Brewing American Lambic: Small Molecule Chemistry of Spontaneously Fermented Coolship Beers

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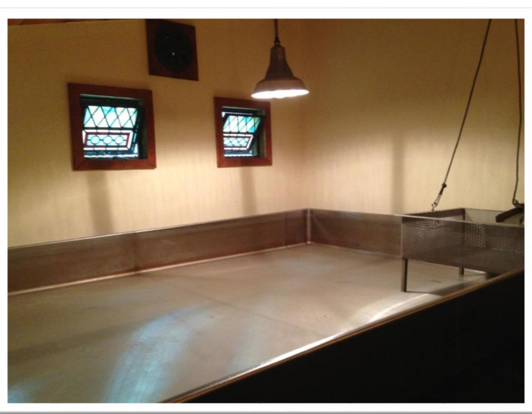
Background

Spontaneously fermented beer has been an integral part of the Belgian brewing tradition for many hundreds of years, and has given rise to a diverse family of Lambic beer, including Gueuze, Kriek, Framboise, Faro and Flanders ales. These beers, share the characteristic dry acidity derived from the "spontaneous" fermentative organisms involved in their production.

Production of many of these beers involves the overnight cooling of unfermented beer (wort) in large, open coolships. During this time the wort is organically inoculated with wild microbes including *Saccharomyces spp., Brettanomyces spp., Acetobacter spp.* and many members of the *Lactobacillaceae.* Over the course of months to years, these wild microbes are responsible for the production of numerous, small flavor-active compounds, all of which contribute to the complex nature of these beers.



Typical Coolship design. Photo Credit: M. Farrell.

Spontaneously fermenting beer at Brewery Timmermans

The microbes involved in fermentation follow a relatively stable pattern (Figure 1). It is therefore likely that studies of the development of the chemistry of coolship ales over time can give insight into the general microbial population of a spontaneously fermented ale. This is possible by tracking and quantifying molecules with specific microbial origins.

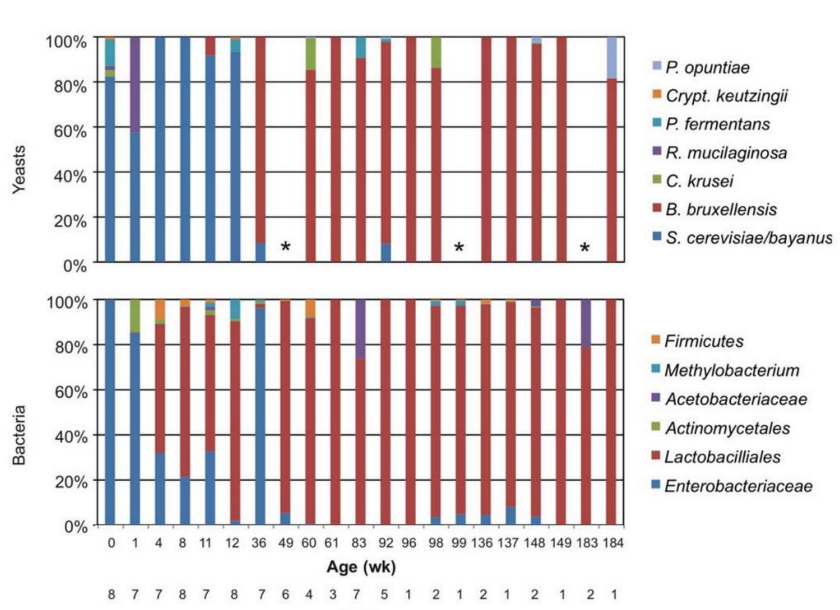


Figure 1: Relative population proportions of yeast (top) and bacteria (bottom) found in the coolship ales produced by the same brewery involved in this study. Figure adapted from Bokulich, N. A. et. al. (2012).

Perhaps the most important flavor-active compounds found in Lambic are the organic acids that contribute to the unique and distinguishing tartness all Lambic beers share. Generally speaking, the majority of the acidity is derived from lactic acid, produced by Lactic Acid Bacteria (LAB), though acetic, succinic, citric and malic acids can contribute to the acids present in a Lambic.

