Identification of Compounds that Impact the Ready-to-drink Coffee Flavor Stability during Storage Using LC-MS Flavoromics

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2021

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Abstract

Coffee is one of the most popular beverages worldwide. Ready-to-drink (RTD) coffee provides consumers a convenient alternative to freshly brewed coffee. In the United States, RTD coffee has become increasingly popular because of the growing demand for convenient beverage options. However, RTD coffee requires additional processing and storage, creating challenges with flavor stability. The current understanding of RTD coffee flavor stability is limited and impedes product innovation. The overall objective of this project was to identify the non-volatile chemical compounds that impact RTD coffee flavor stability during storage using untargeted flavoromics.

In Phase I, untargeted LC/MS flavoromics analysis was applied to identify chemical compounds that were generated during storage and impacted the flavor stability of ready-to-drink (RTD) coffee. Two coffee samples (Arabica and Robusta) prepared in air and under nitrogen were stored over 4 months at 30 ºC. Degree of difference (DOD) sensory evaluation revealed significant changes in the RTD coffee after 1, 2 and 4 months. MS chemical profiles of non-aged and aged RTD coffee samples were modeled against the DOD scores by orthogonal partial least squares (OPLS) with good fit ($R^2Y = 0.966$) and predictive ability ($Q^2 = 0.960$). Five highly predictive chemical features positively correlated to DOD were subsequently identified as 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3-O-feruloylquinic acid, and 5-O-feruloylquinic acid. These

five chlorogenic acids compounds in addition to quinic acid significantly increased in concentration during storage. Sensory recombination tests confirmed that these six acid compounds significantly impacted the flavor stability of RTD coffee during storage, primarily by reducing the pH of the product.

In Phase II, untargeted LC/MS flavoromics analysis was applied to identify chemical compounds that were degraded during storage and impacted the flavor stability of ready-to-drink (RTD) coffee. Ten highly predictive chemical features that negatively correlated with flavor changes were selected based on the multivariate statistical model established in Phase I. Next the compounds were isolated by multi-dimensional LC fractionation and subsequently identified (MS and NMR). Quantitative analysis indicated eight of the ten compounds were significantly ($p<0.05$) degraded during storage. Sensory recombination testing of the eight compounds as a mixture and individually revealed two of the highest predictive compounds, 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide, impacted the flavor stability of RTD coffee at subthreshold concentrations. Furthermore, these two compounds were completely degraded after 4 months of storage.

Overall, this work demonstrated the application of untargeted LC/MS flavoromics to identify compounds and mechanisms that impact RTD coffee flavor stability during storage. Compounds identified in this work provide a novel basis to optimize the preservation of RTD coffee flavor during storage, therefore allowing the development of higher quality RTD coffee products.

Dedication

To my beloved grandmother and parents who have been always supporting me throughout my education and growth

Acknowledgments

First, I would like to express my sincere appreciation to my advisor, Dr. Devin G. Peterson, for his mentorship and guidance during my time at FREC. His passion, patience, support, and trust have been always helping me overcome the challenges that I had in the past 4 years. To me, Devin is more than an advisor; he is also a teacher, a mentor, and a friend.

I would also like to extend my gratitude to my committee members, Dr. Chris Simons, Dr. Jessica Cooperstone, and Dr. Emmanouil Chatzakis, for their valuable inputs and suggestions on my research.

I owe a great debt of gratitude to Dr. Laurianne Paravisini. I would never forget the things she has taught me, as well as the happy time and laughter we had. I am also indebted to Dr. Edisson Tello Camacho for his immeasurably support in this work. Specially thanks to Julie Peterson for her guidance, support, and encouragement.

Many thanks to my lab mates and friends at the OSU. This friendship has carried me through the long journey.

Lastly, I would like to thank the important people in my life. Very special thanks to my father, my mother, and my girlfriend, Dr. Weijia Shi.

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Chapter 1. Literature Review

Coffee is a popular drink that millions of people consume every day. In recent years, ready-to-drink (RTD) coffee has gained popularity because of the growing demand for convenient beverage options. As flavor is one of the most important factors driving coffee consumption, coffee flavor has been extensively researched for years. However, RTD coffee requires the brew to undergo additional processing and storage and therefore creates challenges with flavor stability. There is limited information reported in the literature focused on the flavor of RTD coffee, challenging food manufacturer's ability to produce RTD coffee that maintains flavor quality during storage. Therefore, in this literature review, a summary of coffee flavor and related past research is presented. This review will discuss coffee chemical composition and flavor generation, including flavor formation during roasting. A discussion of the limited research regarding RTD coffee flavor stability during manufacturing and storage will be presented. Finally, coffee research methodologies will be discussed, and untargeted flavoromics will be introduced as an approach to understand the flavor instability of RTD coffee during storage.

1. Coffee

Over time, coffee has spread worldwide as an important agricultural commodity and a popular beverage. Generally, coffee refers to a brewed drink made from the seeds of

the cherries of Coffea plants. There are more than 70 species of Coffea plants; however, only two species are commercially cultivated worldwide: Coffea Arabica and Coffea canephora (Etienne, 2005). The coffee cherries and brewed drinks produced by the two species are known as Arabica and Robusta, respectively. Harvested from Coffea plants, coffee cherries are processed into green coffee beans using either a wet or dry method, which removes the outer layer and pulp. The raw coffee green beans are further roasted, ground, and brewed into various coffee products for consumption.

After its introduction in the 15th century, coffee became one of the most consumed beverages today (Mussatto et al., 2011). In addition to roasted whole and ground coffee beans, coffee products such as single-serve, instant, ready-to-drink (RTD), and coffee-flavored beverages are commonly available. The value of the world coffee market exceeded $200 billion in 2019, and coffee consumption is steadily growing at an annual rate of 2.2 % (ICO, 2019, 2020). In the United States, the current $15.6 billion coffee market is expected to grow through 2024 (Failla, 2019).

Ready-to-drink (RTD) coffee provides consumers a convenient alternative to fresh-brewed. The term RTD coffee encompasses shelf-stable or refrigerated bottled/canned coffee drinks. In general, the manufacturing process of RTD coffee includes coffee extraction, pH adjustment, blending with other ingredients, sterilization, and aseptic filling (Ikeda et al., 2018). RTD coffee and its related beverage products first found enormous success in Japan, where the manufacturing technology and production have been well developed (Clarke & Vitzthum, 2008). In recent years, RTD coffee has gained favor from consumers in the North American market, driving the growth of the coffee market.

According to Mintel's report (Failla, 2019), RTD coffee maintained the fastest growth rate among all coffee products between 2017–2019 in the United States, and it is forecasted that RTD coffee will surpass the current segment leader in coffee sales, roasted coffee, by 2024.

2. Green coffee bean chemical composition and precursors of coffee flavor

The composition of green coffee beans varies depending on species, origin, agricultural practice, and washing and drying process (Farah, 2012). The green coffee bean is mainly comprised of carbohydrates, lipids, proteins, free amino acids, chlorogenic acids, organic acids, caffeine, trigonelline, and minerals (Clarke & Vitzthum, 2008). These compounds serve as precursors in forming the distinctive color and flavor of coffee brew during the roasting process. A summary of the chemical composition of green coffee beans is provided in Table 1.

Table 1. Chemical composition of Arabica and Robusta green coffee bean; values in % of solids (Belitz et al., 2009).

Constituent	Arabica		Robusta	Components
Soluble carbohydrates	9–12.5		6–11.5	
Monosaccharides		0.2–0.5		Fructose, glucose, galactose, arabinose (traces)
Oligosaccharides	6–9		3–7	Sucrose (>90%), raffinose (0–0.9%), stachyose (0–0.13%)
Polysaccharides		3–4		Polymers of galactose (55–65%), mannose (10–20%), arabinose (20–35%), glucose (0–2%)
Insoluble polysaccharides	46–53		34–44	
Hemicelluloses	5–10		3–4	Polymers of galactose (65–75%), arabinose (25–30%), mannose (0–10%)
Cellulose, β(1–4)mannan	41–43		32–40	
Acids and phenols				
Volatile acids		0.1		
Nonvolatile aliphatic acids	2–2.9		1.3–2.2	Citric acid, malic acid, quinic acid
Chlorogenic acid[c]	6.7–9.2		7.1–12.1	Mono-, dicaffeoyl- and feruloylquinic acid
Lignin		1–3		
Lipids	15–18		8–12	
Wax		0.2–0.3		
Oil		7.7–17.7		Main fatty acids: 16:0 and 18:2 (9,12)
N Compounds		11–15		
Free amino acids		0.2–0.8		Main amino acids: Glu, Asp, Asp-NH_2
Proteins		8.5–12		
Caffeine	0.8–1.4		1.7–4.0	Traces of theobromine and theophylline
Trigonelline	0.6–1.2		0.3–0.9	
Minerals		3–5.4		

Carbohydrates constitute about half of the green coffee bean's dry weight being classified in low and high molecular weight. Among them, sucrose is the principal low molecular weight carbohydrate, which makes up greater than 90% of the oligosaccharides in green coffee beans (Table 1). Trace amounts of monosaccharides have also been reported; however, their content is relatively low (Clifford, 1985). Low molecular weight carbohydrates participate in extensive chemical reactions during the roasting process, contributing to the formation of the color and flavor of roasted coffee beans and coffee

brews (Farah, 2012). On the other high molecular weight carbohydrates, including soluble and non-soluble polysaccharides, account for approximately half of the dry weight of green coffee beans (Table 1) (Bradbury & Halliday, 1990). Soluble polysaccharides extracted with hot water during brewing can significantly influence the organoleptic quality of coffee by binding aroma, stabilizing foam, forming sedimentation, and increasing viscosity (Arya & Rao, 2007; Ballesteros et al., 2015). In addition, insoluble polysaccharides make up the majority of the thick cell wall and provide structural support (Redgwell & Fischer, 2006).

Lipid content in green coffee beans varies between Arabica and Robusta species. Triacylglycerols are the main component, accounting for about 75% of the total lipid fraction. The remaining unsaponifiable fraction includes free and esterified diterpene alcohols, free and esterified sterols, and trace amounts of other lipids such tocopherols (Speer & Kölling-Speer, 2006). Diterpenes and their derivatives have gained research attention, not only because of their physiological properties but also their impact on coffee flavor quality (Kurzrock & Speer, 2001; Sittipod et al., 2020).

Proteins represent approximately 11% of the green coffee beans' dry weight. In contrast, free amino acid and peptide contents are relatively low, ranging from 0.3–0.6% and 0.4–0.6% on a dry weight basis, respectively (Arnold & Ludwig, 1996; Ludwig et al., 2000; Montavon et al., 2003). Proteins, peptides, and free amino acids are considered essential precursors in the development of coffee flavor during roasting. The reactions between the carbonyl group of reducing sugars and the amino group of amino acids (including peptides and proteins), known as the Maillard reaction, contribute to the formation of various classes of volatile compounds such as furans, pyrazines, pyridines,

pyrroles, and aldehydes (Farah, 2012). The reaction also forms brown nitrogenous condensation polymers, called melanoidins, which are responsible for coffee color and antioxidant activity (Borrelli et al., 2002).

Chlorogenic acids are the major phenolic compounds in green coffee beans, accounting for 6–12% of the dry basis. Green Robusta coffee beans typically contain 1.5 to 2 times higher chlorogenic acid content than Arabica beans (Bicho et al., 2013; Jeszka-Skowron et al., 2016). From a chemical structure perspective, chlorogenic acids are a family of esters composed of *trans*-cinnamic acids and (-)-quinic acid. The chlorogenic acid family is divided into several subclasses depending on the type and number of cinnamic substituents and the position of the ester bond. A summary of the most representative chlorogenic acid families is presented in Figure 1 (Farah, 2012). Caffeoylquinic acids are the predominant subclass in green coffee beans, with 5-caffeoylquinic acid being the most abundant. The chlorogenic acid family in green coffee beans serve as important precursors in coffee flavor generation, whose total concentration may decrease up to 50% during the roasting process, depending on the roast level (Mills et al., 2013). Their thermal degradation leads to the formation of various phenols and catechols (Farah et al., 2005). Moreover, these acids are thought to contribute directly to bitter taste, sourness, astringency, and other sensory attributes of coffee brew (Campa et al., 2005; Ribeiro et al., 2012; Sunarharum et al., 2014).

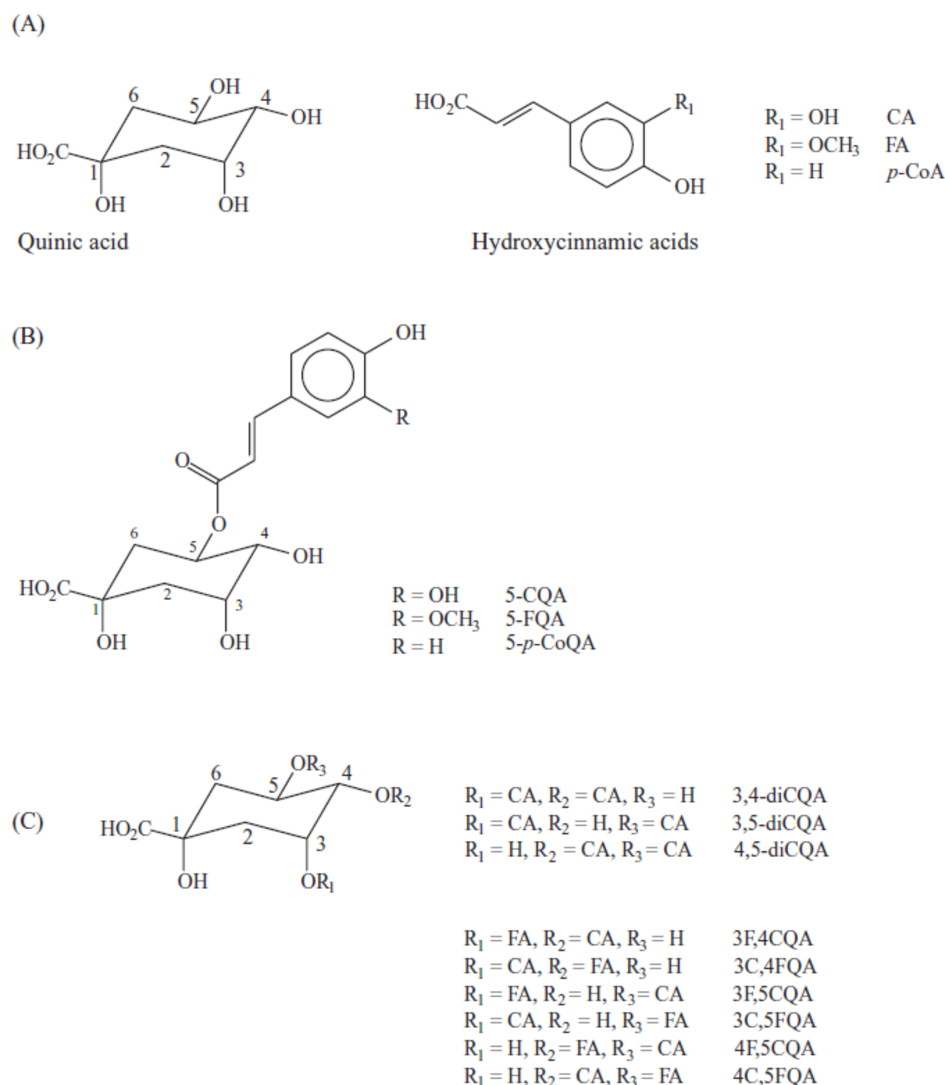


Figure 1. Chlorogenic acid family. (A) Basic moiety of quinic acid and *trans*-cinnamic acid, (B) monoesters of quinic acid with *trans*-cinnamic acid at 5-position, (C) di-esters of quinic acid with *trans*-cinnamic acid (Farah, 2012).CA: caffeic acid; FA: feruloyl acid; CQA: caffeoylquinic acid; FQA: feruloylquinic acid; CoQA: coumaroylquinic acid; diCQA: dicaffeoylquinic acid.

Caffeine and trigonelline are well-known nitrogenous alkaloids in green coffee beans because of their importance on coffee physiological properties and bitterness perception (Farah et al., 2006; Ky et al., 2001; Mazzafera, 1991; Ramalakshmi & Raghavan, 1999). Caffeine is the primary bioactive compound providing central nervous system stimulating effects. However, acute intake of large doses of caffeine may cause adverse side effects on health (Pohler, 2010). Similarly, trigonelline has been demonstrated to present several potential biological activities, including neuroprotective, antidiabetic, antimutagenic, and antithrombotic functions (Mohamadi et al., 2018). Both caffeine and trigonelline contribute to the bitterness in the coffee brew. Moreover, trigonelline serves as a precursor for the formation of some odor-active volatiles such as pyrroles and pyridines (De Maria et al., 1996).

3. Coffee flavor generation during roasting

Although coffee is frequently consumed due to its stimulating effects, there is no doubt that people enjoy drinking coffee because of its distinctive and pleasant flavor (Flament, 2001; Phan & Chambers, 2016). Consumers' coffee purchase decisions depend on various reasons, such as functional characteristics, packaging, branding, and the sensory characteristics of coffee, with the latter being the most influential factor (Wang & Yu, 2016). However, green coffee beans lack the desirable flavor of the coffee brew, and beans must be processed to achieve the desired flavor. Arguably, the roasting process is primarily responsible for the flavor generation of key coffee sensory characteristics, and therefore

the significant influence of the roasting process on coffee flavor has been continuously studied (Eggers & Pietsch, 2001).

The roasting process is comprised of three main phases: an initial drying phase, the actual roasting phase where the chemical composition of green bean changes intensively, and a cooling phase at the end (Buffo & Cardelli-Freire, 2004). The initial drying phase involves loss of water, browning/caramelization, and increased bean volume (Farah, 2012). Most complex pyrolytic reactions related to flavor generation occur during the roasting phase, via mechanisms such as the Maillard reaction, Strecker degradation, breakdown of amino acids, interactions between intermediate products, and degradation of nitrogenous compounds, phenolic compounds, and lipids (Buffo & Cardelli-Freire, 2004). The roasting phase, which varies between 180 °C to 240 °C for 8 to 15 min, generates thousands of volatile and non-volatile flavor compounds. For example, the formation of coffee thiols, a group of volatiles representing the fresh, roasty, and coffee-like notes of fresh coffee brew, is dependent on roasting time and temperature (Baggenstoss et al., 2008). Maillard-type reactions between sulfur-containing amino acids and sugars under high temperatures are primarily responsible for thiol formation (Cerny & Davidek, 2003; Hofmann & Schieberle, 1997; Hofmann & Schieberle, 1998). Other odor active volatiles such as furans, aldehydes, pyrroles, and pyrazines are generated during the roasting process through the Maillard reactions and degradation of carbohydrates, free amino acids, and lipids (Crews & Castle, 2007; de Melo Pereira et al., 2019; Poisson et al., 2009). From a non-volatile perspective, aliphatic acids that contribute to coffee brew acidity are the degradation products of sucrose and polysaccharides (Clarke, 2012; Ginz et al., 2000). Several chlorogenic acid lactones,

which are contributors to the bitterness in the coffee brew, are formed through heat-induced chlorogenic acid lactonization and the subsequent water loss in the roasting phase. Some studies indicated their concentrations have shown strong dependence on the roasting time and temperature (Blumberg et al., 2010). Compared to the other two phases, the cooling phase, which utilizes air or water as the cooling agent, has little impact on coffee flavor generation (Buffo & Cardelli-Freire, 2004).

In general, higher roasting temperatures result in darker roasted coffee beans, which present bitter, burnt, ashy, coffee, and roasty characteristics; on the other hand, lower roasting temperatures produce lighter roasted coffee beans, which typically have sweet, cocoa, nutty, and sour flavor notes (Bhumiratana et al., 2011; Moon & Shibamoto, 2009). While roasting level depends on personal preference and production craftmanship, lighter roasted beans will preserve more of the flavor notes inherent in coffee varieties and geographical origins as compared to dark roasted beans, whose intense coffee-like and bitter flavor notes might mask these characteristics (Bhumiratana et al., 2011; Sunarharum et al., 2014).

4. Coffee flavor

Flavor perception is a multimodal process that involves the combination of olfactory (aroma), gustatory (taste), and somesthetic (touch) stimuli. Olfactory stimuli occur when volatile compounds reach the olfactory epithelium in the nasal cavity through the nose (orthonasal olfaction) or mouth (retronasal olfaction) (Rozin, 1982; Small et al., 2005). The gustatory sensation is activated when non-volatile compounds stimulate

epithelial taste receptor cells in the tongue, palate, and throat (Breslin & Spector, 2008). Finally, the somesthetic sense is the most diverse sensory system of the human body that responds to a variety of mechanical, tactile, thermal, and chemical stimuli (Hollins, 2010). The integration of cues from all these complex sensory systems and their interactions give rise to our everyday experience of food and flavor.

Consumer's enjoyment of coffee flavor is one of the most significant drivers of coffee consumption (Labbe et al., 2015; Samoggia & Riedel, 2018; Sousa et al., 2016). Given the important role that coffee flavor plays in consumer's coffee consumption, a deeper understanding of the complexity of coffee flavor from a chemical perspective will guide better production and preservation of coffee flavor. Hence, efforts to understand the compounds causing the distinctive flavor of coffee have been made for more than a hundred years (Grosch, 1998).

4.1.Coffee aroma

A considerable amount of research has been focused on understanding the key chemical compounds responsible for the coffee aroma and their impact on coffee sensory characteristics. Research on characterizing key aroma compounds (odorants) of coffee beans and brew have advanced with the application of gas chromatography-olfactometry (GC-O) techniques, such as aroma extract dilution analysis (AEDA) and odor activity value (OAV).

