EFFECT OF pH AND TEMPERATURE ON THE BINDING

INTERACTIONS OF CAFFEINE AND CHLOROGENIC ACID

WITH SODIUM CASEINATE

A Thesis

presented to

the Faculty of California Polytechnic State University,

San Luis Obispo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Agriculture,

with Specialization in Dairy Product Technology

By

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May 2013

COMMITTEE MEMBERSHIP

TITLE: EFFECT OF pH AND TEMPERATURE
                                ON THE BINDING INTERACTIONS OF
                                CAFFEINE AND CHLOROGENIC ACID
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ABSTRACT

EFFECT OF pH AND TEMPERATURE ON THE BINDING INTERACTIONS

OF CAFFEINE AND CHLOROGENIC ACID WITH SODIUM CASEINATE

Emily S. Kong

Coffee is a popular and well-loved beverage consumed worldwide by millions of people every day. While most patrons of coffee do so because of its unique and satisfying taste, consumers may be unaware of the potential beneficial health effects it also imparts. The antioxidants found in green coffee beans collectively known as chlorogenic acids (CGA) and caffeine are two of the most abundant bioactive compounds present in coffee. Both these bioactive compounds have been implicated in many studies to impart a wide range of health benefits, from reducing the risk of Type 2 diabetes, to their use as aides in weight management. Indeed, epidemiological studies on people who consume moderate amounts of coffee on a regular basis have unanimously shown benefits to overall health.

While caffeine and CGA are naturally occurring compounds in coffee, their potential in conferring beneficial health effects warrant research into other potential food matrixes that can be used to bind and deliver these bioactive compounds into foods that do not naturally contain them. Milk proteins, specifically caseins, have been shown to be excellent vehicles to both bind and deliver sensitive bioactive compounds of various chemical and physical properties. Caseins have been shown in numerous studies to successfully bind to

molecules such as vitamin D_2, ω-3 polyunsaturated fatty acids (DHA), and iron to name a few. Because caseins exhibit high versatility in binding a variety of molecules, caseins were the milk protein of choice for the experiments in this thesis.

Polyphenols have been the subject of many studies on its binding capacity with milk proteins, but research on the binding capacity of caffeine with caseins is limited. Therefore, the objectives of this thesis are threefold: 1) develop, optimize and validate an HPLC method for the accurate and simultaneous determination of caffeine and CGA, 2) establish a procedure by which caffeine and CGA bind to sodium caseinate, and 3) determine the optimal treatment conditions of pH and temperature to increase binding interactions and speculate on the mechanism of binding for each bioactive compound.

A reversed phase HPLC (RP-HPLC) method was developed and subjected to validation studies with good results in linearity (caffeine R^2 = 0.9992, CGA R^2 = 0.9995) and precision (RSD of caffeine <1%, RSD of CGA <2%). The developed method also demonstrated selectivity for caffeine and CGA. This method was then used to analyze sodium caseinate samples containing caffeine and 5-CGA. The results from these studies have shown that binding interactions between caffeine and sodium caseinate are temperature dependent ($p < 0.01$) whereas binding interactions between CGA and sodium caseinate are influenced by both pH and temperature ($p < 0.01$). Elucidating the binding mechanisms of caffeine and CGA to sodium caseinate and providing a sensitive analytical technique by which these compounds can be accurately quantified may facilitate

future research involving the use of caffeine and CGA in many other facets, as well as promoting its increased use in the dairy industry.

ACKNOWLEDGMENTS

McGaughey for their immeasurable dedication, passion, and enthusiasm. Thank you for tirelessly reviewing my stats outputs, and most of all, for teaching me the values of and sparking my interest in statistics. I would also like to thank Dr. Corinne Lehr for her patience and for her willingness to help with the analytical chemistry portion of this work.

To my fellow graduate students, I would like to thank: Hadi Eshpari, for his kind words of encouragement, his great sense of humor, and most of all, his perseverance. Lauren Collinsworth, for sharing almonds with me during late nights at the DPTC. Melissa Hsu, Valerie Arechiga and Corbin Kembel, my unofficial swim coaches and team UdderSuckers. Tracey Nguyen, for being my neighbor, in every sense of the word, and for going through this journey with me from the beginning. Mariela Poveda and Garrett Walsh, for the welcomed entertainment. Katy Lees, thank you for all your wonderful treats and visits. A very special thanks to Andrea Laubscher, who has always made my life easier from the start; thank you for your positive attitude, calm demeanor, and kind friendship. Your unconditional help and guidance made this thesis project possible. Many thanks also to Dan Zhao, Sean Vink, Vandna Sikand, and Kristy Herbaugh for each shaping my experience at the DPTC. I am thankful to have met and gotten to know every one of you.

To Kelly Carnes and Kathi Reis, you both have changed my life in ways you cannot imagine. Thank you for teaching me what no books could. Thank you

Kelly, for empowering me to become a stronger person, physically and mentally. Thank you Kathi, for your patience, dedication, and constant encouragement; you have irreversibly changed my life for the better. Thank you for being strong, confident women, and inspiring me to be the same. I am forever grateful to have met you both.

To my life before Cal Poly: I would like to thank Dr. David Lechuga-Ballesteros for being a mentor, friend and supporter since before Day 1. Thank you for providing much needed comic relief in tense situations and for your transcending guidance through trying times. I would also like to thank Jon Schroeder, to whom I owe practically all my Word & Excel skills to; they came in very handy during the writing of this thesis. Thank you for teaching me how to be a good analytical chemist. I would like to thank Dr. Melissa Turcotte for her words of wisdom that only a recent PhD graduate could provide, and Dr. Nancy Carrillo for relentlessly encouraging me to pursue a Master's.

I would like to thank my parents for their endless support through every one of my seemingly impulsive decisions. Thank you for always believing that you raised a daughter with her head on straight. Thank you to my brothers and sisters-in-law: Jeff, Jerry, Tam & Rujjira, for always answering my calls, believing in me, and for your unconditional love and support. I am lucky to call you all family. And to my little niece, Hannah, who at just 10 months old has

provided the impetus for finishing graduate school in a timely matter so that I can watch her grow up.

I would like to thank my dear friends, Katherine Fader, Josh Marcley, Vish Patel, Ron Sloat, Randy Spagnola, Diem Tran, and Linda Y for their unconditional love and support. You guys are my strength in times of weakness.

And finally, to Michael Drew. Thank you for being my inspiration. Thank you for moving with me, for many moments of laughter, giving me perspective, letting me watch TV after a bad day, and for always being there. Most of all, thank you for vicariously living through another 2 years of graduate school.

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1.0 INTRODUCTION

The major bioactive compounds of coffee have been studied extensively, with the first isolation of caffeine from green coffee beans performed in 1820 by the German chemist, Friedlieb Ferdinand Rung. Caffeine has been linked to reducing the risk of many metabolic diseases, including diabetes and certain cancers. In addition to caffeine, coffee is an abundant source of phenolic compounds, collectively known as chlorogenic acids (CGA) that have also been shown to impart many crucial health benefits. However, light and oxygen sensitivities of phenolic compounds, or antioxidants, makes transporting these compounds to be incorporated into different food matrixes a technical challenge. Caseins in milk have been reported to be excellent delivery systems via nano-encapsulation because of their high tendency to aggregate into self-associated micelles, or by hydrophobic binding due to its unique amphipathic structure. The associative properties of α_{s1}-, α_{s2}-, and β-caseins via hydrophobic binding can be attributed mainly to the effect of temperature.

Caseins have been specifically shown in previous studies to have excellent binding capacity with a wide variety of bioactive compounds including antioxidants, but literature on their binding capacity with caffeine is limited. The work herein attempts to elucidate the mechanism by which caffeine and CGA bind to casein micelles. First, a procedure will be established to bind these compounds to sodium caseinate. Using this procedure, we can then investigate what effects of pH and temperature are on the binding capacity of sodium

caseinate with these bioactive compounds. Secondly, determining the effects of pH and temperature will allow for speculation on sodium caseinate's mechanism of binding. Heat treatment on caseins can lead to denaturation of the proteins, structural rearrangement, and other conformational changes within the protein which may increase the exposure and availability of hydrophobic sites for interaction with other compounds. Steric interactions and repulsive forces are mainly responsible for maintaining the structure of the micelle and preventing aggregation of the micelles. However, these interactions may also affect casein's binding capacity with other compounds through electrostatic interactions resulting from changes in the ionic strength of the chemical environment.

An analytical method to quantify these compounds by reversed phase HPLC (RP-HPLC) will be developed and validated in an effort to accurately characterize and quantify these compounds as well.

2.0 LITERATURE REVIEW

2.1 Milk proteins

The proteins in milk provide an extremely rich source of nutrition by acting as effective binding agents and delivery systems for sensitive bioactive compounds. Milk proteins are also extremely versatile due to its various functional, physiochemical, and structural properties. Briefly, a summary of the properties of milk proteins, with specific emphasis on caseins, is provided in the following sections.

2.1.1 Overview of milk proteins

Milk is a fluid secreted by all female mammalian species and is the primary source of meeting the nutritional needs for the neonate. Over time, milk proteins have been remarkable at adapting to their evolutionary task of delivering vital nutrients, the most important of which are amino acids: the building blocks of proteins, in addition to calcium and phosphates. As a dietary item, milk has a pleasant taste, flavor, and mouth-feel. In addition, milk is the single most complete food available. It comes as no surprise then, that the individual components in milk have been an attractive area of research, both industrially and academically.

The composition of milk varies across species due to the unique nutritional and physiological requirements necessitated by that species (Table 2.1). Bovine milk contains approximately 3.4% protein; of which approximately 80% are caseins, and the remaining 20% are whey proteins, also known as serum proteins (Fox & McSweeney, 1998). The major role of caseins is to transport

calcium, essential amino acids, and antibodies, among other vital micronutrients, from the mother to the neonate. Therefore, caseins not only possess an important biological function, they are also a highly sought after functional food. Caseins are especially rich in essential amino acids; specifically α_{s2}-caseins are rich in lysine, which plant based proteins are deficient.

Table 2.1. Composition % of milks of some species (Fox, 2003)

Species	Total Solids	Fat	Protein	Lactose	Ash
Human	12.2	3.8	1.0	7.0	0.2
Cow	12.7	3.7	3.4	4.8	0.7
Goat	12.3	4.5	2.9	4.1	0.8
Sheep	19.3	7.4	4.5	4.8	1.0
Pig	18.8	6.8	4.8	5.5	--
Horse	11.2	1.9	2.5	6.2	0.5
Donkey	11.7	1.4	2.0	7.4	0.5
Reindeer	33.1	16.9	11.5	2.8	--
Domestic rabbit	32.8	18.3	11.9	2.1	1.8
Bison	14.6	3.5	4.5	5.1	0.8
Indian elephant	31.9	11.6	4.9	4.7	0.7
Polar bear	47.6	33.1	10.9	0.3	1.4
Grey seal	67.7	53.1	11.2	0.7	--

2.1.2 Functional milk proteins

The principal functional food proteins derived from milk are caseins and whey proteins. The relative ease with which purified milk proteins can be obtained has caused an increase in market consumption as well as research into characterizing the properties of these proteins. Full characterization is required in order to understand and exploit the extensive applications of the functionalities of milk proteins. Because of its unique surface activities, caseins are the functional protein of choice for many food applications including their use as foaming agents, thickening agents, and for their stabilizing properties. Caseins also possess

a high water binding capacity, which is a desirable functional property for use in various foods, such as baked products and cheese products. Prior to their use in foods, caseins have historically been used for non-food applications such as adhesives for wood, plastics, paper coating and leather and synthetic fiber finishing. Caseins are composed of a hydrophobic and a hydrophilic region, making them distinctly amphipathic. Consequently, caseins readily absorb at air-water and oil-water interfaces, giving them exceptional emulsifying properties (Fox & McSweeney, 1998). Furthermore, caseins can be obtained by other isolation and purification methods from skim milk to result in acid casein or rennet casein, by the addition of acid or the enzyme chymosin (also known as rennet), respectively. Caseins can further be processed and dried into caseinates with the addition of sodium hydroxide to yield sodium caseinate, and calcium hydroxide to yield calcium caseinate. The difference in the composition of each type of casein can be seen in Table 2.2.

Table 2.2. Composition of casein and caseinates (Southward, 1985)

Component	Acid casein	Rennet casein	Sodium caseinate	Calcium caseinate
Moisture (%)	11.4	11.4	3.8	3.8
Protein (%)	85.4	79.9	91.4	91.2
Ash (%)	1.8	7.8	3.6	3.8
Lactose (%)	0.1	0.1	0.1	0.1
Fat (%)	1.3	0.8	1.1	1.1
Sodium (%)	< 0.1	< 0.1	1.2 – 1.4	< 0.1
Calcium (%)	4.6 – 5.4	7.3 – 7.7	6.5 – 6.9	6.8 – 7.0
pH	4.3 – 4.6	6.5 – 6.7	6.5 – 6.9	6.8 – 7.0
Solubility in water (%)	0	0	100	90 – 98

2.1.3 Whey proteins

About 20–25% of the proteins in milk are whey proteins, also known as serum proteins. Whey proteins remain soluble at pH 4.6, the isoelectric point of caseins. The major fractions of whey are α-lactalbumin and β-lactoglobulin, representing approximately 20% and 50% of total bovine whey protein, respectively (Fox & McSweeney, 1998). A unique characteristic of α-lactalbumin is its biological role in the synthesis of lactose, interacting with UDP-galactosyl transferase to produce lactose from galactose (Brew, 2003). Other fractions of whey include immunoglobulins (Hurley, 2003), proteose-peptones (Rowland, 1938) and lactoferrin, among other minor milk proteins that also play important biological roles (Lönnerdal, 2003).

Whey proteins are used in a variety of food applications due to their unique functionality within a food system which include stabilization, gelation, and emulsification. Like caseins, whey proteins can be prepared from skim milk by using acid to yield acid whey, or by rennet to yield rennet (or sweet) whey. Further processing of whey by ultrafiltration, diafiltration or adsorption chromatography to remove lactose and salts results in the production of whey protein concentrates (WPC) containing 30–80% protein, and whey protein isolates (WPI) containing about 95% protein (Fox & McSweeney, 1998). Due to the ability to obtain such high-quality proteins, WPC and WPI are extensively used in the food industry for products such as muscle building and recovery protein drinks, in foods designed for weight management, and in prepared foods that provides high protein contents for extended satiety.

2.1.4 Milk proteins as delivery systems for bioactives

Recently, interest in developing functional food systems that can also act as effective carriers and delivery systems of various bioactive molecules has gained much interest. Milk proteins contain chemical and structural versatility that make them ideal candidates for delivery of various bioactive molecules (Benshitrit et al., 2012). The ease with which milk proteins can be obtained by relatively inexpensive measures makes them a suitable target for use as a delivery system on an industrial basis. In addition, milk proteins are natural and are Generally Recognized As Safe (GRAS) for use in the food and pharmaceutical industry. Their unique surface properties allow them to deliver hydrophobic or hydrophilic bioactive molecules. They are able to form complexes with macromolecules and have innate protective qualities vital to delivering and improving the bioavailability of molecules prone to oxidation. Furthermore, milk proteins undergo extensive self-assembly and co-assembly; this causes the formation of micelles in caseins. Natural self-assembly is the basis for nano-encapsulation of nutraceuticals (Semo et al., 2007) thus harnessing this property of milk proteins can prove instrumental for both the food and pharmaceutical industry.

2.2 Caseins

2.2.1 Chemistry of caseins

About 95% of the casein in milk exists as colloidal molecules known as micelles that have a high tendency to aggregate; making them a very suitable vehicle for the delivery of bioactive molecules. Aggregation into large particles

that precipitate readily from solution occur at temperatures between 30–35 °C in acidic conditions. Casein micelles are responsible for the white color of milk due largely to their ability to scatter light. This white color is lost if the casein micelle structure is disrupted by removal of the inorganic constituents in casein, collectively known as colloidal calcium phosphate (CCP). Removal of the CCP results in a very different system from that of the normal milk system, namely the dissociation of the casein micelle structure.

