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Comparative Investigation of Flavors in Red and Brown Flemish Beers: Key-Role of *Brettanomyces* and Torrefied Malts in Ethylphenols Occurrence

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ABSTRACT

Red and brown Flemish sour beers form a distinct class of Belgian beers obtained by mixed (yeast/lactic bacteria) microbial fermentation and often resulting from blending a 1-to-2-year-old beer with a younger one to obtain a balance between acidic character and sweetness. A detailed composition in volatiles (phenols, lactones, esters, alcohols, acids, …) of three beers representative of the red and brown subcategories is presented. GC data were obtained after different extraction procedures, including solvent-assisted flavor evaporation (SAFE) and headspace. The first results showed the influence of *Brettanomyces* yeast on the phenol and ester contents. An efficient *Brettanomyces* activity in the red sour beers (especially in Rodenbach Vintage) was observed, favored by long maturation in wooden casks. This was organoleptically perceived by the horsey flavors brought by 4-ethylguaiacol and 4-ethylphenol, and the solvent-like ethyl acetate through esterase activity. The brown Flemish sour beer (produced in stainless steel fermenters) showed significantly more unreduced 4-vinylguaiacol and 4-vinylphenol, although traces of 4-ethylguaiacol and 4-ethylphenol were also detected (most probably here issued from torrefied malts, as suggested by the opposite substituted phenol/guaiacol ratio).

KEYWORDS

Beer volatiles; *Brettanomyces*; red and brown Flemish beers; SAFE extraction

Introduction

Among Belgian beer specialties, sour beers have received considerable interest in the United States over the last decades, with the local development of production sites mimicking traditional Belgian breweries.[1] This trend, driven largely by the popular books of the British journalist Michael Jackson, was initiated in 1977 by the first edition of a new guide of world beers,[2] in which special attention was paid to Belgian sour beers, followed by a book fully devoted to Belgian beers.[3] These books popularized worldwide the two main categories of Belgian sour beers: on the one hand, lambic beer and its derivatives (e.g., gueuze, kriek), produced in the Senne Valley and the Pajottenland near Brussels; and, on the other hand, Flemish sour beers produced specifically in two provinces of Belgium: West Flanders and East Flanders (not to be confused with today's Flanders Region, which includes additional provinces where the Flemish language is spoken).

Lambic beer requires at least 30% unmalted wheat, together with the usual pilsen malt, and long boiling with aged hop.[4,5] Spontaneous fermentation by yeast and bacteria in ambient air takes place during overnight wort cooling in large, open coolships and is followed by a long fermentation-maturation in oak barrels for at least one year.[4] Gueuze is a blend of a very dry, acidic old lambic (2–3 years old) with a younger lambic (1 year old), the latter providing the sugars needed for carbonation during bottle refermentation.

Flemish sour beers are particularly hard to characterize and classify, as illustrated by widespread confusion, in particular, about their color. Historically, prior to 1977 and Jackson's books, all Flemish sour beers including Rodenbach were called Brown or more exactly "Oud Bruin" (Old Brown in Flemish) because these dark beers were aged for several months to let lactic bacteria (inoculated together with *Saccharomyces*) and possibly wild yeasts carry out their souring and fermentation steps. Jackson introduced the point that West-Flanders sour beers have fruity flavors reminiscent of red wines and are slightly reddish, and this led to calling the beers of that region, particularly Rodenbach, red ales. The dark sour beers of the other province (notably from the area around the city of Oudenaarde), dominated by malt flavors and malt-oriented fruity flavors, were distinguished as brown ales. Today, Liefmans Goudenband beer is generally viewed as the prototypical brown sour beer. The popular but caricatural red-versus-brown distinction, originally favored for commercial reasons, is now sometimes replaced in the academic community with red-brown versus brown.[6]

The mahogany color of Rodenbach beer (80–90 EBC) comes from the use of reddish barley malt (Vienna or Crystal) in addition to pale winter barley malt and 20% maize. Prolonged boiling leads to good protein coagulation and hence a minimal amount of foam in the finished beer.

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Use of old hops explains the moderate bitterness of the beer, less than 10 bitterness units (BU). The (high) fermentation is launched by inoculating a yeast suspension (*Saccharomyces cerevisiae*) with a low level of lactic acid bacteria (level controlled by an acid wash) harvested at the end of the primary fermentation of a previous brew. This primary fermentation, operated at 21 °C, lasts for about one week. Next comes a secondary fermentation of the unfiltered beer, for 4–5 weeks at 15–21 °C in epoxy-covered tanks. Two types of wort are subjected to this sequence of two fermentations,[7] a "light" wort of about 11°P and a "heavy" wort of 13.5°P. After the secondary fermentation, the light beer is cooled to 0 °C to be blended with old beer. The heavy beer is transferred to large wooden casks (10 to 65 m^3) for a tertiary fermentation/maturation that takes 18–24 months. At the end, a small fraction of the heavy beer is sold as Rodenbach Vintage, while the rest is blended with young beer. Rodenbach Grand Cru is a 1/3 young- 2/3 old blend. Microbiological analysis of these successive fermentations[7] indicates that the primary fermentation is essentially alcoholic and related to the action of *S. cerevisiae*, because the population of lactic acid bacteria grows moderately. Then, after a yeast harvest, the secondary fermentation of the light beer is dominated by fermentation/biomass production by lactic bacteria (*Lactobacillus delbrueckki subspec. delbrueckki* and *subspec. bulgaricus*). The lactic fermentation is homo- or heterofermentative and thus linked to production of ethanol, lactic acid, and the latter's ethyl ester. In the heavy beer, yeast continues to dominate during the secondary fermentation. During the third fermentation of « heavy beer », the oak barrels are the source of *Pediococcus parvulus* bacteria and wild *Brettanomyces* yeasts (*B. lambicus* or *B. bruxellensis*).[7] These dominate the further fermentative processes, producing lactic acid, acetic acid and its ethyl ester, and special flavors developed by *Brettanomyces* (e.g., cheesy from short-chain acids, barnyard from 4-ethylguaiacol).[8]

