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Caffeine-milk protein interactions in coffee brews: Effects on the *in vitro* bioaccessibility of caffeine

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Abstract

Coffee is one of the most consumed beverages around the world and is known for a myriad of health benefits. Black coffee is a rich source of bioactive compounds and has high antioxidant activity. Coffee has been consumed in various forms with and without the addition of milk. The impact of milk addition on the phenolic properties, antioxidant activity, and functionality of bioactive components has been an interesting topic among researchers for the past few decades. But to the best of our knowledge, the effect of milk addition to coffee brew on caffeine bioaccessibility has not yet been addressed in the literature. Accordingly, this study aimed to investigate the interactions between caffeine and milk proteins in coffee and the effect on *in vitro* bioaccessibility of caffeine, as well as the antioxidant properties.

A range of analytical techniques was employed to assess these interactions. Ultraviolet (UV) spectroscopy and high-performance liquid chromatography (HPLC) were used to quantify caffeine content in black coffee and milk-based coffee samples at various stages of digestion. Fourier transform infrared (FTIR) spectroscopy was applied to examine the molecular interactions between caffeine and milk proteins, focusing on non-covalent binding mechanisms such as hydrogen bonding and Van der Waals forces. Total phenolic content (TPC) and antioxidant capacity were measured using the Folin-Ciocâlteu assay and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity, respectively. Additionally, particle size distribution and ζ-potential analysis were performed to determine the stability and colloidal properties of the coffee samples. *In vitro* digestion was conducted to simulate gastrointestinal conditions, including the gastric and intestinal phases, to evaluate changes in caffeine bioaccessibility over digestion.

The results demonstrate a significant reduction in caffeine content upon milk addition at all digestion stages, indicating strong interactions between caffeine and milk proteins. UV spectroscopy and HPLC analyses revealed that in black coffee, the initial free caffeine content before digestion was 173.1 µg/mL, which decreased to 90.7 µg/mL post-gastric digestion but increased to 147.9 µg/mL after the intestinal phase, suggesting increased free caffeine concentration at the end of digestion. The free caffeine concentration obtained for milk-based coffee was 142 µg/mL before digestion, and at the end of digestion, it was 108 µg/mL, which indicates that milk addition reduced caffeine recovery. Similarly, the addition of milk reduced the TPC and DPPH radical scavenging activity of pure caffeine and coffee brews. The TPC values for black coffee and milk-based coffee were 133.4 and 94.7 µg gallic acid equivalent

(GAE)/mL, respectively, at the end of digestion. The DPPH antioxidant activity values were 27.42 and 3.54 μg/mL, respectively, for black coffee and milk-based coffee after intestinal digestion. The data from FTIR analysis suggest that non-covalent interactions, such as hydrogen bonding and Van der Waals forces, exist between milk proteins and caffeine, which may affect the functionality of caffeine. Shifts at the major caffeine peak regions (2950-2930 cm^{-1}) indicate the C-H stretching vibrations, suggesting a possibility for hydrogen bonding and Van der Waals interactions. The results from the particle size data are inconclusive in determining the extent of caffeine interactions or bioaccessibility, necessitating further molecular-level and bioavailability studies. Data from ζ-potential measurement reveals that milk-based coffee has a more stable system compared to black coffee after intestinal digestion. This complements other results as higher stability for a colloidal system could be an indication of less availability for further interactions or release of free caffeine. The i*n vitro* digestion studies reveal that the overall bioaccessibility of caffeine decreases when milk is added to coffee brew. The percentage bioaccessibility of caffeine in black coffee and milk-based coffee was 85.8 and 77%, respectively. The findings of this research contribute to the understanding of coffee as a functional beverage and provide insights into how the addition of milk to the coffee brew may affect the functionality.

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Abbreviations

ABTS	2,2'-Azinobis-3-ethylbenzothiazoline-6-sulfonic acid assay
APPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ATR	Attenuated Total Reflectance
AUC	Area Under the Curve
α-La	α-Lactalbumin
β-Lg	β-Lactoglobulin
BSA	Bovine Serum Albumin
CD	Circular Dichroism
CQA	Caffeoylquinic Acid
FQA	Feruloylquinic Acid
CVD	Cardiovascular Disease
DPPH	2,2-Diphenyl-1-picryl-hydrazyl assay
DW	Dry Weight
FTIR	Fourier Transform Infrared
GAE	Gallic Acid Equivalent
GI	Gastrointestinal
HPLC	High-performance Liquid Chromatography
ITC	Isothermal Titration Calorimetry
LDL	Low Density Lipoprotein
MQ	MilliQ
NMR	Nuclear Magnetic Resonance
oxLDL	Oxidized LDL
PBS	Phosphate Buffered Saline
PD	Parkinson’s Disease
TEAC	Trolox Equivalent Antioxidant Capacity
TPC	Total Phenolic Content
UV	Ultraviolet
VOC	Volatile Organic Compounds

1. Introduction

Coffee is the third most widely consumed beverage globally, following water and tea, and is brewed from roasted coffee beans. It serves as one of the most prevalent sources of caffeine and is prepared and enjoyed in various forms, depending on individual preferences. Some of the most popular types of coffee include espresso (a concentrated shot of coffee with an intense flavour and a rich crema layer), americano (an espresso shot diluted with hot water), flat white (an espresso shot combined with steamed milk and a thin layer of foam), latte (a milder alternative to a flat white, containing a higher proportion of steamed milk), cappuccino (comprising equal parts espresso, steamed milk, and foam), and iced coffee (prepared using cold milk and espresso) (Hatzold, 2012). The brewing method significantly influences the characteristics of the final coffee product, as different techniques yield distinct flavour profiles and textures. Drip brewing, also known as filter coffee, involves pouring hot water over ground coffee beans held within a filter paper. Espresso is prepared by forcing hot water through finely ground coffee at high pressure, producing a concentrated and full-bodied beverage. The French press method entails steeping coarsely ground coffee beans in hot water before pressing the grounds with a plunger to extract the liquid. Cold brew, in contrast, requires the prolonged steeping of coffee grounds in cold water over several hours to extract soluble compounds, resulting in a smooth and less acidic beverage. These represent some of the most widely adopted coffee brewing techniques (Derossi et al., 2018; Nosal et al., 2022).

The coffee tree is a member of the genus Coffea in the family *Rubiaceae*. Out of the 80 known coffee species, only two are significant economically, and these are *Coffea arabica* (Arabica) and *Coffea canephora* var. *robusta*. Roasted coffee beans contain polysaccharides, oligosaccharides, proteins, lipids, chlorogenic acid, fatty acids, caffeine, trigonelline, minerals, free amino acids, and melanoidins (Farah, 2012). Caffeine, a white crystalline purine, chemically known as 1,3,7-trimethylxanthine, stands out as the most notable bioactive compound in coffee. The caffeine content in a single 250 mL serving of coffee can vary widely, ranging from 50 to 300 mg (Fredholm et al., 1999). The composition of a cup of coffee is affected by factors such as origin (climate, altitude, and soil type of the plantation region) and variety of coffee beans (Arabica or Robusta), harvesting and processing methods, degree of roasting, grind size, brewing time, method, temperature and pressure, and the ratio of coffee to water (Olechno et al., 2021).

Coffee is known to have myriads of bioactive compounds, such as antioxidants, flavonoids, and polyphenols, and these compounds contribute to several health benefits. With moderate consumption of coffee, several positive effects are seen on human health, but individual responses to coffee may vary. Coffee consumption has resulted in a lowering of the incidence of neurodegenerative diseases, such as Parkinson's Disease (PD) and Alzheimer's disease (Lindsay et al., 2012; Panza et al., 2015). Low dopamine levels are the primary cause of PD (Faudone et al., 2021). Because caffeine appears to preserve the brain cells that create dopamine, several epidemiologic studies demonstrate that increased caffeine intake decreases the chance of developing PD (Lim & Tan, 2012).

Several studies confirm that drinking coffee is associated with reduced risk of various types of cancer. Coffee helps speed up digestion by increasing the synthesis of bile acids, which lowers the chance of exposing colon cells to carcinogens. Coffee also lowers the chance of developing endometrial cancer, leukaemia, skin cancer, prostate cancer, oral cancer, and liver cancer (Nkondjock, 2012; Safe et al., 2023). A few studies, in contrast, have reported that increased coffee consumption has a positive association with oesophageal cancer and an inconclusive effect on other types of cancer. Therefore, the association between coffee consumption and the prevention of cancer is still under study and discussion (Carter et al., 2022; Grosso et al., 2017; Safe et al., 2023; Vitaglione et al., 2012).

Coffee consumption has been associated with a reduced risk of developing Type 2 diabetes. In the short term, caffeine appears to elevate blood sugar levels, but when considering long-term effects, polyphenols and minerals such as magnesium that are present in coffee enhance insulin effectiveness and support glucose metabolism (Matusheski et al., 2012). Studies indicate that individuals who drink coffee are less likely to experience depression than those who do not, as the polyphenols and flavonoids act as antioxidants and anti-depressants. Coffee consumption is also correlated with improved cognitive performance and mood enhancement. Caffeine promotes alertness and wakefulness, inhibits adenosine, and chlorogenic acids protect neural cells against oxidative stress (Yi-Fang & Yumin, 2012).

Milk, a highly nutritious beverage, is consumed worldwide and is secreted by mammals, including sheep, goats, camels, buffaloes, cows, humans, yaks, and others. It is regarded as a complete food, as it contains proteins, fats, carbohydrates, vitamins, and minerals. Milk is an opaque, yellowish-white emulsion of oil in water, characterised by a pleasant flavour and

mouthfeel. It is generally free from toxins, except for aflatoxins, which can be transferred to milk through contaminated cattle feed.

Cows' milk is the most widely consumed globally, although preferences vary by region. For example, camel milk is widely consumed in the United Arab Emirates, buffalo milk is more popular in India and Pakistan, and yak milk is predominantly used by Tibetan communities. Bovine milk consists of more than 85% water, with proteins contributing approximately 2–4% and milk fat accounting for 3–6%. Of the proteins, 80% are caseins, and the remainder includes whey proteins, protease, peptones, and non-protein nitrogen. The pH of fresh bovine milk typically ranges from 6.5 to 6.9, with an isoelectric point of 4.6 for caseins and 5.5 for whey proteins (Fox, 2003; Rashidinejad et al., 2022).

Black coffee beverages, such as short black and Americano, are bitter and are often not appealing to everyone. The first reported person to add milk to coffee was Johan Nieuhof, the Dutch ambassador to China around the 1660s (Choong, 2016). To reduce the astringency, European coffee drinkers also started adding dairy ingredients such as milk or cream to their coffee since milk is abundantly available in cooler regions such as Germany, Switzerland, the UK, the Netherlands, and France. Not only the bitterness but also the acidity and the temperature of the coffee are reduced when milk is added to freshly brewed coffee (Sivetz & Desrosier, 1979). However, the addition of milk has a different effect on the bioactive components of a cup of coffee (Dupas et al., 2006). Bioaccessibility is defined as the "proportion of a nutrient that is chemically and physically available for absorption by the small intestine" (Le Feunteun et al., 2021). Several studies have been carried out on the effects of the addition of skimmed milk, semi-skimmed milk, full cream milk, or heavy cream to understand the effect on bioactive components and bioaccessibility (Niseteo et al., 2012; Quan et al., 2020; Soares et al., 2021; Yu et al., 2021). Overall, based on the findings of the previous research, these effects can be positive, negative, and neutral (Rashidinejad et al., 2022; Ryan & Petit, 2010).

Quan et al. (2020) documented the impact of adding whole milk and skim milk to coffee on antioxidant activities, as well as on the quantities of chlorogenic acids, caffeic acid, ferulic acid, and total phenolic content. A significant reduction in the antioxidant activity of coffee was observed when whole milk and skim milk were added, compared to black coffee. The reduction was more pronounced with the addition of skim milk rather than whole milk. Similarly, a pattern of reduction was reported for individual phenolic content such as chlorogenic acid and

caffeic acid. In contrast, the total phenolic content was slightly higher when whole milk was added to coffee but showed an almost 50% reduction in the case of skim milk compared to black coffee samples. A study carried out by Jeon et al. (2019) on instant coffee samples also reported a reduction in caffeine and chlorogenic acid when compared to coffee samples without milk or cream.

These studies suggest that the addition of milk adversely affects the bioactive properties of coffee. This may indicate that milk components, such as milk proteins and fat, interact with the bioactive components of coffee. Prigent et al. (2003) proposed that polyphenols interact with proteins and form covalent and non-covalent bonds. The hydrophobic association of proteins and polyphenols leads to non-covalent binding, which is relatively unstable and reversible. These are further stabilised by hydrogen bonding. Dupas et al. (2006) suggested that these kinds of interactions can hinder the absorbance of polyphenols and thereby reduce properties, such as antioxidant capacity. Serafini et al. (2003) reported that the antioxidant activity of dark chocolate was higher than milk chocolate when carried out using *in vitro* digestion. Similar types of association can also be expected in coffee between caffeine and milk proteins due to the possible binding sites, such as oxygen groups and hydrogen groups present in the structure of caffeine that can form hydrogen bonds with milk proteins, and carbon groups of both compounds that can form covalent bonds. Sánchez-González et al. (2005) also reported a reduction in antioxidant activity of milk-based coffee.

On the other hand, studies concluding that the addition of milk has a positive impact on the bioefficacy of coffee primarily suggest that it is the milk fat that affects the bioactive components, particularly chlorogenic acids, rather than milk proteins (Alongi et al., 2019). However, a study carried out by Tagliazucchi et al. (2012) implied that milk protein may also interact with polyphenols and that the recovery of these polyphenols was higher after digestion compared to that without milk addition. The findings indicate that milk protein had a protective effect on the polyphenols during digestion, demonstrating that the bioaccessibility of coffee polyphenols was improved by the addition of milk. A study done by Al Doghaither et al. (2017) also suggests that there is a significant increase in the total phenolic content and antioxidant capacity of coffee samples when high-fat evaporated milk and low-fat evaporated milk are added, compared to pure coffee.

To date, numerous research activities have primarily focused on the effect of adding milk and dairy products, such as condensed milk, cream, milk froth, butter, and yogurt, to coffee brews

on the composition of polyphenols, bioaccessibility, and antioxidant capacity (Dupas et al., 2006; Helal et al., 2022; Niseteo et al., 2012; Quan et al., 2020; Soares et al., 2021). People primarily consume coffee for its stimulating effects, as caffeine enhances alertness and reduces fatigue. Understanding the caffeine content in coffee is crucial for managing intake and minimising potential adverse effects, such as anxiety, insomnia, and an increased heart rate (Faudone et al., 2021). As many coffee drinkers consume more than one cup per day and given that caffeine intake has a recommended daily limit, it is important to be aware of the caffeine content in each cup to prevent potential health risks (Fredholm et al., 1999).

Although few studies have investigated the interactions between caffeine and milk proteins as isolated compounds, the impact of milk on caffeine functionality remains an area requiring further exploration (Quan et al., 2020; Santa Rosa et al., 2021; Tenney et al., 2017). Certain groups, such as pregnant women and individuals with specific health conditions, must closely monitor their caffeine intake. By quantifying the bioavailable caffeine in a cup of coffee and considering various influencing factors, such as the addition of milk, individuals can make more informed decisions about their coffee consumption, aligning it with their health goals and tolerance levels. However, the nature of the chemical bonds between milk compounds and caffeine, the potential reversibility of these interactions, and their effect on caffeine bioaccessibility remain unclear.

This research primarily aims to investigate the effect of milk addition on the interactions between caffeine and milk proteins in coffee brews, as well as the effect on antioxidant capacity and total phenolic content. Additionally, an *in vitro* gastrointestinal digestion model is employed to assess the bioaccessibility of caffeine in milk-based coffee beverages. The findings may contribute to the ongoing debate regarding whether milk addition enhances or diminishes the bioactive compounds in coffee, thereby providing clarity on the potential health benefits of milk-based coffee beverages. Moreover, this study is particularly significant as it examines how milk affects the bioaccessibility and efficacy of caffeine, potentially influencing dietary recommendations and consumption habits. The food industry may also benefit from these insights by developing new formulations of coffee beverages that optimise health benefits while catering to consumer preferences.

2. Review of literature

2.1 Background

Coffee is one of the most consumed functional beverages after tea and water around the globe (Ramalakshmi et al., 2009; Soares et al., 2021). Due to its rich aroma and flavour *Coffee arabica* is the most popular type of coffee bean produced around the world. Compared to Robusta, Arabica has a lower caffeine content. Major steps in coffee processing involve harvesting, washing, processing (wet and dry method) de-hulling, fermentation (in the wet method), drying, size grading, colour sorting, roasting, grinding, and extraction/brewing. Factors such as geography, climatic conditions, and processing methods affect the composition of green coffee beans. Roasting has a significant role in altering the bioactive properties and nutritional qualities of a coffee bean (Esquivel & Jiménez, 2012; Pilipczuk et al., 2015). The caffeine content is also reduced upon roasting.

The property of a cup of coffee depends on the method of roasting, grind size, brewing method, time and temperature of brewing, coffee/water ratio, and if milk or milk products are added, the type of milk used (Olechno et al., 2021). The bioactivity changes with these factors, and this needs to be studied to understand the characteristics of a cup of coffee. The bioaccessibility of caffeine can also change with milk addition, which can be determined by *in vitro* digestion studies. Derossi et al. (2018) reported that the grinding level and brewing technique have significant effects on the antioxidant activity and bioactive compounds in a cup of coffee. There is a possibility that caffeine interacts with milk proteins or other components of milk, and then, when it reaches the stomach or intestines, these bonds can break down and release caffeine, making it available to be absorbed into the bloodstream. If this is the case, it could be posited that milk plays a protective role in delivering caffeine to the human body. Another possibility is that milk could interact with caffeine and reduce the ability to cross the blood-brain barrier, thereby diminishing its potential as a central nervous system stimulant and reducing other functional properties.

2.2. General review of coffee

2.2.1. Composition of coffee beans

The major components of coffee beans include minerals, caffeine, trigonelline, lipids, chlorogenic acids, aliphatic acids, oligosaccharides, polysaccharides, amino acids, proteins, and humic acids. The composition of green and roasted arabica robusta and instant coffee beans is given in Table 2.1. The mineral content in coffee beans consists of potassium, calcium,

magnesium, phosphorus, and sulphur. The carbohydrates found in coffee beans are generally simple sugars, which also include reducing sugars. Stachyose, mannose, ribose, raffinose, rhamnose, arabinose, and galactose are found in smaller quantities, and higher levels of glucose and fructose have also been reported. Polysaccharides, otherwise known as glycans, found in green coffee beans include araban and glucan from arabinose and glucose, respectively. Sucrose is known to undergo caramelization reactions when exposed to temperatures exceeding the melting point of 130°C, particularly in the presence of ammonia or proteinaceous substances as employed in the industrial production of caramels. These chemical transformations are likely to occur during the roasting of green coffee, which typically contains around 3-8% sucrose by weight.

The process of caramelization during roasting results in the production of carbon dioxide and water, which are characteristic byproducts of coffee roasting. The loss of dry matter in coffee during roasting varies depending on the roast level, with light roasts experiencing 0-5% loss, medium roasts 5-10%, and dark roasts over 10%. These losses primarily consist of carbon dioxide and water, in addition to other volatile compounds generated during the roasting process. In roasted coffee, these condensed complexes could potentially form covalent bonds with proteins, protein fragments, unchanged polysaccharides, as well as certain components such as chlorogenic acids and their derivatives. These complex structures have been described using different terms such as 'humic acids', 'melanoidins', or simply 'Maillard products'.

Table 2.1 Chemical composition (% db) of green and roasted Arabica and Robusta, and instant coffee powder. Modified from Thompson (1988) and Farah (2012)

Components	Arabica		Robusta		Instant coffee powder
	Green	Roasted	Green	Roasted	
Minerals	3-4.2	3.5-4.5	4-4.5	4.6-5.0	9-10
Caffeine	0.9-1.3	1.1-1.3	1.6-2.4	2.4	4.5-5.1
Trigonelline	0.6-2	0.2-1.2	0.6-0.75	0.3-0.6	-
Lipids	12-18	14.5-20.0	9-13	11-16	-
Total chlorogenic acids	5.5-8	1.2-3.0	7-10	3.9-4.6	5.2-7.4
Aliphatic acids	1.5-2.0	1.1-1.5	1.5-2	1-1.5	-
Oligosaccharides	6-8	0-3.5	5-7	0-3.5	0.7-5.2
Total polysaccharides	50-55	24-39	37-47	-	~6.5
Amino acids	0.5	0	0.8	0	0
Proteins	11-13	13-15	11-13	13-15	16-21
Humic acids	-	16-17	-	16-17	15

db - dry basis

The major nitrogenous components present in coffee are alkaloids, nicotinic acids, trigonelline amino acids, and proteins. The major alkaloid present in coffee is caffeine (Celli & Camargo, 2019). Caffeine, being a weak base, tends to create unstable salts, such as with acetate, where the acetic acid can easily evaporate at the roasting temperature. Although caffeine remains relatively stable in mild acidic and alkaline environments, it can form a range of complexes with various other compounds present in coffee, such as chlorogenic acids or polynuclear aromatics (Ishizu & Tsutsumi, 2012). As green coffee beans undergo a roasting process, a combination of external heat application and exothermic chemical reactions raises the bean temperature to over 200℃. There are different roasting styles, namely, light roast (cinnamon roast, new England roast), medium roast (American roast, city roast), and dark roast (full city roast, Vienna roast, French roast, and Italian roast), all differing in the time-temperature combinations used for roasting (Spiller, 1998). 180-205℃ for 1 to 1.5 min gives light roasted coffee beans, roasting at 210-230℃ for 1.5 to 2 min leads to a darker coffee bean than light roasted coffee beans, and dark roasted coffee is roasted at 240-250℃ for 2 to 2.5 min (Thompson, 1988).

The sensory attributes of coffee, including its taste, aroma, and mouthfeel, are influenced by a complex interplay of chemical compounds formed during processing and roasting. Among these, caffeine plays a crucial role in defining coffee's characteristic bitterness, while polyphenols contribute to the astringency. The intense aroma of coffee arises from a complex blend of chemical compounds that develop during the roasting process, interacting to form the unique sensory experience linked with coffee consumption. A variety of volatile organic compounds (VOCs) and flavour components found in coffee contribute to its distinct aroma and flavour characteristics. Key compounds identified in studies as significant contributors to the aroma of coffee include 3-caffeoylquinic acid (CQA), 4-caffeoylquinic acid, 5-caffeoylquinic acid, 3-O-feruloylquinic acid (FQA), and 5-O-feruloylquinic acid (Celli & Camargo, 2019).

Another compound present in green coffee beans is trigonelline, a white and odourless compound, which has one-quarter of the bitterness of caffeine and is less lethal. Nicotinic acid (a significant source of vitamin B3) found in roasted coffee beans is primarily derived from trigonelline. The LD_{50} value of caffeine is 200 mg/kg, and that of trigonelline is 5000 mg/kg in rats (Fredholm et al., 1999; Tice, 1997). The process of roasting significantly reduces the quantity of trigonelline. Enzymes such as amylase, catalase, protease, lipase, and several others, are found in green coffee beans, but these are inactivated during the roasting process. The amino

acid composition, total protein, and albumin (which is water-soluble) of Brazilian arabica green coffee beans are provided in Table 2.2. The free amino acids present in green coffee beans are degraded by simple pyrolysis, leaving only trace amounts in roasted coffee beans. Part of the aroma is brought about by free amino acids reacting with other compounds and becoming volatile.

Table 2.2 Amino acid composition of total protein and albumin in a Brazilian arabica coffee (Thompson, 1988)

Amino acid	As a percentage of	
	Total protein	Albumin
Alanine	4.96	4.94
Arginine	5.57	5.25
Aspartic acid	10.02	10.60
Glutamic acid	19.16	18.54
Glycine	6.39	6.01
Histidine	2.45	1.88
Isoleucine	3.76	3.46
Leucine	9.84	9.25
Lysine	6.49	6.22
Methionine	2.38	0.42
Phenylalanine	6.59	6.77
Proline	6.37	5.86
Serine	5.23	5.34
Threonine	3.56	3.19
Tyrosine	4.85	3.59
Valine	4.93	5.17
Tryptophan	-	1.47
Ammonia	2.23	2.09

Other major bioactive compounds present in coffee beans include chlorogenic acids (CGA). These are phenols and are generally considered secondary plant products. Phenols present in coffee beans include 2-methylphenol, 3-ethylphenol, vanillin, quinol, 4-ethylcatechol, 4-vinylcatechol, pyrogallol, 3,4-dihydroxycinnamaldehyde, 3,4-dihydroxybenzaldehyde, and 3-hydroxybenzoic acid. Coffee beans also contain free fatty acids, lipids, triglycerides, and tocopherols (Thompson, 1988).

2.2.2. Caffeine and its derivatives

There are several types of methylated dioxopurines, but the most important one is caffeine, or 1,3,7-trimethyl-2,6-dioxopurine (Figure 2.1). The dioxopurines in coffee are mainly caffeine, although small amounts of 3,7-dimethylxanthine (theobromine) and 1,3-dimethylxanthine (theophylline) can also be found. When caffeine is crystallized from water, it usually forms a hydrate which was once thought to have one water molecule per caffeine molecule. Recent

studies show that this hydrate is a 4/5 hydrate with about 6.95% water content. At around 140°C, caffeine changes from the hydrate form to anhydrous caffeine, a shift that can be observed using X-ray diffraction (Thompson, 1988).

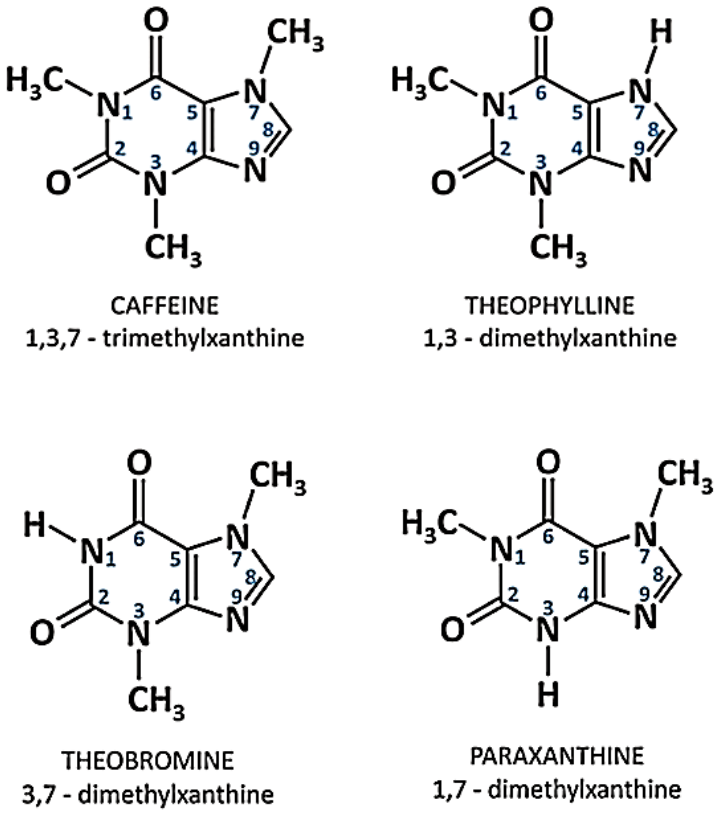


Figure 2.1 Caffeine and its derivatives. Modified from Gramza-Michałowska et al. (2021)

Caffeine dissolves moderately in water (about 4.6% by weight at 40°C), but this can vary depending upon the conditions. Anhydrous caffeine is stable above 52°C, whereas the hydrate form is more stable below this temperature (Thompson, 1988). Because the change between hydrate and anhydrous forms is slow, experiments need to start with the correct form, based on the temperature, to accurately measure solubility.

Understanding the solubility and phases of caffeine is important when making pharmaceuticals and processing food. The stability of caffeine crystals depends upon factors such as humidity and temperature, which are key for industrial crystallisation and processing. The solubility of caffeine in water is given in Table 2.3, and the solubility in different organic solvents is given

in Table 2.4. One of the main factors that determines the effectiveness of any drug is the solubility. The ability of a substance to pass through the gastrointestinal epithelium to the blood stream depends upon the solubility in water (Ribeiro et al., 2012). Therefore, it is important to know the solubility of caffeine in water and other solvents to fully understand its efficacy. Understanding the solubility of caffeine in different organic solvents also helps in the development of techniques for the decaffeination of coffee beans. Extracting wetted green coffee beans in solvents such as chloroform, benzene, or dichloromethane, followed by removing the solvent by steam distillation, removes caffeine from the beans.

Table 2.3 Solubility of caffeine in water (Thompson, 1988)

Temperature (°C)	Solubility (g per 100 g water)
0	0.60
15	1.00
20	1.46
25	2.13
30	2.80
40	4.64
50	6.75
60	9.70
70	13.50
80	19.23

In the human body, caffeine is metabolised into various substances such as paraxanthine, theophylline, and theobromine, each of which affects the body in different ways. Caffeine is absorbed through the lining of the stomach and small intestine within 45 min of ingestion (Rubem et al., 2012). The absorbed caffeine enters the bloodstream and is distributed throughout the body, including the brain. Caffeine can cross the blood-brain barrier and has a stimulatory effect on the central nervous system. Simultaneous with absorption, caffeine that reaches the liver is broken down into paraxanthine (84%), theobromine (12%), and theophylline (4%) (Figure 2.1). These metabolites are carried through the bloodstream throughout the body and finally excreted through urine after reaching the kidneys. Paraxanthine has lipolytic activity, leading to an increase in glycerol and free fatty acid contents in the blood. Theobromine dilates blood vessels and is a diuretic; therefore, it increases the volume of urine. Theophylline is known to smooth the muscles of the bronchi and can, thus, be used to treat asthma. This highlights the complexity and importance of caffeine in scientific research and practical applications (Yi-Fang & Yumin, 2012).