Recently, the explosive diversification and growth of the craft brewing industry in the United States has led to the creation and expansion of the American Wild Ale (AWA) style, which includes beers brewed in a manner nearly identical to that of the traditional Belgian Lambic, excepting geographical differences. In this project, quantitative ^{1}H-NMR methodology and multivariate discriminate analysis was used to investigate and quantify the key "macro-chemistry" of typical American Wild Ales as finished products and over the course of their fermentation. This data was then used to draw conclusions about previously studied microbiological populations[1] participating in the fermentation.

Methods

- Beer was acquired directly from a domestic brewery.
- Vol. =175µL (straight runs) & 500µL (lyophilized)
- Samples were run on a Mercury-VX 300 spectrometer operating at 299.681 MHz.
  - Spectral Parameters: pw=67.5°, d1=5s, at=7s, T=27°C
  - nt=256 (straight runs) / nt=128 (lyophilized)
- Spectra processed in Mnova & ACD
- Chemometrics performed in Eigenvector

Spectra and Assignments

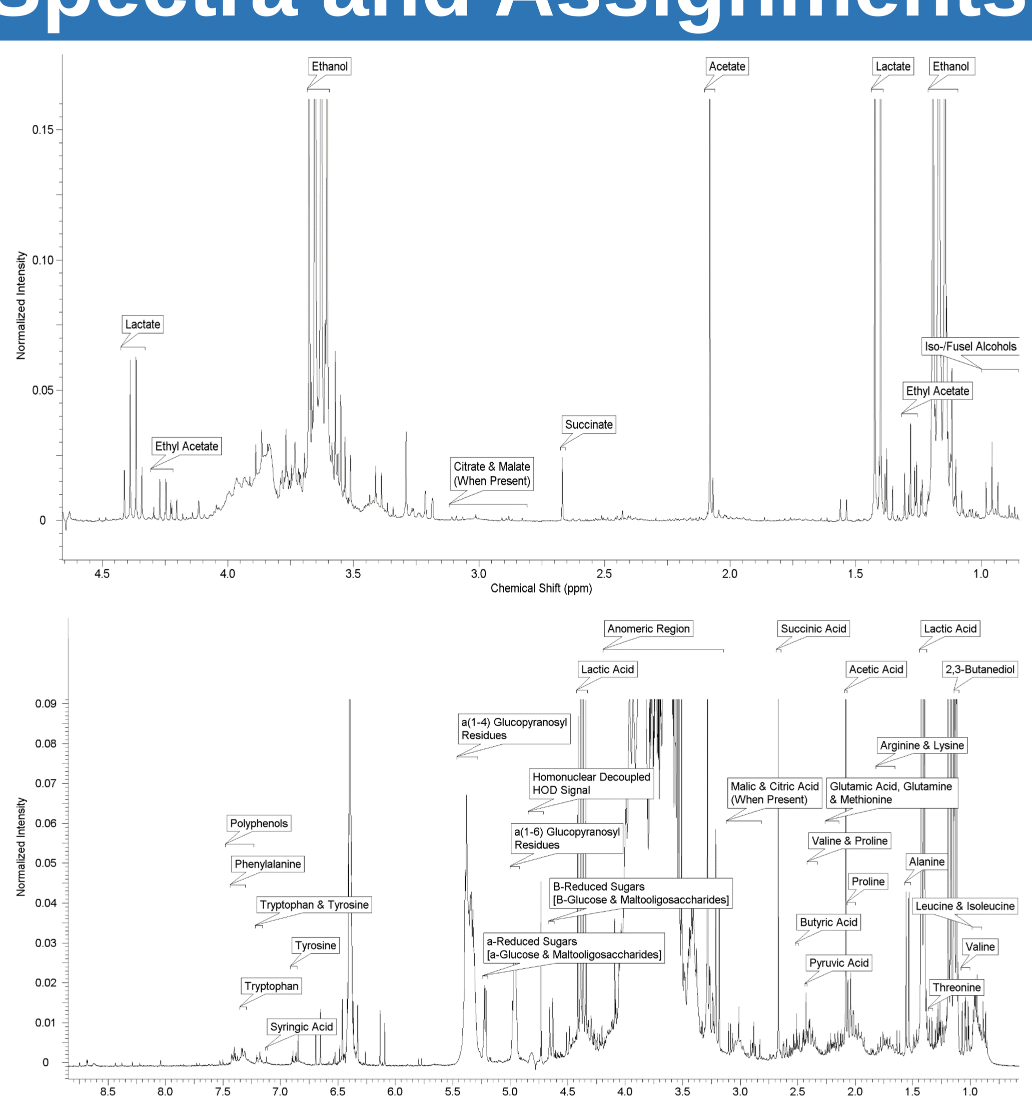


Figure 2: "Straight" analysis (top) and lyophilized analysis (bottom) of an American Gueuze-style beer with peak assignments. Straight analysis samples were used for the quantification of volatile compounds (lactic acid, acetic acid, and ethyl acetate)