Liefmans Goudenband is produced by a variant of the aforementioned recipe, starting with barley malt only. The dark beer color (109 EBC) results from the use of special malts, including a small fraction of torrefied malt. After boiling and rapid cooling, the wort is transferred to open copper casks and inoculated with a mixture of *S. cerevisiae* and lactic bacteria (harvested from a previous brew). A one-week open-air primary fermentation at 20–24 °C allows additional uncontrolled inoculation of lactic or acetic bacteria and wild yeasts, causing already an apparent 4°P attenuation. The beer is then transferred to stainless steel tanks for at least three months. By comparison with Rodenbach maturation with *Brettanomyces* in oak barrels, stainless steel avoids oxygen permeation and the beer remains less acetic, as stated by limited influence of *Brettanomyces*.[1,9] The final beer is obtained by blending the dark beer issued from a long maturation with a young beer.

The acidic character of Flemish and lambic beers comes mainly from lactic and acetic acids.[10] Oak-barrel Rodenbach beer contains 2500–5000 mg/L of lactic acid and about 1500 mg/L of acetic acid.[6] Liefmans contains up to 5000 mg/L of lactic acid and 1000 mg/L of acetic acid. For comparison, commercial lambic beer contains 1500 – 3500 mg/L of lactic acid and 500–1500 mg/L of acetic acid.[11] In fermented beverages, acetic acid is considered detrimental above 1200 mg/L, which explains the usual comment that red ales are more acetic in character than Old Brown beers, whose acidity gives rise to a milder, lactic acid perception.

Few studies have been published on the chemical composition of Belgian sour beer volatiles, except for lam bic.[4,9,11,12] In both lambic and Flemish sour beers, flavors originating from wild yeasts are of high relevance.[8,12,13] The combined action of *Brettanomyces* and other microorganisms needs to be taken into account to explain the origin of some flavors in mixed fermentation beers.[14] Apparently, no detailed chemical information has been published specifically on brown ales. A single study has been published on red ales, in which both the microbial composition and the metabolite composition were addressed.[6] This study involved beer samples taken directly from the oak barrel at the end of maturation at three different breweries (including Rodenbach). In particular, static-headspace gas chromatography coupled to mass spectrometry (GC-MS) was used to determine the concentrations of some volatiles, including esters, alcohols and organic acids. The data from this study reflect some characteristics of successive *Saccharomyces*/*Brettanomyces* fermentations of the wort, in parallel with the competing lactic and acetic fermentation effects. Concentrations of phenols, special malt volatiles, and hop volatiles were not measured.

The purpose of the present work was to conduct a preliminary comparative study of the two main commercial Flemish sour beers: Liefmans Goudenband and Rodenbach, representative of the two subfamilies of Flemish sour beers. For Rodenbach, both the Grand Cru and Vintage beers were analyzed, the latter being close to the samples studied by Snauwaert et al..[6] Using various extraction procedures and various analytical techniques, these three commercial beers were compared with respect to the concentrations of a wide variety of volatiles (including phenols, esters, alcohols, short chain organic acids, and Maillard reaction products) and bitter compounds (iso-alpha acids, tetrahydroisohumulones…).

As detailed in the next section, a wide range of techniques were used: SAFE extraction (ultra-low-pressure distillation) after a preliminary liquid-liquid organic extraction; GC-MS; static headspace analysis of esters and alcohols; hexanol extraction of organic short chain acids followed by gas chromatography-flame ionization detector (GC-FID); and high performance liquid chromatography-ultraviolet (HPLC-UV) separation of iso-alpha acids and analogs. This comparative analysis of volatiles found in the Liefmans Goudenband and Rodenbach beers enabled us to draw some general conclusions.

Experimental

Beer samples

Three red/brown beers were purchased from shops (Brussels, Belgium): 33-cL of Liefmans Goudenband (LG), 33-cL of

Rodenbach Grand Cru (RGC), and 75-cL of Rodenbach Vintage (RV). The RV analyzed had been bottled in 2015 and was opened and studied in early 2021. The LG and RGC samples were analyzed as sold. These three beers were selected as typical of the red Flemish sour beer sub-family (RGC and RV) or of the brown acidic beer subfamily (LG).

