Sweet n’ Sour: How Kombucha Analytes Impact Flavor

Presented by Keisha Harrison, MS

Department of Food Science & Technology
Department of Microbiology
Center for Genome Research and Biocomputing

9/18/2020

Oregon State University

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Fermentation Microbiology Research
Curtin Lab, Oregon State University

Cheese

- Spoilage (molds)

Wine

- Spoilage (*Brettanomyces*)

Beer

- Consistency
- Quality
- Differentiation (terroir?)

Kombucha

- Consistency
- Quality
- Differentiation (terroir?)

Fermentation Research Tools at OSU

To learn about ***culture conditions***, we use **lab-scale fermentations** and **culture-based methods**

To learn about ***product composition*** *and* ***fermentation chemistry,*** we use **enzymatic assays** and **nuclear magnetic resonance [NMR] spectroscopy**

To learn about ***culture identification***, we use **amplicon and shotgun sequencing.**

Curtin Lab Publications [from 2019-]

Barrel-Induced Variation in the Microbiome and Mycobiome of Aged Sour Ale and Imperial Porter Beer

Avi Shayevitz*, Keisha Harrison, and Chris D. Curtin

Department of Food Science and Technology, Oregon State University, Corvallis, OR, U.S.A.

ABSTRACT

Traditional styles of beer that undergo spontaneous fermentation and/or maturation in oak barrels are becoming increasingly popular amongst craft beer consumers. These products are costly to produce, partly because some barrels develop undesirable flavor profiles and are excluded from the final blend. As a first step towards understanding variation in beer quality that occurs during barrel maturation, next-generation sequencing was used to profile the bacterial microbiome and fungal mycobiome of three beers; an American Coolship Ale (ACA), an aged sour ale, and an Imperial Porter. For the aged sour ale, produced across three years, it was observed that while there were significant differences in key lactic and acetic acid bacterial genera according to beer age, within each production batch there was substantial barrel-induced variation in microbiome profiles. Similarly, for the Imperial Porter significant differences were observed in the relative abun-

KEYWORDS

brewing; ecology; fermentation; microbiology; microbiome; mycobiome

Commercial *Saccharomyces cerevisiae* Yeast Strains Significantly Impact Shiraz Tannin and Polysaccharide Composition with Implications for Wine Colour and Astringency

Keren A. Bindon [1,*], Stella Kassara [1], Mark Solomon [1], Caroline Bartel [1], Paul A. Smith [1,2], Alice Barker [1] and Chris Curtin [1,3]

1 The Australian Wine Research Institute, Hartley Grove, Urrbrae, Adelaide, SA 5064, Australia
2 Wine Australia, Industry House, Corner Hackney and Botanic Roads, Adelaide, SA 5000, Australia
3 Department of Food Science and Technology, Oregon State University, 232B Wiegand Hall, Corvallis, OR 97331, USA
* Correspondence: keren.bindon@awri.com.au; Tel.: +1-541-737-1599

Received: 31 July 2019; Accepted: 3 September 2019; Published: 9 September 2019

Abstract: To gain knowledge on the role of *Saccharomyces cerevisiae* yeast strains (and their hybrids) on wine sensory properties, 10 commercially available yeast strains were selected on the basis of their widespread usage and/or novel properties and used to produce Shiraz wines. Significant differences

The Impact of This Research

Through these projects, we aim to address…

Issues with undesired ethanol concentrations

Issues with phenolic and other off-flavors

Issues with stuck/slow fermentations

Leading to

Improvements in batch-to-batch consistency

Improvements in scale-up approaches

Improvements in predictability of fermentation outcomes

NMR Analysis Used to Investigate the Following Topics

- Analyte-Development During Fermentation
- Small-Scale Brand Comparison
- Style Development
- Analysis of Hard Kombucha Analytes
- Comparison of Hard Kombucha to Alcoholic Beverages

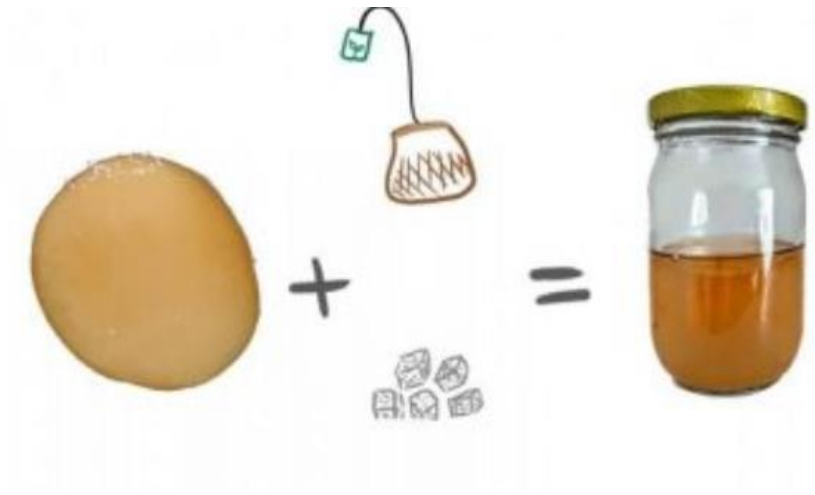
Summary of Kombucha Analyte Experiments

Nuclear magnetic resonance (NMR) is a powerful technique for structure determination. Hence it can also provide better understanding of the complex structures in complicates systems, such as Kombucha.

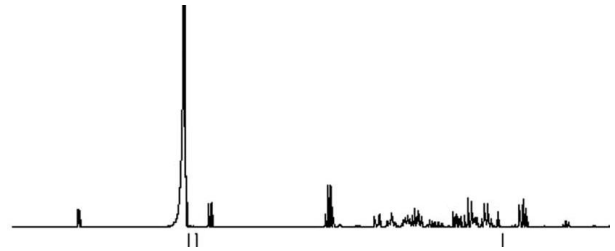
BACKGROUND

Commonly Kombucha Analytes

SNAPSHOT: Kombucha Chemical Composition



Starter culture
Sugar (sucrose, dextrose, fructose)
Tea
Controllable aeration



	Company A	Company B
Acetic acid (g/L)	3.19	0.18
Ethanol (g/L)	35.14	0.05
Ethanol (v/v) %	4.50	0.01
Fructose (g/L)	0.00	0.57
Gluconic acid (g/L)	0.64	0.08
Glucose (g/L)	0.00	0.50
Glycerol (g/L)	2.67	1.62
Lactic acid (g/L)	0.13	0.01
Sucrose (g/L)	0.24	5.79

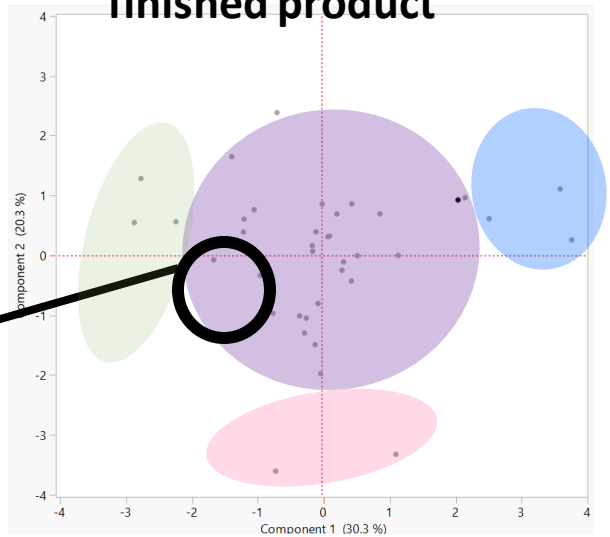
Varied Commercial Kombucha Composition

RESULTS

KBI Analyte Study (n= 39)

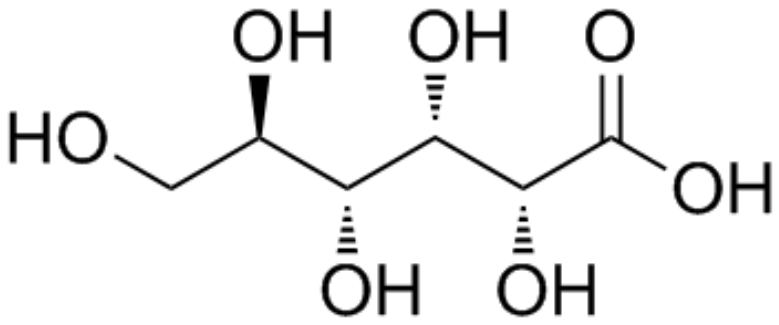
- Sucrose [g/L]….. **0.0 – 42.7**
- Fructose [g/L] ….. **0.1 – 42.9**
- Glucose [g/L] …. 0.3 – 38.7
- Caffeine [mg/L] ….. **0.0 – 66.4**
- Ethanol [v/v%] …. **0.01% - 1.51%**
- Acetic acid [mg/L] ….. **0.0 – 5.9**
- Lactic acid [g/L] ….. **0.0 – 3.2**
- Gluconic acid [g/L]….. **0.1 – 24.0**

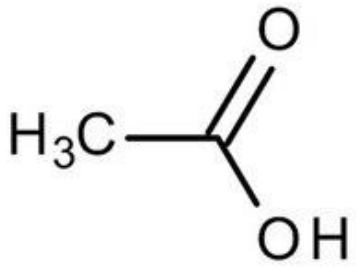
Assuming that we consider the "typical" finished product

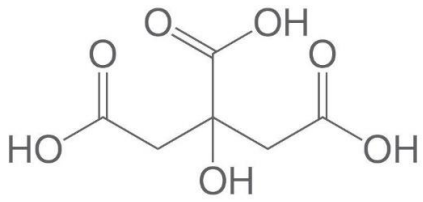


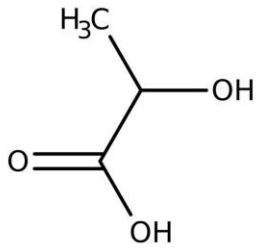
The majority of Kombucha samples group together. Can we define Kombucha by range of fermentation byproducts?

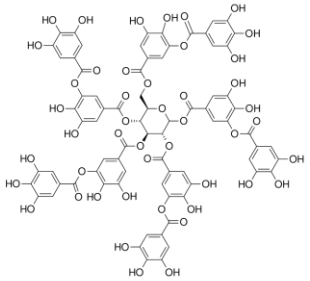
BACKGROUND

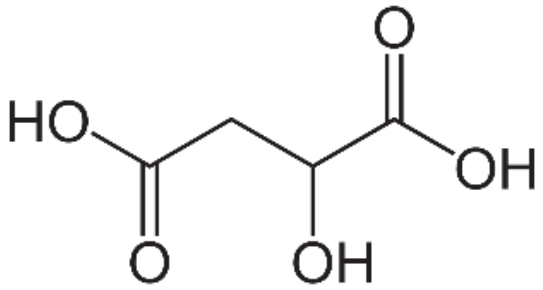












Organic acids that contribute to flavor

- Citric acid, 60mg/l
- Gluconic acid
- Mallic acid
- Tannic acid
- Acetic acid
- Lactic acid
- Gallic acid

BACKGROUND

Organic acids that contribute to flavor

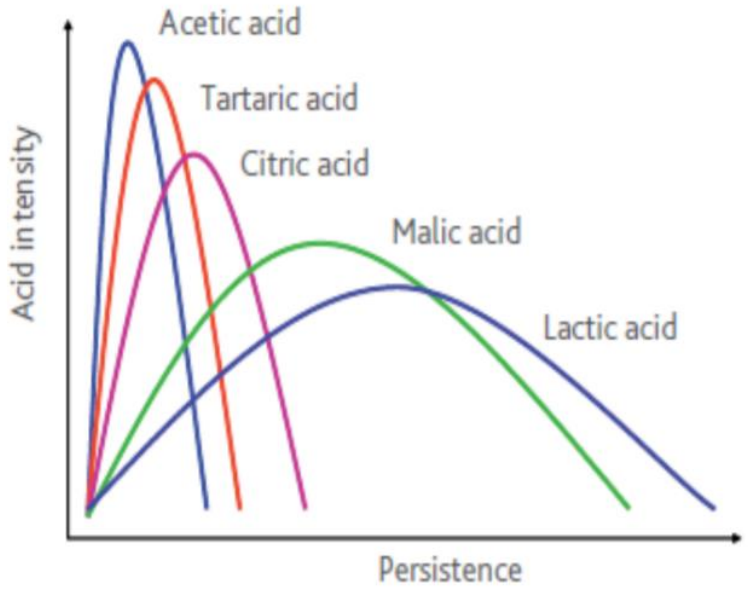


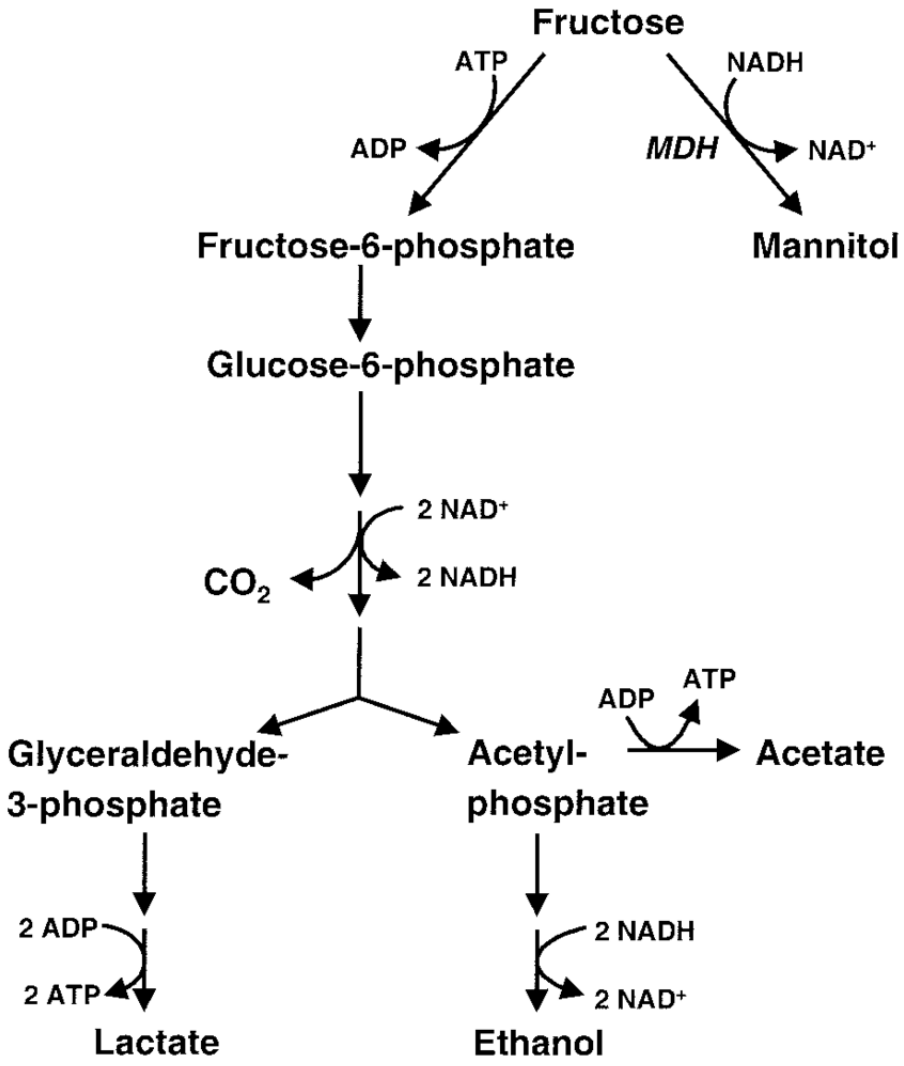
Figure 1.8. Effect of the acids on mouthfeel sensations: Intensity and Persistence. Source: Laffort. Tools acidification in Musts and Wines.

- Citric acid: tart, sour, citrus
- Gluconic acid: sweet, mild acidic bite
- Malic acid: smooth, tart
- Tannic acid: bitter
- Acetic acid: vinegary, sour
- Lactic acid: sharp, tart, crisp
- Gallic acid: astringent

BACKGROUND

What's known about organic acids

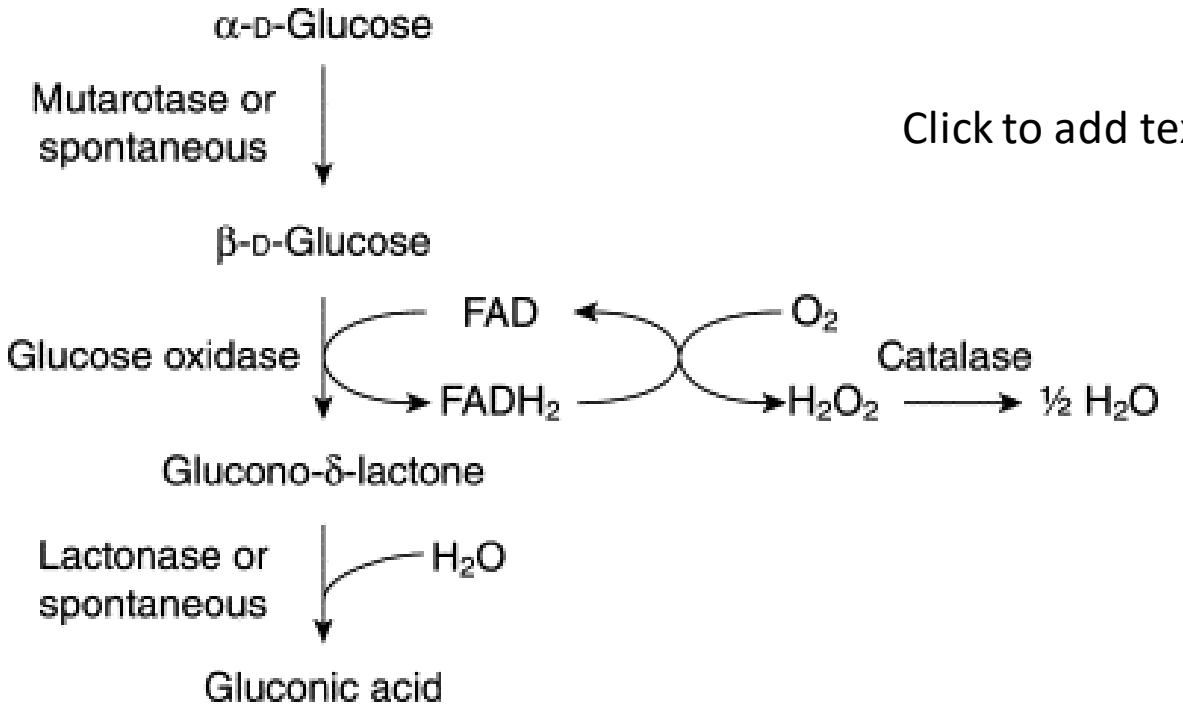
- Fructose and glucose may be metabolized to form Lactic acid, Acetic acid, and Malic acid.
- Acid formation dependent upon oxygen conditions.
- **Survey of commercial Kombucha products reveal trace to null amounts of glucuronic acid**.



BACKGROUND

What's known about organic acids

- Genera of AAB convert glucose into glucuronic acid
- Komagateibacter and Gluconobacter species



Click to add text

Table 1.2. Molecular weight (MW), Protons per Molecule, Equivalent Weight and Multiplying Factor for the main acids in wine. Adapted from Margalit (2012).

