TARGETED AND UNTARGETED CHROMATOGRAPHY–MASS SPECTROMETRY METHODS FOR ALCOHOLIC BEVERAGE ANALYSIS

by

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DISSERTATION

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Abstract

Targeted and Untargeted Chromatography–Mass Spectrometry Methods for Alcoholic Beverage Analysis

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There has been an increased interest in the "craft" alcohol industry in recent years. Contrary to larger corporations that focus on standardizing their recipes for mass production, these small, independent businesses can experiment with ingredients and procedures to create unique and flavorful products. Beer and whiskey in particular have seen a surge in popularity, indicating the public may be interested in a deeper understanding of these beverages and their flavors on the chemical level.

Brewing and distilling rely heavily on chemistry to perform the proper procedures to obtain the desired product, and to understand the interactions of the various ingredients. However, these modern craft beers and whiskies tend to have more intricate flavors than their mass-produced counterparts, making their chemical profiles potentially even more complex. Therefore, advanced analytical techniques are desirable to obtain a more complete view of these popular beverages.

Three studies were conducted using mass spectrometry methods for beer and whiskey flavor analysis. First, a collection of 32 craft beers covering 5 styles were analyzed in a targeted fashion using liquid chromatography quadrupole time-of-flight mass spectrometry to determine if they could be distinguished based solely on the presence or relative content of iso-α-acids

and phenolic compounds. These two classes of compounds were selected due to their influence on beer flavor and stability. The optimized method successfully separated and quantified the monitored analytes using high resolution multiple reaction monitoring. While a few compounds were found to be unique to a style, such as vanillin in stout beers and a higher iso-α-acid content in India pale ales, it was determined that the targeted analytes were not sufficient to confidently distinguish the styles. Therefore, the second study analyzed the same set of beers using untargeted and multivariate techniques to determine which compounds were the most influential in differentiating beer styles. Unsupervised principal component analysis provided visualization of the variance in the 5 styles, confirming that there were indeed differences between them on the chemical level. Further multivariate analysis resulted in a list of possible formulas for the most influential metabolites, and their identities were predicted.

Finally, a third study was conducted to develop a headspace solid phase microextraction gas chromatography mass spectrometry method to simultaneously identify and quantify compounds which cause off-flavors in whiskey. Some of the major contributors to whiskey off-flavors include 2,4,6-trichloroanisole and geosmin, which impart moldy and musty tastes. These compounds, along with two other haloanisoles, were separated and quantified in ten different whiskey samples using the optimized method. The application of these advanced analytical methods could potentially enhance quality control practices and product marketability in the alcohol industry.

Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
DVB	Divinylbenzene
ESI	Electrospray ionization
FID	Flame ionization detection
GC	Gas Chromatography
HCA	Hierarchal cluster analysis
HS	Headspace
IAA	Iso-α-acid
IC	Ion chromatography
IPA	India pale ale
IS	Internal standard
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
MeOH	Methanol
MS	Mass spectrometry
MRM	Multiple reaction monitoring
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
PLS-DA	Partial least-squares discriminant analysis
QC	Quality control
QTOF	Quadrupole–time-of-flight
RF	Random forest
RSD	Relative standard deviation
RT	Retention time

SPME	Solid phase microextraction
TCA	2,4,6-trichloroanisole
TBA	2,4,6-tribromoanisole

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CHAPTER ONE

Introduction to Dissertation

While the creation and consumption of alcoholic beverages can be dated back some 6000 or more years, there is still much we do not understand about their flavor profiles on the chemical level due to the complexity of the beverages' matrix [1]. This is becoming an even more prominent issue with the growing craft alcohol industry, as a wider range of beer and whiskey styles have been created through experimental procedures and ingredients [2,3]. For example, Buffalo Trace Distillery currently has more than 30,000 experimental barrels in their warehouse that vary based on the ingredients used (i.e. mash bill), types of wood, barrel toasts, and more [4]. There are approximately 9,000 craft breweries [5] and 1,700 craft distilleries [6] in the U.S. alone, leaving the industry in a constant state of flux. The wide variety of new beers and whiskies being developed should be accompanied by updated analytical methods.

Analytical techniques have long been used for improving consistency and quality control in brewing and distilling [7]. While these early techniques consisted of predominantly physical measurements, they have expanded over time into a comprehensive set of accredited procedures used throughout the alcohol industry [8,9]. These procedures have been essential to providing reliable and consistent results across different locations. However, as brewing and distilling practices evolve, so too do analytical techniques. It is therefore important to develop and adopt new and improved methods of analysis to keep up with the ever-changing alcohol industry [7]. This would include methods for compounds that have not been previously considered, analyzing a wider range of compounds to gain a more complete view of the flavor

profiles for the innovation of new products, or developing more sensitive and repeatable methods to replace current quality control practices.

Currently, there is a lack of advanced analytical techniques used in the alcohol industry. This is primarily due to the high cost of advanced instruments, the need for skilled technicians to operate them, and the lack of required routine analysis by the government. For example, the Texas Alcoholic Beverage Commission (TABC) allows alcoholic products to be sold on premises without registration, so long as the label contains the product name and alcohol content [10]. Further analysis of the chemical composition can be performed if desired, but is not required. Therefore, many breweries and distilleries will only test for basic parameters such as alcohol by volume (ABV) and pH. However, more advanced analytical techniques would be useful to delve deeper into the chemical composition and flavor profiles. The following chapters discuss primarily chromatography-mass spectrometry methods implemented to better understand the chemical composition and flavor variations of beer and whiskey.

Chapter 2 introduces the ingredients and processes used for brewing, as well as the chemical species introduced from them [11]. The major ingredients include grains, water, hops, and yeast. A comprehensive discussion of the various analytical methods used for beer ingredient and the organic, inorganic, and biological content of the finished product is provided.

As previously stated, the emerging craft beer market has greatly expanded the number of beer styles, of which the Brewers Association currently lists 75 based on ingredients, region of origin, and brewing methods, among others [2]. Many styles tend to overlap when it comes to physical characteristics, such as color and flavor, and so they can be difficult to differentiate

using strictly sensory analysis. Therefore, chapter 3 investigates two classes of beer flavor compounds, iso-α-acids and phenols, as distinguishing features between five different beer styles using targeted LC-QTOF-MS [12]. The optimized method was able to separate and identify the compounds of interest in the beers, which included 12 phenolic compounds and 12 iso-α-acid homologues, using high resolution multiple reaction monitoring (MRM). However, the data revealed that these compounds alone were not enough to distinguish between the beer styles. Therefore, further untargeted analysis was performed on the same beer set to determine which features were most influential in differentiating the styles. The details of these results are given in chapter 4 [13]. The high mass accuracy of the QTOF in conjunction with multivariate techniques, such as principal component analysis, partial least-squares discriminant analysis, random forest, Hierarchal cluster analysis, and analysis of variance, allowed for the five beer styles to be discriminated from one another. Further statistical analysis identified key metabolites from each style, and their formulas and identities were predicted.

Chapter 5 focuses on common fault compounds which cause off-flavors in whiskey. The major contributors to off-flavors include trichloroanisole (TCA), which imparts a moldy taste, and geosmin, which can cause whiskey to taste earthy or musty. TCA and geosmin have low sensory detection thresholds of approximately 5 ng/L and 75 ng/L, respectively. These compounds can lead to large economic losses for distilleries, and so their detection and quantification are important for the industry so that counteractive measures can be taken prior to sale. A HS-SPME-GC-MS method was optimized for these compounds, with the goal of quantifying them in various types of whiskey.

Lastly, chapter 6 summarizes and expands upon the work described here. Potential future studies which could further alcoholic beverage analysis and quality control practices are presented. This includes extending the untargeted LC-QTOF-MS method to incorporate beer substyles, the development of a beer profile database for the rapid categorization of new brews, and tracking whiskey fault compounds throughout the distillation process to determine their exact origin. These prospective studies hold promise for the future of advanced analytical techniques in the alcohol industry.

CHAPTER TWO

A REVIEW OF THE ANALYTICAL METHODS USED FOR BEER INGREDIENT AND FINISHED PRODUCT ANALYSIS AND QUALITY CONTROL

Anderson, H. E., Santos, I. C., Hildenbrand, Z. L., Schug, K. A., A review of the analytical methods used for beer ingredient and finished product analysis and quality control. *Anal. Chim. Acta* 2019, 1085, DOI: 10.1016/j.aca.2019.07.061. [11]

A Review of the Analytical Methods used for Beer Ingredient and Finished Product Analysis and Quality Control

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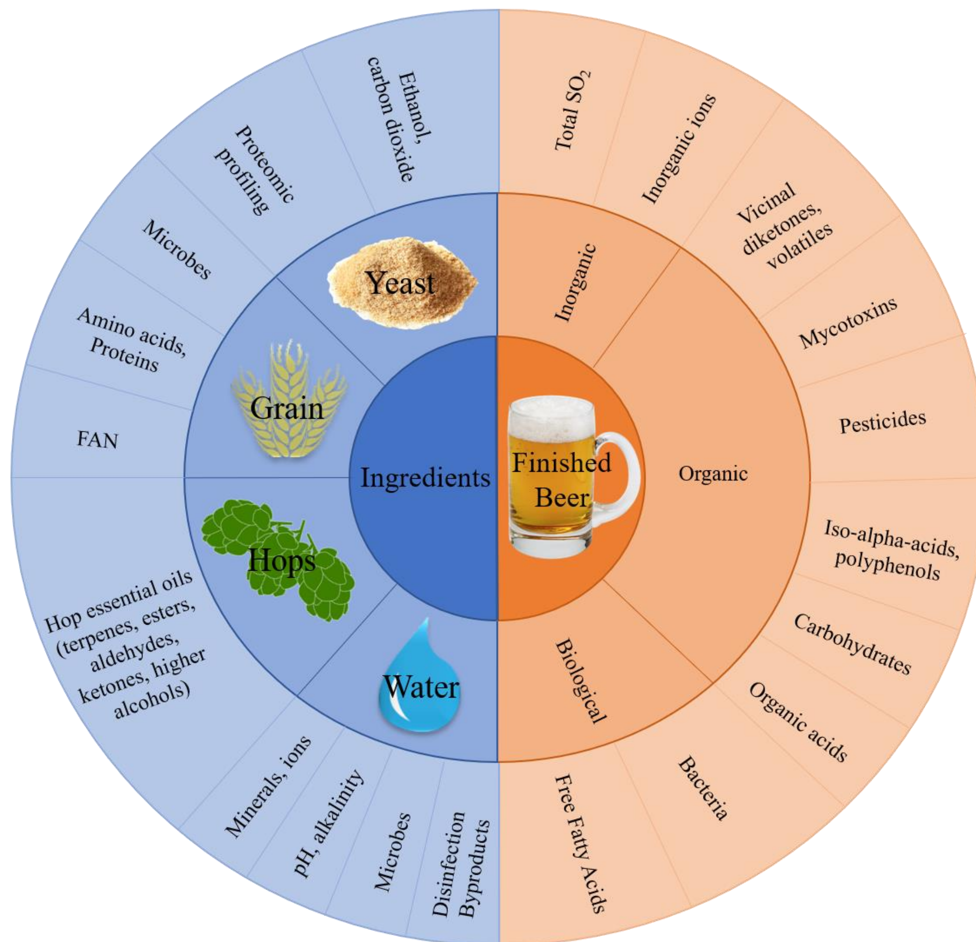

Figure 2-Graphical abstract. Diagram of beer and its ingredients, including the associated constituents that are subject to analysis.

2.1. Abstract

Beer is an incredibly complex beverage containing more than 3,000 different compounds, including carbohydrates, proteins, ions, microbes, organic acids, and polyphenols, among others. Beer becomes even more complex during storage, for over time it may undergo chemical changes that negatively affect the flavor, aroma, and appearance. Thus, it can be expected that maintaining the quality of beer throughout its lifetime is a difficult task. Since it is such a popular drink throughout the world, being familiar with proper analytical techniques for beer evaluation is useful for researchers and brewers. These techniques include, but are not limited to, gas chromatography, liquid chromatography, matrix assisted laser desorption/ionization, capillary electrophoresis, mass spectrometry, ultraviolet-visible spectroscopy, and flame ionization detection. This review aims to summarize the various ingredients and components of beer, discuss how they affect the finished product, and present some of the analytical methods used for quality control.

Keywords: beverage; hops; fermentation; iso-α-acids; bacteria; polyphenols

2.2. Introduction

Beer is the third most popular beverage behind water and tea, and the most consumed alcoholic drink in the world. Although the practice of brewing can be dated back 6000 to 8000 years, identifying and quantifying the various components contained therein is still a challenge due to the complexity of beer [1,14]. Beer is typically brewed using four main ingredients: A starch source (usually malted barley); yeast; hops; and water. Figure 2-1 illustrates the step-wise process of brewing, from the treatment of these ingredients through the different operations required to arrive at the final product [1].

The main purpose of a starch source is to provide sugars for yeast growth and to give body to the beer [15]. The typical grain of choice is barley [16,17], which introduces carbohydrates, saccharides, amino acids, proteins, and vitamins into the beer, the combination of which affect the overall taste, aroma, and color [18–20]. It also contains polyphenols that contribute to antioxidant activity [21,22]. The brewing process begins by mashing the barley in warm water to produce a clear, sweet, brown liquid known as wort. The sugars contained in the wort are needed for fermentation.

The wort is brought to a boil and hops, which account for the bitter flavor and aroma of beer, are added [23]. As the hops are boiled, important bittering agents such as α- and β-acids get extracted. The hops contain a complex mixture of terpenes [24], which provide beer with aromas that are generally described as citrusy, herbal, spicy, flowery, and fruity [25,26].

The next step is fermentation, where yeast is added to consume sugars from the wort and produce carbon dioxide, ethanol, and volatile phenolic compounds which may cause "phenolic

off-flavors" (POF) in the beer [27]. After anywhere from a few days to several weeks of fermentation, the yeast is removed, and the beer is matured to develop more flavor before being filtered and bottled for sale.

Water is arguably the most important ingredient in the brewing process as it composes roughly 90% of a beer's volume. Many factors contribute to water quality, such as the pH, alkalinity, ion, and microbial content, as well as the potential presence of disinfection byproducts (DBPs). Certain beers depend on water from specific regions for their characteristic flavor. Some examples include Guinness®, which was originally brewed with hard water from Dublin, Ireland, and Pilsner Urquell which originated from a soft water region in Pilsen, Czech Republic [20].

All the ingredients used during brewing contain a wide variety of chemical components which will interact during the different stages mentioned. If not carefully controlled, the interactions may cause unwanted characteristics in the finished product, such as off-flavors, staling, lack of foam development, and spoilage [28,29]. The recent surge in popularity of craft brews has created a higher volume of consumers and sparked a renewed interest in a greater variety of beer styles and ingredients [30]. It is therefore more important than ever to guarantee the quality of beer and consistency between batches.

While there are approximately 7000 craft breweries in the U.S., it has been anecdotally estimated that less than 10% of them use a significant amount of sophisticated instrumental analysis to support their beer production. This is primarily because the required instrumentation is too expensive for small breweries to afford, and often calls for trained

technicians to operate. However, in another review covering the analytical methods used for brewing, the author points out that some breweries use either centralized quality control laboratories manned by trained technicians or satellite stations where the analyses are performed by process workers [7], which may be a more affordable option for brewers who wish to perform more in depth quality control. This review also presents comprehensive tables that contain general, microbiological, and sensory analyses that are included in the Brewing Analytes Proficiency Scheme (BAPS), which is an international scheme designed to promote quality in the measurements of common beer analytes [7]. Wider usage of these detailed analytical techniques may help to improve quality control, provide better means for marketing beer in the ever-expanding industry, or even help to innovate new developments in craft brewing. The information presented here has the potential to assist brewers and/or quality control laboratories to determine the best suited method for their analyses, and possibly improve the way ingredients and brewing techniques are selected for a desired beer type.

The present review discusses the main ingredients and products of brewing and beer and overviews the analytical methods available for their analysis and control. First, different compounds found in the brewing ingredients and some of their effects on the taste, aroma, and quality of the final product are presented, as well as the analytical methods from published literature sources used to identify them. Details on the organic, inorganic, and biological content of finished beer is then discussed along with the methods used for quality control.

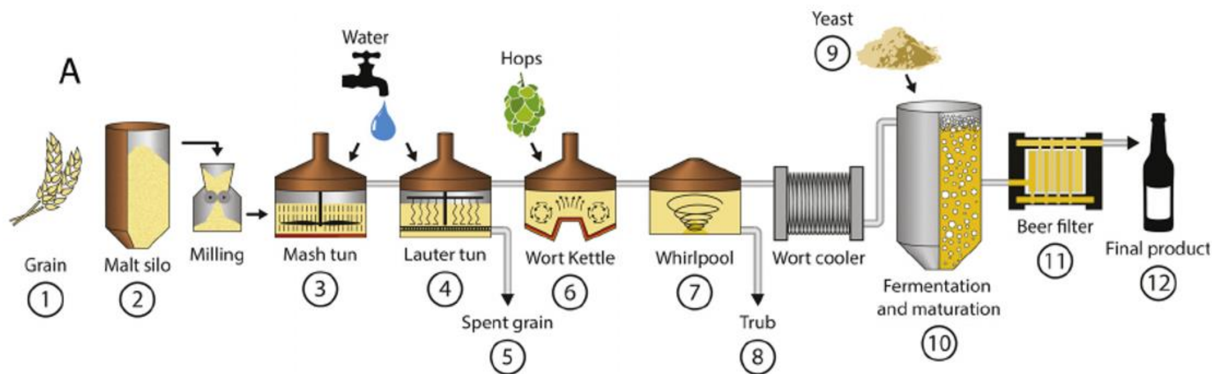

Figure 2-1. Illustration of the steps involved in the brewing process, including when specific ingredients are added [1]. Reprinted with permission from reference 1 (copyright 2018, Elsevier).

2.3. Methods for Ingredient Analysis

The selection of high-quality brewing ingredients is of utmost importance, as these are the source of all constituents affecting the flavor, aroma, and color of beer. In general, brewers rely mostly on sensory analysis when choosing ingredients. More in-depth analysis of the main components prior to or during the brewing process would help brewers set more meaningful ingredient specifications and allow them to better ensure consistency between batches. Some of the methods currently being used in laboratories for ingredient and finished product analysis are summarized in Figure 2-2 and then discussed.

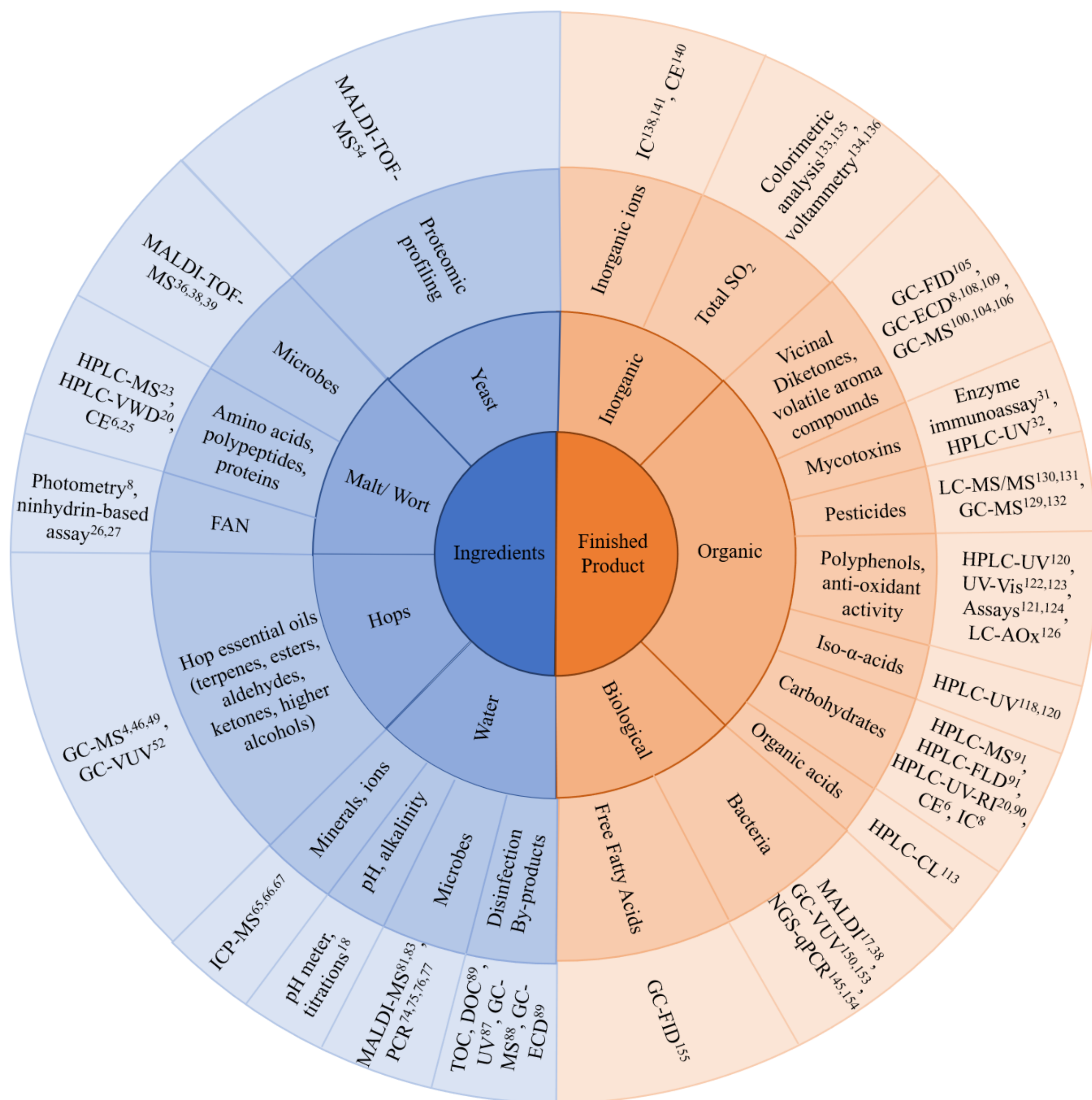

Figure 2-2. Schematic summarizing the analytical methods used for determining various compounds of interest in beer and its ingredients.

2.3.1. Barley/ Malt/ Wort

2.3.1.1. Amino acids, polypeptides, proteins. Some of the nutritional value and stability of beer comes from proteins and amino acids, meaning that protein characterization during the

different stages of brewing can be useful in quality control [18,31,32]. The protein in beer originates from the malted barley, which contains about 10 – 12% protein. A third of that protein is extracted during mashing and many of the larger proteins are removed during the boiling process, leaving the average beer with 0.2 – 0.6 g/100 mL of protein-derived material, primarily in the form of peptides and polypeptides [18]. The exact amounts depend on the preparation and type of beer [32]. Most of the free amino acids present in the wort are taken up by yeast during fermentation. However, proline cannot be assimilated by the yeast and remains in beer at a higher concentration than other amino acids, accounting for a large portion of the low molecular weight nitrogenous material [18,31,33]. Proline residues help to produce aromatic compounds during brewing and in this way can influence the quality [32].

High performance liquid chromatography (HPLC) can be used to analyze amino acids, polypeptides and proteins in beer. Das et al. prepared samples of various Indian rice beers through acid hydrolysis followed by derivatization for analysis by HPLC with a variable wavelength detector (VWD) at 265 nm [31]. It was found that while all samples contained most of the essential amino acids, the concentrations differed significantly due to variations in the ingredients and brewing procedure. It was concluded that the relatively high amino acid content shows that rice beer can be a good source of energy and essential nutrients [31]. Petry-Podgórska et al. used HPLC "shot-gun" analysis to determine protein glycation during the brewing process, with the goal of detecting and locating non-enzymatic glycations in barley malt [34]. Glycation is the non-enzymatic reaction between reducing sugars, such as glucose or fructose, and proteins, lipids, or nucleic acids. The extent of protein glycation is important for beer analysis because it influences the quality of malt and the properties of beer foam.

Following separation by HPLC, the authors used tandem mass spectrometry (MS/MS) to identify the proteins and found that some were resistant to the malting process and thus retained in the final beer. Of the 16 proteins identified in the study, glycation was detected for five of them. Two of the five glycated proteins, protein Z and ns-LTP1 (non-specific lipid transfer protein), are crucial for malt and beer quality. Since these proteins survived the brewing process, they can serve as beer quality markers when monitoring glycation [34]. The sample preparation is quite lengthy, as the proteins must be extracted from the barley grain, incubated to reduce disulfide bridges, alkylated, and then digested enzymatically prior to separation by HPLC, which causes protein glycation analysis to be relatively difficult to perform.

Capillary electrophoresis (CE) can also be used to analyze proteins and amino acids [18]. In one review, Kennedy reported that four fluorescein-labeled amino acids were separated in less than 140 ms with high efficiency by optically gated CE [35]. It was also stated that short-lived species, such as certain conformations of proteins, were able to be detected due to the high-speed separation capability of CE. However, there are some limitations to this method as well. The fast separation times, though advantageous, tend to place greater demand on the detection system and frequently require detection limits below 1 amol. Also, reproducibility is difficult, and temperature differentials that develop within the column can cause uneven migration and band-broadening [36]. Although CE is not typically used for routine analysis, and few brewers have access to one, it may be a useful technique for the rapid analysis of beer quality.

2.3.1.2. Free amino nitrogen (FAN). As previously discussed, wort contains relatively large amounts of amino acids, peptides, and proteins. During the brewing process, yeast is added to

the wort and fermented for several weeks. The yeast requires the uptake of nitrogen in the form of amino acids to synthesize the proteins required for healthy growth [20]. The individual amino acids and small peptides used for yeast cell growth are collectively referred to as free amino nitrogen (FAN) [37,38]. Low FAN concentrations may lead to decreased fermentation performance [39].

The most common method for the determination of FAN in wort is a ninhydrin-based assay [37,38]. However, this method measures the content of ammonia in addition to the FAN [40]. An alternative strategy is to use discrete photometric analysis for colorimetric and enzymatic testing. The FAN can be measured using a rapid two-reagent NOPA (α-amino nitrogen by o-phthaldialdehyde) method, which was developed for the automated discrete analyzer using a blank buffer to eliminate color interference from the sample. This method showed excellent reproducibility between 2.2 and 3.2% [20]. Although FAN is said to be a good index for potential yeast growth and efficiency, it is only a general measurement that is used as a "blunt instrument" for setting wort specifications [37,38].

2.3.1.3. Microbes. Barley contains a complex microbial population of bacteria, filamentous fungi, yeasts, and mold. Although beer is an unfavorable medium due to its antimicrobial properties, some beer-spoilage microorganisms may survive the brewing process and adversely affect the final beer. In addition, fungal metabolites known as mycotoxins may be present, which can make beer unsafe to consume. Mycotoxins are low molecular weight secondary metabolites produced by filamentous fungi that are toxic to animals and humans. Grains used for brewing, such as barley and wheat, have often been associated with contamination by mycotoxins. Particularly, barley is commonly affected by the plant disease *Fusarium* head blight

(FHB) [41], also known as “scab”. In fact, the highest public health concern related to beer consumption is the presence of *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone (ZEN), which are often detected in FHB infected barley [42]. Exposure to DON can cause acute and chronic effects in humans and animals such as immunosuppression, neurotoxicity, teratogenicity, and embryotoxicity [43]. Although some mycotoxins perish during steeping, the growth of *Fusarium* mold and the production of mycotoxins are still possible in the early stages of brewing, and these species may be transferred from malt into the finished beer [44].

Physical, chemical, and biological based technologies can be used to treat barley and suppress mold growth to prevent mycotoxin development in malt [44]. One promising physical technique is electron-beam irradiation of FHB-infected barley. Kottapalli et al. showed that irradiation under optimized parameters could reduce *Fusarium* infection in barley and DON levels in final malts with minimal impact on the overall malt quality [45]. Simple hot water treatment of the barley at 45 ℃ was also shown to reduce infection and DON content [46]. While chemical treatments such as sodium bisulfate, chlorine gas, and ammonia seem quite effective for reducing DON levels in “scabby” barley, these methods can be too severe on the grain quality and leave unwanted residues [44]. Ozonation is a more promising chemical treatment for barley and beer detoxification as it does not leave residuals of any type [46]. Available microbiological control methods work by adding competing fungi as starter cultures to the steeping process. In fact, the use of *Geotrichum candidum* was shown to reduce *Fusarium* infection rates from 86% to 0% in steeped barley. However, the starter cultures of *G. candidum* would need to be monitored for toxin production since certain strains may produce clavinet alkaloids [44].

For microorganisms present in the mash, such as *Bacillus, Pediococcus, Enterobacteriaceae,* and *Lactobacillus*, the preferred approach for detection is the traditional culture method followed by microscopic observations, catalase tests, and polymerase chain reactions (PCR) for positive results [47]. It is typically cost intensive and time consuming to obtain results this way. An alternative approach is the use of matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-TOF-MS), which provides more straightforward and rapid identification of bacteria. This technique involves using a laser to ionize samples into charged molecules that can then be separated based on their mass-to-charge ratio (*m/z*) and identified by comparison to a database. An advantage of using MALDI is its compatibility with various analyzers, while some drawbacks include suppression effects, "sweet spots", and selective ionization [48]. There are several studies which show the use of MALDI-TOF-MS for the identification of beer spoilage microorganisms [47,49,50]. Reyes et al. identified microflora in hops and grains using MALDI-TOF-MS with at least an 85% match to the SARAMIS database [47], and Turvey et al. analyzed beer spoilage bacteria, wild yeast, and fungi for industrial application [49]. Though owning a MALDI-TOF-MS would be somewhat illogical for small breweries, beer samples could be sent to outside laboratories for more detailed microbial analysis using this method. Microbial identification by MALDI-TOF-MS is a very inexpensive process once the instrumentation has been acquired.

2.3.2. Hops

Hops (*humulus lupulus*) give beer its bitter flavor and pleasant aroma, which are the most distinguishable characteristics of the beverage. The bitterness is achieved by isomerizing α-acids into the more soluble iso-α-acids during the boiling of the wort, while the aroma of beer

comes from a mixture of volatile compounds derived from the hop essential oils [24]. Hops also have a favorable influence on the stability of beer foam and have been shown to provide antifungal and antibiotic properties [51,52]. In fact, Reyes et al. observed little to no microbial growth on various agar when analyzing hops because of these bactericidal properties [47].

Another interesting example of these characteristics can be seen in the history of a popular hoppy beer style, India Pale Ale (IPA). In the late 18th and early 19th centuries, India was one of the largest English colonies and housed many emigrants, troops, and sailors who had a large appetite for beer. The beer had to be shipped from England because India's climate was too warm for brewing, but the long voyage between countries tended to cause spoilage [53,54]. To solve this issue brewers began making beer with extra hops and higher alcohol levels, both of which acted as preservatives. This new, very bitter, high alcohol beer style [54] was considered an improvement on the pale ales and porters previously being shipped to India that often arrived stale or soured [53]. Although Europeans began to lose interest over the years, IPAs experienced a huge popularity surge in the American market during the 1970s and is now one of the leading craft beer styles [55] due to its hoppy bitterness, variety of flavors (citrus, floral, etc.), and often high alcohol content.

The bitterness and foamability of beer can be adjusted by adding the hops at different times during the boiling process. When added at the start of the boil, the volatile aroma compounds tend to evaporate and are lost. To avoid this, hops can be added within the last 30 minutes of the boil in a process called "late hopping", which keeps more volatile components in the wort so that they can become part of the finished beer [16,56]. Another option, known as "dry-hopping", involves adding the hops just before packaging so that some of the original

constituents are directly transferred into the beer, giving it a discrete hoppy character [15]. Dogfish Head Craft Brewery utilizes a method they call "continuous doping" in which hops are added to the boiling wort at a consistent rate for various amounts of time. This technique allows the brewer to add large amounts of hops without causing overpowering bitterness. Dogfish Head uses continuous doping to make their 60 Minute, 75 Minute, 90 Minute, and 120 Minute IPAs. Because hop compounds have such a profound effect on the flavor of beer, the essential oil profile of hop samples contain valuable information for brewers.

2.3.2.1. Hop essential oils. There is a wide variety of hop strains that can be used in brewing, and each strain has distinct odor characteristics and volatile compositions, which are affected by geographical location, climate, and agronomical factors [16,57]. The characterization of hop essential oil is a difficult task due to the complexity of the composition. The essential oil comprises approximately 0.5 – 3.0% (v/w) of the hop cones and contains around 1000 terpenes, of which just 400 have been identified [16,26,58–60]. The oil can be divided into two main fractions: Hydrocarbons, of which mono- and sesquiterpenes (built from 2 or 3 isoprene units, respectively) account for 70%, and oxygen containing compounds such as aliphatic esters, aldehydes, ketones, and higher alcohols make up the remaining 30% [16,25,57,58]. Although strains exhibit differences in aroma profiles, the major odor-active terpenes found in analyzed hop varieties are usually myrcene, linalool, humulene, and β-caryophyllene [58,61].

In general, gas chromatography-mass spectrometry (GC-MS) is used for the analysis of essential oils [60]. Many extraction and concentration methods have been developed for sample preparation, including steam distillation, extraction with organic solvents, extraction with liquid carbon dioxide, and stir bar sorptive extraction (SBSE) [24,57–59]. Most of these

techniques require special equipment, large solvent volumes, and extensive sample preparation which increases the risk of analyte loss. Additionally, the solvent extraction and carbon dioxide extraction methods can extract nonvolatile residues along with the essential oils, which may adversely affect the GC column.

