Lincoln University
Te Whare Wānaka o Aoraki
CHRISTCHURCH•NEW ZEALAND

Lincoln University Digital Dissertation

Copyright Statement

Interaction of Milk Proteins with Kiwifruit and Mixed Fruit Polyphenols

---

A Dissertation

submitted in partial fulfilment

of the requirements for the Degree

of Master of Science in Food

Innovation

at

Lincoln University

by

Caren Meyn Alvarez

Lincoln University

2020

Abstract of a Dissertation submitted in partial fulfilment of the requirements for the Degree of Master of Science in Food Innovation.

Interaction of Milk Proteins with Kiwifruit and Mixed Fruit Polyphenols

by

Caren Meyn Alvarez

The biofunctional properties of polyphenol along with the nutritional property of milk makes this system an excellent source of various health benefits. The structural and physiochemical properties of milk proteins facilitate their use as vehichles for bioactives. There is a growing demand for the delivery of antioxidants and its protection to ensure its bioavailability. Milk proteins are known to bind to polyphenols and protect them from the various harsh conditions within the stomach, till its released for absorption. Phenolic profiles of the Kiwifruit and fruit complex extracts showed predominant amounts of catechin, epicatechin and procyanidin B1 and B2, along with smaller amounts of other phenols such as caffeic acid, gallic acid, protocatechuic acid, syringic acid, rutin and quercetin. The HPLC extraction was carried out in various step using 70% acetone followed by 8% TCA precipitation. Addition of milk to theses extracts showed a decrease in antioxidant activity. However, extraction of polyphenols from the milk-polyphenols system did show considerable amounts of antioxidative property in corelation with the amount of phenolics that were extracted. Loading efficiency studies revealed that almost 100% of the polyphenols were bound to the milk proteins.

Keywords: Milk proteins, polyphenols, casein micelles, whey proteins, antioxidant activity, antioxidant assays, polyphenol extraction.

Acknowledgements

I owe my sincere gratitude and appreciation to Dr. Maneesha Mohan for her constant guidance, immense support, countless research articles, encouragement and patience throughout this study.

I extend my sincere thanks to Letitia Stipkovits for her kind hearted assistance and help with all the lab work and calculations. She always had a solution for every help we asked her for.

A massive thanks to Dr. Jenny Zhao for all the help with the HPLC analysis amidst her busy schedule and sharing her expertise on polyphenol extraction.

I especially render my gratitude and thanks to all the department staff members for their support.

Working with a high number of samples would not have been easy if it was'nt for Rajni Adhikari for constantly being there as a part of a team.

My husband , for his unwavering support and tolerance with me through all this.

As everything begins and ends in God, I conclude by thanking God for everything.

Table of Contents

List of Tables

List of Figures

Introduction

There has been an increased interest in providing polyphenol rich products in the human diet keeping mindfull of their health benefits associated with their consumption. Polyphenols are a group of naturally occuring phytochemicals, synthesized as secondary metobolites in most plants. Their basic structure comprises of an hydroxyl group attached to an aromatic ring. Polyphenols are categorized into flavanoids, phenolic acids, stilbenes and lignans. The major source of polyphenols in the human diet are from fruits, vegetables and cereals. Increased production of polyphenols in plants is considered as a defence mechanism against ultraviolet radiation or to evade pathogens (Pandey & Rizvi, 2009). They are the main bioactive compounds responsible for the antioxidant activity of fruits and hence called antioxidants. Antioxidants or 'free radical scavengers' help in neutralizing the harmfull free radicals in our bodies. Among all fruits, berries and red fruits are to known to have considerably high amounts of polyphenols such as anthocyanins, flavan-3-ols, procyanidins, flavanols and cinnamic acid derivatives. However , these compounds are considered to be extensively unstable and highly susceptable to degradation, under varying conditions of temperature, oxygen and pH (Bąkowska, Kucharska, & Oszmiański, 2003). The stability of these compounds is a very important aspect that has to be maintained, to ensure that their desired properties are intact, when consumed (Volf, Ignat, Neamtu, & Popa, 2014). It is important to asses whether the antioxidative properties are retained in food mixtures. A study on cocoa polyphenols in milk does not support the hypothesis of a decrease in bioavailabilty of polyphenols themselves but on the contrary they support the maintanence of its antioxidative properties (Gallo, Vinci, Graziani, De Simone, & Ferranti, 2013)

Milk proteins have been studied on a large scale to investigate milk-polyphenol interactions and the retention of antioxidative properties of the bioactive compounds. Due to the unique structure and property of milk proteins, they are considered as efficient bioactive delivery systems. Milk consists of casein and whey proteins. β-lactoglobulin, a major whey protein, exhibits a unique attraction towards hydrophobic molecules and thus acts as an alterntive transport agent. Studies have shown various binding efficiencies of β-lactoglobulin with Vitamin D (Forrest, Yada, & Rousseau, 2005), tea polyphenols (Kanakis et al., 2011), resveratrol (Liang, Tajmir-Riahi, & Subirade, 2008) and retinol (Puyol, Perez, Ena, & Calvo, 1991). α-lactoglobulin has similar binding efficiencies towards vitamin D, retinol and palmitic acid (Livney, 2010). Various flavanoids like luteolin, tricin, linarin and apigenin shows binding affinity towards bovine serum albumin (BSA) (Fu et al., 2016). Milk caseins are in two forms at room temperature: micelles which are easily removed by centrifugation and small monomers or polymeric units which are not easily seperated by centrifugation. This unique property of caseins in forming micelles depends on the amount of calcium in milk. These calcium-caseinate micelles contain

α, β, k caseins (Waugh, 1958). Casien micelles are also known to bind to bioactive compounds. In aqueous solutions, self association of β-caseins makes it a very efficient nanocarrier for the delivery of bioactive compounds. For the development of novel transport systems of bioactive compounds , self and co- assembling properties of these milk proteins makes them a highly potential delivery system. The first report to be issued supporting the above statement was a study conducted on casein micelles for the delivery of exogenous hydrophobic bioactives (Semo, Kesselman, Danino, & Livney, 2007). Later on Studies were also carried out on the interaction of tea polyphenols with milk protein systems to analyze the impact of milk on the antioxidative capability of tea polyphenols (Langley-Evans, 2000). The results from this study showed a decrease in antioxidant potential of tea polyphenols. This effect was higher when full fat milk was used. Hence it is important to understand the interaction of polyphenols with milk proteins to clarify and understand its bioavailability when consumed.

Owing to the increased consumer demands for nutritious and health beneficial food products, food companies have introduced the idea of fortyfiying food groups that are included in a person's daily diet. One such food group is milk and other dairy products. Milk is an essential beverage that is commonly consumed by children and adults. Milk, in itself contains some antioxidative compounds such as urate, proteins, carotenoids and Vitamins (Calligaris, Manzocco, Anese, & Nicoli, 2004). Milk that is processed and sold in the market undergo various heat stabilization process to achieve milk safety and stability. These heat treatments owe to the degradation of the natural antioxidants present in raw milk (Renner, 1988). Fortifying milk with polyphenols help in providing additional bioactive compounds.

Review of the Literature

1.1 Dietry Polyphenols and their Extracts

Polyphenols are naturally occurring compounds in fruits and vegetables. Fruits like grapes, apple, kiwifruit and berries contains 200-300 mg of polyphenols per 100 grams of fresh weight. These polyphenols contribute to the astringency, bitterness, colour, flavour and oxidative stability in food. Epidemiological studies and associated meta-analysis carried out towards the end of the 20th century suggested that, daily consumption of diets rich in plant polyphenols offered many health benefits like, some protection against the development of cancers, cardiovascular diseases and neurodegenerative diseases (Graf, Milbury, & Blumberg, 2005). Around 8000 polyphenols have been identified in various plants. A common intermediate, phenylalanine or a close precursor gives rise to all plant phenolic compounds. Therefore all the compounds share the same basic structure, consisting of an aromatic ring with an attached hydroxyl group (Kondratyuk & Pezzuto, 2004).

1.1.1 Classification of Polyphenols

Polyphenols can be classified into different groups depending on the number of phenol rings and the structural elements that bind these rings to each other as illustrated in figure 2.1. The different groups are phenolic acids, Flavanoids, Stilbenes and Lignans.

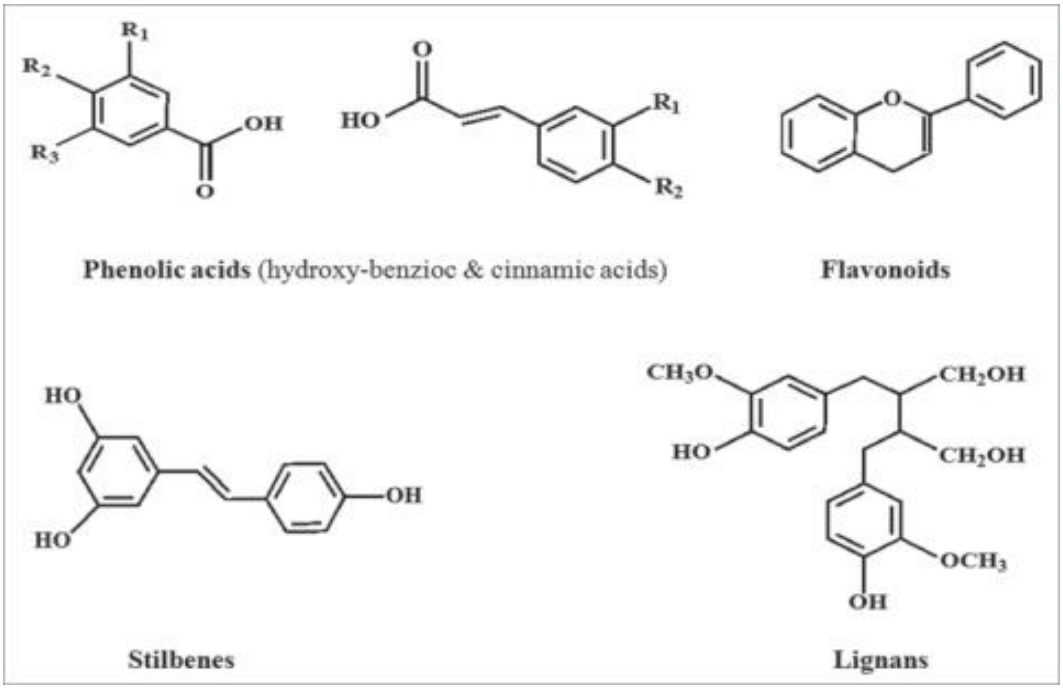


Figure 0.1 Chemical structures of different groups of polyphenols **(Pandey & Rizvi, 2009)**

Different groups of polyphenols along with their subclasses and their respective representative examples have been classified and listed in table 2.1.

Table 0.1 Classification of polyphenols with their representative examples **(Yildirim-Elikoglu & Erdem, 2018)**

Plyphenol Class	Sub-Classification	Representative examples
Flavonoids	Flavanones	Hesperetin, Naringenin
	Flavones	Apigenin, Luteolin
	Flavan-3-ols	Catechin, Epigallocatechingallate
	Flavon-3-ols	Kaempferol, Quercetin
	Isoflavones	Daidzein, Genistein
	Anthocyanidins	Cyanidin, Malvidin
Phenolic Acids	Hydroxybenzoic Acid	Gallic Acid
	Hydroxycinnamic Acid	Ferulic acid
Curcuminoids		Curcumin
Stilbenes		Resveratrol
Tannins		Procyanidin B1
Lignans		Enterodiol

Distribution of polyphenols and its production in plants

There is no uniform distribution of phenolics within the tissue, cellular and subcellular levels of the plant. The cell walls consists of insoluble phenolics while the plant vacoules contain the soluble phenolics. Quercetin is one of the polyphenols that are found in all plant products like fruits, vegetables and cereals, where as Flavanones and isoflvanones are restricted to particular foods (Wink, 1997). Polyphenol content in plants vary depending upon the degree of ripening during the time of harvest, environmental factors, processing and storage. It has been observed that during ripening, phenolic acid content decreases while there is an increase in anthocyanin concentrations (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). Phenolic acids are mainly involved in the response of plants to various stress by lignifying the damaged areas. They also posses antimicrobial properties and hence their concentrations increase after an infection (Parr & Bolwell, 2000).

Storage of food is another factor that affect the polyphenol content. Polyphenol content changes due to oxidation of these compounds during storage. Fruits that are grown in places like New Zealand that do not have an ozone layer, are exposed to high amounts of ultraviolet radiations. As a defense mechanism to the UV radiations, plants produce higher amounts of polyphenol content.