Table 2.4 Solubility of caffeine in organic solvents (Thompson, 1988)

Solvent	*Temperature (°C)*	*Solubility (g per 100 g solvent)*
95% Aqueous ethanol	25	1.32
Ethanol	25	1.88
Ethyl acetate	18	0.73
Methanol	25	1.14
Acetone	30.5	2.32
Benzene	18	0.91
Carbon tetrachloride	18	0.09
Chloroform	17	12.9
Ether	18	0.12
Petroleum ether	15-17	0.03
Trichloroethylene	15	0.76
Dichloroethylene	15	1.82
Toluene	25	0.58
Dichloromethane (methylene chloride)	33	9

2.2.3. Health-promoting properties of coffee

Coffee is reported to have antibacterial properties due to the presence of caffeic acid, chlorogenic acid, and protocatechuic acid. Coffee is a rich source of antioxidants such as caffeic acid, coumaric, sinapinic, chlorogenic, ferulic acids, flavonoids, polyphenols, and caffeine. Morales and Jimenez-Perez (2004) reported that melanoidins in coffee have more antioxidant power than melanoidins in beer. The theophylline in coffee is prescribed for treating asthma; therefore, coffee shows a protective effect on airway function due to caffeine and theophylline (Schwartz & Weiss, 1992). Regular consumption of coffee can result in preventing or reducing the occurrence of asthma and bronchitis.

Coffee is also reported to have a positive effect on Type 1 diabetes patients by reducing the risk of cardiovascular events. The susceptibility of low-density lipoprotein (LDL) oxidation and formation of oxidized LDL (ox-LDL) is potentially decreased by regular coffee consumption. Coffee consumption has been reported to show a strengthening of "in the moment" information processing and one's tendency to engage in risky activities that involve more adrenaline rush. Caffeine, chlorogenic acid, and caffeic acid aid in improving gastrointestinal (GI) and liver health by directly or indirectly releasing gastrin or other GI hormones. Caffeine has a laxative effect and promotes bowel movement. Caffeine also helps to reduce the risk of alcoholic and non-alcoholic liver cirrhosis. A decline in the risk of Parkinson's disease and Alzheimer's disease has also been widely reported due to regular coffee intake (Bisht & Sisodia, 2010).

2.2.3.1. Cardiovascular disease

Studies have extensively investigated the relationship between caffeine consumption and its effect on various cardiovascular outcomes (Prineas et al., 1980), and this has been the subject of extensive research. Caffeine, a widely consumed stimulant found in coffee and other beverages, elicits various physiological responses upon ingestion, including central nervous system stimulation and acute cardiovascular effects such as increased blood pressure, arterial stiffness, and endothelium-dependent vasodilation (Berne et al., 1974). Although caffeine has been associated with an increased metabolic rate and diuresis, its impact on cardiovascular health remains a topic of ongoing investigation. Studies have explored the potential benefits and risks of caffeine consumption on CVD, with findings suggesting a complex interplay that necessitates further exploration to better understand the effects of caffeine intake on cardiovascular outcomes.

Although some studies suggest potential benefits of caffeine consumption, such as neuroprotective properties, there is currently no direct evidence that caffeine or its metabolites impact lipid metabolism or antioxidant activity. Moreover, research findings have not conclusively demonstrated that the caffeine content in coffee provides protective effects against CVD; under certain conditions, it may even pose risks. The intricate interplay between caffeine intake and cardiovascular health necessitates further exploration to better understand the potential impacts of caffeine consumption on cardiovascular outcomes (Robertson & Curatolo, 1982). Faudone et al. (2021) reported that caffeine intake reduces the risk of CVD.

The intricate relationship between caffeine consumption and cardiovascular health presents a multifaceted landscape that requires comprehensive investigation. Although some studies hint at the potential benefits of caffeine consumption, such as neuroprotective properties, the direct impact of caffeine on lipid metabolism, antioxidant activity, and CVD remains inconclusive. The diverse physiological responses triggered by caffeine intake underscore the need for continued research to elucidate the underlying mechanisms of cardiovascular outcomes. Further exploration into the interplay between caffeine consumption and cardiovascular health is essential to inform evidence-based recommendations and strategies for optimizing cardiovascular well-being in individuals who consume caffeine regularly (Bidel & Tuomilehto, 2012).

2.2.3.2. Anticancer activity

The relationship between coffee consumption and cancer risk has gained significant attention due to the diverse array of coffee constituents that may potentially confer protective effects through various biological mechanisms. Coffee, being a primary source of caffeine, has been the subject of interest in cancer research with studies exploring the impact of caffeine on different types of cancer. Some investigations have reported an association of breast cancer with coffee consumption (Baker et al., 2006; Nkondjock, 2012) and a protective effect of coffee constituents, such as cafestol and kahweol, against colorectal cancer (Cavin et al., 2002; Nkondjock, 2012). Cavin et al. (2002) suggest a possible link between caffeine intake and certain cancer types, whereas others have not found a significant association; for example, in a study on coffee consumption and stomach cancer risk Larsson et al. (2006). The intricate relationship between caffeine consumption and cancer risk necessitates further exploration to clarify the mechanisms and potential implications of caffeine on various cancer types.

Coffee consumption has been associated with potential protective effects against cancer through various mechanisms. Reductions in cholesterol, bile acid, and neutral sterol secretion in the colon, as well as increased colonic motility induced by coffee consumption, may directly impact carcinogen exposure in the colon. Additionally, the diuretic effect of coffee consumption, attributed to caffeine, may contribute to reducing the risk of kidney cancer by blocking the secretion of antidiuretic hormones. Studies have indicated that caffeine can both stimulate and suppress tumours, with outcomes varying depending on the type of tumour and the timing of administration. Although some research suggests a potential reduction in cancer risk associated with coffee consumption, particularly in hereditary breast cancer, the effects may be limited to caffeinated coffee consumption.

The relationship between caffeine consumption through coffee and cancer risk is complex, with studies presenting conflicting findings on the potential impact of caffeine on various cancer types. Although some research suggests a possible protective effect of coffee consumption against colorectal, prostate, liver, and endometrial cancers, the mechanisms underlying these effects remain to be fully elucidated (Carter et al., 2022). Further investigation is warranted to better understand the interplay between caffeine intake, coffee consumption, and cancer risk, as well as to explore the potential benefits and risks associated with caffeine in the context of cancer prevention and treatment (Nkondjock, 2012).

2.2.3.3. Antibacterial and antifungal activity

Caffeine has been associated with antibacterial activity, creating interest in a potential role in fighting bacterial infections. The antibacterial properties of caffeine have been explored in various studies carried out by Hosseinzadeh et al. (2006), Raj and Dhala (1965), and Al-Janabi and Ali (2011), with research indicating synergistic effects with other compounds present in coffee. Understanding the antibacterial activity of caffeine is crucial for revealing any therapeutic implications and potential applications in antimicrobial strategies. The study carried out by Al-Janabi and Ali (2011) reveals that caffeine has the potential to inhibit *Staphylococcus aureus* and *Bacillus subtilis*.

Caffeine concentrations of 7 mg/mL and higher are reported to cause an inhibitory effect on the growth of *B. subtilis*, and a concentration of 10 mg/mL and above is needed to suppress the growth of *S aureus.* Seven strains were studied by Al-Janabi and Ali (2011), and among these, *Salmonella typhi, Enterobacter cloacae, E. aerogenes,* and *E. coli* were isolated Gram-negative species; these required a higher concentration of caffeine than the Gram-positive strains studied to inhibit growth. *Paracoccus yeei* was the only strain studied that showed resistance to caffeine. Similarly, a study carried out by Mewaba et al. (2022) reported that caffeine isolated from *Coffea canephora* has inhibitory properties against methicillin-resistant *S. aureus*. These studies emphasise the role of caffeine in contributing to the antimicrobial properties of coffee. Further research is necessary to dig deeper into the mechanisms by which caffeine exerts an antibacterial effect and to explore novel applications for this widely available compound in the realm of antimicrobial strategies.

AlEraky et al. (2022) showed that caffeine has inhibitory and fungicidal effects against *Candida albicans*, with a minimum inhibitory concentration (MIC) of 30 mg/mL and a minimum fungicidal concentration (MFC) of 60 mg/mL. Alfaifi et al. (2020) studied the effect of caffeine and nicotine on *Candida albicans* and revealed that in the presence of 8 mg/mL nicotine and a concentration of 1 to 32 mg/mL caffeine, biofilm formation of *Candida albicans* decreased. These studies indicate that caffeine has promising antibacterial and antifungal activity, making it a potential drug for antibiotics and antifungal formulation and treatment.

2.2.3.4. Antidiabetic activity

Type 2 diabetes, a significant global health concern, is characterized by elevated blood glucose levels (hyperglycaemia) due to defects in insulin secretion and/or action, representing a spectrum of metabolic abnormalities. Symptoms include lethargy, blurred vision, and increased

susceptibility to infections. Prolonged hyperglycaemia can lead to insulin deficiency and damage to various cells, tissues, and organs. Interestingly, there is an inverse relationship between coffee consumption and diabetes, with regular coffee drinkers showing a reduced risk of developing Type 2 diabetes. Studies suggest that this effect may be attributed more to compounds other than caffeine in coffee, as decaffeinated coffee demonstrates a similar impact on diabetes risk (Matusheski et al., 2012; Safe et al., 2023). Matusheski et al. (2012) also considered the effect of milk and/or sugar addition to coffee and reported little or no effect on an association with a reduced risk of diabetes.

2.2.3.5 Neuroprotection

Several epidemiological studies have reported the protective effect of caffeine against several neurogenerative diseases, such as Alzheimer's and Parkinson's diseases (Ritchie et al., 2007; Santos et al., 2010). The main mechanism of caffeine action in the brain is inhibition of adenosine receptors, specifically A_1 and A_{2A} receptors. Adenosine is a neuromodulator that generally lowers insomnia and encourages sleep. Caffeine inhibits inhibitory neurotransmission by opposing these receptors, which increases neuronal firing and increases the release of neurotransmitters, such as glutamate and dopamine. This antagonistic relationship can shield neurons from harm and deterioration, especially in situations such as Parkinson's disease, where studies have demonstrated the potential benefits of blocking the adenosine A_{2A} receptor. It has been demonstrated that caffeine possesses antioxidant qualities that aid in the reduction of oxidative stress and the neutralization of free radicals, both of which are important in the etiology of numerous neurodegenerative illnesses (Ritchie et al., 2007). Caffeine can help shield neurons from harm and preserve cellular integrity by lowering oxidative stress. In addition to reducing neuroinflammation, caffeine can alter the production of inflammatory cytokines. Alzheimer's disease and other neurodegenerative illnesses are characterized by persistent inflammation in the brain. It is possible to preserve neuronal function and halt the advancement of neurodegenerative disorders by reducing inflammation (Faudone et al., 2021; Panza et al., 2015; Safe et al., 2023).

2.2.4 Safety concerns related to coffee consumption.

Although coffee is widely consumed and has been linked to health benefits, there are several safety issues and possible adverse health effects with coffee intake. Depending on a person's sensitivity, underlying medical issues, and coffee intake, these concerns may differ. Sleep disruptions and insomnia are among the most prevalent problems. Excessive coffee consumption, particularly in the afternoon, can seriously disrupt sleep cycles, resulting in

trouble falling asleep and poor-quality sleep. Excessive consumption of caffeine can cause increased jitters, restlessness, and anxiety. These effects are especially noticeable in people who are predisposed to anxiety problems since caffeine may heighten symptoms, including sweating, an elevated heart rate, and uneasiness.

Polyphenols in coffee can prevent non-heme iron (iron derived from plants) from being absorbed, which could cause iron deficiency in susceptible people. For individuals with high blood pressure, caffeine may produce a transient rise in their readings. Coffee consumption can raise the production of stomach acid, which may aggravate acid reflux and heartburn, particularly in people with gastroesophageal reflux disease. Frequent caffeine usage can develop a dependency, and abrupt withdrawal symptoms such as headaches, exhaustion, agitation, and trouble focusing might occur. Consuming too much coffee may hinder the body's ability to absorb calcium, which can affect bone density and raise the risk of osteoporosis. A high caffeine intake during pregnancy has been linked to a higher risk of low birth weight, premature birth, and miscarriage (Chu, 2012; Josiane et al., 2016).

2.3 Milk proteins

Milk is a complex biological fluid rich in proteins, minerals, and fats produced by mammals to nourish and protect their newborns. These nutrients are essential for the growth and development of the neonate (Rashidinejad et al., 2017). The composition of bovine milk is given in Table 2.5. The primary role of milk is to provide the necessary amino acids and minerals that are crucial for building and maintaining muscles and other tissues in newborn mammals. Milk is slightly acidic, with a pH ranging between 6.5 and 6.7, and is sensitive to temperature fluctuations. As a perishable item, it must be refrigerated until consumed. Milk contains calcium phosphate and proteins as its primary buffering agents and is composed of sugar (lactose), fat (triglycerides), proteins, vitamins (A, D, E, B12), and minerals (calcium, magnesium), among other minor components (Fox, 2015). The proteins in milk are categorized into two main types: casein and whey proteins. Casein proteins are important for forming curds during cheesemaking and provide a slow release of amino acids during digestion. At low pH, especially at the pH in the stomach, casein is precipitated by enzymes such as chymosin, which forms a clot and slows the rate of passage through the duodenum, unlike whey proteins, which pass more quickly. Casein also acts as a source of calcium and phosphorous in the human body. Caseins trap insoluble calcium phosphate and disperse it in milk, then deliver it to the human body. Whey proteins are more soluble and quickly digested, providing a rapid supply of amino

acids (Hambraeus & Lonnerdal, 2003). Together, these proteins make milk a complete source of nutrition, supporting the overall health and growth of the newborn.

Table 2.5 Bovine milk composition (Walstra et al., 2006)

Component	Average Content in Milk (% w/w)	Range (% w/w)	Average Content in Dry Matter (% w/w)
Water	87.1	85.3–88.7	—
Solids-not-fat	8.9	7.9–10.0	—
Fat in dry matter	31	22–38	—
Lactose	4.6	3.8–5.3	36
Fat	4.0	2.5–5.5	31
Protein	3.3	2.3–4.4	25
Casein	2.6	1.7–3.5	20
Mineral substances	0.7	0.57–0.83	5.4
Organic acids	0.17	0.12–0.21	1.3
Miscellaneous	0.15	—	1.2

Milk is an oil-in-water type of emulsion with fat globules suspended in the aqueous phase. Milk fat mainly consists of triglycerides (97-98%), plus diacylglycerides, monoglycerides, free fatty acids, phospholipids, and sterols (cholesterol). The fat globules (~0.1-20 μm) contain an outer membrane called milk fat globule membrane (MFGM) and are responsible for keeping the globular structure of fat in the milk. This layer mainly consists of proteins and phospholipids (MacGibbon, 2020). The yellowish tint of milk fat is contributed by carotenoids, and these are responsible for most of the vitamin A activity in milk. Lactones and acetoin found in milk fat are the main contributors to milk flavour, which is more prominent in butter and dried milk (Gu et al., 2022). Milk and dairy products contain several antioxidants, such as tocopherols (vitamin E), low levels of ascorbic acid (vitamin C), superoxidase dismutase, and carotenoids (scavengers of free radicals). Caseins and several Maillard reaction products also show antioxidant properties in milk (Fox, 2015).

The main casein molecules are α-casein (α_{s1} and α_{s2}), β-casein, and κ-casein. Due to a large number of proline groups, casein proteins lack well-defined secondary and tertiary structures compared to whey proteins. The proteins in milk and the general attributes are given in Table 2.6. In the native state, casein in milk exists as a colloidal dispersion of an association of macromolecules. The caseins form a distinct quaternary structure known as the casein micelle of ~200 nm in size and a mass of ~10^8 Da. Although it differs from a typical detergent micelle structure, it similarly involves an association of molecules that gain stability by shielding hydrophobic surfaces from interaction with the aqueous solution. It is formed from the four

main types of caseins in association with calcium phosphate, and stabilised by hydrophobic, hydrophilic, and electrostatic interactions (Walsh et al., 2007). These are also known as calcium-caseinate phosphate complexes mainly because the caseins are bound by calcium and magnesium phosphates to the phosphorylated serine amino acids of the caseins. Caseins are amphiphilic in nature, with both hydrophobic and hydrophilic regions (Walstra et al., 2006). The average isoelectric point of the caseins is 4.6. In the laboratory, HCl or acetic acid is used as a precipitation agent to separate casein, and therefore, it is important to know which buffer is chosen for preparing milk protein solutions. Acetic acid buffer has a pKa of 4.76, and caseins coagulate in acetic acid buffer to form an insoluble gel. On the other hand, caseins and whey proteins are dispersible in phosphate-buffered saline with a neutral pH (pH 7.4).

Different models have been proposed for defining the structure of casein micelles that are classified into three main categories: internal structure model, coat-core model, and sub-micelle model. The coat-core model was proposed by Waugh and Nobel, Payens, and Parry and Carroll, (Noble & Waugh, 1965; Parry & Carroll, 1969; Payens, 1966), suggesting that the micelle structure consists of different protein compositions in the core and on the surface. However, a more recent and comprehensive model was proposed by Dalgleish (2011) (known as the nanocluster model), emphasising the role of calcium phosphate nanoclusters surrounded by casein proteins as the fundamental building blocks of the micelle. According to this model, the casein micelles do not consist of simple subunits or a clear core-shell structure. Instead, they are formed by an aggregation of calcium phosphate nanoclusters stabilized by phosphorylated regions of α_s- and β-caseins, which interact closely with the nanoclusters. This is schematically represented in Figure 2.2. κ-Casein, although only found on the micelle's surface, plays a crucial role in providing steric stabilization by forming a hairy layer that prevents aggregation and stabilizes the micelles through steric and electrostatic repulsion. This model supports a dynamic structure where the micelle's integrity and stability are primarily determined by the interactions of casein proteins with calcium phosphate, allowing for flexibility and responsiveness to environmental changes, such as pH or enzymatic activity. α_{s1}-, β-, and κ-caseins have several hydrophobic residual sites that can bind and stabilise proteins by hydrophobic association (Bhat et al., 2016). Casein micelles can be sedimented by centrifugation at 100,000 × g for 1 h, but whey proteins are non-sedimentable and remain in the supernatant.

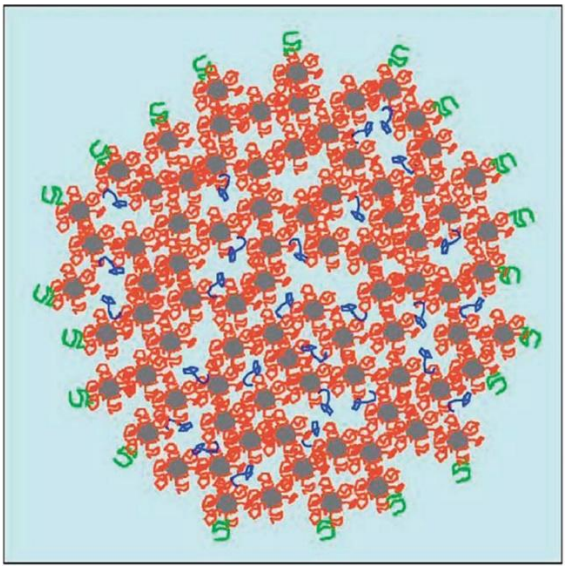


Figure 2.2 Schematic structure of the casein micelle, incorporating calcium phosphate nanoclusters (grey) with their attached caseins (red) and the surface-located κ-casein (green). The "hydrophobically bound" mobile β-casein is shown in blue within the water channels inside the micelle (Dalgleish, 2011)

Table 2.6 Proteins in bovine milk (Walstra et al., 2006)

Protein	mmol/m³ Milk	g/kg Milk	g/100 g Protein	Molar Mass (Da)
Casein	1120	26	78.3	—
α_{s1}-Casein	450	10.7	32	~23600
α_{s2}-Casein	110	2.8	8.4	~25200
β-Casein	360	8.6	26	23983
κ-Casein	160	3.1	9.3	~19550
Serum proteins	~320	6.3	19	—
β-Lactoglobulin	180	3.2	9.8	18283
α-Lactalbumin	90	1.2	3.7	14176

Whey proteins are mainly of two types, α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), as well as others such as immunoglobulins and bovine serum albumin (BSA). Whey proteins are water-soluble in nature and are soluble at pH 4.6. Whey proteins exist as small quaternary structures or monomers in milk. With a diameter of around 3.6 nm, each monomer of β-Lg possesses an essentially globular structure with a tertiary folded structure. Whey proteins are heat sensitive and denature upon heat treatment. β-Lg can bind hydrophobic molecules in a surface calyx. The ionic strength of milk is in the range of 0.07 to 0.08 mol/L and is contributed by the ions present in the milk, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), sodium (Na^{+}), potassium (K^{+}), chloride (Cl^{-}), and phosphate (PO_4^{3-}), amongst other minor ions.

2.4 Effect of milk addition on the functionality of caffeine

Interactions between milk proteins and polyphenols have been extensively researched by Soares et al. (2021), Ramalakshmi et al. (2009), Niseteo et al. (2012), and others for delivering

bioactive compounds. Milk proteins possess unique structural properties and functionalities, are readily accessible, cost-effective, and offer high nutritional value, making them exceptional for bioactive delivery systems. β-Lg exhibits a particular affinity for hydrophobic molecules, making it capable of carrying various substances. Recent investigations have demonstrated that β-Lg can bind to a range of compounds, including retinol, vitamin D, tea polyphenols, and resveratrol. Similarly, α-La has been found to interact with retinol, vitamin D, and palmitic acid.

Whey proteins and individual casein fractions also exhibit the ability to bind diverse bioactive compounds. The self-association of β-casein into micelles in aqueous solutions suggests a potential as a nanocarrier for delivering bioactive molecules. The self-assembly and co-assembly of milk proteins, along with their functional properties, show a potential for developing innovative transport systems for bioactive molecules. An interest in milk protein-polyphenol interactions has risen in recent years, driven by the growing demand for healthier food products. Food companies are increasingly focused on producing functional food items with health-enhancing properties. Dairy products, particularly milk, are considered valuable sources of beneficial substances for both children and adults. Consequently, the development of functional dairy products enriched with plant sterols, peptides, omega-3 fatty acids, and phenolic compounds has gained significant attention in the dairy market (Helal et al., 2022).

Several studies have been carried out to investigate the effect of milk addition to coffee and any changes to the efficacy of bioactive compounds (Rashidinejad et al., 2022). Coffee is bioactive in nature, with antioxidant activity, phenolic activity, and antibacterial, anticarcinogenic, and anti-inflammatory properties. Research on the effects of coffee phenolic compounds reports significant changes in overall coffee bioactivity upon milk addition. Niseteo et al. (2012) reported that milk addition reduced the antioxidant capacity of different coffee brews. Quan et al. (2020) also reported that the total phenolic bioaccessibility of regular coffee and coffee with whole or skim milk was reduced, but the reduction was higher for regular coffee and the least for coffee with skim milk. Dupas et al. (2006), on the other hand, reported that milk addition did not have any significant effect on the antioxidant power of coffee. The major factors that influence the bioactivity of coffee are the amount of milk added, the type and composition of milk, the method of preparation of coffee, and temperature. The possibility of interactions between coffee phenolics or other components such as caffeine and milk protein or fat could account for the changes in the bioactive properties of coffee.

The effects of milk fat on the bioactivity of caffeine have not been much explored to date and are still an open topic for study. Caffeine may bind to milk proteins, especially through hydrophobic bonds with β-lactoglobulin at neutral pH. These interactions could be reversible or irreversible depending on the type of bonds formed. The bioaccessibility of caffeine after interactions with milk components is an important factor in determining whether the addition of milk has an adverse effect on the bioactive properties of caffeine. Milk could act as a protective carrier for caffeine from the harsh, acidic conditions in the stomach and deliver it to other parts of the body, or it could also be a binding agent that prevents caffeine from attaching to other binding sites, thereby blocking the role of caffeine in the body and causing a negative or masking effect on the bioactivity of caffeine. The effect must be explored by carrying out *in vitro* studies under different environmental conditions, such as pH, temperature, amount, and type of milk.

2.4.1 Chemistry of protein-caffeine binding

As discussed earlier, caseins in milk are found as micelles, held together by hydrophobic interactions, hydrogen bonds, and calcium phosphate nanoclusters. The caseins possess numerous proline and limited cysteine residues, resulting in a more randomly coiled configuration compared to whey proteins. This structural characteristic, along with hydrophobic regions, makes caseins prone to self-association, interact with other proteins, and bind to various ligands. α_{s1}-Casein, α_{s2}-casein, and β-casein are phosphoproteins that can precipitate by binding calcium to serine-phosphate residues. In comparison, κ-casein is a glycoprotein containing two cysteine residues and plays a crucial role in stabilizing (both steric and electrostatic) casein micelles in milk.

Whey proteins are rich in sulphur-containing amino acids that form disulfide bonds, primarily cysteine, which stabilize the spherical structure formed by the hydrophobic association. Milk proteins have attracted attention due to interactions with polyphenols, and particularly due to their significance in human nutrition and dietary practices of consuming tea or coffee with milk. Several studies have indicated that the interactions between milk proteins and phenolic compounds can alter protein structure, stability, digestibility, and functional properties, as well as impact upon antioxidative properties and bioavailability of phenolics. In a study carried out by Niseteo et al. (2012) on the effect of different brewing techniques and milk addition on commonly consumed coffee brews, it was observed that decaffeinated espresso black coffee brews showed lesser antioxidant capacity than regular black coffee.

Additionally, decaffeinated instant black coffee samples studied by Niseteo et al. (2012) showed an increase in antioxidant power compared to decaffeinated instant black coffee samples. This indicates that caffeine has a role in the antioxidant activity of coffee brews. Milk addition also reduces the antioxidant activity of coffee. In a study by Santa Rosa et al. (2021), it was reported that β-Lg binds with caffeine molecules in the hydrophobic protein cavities (calyx). This indicates that the type of bond between the protein and caffeine molecules is hydrophobic in nature. The different types of interactions that occur between proteins and polyphenols, the mechanism of interactions, and the changes to functionality are listed by Ozdal et al. (2013) in a review paper. Hydrogen bonding, hydrophobic interactions, and Van der Waals forces (non-covalent forces) are some of the reversible interactions that are formed between different milk proteins and phenolic acids. Further research on caffeine and milk proteins is necessary to fully explore and understand the complexities of the interactions between caffeine and milk proteins.

2.4.2. Factors that affect protein-caffeine complex formation

Caffeine-protein complex formation can be affected by several factors such as temperature during preparation, pH and ionic strength of both caffeine and milk protein solutions, structure of milk proteins, and the caffeine: milk protein ratio. These factors are explained in detail in the following sections. Nevertheless, work done by Tenney et al. (2017) suggests that whey protein isolate does not bind to caffeine and doesn't inhibit the masking effect of caffeine. On the other hand (Santa Rosa et al., 2021) reported that β-Lg forms complexes with caffeine. These contradicting reports open the possibility for further study of caffeine: milk protein interactions and the effects of digestion.

2.4.2.1. Ionic strength and pH

In a study by Stanciuc et al. (2020), it was found that the pH significantly influences the structure and behaviour of β-Lg. At different pH levels, β-Lg undergoes distinct transitions and structural changes that impact upon functionality. For example, at low pH values (around 2 to 3), β-Lg exists as a monomeric structure (denoted as M) due to electrostatic repulsion between dimers and exhibits high stability. As the pH increases, specific transitions occur, such as the N-to-Q (acidic quasi-native (Q) state to the native (N) dimeric state) transition or vice versa between pH 4.5 and 6, leading to volume increases or decreases. A Q-to-N transition involves changes in compactness. At pH 7, β-Lg undergoes an N-to-R (native state to relaxed (R) state) transition, also known as the Tanford transition, resulting in volume decreases and changes in

protein hydration. These transitions are associated with alterations in the intrinsic compressibility and interior packing of the protein. The accessibility of ligands to the calyx (hydrophobic pockets) of β-Lg, a crucial binding site, is pH-dependent and regulated by the conformational changes of the mobile EF loop (residues 85-90). The EF loop opens when Glu^{89} is deprotonated, allowing ligand access to the calyx. Studies have shown that the calyx site is accessible at pH values close to neutral, indicating the importance of pH in ligand binding to β-Lg (Dominguez-Ramirez et al., 2013). This opens a possibility for caffeine binding to β-Lg near neutral pH. Therefore, at around pH 7 (approximately near the pH of milk), caffeine may also interact with β-Lg by hydrophobic association.

In contrast, caffeine may interact less with milk proteins at neutral pH. The pH of a solution has an important role in determining the surface charge of the adsorbent molecule and the degree of ionisation, which in turn has an impact on the adsorption process. At an acidic pH, the adsorbent surface will be positively charged, and negatively charged molecules have a greater propensity to bind to the molecule and less so at basic pH. The pH of a caffeine solution is about 6.55. The average isoelectric point of caseins is 4.6, and milk has a pH of 6.6; therefore, caseins have a net negative charge. Greater repulsion of caseins and negatively charged caffeine molecules will occur at this pH. In studies done by Fakioglu and Kalpakli (2022), it was found that the optimum pH for caffeine adsorption is at pH 2-3 for different solutions. Therefore, the effect of pH on caffeine and milk protein in milk-based coffee can only be confirmed by studying caffeine-milk protein interactions at different pH levels.

2.4.2.3. Temperature

Coffee is typically prepared at temperatures above 60°C. Temperature has an impact on hydrophobic associations and hydrogen bonds. As the temperature increases, the bond length of water molecules increases, the bond angle reduces, and the hydrogen coordination number becomes smaller (Li, 2022). There is an increased possibility of hydrophobic bonds forming as the temperature increases (Ozdal et al., 2013). Proteins also undergo denaturation when heat-treated, and this unfolds the proteins, leading to exposed areas for binding (Li et al., 2021). The solubility of caffeine increases as the temperature increases, and temperature changes can also cause structural changes in the proteins, which affect caffeine-protein interactions. Polyphenol-protein interactions have been reported to show conflicting effects based on the type of protein and polyphenols. BSA shows a significant decrease in binding after heat denaturation, but α-La shows no significant effect on binding to tea polyphenols (Yildirim-

Elikoglu & Erdem, 2017). Santa Rosa et al. (2021) reported that caffeine-β-Lg complex formation is reduced as temperature increases. The effect of temperature on caffeine-milk protein interactions is very relevant as the coffee beverage is consumed mostly as a hot beverage; therefore, temperature is crucial in deciding the fate of caffeine bioaccessibility.