The headspace (HS)-trap method has been used in recent years to reduce sample preparation time and thereby analyte losses. The technique involves placing a sample into a closed vessel (trap) and then heating it using a known temperature profile to release the volatile compounds to the gas phase. The vapor in the trap is then sampled for analysis. The HS-trap system allows a large number of samples to be analyzed in a relatively short time [62], is easily automated [26], and can reduce the detection limit by using up to four trap enrichment cycles [58]. There are multiple cases of successful hop analysis using HS-GC-MS [16,57,60,62]. Aberl et al. studied the essential oil from 24 hop varieties using this method. More than 65 hop volatile compounds were identified, and 21 of them were quantified using analyte specific calibration curves [58]. Because the essential oil compositions vary (for example, bitter hops usually contain higher levels of essential oils than aroma hops), this information can be used to differentiate between hop varieties and choose the best strain for the style of beer being brewed.

While GC-MS currently provides the widest range of applicability for detecting chemical compounds, it is unable to differentiate many isomeric, isobaric, small, or labile compounds. Analyzing terpenes by MS is especially challenging because of their identical chemical formulas and similar fragmentation patterns [63]. An alternative GC detection method applied to the analysis of terpenes is vacuum ultraviolet spectroscopy (VUV), which measures the absorption

of volatile species in the range of 120-240 nm. The advantage of monitoring this wavelength range is that all chemical compounds exhibit unique absorption spectra, which can be used for qualitative analysis or quantification using straightforward Beer-Lambert law principles [64]. Perhaps the most attractive feature of this detector is the ability to deconvolute co-eluting peaks into the additive contributions from two or more analytes. Even overlapping isomers can be easily deconvoluted based on their disparate absorption signatures. Using the VUV data analysis software, Qiu et al. deconvoluted co-eluting terpenes and quantified them by comparison to calibration standards. A portion of a chromatogram from this study can be observed in Figure 2-3, which shows the original overlapping signal along with the deconvolved peaks [63]. Four commercial turpentine oil samples were also analyzed using GC-VUV, which allowed for the detection of 31 terpenes with α-Pinene being the most dominant [63]. Based on this study and the unique qualities of the instrument, VUV may be the ideal detection method for terpenes in the coming years.

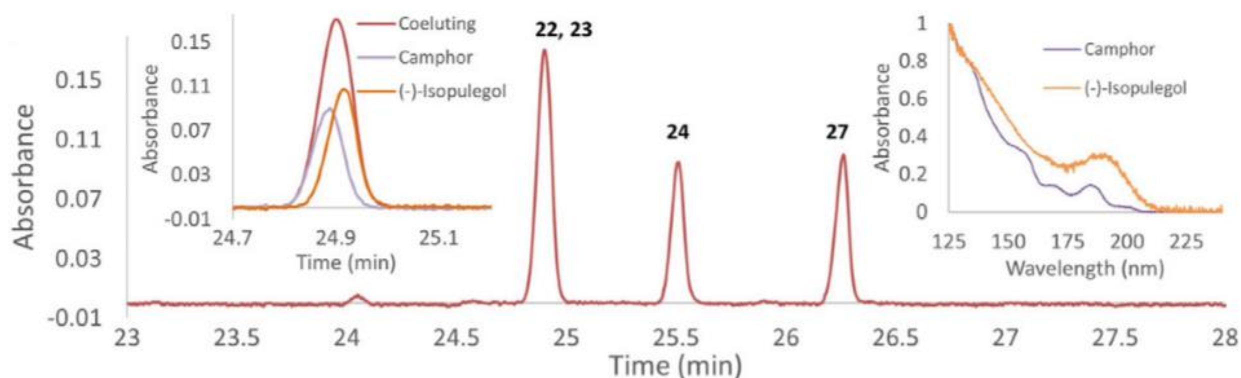

Figure 2-3. Expansion of retention time window of a standard mix of terpenes which includes the spectra of two coeluting terpenes (inset right) and the deconvolved signals (inset left) [63]. Reprinted with permission from reference 63 (copyright 2017, Wiley-VCH).

2.3.3. Yeast

Yeast cells are mainly responsible for the production of ethanol and carbon dioxide during fermentation. Brewing strains are typically divided into top-fermenting *Saccharomyces cervesisiae,* which is used for ales, and bottom-fermenting *Saccharomyces pastorianus* for lagers, although there are other types as well. Choosing an appropriate yeast strain is an important factor for beer quality, yet it has been somewhat neglected in favor of variations in malt and hops [65]. It has been suggested that selecting a suitable brewing yeast is essential to creating a phenolic taste profile in beer, as it has an influence on the final terpene concentration [27,66]. Yeast strain also has an effect on the formation of aroma-active esters [39,67]. In typical lager beers, the ethanol produced by the yeast can increase the retention of aldehydes which reduces "worty-flavor" [68]. Since yeast can have an impact on the final product, it is necessary to develop methods for the fast discrimination of various strains. The analytical methods for the determination of volatile compounds produced by fermentation of the yeast will be discussed later in this paper.

2.3.3.1. Proteomic Profiling. The genus *Saccharomyces* contains yeasts strains, which are closely related, making their identification and differentiation quite difficult. Over the years, these yeasts have been characterized using biochemical and DNA-based methods, which seemed to be insufficient for an exact discrimination within the genus [69]. Phenotypic identification was found to be time- and labor-intensive, and molecular assays can be expensive and complex. Therefore, a desire for a more simple, fast and cost-effective method for classifying brewing yeasts emerged. This need was satisfied using MALDI-TOF-MS, which relies on the generation of "protein fingerprints," which are compared to reference spectra in a well-

characterized library [70]. This technique can obtain species-specific spectral patterns uninfluenced by the age of the culture, the medium used, or growth conditions [69]. Lauterbach et al. utilized MALDI-TOF-MS to establish a database of common brewing yeasts. This database allows the assignment of top- and bottom-fermenting yeasts, as well as strains that could cause beer spoilage. It can also match strains to a specific beer type of preferential use, making it an indispensable tool for brewers [65].

2.3.4. Water

Water composition is possibly the most important parameter in beer [71]. Brewing is a very water-intensive activity, using anywhere from 5-10 times more water than the amount of beer produced [30]. Some of this water is used for beer production – mashing, boiling, filtration, and packaging – but the majority is used for cleaning and ends up down the drain, unless it is reclaimed [72]. Water accounts for roughly 90% of beer and has many different parameters that can affect the flavor, including the pH, alkalinity, metal ion concentrations, microbes, and the presence of disinfection byproducts (DBP). For the past 60 years, brewing textbooks have given the following recommendations for water: It should be clean, pre-boiled to remove temporary hardness, the alkalinity should be less than 50 mg L^{-1}, and the water should contain 50 to 100 mg L^{-1} of calcium [30]. A complete list of approved analytical methods for water analysis under the Clean Water Act can be found on the EPA website [73].

2.3.4.1. pH and alkalinity. The most common and easily monitored parameter of water is the pH, which can be measured using a digital pH meter. Most potable water has a pH around 7.5. Typically, lager beers have a pH in the range 4.00 – 5.00, ales vary between pH 3.00 – 6.00, and sour beers can have a pH as low as 3.30 [74]. The alkalinity of water refers to its capability

to neutralize acid. In other words, it acts as a buffer system by essentially absorbing the excess H^+ ions and protecting the water from fluctuations in pH. In most cases, this buffering system consists of carbon dioxide/ carbonic acid, bicarbonate, and carbonate ($CaCO_3$) in equilibrium [30]. The alkalinity can be measured through titrations using methyl orange as the indicator because its pH is in the same range as the equivalence point for total alkalinity, and it has a distinct color change that can be easily measured. The method can be automated using an autoanalyzer with an applicable range of 10 to 200 mg L^{-1} as $CaCO_3$. One issue with this method is that the turbidity and color of the sample may interfere with the analysis [73]. The water alkalinity varies directly with wort and beer pH. As the alkalinity increases, so does the pH of both the wort and final beer [75], which can be problematic for the flavor profile. Because of this, the maximum alkalinity for brewing water is 100 mg L^{-1} as $CaCO_3$ [30,75].

2.3.4.2. Ions. Water contains many naturally occurring minerals and compounds, as well as manmade contaminants. Ions in brewing water are divided into two main categories: Primary standards, which refer to the safety of the water; and secondary standards, which affect the taste and pH. The primary standards have maximum contaminant levels (MCL) which can be legally enforced while the secondary standards have unenforceable secondary maximum contaminant levels (SMCL). The common primary standards are disinfection byproducts (bromate, haloacetic acids, and trihalomethanes, which will be discussed later), chlorine, nitrate, and nitrite. The common secondary standards are copper, chloride, iron, manganese, silicate, sulfate, and aluminum. There are also unregulated ions that can have an effect on beer, such as boron, magnesium, phosphate, potassium, and calcium [30]. The ions of greatest concern for the quality of beer are calcium, magnesium, sodium, chloride, and sulfate. Calcium,

although flavorless, works to protect, stabilize, and promote enzyme activity in the mash. High levels of magnesium cause an unpleasant sour and bitter taste in beer. Sodium can make the malt character sweeter at low concentrations but can also interact with chloride to give a salty taste. Chloride provides a rounder, fuller, sweeter quality to the beer and is also used to lower the alkalinity. A moderate amount of sulfate is said to increase the "linger time" of the bitterness and accentuate the hop flavor, as well as add to the dryness of some beers [30]. Clearly, the concentrations of these ions need to be measured prior to brewing so that the beer has a pleasant flavor.

Ions in water samples have been analyzed using inductively coupled plasma mass spectrometry (ICP-MS) [76–78]. Although other instruments can be used for metal detection (for example, atomic absorption spectroscopy or x-ray fluorescence (XRF)), ICP-MS is a highly sensitive tool for element-specific analysis and has been proven to be ideal for a wide array of sample types due to the high ion density and temperature of the plasma [77,79]. Other benefits of this technique include its efficiency and specificity due to the low detection limit (ppm), high resolution, and wide linear range. For even better elemental speciation, ICP-MS can be coupled to a separation technique such as capillary electrophoresis (CE) or ion chromatography, which would allow all species (including metals in different oxidation states) to be measured from just one injection [80]. Once the ions are identified, a brewer can treat the water to remove any unwanted substances.

2.3.4.3. Microbes. Many water sources contain microbes – bacteria, viruses, and parasites – that could be harmful to human health. An increased focus on water quality in recent years has led to a need for a simple detection technique for multiple microbes such as coliforms, *E. coli*,

and *Cryptosporidium* [81]. However, direct analysis is difficult and impractical due to the low concentration of microbes in environmental samples. To solve this issue, the water samples are concentrated using techniques, such as ultra-filtration, prior to analysis [82–84]. The sample will usually undergo a secondary concentration via centrifugation, after which the microbes can be identified by real-time polymerase chain reaction (PCR) [85–88] or culturing methods [89]. Although PCR has good detection levels, it is complicated and expensive, while culturing methods tend to be time consuming and not fully specific.

As stated in earlier sections, an alternative for microbial characterization is MALDI-TOF-MS [90,91], which is a powerful identification tool due to its high throughput and versatility [92,93]. Li et al. used a combination of membrane filtration (MF) and vancomycin-conjugated magnetite nanoparticles (VNPs) for the selective separation and concentration of Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus,* etc) in water samples followed by their identification using MALDI-MS. The spectra of *B. cereus* can be observed in Figure 2-4. This method showed acceptable reproducibility and proved to be an easy and reliable approach for the rapid analysis of Gram-positive bacteria in water samples [92]. MALDI-TOF-MS has also been used to identify bacteria in groundwater samples that could have potentially been contaminated by nearby anthropogenic activities, such as oil and gas extraction [94–96]. These studies identified opportunistic pathogenic bacteria [94], denitrifying and heterotrophic bacteria from the Phylum *Proteobacteria* [95], and cultivable organic-degrading bacteria [96]. Groundwater is major source of drinking water in the United States and therefore may be used by some brewers to make their beer. Because these water sources possibly contain harmful bacteria, analyzing them for microbes prior to brewing would ensure that the drinking water is safe to consume.

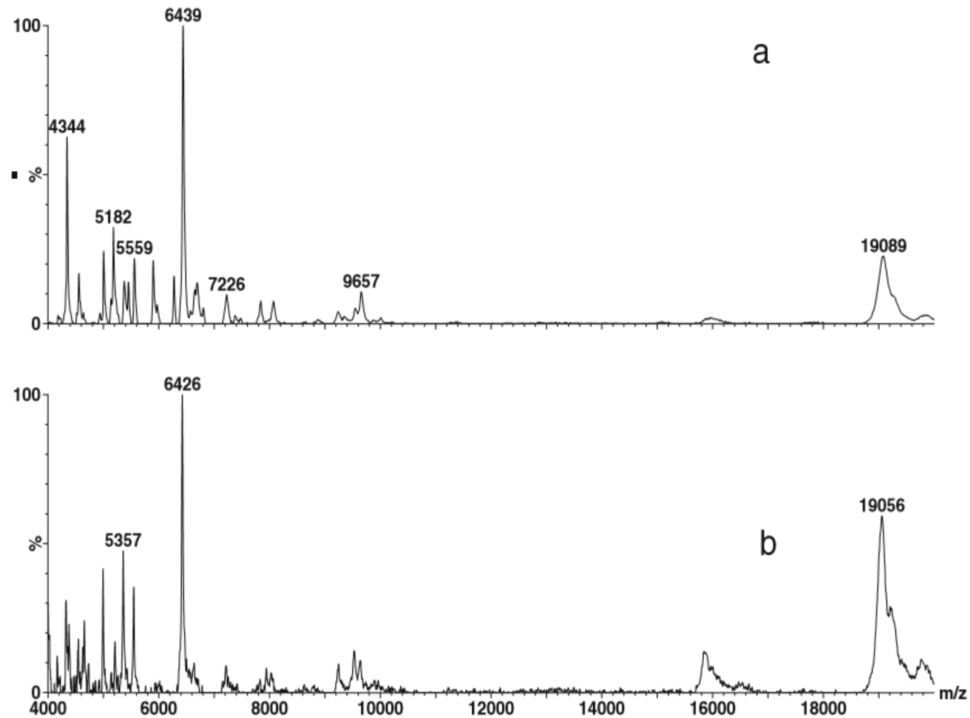

Figure 2-4. MALDI-MS spectra of *B. cereus*: (a) aqueous suspension of approximately 10^8 cfu mL^{-1} (colony-forming unit per milliliter); (b) separated by MF-VNP technique from tap water spiked at $1x10^3$ cfu mL^{-1} [92]. Reprinted with permission from reference 92 (copyright 2010, Springer).

2.3.4.4. Disinfection byproducts. The pathogens in water are controlled by disinfection via chlorination or chloramination, which form a variety of known and unknown disinfection byproducts (DBPs). Some DBPs are carcinogenic and may pose health risks if consumed. The major DBPs are bromate, trihalomethanes (THM), and haloacetic acids. A bromate concentration higher than 0.05 mg L^{-1} in fresh water indicates contamination by industrial waste or pesticides. The specific THMs in brewing water are chloroform, dibromochloromethane, bromodichloromethane, and bromoform. Long term exposure to THMs in animals has been linked to deleterious effects on the central nervous system, liver,

kidneys, and heart. The five most common haloacetic acids in water – monochloroacetic acid, dichloroacetic acid, trichloroacetic acid, monobromoacetic acid, and dibromoacetic acid – are referred to as HAA5 and have been linked to an increased risk of cancer [30]. All of these are primary standards and therefore have legally enforceable maximum contaminant levels (MCL), which are 0.01 mg L^{-1} bromate, 0.06 mg L^{-1} HAA5, and 0.08 mg L^{-1} total THMs in the final beer product [30].

Several models have been developed to predict DBP formation in water. The parameters incorporated into these models include total organic carbon (TOC), dissolved organic carbon (DOC), ultraviolet absorbance at 254 nm (UV_{254}), temperature, pH, and reaction time. The TOC, DOC, and UV_{254} are often used as surrogate measurements of the natural organic matter (NOM). The NOM in water reacts with disinfection agents such as chlorine and chloramine to produce DBPs, and studies have found a correlation between DBPs and NOM content in raw waters [97]. Chen et al. used models based upon DOC, UV_{254} and bromide to predict the formation potentials of various DBPs in both potable and waste waters [98]. The model was determined to be a useful tool for assessing the potentials for THM formation and demonstrated that removing DOC or UV_{254} materials will decrease THM significantly. The model also showed that HAA precursors were UV-absorbing due to their positive relationship with UV_{254} [98]. Based on these results, predictive models seem to be a good way to assess the potential DBP content in water.

Alternative techniques used for DBP analysis include gas chromatography (GC) equipped with either MS [99] in the negative chemical ionization mode or an electron capture detector (ECD) [100], both of which are sensitive and selective for halogenated compounds. Liquid

chromatography-mass spectrometry (LC-MS) can also be used. In fact, the first known DBP – chloroform – was identified using GC-MS in the 1970s, so it has played a key role in water analysis for many years [99]. Drinking water extracts are generally quite complex and may contain as many as 300 compounds, which includes naturally occurring components or pollutants in addition to DBPs, so careful background subtraction is necessary to obtain a clean spectrum using GC-MS. An example of a typical chromatogram for a drinking water extract obtained by GC-MS is shown in Figure 2-5. Although GC-MS has been proven as an effective technique for identifying DBPs, it is limited to the lower molecular weight fraction because higher molecular weight compounds tend to be less volatile. Since GC-MS requires the sample to be in the gas phase, it also has difficulty analyzing most ionic DBPs. However, LC-MS can be used for the analysis of non-volatile, higher molecular weight, and ionic compounds. Conversely, lower molecular weight DBPs are almost impossible to distinguish using LC-MS because of the high chemical background in that region of the chromatogram, though this can be overcome by derivatization [99]. While these methods have successfully classified many DBPs over the years, a considerable percentage of them are still unaccounted for and so more research is required [99]. Once identified, disinfection byproducts are normally removed using reverse osmosis or activated carbon filtration [30].

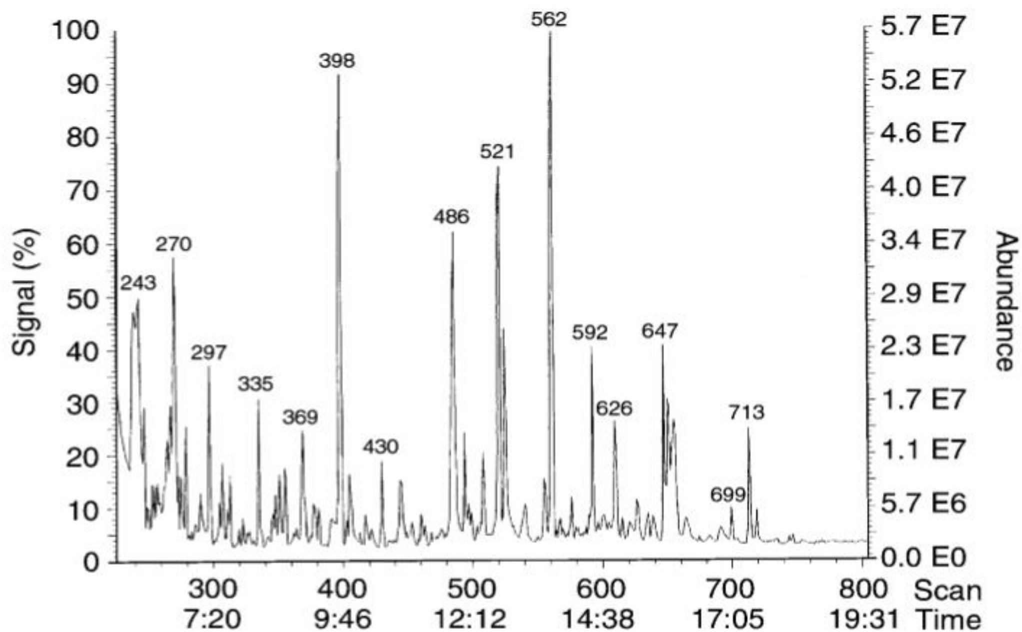

Figure 2-5. A typical GC-MS chromatogram from a drinking water extract. Some peaks represent naturally occurring compounds or pollutants that were originally present in the water sample, while others depict the DBPs formed by the water treatment [99]. Reprinted with permission from reference 99 (copyright 2002, Royal Society of Chemistry).

2.4. Finished Product

2.4.1. Organic Content

2.4.1.1. Carbohydrates. Tracking the carbohydrate content in wort and beer is important in modern brewing, especially for the development of new flavors and selecting raw ingredients. The composition of beer is approximately 3.3 – 4.4% carbohydrates, which comprise 75 – 80% dextrins, 20 – 30% monosaccharides and oligosaccharides, and 5 – 8% pentosans, making carbohydrates the major nonvolatile component in beer [18,19]. Several saccharides remain in beer after fermentation, including monosaccharides (mainly *D*-glucose, *D*-fructose, *D*-ribose, *L*-arabinose, *D*-xylose, and *D*-galactose in trace amounts) which contribute to the sweetness of

the product and comprise approximately 10% of wort carbohydrate. The main disaccharides are maltose and sucrose, while some major trisaccharides are maltotriose, iso-maltose, and raffinose, which have been shown to have several health benefits, such as prevention of heart disease [18,31].

One of the most common methods for the separation and quantitation of carbohydrates in beer is HPLC, which can be coupled with refractive index (RI) [31], ultraviolet (UV) [101], fluorescence (FLD), or mass spectrometric detectors [102]. Castellari et al. proposed a method to quantitate not only carbohydrate classes of compounds in beer, but also organic acids, glycerol, and ethanol in a single-run separation using HPLC. However, direct injection of the samples showed poor resolution due to interferences. In order to avoid this, HPLC was coupled with UV and RI detectors connected in series [101]. This set up was chosen to limit the matrix interference and improve separation by reducing the retention times. The HPLC-UV-RI method allowed for the evaluation of nine different beer compounds with a total analysis time of 35-40 minutes. It was shown to have acceptable accuracy, repeatability, and limit of quantitation (LOQ) for production and quality assurance. According to Plata et al., reference LOQ for carbohydrates by HPLC-RI falls between 39 and 87 mg L^{-1} [103].

Rakete et al. established a novel method for reversed phase HPLC coupled to fluorescence or MS detection, using 1-naphthylamine for precolumn derivatization with sodium cyanoborohydride, to study the changes in the carbohydrate profile during brewing [102]. The choice of derivatization agent allowed for a tremendous increase in fluorescence activity and sensitivity for mass spectrometry. MS detection was able to identify the molecular weight and thus the degree of polymerization of unknown carbohydrates. The limit of detection (LOD)

using fluorescence was determined to be 1.2 μM, which was 100-fold lower than that of another commonly used method: fluorophore-assisted carbohydrate electrophoresis (FACE) [102].

An alternative to HPLC is capillary electrophoresis (CE), which is generally cheaper, gives higher efficiencies, faster separation times, and requires minimal sample preparation [18,104–106]. CE is a versatile technique because of the wide range of applicable analytes, separation modes, and detectors available [107]. Although CE has an intrinsically high resolving power when separating complex carbohydrate samples, two major difficulties must be overcome. First, many carbohydrates lack readily ionizable charged functional units, which hinders their ability to be separated by CE. However, the molecules can be converted to charged species "*in situ*" by complex formation with ions such as borate and metal cations. This allows them to be separated in an electric field by ensuring their differential electromigration. The other issue is the inability of most carbohydrates to absorb or fluoresce, thereby impeding their detection by many techniques. This can be resolved by labeling the functional groups (amino and carboxylic acid groups) of the sugar molecules with UV-absorbing or fluorescent tags, which allows the carbohydrates to be distinguished by absorbance or fluorescence detectors [18].

Carbohydrates can also be analyzed using variations of ion chromatography. This technique has been used to monitor the carbohydrate content throughout the brewing process to determine the extent of fermentation [20]. Fangel et al. describes the preparation and quantification of β-(1->3)(1->4)-glucan in beer samples using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The effectiveness and precision of the method was assessed by comparison to carbohydrate microarrays. It was

shown that HPAEC-PAD has the ability to distinguish oligosaccharides from β-(1->3)(1->4)-glucan and β-(1->4)-glucan, and is suitable for ranking beers based on carbohydrate content [1]. The LOD and LOQ of this method are very low, confirming that it is a selective detection system [19]. The ability of ion-exchange chromatography with pulsed amperometric detection to identify malto-oligosaccharides in beer and fermentable sugars in wort can be observed in Figure 2-6 [20].

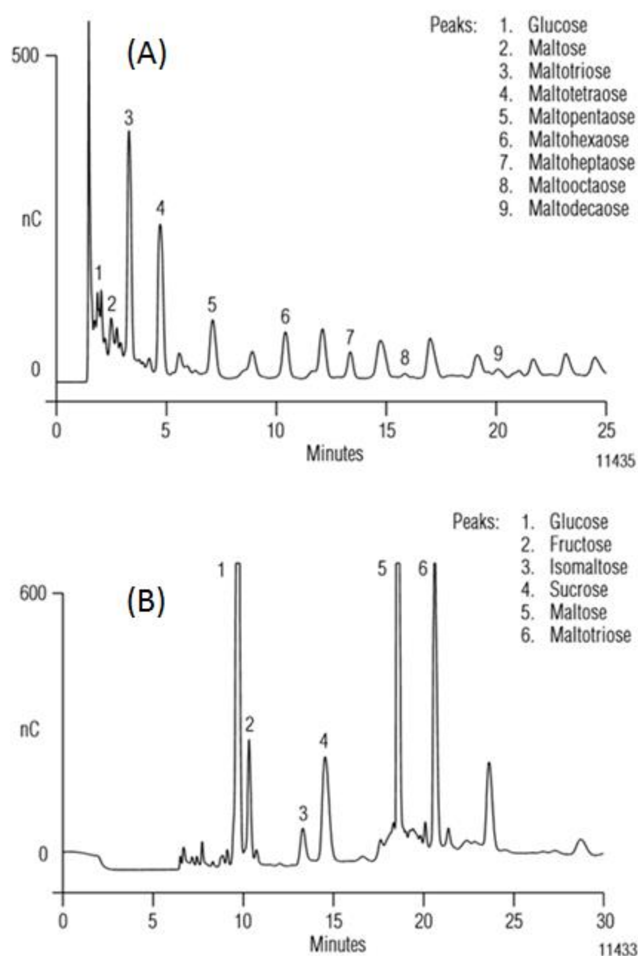

Figure 2-6. Chromatograms showing the separation of (A) malto-oligosaccharides in an American beer sample and (B) fermentable sugars in wort by ion-exchange chromatography with pulsed amperometric detection [20]. Reprinted with permission from reference 20 (copyright 2016, Thermo Fisher).

2.4.1.2. Volatile aroma compounds. Fermentation leads to the production of ethanol and a series of by-products, including other alcohols, carbonyl compounds, esters, aldehydes, and acids [108]. These compounds help to create the aroma and flavor profile of beer, so understanding the nature and concentration of the volatile substances may be incredibly important for the selection of raw materials and yeast strains, as well as for quality control [109]. Gas chromatography-mass spectrometry (GC-MS) is the conventional technique for the detection and identification of volatile aroma compounds. However, beer has nonvolatile components, as well, and direct injection into the GC leads to contamination, so the volatile compounds must first be extracted using headspace (HS) techniques and/or solid phase micro-extraction (SPME) methods [110,111].

Headspace extraction is an effective technique for measuring volatile species in samples that have a complex matrix, such as beer. The method is based on sampling the vapor phase (headspace) above the liquid sample in a closed vial, and then measuring it by GC [112]. Using HS-GC in conjunction with SPME will isolate the volatile sample while greatly reducing the interference of nonvolatile species. SPME eliminates the use of potentially toxic organic solvents and requires minimal sample preparation [111]. HS-SPME-GC has been successfully applied to several aroma-related analyses of beer [109,111,113–115]. Using an optimized HS-SPME-GC-MS method, Cajka et al. obtained relatively good repeatability of peak height measurements within a series of ten consecutive analyses of beer for 45 selected volatile compounds. After observing the GC profiles, several volatile markers were selected and grouped according to classes of alcohols, aldehydes, ketones, esters, carboxylic acids, ethers,

and other compounds. Each group was shown to affect the beer profile in different ways. Esters were characterized by their fruity flavor and were shown to have an impact on the overall flavor balance. Alcohols contributed to the pungent smell and taste of beer, and higher alcohols act as the immediate precursors of the more flavor-active esters. Stale flavors were shown to increase directly with the development of aldehydes and ketones during storage. Carboxylic acids can contribute to multiple odors such has fruity, cheesy, bitter, or rancid. Lastly, ethers caused the development of sweeter aromas like almond and burnt sugar [115]. This method can also be equipped with a flame ionization detector (FID) [111]. Using HS-SPME-GC-FID, Jiao et al. was able to obtain the chromatographic fingerprint of the flavor in various beer samples. After processing the GC data using Principal Component Analysis (PCA) and Hierarchal Clustering Analysis (HCA), the authors classified 28 beer samples by type, taste, and brewery from which they originated [116]. These classifications are presented in Table 2-1. According to Charry-Parra et al., HS-SPME-GC-FID is a simple method which enables it to be used for quality control under normal brewery production conditions [111]. Unfortunately, the FID detector provides no qualitative information about the compounds eluting from the column; identifications must be performed using retention indices or retention time matching with standards.

Table 2-1. List of beer samples analyzed and classified by HS-SPME-GC-FID. The code number was used to indicate main types (T: Traditional, R: Draft, D: Dark), breweries (1, 2, 3, 4), and tastes (a, b, c, d, e, f) [116].

No.	Code	Type	Brewery	Taste
1	T1a	Traditional	HARBIN	ice-pure
2	T1a	Traditional	HARBIN	ice-pure

3	T1a	Traditional	HARBIN	ice-pure
4	T1a	Traditional	HARBIN	ice-pure
5	T2b	Traditional	REEB	original
6	T2b	Traditional	REEB	original
7	T2b	Traditional	REEB	original
8	T2b	Traditional	REEB	original
9	T2b	Traditional	REEB	original
10	R3c	Draft	ASAHI	fine-taste
11	R3c	Draft	ASAHI	fine-taste
12	R3c	Draft	ASAHI	fine-taste
13	R3c	Draft	ASAHI	fine-taste
14	R3c	Draft	ASAHI	fine-taste
15	R3c	Draft	ASAHI	fine-taste
16	R3c	Draft	ASAHI	fine-taste
17	R3c	Draft	ASAHI	fine-taste
18	R3d	Draft	ASAHI	super-dry
19	R3d	Draft	ASAHI	super-dry
20	R3d	Draft	ASAHI	super-dry
21	R3d	Draft	ASAHI	super-dry
22	R3d	Draft	ASAHI	super-dry
23	R4e	Draft	SUNTORY	fresh
24	R4e	Draft	SUNTORY	fresh

25	R4e	Draft	SUNTORY	fresh
26	R4e	Draft	SUNTORY	fresh
27	D2f	Dark	REEB	dark
28	D2f	Dark	REEB	dark

2.4.1.3. Vicinal diketones. Vicinal diketones (VDK) are molecules with two ketone groups on adjacent (vicinal) carbon atoms. The most common ones found in beer are 2,3-butanedione (also called diacetyl) and 2,3-pentanedione, which are responsible for off-flavors associated with deterioration in lagers [20,117]. Although VDKs are produced during fermentation, they are not formed directly by the yeast but instead by a long-chain reaction. During the synthesis of valine and isoleucine, the precursors α-acetolactate and α-acetohydroxybutyrate are excreted by the yeast cells and undergo oxidative decarboxylation to form diacetyl and pentanedione, respectively [117]. Characteristic tastes of diacetyl include sweet butter, caramel, or butterscotch while pentanedione produces honey-like notes in the beer [118]. In addition to producing off-flavors, Tian found that a ratio of VDKs (diacetyl/pentanedione) reflected the degree of contamination in beer. The concentration ($\mu g\ L^{-1}$) of pentanedione was reduced in samples that were seriously contaminated by microbes during fermentation, and a prominent increase of diacetyl was observed at the same time. Therefore, it was determined that a ratio of approximately 1 represented a “normal” beer while contaminated beers had a ratio higher than one [119]. Because these two VDKs are used as target analytes to control beer quality, their quantification should be important to the brewing industry [117].

As with the volatile aroma compounds, HS-SPME-GC is the best method for preparing VDKs for analysis. HS-SPME is a solvent-free sample preparation technique that allows simultaneous sampling, extraction, pre-concentration, and addition of analytes into the GC in a single step, which extends the life of the column and prevents contamination [120]. Several detectors can be used, such as MS, FID, or an electron capture detector (ECD). Leça et al. used HS-SPME-GC-MS to quantify VDKs in beer, which demonstrated high levels of sensitivity and was shown to be simple, rapid, and advantageous for quality control [117]. Although MS and FID are acceptable detectors, ECD may be more suitable for VDK analysis as it is used for detecting compounds with high electron affinity, such as diacetyl and pentanedione, which have the ability to capture electrons [119,120]. Electron capture causes the current between the detector's anode and cathode to reduce, meaning that the analyte concentration is proportional to the degree of electron capture and can be determined by the peak area in a chromatogram [119]. HS-SPME-GC-ECD has been successfully used for the determination of VDKs numerous times [20,119,120]. This detection method was shown to be precise, accurate, selective, and highly sensitive, even for trace analysis. However, issues such as the limited linear dynamic range of ECD must be overcome.

2.4.1.4. Organic acids. Organic acids are end products of both yeast fermentation and bacterial fermentation [20]. These acids are critical to the flavor profile, but due to bacterial fermentation may introduce a sour flavor, which could be intentional when brewing sour beers or unintentional, due to spoilage. The identity and amount of organic acids generated during fermentation is dependent on the yeast strain and brewing protocol used. Organic acids affect the pH and taste (sour, tart, acidic) of beer, and provide positive physiological effects such as

reducing uric acid [121,122]. The presence of these acids may also help to increase the shelf-life of beer by inhibiting the growth of some spoilage bacteria such as *Salmonella* [31].

