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Investigating the Molecular Basis of Coffee Variability through Ultra-High Performance Liquid Chromatography with Tandem Mass Spectrometry Analysis and Melanoidin Characterization

By

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Chapter 1: Why Coffee Chemistry Matters

Wisconsin Institute for Science Literacy (WISL) Dissertation Chapter

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1.1 Personal Motivation

As a senior in high school, my teachers heavily discouraged me from attending a large university, especially as I intended to study science. These comments were not made from malice; my teachers genuinely cared about my success. They had simply watched many students from my background follow that same path and struggle, and they did not want the same to happen to me.

I grew up in a small, low-income town. My high school was poorly funded, which was reflected in the coursework. All my tests were written to be as easy as possible. Notably, my chemistry class did not involve a laboratory component – most of our time was spent watching videos on YouTube. This is not a criticism of my former teachers; they did the best they could with the resources they had.

Despite my lack of preparation, I still chose to attend the University of Illinois at Urbana-Champaign. By most metrics, I was very successful. I graduated with a 4.0 G.P.A. while double majoring in Honors Molecular and Cellular Biology and Chemistry. However, my high school teachers were correct – it was immensely difficult. Many of my colleagues had taken AP and college preparatory courses throughout high school. I had to work twice as hard to keep up, often at the expense of my mental health. I am still working through the mental illness that developed as a result.

At the same time, I recognize that I was privileged in many ways that made my success possible. I was raised to be stubborn and unyielding. I did not have to work while attending high school or college. I did not have to worry about my family – I knew that my parents were healthy, and my siblings would never go hungry. While both of my parents

had unconventional experiences with higher education, they did both have some experience. I was not on my own.

These privileges matter. My success is not a reflection solely of my intelligence – more importantly, it reflects circumstance. My high school peers were just as capable, but many of them lacked necessary resources and support systems.

It is my goal to make science more accessible and less intimidating to individuals from low-income and disadvantaged communities. Everyone deserves a strong scientific education, as well as the preparation and support needed to succeed. My passion for science education has inspired me to focus on scientific research that directly benefits the public, which has led me to study the chemistry of coffee.

1.2 Introduction

I recently acquired a programmable coffee maker with the “delay brew” function, and it has truly changed my life. I’ve never been much of a morning person, but waking up to the smell of coffee has become one of my favorite daily experiences. Coffee may have a hold on my heart, but at least I know I’m not alone. The 2025 National Coffee Association’s report states that two-thirds of American adults drink coffee daily, and American coffee drinkers average about three cups per day.[1] Coffee even accounts for more than 8% of the value of the entire U.S. food service industry.[2] In a country driven by productivity and profit, it is no surprise that coffee consumption is deeply woven into the fabric of our society.

Drinking coffee isn’t just about the energy boost – it’s also a social ritual that facilitates conversation, connection, and collaboration, especially in work settings.

However, as coffee is often used to counteract fatigue, it may also reinforce a culture of overwork and blurred boundaries.

If you are a coffee connoisseur, you likely have a preferred coffee roast and brewing technique – and perhaps even a preferred water source for brewing. But why does this matter? How does changing the roast level, brewing technique, and presence of minerals affect the taste and appearance of coffee? Furthermore, how do these changes affect the health advantages and disadvantages of coffee? Importantly, coffee is more than a drink. **It is a complex chemical system.** To answer these questions, it is necessary to look at coffee at the molecular level.

1.2.1 What's inside your cup?

One single cup of coffee contains over 1,000 unique molecules.[3] These molecules are constantly working together and interacting to generate the smell, taste, and appearance of coffee. These compounds in coffee fall into several categories, including alkaloids, acids, carbohydrates, proteins, and aromatic compounds (**Figure 1**). Alkaloids, like caffeine (**1**), serve as a stimulant and make coffee taste bitter.[4] Coffee tastes sour due to the presence of acids like citric acid (**2**) and astringent due to acids like chlorogenic acid (**3**).[5,6] Conversely, carbohydrates like sucrose (**4**) contribute to sweetness in green coffee beans, but many are broken down during roasting.[7] The smell and flavor of coffee is affected by many cyclic compounds, such as 2,3-dimethylpyrazine (**5**).[8] Cyclic compounds are chemical compounds that contain atoms that form a closed loop or ring. Many compounds in coffee, such as alkaloids and acids, have antioxidant and anti-inflammatory properties. Several acids are also antimicrobial and seem to be protective

against high cholesterol.[5] Thus, understanding the molecules in coffee is very important for human health.

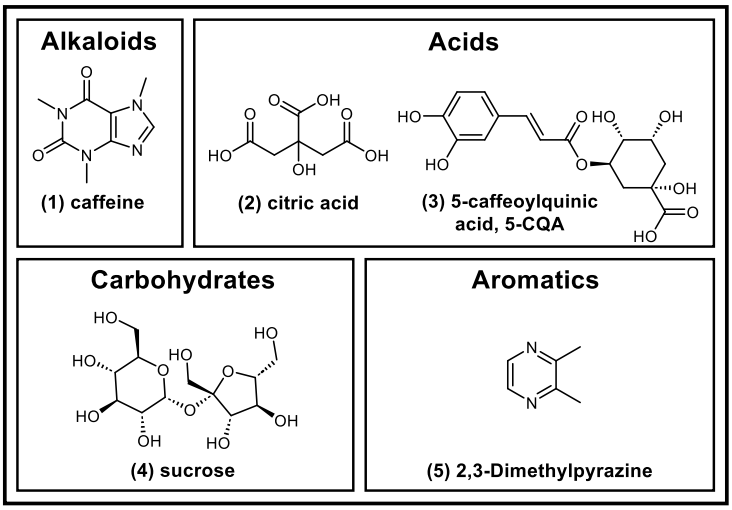

Figure 1 Select compounds found in coffee.