ACID	MW	Protons per Molecule	Equivalent Weight	Multiplying Factor
Tartaric	150	2	75	1.00
Malic	134	2	67	0.89
Lactic	90	1	90	1.20
Succinic	118	2	59	0.79
Fumaric	116	2	58	0.77
Citric	192	3	64	0.85
Acetic	60	1	60	0.80

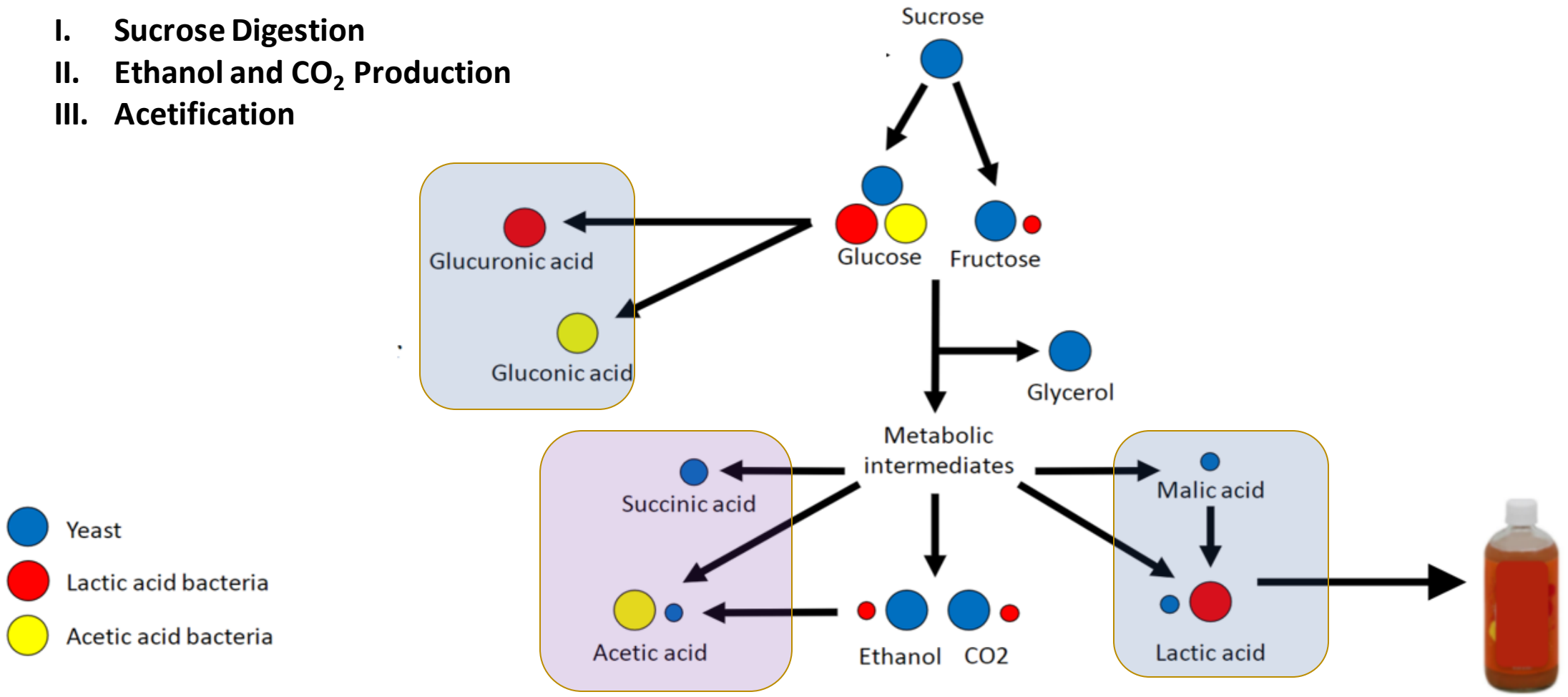
Monitoring Organic Acid Development

- Measurements and Methods of detection
- Titratable Acidity
- Sourness Unit
- Enymatic Assays
- GC-MS
- NMR

BACKGROUND

The SCOBY transforms sweet tea to Kombucha

I. **Sucrose Digestion**
II. **Ethanol and CO_2 Production**
III. **Acetification**

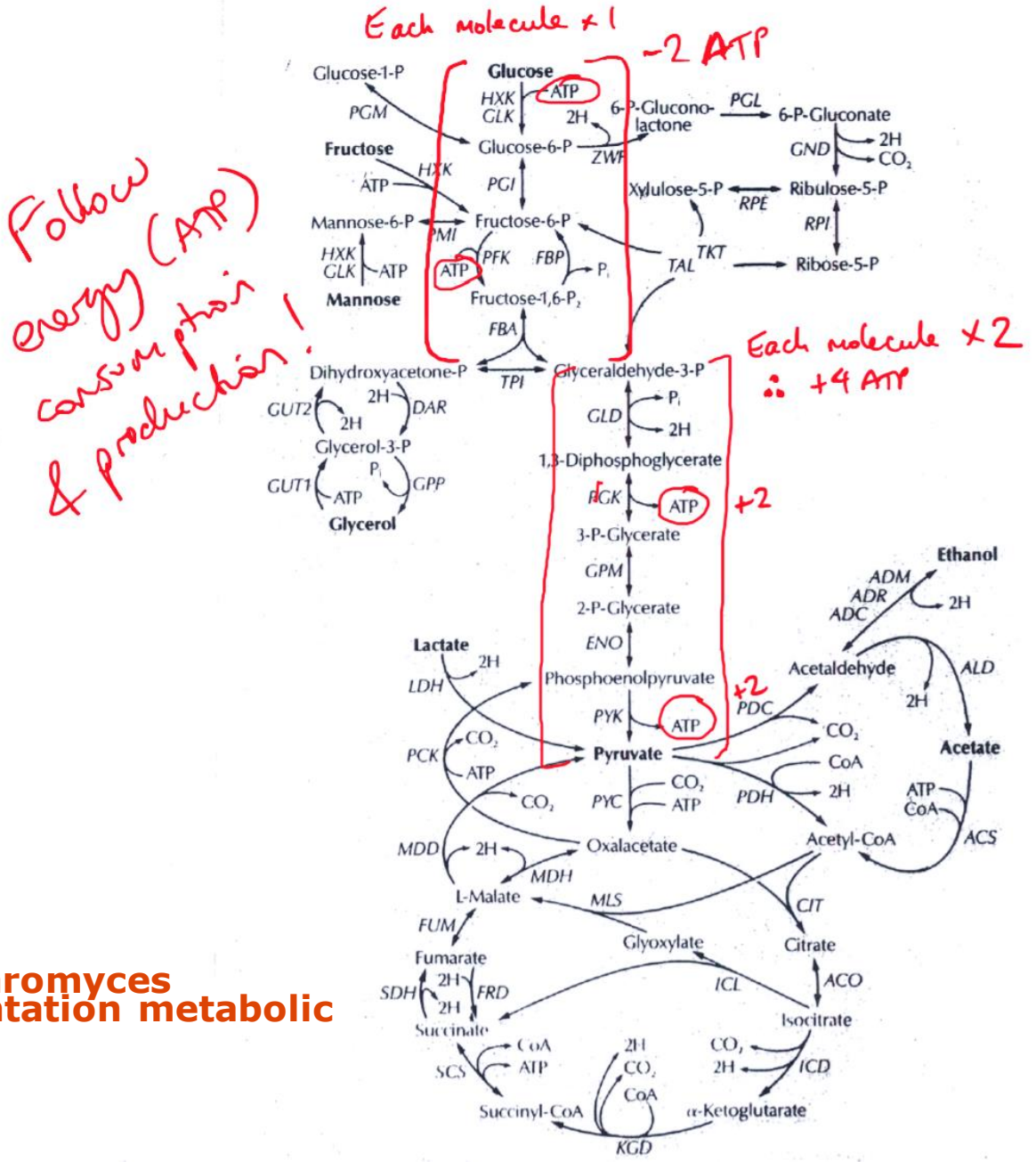


BACKGROUND

Kombucha Metabolism

Alcohol fermentation and reparation are complex metabolic pathways to restore energy to the microbial cell.

Byproducts-organic acids- are formed along the way



Saccharomyces fermentation metabolic cycle

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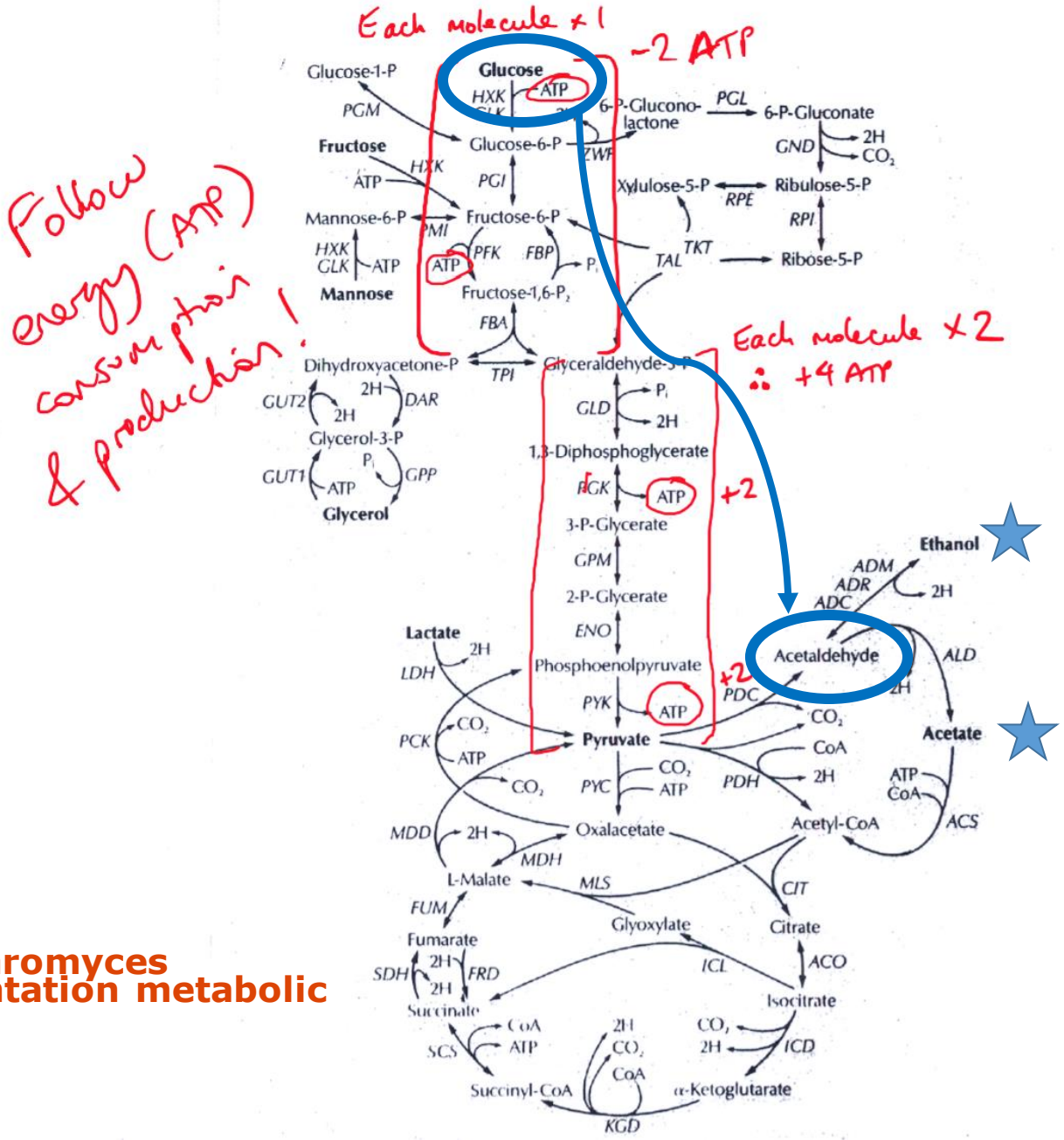
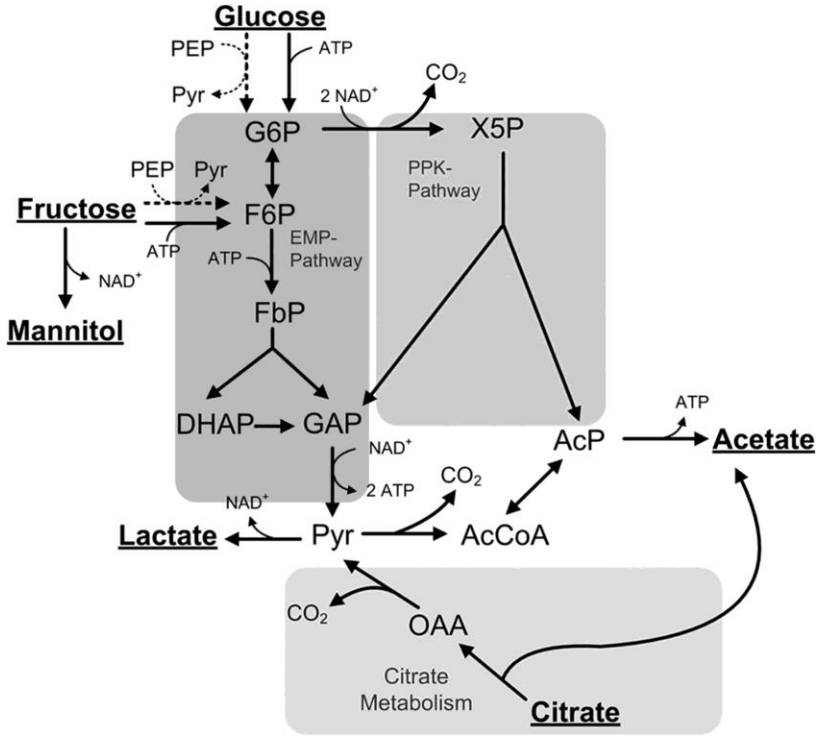


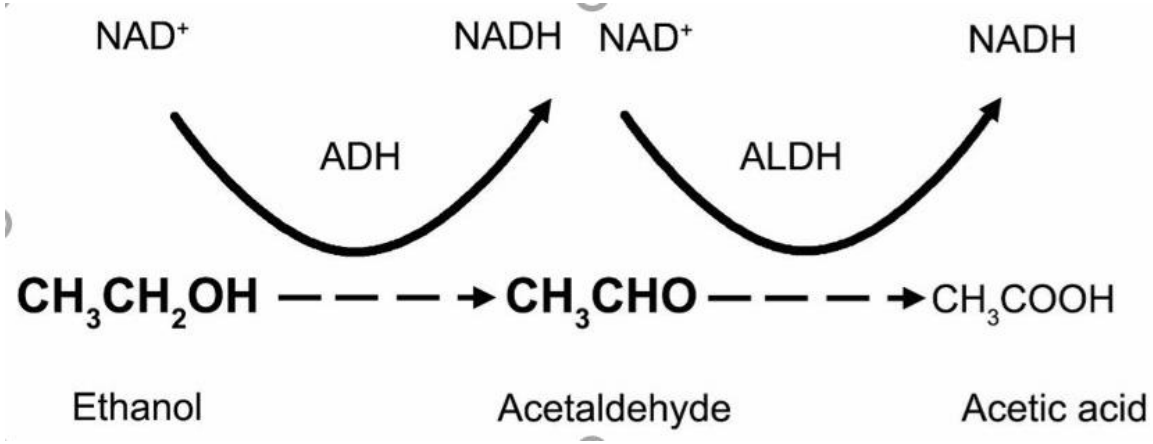
Saccharomyces fermentation metabolic cycle

BACKGROUND

Bacterial Oxidation

- Cause “slow” and “sluggish” fermentations
- **Make the cellulosic biofilm or pellicle** that the bacterial species attach to and become embedded within
- Organic acids contribute “dry, sour” and “pucker, tart” flavors
- Oxidize Ethanol

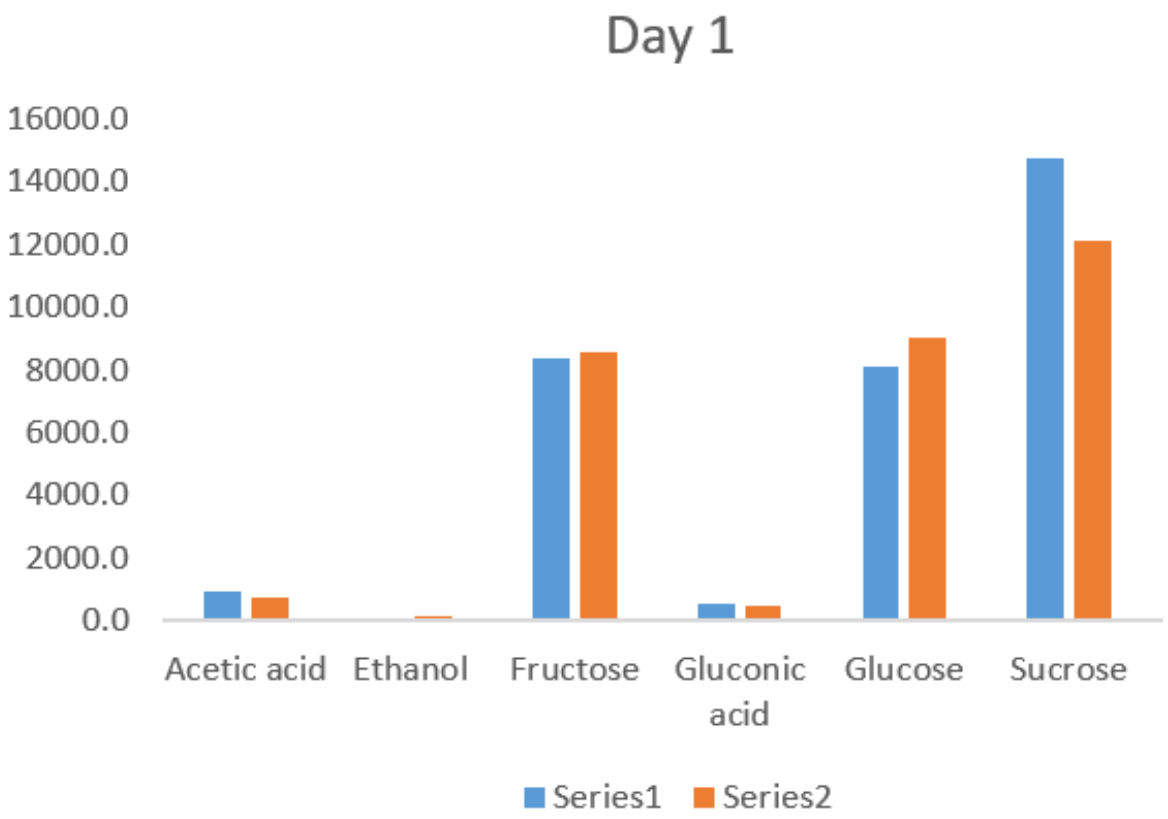


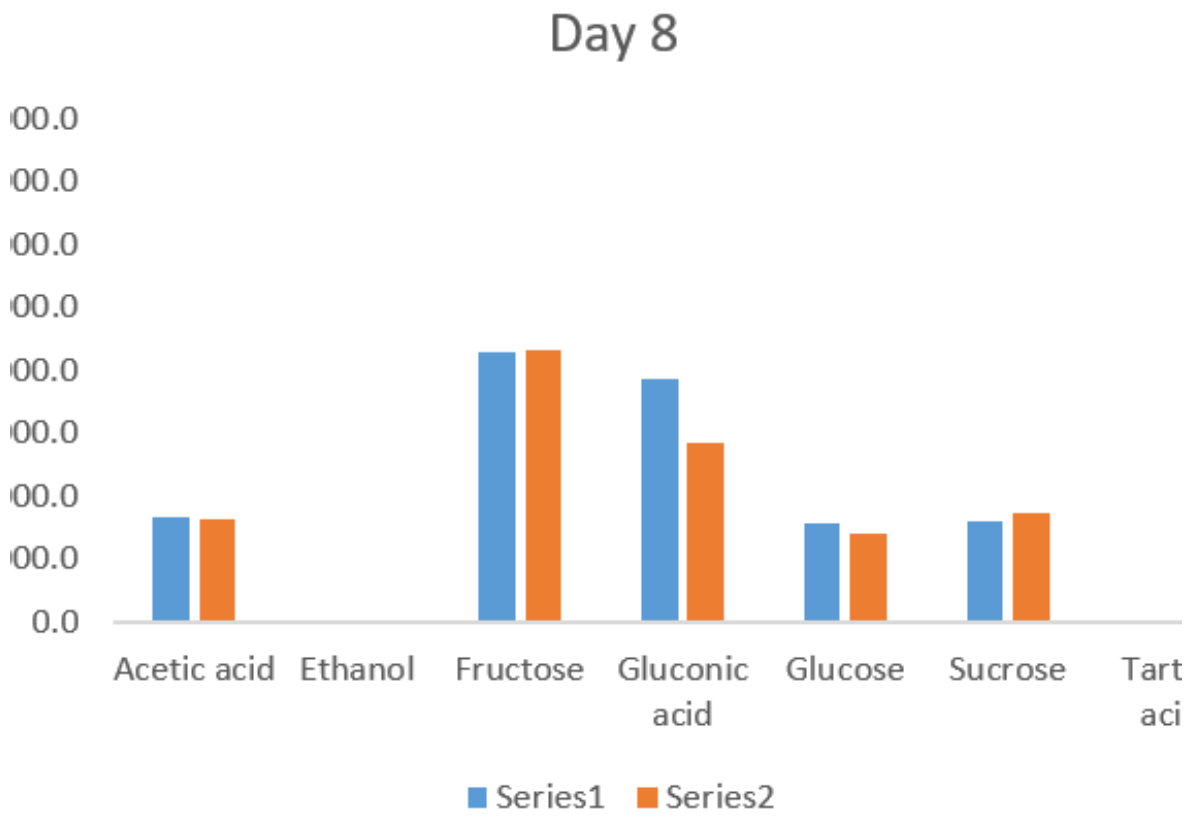


RESULTS

Different AAB, Kombucha Fermentations

Series 1: Gluconobacter oxydans
Series 2: Gluconobacter oxydans, Komagateibacter xylinus





