CHARTING A CHEMICAL ROADMAP OF TERROIR IN CORN—TOOLS FOR SELECTION OF NOVEL VARIETIES FOR WHISKEY

A Dissertation

by

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ABSTRACT

Corn is a vital ingredient to the whiskey industry, most notably as the main ingredient in bourbon whiskey. However, little research exists that explores how genetic, environment, and gene-environment interaction effects (collectively, terroir) impact corn chemistry and ultimately flavor and alcohol yield in whiskey. Here, the impact of terroir on new-make bourbon whiskey, as well as how it can be leveraged for the selection of flavor and alcohol yield in corn, was determined. A novel lab-scale distillation process, high performance liquid chromatography, gas chromatography-mass spectrometry, and quantitative sensory analyses allowed for the identification and quantification of those flavor compounds, aromas, and yield-related metrics that are impacted by terroir.

We report for the first time that alcohol yield, a variety of flavor compounds, and ultimately aroma are indeed impacted by terroir in new-make bourbon whiskey. Certain metabolites in corn, mash, and beer were identified as significant predictors for alcohol yield and flavor chemistry in new-make bourbon whiskey, providing chemical makers that can be implemented in a breeding program. Notably, it appears that benzaldehyde in corn, ferulic/coumaric acid in mash, and total sugar concentration in mash can serve as markers for various flavor compounds and alcohol yield in new-make bourbon whiskey.

DEDICATION

I dedicate this dissertation to my son Geo Arnold. I hope you enjoy a passion for lifelong learning and the happiness it brings. I dedicate this dissertation to my wife Leah Arnold. You displayed a ridiculous amount of patience through the countless hours where I couldn't peel my eyes away from a computer screen. I dedicate this dissertation to my parents Anne and Bobby Arnold. I always knew no matter what my career brought that you would be proud of me. I dedicate this dissertation to my grandfather Walter Arnold. You gave me my first garden, which surely planted the seeds for the ideas researched here. Lastly, I dedicate this dissertation to the bourbon industry veterans in my family. My great-uncles Bobby Panther and Richard Panther, my uncles Rick Panther and Craig Panther, and my grandfather Louis Panther. You paved a trail for me, and I'm proud to carry the torch, from Kentucky to Texas and beyond.

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Contributors

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All other work conducted for the dissertation was completed by the student independently.

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1. INTRODUCTION: THE BIRTH, LOSS, AND REMERGENCE OF TERROIR IN WHISKEY

Whiskey is a distilled spirit made from grain and (almost always) aged in oak barrels. Grain here refers specifically to fruit from the cereal grasses (namely, barley, corn, wheat, and rye), and in some rare cases from the pseudocereals. Whiskey is essentially distilled beer, just as brandy is distilled wine. While whiskey and wine share many of the same overarching production processes, there is one major phenomenon that is championed in wine and largely neglected in whiskey—terroir. While the exact definition of terroir—and its impact on wine and flavor—is debated by connoisseurs, critics, and academics alike, it is widely accepted that the phenomenon is responsible for many of the flavor variations that exist among all wines.

Terroir is a French word that stems from the Latin word *territorium*, which can roughly be translated as territory or an area of land with defined boundaries. The exact definition has evolved over the generations. As early as the seventeenth century, the word terroir simply referred to a territory or a region. But by the nineteenth century it referred to a "small area of land being considered for its qualities or agricultural properties." [1]. In his 1884 collection of essays *Les grotesques*, the French poet and dramatist Théophile Gautier described a hill with thin and rocky soil that produced excellent claret, a type of French rosé wine [2]. He used the word *terroir* as a catch-all for the entire set of characteristics of that hill.

The European Union established a legal definition in 2012. They claimed that products possessing terroir are those "whose quality or characteristics are essentially or exclusively due to a particular geographic environment with its inherent natural and human factors." [3].

From the perspective of these various definitions, terroir appears to refer to all phenomena that occur on the farm, such as, but not limited to soil conditions, topography, climate, fertilizer, pesticides, agronomic techniques, and irrigation. Considered as such, terroir is nothing more than a romantic synonym for the environment effect. However, given that the notion of terroir in wine is inseparable from the grape variety, terroir in whiskey can more accurately be seen as the interplay of how grain varieties express flavor in the context of their environment, which would fall in-line with the gene-environment interaction effect, in addition to the genetic and environment main effects.

Aside from the word itself, the concept of terroir has been rooted and praised in winemaking since at least the 1st century AD [1], and its importance has held true to modern times. Indeed, the wine industry has increasingly based label information and style differentiation on terroir. The situation with whiskey, though, is quite different. While it can be argued that terroir was an important, inescapable consideration in whiskey before American prohibition, it was largely forgotten and ignored throughout the 20th century, as plant breeders, farmers, and distillers focused on agronomic and ethanol yields, often at the expense of flavor [4].

That said, few would argue that whiskey is a beverage whose flavor is low in quality or diversity. On the contrary, whiskey is often viewed as being one of the most complex of beverages. So why, then, should we be concerned about what role terroir might play in whiskey? Perhaps it is nothing more than a marketing buzzword. But if terroir is indeed more than that, and if science can support and elucidate its role in whiskey while concurrently allowing plant breeders, farmers, and distillers to competently pursue it, then terroir might potentially hold the key to unlocking new and forgotten flavors.

While any grain species and the subsequent styles distilled from them can be studied and considered in the context of terroir, this dissertation will focus on corn (*Zea mays* L; commonly maize in much of the world). Arguably, the most popular style of whiskey produced primarily or solely from corn is *bourbon whiskey*, which can only be produced in the United States (US). But it should be noted that many Canadian, Scottish, and Irish *grain whiskies* are also produced from corn, as well as the US style known as *corn whiskey*.

1.1. Background on corn utilization in whiskey production

The US whiskey industry is dominated by whiskey styles that by law must contain corn as the main fermentable substrate, or that by choice use corn as a substantial secondary ingredient. *Bourbon whiskey* (or simply *bourbon*), per the Standards of Identity for Distilled Spirits (Title 27, Part 5, Subpart

C of the US Code of Federal Regulations), must contain at least 51% corn. However, most *bourbon* brands utilize 70–80% corn. *Corn whiskey*—a much less popular yet still important style—must contain at least 80% corn, with the barrel maturation process differentiating *bourbon* and *corn whiskey*. *Rye whiskey* was the most popular style in the US throughout the 18th and 19th centuries, and it has seen a recent resurgence, largely due to the recent rise in Prohibition-era cocktails. This style must contain at least 51% rye, and many *rye whiskey* brands do indeed utilize near the minimum rye requirement, with corn making up anywhere from 30 to 40% of the recipe. *Wheat whiskey* follows the same trend as rye, in that wheat must be the majority grain, but corn is still present at a fairly high percentage.

Ultimately, corn is a vital ingredient in many of the US's most popular whiskeys, and the Canadian whiskey industry is similar in this aspect. Even though rye is often championed in Canadian brands, 90% of the grain used by the Canadian whiskey industry is corn [5]. Being that corn is a grain native to North America that was domesticated in Mexico [6, 7], it is fitting that both the US and Canadian whiskey industries rely primarily on it as the fermentable substrate. In Scotland and Ireland, two of the largest national producers of whiskey, barley grows favorably. Consequently, raw barley is a primary grain in the Irish style known as *pot still whiskey*; and barley's downstream derivative barley malt (or malted barley, or often referred to solely as malt) is the dominant grain for Scotch and Irish *malt whiskeys*. However, corn still has a place in these industries. Scotch and Irish *grain whiskeys*, which are the main component styles used to create Scotch and Irish *blended whiskeys*, were previously made primarily

with corn. In the 1980s, wheat replaced corn in this facet [8]. However, the North British Distillery Company Ltd in Scotland—whose whiskey product is the main component of Johnnie Walker Scotch *blended whiskeys* (top selling Scotch whiskey worldwide) and Famous Grouse (top selling whiskey in Scotland, and top 10 worldwide)—still utilizes corn as their base ingredient (www.thenorthbritish.co.uk [obtained: June 2020]). Also, the Irish *blended whiskey* Jameson (top selling Irish whiskey worldwide) uses corn as the base for its grain whiskey component (www.jamesonwhiskey.com [obtained: June 2020]). Ultimately, although corn is most championed in *bourbon*, it is one of the most prevalent grains in international whiskey production.

Although corn is such an important ingredient in whiskey production, there are few previous reports on how genetics (i.e., variety) and environmental factors (e.g., soil conditions, climate, topography, agronomic management, and seasonal fluctuations) of corn impact flavor—or even just ethanol yield—in whiskey. Prior to this dissertation and the research papers and popular science book (Sections 2-4 below) that came from it, the only other reports found through an extensive literature search were limited to the following: one that focused on agronomic yield relevant to whiskey distillation [9], and four that focused on ethanol yield [10-13]. To our knowledge, no studies have yet been conducted that investigate how corn variety (G), growing environment (E), and/or their interaction (GxE) impact whiskey flavor.

The suitability of different barley and wheat varieties and growing environments for whiskey has received slightly more attention than corn, although the research is still limited. Many of these studies have focused on how G, E, and GxE impact agronomic

yield. There are a few examples in the literature where alcohol yield was investigated [8, 14-18], but none that have addressed flavor in whiskey. There are a limited number reports where flavor was addressed in beer [19-23], however, which is essentially the intermediate step between grain and whiskey. And there are indeed some barley and wheat cultivars that were developed for (either solely or partly) the whiskey industry [24, 25]. But to date, there are no corn cultivars that have been developed or highlighted for whiskey production. The craft distilling movement has revitalized the use of open-pollinated heirloom varieties, and while these heirlooms were created through recurrent selection by farmers who would have likely considered flavor, it's unlikely that they were selected solely for whiskey production.

Current protocol among nearly all large-scale bourbon distilleries (the majority of which are located in Kentucky) is to utilize commodity yellow dent hybrid corn [5], which is commonly referred to as field corn. While large-scale distillers will specify a certain grade (at least #2 in the US, which is a grade that requires certain quality standards set by the United States Department of Agriculture) to ensure acceptable test weight, moisture level, foreign material, and broken/damaged kernels, they will rarely specify the specific variety of corn. And as recently as 2014, only 40% of the 15-20 million bushels of corn used by the Kentucky bourbon industry was grown in their home state. The other 60% came from other corn belt states, primarily Indiana. Regardless, whether of Kentucky or Indiana origin, the corn used by Kentucky bourbon distillers is largely a product of the commodity grain system and with varieties developed for the commodity system. Ultimately, the blending nature of the commodity grain system—

which does not separate varieties and farms—means that flavor nuances from terroir are lost.

Comparing this disregard for variety and growing environment consideration to the wine industry, it would be analogous to winemakers deciding to make a red wine, and instead of requesting or growing a certain red grape cultivar (e.g., merlot, syrah, pinot noir, etc.) and/or vineyard location (e.g., Napa, Bordeaux, Sonoma, etc.), they would only concern themselves with the color of the grape (red) and some general (but not flavor related) quality specifications. Winemakers, of course, do concern themselves with grape variety and vineyard location, and they label their wines accordingly. The diversity of flavors among wine grape cultivars is extensive, and therefore many wines are categorized and labeled as a varietal based on their grape cultivar (e.g., merlot). Those that are not labeled as a varietal are usually labeled by where the vineyards were located (e.g., Napa Valley, and perhaps even more specific, such as the St. Helena appellation within Napa Valley). And some wines are labeled by both the varietal and the vineyard location (e.g., Cabernet sauvignon from Napa Valley).

1.2. Terroir in modern, high-yield, hybrid, genetically similar corn varieties

Even if whiskey distilleries did wish to utilize specific corn varieties from specific farms (i.e., utilize *identity preserved* corn) in an effort to achieve greater and more consistent alcohol yield and flavor, there has been no reported scientific evidence that the effort would produce desirable or meaningful results. Moreover, the scientific literature on the reduced genetic diversity of modern corn varieties might suggest the

opposite, as they might not harbor enough genetic variation for flavor distinctions caused by G, E, and GxE (collectively, terroir) to be realized. As desired agronomic performance traits—such as yield—were pursued in corn, the genetic variability of the species declined [26]. Reports indicate that the majority of recently developed corn inbred lines utilized in American breeding programs are products of a small, stratified, and closed germplasm base [27-29]. Additionally, US Corn Belt germplasm can be traced to a narrow range of populations from only two races—the Northern Flint and the Southern Dent [30, 31].

Alcohol yield and flavor are quantitative traits, with many different genes and environmental stimuli influencing the final phenotype [32, 33]. Alcohol yield is primarily correlated with grain starch concentration, the starch composition (i.e. ratio and composition of amylose and amylopectin), and the starch's propensity to by hydrolyzed by amylases into sugar during mashing [18]. Grain-derived flavors in whiskey can be introduced through multiple pathways. Different sugar, amino acid, fatty acid, and secondary metabolite concentrations and compositions will impact the production of flavor compounds by yeast during fermentation. Also, grain-derived compounds can undergo reactions (such as thermal degradation, chemical and enzymatic oxidation, Maillard reaction, and Strecker degradation) during whiskey processing, which will ultimately deliver flavor compounds to whiskey. Lastly, secondary metabolites (such as pyrrolines, thiazolines, pyrazines) produced by grain can potentially impact whiskey flavor directly [34, 35].

If genetic diversity is limited in the relevant pathways among modern yellow dent hybrids, then variety and environment might not greatly influence alcohol yield and flavor in commodity yellow dent hybrid corn. Conversely, if there is still sufficient genetic diversity among these varieties or elsewhere, then both variety and environment could have an impact on alcohol yield and flavor, as the relevant genes and how they respond to environmental stimuli would vary. Furthermore, we can expect that the genetic diversity of the species at large—for instance, the other nearly 100 recognized races of corn that exist throughout the Americas or 20,739 accessions in the USDA-ARS National Germplasm Repository—might contain novel flavor profiles far beyond what is currently available in commodity yellow dent hybrid corn [36, 37].

That said, while genetically diverse varieties—such as heirlooms, landraces, and other corn open pollinated varieties (OPVs)—might show greater flavor diversity due to terroir, the reality is that most "mainstream" bourbon will continue to be produced from high-yielding hybrid corn varieties because of the low expense and ease of obtainment. While heirloom grains do supply a niche in the whiskey industry, on the farm they might have a grain yield only half of modern hybrid varieties. The reality is that the vast majority of farmers and distilleries—especially those producing tens to hundreds of thousands barrels annually—can't drastically sacrifice yield (especially a 50 percent one) for flavor. Both yield and flavor must be considered as important selection criteria.

Ultimately, the utilization of a specific variety of corn grown in a specific environment will increase cost and require additional logistics for the distillery. It would require the distillery to identify a farmer(s) and identity preserve a silo(s), be it one that

they build and operate, or one that is managed by a grain elevator. To justify such an endeavor, there must be evidence to show that corn variety and environment can significantly impact alcohol yield and whiskey flavor; or in the future marketing potential including sustainable production.

Section 2 will describe the research conducted to explore the potential for terroir to impact the flavor of new-make bourbon whiskeys produced from high-yielding yellow dent hybrid corn varieties grown on three to four farms throughout Texas. Section 3 will reveal a chemical roadmap in whiskey that can be used to as a guide to leverage the terroir effect and select for flavor. Section 4 will explore variations in precursor metabolites and mash/beer and new-make bourbon whiskey produced from genetically diverse varieties. Further, it will provide a proof-of-concept for selection of whiskey flavor through metabolite precursors in grain.

2. ASSESSING THE IMPACT OF CORN VARIETY AND TEXAS ENVIRONMENT ON FLAVOR AND ALCOHOL YIELD IN NEW-MAKE BOURBON WHISKEY*

2.1. Selection of corn varieties and growing environments

This research investigated the variation in alcohol yield and whiskey flavor among three high-yielding yellow dent hybrid corn varieties (commercially available varieties in 2016) grown in three to four different experimental field plots in Texas [38]. Only environments within Texas (the 11th largest producer of corn in the U.S., USDA-NASS) were considered for this research because of the funder's interest in sourcing local, Texas-grown corn. However, four very different environments across different regions within Texas were chosen (Table 1).

*Reprinted with permission via the Creative Commons Attribution license from "Assessing the impact of corn variety and Texas terroir on flavor and alcohol yield in new-make bourbon whiskey" by Arnold et al, 2019. *PloS one*, 14.8, e0220787, Copyright 2019 Arnold et al.

Table 1. Characteristics of the different growing locations

Farm	County	District	Soil Type	Planting Date	Harvesting Date	Plants $hectare^{-1}$	Irrig-ation	RW	Crop Rotation
Texas AgriLife Extension	Calhoun	Coastal Bend	Livia Silt Loam	2/26/2016	8/3/2016	53,987	No	38	Grain Sorghum
Rio Farms	Hidalgo	South	Raymondville Clay Loam	2/18/2016	7/21/2016	57,027	Yes	30	Soybeans
Sawyer Farms	Hill	Central	Houston Black Clay	2/15/2016	8/15/2016	64,218	No	30	Wheat
Texas AgriLife Extension	Hansford	Panhan-dle	Perryton Silty Clay	5/11/2016	10/11/2016	75,012	Yes	30	Soybeans

RW = average row width in inches between rows. Sawyer Farms is the only commercial grower, with other locations being sites of the Texas A&M (TAMU) Corn Variety Testing Program. Reprinted from Arnold et al, 2019.

The goal of this study was to understand the extent to which terroir (G, E, and GxE) can impact alcohol yield and flavor across a range of yellow dent hybrid varieties and Texas environments. This would be infeasible to evaluate at a distillery scale, so a repeatable small batch evaluation procedure first needed to be developed. G, E, and GxE were treated as random effects so that the results can be extrapolated to more situations than just the three varieties and four environments considered here.

2.2. Materials & methods

2.2.1. Mash, beer, and new-make bourbon production and analyses

New-make samples (i.e. unaged whiskey that is the immediate by-product of distillation) were produced from three varieties of yellow dent hybrid corn obtained from the Texas A&M Corn Variety Testing Program and also from one commercial grower.

The three varieties (D57VP51—Dyna-Gro; 2C797—Mycogen Seed; REV25BHR26 Terral Seed) were grown in three different locations in 2016 (Texas AgriLife Extension, Calhoun County, Texas; Rio Farms, Hidalgo County, Texas; Sawyer Farms, Hill County, Texas); an additional location (Texas AgriLife Extension, Hansford County, Texas) was selected to grow one of the varieties (REV25BHR26—Terral Seed). The four farms were chosen in an attempt to highlight the diversity of environments in Texas, all within different districts of the Texas A&M AgriLife Extension Service, consisting of varying soil types and agronomic techniques (Table 1).

For the lab-scale milling, mashing, fermentation, and distillation processes, methods were aligned with a laboratory procedure previously developed by the Scotch Whisky Research Institute (SWRI), known to produce a new-make spirit that is comparable to that produced via industrial instrumentation and processes [10, 39, 40]. Where SWRI methods were created to mimic typical Scotch whisky grain distillery operations, our methods were adapted to more closely simulate typical bourbon whiskey distillery operations.

For processing each batch, whole corn kernel samples were initially sieved through a 0.48 cm round commodity hand sieve (Seedburo Equipment Company) to remove broken kernels. Foreign material and heat-damaged kernels were manually removed via inspection against white paper. The remaining kernels were then milled using a Victoria Plate Mill and then sieved 3X through a 2000 micrometer screen to ensure that the milled grain was fine and consistent from batch-to-batch. A 3 L beaker was filled with 1750 g of carbon-filtered municipal water. A mechanical mixer (100W-

LAB-SM, Gizmo Supply Co.) was used for agitation, and the temperature of the water was brought to 65˚C using a 120V hot plate with infinite heat controls (CSR-3T, Cadco) set to medium. Then 448 g of milled corn and 2 mL of high-temperature alpha amylase (AHA-400, FermSolutions Inc.) were added to the beaker. A cover slip that still allowed the mechanical mixer to operate was placed on top of the beaker to prevent excessive evaporation. The temperature of the mash was brought to 85˚C and held for 1.5 h. After incubation, an ice bath was used to indirectly cool the temperature of the mash to 32˚C. Once 32˚C was achieved, 1.5 mL of glucoamylase (GA-150, FermSolutions Inc.) was added. Immediately after, 0.26 g of active dry yeast (Species: *Saccharomyces cerevisiae*; Strain: RHB- 422, F&R Distilling Co.'s proprietary strain) was added. The same strain was used for all batches, and the concentration of yeast used was based on standard inoculation rates for the whiskey industry, ensuring the role of other microbial organisms was minimal. The mash was further cooled to 24˚C using an indirect ice bath and mixed for an additional 10 min. Using aseptic techniques, pH was recorded with a digital pH meter (pH 220C, EXTECH) and specific gravity was recorded using a digital density meter (SNAP 50 density meter, Anton Paar). Further, a 25 mL sample was removed and stored at -20˚C for high performance liquid chromatography (HPLC) processing. Mixing was then halted, the mash was transferred to a 2.7 L Fernback flask that had been sanitized with Star-San (phosphoric acid based, no rinse sanitizer), and the flask was covered with flame sterilized aluminum foil. Fermentation proceeded for 120 h at room temperature, with pH and specific gravity recorded twice during fermentation, and also at the end of fermentation. Further, 25 mL samples were removed at the same time

points and stored at -20˚C for HPLC processing. Measurements for all 30 treatments were only recorded for Day 0 and Day 5. The treatments recorded for Day 1 (n = 17), Day 3 (n = 15), and Day 4 (n = 27) were chosen at random (Day 2 is not shown due to insufficient data). Three Day 5 outliers were identified based on discrepancies between alcohol yield and ethanol concentration. These outliers were removed from a portion of the analyses. The fermented mash, now called "beer", was frozen at -20˚C.