The pH of milk is approximately 6.7, at this pH casein micelles have a net negative charge and exists as a colloidal dispersion. Caseins are the insoluble proteins obtained by isoelectric precipitation of milk at pH 4.6. Casein can be obtained from milk by isoelectric precipitation or enzymatic (rennet) coagulation. This occurs after separation of the cream layer using centrifugation to produce milkfat products, such as butter and the phospholipid-rich milk fat globular membrane (MFGM). In isoelectric precipitation, the pH of skim milk is lowered from pH 6.7 to pH 4.6 at 20 °C to obtain a casein pellet after centrifugation. In the case of rennet coagulation, there is no change in the pH of milk, rather the enzyme rennet, also known as chymosin, is added. Chymosin cleaves a specific peptide bond in the amino acid sequence of κ-casein, Phe_{105}-Met_{106}, to result in two fragments known as glycomarcopeptide, and *para*-κ-casein. This cleavage has a specific consequence, causing κ-casein to lose its ability to stabilize the casein micelle, resulting in an increase in surface hydrophobicity and consequently the formation of a three-dimensional gel network or clot, in the

presence of calcium ions. This important reaction is essential in the cheese-making process.

In contrast to globular whey proteins, which are relatively hydrophilic, caseins have high surface hydrophobicity owing to the exposure of their hydrophobic residues and relative lack of secondary and tertiary structures. High levels of proline are responsible for the lack of α- and β-structures (Fox & McSweeney, 1998), necessary for formation of secondary and tertiary structures. As noted before, milk proteins are unique in their ability to bind and deliver hydrophobic or hydrophilic molecules. This notable feature can be explained by the uneven distribution of the hydrophobic and hydrophilic residues in the amino acid sequence, which gives caseins a distinctly amphipathic structure (Swaisgood, 2003). Milk proteins can bind small hydrophobic molecules by means of several mechanisms, including: hydrophobic interactions, van der Waals attraction and hydrogen bonds (Livney, 2010).

2.2.2 Physiochemical properties of the fractions of casein

The composition of milk is well known and its individual constituents are well characterized. As such, it has been known that caseins are composed of four individual fractions: α_{s1}-, α_{s2}-, β-, and κ-caseins. κ-caseins play an extremely important physiological role in maintaining the integrity of the micelle structure by stabilizing the caseins in the presence of calcium ions in milk. Under a hypothesized model proposed by Holt (1994), κ-casein forms a "hairy layer" on the surface of micelles and is responsible for stabilizing the micelles sterically and electrostatically from aggregation. If this "hairy layer" is removed, via specific

hydrolysis or collapsed by ethanol, the colloidal stability of the casein micelle is compromised and results in precipitation of the casein (Holt and Horne, 1996). κ-caseins are the least sensitive to calcium salt and remain soluble under all conditions which would precipitate the other casein fractions. α_{s2}-caseins are the most hydrophilic and most calcium sensitive of all the caseins, they precipitate at a Ca^{2+} concentration of less than 2 mM. α_{s1}-caseins are slightly less sensitive to the presence of calcium salt, precipitating in the range of 3–8 mM Ca^{2+}. β-caseins are the most hydrophobic fraction of the caseins and less sensitive yet to calcium salt, precipitating at a range of 8–15 mM Ca^{2+} at 37 °C (Swaisgood, 2003). Of all the casein fractions, β-casein is the most temperature sensitive. Monomers predominate in β-casein at low temperatures (<10–15 °C) but associate via hydrophobic bonding into micelles at increased temperatures (O'Connell, 2003). β-casein associates into soap-like micelles with a hydrophobic core and hydrophilic surface with increases in temperature, indicating that β-casein undergoes a temperature-induced micellization process. Being temperature dependent, this micellization process can be explained predominantly by hydrophobic bonding as the principal associative force, with electrostatic and steric interactions being responsible for maintaining the stability of the micelle and preventing the micelle from aggregating (O'Connell, 2003).

Based on primary structures, α_{s1}- and α_{s2}-caseins are both suggested to be composed of a negatively charged N-terminal hydrophilic domain and a positively charged C-terminal hydrophobic domain. Interactions of the hydrophobic domains are likely to be responsible for the associative nature of the protein via

hydrophobic interactions. Consequently, the associative properties of both protein fractions are highly dependent on the ionic strength and temperature of its environment. It is important to note that association of these proteins may not only be due to hydrophobic interactions exclusively, as dissociation of the protein does not occur at low temperatures, as in the case of β-casein (Swaisgood, 2003). All casein fractions share in common a distinctly amphipathic structure which confers excellent surface properties.

2.2.3 Structural properties of casein

Many hypothesized models of the structure of casein have been developed, though a consensus within the scientific community regarding the presence or absence of secondary or tertiary structures of the casein micelle remains yet to be achieved. In an effort to validate a hypothesized model of the casein micelle, characterization of caseins using advanced imaging techniques have been at the forefront of native casein micelle research. These imagining techniques include, but are not limited to: confocal microscopy (CM), conventional electron microscopy, scanning electron microscopy (SEM), atomic force microscopy (AFM), cryo-transmission electron microscopy (cryo-TEM) and tomographic reconstruction models. Through the combined use of these techniques and years of research resulting in a wealth of new insights into the internal architecture of the casein micelle, certain structural and dynamic properties of the casein micelle can now be visualized and confirmed unequivocally. Work by Harte and coworkers (2011) demonstrated through the use of cryo-TEM that the interior of the casein micelle not only exhibited proteins

in a random arrangement, but that it also contained water-filled cavities and

channels, as well as several hundred high-density nanoclusters (Figure 2.1). The

presence of water-filled channels is not surprising, given that micelles are highly

hydrated and bind approximately 2.0 g H_2O/g protein (Fox, 2003).

Figure 2.1. Cryo-TEM tomograms of casein micelles (Trejo et al., 2011) *A)*
Slabs (4.74 nm) through the centers of 2 micelles from the cryo-TEM tomograms;
B) highlighting of the internal channels in the native casein micelles in the same
image; C) highlighting of the internal cavities in the native casein micelles in the
same image. Scale bar = 200 nm.

2.3 Caseinates

2.3.1   Preparation of sodium caseinate

Several types of commercial caseinates are obtained from the

resolubilization of the casein pellet remaining after isoelectric precipitation.

Caseinates are produced by the addition of one of several dilute alkalis to adjust

the pH of casein to 6.5–7.0 using sodium, potassium, ammonium, or calcium. The

resulting homogenous solution is then spray dried to produce a caseinate powder

(Fox & McSweeney, 1998). Sodium caseinate is the most common manufactured

caseinate due to its high solubility in water and is therefore used in a wide variety

of applications. Sodium caseinate has been exploited in many studies on its

binding and harnessing ability for the preparation of nano-vehicles for bioactive

molecules because of its excellent solubility properties (Livney, 2010).

2.3.2 Sodium caseinate as a delivery system

Sodium caseinate has been established as an excellent vehicle for the

transport of bioactive molecules and in the use as a delivery system through many

previous studies. One such study by Singh and coworkers (2009) investigated

sodium caseinate's ability to bind to iron. Iron is an essential mineral, obtained

only through diet, which aids in the production of hemoglobin present in red

blood cells and is responsible for transporting oxygen and distributing it to cells

throughout the body for metabolism. A lack of iron results in iron deficiency and

anemia. The iron content in milk is approximately 0.2–0.5 mg/iron/L (Sugiarto,

2009) and is considered too low as the recommended daily allowance (RDA)

according to the Center for Disease Control and Prevention (CDC) is about 8

mg/day in most adults; therefore fortification of milk and dairy products is

necessary. Sodium caseinate and whey protein isolate (WPI) were used to

determine the binding affinity of iron to those milk proteins. Their results showed

that sodium caseinate contained more binding sites ($n = 14$) than WPI ($n = 8$) for

binding iron, likely due to the presence of clusters of phosphoserine residues

present in casein that are known to bind well to divalent cations (Sugiarto, 2009).

The number of phosphoserine residues present in casein is different between the

individual fractions of casein. α_{s1}-casein contains the most residues and κ-casein

containing the least, suggesting binding of iron occurs between the individual

fractions, rather than the casein micelle as a whole. The discrepancy in binding

affinities between sodium caseinate and whey protein may be explained by the difference in their chemical structure. Whey proteins are large globular proteins with a relatively compact, rigid structure whereas caseins possess a more open and flexible structure owing to the relative lack of secondary and tertiary structures, which facilitates the binding of various bioactive molecules, including metal ions such as iron. It is important to note that binding affinity is a function of environmental conditions, such as pH and temperature; suggesting binding may be due to electrostatic, hydrophobic, or a combinatory effect of both interactions.

Curcumin is a polyphenolic compound found in the spice turmeric, which has been implicated in decreasing the risk of Alzheimer's disease in India, a country known to use and consume large amounts of turmeric in their traditional foods (Govindarajan, 1980). Curcumin has also been shown to possess anti-cancer, anti-microbial, and anti-inflammatory properties in addition to its antioxidant activity (Maheshwari et al., 2006). However, the low solubility of curcumin in aqueous solutions remains a limitation in effectively delivering the bioactive compound to be metabolized. Binding curcumin to protein complexes, particularly water soluble milk proteins, and delivering the compound to the site where it can be metabolized may possibly mitigate this problem. Corredig and coworkers (2012) have shown that curcumin binds to nonpolar regions of the casein micelle, indicating that binding occurs mainly due to hydrophobic interactions.

Sodium caseinate has also been shown to be effective carriers of many other bioactive compounds of various chemical properties, such as soy

phospholipids (Istarova, 2005), vitamin D_2 (Semo et al., 2007), omega-3 polyunsaturated fatty acids (Zimet et al., 2011), and gum arabic (Ye, 2012).

2.4 Coffee

2.4.1 History and background

Coffee is a popular beverage consumed throughout the world from as far back as the tenth century, though it may have been cultivated in indigenous Ethiopia as early as 575 A.D. (Dews, 1984). According to historical records, the first reliable reference of a caffeine-containing beverage described in a Chinese dictionary is tea around 350 A.D., long before the first mention of coffee. Coffee was consumed as a hot beverage around 1000 A.D. It has a pleasant taste, aroma and is consumed both for its flavor and stimulating effects of caffeine alike. It is a versatile food; its grounds can be incorporated into other foods, it can be sweetened and used in baked goods and of course, it can be brewed and consumed as a beverage. The majority of coffee beverage consumption is from the *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) species. Arabica coffee is considered to have a superior taste to Robusta coffee and is why it represents about 70% of the world market (Lean, 2012) and reaches higher prices in the international market (Gielissen & Graafland, 2009). Robusta beans on the other hand, are often used in instant coffees.

Coffee consumption rates rose from 3.5 kg/person/year to 4.4 kg/person/year in the United States from 1995 to 2007, and from 3.3 kg/person/year to 4.1 kg/person/year in Europe (World Resources Institute, 2011). The desirable effect of caffeine is undoubtedly one of the major contributors to

this increase in coffee consumption. Coffee also contains many micronutrients, such as magnesium, potassium, niacin and vitamin E, all of which could contribute to the overall health benefits of coffee consumption (Higdon & Frei, 2006). However, coffee is composed of over a thousand different compounds that contribute both to its taste and beneficial attributes, yet many of these compounds remain yet to be characterized.

2.4.2 Composition of the coffee fruit

The coffee fruit is composed of several layers that each have a distinct function in protecting the cherry at the center of the bean and are depicted in Figure 2.2. The coffee bean is composed of three distinct layers: a smooth but tough outer skin layer known as the pericarp, followed by a fibrous layer of sweet pulp, called the outer mesocarp, and finally a thin yellowish layer known as the endocarp or parchment. Between the mesocarp and endocarp layers lies a translucent, colorless and viscous layer of mucilage, also known as the pectin layer. The coffee cherry, or endosperm, is covered by a layer known as the silverskin (Belitz et al., 2009; Berbert et al., 2001). Upon ripening of the fruit, the pericarp changes colors ranging from yellow to red, depending on the specific genotype. The amount of caffeine found in the pericarp is approximately two to ten times lower than that of the amount found in the seed. This is primarily because caffeine is only synthesized in the earlier developmental stages of the seed and thus is not continually synthesized during fruit maturation (Esquivel & Jimenez, 2012).

Figure 2.2. Layers in a coffee fruit (Esquivel & Jimenez, 2012).

2.4.3   Processing & effects of roasting on coffee beans

Green coffee beans can be processed by one of two methods, via dry or wet processing. Dry processing involves drying the coffee fruit in the sun before being mechanically hulled, removing the dried husks containing the skin, pulp, mucilage and parchment layers in addition to much of the silverskin in a single step. Wet processing is a much more involved process which includes separation of the ripe and unripe berries in water based on their density. The outer layers of the fruits are removed by pressing the fruit through a screen with water. The middle layers of the fruit are removed next through a fermentation step which hydrolyzes the mucilage layer using enzymes from within the coffee tissues as well as from microorganisms found on the skin of the fruit (Belitz et al, 2009; Vaast et al., 2006). The final hulling step removes the parchment layer from the beans after drying. An additional polishing step is optional to remove the silverskin layer. Though the multistep wet process method takes significantly more time and energy, it has been shown that this method yields higher contents

of CGA and trigonelline, and a lower content of sucrose in comparison to the dry method (Duarte et al., 2010).

Sensory attributes of a coffee beverage such as flavor, aroma and body, are developed during the roasting process and due largely in part by the CGA content in the beans, which gives coffee its characteristic and desirable properties. Therefore, the amount of CGA is a large determinant of coffee cup quality. During processing and roasting, CGAs may be degraded into low molecular weight compounds through hydrolysis, thus dark roast coffee tends to contain a lower amount of CGAs compared to its unroasted or light roast counterpart (Farah & Donangelo, 2006). The sensory profile of roasted coffee is a result of Maillard reactions, a complex series of reactions that take place at high temperatures and occur in the presence of amino acids and reducing sugars. Because CGA contributes to the antioxidative properties of roasted coffee as well, it makes sense that higher temperature of roasting results in a reduction of total CGA content (Moon et al., 2009). Green coffee beans subjected to severe roasting conditions can result in up to 95% loss of CGA (Trugo & Macrae, 1984).

However, studies have also shown that roasted coffee extract have antibacterial activity against several strains of enterobacteria (Almeida et al., 2006) as well as microorganisms such as *Staphylococcus aureus* and *Streptococcus mutans* (Daglia et al., 2007; Dacarro et al., 1995). Further, consumption of roasted coffee beverages may increase populations of *Bifidiobacterium* spp., the beneficial strain of bacteria found in probiotics, which

indicates that coffee consumption may possibly have some prebiotic effects as well (Jaquet et al, 2009).

2.4.4 Coffee bioactives

Coffee has a complex chemical spectrum containing over a thousand compounds, many of which still remain unidentified and thus difficult to assess the possible health benefits or adverse effects upon consumption. Of the characterized compounds, coffee contains varying levels of caffeine depending on the type of bean, with Robusta beans containing significantly more caffeine than Arabica beans. Many consumers of coffee do so because of the stimulating effects and increased alertness imparted by caffeine, which accounts for 1 to 4% of the bean on dry basis, and has a half-life of about 5 hours in most adults (Lean, 2012). Green coffee beans also consist of many phenolic compounds which account for up to 14% on dry matter basis (Esquivel & Jimenez, 2012). These phenolic compounds are esters of *trans*-cinnamic acids and quinic acids. Esterification can occur at positions 3, 4, or 5 and results in several isomers of CGA. Other phenolic compounds found in green coffee beans are caffeic, ferulic and dimethoxycinnamic acids.

Other bioactive compounds that have been reported in green coffee beans are cafestol and kahweol (Figure 2.3). These diterpenes have been implicated in both positive and negative health roles. They have been shown to increase total serum cholesterol levels (Farah & Donangelo, 2006) and production of low density lipoprotein cholesterol (LDL) (Higdon & Frei, 2006). However, kahweol has also been identified as having potential anti-inflammatory effects (Cardenas

et al., 2011). In addition, cafestol shows anti-oxidative properties, based on decreased DNA damage to cells treated with the diterpene (Lee & Jeong, 2007). Diterpenes in coffee may also promote the elimination of carcinogens. However, as with all constituents of coffee, the concentration of these compounds varies greatly depending on the type of bean, roasting procedures and brewing method. Interestingly, these diterpenes have only been found in unfiltered coffee, such as coffee obtained from boiling or by using a French Press.

