale malts and brews. The malting quality data, beer analytical data, sensory data, and beer volatile metabolite profiles of three DH lines were compared to those for the cultivar CDC Copeland. This research tested the ***hypothesis*** that barley genetic variation can, through malt, affect desirable sensory attributes of lager beer, and that these phenotypes are associated with differences in the volatile profiles.

The research methods utilized in these studies included three metabolomics platforms (UHPLC-MS, GC-MS, SPME-GC-MS). These platforms were utilized in conjunction with sensory analysis techniques (e.g.QDA, Projective Mapping/Napping, CATA, etc.) malting

quality analytics (AA, DP, FAN, etc.) and agronomic analytics (heading date, protein, etc.) to evaluate flavor and flavor stability based on sensory and metabolite analysis, determine beer volatile and/or non-volatile metabolite markers for flavor and flavor stability, and determine the co-varying metabolites regarding how malt genotype affects beer flavor and the associations among metabolites that influence beer flavor and flavor stability. There is increasing interest in the study of barley genetics, the influence of GE interactions, and how they affect the malting of barley and the creation of beer. The studies conducted for this thesis identified volatile and non-volatile possible biomarkers for identifying flavor and flavor stability through barley type chosen. These results suggest that, after confirmative study, barley genome identifiers could be used in agriculture to increase certain flavor and flavor stability characteristics when breeding barley for beer production.

Our closing conclusions regard these chapters' contributions to the ability to understand how genetics plays a role in the development of quality and flavor not only in malt, but in beer and distilled spirits. The potential to develop and produce high quality varieties with new and unique flavors while still meeting agronomic and quality standards lends an opportunity to expand the breadth of our knowledge of barley and malt chemistry. Therefore, we serve to arm the industry members with discernable and practical information that leads to informed decision making about a product and the type of product that will be produced.

This research could provide novel methods to predict sensory traits based on volatile and non-volatile metabolite abundances, of use to maltsters and brewers seeking greater understanding of the chemistry and interactions of raw ingredients at a molecular level. Barley malt is an essential raw ingredient to beer flavor and flavor stability. Modern malting and brewing processes should involve a deeper look into barley and malt through metabolomics,

proteomics, lipidomics, and ionomics to understand the associate of amino acids, lipids, alkaloids, volatile compounds, and other unknowns to flavor. This should involve understanding of the composition, as well as the abundance, of the compounds and how they affect beer flavor and flavor stability. Knowledge and understanding of metabolites which are related to genes and environmental circumstances could provide insight to producers of barley for crop improvements or experimental barley lines. This research suggests that designing effective malting, brewing, and distilling schemes based on a deeper understanding of barley, malt, and the finished beer will require moving beyond broad measurements of quality (such as FAN) and discerning the relationship between chemistry and behavior during the process.

Brewers should pay particular attention to malt and malt quality, as it is the interactions between high quality malt and other ingredients (hops, yeast) that create substantial flavor. Brewers should see the need to connect all of the ingredients and to choose only high-quality ingredients of which they know the interactions and results. For example, paying attention to the protein quantity and composition in malt will help the brewer make decisions regarding amounts and composition of hops to add. For example, using a low-quality malt with higher protein will not improve the flavor or character of a beer, but using a lower-protein, higher-quality flavor-forward malt will improve aroma, mouthfeel, foam retention, and overall flavor.

The future of this research involves narrowing the focus from the broad profiles and characterization of the metabolome to now applying the knowledge we have gained to specific experiments. For example, we have a plethora information on the malt metabolome, but a question now should be, how do different processing steps affect that metabolome? How can we manipulate the malting process, through steeping, germination, and kilning to influence the chemistry, which influences flavor and quality? Currently, we are addressing those questions

with experiments that involve manipulating the malting process through increasing germination and kilning times and temperatures, measuring the differences in metabolome, sensory properties, quality, and function. Information is only as good as how you use it. The questions asked by the industry are meant to be answered with applicable, timely, and thoughtful results, communication, and concern.

