Characterization of aroma and flavor compounds present in lambic (gueuze) beer

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ABSTRACT

Lambic beer is one of the oldest beer styles still being brewed in the western world today and the only beer that is still brewed through spontaneous fermentation. Lambic beers are only produced within a 500 km radius of Brussels because of the natural microflora found within the air in that region. Little is known about the chemical composition of lambic beers. The objective of this research were (1) to compare SPME and SAFE for the isolation of flavor and aroma compounds, (2) determine the volatile composition and acids of commercially available lambic gueuze using SPME/GC-MS and HPLC, and (3) determine the major aroma compounds of aging lambic beer using GC-O. In comparing the two extraction methods, both SPME and SAFE were able to identify a similar number of chemical compounds, however SAFE identified a greater number of acid compounds. A total of 50 compounds were identified within the nine commercial brands of lambic beer. HPLC was used in the identification and quantification of acetic and lactic acid. The concentration of acetic acid for the commercial brands ranged from 723 mg/L – 1624 mg/L while lactic acid ranged 995 – 2557 mg/L. GC-O was used in the analysis of aged (3, 6, 9, 12, and 28 month) lambic beer samples. As the samples increased in age, the number of aroma compounds detected by the panelists increased as well. Panelists were only able to detect nine aroma compounds in the three month old sample, while seventeen compounds were detected in the twenty eight month old sample. The research conduct increased the number of volatile and semi-volatile compounds previously identified in lambic beer from twenty-seven up to fifty compounds.

In memory of my grandfathers
Daniel Max Thompson MD,
And
Frances John Crump
Always encouraging me to pursue my dreams and never let anything get in your way. I know they would be very proud of me in all that I have done and will do in the future.

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ABBREVIATIONS

AEDA	aroma extraction dilution analysis
CHARM	Combined hedonic aroma response measurement
DMS	dimethyl sulfide
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
4-EG	4-ethylguaiacol
4-EG	4-ethylphenol
FD	Flavor dilution
FID	Flame ionization detector
GC-MS	gas chromatography-mass spectrometry
GC-O	gas chromatography – olfactometry
HPLC	high performance liquid chromatography
IVA	Isovaleric acid
LRI	linear retention index
MS	Mass spectrometry
OAV	odor active value
PA	polyacrylate
PDMS	polydimethylsiloxane
PEG	Polyethylene glycol
PFPD	Pulse flame photometric detector
SAFE	solvent-assisted flavor evaporation
SDE	simultaneous distillation extraction
SMM	S-methyl methionine
SPME	solid phase microextraction

Chapter 1: Introduction

"The lambic family of beer is not everyone's glass of beer, but no one with a keen interest in alcoholic drink would find them anything less than fascinating. In their "wildness" and unpredictability, these are exciting brews. At their worst, they offer a taste of history" (*1*).

Lambic beer is one of the oldest styles of beer still being produced by spontaneous fermentation in the Western world today (*2*). This style of beer is mainly produced within 500 square kilometers of Brussels and the Payottenland, a valley on the Senne River located on the western side of Brussels. This region seems to have the perfect combination of airborne microorganisms that can create a consistent beer through spontaneous fermentation (*3*). Traditional beers, regardless of whether they are ales or lagers, normally take anywhere from several days to several months to ferment, while lambic beers can take anywhere from one to two years before the fermentation process has been completed (*4, 5*). The lambic brewing industry is in jeopardy because of the very product they produce. The long fermentation period requires breweries to store approximately \$100,000 – \$300,000 worth of product resulting in thousands of dollars in taxes owed before the beer even has time to reach maturity (*2, 3*).

Alcoholic beverages are a complex mixture of volatile, semi-volatile and nonvolatile compounds belonging to several different chemical families including higher alcohols, ethyl esters, fatty acids, ketones, isoamyl esters, aldehydes and ketones, furanic compounds, terpenoids, C13-norisoprenoids, and volatile phenols (*6*). The complex combination of chemical compounds found in alcoholic beverages plays a vital role in their appearance, aroma, flavor, and mouth feel. The combination of taste and olfactory properties are often responsible for the quality, character, and consumer acceptability of alcoholic beverages. Similar to other alcoholic beverages, a beer's aroma is made up of hundreds of different chemical compounds with

different polarities and varying concentrations. The quality of the raw ingredients and the fermentation process play major roles in the chemical composition of a product (*7*).

Alcoholic beverages contain over 800 volatile compounds, however only ten to thirty are generally aroma active (*8-10*). A number of these compounds are found in several different alcoholic products including beer, wine and distilled liquors, however the concentration tends to vary between these beverages. Compounds present in different alcoholic beverages have the ability to affect the aroma and flavor in individual, synergistic, or antagonistic ways. Some volatile compounds contribute to the aroma and flavor of the alcoholic beverage while others just enhance the background profile of the product (*11*). The aliphatic alcohol, n-propanol, amylalcohol (n-pentanol) and aromatic alcohols β-phenyl ethanol, and benzyl alcohol are examples of higher alcohols also known as fusel alcohols, which are found in alcoholic beverages. Fusel alcohols can have either positive or negative effects on both the aroma and flavor of the beverage. Fusel alcohols, at their optimal concentration, can impart a fruity note, however when the concentration exceeds 300 mgL-1, this results in a strong pungent smell and taste. Fatty acids are usually produced in the early stages of the fermentation process. Fatty acids also play an important role in the aroma profile of alcoholic beverages. Acetic acid smells like vinegar, propanoic acid has a goaty smell, butanoic acid (spoiled butter), and lactic acid are short chain fatty acids that are by-products of microorganisms during fermentation.

Ethyl esters (which produce fruity flavors) are produced closer to the end of the fermentation process and are another group of chemical compounds found in beer, wine, and whiskey. Ethyl esters play a huge role in the sensory profile of the overall product. The ethyl esters that typically dominate in beer, wine and whiskey are straight chain fatty acids and acetates which are produced by an enzymatic catalyzed condensation reaction between aycl-CoA

and a higher alcohol (*12*). The rate of ester formation in an alcoholic beverage related to the concentrations of acyl-CoA and fusel alcohols and the overall enzymatic activity affecting the formation and breakdown of the esters. Ethyl acetate (fruity, solvent-like), isoamyl acetate (banana, pear), isobutyl acetate (banana, fruity) ethyl hexanoate (green apple), ethyl octanoate (fruity, soapy), ethyl decanoate (floral, soap), and 2-phenylethyl acetate (honey, fruity, flowery) are all esters that have a significant impact on the aroma profile of an alcoholic beverage compared to less volatile longer chain esters. Based upon the combinations and concentration of esters present, they can have a synergistic effect on the aroma profile of the beer (*6*). Since most esters are present in alcoholic beverages around their odor-detection threshold levels, small changes in their concentration can have a huge impact on the beverages flavor and aroma (*12*).

The overall chemical composition of an alcoholic product such as beer is complex and beer contains many compounds at different of concentrations. These chemical compounds can have different degrees of polarity, and volatility and the wide range of concentrations found within beer or any other alcoholic beverage can be at concentrations of as little as ng/L (ppb) to mg/L (ppm). The overall influence that a compound has on the aromatic profile of a product can vary. Often, compounds present at trace amounts have a greater influence on the aroma profile than those found at higher concentrations (*7*). The key factor is the concentration present relative to the odor detection threshold. Aroma research typically begins with isolating and identifying aroma active compounds in a food sample (*9*). In order to characterize aroma active compounds, volatile compounds must first be separated from nonvolatile compounds (*13*).

Trace amounts of aroma compounds are located throughout the food matrix, making these compounds difficult to extract and concentrate. It can be extremely difficult to isolate trace compounds in foods that contain lipids, proteins, complex carbohydrates, sugars, and water.

Water is one of the most abundant volatile compounds in food, so any procedure that involves placing a vacuum or a distillation process extracts water along with the other volatile compounds within the sample. Most isolation techniques are based upon solubility and volatility of aroma compounds. While solvent extraction is a useful technique, it does have its downfalls. A weakness with this method is that compounds have different partition coefficients and will be extracted at different times and concentrations throughout the process. Another weakness of solvent extraction is that this method will co-extract lipids from the sample which would require further separation prior to analysis (*9*).

The hardest part in determining the volatile compounds responsible for the aroma profile of a food product is finding a suitable method for isolating the odor active compounds (*14*). The method selected for extraction should be able to successfully isolate the compounds that contribute to the overall flavor of the product without altering or causing the formation of artificial compounds. Volatile and semi-volatile compounds in alcoholic beverages have been previously analyzed using a number of different methods: liquid-liquid extraction, solid phase microextraction (SPME) (*15*), simultaneous distillation extraction (SDE) (*16*), and SAFE (solvent-assisted flavor evaporation) (*17*). All of these methods have their disadvantages and advantages.

Simulatneous distillation and extraction (SDE) is one of the oldest and most widely used techniques for separating volatile compounds from nonvolatile components (*16*). SDE is often used in research to isolate volatile compounds because of its versatility and simplicity (*18, 19*). SDE has been used to isolate volatile compounds in beer, spices, fruits and wine (*16, 20-22*). While SDE is a simple and fast aroma extraction technique, because of the elevated temperatures applied during distillation, this may cause the formation of artificial compounds (*19*).

Solid phase microextraction (SPME) is a simple, fast, and solvent-free extraction technique that utilizes a small 1-2 cm piece of fused silica (*23*). SPME was first developed by Pawlisyn in 1997 to analyze environmental samples like air, soil, and water (*15, 24*). Since then, it has been used to analyze volatiles compounds found in foods (*25*). The fused silica fiber can be coated with either a liquid or solid phase to extract and concentrate these compounds. This method is based upon an equilibrium being reached between the volatile compounds present in the headspace above the sample and the concentration on the coated fiber (*23*). Once the fiber has reached equilibrium within the sample, the fiber is then thermally desorbed into a GC carrier gas releasing the volatile compounds to be analyzed. The volatile profile obtained during analysis is based upon the profile of the sample and by sampling parameters. While this method can provide excellent results, the fibers used with this method possess a limited linear range and competition between volatile compounds for binding sites, which could potentially cause errors (*25*).

Direct solvent extraction is a simple and efficient technique used for aroma isolation. A major limitation of this method is that it should not be used with foods that contain lipids, because the aroma compounds as well as the lipids will be extracted. Lipids must be separated from the flavor compounds prior to analysis. Molecular distillation, steam distillation, and dynamic headspace are three techniques currently used to separate aroma compounds from lipids. Solvent extraction can be as simple as placing a food sample into a separatory funnel with a solvent and shaking, or as costly and complicated as using pressure chambers and supercritical CO_2. Supercritical CO_2 has a low boiling point, leaves no residues to interfere with sensory analysis, and has the ability to penetrate food samples. Some of the drawbacks of this method are

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the pressure and cost requirements to perform, small sample size, and the nonpolar nature of carbon dioxide.

Research Objectives

The purpose of this research was to study the aroma and flavor profile of commercially available lambic beer. The overall objective of this research was to help brewers have a greater understanding of what makes up this particular style of beer flavor and aroma. The continual growth of the craft brewing industry within the United States has people looking for unique good quality beer. A number of brewers are fascinated by lambic beers because of its ability to bridge the gap between beer and wine. By unlocking the mystery of what makes a lambic beer unique, hopefully future brewers will be able to successfully make a copy of this particular style of beer without having to wait two years to maturate.

The project has three aims:

I. Compare two extractions methods: SPME (solid phase microextraction) and SDE-SAFE (solvent-assisted flavor evaporation) for the isolation of flavor and aroma compounds in lambic beer.

II. To determine the volatile composition of commercially available lambic gueuze using SPME/GC-MS, and HPLC.

III. Determine the major aroma-impacting compounds of aging lambic beer using GC-O.

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Chapter 2: REVIEW OF THE LITERATURE

History of Lambic Beer

Lambic beer is one of the oldest styles of beer still being brewed in the western world today (*1, 2*). Over the past 2000 years, Belgium has been a major thoroughfare in Europe. Belgium has seen its share of foreign rulers from the Romans, Germans, Dutch, Burundians, Austrians, Spanish, and French with each one contributing something to the Belgian culture including brewing. Some historians believe that the Romans brought with them an ancient brewing techniques. Beer, in ancient times was almost certainly fermented through spontaneous fermentation. Brewers who used this process would expose the wort to the air, allowing it to be inoculated with whatever wild-born yeast and other microorganisms that were present in the air. Only a 500 square kilometer area around Brussels and the Payottenland, a valley on the Senne River on the western side of the city, has the right combination of airborne microorganisms necessary to create a consistent lambic beer through spontaneous fermentation (*3*). Sikaru is an example of a spontaneously fermented beer. It was produced 5000 years ago by Sumerians in Mesopotamia. An ancient Sumerian tablet was found to contain the composition of sikaru on it, which is virtually identical to that of a lambic beer today. A Sumerian brewer would use sixty "silas" of barley malt and thirty six "silas" of a Sumerian wheat variety called épeature. This would equate to 62.5 percent malt and 37.5 percent raw grain. Cantillon, a lambic brewery in Brussels, uses a similar recipe consisting of 830 kg (1,830 pounds) of barley malt and 460 kg (1,014 pounds) of unmalted wheat per 52 HL, which would equate to 65 percent malt and 35 percent raw grain (*1*). One difference that is apparent between a lambic beer and a sikaru beer is that lambic beer utilizes aged hops for flavoring, while ancient Sumerians used spices such as cinnamon to flavor sikaru. Spontaneous fermentation of the sikaru would have most likely been

caused by the yeast *Saccharomyces* and *Schizosaccharomyces.* Similar to a lambic beer, sikaru beer was a luxury product and was often used as payment for workers during ancient times (*1, 3*).

A Beer is Born

Little is known about the origin of the word lambic since there are a number of different theories on how the word came to be. One theory suggests that the word came from one of four Belgian villages: Lembeek, Borcht-Lombeek, Onze-Lieve-Vrouw-Lombeek or Sint-Katelijne-Lombeek. Another possibility is that it came from the Spanish word lambicado, which means "carefully prepared." The creation of lambic beer has been attributed to Duke Jean IV of Brabant who in 1428 decided to experiment with brewing beer. He was tired of drinking the same style of beer so he decided to macerate and boil the barley with hops in a still, known as an alambic. The Duke's experiment was deemed a success and the resulting beer is known today as a lambic.

Old unblended lambic beers are probably similar to the lambics served centuries ago. Unblended lambics are unheard of outside of Brussels and Payottenland. The more common lambics served today are gueuze, faro, and other various lambics sweetened and flavored with fruits.

Gueuze is result of the careful blending of different aged and different tasting gueuze beers (*4, 5*). Gueuze is similar to champagne in that they both undergo a secondary fermentation period in the bottle. The *Method Champenoise* often is attributed to in the 18th century by the Benedictine monk Dom Pérignon. A century later the mayor of Lembecq, who was a brewer and an engineer by the name of Cayaerts, decided to utilize this method to referment their lambic beer in a bottle using this process. Thus, gueuze was born.

Faro comes from the Spanish word for barley wine. Faro is a of young lambic beer sweetened with candy sugar. Fruit lambic are traditionally made with cherries (kriek the Flemish word for cherries) and raspberries (framboise from the French). Peaches, grapes, plums, pineapples and black currants have also been used with some success in lambic beers (*1, 3*).

Lambic Brewing Industry

 Similar to other countries, in Belgium beer is only produced by a few large breweries. The two largest breweries in Belgium are Stella Artois and Maes. The success of these two breweries has not prevented the growth of smaller, artisan breweries from arising. The recent increase in small artisan breweries can be attributed to the increase in specialty beer drinkers. This new trend has caused a number of medium-sized lager breweries to start brewing specialty beers, while the larger breweries continue to take over existing smaller breweries (*1, 3*).

The most well-known lambic brewery in Belgium is Cantillon. Cantillon is not only a working brewery, but a museum as well. The brewery was opened in 1937 by Paul Cantillon with the help of his two sons Robert and Marcel. The brewery, however, is no longer run by the Cantillon family but by Jean Pierce Van Roy, a former school teacher turned brew master. Jean with his wife Claude, turned the brewery into a working museum in 1978.

Another well-known lambic brewery is Lindemans. This brewery began producing lambic beer in 1809 on the Lindeman's farm in Sint-Pieters-Leeuw and is currently the leading exporter of lambic beer to the United States. René Lindemans, the brew-master for Lindemans, combines old world tradition with modern brewing techniques. Lindemans still uses traditional mashing and wort cooling in swallow open air tank.

Lambic brewers are currently jeopardized by the very products they produce because of the time-consuming process required to produce their beer. Lambic beers spend anywhere from a

a year to three years in casks or barrels before they are ready for consumption. Therefore, breweries can have anywhere from $100,000 to over $300,000 in product ageing in a barrel at any one time. The current tax system in Belgium is also very harsh on lambic breweries requiring these breweries to pay taxes on their beer within a year after brewing. This is difficult for breweries because true lambics are required to be aged a minimum of a least one year prior to saling. In the end, brewers owe money to the Belgium government often before the product is sold. Another problem is that lambic brewers are beginning to retire and they do not have anyone to take over the old and artful craft of lambic brewing (*1, 3*).

Characteristics of Lambic Beer

Sensory

Before a person can take their first sip of a lambic or gueuze beer he/she must first recognize that this is no ordinary beer and what constitutes as a defect (volatile acidity, lactic acid, Brettanomyces character) in other styles of beer is considered desirable for this particular beer. Gueuze can range in color from golden yellow for young gueuze to light amber for older ones. Similar to bottle fermentation for champagne, gueuze undergoes a similar process to produce its high level of carbonation and "gassy" mouth feel like champagne. Once the bottle is open, beer gushes out due to the high levels of carbonation resulting in foam whose color falls between the white foam of normal beer and the yellowish foam of sparkling wine. Bulk fermented and filtered lambic beer typically has a lower CO_2 amount (2.0 to 2.4 volumes) than that of commercial ales and lagers (2.4 to 2.8 volumes). Once a glass of gueuze is poured, the higher volatile acidity, which is particular high in this style of beer, gives off a vinegar, goaty, and rancid aroma. The fruity aromas that often come through can be described as apple, melon, and even apricot. Brettanomyces plays a role in the aroma composition of the beer with its horse

and barnyard smells (*1*). The overall concentration of 4-ethylphenol and 4-ethylguiacol, two products of Brettanomyces growth, have a low enough concentration that does not seem to cause the drinker to be disgusted (*6, 7*). The aroma profile of gueuze is balanced by fruity esters plus the woody, vanilla aroma coming from the wooden casks used for fermentation. The wort also provides some key aroma notes to the beer like caramel and/or nutty flavors (*1*).

Upon drinking the very first sip of lambic beer, the drinker often experiences sour, acidic, and at times astringent tastes that surprise people because of the lack of bitterness associated with hops. Because old hops are used, little to no bitterness is found in the finished product. Depending on the age of the beer, sweetness can range from very dry to fairly sweet for a bulk fermented filtered gueuze. Due to the high level of carbonation, lambic beer will normally foam in one's mouth causing a stinging sensation on the tongue and palate. In traditionally made gueuze, the gueuze contains more tannins from the wheat and wood, giving it a thinner taste, while bulk fermented gueuze is thicker and tends to have a smoother mouthfeel. Once the drinker finishes a glass of gueuze, a warming sensation caused by the higher ethanol content will linger, along with the fruity and horse aromas, but a puckering effect caused by the acid and astringency of the beer often occurs (*1*).

Flavor

Flavor plays an important role in the acceptability of a food product. Flavor is a combination of two sensations--odor and taste (*8*). The sensation of taste is made up of sweet, salty, bitter, umami and sour as well as the chemical feelings of cooling, astringency, spicy, bite, metallic, and flavor (*9*). The most important flavor compounds in both lambic and other beers are the higher alcohols and esters, organic acids, dimethyl sulfide, and diacetyl; this is very similar to that of American lagers (*10, 11*).

Fusel Alcohol and Esters.

Ethanol is just one of several alcohols that can be found in beers along with higher alcohols, fusel alcohols, which are considered important by products of fermentation. Esters are also important flavor and aroma compounds in beer. These compounds provided strong and oftentimes penetrating fruity flavors to the beer. Similar to ethanol and fusel alcohols, these compounds are also produced during the main fermentation stage (*12*). Based upon a study by Harrison, it was determined that a number of different alcohols contributed to the flavor of beer: iso-amyl, phenethyl, propyl, and iso-amyl alcohol (2-methylbutanal) (*8*).

The concentration of higher alcohols in lambic beers and other styles of beer are similar, however the concentration of esters are very different (*13*). Ethyl acetate can be found in much higher concentrations in lambic beers than any other styles (*14*). Ethyl acetate has a solvent-like fruity aroma with an odor detection threshold level of 30 ppm (*14, 15*). The average concentration of ethyl acetate is between 8 – 48 ppm for traditional beers, while it is 33.4 – 67.6 ppm for filtered gueuze and 60.9 – 167 ppm in unfiltered gueuze (*14*). Isoamyl acetate is another compound found in lambic beer in levels that differ from traditional beers. In lambic beers, isoamyl acetate is found in much lower concentrations then in traditional beers. In traditional beers isoamyl acetate can range anywhere from 1.2 – 2.8 ppm in lagers and 0.7 – 3.3 in ales (*14, 15*). Ethyl lactate is a compound normally found in whiskey (*16*), wine, sherry (*17*), and cider (*18*), but is not traditionally found in beer. The few times that ethyl lactate has been reported in beer, the overall concentration has been so low that it was assumed that it had no effect on the overall aroma of the beer. That is not the case in lambic beers where the concentration of ethyl lactate has been determined to be 483 ppm, well above the taste threshold of 50 ppm and the odor threshold of 14 ppm (*8, 13*).

The ethyl esters of higher fatty acids, ethyl caprylate, and ethyl caprate, are traditionally found in lambic and gueuze beers. These ethyl esters are normally absent in lagers and present in only small concentrations in ales (*19, 20*). Both ethyl caprylate and ethyl caprate are considered to be typical aroma and flavor compounds of lambic and gueuze beer. The ethyl esters of caproic, caprylic, and capric acids give lambic and gueuze beer its wine and fruity flavor. The threshold values for these compounds are as such 0.2, 0.8 and 1.1 ppm respectively for cproic, caprylic, and capric acids (*14, 18*).

Organic Acids. Pyruvic acid, L-malic acid, acetic acid, and L-lactic acid are found in different concentrations in different styles of beer (*21*). Lambic and gueuze are well known for their high levels of acid. It has been reported that gueuze has a lactic acid concentration of l500 - 3400 ppm and acetic acid had a concentration of approximately 700 - 1200 ppm (*13*). Propionic, isobutryic, and butyric acids are also found in somewhat higher concentrations than in other styles of beer. Ales and lagers have much lower acetic and lactic acid concentrations then gueuze beer. The concentration of acetic acid in ales and lagers tends to range anywhere from 60 – 140 ppm, while lactic acid in gueuze beer ranges between 70 – 200 ppm (*8, 20*). Most people have a flavor threshold of acetic acid at 200 ppm while the flavor detection threshold for lactic acid is 400 ppm (*8, 22*). The taste threshold levels of butyric, propionic, and isobutyric acid are approximately 1, 100, and 200 ppm respectively. Butyric acid is one of the few acids in gueuze beer that is usually found over its threshold concentration (*13*). Butyric acid can be described as having a cheesy or rancid aroma (*14*). Lambic and gueuze beers are known for containing high levels of caprylic (C8) and capric (C10) acids. Capric acid concentration in gueuze beer usually exceeds 2 ppm, which is slightly higher than the concentration found in lagers or ales (*19*).

Methyl Sulfunyl Methane.

Dimethyl sulfide (DMS) is thioether which plays an important role in the flavor of beer and is also the main volatile sulfur compound in beer (*23*). Malt, hops and water can all be possible sources of sulfur. While the primary sources of volatile and semi-volatile compounds do not always come from the raw materials themselves, they can be generated during malting and fermentation (*24, 25*). DMS has an odor detection concentration threshold of approximately 30 – 50 µg/L, and once the concentration reaches between 50 – 100 ppb it starts to affect the taste. DMS becomes very detrimental to the beer when its overall concentration is greater than 100 µg/L because of the cooked vegetable, corn or cabbage like smell it produces (*26, 27*).

DMS can arise three different ways in beer: (1) breakdown of compounds during wort boil and malting (*28*); (2) yeast metabolizing compounds produced during boiling or kilning (*29*); and/or (3) bacterial contamination (*30, 31*). S-methyl methionine (SMM) is a major precursor for DMS. This compound is produced during barley germination, but is later largely destroyed during kilning and boiling (*32*). *Enterobacteria* have been found to possess the ability to produce DMS along with several other sulfur compounds (*33, 34*). Some *Enterobacteria* may also enzymatically produce the precursor SMM, resulting in the spoilage of the wort through excess accumulation of DMS in both lager and ale brews (*10*). White and Parson determined that all yeast that they analyzed were capable of producing DMS and were able to produce larger quantities then microorganisms during the fermentation of lager wort (*35*). Still, yeast have the ability to produce small amounts of DMS even when all the precursors are removed prior to the start of the main fermentation (*10*).

