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Acid and Volatiles of Commercially-Available Lambic Beers

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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 (one year) and older (two to three years) beers. Little is known about the volatiles and semi-volatiles found in commercial samples of gueuze lambic beers. SPME was used to extract the volatiles from nine different brands of lambic beer. GC-MS was used for the separation and identification of the compounds extracted with SPME. The pH and color were measured using standard procedures. A total of 50 compounds were identified in the nine brands. Seventeen of the 50 compounds identified have been previously identified. The 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 microorganism in beer and wine, but important for the flavor characteristics of lambic beer. There were no differences in pH, but there were differences in color between the beer samples.

Keywords: lambic beer; solid-phase microextraction; gas chromatography

1. Introduction

Lambic beer is one of the oldest styles of beer still being brewed today [1]. Eight lambic breweries (Belle Vue, Boon, Cantillon, De Troch, Girardin, Lindemans, Mort Subite, Timmermans), five blenders (De Cam, Drie Fonteinen, Hanssens, Oud Beersel, Tilquin), and two lambic breweries located in West Flanders (Bockor, Van Honsebrouck) are currently producing and selling lambic beer. However, the distribution of this type of beer is very limited within the United States [2]. Many lambic brewers and blenders are in financial trouble because of the time required to produce lambic beer; they can spend up to several years aging in casks or fermentation tanks before they are ready to be sold. This causes breweries to hold onto hundreds of thousands of dollars' worth of inventory while the beer is aging. Another issue that arises from aging lambic beer is that 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 for 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]. However, in the U.S. craft industry, there has been a great deal of interest in complex fermented sour beers in the last few years.

Beer is a complex beverage system made up of volatile and semi-volatile compounds belonging to a number of different chemical classes such as 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 important roles in the appearance, aroma, flavor, and mouthfeel of alcoholic beverages. Consumers judge the quality, character, and acceptability of alcoholic beverages based upon visual, olfactory, and taste properties. The aroma profiles of beer are composed of many different chemical compounds varying in concentrations 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 profiles, while others contribute to the key aroma and flavor characteristics [9]. Compounds with the greatest concentration do not always have the greatest influence on a product's aroma. In actuality, compounds with low concentrations often have the greatest influence on the aroma of a product [10]. What is important is the concentration relative to the odor detection threshold in the beer matrix.

The goal 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 quantify the acids.

2. Materials and Methods

2.1. Chemicals

Ethyl isobutyrate (99% purity), ethyl butyrate (99% purity), ethyl 2-methylbutyrate (99% purity), ethyl isovalerate (99% purity), isobutyl alcohol, 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 purchased from Fisher Scientific (Pittsburg, PA, USA) and used as standards. Acetic acid with a concentration of 0.150 g/L and L-lactic acid with a concentration of 0.204 g/L were purchased from R-Biopharm AG (Darmstadt, Germany). Octanoic and hexanoic acids at concentrations of 5 mg/10 mL were purchased from Fluka Analytical (Sigma-Aldrich, St. Louis, MO, USA). Isobutyric acid with a concentration of 5 mg/10 mL was purchased from Acros Organics (Geel, Belgium). Replicate bottles of gueuze lambic beer samples were purchased from local wine and beer stores in Blacksburg, VA and Athens, GA. The brands were Cuvee Reneé gueuze lambic (LK23JGC 2975 23 November 2012), Oude Gueuze Vieille (30 October 2026 L8304), Hanssens Artisan, Cantillon Gueuze 100% Lambic Bio (3 December 2010 Bottled), 3-Fonteinen (Bottled 23 February 2006), Gueuze Girardin (XO179), Oude Gueuze Boon (Best before 26 January 2025), Gueuze Boon (02 December 2025), and Cantillon-Classic Gueuze (13 November 2009 bottled).

2.2. 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 min to facilitate a sample measurement. 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).

2.3. pH Measurement

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

2.4. Color

Color was measured with the official AOAC 976.08 method using a scanning spectrophotometer (Shimadzu model UV-2550, Columbia, MD, USA) [11].