Results

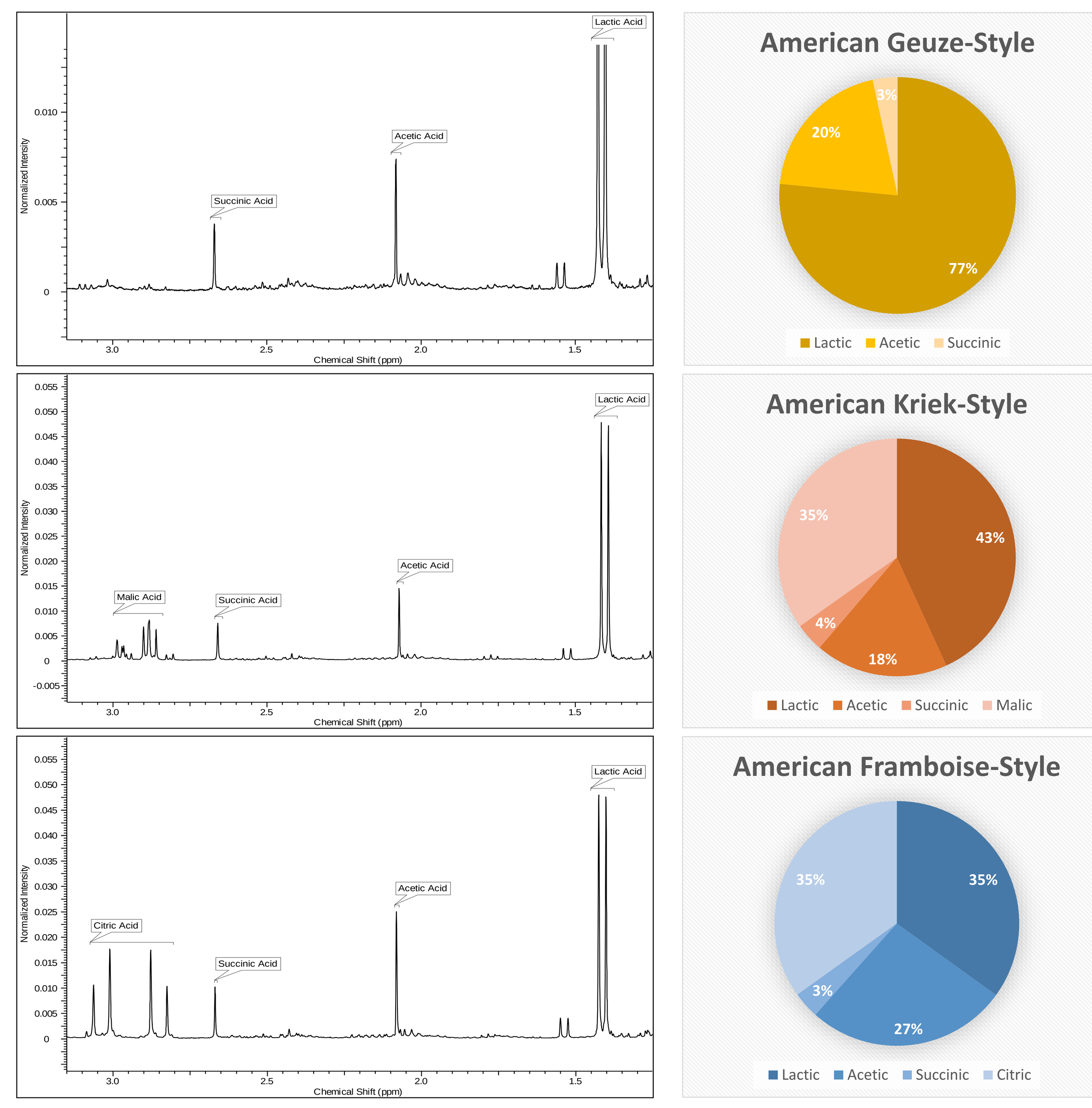


Figure 3: Acid regions and relative acid proportions of 3 different styles of AWA. A Gueuze-style beer (top) is a blend of one, two and three year old beer. A Kriek-style beer (middle) is a 2-year old beer that has been aged on cherries (a high malic acid fruit) for 4 months. A Framboise-style beer (bottom) is a two year old beer that has been aged on raspberries (a high citric acid fruit) for 4 months.