Primary odorants reported in coffee brew include furans, pyrazines, sulfur-containing compounds, aldehydes and ketones, phenols, and others (Blank et al., 1991; Blank et al., 1992; Grosch et al., 2000; Holscher & Steinhart, 1992). More than 800

odorants have been discovered in the coffee aroma; however, studies have shown that the overall coffee aroma profile can be closely mimicked by a model system containing only 24 to 27 odorants (Mayer et al., 1999; Mayer et al., 2000). Thus, differences in the coffee aroma sensory profile between Arabica and Robusta or between various coffee brews are primarily determined by the concentration differences of these potent odorants instead of the appearance of new volatile compounds (Blank et al., 1991). Among the reported potent odorants, sulfur-containing thiols, typically described as having 'coffee' and 'roasty' notes, have a substantial impact on the aroma profile of coffee brew due to their low odor thresholds (Dulsat-Serra et al., 2016; Holscher & Steinhart, 1992). In particular, 2-furfurylthiol, 3-mercapto-3-methyl-1-butyl formate, 2-methoxy-3-iso-propylpyrazine, 3,5-dimethyl-2-ethylpyrazine, 2,3-diethyl-5-methylpyrazine, 4-vinylguaiacol, and β-damascenone have been determined to provide distinctive coffee aroma characters such as roasty, coffee, nutty, earthy, burnt, and sweet caramel-like (Akiyama et al., 2007; Belitz et al., 2009; Blank et al., 1992; Czerny & Grosch, 2000; Semmelroch & Grosch, 1995). The loss of low boiling point odorants such as methanethiol and 2-furfurythiol is responsible for the decrease in aroma freshness in stale coffee (Holscher & Steinhart, 1992).

4.2.Coffee taste

Compared to the extensive number of studies focused on coffee aroma, information regarding the taste-active non-volatiles in coffee is limited. Sourness has been related to the presence of acids in coffee (Balzer, 2001). The pH of coffee brew, and titratable acidity, which refers to the total amount of acids in the coffee brew, have shown correlations to

perceived sourness and acidity (Voilley et al., 1981). An example of the acid content in Arabica coffee brew is shown in Table 2. However, few conclusive research findings have determined which acid or acids are primarily responsible for the perceived sourness. Chlorogenic acids have been described as slightly bitter, sour, astringent, and as having a lingering aftertaste in isolated form, and they have been associated with coffee sourness and astringency (Campa et al., 2005; Trugo & Macrae, 1984; Variyar et al., 2003).

Table 2. Typical coffee acids and acid content of a Colombian Arabica coffee brew (Balzer, 2001).

Acid	Proton	pK^a	Typical content (µmol/100 ml)
Chlorogenic		3.4	96–291
Citric	1	3.14	75–189
	2	4.77	
	3	6.39	
Quinic		3.4	123–242
Phosphoric	1	1.96	65–108
	2	7.21	
	3	12.30	
Formic		3.75	130–159
Acetic		4.73	74–226
Malic	1	3.4	58–76
	2	5.05	
Glycolic		3.83	51–100
Lactic		3.89	22
Pyroglutamic		3.32	27

Bitter is a prominent taste attribute in the coffee brew. Caffeine, a well-known bitter compound, is responsible for approximately 10% of the bitterness of coffee (Voilley et al., 1981). In addition, proline-based diketopiperazines (DKPs), which belong to a group of cyclic dipeptides that have been identified as bitter compounds in many other food

products (Gautschi et al., 1997; Pickenhagen et al., 1975), are considered contributors to coffee bitterness (Ginz & Engelhardt, 2000). In recent years more bitter compounds have been identified in coffee utilizing sensory-guided fractionation, a targeted research approach. Using this approach, several chlorogenic acid lactones were found to be strong bitter compounds in the coffee brew. Chlorogenic acid lactones are formed during the coffee roasting process when chlorogenic acids eliminate a water molecule from the quinic acid moiety and form an intramolecular ester bond (Farah et al., 2005; Scholz & Maier, 1990). These chlorogenic acid lactones show intense bitter-taste activity with threshold levels ranging from 9.8 to 180 μmol/L in water (Frank et al., 2008; Frank et al., 2006) (Table 3). Frank et al. (2007) also reported the presence of bitter-tasting 4-vinylcatechol oligomers in coffee, including 1,3-bis(3′,4′-dihydroxyphenyl) butane, trans-1,3-bis(3′,4′-dihydroxyphenyl)-1-butene, and several hydroxylated phenylindanes with low bitter thresholds ranging between 23 and 178 μmol/L. More recently, a class of (furan-2-yl) methylated benzene diols and triols, along with mozambioside, an Arabica-specific furokaurane glucoside, have also been associated with coffee bitterness (Kreppenhofer et al., 2011; Lang et al., 2015).

Table 3. Human bitter taste threshold of bitter-active chlorogenic acid lactones (Frank et al., 2006).

Compound	Bitter threshold concentration	
	(mg/l)	(µmol/l)
3-*O*-caffeoyl-γ-quinide	13.4	40.0
4-*O*-caffeoyl-γ-quinide	12.1	36.0
5-*O*-caffeoyl-*epi*-δ-quinide	60.5	180.0
4-*O*-caffeoyl-*muco*-γ-quinide	11.2	30.0
5-*O*-caffeoyl-*muco*-γ-quinide	9.7	29.0
3-*O*-feruloyl-γ-quinide	13.7	39.0
4-*O*-feruloyl-γ-quinide	13.7	39.0
3,4-*O*-dicaffeoyl-γ-quinide	4.8	9.8
3,5-*O*-dicaffeoyl-*epi*-δ-quinide	24.9	50.0
4,5-*O*-dicaffeoyl-*muco*-γ-quinide	4.8	9.8

4.3.Interactions between coffee volatiles and non-volatiles

Coffee flavor changes as it cools after brewing, which is often referred to as staling. These changes are typically related to the loss of desirable aroma attributes. The sulfury-roasty notes were observed to decrease shortly after coffee is brewed, hence research interest has been placed on understanding the interactions between the coffee matrix and odor-active thiols that are primarily responsible for the roasty aroma notes in coffee brew (Dulsat-Serra et al., 2016). Notably, the concentrations of odor-active thiols such as 2-furfurylthiol, 3-methyl-2-buten-1-thiol, and 3-mercapto-3-methyl-1-butyl formate significantly decreased, making significant impacts on the sensory perception (Charles-Bernard, Roberts, et al., 2005). The mechanisms behind these observations have been also

studied. From a physical perspective, hydrophobic trapping and salting-out are the main interactions between thiols and the coffee matrix, and from a chemical perspective, the losses of thiols could be partially explained by covalent binding with melanoidins, a Maillard reaction product (Hofmann & Schieberle, 2002). In addition, thermal degradation products of chlorogenic acids such as catechol, 4-ethylcatechol, and hydroxyhydroquinone are also responsible for binding thiol volatile compounds (Müller et al., 2006; Müller & Hofmann, 2005, 2007). Peptides/proteins as well as polysaccharides were also associated with thiol bindling, however, their contribution in the binding process is limited compared to the mechanisms mentioned above (Charles-Bernard, Kraehenbuehl, et al., 2005).

4.4.Ready-to-drink (RTD) coffee flavor

RTD coffee requires coffee brew to undergo additional processing and storage, often creating flavor instability over time. RTD coffee and related products generally require heat treatment and pH adjustment to achieve a longer shelf-life; therefore, it is challenging to maintain the original coffee flavor in RTD coffee (Ikeda, Akiyama, Hirano, Miyazi, et al., 2018).

Research effort has been made to understand the negative influences of the manufacturing process on RTD coffee flavor. For example, the characteristic roasty coffee flavor in RTD coffee decreases significantly during heat treatment (Kumazawa, 2006). It has been reported that heat sterilization impacted the chemical stability of several aroma-active sulfur-containing odorants such as 2-furfurylthiol, methional, and 3-mercapto-3-methylbutyl formate, and pH adjustment during manufacturing affected the release of these

odorants, resulting in the decrease in roasty odor notes of RTD coffee (Kumazawa & Masuda, 2003a, 2003b; Kumazawa et al., 1998). In addition, Murakami et al. (2010) reported that the overall coffee flavor, coffee aroma, and bitterness of canned coffee drinks were weaker in intensity after the sterilization process. Less is known regarding the flavor stability of RTD coffee during storage. Akiyama et al. (2014) reported that the concentrations of 4-vinylguaiacol and pyrazines with nutty-roast odor decreased in aseptically packaged coffee beverages during a 2-week storage period, which significantly affected the retronasal aroma profile. Pérez-Martínez et al. (2008a) monitored the changes of 47 volatile compounds in coffee brew stored at 4°C and 25°C for 30 days and evaluated their influence on the loss of aroma intensity and freshness during storage. From an analytical standpoint, few studies have investigated the changes occurring in the non-volatile fraction of RTD coffee during storage and their corresponding impacts on sensory attributes. Several phenolic compounds have been associated with the generation of sourness, rancid aroma, and astringent aftertaste in coffee brew during storage (Pérez-Martínez et al., 2008b).

Overall, information on the flavor profile of RTD coffee during storage remains fragmentary. The chemical changes in RTD coffee during storage, as well as their corresponding sensory relevance, are still not fully understood. The lack of knowledge necessitates the need for research to better connect the chemical changes in RTD coffee over time with flavor stability during storage.

5. Coffee flavor research methods

Research on coffee flavor has been carried for decades. However, while knowledge on the coffee chemical composition and the sensory relevance of key flavor compounds has greatly expanded, there is still limited information that explains overall coffee flavor from a comprehensive perspective (Sunarharum et al., 2014). Coffee flavor perception is a multimodal system that involves the contribution from individual chemical compounds, the interaction between different chemical components and classes, and the modulating impact from non-sensory active chemicals. With the advancement of research methods and analytical techniques, a deeper understanding of coffee flavor becomes available. In the following section, a discussion of coffee flavor research methods will help identify the existing challenges and discover new research opportunities.

5.1. Targeted flavor analysis

Sensory-guided flavor analysis has been applied to characterize the volatiles and non-volatiles in coffee for decades. Most of the discovery on coffee volatiles was accomplished based on the application of the gas chromatography-olfactometry (GC-O) technique. GC-O combines the gas chromatography separation technique with an olfactometer where the human nose is considered a detector for olfactory attributes. Panelists sniff the eluents from a GC column and record their perceived aromas simultaneously. Information on the eluent’s sensory occurrence, duration, and qualitative descriptor is recorded (Acree et al., 1984), and the corresponding chromatographic peak will be identified by matching retention index and mass spectrum. More sophisticated

techniques based on GC-O such as AEDA (aroma extract dilution analysis), CHARM (combined hedonic aroma response method), and OAV (odor activity value) have been broadly applied to aroma analysis for identifying and ranking the contribution of potent odorants in various food samples (Grosch, 1993; Kesen et al., 2013; Marin et al., 1988; Qian & Reineccius, 2003).

In general, analysis of coffee aroma starts with screening potent odorants by AEDA and CHARM analysis, where results are expressed as FD (flavor dilution) factors. Odorants with a high FD factor are subsequently quantified, and OAV is calculated as a ratio of the concentration of the odorant to its odor threshold. Finally, an aroma recombination model is formulated based on the OAV and quantitative data and then is compared to the actual coffee samples (Grosch, 1998). Mayer and Grosch (2001) prepared a model solution that contains 22 previously identified coffee potent odorants. The similarity between the aroma of the model solution to that of a roasted coffee headspace was scored 2.6 on a scale of 0.0 (no similarity) to 3.0 (identical). After being applied to coffee flavor research, GC-O and the related technology have successfully demonstrated their effectiveness in characterizing coffee aroma profiles (Blank et al., 1992; Czerny & Grosch, 2000; Sanz et al., 2002; Semmelroch & Grosch, 1996). This analysis method has built a fundamental understanding of the coffee aroma profile.

Most research focused on characterizing coffee flavor has primarily centered on the aroma active compounds, as coffee aroma was considered the primary contributor to coffee flavor perception. In contrast, less attention has been paid to coffee taste active compounds, and consequently, information available on coffee tastants is comparatively limited and

fragmentary. In general, taste-active non-volatile compounds in various food products have been analyzed using tasted-guided fractionation methods such as direct scaling and taste dilution analysis (TDA). In direct scaling analysis, the food sample is divided into several individual fractions by preparative high-pressure liquid-chromatography (HPLC); panelists taste each fraction independently and rate the intensity of the sensory attributes of interests. Using directly scaling, Bin and Peterson (2016) discovered the bitter compounds in whole wheat bread crumb, and similarly, Zhang and Peterson (2018) identified novel monosodium L-pyroglutamate (L-MSpG) and monosodium D-pyroglutamate as umami contributors in potatoes and potato chips. In TDA analysis, similar to direct scaling, the food sample is divided into several individual fractions; however, each fraction is independently tasted by panelists and further diluted until no taste activity is reported. TDA was initially introduced to identify the intense bitter compound 1*H*,4*H*-Quinolizinium-7-olate from a complex mixture of Maillard reaction products (Frank et al., 2001). Using TDA, two peptides, PR-7 and YV-8, were found to significantly contribute to the typical umami and kokumi taste characteristics of pufferfish (Zhang et al., 2019). Several important bitter compounds in coffee, such as chlorogenic acid lactones and mozambioside, have been successfully discovered by several authors using taste-guided fractination approaches, as discussed in the prior section. Both direct scaling and TDA have proved to be highly effective approaches to characterize key tastants responsible for specific sensory attributes in many food products, and TDA was especially widely applied to discover coffee taste-active non-volatiles.

Targeted flavor research methodologies have solved many questions in coffee flavor and established the essential links between coffee chemical composition and sensory attributes. Nevertheless, flavor perception is a dynamic process that involves multiple sensory stimuli (Taylor & Roberts, 2004). Although aroma/taste-guided methods are systematic and straightforward in operation, they are limited in scope. It should be noted that the perceived intensity of sensory attributes is not always linear to the concentration of chemical stimuli. Moreover, singly evaluating chemical compounds places the analysis out of context, ignoring the contextual interactions between different components (Ronningen et al., 2017). For instance, flavor modulators, which are odorless/tasteless on their own, could be omitted in such a situation. Consequently, a more comprehensive research approach is needed to comprehend RTD coffee flavor perception from a broader point of view.

5.2.Untargeted flavor analysis - flavoromics

In recent years, metabolomics—an emerging field that aims at the high-throughput characterization of small molecules (usually below 1500 Da)—has become a powerful tool in many research areas, such as human nutrition, pharmaceutical discovery, plant analysis, and food science (Cevallos-Cevallos et al., 2009; German et al., 2005; Hall et al., 2008; Wishart, 2008). The widespread application of this research method relies on rapid separation techniques, including capillary electrophoresis (CE), gas chromatography (GC), and liquid chromatography (LC), as well as the robust and precise compound determination such as vibrational spectroscopy, mass spectrometry (MS), and nuclear magnetic resonance

(NMR) (Dunn et al., 2005). Depending on the purpose, metabolomics analysis could be classified as targeted or untargeted. Targeted metabolomics involves identification and quantification for a specific group of compounds of interest. In contrast, untargeted metabolomics focuses on detecting as many analytes as possible to acquire comprehensive chemical compositional data without necessarily knowing the identity of a specific compound or group(s) of compound(s) (Monton & Soga, 2007). The chemical data acquired from metabolomics experiments is generally combined with statistical tools to perform discriminative, informative, and predictive analysis. As metabolomics analysis allows the simultaneous chemical profiling of large numbers of chemicals in a complex matrix, it offers food researchers a great opportunity to capture more detailed and comprehensive pictures of food chemical composition. This technology has been successfully applied to many fields of food research, including food component analysis, food safety, food quality assurance, and food nutrition (Arapitsas et al., 2016; Cubero-Leon et al., 2018; Mattivi et al., 2006; Ogrinc et al., 2003; Schueuermann et al., 2019; Thompson et al., 2006).

To address some questions that may be difficult to answer using traditional targeted methods, Reineccius (2008) introduced the concepts of untargeted metabolomics to flavor research and named the method flavoromics. Compared to traditional tasted-guided fractionation, flavoromics has two core advantages. First, flavoromics borrows the idea of untargeted analysis that involves chemical profiling of every (or near every) small molecule in a food system; hence, it considers more compounds as potential candidates of chemical stimuli in human sensory perception (unbiased) instead of focusing on certain

groups of compounds that have known flavor activities (biased) (Charve et al., 2011). Second, it is a data-driven approach that establishes the statistical correlation between chemical compounds and sensory perception. Therefore, it could be used as an efficient screening tool that does not require identifying certain compounds at the initial stage of flavor study. These characteristics of flavoromics allow for the discovery of new flavor contributors that would be overlooked by traditional taste-guided fractionation. For example, some poorly-defined flavor terms are hardly associated with specific sensory attributes, creating hurdles for sensory-guided experiments; on the other hand, flavoromics is not restrained by this limitation. Using the flavoromics approach, Ronningen et al. (2017) identified novel flavor compounds nomilin glucoside and ionone glucoside and further confirmed their sensory impact on the 'orange character' and freshness of citrus fruits during storage. Similarly, Sittipod et al. (2019) discovered a group of chlorogenic acid derivatives in coffee brew that significantly impacted the coffee quality and overall cupping scores. More recently, 4-caffeoylquinic acid, 5-caffeoylquinic acid, and 2-*O*-β-D-glucopyranosylatractyligenin were identified as flavor modulators that significantly decreased the bitterness in the coffee brew (Gao et al., 2021). These studies and others highlight the ability of the flavoromics approach to identify flavor modulators that have no taste activity on their own. These compounds would likely have been neglected by traditional taste-guided fractionation methods, as no sensory descriptor could be directly linked with a tasteless compound.

Prior research on understanding RTD coffee flavor has mainly relied on targeted approaches and information characterizing overall RTD coffee flavor is limited (Ikeda,

Akiyama, Hirano, Miyazi, et al., 2018). Particularly, overall RTD coffee flavor is impacted by many aspects of chemical and sensory changes during storage, which are difficult to characterize by targeted approaches. Hence, the flavoromics approach that monitors a wide range of chemical compounds instead of a particular group of compounds can offer new insight on RTD coffee flavor stability during storage. The general workflow of the flavoromics research approach is summarized in Figure 2 and explained in detail as follows.

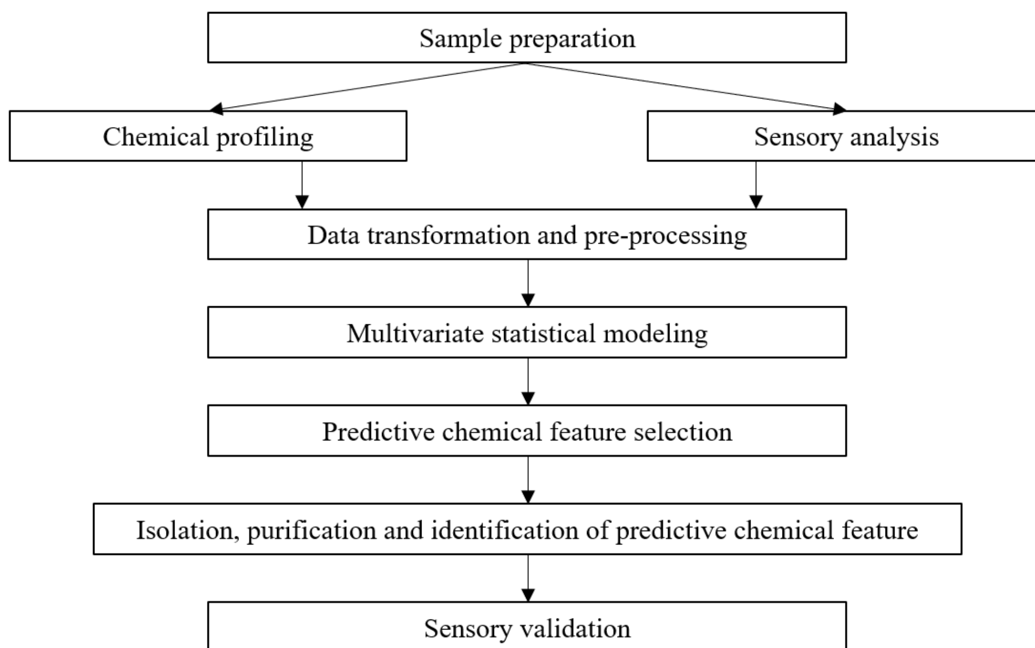


Figure 2. General workflow of flavoromics research approach

5.2.1. Sample preparation

Performing untargeted analysis requires the acquisition of information from as many chemical compounds as possible. However, it is challenging to extract and detect all chemical compounds in a food matrix due to the diversity of physical and chemical properties for compounds (Li et al., 2020). Hence, extra attention should be given to sample preparation in flavoromics work, ensuring a representative chemical profile with an adequate number of features extracted from the sample. The sample preparation method should be quick and straightforward to minimize potential changes and prevent artifacts in the original samples (Cruickshank-Quinn et al., 2014). Depending on the physicochemical properties of samples and the purpose of analysis (volatile or non-volatile), preliminary comparisons between different solvents and extraction methods might be necessary to maximize the amount and concentration of the compounds of interest being extracted (Cevallos-Cevallos et al., 2009). Biological and analytical replicates are often included to ensure reproducibility; processed blanks, which do not contain any samples but have undergone the same sample preparation process, are usually used to identify inferences resulting from sample preparation (Korman et al., 2012). In addition, a quality control (QC) sample, which is prepared by pooling equal aliquots of all samples, is generally used to monitor analytical performance (Godzien et al., 2015).

5.2.2. Chemical profiling and sensory analysis

Chemical profiling

Chemical profiling is usually considered a critical step in untargeted analysis. Separation techniques such as capillary electrophoresis (CE), gas chromatography (GC),

and liquid chromatography (LC) are often required to separate the complex analytes in food matrices. The choice of separation techniques depends on the research purpose and the chemical properties of food samples. In general, LC has been widely applied to acquire the non-volatile chemical profiles in food samples due to its advantages, including superb sensitivity, high flexibility, rapid separation, minimal sample size requirement, and the potential for detecting the largest portion of analytes (Wishart, 2008).

In flavor research, mass spectrometry (MS) is frequently used as a detection method. This choice of method is due to its ability to identify and quantify compounds with high sensitivity and accuracy, as well as the superb adaptability between MS instruments and LC/GC separation modules (Dunn et al., 2013). Other detection techniques that do not require prior separation of compounds, such as NMR (Tang & Hatzakis, 2020), vibrational spectroscopy (Xu et al., 2013), and direct infusion mass spectrometry (DIMS) (Luthria et al., 2008), have also been widely applied to untargeted food analysis, but seldom utilized in flavor research.