1.1.2 Fruit Polyphenols

Fresh fruits are known for its bioactive nutrient content and its importance as dietary antioxidants. Fruits and berries are excellent sources of bioactive compounds like anthocyanins, flavanols, procyanidins and phenolic acids (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002).

Table 0.2 List of polyphenols present in few fruits

Fruit	Class of polyphenol	Polyphenol	Reference
Kiwifruit	Flavan-3-ols	Epicatechin, Catechin, Procyanidin B1, Procyanidin B2	(Wojdyło, Nowicka, Oszmiański, & Golis, 2017) (Ma et al., 2017) (Krupa, Latocha, & Liwińska, 2011)
	Hydroxybenzoic acids	Gallic acid protocatechuic acid	
	Hydroxycinnamic acids	Chlorogenic acid, Neochlorogenic acid, Caffeic acid	
	Flavonols	Quercetin-3-rhamnoside, Quercetin-3-0-Glucoside, Rutin	
Blackcurrant	Anthocyanins	Delphinidin-3-glucoside, Delphinidin -3-rutinoside, Cyanidin- 3-glucoside, Cyanidin-3-rutinoside	(Bakowska-Barczak & Kolodziejczyk, 2011)
	Flavonoid	Myricetin, Quercetin, Rutin, Kaempferol	
Red Grape	Anthocyanins	Malvidin-3-O-glucoside, Petunidin, Cyanidin, Peonidin, Delphinidin	(He, Xu, Zeng, Qin, & Chen, 2016)

A previous study on the phenolic content of blackcurrants stated that black currants consisted of 500-1342mg/100g of total polyphenols, out of which the most predominant polyphenol was anthocyanins accounting for 160-411mg/100g. Anthocyanin are known for their wide range of health benefits which include antioxidant, antimicrobial and anticarcinogenic activities (Han, Shen, & Lou, 2007). Anthocyanins are also the major bioactive compound present in red grape skin extracts. The major anthocyanin in red grape skin extracts (GSAE) is malvidin-3-glucoside (Han et al., 2007) and the remaining anthocyanin forming glycosides are petunidin, cyanidin, peonidin and delphinidin (Ortega-Regules, Romero-Cascales, López-Roca, Ros-García, & Gómez-Plaza, 2006). Berries like blueberry, blackberry and strawberry are well known as 'super fruits' due to their nutraceutical and functional food properties. A study showed that these berries contained a range of phenolic acids, which included gallic acid, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid and cinnamic acid. Apart from phenolic acids, they also exhibited a wide range of flavonoids like luteolin, rutin, myricetin, quercetin, gallocatechin, epigallocatechin, catechin, catechin gallate, malvidin-3-galactoside, malvidin-3-glucoside and cyanidin. Blueberries had higher concentrations of proanthocyanins and anthocyanidins and hence exhibited greater antioxidant properties (Huang, Zhang, Liu, & Li, 2012). Boysenberries are known for its rich mixture of polyphenols which include four anthocyanins and four ellagitannins (Furuuchi, Yokoyama, Watanabe, & Hirayama,

2011). Stability of polyphenols is an important aspect and it seen that they are affected by pH, metal ions, exposure to light, temperature, oxygen and enzymatic activities (Bąkowska et al., 2003).

1.2 Milk Proteins

Milk is a complex mixture containing varying amounts of water, fat, protein and lactose, whereas minor components are minerals, vitamins, enzymes and dissolved gasses. The colloidal phase of milk contains caseins, calcium phosphate and globular proteins. The fat globules in milk are in the form water in oil type of emulsion. Milk is mainly composed of 12.6% milk solids and 87.4% of water. Milk solids comprise of milk fats (3.7%), proteins (3.4%), Lactose (4.8%) and minerals (0.7%). However the percentage of Non-fat milk solids varies for full fat milk and skimmed milk (Fig 2). The major milk proteins are casein and whey proteins. Casein contributes to 80% of the total milk proteins, which can be preciptated at pH4.6, leaving 20% of the proteins back in the solution which are the whey or serum proteins. These two proteins are not homogenous in their composition. The various concentration of these proteins are listed in Table 2.2.

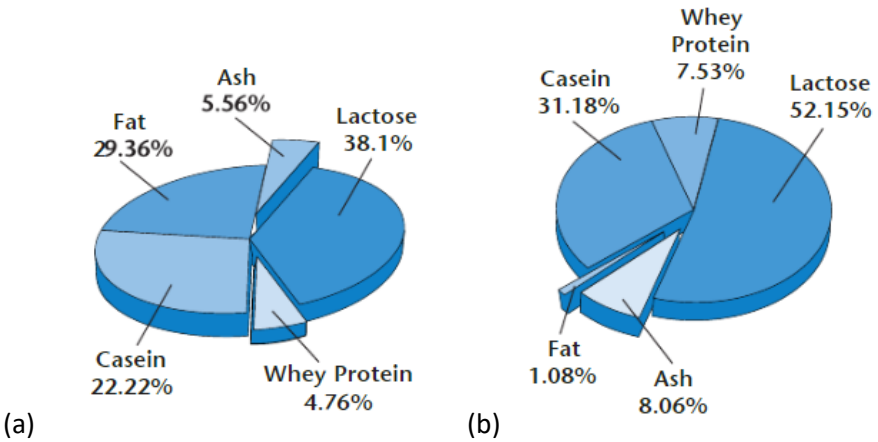


Figure 0.2 Milk solid compostion of (a) Whole Milk (b)skimmed milk **(Coultate, 2009)**

Table 0.3 Concentrations of milk proteins **(Ng-Kwai-Hang, 2011)**

Proteins	Concentrations in Milk (g/l)
Caseins	25.2
α_{s1}-casein (α_{s1}-CN)	10
α_{s2}-casein (α_{s2}-CN)	2.6
β-casein (β-CN)	9.3
Κ-casein (κ-CN)	3.3
Whey Proteins	5.6
β-lactoglobulin	3.2
α-lactalbumin	1.2
Serum albumin	0.4
Immunoglobulin	0.8

1.2.1 Caseins

Caseins are proline rich compounds that can assume any of the several energetically favourable confirmations in solutions, also called open structured rheomorphic proteins. They have distinct hydrophobic and hydrophilic domains the serine-phosphate residue centres present on α_{s1}-CN, α_{s2}-CN and β-CN is for the purpose of calcium sequestration. The κ-CN is a glycoprotein with 2 cysteines connected by intermolecular disulphide bonds. Around 95% of these caseins have a unique ability of self-assembling themselves into aggregates or casein micelles which are spherical colloids with an average measurement of 150nm, exhibiting a molecular mass of 10^6 and $3x10^9$ Da. Around 6% of these compounds are of lower molecular weights called colloidal calcium phosphates (McSweeney & Fox, 2003). These micelles approximately contain 2g of water per of protein and are present in quantities as much as $10^{14} – 10^{16}$ micelles/ml of milk. Due to the high proline content of the open structured caseins, they are susceptible to proteolytic cleavage. In the presence of acid soluble calcium-phosphate, it serves as an excellent target activated release mechanism for the delivery of food in the stomach. This remarkable property of casein micelles makes then excellent nano-vehicle for nutrient delivery.

1.2.2 Whey Proteins

The major whey protein present in milk is the β-lactoglobulin, which is small globular protein. They are arranged an eight stranded, antiparallel β-barrel with a three turn alpha helix on its outer surface. It contains two disulfide bridges, a free thiol group and they exsist as dimers in milk (McSweeney & Fox, 2003). Among the whey proteins, a smaller metaloglobular protein exsists, which is the α-lactalbumin, the second most prevelant whey protien. It consists of four disulphide bridges. Structurally it is similar to lysozyme and requires calcium to attain its functional fold. Bovine serum albumin (BSA) is larger globular protein foun in blood serum as well as milk. It has an alpha helical structure with fiftenn disulfide bridges and a free thiol. Though most of the BSA used in researches are obtained from blood serum, it can also be obtained from milk (J. Li & Yao, 2009). Another minor protein among the whey proteins are lactoferrin, which is a monomeric globular glycoprotein and belongs to the transferrin family. It exhibits antimicrobial properties (Edwards, Creamer, & Jameson, 2009).

1.3 Polyphenol-Milk Protein Interactions

Milk protein and polyphenol binding studies have gained a special interest due the importance of milk and polyphenol nutriotion in the human diet and also due to various dietry habits such as drinking milk, tea, coffee etc. Many studies have revealed that the interactions between milk proteins and polyphenols have caused a change in the stability, structure and functional properties of proteins along with drastic changes in the antioxidative property of the polyphenols and their bioavailability.

1.3.1 Binding Mechanisms Involved in Polyphenol-Milk Protein Interactions

Studies on the binding mechanisms of polyphenols and milk proteins gives us a better understanding about the interactions and the factors affecting them. Various factors affect the strength and affinity of binding of polyphenols and the two possible mechanisms are covalent and non-covalent interactions which can result in reversible and irreversible binding. Hydrogen bonding, hydrophobic interactions and van der waals are non-covalent interactions and they cause reversible protein-polyphenol binding. Covalent bonds formed under certain conditions such as thermal, enzymatic and oxidative formation of o-quinone, are irreversible interactions (Yuksel, Avci, & Erdem, 2010). Hydrogen bonds are formed between electronegative atoms of nitrogen or oxygen and a positively charged hydrogen atom of neighbouring hydroxyl group of polyphenol molecule, while hydrophobic interactions exist between protein amino acids and the aromatic rings of polyphenols. Noncovalent protein–polyphenol interactions are non-covalent and are more common compared to covalent bonds that are formed from a combination of interactions. (O'connell & Fox, 2001). β-lactoglobulin, a major whey protein is also known for its potential as a delivery agent. The interactions that are associated with these proteins and various flavonoids are hydrogen bonding, hydrophobic interactions and van der waals attractions (Kanakis et al., 2011). One study reported the covalent bond formation through -SH group of β-lactoglobulin and cocoa polyphenols (Gallo et al., 2013). The binding mechanism of BSA has also been studied by several researchers. It has been revealed that the hydrophobic pockets present on the BSA are the main sites for binding to several polyphenols. The other interactions involved BSA and polyphenols binding are electrostatic and hydrogen bonding (Khatun, Yasmeen, Kumar, & Subbarao, 2018). The second most important protein in whey is the α-Lactalbumin, where four disulfide bonds stabilizes its structure. There are lesser studies about α-lactalbumin–polyphenol interactions than β-lactoglobulin and BSA. "H-bond formation has been reported to be the main interaction type between α-lactalbumin and various polyphenols such as genistein, kaempferol, trans-resveratrol, and curcumin" (Mohammadi & Moeeni, 2015). Proline residue on proteins are the major sites of polyphenol binding (Williamson, 1994). Due to the high proline content of caseins, its affinity toward polyphenolic compounds is higher than the other milk proteins. Hydrophobic interactions and H-bonding are involved in the binding of polyphenols to caseins which is similar to whey proteins (Mehranfar, Bordbar, & Parastar, 2013). This binding property of the casein is exploited for the use as vehicles for bio actives by forming micelles. A study reported that casein micelles formed complexes with curcumin via hydrophobic interactions which could be an alternative drug formulation for cancer therapy (Sahu, Kasoju, & Bora, 2008). The binding forces involved in milk protein – polyphenol interactions are basically non-covalent, stabilized by H-bonds. The strength, affinity and type of interactions vary with the following factors described below.

1.3.2 Factors affecting the polyphenol-milk binding interactions

Various factors have been reported that affect Polyphenol–protein interactions such as pH, ionic strength and temperature besides the structure of both protein and polyphenols. The wide variety of polyphenol structures and variations in the external conditions makes the evaluation of the binding interactions more complicated.

pH and Ionic strength

The effects of pH on the interactions between polyphenols and milk proteins have been widely studied. A decrease in pH causes changes in the tertiary structure of proteins such as BSA which directly affects the binding of certain phenolics like ferulic acid and chlorogenic acid. With the help of fluorescence quenching, at pH 2.0 resveratrol binding to β-lactoglobulin was found to be same at neutral pH, due to the stability of the β-lactoglobulin at acidic pH (Liang & Subirade, 2012). It was also observed that similar binding characteristics of BSA for catechin, epicatechin, and rutin was not affected at different pH values, whereas a stronger affinity existed for quercetin at higher pH. (Frazier, Papadopoulou, & Green, 2006). In addition, polyphenols were reported to have higher affinity to proteins near isoelectric point of the protein unless the interactions were electrostatically driven (Wang, Ho, & Huang, 2007). Hence, pH dependent changes in the binding affinity of polyphenols is mostly due to the structural and conformational changes in the protein owing to change in pH.