DMS in lambic and gueuze beer most likely comes from the metabolism of Enterobacteria found in the wort (*2*). The maximum concentration of DMS was reported by Van

Oevelen and his colleges to be 450 ppb two weeks after the start of the main fermentation. The high concentration of DMS is quickly lowered by the stripping caused by the formation of CO_2 during the main fermentation. The average concentration of DMS normally found in bottles of gueuze is roughly 54 ppb (ranging from 25 to 75 ppb). There is no significant difference in the levels of DMS found in lambic beers and traditional lagers or ales. When the mean level of DMS is higher than 30 ppb, in a slight cabbage to vegetable-like odor may be reported in the beer (*34*).

Diacetyl and Related Compounds. Diacetyl and pentanedione have similar taste and flavors which can be described as buttery, honey-like, and sweet. Diacetyl can be detected in small quantities in traditional lagers, however strongly hopped or malted beers tend to mask the aroma (*36*). The threshold for diacetyl in lager beers is $0.1 - 0.2$ ppm, while for ales it is $0.1 - 0.4$ ppm (*10*).

As a result of the spontaneous fermentation needed to produce lambic beers, the fermentation process cannot be controlled, which results in large variabilities. Variability between each cask is a result of the pores within the wooden casks and the interaction the beer has with oxygen. Casks that are placed in dry areas or areas that undergo a lot of vibration run the risk of spoilage by acetic acid bacteria. Based upon the study carried out by Van Oevelen and his colleagues, the main aroma characteristics compounds of lambic beers were identified as ethyl lactate, ethyl acetate, acetic acid and lactic acid. Higher levels of both acetic acid and lactic acid are associated with higher amounts of both ethyl acetate and ethyl lactate. Lambic beers also contain high levels of caprylic (C_8) and capric (C_{10}) acids and ethyl caprate. Gueuze beer tends to also have a low level of phenethyl acetate (*13, 19, 34*).

Manufacturing of Lambic Beer

      Spontaneous fermentation has been used in the production of beer, wine, and cider for many centuries. However, spontaneous fermentation has been recently replaced in many cases with a mixture of known microorganisms, allowing the producer to maintain more control over the fermentation process. Lambic and gueuze are two types of beer that are still produced today using spontaneous fermentation of wort (*2, 13*). A Belgium royal decree prohibits the use of transferable cultures requiring brewers to continue to allow the natural microfloral to inoculate the wort (*37*). This particular style of beer is mainly produced within fifteen kilometers of Brussels. (*2, 13*).

      Lambic, gueuze, and other similar styles of beer are complex beers made from a few key components including malted barley, unmalted wheat, aged hops, and fruits. While the ingredients play a vital role in the brewing process, the fermentation process plays a key role in the uniqueness of this style of beer. When comparing American ales and lagers to lambic beer, the key differences between these beers are that lambics are produced by cooling of the wort in open air and utilizing a spontaneous fermentation in wooden barrels.

      A major difference between gueuze/lambic beers and American ales and lagers is the final specific gravity. American ales and lagers range between 1.015 – 1.019 (3.7 – 4.8 degrees Plato), while gueuze and fruit lambics range between 1.008 – 1.048 (2.2 - 12 degrees Plato) which is half that of traditional ales and lagers. Unlike American beers only 50 - 68% of the fermentable sugars are metabolized by the yeast, the real degrees of fermentation (RDF) for lambics and gueuzes varies between 63 – 82%. Reducing sugars (such as maltose) are found in trace amounts to 0.8% in gueuzes, while in fruit reducing sugars can range from trace amounts to

two percent. Fruit lambics tend to have higher amounts of reducing sugars because a few breweries will sweeten the beer with sucrose syrups instead of using real fruit.

Lambic beers contain a wide range of alcohol concentrations. The average ethanol concentrate for Faro is 3.5 percent (2.8 percent w/w), while gueuze varies from 5.3 – 6.2% v/v (4.2 – 5 w/w). Fruit lambics have the highest ethanol average at 6.5 percent v/v (5.2 percent w/w) (*1*).

Traditionally lambic beer is brewed during the colder months of the year, because of potential spoiling of the wort during the warmer summer months. The actual brewing season for the beers normally starts sometime in September and goes to sometime in April (*5*). The wort is cooled overnight in open shallow vessels where yeast and other microorganisms from the environment are able to inoculate the wort. Once the wort has cooled, it is then pumped into either wooden or metal casks that are placed into non air-conditioned warehouses. Temperature variations within these warehouses can vary from 0°C to 25°C (*2, 13*).

Fermentation

Lambic beer is unique in that fermentation is caused by wild yeast and other microorganisms. The fermentation process itself is broken down into four distinct stages with different microorganisms playing a crucial role in each stage, which ultimately complete contributes characteristics to the beer flavor or aroma. The first stage is dominated by the wild yeast *Klacekera apiculata* and enteric bacteria. The second stage is dominated by *Saccharomyces* followed by lactic acid bacteria in the third stage. The fourth and final stage is dominated by Brettanomyces. Figure 2.3 Shows a graphical representation of the flavor development that occurs during the spontaneous production of lambic beer (*2, 3*).

The first stage of fermentation for lambic beer is dominated by enteric bacteria and *K. apiculata.* Fermentation begins three to seven days after the wort has become inoculated with the wild yeast *K. apiculata* and enteric bacteria. *K. apiculata,* reaches a maximum concentration of 10^5 cells/mL within the first week of fermentation, but is quickly out-competed by the Saccharomyces species and *K. apiculata* dies off (*10*). *K. apiculata* will only ferment glucose and not maltose. *K. apiculata* and *Enterobacteriacea* are both fast growing microorganisms and cause the pH of the wort to drop from 5.1 to 4.6 because of the acetic and lactic acids being produced. The diacetyl content during this stage is close to 1 g/L while the dimethylsulfide (DMS) is approximately 500 mg/L. *K. apiculata* has the ability to secrete protease into the wort, which can then break down the protein that did not precipitate during the boil. The amount of protein still left in the wort is higher than for an all barley malt wort, because of the raw wheat used in the mash which has higher protein than barley malt (*1*).

The third stage of fermentation actually overlaps the second stage. This stage starts approximately three to four months after the beer has been brewed. Stage three is dominated by the proliferation of lactic and acetic acid bacteria, with both peaking in cell numbers around six to eight months, which is usually in late spring to early summer when the early morning temperature begins to get warmer. Warmer temperatures are required for the growth of these microorganisms (*1*).

The characteristic sourness of lambic beer can be attributed to the presence of the lactic acid. The majority of the lactic acid bacteria fall within the *Pediococcus* genus. These bacteria can convert sugars into lactic acid. While some strains of *Pediococcus* are beneficial in beer, others have the ability to produce a slime layer that will leave a permanent haziness to the beer that cannot be removed with filtration. Unlike lactic acid bacteria, acetic acid bacteria are

undesirable in this style of beer, because of their ability to convert ethanol into acetic acid causing the beer to become acidic or hard (high volatile acidity). This only becomes a problem if the cask or barrel that the beer is stored in has been damaged or has a leak allowing oxygen to come in contact with the beer. The increase in oxygen nurtures the aerobic *Acetomonas* bacteria allowing it to grow in this once low oxygenated environment.

After about eight months, an increase in the number of yeast cells occurs signifying the start to the four and final stage of the fermentation process. Brettanomyces plays a crucial role in the development of the aromatic profile and even the flavor profile of this beer. A large portion of the aromatic profile of this beer is composed of esters which are by-product of this microorganism's metabolism. *Brettanomyces* produces an enzyme that can convert acids into esters and conversely. The two most influential compounds that are produced are ethyl lactate and ethyl acetate. These two compounds were converted into ester from lactic and acetic acids produced in stage three (*1, 3*).

Microorganisms in Beer

Microorganisms have the ability to produce a wide array of by products which can play an important role in the flavor composition within a food or beverage. In a number of different foods and beverages, the presence of microorganisms can cause off-flavors. However, a number of different foods require the presence of these microorganisms for the development of pleasant, attractive and required flavors. Traditional alcoholic beverages rely on a number of different microorganisms to obtain their flavor profiles (*38*). Belgian's gueuze is one of these products. It is an unique style of beer in that it is not produced from a single pure culture but from a mixture of a number of different microorganisms (*39*). Although microorganisms (other than *Saccharomyces)* regarded as spoilage microorganisms in most beers, this is not the case for

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lambics because these microorganisms play an important role in the overall flavor of this particular style of beer (*10*).

Yeast

The strain of yeast present for the main fermentation plays an important role in determining the level of flavor compounds present in the beer (*20*). In lambic beer, *Saccharomyces* species are the yeast responsible for the main alcoholic fermentation and are responsible for most of the attenuation in the wort. For the first seven months of the brewing process, these microorganisms dominate the microflora of the wort, reaching a population density of $5*10^6$ cells/mL after only three to four weeks. The overall yeast cell population, however, is still significantly lower than that normally found in most commercial top or bottom fermenting beer at 10^8 cells/mL. The two main species of *Saccharomyces* yeast found in lambic wort are *S. cerevisiae* and *S. bayanus.* Both species of saccharomyces can metabolize glucose, maltose, and to some extent maltotriose, the main sugars found in lambic wort (*1*).

The fermentation of lambic wort using traditional brewer's yeast, *Saccharomyces cerevisiae,* reaches an attenuation of approximately 60 – 64% (3.0 – 3.5 plato) which is the normally called the attenuation limit. Unlike other beers, lambic beers go through a secondary fermentation step which increases the attenuation beyond the normal range to what is called the overattenuation or superattenuation stage of 63 – 83%. This is the result of yeast such as *Brettanomyces lambicus* converting the remaining sugars left in the wort (*1, 40*).

Enterobacteriaceae

Enteric bacteria, or enterobacteriacea, are gram negative bacteria (*1*). There are a number of different species of bacteria that make up the enterobacteriaceae family, many of which can be isolated from a number of different ecological niches. Some of these bacterium are harmless while others can be pathogenic to humans, animals, and/or insects. A few of the following genera are included in this family and they are: *Escherichia, Shigella, Yersinia, Morganella,* and *Samonella (41).*

While in other beer styles enterobacteria are considered to be potential spoilage microorganisms, that is not the case in lambic beers. Once the wort has had time to cool down, the enteric bacteria present in the wort reach a very high cell density. The enteric bacteria will reach a maximums concentration of 10^8 cells/mL within two weeks of the wort cooling down (*1, 42-44*). The enterobacteria population decreases once the pH of the wort drops below 4.4 and the ethanol concentration rises above 2% (*45*).

Enterobacteria have the ability to impart a variety of flavors into the wort such as sweet, honey, fruity, vegetables and even fecal. Similar to other brewing microorganisms, enterobacteria have the ability to metabolize the available glucose in the wort not only for growth, but into lactic acid, acetic acid, ethanol, and carbon dioxide. While enterobacteria can metabolize glucose, they are unable to metabolize maltose or maltotriose. The enterobacteria present in the wort have the ability to consume a number of different amino acids and peptides that can temporarily impact the flavor of the beer. One possible reason for the slow start to the main fermentation is because of the depletion of the amino acids in the wort by the enterobacteria (*39*).

Enterobacteria have the ability to produce not only several sulfur compounds, but carbonyls and phenols which all play a role in the aromatic and flavor profile of beer although some of these compounds will disappear during later phases of fermentation. The compounds become entrapped by the CO_2 produced during the main fermentation. DMS is a good example of this phenomenon. Within the first two weeks, the concentration of DMS produced by the enterobacteria exceeds 450 ppb. The concentration of DMS drops to 100 ppb because most of it is stripped away by fermentation gases (*1, 13*).

Lactic Acid Bacteria

Lactic acid bacteria are gram-positive nonsporulating rods or cocci (*41*). These microorganisms are potentially one of the most dangerous spoilage microorganisms in beer because of their microaerophillic nature, ability to tolerate the antiseptic properties of hops, and their ability to survive in 0.5 – 14% ethanol and low pH environments (*10*). The major division that separates *Lactobacillus sp.* from each other is based upon how they metabolize glucose (*46*). Lactic acid bacteria are broken down into four genera: (1) *Lactobacillus* for the rod shaped organisms; (2) *Streptococcus* for the homofermentative facultatively anaerobic cocci; (3) *Leuconostoc* for the heterofermentative cocci that occur in pairs or short chains; and (4) *Pedioccus* contains the homofermentative cocci that divide into pairs and tetrads (*41*).

Once the main fermentation is complete, an increase in the overall bacterial population, specifically lactic acid producing bacteria from the genus *Pediococcus* and some *Lactobacillus* is observed (*1*). After about three to four months, lactic acid starts to develop within the beer. The bacteria reach their maximum numbers at the seven month mark which usually coincides with the beginning of summer (*2*). The warmer temperatures present during aging in the cellars during the summer appears to be essential for the growth of these bacteria. This phenomenon can be

seen again in the second year of fermentation when the summer months come around again. It has been hypothesized that one way to speed up the fermentation process of lambic/gueuze beer would be to increase the ambient temperature in the aging cellar once the main fermentation stage has been completed. Unfortunately, creating temperature controlled aging cellars would be costly for breweries because of the age of the buildings and the overall renovation cost (*1*).

Nevertheless, different species of lactobacilli have different tolerances for the antiseptic properties of hops (*41*). *P. damnous* is impervious to the antiseptic properties of hops which is why this species has the ability to grow in hopped beer. Lactic acid bacteria take glucose and convert it into lactic acid without producing carbon dioxide as a by-product. Lactic acid is the primary component that gives lambic beer its sour taste. Lactic acid bacteria are slow growing microorganisms that have complex nutritional requirements, which is partly why lactic acid bacteria will not reach a very high cell density in the wort. *P. damnous* not only has the ability to produce lactic acid but acetoin and diacetyl which both contribute to the aroma present in this beer (*1, 2, 34*).

Brettanomyces

Brettanomyces, more commonly known as bret, is an asexual, nonsporulating wild form of yeast associated with the spoilage of red wines, beer, and ciders (*47*). Bret is slow growing and it can take several months after the initial fermentation step to be complete before bret is detected (*40*). Brettanomyces was first isolated in 1904 from the stock of a late fermenting English beer (*48*). N. Hjelte Claussen of New Carlsberg Brewery was the first to introduce the name brettanomyces to describe the yeast required to make the English stock ale. It was not until 1920 that *Brettanomyces/Dekkera* was recognized as its own genus (*49*). There are five species

of Brettanomuces/Dekkera and they are *B. custersianus, B. naardenensis, B. nanus, B. anomalus*, and *B. bruxellensis* (*50*).

Bret is a fairly common species of yeast that can be found in a number of different locations and products like wine, beer, cider, wineries, breweries, both wine and brewing equipment, and oak barrels used for aging (*51*). Once bret gets into a brewery, especially an older one, it is extremely difficult to remove, especially since they can be found in the air and within the wood barrels used for fermenting lambics (*1*). Some forms of wild yeast like brettanomyces are typically considered 'niche' contaminates because they come into play only under certain circumstances (*52*).

Brettanomyces is not detected in lambic beer until after about eight months of fermentation. The two strains that dominate lambic and gueuze beer are *B. bruxellensis* and *B. lambicus*. These two strains are present in the beer for another eight months and are the main contributors of the aroma profile for lambic beer (*13*). *B. bruxellensis* is the predominant species found in the breweries that are located within the city limits, while *B. lambicus* is the main yeast for the breweries located in the country (*1*). Lambic beers are both produced from a mixture of yeast cultures, saccharomyces and brettanomyces (*42*). Bret, unlike other yeast, has the ability to ferment much more effectively under aerobic conditions then anaerobic conditions. This property is consistent with this particular yeast's ability to form films or pellicle which can be found on the surface of the beer within the fermentation tank or cask. Brewers will not allow anyone to disturb either the film or the pellicle during the aging process due to the increase risk of oxidation (*1*).

The secondary by products produced by bret have the ability to accumulate to a much greater extent than those of other yeast. The secondary products play a critical role in the sensory

profile of lambic beer. While the secondary products produced by bret account for a small percentage of the total fermented sugars, they still play a critical role in the aroma of this beer regardless of their low concentrations. The main compounds produced are the esters ethyl acetate and ethyl lactate. These esters can be formed enzymatically or chemically. Esterase is the name of the enzyme that can cause the formation of the esters by causing a chemical reaction between ethanol and an organic acid. Brettanomyces displays a higher esterase activity then other yeasts (*Sacchromyces* or *Kloeckera*) (*53*).

In lambic beer bret's ability to synthesis ethylphenols (4-ethylphenol and 4-ethylguaiacol) and vinylphenols are important to the unique characteristics of this particular style (*54*). Bret is the only microorganism that is known to produce ethylphenols. While there are some species of lactic acid bacteria and yeast that can produce ethylphenols in cultured media, none of them have been found to be able to produce them in an actual beverage system (*55*). Brettanomyces is a key player in the flavor profile of lambic and gueuze beer (*2, 52*). The 'bretty' character produces a wide array of different aromas and flavors such as mineral, tobacco, barnyard, leathery, pharmaceutical and smoky (*43, 53*). The aroma compounds produced by bret also have the ability to suppress desirable fruity notes (*56*). The horsy smell produced by bret can vary from slight to very strong. The overall strength of the horsy smell is dependent upon the fermentation conditions. Tetrahydropyridines are the compounds responsible for the horsy smell (*1*). Tetrahydropyridines are produced when the enzyme esterase breaks down ethanol and the amino acid lysine. Lysine is present in the wort and ethanol is formed after the initial fermentation begins, creating an ideal environment for the production of tetrahydropyridines formed by brettanomyces. A small quantity of the horsy flavor is desirable for this particular style of beer (*1, 57*).

Gas Chromatography-Olfactometry

GC-O is a commonly used technique to analyze complex food flavors because of its ability to provide information immediately regarding the presence of a certain aromatic compound within a sample. Researchers have successfully identified over 8,000 volatile compounds present in foods and beverages but typically only a few of these compounds actually make up the characteristic aroma of a food or beverage. Separating the "active" from the "inactive" volatile compounds is possible by combining the human olfactory system with analytical techniques like gas chromatography-olfactometry (GC-O), aroma extraction dilution analysis (AEDA), or CHARM (*58, 59*). Meilgaard was the first to study the effect that the sensory contributions volatile compounds had on beer flavor. Meilgaard's research focused on calculating the odor active value (OAV: ratio of concentration to odor threshold) for over 239 compounds based upon quantitative data. In some GC-O configurations, the effluent is divided between a FID and the sniffing port (*60*). This is because the effluent coming from the GC may be too low in concentration to be detected by the FID. GC-O has been used in characterizing how trace amounts of a compound can impact the aroma of a product (*61*).

The primary reason researchers use GC-O is so they can arrange their list of identified aromatic compounds based upon their importance in the sample. This list can be arranged in a number of different ways (*62*).

Similar to other alcoholic beverages, a beer's particular aroma is made up of a number of different chemical compounds with different polarities and with varying concentrations. The composition of the raw ingredients and the fermentation process play large roles in the chemical composition of a product. Alcoholic fermentation produces a number of chemical by-products besides ethanol, such as esters, carbonyls, alcohols, and acids all of which are influenced by the

quality of the raw ingredients. Some chemical compounds can be present in levels as high as mg/L while others in quantities as low as ng/L. The overall influence a compound has on the aromatic profile of a product can vary. Typically, compounds present in trace amounts have a greater influence over the aroma profile than those found at higher concentrations (*63*).

The human nose is one of the most valuable and sensitive tools currently available to researchers conducting aroma analysis. The theoretical detection limit of the human nose is around 10^{-19} moles, a much lower detection limit then the flame ionization detector (FID) present on the GC. Aroma research begins with isolating and identifying aroma active compounds in a food sample (*64*). For the characterization of aroma active compounds to occur, volatile compounds must first be separated from nonvolatile compounds. Several methods used for separation include solvent extraction, headspace concentration and distillation (*65*).

Gas chromatography-olfactometry (GC-O), also known as gc-sniffing, is an analytical technique that utilizes a human being as the detector to smell the effluents coming from a gas chromatography (GC) to characterize the volatile compounds present in the sample (*63, 66, 67*).

It is believed that GC-O developed shortly after James and Martin published their description of a GC (*66, 68*). It was not until the mid-1960s that Fuller and Guadagni both published papers on the use of gc-sniffing as a viable analytical technique (*69, 70*). The first use of the name "gas chromatography-olfactometry" was not used until 1980 when Takeuchi and his colleagues published their paper linking all experiments that utilized panelists sniffing effluents from a gas chromatograph under a common name (*66, 71*).

A GC is a useful tool in its ability to separate volatile compounds within a sample. However, it is unable to provide any information on the sensory intensity of a desired compound within a sample. It is well accepted that not every single volatile detected by the GC plays a role

in the sensory profile of the sample. Research thus far has shown that generally only 10 – 30 compounds make up the aroma profile of a food sample and determining which of these compounds are aroma active from several hundred can be difficult (*64, 72*). There are a few compounds like sulfur compounds that have a low sensory threshold which are easily detectable by the nose, but may or may not be detected by a GC due to the low quantities present in a sample, indicating further concentration of the compound is needed (*72*).

A combination of both qualitative and quantitative analysis is conducted on every compound that leaves the GC column to determine (1) if the compound is present in the sample is above its sensory threshold; (2) what it smells like; (3) when it is being eluted from the column; and (4) the overall intensity of the odor (*63*).

Gas Chromatography-Olfactometry Methodology

The earliest known work regarding flavor analysis was conducted over 50 years ago by Patton and Josephson (*73*). The purpose of Patton and Josephson's research was to estimate the importance of certain flavor compounds based upon the ratio of the compound to its threshold concentration. The ratio is based upon how much the compound is over its sensory threshold level.

Modern day aroma research begins with selecting isolation and identification techniques that will allow the researcher to identify the compounds present in a food sample (*64*). There are two different sensory techniques used for determining the odor activity of compounds present in foods. These two techniques were developed by two separate research groups, one in the United States and one in Germany: (1) CHARM analysis by Acree and coworkers (*74*) and (2) aroma extraction dilution analysis (AEDA) by Grosch and his coworkers (*75, 76*). These two methods are based upon the detection of the odors present instead of estimating the intensity based upon a

stimulus at super threshold levels (*77*). In both procedures an extract of the food sample is obtained and is diluted either 1:1 or 1:2. Each dilution is then analyzed using a GC-O (*58*).

CHARM

CHARM is an acronym for Combined Hedonic Aroma Response Measurement (*61*). CHARM analysis is similar to AEDA in that they are both GC-O methods that look at odor activity. A big difference between these two techniques is that charm looks at measuring the dilution value throughout the entire time compounds are being eluted from the gas chromatography while AEDA just looks at the maximum dilution value (*60*). When an odor is detected by the panelists, he/she presses a computer mouse for the entire duration that the odor is being detected. Once the odor is no longer detected, the mouse is released. The panelists are then asked to pick a descriptor from a predetermined list. Having trained panelists is essential for this type of analysis since an extended knowledge of different aromas and their descriptors are necessary. With any type of psychophysical experiment, human error or bias can occur when sniffers know too much information about the sample. To limit bias, samples should be randomized. Error can also result due to fatigue, which is why sniffing sessions should last no longer than twenty-five minutes. Since internal standards are not traditionally used with this type of measurement, the chromatography must be precisely reproduced to ensure accuracy. CHARM analysis requires special software and a computer. The software combines the time and duration of each individual sniffer's chromatogram into one final one to create an aromograph with peaks and integrated peaks (CHARM values) (*77*), which are relative to the amount of the chemical compound in the extract (*60*). The CHARM value can be calculated using the following equation $c=d^{n-1}$, where n is equal to the number of times an aroma is smelled by all the panelists and d is the dilution factor (*77*). CHARM analysis has been utilized to determine the

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odor active compounds present in a number of different foods, beverages, and spices such as beer (*78*), coffee (*79, 80*), citrus peel (*81*), coriander (*82*), as well as boiled potatoes (*83*).

Dilution Analysis

When using dilution analysis, an extract is diluted in a series of 1:2 or 1:3 dilutions. Each dilution is then sniffed by a panelist until no significant odor is detected (*66*). A sniffer will begin with the most concentrated sample and proceed down to the most diluted (*77*). When using AEDA, the results are expressed using a flavor dilution (FD) factor (*84*), which is the ratio of the concentration of the effluent in the initial extract to the concentration of the effluent in the most diluted extract in which the effluent in detected by the GC-O. When using AEDA, the FD factor is a relative measurement and is relative to the odor active value of the compound present in air. An advantage of AEDA over CHARM analysis is that no computer or special software is needed--only a pen and paper to write down retention times and odor descriptions, and this is one reason why AEDA is more widely used then charm analysis. To create an AEDA aromagram, the retention time is plotted (retention index or Kovats number) on the x-axis with the maximum dilution value on the y-axis. AEDA has been utilized to identify the odor active compounds in a number of different food products such as cheese (*85*), grapefruit juice (*86*), green and black tea (*87*), popcorn (*88*), wines (*89, 90*), tequila (*91*), and coffee (*84*).

