University of Reading

The effect of thermal denaturation on whey protein derived mouthdrying and investigations into mucoadhesion as a mechanism of action

A thesis submitted by

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Declaration

Declaration: I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Stephanie Bull

Thesis structure

This thesis includes publications and is structured as following:

Chapter 1 Part 1 comprises an unpublished introduction and literature review, excluding in depth discussion of mucoadhesion, as this is covered in Chapter 1 Part 2, a published review of mucoadhesion in food. This was written alongside another PhD student and, as such, includes some information which is more relevant to the topic of their thesis, such as fat replacement and flavour retention. Chapter 2 Part 1 is a supplementary section giving additional information to Chapter 2 Part 2, which is based on the manuscript of a published paper. Chapters 3, 4 and 5 are written as research papers, yet to be submitted for publication. Chapter 6 is the general discussion, where the links between the chapters are clearly discussed.

Abstract

Whey protein is a functional ingredient used in sports nutrition and in the prevention of sarcopenia in older adults. Non-compliance of whey protein fortified beverages in clinical settings can be due to negative sensory attributes, such as a drying sensation. The mechanism of whey protein derived mouthdrying is yet to be fully defined, with a focus on low pH beverages in the literature. This study aimed to: investigate the effect of thermal denaturation on the build-up of drying elicited by neutral pH whey protein concentrate (WPC); determine potential mechanisms; and mitigate drying.

This research investigated the sensory properties of four WPC aqueous samples heated for varying lengths of time using two sensory techniques; quantitative descriptive analysis and sequential profiling. Heating time was found to correlate with higher drying, mouthcoating and chalky intensities, with an increase over repeated consumption and a high persistence of sensation seen for all samples. Heated samples had a larger particle size, which could contribute to the increase in drying observed. The physical build-up of WPC in the mouth was measured using an *in vivo* retention method, which found the most heated sample had the highest retention up to 60 s after the sample had been swallowed. Accessible thiol concentration was seen to increase with heating time, which could lead to interactions with the mucosa, and consequently a higher oral retention. Interactions of WPC with mucin were observed using particle size analysis and spectroscopic analysis; however, interactions were not observed for individual whey proteins, indicating that the complex mix of WPC contributes to the interactions. Polysaccharides were used in an attempt to mitigate drying by blocking mucoadhesive interactions and lubricating the mouth. Although the mitigation of drying was unsuccessful, a reduction in other mouthfeel attributes was observed suggests this is an area requiring further research.

The thesis concludes that both particle size and protein structure are implicated in whey protein derived mouthdrying, supporting the proposal of a mucoadhesive mechanism.

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List of Abbreviations

ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
AUC	Area under the curve (time-intensity)
BSA	Bovine serum albumin
BSM	Bovine submaxillary mucin
c^*	Coil overlap concentration
CD	Circular dichroism
CMC	Carboxymethyl cellulose
CN	Casein
DLS	Dynamic light scattering
FA	Fatty acid
GMP	Glycomacropeptide
HMP	High-methoxyl pectin
HPMC	Hydroxypropylmethyl cellulose
IG	Immunoglobulin
IgG	Immunoglobulin G
Imax	Maximum intensity
LF	Lactoferrin
LP	Lactoperoxidase
MUC5B	High-molecular-weight mucins
MUC7	Low-molecular-weight mucins
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
NPNs	Nonprotein nitrogens
ONS	Oral nutritional supplements
PGA	Propylene glycol alginate
PTS	Proline, threonine, serine
QDA	Quantitative descriptive analysis
RM-ANOVA	Repeated measures analysis of variance
TEM	Transmission electron microscopy
WPC	Whey protein concentrate (80% protein)
WPI	Whey protein isolate (90% protein)
XG	Xanthan gum
α-LA	α-Lactalbumin
β-LG	β-Lactoglobulin

Chapter 1 Part 1. Introduction and literature review

1.1 Study rationale and hypotheses

Whey protein is a functional food ingredient used to aid muscle anabolism, utilised in both sports nutrition and in prevention of sarcopenia. This study aimed to investigate the influence of whey protein denaturation on the build-up of mouthdrying and the underlying mechanism of mouthdrying in whey protein. The hypothesis was that the intensity of mouth drying would increase with denaturation; repeated consumption would lead to a build-up of drying, particularly in denatured samples; and that mucoadhesion would contribute to the build-up of a drying sensation. Whey protein concentrate (WPC) was selected, as a commonly used ingredient in commercial products. In order to understand the mechanism of the perception of mouth drying in whey proteins, the current study examined the sensory properties of repeated consumption of thermally denatured WPC samples; *in vivo* oral retention and structural differences between WPC samples; interactions of both WPC samples and individual whey proteins with salivary mucin; and the potential to mitigate the perception of drying using polysaccharides.

1.2 Literature review

1.2.1 Milk composition

Milk has an essential role in the mammalian diet, providing complete nutrition for the rapidly growing neonate. The consumption of milk is an integral part of human life and is not limited to human breast-milk, but also from a variety of other species. Bovine milk contains more essential amino acids, fatty acids, trace elements, vitamins and minerals than any other food substance (Roadhouse & Henderson, 1950).

Milk is a water-based mixture with a variety of components giving rise to its complexity, and giving it a breadth of nutritional and biological functions. The composition of milk can be summarised as the following (in order of abundance): water, fat, lactose, ash, casein, and whey proteins (summarised in Table 1.1).

The lipids in milk are mostly fatty acids (FAs), which are carboxylic acids with long hydrocarbon chains. Milk contains a large range of FAs, with bovine milk containing more short carbon chain FAs than human milk (Patton, 2005). Fatty acids exist in milk as triglycerides, which form droplets in the water-based milk, allowing the milk to exist as an emulsion.

Lactose is the main carbohydrate present in milk, and is exclusively naturally occurring in milk. Most milk also contains free saccharides, primarily oligosaccharides, however concentrations of saccharides in bovine milk are relatively low (Urashima, Saito, Nakamura, & Messer, 2001).

Ash is a mixture of minerals and salts, the ionic components of which include calcium, sodium, potassium, magnesium, chloride, citrate, phosphate and sulfate. Bovine milk contains higher concentrations of all ions than human milk, except sulfate (Atkinson, Alston-Mills, Lonnerdal, & Neville, 1995), contributing to its high nutritional value.

Table 1.1: A summary of the typical composition of bovine milk(P.F. Fox, Guinee, Cogan, & McSweeney, 2000).

Component	Concentration (g/L)
Water	873
Lipids	37
Lactose	48
Ash	7
Casein	28
Whey proteins	6

The proteins in milk can be largely grouped under two classifications: whey proteins and caseins. Other minor groups of proteinaceous materials within milk are nonprotein nitrogens (NPNs) and proteose peptones. The composition of proteins in bovine milk is summarised in Table 1.2. Whey and casein separate during the coagulation of milk, which occurs during the manufacture of cheese. The properties of caseins vary greatly from those of whey proteins. The defining difference between caseins and whey proteins is the solubility in milk: caseins are insoluble at pH 4.6, whereas whey proteins are soluble. Due to their insolubility, caseins exist as micelles in milk (P. F. Fox & Brodkorb, 2008). Other physical differences can be explained by differences in amino acid composition: caseins lack α- and β-structures due to their high proline content, and are phosphorylated, leading to insolubility, heat stability, the ability to bind metals, and molecular charge; whereas whey proteins are highly structured and generally non-phosphorylated. Another important differentiating characteristic is the sulphur levels in casein and whey proteins. Caseins are low in sulphur, with the low sulphur content of 0.8% coming from methionine, which is unable to form disulphide bonds. Whey proteins are much higher in sulphur content (1.7%), with cysteine and cystine contributing to those levels (Boland, Singh, & Thompson, 2014).

Table 1.2: The composition of proteins in bovine milk (Farrell et al., 2004).

Protein	Concentration (g/L)
α_{s1}-Casein (α-CN)	12-15
α_{s2}-Casein (α-CN)	3-4
β-Casein (β-CN)	9-11
κ-Casein (κ-CN)	2-4
β-Lactoglobulin (β-LG)	2-4
α-Lactalbumin (α-LA)	0.6-1.7
Bovine serum albumin (BSA)	0.4
Immunoglobulins (IGs)	0.45-0.75
Secretory component	0.02-0.1
Lactoferrin (LF)	0.02-0.1

With such a variety of components, it is not surprising that milk has many biological functions. The majority of milk is water, therefore can be an important source of hydration, especially for neonates. Another function of the water is to solubilise the salts, lactose and proteins. Milk exists as an emulsion, with lipids dispersed as globules stabilised by a complex outer structure comprising of proteins and phospholipids (Bracco, Bohren, & Hidalgo, 1972).

The biological function of protein in milk is to provide essential amino acids, amino groups and energy for the growth of the neonate. Due to its abundance of nutrients, milk has been adopted as part of adult human diets (Itan, Powell, Beaumont, Burger, & Thomas, 2009). Milk is important in providing a source of calcium for adolescents and women (Fisher & Dodds, 1958; Soroko, Holbrook, Edelstein, & Barrettconnor, 1994), and the benefits of protein in milk are also important for older adults, in order to increase protein production and prevent sarcopenia (Beasley, Shikany, & Thomson, 2013; Boirie, Morio, Caumon, & Cano, 2014; Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2006; Wall, Cermak, & van Loon, 2014). Milk proteins also include bioactive peptides, aiding brain, heart and immune function (Korhonen, 2009).

1.2.2 Whey proteins

1.2.2.1 Health benefits of whey proteins

Whey protein is the subject of many studies investigating various health benefits. The most commercially applied of these is the basic nutritional provision of essential amino acids for muscle synthesis. The synthesis of muscle requires a source of amino acids; however some protein compositions are a more suitable source than others (Wolfe, 2000). The individual amino acids in a protein structure can influence the utilisation of that protein in muscle

synthesis, of particular interest is leucine, which acts as a nutritional signal for muscle metabolism (Anthony, Anthony, Kimball, & Jefferson, 2001). Whey protein has a high proportion of leucine, ~10%, and therefore stimulates muscle anabolism (Norton, Wilson, Layman, Moulton, & Garlick, 2012). The timing of protein consumption can also maximise bioavailability, which has led to the use of whey proteins in the sports industry before or after exercise (Tipton et al., 2007; Wolfe, 2000). Another commercial application of whey proteins is increasing protein intake in older adults to prevent sarcopenia, due to studies showing that whey protein had a significantly higher impact on protein gain than caseins for older adults (Dangin et al., 2003).

The health benefits of whey protein are not limited to the nutritional value of amino acids in proteins however. Whey proteins have been linked to: reducing blood pressure (Fluegel et al., 2010); having anticarcinogenic effects (Tsuda et al., 2000); modulation of the immune system (Gill, Doull, Rutherfurd, & Cross, 2000); antimicrobial activity (Bruck, Graverholt, & Gibson, 2003; Micke, Beeh, Schlaak, & Buhl, 2001); and reducing the effects of anxiety and stress (Booij, Merens, Markus, & Van der Does, 2006; Yamauchi, Wada, Yamada, Yoshikawa, & Wada, 2006).

1.2.2.2 Processing of whey

Whey is a side-product of the cheese making process, and can take one of two forms. If rennet is used for milk coagulation, then sweet whey is produced (pH > 5.6), whereas if acid is used for coagulation, acid whey is produced (pH < 5.1). Liquid whey obtained from the cheese making industry contains many other constituents including salts, minerals, lactose, fats, and water, which need to be removed in order to concentrate the whey. Current methods for the separation of whey use membrane filtration: ultrafiltration, microfiltration, nanofiltration and reverse osmosis. During membrane filtration the sample is passed through a semi-permeable membrane, which prevents insoluble and high molecular weight compounds from passing through, the molecular weight cut-off depends on the pore size of the membrane. Ultrafiltration rejects larger molecules such as proteins, fats and insoluble salts, which remain in the retentate. During microfiltration the whey proteins pass through the membrane into the permeate, whereas bacteria and particulates are removed by the membrane. Nanofiltration removes molecules with a molecular weight below ~ 1 kDa, and can remove minerals and lactose from whey. During reverse osmosis only water passes through the membrane (Wagner, 2001).

Whey is commercially available in two main forms: whey protein concentrate (WPC) and whey protein isolate (WPI), which are often sold as spray-dried powders to extend shelf-life. Spray drying is a process in which a liquid spray is exposed to a hot dry gas causing the rapid evaporation of water from the droplet surfaces to create a dry powder. The latent heat of

evaporation causes the droplet contents to remain at a low enough temperature to minimise thermal degradation (Deis, 1997).

The majority of dairy products undergo a form of heat treatment to eliminate pathogens and to ensure the safety of the final product. While the heat treatment is essential to ensure safety; thermal processing can alter the functional properties of dairy produce, lowering its nutritional value. There are many competing mechanisms occurring when milk is exposed to heat including chemical reactions, denaturation, and aggregation. The combination of conditions such as time, temperature and pH determine the type and extent of changes that occur.

The basic stages of an example process for whey protein concentrate are outlined in Figure 1.1, showing stages where potential denaturation could occur. Sweet liquid whey is firstly filtered using ultrafiltration at low temperature. A large number of membranes are used to filter proteins and remove fats and salts. This is a key step in determining protein content. The whey is then pasteurised by rapidly heating to 72 °C for a few seconds to destroy pathogens, this is not enough time for denaturation to occur. Reverse osmosis is then used to concentrate the liquid at low temperatures. Evaporators are then used to concentrate the whey protein liquid at temperatures around 65 to 77 °C, this stage is a potential source of denatured protein in the final product. A spray drier is then used to create a powder; due to the nature of spray drying, the temperature inside the droplets containing whey particles will not reach temperatures sufficient to cause denaturation. The final stage is a fluid bed, where emulsifiers such as soya lecithin can be added. The fluid bed can reach temperatures over 100 °C for up to 20 minutes, posing a significant risk of protein denaturation.

Figure 1.1: Flow diagram outlining processing of whey protein concentrate from sweet liquid whey. Stages where denaturation is a potential risk are highlighted in red. These are stages where temperatures may exceed 70 °C for an extended period of time. This is an example of a process used to produce whey protein concentrate, and may vary between manufacturers.

Bleaching is used to improve the overall aesthetic of some whey protein powders, particularly those produced from certain cheeses such as cheddar, using a bleaching agent such as hydrogen peroxide or benzoyl peroxide. The acceptability of appearance must also be weighed against an

acceptable flavour, as the process of bleaching can cause an increase in off-flavours in whey protein (A. J. Fox, Smith, Gerard, & Drake, 2013; Jervis et al., 2012) and denaturation of the whey proteins (Cooney & Morr, 1972; Marshall, 1986).

Changes to the chemical composition of processed whey protein powders continues throughout storage of the product. The functional properties of whey protein powders have been found to change significantly over 6 months of storage, including the solubility, emulsifying and foaming properties, and browning of the powder. These changes are influenced by the level of caseins present, calcium and lactose content, storage temperature, and water activity (Gazi & Huppertz, 2015; Hsu & Fennema, 1989; Lichan, 1983). Off-flavours develop during the storage of whey protein over 8 months. The off-flavours originate from volatiles formed through: lipid oxidation, such as 1-octen-3-ol; degradation of amino acids, such as dimethyl disulphide; and fermentation, such as diacetyl from the fermentation of citrate by lactic acid bacteria (Issa & Qian, 2008; B. J. Wright, Zevchak, Wright, & Drake, 2009).

All of these processes contribute to the overall functional and sensory characteristics of a whey protein powder, and as such are important considerations when designing the manufacture process of a product.

1.2.2.3 Whey protein structures and stability

Table 1.3: Typical composition of sweet whey proteins derived from bovine milk with percentage composition, molecular weight and isoelectric points (Etzel, 2004).

Protein	Content (%)	Molecular weight (kDa)	Isoelectric point
β-Lactoglobulin (β-LG)	48−58	18	5.4
α-Lactalbumin (α-LA)	13−19	14	4.4
Glycomacropeptide (GMP)	12−20	8.6	< 3.8
Bovine serum albumin (BSA)	6	66	5.1
Immunoglobulins (Ig)	8−12	150	5−8
Lactoferrin (LF)	2	77	7.9
Lactoperoxidase (LP)	0.5	78	9.6

Although whey proteins are all soluble proteins from milk, the individual whey proteins are diverse in structure with a range of molecular weights from 8.6 kDa to 150 kDa. Proteins are chains of amino acids, which can form complex folded structures due to intramolecular forces such as sulphur bridges and hydrogen bonds.

A protein's structure can be described using four main categories. The primary structure refers to the order of amino acids in the protein chain. The secondary structure describes the formation of regular structures held together by hydrogen bonding, the most common of these are α-helices, loose coils of amino acids; and β-sheets, folded portions of the chain aligning either parallel or anti-parallel to each other. The overall 3-dimensional shape of the protein is described as the tertiary structure, stabilised by complex combinations of ionic interactions, hydrogen bonding, van der Waals forces, and sulphur bridges. The combination of more than one unit, for example two polypeptide chains, is the quaternary structure of the protein.

The major component of bovine whey protein is β-Lactoglobulin (β-LG), which comprises approximately 50% of the total protein content. β-LG is a lipocalin, a group of proteins that have the ability to bind small hydrophobic molecules (Flower, 1996). β-LG is made up of 162 amino acid residues and has a molecular weight of 18 kDa (Sawyer, 2013), and often exists as a dimer or as an octamer depending on pH, concentration and temperature (Verheul, Pedersen, Roefs, & de Kruif, 1999). β-LG contains two disulfide bonds, and a free but unexposed thiol group (Brownlow, Cabral, et al., 1997).

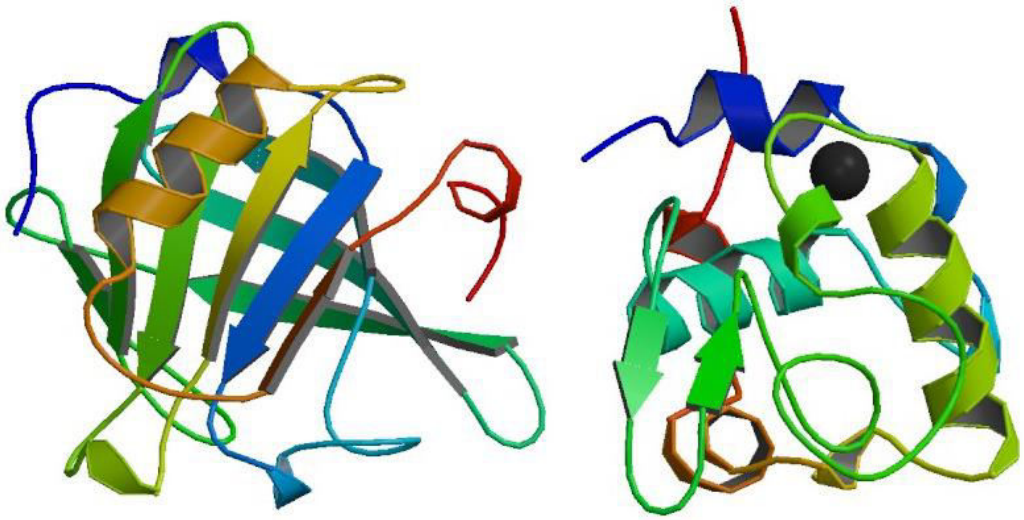

Figure 1.2: Crystal structures of A: bovine β-Lactoglobulin (Brownlow, Morais Cabral, et al., 1997); B: bovine α-lactalbumin (Pike, Brew, & Acharya, 1996).

The next most abundant whey protein in bovine milk is α-lactalbumin (α-LA), a globular protein comprised of 123 amino acid residues with a molecular weight of 14.2 kDa. Bovine α-LA strongly binds cations including Ca^{2+} and K^{+}, and is stabilised by four disulfide bonds (Permyakov & Berliner, 2000). Unlike β-LG, α-LA has no free thiol groups.

Other components of bovine whey include glycomacropeptides (GMP), bovine serum albumin (BSA), immunoglobulin G (IgG), lactoferrin (LF), and lactoperoxidase (LP), the properties of which are summarised in Table 1.3.

Denaturation of a protein refers to the loss of the protein's quaternary, tertiary and secondary structures, resulting in the loss of biological function. The application of heat provides enough energy to break the weakest intramolecular forces thereby resulting in a loss of structure. The large variation in the structure of the whey proteins is reflected in the denaturation behaviour. The most abundant protein in bovine whey, β-LG, has a critical temperature of denaturation of 70 °C (Dewit & Swinkels, 1980), with aggregation occurring when temperatures over 70 °C are sustained (Iametti, DeGregori, Vecchio, & Bonomi, 1996). Above 70 °C, β-LG dimers dissociate, and the protein molecules begin to unfold revealing a free thiol group and hydrophobic residues (Iametti et al., 1996; Qi, Brownlow, Holt, & Sellers, 1995). The free cysteine and disulphide bridge which are exposed, are otherwise shielded from the solvent by an α-helix (Zeiler & Bolhuis, 2015).

Thermal denaturation occurs at a relatively low temperature for α-LA (35 °C), although this increases when bound to calcium. In the presence of available cysteine groups of β-LG or BSA, α-LA forms large oligomers upon denaturation due to disulphide bonding (Havea, Singh, & Creamer, 2001). The denaturation temperatures for the other minor whey proteins are contained in Table 1.4, and range from 35-72 °C.

Table 1.4: Denaturation characteristics for bovine whey proteins under thermal, pressure-driven, and chemical denaturation. (Mazri, Ramos, Sanchez, Calvo, & Perez, 2012; Mazri, Sanchez, Ramos, Calvo, & Perez, 2012; Relkin, 1996; Vermeer & Norde, 2000). Common chemical denaturants are listed for the three most abundant whey proteins (TFE: 2,2,2-trifluoroethanol; GdmCl: guanadinium chloride).

Protein	Denaturation temperature (°C)	Denaturation pressure (MPa)	Common chemical denaturants
β-LG	70	50	TFE, urea, GdmCl
α-LA	35	200	TFE, proteinase-K,
BSA	65	800	Urea, GdmCl
IgG	61	300	-
LF	72	200	-
LP	68	>700	-

Another important factor providing stability for proteins are hydrophobic interactions, the repulsion of water by hydrophobic amino acid residues. The application of pressure can change the structure of water around proteins and weaken hydrophobic interactions with the protein, which results in denaturation (Grigera & McCarthy, 2010). Pressure-driven denaturation of whey proteins occurs at a range of pressures at room temperature, from 50 MPa to over 700 MPa, as summarised in Table 1.4. Chemical denaturation varies depending on the specific denaturant involved, however influential mechanisms include reducing the hydrophobic effect, binding to amino acid moieties, and weakening hydrogen bonding. Some common chemical denaturants for β-LG, α-LA, and BSA are outlined in Table 1.4. The aggregation of isolated whey proteins, in particular β-LG, has been well studied (Elofsson, Dejmek, & Paulsson, 1996; Mehalebi, Nicolai, & Durand, 2008); however when heating whey proteins as a mixture, different denaturation and aggregation behaviour is observed. When heated in the presence of other whey proteins, β-LG forms both homopolymers and heteropolymers (Havea et al., 2001), and in the presence of caseins, large micelles can be formed upon aggregation (Havea, 2006).

1.2.3 Sensory attributes of whey protein

Whey proteins are becoming an increasingly popular functional food, due to associated health benefits such as the provision of amino acids essential for muscle synthesis (Norton et al., 2012). Whey proteins have been widely utilised in sports nutrition (Wolfe, 2000), the prevention of sarcopenia in elderly and malnourished patients (Dangin et al., 2003), and in a newly developing market for general health and lifestyle products (Chungchunlam, Henare, Ganesh, & Moughan, 2014; Fekete, Givens, & Lovegrove, 2013). The successful use of whey proteins as an aid to muscle growth depends on a consistent intake over an extended period of time (Rahemtulla et al., 2005); therefore the sensory properties of whey protein beverages are of significant importance to ensure a sufficient consumption of protein is achieved.

Sensory profiling of whey proteins often includes attributes such as bitter taste, cooked milk, sweet aromatic, soapy, brothy, and metallic. Undesirable sensory attributes present in whey protein have been described as cabbage, cardboard, and astringent (Croissant, Kang, Campbell, Bastian, & Drake, 2009; Whetstine, Croissant, & Drake, 2005; J. M. Wright, Whetstine, Miracle, & Drake, 2006). Childs and Drake (2010) reported that flavours were more objectionable to consumers than astringency, however suggested that both should be the focus of future studies.

1.2.3.1 Mouthfeel of whey protein fortified foods and beverages

Older adults have a heightened awareness of texture, and a tendency to experience a dry mouth sensation (Narhi, 1994; Song, Giacalone, Johansen, Frost, & Bredie, 2016). Studies have shown that the mouthfeel of whey protein beverages contributes to the disliking and, therefore, refusal of whey protein beverages; in one study the textural properties were the main reported reason for 19% of trial discontinuations in older adults (of the 56% who completed the questionnaire) (Gosney, 2003). While many of these beverages also contain casein, whey protein has been shown to contribute more to drying sensations (Withers, Lewis, Gosney, & Methven, 2014). In order to reduce non-compliance, the mouthfeel properties responsible must be addressed. One major textural aspect of whey proteins is astringency, which was described as a 'textural defect' of dairy products in a 1994 review (Lemieux & Simard). Mouthdrying has been found to be perceived to a greater extent by older adults than younger adults (Withers, Gosney, & Methven, 2013). Astringency in whey protein is an issue which many are trying to combat; Wang, Tan, Mutilangi, Plans, and Rodriguez-Saona (2016) developed a portable infrared method for predicting astringency in acidic whey protein beverages.

Drying has also been observed in casein (Harwalkar & Elliott, 1971) with breakdown products of proteolysis as a proposed mechanism (Harwalkar, Boutinmuma, Cholette, McKellar, & Emmons, 1989). More recently, Withers et al. (2014) suggested a change in structure was responsible for casein drying, with larger aggregates observed for the more drying calcium caseinate than sodium caseinate. Casein was also found to bind to the oral mucosa, which could be a potential cause for drying (Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013).

The use of the terms *drying* and *astringency* are often seen as interchangeable, however astringency can be used to cover a range of different mouthfeel sensations (Gawel, Oberholster, & Francis, 2000), or to specifically refer to "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM, 2004), which are not present in whey proteins. In this thesis the observed sensation of the drying of the mouth will simply be referred to as drying, whereas astringency refers specifically to the puckering of the cheeks.

The microstructure of whey protein-rich gels has been shown to influence the chalkiness, adhesiveness and mouthcoating attributes (Cakir et al., 2012). Chalkiness, adhesiveness, and mouthcoating were associated with particulate, coarse stranded and carrageenan continuous structures. The use of fermented whey in yoghurts can alter mouthfeel attributes such as thick, grainy, smooth, softness, stickiness, body and meltdown (Gallardo-Escamilla, Kelly, & Delahunty, 2007; Pang, Deeth, Prakash, & Bansal, 2016; Torres, Janhoj, Mikkelsen, & Ipsen, 2011). Torres et al. (2011) found discrimination for all texture attributes except for

'flourychalky' between experimental factors and their interactions, including: protein concentration, fat content, particle size and degree of protein denaturation. Pang et al. (2016) observed that the addition of whey protein isolate to yoghurt increased thickness and stickiness but reduced smoothness. The addition of whey protein to sauces was investigated, finding the addition of whey protein to tomato sauces increased grainy, oily and pieces mouthfeel attributes. When added to white sauces whey protein altered mouthfeel attributes such as thick, smooth, glutenous, mouthcoating and mouthdrying (Tsikritzi et al., 2015).

1.2.3.2 Flavour of whey protein fortified foods and beverages

The flavour imparted by whey protein is an important consideration when fortifying foods and beverages. Russell, Drake, and Gerard (2006) developed a lexicon for whey protein based on 21 whey protein samples and an additional 29 soy protein samples. The flavour attributes developed were: sweet aromatic, brothy, metallic/meat serum, animal/wet dog, pasta/doughy, cardboard/wet brown paper, soapy, fruity, catty, dirty/soil, yeasty.

The addition of whey protein to white sauces increased the vegetable soup odour (Tsikritzi et al., 2015). Degree of cabbage odour was shown to rely on the type of microparticulated whey protein when types with different particle size and denaturation degree were added to yoghurt (Torres et al., 2011).

Sweet, acid and natural yoghurt flavour were altered by the addition of polysaccharides to whey protein enriched yoghurts, while milky, citrus, rancid, and salty were not significantly altered upon the addition of the polysaccharides chosen: carboxymethyl cellulose, high-methoxy pectin, propylene glycol alginate, and xanthan gum (Gallardo-Escamilla et al., 2007).

The processing of whey protein can result in different sensory profiles. Sweet aromatic, cardboard, cabbage/brothy and soapy were assessed for changes upon acidification of spray dried whey protein, with changes in all dependent on both the pH of samples upon sensory evaluation and the pH of the sample during spray drying (Park, Bastian, Farkas, & Drake, 2014a). A decrease in spray drying temperature increased cardboard flavour (Park, Bastian, Farkas, & Drake, 2014b). The effect of sonication on sensory properties of whey protein was investigated, with flavour attributes developed by a screened panel of: animal, bitter, brothy, cardboard, cereal, chalky, fecal, flour paste, fruity, malty, metallic, pasta, roasted, salty, soapy, sour, sweet, and yeasty (Martini & Walsh, 2012). The source of whey protein can impact the flavour attributes, with whey from cottage cheese having a potato/ brothy flavour, mozzarella whey having a higher sweet aromatic intensity, and cheddar whey being high in cardboard aroma (Smith, Foegeding, & Drake, 2016).

A bitter taste has been recorded in some whey proteins products, particularly in hydrosylated whey (Martini & Walsh, 2012; McGugan, Larmond, & Emmons, 1979; Whetstine et al., 2005; White, Fox, Jervis, & Drake, 2013; J. M. Wright et al., 2006). Whey protein hydrolysate has a distinct bitterness which is a key area of research to reduce in order to increase palatability (Leksrisompong, Gerard, Lopetcharat, & Drake, 2012; Spellman, O'Cuinn, & FitzGerald, 2005).

1.2.4 Mechanisms of protein derived mouthdrying

The nature of the drying sensation elicited by whey proteins is currently unknown, although there have been mechanisms proposed in the literature. As many commercially available whey protein beverages are low pH, the inherent astringency of acidity caused by ionic interactions with salivary proteins (Thomas & Lawless, 1995) not the whey proteins themselves, was suggested as the origin of whey protein beverage drying by Lee and Vickers (2008).

Sano, Egashira, Kinekawa, and Kitabatake (2005) suggested the precipitation of whey protein isolate at its isoelectric point causes astringency in the mouth; the isoelectric point for β-LG is 5.4 (Table 1.3), at which point the repulsive forces between charged protein particles will be lost, and precipitation occurs. A correlation between lowering the pH of whey protein solutions and an increase in both turbidity and drying, was linked to interactions between positively charged whey proteins at low pH and negatively charged salivary proteins (Beecher, Drake, Luck, & Foegeding, 2008). Vardhanabhuti, Kelly, Luck, Drake, and Foegeding (2010) observed that low pH whey protein beverages were more drying than equivalent pH buffer solutions, and therefore charge interactions between whey proteins and salivary proteins contribute to drying.

Interactions between β-LG and salivary proteins at low pH relate to larger particle size and an increase in turbidity, which correlated with increased astringency (Ye, Streicher, & Singh, 2011). The observation that at very low pH (~2.0) no interactions and a lower astringency were observed, was explained by the positive charge on both whey protein and salivary proteins, indicating that the interaction itself contributes towards astringency. Ye et al. (2011) concluded that the involvement of saliva in the precipitation of protein is integral to the sensation of astringency.

A variation of this theory proposes the disruption of salivary structure as the cause for astringency, through the lack of a continuous lubricative layer (Gibbins & Carpenter, 2013). These mechanisms based on the low pH of whey protein beverages do not account for the drying observed in neutral beverages such as oral nutritional supplements, and therefore other mechanisms must be considered in order to account for the drying elicited by whey protein at neutral pH.

1.2.4.1 Mucoadhesion in astringency and mouthdrying

Astringency is a persistent sensation (Courregelongue, Schlich, & Noble, 1999), which would agree with a mucoadhesive mechanism prolonging the oral exposure and therefore the sensation. Mucoadhesion is the adherence of materials to mucosal membranes, which in this context is the proposed binding of whey proteins to the oral mucosa: the cheeks, gums and tongue. Mucoadhesion occurs via intermolecular forces (electrostatic attraction, hydrophobic interactions and hydrogen bonding) and some covalent bonding such as disulphide bond formation (Andrews, Laverty, & Jones, 2009; Smart, 2005; Sosnik, das Neves, & Sarmento, 2014). A detailed review of mucoadhesion can be found in Chapter 1 Part 2. Mucoadhesion has been proposed as a mechanism for astringency derived from stimuli other than dairy proteins.

Polyphenols are thought to produce an astringent sensation by the binding to salivary proteins (Bajec & Pickering, 2008; Gambuti, Rinaldi, Pessina, & Moio, 2006; Nayak & Carpenter, 2008), forming large aggregates (Jobstl, O'Connell, Fairclough, & Williamson, 2004). The cause of astringent sensations by polyphenols is thought to be the binding of polyphenols to the mucins which are bound to mucosal cells, as an increase in saliva flow reduces the astringent response (Nayak & Carpenter, 2008). The binding of polyphenols to mucins forms complexes (Quintero-Florez, Sanchez-Ortiz, Martinez, Marquez, & Maza, 2015), and this process leads to increased mucin sedimentation, aggregation and viscosity, disrupting the salivary mucin network (Davies et al., 2014). Although it is not yet an accepted mechanism, Gibbins and Carpenter (2013) alluded to mucoadhesion as the cause of astringency in a recent review. The interactions of polyphenols with the oral mucosal pellicle and mucosa below is suggested as a cause for the loss of lubrication and increase in friction in the mouth. These findings appear to mirror the mechanism for astringency in polyphenols, as the disruption of the saliva results in an astringent or drying sensation. However, while the interactions between polyphenols and saliva are through hydrophobic interactions and hydrogen bonding (Bennick, 2002); the nature of interactions between whey protein and saliva is yet to be defined.

Chitosan, a mucoadhesive polysaccharide, has been found to elicit an astringent sensation when adsorbed to the oral mucosa (Yakubov, Singleton, & Williamson, 2014). However, not all mucoadhesives cause an astringent response: carboxymethyl cellulose (CMC), a known mucoadhesive, has been found to reduce astringency in soymilk. It was suggested by Courregelongue et al. (1999) that the addition of CMC to soymilk restored salivary lubrication and reduced astringency by binding to astringents, lowering their ability to bind salivary proteins. In a more recent study, the ability of CMC to reduce astringency was more effective than guar gum, xanthan gum or Arabic gum; however all produced the highest response at the highest concentration tested (Troszynska et al., 2010).

Mucoadhesion has been observed in whey protein, leading to its utilisation in drug delivery (Deat-Laine et al., 2013; Rubi Serrano-Cruz, Villanueva-Carvajal, Morales Rosales, Ramirez Davila, & Dominguez-Lopez, 2013). Withers, Cook, et al. (2013) found that milk proteins, β-LG and caseins, bound to porcine oral mucosa *in vitro*, and suggested that this could be the cause of perceived drying in milk protein beverages. Another recent study found structural changes upon mixing β-LG with bovine submaxillary mucin, as observed by nuclear magnetic resonance, circular dichroism, and dynamic light scattering, showing binding between the milk proteins and mucins, which suggests mucoadhesive interactions (Celebioglu et al., 2015). The effect of the mucoadhesion of β-LG on the sensory perception of "astringency" or "drying" is proposed to be the loss of lubrication of saliva, as observed by tribology (Celebioglu, Gudjonsdottir, Chronakis, & Lee, 2016; Vardhanabhuti, Cox, Norton, & Foegeding, 2011). It is proposed that the hydrophobic regions of β-LG interact with the termini of mucin at the air-liquid interface, whereas the hydrophilic regions interact with the glycosylated region of mucin in the bulk liquid (Celebioglu, Kmiecik-Palczewska, Lee, & Chronakis, 2017).

The build-up of drying over repeated consumption (Withers et al., 2014) has been attributed to interactions between whey proteins and the oral mucosa. Interactions between β-LG and mucin were demonstrated to correlate to an increased perception of astringency by Vardhanabhuti and Foegeding (2010), with spectroscopic studies proving these interactions were of mostly hydrophilic nature (Celebioglu et al., 2015). β-LG and LF were found to bind to human oral epithelial cells, indicating a adherence to the oral cavity (Ye, Zheng, Ye, & Singh, 2012).

A positive correlation was found between protein denaturation and mucoadhesive strength in drug delivery (Hsein, Garrait, Beyssac, & Hoffart, 2015). The unfolding of whey proteins during denaturation exposes hydrophobic regions and thiol groups (Iametti et al., 1996), which could therefore increase the strength of mucoadhesive binding. The denaturation of whey proteins has previously been linked to astringency (Josephson, Thomas, Morr, & Coulter, 1967); this may result from increased hydrophobic interactions or disulphide bonds with the oral mucosa which increases mucoadhesion, finally resulting in increased drying sensation (Hsein et al., 2015).

We hypothesise that particle size will increase upon denaturation due to aggregation, and that this will increase drying as has been seen for low pH whey protein (Ye et al., 2011). We hypothesise that the increase in particle size and change in protein structure will increase mucoadhesive strength, and cause retention of whey protein in the oral cavity, resulting in a persistence of drying. Evidence that mouthfeel alteration is elicited by mucoadhesion is limited, however using lubricating mucoadhesives has been shown to influence mouthfeel in milk protein samples. Gallardo-Escamilla et al. (2007) found that CMC and high-methoxy pectin

increased smoothness of fermented whey. The addition of polysaccharides could be considered as a mitigation strategy in the reduction of drying in whey protein fortified beverages.

1.3 Conclusions, hypothesis and objectives

Due to the beneficial uses of whey protein in the prevention of sarcopenia and in sports and lifestyle nutrition, the need for an improvement of texture properties is essential for the complete consumption of products. The build-up of drying in the mouth elicited by whey protein could influence compliance of patients consuming whey protein fortified beverages. The mechanism of drying elicited by whey protein is currently undefined, although interactions with saliva at low pH have been widely recognised and the potential link between whey protein drying and mucoadhesion has been established in the literature. The processing of whey protein can alter the sensory profile, and thermal denaturation of whey protein has been shown to increase mucoadhesive strength of whey protein used in drug delivery.

The majority of literature focuses on drying elicited by whey protein at low pH; however neutral pH whey protein beverages have also been shown to cause a drying sensation which increases with repeated consumption. There is also a lack of research on the effect of whey protein denaturation on drying.

We hypothesise that the thermal denaturation of model whey protein beverages will increase the intensity of the drying sensation, with an increase in build-up over repeated consumption. The hypothesised mechanism for this is an increase in particle size and a change in protein structure leading to stronger mucoadhesive forces, and therefore a longer retention of whey protein in the oral cavity.

To determine the effect of thermal denaturation on the persistence of whey protein mouthdrying, a sequential profiling technique, as used by Withers et al. (2014) will be employed. This temporal method will enable panelists to recreate the normal consumption of whey protein beverages. The aim of the sensory method is to determine whether each sip intensifies the drying sensation, and therefore a continuous method, such as time-intensity, is not required. The sequential profiling method allows each sip to be assessed during the consumption, and as after effects.

To measure the mucoadhesive strength, an *in vivo* oral retention method will be used. Alongside this, interactions between thermally treated whey protein and mucin will be assessed using a number of analytical techniques, to determine the nature of the mucoadhesive mechanism.

A mitigation strategy based on the mechanism suggested by the analysis of whey proteins and mucin mixtures will be developed and tested using sensory profiling. The use of

polysaccharides to compete for mucoadhesion could reduce the sensation of drying in the mouth, creating a more acceptable product for consumers.

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Chapter 1 Part 2. Mucoadhesion: A food perspective

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Abstract

The role of mucoadhesion in the perception and sensory characterisation of food products is becoming more apparent. Traditionally, mucoadhesives are used to enhance drug permeability and retention at mucosal membranes in the body, by adherence to a mucosal membrane formed through various interactions between the mucoadhesive and proteins present in the mucosa. Many polysaccharides used in the food industry as thickeners, emulsifiers, stabilisers and fat replacers also have mucoadhesive properties, and are commonly used in the pharmaceutical industry in drug formulations. More recently, there has been an increasing interest in utilising these polysaccharides as mucoadhesives to modulate the organoleptic properties of food. This review reflects on the recent developments in mucoadhesion and the limited research into the impact of mucoadhesion when designing food formulations and modifying the organoleptic properties of food. It will also outline the areas of food science that could benefit from an understanding of mucoadhesion, mainly focusing on developing an understanding of how mucoadhesion may explain results found from sensory studies involving polysaccharides. Furthermore, possible negative impacts of mucoadhesion in foodstuff will be explored. An overview of methods for the measurement of mucoadhesion is also provided. An understanding of the mucoadhesive nature of polysaccharides may be useful to the food industry with regard to new product design.