1.2.2 Roasting transforms coffee

When harvesting, coffee beans exist within a coffee cherry (**Figure 2A**). To isolate the green bean, coffee cherries are peeled and processed (**Figure 2B**). Prior to roasting, green coffee beans are composed primarily of complex carbohydrates (i.e. sugars).[9] During the roasting process, a series of chemical reactions will break down and recombine sugars, proteins, and organic acids into melanoidins.[10] Melanoidins contribute to the deep brown color of coffee. While many of the formation reactions and individual components of melanoidins are understood, the exact structure of melanoidins is unknown.[11–16] Additionally, the roasting process generates new flavors (caramel, nutty, smoky) and increases coffee bitterness.[17]

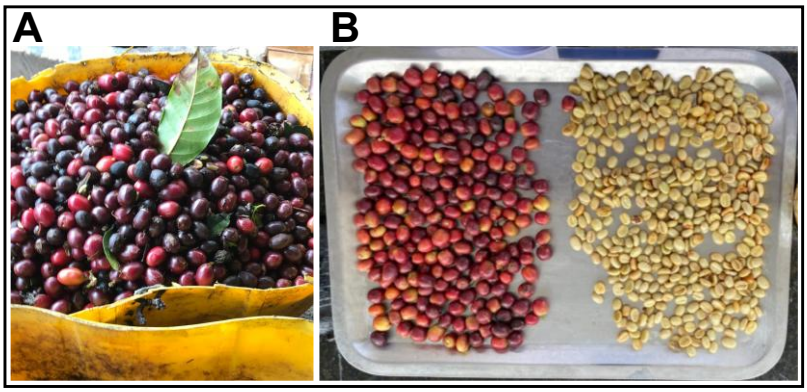

Figure 2 (A) Coffee cherries **(B)** Coffee cherries (left) and washed green coffee beans (right). *Photo Credit: Juan Carlos Carmona-Hernandez, Universidad de Manizales*

1.2.3 Brewing Temperature

The brewing temperature of coffee is very influential in the taste, sight, flavor, and composition of a cup of coffee. Broadly, during coffee brewing (i.e. extraction), water will be used to pull compounds out of the coffee grounds – but not all compounds will be removed equally. Compounds removed will depend on the brewing temperature and the length of the brew. Brewing conditions can take the same beans and transform them into very different drinks.[18]

1.3 Analysis Techniques

1.3.1 Ultra-High Performance Liquid Chromatography Coupled with Mass Spectrometry

Coffee is composed of thousands of molecules.[3] Understanding coffee chemistry requires identifying and quantifying the small molecules present in solution. One tool that is frequently used for such tasks is Mass Spectrometry (MS). MS is often used in

combination with liquid chromatography, which is used to separate complex mixtures. MS allows scientists to “weigh” molecules.

All molecules are made of atoms, and each atom has a specific mass. For example, carbon atoms have a mass of 12 atomic mass units (amu), while oxygen atoms have a mass of 16 amu. To calculate the mass of a molecule, we simply add up the masses of the individual atoms. Carbon dioxide (CO_2), for instance, has a mass of 44 amu (12+16+16 = 44). As a brief note, the total weight of an atom is determined by the number of protons and the number of neutrons present in the atom. It is possible for atoms of a particular type (i.e. carbon) to vary in the number of neutrons, leading to different “isotopes.” For simplicity’s sake, I am not discussing variation in the number of neutrons.

MS functions by giving molecules a charge. Then, the molecules move through the instrument, and their mass-to-charge ratio (m/z) is recorded. Frequently, the charge (z) is either +1 or -1, so the mass of the compound is very close to the mass-to-charge ratio. This process creates a mass spectrum, which serves as a readout of all the masses present in solution.[19]

However, there is a problem: multiple different compounds can share the same mass. For example, both ethanol and dimethyl ether (**Figure 3**) contain 1 oxygen, 2 carbons, and 6 hydrogens, so they both have a mass of 46 amu. If a mass of 46 amu is recorded by MS, it will be unclear if the compound detected was either ethanol or dimethyl ether.

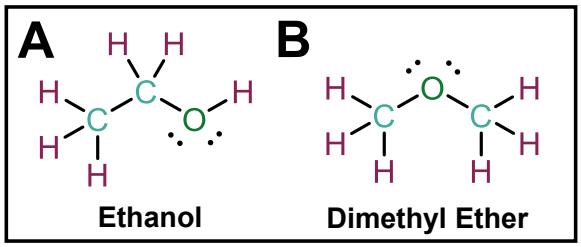

Figure 3 (A) Chemical structure of ethanol **(B)** Chemical structure of dimethyl ether.