BACKGROUND

Some conditions that impact metabolism

Surface Area/Oxygenation

Temperature

FAA

Starting pH

Microbiota

BACKGROUND

Temperature Impacts

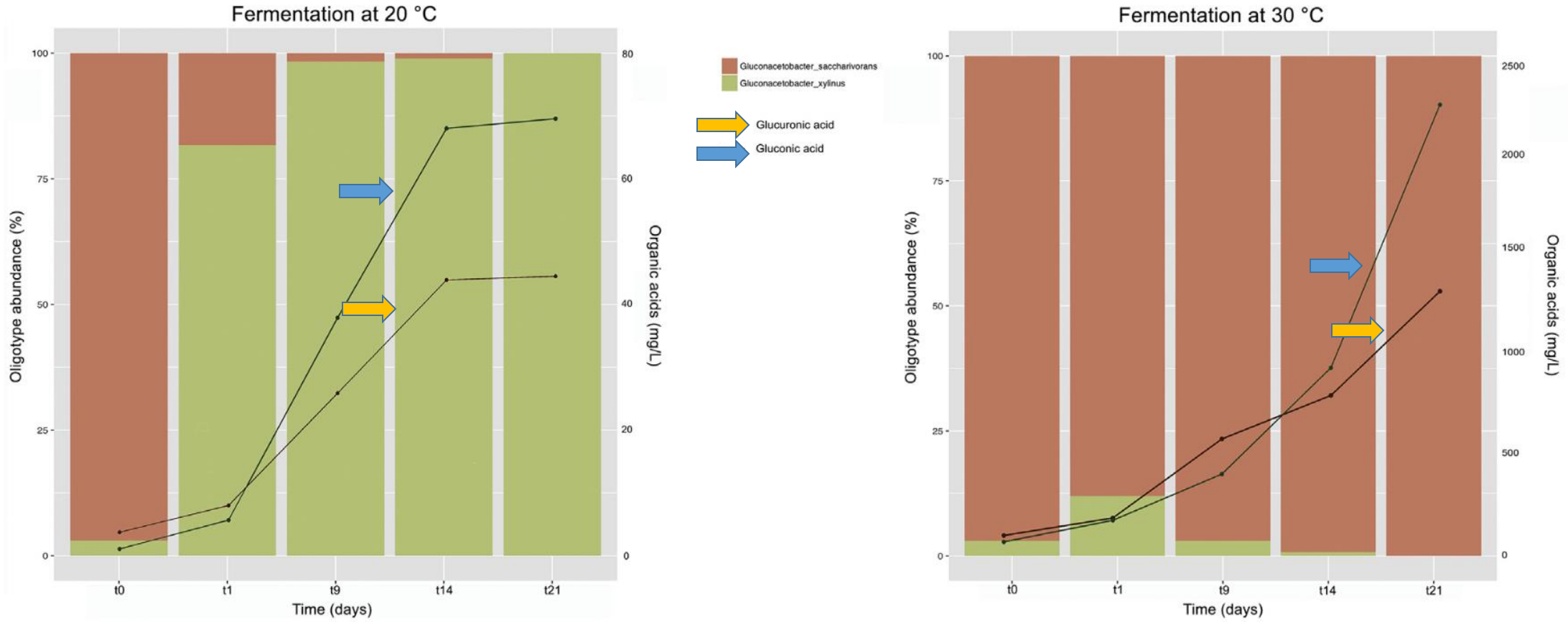


Fig. 3. Stacked bar chart showing the abundance of *Gluconacetobacter* oligotypes and line plot reporting the concentration of gluconic and glucuronic acids in Kombucha tea samples fermented at 20 °C (left panel) or 30 °C (right panel).

De Filippis *et al.* (2018) *Food Microbiology*, 73:11-16.

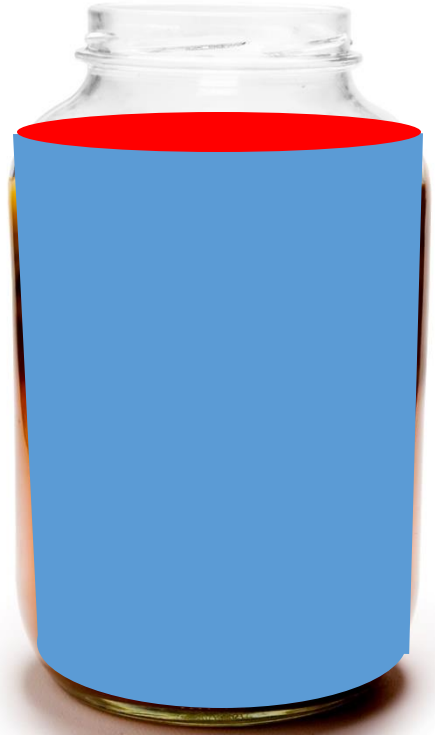
BACKGROUND

Oxygen Requirements

- Not heavily researched, but worth noting that vinegar producers aerate to saturation.

Specific interfacial area as a key variable in scaling-up Kombucha fermentation

Dragoljub Cvetković [a], Siniša Markov [a,*], Mirjana Djurić [a]
Dragiša Savić [b], Aleksandra Velićanski [a]



- Typical 2G kombucha jar SIA ratio = 0.043-0.048
- TA of 4g/L achievable in ~7days @ 28degC

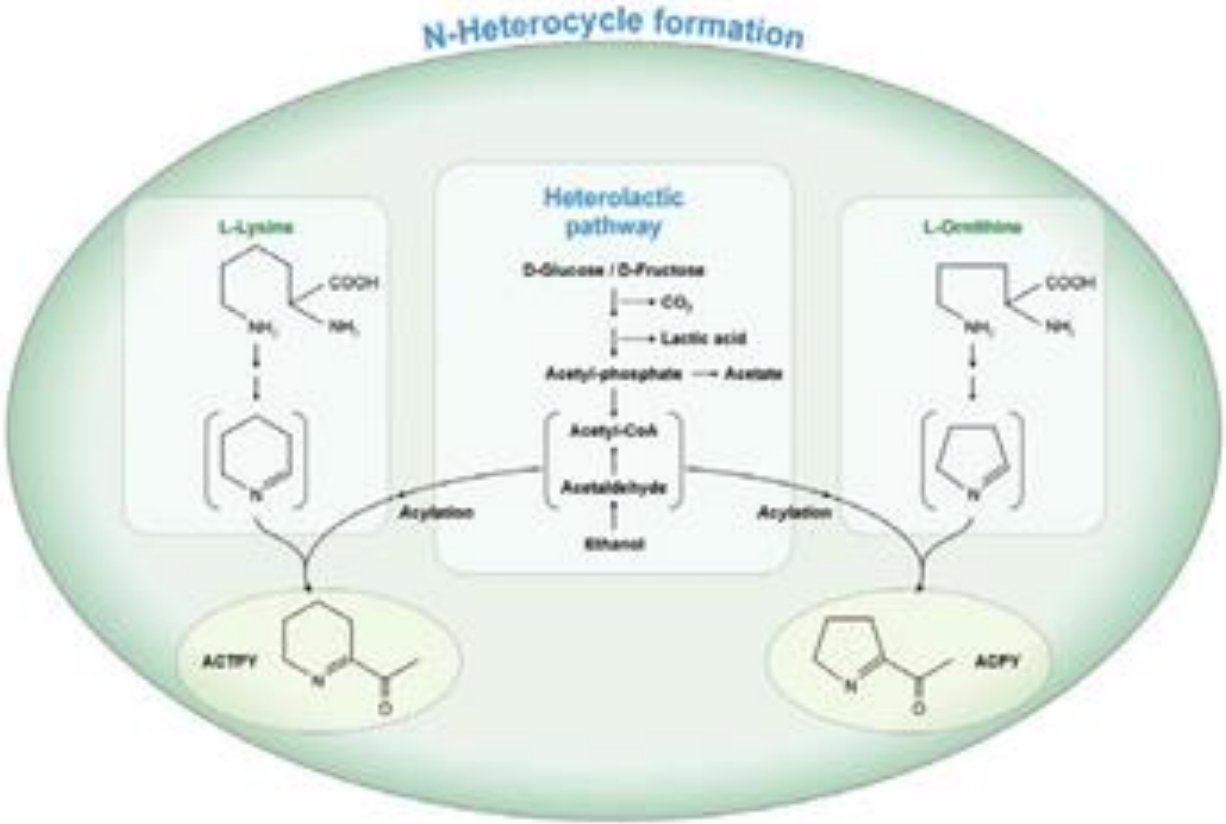
BACKGROUND

Off-flavors

- **Butyric acid**: rancid butter, parmesan cheese, and vomit (2-3 ppm)
- **Usnic acid**: bitter
- **Acetaldehyde**: tart apple
- **N-heterocyclic volatile bases 2-acetyltetrahydropyridine:** mouse taint
- **Proponic acid**: nutty, cheesy
- **2-mercapto-3-methyl-l-butanol**: onion

Compound	Threshold in water (µg/L)	Range in wine (µg/L)
ACTPY	1.6	4.8 – 106
ETPY	150 0.1	2.7 – 18.7
ACPY	0.1	trace 7.8

BACKGROUND



ure 12. A schematic representation of the formation of potent and unpleasant nitrogen-heterocycle 'mousy' off-flavour compounds acetyltetrahydropyridine (ACTPY) and 2-acetyl-1-pyrroline (ACPY)) by some lactic bacteria (based upon Costello and Henschke 2002).

Bacteria diversity may impact fermentation

LAB heterotrohps makes nitrogenous ring associated with mousy off-flavours

Kombucha Styles- Standards of Identity

BACKGROUND

OSU NMR Facility and Instrumentation

- The 800 MHz NMR spectrometer is currently the highest field NMR spectrometer in the state of Oregon

- This spectrometer will primarily be used for the analysis of biological samples, leading to advances in research areas such as Alzheimer's Disease and Cancer

- Facility manager: Dr. Patrick Reardon

METHODS

NMR vs Chromatography Methods

PROS

- More **accessible** instrumentation
- Does not require high level training
- Does not overestimate purity
- Affordable

CONS

- Different functional groups require different columns
- **Multiple standard curves**
- Require different stationary and mobile phases

METHODS

NMR vs Chromatography Methods

PROS

- Easy sample prep
- Single internal standard required
- **Simultaneous analysis of compounds**
- Spectrometer accessible to wide range of functional groups

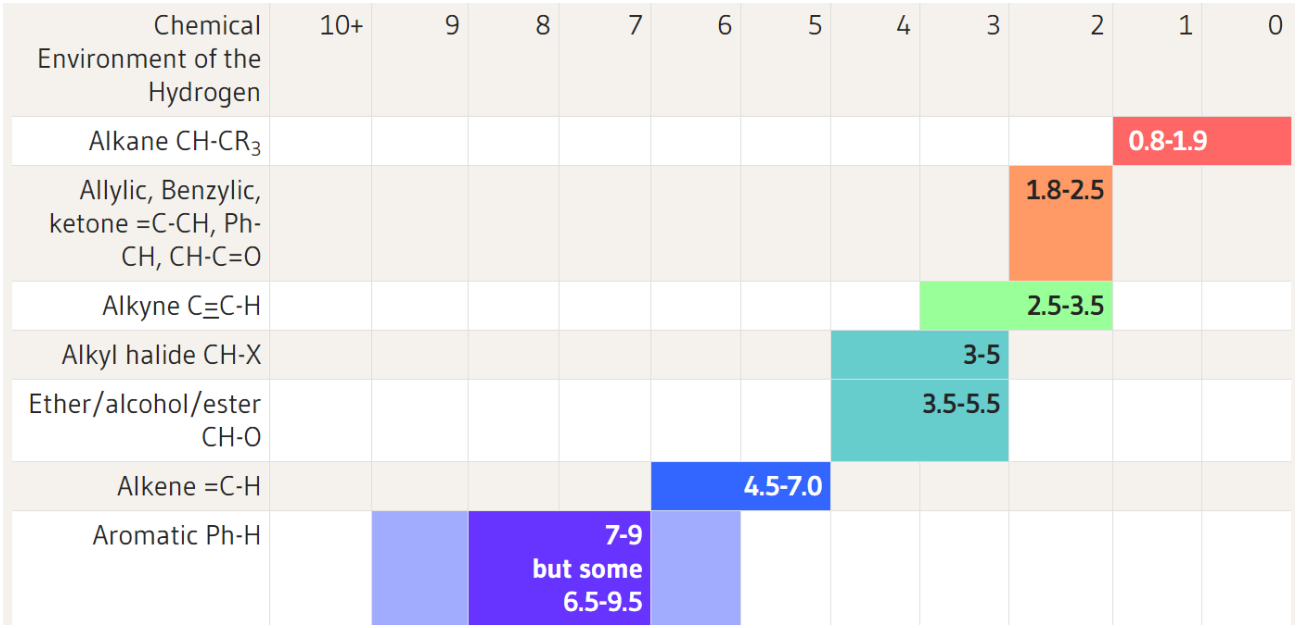
CONS

- Spectrometer is **expensive**
- Not generally accessible
- Requires highly trained professionals
- Requires constant cooling

METHODS

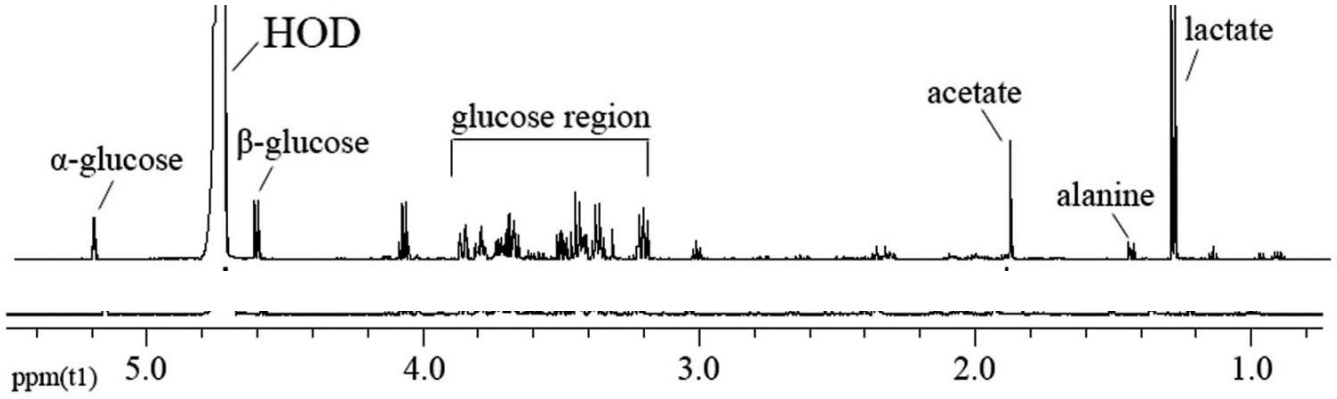
Nuclear Magnetic Resonance

- Quantification by NMR spectroscopy of compounds is often used in the analysis of foods
- The proportionate frequency change of spin flip (due to magnetic field) and irradiation frequency is the **shift**
- **Shift** from a standard compound's frequency in parts per million (δ, ppm) is measured for each compound.
- **This shift can be used to calculate concentration (mg/l)**



METHODS

Nuclear Magnetic Resonance

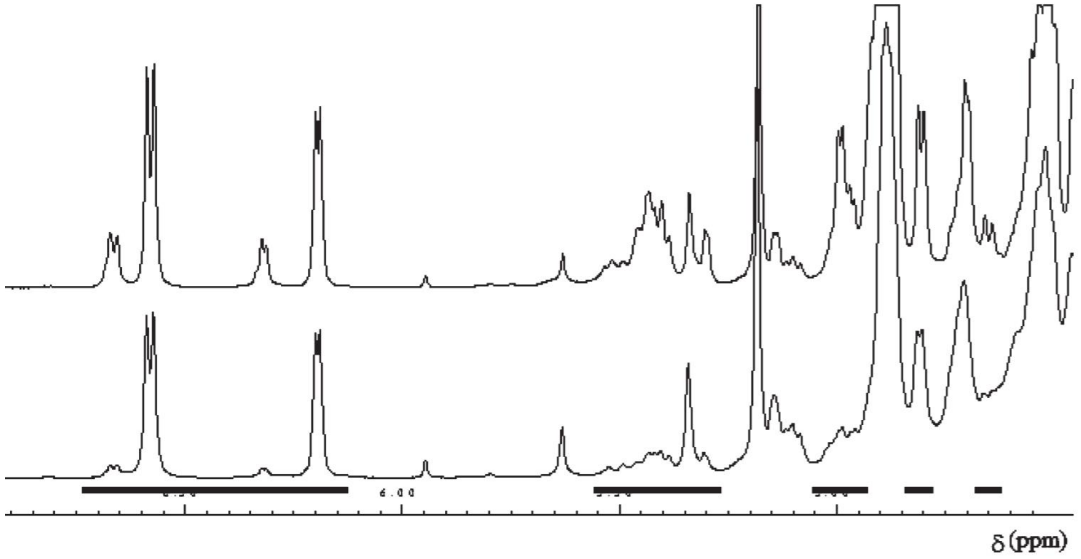


Functional groups of compounds have unique chemical shifts

METHODS

NMR, previous use in the food industry

- In recent years, qNMR is used to verify composition as a consequence of **natural** and **artificial** processes
  - **Natural**: enzymatic, aging, hardening
  - **Artificial**: cooking, refinement
- Used in adulteration/falsification detection, especially on regards to cheapening product with low-cost syrups (i.e. Honey)
- Advantages of this technique in food applications:
- Wide range of chemical species in single experiment
  - Repeatability/reproducibility
  - Simple extraction/prep
- Spectra of “authenticity” can be made, (i.e. Honey)



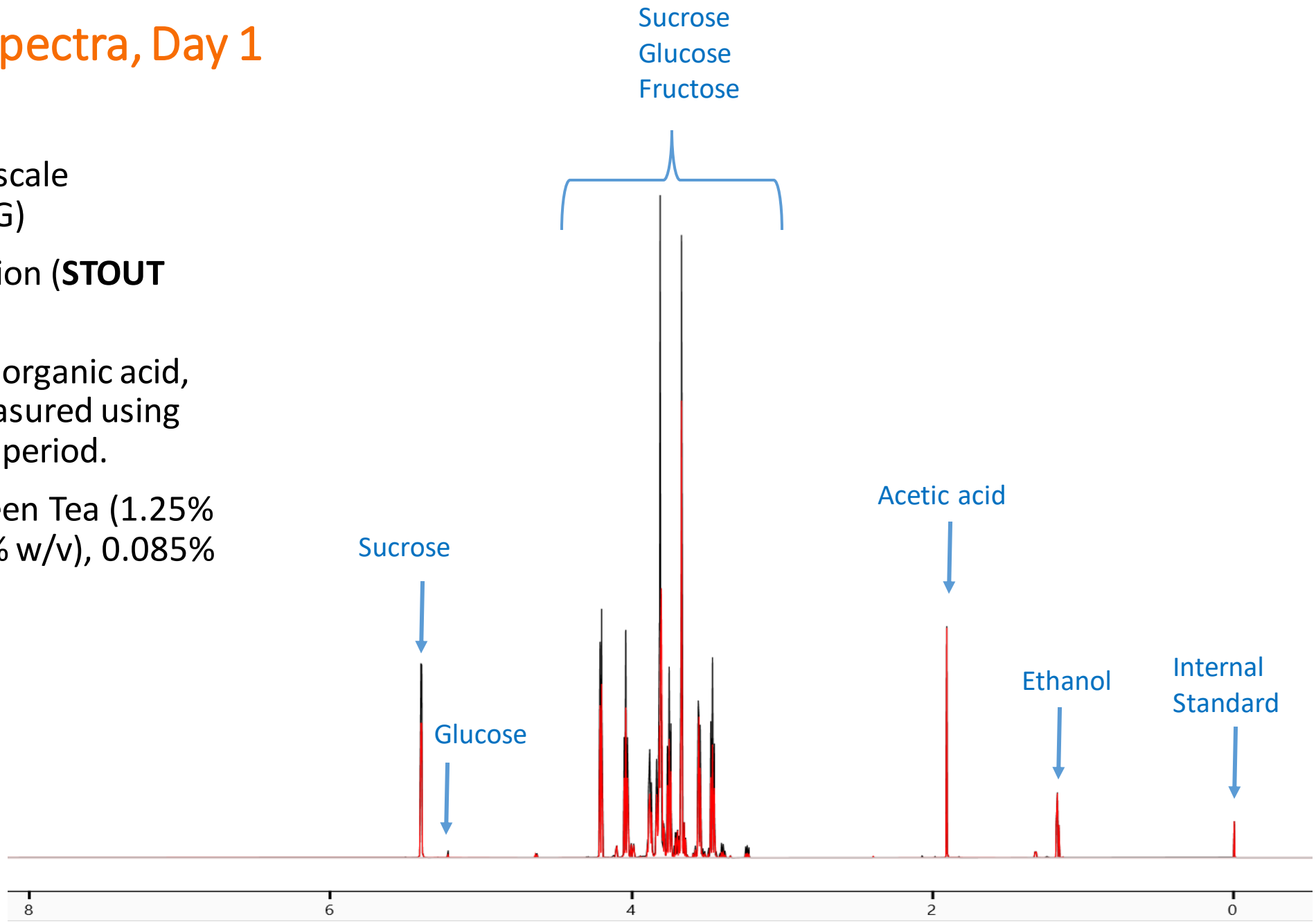
igure 2. Comparison between significant spectroscopic regions of an authentic (lower spectrum) and a 40% adulterated honey (upper spectrum). he highlighted spectroscopic regions are those containing the most correlated variables with CF1 and CF2 during GDA.