Specific gravity, a measure of density, provides an estimate of fermentable substrate (monosaccharides, disaccharaides, and trisaccharides) and unfermentable substrate (dextrin and starch) yielded via the mashing process (Day 0), the level of attenuation (i.e. the conversion of sugars into alcohol and carbon dioxide by yeast) throughout fermentation (Day 1–4), and the level of attenuation at the end of fermentation (Day 5). The specific gravity (or other corresponding measures of density, such as brix and plato) is one of the most common measurements taken in a distillery, and it is especially important to measure after mashing and during fermentation, as it provides quick and robust insight into process efficiencies. However, specific gravity is ultimately tied to soluble dextrins and sugars, which is why we also conducted follow-up HPLC analyses to quantify these compounds individually.

Beer was rapidly thawed, and 1.65 L was added to the stripping still, which was a stainless steel still with an air fan cooled condenser and an electric, indirect heating element (Air Still, Still Spirits). Distillation proceeded until 550 mL of distillate (termed "low-wines") was collected in a grade A volumetric flask. The alcohol concentration by volume of the low-wines was measured using a density meter (DMA 5000 M, Anton

Paar). Using weight, low-wines were diluted to the desired alcohol concentration with the addition of water. The spirit still, which was a copper alembic style still with a worm coil condenser and no innate heating element (heat was be supplied using the Cadco CSR-3T 120V hot plate with infinite heat controls and set to medium for the spirit run), was charged with 500 mL of low-wines. The condenser was filled with ice water. Distillation commenced, and the first 25 mL of distillate (termed the "heads") was collected using a grade A volumetric flask. Using a different grade A volumetric flask, the next 100 mL of distillate (termed the "hearts") was then collected. The condenser was monitored to ensure the temperature of the distillate was consistent from batch-to-batch. The hearts distillate was then stored in Boston round glass bottles with inert caps at room temperature until further processing.

Both stills were cleaned throughout the experiment according to the following methods in order to ensure that the organic residue was not carried-over from batch-to-batch, as well as to ensure that the impact of copper would be consistent from batch-to-batch. These methods were also developed with guidance from the Scotch Whisky Research Institutue. Before experiment commencement and after at least every 3rd distillation, the stainless stripping still was cleaned by distilling 2% (80 mL of 50% caustic topped off to 2 L) caustic solution (50286, Chemstation) for 30 min, then scrubbed with an abrasive pad, and finally washed thoroughly with RO water. Before commencement and after at least every 3rd distillation, the copper spirit still was cleaned by distilling 2% (40 mL of 50% caustic topped off to 1 L) caustic solution (50286, Chemstation) for 15 min. The heat was then turned off and the caustic was soaked for an

additional 15 min, after which the still pot and swan neck were scrubbed with an abrasive pad and washed thoroughly with RO water.

This experimental design resulted in ten treatments (3 corn varieties x 3 environments + 1 corn variety/1 environment [REV25BHR26 from Hansford County, TX]), and each treatment was repeated three times, creating 30 batches total.

2.2.2. HPLC analysis of mash and beer

HPLC was used to detect compounds DP4+ (dextrins), DP3 (maltotriose), maltose, glucose, lactic acid, glycerol, acetic acid, and ethanol in mash and beer samples at various time points. Each of the 30 batches were analyzed at various timepoints with HPLC, with each timepoint being analyzed in triplicates. The HPLC triplicates were assessed to ensure the relative standard deviation was below 0.5% and then averaged to achieve a final value for statistical analysis. Standards were run before every monitored timepoint. The standard for the HPLC was Ethanol Industry HPLC Standard (Midland Scientific Inc., La Vista, NE, USA), and includes the following compounds: DP4+ (dextrins), DP3 (maltotriose), maltose, glucose, lactic acid, glycerol, acetic acid, and ethanol.

All HPLC analyses in this study were executed as described previously [41]. Briefly, samples were centrifuged at 4000 x g using a desktop centrifuge, and then filtered through a 0.22-µm membrane filter. An autosampler vial containing at least 0.5 mL of the sample was analyzed by HPLC using a Shimadzu LT-20AT (Shimadzu USA, Canby, OR). The separations were carried out using a Rezex ROA-Organic Acid H+ 8%

(300Å~7.8 mm, 5 µm, Phenomenex, Torrance, CA, USA). The HPLC analysis was performed in isocratic mode with a mobile phase of 0.005 N sulfuric acid using vacuum sealed, pre-made solvent (Chata Biosystems). The analytes were detected by refractive index (RID-20A, Shimadzu USA, Canby, OR).

2.2.3. New-make bourbon and corn descriptive sensory analysis

A human sensory panel was used in this research. All participants signed a written consent form after being walked through their rights as participants, and the Texas A&M institutional review board specifically approved the study (IRB Number: IRB2016-0842M).

A whiskey lexicon was developed based on 28 commodity spirits (14 whiskeys from different grain origins, 15 miscellaneous spirits) and 21 new-make spirits. The focus was on whiskey and new-make whiskey, but other miscellaneous spirts (cachaça, vodka, rum, ouzo, vermouth, gin, Sambuca, flavored liqueurs, triple sec, and amaretto) were used to cover attributes not commonly found in whiskey or new-make spirits. Other sources used to develop attributes were from new-make spirit published literature [42-57] and existing, published lexicons [58, 59] to encompass alcohol and spirits, but the developed lexicon focused on flavors and aromas found in new-make bourbon. New-make bourbon and corn were evaluated by a 7-member, expert trained whiskey aroma descriptive attribute panel that has over 20 years of experience in descriptive sensory attribute evaluation across food products. Aroma analysis allows for a nearly full assessment of a whiskey's flavor, negating any effects of alcohol ingestion, and

therefore is the main form of sensory evaluation used in the industry [60]. This panel helped develop and was trained using the new-make bourbon lexicon for 31 days followed by a validation trial prior to testing. Following the completion of the new-make bourbon samples, panelists trained for 3 days on corn samples using the new-make bourbon lexicon. Whiskey and corn aroma attributes were measured using a new-make bourbon lexicon (0 = none and 15 = extremely intense) that was specifically developed for this research. After training was complete, panelists were presented three to four new-make samples per day for 8 days, and six corn samples a day for 5 days in a two-hour session. Panelists evaluated new-make samples individually and reached consensus on attributes and intensities.

Prior to the start of each trained panel corn evaluation day, panelists were calibrated using one orientation or "warm up" sample that was evaluated and discussed orally. After evaluation of the orientation sample, panelists were served the first sample of the session and asked to individually rate the sample for each corn/new-make bourbon aroma lexicon attribute. References were available at all times during training and evaluation. Steamed cotton towels were available for cleansing the nasal palette during evaluation of samples. New-make samples were prepared no more than 30 minutes prior to serving by diluting the new-make bourbon (~125 proof, 62.5% alcohol by volume) with double-distilled water to testing strength used in the industry (40 proof, 20% alcohol by volume [60]). Each panelist was served 8 mL of the diluted sample in a nosing glass (grappa or tulip glass), with a watch glass to concentrate volatiles. Corn samples were ground one hour prior to serving. Each panelist was served 10 g of milled

corn sample in a medium snifter glass covered with a watch glass to concentrate volatiles. Samples were identified with random three-digit codes and served in random order.

2.2.4. New-make bourbon and corn flavor compound identification and quantification

Volatiles were captured from the same new-make bourbon and corn samples evaluated by the expert, trained descriptive panel. After samples were prepared for panelists, approximately 80 g of new-make bourbon and 40 g of corn were placed in glass jars (473 mL, new-make; 236 mL, corn) with a Teflon lid under the metal screw-top to avoid off-aromas. The headspace was collected with a solid-phase micro-extraction (SPME) portable field sampler (Supelco 504831, 75 µm Carboxen/ polydimethylsiloxane, Sigma-Aldrich, St. Louis, MO, USA). The headspace above each new-make and corn sample in the glass jar was collected for 2 hours for each sample at room temperature at approximately 21°C; new-make samples were mixed at low speeds on a laboratory stirrer hot plate (Model P.C.- 351,120 V, Corning Glass Works, Corning, NY, USA).

Volatiles were evaluated using a gas chromatograph/mass spectrometer system with dual sniff ports for characterization of aromas (GC-MS/O). This technology provided the opportunity to separate individual volatile compounds, identify their chemical structure and characterize the aroma/flavor associated with the compound. Upon completion of collection, the SPME was injected in the injection port of an Agilent

Technologies (Santa Clara, CA, USA) 7920 series GC where the sample was desorbed at 280°C. The sample was then loaded onto the multi-dimensional gas chromatograph into the first column (30m X 0.53mm ID/ BPX5 [5% Phenyl Polysilphenylene-siloxane] X 0.5 µm, SGE Analytical Sciences, Austin, TX, USA). The temperature started at 40°C and increased at a rate of 7°C/minute until reaching 260°C. Upon passing through the first column, compounds were sent to the second column ([30m X 0.53mm ID; BP20-Polyethylene Glycol] X 0.50 µm, SGE Analytical Sciences, Austin, TX, USA). The gas chromatography column then split into three different columns at a three-way valve with one going to the mass spectrometer (Agilent Technologies 5975 Series MSD, Santa Clara, CA) and two going to the two humidified sniff ports with glass nose pieces heated to 115°C. The sniff ports and software for determining flavor and aroma were part of the AromaTrax program (MicroAnalytics-Aromatrax, Round Rock, TX, USA). The GC-MS/O set-up could host two operators, and to keep a human variable constant, the same two operators always evaluated the volatiles. These two flavor chemistry research technicians underwent sensory training using the lexicon developed here; were trained on aroma identification, quantification, and GC-MS/O operation; and had previously analyzed over 500 hours of GC-MS/O samples. Each operator was trained to accurately use the Aromatrax software to indicate where an aroma event was present. Only those volatile compounds that were present during an aroma event (where any detectable aroma was present at the sniff port) were kept for analysis. Aroma identity was not collected for each of the volatile compounds.

The MS detected ions within 35–300 m/z range in the electron impact mode at 70 eV. Chromatography data was collected in the scan mode (Agilent MSD Chemstation E.02.02.1431 software, Agilent Technologies, Santa Clara, CA, USA). Volatile compounds with at least 1200 total ion counts (area under the curve) and a quality score above 75 (based on its match to the NIST library) and were present during an aroma event were kept for analysis. Units of measure were total ion count (TIC) area under the curve and compound identity was based on the NIST library. For verification of volatile compound identification (via retention times) and quantification, alkane standards (C7 to C30; Catalog #49451-U; Sigma Aldrich, St. Louis, MO, 63103) were run prior to and after experimental samples to verify the retention times and concentrations were consistent among samples.

2.2.5. Proximate analysis of corn

Proximate analyses of corn samples were determined from each variety-environment treatment. Fourier Transform Near-Infrared Reflectance (FT-NIR) Spectroscopy was used for predicted values of protein, starch, and lipid of the corn samples. Whole kernels and ground corn samples were evaluated with a Thermo Scientific Antaris II FT-NIR (Thermo Fischer Scientific) using a sample spinner cup that held approximately 175g of whole kernel corn. Preparation of ground samples was as described previously [61]. Approximately 175 grams of each corn sample were ground to 2 mm using a Polymix PX-MFC 90 D mill (Kinematica Ag, Eschbach, Germany) and further ground using a Cyclone sample mill (UDY Corporation, Fort Collins, CO, USA)

to 1-mm fineness. The first set of 10 whole corn samples were run in triplicate with 128 scans and 10 ground corn samples were run in triplicate with 64 scans at ambient temperature. Reflectance measurements were taken by using a rotating cup that holds approximately 175g of corn over the instrument's integrating sphere module. Approximately, 3000 points across the spectrum, every 4 wave numbers, were collected for each sample scanned at a spectral range between 10,000 to 4,000 cm^{-1}. The predictions were made with calibrations created using primarily Texas grown corn and wet chemistry performed by Ward Laboratories (Kearny, NE, USA). Whole and ground kernel calibrations were developed using the same samples, ground kernel calibrations are better but are also destructive to the grain.

2.2.6. Statistical analyses

The goal was to attribute variability to variety, environment, and the interaction of these effects, as we were attempting to draw conclusions for all possible levels of Texas environments and commercial yellow dent hybrids. Our interest was not solely concerned with the levels of Hansford County, Hidalgo County, Hill County, and Calhoun County for environment; or the levels of D57VP51—Dyna-Gro, 2C797—Mycogen Seed, and REV25BHR26—Terral Seed for variety. Therefore, the data was analyzed as a completely randomized design, using variety, environment, and their interaction as random effects for all Restricted Maximum Likelihood (REML) models using JMP12 (SAS Institute, Inc. Cary, NC, USA). Variance components and percent of total variance were obtained from these REML analyses, which were used to explain the

impact of variety and environment on variation. Correlation probabilities, analysis of variance (ANOVA), and regression analyses were obtained using JMP12 (SAS Institute, Inc. Cary, NC, USA).

2.3. Results & discussion

2.3.1. Corn analysis

Protein, fat, and starch

FT-NIR was used to measure the percentage of protein, fat, and starch (dry basis) in both whole and ground corn kernels. Whole kernel analysis is the most common method used by distillers when analyzing kernels after harvest, before loading into a silo for storage, and upon delivery to the distillery.

Whole corn analysis showed that nearly 85% of the experimental variation in protein among the treatments was due to variety, environment, and interaction effects (Table 2); the rest of the variation was residual, also known as unexplained error variance, and here reported as replicates nested within variety and environment. However, environment was responsible for 0% of the variation in fat and starch variation, and instead variety and interaction effects accounted for over 75% of the variation.

Table 2. Percent of total variance for proximate analysis of corn kernels as determined through REML

Effect	Whole Corn			Milled Corn		
	Protein %	*Fat (Oil) %*	*Starch %*	*Protein %*	*Fat (Oil) %*	*Starch %*
Environment	39.1%	0%	0%	60.8%	49.9%	2.7%
Genetic	26.5%	61.6%	28%	17.4%	32.8%	9.4%
GxE	19.1%	16.6%	48.4%	21.5%	15.5%	85.6%
Error	15.3%	21.8%	23.6%	0.3%	1.8%	2.3%
Sum Total Variance	100%	100%	100%	100%	100%	100%
Units	Mg g^{-1}	Mg g^{-1}	Mg g^{-1}	Mg g^{-1}	Mg g^{-1}	Mg g^{-1}
Mean Value +/- SE	7.86 +/- 0.46	4.30 +/- 0.14	68.02 +/- 0.41	9.35 +/- 0.38	3.21 +/- 0.19	69.01 +/- 0.20
Observations (n)	30	30	30	30	30	30

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Distillers grind kernels in a mill to create a grist prior to mashing. Nearly 100% of the experimental variation in milled corn protein was due to variety, environment, and interaction effects, with residual variation having essentially no role (Table 2). The reduced residual is almost certainly due to greater precision of the milled corn. Unlike with whole corn analysis, environment was responsible for variation in fat, with variety and interaction effects having a lesser but still substantial role. Variation in starch was largely due to interaction effects, with environment, variety, and residual effects playing a small role in variation.

These results for milled corn analysis were not well aligned with whole corn analysis. It is well known that grinding helps to homogenize samples, improving results in near infrared reflectance spectroscopy, and that these particular FT-NIRS calibrations and predictions work better in ground samples than whole samples [61]. While discrepancies in variance components between whole corn and milled corn exist, the proximate analysis results indicate that variety, environment, and interaction effects are responsible for most of the variation in protein, fat, and starch levels. Given that starch ultimately dictates the amount of alcohol that can be produced, and that protein and fat

are potentially important for flavor (e.g., amino acids are important for fusel alcohol production via the Ehrlich pathway [62]), these results suggest that alcohol yield and flavor could be impacted by variety and environment in our samples.

Flavor compounds and aromas in milled corn kernels

Milled corn samples were exposed to GC-MS/O and descriptive sensory analysis techniques. The GC-MS/O detected 52 different flavor compounds that registered an aroma event via olfactometric detection by a trained operator. Descriptive sensory analysis utilized a trained sensory panel to detect and quantify up to 49 different corn kernel aromas. The percent of total variance is reported for flavor compounds (Table 3) and aromas (Table 4) where the residual effect was responsible for no more than ~80% of the variation. For the 44 compounds and 37 aromas detected but not shown random residual error was responsible for most of the variation suggesting a low importance of environment or variety.

Table 3. Percent of total variance for milled corn flavor compound concentrations as determined through REML

Effect	GC-MS/O							
	Acetic Acid	Benzalde-hyde	Hexanal	Methane, thio-bis	1-Hexanol	Ethyl decanoate	3-Dodecen-1-al	Decanal
Environ-ment	0%	11.9%	0%	4%	25.1%	4%	5.5%	0%
Genetic	19.1%	19.9%	0%	0%	0%	17%	6.4%	8%
GxE	0%	37.5%	24.4%	30%	1.6%	1%	10.1%	49.3%
Error	80.9%	30.7%	75.6%	66%	73.3%	78%	78%	42.7%
Sum Total Variance	100%	100%	100%	100%	100%	100%	100%	100%
Units	Ion Count	Ion Count	Ion Count	Ion Count	Ion Count	Ion Count	Ion Count	Ion Count
Mean Value +/- SE	65197.7 +/- 19981.3	6877.6 +/- 2900.2	280095.9 +/- 78261.2	60219.4 +/- 38995.7	18681.67 +/- 12727.3	62152.3 +/- 47293.6	27218.5 +/- 14948.6	4989.4 +/- 3947.3
Observast-ions (n)	30	30	30	30	30	30	30	30
Ion Count = Total ion count (TIC) area under the curve and compound identity was based on the NIST library.								

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Table 4. Percent of total variance for milled corn aroma concentrations as determined through REML

Effect	Descriptive Sensory Analysis											
	Sweet	Sour	Corn	Wood	Oily	Ran-cid	Medici nal	Lea t-her	Bar n	Soa p	Sol-vent	But - yric
Environ-ment	1.8%	10.8%	20%	31.9%	31.2%	34.2%	0%	0%	53.2 %	37.1 %	23.6 %	21.3 %
Genetic	24.6%	0%	0%	0%	0%	4.6%	0%	22.1 %	0%	0%	0%	0%
GxE	0%	20.3%	27.4%	0%	13.4%	0%	30.5%	8.9 %	0%	0%	0%	0%
Error	73.6%	68.9%	51.6%	68.1%	55.4%	61.2%	69.5%	69%	46.8 %	62.9 %	76.4 %	78.7 %
Sum Total Variance	100%	100%	100%	100%	100%	100%	100%	100 %	100 %	100 %	100 %	100 %
Units	Int	Int	Int	Int	Int	Int	Int	Int	Int	Int	Int	Int
Mean Value +/- SE	2.8 +/- 0.2	2.5 +/- 0.3	6.9 +/- 0.3	6.4 +/- 0.3	2.3 +/- 0.2	0.4 +/- 0.2	1.8 +/- 0.1	1.6 +/- 0.2	1.0 +/- 0.4	0.3 +/- 0.2	0.2 +/- 0.1	0.1 +/- 0.1
Observast-ions (n)	30	30	30	30	30	30	30	30	30	30	30	30
Int = Intensity, which corresponds to an aroma intensity, with a scale of 0-15, and was determined by the trained sensory panel.												

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Two compounds where the variation was not largely due to a residual effect appeared especially important—benzaldehyde and decanal. Benzaldehyde possesses a characteristic almond-like aroma [63], and decanal imparts fruity aromas [64]. Interaction effects were responsible for most of the variation found in these two aldehydes.

For the aromas detected via descriptive sensory analysis and highlighted in Table 4, residual effects on average accounted for the majority of the variation (a range of about 50–75%). That said, of the twelve aromas highlighted, the non-residual experimental variation was largely due to environment in seven (Woody, Oily, Rancid, Barnyard, Soapy, Solvent Like, Butyric), variety in two (Overall Sweet, Leather), and the interaction in three (Overall Sour, Corn, Medicinal).

Overall, kernel analysis thus indicated that we might expect variety and environment to influence alcohol yield and flavor. Next, we aimed to process each kernel treatment into mash, beer, and finally new-make bourbon, conducting relevant analyses at each step.

2.3.2. Mash and fermentation analysis

Specific gravity

Analysis of covariance (ANCOVA) was carried out with treatment, hours into fermentation, and the interaction as effects, and specific gravity as the response. A visual of fermentation growth curves, as measured as specific gravity, for each variety among the different environments (Figure 1, Table 5) shows that the different treatments displayed significantly different slopes ($p = 0.0046$). Table 6 shows the percent of total

variance for specific gravity at different points during mashing and fermentation. At all timepoints, the environment was the biggest driver of variation next to the residual variation. The variety in this case contributed meaningful but less overall variation than the environment or replicate variation. This is likely due to the samples being from relatively narrow germplasm. It is important to note that the replicate variation was lowest when the samples were first mashed (Day 0) and highest at the end of fermentation (Day 5). This suggests that the fermentation process normalized the diverse samples, reducing differences due to variety or environment between them.