2.5. Solid Phase Microextraction (SPME)

The extraction and concentration of volatile compounds in commercially available gueuze beer was performed by solid phase microextraction. An SPME fiber (50/30 µm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA, USA) was exposed to the headspace above 4 mL of gueuze beer in 10 mL headspace vials with a Teflon-lined silicone septa (Chromacol, Fisher Scientific) for 30 min at 40 °C with an agitation speed of 250 rpm. Samples were equilibrated at 40 °C for two minutes prior to exposing the fiber. An AOC-5000 Plus (Shimadzu Scientific, Columbia, MD, USA) SPME autosampler was used for the automation of extraction and injection. Volatile compounds were desorbed for five minutes in the injection port of a QP2010 Ultra (Shimadzu, Columbia, MD, USA) 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 column (Shimadzu; 30 m × 0.25 mm i.d. × 0.25 µm film thickness) 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 35 °C held for 5 min and then increased to 225 °C at a rate of 6 °C /min. Once the final temperature of 225 °C was reached, it was maintained for 10 min. The MS was maintained at 200 °C and the sample mass was scanned in the range of 40–800 amu. GCMS was performed to identify the volatile compounds present in commercial samples of gueuze. Peaks were identified using a standardized retention time (retention index values, RI) and the fragmentation spectra of standards and the Wiley 2010 mass spectral library.

Identification

RI and Odor. Volatile compounds were identified based upon their RI values using both polar (DB-Wax) and nonpolar (DB-5) columns (30 m × 0.25 mm i.d., 0.25 µm film; J&W, Folsom, CA, USA). The RI values were compared to literature values. Aliphatic hydrocarbon standards were analyzed in the same manner on both the DB-5 and DB-Wax columns to calculate RI:

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

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 $t_{\mathrm{R}(N+n)}$ are the retention times of the unknown compound, the lower alkane, and the upper alkane, respectively.

2.6. High Performance Liquid Chromatography (HPLC)

An analysis of acids was conducted using an Agilent 1100 Series LC (Agilent Technologies, Santa Clara, CA, USA) with a 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 (C_6H_5) column (Macherey-Nagel, Bethlehem, PA, USA) 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 the injection volume was 5 µL. External standard curves for acetic and L-lactic acid were made at 200–1200 mg/L concentrations in beer.

2.7. Chemical Analysis of Lambic Beers

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 commercially available lambic beer samples. The Enology Service Laboratory analyzed the reducing sugars (grams/L of glucose and fructose) using AOAC 923.09, pH (AOAC 945.10), titratable acidity (AOAC 950.07), and volatile acidity (TTB variation method of AOAC 964.08).

2.8. Statistical Analyses

Statistical analyses were performed with SPSS software for Windows (version 18.0; SPSS Inc., Chicago, IL, USA). 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 $p < 0.05$. Mean values were reported ± standard deviation (SD).

3. Results and Discussion

3.1. pH

All of the lambic beers examined contained relatively high levels of organic acids. The pH values of gueuze, kriek (sour cherries), and framboise (raspberries) were lower than typical American lagers. The pH range previously reported for gueuze lambic beer is 3.20–3.51 [12]. The pH of American lagers tends to range from 3.7 to 4.8 [1]. Gueuze has a lower pH than other beer styles because of the additional microbial activity, resulting in the production of acetic, lactic, and other acids [1]. The presence of acetic or lactic acid bacteria is common and expected in lambic beers; however, in typical beers, these microorganisms are considered spoilage organisms. Lactic acid bacteria produces off-flavors and aromas such as honey or sweet butterscotch provided by the vicinal diketones-2,3-butanedione (diacetyl) and 2,3-pentanedione. Acetic acid bacteria can be hop-insensitive (growth not inhibited by hop antimicrobial components), similar to lactic acid bacteria, and can be responsible for the ropiness of beer [13].

The pH range observed for the nine commercial beers that we examined ranged from 3.23 to 3.62 (Table 1). Hanssens Artisan and 3-Fonteine had the lowest pH values of 3.23 and 3.24, respectively. These samples also had the highest total acidity (Tables 1 and 3). Hanssens Artisan, which had the lowest pH, also had the highest titratable acidity (TA) at 7.83 g/L, while 3-Fonteine had the second highest TA at 5.71 g/L. A significant difference in pH was found between all of the beers (Table 1).

Table 1. Comparison of pH levels for Commercial Lambic (Gueuze) Beers.

Brand	*N*	Subset for Alpha = 0.05			
		1	2	3	4
Hanssens Artisan	12	3.23 ± 0.01^{A}			
3-Fonteinen	12	3.24 ± 0.02^{A}			
Oude Gueuze Boon	6		3.43 ± 0.005^{B}		
Cantillon	6		3.44 ± 0.01^{B}		
Oude Gueuze Ville	9		3.44 ± 0.03^{B}		
Girardin	12		3.44 ± 0.02^{B}		
Gueuze Boon	6			3.52 ± 0.04^{C}	
Cantillon Bio	9			3.53 ± 0.01^{C}	
Cuvee Renée	9				3.62 ± 0.02^{D}