1.4 Introduction

Mucoadhesion has attracted a lot of attention in pharmaceutical research and the pharmaceutical industry, and is therefore well defined and effectively utilised within these fields. In the simplest terms, mucoadhesion is the adhesion of a polymeric material to a mucosal membrane in the body. The polymeric material, containing an active pharmaceutical ingredient (API), adheres to a target mucosa for an extended period of time compared to the API itself, thereby prolonging the API residence on mucosal surfaces, increasing permeation and thus bioavailability for certain APIs (Andrews, Laverty, & Jones, 2009). The importance and interest in developing mucoadhesive formulations has increased as more challenging drugs, such as peptides, proteins and oligosaccharides have been discovered and synthesised. These types of therapeutics are challenging for various reasons, such as their poor solubility, limited uptake, fast breakdown or short half-life. Furthermore, it may be necessary for certain drugs to bypass first pass metabolism and therefore alternative routes such as sublingual administration are sought. The systemic absorption of APIs through diffusion or transport across mucosal surfaces may be enhanced by the addition of mucoadhesives. This is termed polymer-mediated enhancement of API delivery. These controlled release formulations have been researched for many years and subsequently employed in a variety of pharmaceutical applications (Andrews et al., 2009; Khutoryanskiy, 2011, 2014; Knipe, Chen, & Peppas, 2015; Liechty, Kryscio, Slaughter, & Peppas, 2010; Peppas, Thomas, & McGinty, 2009; Salamat-Miller, Chittchang, & Johnston, 2005; Shaikh, Raj Singh, Garland, Woolfson, & Donnelly, 2011).

Mucoadhesives can be utilised in drug formulations to deliver APIs to a variety of target mucosal tissues. These include: the nasal route via sprays, gels and pumps; vaginal or urethral routes using suppositories, pessaries, vaginal rods and gels; and the oral route via buccal and sublingual patches, tablets and gels. One of the most commercially recognised formulations containing mucoadhesives is Gaviscon Liquid®. This product contains sodium alginate, a mucoadhesive polysaccharide, which gels in the presence of Ca^{2+} ions. Due to its mucoadhesive and gel forming abilities, this formulation is used to treat heart burn by coating the esophageal walls with the viscous, mucoadhesive gel, protecting it against the acid rising from the stomach (Richardson, Dettmar, Hampson, & Melia, 2004). The oral route for drug delivery includes targeting formulations to the buccal tissue in the mouth as well as the rest of the gastrointestinal tract (GI), including the esophagus, stomach, small and large intestine. Each of these routes of administration has different mucosal structures and a different secretory mucus composition, which will affect the mucoadhesive's strength of the dosage form.

The recognition and consequent extensive research of mucoadhesion in the pharmaceutical field has led to an excellent understanding of the mechanical, chemical and physical factors involved.

This has subsequently advanced the development of dosage forms, improving the delivery and efficacy of APIs. The ability of mucoadhesives to retain small molecules at mucosal surfaces may prove important to the food industry. The purpose of this review is to enhance the knowledge and understanding of how mucoadhesion may influence organoleptic properties and thus may be utilised in the design of healthier food products. This review will cover: a definition of mucoadhesion and an overview of the oral environment; examples of mucoadhesion in food substances; currently accepted methods for studying mucoadhesion; and influences that mucoadhesion could have on the food industry.

1.5 Mucoadhesion

1.5.1 Oral cavity mucosa

Before exploring the theories of mucoadhesion, a sound comprehension of the target mucosal tissue structure and characteristics is required. Mucosal tissues cover various organs, however since this review is concerned with the incorporation of mucoadhesives into food products, only the oral cavity will be discussed.

The anatomy and histology of the human oral cavity has been described extensively (for a comprehensive guide see Squier and Brogden (2011)), therefore only a brief overview will be provided. The oral mucosa is the moist membrane lining all surfaces of the oral cavity with the exception of the teeth (Figure 1.3). There are three different kinds of oral mucosa, each with characteristics that reflect the role and environment to which they are exposed. The masticatory mucosa is keratinised and covers the gingiva and hard palate. As the name suggests, this mucosa is responsible for masticatory processes and must therefore be tough as it is at risk of abrasions and potential infection from pathogen-harboring food. The rest of the oral cavity is covered with soft, non-keratinised epithelium, called the lining mucosa. The dorsal of the tongue is an exception to this, possessing a specialised mucosa with characteristics of both masticatory and lining mucosae. Mucosal surfaces all have a mucosal secretion, which, in the oral cavity, is the saliva. This is a relatively thin covering of mucus, compared to other areas of the body, between 1 and 100 μm thick (Collins & Dawes, 1987; Smart, 2005; Wolff & Kleinberg, 1998). This serves many roles similar to other mucosal secretions in the body as it protects tissues against mechanical and pathogenic stress. In addition to this, it serves many specialised roles necessary for speech, mastication, bolus formation and deglutition (Humphrey & Williamson, 2001).

The saliva is a highly aqueous solute consisting of around 95% water with the remainder comprising salts and proteins. Mucins are large glycoproteins of particular importance in establishing mucoadhesion, comprising approximately 1.2 mg/mL in healthy individuals (Kejriwal, Bhandary, Thomas, & Kumari, 2014). Mucins are responsible for the highly

viscoelastic nature of all mucosal secretions due to the formation of aqueous, gel-like networks. This viscoelasticity is important, serving as a barrier to foreign substances, slowing diffusion and inhibiting large molecules from penetrating. However, with regard to mucoadhesion, polymer chains that can penetrate into this mucus layer can interact with the mucin resulting in a continuous network of polymer and mucin interactions, strengthening a mucoadhesive joint. Mucins exist as both secretions in the saliva, as well as transmembrane mucins on epithelial cells, which are exposed to the oral cavity. Mucins are integral for the lubrication of the oral cavity, due to their water retaining capacity, enabling all the usual functions of mastication, swallowing and speech. Mucins enable saliva to serve many functions including: acting as a diffusion barrier for nutrients, pathogens and drugs; hydration of the underlying epithelia; and protection from chemical and mechanical damage (Amerongen, Bolscher, & Veerman, 1995; Humphrey & Williamson, 2001; Pedersen, Bardow, Jensen, & Nauntofte, 2002; Veerman, van den Keybus, Vissink, & Amerongen, 1996).

The molecular weights of mucins range from 500 kDa to 20 MDa, however they have a tendency to aggregate and form large supramolecules, driven by hydrophobic interactions of nonpolar groups and the hydrogen bonding of sugar units (Bansil & Turner, 2006). Generally speaking, all mucins are derived from a similar structure and will, to a certain degree, serve the same function of protecting the delicate underlying tissues. However, there is large heterogeneity and diversity between the complex structures of mucins (Mathiowitz, Chickering, & Lehr, 1999) influenced by the variation of the environments to which they are exposed.

Mucins found in the oral cavity can be divided into high-molecular-weight (MUC5B) and low-molecular-weight (MUC7) fractions (Schipper, Silletti, & Vinyerhoeds, 2007; Thomsson et al., 2002). MUC5B mucins are produced by all salivary glands except the parotid gland (Veerman et al., 1996) and has similar characteristics to mucin in other mucosal secretions in the body (Amerongen et al., 1995). The MUC5B mucins are one of the major mucins present in saliva and are associated with the gel-like formation of saliva, which is attributed to entanglements of these mucin molecules with one another (Schipper et al., 2007; Schulz, Cooper-White, & Punyadeera, 2013). The interactions thought to be important for this gel formation include; hydrophobic interactions between the hydrophobic regions of the core proteins (Bromberg & Barr, 2000), van der Waals and hydrogen bonds between oligosaccharide side chains and calcium-mediated crosslinks (Raynal, Hardingham, Sheehan, & Thornton, 2003). MUC7 mucins are thought to be uniquely found in salivary secretions (Amerongen et al., 1995), produced by the submandibular, sublingual and palatine glands (Bolscher et al., 1999).

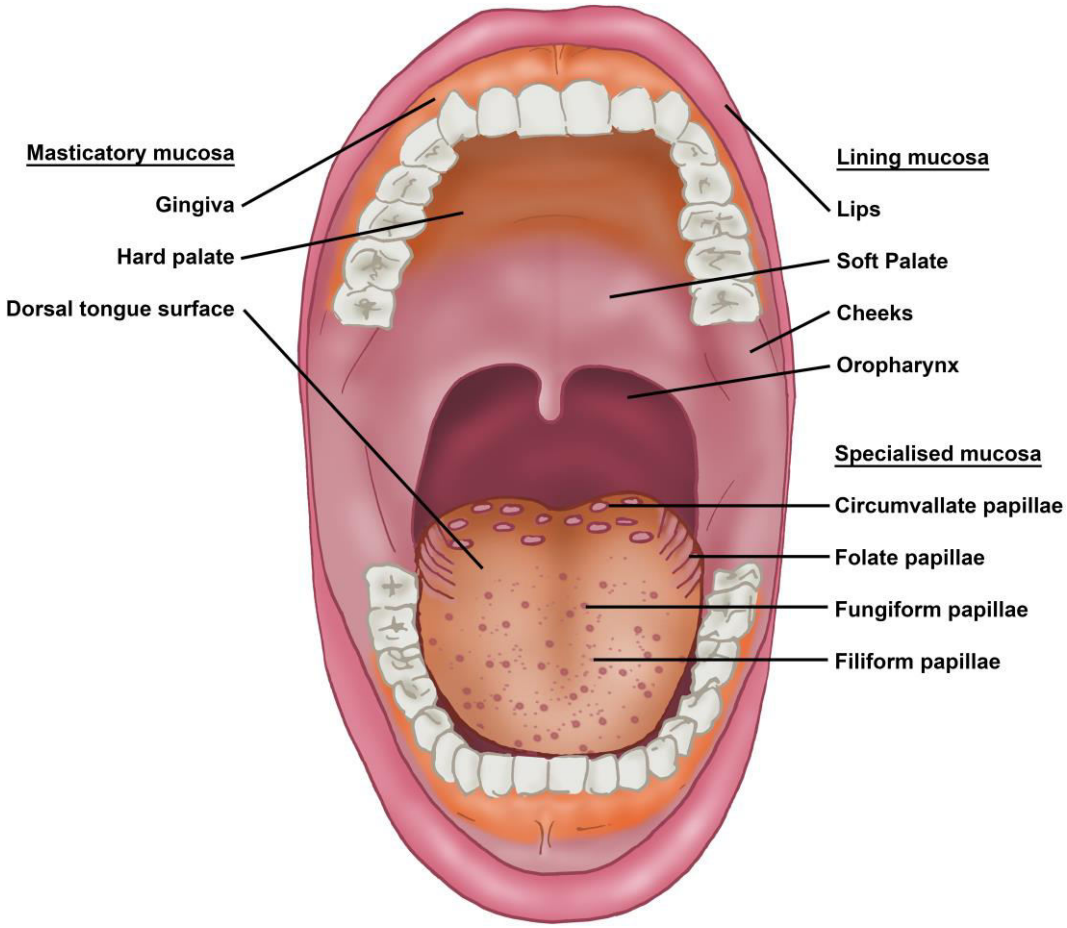

Figure 1.3: Oral mucosa and lingual papillae. Keratinised masticatory mucosa covers the gingiva, hard palate, and dorsum of the tongue. The non-keratinised lining mucosa covers the rest of the mouth surface including: the lips, cheeks, and soft palate. Regions of taste buds in the lingual papillae are covered by a specialised mucosa, containing nerve endings enabling sensory perception. There are four types of lingual papillae: circumvallate papillae are large dome-shaped papillae towards the rear of the tongue, which are surrounded by a serous secretion produced by adjacent Von Ebner's glands; foliate papillae are folds on the sides of the rear of the tongue, contain taste buds, and are covered by non-keratinised mucosa; fungiform papillae are found mostly on the front of the tongue, are covered by non-keratinised mucosa, and contain taste buds; filiform papillae are very small, keratinised, and are the most numerous papillae type, covering most of the dorsal surface, however they do not contain taste buds.

The protein core of mucin is glycosylated by many oligosaccharide side chains covalently linked in areas of clustered proline, threonine and serine (PTS) amino acids (Thomsson et al., 2002). These highly branched oligosaccharides contribute up to 80% of the dry weight of mucin. There is heterogeneity within and between mucin types and the saccharides that glycosylate them, with MUC5B possessing a more diverse range than MUC7 (Thomsson et al., 2002). The *O*-linked chains are initiated with *N*-acetylgalactosamine with up to 20 more residues extending from this. The large variations of sugar units that may be attached include, *N*-acetylglucosamine, *N*-acetylgalactosamine and other glucose, galactose and fructose derived

residues (Mathiowitz et al., 1999). The chains are terminated with sialic acid, sulfonic acid, or *l*-fructose residues, with the first two possessing a net negative charge at neutral pH (Gandhi & Robinson, 1994; Peppas & Sahlin, 1996). Recent studies have confirmed the presence of both types of salivary mucins in the mucosal pellicle that lines the oral epithelia (Morzel, Tai, Brignot, & Lherminier, 2014; Ukkonen et al., 2017). The pellicle is a biological film adhered to the epithelial cells which serves to protect the underlying tissue from abrasions and plays a role in bacterial colonization (Bradway, Bergey, Jones, & Levine, 1989). Mucin biochemistry and properties are covered in more depth elsewhere (Amerongen et al., 1995; M. T. Cook & Khutoryanskiy, 2015; Schulz et al., 2013; Thomsson et al., 2002).

1.5.2 Theories of mucoadhesion

Mucoadhesion occurs due to a range of physicochemical interactions between the polymeric material and the mucosal environment. The properties of the environment, such as the pH and flow rate of the mucosal secretion, will determine polymer-mucin interactions. Generally there are two stages considered to be essential in establishing mucoadhesion (Bodde, 1990; Duchene, Touchard, & Peppas, 1988). Firstly, the initial intimate contact between the polymeric material and the mucosal surface is required. Secondly, the consolidation period can ensue which reinforces the mucoadhesive bonding. There are six main theories of mucoadhesion, which have been proposed and evaluated in the literature (Derjaguin, Aleinikova, & Toporov, 1994; Derjaguin, Toporov, Muller, & Aleinikova, 1977; Gu, Robinson, & Leung, 1988; Huang, Leobandung, Foss, & Peppas, 2000; Jabbari, Wisniewski, & Peppas, 1993; Mikos & Peppas, 1989; Peppas & Buri, 1985). These include: adsorption, wetting, electronic, diffusion, dehydration and mechanical theories (Figure 1.4). These theories can be thought of as complementary, describing different phenomena that occur simultaneously or at different stages of the process, which facilitate mucoadhesion. The theories of mucoadhesion have been reviewed in detail multiple times (Andrews et al., 2009; Khutoryanskiy, 2011; Salamat-Miller et al., 2005; Shaikh et al., 2011; Smart, 2005) and therefore only an outline of the theories governing the interactions and mechanisms of these stages will be provided.

- **Wetting** theory is concerned with polymer spread and ability to swell on the wet mucosal surface. A higher affinity to spread on the mucosa results in stronger mucoadhesion. Typically, the wetting phenomena are important for liquid mucoadhesives.
- **Dehydration** theory describes the process where a material capable of gelling is brought into contact with a moist mucosal membrane. The movement of water from the mucus gel to the water-absorbing material reaches equilibrium and facilitates an adhesive joint. An example of this is the water uptake by a solid dosage form containing

a hydrophilic polymer, such as poly(acrylic acid), when placed on a moist surface. Once in contact with the wet mucosa, the dosage form will rapidly dehydrate the surface and adhesion will occur (Jabbari et al., 1993).

- **Diffusion** theory considers the entanglement of polymer and mucin chains due to interpenetration, allowing for further primary and potentially secondary bonds to form, strengthening the adhesion (Jabbari et al., 1993; Peppas & Buri, 1985).
- **Adsorption** theory considers interactions between the mucosal surface and polymer; including Van der Waals forces, hydrogen bonds, and hydrophobic interactions (Mikos & Peppas, 1989). These non-covalent interactions are likely to form the majority of interactions; however, covalent bonding is possible depending on the chemical properties of the polymer. Thiolated polymers can form disulfide bonds with cysteine groups in mucins via thiol exchange reactions, or the oxidation of free thiol groups (Bernkop-Schnurch, 2005). The protein backbone of some mucins contain large regions high in cysteine residues and low in oligosaccharides, which provide a potential area for strong chemical bonds to occur (Dekker, Rossen, Buller, & Einerhand, 2002).
- **Electronic** theory describes the transfer of electrons between the mucoadhesive and the mucus layer, resulting in the formation of a charged double layer at the interface of the mucin and polymer networks (Derjaguin et al., 1994; Derjaguin et al., 1977).
- **Mechanical** theory describes the effect of contact area on the interaction between the polymer and mucosal surface (Smart, 2014). The effect of this will be particularly relevant in the oral cavity, which has a very thin layer of saliva in some areas; therefore, the mucoadhesive is more likely to contact the rough underlying tissue. Irregular surfaces and micro-cracks give a larger contact area and thus mucoadhesive strength. The papillae on the tongue provide a suitably rough surface and therefore greater surface area for penetration by mucoadhesives.

Buccal mucoadhesion is extensively researched in the pharmaceutical field (Rossi, Sandri, & Caramella, 2005; Salamat-Miller et al., 2005) due to the ease of application and the ability to bypass the first-pass metabolism in drug delivery. Whilst this is an important area to consider, regarding relevance to the food industry, adhesion occurring on the tongue may be more revealing. There has been much interest in the interactions of food emulsions on the tongue including the adhesion exerted by emulsion droplets and how this corresponds to the lubricating properties of the system.

A body of work by Dresslehuis et al and Silletti et al have found that adhesion of emulsion droplets is dependent on the sensitivity to coalescence with a higher sensitivity resulting in a higher retention of fat in the mouth (de Hoog, Prinz, Huntjens, Dresselhuis, & van Aken, 2006; Dresselhuis, de Hoog, Stuart, Vingerhoeds, & van Aken, 2008; Dresselhuis et al., 2007; Dresselhuis, Stuart, van Aken, Schipper, & de Hoog, 2008; Dresselhuis, van Aken, de Hoog, & Stuart, 2008; Silletti, Vingerhoeds, Norde, & van Aken, 2007b; Vingerhoeds, Silletti, de Groot, Schipper, & van Aken, 2009). This body of work is important when considering the perception of fat in foods and when considering ways to reduce fat content whilst maintaining the lubricating mouthfeel.

Recent work suggesting that milk proteins bind to the tongue by mucoadhesive interactions has lead the authors to suggest that mucoadhesion plays a role in creating negative sensory attributes associated with milk products such as drying and astringency (Celebioglu et al., 2015; Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013). Conversely, this adhesion to the tongue may be useful for incorporating mucoadhesive polymers into food products to produce positive sensory results. An example of this application would be the utilisation of mucoadhesives to prolong the retention and consequent perception of tastants on the tongue.

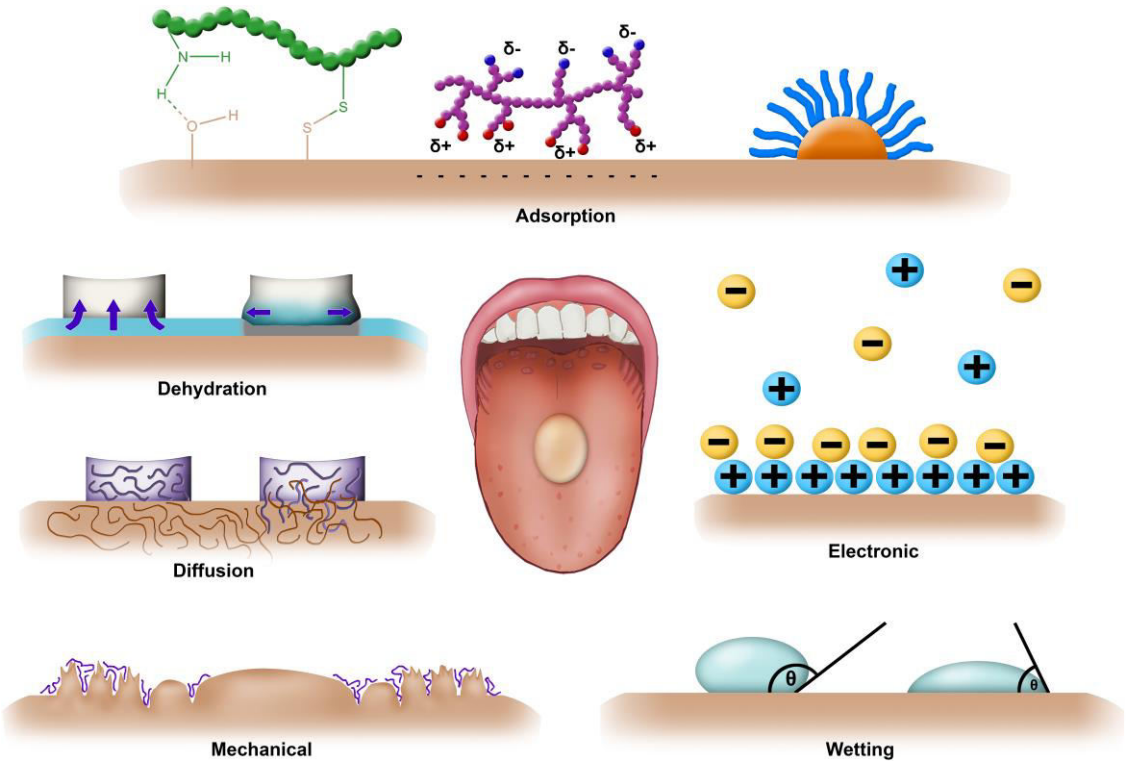

Figure 1.4: The six main mechanisms of mucoadhesion: adsorption, dehydration, diffusion, electronic, mechanical and wetting.Adsorption is depicted by: hydrogen bonding and covalent bonding via disulfide bonds between the mucoadhesive and the mucosa; dipoles interacting with negatively charged mucins; the hydrophobic core of a colloid interacting with the mucosa. The dehydration mechanism occurs when a mucoadhesive absorbs water from the mucosal surface, swelling, gelating and creating a strong mucoadhesive joint. The electronic theory describes the formation of an electronic double layer. The diffusion theory describes the entanglement of polymer chains and mucins. The mechanical theory describes polymers cumulating around physical irregularities in the surface, for example papillae. The wetting mechanism is concerned with the contact angle of a liquid mucoadhesive on the mucosal surface.

1.5.3 Properties of mucoadhesives

The extent of mucoadhesion that a particular polymeric material will exert is dependent on various factors including the size and physicochemical properties of the polymer and the environment in which it will reside. Polymer characteristics such as molecular weight and viscosity in solution show positive correlation with mucoadhesive strength (Chen & Cyr, 1970; D. Tiwari, Goldman, Sause, & Madan, 1999). The amount of initial bonds formed is dependent on the ability to diffuse into the mucus layer, therefore, higher polymer flexibility results in better diffusion into the mucus network and consequently stronger mucoadhesion (Gu et al., 1988). Along with flexibility, hydrogen-bonding moieties are essential for strong mucoadhesion, enabling interactions between the polymer and the mucin oligosaccharide hydroxyl groups (H. Park & Robinson, 1987). The ionic state of a polymer, which can influence the degree of mucoadhesion, is dependent on the pH of the medium in which it resides, which

varies among different mucosal environments. In the case of oral mucoadhesion the medium is saliva where the pH is typically between 7.0 and 7.5 or slightly acidic between 5.9 and 7 dependent on disease state (Fenoll-Palomares et al., 2004). Anionic polymers such as some polysaccharides possessing carboxyl groups will be partially negatively-charged at a near neutral pH; their strong mucoadhesive properties could be due to hydrogen bonding and Van der Waals forces (Peppas & Buri, 1985). Cationic polymers, such as chitosan, which possess amino functional groups (pK_a ~ 6.5), are also strong mucoadhesives. Due to the relatively high pK_a, chitosan forms a gel in acidic conditions, such as those found in the stomach. However, chitosan is insoluble at neutral pH, and therefore is suitable for oral delivery of APIs targeting the GI tract, as it is insoluble in saliva (Sogias, Williams, & Khutoryanskiy, 2008). Non-charged polymers such as starch or dextran generally exhibit poorer mucoadhesive properties compared to polyelectrolytes (Khutoryanskiy, 2011).

Thiolated polymers, which can be either cationic or anionic, form mucoadhesive bonds via disulfide bonding, therefore the concentration of thiolate ions is the key factor in forming mucoadhesive interactions. In situ cross-linking of thiomers could also contribute to their mucoadhesive properties, as disulfide bonds within the polymer, strengthening bonds made with the mucosa. Another important factor in determining thiomer mucoadhesive strength is the molecular mass of the polymer chains. A detailed review on thiomer mucoadhesion was provided by Bernkop-Schnurch (2005).

The concentration of polymer is an important consideration for optimum mucoadhesion. If the concentration is too low the interaction between polymer and mucin is unstable (Peppas & Buri, 1985), whereas too high will result in the polymer network being impervious to the solvent resulting in a lack free polymer chains to diffuse into the mucus interface, due to their highly coiled and compact structure (Salamat-Miller et al., 2005). Hydration of the polymer chains within the mucus layer is influenced largely by the concentration and is required for the polymer to expand and form a network with the mucus to form a strong adhesive joint. Salivary flow and constituents can vary considerably between individuals (Fenoll-Palomares et al., 2004) and therefore may explain some of the variability in mucoadhesion test results obtained in the literature, as the hydration of the dosage form and the solutes in the solvent will impact mucoadhesive strength (Stecker, Swift, Hodges, & Erickson, 2002; Yehia, El-Gazayerly, & Basalious, 2008).

1.6 Mucoadhesives and food

The purpose of this review is to introduce the relevance of mucoadhesion in sensory perception and nutritional quality of food products and discuss the potential impact. The mucoadhesive properties of food ingredients may be important in explaining perceptual changes when

redesigning food. Although mucoadhesion *per se* is seldom investigated as an influencing factor to explain outcomes reported within food and sensory science research, attributes such as mouthcoating, stickiness and creaminess are more than likely pertaining to this phenomenon. The phenomenon is becoming increasingly recognised and investigated in the literature (Juan-Mei & Shao-Ping, 2016; Malone, Appelqvist, & Norton, 2003a) and has been implicated in considering the astringency of tannins (Gibbins & Carpenter, 2013) and drying nature of milk proteins (Withers, Gosney, & Methven, 2013). Furthermore, Silletti and Dresselhuis have studied interactions between emulsions and the oral cavity with regard to the adhesive interactions (Dresselhuis, de Hoog, Stuart, & van Aken, 2008; Dresselhuis et al., 2007; Dresselhuis, van Aken, et al., 2008; Silletti et al., 2007b; Silletti, Vingerhoeds, Van Aken, & Norde, 2008). Mucoadhesion can be utilised for the delivery of nutrients to the body, much like in pharmaceutical delivery, whereby the prolonged exposure by mucoadhesion of bioactive components in food can increase absorption in the GI tract. The use of mucoadhesives for the enhanced delivery of bioactives and nutrients in foods has been documented and discussed several times (Cirillo, Spizzirri, & Iemma, 2015; Garti, 2008; Sabliov, Chen, & Yada, 2015) and this review will not go into detail of this area as there is a large overlap with pharmaceutical drug delivery findings. This review aims to propose mucoadhesion as a mechanism important to the food industry, aside from nutraceutical delivery.

This review is not intended as a replacement for existing theories or assumptions, rather a consideration that may provide further explanation for observations. More precisely, this section of the review will explore: mucoadhesive polysaccharides that are currently used in the food industry for properties besides mucoadhesion; potential benefits of mucoadhesives, including prolonged flavour delivery and improved texture of manufactured products; and potential drawbacks of mucoadhesion in food products.

1.6.1 Mucoadhesive polysaccharides

Polysaccharides are commonly used in the food industry as thickeners, gelling agents, stabilisers, emulsifiers, and binders. They are most commonly used in liquid or semi-solid dairy products, meat products, sauces and confectionary (Gidley & Reid, 2006). The impact of adding polysaccharides to food products to the structure and sensory perception of a food product is gathering interest (Boland, Delahunty, & van Ruth, 2006; Gonzalez-Tomas, Bayarri, Taylor, & Costell, 2008; Koliandris, Lee, Ferry, Hill, & Mitchell, 2008; Tromelin, Merabtine, Andriot, Lubbers, & Guichard, 2010; van Vliet, van Aken, de Jongh, & Hamer, 2009). The fact that many of these polysaccharides are mucoadhesive has rarely been reported in the literature as an influencing phenomenon to consider when investigating the results obtained with regards to flavour release and sensory perception.

High viscosity is a common property of many polysaccharides in aqueous solutions. This property has made them an attractive ingredient to use in manufactured liquid and semi-solid products to add bulk and improve texture, stability, and appearance. These polysaccharides include: carboxymethyl cellulose (CMC), pectin, alginate, xanthan gum (XG), guar gum and carrageenan. They come from a variety of sources and exhibit diverse chemical properties. Many of these polysaccharides have been evaluated as mucoadhesives, and are reported extensively in pharmaceutical literature (Fuongfuchat, Jamieson, Blackwell, & Gerken, 1996; Klemetsrud, Jonassen, Hiorth, Kjoniksen, & Smistad, 2013; Rossi, Bonferoni, Ferrari, Bertoni, & Caramella, 1996; Rossi et al., 1995; Thirawong, Kennedy, & Sriamornsak, 2008; Thirawong, Nunthanid, Puttipipatkhachorn, & Sriamornsak, 2007) and are utilised for their mucoadhesive capability in various pharmaceutical applications. The impact that the mucoadhesive nature of many of these commonly used polysaccharides may have on the sensory perception of food will be discussed.

Mucoadhesive strength is a continuum dependent on: the polymer chemistry and molecular weight; dosage form (e.g. particulates, tablets, films, liquids etc); other ingredients present in the formulation; and how it is being measured. Studies investigating the best formulation for mucoadhesives will often use a combination of polysaccharides to produce an optimum formulation, comparing different polymers in one study. Therefore, it is impossible to attribute a definitive value of mucoadhesive strength to any particular mucoadhesive, as the variables are seemingly infinite. Grabovac, Guggi, and Bernkop-Schnurch (2005) published a study of the nineteen most commonly used mucoadhesive polymers and conducted a large study comparing the difference in small intestine mucoadhesive strength, giving a guide to the mucoadhesive strength of commonly used polysaccharides. As this review is concerned with in-mouth mucoadhesion only, Table 1.5 outlines the mucoadhesive strength and ranking of commonly used polysaccharides in the food industry based on type of formulation tested. The studies referenced are restricted to those that have investigated buccal or gingival mucoadhesion. As can be seen in Table 1.5 there are a variety of polysaccharides that have been assessed for mucoadhesion, each with differing results depending on the formulation.

1.6.1.1 Polysaccharides as fat replacers

Polysaccharides are a popular ingredient in reduced fat products as they add bulk and increase viscosity, whilst contributing fewer calories than fat. Fat plays a significant role in the overall sensory experience and thus, satisfaction and acceptability of the food product. As well as structural impacts with regards to providing hydrophobic matrices, fat affects all sensory aspects of food including appearance, texture, mouthfeel and flavour profile. Fat is not only a source of flavour itself, but contributes to the temporal release and perception of flavours in the food

matrix. Additionally, mounting evidence is suggesting that fatty acids should be regarded as the sixth basic taste (Running, Mattes, & Tucker, 2013). Therefore, reducing fat content of a food will undoubtedly alter these aspects, which must be characterised in order to rectify them. From here on the term "flavour" will refer to both taste (tastants) and aroma (volatile compounds) perception.

The food choice of consumers is influenced by many factors (Furst, Connors, Bisogni, Sobal, & Falk, 1996); however, ultimately consumers select food because they like the taste and an important factor in this is a high quality, balanced flavour profile (Verbeke, 2006). Consumers can become highly attuned to flavour imbalances, especially in familiar products, so maintaining a sensory balance is integral. Due to this, it is important to consider the impact to the food microstructure, flavour release and subsequent physiological perception of aroma and taste when attempting to develop lower fat alternatives with polysaccharides. A lower fat content will reduce the binding of lipophilic aroma compounds to the food matrix, whilst the increase of water content to counterbalance this will relatively dilute tastants and more hydrophilic aromas, leading to alterations in flavour perception.

There are many examples of this change in flavour perception in the literature. Shamil, Wyeth, and Kilcast (1991) used a time intensity study to compared the sensory profiles of reduced-fat cheese and salad cream to their full-fat counterparts. They found that maximum intensity and total intensity perceived (area under the curve, AUC) of bitterness, sharpness and astringency was higher in reduced fat products. Saltiness on the other hand was reduced in the lower fat products. Since then other studies regarding salt perception and thickeners in low fat systems have shown similar results and is thought to be due to the relative dilution of hydrophilic compounds when fat is reduced (D. J. Cook, Linforth, & Taylor, 2003). More recent studies investigated the effects of different fat levels in oil in water emulsions and dairy desserts on flavour release *in vivo* and perception (Arancibia, Castro, Jublot, Costell, & Bayarri, 2015; Arancibia, Jublot, Costell, & Bayarri, 2011). Their findings show that the release and perception of a lipophilic aroma compound, linalool, was quicker when fat was reduced; whereas the release and perception of a more hydrophilic compound (cis-3-hexen-1-ol) was less effected by fat but depended on the thickness of the medium.

As fat content is reduced, rate of release of lipophilic aroma compounds is increased, which alters time intensity flavour perception (Malone, Appelqvist, Goff, Homan, & Wilkins, 2000). Generally, reducing fat not only impacts the initial intensity of aroma but also the intensity over time, usually resulting in the former being initially higher and the latter diminished. Aroma perception in high fat foods is generally lower in intensity but sustained over a longer period of time, compared to an initial burst of intense aroma that rapidly disappears in lower fat

counterparts. This can result in an unbalanced flavour profile; therefore, attempts at controlling the release of lipophilic flavour compounds have been made by encapsulation of these compounds (Malone & Appelqvist, 2003; Malone et al., 2000).

Another barrier for the food industry to overcome with regard to fat reduction is maintaining the creamy, fatty mouthfeel associated with higher fat products. This is a particularly difficult endeavor as it is not entirely certain what aspects of a food product are associated with the perception of these attributes but adhesion and spreading over oral surfaces is thought to be important (Dresselhuis, Stuart, et al., 2008; Dresselhuis, van Aken, et al., 2008). Whilst there is a relationship between creaminess perception and viscosity in liquid and semi-solid food (Akhtar, Stenzel, Murray, & Dickinson, 2005), there is mounting evidence that this is not the only important aspect (Malone et al., 2003a; Richardson-Harman et al., 2000; Verhagen, Rolls, & Kadohisa, 2003). Frictional forces between the food, saliva and oral mucosa may be equally as important. The lubrication of oral surfaces has been of great interest to many researchers in this field in an attempt to identify the mechanisms important for an enhanced perception of fattiness in lower fat products. In particular relevance to the mucoadhesion discussion, Dresslehuis et al. identified that the adhesion, spreading and coalescence of emulsion droplets on oral tissues is important in reducing the in-mouth frictional forces and thus enhancing the lubricating properties (Dresselhuis, de Hoog, Stuart, Vingerhoeds, et al., 2008; Dresselhuis et al., 2007; Dresselhuis, Stuart, et al., 2008; Dresselhuis, van Aken, et al., 2008). As some mucoadhesive polysaccharides also enhance lubrication, a better understanding and employment of mucoadhesives may lead to better product design with respect to these properties (Malone et al., 2003a; Stokes, Macakova, Chojnicka-Paszun, de Kruif, & de Jongh, 2011).

Table 1.5: A summary of the characteristics of various food polymers and the mucoadhesive properties found in the literature.

Polymer type	Characteristics	Mucoadhesion studies
Acacia gum	Also known as gum Arabic, a complex mixture of glycoproteins and polysaccharides.	Few studies to date have been produced with acacia gum; however one study found it to be a very weak mucoadhesive in a patch formulation [(a)].
Carboxymethyl cellulose (CMC)	An anionic polysaccharide produced by reacting alkali cellulose with sodium monochloroacetate. Comes in varying degrees of substitution of hydroxyl groups.	CMC has been the subject of many mucoadhesive studies as it is a good mucoadhesive in both solid [(b, c, e, f, g)], liquid [(d)] and gel [(h, i)] formulations.
Carrageenan	A linear sulphated polysaccharide that	Carrageenan is not widely studied for oral

	forms helical structures. The chain is made up of repeating units of galactose and 3,6 anhydrogalactose. The degree of sulfation can differ: and is denoted by the prefix (kappa, iota, lambda).	mucoadhesion, but has been found to be moderately mucoadhesive [f]. This polysaccharide with charged sulphur groups has potential to be a good mucoadhesive.
Carboxymethyl starch	An anionic derivative of starch with carboxylic group.	Ionic derivatives of starch have shown good mucoadhesion in solid form [c].
Chitosan	A cationic, linear polysaccharide composed of randomly linked D-glucosamine and N-acetyl-D-glucosamine. Made by treating chitin shells of crustaceans with alkaline substances.	Chitosan is one of the most extensively studied mucoadhesives and is a good mucoadhesive, particularly in solid form when studied for the oral cavity [j, k, g].
Guar gum	A non-ionic, branched polysaccharide composed of galactose and mannose sugars. Produced from the endosperm of guar beans.	Guar gum has been found to enhance the mucoadhesion of solid formulations when with a mixture of other mucoadhesive polymers [l]. Studies have found guar gum to range from being a relatively poor mucoadhesive [m] to exhibiting good mucoadhesion [n].
Gellan gum	Anionic polysaccharide made of repeating tetrasaccharide units of two D-glucose residues, one L-rhamnose and one D-glucuronic acid.	In solid form, gellan gum has been found to be a weak mucoadhesive in the oral cavity [o].
Hydroxyethyl cellulose (HEC)	A non-ionic polysaccharide made by reacting ethylene oxide with alkali cellulose.	In solid form HEC has been found to exert low mucoadhesive strength [b] but in gels exhibits moderate mucoadhesion [h, i].
Hydroxypropyl cellulose (HPC)	A non-ionic cellulose ether in which some hydroxyl groups in the repeating glucose units have been hydroypropylated using propylene oxide.	HPC has been found to show moderate mucoadhesive strength [e].
Hydroxypropyl-methyl cellulose (HPMC)	A non-ionic cellulose ether in which some hydroxyl groups in the repeating glucose units have been replaced with hydroxyproply or methyl groups.	There are mixed results obtained for HPMC with some showing strong [n] to moderate mucoadhesion [b, e, g] in solid for and good [p] to weak mucoadhesive strength in gel form [i].
Pectin	An anionic heteropolysaccharide rich in galacturonic acid. In nature, 80% of the carboxyl groups of galacturonic	Pectin has been found to show good mucoadhesion in solid and liquid formulations [d, j, k, g]. The different degrees

	acid are esterified with methanol, however, this can be artificially manipulated to change the behavioural properties in food and pharmaceutical applications. In particular, low methoxyl pectin and amidated pectin gel in the presence of Ca+ ions.	of esterification have all been shown to be relatively mucoadhesive [(q)].
Sodium alginate (SA)	An ionic polysaccharide found in cell walls of brown algae. It is a linear copolymer with homopolymeric blocks of mannuronate (M) and guluronate (G). This M:G ratio is important in determining the polymers properties. SA gels in the presence of Ca+ ions.	SA has been studied multiple times for its mucoadhesive abilities and is generally regarded as an excellent mucoadhesive in both solid [(b, o, c)] and liquid formulations [(d)].
Xanthan gum (XG)	An anionic polysaccharide composed of pentasaccharide repeat units of glucose, mannose and glucuronic acid.	Xanthan gum has mixed results with regard to its mucoadhesive strength with some studies of buccal patches showing poor mucoadhesion [(r, s)], whereas others found it was an excellent mucoadhesive in tablet form [(m)].

(a) (Guo, 1994); (b) (Yehia, El-Gazayerly, & Basalious, 2009); (c) (Juliano, Gavini, Cossu, Bonferoni, & Giunchedi, 2004); (d) (Ali & Bakalis, 2011); (e) (Alanazi, Rahman, Mahrous, & Alsarra, 2007); (f) (Eouani, Piccerelle, Prinderre, Bourret, & Joachim, 2001); (g) (Nafee, Ismail, Boraie, & Mortada, 2004); (h) (Jones, Woolfson, & Brown, 1997); (i) (Fini, Bergamante, & Ceschel, 2011); (j) (Kaur & Kaur, 2012); (k) (Hagesaether, Hiorth, & Sande, 2009); (l) (S. Tiwari, Singh, Rawat, Tilak, & Mishra, 2009); (m) (C. R. Park & Munday, 2004); (n) (Sai Krishna, John, & Syed, 2014); (o) (Remunan-Lopez, Portero, Vila-Jato, & Alonso, 1998); (p) (Ceschel et al., 2002); (q) (Thirawong et al., 2007); (r) (Burgalassi, Panichi, Saettone, Jacobsen, & Rassing, 1996); (s) (Abu-Huwaij, Obaidat, Sweidan, & Al-Hiari, 2011).