Figure 1. Fermentation growth curves using specific gravity as response factor

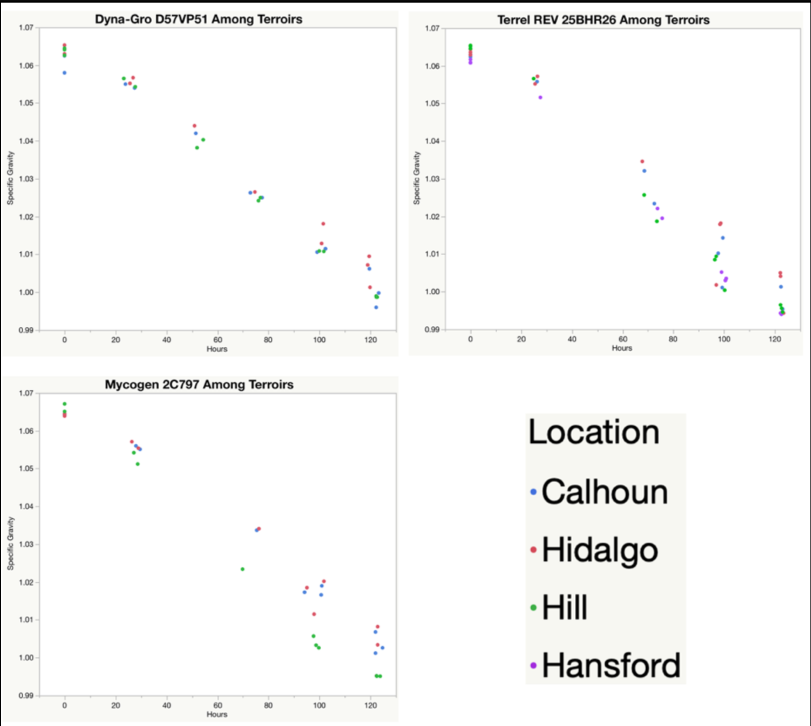

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Table 5. Effect tests from ANCOVA of specific gravity x hours of fermentation

Source	Degrees Freedom	Sum of Squares	F Ratio	Prob > F
Treatment	9	0.00094879	7.8261	<.0001*
Hours	1	0.08245726	6121.289	<.0001*
Treatment*Hours	9	0.00034847	2.8743	0.0046*

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Table 6. Percent of total variance for specific gravity measurements of mash and fermentation as determined through REML

Effect	Day 0 (Mash)	Day 1	Day 3	Day 4	Day 5
Environment	45.4%	36.7%	54.3%	36.1%	35%
Genetic	16.2%	0%	1.4%	4.7%	11.5%
GxE	9.5%	9.3%	0%	9.5%	0%
Error	28.9%	54%	44.3%	49.7%	53.5%
Sum Total Variance	100%	100%	100%	100%	100%
Units	Specific Gravity	Specific Gravity	Specific Gravity	Specific Gravity	Specific Gravity
Mean Value +/- SE	1.1 +/- 0.0	1.05 +/- 0.0	1.03 +/- 0.0	1.0 +/- 0.0	0.99 +/- 0.0
Observations (n)	30	17	15	27	30

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Dextrins, sugars, and ethanol

While specific gravity is a rapid and informative measurement, it does not discriminate between the various types of sugars. Further, it does not provide a direct measurement of ethanol concentration. HPLC is able to effectively separate and quantify DP4+ (dextrins), DP3 (maltotriose), maltose, glucose, and ethanol. Table 7 shows the variance components for these compounds at Day 0 and Day 5.

Table 7. Percent of total variance for DP4+, DP3, maltose, glucose, and ethanol at Day 0 and Day 5 (with and without outliers removed) of fermentation as determined through REML

Effect	*Day 0*				
	DP4+	**DP3**	**Maltose**	**Glucose**	**Ethanol**
Environment	0%	12.1%	0%	0%	ND
Genetic	6%	0%	0%	5%	ND
GxE	57%	20.6%	68.6%	8.3%	ND
Error	37%	67.3%	31.4%	86.7%	ND
Sum Total Variance	100%	100%	100%	100%	ND
Units	wt/vol	wt/vol	wt/vol	wt/vol	% ABW
Mean Value +/- SE	2.5 +/- 0.2	0.1 +/- 0.0	2.9 +/- 0.2	7.9 +/- 0.2	
Observations (n)	26	26	26	26	26
Effect	*Day 5 (with outliers)*				
	DP4+ (wt/vol)	**DP3 (wt/vol)**	**Maltose (wt/vol)**	**Glucose (wt/vol)**	**Ethanol (% ABW)**
Environment	21.6%	0%	38.9%	40.1%	5.5%
Genetic	0%	0%	5.1%	10.6%	0%
GxE	0%	0%	0	0%	5.5%
Error	78.4%	100%	56%	49.3%	89%
Sum Total Variance	0.000212	100%	100%	100%	100%
Units	wt/vol	wt/vol	wt/vol	wt/vol	% ABW
Mean Value +/- SE	0.1 +/- 0.0	0.03 +/- 0.0	0.2 +/- 0.0	0.9 +/- 0.4	6.7 +/- 0.2
Observations (n)	30	30	30	30	30
Effect	*Day 5 (outliers removed)*				
	DP4+ (wt/vol)	**DP3 (wt/vol)**	**Maltose (wt/vol)**	**Glucose (wt/vol)**	**Ethanol (% ABW)**
Environment	45.8%	0%	45.9%	36.8%	38.9%
Genetic	7.7%	0%	1.2%	10.3%	19.7%
GxE	0%	0%	0%	0%	0%
Error	46.5%	100%	52.9%	52.9%	41.4%
Sum Total Variance	100%	100%	100%	100%	100%
Units	wt/vol	wt/vol	wt/vol	wt/vol	% ABW
Mean Value +/- SE	0.1 +-/ 0.0	0.03 +/- 0.0	0.2 +/- 0.0	0.9 +/- 0.4	6.8 +/- 0.2
Observations (n)	27	27	27	27	27

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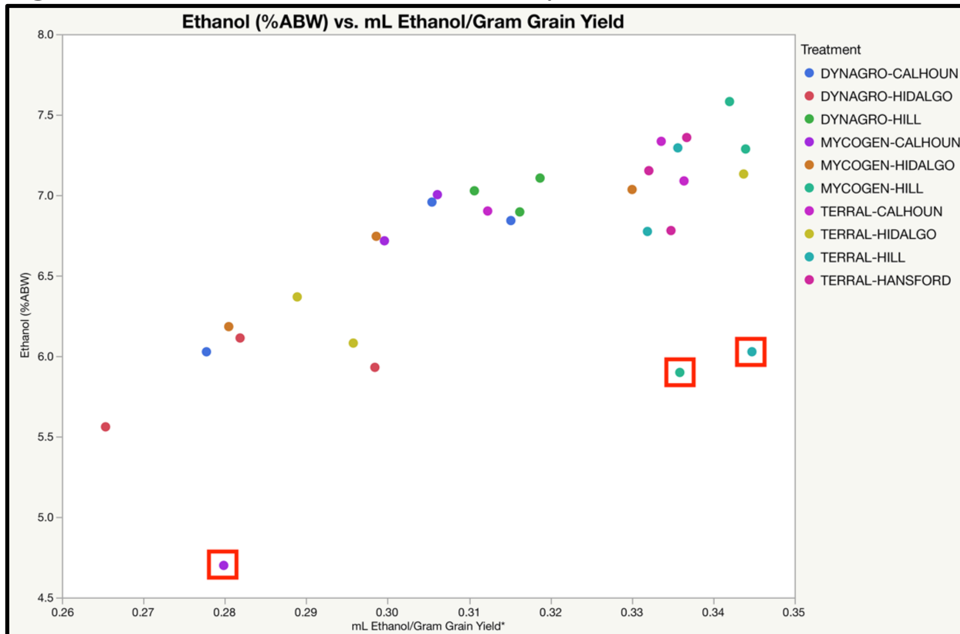
At Day 0, post-mashing, much of the variation in dextrins and maltose was due to the interaction effect. Maltotriose shows a similar result, although to a lesser extent. The residual was responsible for most of the variation in glucose.

It was somewhat surprising that the ethanol concentration at Day 5, post-fermentation, did not show variation due to environment or variety. As reported later alcohol yield did show variation due to variety and environment. As explained in materials and methods, three outliers (Figure 2) were identified in the data. Removing

these outliers, the results showed that environment and variety were responsible for 39% and 20% of the experimental variation, respectively. This better aligns with the impact of environment and variety on alcohol yield variation. Further, much of the variation in the concentrations of dextrins, maltotriose, and glucose at Day 5 was due to environment. The results from mash and fermentation analysis suggest that variety and environment do impact starting fermentable extract and attenuation, which can impact alcohol yield.

Figure 2. Ethanol % in beer vs. ethanol yielded after distillation



Red boxes denote the three outlier data points that were removed for subsequent REML analysis (Table 10).

2.3.3. New-make bourbon analysis

Alcohol yield

While starch levels in corn, sugar yields during mashing, and alcohol production during fermentation are important measurements for assessing alcohol yield, distillers ultimately determine yield through measurement after distillation. As is described, each treatment was exposed to identical mashing, fermentation, and distillation procedures. After distillation, the milliliters of ethanol per gram of corn was measured (Table 8). Both environment and variety were responsible for 32% and 24% of the experimental variation, respectively. The conservative REML best linear unbiased predictions ranged from 0.29 ml ethanol per gram of grain yield (Dyna-Gro, Monte Alto) to 0.34 (Terrel, Sawyer Farms), which would mean 17% more corn would need to be purchased for the same whiskey production.

Table 8. Percent of total variance for alcohol yield of new-make bourbon as determined through REML

Effect	mL EtOH / Gram Corn
Environment	32.3%
Genetic	24.1%
GxE	1.2%
Error	42.4%
Sum Total Variance	100%
Units	mL
Mean Value +/- SE	0.31 +/- 0.0
Observations (n)	30

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Multivariate analysis (Table 9, Table 10) shows that starch percentage, total extract (the sum of DP4+, DP3, maltose, and glucose), ethanol (%ABW), and the ultimate alcohol yield all possess statistically significant correlations. This is important, as it further supports the notion that variation from variety and environment across starch percentage in the corn, total extract post-mashing, and ethanol (%ABW) post-fermentation can ultimately impact ethanol yield post-distillation.

Table 9. Pearson correlations and probabilities (Prob > F) among starch concentration in corn, ethanol (%ABW) post-fermentation, and ethanol yield post-distillation

	Starch % Dry Basis	Ethanol (%ABW)	mL EtOH/Gram Corn
Starch % Dry Basis	—	-0.2543^{NS}	-0.4028**
Ethanol (%ABW)	0.2543^{NS}	—	0.6708***
mL Ethanol/Gram Grain Yield	-0.4028**	0.6708***	—

Observations (n) = 60. Values reported are strength and direction of correlation (R). *, **, *** indicate statistical significance at the 10%, 5%, and 1% level respectively. Given the limited sample size (n = 30), we consider the 10% level to be practically useful and significant. NS indicate non-significant differences. Reprinted from Arnold et al, 2019.

Table 10. Pearson correlations and probabilities (Prob > F) among starch concentration in corn, total extract post-mashing (Day 0), ethanol (%ABW) post-fermentation (Day 5), and ethanol yield post-distillation

	Starch % Dry Basis	Total Extract	Ethanol (%ABW)	mL EtOH/Gram Corn
Starch % Dry Basis	—	-0.4860**	-0.2639^{NS}	-0.3123^{NS}
Total Extract	-0.4860**	—	0.4285**	0.5549***
Ethanol (%ABW)	-0.2639^{NS}	0.4285**	—	0.6575***
mL Ethanol/Gram Grain Yield	-0.3123^{NS}	0.5549***	0.6575***	—

Obersvations (n) = 56. Four batch replicates were not included for total extract post-mashing (Day 0) HPLC analysis (Mycogen-Hidalgo Batch 2, Mycogen-Hidalgo Batch 3, Terral-Calhoun Batch 3, and Terral-Hansford Batch 3) due to loss of sample during HPLC analysis. Values reported are strength and direction of correlation (R). *, **, *** indicate statistical significance at the 10%, 5%, and 1% level respectively. Given the limited sample size (n = 56), we consider the 10% level to be practically useful and significant. NS indicate non-significant differences. Reprinted from Arnold et al, 2019.

Table 11. Percent of total variance for new-make bourbon flavor compound concentrations as determined through REML

Effect	GC-MS/O								
	Isoamyl acetate	2-methylbutyl decanoate	Ethyl 2-nonenoate	2-tridecanone	2,4-decadienal	Ethyl sorbate	Isopentyl hexanoate	Ethyl acetate	4-vinylanisole
Envir.	9.8%	0%	67.9%	0.9%	29.7%	1.2%	21.8%	24.6%	47.9%
Genetic	3.6%	0%	0%	25.6%	0%	2.5%	0.7%	0%	15.1%
GxE	23.2%	49.2%	0%	0%	0%	15%	0%	2.8%	16.1%
Error	63.4%	50.8%	32.1%	73.5%	70.3%	81.3%	77.5%	72.6%	20.9%
Mean Value +/- SE	189638.3 +/- 56844.9	38039.0 +/- 38039.0	96846.9 +/- 55953.4	30039.3 +/- 20075.5	126750.6 +/- 35235.9	32691.29 +/- 5571.4	30471.4 +/- 17721.7	679113.7 +/- 122738.8	445662.2 +/- 114215.8

Effect	GC-MS/O								
	Ethyl decanoate	Ethyl dodecanoate	Acetal	Styrene	Ethyl undecanoate	(*E*)-2-heptenal	2-methyl-5-isopropenylfuran	Ethyl (*E*)-2-octenoate	Ethene, ethoxy-
Envir.	43.1%	3.1%	29.4%	6.7%	64.4%	18.1%	0%	49.4	0%
Genetic	21.8%	3.6%	17.6%	35.3%	8%	22.4%	20.8%	0%	1.5%
GxE	0%	17.1%	0%	1.5%	4.5%	0%	1.7%	0%	35.5%
Error	35.1%	76.2%	53%	56.5%	23.1%	59.5%	77.5%	50.6%	63%
Mean Value +/- SE	51696255 +/- 10687333	3899065.2 +/- 1024933	131682.7 +/- 66275.5	1013993.7 +/- 436857.9	183871.0 +/- 83917.6	67719.8 +/- 38377.9	10263.4 +/- 8670.2	196254.5 +/- 79899.0	3746.1 +/- 3654.0

Effect	GC-MS/O								
	Ethyl trans-4-decenoate	Ethyl heptanoate	Ethyl hexanoate	Napthalene	Nonanal	Ethyl nonanoate	(*E*)-2-nonenal	Isoamyl octanoate	Ethyl octanoate
Envir.	68.6%	21.2%	21.6%	21.8%	6.9%	39.1%	9.6%	34.6%	7.4%
Genetic	5.2%	11.5%	10.4%	0%	24.1%	12.2%	18.5%	9.9%	32.2%
GxE	0%	0%	0%	10.9%	8.9%	0%	0%	0%	4.3%
Error	26.2%	67.3%	68%	67.3%	60.1%	48.7%	71.9%	55.5%	56%
Mean Value +/- SE	2335925.5 +/- 705858.2	275450.2 +/- 83277.6	897135.5	19611.0 +/- 17922.4	350703.97 +/- 118026.4	2551498.7 +/- 743395.8	303987.0 +/- 72030.9	1104644.7 +/- 289180.2	22363759 +/- 6611977

Effect	GC-MS/O								
	Ethyl hept-2-enoate	2-Nonanone	2-octenal, (E)-	2-undecanone	Phenylethyl alcohol	Acetophenone	2-pentylfuran	Ethyl (E)-4-hexenoate	Cedr-8-ene
Envir.	32.1%	5%	26.2%	23.2%	16.2%	9.1%	37.3%	0%	72.5%
Genetic	0%	17.1%	0%	1.4%	0%	0%	8%	19%	0%
GxE	4.5%	10%	0%	63.1%	27.5%	42%	0%	28.2%	0%
Error	63.4%	67.9%	73.8%	12.3%	56.3%	48.9%	54.7%	52.8%	27.5%
Mean Value +/- SE	52677.9 +/- 20017.3	11597.9 +/- 7730.7	102855.3 +/- 41003.2	19602.0 +/- 20491.3	307577.2 +/- 87401.5	5469.8 +/- 4026.9	117483.8 +/- 61497.9	15337.2 +/- 8193.0	117628.7 +/- 116189.1

Envir. = Environment. Values are total ion count area under the curve. Compound identity was based on the NIST library. N = 30 for each compound. Reprinted from Arnold et al, 2019.

Table 12. Percent of total variance for new-make bourbon aroma concentrations as determined through REML

Effect	Descriptive Sensory Analysis												
	Alcohol	Sweet	Sour	Grain Com-plex	Corn	Malt	Wood	Musty	Mola-sses	Anise	Lactic Acid	Stale	Pung-ent
Environ-ment	0.%	4.7%	0%	0%	0%	35.9%	8.8%	0%	0%	63%	0.6%	14.4%	5.8%
Genetic	25.9%	21.9%	20.8%	26.6%	2.6%	0.4%	34.2%	14.6%	19.6%	0%	0%	0.9%	32.2%
GxE	24.4%	0%	12%	0%	42.5%	0%	0%	8%	0%	0%	48.6%	9%	7.2%
Error	69.7%	73.4%	67.2%	73.4%	54.9%	63.7%	57%	77.4%	80.4%	37%	50.8%	75.7%	54.8%
Mean Value +/- SE	6.6 +/- 0.2	2.6 +/- 0.2	3.1 +/- 0.3	5.3 +/- 0.2	5.1 +/- 0.2	3.6 +/- 0.2	3.9 +/- 0.3	2.9 +/- 0.2	1.2 +/- 0.3	0.3 +/- 0.2	1.9 +/- 0.2	2.4 +/- 0.1	4.0 +/- 0.2

Values correspond to an aroma intensity, with a scale of 0-15, and was determined by the trained sensory panel. N = 30 for each aroma. Reprinted from Arnold et al, 2019.

Flavor compounds and aromas

Samples of new-make bourbon were exposed to GC-MS/O and descriptive sensory analysis techniques. GC-MS/O detected 68 different compounds (16 more than the milled corn) that registered an aroma event via olfactometric detection by a trained operator. Sensory analysis utilized a trained panel to detect and quantify up to 54 different new-make whiskey aromas. Table 11 and Table 12 above provide the percent of total variance for those flavor compounds and aromas where the residual effect was responsible for no more than ~80% of the variation.

Of the thirty-six flavor compounds identified in Table 11 where the concentration showed substantial variance beyond the residual (i.e. more than ~20% of the total variance), 50% were esters, 14% were aldehydes, and 11% were ketones. The fact that such a large percentage of esters displayed variation due to variety and environment is encouraging, as esters are also important flavor contributors in new-make whiskey, usually contributing fruity characteristics. Aldehydes and ketones are also important flavor contributors, providing fruity, floral, grassy, and fatty aromas.

Thirteen aromas were detected via Spectrum sensory analysis where the residual effect was not responsible for more than ~80% of the total variance component (Table 12). Variance was largely due to environment in three (Malt, Anise, Stale), variety in seven (Sweet, Sour, Grain Complex, Woody, Musty Earthy, Molasses, Prickle Pungent), and the interaction of environment and variety in three (Alcohol, Corn, Lactic Acid).

Many of the flavor compounds identified in Table 11 have been reported previously as being important contributors to flavor in bourbon. Poisson and Schieberle utilized aroma extract dilution analysis (AEDA), quantitative measurements, aroma recombination, and omission studies to identify the most odor-active flavor compounds in whiskey [55, 56]. From the compounds they identified, the following were also identified in this report (Table 11), grouped according to compound class: *esters*—isoamyl acetate, ethyl acetate, ethyl hexanoate, ethyl octanoate; *aldehydes*—(*E*)-2-heptenal, nonanal, (*E*)-2-nonenal, 2,4-decadienal; *fusel alcohol*—phenylethyl alcohol; and acetal. According to Poisson and Schieberle, the esters listed contribute fruity flavors. The aldehydes (*E*)-2-heptenal, (*E*)-2-nonenal, and 2,4-decadienal contribute fatty and green flavors, and nonanal contributes soapy flavors. Phenylethyl alcohol is known for imparting rose and floral aromas. Acetal (also called 1,1-diethoxyethane) contributes fruity and ethereal flavors.

The only flavor compound found in both milled corn and new-make from Table 3 and Table 11 where the respective concentrations showed substantial variance beyond the residual was ethyl decanoate. Ethyl decanoate has previously been identified in bourbon [65], described as having a fruity, apple aroma. Importantly, combined among

all new-make samples, ethyl decanoate had the highest peak area value (averaged 48 million across samples, more than double the next highest ethyl octanoate with 22 million) out of the sixty-eight flavor compounds detected. Combined among all corn samples, ethyl decanoate had the ninth highest peak area value (averaged 60,421 across samples, hexanal was the highest with 280,095) out of fifty-two flavor compounds detected. However, ethyl decanoate concentration in corn did not show significant correlation to ethyl decanoate concentration in new-make. This might suggest that ethyl decanoate present in corn is altered during the mashing, fermentation, or distillation processes of whiskey production. Another likely possibility is that yeast production of decanoic acid and/or ethyl decanoate is impacted by other compositional aspects of corn, and these aspects can negate varying contributions from the corn kernels themselves.

While the presence and concentration of certain flavor compounds can correlate with aroma flavors and concentrations, this is not always the case. As pointed out by Poisson and Schieberle, more than 300 compounds have been identified in whiskey, yet only a subset of these (likely 30 to 60) are important for flavor. Therefore, we aimed to determine if there were any important correlations between flavor compounds and aroma in new-make. First, looking at each relationship between flavor compounds and aroma individually, moderate to no correlations were found in most cases and nothing was identified that warranted discussion. However, instead of considering each aroma individually, we grouped them into two categories, denoted as “good” and “bad” aromas and summed the individual aroma concentrations generated by the Spectrum method. These good and bad categories corresponded to aromas that are typically deemed

desirable and undesirable, respectively, in new-make bourbon. Further, we considered all detected flavor compounds and aromas, not just those reported in Table 11 and Table 12.

Of the 68 new-make bourbon flavor compounds identified by GC-MS/O, seven were found to possess both statistical (i.e. p-values) and practical (i.e. effect sizes) significance with the summed value Total Aroma Units—Good (Table 13). Four of these seven flavor compounds were esters (isoamyl acetate, ethyl nonanoate, ethyl octanoate, and ethyl (*E*)-4-hexenoate), known to impart desirable fruity flavors to whiskey. Nonanal imparts soapy characteristics, which is typically deemed a desirable aroma contributor to a whiskey's flavor. Acetaldehyde was the only flavor compound that showed a negative correlation to Total Aroma Units—Good. This is not surprising, as high-levels of acetaldehyde impart astringent, solvent, and green apple flavors. The majority of this compound is typically discarded during distillation, at the discretion of the distiller's judgment. Styrene is usually attributed to phenolic and plastic flavors. While in isolation these flavors are negative, a certain level of phenolic nuances is usually desired in whiskey.