Temperature

Temperature changes like pH change causes various structural and conformational changes in protein, which indirectly affects the polyphenol binding property. A study on the effect of temperature on binding of chlorogenic acid to BSA, found that the amount of bound chlorogenic acid decreased with increasing temperatures from 5 °C to 60°C (Prigent et al., 2003). They also reported that heat denaturation of BSA significantly reduced the binding. Contradictory to this study, another study reported a increased binding affinities of EGCG to BSA at high temperatures due to the exposure of hydrophobic parts caused by unfolding, which resulted in higher levels of EGCG binding on the surface of BSA (Wang et al., 2007). β-lactoglobulin also showed a higher affinity to EGCG in its pre-heated form compared to the native state. Similarly, resveratrol was reported to interact with β-lactoglobulin at the outer surface of the thermally denatured protein with a higher affinity (Shpigelman, Israeli, & Livney, 2010). Contrarily, Rawel et al. reported that the binding affinity of quercetin and chlorogenic acid to BSA was diminished after heat denaturation, which could be a result of the polymerization of BSA molecules leading to a decrease in available surface area. As observed from the contradictory results, the effects of temperature on polyphenol–milk protein interactions may be different depending on the structure of the protein and the main driving force of binding. Several heat treatment norms are applied in the dairy industry during production of various products. Thermal denaturation of whey

proteins in milk has an impact on techno-functional properties of dairy products. Therefore, the knowledge on the effects of temperature and the thermal denaturation of milk proteins on polyphenol binding is critical especially for optimization of process conditions when polyphenol-rich dairy products are desired to be manufactured (Rawel, Meidtner, & Kroll, 2005).

Type of polyphenol and its structure

The binding characteristics and affinities of polyphenols to milk proteins are affected by the type of polyphenol, which differ in molecular weight, hydrophobicity, molecular flexibility, methylation, hydroxylation and glycosylation. It has been reported that, with increase in molecular weights of polyphenol, the binding affinity towards protein also increases (Hasni et al., 2011). Soares, Mateus, and De Freitas (2007) supported this report by explaining the formation of more number of bonds by heavier polyphenols within the binding sites of the BSA. The effects of polyphenol structures on binding to BSA was evaluated by Papadopoulou, Green, and Frazier (2005) and reported that , due to the presence of a ketone group, quercetin and its glycoside binds to BSA more strongly than catechin and epicatechin. Soares et al. reported that presence of a galloyl group on catechins enhances the binding affinity to BSA and similar results were observed by Skrt, Benedik, Podlipnik, and Ulrih (2012). However, another study conducted by Bohin, Vincken, van der Hijden, and Gruppen (2012) was not able to find a measurable interaction between BSA and catechin or epicatechin whereas an extra galloyl group on EGCG lead to a significant interaction. Kanakis et al. (2011) also found an enhanced interaction between tea catechins and β-lactoglobulin in the presence of galloyl groups. Hydroxylation of polyphenols has been reported to influence the binding strength of polyphenols to milk proteins. Xiao, Cao, Wang, Yamamoto, and Wei (2010) reported higher binding constants and number of binding sites between flavones and BSA when the ring A is hydroxylated. A positive correlation between the number of –OH groups of catechins and binding affinity to β- lactoglobulin, β-casein, and α-casein was also mentioned in the literature. A comprehensive study was carried out by Xiao et al. (2011) who evaluated the effect of hydroxylation on binding of different class of flavonoids to milk proteins. The authors concluded that, hydroxylation on the ring A of flavanones significantly improved the affinities for milk proteins whereas slightly reducing effect was observed for isoflavones. Binding affinities of flavones and flavanols were slightly enhanced with increasing hydroxylation on rings A and B. On the other hand, methylation of flavonoids had reportedly led to a slight decrease in binding affinity to milk proteins.

Protein Structure

Since protein–polyphenol interactions are particularly related to surface properties of proteins, not only polyphenol structure is important, but protein conformation is also critical for the nature of binding reactions. Intrinsically unstructured proteins have been reported to have a higher affinity than more closed and globular proteins due to the reduced accessibility of amino acid residues of the latter

(de Freitas & Mateus, 2001) However, BSA, having specific binding cavities for polyphenolic substances despite its globular structure, seems to be anomalous in this respect. Amino acid composition, specifically prolyl residues and proline repeats, have been reported to play a critical role for polyphenol binding properties of proteins (Mehranfar et al., 2013). Although proline residues are not the only possible binding sites for polyphenols, there are considerable number of studies showing the significance of these residues on protein–polyphenol interactions. Binding of polyphenols to other amino acid residues such as phenylalanine, histidine, and arginine have been also reported in literature (Bohin et al., 2012). Besides the sequence, presence of bulky amino acids oriented in a close position to binding sites may reduce the accessibility for polyphenols (Richard, Vitrac, Merillon, & Monti, 2005).. Catechin and epicatechin were found to bind at the surface near to Trp134 residue of BSA which was confirmed with the perturbation of the environment of Trp 134 upon binding (Roy et al., 2012). β-Lactoglobulin exists as a mixture of monomers and dimers at neutral pH. Binding of ligands to β-Lg has been reported to depend on the oligomeric state of the protein, which is related to environmental factors such as pH and temperature. β-Lactoglobulin is a small globular protein folded into a calyx formed via 8 antiparallel β-strands and an α-helix at the outer surface. It has been reported that β-lactoglobulin has 3 possible ligand binding sites; the outer surface of β-lactoglobulin near Trp19–Arg124, the internal cavity of β-barrel and the surface hydrophobic pocket between α-helix and β-barrel. Gholami and Bordbar (2014) reported that the binding of naringeninto β-lactoglobulin occurs on the outer surface of the protein but not in the central cavity, similar to the results of Riihimaki et al. (2008) who studied the binding of myricetin and daidzein to β-lactoglobulin. Binding sites for EGCG on β-lactoglobulin was found to be comprised of mainly aromatic residues besides negatively and positively charged residues. Gallo and coworkers (2013) carried out a detailed study on the interactions between cocoa polyphenols and β-lactoglobulin and the effects of some modifications of the protein on polyphenol binding reactions were also investigated. The results revealed that the cocoa polyphenols specifically bound to free cysteine (Cys) residue and alkylation of this residue prevented the interaction whereas in case of lactosylation of β-lactoglobulin, polyphenols bind to the same Cys residue as in native form of the protein. Bovine α-lactalbumin has two main domains connected via two disulfide bonds; α- domain consists of two 310 and three α-helices whereas β-domain consists of three-stranded antiparallel β-sheet. Bovine α-lactalbumin is known to bind metal cations, which is a characteristic ability of this protein. Mohammadi and Moeeni (2015) reported interactions between bovine α-lactalbumin and different flavonoids (curcumin, resveratrol, genistein, and kaempferol), Milk caseins are considered as unique carriers for polyphenols due to high proline content and self-association properties. Although casein fractions are almost similar in size, molecular weight, and net charge they differ in their degree of un-foldedness which is attributed to different functional properties. It is well known that β-casein can form reversible micelles at low concentrations of 21 μmol/L (O'connell & Fox, 2001). Formation of micelles was considered to affect the interactions in two

ways: (i) reducing the binding of polyphenols via reduced accessibility of amino acid residues and (ii) enhancing binding with formation of more hydrophobic environment inside micelles. EGCG showed higher affinity to β-casein among other proteins such as α-lactalbumin, β-lactoglobulin, lysozyme, ovalbumin, and gelatin and prevailing as a carrier for flavonoids (Bohin et al., 2012). Binding sites on α-casein and β-casein for resveratrol, genistein and curcumin were reported to be different for each casein fraction and the stability of the β-casein–flavonoid complexes was found to be higher (Bourassa, Bariyanga, & Tajmir-Riahi, 2013). Based on the general estimation, β-casein should have higher affinity for polyphenols than that of α-casein because of the higher proline content and higher number of proline repeats of the former which is in accordance with the results of Hasni et al. who found a higher binding affinity of EC and EGCG for β-casein compared to α-casein due to the more hydrophobic nature of β-casein. However, Bohin et al. (2012) reported that β-casein did not consistently show a higher affinity for flavan-3-ols speculating on the importance of other factors that could affect the binding strength of polyphenols. There are some well accepted hypotheses such as high number of proline residues and a more open structure compared to globular proteins enhances binding interactions between proteins and phenolics. Caseins, having a high proline content and flexible structure, could be valuable vehicles for binding of phenolic compounds. Several phenolic compounds were reported to bind caseins with a high affinity. However, more recent studies on casein–polyphenol interaction studies showed that there might be more factors other than the proline residues, which should be investigated in detail via sophisticated techniques. Whey proteins are also found to bind several phenolics. The hydrophobic cleft of globular proteins is generally considered as a binding loop for ligand binding, but several researchers reported that polyphenols may also bind to the surface of whey proteins. The specificity of binding of polyphenols to milk proteins is not fully understood yet. However, it is obvious that the structure of both polyphenols and proteins are key factors affecting the binding interactions.

Polyphenol-Protein Ratio

To obtain a stable protein complex, the polyphenol: protein ratio should be high enough to form multiple noncovalent interactions. Precipitation of proteins upon binding of polyphenols have been studied in detail and a model was proposed for precipitation based on the protein/polyphenol ratio (Pascal et al., 2007). According to this model, at low protein concentrations, the agglomeration process depends on the flavonoid concentration. Binding sites on the protein are saturated with flavonoids and with increasing flavonoid concentration, they form bridges between saturated polypeptide chains until finally large aggregates are formed at very high flavonoid concentrations. For high protein concentrations, flavonoids form bridges before the saturation point, which leads to aggregate formation at lower flavonoid concentrations.

1.4 Objective of this Study

There has been a lot of studies conducted on milk proteins and polyphenols , however, a little has been contributed to the interaction of polyphenol rich fruit extracts with milk proteins, polyphenol bioavailabilty and the loading efficiencies in milk.

Keeping in mind, the issues outlined above, the objective of this research was to examine the antioxidative activity of the polyphenols present in polyphenol-milk systems and their loading efficiencies in whey and casein proteins present in milk.

Materials and Methods

1.5 Materials

Two fruit extracts were used in this research. The kiwi fruit skin extract was procured from Digesten New Zealand, and the fruit complex extract was purchased from Oxi-fend New Zealand. The fruit complex comprised of the extracts from grapes (*Vitis vinifera)*, Blackcurrant (*Ribes nigrum),* Boysenberry (*Rubus ursinus),* kiwifruit (*Actinidia deliciosa).* Pams trim milk was purchased from a local supermarket in New Zealand. All the other chemicals and solvents used were analytical grade and purchased from Sigma-Aldrich chemicals. The standards used were HPLC grade and purchased from Sigma-Aldrich, New Zealand.

1.6 Sample preparation

1.6.1 Polyphenol extract stock solution

Stock solutions of Kiwifruit extract (K) powder and fruit complex extract (F) powder were prepared in concentrations of 50mg of polyphenol/ml. Kiwifruit extract had an initial total phenolic content (TPC) of 89mg/ gram of extract powder (TPC data provided by Digesten, NZ). In order to attain the required concentration of polyphenol in the stock solution, 0.56g of the extract was dissolved in 1ml of Phosphate buffer (pH 6.5). similarly, fruit complex extract had an initial total phenolic content of 594mg/gram of extract powder (TPC data provided by Oxi-fend, NZ). A stock solution of 0.084g of fruit complex extract was dissolved in 1ml phosphate buffer (pH 6.5). Preparation of the stock solution in PBS buffer with pH 4.6, is to imitate the milk system, where milk is at a pH of 6.5 (Gallo et al., 2013). The fruit extract powder was dissolved in PBS buffer by stirring it using a magnetic stirrer for 15minutes. It was then placed on a vortex mixer for 10 minutes and centrifuged at 5000g for 20min at 20°C. The supernatant was collected, and the pH adjusted to 6.5 using 0.1N NaOH and stored at 4°C.

1.6.2 Polyphenol-Milk systems

The polyphenol to protein ratio chosen for initial loading was 1:40, which is 24mg of polyphenol for every 1gram of protein (da Silva, Matumoto-Pintro, Bazinet, Couillard, & Britten, 2015). Considering standard trim milk to have 3.2% of proteins per 100ml, 1.6ml of the polyphenol stock solution was added to 98.7ml of milk. The control samples consisted of 1.6ml of the polyphenol stock solution and 98.7ml of PBS buffer (pH 6.6). The milk-polyphenol and the control samples were placed in a water bath shaker at 45°C for 10min at 65rpm to ensure proper dissolution. Samples were freeze dried and stored at -2°C.