1.6.1.2 Flavour retention modulated by mucoadhesives

The perception of flavour is complex, however, in the simplest terms it is a combination of the senses of smell and taste. Of course there are other influencing factors on the perception of flavour, such as texture (Koliandris et al., 2008), temperature, health, memory and emotional states; however the physiological interactions concern the mouth and nose. The release of aroma and taste compounds from food is initiated by the breakdown of the matrix upon mastication and dilution with the saliva. Therefore, flavour release and perception is largely dependent on the matrix with which these compounds reside and their interactions with the saliva and mucosa.

Polysaccharide thickeners are known to alter perception and release of both tastants and aroma molecules (Shamil et al., 1991). Perception of tastants is primarily influenced by their ability to travel through the food matrix and saliva, diffusing into the taste bud lumen to activate taste

receptor cells. Conversely, aroma compounds are released due to masticatory processes breaking up the food matrix allowing these compounds to escape and be mixed with the saliva. Depending on the hydrophobicity and volatility of these compounds they will travel to the nasal cavity upon swallowing, where aroma is perceived by the olfactory bulb via signals received from nerve endings in the nasal cavity, which are coated in olfactory mucosa (Figure 1.5). The eventual perception will, therefore, largely depend on the affinity of the aroma compound for the food matrix and saliva. In addition to these factors, aroma compounds themselves can adsorb directly to oral and pharyngeal mucosa (Esteban-Fernandez, Rocha-Alcubilla, Munoz-Gonzalez, Victoria Moreno-Arribas, & Angeles Pozo-Bayon, 2016; Taylor, 2002) or to food residues adsorbed to the mucosa (Malone, Appelqvist, & Norton, 2003b; Salles et al., 2011). Furthermore, the expiration of breath after swallowing the food bolus facilitates the transport of these compounds retronasally to olfactory receptors, this can occur for a prolonged period once the food has been swallowed (Salles et al., 2011). This mechanism is responsible for the aroma persistence of certain foods as opposed to the first aroma impression when the food is still in the mouth.

Flavour compounds vary significantly in their chemical structure and their target receptors. Tastants require access to taste buds, predominantly on the tongue, and aroma compounds need to be released from the food matrix in order to travel to the olfactory epithelium. The heterogeneity of these molecules, ranging from highly charged metal ions to polar hexose sugars to lipophilic aromatic rings, makes it impossible for a universal theory describing the matrix changes affecting their perception and release. For example, saltiness is perceived due to the direct uptake of sodium ions into sodium channels in taste bud receptor cells. As sodium ions are small and hydrophilic, they will reside in aqueous solutions and preferentially move to the saliva components during consumption of a high fat food, thereby increasing the perception. On the other hand, aroma molecules are volatile with a tendency to be lipophilic, so have lower affinity for saliva and mucosa. Therefore, during the consumption of high fat products, the aroma compound will reside with the food matrix and be released more slowly. To detail the effects of all possible flavour compound and polysaccharide interactions would be too extensive for this review to cover; therefore, a selection of examples will be presented.

There are numerous studies investigating the influence of polysaccharides in food on viscosity, *in vitro* and *in vivo* release, and sensory perception. The effect that any one particular polysaccharide will have on a food will depend largely on the food matrix, the concentration (and thus viscosity) and state of the polysaccharide. Investigations into the adhesive nature of the polysaccharides rarely advance further than the assessment of attributes such as mouthcoating or stickiness. The vast amount of literature using an exhaustive combination of

polysaccharides, viscosity grades, concentrations, matrix constituents makes it difficult to draw any real conclusions of the effect of mucoadhesion in these findings, as this aspect is seldom assessed or discussed. A review by Kuo and Lee (2014) gives a good overview of how salt perception is altered by polysaccharides amongst other aspects of food formulations. Table 1.6 outlines some of the studies that compare various polysaccharide thickeners and the effect they have on sensory perception of various aromas and tastants. This table is not exhaustive but is to illustrate the vast combinations of polysaccharides, flavours and food matrices studied in the literature.

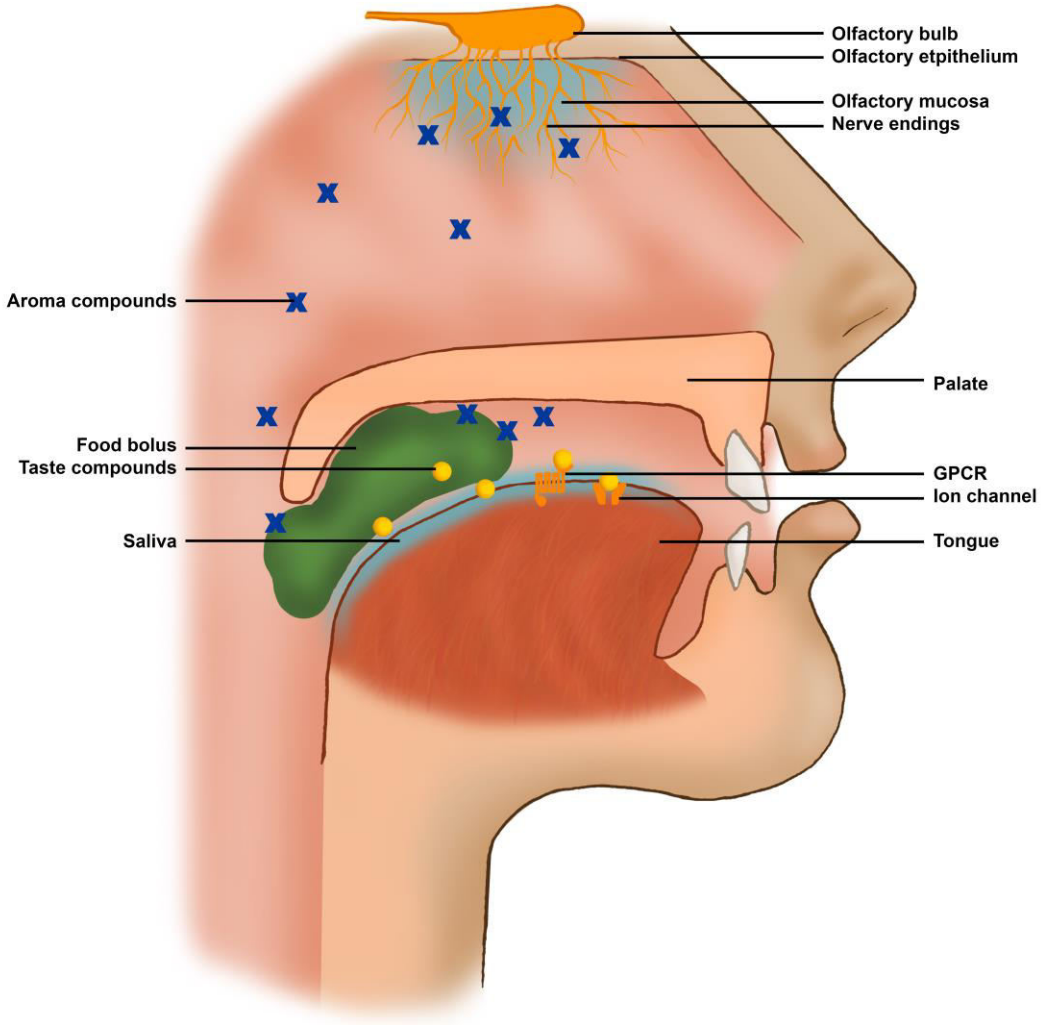

Figure 1.5: The routes of aroma (blue x) and taste (yellow ●) compounds from a food bolus are shown.Taste compounds are perceived on the tongue via receptors such as ion channels and G-protein coupled receptors (GPCRs). The surface of the tongue is covered by a mucosal membrane, and saliva, which contains mucins. The interaction of the food matrix with mucosal surfaces could vary the perception of different taste compounds. Aroma compounds are volatile and are perceived retronasally via nerve receptors, which lead to the olfactory bulb. The olfactory epithelium is coated with olfactory mucosa, so the mucoadhesion of volatile compounds could increase flavour perception.

Malkki, Heinio, and Autio (1993) alluded to mucoadhesion as an explanation for their findings on flavour release and perception in polysaccharide thickened solutions. They compared three thickeners, CMC, oat gum and guar gum with respect to their impact on sweetness and aroma perception over time. They found that oat gum prolonged the perception of sweetness and they proposed that adherence of the solution to the taste buds for longer could provide an explanation for this, although they did not carry out any experiments to test this. The viscosities were matched at the shear rate of 50 s^{-1}, which is considered to be the shear rate of the mouth and oat gum showed the weakest shear thinning behavior indicating that at lower shear rates, the viscosity would be lower than the other two samples. This could affect mass transfer of glucose molecules to the receptors; however, they do report that even the most viscous sample was sweeter than the least viscous CMC and guar samples. Interestingly they also found that oat gum solutions had the lowest aroma perception over time. This may suggest that the benefit obtained from adherence of the matrix at taste buds, prolonging tastant perception, may be at the cost of aroma release from the matrix of the food. However, there was no control used for aroma perception data so it is difficult to draw this conclusion as all polysaccharides may have altered perception over time compared to the aroma compounds in water.

This effect could be advantageous in low fat systems where flavour is unbalanced. Taste-aroma interactions have been documented in the literature with the former usually enhancing the latter in congruent pairings (D. J. Cook, Linforth, et al., 2003; Hort & Hollowood, 2004; Niimi et al., 2014). This interaction could be taken advantage of intelligent food design, using mucoadhesives to enhance flavour through this mechanism. The retention of tastants in close proximity to taste buds, thereby enhancing their taste, and aroma compounds being released more slowly, could result in flavour being perceived more intensely and sustained over time, to mimic the sensory profile of a more traditional high fat food matrix.

Gallardo-Escamilla et al. (2007) investigated the sensory impact of various polysaccharides in a fermented whey drink. The selected polysaccharides were high methyl-ester pectin, propylene glycol alginate (PGA), CMC and XG. The concentrations used were of equivalent viscosities when added to the whey product, although the authors recognise the high shear rate used to match viscosity may have affected results. They found that the presence of all polysaccharides reduced the overall typical yoghurt aroma released in headspace analysis, however, perception data showed only a significant decrease when thickened with PGA. The perception of acidity was decreased in all samples (except XG) compared to the control, and sweetness was perceived to be higher in the CMC and PGA samples (Gallardo-Escamilla et al., 2007). The study emphasises the complex relationship between thickener, flavour perception and flavour release.

Table 1.6: An outline of the effect of various polysaccharides on the sensory perception of taste, flavour, and mouthfeel in studies on different food matrices and models.The effect of the polysaccharide is indicated by ↑ (increase in perception), and ↓ (decrease in perception). c* denotes the coil overlap concentration; AUC refers to the area under a time-intensity curve; Imax refers to the maximum intensity during a time-intensity profile.

Food matrix	**Polysaccharide(s) used**	**Effect on sensory perception**
Fermented whey drink [(a)]	Propylene glycol (PG) alginate, CMC, high-methoxy pectin, XG	CMC and PG alginate ↑ sweetness and ↓ acidity and yoghurt attributes compared to other polysaccharides and control. Mouthcoating was most strongly associated with CMC
Custard dessert [(b)]	CMC with varying viscosity grades and concentrations used	Increasing concentration and viscosity ↓ sweetness perception and ↑ the in-nose total release and Imax of ethyl butyrate, ethyl 3-methylbutanoate, ethyl hexanoate compared to lower concentration of the same viscosity grade.
Gels with differing rigidities [(c)]	Pectin, gelatin	Increased gel rigidity ↓ in-nose release rates, perception of odour, strawberry flavour and sweetness but ↑ total release and intensity for hexanal, ethyl butanoate, ethyl 3-methylbutanoate and ethyl hexanoate. Pectin gels ↑ AUC and Imax compared to gelatin gels for all aromas.
Pastes with differing viscosities at a shear rate of 50 s^{-1} [(d)]	HPMC, starches: wheat, waxy maize, and modified waxy maize	HMPC ↓ salt and basil flavour perception compared to all starches. Waxy maize starch ↓ salt and basil flavour compared to other starches.
Dairy dessert containing carrageenan and starch [(e)]	Pectin - with differing Ca+ reactivities	Perception of adhesiveness ↑ in desserts with pectin compared to control without. Sweetness and vanilla perception were unaltered.
Lemon flavoured dairy dessert [(f, g, h)]	CMC, modified starch	CMC ↓ linalool and cis-3-hexen-1-ol *in vivo* aroma release compared to samples thickened with starch but had a similar release to the fat only samples. CMC ↓ overall flavour and sweetness perception compared to starch samples.
Aqueous solutions with aspartame [(i)]	CMC, SA	CMC ↓ sweetness perception of aspartame, particularly beyond c*. SA did not have an effect on sweetness perception.

(a) (Gallardo-Escamilla et al., 2007); (b) (van Ruth, de Witte, & Uriarte, 2004); (c) (Boland et al., 2006); (d) (Ferry et al., 2006); (e) (Arltoft, Madsen, & Ipsen, 2008); (f) (Arancibia et al., 2011); (g) (Arancibia, Costell, & Bayarri, 2013); (h) (Arancibia et al., 2015); (i) (Xue et al., 2014).

Mucoadhesion may explain part of the results in this study, as the enhanced sweetness found by adding known mucoadhesives (pectin, CMC and alginate) could play an important role in prolonging the residence of the sugar molecules in close proximity to the taste receptors. Bayarri, Chulia, and Costell (2010) also found that carrageenan enhanced the perception of sweetness and vanilla aroma intensity in model fat-reduced custards compared to a full-fat counterpart.

Hydroxypropylmethyl cellulose (HPMC) is a non-ionic, semisynthetic polysaccharide and is a relatively weak mucoadhesive in the oral cavity compared to other polysaccharides such as chitosan and CMC (Nafee et al., 2004). This polysaccharide is used in many studies as a viscosity modifier. Studies have found that this thickener decreases the perception of saltiness, sweetness and aroma compounds in liquid systems due to the enhancement in viscosity (D. J. Cook, Linforth, et al., 2003; Hollowood, Linforth, & Taylor, 2002). These studies found that by increasing the concentration of HPMC, above the coil overlap concentration (*c**), a decrease in perception of tastants and aromas was observed. c* refers to the concentration above which polysaccharide molecules physically interact, and is determined by a sharp increase in viscosity after this point. The authors propose that the reduction in taste intensity was due to entrapment of the compounds within the polymer network, slowing the mass transfer to taste buds. The atmospheric pressure ionisation mass spectrometry data found that the *in vivo* aroma release concentrations were no different between samples with differing viscosities. The authors concluded that this was due to aroma-taste interactions, where a decrease in the perception of saltiness or sweetness decreased the perception of the congruent aromas, even though the same amount of aroma may be delivered to the nasal cavity (D. J. Cook, Linforth, et al., 2003; Hollowood et al., 2002). The role of mucoadhesion was not tested within these experiments, however, the apparent decrease in salt and sweet perception may be explained by the fact that HPMC is non-ionic and therefore may not interact with the tastant compounds compared to ionic mucoadhesives such as CMC. Therefore, the salt and sugar molecules may favour partitioning into the salivary phase during mastication and be swallowed before activating taste receptors on the tongue that may be shielded by the viscous polysaccharide.

There is abundant research in the field of viscosity, thickeners and flavour perception and release. However, most studies investigating these parameters use a model thickener and do not necessarily consider the differences between thickener types. Much like the differing strengths of mucoadhesion each thickener will possess, the interaction between the thickener and flavour molecules will differ. Therefore, it is difficult to draw conclusions about the role mucoadhesion plays in many of these studies, as the mucoadhesive strength of the thickeners is not measured. This is a limitation as the mucoadhesive strength of the thickeners could be a factor in the

difference in aroma release between different thickeners, which is only assessed as the sensory perception of adhesiveness (D. J. Cook, Hollowood, Linforth, & Taylor, 2003; Ferry et al., 2006; Gallardo-Escamilla et al., 2007; Yang, Young-Suk, Sang-Ho, & Kwang-Ok, 2014).

1.6.1.3 Polysaccharide mucoadhesion and texture

Trained sensory panels often describe the textural aspects of high fat foods as creamy, fatty, slippery, oily and smooth; dependent on the type of food. It can be difficult for panellists to distinguish between these types of words; partly due to the difficulty in classifying these perceptions by experimental means. Factors including rheology, tribology, colloidal behaviour and flavour all have an important influence.

As mentioned previously, fat serves many purposes in food with many textural cues that are difficult to mimic without it. Emulsions are designed with this in mind in an attempt to mimic the lubricating, thick and creamy properties that fat imparts (Malone et al., 2003a; van Aken, Vingerhoeds, & de Hoog, 2007; van Aken, Vingerhoeds, & de Wijk, 2011). These studies highlight the importance of thin film rheology and tribology as well as bulk rheology when comparing thickeners to fuller fat systems. In order to understand perceived textural changes to food when incorporating mucoadhesives, it is vital to establish a way to characterise these changes. Malone et al. (2003a) studied the adsorption to a mucin-coated film of oil-in-water emulsions in comparison to an oil-in-water emulsion with chitosan. They found that the addition of the mucoadhesive, chitosan, enhanced the affinity of the oil to the mucin film. The authors note that the presence of chitosan resulted in an astringent mouthfeel when given to a trained sensory panel, which was attributed to chitosan binding to mucin molecules causing precipitation (Malone et al., 2003a). This is one of the few studies, which attempts to directly employ mucoadhesives as a way to modulate the organoleptic properties of food by texture modulation. There are, of course, many other studies into the textural aspects of liquid, semi-liquid and semi- solid foods, some of which specifically investigate the interaction between the food and mucosa (Canon, Giuliani, Pate, & Sarni-Manchado, 2010; Esteban-Fernandez et al., 2016; van Aken et al., 2007; van Aken et al., 2011). Many of these refer to the specific interactions of flavour molecules with the food matrix and oral anatomy, however, select studies have investigated the influence of hydrocolloids on the textural perception of emulsions (Silletti, Vingerhoeds, Norde, & Van Aken, 2007a; van Aken et al., 2007; van Aken et al., 2011).

Most of the studies regarding the effect of polysaccharide thickeners on texture are focused on liquid products (van Vliet et al., 2009; Wendin & Hall, 2001; Wendin, Solheim, Allmere, & Johansson, 1997). The nature of the food matrix is of paramount importance when considering the effect of mucoadhesive polysaccharides. The literature to date has focused on analyzing the sensory impact of polysaccharides on liquid and semi-solid products, as this is where their

viscosity and emulsifying properties can be utilised most effectively. However, the results from these studies, and the likely role of mucoadhesion in contributing to the changes in sensory perception, may generate interest in incorporating these mucoadhesives into dry food products. For many mucoadhesives, the solid form has the highest mucoadhesive strength, due to swelling and spreading behavior upon contact with the moist mucosal surface of the oral cavity. This results in a strong, lubricating, adhesive joint. To date, and to the best of the authors' knowledge, there are no studies investigating mucoadhesive polysaccharides in dry, solid food products.

1.6.2 Mucoadhesion by native compounds in food

When considering mucoadhesion in foods, added ingredients are not the only substances to consider as mucoadhesive; compounds found naturally in food products can also have mucoadhesive properties, and therefore the chemical nature of whole food matrices and products must be considered. Most polysaccharides are added to foods as functional ingredients; however pectin is found naturally in fruit such as pears, plums and citrus fruits. There is also evidence that other substances occurring naturally in food have mucoadhesive abilities, such as some polyphenols, proteins and flavour compounds. A summary of mucoadhesive studies in food substances is outlined in Table 1.7.

Table 1.7: A summary of mucoadhesive studies in food systems.

Food substance	Methods	Findings
Olive oil [(a)]	*In vitro* binding assay, turbidimetry [(a)]	Mucins bind with polyphenols to form complexes [(a)]
Chewing gum [(b)]	*In vivo* retention method [(b)]	Retention of flavour compounds in the oral cavity [(b)]
Rape seed and skin [(c)]	*In vitro* binding assay, SDS-PAGE [(c)]	Astringency is caused by the binding of polyphenols to salivary proteins [(c)]
Tea [(d, e)]	*In vitro* methodology [(d, e)]; sensory evaluation [(d)]; atomic force microscopy, particle tracking microrheology [(e)]	Polyphenols from black tea bind to salivary proteins [(d)] Polyphenols from green tea reorganise the salivary mucin network [(e)]
Milk proteins [(f, g, h, i, j, k, l)]	*Ex vivo* retention method, fluorescent microscopy [(f)]; NMR, CD [(g)]; tribology [(h)]; turbidimetry, viscometry, *ex vivo* wash-off method [(j)]; *in vivo* retention [(l)]; DLS [(g, k)]; zeta-potential [(g, k)]; rheology [(f, i, k)]; sensory evaluation [(i, k, l)]	Caseins and β-lactoglobulin bind to oral mucosa [(f)] Structural changes indicate interaction between submaxillary mucin and β-lactoglobulin [(g)] β-lactoglobulin causes loss of salivary lubrication [(h)] Mucoadhesives alter mouthfeel when added to fermented whey [(i)] and yoghurt [(j)] Build-up of mouthdrying with repeated consumption of whey protein [(k)] Astringency caused by the flocculation of whey proteins and saliva [(l)]
Wine [(m)]	*In vivo* retention method, GC-MS [(m)]	Strength of aroma-mucosa interactions dominates over aroma amount [(m)]

(a) (Quintero-Florez, Sanchez-Ortiz, Martinez, Marquez, & Maza, 2015); (b) (Hussein, Kachikian, & Pidel, 1983); (c) (Gambuti, Rinaldi, Pessina, & Moio, 2006); (d) (Nayak & Carpenter, 2008); (e) (Davies et al., 2014); (f) **(Withers, Cook, et al., 2013)**; (g) (Celebioglu et al., 2015); (h) (Vardhanabhuti, Cox, Norton, & Foegeding, 2011); (i) (Gallardo-Escamilla et al., 2007); (j) (Pang, Deeth, Prakash, & Bansal, 2016); (k) (Bull et al., 2017); (l) (Vingerhoeds et al., 2009); (m) (Esteban-Fernandez et al., 2016).

1.6.2.1 Mucoadhesion in astringency and mouth drying

Polyphenols are a group of compounds, found in plant-derived foods such as wine and tea, which can elicit an astringent sensation in the mouth. Astringency is defined as "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM, 2004). Astringency is a persistent sensation (Courregelongue, Schlich, & Noble, 1999), which would agree with a mucoadhesive mechanism prolonging the oral exposure to the sensation. Chitosan has been found to elicit an astringent sensation when adsorbed to the oral mucosa (Yakubov, Singleton, & Williamson, 2014). However, not all mucoadhesives cause an astringent response: CMC, a known mucoadhesive, has been found to reduce astringency (Courregelongue et al., 1999; Troszynska et al., 2010), possibly caused by a competition for mucin binding between CMC and polyphenols.

Polyphenols are thought to produce an astringent sensation by the binding to salivary proteins (Bajec & Pickering, 2008; Gambuti et al., 2006; Nayak & Carpenter, 2008), forming large aggregates (Jobstl, O'Connell, Fairclough, & Williamson, 2004). The mechanism of the binding of polyphenols to mucins to produce an astringent sensation is thought to be with those mucins bound to mucosal cells, as an increase in saliva flow reduces the astringent response (Nayak & Carpenter, 2008). The binding of polyphenols to mucins forms complexes (Quintero-Florez et al., 2015), and this process leads to increased mucin sedimentation, aggregation and viscosity, disrupting the salivary mucin network (Davies et al., 2014). Although it is not yet an accepted mechanism, Gibbins and Carpenter (2013) alluded to mucoadhesion as the cause of astringency in a recent review.

Astringency, or "mouth drying" is often negatively associated with milk and dairy products (Lemieux & Simard, 1994). Mucoadhesion has been observed in whey protein and a positive correlation was found between protein denaturation and mucoadhesive strength (Hsein, Garrait, Beyssac, & Hoffart, 2015). Withers, Cook, et al. (2013) found that milk proteins, β-lactoglobulin (β-LG) and caseins, bound to oral mucosa *in vitro*, and suggested that this could be the cause of drying in milk protein beverages. Another recent study found structural changes upon mixing β-LG with bovine submaxillary mucin, as observed by nuclear magnetic resonance (NMR), circular dichroism (CD), and dynamic light scattering (DLS), showing binding between the milk proteins and mucins, which suggests mucoadhesive interactions (Celebioglu et al., 2015). The effect of the mucoadhesion of β-LG on the sensory perception of "astringency" or "drying" is proposed to be the loss of lubrication of saliva, as observed by tribology (Vardhanabhuti et al., 2011). The flocculation of whey proteins with saliva has also been linked to astringency (Vingerhoeds et al., 2009). These findings appear to mirror the mechanism for

astringency in polyphenols, as the disruption of the saliva results in an astringent or drying sensation.

Evidence of mucoadhesion altering mouthfeel effects is limited, however using lubricating mucoadhesives, such as CMC and HMP, has been shown to influence mouthfeel in milk protein samples (Gallardo-Escamilla et al., 2007; Pang et al., 2016).

Aroma adsorption to the oral mucosa has been investigated extensively. A study by Hussein et al. (1983) was one of the first to investigate the effect of aroma persistence after consumption. In this study, participants rinsed their mouths after 1 and 5 minutes post-consumption, and measured the amount of volatile left in the mouth. The authors found the most persistent aromas to be menthol and anethole; however, it was unclear whether the extraction technique was suitable to remove all volatile compounds, especially those adhered to the mucosa. More recently, Esteban-Fernandez et al. (2016) used intra-oral SPME/GC-MS to investigate wine "after-aroma". The authors found that the strength of the aroma-mucosa interactions was more important that the actual amount of aroma adsorbed.

1.7 Methods for the measurement of mucoadhesion

Mucoadhesion can be evaluated using *in vitro* methods to directly measure the adhesion of a substance to a mucosal tissue, or a mucosa-mimetic material, such as a hydrogel (M. T. Cook, Smith, & Khutoryanskiy, 2015). Alternatively, physical properties can be measured as an indication of mucoadhesive strength. An in-depth summary of methods can be found in other reviews (Davidovich-Pinhas & Bianco-Peled, 2010, 2014; Khutoryanskiy, 2011; Yakubov et al., 2014) but a brief summary will be given here.

1.7.1 *In vitro* methods

Tensile methods measure the force required to cause detachment between a mucoadhesive and a mucosal tissue. The mucoadhesive is placed on the platform of an automatic tensile instrument (commonly a texture analyser or tensiometer) and put into contact with the mucosal tissue; a detachment profile can then be measured. The main limitation of this technique is that there are many factors capable of affecting the results of the experiment: the mucosa environment, testing speed, and how the initial contact is formed (Tobyn, Johnson, & Dettmar, 1997). An alternative method uses a rotating disc to measure the time taken for detachment, which is useful for solid mucoadhesives, and the results tend to correlate to those obtained by the tensile method (Grabovac et al., 2005).

The tensile and rotating-disc methods are limited by the lack of saliva flow, which is considered using the flow-through method, first described by Ranga Rao and Buri (1989). The

mucoadhesive is applied to the mucosal tissue and a biological flow is simulated, using a media such as artificial saliva or buffer to wash the mucosal tissue (see Figure 1.6). Analysis can be performed on the wash-off fluid, or on the mucosal tissue to monitor retention (Cave, Cook, Connon, & Khutoryanskiy, 2012). This method can be considered as an *ex vivo* technique, when the irrigation media closely resembles the composition of the mucosal secretion (Madsen, Sander, Baldursdottir, Pedersen, & Jacobsen, 2013). When using actual saliva, further considerations include the variation in composition and flow between individuals, which can complicate *in vitro* methodology; therefore a standardised procedure is important (Schipper et al., 2007).

Tribological methods are used to measure lubrication and friction between two surfaces and can be related to sensory properties associated with mucoadhesion, (Phuong, Bhesh, & Sangeeta, 2016; Prakash, Tan, & Chen, 2013). They can be adapted to use mucosal tissues to measure lubrication *in vitro* (Dresselhuis, de Hoog, Stuart, & van Aken, 2008).

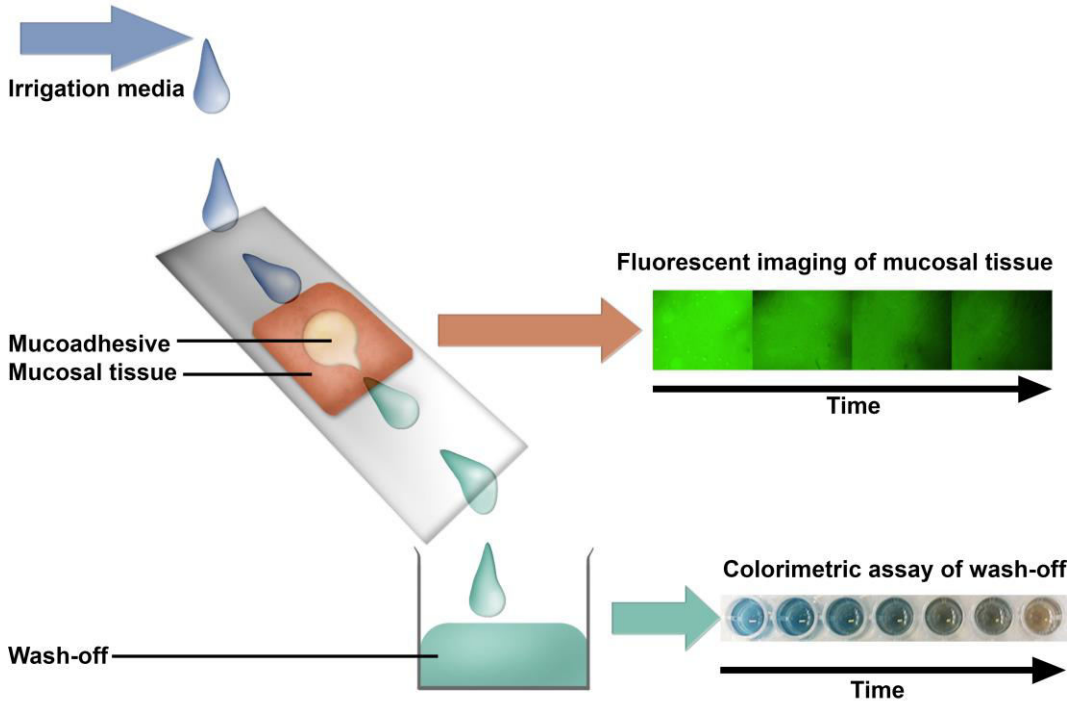

Figure 1.6: An example of a flow-through retention experiment used to measure mucoadhesion. A mucosal tissue is placed on an angled slide, the mucoadhesive is placed on the mucosal surface and the system is washed with a suitable irrigation media, for example artificial saliva. Retention can be measured by either: observing the tissue, for example using fluorescent labelling; or by measuring the concentration of mucoadhesive in the wash-off.

1.7.2 Physical techniques

Rheological synergism is an effect observed when the addition of a mucoadhesive polymer solution increases the viscosity and elastic modulus rheology of a mucin solution more than expected by addition of the respective viscosities of the separate solutions. It has been used as a method for evaluating mucoadhesive strengths of polymers (Ivarsson & Wahlgren, 2012; Rossi et al., 1995; Thirawong et al., 2008). Ivarsson and Wahlgren (2012) found inconsistencies between tensile and rheological methods for the assessment of mucoadhesion. They found that the ranking order of polymers from most to least mucoadhesive inverted when rheological experiments took place. This evidence shows that rheological experiments should not be used alone when attempting to measure the mucoadhesive nature of polymers.

The importance of understanding the physical and chemical interactions leading to mucoadhesion has been previously highlighted (Peppas & Huang, 2004). Mucin powders are commercially available, and interactions between mucins and mucoadhesives can be studied using a variety of techniques. Turbidimetric methods can give an indication of mucoadhesion as mucin/mucoadhesive particles aggregate and increase solution turbidity (Sogias et al., 2008). Studying the change in surface charge of mucin particles by zeta potential and DLS measurements has also been used to measure mucoadhesion (Takeuchi et al., 2005).

Other techniques implemented for the study of mucoadhesion include: surface plasmon resonance (Takeuchi et al., 2005); NMR; CD (Celebioglu et al., 2015); x-ray photon spectroscopy; differential scanning calorimetry (Patel et al., 2003); and isothermal titration calorimetry (Albarkah, Green, & Khutoryanskiy, 2015; Zhao et al., 2012).

1.8 Concluding remarks

The understanding of mucoadhesion in food substances could have many impacts on the food industry, whether mucoadhesives are added as a functional ingredient, or whether native mucoadhesives in the food are manipulated to control sensory properties. By understanding the properties of mucoadhesive food components, a higher level of control could be achieved in the texture and flavour of a food product. Mucoadhesion could also play a significant role in the future of low-fat foods utilising fat replacers.

Furthermore, many mucoadhesive polysaccharides are not just adhesive to mucosa but also non-biological surfaces, which could be utilised in food manufacturing processes to topically adhere flavourings to foods. This could result in reduced costs and higher consumer satisfaction due to a reduction in the loss of flavouring on the product and subsequently more flavour delivered to the consumer.

In conclusion, mucoadhesion is an important consideration for food researchers and product developers and has the potential to be utilised in enhancing the organoleptic properties of foods. The impact of mucoadhesive ingredients on sensory perception is beginning to be elucidated, as outlined in this review; however further research in this area is required for a better understanding. Native ingredients such as proteins and polyphenols should be investigated to provide an in-depth understanding of the mechanism of adhesion in the oral mucosa. Other research should focus on the mechanisms involved in flavour retention in mucoadhesive matrices and the subsequent release. As static measurements of flavour perception are likely to miss any prolonged perception caused by slowing the release of flavour compounds, temporal methods are required to study the impact of mucoadhesives on flavour release and perception.

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Author contribution

SLC and SPB wrote the manuscript; LM, JKP and VVK edited the manuscript; and SPB produced the illustrations.

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Chapter 2 Part 1. Sensory method development

Abstract

This preliminary part of Chapter 2 summarises the development of the sensory methods that are subsequently used in Chapter 2 Part 2 and Chapter 5. The aim was to have two sensory methods; one profiling method to describe and quantify all characteristics of whey protein model beverages, and secondly a temporal method to track changes in the perception of the sensory attributes over repeated consumption. The development of such methods required model whey protein beverages that differed in their sensory characteristics. Therefore, different heat treatments of whey protein concentrate were used and particle size of the resulting samples was quantified by dynamic light scattering. Quantitative Descriptive Analysis was able to determine differences between whey protein model beverages unheated and heated at 70 °C, and sequential profiling was able to detect differences over repeated consumption.

2.1 Method development

In order to develop a sample preparation protocol, a range of denaturation times (0, 5, 8, 12, 15 and 20 min at 70 °C) were trialled, and pH values and particle size were measured, as detailed below. A sensory method was used adapted from Methven et al. (2010), utilising both Qualitative Descriptive Analysis (QDA) and sequential profiling. This was trialled on two of the samples trialled: WPC00 (unheated) and WPC20 (heated at 70 °C for 20 min), 10% (w/v) solutions, one unheated and three heated at 70 °C for 20 minutes, respectively, as these represented either end of the analysed range.

2.1.1 Materials

The whey protein concentrate (WPC) used was Volactive Ultrawhey 80 Instant (Volac International Limited, Orwell, Royston, UK), a dry powder with a protein content of 80% minimum, and containing soy lecithin (0.5% maximum) as an emulsifying agent. The remaining 20% contains moisture, fat, lactose, and minerals. The WPC was from a sweet cheese whey source, and was spray dried after cross-flow membrane ultrafiltration, as shown in Chapter 1 Figure 1.1. There is potential for denaturation of the product during processing, and therefore the WPC powder will not be completely comprised of native protein. The product was not bleached and was kept for a maximum of 18 months from production date in a cool dry location.

Crackers (Carr's Table Water Biscuits, United Biscuits, London, UK) were used as palate cleansers in sensory profiling.

2.1.2 Sample preparation

WPC beverages were prepared by addition of WPC powder to water (500 mL in 800 mL glass beaker, 10% w/v, deionised water). Samples were stirred for 30 min at room temperature (25 ± 2 ^{0}C). After stirring, denatured samples were stirred further while being heated using a water bath (70 °C; 0, 5, 8, 12, 15 and 20 min), then cooled in a water bath and allowed to fully hydrate overnight at 4 °C. Samples were allowed to reach 70 °C before timing commenced; all samples reached 70 °C in 12 ± 4 min. An unheated sample was selected as a control sample which had undergone no heat treatment, with exception of heat processes during the production of WPC powder from liquid whey. Samples were not pH adjusted, in order to observe the effects of heating on neutral model whey protein beverages.

2.1.2.1 Heating conditions

WPC samples were heated using a water bath at 70 °C, chosen to represent the denaturation temperature of β-LG (Dewit & Swinkels, 1980), the most abundant whey protein in bovine milk. Sample temperature was monitored using a temperature probe placed in the centre of the sample. To observe a range of different denaturation steps, the sample was heated at 70 °C until aggregation occurred after 20 minutes. As substantial aggregation makes the sample unacceptable for sensory analysis, the maximum heating time was set as 20 min. An unheated sample was included to act as a control in comparison to the heated samples. Interval time points were selected to observe the effects of heating at intermediate stages.

2.1.2.2 Dynamic light scattering

WPC samples were diluted 100 times in water (HPLC grade water) for dynamic light scattering (DLS) analysis and measurements were performed using Nano-S Zetasizer (Malvern Instruments, UK) at 30 °C, with an equilibration time of 60 s. Samples were collected using a minimum of 10 runs with 10 s per run. Data was quality checked by the software and if minimum criteria were not met, a further experiment would be added on to the dataset.

DLS uses light scattering to predict the size of particles in solution or suspension, using the rate of diffusion due to Brownian motion. DLS uses algorithms to produce a size distribution which can be displayed as a function of intensity, number or volume. Intensity of scattering is proportional to particle size, and therefore an intensity distribution will be biased towards larger particles in solution and may over exaggerate their presence. By observing volume and number distributions, the significance of larger peaks in polydisperse solutions can be determined.

2.1.3 Sensory method development

A trained sensory panel of experts in profiling techniques (n = 9; 8 females, 1 male) had a minimum of 6 months general training, including training on mouthfeel, astringency, oral nutritional supplements (beverages containing whey protein) and sequential profiling. The panel were given further training on WPC profiling and sequential profiling (minimum 4 hours). Sensory profiling was carried out at room temperature (22 ± 2 °C) in isolated booths, training and vocabulary development was carried out in a discussion room. Unheated WPC (WPC00) and WPC heated for 20 min (WPC20) were selected for sensory profiling, as the start and end-points of the heating process.

Ethics are not required when using the commercial sensory panel (MMR Research Worldwide) if commercially available standard food items are being presented in a standard format. This consent is covered in the contract of employment for all panellists (MMR Research Worldwide).

2.1.3.1 Quantitative descriptive analysis development

Quantitative descriptive analysis (QDA) (Stone, Sidel, Oliver, Woolsey, & Singleton, 1974) was performed using a consensus vocabulary developed by the panel during training (35 attributes; 3 appearance, 6 odour, 6 taste, 7 flavour, 7 mouthfeel, 6 after effects, shown in Appendix 2.1. WPC00 and WPC20 were evaluated in duplicate according to a balanced design, using blind coded samples, and unstructured line scales (0 – 100) with appropriate anchors (nil to extreme). Four samples were presented monadically in opaque white cups (20 mL) per session, crackers and filtered tap water were provided as palate cleansers between samples during an enforced break between samples (2 min). Evaluation was carried out under artificial daylight. Data was captured using Compusense at-hand (Compusense, Ontario, Canada).

2.1.3.2 Sequential profiling method development

Sequential profiling was carried out to establish the build-up of seven sensory attributes over repeated consumption of eight aliquots (5 mL) of samples, scoring after consumption of each aliquot (T0), and following 30 (T30) and 60 s (T60) time delays, as described by Methven et al (2010), (Compusense at-hand, Ontario, Canada). Thus there are eight aliquots tasted consecutively for each of two samples (WPC00 and WPC20), scored at three time points (T0, T30 and T60) as demonstrated in Figure 2.1. The seven attributes scored on unstructured line scales (0 – 100) were bitter, sour, metallic, cooked milk flavour, mouthcoating, chalky and drying. Samples were coded with three-digit numbers and all eight aliquots of one sample were presented together with the same code, the panellists were not blinded to the sequential nature of the evaluation. Warm filtered tap water and unsalted crackers were provided as palate cleansers in the 2 minute enforced break between samples; however panellists were instructed

not to use these between the eight aliquots of the same sample. Panellists were instructed to consume the total volume of each aliquot and to coat the mouth with the sample before swallowing. Evaluation was carried out under red lighting and aliquots were served in opaque black cups to mask appearance differences between samples.