Thankfully, we can still differentiate these molecules by MS – we just have to do a bit more work. After the detector collects the aforementioned data, molecules can be diverted to a different chamber, where they break into small pieces as a result of being bombarded with high-energy gas molecules. Molecules will break apart differently based on how the atoms were originally connected. The mass-to-charge data for these fragments will be collected by the detector, and the MS2 spectrum is generated.[20,21]

To better understand fragmentation, imagine that the two block structures in **Figure 4** are molecules. Both molecules are made up of 1 long brick, 2 medium bricks, and 4 short bricks, so they would not be distinguishable after the first phase of MS (MS1). However, the bricks are connected differently in block A vs. block B. In the second phase of MS (MS2), we see that these blocks will break apart (fragment) in different ways based on the connectivity of the bricks. Studying these fragmentation patterns helps to identify and differentiate block A and block B.

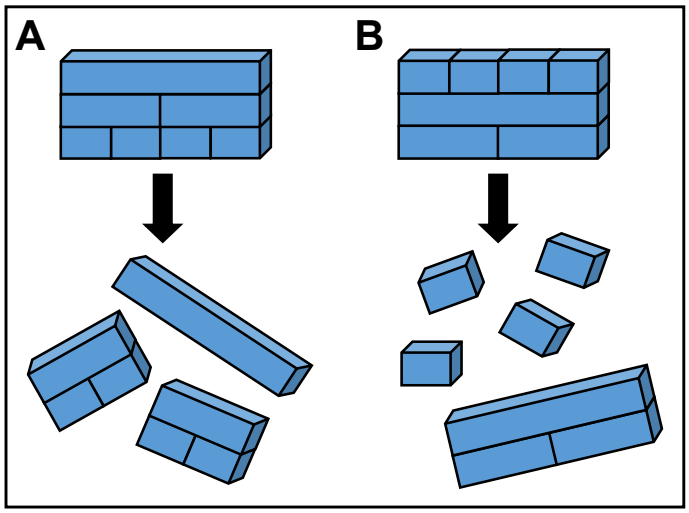

Figure 4 Blocks formed from identical components but with differing connectivity will break apart differently.

1.3.2 Total Phenolic Content

Coffee is rich in antioxidants, which are beneficial to human health. Compounds called polyphenols are a type of antioxidant. Polyphenols are naturally found in plants, and they protect plants from UV rays, pests, and disease.[22] Polyphenols are also responsible for the bright colors in many fruits and vegetables.[23] When we eat plants containing polyphenols, these polyphenols protect our cells, too.[22]

In all multi-cellular organisms, normal processes generate very reactive molecules known as free radicals. At high amounts, free radicals can damage cells by reacting with DNA and protein. Polyphenols and other antioxidants react with free radicals, making it less likely that these molecules will cause cellular damage.[24,25]

Because antioxidants are relevant to human health, it is useful to be able to quantify the amount of antioxidants in a coffee solution. One possible method for antioxidant quantification is the Folin-Ciocalteu (F-C) assay. As many antioxidants in

coffee are polyphenols (**Figure 5**), the total antioxidant capacity of a solution is frequently used to estimate the total phenolic content of a solution.[26]

The F-C assay uses a pre-mixed combination of phospho-metallic acids. Phospho-metallic acids consist of metals that are bound to phosphorus, and they are really good at accepting electrons. On the contrary, antioxidants are really good at donating electrons. While the exact process is a bit complex, the key idea is that the antioxidants will donate electrons to the phospho-metallic acids via a redox (reduction-oxidation) reaction, which causes the phospho-metallic acids to change in color from yellow to blue. Conveniently, the intensity of the blue color corresponds to the degree of reaction with antioxidants, and I can quantify the intensity of blue color by measuring how much light the solution can absorb at a specific wavelength (765 nm).[27–29] To make the results comparable across experiments, a standard compound called gallic acid is used (**Figure 5**). Antioxidant activity is often reported in gallic acid equivalents (GAE).[30]

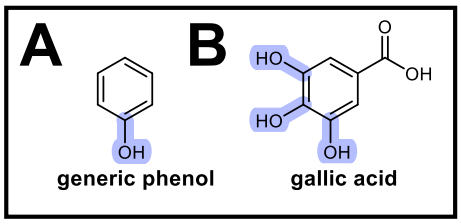

Figure 5 Chemical structures of **(A)** generic phenol and **(B)** gallic acid. The bonds colored purple are phenolic bonds.

1.4 Results and Discussion

1.4.1 How does origin and roast level affect coffee composition?

Colombia is a major contributor to the global coffee market. In the 2024/2025 season, Colombia generated 13.2 million 60-kg bags of coffee, ranking third globally.[31] This amount of coffee would completely fill over 500 Olympic-sized swimming pools. As of March 2023, specialty coffee accounted for 52% of Colombian coffee exports – which is a 15% increase from 2010.[32–34] As the Colombian market transitions to more specialized coffee, more rigorous seed selection, processing, harvesting, and drying is required.[34,35] Colombian specialty coffees are often produced by smallholder farmers and family roasters. Thus, it is important to understand how these specialty coffees compare to coffees made by large manufacturers for the U.S. market.