Table 13. Pearson correlations and probabilities (Prob > F) of new-make bourbon flavor compounds with Total Aroma Units—Good and Total Aroma Units—Bad

	Isoamyl acetate	**Acetalde-hyde**	**Nonanal**	**Ethyl Nonan-oate**	**Ethyl Octan-oate**	**Styrene**	**Ethyl (*E*)-4-hexenoate**	**(*E*)-2-nonenal**
Total Aroma Units—Good	0.3352*	-0.3846**	0.3734**	0.3182*	0.4270**	0.4542**	0.5067***	0.2991^{NS}
Total Aroma Units—Bad	0.1558^{NS}	0.0309^{NS}	0.1100^{NS}	0.2194^{NS}	0.2282^{NS}	0.1019^{NS}	0.0061^{NS}	0.4669***

n = 30. Values reported are strength and direction of correlation (R). *, **, *** indicate statistical significance at the 10%, 5%, and 1% level respectively. Given the limited sample size (n = 30), we consider the 10% level to be practically useful and significant. NS indicate non-significant differences. Reprinted from Arnold et al, 2019.

Likewise, of the 68 new-make bourbon flavor compounds identified by GC-MS/O, only (E)-2-nonenal was found to possess both statistical and practical significance with the summed value Total Aroma Units—Bad (Table 13). Given that it is known to harbor aromas of cardboard, staleness, and body odor [66], it is not surprising that increased levels of (*E*)-2-nonenal led to undesirable aromas in the new-make bourbon samples considered here.

All of the bourbon new-make flavor compounds listed in Table 13 were also highlighted in Table 11, with their concentrations showing substantial variance beyond the residual. This indicates that certain new-make bourbon flavor compounds that are significantly correlated with overall desirable and undesirable flavors in new-make bourbon also show concentration variations due to variety and environment. Further, of the eight bourbon new-make flavor compounds listed in Table 13, only three of them (ethyl nonanoate, styrene, and ethyl (*E*)-4-hexenoate) were not listed by Poisson & Schieberle as being important contributors in bourbon.

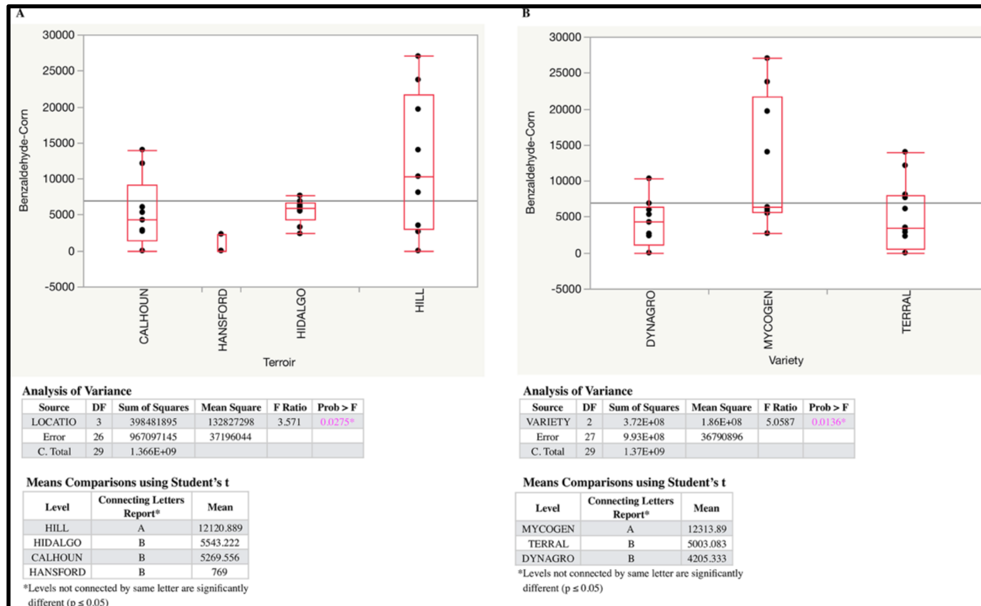
Next, we aimed to determine if any milled corn flavor compounds correlated with the new-make bourbon flavor compounds identified in Table 13. We found benzaldehyde concentration in milled corn correlated with both statistical and practical significance to isoamyl acetate (R = 0.5148***), nonanal (R = 0.4790***), styrene (R = 0.4221**), and ethyl octanoate (R = 0.5042***) concentrations in new-make bourbon. Ethyl octanoate is especially interesting, due to its strong correlation to Total Aroma Units—Good in new-make bourbon. Further, among the 68 flavor compounds identified in new-make bourbon, ethyl octanoate had the second highest total peak area value for the sum of all measured samples (n = 30). In general, benzaldehyde concentration in corn statistically and practically correlated with a number of other flavor compound concentrations in new-make bourbon, such as: isoamyl alcohol (R = 0.3850**), benzene, 1-ethenyl-4-methoxy (R = 0.4494**), ethyl-trans-4-decenoate (R = -0.4261**), and phenylethyl alcohol (R = 0.4380**), some of which were noted in Table 11 and highlighted by Poisson and Schieberle to be important contributors to flavor in bourbon. Importantly, benzaldehyde concentration in corn kernel did not correlate with (*E*)-2-nonenal concentration in new-make bourbon.

Benzaldehyde concentration in milled corn was, however, not significantly correlated with Total Aroma Units—Good in new-make bourbon (R = 0.2837^{NS}). However, once a single outlier was removed, the correlation improved (R = 0.3620*). Given that benzaldehyde concentration in corn is greatly influenced by variety and environment (Table 3), and is readily measured by GC-MS without the expense of

creating new-make or conducting sensory analysis, it might have practical use in selecting improved corn for whiskey.

In recent years, it has become common for chefs, bakers, maltsters, and brewers to collaborate with plant breeders in an effort to breed and select for crop varieties that deliver new or forgotten flavors [67-70]. It is our belief that breeding and better selecting corn growing locations for specific compounds, such as increased benzaldehyde concentrations, has potential to deliver improved corn that possess heightened and desirable flavors in new-make bourbon. In this study, 2C797—Mycogen Seed benzaldehyde concentration was found to be significantly higher than the other two varieties (Figure 3B). Here we found corn from the Hill county environment contained significantly higher concentrations of benzaldehyde than the other three environments (Figure 3A).

Figure 3. ANOVA and mean comparisons of benzaldehyde concentrations in corn



Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
LOCATIO	3	398481895	132827298	3.571	0.0275*
Error	26	967097145	37196044		
C. Total	29	1.366E+09			

Means Comparisons using Student's t

Level	Connecting Letters Report*	Mean
HILL	A	12120.889
HIDALGO	B	5543.222
CALHOUN	B	5269.556
HANSFORD	B	769

*Levels not connected by same letter are significantly different ($p \leq 0.05$)

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
VARIETY	2	3.72E+08	1.86E+08	5.0587	0.0136*
Error	27	9.93E+08	36790896		
C. Total	29	1.37E+09			

Means Comparisons using Student's t

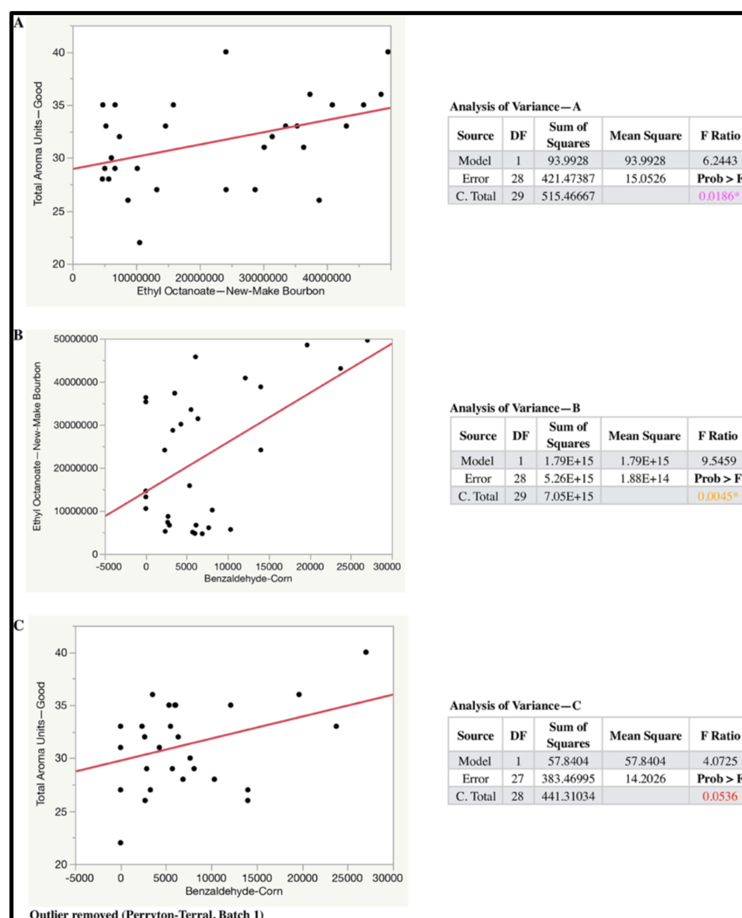
Level	Connecting Letters Report*	Mean
MYCOGEN	A	12313.89
TERRAL	B	5003.083
DYNAGRO	B	4205.333

*Levels not connected by same letter are significantly different ($p \leq 0.05$)

The dependent variable is peak area. (A) Individual peak areas for each environment, accompanied with ANOVA and mean comparison analyses. (B) Individual peak areas for each variety, accompanied with ANOVA and mean comparison analyses. Reprinted from Arnold et al, 2019.

To show the progression of analyses that elucidate how desirable aromas in new-make bourbon can be linked to flavor compounds in new-make bourbon and corn Figure 4, shows the individual linear regressions and ANOVA results.

Figure 4. Benzaldehyde in corn and ethyl octanoate new-make bourbon are linked to desirable aromas in new-make



Analysis of Variance—A

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	93.9928	93.9928	6.2443
Error	28	421.47387	15.0526	Prob > F
C. Total	29	515.46667		0.0186*

Analysis of Variance—B

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	1.79E+15	1.79E+15	9.5459
Error	28	5.26E+15	1.88E+14	Prob > F
C. Total	29	7.05E+15		0.0045*

Analysis of Variance—C

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	1	57.8404	57.8404	4.0725
Error	27	383.46995	14.2026	Prob > F
C. Total	28	441.31034		0.0536

(A) Ethyl octanoate concentration in new-make is positively correlated with Total Aroma Units—Good concentration. (B) Benzaldehyde concentration in corn is positively correlated with ethyl octanoate concentration in new-make bourbon. (C) Benzaldehyde concentration in corn is positively correlated with Total Aroma Units—Good concentration. Reprinted from Arnold et al, 2019.

2.4. Conclusions

To our knowledge, this is the first report to investigate the impact of variety and environment on flavor and alcohol yield in new-make bourbon. Our findings suggest that even among high-yielding yellow dent corn hybrid varieties, variations in flavor and alcohol yield potential still occur and can be targeted. Further, we showed that the

different Texas environments impacted both flavor and alcohol yield. Lastly, our results suggest that it is possible to select for the trait of flavor based on chemical markers in corn, even when those chemical markers are only precursors to, and/or correlated with, flavor compounds and sensory notes in whiskey.

3. CHARTING A CHEMICAL ROADMAP OF TERROIR IN WHISKEY THROUGH WINE

While the research in Section 2 revealed thirty-six flavor compounds that are meaningfully influenced by variety, environment, and/or the interaction, we were limited in determining which compounds were meaningful contributors to overall flavor, much less which specific aromas. Published literature suggests that even though there are hundreds of different compounds in whiskey of varying classes—organic acids, esters, ketones, aldehydes, terpenes, pyrazines, acetals, alcohols, lactones, sulfur compounds—only thirty to sixty of these are responsible for the majority of flavor in whiskey [55, 56, 71-73]. Therefore, it's unlikely that all thirty-six flavor compounds from Table 11 are important for flavor in whiskey. For plant breeders, agronomists and distillers to target specific and heightened flavors in whiskey via direct or indirect selection of chemical markers in grain, a roadmap is necessary to reveal which flavor compounds to focus on, their origin, and lastly how they might be influenced by some aspect of terroir.

If terroir is to be elucidated in whiskey, it is logical to use wine as a model system for comparison since more is known. While literature suggests little research exists for whiskey, wine has received substantial scientific investigation and publication from academics and industry alike. Therefore, a homology comparison between wine and whiskey (and even beer and whiskey in some cases) might help to elucidate whiskey terroir; flavor compounds in wine which have been studied at length and shown to be impacted by terroir (again, genetics, environment, and/or their interaction) can be

compared to flavor compounds in whiskey. If wine and whiskey share many of the same—or closely related—flavor compounds, then this similarity might provide a roadmap for chemical markers in grain that can be targeted for selection in breeding programs.

3.1. Merging the chemical roadmaps

The flavor compounds of wine come from the grape, the fermentation byproducts, and—if barrel aging is employed—the oak barrel. This is true regardless of the style of wine. While bottle aging (or even aging in stainless-steel tanks before bottling) will manipulate and change flavor compounds—through chemical reactions—it does not introduce any original organic matter (i.e., flavor compounds).

In the same way, the flavor compounds in whiskey come from the grain, fermentation byproducts, and the oak barrel. While water can technically introduce flavor compounds (e.g., flavors that come from compounds like geosmin and 2-methylisoborneaol), more often than not water is filtered through activated carbon to remove any organic compounds and off-flavors. And similar to bottle aging in wine, while distillation will manipulate and change flavor compounds—through chemical reactions—the process does not actually introduce any original organic matter (i.e., flavor compounds).

Table 14 below shows the key flavor compounds in bourbon, rye, and malt whiskeys, as elucidated by aroma extract dilution analysis (AEDA). In conjunction with gas chromatography, olfactometry, and mass spectrometry, this technique allows for the

assignment of a flavor dilution (FD) value to each identified compound [55, 72, 73]. High FD values suggests a greater overall contribution to the flavor of a food or beverage. Further, the FD values calculated for the bourbon and rye whiskey research were followed-up with aroma recombinate studies, to confirm the importance of the flavor compounds with high FD values [55, 56, 72].

Along with the flavor compounds identified from these three whiskey AEDA papers, those flavor compounds identified in Section 2 and its respective journal publication are also specified in Table 14. Further, the aromas of each individual compound, as well as their proposed origin among the three sources of flavor in whiskey (grain, fermentation by-products, and oak) are noted. Of utmost importance, the last column references existing literature which documents how some aspect of terroir (genetics, environment, and/or their interaction) in wine and beer influences the presence and/or concentration of each compound.

Table 14. Charting the chemical roadmap of terroir in whiskey

Compound Class	Important Flavor Compound in Bourbon[55]	Important Flavor Compound in Rye Whiskey[72]	Important Flavor Compound in Malt Whisky[73]	Aroma	Origin(s)	Impacted by Terroir in Wine or Beer
Acetal	1,1-diethoxyethane†	—	1,1-diethoxyethane†	*Sweet, Green, Ethereal*	Fermentation, Maturation	Wine[74, 75]
Aldehyde	2,4-nonadienal	—	—	*Fatty, Melon*	Grain	—
	2,6-nonadienal	—	—	*Cucumber*		Wine[76] / Beer[22]
	2-decenal	—	—	*Orange, Floral*		—
	2-heptenal†	—	2-heptenal†	*Apple, fatty*		Wine[77, 78] / Beer[79]
	2-nonenal†	—	—	*Stale Bread, Cardboard*		Wine[80] Beer[79]
	2-methylbutanal	—	—	*Chocolate*		Wine[74, 80] / Beer[79, 81]
	2,4-decadienal†	—	—	*Meaty, Fatty*		Wine[77]
	Isobutyraldehyde	—	—	*Grainy*		Wine[82] / Beer[79]
	Isovaleraldehyde	—	—	*Chocolate*		Wine[74, 80] / Beer[79]
	Nonanal†	—	—	*Soapy, Fatty*		Wine[83-86]
	Acetaldehyde*	Acetaldehyde*	—	*Green Apple*	Fermentation, Maturation	Wine[87, 88] / Beer[79]
Ester	—	—	Ethyl laurate†	*Floral, Waxy*	Fermentation	Wine[89-91]
	—	—	Ethyl undecanoate†	*Soapy, Waxy*		—
	2-phenethyl propionate	—	—	*Floral, Rose, Sweet*		—
	Ethyl 2-methylbutyrate*	—	—	*Apple*		Wine[74, 92, 93]
	Ethyl 2-phenylacetate	—	—	*Cocoa, Honey, Floral*		Wine[86]
	Ethyl butyrate*	Ethyl butyrate*	—	*Pineapple*		Wine[74, 84, 86, 93-97]
	Ethyl cinnamate	Ethyl cinnamate	—	*Cinnamon*		Wine[98-101]
	Ethyl hexanoate*†	Ethyl hexanoate*†	—	*Fruity, Apple*		Wine[74, 84-86, 93, 96, 97, 102-104]
	Ethyl isobutyrate*	Ethyl isobutyrate*	—	*Citrus, Straw-berry*		Wine[95, 99, 103, 105]

Table 14 Continued

Compound Class	Important Flavor Compound in Bourbon[55]	Important Flavor Compound in Rye Whiskey[72]	Important Flavor Compound in Malt Whisky[73]	Aroma	Origin(s)	Impacted by Terroir in Wine or Beer
Ester	Ethyl isovalerate*	Ethyl isovalerate*	Ethyl isovalerate*	*Apple, Pineapple*	Fermentation	Wine[74, 95, 97, 105]
	Ethyl octanoate*†	—	—	*Fruity, Floral, Banana, Pineapple*		Wine[74, 83, 86, 93, 97, 104]
	Ethyl pentanoate	—	—	*Apple, Pineapple*		Wine[93, 95, 96]
	Ethyl propanoate	Ethyl propanoate	—	*Grape*		Wine[74, 88, 106]
	Isoamyl acetate*†	Isoamyl acetate*†	Isoamyl acetate*†	*Banana*		Wine[74, 86, 95, 96, 104, 107]
	Phenylethyl acetate	Phenylethyl acetate	—	*Honey*		Wine[86, 95, 97, 104] / Beer[108]
	Ethyl acetate*†	—	—	*Fruity, Ethereal*	Fermentation, Maturation	Wine[74, 86, 95-97, 104] / Beer[79]
	—	Ethyl vanillate	—	*Vanilla, Sweet*		Wine[109-112]
Fusel Alcohol	—	—	Isopropyl alcohol	*Alcohol, Solvent*	Fermentation	Wine[97]
	Isoamyl alcohol*	Isoamyl alcohol*	Isoamyl alcohol*	*Banana*		Wine[74, 86, 96, 104] / Beer[79]
	Isobutanol	Isobutanol	Isobutanol	*Wine, Vinous*		Wine[86, 97, 113, 114] / Beer[79]
	Phenethyl alcohol*†	Phenethyl alcohol*†	Phenethyl alcohol*†	*Floral, Rose*		Wine[86, 95-97]
Ketone	4-methylacetophenone	—	—	*Floral, Hawth-orne*	Grain	Wine[115]
	Diacetyl*	—	—	*Buttery*	Fermentation	Wine[116, 117] / Beer[79]
Lactone	*trans*-whiskey lactone	*trans*-whiskey lactone	*trans*-whiskey lactone	*Coconut, Celery*	Maturation	—
	cis-whiskey lactone	*cis*-whiskey lactone	*cis*-whiskey lactone	*Coconut, Oak*		—
	Sotolon	—	—	*Caramel, Curry*		—
	6-dodeceno-γ-lactone	—	—	*Peach*	Grain, Fermentation	Wine[117, 118]
	γ-decalactone	—	γ-decalactone	*Coconut, Peach*		Wine[97, 119, 120]
	γ-dodecalactone	—	—	*Coconut, Peach*		Wine[77, 120]

Table 14 Continued

Compound Class	Important Flavor Compound in Bourbon[55]	Important Flavor Compound in Rye Whiskey[72]	Important Flavor Compound in Malt Whisky[73]	Aroma	Origin(s)	Impacted by Terroir in Wine or Beer
Lactone	γ-nonalactone	γ-nonalactone	—	*Coconut, Peach*	Grain, Fermentation	Wine[97, 100, 120, 121]
	δ-nonalactone	—	—	*Peach*		Wine[120, 122]
Methoxypyrazine	2-isopropyl-3-methoxypyrazine	—	—	*Earthy*	Grain	Wine[123-125]
Organic acid	—	Acetic acid	—	*Vinegar*	Fermentation, Maturation	—
	—	Butyric acid*	—	*Rancid*	Fermentation	Wine[92]
	—	Isovaleric acid*	—	*Cheesy*		Wine[100, 126, 127]
	Phenylacetic acid	Phenylacetic acid	—	*Honey, Floral*	Grain, Fermentation	Wine[77, 97, 128]
NorisoprenoidTerpene	α-damascone	—	—	*Cooked apple*	Grain	—
	β-damascenone*	β-damascenone*	—	*Cooked apple*		Wine[74, 86, 93, 95, 97, 104, 129-132]
	β-ionone	β-ionone	—	*Violets*		Wine[93, 97, 133-135]
Sulfide	Dimethyl sulfide	—	—	*Cooked corn*	Grain, Fermentation	Wine[136-138] / Beer[139, 140]
Volatile Phenol	—	—	Benzenol	*Phenolic*	Grain	—
	—	—	4-ethyl-2-methyl phenol	*Phenolic*		—
	—	*p*-cresol	*p*-cresol	*Band-Aid*		—
		—	4-propylguaiacol	*Clove*		
	—	4-vinylguaiacol	4-vinylguaiacol	*Spice, Clove*	Grain, Fermentation	Wine[93, 131, 141-143] / Beer [19]
	4-ethylguaiacol	4-ethylguaiacol	4-ethylguaiacol	*Phenolic, Smoky, Bacon*		Wine[93, 131, 143, 144]
	4-ethylphenol	4-ethylphenol	4-ethylphenol	*Band-Aid, Smoky*		Wine[93, 131, 143, 144]

Table 14 Continued

Compound Class	Important Flavor Compound in Bourbon[55]	Important Flavor Compound in Rye Whiskey[72]	Important Flavor Compound in Malt Whisky[73]	Aroma	Origin(s)	Impacted by Terroir in Wine or Beer
	Eugenol	Eugenol	—	*Clove*	Grain, Maturation	Wine[93, 97, 143]
	Guaiacol	Guaiacol	Guaiacol	*Woody, Smoky*		Wine[93, 97, 131, 143]
	Vanillin	Vanillin	—	*Vanilla*	Maturation	—
	—	Syringaldehyde	—	*Sweet, Green*		—
	—	Syringol	—	*Sweet, Smoky*		—

* Denotes one of the eighteen flavor compounds responsible for the global vinous odor in wine [71].
† Denotes one of the thirty-six flavor compounds from Section 2 (and its respective journal publication) shown to be meaningfully impacted by terroir in whiskey [38].