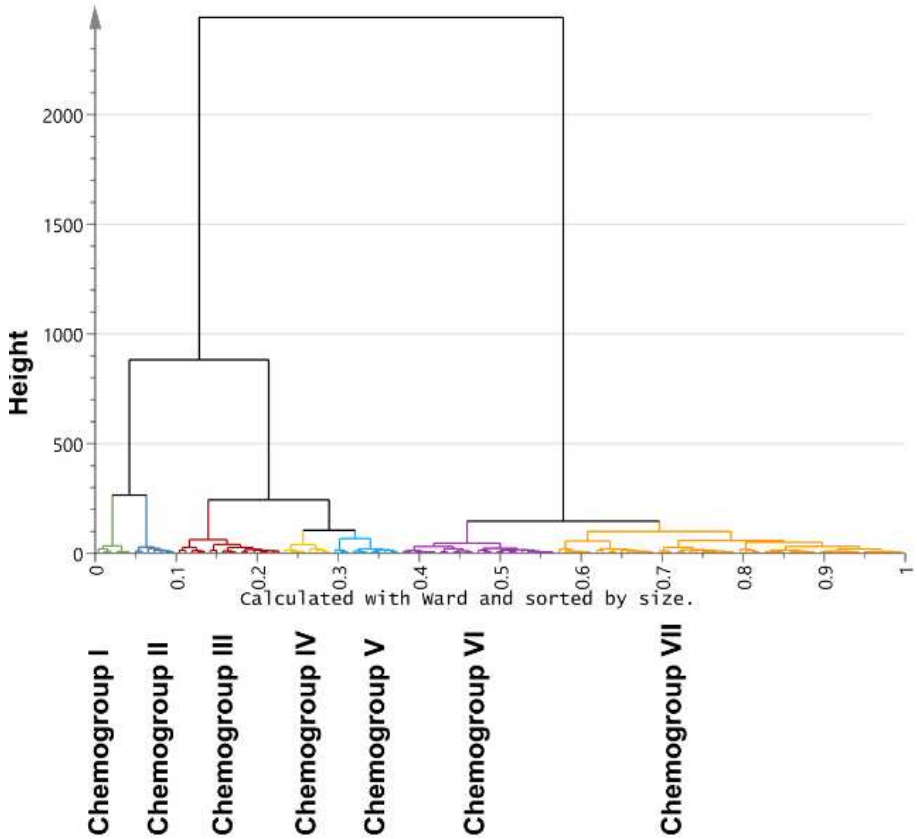

Figure S.1 The dendrogram (tree plot) displays the results of a cluster analysis from either hierarchical cluster analysis (hca). the plot shows the number of clusters as a function of the vertical coordinate of height and sorted by Ward linkage. The vertical axis indicates the loss in the variance increase when clusters are merged. The scale on the vertical axis is arbitrary. The dendrogram is colored and labeled by chemogroup.