2.4.2.4. Caffeine: protein ratio

The protein: caffeine ratio is one of the important factors in determining the possibility of interactions between caffeine and milk proteins and the type and strength of the bonds formed. A sufficient concentration of caffeine compared to protein is necessary to induce a significant degree of interactions. Santa Rosa et al. (2021) studied the binding of caffeine with β-Lg and found that caffeine interacted with β-Lg. Based on the method of analysis it was observed by fluorescence spectroscopy that β-Lg interacted with caffeine to form a 1:1 stoichiometric complex, and a different stoichiometric ratio of 1:3 using isothermal titration calorimetry. Isothermal titration calorimetry is an efficient method to study the effect of concentration on protein-ligand binding. Xin et al. (2020) studied the interactions between β-Lg and selected phenolic acids and reported that as the concentration of the phenolic acid increased, binding with β-Lg also increased. In a practical context, coffee beverages, particularly flat whites, cappuccinos, and lattes, have a milk-to-coffee ratio of 2:1. The amount of protein present in a cup of these type of coffee is more than the amount of caffeine. Consequently, most of the caffeine could bind to all possible protein sites. Another possibility is that due to the high protein concentration, self-association of proteins may occur, reducing the number of binding sites available for other ligands. It is crucial to understand the effect of the caffeine-to-protein ratio as this affects the interactions between caffeine and milk protein, thereby impacting the nutritional and functional properties of both components.

2.5. The effect of interactions on the functionality and health-promoting effects of caffeine

The consensus in the scientific literature suggests that milk components, such as milk fat and milk proteins, can bind to polyphenols in tea and coffee brews and that this will have an impact on the bioactive efficacy of caffeine, CQAs, and caffeic acid (Al-Shabib et al., 2023; Ali et al., 2013; Stanciuc et al., 2020). This can indeed affect the functionality of the coffee brews. Properties such as antibacterial activity, anticarcinogenic activity, antidiabetic effect, and improved cognitive function are characteristics of caffeine when coffee is consumed (Rashidinejad et al., 2022). Milk addition might have a significant impact on these activities,

and this can be explained by the interactions of caffeine with milk proteins. These can be studied by antioxidant assays and caffeine content evaluation.

The effect of milk addition on coffee and the impact upon structure, composition, and functionality of caffeine has yet to be explored. Caffeine binding to milk proteins, such as β-Lg, has been reported by Santa Rosa et al. (2021) and Tenney et al. (2017). Tenney et al. (2017) reported that caffeine was bound minimally to whey protein isolates, and there was no significant effect on the bitterness of caffeine upon combining whey protein isolates with caffeine. Santa Rosa et al. (2021) also reported that caffeine and β-Lg interact, and the types of bonds formed are hydrophobic and hydrophilic, depending upon the temperature of interactions. At 294.2 K, the C-C bonds are in the *trans* conformation; thus, the unfolded β-Lg is organised into a linear form, and there will be more exposure to hydrophobic amino acids where caffeine can bind. At 310.6 K, C-C bonds are converted to the *cis* form, and the hydrophobic regions will become partially folded and less available for caffeine to bind. In this case, caffeine may bind to hydrophilic sites. These bonds are reversible; therefore, milk proteins have the potential to act as a delivery agent for caffeine to the body (Santa Rosa et al., 2021).

2.6. The effect of interactions on the structural changes of proteins

The impact of caffeine on the structure of milk proteins is not yet fully understood. Several studies of proteins and polyphenols suggest that α-casein has a lower affinity towards polyphenols than β-casein (Al-Hanish et al., 2016; Li et al., 2020; Xiao et al., 2011). This can be attributed to the more hydrophobic nature of β-casein. A similar effect can be anticipated with caffeine due to the potential for hydrophobic interactions between caffeine and proteins. Conformational changes were also observed in casein proteins, with an increase in random coils. Hydrophobic interactions between β-Lg and polyphenols were also identified. Caffeine can form similar interactions with β-Lg due to the C=O groups present in the structure. As a result of these interactions, there will be changes in the conformation of both caffeine and β-Lg with vibrations or stretching in the C-H and/or C=O bonds in the caffeine molecules.

The overall molecule size (due to changes in the C=O bond length), charge, and optical activity of caffeine can also change because of these interactions. A stronger structural stabilisation was noted for β-Lg when it interacted with polyphenols (Ozdal et al., 2013). Understanding the structural changes in caffeine and milk proteins is important due to their effect on digestibility and bioaccessibility. The structure of caffeine is significant as it is a neurostimulator and acts as a ligand when binding to CNS adenosine receptors.

2.7. Bioavailability of caffeine from coffee

The effect of milk addition on the bioaccessibility and bioavailability of caffeine is still largely unknown. It has been reported that there is a significant reduction in the amount of several phenolic compounds when milk is added to coffee and tea (Jeon et al., 2019; Quan et al., 2020). Therefore, milk addition has a negative, or masking, effect on the functional groups of phenolics in coffee. However, several other studies state that milk addition can have a neutral or positive impact on the functional groups of coffee. This could be due to the ability of milk proteins to act as a carrier for delivering the functional groups to target areas in the human gut and providing protection from the harsh conditions in the stomach, thereby improving bioavailability and bioefficacy (Alongi et al., 2019; Renouf et al., 2010). A similar effect can be seen for caffeine. It must also be noted that milk proteins are bioactive compounds and can improve the nutritional properties of the coffee beverage.

The likelihood of forming irreversible bonds between caffeine and milk proteins is lower compared to other phenolic compounds present in milk (Ozdal et al., 2013). This is due to the functional groups in caffeine, which are amide groups, methyl groups, and an imide group, which tend to form reversible interactions. As a result, the amount of caffeine that reaches the target organs could conceivably be similar to the initial amount of caffeine. At a pH of 2-3 in the gastric phase of digestion, there is a chance of stronger interactions between caffeine and milk protein, as discussed in Section 2.4.2.1. During the gastric phase of digestion, the probability of the amount of caffeine remaining the same is higher. When caffeine reaches the intestinal phase, with a pH of around 7, the bonds between caffeine and milk protein slowly dissociate to form the original compounds, making them available for absorption through the intestinal epithelium (Ozdal et al., 2013).

It is necessary to carry out *in vitro* gastrointestinal digestion experiments to determine the amount of caffeine recovered in each phase of digestion. This will shed light on the bioaccessibility of caffeine with the addition of milk. However, to understand the bioavailability of caffeine, *in vivo* experiments need to be carried out. It is essential to understand the bioaccessibility and bioactivity of caffeine to determine if the addition of milk diminishes the bioactive compounds in coffee. This has been a topic of discussion over the past few decades, as coffee and tea are two of the most popular beverages consumed worldwide.

2.8. The effect of caffeine-protein interactions on beverage/infusion properties

Black coffee has a rich aroma, a defining characteristic feature of coffee brews, due to the volatile compounds present in the coffee. Roasting coffee increases the level of melanoidin compounds. Roasting can also influence the aroma of coffee beans due to the myriads of volatile compounds generated. A shot of coffee has a rich crema on top if brewed using an espresso machine. It has a strong flavour and astringent mouthfeel due to the phenolic compounds and caffeine. The bitterness and astringency of coffee can be reduced by the addition of milk or dairy products. The sensory properties of coffee are significantly affected by milk addition. Black coffee brews have a water-like consistency, but when milk is added, coffee turns more viscous and has a frothy texture. The extent depends upon the type of milk or dairy products used. Some people prefer alternative kinds of milk mimetics over cows' milk, and this can also affect the flavour. Milk fat gives a richer texture to the coffee, adding a slight buttery flavour.

Caffeine can bind to both milk fat and protein, thereby reducing the bitterness of the coffee. The chance of binding to α- and β-caseins is higher because these proteins are more hydrophobic (Li et al., 2020). However, a study carried out by Tenney et al. (2017) to reduce the bitterness of caffeine using whey protein isolates concluded that there was no significant reduction of the bitterness of caffeine as only a small amount of whey proteins were bound to caffeine. Other volatile compounds can also bind to milk proteins and fats and lead to a reduction in the aroma intensity of coffee. These changes depend upon how much the milk is heated and frothed. Milk can have a silky-smooth texture when steamed for making flat white and lattes. The main impact on the sensory properties due to caffeine-milk protein interactions is on the bitterness and the body of the coffee, giving it a velvety texture and making the beverage feel more luxurious and fuller-bodied. The aftertaste left on the palate will also be more pleasant with reduced astringency.

2.9. Evaluation of methods for studying caffeine-milk protein interactions

2.9.1. Quantification of caffeine content

2.9.1.1. UV spectroscopy

Caffeine content can be determined analytically using UV spectroscopy in a variety of materials, including drinks, medications, and biological fluids. This technique is based on the quantifiable absorption of UV light by caffeine molecules, which allows one to ascertain the quantity of caffeine. Caffeine molecules in a solution absorb energy when exposed to UV light,

which causes an electronic transition from the ground state to the excited state. Li et al. (2020) used a UV spectrometer to quantify the phenolic acids. The sample solutions were prepared and measured in a range from 200 to 400 nm using a 1 cm quartz cuvette. Caffeine standards were prepared, and the calibration curve was plotted to determine the concentration of the unknown samples. Although this technique is simple and less sophisticated, it has several disadvantages. Coffee contains various compounds that can absorb UV light at similar wavelengths as caffeine. This can lead to overlapping spectra and inaccurate quantification of caffeine. It may not be sensitive enough to detect low concentrations of caffeine accurately. Furthermore, extensive sample preparation may be required to remove interfering substances and extract caffeine, which can be time-consuming and may lead to sample loss or contamination. UV spectroscopy provides limited information about the chemical structure of the analyte, making it difficult to confirm the presence of caffeine without complementary techniques such as HPLC or mass spectrometry.

2.9.1.2. High-performance liquid chromatography (HPLC)

HPLC is a fast, flexible, and reproducible method for both qualitative and quantitative analysis of liquid samples. HPLC separates the components of a liquid sample based on polarity using a column, and the components can be identified based on the retention time from the plot generated by the software after passing through the detector. The area under each peak also helps to quantify the amount of each component. The solvents used for HPLC analysis must be of high purity. The sample and the solvents must be free from suspended particles or chemical impurities to avoid clogging the column or presenting spurious peaks. To eliminate suspended particles, the sample must first be sonicated and filtered through a Millipore-type filter before injecting it into the sample port. Normal phase HPLC and reverse phase HPLC are the most used types of HPLC, and this categorisation is based on the polarity of the column packing. Caffeine, theobromine, and theophylline content of roasted Arabica and Robusta coffee beans were quantified by Huck et al. (2005) using liquid chromatography.

2.9.2. Evaluation of total phenolic content

2.9.2.1. Folin-Ciocâlteu method

A commonly used colorimetric assay for determining the total phenolic (TP) content of different samples is the Folin-Ciocâlteu method. This procedure is based on the reduction of the Folin-Ciocâlteu reagent by phenolic chemicals in an alkaline environment, producing a blue complex that can be detected using spectrophotometry. This method is very sensitive and even low concentrations of phenolic compounds in the sample could be detected. When performed

under standardized conditions, it provides consistent and reproducible results. A technique modified from that of Quan et al. (2020) was used to assess TP content. Gallic acid equivalents (GAE) in milligrams (mg) per litre were used to express the results.

2.9.3. Evaluation of antioxidant activity

2.9.3.1. ABTS assay

The ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) assay is a commonly used colorimetric assay for determining antioxidant potential. The basis of this assay is the capacity of antioxidants to inhibit the ABTS radical cation ($ABTS^{\bullet+}$) generated by the oxidation of ABTS by potassium persulfate, resulting in a spectrophotometrically measurable reduction in colour intensity at 734 nm. Since the ABTS•+ radical reacts quickly with both natural and synthetic antioxidant chemicals in food components, TEAC tests enable the detection of a wide range of antioxidant substances. Although the sample for which antioxidant activity is tested frequently affects the pH value, the TEAC antioxidant test can be performed throughout a broad pH range. This is because the reaction process might change depending on the pH, for example, an acidic environment can enhance electron transport. Approaches for determining hydrophilic and lipophilic antioxidant activity were developed as a result of ABTS•+ solubility in buffered and organic environments. The aggregate of these approaches provided an accurate assessment of the products' antioxidant capacity. The TEAC test is inexpensive and easy to use. The ABTS assay of coffee samples was determined using the method carried out by Quan et al. (2020). The absorbance was then measured at 734 nm using a UV spectrophotometer. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) in mmol Trolox per litre.

2.9.3.2. DPPH assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a quick, easy, and accurate way to measure antioxidant activity. This test assesses the DPPH radical's decrease, revealing substantial details about a substance's capacity to scavenge free radicals. The basis of this assay is the reduction of the deep violet-coloured, stable free radical DPPH to a yellow-coloured diphenyl picrylhydrazine. As antioxidants interact with DPPH, it is converted to DPPH-H, with decreased absorbance. The degree of discolouration reveals the capacity of an antioxidant to scavenge hydrogen by donating energy. Derossi et al. (2018) carried out the DPPH assay of coffee samples by adding 100 µL of sample to 900 µL of DPPH solution prepared in 99.5% ethanol and kept in the dark for 2 h. The colour reduction was determined by measuring

absorbance at 517 nm. Trolox was used as a calibration standard, and results were reported as TEAC in mg Trolox per mL of brew.

2.9.3.3. Ferric reducing antioxidant power (FRAP) assay.

A common technique for determining antioxidant potential is the Ferric Reducing Antioxidant Power (FRAP) assay, which may be used to evaluate food extracts, drinks, and biological materials. Antioxidants in an acidic environment reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions, forming a blue-coloured complex that can be detected spectrophotometrically at 593 nm. Ryan and Petit (2010) estimated the antioxidant capacity using FRAP assay. The FRAP reagent was prepared by mixing 10 mmol/L of 2,4,6-tripryridyl-s-triazine with 40 mmol/L of hydrochloric acid, 300 mmol/L of acetate buffer (pH 3.6), and 20 mmol/L of ferric chloride and combined in a 10:1:1 ratio. The sample and reagent were incubated at 37°C for 4 min to carry out the FRAP assay. In addition to being incubated at 37°C, a reagent blank was used to measure absorbance at 593 nm. The total antioxidant capacity of the samples was assessed in comparison to ferrous sulphate, which has a known FRAP value of 1000 μmol/L.

2.9.3.4. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay evaluates antioxidant potential by measuring the capability to scavenge peroxyl radicals produced by a particular source, such as AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride). Over time, there is a decrease in fluorescence due to the reaction between these peroxyl radicals and a fluorescent probe, usually fluorescein. If an antioxidant is present, it inhibits the degradation of the probe by competing with peroxyl radicals. The difference in integrated areas under the decay curves (AUC) for the fluorescein solution produced, regardless of the compound of interest, is used to evaluate the antioxidant capability. This represents the idea that lag time, rate, and total inhibition are all contained in one value. As a result, the assay evaluates the antioxidant under test capacity rather than just reactivity. Ramalakshmi et al. (2009) used Trolox standard solutions of 6.5 to 50 μM concentration, which were prepared from 2 mM stock solution. A fluorescein sodium salt solution (8.16 μM) was used to prepare a working standard of concentration 81.6 nM. An AAPH solution of 200 mM was also prepared. The sample or standard (25 μL) was added to 150 μL of fluorescein and incubated for 30 min in the dark at 37°C. AAPH solution (25 μL) was added to initiate the oxidation reaction. The fluorescence intensity was measured immediately at regular intervals from 60 to 120 min. The excitation wavelength was 485 nm, and the emission wavelength was 520 nm. Results were expressed as TEAC in μmol Trolox equivalent per litre.

2.9.4. Determination of interactions between milk proteins and caffeine

2.9.4.1. Fourier transform infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) is a technique used for analysing the secondary structure of proteins. FTIR helps in determining the functional groups present in a compound. The sample absorbs the light which will cause vibrations in the molecules based on the functional groups present. The non-absorbed wavelengths are transmitted to the detector which generates a graph with a time domain which is then converted to a frequency domain plot (Al-Hanish et al., 2016). The graph of intensity against wavenumber helps to identify the functional groups and types of vibrations in the compound. FTIR spectra were obtained using the procedure of Li et al. (2020) with a resolution of 4 cm^{-1} using the attenuated total reflection technique, yielding 64 scans from 4000-400 cm^{-1}. The absorbance spectra of the buffer were reduced from the sample spectra, and baseline corrections were made in the 1600–1700 cm^{-1} range to produce the amide I band. These parameters were used to guide a curve-fitting technique that identified which Gaussian-shaped curves best fit the original protein spectra. Once specific bands have been identified, the percentages of each secondary structure of the protein were ascertained by comparing the relative areas of each component band.

2.9.4.2. Particle size and ζ-potential

The stability and behaviour of charged colloidal dispersions in a liquid media can be inferred from the ζ-potential. Particle surfaces may acquire a charge through mechanisms such as the dissociation of surface groups or the adsorption of ions. Dispersion in water results in an electrical double layer around the particle, which consists of two parts: the Stern layer, where counterions are tightly bound to the particle surface, and the diffuse layer, where a cloud of ions is loosely associated with the particle. The ζ-potential is the electrical potential measured at the boundary between this diffuse layer and the bulk liquid (the plane of shear), representing the difference in potential between the hydrodynamic plane of shear and a point infinitely distant from the particle.

2.9.5. Evaluation of in vitro bioaccessibility of caffeine

Living beings consume food containing carbohydrates, fats, proteins, minerals, and vitamins to meet their nutritional requirements. These nutrients are broken down into simpler molecules within the body and processed by various organs. The process of digestion is very complex, which requires mechanical and chemical processes and varies according to species. Several studies have been carried out in the past to understand these mechanisms, and *in vitro* methods have been developed to mimic the process of digestion.

In vitro and *in vivo* digestion models have been developed and utilised to simulate human food digestion, enabling the investigation of the amount of various nutrients absorbed by the human body and to study of the bioaccessibility and bioavailability of different food components. Bioaccessibility refers to the quantity of a food component released into the gastrointestinal tract that is available for absorption. Bioavailability defines the amount of food that is absorbed into the circulatory system and available for physiological functions (Grundy et al., 2024). There are several types of *in vitro* digestion models: static, semi-dynamic, and dynamic being the three main types used by researchers.

An *in vitro* digestion model comprises three major phases: the oral phase, the gastric phase, and the intestinal phase. Carbohydrate digestion primarily occurs in the mouth, protein digestion in the stomach, and fat digestion in the stomach and intestines, with most fat digestion taking place in the intestines (Julie et al., 2024; Omer & Chiodi, 2024; Trommelen et al., 2021). Alkaloids are mainly digested in the stomach and intestine, metabolised in the liver, and excreted through urine. Caffeine, an alkaloid, is absorbed through the lining of the stomach and small intestine. Absorption is typically complete within 45 min after ingestion (Fredholm et al., 1999). Approximately 90% of the caffeine is cleared from the stomach within 20 min of ingestion, and peak plasma concentration is reached within approximately 1 to 1.5 h (Heckman et al., 2010).

Caffeine is metabolised in the liver. This metabolic process converts caffeine into three primary metabolites: paraxanthine, theobromine, and theophylline (Rubem et al., 2012). A static *in vitro* digestion was carried out using the harmonized INFOGEST method developed by Minekus et al. (2014). In the oral phase, 5 mL of the sample was mixed with 1 mL of simulated salivary fluid (SSF) containing 112.6 mg KCl, 50.36 mg KH_2PO_4, 114.2 mg $NaHCO_3$, 3.1 mg $MgCl_2(H_2O)_6$, and 0.6 mg $(NH_4)_2CO_3$. The pH was adjusted to 7 with 1 mol/L NaOH, followed by 75 µL of $CaCl_2$ (441 mg) and 307 µL of salivary α-amylase (75 U/mL), and diluted to 15 mL with deionized water. Samples were incubated at 37°C for 10 min, then cooled on ice. In the gastric phase, 1 mL of simulated gastric fluid (SGF) containing 51.5 mg KCl, 12.3 mg KH_2PO_4, 210 mg $NaHCO_3$, 2 mg $MgCl_2(H_2O)_6$, 4.81 mg $(NH_4)_2CO_3$, and 274 mg NaCl was added. The pH was adjusted to 3 with 5 mol/L HCl, followed by 15 µL $CaCl_2$ and 100 µL pepsin (2000 U/mL). The volume was adjusted to 30 mL with deionized water and incubated at 37°C for 2 h, then cooled on ice. In the intestinal phase, 500 µL of simulated intestinal fluid (SIF) containing 50.73 mg KCl, 10.9 mg KH_2PO_4, 714 mg $NaHCO_3$, 6.71 mg NaCl, and 222.7 mg

$MgCl_2•(H_2O)_6$ was added. The pH was adjusted to 7 with 1 mol/L NaOH, followed by 80 μL $CaCl_2$, 500 μL bile, and 100 μL pancreatin (2000 U/mL). The volume was adjusted to 40 mL with deionized water, incubated at 37°C for 2 h, then cooled on ice.

2.10. Review summary

Given the global popularity of coffee as a beverage, it is pertinent to examine the role of each component within it, particularly those that are bioactive and that have an impact on human health. Numerous studies have been carried out worldwide on the composition of coffee beans, gaining insights into the factors that influence the composition of coffee components. These include the effect of species, degree of roasting, geographical factors, brewing methods, and the coffee-to-water ratio (Olechno et al., 2021). The bioactive properties and nutritional benefits of these coffee components have been the subject of study for many years. In addition to the health-promoting properties of coffee discussed in Section 2.2.3., coffee is also recognised as a beverage with a stimulatory effect on the central nervous system, aiding individuals in staying awake (Chu, 2012; Grosso et al., 2017; Nawrot et al., 2003). This cognitive effect contributes to coffee's worldwide popularity (Dews, 1982; Panza et al., 2015). The primary bioactive components of coffee, particularly phenolic compounds and caffeine have been extensively reviewed (Nosal et al., 2022; Pilipczuk et al., 2015). The influence of various factors on these components' bioactive properties has also been the focus of several investigations (Josiane et al., 2016; Niseteo et al., 2012; Ramalakshmi et al., 2009; Soares et al., 2021).

Coffee is consumed both black and with the addition of various components such as milk and other dairy products (e.g., cream), which may affect its composition, nutritional factors, antioxidant potential, phenolic properties, digestibility, and the bioaccessibility of its bioactive compounds. The primary effect of adding milk on phenolic compounds, as well as their bioaccessibility, bioavailability, and antioxidant capacity, has been studied by numerous researchers (Bhagat et al., 2019; Dupas et al., 2006; Helal et al., 2022; Quan et al., 2020). However, as a result of this review, a research gap was identified in the effect of milk components (protein and fat) on caffeine bioaccessibility. Accordingly, this study was conducted under the hypothesis that caffeine interacts with milk proteins, and thus, the addition of milk negatively impacts the antioxidant activity, total phenolic content, and the *in vitro* bioaccessibility of caffeine in coffee beverages, which may, in turn, diminish its health-promoting properties. Understanding health promoting properties of caffeine is outside the scope of this thesis.

3. Materials and methodology

3.1. Materials

Bags of roasted Arabica coffee beans (2 × 1 kg) were purchased from Arrosta Coffee Roasting Co. (Palmerston North, New Zealand). The beans were of single origin from the Medellin region of Colombia. The beans had flavour notes of cocoa, spices, and nuts. Homogenised full-fat milk (Anchor Blue^{TM} milk) processed by Anchor Dairy (Fonterra Co-operative Group) was purchased from a local supermarket (New World, Palmerston North, New Zealand). Figure A1.1 of Appendix 1 illustrates the raw materials used for the experiment. The composition of the milk is provided in Appendix 1 (Table A1.1). Coffee filter paper and a manual coffee dripper were purchased from a local supermarket (PAK'nSAVE, Palmerston North, New Zealand). κ-Casein (≥70%) polyacrylamide gel electrophoresis (PAGE) and Folin-Ciocâlteu reagents were purchased from Merck (Bayswater, WA, Australia). The former was stored at -20°C and the latter at 4°C. 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), caffeine (≥90%), gallic acid (≥98.5%), Trolox (≥97%), bile salts, pepsin, amylase, pancreatin, α_{s1}-casein (≥70%), β-casein (≥98%), α-La (≥85%), and β-Lg (≥90%) (Merck Life Science Ltd, Auckland, New Zealand) were already present in the laboratory. For HPLC analysis, HPLC-grade reagents were used.

3.2. Methods

3.2.1. Preparation of caffeine and milk protein solutions

Caffeine stock solution (1 mg/mL) and subsequent standards were prepared using caffeine powder (purity ≥99%; anhydrous, MW=194.19 g/mol, procured from Merck Life Science Ltd) by dissolving in PBS buffer at pH 7.4. α-Casein, β-casein, κ-casein, α-LA, and β-LG were used to prepare individual protein solutions with a concentration of 3 mg/mL using PBS buffer. The caffeine-milk protein mixture samples were prepared by adding caffeine (0.3 mg/mL) and each protein (3 mg/mL) at a 1:2 ratio. All the solutions were stored at 4°C when used within 2 days of preparation. For long-term storage, samples were frozen and stored at -20°C.

3.2.2. Preparation of coffee brews

Bean samples were ground using a coffee grinder and manually sieved through a stainless-steel sieve with a mesh size of 600 μm before brew preparation. To simulate a standard coffee brew preparation, 10 g of coffee samples were used for the preparation of 160 mL of coffee brews (using distilled water heated to 95°C). The preparation technique is illustrated in Figure A1.2 of Appendix 1. The ground coffee was vacuum packed and stored as 10 g packets/sachets at

22℃ to avoid any changes to the physical or chemical characteristics of the coffee powder during the storage period throughout the experiments.

Brewing was carried out using the drip coffee method. Ground coffee (10 g) was transferred to a coffee filter paper and placed on a beaker using a plastic filter paper holder. Water (40 mL) was poured within a time frame of 20 seconds in a circular motion and left to drip for 1 min, and the procedure was repeated until a total of 170 mL of water at 95℃ was used for brewing (160 mL of brew was recovered after filtration). The coffee brew was divided into four equal portions, and 80 mL of homogenised and standardised milk heated to 95℃ was added to three of the four 40 mL coffee brews to obtain 120 mL coffee samples (1:2 coffee: milk ratio), and the 80 mL of hot water was added to the fourth sample (Figure A1.2). The brewing was carried out in triplicate, generating nine coffee-milk samples for further analyses.

3.3. Identification of caffeine-milk protein interactions in coffee

3.3.1. UV-spectroscopy

The caffeine content of the samples was estimated using a spectral scan in the UV range as suggested by Li et al. (2020). The samples were centrifuged and extracted, and the absorbance was measured at 273 nm using a quartz cuvette with a path length of 10 mm by placing it on a UV spectrophotometer (Thermo Fisher Scientific, Genesys 10-S, Waltham, MA, USA). The caffeine standard curve was plotted using the absorbance of caffeine standards (0.002-0.04 mg/mL). The concentration of the unknown samples was calculated using the equation obtained from the standard curve. All the measurements were made in triplicate.

3.3.2. High-performance liquid chromatography (HPLC) analysis

The caffeine concentration was determined using an HPLC method adopted by Huck et al. (2005) with slight modifications. The samples were analysed using Vanquish, UHPLC, (Thermo Fisher, Waltham, MA, USA) at 25℃ using a Kinetex 2.6 µm XB-C18 100 Å, LC column 100 x 4.6 mm (Phenomenex, Torrance, CA, USA) and connected with a microarray detector set at 215 nm. The samples were ultracentrifuged at 100,000 × g and then diluted two-fold using PBS buffer. The diluted samples were then extracted using an equal volume of 0.1% orthophosphoric acid in acetonitrile mixed using a vortex mixer and left to separate for 30 min. The samples were again centrifuged using a microcentrifuge at 22,000 × g. The supernatant was collected and filtered using a 0.22 µm Millipore syringe filter. The mobile phase was composed of solvent A with 0.1% orthophosphoric acid in water and solvent B with 0.1% orthophosphoric acid in acetonitrile. The injection volume was set at 10 µL with a flow rate of

1.5 µL/min. The elution gradient employed was 0-10% B over 0.45 min, 20% B at 2.68 min, 90% B at 3.34 min, and then holding for 3.8 min, returning to 10% by the end of 5.9 min, and then back to initial conditions within 6 min from the start. The calibration curve was plotted using a caffeine standards curve (5-320 µg/mL). The baseline was corrected using the Thermo Scientific™ Chromeleon Chromatography Data System 7, and the data was collected.

3.3.3. Total phenolic content (TPC)

Total phenolic content was determined spectrophotometrically based on a modified method of Quan et al. (2020) using the Folin-Ciocâlteu reagent. Sample (1 mL) was added to 0.5 mL Folin-Ciocâlteu reagent, and 7.5 mL of PBS buffer was added. The solution was incubated for 10 min, and 1.5 mL of 20% Na_2CO_3 solution was added, which was then maintained in the dark for 2 h. The absorbance was measured at 765 nm. Calibration standards were prepared using gallic acid standards with a range of concentrations from 0.015-0.09 mg/mL. The results were expressed in gallic acid equivalents per litre.

3.3.4. DPPH radical-scavenging activity

The DPPH radical scavenging activity was carried out using the method of Xiao et al. (2011) with modifications. Briefly, Trolox standards were prepared with concentrations from 0.005-0.025 mg/mL in PBS buffer. DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was prepared in ethanol and left in a dark, cool place for 3 h. The absorbance of DPPH solutions was adjusted to 0.71±0.02 at 515 nm. The sample (1 mL) was added to 2.5 mL of DPPH solution prepared in ethanol, and 2 mL of PBS buffer was also added to it and left in a dark place for 30 min. The absorbance was measured using a quartz cuvette with a 1 cm path length and placed in a UV spectrophotometer set at a wavelength of 515 nm. A standard curve was plotted using Trolox standards, and the results were expressed as µmol of Trolox per mL of sample.

3.3.5. Fourier transform infrared (FTIR) analysis

The FTIR spectroscopic measurements of the samples were taken using the protocol followed by Zhang et al. (2014). OMNIC software (omnic32) and FTIR spectrophotometer (Thermo Scientific, Nicolet iS5 with iD7 ATR) were used for FTIR analysis. Briefly, the resolution of the infrared spectra was set at 8 cm^{-1} by using the attenuated total reflectance (ATR) method, acquiring 60 scans. The background was run using a PBS buffer and was reduced from the sample spectra. Baseline correction was carried out in the 1600-1700 cm^{-1} range to obtain the amide I band. The absorbance spectra were then converted to transmittance data by using the

same software. The data was collected, and Originlab software was used to plot the data and for further analysis and comparison.