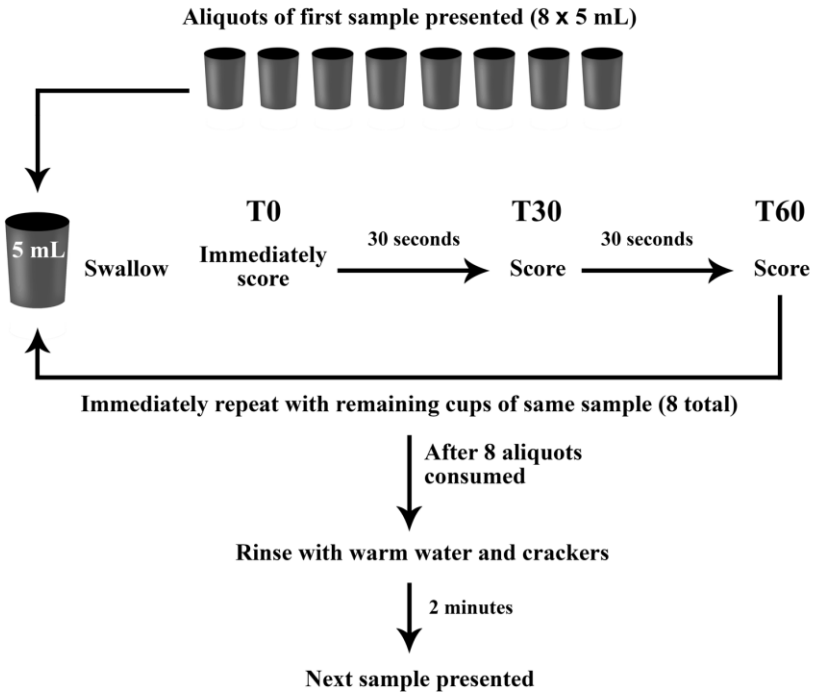

Figure 2.1: Flow diagram showing scheme of sequential profiling. 8 aliquots (each 5 mL) of one sample are presented together, one sample is consumed and immediately scored after swallowing, after a 30 s break the sample is scored again, and after another 30 s break the sample is scored, totalling three scoring times for each aliquot (T0, T30 and T60). After this is repeated for all 8 aliquots, the panellist is given 2 minutes to cleanse with warm water and crackers before being presented 8 aliquots of the next sample.

2.1.4 Statistical analysis

SENPAQ (version 5.01) was used to carry out analysis of variance (ANOVA) of QDA data. XLSTAT (version 2012.1.01) was used to carry out three-way repeated measures ANOVA (RM-ANOVA) on the sequential profiling data using sample (n = 2), assessors (n = 9) and repeated consumption (n = 8) as explanatory variables.

To account for differences between panellists in QDA, the error is divided by the assessor interaction; however this is not possible when analysing repeated measures data from sequential profiling. Panel performance was monitored for both QDA and sequential profiling using SenPAQ (QI Statistics, UK).

2.2 Results and discussion

2.2.1.1 Heating profile

In the 500 mL sample used, temperature reached 70 °C after 20 min, remained at this temperature until removed from the water bath, before decreasing to room temperature over 10 min in a cold water bath, as shown in Figure 2.2 (averages for processing triplicates shown) alongside theoretical denaturation temperatures for individual whey proteins. Subsequent sample preparations used the same sample volume (500 mL) and container type and size (800 mL glass beaker), and temperature profiles were monitored to ensure consistency.

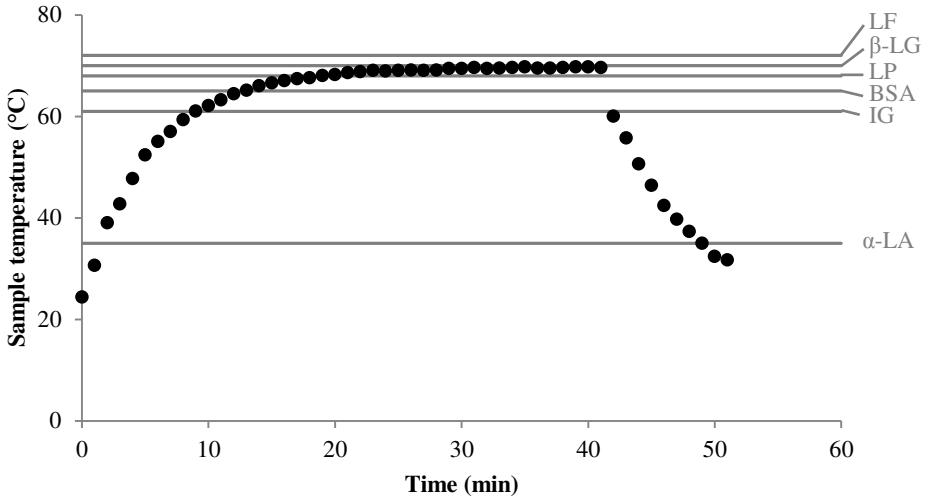

Figure 2.2: Sample temperature when heated at 70 °C. Once the target temperature has been reached, it is maintained for 20 min before cooling. Minimum denaturation temperatures are shown for lactoferrin (LF), β-lactoglobulin (β-LG), lactoperoxidase (LP), bovine serum albumin (BSA), immunoglobulin (IG) and α-lactalbumin (α-LA) (Relkin, 1996; Vermeer & Norde, 2000).

2.2.1.2 Dynamic light scattering

An increase in particle size (z-average) was observed with a longer heating time, with a particle size maximum at 15 minutes, shown in Table 2.1. Unheated WPC was included as a standard, with 20 minutes being the maximum heating time before severe aggregation occurred, leaving the sample unpalatable and unsuitable for sensory analysis. 5 and 10 minutes were selected as intervals between this time which would cover a range of particle sizes. Particle size and polydispersity increased with heating time (Appendix 2.1).

Table 2.1: Z-averages of WPC heated at 70 °C for varying lengths of time . Error represents ± 2 standard deviation.

Heating time (min)	Z-average (d.nm)
0	210 ± 10
5	293 ± 11
8	365 ± 9
12	441 ± 18
15	451 ± 29
20	368 ± 6

2.2.1.3 Quantitative descriptive analysis results

QDA evaluation of WPC00 and WPC20 found significant differences between samples for 21 of the 35 attributes rated: 3 appearance; 4 odour; 1 taste; 4 flavour; 5 mouthfeel; 4 after effects. Significant differences were seen for mouthfeel and aftertaste attributes associated with drying (Figure 2.3), with higher scores in WPC20 for drying, chalky, furring and mouthcoating. As the sequential profiling method could assess a maximum of seven attributes, a selection was based on the QDA results. The seven attributes chosen were bitter, sour, metallic, cooked milk flavour, mouthcoating, chalky and drying. Cooked milk was selected as a high scoring flavour and odour attribute, which showed significant differences between samples in odour, and almost significant differences for flavour (Figure 2.3), with WPC20 scoring higher in both. Bitter and metallic are negative attributes associated with whey protein beverages (Martini & Walsh, 2012; Whetstine, Croissant, & Drake, 2005), and were selected as basic taste controls for sequential profiling due to the lack of significant differences found between WPC00 and WPC20. During discussion, mouthcoating, chalky and drying were highlighted as distinctive attributes found in the samples, and significant differences were observed through QDA for all three attributes (Figure 2.3). Although furring was identified as significant, some panellists found the attribute difficult to define, and upon further discussion, it was excluded from sequential profiling.

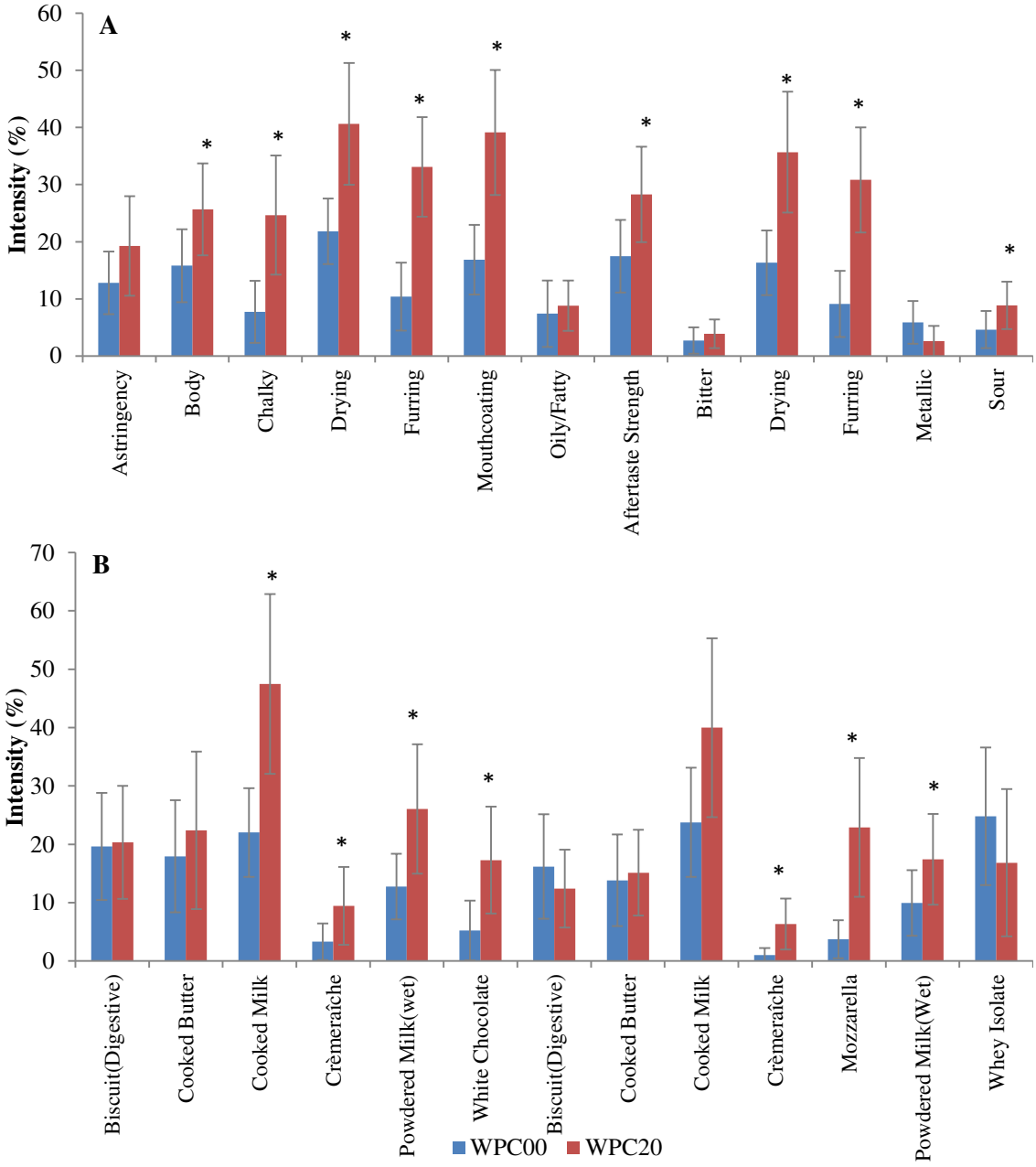

Figure 2.3: QDA intensities for A: mouthfeel and after effect, B: odour and flavour attributes for WPC00 and WPC20 samples. *Significantly different scores between samples ($p \leq 0.05$) from ANOVA.

2.2.1.4 Sequential profiling results

Significant differences were observed by sequential profiling between samples for sour, cooked milk, mouthcoating, chalky and drying (T0, T30, T60); and for metallic (T60) as summarised in Table 2.2. Sour, cooked milk flavour, drying, chalky and mouthcoating were observed to be higher for WPC20 than WPC00, which is consistent with QDA data. WPC00 scored higher metallic intensities than WPC20, with an increase with consumption for T60. Mean intensities are shown in Appendix 2.2.

Significant increases were observed over repeated consumption of aliquots for drying (T0, T30, T60; shown in Figure 2.4), mouthcoating (T30, T60), and cooked milk (T60). Drying was also seen to retain high intensity in T30 and T60 scores in relation to T0 scores. This persistence of intensity is indicative of a build-up of these sensations in the mouth.

Table 2.2: Statistical significance (*p*-values) from RM-ANOVA of sequential profiling for differences between WPC00 and WPC20 samples, and across repeated consumption: during consumption (T0), 30 s and 60 s after tasting (T30 and T60). *Significant difference ($p \leq 0.05$).

Attribute	T0		T30		T60	
	Sample	**Consumption**	**Sample**	**Consumption**	**Sample**	**Consumption**
Bitter	0.702	0.155	0.321	0.147	0.341	0.424
Metallic	0.072	0.769	0.105	0.874	< 0.0001*	0.912
Sour	< 0.0001*	0.924	< 0.0001*	0.830	< 0.0001*	0.064
Cooked milk	< 0.0001*	0.693	< 0.0001*	0.417	< 0.0001*	0.008*
Mouthcoating	< 0.0001*	0.387	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*
Chalky	< 0.0001*	0.622	< 0.0001*	0.680	< 0.0001*	0.984
Drying	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*	< 0.0001*

Positive correlations (values > 0.9) were found between the following attributes at each tasting time (Appendix 2.4). T0: drying and cooked milk, drying and mouthcoating, mouthcoating and sour, mouthcoating and chalky, mouthcoating and cooked milk, chalky and sour, chalky and cooked milk, cooked milk and sour. T30: drying and mouthcoating, chalky and sour, mouthcoating and cooked milk, cooked milk and sour. T60: drying and cooked milk, drying and mouthcoating, mouthcoating and cooked milk, chalky and sour, cooked milk and sour.

Strong correlations of attributes associated with heating with cooked milk flavour are expected as this is a distinct attribute of heated WPC caused by the modification of flavour by heating. More correlations were observed at T0 than T30 and T60, which show that not all attributes have prolonged sensations which correlate to the scoring during consumption. This shows the need to study the aftereffect scoring alongside the scoring during consumption. Drying and mouthcoating were correlated during consumption and both aftereffect scores, future training on these attributes is needed to ensure the attributes are separate to all panellists.

Panel performance was monitored and assessor interactions were observed for all attributes with the exception of bitter and metallic (Appendix 2.5). Further panel training was required before further experiments to reduce attribute interactions. The use of unstructured line scales leads to a natural variation in use of the scale, particularly as panellists were asked to score the sample in relation to their experience of milk-based beverages. The panel used 50% of the scale on

average, which arose from the difference in use of the scaling system per person, and the lack of extreme sensations experienced from the WPC beverages in relation to other milk based drinks.

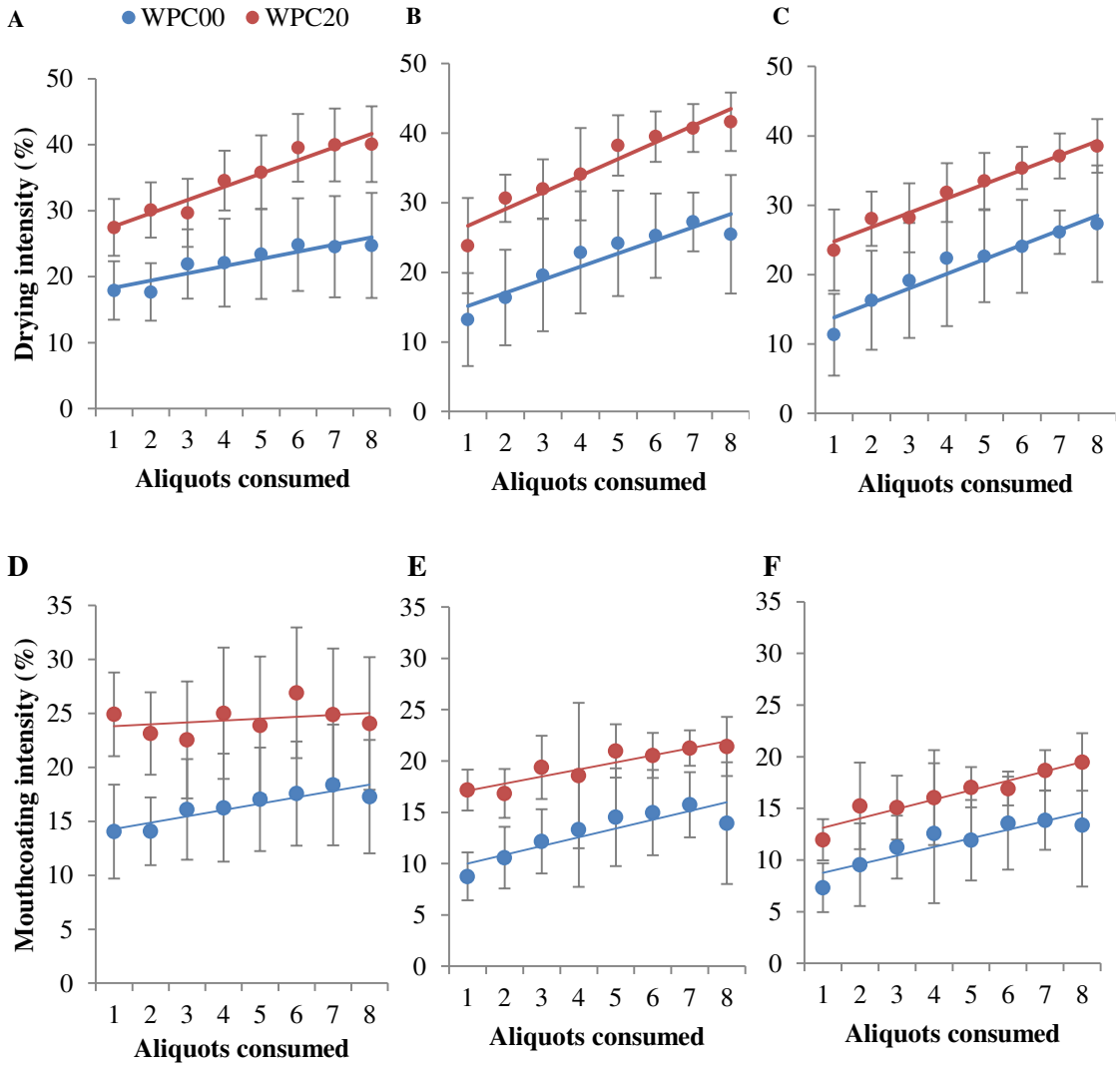

Figure 2.4: Mean intensities scored during consumption from sequential profiling of WPC samples over 8 repeated consumptions. A: drying T0; B: drying T30; C: drying T60; D: mouthcoating T0; E: mouthcoating T30; F: mouthcoating T60.. Error bars represent ± 2 SEM.

2.3 Conclusion

A general increase in particle size was observed with heating time, with a maximum at 15 min. In order to represent a range of samples, heating times chosen for further experiments were: 0, 5, 10 and 20 min, to represent a range including unheated and representing a range of different particle sizes.

The sensory results showed significant differences were detectable between WPC00 and WPC20. Sequential profiling was a suitable method to observe an increase in intensity over repeated consumption, with significant differences between the two samples. Drying and

mouthcoating were found to significantly increase over repeated consumption and to persist between sips.

The sensory methods used in this section will be implemented in Chapter 2 Part 2 using WPC heated for varying times at 70 °C (0, 5, 10, 20 min) to compare the effect of heating on mouthfeel attributes, with a focus on the build-up of drying in the mouth.

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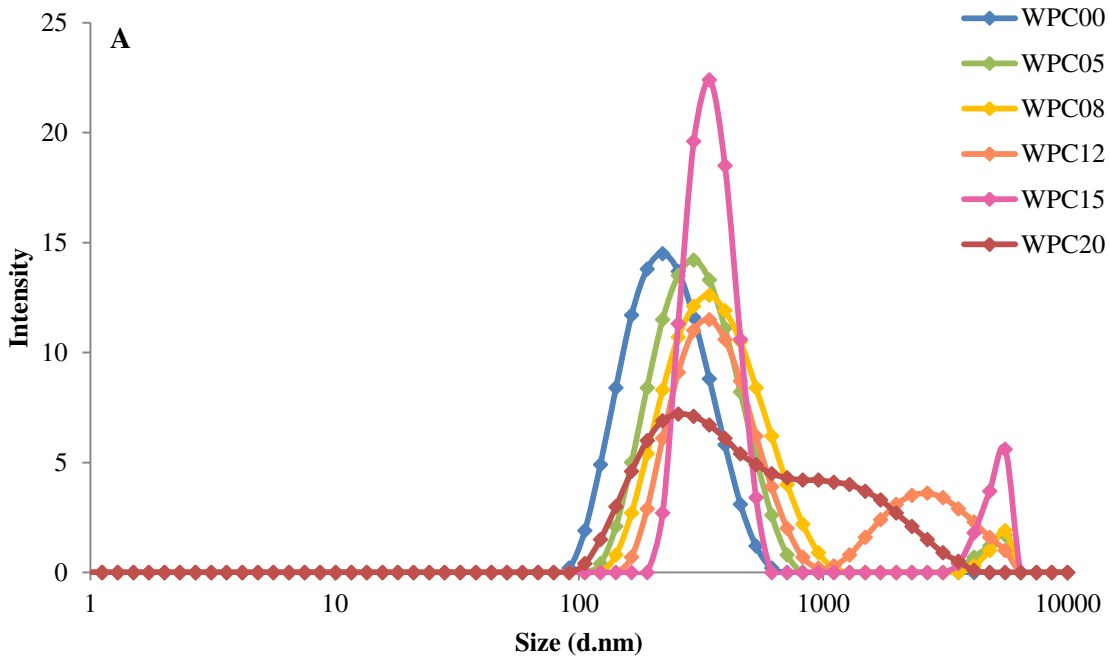
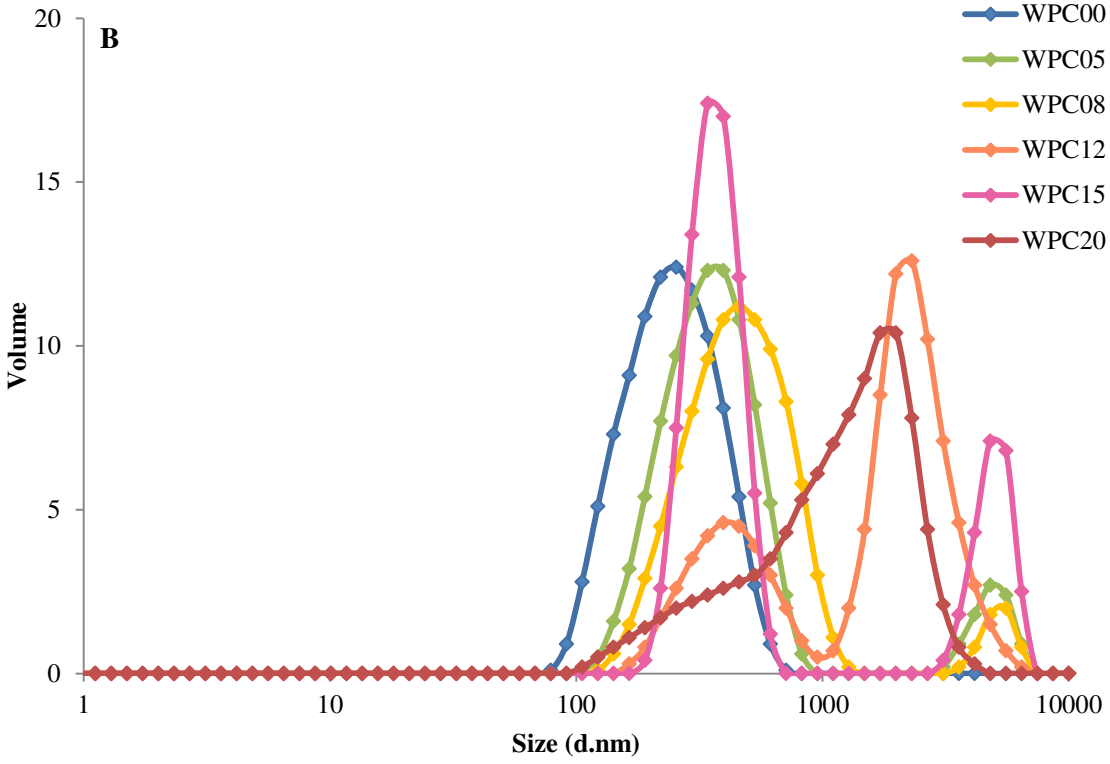
Appendix

Appendix 2.1: Size distribution of WPC heated at 70 °C for varying lengths of time shown as a function of A: intensity; and B: volume. Volume distributions are shown as a contrast to intensity distributions as they tend to skew towards smaller sizes, whereas intensity distributions are skewed towards larger sizes. Particle size is shown as a logarithmic scale.





Appendix 2.2: QDA attributes and reference descriptions or standard, with results for WPC00 and WPC20. * significant differences between samples ($p \leq 0.05$).

Modality	Attribute	Reference description	WPC00	WPC20
Appearance	Beige colour	Degree of beige colour intensity	14.8	47.8*
Appearance	Body	Fullness of sample	30.3	20.0*
Appearance	Opacity	Overall opacity of sample	95.0	81.2*
Odour	Biscuit (baked cereal)	Baked cereal element of a digestive biscuit	20.3	19.6
Odour	Cooked butter	Unsalted butter fully melted	22.4	17.9
Odour	Cooked milk	Semi-skimmed milk heated in a microwave for 3 mins	47.5	22.0*
Odour	Crème fraîche	Crème fraîche	9.5	3.3*
Odour	Powdered milk (wet)	10% skim powdered milk in deionised water	26.1	12.8*
Odour	White chocolate	White chocolate (Nestlé, Milkybar)	17.3	5.2*
Taste	Sour	Citric acid (0.76 g/L)	11.9	6.7*
Taste	Bitter	Quinine (0.04 g/L)	4.7	6.6
Taste	Metallic	Iron (II) sulfate (0.0036 g/L)	2.8	7.0*
Taste	Salty	Sodium chloride (1.19 g/L)	1.5	3.4
Taste	Sweet	Sucrose (5.76 g/L)	12.1	10.5
Taste	Umami	Monosodium glutamate (0.29 g/L)	4.0	7.5
Flavour	Biscuit (baked cereal)	Baked cereal element of a digestive biscuit	12.4	16.2
Flavour	Cooked butter	Unsalted butter fully melted	15.1	13.8
Flavour	Cooked milk	Semi-skimmed milk heated for 3 min	40.0	23.8
Flavour	Crème fraîche	Crème fraîche	6.3	1.0*
Flavour	Mozzarella	Buffalo mozzarella	22.9	3.7*
Flavour	Powdered milk (wet)	10% skim powdered milk in deionised water	17.4	10.0*
Flavour	Whey isolate	WPI90 (5% in deionised water; Volac)	16.8	24.8
Mouthfeel	Astringency	Puckering of the cheeks	19.2	12.8
Mouthfeel	Body	Fullness of sample	25.7	15.8*
Mouthfeel	Chalky	Dry fine insoluble powder	24.7	7.7*
Mouthfeel	Drying	The absorbance of moisture from the mouth	40.6	21.8*
Mouthfeel	Furring	Rough ‘furry’ texture on tongue and mouth	33.1	10.4*
Mouthfeel	Mouthcoating	Degree of coating of the mouth	39.1	16.9*
Mouthfeel	Oily/fatty	Degree of oily sensation	8.8	7.4
Aftereffect	Aftertaste strength	The strength of the overall aftertaste	28.3	17.5*
Aftereffect	Bitter	Quinine (0.04 g/L)	3.9	2.7
Aftereffect	Drying	The absorbance of moisture from the mouth	35.7	16.3*
Aftereffect	Furring	Rough ‘furry’ texture on tongue and mouth	30.8	9.1*
Aftereffect	Metallic	Iron (II) sulfate (0.0036 g/L)	2.6	5.9
Aftereffect	Sour	Citric acid (0.76 g/L)	8.9	4.6*

Appendix 2.3: Mean intensities for WPC00 and WPC20 sequential profiling data. Superscript letters in a column indicate significantly different groupings ($p \leq 0.05$).

	Aliquot	Sour	Metallic	Bitter	Cooked Milk	Mouthcoating	Drying	Chalky
WPC00	1	8.9^{ab}	6.9^{a}	5.7^{a}	20.9^{bc}	14.1^{c}	17.9^{d}	4.3^{c}
	2	9.7^{ab}	5.8^{a}	4.6^{a}	18.4^{c}	14.1^{c}	17.7^{d}	4.2^{c}
	3	8.2^{b}	6.4^{a}	4.6^{a}	20.9^{bc}	16.1^{bc}	21.9^{cd}	5.0^{c}
	4	9.9^{ab}	7.8^{a}	5.6^{a}	21.0^{bc}	16.3^{bc}	22.1^{cd}	4.7^{c}
	5	8.4^{b}	8.0^{a}	5.6^{a}	21.2^{bc}	17.0^{bc}	23.4^{bcd}	5.6^{bc}
	6	10.1^{ab}	8.1^{a}	5.8^{a}	22.2^{abc}	17.6^{bc}	24.8^{bcd}	4.6^{c}
	7	10.8^{ab}	8.5^{a}	4.5^{a}	23.1^{abc}	18.4^{abc}	24.5^{bcd}	5.2^{c}
	8	9.4^{ab}	8.7^{a}	4.8^{a}	21.0^{bc}	17.3^{bc}	24.7^{bcd}	4.6^{c}
WPC20	1	18.6^{a}	6.6^{a}	6.3^{a}	30.2^{abc}	24.9^{ab}	27.5^{abcd}	14.4^{ab}
	2	16.9^{ab}	6.5^{a}	4.8^{a}	31.1^{abc}	23.1^{abc}	30.1^{abcd}	14.9^{a}
	3	18.1^{ab}	6.2^{a}	4.4^{a}	33.8^{ab}	22.6^{abc}	29.7^{abcd}	14.9^{a}
	4	17.0^{ab}	5.5^{a}	4.8^{a}	33.4^{ab}	25.0^{ab}	34.6^{abc}	15.7^{a}
	5	14.8^{ab}	5.8^{a}	4.9^{a}	33.8^{ab}	23.9^{ab}	35.8^{ab}	15.5^{a}
	6	16.4^{ab}	4.9^{a}	5.6^{a}	32.2^{ab}	26.9^{a}	39.5^{a}	15.9^{a}
	7	16.9^{ab}	5.7^{a}	5.0^{a}	34.7^{a}	24.9^{ab}	40.0^{a}	12.2^{abc}
	8	17.1^{ab}	6.1^{a}	4.0^{a}	32.5^{ab}	24.1^{ab}	40.1^{a}	10.8^{abc}

Appendix 2.4: Correlation matrices for sequential profiling attributes. T0, T30 and T60 correlation matrices shown separately.

T0	Drying	Cooked Milk	Sour	Chalky	Mouthcoating	Bitter	Metallic
Drying	-	0.90	0.79	0.78	0.92	-0.23	-0.59
Cooked Milk	0.90	-	0.94	0.94	0.95	-0.19	-0.66
Sour	0.79	0.94	-	0.93	0.93	-0.12	-0.63
Chalky	0.78	0.94	0.93	-	0.93	-0.06	-0.70
Mouthcoating	0.92	0.95	0.93	0.93	-	-0.06	-0.61
Bitter	-0.23	-0.19	-0.12	-0.06	-0.06	-	0.20
Metallic	-0.59	-0.66	-0.63	-0.70	-0.61	0.20	-

T30	Chalky	Sour	Cooked Milk	Drying	Mouthcoating	Bitter	Metallic
Chalky	-	0.93	0.89	0.72	0.79	-0.20	-0.66
Sour	0.93	-	0.91	0.78	0.88	0.01	-0.55
Cooked Milk	0.89	0.91	-	0.86	0.92	-0.05	-0.43
Drying	0.72	0.78	0.86	-	0.97	0.07	-0.41
Mouthcoating	0.79	0.88	0.92	0.97	-	0.14	-0.46
Bitter	-0.20	0.01	-0.05	0.07	0.14	-	0.38
Metallic	-0.66	-0.55	-0.43	-0.41	-0.46	0.38	-

T60	Chalky	Sour	Mouthcoating	Drying	Cooked Milk	Bitter	Metallic
Chalky	-	0.92	0.80	0.81	0.80	-0.25	-0.78
Sour	0.92	-	0.88	0.89	0.93	0.02	-0.59
Mouthcoating	0.80	0.88	-	0.99	0.95	0.15	-0.46
Drying	0.81	0.89	0.99	-	0.93	0.18	-0.46
Cooked Milk	0.80	0.93	0.95	0.93	-	0.22	-0.45
Bitter	-0.25	0.02	0.15	0.18	0.22	-	0.58
Metallic	-0.78	-0.59	-0.46	-0.46	-0.45	0.58	-

Appendix 2.5: Panel performance by attribute and time point. Assessor by sample interactions are shown, *p*-values show a significant interaction when $p < 0.05$.

Attribute	Time point	Assessor*Sample *p*-value
Bitter	T0	0.311
	T30	0.711
	T60	0.272
Chalky	T0	<0.0001
	T30	0.004
	T60	0.008
Cooked milk	T0	<0.0001
	T30	0.048
	T60	0.175
Drying	T0	<0.0001
	T30	0.010
	T60	0.418
Metallic	T0	0.998
	T30	0.770
	T60	0.284
Mouthcoating	T0	0.001
	T30	0.0001
	T60	0.092
Sour	T0	<0.0001
	T30	<0.0001
	T60	0.0002

Chapter 2 Part 2. Whey protein mouth drying influenced by thermal denaturation

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Experiments were carried out by S. P. Bull with the assistance of Y. Hong, a master's student under S. P. Bull and L. Methven's supervision. V.V. Khutoryanskiy, J. K. Parker, M. Faka, and L. Methven edited the manuscript.

Abstract

Whey proteins are becoming an increasingly popular functional food ingredient. There are, however, sensory properties associated with whey protein beverages that may hinder the consumption of quantities sufficient to gain the desired nutritional benefits. One such property is mouth drying. The influence of protein structure on the mouthfeel properties of milk proteins has been previously reported. This paper investigates the effect of thermal denaturation of whey proteins on physicochemical properties (viscosity, particle size, zeta-potential, pH), and relates this to the observed sensory properties measured by qualitative descriptive analysis and sequential profiling. Mouthcoating, drying and chalky attributes built up over repeated consumption, with higher intensities for samples subjected to longer heating times ($p \leq 0.05$). Viscosity, pH, and zeta-potential were found to be similar for all samples; however particle size increased with longer heating times. As the pH of all samples was close to neutral, this implies that neither the precipitation of whey proteins at low pH, nor their acidity, as reported in previous literature, can be the drying mechanisms in this case. The increase in mouth drying with increased heating time suggests that protein denaturation is a contributing factor and a possible mucoadhesive mechanism is discussed.

2.4 Introduction

Whey proteins are becoming an increasingly popular functional food, due to associated health benefits such as the provision of amino acids essential for muscle synthesis (Norton, Wilson, Layman, Moulton, & Garlick, 2012). Recently, whey proteins have been widely utilised in sports nutrition (Wolfe, 2000), the prevention of sarcopenia in elderly and malnourished patients (Dangin et al., 2003), and in a newly developing market for general health and lifestyle products (Chungchunlam, Henare, Ganesh, & Moughan, 2014; Fekete, Givens, & Lovegrove, 2013). The successful use of whey proteins as an aid to muscle growth depends on a consistent intake over an extended period of time (Rahemtulla et al., 2005); therefore the sensory properties of whey protein beverages are of significant importance to ensure a sufficient consumption of protein is achieved. Studies have shown that the mouthfeel of whey protein beverages contributes to the disliking and, therefore, refusal of whey protein beverages, with textural properties being the main reason for 19% of trial discontinuations (of the 56% who completed the questionnaire) (Gosney, 2003). In order to reduce this figure, the mouthfeel properties responsible must be addressed. One major textural aspect of whey proteins is astringency, which was described as a 'textural defect' of dairy products in a 1994 review (Lemieux & Simard). The use of the terms *drying* and *astringency* are often seen as interchangeable, however astringency can often be used to cover a range of different mouthfeel sensations (Gawel, Oberholster, & Francis, 2000), or to specifically refer to "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM, 2004), which are not present in whey proteins. In this paper the observed sensation of the drying of the mouth will simply be referred to as drying, whereas astringency refers specifically to the puckering of the cheeks.

The nature of the drying sensation elicited by whey proteins is currently unknown, although there have been mechanisms proposed in the literature. As many commercially available whey protein beverages are low pH, the inherent astringency of acidity not the whey proteins themselves, was suggested as the origin of whey protein beverage drying (Lee & Vickers, 2008). An alternative theory is that the interactions between positively charged whey proteins at low pH and negatively charged saliva proteins causes whey protein beverage drying. This can explain the observed correlation between the lowering the pH of whey protein solutions and an increase in both turbidity and drying (Beecher, Drake, Luck, & Foegeding, 2008), and the observation that low pH whey protein beverages are more drying than equivalent pH buffer solutions (Vardhanabhuti, Kelly, Luck, Drake, & Foegeding, 2010). A more recent study elaborates on this theory by proposing that the contribution of salivary proteins to whey protein aggregates at low pH in the mouth reduces the amount of salivary proteins available for oral lubrication; this therefore creates the drying sensation (Ye, Streicher, & Singh, 2011). A

variation of this theory proposes the disruption of salivary structure as the cause for astringency in whey protein (Vardhanabhuti, Cox, Norton, & Foegeding, 2011).

Another proposed mechanism is linked to the binding of whey proteins to the oral mucosa (Celebioglu et al., 2015). A study supporting this mechanism found that two whey proteins, β-lactoglobulin (β-LG) and lactoferrin, bound to human oral epithelial cells (Ye, Zheng, Ye, & Singh, 2012). An in vitro study, measuring the binding of two milk proteins, β-LG and casein, to porcine mucosa using fluorescence microscopy, attributed the drying sensation to mucoadhesion (Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013). Mucoadhesion is the adherence of materials to mucosal membranes, which in this context is the binding of whey proteins to the oral mucosa: the cheeks, gums and tongue. Mucoadhesion occurs via intermolecular forces (electrostatic attraction, hydrophobic interactions and hydrogen bonding) and some covalent bonding such as disulphide bond formation (Andrews, Laverty, & Jones, 2009; Smart, 2005; Sosnik, das Neves, & Sarmento, 2014). The unfolding of whey proteins during denaturation exposes hydrophobic regions and thiol groups (Iametti, DeGregori, Vecchio, & Bonomi, 1996), which could therefore increase the strength of mucoadhesive binding.

As whey proteins are heated they undergo thermal denaturation. This occurs at various temperatures due to the structural differences between the individual proteins in whey. The most abundant protein in bovine whey, β-LG, has a critical temperature of denaturation of 70 °C (Dewit & Swinkels, 1980), with aggregation occurring when temperatures over 70 °C are sustained (Iametti et al., 1996). The denaturation of whey proteins has previously been linked to astringency (Josephson, Thomas, Morr, & Coulter, 1967); this may result from increased hydrophobic interactions or disulphide bonds with the oral mucosae which increase mucoadhesion, finally resulting in increased drying sensation (Hsein, Garrait, Beyssac, & Hoffart, 2015). We hypothesise that particle size will increase upon denaturation due to aggregation, that this will not affect particle charge, but that it will have an effect upon the sensory perception of the sample.

The present study aimed to explore the relationship between denaturation of whey proteins and sensory attributes related to mouth drying. The build-up of sensory properties was analysed using sequential profiling as an indication of potential mucoadhesion.

2.5 Materials and methods

The whey protein concentrate (WPC) used was Volactive Ultrawhey 80 Instant (Volac International Limited, Orwell, Royston, UK), a dry powder with a protein content of 80% minimum, and containing soy lecithin (0.5% maximum) as an emulsifying agent. The remaining

20% contains moisture, fat, lactose, and minerals. Crackers (Carr's Table Water Biscuits, United Biscuits, London, UK) were used as palate cleansers in sensory profiling.