High performance liquid chromatography (HPLC) is a popular technique for organic acid analysis due to its speed, stability, and simplicity of sample preparation. Using HPLC, Montanari et al. determined the total content of organic acids to be between 451 mg L^{-1} and 712 mg L^{-1} in four different beer samples [122]. The common organic acids found included lactic, tartaric, malic, acetic, citric, and succinic acid, with lactic acid typically having the highest concentration [31,122]. When used for the analysis of fruits and juices, which contain many of the same organic acids as beer, the method was shown to have adequate performance [123]. For example, the analytical figures of merit for the determination of tartaric acid were: Linearity (r^2) of >0.999; relative standard deviation (RSD) values between 0.38 and 1.28% for repeatability and 0.85 to 1.59% for reproducibility; a LOD of 0.72 µg mL^{-1} and a LOQ of 2.40 µg mL^{-1} [123]. However, the ability of HPLC to determine trace levels of organic acids in beer has been limited by the inadequate selectivity and sensitivity provided by conventional liquid chromatographic detectors. This includes absorbance and fluorescence detectors because organic acids lack strong chromophores or fluorophores. Pèrez-Ruiz et al. attempted to solve these issues using a novel post-column-reaction detection system consisting of chemiluminescence (CL) and photochemical reactions [124]. CL detection is very sensitive due to the absence of a light source as this reduces noise and eliminates Rayleigh and Raman scattering, allowing photon detectors to operate at high gain to improve the signal to noise ratio. Many photochemical reactions have been adapted as post-column detection schemes in liquid chromatography because of their selectivity and specificity. Comparison of beer chromatograms using

photochemical-CL detection and absorbance detection shows that the absorbance chromatogram was much more complex, had poorly formed peaks, and the baseline was not stable, which can be observed below in Figure 2-7. Using the combined HPLC-photochemical-CL detection system on four different beers, the authors detected lactic acid (559-631 mg L^{-1}), malic acid (40-68 mg L^{-1}), tartaric acid (0-24 mg L^{-1}), oxalic acid (12-25 mg L^{-1}), and citric acid (74-202 mg L^{-1}) with high sensitivity and selectivity [124].

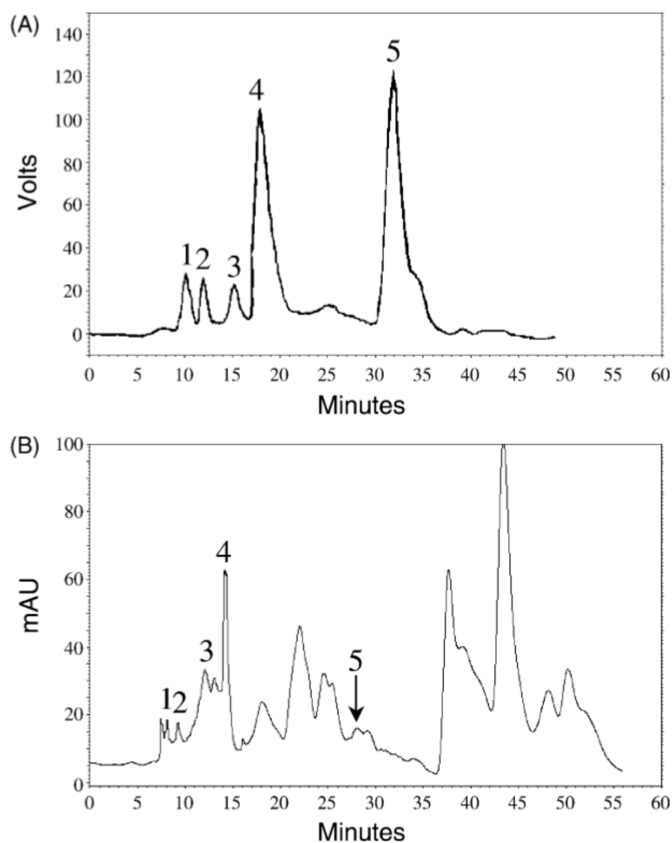

Figure 2-7. Chromatograms of a beer sample analyzed by (A) photochemical-CL detection and (B) absorbance detection at 210nm. The numbered peaks represent (1) oxalic acid, (2) tartaric acid, (3) malic acid, (4) lactic acid and (5) citric acid [124]. Reprinted with permission from reference 124 (copyright 2004, Elsevier).

2.4.1.5. Iso-α-acids. The bitterness of finished beer is derived from precursors present in the hops, the most essential of which are α-acids (*humulones*) and β-acids (*lupulones*). According to De Keukeleire, the most important chemical conversion during the wort boil is the thermal isomerization of the α-acids into iso-α-acids via an acyloin-type ring contraction [15], as seen in Figure 2-8. Iso-α-acids impart about 80% of the beer bitter taste and can vary in concentration, from 15 mg L^{-1} in typical American lagers to almost 100 mg L^{-1} in very bitter English ales [15]. The degree of isomerization and amount of bitterness are directly related and are highly dependent on the hop strain used and the amount of boiling time [125]. In addition to bitterness, iso-α-acids are of interest due to their influence on foam stability and their bacteriostatic effects [126]. These acids have also been shown to have potential positive health effects, such as improving cognitive decline (dementia) induced by high fat diets [127] and reducing liver tumor formation in mice [128].

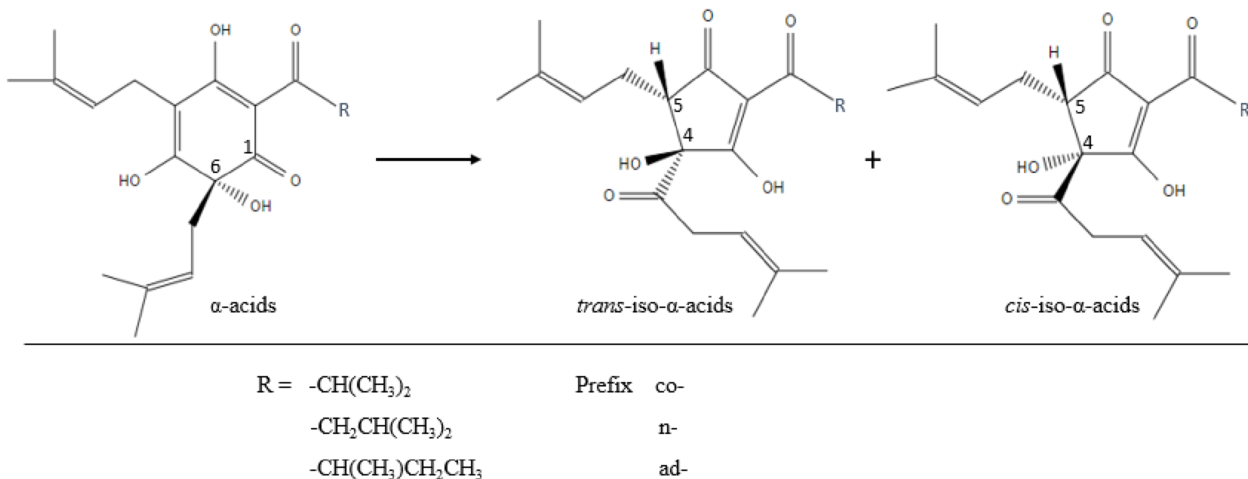

Figure 2-8. Conversion of α-acids to iso-α-acids via an acyloin-type ring contraction.

The most widely used methodology for beer bitterness determination is based on measuring the UV absorption of an iso-octane extract of acidified beer at 275 nm [129]. Other

common methods include automated discrete photometry [20], electric tongue [130], and lead conductance [126]. However, these methods can be time consuming, complex, and lack specificity. Therefore, HPLC has been increasingly applied for the quantitative determination of iso-α-acids. Applying an optimized HPLC method to iso-α-acids has several advantages. For example, it is able to demonstrate differences in the behavior of individual iso-α-acids as a function of wort boiling, differences in α-acids isomerization kinetics, and differential behavior of individual iso-α-acids with respect to foam portioning and beer storage. There are some disadvantages to using this technique, as well. Iso-α-acids tend to interact with trace metals during the chromatographic run, which has been reported to cause poor resolution and recovery of the analytes. These issues can be avoided by using high quality solvents and a thoroughly demineralized column [129].

2.4.1.6. Polyphenols and Antioxidant Activity. Polyphenols are a class of readily oxidizable compounds capable of preventing the oxidation of other molecules, meaning they exhibit antioxidant potential. In addition, polyphenols can influence the colloidal stability, color, flavor (bitterness, astringency, harshness), and shelf-life of beer [121]. Most of the polyphenols are derived from the malt (roughly 70%) while the other 30% are derived from the hops, the exact amount depending on the hop strain and the point at which they are added [52,131,132]. Phenolic constituents of beer cover a large structural variety and belong to the classes of simple phenols, benzoic and cinnamic acid derivatives, coumarins, catechins, (prenylated) chalcones, and flavonoids [52]. Some common phenolic components in beer include flavan-3-ols and their condensed products, the proanthocyanidins [52], which have been shown to determine the colloidal stability [15]. This stability refers to the propensity of beer to produce hazes through

the complexation of polyphenols and proteins, which leads to the formation of finely dispersed precipitates [20]. Undesirable haziness caused by the polyphenol-protein interactions can be removed using cold filtration or adsorptive resins such as polyvinylpyrrolidine (PVPP). Although polyphenol removal undoubtedly improves the shelf stability of beer in terms of haze formation, PVPP is not selective for pulling out exclusively haze active polyphenols and can therefore lead to losses of flavor active polyphenols, as well [21,131].

To prevent unwanted haze formation, the total polyphenol content (TPC) should be analyzed [20]. Colorimetric reactions in conjunction with UV-Vis spectrophotometric methods are widely used for total polyphenol measurements because of the low cost, ease of operation, and quick analysis time. A well-established method for determining TPC involves the oxidation of polyphenolic compounds by the Folin-Ciocalteu reagent (a mixture of tungstate and molybdate) in an alkaline medium to form a colored complex which can then be quantified spectrophotometrically [133]. In most of these studies either tannic acid or gallic acid were used as reference compounds for the calibration curve, although tannic acid was shown to exhibit better recovery rates and less interference from the sample [134]. One drawback of this method is that it involves phosphotungstic or phosphomolybdic acids, which results in non-recyclable waste. In addition, some reducing agents such as citric acid, sulfites, or simple sugars may be present in the beer sample, which can interfere with the reagent [133]. Results of the studies showed that TPC varied significantly depending on the beer type. Piazzon et al. found the trend to be as follows: Dealcoholized < lager < pilsner < wheat < ale < abbey < bock [132]. It was also determined that more hoppy beers contained a higher polyphenol content [20], which should be expected since a portion of the polyphenols originate from the hops.

Phenolic compounds are by far the most common dietary antioxidants in the average human diet [132]. An important group of these found in beer are the flavan-3-ols [52], which behave as antioxidants via scavenging of free radicals and chelate transition metals, as well as mediate and inhibit enzyme activity [21]. When comparing beers with varying antioxidant activity, those which were more abundant in phenolic antioxidants exhibited higher quality in terms of foamability, thermal and oxidative stability [21], as well as a longer shelf life [132]. The shelf life is believed to be increased due to the antioxidants counteracting the autoxidative mechanisms of the iso-α-acids [135]. It has also been reported that the antioxidant content of beer has positive health effects in humans when consumed in moderation, such as increasing the plasma antioxidant and anticoagulant activities and improving plasma lipid levels [132].

There are several different methods that have been used to track the antioxidant activity of beer polyphenols. Spreng et al. applied activity-guided fractionation in combination with oxygen radical absorbance capacity (ORAC), hydrogen peroxide scavenging (HPS), and linoleic acid (LA) assays to a pilsner-style beer in order to locate the key antioxidants. LC-TOF-MS followed by 1D/2D NMR (nuclear magnetic resonance) spectroscopy experiments were also performed, which allowed the structural identification of 31 antioxidants. This was the first study to show that hordatines, saponarin and quercetin-3-O-β-D-(6”-O-malonyl)glucoside exhibited the highest antioxidant activities in beer [135]. Another applicable assay is the ferric reducing antioxidant power (FRAP) assay, which measures the antioxidant potential in a sample by monitoring the reduction of a ferric-tripyridyltriazine complex to its colored, ferrous form in the presence of antioxidants. Piazzon et al. used the FRAP assay to measure the antioxidant activity of seven beer types, with five samples of different brands for each type. Results showed

a strong correlation between TPC measured by the Folin-Ciocalteu method and antioxidant activity measured by the FRAP assay (r = 0.92), and a very similar trend in beer type was determined: Dealcoholized < pilsner < lager < wheat < ale < abbey < bock. The activities were expressed as micromoles of Fe^{2+} per liter of beer and ranged from 1525 µmol L^{-1} for dealcoholized beer to 4663 µmol L^{-1} for bock beers [132]. One limitation of the FRAP test is that the reaction is nonspecific, so the ferric-tripyridyltriazine reduction could be caused by any compound with a suitable redox potential [136]. Assay-guided fractionation procedures also tend to be time-consuming.

Several sensitive post column HPLC methods for antioxidant analysis have been developed in recent years and are meant to screen for antioxidant activity in a more direct and rapid fashion than traditional bulk assays. One such method, called liquid chromatography-antioxidant (LC-AOx), can be used to separate beer polyphenols via HPLC and then evaluate the antioxidant contribution of the individual components. A stable model free radical system, such as 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$), is required for this technique because it shows a decrease in absorbance at 734 nm when reacted with a reducing agent. This approach may prove to be more sensitive toward reactive antioxidants than traditional HPLC with UV detection. Leitao et al. applied LC-AOx followed by UV-Vis detection to track the antioxidant activity of beer extracts throughout the stages of the brewing process (brewing, boiling, and fermentation). They found that while the total antioxidant activity remained consistent, the polyphenolic content showed a three-fold increase following hopping and fermentation [137]. However, the authors attributed this increase to better extraction of the compounds due to the presence of ethanol after fermentation; they did not

detect polyphenol content in hop extracts with the LC-AOx method. Based on these observations, this method may only be suitable for antioxidant activity and not polyphenol analysis.

2.4.1.7. Mycotoxins. As previously stated, mycotoxins are toxic secondary metabolites produced by fungi that may have acute and chronic effects on humans and animals. In addition to analyzing barley for mycotoxins, it is also recommended to determine the amount in finished beer to ensure the concentrations are below acceptable limits. Current European regulations on mycotoxins set maximum levels of deoxynivalenol (DON) and zearalenone (ZEN) in cereal based products (i.e. beer) as 750 $\mu g\ L^{-1}$ and 75 $\mu g\ L^{-1}$, respectively [46]. As of 2010, the FDA advisory level of DON in grain by-products for human consumption is 1 $mg\ L^{-1}$ [138].

Bryła et al. analyzed beer for the occurrence of mycotoxins using HPLC-UV at 218 nm. The method was shown to have a LOD of 1.3 $\mu g\ L^{-1}$ and satisfactory ranges for recovery and repeatability (precision; RSD). In this study, DON was present in 83% of samples at an average concentration of 9.0 ± 12.7 $\mu g\ L^{-1}$, which could be regarded as safely low [43]. Another way to analyze for the occurrence of mycotoxins is using enzyme immunoassays as rapid screening methods. Bauer et al. used this technique to investigate not only DON and ZEN levels, but also ergot alkaloids and alternariol (AOH) in German beers. One benefit of this method was that only DON required extraction to reduce the minimum sample dilution factor and achieve a sensitive LOD. For all other samples, only dilution and pH adjustment were necessary. The detection limits were 2.1 $\mu g\ L^{-1}$ DON, 0.14 $\mu g\ L^{-1}$ ZEN, 0.06 $\mu g\ L^{-1}$ ergot alkaloids (measured as ergometrine equivalents), and 0.18 $\mu g\ L^{-1}$ AOH. Results showed that 75% of beer samples contained DON, 93% were positive for ergot alkaloids, while ZEN and AOH were detected in all

samples. Despite the frequency of occurrence, concentrations of all the analytes were below European Union tolerable daily intake and it was therefore concluded that beer is not a major source of intake of these toxins [42]. While it seems that most beers contain acceptably low levels of mycotoxins, confirming this through analysis is still suggested.

2.4.1.8. Pesticides. The grains and hops used in brewing are susceptible to attack by insects, such as hop aphids and two-spotted spider mites, as well as microbial pathogens. To prevent crop damage due to these pests, different combinations of pesticides are widely used at many stages of cultivation and post-harvest storage [139]. While these pesticides are necessary for crop protection, they may be carried over into the beer during the brewing process and potentially harm the consumer. It is therefore desirable to have a comprehensive method to detect the different types of compounds used, such as glyphosate, glufosinate, chlorfenapry, quinoxyfen, and fenarimol, to name a few.

The most commonly used method for pesticide analysis in beer is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [140–142], although GC-MS has also been used [139,143]. Nagatomi et al. developed an LC-MS/MS method to simultaneously analyze beer for glyphosate, glufosinate, and their metabolic products without requiring any conversion steps. This method was used to analyze 15 commercial canned beer samples. Although traces of glyphosate were found in four of the samples, they were not quantified at a level greater than the LOQ of 10 μg kg^{-1}, which was deemed sufficiently low [142]. Inoue et al. used LC-MS/MS to track the fate of pesticides throughout the brewing process. This was performed by artificially contaminating ground malt samples with a variety of pesticides and then analyzing the residual ratios in unhopped wort, spent grain, cooled wort, and the finished beer. Some key

conclusions were that most of the pesticides became reduced in content after mashing, and only a few pesticides remained at large ratios in the beer. The pesticides that carried over into the beer were methamidophos, 2-(1-naphthyl)acetamide, imazaquin, fluoroxypyr, flumetsulam, thiamethoxam imibenconazole-desbenzyl, imidacloprid, and tebuthiuron, so special care should be taken with the use of these pesticides on brewing grains and hops [141].

2.4.2. Inorganic Content

2.4.2.1. Total SO_2. Most of the sulfur dioxide (SO_2) in beer originates from yeast metabolism or any exogenous sulfite added before packaging [144]. SO_2 contributes to an increase in flavor stability by reacting with carbonyl compounds (usually aldehydes) to form α-hydroxysulfonates, which are not flavor active and thus increase the flavor threshold of the carbonyls that are responsible for stale, unpleasant tastes in beer [20,145]. SO_2 also acts as an antioxidant and antimicrobial agent at high concentrations. However, a small portion of the population suffers from sulfite sensitivity, the side effects of which include a large array of dermatological, pulmonary, gastrointestinal, and cardiovascular symptoms [146]. Therefore, SO_2 levels must be controlled to avoid these adverse effects on human health [147]. Both US and EU regulations state that total SO_2 content must be included on the labelling of alcoholic beverages if detected at a level of 10 mg L^{-1} or higher [146].

One of the most common ways to determine total SO_2 in food and beverages is colorimetric analysis, which is the recommended procedure by the American Society of Brewing Chemists (ASBC) [148]. This method involves hydrolysis of bound SO_2 with an alkali solution followed by the reaction between SO_2, p-rosaniline, and formaldehyde to form a colored solution after

about 30 minutes. The absorbance of this complex can then be measured at 550 nm [147]. Although this method has been widely used due to its precision and ability to measure both free and total SO_2, the overall procedure is time consuming and involves handling p-rosaniline, a potential carcinogen [144]. These issues can be reduced by adapting a method using flow injection analysis (FIA). This technique involves adding the beer sample into a mixing chamber with sodium hydroxide (NaOH) to release bound sulfites prior to injection. Sulfuric acid is introduced to lower the pH and convert existing equilibrium products into gaseous sulfur dioxide, which is isolated by diffusion across a gas permeable membrane. The SO_2 is then reacted with p-rosaniline and formaldehyde to form the colored product, which is measured using a spectrophotometer [146]. The p-rosaniline is introduced using the merging zones technique, which reduces its consumption by adding only the required amount for the reaction into the system as opposed to a continuous flow through the manifold. Compared to the original procedure, the FIA system required no sample preparation, provided accurate and precise results with higher sampling rates, and the consumption of p-rosaniline was reduced ten-fold [144].

Voltammetric methods have also been used for the determination of free and total SO_2 in beer. The first step involves diluting beer samples and adding NaOH to make the solution alkaline, which causes the decomposition of adducts between SO_2 and carbonyls and converts hydrogen sulfite to sulfite. The volatile aldehydes (mainly acetaldehyde) are purged from the sample using nitrogen, collected in an appropriate electrolyte trapping solution, derivatized with hydrazine, and then voltammetrically determined. The remaining beer sample is acidified to convert all sulfite into SO_2, which is then transferred by nitrogen gas into a trapping solution,

and the total SO_2 concentration is determined by voltammetry. Because acetaldehyde represents approximately 95% of all the aldehydes in beer and the acetaldehyde-hydrogensulfite adduct is very stable, the free SO_2 concentration can be calculated as the difference between total SO_2 and acetaldehyde concentrations [145,147]. This method showed a RSD of about 2.1% for total SO_2 concentrations typically found in beer [145]. Voltammetric determinations seem to agree with those obtained by the p-rosaniline reference method, with the advantage of excluding the use of toxic reagents (p-rosaniline) and providing more accurate and precise results [146].

2.4.2.2. Ions. The inorganic compounds found in beer are metal cations, trace metals and anions, which influence the drink's clarity and salty taste. These compounds can originate from several places, such as the raw materials (usually malt, since most brewing water is deionized to standardize the quality before adding back the requisite ions), brewing construction materials, or processing and packing of the finished beer. Concentrations of inorganic components generally range from 0.5 – 2 g L^{-1} [18]. Chloride and sulfate are the ions of highest interest, as chloride provides mellowing and fullness to the flavor while sulfate enhances the dry character of a beer. Some other commonly found ions include nitrate, phosphate, iron, copper, zinc, manganese, nickel and aluminum. Inorganic ion analysis is typically performed by ion chromatography (IC) or capillary electrophoresis (CE) [20,106,107,149–153], although atomic emission spectrometry with inductively coupled plasma (ICP-AES) can also be used to determine the inorganic ions in beer [28].

Ion chromatography was used specifically for the determination of inorganic ions when it was first introduced in 1975, but later broadened to include organic ions, other separation

methods (i.e. ion exclusion), simultaneous separation of anions and cations, and a variety of detectors. It has been used extensively for the determination of various inorganic ions in alcoholic beverages such as beer, wine and vodka [152]. Zeng et al. successfully applied IC with acidified aqueous mobile phases and non-suppressed conductor detection for the determination of cations in beer. The chromatogram for one sample is presented in Figure 2-9. This technique showed satisfactory sensitivity, detection limits, and reproducibility for the ions of interest. One advantage of this method was that it allowed the beer samples to be injected directly after dilution with no other preparation [149]. IC is useful for analyzing aluminum in beer [154], as well as anions such as chloride, sulfate, nitrate, and phosphate [153].

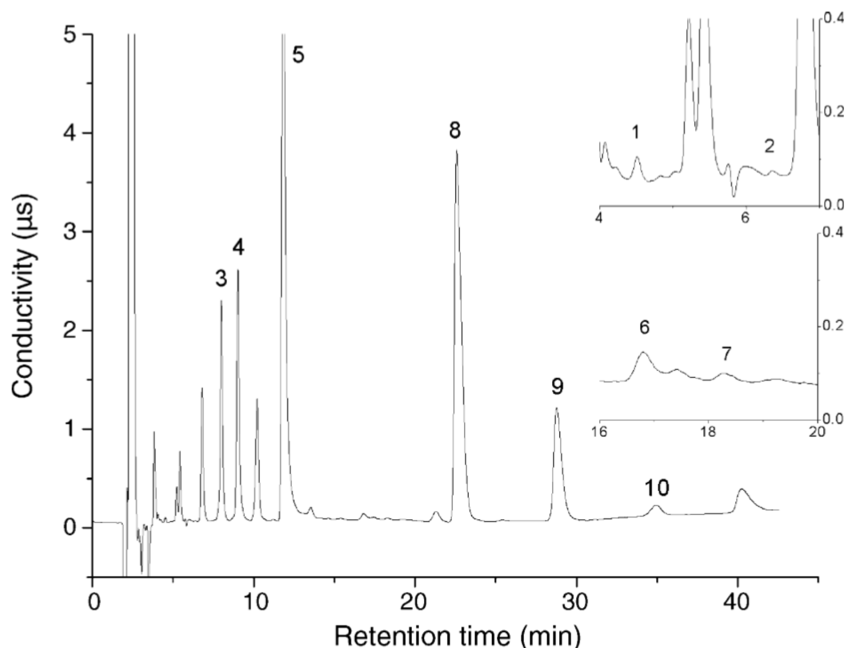

Figure 2-9. Chromatogram of a beer sample obtained by IC with non-suppressed conductor detection. Peak identities: 1 = copper; 2 = sodium; 3 = ammonium; 4 = potassium; 5 = nickel; 6 = zinc; 7 = cobalt; 8 = magnesium; 9 = calcium; 10 = strontium [149]. Reprinted with permission from reference 149 (copyright 2006, Elsevier).

Capillary electrophoresis, specifically capillary zone electrophoresis (CZE) with UV detection, has also been used for inorganic ion analysis in multiple studies. Klampfl investigated the use of CZE equipped with a conductivity detector in series with a fixed-wavelength UV detector operated at 254 nm for the analysis of inorganic anions in four different types of beer. By using both detectors simultaneously, it was possible to perform quantitation using the best detection method for each type of analyte. For example, chloride and sulfate were better suited for detection by conductivity while phosphate favored UV detection. This approach resulted in excellent limits of detection ranging from 0.02 mg L^{-1} for chloride and 0.41 mg L^{-1} for phosphate, and RSD values between 0.5 and 6.6% for the analytes of interest. Sample preparation was simple, requiring only dilution and 15 minutes of degassing [151]. CZE was shown to be a simple and reliable method for the simultaneous determination of inorganic and organic anions, amino acids, and carbohydrates in pineapple and soy sauce samples [150], and this approach seems promising for beer analysis, as well.

When comparing IC and CE, both methods were found to have certain advantages over the other. The selectivity of IC is dependent on the choice of stationary phase, of which there are more than 150 commercially available, while CE selectivity depends on electrophoretic mobilities of the analytes and is therefore much more difficult to manipulate. IC is also considerably more sensitive under routine conditions (direct injection) and can achieve RSD (precision) of 1% or less, while the RSD of CE is typically between 3 and 5%. Some areas where CE excels are the speed of analysis, cost of consumables, and separation efficiency, which is 50 times higher than that of IC. Despite their differences, the separation selectivities of these

techniques are complementary in that a sample separation which is problematic in one technique is often relatively straightforward in the other [107]. When used in tandem, the interferences from other ions are decreased and peak identities can be confirmed, which is advantageous [106]. Although ion chromatography is an older and more developed technique, the application of capillary electrophoresis for inorganic anion determination is growing and may partially replace IC in the future [106].

2.4.3. Biological

2.4.3.1. Bacteria. Lambic sour beer is one of the oldest types currently being brewed and has been the topic of many bacterial analysis studies. Although lambic beers are made only in Belgium, sour beers in general are currently attracting interest in other parts of the world. American craft breweries, for example, have started brewing American coolship ales by mimicking the lambic beer production method [155]. The sour character of these beers stems primarily from the metabolic activities of various yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) [29,155–157]. However, these bacteria can lead to unwanted effects such as beer spoilage, turbidity, acidity, and off flavors [158]. Therefore, the characterization of beer spoilage bacteria is imperative to brewers for quality control purposes. Previous microbial studies on beers used phenotypic identification techniques only, which recent studies revealed to have inadequate taxonomical resolution for the species-level identification of yeasts, LAB, and AAB [155].

One alternative for classifying bacteria in beer is MALDI-TOF-MS, which can differentiate organisms at the genus, species, or strain level [159]. Studies have identified bacterial cells by

comparing the MALDI-TOF-MS spectra obtained from simple microbial mixtures and cultured bacterial cells against a library of known spectral fingerprints obtained from intact bacterial cells [160]. Alternatively, proteins can be desorbed from whole cells of microorganisms by MALDI to generate spectra containing unique biomarkers, which may then be compared to a protein database [49,92,159].

For the identification of beer-spoilage bacteria by MALDI-TOF-MS, Wieme et al. constructed an extensive database composed of more than 4200 mass spectra, including replicates derived from 273 AAB and LAB, covering a total of 52 species, grown on at least three growth media. The spoiled beer samples contained too few bacterial cells to allow direct detection and classification through MALDI-TOF-MS, so the bacteria had to undergo enrichment and isolation prior to analysis. The resulting identifications were verified using sequence analysis of protein coding genes. Peak-based numerical analysis of the MALDI spectra allowed a straightforward species identification of 327 out of the 348 collected isolates (94%) from spoiled beer samples. Table 2-2 shows the results of this study [29]. Due to the low consumable cost, high-throughput, and accurate identification of the bacteria, this technique was determined to be appropriate for routine microbial quality control in the brewing industry. However, the high initial cost and subsequent maintenance costs of a MALDI-TOF instrument may prove too expensive for small and medium-size breweries, so cheaper options would be beneficial. Variations of MALDI-TOF-MS used specifically for the bacterial analysis of lambic sour beers were described by Spitaels et al. [155,156].

Table 2-2. Results of 32 identified isolates representing 15 clusters of 348 beer-spoilage isolates from the study by Wieme [29]. The type strain which had the highest sequence similarity with respect to the query sequence is annotated in the designated column.

Cluster Representatives			BS	Query Sequence		Type strain with highest pairwise sequence similarity to query sequence		
Cluster 1	74 isolates							
Acetobacter cerevisiae/		R-49601	10	*rpoB*	KF910097	*A. cerevisiae*	98.3%	KF537492
Acetobacter malorum[1]		R-49602	11	*rpoB*	KF910098	*A. cerevisiae*	98.3%	KF537492
Cluster 2	7 isolates							
Acetobacter fabarum		R-50650	37	*dnaK*	KF910092	*A.fabarum*	98.5%	HG329542
Cluster 3	11 isolates							
Acetobacter indonesiensis		R-50362	37	*rpoB*	KF910108	*A. indonesiensis*	97.4%	KF537503
		R-50645	37	*rpoB*	KF910109	*A. indonesiensis*	97.4%	KF537503
Cluster 4	8 isolates							
Acetobacter orleanensis		R-49862	14	*rpoB*	KF910101	*A. orleanensis*	99.4%	KF537507
Cluster 5	1 isolate							
Acetobacter persici		R-50064	14	*rpoB*	KF910096	*A. persici*	98.7%	KF537531
Cluster 6	7 isolates							
Gluconobacter cerevisiae		R-50419	36	*dnaK*	KF910090	*G. kondonii*	98.4%	HG329571
			36	*groEL*	HG329605	*G. kanchanaburiensis*	97.2%	HG329598
			36	*rpoB*	KF910104	*G. kondonii*	89.0%	HG329607
Cluster 7	2 isolates							
Gluconobacter sp.		R-50361	37	*dnaK*	KF910093	*G.uchimurae*	97.9%	HG329581
			37	*rpoB*	KF910110	*G.roseus*	95.2%	HG329613
Cluster 8	7 isolates							
Gluconobacter cerinus		R-50416	36	*rpoB*	KF910102	*G. cerinus*	98.2%	FN391790
		R-50417	36	*dnaK*	KF910091	*G. cerinus*	98.0%	FN391644
		R-50417	36	*rpoB*	KF910103	*G. cerinus*	98.1%	FN391790
Cluster 9	19 isolates							
Gluconobacter japonicus		R-50363	37	*rpoB*	KF910105	*G. japonicus*	98.9%	HG329615
		R-50643	37	*rpoB*	KF910107	*G. japonicus*	99.0%	HG329615
Cluster 10	5 isolates							
Gluconobacter oxydans		R-49860	6	*rpoB*	KF910112	*G. oxydans*	99.9%	FN391799
		R-49861	6	*rpoB*	KF910110	*G. oxydans*	99.9%	FN391799
Cluster 11	21 isolates							
Lactobacillus backii		R-49483	4	*pheS*	KF910133	*L. backii*	99.6%	AB769496
		R-49484	4	*pheS*	KF910132	*L. backii*	99.7%	AB769496
		R-50069	4	*pheS*	KF910147	*L. backii*	99.5%	AB769496
Cluster 12	100 isolates							
Lactobacillus brevis[2]		R-49531	11	*pheS*	KF910129	*L. brevis*	89.9%	AM087680
		R-49856	15	*pheS*	KF910143	*L. brevis*	99.0%	AM087680
		R-49857	15	*pheS*	KF910144	*L. brevis*	99.0%	AM087680
		R-49864	10	*pheS*	KF910155	*L. brevis*	98.9%	AM087680
		R-49877	16	*pheS*	KF910146	*L. brevis*	98.9%	AM087680

		R-49879	13	*pheS*	KF910156	*L. brevis*	98.9%	AM087680
Cluster 13	59 isolates							
Lactobacillus malefermentans		R-49868	12	*pheS*	KF910142	*L. malefermentans*	99.8%	AM263505
		R-50644	37	*pheS*	KF910149	*L. malefermentans*	99.7%	AM263505
		R-50646	37	*pheS*	KF910150	*L. malefermentans*	99.7%	AM263505
		R-50647	37	*pheS*	KF910151	*L. malefermentans*	99.7%	AM263505
		R-50649	37	*pheS*	KF910152	*L. malefermentans*	99.7%	AM263505
Cluster 14	5 isolates							
Pediococcus claussenii		R-49863	7	*pheS*	KF910145	*P. claussenii*	100.0%	AM899832
Cluster 15	22 isolates							
Pediococcus inopinatus		R-50648	37	*pheS*	KF910153	*P. inopinatus*	100.0%	AM899821
		R-50651	37	*pheS*	KF910154	*P. inopinatus*	100.0%	AM899821

BS = beer sample

[1] Also retrieved from BS5, BS6, BS9, and BS35.

[2] Also retrieved from BS4 and BS14.