1.6.3 Experimental design

The experimental design consisted of two treatments which were the control samples and milk-polyphenol samples mixed at two concentration of 1:40 (polyphenol: Protein) and 1:0 (Polyphenol: protein) ratio. Duplicates of each treatment was also considered. The control samples were devoid of protein. Table 3.1 illustrates the list of samples, their details and sample Ids.

Table 3.1 List of sample ID and sample details

Sample ID	Sample Details
CT-K1 A	CT – Control: no protein (PBS buffer)
CT-K1 B	M – Milk as the source of protein
CT-F1 A	K- Kiwifruit extract
CT-F1 B	F – Fruit complex extract
M-K1 A	1 - 1:40 (Polyphenol: Protein)
M-K1 B	3 – 1:0 (Polyphenol: protein)
M-K3 A	A – Duplicate 1
M-K3 B	B – Duplicate 2
M-F1 A	
M-F1 B	
M-F3 A	
M-F3 B	

1.6.4 Polyphenol extraction

From previous trials with polyphenol-Milk systems, acetone extractions had better phenolic content than methanol extracts. The extraction procedure was carried out with acetone: water (70:30, v/v), with some modifications based on a previous study (Cebeci & Şahin-Yeşilçubuk, 2014). In the first extraction step, 2g of the freeze-dried sample was extracted with 20ml acetone: water (70:30, v/v) by stirring the samples on a magnetic stirrer for 1 hour. It was then centrifuged at 4000g for 30min at 4°C. The supernatants were collected. In order to precipitate out the proteins completely, the supernatant was treated with 8% trichloroacetic acid (TCA) (Grimbleby & Ntailianas, 1961). It was then centrifuged at 5000g for 20minutes at 4°C. Half of the supernatant was collected and passed through a syringe filter (0.4micron) for HPLC profile determination. The rest of the supernatant was stored at -2°C, which was used for determining the antioxidant activity and total phenolic content.

1.6.5 Loading efficiency studies

Loading efficiency studies helps in determining the amount of polyphenols that are bound to the casein and whey protein and the amount of unbound polyphenols. To carry out this study, 2g of the freeze-dried samples was mixed with 5ml of RO water (initially added 4ml of water and gradually increased to the minimum amount of water required for hydration of the sample). Samples were stirred for 1

hour using a magnetic stirrer. The pH was reduced to 4.6 using 0.1N HCL to facilitate casein precipitation (Livney, 2010). It was then centrifuged at 3000g for 15minutes at 20°C. 0.5ml of supernatant was then mixed with 1.5ml acetone (100% - to avoid further dilution with water). It was then stirred for one hour and then treated with 8% TCA, centrifuged at 4000g for 30minutes at 4°C. The supernatant was collected and passed through a syringe filter (0.4 micron) for HPLC analysis, while the rest of the supernatant was analyzed for total phenolic content and antioxidation activity. The total amount of phenol present in the control samples (no proteins) minus the total phenols present in the supernatant of the milk-polyphenol samples would give us total phenols bound to the milk proteins.

1.6.6 HPLC determination of individual polyphenols

For HPLC analysis, the acetone extracts were used. The HPLC system was an Agilent HPLC 2 system, comprising of a quaternary pump, DAD and FLD detectors. The column used was an EXL-1110-1546U, ACE 3µ C18-PFP 150X4.6mm (Advanced Chromatography Technologies, Aberdeen, Scotland) and its temperature was maintained at 20°C. The solvent systems were, A (0.05M $NH_4H_2PO_4$, pH=2.6), B (100% Acetonitrile) and C (0.2M H_3PO_4, pH=1.5). The elution system was as follows; 0-2min 100% A, 2-5min 93.6% A and 6.4% B, 5-17min 2.8% A and 11.2% B, 17-22min 3.6% A and 14.4% B, 22-29.5min 4.2% A and 16.8% B, 29.5min- 55min 6.6% A and 26.4% B, 55-70min 10% A and 40% B, 70-75min 10% A and 40% B, 75-78min 36% A and 64% B, 78-81min 36% A and 64% B, 81-90min 100% A. the flow rate was maintained at 0.8ml/min with an injection volume of 10 µl. Detection was carried out at 280nm, 320nm, 360nm, 520nm. Scan from 220nm to 600nm and stored the spectrum.

1.6.7 Total phenolic content

The total polyphenols content of the extracts was determined using the Folin–Ciocalteu colorimetric method as described by Singleton et al. (1999) with modifications (Waterhouse, 2002). Standard solutions of gallic acid were prepared at 0, 12.5, 25, 50, 75, 100 and 150 µg/ml in 70% acetone (as the samples were extracted with acetone). 0.25ml of each standard and sample extract were placed in cuvettes, to which 1.25 ml of 0.2N Folin-Ciocalteu reagent and 1ml of 7.5% sodium carbonate was added and mixed well. The standards and samples were prepared in triplicates. The cuvettes were incubated for 2hours at room temperature in a dark place. Using a spectrophotometer, the absorbance of the standards and samples were measured at 760nm. The blank used here was 70% acetone. The results were expressed as mg of gallic acid equivalents per 100g of the extract. Data was reported as a mean of the two out the three closest values.

1.6.8 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of the sample extracts was determined using the Trolox equivalent antioxidant capacity (TEAC) with DPPH radicals (Yen & Chen, 1995). Standard solutions of Trolox with

concentrations of 0, 12.5, 25, 50, 75 and 100 µmol Trolox. 0.025ml of sample and standard solution was placed into 96 well microplates, to this 0.050ml of 0.1mM DPPH solution and 0.075ml of acetone was added. The absorbance was recorded at 517nm using a BMG Omega plate reader. The blank used here was 70% acetone. The samples and standards were assayed in triplicates and the results were expressed as µmol of Trolox equivalents per gram of sample extract.

1.6.9 ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) assay

The antioxidant activity of the sample extracts was also determined using Trolox equivalent antioxidant capacity (TEAC) with ABTS radical (Re et al., 1999). In order to carry out this assay, 7mM ABTS stock solution (10ml) was prepared in water. A 10mM potassium persulphate stock solution was also prepared in water. In a volumetric flask, 9.5ml of 7mM ABTS stock solution and 245µl of 100mM potassium persulphate solution was mixed and made up to 10ml with water. The flask was covered with aluminum foil to protect it from light and the reaction was allowed to stand overnight for at least 16 hours in the dark at room temperature. The following day the ABTS radical reagent solution was diluted with PBS pH 7.4 to produce an absorbance of 0.70 (±0.02) at 734nm.

Standard solutions of Trolox was prepared in the same range of 0, 12.5, 25, 50, 75,100 and 200µmol Trolox. To 3ml of the diluted ABTS radical reagent solution, 300µl of each Trolox standard and sample extract was added into cuvettes and mixed well. The reagent- sample mixtures were kept for incubation for 6 minutes at room temperature. The absorbance was recorded at 734nm with the blank as 70% acetone. The samples and standards were assayed in triplicates and the results expressed as µmol of Trolox equivalents per gram of sample extract.

1.6.10 Statistical analysis

This experimental design consisted of duplicates. All the assays were carried out as sample triplicates and the data was expressed as mean ± the standard deviation. Statistical analysis was carried out by ANOVA using the general linear model in Minitab 9. Significant differences ($P<0.05$) between the means were identified by Tukey comparison tests.

Results and Discussions

Acetone extracted samples and samples that were extracted for loading efficiency studies were analyzed for its polyphenol content by HPLC profiling and total phenolic content assay. The antioxidation activity was measured using DPPH and ABTS assays.

1.7 HPLC and TPC determination of polyphenols.

The standards used for the HPLC which helped in quantifying the individual polyphenols in the extracted samples are gallic acid, protocatechuic acid, castalagin, procyanidin B1, hydrobenzoic acid, cyanidin-3-sophoroside, delphinidin-3-rutinoside, catechin, vannilic acid, procyanidin B2, caffeic acid, syringic acid, epicatechin, malvidin-3-O-glucoside, epigallocatechin gallate, p-coumaric acid, ferulic acid, rutin, cinnimic acid and quercetin. The individual polyphenols present in the control samples (Kiwifruit and mixed fruit extract) are shown in Figure 4.1.

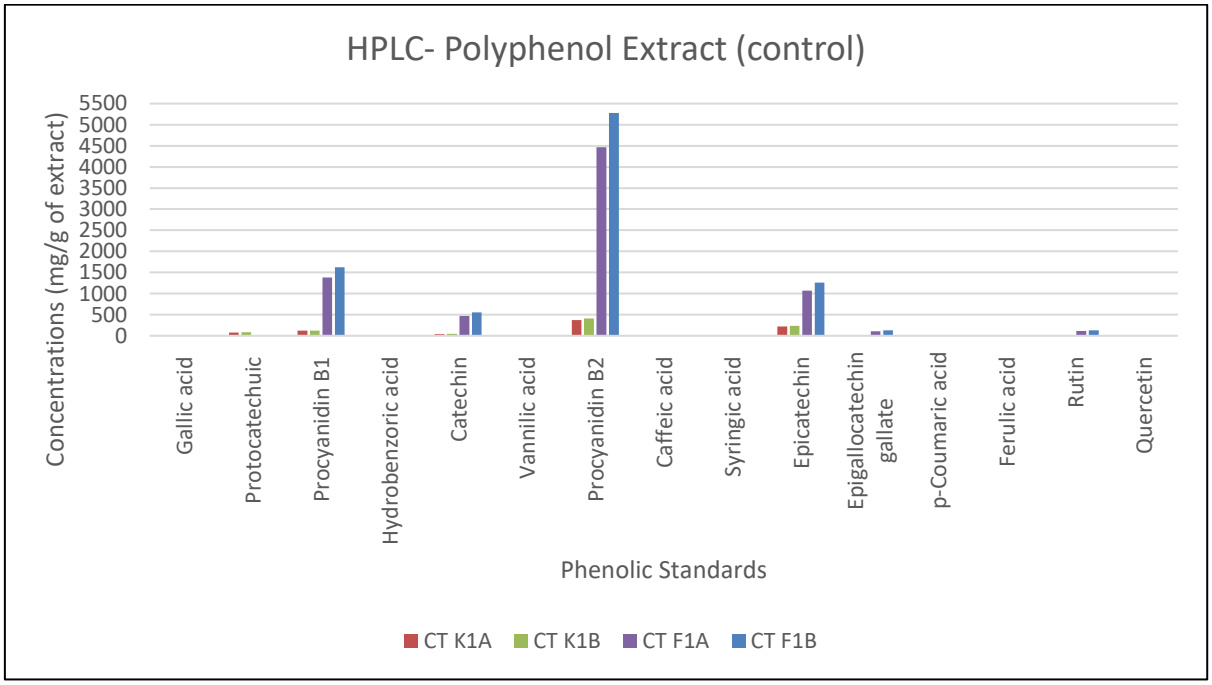


Figure 0.1 HPLC profile of individual polyphenols present in the control samples (kiwifruit and fruit complex extracts).

The major components in kiwifruits and mixed fruit extract containing grapes, blackcurrant, boysenberry and kiwifruit, are bioactive compounds. The above graph shows the various polyphenols detected in the kiwifruit extract (CT K1A and CT K2B: experimental duplicates) which were Procyanidin B1 and B2, catechin, epicatechin, protocatechuic acid, vanillic acid, caffeic acid, syringic acid, epigallocatechin gallate, ferulic acid and rutin. The two most prominent phenolics identified were

catechins and procyanidin B2, which is in accordance with a previous study (H.-Y. Li et al., 2018). However, they had a total of 12 polyphenols that were identified via HPLC and tested in various types of kiwifruit, hence the results varied in terms of individual polyphenols. Another reason for the identification of all 12 polyphenols was because the whole kiwifruit pulp was used, while our extracts were obtained from the skin of the fruit. However, the major and the most prominent polyphenols were identified and the others in trace amounts. The fruit complex extracts (CT F1A and CTF1B: experimental duplicates) showed the presence of Gallic acid, procyanidin B1, procyanidin B2, catechin, epicatechin, epicatechin gallate, hydrobenzoic acid, syringic acid, p-coumaric acid, quercetin and rutin. Higher quantities of procyanidin B1 and B2, catechin and epicatechins were quantified, as these polyphenols are commonly present among grapes, blackcurrant, boysenberry and kiwifruits (Olas, 2018). Since the polyphenol identification were in accordance to previous studies, our extraction methods and solvent were efficient enough to dissolve most of the polyphenols. Furthermore, to properly understand the total amount of phenolics, each extract can contribute, the total phenolic content was analyzed and illustrated in figure 4.2.