To understand the effect of origin on coffee composition, I compared three Colombian coffee samples (CM1, CM2, CM3) and three U.S. market coffee samples (UL1, UM1, UD1), listed in **Table 1**, to understand how differences in processing and market affect the underlying chemistry.

Table 1: Summary of coffee sample descriptors.

Market Name	Code[1]	Roast Level	Origin	Bean
Café Arte	CM1	Medium	Colombia	Arabica
Café Barranquero	CM2	Medium	Colombia	Arabica
Café Los Herrera	CM3	Medium	Colombia	Arabica
Starbucks® Veranda Blend®	UL1	Light	Latin America	Arabica
Starbucks® Pike's Place® Roast	UM1	Medium	Latin America	Arabica
Starbucks® Caffé Verona®	UD1	Dark	Asia/Pacific	Arabica

[1]The first letter of the abbreviation represents the commercial origin of the coffee sample (Colombia or the United States); the second letter represents the roast (light, medium, or dark).

1.4.1.1 Coffee Melanoidin Content (MC), pH, Total Phenolic Content (TPC), and Titratable Acidity (TA)

A brief note about statistics: in several of the following bar graphs, letters appear above each bar. I generated these letters by having a computer program run a “Tukey’s Multiple Comparison Test” on specific data points. After this test had been run, the program assigned different letters to samples that were meaningfully different, but the same letter to samples that were not meaningfully different. Samples were meaningfully different when there was a $p \leq 0.05$. A p value less than 0.05 means that there is a less than 5% chance that the result was solely due to random chance.

Broadly speaking, darker roasts contain more “roasting products,” while lighter roasts keep more of the original coffee bean compounds. As previously mentioned, one roasting product formed is melanoidins. Because the brown color in coffee comes primarily from melanoidins, I can use the intensity of the brown color (i.e. amount of light a coffee sample absorbs at 410 nm) to estimate the melanoidin content.[36–39] Detailed in **Figure 6A**, I found that melanoidin content is similar between the three Colombian medium roasts and the U.S. market light roast. Furthermore, the melanoidin content is similar between the U.S. market medium and dark roasts. Interestingly, I found all three Colombian samples had fewer melanoidins than the U.S. market medium roast sample, indicating that a medium roast in Colombia is more like a light roast on the U.S. market.

Knowing the acidity of a coffee solution is very useful, especially as coffee connoisseurs tend to value more acidic coffees.[40] The acidity of a coffee solution can be measured in two different ways, and both techniques measure something slightly different. pH measures how acidic coffee is at a specific moment, whereas titratable

acidity measures the total possible acidity of coffee. pH exists on a logarithmic scale, which means that a pH of 4 is ten times as acidic as a pH of 5 and 100 times as acidic as a pH of 6. **Figure 6B** depicts the average pH for the six coffee samples. Collectively, I found that U.S. market samples are less acidic (i.e. more basic) than Colombian coffee samples. My data in **Figure 6C** shows that samples with a lower pH have a greater total possible acidity. Importantly, both pH and titratable acidity measure acidity at the molecular level. Not all acids result in a sour taste, so pH and titratable acidity are not necessarily a metric of coffee taste.

Lastly, **Figure 6D** depicts a comparison in the total phenolic content, as measured by the Folin-Ciocalteu assay (described above in 1.3.2). I found that my Colombian samples had a greater abundance of polyphenols than my U.S. market samples. Coffees with more polyphenols often taste more acidic, more complex, and more astringent.[41]