3.1.1 Setting a course for selection

In Table 14, the eighteen compounds responsible for the global vinous odor in wine are marked with *. This global odor—that is shared by all wines—can be further defined as “slightly sweet, pungent, alcoholic, and a little bit fruity.”, as is noted in a 2007 research article in the *Proceedings of the Thirteenth Australian Wine Industry Technical Conference* [71]. Research from Vicente Ferreira’s lab has shown that seventeen of these eighteen compounds come solely from fermentation—primarily, a mixture of fusel alcohols (also called fusel oils or higher alcohols), esters, fatty acids, diacetyl and acetaldehyde. Only β-damascenone is derived from grapes directly. All other compounds responsible for flavors beyond the global odor (known as impact compounds), regardless of their source, create their effects through individual or synergistic interactions.

When deciding which flavor compounds to target for selection, evidence suggests that it might make more sense to focus on those impact compounds that are both shared between wine and whiskey and derived from grapes versus the eighteen from the global vinous odor group. This is because Ferreira and his team actually found that the mixture of global odor compounds has a certain innate buffer to it, meaning that the flavor does not change (or changes very little) if one compound is present at very low concentrations or dosed back in at exaggerated levels. So, while the global odor compounds are crucial to creating the flavor of wine (and maybe whiskey), they will always be present in wine—regardless of any impact from terroir—and the concentrations of each can vary widely while still collectively delivering the same vinous flavor.

There are, however, two potential exceptions—isoamyl acetate and β-damascenone. Ferreira found that when the former was omitted, the global odor compound mixture experienced a noticeable decrease in fruity flavor. When the latter was omitted, the mixture experienced a noticeable decrease in overall flavor intensity. And indeed, isoamyl acetate and β-damascenone are reported to be important contributors to flavor in all the whiskey styles (Table 14). Further, the results in Section 2 show that the concentration of isoamyl acetate (among many other esters) in new-make bourbon is meaningfully influenced by terroir (Table 11). So, isoamyl acetate and β-damascenone might still be prime selection targets. Indeed, isoamyl acetate has even been cited as "the only ester capable of imparting its characteristic aroma nuance to wines." [71].

In general, however, it will make most sense to focus on those compounds in Table 14 that are not a part of the eighteen compounds responsible for the global odor of wine, but that are indeed shown to be impacted by terroir in wine or beer. While we can't determine for sure if some derivation of the global odor group is responsible for the global odor of whiskey—or if their buffering characteristics also occur in whiskey—focusing on those impact compounds in wine that are influenced by terroir and also important for flavor in whiskey should provide the best chance of success to select for flavorful and diverse grain varieties.

3.2 Origins of flavor & terroir insights

Below, the specific origins (either previously reported or hypothesized here) of the grain-derived flavor compounds listed in Table 14 are discussed. Further, an extensive literature search was done to identify which of these compounds in whiskey have also been identified in wine (and sometimes beer) and whose presence and concentration are impacted by terroir. Lastly, those compounds that are highlighted in both Table 11 and Table 14 are noted.

Acetals

1,1-diethoxyethane (often referred to as just acetal) was the sole acetal identified in any of the three reports. It arises from the condensation of ethanol and acetaldehyde through both chemical and biochemical pathways [145]—during fermentation, distillation, and maturation—and it is a major component of flavor in whiskey [146].

The extent that its presence is due to chemical versus biochemical means has not been determined. Reports show that grape variety [75] and fungicide treatment [74] significantly impact the concentration of 1,1-diethoxyethane in wine. Lastly, Table 11 shows that environment and genetics accounted for 29.4% and 17.6%, respectively, of the variation in new-make bourbon.

Aldehydes

Research shows that all but one of the aldehydes identified are largely or solely derived from grain [147]. The high temperatures of the malting, mashing, and distillation process induce Strecker degradations of amino acids and oxidation of fatty acids, which produce a variety of different aroma-active aldehydes.

One group of grain-derived aldehydes arise from fatty acid oxidation. 2,6-nonadienal, 2-heptenal, 2,4-decadienal, 2-nonenal, and 2-nonenal's derivative nonanal are all reported in wine, and their concentrations are impacted by some aspect of terroir, from grape variety to vineyard conditions to vintage variations. For example, one report showed that 2,6-nonadienal had an FD factor in merlot wine double that of 'cabernet sauvignon, and four times that of cabernet franc and cabernet gernischt [76]. Further, the presence of 2-heptenal and 2-nonenal in a crude beer mash depended on the variety of barley [79].

The other group of grain-derived aldehydes arise from Strecker degradations of amino acids: 2-methylbutanal from isoleucine, isobutyraldehyde from valine, and isovaleraldehyde from leucine. Another study of beer found that among fourteen

different beer mashes—each with their own specific variety of barley or wheat—2-methylbutanal was detected in only two of them [81]. Another found that while isobutyraldehyde and isovaleraldehyde were present in a selection of ten barley varieties, the concentrations were significantly higher in the French and Australian varieties as opposed to the Canadian and Chinese ones [79].

Table 11 and Table 14 share the following aldehydes: 2,4-decadienal, 2-heptenal, 2-nonenal, nonanal. Across all, 30% to 40% of the variation present in new-make bourbon was due to environment, genetics, and/or the interaction.

Acetaldehyde is the single non-grain derived compound in the aldehyde class. Acetaldehyde is produced by yeast as an intermediate in ethanol production during the fermentation process. Acetaldehyde concentrations can also increase in whiskey due to the oxidation of ethanol during maturation. A report in beer showed that acetaldehyde concentration was significantly impacted by barley variety and environment [79]. In wine, acetaldehyde was one of eleven compounds that could discriminate different red wine varietals from Valencia, Spain [88]. In sparkline wines, acetaldehyde levels are significantly higher when the grapes come from low-yield vineyards as opposed to high-yield [87]. Lastly, for direct evidence in whiskey, the results from Section 2 found that terroir was responsible for 15% of the variation present among the new-make bourbon samples.

Ultimately, while acetaldehyde is not derived from grain directly and instead is a by-product of fermentation, research shows that terroir can still influence its presence and concentration in wine and beer. However, this is not entirely surprising. The nutrient

composition of must or juice (in wine) and mash or wort (in beer and whiskey) does have influence over the production of fermentation-derived flavor compounds. What nutrients a grape contributes to a must or juice, or that a grain contributes to a mash or wort, is a quantitative trait that is dictated by genetics, environment, and/or their interaction. What this means is that terroir can potentially influence the production of flavor compounds produced by yeasts during fermentation.

Esters, Fusel Alcohols, & Organic Acids

While esters and fusel alcohols are indeed synthesized by grapes and grains, they are present at such low concentrations that their effect on a wine's flavor (and whiskey's, presumably) is insignificant [148]. Therefore, their presence in wine, beer, and whiskey is derived from fermentation and/or chemical reactions during fermentation, distillation, and maturation. Further, the other organic acids identified in Table 14 are mainly derived from fermentation. The exceptions are acetic acid, which is a potential byproduct of hemicellulose thermal degradation during the charring of oak barrels; and phenylacetic acid, which can originate from oxidation of the Strecker-aldehyde phenylacetaldehyde. Ultimately, though, these three compound classes that are crucial to flavor—especially esters, which contribute much of the fruity and floral aromas to wine, beer, and spirits—are largely not derived from grapes or grains directly.

Fusel alcohols and organic acids can both be produced from amino acids via the Ehrlich pathway [149]. The catabolism begins with the transamination of an amino acid into an α-keto acid and then subsequent decarboxylation into an aldehyde. From here,

the pathway can proceed via oxidation or reduction, producing a higher alcohol or organic acid, respectively. Oxidation serves as a means of regenerating NAD produced from ethanol fermentation back to useable NADH, and it accounts for 90% or more of the pathway under anaerobic fermentation. Therefore, while it's possible for organic acids to be produced by the Ehrlich pathway, in an anaerobic ethanol fermentation, the majority of those amino acids that enter the pathway will be converted into fusel alcohols.

Three non-volatile fixed acids account for the majority of acids in wine: malic, citric, and tartaric acids. The majority of the other various organic acids in wine are produced by yeasts and bacteria during fermentation. Given that fixed acids—such as malic, citric, and tartaric acids will not evaporate during distillation, it's likely that most of the organic acids important to flavor in whiskey are derived from yeast and bacterial fermentation. Some may arise from the reductive pathway of the Ehrlich pathway, as stated above. But others—such as lactic, pyruvic, acetic, butyric, and isovaleric acids—will be produced as intermediate or final by-products of yeast (including other genera and species beyond *Saccharomyces cerevisiae*, such as *Brettanomyces*) and bacterial glucose metabolism [150, 151].

The biochemical production of esters occurs in the cytoplasm of a yeast or bacterial cell from enzymatic condensation reactions—called *esterification*—of organic acids and alcohols (both ethanol and fusel alcohols). As discussed above, the precursor organic acids and alcohols of esters are themselves produced by upstream metabolic pathways that start with sugars and amino acids. For example, higher alcohols produced

via the Ehrlich pathway can be converted into their corresponding acetate esters via acetyl-CoA [152]. Esterifications can also happen via chemical means during fermentation, distillation, or maturation. However, the basal alcohols and acids involved in these reactions are still largely derived from yeast fermentation.

Before considering the impact of terroir on fusel alcohols, organic acids, and esters, it's worth noting that yeast strain, bacterial community, and fermentation temperature/length are well established influencers on their presence and concentration. Indeed, one of the first gas chromatography studies in wine was monumental for showing that different yeast strains produced varying levels and types of higher alcohols [153]. While it had been known through practice and sensory analysis studies that different yeast strains created different flavors, this early work actually revealed some of the compounds that were responsible for the variation.

However, as previously discussed, terroir can still impact flavor compounds which derive indirectly from grain, such as fermentation by-products. For example, while all grapes and grains contain proteins, the concentrations and compositions will vary among varieties and species. As amino acids are taken up by yeast cells, they can be converted into fusel alcohols and esters (and to a much lesser extent organic acids) via the Ehrlich pathway. Therefore, it can be reasoned that grapes and grains with higher concentrations of proteins may end up producing wines and whiskeys with heightened fruity and floral notes from fusel alcohols and esters compared to grapes and grains with lower levels of proteins and amino acids. Indeed, reports in wine show that increased nitrogen in the soil leads to increased ester concentrations in wine. Further, the specific

composition of the proteins, and therefore the amino acids, will also lead to varying flavors. For example, the amino acid leucine will be metabolized into isoamyl alcohol and ethyl isovalerate, while the amino acid valine will be metabolized into isobutyl alcohol and ethyl isobutyrate.

The specific make-up of sugars will also influence fermentation by-products. In must or juice, glucose and fructose sugars dominate. In mash or wort, maltose dominates, but it is complemented by glucose and maltotriose. Research has shown that there are no significant differences in ester and fusel alcohol concentrations between glucose and fructose metabolization. However, higher alcohol concentration disproportionately increases when the ratio of sucrose increases; and conversely, higher alcohol and ester concentrations decrease as maltose ratios increase [154]. Why exactly different sugar metabolisms lead to varying levels of higher alcohols and esters is still unknown.

Ultimately, it has been found in wine that vineyard location, grape variety, and agronomic management can all impact the presence and concentration of more than a dozen esters, fusel alcohols, and organic acids from Table 14. In beer, barley variety has been reported to impact the presence and concentration of phenylethyl acetate, ethyl acetate, isoamyl alcohol, and isobutanol.

The only fusel alcohol shared between Table 11 and Table 14 is phenethyl alcohol, with the environment and the gene x environment interaction accounting for 16.2% and 27.5% of the variation in new-make bourbon, respectively. No organic acids were identified in Table 11. The shared esters were ethyl laurate, ethyl undecanoate,

ethyl hexanoate, ethyl octanoate, isoamyl acetate, and ethyl acetate. Across all, 25% to 80% of the variation present in new-make bourbon was due to environment, genetics, and/or the interaction.

Ketones

4-methyacetophenone was found to discriminate cabernet sauvignon wines from different Australian geographic indications. However, it is scarcely reported in literature, and its origins are not known.

Diacetyl is a well-known flavor compound in an array of food and beverages, providing a distinct buttery aroma. At heightened concentrations, it is typically regarded as a taint compound. But in some styles—such as chardonnay—and at acceptable levels, it is desirable. Its presence in wine, beer, and whiskey can come from chemical reactions and/or microbial biosynthesis during fermentation. In one pathway, acetolactate—an intermediate in valine biosynthesis—produced by yeast will leak out of the cell and into the fermentation medium. Acetolactate is then chemically converted via oxidation to diacetyl. In a second pathway, diacetyl is biosynthesized by yeasts and bacteria during fermentation. When diacetyl levels are elevated to the point of concern, unintentional bacterial infection is usually the cause.

Fermentation temperature/length, level of oxygen at the start of fermentation, and yeast strain can all impact the presence and concentration of diacetyl. However, it’s also been reported that the concentration of valine and other amino acids in barley malt can impact the prevalence of diacetyl [155]. And indeed, a report in beer has shown that

barley variety does significantly impact diacetyl levels. No ketones were shared between Table 11 and Table 14.

Lactones

Of the lactones identified, at least four are derived either directly or indirectly from grain: δ-nonalactone, γ-nonalactone, γ-decalactone, and γ-dodecalactone. All four of these lactones carry coconut, peach, and creamy sweet flavors. While δ-nonalactone, γ-decalactone, and γ-dodecalactone are produced by yeast from fatty acid precursors during fermentation, its reported that γ-nonalactone is formed by the lipoxygenase oxidation of grain-derived linoleic acid during mashing [156, 157]. In wine, γ-nonalactone has been called the "hidden key wine odorant"[158]. Again, vineyard location, grape varietal, and agronomic management can impact the presence and concentration of lactones in wine. No lactones were identified in Table 11.

Methoxypyrazines

Among whiskeys, 2-isopropyl-3-methoxypyrazine (IPMP) was only identified in the bourbon report. In wine, it is an important contributor of potato, earthy, and asparagus aromas in certain wines. While some yeast species and strains—and even the vineyard pest Lady Beetle (*Harmonia axyridis*) [159]—can produce IPMP, its presence in wine is believed to derive primarily from the grape berry itself and its amino acid precursors. IPMP levels are reported to be highest in cabernet sauvignon gapes, with levels dependent on a combination of environmental factors, with increased sunlight,

temperature, and water stress leading to lower concentrations [123, 125]. Vineyard location is also influential, with New Zealand sauvignon blanc being significantly higher in IPMP than Australian [123]. While the origin of IPMP in bourbon or other whiskeys has not been reported, it is indeed possible that it—as well as other potentially important methoxypyrazines—originate in the grain.

Norisoprenoid Terpenes

Norisoprenoid terpenes in wine and whiskey are reported to be produced from the degradation of carotenoids—especially carotenes and luteins—in grapes and grains, respectively [132]. In grapes, these flavorless precursors are bound to sugars in the fruit but are released during fermentation and develop into floral norisoprenoid terpenes. Table 14 cites multiple research reports showing that the composition and concentration of carotenoids in grapes is influenced by grape variety, soil characteristics, climate, and viticultural practices. For example, more sunlight in the vineyard appears to encourage the development of most norisoprenoid terpenes, such as β-ionone. Interestingly, this is the opposite of β-damascenone, which is reported to be heightened in conditions with less sunlight [130]. No norisoprenoid terpenes were identified in Table 11.

Sulfides

Dimethyl sulfide in wine, beer, and whiskey arises from two sources: the thermal degradation of S-methyl methionine (SMM) in grain during malting, mashing, and distillation; or the reduction of dimethyl sulfoxide (DMSO)—which itself is derived

from the breakdown of SMM—by yeasts [140, 160]. Grape and grain varieties appear to be most influential in determining levels of dimethyl sulfide in wine and beer.

Volatile Phenols

Volatile phenols impart flavors that range from *smoky* and *medicinal* to *barnyard* and *sweaty saddle* to *vanilla* and *sweet spice*. They are a diverse set of compounds, and the human nose is especially sensitive to their presence. Volatile phenols can come from fermentation byproducts, from malts that were dried using smoke (such as peat smoke or wood smoke), from thermal degradation of grain constituents during high-temperature malting, mashing, or distillation, and from oak maturation. Regardless, they form from the thermal degradation or microbial metabolism of the hydroxycinnamic acids that are the basal building blocks of lignin and lignan in grape, grain, and oak.

From Table 14, 4-vinylguaiacol, 4-ethylguaiacol, and 4-ethylphenol are certainly promising in the context of terroir, as they likely are derived from grain and not peat smoke or oak. 4-vinylguaiacol and 4-vinylphenol are well-known flavor compounds in whiskey, beer, and wine. The characteristic clove, spice, and phenolic flavors of Hefeweizens, witbiers, and saisons are largely due to these two volatile phenols. 4-vinylguaiacol is formed through the thermal degradation or metabolism of ferulic acid, and 4-vinylphenol is formed through the thermal degradation or metabolism of coumeric acid. Only certain yeast species and strains can produce the necessary enzymes to break down ferulic acid and coumeric acid into their vinyl phenol derivatives. The

Saccharomyces cerevisiae yeast strains used to make Hefeweizens, witbiers, and saisons typically have the genetic machinery to produce these necessary enzymes.

4-ethylguaiacol and 4-ethylphenol result from either the thermal degradation or metabolism of their vinyl phenol precursors. The former, 4-ethylguaiacol occurs during malting, mashing, or distillation. The latter, 4-ethylphenol occurs during fermentation, and is typically attributed to the wild yeast *Brettanomyces*, which can produce the enzymes necessary to metabolize 4-vinylguaiacol and 4-vinylphenol into 4-ethylguaiacol and 4-ethylphenol, respectively.

Multiple reports in wine show that 4-vinylguaiacol, 4-ethylguaiacol, 4-vinylphenol, and 4-ethylphenol are impacted by vineyard location, grape varietal, and agronomic management in both presence and concentration. Further, a report showed that the hydroxycinnamic acid precursors for 4-vinylguaiacol and 4-vinylphenol varied widely based on the variety of barley, where the barley grew, and the agronomic techniques employed [19].

Eugenol and guaiacol are often attributed to oak barrel maturation. However, it's likely that they can originate in grain from thermal degradation of hydroxycinnamic acids. Table 15 shows drastically different concentrations for eugenol, guaiacol, and 4-ethyguaiacol in rye whiskey as opposed to bourbon. Given that bourbon and rye whiskey are matured in nearly identical barrels, this suggests that the varying levels of rye in their recipes are responsible for the elevated levels.

No volatile phenols were identified in Table 11. However, the closely related cinnamic acid derivatives styrene and 4-vinylanisole were.

Table 15. Concentration differences for three volatile phenols between bourbon and rye whiskey

Compound Class	Flavor Compound	Concentration in Bourbon (ppb) [56]	Concentration in Wild Turkey Rye (ppb) [72]	Concentration in Rittenhouse Rye (ppb) [72]
Volatile Phenol	4-ethylguaicol	59	2180	187
	Eugenol	240	583	993
	Guaiacol	56	3760	3150

3.3 Insights into chemical variations among different whiskey styles

While there is considerable overlap in the flavor compounds important to bourbon, rye, and malt whiskeys, it is necessary to hypothesize both why this overlap occurs, as well as what causes some flavor compounds to be specific to only one or two of the styles. Doing so should help facilitate which flavor compounds to select for in grain.

While the data is limited, Table 14 and Table 15 provide some evidence that volatile phenols are more prevalent in rye whiskeys than in bourbon. Anecdotally, rye whiskey is known to possess higher levels of spice, phenolic, and clove notes as compared to bourbon, which does indeed align with a heightened level of volatile phenols. Given that the rye whiskeys and bourbon from Table 15 would have been matured in very similar barrels—per U.S. law which states both styles must be aged in new, charred oak barrels—it's probable that some of the differences in the three compounds listed are due to grain.

Hydroxycinnamic acids in grain are precursors to volatile phenols, which can form through thermal degradation or microbial metabolism. In grain they can exists in

either bound or soluble form, and pending which types are measured (or if both are measured), reports have varied widely on concentrations across different grain species [161, 162]. It is unknown to what extent that bound hydroxycinnamic acids are released during mashing, fermentation, or distillation and therefore susceptible to thermal degradation or microbial metabolism. What has been reported, however, is that regardless of the extraction method used, the concentration of lignan in the bran of rye is drastically higher than in the bran of corn (6,000 to 8,000 μg/100 g compared to 1,000 μg/100 g) [163].