Time Intensity

McDaniel and co-workers developed another GC-O technique called OSME (*92-94*). A major difference between OSME and AEDA is that OSME, while still depending on the evaluation of a group of panelists, utilizes only a single concentration of the extract detected by the GC-O. AEDA is based upon odor detection threshold while OSME is based upon perceived intensity. OSME, or time intensity, looks at the estimated odor intensity of a compound based

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upon time. Panelists move a resistor bar based upon the intensity of the effluent coming off the GC as well as write a descriptor pertaining to the effluent. A trained sniffer will rate the intensity of the effluent coming off the GC using an electronic time-intensity scaling device based on a scale of 15 cm where 0 = none, 7 = moderate, 15 = extreme. The actual scaling device is coupled with a computer data handling software that has the ability to produce FID-style aromagrams known as osmegrams.

Time intensity GC-O is not as popular a technique as dilution analysis because of the additional hardware (intensity transponder) and software requirements necessary to receive the additional data from the transponder and GC. ChromPerfect is a type of chromatographic software currently available and can rapidly provide a visualization of the FID chromatogram and the sniffer's GC/O response (*58, 77*). Time intensity has been used in the analysis of a number of different food products such as blackberries (*95*), hop oil (*96*), wines (*92*), and citrus products (*97-99*).

Solid Phase Microextraction (SPME)

Solid phase microextraction (SPME) is a relatively new extraction technique for the rapid, solventless extraction of volatile and semi-volatile organic compounds present in the headspace above a solid or liquid sample (*100, 101*). This technique utilizes a small 1-2 cm piece of fused silica (*102*). SPME was first developed by Pawlisyn in 1997 to analyze environmental samples like air, soil, and water (*103, 104*). Since then it has been used to analyze volatiles compounds found in a variety of foods (*105*).

SPME is based on reaching an equilibrium between the volatile compounds present in the headspace above the sample and the concentration on the coated fiber (*102*). Once the fiber has reached equilibrium with the sample, the fiber is then thermally desorbed into a GC carrier gas

releasing the volatile compounds to be analyzed. The volatile profile obtained during analysis is based upon the profile of the sample and how the sampling parameters are controlled. While quantification is possible with this technique, every step in the process must be carefully controlled to ensure consistent results. Incorporating internal standards into the sample matrix and always adhering to specific extraction times will ensure proper quantification of the sample. Every organic compound will behave differently with the SPME fiber based upon its volatility, polarity, organic/water partition coefficient, volume of the sample or headspace volume, agitation speed, pH of the solution and the extraction temperature (*100*).

    SPME Process. Samples are placed into a vial or similar container and sealed with a septum type cap. Fibers should be conditioned prior to their initial use because the polymer phase has the ability to absorb chemical compounds from the laboratory air potentially resulting in large background noise on the chromatogram. Regardless whether the sample is liquid or solid, the needle must first penetrate the septum of the sampling vial before extraction can begin. For liquid sampling, the fiber is extended into the sample during extraction. For headspace sampling, the fiber is extended into the vapor phase above the solid or liquid sample. When penetrating the septum of the vial, care needs to be taken not to bend the needle. Using a clean needle to puncture the septa of the sampling vial and the GC injection port is one way to reduce the risk of bending needles.

    Placing a small stir bar into the sampling vial could potentially help the sample reach equilibrium faster. After the fiber has been exposed to the sample for a predetermined amount of time (1 – 20 minutes), the fiber is retracted back into the needle before being removed from the septum. Once removed from the septum, the needle is then inserted into the GC port for 1 – 8 minutes. The analytes that were absorbed to the fiber are thermally desorbed. The penetration

depth of the needle into the GC injection port is not critical as long as the exposed fiber is extended into the heated zone of the injection system. When trying to analyze samples with trace amounts of a particular analyte, using a preconcentration step can help ensure proper identification of compounds present in a sample. To ensure that the preconcentration step is not wasted, the researcher should use as small as possible a split ratio (10:1) the split/splitless capillary injection port. The injection port liner should be no greater than 1 mm in diameter to help provide sharper peaks for some of the higher boiling volatile compounds. Although a standard split liner packed with glass wool can provided satisfactory chromatography results, care must be taken to ensure that the SPME fiber does not come into contact with the glass wool when exposed during the thermal heating process. Extending the fiber into the glass wool could lead to a broken or damaged fiber (*100*).

SPME Fiber.

The original fiber used by Pawliszyn was coated with polydimethylsiloxane (PDMS) to extract the volatile and semi-volatile compounds present in water samples. A number of different coatings are available that can add to extraction capabilities including carboxen, divinyl benzene, polyacrylate, and carbowax (polyethylene glycol; PEG). Commercially available SPME fibers can be coatedwith one type of coating or combined with multiple coatings (*77*). The fused silca fiber can be coated with either a liquid or solid phase to extract and concentrate these compounds (*102*). For general use, using a nonpolar thick film fiber can provide high sensitivity for most compounds (*100*). If the compounds of interest in a sample have a molecular weight less than 125, the manufacturer recommends using the 85 µm Carbowax/PDMS fiber. Polarity plays a critical role in the efficiency of extraction for compounds with a molecular weight greater than 125. When the compounds of interest in a sample have molecular weights greater than 125, it is

recommended that polyethylene glycol (PEG), polyacrylate (PA), and DVB/Carboxen fibers

should be used for extraction (*106*). Along with different polarities, fibers can have different

thickness. Using a fiber with a thicker coating has the advantage of allowing for more analyte to

be loaded onto the liquid coating, thus allowing more analyte to be used for analysis and

detection. There are three different thickness commercially available at this time 100 μm, 30

μm, and 7 μm. The larger 100 μm fiber has the ability to absorb a greater number of analytes

then any other thickness however it may not release the analytes as easily as other fibers would.

This can be seen when you have subsequent carryover from one sample to the next. The intended

use for the 30 μm thick fiber is to analyze semi-volatile compounds while the 7 μm thick fiber is

intended to be used for immersing and extracting semi-volatile compounds with a molecular

weight greater than 250 (*77*). If one is interested in extracting higher boiling compounds like

polyaromatic hydrocarbons, higher temperatures are required to thermal desorb the analytes from

the fiber into the GC injection port. In general, the thicker the fiber, the longer it will take before

equilibrium can be reached; however, an increase in sensitivity is possible due to the amount of

analyte that can adhere to the fiber (*100*).

As more and more researchers move towards using automated sampling systems similar

to the CTC robotic auto sampler, new metal alloy fibers are being developed. The reason for this

is that the CTC auto sampler has the ability to agitate samples while exposing the fiber located

either in the sample or above the headspace. The advantage of using this new fiber is that the

core material allows for more durability due to the consistent agitation of the fiber during

exposure when using the CTC auto sampler (CTC, Zwingen, Switzerland) (*77*).

Fibers also have the ability to come in different needle gauge sizes. The smaller 24 gauge

needle is typically used for the extraction and injection using a manual holder and silicon

polymer septum GC inlet port. The purpose of using a smaller gauge needle is to decrease the

septum coring that can occur with larger gauge needles. The 23 gauge needle is recommended

when using the Merlin Microseal (Merlin Instruments, Half Moon Bay, CA USA). An advantage

the Merlin Microseal has over the silicon polymer spetum GC inlet port is that it has a longer

shelf life (1 year or 25,000 injections) and does not contaminate the GC with shaved silica

particles. This size needle prevents leakage when the needle is being desorbed while inserted into

the injection port (*77*).

Prior to their initial use, SPME fibers should first be conditioned as described by the

manufacturer. The purpose of this is to remove any chemical compounds that could have adhered

to the fiber during manufacturing or from the environment itself, because these compounds can

obscure chromatographic peaks. Additional bake out sessions are recommended to remove

chemical compounds that could have adhered to the fiber during consistent use. In addition to

brief daily bake out sessions, it is suggested that blanks be used during sampling to ensure that

there is no carryover effect from one sample to the next when using an auto sampler.

One advantage of using SPME over traditional static headspace extraction is that SPME

is not limited by the volatile compounds partitioning into the headspace. SPME results are

dependent upon two partitioning coefficients not like the one for static headspace. The first

partition coefficient is the same as that of static headspace. This is based upon the partition

between the sample and the sample headspace. This partitioning main application is to determine

the amount of time required to go from either a solid or liquid sample phase into the headspace.

It is also used to determine how long the sample should be allowed to reach equilibrium before

penetrating the seal of the septum to start the extraction process. If the time and temperature is

not kept consistent from sample to sample, the headspace concentration values will vary from sample to sample thus decreasing overall accuracy and precision.

It is essential that equilibrium be reached between the samples and headspace because the concentration of the analyte will be lowered. Unfortunately, most procedures do not allow for the sample to reach equilibrium, requiring precise control over temperature and time for reproducibility. There are ways to increase headspace analyte concentration by increasing the sampling temperature or by adding salt to the aqueous sample to cause a change in the ionic strength. However salt does not always work (*107*).

The second partition is based upon the amount of time the fiber is exposed to the sample. Another important factor is the competition between the different chemical compounds present in the headspace and their ability to adhere to the fiber (*77*).

One advantage of using SPME over other sampling techniques is that no solvent is required and extraction of the compounds can occur without heating the sample. This can greatly reduce the chance of creating chemical artifacts (*100*).

Simultaneous Distillation Extraction (SDE)

Simultaneous distillation extraction is one of the oldest separation techniques used today to separate volatile compounds from non-volatile compounds (*110*). The development of sophisticated extraction and distillation methods to separate volatile compounds present in a food matrix from non-volatile ones has baffled researchers for centuries. Finding a viable separation technique is always the challenge for researchers who are interested in identifying and quantifying food compounds. Separation techniques should meet certain requirements: (1) extraction techniques should not exclude compounds that play a vital role in the overall flavor of the food; (2) extraction technique should not alter key compounds; and (3) separating non-

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volatile compounds that could potentially interfere with chromatograph techniques is also essential.

SDE is widely used by a number of flavor researchers because of its versatility. SDE is a simple fast aroma extraction technique, however because of the elevated temperature experienced during distillation, it can potentially lead to the formation of artificial compounds (*110*). Water is a major volatile found in food which makes distillation a reasonable choice for isolation. The presence of carbohydrates, lipids, and proteins could potentially interfere with the distillation process because of the formation of foam and sticky gel. Antifoaming agents can be used to prevent foaming or by keeping the temperature below 50ºC, because starches began to gelatinize around 65 ºC. Proteins can denature, but they tend not to do so below 50 ºC (*60*).

Solvent Assisted Flavor Evaporation (SAFE)

In a 1999 paper written by Engel and his co-workers, he describes a method that couples vacuum distillation, cold trapping and solvent extraction for solid samples (*110*). Solvent extraction is required for sold samples like popcorn (*111*), coffee, and bread (*75*). This particular method has been proven to be superior to the traditional SDE method because of its better temperature control and speed. Solvent extraction is safer than other methods that utilize high vacuum distillation or cold trapping techniques. Unlike SDE, which can lead to the formation of artificial compounds, SAFE products an extract that is a close approximation of the original flavor (*77, 112*).

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Table 2.1 Description of all the styles of lambic beer currently available on the commercial market.

Style	Descriptor
Lambic	A beer style that is produced through spontaneous fermentation from 30% unmalted wheat, with a wort gravity of at least 1.020 (5 degree Plato) traditionally in oak casts.
Faro	A blend of young lambic from made from moderate-gravity wort sweetened with candy sugar
Fox lambic	Young lambic
Framboise	A fruit derivative of a lambic macerated with raspberries in a young blended lambic
Gueuze	Is a blend of one, two, three year old lambics blended together. One type is refermented or bottle-fermented, while another type is filtered or bulk-fermented in tanks
Kriek	A fruit derivative of a lambic macerated with sour cherries in a blended young lambic
Muscat	A fruit derivative of a lambic made by macerated grapes in a blended young lambic
Pêche	A fruit derivative of a lambic made by macerated peaches in a blended young lambic
Vieux Lambic	Old lambic or aged lambic aged three years in a wooden cask and one year in the bottle.

CHAPTER 3: COMPARISON OF TWO EXTRACTION TECHNIQUES, SPME AND SDE SAFE FOR THE ANALYSIS OF LAMBIC BEER

Abstract

Lambic beers are the only beers currently being brewed that undergo a natural fermentation process. Lambic beers are only produced within 500 square kilometers of Brussels and Payottenland, a valley on the Senne River located on the western side of Brussels. This particular region appears to have the perfect combination of airborne microorganisms required to make a consistent beer through spontaneous fermentation (*1*). Limited research is currently available describing the volatile and semi-volatile compounds present in lambic beers. The purpose of this study was to compare two extraction techniques: SPME (solid-phase microextraction) and SDE (simultaneous distillation and extraction), coupled with SAFE (solvent assisted flavor evaporation), for the isolation of flavor compounds present in commercial available lambic gueuze beer using GC-MS, SPME-PHPD (photometric pulse flame detector) and GC-O (OSME or AEDA) techniques. A total of 101 compounds were identified using two different extraction techniques (63 volatile compounds and 38 sulfur compounds). SPME was able to identify 45 of the 63 volatile compounds, while SDE/SAFE was able to identify 46 of the 63 volatile compounds. The major volatile chemical classes were alcohols, acids, phenols, ketones, and esters. Each method was able to identify compounds that the other one could not. In comparing the two extraction methods for GC-O using SPME, 28 volatile compounds were identified. SDE/SAFE was able to identify 32 volatile compounds, six of which were found using both extraction methods. Ethanol was found to be the highest ranking odor active compound using SPME, followed by 1-(furan-2-yl)-ethanone, unknown sulfur, ethyl isobutyrate, and 3-methylbutanoic acid. Phenyl methanol and 2-methyl-butanoic acid both had

flavor dilution (FD) factors of 729, followed by 3,5-dimethyl-1,2,4-trithiolane and 4-ethylphenol with FD factors of 243.

Introduction

Alcoholic beverages are a complex mixture of volatile and semi-volatile compounds belonging to several different chemical families including higher alcohols, ethyl esters, fatty acids, higher alcohol, isoamyl esters, carbonyl compounds, furanic compounds, terpenoids, C13-norisoprenoids, and volatile phenols (*2*). The complex combination of chemical compounds found in alcoholic beverages plays a vital role in the appearance, aroma, flavor, and mouthfeel. The combination of taste and olfactory properties are often responsible for the quality, character, and consumer acceptability of alcoholic beverages. Similar to other alcoholic beverages, a beer's aroma is made up of hundreds of different chemical compounds having different polarities and concentrations (*3*).

Alcohol beverages contain over 800 volatile compounds, but only ten to thirty of them are generally aroma active (*4-6*). Compounds present in different alcoholic beverages have the ability to affect the aroma and flavor individually, synergistically, or antagonistically. Some volatile compounds contribute to the aroma and flavor of the alcoholic beverage while others enhance the background profile of the product (*7*). Typically, compounds present in trace amounts have a greater influence on the aroma profile then those found at higher concentrations (*3*).

The most critical step in determining which flavor compounds contribute to the aroma of a food product is selecting an extraction method that has the ability to isolate all aroma active compounds. The extraction technique selected should ensure the isolation of all characteristic compounds that play vital roles in the aroma of the product without resulting in the formation of artifacts (*8*). SDE (simultaneous distillation and extraction) (*9*), SPME (solid-phase microextraction) (*10*), and SAFE (solvent-assisted flavor evaporation) (*11*) are three different

extraction techniques currently used by researchers. SDE is one of the oldest extraction techniques and is still used because of its simplicity and versatility; however, because of the elevated temperature applied during extraction this technique can potentially lead to the creation of artificial compounds not normally present (*12*). SPME utilizes a 2 cm piece of fused silica that has either a liquid or solid coating to extract and concentrate compounds. Because of the ease of use and ease of automation, SPME is commonly used for the isolation of aroma compounds as well as monitoring quality changes during storage (*8*). When using SPME precise control over time and temperature is essential for this extraction technique to ensure proper quantification of volatile and semi-volatile compounds (*13*). SAFE, another isolation technique that can be coupled with SDE, utilizes low pressure to extract volatiles at low temperatures to prevent the formation of artificial compounds (*14*).

The purpose of this study was to compare two extraction techniques, SPME (solid-phase microextraction) and SDE (Simultaneous distillation and extraction) coupled with SAFE (solvent assisted flavor evaporation), for the isolation of flavor compounds present in commercial samples of gueuze beer using GC-MS and GC-O (OSME or AEDA) techniques.

Materials and Methods

Simultaneous Distillation Extraction and Solvent-Assisted Flavor Evaporation

Materials. Lambic gueuze beer samples were purchased from a local wine and beer store in Blacksburg, VA. The brands were Cuvee Reneé (LK23JGC 2975 23 Nov 2012),

Chemicals. Diethyl ether (anhydrous, 99.8%), sodium chloride (99%), *n*-alkane standards (C6-C30), 2-methyl-3-heptanone (internal standard for neutral fraction), and 2-ethylbutric acid (internal standard for acidic fraction was purchased from Aldrich Chemical Co. (St. Louis, MO). Sodium sulfate (99%) and hydrochloric acid (36.5%) was obtained from Fisher

Scientific (Pittsburg, PA). Deodorized distilled water was prepared by boiling glass-distilled water down to two-thirds of its original volume.

Simultaneous Distillation Extraction.

SDE-SAFE extraction method was conducted at the Agricultural Bioprocess Laboratory of Dr. Keith Cadwallader at the University of Illinois Champaign-Urbana.

Lambic beer (375 mL) sample was poured into the sample side of the continuous solvent extraction apparatus. Diethyl ether (150 mL) was used as the extraction solvent. Continuous extraction was conducted over a twenty-four hour period at 45ºC. The extract was then transferred into the solvent flask and concentrated to 100 mL on a Vigreux column at 45ºC.

Solvent Assisted Flavor Evaporation (SAFE)

Volatile compounds were separated from the lambic beer extract using solvent-assisted flavor evaporation (SAFE). The SAFE system was composed of two liquid nitrogen-cooled traps (receiving and waste), transfer head and a 1 L round bottom flask. This system was operated under high vacuum (approximately 10^{-5} Torr) when extracting volatile compounds from the sample. The lambic beer extract (100 mL) was slowly fed into upper portion of the transfer head.

Separation of the lambic beer extract occurred when aliquots of the sample were dropped into the round bottom flask that was partially submerged in a warm (50ºC) water bath. The extraction process took approximately 2 hours. Separated volatiles passed through the separation head into the receiving tube where they condensed and froze because of the sudden drop in temperature. Once the separation was completed, the receiving tube was removed and allowed to thaw out at room temperature for 30 minutes before proceeding with the fractionation of the lambic beer.

Before separating the extract into the acidic, neutral and basic fraction, the SAFE extract was concentrated to 30 mL using a Vigreux column attached to a 125 mL separatory funnel submerged in a 40ºC water bath. After concentrating the extract, it was washed with aqueous Na_2CO_3 (0.5 mol/L, 3 x 20 mL), and the ether layer containing the neutral and basic volatile compounds was collected. The aqueous layer was washed with diethyl ether (2 x 10 mL) and then acidified to pH 2 using 10% (w/v) aqueous HCl solution saturated with NaCl. The aqueous layer was then washed with diethyl ether (3 x 20 mL). The diethyl ether layer was collected and the aqueous layer discarded. The ether layer containing the acidic volatiles was washed with saturated NaCl (2 x 15 mL) and collected in a 50 mL separatory funnel. The diethyl ether layer was concentrated to 10 mL using a Vigreux column attached to the 50 mL separatory funnel submerged in a 43ºC water bath. The diethyl ether layer was then dried over anhydrous Na_2SO_4 and further concentrated to 2 mL. The 2 mL acidic fraction was then transferred into a 200 µL vial and concentrated to 150 µL using a gentle stream of nitrogen gas.

The original diethyl ether layer from above that contained the neutral base fraction was washed with saturated NaCl (2 x 10 mL) and the diethyl ether layer collected while the saturated NaCl layer was discarded. The diethyl ether layer was concentrated to 10 mL using a Vigreux column attached to a 50 mL separatory funnel submerged in a warm (43ºC) water bath. The diethyl ether layer was then dried over anhydrous Na_2SO_4 and concentrated again to 2 mL in the warm water bath. The 2 mL neutral basic fraction was then transferred into a 200 µL vial and concentrated down to 150 µL using a gentle stream of nitrogen gas. The acidic and neutral basic fractions were stored at -70 ºC until analysis.

Aroma Extract Dilution Analysis (AEDA)

The acidic and neutral basic aroma extracts were diluted using diethyl ether at a 1:3 ratio as previously described (*15*). Each dilution was kept in 1.5 mL clear glass vial with PTFE-lined screw cap and kept at -70ºC until analyzed. Flavor dilution (FD) factors were used to identify the most intense odorants in the flavor extracts. The FD factor of an odorant is based upon the ratio of the initial concentration within the highest dilution at which a panelists is able to detect the odorant by GC-O (*16*).

The GC-O system was the same as previously described (*17*).

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS was utilized to separate the acidic and neutral base fractions obtained by SAFE. Both extractions were injected using a cool on column method (+3 temperature tracking method) using a 6890 GC/5973 mass selective detector (MSD) (Agilent Technologies Inc.) (*14*). Extracts were separated using both a polar capillary column (Stabilwax-DA 30 m x 0.25 mm id 0.5 µm film; Restek, Bellefonte, PA) and a nonpolar column (RTX-5MS, 30 m x 0.25 mm id.; 0.5 µm film; Restek, Bellefonte, PA). Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC oven had an initial temperature of 35º C, which was held there for 5 minutes and then increased to 225º C at a rate of 6º C/min. Once the final temperature of 225º C was reached, it was maintained for 10 minutes. The mass spectrum detector conditions have been previously described (*14*).

Identification

Retention Index (RI) and Odor quality. Volatile compounds were identified based upon their odor descriptions and RI values on both polar and nonpolar columns. Values were compared to those previously report in the literature. Solutions of alkane standards were analyzed in the same manner on both the DB-5 and the RTX-S to calculate the RI:

$$RI = 100N + 100n(t_{Ra} - t_{Rn})/(t_{R(N+n)} - t_{RN})$$

N is the carbon number of the lowest alkane and n is the difference between the carbon number

of the two n-alkanes that are bracketed between the compound; t_{Ra}, t_{Rn}, and $t_{R(N+n)}$ are the

retention times of the unknown compound, the lower alkane, and the upper alkane.

Volatile Extraction by Solid Phase Microextraction (SPME)

This procedure was conducted at the Citrus Research and Education Center of the

University of Florida in the laboratory of Dr. Russell L. Rouseff located in Lake Alfred, FL.

Volatile compounds were extracted using solid phase microextraction (SPME) from

Lindeman's Cuveé Rene lambic (gueuze) beer. Compounds were identified using gas

chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC-O).

Each aliquots (10 mL) of lambic beer sample was analyzed in triplicate and placed into 40 mL

glass vials with plastic screw caps and Teflon coated septa. Samples were equilibrated for 30

minutes in a 40°C water bath. A SPME fiber (50/30 um DVM/Carboxen/PDMS; 2 cm; (Supelco,

Bellefonte, Pa., U.S.A.) was exposed to the beer headspace for 30 minutes at 40°C then inserted

into Agilent 7890H GC injection port for 8 minutes in splitless mode at 220°C to desorb the

volatiles. A nonpolar (XP-5) 60m x 0.25mm id 0.5µm column was used with He as the carrier

gas at a flow rate of 2.0 mL/min. The effluent coming off the column was split 1:2 into an FID

and a sniffing port (Datu, Geneva, NY). The odor and the intensity of the compounds were

analyzed using a time-intensity approach with a single assessor. The assessor smelled each

sample twice. The aroma intensity for each compound was continuously recorded using a

potentiometer. Chrom Perfect version 5.0.0, Justice Laboratory Software (Justice Innovations,

Inc., Palo Alto, CA) was used to record and integrate both the olfactory data and FID. A

compound was deemed aroma active if it is picked up by the assessor in two out four of the GC-

O runs. The intensity of each compound was normalized so that the peak with the highest

intensity received a score of 100. The normalized intensities of all the runs pertaining to all the

compounds were then averaged. If a compound was detected by the assessor, they were then

asked to rate the compound on an intensity scale of 1 to 5 as a way to estimate the intensity, with

1 meaning "slightly" and 5 meaning "very potent" aroma. Compounds were identified using

linear retention indices (LRI) calculated using the retention time and data of alkane standards ran

under similar conditions.