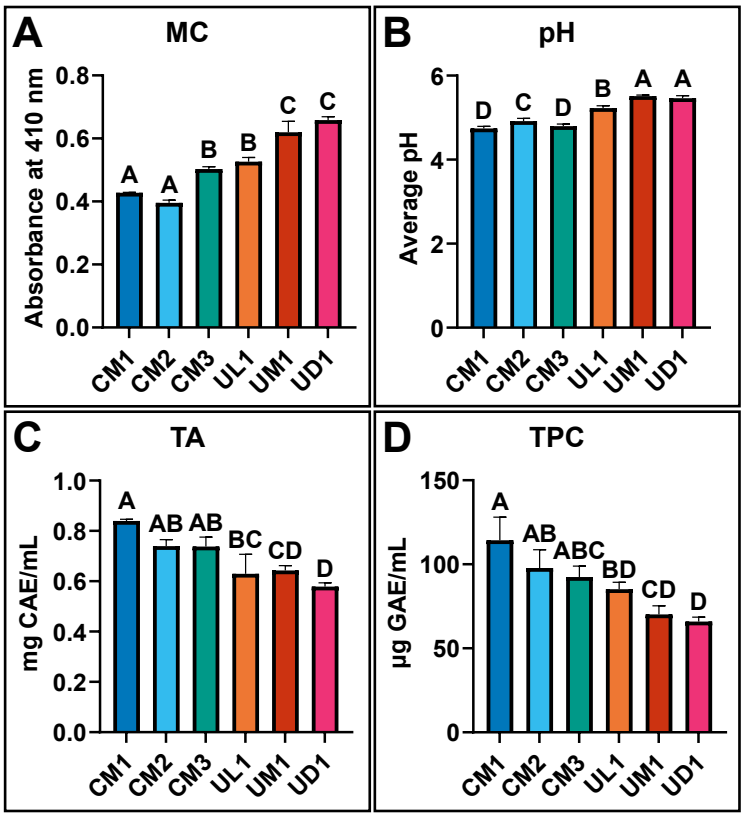

Figure 6 Comparison of coffee **(A)** melanoidin content (MC), **(B)** pH, **(C)** titratable acidity (TA), and **(D)** total phenolic content (TPC). *Data are expressed as means ± standard deviations of triplicate determinations of each sample. Statistical significance levels by one-way ANOVA were $p < 0.0001$ for all data plots. Letter designations (A-D) were generated via Tukey's multiple comparisons test, where different letters represent sample groups with statistically significant differences in means, $p \leq 0.05$.* **(C)** TA was reported in mg of citric acid equivalents (CAE) per mL. **(D)** TPC was measured by the Folin-Ciocalteu assay and is expressed as µg of gallic acid equivalents (GAE) per mL of coffee.

1.4.1.1 Identifying and Quantifying Specific Small Molecules in Light and Dark Roast Coffee Samples

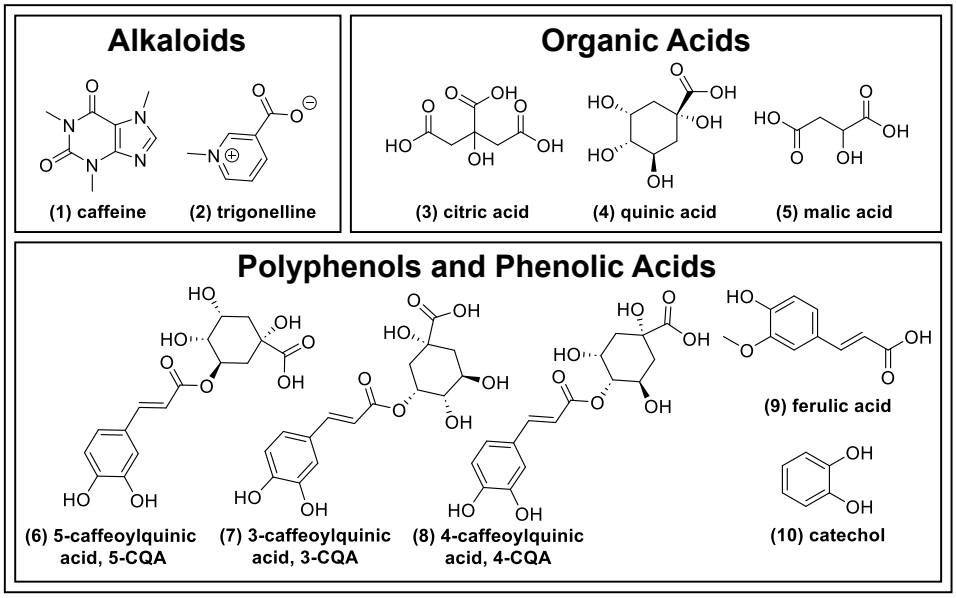

Figure 7 Chemical structures of compounds found in coffee.

MS can be used to identify and quantify specific small molecules in coffee. **Figure 7** shows the chemical structures of ten different compounds that I detected in my coffee samples. These compounds contribute to coffee taste[5,42] and are associated with potential health benefits. Furthermore, **Figures 8-10** depict the abundance of these compounds in my coffee samples, detailing roast-based and market-based differences. Understanding differences in compound abundance will improve understanding of the health advantages and disadvantages of coffee, as well as the flavor profile.

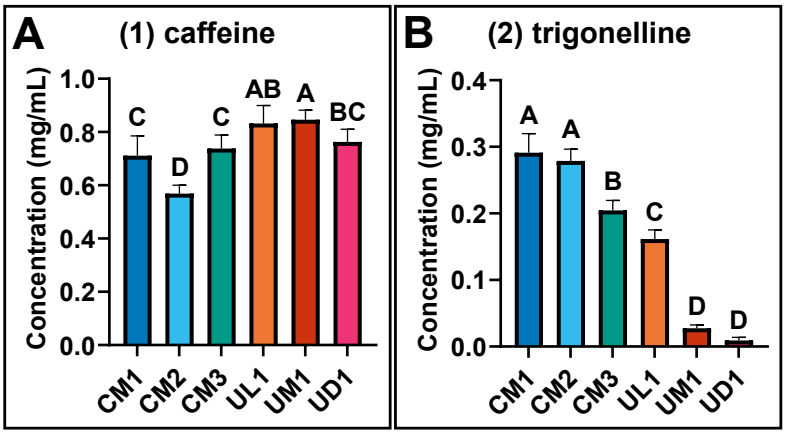

Figure 8 Concentration (mg/mL) of **(A)** (1) caffeine and **(B)** (2) trigonelline in brewed coffee samples. *Data are expressed as means ± standard deviations of triplicate determinations of each sample. Statistical significance levels by one-way ANOVA were $p < 0.0001$ for both alkaloids. Tukey's multiple comparisons tests were used for post hoc analysis, where different letters represent sample groups with statistically significant differences in means, $p \leq 0.05$.*