Chemical standards

Dichloromethane, absolute ethanol, methanol, acetonitrile, 37% hydrochloric acid, sodium hydroxide, and citric acid monohydrate were purchased from VWR International (Leuven, Belgium). The isohumulone standard (ICS-I3) and the tetrahydroisohumulone standard (ICS-T2) were purchased from Labor Veritas Co. (Zürich, Switzerland). Hexan-1-ol, oct-1-en-3-ol, propan-1-ol, 2,3,5-trimethylpyrazine, 2,6-dimethylpyrazine, 2-acethylthiophene, 2-phenylethanol, 2-phenylethyl acetate, 4-ethylguaiacol, 4-ethylphenol, 4-methylguaiacol, 4-methylphenol, 4-vinylguaiacol, 5-methylfurfural, acetovanillone, benzaldehyde, decanoic acid, ethyl acetate, ethyl butanoate, ethyl decanoate, ethyl heptanoate, ethyl hexanoate, ethyl leucate, ethyl octanoate, eugenol, furfural, guaiacol, hexanoic acid, isoamyl acetate, isoamyl alcohol, isoamyl propionate, isobutanol, isobutyl acetate, isovaleric acid, maltol, methional, nonanoic acid, oak lactone, octanoic acid, pentanoic acid, 2-phenylacetaldehyde, syringaldehyde, vanillin, γ-decalactone, γ-nonalactone, and δ-decalactone were bought from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Tabanone was purchased from Vigon International (East Stroudsburg, PA, U.S.A.). Milli-Q water was used (Millipore, Bedford, MA, U.S.A.).

Isolation of the volatiles

SAFE followed by GC-MS analysis

The SAFE method extracts volatile components from the matrix by means of a high-vacuum and low-temperature distillation.[15] Following the protocol of Dusart et al.,[16] 50-mL sample of degassed beer was spiked with 150 µL of 2-acethylthiophene (8 mg/L) as internal standard (IST). The beer was then mixed vigorously with 75 mL of bidistilled dichloromethane for 20 min. The resulting emulsion was centrifuged at 2264 g for 20 min. The aqueous phase was discarded; the organic phase was dried over anhydrous sodium sulphate. The organic phase was then introduced into the dropping funnel of the SAFE vessel. The conditions for the SAFE analyses were the following: water bath temperature 40 °C, pressure below 10^{-3} Pa, and the apparatus body at 30 °C. The distillate was continuously recovered in the liquid-nitrogen-cooled SAFE flask for 15 min distillation, followed by an extraction with distilled water (3 × 25 mL) to remove any residual alcohol. The extract was dried over anhydrous sodium sulfate. The extract was then concentrated to 500 µL in a Kuderna-Danish apparatus at 45 °C. Extracts were stored at −80 °C until analysis by gas chromatography-electron ionization-mass spectrometry (GC-EI-MS).

SAFE extracts were analyzed with a wall-coated open tubular apolar capillary column (CP-Sil 5 CB, 50 m × 0.32 mm i.d., 1.2 µm film thickness) on an Agilent 7890B gas chromatograph. Injections (1 µL) were carried out at 250 °C in splitless mode. The carrier gas was helium, and the pressure was set at 65 kPa. The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 250 °C (held for 30 min) at 3 °C/min. The column was connected to a single quadrupole mass spectrometer (Agilent 5977B MSD) operating in selected ion monitoring (SIM) mode with electron ionization at 70 eV.

The concentration of a volatile X (C_X) was determined as:

$$C_X = C_{IST}(CR_{IST} / CR_X)\left(\%RecovIST / \%RecovX\right)A_X / A_{IST}$$

in which C_{IST} is the IST concentration in beer; A_X and A_{IST} are the peak areas of X and IST, respectively; CR_X and CR_{IST} are the X and IST response coefficients, respectively; and %RecovX or %RecovIST is the recovery factor for X or IST, respectively.

To avoid complete standard addition procedure, ($\%Recov_{IST}/\%Recov_X$) was set at 1 (ratio between 0.8 and 1.2 for most X species[16]). For commercially available compounds, calibrations with respect to IST were constructed for each X species. For commercially unavailable compounds (symbolized by + in Tables), IST equivalent concentrations (in which, ($CR_{IST}/CR_{X)}$ was set at 1) are given.

Static headspace analysis of esters and alcohols

Prior to analysis, beers were stored for 2 h at 4 °C to avoid excessive foaming. A 40 µL sample of IST (2-pentanol at 2500 mg/L) and sodium chloride in excess (2 g) were added to 5 mL of beer in a headspace vial. Those were incubated at 60 °C and automatically shaken for 30 min before injection of 500 µL of the headspace (Automatic injector CTC Analytics Combibal, Hamilton 2.5-mL syringe). The following compounds were analyzed: propan-1-ol, ethyl acetate, isobutanol, isoamyl alcohol, isoamyl acetate, ethyl hexanoate, ethyl octanoate, and ethyl decanoate. The GC-MS parameters were the same as described in the previous section, except for the temperature program: start at 32 °C for 5 min, then a rise from 32 to 140 °C at 8 °C/min, from 140 to 180 °C at 15 °C/min, and was finally held at 180 °C for 30 min.

The Liefmans Goudenband beer was selected for a standard addition procedure. Standard addition slopes were then used for the quantification of Rodenbach beers, analysis being performed in duplicate.

n-Hexanol extraction of short-chain fatty acids

Isovaleric, pentanoic, hexanoic, octanoic, and decanoic acids, together with two 2-phenylethyl acetate and β-phenylethanol, were extracted from beers according to Silva Ferreira et al..[17] Ten milliliters of degassed beer was

mixed with 100 µL of internal standard (1000 mg/L nonanoic acid) in a 20-mL glass flask and shaken for 10 s. Then, 300 µL of n-hexanol was added and the flask shaken for 10 min. The hexanol layer was recovered by a first manual shaking and then a first centrifugation at 3000 rpm for 5 min. The organic phase and emulsion were then transferred to an Eppendorf tube and subjected to a last centrifugation at 11500 rpm for 5 min. The clean n-hexanol layer was transferred to a GC vial and stored at 4 °C prior to analysis by GC-FID. One microliter of each n-hexanol extract was analyzed with an Agilent 6890 N gas chromatograph equipped with a split injector (split ratio 73.6) maintained at 240 °C. Fatty acids were separated with a wall-coated open tubular (WCOT) polar CP-FFAP CB capillary column (25 m x 0.32 mm i.d., 0.3 µm film thickness). The carrier gas was nitrogen, and the pressure was set at 10 kPa. The oven temperature was programmed to start at 125 °C for 10 min and then to rise to 160 at 1.6 °C/min and finally to 250 °C at 15 °C/min; this temperature was held for 15 min. The column was connected to an FID (set at 250 °C with inlet of 21 mL/min H_2 and 210 mL/min air). For the nonanoic acid IST, the retention index (RI) was 1602.