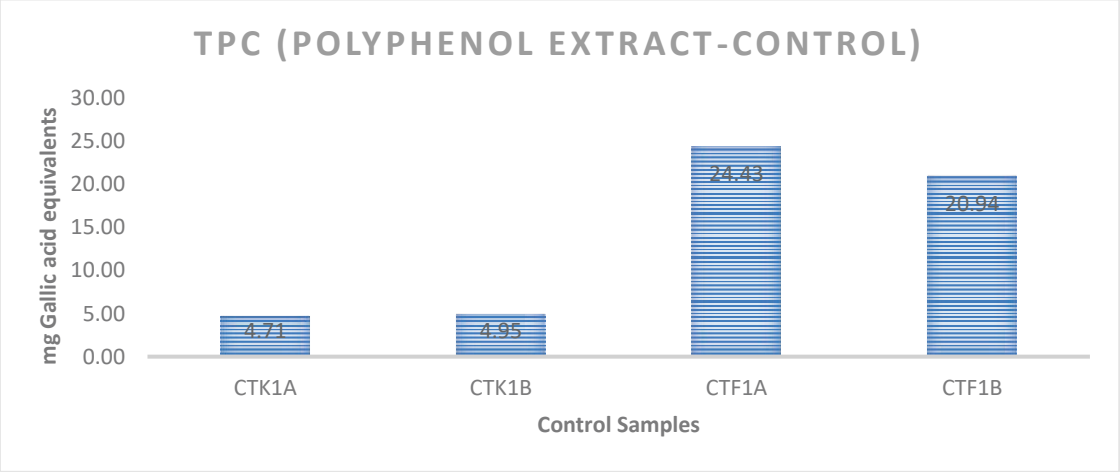


Figure 0.2 Total phenolic content of the control samples.

The equation from the gallic acid standard curve was Y=0.0126x, R^2 = 0.9912. From the ANOVA and regression analysis, P<0.05 (Appendix A.1). This shows that there are significant differences between the means. The tukey comparison method signifies that the significant differences are between the fruit complex control sample, the Milk-Fruit complex polyphenol sample and the rest of other samples.

The total phenolic content of the kiwifruit extract ranges from 4.71±0.07 to 4.95±0.13 mg GAE/g of extract, while the total phenolic contribution of the fruit complex ranges from 20.94 ±0.20 to 24.43±0.16. When we compare the total phenolic content of the control samples with the milk – polyphenol systems, we can see that there is a decrease in the quantity of polyphenols for the milk-fruit complex system, while the Milk- kiwifruit extract had an additive effect, as shown in figure 4.3.

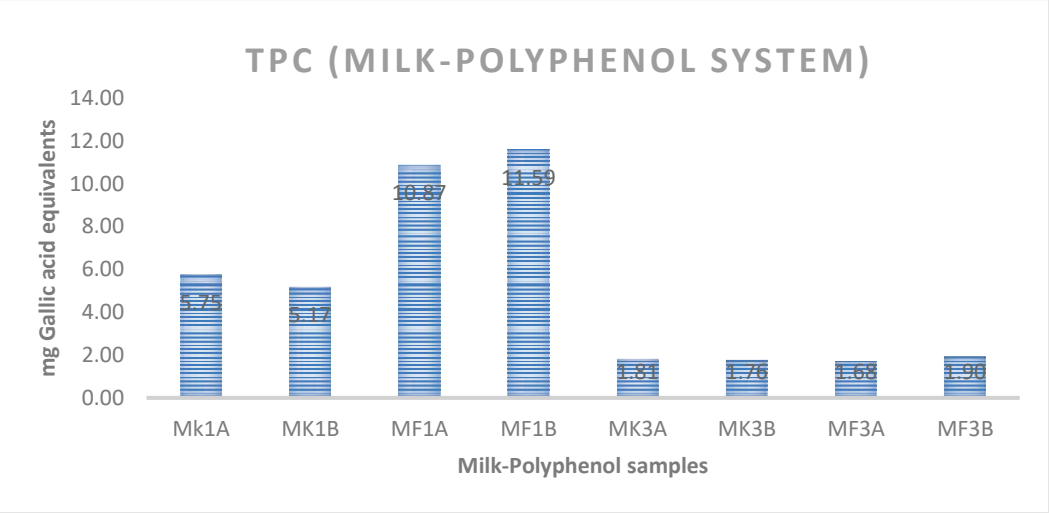


Figure 0.3 Total phenolic content of the Milk with kiwifruit and mixed fruit extract

In the above figure samples named Mk3A, Mk3B, MF3A and MF3B are just milk samples with no polyphenol content. The phenolic content of the milk samples was also determined to assess the amount of phenols contributed by the milk alone which ranges from 1.81±0.13 mg GAE/g of samples to 1.90±0.00 mg GAE/g of samples. This is due to the fact that milk proteins like caseins and whey act as polyphenols in the total phenolic content assay (Rival, Boeriu, & Wichers, 2001). The phenolic content in the MK1A and MK1B samples is an accordance with the phenolic content of the kiwifruit extract (CTK1A and CTK1B) keeping in mind the phenolic content contributed by the milk alone. On the contrary the milk – fruit complex extracts exhibited only half of the phenolic content in comparison with the Fruit complex extract. This can be further explained by the HPLC data of the milk – polyphenol samples.

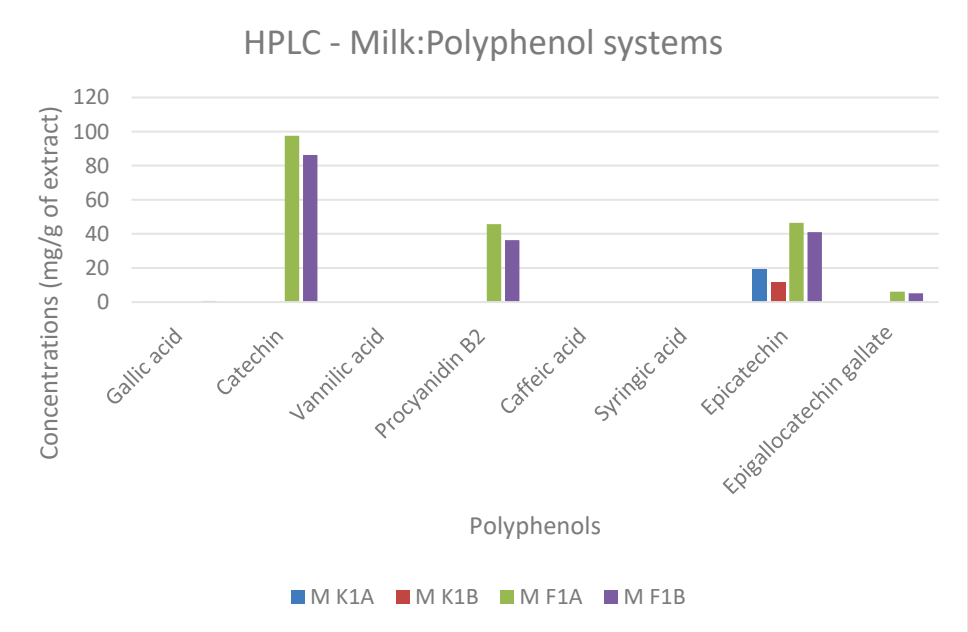


Figure 0.4 HPLC data for the milk-polyphenol systems

The above graph shows that the prominent phenols like catechins, epicatechin, procyanidin B2 and epigallocatechin gallate present in the fruit complex are still retained, while the rest of the minor phenolic compounds have not been detected. The catechins being detected in the milk polyphenol system is in contradictory with a previous study (Cebeci & Şahin-Yeşilçubuk, 2014). The reason could be the difference in the HPLC extraction procedure where they used methanol and formic acid as solvents while we used acetone as our extraction solvent. The use of 8% TCA (trichloroacetic acid) could also be an added advantage in retaining the catechins and precipitating out the proteins. A previous trial was conducted on the fruit polyphenol extracts with methanol and formic acid as solvents, but there was no polyphenol detection in the HPLC run. This shows that acetone would be a better solvent for polyphenol extraction than methanol which holds true according to previous studies (Turkmen, Sari, & Velioglu, 2006). The catechins and the procyanidins belong to the same group of flavanols and hence share the same binding abilities to milk proteins (Kanakis et al., 2011).

1.7.1 Loading Efficiency studies

These studies were carried out to quantify the total phenolics that were bound to the milk protein. These amounts can be calculated by comparing the total phenolics in the control samples and the phenolics present in the supernatant after the precipitation of the casein and the whey proteins. The total phenol content of the milk – kiwifruit extract samples is in the range of 7.94±0.49 mg GAE/g of extract to 6.70±0.07 mg GAE/g of extract. TPC of the milk-fruit complex samples was in the range of 6.67±0.88 mg GAE/g of extract to 8.18±0.14 mg GAE/g of extract as shown in figure 4.5. The expected values were assumed to be much lower than the TPC of MK1 A, MK1b, MF1A and MF1B samples in order to prove that the milk proteins bind to these polyphenols. On the contrary the TPC values were approximately more or less close to the expected values.

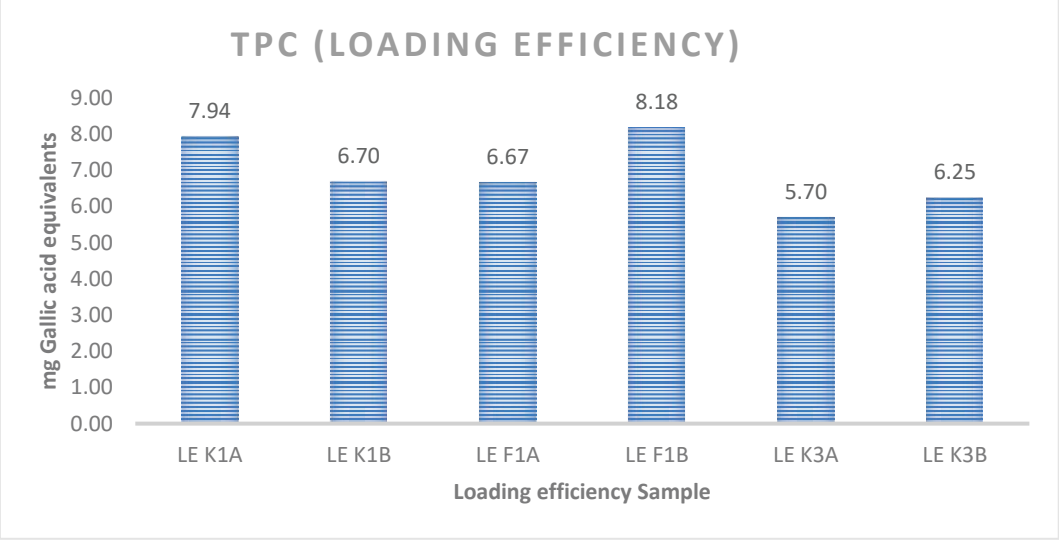


Figure 0.5 Total phenolic content determination of milk-polyphenol samples to study their loading efficiency.

However, the graph also shows that the loading efficiency studies on pure milk samples (LE K3A and LE K3b) also contributed to the phenolic content as stated for the earlier results. In order to understand this better the HPLC determination of these loading efficiency samples were conducted and displayed in figure 4.6.

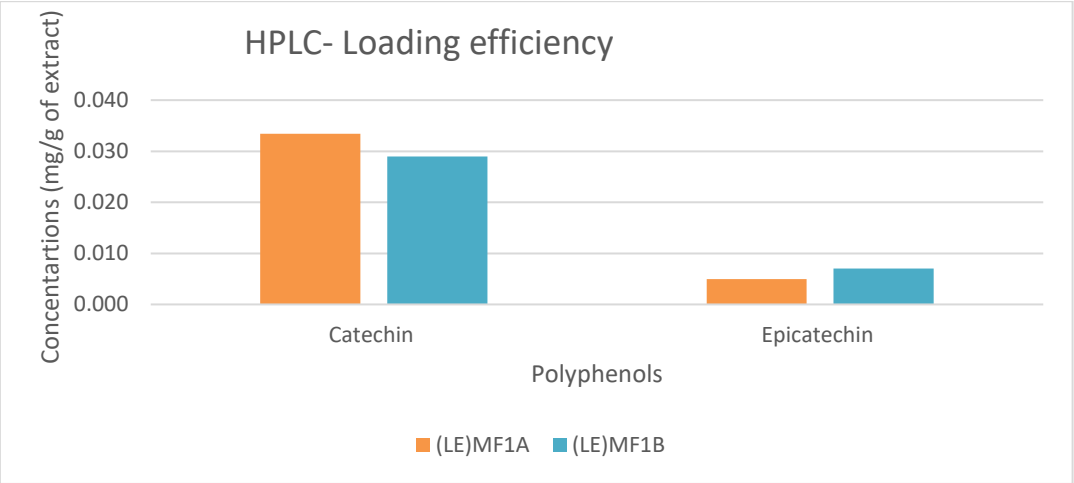


Figure 0.6 HPLC analysis of the samples extracted for loading efficiency studies. (LE – Loading efficiency)

From the HPLC data, two points can be firmly put forward which are: the phenolic content exhibited by these samples in the TPC assay is contributed by the milk proteins, as there was no polyphenol detection via HPLC as per App 3 (Appendix). Though catechin and epicatechin were detected for the milk- fruit complex samples, their values from the HPLC data was negligibly very small compared to the TPC data. A previous study by Cebeci and Şahin-Yeşilçubuk (2014) on blueberry and milk systems also showed a reduction in phenolic content. From our data we can say that there was 100% loading of our polyphenols on the milk proteins. This can be explained further by assessing their radical scavenging activity by DPPH assay.