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

3.2. Color

Lambic (gueuze) beer can exhibit a wide range of colors, from golden yellow for young lambic beer to light amber for older (two to three years) beer. Gueuze typically ranges in color from 8 to 13 degrees SRM (Standard Reference Method) [1,13]. The color for the beer analyzed ranged from SRM 6.85 to 10.25 (Table 2). The table shows that there were significant differences in the sample color ($p < 0.05$) between samples. A significant difference between the color of Oude Gueuze Vieille and Girardin was observed. Oude Gueuze Vieille had a color value of 6.85 while Girardin had a color value of 10.25. Table 2 reports the color values of the individual experimental units. The data show 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 and 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 [1,2]. Additional color formation comes directly from the wooden casks themselves, either from the wood or from oxidation during the fermentation and maturation process [1]. It is not unusual for wort used in lambic beer to be boiled for four or more hours while 60 min is typical for an American lager.

Table 2. Color comparison for commercial lambic (gueuze) beers.

Brand	*N*	Means
Oude Gueuze Vieille	4	6.85 ± 1.21 [a]
Cantillon Bio	3	7.29 ± 0.26 [ab]
Cantillon	2	8.09 ± 0.49 [ab]
Oude Gueuze Boon	2	8.26 ± 0.19 [ab]
3-Fonteinen	4	8.46 ± 0.13 [abc]
Gueuze Boon	2	8.86 ± 0.13 [bc]
Hanssens Artisan	4	9.06 ± 0.33 [bc]
Girardin	2	10.26 ± 0.15 [c]

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

3.3. Titratable Acidity, Residual Sugar, Lactic Acid, Volatile Acidity, and Ethanol

Because of the high attenuation rate found in gueuze lambic beer, small to trace amounts of reducing sugars were found (Table 3). In prior research [14], only trace amounts (0.8% *w*/*v*) were reported. The amount of reducing sugars in the eight commercial beers tested ranged from 0.7% *w*/*v* to 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 and 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 17.27 g/L, where Oude Artisan had the highest volatile acidity, while Boon had the lowest. Volatile acidity refers to the organic acids (such as acetic or butyric acids) that are more volatile or are more easily vaporized than non-volatile or fixed acids. Total acidity (g/L) for the lambic (gueuze) beer ranged from 2.62 to 7.83 g/L with Oude Boon exhibiting the lowest value and Oude Artisan having the highest value. Ethanol ranged from 5.64% to 7.16%. Ethanol concentration for gueuze beers has been previously reported to range between 4.25% and 5.20% [14].

Table 3. Chemical measurements of lambic beer samples.

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 ± 0.07	9.86 ± 0.15	11.15 ± 1.53	6.06
3 Fonteine	*n* = 2	1.2	5.71 ± 0.32	12.73 ± 0.71	7.22 ± 1.17	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	0.70 ± 0.14	7.83 ± 0.91	17.47 ± 2.03	17.27 ± 1.65	5.66
Oude Gueuze Vieille	*n* = 2	1.65 ± 0.07	2.74	6.10 ± 0.01	5.71 ± 0.07	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).

3.4. 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 able to provide more complete volatile profiles, due to the wider range of volatile and semi-volatile compounds detected. A study comparing SPME with continuous liquid-liquid extraction/solvent-assisted flavor evaporation (CLLE/SAFE) for lambic beer reported that SPME recovered more esters but CLLE/SAFE recovered a greater number of acid compounds [16]. Others have shown that SPME-GCMS is useful for analysing volatiles in a wide range of beer styles [17].

3.5. Volatile and Semivolatile Compounds

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). Compounds that could not be identified by comparing their retention index values were marked as tentatively identified. The 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 beer. Seventeen compounds have been reported by both Van Oevelen et al. [12] and Spaepen et al. [18]. 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 [18] 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. Rossi et al. [17] used SPME to characterize the volatiles in different types of beer (lambic was not analysed) and reported that volatile fingerprints could be characterized based on the type of fermentation (top versus bottom) and style.

The major chemical classes that account for gueuze lambic beer were alcohols, acids, esters, phenols, aldehydes, and sulfur compounds. The production of alcohols in beer is a result of yeast metabolism [19]. Of the 50 compounds identified, eight were alcohols. Phenethyl alcohol, isoamyl alcohol, and isobutanol have been previously reported [12] in lambic beer. The compounds 2-methyl-1-butanol [20], 1-hexanol [21], heptyl alcohol [22], 1-octanol [22], 2-nonanol [21], and 1-decanol [22] have been previously reported in beer, but not lambic beer.