Sensory analysis

Paralleling chemical profiling, sensory analysis provides complementary information to chemical data. Thanks to the advancements of sensory science, a variety of sensory evaluation methods could be utilized to accurately record the sensory changes corresponding to chemical changes (McCain‐Keefer et al., 2020). Similar to instrumental analysis, the choice of sensory methods also depends on the research purpose. Same-different tests are generally applied to detect overall sensory differences, as they provide a sensitive measurement of any sensory change (Pecore et al., 2006). The use of the same-

different test can match well with the untargeted chemical profiling, which takes a wider range of chemical stimuli and sensory responses into consideration. The degree of difference (DOD) test is an extension of the same-different test (Aust et al., 1985; Bi, 2008). In the DOD test, panelists are asked to rate the degree of difference of a given sample compared to a control sample, using a scale that ranges from 0 (identical) to *m* (extremely different; $m > 2$) (Bi, 2008). The DOD test is helpful as it can provide an estimation on the magnitude of the perceptible overall sensory difference; however, it is still necessary to identify the cause of the difference as this method does not give information on the specific sensory attribute causing the difference (Aust et al., 1985; Costell, 2002).

5.2.3. Data transformation and pre-processing and multivariate statistical modeling

Chemical profiling data acquired from analytical platforms contains qualitative and quantitative information of the metabolic features in food samples, which requires data transformation and pre-processing before being used as input for subsequent statistical analysis. Usually, analytes acquired from analytical platforms are referred to as chemical features. Data transformation of chemical profiling data typically includes several spectral processing steps such as baseline correction, noise filtering, deconvolution, peak alignment, feature detection, and integration (Alonso et al., 2015). Data transformation and pre-processing have been shown to significantly improve the performance of multivariate statistical analysis (MVA) (Son et al., 2008). In addition, data pre-processing can be used

as a useful tool to evaluate chemical profiling methods, which benefits the collection of high-quality data.

In flavoromics, MVA is primarily employed to establish correlations between chemical profiling data and sensory responses. The most common multivariate statistical models used in metabolomics studies are principal component analysis (PCA) and partial least squares (PLS) (Eriksson et al., 2013; Sumner et al., 2005). PCA, as a basic unsupervised multivariate model, captures the latent structures of a dataset. Each latent structure is a linear combination of variables, called principal components, and each principal component maximizes the variation within observations. Upon visualization (usually known as PCA plot), the distance between projections of observations on the plane of a component elucidates the scope of variation between these observations. Therefore, PCA is a practical tool for describing natural grouping and clustering, assessing data quality, and identifying outliers (Bro & Smilde, 2014). In contrast, PLS is a supervised model that allows discrimination between classes of observations according to the relationships between predictors, **X**, and responses, **Y**. The ability of PLS to distinguish relevant information from large numbers of noisy variables (and observations) makes it a powerful tool for the interpretation of complicated metabolomics research problems (Wold et al., 2001).

Different model metrics, including R^2X, R^2Y, and Q^2, can be applied to evaluate model quality. R^2X and R^2Y represent the proportion of variation explained by the model in variables X and Y, respectively. Thus, higher values of R^2X and R^2Y are desired, as the model takes more comprehensive chemical and sensory information into consideration. Q^2

is an indicator of the model's predictive ability. The higher the value of Q^2, the better predictive ability the model will have (Wold et al., 2001). In general, values of Q^2 higher than 0.75 are considered acceptable quality in the literature. The permutation test can be applied to evaluate the over-fitting in the models, during which a small value of the permutated Q^2 is desirable (Eriksson et al., 2008).

5.2.4. Predictive chemical feature selection

The chemical profiling of food systems usually results in large numbers of chemical features. It is a practical issue to identify a few critical chemical features that are most influential on flavor perception. Chemical features can be positively or negatively correlated to the **Y** variable depending on the research purpose. Selecting features based on the VIP (variable importance in the projection) scores from PLS modeling is one of the most commonly used strategies, where features with VIP scores greater than one are usually considered significant contributors. However, when multicollinearity is present among variables, the 'greater than one rule' may not be adequate, and combination with other variable selection strategies is recommended (Chong & Jun, 2005). A complementary tool for chemical feature selection is the S-plot. The S-plot provides visualization of the variable's influence in a model according to the covariance (p[1]) and correlation (p[corr1]) of each variable. Covariance represents the magnitude of changes of a variable among all observations, and correlation indicates the linear relationship between two variables. A higher covariance indicates higher contribution, while a higher correlation represents better reliability (Wiklund et al., 2008). Other assessment strategies such as univariate analysis,

ANOVA, cross-validation score, and loading plot are also available; however, they should be used with caution on a case-by-case basis.

5.2.5. Sensory validation and identification of predictive chemical features

Multivariate analysis establishes the correlations, which do not necessarily lead to causative relationships. Even though untargeted analysis is employed as a screening tool at the initial stage of research, additional verification is still essential to properly link chemical inputs with sensory outcomes. Sensory validation must be done to determine if a selected chemical feature may truly contribute to sensory perception (Charve et al., 2011). In flavor analysis, non-volatile predictive chemical features are often isolated from the food matrix and purified through multidimensional fraction, which allows the sensory validation and identification of these features (Gao et al., 2021; Sittipod et al., 2019; Sittipod et al., 2020). Once the isolated chemical features are greater than 90% purity, sensory validation is performed by recombining a compound (predictive feature) or a set of compounds with a control sample. The concentrations of compounds added are determined by the concentration difference between the samples that present variations in their sensory attributes of interest. Paralleling the sensory validation, the compound(s) can be identified using multiple analytical platforms. Identification of volatile compounds is straightforward since the library of mass spectra and linear retention indices have been thoroughly established. On the contrary, extra effort is needed to identify non-volatile compounds due to the limited online database. As mentioned, non-volatile compounds usually require isolation and purification from the food matrix if commercial authentic standards are not

available. Structural elucidation techniques such as tandem mass spectrometry and NMR are useful tools for the identification process.

6. Summary

In this literature review, a brief introduction to coffee flavor was presented. Coffee flavor is still a challenging research topic due to the complexity of coffee chemistry and the limitations inherent in traditional flavor research methodologies. Particularly, the lack of relevant knowledge regarding the non-volatile chemical changes in RTD coffee presents a research opportunity to better understand RTD coffee flavor stability during storage. As the growing market of RTD coffee emphasizes the demand for longer flavor shelf-life, understanding RTD coffee flavor stability will provide guidance for flavor preservation.

Research over the past three decades has provided a volume of knowledge on coffee flavor and built the fundamental basis of coffee flavor chemistry. Nonetheless, research progress has reached a bottleneck when the analysis involves complex flavors that require consideration of more compounds as potential candidates for sensory stimuli. As RTD coffee flavor stability involves the changes of aroma, taste, and somatosensation, it necessitates the need for an innovative and comprehensive approach to expanding our knowledge on RTD coffee flavor stability. Hence, the following chapters will demonstrate the application of untargeted flavoromics analysis to characterize RTD coffee flavor stability during storage.

Chapter 2. Identification of non-volatile compounds that are generated during storage and impact flavor stability of ready-to-drink coffee

Abstract

Untargeted LC/MS flavoromics analysis was applied to identify chemical compounds generated during storage that impacted the flavor stability of ready-to-drink (RTD) coffee. Two coffee samples (Arabica and Robusta) prepared in air and under nitrogen were stored over 4 months at 30 ºC. Degree of difference (DOD) sensory evaluation revealed significant flavor changes in the RTD coffee after 1, 2 and 4 months. MS chemical profiles of non-aged and aged RTD coffee samples were modeled against the DOD scores by orthogonal partial least squares (OPLS) with good fit ($R^2Y = 0.966$) and predictive ability ($Q^2 = 0.960$). Five highly predictive chemical features positively correlated to DOD were subsequently identified as 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3-O-feruloylquinic acid, and 5-O-feruloylquinic acid. These five chlorogenic acid compounds in addition to quinic acid significantly increased in concentration during storage. Sensory recombination tests confirmed that these six acid compounds significantly impacted the flavor stability of RTD coffee during storage, primarily by reducing the pH of the product.

1. Introduction

First introduced in the 15th century, coffee is a popular beverage that has become one of the most important agricultural commodities worldwide (Mussatto et al., 2011). The value of the world coffee market is estimated to exceed $200 billion, and coffee consumption is steadily growing at an annual rate of 2.2 % (ICO, 2019, 2020). In 2019 the United States coffee market was valued at $15.6 billion and expected to grow 22.7% through 2024, driven in large part by the fast growth rate of ready-to-drink (RTD) products (Failla, 2019). In general, the term RTD coffee encompasses shelf-stable or refrigerated bottled/canned coffee drinks. RTD coffee has become increasingly popular because of the growing demand for convenient beverage options (Wang & Yu, 2016). It is forecasted that RTD coffee will surpass roasted coffee to become the largest segment in US coffee sales by 2024 (Failla, 2019).

Coffee and RTD coffee products are favored worldwide not only due to their stimulating effects but also their distinctive and pleasant flavor (Flament, 2001; Phan & Chambers, 2016). Consumer's coffee purchase decisions are dependent on several factors, including functionality, packaging, branding, and sensory characteristics. Flavor has been identified as the most influential factor for purchase decisions (Samoggia & Riedel, 2018; Wang & Yu, 2016). As a result, focus has been placed on the development of manufacturing processes and preservation strategies that deliver desirable, high-quality coffee flavors. For decades the chemical basis and sensory properties of coffee flavor have been of research interest.

Coffee flavor is a complex combination of aroma, taste, and somatosensation (Sunarharum et al., 2014). Most research has focused on the discovery of key aroma and taste compounds that contribute to the flavor profile of coffee beans and brews (Blank et al., 1991; Blank et al., 1992; Frank et al., 2007; Ginz & Engelhardt, 2000; Grosch et al., 2000), however, literature specifically focused the flavor of RTD coffee is limited. RTD coffee requires additional processing and storage and therefore faces challenges with flavor stability (Ikeda, Akiyama, Hirano, Miyaji, et al., 2018). For example, the decrease in roasty odor notes of RTD coffee after heat processing has been attributed to the degradation of unstable odorants containing the thiol functional group (Kumazawa, 2006; Kumazawa & Masuda, 2003b). Similarly, Murakami et al. (2010) reported that the overall coffee flavor, coffee aroma, and bitterness of canned coffee drinks were weaker in intensity after the sterilization process. During storage, freshly prepared coffee brew packaged in aseptic glass bottles has been found to develop several unpleasant attributes such as rancid aroma, sourness, and an astringent aftertaste, as well as exhibit the loss of aroma intensity and freshness (Pérez-Martínez et al., 2008a, 2008b); however, the connection between chemical changes and the corresponding impacts on sensory attributes of RTD coffee during storage is still limited. Moreover, from an analytical standpoint, less information has been reported regarding changes that occur in the non-volatile fraction of coffee brew during storage (Pérez-Martínez et al., 2008b). The lack of relevant knowledge opens the research opportunity to better understand RTD coffee flavor stability.

Traditionally, the discovery of flavor compounds in foods has relied on targeted approaches, which primarily focus on evaluating singular compounds in a unimodal

sensory response (Frank et al., 2001; Grosch, 1993; Ottinger et al., 2001); however, these approaches can overlook the effects of contextual interactions and flavor modulators. In recent years, an untargeted flavor approach named flavoromics has been applied to understand the chemical drivers of flavor properties in different complex food matrices (Andujar-Ortiz et al., 2015; Charve et al., 2018; Cong, Schwartz, & Peterson, 2021). Flavoromics combines comprehensive chemical profiling with statistical analysis to establish correlations between chemical components and sensory responses (Reineccius, 2008). Using flavoromics, several tasteless compounds that modulated coffee flavor were discovered (Gao et al., 2021; Sittipod et al., 2019). In addition, Ronningen et al. (2017) identified the novel flavor compounds nomilin glucoside and ionone glucoside, and successfully related their sensory impact to the loss of orange freshness during aging. Hence, this flavoromics approach can offer new insights into chemical drivers that impact RTD coffee flavor stability over time.

This study aimed to apply untargeted flavoromics analysis to identify non-volatile chemical compounds that impact the flavor stability of RTD coffee during storage. In the first of this two-phase study, compounds that were generated during storage or positively correlated to flavor change were investigated. LC/MS chemical profiling with multivariate statistical analysis (MVA) was utilized to correlate RTD coffee compounds with overall flavor changes. Highly predictive markers were selected, purified, and identified, and their sensory relevance was confirmed by a sensory recombination experiment.

2. Material and methods

2.1. Chemicals and Materials

Optima-grade formic acid, acetonitrile, methanol, acetone, and food-grade hydrochloric acid (HCl) were purchased from Fisher Scientific (Waltham, MA). Quinic acid, methylparaben, deuterated methanol, and deuterated water were purchased from Millipore Sigma (Burlington, MA). 3-caffeoylquinic acid (3-CQA), 4-caffeoylquinic acid (4-CQA), and 5-caffeoylquinic acid (5-CQA) were purchased from BOC Sciences (Shirley, NY). Nanopure water was purified through Barnstead Nanopure Diamond Water Purification System (Thermo Fisher, Dubuque, IA). Leucine enkephalin was purchased from Waters Co. (Milford, MA). Organic green Arabica and Robusta coffee beans were sourced from local suppliers (Columbus, OH).

2.2. Ready-to-drink coffee samples and storage conditions

The Arabica and Robusta green coffee beans were roasted to the light-roast level corresponding to a roast color of 100 CTN (Jupiter Tangential Roaster, Probat, Emmerich, Germany). Then the roasted beans were ground into coarse grounds with particle size d' = 2.4 mm. Coffee brews were extracted with deoxygenated and deionized water at 85 °C using a French press coffee maker with a 0.037 mm mesh screen. The extract was decanted to remove surface oil and ground particles and then poured into a steel can (approximately 180 mL) and sealed. The brewing and extraction process of the RTD coffee samples was conducted under two processing conditions to investigate the effect of oxygen during storage: samples made under an open-air condition were considered air-headspace RTD coffee samples, while those made in a nitrogen glove box were considered nitrogen-flushed

RTD coffee samples. All sealed cans were retorted at 125 °C for 5 min for sterilization. After preparation, a quarter of the RTD coffee samples was stored in a -40 °C freezer to mimic non-aged samples, and the rest was aged at 30 °C in a Barnstead Lab-line incubator (Lab-line/Barnstead, Dubuque, IA) for 1, 2, and 4 months. After storage, samples were kept at -40 °C for instrumental and sensory analysis. In the following discussion, the frozen 0-month RTD coffee was referred to as the non-aged RTD coffee sample. The combination of sample conditions (2 coffee species × 2 processing conditions × 4 storage time points) gave a total of 16 independent samples (n = 16).

2.3. Degree of difference (DOD) sensory evaluation

A Degree of Difference (DOD) sensory test was used to evaluate the overall flavor differences between non-aged and aged RTD coffee at 1, 2, and 4 months. Fourteen trained panelists (6 males and 8 females, ages 23 to 44) were recruited at the Ohio State University to participate in the test. Panelists evaluated each RTD coffee variety (Arabica air-headspace, Arabica nitrogen-flushed, Robusta air-headspace, Robusta nitrogen-flushed) conditions in separate sessions, for a total of 4 sessions over 4 days. During each session, RTD coffee samples in steel cans were warmed in a hot water bath and then poured into air pots to maintain serving temperature (60 °C to 65 °C). Seventy mL of each sample (1.6% total solid content) was served in 3 oz black ceramic cups. Panelists were given a set of RTD coffee samples that consisted of a control sample (non-aged) and 4 test samples (1 non-aged as blind control and 3 aged samples from 1, 2, and 4 months). The serving order of the test samples was randomized and balanced. Panelists were asked to make a series of 4 comparisons between the control sample and each test sample. For each

comparison, panelists tasted the control first and then the test sample. After tasting the samples, panelists placed the cup on a large printed DOD scale which was used as a visual representation of the size of the flavor difference between the control and the test sample (Figure 3). The DOD scale ranged from 0 to 10 points, with descriptors below the numbers explaining the size of difference (Aust et al., 1985; Bi, 2008). Panelists followed the same procedure for all 4 comparisons and entered their results into Compusense Cloud sensory analysis software (Compusense, Guelph, ON, Canada). Panelists were also asked to describe the sensory attributes that were observed to be contributing to the flavor differences. Unsalted crackers and water were provided for panelists to cleanse their palate between samples. Study protocols were approved by the OSU Institutional Review Board (2017H0072).

<table>
<tr><td>0</td><td>1</td><td>2</td><td>3</td><td>4</td><td>5</td><td>6</td><td>7</td><td>8</td><td>9</td><td>10</td></tr>
<tr><td>None</td><td colspan="2">Very Little</td><td colspan="2">Little</td><td colspan="2">Medium</td><td colspan="2">Strong</td><td colspan="2">Extreme</td></tr>
</table>

Figure 3. Degree of Difference (DOD) scale. The DOD scale was provided to panelists in printed form and displayed in the Compusense Cloud sensory analysis software.

2.4. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) chemical profiling

The extraction method was optimized to extract as many chemical features as possible from RTD coffee samples. Sample clean-up was automated by a Hamilton MicroLab Star Plus Liquid Handling System (Hamilton Robotics, Reno, NV). In brief, all

16 RTD coffee samples (600 µL, 1.6% total solid content) were diluted with nanopure water (400 µL), and the mixture was loaded onto an Oasis HLB 96-well plate cartridge (Waters Co., Milford, MA, USA) for solid-phase extraction (SPE). 500 µL of 5% methanol/water (v/v) was subsequently used to remove salts and highly polar compounds. Finally, 200 µL of 95% acetonitrile/water (v/v) was used to elute analytes retained on the cartridge. The eluent was further diluted 1:4 with nanopure water prior to UPLC-MS analysis. A quality control (QC) sample was developed by mixing an equal amount (1 mL) of all RTD coffee samples and prepared with the same sample clean-up protocol.

Non-volatile chemical profiling was performed using ultra-performance liquid chromatography coupled with a time-of-flight (Q-ToF) mass spectrometer (Waters Acquity H-Class quaternary solvent manager with Waters Synapt G2-S mass spectrometer, Waters Co.). Chromatographic separation was achieved using a reverse-phase Cortecs C18+ column (1.6 µm, 2.1 × 100 mm, Waters Corp.) held at 40 °C. The mobile phase consists of (A) water, (B) acetonitrile, and (C) 5% formic acid in water (v/v) at a flow rate of 0.5 mL/min. Injection of 2 µL RTD coffee analytes went through a gradient as follow: 0-0.5 min, 5% B; 0.5-11 min, 5-50% B; 11-12.5 min, 50-95% B; 12.5-14 min, 95% B; 14-15 min, 95-5% B; 15-16 min, 5% B; C was constantly held at 2%. Electrospray ionization (ESI) was operated in negative mode with capillary voltage of 2.5 kV, cone voltage of 35 V, source temperature of 120 °C, desolvation temperature of 450 °C, cone gas flow of 120 L/h, desolvation gas flow of 800 L/h, and nebulizer pressure of 6.0 bar. Continuum mass spectral data were collected with 0.3 sec scan time over a mass range of 50 to 1200 m/z.

Leucine enkephalin (m/z 556.2771) was infused every 30 sec as an internal standard for mass correction.

RTD coffee samples were prepared with 2 biological and 2 technical replicates. All injections were performed in randomized order. A water blank, a column standard (mixture of 4 parabens), and the QC sample were injected after running every 10 samples to monitor analytical performance.

2.5.Multivariate statistical analysis (MVA)

Chromatographic and spectral data were converted into statistical variables by deconvolution, ion extraction, and integration using Progenesis QI software (Nonlinear Dynamics, Durham NC). Feature extraction criteria were set to medium sensitivity in the software. Each chemical feature was reported as a retention time-mass/charge ratio (RT_m/z) with ion abundance. Chemical features exported from Progenesis QI were further processed in the R program to filter out noise based on ion intensity threshold (> 500 counts) and coefficient of variance between all replicates (< 30%). Multivariate data analysis was performed with two RTD coffee biological replicates and pooled QC sample by using SIMCA-P+ 14.1 (Umetrics, Umea, Sweden). Principal component analysis (PCA) and orthogonal partial least squares (OPLS) models were generated with chemical and sensory data in Pareto-scaling. In the OPLS model, DOD scores of RTD coffee samples were assigned as Y variable, and chemical features (RT_m/z by ion abundance) were assigned as X variables. The predictive variable of importance (VIPpred) scores and S-plot were subsequently generated to select highly significant predictive chemical features. The features discussed in the current chapter were referred to as positively correlated features

because their concentrations in RTD coffee samples increased as the degree of flavor differences increased over time

2.6. Off-line Multidimensional Preparative-Liquid Chromatography/Mass Spectrometry (Prep-LC/MS) Fractionation

According to the model's predictive ability, top chemical features were selected, isolated, or purchased to investigate their impact on the flavor stability of RTD coffee during storage in a sensory study. Commercial standards of three compounds (RT_m/z) 2.68_353.1, 3.22_353.1, and 3.36_353.1 were purchased from BOC Sciences (Shirley, NY) and the other two selected chemical features (RT_m/z) 3.59_367.1 and 4.29_367.1 were isolated from the coffee brew as follows.