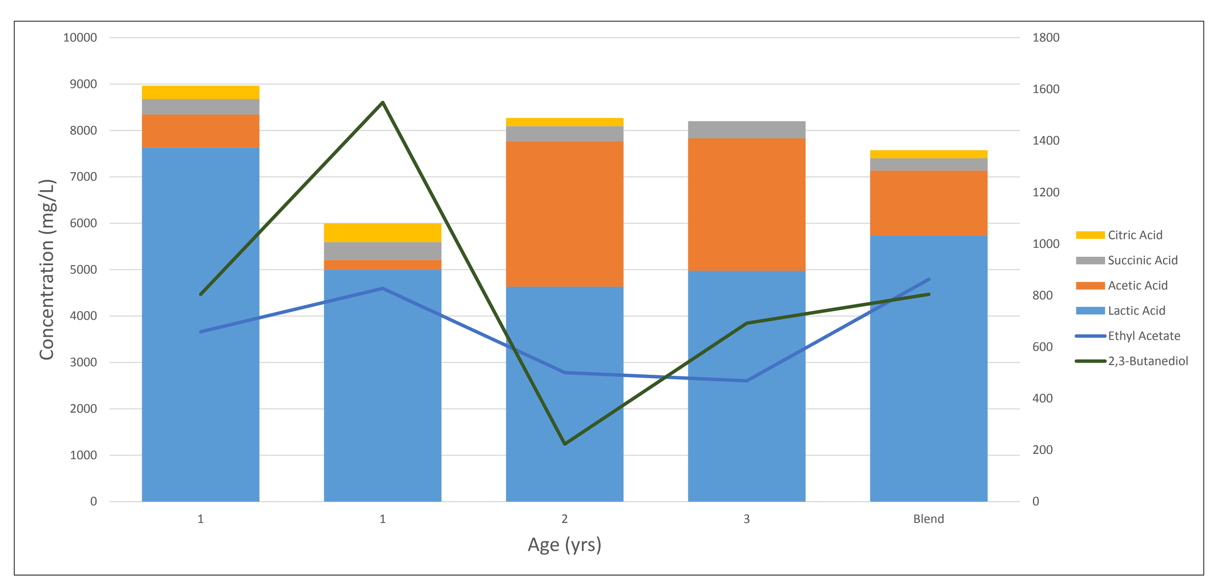


Figure 4: Absolute concentrations of major components found in AWA (Acids: left y-axis, Others: right y-axis). The age of the beers refers to the fermentation time (in years) for the respective barrels sampled, as all samples came from separate batches. The 1 & 3 year old barrels were brewed in the Winter (November) and the 2 year old barrel was brewed in the Spring (May). Blend refers to the finished Gueuze-style AWA (Figure 3, top).