Twenty-three esters 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 [12,18,23]. An additional fifteen ethyl esters were detected using SPME. These compounds are shown in Table 4.

Acids play a vital role in the aroma and flavor profiles 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 [12,18]. With the exception of isovaleric and valeric acids, all have been previously reported in gueuze lambic beer, often associated with the aged hops used. Isovaleric and valeric acid, however, have been reported in other styles of beer [19].

Table 4. Chemicals identified within the commercial brands.

Chemical	LRI	Confirmed	Cuvee Renee	Oude Gueuze Vielle	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon	Compounds
Ethyl acetate	587	628	x	x	x	x	x	x	x	x	x	ester
Propanoic acid	637	668					x			x		acid
Isoamyl alcohol	683	734	x	x	x	x	x	x	x	x	x	alcohol
2-methyl-1-butanol	689	744	x	x	x	x	x	x	x	x	x	alcohol
Isobutyl acetate	752	776	x	x	x	x	x	x	x	x	x	esters
Ethyl isobutyrate	764	756	x	x	x	x	x	x	x	x	x	ester
Ethyl butyrate	804	800	x	x	x	x	x	x	x	x	x	ester
Furfural (2-furanal)	833	829		x	x	x		x	x	x	x	heterocyclic aldehyde
Isovaleric acid (3-Methylbutanoic acid)	851	854	x	x	x	x	x		x			Acid
Butanoic acid, 2-methyl-, ethyl ester (ethyl 2-methyl butyrate)	853	846	x	x	x	x	x	x	x	x	x	ester
Butanoic acid, 3-methyl-, ethyl ester	857	854	x	x	x	x	x	x	x	x	x	ester
2-Furanmethanol	860	866	x	x	x	x	x			x	x	furfuyl alcohol
Hexanol	873	880	x	x	x	x	x	x	x		x	alcohol
Isoamyl Acetate	880	876	x	x	x	x		x	x	x	x	ester
1-Butanol, 2-methyl-, acetate	882	880	x	x	x	x	x	x	x	x	x	ester
Styrene	888	893	x	x	x	x	x			x		benzene
Lactic Acid	906		x					x	x			acid
1-(2-furanyl)-Ethanone	910	910	x			x						ketone
5,5-Dimethyl-2(5H)-furanone	952	951		x	x	x	x	x	x	x		ketone
Heptyl alcohol	953	962	x	x	x	x		x	x	x	x	alcohol
Ethyl isohexanoate	966	968	x	x	x	x	x	x	x		x	ester
1-Propanol, 3-(methylthio)-	977	978	x	x	x	x						sulfur
Hexanoic acid (Caproic acid)	986	1019	x	x	x	x		x				acid
Hexanoic acid, ethyl ester	998	996	x	x	x	x	x	x	x	x	x	ester
Isoamyl lactate	1067	ND	x	x	x	x	x	x	x	x	x	ester
Octanol	1070	1072	x	x	x	x	x	x	x	x	x	alcohol
Heptanoic acid, ethyl ester	1097	1097	x	x	x	x	x	x	x		x	ester
2-Nonanol	1099	1098	x	x	x	x	x	x	x	x		alcohol
Nonanal	1102	1104	x	x	x		x	x		x	x	aldehyde
Valeric Acid	1104			x								acid
Isopentyl 3-methylbutyrate (Butanoic acid, 3-methyl-, 3-methylbutyl ester)	1104	1103			x	x	x		x			ester
Phenylethyl Alcohol	1110	1118	x	x	x	x	x	x	x	x	x	alcohol
2-ethyl-hexanoic acid	1119	1129	x	x	x							acid
Ethyl benzoate (Benzoic acid etyl ester)	1166	1170	x	x	x	x	x	x	x	x	x	ester
4-ethylphenol	1166	1169	x	x	x	x	x	x	x	x	x	phenolic
Octanoic acid	1180	1179	x	x	x	x	x	x	x	x	x	acid
Octanoic acid, ethyl ester (ethyl caprylate)	1197	1198	x	x	x	x	x	x	x	x	x	ester
Decanal	1204	1209	x	x	x	x	x	x	x	x	x	aldehyde
Benzeneacetic acid, ethyl ester	1244	1244	x	x	x	x	x	x	x	x	x	ester
Isopentyl hexanoate (Isoamyl caproate)	1249	1254	x	x	x	x	x	x	x	x	x	ester
β-Phenethyl acetate (Acetic acid, 2-phenylethyl ester)	1255	1260	x	x	x	x	x	x	x	x	x	ester
Decanol	1271	1272	x			x	x	x				alcohol
p-Ethylguaiacol	1278	1287	x	x	x	x	x	x	x	x	x	phenol
Nonanoic acid, ethyl ester	1295	1297	x	x	x	x	x	x	x		x	ester
Decanoic acid	1367	1373	x	x	x	x	x	x			x	acid
Ethyl 9-decenoate	1386	ND	x	x	x	x		x		x	x	ester
Decanoic acid, ethyl ester (Ethyl decanoate)	1394	1398	x	x	x	x		x	x	x	x	ester
Octanoic acid, 3-methylbutyl ester	1455	1450	x	x	x	x	x	x	x	x		ester
Ethyl dodecanoate	1594	1593	x	x	x	x		x	x		x	ester
Acetic acid			x	x	x	x		x	x	x	x	Acid
Isobutanol (1-Propanol, 2-methyl-)		647		x			x	x	x	x	x	alcohol