Three hundred grams of fresh coffee grounds were added to 3 L of 80% methanol/water (v/v). The mixture was stirred at room temperature for 12 h and then filtered through a Whatman grade 4 filter paper (GE Healthcare, Buckinghamshire, UK) and a 5-kDa ultrafiltration membrane (Millipore Sigma, Burlington, MA). Solid-phase extraction (SPE) was performed to clean up filtered coffee. Specifically, 200 mL filtered coffee was loaded onto an Oasis HLB 6 g bed cartridge (Waters, Milford, MA). 100 mL of 5% methanol/water (v/v) was used to wash the cartridge, and 100 mL of 95% methanol/water (v/v) was used to elute analytes from the cartridge. The SPE process was repeated for several cartridges to increase extraction yield. The eluent was subsequently freed from the solvent using a Rocket Synergy Purge (Genevac, Ipswich, UK) and lyophilized. The lyophilized sample was reconstituted to approximately 500 mg/L in 20% methanol/water (v/v), filtered through a PTFE 0.45-µm filter, and then injected into the

Prep LC-MS fractionation system (Waters 2545 binary pump and TQD mass spectrometer coupled with 2767 fraction collector). First dimension isolation was achieved using an Xbridge Prep C18 column (5 µm, 50 mm × 50 mm, Waters Corp.). The mobile phase was maintained at a 100 mL/min flow rate using a binary solvent system of 0.1 % formic acid in water (A) and methanol (B). The gradient was set as follow: 0-0.5 min, 5% B; 0.5-1.5 min, 5-27% B; 1.5-5.5 min, 27% B; 5.5-8.5 min, 27-50% B; 8.5-10 min, 50-95% B; 10-13 min, 95%; 13.01-15 min, 5% B. First-dimension fractions were pooled, freed from solvent, and lyophilized before reconstituting to approximately 500 mg/L in 10% methanol and filtering through a 0.45-µm filter.

To achieve better purity, second dimension HPLC fractionation was performed on an Atlantis T3 OBD column (5 µm, 50 mm × 250 mm, Waters Corp.) using a mobile phase consisting of (A) water with 0.1% formic acid and (B) acetone with 0.1% formic acid. The gradient was set at 100 mL/min as follows: 0-0.5 min, 5% B; 0.5-1 min, 5-20% B; 1-30 min, 20% B; 30-31 min, 20-95% B; 31-34 min, 95% B; 34.01-37 min, 5% B. The same column and gradient were applied to both features. The second-dimension fractions were handled using the same protocol as the first-dimension fractions.

To achieve purity greater than 90%, a third dimension fractionation was performed utilizing a Xselect CSH Phenyl-Hexyl OBD prep column (5 µm, 10 × 250 mm, Waters Corp.) on a semi-prep scale. The mobile phase consists of (A) water with 0.1% formic acid and (B) acetone with 0.1% formic acid at a flow rate of 7 mL/min. The gradient was optimized as follow: 0-1 min, 12% B; 1-23 min, 12-20% B; 23-25 min, 20-95% B; 25-27.5

min, 95%; 27.51-30 min, 5% B. The same column and gradient were applied to both features.

The TQD mass spectrometer was operated under negative ESI mode using the following settings: capillary voltage = 2.5 kV, cone voltage = 30 V, source temperature = 150 °C, and desolvation gas temperature = 400 °C. The time-based collection was applied to collect the first-dimension fractions within the retention time range of each targeted feature. Mass-triggered collection under single ion monitoring (SIR) mode was used to collect the 2nd and 3rd dimension fractions. After each collection, pooled fractions were injected to Synapt G2-S UPLC-QTof-MS (Waters Co.) to ensure accurate collection of targeted chemical features. Purity was calculated based on total ion chromatogram peak area under both positive and negative ESI modes. Purified chemical features proceeded to sensory recombination and NMR experiments when the calculated purity was greater than 90%.

2.7. Quantification of positively correlated predictive compounds by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS)

The concentration of compounds 1-6 (Table 4) were quantified in the non-aged and 4-month aged Arabica nitrogen-flushed RTD coffee samples using a UPLC Waters Acquity H-Class system coupled with TQS mass spectrometer (Waters Corp.). Quantification was carried out with 5-point standard addition calibration curves (in triplicate) and displayed good linearity of all the compounds (R2 > 0.98). Sample preparation was performed as described in Section 2.4 with the addition of 100 mg/L

methylparaben as an internal standard. Quantitative analysis was conducted using an Acquity H-class UPLC system (Waters Co., MA) coupled with a Xevo TQ-S mass spectrometer (Waters Co., MA) in multiple reaction monitoring (MRM) acquisition mode. Chromatographic separation of targeted compounds was achieved using a Cortecs UPLC T3 column (1.6 µm, 2.1 × 100 mm, Waters Corp.). The mobile phase consists of (A) water with 0.1% formic acid (v/v) and (B) acetone with 0.1% formic acid at a flow rate of 0.5 mL/min. The gradient was optimized as follow: 0-0.5 min, 5% B; 0.5-10 min, 5-15% B; 10-10.5 min, 15-20% B; 10.51-13 min, 95%; 13.01-16 min, 5% B. The concentration of quinic acid was also quantified using the same protocol with modification on the gradient as follows: 0-3 min, 0% B; 3-5min, 0-40% B; 5.01-7 min, 95% B; 7.01-9, 0% B. The mass spectrometer was operated under negative ESI mode with a capillary voltage of 3 kV, cone voltage of 30 V, desolvation temperature of 550 °C, source temperature of 150 °C, desolvation gas flow of 1000 L/h, and cone gas flow rate of 150 L/h. Multiple reaction monitoring (MRM) transition was optimized for each compound and presented in Table 4. MRM transition of methylparaben (internal standard) was monitored as m/z 150.95 → 91.85 with a cone voltage of 20 V and collision energy of 18 V.

2.8.Sensory recombination of RTD coffee

The non-aged Arabica nitrogen-flushed RTD coffee sample (pH = 4.92) was used as a control sample for the sensory recombination test. A decrease of pH in the RTD coffee sample was observed during storage; hence, two RTD coffee models were prepared to evaluate the effects of the highly predictive chemical features and the pH, separately. The RTD coffee model 1 was prepared by only adding food-grade HCl to reach the pH of an

aged sample (pH = 4.76), representing a pH adjusted control sample. The RTD coffee model 2 was prepared by spiking a mixture of compounds 1-6 into the control sample to match the concentration of these compounds in a 4-month aged Arabica nitrogen-flushed RTD coffee sample (see Table 4). The addition of compounds 1-6 dropped the pH of the non-aged RTD coffee to 4.79, which was further adjusted using food-grade HCl to mimic the pH of an aged sample (pH = 4.76).

Fourteen trained panelists (6 males and 8 females, ages 23 to 44) from the Ohio State University participated in the sensory recombination test. The RTD coffee control sample, model 1, and model 2 were kept in a hot water bath to maintain serving temperature (60 °C to 65 °C). Panelists were served 5 mL samples in 1-oz black cups. Panelists were asked to evaluate 3 pairs of RTD coffee samples following the same DOD protocol described in Section 2.3. In order to maintain serving temperature, panelists were given one pair of samples at a time; each pair consisted of a control sample and a test sample (control sample as blind control, model 1, or model 2). The serving order of test samples was randomized and balanced. All data were recorded using Compusense Cloud software (Compusense, Guelph, ON, Canada). Unsalted crackers and water were provided for panelists to cleanse their palate between samples. Study protocols were approved by the OSU Institutional Review Board (2021B0121).

2.9.Nuclear magnetic resonance (NMR)

The two selected chemical features that could not be compared to authentic commercial standards were identified using Nuclear Magnetic Resonance spectroscopy (NMR). NMR analysis was performed on a Bruker Advance III HD Ascend spectrometer

equipped with a 5-mm triple resonance observe TXO cryoprobe with z-gradients, operating at 700 MHz for the 1H nucleus and 176 MHz for the 13C nucleus (Bruker BioSpin, Rheinstetten, Germany). Instruments were calibrated using the residual undeuterated solvent as an internal reference CD3OD 1H NMR = 3.31 ppm, 13C NMR = 49.0 ppm. Deuterated methanol-d4 was used as a solvent to dissolve purified compounds 4 (3.59_367.1) and 5 (4.29_367.1), and NMR data are presented here.

3-O-Feruloylquinic acid (3.59_367.1): ^{1}H NMR (700 MHz, MeOD) 7.66 (d, *J* = 15.9 Hz, 1H), 7.20 (d, *J* = 2.0 Hz, 1H), 7.08 (dd, *J* = 8.1, 2.5 Hz, 1H), 6.81 (dd, *J* = 8.2, 2.5 Hz, 1H), 6.40 (d, *J* = 15.9 Hz, 1H), 5.36 (dt, *J* = 6.1, 3.6 Hz, 1H), 4.12 (td, *J* = 8.2, 3.9 Hz, 1H), 3.90 (s, 3H), 3.69 (dd, *J* = 7.8, 3.3 Hz, 1H), 2.31 (ddd, *J* = 11.6, 6.1, 2.8 Hz, 1H), 2.21 (ddd, *J* = 14.1, 6.9, 3.6 Hz, 1H), 2.10 (d, *J* = 11.6 Hz, 1H), 2.05 (m, 1H). ^{13}C NMR (176 MHz, MeOD) δ 175.7, 167.9, 149.4, 147.8, 146.7, 127.9, 124.1, 115.7, 115.0, 111.7, 75.1, 72.6, 70.2, 68.9, 56.4, 37.8, 36.9.

5-O-Feruloylquinic acid (4.29_367.1): ^{1}H NMR (700 MHz, MeOD) δ 7.63 (d, *J* = 15.9 Hz, 1H), 7.20 (s, 1H), 7.08 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.81 (dd, *J* = 8.8, 2.6 Hz, 1H), 6.39 (d, *J* = 15.9 Hz, 1H), 5.46 (ddd, *J* = 11.6, 9.9, 5.0 Hz, 1H), 4.12 (q, *J* = 3.3 Hz, 1H), 3.90 (s, 3H), 3.66 (dd, *J* = 9.8, 3.1 Hz, 1H), 2.28 (ddd, *J* = 12.6, 5.0, 3.0 Hz, 1H), 2.20 – 2.15 (m, 1H), 2.11 (dt, *J* = 14.3, 3.2 Hz, 1H), 1.97 (dd, *J* = 14.3, 2.9 Hz, 1H). ^{13}C NMR (176 MHz, MeOD) δ 182.7, 168.9, 150.5, 149.4, 146.6, 127.9, 124.0, 116.4, 115.9, 111.7, 80.7, 75.2, 73.2, 72.5, 56.4, 39.7, 37.9.

2.10. Data analysis

Statistical analysis was conducted with SPSS Statistics version 25 (IBM, Armonk, NY). DOD scores of each RTD coffee variety were analyzed by one-way ANOVA; when a significant difference was observed ($p < 0.05$) post-hoc LSD was performed between all samples, and 1-sided Dunnett's test was used to compare between the blind control (non-aged) and aged samples (1, 2, 4 months aged). Student's T-test was applied to analyze quantification data of non-aged and 4-month aged Arabica nitrogen-flushed RTD coffee samples.

3. Results and discussion

3.1. Degree of difference (DOD) sensory evaluation

The main goal of this study was to explore the chemical changes that impact the flavor stability of ready-to-drink (RTD) coffee during storage. RTD coffee samples were prepared from two coffee species (Arabica and Robusta) under different processing conditions (air versus nitrogen), which were selected to provide sample variation in chemical composition, allowing for more comprehensive data to enable the identification of universal chemical drivers of RTD coffee flavor stability. Different coffee species (Arabica and Robusta) have been reported to present different flavor attributes that directly affect the flavor quality of coffee brew (Ky et al., 2001; Nebesny & Budryn, 2006). In addition, the presence of oxygen has been shown to lead to the oxidation of unsaturated free fatty acids, resulting in unpleasant aroma and taste attributes in coffee (Kreuml et al., 2013).

Degree of difference (DOD) sensory evaluation was initially performed to characterize the overall flavor changes that occurred in RTD coffee during 4-month storage (Figure 4). As shown, the blind control samples were rated between 0.8 and 1.4 out of 10, corresponding to *none* to *very little* difference as explained on the DOD scale. The blind control samples successfully helped to detect the baseline in panelists' sensory responses (Aust et al., 1985) and ensured that panelists performed well in recognizing the real differences that existed between non-aged and aged samples. Overall, the results indicated that the largest degree of flavor change occurred within the first month of storage. The DOD scores for the 1-month samples ranged between 3.9 to 5.3, corresponding to a *little* to *medium* difference. For the majority of samples, the difference scores began to level out at 2-4 months at 5.9 to 6.9, corresponding to a *medium* to *strong* difference. Subsequently, ANOVA and post-hoc analysis (LSD and Dunnett's test) were applied to each RTD coffee variety to analyze DOD scores. In brief, significant differences ($p < 0.05$) were observed between the non-aged samples (blind controls) and all aged samples (1-month, 2-month, and 4-month) (Figures 4A-4D); however, not all the aged samples were significantly different from each other. For the Arabica air-headspace and nitrogen-flushed RTD coffees (Figures 4A and 4B), the 4-month samples were significantly more different than the 1-month samples. For the Robusta air-headspace sample (Figure 4C) the 2-month and 4-month samples were significantly more different than the 1-month sample. For the Robusta nitrogen-flushed sample (Figure 4D) the 4-month sample was significantly more different than the 1 and 2-month samples. Although sensory comparison was not carried out between

different RTD coffee varieties, it could be noticed that RTD coffee samples from different coffee species and processing conditions presented a similar DOD trend during storage.

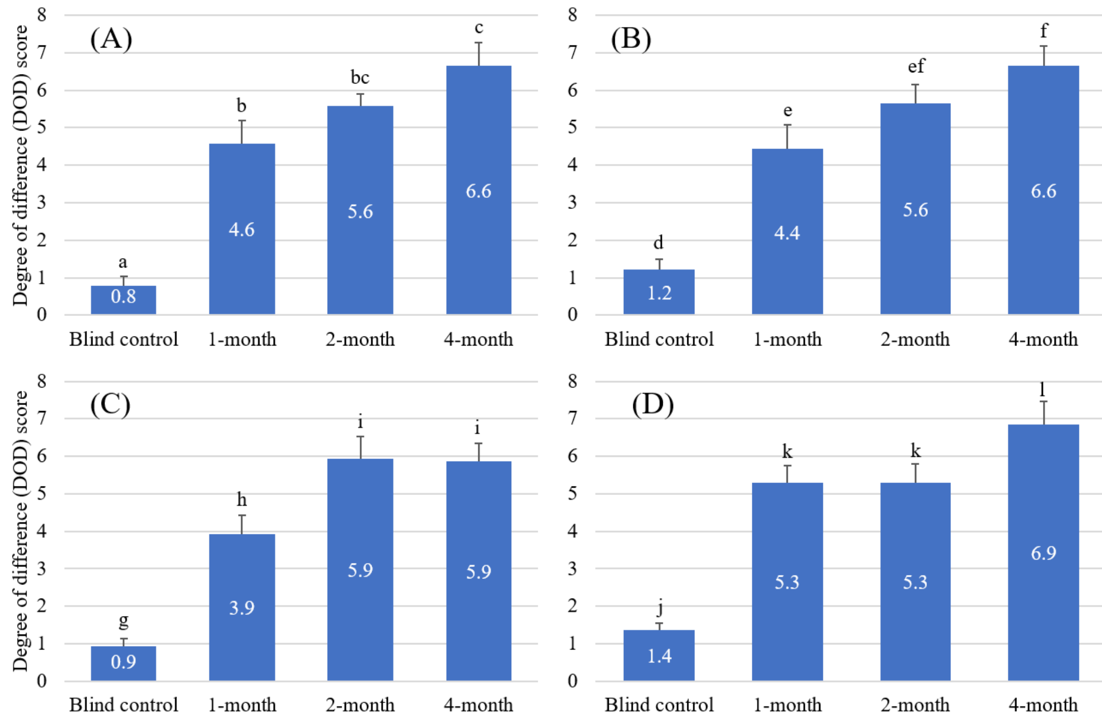


Figure 4. Mean degree of difference (DOD) scores of RTD coffee samples with standard error, on a 10-point scale (A) Arabica air-headspace (B) Arabica nitrogen-flushed (C) Robusta air- headspace (D) Robusta nitrogen-flushed; within each RTD coffee variety, different letters represent significant differences in DOD scores according to post hoc LSD test ($p < 0.05$); n = 14.

In addition to DOD scores, panelists (n=14) provided qualitative comments on the flavor differences observed between RTD coffee samples. In general, panelists indicated

that sourness was one of the major drivers for the DOD scores. For example, “more sour” was mentioned 12 times, followed by “more fruity” 6 times, “more astringent” 3 times, and “less coffee aroma” 3 times for the 4-month aged Arabica nitrogen-flushed sample. Given that sourness is known to be impacted by the pH and acidity in coffee (Clarke, 2012), the pH values of RTD coffee samples were measured. The non-aged coffee decreased from pH 4.92 to 4.76 in the 4-month aged Arabica nitrogen-flushed RTD coffee sample; similar results were observed in other RTD coffee samples. This observation was in agreement with previous studies that reported a decrease in pH and the development of sour taste in coffee brew during 60 days of storage (Pérez-Martínez et al., 2008b; Rosa et al., 1990). Anese and Nicoli (2003) reported a zero-order kinetic change of [H^+] concentration in RTD coffee during storage and suggested that the rate of pH decrease was not affected by the presence of oxygen. Thus, in the current study, it was expected the noted increased sour attributes and reduction in pH values during storage would originate from the generation of acidic compounds. To investigate the compounds that contributed to the development of sour taste and overall flavor changes of aged RTD coffee, DOD scores were modeled with chemical data in the following multivariate analysis.

3.2.Multivariate statistical modeling

Multivariate statistical modeling was applied to establish correlations between chemical profiles and flavor changes of RTD coffee during storage. Specifically, non-volatile chemical profiling data of RTD coffee samples collected through the UPLC-MS platform were converted into a total of 1489 chemical features, while the DOD scores were used as predictive variables and modeled against chemical features.

Initially, an unsupervised principal component analysis (PCA) was employed to assess data quality and investigate the natural clustering of RTD coffee samples (Figure 5a). RTD coffee samples were colored according to the month of storage. As seen, the first principal component (PC) separated the RTD coffee samples based on coffee species, which explains 73% of the total chemical variations. It was expected that the foremost chemical variations came from the coffee species as Arabica and Robusta have been reported to be chemically different in a wide range of compounds (Bertrand et al., 2008; Bicho et al., 2013; Calvini et al., 2017; El-Abassy et al., 2011). Following the species variations, the storage effect determined the second PC's clustering, accounting for 14% of the total chemical variation. It could be seen that the non-aged RTD coffee samples separated from the aged samples, while aged samples from different months of storage nearly clustered together. This result indicated that, from a storage perspective, the most distinctive chemical changes happened during the first month, and after which, the chemical changes kept increasing but at a slower rate. RTD coffee samples changed chemically during storage, matching the trend of DOD scores, which demonstrate the impact of chemical changes on the flavor changes in RTD coffee. In addition, two replicates of QC samples overlapped together, indicating good instrument performance during chemical profiling. Good model metrics were achieved ($R^2X = 0.926$, $Q^2 = 0.887$), and sample clustering reflected their natural properties, meaning that chemical variations within RTD coffee samples were adequately captured by UPLC-MS profiling and correctly characterized by PCA modeling.

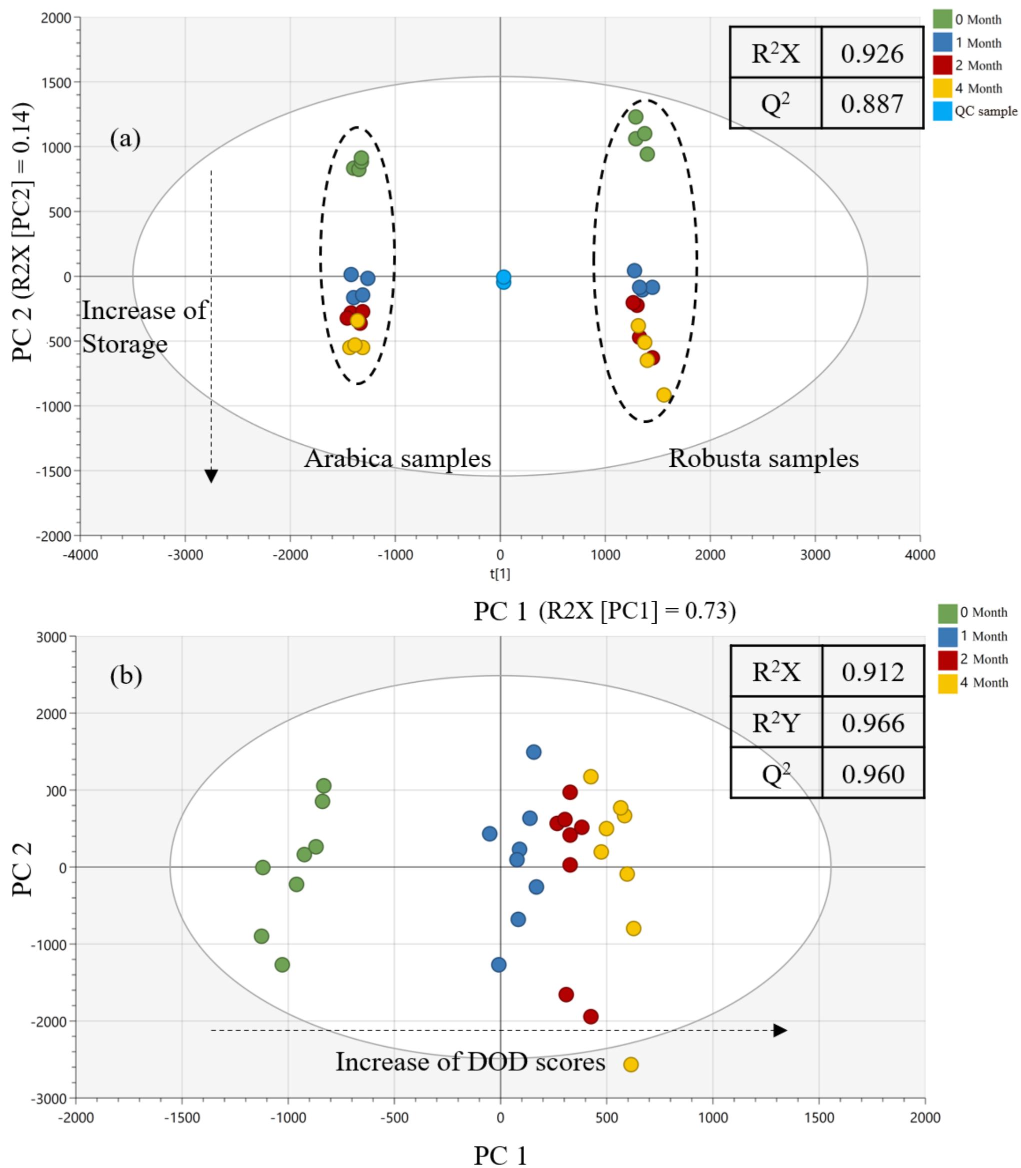


Figure 5. Multivariate analysis of LC-MS profiles for RTD coffee samples with duplicates (a) PCA score plot color-coded by storage time (b) OPLS score plot for the LC-MS profile versus degree of difference (DOD) score, color-coded by storage time. Models were generated with two RTD coffee biological replicates.