3.3.6. Particle size and ζ-potential measurement using dynamic light scattering

The ζ-potential and particle size of the coffee samples were analysed using a Zetasizer Pro (DTS 1235 ζ-potential Transfer Standard, Malvern Instruments Ltd., Worcestershire, UK). The solutions were diluted and filtered using a 0.22 μm Millipore filter and transferred to a DTS1070 disposable folded capillary cell (Silva et al., 2014). The particle size and ζ-potential were measured using the ZS Xplorer version 3.2.0 software. The temperature was set at 25℃ for measuring samples. The refractive index was set at 1.33. All the samples were scanned in triplicate, and data were collected (Post et al., 2012).

3.4. Investigation on bioaccessibility of caffeine in coffee

3.4.1. *In vitro* gastrointestinal digestion

Static *in vitro* digestion was carried out following the harmonized INFOGEST method developed by Minekus et al. (2014), with modifications. Briefly, KCl (3.73 g), KH_2PO_4 (6.8 g), $NaHCO_3$ (8.4 g), $MgCl_2•(H_2O)_6$ (3.05 g), $(NH_4)_2CO_3$ (4.8 g), NaCl (11.7 g), $CaCl_2•(H_2O)_2$ (4.41 g), 1M NaOH (4 g), and 6M HCl (21.9 g) stock solutions were prepared in 100 mL Milli-Q water for preparing simulated digestive fluids. KCl stock (15.1 mL), 3.7 mL KH_2PO_4, 6.8 mL $NaHCO_3$, 0.15 mL $MgCl_2•(H_2O)_6$, and 0.06 mL $(NH_4)_2CO_3$ were mixed, and 0.09 mL HCl was added to form 400 mL 1.25× Simulated Salivary Fluid (SSF). The sample (5 mL), 4 mL of SSF, 0.025 mL $CaCl_2$, and 0.75 mL salivary α-amylase (75 U/mL) were added to the flask. The pH was adjusted to 7 using NaOH, and the final volume was adjusted to 10 mL using Milli-Q water. The sample was agitated at 37℃ in a double-jacketed flask for 10 min to complete the oral phase of digestion.

The oral phase of digestion was terminated by reducing to pH 3 using 6M HCl. In the same flask with a 10 mL sample, 8 mL simulated gastric fluid (SGF) was added along with 0.005 mL $CaCl_2$, 1.6 mL pepsin (2000 U/mL), and the final volume was adjusted to 20 mL using Milli-Q water. KCl stock solution (6.9 mL), 0.9 mL KH_2PO_4, 12.5 mL $NaHCO_3$, 0.4 mL $MgCl_2•(H_2O)_6$, 0.5 mL $(NH_4)_2CO_3$, and 11.8 mL NaCl were mixed and made up to 400 mL to make 1.25× SGF. The samples were agitated for 2 h and maintained at 37℃ at pH 3 to simulate gastric conditions. After 2 h, the digestion ceased by raising the pH above 6 using 1M NaOH, and samples were collected for further analysis and testing. The samples were immediately placed in an ice bath. Simulated intestinal fluid (SIF) was made by mixing 6.8 mL of KCl stock solution, 0.8 mL

KH_2PO_4, 42.5 mL $NaHCO_3$, 1.1 mL $MgCl_2•(H_2O)_6$, and 9.6 mL NaCl made up to a final volume of 400 mL. To initiate the intestinal phase digestion, 8 mL of the gastric phase sample was added to another flask, followed by the addition of 3.2 mL of SIF, 0.016 mL of $CaCl_2$, 1.2 mL of bile salt solution (160 mM), 2 mL of pancreatin (2000 U/mL) and final pH and volume was adjusted to 7 and 16 mL, respectively. The intestinal phase was maintained under these conditions for 2 h at 37°C. To stop the intestinal phase digestion, the pH was increased to 9, and samples were placed in an ice bath. All digested samples were stored at -20°C until further analysis. PBS buffer was also subjected to digestion along with the sample as a control. As digestion progresses through its various phases, the samples undergo dilution, which was accounted for using standardised dilution factors. These factors enabled accurate comparison with the initial concentrations and were consistently applied across all tests.

In the study, the bioaccessibility of caffeine was calculated using the following equation:

$$Bioaccessibility\ of\ caffeine\ (\%) = \frac{Concentration\ of\ free\ caffeine\ post-intestinal\ digestion}{Initial\ cocentration\ of\ caffeine\ before\ digestion} \times 100 \qquad (3.1)$$

The percentage change after each digestion phase was also calculated for comparative study, and the formula used for calculating percentage change was as follows:

$$Percentage\ change = \frac{(Caffeine\ content\ in\ the\ later\ phase - Caffeine\ content\ in\ the\ earlier\ phase)}{Caffeine\ content\ in\ the\ earlier\ phase} \times 100 \qquad (3.2)$$

4. Results and discussion

4.1. Caffeine content

This study aimed to determine whether caffeine interacts with milk proteins in both their pure forms and within a coffee matrix when milk is added. Additionally, it sought to examine the effects of digestion on coffee and milk-based coffee samples, specifically assessing whether the free caffeine concentration decreases due to the digestion environment by simulating gastric and intestinal digestion, i.e., whether there is any negative effect of milk addition on the *in vitro* bioaccessibility of caffeine. The caffeine concentration in samples post-gastric and post-intestinal digestion was compared with that of black coffee prior to digestion. Furthermore, the corresponding results for each phase of black coffee were compared against milk-based coffee to evaluate the impact of milk addition. It was hypothesised that caffeine interacts with milk proteins in a manner similar to polyphenol-milk protein interactions reported previously (Niseteo et al., 2012; Quan et al., 2020; Soares et al., 2021). Such interactions are expected to reduce the concentration of free caffeine, which has biological significance, as binding with milk proteins may decrease the immediate availability (bioaccessibility) of caffeine for absorption, potentially altering its absorption rate and physiological effects.

Figures 4.1, 4.2, 4.4, and 4.5 present the caffeine content in control and caffeine-protein mixed samples at various stages of digestion. In these analyses, 100 μg/mL of caffeine was used. A slight reduction in caffeine content (<1.8 μg/mL) in the control samples prior to digestion may be attributed to experimental error. A significant reduction ($p<0.05$) in caffeine content in the control samples following the gastric phase suggests that digestion influences caffeine levels, a finding consistent with the studies of Soares et al. (2021) and Khochapong et al. (2021). Soares et al. (2021) proposed that the reduction in caffeine content after gastric digestion results from interactions between caffeine and digestive enzymes. The conformational changes in proteins under acidic conditions may expose binding sites, thereby facilitating stronger interactions between caffeine and milk proteins. However, variations in binding affinity among different protein types require further investigation.

This study does not focus on the amount of caffeine absorbed during each digestion phase or the proportion of caffeine carried over to subsequent phases; rather, it examines the free caffeine present in the supernatant, which is readily available for intestinal absorption (Pirmohammadi et al., 2023; Reginald & William, 2002). Animal studies are necessary to determine the extent of caffeine absorption into the bloodstream. The caffeine bound to proteins was removed via

centrifugation and is not considered in this study; however, the entire digesta was carried over to subsequent digestion phases.

The absence of significant differences in caffeine content in the control sample post-intestinal digestion, compared with that observed after gastric digestion, suggests that caffeine exhibits a strong affinity for digestive enzymes even under neutral conditions. However, caffeine interactions with milk proteins appear more pronounced under acidic conditions. No significant reduction in caffeine content was observed in caffeine-protein mixed samples prior to digestion, indicating that pre-digestive conditions are less conducive to caffeine-protein interactions. This may be attributed to the lack of an acidic and enzymatic environment necessary to facilitate protein denaturation and binding. Environmental factors such as pH and ionic strength play a crucial role in bond formation and require further investigation.

A statistically significant ($p<0.05$) reduction in caffeine content was observed in both caffeine/α-casein (39.04 μg/mL) and caffeine/β-casein (41.94 μg/mL) mixtures after gastric digestion, compared with the caffeine control (55.06 μg/mL). This reduction may result from interactions between caffeine, proteins, and digestive enzymes. Gastric conditions appear to destabilise caffeine, inducing conformational changes that enhance its ability to bind with proteins (Fakioglu & Kalpakli, 2022). The increase in free caffeine concentration at the end of the intestinal phase suggests that caffeine-protein interactions are weaker under neutral pH conditions. By the end of intestinal digestion, the caffeine content in these mixtures was comparable to that of the control, indicating that caffeine interacts more readily with digestive enzymes than with milk proteins during the intestinal phase. Further molecular-level studies are needed to elucidate these interactions.

In the case of the caffeine/κ-casein mixture, greater interactions were observed before digestion compared with the control. The caffeine content remained stable after both gastric and intestinal digestion, suggesting that digestive fluids bind more effectively to caffeine than milk proteins under these conditions. This indicates that caffeine/κ-casein complexes are more stable than caffeine/α-casein or caffeine/β-casein, highlighting the strong affinity of κ-casein for caffeine. κ-Casein differs significantly from α- and β-caseins due to glycosylation and lower hydrophobicity (Dalgleish, 2011). The stability of κ-casein may be due to the structural stability across digestion phases, which makes it less prone to enzymatic breakdown compared to other caseins. For caffeine/α-La and caffeine/β-Lg mixtures, a reduction in caffeine content was observed at every stage of digestion (Dominguez-Ramirez et al., 2013). However, it remains

unclear whether the bound caffeine remains bioavailable. Further research is needed to determine the bioavailability of caffeine when milk is added to coffee brews.

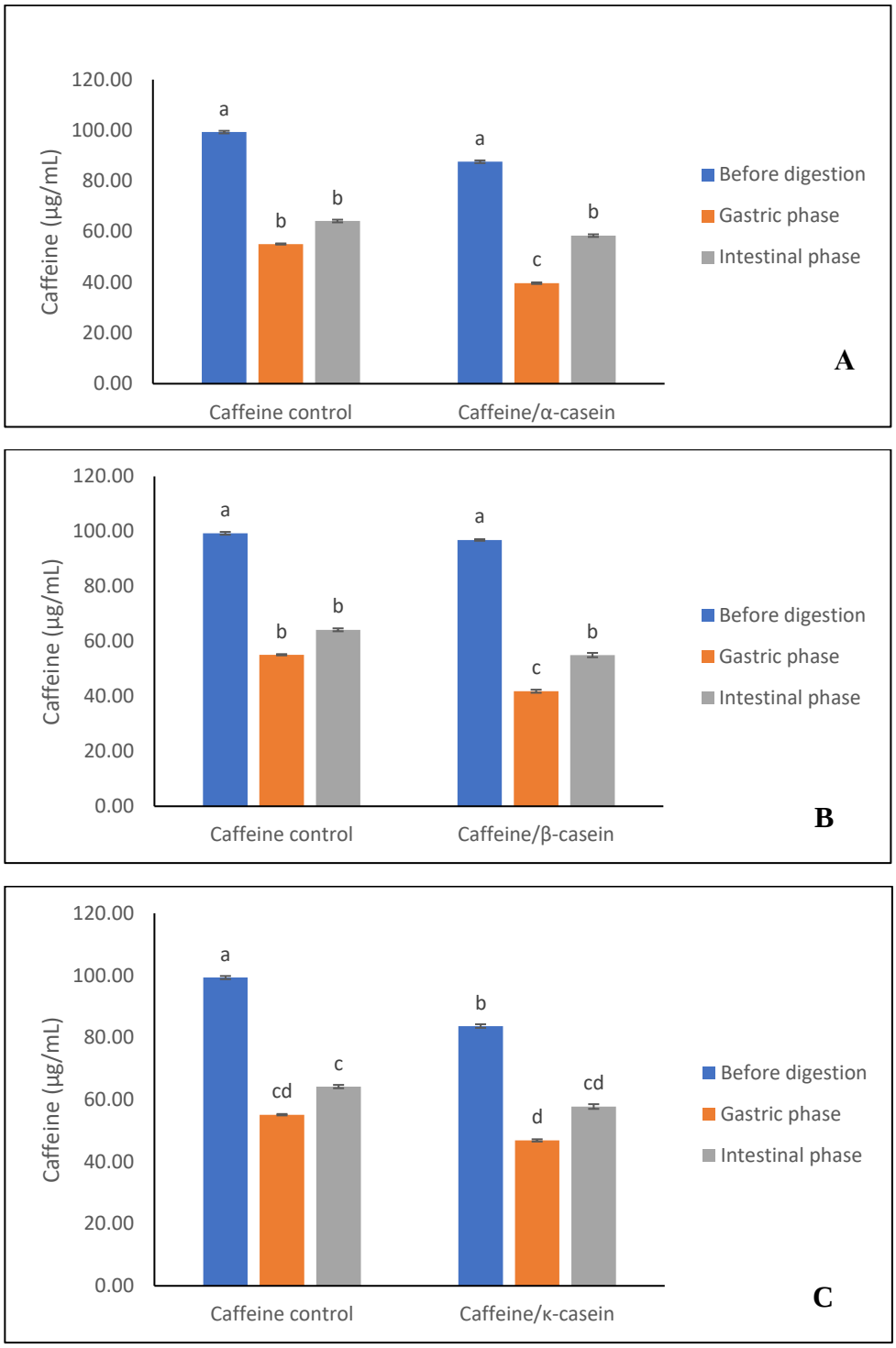


Figure 4.1 The free caffeine content of caffeine-pure milk casein protein mixtures at different digestion stages compared against the control, obtained through the UV spectroscopy method: Caffeine/α-casein (A), Caffeine/β-casein (B), Caffeine/κ-casein (C). Values are the mean±SD (n=6). Columns with different superscripts (a-d) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

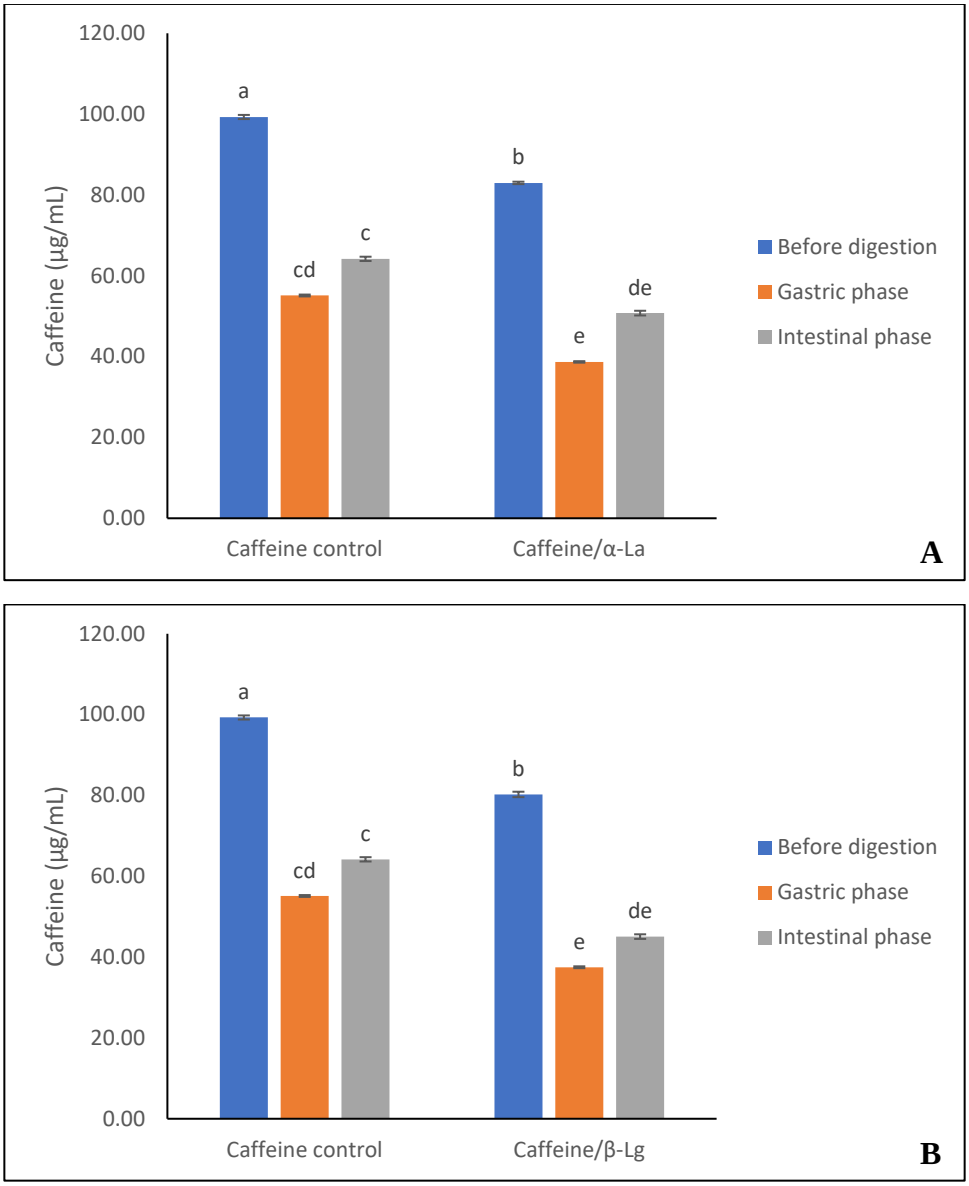


Figure 4.2 The free caffeine content of caffeine-pure milk whey protein mixtures at different digestion stages compared against the control, obtained through the UV spectroscopy method: Caffeine/α-lactalbumin (A) and Caffeine/β-lactoglobulin (B). Values are the mean±SD (n=6). Columns with different superscripts (a-e) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

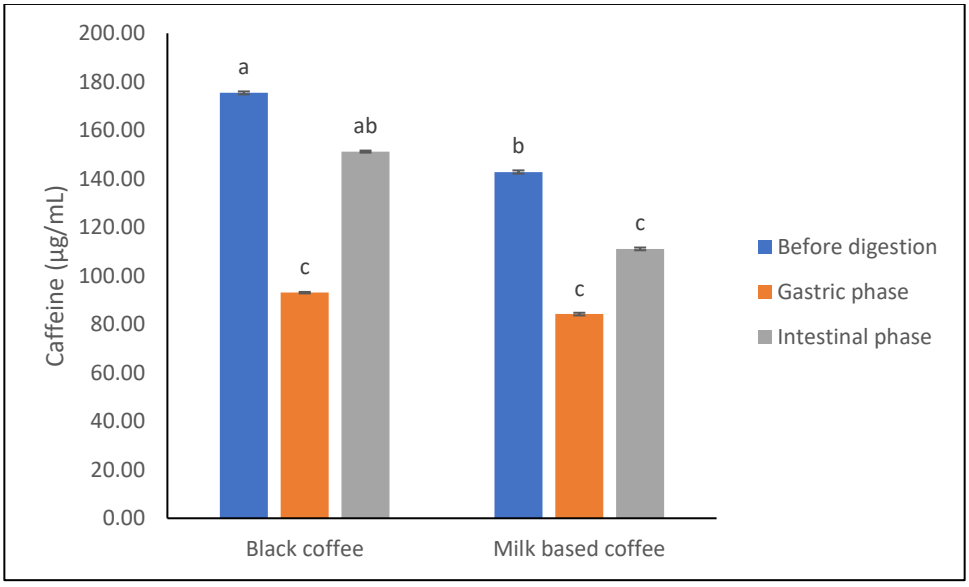


Figure 4.3 The free caffeine content of black coffee and milk-based coffee at different digestion stages obtained through the UV spectroscopy method. Values are the mean±SD (n=6). Columns with different superscripts (a-c) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA. The pH of black coffee, milk, and milk-based coffee before digestion was 4.9, 6.7, and 6.4, respectively. The pH was 3 and 7 after the gastric and intestinal phases, respectively, for all the samples.

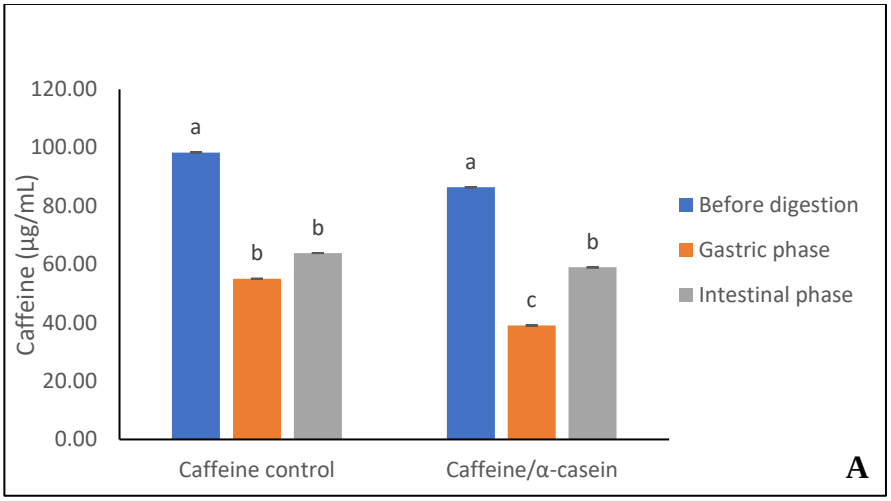
Figures 4.3 and 4.6 show the caffeine content in black coffee and milk-based coffee analysed using UV and HPLC methods at different stages of digestion. Caffeine is known to interact with chlorogenic acid and other compounds in coffee (Ishizu & Tsutsumi, 2012). In black coffee, the caffeine content measured before digestion (173.1 µg/mL) represents only the free caffeine present in the sample. This measurement does not account for the total caffeine content, as caffeine is known to form complexes with polyphenols (Ishizu & Tsutsumi, 2012). These complexes are removed during centrifugation, resulting in an underestimation of the total caffeine in the sample. Considering this, the caffeine content in black coffee before digestion is taken as a reference point for comparing caffeine levels in digested samples and milk-based coffee samples to understand the effect of digestion as well as milk addition on the caffeine content in the coffee samples.

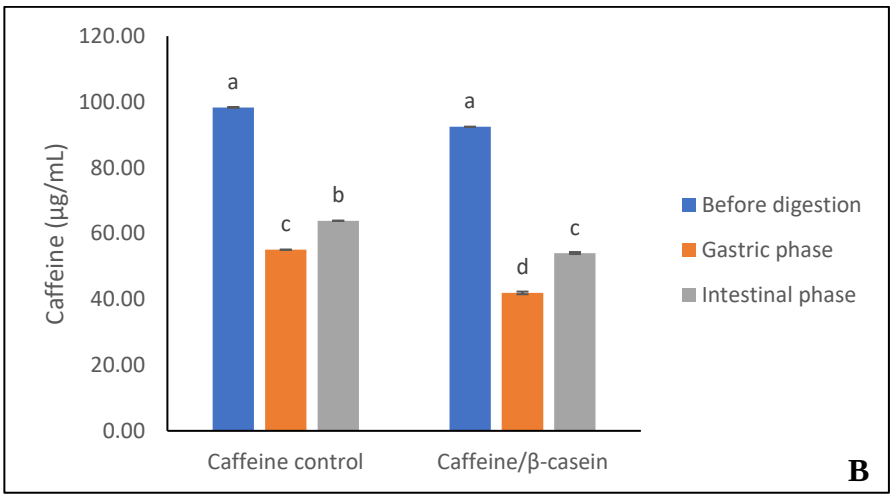
A progressive reduction in caffeine levels is observed in black coffee after each digestion phase, with the lowest content recorded post-gastric digestion (90.7 µg/mL) (Khochapong et al., 2021). This suggests that gastric conditions (acidic environment) enhance the interaction potential of casein (Fakioglu & Kalpakli, 2022). Caffeine interacted with polyphenols and other components in coffee, and the amount of free caffeine was lower in the gastric phase. However,

higher levels of free caffeine were detected after the intestinal phase (147.9 µg/mL), indicating increased availability at the end of digestion.

In contrast, the addition of milk to coffee significantly reduced caffeine content at all stages of digestion compared to black coffee. This implies potential interactions between caffeine and milk proteins or other components, such as fats. The percentage increase in the free caffeine content post-intestinal digestion is lower than that of black coffee because milk proteins forms more stable complexes with caffeine than caffeine-polyphenolic complexes, which could resist breakdown during digestion. The findings highlight that black coffee contains more readily accessible caffeine for absorption, whereas milk incorporation diminishes the amount of free caffeine available.

Although there are stable protein-caffeine complexes formed after digestion, the increase in caffeine concentrations after intestinal digestion suggests the reversibility of bonds and that bound caffeine could still be bioaccessible as well as bioavailable, but the pharmacokinetics of caffeine could be delayed. Milk proteins may function as a carrier for caffeine; therefore, it cannot be concluded that the effect of milk addition is positive or negative until the bioavailability of bound caffeine is determined using animal or human *in vivo* studies. The systemic circulation of caffeine and absorption dynamics could be assessed by carrying out *in vivo* studies using isotope-labelled caffeine.





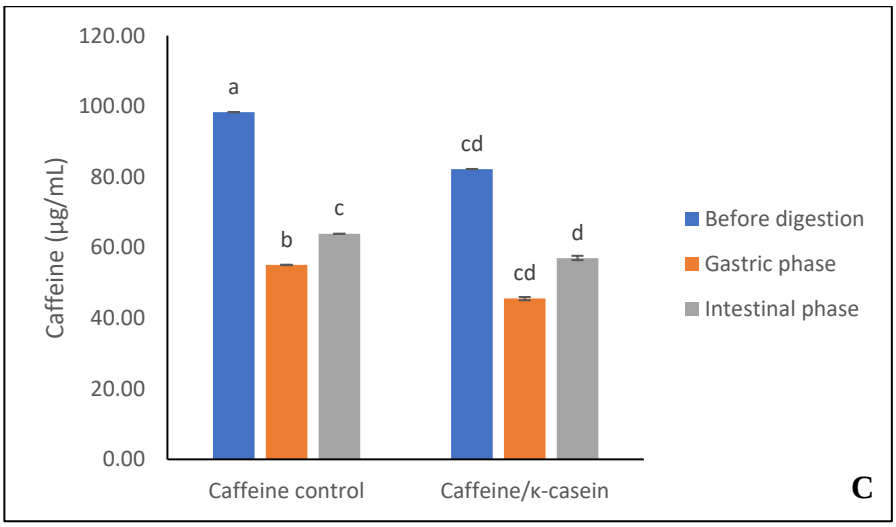


Figure 4.4 The free caffeine content of caffeine-pure milk casein protein mixtures at different digestion stages compared against the control obtained through HPLC analysis: Caffeine/α-casein (A), Caffeine/β-casein (B), Caffeine/κ-casein (C). Values are the mean±SD (n=6). Columns with different superscripts (a-d) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

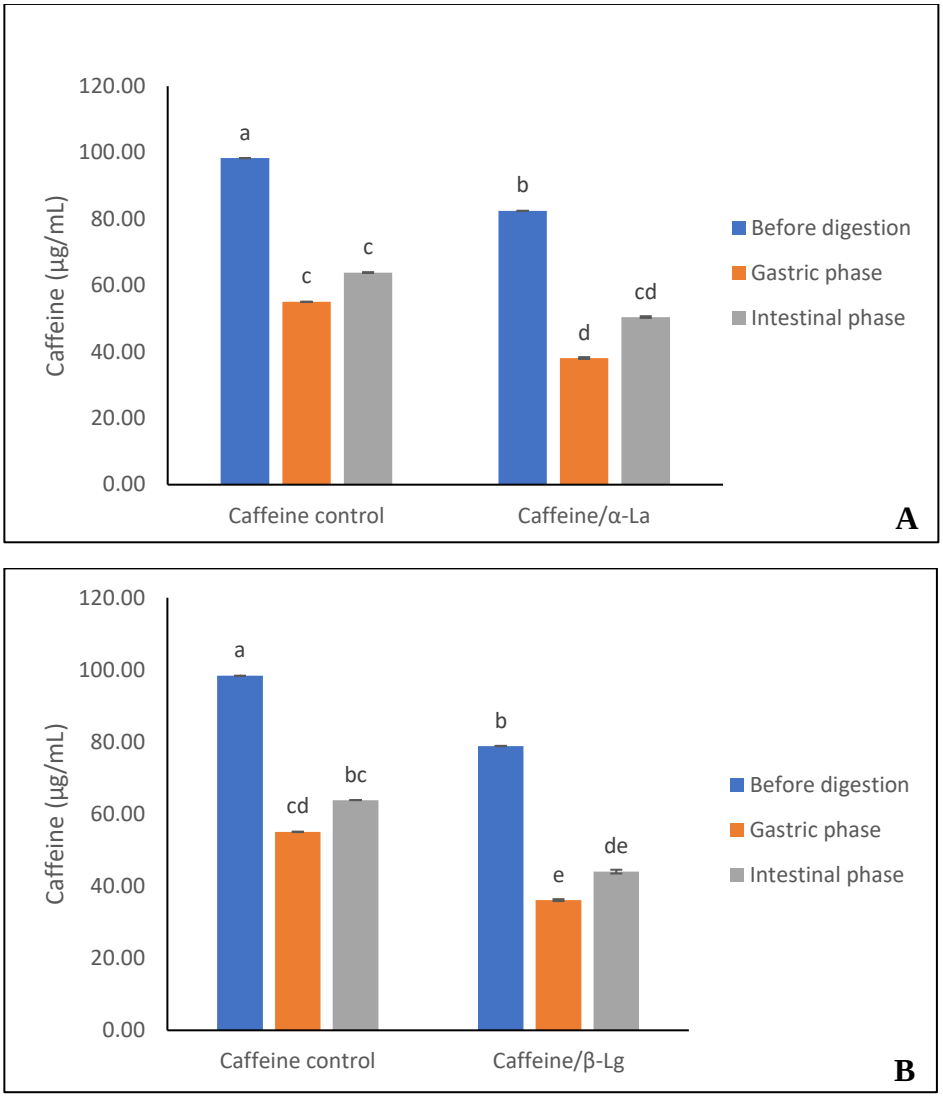


Figure 4.5 The free caffeine content of caffeine-pure milk whey protein mixtures at different digestion stages compared against the control obtained through HPLC analysis: Caffeine/α-lactalbumin (A) and Caffeine/β-lactoglobulin (B). Values are the mean±SD (n=6). Columns with different superscripts (a-e) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

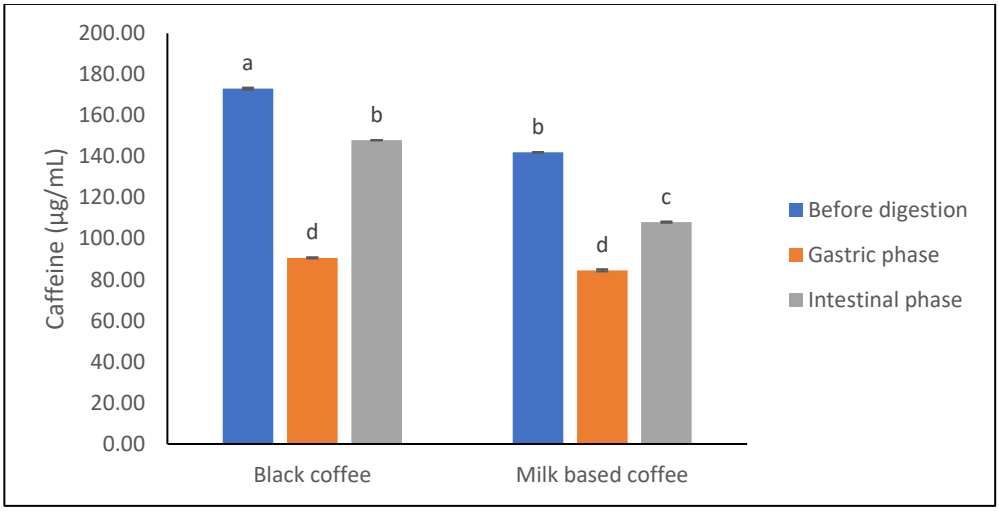


Figure 4.6 The free caffeine content of black coffee and milk-based coffee at different digestion stages obtained through HPLC analysis. Values are the mean±SD (n=6). Columns with different superscripts (a-d) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA. The pH of black coffee, milk, and milk-based coffee before digestion was 4.9,6.7, and 6.4, respectively. The pH was 3 and 7 after the gastric and intestinal phases, respectively, for all the samples.