External standard calibration curves prepared in distilled water were used to quantify isovaleric acid (IVA), ethyl octanoate, 4-ethylphenol (4EP), 4-ethylguaiacol (4EG), ethyl caprylate, octanol, ethyl undecanoate, and ethyl acetate (Table 5). Isovaleric acid has been previously reported in beer, but not in lambic beers [19]. Isovaleric acid, 4-ethylphenol and 4-ethylguaiacol are key components in the overall aroma of *Brettanomyces* [24]. The concentration of isovaleric (3-methylbutyric acid) acid for gueuze lambic beer ranged from 1.92 mg/L for Oude Gueuze Vieille–3.01 mg/L for Cuvee Renée. Isovaleric acid was found in six of the nine commercial beers (Cuvee Renée, Oude Gueuze Vieille, Cantillion, Cantillion Bio, Girardin, and Oude Boon). When comparing the means for all the brands, no difference was found for IVA. Both 4-ethylphenol and 4-ethylguaiacol are known by-products of the yeast species *Brettanomyces*. 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 it was found at 0.28 mg/L for both Girardin and Oude Boon. Table 5 includes a comparison of 4-ethylphenol levels in commercial brands. The sensory detection threshold for 4-ethylphenol is reportedly 425 μg/L, and 4-ethylguaiacol has a sensory threshold of 100 μg/L [25,26]. The 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 5). When 4-ethylphenol is in the presence of 4-ethylguaiacol, the sensory threshold for 4-ethylphenol is lower [27]. The ratio of 4EP to 4EG is most often reported as 10:1. The ratio, however, can vary between regions and wines [5,25]. Little is known about the ratio of 4EP:4EG in lambic beers, but in our samples, we obtain a ratio of 0.33:1.

Ethyl octanoate (ethyl caprylate) was the fourth compound quantified. Ethyl octanoate has been previously reported in the literature as being found in lambic beer. The concentration of ethyl octanoate found within the literature was reported to be 0.16–0.59 mg/L [23]. Ethyl octanoate was found within all nine commercial brands tested. The concentration of ethyl octanoate ranged from 1.36 mg/L for 3 Fonteinen to 5.72 mg/L for Cantillion. When comparing the means, a difference was found between the different brands (Table 5).

Octanol has been previously reported in beer [22,28,29], 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 5). Ethyl undecanoate has been reported in wine [30], brandy [31], whiskey [32], cognac [33], and rum [34], but not beer. Ethyl undecnaote was detected in four of the nine brands. The range for ethyl undecanoate 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 Vieille 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 5).

Ethyl acetate is one of the twenty-seven compounds previously identified in lambic beer [12,23]. 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 to 67.6 mg/L [12]. The concentration of ethyl acetate in the commercial lambic beers ranged from 11.82 to 66.89 mg/L. Boon had the lowest concentration of ethyl acetate and Hanssens Artisan had the highest concentration (Table 5).

Table 5. Quantification of Compounds for Commercial Lambic Beers.