The Liefmans Goudenband beer was again selected for standard addition procedure (curves further used for both Rodenbach beers).

HPLC analysis of iso-alpha acids

The *cis* and *trans* co- and n-isohumulones and tetrahydroisohumulones were analyzed in red and brown Flemish beers by HPLC-UV.[18]

Beer samples devoid of yeast were degassed by shaking and diluted twice in methanol. After 15 min, the mixture was filtered through a Chromatil polyester filter (0.45 µm, Macherey-Nagel, Düren, Germany).

Separation was performed on two C8 columns in tandem: Zorbax Eclipse XDB-C8 150 × 4.6 mm, 5 µm and Zorbax Eclipse XDB-C8 150 × 4.6 mm, 3.6 µm (Agilent Technologies, Santa Clara, CA, U.S.A.), with the binary solvent system of Analytica-EBC method 9.47 with A: methanol; B: 1% aqueous citric acid (pH 7.0): acetonitrile (70:30). Gradient elution was as follows: 15% A for 5 min, increasing A to 80% over 25 min, and keeping 80% A for 3 min. The column temperature was maintained at 35 °C, the flow rate at 1.0 mL/min, and the injection volume was 50 µL. Chromatograms were recorded throughout elution with the Empower software (Build 1154, Waters Corporation, Milford, MA, U.S.A.). The selected chromatogram reports the absorbance at 270 nm, characteristic of isohumulones and tetrahydroisohumulones (tetra). Calibration curves gave a common factor for isohumulones and another one for tetrahydroisohumulones.

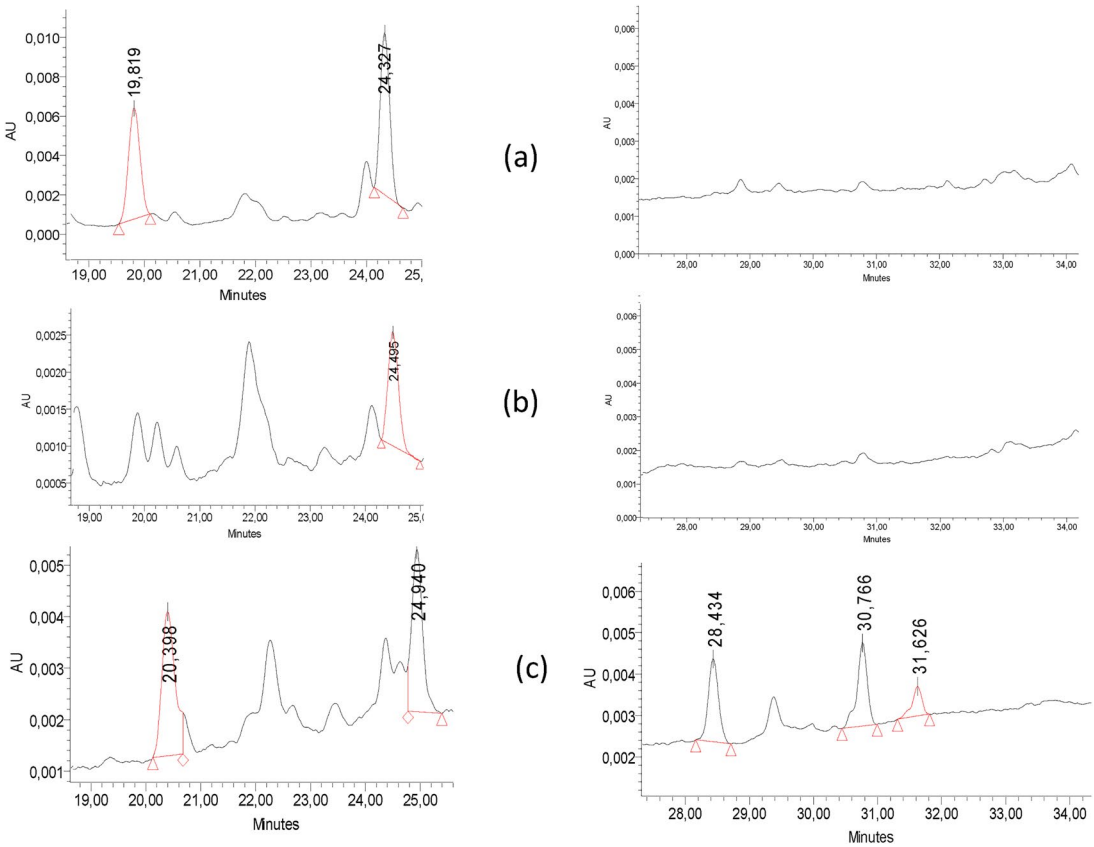

Figure 1. High performance liquid chromatography (HPLC) chromatograms of isohumulones (left) and tetrahydroisohumulones (right) for Rodenbach Grand Cru (a), Rodenbach Vintage (b), and Liefmans Goudenband (c).