GC-MS was used to confirm the identity of the aroma active volatiles identified during

the GC-O experiment. The volatile compounds was desorbed by inserting the fiber directly into

the injection port of the GC, which was maintained at 220° C, for eight minutes. Volatiles were

separated and analyzed using a 60m x 0.25mm id 0.5μm wax column with helium carrier gas

flow rate of 2.0 mL/min. The GC oven had an initial temperature of 40°C, held there for 2

minutes, and then increased to 240° C at a rate of 7° C/min and held for 9.50 minutes. The MS

was maintained at 240° C and the sample mass was set to scan between 40-300 m/z in the

positive ion mode. Chromatographic peaks were identified using both standardized retention

time (LRI values) and fragmentation spectra of standards and compared to NIST 2005 spectral

library (*18*).

GC-PFPD Identification of Sulfur Compounds

SPME was used as the extraction technique to analyze the volatile sulfur compounds

within Lindeman's Cuveé Renee. An Agilent 7890A gas chromatograph equipped with sulfur-

specific 5380 pulsed flame photometric detector (PFPD) (model 5380 pulsed flame photometric

detector (PFPD), OI Analytical Co., College Station, TX) was used in the analysis of the sulfur

compounds within the lambic beer. Compounds were separated using a Stable Wax column (30

m x 0.32 mm id. cross-linked polyethyleneglycol, 0.50 μm film thickness, Restek, Bellefonte,

PA). Helium was the carrier gas at a flow rate of 1.5 mL/min. The initial oven temperature was set to 35ºC, held there for one minute, then increased at a rate of 3ºC/min to 65ºC, then increased at a rate of 6ºC/min to 170ºC, and finally increased at 10ºC/min to a final temperature of 240ºC with a hold time of five minutes. The GC injector temperature was set to 200ºC and the detector temperature was 250ºC. The sulfur gate was set to 6 - 24.9 ms, and the pulse frequency was 3 pulses/s. Retention indices were calculated using standard alkanes C_5 - C_{25}.

Time-Intensity Olfactometry Data Acquisition and Analysis, Identification

The assessor was asked to indicate aroma intensity continuously throughout the entire chromatographic run using a linear potentiometer. The potentiometer has a pointer that has the ability to move across 10-cm to indicate aroma intensity. The device has an output of $0 - 1.0$ V connected to the Chrom Perfect A/D board, the software digitally recorded the time and intensity. The chromatographic software used the information collected to create an aromagrams and to calculate olfactometry peak area, peak height, peak duration, and kovats index (KI or LRI) for each individual compound. The assessor was asked to analyze two commercial gueuze samples from the same brewery, each sample in duplicate. Each bottle of beer was analyzed twice by the assessor. Mean aroma intensities for each odorant were calculated by averaging the peak height for four runs. Aroma-active compounds were defined as ones that were detected by the assessor fifty percent of the time, shared similar descriptions, as well as similar retention times.

RI and Odor quality. Volatile compounds were identified based upon their odor descriptions and RI values on both a polar and nonpolar column. Values were also compared to those previously reported. Solutions of hydrocarbons were analyzed in the same manner on both the DB-5 and the RTX-S to calculate RI:

$$RI = 100N + 100n(t_{Ra} - t_{Rn})/(t_{R(N+n)} - t_{RN})$$

N is the carbon number of the lowest alkane and n is the difference between the carbon number

of the two n-alkanes that are bracketed between the compound; t_{Ra}, t_{Rn}, and $t_{R(N+n)}$ are the

retention times of the unknown compound, the lower alkane, and the upper alkane.

Results and Discussion

Identification of Aroma Compounds from Gueuze Beer using SPME/GC-MS and SDE/SAFE

The use of SPME to extract volatile and semi-volatile compounds from beer has been

used for years (*19*). Rodriques et al. (*2*) reported that DVB/CAR/PDMS was able to provide a

more complete profile due to the wider range of chemical compounds detected and higher signal

intensities. SAFE/SDE has been used for the extraction and identification for a number of

different fermented products such as rum (*20*), Chinese Sinkiang fermented camel milk (*21*), and

rice wine (*22*).

In this work, aroma compounds were identified using two common extraction techniques:

SPME and SAFE and both were coupled with GC-MS using Carbowax columns. A total of 64

aroma compounds were identified using a combination of retention index and mass spectral

matching against library standards (Table 3.1). Compounds that could not be identified by

comparing retention index values and mass spectra library were marked as tentatively identified.

Compounds identified belonged to several different chemical classes (ketones, acids, alcohols,

benzene, and phenols). Of the 65 compounds, 17 esters, 15 acids, 12 alcohols, 5 aldehyde, 2

ketones, 2 phenols, 1 thiol, 1 furan, and 1 alkane were identified by GC-MS.

Forty-three of the 62 aroma compounds identified have not been previously reported in

lambic beer. Van Oevelen et al. (*23*) reported finding acetic acid, lactic acid, butyric acid,

propionic acid, isobutyric acid, propanol, butanol, isobutanol, isoamyl alcohol, n-amyl alcohol,

phenethyl alcohol, ethyl acetate, and ethyl lactate. Spaepen et al. (*24*) reported finding hexanoic acid, octanoic acid, decanoic acid, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate, and phenethyl acetate.

When comparing the two extraction methods to each other, each extraction method was unable to identify 17 volatile compounds. SPME was unable to extraction seven acids (lactic, propanoic, isobutyric, butyric, valeric, heptanoic, and hexadecanoic), six alcohols (propanol, butanol, isoamyl alcohol, 2-heptanol, hexanol, 4-methyl-3-pentenol), two furans (1-(furan-2-yl)-ethanone, 2-furanmethanol), one thiol (3-methylthio-1-propanol), and one aldehyde (benzeneacetaldehyde) that SDE-SAFE was able to extract. Engel et al (*11*) reported that SAFE is a much more efficient extraction technique for isolating less volatile and polar compounds like acids and alcohols. SPME was able to extract 16 compounds that SAFE was unable to identify. SPME was able to identify nine esters (ethyl 4-methylpentanoate, ethyl nananoate, ethyl 9-decenoate, ethyl undecanoate, ethyl dodecanoate, ethyl tetradecanoate, ethyl hexadecanoate) compounds, three alcohols (octanol, decanol, 2-nonanol), one ketone (4-methyl-2-pentanone), one aldehyde (decanal), ketone (α-ionone), acid (isobutanoic), phenol (4-vinylguaiacol), and calamenene.

Alcohols (36.2% of the total volatiles), acids (7.3%), and esters (48%) account for the majority 91% of the volatile compounds identified in gueuze beer. Alcohols are produced as a by-product of yeast metabolism (*25*). Ethanol (27.9% of the total alcohols), phenethyl alcohol (4.3%), and isoamyl alcohol (3.5%) were the three major alcohols identified. Esters were the dominant class in terms of total amount for SPME for gueuze beer. Among the 17 detected esters, ethyl octanoate (23.0% of the proportion percentage to esters), ethyl decanoate (14.1%), ethyl dodecanoate (2.3%), and ethyl hexanoate (2.1%), were the major ester compounds

identified. The concentration of esters are dependent upon several factors: fermentable sugars, fermenting temperature, and yeast strains (*25*). Esters are an important group of volatile compounds produced during yeast fermentation. Ethyl lactate, one of the ester compounds detected by both isolation techniques, is a byproduct of *Lactobacillus* sp and related to lactic acid fermentation (*26*).

After alcohols and esters, acids are another major group of compounds present in gueuze beer. A total of 15 acids were detected. Among them were acetic acid (0.2% of the total acids identified), decanoic acid (1.5%), butyric acid and hexanoic acid (0.2%), and octanoic acids (5.0%).

GC-PFPD Identification of Sulfur Compounds

Sulfur compounds contribute to the aroma and flavor of a number of different food products including beer (*27*). However, because of their low sensory thresholds, sulfur compounds can easily become unpleasant and cause off-flavors and aromas (*28*). Malt, hops and water are three possible sources of sulfur compounds. However the majority of the volatile and semi-volatile compounds do not traditionally come from the raw materials, but are produced during fermentation (*25*). A number of sulfur compounds have been reported in beer, and the two main volatile sulfur compounds are dimethyl sulfide (DMS) (*29*) and methiol (*27*). Several other sulfur compounds are only found in trace amounts (*30*).

A pulse flame photometric detector (PFPD) utilizes a flame source and a gas flowing at a rate that is unable to maintain a continuous flame, in contrast to that of a flame ionization detector (FID). The cycle is repeated between 2 - 4 times a second. Selectivity is based upon the appropriate filter (*31*).

The PFPD is currently the most popular commercially available detector for the selective measurement of sulfur compounds. SPME can be used in the extraction of sulfur compounds varying across a wide range of boiling points and polarities. Identification of sulfur compounds using SPME-GC-PFPD is extremely difficult due to the low concentration of sulfur compounds typically present and the relatively higher concentration of non-sulfur compounds (27). Compounds were tentatively identified using their retention times to calculate their linear retention index (LRI), also known as the Kovats Index.

Currently, there is limited research available describing the entire range of sulfur compounds present in gueuze beer. Van Oevelen et al. (23) reported finding dimethyl sulfide; however, no other sulfur compounds were identified in gueuze beer. Hill et al. (27) reported finding over thirteen sulfur compounds in European pilsner and lagers.

In this study, the PFPD detected thirty-eight sulfur peaks in Lindeman's Cuveé Rene, a commercially available gueuze beer. The retention time and LRI value obtained for unknown peaks were compared to values previously reported in the literature coming from Dr Rouseff laboratory pertaining to sulfur compounds. Of the thirty-eight compounds detected, five of them have been previously reported in beer (27), one in wine (32), twenty-nine were identified for the first time in gueuze beer, and three could not be identified. The use of MS for identification is extremely difficult for sulfur compounds because of their low concentrations found in beer. The PFPD detected sulfur compounds were tentatively identified using their LRI values. The thirty eight compounds are shown in Table 3.2. Two peaks in Table 1 were reported as unknown, but did produce a sulfur detector response. Based upon the retention times, these two compounds were tentatively identified as 2-methylthiacylopentane and 3-(methylthio)-pyridine. Further research is required to confirm the identification of all the compounds listed in Table 3.2.

SPME/GC-O

Table 3.3 contains the information on the 28 aroma-active compounds present in a commercially available (gueuze) lambic beer. Compounds that were detected in fifty percent of all GC-O samples at the same retention time with similar aroma descriptors were considered to play a role in the aroma of lambic (gueuze) beer. Tentative identification was made comparing similar LRI values for known aroma compounds with similar descriptors. Compounds were ranked based upon the average of the intensity perceived by the panelist per each chemical.

The five highest ranking chemical groups for aroma intensities were: alcohol, acid, ester, thiol, and furan. The most intense odorants perceived was ethanol, followed by 1-(furan-2-yl)-ethanone. While this is the first time that 1-(furan-2-yl)-ethanone has been identified in beer, it has been identified in sweet corn products (*33*), guava fruits and canned puree (*34*), and Castanopsis flower (*35*). It should be noted that 1-(furan-2-yl)-ethanone was identified based on the mass spectrum and LRI, value which were obtained using the SAFE/SDE method. A peak at the same LRI was seen when using SPME as the extraction technique, however not enough of the compound was present for MS identification. An unknown sulfur compound had the third highest aroma intensity. This compound was identified as a sulfur compound based upon its aroma descriptors (musty, moldy/decay) and comparing the LRI values and the peaks detected when using the GC-PFPD. Ethyl isobutyrate was the fourth highest ranking aroma compound perceived with an odor description of bubble gum or sweet. This compound has been identified in Jamaican rum (*36*) and Bavarian Pilsner style beer (*37*). This is the first time, however, that this compound has been identified in lambic beer. The fifth highest odorant perceived was isovaleric (3-methylbutanoic) acid. The odor descriptor for isovaleric acid is rancid or acidic.

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This compound has also been identified as being one of several compounds produced by the wild yeast species, *Brettanomyces* (*38*).

A single expert assessor of lambic beer was used in the sensory analysis of the lambic beer due to the inherent variation between multiple assessors. Acree et al determined that the variation between individual assessors was greater than the variation between either the age or the sex of the panelists (*39*).

AEDA

The major volatiles that eluted with an odor are shown in Table 3.4 sweaty, fruity, and spicy odors. Sniffing of serial dilutions (AEDA) resulted in 32 odor-active compounds, which ranged in FD factor range of 1 - 729. The highest FD factors were found for 2-methyl-butanoic acid (sweaty) and phenylmethanol (solvent/aromatic). Isovaleric acid (rancid, sweaty) and 4-ethylphenol (must) both had the next highest ranking FD factors of 243. The volatile acidity present in this particular style is usually high and produces a vinegar-like and cheesy aroma.

The odor active compounds were separated into acidic and neutral basic volatile compound fractions. Within the acidic fraction a total of 18 compounds were detected by using the AEDA method. The highest odor active compound for the acidic fraction (AF) was 2-methylbutanoic acid (sweaty) with a FD factor of 729, followed by isovaleric acid (rancid) with an FD value of 243. A few of the odor descriptors for compounds in the AF were sweat, rancid, Odor active compounds acetic acid (vinegar) (*40*), decanoic acid (rancid, fat) (*24*), furaneol (carmel) (*41*), hexanoic acid (sweat) (*40*), butyric acid (*40*) (rancid, cheese), propanoic acid (pungent) (*23*), and 2-methylbutanoic acid (sweaty) (*42*) have all been identified in beer and were previously identified in lambic beer. Decanoic, hexanoic, butyric and propanoic acids have

also been identified in lambic beer (*23, 24, 43*). The concentration of organic acids in lambic beer can range from three to eight times higher than for a typically American Lager (*44*).

The Neutral/Basic fraction (NBF) accounted for 22 of the 40 volatile compounds identified within the NBF fraction using AEDA method. Phenylmethanol (FD - 729) and 3,5-dimethyl-1,2,4-trithiolane (FD - 243) were identified as having the highest FD factor within the NBF. Some of the odor descriptors for the identified compounds were fruity, malty, honey, and spice. Isoamyl acetate (*23*), ethyl octanoate (*24*), and ethyl dodecanoate (*24*) had lower FD values for the NBF and have all been identified previously in lambic beer. Prior to 2005, ethyl 4-methylpentanoate had never been identified as playing a role in the odor activity of beer. Using SDE/SAFE, Fritsch et al (*37*) was able to determine the presence of ethyl 4-methylpentanoate while others had not been able to do so.

4-Ethylphenol and 4-ethylguaiacol, two compounds commonly associated with the wild yeast *Brettanomyces sp.* that can cause wine, beer, and ciders to spoil (*45*), was shown to play a role in the odor activity of this particular style of beer. The FD factor for 4-ethylphenol was 243, tied for the second highest FD factor in the NBF, and 4-ethylguaiacol had an FD factor of 81. Van Oevelen et al (*43*) and Boulton and Quain (*46*) all have indicated that these two compounds play a vital role in the aroma of lambic beer. Our results support the role of these two compounds in the overall aroma of lambic beer.

SPME vs. SDE/SAFE with GC-O

When using SPME as the extraction technique for GC-O, 28 volatile compounds where identified, while when using SDE/SAFE, 32 volatile compounds were found. In comparing the two methods, 4-guaiacol, ethyl 4-methylpentanoate, ethyl octanoate, hexanoic acid, ethyl dodecanoate, and nonanoic acid were detected by the three panelists for SDE-SAFE and the

single assessor for the SPME procedure. SDE/SAFE was able to identify nine organic acids, while SPME was only able to identify four. In comparing the two extraction methods, SPME was able to identify seven esters, while SDE/SAFE was able to identify five esters. Three alcohols were extracted using SPME, while eight alcohols were extracted using SDE/SAFE.

Conclusion

In this study, the volatile compounds of commercially available gueuze beer were extracted using SPME and SAFE and analyzed using a GC-MS. A total of 101 compounds, 63 volatile compounds and 38 sulfur compounds were identified. Further studies are needed to confirm the identification of the proposed volatile and sulfur compounds identified in this study. The two extraction techniques provided similar numbers regarding the quantity of compounds identified using GC-MS and GC-O.

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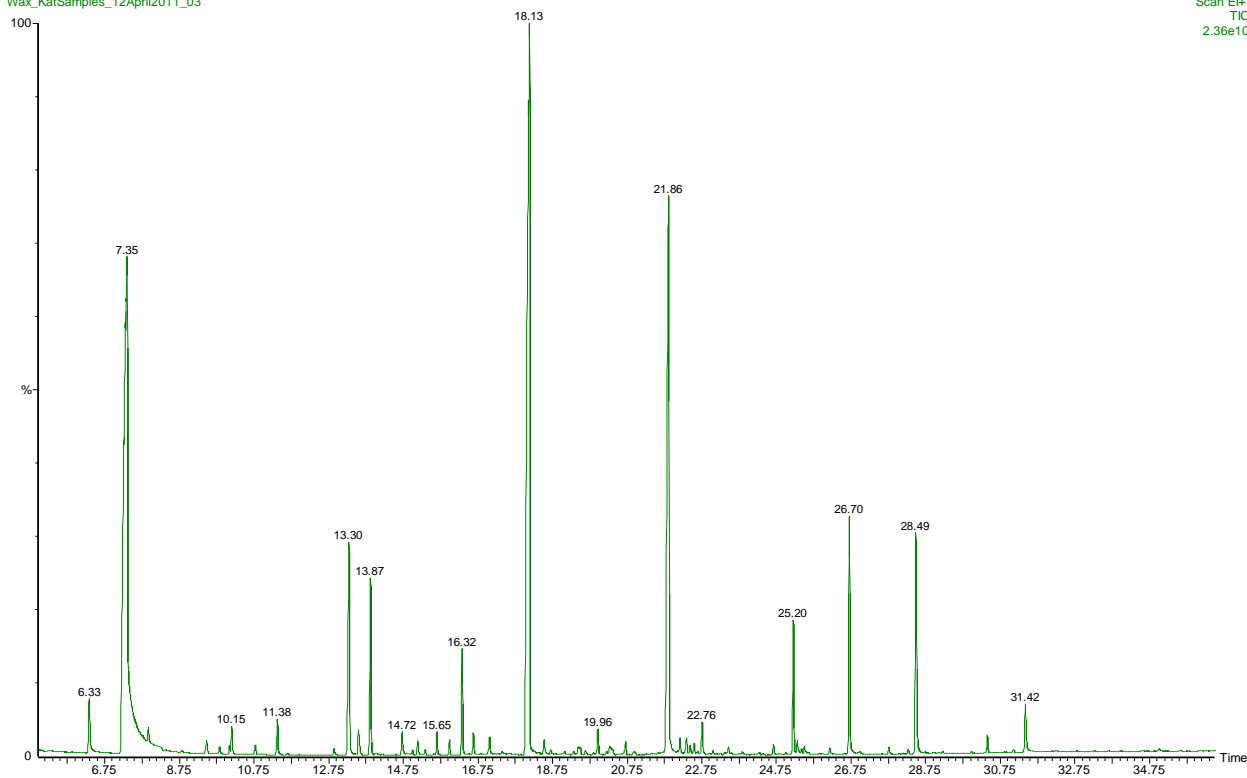
Figure 3.1: GC-MS Chromatograph of lambic beer using SPME
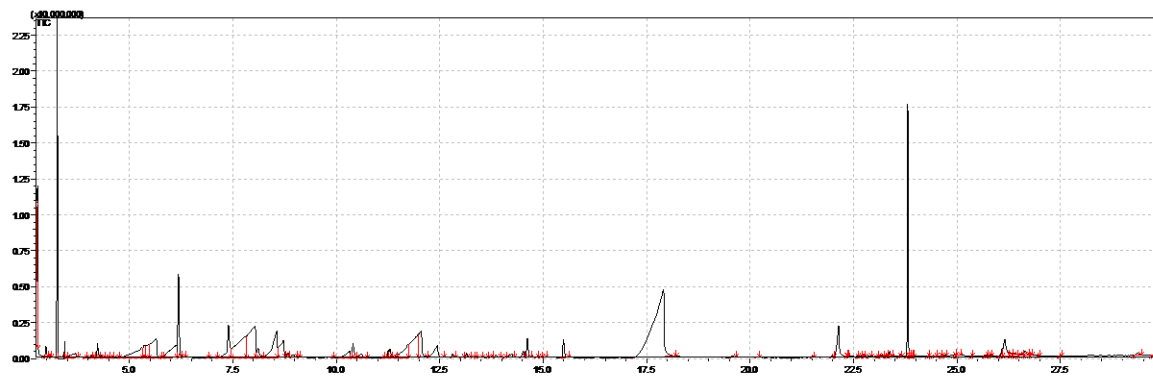
 Figure 3.2 GC-MS Chromatography of Lambic Beer using SDE/SAFE Extraction
(Acidic Fraction)
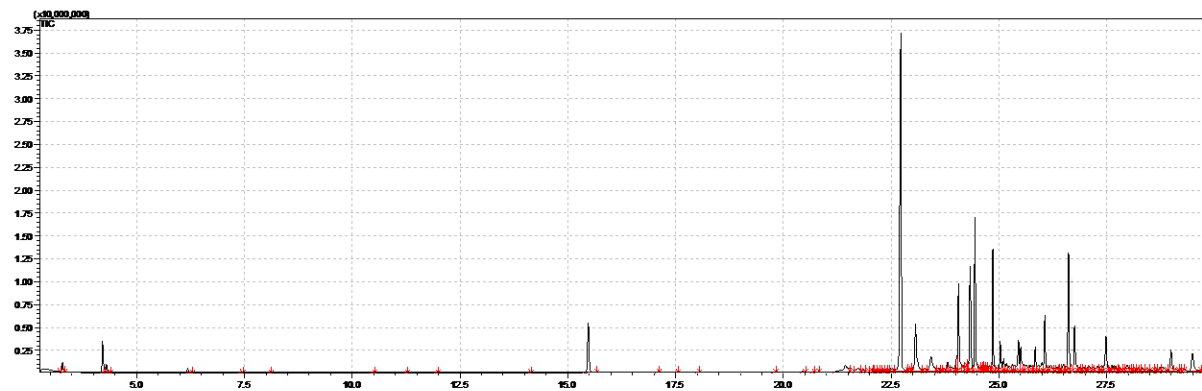
 Figure 3.3 GC-MS Chromatography of Lambic Beer using SDE/SAFE Extraction
(Neutral/Basic Fraction)

Table 3.1: Identification of flavor compounds present in lambic (gueuze) beer using SPME and SAFE-SDE as extraction methods.

				Method		Identification	Reference
Chemical Compound	**Previously Identified**	**LRI Wax**	**LRI Confirmed**	**SPME**	**SAFE-SDE**		
Lactic acid	(23)	*			x	MS	
Ethyl acetate	(24)	900		x	x	MS, RI	
Ethanol		954		x	x	MS, RI	
Ethyl isobutyrate		982	984	x	x	MS, RI	(47)
4-methyl-2-pentanone		1025		x		MS, RI	
Benzeneacetaldehyde		*	962		x	MS,RI	(20)
Propanol	(23)	1035	1036		x	MS	
Ethyl butanoate		1051	1047	x	x	MS, RI	(48)
Ethyl 2-methylbutanoate		1065	1060	x	x	MS, RI	(49)
Ethyl 3-methylbutanoate		1077	1077	x	x	MS, RI	(49)
Isobutyl alcohol	(23)	1106		x	x	MS, RI	
Isoamyl acetate	(23)	1136	1132	x	x	MS, RI	(32)
Butanol	(23)	1138	1138		x	MS,RI	
Ethyl 4-methylpentanoate		1198	1181	x		MS,RI	(47)
Isoamyl alcohol	(23)	1216		x	x	MS, RI	
Ethyl hexanote		1243	1244	x	x	MS, RI	(50)
Styrene		1283	1273	x	x	MS, RI	(51)
2-heptanol		1313	1331		x	MS, RI	(52)
Ethyl lactate	(23)	1343		x	x	MS	
Ethyl heptanoate	(24)	1345		x	x	MS	
Hexanol		1353	1351		x	MS,RI	(53)
4-Methyl-3-pentenol		1388			x	MS, RI	
Nonanal		1416	1415	x	x	MS, RI	(48)
1-(furan-2-yl)-ethanone		1434	1434		x	MS, RI	(35)
Ethyl octanoate	(24)	1454	1446	x	x	MS, RI	(50)
Acetic acid	(23)	1475	1477	x	x	MS, RI	(48)
α-ionone		1484		x		MS	
Pentadecane		1501	1500	x		MS,RI	
Decanal		1524	1538	x		MS, RI	(54)
2-nonanol		1527	1532	x		MS, RI	(55)

Ethyl nananoate		1552	1528	x		MS, RI	(56)
Propanoic acid	(23)	1565	1523		x	MS,RI	(57)
Isobutyric acid (2-methylpropanoic acid)	(23)	1570	1584		x	MS, RI	(50)
Octanol		1570	1566	x		MS, RI	(58)
Isobutanoic acid		1593	1588	x		MS, RI	(48)
Butyric acid	(23)	1634	1628		x	MS, RI	(49)
Ethyl decanoate	(24)	1658	1630	x	x	MS, RI	(57)
2-Furanmethanol		1661	1661		x	MS, RI	(59)
Valeric acid		1671			x	MS	
Isovaleric acid (3-Methylbutanoic acid)		1691	1691	x	x	MS, RI	(48)
Ethyl succinate		1697	1690	x	x	MS, RI	(60)
Ethyl 9-decenoate		1710	1694	x		MS, RI	(60)
3-(Methylthio)-1-propanol		1715	1738		x	MS,RI	(50)
Ethyl undecanoate		1756		x		MS	
Decanol		1773	1748	x		MS, RI	(61)
Ethyl dodecanoate		1858	1822	x		MS,RI	(62)
Hexanoic acid	(24)	1865	1863	x	x	MS, RI	(50)
Benzyl alcohol		1876		x	x	MS	
Calamenene		1883	1837	x		MS	
Phenethyl alcohol	(23)	1956	1940	x	x	MS, RI	(48)
Heptanoic acid		1965	1990		x	MS, RI	(63)
Hexadecanoic acid		*	1984		x	MS,RI	(64)
4-ethylguaiacol		2033	2048		x	MS,RI	(32)
Furaneol		2033	2039		x	MS, RI	(49)
Ethyl Myristate (Ethyl tetradecanoate)		2064	2034	x		MS	(62)
Octanoic acid	(24)	2079	2083	x	x	MS, RI	(50)
Nonanoic acid		2185	2202	x	x	MS,RI	
P-Ethylphenol		2216	2205	x	x	MS, RI	(48)
4-vinylguaiacol		2249	2223	x		MS, RI	(48)
Ethyl palmitate (Ethyl hexadecanoate)		2267	2229	x		MS, RI	(62)
Decanoic acid	(24)	2289	2296	x	x	MS, RI	(50)

Ethyl hydrogen succinate		2412	2360	x		MS, RI	(*20*)
Dodecanoic acid		2501	2517	x	x	MS, RI	(*48*)
2-Phenylacetic acid		2547	2574		x	MS,RI	(*49*)

Linear Retention Index (LRI)

*Compounds were identified comparing LRI values for DB-5 column

Compounds were identified based upon their LRI (RI) values and mass spectrum. (MS).