Employing complementary analytical techniques is essential to ensure reliable and accurate results when analysing the interactions of caffeine with milk proteins and subsequent bioaccessibility during different phases of digestion. Both UV spectroscopy and HPLC provide valuable insights, each offering distinct advantages. UV spectroscopy is widely used due to its simplicity and ability to provide an overview of changes in caffeine concentration across digestion phases. However, its broader detection range can sometimes lack the specificity required to differentiate closely related compounds in complex matrices. In contrast, HPLC offers high precision and sensitivity, making it the preferred technique for accurately quantifying free caffeine in systems such as milk-based coffee, where multiple components may interfere with measurements (Dhole et al., 2012; Wang et al., 2019).

The trends observed in UV spectroscopy align with HPLC results, reinforcing the robustness of the data. However, slight discrepancies between the two methods may arise due to differences in sensitivity and specificity. HPLC detects caffeine by separating individual components within the column, whereas UV spectroscopy measures the absorbance value of any compounds present in the detected region, making it difficult to determine whether the signal originates solely from caffeine or includes other components. These methodological considerations underscore the importance of using complementary techniques to validate findings in complex food systems, ensuring a comprehensive understanding of caffeine dynamics.

4.2. Total phenolic content

Figures 4.7 and 4.8 compare the TPC values of caffeine-protein mixed samples against control and at each stage of digestion. A decrease in TPC values can be observed after each stage of digestion for caffeine/α-casein. The TPC reduced to 42 μg GAE/mL from 83 μg GAE/mL at the end of digestion with an almost 50% reduction. However, the TPC values of caffeine remain the same at all digestion stages. α-Casein control also shows a decrease after the gastric phase, but it remained unchanged in the intestinal phase. The decrease in the samples could be due to the effect of a decrease in the protein's phenolic activity. Similarly, caffeine/α-La showed a reduction in TPC after each phase of digestion. Conversely, intestinal phase samples of caffeine/β-casein and caffeine/κ-casein had similar TPC values compared to the gastric phase samples, and an increase was observed for caffeine/β-Lg. This could be because some of the bonds formed, such as hydrogen bonds or hydrophobic interactions, might be reversible and could have released some of the caffeine and made it available for phenolic activity in the intestinal phase (Dupas et al., 2006; Spencer et al., 1988).

The least reduction in TPC content occurred for caffeine/β-Lg samples after digestion, with only a 12% reduction compared to that before digestion. In the caffeine/α-casein complex, caffeine may promote stronger aggregation and binding of polyphenols to the protein, leading to a higher reduction in TPC on the other hand, in the caffeine/β-Lg complex, caffeine may not significantly alter the polyphenol-protein interactions, resulting in less reduction in phenolic content. The structural difference in milk proteins has an impact on the TPC of the sample and how caffeine influences it.

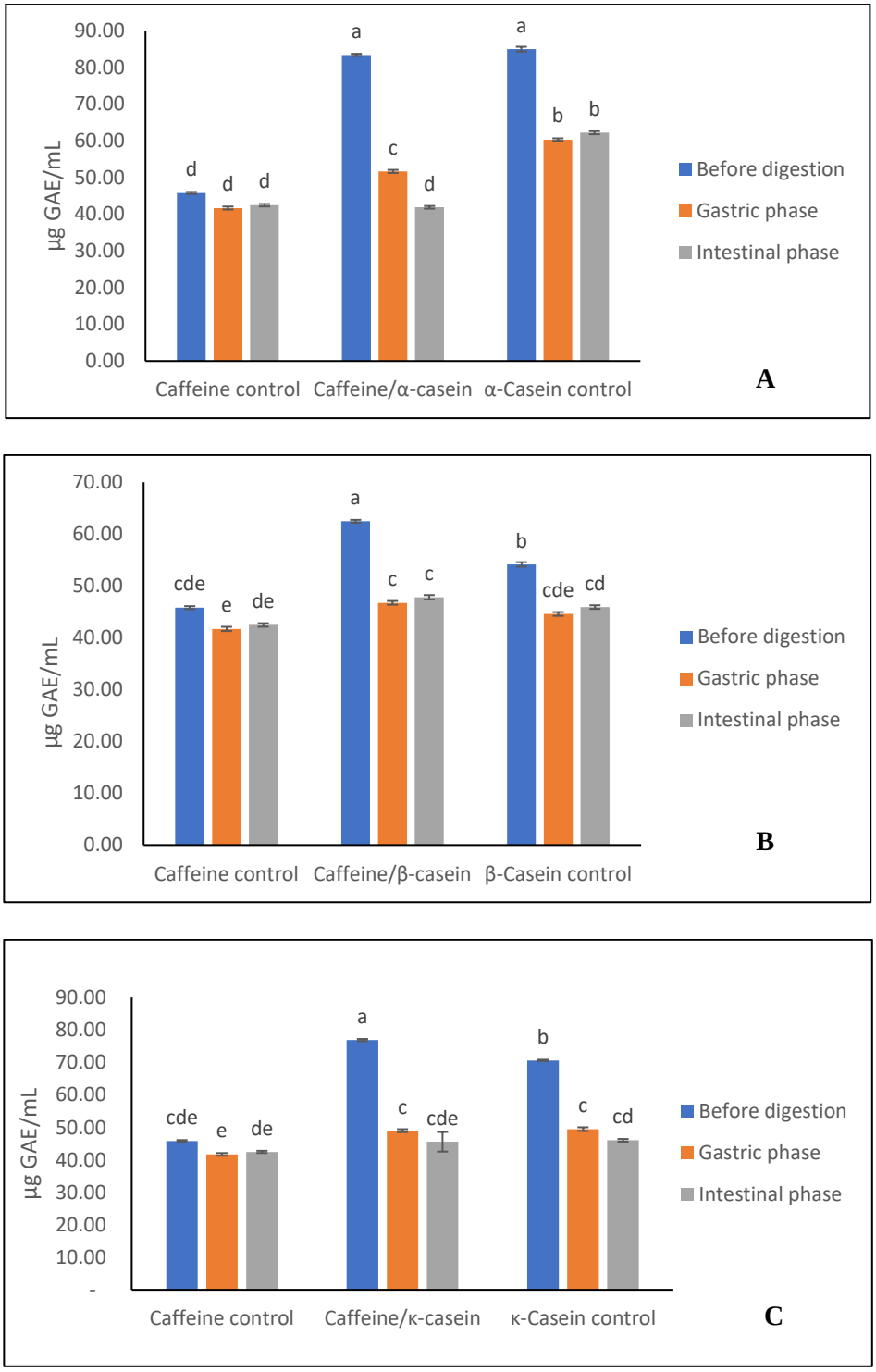


Figure 4.7 The total phenolic content of caffeine with pure milk casein proteins at different digestion stages compared against the controls: Caffeine/α-casein (A), Caffeine/β-casein (B), and Caffeine/κ-casein (C). Values are the mean±SD (n=6). Columns with different superscripts (a-e) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

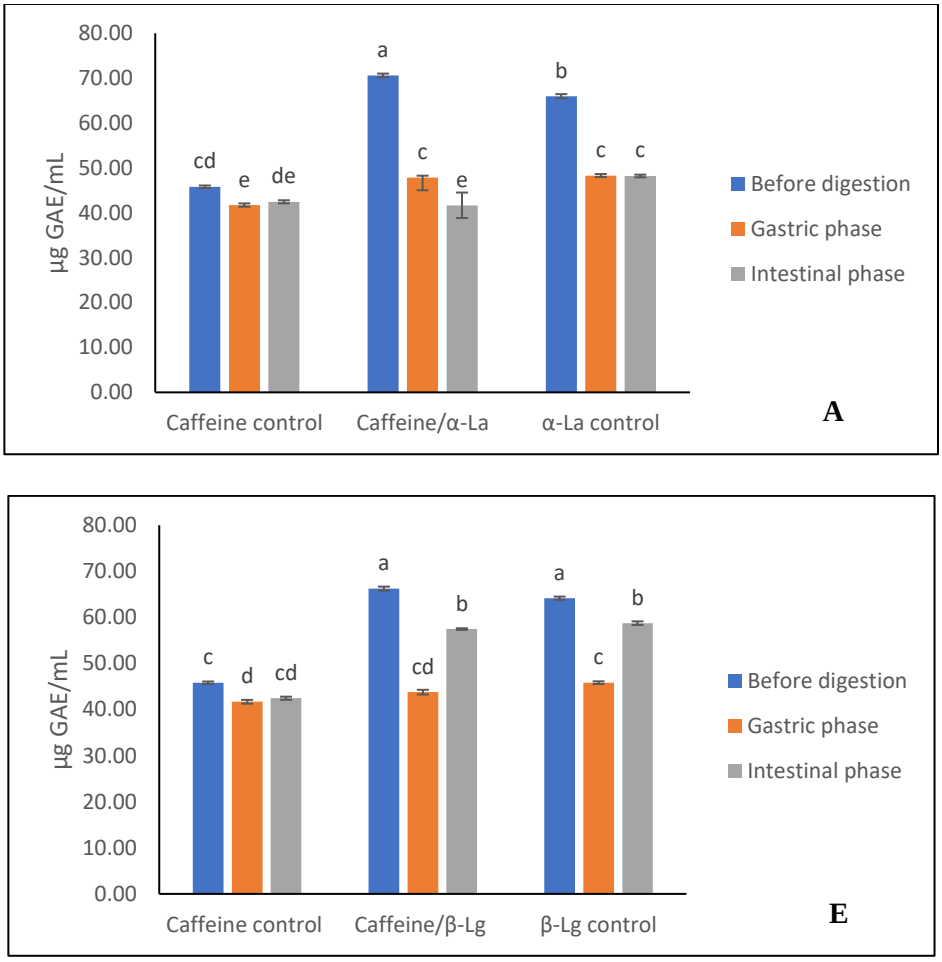


Figure 4.8 The total phenolic content of caffeine with pure milk whey proteins at different digestion stages compared against the controls: Caffeine/α-lactalbumin (A) and Caffeine/β-lactoglobulin (B). Values are the mean±SD (n=6). Columns with different superscripts (a-e) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

Figure 4.9 depicts the TPC of black coffee, milk, and milk-based coffee samples before digestion, after the gastric phase, and after the intestinal phase. All the samples were centrifuged before testing. The statistical analysis shows that there is no significant difference in the TPC values when compared against each digestion phase for each type of sample. The error bars represent standard error rather than confidence interval, which for particular data looks larger even though the group means are not statistically significant. As a result, while a visual trend of increase is observed, statistical analysis confirmed that the differences were not significant. Milk shows an apparent increase in TPC values after each digestion stage, but the difference is not statistically significant. Black coffee also had similar results but higher than for milk and milk-based coffee samples, which is comparable to the values reported by Niseteo et al. (2012).

These authors reported that regular black coffee has a higher TPC value than coffee with added milk, and decaf coffee had a lower TPC than regular black coffee brews. However, the reason for the decaf coffee brew showing a lower TPC value was not provided. The decrease in values of milk-based coffee samples compared to black coffee might be due to the complex formation between caffeine and polyphenols in milk-based coffee samples with milk proteins (Dubeau et al., 2010). In the case of the current research, since the samples were centrifuged before analysis, whether the complexes show phenolic activity remains uncertain. Compared to the TPC values obtained by studying caffeine and pure proteins, it can be concluded that polyphenols found in coffee have more influence on the phenolic activity than caffeine, as caffeine is an alkaloid and not a polyphenol.

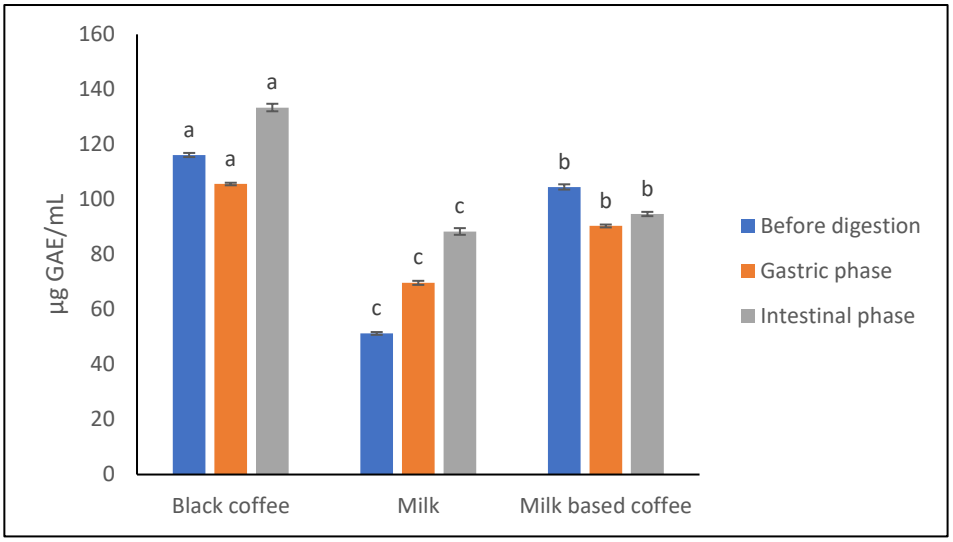


Figure 4.9 The total phenolic content of black coffee, milk, and milk-based coffee at different digestion stages. Values are the mean±SD (n=6). Columns with different superscripts (a-c) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

4.3. Antioxidant capacity

Figures 4.10 and 4.11 show the Trolox equivalent antioxidant capacity (TEAC) values of caffeine-protein mixed samples compared against their control at each phase of digestion. Caffeine showed a higher antioxidant capacity at the end of digestion (7 μg/mL) compared to what was observed in the native state (2.1 μg/mL). This could be due to the presence of digestive enzymes, which may make caffeine more available for reaction with the 2,2-diphenyl-1-picryl-hydrazyl reagent, causing more reduction of the reagent. No significant difference was observed in the values obtained for caffeine/α-casein when compared against the caffeine control or across the digestion stages. The case for caffeine/α-La is similar. However, an increase in the TEAC values was obtained for caffeine/β-casein at the end of intestinal digestion (5.1 μg/mL), compared to what was observed before digestion (2.9 μg/mL).

A comparable pattern was identified in the interactions between caffeine and κ-casein as well. This could be due to the formation of complexes, through hydrophobic interactions or hydrogen bonding, between the caffeine and proteins after mixing. As digestion progresses, the proteins are broken down, and the resulting digesta, with more caffeine released into the system, show higher antioxidant activity. Conversely, the caffeine/β-Lg mixture has higher antioxidant activity before digestion, and the digestive conditions reduced the antioxidant activity. Digestive conditions may not be favourable for β-Lg to exhibit antioxidant activity due to possible conformational changes during gastric digestion. To summarise, different proteins interact with caffeine differently, and the effect of digestive conditions on TEAC also varies depending upon the type of protein binding to caffeine.

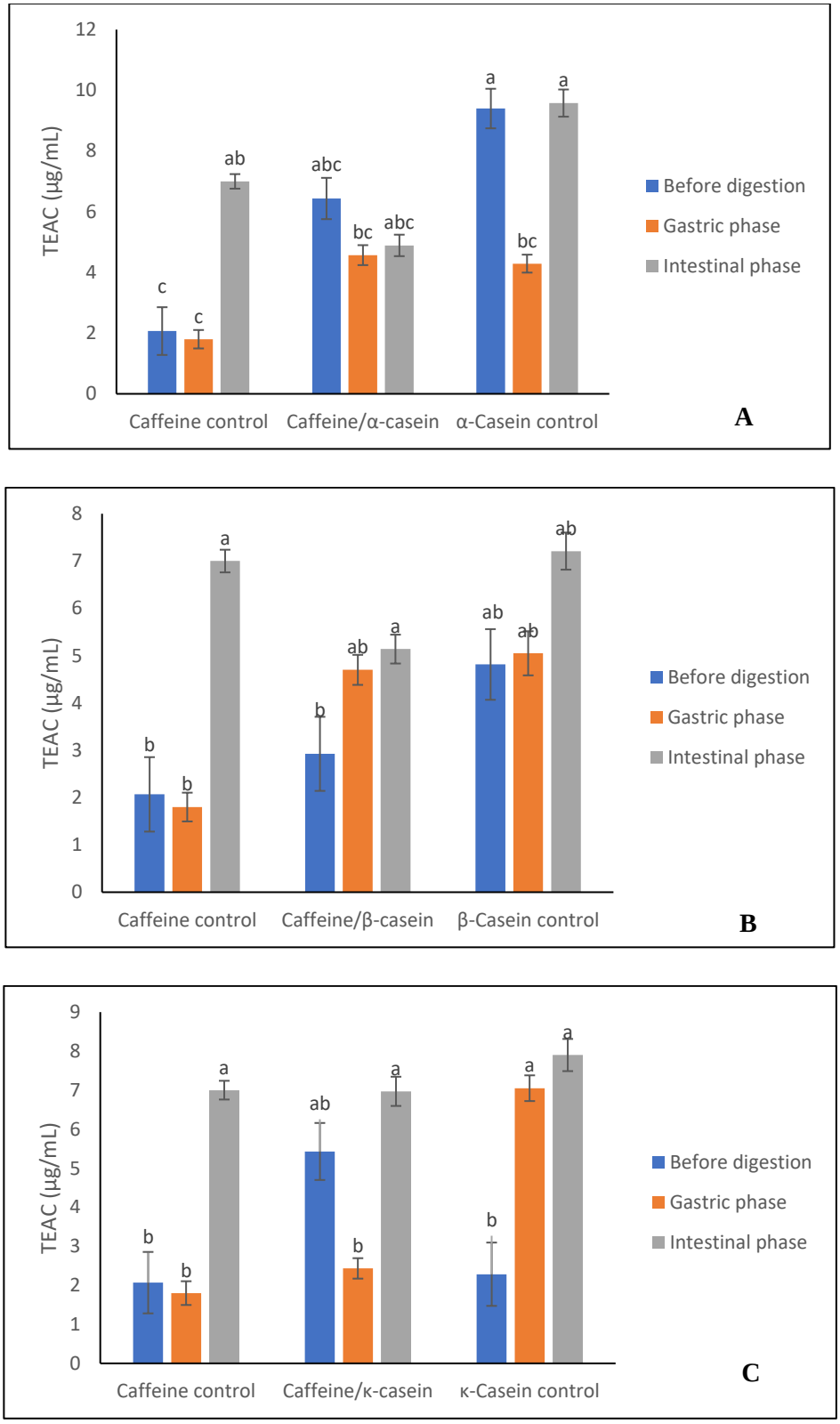


Figure 4.10 The Trolox equivalent antioxidant capacity (DPPH method) of caffeine with pure milk casein proteins at different digestion stages compared against the controls: Caffeine/α-casein (A), Caffeine/β-casein (B), Caffeine/κ-casein (C). Values are the mean±SD (n=6). Columns with different superscripts (a-c) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

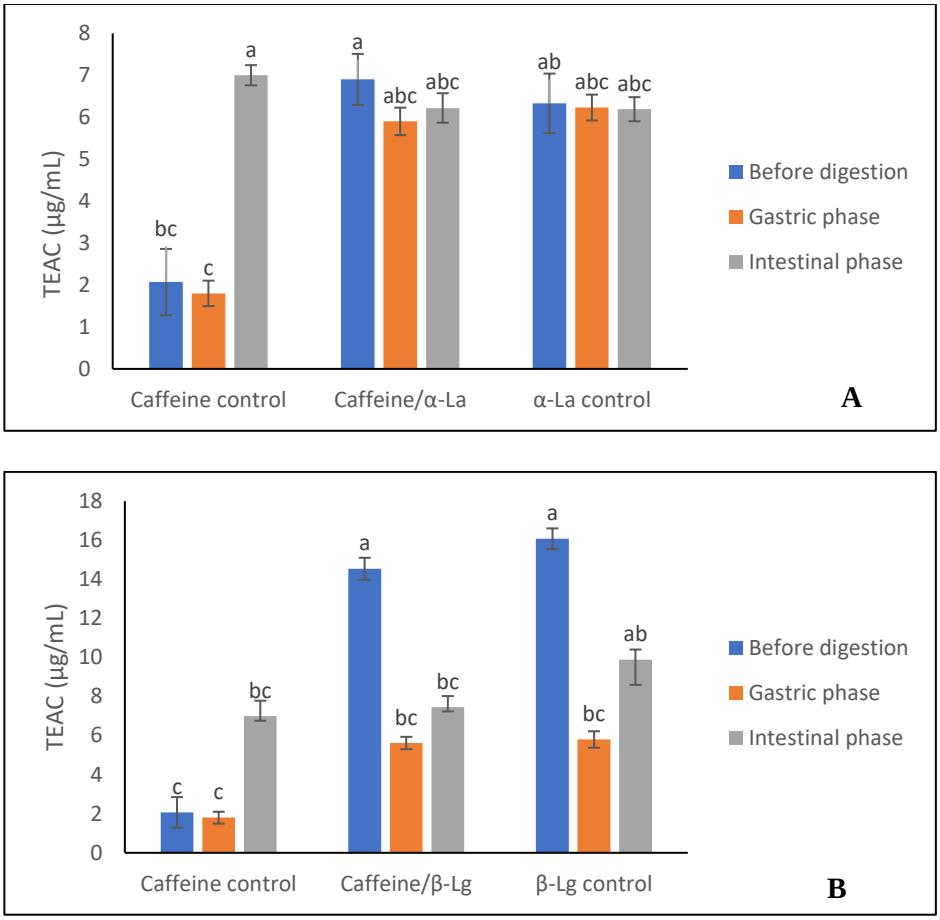


Figure 4.11 The Trolox equivalent antioxidant capacity (DPPH method) of caffeine with pure milk whey proteins at different digestion stages compared against the controls: Caffeine/α-lactalbumin (A) and Caffeine/β-lactoglobulin (B). Values are the mean±SD (n=6). Columns with different superscripts (a-c) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

Figure 4.12 shows the TEAC values for black coffee, milk, and milk-based coffee samples at three stages: before digestion, after the gastric phase, and after the intestinal phase. Black coffee exhibits the highest antioxidant potential, with milk addition significantly reducing the TEAC of the sample ($p<0.05$). Niseteo et al. (2012) also reported higher antioxidant values for plain coffee than coffee with added milk. Dubeau et al. (2010) reported a reduction in the antioxidant capacity of tea upon milk addition. These authors attributed the inhibitory effect to the formation of covalent and non-covalent interactions between tea polyphenols and caseins. Ramalakshmi et al. (2009) identified caffeine as one of the major antioxidant components in coffee, along with chlorogenic acids and phenolic compounds. Therefore, caffeine can bind with milk proteins through non-covalent interactions.

Digestion did not exert a notable effect on antioxidant activity, as TEAC values remained consistent across all samples throughout the digestive process. This stability suggests that compounds responsible for antioxidant activity—such as chlorogenic acids, caffeine, and melanoidins—may interact with milk components, including proteins, fats, and minerals, potentially leading to reduced antioxidant capacity in milk-based coffee. In natural human digestion, all ingested material passes through the stomach and reaches the intestine, allowing for comprehensive absorption and interactions with digestive enzymes. However, in this study, the analysis was carried out on centrifuged samples, which may have led to the removal of certain portions of the digesta. These removed components might possess antioxidant properties, thus raising ambiguity regarding whether the observed reduction in antioxidant activity reflects a true decrease or results from sample preparation. The eliminated fractions could also contain antioxidants, suggesting that milk addition might not inherently diminish the bioactive properties of coffee. Nevertheless, the findings indicate that milk addition appears to reduce the measured antioxidant activity, suggesting a potential negative impact on these bioactive compounds.

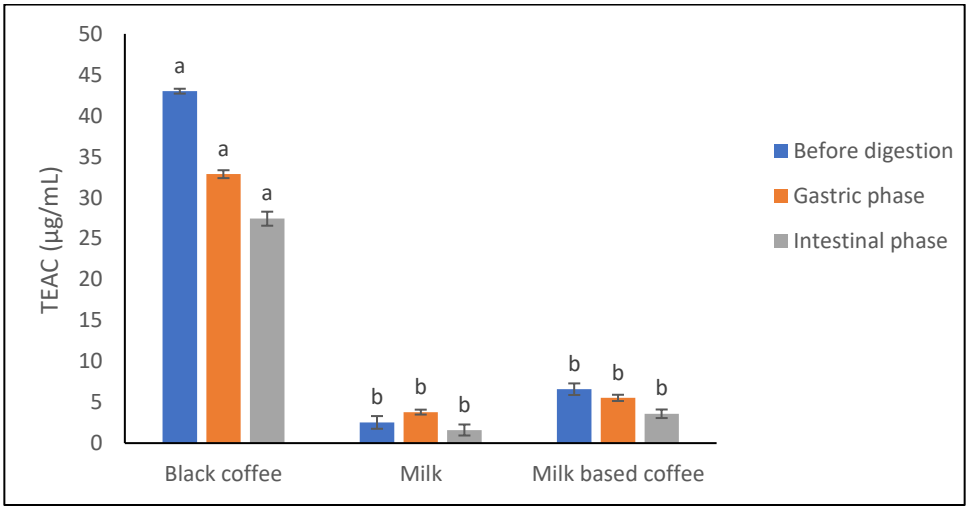


Figure 4.12 The Trolox equivalent antioxidant capacity (DPPH method) of black coffee, milk, and milk-based coffee at different digestion stages. Values are the mean±SD (n=6). Columns with different superscripts (a, b) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

4.4. FTIR analysis

FTIR spectra were recorded to understand, at a deeper level, the conformational changes in the protein structure upon interactions with caffeine. The amide I (~1600-1700 cm^{-1}) and amide II (~1500-1600 cm^{-1}) bands are very sensitive regions, and a shift in the region indicates protein folding or denaturation. The amide I region primarily arises from C=O stretching vibrations. This region is influenced by hydrogen bonding and the surrounding environment of the proteins (Stuart, 2004). The amide I region is more sensitive than the amide II region. Another region that is relevant to studying the interactions of caffeine and milk protein is the wavenumber region 2982-2825 cm^{-1} (Weldegebreal et al., 2017). This region is correlated with the symmetrical and asymmetrical stretching of C-H bonds of the methyl group in the caffeine molecule.

Figure 4.13 shows the FTIR spectra of the caffeine-milk protein mixture and its respective controls at different stages of digestion. The caffeine-protein mixture before digestion has a peak at the same wavenumber as the respective control in the amide I region, except for caffeine/α-La, which showed a peak shift. This indicates a change in the α-lactalbumin conformation, possibly due to hydrogen bonding between the protein and caffeine. In the amide II region, all the mixture samples showed a peak shift, suggesting that the structure of proteins is affected by the addition of caffeine.

At the major caffeine peak (2950-2930 cm^{-1}), only caffeine/β-Lg has a peak shift compared to both the controls. However, all the mixed samples have a peak at a lower wavenumber compared to the caffeine control. A shift to a lower wavenumber indicates weaker C-H bonds or increased bond length. This occurs when the C-H bond is involved in hydrogen bonding and interactions due to Van der Waals forces (Pochec et al., 2022; Robert et al., 2008). A larger shift could be observed in the 2820-2850 cm^{-1} region, suggesting significant structural changes and strong hydrogen bonding.

After the gastric digestion, a major shift has occurred for caffeine/α-La and caffeine/β-Lg compared to the control, which indicates that gastric conditions are more suitable for whey proteins to interact with caffeine than caseins. Major shifts in peaks are not present in the samples after the intestinal phase, but a few minor shifts could be observed, indicating the possibility of interactions even after intestinal digestion. The spectral data is summarised in Table 4.1.