Figure 2.3. Chemical structures of cafestol and kahweol (Higdon & Frei, 2006) *R = H: free diterpene; R = fatty acid: diterpene ester*

2.4.5 Caffeine

The chemical name for caffeine is 1, 3, 7-trimethylxanthine (Figure 2.4) and is a purine alkaloid that is naturally occurring in coffee beans. Caffeine is essentially completely metabolized by the liver into its primary metabolite, paraxanthine, and is catabolized to form xanthine and theobromine. Physiological effects of caffeine include central nervous system stimulation, increased awareness and metabolic rate, increased concentration, and acute elevation of blood pressure, in addition to diuresis (Higdon & Frei, 2006). These effects can be felt relatively soon after ingestion. Caffeine is absorbed rapidly and almost

completely in the stomach and small intestine and distributed to all tissues, including the brain, which is likely why stimulation of the central nervous system occurs. Additional evidence on the stimulatory effects of caffeine is due to its antagonism of A_1 and A_{2A} subtypes of the adenosine receptors (James, 2004). Adenosine (Figure 2.4) is a constituent of adenosine triphosphate (ATP) and acts as an intermediary in a wide range of metabolic pathways, including behavioral influences of xanthines. The effects of adenosine are opposite that of caffeine, suggesting an antagonistic relationship and resulting in effects of caffeine that are generally stimulatory. This supports the hypothesis that xanthines act behaviorally by blocking adenosine receptors to elicit locomotive enhancement (Dews, 1984).

As a natural product, caffeine has been used as a major ingredient in food products, including soft drinks, baked goods, dairy desserts, and candy, as well as an active ingredient in many pharmaceuticals. For instance, the use of caffeine in analgesics may be due to the fact that caffeine has been found to decrease the systemic elimination of acetaminophen and increase the bioavailability of aspirin (Higdon & Frei, 2006). The wide versatility of caffeine gives impetus to research investigating its use as an ingredient in foods, as well as into its metabolism and positive or adverse effects to health upon ingestion.

Figure 2.4. Chemical structures of caffeine and adenosine

2.4.6 Chlorogenic acid (CGA)

Coffee is also an abundant source of polyphenolic compounds, which commonly act as antioxidants. Structurally, polyphenols are characterized by having one or more aromatic rings with one or more hydroxyl groups attached. Phenolic compounds are secondary metabolites that are ubiquitous constituents involved in a higher plants defense mechanism against environmental stress. Conditions such as severe weather, excess ultraviolet (UV) or high visible light, and water stress can directly impact the amount of phenolic compounds produced by a plant (Farah & Donangelo, 2006). The amounts of phenolic compounds present in coffee beans thus varies depending largely on cultivar, degree of maturation, and are attributed to a lesser extent by agricultural practices (Clifford, 1985).

Present as a family of esters formed between *trans*-cinnamic acids and quinic acid, the phenolic fraction of coffee is collectively known as chlorogenic acids (CGA). CGAs have been found on the surface within the cuticular wax and also in the cytoplasm of the coffee seed. CGA is a broad classification of the

antioxidants present in coffee which also include: caffeoylquinic acids (CQA), dicaffeoylqunic acids (diCQA), feruloylqunic acids (FQA), *p*-coumaroylquinic acids (*p*CoQA) and mixed diesters of caffeic and ferulic acids with quinic acid, each group containing at least three isomers. The nine main isomers of CGA are: 5-CQA, 4-CQA and 3-CQA; 3,5-diCQA, 4,5-diCQA and 3,4-diCQA, 5-FQA, 4-FQA and 3-FQA (Farah & Donangelo, 2006). CGA and its isomers make up the main components of the phenolic fraction of the unroasted, green coffee beans, reaching levels of up to 14% on dry matter basis (Farah & Donangelo, 2006). In green coffee beans, 5-CGA (Figure 2.5) alone accounts for about 56–62% of total CGA, with 4-CGA and 3-CGA isomers accounting for up to 10% each of total CGA (Farah & Donangelo, 2006). As such, esters of CGA are usually formed on carbon-5 of quinic acid but also on carbons-3 and 4, and less commonly on carbon-1. Roasting causes isomerization of CGA resulting in a substantial decrease in substitution at the carbon-5 position and an increased level of substitution at the carbon-3 and 4 positions. (Trugo & Macrae, 1984; Leloup et al., 1995, Farah et al., 2005). The processing conditions, such as temperature, as well as brewing conditions (water temperature, water to bean ratio) also has a marked effect on the amount of CGA that is extracted from the coffee bean.

Figure 2.5. Chemical structure of 5-CGA

2.5 Health benefits of coffee bioactives

2.5.1   Health benefits of caffeine

Coffee consumption is undoubtedly made popular by the increased

alertness and stimulating effects of caffeine. It is the beneficial health effects of

coffee bioactive compounds that make it a functional food. Due to this

functionality and the increase in awareness of health and nutrition of foods, it is

imperative that these individual components be studied extensively for any

beneficial as well as adverse effects upon ingestion. Caffeine has been well

characterized and has been the focus of many areas of research, with

epidemiological studies being at the forefront of investigating the associations

between caffeine and potential health benefits in those who consume coffee.

Some of the health benefits of caffeine include reduced risk of depression in

women with a mean age of 63 years that regularly consume coffee (Lucas et al.,

2011) as well as reduced cognitive decline in older women. Caffeine in coffee has

also been observed to be inversely associated with the risk of Parkinson's disease

in men in case-control studies (Hernan et al., 2002). The results from these studies

failed to show a correlation between coffee consumption in women and a

decreased risk of Parkinson's disease. These results may be attributed to the modifying effect of estrogen replacement therapy inhibiting cytochrome CYP1A2, which is also responsible for caffeine metabolism (Pollock et al., 1999).

2.5.2 Health benefits of chlorogenic acid

CGAs have numerous beneficial health properties due to their potent antioxidant activity, some of which include hepatoprotective, hypoglycemic and antiviral activities in addition to cell vitality (Cheng, 2011).

Epidemiological studies have suggested that the major phenolic compounds in coffee, caffeic acid (CA) and CGA, and regular coffee consumption have an inverse correlation with the incidence of Type 2 diabetes (Greenberg et al., 2005). One of the causative factors of Type 2 diabetes may be due to misfolding of human islet amyloid polypeptides (hIAPP). Huang and coworkers (2011) have investigated the inhibitory effects of caffeine, CA, and CGA on toxic aggregation of hIAPP. This study has immense implications since Type 2 diabetes constitutes 90–95% of diabetic patients worldwide (Clark et al., 1987). These studies concluded that while caffeine appeared not to have a significant inhibitory effect on the formation of lineal hIAPP fibrils, when compared to the major polyphenolic compound (-) –epigallocatechin 2-gallate (EGCG) found in tea, CA and CGA did have a marked inhibitory effect as detected by tioflavin-T (ThT) fluorescent dye binding studies and TEM imaging of the aggregates (Figure 2.6). The concentration of the coffee bioactive compound to hIAPP was also observed to affect the extent of inhibition. Since these studies were conducted in vitro, many physiological events such as

metabolism of the coffee bioactives into their metabolites and varying

concentrations of hIAPP present in the body, the data from these studies suggest

that regular coffee consumption may inhibit hIAPP amyloid formation, thus

lowering the risk of Type 2 diabetes. A significant inverse association between

the risk of Type 2 diabetes and coffee consumption has also been observed in

many other epidemiological studies (Salazar-Martinez et al., 2004, Rosengren et

al., 2004).

Figure 2.6. TEM images of hIAPP co-incubated with different compounds (Cheng, 2011). *(A) hIAPP; (B) 1:1 mixture of hIAPP and EGCG; (C, E, G) equimolar amounts of caffeine, CA, and CGA; (D, F, H) 5-fold molar excess of caffeine, CA, and CGA.*

Inflammation has long been associated with a plethora of cardiovascular

diseases, which include atherosclerosis, cancer, obesity, and diabetes (Lai et al.,

2012). Cardiovascular disease continues to be the leading cause of death in developed countries such as the United States. Environmental factors such as diet have an immense impact on general health, one of which is modulating inflammation. Inflammation is a response triggered by noxious stimuli caused by either physical or chemical trauma. Phenolic compounds such as CGA have been shown to attenuate inflammation and confer anti-inflammatory effects (Frost-Meyer & Logomarsino, 2012). Since polyphenols are extensively metabolized in the body, consuming polyphenol-rich foods can help mitigate the effects of chronic inflammation and aid in the prevention of cardiovascular diseases. However, one main disadvantage of polyphenol consumption is their relatively low bioavailability in the body and thus remains a technological challenge. Developing functional foods that effectively carry and deliver polyphenols to the site where they can exert their antioxidant potential has gained considerable attention.

2.6 Techniques of analysis

Because of the large variation in the amount of bioactive compounds present in coffee beans, it is imperative to develop reliable and sensitive methods for establishing levels of these bioactive compounds. Given the discrepancy between results obtained by high resolution chromatographic methods and by less sophisticated methods, the need to continually develop and optimize sensitive methods of detection and quantification is of paramount importance. Many compounds in coffee remain uncharacterized due largely to lack of commercial standards and similarities of their chemical structures. Consequently, sensitive

methods for proper identification and quantification are in high demand. Briefly, a summary of common methods employed in the isolation, determination and quantification of the coffee bioactive compounds, caffeine and CGA, will be discussed below. Methods of protein determination will also be reviewed.

2.6.1   High Pressure Liquid Chromatography (HPLC)

Separation of compounds using HPLC is achieved by exploiting differences in polarity. Typically, compounds are carried via an aqueous solution of low ionic strength and attached to a stationary phase. There are many chromatographic methods used for separating compounds including: normal phase, reversed phase, ion-exchange, size-exclusion or gel permeation, metal interaction, and affinity chromatography. In the most commonly used method, reversed phase HPLC (RP-HPLC), the stationary phase is a column composed of a matrix of polar silica gel substituted with nonpolar hydrocarbon chains (e.g. C_8 or C_{18} alkyl chains). Compounds are carried through the column using a polar mobile phase and elution is obtained by increasing the hydrophobicity of the mobile phase. Compounds are driven by hydrophobic interactions to adsorb to the stationary phase (Swadesh et al., 1993). The compound of interest, the analyte, is then eluted based on its polarity with the most polar compounds eluting first and the less polar compounds eluting last. Nonpolar compounds have an affinity to interact with the relatively nonpolar hydrocarbon chains attached to the stationary phase and are retained longer than more polar compounds. The dimensions of a column, length and diameter, as well as the particle size of the packing material have a large effect on separation and resolution of analyte peaks and on

equilibration time. Therefore, the analytical column often has an impact on the performance of a chromatographic method.

Chromatographic conditions may employ the use of isocratic or gradient elution. In isocratic elution, a single mobile phase remains at a fixed concentration throughout the analysis. In gradient elution, several mobile phases of various ionic strengths are mixed together by a high pressure pump and the concentration of the mobile phases varies throughout the run. Gradient elution is preferred over isocratic elution if determination of several analytes within a single sample is needed.

There are several types of detectors used in HPLC analysis, each having the ability to detect different types of molecules. UV-detectors are commonly used to detect an array of small organic molecules that possess a chromaphore, a part of their chemical structure that has the ability to absorb UV-light (e.g. conjugated double bonds or aromatic rings). RP-HPLC has also been extended for the analysis of macromolecules, such as proteins. A major limitation to UV-detectors is being able to measure only at one specified wavelength. Hence if there are several analytes in a sample that absorb at different wavelengths, using a UV-detector would mean having to inject the sample multiple times at different wavelengths to completely analyze the sample. A solution to this problem is to use a photo-diode array (PDA) detector. PDA detectors are capable of analyzing a sample at multiple wavelengths, allowing one sample with multiple analytes to be analyzed at once, saving time and money.

Several methods exist for the simultaneous determination of purine alkaloids and polyphenols by HPLC (Samanidou et al., 2012, Ramalakshmi et al., 2011, Trugo et al., 1991). More sophisticated and sensitive methods of analysis, such as tandem mass spectroscopy, have also been employed for the determination of polyphenols in green coffee beans (Alonso-Salces et al., 2009). Caffeine in various coffee brews have also been determined by a rapid method known as direct analysis in real time (DART) ion source coupled to high-resolution time-of-flight mass spectrometry (TOFMS) (Danhelova, et al., 2012). This sensitive method is not only capable of detecting and quantifying caffeine, it is also able to detect other polar compounds isolated from Arabica and Robusta coffee brews, measured in both positive and negative ion modes (Figure 2.7). Rapid and powerful methods of analysis such as DART-TOFMS may be used in the future in assessing the authenticity of coffee. Selection of the method and technique depends on the level of sensitivity needed for an analysis, and the availability of the instruments. However, analytical methods are often compared to results obtained by HPLC (Trugo et al., 1991) due to the simplicity of sample preparation, accurate results, availability and affordability of the instrument, and relatively low level of training required to complete an analysis.

Figure 2.7. DART-TOFMS spectra (Danhelova et al., 2012). (*m/z 100–450) acquired in DART positive and negative ion modes of the extracts of roasted ground Arabica and Robusta coffee brew.*

2.6.2 Oxygen Radical Absorbance Capacity (ORAC) assay of antioxidants

Antioxidants are an important class of compounds used as preservatives in foods. They are radical inhibitors that prevent the oxidation of foods by preventing unwanted radical reactions by reactive oxygen species (ROS). An excess of ROS in the body leads to cellular injury and damage to proteins, which in turn plays a role in the development of many diseases, such as diabetes, cardiovascular disease, and neurodegenerative diseases. Consumption of antioxidants from food sources is one way to decrease ROS-induced oxidative stress in the body. In order to evaluate whether a particular food is an adequate source of antioxidants, it is important to assess the antioxidant capacity of its extracts. A classical tool for measuring antioxidant capacity is the Oxygen

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Radical Absorbance Capacity (ORAC) assay. The ORAC assay is capable of measuring the antioxidant capacity of biomolecules from a variety of samples, making it an extremely versatile assay. In addition to its versatility, the assay can be performed with relative ease. An ORAC assay kit obtained from Cell Biolabs, Inc. (San Diego, CA) is a fast and reliable kit for the direct measurement of the antioxidant capacity from various food extracts. Once the appropriate reagents are prepared, sample preparation in a multi-well microtiter plate is performed by adding the sample and a fluorescein solution to each well in a 1:6 ratio and incubating at 37 °C for 30 minutes. The fluorescein solution is a fluorescent probe which is oxidized by peroxyl radicals. A solution of free radical initiator is added to each well and mixed immediately prior to being placed in a microplate reader at 37 °C and analyzed with an excitation wavelength of 480 nm and emission wavelength of 520 nm (Figure 2.8). The free radical initiator produces the peroxyl radicals to quench the fluorescent probe over time. Therefore, the antioxidants present in the sample works to block the peroxyl radical oxidation of the fluorescent probe and the antioxidant capacity of the sample is measured by comparison with a standard curve of vitamin E, measured in Trolox^{TM} equivalents (TE). Another advantage of the ORAC assay is its ability to combine both inhibition time and degree of inhibition into a single quantity, the only method thus far capable of providing these results (Ramalakshmi et al., 2011).

Figure 2.8. ORAC assay principle. *ORAC assay of a sample is calculated by subtracting the area under the curve (AUC) of the blank from the AUC of the sample to obtain the net AUC (Cell Biolabs, Inc.).*

2.6.3   Protein analysis

Numerous methods for the quantification of protein have been developed. The Official Methods of Analysis of AOAC International include the Kjeldahl, Dumas, and infrared spectroscopy methods (AOAC, 2000). The Kjeldahl method (AOAC, 995.04), developed in 1883 by Johann Kjeldahl, involves digestion of protein with sulfuric acid and conversion of the protein nitrogen to ammonium sulfate by oxidation with potassium permanganate, neutralization of the digest, and finally titration to obtain the percent nitrogen. A conversion factor is then used to convert percent nitrogen to percent protein. Although the Kjeldahl method for protein analysis is an AOAC method and commonly used, it can be quite time consuming and requires the use of hazardous chemicals. An alternative to the Kjeldahl method is the Dumas method (AOAC, 992.23), developed in 1831 by Jean-Baptiste Dumas. Samples are weighed into a tin capsule and placed in a combustion reactor equipped with a built in gas-chromatograph (GC). The nitrogen released by the sample is then collected and measured directly by the GC. This automated process takes considerably less time to analyze samples, and

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requires no hazardous chemicals. However, like the Kjeldahl method, the Dumas method measures total organic nitrogen and therefore does not distinguish between protein and non-protein nitrogen (NPN). Infrared spectroscopy is used to measure the absorption of radiation by peptide bonds to estimate the protein concentration in a food sample (AOAC, 997.06).

Another alternative method for the quantification of protein in a sample is the Bicinchoninic acid (BCA) method. The BCA method exploits the ability of proteins to reduce Cu(II) to Cu(I) under alkaline conditions (Smith et al., 1985). A purple-colored product is obtained by the chelation of two molecules of BCA with one cuprous ion. The color formed is proportional to the protein concentration in a sample as measured by a spectrophotometer at 562 nm. The concentration of protein in a sample is then compared to a standard curve of known concentrations of a protein such as Bovine Serum Albumin (BSA). Therefore, the BCA assay is used as a colorimetric method for the determination and quantification of total protein in a sample. This is a simple and widely used, albeit time sensitive method of protein analysis.