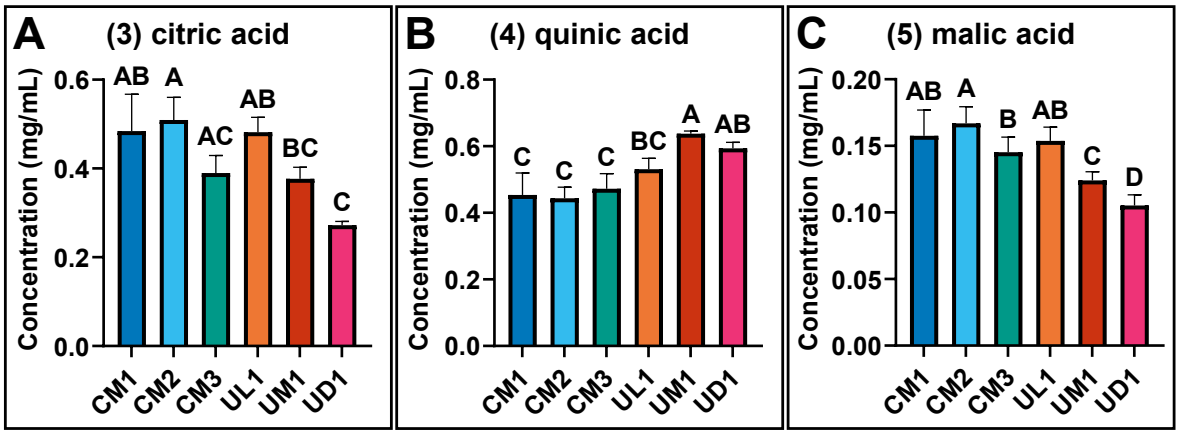

Figure 9 Concentration (mg/mL) of **(A)** (3) citric acid, **(B)** (4) quinic acid, and **(C)** (5) malic acid in brewed coffee samples. *Data are expressed as means ± standard deviations of triplicate determinations of each sample. Statistical significance levels by one-way ANOVA were p = 0.0003 for (3) citric acid, p = 0.0002 for (4) quinic acid, and p < 0.0001 for (5) malic acid. Tukey's multiple comparisons tests were used for post hoc analysis, where different letters represent sample groups with statistically significant differences in means, p ≤ 0.05.*