Another option for microbial analysis is the use of fatty acids, which are essential components in cell membranes of bacteria. Similar to protein profiles, fatty acid profiles are unique from one bacterial species to the next and can be used for their identification by comparison to a database. The Microbial Identification System (MIS) produced by Microbial ID (MIDI, Newark, DE, USA) is a commercially available library widely used to identify microorganisms by their fatty acid profiles [161]. The fatty acids are generally identified and quantified using GC coupled with either MS or FID detectors [162,163]. A recently developed technique, GC-VUV, provides comparable information to GC-MS but can also deconvolve coeluting peaks by overlapping absorption spectra, which would be advantageous for complex beer samples. Santos et al. used GC-VUV to determine bacteria fatty acid methyl esters

(FAMEs), which were used to identify and discriminate environmental bacteria based on their fatty acid profiles [161]. Santos et al. also described a comparative study in which GC-VUV and MALDI-TOF-MS were used for fatty acid and protein compositions of bacteria in water samples as an indicator of environmental stress [164]. It was determined that the two methods could be used as complementary tools for environmental analysis. Although no studies have been found which apply GC-VUV to bacterial analysis in beer, it seems like an interesting prospect for future work.

In addition to identifying the bacteria, brewers may also want to quantitate the amount present in their beer. A promising method for bacterial population analysis is next-generation sequencing (NGS) combined with a quantitative polymerase chain reaction (qPCR) assay. Takahashi et al. used NGS-qPCR to track the total amounts of bacteria during fermentation and maturation and found that the populations ranged from approximately 10^3 cells mL^{-1} to 10^4 cells mL^{-1} in beer samples. They also determined that a larger bacterial population was present in malt than in barley, and that the microbial community of barley changes during the malting process [165]. Another study by Takahashi et al. involved applying NGS-qPCR for the quantification of LAB and AAB cell densities in 37 craft beer samples. The results of this study were compared to those obtained by species-specific qPCR, a more established method, and proved to be consistent [157]. Thus, this technique appears to be practical for analyzing bacterial communities based on LAB and AAB cell densities in beer.

2.4.3.2. Fatty Acids. The fatty acid profile of beer is affected by the raw materials (malt and hops) and mashing process used, as well as yeast metabolism and maturation [166,167]. Fatty acid content can be applied not only for bacterial analysis, but also for determining beer quality. Although they are only minor constituents, fatty acids can have adverse effects on beer flavor, such as staling. Medium-chain fatty acids like hexanoic, octanoic, and decanoic acids are responsible for rancid or goaty flavor characteristics. Furthermore, long-chain unsaturated fatty acids, such as linoleic and linolenic acids, may lead to the formation of an aging flavor due to their oxidative degradation [167]. An unexpected increase in their content, coupled with poor storage conditions, can result in an undesirable stale flavor [168]. There is also an interesting relationship between fatty acids and over-foaming volume. Saturated fatty acids are known to promote gushing (i.e. spontaneous over-foaming) whereas unsaturated fatty acids tend to suppress the over-foaming volume [169]. Because of their effects on various aspects of beer quality, a suitable and reliable method for fatty acid analysis is desirable.

As stated in the previous section, fatty acids are typically quantified using gas chromatography, which has been used to study the changes in beer volatile compounds during storage since the 1960s [168]. Detection by either MS or FID is acceptable. These GC methods can use various sample preparation techniques, which involve a crucial extraction step that often needs complex procedures. Previous works have used steam distillation and liquid-liquid extraction techniques, which are both time-consuming and labor intensive. Additionally, liquid-liquid extraction requires large volumes of organic solvents and suffers from problems of emulsion formation, which may lead to analyte losses [166]. More modern procedures are based on solid-phase extraction strategies, such as solid-phase microextraction (SPME) and stir

bar sorptive extraction (SBSE), which are generally faster and minimize the use of organic solvents, leading to better recovery and high reproducibility [167]. Bravi et al. quantitatively determined the fatty acids in beer using a GC-FID method with liquid– liquid cartridge extraction and SPE purification, which avoided emulsion formation and facilitated the efficient extraction of fatty acids present in low amounts. However, the fatty acids had to be derivatized to their methyl esters (FAMEs) before being injected into the GC, which complicates the sample preparation procedure. On average, the most representative fatty acids were determined to be unsaturated oleic and linoleic acids, which should inhibit the over-foaming volume [166]. The method was shown to be applicable to a wide range of fatty acids in different beer samples, making it a suitable choice for this type of analysis.

2.5. Conclusions

The complex composition of beer can be attributed to the presence of various classes of compounds, some of which originate from the raw materials while other develop through interactions during the brewing process. These components can affect the beverage in a multitude of ways, ranging from the flavor, to foaming ability, to whether or not it is safe to consume. Therefore, being familiar with common analytical methods for beer and ingredient analysis may help brewers to produce higher quality beers.

There is currently a lack of advanced analytical techniques used in routine brewing operations. This is primarily due to the high initial cost of analytical instruments and the need for a skilled technician to operate them. Despite these drawbacks, their use could provide greater consistency for quality control and perhaps add to a more unique marketing approach.

For example, the widespread introduction of MALDI-TOF-MS to the brewing scene would help to categorize beers based on their microbial content, while the ability of GC-VUV to separate and quantify terpenes may increase the marketability of certain hops and hoppy beers. However, due to their expensive nature, there is a need for cheaper and simpler techniques that give comparable information to these more advanced instruments.

While some products exist that allow in-house brewing quality control, these usually only test for basic parameters such as alcohol by volume (ABV), bitterness (IBU), pH, color, and possibly a few in-depth specifications like FAN, VDKs, and total SO_2. It does not appear that testing for potential hazards like mycotoxins, pesticides, and harmful bacteria is very common. In fact, the only analysis required by the State of Texas prior to serving beer is ABV, so brewers are not legally obligated to report any other compounds present in their product. This could be another reason why advanced analytical technologies are not more widespread in a field that uses a lot of chemistry and biochemistry. Although it is apparent that the content of beer has been analyzed for many years, there is still room for improvement and growth by making analytical instruments more available to brewers.

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CHAPTER THREE

TARGET PROFILING OF BEER STYLES BY THEIR ISO-α-ACID AND PHENOLIC CONTENT USING LIQUID CHROMATOGRAPHY-QUADRUPOLE-TIME-OF-FLIGHT-MASS SPECTROMETRY

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Target Profiling of Beer Styles by their Iso-α-acid and Phenolic Content using Liquid Chromatography-Quadrupole-Time-of-Flight-Mass Spectrometry

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3.1. Abstract

Beer styles show wide variation in color, flavor, and clarity, due to differences in their chemical content. Some of the major flavor compounds in beer are iso-α-acids and phenolic compounds. These were investigated as potentially discerning features between beer styles. A selection of thirty-two American beers covering five styles were analyzed using liquid chromatography quadrupole time-of-flight mass spectrometry, which resulted in high mass accuracy chromatograms of the studied analytes. Distinctions between the presence or relative concentrations of certain compounds were observed and related back to brewing ingredients and procedures. For example, vanillin was only observed in stout beers due to the use of roasted barley malts for brewing, while chlorogenic acid isomers were found in two sours at relatively high concentrations (189 mg/L and 34 mg/L) because of the fruits used to flavor the beers. Distinctions were further confirmed using multivariate analysis techniques, which separated three of the five beer styles (India pale ales, stouts, and sours).

Keywords: craft beer; high resolution mass spectrometry; iso-α-acids; phenolic acids; multivariate analysis

3.2. Introduction

The brewing of beer can be traced back some 6,000 to 8,000 years ago. These primitive beers probably had a sweet, malty body flavored with herbs, spices, or fruits, which is quite dissimilar to the drink we are familiar with today [14,170]. Multiple styles have emerged over time, ranging from light and crisp pilsners, to dark and malty porters, to hazy and hoppy IPAs (India pale ale). Despite exhibiting vastly different color and flavor profiles, modern beer styles are brewed using the same four basic classes of ingredients: a starch source (such as barley or wheat), water, hops, and yeast. The recent surge in small, independent breweries in the United States has introduced more experimentation with ingredients and brewing procedures to create contemporary, complex, and flavorful beers.

From 2018 to 2019, the number of independent breweries in the U.S. increased by 9.1% for a total of 8,386, and their beers now account for 13.6% of the beer market by volume [5]. These statistics reflect the growing interest in specialty beers and illustrates the potential desire for reliable methods of flavor profiling. Flavor profiling on the chemical level can be challenging due to the complexity of beer, which contains a wide variety of components such as carbohydrates, proteins, microbes, secondary metabolites, sulfur dioxide, and, of course, ethanol [11]. Perhaps the most interesting components are the isomerized alpha acids (iso-α-acids) and phenolic compounds. These are present in beers in relatively low concentration but are widely believed to contribute the largest influence on beer flavor and stability [171].

Iso-α-acids originate from humulones (α-acids) in the cone-shaped flowers of the hops vine (*Humulus lupulus*, L. Cannibinaceae). Homologues of α-acids include cohumulone, n-humulone, and adhumulone, which differ only in the nature of the saturated acyl side chain [131]. When

hops are added during the boiling stage of brewing, the α-acids become thermally isomerized to produce *trans*- and *cis*- stereoisomers of iso-α-acids (isocohumulone [iCH], isohumulone [iH], and isoadhumulone [iAH]) [15]. The iso-α-acids (IAA) are major contributors to the characteristic bitter flavor of beer, with the *cis*-isomers generally contributing more bitterness than the *trans*-isomers [172]. The light stability of beer can be improved by converting IAAs to their reduced forms by hydrogenation and reduction reactions [15]. The three types of reduced iso-α-acids include dihydro- (also called "rho", [ρ]-), tetrahydro-, and hexahydro-iso-α-acids (RiAA, TiAA, HiAA, respectively), which differ by the number of hydrogen atoms incorporated during reduction (Figure 3-S1) [172]. Both RiAA and HiAA have an extra chiral center, resulting in multiple enantiomeric forms.

Polyphenols and phenolic acids primarily originate from barley and hops and contribute to the flavor, color, body, and colloidal stability (i.e. haziness) of beer [15,20,172]. While some phenols add desirable flavor characteristics, such as clove in a Hefeweizen, others create unwelcome off-flavors. Some have been described as tasting like plastic, adhesive bandages, or sweaty horse blanket [173]. Phenolic compounds present in beer include classes of phenolic acids, simple phenols, flavonoids, hydroxycoumarins, proanthocyanidins, and tannins [121,174].

Over the past few decades, liquid chromatography-mass spectrometry (LC-MS) has been the dominant technique applied to the separation and characterization of both the phenolic compounds and iso-α-acids in beer, due to its ability to quantify and identify analytes at low concentrations in the presence of interferences [175], of which beer has many. In this respect, LC-MS may be a more appropriate alternative to the standard UV detection methods currently used by brewers in order to more fully understand the presence of specific chemical compounds

of interest. Using a quadrupole time-of-flight (QTOF) mass analyzer allows for higher accuracy ion mass measurements and introduces the potential for predicting unknown compounds that may be responsible for the variations in flavor.

The usage of advanced analytical techniques for flavor profiling in the brewing industry could potentially assist in the innovation of new flavors, improve quality control practices, or provide brewers with a better understanding of how the ingredients and processes affect their finished product. There is also an opportunity to expand the marketability of beers by providing deeper insight into their flavor profiles. Of course, LC-MS represents a much larger capital investment than a UV-Vis instrument, and it is more complicated to operate. However, if one desires to parse the vast degree of chemical variation in the products produced by today's craft brewing industry, instrumentation with much higher specificity is needed, and LC-MS is one obvious choice for this task.

Due to their significant impact on flavor and stability, the IAA and phenols in beer have been studied extensively [132,176–178], but primarily in the context of their contributions to bitterness [15,179]. This study aims to simultaneously analyze the IAA and phenolic compounds in beer using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS), and to use the chemical profiles to distinguish between the flavor characteristics of various U.S. beer styles. Multivariate analysis was conducted on the mass spectral data, which further illustrated the variability between some beer styles.

3.3. Materials and Methods

3.3.1. *Materials*

A single bottle or can of thirty-two U.S. commercial craft beers were purchased from local stores. Five different styles (IPA [I], blonde [Bl], stout [St], wheat [W], and sour [S]) from 22 breweries were represented (Table 3-S1). The beers were freshly opened, and 30 mL were transferred and immediately degassed for 30 min by sonication. Degassed samples were then diluted 50% with water prior to injection. Excess beer was stored at 5 ^{o}C for two days before no longer being considered viable.

3.3.2. *Chemicals and reagents*

All reagents were of LC-MS grade. Water (H_2O), methanol (MeOH), and acetonitrile (ACN) were obtained from Honeywell (Muskegon, MI, USA). Formic acid (98-100%) was purchased from EMD Millipore (Billerica, MA, USA).

Phenolic reference standards including vanillin (99%), 4-hydroxybenzoic acid (99%), caffeic acid (98%), quercetin (95%), naringin (95%), 4-hydroxycoumarin (98%), myricetin (98%), 4-hydroxy-3-methoxycinnamic acid (99%), (+)-catechin (99%), esculin (European Pharmacopoeia reference standard), and chlorogenic acid (European Pharmacopoeia reference standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenolic standards were chosen based on previous studies involving phenolic compounds in beer [178].

Four variations of the American Society of Brewing Chemists (ASBC) international calibration standards (ICS) for HPLC analysis of isomerized and reduced isomerized α-acids were

purchased from Labor Veritas (St. Paul, MN, USA) which are listed in Table 3-S2. The IAA, RiAA, and HiAA calibration standards were obtained as a purified preparation of the dicyclohexylamine (DCHA) salts. The International Subcommittee for Isomerized Hop α-Acids Standards determined the total percentage of iso-α-acids present in the standards.

3.3.3. *Preparation of Standard Solutions*

Stock solutions of 1 mg/mL were prepared for the phenolic and iso-α-acid standards. All stock solutions were made in acetonitrile, except for HiAA and RiAA, which were insoluble in ACN and therefore, had to be prepared in methanol. Solutions were stored at 5 ^{o}C. Amber vials were used to decrease light exposure.

Calibration curves for each phenolic standard were created and contained at least 7 points, run in triplicate, and spanned over two orders of magnitude (Figure 3-S2). Quality control checks were run at low, medium, and high concentration levels, at concentrations distinct from those used for the calibration curve, for each compound (Table 3-S3). The concentration points used for the calibration curves are listed in Table 3-S4. Limit of detection (LOD), limit of quantitation (LOQ), and R^2 were calculated for each curve. Lack-of-fit tests were performed for each curve to assess the linearity. The concentration of each analyte was then calculated for each of the beer samples and average phenolic concentrations, along with standard deviations, were calculated using injection replicates for each beer sample ($n = 3$) and then averaged for each style of beer.

3.3.4. *Instrumentation*

Analyses were carried out on a Shimadzu Nexera X2 liquid chromatograph equipped with two solvent delivery pumps (LC-30AD), an online degassing unit (DGU-20A5R), autosampler (SIL-30AC), column oven (CTO-20AC), system controller (CBM-20A), and quadrupole time-of-flight (QTOF) mass spectrometer (LCMS-9030) (Shimadzu Scientific Instruments, Inc., Columbia, MD). Instrument control and data acquisition were performed using Realtime Analysis, and data integration and further evaluation were performed using DataAnalysis (LabSolutions Chromatography Workstation, v.5.97, Shimadzu Corp.) and Excel (Microsoft Office, v.16.0.4993.1002, Microsoft Corp., Redmond, Washington, USA). Separation was achieved using a Restek Raptor C18 column (100 mm x 2.1 mm x 2.7 µm) (Restek Corporation, Bellefonte, PA). Injections of 1 µL were used. Mobile phases consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Gradient elution was performed at a flow rate of 0.4 mL min^{-1}, as follows: 0-1 min, 5% B; from 1-5 min, 5 – 75% B; from 5-9 min, 75 – 95% B; and from 9.01-15 min, a step gradient back to 5% B for re-equilibration. The back-pressure of the LC-MS system varied due to the use of gradient elution, but was typically around 3000 psi.

An electrospray ionization (ESI) source was used in negative ionization mode under the following conditions: nitrogen nebulizing gas and drying gas flows were 2 L min^{-1} and 10 L min^{-1}, respectively; the desolvation line temperature was 250 °C and the heat block temperature was 400 °C; the negative interface voltage was -3.5 kV. High-resolution multiple reaction monitoring (MRM) optimizations were performed for each of the analytical standards. In addition to monitoring MRM transitions at optimized collision energy for each targeted analyte, full scan MS data was collected in ESI negative ionization mode during data acquisition.

3.3.5 *Statistical Analysis*

The final peak list containing the relative peak area of the detected compounds for each sample was used to conduct multivariate analysis on each beer style Multivariate analysis was performed using MetaboAnalyst 4.0 online (Quebec, Ca). Prior to statistical analysis, the data was normalized using auto-scale. No further data transformation was performed.

3.4. Results and Discussion

3.4.1. *Quantitation*

Negative ionization mode was used due to its higher response yield for the targeted analytes. Optimized MRM transitions (Tables 3-S5 and 3-S6) were monitored for each standard. A representative standard chromatogram is shown in Figure 3-1.

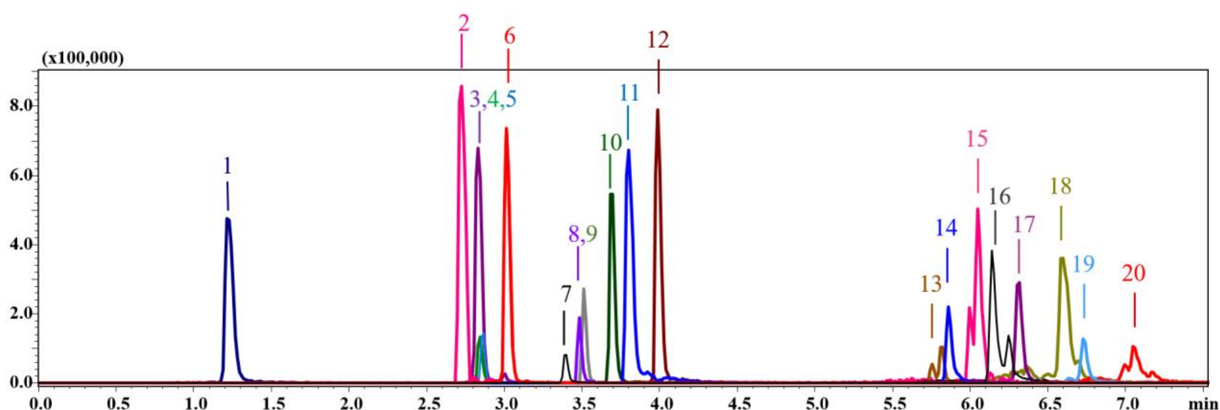

Figure 3-1. LC-QTOF-MS MRM chromatogram of targeted phenolic and IAA standards. 1. Gallic acid [dark blue], 2. Esculin [pink], 3. Chlorogenic acid [purple], 4. Benzoic acid [green], 5. Catechin [teal], 6. Caffeic acid [red], 7. Vanillin [black], 8. Cinnamic acid [lilac], 9. Naringin [grey], 10. Myricetin [dark green], 11. Hydroxycoumarin [blue], 12. Quercetin [brown], 13. *c*-RiCH [light brown], 14. *t*-iCH [blue], 15. *c*-RiAH and *c*-RiH [pink], 16. *t*-iAH and *t*-iH [black], 17. *c*-HiCH [purple], 18. *c*-HiAH and *c*-HiH [olive green], 19. *c-/t*-TiCH [cyan], 20. *c-/t*-TiAH and *c-/t*-TiH [red]

Calibration curves were created for the phenolic compounds, and their attributes are listed in Table 3-S3. The limit of detection (LOD) was determined empirically by analyzing a low concentration point, and then halving that concentration until the analyte signal could no longer be detected. The lowest concentration that could be reliably detected was then reported as the LOD. The limit of quantitation (LOQ) was then calculated as 3.3 times the LOD. All calibration curves had an R^2 value greater than 0.995, and both LOD and LOQ were below the range expected to be observed in the beer samples for all standards.

The chromatograms of the MRMs further illustrate some of the differences in chemical content within, shown in Figures 3-2 and 3-S3. Differences were often due to elevated concentrations of the phenolic compounds and their isomers (Figure 3-S3, 3-S4). The IPAs (Figure 3-S3A) and blondes (Figure 3-S3B) appeared to have the least variation within the style, while the sours (Figure 3-S3E) showed the most variation. This figure also highlights the chlorogenic acid, catechin, and benzoic acid isomers, as multiple peaks can be observed for these analytes in almost every beer. Specifically, the stouts (Figure 3-S3C) and sours (Figure 3-S3E) show differing ratios of chlorogenic acid isomers within the style. With the exception of the wheat beers (Figure 3-S3D), all the styles showed a more intense peak for a benzoic acid isomer (RT 3.780 min) rather than the standard (RT 2.592 min). When observing the IAA profile of the sour beers (Figure 3-S3E), it is apparent that they have additional iCH (RT 6.261 min) and iAH (RT 6.626 min and 6.768 min) isomers that were not present in the standards or other beer styles.

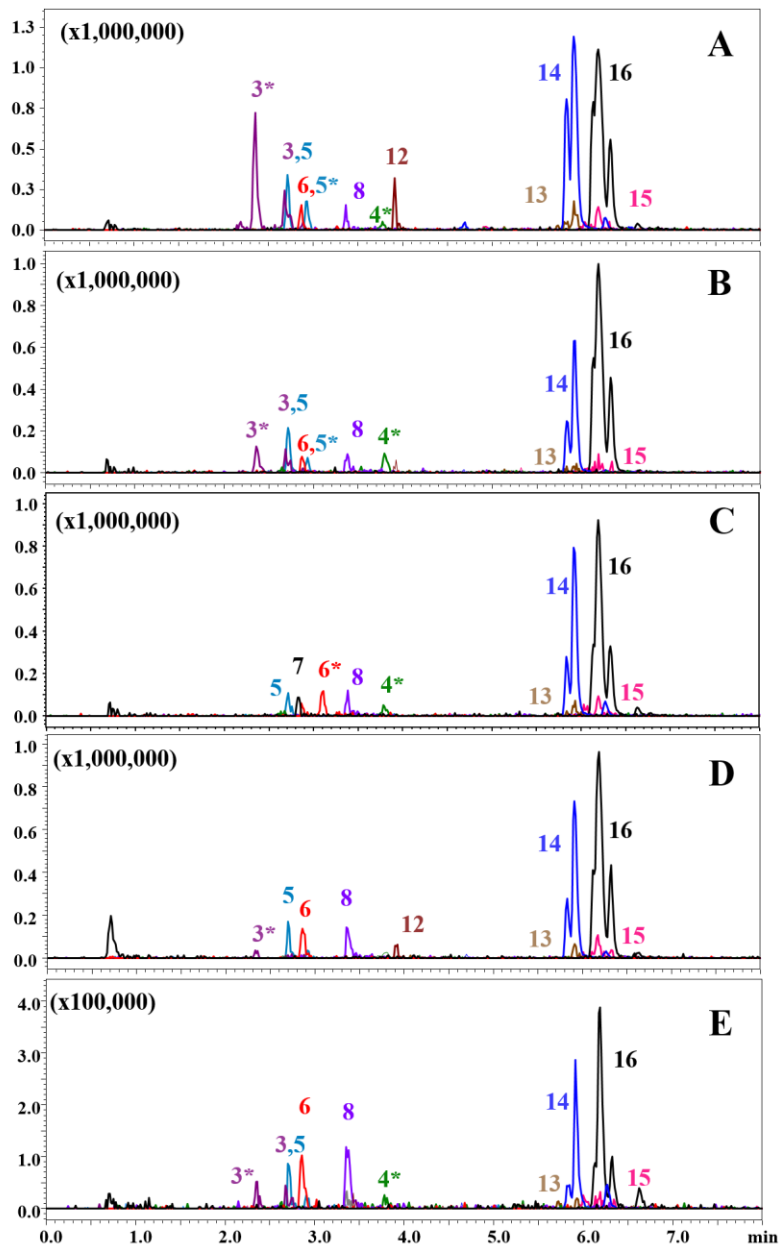

Figure 3-2. Representative LC-QTOF-MS MRM chromatograms for each beer style; [A] IPA beer #4 ; [B] blonde beer #3; [C] stout beer #4 ; [D] wheat beer #1; and [E] sour beer #4; 3. chlorogenic acid [purple] (3* isomer), 4*. Benzoic acid isomer 5. catechin [teal] (5* isomer), 6. caffeic acid [red] (6* isomer), 7. vanillin [black], 8. cinnamic acid [lilac], 12. quercetin [brown], 13. RiCH [light brown]; 14. iCH [blue]; 15. RiAH and RiH [pink]; 16. iAH and iH [black]. *Signals for phenol and reduced IAA MRMs were increased by a factor of 10. The y-axes (intensity) differ between styles.*

3.4.2 *Phenolic Compounds*

Total concentrations were compared to evaluate relative phenolic content between beer styles (Figure 3-3). For the analytes that showed multiple isomers, only the peak with the same retention time as the standard was quantified, although this was not always the most abundant isomer. An example of this can be seen in Figure 3-S5, which shows the total percentages of the chlorogenic acid and catechin isomers present in the beer samples. The retention time of the most abundant catechin isomer matched the standard, (+)-catechin, and accounted for 79% of the total area from the relevant MRM transitions. The most abundant chlorogenic acid isomer (61%) did not match the standard, which accounted for only 37% of the total area.

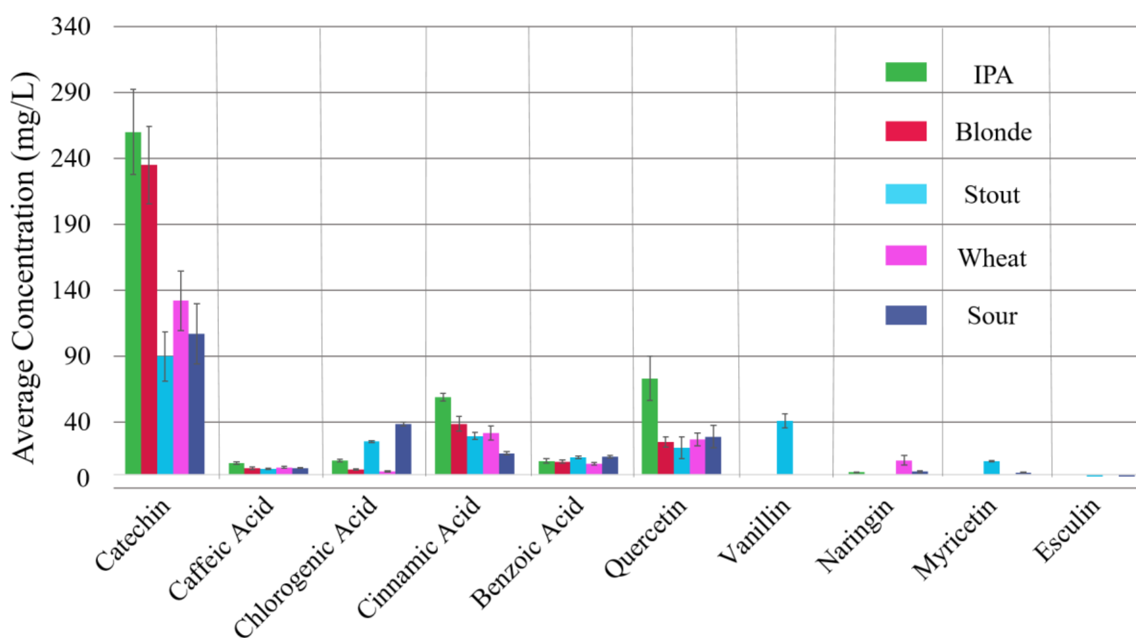

Figure 3-3. Averaged concentrations (mg/L) of targeted phenolic compounds by each beer style; IPA [green] (n = 18), blonde [red] (n = 18), stout [cyan] (n = 21), wheat [pink] (n = 21), sour [blue] (n = 18). *Concentrations in parts per million, error bars represent average standard deviation.*

One of the targeted compounds, 4-hydroxycoumarin, was not found in any of the samples. Esculin was found in two beers, but the concentrations were barely above the LOD. All other phenolic compounds were found in at least one beer style.

Vanillin was only found in stouts, myricetin in stouts and sours only, and naringin in IPA, wheats, and sours only. Catechin, caffeic acid, chlorogenic acid, cinnamic acid, benzoic acid, and quercetin were found in all beer styles. Similar averaged concentration for both caffeic acid and benzoic acid were observed for all beer styles, but alternatively, total concentrations for all other phenolic compounds were different between each beer type.

Catechin concentrations were generally between 3-5 times higher than any other phenolic in all beer styles. The average concentration, shown in Figure 3-3, for catechin in the IPAs (260 mg/L) and blondes (235 mg/L) was nearly double that of the next highest beer style (wheats, 132 mg/L).

In general, IPAs had higher concentrations of cinnamic acid and quercetin than the other beer styles. Averaged concentrations of quercetin (73 mg/L) were at least two times higher in IPAs and naringin (11 mg/L) appeared more prominent in wheat beers than any other beer styles. Chlorogenic acid was higher in stouts and sours. Besides containing higher amounts of catechin, there were no distinguishing phenolic compounds for the blondes.

Average phenolic concentrations were compared between individual beers and are shown in Figure 3-S6. Catechin (Figure 3-S6A) and caffeic acid (Figure 3-S6B) were the only phenolics found in every beer sample. Chlorogenic acid (Figure 3-S6C), cinnamic acid (Figure 3-S6D), benzoic acid (Figure 3-S6E), and quercetin (Figure 3-S6F) were present in most of the

samples. The remaining four phenolic compounds were only detected in about a quarter of the beers that were studied.

When averaged by beer style, it had appeared that naringin was more prominent in wheat beers than the other styles; however, while this compound is in high concentration in three of the wheat samples, it was not detected in the other four (Figure 3-S6G). Similarly, chlorogenic acid appears to be more concentrated in sour beers but was only present in high amounts in two out of the six samples (Figure 3-S6C). Myricetin was only present in three stouts and one sour (Figure 3-S6H), and esculin was only detected in one stout and one sour beer (Figure 3-S6J). Alternatively, vanillin (Figure 3-S6I) was found in significant concentrations in all 7 stout samples.

The chlorogenic acid, catechin, and benzoic acid isomers observed in the beer samples are suspected to have originated from the ingredients used for brewing. Many of the ingredients are biological materials (i.e. barley, yeast, fruits, etc.) that contain complex mixtures of closely related isomers. For example, catechin has four major diastereomers; (+)-catechin, (-)-catechin, (+)-epicatechin, and (-)-epicatechin, as well as various galloyl-derivatives [180], some of which have been previously identified in beer extracts [181]. In addition, several chlorogenic acid (5-CGA) isomers, such as pseudochlorogenic acid (1-CGA), neochlorogenic acid (3-CGA), and cryptochlorogenic acid (4-CGA) have been identified in plant materials that can be used in brewing, including coffee, pears, cherries, apricots, and oranges [182,183]. Out of the six IPAs studied, five were described as having orange or citrusy flavors (Table 3-S1). In multiple studies, chlorogenic acid has been determined to be one of the major phenolic acids in oranges and other citrus fruits [184–186]. Interestingly, the IPA with lowest concentration of chlorogenic acid was the only one not described as exhibiting a citrus taste (IPA #5).

Two of the sour beers showed significant amounts of chlorogenic acid isomers. Figure 3-4 represents sour beers #1 and #6, which were brewed with blackberries and cherries, respectively. The major peak in sour beer #1 (Figure 3-4A) aligns with the chlorogenic acid standard (5-CGA), which is one of the predominant hydroxycinnamic acid derivatives present in blackberries [187]. Blackberries also contain neochlorogenic acid (3-CGA), which is a major hydroxycinnamic acid found in cherries [188]. The retention time of the smaller peak in sour beer #1 matches the major peak in sour beer #6 (Figure 3-4B), and so this is hypothesized to be the neochlorogenic acid (3-CGA) isomer.

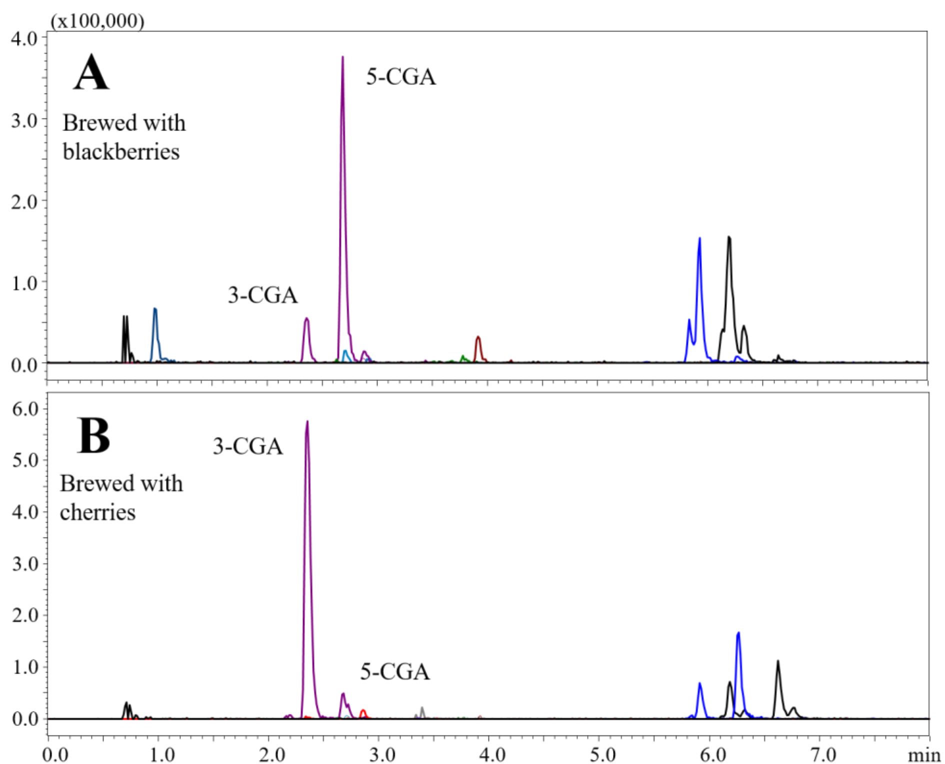

Figure 3-4. LC-QTOF-MS MRM chromatograms for iso-α-acids and chlorogenic acid for representative sour beers 1 (A) and 6 (B); showing relative content of chlorogenic acid at 2.38 min and 2.70 min [purple] to the IAA peaks; two peak apex for iCH [blue] and three peak apex for iAH/iH [black].