2.5.1 Preparation of whey protein beverages

WPC beverages were prepared by addition of WPC powder to water (10% w/v, deionised water). The dilution selected is recommended for many commercially available powders, and represents a serving of 20 g of protein per 250 mL portion, which has been linked to nutritional benefits (Tipton et al., 2007). All samples were stirred for 30 min at room temperature (25 ± 2 °C). A native sample was then stirred for a further 60 min at room temperature (WPC00). Three samples were stirred while being heated in a water bath set at 70 °C for 5, 10 and 20 min (WPC05, WPC10, and WPC20 respectively). A heating temperature of 70 °C was selected as the critical temperature of denaturation for β-LG, the most abundant whey protein (Dewit & Swinkels, 1980). The samples were cooled in a water bath then allowed to hydrate overnight at 4 °C. The pH of all samples ranged from 6.5 to 6.7 (Mettler Toledo SevenEasy, Switzerland; 22 ± 3 °C) and absorbance of light (680 nm; diluted 50 times in water) was measured to quantify sample opacity (Table 2.3). Measurements were performed in triplicate on each of three processing replicates prepared on three separate days.

2.5.2 Instrumental analysis methods

All instrumental measurements were performed in triplicate on each of three processing replicates prepared on three separate days.

2.5.2.1 Rheology

Rheological properties of WPC samples were analysed using an oscillatory rheometer (AR2000, TA Instruments, USA) fitted with a 40 mm diameter rotating plate adjusted to 37 °C. Samples were placed on the lower plate surface and equilibrated to 37 °C. Strain sweeps of the samples were obtained by applying oscillation at a frequency of 2 Hz for strain values ranging from 0.01 to 10 in 12 steps. A strain of 1% was then chosen in the linear viscoelastic region for a frequency sweep, where the frequency was varied from 0.1 to 10 Hz in 25 steps.

2.5.2.2 Dynamic light scattering

WPC samples were diluted 100 times in water (HPLC grade water) for dynamic light scattering (DLS) analysis and measurements were performed using Nano-S Zetasizer (Malvern Instruments, UK) at 30 °C, with an equilibration time of 60 s.

To determine whether any sample sedimentation occurred during the time taken to perform a sensory evaluation, WPC samples were left to stand for 1 hour, and then the upper 1 mL and lower 1 mL were assessed using the DLS technique described above.

2.5.2.3 Zeta-potential

WPC samples were diluted 100 times in water (HPLC grade water) for ζ-potential measurements, which were performed using Nano-S Zetasizer (Malvern Instruments, UK) at 30 °C with an equilibration time of 60 s.

2.5.3 Sensory methods

A trained sensory panel of experts in profiling techniques (n = 11; 10 female, 1 male), with a minimum of 6 months training, were given further training on WPC profiling and sequential profiling (minimum 5 hours). Training needs were assessed from previous panel performance monitoring in Chapter 2 Part 1 (Appendix 2.5). Sensory evaluation was carried out at room temperature (25 ± 2 °C) in isolated booths.

2.5.3.1 Quantitative descriptive analysis

Quantitative descriptive analysis (QDA) (Stone, Sidel, Oliver, Woolsey, & Singleton, 1974) was performed using a consensus vocabulary developed by the panel during training (34 attributes; 3 appearance, 6 odour, 6 taste, 6 flavour, 6 mouthfeel, 6 aftereffects, Appendix 2.3). The panel assigned mouthfeel characteristics in order to separate important attributes describing distinct sensations. These consensus mouthfeel attributes were: body; furring, the roughening of the tongue; chalky, to describe the sensation of particulate matter; mouthcoating; astringency, specific to the puckering of the cheeks; and drying, to describe the sensation of the reduction of saliva in the mouth.

WPC samples were evaluated in duplicate according to a balanced design using unstructured line scales with appropriate anchors. Samples were presented monadically in opaque white cups (20 mL), crackers and warm filtered tap water were provided as palate cleansers between samples during an enforced break (2 min). Evaluation was carried out under artificial daylight.

2.5.3.2 Sequential profiling

Sequential profiling was carried out to establish the perception of seven sensory attributes over repeated consumption of eight aliquots (5 mL) of samples, with 1-minute breaks between aliquots. Samples were scored after consumption of each aliquot (T0), and following 30 (T30) and 60 s (T60) time delays, as described by Methven et al. (2010) (Compusense at-hand, Ontario, Canada). Thus there are eight aliquots tasted for each of four samples (WPC00, WPC05, WPC10 and WPC20), scored at three time points (T0, T30 and T60).

The seven attributes scored were bitter, sour, metallic, cooked milk flavour, mouthcoating, chalky and drying. The maximum number of attributes that we recommend to score within one sequential profiling session is 7, determined through training with the panel. These were chosen

carefully from the full QDA profile. Bitter, sour and metallic are taste attributes associated with whey protein beverages (Martini & Walsh, 2012; Whetstine, Croissant, & Drake, 2005). Cooked milk flavour was selected as this attribute showed significant differences between samples in the QDA data as both an odour and flavour attribute. Mouthcoating, chalky and drying were selected by the panel as dominant mouthfeel attributes, and the QDA data showed increases upon heating for all three attributes.

Samples were coded with three-digit numbers and all eight aliquots of one sample were presented together with the same code; the panellists were not blinded to the sequential nature of the evaluation. Warm filtered tap water and crackers were provided as palate cleansers in the 2 minute enforced break between samples; however panellists were instructed not to use these between the eight aliquots of the same sample. Panellists were instructed to consume the total volume of each aliquot and to coat the mouth with the sample before swallowing. Two samples were scored in each session. Evaluation was carried out under red lighting and aliquots were served in opaque black cups to mask appearance differences between samples. Nine of the trained panellists were present for sequential profiling.

2.5.4 Statistical analysis

SENPAQ (version 5.01) was used to carry out analysis of variance (ANOVA) of QDA data. IBM SPSS Statistics (version 21) was used to carry out three-way repeated measures ANOVA (RM-ANOVA) on the sequential profiling data using sample (n = 4), assessors (n = 9) and repeated consumption (n = 8) as explanatory variables. Analytical data were analysed by one-way ANOVA using IBM SPSS Statistics (version 21). Tukey's test was used for any posthoc analysis. To account for differences between panellists in QDA, the error is divided by the assessor interaction; however this is not possible when analysing repeated measures data from sequential profiling. Panel performance was monitored for both QDA and sequential profiling using SenPAQ (QI Statistics, UK) (Appendix 2.9).

Rates of incline were calculated for sequential profiling data using the gradient of the line of best fit across repeated consumption using IBM SPSS Statistics (version 21). Relative strengths of aftereffect were calculated to give the aftereffect scores as a percentage of the T0 score in order to indicate the intensity of aftereffect in comparison to the intensity scored during consumption:

$$Relative\ strength = \frac{Tn}{T0} \times 100\%$$

where n = 30 or 60.

2.6 Results

2.6.1 Instrumental analysis

A general increase in absorbance at 680 nm was seen with heating time, indicating an increase in turbidity, which would be expected due to an increase in aggregation. pH values were consistent across all samples, and were near-neutral pH (Table 2.3).

Table 2.3: Heating time, pH, and absorbance of light at 680 nm (samples diluted 50 times in water) for WPC samples.Errors represent ± 2 standard deviations. Superscript letters in a column indicate significantly different groupings ($p \leq 0.05$).

Sample	Heating time at 70 °C (min)	pH	Absorbance at 680 nm
WPC00	0	6.60 ± 0.04[a]	0.098 ± 0.021[a]
WPC05	5	6.62 ± 0.04[a]	0.149 ± 0.025[b]
WPC10	10	6.63 ± 0.04[a]	0.170 ± 0.023[b]
WPC20	20	6.64 ± 0.03[a]	0.222 ± 0.088[b]

2.6.1.1 Rheology

WPC samples were found to have similar viscosities at frequencies between 0.1 and 10 Hz (Figure 2.5), showing no significant difference between them ($p > 0.05$).

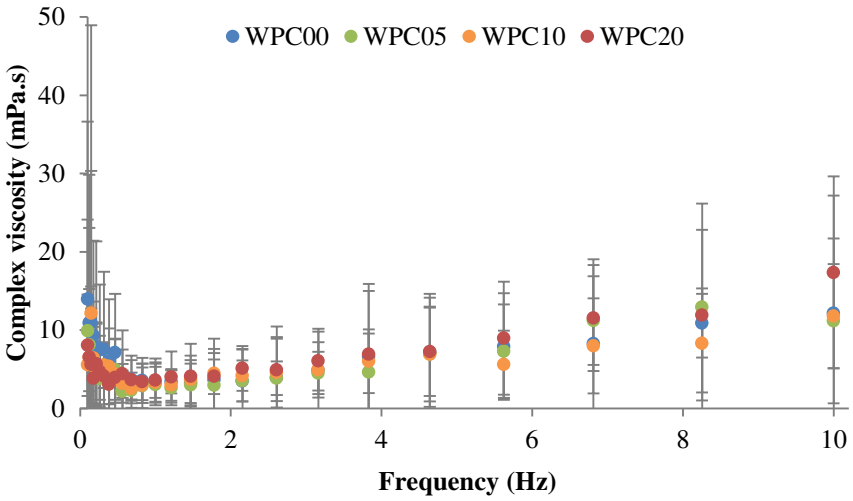

Figure 2.5: A frequency sweep at a strain of 1% for WPC samples, showing rheological behaviour across a frequency range of 0.1 to 10 Hz. Error bars represent ± 2 standard deviations.

2.6.1.2 Dynamic light scattering and ζ-potential measurements

A general increase in particle size diameter (z-average) with an increase in heating time was observed, significant differences were found between all samples, with the exception of WPC05 and WPC10 ($p \leq 0.05$). No significant difference was found between Z-average values of the upper and lower 1 mL of sensory samples (taken from a 5 mL sample). Sample charges were determined to be negative for all WPC samples, with no significant difference between ζ-potential magnitudes ($p > 0.05$). These findings are summarised in Table 2.4.

Table 2.4: The z-averages of WPC samples measured from the bulk, upper 1 mL and lower 1 mL of samples allowed to stand, as measured by DLS; and the ζ-potentials of WPC samples. Errors represent ± 2 standard deviations.

Sample	Z-average (nm)			ζ -potential (mV)
	Bulk sample	Upper sample	Lower sample	
WPC00	220 ± 16	224 ± 11	219 ± 11	-27.7 ± 3.1
WPC05	272 ± 15	293 ± 15	282 ± 10	-26.7 ± 2.6
WPC10	288 ± 19	299 ± 26	289 ± 24	-27.0 ± 3.9
WPC20	317 ± 71	335 ± 25	321 ± 21	-26.2 ± 4.0

2.6.2 Sensory data

2.6.2.1 QDA data

Of the 34 attributes evaluated, 15 were significantly different between samples, as outlined in Appendix 2.3. The appearance attributes were important, as any visual differences between samples would require masking for an unbiased evaluation. Significant differences were found for both beige colour and body appearance attributes, therefore red light was a requirement for further evaluation of samples by sequential profiling. Taste attributes showed little or no change across samples with increasing heating times. Across biscuit (baked cereal), cooked butter and cooked milk odour and flavour attributes, WPC00 had a higher intensity score than WPC05, however there was an upward trend in intensity across WPC05 to WPC10 and WPC20 (scores for odour attributes shown in Figure 2.6). A significant increase in intensity of mouthfeel attributes was seen for samples with longer heating times, including drying but with the exception of astringency (see Figure 2.7).

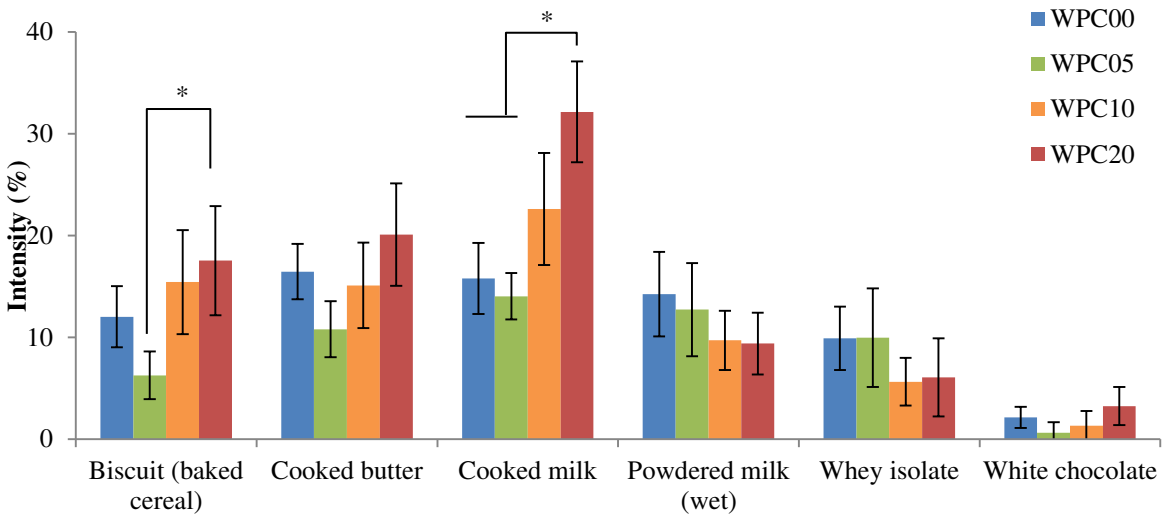

Figure 2.6: QDA intensities for selected odour attributes for WPC samples. Error bars represent ± 2 standard error of the mean. *Significantly different scores between samples ($p \leq 0.05$) calculated through ANOVA.

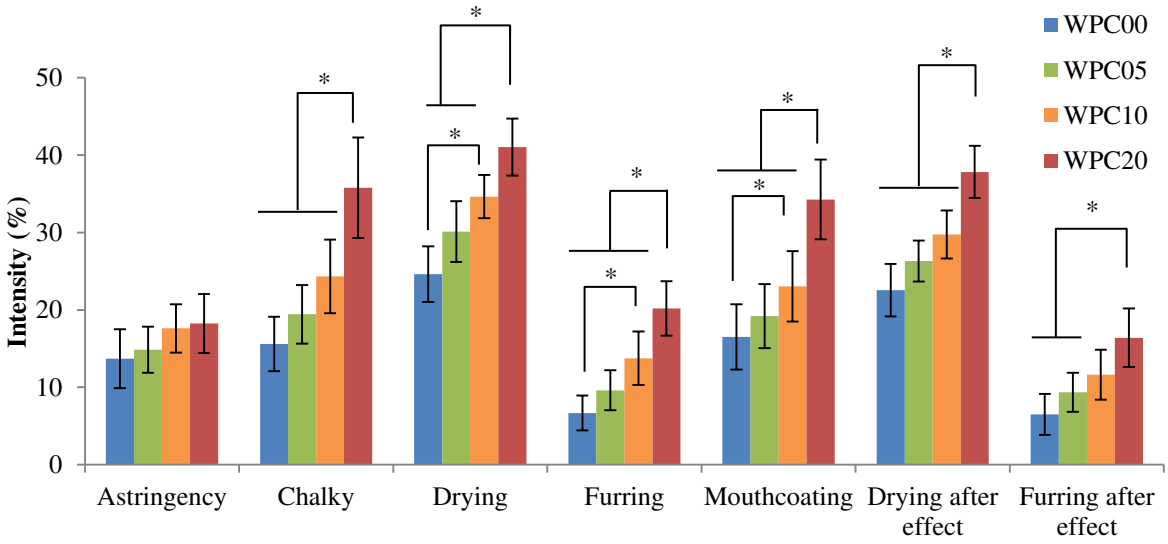

Figure 2.7: QDA intensities for mouthfeel and aftereffect attributes related to mouth drying for WPC samples. Error bars represent ± 2 standard error of the mean. *Significantly different scores between samples ($p \leq 0.05$) calculated through ANOVA.

2.6.2.2 Sequential profiling data

Data from sequential profiling was collected to observe the change in intensity of attributes over repeat consumption of 40 mL of WPC samples. Significant differences ($p \leq 0.05$) between WPC samples were found overall for drying, mouthcoating and chalky attributes: WPC00 had a significantly lower drying score than the heated WPC samples; WPC10 and WPC20 were found to have significantly higher mouthcoating intensities than WPC05, which had a significantly higher mouthcoating intensity than WPC00. A general increase in chalky intensity was observed for samples with longer heating times (Figure 2.8). These data support those collected by QDA.

These attributes were also found to increase significantly with repeated consumption, with rates of incline (Δ intensity/aliquot) ranging from 0.7 (WPC00 chalky T0) to 2.9 (WPC10 drying T60) (average rates of incline shown in Table 2.5). These attributes were all positively correlated with correlation coefficients greater than 0.9 (Appendix 2.8).

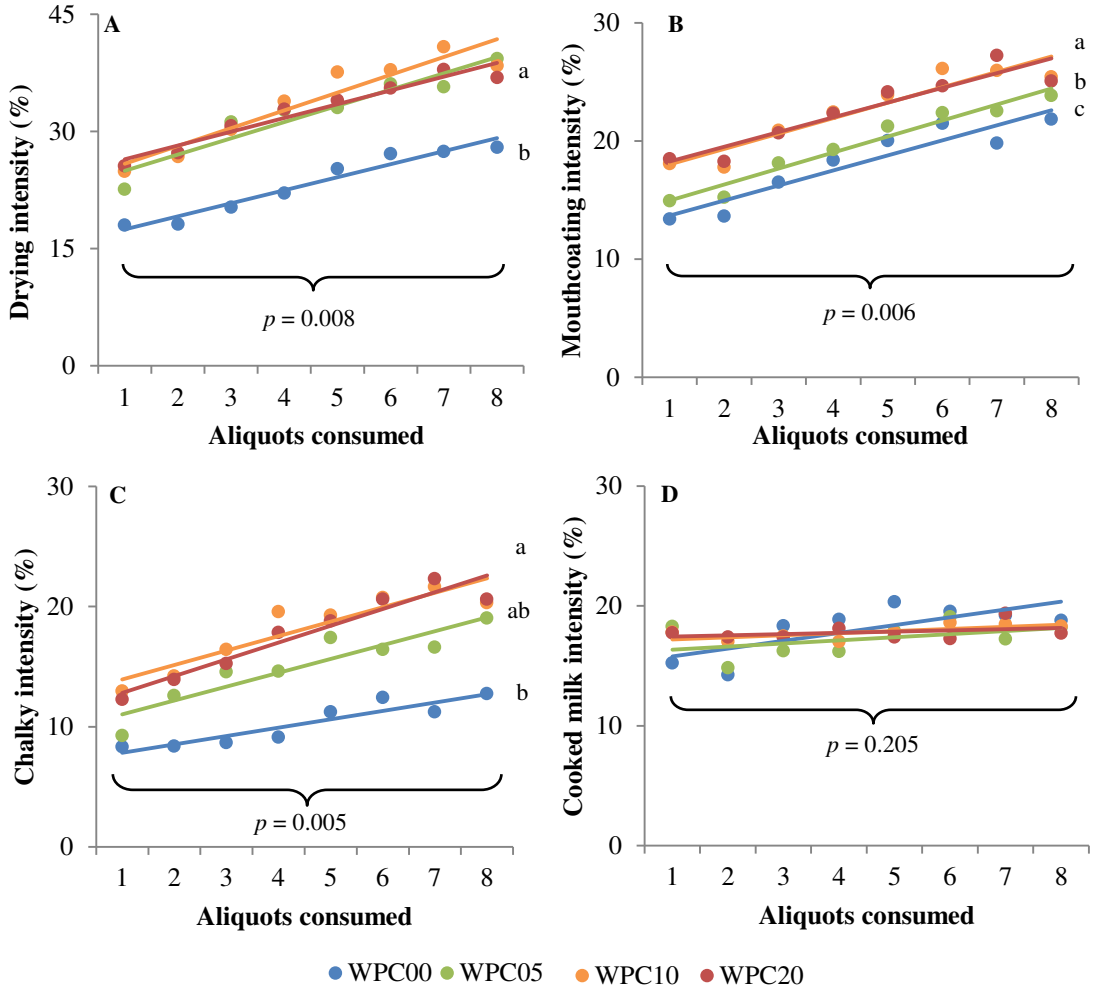

Figure 2.8: Mean intensities scored during consumption (T0) from sequential profiling of WPC samples over 8 repeated consumptions. Letters denote significantly different sample groupings as calculated by RM-ANOVA; *p*-values are shown for significant changes over repeated consumption. A: drying. Rates of incline: WPC00, 1.7 (R^2 = 0.951; WPC05, 2.1 (R^2 = 0.919); WPC10, 2.3 (R^2 = 0.898); WPC20, 1.8 (R^2 = 0.942). B: mouthcoating. Rates of incline: WPC00, 1.3 (R^2 = 0.882); WPC05, 1.4 (R^2 = 0.959); WPC10, 1.3 (R^2 = 0.891); WPC20, 1.3 (R^2 = 0.886). C: chalky. Rates of incline: WPC00, 0.7 (R^2 = 0.855); WPC05, 1.2 (R^2 = 0.850); WPC10, 1.2 (R^2 = 0.844); WPC20, 1.4 (R^2 = 0.920). D: cooked milk. Rates of incline: WPC00, 0.7 (R^2 = 0.549); WPC05, 0.3 (R^2 = 0.204); WPC10, 0.2 (R^2 = 0.492); WPC20, 0.1 (R^2 = 0.125).

Table 2.5: Significance levels over repeat consumption for tasting (T0) and aftertaste at 30 (T30) and 60 s (T60), from RM-ANOVA of sequential profiling data. The average rates of incline (Δ intensity/aliquot) are shown beside significance levels. Significance levels between samples are shown: no significant difference; and a significant difference, $p \leq 0.05$ (*).

Attribute	Sample significant differences			Rate of incline over repeat consumption		
	T0	T30	T60	T0	T30	T60
Cooked Milk	ns	ns	ns	ns (0.3)	ns (0.4)	* (0.4)
Sour	ns	ns	ns	ns (0.2)	* (0.5)	* (0.5)
Metallic	ns	ns	ns	ns (0.1)	* (0.2)	* (0.2)
Bitter	ns	ns	ns	* (0.4)	* (0.4)	* (0.5)
Chalky	*	*	*	* (1.1)	* (1.3)	* (1.3)
Drying	*	*	*	* (1.9)	* (2.4)	* (2.4)
Mouthcoating	*	*	*	* (1.3)	* (1.6)	* (1.6)

In contrast, cooked milk, bitter, sour, and metallic attributes showed no significant differences between samples neither during consumption nor during aftertaste ratings (Table 2.5). Some significant differences (detail in Table 2.5) were seen for these attributes across repeated consumption: bitter T0, T30, T60; metallic and sour T30, T60; cooked milk T60. Rates of incline (Δ intensity/aliquot) ranged from 0.2 (WPC00 metallic T0) to 0.7 (WPC20 cooked milk T30) (Table 2.5).

To quantify the relative strength of the intensity scores for aftertaste results, the mean intensity scores at T30 and T60 were calculated as percentages of the equivalent T0 score (Table 2.6). This provides a comparison of how much the attribute intensity increased or decreased post consumption. These results showed high aftertaste scores for drying (95-112% intensity compared to the T0 score), mouthcoating (89-104%), chalky (83-101%), and bitter (81-96%); and lower aftertaste scores for metallic (63-91%), sour (64-82%) and cooked milk (56-77%); (drying and cooked milk represented graphically in Figure 2.9).

Table 2.6: Relative strength of aftertaste, expressed as a percentage of the T0 score, for T30 and T60. Values are shown for the eighth aliquot scores for all attributes.

Attribute	T30				T60			
	WPC00	WPC05	WPC10	WPC20	WPC00	WPC05	WPC10	WPC20
Cooked Milk	65%	65%	62%	77%	61%	59%	56%	71%
Sour	73%	82%	80%	65%	71%	64%	70%	59%
Metallic	63%	89%	84%	87%	63%	76%	91%	63%
Bitter	91%	94%	74%	83%	87%	84%	81%	96%
Chalky	98%	92%	101%	95%	94%	89%	97%	83%
Drying	103%	99%	105%	112%	96%	95%	101%	97%
Mouthcoating	92%	104%	99%	101%	89%	99%	95%	96%

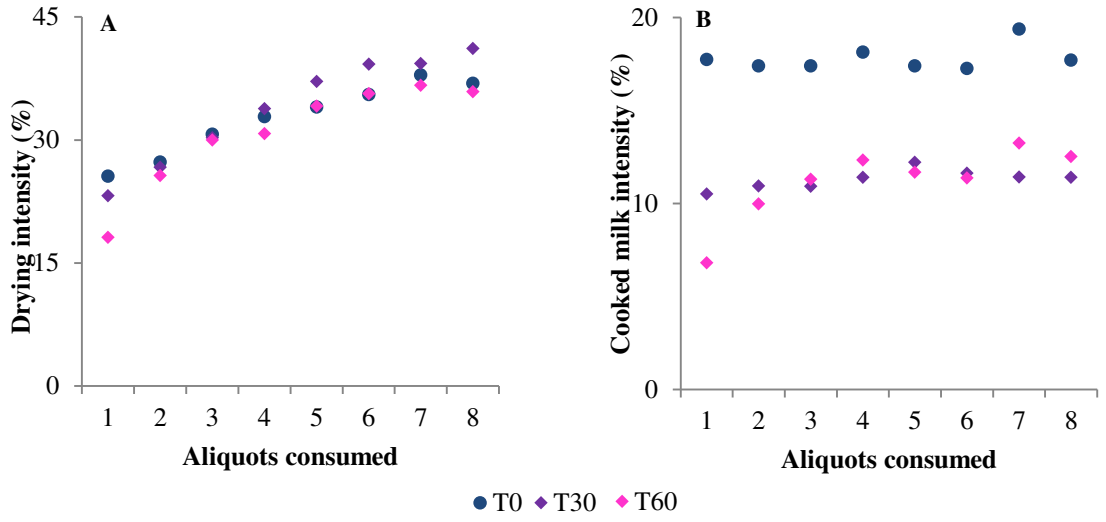

Figure 2.9: Mean intensities scored for WPC20 showing T0, T30, and T60 as separate data sets for A: drying, where little difference is seen between T0, T30 and T60; and B: cooked milk, where a decrease in intensity is observed for T30 and T60 in comparison to T0.

2.7 Discussion

The range of significantly different attributes between samples evaluated by QDA shows that the heat treatment of WPC samples can significantly affect the sensory profile. For example, as little heating as possible would be recommended in order to decrease the amount of mouth drying sensation in a product; whereas heating a sample at 70 °C would be preferable to

increase the amount of cooked milk odour in a product. The majority of significant differences were found in appearance, mouthfeel and aftereffect attributes (Appendix 2.3).

Odour and flavour attributes which showed significant differences between samples were: biscuit (baked cereal) (odour only); cooked butter (flavour only); and cooked milk (both flavour and odour). These attributes followed a trend of a general increase in intensity with samples that had longer heating times, with the exception of WPC00, which scored higher than WPC05, and in some cases WPC10 or WPC20 (Figure 2.6). The proposed reason for this is that upon initial heating, existing volatile flavour molecules are initially lost as the vapour pressure of the sample is increased; therefore a decrease in flavour and odour attributes is seen between WPC00 and WPC05. However upon further heating of the sample, new volatile molecules are created through the release of thiol compounds from denatured proteins, the pyrolysis of sugar, and the degradation of amino acids, among other heat-induced mechanisms (Calvo & de la Hoz, 1992). Further experiments would be required to study the changes in flavour chemistry upon heating.

The differences in appearance attributes for samples are important as these required masking for sequential profiling. As a result, samples were presented monadically in opaque black cups under red lighting. The upper and lower 1 mL of sensory samples were analysed using DLS to ensure that sedimentation was not significant over the time taken to evaluate samples. There was no significant difference found between samples, and therefore no sedimentation effect was likely to have occurred during sensory evaluation.

The samples needed to have similar viscosities in order to control the sensory experiments, as differences in viscosity can affect sensory perception of both mouthfeel and texture attributes (Courregelongue, Schlich, & Noble, 1999), however in previous research by Beecher et al., viscosity was not found to have an effect on drying (2008). The rheological analysis of WPC samples showed no difference between samples over frequencies of 0.1 to 10 Hz, corresponding to oral shear rates predicted in the mouth (Cutler, Morris, & Taylor, 1983; Shama & Sherman, 1973). This is partially reflected by the QDA results for the body mouthfeel attribute: no significant differences were found between samples with the exception of WPC20, which scored higher than the other samples.

The QDA found that all mouthfeel attributes showed a general trend of increasing attribute intensity with samples that had undergone longer heating times, and all mouthfeel attributes except astringency showed some differentiation between samples (Figure 2.7; Appendix 2.3). We concluded that chalky, drying, furring and mouthcoating all increased upon heating. These attributes were all positively correlated. The sequential profiling results also concluded that

chalky, mouthcoating and drying increased with repeated consumption, which could contribute to the drying sensations preventing full consumption of whey protein beverages (Gosney, 2003).

The significant difference found for drying between WPC00 and the three heated samples (WPC05, WPC10 and WPC20) proves the hypothesis that drying increases with heating. This finding also indicates that samples which have been heated for over 5 min at 70 °C have a significantly increased intensity of drying, which could be caused by the denaturation of whey proteins at these conditions.

The sequential profiling results for mouthcoating support a mechanism of whey protein mucoadhesion. Samples showed significant differences for mouthcoating: WPC05 scored significantly higher than WPC00; and WPC10 and WPC20 scored significantly higher than WPC05. These differences indicate that mouthcoating significantly increases before 5 to 10 minutes of heating at 70 °C, however there is no difference seen for further heating from 10 to 20 min. This increase in mouthcoating could be due to an increase in mucoadhesion caused by the denaturation of protein in the samples (Hsein et al., 2015). The increase of mouthcoating and drying over repeated consumption also supports the mucoadhesion theory, as the observed build-up of these attributes suggests a physical increase of sensation-causing substance in the mouth, which would be consistent with mucoadhesion. The aftertaste intensities (T30 and T60) are high for chalky, mouthcoating and drying, demonstrating that these sensations are just as prominent once the sample has been swallowed. This has been previously reported in whey-rich ingredients by Withers et al. (2014), and could be due to mucoadhesion of the whey proteins.

DLS can be used to measure the average particle size in solution, and to determine the change in particle size upon aggregation of a sample. The aggregation of isolated whey proteins, in particular β-LG, has been well studied using light scattering techniques (Elofsson, Dejmek, & Paulsson, 1996; Mehalebi, Nicolai, & Durand, 2008); however when heating WPC, which contains a mixture of the different whey proteins, and other constituents such as lactose, fats and minerals, different denaturation and aggregation behaviour is observed. When heated in the presence of other whey proteins β-LG forms both homopolymers and heteropolymers (Havea, Singh, & Creamer, 2001), and in the presence of caseins large micelles can be formed upon aggregation (Havea, 2006). This formation of large particles could explain the large particle sizes observed for the WPC samples, in comparison to those formed by isolated proteins. The z-averages calculated from DLS measurements give an indication to the average particle size in WPC samples, however as the sample is unlikely to be monodisperse due to the range of constituents in the mixture, these values are provided only as a comparative guide to the change observed upon heating of samples. A positive correlation was observed between the heating time and z-average particle size, with significant differences found between samples. This

increase is likely to be caused by an increase in the size of aggregates caused by higher levels of denaturation.

The observation that larger particle sizes can contribute to the "astringency" of whey protein has been previously reported by Ye and Singh (2011). The increase observed in chalky upon heating could be caused by the increase in particle size with longer heating times, however the increase in chalky over repeated consumption indicates that particle size is not the sole contributor to mouthfeel attributes in the samples, which could be caused by a build-up of these particles by mucoadhesion.

All WPC samples were found to have a negative charge with ζ-potentials of similar magnitude, which is expected due to the similar pH of samples. As the samples have similar ζ-potentials, it is unlikely that the differences in drying perception between WPC samples in this study arise from electrostatic interactions with saliva proteins, as predicted by Ye et al. (2011). Although the electrostatic interactions could still be occurring, the differences between the samples must be caused by another mechanism.

2.8 Conclusions

Whey protein samples were heated for varying times (0, 5, 10 and 20 min; 70 °C) and the pH, viscosity, particle size, and ζ-potential were measured. All WPC samples were found to have similar pH, viscosity and ζ-potentials, indicating that previously proposed mechanisms for whey protein drying based on these properties (Lee & Vickers, 2008; Vardhanabhuti et al., 2010; Ye et al., 2011) cannot explain the changes in drying and related attributes which varied significantly between WPC samples. The z-averages of WPC samples increased with longer heating times, indicative of aggregation caused by the denaturation of a mixture of whey proteins (Havea et al., 2001).

Drying, mouthcoating and chalky attributes were found to increase for samples with longer heating times, with the intensity of these attributes building up with the repeated consumption of sample. These findings are compatible with the proposed mechanism of mucoadhesion as the source of whey drying, supported by previous studies (Withers et al., 2013), and denaturation increasing mucoadhesive strength (Hsein et al., 2015).

Further research is required to determine the mucoadhesive properties of whey proteins. Investigations will be carried out to establish the mechanism of action for the adhesion of whey proteins to the oral mucosa, how this is influenced by protein structure and denaturation, and how a drying sensation is elicited by this mechanism.

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Appendices

Appendix 2.6: QDA attributes and reference descriptions or standard, with results for WPC samples.Superscript letters in a column indicate significantly different groupings ($p \leq 0.05$).

Modality	Attribute	Reference description	WPC00	WPC05	WPC10	WPC20
Appearance	Beige colour	Degree of beige colour intensity	40.2 ^{a}	26.5 ^{b}	21.3 ^{bc}	13.1 ^{c}
Appearance	Body	Fullness of sample	20.5 ^{b}	23.1 ^{ab}	24.5 ^{ab}	27.4 ^{a}
Appearance	Opacity	Overall opacity of sample	82.9	86.5	85.2	89.7
Odour	Biscuit (baked cereal)	Baked cereal element of a digestive biscuit	12.0 ^{ab}	6.3 ^{b}	15.4 ^{ab}	17.5 ^{a}
Odour	Cooked butter	Unsalted butter fully melted	16.5	10.8	15.1	20.1
Odour	Cooked milk	Semi-skimmed milk heated in a microwave for 3 mins	15.8 ^{b}	14.0 ^{b}	22.6 ^{b}	32.1 ^{a}
Odour	Powdered milk (wet)	10% skim powdered milk in deionised water	14.2	12.7	9.7	9.4
Odour	Whey isolate	WPI90 (5% in deionised water; Volac)	9.9	10.0	5.6	6.1
Odour	White chocolate	White chocolate (Nestlé, Milkybar)	2.1	0.6	1.3	3.3
Taste	Sour	Citric acid (0.76 g/L)	14.2	14.1	15.3	17.2
Taste	Bitter	Quinine (0.04 g/L)	15.5	13.0	15.1	15.0
Taste	Metallic	Iron (II) sulfate (0.0036 g/L)	14.3	13.1	13.6	11.3
Taste	Salty	Sodium chloride (1.19 g/L)	2.4	1.6	3.4	2.8
Taste	Sweet	Sucrose (5.76 g/L)	5.8 ^{ab}	5.0 ^{b}	6.0 ^{ab}	8.8 ^{a}
Taste	Umami	Monosodium glutamate (0.29 g/L)	2.6	1.4	2.2	2.9
Flavour	Biscuit (baked cereal)	Baked cereal element of a digestive biscuit	11.2	8.0	11.9	11.8
Flavour	Cooked butter	Unsalted butter fully melted	13.4 ^{a}	6.5 ^{b}	8.1 ^{ab}	12.8 ^{ab}
Flavour	Cooked milk	Semi-skimmed milk heated for 3 min	15.6 ^{b}	12.3 ^{b}	19.3 ^{ab}	25.3 ^{a}
Flavour	Powdered milk (wet)	10% skim powdered milk in deionised water	12.7	11.7	11.2	11.2
Flavour	Whey isolate	WPI90 (5% in deionised water; Volac)	8.9	9.0	7.2	3.8
Flavour	White chocolate	White chocolate (Nestlé, Milkybar)	2.0	0.5	1.5	1.8
Mouthfeel	Astringency	Puckering of the cheeks	13.7	14.9	17.6	18.2
Mouthfeel	Body	Fullness of sample	17.2 ^{b}	18.0 ^{b}	18.5 ^{b}	30.2 ^{a}
Mouthfeel	Chalky	Dry fine insoluble powder	15.6 ^{b}	19.4 ^{b}	24.3 ^{b}	35.8 ^{a}
Mouthfeel	Drying	The absorbance of moisture from the mouth	24.6 ^{c}	30.1 ^{bc}	34.6 ^{ab}	41.0 ^{a}
Mouthfeel	Furring	Rough ‘furry’ texture	6.7 ^{c}	9.6 ^{bc}	13.8 ^{b}	20.2 ^{a}

		on tongue and mouth				
Mouthfeel	Mouth-coating	Degree of coating of the mouth	16.5 [c]	19.2 [bc]	23.0 [b]	34.3 [a]
Aftereffect	Aftertaste strength	The strength of the overall aftertaste	18.4	17.5	20.7	21.9
Aftereffect	Bitter	Quinine (0.04 g/L)	6.2	6.3	7.1	9.0
Aftereffect	Drying	The absorbance of moisture from the mouth	22.5 [b]	26.3 [b]	29.7 [b]	37.8 [a]
Aftereffect	Furring	Rough 'furry' texture on tongue and mouth	6.5 [b]	9.4 [b]	11.6 [ab]	16.4 [a]
Aftereffect	Metallic	Iron (II) sulfate (0.0036 g/L)	10.7	8.2	7.9	8.2
Aftereffect	Sour	Citric acid (0.76 g/L)	3.9 [b]	4.9 [ab]	5.7 [ab]	7.4 [a]

Appendix 2.7: . The rate of incline (Δ intensity/aliquot) for each attribute shown by sample and time point (T0, T30 and T60).

	Rate of incline over repeat consumption											
Attribute	**WPC00**			**WPC05**			**WPC10**			**WPC20**		
	T0	**T30**	**T60**	**T0**	**T30**	**T60**	**T0**	**T30**	**T60**	**T0**	**T30**	**T60**
Cooked Milk	0.7	0.5	0.5	0.3	0.2	0.3	0.2	0.1	0.2	0.1	0.7	0.7
Sour	0.2	0.5	0.5	0.3	0.6	0.4	0.1	0.5	0.6	0.2	0.3	0.5
Metallic	-0.2	0.2	0.2	0.3	0.4	0.3	0.1	0.2	0.2	0.1	0.1	0.1
Bitter	0.3	0.4	0.5	0.6	0.5	0.5	0.3	0.3	0.4	0.3	0.4	0.6
Chalky	0.7	0.9	1.0	1.2	1.4	1.3	1.2	1.5	1.7	1.4	1.4	1.2
Drying	1.7	2.0	1.9	2.1	2.2	2.3	2.3	2.8	2.9	1.8	2.6	2.4
Mouthcoating	1.3	1.5	1.5	1.4	1.8	1.7	1.3	1.7	1.9	1.3	1.6	1.5

Appendix 2.8: Correlation matrices for sequential profiling attributes. T0, T30 and T60 correlation matrices shown separately.