Subsequently, a supervised orthogonal partial least squares (OPLS) model was generated to distinguish the variations in chemical profiling data (X variables) that were correlated with DOD scores (Y variable). As shown in Figure 5b, chemical profiling data of RTD coffee samples presented highly correlated linearity to DOD scores with great goodness of fit ($R^2Y = 0.966$) and high predictive power ($Q^2 = 0.960$). In general, PLS models with $Q^2 > 0.5$ are considered as acceptable predictivity in the untargeted metabolomics studies (Triba et al., 2015); however, higher values indicate stronger correlation and better predictivity (Eriksson et al., 2013; Wold et al., 2001). Additionally, a permutation test was included to ensure that model had no overfitting effects (permuted $R^2 = 0.315$, permuted $Q^2 = -0.565$) (Eriksson et al., 2013).

To identify compounds that impacted the flavor of RTD coffee samples during storage, chemical features that were highly predictive of the DOD scores were selected based on the predictive variable of importance scores (VIPpred) and S-plot (Figure 6). The VIPpred scores explain the contribution of X variables (chemical features) in predicting Y variable (DOD scores) (Galindo-Prieto et al., 2014). Typically, X variables with VIPpred scores > 1 are considered significant contributors (Chong & Jun, 2005). The S-plot provides visualization of the variable's influence in a model according to the covariance (magnitude of changes, p[1]) and correlation coefficient (correlation to Y, p[corr1]) of each X variable. Similar criteria have been successfully applied to select predictive features in untargeted flavoromic studies (Charve et al., 2011; Cong, Schwartz, Tello, et al., 2021; Ronningen et al., 2017; Sittipod et al., 2019). In this study, compounds that were positively correlated to DOD scores during storage were investigated to focus on compounds

generated during storage. Among the top ten VIPpred features for the PLS model, five positively correlated features were selected (score 3.7-5.6) and are shown in Figure 6 and reported in Table 4 (compounds 1-5).

Table 4. Highly predictive positively correlated compounds of sensory changes for nitrogen flushed Arabica RTD coffee during storage and quinic acid

Compound	Chemical feature (RT_m/z)	VIPpred score	MRM transition (collision energy)	Compound identity	Compound concentration (mg/L) [i]		Sample concentration difference (mg/L)	% Change (concentration)
					non-aged RTD coffee	4-mo aged RTD coffee		
1	2.68_353.1	3.7	353.1 → 191.1 (20)	3-caffeoylquinic acid	172.9[a]	254.4[b]	81.5	47.1
2	3.22_353.1	5.6	353.1 → 179.0 (18)	5-caffeoylquinic acid	207.8[a]	266.6[b]	58.8	28.3
3	3.36_353.1	4.7	353.1 → 191.1 (18)	4-caffeoylquinic acid	165.2[a]	209.5[b]	44.3	26.8
4	3.59_367.1	4.1	367.1 → 134.0 (34)	3-O-feruloylquinic acid	87.8[a]	106.3[b]	18.5	21.1

Continued

Table 4 Continued

5	4.29_367.1	4.5	367.1 → 191.0 (16)	5-O-feruloylquinic acid	56.9[a]	66.2[b]	9.3	16.3
6	NA	Targeted[ii]	191.1 → 84.9 (22)	Quinic acid	1258.9[a]	1427.8[b]	168.9	13.4

i. Different letters indicate significant differences in compound concentration according to Student's T-test ($p < 0.05$);

ii. The compound was analyzed by targeted analysis.

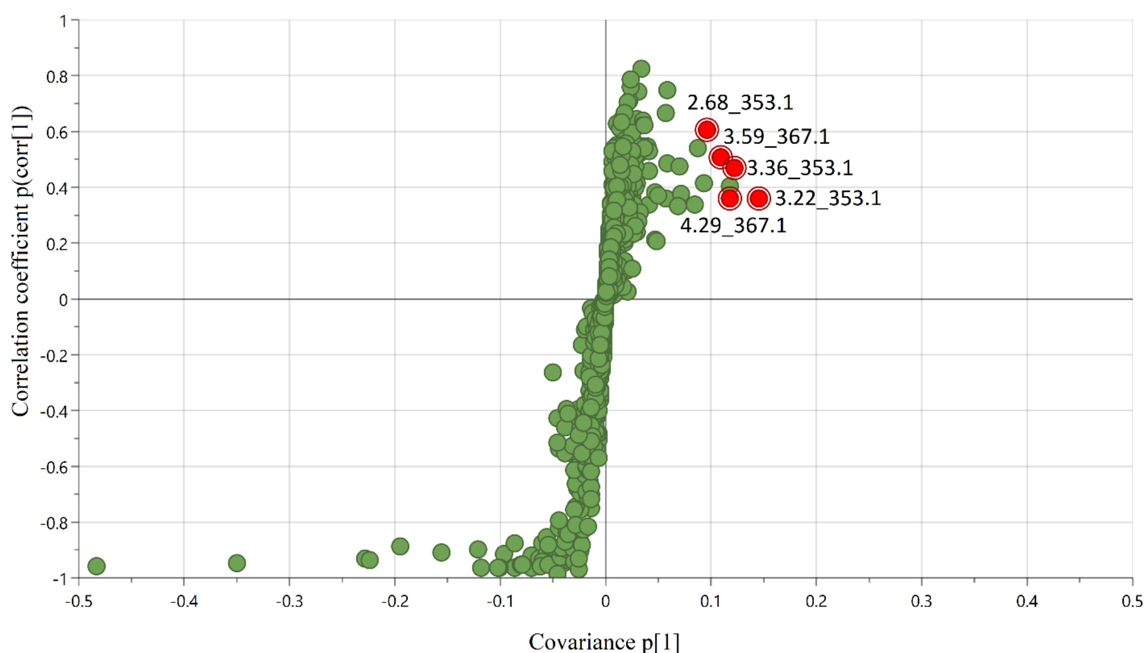


Figure 6. S-plot from OPLS model for DOD during storage; highlighted dots represent selected positively correlated chemical markers of interest.

3.3.Targeted analysis of organic acids

Because of the expected importance of the generation of acidic compounds on the noted DOD sensory scores during storage of the coffee samples, limitations of the untargeted LC/MS profiling method used in the current study to characterize the acidic compounds were considered. It was anticipated sample clean-up losses and limited reverse-phase chromatographic separation resulted in some hydrophilic organic acids being not adequately included in the untargeted chemical profiling and the statistical modeling. Therefore, a targeted analysis of well-known hydrophilic non-volatile organic acids in the samples was also conducted. Organic acids are an important chemical

component of coffee, which account for about 6% of the roasted coffee beans' weight and contribute to the pH and titratable acidity in the coffee brew (Clarke, 2012). Important organic acids in coffee include but are not limited to citric acid, quinic acid, malic acid, formic acid, acetic acid, phosphoric acid, and chlorogenic acids (Clarke & Vitzthum, 2008; Illy & Viani, 2005). In the current study, the non-volatile highly polar organic acids including citric acid, quinic acid, malic acid, and phosphoric acid were monitored by targeted analysis (chlorogenic acids were monitored by the untargeted analysis). Quantitative and statistical analysis of these four acid compounds reported that only quinic acid presented significant concentration differences ($p < 0.05$) between non-aged and aged samples, which is shown in Table 4. Consequently, quinic acid was also selected, in addition to the positively correlated predictive features 1-5 (Table 4) for further investigation.

3.4.Identification of positively correlated predictive features

Compounds 1, 2 and 3, with VIPpred of 3.7, 5.6, and 4.7 respectively (Table 4) displayed the same accurate mass parent ion $[M-H]^-$ at m/z 353.0882, which corresponds to a molecular elemental composition of $C_{16}H_{18}O_9$ (Δmass = 0.67 ppm), indicating the three compounds were isomers. MS/MS fragmentation also revealed a common product ion of m/z 191.1 after losing 162.0 mass units. This fragmentation pattern matched the cleavage of an ester bond between quinic acid and caffeic acid moieties in a caffeoylquinic acid molecule (Clifford et al., 2003). Hence, features 2.68_353.1, 3.22_353.1, and 3.36_353.1 (Table 4) were compared to authentic commercial standards by matching retention time, accurate mass, and MS/MS fragmentation; and compounds 1, 2 and 3 were identified as 3-

caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA), and 4-caffeoylquinic acid (4-CQA), respectively (Table 4).

Compounds 4 and 5 with a VIPpred of 4.1 and 4.5, respectively had a calculated molecular elemental composition $C_{17}H_{20}O_9$ (Δmass = 0.74 ppm) assigned to parent ion $[M-H]^-$ with an accurate mass m/z 367.1037. The MS/MS fragmentation indicated the structure contained a ferulic acid moiety (m/z 193.1). According to the elemental composition and fragmentation pattern, it was speculated that these two features belonged to the feruloylquinic acid family (Kuhnert et al., 2010). However, authentic commercial standards were not available, thus 1D and 2D NMR analysis was required for positive identification. Compounds 4 and 5 were isolated from the coffee samples through multiple dimensions of LC fractionation as was described in section 2.6, with > 90% purity, for NMR analysis. The ^1H NMR data showed similar key chemical shifts and coupling constants, indicating structural isomerism between these compounds. Compound 4 showed three aromatic signals at δ 7.20 (d, J = 2.0 Hz, 1H), δ 7.08 (dd, J = 8.1, 2.5 Hz, 1H), and δ 6.81 (dd, J = 8.2, 2.5 Hz, 1H), indicating the trisubstituted ring moiety. Also, two olefinic signals at δ 7.66 (d, J = 15.9 Hz, 1H), and δ 6.40 (d, J = 15.9 Hz, 1H), revealed the *E* geometry in the double bond in the vinylcatechol group. The key signals attributed to three carbinolic methines at δ 5.36 (dt, J = 6.1, 3.6 Hz, 1H), δ 4.12 (td, J = 8.2, 3.9 Hz, 1H), and δ 3.69 (dd, J = 7.8, 3.3 Hz, 1H) confirmed the caffeic acid moiety. All ^1H and ^{13}C NMR data analysis of compound 4 and the match with the data reported in the literature allowed for the identification of this compound as 3-O-Feruloylquinic acid (Dokli et al., 2013). Likewise, compound 5 presented similar key NMR signals, scilicet, three aromatic signals

located at δ 7.20 (s, 1H), δ 7.08 (dd, J = 8.2, 2.0 Hz, 1H), and δ 6.81 (dd, J = 8.8, 2.6 Hz, 1H), two olefinic signals resonating at δ 7.63 (d, J = 15.9 Hz, 1H) and δ 6.39 (d, J = 15.9 Hz, 1H), indicating *E* configuration, and the same three carbinolic methines groups located at δ 5.46 (ddd, J = 11.6, 9.9, 5.0 Hz, 1H), δ 4.12 (q, J = 3.3 Hz, 1H), and δ 3.66 (dd, J = 9.8, 3.1 Hz, 1H). All ^{1}H and ^{13}C NMR data analysis of compound 5 matched the data reported in the literature for compound 5-O-Feruloylquinic acid (Dokli et al., 2013), allowing the structural identification and confirming the structural isomerism between compounds 4 and 5.

3.5.Quantification of positive correlated predictive features in RTD coffee samples

Since RTD coffee samples made from different species and processing conditions exhibited similar trends in chemical and flavor change during storage (Figure 4), the Arabica nitrogen-flushed RTD coffee was selected as a representative sample for the following quantification and sensory recombination testing. The concentrations of compounds 1-6 were quantified in the non-aged and 4-month aged samples, shown in Table 4.

The concentrations of the acidic compounds 1-5 significantly ($p < 0.05$) increased during storage, as expected based on the noted decrease in pH for RTD coffee samples during storage. Quinic acid showed the largest absolute concentration change by increasing 169 mg/L over 4 months, while 3-caffeoylquinic acid presented the largest percentage change of 47%. An increase of quinic acid concentration after brewing has been reported, which is generally attributed to the breakdown from chlorogenic acids and the hydrolysis of quinic acid lactones (Clarke & Vitzthum, 2008; Clarke & Macrae, 1988; Rosa et al.,

1990). In a similar way, hydrolysis of the intramolecular ester bond in chlorogenic acid lactones has also been observed in the coffee brew (Bennat et al., 1994; Karin Kraehenbuehl et al., 2017; Schrader et al., 1996), which to some extent explained the increase in chlorogenic acid concentration including caffeoylquinic acids (CQAs) and feruloylquinic acids (FQAs). More recently, it was reported quinic acid and chlorogenic acids are incorporated into low molecular weight coffee brew melanoidins during the roasting process (Bekedam, Roos, et al., 2008; Bekedam, Schols, et al., 2008; Moreira et al., 2015; Moreira et al., 2017). The subsequent release of acids from melanoidins during storage may explain in part the acidification of RTD coffee during storage.

The influence of compounds 1-6 on RTD coffee acidification during storage was evaluated. Compounds 1-6 were added to the non-aged Arabica nitrogen-flushed sample to match the concentration of the 4-month aged sample (Table 4). The pH dropped from 4.92 to 4.79, which accounted for 81% of the total pH decrease compared to a 4-month aged sample at pH 4.76. The remaining 19% (0.3 pH units) is likely related to the increasing concentration of other acids not identified.

3.6. Sensory recombination of positively correlated features with RTD coffee samples

The sensory impact of the highly predictive positively correlated compounds 1-5 as well as the additional targeted acidic compound 6 (Table 4) were further investigated. The DOD scores of two aged RTD coffee recombination models were compared to the non-aged RTD coffee sample. Model 1 was the control sample that was pH adjusted to mimic the RTD coffee after 4 months storage, while model 2 was also pH adjusted but

additionally contained the higher concentrations of compounds 1-6 as reported in the aged coffee sample (Table 4). As shown in Figure 7, the DOD score of the blind control sample was rated as 0.2, indicating the good performance of the trained panelists (no detectable difference). Both models 1 and 2 with DOD scores of 2.7 and 3.1, respectively, were significantly different from the blind control sample (non-aged) at $p < 0.05$. The DOD scores for models 1 and 2 were not significantly different from each other and corresponded to a *little* difference on the DOD scale.

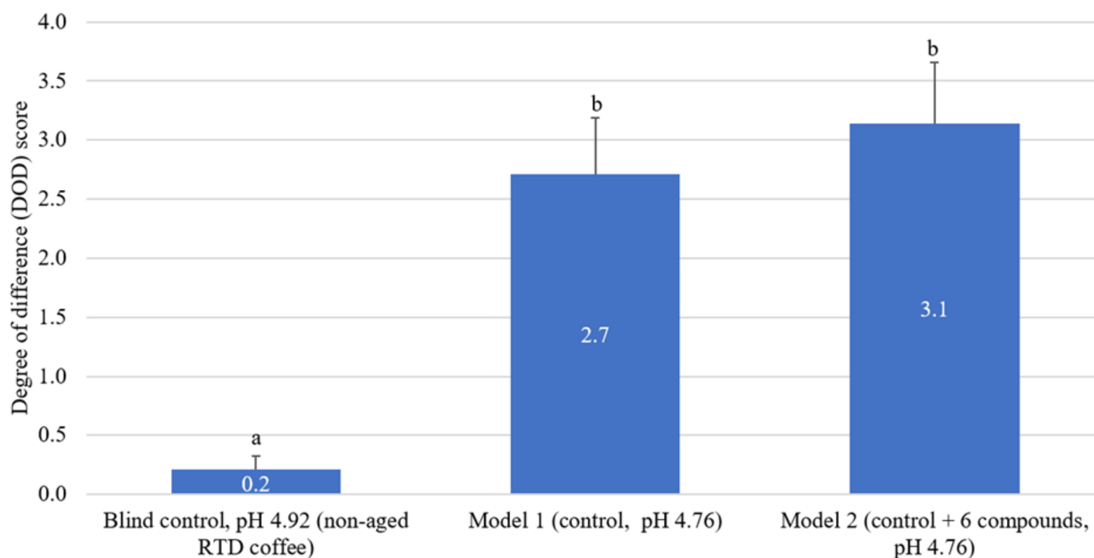


Figure 7. Mean degree of difference (DOD) scores with standard error for blind control and recombination models 1 (control, pH adjusted) and model 2 (control, pH adjusted with the addition of 81.5, 58.5, 44.3, 18.5, 9.3 and 168.9 mg/L of 3-caffeoylquinic acid, 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3-O-feruloylquinic acid, 4-O-feruloylquinic

acid and quinic acid, respectively); letters represent significant differences in DOD scores according to 1-sided Dunnett's test ($p < 0.05$); n = 14, DOD scores ranged 0-10

Results from model 1 (Figure 7) indicated that the decrease of pH that occurred during storage significantly changed the overall flavor of the RTD coffee sample. Panelists (10 out of 14) mentioned that sourness was the primary reason for the difference between the control sample and model 1. The sensory evaluation for the 4-month aged Arabica nitrogen-flushed RTD coffee samples received a DOD score of 6.6 (Figure 4), while recombination model 1 received a DOD score of 2.7, accounting for about 40% of the coffee samples DOD score. In general, the pH of Arabica coffee brew ranges from 4.85 to 5.13 (Leroy et al., 2006; Rao & Fuller, 2018), and a pH of 4.8 or higher is considered a critical value for acceptable coffee quality (Rosa et al., 1990). A change in coffee brew pH of 0.1 units has resulted in significant differences in perceived sourness (Woodman, 1985). In the current study, a decrease in coffee pH of 0.16 units (4.94 to 4.76) was reported to significantly impact the sensory profile of the sample. Similar to the sensory DOD analysis of the non-aged to aged coffee samples, panelists indicated that sourness was one of the primary drivers for overall flavor changes in the recombination model 1 (Figure 7). These results indicated that sourness development in the RTD coffee was associated with the decrease of pH over time, which significantly contributed to the overall flavor changes during storage.

In addition to the impact of pH change on flavor stability of RTD coffee during storage, the influence of the increased concentration of the weak acids on sensory DOD

scores was also evaluated in model 2 (Figure 7). Sourness in coffee has been related to both the pH value and total acidity (Clarke & Vitzthum, 2008). Model 2 was not reported to be significantly different from model 1 (Figure 7) in DOD score indicating the increased acid concentration during storage did not impact the flavor stability due to a higher total acidity content but rather by the direct change in pH (Table 4).

Although the panelists indicated sour was the primary difference observed between the samples, in addition to sourness, quinic acid and chlorogenic acids have been associated with coffee flavor attributes such as bitterness, astringency, and lingering aftertaste in past studies (Buffo & Cardelli-Freire, 2004; Seninde & Chambers, 2020; Sunarharum et al., 2014). Quinic acid was reported to exhibit an aspirin-like bitter taste at a threshold level of 10 mg/L (Frank et al., 2006). Moreover, quinic acid was found to be associated with astringency perception and lingering aftertaste in cranberry juice (Peleg & Noble, 1999) and fruit pulps (Marsh et al., 2006). In the current study, comparing the increase of quinic acid from 1259 mg/L to 1429 mg/L during storage (Table 4) quinic acid possibly contributed to the overall flavor of RTD coffee (bitterness, astringency). However, the increased concentration of quinic acid during storage was not found to increase the DOD beyond the impact of pH change (Figure 7). Compounds 1-5 (CQAs and FQAs) belong to the chlorogenic acid family, which is also known to affect the sourness, bitter taste, astringency, and other sensory attributes of coffee brew (Campa et al., 2005; Trugo & Macrae, 1984; Variyar et al., 2003). Similarly, the increased amount of these chlorogenic acids in the RTD coffee during storage was not shown to impact the DOD beyond the influence of pH (Figure 7). Higher chlorogenic acid content in coffee has been shown to

be associated with less bitterness (Gao et al., 2021; Wei et al., 2014) and more astringency (Borsato et al., 2011; dos Santos Scholz et al., 2018). However, the higher concentrations of quinic acid and chlorogenic acids observed in the aged samples (Figure 7) did not significantly impact the reported DOD beyond the impact of sample pH.

Changes in the aroma were also reported in the aged samples however they were less frequently noted than taste (sour). This study focused specifically on the non-volatile flavor changes. However, changes in the aroma volatility and stability would be expected. Some potent well-known coffee odorants such as 2-furfurylthiol and 3-mercapto-3-methylbutyl esters have shown pH dependence in RTD coffee drinks (Kumazawa & Masuda, 2003a, 2003b).

4. Conclusion

A novel understanding of RTD coffee generation pathways during storage was identified. Five specific chlorogenic acid compounds 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3-O-feruloylquinic acid, and 5-O-feruloylquinic, in addition to quinic acid, were reported to be generated during storage and reduced the product pH, directly impacting the flavor of RTD coffee. Untargeted LC/MS flavoromics analysis was successfully applied to define compounds that impact the flavor stability of RTD coffee.

Chapter 3. Identification of non-volatile compounds that are degraded during storage and impact flavor stability of ready-to-drink coffee

Abstract

Untargeted LC/MS flavoromics analysis was applied to identify chemical compounds that degraded during storage and impact the flavor stability of ready-to-drink (RTD) coffee. MS chemical profiles for sixteen ready-to-drink (RTD) coffee samples stored for 0, 1, 2 and 4 months at 30 ºC were modeled against the sensory degree of difference (DOD) scores by orthogonal partial least squares (OPLS) with good fit and predictive ability. Ten highly predictive chemical features that negatively correlated with flavor changes of RTD coffee during storage were subsequently identified (MS and NMR). Quantitative analysis indicated eight of the ten compounds were significantly ($p<0.05$) degraded during storage. Sensory recombination testing of the eight compounds as a mixture and individually revealed two compounds, 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide, impacted the flavor stability of RTD coffee at subthreshold concentrations. Furthermore, these two compounds were completely degraded after 4 months of storage.