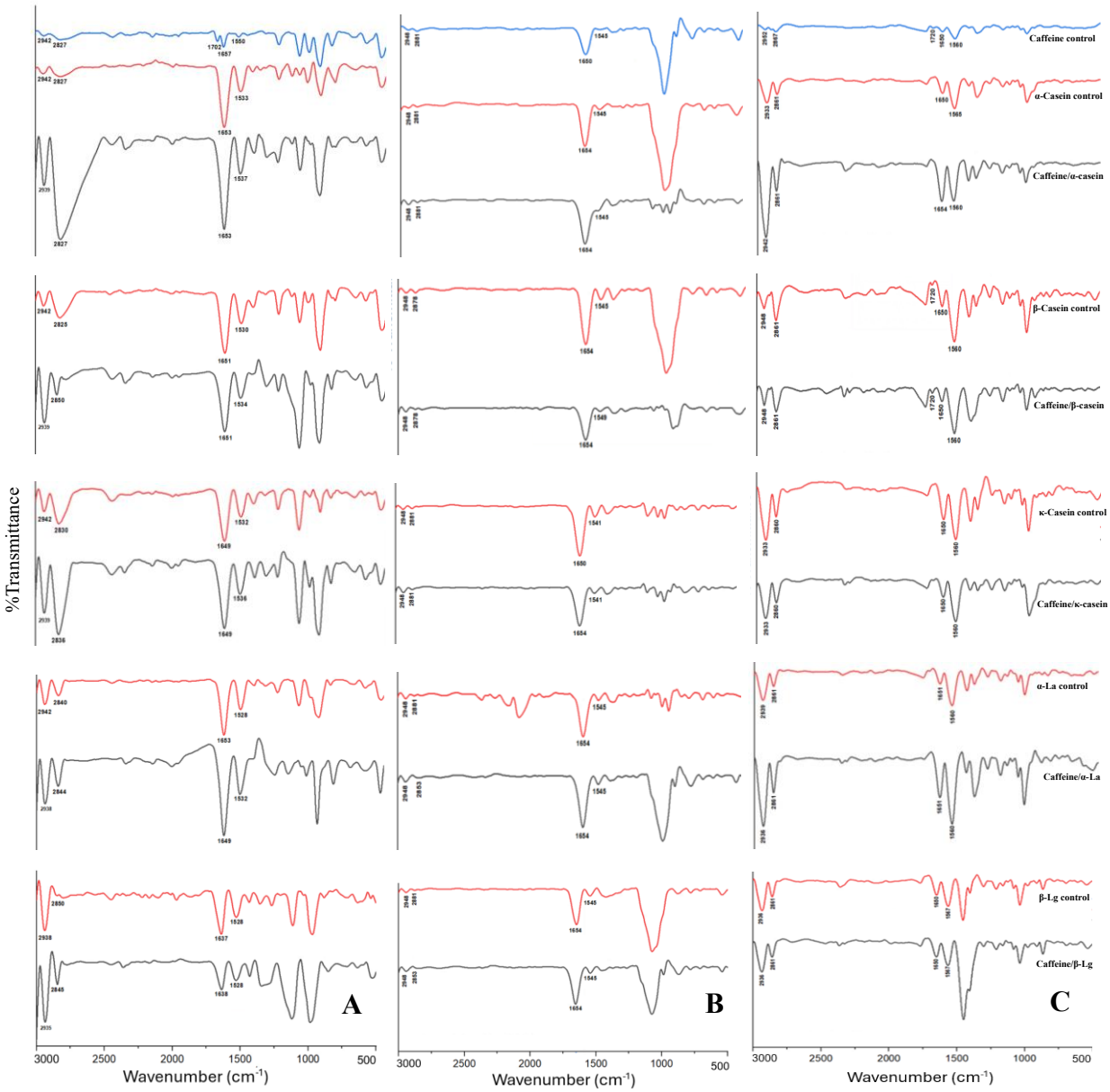


Figure 4.13 FTIR spectra of pure caffeine-milk protein before digestion (A), after gastric phase (B), and intestinal phase (C), compared against its respective controls.

Figure 4.14 represents the FTIR spectra of coffee and milk samples. From the data, it is evident that a significant shift has occurred when milk is added to a coffee brew. Changes in peaks 1 and 2 in the sample before digestion indicate that C-H stretching vibrations may have taken place with an increase in bond length. Hydrogen bonding and Van der Waals forces could cause changes in the bond length. Stretching vibrations in the C=O region (1700-1500 cm^{-1}) have also occurred, which could be an indication of hydrogen bond formation with the proteins in milk. Similarly, there are changes in the methyl groups region (2840-2820 cm^{-1}) in milk-based coffee after gastric and intestinal digestion compared to black coffee samples. However, further

analysis is necessary to understand whether these interactions are reversible. It is still not clear whether caffeine is bioavailable after digestion.

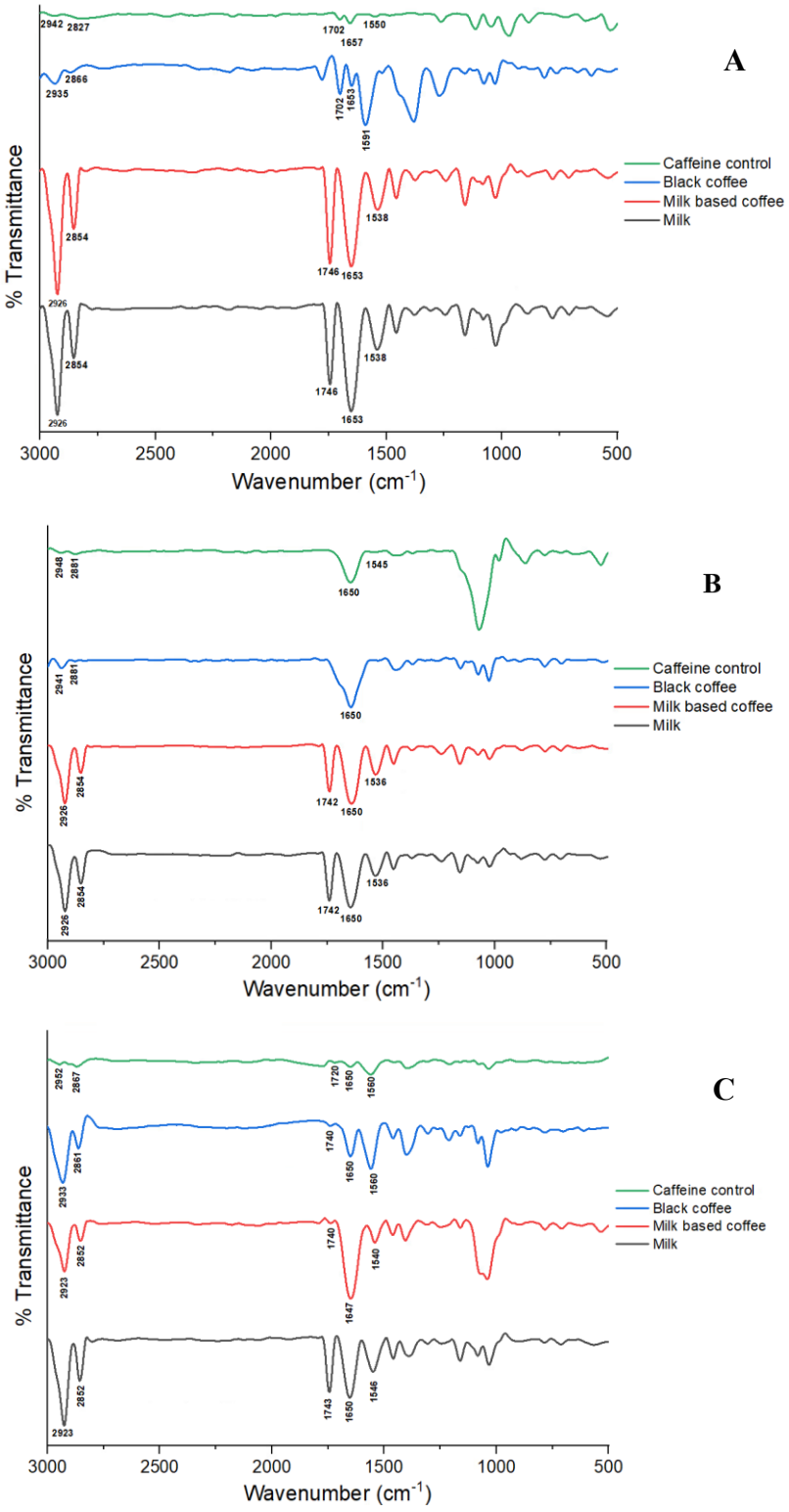


Figure 4.14 FTIR spectra of pure caffeine, black coffee, milk-based coffee, and milk before digestion (A), after gastric phase (B), and intestinal phase (C).

Table 4.1 FTIR spectral assignment: Major peaks, vibrations, and sample-specific observations

Region (cm^{-1})	Description	Type of Bonds	Type of Vibration	Sample
1500-1600	Amide II region - sensitive to protein structure, arises from N-H bending and C-N stretching vibrations.	N-H and C-N	N-H bending and C-N stretching	All samples before digestion, milk-based coffee samples before digestion, and after intestinal phase.
1600-1700	Amide I region - sensitive to protein folding and denaturation, primarily arises from C=O stretching vibrations.	C=O (carbonyl) with hydrogen bonding influence	C=O stretching	Caffeine/α-lactalbumin before digestion
2825-2982	Symmetrical and asymmetrical stretching of C-H bonds in methyl groups of caffeine.	C-H (methyl groups)	Symmetrical and asymmetrical C-H stretching.	All samples before digestion, caffeine/α-lactalbumin and caffeine/β-lactoglobulin samples after gastric phase, milk-based coffee samples before digestion, and after gastric phase.
2930-2950	Major caffeine peak shift indicates weaker C-H bonds or hydrogen bonding interactions.	C-H (methyl groups)	C-H stretching	All samples before digestion, milk-based coffee samples before digestion, and after gastric phase.

4.5. Particle size and ζ-potential

Table 4.2 provides the particle size of black coffee, milk, and milk-based coffee samples at different stages of digestion. The particle size distribution curves, generated using ZS Xplorer version 3.2.0 software, are presented in Appendix 5. A multimodal particle size distribution was observed for all samples before digestion, which persisted throughout digestion except in black coffee. After gastric digestion, black coffee exhibited a monomodal particle size distribution, a trend also reported by Alongi et al. (2019) during the *in vitro* digestion of coffee brews.

The particle size in black coffee is primarily influenced by compounds such as chlorogenic acid, caffeine, and melanoidins, which contribute to the smaller-sized particles, while polysaccharides and calcium-polyphenol aggregates account for the larger particles (Okubo et al., 2008). The intensity-weighted size distribution curve in Figure A5.1 in Appendix 5 reveals smaller particles dominating in number, as indicated by a taller peak for smaller-sized particles despite the curve's bias toward larger particles. This type of analysis, which is highly sensitive to particle aggregation, is particularly useful for examining interactions within the sample.

As digestion progresses, the larger particles in black coffee break down into smaller ones, resulting in a reduction in average particle size by the end of digestion (Alongi et al., 2019). The average particle size observed after gastric digestion is larger than that before digestion and after the intestinal phase. This suggests the possibility that gastric conditions promote interactions between smaller compounds and digestive enzymes. However, supporting evidence from caffeine analysis, antioxidant assays, and phenolic content measurements indicates that gastric conditions favour interactions between smaller compounds and digestive enzymes.

The subsequent reduction in particle size during the intestinal phase suggests the presence of more free caffeine and other smaller components, consistent with these findings. For milk samples, Peak 1 in the particle size distribution is mainly attributed to caseins, whereas larger particles correspond to fats (Alongi et al., 2019; Gallier et al., 2012; Gaucher et al., 2008; Tunick et al., 2016). During digestion, fats and proteins break down, forming smaller and more stable complexes, as observed in milk-based coffee. When milk is added to coffee, the average particle size becomes smaller than that of black coffee or milk alone. This is consistent with the results obtained by Okubo et al. (2008). Fat particles remain relatively unchanged in size, whereas caseins and other milk proteins are primarily responsible for interactions with coffee

components. However, it remains unclear whether caffeine or polyphenols specifically interact with milk proteins based on the current results.

Table 4.2 The particle size of black coffee, milk, and milk-based coffee samples at different digestion stages. The values are the mean±SD (n=9).

Digestion stage	Sample	Size distribution (nm)		
		Peak 1	Peak 2	Peak 3
Before digestion	Black coffee	576±11	4590±40	0
	Milk	320±24	5040±50	0
	Milk-based coffee	220±25	5136.±40	0
Gastric phase	Black coffee	628±27	0	0
	Milk	810±30	144±23	0
	Milk-based coffee	254±21	56±11	0
Intestinal phase	Black coffee	446±20	0	0
	Milk	248±13	67±20	0
	Milk-based coffee	431±8	88±18	4895±40

The ζ-potential of black coffee, milk, and milk-based coffee after three phases of *in vitro* digestion is shown in Figure 4.15. The samples were tested for ζ-potential before digestion at the natural pH, which is given in Table A5.1 in Appendix 5. For black coffee, the observed ζ-potential was -10.94±0.41 mV at a pH of 4.94±0.03 which is less than the values reported by Okubo et al. (2008) and Feng et al. (2023). Okubo et al. (2008) observed a ζ-potential of -24 mV for coffee without cream, and Feng et al. (2023) recorded a value of -42 mV for coffee brew. The pH, dilution factor, temperature, and method of preparation of coffee brew influence the observed values of ζ-potential (Cano-Sarmiento et al., 2018). The pH at which Okubo et al. (2008) and Feng et al. (2023) measured ζ-potential was not mentioned in the articles. Alongi et al. (2019) reported the ζ-potential of coffee brews before and after *in vitro* digestion were -17.5±1.3 mV and −32.5±1.6 mV, respectively. These authors suggest that the higher stability after digestion could be due to the presence of bile salts and other digestive enzymes and products. A similar trend could also be observed in the results obtained with a higher ζ-potential value after the end of intestinal digestion. The ζ-potential values in the gastric phase were positive, which was similar to the values observed for milk and milk-based coffee samples. Few studies have been carried out to understand the impact of gastric phase digestion on the ζ-potential of black coffee.

The ζ-potential of milk was studied by several authors, and values for casein and milk fat globules were reported for skim milk, whole milk, and reconstituted milk (Alongi et al., 2019; Gallier et al., 2012; Kühnl et al., 2010; Kytariolos et al., 2013; Post et al., 2012; Sun et al., 2022; Tunick et al., 2016; Wu, 2014). The ζ-potential values obtained for milk were almost similar to the values reported by Post et al. (2012), Tholstrup et al. (2007), and Tunick et al.

(2016). The ζ-potential of milk samples was measured at pH 6.71±0.03, and all samples were diluted 100-fold before measurement at 25°C. The ζ-potential of milk was due to the presence of particulate casein micelles and milk fat globules. Casein micelles are also broken down into peptides and other digestion products by the end of digestion. Milk tends to show a higher ζ-potential value than black coffee and milk-based coffee because milk fat is surrounded by a protein-phospholipid membrane, preventing coalescence and ensuring the stability of the fat phase, and casein micelles stabilised by calcium phosphate bridge which makes it a more stable structure compared to black coffee and milk-based coffee.

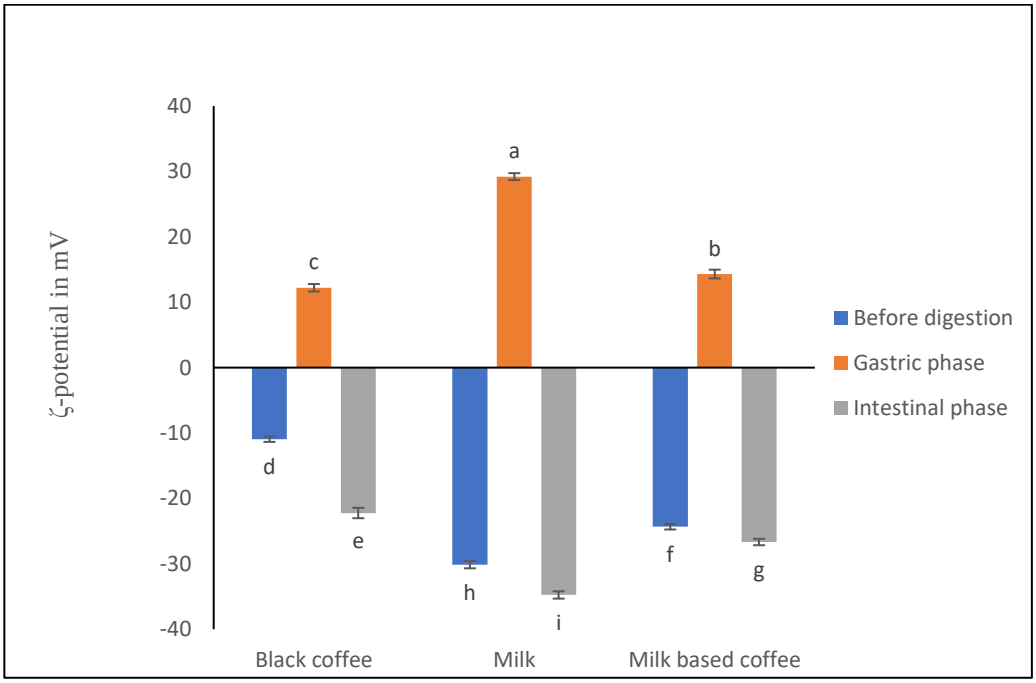


Figure 4.15 The ζ-potential of black coffee, milk, and milk-based coffee before digestion, after gastric digestion, and after intestinal digestion. Values are the mean±SD (n=9). Columns with different superscripts (a-i) are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA

The ζ-potential values of milk-based coffee before digestion were more negative than black coffee but less so compared to milk, indicating that the stability of coffee brew is improved by the addition of milk. Even after gastric and intestinal digestion, the milk-based coffee showed a higher absolute ζ-potential value, and the difference is statistically significant ($p<0.05$). The pH of milk-based coffee samples was 6.43±0.03 at the time of measuring ζ-potential. The pH was maintained at either 3 or 7 for all the samples after gastric and intestinal digestion,

respectively. Alongi et al. (2019) studied the effect of milk addition on the coffee brew and observed that the ζ-potential values of coffee with milk were higher compared to black coffee brew, which approximates the experimental outcomes of this study. There was no statistically significant difference in values obtained for undigested and digested coffee samples with low-fat milk (Alongi et al., 2019). However, the ζ-potential value showed a significant increase after digestion when the fat percentage was 7.1%, indicating that fat globules play a more significant role in stabilising coffee colloids rather than milk proteins.

However, the role of colloidal stability on caffeine bioaccessibility cannot be determined by just measuring the ζ-potential. Some authors claim that a more stable digesta improves the bioaccessibility of bioactive compounds in coffee (Alongi et al., 2019; Otemuyiwa et al., 2017), whereas other studies claim that higher stability leads to reduced bioaccessibility, primarily due to the binding of the components to milk proteins (Duarte & Farah, 2011; Dupas et al., 2006). In a more stable colloidal system, the caffeine could remain bound to the milk proteins, leading to an initially reduced bioaccessibility. However, as digestion progresses, the milk proteins are broken down, and the bound caffeine is slowly released into the bloodstream, thus gradually increasing the bioavailability, leading to caffeine having a prolonged effect on the body. This sustained release is beneficial for the longer-lasting effect of caffeine.

For more rapid caffeine absorption or a quick energy boost, a less stable colloidal system would be preferable as more caffeine would be available to be immediately absorbed into the bloodstream. However, there is also a possibility of faster precipitation of protein-caffeine complexes due to higher instability at a reduced pH. If this precipitate is not well digested or absorbed, it could lead to reduced overall bioavailability of caffeine; this is best determined using *in vivo* digestion studies. Understanding these interactions is crucial as it can influence dietary recommendations and consumption habits, particularly for individuals seeking specific health benefits from their coffee consumption. Additionally, the food industry could leverage these findings to develop new formulations of coffee beverages that optimise the stability and bioaccessibility of caffeine, catering to consumer preferences and enhancing the health benefits of their products.

4.6. *In vitro* antioxidant activity and bioaccessibility

The main aim of this study was to determine if caffeine interacts with milk proteins and, if so, to understand if these interactions affect the bioactivity, antioxidant properties, and *in vitro* bioaccessibility of caffeine. The results reveal that the *in vitro* antioxidant capacity of pure caffeine increased after digestion, whereas for black coffee, it remained unchanged throughout digestion. However, the addition of milk reduced the antioxidant activity of caffeine, likely due to interactions between caffeine and milk proteins, The antioxidant activity declined significantly by the end of digestion, except when β-casein and κ-casein were used. This aligns with findings from other studies which report a similar reduction in the antioxidant activity of polyphenols when milk is added to tea or coffee or when polyphenols interact with milk proteins (Dubeau et al., 2010; Liu et al., 2015; Niseteo et al., 2012; Sánchez-González et al., 2005; Zorilla et al., 2011).

Most research examining the *in vitro* antioxidant activity of polyphenols affected by milk proteins supports the hypothesis that protein–polyphenol interactions decrease the antioxidant activity of polyphenols (Niseteo et al., 2012; Sánchez-González et al., 2005; Yildirim-Elikoglu & Erdem, 2017); however, the exact mechanisms through which non-covalent interactions with milk proteins reduce the antioxidant activity of polyphenols remain poorly understood. Furthermore, the lack of standardised antioxidant activity assays in different studies challenges the comparison of results and underscores the need for a universally accepted protocol for such assessments.

In this study, the amount of free caffeine recovered before digestion was observed to decrease in samples where κ-casein, α-LA, and β-LG were mixed with caffeine. This suggests that caffeine interacts with these milk proteins even before digestion. Additionally, digestion itself reduced the amount of free caffeine in control samples, potentially due to interactions between caffeine and digestive enzymes. At the end of digestion, the recovery of free caffeine in samples containing α-casein, β-casein, and κ-casein was comparable to that of control caffeine samples, indicating that caffeine exhibits a higher affinity for whey proteins than caseins during digestion. This observation implies that the interactions between caffeine and whey proteins may lead to a greater reduction in unbound caffeine, likely impacting bioactivity. Similarly, a reduction in free caffeine content was observed for black coffee during digestion, which became more pronounced with the addition of milk.

The primary aim of this research was to estimate the *in vitro* bioaccessibility of caffeine in coffee brews with the addition of milk, in order to determine whether milk reduces caffeine bioaccessibility in such beverages. Based on previous studies investigating the bioaccessibility of polyphenols in tea and coffee following milk addition, it was hypothesised that milk addition would similarly reduce the bioaccessibility of caffeine in coffee (Duarte & Farah, 2011; Dupas et al., 2006; Otemuyiwa et al., 2017). The caffeine concentration obtained from UV spectroscopic analysis and HPLC were used to calculate the *in vitro* bioaccessibility (%) of caffeine and is summarised in Table 4.3. The percentage bioaccessibility of caffeine in black and milk-based coffee was calculated using equation 3.1 and the results obtained were 85.8±0.4 and 77.0±0.9%, respectively. The lower value for milk-based coffee suggests that milk addition negatively impacted the bioaccessibility of caffeine. Similar findings that milk addition slightly reduced coffee polyphenol absorption in the body were reported by Dupas et al. (2006) and Duarte and Farah (2011). When Lamothe et al. (2014) studied the effect of the dairy matrix on polyphenols, they observed a reduction in polyphenol recovery upon adding milk to tea extracts. The interactions of caffeine with milk proteins are the primary cause of this reduction in bioaccessibility. Milk proteins also reduce the absorption of caffeine in the small intestine. These findings suggest that black coffee provides more readily available caffeine than milk-based coffee.

One limitation of this study is that bound compounds, which were removed through centrifugation and filtration, were not included in the quantification of caffeine. This is a critical consideration, as bound caffeine in the human body would remain in circulation and could potentially be transported to target organs, with proteins acting as carriers for delivery. If these bonds are reversible, proteins may facilitate the transport of caffeine to target tissues while retaining bioactivity. However, if the bonds are irreversible, the effect of caffeine may be diminished, thereby reducing its bioactivity. Since bound caffeine was excluded from the analysis, only free caffeine was considered in this study.

Table 4.3 summarises the data obtained from HPLC analysis for black coffee and milk-based coffee, including the percentage change (calculated using equation 3.2) across various digestion phases for each sample. Table 4.4 provides a comparison between black coffee and milk-based coffee at corresponding stages of digestion, along with the percentage change observed.

In black coffee, the acidic conditions of the gastric phase may have contributed to the observed 47.6% reduction in free caffeine concentration after gastric digestion compared to pre-digestion

levels. The acidic environment influences the solubility of caffeine. The subsequent increase of 63.2% in free caffeine concentration after intestinal digestion suggests that caffeine becomes more bioavailable in the neutral or alkaline conditions of the intestinal phase. In milk-based coffee, a similar reduction of 40.5% was observed after gastric digestion; however, the percentage increase in free caffeine concentration post-intestinal digestion was lower (27.8%) compared to that of black coffee. This indicates that caffeine-milk protein interactions are stronger and less reversible than caffeine interactions with digestive enzymes or chlorogenic acids (Dupas et al., 2006; Ishizu & Tsutsumi, 2012; Kashani-Amin et al., 2013). The reduction in free caffeine concentration observed in each digestion phase, when comparing black coffee and milk-based coffee, further supports the hypothesis that caffeine interacts with milk proteins.

Table 4.3 Caffeine content (µg/mL) obtained from HPLC analysis, percentage change obtained when compared across each digestion phase, and bioaccessibility of caffeine (%) in black coffee and milk-based coffee.

Sample		Before digestion vs. gastric phase	Gastric phase vs. intestinal phase	Before digestion vs. intestinal phase
Black coffee	Caffeine content (µg/mL)	173.07→90.66	90.66→147.92	173.07→147.92
	Percentage change (%)	-47.6	+63.2	-14.5
	Bioaccessibility of caffeine (%)			85.8±0.4
Milk-based coffee	Caffeine content (µg/mL)	141.99→84.57	84.57→108.05	141.99→108.05
	Percentage change (%)	-40.5	+27.8	-23.9
	Bioaccessibility of caffeine (%)			77.0±0.9

Table 4.4 Caffeine content (µg/mL) of black coffee and milk-based coffee obtained from HPLC analysis and percentage change obtained when compared to the corresponding digestion phase for both samples

	Caffeine content (µg/mL)		Percentage change (%)
	Black coffee	Milk-based coffee	
Before digestion	173.07	141.99	-17.9
Gastric phase	90.66	84.57	-6.7
Intestinal phase	147.92	108.05	-26.9

In vivo studies are essential to fully understand the physiological effects of caffeine when milk is added to coffee. Such studies would account for the absorption kinetics of bound caffeine, transport to target organs, and the bioavailability of both free and bound forms. This current research primarily focuses on determining the interactions between caffeine and milk proteins by quantifying the amount of free caffeine recovered after each digestion phase and examining how digestion influences these interactions. Future research integrating *in vivo* models is crucial to provide a comprehensive understanding of the bioactivity and bioaccessibility of caffeine in milk-based coffee.

5. Conclusions and future recommendations

5.1 Conclusions

This thesis examines the impact of the addition of milk to brewed coffee on the bioaccessibility of caffeine and the antioxidant activity of coffee. An *in vitro* gastrointestinal digestion model was chosen to study the effect of digestion on black coffee and milk-based coffee. The effect of milk addition on interactions of various phenolic compounds in coffee and tea has been studied previously (Niseteo et al., 2012; Quan et al., 2020; Ramalakshmi et al., 2009; Soares et al., 2021), but the impact of milk addition has not been discussed anywhere nor was considered in any of the previous studies. Based on the results from the corresponding analyses in this thesis (UV spectroscopy, HPLC, TPC, DPPH, FTIR, particle size, and ζ-potential), evidence was provided that caffeine interacts with milk proteins, shown by a reduction in free caffeine content at various digestion stages (before digestion, after gastric and intestinal phases).

Caffeine content analysis of pure caffeine, milk proteins, and coffee by UV spectroscopy and HPLC suggests that caffeine interacts with milk proteins, forming complexes that reduce free caffeine content compared to the control samples. The free concentration of caffeine decreased as digestion proceeded, indicating that milk proteins reduce the amount of free caffeine available in the matrix, which aligns with the findings of Soares et al. (2021) and Khochapong et al. (2021), confirming the robustness of the current findings. Gastric conditions are the most suitable environment for caffeine-milk protein interactions, with the least amount of free-caffeine concentration obtained after this phase. At the end of intestinal digestion, more free caffeine was retained, indicating that the binding reactions are reversible (Yildirim-Elikoglu & Erdem, 2017). The results from TPC and DPPH also support the hypothesis that milk addition reduces the overall bioactivity of the compounds in black coffee and digestion of the samples also seems to reduce the bioactivity and antioxidant potential of the compounds in coffee (Dubeau et al., 2010; Sánchez-González et al., 2005; Zorilla et al., 2011). In particular, the bioactivity of caffeine was also reduced by milk-protein interactions. From the FTIR data, it is evident that caffeine interacts with milk proteins with peak shifts, suggesting the possibility of Van der Waals interactions and hydrogen bonding between caffeine and milk proteins. The data obtained from particle size measurement and ζ-potential measurements also suggest that milk addition negatively impacts the bioaccessibility of caffeine in coffee by making it more stable in association with milk proteins (Okubo et al., 2008). This stability may render the compounds more inert and reduce the rate of absorption into the human body.

The bioaccessibility of caffeine in black coffee and milk-based coffee was 85.8±0.4 and 77.0±0.9%, respectively. This suggests that milk addition negatively affected the bioaccessibility of caffeine. The primary focus of this research was to understand the effect of milk addition to coffee on the bioaccessibility of caffeine, which was hypothesised to reduce bioaccessibility. The interactions of caffeine with milk proteins were a factor in this reduction, and evidence was provided by studying the effect of individual milk proteins with caffeine. Even though the results show a reduction in free caffeine content after milk addition to black coffee, it is important to note that the removal of denser particles during centrifugation would exclude caffeine that might be present in this fraction from analysis. Accordingly, it cannot be conclusively stated that milk addition reduced caffeine bioaccessibility. Further analysis of the precipitate by dissolving the complexes in a solvent to release the bound caffeine and quantifying it using liquid chromatography combined with mass spectroscopy or using methods such as isotope-labelling is necessary to determine if caffeine is present, and this needs to be studied further to understand the effect of milk addition on black coffee during digestion.

In the current study, an *in vitro* digestion model was used to investigate the concentration of free caffeine; however, several limitations inherent to this approach should be addressed in future research. One significant drawback is that the present study only analysed the caffeine content at the endpoints of gastric and intestinal digestion phases. Although this provides a snapshot of caffeine concentration at those specific stages, it fails to capture the dynamic changes that occur throughout the digestion process. Future studies should conduct a time-resolved analysis, estimating caffeine content at regular intervals (e.g., every 15 min) during digestion. This approach would provide a more comprehensive understanding of how digestion impacts caffeine bioaccessibility over time. Additionally, varying the duration of each digestion phase could reveal how changes in gastric and intestinal transit times influence the release and absorption of caffeine.