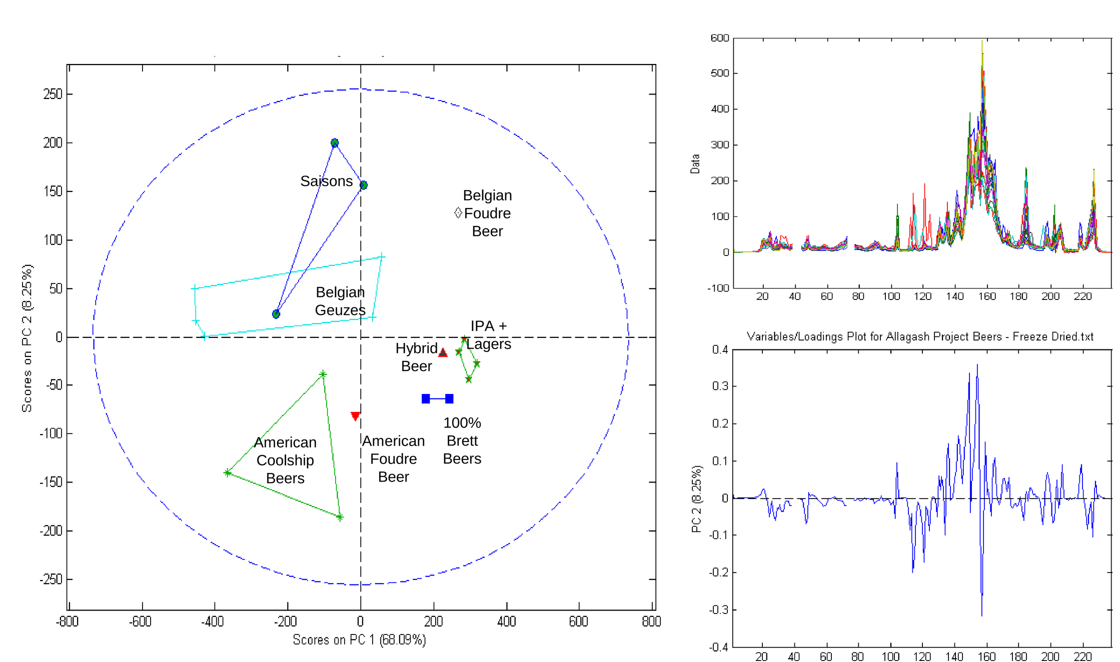
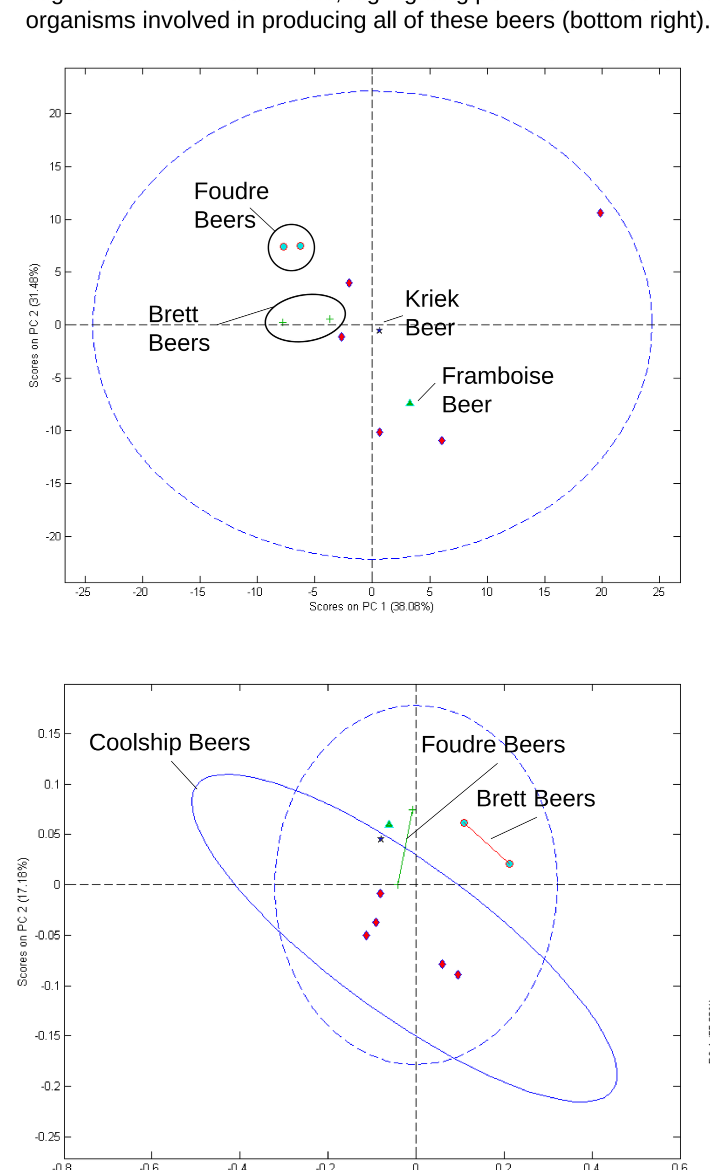