1. Introduction

Coffee is one of the most popular beverages in the world, with an estimated consumption of 3 billion cups every day (Samper et al., 2017). The global coffee industry was valued at more than $200 billion in 2019 (ICO, 2019). In the United States, the coffee market is valued at $15 billion and still growing (Bryant, 2020). The market includes the sales of roasted coffee beans, instant coffee, ready-to-drink (RTD) coffee, and coffee beverages. Among all coffee products, RTD coffee maintained the fastest growth rate between 2017 to 2019 in the US, strongly contributing to the total growth of the coffee market (Failla, 2019).

Coffee flavor quality is one of the most significant drivers of consumption (Labbe et al., 2015; Samoggia & Riedel, 2018; Sousa et al., 2016). The flavor of coffee is the result of a complex mixture of chemical compounds, which are impacted by several factors such as coffee species, geographical origins, agricultural practices, post-harvesting processing methods, roasting, brewing techniques, and storage (Buffo & Cardelli-Freire, 2004; Sunarharum et al., 2014).

The flavor attributes of RTD coffee are known to be affected by manufacturing processes and storage. Previous research efforts have primarily focused on the negative influences of the manufacturing process on RTD coffee flavor. The characteristic roasty notes of RTD coffee flavor have been found to decrease significantly during aseptic heat treatment (Kumazawa, 2006). The stability of several well-known coffee sulfur-containing odorants such as 2-furfurylthiol, methional, and 3-mercapto-3-methylbutyl formate are sensitive to heat sterilization; moreover, the volatility of these odorants is affected by pH

adjustment during RTD coffee production (Kumazawa & Masuda, 2003a, 2003b; Kumazawa et al., 1998). Murakami et al. (2010) reported that RTD coffee presented less intense aroma and taste profiles after thermal treatment compared to freshly brewed coffee. Given the important role that coffee flavor plays in consumption, an improved understanding of the chemical basis for coffee flavor quality in RTD products and flavor stability during storage is needed for product improvement strategies.

There are a limited number of studies that have focused on the flavor stability of RTD coffee during storage, and they have primarily focused on changes in aroma attributes. Akiyama et al. (2014) reported that the concentrations of 4-vinylguaiacol and pyrazines with nutty-roast odor decreased in aseptically packaged coffee beverages during a 2-week storage period, which significantly affected the retronasal aroma profile. Pérez-Martínez et al. (2008a) monitored the changes of 47 volatile compounds in coffee brew stored at 4 °C and 25 °C for 30 days and evaluated their influence on the loss of aroma intensity and freshness attributes during storage. Conversely, limited information is known about the stability of the non-volatile flavor profile in RTD coffee during storage. Several phenolic compounds have been associated with the generation of sourness, rancid flavor, and astringent aftertaste in coffee brew during storage (Pérez-Martínez et al., 2008b). As the growing market for RTD coffee emphasizes the demand for longer flavor shelf-life, it is essential to understand the non-volatile chemical basis of RTD coffee flavor stability to provide better RTD coffee flavor.

Historically, the characterization of compounds that impact RTD flavor has relied on traditional targeted analytical methods. However, targeted flavor analysis can overlook

contextual interactions or flavor modulators that are odorless/tasteless on their own but impact flavor perception. Thus, to build on prior findings a research approach to more comprehensively identify flavor changes of RTD coffee during storage is needed. Reineccius (2008) introduced the concept of an untargeted approach to flavor characterization called flavoromics. The advantage of untargeted flavoromics lies in its ability to monitor a wide range of chemical compounds instead of a particular group of compounds (Charve et al., 2011). Using flavoromics, Sittipod et al. (2019) identified 3-O-caffeoyl-4-O-3-methylbutanoylquinic acid and its corresponding lactone in coffee that positively associated with overall coffee cup quality. Similarly, Gao et al. (2021) found that a tasteless compound, 2-O-β-D-glucopyranosyl-atractyligenin, could modulate bitterness in the coffee brew. Hence, flavoromics can provide new insight to characterize RTD coffee stability during storage.

In the second phase of this two-phase study, untargeted LC/MS flavoromics analysis was applied to identify non-volatile compounds that were degraded during the storage of RTD coffee and impacted flavor stability. Multivariate analysis was utilized to establish the correlation between non-volatile chemical profiling data and overall flavor changes. Highly predictive negatively correlated compounds of RTD coffee flavor changes were selected from statistical models, purified using multidimensional fractionation, and identified by MS and NMR. Finally, sensory recombination was performed to validate the sensory relevance of the identified compounds on RTD coffee flavor stability.

2. Materials and methods

2.1. Chemicals and Materials

Nanopure water was purified using a Barnstead Nanopure Diamond Water Purification System (Thermo Fisher, Dubuque, IA). UPLC-grade formic acid, acetonitrile, methanol, and acetone were purchased from Fisher Scientific (Waltham, MA). Methylparaben, deuterated methanol, and deuterated water were purchased from Millipore Sigma (Burlington, MA). Leucine enkephalin and Oasis HLB cartridges (10mg and 6g) were purchased from Waters Co. (Milford, MA). Organic green Arabica and Robusta coffee beans were sourced from local suppliers (Columbus, OH).

2.2. Ready-to-drink (RTD) coffee samples

RTD coffee samples were prepared as described in Chapter 3 Section 2.2. In brief, RTD coffee samples were made from 2 coffee species (Arabica and Robusta) under 2 processing conditions (air-headspace and nitrogen flushed) and subsequently aged for 4 months (storage time points: 0, 1, 2, 4). All samples after storage were kept at -40 °C for instrumental and sensory analysis. The combination of sample conditions gave a total of 16 independent RTD coffee samples (n=16). In the following discussion, the frozen 0-month RTD coffee was considered a non-aged RTD coffee sample.

2.3. Sensory evaluation by the Degree of Difference (DOD) test

The overall flavor differences between non-aged and aged RTD coffee from 1, 2, and 4 months were measured using a degree of difference (DOD) test, as described in detail in Chapter 3 Section 2.3. In summary, fourteen trained panelists (6 males and 8 females, ages 23 to 44) evaluated the overall flavor change of RTD coffee flavor between non-aged

and aged RTD coffee samples from 1, 2, and 4 months, according to a DOD scale (Figure 8). All results were entered into Compusense Cloud sensory analysis software (Compusense, Guelph, ON, Canada). Study protocols were approved by the OSU Institutional Review Board (2017H0072).

0	1	2	3	4	5	6	7	8	9	10
None	Very Little		Little		Medium		Strong		Extreme	

Figure 8. Degree of Difference (DOD) scale. The DOD scale was provided to panelists in printed form and displayed in the Compusense Cloud sensory analysis software.

2.4. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) chemical profiling

The non-volatile chemical profiling was conducted with reverse-phase Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). Sample clean-up procedure, chromatographic separation methods, and mass spectrometer settings were presented in Chapter 3 Section 2.4. In summary, all RTD coffee samples were prepared with 2 biological replicates, and each biological replicate was injected twice on the UPLC system. All injections were performed in randomized order. After running every 10 samples, a water blank, a column standard, and a QC sample were injected to monitor analytical performance.

2.5.Multivariate statistical analysis (MVA)

Data processing, including retention time alignment, deconvolution, ion extraction, and integration, was performed using the Progenesis QI software (Nonlinear Dynamics, Durham NC). Each chemical feature was reported as a retention time-mass/charge ratio (RT_*m/z*) with ion abundance. Data filtering based on ion intensity cutoff (> 500 counts) and coefficient of variance between replicates (< 30%) were applied to chemical features by running an R script (version 3.5.2, R Foundation, Vienna, Austria). Multivariate data analysis was then conducted with two RTD coffee biological replicates using SIMCA-P+ 14.1 (Umetrics, Umea, Sweden). Principal component analysis (PCA) and orthogonal partial least squares (OPLS) models were built with Pareto-scaling. Specifically, the X variables and Y variables in the OPLS model were the chemical features (RT_*m/z* by ion abundance) and the DOD scores of RTD coffee samples, respectively. Then five chemical features with high predictability of RTD coffee overall flavor changes during storage were selected based on the predictive variable of importance (VIPpred) scores and S-plot. The concentrations of these chemical features decreased during storage as larger overall flavor changes were observed within RTD coffee samples. Hence, they were referred to as negatively correlated predictive features.

2.6.Off-line Multidimensional Preparative-Liquid Chromatography/Mass Spectrometry (Prep-LC/MS) Fractionation

Based on the multivariate statistical modeling, top predictive chemical features were selected and isolated for identification and sensory validation. Five selected chemical

features (RT_*m/z*) 3.92_335.1, 4.07_335.1, 4.24_335.1, 5.22_349.1, and 5.28_349.1 were isolated from the coffee brew as follows.

Three hundred grams of fresh coffee grounds were added to 3 L of 80% methanol/water (v/v). The mixture was stirred at room temperature overnight and then filtered through filter paper (GE Healthcare, Buckinghamshire, UK) and a 5-kDa ultrafiltration membrane (Millipore Sigma, Burlington, MA). Sample clean-up was performed with an Oasis HLB 6 g bed cartridge (Waters, Mil-ford, MA). Briefly, 200 mL of filtered coffee was loaded onto the cartridge. Then 100 mL of 5% methanol/water (v/v) was used to wash the cartridge, and 100 mL of 95% methanol/water (v/v) was used to elute analytes from the cartridge. Multiple cartridges were used at the same time to increase extraction yield. The eluent from different cartridges was pooled and freed from the solvent using a Rocket Synergy Purge (Genevac, Ipswich, UK). After freeze-drying, the eluent was reconstituted to approximately 500 mg/L in 20% methanol/water (v/v), filtered through a PTFE 0.45-µm filter, and then injected into the Prep LC-MS fractionation system (Waters 2545 binary pump and TQD mass spectrometer coupled with 2767 fraction collector).

First dimension isolation was performed using an Xbridge Prep C18 column (5 µm, 50 mm × 50 mm, Waters Corp.) with a mobile phase consisting of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid at a flow rate of 100 mL/min. The gradient was set as follow: 0-0.5 min, 5% B; 0.5-1.5 min, 5-27% B; 1.5-5.5 min, 27% B; 5.5-8.5 min, 27-50% B; 8.5-10 min, 50-95% B; 10-13 min, 95%; 13.01-15 min, 5% B. During first dimension fractionation, features 3.92_335.1, 4.07_335.1,

4.24_335.1 were collected into one fraction (fraction 1), while features 5.22_349.1, and 5.28_349.1 were collected into another (fraction 2). These fractions were freed from solvent, lyophilized, and then reconstituted in 10% methanol (v/v).

Second dimension fractionation was performed using an Atlantis T3 OBD column (5 µm, 50 mm × 250 mm, Waters Corp.) using a mobile phase consisting of (A) water with 0.1% formic acid and (B) acetone with 0.1% formic acid at a flow rate of 100 mL/min. The gradient was optimized to separate each selected chemical feature. For fraction 1 from the first dimension (containing features 3.92_335.1, 4.07_335.1, and 4.24_335.1), the gradient was set as follows: 0-0.5 min, 5% B; 0.5-1 min, 5-20% B; 1-30 min, 20% B; 30-31 min, 20-95% B; 31-34 min, 95% B; 34.01-37 min, 5% B. During second dimension fractionation, a total of five structural isomers with *m/z* 335.1 were observed (Table 5). These structural isomers were co-eluted in the chemical profiling due to the rapid chromatographic gradient used; therefore, they showed up as only three chemical features in the multivariate statistical analysis. To achieve better purity of selected chemical features, these five structural isomers were divided into two independent fractions (fractions 3 and 4) for a higher dimension of separation.

For fraction 2 from the first dimension (containing 5.22_349.1, and 5.28_349.1), the gradient was as follows: 0-0.5 min, 5% B; 0.5-1 min, 5-13% B; 1-28.5 min, 13-14% B; 28.5-29 min, 20-95% B; 29-31.5 min, 95% B; 31.51-34 min, 5% B. Similarly, a total of five structural isomers with *m/z* 349.1 were observed (Table 5), and they were divided into two independent fractions (fractions 5 and 6). All fractions collected from second

dimension fractionation were freed from solvent, lyophilized, and then reconstituted to approximately 500 mg/L in 20% methanol (v/v).

To achieve purity greater than 90%, third dimension fractionation was performed using an Xbridge Prep Shield RP18 column (5 µm, 10 ×250 mm, Waters Corp.) on a semi-prep scale. The gradient of (A) water with 0.1% formic acid and (B) acetone with 0.1% formic acid at a flow rate of 5 mL/min was specifically optimized to separate each structural isomer. In summary, a total of 10 compounds were isolated from fractionation and listed in Table 5. The gradient used to achieve high purity for compounds 1-10 was as follow: for compounds 1-3 (fraction 3), 0-1 min, 14% B; 1-33 min, 14-16% B; 33-34 min, 16-95% B; 34-37 min, 95%; 37.01-40 min, 5% B; for compounds 4 and 5 (fraction 4), 0-1 min, 18% B; 1-14.5 min, 18-22% B; 14.5-15.5 min, 22-95% B; 15.5-18 min, 95%; 18.01-21 min, 5% B; for compounds 6-8 (fraction 5), 0-1 min, 13% B; 1-43 min, 13-17% B; 43-44 min, 17-95% B; 44-47 min, 95%; 47.01-49 min, 5% B; for compounds 9 and 10 (fraction 6), 0-1 min, 18% B; 1-27 min, 18-26% B; 27-28 min, 26-95% B; 28-30 min, 95%; 30.01-33 min, 5% B.

The time-based collection was applied to the first-dimension fractionation, and mass-triggered collection under single ion monitoring (SIR) mode was applied to the second and third dimension fractionations. The settings for the TQD mass spectrometer were as follows: negative ionization mode, capillary voltage = 2.5 kV, cone voltage = 30 V, source temperature = 150 °C, and desolvation gas temperature = 400 °C. After collection, the purity of each collected feature was confirmed using Synapt G2-S UPLC-

QToF/MS (Waters Co.), where purity was calculated based on total ion chromatogram peak area under both positive and negative ESI modes.

2.7. Quantification by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS)

According to chemical profiling and sensory evaluation, similar trends in chemical and overall flavor changes during storage were observed within RTD coffee samples made from different species and processing conditions. Hence, the Arabica nitrogen-flushed RTD coffee samples were used as a representative sample for quantification. The concentrations of compounds 1-10 (Table 5) were determined in 0- and 4-month aged Arabica nitrogen-flushed RTD coffee samples using a UPLC Waters Acquity H-Class system coupled with TQS mass spectrometer (Waters Corp.). Sample preparation was performed following the protocol as described in Section 2.4 with the addition of 100 mg/L methylparaben as an internal standard. Chromatographic separation of targeted compounds was achieved on a Cortecs UPLC T3 column (1.6 µm, 2.1 × 100 mm, Waters Corp.) with a mobile phase consisting of (A) water with 0.1% formic acid (v/v) and (B) acetone with 0.1% formic acid at a flow rate of 0.5 mL/min. For compounds 1-5, the gradient was as follows: 0-0.5 min, 5% B; 0.5-10 min, 5-15% B; 10-10.5 min, 15-20% B; 10.51-13 min, 95%; 13.01-16 min, 5% B. For compounds 6-10, the gradient was as follows: 0-0.5 min, 5% B; 0.5-15 min, 5-17% B; 15.01-18 min, 95%; 18.01-21 min, 5% B.

Quantification was carried out using 5-point standard addition calibration curves (in triplicate) and displayed good linearity of all the compounds ($R^2 > 0.99$). Two multiple reaction monitoring (MRM) transition methods were optimized for each group of

isomers and presented in Table 5. Methylparaben was used as an internal standard and monitored as *m/z* 150.95 → 91.85 with a cone voltage of 20 V and collision energy of 18 V. The settings for the TQS triple quadrupole mass spectrometer were as follows: negative ESI mode, capillary voltage = 3 kV, cone voltage = 30 V, desolvation temperature = 550 °C, source temperature = 150 °C, desolvation gas flow = 1000 L/h, and cone gas flow rate = 150 L/h.

Quantification results were statistically analyzed by Student's T-test. Compounds that showed significant concentration differences between the 0- and 4-month Arabica nitrogen-flushed RTD coffee samples were included in the sensory recombination.

2.8.Sensory recombination of RTD coffee

2.8.1. Sensory recombination of a mixture of compounds

Sensory recombination testing first evaluated the impact of a mixture of all compounds and then evaluated the impact of the individual compounds. As the concentrations of negatively correlated compounds decreased from 0 to 4th months, the 4-month aged Arabica nitrogen-flushed RTD coffee sample was used as the control sample for the sensory recombination tests. Only compounds that showed significant concentration changes during storage were included in the sensory recombination testing (Table 5). The RTD coffee model 1 was prepared by spiking a mixture of compounds 1, 3-6, and 8-10 into the control sample matching the concentrations of these compounds in the non-aged Arabica nitrogen-flushed RTD coffee sample (see Table 5).

Fourteen trained panelists (6 males and 8 females, ages 23 to 44) from the Ohio State University participated in the sensory recombination test. The RTD coffee control

sample and model 1 were kept in a hot water bath to maintain serving temperature (60 °C to 65 °C). Five mL of each sample was served in 1-oz black cups. Panelists were asked to evaluate 2 pairs of RTD coffee samples following the same DOD protocol described in Section 2.3. In order to maintain serving temperature, panelists were given one pair of samples at a time; each pair consisted of a control sample and a test sample (control sample as blind control or RTD coffee model 1). The serving order of test samples was randomized and balanced.

2.8.2. Sensory evaluation of individual compounds

Following the same protocol as described above, the control sample was spiked with each individual compound and evaluated by panelists in subsequent sessions. Compounds 1 and 3-5 were evaluated in one session, and compounds 6 and 8-10 were evaluated in another session. During each session, panelists were asked to evaluate 5 pairs of RTD coffee samples following the DOD protocol. Similarly, panelists were given one pair of samples at a time; each pair consisted of a control sample and a test sample. The serving order of test samples was randomized and balanced. All data were recorded using Compusense Cloud software (Compusense, Guelph, ON, Canada). Unsalted crackers and water were provided for panelists to cleanse their palate between samples. Study protocols were approved by the OSU Institutional Review Board (2021B0121).

2.9. Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) was used to identify compounds 1-10. 1D and 2D NMR experiments were performed on a Bruker Advance III HD Ascend spectrometer equipped with a 5-mm triple resonance observe TXO cryoprobe with z-

gradients, operating at 700 MHz for the ^{1}H nucleus and 176 MHz for the ^{13}C nucleus (Bruker BioSpin, Rheinstetten, Germany). 50/50 deuterated water/deuterated methanol-d4 (v/v) was used as a solvent to dissolve purified compounds 1-10. Instruments were calibrated using the residual undeuterated solvent as an internal reference CD_3OD ^{1}H NMR = 3.31 ppm, ^{13}C NMR = 49.0 ppm. ^{1}H and ^{13}C NMR data are presented here:

5-O-caffeoyl-muco-γ-quinide (compound 1): ^{1}H NMR (700 MHz, MeOD) δ 7.54 (d, *J* = 15.9 Hz, 1H), 7.03 (d, *J* = 2.1 Hz, 1H), 6.94 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.77 (d, *J* = 8.2 Hz, 1H), 6.16 (d, *J* = 15.9 Hz, 1H), 5.13 (t, *J* = 6.2 Hz, 1H), 4.73 (q, *J* = 5.3 Hz, 1H), 3.96 (d, *J* = 4.0 Hz, 1H), 2.48 (m, 1H), 2.37 (m, 2H), 2.05 (dd, *J* = 14.5, 3.1 Hz, 1H); ^{13}C NMR (176 MHz, MeOD) δ 180.3, 167.4, 149.7, 147.8, 146.8, 127.6, 123.0, 116.4, 115.2, 114.6, 77.9, 73.5, 72.7, 67.1, 39.5, 38.6.

4-O-caffeoyl-muco-ɣ-quinide (compound 2): ^{1}H NMR (700 MHz, MeOD) δ 7.61 (d, *J* = 15.8 Hz, 1H), 7.08 – 7.05 (m, 1H), 6.98 (dd, *J* = 8.1, 1.9 Hz, 1H), 6.79 (d, *J* = 8.1 Hz, 1H), 6.30 (d, *J* = 15.9 Hz, 1H), 5.07 (d, *J* = 4.1 Hz, 1H), 4.13 (d, *J* = 5.0 Hz, 1H), 2.46 – 2.37 (m, 2H), 2.30 (dd, *J* = 13.9, 5.3 Hz, 1H), 2.11 – 2.06 (m, 1H); ^{13}C NMR (176 MHz, MeOD) δ 180.0, 167.3, 149.9, 148.1, 146.8, 127.5, 123.2, 116.5, 115.2, 114.0, 75.5, 72.3, 70.4, 69.3, 42.4, 39.6.

5-O-caffeoyl-epi-δ-quinide (compound 3): ^{1}H NMR (700 MHz, MeOD) ^{1}H NMR (700 MHz, MeOD) δ 7.60 (d, *J* = 15.9 Hz, 1H), 7.05 (d, *J* = 8.3 Hz, 1H), 6.98 (d, *J* = 7.4 Hz, 1H), 6.79 (d, *J* = 8.0 Hz, 1H), 6.29 (d, *J* = 15.8 Hz, 1H), 5.21 (dq, *J* = 9.2, 4.4 Hz, 1H), 4.66 (d, *J* = 4.4 Hz, 1H), 4.48 (d, *J* = 9.7 Hz, 1H), 2.53 – 2.50 (m, 1H), 2.37 (q, *J* = 6.2, 5.0

Hz, 1H), 1.83 – 1.76 (m, 2H); ^{13}C NMR (176 MHz, MeOD) δ 177.4, 167.7, 149.9, 148.1, 146.9, 127.4, 123.2, 116.5, 115.2, 113.9, 79.8, 70.0, 67.0, 64.6, 42.8, 38.0.