Moreover, although *in vitro* models are valuable for simulating digestion, these cannot fully replicate the complexities of *in vivo* systems. For instance, understanding the half-life of caffeine, the time profile for caffeine to be released from the stomach and intestine, and the eventual absorption into the bloodstream requires *in vivo* studies. Such studies are crucial for determining the bioavailability of caffeine, which includes not only release during digestion but also absorption and metabolism. Besides that, the pharmacological effects of caffeine, such as the impact on alertness or cardiovascular health, depend upon the amount of caffeine that

reaches target tissues. Future *in vivo* studies should focus more on how the addition of milk affects the timing and extent of the physiological effects of caffeine.

Additionally, understanding how caffeine is metabolized in the liver and converted into active metabolites, such as paraxanthine, theobromine, and theophylline, is crucial to further understanding the health benefits. Milk addition affects the production of these metabolites, which in turn contributes to the overall pharmacological profile of caffeine. The duration and intensity of these physiological effects are potentially affected by caffeine metabolites. Milk proteins and fat may alter the rate at which caffeine is metabolized or the proportions of these metabolites formed. Investigating these processes *in vivo* will provide deeper insights into the interactions between caffeine and milk and the implications for caffeine metabolism and physiological efficacy.

5.2 Future recommendations

Future studies should incorporate advanced analytical techniques such as fluorescence spectroscopy, isothermal titration calorimetry (ITC), and molecular docking analysis to further elucidate caffeine-milk protein interactions. Fluorescence spectroscopy is a powerful tool for assessing the strength and nature of bonds between caffeine and milk proteins. It can detect conformational changes in protein structure upon complex formation, providing insights into how these interactions may influence functional properties. Additionally, fluorescence spectroscopy can evaluate the stability of caffeine-protein interactions under varying conditions, such as pH and temperature, which is critical for understanding their behaviour during digestion. This technique's ability to quantify bioaccessible caffeine in real-time makes it particularly suitable for time-resolved studies, offering deeper insights into the dynamic behaviour of these complexes.

ITC, on the other hand, provides thermodynamic insights into caffeine-protein interactions by measuring key parameters such as binding affinity, enthalpy, and entropy. These data help to determine the forces driving these interactions, such as hydrogen bonding and hydrophobic effects. Unlike fluorescence spectroscopy, ITC is particularly effective for studying the impact of varying protein concentrations on binding stoichiometry, allowing for the identification of potential saturation points in caffeine binding. Moreover, ITC can assess the influence of temperature on these interactions, providing valuable data on the thermal stability of caffeine-protein complexes under different physiological and processing conditions.

Molecular docking analysis offers a computational approach to identifying specific binding sites on milk proteins where caffeine molecules attach. This method reveals detailed interaction profiles, such as hydrogen bonding and hydrophobic interactions, which are challenging to capture experimentally. Docking studies can also compare binding affinities among different protein variants, helping to pinpoint which proteins play the most significant role in caffeine binding. Furthermore, molecular docking provides a visual representation of experimental findings, facilitating molecular-level interpretations of caffeine-milk protein interactions.

Although these techniques share the common goal of characterising caffeine-protein interactions, each offers unique advantages. Fluorescence spectroscopy is invaluable for real-time monitoring and structural analysis, ITC excels in thermodynamic and stoichiometric characterisation, and molecular docking provides precise insights into binding mechanisms at the molecular level. Together, these methods can offer a comprehensive understanding of the factors influencing caffeine bioaccessibility and interaction dynamics, guiding more targeted future research.

In this study, caffeine-milk protein interactions were investigated using a fixed milk-to-coffee ratio (2:1). Future research should explore the effects of varying milk concentrations on caffeine bioaccessibility and protein-caffeine complex formation. Adjusting the milk concentration may provide deeper insights into how different levels of milk affect the extent of caffeine-protein interactions and the overall bioactive profile of the beverage. This could reveal concentration-dependent trends that were not addressed in the current study, thereby enhancing our understanding of how milk composition influences coffee's functional properties.

Additionally, this study maintained a constant protein concentration when examining caffeine interactions. Future studies should investigate how variations in protein concentration affect caffeine binding. Since protein availability directly impacts caffeine interactions, altering protein concentration may significantly influence binding dynamics and the overall functionality of these complexes. Such studies would provide a more comprehensive understanding of how protein levels affect caffeine bioaccessibility.

Moreover, this study examined caffeine-protein interactions under a single pH condition (pH 7.4 in PBS buffer). Since coffee naturally has a lower pH, future research should assess these interactions under varying pH conditions to determine how the acidic environment of coffee influences free caffeine concentration and its interactions with milk proteins during digestion. Understanding the stability of caffeine-milk protein complexes under different pH conditions

could provide insights into caffeine release during various digestive phases, which is essential for understanding its bioavailability and physiological effects.

Another potential avenue for research is examining caffeine-milk protein interactions in skim milk. By removing the influence of milk fat, researchers can isolate the specific effects of proteins on caffeine binding. Additionally, the role of milk fat in these interactions could be explored using milk with varying fat concentrations. This approach would help determine whether milk fat enhances or inhibits caffeine bioaccessibility and its potential impact on the functional properties of milk-based coffee.

Future research could also investigate how additional factors, such as milk temperature, brewing time, and coffee bean type (e.g., Arabica vs. Robusta), influence caffeine content and its interactions with milk proteins. Variations in milk temperature may alter protein-caffeine binding kinetics, while differences in brewing time could affect the extraction of bioactive compounds. These variables should be examined to assess the robustness of the conclusions drawn from this study. Additionally, the distinct chemical compositions of Arabica and Robusta coffee beans may result in unique interaction patterns with milk components. Investigating these variables would provide a more nuanced understanding of how different preparation methods and coffee types impact caffeine-protein interactions and the beverage's functional profile.

The addition of milk to coffee enhances its sensory attributes, making it more palatable for many consumers. However, it also significantly influences free caffeine content. This study found that at a 2:1 milk-to-coffee ratio, free caffeine content decreased during digestion compared to that before digestion and also when compared to black coffee samples, potentially reducing the bioactivity of the coffee brew. This reduction highlights the importance of understanding caffeine-milk interactions, as they may have implications for caffeine's physiological effects.

These findings are particularly relevant as they offer insights into how milk affects caffeine bioaccessibility and efficacy. The results have implications for dietary recommendations and coffee consumption habits, particularly for individuals who need to monitor their caffeine intake. By understanding these interactions, consumers can make informed choices that align with their health goals and caffeine tolerance. Moreover, the food industry could leverage these findings to develop new coffee formulations that optimise health benefits. By applying this knowledge, manufacturers can create milk-based coffee products that retain desirable sensory

properties while maximising caffeine's bioaccessibility and functional benefits. This could lead to innovative coffee beverages with enhanced stability, improved bioaccessibility, and tailored health benefits, catering to a wide range of consumer preferences.

Lastly, future research should continue addressing these treatment variables to build a more comprehensive understanding of caffeine-milk protein interactions. Employing both advanced *in vitro* models with time-resolved analysis and *in vivo* studies would provide a more holistic view of caffeine bioaccessibility, bioavailability, and pharmacokinetics. Such research would offer valuable insights into how milk addition influences the physiological and pharmacological effects of caffeine, ultimately contributing to more informed dietary and industrial applications.

6. References

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Appendices

Appendix 1

Samples and experimental setup.

Figure A1. 1 Coffee beans and milk used for the study

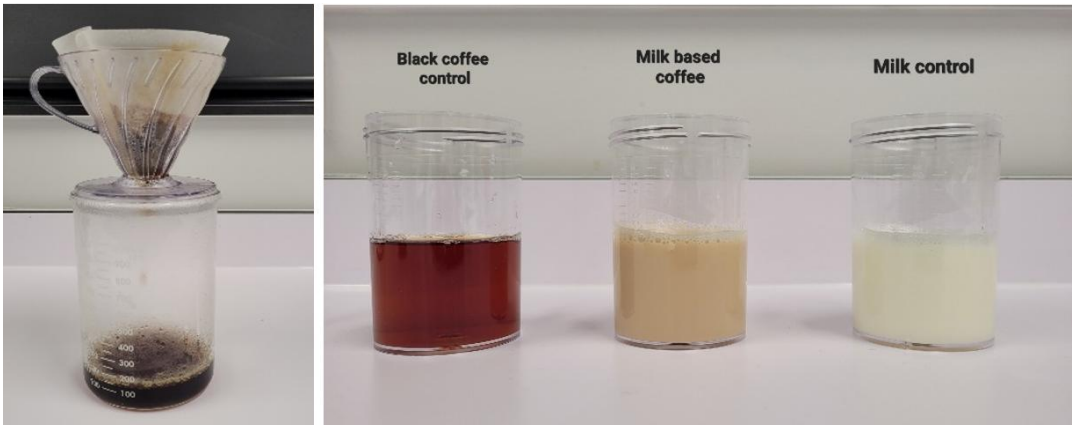


Figure A1. 2 Preparation technique of the coffee brew (drip brewing method) and coffee samples and milk used for testing

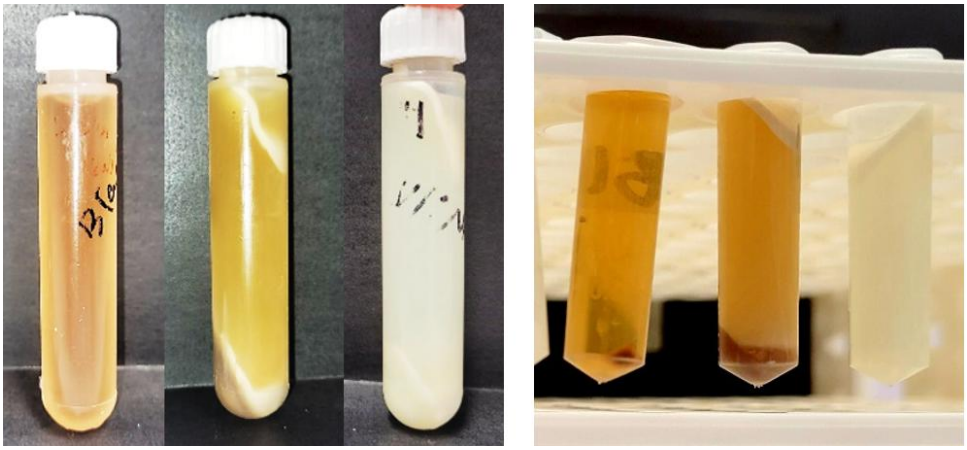


Figure A1. 3 Black coffee, milk-based coffee, and milk after ultracentrifugation

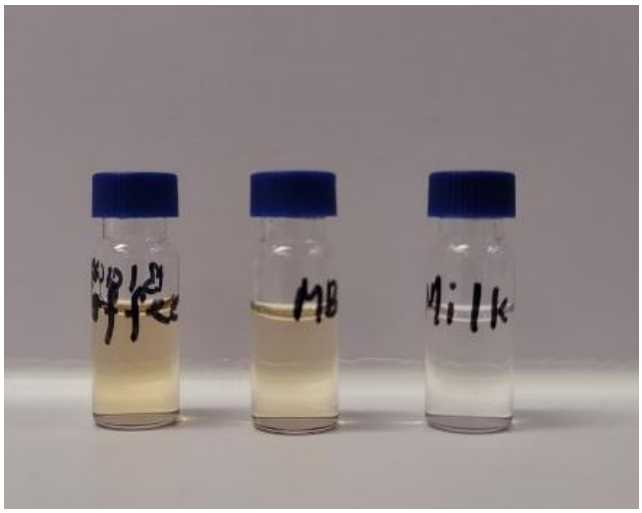


Figure A1. 4 Black coffee, milk-based coffee, and milk samples after ultracentrifugation, extraction using acetonitrile, and filtration for HPLC analysis

Table A1. 1 Anchor blue nutritional information

Composition	Average quantity per 100 mL
Energy	263 kJ (63 calls)
Protein	3.3 g
Fat - Total	3.4 g
-Saturated	2.3 g
Carbohydrates	4.8 g
-sugars	4.8 g
Sodium	40 mg
Calcium	117 mg
Vitamin A	43 µg
Riboflavin (B_2)	0.2 mg

Appendix 2

Data related to the UV spectroscopy and HPLC analyses.

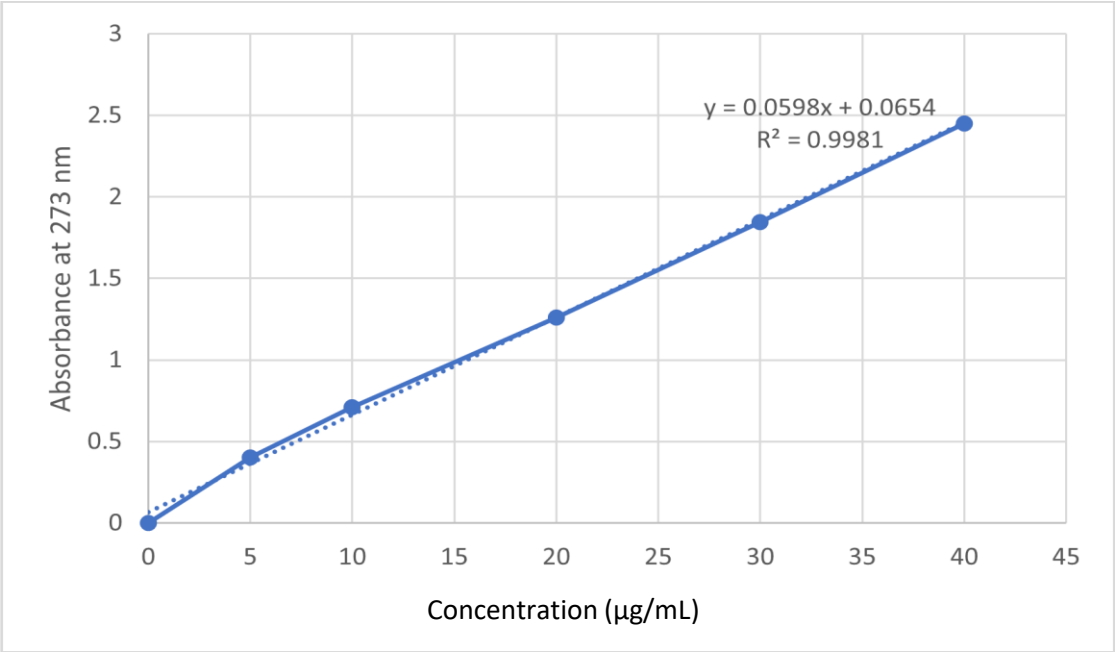


Figure A2. 1 A calibration curve for different concentrations of caffeine using UV spectroscopy for determination of caffeine content of the samples

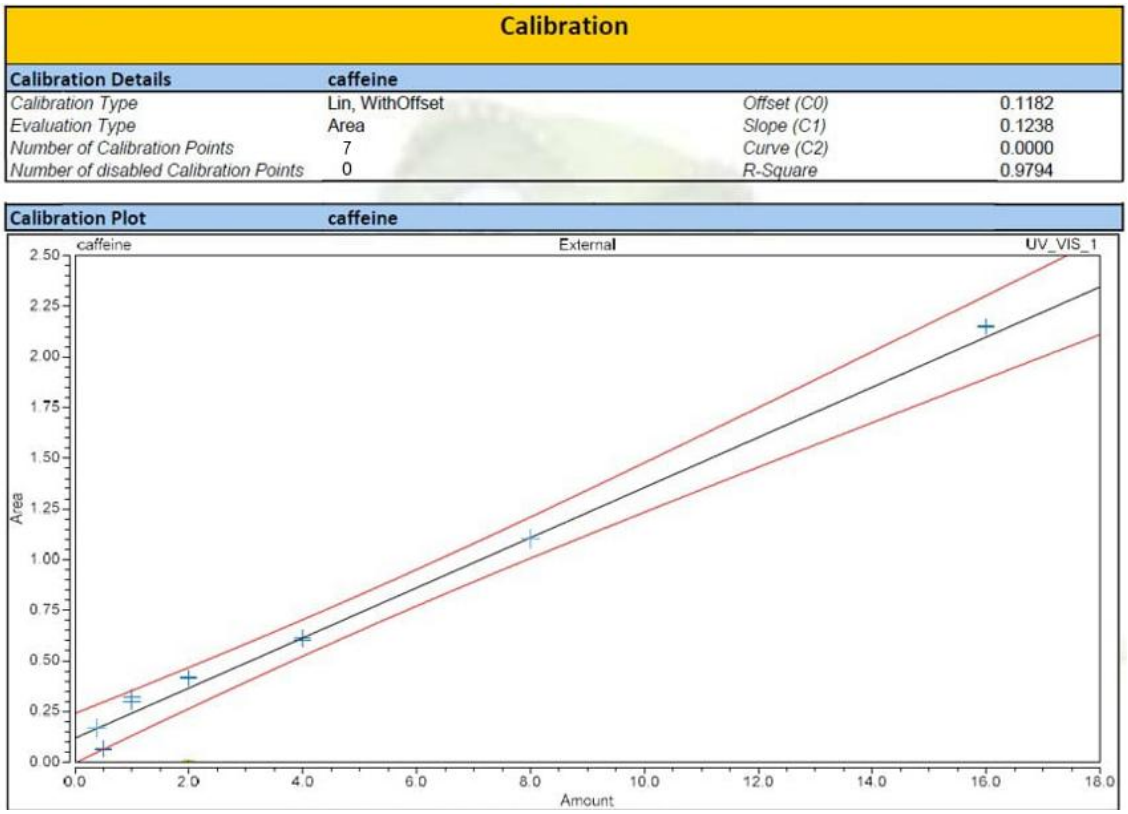
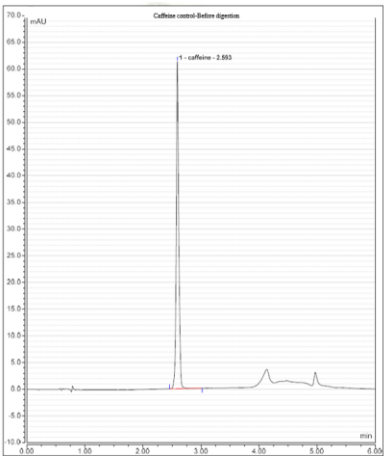
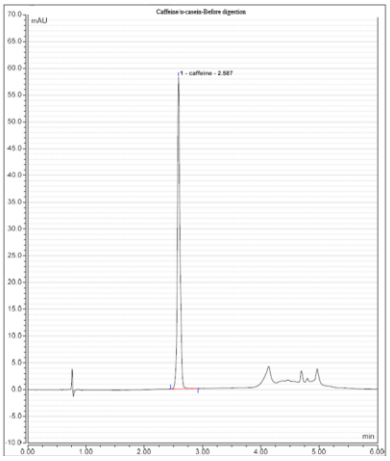


Figure A2. 2 A calibration curve for different concentrations of caffeine for determination of caffeine content of the samples using HPLC method obtained from Chromeleon software


Caffeine control-Before digestion
mAU
1 - caffeine - 2.593
min


Caffeine/α-casein-Before digestion
mAU
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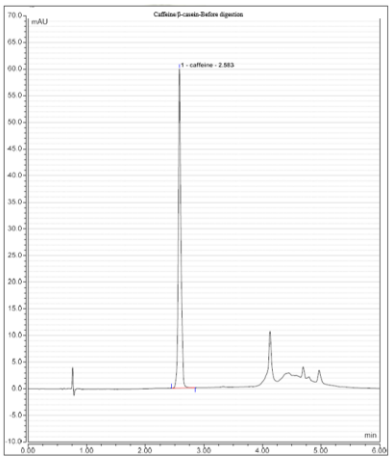
min


Caffeine/β-casein-Before digestion
mAU
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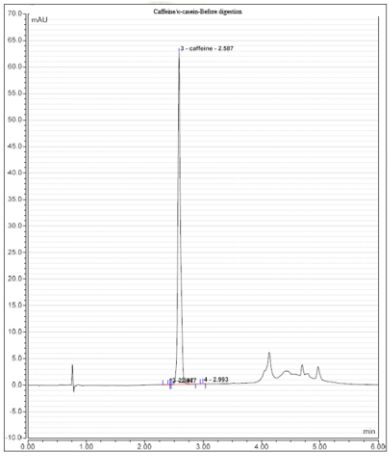
min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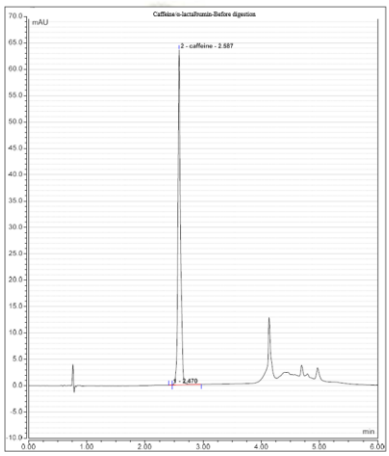
mAU
3 - caffeine - 2.587
4 - 2.993
min


Caffeine/α-lactalbumin-Before digestion
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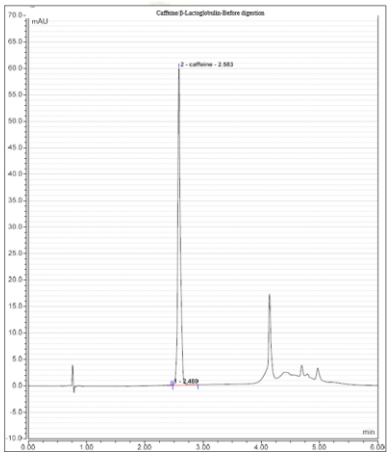
min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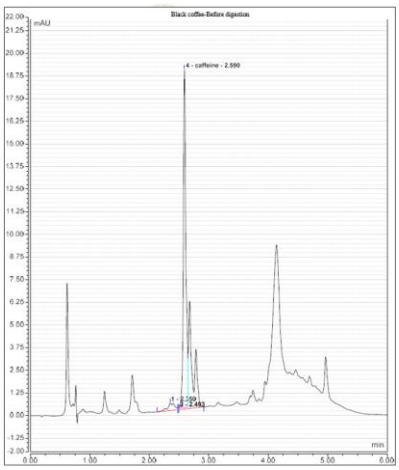
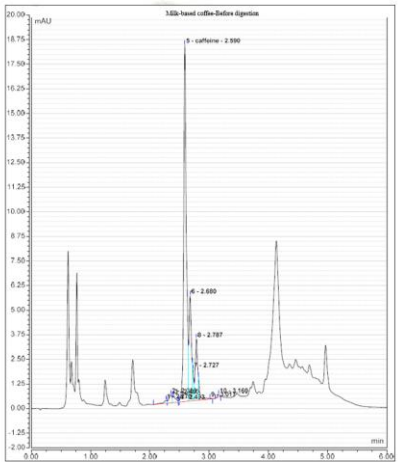
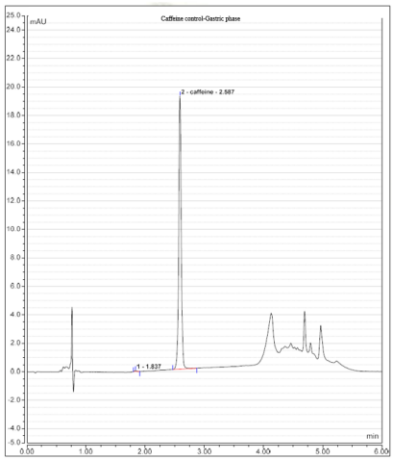
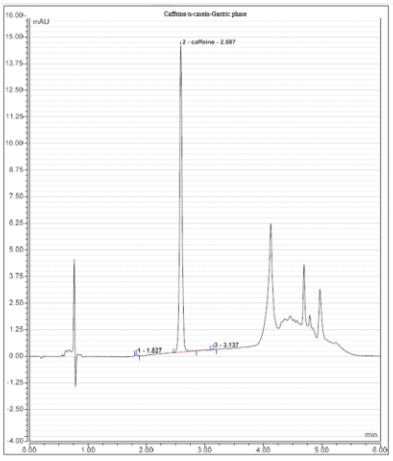
2.489
min


Black coffee-Before digestion
mAU
4 - caffeine - 2.590
min


Milk-based coffee-Before digestion
mAU
5 - caffeine - 2.590
6 - 2.680
8 - 2.787
2.727
min


Caffeine control-Gastric phase
mAU
2 - caffeine - 2.587
1 - 1.837
min


Caffeine/α-casein-Gastric phase
mAU
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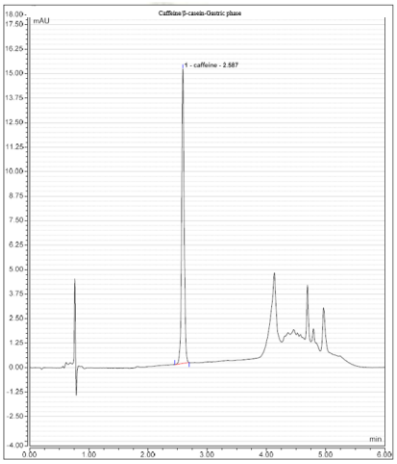
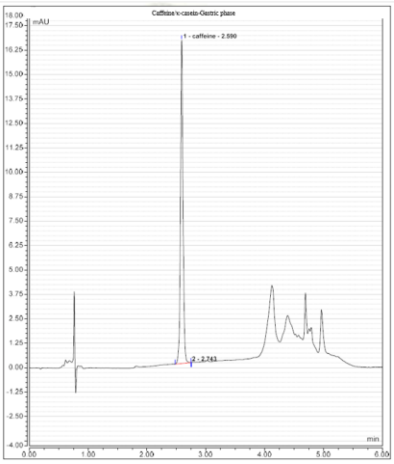
1 - 1.827
2 - 3.137
min


Caffeine/β-casein-Gastric phase
mAU
1 - caffeine - 2.587
min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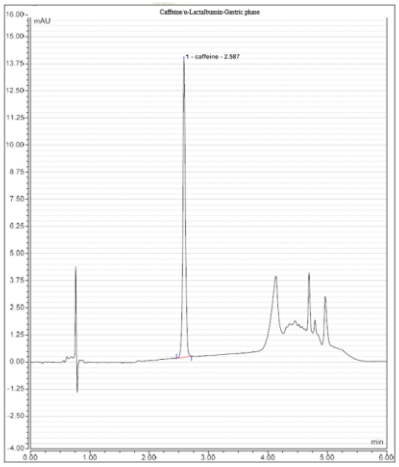
mAU
1 - caffeine - 2.590
2 - 2.763
min


Caffeine/α-Lactalbumin-Gastric phase
mAU
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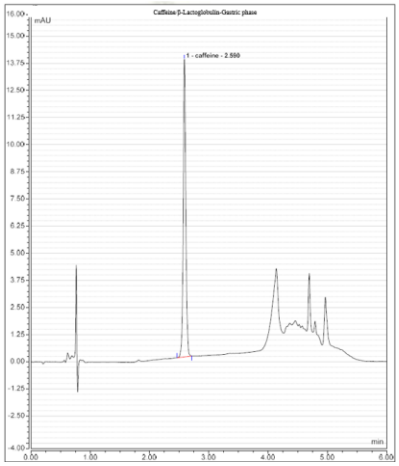
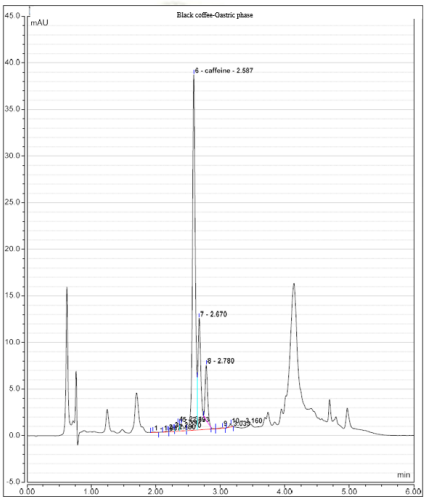
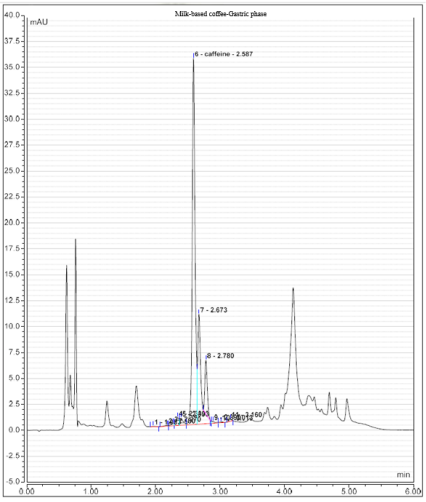
min


Caffeine/β-Lactoglobulin-Gastric phase
mAU
1 - caffeine - 2.590
min


Black coffee-Gastric phase
mAU
6 - caffeine - 2.587
7 - 2.670
8 - 2.780
min


Milk-based coffee-Gastric phase
mAU
6 - caffeine - 2.587
7 - 2.673
8 - 2.780
min

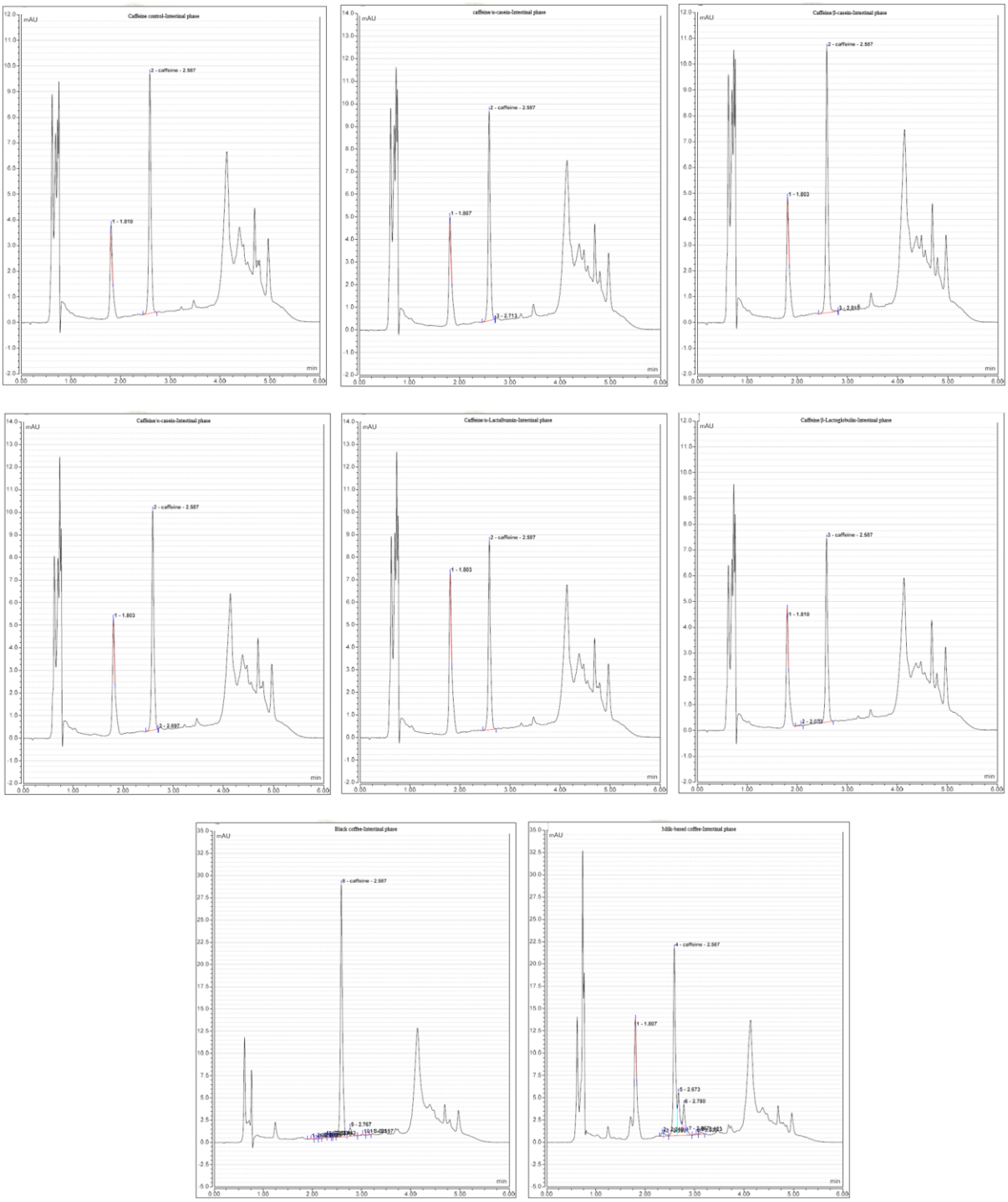


Figure A2. 3 The chromatograms of all samples obtained by HPLC analysis at different digestion stages.