T0	Chalky	Drying	Mouthcoating	Bitter	Cooked Milk	Sour	Metallic
Chalky	-	0.95	0.91	0.66	0.19	0.32	-0.16
Drying	0.95	-	0.90	0.74	0.27	0.27	-0.18
Mouthcoating	0.91	0.90	-	0.72	0.52	0.47	0.01
Bitter	0.66	0.74	0.72	-	0.29	0.47	0.03
Cooked Milk	0.19	0.27	0.52	0.29	-	0.34	0.02
Sour	0.32	0.27	0.47	0.47	0.34	-	0.12
Metallic	-0.16	-0.18	0.01	0.03	0.02	0.12	-

T30	Chalky	Drying	Bitter	Mouthcoating	Metallic	Sour	Cooked Milk
Chalky	-	0.96	0.77	0.94	0.62	0.60	0.48
Drying	0.96	-	0.84	0.96	0.66	0.68	0.57
Bitter	0.77	0.84	-	0.82	0.68	0.71	0.54
Mouthcoating	0.94	0.96	0.82	-	0.75	0.78	0.66
Metallic	0.62	0.66	0.68	0.75	-	0.71	0.46
Sour	0.60	0.68	0.71	0.78	0.71	-	0.70
Cooked Milk	0.48	0.57	0.54	0.66	0.46	0.70	-

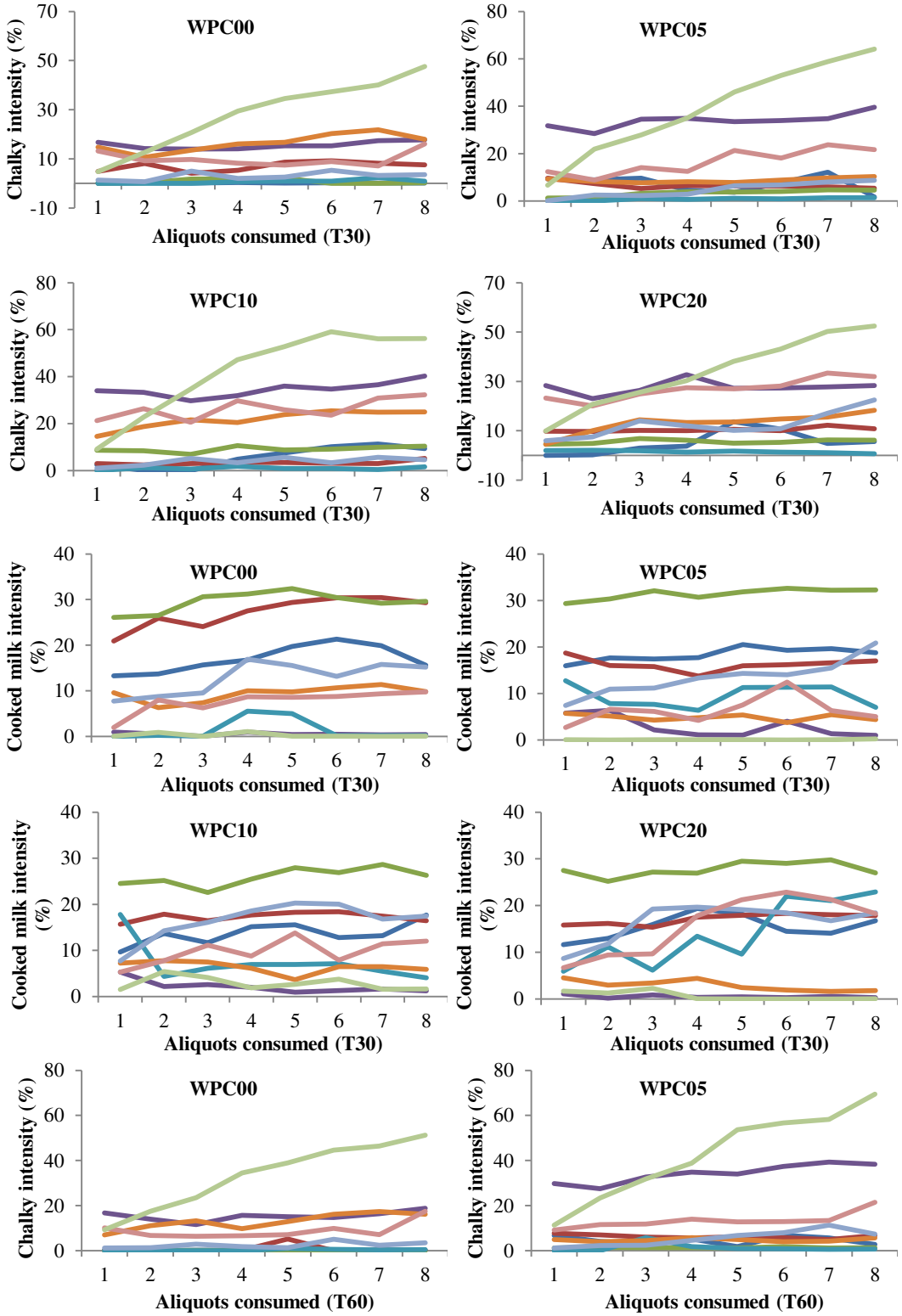
T60	Chalky	Drying	Mouthcoating	Bitter	Metallic	Sour	Cooked Milk
Chalky	-	0.96	0.93	0.83	0.66	0.60	0.56
Drying	0.96	-	0.96	0.89	0.69	0.65	0.65
Mouthcoating	0.93	0.96	-	0.89	0.76	0.77	0.74
Bitter	0.83	0.89	0.89	-	0.69	0.68	0.75
Metallic	0.66	0.69	0.76	0.69	-	0.68	0.57
Sour	0.60	0.65	0.77	0.68	0.68	-	0.78
Cooked Milk	0.56	0.65	0.74	0.75	0.57	0.78	-

Appendix 2.9: Panel performance by attribute and time point for all four WPC samples. Assessor by sample and sample by repeat interactions are shown, *p*-values show a significant interaction when $p < 0.05$.

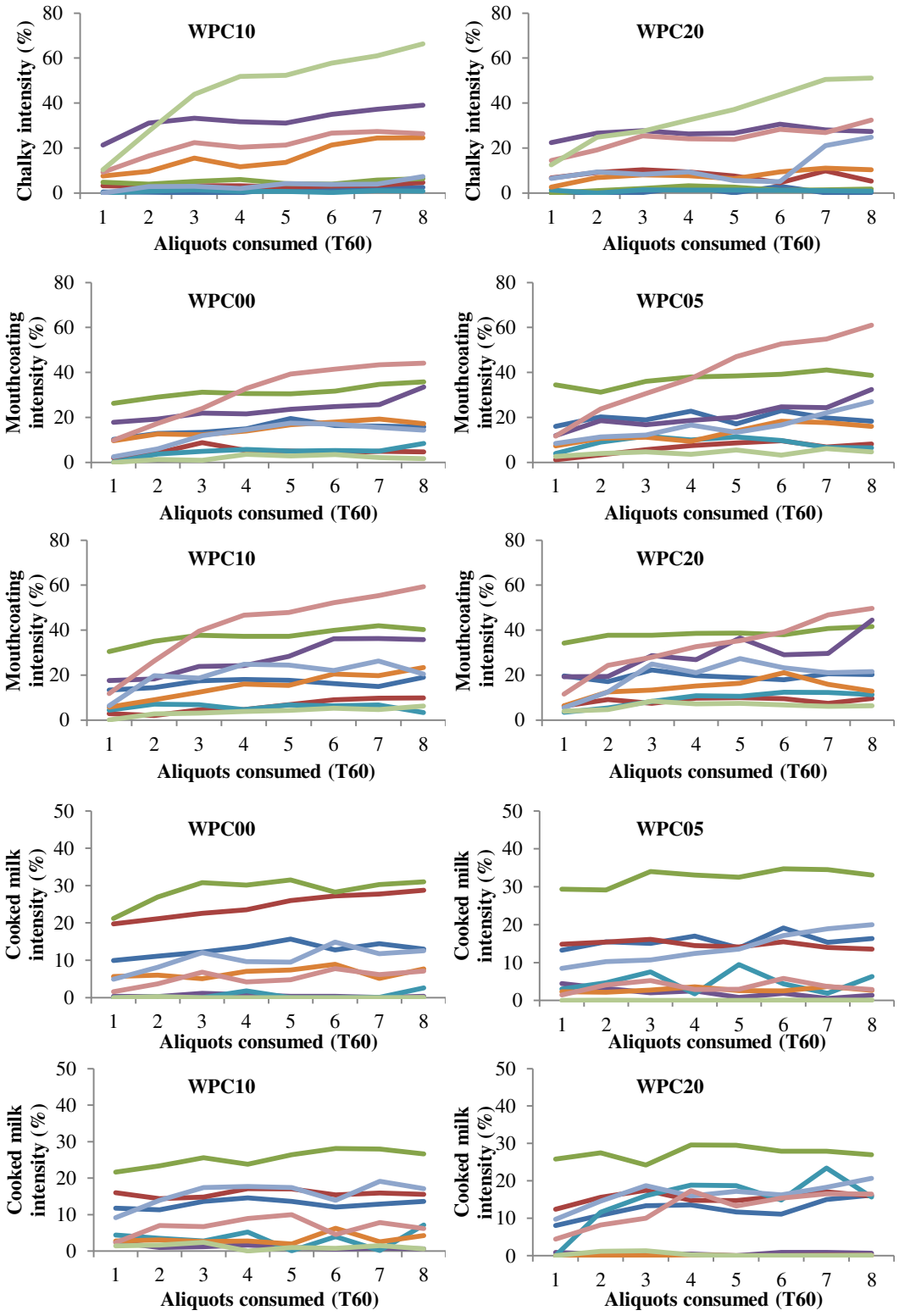
Attribute	Time point	Assessor*Sample *p*-value	Sample*Repeat *p*-value
Bitter	T0	1.000	0.395
	T30	1.000	0.448
	T60	1.000	0.264
Chalky	T0	0.736	0.412
	T30	0.021	0.849
	T60	0.001	0.950
Cooked milk	T0	1.000	0.788
	T30	0.010	0.555
	T60	<0.0001	0.157
Drying	T0	0.981	0.538
	T30	0.902	0.752
	T60	0.384	0.441
Metallic	T0	0.980	0.226
	T30	0.995	0.415
	T60	0.999	0.577
Mouthcoating	T0	1.000	0.876
	T30	0.328	0.777
	T60	0.033	0.863
Sour	T0	0.983	0.186
	T30	1.000	0.287
	T60	1.000	0.075

No assessor interactions were observed across T0, however significant interactions were seen for: T30 chalky and cooked milk; T60 chalky, cooked milk and mouthcoating. These are shown in Appendix 2.10, and it can be seen that while assessors show different use of scale, there is no change in order of effect. It is noted that the panellists used the scales to different degrees, as they were instructed to judge attributes on their experiences. Individual differences in perception and physiological factors such as saliva flow will affect these scores.

Appendix 2.10: Mean scores for panellist repeats over repeated consumption at T30 and T60. Chalky and cooked milk are shown for T30 and chalky, mouthcoating and cooked milk are shown for T60 as they had significant assessor interactions. Each line represents one panellist.



Appendix 2.10 continued: Mean scores for panellist repeats over repeated consumption at T30 and T60. Chalky and cooked milk are shown for T30 and chalky, mouthcoating and cooked milk are shown for T60 as they had significant assessor interactions. Each line represents one panellist.



Chapter 3 Oral retention of thermally treated whey protein and an investigation of mechanisms

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Abstract

This study investigated the *in vivo* retention of whey protein in the oral cavity by measuring the concentration of protein in expectorated saliva at intervals over 5 minutes post swallow. Heated whey protein was shown to have a higher retention time in the oral cavity compared to unheated whey protein up to 1 minute post swallow. The structural changes on thermal treatment of whey protein concentrate were studied using nuclear magnetic resonance and circular dichroism to determine whether any structural differences were apparent which may lead to mucoadhesion. Accessible thiol groups were analysed using Ellman's assay, and free ionic calcium was measured, concluding that the increase in free thiol associated with thermal processing of whey protein concentrate may contribute to mechanisms leading to oral retention of whey protein, while no change in free ionic calcium was observed.

3.1 Introduction

Whey protein is often heated during processing; both during the production of a spray dried powder, and subsequently during the incorporation into finished products such as drinks and bars. The heating of whey protein at high temperatures can cause denaturation, which is linked to an increase in the perception of mouthdrying (Chapter 2 Part 2). Whey protein provides an important source of protein to patients at risk of sarcopenia; however an increase in mouthdrying has been linked to a reduction in compliance (Gosney, 2003; Withers, Gosney, & Methven, 2013). Mouthdrying in whey protein has been attributed to interactions with salivary proteins (Vardhanabhuti, Cox, Norton, & Foegeding, 2011; Vardhanabhuti & Foegeding, 2010; Ye, Streicher, & Singh, 2011), and more recently to interactions with the oral mucosa, a phenomenon known as mucoadhesion (Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013; Ye, Zheng, Ye, & Singh, 2012). An *in vitro* dynamic model found an increase in turbidity associated with the addition of whey protein isolate to artificial or whole human saliva, which was related to higher scores of astringency (Andrewes, Kelly, Vardhanabhuti, & Foegeding, 2011), supporting a mechanism of mucoadhesion as the cause of mouthdrying in whey protein beverages.

Whey protein concentrate (WPC) is a spray-dried powder of 80% protein, which additionally contains lactose, calcium and lipids. As a complex mixture, there are many factors which affect WPC denaturation. When heating WPC, the different components are able to interact and influence the denaturation of proteins: for example, β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and bovine serum albumin (BSA) form both homopolymers and heteropolymers when heated together (Havea, Singh, & Creamer, 2001). These polymers form large particles, which could lead to an increase in astringency (Ye et al., 2011). While changes in particle size occur upon heating, changes in whey protein particle shape can also be observed in whey proteins, as particles go from spherical to fibrillar structures (Krebs, Devlin, & Donald, 2009), which could increase mucoadhesion through entanglement of the fibrils with mucosal proteins (Jabbari, Wisniewski, & Peppas, 1993; Peppas & Buri, 1985).

The presence of calcium in WPC also affects thermal denaturation and aggregation through calcium bridges between negative charges on proteins, shielding of negative charges, and increasing hydrophobic interactions (Havea, Singh, & Creamer, 2002). The increased interactions due to calcium, lead to fewer disulfide bonding during aggregation, and larger particle sizes (Riou, Havea, McCarthy, Watkinson, & Singh, 2011), both of which may affect mucoadhesive strength.

The denaturation of proteins can alter the number of free accessible thiol groups due to protein unfolding, disulfide bond formation, and aggregation. The total thiol content of β-LG increases

upon heating at pH 3, but decreases at pH 5 – 7, as there are more sulfhydryl groups at low pH (Rahaman, Vasiljevic, & Ramchandran, 2015), due to the unfolding of the protein, revealing a buried cysteine residue (Zeiler & Bolhuis, 2015). Polymers containing thiol groups can form disulfide bonds with mucosal surfaces, leading to increased mucoadhesive strength (Bernkop-Schnurch, 2005); therefore altering the number of free accessible thiols in WPC through thermal denaturation could affect mucoadhesive interactions.

Mucoadhesion can occur through many mechanisms: covalent bonding, including formation of disulfide bridges (Bernkop-Schnurch, 2005); non-covalent interactions, such as Van der Waals forces, hydrogen bonding and hydrophobic interactions (Mikos & Peppas, 1989); and electrostatic forces (Derjaguin, Aleinikova, & Toporov, 1994; Derjaguin, Toporov, Muller, & Aleinikova, 1977). Mucoadhesion can be measured using a range of techniques: physical techniques, such as rheology, change in particle size and charge, and change in structure observed by circular dichroism (CD) (Celebioglu et al., 2015; Takeuchi et al., 2005; Thirawong, Kennedy, & Sriamornsak, 2008); *in vitro* methods, such as measuring wash-off and tribology methods (Cave, Cook, Connon, & Khutoryanskiy, 2012; Dresselhuis, de Hoog, Stuart, & van Aken, 2008); and *in vivo* oral retention methods (Cook, Woods, Methven, Parker, & Khutoryanskiy, 2018).

CD has been used to study whey protein structure by measuring the effect of chiral samples on circularly polarised light to predict secondary structural features. CD has been used in the literature both for individual whey proteins (Celebioglu et al., 2015; Chandrapala, Zisu, Kentish, & Ashokkumar, 2012; Chen et al., 2005; Vermeer & Norde, 2000; Wada, Fujita, & Kitabatake, 2006; Wijesinha-Bettoni et al., 2007), and for whey protein mixtures, such as isolate or concentrate (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Liu & Zhong, 2013; Tomczynska-Mleko et al., 2014). Effects of thermal treatment on whey protein has been observed using CD, showing a decrease in α-helical structure and an increase in unfolding upon heating (Tomczynska-Mleko et al., 2014; Wada et al., 2006; Wijesinha-Bettoni et al., 2007). CD results in this study found a decrease in the near-UV CD spectra with increased heating time at 70 °C, corresponding to aromatic moieties, and a change in the α-helical region of the far-UV CD spectra.

The factors affecting mucoadhesion are varied, especially in a system as complex as WPC. This study aims to investigate the effect of thermal processing on the retention time of whey protein concentrate beverages in the oral cavity, and the structural and physicochemical characteristics which may underpin the oral retention. It is hypothesised that WPC with higher heating times will have higher oral retention, which may be caused by either increased free ionic calcium,

increased accessible thiol groups, or a change in secondary or tertiary structure leading to increased intermolecular interactions.

The aim of this study is to measure the oral retention of thermally treated model whey protein concentrate beverages, in order to determine a relationship between thermal denaturation and mucoadhesion. The structural and physicochemical characteristics of the samples were analysed to investigate the underlying mechanism of adhesion.

3.2 Materials and methods

The whey protein concentrate (WPC) used was Volactive Ultrawhey 80 Instant (Volac International Limited, Orwell, Royston, UK), a dry powder with a minimum protein content of 80%, and containing soy lecithin (0.5% maximum) as an emulsifying agent. The remaining 20% contains moisture (5%), fat (7%), lactose (4%), and minerals.

DTNB (5,5-dithio-bis-(2-nitrobenzoic acid), deuterium oxide, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, and L-cysteine hydrochloride monohydrate were supplied by Sigma-Aldrich (Dorset, UK).

3.2.1 Preparation of whey protein beverages

Model WPC beverages were prepared by addition of WPC powder to water (10% w/v, deionised water). All samples were stirred for 30 min at room temperature (25 ± 2 ^{0}C). A native sample was then stirred for a further 60 min at room temperature (WPC00). Three samples were stirred while being heated in a water bath set at 70 °C for 5, 10 and 20 min (WPC05, WPC10, and WPC20 respectively). The samples were cooled in a water bath until they reached room temperature, then allowed to hydrate overnight at 4 °C. The pH of all samples ranged from 6.5 to 6.7 (Mettler Toledo SevenEasy, Switzerand; 22 ± 3 ^{0}C).

3.2.2 In vivo protein retention method

An *in vivo* retention study was used as a measure of mucoadhesion. Five healthy volunteers were recruited; four males and one female, aged between 25 and 30. Before consumption, each volunteer rinsed their mouth with a salt solution (1% w/v) to clear the mouth of any particulate matter. Saliva was collected for each sample and time point in triplicate during separate sessions. Three samples were selected from Chapter 2 (WPC00, WPC05, and WPC20) to represent a range of sensory attributes associated with drying and physical characteristics.

The study was given a favourable opinion for conduct by the University of Reading, School of Chemistry, Food and Pharmacy (study number 27/15, Appendix 3.1 & 3.2). Previous studies

have indicated that 5 subjects is sufficient for such *in vivo* studies where difference in retention due to sample differences were greater than inter-individual differences (Cook et al., 2018).

3.2.2.1 Saliva collection

During each session, each volunteer was presented with one type of sample (WPC00, WPC05 or WPC20) to avoid crossover effects. A blank sample of saliva was collected from each participant before consuming any sample. The volunteer was presented with 5 mL of the sample and instructed to swill it around their mouth for 10 s before swallowing, after which a countdown timer was started. The volunteer was prompted to expectorate their saliva into a collection tube for analysis. The timer was randomised to count down from 5, 10, 20, 30, 45, 60, 120, 180, 240 or 300 s. Volunteers rinsed with warm water during a 2 min enforced break between sample consumption. Only one sample was presented per session, with one aliquot per randomised expectoration time. Volunteers recorded number of swallows of saliva between swallowing the sample and expectoration ($\leq$1 min^{-1}). Collection tubes were weighed before and after expectoration to monitor saliva weight for each volunteer.

3.2.2.2 Protein quantification

Protein concentration was determined using the Bradford microplate assay in triplicate (Bradford, 1976; Zor & Seliger, 1996), giving a total of 9 readings per volunteer per time point for each of three samples (Epoch, Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA).

The total amount of whey protein for each sample was calculated as the sample weight (assuming the density of saliva is 1 g mL^{-1}) multiplied by the protein concentration determined by the Bradford assay, subtracting each volunteer's baseline. The baseline was calculated as the average protein concentration in the volunteer's blank saliva sample multiplied by the weight of the sample weight.

3.2.3 Free ionic calcium measurement

Free ionic calcium in WPC samples was measured using a calcium selective ion electrode (Sentek, Essex, UK) and pH meter as described by Lin, Lewis, and Grandison (2006). Measurements were performed in triplicate on each of three processing replicates at ambient temperature (20.3 ± 0.05 °C). Calibration of the electrode was performed using 0.5, 1, 2.5 and 5 mM solutions of $CaCl_2$ in a dilution standard comprising 13.5 mM imidazole and 67.5 mM KCl.

3.2.4 Accessible thiol group measurement

Accessible thiol content of WPC samples was measured using an adaptation of Ellman's assay (Bravo-Osuna, Teutonico, Arpicco, Vauthier, & Ponchel, 2007; Withers, Cook, et al., 2013).

10% WPC samples were diluted using a phosphate buffer (0.1 M, pH 8) to reach a final concentration of 2 mg/mL. DTNB was dissolved in phosphate buffer (0.3 mg/ML) and added to the dilute samples in a ratio of 1:1. The treated samples were left to incubate in the dark for 2 hours before absorbance at 412 nm was measured (Epoch, Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA). Cysteine hydrochloride standards (25 – 750 μM in phosphate buffer) were used to establish a standard curve, from which the thiol content of the samples was calculated. Measurements were performed in triplicate on each of three processing replicates.

3.2.5 Circular dichroism

CD spectra were recorded using a Chirascan CD Spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK) in both near and far-UV ranges. Measurements were performed in triplicate on three processing replicates for each sample; the spectrum of the solvent was subtracted from the average of the triplicate scans of samples.

Near-UV spectra were recorded over a range of 450 down to 260 nm using a 1 mm pathlength cuvette with a step size of 2 nm. For the near-UV spectra, WPC samples were diluted to obtain a total concentration of 1% (w/v). Far-UV spectra were recorded over a range of 280 to 185 nm using a 0.1 mm pathlength cuvette with a step size of 1 nm. For this wavelength range, WPC samples were diluted to obtain a total concentration of 0.1% (w/v).

3.2.6 Nuclear magnetic resonance

Samples of WPC00, WPC05, WPC10, and WPC20 were prepared at concentrations of 10 mg/mL in a mixture of deuterium oxide and water (10% D_2O, 90% H_2O; Sigma-Aldrich). ^1H NMR spectra were recorded for all samples. 2D NOESY spectra were collected for all samples to confirm the appearance of peaks in overlapped ^1H spectra of WPC samples. A standard pre-saturation sequence was used to suppress water signals. All spectra were recorded at 25°C on a 700 MHz Bruker Avance III spectrometer (Bruker, Billerica, MA, USA).

3.2.7 Statistical analysis

IBM SPSS Statistics (version 21) was used to carry out three-way repeated measures analysis of variance (RM-ANOVA) on the in vivo retention data using sample (n = 3), assessors (n = 5) and time (n = 10) as explanatory variables. Analytical data were analysed by one-way ANOVA using IBM SPSS Statistics (version 21). Multiple pairwise comparisons were also carried out using IBM SPSS Statistics (version 21) using Tukey's HSD test ($p \leq 0.05$).

3.3 Results

3.3.1 In vivo protein retention method

All expectorated samples showed a general trend of initially increasing in protein concentration, followed by a gradual decrease, with a plateau reached at approximately 3 min post swallow (Figure 3.1, 180 s). WPC20 was significantly higher in protein in the expectorated samples overall compared to WPC00 ($p = 0.007$), tending to give higher protein weights over the first 60 s. While WPC20 peaked at 30 s; WPC05 peaked at 10 s before gradually declining. Average saliva weights for each time point are shown in Figure 3.2. No significant differences were found between samples or across time points through pairwise comparisons.

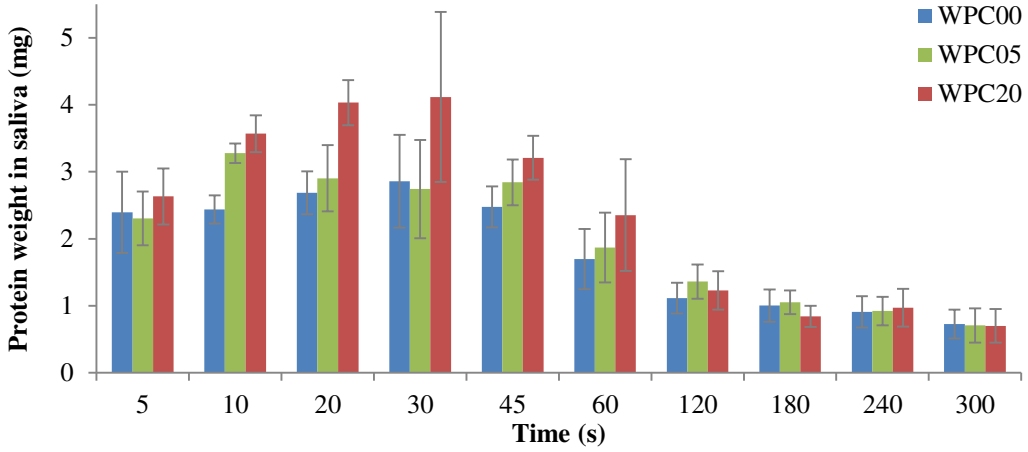

Figure 3.1: Average protein weight in expectorated saliva after consumption of 5 mL of sample over 5 min (all time points collected were independent). Average baseline saliva protein weight is subtracted for each volunteer. Saliva is assumed to have a density of 1 g mL^{-1}. Error bars represent ± 2 standard error of the mean.

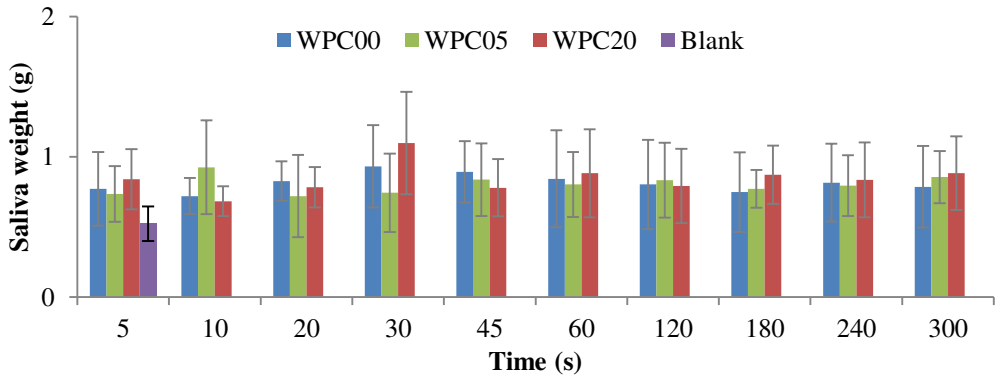

Figure 3.2: Average saliva weights of all volunteers over 5 min of collection. Error bars represent ± 2 standard error of the mean.

3.3.2 Free ionic calcium and accessible thiol concentration

No significant difference was observed in free ionic calcium between the samples, and as such it is unlikely that calcium will have an effect on the mucoadhesive strength of the WPC samples. Free thiol groups can increase as a result of protein denaturation, and indeed the free thiol concentration measured did increase significantly with heating time of the WPC (Table 3.1).

Table 3.1: Free ionic calcium and accessible thiol concentrations in 10% w/v WPC samples. Mean values ± 2 standard deviations. Superscript letters in a column indicate significantly different groupings ($p \leq 0.05$).

Sample	Ca^{2+} concentration (mM)	Accessible thiol concentration (mM)
WPC00	3.45 ± 0.53 [a]	4.03 ± 0.44[a]
WPC05	3.41 ± 0.32 [a]	5.50 ± 0.66[b]
WPC10	3.38 ± 0.26 [a]	5.57 ± 0.95[b]
WPC20	3.08 ± 0.57 [a]	6.87 ± 0.79[c]

3.3.3 Circular dichroism

Far-UV CD spectra were collected on more dilute samples than near-UV spectra to reduce light scattering and improve signal. Spectra for all samples contained a broad peak at 208 nm with an inflection around 220 nm; although the intensity of the 208 nm peak increased with heating WPC from 0 to 5 and 10 minutes, after 20 minutes of heating the peak was the same as for the native sample.

Higher concentrations were used for near-UV CD spectra to allow observation of characteristics in this wavelength region, as excessive light scattering was not an issue. A change in structure was observed upon heating in the aromatic region (260 – 310 nm); with a reduction in the peak size for samples with higher heating times (Figure 3.3).

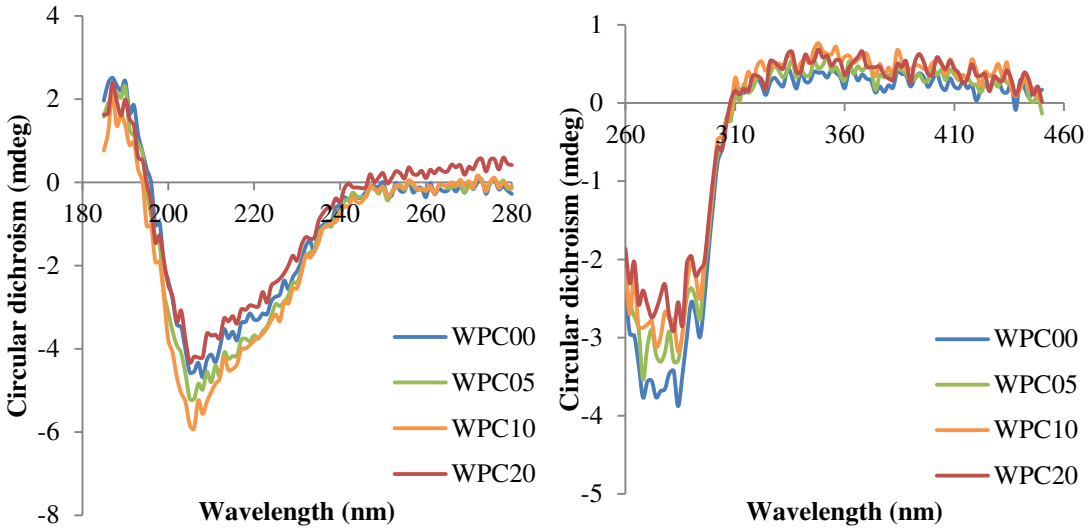

Figure 3.3: Far-UV (left) and near-UV (right) CD spectra comparing WPC00, WPC05, WPC10 and WPC20.

In order to compare samples with unknown concentrations of specific proteins, spectra were normalised by the area between 0 and the CD curve. These normalised spectra were each subtracted from WPC00 spectra in order to compare the differences observed upon heating of the samples for different times. Spectra in Figure 3.4 display error bars around zero as a measure of significant differences between samples.

Far-UV CD spectra found an increase in signal around 180 – 210 nm, with larger differences seen for WPC10, and fewer significant differences between WPC20 and WPC00. An increase in negative signal was observed for WPC20 between 250 and 280 nm in comparison to WPC00 and WPC10. Near-UV CD spectra showed significant differences moving further towards lower wavelengths with increasing heating time, with larger differences observed for WPC10 and WPC20 (Figure 3.4).

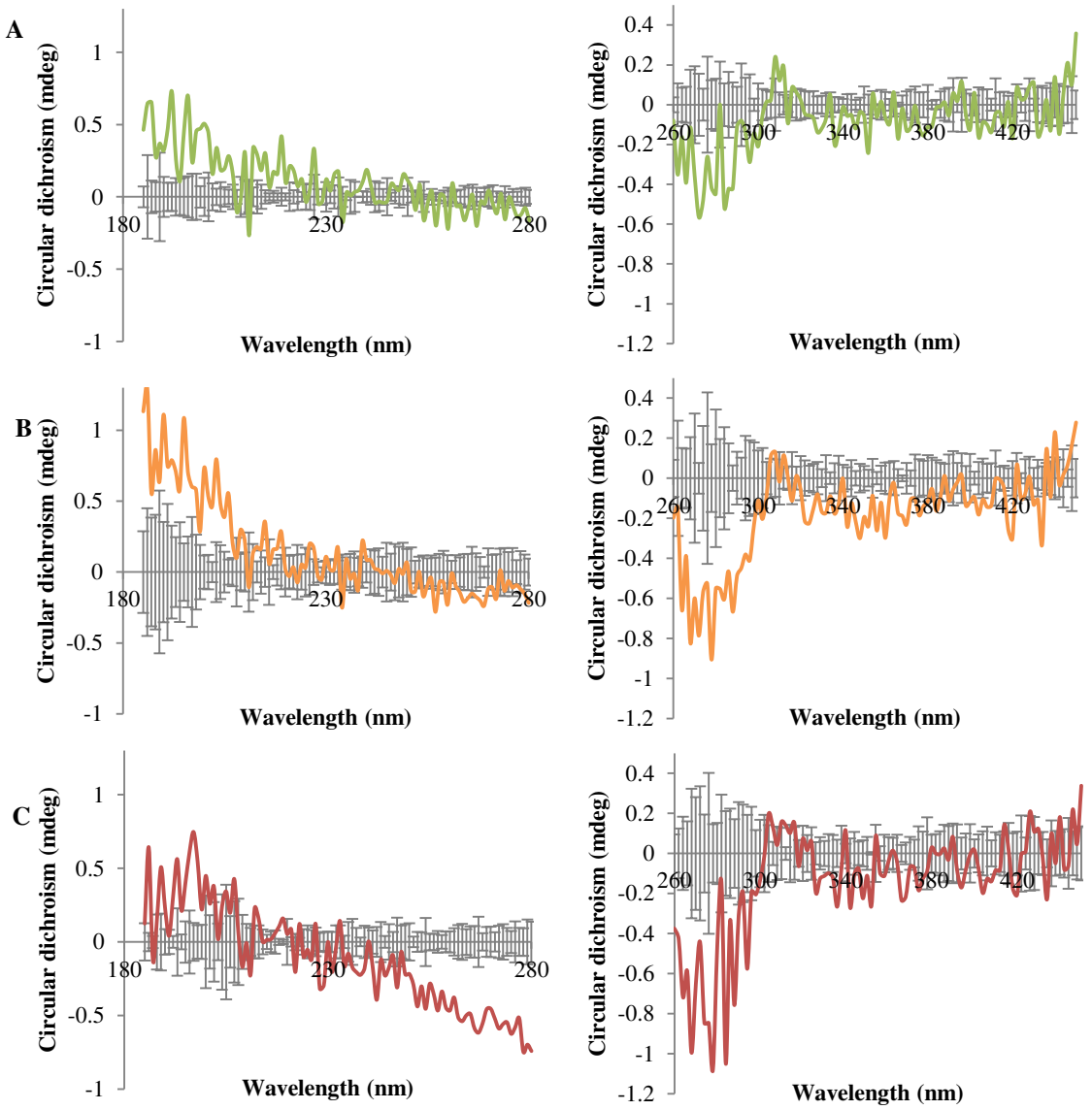

Figure 3.4: Far-UV (left) and near-UV (right) CD difference spectra of normalised WPC00 spectrum minus other WPC sample spectra. A: WPC05; B: WPC10; C: WPC20. Error bars represent ± 2 standard deviations and give an indication of significant difference between the samples.

3.3.4 Nuclear magnetic resonance

Full ^{1}H NMR spectra are given in Appendix 1; two regions (8.8 – 8.0 ppm; 3.2 – 1.8 ppm) are enlarged in Figure 3.5. The yellow bands highlight regions where spectral differences exist between the four samples. Structural differences between WPC00 and the three heated samples were inferred from ^{1}H NMR, using 2D NOESY spectra to confirm. Three peaks were present only in WPC00 (δ 8.35, 2.31, 1.82); one peak was more pronounced in WPC00 (δ 3.16); and one less pronounced in WPC00 (δ 3.10). A trend of increasing peak intensity with heating time was observed in three areas (δ 2.61, 2.59, 2.41). Differences between multiplets were seen, with similarities between WPC05 and WPC10 (δ 8.89 – 8.50, 8.17 – 8.29). The differences between

WPC00 and heated samples indicate a conformational change occurs upon heating the sample, with differences occurring with samples that were heated for longer.

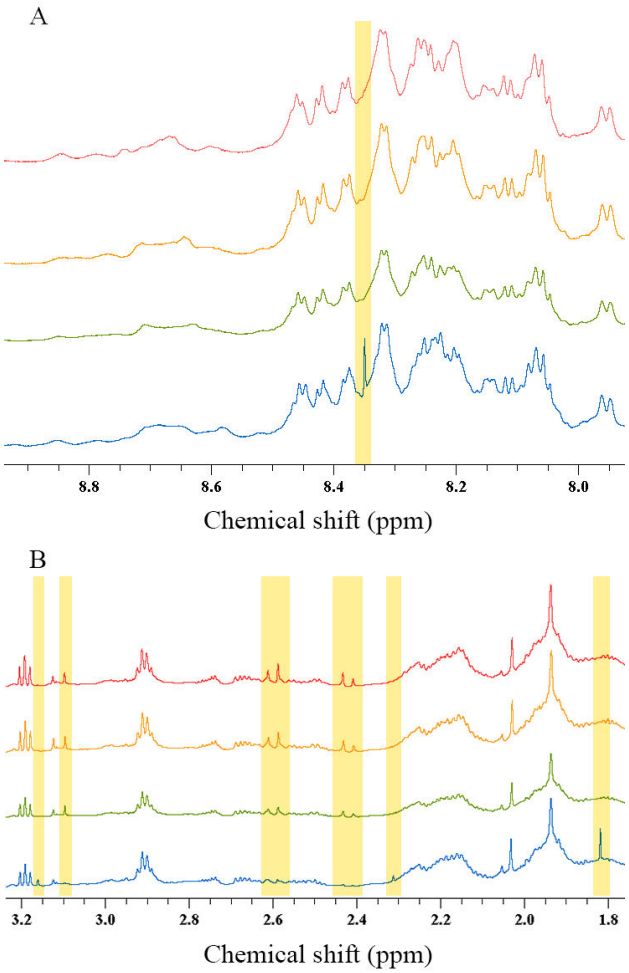

Figure 3.5: ^{1}H NMR spectra of WPC samples (A: 8.8 – 8.1 ppm; B: 3.2 – 1.8 ppm); WPC00 (blue), WPC05 (green), WPC10 (orange) and WPC20 (red). Differing peaks highlighted for clarity.

3.4 Discussion

Thermally treated whey protein gives an increased perception of mouthdrying, which builds up over repeated consumption (Chapter 2 Part 2), which is consistent with mechanisms of mucoadhesion (Vardhanabhuti & Foegeding, 2010; Withers, Cook, et al., 2013). This build-up of a drying sensation could be explained by an accumulation of WPC in the oral cavity. The *in vivo* oral retention results showed retention of WPC in the mouth for up to one minute after just one 5 mL sip; repeated consumption could lead to greater protein building up, and a prolonged drying sensation. The higher protein weights observed in saliva for WPC20 indicate the presence of more protein in the mouth than WPC00; this correlates with a higher sensory score for drying, mouthcoating, and chalky in WPC20, alongside larger particle sizes (Chapter 2 Part 2). WPC05 was not found to be significantly different to either WPC00 or WPC20. The protein weight in expectorated saliva increases initially, presumably due to the release of protein from

the mucosal surfaces into the saliva. After the maximum, the protein levels in the saliva decrease as the WPC is removed from the oral mucosa and swallowed.

Andrewes et al. (2011) found similar results when studying whey protein isolate (WPI) and whole saliva *in vitro*. They measured turbidity, with maximum turbidity measured 60 s after simulating swallowing 5 mL of acidic WPI solution. Due to the acidic nature of the WPI samples used, the clear samples became turbid upon the increased pH caused by the constant addition of saliva. The WPC samples used in the current study had a neutral pH (6.5 – 6.7); therefore changes observed are not related to pH-driven aggregation.

Average saliva weights were consistent across time points, with no significant difference found across the samples, showing that the effects observed are not due to an increased amount of stimulated saliva; however, a possible change in saliva composition must be acknowledged. Salivary proteins have been found to increase in concentration when an astringent compound was present in the mouth (Dinnella, Recchia, Vincenzi, Tuorila, & Monteleone, 2010), and this could contribute to the higher protein weights after consuming WPC; however this has only been shown using polyphenol astringents, which have a different mechanism of action to the mouthdrying caused by dairy proteins.

The volunteers used in this study were healthy young adults (aged 25 – 30). Older adults (aged 65 and older) have saliva with higher levels of protein, potassium, chlorine, amylase, lysozyme, albumen and secretory immunoglobulin (Nagler & Hershkovich, 2005b). Older adults also have a reduced salivary flow rate in comparison to younger adults (Nagler & Hershkovich, 2005a). The type of proteins present in saliva has also shown to vary with age in women (Ambatipudi, Lu, Hagen, Melvin, & Yates, 2009). These factors could affect mucoadhesion of WPC in many ways: a reduced salivary flow would expose more mucosal tissue to the WPC, leaving it available to adhere; a decreased flow rate would also reduce the rate of clearing in the mouth; an increased proportion of salivary proteins could allow more interactions to occur, increasing mucoadhesion; different salivary proteins may interact more strongly, and therefore alter the degree and mechanism of mucoadhesion.

The far-UV CD spectra for WPC samples in this study were similar to those observed previously for WPI (Liu & Zhong, 2013; Tomczynska-Mleko et al., 2014) with a peak around 208 nm, corresponding to an α-helix, and a broad peak around 220 nm, characteristic of β-sheets (Greenfield, 2006). The increase in peak intensity at 208 nm with heating time from WPC00 to WPC10 shows a tendency to develop a random coil conformation, as previously observed by Tomczynska-Mleko et al. (2014). The decrease in peak intensity for WPC20, to

below that of WPC00 could be due to a confounding factor, such as an increase in turbidity caused by aggregation.