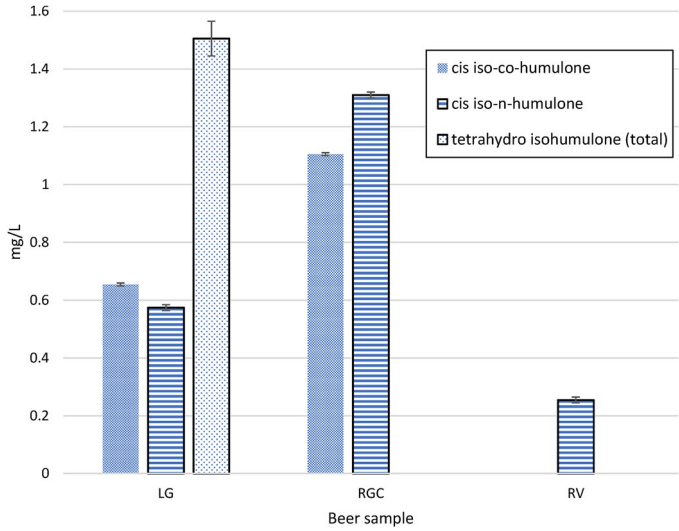

Figure 2. Concentrations of isohumulones and tetrahydroisohumulones in Liefmans Goudenband (LG), Rodenbach Grand Cru (RGC), and Rodenbach Vintage (RV).

Results

Iso-alpha acids

Before investigating the flavors of the brown and two red Flemish beers, HPLV-UV analyses were performed to compare their composition in terms of bitter compounds.

As depicted in Figures 1 and 2 and as expected, *cis*-iso-co-humulone (eluting at 20 min) and *cis*-iso-n-humulone (eluting at 24.5 min) were detected at low levels (both at 0.6 mg/L in LG and at 1.2 mg/L in RGC). No *cis*-iso-co-humulone was detected, and only 0.25 mg/L *cis*-iso-n-humulone was found in RV, aged for two years in an oak barrel and then for six years in a bottle. Similar measurements recently performed on three gueuze beers have shown the total absence of isohumulones in them.[18] The results for red/brown ales confirmed the low bitterness of these acidic beers, which was not surprising given the choice of old hops mainly to limit Gram + lactic bacteria during fermentation. In LG, 1.5 mg/L tetrahydroisohumulones (eluting at 28.4, 30.8 , and 31.6 min) had been added, most probably to improve its foam stability.

Phenols and lactones

Table 1 reports concentrations of a set of phenols and lactones in the red/brown beers investigated, as obtained by the SAFE-GC-MS procedure. In this table and the following ones, most of the perception thresholds in beer are taken from Appendix 1 in Miller.[19]

In the three beers investigated, 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG) were detected in the 54–384 µg/L range, usually considered in a brewery as markers of the presence of *Brettanomyces* in the fermentation process, because this yeast produces the enzyme vinyl phenol reductase catalyzing conversion of the vinyl form of the phenol to its ethyl form.[8,13] The concentrations of 4EP and 4EG were lower than in lambics, [280–1130] µg/L for 4EP and [520–5800] µg/L for 4EG.[13,20] The presence of *Brettanomyces* in Rodenbach beers is well documented: *B. lambicus* and *B. bruxellensis* have been identified and found to be active during the tertiary fermentation, which takes place for two years in oak barrels.[7] The higher concentrations of the ethyl forms in Rodenbach Vintage is likely related to the fact that it has aged for two years and that no young beer had been added, in contrast to Rodenbach Grand Cru, which is a blend with one-third young beer. In addition, the latter is filtered and pasteurized. Hence, *Brettanomyces* activity can continue in Rodenbach Vintage, which seems not to be pasteurized.

In both Rodenbach beers, the 4EG concentration was, as expected, higher than the 4EP concentration.[12] In LG, in which *Brettanomyces* are not active, the opposite was true (more 4EP than 4EG) with both compounds below their thresholds (150 µg/L for 4EP and 130 µg/L for 4EG). In that case, torrefied malt was most probably the main origin of the ethyl phenols.[21] This is in concordance with the presence of the unreduced form 4-vinylphenol (4VP) and 4-vinylguaiacol (4VG) found at higher levels in LG (8 and 7 µg/L against <3 and <8).

As depicted in Figure 3, both the *cis*- and the *trans*-oak lactone isomers were found in the beers investigated here (LG given as an example, by comparison with a standard mixture). Oak lactone totaled about 20 µg/L for the two isomers, far below their perception threshold of 150 and 830 µg/L for *cis*- and *trans*-oak lactone, respectively. The presence of oak lactone in Rodenbach can naturally be related to oak barrel aging, but the similar quantities found in Liefmans Goudenband are surprising. In the latter case, contact with oak wood does not occur during aging, because Liefmans beer is aged in stainless steel fermenters.

The presence of tabanone (5 isomers of megastigmatrien-3-one) in beers was also investigated, because this molecule is often found in wines aged in wood barrels. Tabanone results from wood toasting in barrel production

Table 1. Concentrations (µg/L) of phenols and lactones in Liefmans Goudenband (LG), Rodenbach Grand Cru (RGC), and Rodenbach Vintage (RV).