1.8 DPPH radical scavenging activity

The radical scavenging activity was expressed in terms of µmol of Trolox equivalents per gram sample weight. Appendix A.2 reports the means ± the standard deviation. This methodology makes use of the stable free radical, DPPH which is purple in colour. Loss of colour indicates that the free radicals have been scavenged by the antioxidants added (Holtz, 2009). The equation from the Trolox standard was $y=-0.0012x + 0.1411$, $R^2 = 0.9846$. From the ANOVA and regression analysis, $P<0.05$. This shows that there are significant differences between the means. The tukey comparison method signifies that the significant differences are between the fruit complex control sample, the Milk-Fruit complex polyphenol sample and the rest of other samples.

Radical scavenging activity was measured for the control samples (No proteins), milk-polyphenol system, milk samples and the samples extracted for loading efficiency studies (LE). The results of the milk samples (MK3A, MK3B, MF3A and MF3B) were not reasonable and had high absorbance values more than the blank and hence they were considered to have no antioxidants, which is also in accordance with the HPLC results for the same (appendix A.1). Figure 4.7 shows the radical scavenging activities of the control and the milk-polyphenol samples.

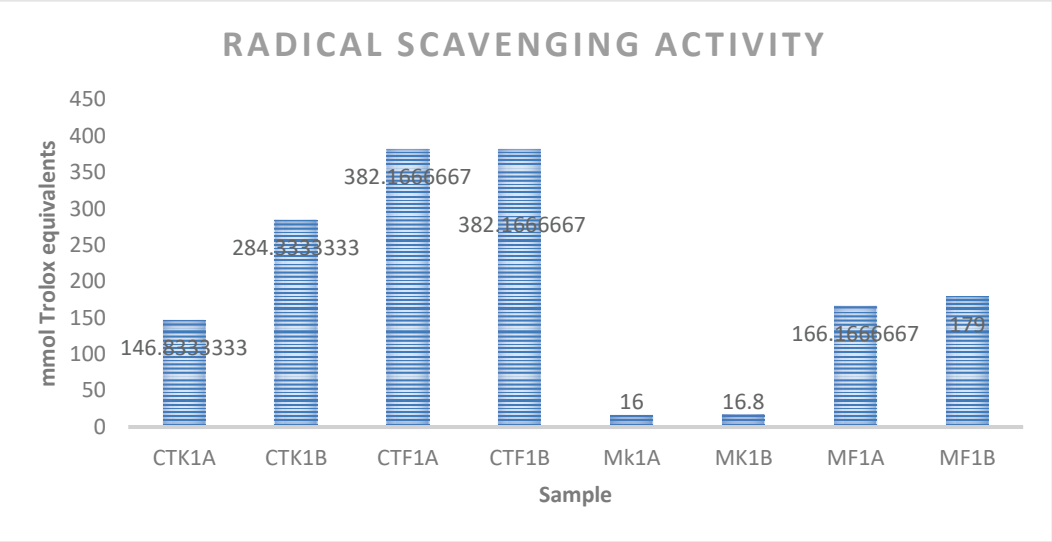


Figure 0.7 Antioxidant activity of the control and milk-polyphenol samples

From the HPLC results of the control samples and the DPPH assay, procyanidin B2 is the major contributor to radical scavenging activity of kiwifruit extract. This is contradictory to one of the studies where they have mentioned that chlorogenic acid is the major contributor to the antioxidant activity. In this similar study they have also mentioned that the predominant phenolic groups were the flavon-3-ols which is accordance to our results (H.-Y. Li et al., 2018). The difference in the results is due to the fact that, in their study they used the pulp of the fruit while we worked on the skin extract. The radical scavenging activity of the Milk – Kiwifruit sample were very less compared to the TPC results, which suggest that around 10% of the activity was retained in the milk complex. While the milk-fruit complex systems showed around 50% of the activity in comparison with the control samples. This was in accordance with the total phenol content of the fruit complex extract and their milk-polyphenol system.

To understand the loading efficiency, the radical scavenging assay of the samples were analyzed (Appendix A.3) and represented as in Figure 4.8. The results of the LE MK1A and LE MK1B were not reasonable and had higher absorbance than the blanks and hence considered to have zero antioxidants. While the milk-fruit complex samples showed some amounts antioxidant activity. From

the respective HPLC and TPC values of the milk-fruit complex samples, the antioxidant activity was contributed by the catechins.

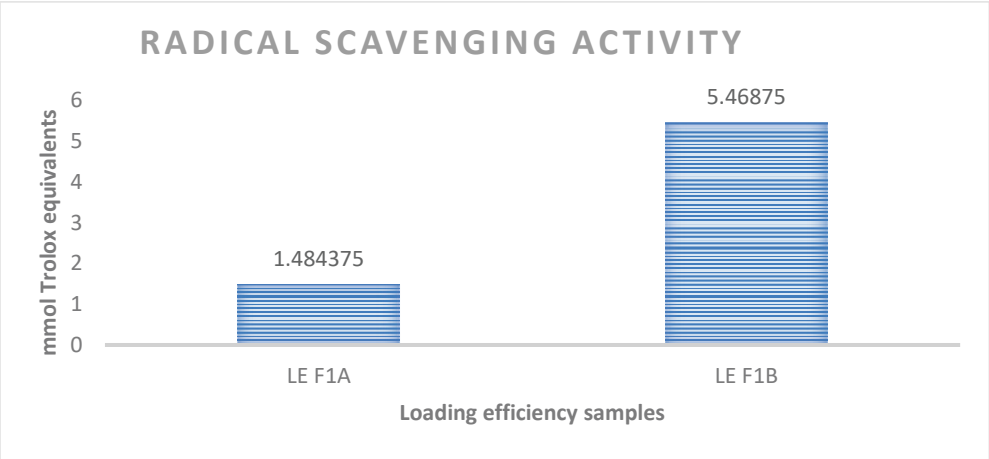


Figure 0.8 antioxidant activity of the samples for loading efficiency studies.

From the HPLC data in Figure 4.6, we can say that the major contributor to the antioxidant activity exhibited by the fruit complex system (Figure 4.8) is the catechins. A previous study explains the type and extent of catechin interaction with the individual milk proteins in a milk-cocoa model system (Gallo et al., 2013). This study explained a decrease in antioxidant activity due to the interaction of catechins with milk proteins, majorly the whey proteins. In contradiction to this study, Rashidinejad, Birch, Sun-Waterhouse, and Everett (2013) explained the interaction of grape falvon-3-0ls with cheese curds and the increase in antioxidant activity when catechins were added to cheese curd. At lower pH the caseins precipitate out leaving the whey proteins in the solutions. An increase in antioxidant activity can mean that the catechins were not bound to the whey proteins. Our study shows that there is a major decrease in antioxidant activity in milk – polyphenol systems while Gallo and his co-workers (2013) stated that only a small part of the protein interacted with the polyphenol. However, our results support our hypothesis of complete loading or binding of polyphenols with milk proteins.

1.9 Antioxidant activity by ABTS assay

The antioxidant activity of the samples was also analyzed using ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)). The ABTS assay is based on the scavenging of long-life radical cation $ABTS^{\cdot+}$. scavenging of these radicals causes a decrease in absorbance at 658nm. This assay is basically carried out with the standard Trolox being prepared in 70% methanol. Since our extracts used acetone, the ABTS assay was carried out using 70% acetone instead of methanol. The results obtained for this assay during this study were not accurate and the values obtained for the Trolox calibration curve were not linear. There are several reasons to explain this discrepancy. It could be due to a wrong methodology followed or more likely the effect of the extraction solvent on ABTS. The solvent used in our extracts was 70% acetone. A previous study was conducted on the effect of various solvents on

ABTS assay (Pérez-Jiménez & Saura-Calixto, 2006). They stated that the type of solvent and its polarity affected the single electron transfer (SET) and hydrogen atom transfer (HAT), which are the main aspects in measurement of antioxidation capacity. ABTS assay was the second most sensitive assay in terms of the solvent used. Another study also stated that acetone had the capability of transforming ABTS into its reduced forms which significantly affected the radical scavenging activity (Liu, Zhou, Wu, Sun, & Chen, 2015).

Conclusions and Future Scope

It is difficult to draw definite conclusions from the data obtained from the HPLC, total phenol content and antioxidation activity assays. However, the results clearly reveal that most of the polyphenols bind completely to the milk proteins. Our results also provide us with an idea about the required amounts of polyphenols to be added to milk, so that enough amounts can be extracted which are over the detection limits. It is apparent that polyphenol losses have occurred in most samples. The sample preparation methods and initial polyphenol concentrations may have caused low recoveries. The use 70% acetone as an extraction solvent gave better HPLC results than the methanol extraction of polyphenols. However, few modifications with the extraction method could increase the chances of the bound polyphenols to be released into the solvent. Due to the time constrains of this study, the extractions methods were assumed and not fully established before use. However, there is a strong binding interaction between the milk protein and the polyphenols. From the extraction methods and the results of the antioxidative activity of the extracted polyphenols from the milk-polyphenol system clearly states that there is a considerable retention of the functional properties of the polyphenols. A decrease in these properties owes to insufficient extraction of these polyphenols from the milk proteins. Bioavailability studies can further help in assessing the retention of antioxidant activities of the polyphenols.

References

Bakowska-Barczak, A. M., & Kolodziejczyk, P. P. (2011). Black currant polyphenols: Their storage stability and microencapsulation. *Industrial crops and products, 34*(2), 1301-1309.

Bąkowska, A., Kucharska, A. Z., & Oszmiański, J. (2003). The effects of heating, UV irradiation, and storage on stability of the anthocyanin–polyphenol copigment complex. *Food chemistry, 81*(3), 349-355.

Bohin, M. C., Vincken, J.-P., van der Hijden, H. T., & Gruppen, H. (2012). Efficacy of food proteins as carriers for flavonoids. *Journal of agricultural and food chemistry, 60*(16), 4136-4143.

Bourassa, P., Bariyanga, J., & Tajmir-Riahi, H. (2013). Binding sites of resveratrol, genistein, and curcumin with milk α-and β-caseins. *The Journal of Physical Chemistry B, 117*(5), 1287-1295.

Calligaris, S., Manzocco, L., Anese, M., & Nicoli, M. C. (2004). Effect of heat-treatment on the antioxidant and pro-oxidant activity of milk. *International Dairy Journal, 14*(5), 421-427.

Cebeci, F., & Şahin-Yeşilçubuk, N. (2014). The matrix effect of blueberry, oat meal and milk on polyphenols, antioxidant activity and potential bioavailability. *International journal of food sciences and nutrition, 65*(1), 69-78.

Coultate, T. P. (2009). *Food: the chemistry of its components*: Royal Society of Chemistry.

da Silva, D. F., Matumoto-Pintro, P. T., Bazinet, L., Couillard, C., & Britten, M. (2015). Effect of commercial grape extracts on the cheese-making properties of milk. *Journal of dairy science, 98*(3), 1552-1562.

de Freitas, V., & Mateus, N. (2001). Structural features of procyanidin interactions with salivary proteins. *Journal of agricultural and food chemistry, 49*(2), 940-945.

Edwards, P., Creamer, L., & Jameson, G. (2009). Structure and stability of whey proteins In: Milk Proteins from Expression to Food Thompson A, Boland M, Singh H eds. In: Academic Press San Diego, CA p.

Forrest, S. A., Yada, R. Y., & Rousseau, D. (2005). Interactions of vitamin D3 with bovine β-lactoglobulin A and β-casein. *Journal of agricultural and food chemistry, 53*(20), 8003-8009.