Figure 3-S6C shows that chlorogenic acid was also present in high amounts in two of the stout beers. Stout #6 was brewed with raspberries, and Teng et al. found that chlorogenic acid was one of the major polyphenols present in raspberry extract [189]. Stout #2 contained the highest amount of chlorogenic acid (87 mg/L) and catechin (158 mg/L), shown in Figure 3-S6A, for that style. This was a white stout, which are generally brewed with cold-steeped coffee and cacao nibs [190]. Coffee is known to contain a high amount of chlorogenic acid (2 – 5 g/100 g) [191], and (+)-catechin has been identified as a major taste-active polyphenol in roasted cocoa nibs [192]. It is expected that many other compounds present in the ingredients would form derivatives during the brewing process. The presence of isomers, derivatives, and unknown compounds results in complex chemical profiles for different craft beers.

3.4.3 *Iso-α-Acids*

One of the most noticeable differences between styles is the intensity of the IAA. The IPAs consistently had the highest amount of IAA, sour beers had the lowest, and the other three styles had roughly the same (Figure 3-S3). This result was expected for the IPAs, as they are typically brewed with higher amounts of hops (from which the IAA originate), giving them a more prominent bitter flavor than the other styles. Conversely, sour beers focus on a more sour, acidic flavor rather than bitterness. The sourness stems from beer microorganisms, such as lactic acid-producing bacteria, acetic acid-producing bacteria, and *Brettanomyces* yeasts, which are desirable for this beer style. However, the IAA from hops exhibit antimicrobial activity that can prevent souring [193]. Therefore, a smaller dosage of hops is generally used when brewing sour beers, which leads to a lower concentration of IAA.

The application of the developed method on the ICS-I4 standard provided distinct peaks for all three major *trans*- isomers of IAA, as shown in Figure 3-5A. Multiple peaks were observed for each IAA when the method was applied to the beer samples. Since beer is produced from materials containing biologically synthesized components, the *cis*- isomers, as well as additional minor IAA homologues, would be expected to be present in addition to the major *trans*- isomers. Therefore, not all of the individual IAAs could be fully resolved, and Figure 3-5B shows that the current method results in at least five partially resolved peaks for the six expected major isomers of the IAAs. Because these extra peaks could not be distinguished, all the peaks that fell within the appropriate retention time and under the same MRM would be integrated together using either the iCH transition or the iH/iAH transition.

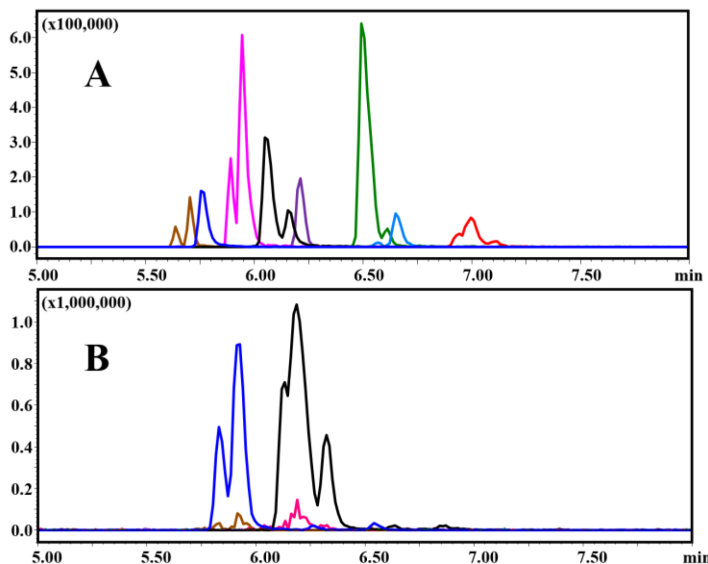

Figure 3-5. LC-QTOF-MS MRM chromatograms for (A) IAA and reduced IAA (RiAA, HiAA, TiAA) standards at 10 mg/L and (B) a representative beer, IPA #1 (I_1): Panel A shows a single peak for iCH [blue] and two peaks for iAH and iH [black]. The beer sample in panel B reveals two peaks for iCH at 5.82 min and 5.95 min [blue], and three peaks for iAH and iH at 6.15 min, 6.20 min, and 6.35 min [black], which was observed in most of the beer samples. The reduced IAA peaks are shown as RiCH [light brown], RiAH and RiH [pink], HiCH [purple], HiAH and HiH [green], TiCH [cyan], and TiAH and TiH [red].

The IAA were present in a significant amount in every analyzed beer sample. One of the three reduced forms (RiAA) were found in most of the beer samples (Figure 3-5B), although at a lower quantity than the IAA. The other two reduced forms of IAA (TiAA and HiAA) were not present in substantial amount in any of the beers.

Qualitative comparison of the IAA peak areas to the phenolic compounds showed that iCH and iH/iAH were by far the most abundant in almost every beer sample. The only exceptions were two sour type beers (Figure 3-4), in which the peak intensity of chlorogenic acid exceeded the intensity of the IAAs.

3.4.4 *Principal Component Analysis*

Samples were evaluated to determine if the style of beer could be classified based on the phenolic and IAA content. The PCA model was able to differentiate between IPAs, stouts, and sours. In Figure 3-6A, principal component (PC) 1 accounts for 29% of the total variance (54.6%) within the first 3 PCs. IPAs and sours were the easiest beer styles to separate. As shown in the HCA in Figure 3-6B, blondes and wheats were not able to be separated, while IPAs had the largest IAA content.

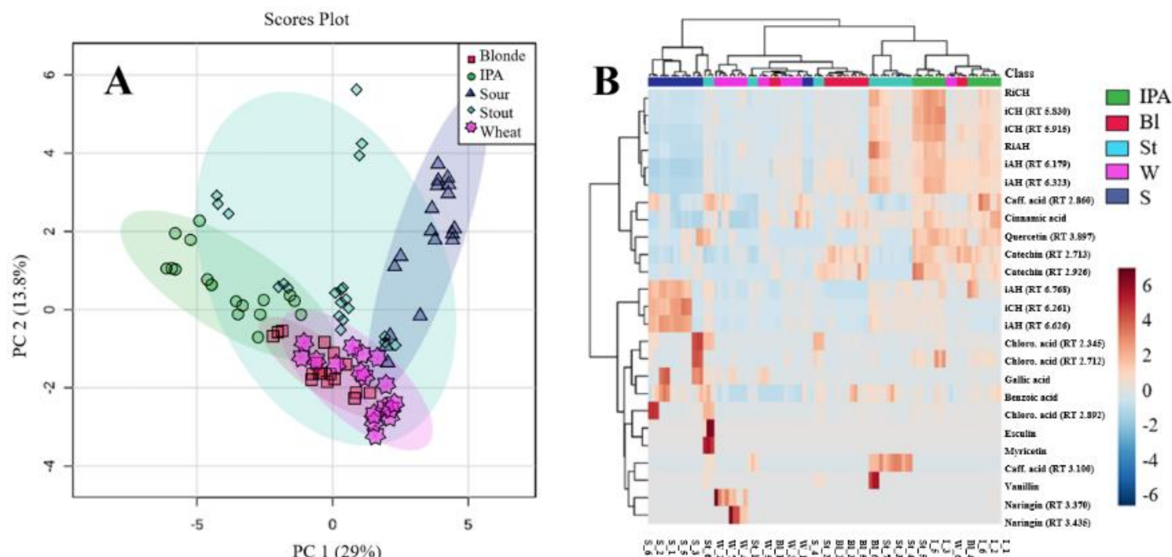

Figure 3-6. **(A)** Principal component analysis of the beer samples evaluated by LC-QTOF-MS. The x-axis represents principal component 1 (PC1) and the y-axis represents principal component 2 (PC2). Blonde (red square), IPA (green circle), sour (dark blue triangle), stout (light blue diamond), wheat (pink asterisk). (**B)** The heat map represents the diversity of the metabolites of the samples.

3.5. Conclusions

This study was the first to profile multiple U.S. beers by simultaneously analyzing IAA and phenolic compounds using LC-QTOF-MS, followed by multivariate analyses, with the goal of finding compounds that can be used to distinguish the variation between styles in order to determine a way to have quality assurance for beer style confirmation. Multivariate analysis techniques were able to separate three of the five beer styles; however, it was not able to fully separate the beer styles.

One of the most interesting results was that only stouts contained vanillin, a flavor component of roasted barley malts, which are often used for brewing dark beers such as porters and stouts [194]. While vanillin seems promising for differentiating stouts from other styles, the

variation in other phenolic compounds studied were insufficient to be individually considered as distinguishing markers. Although it seems that cinnamic acid, quercetin, and catechin may be good markers for IPAs, additional confirmation would be required. Trends were observed between some of the phenolic compounds, namely chlorogenic acid, and the ingredients used for brewing certain beers. The IAAs were qualitatively observed to be most prominent in the IPAs and least prominent in sour beers, which agrees with the ingredients and brewing procedures utilized for these styles.

However, it was determined that targeted analysis alone was insufficient for profiling beer styles due to the overwhelming presence of isomers and unknown compounds, and so further untargeted and multivariate analysis would be required to predict which compounds were most responsible for the variation between styles.

Acknowledgements

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3.6. Supporting Information

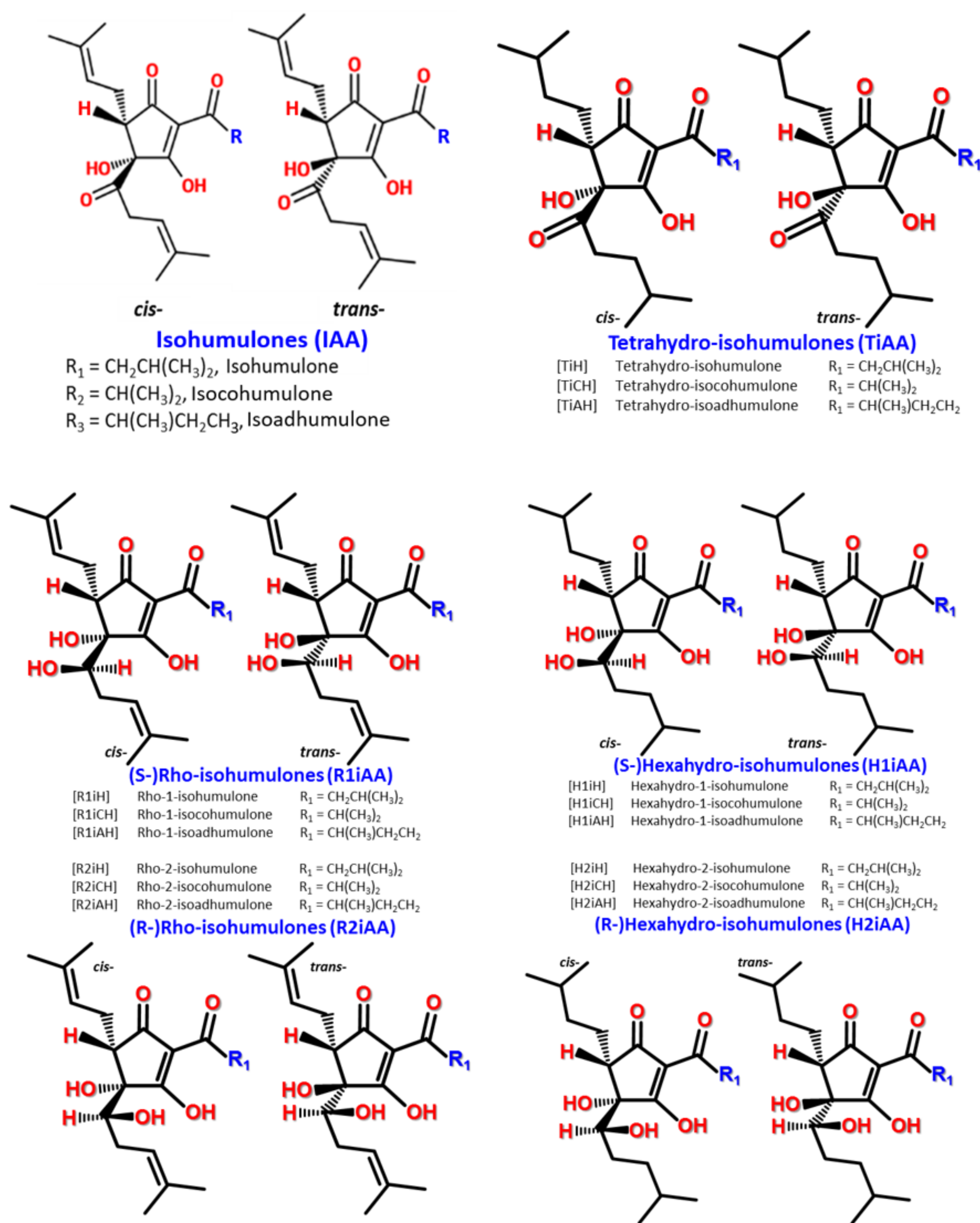

Figure 3-S1. Structures of the iso-α-acids and reduced iso-α-acids.

Table 3-S1. Complete list of beers used for this study.

Brewer	Brewer Location	Label Type	IBU	Sample ID
Community Brewing	Dallas, TX	IPA	50	I_1
Ballast Point Brewing	San Diego, CA	IPA	50	I_2
Nine Band Brewing	Allen, TX	IPA	63	I_3
Cigar City Brewing	Tampa, FL	IPA	65	I_4
Community Brewing	Dallas, TX	IPA	85	*I_5
New Belgium Brewing	Fort Collins, CO	IPA	42	I_6
Deep Ellum Brewing	Dallas, TX	Blonde	23	Bl_1
Real Ale Brewing	Blanco, TX	Blonde	23	*Bl_2
HopFusion Ale Works	Fort Worth, TX	Blonde	17	Bl_3
Legal Draft Beer	Arlington, TX	Blonde	18	Bl_4
Karbach Brewing	Houston, TX	Blonde	20	Bl_5
Wild Acre Brewing	Fort Worth, TX	Blonde	15	Bl_6
Legal Draft Beer	Arlington, TX	Stout	30	St_1
New Holland Brewing	Holland, MI	Stout	35	St_2
HopFusion Ale Works	Fort Worth, TX	Stout	34	St_3
Deep Ellum Brewing	Dallas, TX	Stout	23	St_4
Left Hand Brewing	Longmont, CO	Stout	25	St_5
New Holland Brewing	Holland, MI	Stout	N/A	*St_6
Lakewood Brewing	Garland, TX	Stout	56	*St_7
Boulevard Brewing	Kansas City, MO	Wheat	14	*W_1
Blue Moon Brewing	Golden, CO	Wheat	9	*W_2
Shannon Brewing	Keller, TX	Wheat	15	W_3
Legal Draft Beer	Arlington, TX	Wheat	13	W_4
Blue Moon Brewing	Golden, CO	Wheat	33	*W_5
Bell’s Brewery	Comstock, MI	Wheat	N/A	*W_6
Avery Brewing	Boulder, CO	Wheat	22	W_7
Division Brewing	Arlington, TX	Sour	4	S_1
Community Brewing	Dallas, TX	Sour	5	S_2
DESTIHL Brewery	Normal, IL	Sour	18	S_3
Dogfish Head Brewing	Milton, DE	Sour	10	S_4
DESTIHL Brewery	Normal, IL	Sour	12	S_5
Prairie Artisan Ales	Krebs, OK	Sour	N/A	S_6

All except 8 beers were purchased in 12 fl oz cans. *denotes 12 fl oz amber bottle. N/A denotes IBU not specified on label. Style and flavor descriptions were pulled from brewers’ websites and “Untappd” app.

Table 3-S2. Pertinent information about the IAA standards. Structures and full names can be found in Figure 3-S1.

Standard	Abbrev.	Total IAA content (w/w)	trans-	cis-	Expected number of peaks
iso-α-acid (ICS-I4)	IAA	65.2%	t-iCH, t-iH, t-iAH	N/A	3
rho-iso-α-acid (ICS-R3)	RiAA	65.0%	N/A	c-R1iCH, c-R2iCH, c-R1iH, c-R2iH, c-R1iAH, c-R2iAH	6
hexahydroiso-α-acid (ICS-H2)	HiAA	65.9%	N/A	c-H1iCH, c-H2iCH, c-H1iH, c-H2iH, c-H1iAH, c-H2iAH	6
tetrahydroiso-α-acid (ICS-T3)	TiAA	99.4%	t-TiCH, t-TiH, t-TiAH	c-TiCH, c-TiH, c-TiAH	6

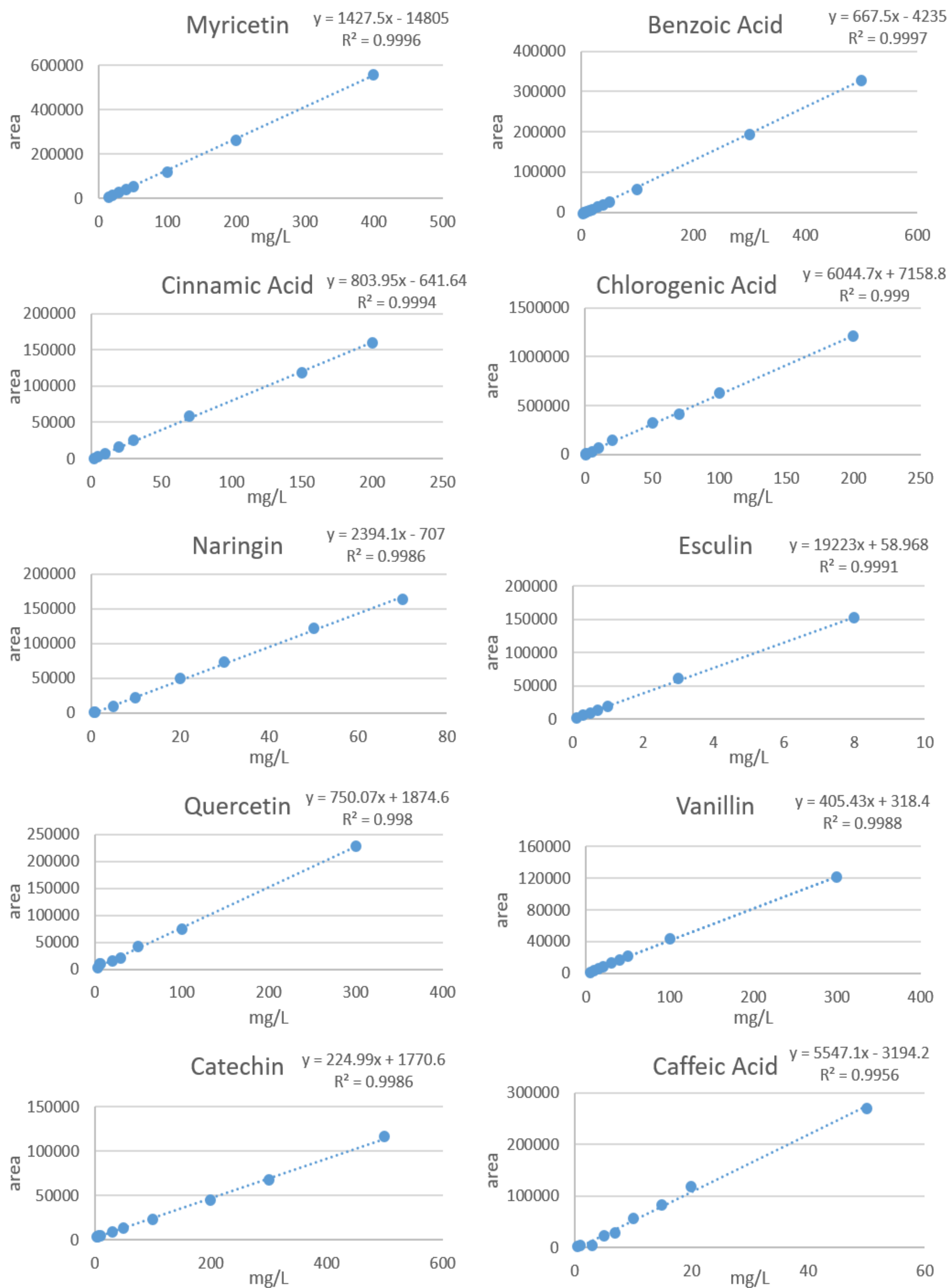

Figure 3-S2. Calibration curves for the phenolic standards. Concentrations are given in mg/L.

Table 3-S3. Calibration curve information, including R^2, quality control (QC) concentrations, limit of detection (LOD), and limit of quantitation (LOQ) for targeted phenolic compounds. The QC concentrations, LODs, and LOQs are given in mg/L.

Compound	**R^2**	**QC concentrations (mg/L)**	**LOD**	**LOQ**
Myricetin	0.999	Low = 8, Medium = 25, High = 300	3	9.9
Benzoic Acid	0.999	Low = 8, Medium = 25, High = 400	3	9.9
Cinnamic Acid	0.999	Low = 3, Medium = 40, High = 180	2	6.6
Chlorogenic Acid	0.999	Low = 3, Medium = 40, High = 180	0.5	1.65
Naringin	0.999	Low = 3, Medium = 15, High = 40	0.7	2.31
Esculin	0.999	Low = 0.85, Medium = 4, High = 7	0.1	0.33
Quercetin	0.998	Low = 2, Medium = 25, High = 200	3	9.9
Vanillin	0.998	Low = 8, Medium = 35, High = 400	5	16.5
Catechin	0.998	Low = 6, Medium = 150, High = 400	3	9.9
Caffeic Acid	0.996	Low = 2, Medium = 8, High = 30	0.5	1.65

Table 3-S4. Concentration levels (mg/L) applied to each calibration curve for the phenolic standards. A = Myricetin, B = Benzoic Acid, C = Cinnamic Acid, D = Chlorogenic Acid, E = Naringin, F = Esculin, G = Quercetin, H = Vanillin, I = Catechin, J = Caffeic Acid.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Point 1	3	3	2	0.5	0.7	0.1	3	5	3	0.5
Point 2	15	5	5	0.7	1	0.3	5	10	5	1
Point 3	20	7	10	1	5	0.5	7	15	7	3
Point 4	30	10	20	5	10	0.7	20	20	10	5
Point 5	40	15	30	10	20	1	30	30	30	7
Point 6	50	20	70	20	30	3	50	40	50	10
Point 7	100	30	150	50	50	8	100	50	100	15
Point 8	200	40	200	70	70		300	100	200	20
Point 9	400	50		100				300	300	50
Point 10		100		200				500	500	
Point 11		300								
Point 12		500								

Table 3-S5. Targeted Phenolic Compounds multiple reaction monitoring (MRM) transitions, masses [Da], and collision cell energy (CE) [V] used in LC-QTOF-MS targeted analysis. Product ion scan mode was used over a range of collision energies to obtain the two most abundant product ions and the optimal CE for each analyte.

Analyte	Standard Retention Time (min)	Parent Mass	Product Ions	CE [V]
Catechin	2.647	289.0708	123.0441, 203.0702	22.0
Caffeic Acid	2.801	179.0344	134.0383, 135.0465	21.0
Chlorogenic Acid	2.598	353.0928	161.0252, 191.0559	22.0
Cinnamic Acid	3.310	193.0497	134.0382, 178.0288	18.0
Benzoic Acid	2.592	137.0239	65.0387, 93.0345	25.0
Quercetin	3.882	301.0336	151.0014, 178.9998	21.0
Naringin	3.367	579.1631	151.0008, 271.0563	28.0
Myricetin	3.564	317.0288	137.0248, 151.0043	19.0
Vanillin	3.187	151.0395	108.0223, 136.0175	19.0
Hydroxycoumarin	3.655	161.0245	117.0341	21.0
Gallic Acid	1.069	169.0138	79.0180, 125.0222	18.0
Esculin	2.457	339.0713	133.0299, 177.0216	22.0

Table 3-S6. Targeted Isomerized and reduced isomerized alpha acids multiple reaction monitoring (MRM) transitions, masses [Da], and collision cell energy (CE) [V] used in LC-QTOF-MS targeted analysis. Product ion scan mode was used over a range of collision energies to obtain the two most abundant product ions and the optimal CE for each analyte. Only the retention times for the isomers with standards are given. The IAA standard contained only the trans isomers, while the RiAA and HiAA standards contained only cis isomers.

Analyte	Group	ID	Standard Retention Time (min)	Parent Mass	Product Ions	CE [V]
trans-isohumulone	IAA	t-iH	6.089	361.2011	96.0758, 265.1474	15.0
cis-isohumulone		c-iH		361.2011	96.0758, 265.1474	15.0
trans-isocohumulone		t-iCH	5.675	347.1852	182.0597, 251.1311	21.0
cis-isocohumulone		c-iCH		347.1852	182.0597, 251.1311	21.0
trans-isoadhumulone		t-iAH	5.968	361.2011	96.0758, 265.1474	15.0
cis-isoadhumulone		c-iAH		361.2011	96.0758, 265.1474	15.0
trans-rho-isohumulone	RiAA	t-RiH		363.2179	196.0766, 247.1367	22.0
cis-rho-isohumulone		c-RiH	6.113	363.2179	196.0766, 247.1367	22.0
trans-rho-isocohumulone		t-RiCH		349.2015	182.0598, 233.1202	21.0
cis-rho-isocohumulone		c-RiCH	5.755, 5.821	349.2015	182.0598, 233.1202	21.0
trans-rho-isoadhumulone		t-RiAH		363.2179	196.0766, 247.1367	22.0
cis-rho-isoadhumulone		c-RiAH	6.020	363.2179	196.0766, 247.1367	22.0
trans-tetra-isohumulone	TiAA	t-TiH	7.185	365.2324	249.1523, 267.0000	25.0
cis-tetra-isohumulone		c-TiH	7.185	365.2324	249.1523, 267.0000	25.0
trans-tetra-isocohumulone		t-TiCH	6.450	351.2165	239.1316, 253.0000	22.0
cis-tetra-isocohumulone		c-TiCH	6.542	351.2165	239.1316, 253.0000	22.0
trans-tetra-isoadhumulone		t-TiAH	7.000	365.2324	249.1523, 267.0000	25.0
cis-tetra-isoadhumulone		c-TiAH	7.050	365.2324	249.1523, 267.0000	25.0
trans-hexa-isohumulone	HiAA	t-HiH		367.2483	223.1729, 249.1526	25.0
cis-hexa-isohumulone		c-HiH	6.700	367.2483	223.1729, 249.1526	25.0
trans-hexa-isocohumulone		t-HiCH		353.2324	235.1333, 253.0000	22.0
cis-hexa-isocohumulone		c-HiCH	6.321	353.2324	235.1333, 253.0000	22.0
trans-hexa-isoadhumulone		t-HiAH		367.2483	223.1729, 249.1526	25.0
cis-hexa-isoadhumulone		c-HiAH	6.500, 6.600	367.2483	223.1729, 249.1526	25.0

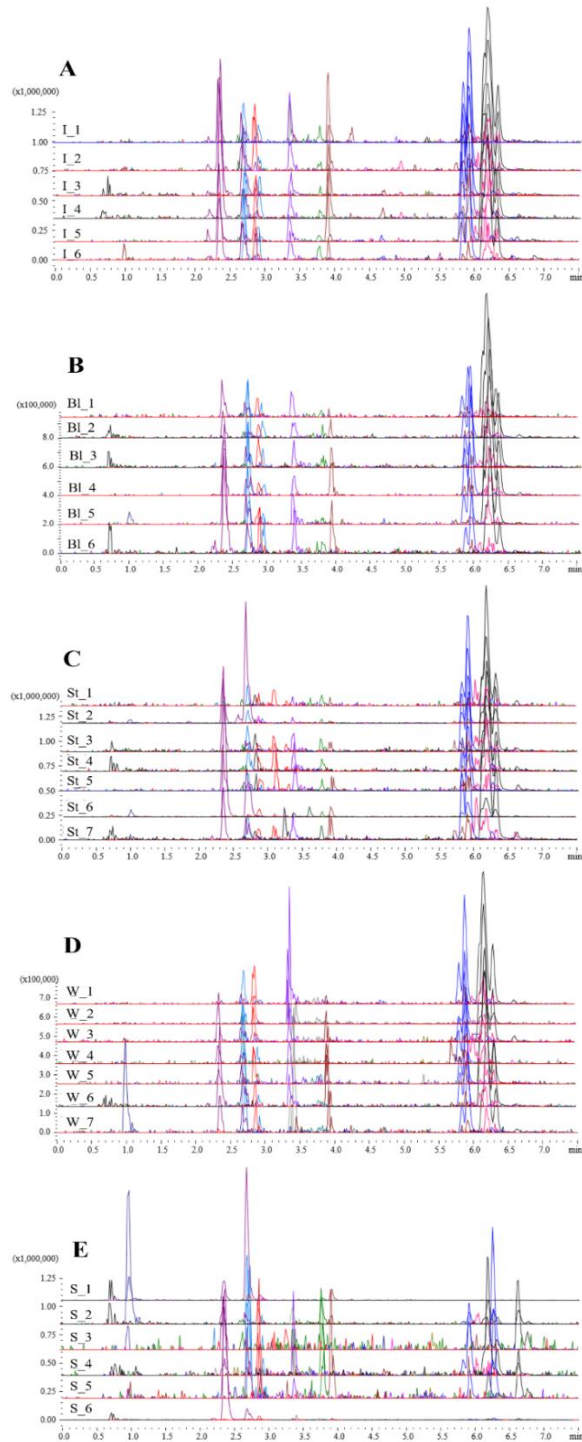

Figure 3-S3. Stacked MRM chromatograms for each beer style. [A] IPAs; [B] Blondes; [C] Stouts; [D] Wheats; [E] Sours. Gallic acid [dark blue], Chlorogenic acid [purple], Catechin [teal], Caffeic acid [red], Vanillin [black], Cinnamic acid [lilac], Naringin [grey], Benzoic acid [green], Quercetin [brown], RiCH [light brown], iCH [blue], RiAH and RiH [pink], iAH and iH [black].

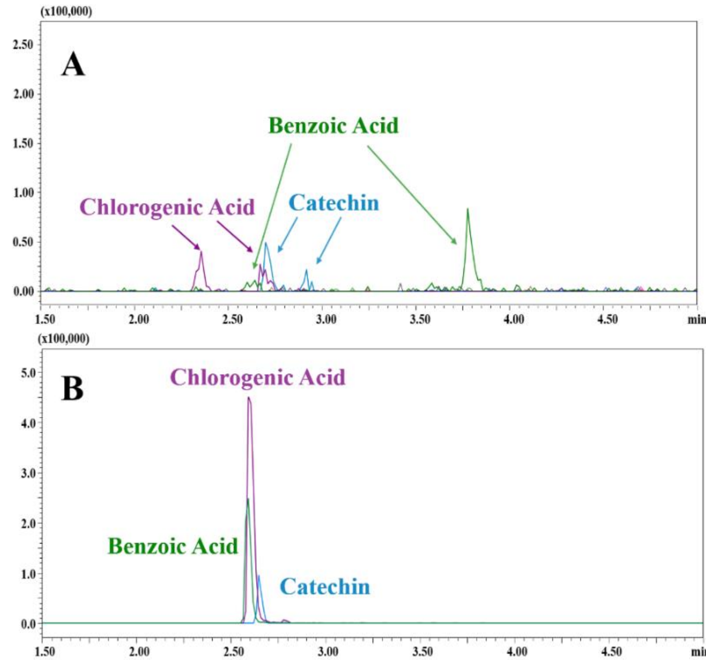

Figure 3-S4. Representative LC-QTOF-MS chromatograms for (A) sour beer #2 (S_2) and (B) phenolic standards. The targeted phenols in beer S_2 show at least two peaks for each MRM; chlorogenic acid at 2.35 min & 2.64 min [purple], catechin at 2.65 min & 2.90 min [blue], and benzoic acid at 2.59 min & 3.78 min [green].

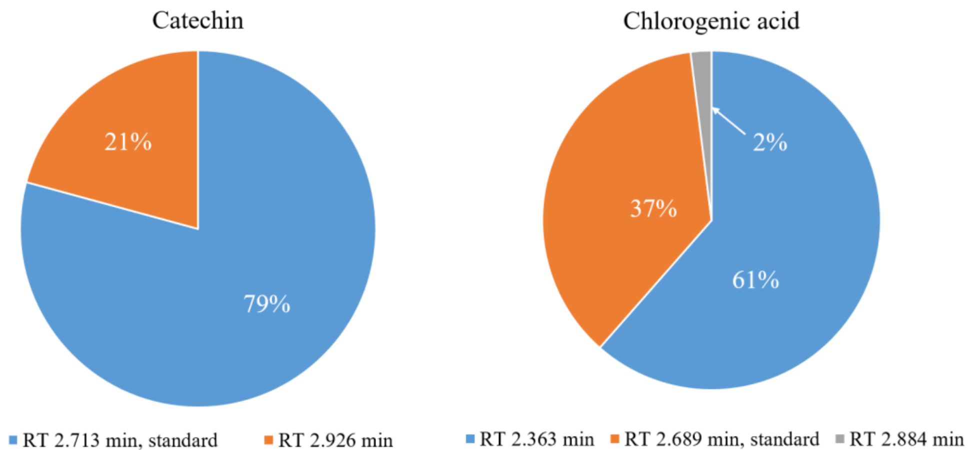

Figure 3-S5. Pie charts showing the total percentage of catechin and chlorogenic acid isomers present in the beer samples. The catechin isomer at 2.713 min (blue) and the chlorogenic acid isomer at 2.689 min (orange) match their standards. Only these isomers were quantified and used for the bar graphs in Figure 3-3 and Figure 3-S6.