2.7 Justification for work

Given the increase in coffee beverage consumption in addition to an increase in popularity of incorporating pure extracts of coffee bioactive compounds into a wide variety of food products, it is necessary, if not imperative to develop analytical methods using sophisticated instruments that are both fast and reliable. Rapid methods of analysis facilitates the use of these compounds in ways that may not have been previously possible, such as using milk proteins to

bind and deliver into different food matrixes. Limited research has been done on the binding capacity of sodium caseinate with the major alkaloid in coffee, caffeine. Given the numerous health benefits conferred by caffeine and regular consumption of caffeine and antioxidant containing beverages such as tea and coffee; much attention has been attracted to projects involving the use of both these compounds. However, incorporation of these compounds has proven a technical challenge due to their susceptibility to oxidation. Binding these compounds with a protective vehicle for transport; such as casein micelles in milk proteins, may mitigate this challenge.

The objectives of this thesis are to first develop and validate an HPLC method for the accurate and simultaneous determination of caffeine and CGA in sodium caseinate samples. Next, is to establish a procedure by which caffeine and CGA can be bound to sodium caseinate and investigate the effects of pH and temperature on its binding capacity. And last is to, determine the conditions that result in increased binding interactions and estimate the binding capacity of sodium caseinate with these bioactive compounds and speculate on their binding mechanism. Knowledge gained from these studies could promote the use of milk proteins in even more functional ways by elucidating the mechanisms and conditions by which proteins bind with these compounds and how changes in the chemical environment can increase binding interactions. Moreover, these studies aim to promote the use of dairy proteins with the health promoting constituents of coffee and to provide a means by which accurate and reliable quantification of these compounds can be achieved.

3.0 MATERIALS AND METHODS

3.1 Development of a method for the simultaneous determination of caffeine and CGA by reversed phase High Pressure Liquid Chromatography (RP-HPLC)

3.1.1   HPLC Method optimization for the analysis of caffeine and CGA in green coffee extracts

3.1.1.1   Mobile phase gradient

Mobile Phase A (MPA) was prepared in a 90:10:0.1 (v/v/v) of MilliQ Nanopure water/acetonitrile/formic acid. MilliQ Nanopure water (900 mL) was transferred to a suitable media bottle and 1.0 mL of formic acid (97% ACS grade, Acros Organics) was added and mixed well. The solution was then filtered through a polyethersulfone (PES) membrane filter (Cat # 431118, 0.22 μm, Corning Inc.) under vacuum. Acetonitrile (100 mL) (HPLC grade, Fisher Scientific) was then added to the solution and mixed well. Mobile Phase B (MPB) was prepared in a 75:25:0.1 (v/v/v) of MilliQ Nanopure water/acetonitrile/formic acid. MilliQ Nanopure water (750 mL) was transferred to a suitable media bottle and 1.0 mL of formic acid (97% ACS grade, Acros Organics) was added and mixed well. The solution was then also filtered through a PES membrane filter (Cat # 431118, 0.22 μm, Corning Inc.) under vacuum. Acetonitrile (250 mL) was then added to the solution and mixed well. The HPLC unit used (Shimadzu Prominence, CBM-20A) was equipped with an autosampler (SIL-20A), column oven (CTO-20A), and a photo-diode array (PDA) detector (SPD-M20A). The column employed was a Shimadzu C_{18} reversed phase (RP) column (Shimadzu,

Japan) with dimensions 4.6 mm x 150 mm. A 50 μL aliquot of the sample was injected at a flow rate of 0.6 mL/min.

Original chromatographic conditions employed for the determination of caffeine in green coffee extracts resulted in co-eluting peaks (Figure 3.1).

Figure 3.1. HPLC chromatogram of caffeine analysis (280 nm) from a green coffee extract diluted 1:50 with MPA.

Adequate peak separation and resolution is essential for proper peak integration and thus obtaining accurate results. Adjusting the gradient profile, in combination with other adjustments to the chromatographic conditions, attempts to effectively separate the peak co-eluting with caffeine. The original parameters for gradient elution, gradient "A", is depicted in Table 3.1. Table 3.2 shows gradient "B", the first attempt at resolving the caffeine peak by increasing to 12% solvent B at t = 15 minutes, 80% solvent B at t = 35 minutes and finally 100% solvent B at t = 37 minutes. This is in contrast to a slow linear increase to 100% solvent B at t = 43 minutes in the original gradient parameters. Gradient "B" also shortens the total run time to 62 minutes. Gradients "C" and "D" (Table 3.3 and Table 3.4) were slight modifications of gradient "B" at t = 15 minutes, holding all other

conditions of the gradient profile the same. Gradient "E" (Table 3.5) employs the use of a step-gradient profile, increasing the percentage of solvent B quicker and held at 20% over a longer period of time. Table 3.6 shows the final gradient profile with a total run time of 60 minutes. Figure 3.2 shows all proposed gradient profiles in a graphical form. Mobile phase A was held at 100% for 3 minutes, and then changed by a linear gradient to 100% B over 30 minutes. The mobile phase was held at 100% B for 7 minutes, and a linear return to 100% A was made over 5 minutes. The mobile phase was held at 100% A for 18 minutes for re-equilibration of the column prior to injecting a new sample. The composition of MPA and MPB remained unchanged from the original chromatographic conditions. Determination of the optimal gradient elution profile will be discussed in the results & discussion section of this thesis.

Table 3.1. Time program of gradient "A"

Time (min)	% Solvent B
0.01	0.0
3.00	0.0
43.00	100.0
50.00	100.0
60.00	0.0
75.00	0.0
76.00	End

Table 3.2. Time program of gradient "B"

Time (min)	% Solvent B
0.01	0.0
3.00	0.0
15.00	12.0
35.00	80.0
37.00	100.0
42.00	100.0
47.00	0.0
62.00	0.0
63.00	End

Table 3.3. Time program of gradient "C"

Time (min)	% Solvent B
0.01	0.0
3.00	0.0
15.00	10.0
35.00	80.0
37.00	100.0
42.00	100.0
47.00	0.0
62.00	0.0
63.00	End

Table 3.4. Time program of gradient "D"

Time (min)	% Solvent B
0.01	0.0
3.00	0.0
15.00	20.0
35.00	80.0
37.00	100.0
42.00	100.0
47.00	0.0
62.00	0.0
63.00	End

Table 3.5. Time program of gradient "E"

Time (min)	% Solvent B
0.01	0.0
3.00	0.0
4.00	20.0
15.00	20.0
35.00	80.0
37.00	100.0
42.00	100.0
47.00	0.0
62.00	0.0
63.00	End

Table 3.6. Time program of final gradient profile

Time (min)	% Solvent B
0.01	0
3.00	0
30.00	100
37.00	100
42.00	0
59.00	0
60.00	End

Figure 3.2. Gradient profiles for separation of caffeine by HPLC.

3.1.1.2 Analytical column

The original analytical column used for the analysis of caffeine and CGA in green coffee extracts was a Shimadzu C_{18} reversed phase (RP) column (Shimadzu, Japan) with dimensions 4.6 mm x 150 mm. Subsequent analyses using this column afforded poor peak separation, poor baseline resolution at 280 nm and consequently, improper caffeine peak integration. To mitigate these issues resulting in inaccurate quantification of caffeine, an Alltech Platinum RP C_{18} Extended Polar Selectivity (EPS) (Grace Davison Discovery Sciences) column with dimensions 4.6 mm x 250 mm and particle size of 5 μm (Figure 3.3) was

selected due to its increased polarity and length which is known to improve peak

separation. Aliquots of 50 μL samples of green coffee extract diluted 1:400 with

MPA were injected using the original analytical column and then with the new

analytical column, holding all other chromatographic conditions constant. A

comparison of the ability of the analytical columns to effectively separate,

resolve, and result in proper peak integration was done visually by overlaying the

chromatograms through the use of EZ-Start 7.3 Chromatography Software

(Shimadzu, Japan). Justification of using the Alltech analytical column in place of

the Shimadzu analytical column will be provided in the results & discussion

section of this thesis.

Figure 3.3. HPLC analytical columns. *Top = Shimadzu RP C_{18} column, dimensions 4.6 mm x 150 mm; bottom = Alltech Platinum RP C_{18} EPS column, dimensions 4.6 mm x 250 mm.*

3.1.1.3   Column temperature

The column oven temperature in the original chromatographic conditions

was set at 40 °C. As part of further optimizing peak separation, column oven

temperature was investigated in the range of 35 °C, 40 °C, 45 °C and 50 °C. A

single sample of green coffee extract diluted 1:800 with MPA was injected at the

above temperatures using the new analytical column, holding all other chromatographic conditions constant. The samples were analyzed at both 280 nm and 330 nm for separation of caffeine and CGA across all temperatures. Peak separation was evaluated visually by overlaying the chromatograms using EZ-Start 7.3 Chromatography Software (Shimadzu, Japan). Determination of the optimal column oven temperature will be discussed in the results & discussion section of this thesis.

3.1.2 Development of a method for the simultaneous determination of caffeine and CGA in green coffee extracts by RP-HPLC

The simultaneous determination of caffeine and 5-CGA was performed using reversed phase High Pressure Liquid Chromatography (RP-HPLC). The HPLC unit (Shimadzu Prominence, CBM-20A), depicted in Figure 3.4 was equipped with an autosampler (SIL-20A), column oven (CTO-20A), and a photo-diode Array (PDA) detector (SPD-M20A). The column employed was a C_{18} EPS silica column with dimensions of 4.6 mm x 250 mm, and particle size of 5 μm (Grace Davison Discovery Sciences). A C_{18}-EPS cartridge system guard column was also used containing the same packing material as the analytical column, with dimensions 7.5 mm x 3 mm (Grace Davison Discovery Sciences). Two mobile phases were mixed by a serial dual-piston pump (LC-20AT). The composition of MPA was 90:10:0.1 (v/v/v) MilliQ Nanopure water/acetonitrile/formic acid while the composition of MPB was 75:25:0.1 (v/v/v) MilliQ Nanopure water/acetonitrile/formic acid. The column was equilibrated at a column oven temperature of 40 °C in MPA for 15 minutes and at a flow rate of 0.60

mL/minute. 50 μL of the sample was injected and the mobile phase was held at 100% A for 3 minutes, and then changed by a linear gradient to 100% B over 30 minutes. The mobile phase was held at 100% B for 7 minutes, and a linear return to 100% A was made over 5 minutes. The mobile phase was held at 100% A for 18 minutes for re-equilibration of the column prior to injecting a new sample. The total analysis run time was 60 minutes. The column was stored and washed in 100% acetonitrile. Caffeine was analyzed at a wavelength of 280 nm while 5-CGA was analyzed at a wavelength of 330 nm. Peak integration was performed using EZ-Start 7.3 Chromatography Software (Shimadzu, Japan). The developed analytical method was subsequently subjected to validation studies, discussed in the next section.

Figure 3.4. Shimadzu Prominence HPLC unit. *A) degassing unit (DGU-20A$_3$); B) pumps (LC-20AT); C) autosampler (SIL-20A); D) communication bus module (CBM-20A); E) photo-diode array detector (SPD-M20A); F) column oven (CTO-20A).*

3.2 HPLC Method validation studies

3.2.1   Linearity

Calibration curves of the analytes, caffeine and 5-CGA, were constructed

using commercial standards purchased from Sigma Aldrich (St. Louis, MO). A

1 mg/mL stock standard solution of each analyte was prepared in deionized (DI)

water. The stock standard was then syringe filtered through a nylon filter (13 mm,

0.45 μm, Fisher Scientific). Serial dilutions of each standard were prepared to

obtain standard concentrations of 0.125, 0.0625, 0.0313, 0.0156, 0.00781 mg/mL.

The standards were analyzed by RP-HPLC according to the newly developed

analytical method mentioned previously. The peak areas were integrated using

EZ-Start 7.3 Chromatography Software (Shimadzu, Japan). A calibration curve was constructed by plotting the peak areas of each dilution of the standard against its known concentration in Excel 2010 (Microsoft).

3.2.2 Precision

Commercial standards of caffeine and 5-CGA were used to prepare a 1 mg/mL stock standard solution. The stock standard was then syringe filtered through a nylon filter (13 mm, 0.45 μm, Fisher Scientific). Serial dilutions in DI water of each standard were prepared to obtain standard concentrations of 0.125, 0.0625 and 0.0313 mg/mL. Each concentration of the caffeine and 5-CGA standards were injected five times, from five separate amber HPLC vials, on a single day to result in 15 total determinations for each analyte. The standards were analyzed by RP-HPLC according to the newly developed analytical method mentioned previously. The peaks of each standard were integrated using EZ-Start 7.3 Chromatography Software (Shimadzu, Japan) and the relative standard deviation (% RSD) of the peak areas were analyzed using Excel 2010 (Microsoft).

3.2.3 Selectivity

Preparation of a control sample containing all but the added bioactive compounds using the same sample procedure is necessary to establish selectivity of a method. A 3.4% (w/v) sodium caseinate control sample was prepared by dissolving 6.8 g of sodium caseinate (lot# ZC0027, 65% protein, Spectrum Chemicals) in 50 mL of DI water while vigorously stirring with a stir bar. The solution was allowed to stir for approximately 1 h, or until all the sodium caseinate had dissolved and resulted in a homogenous solution. An additional 50

mL of DI water was then added to result in a 3.4% (w/v) solution. Procedures for preparing sodium caseinate samples with caffeine and 5-CGA, outlined later, followed the above procedures, except the additional 50 mL of DI water was replaced with a 50 mL solution of caffeine or 5-CGA. Isoelectric precipitation of the sodium caseinate control sample was achieved by adjusting the pH of the sample to pH 4.6 using 0.1 N HCl (Fisher Scientific) at 30 °C. The sample was then centrifuged using an Eppendorf 5810 centrifuge (Hamburg, Germany) at 3,000 rpm, 20 °C for 15 min. The sample supernatant was then syringe filtered through a nylon filter (13 mm, 0.45 μm, Fisher Scientific) and analyzed by RP-HPLC according to the newly developed analytical method mentioned previously.

3.3 Binding interactions of caffeine and 5-CGA with sodium caseinate

3.3.1 Establishing a procedure for binding caffeine to sodium caseinate

Given the limited research on the binding of caffeine and CGA to sodium caseinate, it was necessary to establish a protocol in order to carry out any further experiments. A procedure was established to bind caffeine and 5-CGA to sodium caseinate according to the methodology described by Zimet et al., (2011) with a few modifications. The molar ratio of sodium caseinate to caffeine was 1:1 and the molar ratio of sodium caseinate to 5-CGA was also 1:1.

3.3.1.1 Preparation of caffeine stock standard

A 0.264 mg/mL (1.36×10^{-3} M) caffeine standard (lot#BCBF3955V, HPLC grade, Sigma Aldrich) was prepared in a volumetric flask dissolved in and diluted to volume with DI water. The same lot of caffeine standard was used throughout all experiments to reduce variations in the response.

3.3.1.2   Preparation of sodium caseinate sample

A 6.8% (w/v) solution of sodium caseinate (lot# ZC0027, 65% protein, Spectrum Chemicals) was prepared by slowly adding approximately 34 g of sodium caseinate to a beaker containing 500 mL of DI water while vigorously stirring with a stir bar to create a vortex. This procedure was found to be more effective at dissolving the caseinate powder without adhering to the sides of the beaker. The solution was allowed to stir for approximately 1 h, or until completely dissolved. Then 50 mL aliquots of the sodium caseinate solution was transferred into separate beakers and the combination of the treatment factors pH (4.4, 6.7) and temperature (25 °C, 72 °C) were applied. Samples treated at 25 °C were left at room temperature whereas samples treated at 72 °C were heated on a hot plate. The pH of the samples was then adjusted to pH 4.4 or 6.7 using 0.1 N HCl (Fisher Scientific) or 0.1 N NaOH (Fisher Scientific). All subsequent experiments were performed using the same batch of sodium caseinate to reduce variations in the response. These procedures were also followed for experiments using 5-CGA.

3.3.1.3   Preparation of 1:1 M ratio sodium caseinate to caffeine samples

Caffeine stock standard solution (50 mL) was slowly added dropwise to a 50 mL aliquot of the homogenous 6.8% (w/v) sodium caseinate solution applied with the treatments mentioned above while stirring to prepare a 3.4% (w/v) caseinate sample with caffeine. The resulting sample had a final molar ratio of 1:1 sodium caseinate:caffeine. A control sample of 3.4% (w/v) sodium caseinate was

prepared by adding 50 mL of DI water to a 50 mL aliquot of the 6.8% (w/v)

sodium caseinate solution in a separate beaker while stirring at room temperature.