Bertelli, Davide, et al. "Detection of honey adulteration by sugar syrups using one-dimensional and two-dimensional high-resolution nuclear magnetic resonance." *Journal of agricultural and food chemistry* 58.15 (2010): 8495-8501.

RESULTS

Reading NMR Spectra, Day 1

- **Day 1**, medium-scale fermentation (5G)
- Open fermentation (**STOUT tanks**)
- Residual sugars, organic acid, and ethanol measured using NMR over 7 day period.
- Day 0: Black/Green Tea (1.25% w/v), Sugar (15% w/v), 0.085% (v/v) Acetic acid

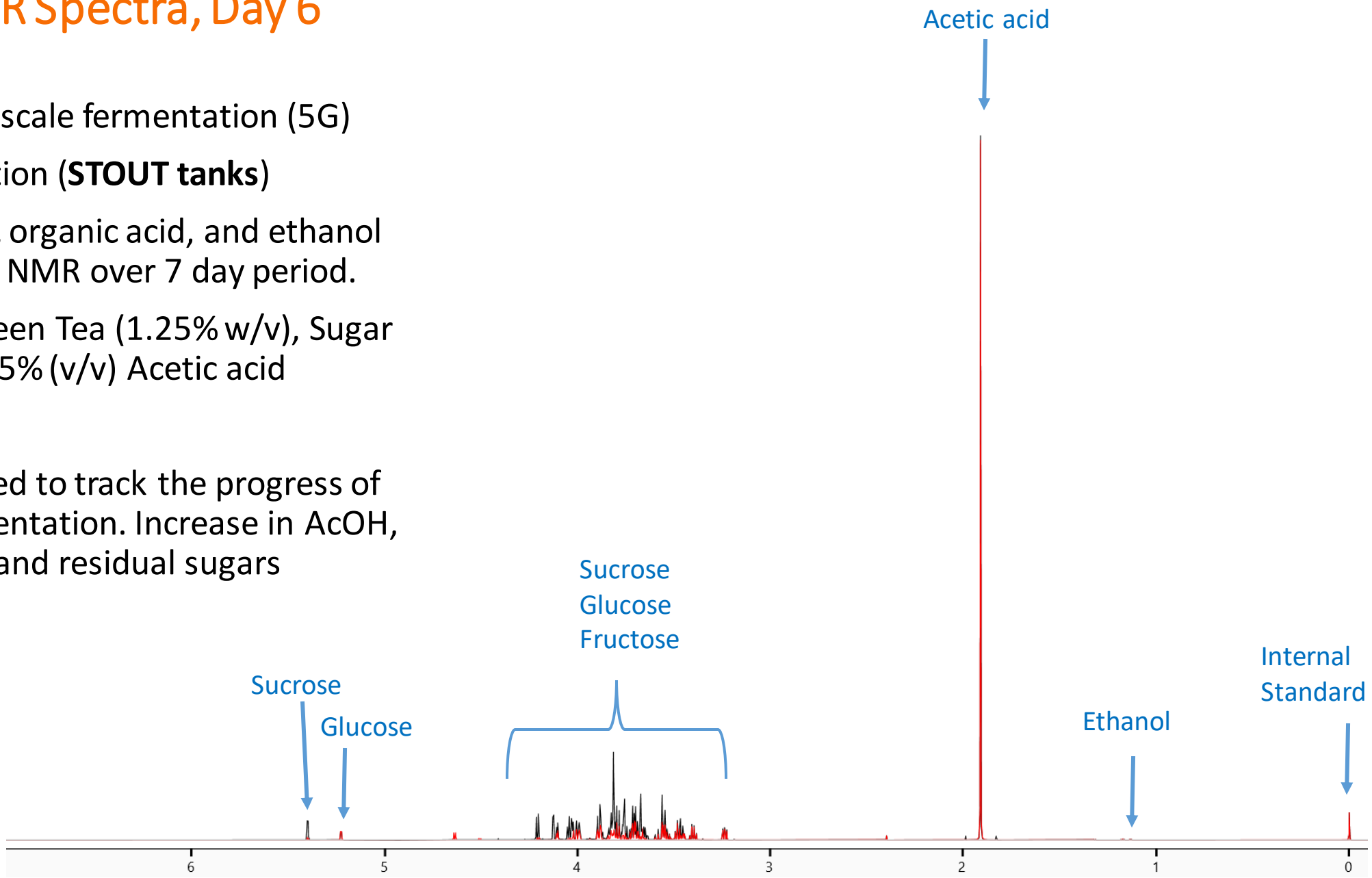


RESULTS

Reading NMR Spectra, Day 6

- **Day 6**, medium-scale fermentation (5G)
- Open fermentation (**STOUT tanks**)
- Residual sugars, organic acid, and ethanol measured using NMR over 7 day period.
- Day 0: Black/Green Tea (1.25% w/v), Sugar (15% w/v), 0.085% (v/v) Acetic acid

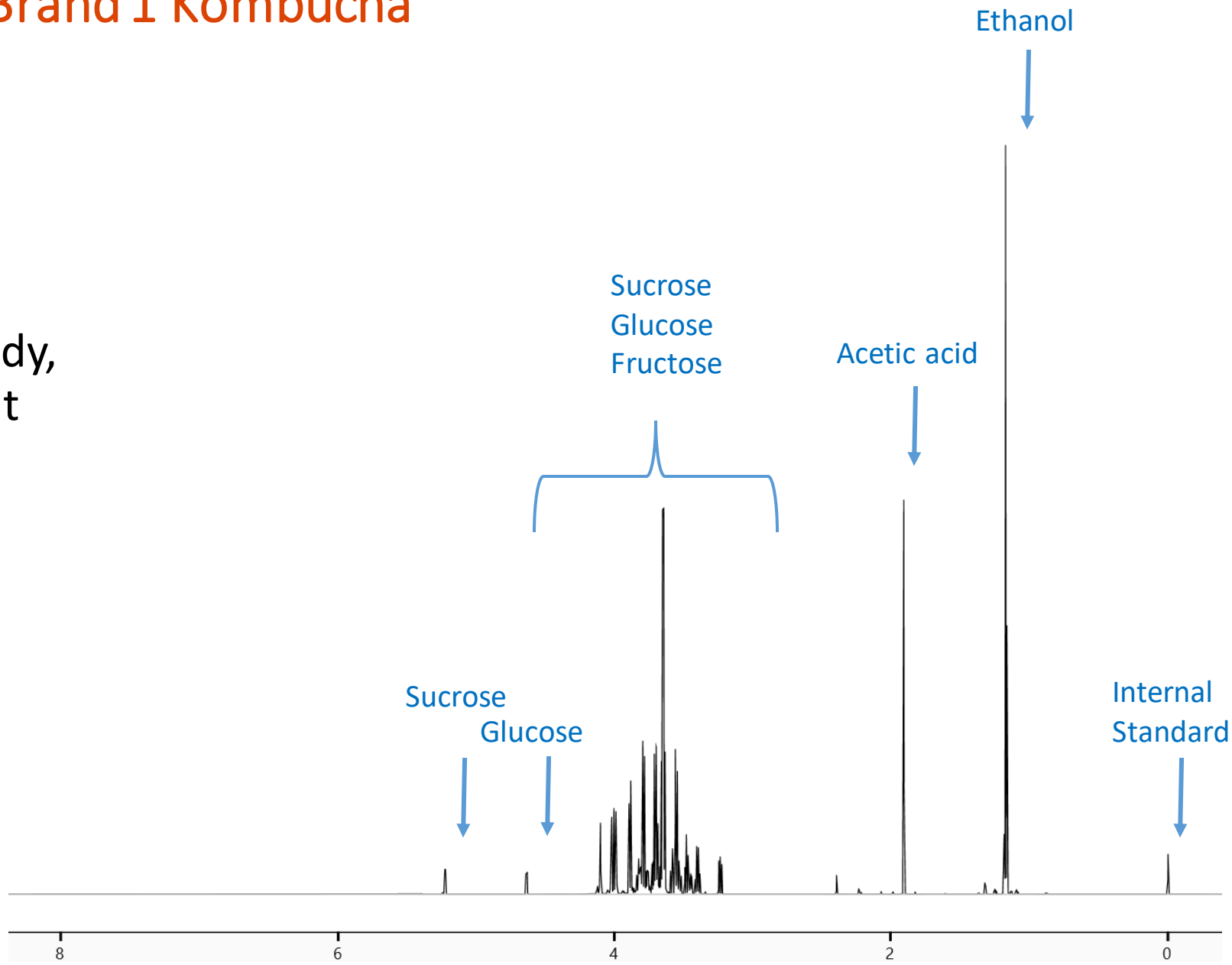
Spectra can be used to track the progress of a Kombucha fermentation. Increase in AcOH, decrease in EtOH and residual sugars



RESULTS

Reading NMR Spectra, Brand 1 Kombucha

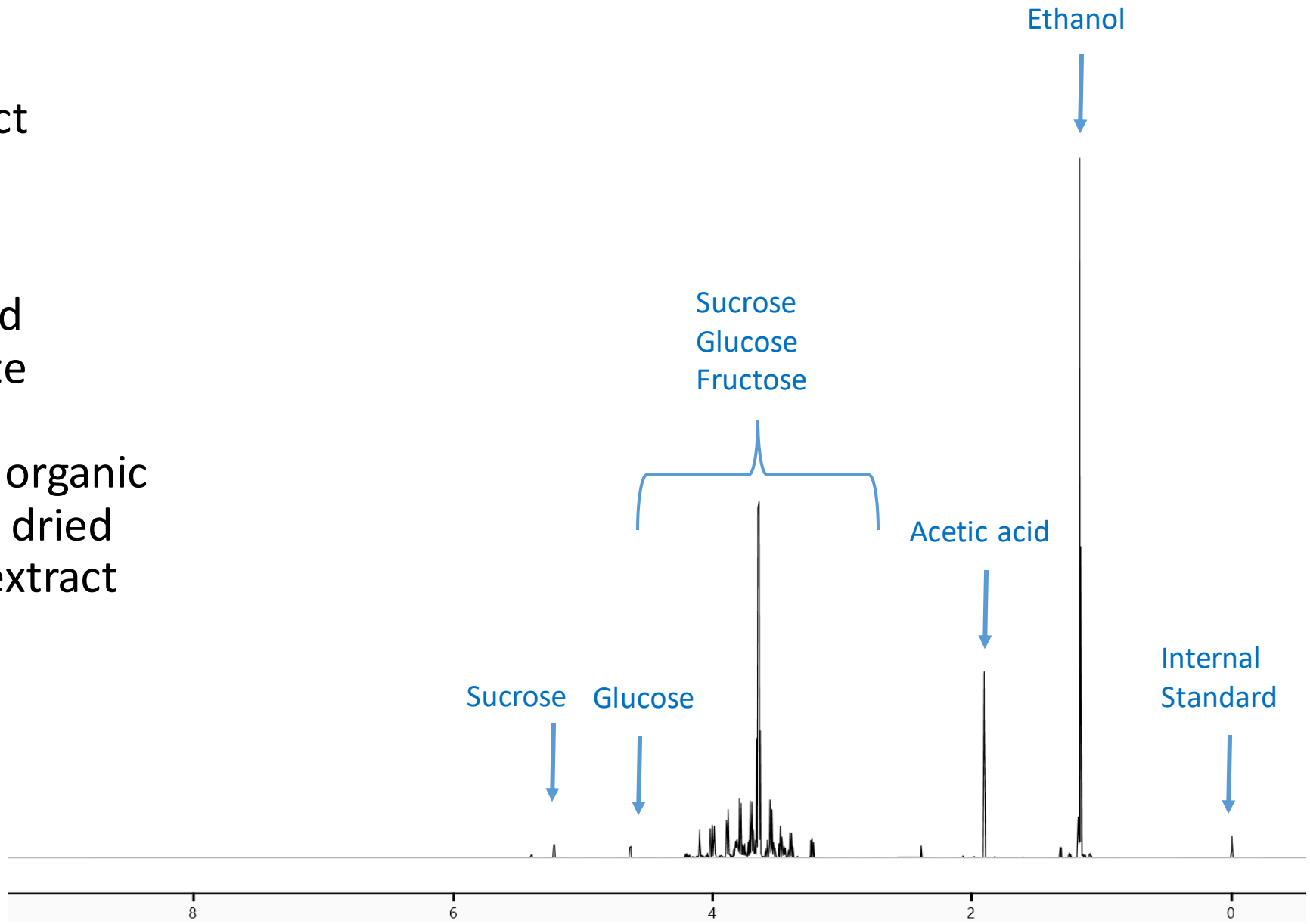
- Commerical Product
- Kombucha Styles, Pilot Study
- Described as, "Light body, fresh melon taste, great any time"



RESULTS

Reading NMR Spectra, Brand 2 Kombucha

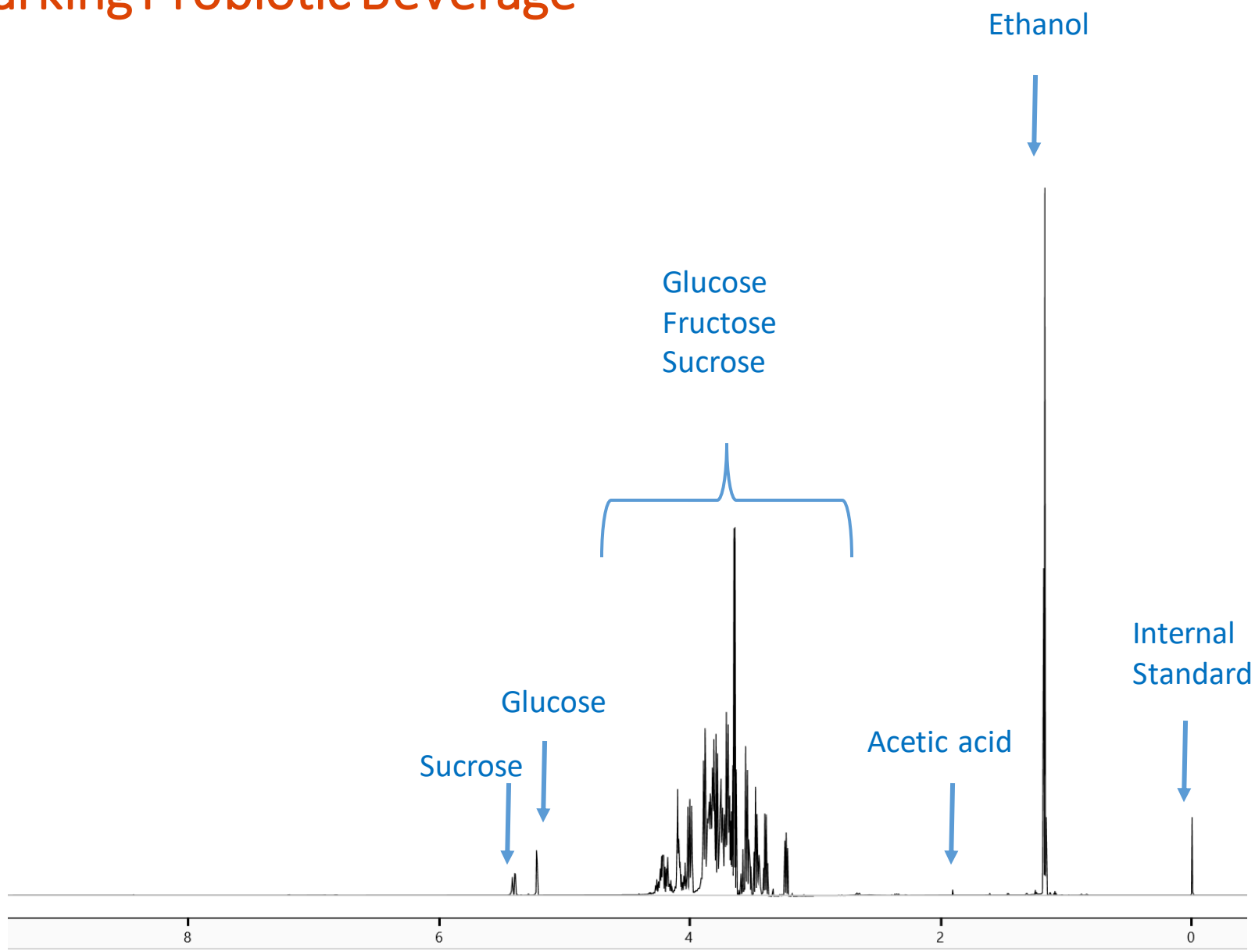
- Commerical Product
- Kombucha Styles, Pilot Study
- **Ingredients:** filtered water, organic white peony tea, organic kombucha culture, organic cane sugar, organic dried ginger and ginger extract



RESULTS

Reading NMR Spectra, Sparking Probiotic Beverage

- Commercial Product
- Kombucha Styles, Pilot Study
- Sparkling Prebiotic Tonic, Ginger Turmeric Cayenne Flavor

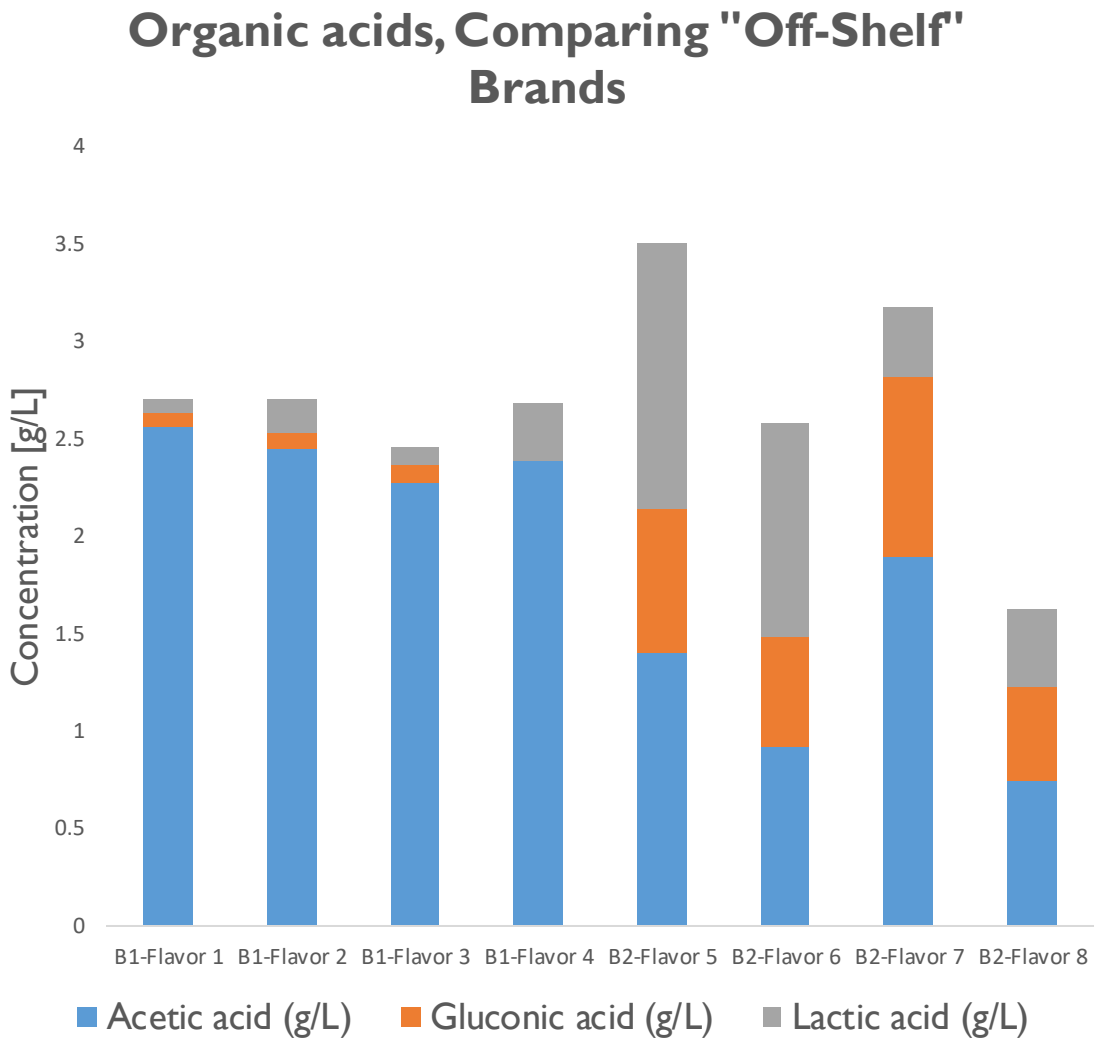


Kombucha Styles- Standards of Identity

RESULTS

NMR-H in off-shelf brand comparisons

- **B1:** Off shelf Brand 1, **B2:** Off shelf Brand 2
- Four flavours from 2 brands were processed for NMR analysis of residual sugar
- [AcOH] and [GlucOH] is lower in B1 than B2 samples.
- Ratio of organic acids may be potentially be used to define styles or characterize brand differences.
- Styles and a flavour “lexicon” is necessary in defining Kombucha