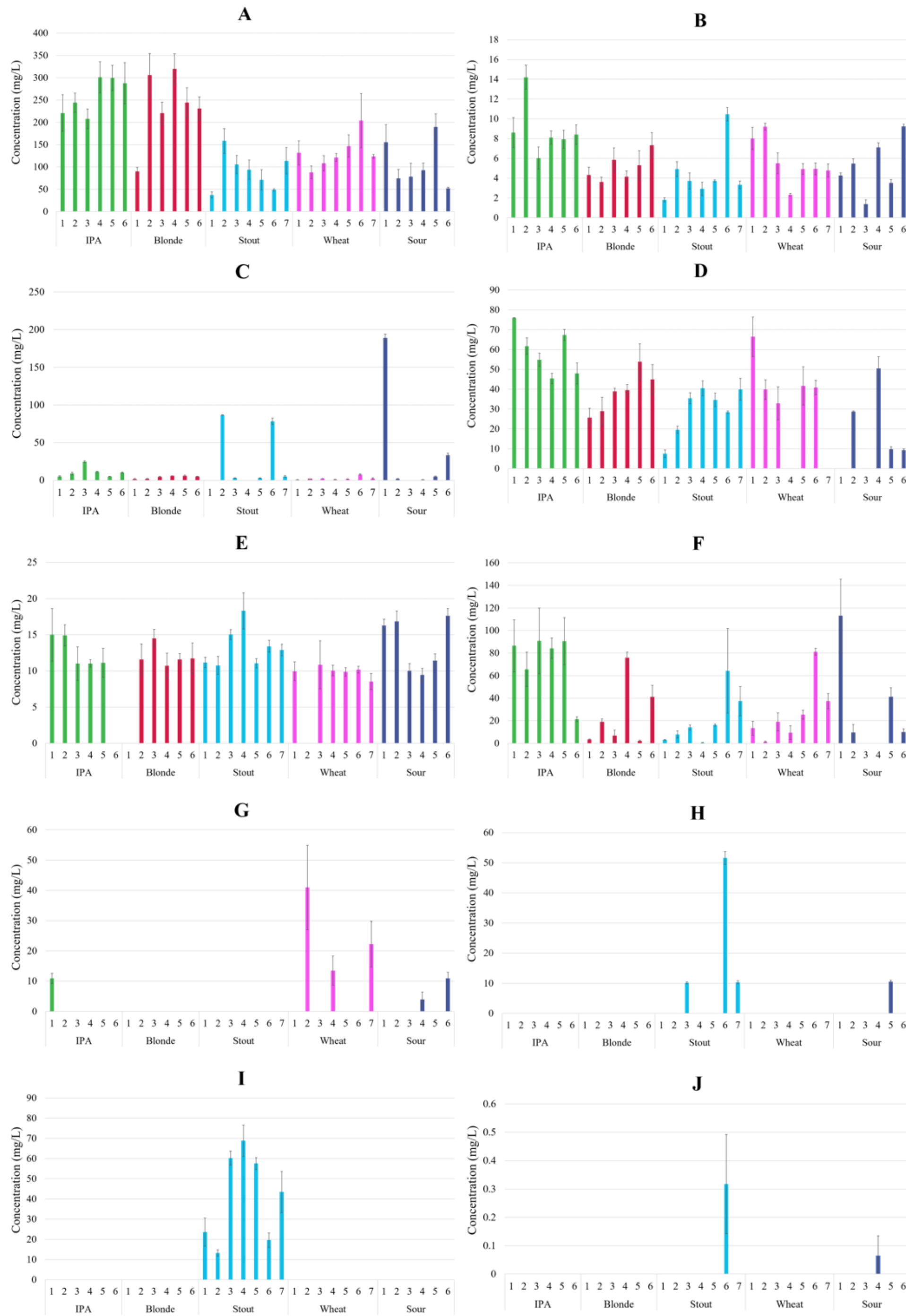

Figure 3-S6. Averaged concentration (mg/L) for individual beers of [A] catechin, [B] caffeic acid, [C] chlorogenic acid, [D] cinnamic acid, [E] benzoic acid, [F] quercetin, [G] naringin, [H] myricetin, [I] vanillin, and [J] esculin. Individual beers grouped by style; IPAs [green], blonde [red], stouts [cyan], wheats [pink], and sours [blue]. *Average concentrations in parts per million. Error bars represent average standard deviation for triplicate measurements.*

CHAPTER FOUR

PROFILING OF CONTEMPORARY BEER STYLES USING LIQUID CHROMATOGRAPHY-QUADRUPOLE-TIME-OF-FLIGHT-MASS SPECTROMETRY, MULTIVARIATE ANALYSIS, AND MACHINE LEARNING TECHNIQUES

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Profiling of Contemporary Beer Styles Using Liquid Chromatography-Quadrupole-Time-of-Flight-Mass Spectrometry, Multivariate Analysis, and Machine Learning Techniques

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4.1. Abstract

Although all beer is brewed using the same four classes of ingredients, contemporary beer styles show wide variation in flavor and color, suggesting differences in their chemical profiles. A selection of 32 beers covering five styles (India pale ale, blonde, stout, wheat, and sour) were investigated to determine chemical features, which discriminate between popular beer styles. The beers were analyzed in an untargeted fashion using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). The separation and detection method were tuned to include compounds from important beer components, namely iso-α-acids and phenolic compounds. Due to the sheer number of unknown compounds in beer, multivariate analysis and machine learning techniques were used to tentatively identify some of the compounds most influential in distinguishing beer styles. It was determined that while many phenols and iso-α-acids were present in the beers, they were not the compounds most responsible for the variations between styles. However, it was possible to discriminate each beer style using multivariate analysis. Principal component analysis (PCA) was able to separate and cluster the individual beer samples by style. Partial least squares – discriminant analysis (PLS-DA) provided a list of key features, which allowed for formula predictions of the most influential metabolite from each style. Machine learning models accurately classified patterns in the five beer styles, indicating that they can be precisely distinguished by their nonvolatile chemical profile.

Key words: Craft beer, high resolution mass spectrometry, principal component analysis, partial least squares – discriminant analysis, untargeted analysis

4.2. Introduction

Despite being one of the oldest and most popular alcoholic beverages across the globe, the identification and classification of beer components still proves to be a challenge due to its high complexity [1,11]. While large industrial brewing companies have focused on standardizing their recipes for mass-production, the contemporary small-batch (so called, "craft") brewing industry focuses instead on experimental ingredients and procedures to produce even more unique and flavorful beers, which augments the already complex profile [195]. Some beer styles are easily distinguished through physical observations, but the innovation of modern beers by brewers has caused the lines between other styles to blur, making it more difficult to determine into which style a beer fits [196]. For example, American IPA and American Pale Ale, stouts and porters, German Pilsner and Munich Helles, are sometimes so similar in color and flavor that they are difficult to distinguish through purely physical observation. By finding commonalities of beers within the same style on a chemical level, one could more easily determine how to categorize new brews.

Over 75 beer styles exist that can exhibit vast differences in flavor, aroma, and color [2] of this fermented beverage. Despite the wide variations between styles, all beers are brewed with malted grains (typically barley), hops, water, and yeast, thus using the same general procedures and classes of ingredients. Some of the more common styles include India pale ales (IPA), blondes, stouts, wheats, and sours. IPAs are brewed with higher amounts of hops, which contribute a more intense bitter flavor. Blondes are usually light, easy drinking beers with low hop bitterness. Stouts are brewed using roasted grains which results in an incredibly dark color, and usually contain notes of coffee, chocolate, or vanilla flavors. As the name suggests, wheat beers use

wheat instead of barley as the primary brewing grain. Sour beers focus of a more acidic flavor by utilizing lactic acid-producing bacteria, acetic acid-producing bacteria, or *Brettanomyces* yeasts [2]. These examples show that slight changes in ingredients and procedures can lead to tremendous variations in flavors and fragrances.

One of the ingredients brewers commonly experiment with, both in strain and quantity, are hops, which contribute heavily to the flavor and aroma [15]. Hops introduce a class of compounds known as α-acids. During the wort boil, the α-acids become thermally isomerized to iso-α-acids (IAA), which are largely responsible for the characteristic bitterness of beer, as well as inhibit the growth of Gram-positive bacteria [15]. Another class of chemicals that can affect the flavor of beer are phenolic compounds, which includes polyphenols, phenolic acids, and flavanols, among others [121,197]. Phenolic compounds originate from brewing plant materials, primarily barley and hops [197], but also from many types of fruit that may be used such as cherries, apricots, and oranges [198]. The composition of phenols in beer can vary greatly depending on the ingredients used. In addition to flavor, phenolic compounds enhance beer stability and exhibit antioxidant activity [21]. Since both the IAA and phenols are major contributors to beer flavor, they have been studied extensively and are known to vary considerably in abundance and type between the beer styles [12,131,132,176–179].

Due to the highly complex nature of beers, targeted analysis with IAA and phenolic compounds alone are not sufficient to be able to differentiate and categorize styles of beer [12]. There are potentially hundreds of other compounds contained in beer that cause differences in styles. Using untargeted analysis and associated techniques would provide the ability to identify some of the key components, which are most influential in differentiating the styles, using

multivariate analysis. Principal component analysis (PCA) can be used as a visualization tool to discriminate between beer styles, followed by partial least squares – discriminant analysis (PLS-DA) to identify key features.

Moreover, machine learning techniques have gained great success to recognize complex patterns in high dimensional variable space. Different machine learning techniques have been applied to solve challenging data analysis problems in chemistry [199,200]. Specifically, supervised machine learning techniques, such as support vector machine (SVM), neural networks (NN), and random forests (RF), can be used to learn complex data patterns and construct pattern representations to separate and discriminate different chemical data types. In fact, due to its ready availability and complexity, beer has often been used as development and proving ground for various multivariate and chemometric data treatment strategies [201–204].

The objective of this study was to profile different beer styles using liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) in combination with statistical techniques. These advanced data science techniques could potentially give a more complete picture of the complexity of beer in a way that sensory analysis alone cannot. The combined application of PCA, PLS-DA, and various machine learning methods allowed beer styles to be precisely distinguished from one another by identifying the specific compounds responsible for their diversity. This approach could prove valuable for the rapid categorization of new brews, and accentuate the defining characteristics between similar styles, improving our understanding of how ingredients affect the flavor, color, and other properties of this popular beverage. Focusing on providing enhanced ability to distinguish beer styles based solely on their chemical composition can ultimately lead to the development of quality assurance of styles of beer.

4.3. Materials and Methods

4.3.1. *Materials*

A single bottle or can of thirty-two U.S. commercial craft beers were purchased from local stores. Five different styles (IPA [I], blonde [Bl], stout [St], wheat [W], and sour [S]) from 22 breweries were represented (Table 4-S1). The beers were freshly opened, and 30 mL were transferred and immediately degassed for 30 minutes by sonication. Degassed samples were then diluted 50% with water prior to injection. Excess beer was stored at 5 ^{o}C for no more than two days.

4.3.2. *Chemicals and reagents*

All reagents were of LCMS grade. Water (H_2O), methanol (MeOH), and acetonitrile (ACN) were obtained from Honeywell (Muskegon, MI, USA). Formic acid (98-100%, LCMS grade) was purchased from EMD Millipore (Billerica, MA, USA).

Reference standards including vanillin (99%), 4-hydroxybenzoic acid (99%) (referred to as benzoic acid in the paper), caffeic acid (98%), quercetin (95%), naringin (95%), 4-hydroxycoumarin (98%), myricetin (98%), 4-hydroxy-3-methoxycinnamic acid (99%) (referred to as cinnamic acid in the paper), (+)-catechin (99%), esculin (European Pharmacopoeia reference standard), and chlorogenic acid (European Pharmacopoeia reference standard) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenolic standards were chosen based on previous studies involving phenolic compounds in beer [178].

Four variations of the American Society of Brewing Chemists (ASBC) international calibration standards (ICS) for HPLC analysis of isomerized and reduced isomerized α-acids were purchased from Labor Veritas (St. Paul, MN, USA) and are listed in Table 4-1. The iso-α-acid (IAA), rho-iso-α-acid (RiAA), and hexahydro- iso-α-acid (HiAA) calibration standards were obtained as a purified preparation of the dicyclohexylamine (DCHA) salts. The International Subcommittee for Isomerized Hop α-Acids Standards determined the total percentage of iso-α-acids present in the standards.

Table 4-1. Pertinent information about the iso-α-acid standards.

Standard	Abbrev.	Total IAA content (w/w)	*trans-*	*cis-*
iso-α-acid (ICS-I4)	IAA	65.2%	*t*-iCH, *t*-iH, *t*-iAH	N/A
rho-iso-α-acid (ICS-R3)	RiAA	65.0%	N/A	*c*-R1iCH, *c*-R2iCH, *c*-R1iH, *c*-R2iH, *c*-R1iAH, *c*-R2iAH
hexahydroiso-α-acid (ICS-H2)	HiAA	65.9%	N/A	*c*-H1iCH, *c*-H2iCH, *c*-H1iH, *c*-H2iH, *c*-H1iAH, *c*-H2iAH
tetrahydroiso-α-acid (ICS-T3)	TiAA	99.4%	*t*-TiCH, *t*-TiH, *t*-TiAH	*c*-TiCH, *c*-TiH, *c*-TiAH

4.3.3. *Preparation of Standard Solutions*

Stock solutions of 1 mg mL^{-1} were prepared for the phenolic and iso-α-acid standards. All stock solutions were prepared in acetonitrile, except for HiAA and RiAA, which were insoluble in ACN and thus, were prepared in methanol. Solutions were stored at 5 ^{o}C. Amber vials were used in order to decrease light exposure.

4.3.4. *Preparation of Quality Controls and Samples*

In an attempt to limit the influence of instrument variation on sequentially run sample data, special attention was paid to the order of samples analyzed, as well as the inclusion of pooled samples referred to as QCs [205]. The experimental design, found in Table 4-S2, included 10 quality controls (QC). Five of the QCs were based on beer style, and were prepared by mixing equal parts of each beer from a given style (i.e. IPA-QC, Blonde-QC, Stout-QC, Wheat-QC, and Sour-QC). An "All-QC" was prepared by mixing equal parts of all the beer samples. Lastly, 4 QCs were prepared based on groups. Beers were clustered (one beer from each style) into seven quality control (QC) groups [A – G] to ensure that each beer style was not analyzed sequentially, and to allow the systematic creation of quality controls to assist in the visualization of variance within the data set. The group QCs were prepared by mixing equal parts of each beer from the group. All QCs were prepared by diluting the applicable beer mixture by 50% with water. The pooled samples were also used to provide quality assurance that the variation detected was not based on instrumentation, and as a representation of the key features for a given style.

4.3.5. *Instrumentation*

Analyses were carried out on a Shimadzu Nexera X2 ultra high-performance chromatograph equipped with two solvent delivery pumps [LC-30AD], online degassing unit [DGU-20A5R], autosampler [SIL-30AC], column oven [CTO-20AC], system controller [CBM-20A], and quadrupole time-of-flight (QTOF) mass spectrometer [LCMS-9030] (Shimadzu Scientific Instruments, Inc., Columbia, MD). Separation was achieved using a Restek Raptor C18 column (100 mm x 2.1 mm x 2.7 µm) (Restek Corporation, Bellefonte, PA). Injection volume was 1 µL.

Mobile phases consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Gradient elution was performed at a flow rate of 0.4 mL min^{-1}, as follows: 0-1 min, 5% B; from 1-5 min, 5 – 75% B; from 5-9 min, 75 – 95% B; and from 9.01-15 min, a step gradient back to 5% B for re-equilibration.

Electrospray ionization (ESI) was performed in the negative ionization mode. The MS data was collected under the following ESI conditions: nitrogen nebulizing gas and drying gas flows were 2 L min^{-1} and 10 L min^{-1}, respectively; the desolvation line temperature was 250℃ and the heat block temperature was 400℃; the interface (spray) voltage was -3.5 kV. MRM transitions were optimized for each of the analytical standards, and these settings are detailed in Tables 4-S3 and 4-S4. Samples were also analyzed using data independent acquisition (DIA) in negative ionization mode for untargeted analysis. The untargeted method contained a series of events with the precursors in 15 m/z increments ranging from 100-1000, with quadrupole 1 (Q1) set with a resolution of 16 to allow for overlap between each precursor. Finally, a collision energy of 25 eV with 17 eV CE spread was used during analysis. The time-of-flight detector was set to scan through various m/z ranges shown in Table 4-S5.

4.3.6. *Data Processing, Statistical Analysis, and Visualization*

LabSolutions was used to acquire the data which were then exported as mzML files and imported into MS Dial (v. 4.00, Yokohama City, Japan). Data processing was performed using MS Dial. The following parameter were used: minimum peak height of 100 counts, mass width of 0.1 Da, mass tolerance of 0.02 Da. A retention time tolerance of 0.1 min and a retention time range of 0-9 min were used as well. The processed data were normalized in MS Dial using the total ion

chromatogram (TIC). Results were further exported as a .txt file from MS Dial for further statistical analysis.

Microsoft Excel was used to clean the exported MS dial results before statistical analysis. Features with a less than a 30% difference from the blank averages were removed from the QCs and samples. Additionally, features with a greater than 30% RSD for each group were removed [205].

After initial data processing and cleaning, data was imported into Metabolanalyst and the areas were normalized using auto-scaled (mean-centered and divided by the standard deviation of each variable). No further data transformation was performed.

MetaboAnalyst 5.0 online (Quebec, Ca) was used to perform statistical analysis. One-way analysis of variance (ANOVA, *p-value* < 0.05), Random Forest and PLS-DA were used to determine significant features. MS Finder (v.3.20, Yokohama City, Japan) was used to predict the formula of the most influential metabolites.

MS Finder was used to predict chemical ontologies and formulas. An isotopic tolerance of 20% was used in addition to checking the element ratio and the probability of the elements. Elements included in the search were C, H, O, N, P, and S. Database hits for food and natural products were preferentially evaluated [206]. Each experimental MS/MS spectrum was compared to theoretical fragments calculated on known compounds retrieved from structure databases [207].

4.3.7 *Machine Learning*

The LC-MS data contains high resolution of information including retention time, ion mass spectral data (m/z), and peak areas. To standardize the data structure to train pattern classification models, the LC-MS data was processed and transformed as a 2-dimentional data using a bucketing method similar to the preprocessing step of the PCA analysis. Specifically, for data of each beer, the time domain is from 0 min to 9 min and the m/z domain is from 100 to 1,000. The bucket resolutions for both time domain and m/z domain are 100. For each bucket, the peak areas covered by the bucket was aggregated as the feature of the bucket. In total, there are 100 x 100 = 10,000 features extracted to represent each beer data sample. Then, two popular supervised machine learning models Naive Bayes [208] and random forests [209] were employed to learn chromatography feature patterns and discriminate different types of beers. Given the limited number of beer samples, it imposed challenges for the machine learning models to capture discriminative patterns in a high feature dimension of 10,000. Thus, we also performed PCA to transform the raw features into a low-dimensional PCA component space for the pattern classification study.

4.4. Results and Discussion

4.4.1. *Targeted Analysis*

A targeted analysis of IAA and phenolic compounds was performed for each of the beer samples. A representative standard chromatogram is shown in Figure 4-S1. Comparison of the MRM traces from the beer samples revealed that a few of the phenolic compounds, such as

chlorogenic acid, appeared as multiple peaks in the beer samples, when only one peak was observed in the standard (Figure 4-S1 and 4-S2). Many of the ingredients used in brewing are biological materials (i.e. yeast, barley, fruits, etc.), which contain complex mixtures of closely related isomers of certain compounds.

Chlorogenic acid (5-CGA) is among these, as multiple isomers such as pseudochlorogenic acid (1-CGA), neochlorogenic acid (3-CGA), and cryptochlorogenic acid (4-CGA) have been identified in various plant materials [182,183]. Therefore, chlorogenic acid and its isomers can be tied back to some brewing ingredients. Figure 4-S2A represents sour beer #1 (S_1), which was brewed with blackberry puree to add flavor. The predominant hydroxycinnamic acid derivatives found in blackberries are chlorogenic acid (5-CGA) and neochlorogenic acid (3-CGA) [187]. In the chromatogram, the major peak aligns with the chlorogenic acid standard (5-CGA), while the smaller peak matches the major peak in Figure 4-S2B, which was hypothesized to be 3-CGA (neochlorogenic acid). Figure 4-S2B represents sour beer #6 (S_6), which is aged on cherries [210]. Sweet cherries are reported to contain neochlorogenic acid (3-CGA) as one of the major hydroxycinnamic acids [188]. The major chlorogenic acid peak in the chromatogram is not 5-CGA (the same chlorogenic acid as the standard), so it is likely that it is 3-CGA, the neochlorogenic acid isomer [12].

Principal component analysis (PCA) and hierarchical cluster analysis (HCA) were used to evaluate the potential of using targeted metabolites to distinguish between the beer styles. As expected, Figure 4-1B shows there is an increased level of IAA in the IPAs. Diverse levels of IAA can be seen in the blondes, stouts, and wheats, while negligible amounts were detected in the sours. Elevated levels of catechin and its isomer were seen in B_4 and W_6, which caused it to

cluster near the IPAs on the heatmap. However, despite the diversity observed in the targeted analysis, it can be seen in Figure 4-1 that the beers could not be differentiated using targeted analysis.

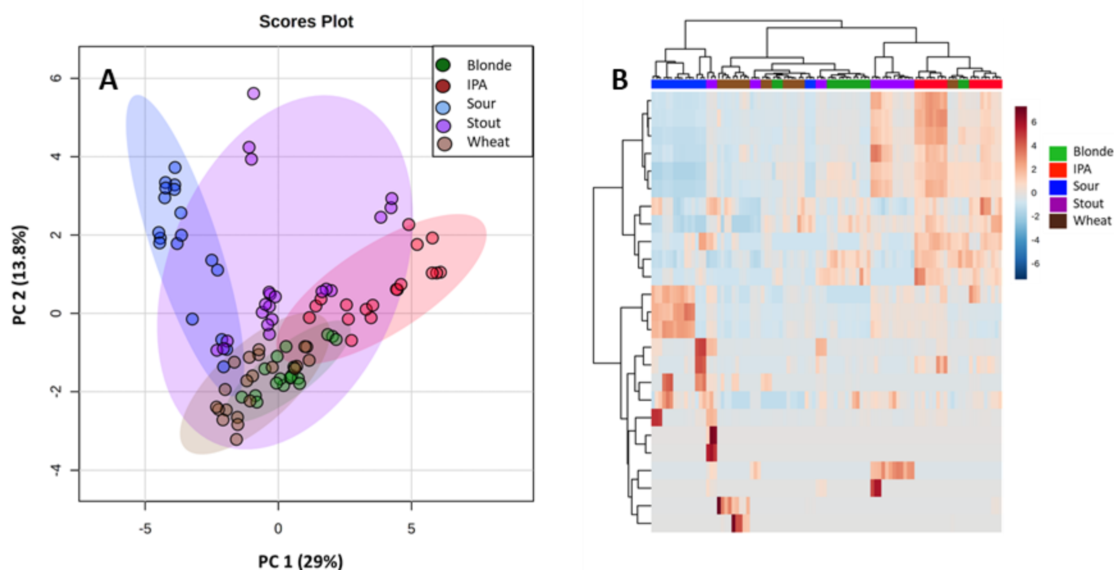

Figure 4-1. **A** Shows a representative PCA and **B** HCA for the targeted compounds detected using MRM in 32 commercially available beer samples [12].

4.4.2. *Statistical Analysis*

Untargeted metabolomics enable the analysis of a wide range of metabolites, and therefore is an ideal tool to highlight key metabolites to differentiate between beer styles. The mass spectral data collected for beer samples was analyzed, and multiple models were assessed to evaluate the influence of instrumental variance.

4.4.2.1. *Samples and Quality Controls*

There were no correlations seen between blanks and quality controls or samples (Figure 4-S3). A PCA model was used to evaluate the variance before and after removing features with less than a 30% difference from the blanks. Prior to data cleaning, PC 1, 2, and 3 explained 26.7% of the variance. However, after data cleaning PC1 increased from 14.3% to 17% variance. Additionally, the samples are clearly distributed across PC2, with some samples having positive and some having negative correlations to PC2. This suggests that there is variance within the samples that is not observed within the blanks.

Two more unsupervised PCA models were created for the analysis of the beer samples. In Figure 4-2A, PC1, PC2, and PC3 represent a total variance of 23%, with each component contributing 11.2%, 7.1%, and 5%, respectively. The points for QC All [yellow], which is a mixture of all the beer samples, are clustered near the zero points for PC1, PC2, and PC3, as would be expected of a composite sample. Additionally, five distinct groupings of the samples and QCs can be seen when marked according to beer style: IPAs [red]; blondes [green]; stouts [purple]; wheats [brown]; and sours [blue]. The highly diverse nature of sour samples is already visible in the initial models that include the QCs and samples (Figure 4-S3 and Figure 4-2). The QCs for 4 beer styles (IPA_QC, Bl_QC, St_QC, W_QC) each clustered at the center of a small cloud of the corresponding samples (Figures 4-S3 and 4-2). Sours showed the greatest variance within the style compared to the others, which is represented by both the lack of clustering of the samples and the fact that S_QC is located close to All_QC, near the zero point of PC1, PC2, and PC3. This clearly illustrates that the QCs, which are a mixture of the beers from each style, share all the variance within a specific style.

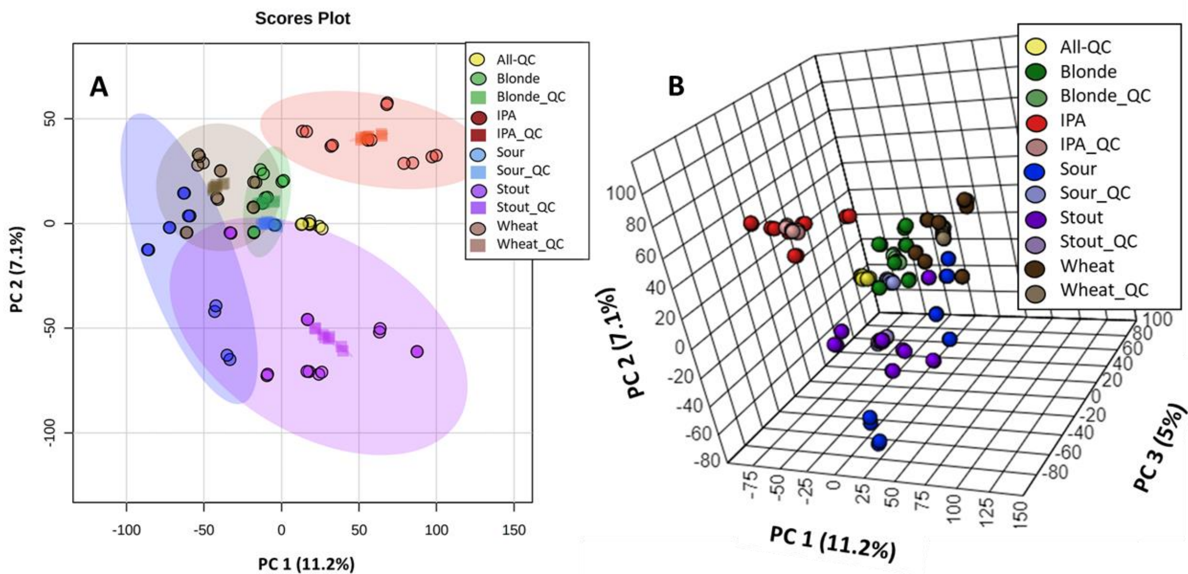

Figure 4-2. PCA for 32 commercial beers and pooled quality controls. Points are colored by beer style for visual clarity. **[A]** PCA 2D model while **[B]** is a 3D model. Colored by beer style: India pale ale (IPA) [red]; blonde (Bl) [green]; stout (St) [purple]; wheat (W) [brown]; sour (S) [blue]; and quality control of all beer samples (All_QC).

4.4.2.2. *Samples Only*

After removing the QCs, the same trends are visible (Figure 4-3). PC1 explains the greatest amount of variance between the individual beer samples (9.7%), with a total variance of 21.3% for PC1, 2 and 3. IPA is the most unique style of beer, being distinctly separated from all other beer styles. Despite the increased ability to differentiate between beer styles, there are still challenges to separate blondes and wheats. Additionally, due to the variation within the styles themselves, outliers can be observed. For example, St_6 is shown to cluster with the blondes in Figure 4-3B. Moreover, the heatmap reveals two sours, S_3 and S_5, that are correlated to stouts and IPAs. The reason behind these outliers could not yet be determined.

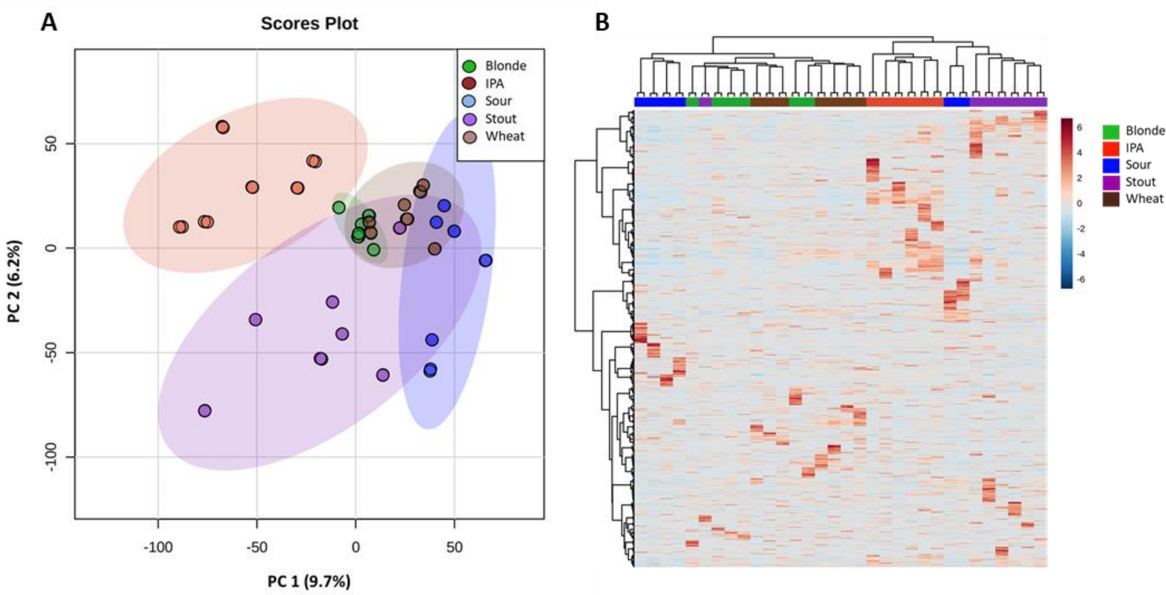

Figure 4-3. (A) PCA and **(B)** HCA for 32 commercial beers. Points are colored by beer style for visual clarity. **[A]** PCA 2D model while **[B]** is a 3D model. Colored by beer style: India pale ale (IPA) [red]; blonde (Bl) [green]; stout (St) [purple]; wheat (W) [brown]; and sour (S) [blue].

4.4.2.3 *Pooled Quality Controls*

Based on the goal of using chemical analysis for style classification, quality assurance, and the variation within the samples of a style, pooled samples were used as a representative style sample. Since the QCs are a combination of each sample, the key defining features for each style will thus be accentuated and yield an improved ability to differentiate between beer styles (Figure 4-S4). In order to determine the key features, the QCs data was evaluated using ANOVA, Random Forest and PLS-DA. It was further evaluated to determine if the top 1,000 features from each analysis were shared by the three statistical approaches (Figure 4-4), in order to make the data manageable. Three statistical approaches offered in Metaboanalyst, a univariate (ANOVA) and two multivariate (PLS-DA and RF), were used in an attempt to remove potential biases from each test and thus reduce the number of key features investigated [211,212]. The profile of each

peak was investigated to assure gaussian shapes. Such approach enabled the reduction of the data from 22,200 features to a final dataset of 54 features that have distinctives retention times and accurate masses (Table 4-2 and 4-S6).

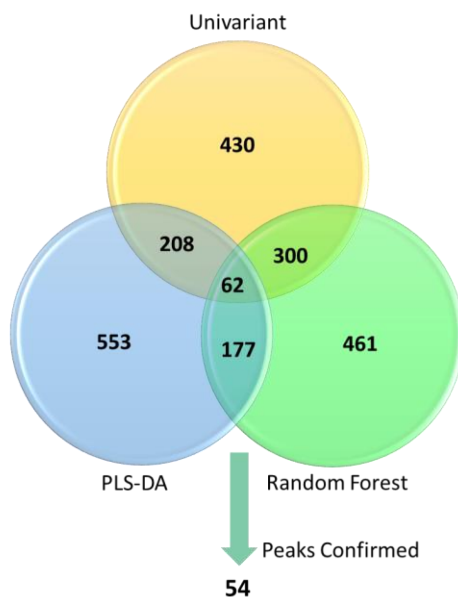

Figure 4-4. Workflow for the feature selection, using ANOVA, PLS-DA, and RF, to select influential metabolites able to differentiate between the beer styles.