The near-UV CD spectra showed differences in the structure of WPC heated at different time points, with a decrease in the near-UV peak (260 – 300 nm) corresponding to aromatic moieties. This is reflected in some of the differences between WPC00 and heated WPC samples seen in ^{1}H NMR spectra at chemical shifts corresponding to aromatic moieties. These changes could be due to denaturation of the proteins leading to a change in secondary structure, affecting amino acid side groups with aromatic character. Although the aromatic groups may not directly influence mucoadhesion, the change in structure could lead to the exposure of thiol groups, hydrophobic regions, or functional groups able to form hydrogen bonds, which could increase mucoadhesive strength.

To the authors' knowledge, no literature exists showing near-UV CD spectra for WPC or WPI; however, near-UV spectra exist for β-LG, α-LA (Mercade-Prieto, Paterson, & Wilson, 2007; Moro, Baez, Busti, Ballerini, & Delorenzi, 2011; Rodiles-Lopez et al., 2010; Wijesinha-Bettoni et al., 2007). Moro et al. (2011) showed a reduction in peaks within the aromatic region (285 and 292 nm) of β-LG upon heating at 85 ^{0}C due to a tryptophan residue absorbance (Trp19) reflecting structural changes of the β-barrel within the protein (Matsuura & Manning, 1994). The near-UV CD spectrum for α-LA, reported by Wijesinha-Bettoni et al. (2007) contained a peak at 270 nm, which disappears upon the unfolding of the protein after heat treatment. The results in this study are consistent with these findings as the broad negative peak in near-UV spectra of WPC samples occurs around 260 – 310 nm, with minima appearing at around 270, 285 and 290 nm. This negative peak decreases in size with heating time, indicating a structural change within the β-barrel of β-LG and a change in tertiary structure.

As no significant difference was observed in free ionic calcium concentrations between WPC samples, this is an unlikely cause for the increased retention in WPC20; however, it does not discount that calcium binding to mucin could contribute to the drying mechanism in all WPC samples equally.

There was an increase in accessible thiol groups with longer heating times, which could increase mucoadhesive strength due to formation of disulfide bridges with mucosal surfaces. This increase in thiol may be due to conformational changes upon denaturation as observed by CD and NMR. Sava, Van der Plancken, Claeys, and Hendrickx (2005) found that surface thiol groups of β-LG increased with heating time at 70 °C at neutral pH, consistent with the findings in the present study with WPC. This increase in accessible thiols and therefore mucoadhesive potential could be an underlying cause of the increase in oral retention observed for WPC20.

3.5 Conclusions

Whey protein samples were heated for varying times (0, 5 and 20 min; 70 °C) and the retention of protein in the mouth was measured using an *in vivo* technique. Higher protein levels were found in saliva expectorated after consuming WPC20 than WPC00, indicating that protein in WPC20 is retained longer in the mouth, which could be explained by mucoadhesion. The findings of a higher sensory score in drying, mouthcoating and chalky for WPC20 (Chapter 2.2) could be explained by the increase of protein expectorated over 60 s post consumption. An increase in accessible thiol concentration with heating time is consistent with the proposed mechanism of mucoadhesion as the source of whey drying, This is supported by the adhesion of whey protein to the oral mucosa (Withers, Cook, et al., 2013), the denaturation of whey protein increasing mucoadhesive strength (Hsein, Garrait, Beyssac, & Hoffart, 2015), and by the conformational changes inferred by the CD and NMR spectra found on heating WPC in this study.

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Appendices

Appendix 3.1: Ethics application form for *in vivo* oral retention experiment.

University of Reading

School of Chemistry, Food and Nutritional Sciences and Pharmacy
Research Ethics Committee

Application Form

SECTION 1: APPLICATION DETAILS

1.1
Project Title:

Date of Submission: 22/02/16 Proposed start date: February 2016 Proposed End Date: July 2016

1.2
Principal Investigator: Dr Lisa Methven

Office room number: 2.65b Internal telephone: 0118 378 8714

Email address: l.methven@reading.ac.uk Alternative contact telephone:
(Please note that an undergraduate or postgraduate student cannot be a named principal investigator for research ethics purposes. The supervisor must be declared as Principal Investigator)
Other applicants
Name: Stephanie Bull; Student Institution/Department: Food; Email: s.p.bull@pgr.reading.ac.uk.

Name: Prof Vitaliy Khutoryanskiy; Staff Institution/Department: Pharmacy ; Email : v.khutoryanskiy@reading.ac.uk

Name: Dr Jane Parker; Staff Institution/Department: Food & Nutritional Sciences ; Email : j.k.parker@reading.ac.uk

….

1.3
Project Submission Declaration

I confirm that to the best of my knowledge I have made known all information relevant to the Research Ethics Committee and I undertake to inform the Committee of any such information which subsequently becomes available whether before or after the research has begun.

I understand that it is a legal requirement that both staff and students undergo Criminal Records Checks when in a position of trust (i.e. when working with children or vulnerable adults).

I confirm that a list of the names and addresses of the subjects in this project will be compiled and that this, together with a copy of the Consent Form, will be retained within the School for a minimum of five years after the date that the project is completed.

Signed…… (Principal Investigator) Date:…… …… (Student) Date:…… …… (Other named investigators) Date:…… …… (Other named investigators) Date:……
1.4 **University Research Ethics Committee Applications** Projects expected to require review by the University Research Ethics Committee must be reviewed by a member of the School research ethics committee and the Head of School before submission. Signed…… (Chair/Deputy Chair of School Committee) Date:…… Signed…… (Head of Department) Date:…… Signed…… (SCFP Ethics Administrator) Date:……

2

SECTION 2: PROJECT DETAILS

2.1

Lay summary

Please provide a summary of the project in non-specialist terms, which includes a description of the scientific background to the study (existing knowledge), the scientific questions the project will address and a justification of these. Please note that the description must be sufficient for the committee to take a reasonable view on the likely scientific rigour and value of the project.

Whey proteins are a nutritional supplement derived from milk used to aid the maintenance and growth of muscle mass. The use of whey protein in the prevention of sarcopenia, or muscle wasting, relies on the consumption of relatively large portions of whey protein beverages (approximately 200 mL per serving). Negative sensory aspects of whey protein, such as the sensation of drying in the mouth, prevent full consumption, and therefore the full clinical benefits are not realised.

A phenomenon known as mucoadhesion is employed in the pharmaceutical industry to enhance the retention of drugs at specific sites of the body. Mucoadhesion describes the adherence of materials to mucosal membranes. Mucosal membranes are the moist surfaces in the body, which are exposed to the external environment. Of interest to this project is the oral mucosa and the interactions it may have with whey proteins. For the interest of the project, whey protein solutions (10% w/v, deionised water) will be assessed for mucoadhesive properties in the mouth.

The adherence of whey proteins to the oral mucosa may cause the mouth drying sensation elicited by whey protein beverages, and understanding this is the first step in the mitigation of this drying to improve consumer acceptance.

(*This box may be expanded as required*)

2.2

Procedure

Please briefly describe what the study will involve for your participants and the procedures and methodology to be undertaken (*you may expand this box as required*).

Students and staff of the University of Reading will be recruited to take part in this study for a monetary reward. This study aims to assess the adhesion of whey protein to the oral muscosa within aqueous solutions. This study will help to determine if whey proteins adhere to the oral mucosa.

Aqueous solutions with whey protein concentrate (80% protein) will be made up at 10% w/v. The panel will swill their mouths with the sample before swallowing the sample. After set periods of time the panellists will be asked to spit their whole saliva into sealed tubes provided.

(Note: All questionnaires or interviews should be appended to this application)

2.3

Where will the project take place?

Room 2-32, sensory booths, Food Biosciences Building, The University of Reading, Whiteknights Campus
……

2.4

Funding

Is the research supported by funding from a research council or other *external* sources (e.g. charities, business)? Yes

If Yes, please give details:

BBSRC and Volac International Ltd (Orwell, UK)

Please note that *all* projects (except those considered as low risk, which would be the decision of the School's internal review committee and require Head of Department approval) require approval from the University Research Ethics Committee.

2.5

Ethical Issues

Could this research lead to any risk of harm or distress to the researcher, participant or immediate others? Please explain why this is necessary and how any risk will be managed.

There may be some very mild distress of the panellists due to the spitting protocol but we will make sure they are as comfortable as possible with the proceedings.

Another issue to consider is the handling and disposure of saliva. The saliva will be stored in sealed tubes immediately after the panellists are finished. These will then be tested using an appropriate technique to measure the analytes (Bradford assay). The saliva solutions will be disposed of in the sluice provided in the nutrition unit of the food biosciences building.

Human Tissue Act 2004

Dr Lisa Methven has been trained by the Designated Individual, Prof J Lovegrove, in the procedures needed to comply with the Act and the other investigators/researchers will be made aware of the requirements of the Act. The relevant tissue samples (i.e. saliva swabs) will be stored under appropriate conditions in the designated and secure HTA storage facility in Department of Food and Nutritional Sciences.

(*this box may be expanded as required*)

2.7

Payment

Will you be paying your participants for their involvement in the study? Yes

If yes, please specify and justify the amount paid

The participants will be paid cash in hand at the rate of £10 per hour, so if a session lasts ½ an hour they will be paid £5. They will sign in and out on a sheet provided at each session.

Note: excessive payment may be considered coercive and therefore unethical. Travel expenses need not to be declared.

2.8

Data protection and confidentiality

4

What steps will be taken to ensure participant confidentiality? How will the data be stored?
Panellists will be assigned a study number and all samples will be labelled accordingly.

2.9

Consent

Please describe the process by which participants will be informed about the nature of the study and the process by which you will obtain consent

N/A

Please note that a copy of consent forms and information letters for all participants must be appended to this application.

2.10

Genotyping

Are you intending to genotype the participants? Which genotypes will be determined?

N/A

Please note that a copy of all information sheets on the implications of determining the specific genotype(s) to be undertaken must be appended to this application.

SECTION 3: PARTICIPANT DETAILS

3.1

Sample Size

How many participants do you plan to recruit? Please provide a suitable power calculation demonstrating how the sample size has been arrived at or a suitable justification explaining why this is not possible/appropriate for the study.

We will recruit 5 participants as this number is often used in the literature to measure similar phenomena.

3.2

Will the research involve children or vulnerable adults (e.g. adults with mental health problems or neurological conditions)?

No

If yes, how will you ensure these participants fully understand the study and the nature of their involvement in it and freely consent to participate?

N/A

5

	(Please append letters and, if relevant, consent forms, for parents, guardians or carers). Please note: information letters must be supplied for all participants wherever possible, including children. Written consent should be obtained from children wherever possible in addition to that required from parents.
3.3	Will your research involve children under the age of 18 years? No Will your research involve children under the age of 5 years? No
3.4	Will your research involve NHS patients, Clients of Social Services or will GP or NHS databases be used for recruitment purposes? No Please note that if your research involves NHS patients or Clients of Social Services your application will have to be reviewed by the University Research Ethics Committee and by an NHS research ethics committee.
3.5	**Recruitment** Please describe the recruitment process and append all advertising and letters of recruitment. **N/A**

Important Notes

1. The Principal Investigator must complete the Checklist in Appendix A to ensure that all the relevant steps and have been taken and all the appropriate documentation has been appended.

2. If you expect that your application will need to be reviewed by the University Research Ethics Committee you must also complete the Form in Appendix B.

3. For template consent forms, please see Appendices C.

4. For examples of information letters, see Appendices D

Appendix A: Application checklist
This must be completed by an academic staff member (e.g. supervisor)

Please tick to confirm that the following information has been included and is correct.
Indicate (N/A) if not applicable:

Information Sheet

Is on headed notepaper	☐	
Includes Investigator's name and email / telephone number	☐	
Includes Supervisor's name and email / telephone number	☐	
Statement that participation is voluntary	☐	
Statement that participants are free to withdraw their co-operation	☐	
Reference to the ethical process	☐	
Reference to Disclosure	☐	N/A ☐
Reference to confidentiality, storage and disposal of personal information collected	☐	
Consent form(s)	☐	

Other relevant material

Questionnaires	☐	N/A ☐
Advertisement/leaflets	☐	N/A ☐
Letters	☐	N/A ☐
Other (please specify)	☐	N/A ☐

Expected duration of the project (months) ☐

Name (print) …… Signature ……

7

Appendix 3.2: Consent form for *in vivo* retention volunteers.

Project code: 27/15

APPENDIX A

Volunteer Information Sheet

Sensory Perception and Mucoadhesion Study

You are invited to take part in a short-term study at The Sensory Science Centre, University of Reading. Information is provided on the study design and background information. Please read carefully and if you have any queries or questions regarding this study please don't hesitate to contact the investigator/ researcher.

Background

Whey protein is naturally derived from milk and used in nutritional supplements to aid muscle growth. Whey proteins have been shown to have mucoadhesives abilities, meaning that they adhere to the mucosal surfaces of the mouth for a prolonged period of time. This effect is utilised in pharmaceutics to retain drugs at mucosal surfaces to enhance their uptake into the body. The ability of these proteins to retain at mucosal surfaces may prove to be important to their sensory properties, and therefore consumer acceptance. This study aims to investigate the mucoadhesive properties of whey protein solutions.

What the study involves

The study will continue over several weeks lasting between 30 and 60 minutes for each day. Each panellist will be provided an isolated sensory booth, fresh water and palate cleansers to use between samples. Panellists will be prompted to rinse their mouths thoroughly between samples.

The participants will use Compusense software for the duration of the experiment. As each sample is given, the time that the panellist must allow between taking the sample in their mouth and spitting their whole saliva out will be told to them. They will be trained when to swallow and how many times during each experiment.

Participants will be presented with samples monadically and will be prompted when to take samples. At the start of each session a baseline saliva sample will be taken. Panellists will be asked to rinse their mouths with water and spit their whole saliva into a sealed tube which will be provided.

Participants will hold a 5 mL sip of sample in their mouth for 10 seconds. They will then swallow the sample or spit the sample into a spittoon provided. After this the timer will start on Compusense when the participant will wait for the appropriate amount of time before cleaning their tongues with their teeth and spitting their whole saliva into a labelled, sealed tube. The tubes will then be collected by the investigator/ researcher and labelled appropriately.

Participants will then be presented with the next samples one after the other with the same protocol to follow, however, the length of time until the final spitting takes place will change.

Project code: 27/15

What will be measured in the samples collected?
The saliva will be contained in a seal tube with deionised water to dilute the contents of the saliva. This fluid will then be used to analyse the protein concentration present in the saliva.

Risks and confidentiality
All samples that panellists will consume are of food grade standard and carry no health risks, except to those that are dairy or whey protein intolerant. There is no risk associated with swallowing any of the foods given and therefore there is no cause for concern, unless you are dairy intolerant. The sessions will be spread over 10 days to limit the amount the participants have to sample each time. This will reduce fatigue and boredom as there are many samples to get through over many different times.
Records of panellists will be kept confidential and a participant number will be given to each panellist to avoid any confidentiality issues. No identifiable individual results will be used, published or presented in scientific meetings. Your records will be kept by the investigators for five years. However, these records will only contain an identification number and code while information matching volunteer names with identification numbers/codes will be kept separately from these files within the School of Food, Chemistry and Pharmacy. The only time data will be matched with volunteer names is for those volunteers that request to have their personal results presented/discussed with them.

If you have any questions or would like further information please contact the study investigators, or Dr Lisa Methven.

Dr Lisa Methven
Lecturer in Food and Sensory Science,
Department of Food and Nutritional Sciences, University of Reading.
l.methven@reading.ac.uk
0118 378 8714

Project code: 27/15

University of Reading

School of Chemistry, Food and Pharmacy
Research Ethics Committee

Consent Form

I have read and had explained to me by Stephanie Bull the accompanying Information Sheet relating to the project entitled "Sensory Mucoadhesion Study".

I have had explained to me the purposes of the project and what will be required of me, and any questions I had, have been answered to my satisfaction. I agree to the arrangements described in the Information Sheet in so far as they relate to my participation.

1. I understand that participation is entirely voluntary and that I have the right to withdraw from the project at any time without giving reason, and that this will be without detriment to any care or services I may be receiving or may receive in the future.

2. This project has been subject to ethical review, according to the procedures specified by the School of Chemistry, Food and Pharmacy Research Ethics Committee, and has been given a favourable ethical opinion for conduct.

3. I have received a copy of this Consent Form and of the accompanying Information Sheet.

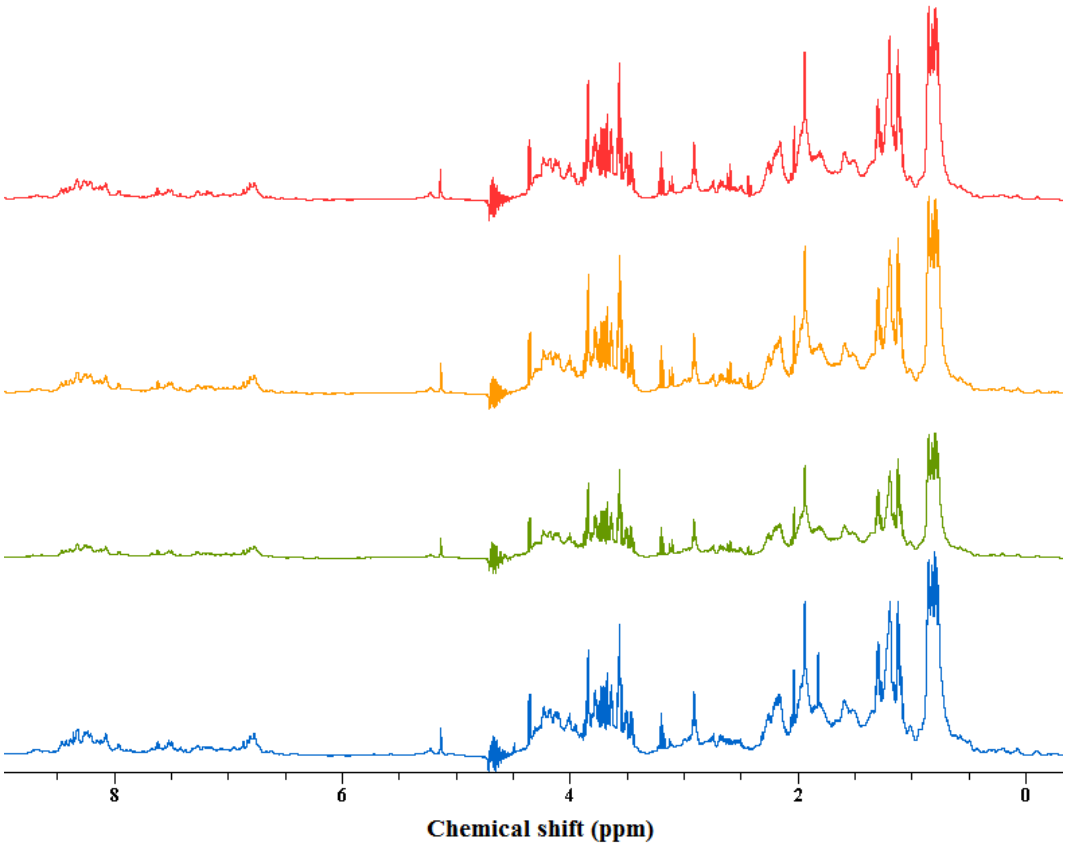
4. I confirm that I do not have a dairy or whey protein intolerance.

Name ……

Signed ……

Date ……

Appendix 3.3: Full ^{1}H NMR spectra of WPC samples; WPC00 (blue), WPC05 (green), WPC10 (orange) and WPC20 (red).



Chapter 4 Interactions between bovine submaxillary mucin and thermally treated whey protein

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Abstract

Thermally treated whey protein concentrate and individual whey proteins (beta-lactoglobulin, alpha-lactalbumin, lactoferrin, lactoperoxidase, bovine serum albumin, immunoglobulin) were mixed with bovine submaxillary mucin in order to observe interactions with mucins indicative of mucoadhesive effects. Circular dichroism showed differences between the sum of whey protein spectra and mucin spectra, and the spectra for the mixture of whey protein with mucin. This indicates an interaction between the whey protein and the mucin, leading to a change in secondary structure. No change in circular dichroism was observed for individual whey proteins, suggesting that the interactions between whey protein concentrate and mucin are due to the complex mixture of proteins. Nuclear magnetic resonance found differences between the summed spectra and mixtures of whey protein concentrate and mucin, indicating a tertiary structural change due to interactions. Particle size was found to increase upon mixing with mucin, with higher sizes resulting from longer whey protein heating times. These results demonstrate that whey protein interacts with bovine submaxillary mucin and this implies that mucoadhesion of whey protein occurs in the oral cavity.

4.1 Introduction

There is an increasing interest in whey protein as a functional food ingredient, with uses in sports nutrition (Tipton et al., 2007) and the prevention and treatment of sarcopenia, particularly in older adults (Beasley, Shikany, & Thomson, 2013). An obstacle in the treatment of sarcopenia using whey protein beverages, is the lack of compliance among patients, with a mean muscle wastage of 63% (Gosney, 2003). One factor identified to reduce liking in whey protein beverages is astringency, or mouth drying (Lemieux & Simard, 1994), which has been shown to be perceived more intensely by older adults than younger adults (Withers, Gosney, & Methven, 2013).

Whey protein is a mixture of soluble proteins from bovine milk: β-lactoglobulin (β-LG), α-lactalbumin (α-LA), glycomacropeptides (GMP), bovine serum albumin (BSA), immunoglobulins, lactoferrin (LF), and lactoperoxidase (LP). These proteins range in molecular weight from 8 to 150 kDa (Etzel, 2004), and denature at temperatures between 35 and 70 °C (Mazri, Ramos, Sanchez, Calvo, & Perez, 2012; Mazri, Sanchez, Ramos, Calvo, & Perez, 2012; Relkin, 1996; Vermeer & Norde, 2000).

The mechanism of action for whey protein drying is currently undefined, although there are many theories of contributing factors. The acidity of whey protein beverages has been attributed with causing astringency (Lee & Vickers, 2008), as acids are inherently astringent. Alternatively, the low pH of many whey protein beverages has been shown to contribute to astringency, as charge interactions between whey protein and salivary proteins are enhanced by low pH (Vardhanabhuti, Kelly, Luck, Drake, & Foegeding, 2010; Ye, Streicher, & Singh, 2011).

The interactions of whey protein in the oral cavity have been explored by considering many different factors. Vardhanabhuti and Foegeding (2010) studied the interactions between low pH β-LG and salivary mucin using SDS-PAGE and turbidity as a measurement of interactions. The binding of milk proteins to mucosa was studied using a fluorescent wash-off method by Withers, Cook, Methven, Gosney, and Khutoryanskiy (2013), finding high retention of β-LG and suggesting this as a possible cause of drying. The interactions between mucin and β-LG have also been studied using spectroscopic methods by Celebioglu et al. (2015), finding strong evidence for interactions in the hydrophilic regions of the proteins. The astringency of whey protein beverages has also been attributed to the binding of β-LG and LF to buccal cells (Ye, Zheng, Ye, & Singh, 2012).

The link between astringency and the interactions with mucosa point to a mechanism of mucoadhesion, which would increase retention of whey protein in the oral cavity. This is

supported by the increase in whey protein mouth drying over repeated consumption (Withers, Lewis, Gosney, & Methven, 2014). Chapter 3 discusses the retention of whey protein in the oral cavity, with higher retention times observed for whey protein heated for longer. The cause of the drying sensation due to the presence of protein in the mouth has been suggested to be due to the disruption of salivary lubrication (Vardhanabhuti, Cox, Norton, & Foegeding, 2011).

The denaturation of whey protein results in structural changes, which can influence the extent of interaction with mucin. Whey protein has been found to have higher mucoadhesive strength when denatured (Hsein, Garrait, Beyssac, & Hoffart, 2015). The effects of drying have been shown to increase with thermal processing, of whey protein (Chapter 2 Part 2), which indicates that the increase in drying could be due to higher mucoadhesion.

As β-LG denatures, the unfolding protein exposes a free cysteine residue and a disulfide bridge (Zeiler & Bolhuis, 2015). Increasing free thiol content can contribute to mucoadhesion, as disulfide bonds are formed between the mucoadhesive material and the mucosa (Bernkop-Schnurch, 2005). As β-LG denatures further, aggregation occurs, which in the presence of other whey proteins, α-LA and BSA, creates heteropolymers (Havea, Singh, & Creamer, 2001). Large particles form upon aggregation of whey protein, and interactions with mucin can cause larger particles to form (Celebioglu et al., 2015).

To better understand the structural changes occurring during denaturation and interactions with mucin, circular dichroism (CD) and nuclear magnetic resonance (NMR) can be used. Celebioglu et al. (2015) used CD to study the effect of mucin on the secondary structure of β-LG. To establish effects on the tertiary structure of β-LG when mixed with mucin, NMR was also used, and residues were suggested as interacting moieties of BSM. The effects of heat treatments on the secondary structure of whey protein has been investigated using CD for whey protein isolate, β-LG and α-LA (Tomczynska-Mleko et al., 2014; Wada, Fujita, & Kitabatake, 2006; Wijesinha-Bettoni et al., 2007). This present study investigates the effect of mixing mucin with whey protein concentrate and isolated whey proteins (β-LG, α-LA, IgG, BSA, LF, LP) on both near-UV and far-UV CD spectra.

This study aims to investigate the interactions of mucin with thermally treated whey protein model beverages and isolated whey proteins in order to explore the mechanism of action further. Free accessible thiol groups were measured for whey protein samples and compared to corresponding mixtures with bovine submaxillary mucin (BSM). Particle size and ζ-potential were assessed to characterise changes in particle size and stability upon mixing with BSM. CD and NMR were used to monitor structural changes when mixing samples with mucin. 1:1

mixtures of whey protein samples and BSM were used in order to assess the molecular interactions of the system.

4.2 Materials and methods

The whey protein concentrate (WPC) used was Volactive Ultrawhey 80 Instant (Volac International Limited, Orwell, Royston, UK), a dry powder with a minimum protein content of 80%, and containing soy lecithin (0.5% maximum) as an emulsifying agent. The remaining 20% contains moisture (5%), fat (7%), lactose (4%), and minerals.

Bovine β-LG, bovine α-LA, bovine serum immunoglobulin G (IgG), BSA, bovine LF, bovine LP, BSM (Type I-S) , DTNB (5,5-dithio-bis-(2-nitrobenzoic acid), deuterium oxide, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, and L-cysteine hydrochloride monohydrate were supplied by Sigma-Aldrich (Dorset, UK).

4.2.1 Preparation of whey protein beverages

Model WPC beverages were prepared by addition of WPC powder to water (10% w/v, deionised water). All samples were stirred for 30 min at room temperature (25 ± 2 ^{0}C). A native sample was then stirred for a further 60 min at room temperature (WPC00). Three samples were stirred while being heated in a water bath set at 70 °C for 5, 10 and 20 min (WPC05, WPC10, and WPC20 respectively). The samples were cooled in a water bath then allowed to hydrate overnight at 4 °C. The pH of all samples ranged from 6.5 to 6.7 (Mettler Toledo SevenEasy, Switzerland; 22 ± 3 ^{0}C).

4.2.2 Accessible thiol group measurement

Accessible thiol content of samples was measured using an adaptation of Ellman's assay (Bravo-Osuna, Teutonico, Arpicco, Vauthier, & Ponchel, 2007; Withers, Cook, et al., 2013). 10% WPC samples were diluted using a phosphate buffer (0.1 M, pH 8) to reach a final concentration of 2 mg/mL. 2 mg/mL β-LG and BSM were dissolved in phosphate buffer. BSM was mixed with WPC samples and β-LG at a ratio of 1:1 with a total concentration of 2 mg/mL in phosphate buffer. DTNB (5,5-dithio-bis-(2-nitrobenzoic acid) was dissolved in phosphate buffer (0.3 mg/ML) and added to the dilute samples in a ratio of 1:1. The treated samples were left to incubate in the dark for 2 hours before absorbance at 412 nm was measured (Epoch, Microplate Spectrophotometer, BioTek Instruments, Inc., Winooski, VT, USA). Cysteine hydrochloride standards (25 – 750 μM in phosphate buffer) were used to establish a standard curve, from which the thiol content of the samples was calculated. Measurements were performed in triplicate on each of three processing replicates.

4.2.3 Dynamic light scattering and zeta-potential

WPC samples were diluted to a total concentration of 0.1% (w/v, HPLC grade water). Solutions of β-LG (0.1% w/v, deionised water), and BSM (0.1% w/v, deionised water) were prepared. Mixtures of BSM with WPC and βLG solutions were in a 1:1 ratio to give a total concentration of 0.1% (w/v). Measurements were performed in triplicate on each of three processing replicates using Nano-S Zetasizer (Malvern Instruments, UK) at 30 °C, with an equilibration time of 60 s.

4.2.4 Circular dichroism

Circular dichroism (CD) spectra were recorded using a Chirascan CD Spectrophotometer (Applied Photophysics Ltd., Leatherhead, UK) in both near and far-uv ranges. Measurements were performed in triplicate on three processing replicates for each sample; the spectrum of the solvent was subtracted from the average of the triplicate scans of samples.

Near-UV spectra were recorded over a range of 450 to 260 nm using a 1 mm pathlength cuvette with a step size of 2 nm. For the near-UV, WPC samples were diluted to obtain a total concentration of 1% (w/v). Solutions of β-LG, α-LA, BSA, IgG, LF, LP and BSM (1% w/v, deionised water), were prepared. WPC samples, and individual whey protein solutions were mixed with BSM solutions in a 1:1 ratio, then diluted to give a total concentration of 2% (w/v).

Far-UV spectra were recorded over a range of 280 to 185 nm using a 0.1 mm pathlength cuvette with a step size of 1 nm. WPC samples were diluted to obtain a total concentration of 0.1% (w/v). Solutions of β-LG, α-LA, BSA, IgG, LF, LP and BSM (0.1% w/v, deionised water), were prepared. For this wavelength range, WPC samples, and individual whey protein solutions were mixed with BSM solutions in a 1:1 ratio, then diluted to give a total concentration of 0.2% (w/v).

4.2.5 Nuclear magnetic resonance

Samples of WPC00, WPC05, WPC10, WPC20, β-LG and BSM were prepared at concentrations of 10 mg/mL in a mixture of deuterium oxide and water (10% D_2O, 90% H_2O). WPC00, WPC05, WPC10, WPC20 and β-LG samples were also mixed with BSM at a ratio of 1:1. ^1H spectra were recorded for all samples. NOESY spectra were collected for all samples, excluding WPC05 and WPC10, and the WPC samples that had been mixed with BSM. Sum spectra of individual samples were constructed and compared to the spectra of the corresponding BSM mixtures (TopSpin 3.7). A standard pre-saturation sequence was used to suppress water signals. NOESY spectra were used to confirm the presence of peaks in ^1H spectra of WPC samples.

4.2.6 Statistical analysis

All data was analysed by one-way ANOVA using IBM SPSS Statistics (version 24).

4.3 Results

4.3.1 Circular dichroism

CD data showed a reduction in the signal intensity in the aromatic region (260 – 310 nm) in near-UV spectra for all WPC samples upon mixing with BSM (Figure 4.1). No differences were observed in far-UV spectra of WPC samples mixed with BSM (Figure 4.2). No differences were observed when individual whey proteins were mixed with BSM in either near or far-uv (Figure 4.2, Appendix 4.1 & Appendix 4.2). Simulated CD spectra of WPC were created using weighted values from the spectra of individual proteins (Figure 4.2). Weightings were as follows: 53% β-LG; 16% α-LA; 10% IgG; 6% BSA; 2% LF; 0.5% LP as averages of reported content of each protein in whey protein (Etzel, 2004).

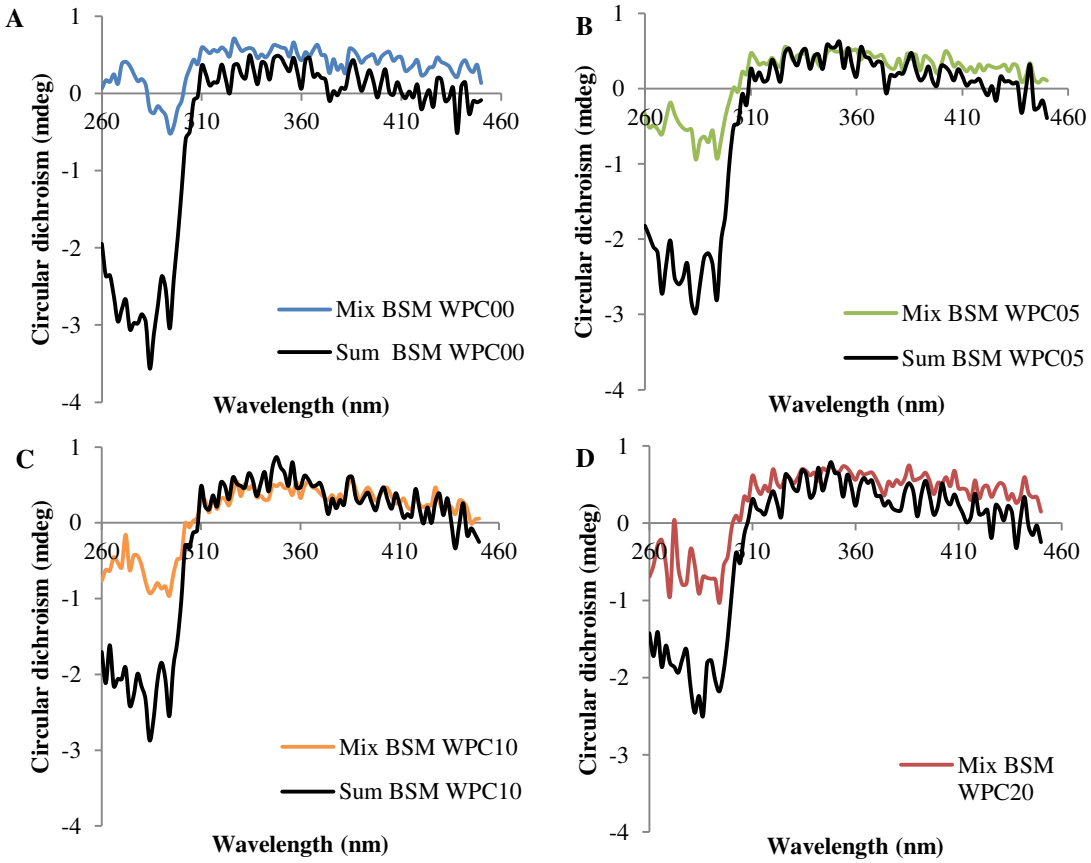

Figure 4.1: Near-UV CD spectra comparing the 1:1 mixture of WPC samples with BSM, and the sum of the individual spectra of BSM and WPC samples. A: WPC00, B: WPC05, C: WPC10, D: WPC20.

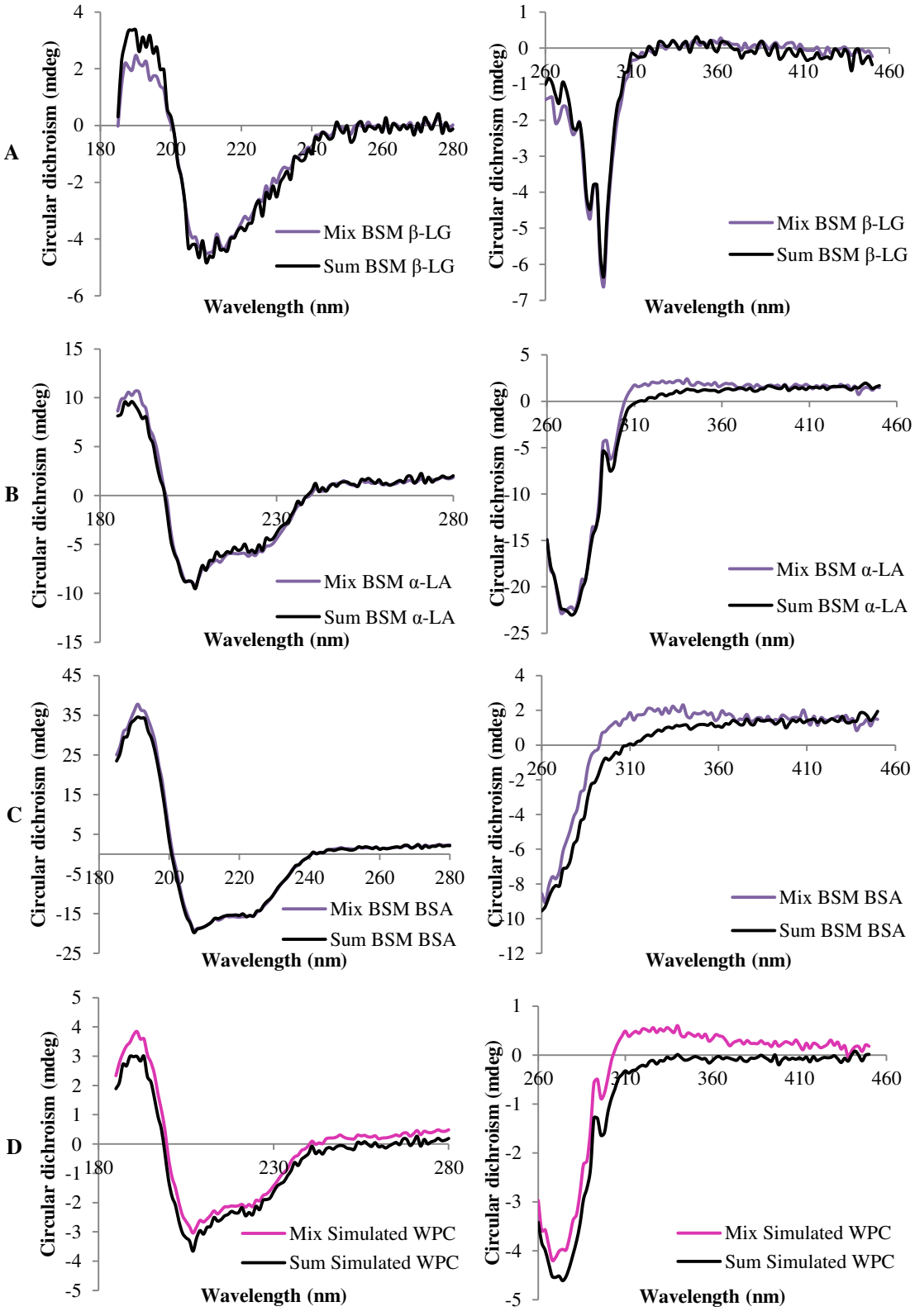

Figure 4.2: Far-UV (left) and near-UV (right) CD spectra comparing the 1:1 mixture of individual whey proteins with BSM, and the sum of the individual spectra of BSM and proteins. Simulated CD spectra of WPC comprised of weighted CD spectra of the individual proteins shown in Figure 4.2, Appendix 4.1 and Appendix 4.2 are shown. A: β-lactoglobulin (β-LG), B: α-lactalbumin (α-LA), C: bovine serum albumin (BSA), D: simulated WPC.

4.3.2 Nuclear magnetic resonance

^{1}H NMR spectra showed differences between the sum of WPC and β-LG samples with BSM, and the spectra of the mixture of WPC samples with BSM (Figure 4.3). Differences are seen in three main regions: 4 - 3.5 ppm; 2.1 – 1.9 ppm; 1.5 – 0.5 ppm. 2D NOESY data (not shown) was used to confirm the presence of peaks.

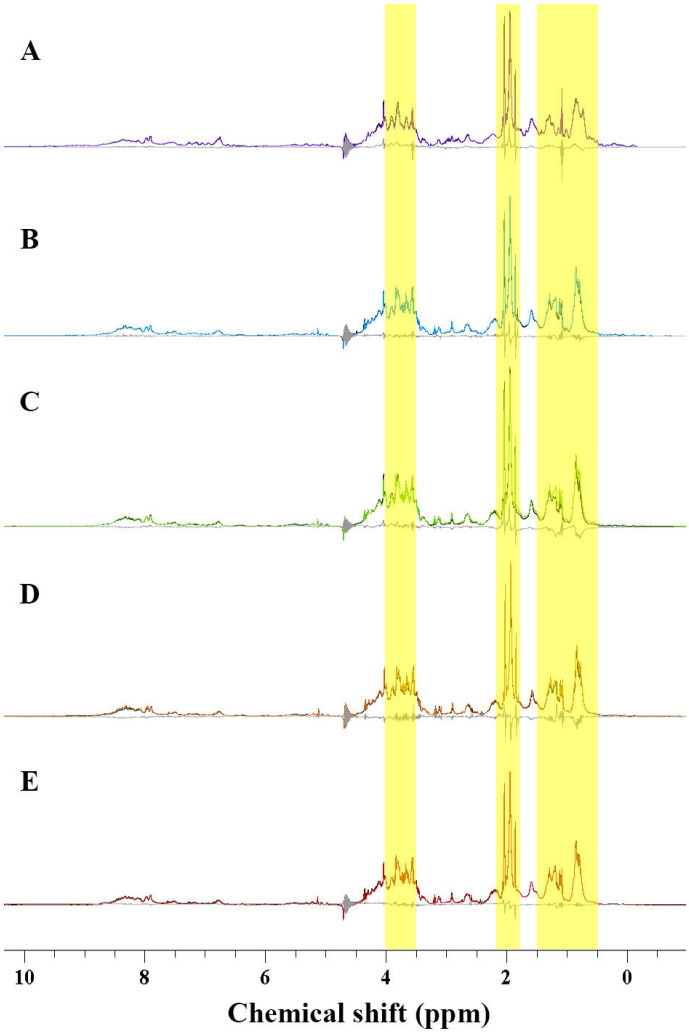

Figure 4.3: NMR spectra showing β-LG or WPC BSM mixtures (coloured), sum spectra of WPC sample and BSM (black), and difference spectra between the two (grey). A: β-LG, B: WPC00, C: WPC05, D: WPC10, E: WPC20.