Compound	RI on CP- Sil5 CB	SIM ions	Flavor	Perception threshold in beer (µg/L)	Concentrations (µg/L)[c]		
					LG	RGC	RV
4-Ethylguaiacol	1261	137,152	Phenolic, leather, smoked	130	54.0	236.0	384.0
4-Ethylphenol	1140	107,122	Medicinal, horsy	150	123.0	100.0	159.0
4-Methylguaiacol	1180	123,138	Spicy, phenolic	20	0.3	0.3	0.4
4-Methylphenol	1046	107,108	Smoke, medicinal	20	0.6	0.8	1.7
4-Vinylguaiacol	1294	135,150	Clove	125	17.0	2.2	7.7
4-Vinylphenol	1198	91,120	Phenolic, medicinal	170	7.6	2.5	0.9
Acetovanillone	1455	151,166	Vanilla	500	20.5	22.9	26.0
cis-Oak lactone	1290	71,99	Coconut, woody	150	10.1	3.4	7.7
Eugenol	1335	149,164	Clove, honey, spicy	200	10.0	2.2	3.0
Guaiacol	1063	109,124	Smoke, sweet	70	2.6	2.8	4.4
Syringaldehyde	1638	167,182	Vanilla	500	312.0	38.0	87.5
Tabanone	[a]	133,190	Cigar, tobacco	n/a	n.d.	n.d.	n.d.
trans-Oak lactone	1259	71,99	Coconut, woody	830	8.1	3.8	10.0
Vanillin	1370	81,151	Vanilla	50	38.0	11.8	82.7
γ-Decalactone	1439	85,128	Coconut	400	1.6	1.8	1.1
γ-Nonalactone	1331	85,100	Coconut	30	59.2	73.0	79.0
δ-Decalactone	1467	99,114	Peach	100[b]	1.3	2.3	2.0
δ-Dodecalactone	1688	71,99	Olive, apricot	10	2.3	3.4	2.7

RI = retention index; n.d. = not detected; n/a = not available

[a] Tabanone had five isomers with RI 1554, 1569, 1571, 1606, and 1619.

[b] The perception threshold of δ-decalactone is in water.

[c] For the concentrations listed, the coefficients of variation between duplicates (triplicates for Liefmans Goudenband) are below 12%.

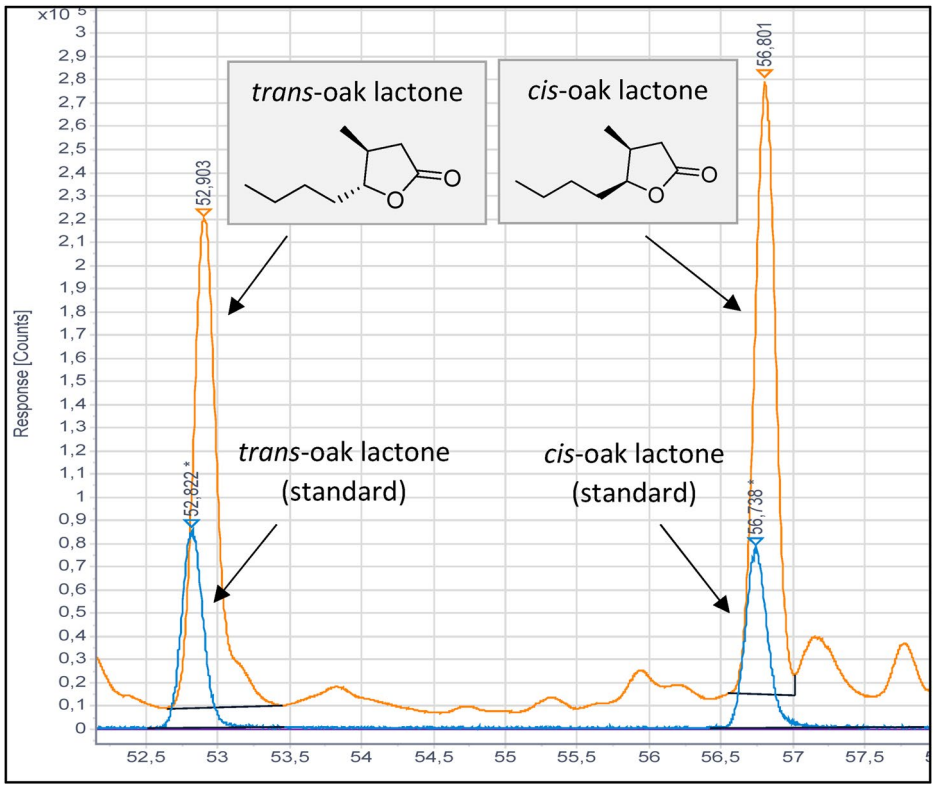

Figure 3. Gas chromatography-mass spectrometry (selective ion monitoring) [GC-MS(SIM)] chromatograms of *cis* and *trans*-oak lactones in Liefmans Goudenband (LG) beer compared with commercial standards.

and is probably produced by degradation of carotenoids.[22] The SIM GC-MS approach did not reveal any tabanone in any of the investigated beers, including Rodenbach beers, in which contact with oak wood is long.

Conversely, all the typical phenols issued from thermal degradation of wood (vanillin, acetovanillone, syringaldehyde, and eugenol[23]) were evidenced in both Rodenbach beers. Some of these, more surprisingly, were even found in Liefmans Goudenband (possibly issued from special

malts). The vanillin concentration, especially in RV (83 µg/L), was probably partly responsible for the wood flavor found in this beer.