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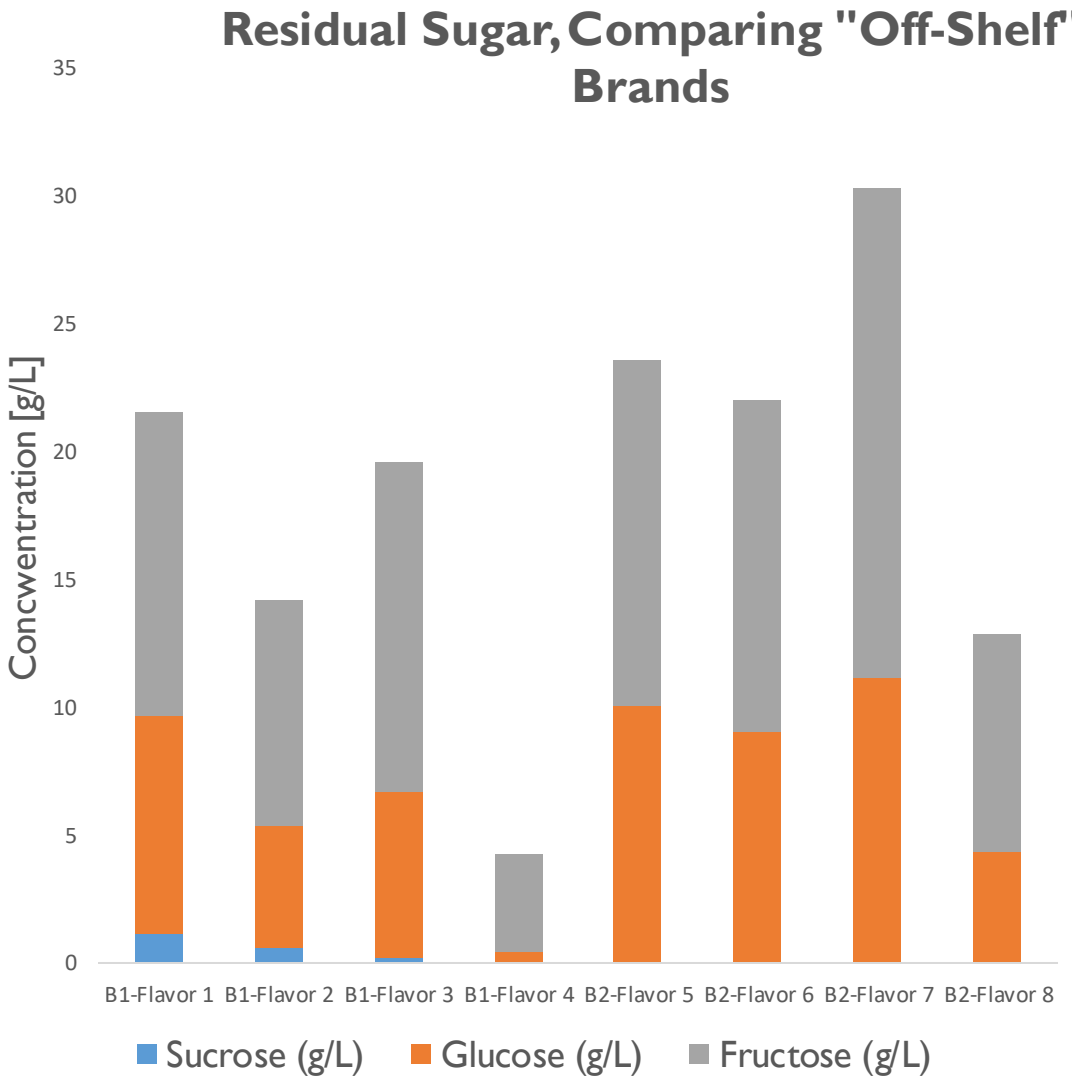
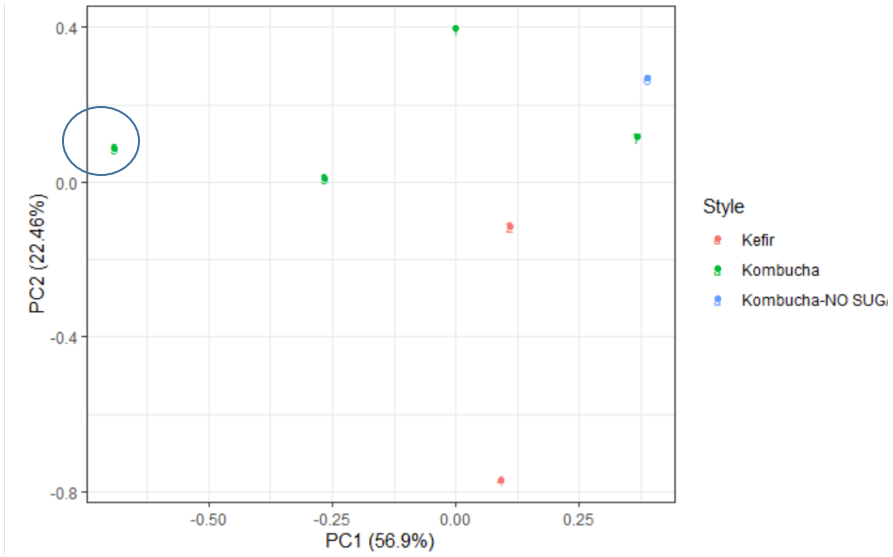
- **B1:** Off shelf Brand 1, **B2:** Off shelf Brand 2
- Four flavours from 2 brands were processed for NMR analysis of residual sugar
- Sucrose is low in comparison to glucose and fructose in all samples.
- Sucrose negligible in B2 samples

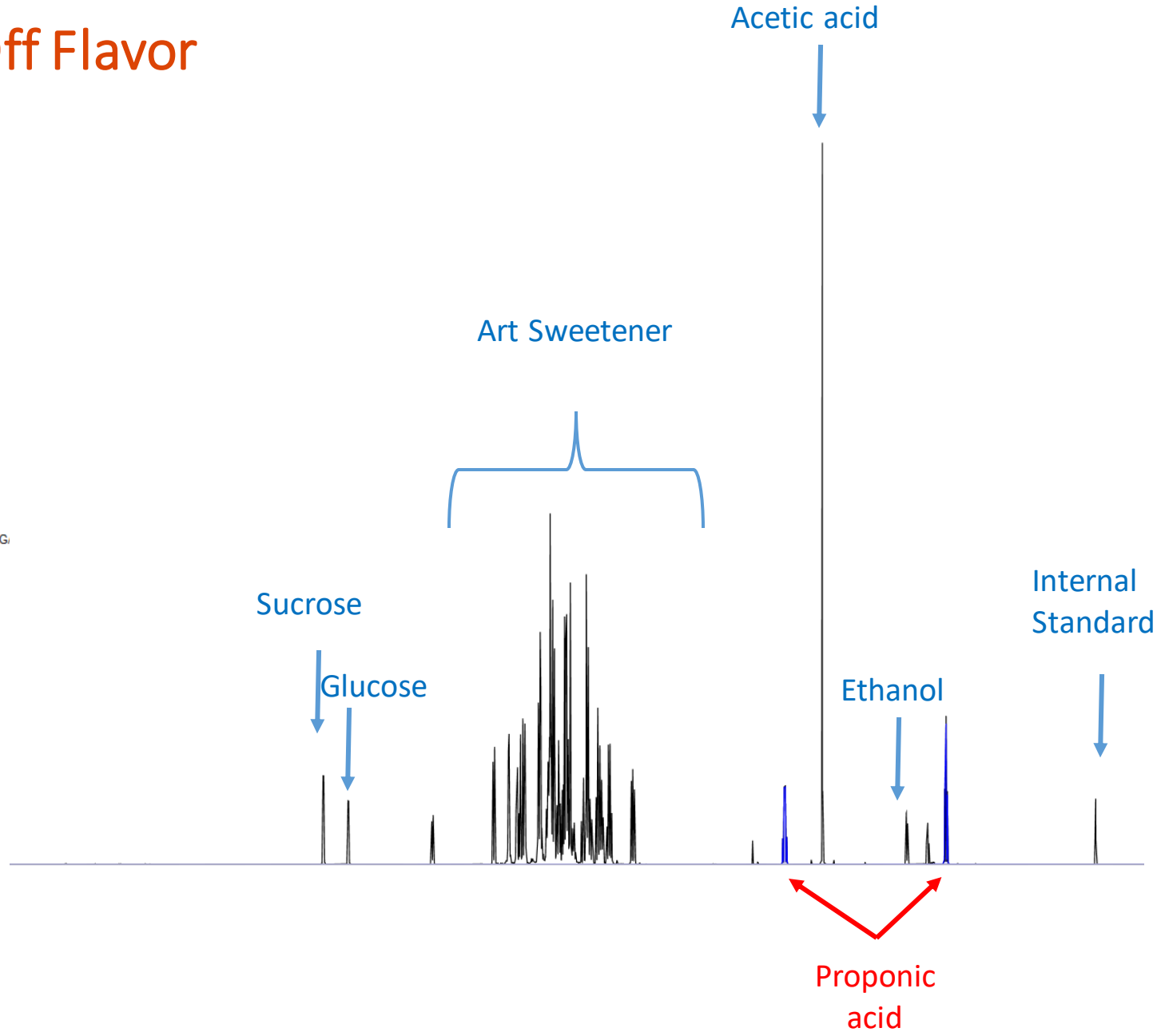
Ratio of **simple sugars**:**sucrose** may potentially be used to define styles



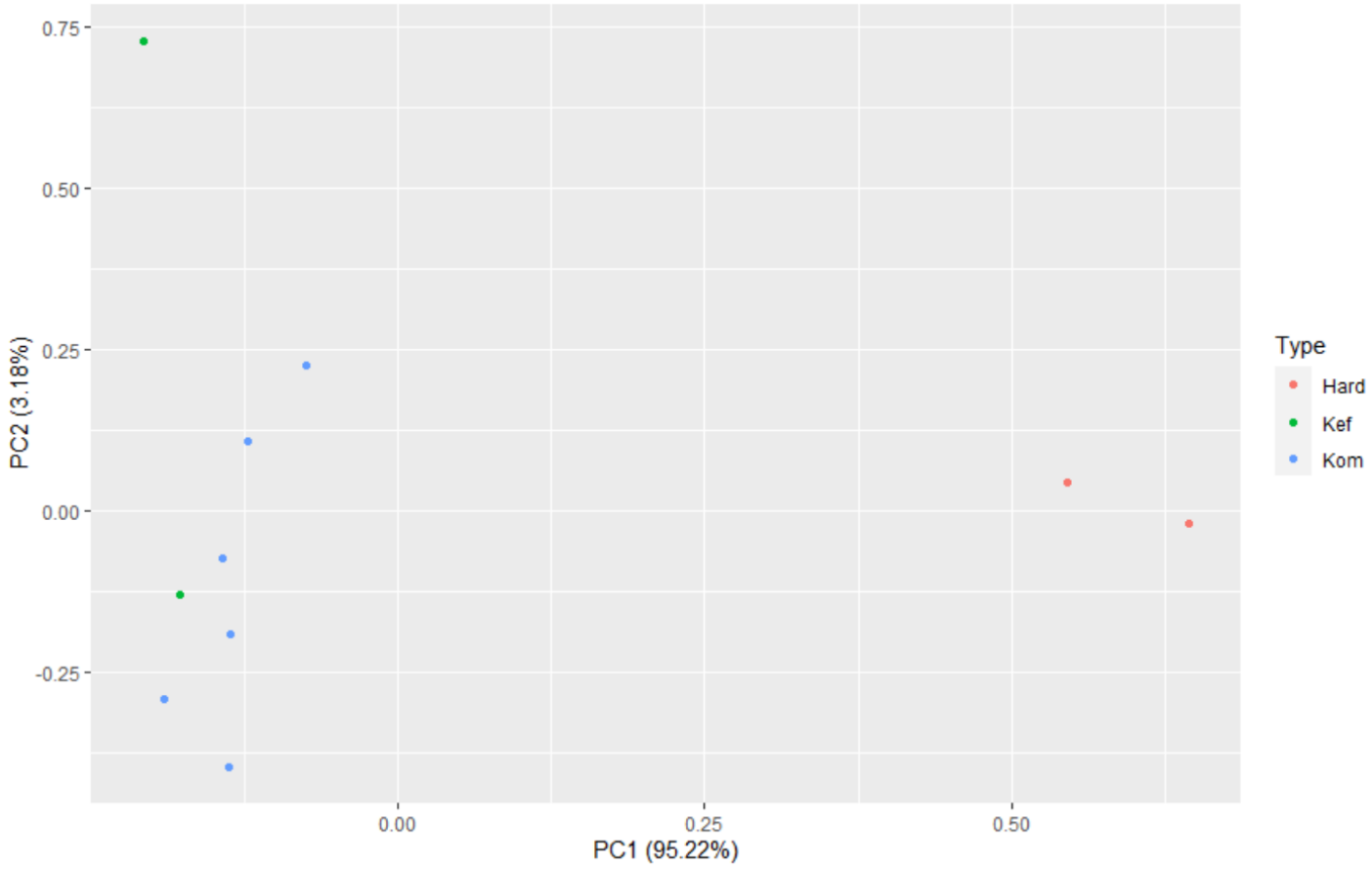
RESULTS

Reading NMR Spectra, Off Flavor





RESULTS

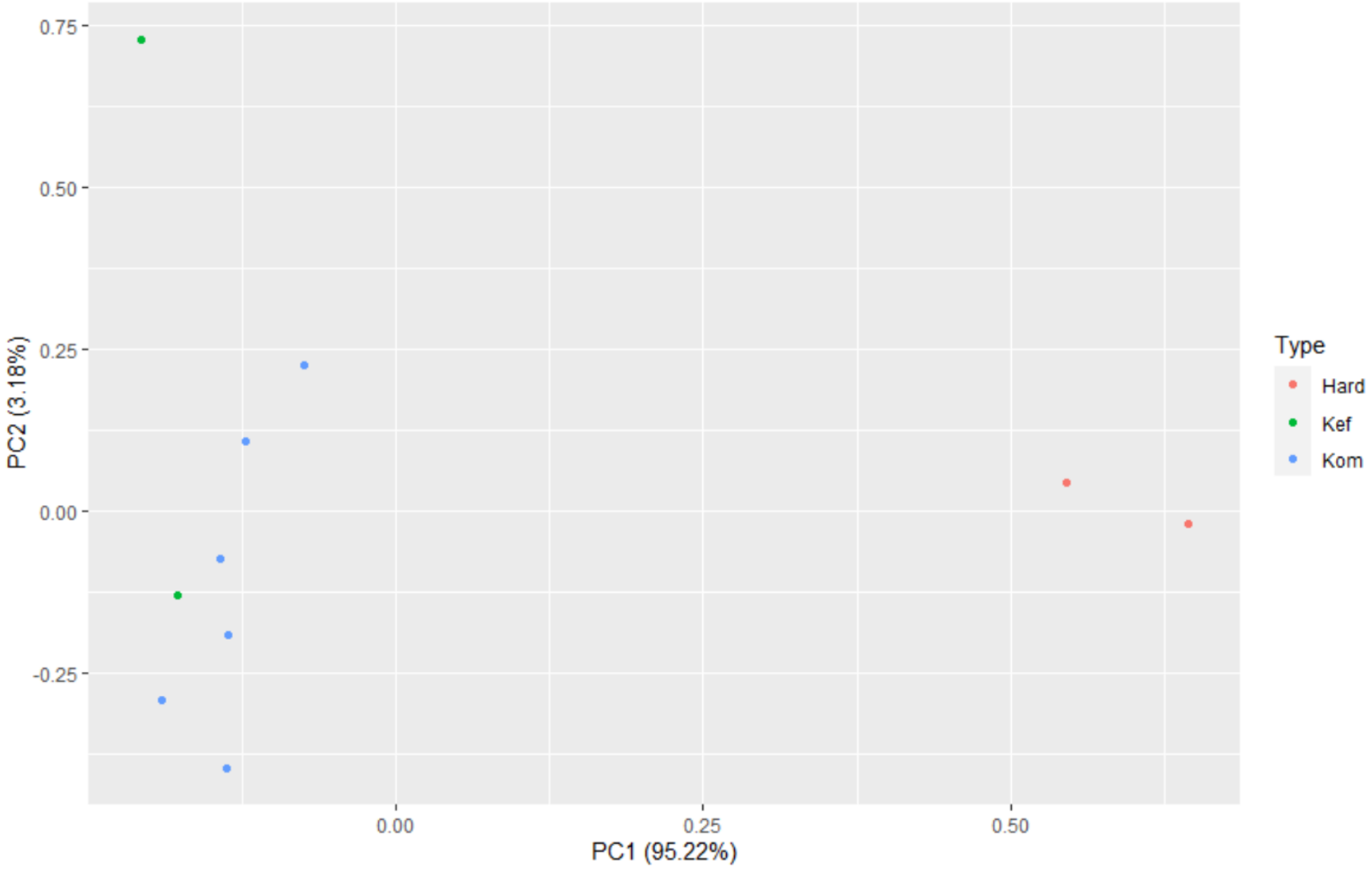


How To Read A PCA Plot

- PCA performed based on their "n" variables. The result will be a 2D plot with n dots
- Dots are mixed or graphically separated along the projected plane.
- If they are very mixed it means that groups are similar (there is no treatment effect).
- If separated along the projected plane, it means that the groups are different