Compound mg/L	Cuvee Renée	Oude Gueuze Vielle	Cantillon	Hanssens Artisan	Cantillon Bio	3 Fonteinen	Girardin	Oude Boon	Boon
Isovaleric acid	3.01 ± 1.02	1.92 ± 0.06	2.15 ± 0.0	—	2.94 ± 0.21	—	2.95 ± 0.37	2.3 ± 0.23	—
Ethyl octanoate	5.67 ± 1.53 ^{A}	2.68 ± 1.88 ^{AB}	5.72 ± 1.89 ^{A}	2.74 ± 1.24 ^{BCD}	4.52 ± 1.54 ^{ABC}	1.36 ± 1.32 ^{D}	2.22 ± 1.32 ^{CD}	1.66 ± 1.32 ^{D}	1.62 ± 1.86 ^{D}
4-Ethyl phenol	1.13 ± 0.02 ^{E}	0.57 ± 0.02 ^{G}	1.08 ± 0.08 ^{EF}	0.57 ± 0.07 ^{G}	0.96 ± 0.06 ^{F}	0.44 ± 0.03 ^{H}	0.28 ± 0.02 ^{I}	0.28 ^{I}	0.32 ^{HI}
4-Ethyl guaiacol	5.77 ± 0.08 ^{J}	1.06 ± 0.09 ^{L}	2.44 ± 0.07 ^{K}	1.36 ± 0.31 ^{L}	2.1 ± 0.23 ^{K}	0.99 ± 0.06 ^{LM}	1.08 ± 0.15 ^{L}	0.52 ± 0.01 ^{M}	0.97 ^{LM}
Octanol	0.041 ^{O}	0.031 ± 0.01 ^{O}	0.052 ± 0.01 ^{O}	0.084 ± 0.01 ^{N}	0.025 ^{O}	0.034 ± 0.01 ^{O}	0.031 ± 0.01 ^{O}	0.02 ^{O}	0.02 ^{O}
Ethyl undecanoate	46.0 ± 9.83 ^{P}	16.3 ± 5.78 ^{QR}	28.8 ± 1.7 ^{PQ}	—	8.6 ± 1.0 ^{R}	—	—	—	—
Ethyl Acetate	ND	22.3 ± 0.95 ^{V}	28.4 ± 1.09 ^{U}	66.9 ± 4.36 ^{S}	46.9 ± 0.29 ^{T}	22.1 ± 0.75 ^{V}	21.4 ± 2.04 ^{V}	17.0 ± 0.22 ^{VW}	11.8 ± 0.22 ^{W}

Means followed by same superscript are not significantly different at the 0.05 level experiment-wise using Tukey-Kramer HSD. ND signifies not detected.

3.6. 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 to the sourness. All organic acids have their own characteristic flavor, aroma, and taste [35–37]. 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 [36]. Third, the organic acids present in beer can aid in prolonging the shelf life by providing the beer with a strong buffering capability [36,38]. Acetic acid has a flavor threshold of 200 ppm, while lactic acid has a flavor threshold of 400 ppm [26,39].

Acetic and L-lactic acid were found in varying concentrations within different styles of lambic beer [39]. 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 6. 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 to 140 ppm acetic acid. The concentration of acetic acid in gueuze beer can range between 500 and 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 ($p > 0.05$).

Table 6. Comparison of Acids within Commercial Brands of Lambic Beer.

Brand	Acetic Acid (mg/L) *	Lactic Acid (mg/L)
Cuvee Renee	916 ± 12.02	2557 ± 8.26
Oude Gueuze Villie	1019 ± 211.43	1094 ± 13.54
Cantillion	1224 ± 1.41	1417 ± 184.25
Hanssens Artisan	1642 ± 847.82	1389 ± 55.64
Cantillon Bio	1473 ± 79.21	1658 ± 890.05
3 Fonteinen	1204 ± 129.78	1294 ± 61.2
Girardin	1499 ± 109.53	1403 ± 96.95
Oude Boon	723 ± 8.48	1228 ± 14.12
Boon	1137 ± 13.44	995 ± 18.55

* No difference was found between the brands.

The concentration of lactic acid in gueuze beer can range between 1500 and 3500 mg/L, while typical American lagers tend to have much lower concentrations, around 40–150 ppm [1]. Table 6 shows a comparison of means for lactic acid. The concentration of lactic acid ranged from 1098 to 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. 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.

The origins of the volatiles in gueuze lambic include raw materials, and chemical/microbial changes occurring during fermentation and aging. Spitaels et al. [40,41] reported a consistent core microbial population including *Pediococcus damnosus*, *Dekkera anomala*, *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, and *S. pastorianus* during production to just *D. bruxellensis* after production. *Brettanomyces* and *Dekkera* are used interchangeably with *Dekkera* most often used to describe the spore form of the yeast. Extended aging was associated with higher levels of ethyl lactate and lower levels of isoamyl acetate and ethyl decanoate with aging. Thus, the differences in some of the volatiles that we have observed in our samples could represent production or age differences.

4. Conclusions

In this study, the volatile and semi-volatile compounds of nine commercial brands of lambic (gueuze) beer were identified using SPME-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. Ethyl acetate was found at 11.8–66.9 mg/L and 4EG at 0.52–5.77 mg/L. Acetic and lactic acids were identified and quantified using HPLC with ranges observed from 723–1642 mg/L and 995–2557, respectively. The results show the range of typical values for volatile aroma compounds and acids in Belgian lambic beers and can help clarify the reasons for some of the variation in flavor characteristics observed in commercial products.