A caffeine control sample (0.132 mg/mL concentration) was also prepared by

adding 50 mL of DI water to 50 mL of caffeine stock standard at room

temperature. The samples were then allowed to stir for 1 h, with the temperature

of the heated samples maintained between 65–75 °C. At the end of the hour, the

pH of the samples were measured and recorded. All samples were prepared in

duplicate.

3.3.1.4 Analytical fractionation

Isoelectric precipitation of the caseins in the samples was achieved by

heating the samples prepared at 25 °C to 30–35 °C on a hot plate while stirring

and slowly adjusting the pH to 4.6 using 0.1 N HCl or 0.1 N NaOH. Samples

prepared at 72 °C were cooled to 30–35 °C, and then the pH of the samples were

adjusted to pH 4.6 using 0.1 N HCl or 0.1 N NaOH. The entire sample was then

quantitatively transferred into suitable centrifuge bottles and centrifuged at 3,000

rpm, 20 °C for 15 min to obtain a casein pellet. The volume of the resulting

supernatant was measured. These procedures were also followed for experiments

using 5-CGA. All samples were wrapped in foil to protect from light degradation.

3.3.2 Establishing a procedure for binding 5-CGA to sodium caseinate

3.3.2.1 Preparation of 5-CGA stock standard

A 0.482 mg/mL 5-CGA (lot#SLBB6914V, >95% titration, Sigma Aldrich)

standard was prepared in a volumetric flask dissolved in and diluted to volume with DI

water. The same lot of 5-CGA was used throughout all experiments to reduce variations in the response.

3.3.2.2   Preparation of 1:1 M ratio sodium caseinate to 5-CGA samples

5-CGA stock standard solution (50 mL) was added dropwise to a 50 mL aliquot of the homogenous 6.8% (w/v) sodium caseinate solution applied with the treatments mentioned previously while stirring to prepare a 3.4% (w/v) caseinate sample with 5-CGA. The resulting sample had a final molar ratio of 1:1 sodium caseinate:5-CGA. A control sample of 3.4% (w/v) sodium caseinate was prepared by adding 50 mL of DI water to 50 mL of the 6.8% (w/v) sodium caseinate solution in a separate beaker while stirring at room temperature. A 5-CGA control sample (0.241 mg/mL concentration) was also prepared by adding 50 mL of DI water to 50 mL of stock 5-CGA standard at room temperature. The sample was then allowed to stir for 1 h with the temperature of heated samples maintained between 65–75 °C. At the end of the hour the pH of the samples were measured and recorded. All samples were prepared in duplicate.

3.3.2.3   Analytical fractionation

Procedures for isoelectric precipitation of caseins in samples containing 5-CGA followed the same methodology as described previously for isoelectric precipitation of caseins in samples containing caffeine, outlined above.

3.4 Experimental design

A Generalized Randomized Complete Block Design (GRCBD), blocked by day, was employed in a 2^2 full factorial design with two replicates per block.

The treatment factors were pH and temperature, each at two levels (Table 3.7). The experimental unit was a sample of a 1:1 M ratio of sodium caseinate to caffeine or 5-CGA. Treatments were randomly assigned to the experimental unit using Excel 2010 (Microsoft) (Table 3.8 & Table 3.9). All treatments were applied to aliquots of the 6.8% (w/v) sodium caseinate solution prior to adding any caffeine or 5-CGA. The response was the concentration of bioactive compound detected in the supernatant after centrifugation using HPLC.

Table 3.7. Levels of the treatment factors

Treatment	Levels
pH	4.4, 6.7
Temperature	25°C, 72°C

Table 3.8. Randomization of treatments to experimental unit in caffeine samples

Run Order	pH	Temperature (°C)	Block (Day)
1	6.7	25	1
2	6.7	25	1
3	6.7	72	1
4	4.4	25	1
5	4.4	72	1
6	4.4	72	1
7	6.7	72	1
8	4.4	25	1
9	6.7	25	2
10	4.4	72	2
11	6.7	72	2
12	4.4	25	2
13	6.7	72	2
14	6.7	25	2
15	4.4	25	2
16	4.4	72	2

Table 3.9. Randomization of treatments to experimental unit in 5-CGA samples

Run Order	pH	Temperature (°C)	Block (Day)
1	6.7	25	1
2	6.7	72	1
3	6.7	25	1
4	4.4	72	1
5	4.4	25	1
6	4.4	25	1
7	6.7	72	1
8	4.4	72	1
9	4.4	25	2
10	6.7	25	2
11	4.4	72	2
12	6.7	72	2
13	4.4	25	2
14	6.7	72	2
15	6.7	25	2
16	4.4	72	2

3.5 Application of established protocol and optimal treatment conditions on green coffee extract

A permeate extract of green coffee was obtained from the liquid extraction of Arabica green coffee beans from Brazil using water. The extract was passed through a filtration system equipped with a membrane filter with a 5,000 molecular weight cut-off. Lastly, the extract was passed through an ultrafiltration system (GEA RT-12) using the maximum cross flow ratio setting to achieve a flow rate of ~20 L/min. A clear, yellow permeate was obtained and used as the green coffee extract for further experiments (Figure 3.5). A 6.8% (w/v) sodium caseinate solution (50 mL) was prepared as previously described and heated to 72 °C on a hotplate while stirring. The pH of the solution was then adjusted to pH 6.7 using 0.1 N HCl. Then 50 mL of the green coffee extract was then slowly added to the sodium caseinate solution dropwise while stirring. The sample was allowed to stir for 1 h with the temperature maintained between 65–75 °C. The beaker used to contain the sample was wrapped in foil to protect from light degradation. The sample was cooled to 30–35 °C before adding 0.1 N HCl to the isoelectric point (pH 4.6) while stirring. The entire contents of the sample were quantitatively transferred into a suitable centrifuge bottle and centrifuged at 3,000 rpm, 20 °C for 15 min. The volume of the supernatant was measured using a graduated cylinder. The sample supernatant was retained for HPLC and BCA analysis. Each sample was syringe filtered through a nylon filter (13 mm, 0.45 µm, Fisher Scientific) and transferred into amber HPLC vials for analysis. The sample was prepared in triplicate.

Figure 3.5. Permeate of green coffee extract obtained after ultrafiltration.

3.6 Statistical analysis

A Generalized Randomized Complete Block Design (GRCBD) blocked by day was employed in a 2^2 full factorial design performed in duplicate. The treatment factors were pH (4.4, 6.7) and temperature (25 °C, 72 °C). The response was the concentration of caffeine or 5-CGA detected in the supernatant of samples using HPLC. All data analysis was carried out using the statistical software, JMP Pro 10 (SAS Institute Inc.) with significance at $p < 0.01$.

3.7 HPLC analysis of caffeine and 5-CGA

HPLC analysis of caffeine and 5-CGA was performed following the developed method outlined previously in this thesis. All samples were syringe filtered through a nylon filter (13 mm, 0.45 μm, Fisher Scientific) and a 1.0 mL aliquot was transferred into amber HPLC vials for analysis. Samples were analyzed in the randomized order previously determined for sample preparation

(see Tables 3.8 and 3.9). All samples were analyzed on the same day of preparation to minimize any degradation of the bioactive compound. Peak integration was performed using EZ-Start 7.3 Chromatography Software (Shimadzu, Japan). All data was collected and analyzed using Excel 2010 (Microsoft). The concentration of caffeine and 5-CGA in the samples was calculated through the use of standard calibration curves of each compound prepared at known concentrations.

3.8 BCA protein assay

Bicinchoninic acid (BCA) analysis was performed following instructions provided by the Pierce BCA^{TM} Protein Assay Kit (No. 23227, Rockford, IL). A standard curve was prepared using Bovine Serum Albumin (BSA) (Product No. 23209, 2 mg/mL concentration) standard in 0.9% saline and 0.05% sodium azide in the concentration range of 2.0–0.025 mg/mL in DI water. DI water was also used as the blank. The working reagent (WR) was prepared by mixing Reagent A (Product No. 23228, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) with Reagent B (Product No. 1859078, containing 4% cupric sulfate) in a 50:1 ratio, respectively.

Samples were randomly selected from each treatment combination of caffeine and 5-CGA with sodium caseinate samples for BCA analysis (Table 3.10), along with the sodium caseinate solution (6.8% (w/v)) and sodium caseinate control (3.4% (w/v)) containing neither caffeine nor 5-CGA. The sodium caseinate solution was diluted three times (1:25, 1:50 and 1:100) using DI

water to ensure a measurable absorbance was obtained. All other samples were analyzed without any further dilution. A 1:20 sample:WR ratio was used for the preparation of BCA samples. 2.0 mL of WR was added into separate 15 mL Falcon tubes. An aliquot of 100 µL of each standard and sample was then added separately to the tubes, capped and mixed well. The samples were incubated at 37 °C for 30 minutes, cooled to room temperature, and measured using a SpectraMax Plus spectrophotometer (Sunnyvale, CA) at 562 nm within 10 minutes. All samples were analyzed in duplicate. The absorbance of the blank solution was subtracted from the absorbance of each standard and sample prior to analyzing the data in Excel 2010 (Microsoft).

Table 3.10. Randomly selected samples and their treatment combinations

Sample	pH	Temperature (°C)
Sodium caseinate & caffeine	6.7	25
	6.7	72
	4.4	25
	4.4	72
Sodium caseinate & 5-CGA	6.7	25
	6.7	72
	4.4	25
	4.4	72

4.0 RESULTS AND DISCUSSION

4.1 Development of a method for the simultaneous determination of caffeine and CGA in green coffee extracts by RP-HPLC

4.1.1 Determination of HPLC chromatographic conditions

The principal objective of these experiments was to develop an HPLC method for the quantification of caffeine and 5-CGA in green coffee extracts. Eventually, this method would be extended to sodium caseinate samples containing both these analytes. Caffeine and 5-CGA are both of relatively low molecular weights (< 2000) and are both soluble in the mobile phases employed indicating that RP-HPLC is a suitable choice for quantification. Three main parameters of the chromatographic conditions evaluated for optimization were: gradient profile, analytical column, and column temperature. Optimization within each of these conditions resulted in the final developed RP-HPLC method.

4.1.1.1   Gradient profile

Four variations of the original gradient profile were evaluated for the purpose of separating caffeine and its co-eluting peak, in addition to reducing total run time. While only changing the gradient profile did not result in successful separation of the caffeine peak, the total run time was decreased from 72 min to 60 min (Figure 4.1). Eluent strength decreases with an increase in polarity of the mobile phase. Thus the reduction in time to reach 100% solvent B in the final gradient profile provided better separation of the caffeine and co-eluting peak when combined with optimization to the column and column temperature. In addition, a closer inspection of Figure 4.1 shows a slight increase

in peak sharpness was observed in the final gradient profile when compared to the original (Figure 4.2). The other variations of the gradient profiles B–E resulted in only slight changes from the original gradient profile (chromatograms not shown).

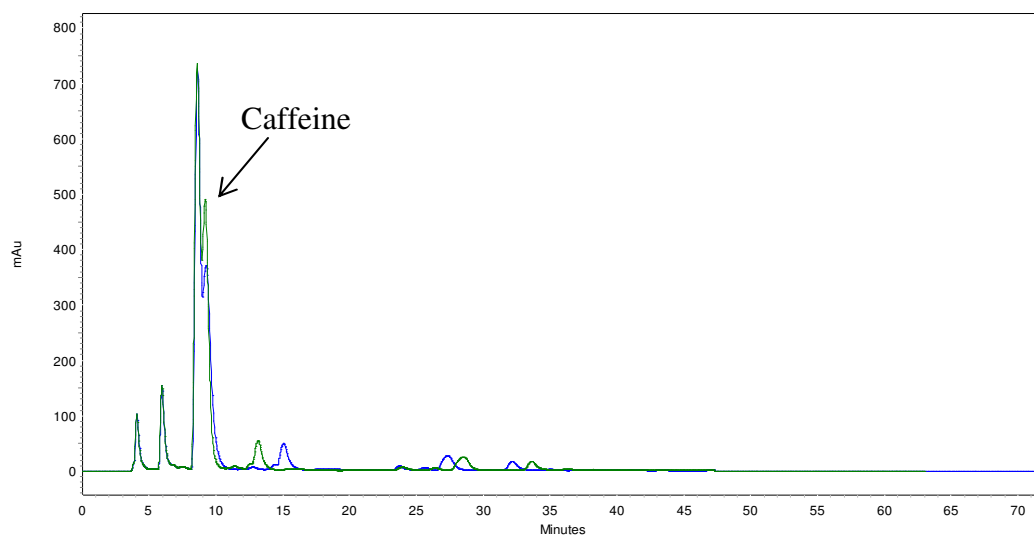

Figure 4.1. Overlay of green coffee extract. *Sample diluted 1:50 with MPA analyzed at 280 nm. The blue trace is the original gradient profile with total run time 72 min, the green trace is the new gradient profile with total run time 60 min.*
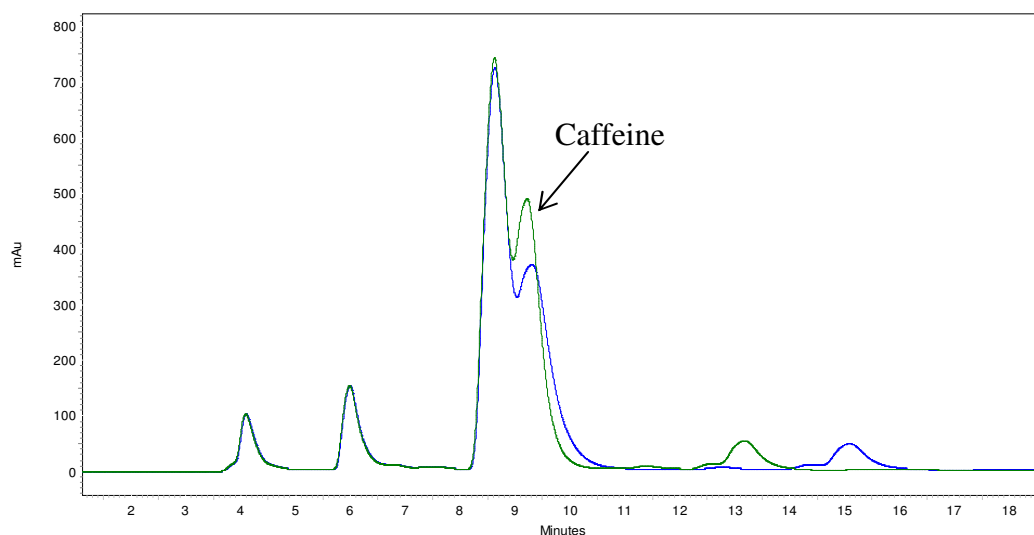

Figure 4.2. Slight peak sharpening observed in caffeine peak using new gradient profile (green trace) vs. old gradient profile (blue trace).

4.1.1.2   Analytical column

The next parameter evaluated was the analytical column. The packing material in both columns was composed of a bonded silica surface covalently attached with octadecyl (C_{18}) chains, resulting in a nonpolar stationary phase. A nonpolar stationary phase used in RP-HPLC concurrent with gradient elution provides rapid elution of polar compounds carried via a polar mobile phase and slower elution of nonpolar compounds that have a greater affinity to interact with the nonpolar bonded silica surface. Therefore, increasing the length of the nonpolar stationary phase results in enhanced separation of polar and nonpolar (or slightly less polar) compounds by increasing interactions sites available to the nonpolar analyte. An increase of just 100 mm of the column length from the Shimadzu C_{18} RP column (length 150 mm) to the Alltech Platinum C_{18} RP-EPS column (length 250 mm), as well as the difference in packing material of the columns provided superior separation of the caffeine peak (Figure 4.3).

Figure 4.3. Overlay of green coffee extract (diluted 1:400 with MPA) using Shimadzu (green trace) and Alltech column (blue trace) for caffeine analysis.

4.1.1.3   Column temperature

Control of column temperature in HPLC is essential for improved retention time reproducibility and precision of a quantitative analysis. Increases in column temperature provide a decrease in retention time and improves baseline resolution by accelerating diffusion of solutes. However, increases in column temperature generally also results in quicker degradation of the stationary phase thereby decreasing column lifetime. Therefore, it is imperative to determine the optimal column temperature for adequate elution and resolution of an analyte that will also result in greatest column lifetime. Four column temperatures: 35 °C, 40 °C, 45 °C, and 50 °C were investigated in an attempt to optimize elution time of the caffeine and 5-CGA peaks concurrent with the optimized gradient profile and updated selection of the analytical column (Figure 4.4 & Figure 4.5).