Table 3.2: Identification of gueuze beer sulfur volatiles from photometric flame pulse detector (PFPD) data

Sulfur Chemical Compound	DB-Wax LRI	LRI Confirmed	Reference	Aroma Descriptors
hydrogen sulfide	698	691	(*65*)	
Carbon Disulphide	726	722	(*66*)	
Dimethyl Sulfide (DMS)	740	736	(*66*)	cabbage, sulfur, gasoline
1-propanethiol	771	771	(*58*)	
Unknown sulfur	779			
methional	909			cooked potato
dimethyl disulfide (DMDS)	1069	1071	(*49*)	onion, cabbage, putrid
3-Methylthiophene	1096	1098	(*66*)	
2-Methylthiacylopentane	1114	1117	(*58*)	
Methyl thiopropionate	1142	1132	(*67*)	
2-methyl thiazole	1273	1268	(*65*)	
dimethyl trisulfide (DMTS)	1397	1399	(*18*)	sulfur, fish, cabbage
Methyl 2-(methylthio)-acetate	1404	1402	(*68*)	
Methyl Thiohexanoate	1411	1417	(*18*)	
Methional	1470	1465	(*65*)	
2,5-Dithiahexane	1484	1479	(*69*)	
3-Mercapto-3-methylbutyl formate	1497	1497	(*70*)	
2-Methyltetrahydrothiophen-3-one	1513	1509	(*71*)	
2-Ethylthiazolidine	1520	1515	(*58*)	
4-mercapto 4-methyl-2-pentanol	1539	1522	(*66*)	
methyl 3-(methylthio)propionate	1556	1554	(*18*)	
ethyl 3-methylthio propionate	1581	1584	(*18*)	
1-p-Menthene-8-thiol	1592	1580	(*66*)	

Sulfur	DB-Wax	LRI	Reference	Aroma Descriptors
3,5-Dimethyl-1,2,4-trithiolane	1606	1602	(*58*)	
Dihydro-2-(3H)-thiophenone	1616	1615	(*71*)	
Methyl thioctanoate	1643	1641	(*18*)	
3-Mercapto-3-methylbutan-1-ol	1657	1658	(*72*)	
2-Acetylthiazole	1662	1660	(*55*)	
Unknown sulfur	1671			
4-Methylthiazole	1684	1681	(*55*)	
3-Mercaptohexyl acetate	1736	1735	(*32*)	
Unknown sulfur	1742			
3-(Methylthio-pyridine	1802	1803	(*69*)	
3-Mercapto-1-hexanol	1839	1828	(*66*)	
Benzothiazole	1962	1951	(*73*)	
Unknown sulfur	1971			
Unknown sulfur	2046			

Linear Retention Index (LRI)

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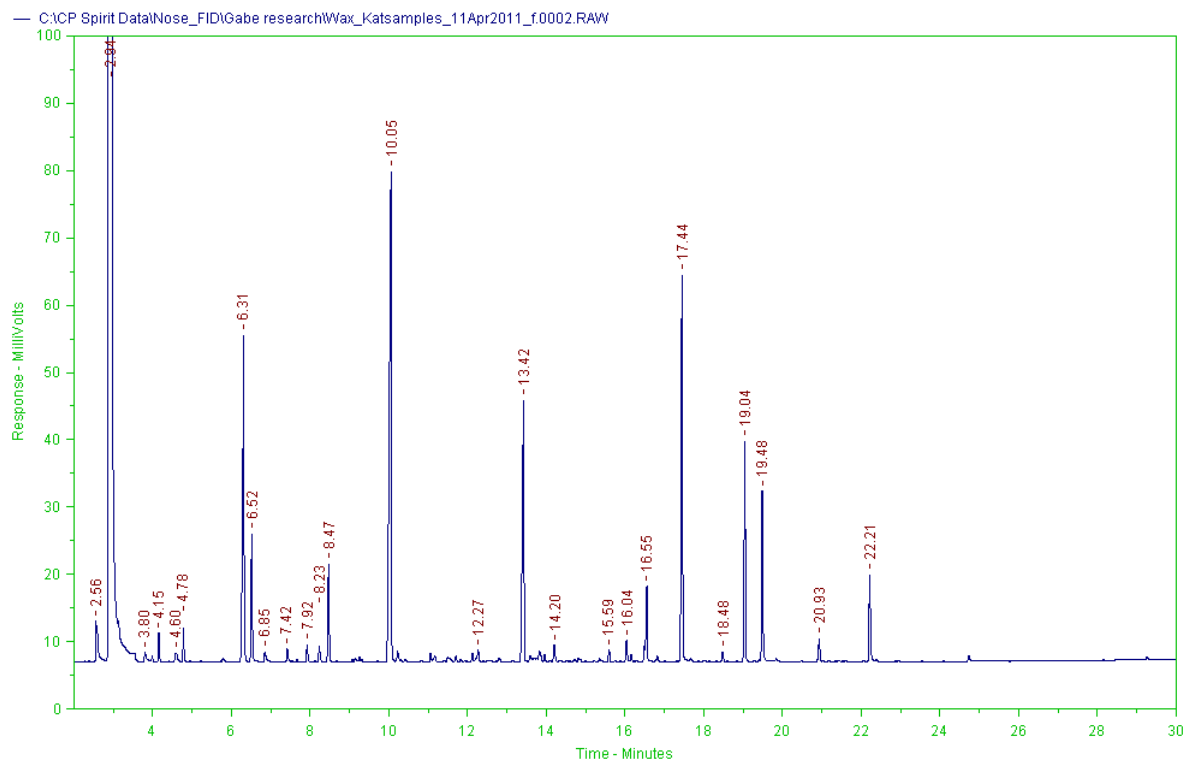
Figure 3.4: GC-O aromagraph for lambic beer using SPME as extraction technique

Table 3.3: Tentative identification of aroma-active compounds in commercial available Gueuze beer based upon FD factors using SDE-SAFE extraction

LRI (WAX)	Compound	Descriptor	Ranking[a]
951	Ethanol	alcohol/ethanol	1
972	Ethyl Isobutyrate	bubble gum	4
1011	**2-butanol**	**oily, wine-like**	**21**
1034	Methyl 3-methylbutanoate	fruity	26
1047	Ethyl Butanoate	fruity, banana, pineapple	23
1063	Ethyl 2-methylbutanoate	sweet, fruity, strawberry	15
1094	3-Methylthiophene	moldy/decay	12
1111	2-Methylthiacylopentane	stinky, stale	27
1180	Ethyl 4-methylpentanoate	fruity	28
1220	Unknown Sulfur	musty, moldy/decay	3
1282	Styrene	pungent,glue	13
1295	Octanol	moss, mushroom	14
1430	**1-(Furan-2-yl)-ethanone**	**balasmic-cinnamic**	**2**
1443	Ethyl Octanoate	musty	16
1493	Unknown Compound 1	fruity	24
1633	Butanoic acid	rancid, cheesy, sweaty	6
1662	Unknown Compound 2		10
1669	2-methylbutanoic acid	overripe fruit, sweaty	18
1685	3-methylbutanoic acid	sweaty, cheesy, rancid	5
1724	dodecanal	fatty, waxy	20
1778	2-Undecanl	sweet	8
1805	3-(Methylthio)-pyridine	sulfur	17
1851	ethyl dodecanoate	fruity	19
1859	hexanoic acid	pungent, cheese, rancid	25
1905	Unknown Compound 3		7
2019	4-ethylguaiacol	clove, spice	22
2052	Unknown Compound 4		9
2177	nonanoic acid	green	11

*Linear Retention Index (LRI) based upon a DB-wax column
[a]Ranking: Compounds were ranked on their average intensity. Compounds with a rank of 1 has the most important odorant and 28 being the least.
Bold letters indicate tentative identification of a compound
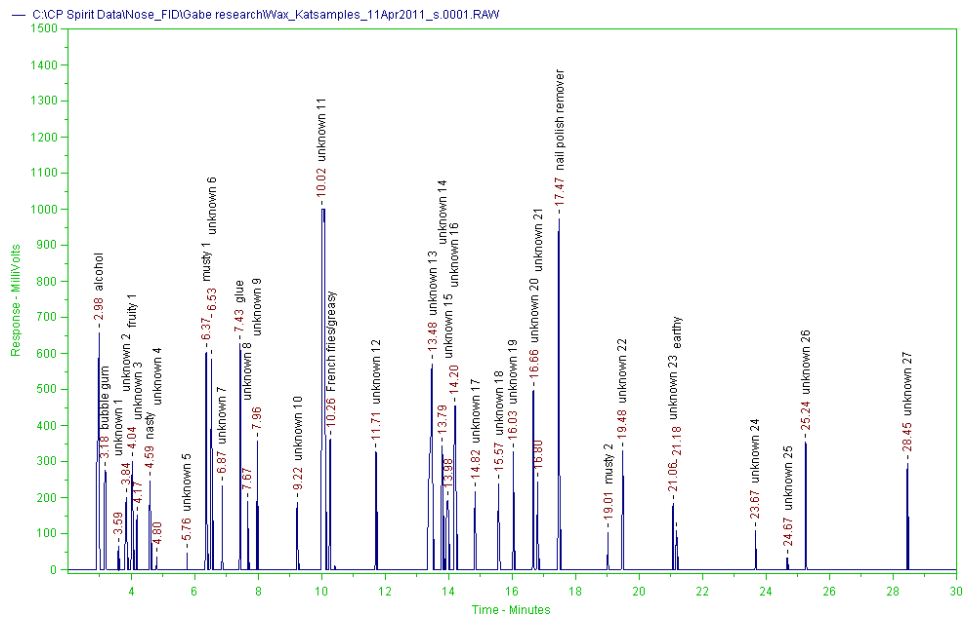
Figure 3.5: GC-O Chromatogram of Commercial Available Gueuze Beer using OSME

Table 3.4. Flavor Dilution values of Volatile Compounds in Lambic Beer

no.	Compound	descriptor	Conc.	RI	FD
1	propanol	pungent	NB	1036	81
2	Ethyl butanoate	pungent, fruit	NB	1047	9
3	Ethyl 2-methylbutanoate	sweet, fruity, berry	NB	1060	27
4	Ethyl 4-methylpentanoate	fruit	NB	1108	3
5	Isoamyl Acetate	banana	NB	1118	1
6	2-methylpropanol	solvent, bitter	NB	1125	3
7	Butan-1-ol	Fruity	NB	1138	1
8	2-methyl-1-butanol	malt	AC	1206	81
9	3-methyl-1-butanol	malt, burnt	NB	1206	81
10	Isoamyl Alcohol		NB	1216	81
11	Ethyl Octanoate	menthol	NB	1422	1
12	Acetic Acid	sour	AC	1475	81
13	propanoic acid	pungent, rancid	AC	1552	3
14	3,5-Dimethyl-1,2,4-trithiolane	potato	NB	1606	243
15	butyric acid	rancid, cheese, sweat	AC	1628	9
16	2-methyl-Butanoic acid	sweaty, overripe fruity	AC	1667	729
17	Isovaleric acid	sweat, acid, rancid	AC	1691	243
18	3-Mercaptohexyl acetate	sulfur	NB	1715	9
19	Phenethyl acetate	rose, honey	NB	1810	3
20	Unknown	sweaty	AC	1833	3
21	Ethyl dodecanoate	mango-like	NB	1858	1
22	hexanoic acid	sweat	AC	1863	3
23	Phenylmethanol	aromatic	NB	1876	729
24	Phenylacetic acid	honey, flower	AC	1908	9
25	heptanoic acid	rancid	AC	1965	3
26	Furaneol	caramel	AC	2022	27
27	4-ethylguaiacol	clove-like, flowery	NB	2033	81
28	octanoic acid	sweat, cheese	AC	2079	27
29	nonanoic acid	green	AC	2191	3
30	4-ethylphenol	must	NB	2205	243
31	n-Decanoic acid	rancid, fat	AC	2289	1
32	2-Phenylethanol	honey, spice, rose, lilac	AC	2547	1

CHATPER 4: ANALYSIS OF THE VOLATILE AND SEMI-VOLATILE COMPOUNDS

PRESENT IN COMMERCIAL AVAILABLE BEER USING GC-MS AND HPLC

Abstract

Lambic beer is the oldest style of beer still being produced in the Western world using spontaneous fermentation. Gueuze is a style of lambic beer prepared by mixing young (1 year) with older beers. Little is known about the volatiles and semi-volatiles found in commercial brands of lambic (gueuze) beers. SPME was used to extract the volatiles and semi-volatiles from nine different brands of lambic beer. GC-MS was used for the separation and identification of the compounds extracted with SPME. pH and color of the beers were measured using standard procedures. A total of 50 compounds were identified within the nine brands. Seventeen of the 50 compounds identified have been previously identified. Compounds identified included a number of different chemical groups such as acids, alcohols, phenols, ketones, aldehydes, and esters. Ethyl acetate, 4-ethylphenol, and 4-ethylguaiacol are known by-products of the yeast *Brettanomyces*, which is normally a spoilage microorganisms in beer and wine but important for flavor characteristics of lambic beer. There were no differences in pH but there were differences in color between the beer samples.

Introduction

Lambic beer is one of the oldest styles of beer still being brewed today (*1*). There are currently eight lambic breweries (Belle Vue, Boon, Cantillon, De Troch, Girardin, Lindemans, Mort Subite, Timmermans), five blenders (De Cam, Drie Fonteinen, Hanssens, Oud Beersel, and Tilquin), and two lambic breweries located in West Flanders (Bockor, Van Honsebrouck) that are currently producing and selling lambic beer. However distribution of this type of beer is very limited within the United States (*2*). Many lambic brewers and blenders are in financial trouble because the very products they make putting their business at risk because of the time required to produce lambic beer. Lambic beers can spend anywhere from a few weeks to several years aging in casks or fermentation tanks before they are ready to be sold. This can be an issue since breweries are holding onto hundreds of thousands of dollars' worth of inventory while the beer is aging. Another issue that arises from aging lambic beer is the current tax system in place forces brewers to pay taxes on their beer within a year of being produced. This is a problem since true lambic beers are required to age a minimum of one year. Oftentimes, brewers are in debt to the government before the beer is even sold. The art and craft of making lambic beer is also dying, because few people are willing to take the place of retiring brewers (*1, 3*).

Beer is a complex beverage system made up of volatile and semi-volatile compounds belonging to a number of different chemical classes such as fusel alcohols, ethyl esters, fatty acids, higher alcohol acetates, isoamyl esters, carbonyl compounds, furanic compounds, terpenoids, C13-norisoprenoids, and volatile phenols (*4*). Many chemical compounds play an important role in the appearance, aroma, flavor, and mouthfeel of alcoholic beverages. Consumers judge the quality, character, and acceptability of alcoholic beverage based upon

olfactory and taste properties. The aroma profile of beer is made up of a number of different chemical compounds varying in concentration and polarity (*5*).

Similar to other alcoholic beverages, beer is made up of a large number (~800) of volatile and semi-volatile compounds, however only ten to thirty are aroma active (*6-8*). Different flavor compounds can affect the aroma and flavor individually, synergistically, or antagonistically and not all compounds affect the aroma of a product equally. Some compounds enhance the background profile, while others contribute to the aroma and flavor characteristics (*9*). It is not always the case that compounds with the greatest concentration have the greatest influence on a product. In actuality, it is the compounds with the lowest concentration often have the greatest influence on the aroma of a product (*10*).

The aim of this study was to compare the chemical and volatile compositions of commercially available lambic beers using GC-MS and HPLC. GC-MS was utilized to analyze the volatiles while HPLC was used to look at the acid compounds.

Materials and Methods

Materials

Ethyl isobutyrate (99% purity), ethyl butyrate (99% purity), ethyl 2-methylbutyrate (99% purity), ethyl isovalerate (99% purity), isobutyl alcohol certified by ACS, iso-amyl alcohol, styrene, nonyl aldehyde (95% purity), ethyl caprylate (99% purity), n-pentadecane (99% purity), decanal, ethyl nonanoate, 1-octanol (99% purity), isobutryic acid (99% purity), mono-ethyl succinate (95% purity), ethyl undecanoate (97% purity), 1-decanol (99% purity), ethyl dodecanoate (98% purity), hexanoic acid (99% purity), n-nonanoic acid (97% purity), decanoic acid (99% purity), lauric acid (99.5% purity) and (-)-ethyl lactate were

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purchased from Fisher Scientific (Pittsburg, PA, USA) and used as standards. Acetic acid with a concentration of 0.150g/L and L-lactic acid with a concentration of .204g/L were purchased from R-Biopharm AG (Darmstadt, Germany). Octanoic acid and hexanoic acid, both having a concentration of 5mg/10mL, were purchased from Fluka Analytical (Sigma-Aldrich, St. Louis, MO). Isobutyric acid with a concentration of 5mg/10mL was purchased from Acros Organics (Geel, Belgium. Lambic beer samples were purchased from a local wine and beer store in Blacksburg, VA and Athens,GA. The brands were Cuvee Reneé (LK23JGC 2975 23 Nov 2012), Oude Gueuze Vieille (30-10-2026 L8304), Hanssens Artisan, Cantillon Gueuze 100% Lambic Bio (3-Dec 2010 Bottled), 3-Fonteinen (Bottled Feb 23, 2006), Gueuze Girardin (XO179), Oude Gueuze Boon (Best before 26-1-2025), Gueuze Boon (02-12-2025), and Cantillon – Classic Gueuze (13 – Nov 2009 bottled).

Sample Preparation

The purchased beer was stored at room temperature before analysis. Beer was degassed using an ultrasonic bath (Model FS20, Fisher Scientific) for 10 minutes. After the beer was sonicated, it was filtered using a 5 mL syringe with a 0.45 µm filter (Fisherbrand MCE, mixed cellulose ester, Cat 09-719B).

pH Measurement

pH was measured in triplicate for all bottles of beer immediately after the bottles were opened. pH measurements were conducted using an Accumet XL20 probe which was calibrated before use (Fisher Scientific, Pittsburg, PA).

Color

Color was measured with the official AOAC 977.50 method using a scanning spectrophotometer (Shimadzu model UV-2550, Columbia, MD) (*11*).

Solid Phase Microextraction (SPME)

Extraction and concentration of volatile compounds in commercially available gueuze beer was performed by solid phase microextraction. A SPME fiber (50/30 um DVM/Carboxen/PDMS, 2 cm, Supelco, Bellefonte, PA) was exposed to the headspace above 4 mL of gueuze beer in 10ml headspace vials with Teflon-lined silicone septa (Chromacol, Fisher Scientific) for 30 minutes at 40°C with an agitation speed of 250 rpm. Samples were equilibrated for two minutes prior to exposing the fiber. An AOC-5000 Plus (Shimadzu Scientific, Columbia, MD) SPME autosampler was used for automation of extraction and injection. Volatile compounds were desorbed for five minutes in the injection port of a QP2010 Ultra (Shimadzu, Columbia, MD) gas chromatography equipped with a GCMS-QP2010 Ultra gas chromatograph - mass spectrometer. The injection port was set to 250°C, and all injections were made in splitless mode using a narrow bore, deactivated glass insert. Volatile compounds were separated using a nonpolar (SHRXI-5MS) 30m * 0.25mm id * 0.25 μm film thickness column with He as the carrier gas at a flow rate of 2.0 mL/min (linear velocity 53.8 cm/sec). The GC oven temperature program was 35º C held for 5 minutes and then increased to 225º C at a rate of 6º C/min. Once the final temperature of 225º C was reached, it was maintained for 10 minutes. The MS was maintained at 200°C and sample mass was scanned in the range of 40 – 200 amu. GCMS was performed to identify the volatile compounds present in commercial samples of gueuze. Peaks were identified using standardized retention time (retention index values, RI) and fragmentation spectra of standards and the Wiley 2010 mass spectral library.

Identification

RI and Odor quality. Volatile compounds were identified based upon their odor descriptions and RI values using both polar (DB-Wax) and nonpolar (DB-5) columns. Values were compared to

literature values. Solutions of hydrocarbons were analyzed in the same manner on both the DB-5

and DB-Wax columns to calculate RI:

$$RI = 100N + 100n \ (t_{Ra} - t_{Rn})/(t_{R(N+n)} - t_{RN})$$

N is the carbon number of the lowest alkane and n is the difference between the carbon number

of the two n-alkanes that are bracketed between the compound; t_{Ra}, t_{Rn}, and $t_{R(N+n)}$ are the

retention times of the unknown compound, the lower alkane, and the upper alkane.

High Performance Liquid Chromatography (HPLC)

Analysis of acids was conducted using an Agilent 1100 Series LC (Agilent Technologies,

Santa Clara, CA) with micro degasser, quaternary pump, autosampler, thermostated column

oven, and a diode array detector (DAD). A 5 µm 250 mm * 4.6 mm (i.d.) nucleosil phenyl

(C6H5) column (Macherey-Nagel, Bethlehem PA) was used at 20°C. The mobile phase

consisted of 10 mM aqueous phosphate buffer at pH 2.5. The wavelength range of 200 – 400 nm

was recorded using the DAD and used for spectral analysis. The flow rate was 1.0 mL/min and

an injection volume was 5 µL. External standard curves for acetic and L-lactic acid were made at

200, 400, 800, 1000, and 1200 mg/L concentrations in beer.

Chemical Analysis of lambic beer

The Enology Service Laboratory at Virginia Polytechnic Institute and State University

(Virginia Tech) is a part of the Wine/Enology Grape Chemistry Group. This is a full service

laboratory that was able to aid in the chemical analysis of the commercial available lambic

beersamples. The Enology Service Laboratory analyzed the beer for reducing sugars, total

acidity, lactic acid, and volatile acidity using standard methods.

Statistical Analyses

Statistical analyses were performed with SPSS software for Windows (version 18.0; SPSS Inc., Chicago, IL). Statistical analysis of the data for pH, color, and quantified compounds was performed by one-way analysis of variance with the linear model. Tukey-Kramer HSD was used to compare the least square means of separation. Brands were considered significant at $p < 0.05$. Data values were reported as means ± SD.

Results and Discussions

pH

All lambic beer styles contain high levels of organic acids. The pH of gueuze, kriek (sour cherries), and framboise (raspberries) all have lower pH levels than typical American lagers. The pH range previously reported for gueuze ranged from $3.20 – 3.51$ (*12*). The pH of American lagers tends to range anywhere from 3.7 - 4.8. Gueuze has a lower pH than other beer styles because of the additional microbial activity resulting in the production of acetic and lactic acid (*1*).The presence of acetic or lactic acid bacteria are common and accepted in lambic beers, however in America beers these microorganisms are spoilage organisms. Lactic acid bacteria produces off-flavors and aromas such as honey or sweet butterscotch provided by the chemical compounds diacetyl and vicinal diketones. Acetic acid bacteria can be hop-insensitive similar to lactic acid bacteria and can be responsible for the ropiness of beer (*13*). The pH range for the nine commercial beers ranged from $3.23 – 3.62$ (Figure 4.1). Hanssens Artisan and 3-Fonteine had the lowest pH values of 3.23 and 3.24. These samples also had the highest total acidity (Table 4.3). Hanssens Artisan, which had the lowest pH, also had the highest total acidity at 7.83 g/L, while 3-Fonteine had the second highest total acidity concentration at 5.71 g/L. A significant difference in pH was found between all the beers (Figure 4.1).