Lignans are a ubiquitous group of dimeric (although higher oligomers exists) phenolic metabolites and essential for plant defense versus structure. The more well-known secondary metabolite lignin, conversely, is polymeric and essential for structural reinforcement and water conduction of plants. However, both are built from the same hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, and 5-hydroxyferulic) [164].

So, while it's speculative, there is some evidence that increased levels of accessible (i.e., not bound and resistant to degradation or metabolism) hydroxycinnamic acids in grain can lead to increased levels of volatile phenols in whiskey.

It is also worth noting that the malt whiskey from Table 14 was produced from malted barley that had been dried with peat, which is partially decayed organic matter that can be used as a fuel source. Given that the material is derived from plants and only partially decomposed, the burning of peat leads to highly aromatic smoke that is distinctly heightened in volatile phenols. Therefore, malted barley produced using peat does indeed provide heightened and unique levels of volatile phenols to whiskey. This

might explain that why volatile phenols were crucial to the flavor of both the rye and malt whiskeys, there were indeed some differences in the types of volatile phenols present.

It is also apparent from Table 14 that aldehydes are likely more important to flavor in bourbon than in rye whiskey. The majority of these aldehydes derive from the oxidation of fatty acids during malting, mashing, or distillation. While fat/oil content can vary widely depending on variety and environment, reports generally show that corn contains more fat/oil than rye [165-167]. So, while again it's speculative, the presence of more fat in corn might explain why there are more aldehydes in bourbon than in rye whiskey.

We also see that organic acids are more prevalent in rye whiskey than in bourbon whiskey. One explanation might be a heightened level of bacteria in a rye mash fermentation than a bourbon mash fermentation. In a bourbon mash, the corn is cooked at temperatures between 85°C to 100°C to effectively gelatinize its crystalline starch. In a rye whiskey mash, the rye is usually cooked at a much lower temperature—64°C to 68°C—as its starch is not as tough to gelatinize. Compared to rye whiskey mash temperatures, the elevated ranges used to cook corn in a bourbon mash will lyse more of the vegetative and sporulating bacteria. However, many bacteria—including both non-spore forming (such as *Lactobacillus*) and spore-forming (such as *Clostridium*)—survive the temperatures of a rye mash [168]. So rye whiskey fermentations can contain higher concentrations of bacteria, which typically produce elevated levels of organic acids compared to yeast during fermentation. This is especially true of bacteria common to

whiskey fermentations, namely lactic acid bacteria and acetic acid bacteria. Indeed, the make-up of *Lactobacillus* species and strains has been shown to be specific and unique to individual distilleries, and appear to contribute to "house flavors" in whiskey [169, 170].

Ultimately, those differences in flavor compounds that do exist in whiskey can be attributed to a number of factors. Some are related to terroir, and some are not. But what is apparent is that grain-derived flavor compounds can originate either from primary metabolites, as is the case with fatty acids and amino acids, or from secondary metabolites, as is the case with carotenoids and hydroxycinnamic acids. By developing this chemical roadmap, plant breeders and distillers should be able to determine which metabolites in grain—be them precursors or end products—are most suitable for selection.

3.4 From theory to practice

Section 2 provided direct evidence that grain variety and growing environment can impact the presence and concentration of both flavor compounds and aromas in new-make bourbon whiskey. This section used previously published literature to build a roadmap of those compounds that are both known to be important for flavor in whiskey and which have also been reported to be impacted by terroir in wine and beer. As revealed in Table 14, for nearly every flavor compound listed, there are reports showing that some aspect of terroir—from variety to vineyard location to agronomic management—can influence flavor presence and concentration in wine or beer. That

said, while this dissertation already provides support that terroir impacts flavor in whiskey, both through experimental and analogical evidence, what has not been presented is how plant breeders can use this information to select for grain varieties that will impart tailored, diverse, and heightened flavors to whiskey.

For selection, it is not practical for plant breeders to identify and quantify flavor compounds in whiskey, even new-make whiskey. The main reason for this is that the process of converting grain into new-make whiskey is low-throughput to the point that the sample numbers needed for selection are not practically achievable. However, converting grain into mash—and even beer—via the use of high throughput, commercially available mash baths would allow for the sample numbers necessary for selection. Therefore, it's possible that metabolite precursors and/or flavor compounds can be identified and quantified in mash and/or beer and the data can be used for selection of improved varieties. A proof-of-concept experiment is first necessary to show that precursor metabolites in mash and/or beer do indeed correlate with flavor compounds in whiskey if successful this could likely be used within a breeding program. Such a study is detailed in Section 4.

4 A PROOF OF CONCEPT—ASSESSING VARITATIONS AND CORRELATIONS OF HYDROXYCINNAMIC ACIDS IN MASH/BEER AND THEIR DOWNSTEAM VOLATILE PHENOLS IN NEW-MAKE BOURBON

4.1 Choosing a chemical marker target for proof-of-concept in bourbon

Two distinct classes of metabolites are apparent targets from Table 14 which could serve as metabolite markers for selection of flavor in grain—primary and secondary. The primary metabolites are fatty acids, amino acids, and starch. The secondary metabolites are methoxypyrazines, carotenoids, and hydroxycinnamic acids.

Selection could also take two different approaches—either for the metabolite group as a whole (e.g., amino acids or carotenoids), or for specific metabolites within the group (e.g., the amino acid valine or the carotenoid lutein). The former would arguably impact a greater number of flavor compounds, which could be either desired or unwanted. The latter would allow for the selection of specific flavor compounds.

If selection is done for a primary metabolite class, whether the group as a whole or a select few, it may result in the decrease in concentration of one or both of the two other primary metabolite classes if both classes are not well monitored. For example, in corn, reports have shown that there is a negative correlation between fatty acid (i.e., oil) and starch content [171, 172]. The same negative correlation exists between amino acids (i.e., protein) and starch [173]. It remains unproven that this is a pleiotropic (genetic) tradeoff, as opposed to correlation from combining different types of corn, which could be broken through breeding. Assuming such tradeoffs are real, it might be a positive

trade-off for flavor. But lower starch content may translate to less alcohol per unit of grain. As yield in the whiskey-making process is a critical metric for efficiency, many distilleries—especially large commercial operations as opposed to small craft ones—would not quickly accept a grain variety that provides less alcohol per unit grain, even if the flavor was improved.

When we consider the flavor compounds from Table 14 identified in the bourbon research and the differences in volatile phenol concentrations between bourbon and rye whiskey in Table 15, it suggests that the selection for heightened levels of lignans and lignans—or more specifically, the hydroxycinnamic acids they are built from—would result in bourbon with a greater prevalence of desired volatile phenols than is produced with modern corn varieties. Further, selection for increased secondary metabolites such as hydroxycinnamic acids might be less likely to negatively correlate with yield (be it agronomic or alcohol). While little published evidence exists to actually support this claim, research from Harry Klee's group in tomatoes has suggested that selection for secondary metabolites should not impact primary metabolites, given that their concentrations are typically much lower [174]. Further, a recent report in corn from Martin O. Bohn's group at the University of Illinois has shown that the concentrations of ferulic acid and *p*-coumaric acid (two types of hydroxycinnamic acids) did not correlate with grain yield or test weight [162].

The analysis of hydroxycinnamic acids in mash and beer is rapidly achieved using routine HPLC methods, making them good targets for high-throughput phenotyping. The remaining portion of this section will detail a proof-of-concept

experiment to investigate if (1) hydroxycinnamic acid concentration in mash and/or beer varies based on the G effect; and (2) if hydroxycinnamic acids in mash and/or beer correlate with volatile phenols in new-make bourbon.

4.2 Materials & methods

4.2.1 Mash, beer, and mew-make bourbon production & kinetics

While the overall lab-scale approach to new-make bourbon production in Section 2 was adhered to here, certain improvements to the method were made. Such improvements are noted below in Table 16. Images of the mashing and distillation experimental set-up are in Figure 5.

Figure 5. Bench top set-ups for new-make whiskey production

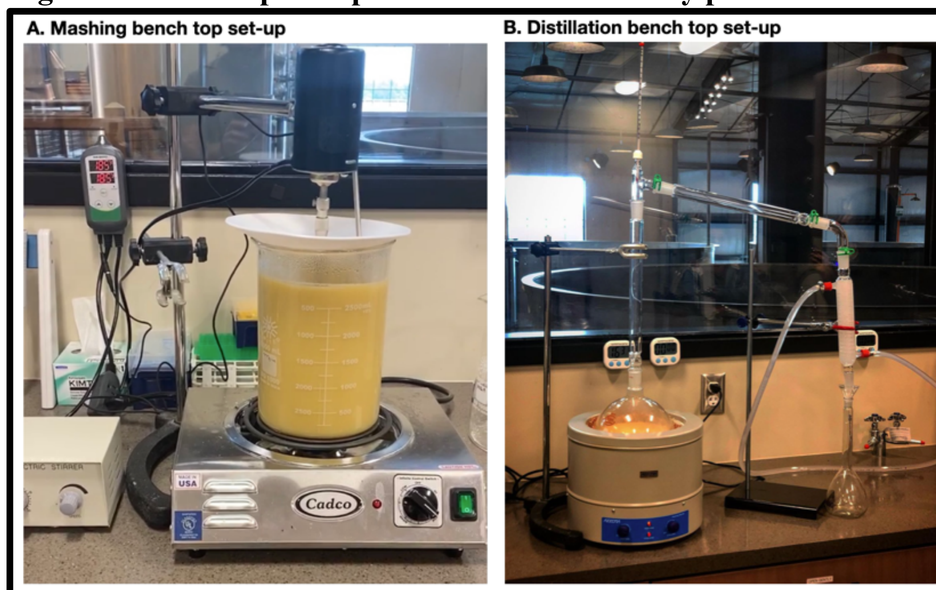


Table 16. Improvements made in experimental techniques for lab-scale new-make bourbon production

Process	Section 2 Technique	Section 4 Technique	Reason for Improvement
Milling	Hand cranked mill with unprecise ability for setting tightness	Automatic mill with precise tightness settings	Improves particle size consistency of grist
Fermentation	Room temperature	Incubation temperature	Fermentation is impacted by ambient temperature; the use of an incubator will increase fermentation consistency
Distillation pot	Non-stir pot	Stir pot	The ability to stir the beer during low-wines distillation ensures a more consistent run, as well as deters grain solids from settling to bottom and burning
Condenser	Non-temperature controlled	Temperature controlled	Controlling condenser temperature can lead to a more consistent distillation
Low-wines % alcohol by volume for spirit distillation	Not normalized across batches	Normalized across batches	Normalizing low-wines proof across all batches for spirit distillation negates flavor variations that may arise due to ratio of heads:hearts:tails

New-make bourbon was produced from four varieties of corn from Clarkson Grain Company that were grown in the same general vicinity and under similar conditions in Illinois (Table 17). Not all aspects of the environment could be controlled nor would be expected to be consistent. However, in general, locations were either in the same county or very close (Macon, IL is only forty-five miles from Piatt, IL). All farms

were dryland but rainfall tends to be sufficient to not limit crop growth. It should be noted, however, that rainfall in Illinois during the 2019 growing season (of which the corn samples used in this section derived from) was six to twenty inches higher than normal.

Lastly, it's important to note again that this research was not only concerned with elucidating the effects of G on hydroxycinnamic acids in mash and beer and volatile phenols in new-make whiskey. The goal was also to assess correlations between hydroxycinnamic acids in mash and/or beer with volatile phenols in new-make bourbon. The use of multiple varieties with distinct genetics and differing environments provides a robustness to the proof-of-concept that chemical markers in grain can be used to select for flavor in whiskey, if indeed correlations are identified.

Table 17. Varieties, Locations, & Growing Conditions

Corn Variety	Location	Soil Type	Planting Date	Harvest Date	Plants per acre	Irrigation type	Average row width	Crop rotation
White	Piatt, IL	Variable soils	N/A	N/A	Varies	Non-irrigated	76 cm	Corn/soy
Blue	Piatt, IL	Drummer Flanagan	6/3/19	11/4/19	30,000	Non-irrigated	76 cm	Corn/soy
Yellow waxy	Macon, IL	Drummer Flanagan	6/10/19	12/2/19	32,000	Non-irrigated	76 cm	Corn/soy/alfalfa
Red	Piatt, IL	Variable soils	N/A	N/A	24,000	Non-irrigated	76 cm	Corn/soy/wheat/alfalfa

For processing each batch, kernels were milled using a Mockmill 200 Stone Grain Mill (Mockmill USA) and then sieved 3X through a 2000 micrometer screen to ensure that the milled grain was fine and consistent from batch-to-batch. A 3 L beaker was filled with 2180 g of carbon-filtered municipal water.

A mechanical mixer (100W-LAB-SM, Gizmo Supply Co.) was used for agitation, and the temperature of the water was brought to 45˚C using a 120V hot plate with infinite heat controls (CSR-3T, Cadco) set to medium. Then 480 g of milled corn and 2 mL of high-temperature alpha amylase (AHA-400, FermSolutions Inc.) were added to the beaker. A cover slip that still allowed the mechanical mixer to operate was placed on top of the beaker to prevent excessive evaporation. The temperature of the mash was brought to 85˚C and held for 1 h.

After incubation, an ice bath was used to indirectly cool the temperature of the mash to 32˚C. Once 32˚C was achieved, 1.5 mL of glucoamylase (GA-150, FermSolutions Inc.) was added. Immediately after, 0.3 g of active dry yeast (Species: *Saccharomyces cerevisiae*; Strain: RHB- 422, F&R Distilling Co.'s proprietary strain) was added. The same strain was used for all batches, and the concentration of yeast used was based on standard inoculation rates for the whiskey industry, ensuring the role of other microbial organisms was minimal. The mash was further cooled to 24˚C using an indirect ice bath and mixed for an additional 10 min. Using aseptic techniques, pH was recorded with a digital pH meter (pH 220C, EXTECH) and specific gravity (SG) was recorded using a digital density meter (SNAP 50 density meter, Anton Paar). The beaker was covered with flame sterilized aluminum foil, weighed, and placed inside a

temperature-controlled incubator. Fermentation proceeded for 120 h at 24˚C. At the end of fermentation, pH, SG, and weight were again measured.

The beer post-fermentation was immediately transferred for distillation. The entire volume of beer from each finished fermentation was added to the stripping still, which was a glass apparatus still with a round bottom flask as the pot, a stirring heating mantle as the heat source, and a temperature-controlled condenser.

After beer was added to the round bottom flask, a magnetic stirrer was also added, and the distillation apparatus was connected. Consistent stir speeds, heat, and condenser temperature (20˚C) were applied for each batch. Distillation proceeded until 750 mL of distillate (termed “low-wines”) was collected in a grade A volumetric flask. The alcohol concentration by volume of the low-wines was measured using a density meter (DMA 5000 M, Anton Paar). Using weight, low-wines were diluted to the desired percent alcohol by volume (% ABV, which is equivalent to the ethanol concentration) with the addition of water, ensuring that the concentration of ethanol in the low-wines was consistent for each batch.

The spirit still was also a glass apparatus still with a round bottom flask as the pot, a stirring heating mantle as the heat source, and a temperature-controlled condenser. The spirit still was charged with 750 mL of low-wines at the same ethanol concentration (22.5%). A magnetic stirrer was added, followed by 5 g of copper powder. Copper is an important component of plant-scale distillation systems, as it reduces the levels of malodourous sulfur compounds [40]. Given that the glass distillation apparatus used here does not contain copper, copper powder was instead added directly to the low-wines.

Consistent stir speeds, heat, and condenser temperature (20˚C) were applied for each batch. Distillation proceeded until 25 mL of "heads" and 175 mL of "hearts" were collected using grade A volumetric flask. The alcohol concentration by volume of the low-wines was measured using a density meter (DMA 5000 M, Anton Paar). The hearts distillate was then stored in Boston round glass bottles with inert caps at room temperature until further processing.

This experimental design resulted in four treatments (four corn varieties), and each treatment was processed in triplicate, creating 12 batches total. Coefficient of variations (CV's) among varieties comparing the lab techniques for new-make bourbon production metrics in Section 2 and this section are below in Table 18. While improvements were not drastic, the improved techniques did generally improve CV's.

Table 18. Comparing replicate experimental errors for lab-scale new-make bourbon production techniques

Section 2				
	Coefficient of Variations %			
Variety	Mash Specific Gravity	Beer Specific Gravity	Low-Wines % ABV	New-Make % ABV
Dyna-Gro	0.1987%	0.4639%	6.4804%	1.0182%
Terrel	0.1496%	0.4059%	5.5667%	1.6692%
Mycogen	0.1167%	53.1895%	8.1491%	1.0925%
Section 4				
	Coefficient of Variations %			
Variety	Mash Specific Gravity	Beer Specific Gravity	Low-Wines % ABV	New-Make % ABV
Blue	0.0197%	0.3009%	5.2239%	0.5732%
Red	0.0795%	32.0873%	4.5567%	2.5690%
White	0.0868%	54.7767%	5.9894%	0.4690%
Waxy yellow	0.0144%	0.0154%	0.5977%	1.0693%

4.2.2 **Analysis of hydroxycinnamic Acids in Mash and Beer**

Hydroxycinnamic acid analysis in mash and beer samples was adapted from a previously published report from researchers at the Guinness Brewing Worldwide Research Centre [175], as described below.

HPLC was performed on a system consisting of a Waters model 510 HPLC pump, a Waters model 710B WISP automatic sample injector, a Waters model 460 electrochemical detector, a Shimadzu model RF-535 fluorescence HPLC monitor, and a Waters Maxima data acquisition and peak integration software system. Chromatographic separation was achieved using a 25-cm X 4-mm i.d. Poroshell EC C-18 10 μm column (Machery-Nagel, Duren, Germany) and a Waters Guard-Pak guard column containing a disposable insert packed with Nova-Pak C silica. The mobile phase consisted of H2O/CH3OH/H3PC4 (480:510:10 by vol) pumped at a flow rate of 1 mL/min at room temperature. Column temperature was 30˚C. Chromatograms were obtained by fluorescence monitoring using a detection excitation wavelength of 200-400 nm.

Standard solutions of ferulic acid, p-coumeric acid, and o-coumeric acid were prepared in the range of 0.1-4.0 mg/L. Standard solutions were injected under the described chromatographic conditions.

Mash and beer samples were filtered through 0.45 μm syringe filters into autosampler vials. The vials were capped and frozen at -20˚C until analysis.

While standards of ferulic acid, p-coumaric acid, and o-coumaric acid were used for identification and quantification three other peaks were clearly resolved and

quantified (via peak area) in the chromatograms, and are noted as unknown A, unknown B, and unknown C in tables below.

4.2.3 Analysis of Flavor Compounds in New-Make Bourbon

Immersion Thin Film-SPME-GC-TOFMS

For each sample, a 1 g sample of whiskey, 9 mL distilled water, and 5 μL 2-undecanone internal standard (0.025 μg/μL) were added to a 10 mL glass GC vial along with a PTFE micro-stirbar and fitted with a PDMS/DVB (on Carbon Mesh) thin film (TF)-SPME device and capped. As one internal standard was used, only semi-quantification was achieved. However, this is still suitable for sample-to-samples and correlations analyses. The sample was stirred 1hr at 900 rpm. The TF-SPME membrane was removed, rinsed with DI water, dried with a lintless cloth, and then thermally desorbed at 250°C with the GERSTEL Thermal Desorption Unit (TDU) into a glass baffled glass inlet liner while volatiles were cryotrapped at a temperature of -100°C. Cryotrapped chemicals were then released from the liner and into the GC capillary column by rapid heating of the liner to 260°C. Volatiles were injected into an Agilent 30 m x 0.25 mm x 1.4 m DB-624 capillary column and analyzed on a Leco BT GC-TOFMS.

Thermal desorption parameters used for TF-SPME

The Programmed Temperature Vaporization (PTV) Solvent Vent mode was used at a flow of 60mL/ min. The GERSTEL TDU initial temperature was 40°C with a 0.4min delay time; the TDU was ramped at 60°C/min to 250°C with a 4min hold time. TDU transfer line temperature was 300°C. The GERSTEL Cooled Injection System (CIS) was fitted with a baffled liner. Cryo liquid nitrogen cooling of the CIS injector was used with an initial temperature of -100°C and an equilibration time of 0.5min. The CIS was then ramped to 260°C at 12 °C/s with a hold time of 3min. Injections were made in splitless mode.

4.2.4 Statistical analysis

Statistical analyses were conducted in JMP Pro statistical software (version 15, SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) in a completely randomized designs was performed to assess significant variations for all metrics. Post-hoc Student's t-test was used for mean comparisons. Pearson's correlation analysis was used to investigate relationships between metabolites in mash/beer and flavor compounds in new-make bourbon.

4.3 Results & discussion

4.3.1 Assessing variation of brewing and fermentation kinetics.

It was important to monitor brewing and fermentation kinetics, as any significant

variations between the same varieties (i.e., the replications) or among different varieties might signify experimental error that could lead to inconsistent flavor chemistries that are not due to grain.

Table 19 below shows that overall, brewing and fermentation kinetics were similar between replicates and among varieties. Critically, starting and final weights, beer pH, and new-make % ABV were not significantly different. Weight is an indicator of volume, and the data show that no experimental error occurred for ensuring that water and grain amounts were consistent among batches. Beer pH is an indicator of potential contamination, and average pH was 4.10 ± 0.1. This indicates that no contamination was present among batches, which is marked by a pH lower than 3.8. New-make % ABV is an indicator of consistency during the spirit run, which is critical, as experimental variations here will definitely lead to flavor compound variations, especially in those compounds associated with the latter portion of a distillation run (namely, fusel alcohols and volatile phenols).