Lastly, guaiacol (3–4 µg/L) and 4-methylphenol (1 µg/L) were found far below their thresholds in all three beers. The presence of these compounds has been related to the use of special malts.[21] Only torrefied malts allow reaching above-threshold levels in beer.

Aliphatic esters and alcohols

Data on esters and alcohols in the three investigated beers are gathered in Table 2 (headspace, SAFE, or n-hexanol extraction, according to the compound).

Ethyl acetate (headspace technique) was found at concentrations ranging from 100 mg/L (LG) to 237 mg/L (RV), significantly higher than in traditional top fermentation beers (8–48 mg/L) and filtered gueuzes (33–67 mg/L) but similar to the levels found in unfiltered gueuzes (61–167 mg/L).[11] These values were expected in the presence of *Brettanomyces*, as mentioned by Snauwaert et al.[6]

Conversely, isoamyl acetate, which is typically around 1–4 mg/L in top fermentation beers,[24] was found here at lower concentrations in both Rodenbach beers (0.3–0.4 mg/L). Probably because of a much lower level of *Brettanomyces*, a more usual value (1.73 mg/L) characterized the LG beer.

Isoamyl alcohol was found at 81–170 mg/L in all beers. These data are in good agreement with previous measurements on Rodenbach beer,[6] in which isoamyl acetate was found at a slightly higher level than measured here (0.88 mg/L), while the isoamyl alcohol concentration was 79 mg/L.

The n-hexanol extraction-GC revealed a similar trend for 2-phenylethyl acetate and its corresponding alcohol. The compound 2-phenylethanol was found in both the Rodenbach and Liefmans beers, at similar levels (35–42 mg/L), while 2-phenylethyl acetate was absent from both Rodenbach beers but occurred at 0.09 mg/L in Liefmans beer.

Snauwaert et al.,[6] who also investigated Flemish sour beers matured in oak barrels (including, most probably, Rodenbach but not Liefmans), found no 2-phenylethyl acetate (in agreement with our observation) nor any trace of 2-phenylethanol.

The low isoamyl acetate and 2-phenylethyl acetate concentrations observed in the Rodenbach beers are well-documented signs of the action of *Brettanomyces* esterases, which degrade them and hence indirectly favor ethyl acetate.[8,13]

Ethyl hexanoate, ethyl octanoate, and ethyl decanoate were analyzed by the headspace procedure. Typical values were obtained, reflecting the primary *Saccharomyces* fermentation, just above the corresponding perception thresholds.

Interestingly, the mushroom/earthy/musty odor-like oct-1-en-3-ol (200 µg/L) reached its threshold in LG beer. This compound, formed by enzymatic degradation of linoleic acid, is typically produced by many fungal species and is often considered as an off-flavor.[25] Its presence could be due to a natural inoculation of fungi along with the inoculation of yeasts and lactic acid bacteria in the open copper casks.

Short chain fatty acids

Cheesy isovaleric acid can be synthesized by *Brettanomyces* through L-leucine metabolization. It is known as the *Brettanomyces* flavor in wine.[13] It could also increase the overall perception of volatile phenolic compounds. With 1 mg/L in LG and RGC and 1.5 mg/L in RV, these values

Table 2. Concentrations (mg/L) of aliphatic esters and alcohols in Liefmans Goudenband (LG), Rodenbach Grand Cru (RGC), and Rodenbach Vintage (RV).[a]

				Concentrations (mg/L)[c]		
Compound	RI[b]	SIM ions	Perception threshold mg/L in beer	LG	RGC	RV
Ethyl acetate**	577	61, 70	30.00	101.50	132.00	237.00
Ethyl butanoate (butyrate)*	741	71, 88	0.40	0.01	0.02	0.01
Ethyl hexanoate (caproate)**	975	88, 99	0.21	0.28	0.13	0.21
Ethyl heptanoate*	1079	88, 113	0.40	n.d.	0.002	n.d.
Ethyl octanoate (caprylate)**	1181	88, 127	0.90	0.18	0.33	0.37
Ethyl decanoate (caprate)**	1382	88,101	1.50	n.d.	0.06	0.03
Isobutyl acetate*	792	56, 73	1.60	1.05	1.60	4.26
Isoamyl acetate**	853	70, 87	1.20	1.73	0.39	0.32
2-Phenylethyl acetate***	1267	–	3.80	0.09	n.d.	n.d.
Isoamyl propionate*	952	57, 70	0.70	0.09	n.d.	n.d.
Ethyl leucate*	1039	69, 87	n/a	0.16	0.38	0.59
Propan-1-ol**	557	42, 59	800	6.41	13.40	53.70
Isobutanol **	603	43, 74	200	52.45	8.70	1.10
Isoamyl alcohol**	728	55, 70	70	169.28	81.10	115.90
Hexan-1-ol *	849	56, 69	400	n.d.	n.d.	0.01
Oct-1-en-3-ol *	962	57, 72	0.20	0.20	n.d.	n.d.
2-Phenylethanol***	1387	–	50.0	42.30	38.10	35.20

RI = retention index; SIM = selected ion monitoring; n/a = not available.

[a] Data was obtained by solvent-assisted flavor evaporation-gas chromatography-mass spectrometry (SAFE-GC-MS) (*), headspace GC-MS (**), or n-hexanol extraction GC-flame ionization detector (FID) (***).

[b] RI was obtained on a CP-Sil5 CB column for SAFE and headspace extractions and on a FFAP CB column for n-hexanol extractions.