Figure 4.4. Overlay of caffeine peak at 280 nm. *From left to right: caffeine peak measured at 50 °C, 45 °C, 40 °C, and 35 °C.*

Figure 4.5. Overlay of 5-CGA peak at 330 nm. *From left to right: CGA pkea measured at 50 °C, 45 °C, 40 °C, and 35 °C.*

Elution of the caffeine peak was observed to decrease from approximately RT = 27 min at 35 °C to approximately RT = 22 min at 50 °C, a total reduction of 5 minutes in retention time. A slight general increase in peak sharpness and resolution was also observed with an increase in column temperature. In addition, improved peak separation was observed in an unknown peak containing a shoulder at approximately RT = 2.5 min at 50 °C compared with the same sample injected at 40 °C (Figure 4.6). However, as mentioned previously, there must be a balance between retention time and column lifetime. Therefore, column temperature at 40 °C was determined the optimal temperature that provided adequate peak elution and resolution without subjecting the column to severe degradation at the higher temperatures investigated.

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Figure 4.6. Separation of unknown peak with shoulder at 50°C (fully resolved) compared to 40°C (co-eluting) at 330 nm.

4.2 HPLC Method Validation

4.2.1 Linearity

Linearity is the test of a method's ability to quantitatively analyze a sample and obtain results that are directly proportional to a known concentration of the analyte within that sample. Many factors such as age of the detector, adsorption of the analyte to the surface of the sample vial, degradation of the sample upon sitting, and phase separation of the sample can lead to under or over estimation of the amount of the analyte in a sample and can result in nonlinear results. Therefore, the importance of verifying the linearity of a method is a crucial step in method development.

The linearity study was carried out by preparing a stock caffeine and 5-CGA standard solution of high concentration (1 mg/mL) and performing serial dilutions using a micropipette to a known concentration range resulting in five standards (Table 4.1). A graph of peak area vs. concentration was plotted and a

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least squares fit regression line was constructed (Figure 4.7 and Figure 4.8). A

correlation coefficient $R^2 \geq 0.980$ is considered an acceptable result for linearity.

A correlation coefficient $R^2 < 0.980$ indicates nonlinearity of the method. The R^2

value for caffeine and 5-CGA was 0.9992 and 0.9995, respectively. This

demonstrates linearity of the developed RP-HPLC method.

Table 4.1. Concentration range and peak areas of caffeine and 5-CGA used in linearity study

Dilution	Concentration (mg/mL)	Caffeine peak area	5-CGA peak area
1:8	0.125	21909584	27548226
1:16	0.0625	11563744	13116460
1:32	0.0313	5869057	6249741
1:64	0.0156	2946908	2993404
1:128	0.00781	1474020	1441236

Figure 4.7. Graph of caffeine peak area (mAU) vs. concentration (mg/mL) in linearity study. $R^2 = 0.9992$.

Figure 4.8. Graph of 5-CGA peak area (mAU) vs. concentration (mg/mL) in linearity study. $R^2 = 0.9995$.

4.2.2 Precision

Precision is a measure of the closeness of agreement (degree of scatter) between a series of measurements of the same homogeneous sample analyzed at set chromatographic conditions. Intra-assay precision, or within-day precision, was evaluated for the precision study. Five separate injections of three standard concentrations of caffeine and 5-CGA were each measured within the same day (Figure 4.9). The relative standard deviation (% RSD) between the five injections of each standard concentration was calculated to determine the precision of the method. Acceptable precision ranges for HPLC methods are between <2–10%, with <2% RSD being a method with great precision, <5% RSD being a method with good precision, and <10% RSD being a method with tolerable precision. An RSD value >10% indicates a method with poor precision. The average RSD was <1% and <2% for caffeine and 5-CGA, respectively. These results indicate great precision of the developed method (Appendix A).

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Figure 4.9. Overlay of caffeine standard at three concentrations. *Five injections for each sample, RSD < 1% for all concentrations. Note chromatogram zoomed in to show time between 17–28 min only (total run time 60 min).*

4.2.3 Selectivity

Selectivity of a method is the measure of a method's ability to selectively detect the analyte from interferences present in the sample matrix. This is achieved through analyzing a sample containing the analyte and a sample containing only the sample matrix (the sample blank). A lack of response or detection in the sample matrix indicates a method's selectivity for the analyte. A sample of 1:1 M ratio sodium caseinate:caffeine and 1:1 M ratio sodium caseinate:5-CGA was analyzed along with the sodium caseinate control sample (the sample blank) containing only the sample matrix. Selectivity of the method was determined through a visual comparison of the chromatograms. The method correctly identified and quantified caffeine (Figure 4.10) and 5-CGA (Figure 4.11) in each sample but did not detect any peaks in the sample matrix at 280 nm or 330 nm (Figure 4.12 & Figure 4.13). This indicates the developed method is

selective both for caffeine and 5-CGA in the presence of a sodium caseinate

matrix.

Figure 4.10. Sample chromatogram of 1:1 M ratio sodium caseinate:caffeine. *Method correctly identified caffeine peak at RT = 21.5 min.*

Figure 4.11. Sample chromatogram of 1:1 M ratio sodium caseinate:5-CGA. *Note: peaks 3-CGA (RT ~13.3 min) and 4-CGA (RT ~24.5 min) are known degradation products of 5-CGA. Undetected peaks are labeled in parentheses at their retention times. Method correctly identified 5-CGA peak at RT = 15.5 min.*

Figure 4.12. Sodium caseinate matrix (sample blank) measured at 280 nm. *Undetected peaks are labeled with parenthesis at their retention time.*

Figure 4.13. Sodium caseinate matrix (sample blank) measured at 330 nm. *Undetected peaks are labeled with parenthesis at their retention time.*

4.3 Experimental Design

A Generalized Randomized Complete Block Design (GRCBD), blocked by day, was used as the design structure for these experiments. Blocked designs are used to control a nuisance factor; a known extraneous source of variation other than the treatment factors that affect the response. In these experiments, the day the experiments were performed was expected to have some effect on the response, therefore it was chosen as the random blocking factor. Complete block designs contain each treatment randomly assigned to at least one experimental

unit in each block, and do not have any replication within blocks. A GRCBD was employed because all samples were prepared in duplicate, therefore generalized RCBDs allow for replication within blocks. RCBDs reduce variability of treatment comparisons by allowing them to be made on more similar units with a block, thus ensuring more accurate results. The GRCBD model equation is shown in Equation 4.1.

Equation 4.1 GRCBD model equation

$$y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_k + \varepsilon_{ijkl}$$

Where: y_{ijkl} = the quantitative response, concentration of bioactive compound at the ith and j^{th} treatment levels, k^{th} block and l^{th} replicate

μ = mean concentration of bioactive compound

α_i = main effect of the ith pH treatment level

β_j = main effect of the jth temperature treatment level

$(\alpha\beta)_{ij}$ = interaction effect of the ith pH and j^{th} temperature treatment levels

γ_k = block effect from the kth block

ε_{ijkl} = random error term, we assume $\varepsilon_{ijkl} \sim IN(0, \sigma^2)$

A two-series 2^k factorial design with k factor level combinations is often employed during screening experiments. Screening experiments attempt to identify which factors, out of k factors, significantly affect the response. Generally, levels of a factor are considered "low" and "high" to observe effects of the treatments on the response using extreme values in both directions. If the

objective of the investigator is to optimize treatment conditions that significantly

affect the response, more experiments can be redesigned holding insignificant

factors constant while varying significant factors at more levels. The model

equation for a two-series 2^k design is shown in Equation 4.2. For 2^k designs, each

model term has 1 degree of freedom (df). The df for treatment factors used in

these experiments are shown in Table 4.2.

Equation 4.2 Model equation for a two-series 2^k factorial design

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

Where: y_{ijk} = the quantitative response, concentration of bioactive

compound at the ith and j^{th} treatment levels and k^{th} replicate

μ = mean concentration of bioactive compound

α_i = main effect of the ith pH treatment level

β_j = main effect of the jth temperature treatment level

$(\alpha\beta)_{ij}$ = interaction effect of the ith pH and j^{th} temperature treatment

levels

ε_{ijk} = random error term, we assume $\varepsilon_{ijk} \sim IN(0, \sigma^2)$

Table 4.2. ANOVA table and df of treatment levels

Source	df
pH	1
Temperature	1
pH*Temperature	1
Block (day)	1
Error	11
Total	15

4.4 Binding caffeine & 5-CGA to sodium caseinate

4.4.1 Binding caffeine to sodium caseinate trials

To estimate the amount of binding that occurred between sodium caseinate and caffeine, a sample of the supernatant from each treatment combination was analyzed by RP-HPLC on the same day they were prepared. The concentration of caffeine recovered in the supernatant of each sample was calculated using a caffeine calibration curve, constructed prior to the sample analysis using a least squares regression line (Figure 4.14). The final caffeine concentration detected in the supernatant of each sample is shown in Figure 4.15. The concentration of caffeine was then converted to the percent of caffeine recovered from the known amount that was added to each sample, with the caffeine control sample containing 100% recovery of the added caffeine. The percentage of caffeine that was bound to sodium caseinate was then estimated based on the calculated difference between the initial amount of caffeine added and the amount of that was recovered in the supernatant. Therefore, the lesser amount of caffeine detected in the supernatant suggests more binding had occurred with the sodium caseinate.

Figure 4.14. Caffeine calibration curve of peak area (mAU) vs. concentration (mg/mL). $R^2 = 0.9924$.

Figure 4.15. Concentration of caffeine detected in the supernatant of all samples prepared on Day 1 and Day 2. *Caffeine control represents a caffeine standard solution with no sodium caseinate. Error bars represent standard error of each sample.*

The concentration of caffeine detected in the supernatant of each sample was analyzed with an overall significance level at $\alpha_E = 0.05$. A Bonferroni adjustment was performed to control Experimentwise (EW) error rate, thereby decreasing the rate of Type 1 error and resulting in an individual significance

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level of $\alpha_I = 0.0167$. There was not enough evidence to conclude that there was

any significant interaction effect of pH and temperature on the true mean

concentration of caffeine recovered in the samples ($p = 0.5597$). Since there was

no significant interaction effect ($p > 0.01$), interpretation of the main effects of pH

and temperature may proceed. There was also not enough evidence to conclude

that the main effect of pH had a significant effect on the true mean concentration

of caffeine recovered in the samples ($p = 0.8983$), however the main effect of

temperature did have a significant effect on the true mean concentration of

caffeine recovered in the samples ($p = 0.0008$). Samples prepared at 72 °C

contained significantly less caffeine than samples prepared at 25 °C, an average of

0.011 mg/mL less which corresponds to a calculated percentage of approximately

9% more caffeine bound at 72 °C than at 25 °C (Figure 4.16).

Figure 4.16. Least square means of caffeine concentration in supernatant of
samples prepared at 25 °C and 72 °C.

With 95% overall confidence, the effect of temperature at 72 °C on the

mean caffeine concentration is between 0.00418 mg/mL and 0.01765 mg/mL less

than the effect of temperature at 25 °C on the mean caffeine concentration detected in the sample supernatant (Appendix B). The percent recovery of caffeine in samples prepared at 72 °C ranged between 65–80%, whereas samples prepared at 25 °C had a percent caffeine recovery ranging from 79–84%. Therefore, a calculated percentage of approximately 20–35% of the added caffeine was estimated to have been bound to sodium caseinate in samples prepared at 72 °C. This corresponds to a concentration of caffeine between 0.0303–0.0523 mg/mL with a calculated average of 0.0413 mg/mL caffeine bound to sodium caseinate. This is compared to a calculated percentage of approximately 16–21% caffeine bound in samples prepared at 25 °C. This corresponds to a concentration of caffeine bound to sodium caseinate between 0.0231–0.0319 mg/mL with a calculated average of 0.0275 mg/mL caffeine bound to sodium caseinate at 25 °C (Figure 4.17). Therefore, it was determined that 72 °C was the best of the two temperatures investigated that had a significant effect on the amount of caffeine recovered in the sample supernatant and thus also had a significant effect on the extent of binding between caffeine and sodium caseinate. The data was also analyzed for any violations of the ANOVA model assumptions (normality, equal variances). Because the experimental design was balanced, no violations of the model assumptions were detected (Appendix D).

Figure 4.17. Calculated average concentration of caffeine bound to sodium caseinate at 25 °C and 72 °C. *Caffeine control represents the average theoretical amount of caffeine bound to sodium caseinate at 100%.*

Temperature was the only treatment that had a significant effect on the concentration of caffeine detected in the samples. The results from these experiments have also shown that optimal binding interactions between caffeine and sodium caseinate is temperature dependent, with a higher temperature resulting in greater binding. This suggests that the most likely mechanism for binding caffeine to sodium caseinate is via hydrophobic interactions. Hydrophobic interactions are highly temperature dependent, and because pH was not observed to have a significant effect on caffeine concentration, the binding mechanism was not likely due to electrostatic interactions. Therefore, conformational changes to the casein structure, such as denaturation of the protein at high temperatures, may increase binding sites available to caffeine.

4.4.2 Binding 5-CGA to sodium caseinate trials

The estimated amount of 5-CGA that was bound to sodium caseinate was measured in the same way as previously described with caffeine samples. A calibration curve of 5-CGA was also constructed prior to any sample analysis and used to calculate the concentration of 5-CGA detected in sample supernatants (Figure 4.18). The final 5-CGA concentration detected in the supernatant of each sample is shown in Figure 4.19.

Figure 4.18. 5-CGA calibration curve of peak area (mAU) vs. concentration (mg/mL) at 330 nm. $R^2 = 0.9958$.

Figure 4.19. Concentration of 5-CGA detected in the supernatant of all samples prepared on Day 1 and Day 2. *5-CGA control represents a 5-CGA standard solution with no sodium caseinate. Error bars represent standard error of each sample.*

At an individual significance level of $\alpha_I = 0.0167$, there was enough evidence to conclude that there was a significant interaction effect of pH and temperature on the true mean 5-CGA concentration detected in the supernatant of samples ($p = 0.0066$). Though the main effect of temperature also had a significant effect on the true mean 5-CGA concentration ($p < 0.0001$), main effects should not be interpreted if interaction effects are significant. Therefore, the remainder of the statistical analysis will only focus on the interaction effect of pH and temperature. There was not enough evidence to conclude that the main effect of pH had a significant effect on the true mean 5-CGA concentration detected in the sample supernatant ($p = 0.2631$).

A post-hoc analysis, Tukey's Honestly Significant Difference (HSD) pairwise comparison was performed after completion of the ANOVA statistical analysis to determine which treatment combinations of pH and temperature

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resulted in significantly different and increased binding interactions of 5-CGA to sodium caseinate (Appendix C). The treatment combinations pH 6.7 and 72 °C resulted in the lowest average 5-CGA concentration detected in the sample supernatant and was thus the best of the investigated conditions for binding 5-CGA to sodium caseinate. The percentage of 5-CGA recovered in the sample supernatants at pH 6.7 and 72 °C ranged between 46–52%, therefore the calculated percentage of 5-CGA bound to sodium caseinate was estimated to be between 48–54% of the original amount of 5-CGA added. This corresponds to a calculated concentration of 5-CGA bound to sodium caseinate between 0.1159–0.1311 mg/mL with a calculated average of 0.1235 mg/mL 5-CGA bound. Samples treated at pH 6.7 and 25 °C had a calculated concentration of 5-CGA bound to sodium caseinate that ranged between 0.0779–0.0903 mg/mL resulting in a calculated average of 0.0841 mg/mL 5-CGA bound (Figure 4.20). Compared to samples treated at pH 6.7 and 25 °C which resulted in a 5-CGA percent recovery ranging from 62–68%, the treatment conditions at pH 6.7 and 72 °C resulted in a calculated average increase of approximately 16.5%, or approximately 0.0437 mg/mL more 5-CGA bound to sodium caseinate. This data was also analyzed for any violations of the ANOVA model assumptions (normality, equal variances). Because the experimental design was balanced, no violations of the model assumptions were detected. (Appendix E).