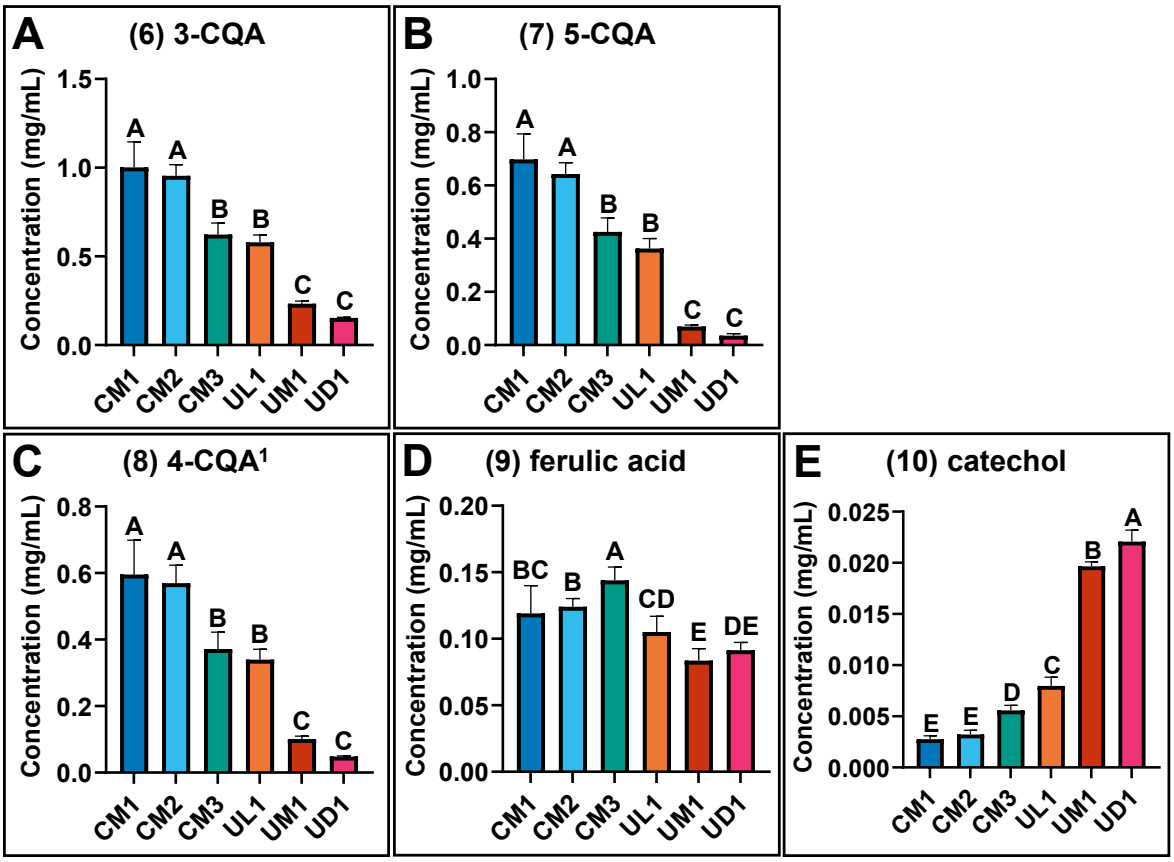

Figure 10 Concentration (mg/mL) of **(A)** (6) 3-CQA, **(B)** (7) 5-CQA, **(C)** (8) 4-CQA[1], **(D)** (9) ferulic acid, and **(E)** (10) catechol in brewed coffee samples. *Data are expressed as means ± standard deviations of triplicate determinations of each sample. Statistical significance levels by one-way ANOVA were $p < 0.0001$ for all compounds. Tukey's multiple comparisons tests were used for post hoc analysis, where different letters represent sample groups with statistically significant differences in means, $p \leq 0.05$. [1]4-CQA is expressed as 5-CQA equivalents.*

I found that trigonelline (2, **Figure 8**), malic acid (5, **Figure 9**), chlorogenic acids (6, 7, 8, **Figure 10**), and ferulic acid (9, **Figure 10**) were more abundant in lighter roasts than darker roasts. Chlorogenic acids (6, 7, 8, **Figure 10**), and ferulic acid (9, **Figure 10**) are polyphenolic acids. Thus, they are associated with reducing damage to cells,

inflammation, and the risk of chronic disease.[24,25] Malic acid influences the fruit flavors in coffee.[42] Trigonelline is not currently associated with any physiological functions in humans,[43] but it has many possible health benefits. There is evidence that suggests trigonelline is able to stop bacterial infections and prevent the progression of cancer,[44] as well as lower cholesterol[45] and blood sugar.[46]

Conversely, I found that quinic acid (4, **Figure 9**), and catechol (10, **Figure 10**) were more abundant in darker roasts than lighter roasts. Both quinic acid and catechol are expected to be more abundant in darker roasts, as both are products of roasting-induced chlorogenic acid degradation.[47,48] Quinic acid is a contributor to the bitter and astringent taste associated with dark roasts.[49] Catechol, however, has a harsh and rubber-like odor that rarely is observed in coffee.[47]

The relationship between caffeine (1, **Figure 8**) content and roast level is a bit more difficult to understand. Typically, caffeine is more abundant in lighter roasts than darker roasts, as roasting leads to some caffeine degradation, but the difference is minimal.[50,51] Previously, I showed that CM1 and CM2 have the lowest melanoidin content out of all the samples (**Figure 6A**). As melanoidins are formed during roasting, the samples with the lowest melanoidin content are the least roasted. So, I would expect caffeine content to be highest in CM1 and CM2, but this is not what I observed. This indicates that there are other factors (i.e. bean processing or brewing method) influencing caffeine content. Increased caffeine consumption in humans often results in improved memory and protection against neurodegenerative diseases, but it can have a negative effect on heart rate, anxiety, insomnia, and digestive issues.[52]

1.4.2 How does brewing temperature affect coffee composition?

As previously mentioned, the brewing temperature selected can result in changes in the compounds that end up in your final coffee product. To better understand the effect of brewing temperature, I compared four samples, detailed in the table below (**Table 2**). All these coffee samples originated from the same bean and processing strategy, meaning that any differences in these samples are a result of brewing temperature or roasting time.

Table 2 Summary of coffee samples.

Coffee Sample	Roast	Brewing Temperature
LC	Light	Cold
LH	Light	Hot
DC	Dark	Cold
DH	Dark	Hot

1.4.2.1 Grouping Coffee Samples Together to Identify Trends

UHPLC-MS/MS can be used to identify and quantify specific small molecules in coffee. However, UHPLC-MS/MS can detect thousands of compounds in a single coffee sample, resulting in a massive amount of data. It would be very difficult and immensely time-consuming to identify and quantify every single molecule detected. Conveniently, there are strategies that I can employ that will streamline my data and allow me to look for overarching trends.

One such method is principal component analysis (PCA) (**Figure 7**). I perform PCA by uploading all the MS data I collect to an analytical computer program. Then, the program will analyze and sort the samples based on the similarities and differences

detected in their chemical composition. Samples that are closer together on a PCA plot are more compositionally similar. Every “dot” on the PCA plot represents a different sample; dots that are the same color represent replicates of the same type of sample. The shaded ellipses represent statistical significance. For example, if I were to test any LH coffee sample, there is a 90% chance the dot would appear in the area colored teal.