[c] For the concentrations listed, coefficients of variation between duplicates were below 25%.

are in the range of those found in usual top fermented beers, where this compound can be issued from bitter iso-humulones. In lambic beers, values up to 2–3 mg/L have been recorded (Table 3).

Conversely, hexanoic acid was found at much higher concentrations (10–12 mg/L) than is usual in top fermented beers (2–4 mg/L),[24] and it was well above its odor threshold of 8 mg/L.

Although less obvious, the same trend was seen for octanoic acid (5–6 mg/L) and decanoic acid (up to 2 mg/L in LG), which are more commonly found, respectively, in the range of 3–4 mg/L and <1 mg/L.

Flavors from malt

SAFE-GC-MS was used to detect a wide variety of volatiles issued directly from malt (Maillard and Strecker reactions) and transferred to the beer as such or issued from non-enzymatic reactions involving precursors from malt.

The LG beer was found to contain the largest proportion of pyrazines. This can be related to the use of torrefied malts and also to its color (109 EBC vs. 80–90 EBC for Rodenbach beers). Yet even in the case of LG, they remained below their sensory thresholds (Table 4).

Conversely, many oxygen heterocycles including furfural (62–562 µg/L), 5-hydroxymethyl-furfural (1–434 µg/L), maltol (4453 µg/L in LG), and furaneol (39–122 µg/L) are often found above their thresholds. Among them, all were found to be more concentrated in RV beer, except maltol, which reached its sensory threshold only in LG, and furaneol, which can arise through maltol metabolism.

Among the Strecker aldehydes, 2-phenylacetaldehyde was found above its threshold in RGC beer only (204 µg/L).

Table 3. Concentrations (mg/L) of short chain fatty acids in Liefmans Goudenband (LG), Rodenbach Grand Cru (RGC), and Rodenbach Vintage (RV).

			Concentrations (mg/L)[a]		
Compound	RI on FFAP CB	Perception threshold in beer (mg/L)	LG	RGC	RV
Isovaleric acid	1028	1.5	0.98	0.96	1.53
Pentanoic acid	1148	8.0	0.27	n.d.	n.d.
Hexanoic acid	1309	8.0	10.03	11.28	11.8
Octanoic acid	1521	13.0	5.93	6.17	5.56
Decanoic acid	1671	10.0	2.02	1.29	0.71

RI = retention index; n.d. = not detected.
[a] For the concentrations listed, the coefficients of variation between duplicates were below 10%.

Conclusion

The volatile composition of Liefmans Goudenband and two Rodenbach beers was studied. Overall, the data indicated that the volatiles of these beers were quite similar qualitatively and often quantitatively. This was likely to be due to the similar nature of the inoculum (*Saccharomyces* yeast and lactic bacteria) launching the fermentation processes and the use of torrefied malt in the former, which partially balanced the absence of *Brettanomyces* and wood. Yet, the 4EP and 4EG concentrations were below their corresponding perception thresholds in Liefmans Goudenband, with, moreover, an opposite ratio between 4-ethylphenol/guaiacol. The absence of *Brettanomyces* in LG was also reflected by less acetic acid, and a lesser decrease in isoamyl acetate and 2-phenylethyl acetate.

The presence of oak lactone in LG at levels similar to those observed in Rodenbach beers was unexpected, even though their concentrations were below the corresponding perception thresholds. Oak lactone precursors could also be issued from torrefied malts.

Lastly, the more pronounced caramel, coffee malt odors of the LG beer were consistent with the above-threshold maltol and furaneol concentrations, significantly higher than in the Rodenbach beers. The Vintage version of the latter, in contrast, was found to contain more furfural and 5-methylfurfural.

To confirm these preliminary results, future research with brewing tests should investigate individual effects of *Brettanomyces* and torrefied malts on standardized products.

Table 4. Concentrations (µg/L) of special-malt-derived volatiles in Liefmans Goudenband (LG), Rodenbach Grand Cru (RGC), and Rodenbach Vintage (RV).

				Concentrations (µg/L)[b]		
Compound[a]	RI on CP-Sil5 CB	SIM ions	Perception threshold in beer (µg/L)	LG	RGC	RV
2,3-Dimethylpyrazine [+]	899	67, 108	800	n.d.	2.3	5.0
2,3,5-Trimethylpyrazine	980	81, 122	830	5.6	2.7	7.7
2,5-Dimethylpyrazine [+]	894	81, 108	3000	13.9	n.d.	6.1
2,6-Dimethylpyrazine	893	108, 109	3000	29.9	13.6	13.8
Furfuryl alcohol [+]	830	97, 98	3,000,000	200	72	46
Furfural	810	95, 96	15	62	55	562
5-Methylfurfural	931	109, 110	1174	67	27.5	216
5-Hydroxymethylfurfural [+]	1188	97, 126	35,000	1.2	26.5	434
Maltol	1087	71, 126	3000	4453	2381	2711
Furaneol [+]	1025	57, 128	30	122	46	39
Methional	872	76, 104	15	6	10	10
Benzaldehyde	938	77, 106	515	3.9	27	3.3
2-Phenylacetaldehyde	1018	91,120	105	42	204	23

RI = retention index; SIM = selected ion monitoring; n.d. = not detected.
[a] For unavailable compounds, concentrations are given in IST equivalent concentrations (these cases are marked as[+]).
[b] For the concentrations listed, the coefficients of variation between duplicates were below 40%.

Disclosure statement

The authors report there are no competing interests to declare.

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