Color

Lambic (gueuze) beer can exhibit a wide range of colors from golden yellow for young gueuze to light amber for older (2-3 years) gueuze. Gueuze typically ranges in color from 8 - 13 degrees SRM (Standard Reference Method) (*1, 13*). Color was measured using the SRM. The color range for the beer analyzed varied from 6.85 – 10.25 (Table 4.2). The table shows there were significant differences in the sample color ($p < .05$) between samples. A significant difference between the color of Oude Gueuze Villie and Girardin was observed. Oude Gueuze Viellie has a color value of 6.85 while Girardin had a color value of 10.25. Figure 4.2 depicts the color values of the individual experimental units. The Figure shows that most of the brands have similar SRM color values with the exception of Girardin. American lagers tend to be lighter in color than lambic beers; American lagers ranging between 2 - 5 degrees SRM (*1*). In lambic beers, little color comes from the unmalted wheat used in the mash. The majority of the color comes from the lengthy boiling of the wort producing Maillard reaction between amines and sugars resulting in melanoidins and caramel. Additional color formation comes directly from the wooden casks themselves either from the wood or from oxidation during the fermentation and maturation process of lambic beer (*1*).   It is not unusual for wort used in lambic beer to be boiled 4 or more hours while 60 minutes is typical for an American lager.

Titratable acidity, residual sugar, lactic acid, volatile acidity and ethanol

Table 4.3 is the data collected by the Enology Service Laboratory at Virginia Tech. Because of the high attenuation rate found in lambic (gueuze) beer, small to trace amounts of reducing sugars were found. In prior research, only trace amounts (0.8% w/v) (*14*) were reported. The amount of reducing sugars in the eight commercial beers tested ranged from 0.7 - 1.8 % w/v. Beers sweetened with syrups tend to contain a higher percentage of reducing sugars (2% w/v)

because these beers tend to undergo a limited secondary fermentation and are quickly filtered and pasteurized once the fermentation process is complete (*1*). Cantillon and Boon both contained the highest percentage of reducing sugars at 1.8%w/v, while Oude Artisan had the lowest at 0.7% w/v. A gueuze that is called "Oude" is considered an old gueuze that has been allowed to ferment for three years, unlike traditional gueuzes that are fermented for two years. The lactic acid (g/L) measured for the lambic (gueuze) beers ranged between 3.67 - 17.47 g/L. Oude Artisan contained the highest lactic acid at 17.47 g/L while Cantillon had the lowest at 3.67 g/L. The volatile acidity for the lambic (gueuze) beer ranged from 3.97 g/L to 17.27g/L, Oude Artisan had the highest volatile acidity, while Boon had the lowest. Volatile acidity refers to the organic acids that are more volatile or are easily vaporized than non-volatile or fixed acids. Total acidity (g/L) for the lambic (gueuze) beer ranged from 2.62 - 7.83 g/L with Oude Boon being the lowest and Oude Artisan having the highest. Ethanol ranged from 5.64 – 7.16%. Ethanol concentration for gueuze beers have been previously reported to range between 4.25 – 5.20% (*14*).

Solid Phase Microextraction Analysis of Volatiles

SPME has been used as an extraction technique for volatile and semi-volatile compounds in beer (*15*). The DVB/CAR/PDMS SPME fiber was reported by Rodriques et al (*4*) as being able to provide a more complete volatile profile, due to the wider range of volatile and semi-volatile compounds detected.

A total of 50 aroma compounds were identified by SPME-GCMS using a combination of retention index and mass spectral matching against library standards (Table 4.3). Compounds that could not be identified by comparing their retention index values were marked as tentatively

identified. Compounds identified belonged to a number of different chemical groups (ketones, acids, alcohols, and phenols).

Thirty-three of the 50 compounds identified have not been previously reported in gueuze lambic. Seventeen compounds have been reported by both Van Oevelen et al. (*12*) and Spaepen et al. (*16*). The compounds previously reported by Van Oevelen et al. (*12*) were acetic acid, lactic acid, butyric acid, propionic acid, isobutyric acid, propanol, butanol, isobutanol, isoamyl alcohol, amyl alcohol, phenethylalcohol, ethyl acetate, and ethyl lactate. Spaepen and his colleagues (*16*) reported finding caproic (hexanoic) acid, caprylic (octanoic) acid, capric (decanoic) acid, isoamyl acetate, ethyl caproate (hexanoate), ethyl caprylate (octanoate), ethyl caprate (decanoate), and phenethyl acetate.

The major chemical classes that account for lambic (gueuze) beer are alcohols, acids, esters, phenols, aldehydes, and sulfur compounds. The production of alcohols in beer are a result of yeast metabolism (*17*). Of the fifty compounds identified, eight were alcohols. Phenethyl alcohol, isoamyl alcohol, and isobutanol have been previously reported alcohol compounds (*12*) in lambic beer. 2-methyl-1-butanol (*18*), 1-hexanol (*19*), heptyl alcohol (*20*), 1-octanol (*20*), 2-nonanol (*19*), 1-decanol (*20*) are all chemical compounds that have been previously reported in beer, but not lambics.

Twenty-three esters compounds were detected using SPME GC-MS. In prior research, only seven have been previously reported and they are ethyl acetate, lactate, butyrate, caproate, caprylate, caprate, and phenethyl acetate (*12, 16, 21*). An additional fifteen ethyl esters were detected using SPME. These compounds can be found in Table 4.3.

Acids play a vital role in the aroma and flavor profile of lambic beer. A total of seven acids were identified using SPME GC-MS. The acids identified were acetic, lactic, isovaleric

hexanoic, valeric, octanoic, and decanoic acid (*12, 16*). With the exception of isovaleric and valeric acids, all have been previously reported in gueuze beer. Isovaleric and valeric acid, however, have been reported in other styles of beer (*17*).

External standards were used to quantify isovaleric acid (IVA), ethyl octanoate, 4-ethylphenol (4EP), 4-ethylguaiacol (4EG), ethyl caprylate, octanol, ethyl undecanoate, and ethyl acetate (Table 4.5). Isovaleric acid has been previously reported in beer before, but never in lambic beers (*17*). Isovaleric acid, 4-ethylphenol and 4-ethylguaiacol are key components in the overall aroma of *Brettanomyces (22)*. The concentration of isovaleric (3-methylbutyric acid) acid for gueuze beer ranged from 1.92 mg/L for Oude Gueuze Vielle – 3.01 mg/L for Cuvee Renée. Isovaleric acid was found in six of the nine commercial beers (Cuvee Renée, Oude Gueuze Vielle, Cantillion, Cantillion Bio, Girardin, and Oude Boon). In comparing the means for all the brands, no difference was found for IVA. 4-ethylphenol and 4-ethylguaiacol are known by-products of the wild yeast species *Brettanomyces*. Both compounds have been previously reported in the literature. Neither compound, however, has been quantified for lambic beers. The concentration of 4-ethylphenol ranged from 0.28 mg/L to 1.13 mg/L. Cuvee Renée had the highest concentration of 4-ethylphenol at 1.13 mg/L and 0.28 mg/L for both Girardin and Oude Boon. Table 4.6 is a comparison of means for 4-ethylphenol for commercial brands. 4-ethylphenol has a sensory threshold of 425 µg/L and 4-ethylguaiacol has a sensory threshold of 100 µg/L. 4-Ethylguaiacol concentration ranged from 0.52 mg/L to 5.77 mg/L. Oude Boon was found to have the lowest concentration of 4EG within the commercial brands, while Cuvee Renée had the highest concentration of 4EG at 5.77 mg/L. (Table 4.7). When 4-ethylphenol is in the presence of 4-ethylguaiacol, the sensory threshold for 4-ethylphenol is lower. The ratio of

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4EP to 4EG is most often reported as 10:1. The ratio, however, can vary between regions and wines (*5, 23*). Little is known about the ratio of 4EP:4EG in lambic beers.

Ethyl octanoate (ethyl caprylate) was the fourth compound quantified. Ethyl octanoate has been previously reported in the literature as being found within lambic beer. The concentration of ethyl octanoate found within the literature was reported to be 0.16 – 0.59 mg/L (*21*). Ethyl octanoate was found within all nine commercial brands tested. The concentration of ethyl octanoate ranged from 1.36 mg/L for 3 Fonteinen – 5.72 mg/L for Cantillion. When comparing the means, a difference was found between the different brands (Table 4.8).

Octanol has been previously reported in beer (*20, 24, 25*), but never specifically lambic beers. The concentration of octanol ranged from 0.025 mg/L to 0.084 mg/L. Oude Boon, Boon, and Cantillion Bio all had a concentration of 0.025 mg/L while, Hanssens Artisan had the highest concentration of 0.084 mg/L (Table 4.9).

Ethyl undecanoate has been reported in wine (*26*), brandy (*27*), whiskey (*28*), cognac (*29*), and rum (*30*), but not beer. Ethyl undecnaote was detected in four of the nine brands. The range for ethyl undecnoate was 8.6 mg/L to 46.02 mg/L. Cantillion Bio had the lowest concentration of ethyl undecanoate at 8.6 mg/L, Oude Gueuze Vielle was next at 16.72 mg/L, Cantillion was third at 28.87 mg/L, and Cuvee Renée had the highest at 46.02 mg/L. (See Table 4.10).

Ethyl acetate is one of the twenty-seven compounds previously identified in lambic beer (*12, 21*). Ethyl acetate was identified in all of the commercial brands of lambic beers. The highest concentration of ethyl acetate previously reported in the literature for lambic beer was 539.8 mg/L. The average concentration for refermented gueuze was 60.9 – 167 mg/L, while filtered gueuze ranged from 33.4 – 67.6 mg/L (*12*). The concentration of ethyl acetate in the

commercial lambic beers ranged from 11.82 – 66.89 mg/L. Boon had the lowest concentration of ethyl acetate and Hanssens Artisan had the highest concentration. (Table 4.11).

Organic acids

The organic acids present in beer play important roles in aroma and taste. First, organic acids are one of the primary groups of compounds that contribute sourness. All organic acids have their own characteristic flavor, aroma, and taste (*31-33*). Citric acid possesses a fresh acid flavor, which is very different from that of malic acid, while succinic has both a salty and bitter flavor in addition to its sourness. Second, acids can help protect beer from harmful microorganisms by decreasing the pH (*32*). Third, the organic acids present in beer can aid in prolonging the shelf life by providing the beer with a strong buffering capability (*32, 34*). Acetic acid has a flavor threshold of 200 ppm, while lactic acid has a flavor threshold of 400 ppm (*35, 36*).

Acetic and L-lactic acid were found in varying concentrations within different styles of lambic beer (*37*). It has been reported that the concentration of lactic can be as high as 10,000 mg/L for lactic in ropy lambics and 1200 mg/L for acetic lambics (*12*).The comparison of acetic and lactic acid found in commercial lambic beer can be found in Table 4.12. In comparison to gueuzes, ales and lagers have a much lower concentration of acetic and lactic acid. Ales and lagers normally contain anywhere from 60 – 140 ppm concentration of acetic acid. The concentration of acetic acid in gueuze beer can range between 500 – 1500 mg/L. The concentration of acetic acid in the commercial samples ranged from 723 mg/L for Oude Boon to 1642 mg/L for Hanssens Artisans. There was no difference in acetic acid concentration between the different brands (p>.05).

The concentration of lactic acid in gueuze beer can range between 1,500 – 3,500 mg/L while typical American lagers tend to have much lower concentrations, around 40 – 150 ppm (*1*). Table 4.12 shows a comparison of means for lactic acid. The concentration of lactic acid ranged from 1098 – 2979 mg/L. Cantillon Bio had the highest level of lactic acid at 2979 mg/L followed by Cuvee Renée at 2563 mg/L. Oude Gueuze Villie had the lowest concentration of lactic acid at 1098 mg/L. Values with the same superscript are not significantly different (p>.05). Based upon the comparison of means, Cantillon Bio is significantly different from Girardin, Cantillion, Hanssens Artisans, 3-Fonteinen, Boon, Oude Boon, and Oude Gueuze Viellie. Cuvee Renée was found not to be significantly different from any of the other brands.

Conclusion

In this study, the volatile and semi-volatile compounds of nine commercial brands of lambic (gueuze) beer were identified using GC-MS and HPLC. A total of 50 volatile and semi-volatile compounds were identified in the nine commercial brands. Of the 50 compounds identified, seventeen of them have been previously identified in the literature. Acetic and lactic acid were identified and quantified using HPLC.

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Table 4.1 Comparison of pH levels for commercial lambic (gueuze) beers.

	Brand	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a,b]	Hanssens Artisan	12	3.2375^A			
	3-Fonteinen Oude Gueuze	12	3.2425^A			
	Boon Cantillon Oude Gueuze	6		3.4367^B		
	Ville	6		3.44^B		
	Girardin	9		3.4433^B		
	Gueuze	12		3.4483^B		
	Boon Cantillon Bio	6			3.5233^C	
	Cuvee Renée	9			3.5367^C	
		9				3.6233^D
	Sig.		1	0.964	0.925	1

Means followed by same superscript are not significantly different at the 0.05 level experimentwise using Tukey-Kramer HSD

Figure 4.1 Comparison of means for pH based upon different commercial brands
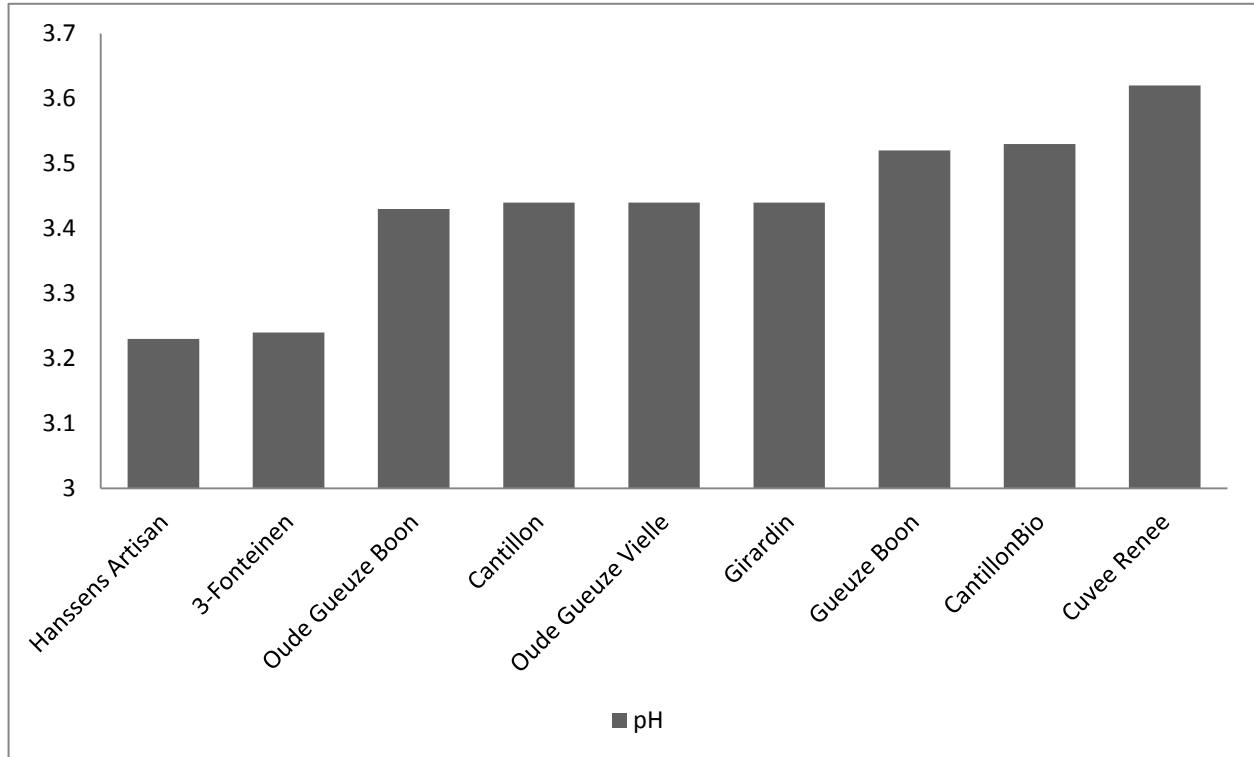
Figure: 4.2 Graphical representation of all the pH values for the commercial gueuze beers
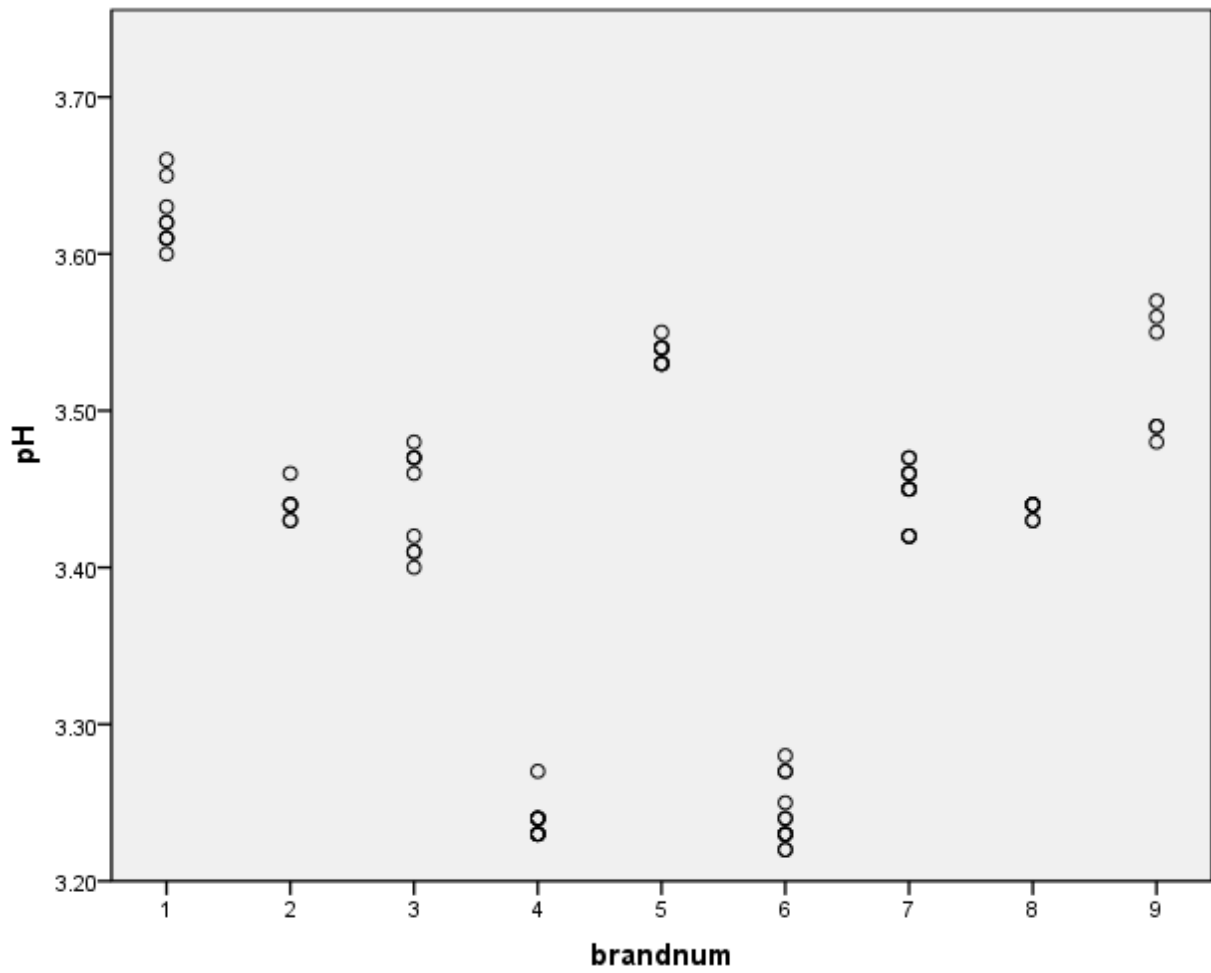
Numbers represent different brands: 1. Cuvee Renée, 2. Cantillon 3. Oude Gueuze Viellie, 4. Hanssens Artisan, 5. Cantillion Bio, 6. 3-Fonteinen, 7. Girardin, 8. Oude Gueuze Boon, 9. Boon

Table 4.2 Color comparison for commercial lambic (gueuze) beer

Brand	N	Means
Oude Gueuze Ville	4	6.8525^a
Cantillon Bio	3	7.29^{ab}
Cantillon	2	8.085^{ab}
Oude Gueuze Boon	2	8.255^{ab}
3-Fonteinen	4	8.46^{abc}
Gueuze Boon	2	8.86^{bc}
Hanssens Artisan	4	9.055^{bc}
Girardin	2	10.255^c

Means followed by same superscript are not significantly different at the 0.05 level

experimentwise using Tukey-Kramer HSD

Figure 4.3 Comparison of means for color of commercial lambic (gueuze) beer
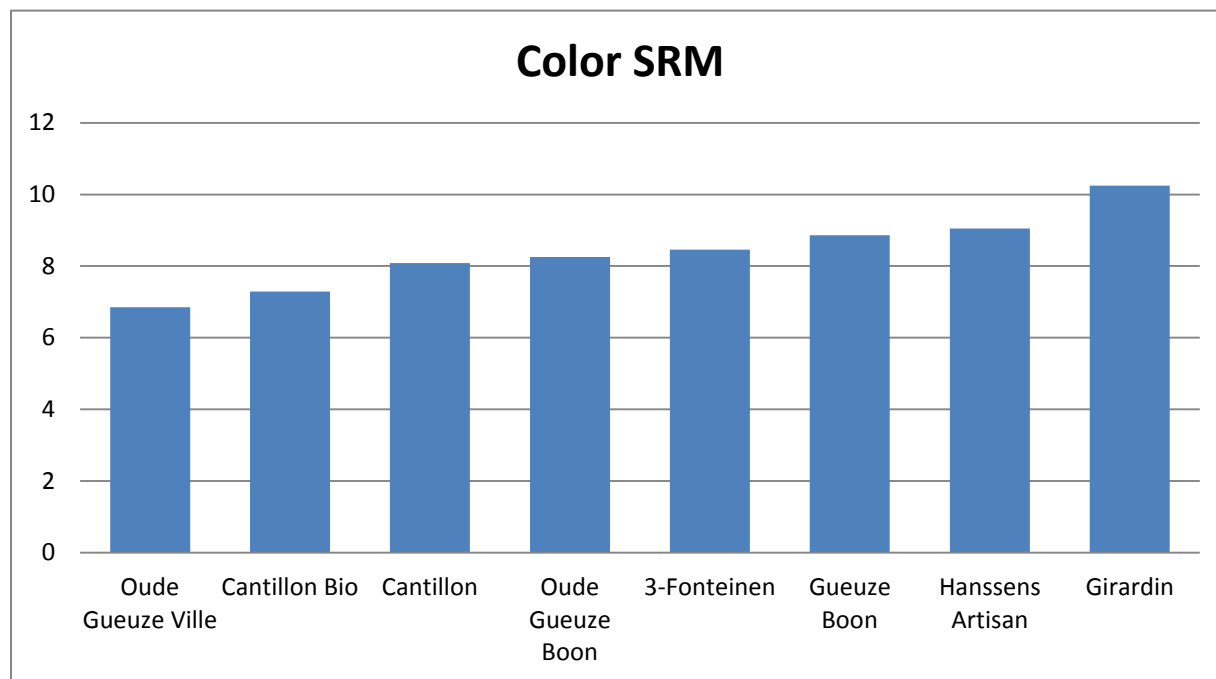
Figure 4.4 All color values for the commercial beer
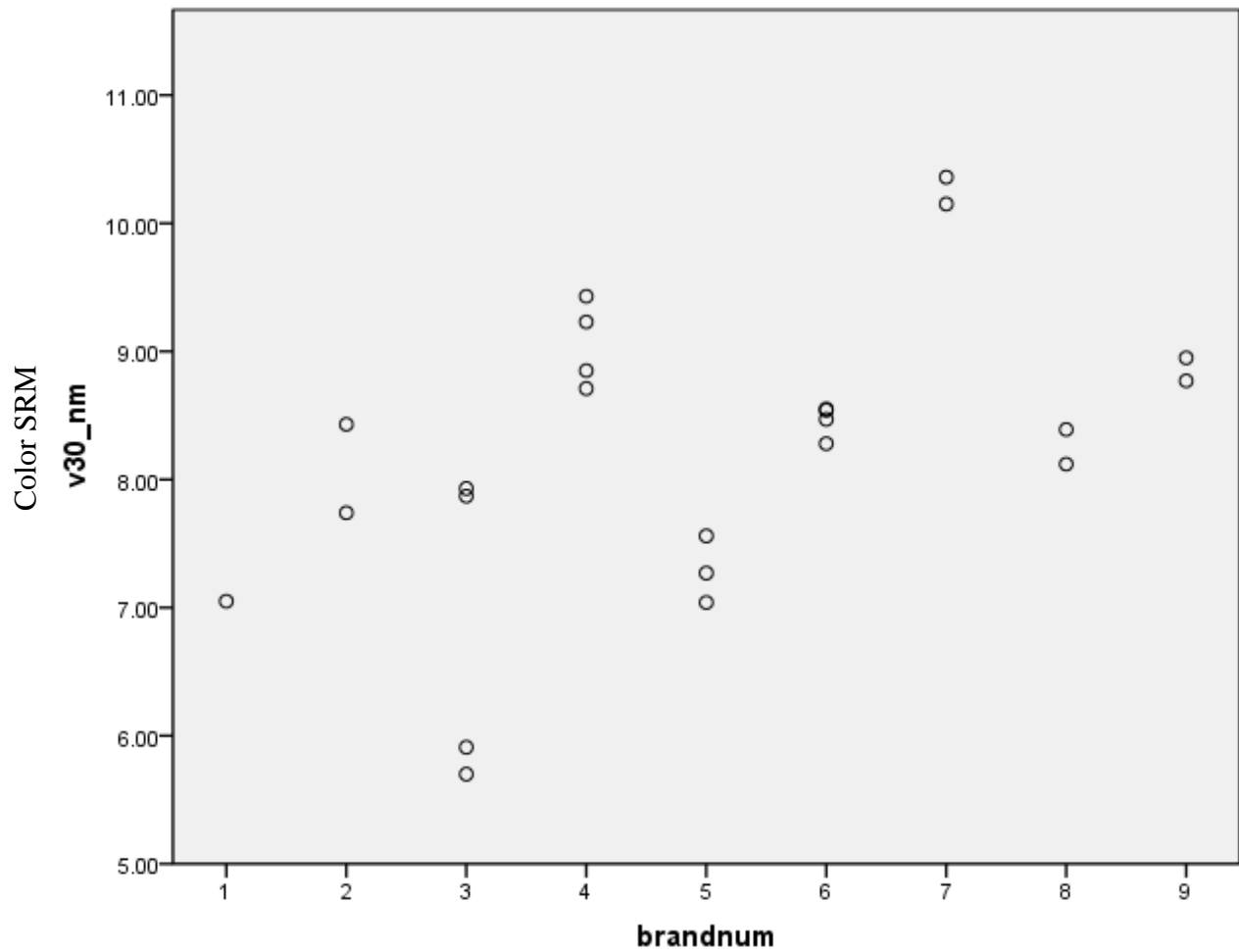
Numbers represent different brands: 1. Cuvee Renée, 2. Cantillon 3. Oude Gueuze Viellie, 4.