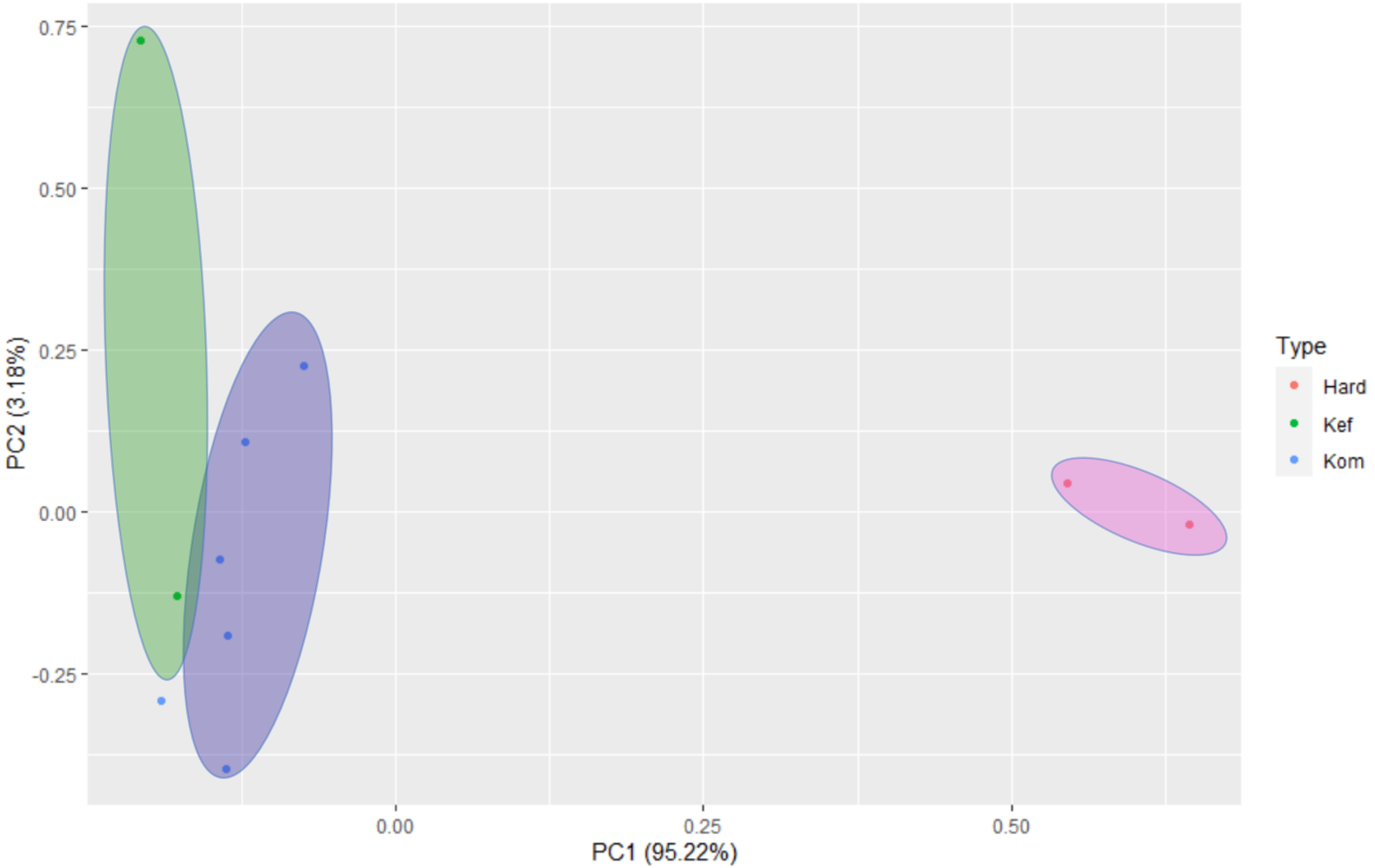
RESULTS

Distinguishing Fermented Beverages



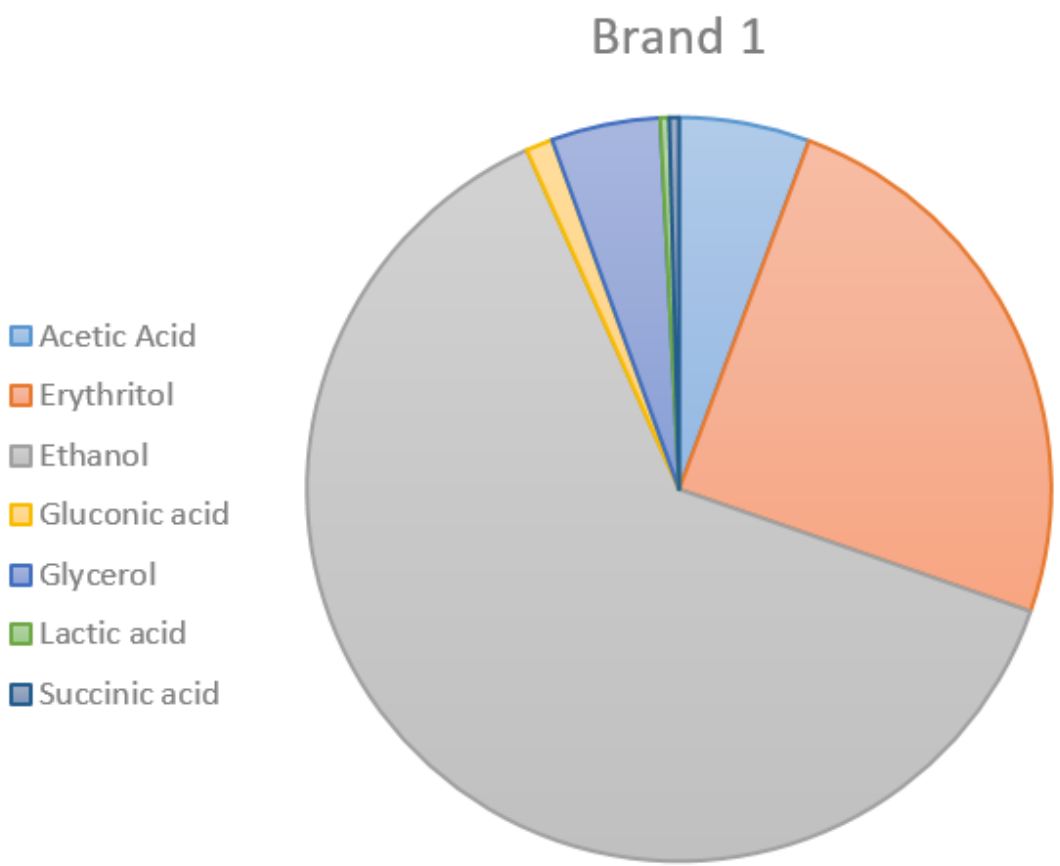
RESULTS

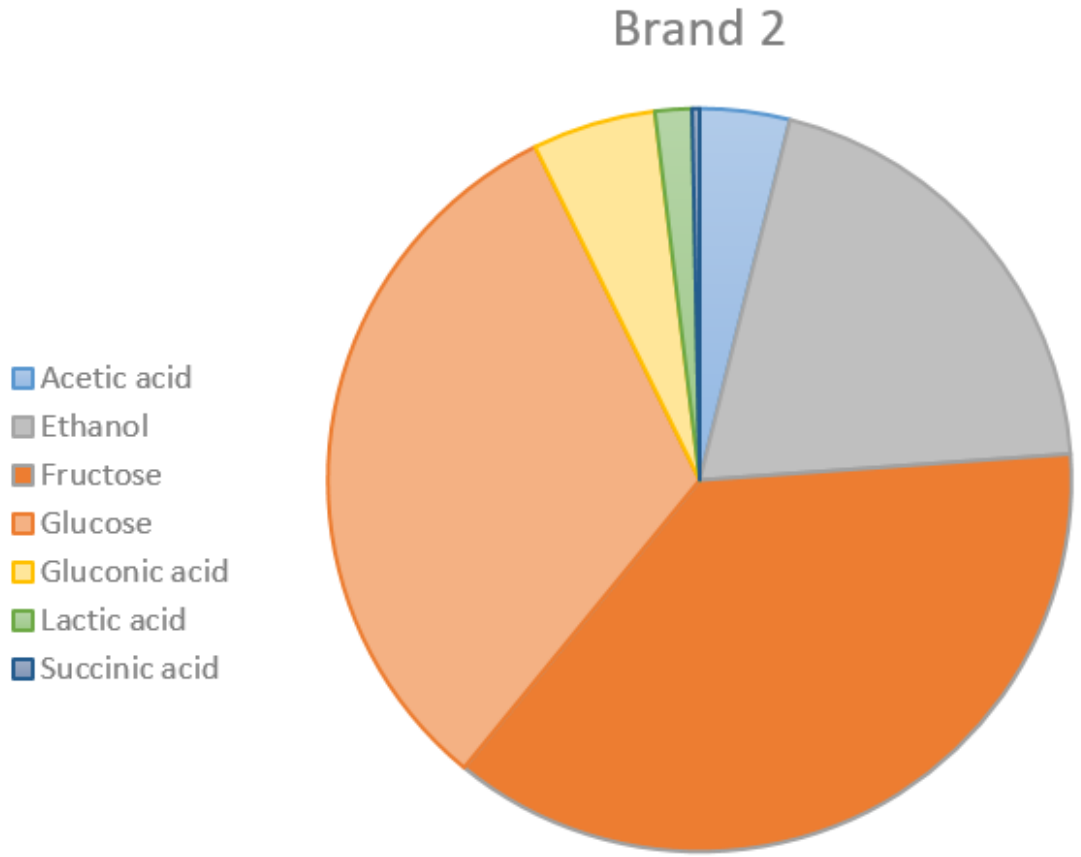
Distinguishing Fermented Beverages



RESULTS

Hard Kombucha- Understanding "Style" Differences



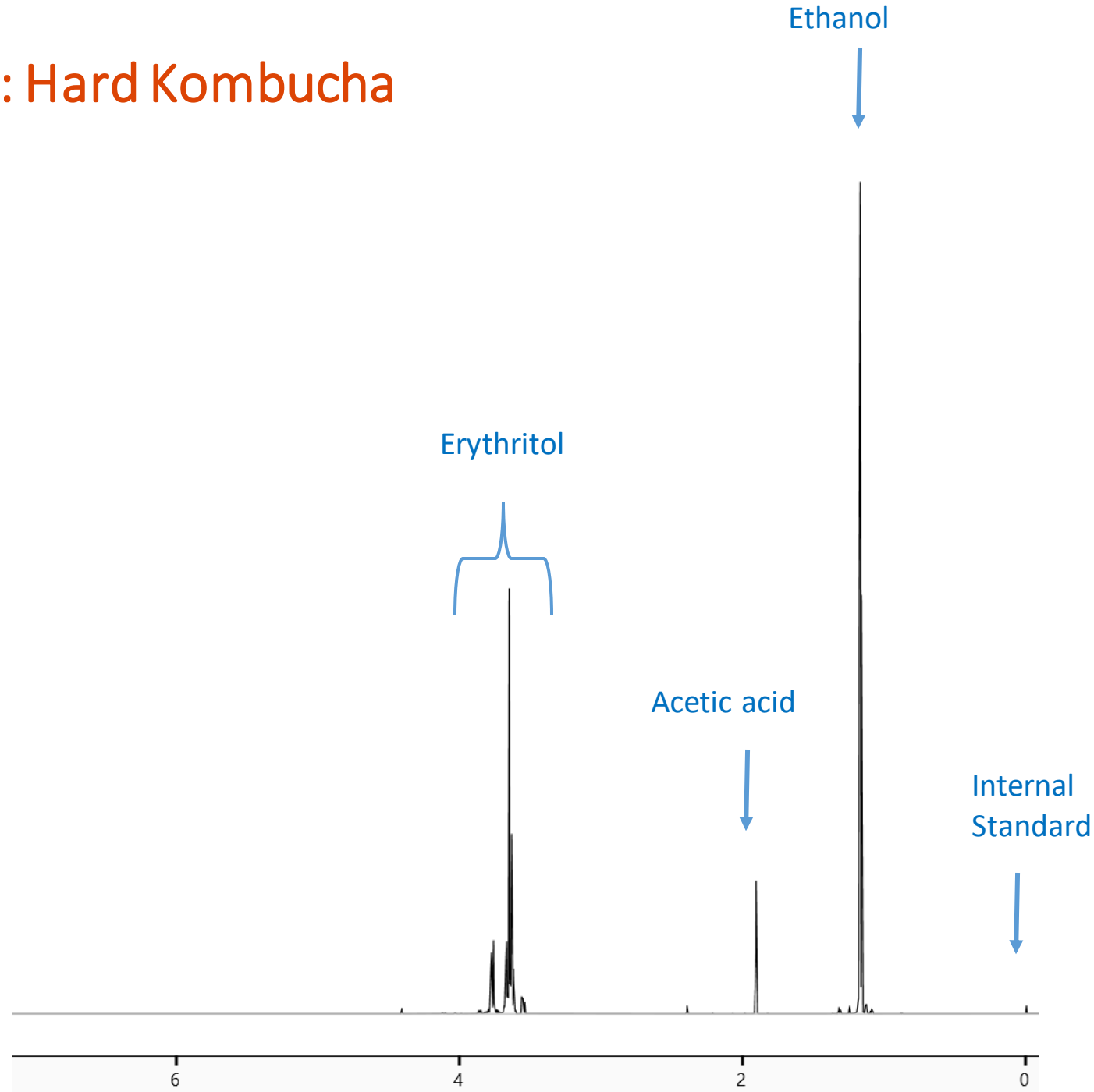


*"….**a sweet** yet biting profile making this **flavor** equal parts refreshing and tart..."*

RESULTS

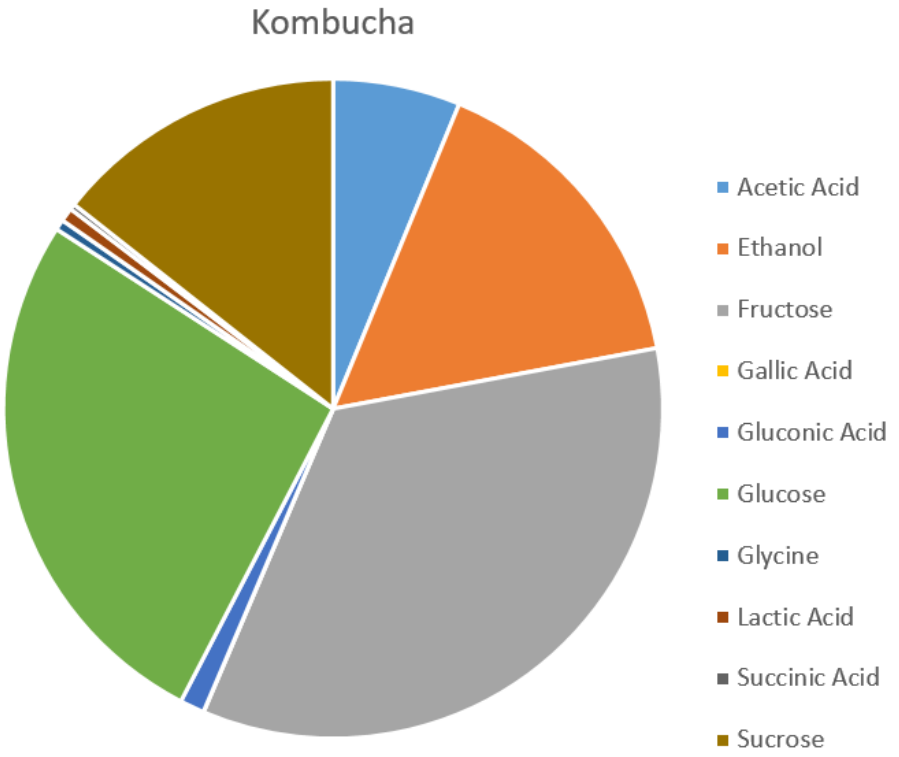
Reading NMR Spectra, Brand 3: Hard Kombucha

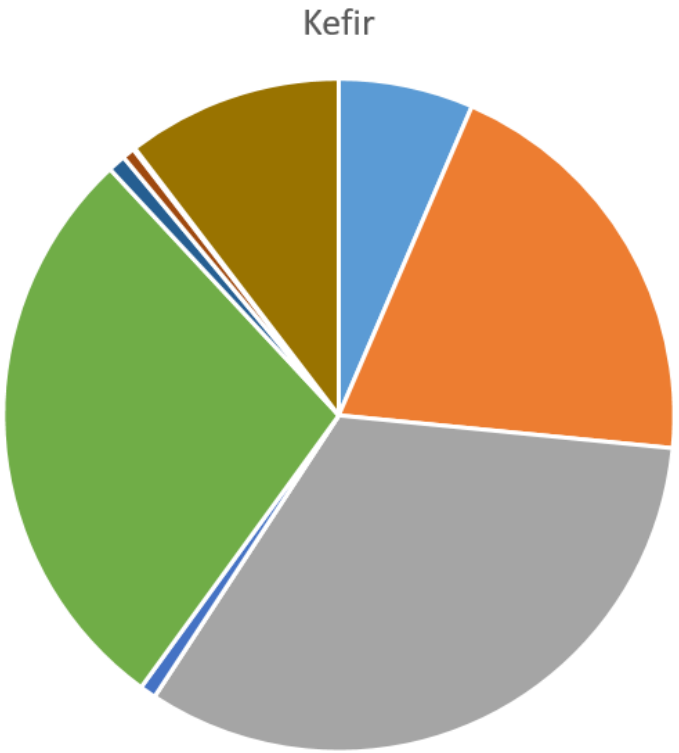
- Commercial Product
- Kombucha Styles, Pilot Study
- Hard Kombucha
- 7.0% EtOH
- Description: "Earthy and familiar with a crisp, cool taste that leaves the palate clean and refreshed."