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References

1. Guinard, J.X. *Lambic*; Brewers Publications: Boulder, CO, USA, 1990; p. 159.
2. Van den Steen, J. *Gueuze & Kriek: The Secret of Lambic*; Lannoo Publishers: Tielt, Belgium, 2011; p. 224.
3. De Keersmaecker, J. The mystery of lambic beer. *Sci. Am.* **1996**, *275*, 74. [CrossRef]
4. Rodrigues, F.; Caldeira, M.; Camara, J.S. Development of a dynamic headspace solid-phase microextraction procedure coupled to gc-qmsd for evaluation the chemical profile in alcoholic beverages. *Anal. Chim. Acta* **2008**, *609*, 82–104. [CrossRef] [PubMed]
5. Ferreira, V.; Ortin, N.; Escudero, A.; Lopez, R.; Cacho, J. Chemical characterization of the aroma of grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *J. Agric. Food Chem.* **2002**, *50*, 4048–4054. [CrossRef] [PubMed]
6. Ferreira, V.; Lopez, R.; Cacho, J.F. Quantitative determination of the odorants of young red wines from different grape varieties. *J. Sci. Food Agric.* **2000**, *80*, 1659–1667. [CrossRef]
7. Reineccius, G. *Flavor Chemistry and Technology*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2006.
8. Taylor, A.J. *Food Flavour Technology*; Sheffield Academic Press: Boca Raton, FL, USA, 2002; p. 302.
9. Romano, P.; Fiore, C.; Paraggio, M.; Caruso, M.; Capece, A. Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* **2003**, *86*, 169–180. [CrossRef]
10. Plutowska, B.; Wardencki, W. Application of gas chromatography-olfactometry (gc-o) in analysis and quality assessment of alcoholic beverages—A review. *Food Chem.* **2008**, *107*, 449–463. [CrossRef]
11. American Society of Brewing Chemists (ASBC). *Methods of Analysis, Online. Beer Method 10. Spectrophotometric Color Method*; Approved 1958 rev; American Society of Brewing Chemists: St. Paul, MN, USA, 2015.
12. Van Oevelen, D.; Delescaille, F.; Verachtert, H. Synthesis of aroma components during spontaneous fermentation of lambic and gueueze. *J. Inst. Brew.* **1976**, *82*, 322–326. [CrossRef]
13. Goldammer, T. *The Brewer's Handbook the Complete Book to Brewing Beer*, 2nd ed.; Apex: Clifton, NJ, USA, 2008; p. 480.
14. Plevoets, C.; Van Ginderachter, S. *Gueuze*; Musee Bruxellois de la Gueze: Brussels, Belgium, 1988; p. 8.
15. Constant, M.; Collier, J. Headspace gas chromatography profiles of fruit-flavored malt beverages using solid-phase microextraction. *J. Am. Soc. Brew. Chem.* **1997**, *55*, 112–118.
16. Thompson-Witrick, K.A.; Rouseff, R.L.; Cadawallader, K.R.; Duncan, S.E.; Eigel, W.N.; Tanko, J.M.; O'Keefe, S.F. Comparison of two extraction techniques, solid-phase microextraction versus continuous liquid–liquid extraction/solvent-assisted flavor evaporation, for the analysis of flavor compounds in gueuze lambic beer. *J. Food Sci.* **2015**, *80*, C571–C576. [CrossRef] [PubMed]
17. Rossi, S.; Sileoni, V.; Perretti, G.; Marconi, O. Characterization of the volatile profiles of beer using headspace solid-phase microextraction and gas chromatography-mass spectrometry. *J. Sci. Food Agric.* **2014**, *94*, 919–928. [CrossRef] [PubMed]