Figure 5: Results of Principal Component Analysis of 20 individual beers spanning 8 separate styles. Though only ~76% of the variance is explained in PCA-1 & PCA-2, samples segregate effectively by style (left). Integrals were taken from 0.5-9.5 ppm to every 0.02ppm, excluding ethanol, lactic acid, acetic acid and maleic acid (qNMR standard) and normalized to 1000. For this PCA the aromatic region was also excluded (~5.7ppm-9.5ppm) (top right). Loadings on PCA 2 indicate discrimination based on sugars and non-volatile acids, highlighting potential metabolic differences between the variety of organisms involved in producing all of these beers (bottom right).



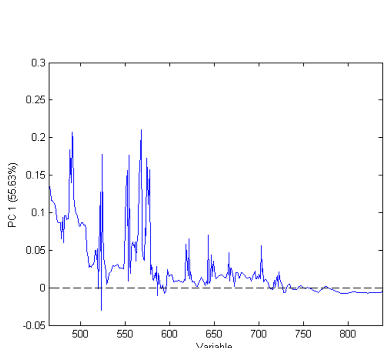


Figure 6: PCA Analysis of AWA based on whole spectra (top left). Whole spectra were correlated based on all resonances excluding ethanol. Correlations based on the whole spectrum were strongly influenced by maltodextrin and lactic acid resonances. Unlabeled data points (diamonds) represent coolship beers. Circles drawn are for labelling purposes only, and are not statistically significant. Dotted line indicates the 95% confidence limit. Correlation of the aromatic region yielded a much more effective separation of samples (bottom left). Unmarked samples represent Framboise Beer (triangle) and Kriek Beer (star). The strongest correlations for the aromatic analysis were due to aromatic amino acids (i.e. Phenylalanine) and polyphenolic resonances (below).

Conclusions

The majority of the acidity in an AWA is derived from the three main acids: Lactic, Acetic and Succinic acids, and the addition of fruit is apparent in the spectra, as organic acids characteristic of respective fruits are obvious (Figure 3).

As demonstrated in Figure 4, raw quantitative analysis of AWA over the course of fermentation demonstrates the large variance found in spontaneously fermented ales. It is easy to infer that the method of brewing is authentic from this data alone.