3-O-caffeoyl-γ-quinide (compound 4): ^{1}H NMR (700 MHz, MeOD) δ 7.62 (d, *J* = 15.9 Hz, 1H), 7.05 (d, *J* = 2.1 Hz, 1H), 6.96 (dd, *J* = 8.2, 2.1 Hz, 1H), 6.78 (d, *J* = 8.2 Hz, 1H), 6.30 (d, *J* = 15.9 Hz, 1H), 4.91 (ddd, *J* = 11.4, 6.8, 4.3 Hz, 1H), 4.74 (m, 1H), 4.29 (t, *J* = 4.6 Hz, 1H), 2.56 (d, *J* = 11.6 Hz, 1H), 2.30 (ddd, *J* = 11.6, 6.1, 2.8 Hz, 1H), 2.16 (ddd, *J* = 11.8, 6.7, 2.8 Hz, 1H), 2.09 (t, *J* = 11.7 Hz, 1H); ^{13}C NMR (176 MHz, MeOD) δ 178.9, 167.9, 149.7, 147.5, 146.8, 127.7, 123.0, 116.4, 115.1, 114.6, 77.6, 73.0, 70.1, 64.8, 37.7, 36.8.

4-O-caffeoyl-γ-quinide (compound 5): ^{1}H NMR (700 MHz, MeOD) δ 7.64 (d, *J* = 15.8 Hz, 1H), 7.08 (d, *J* = 2.1 Hz, 1H), 6.99 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.79 (d, *J* = 8.0 Hz, 1H), 6.36 (d, *J* = 15.9 Hz, 1H), 5.30 (t, *J* = 4.8 Hz, 1H), 4.88 (d, *J* = 5.3 Hz, 1H), 3.97 (ddd, *J* = 11.5, 6.6, 4.7 Hz, 1H), 2.36 (ddd, *J* = 11.7, 5.7, 2.7 Hz, 1H), 2.16 (s, 2H), 1.97 (t, *J* = 11.9 Hz, 1H). ^{13}C NMR (176 MHz, MeOD) δ 178.7, 168.0, 149.8, 147.9, 146.8, 127.6, 123.1, 116.5, 115.2, 114.4, 75.1, 73.0, 68.4, 66.1, 40.4, 38.5.

5-O-feruloyl-muco-γ-quinide (compound 6): ^{1}H NMR (700 MHz, MeOD) δ 7.60 (d, *J* = 15.9 Hz, 1H), 7.18 (d, *J* = 2.0 Hz, 1H), 7.07 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.81 (d, *J* = 8.2 Hz, 1H), 6.26 (d, *J* = 15.9 Hz, 1H), 5.15 (dd, *J* = 5.4, 1.4 Hz, 1H), 4.74 (ddd, *J* = 5.9, 4.2, 1.3 Hz, 1H), 3.97 (d, *J* = 4.3 Hz, 1H), 3.90 (s, 3H), 2.50 (d, *J* = 11.6 Hz, 1H), 2.44 – 2.37 (m, 2H), 2.06 (ddt, *J* = 14.3, 2.9, 1.4 Hz, 1H); ^{13}C NMR (176 MHz, MeOD) δ 180.4, 170.3, 167.4, 149.4, 147.7, 127.6, 124.4, 116.4, 114.9, 111.6, 78.0, 73.6, 72.8, 67.1, 56.4, 39.5, 38.6.

4-O-feruloyl-muco-γ-quinide (compound 7): ^{1}H NMR (700 MHz, MeOD) δ 7.68 (d, *J* = 15.8 Hz, 1H), 7.23 (d, *J* = 2.0 Hz, 1H), 7.11 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.82 (d, *J* = 8.1 Hz, 1H), 6.41 (d, *J* = 15.9 Hz, 1H), 5.09 (d, *J* = 4.1 Hz, 1H), 4.13 (dd, *J* = 5.4, 1.6 Hz, 1H), 3.90 (s, 3H), 2.47 – 2.41 (m, 1H), 2.41 (d, *J* = 11.6 Hz, 1H), 2.30 (dd, *J* = 13.8, 5.3 Hz, 1H), 2.09 (dd, *J* = 13.0, 2.3 Hz, 1H). ^{13}C NMR (176 MHz, MeOD) δ 180.0, 170.3, 167.3, 149.4, 148.0, 127.4, 124.4, 116.5, 114.4, 111.8, 75.5, 72.3, 70.4, 69.4, 56.4, 49.5, 42.4, 39.6.

5-O-feruloyl-epi-δ-quinide (compound 8): ^{1}H NMR (700 MHz, D_2O) δ 7.71 (d, *J* = 15.6 Hz, 1H), 7.26 (s, 1H), 7.19 (d, *J* = 8.0 Hz, 1H), 6.83 (d, *J* = 8.2 Hz, 1H), 6.38 (d, *J* = 15.8 Hz, 1H), 5.24 (qd, *J* = 8.2, 7.0, 4.3 Hz, 1H), 4.85 (m, 1H), 4.65 (m, 1H), 3.88 (s, 3H), 2.69 (dd, *J* = 14.0, 9.4 Hz, 1H), 2.49 (ddd, *J* = 13.7, 10.0, 3.4 Hz, 1H), 1.98 (dd, *J* = 13.9, 3.9 Hz, 1H), 1.86 – 1.81 (m, 1H).

3-O-feruloyl-γ-quinide (compound 9): ^{1}H NMR (700 MHz, MeOD) δ 7.70 (d, *J* = 15.9 Hz, 1H), 7.21 (d, *J* = 2.0 Hz, 1H), 7.10 (dd, *J* = 8.2, 2.0 Hz, 1H), 6.82 (d, *J* = 5.3 Hz, 1H), 6.40 (dd, *J* = 15.9, 0.9 Hz, 1H), 4.75 (t, *J* = 5.5 Hz, 1H), 3.90 (s, 3H), 2.56 (d, *J* = 11.6 Hz, 1H), 2.31 (ddd, *J* = 11.6, 6.1, 2.8 Hz, 1H), 2.24 – 2.15 (m, 2H). ; ^{13}C NMR (176 MHz, MeOD) δ 182.9, 170.0, 169.0, 150.5, 149.3, 146.8, 127.9, 124.0, 116.4, 115.9, 111.7, 80.6, 79.8, 70.5, 66.2, 56.4, 42.3, 37.9.

4-O-feruloyl-γ-quinide (compound 10): ^{1}H NMR (700 MHz, MeOD) δ 7.69 (d, *J* = 15.9 Hz, 1H), 7.22 (s, 1H), 7.09 (dd, *J* = 8.3, 2.0 Hz, 1H), 6.81 (d, *J* = 8.1 Hz, 1H), 6.40 (d, *J* = 15.9 Hz, 1H), 5.31 (t, *J* = 4.8 Hz, 1H), 4.91 (m, 1H), 3.91 (m, 1H), 3.89 (s, 3H), 2.56

(d, J = 11.6 Hz, 1H), 2.31 (ddd, J = 11.6, 6.1, 2.8 Hz, 1H), 2.17 (ddd, J = 12.0, 6.5, 2.9 Hz, 1H), 2.10 (d, J = 11.7 Hz, 1H).

2.10. Data analysis

Statistical analysis was performed using SPSS Statistics version 25 (IBM, Armonk, NY). Quantification data were analyzed by Student's T-test. DOD scores of each RTD coffee variety were analyzed by one-way ANOVA; when a significant difference was observed ($p < 0.05$), post-hoc LSD was performed between all samples, and 1-sided Dunnett's test was used to compare between the blind control (non-aged) and aged samples (1, 2, 4 months aged).

3. Results and discussion

3.1. Sensory evaluation by the degree of difference (DOD) test

This study aimed to identify non-volatile compounds that were degraded during storage and impacted the flavor stability of ready-to-drink (RTD) coffee. Two coffee species (Arabic and Robusta) were prepared in air and under nitrogen, aseptically processed and stored over 4 months at 30 ºC. The flavor changes of RTD coffee during storage were measured by a degree of difference (DOD) test, as previously discussed in chapter 2. In general all aged RTD coffee samples (1-month, 2-month, and 4-month) were found to be significantly different ($p < 0.05$) from the non-aged samples (control). For all coffee samples, the DOD scores increased over the 4 months of storage and the largest flavor changes occurred between the non-aged and the 1-month aged sample. The DOD scores for the specific Arabica nitrogen-flushed RTD coffee for the blind control (non-

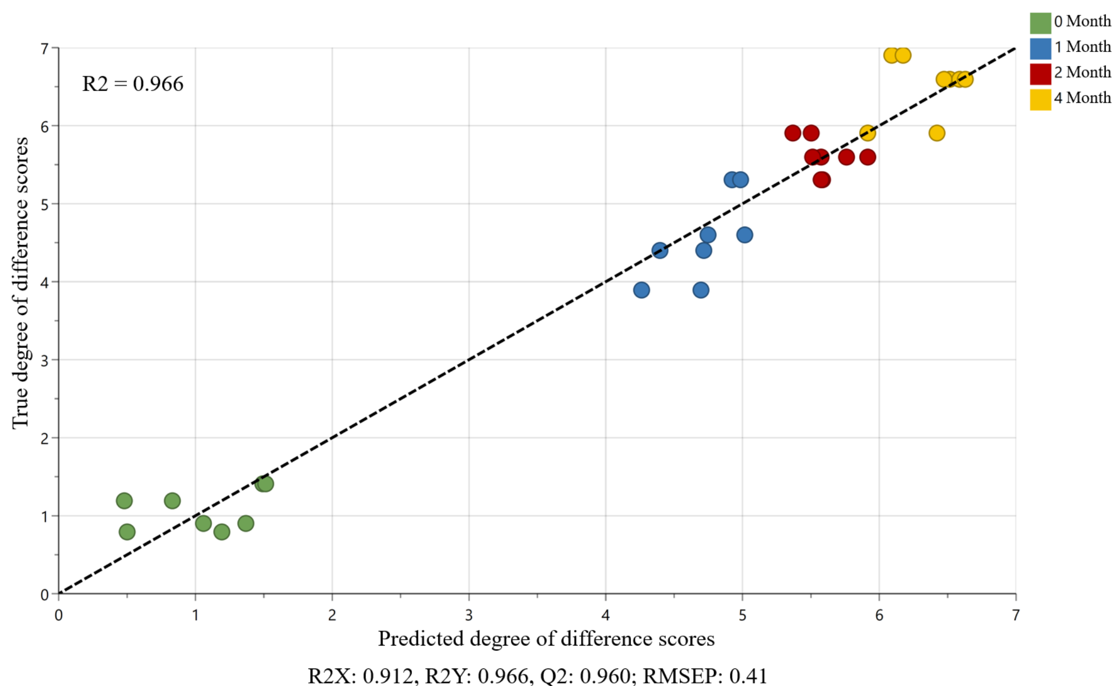
aged), 1-month, 2-month and 4-month aged samples were 1.2, 4.4, 5.6 and 6.6, respectively.

In chapter 2, compounds that were generated during storage and impacted flavor stability were identified and reported to contribute to a DOD score of 3.1, corresponding to *little* difference as compared to the 4-month aged RTD sample with a DOD score of 6.6 corresponding to a *medium* difference. To further understand the chemical changes that impact the flavor stability of RTD coffee during storage, compounds that degraded during storage or were negatively correlated based on the multivariate statistical model were selected and their sensory relevance determined.

3.2.Multivariate statistical analysis

Compounds that negatively correlated with the flavor changes of RTD coffee during storage were explored by modeling a total of 1489 non-volatile chemical features against the DOD scores. An initial unsupervised principal component analysis (PCA) confirmed that high-quality chemical data was obtained from UPLC-MS profiling. Subsequently, a supervised orthogonal partial least squares (OPLS) model was built to investigate the contribution of chemical features to the DOD scores. The results of multivariate statistical analysis were previously discussed in Chapter 2 Section 3.2. To summarize, the PCA showed that high-quality non-volatile chemical data was obtained from untargeted UPLC-MS profiling ($R^2X = 0.926$, $Q^2 = 0.887$), and OPLS was built with the goodness of fit and high predictability ($R^2X = 0.912$, $R^2Y = 0.966$, $Q^2 = 0.960$). The predictive power was further illustrated in Figure 9. Predicted DOD scores from the OPLS model were plotted against the true DOD scores, showing a root mean squared error of

prediction (RMSEP) of 0.4 out of a total of 10-points. In the previous chapter, compounds that positively correlated with flavor changes of RTD coffee during storage were selected based on the predictive variable of importance scores (VIPpred) and S-plot, and their sensory relevance was successfully validated. Following the same selection criteria (highest VIPpredictive scores), five highly predictive chemical features (RT_m/z) 3.92_335.1, 4.07_335.1, 4.24_335.1, 5.22_349.1, and 5.28_349.1 were selected (Figure 10) and are shown in Table 5. These features were compounds that negatively correlated to DOD scores indicating they degraded in RTD coffee samples over time.



R2X: 0.912, R2Y: 0.966, Q2: 0.960; RMSEP: 0.41

Figure 9. Plot of the predicted degree of difference (DOD) scores versus true DOD scores from the OPLS model; coffee biological replicates; color-coded by storage time.

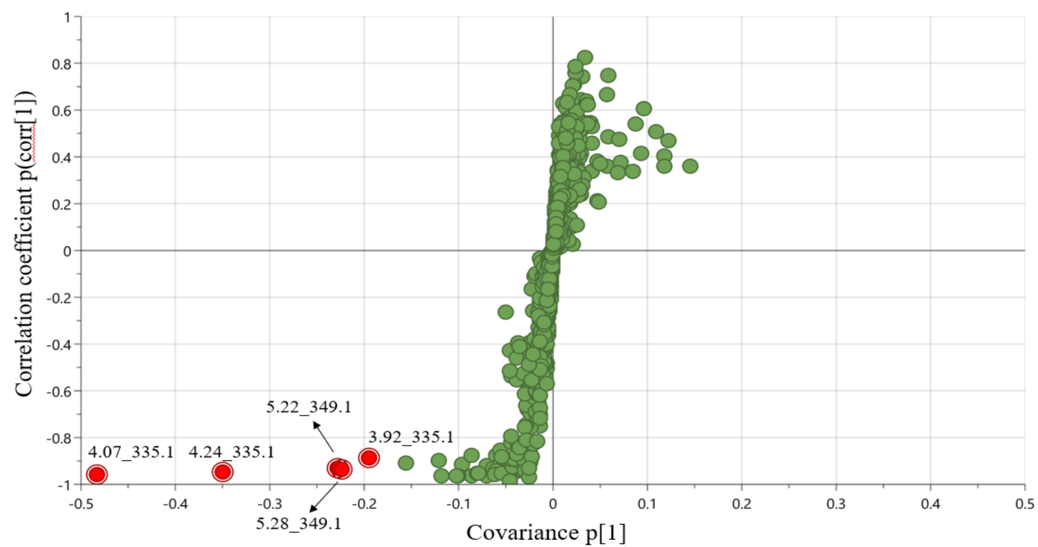


Figure 10. S-plot from OPLS model for sensory changes during storage; highlighted dots represent selected negatively correlated chemical features of interest.

Table 5. Highly predictive negatively correlated compounds of sensory changes for RTD coffee during storage

Compound	Chemical feature (RT_m/z)	VIPpred score	MRM transition (collision energy)	Compound identity	Compound concentration (mg/L) [1]		Sample concentration difference (mg/L)	% Change (concentration)
					Non-aged RTD coffee	Aged RTD coffee		
1	3.92_335.1	7.5	335.1 → 161.0 (26)	5-O-caffeoyl-muco-γ-quinide	8.5[a]	2.6[b]	5.9	69%
2	4.07_335.1 1st isomer	18.6	335.1 → 161.0 (26)	4-O-caffeoyl-muco-ɣ-quinide	22.4[a]	21.7[a]	0.7	3%
3	4.07_335.1 2nd isomer	18.6	335.1 → 161.0 (26)	5-O-caffeoyl-epi-δ-quinide	10.8[a]	2.4[b]	8.4	78%
4	4.07_335.1 3rd isomer	18.6	335.1 → 161.0 (26)	3-O-caffeoyl-ɣ-quinide	5.9[a]	0.0[b]	5.9	100%
5	4.24_335.1	13.5	335.1 → 161.0 (26)	4-O-caffeoyl-ɣ-quinide	6.5[a]	0.0[b]	6.5	100%

Continued

Table 5 continued

6	5.22_349.1 1st isomer	8.8	349.1 → 173.0 (18)	5-O-feruloyl-muco-γ-quinide	2.9^{a}	0.5^{b}	2.4	83%
7	5.22_349.1 2nd isomer	8.8	349.1 → 173.0 (18)	4-O-feruloyl-muco-γ-quinide	3.4^{a}	2.7^{a}	0.7	21%
8	5.28_349.1 1st isomer	8.6	349.1 → 173.0 (18)	5-O-feruloyl-epi-δ-quinide	3.1^{a}	0.0^{b}	3.1	100%
9	5.28_349.1 2nd isomer	8.6	349.1 → 173.0 (18)	3-O-feruloyl-γ-quinide	4.3^{a}	0.1^{b}	4.2	98%
10	5.28_349.1 3rd isomer	8.6	349.1 → 173.0 (18)	4-O-feruloyl-γ-quinide	3.7^{a}	0.1^{b}	3.6	97%

Different letters indicate significant differences between non-aged and aged RTD coffee compound concentration according to the Student's T-test ($p < 0.05$);

3.3.Identification of negatively correlated predictive features

The top five predictive features were selected based on VIP scores and after fractionation were reassigned to ten compounds as a result of several co-eluding structural isomers. For example, feature 4.07_335.1 (retention time_*mz*) after multi-dimensional LC fractionation resulted in three structural isomers being isolated. A list of the ten compounds isolated from multidimensional purification are shown in Table 5 and each compound was analyzed using tandem MS/MS and NMR experiments for identification.

Compound 1 (Table 5) presented a molecular ion peak $[M-H]^-$ at *m/z* 335.0773, leading to a molecular elemental composition of $C_{16}H_{16}O_8$ (Δmass = 0.55 ppm). The MS/MS fragmentation under negative ESI mode produced major fragments at *m/z* 135, *m/z* 161, and *m/z* 179, which agreed with the fragmentation pattern of caffeoylquinic acid lactones. The fragment at *m/z* 179 corresponded to a caffeic acid moiety, and signal at *m/z* 135 was assigned to the fragmentation product of vinyl-3,4-diphenol moiety (Asamenew et al., 2019; De Rosso et al., 2018). Given the presence of multiple structural isomers, the purified form of feature 3.92_335.1 was analyzed by NMR. The ^1H NMR showed characteristic signals of the vinyl-3,4-diphenol moiety, among them three aromatic signals at δ 7.03 (d, *J* = 2.1 Hz, 1H), δ 6.94 (dd, *J* = 8.2, 2.0 Hz, 1H), and δ 6.77 (d, *J* = 8.2 Hz, 1H), indicating the trisubstituted ring. In addition, two olefinic proton signals resonating at δ 7.54 (d, *J* = 15.9 Hz, 1H), and δ 6.16 (d, *J* = 15.9 Hz, 1H), confirmed the double bond and the coupling constant *J* = 15.9 Hz revealed the *E* geometry. Additional signals correlated with the quinic lactone moiety, three key signals attributed to carbinolic methines at δ 5.13 (t, *J* = 6.2 Hz, 1H), δ 4.73 (q, *J* = 5.3 Hz, 1H), and δ 3.96 (d, *J* = 4.0 Hz,

1H) and the two methylene signals located at δ 2.48 (m, 1H), δ 2.37 (m, 2H), and δ 2.05 (dd, J = 14.5, 3.1 Hz, 1H) confirmed the moiety. The ^{13}C NMR signals were also in agreement with the caffeoylquinic acid lactones, the carboxyl group of the lactone was confirmed by the signal located at δ 180.3 ppm and the carboxyl group of the ester was defined by signal at δ 167.4 ppm. Two additional signals δ 149.7 ppm and δ 147.8 ppm were assigned to two quaternary phenolic carbons in the 3,4-diphenol moiety. The signals resonating at δ 77.9 ppm and δ 73.5 ppm were consistent with the quaternary carbinolic carbon and the hydroxymethine that support the lactone group in the quinic lactone moiety, respectively. Finally, all ^{1}H and ^{13}C NMR data analysis of compound 1 and the match with the data reported in the literature allowed conclusive identification of this compound as 5-O-caffeoyl-muco-γ-quinide (Figure 11), previously reported in the literature (Frank et al., 2006).

Compounds 2 – 5 (Table 5) showed the same molecular ion peak $[M-H]^-$ at *m/z* 335.0773, corresponding to the same molecular elemental composition of $C_{16}H_{16}O_8$ (Δmass = 0.55 ppm). Likewise, MS/MS fragmentation of these compounds shared similar patterns as 5-O-caffeoyl-muco-γ-quinide (compound 1) with slight differences in the abundance of daughter ions. Hence, compounds 2 – 5 were established as structural isomers of compound 1, therefore, were also purified and analyzed by NMR experiments. The ^{1}H and ^{13}C NMR experiments of compounds 2 – 5 showed similar key chemical shifts and coupling constants as compound 1, indicating structural isomerism among these compounds. Thus, comparison of the NMR data with published literature led to the identification of these compounds as 4-O-caffeoyl-muco-ɣ-quinide (compound 2), 5-O-

caffeoyl-epi-δ-quinide (compound 3), 3-O-caffeoyl-ɣ-quinide (compound 4), and 4-O-caffeoyl-ɣ-quinide (compound 5) (Frank et al., 2008; Frank et al., 2006; Gao et al., 2021).