18. Spaepen, M.; Vanoevelen, D.; Verachtert, H. Fatty-acids and esters produced during spontaneous fermentation of lambic and gueuze. *J. Inst. Brew.* **1978**, *84*, 278–282. [CrossRef]
19. Nykänen, L.; Suomalainen, H. *Aroma of Beer, Wine, and Distilled Alcoholic Beverages*; Kluwer Boston: Dordrecht, The Netherlands; Boston/Hingham, MA, USA, 1983.
20. Strating, J.; Venema, A. Gas-chromatographic study of an aroma concentrate from beer. *J. Inst. Brew* **1961**, *37*, 525–528. [CrossRef]
21. Tressl, R.; Friese, L.; Fendesack, F.; Koppler, H. Gas chromatographic mass spectrometric investigation of hop aroma constitutents in beer. *J. Agric. Food Chem.* **1978**, *26*, 1422–1426. [CrossRef]
22. Drawert, F.; Tressl, R. Beer aromatics and their origin. *Tech. Q. Master Brew. Assoc. Am.* **1972**, *9*, 72–76.
23. Van Oevelen, D.; Spaepen, M.; Timmermans, P.; Verachtert, H. Microbiological aspects of spontaneous wort fermentation in production of lambic and gueuze. *J. Inst. Brew.* **1977**, *83*, 356–360. [CrossRef]
24. Goode, J. Brettanomyces. In *The Science of Wine, from Vine to Glass*; University of California Press: Berkeley, CA, USA, 2005; pp. 136–143.
25. Ferreira, V.; Aznar, M.; Lopez, R.; Cacho, J. Quantitative gas chromatography-olfactometry carried out at different dilutions of an extract. Key differences in the odor profiles of four high-quality spanish aged red wines. *J. Agric. Food Chem.* **2001**, *49*, 4818–4824. [CrossRef] [PubMed]
26. Harrison, G.A. Flavour of beer—Review. *J. Inst. Brew.* **1970**, *76*, 486. [CrossRef]
27. Cook, A.H.; Kirsop, B.H. The content of some organic acids in beer and other fermentation media. *J. Inst. Brew.* **1974**, *80*, 474.
28. Buttery, R.G.; Black, D.R.; Lewis, M.J.; Ling, L. A study of the fate of volatile hop constitutes in beer. *J. Food Sci.* **1967**, *32*, 414–419. [CrossRef]
29. Strating, J.; van Eerde, P. The staling of beer. *J. Inst. Brew.* **1973**, *79*, 414–415. [CrossRef]
30. Chaudhary, S.S.; Webb, A.D.; Kepner, R.E. Glc investigation of the volatile compounds in extracts from sauvignon blanc wines from normal and botrytised grapes. *Am. J. Enol. Vitic.* **1968**, *19*, 6–12.
31. Suomalai, H.; Nykanen, L. The formation of aroma compounds by yeast in sugar fermentation. *Suomen Kemistilehti B* **1964**, *37*, 230–232.
32. Williams, A.A.; Tucknott, O.G. The volatile components of scotch whisky. *J. Sci. Food Agric.* **1972**, *22*, 1–7. [CrossRef]
33. Schreier, P.; Drawert, F.; Winkler, F. Composition of neutral volatiles constitutes in grape brandies. *J. Agric. Food Chem.* **1979**, *27*, 365–372. [CrossRef]
34. Liebich, H.M.; Koenig, W.A.; Bayer, E. Analysis of the flavor of rum by gas-liqued chromatography and mass spectrometry. *J. Chromatogr. Sci.* **1970**, *8*, 527–533. [CrossRef]
35. Siebert, K.J. Chemometrics in brewing—A review. *J. Am. Soc. Brew. Chem.* **2001**, *59*, 147–156.
36. Whiting, G.C. Organic acid metabolism of yeasts during fermentation of alcoholic beverages—A review. *J. Inst. Brew.* **1976**, *82*, 84–92. [CrossRef]
37. Xiang, Y.; Li, Y.; Li, Q.; Gu, G. Factors influencing the content of organic acids in final malt. *J. Am. Soc. Brew. Chem.* **2006**, *64*, 222–227.
38. Bamforth, C.W. Ph in brewing: An overview. *Tech. Q. Master Brew. Assoc. Am.* **2001**, *38*, 1–9.
39. Engan, S. Organoleptic threshold values of some organic-acids in beer. *J. Inst. Brew.* **1974**, *80*, 162–163. [CrossRef]
40. Spitaels, F.; Wieme, A.D.; Janssens, M.; Snauwaert, I.; Aerts, M.; Van Landschoot, A.; De Vuyst, L.; Vandamme, P. Microbiota and metabolites of aged gueuze beers converge to the same composition. *Food Microbiol.* **2015**, *47*, 1–11. [CrossRef] [PubMed]
41. Spitaels, F.; Wieme, A.D.; Janssens, M.; Aerts, M.; Van Landschoot, A.; De Vuyst, L.; Vandamme, P. The microbial diversity of an industrially produced lambic beer shares members of a traditionally produced one and reveals a core microbiota for lambic fermentation. *Food Microbiol.* **2015**, *49*, 23–32. [CrossRef] [PubMed]