Table A2. 1 The caffeine content (µg caffeine/mL) of caffeine/α-casein at different digestion stages compared against the control obtained through UV spectroscopy and HPLC analysis. Values are the mean±SD (n=6). Values with different superscripts (a-c) for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	UV	HPLC
Before digestion	Caffeine control	99.3±0.5^{a}	98.33±0.04^{a}
	Caffeine/α-casein	87.6±0.5^{a}	86.41±0.03^{a}
Gastric phase	Caffeine control	55.12±0.23^{b}	55.06±0.07^{b}
	Caffeine/α-casein	39.7±0.3^{c}	39.04±0.03^{c}
Intestinal phase	Caffeine control	64.2±0.5^{b}	63.87±0.03^{b}
	Caffeine/α-casein	58.4±0.5^{b}	58.96±0.03^{b}

Table A2. 2 The caffeine content (µg caffeine/mL) of caffeine/β-casein at different digestion stages compared against the control obtained through UV spectroscopy and HPLC analysis. Values are the mean±SD (n=6). Values with different superscripts (a-c) and (a-d) respectively for each result is significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA

Digestion phase	Samples	UV	HPLC
Before digestion	Caffeine control	99.3±0.5^{a}	98.33±0.04^{a}
	Caffeine/β-casein	96.87±0.30^{a}	92.43±0.03^{a}
Gastric phase	Caffeine control	55.12±0.23^{b}	55.06±0.07^{c}
	Caffeine/β-casein	41.9±0.6^{c}	41.94±0.40^{d}
Intestinal phase	Caffeine control	64.2±0.5^{b}	63.87±0.03^{b}
	Caffeine/β-casein	55.0±0.8^{b}	54.06±0.30^{c}

Table A2. 3 The caffeine content (µg caffeine/mL) of caffeine/κ-casein at different digestion stages compared against the control obtained through UV spectroscopy and HPLC analysis. Values are the mean±SD (n=6). Values with different superscripts (a-d) for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	UV	HPLC
Before digestion	Caffeine control	99.3±0.5^{a}	98.33±0.04^{a}
	Caffeine/κ-casein	83.7±0.6^{b}	82.22±0.03^{cd}
Gastric phase	Caffeine control	55.12±0.23^{cd}	55.06±0.07^{b}
	Caffeine/κ-casein	46.9±0.4^{d}	45.5±0.5^{cd}
Intestinal phase	Caffeine control	64.2±0.5^{c}	63.87±0.03^{c}
	Caffeine/κ-casein	57.8±0.8^{cd}	57.03±0.60^{d}

Table A2. 4 The caffeine content (µg caffeine/mL) of caffeine/α-lactalbumin at different digestion stages compared against the control obtained through UV spectroscopy and HPLC analysis. Values are the mean±SD (n=6). Values with different superscripts (a-e) and (a-d) respectively for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	UV	HPLC
Before digestion	Caffeine control	99.3±0.5^{a}	98.33±0.04^{a}
	Caffeine/α-La	83.0±0.3^{b}	82.45±0.05^{b}
Gastric phase	Caffeine control	55.12±0.23^{cd}	55.06±0.07^{c}
	Caffeine/α-La	38.70±0.19^{e}	38.1±0.3^{d}
Intestinal phase	Caffeine control	64.2±0.5^{c}	63.87±0.03^{c}
	Caffeine/α-La	50.8±0.6^{de}	50.5±0.3^{cd}

Table A2. 5 The caffeine content (µg caffeine/mL) of caffeine/β-lactoglobulin at different digestion stages compared against the control obtained through UV spectroscopy and HPLC analysis. Values are the mean±SD (n=6). Values with different superscripts (a-e) for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	UV	HPLC
Before digestion	Caffeine control	99.3±0.5^{a}	98.33±0.04^{a}
	Caffeine/β-Lg	80.3±0.7^{b}	78.87±0.05^{b}
Gastric phase	Caffeine control	55.12±0.23^{cd}	55.06±0.07^{cd}
	Caffeine/β-Lg	37.5±0.2^{e}	36.1±0.3^{e}
Intestinal phase	Caffeine control	64.2±0.5^{c}	63.87±0.03^{bc}
	Caffeine/β-Lg	45.1±0.6^{de}	44.0±0.5^{de}

Table A2. 6 The caffeine content (µg caffeine/mL) of black coffee and milk-based coffee at different digestion stages. Values are the mean±SD (n=6). Columns with different superscripts (a-c) and (a-d) respectively for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	UV	HPLC
Before digestion	Black coffee	175.4±0.6^{a}	173.1±0.5^{a}
	Milk-based coffee	142.8±0.7^{b}	141.99±0.06^{b}
Gastric phase	Black coffee	93.04±0.30^{c}	90.7±0.3^{d}
	Milk-based coffee	84.2±0.6^{c}	84.6±0.5^{d}
Intestinal phase	Black coffee	151.2±0.4^{ab}	147.9±0.1^{b}
	Milk-based coffee	111.1±0.6^{c}	108.1±0.3^{c}

Appendix 3

Data related to the total phenolic content determination and DPPH analysis.

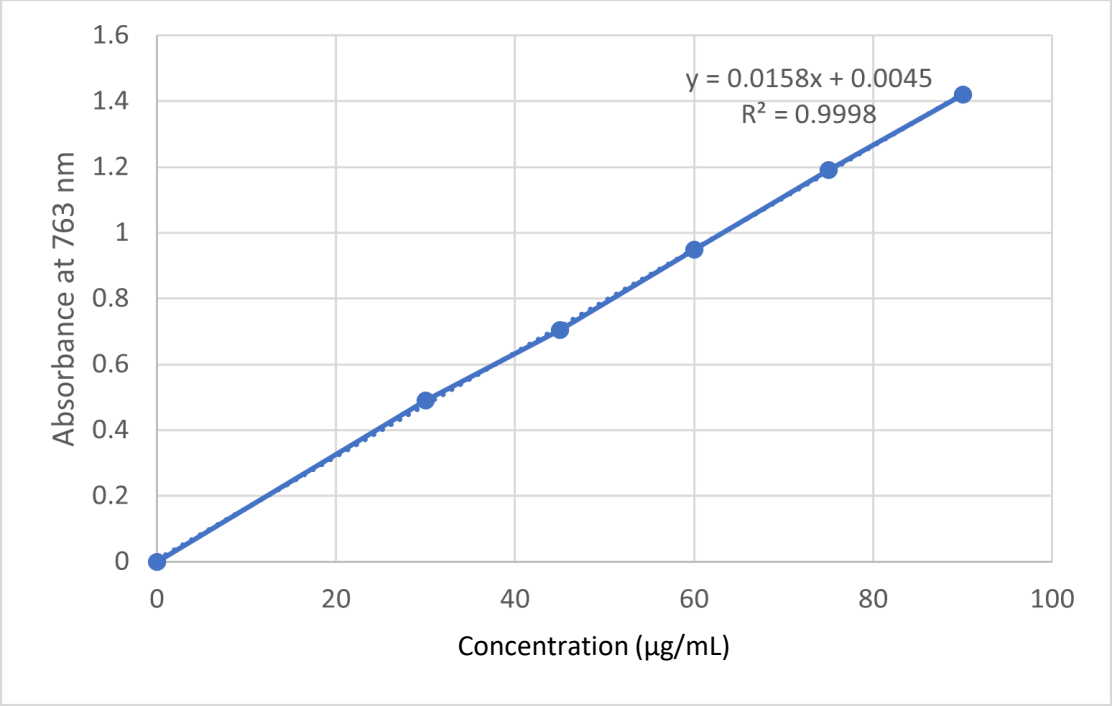


Figure A3. 1 A calibration curve for different concentrations of gallic acid using the Folin-Ciocâlteu method for total phenolic content determination of samples

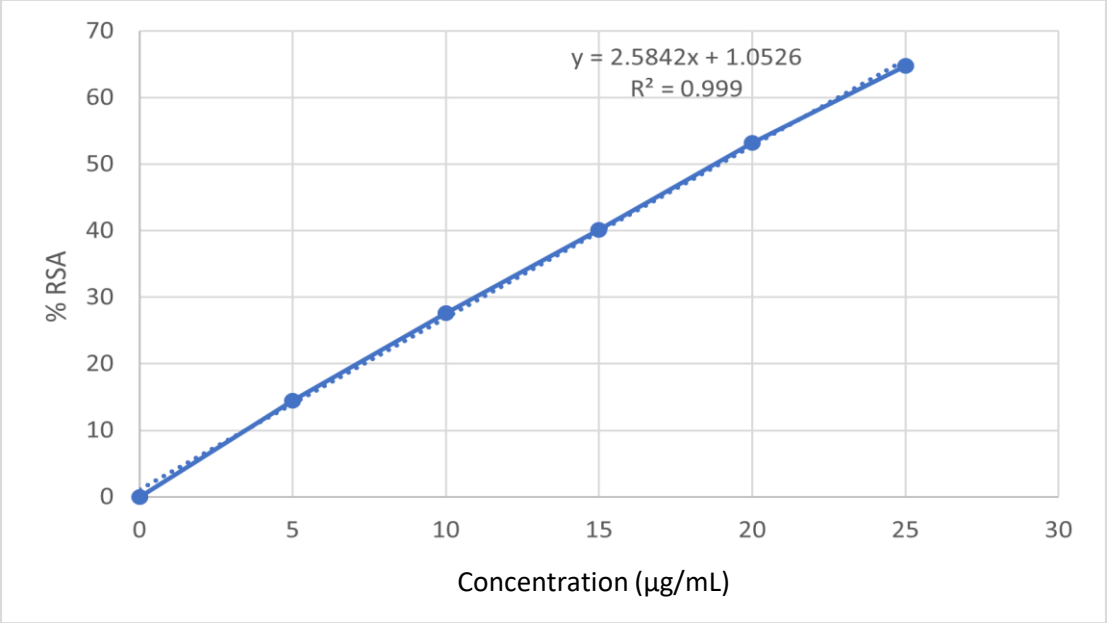


Figure A3. 2 A curve plotted by calculating the % radical scavenging activity of different concentrations of Trolox determined using the DPPH method.

Table A3. 1 The total phenolic content (in μg GAE/mL) and Trolox equivalent antioxidant capacity (in μg/mL) (DPPH method) of caffeine with α-casein at different digestion stages compared against the controls. Values are the mean±SD (n=6). Values with different superscripts (a-d) and (a-c) respectively for each result are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Sample	TPC	DPPH
Before digestion	Caffeine control	45.8 ± 0.3^{d}	2.8 ± 0.8^{c}
	Caffeine/α-casein	83.4 ± 0.3^{a}	6.4 ± 0.7^{abc}
	α-Casein control	85.0 ± 0.7^{a}	9.4 ± 0.7^{a}
Gastric phase	Caffeine control	41.7 ± 0.4^{d}	1.8 ± 0.3^{c}
	Caffeine/α-casein	51.7 ± 0.4^{c}	4.6 ± 0.3^{bc}
	α-Casein control	60.4 ± 0.4^{b}	4.3 ± 0.3^{bc}
Intestinal phase	Caffeine control	42.5 ± 0.3^{d}	7.0 ± 0.3^{ab}
	Caffeine/α-casein	41.9 ± 0.4^{d}	4.9 ± 0.4^{abc}
	α-Casein control	62.2 ± 0.4^{b}	9.6 ± 0.5^{a}

Table A3. 2 The total phenolic content (in μg GAE/mL) and Trolox equivalent antioxidant capacity (in μg/mL) (DPPH method) of caffeine with β-casein at different digestion stages compared against the controls. Values are the mean±SD (n=6). Values with different superscripts (a-e) and (a, b) respectively for each result are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Sample	TPC	DPPH
Before digestion	Caffeine control	45.8 ± 0.3^{cde}	2.8 ± 0.8^{b}
	Caffeine/β-casein	62.5 ± 0.3^{a}	2.9 ± 0.8^{b}
	β-Casein control	54.2 ± 0.4^{b}	4.8 ± 0.8^{ab}
Gastric phase	Caffeine control	41.7 ± 0.4^{e}	1.8 ± 0.3^{b}
	Caffeine/β-casein	46.7 ± 0.4^{c}	4.7 ± 0.3^{ab}
	β-Casein control	44.6 ± 0.4^{cde}	5.1 ± 0.5^{ab}
Intestinal phase	Caffeine control	42.5 ± 0.3^{de}	7.01 ± 0.19^{a}
	Caffeine/β-casein	47.8 ± 0.4^{c}	5.1 ± 0.3^{a}
	β-Casein control	45.9 ± 0.4^{cd}	7.2 ± 0.4^{ab}

Table A3. 3 The total phenolic content (in μg GAE/mL) and Trolox equivalent antioxidant capacity (in μg/mL) (DPPH method) of caffeine with κ-casein at different digestion stages compared against the controls. Values are the mean±SD (n=6). Values with different superscripts (a-e) and (a, b) respectively for each result are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Sample	TPC	DPPH
Before digestion	Caffeine control	45.8 ± 0.3^{cde}	2.8 ± 0.8^{b}
	Caffeine/κ-casein	76.9 ± 0.3^{a}	5.4 ± 0.7^{ab}
	κ-Casein control	70.61 ± 0.19^{b}	2.3 ± 0.8^{b}
Gastric phase	Caffeine control	41.7 ± 0.4^{e}	1.8 ± 0.3^{b}
	Caffeine/κ-casein	49.0 ± 0.5^{c}	2.4 ± 0.3^{b}
	κ-Casein control	49.5 ± 0.6^{c}	7.1 ± 0.3^{a}
Intestinal phase	Caffeine control	42.5 ± 0.3^{de}	7.01 ± 0.19^{a}
	Caffeine/κ-casein	45.6 ± 3.3^{cde}	7.0 ± 0.4^{a}
	κ-Casein control	46.1 ± 0.4^{cd}	8.0 ± 0.4^{a}

Table A3. 4 The total phenolic content (in µg GAE/mL) and Trolox equivalent antioxidant capacity (in µg/mL) (DPPH method) of caffeine with α-La at different digestion stages compared against the controls. Values are the mean±SD (n=6). Values with different superscripts (a-e) and (a-c) respectively for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Sample	TPC	DPPH
Before digestion	Caffeine control	45.8±0.3^{cd}	2.8±0.8^{bc}
	Caffeine/α-La	70.6±0.4^{a}	6.9±0.6^{a}
	α-La control	65.9±0.5^{b}	6.3±0.7^{ab}
Gastric phase	Caffeine control	41.6±0.4^{e}	1.8±0.3^{c}
	Caffeine/α-La	47.8±0.4^{c}	5.9±0.3^{abc}
	α-La control	48.2±0.4^{c}	6.2±0.3^{abc}
Intestinal phase	Caffeine control	42.4±0.4^{de}	7.01±0.19^{a}
	Caffeine/α-La	41.6±2.9^{e}	6.2±0.4^{abc}
	α-La control	48.2±0.3^{c}	6.2±0.3^{abc}

Table A3. 5 The total phenolic content (in µg GAE/mL) and Trolox equivalent antioxidant capacity (in µg/mL) (DPPH method) of caffeine with β-Lg at different digestion stages compared against the controls. Values are the mean±SD (n=6). Values with different superscripts (a-d) and (a-c) respectively for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Sample	TPC	DPPH
Before digestion	Caffeine control	45.8±0.3^{c}	2.1±0.8^{c}
	Caffeine/β-Lg	66.2±0.4^{a}	14.5±0.6^{a}
	β-Lg control	64.2±0.4^{a}	16.1±0.5^{a}
Gastric phase	Caffeine control	41.7±0.4^{d}	1.8±0.3^{c}
	Caffeine/β-Lg	43.8±0.5^{cd}	5.6±0.3^{bc}
	β-Lg control	45.8±0.23^{c}	5.8±0.4^{bc}
Intestinal phase	Caffeine control	42.5±0.3^{cd}	7.01±0.19^{bc}
	Caffeine/β-Lg	57.45±0.19^{b}	7.46±0.18^{bc}
	β-Lg control	58.8±0.4^{b}	9.9±1.3^{ab}

Table A3. 6 The total phenolic content (in µg GAE/mL) and Trolox equivalent antioxidant capacity (in µg/mL) (DPPH method) of black coffee, milk, and milk-based coffee at different digestion stages. Values are the mean±SD (n=6). Values with different superscripts (a-c) and (a, b) respectively for each result are significantly different (p<0.05) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	TPC	DPPH
Before digestion	Black coffee	116.2±0.8^{a}	43.±0.30^{a}
	Milk	51.3±0.5^{c}	2.5±0.8^{b}
	Milk-based coffee	104.45±1.05^{b}	6.6±0.7^{b}
Gastric phase	Black coffee	105.6±0.5	32.9±0.5^{a}
	Milk	69.7±0.7^{c}	3.8±0.3^{b}
	Milk-based coffee	90.4±0.5^{b}	5.5±0.4^{b}
Intestinal phase	Black coffee	133.4±1.4^{a}	27.4±0.9^{a}
	Milk	88.42±1.19^{c}	1.6±0.7^{b}
	Milk-based coffee	94.7±0.8^{b}	3.6±0.5^{b}

Appendix 4

Data related to the FTIR spectroscopy analysis.

Table A4. 1 FTIR spectral data (wavenumber in cm^{-1}) of pure caffeine control, milk protein control, and caffeine-milk protein mixture before digestion

Sample	Peak 1	Peak 2	Peak 3	Peak 4
Caffeine control	2942	2827	1657	1550
α-Casein control	2942	2827	1653	1543
Caffeine/α-casein	2939	2827	1653	1539
β-Casein control	2942	2825	1651	1530
Caffeine/β-casein	2939	2850	1651	1534
κ-Casein control	2942	2830	1649	1532
Caffeine/κ-casein	2939	2836	1649	1536
α-Lactalbumin	2942	2840	1653	1528
Caffeine/α-La	2938	2844	1649	1532
β-Lactoglobulin	2938	2850	1637	1528
Caffeine/β-Lg	2935	2845	1638	1528

Table A4. 2 FTIR spectral data (wavenumber in cm^{-1}) of pure caffeine control, milk protein control, and caffeine-milk protein mixture after gastric digestion

Sample	Peak 1	Peak 2	Peak 3	Peak 4
Caffeine control	2948	2881	1650	1545
α-Casein control	2948	2881	1654	1545
Caffeine/α-casein	2948	2881	1654	1545
β-Casein control	2948	2878	1654	1545
Caffeine/β-casein	2948	2878	1654	1549
κ-Casein control	2948	2881	1650	1541
Caffeine/κ-casein	2948	2881	1654	1541
α-Lactalbumin	2948	2881	1654	1545
Caffeine/α-La	2948	2853	1654	1545
β-Lactoglobulin	2948	2881	1654	1545
Caffeine/β-Lg	2948	2853	1654	1545

Table A4. 3 FTIR spectral data (wavenumber in cm^{-1}) of pure caffeine control, milk protein control, and caffeine-milk protein mixture after intestinal digestion

Sample	Peak 1	Peak 2	Peak 3	Peak 4
Caffeine control	2952	2867	1650	1560
α-Casein control	2933	2861	1650	1565
Caffeine/α-casein	2942	2861	1654	1560
β-Casein control	2948	2861	1650	1560
Caffeine/β-casein	2948	2861	1650	1560
κ-Casein control	2933	2860	1650	1560
Caffeine/κ-casein	2933	2860	1650	1560
α-Lactalbumin	2939	2861	1651	1560
Caffeine/α-La	2936	2861	1651	1560
β-Lactoglobulin	2936	2861	1650	1567
Caffeine/β-Lg	2936	2861	1650	1567

Table A4. 4 FTIR spectral data (wavenumber in cm^{-1}) of pure caffeine, black coffee, milk and milk-based coffee protein before digestion

Sample	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Caffeine control	2942	2827	1702	1657	1550
Black coffee	2935	2866	1702	1653	1591
Milk	2926	2854	1746	1653	1538
Milk-based coffee	2926	2854	1746	1653	1538

Table A4. 5 FTIR spectral data (wavenumber in cm^{-1}) of pure caffeine, black coffee, milk, and milk-based coffee protein after gastric digestion

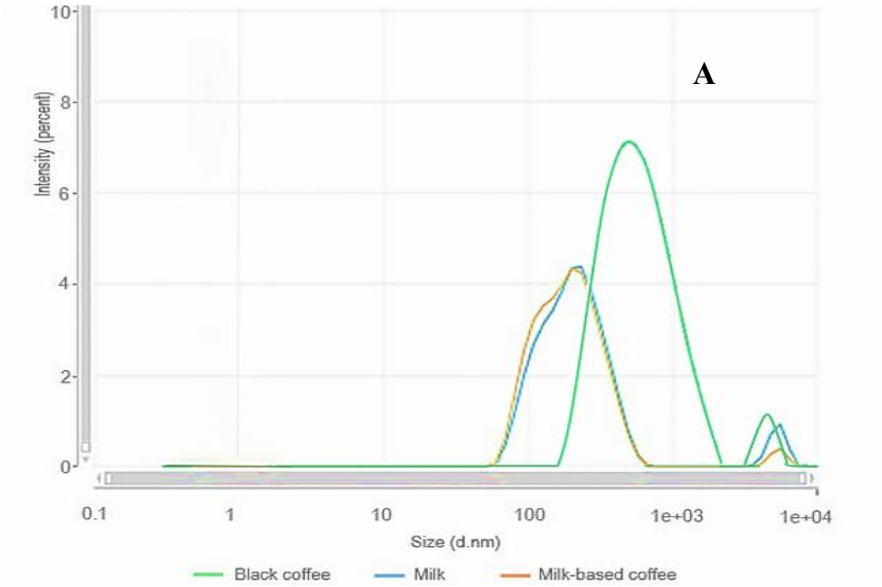
Sample	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Caffeine control	2948	2881	-	1650	1545
Black coffee	2941	2881	-	1650	-
Milk	2926	2854	1742	1650	1536
Milk-based coffee	2926	2854	1742	1650	1536

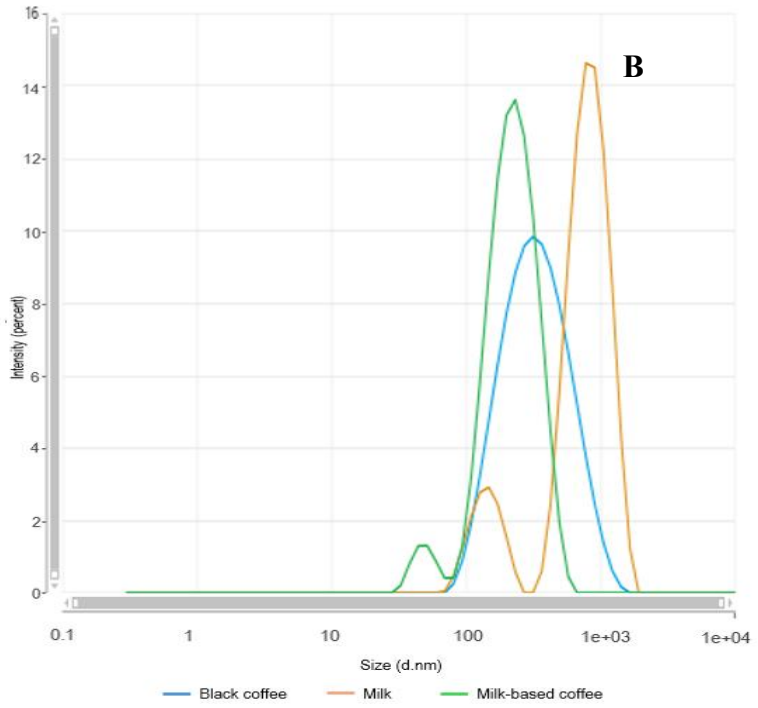
Table A4. 6 FTIR spectral data (wavenumber in cm^{-1}) of pure caffeine, black coffee, milk, and milk-based coffee protein after intestinal digestion

Sample	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5
Caffeine control	2952	2867	1720	1650	1560
Black coffee	2933	2861	1740	1650	1560
Milk	2923	2852	1743	1650	1546
Milk-based coffee	2923	2852	1740	1647	1540

Appendix 5

Data related to particle size analysis and ζ-potential measurement.





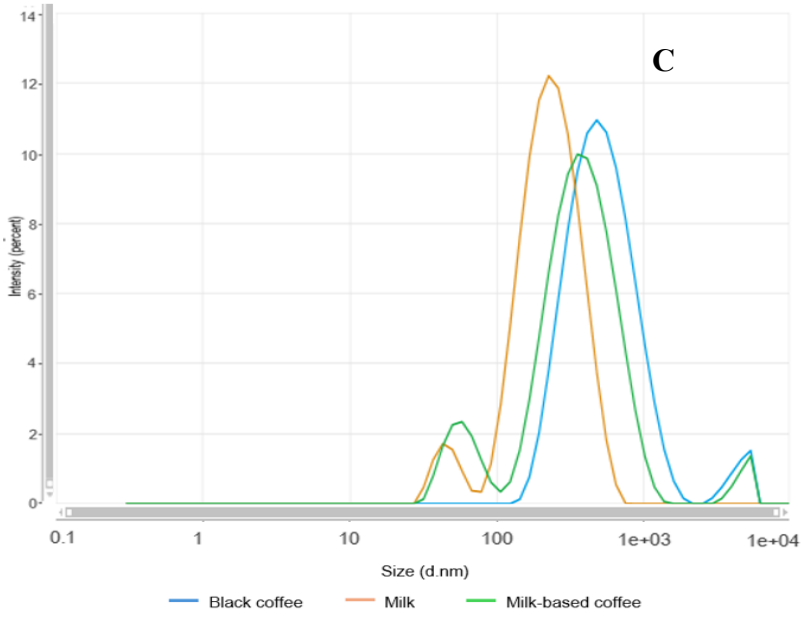


Figure A5. 1 The particle size distribution of black coffee, milk, and milk-based coffee before digestion (A), after gastric phase (B), and after intestinal phase (C).

Table A5. 1 The pH and ζ-potential of black coffee, milk, and milk-based coffee at different digestion stages with their respective pH. Values are the mean±SD (n=6). Values with different superscripts (a-i) for the result are significantly different ($p<0.05$) from each other and were compared using the Tukey test in one-way ANOVA.

Digestion phase	Samples	pH	ζ-potential (mV)
Before digestion	Black coffee	4.94±0.19	-10.9±0.4^{d}
	Milk	6.71±0.03	-30.1±0.6^{h}
	Milk-based coffee	6.43±0.03	-24.3±0.4^{f}
Gastric phase	Black coffee	2.99±0.03	12.2±0.6^{c}
	Milk	3.00±0.04	29.2±0.5^{a}
	Milk-based coffee	3.01±0.03	14.3±0.7^{b}
Intestinal phase	Black coffee	6.99±0.06	-22.2±0.8^{e}
	Milk	6.99±0.03	-34.7±0.6^{i}
	Milk-based coffee	7.00±0.04	-26.7±0.5^{g}

Appendix 6

Declaration of usage of AI software throughout the thesis

I have utilised ChatGPT Version 4o in various sections of my thesis, including introduction (1), health promoting benefits (2.2.3), effects of milk addition on the functionality of caffeine (2.4), bioavailability of caffeine from coffee (2.7), evaluation of methods for studying caffeine-milk protein interactions (2.9), results and discussion (4), and conclusions and future recommendations (5), while ensuring that all AI-assisted outputs are critically reviewed and ethically integrated into my research. As a responsible researcher, I acknowledge that I have used ChatGPT only to overcome writer's block and to identify grammar or vocabulary errors to polish the contents that was originally written by myself.