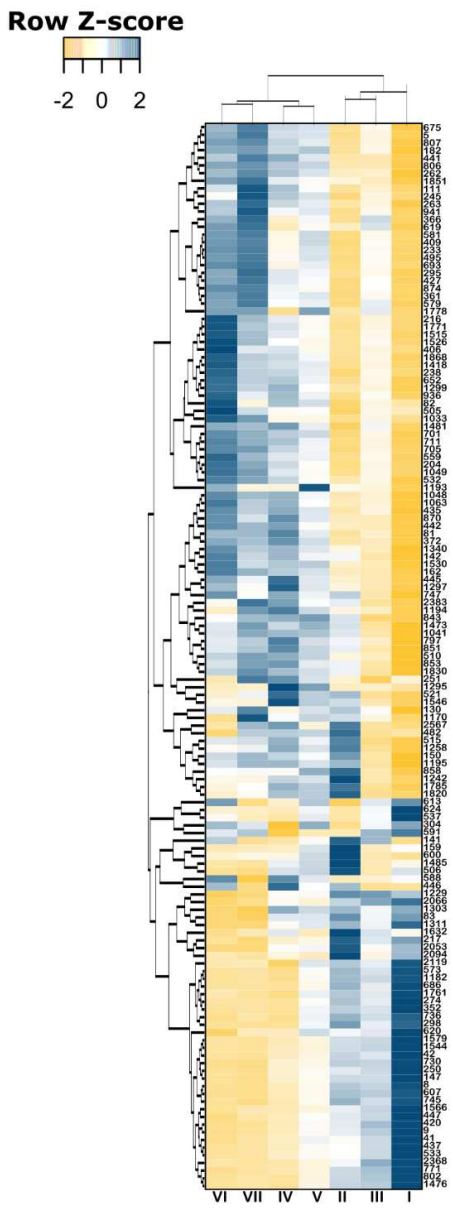

Figure S.2 Heatmap of Z-scored abundances. Prior to heatmapping, metabolite data were normalized within each variety via z-transformation normalized peak area - mean/standard deviation of total peak area of each metabolite). The resulting z-scores were converted into colors and grouped using hierarchical clustering. Heat maps with hierarchical clustering were built within chemicals classes, as described as 140 lipids. The color in each cell represents the z-transformed abundances of the averaged replicates (n = 2) per malt sample. Z-transformation was based on the mean abundance and standard deviation of the metabolite across all samples.

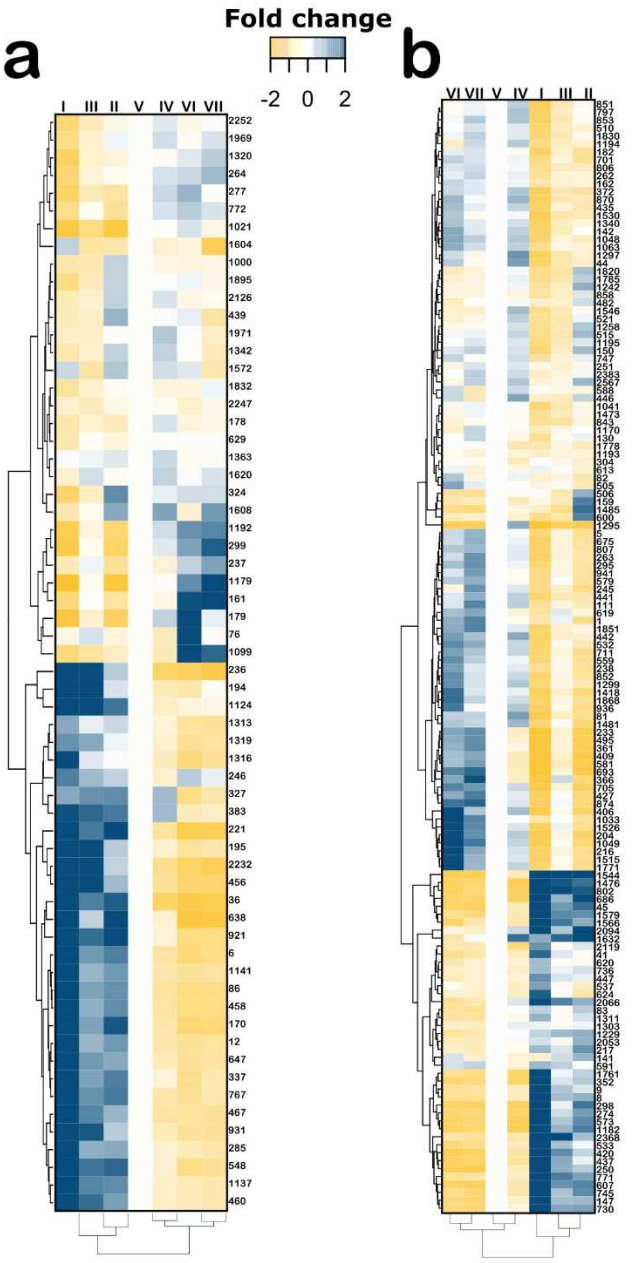

Figure S.3 Heatmap of fold change (FC) among chemogroups. Fold change (FC) for all chemogroups was calculated among all chemogroups compared to the control chemogroup (Chemogroup V). Among chemogroups, FC was calculated as the ratio of the average mean of that chemogroup divided by the control chemogroup for each metabolite. Heatmaps of (a) non-lipids and (b) lipids compared to the control chemogroup (Chemogroup V).