Compounds 6 – 7 (Table 5) with the molecular elemental composition of $C_{17}H_{18}O_8$ (accurate mass *m/z* 349.0932, Δmass =0.86) were two structural isomers that co-eluded during chemical profiling and were further separated by multidimensional fractionation. The MS/MS fragmentation of compound 6 exhibited main fragments at *m/z* 175 and *m/z* 193, which were typical fragments of ferulic acid moiety (Clifford et al., 2006; Kuhnert et al., 2010). Further, NMR analysis of compound 6 showed similar chemical shift signals as compound 1. The ^{1}H NMR showed characteristic aromatic signals at δ 7.18 (d, *J* = 2.0 Hz, 1H), δ 7.07 (dd, *J* = 8.2, 2.0 Hz, 1H), and 6.81 (d, *J* = 8.2 Hz, 1H), one double bond attributed to the signals at δ 7.60 (d, *J* = 15.9 Hz, 1H), and δ 6.26 (d, *J* = 15.9 Hz, 1H) with *E* configuration. Also, the NMR signals corresponding to the quinic lactone moiety were determined, three carbinolic methines at δ 5.15 (dd, *J* = 5.4, 1.4 Hz, 1H), δ 4.74 (ddd, *J* = 5.9, 4.2, 1.3 Hz, 1H), δ 3.97 (d, *J* = 4.3 Hz, 1H) and two methylene signals at δ 2.50 (d, *J* = 11.6 Hz, 1H), 2.44 – 2.37 (m, 2H), 2.06 (ddt, *J* = 14.3, 2.9, 1.4 Hz, 1H). The only difference between compound 6 and compound 1 was the additional methoxy group attributed to the signal located at δ 3.90 (s, 3H), which confirmed the ferulic acid moiety in the structure of compound 1. The ^{13}C NMR signals were also in agreement with the ferulic acid lactones, scilicet the lactone carbon was confirmed by the signal located at δ 180.4 ppm and the carboxyl group of the ester by the signals at δ 170.3 ppm. Further, the methoxy group was confirmed by the presence of the signal resonating at δ 56.4 ppm. Lastly, the ^{1}H and ^{13}C NMR data analysis of compound 6 and the comparison with the data

reported in the literature allowed the identification of compound 6 as 5-O-feruloyl-muco-γ-quinide (Figure 11), previously reported in the literature (Frank et al., 2006).

In the same way, other structural isomers of *m/z* 349.1 (compounds 7 – 10) that co-eluded during chemical profiling were individually identified based on the MS/MS fragmentation and NMR analysis. Thus, comparison of the NMR data with published literature led to the identification of these compounds as 4-O-feruloyl-muco-γ-quinide (compound 7), 5-O-feruloyl-epi-δ-quinide (compound 8), 3-O-feruloyl-γ-quinide (compound 9), and 4-O-feruloyl-γ-quinide (compound 10) (Frank et al., 2006).

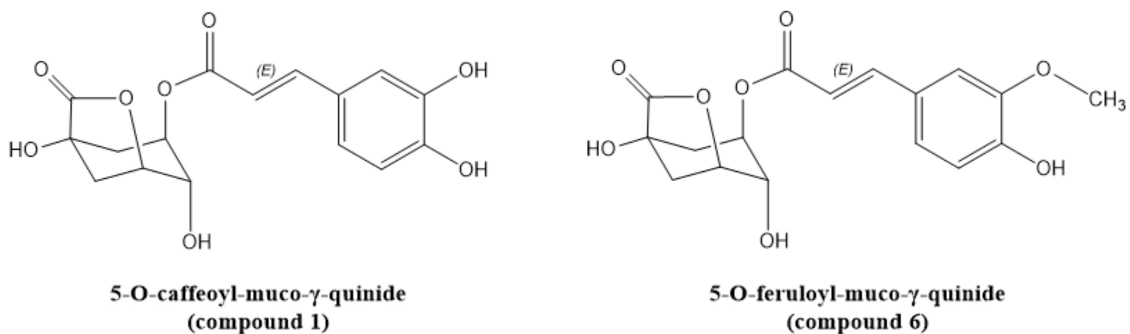


Figure 11. Stereostructures of compounds 1 and 6.

3.4. Quantification of negatively correlated predictive features in RTD coffee samples

The Arabica nitrogen-flushed RTD coffee samples were used in the quantification and sensory recombination, as RTD coffee samples made from different coffee species and processing conditions followed a similar trend of chemical and flavor changes during

storage. The concentrations of compounds 1-10 were quantified in the non-aged sample (control) and 4-month aged samples, as shown in Table 5. Data analysis showed that the concentrations of compounds 1, 3-6, and 8-10 significantly ($p < 0.05$) decreased during storage, whereas no changes were observed for compounds 2 and 7 that co-eluted with other structural isomers in the chemical profiling. Therefore, compounds 2 and 7 were removed from further investigation as they were not considered predictive compounds negatively correlated to changes in flavor over storage. Compounds 1-10 (caffeoyl- and feruloylquinic acid lactones) belong to the chlorogenic acid lactone (CGL) family, which are formed from the corresponding chlorogenic acids during the roasting process (Farah & Donangelo, 2006; Kaiser et al., 2013; Scholz & Maier, 1990). Quantification results show that the concentrations of eight CGLs decreased between 69-100% in the RTD coffee matrix during the 4 months of storage. This result was in line with previous observations that CGLs undergo hydrolysis of the intramolecular lactone, resulting in decreased concentration in coffee brews (Bennat et al., 1994; Schrader et al., 1996). In the following section, the flavor impact of CGLs was investigated by sensory recombination testing.

3.5. Sensory recombination of negatively correlated features with RTD coffee samples

Based on the quantification results, only the eight compounds that showed significant changes in concentration during storage were included in sensory recombination. Initially, an RTD coffee model (1) was prepared by adding compounds 1, 3-6, and 8-10 into a 4-month aged RTD coffee sample to mimic the concentrations in the non-aged sample (Table 5). When RTD coffee model 1 was compared to an aged RTD

coffee sample (Figure 12), the DOD score for model 1 was significantly different ($p < 0.05$) from the control, and the difference was rated as 2.9 corresponding to *little* difference. Subsequently, recombination models with each individual compound were evaluated in the aged RTD coffee sample at the respective concentrations of the non-aged sample (Table 5). The results showed (see Figure 13) that the addition of two of the eight individual compounds, 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide, caused a significant difference ($p < 0.05$) from the blind control according to a 1-sided Dunnett's test. Specifically, RTD coffee models 4 and 5 were rated with DOD scores of 2.3 and 2.5 respectively, corresponding to a *little* difference (Figure 13). Among all the eight CGLs predictive for the DOD scores (Table 5), 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide had the highest VIP scores of 18.6 and 13.5, respectively, and were identified as the main contributors to the flavor instability of RTD coffee during storage.

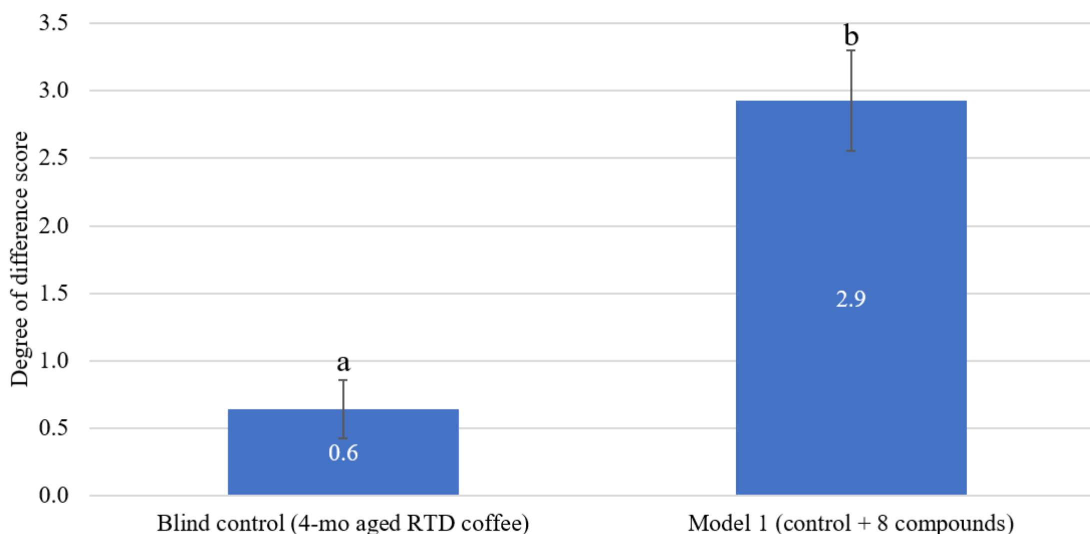


Figure 12. Mean degree of difference (DOD) scores (scale 1-10) with standard error for blind control and recombination model (control + 5.9, 8.4, 5.9, 6.5, 2.4, 3.1, 4.2, 3.6 mg/L of 5-O-caffeoyl-muco-γ-quinide, 5-O-caffeoyl-epi-δ-quinide, 3-O-caffeoyl-ɣ-quinide, 4-O-caffeoyl-ɣ-quinide, 5-O-feruloyl-muco-γ-quinide, 5-O-feruloyl-epi-δ-quinide, 3-O-feruloyl-γ-quinide, 4-O-feruloyl-γ-quinide, respectively); different letters represent significant differences in DOD scores according to Student T-test at $p < 0.05$; $n = 14$

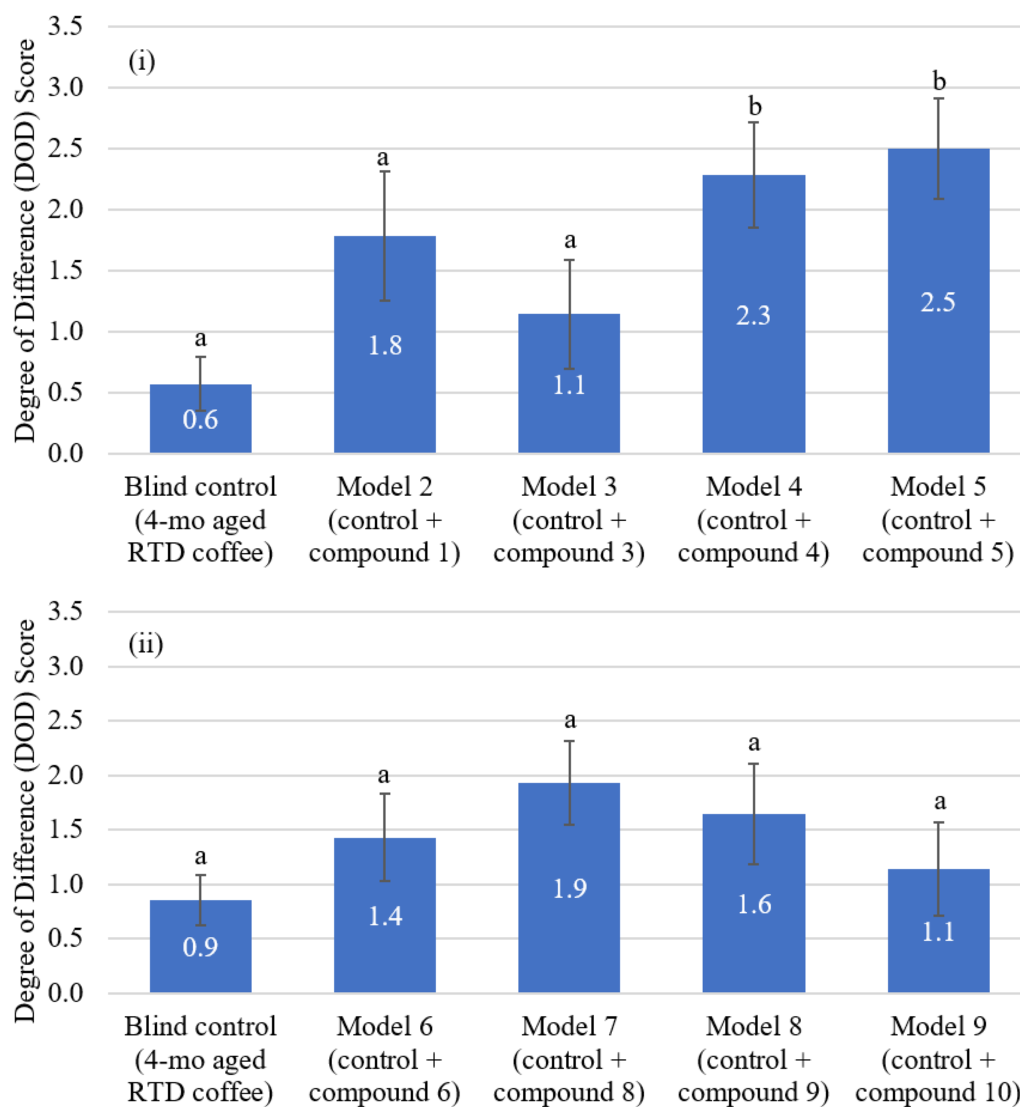


Figure 13. Mean degree of difference (DOD) scores (scale 1-10) with standard error for sensory recombination sample set (i) blind control and recombination models 2-5 and (ii) blind control and recombination models 6-9; compounds 1, 3, 4, 5, 6, 8, 9, 10 are 5-O-caffeoyl-muco-γ-quinide, 5-O-caffeoyl-epi-δ-quinide, 3-O-caffeoyl-γ-quinide, 4-O-caffeoyl-γ-quinide, 5-O-feruloyl-muco-γ-quinide, 5-O-feruloyl-epi-δ-quinide, 3-O-feruloyl-γ-quinide, 4-O-feruloyl-γ-quinide, added at added at 5.9, 8.4, 5.9, 6.5, 2.4, 3.1,

4.2, 3.6 mg/L, respectively; different letters represent significant differences in DOD scores according to 1-sided Dunnett's test at $p < 0.05$; $n = 14$

Both 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide, along with other CGLs in Table 5, have been reported as bitter-tasting compounds in coffee (Frank et al., 2008; Frank et al., 2007; Frank et al., 2006). K. Kraehenbuehl et al. (2017) reported that enzymatic hydrolysis of these CGLs in a coffee model system resulted in the reduction of bitter intensity. Bitter threshold values in water have been reported at 13.4 mg/L for 3-O-caffeoyl-ɣ-quinide and 12.1 mg/L for 4-O-caffeoyl-ɣ-quinide (Frank et al., 2006). In the current study, the concentrations of 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide present in the non-aged RTD coffee were 5.9 and 6.5 mg/L, below the published bitter thresholds. However, significant flavor changes were observed from the loss of these compounds in the 4-month aged RTD coffee (Figure 13i) indicating sub-threshold activity.

Subthreshold tastants have also been reported to affect flavor perception through enhancement and suppression effects. For example, bitter-tasting L-phenylalanine and L-tyrosine appeared to enhance the umami taste of MSG/NaCl mixtures at subthreshold concentrations (Lioe et al., 2005). Others have reported subthreshold concentrations of astringent phenolic acid ethyl esters and flavanols contributed to red wine bitterness (Hufnagel & Hofmann, 2008). Even though the addition of 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide were not observed to directly affect bitter perception in this RTD coffee, other taste attributes may be affected. In addition, subthreshold multimodal interactions of aroma and taste have also been demonstrated (Dalton et al., 2000; Fujimaru

& Lim, 2013; Ito & Kubota, 2005; Labbe & Martin, 2009). In the current study, the degree of overall flavor change was measured. Panelists focused on evaluating the size of differences rather than rating the intensity of any specific sensory attributes. During the evaluation, panelists respond to any perceived changes of aroma, taste, or somesthetic perceptions between the control and test samples. Review of the panelist's comments (n = 14) indicated model 4 (3-O-caffeoyl-ɣ-quinide, Figure 12i) was described as "less sour" 5 times, followed by "more bitter" 3 times, and "changes in coffee aroma" 2 times. Similarly, model 5 (4-O-caffeoyl-ɣ-quinide, Figure 13i) was described as "less sour" 8 times, followed by "more bitter" 2 times, and "changes in coffee aroma" 2 times. Hence, 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide likely induced taste interactions as well as cross-modal interactions that impacted the overall flavor of RTD coffee.

Comparing the sensory recombination DOD score for model 1 of 2.9 (Figure 12) with the 4-month aged coffee sample (6.6), it can be seen that decreasing concentrations of 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide over time contributed to the overall flavor difference between in the Arabica nitrogen-flushed RTD coffee samples during storage. As discussed, the decreased concentration of CGLs may be due to the hydrolysis into their corresponding chlorogenic acids. This observation is aligned with the findings in Chapter 2, where the generation of five chlorogenic acids was reported to contribute to the overall flavor changes in RTD coffee during storage. The sensory relevance of the degradation of 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide, as well as the generation of 5 chlorogenic acids, were separately confirmed by the sensory recombination testing. Although the flavor impacts of these two groups of compounds

could not be simply model together, it would be expected both contributed to RTD coffee flavor stability during storage.

4. Conclusion

A novel understanding of RTD coffee degradation pathways during storage was identified. The loss of 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide over 4 months were found to significantly impact the RTD coffee flavor at the subthreshold concentrations. These results demonstrated the success of applying flavoromics to understand the complex mechanisms of RTD coffee flavor stability during storage.

Chapter 4. Overall Summary

In this study, untargeted flavoromics was successfully employed to characterize the RTD coffee flavor stability during storage. The flavor changes in RTD coffee over time were investigated from a comprehensive point of view by combining multiple research tools, including sensory evaluation, state-of-the-art analytical platforms, and multivariate statistical modeling.

Initially, RTD coffee samples were prepared from two coffee species (Arabica and Robusta) under two processing conditions (air versus nitrogen). As coffee species and the presence of oxygen are known factors contributing to the complexity of coffee flavor, the combination of sample conditions provided variations in chemical composition, allowing for more comprehensive data to enable the identification of universal chemical drivers of RTD coffee flavor stability.

After RTD coffee samples were prepared and aged for 4 months, the flavor changes were measured using the degree of difference (DOD) test. The DOD test allowed panelists to focus on the overall flavor differences instead of specific sensory attributes and converted the perceivable sensory differences into numeric scores. In summary, the sensory evaluation results indicated that the most apparent degree of flavor changes occurred within the first month of storage and continued to increase at a slower rate through 4 months of

storage. Specifically, significant differences ($p < 0.05$) were observed between the non-aged samples and aged samples; however, not all the aged samples were significantly different from each other. Although sensory comparison was not directly conducted between different RTD coffee varieties, a similar trend of overall flavor changes was observed among RTD coffee samples from different coffee species and processing conditions.

Paralleled with sensory evaluation, the non-volatile chemical fingerprints of RTD coffee were collected through untargeted chemical profiling. The advantage of untargeted chemical profiling lies in its ability to monitor a wide range of chemical compounds simultaneously. Initially, the sample preparation and chromatographic separation method were optimized to extract as many chemical features as possible from RTD coffee samples. Then, the chemical fingerprints of RTD coffee were successfully profiled by a combination of reverse-phase liquid chromatography and a time-of-flight mass spectrometer. Finally, the chromatographic and spectral data went through feature extraction and noise filtration and were converted into a total of 1489 chemical features.

Subsequently, multivariate statistical models were generated using chemical fingerprints and sensory data. Principal component analysis ($R^2X = 0.926$, $Q^2 = 0.887$) confirmed that chemical variations within RTD coffee samples were adequately captured by UPLC-MS profiling and correctly characterized by PCA modeling. Furthermore, high quality orthogonal partial least squares model ($R^2X = 0.912$, $R^2Y = 0.966$, $Q^2 = 0.960$) was developed to establish correlation between chemical fingerprints and DOD scores. On average, the predicted DOD scores from the OPLS model were only 0.4 points different

from the actual DOD scores on a 10 points scale. The high predictivity of multivariate statical models granted confidence in selecting chemical features highly predictive to the RTD coffee overall flavor changes.

Predictive chemical features that were not commercially available were isolated from the coffee matrix using off-line multidimensional preparative fractionation. During the fractionation process, several structural isomers of the selected predictive chemical features were observed. These structural isomers were individually isolated, and their structure and identity were further elucidated using Nuclear Magnetic Resonance spectroscopy. The application of multidimensional fractionation enabled the orthogonal separation of co-eluted compounds, exhibiting its ability to provide complementary information to untargeted chemical profiling. Furthermore, the isolation of predictive chemical features allowed the flavoromics analysis to perform subsequent sensory recombination study, which moved a step further than the regular untargeted study by adding a validation experiment.

As panelists indicated in the initial sensory evaluation that sourness was one of the major drivers for overall flavor changes in RTD coffee, the importance of acidic compounds on RTD coffee flavor was considered. It was anticipated that the sample clean-up and the reverse-phase chromatographic separation resulted in some hydrophilic organic acids being not adequately included in the untargeted chemical profiling and the statistical modeling. Therefore, the concentrations of several well-known hydrophilic non-volatile organic acids were monitored in RTD coffee samples using targeted analysis. Results showed that only the concentration of quinic acid changed significantly in RTD coffee

during the 4-month storage. Hence, quinic acid was also considered as a predictive compound and included in sensory validation.

Finally, the sensory validation experiment was carried out by recombining the predictive compounds with the RTD coffee matrix. The sensory recombination results revealed that two groups of compounds directly or indirectly contributed to the overall flavor changes in RTD coffee during storage. First, five chlorogenic acids and quinic acid, which were generated over time, directly contributed to the overall flavor changes and sourness development in RTD coffee during storage, primarily by reducing the pH of the RTD coffee matrix. Second, 3-O-caffeoyl-ɣ-quinide and 4-O-caffeoyl-ɣ-quinide, which are degraded over time, impacted the overall RTD coffee flavor at subthreshold concentrations, indicating indirect contribution to RTD coffee flavor stability by flavor interactions. Subthreshold compounds were likely overlooked by the traditional taste-guided research approaches, as they would be considered taste inactive due to the low concentrations. However, the untargeted flavoromics allowed the discovery of the flavor activity of subthreshold compounds as well as flavor interactions, which highlighted the successful application of this new research approach to understand complex mechanisms of flavor perception.

In the current study, five chlorogenic acids, quinic acid, and two chlorogenic acid lactones were identified as significant contributors to RTD coffee stability during storage. Their impacts on overall RTD coffee flavor were confirmed by sensory recombination; however, their influences on specific RTD coffee sensory attributes were not further discussed. Future work can utilize descriptive sensory analysis tools to understand their

sensory relevance on specific RTD coffee sensory attributes, offering more detailed and complete information on RTD coffee flavor changes during storage.

In summary, this work demonstrated the application of untargeted LC/MS flavoromics to identify compounds and mechanisms that impact RTD coffee flavor stability during storage. Compounds identified in this work provide a novel basis to optimize the preservation of RTD coffee flavor during storage, therefore allowing the development of higher quality RTD coffee products.

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