Figure 4.20. Calculated average concentration of 5-CGA bound to sodium caseinate at 25 °C and 72 °C, each at pH 6.7. *5-CGA control represents the average theoretical amount of 5-CGA bound to sodium caseinate at 100%.*

The results from these experiments show that the treatment combination of pH at 6.7 and temperature at 72 °C provided the best treatment combination for binding 5-CGA to sodium caseinate of all treatments investigated. This suggests that casein with a net negative charge at pH 6.7 and some extent of protein denaturation at 72 °C may increase binding sites available for 5-CGA. Both hydrophobic and electrostatic interactions are likely to be the mechanism of binding for 5-CGA to sodium caseinate as both pH and temperature had some influence on binding behavior.

4.4.3 Binding caffeine & 5-CGA present in green coffee extract to sodium caseinate trials

After determining the optimal conditions of the investigated treatments for binding pure standards of caffeine and 5-CGA to sodium caseinate, we were interested to see whether these conditions would result in a similar extent of

binding from a sample of green coffee extract that naturally contained both these compounds. The green coffee extract also contained numerous other unidentified compounds, thus this trial would demonstrate any interferences or competition of binding to sodium caseinate.

Immediately upon addition of the green coffee extract to the sodium caseinate solution, the color of the solution turned from opaque white to dark gray-green. The color of the solution continued to develop while being stirred for 1 h, turning into a dark emerald green color (Figure 4.21). During isoelectric precipitation with 0.1 N HCl, the sample solution returned to a bright white color and once the isoelectric point was reached (pH 4.6) the supernatant turned to a clear, light green color (Figure 4.22). The precipitated caseins contained a greenish hue in comparison to its usual bright white color. This indicated that some compounds responsible for the color of green coffee had been bound to sodium caseinate, possibly including 5-CGA as samples containing pure 5-CGA in previous studies resulted in a similar color change. Once the green coffee casein pellet was dried and compared to a control casein pellet, a noticeable green color remained in the green coffee pellet while no color change was observed in the control (Figure 4.23).

Figure 4.21. Sodium caseinate solution before addition of green coffee extract (left) and after addition of green coffee extract (right).

Figure 4.22. Sample of sodium caseinate bound with green coffee extract after isoelectric precipitation at pH 4.6 and 30 °C.

Figure 4.23. Casein pellets after centrifugation. *Sodium caseinate control pellet (left) and sodium caseinate with green coffee extract pellet (right).*

The concentration of caffeine and 5-CGA detected in the supernatant of sodium caseinate with green coffee extract samples was comparable to results using commercial standards. Each sample was prepared in triplicate (Figure 4.24 and Figure 4.25). The average percentage of caffeine bound to sodium caseinate was 16.51% (±0.04) corresponding to a calculated average concentration of 0.1605 mg/mL caffeine bound from the total caffeine concentration of 0.9722 mg/mL in the pure green coffee extract. The average percentage of CGA bound to sodium caseinate was 47.90% (±0.04), corresponding to a calculated average concentration of 2.098 mg/mL CGA bound from the total CGA concentration of 4.379 mg/mL in the pure green coffee extract (Figure 4.26). These results confirmed the optimal binding conditions determined for each respective bioactive compound in previous studies using commercial standards. However, it is important to note that results from these experiments represent only an approximation, a calculated average concentration of the bioactive compound estimated to have been bound to sodium caseinate. Without direct measurement of

the casein pellet for the concentration of bioactive compound actually bound, there is no way of definitively assessing the true concentration of the bound material.

There appeared to be no detectable interfering compounds in the green coffee extract that inhibited binding of caffeine or 5-CGA. A combination of hydrophobic and electrostatic interactions is also likely to be the binding mechanism between caffeine and 5-CGA to sodium caseinate from green coffee extracts.

Figure 4.24. Concentration of caffeine detected in the supernatant of green coffee extract with sodium caseinate samples. *Error bars represent standard deviation of each sample.*

Figure 4.25. Concentration of 5-CGA detected in the supernatant of green coffee extract with sodium caseinate samples. *Error bars represent standard deviation of each sample.*

Figure 4.26. Calculated average concentration of caffeine and total CGA bound to sodium caseinate from green coffee extract at optimal treatment conditions pH 6.7 & 72 °C. *Controls represent the amount of bioactive compound detected in a pure green coffee extract sample.*

4.5 HPLC trials

The concentration of caffeine and 5-CGA bound to sodium caseinate was

determined through calculating a difference between the added concentration of

bioactive compound in a sample and the concentration recovered in the supernatant through HPLC analysis. A commercial standard of caffeine was injected and measured at 280 nm to determine the retention time of caffeine (Figure 4.27). Likewise, a commercial standard of 5-CGA was injected and measured at 330 nm to determine the retention time of 5-CGA (Figure 4.28). Green coffee extract samples contained CGA isomers with known retention times from previous studies. Retention times of all compounds are summarized in Table 4.3.

Figure 4.27. Caffeine standard (1:16 dilution) measured at 280 nm.

Figure 4.28. CGA standard (1:16 dilution) measured at 330 nm.

Table 4.3. Retention times and relative retention times of caffeine and CGA isomers

Analyte	Retention Time (min)	Relative Retention Time (min)
Caffeine	21.4	1.000
3-CGA	13.2	0.617
4-CGA	23.2	1.080
5-CGA	15.5	1.000
3-FQA	29.4	1.900
4-FQA	30.9	1.990
5-FQA	34.7	2.240

In sodium caseinate samples containing caffeine, a single caffeine peak was observed at approximately RT = 21.5 min, with no other impurity or degradation peaks observed in the chromatogram (Figure 4.29). In sodium caseinate samples containing 5-CGA, a large 5-CGA peak was observed at approximately RT = 15.5 min, along with two minor peaks constituting 3-CGA (RT = 13.2 min) and 4-CGA (RT = 23.2 min) that are likely due to degradation of CGA upon heating (Figure 4.30). It is important to note that degradation compounds were mostly observed in samples treated at 72 °C which is consistent with the behavior of CGAs upon heating.

Figure 4.29. Sample chromatogram of 1:1 M sodium caseinate:caffeine sample (pH 6.7, 72 °C).

Figure 4.30. Sample chromatogram of 1:1 M sodium caseinate:5-CGA sample (pH 6.7, 72 °C). *Unknown peak detected at RT ~ 9.5 min. Undetected peaks are labeled in parentheses.*

In green coffee extract samples, caffeine was observed at the expected RT, but the sample also contained many other peaks with a comparable peak height to caffeine that were not observed in the blank or sodium caseinate control and were thus not identified (Figure 4.31). These peaks were also not integrated nor used in the data analysis. 3-CGA, 4-CGA and 5-CGA isomers were observed in larger quantities in the green coffee extract samples with sodium caseinate. The peak areas of all CGA isomers were added together to quantify the total CGA concentration in samples. A sample of the pure green coffee extract with no sodium caseinate afforded all observable CGA isomers (Figure 4.32).

Figure 4.31. Sodium caseinate and green coffee extract sample at 280nm (pH 6.7, 72 °C, rep 3).

Figure 4.32. Sample chromatogram of green coffee extract only measured at 330 nm.

4.6 BCA trials

BCA trials were performed to confirm the absence of interference from soluble proteins remaining in the sample supernatant. The concentration of protein present in each sample was calculated using a standard curve of BSA, constructed prior to sample analysis (Figure 4.33). The absorbance of the blank solution was subtracted from the absorbance of each standard and sample to calculate the final protein concentration. The amount of protein from each sample ranged between 1–6% of the original sodium caseinate solution and was thus not

considered to be an interfering substance during caffeine or 5-CGA quantification

(Table 4.4 and Table 4.5). In addition, a sodium dodecyl sulfate polyacrylamide

gel electrophoresis (SDS-PAGE) analysis was run to qualitatively assess the

amount of impurities (i.e. whey proteins) present in the original sodium caseinate

powder used. The SDS-PAGE eletrophoretogram depicted in Figure 4.34 shows

sodium caseinate with various dilutions that contained impurities present in the

powder at relatively low intensities compared to sodium caseinate. This

qualitative assessment showed any impurities or soluble proteins that remained in

the supernatant after isoelectric precipitation of the caseins did not total to a

significant amount and thus would not affect any interpretation of the data from

these experiments.

Figure 4.33. BSA standard curve of absorbance at 562 nm vs. concentration
(mg/mL). $R^2 = 0.9980.$

Table 4.4. BCA protein assay of 1:1 M sodium caseinate:caffeine samples

Repetition	pH	Temperature (°C)	Concentration (mg/mL)	Protein (%)
1	6.7	25	0.2756	1.01 ± 0.00
2	6.7	25	0.3018	1.11 ± 0.00
1	6.7	72	0.3790	1.39 ± 0.00
2	6.7	72	0.3671	1.35 ± 0.00
1	4.4	25	0.2566	0.94 ± 0.00
2	4.4	25	0.3077	1.13 ± 0.00
1	4.4	72	0.3006	1.10 ± 0.00
2	4.4	72	0.3434	1.26 ± 0.00

Table 4.5. BCA protein assay of 1:1 M sodium caseinate:5-CGA samples

Repetition	pH	Temperature (°C)	Concentration (mg/mL)	Protein (%)
1	6.7	25	1.3120	4.82 ± 0.00
2	6.7	25	1.2894	4.74 ± 0.00
1	6.7	72	1.5319	5.63 ± 0.00
2	6.7	72	1.5461	5.68 ± 0.00
1	4.4	25	1.2133	4.46 ± 0.00
2	4.4	25	1.2407	4.56 ± 0.00
1	4.4	72	1.4035	5.16 ± 0.00
2	4.4	72	1.4510	5.33 ± 0.00

Figure 4.34. SDS-PAGE eletrophoretogram of sodium caseinate with various dilutions used in all experiments. *Lane 1 = most dilute sample of sodium caseinate; lane 6 = least dilute sample of sodium caseinate. Impurities become less evident in diluted samples. The level of intensity of impurities in all lanes is markedly less than that of sodium caseinate. The far right lane depicts the molecular marker (ladder).*

5.0 CONCLUSIONS

Rates of coffee consumption worldwide continue to increase due to the uniquely satisfying flavor of coffee. Though most coffee consumers may not be aware of it, the bioactive compounds in coffee actually serve many important physiological functions in maintaining overall health. These benefits include hepatoprotective, hypoglycemic, and antiviral activity. Clearly, these health benefits warrant further investigation into exploiting their beneficial activities and imparting them from foods other than from coffee. Milk proteins have been remarkable at adapting to their evolutionary task of binding and transporting vital micronutrients from the mother to the neonate, thus the binding capacity of casein to caffeine and CGA and its use as a bioactive transport vehicle was the primary objective of this thesis. However, a procedure was first established to bind caffeine and CGA to sodium caseinate by determining the optimal chemical environment (pH and temperature) that would result in increased binding interactions based on the investigated treatment conditions. The steps in the developed procedure are both simple to carry out and relatively efficient thus providing a protocol that is neither time consuming nor labor intensive. The main effect of temperature was shown to have a significant effect on the concentration of caffeine detected in the supernatant and therefore the concentration that remained bound to sodium caseinate ($p < 0.01$). This suggests that binding of caffeine to sodium caseinate is likely via hydrophobic interactions. Conversely, the interaction effect of pH and temperature was shown to have a significant effect on the concentration of CGA detected in the supernatant and therefore the

concentration that remained bound to sodium caseinate ($p < 0.01$). This suggests the binding mechanism for CGA is likely a result of both electrostatic and hydrophobic interactions with sodium caseinate. The main effect of pH alone was not found to be statistically significant ($p > 0.01$) for either caffeine or CGA, indicating that electrostatic interactions alone are not likely to contribute to the binding mechanism. However, the main effect of pH may not have been statistically significant due to the fact that all samples were standardized to a final pH at 4.6 to precipitate the caseins. This may affect any reversible binding events that originally occurred at the initial investigated levels of pH. Essentially, the binding procedures provided herein may not have sufficiently shown the true effects of pH due to the inability to measure binding that occurred at the investigated levels of pH but rather, these procedures allowed only for binding measurements after isoelectric precipitation of the caseins, and therefore an estimated amount of binding that occurred at the final pH of 4.6.

The difference in binding mechanisms between caffeine and 5-CGA to sodium caseinate could be attributed to the fundamental differences in their chemical structure. Compared to caffeine, 5-CGA contains a carboxylic acid (–COOH) functional group which can remain protonated or easily deprotonated by being in a chemical environment with a pH greater than its pKa (~3.5). Because the pKa of 5-CGA is relatively low, changes in its ionic state can be easily influenced by a smaller change in the pH of its chemical environment and thus influence binding with other molecules by electrostatic interactions. Conversely, the pKa of protonated amines are ~14 thus much larger changes to

the pH of its chemical environment are needed to influence electrostatic interactions. The small difference in electronegativity between the bonded atoms in the caffeine molecule makes it a relatively nonpolar compound. Thus it is plausible that caffeine binds to hydrophobic regions of the casein micelle once they have been exposed upon denaturation at higher temperatures. The prevalent binding mechanism between caffeine and the nonpolar regions of sodium caseinate are thus likely via hydrophobic interactions.

Application of the optimal experimental conditions on a sample of green coffee extract which contained naturally occurring caffeine and CGA was performed to verify comparable binding capacity and reproducibility of the method as sodium caseinate with commercial standards of the bioactive compounds. Results from these experiments were in good agreement with the results from binding commercial standards of caffeine and 5-CGA to sodium caseinate. Simultaneous determination of caffeine and 5-CGA was achieved through the development, optimization, and validation of an analytical procedure by RP-HPLC. This method used common reagents that are readily accessible by most laboratories and provided a sensitive method for accurately quantifying both caffeine and CGA through demonstrating good results from validation studies that included: linearity, intra-assay precision, and selectivity.

Results from these experiments provide but a modest contribution to the immense potential applications of coffee bioactive compounds in dairy products. Indeed, the procedures provided herein for binding caffeine and 5-CGA to sodium caseinate serves as a preliminary starting point for many more research endeavors

involving these health-promoting and versatile compounds. The continual development of sensitive analytical techniques for accurate quantification of molecules is needed by researchers across all disciplines of science, both in academia and industry. Thus through active collaboration of scientists from interdisciplinary backgrounds, such as those involved in this thesis work, insights into additional ways of incorporating the ever versatile casein micelle with other health promoting molecules is promising.