Hanssens Artisan, 5. Cantillion Bio, 6. 3-Fonteinen, 7. Girardin, 8. Oude Gueuze Boon, 9. Boon

Figure 4.5 SRM Color Graph with Corresponding Values
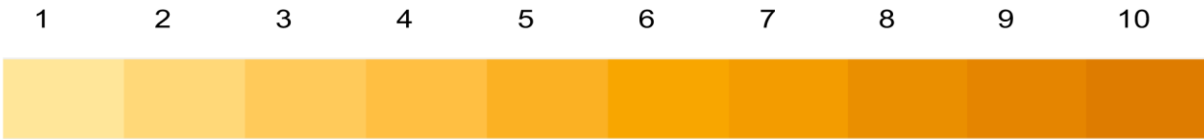
Table 4.3 Data collected by the Enology Service Laboratory

Name	Sample Size	RS % w/v	TA (g/L)	TA – Lactic Acid (g/L)	VA (g/L)	Ethanol %
Cantillon	n = 1	1.8	3.29	3.67	12.59	5.64
Cantillon Bio	n = 2	1.2	4.42	9.86	11.15	6.06
3 Fonteine	n = 2	1.2	5.71	12.73	7.22	6.39
Girardin	n = 1	.90	4.96	11.07	6.30	6.43
Boon	n = 1	1.8	2.71	6.06	3.97	6.02
Oude Boon	n = 1	1.2	2.62	5.85	4.92	7.16
Hansanns Artisan	n = 2	.70	7.83	17.47	17.27	5.66
Oude Gueuze Vielle	n = 2	1.65	2.74	6.10	5.71	6.5

RS – residual sugar

TA - Total Acidity (g/L) – was calculated as lactic acid equivalent

Lactic acid (g/L)

VA - Volatile acidity (g/L)

Table 4.4 Comaprison of Chemical Compounds found in Commerically Available Lambic Beer

Chemicals	LRI	Confirmed	Cuvee Renee	Oude Gueuze	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon
Acetic acid			x	x	x	x	x	x	x	x	x
Ethyl Acetate	587		x	x	x	x	x	x	x	x	x
Propanoic Acid	637	668					x			x	
Isobutanol (1-Propanol, 2-methyl-)		647		x			x	x	x	x	x
3-methyl-1-Butanol (Isoamyl Alcohol)	683	734	x	x	x	x	x	x	x	x	x
2-methyl-1-Butanol	689	744	x	x	x	x	x	x	x	x	x
ethyl isobutyrate (Propanoic acid, 2-methyl-, ethyl ester)	764	756	x	x	x	x	x	x	x	x	x
Isobutyl Acetate	752	776	x	x	x	x	x	x	x	x	x
Butanoic acid, ethyl ester (Ethyl butyrate)	804	800	x	x	x	x	x	x	x	x	x
Furfural (2-furanal)	833	829	x	x	x	x		x	x	x	x
Isovaleric acid (3-Methylbutanoic acid)	851	854	x	x	x	x	**x**		x	x	
Butanoic acid, 2-methyl-, ethyl ester (ethyl 2-methyl butyrate)	853	846	x	x	x	x	x	x	x	x	x
Butanoic acid, 3-methyl-, ethyl ester	857	854	x	x	x	x	x	x	x	x	x
2-Furanmethanol	860	866	x	x	x	x	x		x	x	x

Table 4.4 Comaprison of Chemical Compounds found in Commerically Available Lambic Beer
-Continued-

Chemicals	LRI	Confirmed	Cuvee Renee	Oude Gueuze Vielle	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon
1-hexanol	873	880	x	x	x	x	x	x	x		x
1-Butanol, 3-methyl-, acetate (Isoamyl Acetate)	880	876	x	x	x	x	x	x	x	x	x
1-Butanol, 2-methyl-, acetate	882	880	x	x	x	x	x	x	x	x	x
Styrene	888	893	x	x	x	x	x		x	x	
Lactic Acid	906		x					x	x		
1-(2-furanyl)-Ethanone	910	910	x			x					
5-Dimethyl-2(5H)-furano	952	951		x	x	x	x	x	x	x	
Heptyl alcohol	953	962	x	x	x	x		x	x	x	x
Pentanoic acid, 4-methyl-, ethyl ester (Ethyl Isohexanoate)	966	968	x	x	x	x	x	x	x		x
1-Propanol, 3-(methylthio)-	977	978	x	x	x	x					
Hexanoic acid (Caproic acid)	986	1019	x	x	x	x		x			
Hexanoic acid, ethyl ester	998	996	x	x	x	x	x	x	x	x	x
Isoamyl lactate	1067	ND	x	x	x	x	x	x	x	x	x
1-octanol	1070	1072	x	x	x	x	x	x	x	x	x
Heptanoic acid, ethyl ester	1097	1097	x	x	x	x	x	x	x	x	x
2-Nonanol	1099	1098	x	x	x		x	x	x	x	
Nonanal	1102	1104	x	x	x	x	x	x	x	x	x

Table 4.4 Comaprison of Chemical Compounds found in Commerically Available Lambic Beer -Continued-

Chemicals	LRI	Confirmed	Cuvee Renee	Oude Gueuze Vielle	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon
Isopentyl 3-methylbutyrate (Butanoic acid, 3-methyl-, 3-methylbutyl ester)	1104	1103			x	x	x		x		
Valeric Acid	1104			x							
Phenylethyl Alcohol	1110	1118	x	x	x	x	x	x	x	x	x
2-ethyl-hexanoic acid	1119	1129	x	x	x	x	x	x	x	x	x
4-ethylphenol	1166	1169	x	x	x	x	x	x	x	x	x
Ethyl benzoate (Benzoic acid etyl ester)	1166	1170	x	x	x	x	x	x	x	x	x
Octanoic acid	1180	1179	x	x	x	x	x	x	x	x	x
Octanoic acid, ethyl ester (ethyl caprylate)	1197	1198	x	x	x	x	x	x	x	x	x
Decanal	1204	1209	x	x	x	x	x	x	x	x	x
Benzeneacetic acid, ethyl ester	1244	1244	x	x	x	x	x	x	x	x	x
Isopentyl hexanoate (Isoamyl caproate)	1249	1254	x	x	x	x	x	x	x	x	x
β-Phenethyl acetate (Acetic acid, 2-phenylethyl ester)	1255	1260	x	x	x	x	x	x	x	x	x
1-Decanol	1271	1272	x			x	x	x			
p-Ethylguaiacol	1278	1287	x	x	x	x	x	x	x	x	x

Table 4.4 Comparison of Chemical Compounds found in Commerically Available Lambic Beer -Continued-

Chemicals	LRI	Confirmed	Cuvee Renee	Oude Gueuze Vielle	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon
Nonanoic acid, ethyl ester	1295	1297	x	x	x	x	x	x	x		x
Decanoic acid	1367	1373	x	x	x	x	x	x			x
Ethyl 9-decenoate	1386	ND	x	x	x	x		x		x	x
Decanoic acid, ethyl ester (Ethyl decanoate)	1394	1398	x	x	x	x		x	x	x	x
Octanoic acid, 3-methylbutyl ester	1455	1450	x	x	x	x	x	x	x	x	
Ethyl undecanote	1515	1498	x	x	x		x				
Ethyl dodecanoate	1594	1593	x	x	x	x		x	x		x

LRI - linear retention index
Bold compounds denotes compounds previously reported

Table 4.5 Quantification of compounds for commercial lambic beers

Compound	Cuvee Renée	Oude Gueuze Vielle	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon
Isovaleric acid (mg/L)*	3.01	1.92	2.15	---	2.94	---	2.95	2.3	---
Ethyl octanoate (mg/L)+	5.67	2.68	5.72	2.74	4.52	1.36	2.22	1.66	1.62
4-ethylphenol (mg/L)+	1.13	0.57	1.08	0.57	0.96	0.44	0.28	0.28	0.32
4-ethylguaiacol (mg/L)+	5.77	1.06	2.44	1.36	2.1	0.99	1.08	0.52	0.97
Octanol (mg/L)+	0.041	0.031	0.052	0.084	0.025	0.034	0.031	0.025	0.025
Ethyl undecanoate (mg/L)+	46.02	16.723	28.875	---	8.6	---	---	---	---
Ethyl Acetate (mg/L)+	D	22.33	28.4	66.89	46.9	22.06	21.42	17.03	11.82

* no difference was when comparing the means

+ A difference was found between the means when using Tukey's HSD test.

Table 4.6 Comparison of means for 4-ethylguaiacol

Brand		Mean (mg/L)	SE
Cuvee Renée	A	5.77	0.1
Cantillion	B	2.44	0.12
Cantillion Bio	B	2.09	0.1
Hanssens Artisan	C	1.34	0.08
Girardin	C	1.07	0.08
Oude Gueuze Viellie	C	1.06	0.08
3 Fonteinen	C D	0.98	0.08
Boon	C D	0.97	0.12
Oude Boon	D	0.52	0.12

Means followed by same superscript are not significantly different at the 0.05 level

experimentwise using Tukey-Kramer HSD

Table 4.7 Comparison of means for 4-ethylphenol for commercial brands

Brand		Mean (mg/L)	SE
Cuvee Renée	A	1.13	0.024
Cantillion	A B	1.08	0.029
Cantillion Bio	B	0.96	0.024
Oude Gueuze Viellie	C	0.57	0.021
Hanssens Artisans	C	0.57	0.021
Fonteinen	D	0.43	0.021
Boon	D E	0.32	0.029
Girardin	E	0.27	0.021
Oude Boon	E	0.27	0.029

Means followed by same superscript are not significantly different at the 0.05 level

experimentwise using Tukey-Kramer HSD

Table 4.8 Comparison of means for ethyl octanoate

Brand		Mean (mg/L)	SE
Cantillion	A	5.715	0.628
Cuvee Renée	A	5.673	0.512
Oude Gueuze Viellie	A B	5.02	0.628
Cantillion Bio	A B C	4.523	0.512
Hanssens Artisan	B C D	2.882	0.44
Girardin	C D	2.222	0.44
Oude Boon	D	1.66	0.44
Boon	D	1.625	0.62
3 Fonteinen	D	1.36	0.44

Means followed by same superscript are not significantly different at the 0.05 level

experimentwise using Tukey-Kramer HSD

Table 4.9 Comparison of means for octanol for commercial brands

Brand		Mean (mg/L)	SE
Hanssens Artisan	A	0.084	0.004
Cantillion	B	0.052	0.006
Cuvee Renée	B	0.041	0.004
3 Fonteinen	B	0.034	0.004
Oude Gueuze Viellie	B	0.031	0.004
Girardin	B	0.031	0.004
Cantillion Bio	B	0.025	0.004
Oude Boon	B	0.025	0.006
Boon	B	0.022	0.006

Means followed by same superscript are not significantly different at the 0.05 level experimentwise using Tukey-Kramer HSD

Table 4.10 Comparison of means for ethyl undecanoate

Brand		Mean (mg/L)	SE
Cuvee Renée	A	46.02	3.55
Cantillion	A B	28.875	3.55
Oude Gueuze Viellie	B C	16.723	4.34
Cantillion Bio	C	8.60	3.55

Means followed by same superscript are not significantly different at the 0.05 level

experimentwise using Tukey-Kramer HSD

Table 4.11 Comparison of means for Ethyl acetate

Brand		Mean (mg/L)	SE
Hanssens Artisan	A	66.89	0.004
Cantillion	B	46.90	0.006
Cantillion Bio	C	28.40	0.004
Oude Gueuze Viellie	D	22.33	0.004
3 Fonteinen	D	22.06	0.004
Girardin	D	21.42	0.004
Oude Boon	D E	17.03	0.004
Boon	E	11.82	0.006

Means followed by same superscript are not significantly different at the 0.05 level

experimentwise using Tukey-Kramer HSD

Table 4.12 Comparison of acids within commercial brands of lambic beer

Brand	Acetic Acid (mg/L)*	Lactic Acid (mg/L)
Cuvee Renée	916	2557
Oude Gueuze Villie	1019	1094
Cantillion	1224	1417
Hanssens Artisan	1642	1389
Cantillon Bio	1473	1658
3 Fonteinen	1204	1294
Girardin	1499	1403
Oude Boon	723	1228
Boon	1137	995

*No difference was found between the brands

Table 4.13 Comparison of Means for Lactic Acid

Brand		Lactic Acid (mg/L)
Cantillon Bio	A	2979
Cuvee Renée	A B	2563
Girardin	B	1618
Cantillion	B	1534
Hanssens Artisan	B	1421
3-Fonteinen	B	1361
Boon	B	1253
Oude Boon	B	1237
Oude Gueuze Viellie	B	1098

Means followed by same superscript are not significantly different at the 0.05 level experimentwise using Tukey-Kramer HSD

CHAPTER 5: ANALYSIS OF THE DEVELOPMENT OF FLAVOR AND AROMA COMPOUNDS OF LAMBIC BEER THROUGHOUT THE FERMENTATION PROCESS

Abstract

Lambic beer is one of the oldest styles of beer still being produced today using spontaneous fermentation. Gueuze is a style of lambic beer that blends "young" (1 year old) and aged (2+ years old) beers. Little is known about the development of the volatile and semi-volatile compounds in lambic beer during aging. SPME with GCMS was used for extraction and identification of volatile and semi-volatile compounds from 3, 6, 9, 12, and 28-month-old samples of lambic beer. Compounds were identified using standardized retention time and mass spectra of standards. GC-O was used to characterize the aroma profiles of the samples. A total of 42 compounds were identified using GC-MS. Seventeen of the 42 compounds identified in the various aged samples have been previously reported in lambic beer. Ethyl lactate, ethyl acetate, 4-ethylphenol and 4-ethylguaiacol were identified in the 9, 12, and 28 month old samples. These four compounds have been linked to the microorganism *Brettanomyces*. Twenty-one aroma active compounds were identified using GC-O. As the age of the gueuze samples increased, a larger number of aroma compounds were identified by the panelists; compounds identified increased from seven for the 3 month old samples to nine for the 6 month old samples, and eleven for both the nine and twelve month old samples, and seventeen for the twenty eight month old samples.

Introduction

Spontaneous fermentation has been used in the traditional manufacturing of beer, wine and apple cider. The use of spontaneous fermentation is currently rarely used for beer manufacture because of the lack of control over the fermentation process; however, lambic beer is still being produced through spontaneous fermentation. All lambic beers are traditionally produced within fifteen kilometers of Brussels (*1, 2*).

The brewing season for lambics begins in September and continues until sometime in April (*3*). The tradition is to brew lambic beers during the colder months of the year to prevent the potential spoiling of the wort during the warmer summer months. The wort is allowed to cool overnight in open shallow trays and is inoculated by the natural microfloral present in the surrounding environment. Once the wort has finished cooling overnight, it is pumped into either metal or wooden casks and stored in non-air-conditioned warehouses. The temperature in these warehouses can vary from 0°C to 25°C (*1, 2*).

Lambic beers are unique in that they are still being produced today through spontaneous fermentation. The fermentation process of lambics can be divided into four distinct stages, with microorganisms contributing key flavor and aroma compounds within each stage. *Klacekera apiculata* (a wild yeast) and enteric bacteria dominate the first stage of fermentation. The secondary stage of the fermentation process is dominated by *Sacchromyces sp.* followed by lactic acid bacteria (LAB) in the third stage. *Brettanomyces*, also known as bret, is a wild yeast often associated with the spoilage of red wines and ciders. In lambic beers, bret imparts expected, positive flavor characteristics and bret dominates the fourth and final stage of fermentation (*4*).

As noted earlier, the initial stage of fermentation for lambic beer is dominated by enteric bacteria and the yeast *K. apiculata.* The actual fermentation process begins three to seven days

after the wort has been inoculated. *K. apiculata* quickly reaches its maximum concentration of 10^5 cells/mL within the first week of fermentation, but is quickly out-competed by *Saccharomyces*. *K. apiculata* only has the ability to ferment glucose and not maltose or more complex carbohydrates. *Enterobacteriacea* and *K. apiculata* are both fast growing microorganisms and they cause the pH of the wort to drop from around 5.1 to 4.6. This pH drop is due to the production of acetic and lactic acids (*5*).

The second and third stages of fermentation actually overlap one another. The third stage of fermentation begins three to four months after the beer has been brewed. The third stage is dominated by lactic and acetic acid bacteria both peaking around six to eight months which usually correlates with the warmer summer months. Warmer temperatures are essential for the growth of both lactic and acetic bacteria (*5*).

The sourness associated with lambic beers can be contributed to the presence of lactic acid. The majority of the lactic acid present in lambic beer fall within the *Pediococcus* genus. While some strains of *Pediococcus* have been found to be beneficial in beer, others have the ability to produce a "ropy" surface layer that will leave the beer with a permanent haziness. While lactic acid is desirable, acetic acid bacteria are undesirable in this particular style of beer because acetic acid bacteria have the ability to convert ethanol into acetic acid causing the beer to become acidic or hard. This only becomes an issue if the casks or barrels being used to store the beer are damaged or have leaks that allow oxygen to come in contact with the beer.

The fourth and final stage is marked by an increase in the number of yeast cells. *Brettanomyces* plays an essential role in the development of the aromatic and flavor profile of lambic beer. Esters play an important role in the aromatic profile of this particular style of beer

which are by-products of this microorganism. The two most influential esters produced are ethyl lactate and ethyl acetate, which originate from lactic and acetic acids during stage three (*5, 6*).

Materials and Methods

Sample Acquisition and Storage. Different stages of gueuze beer were obtained from the brewmaster at Lindemans, a traditional lambic brewery located in Vlezenbeek, near Brussels, Belgium. Samples collected were 3, 6, 9, 12, and 28 months old. Samples were taken from corresponding storage tanks within the brewery and placed into 120 mL sampling cups. Samples were stored in a -3°C refrigerator prior to analysis.

Gas Chromatography Olfactometry. An experienced 3-person (1 female and 2 males) sensory panel consisting of students at Virginia Tech was used to evaluate the different stages of fermenting gueuze beer. The panelists were trained in eight, 15 minute sessions before the study began. Thirteen pure aroma compounds associated with gueuze beer were selected and used to train the panelists. The aromas selected were: medicinal/barnyard, spice, sweaty, fruity, green, banana, brandy, banana-pineapple, citrus, vinegar, rancid, cheesy, and pineapple. The panel was approved by the Virginia Tech Institutional Review Board (IRB #11-364).

Gas Chromatography Olfactometry. Extraction and concentration of the volatile and semi-volatile compounds were conducted using a solid phase microextraction fiber (SPME). A divinylbenzene, Carboxen, polydimethylsiloxane (DVB/CAR/PDMS, SKU 57348-U) coated fiber (Supelco, Bellefonte, PA) was exposed approximately 1 cm above the headspace for 30 minutes at 40°C while a magnetic bar continued to stir the sample. Volatile and semi-volatile compounds were desorbed in the injector port of the gas chromatograph with olfactometry detector, which consisted of a HP 5890A GC (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame ionization detector (FID) and a sniffing port (ODOII; SGE Inc. Austin, TX). The

injector temperature was set to 250°C and all injections were made in the splitless mode. Compounds were separated using a 30m x 0.25-mm i.d. (0.25 μm film thickness) capillary column (DB-5ms; J&W Scientific, Folson, CA) using helium as the carrier gas with a flow rate of 1.0 ml.min^{-1} (linear flow velocity ~ 25 cm/sec). The effluent coming from the column was split 1:1 between the FID and the sniffing port using deactivated fused silica capillaries (1-m length x 0.32 μm i.d.). Chromatograms were recorded using a HP 3396A integrator (Hewlett-Packard Co., Palo Alto, CA).

Three panelists were asked to participate in assessing the odor profiles of lambic beer. Five milliliters of beer was placed in a 15 mL glass vial fitted with a Teflon-lined cap. The sample was heated to 40°C using an 'RTC basic' heater with an ETS D4 Fuzz Controller (IKA Werke, Wilmington, NC) while being stirred using a 4 mm stir bar. An SPME fiber (50/30 μm DVB/CAR/PDMS) on a 2 cm StableFlex fiber (Supelco Bellefonte, PA) was manually inserted into the vial and exposed for 30 minutes. A DB-5 capillary column (30 m * 0.25 mm id * 0.25 μm film thickness) was used for separating the volatile compounds. The same time and temperature methodology used for the GC-MS was also utilized here. The effluent coming from the GC column was split 1:1 ratio between the FID and the sniffing port. Integration was done using a HP 3396A Integrator (Hewlett-Packard, Palo Alto, CA USA).

Solid Phase Microextraction. Extraction and concentration of volatile compounds found in commercially available gueuze beer (Cuvée René, Brouwerij Lindemans) was performed by solid phase microextraction (SPME). A SPME fiber (50/30 um DVM/Carboxen/PDMS, Supelco, Bellefonte, Pa., U.S.A.) was exposed to the headspace above 4 mL of gueuze beer for 30 minutes at 40°C with an agitation speed of 250 rpm. Samples were equilibrated for two minutes prior to exposing the fiber. An AOC-5000 plus autosampler (Shimadzu, Columbia, MD) was used for

automation of SPME extractions. Volatile compounds were desorbed for five minutes in the injection port of a model QP2010 Ultra gas chromatograph - mass spectrometer (Shimadzu, Columbia, MD). The injection port was set to 250°C and all injections were made in splitless mode. Volatile compounds were separated on a nonpolar (SHRXI-5MS, Shimadzu, 30m * 0.25mm id * 0.25 μm film thickness) column with He as the carrier gas at a flow rate of 2.0 mL/min (linear velocity of 53.8 cm/sec). The GC oven temperature program was set to 35º C and held for 5 minutes, and then increased to 225º C at a rate of 6º C/min. Once the final temperature of 225º C was reached, it was maintained for 10 minutes. The MS was maintained at 200°C and sample mass was scanned in the range of 40-200 amu. The SPME-GCMS was performed on the different gueuze samples (3, 6, 9, 12, and 28 month old). Chromatographic peaks were identified using both standardized retention time (Kovats values) and fragmentation spectra of standards and the Wiley 2010 library.