Frazier, R. A., Papadopoulou, A., & Green, R. J. (2006). Isothermal titration calorimetry study of epicatechin binding to serum albumin. *Journal of pharmaceutical and biomedical analysis, 41*(5), 1602-1605.

Fu, L., Sun, Y., Ding, L., Wang, Y., Gao, Z., Wu, Z., . . . Bi, Y. (2016). Mechanism evaluation of the interactions between flavonoids and bovine serum albumin based on multi-spectroscopy, molecular docking and Q-TOF HR-MS analyses. *Food chemistry, 203*, 150-157.

Furuuchi, R., Yokoyama, T., Watanabe, Y., & Hirayama, M. (2011). Identification and quantification of short oligomeric proanthocyanidins and other polyphenols in boysenberry seeds and juice. *Journal of agricultural and food chemistry, 59*(8), 3738-3746.

Gallo, M., Vinci, G., Graziani, G., De Simone, C., & Ferranti, P. (2013). The interaction of cocoa polyphenols with milk proteins studied by proteomic techniques. *Food research international, 54*(1), 406-415.

Gholami, S., & Bordbar, A.-K. (2014). Exploring binding properties of naringenin with bovine β-lactoglobulin: A fluorescence, molecular docking and molecular dynamics simulation study. *Biophysical chemistry, 187*, 33-42.

Graf, B. A., Milbury, P. E., & Blumberg, J. B. (2005). Flavonols, flavones, flavanones, and human health: epidemiological evidence. *Journal of medicinal food, 8*(3), 281-290.

Grimbleby, F., & Ntailianas, H. (1961). Binding of trichloroacetic acid by protein. *Nature, 189*(4767), 835-836.

Han, X., Shen, T., & Lou, H. (2007). Dietary polyphenols and their biological significance. *International Journal of Molecular Sciences, 8*(9), 950-988.

Hasni, I., Bourassa, P., Hamdani, S., Samson, G., Carpentier, R., & Tajmir-Riahi, H.-A. (2011). Interaction of milk α-and β-caseins with tea polyphenols. *Food chemistry, 126*(2), 630-639.

He, Z., Xu, M., Zeng, M., Qin, F., & Chen, J. (2016). Interactions of milk α-and β-casein with malvidin-3-O-glucoside and their effects on the stability of grape skin anthocyanin extracts. *Food chemistry, 199*, 314-322.

Holtz, R. W. (2009). In vitro methods to screen materials for anti-aging effects. In *Skin aging handbook* (pp. 329-362): Elsevier.

Huang, W.-y., Zhang, H.-c., Liu, W.-x., & Li, C.-y. (2012). Survey of antioxidant capacity and phenolic composition of blueberry, blackberry, and strawberry in Nanjing. *Journal of Zhejiang University Science B, 13*(2), 94-102.

Kanakis, C., Hasni, I., Bourassa, P., Tarantilis, P., Polissiou, M., & Tajmir-Riahi, H.-A. (2011). Milk β-lactoglobulin complexes with tea polyphenols. *Food chemistry, 127*(3), 1046-1055.

Khatun, S., Yasmeen, S., Kumar, A., & Subbarao, N. (2018). Calorimetric, spectroscopic and molecular modelling insight into the interaction of gallic acid with bovine serum albumin. *The Journal of Chemical Thermodynamics, 122*, 85-94.

Kondratyuk, T. P., & Pezzuto, J. M. (2004). Natural product polyphenols of relevance to human health. *Pharmaceutical biology, 42*(sup1), 46-63.

(b) Krupa, T., Latocha, P., & Liwińska, A. (2011). Changes of physicochemical quality, phenolics and vitamin C content in hardy kiwifruit (Actinidia arguta and its hybrid) during storage. *Scientia Horticulturae, 130*(2), 410-417.

Langley-Evans, S. C. (2000). Antioxidant potential of green and black tea determined using the ferric reducing power (FRAP) assay. *International journal of food sciences and nutrition, 51*(3), 181-188.

Li, H.-Y., Yuan, Q., Yang, Y.-L., Han, Q.-H., He, J.-L., Zhao, L., . . . Wu, D.-T. (2018). Phenolic profiles, antioxidant capacities, and inhibitory effects on digestive enzymes of different kiwifruits. *Molecules, 23*(11), 2957.

Li, J., & Yao, P. (2009). Self-assembly of ibuprofen and bovine serum albumin− dextran conjugates leading to effective loading of the drug. *Langmuir, 25*(11), 6385-6391.

Liang, L., & Subirade, M. (2012). Study of the acid and thermal stability of β-lactoglobulin–ligand complexes using fluorescence quenching. *Food chemistry, 132*(4), 2023-2029.

Liang, L., Tajmir-Riahi, H., & Subirade, M. (2008). Interaction of β-lactoglobulin with resveratrol and its biological implications. *Biomacromolecules, 9*(1), 50-56.

Liu, H., Zhou, P., Wu, X., Sun, J., & Chen, S. (2015). Radical scavenging by acetone: A new perspective to understand laccase/ABTS inactivation and to recover redox mediator. *Molecules, 20*(11), 19907-19913.

Livney, Y. D. (2010). Milk proteins as vehicles for bioactives. *Current opinion in colloid & interface science, 15*(1-2), 73-83.

Ma, T., Sun, X., Zhao, J., You, Y., Lei, Y., Gao, G., & Zhan, J. (2017). Nutrient compositions and antioxidant capacity of kiwifruit (Actinidia) and their relationship with flesh color and commercial value. *Food chemistry, 218*, 294-304.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *The American journal of clinical nutrition, 79*(5), 727-747.

McSweeney, P. L., & Fox, P. F. (2003). *Advanced dairy chemistry* (Vol. 1): Springer.

Mehranfar, F., Bordbar, A.-K., & Parastar, H. (2013). A combined spectroscopic, molecular docking and molecular dynamic simulation study on the interaction of quercetin with β-casein nanoparticles. *Journal of Photochemistry and Photobiology B: Biology, 127*, 100-107.

Mohammadi, F., & Moeeni, M. (2015). Study on the interactions of trans-resveratrol and curcumin with bovine α-lactalbumin by spectroscopic analysis and molecular docking. *Materials Science and Engineering: C, 50*, 358-366.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, Rubus, and Ribes. *Journal of agricultural and food chemistry, 50*(3), 519-525.

Ng-Kwai-Hang, K. (2011). Milk Proteins| Heterogeneity, Fractionation, and Isolation.

O'connell, J., & Fox, P. (2001). Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. *International Dairy Journal, 11*(3), 103-120.

Olas, B. (2018). Berry phenolic antioxidants–implications for human health? *Frontiers in pharmacology, 9*, 78.

Ortega-Regules, A., Romero-Cascales, I., López-Roca, J. M., Ros-García, J. M., & Gómez-Plaza, E. (2006). Anthocyanin fingerprint of grapes: environmental and genetic variations. *Journal of the Science of Food and Agriculture, 86*(10), 1460-1467.

Pandey, K. B., & Rizvi, S. I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative medicine and cellular longevity, 2*(5), 270-278.

Papadopoulou, A., Green, R. J., & Frazier, R. A. (2005). Interaction of flavonoids with bovine serum albumin: a fluorescence quenching study. *Journal of agricultural and food chemistry, 53*(1), 158-163.

Parr, A. J., & Bolwell, G. P. (2000). Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *Journal of the Science of Food and Agriculture, 80*(7), 985-1012.

Pascal, C., Poncet-Legrand, C., Imberty, A., Gautier, C., Sarni-Manchado, P., Cheynier, V., & Vernhet, A. (2007). Interactions between a non glycosylated human proline-rich protein and flavan-3-ols are affected by protein concentration and polyphenol/protein ratio. *Journal of agricultural and food chemistry, 55*(12), 4895-4901.

Pérez-Jiménez, J., & Saura-Calixto, F. (2006). Effect of solvent and certain food constituents on different antioxidant capacity assays. *Food research international, 39*(7), 791-800.

Prigent, S. V., Gruppen, H., Visser, A. J., Van Koningsveld, G. A., De Jong, G. A., & Voragen, A. G. (2003). Effects of non-covalent interactions with 5-O-caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins. *Journal of agricultural and food chemistry, 51*(17), 5088-5095.

Puyol, P., Perez, M. D., Ena, J. M., & Calvo, M. (1991). Interaction of bovine β-lactoglobulin and other bovine and human whey proteins with retinol and fatty acids. *Agricultural and biological chemistry, 55*(10), 2515-2520.

Rashidinejad, A., Birch, E. J., Sun-Waterhouse, D., & Everett, D. W. (2013). Effects of catechin on the phenolic content and antioxidant properties of low-fat cheese. *International journal of food science & technology, 48*(12), 2448-2455.

Rawel, H. M., Meidtner, K., & Kroll, J. (2005). Binding of selected phenolic compounds to proteins. *Journal of agricultural and food chemistry, 53*(10), 4228-4235.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine, 26*(9-10), 1231-1237.

Renner, E. (1988). Storage stability and some nutritional aspects of milk powders and ultra high temperature products at high ambient temperatures. *Journal of Dairy Research, 55*(1), 125-142.

Richard, T., Vitrac, X., Merillon, J., & Monti, J. (2005). Role of peptide primary sequence in polyphenol–protein recognition: An example with neurotensin. *Biochimica et Biophysica Acta (BBA)-General Subjects, 1726*(3), 238-243.

Riihimaki, L. H., Vainio, M. J., Heikura, J. M., Valkonen, K. H., Virtanen, V. T., & Vuorela, P. M. (2008). Binding of phenolic compounds and their derivatives to bovine and reindeer β-lactoglobulin. *Journal of agricultural and food chemistry, 56*(17), 7721-7729.

Rival, S. G., Boeriu, C. G., & Wichers, H. J. (2001). Caseins and casein hydrolysates. 2. Antioxidative properties and relevance to lipoxygenase inhibition. *Journal of agricultural and food chemistry, 49*(1), 295-302.

Roy, D., Dutta, S., Maity, S. S., Ghosh, S., Roy, A. S., Ghosh, K. S., & Dasgupta, S. (2012). Spectroscopic and docking studies of the binding of two stereoisomeric antioxidant catechins to serum albumins. *Journal of Luminescence, 132*(6), 1364-1375.

Sahu, A., Kasoju, N., & Bora, U. (2008). Fluorescence study of the curcumin− casein micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules, 9*(10), 2905-2912.

Semo, E., Kesselman, E., Danino, D., & Livney, Y. D. (2007). Casein micelle as a natural nano-capsular vehicle for nutraceuticals. *Food hydrocolloids, 21*(5-6), 936-942.

Shpigelman, A., Israeli, G., & Livney, Y. D. (2010). Thermally-induced protein–polyphenol co-assemblies: beta lactoglobulin-based nanocomplexes as protective nanovehicles for EGCG. *Food hydrocolloids, 24*(8), 735-743.

Skrt, M., Benedik, E., Podlipnik, Č., & Ulrih, N. P. (2012). Interactions of different polyphenols with bovine serum albumin using fluorescence quenching and molecular docking. *Food chemistry, 135*(4), 2418-2424.

Soares, S., Mateus, N., & De Freitas, V. (2007). Interaction of different polyphenols with bovine serum albumin (BSA) and human salivary α-amylase (HSA) by fluorescence quenching. *Journal of agricultural and food chemistry, 55*(16), 6726-6735.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food chemistry, 99*(4), 835-841.

Volf, I., Ignat, I., Neamtu, M., & Popa, V. I. (2014). Thermal stability, antioxidant activity, and photo-oxidation of natural polyphenols. *Chemical Papers, 68*(1), 121-129.

Wang, X., Ho, C.-T., & Huang, Q. (2007). Investigation of adsorption behavior of (−)-epigallocatechin gallate on bovine serum albumin surface using quartz crystal microbalance with dissipation monitoring. *Journal of agricultural and food chemistry, 55*(13), 4987-4992.

Waterhouse, A. L. (2002). Determination of total phenolics. *Current protocols in food analytical chemistry, 6*(1), I1. 1.1-I1. 1.8.

Waugh, D. F. (1958). The interactions of α s-β-and κ-caseins in micelle formation. *Discussions of the Faraday Society, 25*, 186-192.

Williamson, M. P. (1994). The structure and function of proline-rich regions in proteins. *Biochemical journal, 297*(Pt 2), 249.

Wink, M. (1997). Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. In *Advances in botanical research* (Vol. 25, pp. 141-169): Elsevier.