4.4.2.4. *Tentative Identification of Features of Merit*

Although MS/MS spectra were generated for most of the features, the ability to identify them is limited [213]. On average, less than 20% of compounds are identified in most untargeted publications due to limited availability of spectral data in databases [206,207]. Since reference mass spectra were not available, molecular formulas were predicted for features using MS-Finder from the precursor ion using the accurate mass, isotope ratio, and product ion information [207]. Theoretical fragmentation was generated for all highlighted features in Table 4-2, which allowed for predicted molecular formulas and supported the structure elucidation process, thus reaching

a level 3 identification based on the Metabolomics Standard Initiative (MSI) [206,214]. However, since the fragmentation of small molecules is still not well understood, theoretical fraction was not available to support all predictions [207]. Therefore, the remaining key features, many of which did not have a theoretical fragmentation available for comparison and thus ranking at a level 4, are listed in Table 4-S6.

The key features listed in Table 4-2 were researched further to determine what their tentative identification could be. Keto acids act as intermediates in the formation of higher alcohols from *Saccharomyces cerevisiae*, a common brewing yeast, during wort fermentation [215]. Based on the mass and structural classification, feature 100 could be 2-oxoadipate, as this has been previously identified in *S. cerevisiae* [216]. Feature 101, a tricarboxylic acid, could possibly be cis-2-methylaconitate. This compound was elevated in sours and has been shown to exist in bacteria [217], which are utilized to give sour beers their characteristic flavor. Feature 102, a methoxyphenol found in IPAs, may be methoxyeugenol. Eugenol is known to impart a spicy, clove character in ales [218]. If present in IPAs, it could have possibly been converted to methoxyeugenol during the brewing process. Feature 107 shows that naphthalenes were more prominent in wheat beer, which could be because naphthaleneacetic acid is widely used to increase crop yield and promote growth of wheat and other cereal crops [219–221]. Feature 113 was predicted to belong to the flavone structural class and was found in wheat beers. Various flavones have been identified in wheat and cereal grains [222], which adds confidence to this prediction, but the particular flavone belonging to this feature has not yet been identified. Depsides and depsidones, feature 114, were predicted to be key features in IPAs and blondes. This may be due to the presence of gallotannins, a beer stabilizer [223,224], in which

the galloyl moieties are linked by depside bonds [225]. Tannins are introduced to beer in the form of hops [226,227], which are used more heavily in the brewing of IPAs. The other key features are still in the process of being identified.

Table 4-2. Tentative identification of key feature for beer style quality assurance. Key features were a trifecta match in the top 1,000 most influential features for RF, PLS-DA, and ANOVA. Feature denoted with an * were a trifecta match in the top 500 features. All aasif=ned adducts listed are [M-H]-.

Assigned Number	Style		RT (min)	Accurate Mass	Formula	Error (mDa)	Structural ontology
100	Blonde, IPA	*	0.92	159.0317	$C_6H_8O_5$	-1.8	Medium-chain keto acids and derivatives
101	Sour (elevated levels)		0.83	187.0277	$C_7H_8O_6$	-2.9	Tricarboxylic acids and derivatives
102	IPA		5.26	193.0906	$C_{11}H_{14}O_3$	-3.5	Methoxyphenols
103	Sour (elevated levels)		2.29	219.0542	$C_{14}H_8N_2O$	2.2	Indolonaphthyridine alkaloids
104	Wheat		0.65	256.1693	$C_{17}H_{23}NO$	1.4	Styrenes
105	Blonde		1.25	304.1088	$C_{18}H_{15}N_3O_2$	0.4	Quinazolinamines
106	Blonde, IPA		5.38	317.2608	$C_{20}H_{34}N_2O$	-1.0	Aminopiperidines
107	Wheat	*	2.83	369.2702	$C_{22}H_{34}N_4O$	-4.2	Naphthalenes
108	IPA		8.33	387.2244	$C_{17}H_{32}N_4O_6$	0.5	Aminocyclitol glycosides
109	Stout, Wheat		2.74	388.0950	$C_{21}H_{15}N_3O_5$	-1.1	Diarylethers
110	Sour, Wheat	*	7.06	417.2718	$C_{24}H_{38}N_2O_4$	4.1	Diterpenoids
111	Wheat	*	2.47	450.3019	$C_{29}H_{41}NO_3$	-0.5	Steroid esters
112	Stout, Sour, Wheat	*	1.02	481.2138	$C_{30}H_{30}N_2O_4$	-0.5	Pyranoquinolines
113	Wheat		0.68	482.1287	$C_{21}H_{25}NO_{12}$	1.7	Flavones
114	Blonde, IPA	*	0.60	566.1679	$C_{29}H_{29}NO_{11}$	-1.1	Depsides and depsidones

From the untargeted analysis of beer metabolites using the pooled QCs, features allowing the differentiation between styles, based on their presence or absence, were identified. Plots were generated to visualize and compare the influential metabolites between beer styles

(Figure 4-S5). Just as one physical characteristic does not define a beer (i.e. taste, aroma, color, mouthfeel), it would require a combination of key features to chemically identify a style of beer. For example, a combination of the presence of metabolite numbers 100 and 112, in addition to the absence of 109 which was found in the other 4 styles, could be a potential indicator that a beer would qualify as an IPA, as seen in Figure 4-S5. As expected, due to the easy-drinking and mild flavor characteristics of blondes [2], there were a limited number of metabolites classified as key features in this style. However, the presence of 100, 106, and 114, which are shared features with IPAs, in combination with the presence of metabolite 105, could be developed into a targeted focus for characterizing blondes. Alternatively, stouts, sours, and wheats share some key features. The classification of these could be differentiated from blondes and IPAs by the presence of 101 and 103. Furthermore, stouts and sours can be characterized through the presence of metabolite 109 in stouts and the absence in sours. Moreover, the presence of feature 110 in sours and its absence in stouts can provide a distinction between the styles. Finally, wheats have a few additional features, 107 and 113, that would not be expected in stouts or sours.

4.4.3. *Pattern Classification Analysis*

In order to perform a pattern classification analysis, data from each beer was processed and represented by a feature vector of 10,000 with a resolution of 100 bins in retention time and m/z domains, as described previously. There were 64 samples (two replicates of 32 beers) in total for 5 beer styles. The supervised learning methods used in this study were Gaussian Naïve Bayes (GNB) [208] and Random Forest Classifier (RF) [209] for multiclass pattern classification. For each method, 5-fold cross validation was performed on raw high dimensional feature space and also

on the reduced PCA space. The cross-validation accuracies of each method are summarized in Table 4-3. The GNB model obtained a testing classification accuracy of 70% using the extracted 10,000 features, while it achieved 100% accuracy using the top 3 PCA components. This shows that the GNB model learned discriminative patterns to classify different styles of beers accurately in the low dimensional PCA space. It is notable that the RF model achieved 100% or close to 100% accuracies in both high dimensional feature space and the low dimensional PCA component space. The RF model showed excellent learning capability in a high feature dimension and low sample size setting. In summary, the high classification accuracies indicate that the studied five styles of beer can be characterized by the extracted features and can be precisely distinguished using machine learning models. This provides strong evidence for further investigation on beer styles and their nonvolatile chemical makeup characteristics.

Table 4-3. The 5-fold cross validation classification performance for the five beer styles

Methods	extracted features	3 components	5 components	10 components
Naive Bayes	0.70	1.00	0.98	0.98
Random Forest	1.00	0.975	1.00	1.00

4.5. Conclusions

Beer is a complex matrix containing hundreds of compounds. Due to the large number of unknown components, targeted analysis alone is insufficient to obtain a full picture of what distinguishes these popular styles, and so untargeted techniques, including PCA and machine

learning, were utilized. The untargeted LC-QTOF-MS method and the use of univariate and multivariate statistical analysis demonstrated advantageous to differentiate the beer styles, in addition to highlighting 54 specific metabolites. Molecular formulas and structural characteristics of the metabolites were predicted thanks to the use of high-resolution MS. This combination of untargeted analysis and statistical methods provided evidence that the proof of concept of using chemical analysis as a beer style quality assurance tool is feasible.

The investigation of an expanded beer collection would be valuable to further develop quality assurance of beer styles. In addition, the chemical validation of the putatively identified metabolites listed in Table 4-2 would enable to validate their performances to discriminate between the beer styles, and to further develop faster targeted methods to assess beer style quality. Moreover, evaluation of a multifaceted data set using collected results from the analysis of volatiles and nonvolatiles, and further evaluation of the ability to distinguish beer styles using advanced data science techniques is being pursued.

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4.6. Supporting Information

Table 4-S1. Beers evaluated in this study. All except 8 beers were purchased in 12 fl oz cans. *denotes 12 fl oz brown bottle. N/A denotes IBU not specified on label. Style and flavor descriptions were sourced from brewers' websites and "Untappd" app.

Brewer	Brewer Location	Label Brand	Label Type	Style Details and Flavor Descriptions	IBU	Sample ID
			India Pale Ale			
Community Brewing Co	Dallas, TX	Citra Slice	IPA	Citrus IPA, citrus, lemon, orange peel	50	I_1
Ballast Point Brewing Co	San Diego, CA	Fathom	IPA	West-coast style IPA, crisp, orange	50	I_2
Nine Band Brewing Co.	Allen, TX	Hellwind	IPA	Hoppy, bitter, citrus	63	I_3
Cigar City Brewing Co	Tampa, FL	Jai Alai	IPA	Citrus, clementine, orange peel	65	I_4
Community Brewing Co	Dallas, TX	Mosaic IPA	IPA	Hoppy, smooth, strong, sweet	85	*I_5
New Belgium Brewing Co	Fort Collins, CO	VooDoo Ranger Juicy Haze	IPA	Citrus, juicy, smooth	42	I_6
			Blonde			
Deep Ellum Brewing Co	Dallas, TX	Dallas Blonde	Blonde	Light, smooth, clean	23	Bl_1
Real Ale Brewing Co	Blanco, TX	Firemans #4	Blonde	Light, smooth, clean, hoppy	23	*Bl_2
HopFusion Ale Works	Fort Worth, TX	Feisty Blonde	Blonde	Honey and vanilla golden ale, sweet	17	Bl_3
Legal Draft Beer Co	Arlington, TX	Legal Blonde Lager	Blonde	Helles Lager, light, smooth, clean, malty	18	Bl_4
Karbach Brewing Co	Houston, TX	Love Street	Blonde	Kölsch style, light, smooth, clean	20	Bl_5
Wild Acre Brewing Co	Fort Worth, TX	Texas Blonde	Blonde	Light, smooth, floral, hoppy	15	Bl_6
			Stout			
Legal Draft Beer Co	Arlington, TX	Chief Justice Stout	Stout	Dark, smooth, coffee, roasty	30	St_1
New Holland Brewing Co	Holland, MI	Dragon's Milk White	Stout	White stout, bourbon barrel-aged, cocoa, coffee, vanilla, light, velvety	35	St_2
HopFusion Ale Works	Fort Worth, TX	Fur Slipper	Stout	Imperial milk stout, sweet, smooth, dark, rich, hazelnut, chocolate, toffee	34	St_3
Deep Ellum Brewing Co	Dallas, TX	Local Legend	Stout	Sweet, smooth, milk, coffee	23	St_4
Left Hand Brewing Co	Longmont, CO	Milk Stout	Stout	Smooth, milk, creamy, sweet, coffee	25	St_5
New Holland Brewing Co	Holland, MI	Dragon's Milk Raspberry Hibiscus	Stout	Raspberry and hibiscus, bourbon barrel-aged, vanilla, aromatic, floral	N/A	*St_6

Lakewood Brewing Co	Garland, TX	The Temptress	Stout	Imperial milk stout, smooth, milk, creamy, sweet, chocolate, low carbonation	56	*St_7
			Wheat			
Boulevard Brewing Co	Kansas City, MO	Unfiltered Wheat	Wheat	Unfiltered, light, smooth, clean, sweet, citrus	14	*W_1
Blue Moon Brewing Co	Golden, CO	Belgian White	Wheat	Coriander, orange peel, light, smooth, sweet, fruity, citrus	9	*W_2
Shannon Brewing Co	Keller, TX	Crystal Wheat	Wheat	Kristallweizen, light, clean, smooth, clove, banana, crisp	15	W_3
Legal Draft Beer Co	Arlington, TX	Hung Jury	Wheat	Hefeweizen, light, smooth, banana, clove, sweet	13	W_4
Blue Moon Brewing Co	Golden, CO	Mango Wheat	Wheat	Mango, fruity, sweet, smooth	33	*W_5
Bell's Brewery	Comstock, MI	Oberon	Wheat	Light, smooth, spring, sweet, hoppy	N/A	*W_6
Avery Brewing Co	Boulder, CO	White Rascal	Wheat	Witbier, coriander, curacao orange, spicy/zesty, fruity, light, smooth, sweet	22	W_7
			Sour			
Division Brewing Co	Arlington, TX	Elder Bramble	Sour	Elderflower, blackberry, fruity, tart, sour, clean	4	S_1
Community Brewing Co	Dallas, TX	Berliner Berry	Sour	Fruited sour, berlinerweisse brewed with boysenberry, tart, refreshing	5	S_2
DESTIHL Brewery	Normal, IL	Flanders Red	Sour	Wild sour, sour, tart, dry, fruity, sweet, cherry	18	S_3
Dogfish Head Brewing Co	Milton, DE	Sea Quench Ale	Sour	Session sour, sour, tart, citrusy, salty	10	S_4
DESTIHL Brewery	Normal, IL	Synchopathic	Sour	Wild sour, sour, tart, dry, grapefruit, orange, lemon, tangerine, pineapple, pine	12	S_5
Prairie Artisan Ales	Krebs, OK	Vape Tricks	Sour	Cherry, sour, tart, light, sweet	N/A	S_6

Table 4-S2. The batch was run in a continuous sequence of beers and quality controls (pooled samples). Quality controls (QCs) for each class of beer, India Pale Ales (IPA), Blonde (Bl), Stouts (St), Wheats(W), Sours (S), and All (A) were prepared for the PCA analysis by mixing equal parts of each beer and diluting by 50%. A QC for group of the beers was prepared in the same manner.

Order	ID	Order	ID	Order	ID	Order	ID	Order	ID
1	Blank	14	Bl_QC	27	St_1	40	St_5	53	S_QC
2	I_3	15	St_QC	28	W_1	41	W_4	54	All_QC
3	Bl_6	16	W_QC	29	S_5	42	S_6	55	Blank
4	St_6	17	S_QC	30	QC_CD	43	I_5	56	St_2
5	W_5	18	All_QC	31	IPA-QC	44	Bl_4	57	W_7
6	S_4	19	Blank	32	Bl_QC	45	St_3	58	QC_G
7	I_4	20	I_1	33	St_QC	46	W_3	59	IPA-QC
8	Bl_1	21	Bl_2	34	W_QC	47	S_1	60	Bl_QC
9	St_7	22	St_4	35	S_QC	48	QC_EF	61	St_QC
10	W_6	23	W_2	36	All_QC	49	IPA-QC	62	W_QC
11	S_2	24	S_3	37	Blank	50	Bl_QC	63	S_QC

12	AB_QC	25	I_2	38	I_6	51	St_QC	64	All_QC
13	IPA_QC	26	Bl_3	39	Bl_5	52	W_QC	65	Blank

Table 4-S3. Targeted phenolic compounds multiple reaction monitoring (MRM) transitions used in LCMS-QTOFMS targeted analysis. The masses are given as the deprotonated ion, $[M\text{-}H]^-$.

Analyte	Formula	Standard RT (min)	Precursor Mass (Da)	Product Ions	CE [V]
Catechin	$C_{15}H_{13}O_6$	2.647	289.07	123.0441, 203.0702	22.0
Caffeic Acid	$C_9H_7O_4$	2.801	179.03	134.0383, 135.0465	21.0
Chlorogenic Acid	$C_{16}H_{17}O_9$	2.598	353.09	161.0252, 191.0559	22.0
Cinnamic Acid	$C_{10}H_9O4$	3.310	193.04	134.0382, 178.0288	18.0
Benzoic Acid	$C_7H_5O_3$	2.592	137.02	65.0387, 93.0345	25.0
Quercetin	$C_{15}H_9O_7$	3.882	301.03	151.0014, 178.9998	21.0
Naringin	$C_{27}H_{31}O_{14}$	3.367	579.17	151.0008, 271.0563	28.0
Myricetin	$C_{15}H_9O_8$	3.564	317.02	137.0248, 151.0043	19.0
Vanillin	$C_8H_7O_3$	3.187	151.03	108.0223, 136.0175	19.0
Hydroxycoumarin	$C_9H_6O_3$	3.655	161.02	117.0341	21.0
Gallic Acid	$C_7H_5O_5$	1.069	169.01	79.0180, 125.0222	18.0
Esculin	$C_{15}H_{15}O_9$	2.457	339.07	133.0299, 177.0216	22.0

Table 4-S4. Targeted Isomerized and reduced isomerized alpha acids multiple reaction monitoring (MRM) transitions in LC-QTOF-MS targeted analysis. Only the retention times for the isomers with standards are given. The IAA standard contained only the trans isomers, while the RIAA and HIAA standards contained only cis isomers.

Analyte	Group	ID	Standard RT (min)	Precursor Mass (Da)	Product Ions	CE [V]
trans-isohumulone	IAA	t-iH	6.089	361.20	96.0758, 265.1474	15.0
cis-isohumulone		c-iH		361.20	96.0758, 265.1474	15.0
trans-isocohumulone		t-iCH	5.675	347.19	182.0597, 251.1311	21.0
cis-isocohumulone		c-iCH		347.19	182.0597, 251.1311	21.0
trans-isoadhumulone		t-iAH	5.968	361.20	96.0758, 265.1474	15.0
cis-isoadhumulone		c-iAH		361.20	96.0758, 265.1474	15.0
trans-rho-isohumulone	RIAA	t-RIH		363.22	196.0766, 247.1367	22.0
cis-rho-isohumulone		c-RIH	6.113	363.22	196.0766, 247.1367	22.0
trans-rho-isocohumulone		t-RICH		349.20	182.0598, 233.1202	21.0
cis-rho-isocohumulone		c-RICH	5.755, 5.821	349.20	182.0598, 233.1202	21.0
trans-rho-isoadhumulone		t-RIAH		363.22	196.0766, 247.1367	22.0
cis-rho-isoadhumulone		c-RIAH	6.020	363.22	196.0766, 247.1367	22.0
trans-tetra-isohumulone	TIAA	t-TIH	7.185	365.23	249.1523, 267.0000	25.0
cis-tetra-isohumulone		c-TIH	7.185	365.23	249.1523, 267.0000	25.0
trans-tetra-isocohumulone		t-TICH	6.450	351.22	239.1316, 253.0000	22.0
cis-tetra-isocohumulone		c-TICH	6.542	351.22	239.1316, 253.0000	22.0
trans-tetra-isoadhumulone		t-TIAH	7.000	365.23	249.1523, 267.0000	25.0
cis-tetra-isoadhumulone		c-TIAH	7.050	365.23	249.1523, 267.0000	25.0
trans-hexa-isohumulone	HIAA	t-HIH		367.25	223.1729, 249.1526	25.0
cis-hexa-isohumulone		c-HIH	6.700	367.25	223.1729, 249.1526	25.0
trans-hexa-isocohumulone		t-HICH		353.23	235.1333, 253.0000	22.0
cis-hexa-isocohumulone		c-HICH	6.321	353.23	235.1333, 253.0000	22.0
trans-hexa-isoadhumulone		t-HIAH		367.25	223.1729, 249.1526	25.0
cis-hexa-isoadhumulone		c-HIAH	6.500, 6.600	367.25	223.1729, 249.1526	25.0

Table 4-S5. DIA Acquisition parameters. Q1 resolution was set at 16 with the precursor for each event in 15 m/z increments. The ToF detector was set to scan various ranges.

Event	Precursor	Begin ToF Scan	End ToF Scan	Event	Precursor	Begin ToF Scan	End ToF Scan
1	Scan	100	1000	32	550	100	560
2	100	100	110	33	565	100	575
3	115	100	125	34	580	100	590
4	130	100	140	35	595	100	605
5	145	100	155	36	610	100	620
6	160	100	170	37	625	100	635
7	175	100	185	38	640	100	650
8	190	100	200	39	655	100	665
9	205	100	215	40	670	100	680
10	220	100	230	41	685	100	695
11	235	100	245	42	700	100	710
12	250	100	260	43	715	100	725
13	265	100	275	44	730	100	740
14	280	100	290	45	745	100	755
15	295	100	305	46	760	100	770
16	310	100	320	47	775	100	785
17	325	100	335	48	790	100	800
18	340	100	350	49	805	100	815
19	355	100	365	50	820	100	830
20	370	100	380	51	835	100	845
21	385	100	395	52	850	100	860
22	400	100	410	53	865	100	875
23	415	100	425	54	880	100	890
24	430	100	440	55	895	100	905
25	445	100	455	56	910	100	920
26	460	100	470	57	925	100	935
27	475	100	485	58	940	100	950
28	490	100	500	59	955	100	965
29	505	100	515	60	970	100	980
30	520	100	530	61	985	100	995
31	535	100	545	62	1000	100	1010

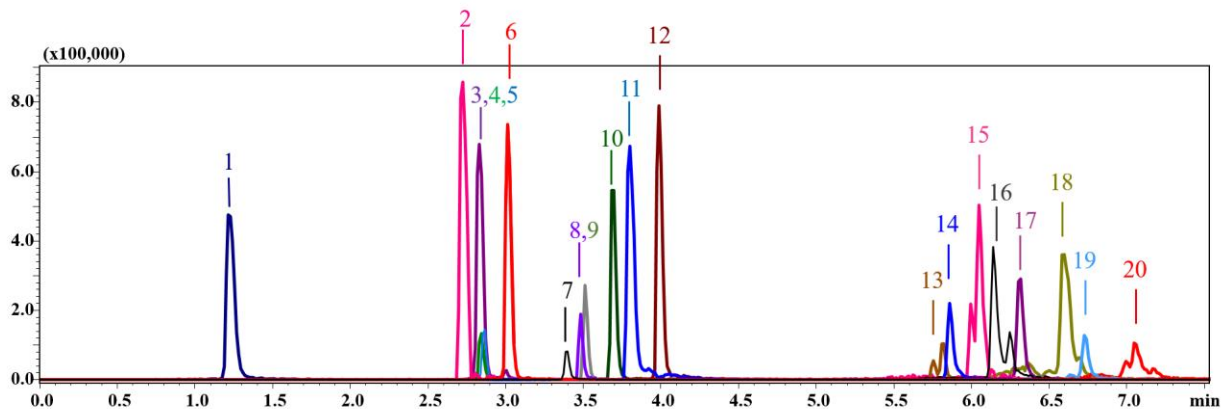

Figure 4-S1. LC-QTOF-MS MRM chromatogram of targeted phenolic and IAA standards. 1. Gallic acid (dark blue), 2. Esculin (pink), 3. Chlorogenic acid (purple), 4. Benzoic acid (green), 5. Catechin (teal), 6. Caffeic acid (red), 7. Vanillin (black), 8. Cinnamic acid (lilac), 9. Naringin (grey), 10. Myricetin (dark green), 11. Hydroxycoumarin (blue), 12. Quercetin (brown), 13. *c*-RiCH (light brown), 14. *t*-iCH (blue), 15. *c*-RiAH and *c*-RiH (pink), 16. *t*-iAH and *t*-iH (black), 17. *c*-HiCH (purple), 18. *c*-HiAH and *c*-HiH (olive green), 19. *c-/t*-TiCH (cyan), 20. *c-/t*-TiAH and *c-/t*-TiH (red).

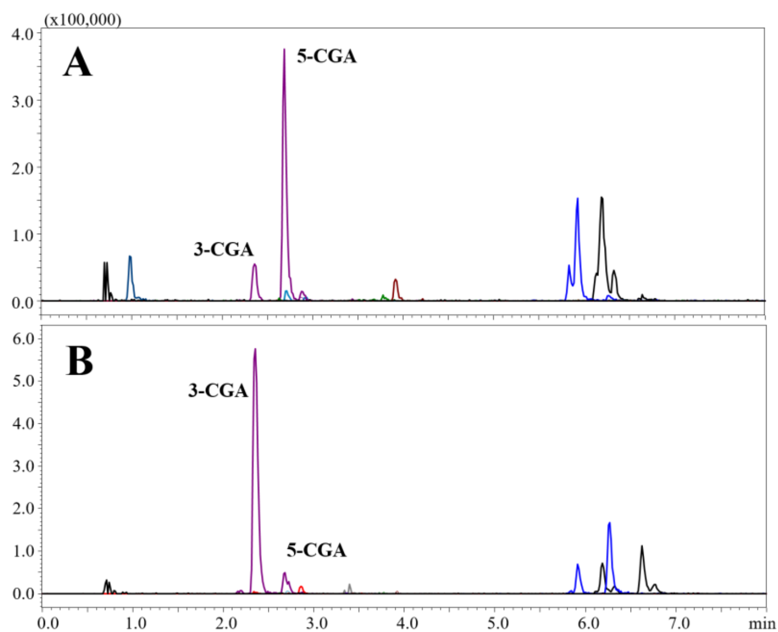

Figure 4-S2. MRM of sour beers S_1 (A) and S_6 (B), showing peaks for chlorogenic acid and its isomers (purple), isocohumulone (blue), and isoadhumulone (black).

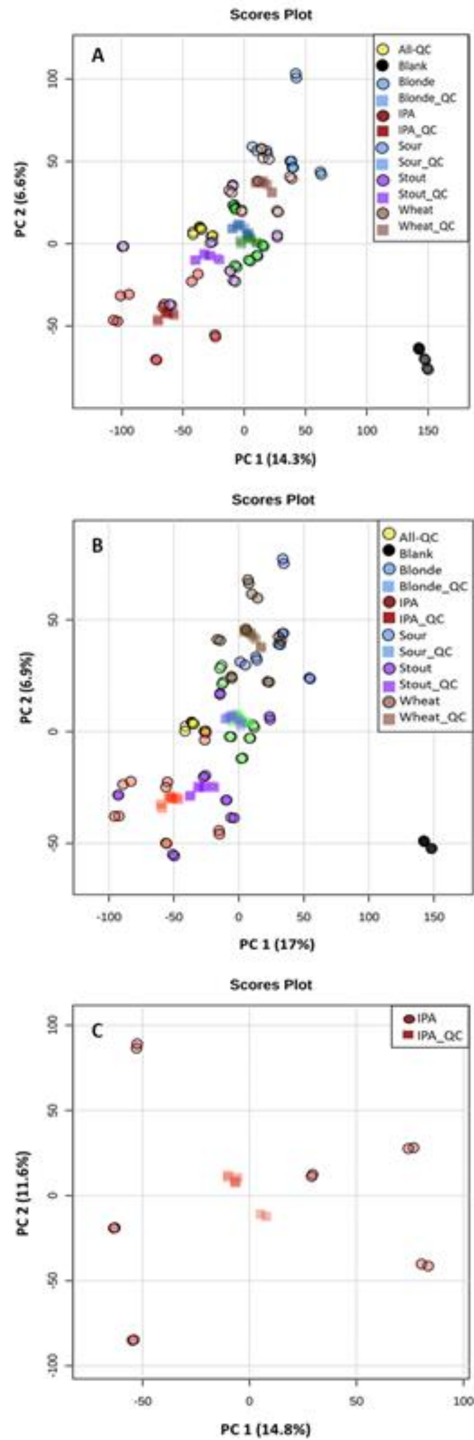

Figure 4-S3. A (Top). PCA scores plot using PC1 and 2 for 32 commercial beers and quality controls (colored) vs Blanks (black) pre data cleaning **(A top)** and post data cleaning **(B middle)**. Showing clear separation between the blanks and beer samples using PC1 (14.3% and 17, respectively). The distribution of samples across PC2 (6.6%) shows variation within the beer samples in which is not explained by the variation seen in PC1. **C (Bottom)** PCA plot for IPA and the IPA_QCs.

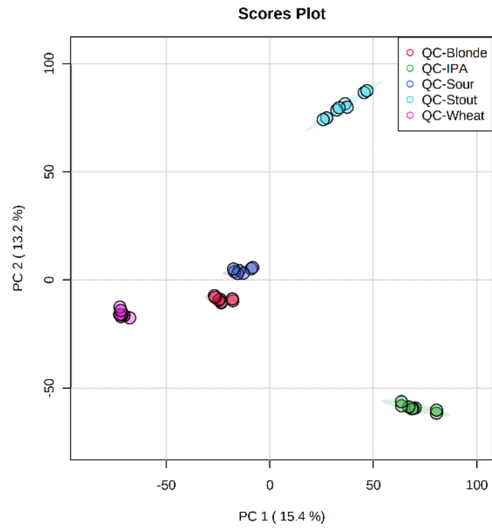

Figure 4-S4. PCA scores plot of pooled samples (QCs)

Table 4-S6. Tentative identification of key feature for beer style quality assurance not represented in manuscript. Key features were a trifecta match in the top 1,000 most influential features for RF, PLS-DA, and ANOVA. Feature denoted with an * were a trifecta match in the top 500 features.

	RT (min)	Accurate Mass	Assigned Adduct	Formula	Error (mDa)
	1.00	169.0165	[M-H]-	$C_7H_6O_5$	-2.3
	2.65	175.0641	[M-H]-	$C_7H_{12}O_5$	-2.9
	0.82	282.1642	[M-H]-	$C_{17}H_{21}N_3O$	-3.0
	2.93	289.0762	[M-H]-	$C_{15}H_{14}O_6$	-4.4
	4.43	306.1766	[M-H]-	$C_{18}H_{21}N_5$	-4.2
	2.57	313.0609	[M-H]-	$C_{19}H_{10}N_2O_3$	1.0
*	1.98	313.0619	[M-H]-	$C_{19}H_{10}N_2O_3$	0.0
	2.69	316.1932	[M-H]-	$C_{19}H_{27}NO_3$	-1.4
	0.78	320.1036	[M-H]-	$C_{18}H_{15}N_3O_3$	0.6
	2.71	326.0934	[M-H]-	$C_{20}H_{13}N_3O_2$	0.1
	2.16	342.1736	[M-H]-	$C_{20}H_{25}NO_4$	-2.5
	6.56	363.1876	[M-H]-	$C_{18}H_{28}N_4O_2S$	-1.6
	2.83	369.0895	[M-H]-	$C_{19}H_{18}N_2O_4S$	2.0
	4.02	375.2743	[M-H]-	$C_{20}H_{40}O_6$	0.9
	5.31	377.2860	[M-H]-	$C_{27}H_{38}O$	-1.0
	5.29	377.2862	[M-H]-	$C_{27}H_{38}O$	-1.2
	0.67	384.1589	[M-H]-	$C_{25}H_{23}NO_3$	1.6
	6.11	389.2401	[M-H]-	$C_{21}H_{34}N_4OS$	-2.0
	7.90	401.2674	[M-H]-	$C_{20}H_{38}N_2O_6$	-1.7
	6.64	403.2186	[M-H]-	$C_{21}H_{32}N_4O_2S$	-1.3
	6.61	403.2191	[M-H]-	$C_{21}H_{32}N_4O_2S$	-1.8
	0.60	404.1113	[M-H]-	$C_{19}H_{23}N_3O_3S_2$	-0.5
	2.91	418.1068	[M-H]-	$C_{19}H_{21}N_3O_6S$	1.0
	4.08	423.2940	[M-H]-	$C_{21}H_{40}N_6OS$	-2.8
	6.56	431.1702	[M-H]-	$C_{23}H_{28}O_8$	0.9
	4.02	439.2898	[M-H]-	$C_{29}H_{36}N_4$	-3.1
*	3.29	463.0962	[M-H]-	$C_{24}H_{20}N_2O_6S$	0.7
	2.88	466.2392	[M-H]-	$C_{23}H_{37}N_3O_5S$	-1.1
	1.05	467.1974	[M-H]-	$C_{21}H_{32}N_4O_6S$	-0.4
	0.68	482.1287	[M-H]-	$C_{28}H_{21}NO_7$	-4.2
	1.87	490.1786	[M-H]-	$C_{30}H_{25}N_3O_4$	-1.4
*	0.68	512.2198	[M-H]-	$C_{30}H_{31}N_3O_5$	-0.7
	2.64	557.3751	[M-H]-	$C_{41}H_{50}O$	3.8
*	2.09	593.1401	[M-H]-	$C_{25}H_{30}N_4O_9S_2$	-2.0
*	1.53	593.1403	[M-H]-	$C_{29}H_{26}N_2O_{12}$	1.0
	1.34	609.2742	[M-H]-	$C_{35}H_{38}N_4O_6$	-2.3

	1.50	614.3015	[M-H]-	$C_{34}H_{41}N_5O_6$	-3.1
	3.78	639.2055	[M-H]-	$C_{33}H_{36}O_{13}$	2.8
*	3.45	655.2006	[M-H]-	$C_{33}H_{36}O_{14}$	2.6

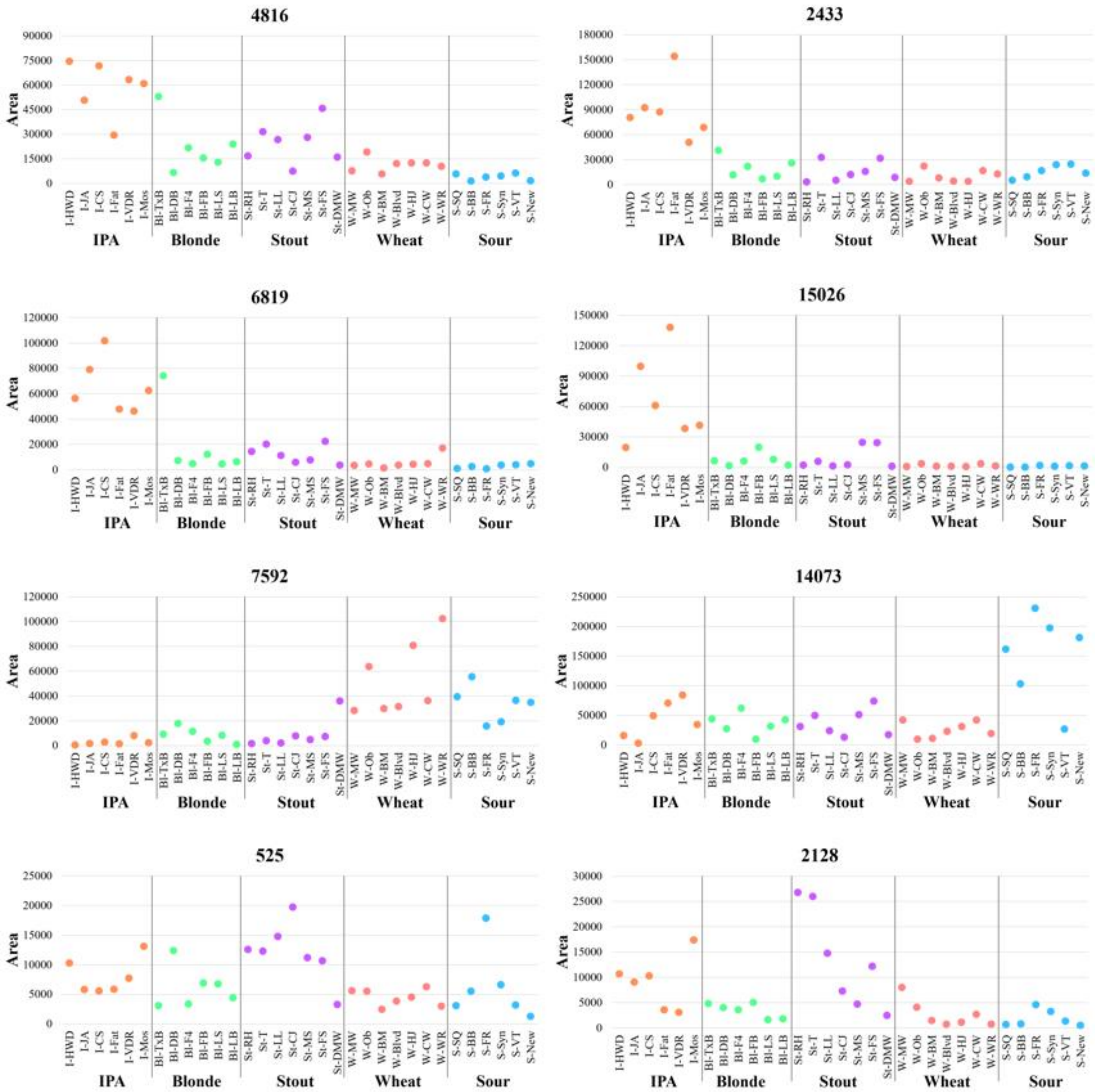

Figure 4-S5. A comparison of the average peak heights of some key features in the individual beers, separated by style.