Identification of Volatiles. Kovats Retention Index (KI) was used to help identify the volatile compounds present in the beer samples. A mixture of n-parafins (C5 – C26) ASTM D2287 Quantitative Calibration Solution in carbon disulfide (Suplico, Bellefonte, PA, USA) was used in determining the KI values for the volatile compounds eluded by the GC-O. The databases Flavornet (http://www.flavornet.org/flavornet.html) and Pherobase (http://www.pherobase.com/) were used to aid in identifying the compounds based upon standardized retention and aroma.

GCMS was used to aid in the identification of the volatile compounds a Shimadzu GC-2010 Plus (Shimadzu, Columbia, MD, USA) with a GCMS-QP2010 Ultra mass selective detector a SHRXI-5MS column composed of 5% phenyl/95% dimethyl polysiloxane (30m x 0.25 mm id x 0.25 μm film thickness) equipped with GCMSsolution.

Results and Discussion

SPME GC-MS

The use of SPME as a viable extraction technique for volatile and semi-volatile compounds for food and alcoholic beverages and has been widely used for a number of years (*7-10*). DVB/CAR/PDMS was reported as being able to produce a more complete profile of the volatile and semi-volatile compounds detected (*11*).

SPME/GC-MS was used for the extraction of the volatile and semi-volatile compounds found in the gueuze samples. A total of 42 compounds were identified using a combination of retention index and mass spectral matching against library standards (Table 5.1). A number of different chemical groups (ketones, acids, alcohols, aldehydes, thiols, furans, and phenols) were identified within the samples.

Thirty-one of the 42 compounds identified have not been previously reported in lambic beer. Eleven compounds identified have been reported by Van Oevelen et al. (*1*) and Spaepen et al. (*12*). The compounds previously reported by Van Oevelen et al. (*1*) were isoamyl alcohol, phenethyl alcohol, ethyl acetate, and ethyl lactate. Spaepen and his colleagues (*12*) reported finding caproic (hexanoic) acid, caprylic (octanoic) acid, capric (decanoic) acid, ethyl caprylate (octanoate), ethyl caprate (decanoate), and phenethyl acetate. The identified compounds belonged to a number of different chemical classes, for example alcohols, acids, esters, and phenols.

Alcohols are produced as a byproduct of yeast metabolism (*13*). Of the forty-two compounds identified, eight were alcohols. Phenethyl alcohol has been previously reported (*1*) in lambic beer. The other alcohols 2-methyl-1-butanol (*14*), 1-hexanol (*15*), heptyl alcohol (*16*), 1-

octanol (*16*), 2-nonanol (*15*), decanol (*16*), isopentyl alcohol, and nonanol (*17*) have been previously reported in beer, but never in lambics or gueuze beers.

The secondary by products produced by the yeast *Brettanomyces* play a greater role in the aroma and flavor profile of lambic beer than traditional brewer's yeast, *Saccharomyces*. The main chemical compounds produced by *Brettanomyces* are the esters, ethyl acetate and ethyl lactate. Ethyl acetate and ethyl lactate can be formed enzymatically or chemically. Esterase is the enzyme used in the chemical reaction between ethanol and an organic acid to produce these esters. *Brettanomyces* displays a higher esterase activity then other yeasts (*Sacchromyces* or *Kloeckera*) (*18*).

Brettanomyces has been linked to the synthesis of ethylphenols (4-ethylphenol and 4-ethylguaiacol) and vinylphenols (*19*). Bret is currently the only known microorganism linked to the development of ethylphenols. Some species of lactic acid have been known to produce ethylphenols in media but not in actual beverage systems (*20*). *Brettanomyces* is a key player in the sensory profile of lambic and gueuze beer (*2, 21*). The "bretty" character is associated with number of different aromas and flavors such as mineral, tobacco, barnyard, leather, pharmaceutical and smoky (*18, 22*). Compounds produced by *Brettanomyces* have the ability to suppress a number of the desirable fruity ester aromas (*23*). The horsey aroma can vary from slight to very strong. The strength of the horsey aroma is dependent upon the fermentation conditions. Tetrahydropyridines are the compounds associated with the horsy smell. Tetrahydropyridines are produced from ethanol and the amino acid lysine from the wort (*5, 24*).

GC-O

Gas chromatography-olfactometry was used to compare the volatile profiles of gueuze beer of different ages (Table 5.2). Compounds that were detected in fifty percent of the GC-O

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samples at the same retention time with similar aroma descriptors were considered to play a role in the aroma of different (3, 6, 9, 12, 28 month) samples. Compounds were ranked based upon their average intensity (very strong, strong, medium, weak, and very weak) perceived by the three panelists for each compound.

3 Month Old Samples

Eight compounds were identified using GC-O (Table 5.3). The eight compounds identified fell within three major chemical groups (acids, alcohols, and esters). The three acid compounds identified were propionic acid, isovaleric acid, and hexanoic acid. Isovaleric acid was the highest ranking acid identified for the 3 month sample. Isovaleric acid received an odor intensity ranking of medium. Propanoic and hexanoic acid both received an odor ranking of weak.

The next major chemical group identified was alcohols. The three compounds identified were 3-methylbutanol, hexanol, and octanol. Similar to isovaleric acid, hexanol had an odor intensity ranking of medium. 3-methylbutanol and octanol both received an odor intensity ranking of weak.

Ethyl isohexanoate and ethyl butyrate were the two ester compounds identified by the three trained panelists. Ethyl butyrate has been previously reported in lambic beer. While ethyl isohexanoate has not been previously reported in lambics, it has been reported in other styles of beer. Ethyl isohexanoate had the highest odor intensity ranking of the two compounds with a ranking of weak/medium.

6 Month Old Sample

In the six month sample, only nine compounds were identified (Table 5. 4). Only one more compound was identified in the six month old sample than in the three month old sample.

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When comparing the two samples, five of the nine compounds identified were also found in the three month old samples. Propionic acid, isovaleric acid, ethyl butyrate, hexanoic acid, and octanol were identified in both the three and six month old samples. Unlike the three month old sample, four chemical groups were identified: alcohols, acids, esters and aldehyde.

The same three acid compounds identified in the six month old sample were found in the three month old sample. The acids identified were propionic, isovaleric, and hexanoic acid. Hexanoic acid was not only the highest ranking acid, but one of the highest ranking compounds overall. Hexanoic acid is described as having a sweaty aroma. The panelists ranked hexanoic acid in the six month old sample as having an odor intensity ranking of medium, which was higher in the six month sample then in the three month old sample. Propionic acid received the same odor intensity ranking of weak for both the three and six month old samples. Isovaleric acid received a lower ranking in the six month old sample than in the three month old sample. However, in the three month old sample, isovaleric acid was tied with hexanol for having the highest odor intensity ranking.

The next major chemical group identified was alcohols. A total of two alcohols were identified as being odor active. The alcohol compounds identified were 2-methylbutanol and octanol. 2-methylbutanol was the highest ranking odor active alcohol identified as well as the highest ranking compound overall. 2-methylbutanol had an odor intensity ranking of strong. Octanol, the only other alcohol identified, received an odor intensity ranking of very weak. This is lower than the three month old sample, where the intensity was described as weak.

Three ester compounds play a role in the odor profile of the six month old samples. The three esters identified were ethyl butyrate, ethyl heptanoate, and ethyl benzoate. Ethyl butyrate has an aroma that can be described as bread. Ethyl butyrate was tied with hexanoic acid for the

second highest odor active compound for all the compounds identified, both of which received an odor intensity ranking of medium. Ethyl heptanoate was the second highest ranking ester compound with a ranking of weak. Ethyl benzoate was the lowest ranking ester compound as well as the lowest ranking compound overall for the six month old sample with an odor intensity ranking of very weak. Ethyl benzoate is described as having a fruity aroma.

Heptanal was the only aldehyde identified for this sample. Heptanal was one of three compounds that received an odor intensity ranking of medium. 2-methylbutanol was the only compound that was ranked higher in overall odor intensity. Heptanal is described as having a fruity aroma.

Nine Month Old Sample

Eleven compounds were identified for the nine month old sample (Table 5.5). The eleven compounds identified fell within seven chemical groups: acid, alcohol, vicinal diketone, phenol, furan, aldehyde and ester. The three strongest odor active compounds came from the acid, phenol, and aldehyde group and those compounds were isovaleric acid, 4-ethylphenol, and decanal. These three compounds all received an odor intensity ranking of medium. Isovaleric acid, hexanoic acid, and octanol were all found in both the nine month and six month old samples. Similar to the six month old sample, isovaleric acid was one of the highest ranking odor compounds. The descriptor used to identify the aroma of isovaleric acid is rancid. Hexanol was found in the three month old sample but not the six month old sample.

Three alcohol compounds were identified to have aroma active properties. The three alcohol compounds were 3-methylbutanol, hexanol, and octanol. Hexanol and octanol were the highest ranking odor active alcohols identified with an odor intensity ranking of weak. 3-methylbutanol had an odor intensity ranking of very weak.

Isovaleric acid and hexanoic acid are the two acid compounds identified in this sample. Isovaleric acid was ranked as one of three compounds with the highest odor activity. Isovaleric acid had an odor intensity ranking of medium, while hexanoic acid received an odor intensity ranking of weak. Both isovaleric and hexanoic acid were identified in the 3, 6, and 9 month old samples.

Two ester compounds were identified in the nine month old sample. Ethyl acetate and ethyl lactate were the two ester compounds identified by the three trained panelists. Ethyl acetate was ranked as very weak aroma odor intensity, while ethyl lactate was ranked slightly higher at weak.

A single compound was identified in phenol, ketone, furan, and aldehyde classes. The only phenolic compound identified was 4-ethylphenol. 4-ethylphenol was one of the highest ranking odor active compounds for the nine month old sample with an odor intensity of medium. Diacetyl is a vicinal diketone described as having a buttery aroma. Diacetyl was ranked as having a weak odor intensity. Furfural is the only furan compound that exhibited aroma active for the nine month old sample. Furfural is described as having a bread-like aroma with a weak odor intensity ranking for this particular sample. Decanal was the only aldehyde identified for this sample. Decanal is described as having an orange or citrus like aroma. Decanal was one of three compounds with the highest ranking odor activity for this sample with an odor intensity of medium.

Twelve Month Old Sample

Eleven compounds were identified for the twelve month old sample (Table 5.6). The eleven compounds identified can be separated into one of the seven chemical groups identified: acid, alcohol, vicinal diketone, phenol, furan, aldehyde and ester. The strongest odor active

compound was 4-ethylphenol with an odor active ranking of strong. Dimethyl sulfide, a thiol, was one of four compounds that the panelists ranked as having an odor intensity ranking of medium. Dimethyl sulfide has an odor descriptor of rotten and sulfury.

Three acid compounds were detected by the panelists and they were propionic, isovaleric, and hexanoic acid. Hexanoic and isovaleric acid are the only two compounds detected in the three, six, nine, and twelve month old samples. Propionic and isovaleric acid were both ranked as having an odor intensity of weak. Hexanoic acid was one of four odor active compounds that had an odor intensity ranking of medium.

Ethyl butyrate and ethyl heptanoate are the two ester compounds that were identified as odor active. Ethyl heptanoate is described as having a fruity aroma. The odor active ranking of ethyl heptanoate was medium. Ethyl butyrate the second ester compound identified by the panelists had an odor intensity ranking of weak.

Hexanol and 2-methylbutanol were the two alcohol compounds identified in this particular sample. 2-methylbutanol was the highest ranking alcohol. The odor intensity ranking for 2-methylbutanol was medium, while hexanol was ranked as very weak.

Diacetyl, a vicinal diketone, was identified in both the nine and twelve month old samples. Similar to the nine month old sample, both were ranked as having an odor intensity ranking of weak. Decanal was the only aldehyde identified for this particular sample. Similar to diacetyl, decanal was ranked as having a weak odor intensity

28 Month Old Sample

Seventeen aroma compounds were identified using GC-O (Table 5.7). The seventeen aroma compounds identified by the three panelists can be broken down into eight chemical groups: acid, alcohol, vicinal diketone, phenol, furan, aldehyde, and thiol. The strongest aroma

compound identified was 2-methylbutanol followed by 3-methylbutanol. These two compounds contributed the malty and burnt aroma found in the beer. Both octanol and hexanol are also from the chemical group alcohol. Octanol received the third highest ranking for the alcohol group. Octanol had an odor intensity ranking of medium, while hexanol was ranked as weak. Phenethyl alcohol was the lowest ranking alcohol compound identified. Phenethyl alcohol is used in the perfume industry for its pleasant sweet and rose like aroma (*25*).

Isovaleric and hexanoic acid were the two acid compounds identified in the twenty eight month old sample. Both isovaleric and hexanoic acid were identified in all five samples (3, 6, 9, 12 and 28 month old). Hexanoic acid was one of the highest ranking odor active compounds for the 28 month old sample. The odor intensity ranking for hexanoic acid was very strong, followed by isovaleric acid at strong.

Ethyl butyrate, ethyl isohexanoate, ethyl phenyl acetate, ethyl lactate, and ethyl heptanoate were the five ester compounds identified. Ethyl lactate had an odor intensity ranking of weak, while ethyl butyrate, ethyl isohexanoate, ethyl phenyl acetate, and ethyl heptanoate were ranked as very weak.

Dimethyl sulfide (DMS) was the only thiol compound identified. Dimethyl sulfide was perceived as having an odor intensity ranking of medium. Diacetyl is a vicinal diketone with an odor intensity ranking of weak. Furural, a furan, was one of the higher ranking odor active compounds with an odor intensity of medium.

Conclusion

In this study, the volatile and semi-volatile compounds of 3, 6, 9, 12, and 28 month old gueuze samples were extracted using SPME and analyzed using GC-MS and GC-O. A total of 42 compounds were identified using GC-MS. GC-O was used to analyzed 3, 6, 9, 12, and 28 month

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samples. Isovaleric acid, hexanoic acid, and propionic acid were identified in the 3, 6, 9, 12, and

28 month samples. As the age of the sample increased, so did the number of compounds detected

by the panelists.

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Table 5.1 Chemical compounds identified in aging lambic beer using GC-MS

	LRI	Literature	3	6	9	12	28
					Months		
Ethyl acetate	587	628	x	x	x	x	x
Ethanol	668	668	x	x	x	x	x
Isopentyl alcohol	700	734	x	x	x	x	x
2-methylbutanol,	744	744	--	x	x	x	x
Propanoic acid, 2-hydroxy-, ethyl ester (Ethyl lactate)	821	ND	--	--	x	x	x
Butanoic acid, 3-methyl-, ethyl ester (Ethyl isovalerate)	862	854	**--**	x	x	x	**x**
Isovaleric acid	861	877	x	--	--	--	--
Isoamyl acetate	884	876	x	x	x	x	x
2-Methyl-1-butyl acetate	889	885	--	--	--	--	x
2-heptanone	894	889	--	x	x	x	x
Heptan-2-ol	905	905	--	x	x	x	x
Amyl acetate	917	915	--	x	x	x	
2(5H)-Furanone, 5,5-dimethyl-	954	951	--	--	--	--	x
Heptan-1-ol	965	969	--	--	--	x	
Ethyl isohexanoate	968	969	x	x	x	**x**	x
Caproic acid	1000	996	x	x	--	--	x
Ethyl caprate	1000	996	x	x	x	x	x
Ethylhexanol	1031	1029	x	--	--	--	--
Isoamyl lactate	1069		x	x	x	x	x
2,5-Dimethyl-4-hydroxy-3(2H)-furanone	1083	1090	x	--	--	--	--
Heptanoic acid, ethyl ester	1097	1099	x	--	--	--	x
2-Nonanol	1102	1098	--	x	x	x	x
Nonanal	1103	1104	x	--	--	x	
Phenylethyl alcohol	1112	1118	x	x	x	x	x
2-ethyl-hexanoic acid	1122	1129	--	x	x	--	x
4-ethylphenol	1166	1169	x	x	x	x	x

	LRI	Literature	Months				
			3	6	9	12	28
Nonanol	1172	1171	x	--	--	--	--
Octanoic acid	1180	1179	x	--	--	--	x
L-.alpha.-Terpineol	1190	1195	x	x	x	x	--
Octanoic acid, ethyl ester	1198	1198	x	x	x	x	x
Decanal	1198	1195	x	x	x	x	x
Benzeneacetic acid, ethyl ester	1245	1244	x	x	x	x	x
β-Phenethyl acetate (Acetic acid, 2-phenylethyl ester)	1255	1260	x	x	x	x	x
Decanol	1272	1272	x	x	x	x	x
4-ethylguaiacol	1279	1287	x	x	x	x	x
Nonanoic acid, ethyl ester	1296	1297	x	x	x	x	x
Decanoic acid	1366	1373	x	--	--	--	x
Decanoic acid, ethyl ester	1395	1398	x	x	x	x	x
Octanoic acid, 3-methylbutyl ester (Isoamyl Octanoate)	1445	1455	x	x	--	--	x
Octanoic acid, 2-methylbutyl ester	1444	ND	x	--	--	--	x
Undecanoic acid, ethyl ester	1494	1494	x	--	x	x	x

LRI – linear retention index

Literature – LRI values correlating with previous identified compound at that specific LRI

x – denotes compound was identified

-- – denotes compound was not identified

Bold compounds have been previously identified

Table 5.2 Aroma compounds identified for aging lambic beer using GC-O

LRI	Compound	Age (months)					Descriptor
		3	6	9	12	28	
506	Diemethyl sulfide	--	--	--	x	x	sulfur, rotten
593	Diacetyl	--	--	x	x	x	buttery
587	Ethyl acetate	--	--	x	--	--	fruity
658	Propanoic acid	x	x	x	x	x	rancid
727	2-methyl-1-Butanol	--	x	--	x	x	burnt
745	3-methyl-1-Butanol	x	--	x	--	x	malt
790	Ethyl butyrate	x	x	--	x	x	apple
833	Furfural (2-furanal)	--	x	x	--	x	bread
875	Isovaleric acid	x	x	x	x	x	rancid
880	Hexanol	x	--	x	x	x	green
902	Heptanal	--	x	--	--	x	rancid
946	Ethyl isohexanoate	x	--	--	--	x	fruit
1010	Ethyl lactate	--	--	x	--	x	fruit
1019	Hexanoic acid	x	x	x	x	x	sweaty
1065	Octanol	x	x	x	--	x	chemical
1098	ethyl heptanoate	--	x	--	x	x	fruit
1166	ethyl benzoate	--	x	--	--	--	fruity
1168	ethyl phenol	--	--	x	x	x	musky
1204	Decanal	--	--	x	x	--	orange peel
1241	Benzeneacetic acid, ethyl ester	--	--	--	--	x	fruit
1253	β-Phenethyl acetate (Acetic acid, 2-phenylethyl ester)	--	--	--	--	x	rose, sweet

*LRI – linear retention index

x – denotes compound was identified

-- – denotes compound was not identified

Table 5.3 Odor profile and ranking for 3 month old lambic beer

LRI	Compound	Descriptor	Ranking
658	Propionic acid	rancid	weak
745	3-methylbutanol	malt	weak
790	Ethyl butyrate	apple	very weak
875	Isovaleric acid	rancid	medium
880	Hexanol	green	medium
946	Ethyl isohexanoate	fruit	weak/medium
1019	Hexanoic acid	sweaty	weak
1065	Octanol	chemical	weak

*LRI – linear retention index

Table 5.4 Odor profile and ranking for 6 month old lambic beer

LRI	Compound	Descriptor	Ranking
658	Propionic acid	rancid	weak
727	2-methyl-1-Butanol	malt	strong
790	Ethyl butyrate	bread	medium
875	Isovaleric acid	rancid	weak
902	Heptanal	fruit	medium
1019	Hexanoic acid	chemical	medium
1065	Octanol	fruit	very weak
1098	Ethyl heptanoate	musky	weak
1166	Ethyl benzoate	fruity	very weak

*LRI – linear retention index

Table 5.5 Odor profile and ranking for 9 month old lambic beer

LRI	Compound	Descriptor	Ranking
593	Diacetyl	buttery	weak
587	Ethyl acetate		very weak
745	3-methyl-1-Butanol	malt	very weak
833	Furfural (2-furanal)	bread	weak
875	Isovaleric acid	rancid	medium
880	Hexanol	green	weak
1010	Ethyl lactate	fruit	weak
1019	Hexanoic acid	sweaty	weak
1065	Octanol	chemical	weak
1168	ethyl phenol	musky	medium
1204	Decanal	organge	medium

*LRI – linear retention index

Table 5.6 Odor profile and ranking for 12 month old lambic beer

LRI	Compound	Descriptor	Ranking
506	Diemethyl sulfide	sulfur, rotten	medium
593	Diacetyl	buttery	weak
658	Propanoic acid	rancid	weak
727	2-methylbutanol	burnt	medium
790	Ethyl butyrate	apple	weak
875	Isovaleric acid	rancid	weak
880	Hexanol	green	very weak
1019	Hexanoic acid	sweaty	medium
1098	Ethyl heptanoate	fruit	medium
1168	Ethyl phenol	musky	strong
1204	Decanal	Organge	weak

*LRI – linear retention index

Table 5.7 Odor profile and ranking for 28 month old lambic beer

LRI	Compound	Descriptor	Ranking
506	Diemethyl sulfide	sulfur, rotten	medium
593	Diacetyl	buttery	weak
727	2-methylbutanol	burnt	very strong
745	3-methylbutanol	malt	strong
790	Ethyl butyrate	apple	very weak
833	Furfural (2-furanal)	bread	medium
875	Isovaleric acid	rancid	strong
880	Hexanol	green	weak
902	Heptanal	rancid	weak
946	Ethyl isohexanoate	fruit	very weak
1010	Ethyl lactate	fruit	weak
1019	Hexanoic acid	sweaty	very strong
1065	Octanol	chemical	medium
1098	ethyl heptanoate	fruit	very weak
1168	ethyl phenol	musky	weak
1241	Benzeneacetic acid, ethyl ester	fruit	very weak
1253	β-Phenethyl acetate (Acetic acid, 2-phenylethyl ester)	rose, sweet	very weak

*LRI – linear retention index

CHAPTER 6: CONCLUSION

Lambic beer is the only beer still being produced today through spontaneous fermentation. Prior research pertaining to this particular style of beer focuses mainly on the development of the microorganisms involved in the fermentation process. Minimal prior research has been conducted looking at the volatile and semi-volatile compounds present in lambic beers. SPME and SDE-SAFE are common extraction techniques used in the analysis of volatile and semi-volatile compounds. A total of 101 compounds were identified using SPME and SDE-SAFE. Of the 101 compounds, 38 sulfur compounds and 63 compounds made up of other chemical groups. In comparing the two techniques to each other SPME was able to identify a greater number of ester compounds, while SDE-SAFE was able to identify a wider range of acid compounds. OSME and AEDA were used in the analysis of the aroma compounds found in Cuvee Renée. A total of twenty eight aroma compounds were identified using OSME. AEDA, a dilution technique was used to identify thirty one aroma compounds.

SPME was selected as a viable extraction technique for the analysis of the volatile and semi-volatile compounds present in nine commercial lambic beers. This extraction technique was selected over others because of the ease of use and the ability to use an automatic sampling system. Fifty compounds were identified using a GC-MS within the nine commercial beer samples. HPLC was used in the identification and quantification of acetic and lactic acid found within the commercial brands of lambic beer. The concentration of acetic acid ranged from 723 – 1624 mg/L whiles the concentration of lactic acid 995 – 2557 mg/L. The pH range for the nine commercial beers was 3.23 – 3.62. Color was also analyzed using a scanning spectrophotometer. Color was measured using the standard reference method. Color ranged from 6.85 – 10.25 which correlates to the golden yellow for a younger lambic and a light amber for older gueuzes.

Gueuze is a style of lambic beer that is made up of young (1 year old) and aged (2+ years old) beer. Little is known about the development of the volatile and semi-volatile compounds during aging. SPME with GC-MS was used for the extraction and identification of volatile and semi-volatile compounds within 3, 6, 9, 12, and 28-month-old samples. A total of 42 compounds were identified using GC-MS within the aging samples. Seventeen of the 42 compounds identified within the aging samples have been previous reported in the literature. GC-O was used to identify the development of the aroma active compounds over the course of the fermentation process. A total of twenty one compounds were identified overall, the fewest compounds were identified in the 3 month with only nine compounds and the greatest number of compounds were identified for the 28 month old sample.

Lambic brewers are still using the same techniques today to produce this particular style of beer. Little is known regarding the development of the volatile and semi-volatile compounds produced during the aging process. The analysis of the commercial brands was able to expand upon the currently knowledge of the volatile and semi-volatile profile of lambic beer. Prior research was only able to identify twenty-five compounds, while this research was able to identify over 50 compounds. By knowing the chemical composition of lambic beer, brewers would be better able to control the process and obtain the desired aroma and flavor compounds. Due to the extensive aging process few brewers in the United States are willing to try to mimic this particular style of beer. Further research is required to analysis the volatile and semi-volatile compounds during the aging process to gather a greater understand of the development of these compounds.