Mash SG was significantly different among varieties, and this is in-line with the results in Section 2, where variety accounted for significant differences in mash SG. Mean comparisons ($p<0.05$; t-test) showed the SG of waxy yellow was significantly higher than the other varieties. Red, white, and blue were not significantly different from each other. As expected, the beer % ABV (calculated), low-wines % ABV, and total ethanol yield values of waxy yellow were therefore significantly higher than the other varieties. However, normalization of each batch with water prior to the spirit run deterred significant differences in new-make % ABV. As mentioned, this was critical for

minimizing experimental error that would impact flavor chemistry. Given the lack of experimental error, especially for new-make % ABV, it was determined that samples were processed in a consistent enough manner that any flavor chemistry differences in mash, beer, and/or new-make bourbon would be due to the grain versus any other ingredient or process.

Table 19. Distributions, mean comparisons and ANOVA results for brewing and fermentation kinetics

Variable	Blue	White	Yellow waxy	Red	Prob > F (0.05)
Mash Weight (g)	2592.0 ± 51.1[a]	2554.3 ± 35.4[a]	2593.7 ± 58.3[a]	2556.7 ± 5.8[a]	0.5446
Mash pH	6.09 ± 0.0[b]	6.13 ± 0.1[ab]	6.22 ± 0.1[a]	6.12 ± 0.0[b]	0.0577
Mash Specific Gravity	1.055 ± 0.0[b]	1.056 ± 0.0[b]	1.058 ± 0.0[a]	1.055 ± 0.0[b]	0.0029
Beer Weight (g)	2350.8 ± 12.8[a]	2343.3 ± 44.2[a]	2335.3 ± 4.3[a]	2350.9 ± 4.5[a]	0.8188
Beer pH	4.18 ± 0.1[a]	4.03 ± 0.0[b]	4.10 ± 0.0[ab]	4.10 ± 0.1[ab]	0.1788
Beer Specific Gravity	0.997 ± 0.0[ab]	1.000 ± 0.0[a]	0.993 ± 0.0[b]	0.999 ± 0.0[a]	0.1357
Beer %ABV (Calculated)	7.7 ± 0.4[ab]	7.5 ± 0.0[b]	8.6 ± 0.0[a]	7.4 ± 0.3[b]	0.0645
Low-Wines % ABV	24.1 ± 1.3[b]	24.0 ± 1.4[b]	26.8 ± 0.2[a]	23.0 ± 1.0[b]	0.0135
Total Ethanol Yield (mL)	180.4 ± 9.4[b]	179.97 ± 10.8[b]	201.1 ± 1.2[a]	172.3 ± 7.9[b]	0.0135
New-Make % ABV	69.75 ± 0.4[a]	69.6 ± 0.3[a]	69.17 ± 0.7[a]	69.70 ± 1.8[a]	0.8861

Means ± standard deviations; different letters (a or b) are indicative of statistical significance ($p<0.05$; t-test); and probabilities of ANOVA are given.

4.3.2 Assessing variation in hydroxycinnamic acid concentrations in mash and beer among varieties

Three hydroxycinnamic acids (ferulic, *p*-coumaric, and *o*-coumaric) and three unknown compounds were identified and quantified in mash and beer samples via HPLC-PDA. Table 20 and Table 21 below shows means, standard deviations, mean comparisons for significant differences, and ANOVA probabilities for mash and beer, respectively. It is well studied and generally accepted that in wine and beer, ferulic acid and *p*-coumaric acid are direct precursors to 4-vinylguaiacol and 4-vinylphenol, respectively. These flavor compounds can be produced via thermal decarboxylation during mashing and distillation, or by enzymatic decarboxylation during fermentation [176]. *o*-coumaric acid's role as a precursor is less clear, although reports do show that it can be converted to salicylaldehyde in tobacco [177].

There were a number of significant differences to note in mash. Ferulic acid levels in mash made from both the blue and yellow waxy varieties were significantly higher than that of the red or white varieties. Unknown A levels were significantly higher in mash made from the yellow waxy, whereas unknown C levels were significantly higher in mash from the blue variety.

Significant differences in beer were different than that of mash. Ferulic acid and unknown A levels in beer were no longer significantly different among varieties. However, levels of unknown B were significantly higher in beer made from the blue and yellow waxy varieties. And as in mash, beer made from the blue variety had significantly higher levels of unknown C.

Table 20. Distributions, mean comparisons and ANOVA results for hydroxycinnamic acid concentrations in mash

Metabolite	Blue	White	Yellow waxy	Red	Prob > F (0.05)
Ferulic acid (ppb)	6.13 ± 0.46[a]	4.07 ± 0.33[c]	6.50 ± 0.59[a]	5.07 ± 0.48[b]	0.0009
p-Coumaric acid (ppb)	0.44 ± 0.11[ab]	0.27 ± 0.0[b]	0.68 ± 0.23[a]	0.57 ± 0.34[ab]	0.1839
o-Coumaric acid (ppb)	0.95 ± 0.36[a]	0.78 ± 0.25[a]	1.14 ± 0.03[a]	1.24 ± 0.70[a]	0.5609
Unknown A (peak area)	427 ± 11.27[b]	280 ± 20.78[d]	548.7 ± 18.44[a]	356.3 ± 24.44[c]	<.0001
Unknown B (peak area)	697.7 ± 180.51[a]	303 ± 63.55[b]	380 ± 304.88[ab]	249 ± 70.06[b]	0.0655
Unknown C (peak area)	367.33 ± 49.32[a]	58 ± 7.81[c]	143.33 ± 26.50[b]	133.33 ± 19.09[b]	<.0001

Means ± standard deviations; different letters (a, b, c, or d) are indicative of statistical significance ($p<0.05$; t-test); and probabilities of ANOVA are given.

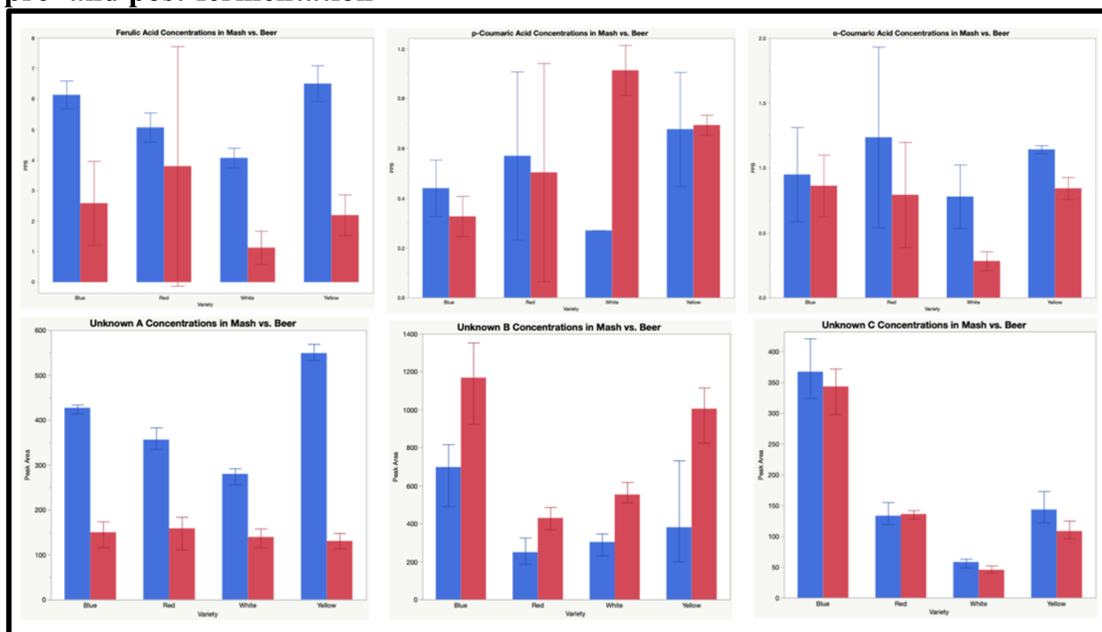
Table 21. Distributions, mean comparisons and ANOVA results for hydroxycinnamic acid concentrations in beer

Metabolite	Blue	White	Yellow waxy	Red	Prob > F (0.05)
Ferulic acid (ppb)	2.58 ± 1.37[a]	1.13 ± 0.54[a]	2.19 ± 0.67[a]	3.80 ± 3.92[a]	0.5235
p-Coumaric acid (ppb)	0.33 ± 0.08[b]	0.91 ± 0.10[a]	0.69 ± 0.04[ab]	0.50 ± 0.44[ab]	0.0644
o-Coumaric acid (ppb)	0.86 ± 0.23[a]	0.28 ± 0.07[b]	0.84 ± 0.09[a]	0.79 ± 0.41[a]	0.0540
Unknown A (peak area)	150 ± 30.27[a]	139.67 ± 21.50[a]	130.67 ± 17.00[a]	158.67 ± 41.31[a]	0.6754
Unknown B (peak area)	1169 ± 220.91[a]	553 ± 57.26[b]	1005.3 ± 157.51[a]	429.33 ± 58.59[b]	0.0006
Unknown C (peak area)	343.3 ± 39.72[a]	45.3 ± 5.86[c]	108.3 ± 14.98[b]	136 ± 7.21[b]	<.0001

Means ± standard deviations; different letters (a, b, or c) are indicative of statistical significance ($p<0.05$; t-test); and probabilities of ANOVA are given.

It is interesting to note the differences that exist between mash and beer. One potential reason may be linked to the fact that hydroxycinnamic acids can undergo conversion—via chemical degradation or metabolism—during fermentation. As shown in Figure 6, ferulic acid, *o*-coumaric acid and unknown A showed consistent drops in concentration from mash to beer. One potential reason is that the yeast strain used in this study is assumed (based on sensory analysis, not with genetic confirmation) to contain the genetic machinery to metabolize hydroxycinnamic acids into volatile phenols. Unknown B showed increases across all varieties from mash to beer, whereas unknown A levels stayed relatively constant. Interestingly, *p*-coumaric acid showed a consistent and substantial increase from mash to beer for the white variety, whereas the other varieties showed slight decreases or increases.

Figure 6. Hydroxycinnamic acid and unknown compound concentration kinetics pre- and post-fermentation



Blue bars represent concentrations in mash pre-fermentation and red bars represent concentrations in beer post-fermentation. Means are relayed with ranges as error bars.

It has been reported that G and GxE significantly impact hydroxycinnamic acid and lignin concentration in corn [178]. Such reports, coupled with the data above, provide support to the idea that selection for increased or decreased levels of hydroxycinnamic acids in corn is possible. However, for them to serve as chemical markers for flavor in whiskey, it will be important to understand how/if significant correlations exist between them and flavor compounds. This investigation is detailed in the following section.

4.3.3 Assessing variations and correlations of volatile phenols (and related compounds) in new-make bourbon

Immersion TF-SPME-GC-TOFMS analysis identified and quantified seventeen volatile phenols and closely related compounds/derivatives (Table 22). Those that are also present in the chemical roadmap (Table 14) are *p*-cresol, 4-ethylphenol, 4-ethylguaiacol, 4-vinylguaiacol, and vanillin. Subsequent correlation analyses were carried out to determine if hydroxycinnamic acids in mash (Table 23) or beer (Table 24) significantly correlated with any of the flavor compounds listed in Table 22. Given the limited sample size ($n = 12$), a correlation coefficient (R) of at least ±0.7 is statistically necessary (assuming $\alpha = 0.05$ and $\beta = 0.20$) to determine if it differs from zero [179]. That said, all significant correlations, regardless of R, are still noted in Table 23 and Table 24.

4-ethylphenol concentrations were significantly higher in new-make bourbon made from the yellow waxy variety compared to those made from the blue or red variety. 4-ethylphenol is a derivative of *p*-coumaric acid via 4-vinylphenol. While *p*-coumaric levels were indeed highest in mash made from the yellow corn variety, it was not significantly so compared to mashes made from the blue and red varieties. Surprisingly, there were no significant correlations present between hydroxycinnamic acids in mash or beer and 4-ethylphenol in new-make bourbon. However, when a single outlier was removed, *p*-coumaric acid in beer did significantly predict 4-ethylphenol ($R=0.6087$, p value $= 0.0469$). And when two outliers were removed, *p*-coumaric acid in

mash also significantly predicted 4-ethylphenol (R=0.6415, p value = 0.0456). This suggests that our limited sample size might limit interpretation of correlation analyses.

4-ethylguaiacol concentrations were significantly higher in new-make bourbon made from the yellow waxy variety as compared to the other varieties. 4-ethylguaiacol is a derivative of ferulic acid via 4-vinyguaiacol. Ferulic acid levels were indeed highest in mash made from the yellow corn variety, although not significantly so compared to the blue variety. Further, ferulic acid was not a significant predictor of 4-ethylguaiacol concentration in new-make bourbon. Correlation analysis did show that both *p*-coumaric acid and unknown A in mash were significant predictors of 4-ethylguaiacol in new-make bourbon. And unknown A in mash made from the yellow corn variety were significantly higher than the other varieties. While *p*-coumaric acid is typically reported as a precursor to 4-vinylphenol and its ethyl derivative, it's worth noting that *p*-coumaric acid is converted into ferulic acid during lignin/lignan biosynthesis via hydroxylation (into the intermediate caffeic acid) and then methylation. Whether or not *p*-coumaric acid can be converted chemically ferulic acid and eventually 4-vinylguaiacol and/or 4-ethylguaiacol from the high temperatures of mashing and distillation has not been explored.

The ANOVA for 4-vinylguaiacol was not significant at 5%. However, mean comparison did show that levels in new-make bourbon made from the red variety were significantly higher than that of the blue variety. However, this data does not align with the ferulic acid data above. Further, no significant correlations were identified, except for when three outliers were removed, at which point ferulic acid in mash did significantly predict 4-vinyguaiacol (R=0.7012, p value = 0.0353).

Vanillin is usually attributed to oak barrel maturation. However, given that it is a degradation product of lignin/lignan, it can also come from grain. Significant correlations in the negative direction were identified between unknown B and unknown C in both mash and beer and vanillin in new-make bourbon. That said, the levels are likely too low to meaningfully impact flavor after maturation, and therefore selection for increased levels of unknown C in grain should not exert an influence of flavors from vanillin in the final whiskey product.

There were other significant variations and correlations beyond those found in Table 14. While their importance to flavor in whiskey is not confirmed in the literature, this does not mean they should be ignored or warrant no further investigation. Lastly, it's important to emphasize is that while selection for increased levels of hydroxycinnamic acids might lead to increased levels of some flavor compounds, the data show that it will also lead to a decrease in others. The example with vanillin mentioned above is one example. Another is styrene, which showed a significant correlation with all hydroxycinnamic acids in either mash and/or beer. However, each correlation for styrene was in the negative direction, whereas correlations for 4-ethylphenol, 4-ethylguaiacol, and 4-vinylguaiacol were in the positive direction. Styrene is pleasantly sweet and phenolic at low concentrations, and Table 13 showed that styrene possessed a positive correlation with the "GOOD" aroma category. However, at excessively high levels, styrene can impart an undesirable plastic flavor. So, styrene's role as a positive or negative flavor compound is—like so many other compounds—concentration dependent. Regardless, this highlights that for effective selection, we will also need an

understanding of desirable ranges for all important flavor compounds that may be impacted the concentration of precursor metabolites.

Ultimately, while the data is not completely aligned, these results do show that hydroxycinnamic acids in mash or beer can be used as predictors of—and chemical markers for—volatile phenols (and closely related derivatives) in new-make bourbon.

Table 22. Distributions, mean comparisons and ANOVA results for volatile phenol (and related compound*) concentrations in new-make bourbon

Compound (ppb)[†]	Aroma	Blue	White	Yellow waxy	Red	Prob > F (0.05)
m-Cresol	*Spicy, Smoky*	0.34 ± 0.05^{a}	1.50 ± 0.75^{a}	1.35 ± 1.70^{a}	0.90 ± 0.66^{a}	0.5138
4-ethylphenol	*Band-Aid, Smoky*	0.30 ± 0.27^{b}	0.79 ± 0.39^{ab}	1.52 ± 0.62^{a}	0.34 ± 0.32^{b}	0.0246
p-Cresol	*Band-Aid*	0.18 ± 0.31^{a}	0.38 ± 0.65^{a}	0 ± 0^{a}	0.01 ± 0.01^{a}	0.5664
3,5-Dimethylphenol	*Phenolic*	0.38 ± 0.36^{b}	1.94 ± 0.30^{a}	1.03 ± 0.94^{ab}	0.63 ± 0.56^{b}	0.0544
Salicylaldehyde	*Medicinal*	8.79 ± 7.8^{a}	13.92 ± 1.63^{a}	15.22 ± 4.88^{a}	12.51 ± 11.15^{a}	0.7326
2-Methoxy-3-methylphenol	*Clove*	68.07 ± 59.26^{a}	43.25 ± 39.97^{a}	70.00 ± 68.15^{a}	46.48 ± 40.27^{a}	0.8881
4-Vinylanisole*	*Sweet*	3.95 ± 4.96^{b}	0.57 ± 0.98^{b}	15.29 ± 3.01^{a}	1.18 ± 2.04^{b}	0.0013
2-Isopropylphenol	*Medicinal*	0.45 ± 0.00^{a}	0.41 ± 0.36^{a}	0.41 ± 0.38^{a}	0.45 ± 0.39^{a}	0.9981
4-Ethylguaiacol	*Phenolic, Smoky, Bacon*	0.21 ± 0.06^{b}	0.48 ± 0.51^{b}	1.72 ± 0.99^{a}	0.48 ± 0.42^{b}	0.0555
4-Vinylguaiacol	*Spice, Clove*	25.48 ± 44.13^{b}	27.29 ± 24.11^{ab}	67.11 ± 11.36^{ab}	75.00 ± 9.83^{a}	0.0986
Piperonal	*Floral*	0 ± 0^{a}	0.31 ± 0.31^{a}	0.18 ± 0.16^{a}	0.09 ± 0.15^{a}	0.3097
Vanillin	*Vanilla*	0.24 ± 0.21^{b}	0.58 ± 0.13^{ab}	0.58 ± 0.13^{ab}	0.92 ± 0.25^{a}	0.0154
Coumarin*	*Vanilla*	0.09 ± 0.11^{a}	0 ± 0^{a}	0.03 ± 0.05^{a}	0.10 ± 0.11^{a}	0.3921
Sec-Butyl salicylate	*Floral*	0.09 ± 0.02^{a}	0.20 ± 0.10^{a}	0.14 ± 0.13^{a}	0.12 ± 0.12^{a}	0.6263
*Styrene**	*Sweet, Phenolic*	16.90 ± 0.82^{b}	31.87 ± 3.57^{a}	22.04 ± 9.55^{ab}	25.34 ± 11.25^{ab}	0.1869
α-Methylstyrene*	*Phenolic*	0.31 ± 0.04^{ab}	0.69 ± 0.20^{a}	0.15 ± 0.27^{b}	0.27 ± 0.34^{ab}	0.1122

Means ± standard deviations; different letters (a, or b) are indicative of statistical significance ($p<0.05$; t-test); and probabilities of ANOVA are given. [†] Semi-quantification, as only one internal standard was used, as indicated in materials and methods. * indicates a compound that is not a volatile phenol, but instead closely related and derived from cinnamic acids. Those compounds underlined are present in the chemical roadmap (Table 14). Styrene in *italics* was shown to positively correlate with "GOOD" aroma category (Table 13).

Table 23. Significant pairwise correlations between hydroxycinnamic acids in mash and volatile phenols in new-make bourbon.

Response	Predictor	Correlation (R)	P Value
m-Cresol	Unknown C	-0.5142	0.0872
3,5-Dimethylphenol	Unknown C	-0.5845	0.0460
Salicylaldehyde	*o*-Coumaric acid	-0.7081	0.0100
4-Vinylanisole	Ferulic acid	0.6884	0.0133
4-Vinylanisole	Unknown A	0.8595	0.0003
2-Isopropylphenol	*p*-Coumaric acid	0.6945	0.0852
4-Ethylguaiacol	*p*-Coumaric acid	0.6945	0.0122
4-Ethylguaiacol	Unknown A	0.6012	0.0387
Piperonal	Unknown B	-0.4987	0.0988
Vanillin	Unknown B	-0.6298	0.0282
Vanillin	Unknown C	-0.6398	0.0251
Styrene	Ferulic acid	-0.7132	0.0092
Styrene	*p*-Coumaric acid	-0.5674	0.0543
Styrene	Unknown A	-0.4992	0.0985
Styrene	Unknown B	-0.6080	0.0359
Styrene	Unknown C	-0.6112	0.0347
α-Methylstyrene	Ferulic acid	-0.6788	0.0152
α-Methylstyrene	*p*-Coumaric acid	-0.7751	0.0031
α-Methylstyrene	*o*-Coumaric acid	-0.6122	0.0344
α-Methylstyrene	Unknown A	-0.6543	0.0210

Table 24. Significant pairwise correlations and linear regression significance between hydroxycinnamic acids in beer and volatile phenols in new-make bourbon.

Response	Predictor	Correlation (R)	P Value
m-Cresol	Unknown C	-0.4998	0.0980
3,5-dimethylphenol	*p*-Coumaric acid	0.7310	0.0069
3,5-dimethylphenol	Unknown C	-0.6100	0.0352
Salicylaldehyde	*o*-Coumaric acid	-0.6473	0.0229
Salicylaldehyde	Unknown A	-0.6197	0.0316
Piperonal	*p*-Coumaric acid	0.6251	0.0297
Vanillin	Unknown B	-0.6836	0.0142
Vanillin	Unknown C	-0.5690	0.0535
Styrene	Ferulic acid	-0.5868	0.0449
Styrene	*o*-Coumaric acid	-0.6348	0.0266
Styrene	Unknown A	-0.5377	0.0714
Styrene	Unknown B	-0.6075	0.0361
Styrene	Unknown C	-0.5895	0.0437
α-Methylstyrene	Ferulic acid	-0.5189	0.0839
α-Methylstyrene	*o*-Coumaric acid	-0.7807	0.0027

4.3.4 Implications for a plant breeding program

While the data above is not entirely clear, the results do suggest that levels of hydroxycinnamic acids positively correlate with desirable volatile phenols in whiskey. Further, a recent report showed that among 12 commercially important corn inbred lines and 66 hybrids derived from their crosses, ferulic acid and *p*-coumaric acid concentration were highly heritable, and a majority of the genetic variation was additive [162]. Further ANOVA analysis from the same study showed that G, E, and GxE (across three years) effects were significant for ferulic acid and *p*-coumaric acid concentrations, with G being responsible for most of the variation. Their results are indeed encouraging and suggests that we can select for hydroxycinnamic acids in corn. What breeding approach we pursue will depend on the corn population of interest.