RESULTS

Sparkling Probiotic vs Kombucha



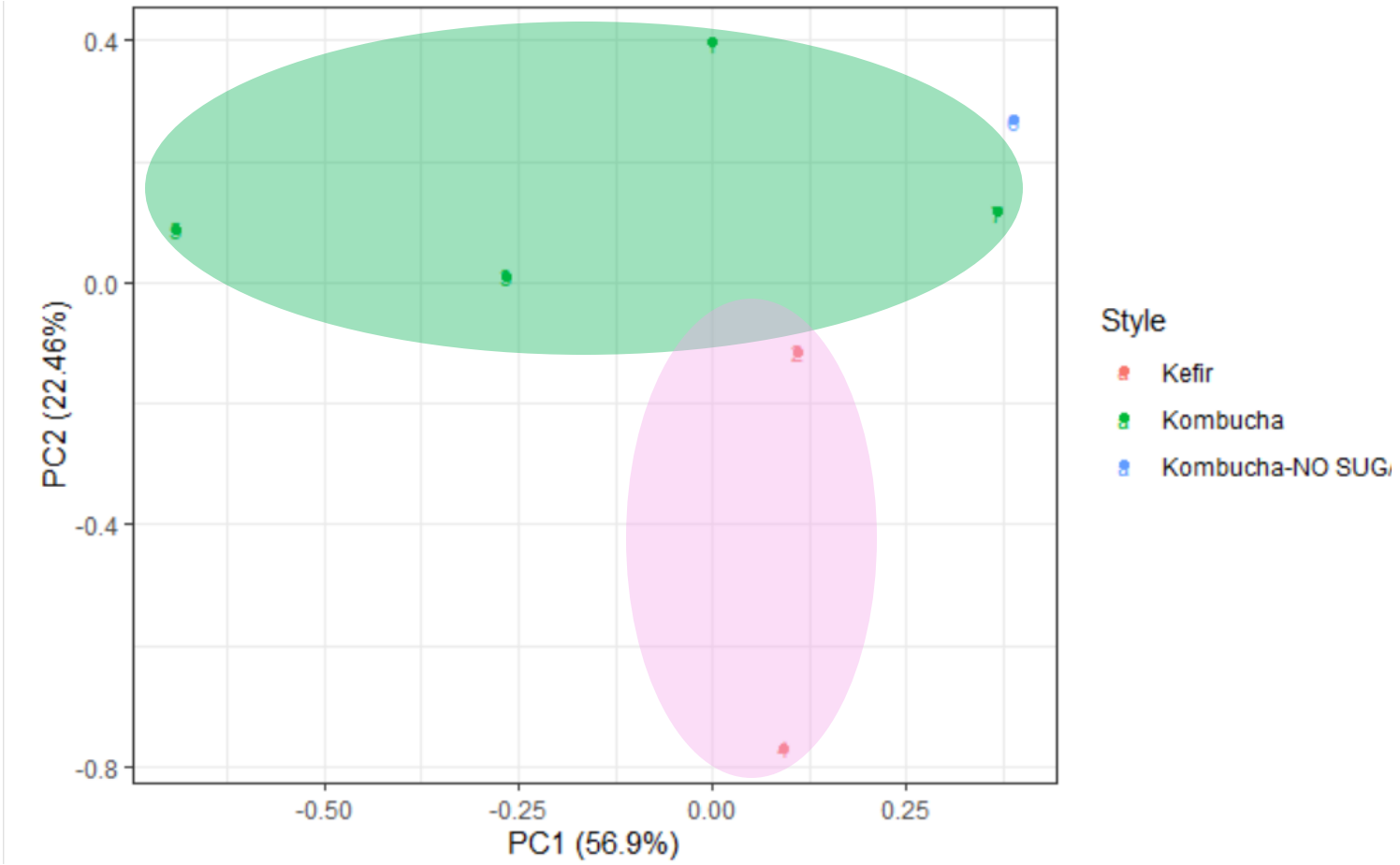


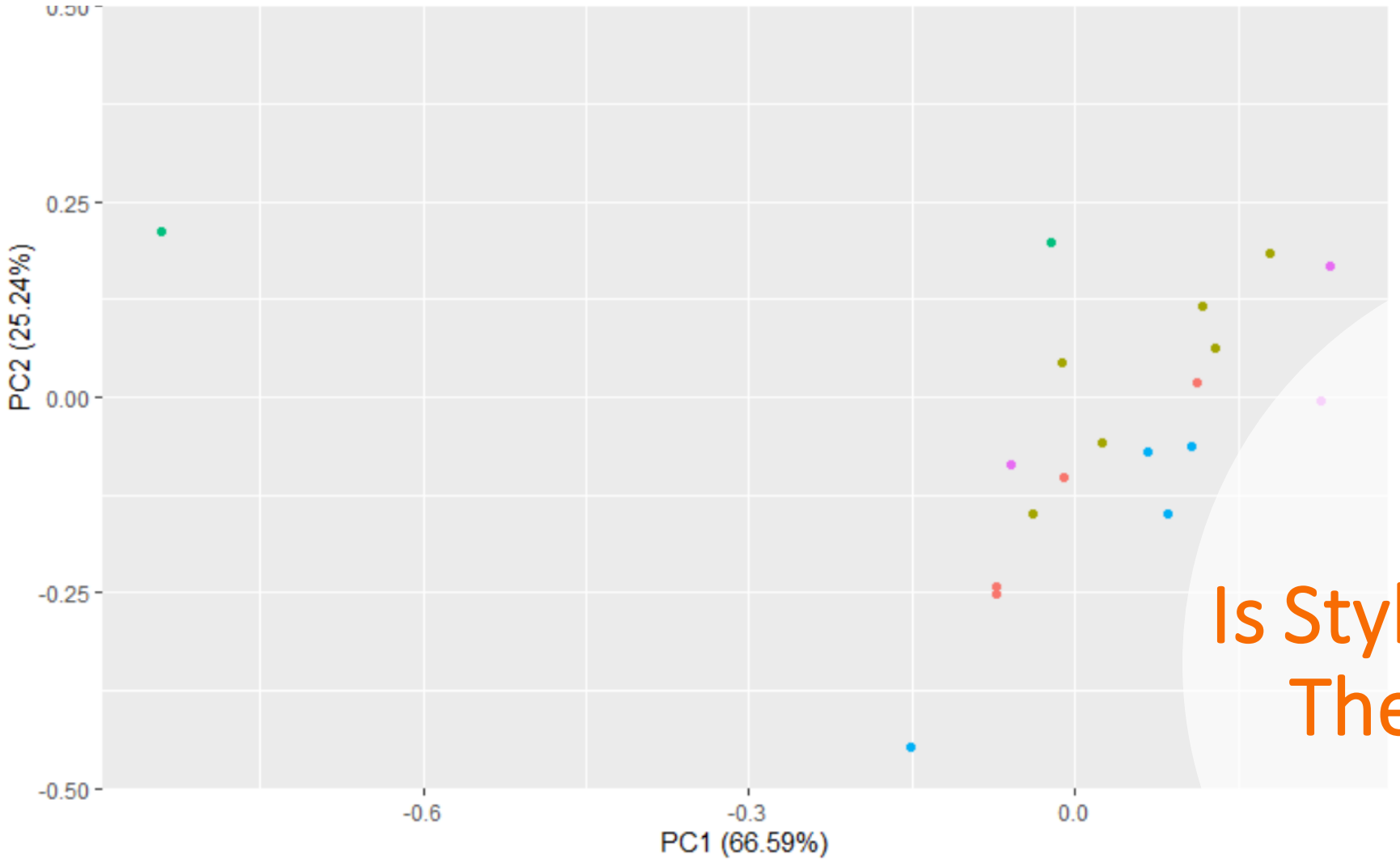
		mg/l
Analyte	**Kombucha**	**Kefir**
Acetic Acid	1815.889	753.05
Ethanol	4593.7105	2327.162
Fructose	9989.983	3792.129
Gallic Acid	3.966	0
Gluconic Acid	348.958	90.225
Glucose	7707.516	3250.555
Glycine	149.023	98.884
Lactic Acid	195.803	68.816
Succinic Acid	80.4695	11.033
Sucrose	4177.3005	1206.411

RESULTS

NMR distinguishing Kombucha from similar fermented products: Sparkling Probiotic Beverage vs Kombucha

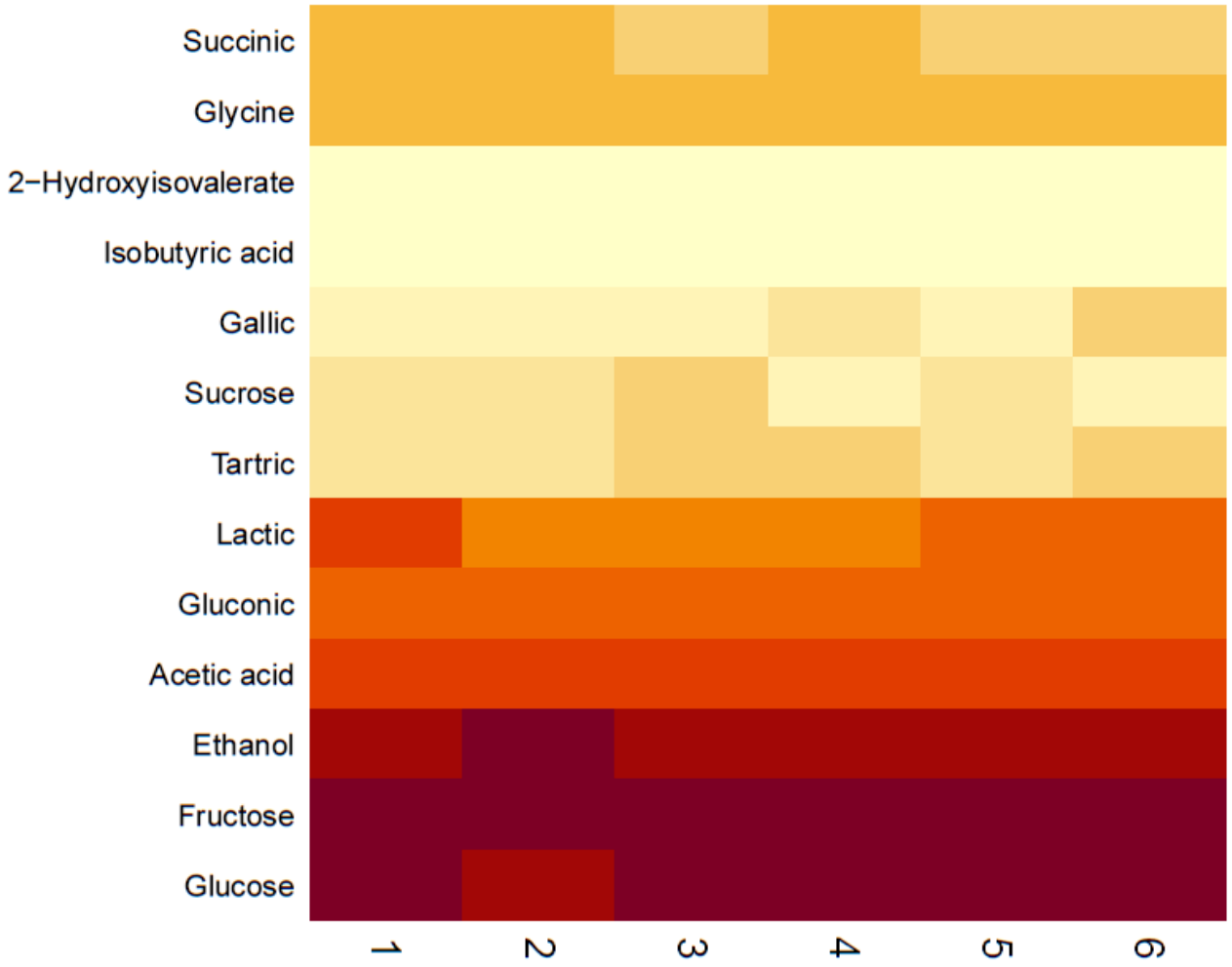
Samples	Style	Flavor
KS1.cnx	Kombucha	Ginger Lemon
KS2.cnx	Kefir	Ginger Gold
KS3.cnx	Kombucha	Honeydew
KS4.cnx	Kefir	Lemon Cayenne
KS5.cnx	Kombucha	Ginger Root
KS6.cnx	Kombucha-NO SUGAR	Ginger Lemonade
KS7.cnx	Kombucha	Ginger Lemon





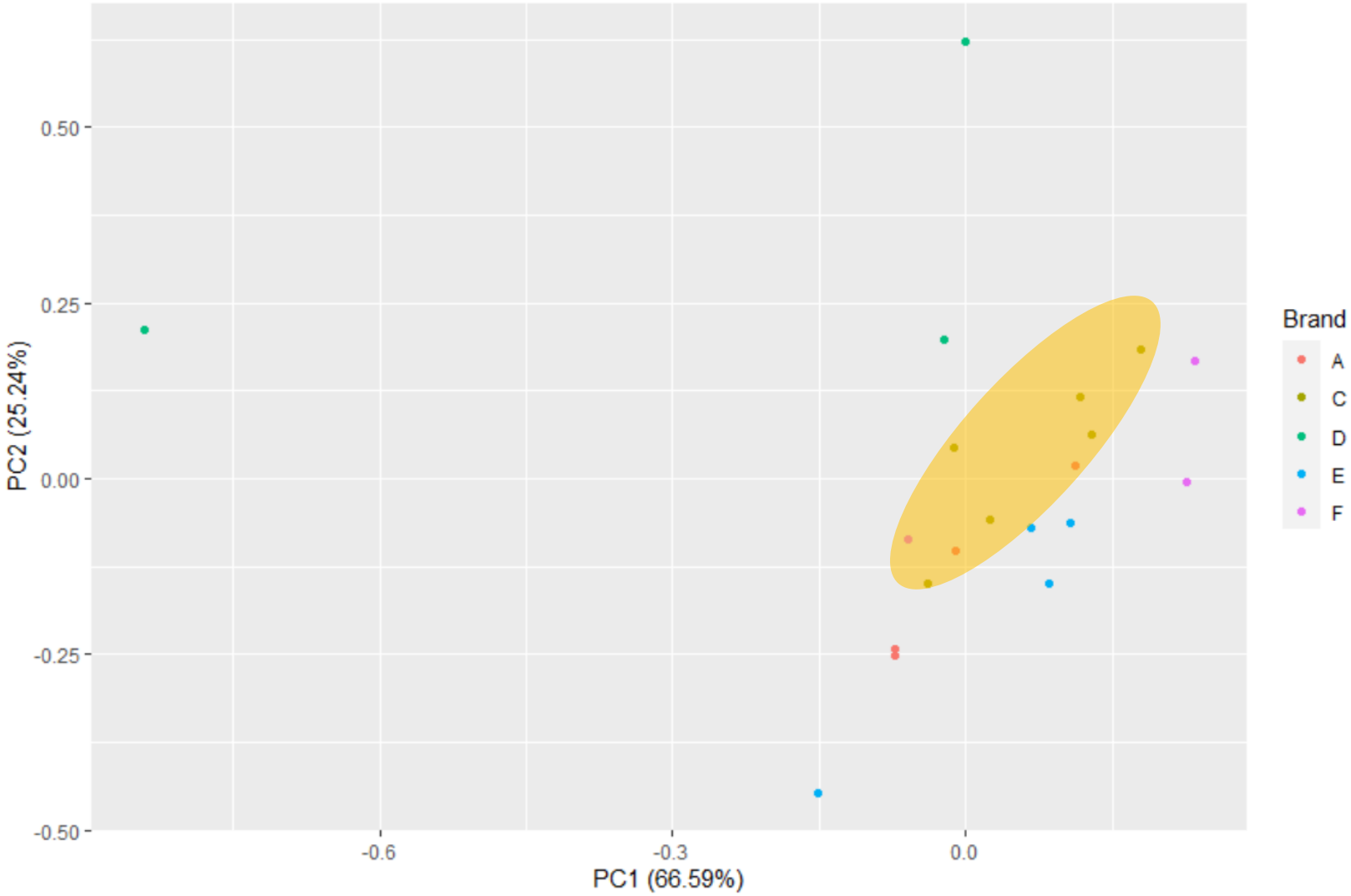
Is Style Defined By The Basis of a Brand?

Analyte Differences- within brand

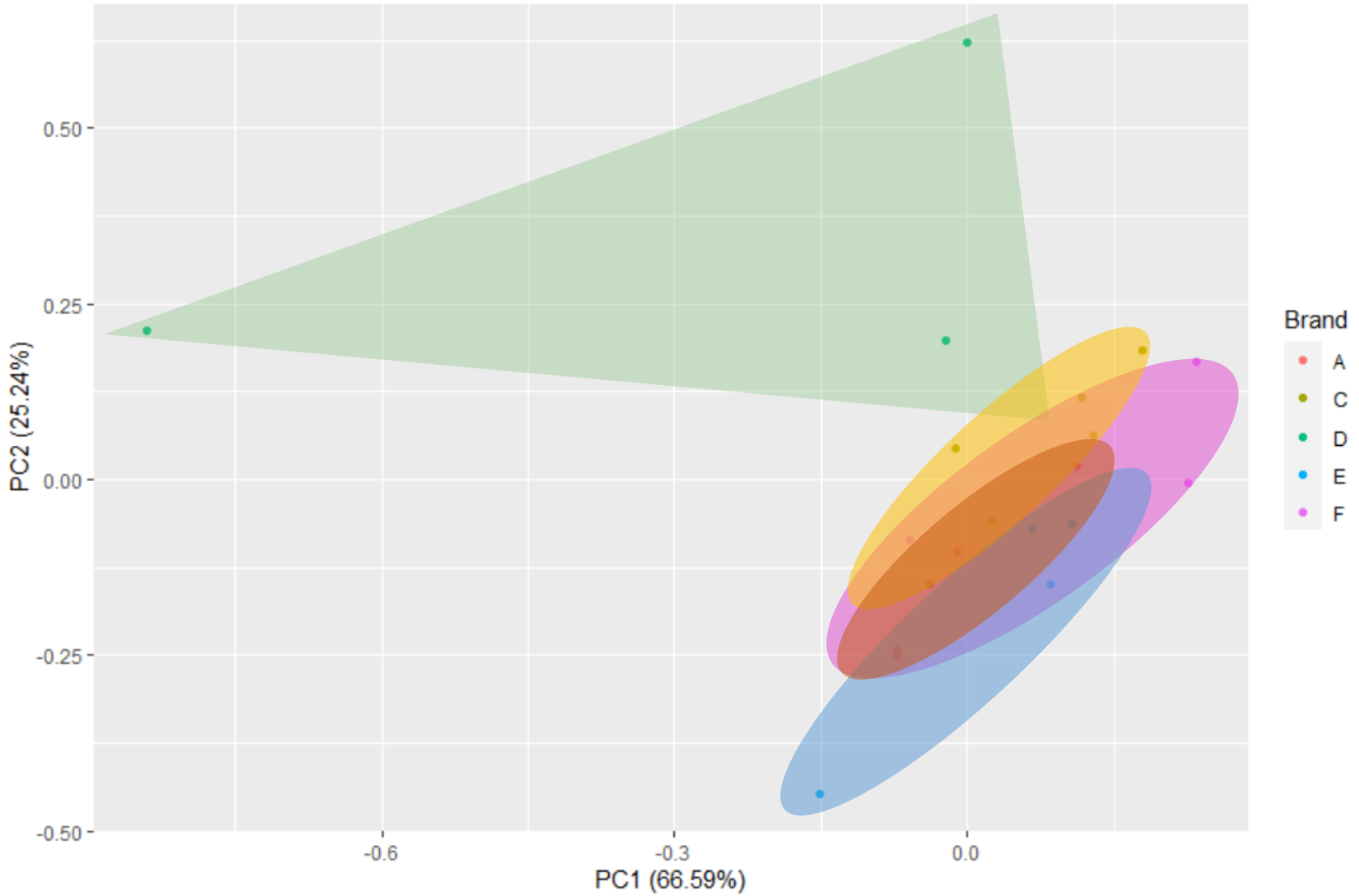


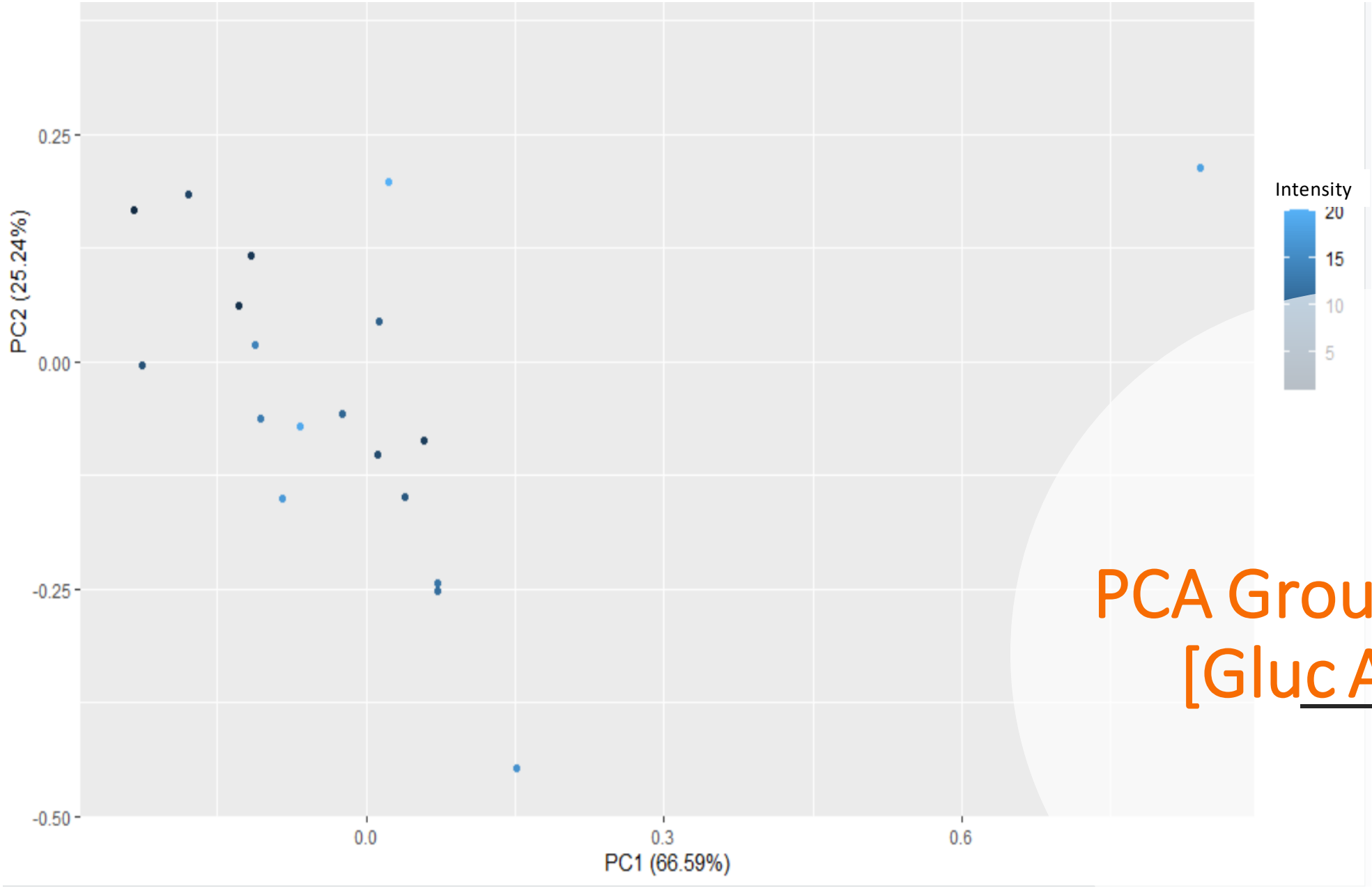
	Sweet	Sour	Tart
Flavor 1	14	15	15
Flavor 2	12	13	13
Flavor 3	14	13	13
Flavor 4	12	13	12
Flavor 5	17	14	15
Flavor 6	7	12	15

Brand Differences?