Wojdyło, A., Nowicka, P., Oszmiański, J., & Golis, T. (2017). Phytochemical compounds and biological effects of Actinidia fruits. *Journal of Functional Foods, 30*, 194-202.

Xiao, J., Cao, H., Wang, Y., Yamamoto, K., & Wei, X. (2010). Structure–affinity relationship of flavones on binding to serum albumins: Effect of hydroxyl groups on ring A. *Molecular nutrition & food research, 54*(S2), S253-S260.

Xiao, J., Mao, F., Yang, F., Zhao, Y., Zhang, C., & Yamamoto, K. (2011). Interaction of dietary polyphenols with bovine milk proteins: molecular structure–affinity relationship and influencing bioactivity aspects. *Molecular nutrition & food research, 55*(11), 1637-1645.

Yen, G.-C., & Chen, H.-Y. (1995). Antioxidant activity of various tea extracts in relation to their antimutagenicity. *Journal of agricultural and food chemistry, 43*(1), 27-32.

Yildirim-Elikoglu, S., & Erdem, Y. K. (2018). Interactions between milk proteins and polyphenols: Binding mechanisms, related changes, and the future trends in the dairy industry. *Food reviews international, 34*(7), 665-697.

Yuksel, Z., Avci, E., & Erdem, Y. K. (2010). Characterization of binding interactions between green tea flavanoids and milk proteins. *Food chemistry, 121*(2), 450-456.

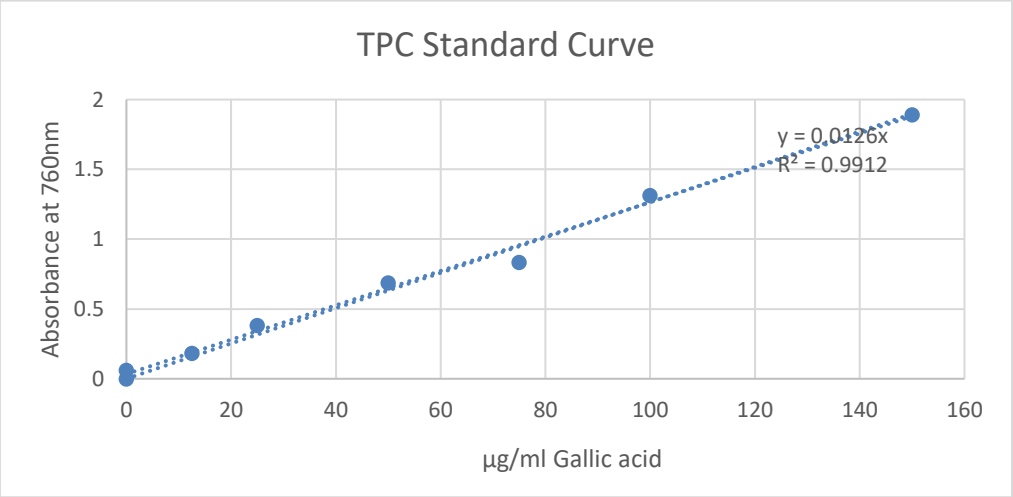
Appendix A

A.1 Total Phenolic content (TPC)

The total phenolic content was expressed in terms of mg Gallic acid equivalents. (LE: Loading efficiency extracts)

samples	Samples diluted*10 (mg GA equivalents)		mg GA equivalents/g of extract		Mean±Std dev (mg GA equivalents /g of extract)
	1	2	1	2	
CTK1A	116.67	119.05	4.67	4.76	4.71±0.07
CTK1B	126.19	121.43	5.05	4.86	4.95±0.13
CTF1A	607.94	613.49	24.32	24.54	24.43±0.16
CTF1B	526.98	519.84	21.08	20.79	20.94±0.20
Mk1A	148.41	138.89	5.94	5.56	5.75±0.27
MK1B	126.98	131.75	5.08	5.27	5.17±0.13
MF1A	275.40	268.25	11.02	10.73	10.87±0.20
MF1B	289.68	289.68	11.59	11.59	11.59±0.00
MK3A	47.62	42.86	1.90	1.71	1.81±0.13
MK3B	43.65	44.44	1.75	1.78	1.76±0.02
MF3A	42.06	42.06	1.68	1.68	1.68±0.00
MF3B	47.62	47.62	1.90	1.90	1.90±0.00
LE K1A	132.54	121.43	8.28	7.59	7.94±0.49
LE K1B	107.94	106.35	6.75	6.65	6.70±0.07
LE F1A	96.83	116.67	6.05	7.29	6.67±0.88
LE F1B	132.54	129.37	8.28	8.09	8.18±0.14
LE K3A	89.68	92.86	5.61	5.80	5.70±0.14
LE K3B	97.62	102.38	6.10	6.40	6.25±0.21

Standard curve obtained for TPC, using gallic acid standard solutions



Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
SampleID	9	645.457	71.717	68.70	0.000
Error	8	8.351	1.044		
Total	17	653.808			

Grouping Information Using the Tukey Method and 95% Confidence

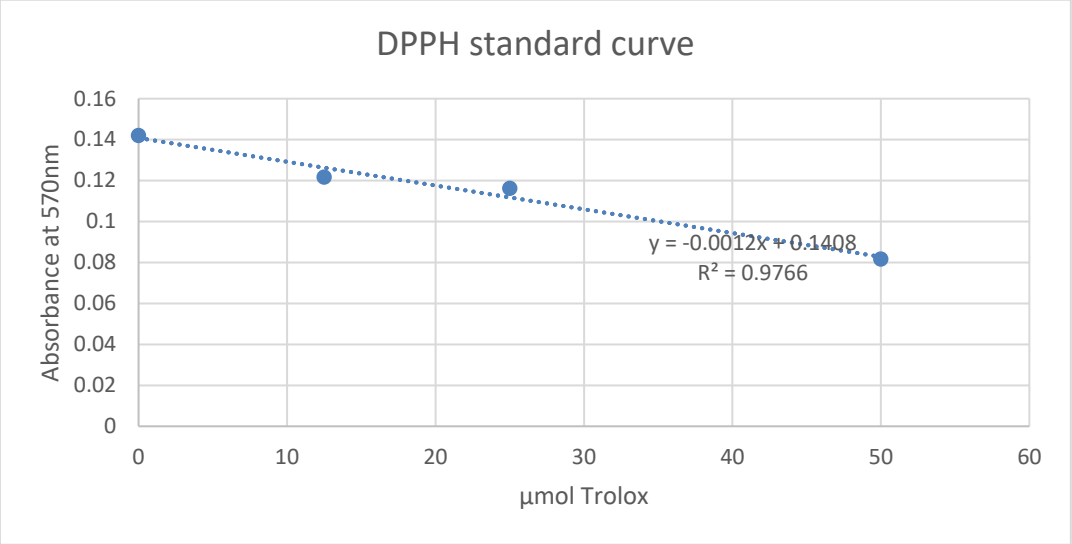
SampleID	N	Mean	Grouping		
CTF1	2	22.6984	A		
MF1	2	11.3016		B	
LEMK1	2	7.5149		B	C
LEMF1	2	7.1677		B	C
Mk1	1	5.9365		B	C D
LEMK3	2	5.8532			C D
MK1	1	5.0794			C D
CTK1	2	4.8571			C D
MK3	2	1.8254			D
MF3	2	1.7937			D

Means that do not share a letter are significantly different.

A.2 Radical scavenging activity (DPPH assay)

samples	Concentration of diluted samples (µmol trolox from std curve)		Concentartin in mmol trolox equivalents /g of extract		Conc (mmol trolox equivalents / gram of extract)
	1st conc.	2nd conc.	1st conc.	2nd conc.	
CTK1A	365.83	368.33	146.33	147.33	146.83±0.70
CTK1B	711.67	710.00	284.67	284.00	284.33±0.47
CTF1A	953.33	957.50	381.33	383.00	382.17±1.17
CTF1B	953.33	957.50	381.33	383.00	382.17±1.17
Mk1A	37.50	42.50	15.00	17.00	16.00±1.41
MK1B	43.33	40.67	17.33	16.27	16.80±0.75
MF1A	416.67	414.17	166.67	165.67	166.17±0.70
MF1B	445.00	450.00	178.00	180.00	179.00±1.41
MK3A	-10.83	-20.83	-4.33	-8.33	-6.33
MK3B	-16.67	-14.17	-6.67	-5.67	-6.17
MF3A	-0.83	-55.83	-0.33	-22.33	-11.33
MF3B	-33.33	-60.83	-13.33	-24.33	-18.83
LE K1A	-8.33	-62.50	-0.52	-3.91	-2.21
LE K1B	-50.00	-8.33	-3.13	-0.52	-1.82
LE F1A	33.33	14.17	2.08	0.89	1.48±0.84
LE F1B	75.83	99.17	4.74	6.20	5.47±1.03
LE K3A	-491.67	-326.67	-30.73	-20.42	-25.57
LE K3B	-274.17	-408.33	-17.14	-25.52	-21.33

Standard curve obtained from DPPH assay using Trolox standard solutions



Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
SampleID	9	328546	36505	30.81	0.000
Error	8	9480	1185		
Total	17	338026			

Grouping Information Using the Tukey Method and 95% Confidence

SampleID	N	Mean	Grouping		
CTF1	2	383.000	A		
CTK1	2	215.667		B	
MF1	2	172.833		B	C
Mk1	1	17.000			C D
MK1	1	16.267			C D
LEMF1	2	3.542			D
LEMK1	2	-2.214			D
MK3	2	-7.000			D
LEMK3	2	-22.969			D
MF3	2	-23.333			D

Means that do not share a letter are significantly different.

A.3 HPLC Profiling of the samples using phenolic standards

App 3.1 HPLC profile of the control and the milk-polyphenol samples.

	Concentration in ppm				Concentration (mg/ gram of extract)			
Standards	**CT K1A**	**CT K1B**	**CT F1A**	**CT F1B**	**CT K1A**	**CT K1B**	**CT F1A**	**CT F1B**
Gallic acid	0	0	0.06	0.11	0	0	0.6	1.1
Protocatechuic	7.73	8.35	0.49	0.66	77.3	83.5	4.9	6.6
Procyanidin B1	11.89	12.08	138.41	162.05	118.9	120.8	1384.1	1620.5
Hydrobenzoric acid	0	0	0.03	0.11	0	0	0.3	1.1
Catechin	4.13	4.8	47.19	55.55	41.3	48	471.9	555.5
Vannilic acid	0.3	0.39	0	0	3	3.9	0	0
Procyanidin B2	37.46	41.3	446.45	527.69	374.6	413	4464.5	5276.9
Caffeic acid	0	0.03	0.34	0.15	0	0.3	3.4	1.5
Syringic acid	1.65	1.86	0.22	0.32	16.5	18.6	2.2	3.2
Epicatechin	21.7	23.95	106.64	125.89	217	239.5	1066.4	1258.9
Epigallocatechin gallate	0.31	0.19	10.72	12.62	3.1	1.9	107.2	126.2
p-Coumaric acid	0	0	0.66	0.6	0	0	6.6	6
Ferulic acid	0.04	0.08	0.96	1.15	0.4	0.8	9.6	11.5
Rutin	0.25	0.25	11.68	13.3	2.5	2.5	116.8	133
Quercetin	0	0	0.45	0.25	0	0	4.5	2.5

App 3.2 HPLC profile of the loading Milk-Polyphenol samples

Phenolic Standards	Concentration in ppm				Concentration (mg/ gram of extract)			
	M K1A	**M K1B**	**M F1A**	**M F1B**	**M K1A**	**M K1B**	**M F1A**	**M F1B**
Gallic acid	0	0	0	0.07	0	0	0	0.7
Catechin	0	0	9.76	8.62	0	0	97.6	86.2
Vannilic acid	0	0	0	0	0	0	0	0
Procyanidin B2	0	0	4.57	3.63	0	0	45.7	36.3
Caffeic acid	0	0	0	0	0	0	0	0
Syringic acid	0	0	0	0	0	0	0	0
Epicatechin	1.9	1.18	4.65	4.11	19	11.8	46.5	41.1
Epigallocatechin gallate	0	0	0.61	0.51	0	0	6.1	5.1

App 3.3 HPLC profile of the loading efficiency samples

Phenolic standards	Concentration in ppm						Concentration in mg/g of extract	
	(LE)M K1A	**(LE)M K1B**	**(LE)M F1A**	**(LE)M F1B**	**(LE)M K3A**	**(LE)M K3B**	**(LE)MF1A**	**(LE)MF1B**
Catechin	0	0	2.09	1.81	0	0	0.033	0.029
Epicatechin	0	0	0.31	0.44	0	0	0.005	0.007