- Varying levels of 2,3-butanediol (a flavor-neutral product of enteric bacterial fermentation) is indicative of the relative population size of *Enterobacteriaceae* in AWA.
  - These bacteria were found in large numbers at the beginning of AWA fermentation (Figure 1), and are commonly found in all Lambic-style fermentations[2].
  - An increased amount of 2,3-butanediol means less simple sugars were available for fermentation into acids, which would partially explain the trends observed in acid vs. 2,3-butanediol concentrations observed in Figure 4.
  - The lowest level of 2,3-butanediol was observed in the beer brewed in the Spring. This could potentially give insight into the relative number of ambient *Enterobacteriaceae* in Spring vs. Winter.
- Between year 1 & 2, the amount of acetic acid increases dramatically.
  - This is most likely due to the increase in the relative proportion of *Brettanomyces spp.* (Figure 1), which is an oxidative yeast capable of producing large amounts of acetic acid[3].
  - As fermentation continues, oxygen will slowly diffuse through the porous aging vessels (oak barrels)[4], providing a substrate for the oxidation of ethanol to acetic acid.
    - Based on this data, it would seem that after one year, the CO_2 "cushion" caused by active fermentation is reduced, facilitating oxygen diffusion & acetic acid formation.
  - Because of the powerful flavor potential of acetic acid[5], blenders may choose to minimize the concentration by mixing in larger proportions of younger beers (Figure 4, Blend), though this is entirely qualitative from the perspective of the blender.
  - The absence of acetic acid in one year old beer is indicative of a microbial *terroir* low in *Acetobacteriaceae*, which is ideal in the production of sour alcoholic beverages. This assumption is confirmed by the previous study (Figure 1).
- Though no trend was obvious through ethyl acetate (EtAc) quantitation, it is known that EtAc in beer decreases in concentration over time[6].
  - Because EtAc is the most common ester found in beer, and is responsible for imparting a "fruity" aroma[7] it is obvious from the data that EtAc plays an important role in sensory perception of AWA, as the highest concentration of EtAc was found in the blended product.
    - It is also possible that the increased EtAc levels are due to the bottle conditioning of AWA.
- No obvious trends were observed in citric or succinic acids.
- PCA analysis of full lyophilized spectra successfully discriminated 20 individual beers across 8 styles
  - Correlations were dependent mostly on sugars, as volatiles (acetic acid, lactic acid & ethanol) were partially lost during freeze drying, and were thus excluded.
- PCA analysis of AWA based on whole spectra successfully separated 4 of the 5 styles, but couldn't separate coolship ales from the other styles.
- PCA analysis of the aromatic region of AWA successfully separated all styles, including the coolship ales.
  - These resonances are currently largely unassigned, but include amino acids (phenylalanine, histidine etc.), flavonoids (catechin), polyphenols & benzoic acids (hydroxycinnamic acids, syringic acid etc.).
  - Correlations in this region could potentially be caused by pH dependent peak movement, but differences between samples was minimal.
  - Coolship beers occurred closest to Foudre beers (brewed with mixed *Lactobacillus* culture), demonstrating that the majority of the variation within the aromatic region is due to contributions by resident prokaryotes.
    - Because Brett Beers (brewed with 100% *Brettanomyces* culture) were more divergent (relative to Coolship & Foudre beers), it is safe to assume the majority of aromatic discrimination was not due to the activity of resident yeasts.

Future Goals & Acknowledgements

The future of this research project will involve the expansion of spectra and data sets to further explain the chemistry of AWA production.

- Collect samples from single barrels as a time-course study, as samples from multiple barrels demonstrate the exciting variability in AWA fermentation.
- Collect samples from multiple barrels of identical batches and use PCA to examine non-evident barrel-to-barrel divergences.
- Collect samples from separate batches and use PCA to discriminate samples based on brewing season.
- Expand these methods to study the overall process of brewing traditional beers (on-going)
  - "From Mash to Bottle: Application of NMR to the Brewing Process"
  - In conjunction with Yard's Brewing Company, Philadelphia, PA.

- **I would like to thank the brewers associated with this project for furnishing samples, and John C. Edwards & Edward R. Zartler for having an acceptable taste in fine ales and the patience to teach a molecular biologist the ropes of NMR.**

References:
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