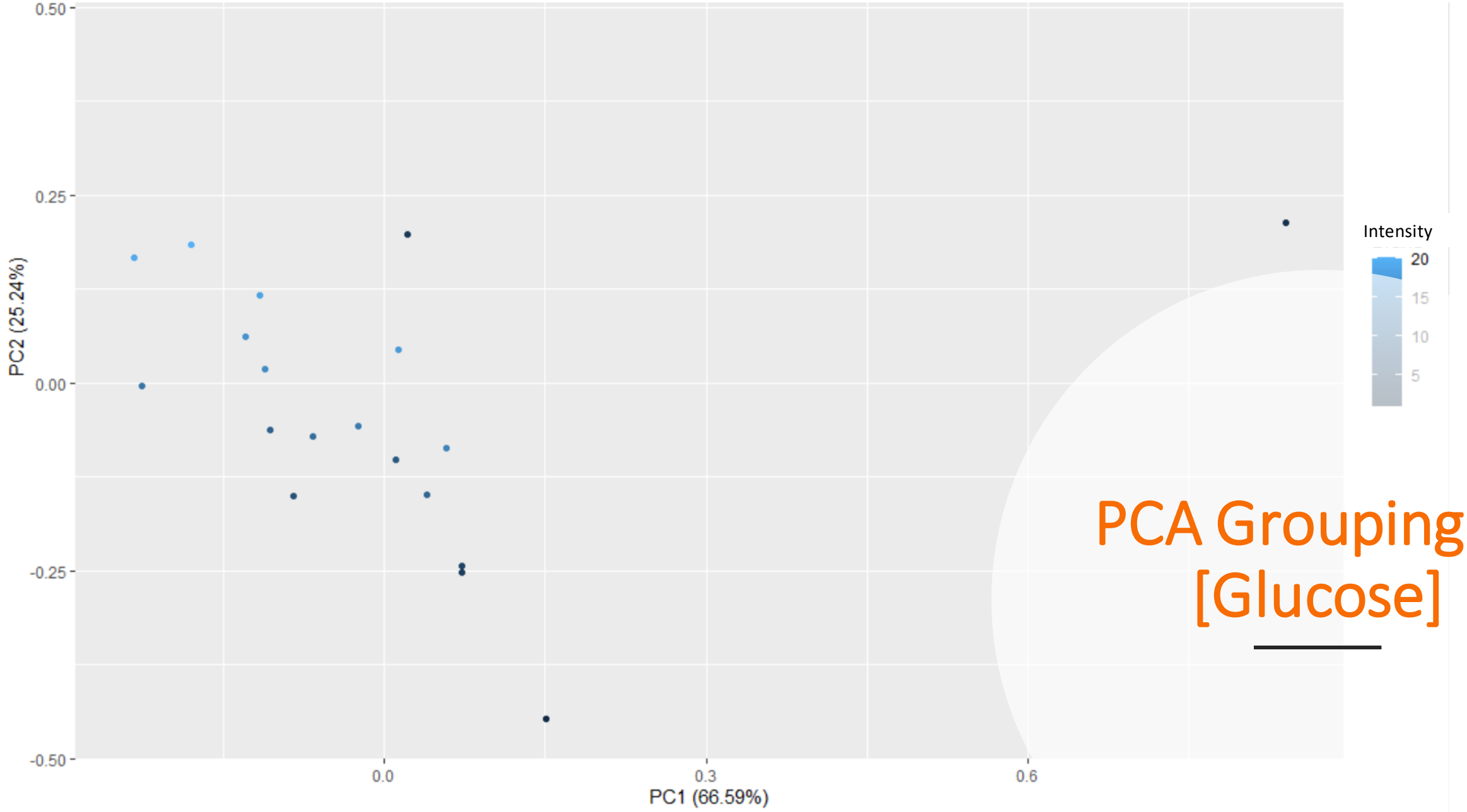


Brands Do Not Determine Analyte Profile

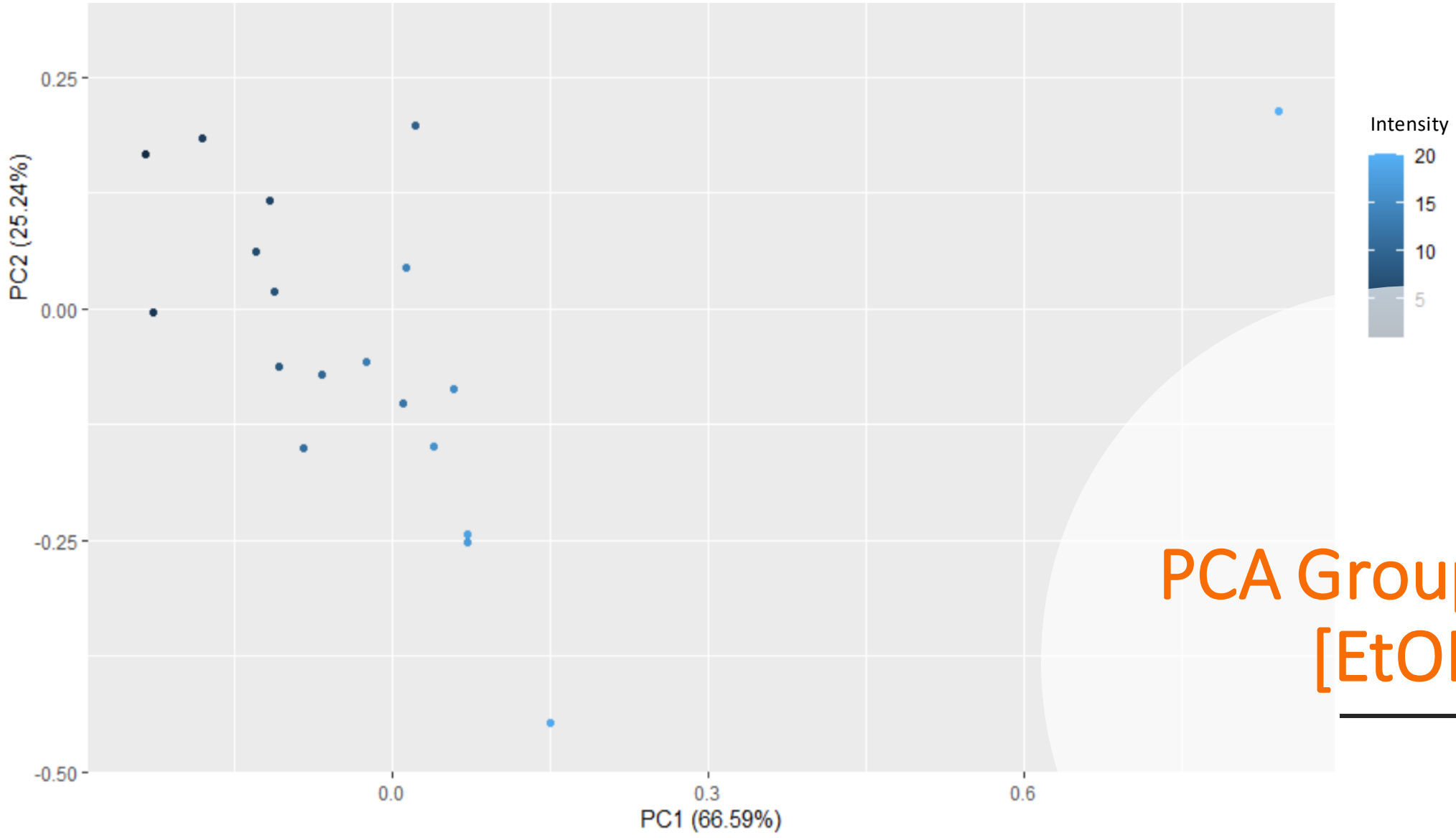




PCA Grouping by [Gluc Acid]

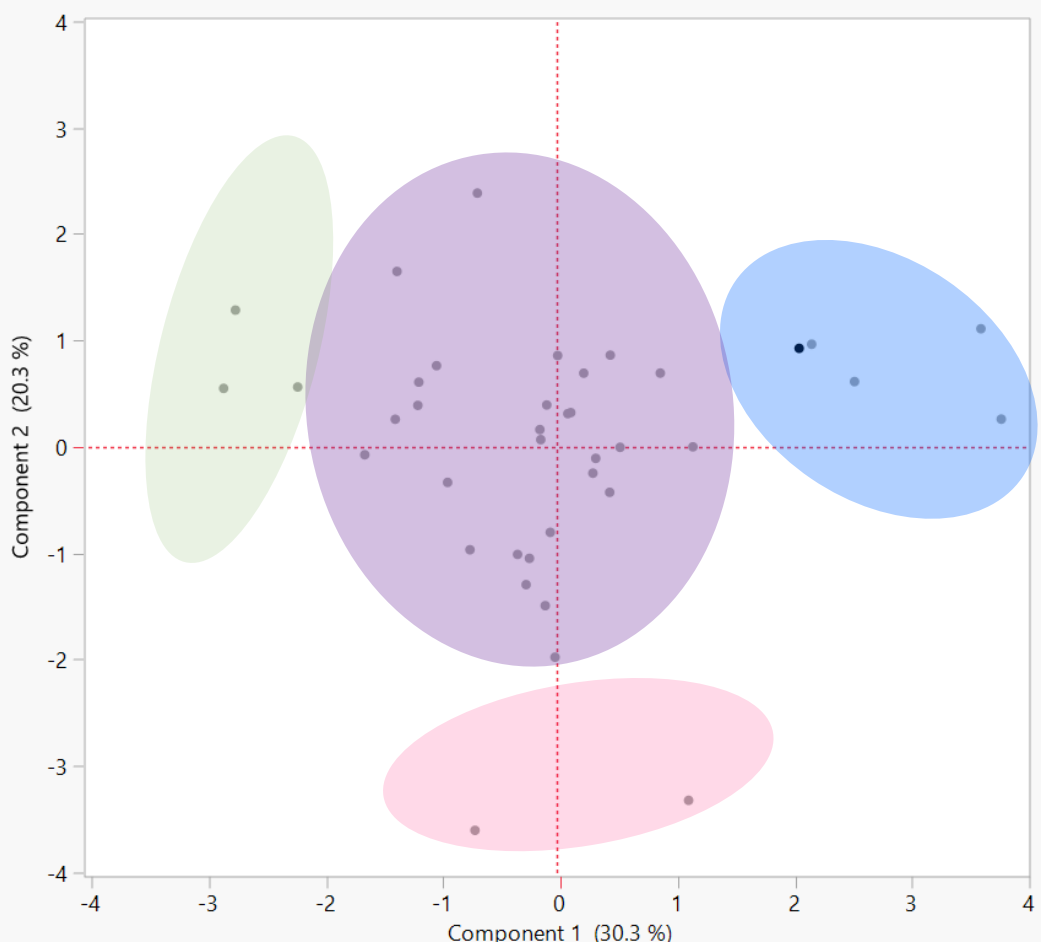


PCA Grouping by [Glucose]



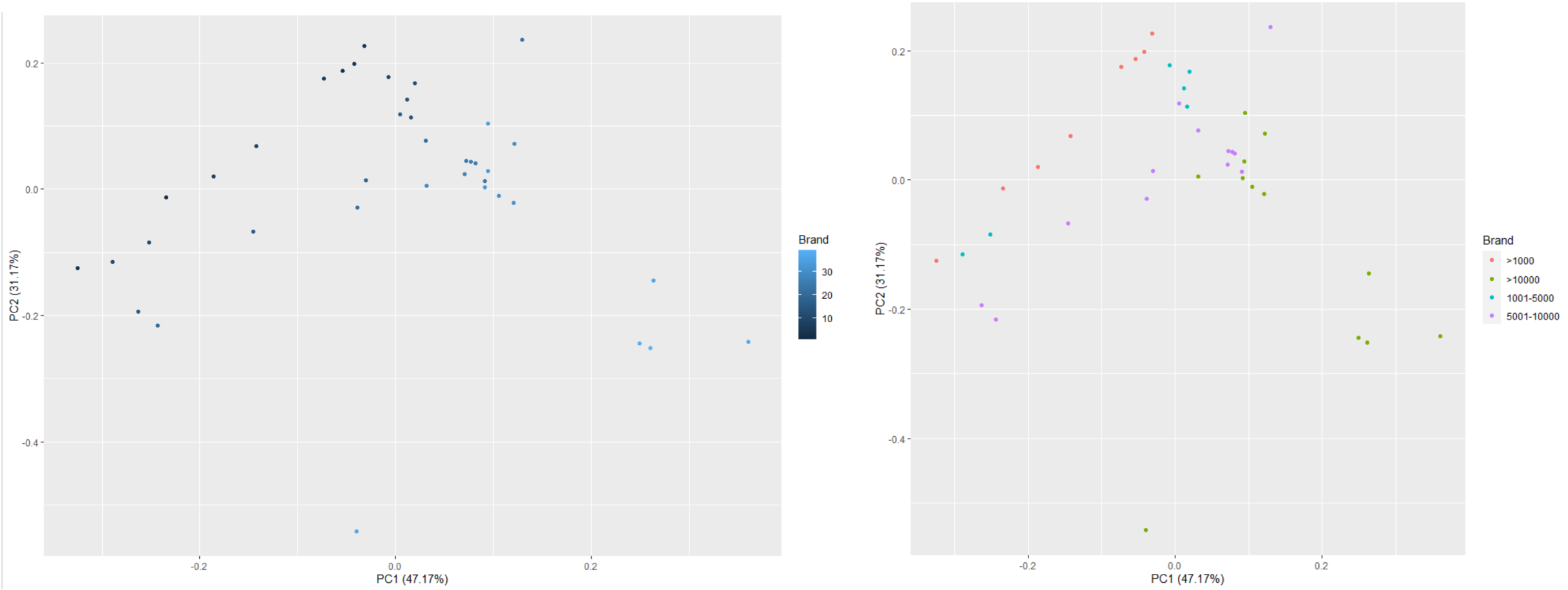
PCA Grouping by [EtOH]

Does [Glucose] Drive Style Designations From the 2018 Analyte Study?



Previous Findings....

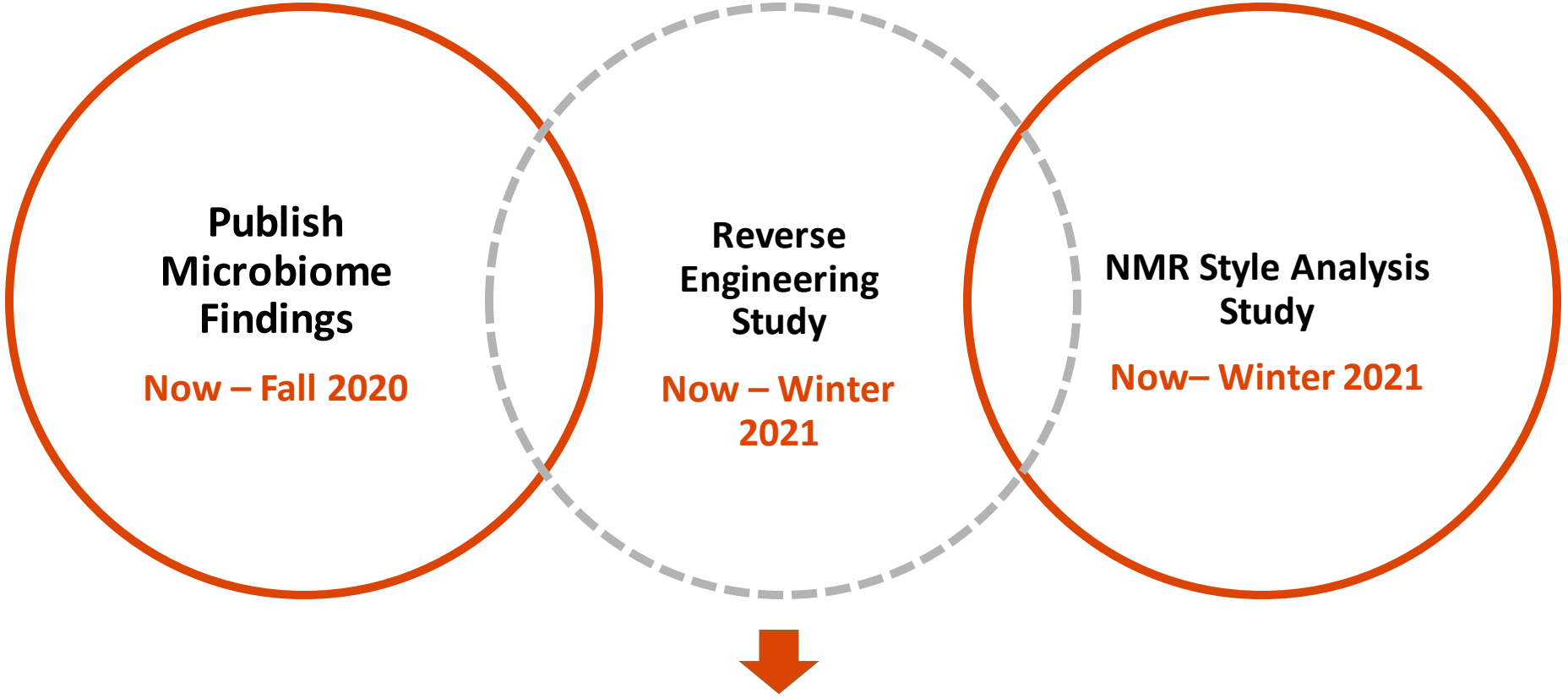
Using Glucose as a Style Designator



Summary

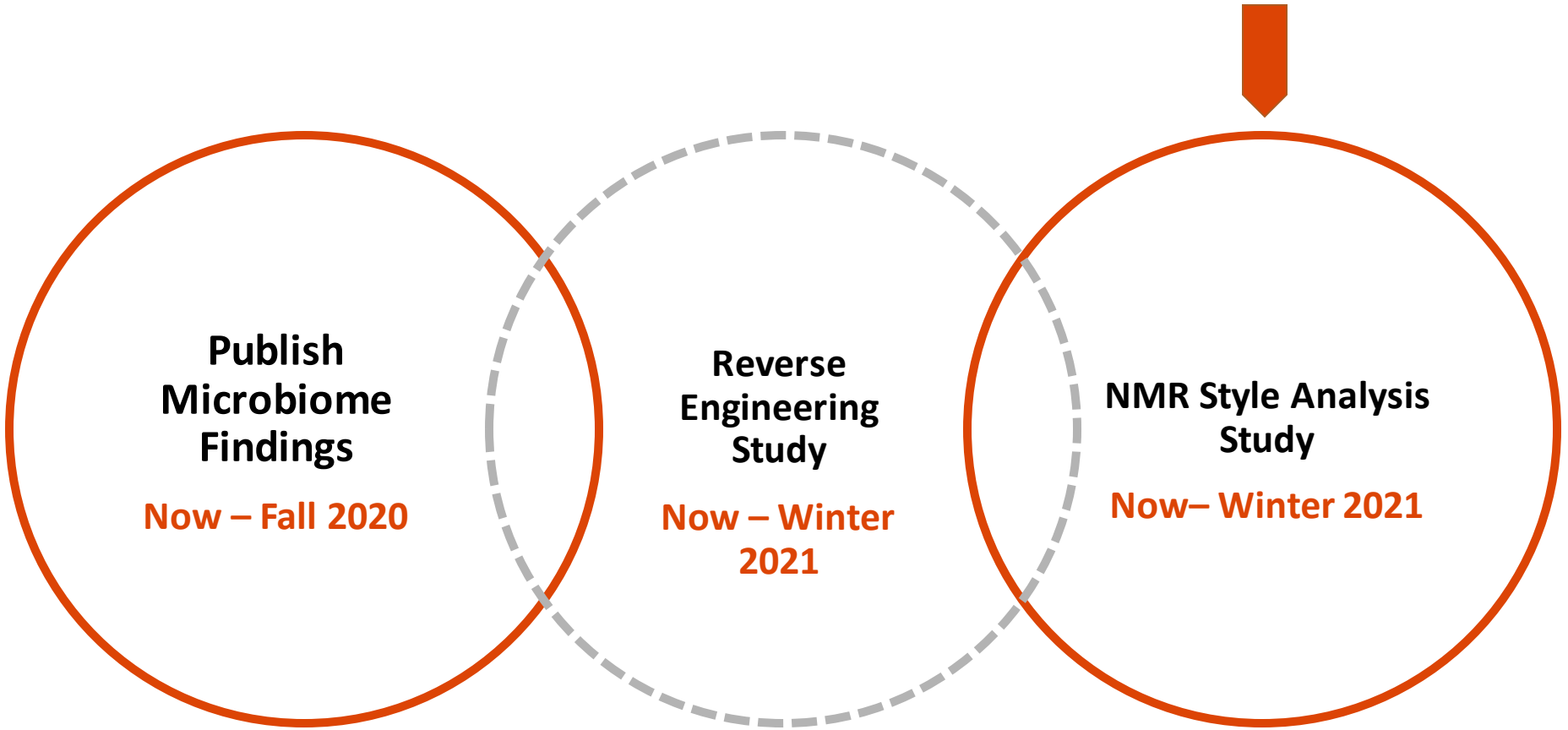
- [Glucose] may be used to identify styles of Kombucha
- NMR spectral fingerprints may be used to identify off-flavors
- Sesnory testing of flavors MUST be used to enhance style development

What are the next steps for Kombucha Research?



Interested in participating? Contact Keisha.Harrison@oregonstate.edu.

Where to get involved?



Interested in participating? Contact Keisha.Harrison@oregonstate.edu.

Thank you for Listening

Presented by Keisha Harrison, MS

Department of Food Science & Technology
Department of Microbiology
Center for Genome Research and Biocomputing

Questions?
Or
Comments?

Oregon State
University