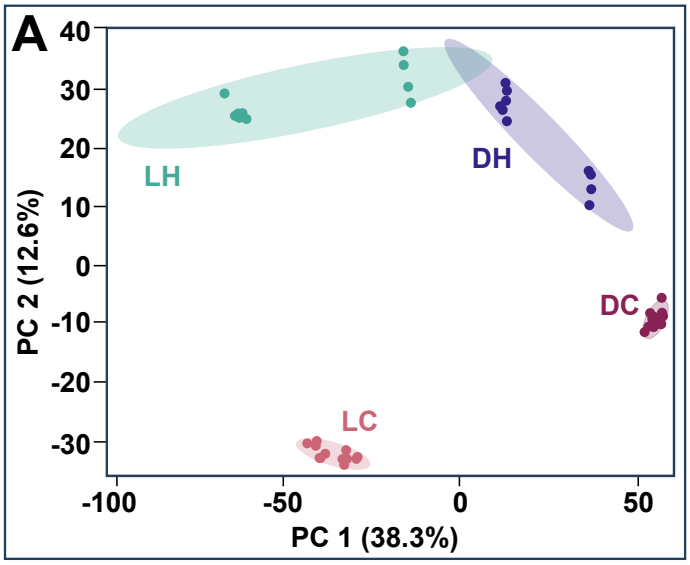

Figure 7. Multivariate analysis of brewed coffee with different roast levels and brewing temperatures depicted by principal component analysis (PCA) scores plot. Principal component 1 (PC 1) and PC2 account for a combined 50.3% of the variability among the different coffee samples. The ellipses correspond to a 90% confidence of assignment.

Another way I can analyze the differences between coffee samples is by determining how many small molecules significantly differ in concentration between any two samples. If the percentage is large, the two coffees being compared are quite distinct. In **Table 3**, DH & LH, LC & LH, and DH & DC have very minimal differences in the

abundance of detected compounds. Interestingly, LC and DC are the most distinct samples, with 10.6% of compounds detected differing in concentration between samples.

Table 3 The percentage of total compounds that significantly differed in concentration between any two samples**,** $p \leq 0.05$.

	LC	**LH**	**DC**	**DH**
LC		0.8	10.6	2.4
LH	0.8		3.1	2.1
DC	10.6	3.1		0.8
DH	2.4	2.1	0.8	

Lastly, I looked at which compounds differ in abundance between two samples by using a volcano plot, where every dot on the plot represents a unique compound. In **Figure 9A**, compounds colored pink are at least twice as abundant in LC relative to LH, whereas compounds colored teal are at least twice as abundant in LH relative to LC. **Figure 9B** shows similar results when comparing DC and DH.

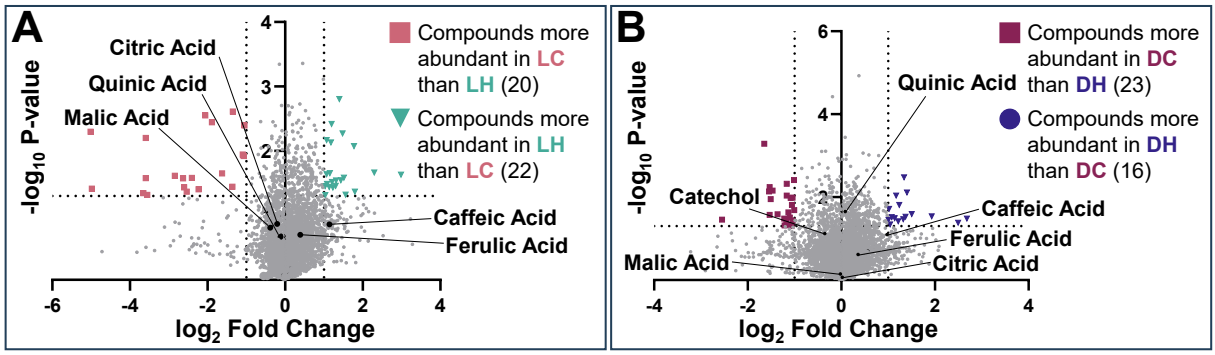

Figure 9 Volcano plot depicting differences in compound composition between **(A)** LC and LH and **(B)** DC and DH. Each point represents a compound. Compounds above y = 1.3 have $p \leq 0.05$. Compounds to the left of x = -1 or to the right of x = 1 have a > 2-fold change in abundance.

1.5 Conclusions

Coffee is a complex chemical system, and this complexity makes it difficult to research and holistically understand. However, scientists can use tools like mass spectrometry (MS), as well as experiments to measure polyphenolic content and melanoidin content, to begin to understand this complexity.

I researched the effects of roast level, origin, and brewing temperature on coffee composition. I found that definitions of roast level are not consistent across different regions, as a Colombian medium roast is more like a U.S. market light roast than a U.S. market medium roast. I've learned that samples with a higher melanoidin content will often have a higher pH and fewer acidic molecules present in the coffee. In studying the effects of brewing temperature, I verified that both brewing temperature and roast level influence coffee composition. Only 0.8% of compounds differed significantly in abundance between

cold brew and hot brew for both light and dark roasts. Interestingly, it seems that roast level is a more significant factor in the composition of cold brew than hot brew, as only 2.1% of detected compounds differed significantly between LH & DH, but 10.6% of detected compounds differed significantly between LC & DC.

When talking about my research with others, I'm frequently asked "What kind of coffee should I be drinking? What is the best/healthiest?" And, truthfully, I don't know. There is still so much the scientific community must learn about the human body and coffee composition before that question can be answered. So, for now, I'd say that the best coffee for you to drink is the coffee that you enjoy the most.

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