CHAPTER FIVE: Determination of 2,4,6-trichloroanisole, geosmin, and other fault compounds in whiskey using headspace–high capacity solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS)

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5.1. Abstract

In this study, we present a method for the simultaneous assay of common fault or off-flavor compounds in whiskey using headspace–solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME-GC-MS). Some of the major contaminants in whiskey include geosmin, 2,4,6-trichloroanisole (TCA), and other haloanisoles, which impart musty and moldy flavors at sensory thresholds in the low ng/L range. The limits of detection of geosmin, TCA, 2,4,6-tribromoanisole (TBA), and 4-bromoanisole were 50 ng/L, 5 ng/L, 50 ng/L, and 5 ng/L, respectively, which are near the sensory thresholds for the compounds. Accuracies and precisions were calculated for each analyte based on triplicate measurements of low, medium, and high quality control levels. The accuracies, reported in percent error, ranged from 0.9% to 17.0%, and the precisions ranged from 1.3% to 16.7%, expressed as the coefficient of variation. The optimized method, described herein, quantified these fault compounds in multiple Scotch and Kentucky Straight Bourbon Whiskies, allowing for routine quality control.

Key Words: Whiskey, TCA, geosmin, fault compounds, SPME

Abbreviations: TCA, TBA, SPME, HS, MRM, ABV

5.2. Introduction

Whiskey is a distilled spirit made from fermented grains such as barley, rye, corn, and wheat. It is typically aged in oak barrels, where the spirit extracts flavors and a brown pigment from the wood. A myriad of chemical components gives whiskey pleasant flavor notes, often described as oaky, sweet, floral, fruity, herbal, spicy, and smoky. However, undesirable off-flavors can also be introduced. Some of the main contributors include TCA (2,4,6-trichloroanisole) and geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol). The structures of these compounds can be found in Figure 5-S1.

TCA and other haloanisoles, such as 2,4,6-tribromoanisole (TBA), can impart a musty or moldy taste to whiskey and other beverages [228–230]. TCA is a common off-flavor found in wines, and is often called “cork taint” due to its association with cork-related contamination [229,231–233]. While far less common, TCA has also been identified in wines and spirits that had no contact with corks. In such cases, barrels made of new oak wood were identified as the source of contamination [229,234]. TCA and TBA can be detected through sensory analysis at very low concentrations, ranging from 2 – 6 ng/L in wine [235,236]. Reported sensory thresholds for these haloanisoles in whiskey could not be found, but it has been observed that alcoholic beverages aged in wood or with higher alcohol content make the recognition of TCA more difficult, thereby raising the sensory threshold [237,238].

Geosmin imparts an unpleasant, earthy, musty taste to beverages [230,233,239]. It can be introduced through contamination of the water [240], grains [241], or barrel used [230]. In wine, geosmin has a sensory detection limit of 60 – 90 ng/L [242,243]. As with TCA, this threshold may be raised in whiskey due to the higher alcohol content.

Although off-flavors are noticeable at low concentrations, even experienced tasters can find it challenging to detect and distinguish between TCA and geosmin near the sensory threshold [230,237]. Sensory analysis in conjunction with chemistry allows these fault compounds to be confidently identified, even at low concentrations. Fault compounds can be removed through filtration with activated carbon [228,237], although this technique will strip some of the desirable flavor characteristics as well. It is important for the alcohol industry to identify what compound is responsible for undesirable characteristics so that counteractive measures can be taken prior to bottling, and substantial economic loss can be avoided.

Solid phase microextraction (SPME) followed by gas chromatography mass spectrometry (GC-MS) has been used extensively to identify TCA, geosmin, and other off flavors in aqueous samples [230,233,239–241,244,245]. This highly automated technique is beneficial due to its simplistic sample preparation, extensive MS library, and sensitivity, allowing for the detection of these compounds, at or below their sensory threshold [246]. While a multitude of studies have been performed on TCA and geosmin in wine, far less have been conducted on whiskey. Although some distilleries already screen their product for TCA and geosmin, they often must use separate analytical labs and methods for the two compounds. In this study, we aim to develop an optimized HS-SPME-GC-MS method for the simultaneous analysis of TCA and geosmin, as well as two other haloanisoles, in whiskey for routine quality control purposes.

5.3. Materials and Methods

5.3.1. Chemicals and Reagents

Standards of 2,4,6-trichloroanisole, 2,4,6-tribromoanisole, geosmin (100 mg/L in methanol), 4-bromoanisole, and 3-chlorotoluene (internal standard) were purchased from Sigma Aldrich (St. Louis, MO, USA). LCMS grade methanol was obtained from Honeywell (Muskegon, MI, USA). Sodium chloride (certified ACS) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ethanol (200 proof) was purchased from Decon Laboratories, Inc. (King of Prussia, PA). Millipore water was retrieved from an Aries high purity water system (Camden, NJ, USA).

5.3.2. Method Validation

Stock solutions of 1000 mg/L were prepared in methanol for all compounds. Working solutions of 100 µg/L and 1 µg/L were made in methanol and used to prepare calibration levels and quality controls. Solutions were always freshly prepared before use.

Calibration curves were created for each monitored analyte. The calibration solutions were prepared in 20 mL headspace (HS) vials (Restek Corporation, Bellefonte, PA) containing 3 grams of sodium chloride, 25 µL of the internal standard (at 500 ng/L), each analyte, and diluted to a total of 5 mL with ultrapure water and 5% ethanol. Quality control samples were prepared and measured at low, medium, and high concentration levels, distinct from those used in the curves. These were prepared in the same manner as the calibration points, but using separately prepared stock solutions of the analytes. Limit of detection (LOD), limit of quantitation (LOQ), percent relative standard deviation (%RSD), and the correlation factor (R^2)

were calculated using the calibration data. Three replicates of each QC level were used to evaluate accuracy and precision.

5.3.3. Samples

Samples of whiskey were obtained from a local liquor store, which included five scotch whiskies and five Kentucky straight bourbon whiskies. These were prepared for analysis by diluting to 5% ABV (alcohol by volume) with ultrapure water in a HS vial containing 3 grams of sodium chloride and 25 µL of the internal standard. Each whiskey was analyzed six times; three non-spiked samples followed by three samples spiked with 450 ng/L geosmin and TBA and 45 ng/L TCA and 4-bromoanisole.

5.3.4. Instrumentation

The fault compounds were assayed simultaneously. Analyses were conducted using a Shimadzu GCMS-TQ8030 gas chromatograph – triple quadrupole mass spectrometer equipped with an AOC-6000 autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD) for automated headspace SPME extraction and desorption. The volatile portion of the samples was extracted using a SPME Arrow with a diameter of 1.10 mm and a divinylbenzene/polydimethylsiloxane (DVB/PDMS) fiber (thickness: 120 µm). The SPME Arrow was supplied by Restek Corporation (Bellefonte, PA). The HS-SPME conditions were 40 ℃ extraction temperature, 10 minute extraction time, incubation time of 2 minutes, and 1 minute desorption time.

Separation was achieved using a non-polar Rxi-5Sil MS column (30 m x 0.25 µm x 0.25 mm) from Restek. Helium was used as the carrier gas, with a flow rate of 1.69 mL/min and a linear

velocity of 47.2 cm/sec. The injection temperature was 280°C. Splitless injection was used during desorption; the split vent was opened to a split ratio of 5:1 after a 1 minute hold time in splitless mode. The temperature program started at 50 °C (isothermal hold for 3 minutes), ramped to 180 °C at a rate of 8 °C/min (isothermal hold for 11 minutes), and then ramped to 280 °C at a rate of 25 °C/min (isothermal hold for 5 minutes).

The ion source temperature of the MS was 230 °C, and a solvent cut time of 1 min was used. The analytes were measured in MRM mode (multiple reaction monitoring) using the transitions listed in Table 5-1.

Table 5-1. List of the analytes used in this study, their MRM transitions, and retention times.

Analyte	Target ion	Transition 1	Transition 2	Retention Time
TCA	212	197	169	21.5 min
Geosmin	182	112	125	23.7 min
TBA	346	331	303	30.0 min
4-bromoanisole	187	171	143	18.9 min
3-chlorotoluene (IS)	126	91	65	13.5 min

5.4. Results and Discussion

5.4.1. SPME optimization

Optimization studies were centered on TCA and geosmin, as these are more common fault compounds and the focal point of this study. TBA and 4-bromoanisole were also considered, but they did not carry as much influence on the final choice of parameters. Various SPME parameters were optimized based on peak area, using a whiskey sample spiked with 45 ng/L TCA, 450 ng/L geosmin, 450 ng/L TBA, and 45 ng/L 4-bromoanisole, which was 9x their

respective LODs. The studied parameters included alcohol content of the sample, salt addition, extraction time, and desorption time. Only one variable was changed at a time.

The alcohol content of the whiskey samples was studied at 20%, 10%, and 5% by volume. This was determined by halving the proof of the whiskey to obtain the ABV, and then diluting with water to the appropriate percentage of alcohol. The effect of the alcohol content on both the SPME fiber and analytes was investigated. The SPME fiber did not experience swelling at any of the tested alcohol percentages. However, all the studied analytes experienced matrix effects at 20% alcohol, as seen in Figure 5-1. It was observed that 5% alcohol produced the highest analyte response, and so this percentage was used for all future studies.

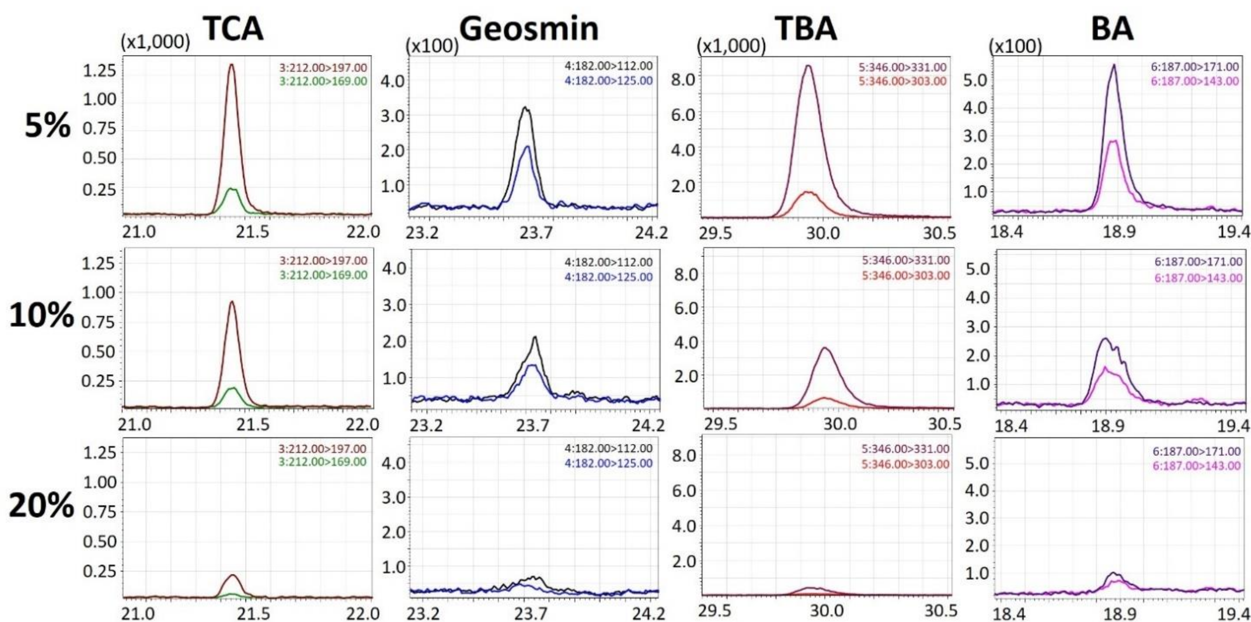

Figure 5-1. Effect of alcohol content on TCA, geosmin, TBA, and 4-bromoanisole (BA). The analytes were spiked into a whiskey sample at 9x their respective LODs. The MRMs for the individual analytes are presented on the same scale.

In headspace sampling, an inorganic salt is often added to the sample to increase its concentration in the headspace [247]. In this study, sodium chloride (NaCl) was used. Salt

addition of 1.5 grams, 3 grams, and 6 grams were investigated. The addition of 3 grams NaCl resulted in the highest recovery of the analytes.

Different extraction times (4 min, 10 min, 15 min, and 20 min) and desorption times (10 s, 1 min, 3 min, 6 min) were evaluated. It was determined that 4 min was not long enough for geosmin extraction. There were no significant differences in extraction efficiency between 10, 15, or 20 min, and thus 10 min was used as the extraction time to increase the speed of the analysis. In addition, a 1 min and 3 min desorption time resulted in the best recoveries of the analytes, and so 1 min was chosen for time efficiency.

5.4.2. Quantitation and Method Validation

Calibration curves were created for each compound, and 3-chlorotoluene was used as the internal standard at 500 ng/L, which was between the medium QC concentrations used for the analytes. Each curve contained at least 7 calibration levels, run in triplicate, and covered 2 orders of magnitude. The concentrations spanned over the range expected to be observed in contaminated whiskies.

The calibration data was used to determine limit of detection (LOD), limit of quantitation (LOQ), percent relative standard deviation (%RSD), and the correlation factor (R^2) for each analyte. Accuracies and precisions were calculated for the QCs. These attributes are listed in Table 5-2, and the concentration levels and QCs for each calibration curve are given in Table 5-S2.

The limit of detection was determined by running a low concentration point, and then halving the concentration until the signal-to-noise ratio of the peak was greater than or equal

to three. The limit of quantitation was taken as 3.3 times the LOD. The %RSD was calculated using triplicate measurements of each calibration level, and then averaged for the analytes. A %RSD lower than 25% was deemed acceptable. Three replicates of quality control levels representing low, medium, and high concentrations for each analyte were used to evaluate the precision and accuracy. The accuracies were calculated as the percent error, and precisions are expressed as the coefficient of variance (CV).

Table 5-2. Calibration curve information for the targeted analytes, including LOD, LOQ, correlation factor, and % RSD, as well as the accuracies (%error) and precisions (CV) for the QCs. LOD and LOQ are given in ng/L. QC levels for TCA and 4-bromoanisole were 25, 250, and 2000 ng/L. QC levels for geosmin and TBA were 100, 750, and 3000 ng/L.

	TCA			**Geosmin**			**TBA**			**4-bromoanisole**		
LOD	5			50			50			5		
LOQ	16.5			165			165			16.5		
R^2	0.9961			0.9974			0.9978			0.9965		
% RSD	21.1%			23.7%			18.5%			17.7%		
QC Levels	**L**	**M**	**H**	**L**	**M**	**H**	**L**	**M**	**H**	**L**	**M**	**H**
Accuracy	14.6	2.8	7.7	13.7	3.6	15.0	12.9	1.1	0.9	11.5	17.0	4.9
Precision	16.7	3.9	2.3	12.2	4.4	1.3	16.7	1.4	1.3	5.0	7.5	1.7

5.4.3. Whiskey Samples

The odor characteristics and possible origins of the compounds analyzed in this study are summarized in Table 5-3. Odor descriptions and origins of 4-bromoanisole were not found in literature, and therefore not included in the table.

Table 5-3. Possible origins and odor descriptions of the studied fault compounds.

Compound	**Origin**	**Odor**	**References**
TCA	Fungi contamination of cork, oak wood, water	Moldy, musty	[228–234,237,248]
Geosmin	Wet grains, water, moldy barrel	Earthy, musty	[230,233,237,239,240,249]
TBA	Fungi contamination of cork, oak wood, water	Moldy, mushroom	[228,229,236,237,245,250,251]

The method successfully separated the monitored analytes. Representative TIC and MRM chromatograms of a spiked Scotch whisky and Kentucky Straight Bourbon whiskey are given in Figure 5-2. Non-spiked samples were analyzed in triplicate, followed by triplicate spiked samples containing 450 ng/L of geosmin and TBA and 45 ng/L of TCA and 4-bromoanisole.

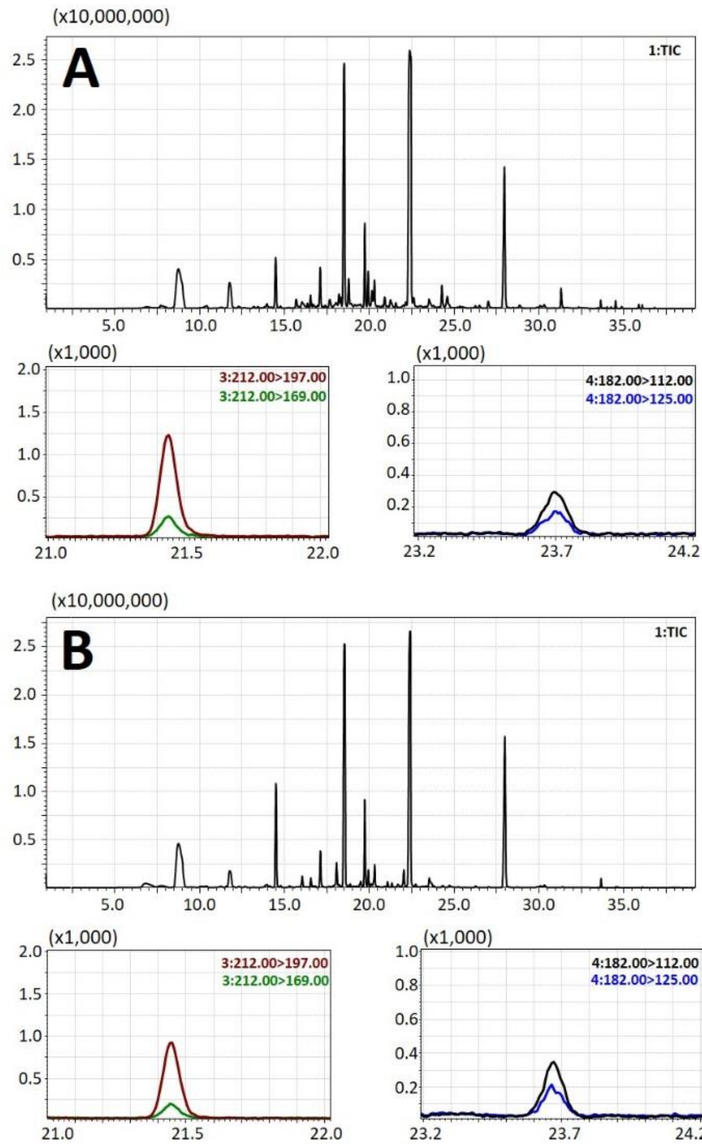

Figure 5-2. A. Represents the full TIC scan of a 92 proof Scotch whisky sample, and B. shows the full TIC scan of an 80 proof Kentucky straight bourbon whiskey sample. The TCA and geosmin MRMs of the spiked whiskey samples are shown below their respective TIC scans.

Distilleries may experience consistent or sporadic contamination from TCA and geosmin. Depending on how the contamination is introduced, these fault compounds can range from 1 ng/L to upwards of 2000 ng/L. Carbon filtration can greatly reduce or completely remove these off-flavors. The concentration threshold for filtration depends on the compound, the proof of

the whiskey, and the distillery. Figures 5-3A and 5-3B show a whiskey sample spiked with 45 ng/L TCA, and Figures 5-3C and 5-3D show the same whiskey after carbon filtration. It can be seen by comparing panels B and D that filtration successfully lowered the amount of TCA, reducing it from 45 ng/L to 20.5 ng/L. However, a decrease in peak intensity can be observed between the TICs in panels A and C, as well as the loss of later eluting peaks. While the reduction of TCA is necessary, some other, desirable flavors may also be stripped from the whiskey during carbon filtration. The ability to detect and quantify fault compounds at the filtration threshold would allow a distillery to determine if filtration is essential, or if it can be avoided to preserve the other flavor notes.

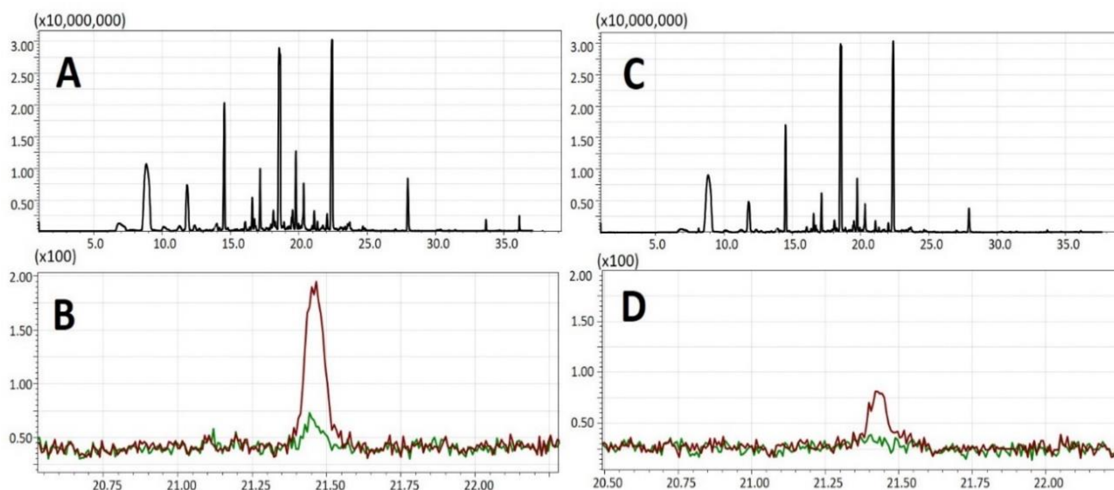

Figure 5-3. A and B represent a whiskey sample spiked with TCA. C and D show the same sample after carbon filtration.

Over the course of this research, 10 whiskey samples from different distilleries were analyzed for contamination by common fault compounds, and all were found to be free of the targeted analytes. Since these whiskies were already bottled for sale, it is assumed that any off-flavors had been previously removed by the distillers. However, spiked samples were used as

surrogate contaminated whiskies, which allowed for the proposed method to be validated. It was determined that this method can successfully detect and quantify TCA and geosmin, as well as other haloanisoles, at low concentrations in contaminated whiskies.

5.5. Conclusions

Distilleries rely on the consistent quality of their product for success in the competitive alcoholic beverage industry. Contamination of whiskies by TCA and geosmin can present a large problem for these companies if not properly addressed, as they impart musty, moldy, or earthy flavors at low sensory thresholds. These fault compounds can be introduced through the grains, water, or barrel used to make whiskey, and so their exact origin can be difficult to determine and remedy. It is therefore essential to detect and quantify common fault compounds in whiskey prior to bottling, so that filtration can be performed, and the consumer can enjoy a fault-free final product.

A HS-SPME-GC-MS method was developed and optimized for the quantitation of TCA and geosmin in various types of whiskey for routine quality control purposes. Each compound has a concentration threshold at which they must be removed by carbon filtration, so accurate quantitation methods are of high importance to distillers. The method described here simultaneously detected and quantified TCA and geosmin, as well as two other haloanisoles that may be present in whiskey. Expanding this method to include other fault compounds, such as tetrachloroanisole, ethyl carbamate, and 2-methylisoborneol, could be incredibly valuable for the alcohol industry and is currently being pursued.

Acknowledgements

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5.6. Supporting Information

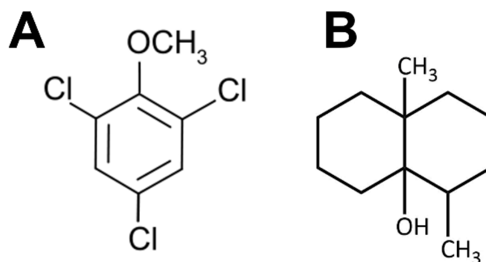

Figure 5-S1. Structures of (A) 2,4,6-trichloroanisole (TCA) and (B) *trans*-1,10-dimethyl-*trans*-9-decalol (geosmin)

Table 5-S1. List of whiskeys used in this study and their alcohol content.

Sample Number	Type	Proof	ABV
1	Scotch Whisky	80	40
2	Scotch Whisky	80	40
3	Scotch Whisky	92	46
4	Scotch Whisky	80	40
5	Scotch Whisky	80	40
6	Kentucky Straight Bourbon Whiskey	100	50
7	Kentucky Straight Bourbon Whiskey	86	43
8	Kentucky Straight Bourbon Whiskey	80	40
9	Kentucky Straight Bourbon Whiskey	80	40
10	Kentucky Straight Bourbon Whiskey	90	45

Table 5-S2. Concentration levels and quality controls (ng/L) applied to each calibration curve for the studied fault compounds.

	TCA	Geosmin	TBA	4-bromoanisole
Point 1	5	50	50	5
Point 2	10	75	75	10
Point 3	50	250	250	50
Point 4	100	500	500	100
Point 5	500	1000	1000	500
Point 6	1000	2000	2000	1000
Point 7	3000	4000	4000	3000
Point 8		5000	5000	4000
Low QC	25	100	100	25
Medium QC	250	750	750	250
High QC	2000	3000	3000	2000

CHAPTER SIX: Summary and Future Work

Beer and whiskey have been widely enjoyed throughout the world for many years. The recent expansion of the craft alcohol industry has introduced new and unique flavors to these timeless beverages. The analytical techniques and methods used for quality control or exploratory purposes should evolve and improve alongside them.

Craft breweries, particularly in the U.S., have seen a significant amount of growth in the past decade, improving upon existing styles and leading to the development of countless new brews. It is expected that the industry could benefit from the rapid analysis and classification of newly created beers for quality assurance purposes. A targeted LC-QTOF-MS method was developed and implemented to determine if five common beer styles could be distinguished from each other based on their iso-α-acid and phenolic content. Calibration curves for ten phenolic compounds including catechin, caffeic acid, chlorogenic acid, vanillin and quercetin were obtained and used to quantify the phenols in beer. Acceptable linearity, limit of detection and limit of quantitation were achieved using the optimized method. Various iso-α-acid stereoisomers and homologues were also identified and compared between styles, with IPAs and sours consistently containing the highest and lowest amounts, respectively. Although a few distinguishing features were identified, such as vanillin in stouts and catechin in IPAs, it was determined that targeted analysis alone was insufficient for the discrimination of beer styles. Therefore, further untargeted analysis was performed on the same beer samples.

The high mass accuracy of the QTOF allowed for untargeted analysis to be performed, which gave a broader view of the beers' nonvolatile profiles. This was followed by various

multivariate analyses and machine learning techniques. PCA illustrated the variance within the data set, which gave evidence that there was some difference on the chemical level between these styles. This was confirmed using three statistical approaches, ANOVA, PLS-DA, and Random Forest, which resulted in a list of formula predictions for the 54 most influential metabolites. These key features were further researched to attain tentative identifications. This study indicates that advanced data science techniques could be a useful tool for beer style quality assurance.

This quality assurance method could be further developed by performing a similar study on an expanded beer list, perhaps on substyles within a style. For example, IPAs can be categorized as an American IPA, West Coast IPA, red IPA, black IPA, hazy IPA, double IPA, triple IPA, double dry-hopped (DDH) IPA, imperial IPA, and so on. Beers within the same style would be much more difficult to differentiate using sensory analysis than the five studied styles, as these were quite visually and aromatically distinct. Performing multivariate analysis on a single type of beer could help to identify more specific metabolites that are exclusive to a style. This could be augmented by the development of a database containing the unique profiles of various beer styles. The database would be established by performing "chemical multi-fingerprinting" to obtain profiles for the beers using assorted analytical techniques. This would include LC-MS for the nonvolatile compounds, GC-MS for the volatile portion, and MALDI-TOF-MS for the biological profile of a style. The biological section would be particularly interesting for the sour beers, which rely on lactic acid-producing bacteria, acetic acid-producing bacteria, and *Brettanomyces* yeast for their flavor. Ideally, a beer could be analyzed on one or more instruments and its profile compared to the database for rapid and confident categorization.

There is also the potential to identify unique characteristics of a beer, which could be utilized for marketing purposes. Furthermore, a database could be beneficial for quality control labs to screen for fault compounds that contribute to off-flavors.

Fault compounds can be economically problematic in distilling as well as brewing. Some of the most common faults found in whiskey include TCA and geosmin, which cause moldy, musty, or earthy smells and flavors. Both compounds have sensory thresholds in the low ng/L range, and so sensitive detection methods are required for their analysis. A HS-SPME-GC-MS method was developed to identify and quantitate TCA and geosmin, as well as other fault compounds, in scotch and Kentucky straight bourbon whiskies. Multiple reaction monitoring was used to reduce background noise and increase sensitivity for the analytes. Calibration curves were made for each compound with acceptable linearity, limit of detection and quantitation, and %RSD. The method was able to quantify the fault compounds near their sensory threshold, which could be advantageous to distilleries. If these compounds were detected above a certain concentration in a sample, the distillery would be notified so that the contaminated whiskey could be carbon filtered to remove the faults before being bottled for sale.

While TCA and geosmin are common fault compounds, there are a number of others that could also be present in whiskey. Therefore, expanding this method to include a wider variety of off-flavors, such as 2-methylisoborneol and other chloroanisoles, is being pursued. This would allow distillers to simultaneously screen for a broad range of fault compounds, which may have been overlooked during sensory analysis. Additionally, TCA and geosmin could be tracked throughout the distillation process to determine their origin, be it from wet grains,

contaminated barrels, or the water used. The ability to verify their source could be incredibly beneficial to distilleries that have experienced consistent contamination from these fault compounds, permitting them to remedy the infected source and avoid contamination in future batches. This would ultimately save the time and resources required to filter the product.

The continual advancement of alcoholic beverage analysis will ensure that these products remain safe and enjoyable to the consumer, and economically viable for the producer.

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BIOGRAPHICAL INFORMATION

Hailee Ratcliffe received her Bachelor of Science degree in Chemistry from Texas State University in 2017 (as Hailee Anderson). While there, she worked as an undergraduate research assistant for two years in Dr. William Brittain's Organic Chemistry lab, where she developed an azobenzene-derived photoresponsive hydrogel. Hailee began her Ph.D. journey shortly after at the University of Texas at Arlington. She joined Dr. Kevin Schug's Analytical Chemistry lab in 2018, where she discovered an interest in the science of brewing and distilling. Since then, her dissertation research has focused on the application of mass spectrometry methods for the analysis of flavor compounds in craft beer and whiskey. She collaborated with a local brewery on her beer projects, and completed an internship with a growing distillery in Fort Worth, TX. She graduates with her Ph.D. in Chemistry from the University of Texas at Arlington in August of 2021 and plans to start a career in the alcoholic beverage industry.

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CHAPTER 2 – A review of the analytical methods used for beer ingredient and finished product analysis and quality control

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CHAPTER 3 – Target profiling of beer styles by their iso-α-acid and phenolic content using liquid chromatography–quadrupole time-of-flight–mass spectrometry

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CHAPTER 4 – Profiling of contemporary beer styles using liquid chromatography quadrupole time-of-flight mass spectrometry, multivariate analysis, and machine learning techniques

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