6.0 DIRECTIONS FOR FUTURE RESEARCH

- Explore additional levels of the main effects of pH and temperature to observe for possible trending in the extent of binding between caffeine and 5-CGA with sodium caseinate

- Hold insignificant factors constant while varying significant factors at larger ranges and employing a response surface model (RSM) to determine optimum treatment levels

- Vary the molar ratio of coffee bioactive to sodium caseinate beyond a 1:1 M ratio to observe any differences in the effects of using different concentrations of each compound

- Investigate whether a specific fraction of casein is responsible for binding caffeine and 5-CGA by using commercial standards of α- and β-caseins

- Direct measurement of the casein pellet for the amount of bound bioactive compound

- Utilize additional characterization techniques such as dynamic light scattering (DLS) particle size analysis on changes to the casein micelle size and structure

- Prepare heated samples using a stirring water bath to maintain better temperature control compared to using a hot plate

- Study whether the same binding interactions occur with other bioactive compounds in coffee, such as caffeic acid

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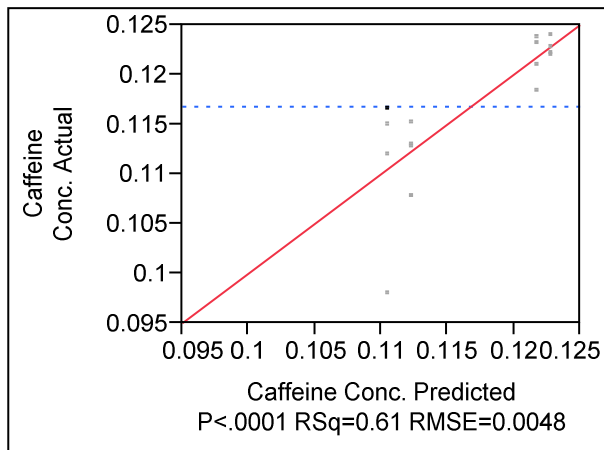
Appendix A: Data for precision studies on caffeine and 5-CGA

Sample Name	Known Conc. (mg/mL)	Observed Conc. (mg/mL)	Peak Area	Average Peak Area	Relative Standard Deviation (% RSD)	Precision Range
Caff A1	0.0625	0.071913	11,871,663			
Caff A2	0.0625	0.071901	11,869,737			
Caff A3	0.0625	0.071968	11,879,946	11,875,780	0.05	<1%
Caff A4	0.0625	0.071936	11,875,076			
Caff A5	0.0625	0.071985	11,882,476			
Caff B1	0.03125	0.033021	6,002,144			
Caff B2	0.03125	0.033018	6,001,681			
Caff B3	0.03125	0.033010	6,000,485	6,000,843	0.04	<1%
Caff B4	0.03125	0.032986	5,996,814			
Caff B5	0.03125	0.033027	6,003,092			
Caff C1	0.015625	0.013204	3,011,482			
Caff C2	0.015625	0.013207	3,011,870			
Caff C3	0.015625	0.013206	3,011,713	3,012,412	0.05	<1%
Caff C4	0.015625	0.013229	3,015,277			
Caff C5	0.015625	0.013206	3,011,719			

Sample Name	Known Conc. (mg/mL)	Observed Conc. (mg/mL)	Peak Area	Average Peak Area	Relative Standard Deviation (% RSD)	Precision Range
CGA A1	0.0625	0.065321	14,860,698			
CGA A2	0.0625	0.065782	14,978,295			
CGA A3	0.0625	0.065130	14,811,928	14,829,745	0.75	<1%
CGA A4	0.0625	0.065207	14,831,589			
CGA A5	0.0625	0.064558	14,666,214			
CGA B1	0.03125	0.034415	6,978,188			
CGA B2	0.03125	0.034718	7,055,321			
CGA B3	0.03125	0.034776	7,070,062	7,007,503	0.74	<1%
CGA B4	0.03125	0.034312	6,951,698			
CGA B5	0.03125	0.034431	6,982,247			
CGA C1	0.015625	0.019591	3,197,140			
CGA C2	0.015625	0.019706	3,226,576			
CGA C3	0.015625	0.019664	3,215,890	3,210,977	1.33	1.33%
CGA C4	0.015625	0.019403	3,149,330			
CGA C5	0.015625	0.019861	3,265,950			

Appendix B: Statistical analysis by JMP – binding caffeine to sodium

caseinate trials

Caffeine Conc. Actual vs Caffeine Conc. Predicted
P<.0001 RSq=0.61 RMSE=0.0048

Summary of Fit

RSquare	0.611634
RSquare Adj	0.514543
Root Mean Square Error	0.00478
Mean of Response	0.116781
Observations (or Sum Wgts)	16

Parameter Estimates

Term	Estimate	Std Error	DFDen	t Ratio	Prob>|t|
Intercept	0.1167813	0.000906	1	128.86	0.0049*
pH[4.4]	-0.000156	0.001195	11	-0.13	0.8983
Temperature[25]	0.0054562	0.001195	11	4.57	0.0008*
pH[4.4]*Temperature[25]	0.0007187	0.001195	11	0.60	0.5597

Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
pH	1	1	11	0.0171	0.8983
Temperature	1	1	11	20.8471	0.0008*
pH*Temperature	1	1	11	0.3618	0.5597

pH
Leverage Plot

pH Leverage, P=0.8983

Least Squares Means Table

Level	Least Sq Mean	Std Error
4.4	0.11662500	0.00149978
6.7	0.11693750	0.00149978

LS Means Plot

pH

LSMeans Differences Student's t

α=0.017

LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	4.4	6.7
4.4	0 0 0 0	-0.0003 0.00239 -0.007 0.00642
6.7	0.00031 0.00239 -0.0064 0.00705	0 0 0 0

Level		Least Sq Mean
6.7	A	0.11693750
4.4	A	0.11662500

Levels not connected by same letter are significantly different.

Temperature
Leverage Plot

Temperature
Leverage, P=0.0008

Least Squares Means Table

Level	Least Sq Mean	Std Error
25	0.12223750	0.00149978
72	0.11132500	0.00149978

LS Means Plot

LSMeans Differences Student's t

α=0.017
LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	25	72
25	0 0 0 0	0.01091 0.00239 0.00418 0.01765
72	-0.0109 0.00239 -0.0176 -0.0042	0 0 0 0

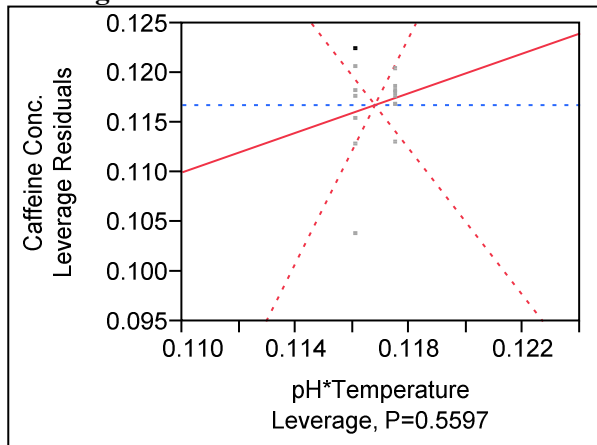| Level		Least Sq Mean
25	A	0.12223750
72	B	0.11132500

Levels not connected by same letter are significantly different.

pH*Temperature
Leverage Plot

Least Squares Means Table

Level	Least Sq Mean	Std Error
4.4,25	0.12280000	0.00225952
4.4,72	0.11045000	0.00225952
6.7,25	0.12167500	0.00225952
6.7,72	0.11220000	0.00225952

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LS Means Plot

pH*Temperature
LSMeans Differences Tukey HSD
α=0.017
LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	4.4,25	4.4,72	6.7,25	6.7,72
4.4,25	0	0.01235	0.00112	0.0106
	0	0.00338	0.00338	0.00338
	0	-4e-5	-0.0113	-0.0018
	0	0.02474	0.01351	0.02299
4.4,72	-0.0123	0	-0.0112	-0.0018
	0.00338	0	0.00338	0.00338
	-0.0247	0	-0.0236	-0.0141
	0.00004	0	0.00116	0.01064
6.7,25	-0.0011	0.01123	0	0.00947
	0.00338	0.00338	0	0.00338
	-0.0135	-0.0012	0	-0.0029
	0.01126	0.02361	0	0.02186
6.7,72	-0.0106	0.00175	-0.0095	0
	0.00338	0.00338	0.00338	0
	-0.023	-0.0106	-0.0219	0
	0.00179	0.01414	0.00291	0

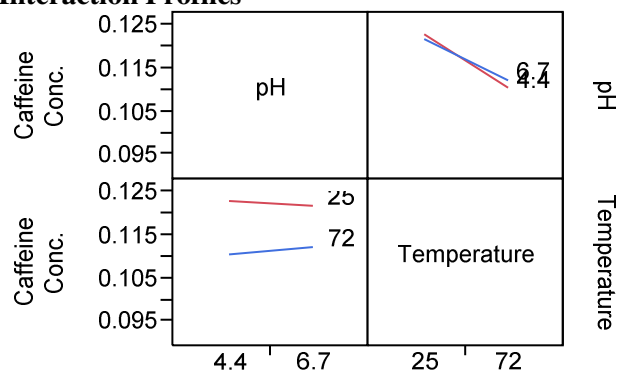| Level		Least Sq Mean
4.4,25	A	0.12280000
6.7,25	A	0.12167500
6.7,72	A	0.11220000
4.4,72	A	0.11045000

Levels not connected by same letter are significantly different.
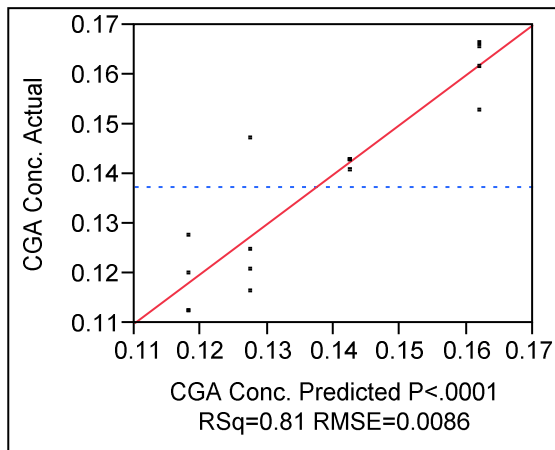
Day
Least Squares Means Table

Level	Least Sq Mean	Std Error
1	0.11745078	0.00061075
2	0.11611172	0.00061075

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Interaction Profiles

Appendix C: Statistical analysis by JMP – binding 5-CGA to sodium

caseinate trials

Summary of Fit

RSquare	0.812019
RSquare Adj	0.765024
Root Mean Square Error	0.008564
Mean of Response	0.1374
Observations (or Sum Wgts)	16

Parameter Estimates

Term	Estimate	Std Error	DFDen	t Ratio	Prob>|t|
Intercept	0.1374	0.0014	1	98.14	0.0065*
pH[4.4]	-0.002525	0.002141	11	-1.18	0.2631
Temperature[25]	0.014675	0.002141	11	6.85	<.0001*
pH[4.4]*Temperature[25]	-0.00715	0.002141	11	-3.34	0.0066*

Fixed Effect Tests

Source	Nparm	DF	DFDen	F Ratio	Prob > F
pH	1	1	11	1.3910	0.2631
Temperature	1	1	11	46.9847	<.0001*
pH*Temperature	1	1	11	11.1535	0.0066*

pH
Leverage Plot

Least Squares Means Table

Level	Least Sq Mean	Std Error
4.4	0.13487500	0.00255803
6.7	0.13992500	0.00255803

LS Means Plot

LSMeans Differences Student's t

α=0.017

LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	4.4	6.7
4.4	0	-0.0051
	0	0.00428
	0	-0.0171
	0	0.00702
6.7	0.00505	0
	0.00428	0
	-0.007	0
	0.01712	0

Level		Least Sq Mean
6.7	A	0.13992500
4.4	A	0.13487500

Levels not connected by same letter are significantly different.

Temperature
Leverage Plot

Temperature Leverage, P<.0001

Least Squares Means Table

Level	Least Sq Mean	Std Error
25	0.15207500	0.00255803
72	0.12272500	0.00255803

LS Means Plot

Temperature

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LSMeans Differences Student's t

α=0.017

LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	25	72
25	0 0 0 0	0.02935 0.00428 0.01728 0.04142
72	-0.0294 0.00428 -0.0414 -0.0173	0 0 0 0

Level		Least Sq Mean
25	A	0.15207500
72	B	0.12272500

Levels not connected by same letter are significantly different.

pH*Temperature
Leverage Plot

Leverage, P=0.0066

Least Squares Means Table

Level	Least Sq Mean	Std Error
4.4,25	0.14240000	0.00396366
4.4,72	0.12735000	0.00396366
6.7,25	0.16175000	0.00396366
6.7,72	0.11810000	0.00396366

LS Means Plot

pH*Temperature
LSMeans Differences Tukey HSD
α=0.017
LSMean[i] By LSMean[j]

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	4.4,25	4.4,72	6.7,25	6.7,72
4.4,25	0	0.01505	-0.0194	0.0243
	0	0.00606	0.00606	0.00606
	0	-0.0071	-0.0415	0.0021
	0	0.03725	0.00285	0.0465
4.4,72	-0.0151	0	-0.0344	0.00925
	0.00606	0	0.00606	0.00606
	-0.0372	0	-0.0566	-0.0129
	0.00715	0	-0.0122	0.03145
6.7,25	0.01935	0.0344	0	0.04365
	0.00606	0.00606	0	0.00606
	-0.0028	0.0122	0	0.02145
	0.04155	0.0566	0	0.06585
6.7,72	-0.0243	-0.0092	-0.0437	0
	0.00606	0.00606	0.00606	0
	-0.0465	-0.0314	-0.0658	0
	-0.0021	0.01295	-0.0215	0

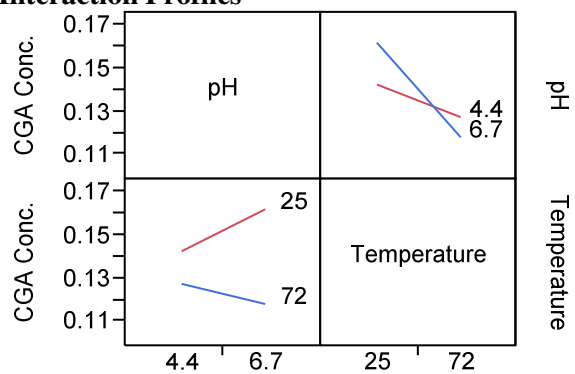| Level				Least Sq Mean
6.7,25	A			0.16175000
4.4,25	A	B		0.14240000
4.4,72		B	C	0.12735000
6.7,72			C	0.11810000

Levels not connected by same letter are significantly different.

Day
Least Squares Means Table

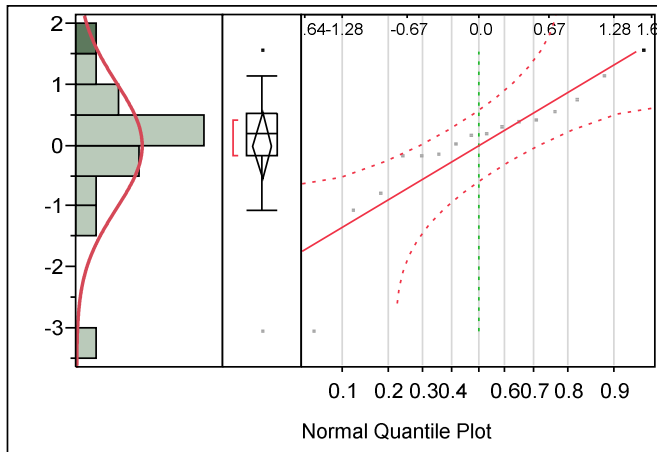| Level	Least Sq Mean	Std Error
1	0.13552606	.
2	0.13927394	.

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Interaction Profiles

Appendix D: Statistical analysis by JMP – model assumptions (caffeine)

Normality

Distributions
Studentized Resid Caffeine Conc.

Normal(-4e-15,1.0328)

Quantiles

100.0%	maximum	1.53725
99.5%		1.53725
97.5%		1.53725
90.0%		1.24456
75.0%	quartile	0.51037
50.0%	median	0.17217
25.0%	quartile	-0.1706
10.0%		-1.6688
2.5%		-3.0376
0.5%		-3.0376
0.0%	minimum	-3.0376

Summary Statistics

Mean	-4.08e-15
Std Dev	1.0327956
Std Err Mean	0.2581989
Upper 95% Mean	0.5503379
Lower 95% Mean	-0.550338
N	16

Fitted Normal
Parameter Estimates

Type	Parameter	Estimate	Lower 95%	Upper 95%
Location	μ	-4.08e-15	-0.550338	0.5503379
Dispersion	σ	1.0327956	0.7629311	1.5984486

-2log(Likelihood) = 45.4386494007571

Equal Variances
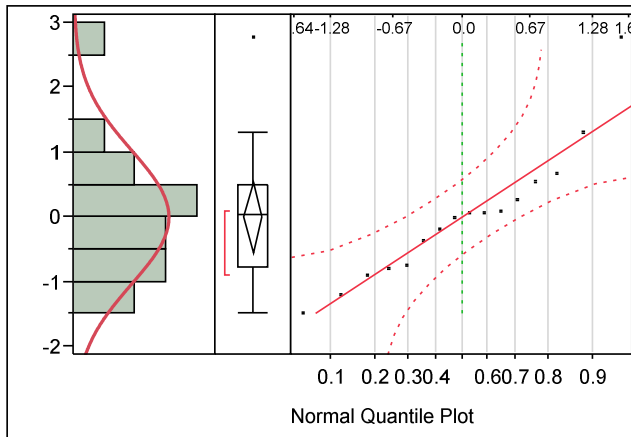
Residual by Predicted Plot

Appendix E: Statistical analysis by JMP – model assumptions (5-CGA)

Normality

Distributions
Studentized Resid CGA Conc.

Normal(1.1e-15,1.0328)

Quantiles

100.0%	maximum	2.75655
99.5%		2.75655
97.5%		2.75655
90.0%		1.73614
75.0%	quartile	0.47497
50.0%	median	0.02418
25.0%	quartile	-0.791
10.0%		-1.2919
2.5%		-1.4854
0.5%		-1.4854
0.0%	minimum	-1.4854

Summary Statistics

Mean	1.055e-15
Std Dev	1.0327956
Std Err Mean	0.2581989
Upper 95% Mean	0.5503379
Lower 95% Mean	-0.550338
N	16

Fitted Normal
Parameter Estimates

Type	Parameter	Estimate	Lower 95%	Upper 95%
Location	M	1.055e-15	-0.550338	0.5503379
Dispersion	Σ	1.0327956	0.7629311	1.5984486

-2log(Likelihood) = 45.4386494007507

Equal Variances

Residual by Predicted Plot