4.3.4.1 Breeding for hydroxycinnamic acids in open-pollinated corn

Over the last hundred years, breeding in corn has focused largely on hybrid varieties. However, the craft whiskey industry has revived the use of heirloom, open-pollinated varieties due to their proposed flavor advantages. As has been discussed at length in this dissertation, flavor has not been selected for in modern corn hybrid varieties. Alternatively, open-pollinated heirlooms were selected—to some extent—for flavor. That said, flavor improvement in heirlooms is still possible, and hydroxycinnamic acids are a prime target. However, in addition to selecting for flavor, yield improvement in heirlooms is needed in order for them to be seriously considered

by distilleries. Therefore, a multiple-trait selection approach for both yield and hydroxycinnamic acids should be pursued.

The hickory king white corn variety is one heirloom variety being trialed for use by distilleries. However, a recent study from the University of Kentucky showed that in a 2019 trial, hickory king white yields were ~37% of two modern hybrids [180]. Further, a 2015 study showed that a white corn variety had nearly half the concentration of total phenols as a morado variety [61]. Therefore, improvement of both yield and hydroxycinnamic acid content in hickory king white is needed.

Ideally, the original population for the breeding program would be combined from multiple hickory king white populations, realizing that different sources of this same heirloom variety may have wide genetic variation. Modified ear-to-row selection (a form of recurrent half-sib selection) as described previously for the open-pollinated heirloom hays golden variety could be employed [181, 182]. The procedure is detailed below. Given this method yields half-sib families, additive genetic variance and narrow-sense heritability can be calculated, proving further insights into the potential for selection of hydroxycinnamic acid concentration.

<u>Each Season Follows Same Procedure</u>

1. 190 ears are harvested from a random-mating population of hickory king white. Each ear becomes a separate entry in the yield and hydroxycinnamic acid evaluations of season 1.

2. Seed from the original parent population and five modern hybrids are used as checks, creating 196 total entries.
3. Three locations will be chosen in Texas: Sawyer Farms, College Station, and Fort Worth.
4. At each location, one replication of the 196 entries will be evaluated in a 14 x 14 triple lattice design.
5. At the primary location (Sawyer Farms), a crossing block scheme will be arranged of 4 female / 2 male rows. Female rows are detasseled and represent each of the 196 entries. Male rows are a bulk of seed from all half-sib families in the test. During growth, per row, the tip of the ears of five plants with the best appearance are spray painted red. Rows are harvested by hand. All ears are evaluated for yield. The non-marked ears are evaluated for hydroxycinnamic acid concentration via FT-NIR [61] or HPLC-PDA as described in this dissertation. The marked ears are saved.
6. At the other two locations, each of the 190 entries are bulked and evaluated for yield and hydroxycinnamic acid concentration.
7. Data for yield and hydroxycinnamic acid concentration are summarized for the three locations, and the best 38 of the 190 half-sib families are selected.
8. The 5 marked ears from each of the selected 38 families constitute the cycle 1 population. Seed from each ear will be a different half-sib family in the next cycle of selection, once again making 190 entries.

9. The cycle is repeated each season. Once desirable gains are made, seed can be bulked and open-pollinated to obtain the synthetic cultivar. Or, if selection will continue into perpetuity, the remnant seed from each cycle could also be used as the synthetic cultivar.

4.3.4.2 Breeding for hydroxycinnamic acids in hybrid corn

While recurrent selection of heirloom, open-pollinated corn varieties should lead to higher-yielding, more flavorful synthetic cultivars, the reality is that due to heterosis, hybrid corn will continue to possess top agronomic potential. However, recent reports show that there is still sufficient genetic variation present even within commercially important inbred corn lines for the improvement of hydroxycinnamic acid concentrations. Specifically, when 12 elite inbred lines and 66 F1 hybrids derived from their crosses were assessed, the researchers found that the broad sense and narrow heritabilities for ferulic acid were 86.6% and 68.7%, respectively; and the broad sense and narrow heritabilities for *p*-coumaric acid were 87.8% and 79.3%%, respectively [162]. A standard approach for a commercial breeding program for hybrid maize is outlined below. This approach utilizes double haploids. If such an approach is not available, then inbreds will need to be generated through traditional techniques, such as the single seed descent or pedigree methods.

Summer 1

Grow 80 F2 populations derived from elite inbreds. Select 50 plants from each population and cross to a haploid inducer.

Winter 1

In winter nursey, produce double haploids and generate 4000 inbred lines. Immediately after in second winter nursery, self inbreds to increase seed. Choose the top 3000 inbred lines based on yield and hydroxycinnamic acid concentrations.

Summer 2

Cross the 3000 inbred lines to an inbred tester. Ideally this would be an elite inbred with a low hydroxycinnamic acid concentration in order to increase testcross variance.

Summer 3

Un-replicated trials of the 3000 testcrosses at 6-8 locations.

Winter 3

Select 400 inbred lines based on testcross yield and hydroxycinnamic acid concentration. Cross to 3 inbred testers each.

Summer 4

Un-replicated trials of the 1200 testcrosses at 8-12 locations.

Winter 4

Select 40 inbred lines based on testcross yield and hydroxycinnamic acid concentration. Cross to 5 elite inbreds each.

Summer 5

Yield and hydroxycinnamic acid concentration trials of experimental hybrids at 15-40 locations.

Summer 6

Yield and hydroxycinnamic acid concentration trials of advanced hybrids at 20-75 locations. This will be accompanied by on-farm strip tests at 30-500 locations.

Summer 7

On-farm strip tests of precommercial hybrids at 50-1500 locations. Release 0-2 new hybrids in the fall.

A final approach to consider would be to take advantage of both the high phenol content shown to exist in open-pollinated, colored corn as well as the yield advantages of heterosis. To do this, inbred lines derived from multiple heirloom corn varieties could be used in testcrosses, either with elite inbreds and/or with each other. The blue corn variety from Clarkson grain used in this section was created through the inbreeding and crossing of existing open-pollinated blue corn varieties.

It's important to note that while it would be impractical to process thousands (or even just hundreds) of entries/inbreds/hybrids into new-make whiskey and assessed for volatile phenol concentration, it is entirely possible to process this number into mash and assess for hydroxycinnamic acid concentration. The reason is that high-throughput means exists for mash production and hydroxycinnamic acid analysis via automated mash-baths and HPLC. This emphasizes the importance of the research in this section—

as well as the chemical roadmap in Section 3—and why further research should continue to explore which metabolite markers in grain are most useful for flavor selection in whiskey

4.3.5 Beyond hydroxycinnamic acids and volatile phenols

Volatile phenols are of course not the only compounds that should be considered for flavor. Indeed, the immersion TF-SPME-GC-TOFMS analysis of new-make bourbon detailed in the materials and methods identified and quantified many other compounds beyond volatile phenols. Those flavor compounds that showed significant concentration differences due to variety are detailed in Table 25 below. Importantly, many of the flavor compounds in the table are also present in the chemical roadmap from Section 3 (Table 14), were reported to be impacted by terroir in Section 2 (Table 11), or both.

It's also very interesting to note that a number of compounds significantly impacted by variety here were not in Section 2. This provides some support to the notion that terroir's impact will be more meaningful among genetically diverse varieties and environments.

The unknown compounds identified in the mash and beer samples were checked for correlations with the flavor compounds listed in Table 25. Again, given the limited sample size ($n = 12$), a correlation coefficient (R) of at least ± 0.7 is statistically necessary (assuming $\alpha = 0.05$ and $\beta = 0.20$) to determine if it differs from zero. There were a number of correlations between the unknown compounds in mash (Table 26) and

beer (Table 27) and the flavor compounds listed in Table 25. Many of these correlations are for compounds found in the chemical roadmap.

As we look to the future of breeding new varieties for flavor in the context of specific environments, it will make sense to focus on those compounds in Table 22 and Table 25 that are both present in the chemical roadmap and shown (to any extent) to correlate with precursor metabolites in mash and beer. That said, the other compounds identified in this dissertation are potentially still important and worthy of consideration.

Table 25. Distributions, mean comparisons and ANOVA results for flavor compound (other than volatile phenol) concentrations in new-make bourbon

Compound Class	Compound (ppb) †	Blue	White	Yellow waxy	Red	Prob > F (0.05)
Acetal	***<u>Acetal</u>***	651.7 ± 169.8^{a}	291.3 ± 281.6^{bc}	469.9 ± 131.2^{ab}	17.5 ± 30.3^{c}	0.0129
	1,1-Diethoxyheptane	0 ± 0^{b}	2.1 ± 3.0^{b}	1.9 ± 1.7^{b}	13.6 ± 2.6^{a}	0.0002
Organic acid	Octanoic acid	75.5 ± 13.5^{ab}	42.0 ± 29.1^{b}	41.4 ± 30.9^{b}	114.9 ± 19.1^{a}	0.0176
	Nonanoic acid	42.1 ± 9.4^{b}	17.6 ± 21.0^{bc}	0 ± 0^{c}	104.6 ± 18.3^{a}	0.0001
	3-Decenoic acid	0.5 ± 0.8^{b}	0.1 ± 0.2^{b}	0 ± 0^{b}	5.1 ± 0.9^{a}	<0.0001
	Undecanoic acid	1.5 ± 2.7^{ab}	0 ± 0^{b}	0.6 ± 0.6^{b}	4.0 ± 0.5^{a}	0.0339
	Benzoic acid	0.6 ± 1^{b}	1.6 ± 0.6^{a}	0.6 ± 0.5^{b}	2.0 ± 0.4^{a}	0.0080
Ester	***<u>Ethyl acetate</u>***	158.2 ± 91.8^{c}	368.7 ± 181.7^{b}	612.3 ± 40.7^{a}	367.5 ± 60.3^{b}	0.0064
	Ethyl sorbate	8.6 ± 2.0^{b}	8.9 ± 1.0^{b}	20.3 ± 6.8^{a}	4.7 ± 2.4^{b}	0.0050
	Octyl acetate	0 ± 0^{b}	0 ± 0^{b}	10.7 ± 2.4^{a}	4.2± 7.2^{ab}	0.0259
	Isopentyl hexanoate	4.8 ± 0.9^{b}	2.4 ± 2.1^{bc}	7.9 ± 0.8^{a}	1.9 ± 1.3^{c}	0.0025
	Ethyl nonanoate	224.2 ± 41.8^{b}	184.4 ± 9.7^{b}	283.7 ± 22.2^{a}	204.0 ± 16.8^{b}	0.0072
	Ethyl trans-4-decenoate	62.4 ± 3.5^{a}	55.6 ± 5^{ab}	31.2 ± 3.6^{c}	52.5 ± 6.4^{b}	0.0002

Table 25 Continued

Compound Class	Compound (ppb) †	Blue	White	Yellow waxy	Red	Prob > F (0.05)
	Ethyl-2,4-decadienoate	23.8 ± 3.4[a]	16.5 ± 1.7[b]	7.5 ± 1.7[c]	20.6 ± 1.4[ab]	<0.0001
	Ethyl dodecanoate	75.4 ± 34.3[b]	92.2 ± 10.8[b]	150.1 ± 25.8[a]	74.4 ± 19.5[b]	0.0153
	Isoamyl decanoate	5.2 ± 1.0[b]	5.6 ± 0.1[ab]	7.6 ± 1.6[a]	4.5 ± 1.1[b]	0.0409
	2-Phenylethyl hexanoate	64.0 ± 8.3[b]	66.4 ± 5.8[b]	148.9 ± 30.9[a]	51.6 ± 17.9[b]	0.0007
	Ethyl tetradecanoate	1.6 ± 0.4[b]	2.3 ± 0.5[b]	4.2 ± 0.9[a]	1.6 ± 0.4[b]	0.0021
	Menthyl valerate	2.6 ± 2.3[ab]	1.5 ± 2.7[b]	0.4 ± 0.7[b]	6 ± 0.8[a]	0.0272
Ketone	Benzophenone	0.4 ± 0.3[b]	0.6 ± 0[ab]	0 ± 0[c]	0.7 ± 0.1[a]	0.0041
	Dihydropseudoionone	26.3 ± 2.4[c]	37.7 ± 3.4[b]	126.3 ± 3.2[a]	26.7 ± 1.6[c]	<0.0001
Lactone	γ-Decalactone	1.0 ± 0.8[c]	1.9 ± 0[b]	1.9 ± 0.4[bc]	3.0 ± 0.5[a]	0.0081
Aldehyde	Octanal	15.0 ± 1.2[b]	34.2 ± 1.8[a]	15.4 ± 5.0[b]	27.4 ± 14.0[ab]	0.0355
	Decanal	11.2 ± 3.5[b]	16.7 ± 6.8[b]	10.7 ± 3.0[b]	25.7 ± 1.7[a]	0.0075
	2,4-Nonadienal	47.0 ± 10.0[a]	31.4 ± 7.7[ab]	7.3 ± 6.5[c]	22.5 ± 20.9[bc]	0.0279
	(E,Z)-2,4-Decadienal	174.8 ± 11.0[a]	77.3 ± 6.0[b]	9.4 ± 3.0[d]	49.8 ± 21.5[c]	<0.0001
	(E,E)-2,4-Decadienal	347.5 ± 182.8[a]	327.3 ± 25.6[a]	65 ± 22.3[b]	165.8 ± 144.7[ab]	0.0539
	2-Undecenal	24.6 ± 3.2[a]	10.3 ± 8.9[b]	0 ± 0[c]	0 ± 0[c]	0.0006
Fusel alcohol	**Isoamyl alcohol**	64.6 ± 25.2[b]	58.9 ± 99.4[b]	1317.0 ± 485[a]	103.0 ± 107[b]	0.0006
	1-Vinylhexanol	4.6 ± 4.2[a]	0 ± 0[b]	0 ± 0[b]	0 ± 0[b]	0.0546
Furan	5-Methylfurfural	0 ± 0[b]	0 ± 0[b]	0.8 ± 0.7[b]	0 ± 0[b]	0.0544

Table 25 Continued

Compound Class	Compound (ppb) †	Blue	White	Yellow waxy	Red	Prob > F (0.05)
Terpene	Estragole	0.7 ± 0.1^{b}	0.1 ± 0.1^{b}	0 ± 0^{b}	0.6 ± 0.2^{a}	0.0005
	γ-Terpinene	5.7 ± 5.0^{a}	0 ± 0^{b}	0 ± 0^{b}	6.4 ± 1.7^{a}	0.0261
	Shisool	13.8 ± 0.2^{b}	6.2 ± 2.6^{d}	10.1 ± 0.9^{c}	16.6 ± 0.4^{a}	<0.0001
	Dihydro-β-ionone	0 ± 0^{b}	0 ± 0^{b}	5.4 ± 0.7^{a}	0 ± 0^{b}	<0.0001
	β-ionone	0.2 ± 0.4^{b}	1.0 ± 0.7^{b}	15.0 ± 2.6^{a}	0.2 ± 0.3^{b}	<0.0001
	β-Damascenone	20.4 ± 1.1^{b}	24.3 ± 4.9^{ab}	18.4 ± 0.7^{b}	29.6 ± 4.9^{a}	0.0197

Means ± standard deviations; different letters (a, b, c, or d) are indicative of statistical significance ($p<0.05$; t-test); and probabilities of ANOVA are given. † Semi-quantification, as only one internal standard was used, as indicated in materials and methods. Those compounds underlined are present in the chemical roadmap (Table 14). Those compounds in *italics* were reported to be impacted by terroir in Section 2 Table 11). Those compounds in bold were not significantly impacted by genetics in Section 2.

Table 26. Significant pairwise correlations between unknown compounds in mash and flavor compounds in new-make bourbon.

Response	Predictor	Correlation (R)	P Value
Isopentyl hexanoate	Unknown A	0.8567	0.0004
Ethyl nonanoate	Unknown A	0.847	0.0005
β-Ionone	Unknown A	0.8102	0.0014
2-Phenylethyl hexanoate	Unknown A	0.7908	0.0022
Ethyl tetradecanoate	Unknown A	0.7559	0.0045
Octanal	Unknown A	-0.7299	0.007
Ethyl sorbate	Unknown A	0.6996	0.0113
Ethyl trans-4-decenoate	Unknown A	-0.6617	0.0191
Ethyl dodecanoate	Unknown A	0.6352	0.0264
Benzoic acid	Unknown A	-0.6295	0.0283
Damascenone	Unknown A	-0.6159	0.033
Isoamyl decanoate	Unknown A	0.6084	0.0358
Octyl acetate	Unknown A	0.6084	0.0464
γ-Decalactone	Unknown B	-0.688	0.0134
2-Undecenal	Unknown B	0.6839	0.0346
(E,Z)-2,4-Decadienal	Unknown B	0.6000	0.0392
Decanal	Unknown B	-0.5825	0.0469
(E,Z)-2,4-Decadienal	Unknown C	0.7400	0.0059
2-Undecenal	Unknown C	0.6839	0.0142
Acetal	Unknown C	0.6408	0.0247
γ-Decalactone	Unknown C	-0.5853	0.0456
Octanal	Unknown C	-0.5803	0.0479
Ethyl Acetate	Unknown C	-0.5796	0.0482

Table 27. Significant pairwise correlations between unknown compounds in beer and flavor compounds in new-make bourbon.

Response	Predictor	Correlation (R)	P Value
γ-Decalactone	Unknown B	-0.7962	0.0019
Decanal	Unknown B	-0.7890	0.0023
Acetal	Unknown B	0.7837	0.0026
Benzoic acid	Unknown B	-0.7332	0.0067
Damascenone	Unknown B	-0.7102	0.0096
1,1-Diethoxyheptane	Unknown B	-0.7093	0.0098
Octanal	Unknown B	-0.6722	0.0166
Estragole	Unknown B	-0.6563	0.0205
Isopentyl hexanoate	Unknown B	0.6196	0.0316
Ethyl nonanoate	Unknown B	0.6096	0.0353
(E,Z)-2,4-Decadienal	Unknown C	0.7743	0.0031
2-Undecenal	Unknown C	0.6698	0.0172
Ethyl-2,4-decadienoate	Unknown C	0.6197	0.0316
Ethyl Acetate	Unknown C	-0.6162	0.0329

4.4 Conclusion

The research within this section has shown for the first time that hydroxycinnamic acids in corn-based mash and beer can act as significant predictors of volatile phenols in new-make bourbon. Further, a number of the volatile phenols identified are present in the chemical roadmap of terroir, and therefore are very likely to be important for shaping a whiskey's flavor. Given that volatile phenols are underrepresented in bourbon as compared to whiskeys produced from rye, selection for hydroxycinnamic acids in corn may lead to untapped flavor diversity and intensity.

The research within this section also confirmed that many of the flavor compounds in the chemical roadmap—from nine different compound classes—are significantly impacted by variety. And again, certain metabolites in mash and beer (although they are unknown at this time) show significant correlations with these flavor compounds.

The importance of identifying precursors in mash or beer for selection of flavor in whiskey cannot be overstated. Given that the conversion of grain into whiskey is low-throughput, prone to experimental variation, and requires significant portions of grain per sample (400 grams at a minimum), this research is a crucial step in making the trait of flavor amenable to selection by plant breeders.

While the conversion of grain into mash and beer is relatively high-throughput, it would be ideal to identify markers in the grain itself that can be measured via non-destructive measures. Rapid, non-destructive spectroscopy methods have been shown to identify and quantify lignin and phenols in grain kernels [61, 183], but whether or not

the techniques are specific or sensitive enough to effectively select for the predictors shown here or other measures of flavor in whiskey is unknown. That said, mash or beer may ultimately be a preferred medium for selection, largely because they are suitable for follow-up sensory analysis, which may provide additional insights and facilitate selection. Given that most of the grain-derived flavor compounds in whiskey are not flavor-active in the raw grain, sensory analysis of raw grain is not necessarily informative.

5 CONCLUSIONS

The debate as to whether or not terroir exists in whiskey has been ongoing for decades, and it likely will continue in some form for the foreseeable future. However, this dissertation provides for the first time a scientific foundation to the terroir phenomenon in whiskey, as well as how it can be leveraged for the selection of flavor. Furthermore, a recent publication from Waterford Distillery, Oregon State, and Teagasc also demonstrated terroir in malt whiskey, confirming our results [184].

Future research should expand on the approaches taken here to validate these findings and identify additional chemical markers in grain, mash, or beer that can be readily measured and used for selection in a breeding process. Will the composition and concentration of carotenoids predict norisoprenoid terpenes? Can we select for starch composition and tailor the specific make-up of sugars in the mash, achieving a desired fusel alcohol profile in the process? Can we increase the concentrations of specific amino acids and fatty acids in grain without negatively impacting starch levels? Will methoxypyrazines, which are so crucial for flavor in certain wines, emerge as equally important flavor compounds in certain whiskeys? And will we ever employ agronomic techniques on the farm that are specifically done to influence the concentration of flavor compounds and/or their precursors, as is done in the vineyard for wine?

While there are many future research endeavors ahead before we fully elucidate and leverage terroir, the chemical roadmap outlined in this dissertation should provide a

framework that chemists, plant breeders, farmers, and distillers can use for the foreseeable future.

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