4.3.3 Accessible thiol groups

Free accessible thiol concentrations were calculated using a 6-point calibration curve. Individual sample values are compared to the mixture of samples with BSM in Table 4.1, with the average of individually measured BSM values subtracted. There was no observed difference in free accessible thiol groups upon mixing with BSM for WPC samples or β-LG (Table 4.1).

Table 4.1: Accessible thiol concentration for samples of WPC and β-LG as individual samples and as mixtures with BSM with the individual BSM value subtracted. Errors represent ± 2 standard deviations. Superscript letters across both columns indicate significantly different groupings ($p \leq 0.05$).

Sample	Accessible thiol concentration (mM)	
	Individual sample	**Mixed with BSM – BSM average**
WPC00	4.0 ± 0.2^{a}	4.0 ± 0.4^{a}
WPC05	5.9 ± 0.3^{b}	6.0 ± 0.5^{b}
WPC10	6.0 ± 0.5^{b}	6.4 ± 0.6^{bc}
WPC20	7.8 ± 0.4^{c}	8.4 ± 0.5^{c}
β-LG	4.3 ± 0.2^{a}	4.1 ± 0.4^{a}

4.3.4 Dynamic light scattering and zeta-potential

Upon mixing with BSM, particle size distribution was shown to shift to higher particle sizes between that of the WPC sample and isolated β-LG. A decrease in particle size distribution was seen upon mixing in comparison to isolated BSM, with larger effects seen for WPC samples than β-LG, as shown in Figure 4.4.

All samples had negative ζ-potential values, ranging from -37.2 to -17.8 mV. Mixing samples with BSM increased the magnitude of the negative ζ-potential for all WPC samples and β-LG (Table 4.2).

Table 4.2: ζ-potential values for WPC samples and β-LG mixed with BSM (1:1) compared to values for the sample . Error bars represent ± 2 standard deviation. Superscript letters across both columns indicate significantly different groupings ($p \leq 0.05$).

Sample	ζ-potenital (mV)	
	Individual sample	**Sample mixture BSM 1:1**
WPC00	-27.7 ± 3.1^{bc}	-34.3 ± 3.2^{cd}
WPC05	-26.7 ± 2.6^{b}	-32.3 ± 2.0^{c}
WPC10	-27.0 ± 3.9^{b}	-31.9 ± 1.2^{c}
WPC20	-26.2 ± 4.0^{b}	-31.2 ± 1.7^{c}
β-LG	-17.8 ± 5.3^{a}	-37.2 ± 1.7^{d}
BSM	-36.5 ± 3.1^{d}	-

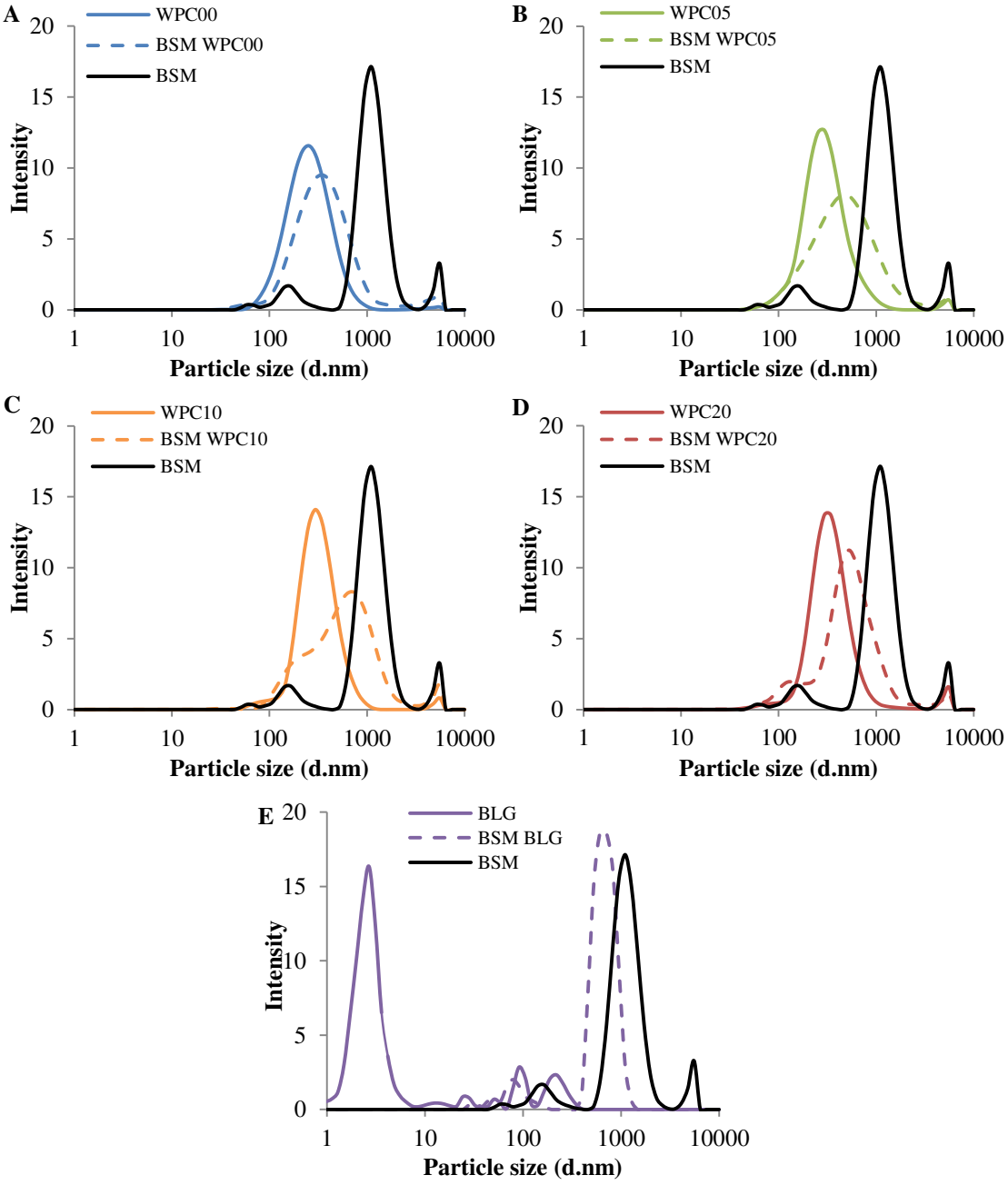

Figure 4.4: Particle size distribution of WPC and β-LG samples in isolation and as a 1:1 mixture with BSM. Particle size is shown as a logarithmic scale and BSM size distribution is shown for clarity. A: WPC00, B: WPC05, C: WPC10, D: WPC20, E: β-LG.

4.4 Discussion

BSM and β-LG have been shown to interact by spectroscopic methods (Celebioglu et al., 2015); however no evidence has previously been shown supporting the interactions occurring when WPC is mixed with mucin. ^{1}H NMR can be used to study the tertiary structural changes observed upon mixing WPC with BSM. The NMR spectra for WPC-BSM mixtures correlates closely to those reported by Celebioglu et al. (2015). The ^{1}H NMR spectra of β-LG has been previously fully assigned by Uhrinova et al. (1998), and α-LA by Alexandrescu et al. (1992); while ovine submaxillary mucin has been fully assigned by Gerken (1986), only oligosaccharide fractions of bovine submaxillary mucin have been previously studied using ^{1}H NMR (Chai et al., 1992). The NMR spectra in this study were used to compare the difference between the sum spectra and the spectra of the mixture of WPC samples and BSM, and a detailed assignment was not undertaken. The difference between the generated sum spectra and the spectra of the mixture with BSM showed differences in three main regions for β-LG and all WPC samples: between 4.3 and 3.3 ppm, 2.1and 1.9 ppm; and from 1.5 to 0.6 ppm. No differences in spectra were observed in the aromatic region, unlike in the CD spectra, which could mean that the changes in aromatic moieties are affected by the alteration of secondary structure characteristics upon interactions with BSM, but are not themselves interacting with BSM. The functional groups of BSM correlating to the differences in the spectra are linked to the mucin glycan groups, while the core backbone residues near 0 ppm are not shown to interact, this agrees with findings by Celebioglu et al. (2015). The interacting regions of WPC can be correlated to the ^{1}H assignment of β-LG by Uhrinova et al. (1998), with differences occurring in regions corresponding to methyl groups, protons in side chains including H^{β}, and H^{α} in α-helices and β-sheets. The NMR results show that the exposed side chains of whey proteins interact with the glycosylated side chains of mucin, with differences seen for β-LG and for WPC samples heated for varying times. It appears that WPC20 had the fewest differences when mixed with BSM, which could correspond to the findings that WPC20 has the largest particle sizes (Chapter 2 Part 2) and reversed the trend in a decreasing far-UV peak (Chapter 3). The structure of WPC20 may have changed to a point where aggregation occurs more readily and interactions caused by particle size may outweigh interactions with specific moieties.

The use of CD to elucidate differences in secondary and tertiary structure has been shown to be useful for BSM-WPC mixtures. While no interactions were evidenced using CD between isolated whey proteins and BSM, there appeared to be an interaction when WPC samples were mixed with BSM at 1:1 ratio. This is further demonstrated by the simulated WPC spectra composed of weighted isolated protein spectra, which showed little difference between the simulated sum spectra and the simulated mixture spectra, in comparison to the WPC samples. The simulated spectra of WPC showed a good correlation with the general shape of the WPC

CD spectra, with peaks and troughs at similar wavelengths. The change in spectra upon mixing WPC with BSM is located in the aromatic region (260-310 nm), with a reduction in negative signal magnitude. The mixture of BSM shows a shift in the remaining small peak towards higher wavelengths, with a small signal around 290 nm, which could correspond to a tryptophan residue in the β-barrel of β-LG (Matsuura & Manning, 1994). The lack of evidence for aromatic residue interactions in NMR suggests that the changes observed for tryptophan could result as a consequence of interactions with other residues within the β-barrel. The reduction in signal intensity around 270 nm could correspond to a loss in structure associated with α-LA (Wijesinha-Bettoni et al., 2007). As described in Chapter 3, there is a reduction in the near-UV CD signal with heating time; therefore, the decrease in difference between the sum spectra and the mix of WPC and BSM with heating time would be expected.

The CD results indicate that the interactions between WPC and BSM are due to the mixture of proteins, not one individual protein; and as such, the system cannot be represented by studying isolated β-LG or α-LA alone. The interactions between WPC and BSM could be due to physical interactions due to the particle size, which increases with heating time. As the ζ-potential of both BSM and all WPC samples are negative under these conditions, it is unlikely that interactions are caused by electrostatic attractions. The presence of impurities, such as calcium, may affect the structure of proteins, leading to different intermolecular interactions, such as hydrophobic interactions and the formation of disulfide bridges. Disulfide bonds may form between thiol groups on WPC and BSM, causing larger particles to form and leading to a drying sensation. As discussed in Chapter 3, WPC samples with longer heating times had higher accessible thiol concentration, and therefore had the potential to form more disulfide bonds, which may lead to more mucoadhesion and drying. Accessible thiol concentrations in WPC and β-LG samples were not found to change upon mixing with BSM. This could indicate that no free thiol groups were exposed on WPC particles upon interacting with BSM; however this could also be a result of any exposed groups interacting with mucin thiol entities, and therefore not being available for the Ellman's assay. Therefore, no conclusions can be drawn as to whether disulfide interactions exist between WPC and BSM.

As WPC samples are heated, an increase in particle size is observed, as discussed in Chapter 2 Part 2. Upon mixing with WPC, a reduction in particle size distribution is seen in comparison to BSM alone. The reduction in BSM particle size upon mixing with WPC samples could be due to a contraction of mucin particles in the presence of calcium ions (Su et al., 2009). These samples have been previously shown to contain the same concentration of free ionic calcium (Chapter 3); therefore, this contraction should happen to the same degree for all samples if the change in particle sizes were due to mucin-calcium binding alone. There is, however, an

increase in the particle size of mixtures of BSM and WPC samples correlating to longer heating times. The shifting of the particle size distribution of WPC-BSM mixtures to higher diameters with longer heating times could result from a higher degree of interactions between WPC and BSM particles, leading to larger particle sizes, and a wider size distribution. The particle size distribution of BSM when mixed with β-LG does not widen, with a similar distribution as the sum spectra; however, the β-LG peak around 3 nm disappears upon mixing with BSM. DLS is biased towards larger particles, so the presence of BSM will obscure the much smaller β-LG particles, so it is not possible to infer that there are no particles of that size remaining.

4.5 Conclusions

WPC was shown to interact with BSM using CD, NMR, particle size distributions. The individual proteins in whey protein were also analysed using CD and found to show little or no structural change when mixed with BSM. These results indicate that no single isolated protein in WPC is responsible for interactions with BSM, and that the mixture of proteins present in WPC may cause complex interactions with BSM leading to structural changes observed using CD and NMR. While CD indicated a change in secondary structure associated with a tryptophan residue, NMR showed no differences in the aromatic region of the spectra, indicating that the change in the secondary structure may be caused by interactions between BSM and other residues in the β-barrel of β-LG. Interactions between BSM and WPC appear to occur between exposed residues of WPC and glycosylated side chains of BSM. The interactions evidenced here indicate the mucoadhesive nature of WPC, which has been linked to a drying sensation upon repeated consumption of whey protein beverages.

Acknowledgements

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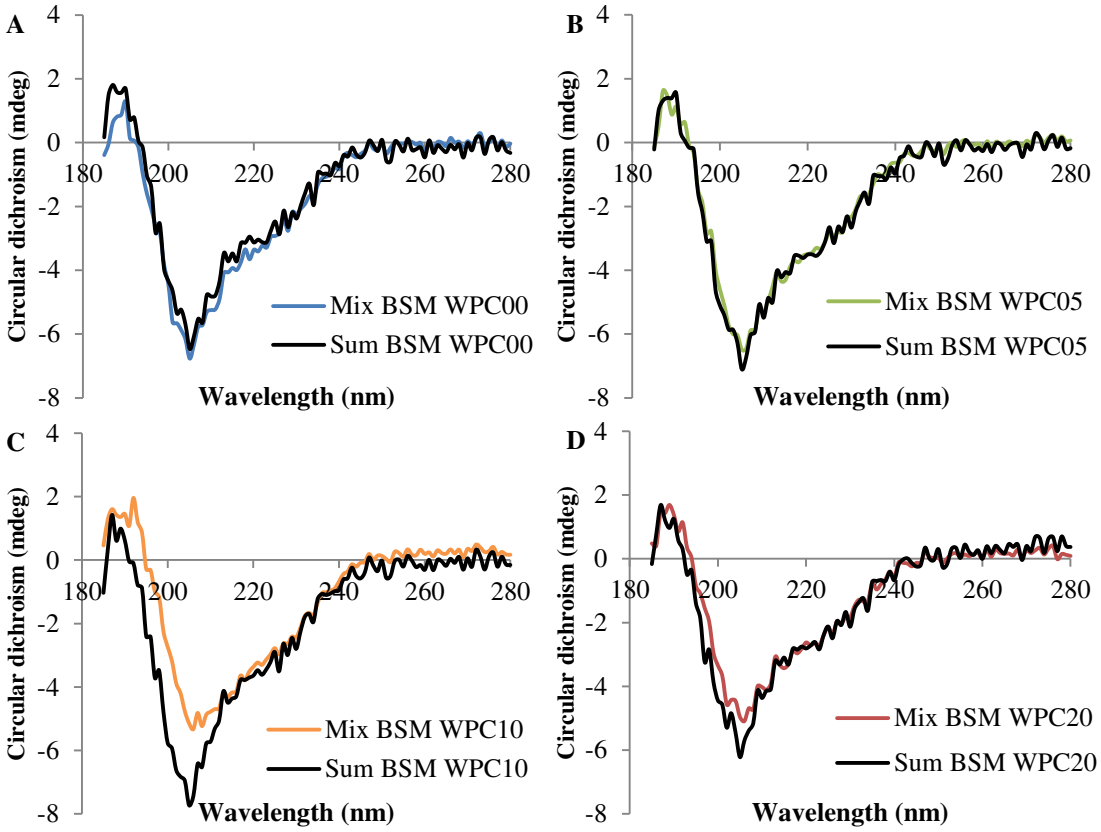
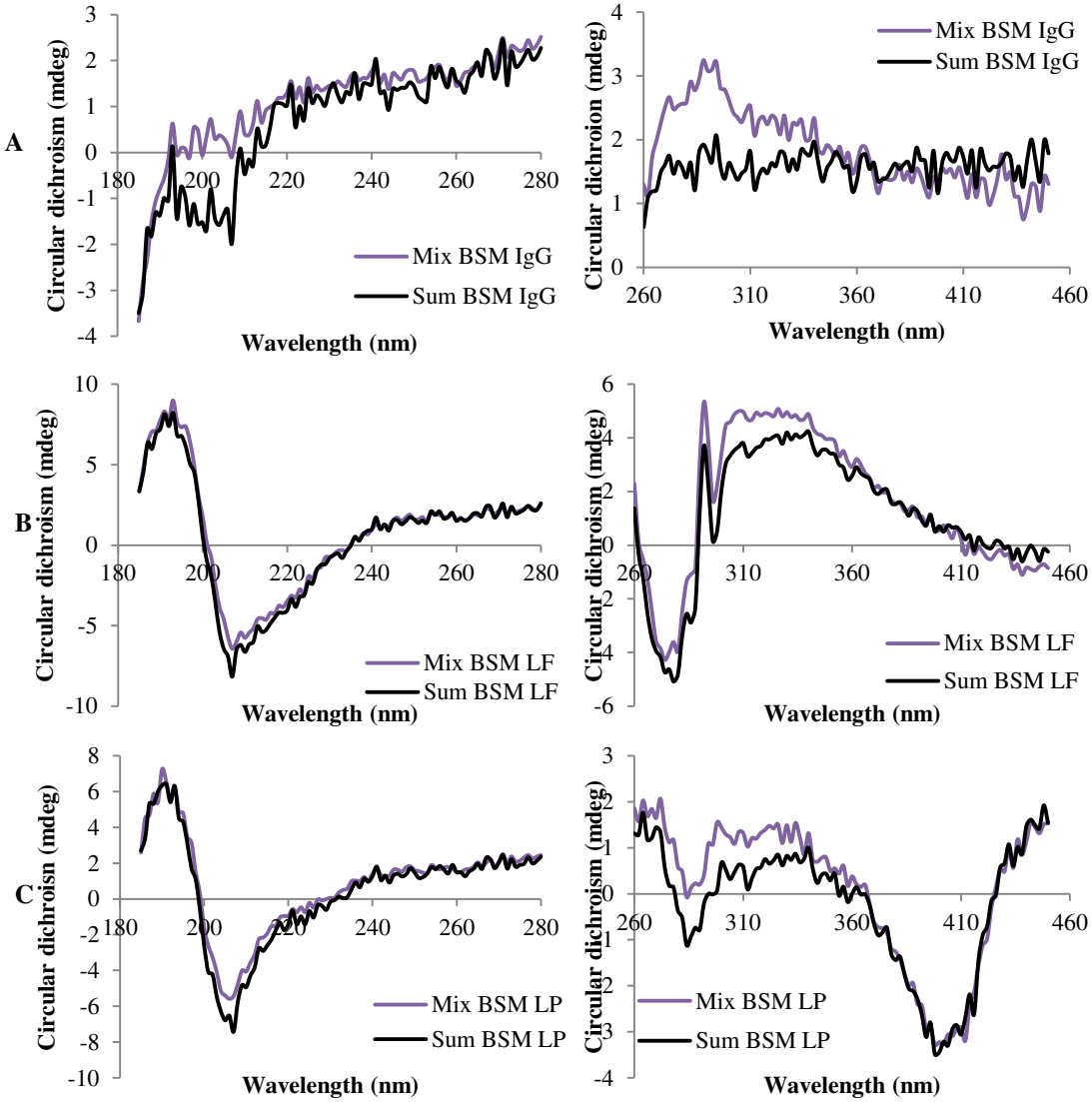
Appendices

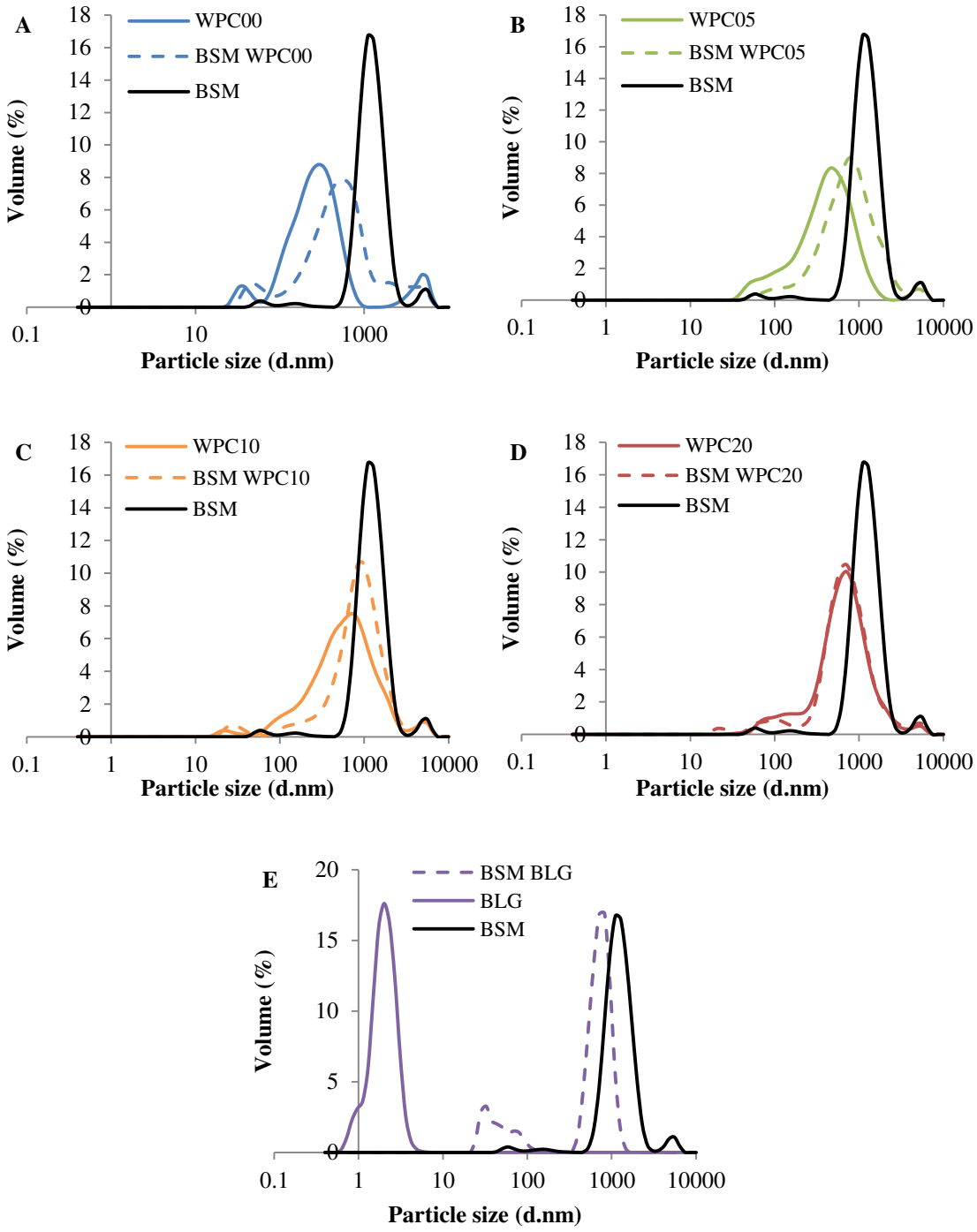
Appendix 4.1: Near-UV CD spectra comparing the 1:1 mixture of WPC samples with BSM, and the sum of the individual spectra of BSM and WPC. A: WPC00, B: WPC05, C: WPC10, D: WPC20.



Appendix 4.2: CD spectra comparing the 1:1 mixture of individual whey proteins with BSM and the sum of the individual spectra of BSM and proteins. Far-UV (left) and near-UV (right)**.** Individual whey proteins shown are: A: immunoglobulin (IgG), B: lactoferrin (LF), C: lactoperoxidase (LP).



Appendix 4.3: Particle size distribution by volume of WPC and β-LG samples in isolation and as a 1:1 mixture with BSM. Volume distributions are shown as a contrast to intensity distributions as they tend to skew towards smaller sizes, whereas intensity distributions are skewed towards larger sizes. Particle size is shown as a logarithmic scale and BSM size distribution is shown for clarity. A: WPC00, B: WPC05, C: WPC10, D: WPC20, E: β-LG.



Chapter 5 Effects of polysaccharides on the mouthfeel and structure of whey protein beverages

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Abstract

This chapter investigates the addition of two polysaccharides, pullulan and carboxymethyl cellulose (CMC), to native and denatured whey protein concentrate to determine the potential mitigating effects of polysaccharides on the mouthfeel attributes of whey protein beverages. The effect on physicochemical properties were characterised by viscosity, particle size, zeta-potential, and transmission electron microscopy. Sensory attributes were defined by qualitative descriptive analysis and changes in mouthfeel over repeated consumption were measured by sequential profiling. A large increase in particle size was observed upon addition of CMC and pullulan to denatured whey protein; this was caused by aggregation of the protein and polysaccharide bridging, as shown by TEM. Pullulan was shown to reduce the intensity of chalky mouthfeel in heated whey protein, demonstrating the potential for polysaccharides to modulate negative sensory attributes associated with whey protein.

5.1 Introduction

Whey protein is a frequently used functional ingredient in sports nutrition, and in the prevention of sarcopenia in older adults. In order to maximise the benefits of whey protein, a significant quantity must be consumed; something which is an issue for clinical patients due to negative sensory attributes causing non-compliance, with textural attributes contributing significantly (Gosney, 2003). One such attribute is mouthdrying, caused by the whey proteins (Lemieux & Simard, 1994), which has been shown to increase over repeated consumption (Chapter 2 Part 2).

Mouthdrying in whey protein has been linked to the interactions with saliva (Ye, Streicher, & Singh, 2011) and the oral mucosa (Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013). Denatured whey protein has been shown to cause more drying, partially attributed to larger particle sizes (Chapter 2 Part 2), and have a higher mucoadhesive strength when used in drug delivery (Hsein, Garrait, Beyssac, & Hoffart, 2015). Previous work has shown an increase in oral retention linked to whey protein with higher levels of denaturation and higher free accessible thiols, resulting in higher mouthfeel scores; hence indicating mucoadhesion as a mechanism for mouthdrying in whey protein beverages (Chapter 3).

Chalkiness is a textural defect of milk products, described as the detection of "numerous, extremely fine, undissolved particles" (Lemieux & Simard, 1994), which is often associated with astringency or drying. The authors previously found drying, chalky and mouthcoating all increased with increased heating time and across repeated consumption of whey protein beverages (Chapter 2 Part 2).

While chalkiness is often described as synonymous to astringency (Charalambous, 1980), the mouth-feel wheel developed by Gawel, Oberholster, and Francis (2000) describes astringency using a vocabulary of 33 words under 7 categories. Using this terminology, chalky falls under particulate; whereas drying is a separate category containing numbing, parching and dry. Little work has been done to characterise the cause of chalky mouthfeel in whey protein fortified foods and milk products, although chalky flavour has been linked to oxidation products in skimmed milk (Clark, 2009).

The modification of milk product texture can be achieved using polysaccharides (Bayarri, Chulia, & Costell, 2010; Gallardo-Escamilla, Kelly, & Delahunty, 2007; Nguyen, Kravchuk, Bhandari, & Prakash, 2017), which are commonly used to improve stability (Du et al., 2009; Huan, Zhang, & Vardhanabhuti, 2016; Koupantsis & Kiosseoglou, 2009; Vardhanabhuti, Yucel, Coupland, & Foegeding, 2009). A summary of reported effects of polysaccharides on dairy sensory properties is shown in Table 5.1.

Table 5.1: Effects of polysaccharides on sensory properties of milk products.

Polysaccharide	Effects
Carageenan	↑ Chalkiness; ↓ thickness (0.01% in skim milk yoghurt) [(b)].
	↑ Vanilla flavour and sweetness; (1.5% in low fat dairy dessert) [(d)].
Carboxymethyl cellulose (CMC)	↑ Sweetness, smooth mouthfeel and thick mouthfeel; ↓ acid taste (0.16% in fermented whey) [(a)].
High-methoxyl pectin (HMP)	↑ Smooth mouthfeel and thick mouthfeel (0.53% in fermented whey)[(a)].
Pectin	↑ Graininess; ↓ aqueousness (0.6% in yoghurt) [(c)].
Propylene glycol alginate (PGA)	↑ Sweetness, gritty mouthfeel and thick mouthfeel; ↓ yoghurt flavour, acid taste and smooth mouthfeel (0.32% in fermented whey) [(a)].
Starch	↑ Chalkiness, thickness, lumpiness, stickiness and residual coating (0.01% in skim milk yoghurt) [(b)].

(a) (Gallardo-Escamilla et al., 2007); (b) (Nguyen et al., 2017); (c) (Arltoft, Madsen, & Ipsen, 2008); (d) (Bayarri et al., 2010).

Many food products containing whey protein also contain thickeners to achieve a palatable thickness, especially for older adults or those with dysphagia. There are various polysaccharides with differing characteristics which have been investigated in the literature. Pectin added to yoghurt has been shown to increase graininess and reduce 'aqueousness' (Arltoft et al., 2008). Interactions between pectin and whey protein have been shown to be stable at pH 4.5 due to electrostatic interactions with β-lactoglobulin (β-LG) (Gentes, St-Gelais, & Turgeon, 2010), while stable complexes between whey protein and high-methoxyl pectin (HMP) were achieved at pH 7 using a heat setting treatment (Wagoner & Foegeding, 2017). HMP interactions with β-LG are also influenced by hydrogen bonding (Girard, Turgeon, & Gauthier, 2002).

Dextran interacts with β-LG at neutral pH due to positively charged regions on β-LG and negatively charged dextran sulfate, which reduces the heat stability of β-LG (Vardhanabhuti et al., 2009). Xanthan gum (XG) is commonly used to form complexes with whey protein to create fat replacers. The size of XG-whey protein complexes is heavily influenced by the protein polysaccharide ratio, with larger complexes forming at higher protein concentration (Laneuville, Paquin, & Turgeon, 2000). When added to set yoghurt, XG and carrageenan caused an increase in chalkiness, however carrageenan reduced lumpiness and increased smoothness (Nguyen et al., 2017). Carboxymethyl cellulose (CMC) is an anionic polysaccharide used to stabilise food and create emulsifying complexes with whey protein (Girard, Turgeon, & Paquin, 2002; Huan

et al., 2016). CMC interacts through electrostatic forces with whey protein at neutral pH, however these interactions are disrupted in an oil-in-water emulsion at neutral pH (Koupantsis & Kiosseoglou, 2009). Chitosan is a commonly used mucoadhesive, which interacts with whey protein above pH 5 (Laplante, Turgeon, & Paquin, 2005). Pullulan is a non-mucoadhesive polysaccharide which has been used to encapsulate pharmaceutical ingredients alongside whey protein (Cabuk & Harsa, 2015).

Mucoadhesive polysaccharides were hypothesised to reduce the intensity of mouth drying imparted by whey protein, by competitively interacting with the mucosa, and forming a lubricative layer. In choosing polysaccharides to combine with whey protein beverages, it is important to consider potential negative sensory attributes they may impart. In fermented whey, PGA and CMC decreased acid taste by reducing the mobility of tastants and hence their interaction with taste receptors. Headspace analysis of flavour volatiles revealed a decrease in key flavour compounds for all hydrocolloids, as the increased thickness reduced mobility and release of volatiles into the headspace (Gallardo-Escamilla et al., 2007). Different polysaccharides had varying effects on mouthfeel attributes, demonstrating that the characteristics of the polysaccharide chosen to thicken milk products affect more than just the thickness (Table 5.1).

In order to develop a mitigation strategy for reducing the negative mouthfeel attributes in WPC beverages, a range of polysaccharides were considered. Mucoadhesive polysaccharides were hypothesised to competitively interact with the mucosa, decreasing the amount of whey protein mucoadhesion derived drying. In choosing a mucoadhesive polysaccharide, the main limitations were suitability for a food product due to taste and texture.

When deciding upon polysaccharides to use, a range of food grade options were informally tested: sodium CMC, sodium alginate, pectin, and pullulan. Sodium alginate had a low solubility in water and produced a very thick mixture, due to gelation of alginate in the presence of calcium ions. Pectin was trialled using four varieties: classic citrus pectin, classic apple pectin, citrus amid pectin, (Herbstreith & Fox, Germany), and citrus Unipectine (Cargill, Minnesota, US). All pectin samples produced large hard particles when mixed with WPC, and had off flavours including acidic, citrus, and grainy. CMC mixed with WPC gave a salty taste and glue-like texture. Pullulan did not impart much flavour but added a thick and gluey texture. CMC was selected as a suitably soluble mucoadhesive polysaccharide, and pullulan was selected as a suitable soluble non-mucoadhesive polysaccharide. Pullulan and CMC were trialled at concentrations from 0.1% to 2% in WPC00. Concentrations were selected based on physical and sensory thickness of sample, with a range of 0.2 to 0.5% selected for CMC and 0.25 to 1% selected for pullulan. Final concentrations selected were below the critical coil

overlap concentration (c^*). While polysaccharides present in concentrations above c^* have been more successful in altering sensory attributes (Troszynska et al., 2010; Xue et al., 2014), polysaccharides over this concentration are far more viscous. While the aim was to match viscosities, CMC caused a large increase in viscosity for all concentrations attempted, but at concentrations below 0.25%, sensory differences were not detectable. Pullulan produced a distinctive off-flavour once added at concentrations over 1%; therefore these concentrations were selected as the closest in viscosity.

The aims of this study were to assess polysaccharides as part of a potential mitigation strategy to reduce mouthdrying attributes in whey protein beverages and to characterise the physicochemical properties of the protein-polysaccharide mixtures for heated and unheated whey protein concentrate.

5.2 Materials and methods

The whey protein concentrate (WPC) used was Volactive Ultrawhey 80 Instant (Volac International Limited, Orwell, Royston, UK), a dry powder with a protein content of 80% minimum, and containing soy lecithin (0.5% maximum) as an emulsifying agent. The remaining 20% contains moisture, fat, lactose, and minerals. Low viscosity sodium CMC was provided by Akzonobel (Arrhem, the Netherlands). Pullulan was provided by Nagase Group (Düsseldorf, Germany). Crackers (Carr's Table Water Biscuits, United Biscuits, London, UK) were used as palate cleansers in sensory profiling.

5.2.1 Preparation of whey protein beverages

WPC beverages were prepared as described in Chapter 2 Part 2: by the addition of WPC powder to water (10% w/v, deionised water). The dilution selected is recommended for many commercially available powders, and represents a serving of 20 g of protein per 250 mL portion, which has been linked to nutritional benefits (Tipton et al., 2007). All samples were stirred for 30 min at room temperature (25 ± 2 ^{0}C). A native sample was then stirred for a further 60 min at room temperature (WPC00). Another sample was stirred while being heated in a water bath set at 70 °C for 20 min (WPC20). The samples were cooled in a water bath then allowed to hydrate overnight at 4 °C. CMC was added to samples of WPC00 and WPC20 at a total concentration of 0.25%, and pullulan was added to separate samples of WPC00 and WPC20 at a total concentration of 1%. Measurements were performed in triplicate on each of three processing replicates prepared on three separate days.

5.2.2 Sensory methods

A trained sensory panel of experts in profiling techniques (n = 11; 10 female, 1 male), with a minimum of 6 months training, were given further training on WPC profiling and sequential

profiling (minimum 4 hours). Sensory evaluation was carried out at room temperature (22 ± 2 ^{0}C) in isolated booths.

5.2.2.1 Quantitative descriptive analysis

Quantitative descriptive analysis (QDA) (Stone, Sidel, Oliver, Woolsey, & Singleton, 1974) was performed using a consensus vocabulary developed by the panel during training (23 attributes; 6 taste, 4 flavour, 6 mouthfeel, 7 aftereffects). The panel assigned mouthfeel characteristics in order to separate important attributes describing distinct sensations. These consensus mouthfeel attributes were: body, furring, chalky, mouthcoating, drying and salivating. All attribute descriptors or references are summarised in Appendix 5.1.

WPC samples were evaluated in duplicate according to a balanced design using unstructured line scales (0 - 100) with appropriate anchors (nil to extreme). Samples were presented monadically in 20 mL cups, crackers and warm filtered tap water (40 °C) were provided as palate cleansers between samples during an enforced break (2 min). Evaluation was carried out under red lighting.

5.2.2.2 Sequential profiling

Sequential profiling was carried out to establish the perception of six sensory attributes over repeated consumption of eight aliquots (5 mL) of samples, with 1-minute breaks between aliquots. Samples were scored after consumption of each aliquot (T0), and following 30 (T30) and 60 s (T60) time delays, as described by Methven et al. (2010) (Compusense at-hand, Ontario, Canada). Thus there were eight aliquots tasted for each of six samples (WPC00, WPC20, WPC00 pullulan, WPC20 pullulan, WPC00 CMC, and WPC20 CMC), scored at three time points (T0, T30 and T60).

The six attributes scored were sour, metallic, creamy flavour, mouthcoating, chalky and drying. These were chosen carefully from the full QDA profile. Sour and metallic are taste attributes associated with whey protein beverages (Martini & Walsh, 2012; Whetstine, Croissant, & Drake, 2005). Creamy flavour was selected as this attribute showed significant differences between samples in the QDA data. Mouthcoating, chalky and drying were selected by the panel as dominant mouthfeel attributes.

Samples were coded with three-digit numbers and all eight aliquots of one sample were presented together with the same code; the panellists were not blinded to the sequential nature of the evaluation. Warm filtered tap water and crackers were provided as palate cleansers in the 2 minute enforced break between samples; however panellists were instructed not to use these between the eight aliquots of the same sample. Panellists were instructed to consume the total

volume of each aliquot and to coat the mouth with the sample before swallowing. Two samples were scored in each session. Evaluation was carried out under red lighting and aliquots were served in opaque black cups to mask appearance differences between samples. Ten of the trained panellists were present for sequential profiling (n = 10; 10 female).

5.2.3 Instrumental analysis methods

All instrumental measurements were performed in triplicate on each of three processing replicates prepared on three separate days.

5.2.3.1 TEM

Samples were diluted 100 fold using water (HPLC grade) and a 300-mesh Formvar/carbon-coated copper grid (Agar Scientific, UK) was floated on a drop of sample for one minute before being dried using filter paper, then stained for 30 s using 2% uranyl acetate (used to stain proteins and lipids), and finally dried with filter paper before observation using TEM (JEM-2100Plus, JEOL, Japan).

5.2.3.2 Rheology

Rheological properties of WPC samples were analysed using an oscillatory rheometer (AR2000, TA Instruments, USA) fitted with a 40 mm diameter rotating plate adjusted to 37 °C. Samples were placed on the lower plate surface and equilibrated to 37 °C. Strain sweeps of the samples were obtained by applying oscillation at a frequency of 2 Hz for strain values ranging from 0.01 to 10 in 12 steps. A strain of 1% was then chosen in the linear viscoelastic region for a frequency sweep, where the frequency was varied from 0.1 to 10 Hz in 25 steps.

5.2.3.3 Dynamic light scattering

Samples were diluted 100 times in water (HPLC grade water) for dynamic light scattering (DLS) analysis and measurements were performed using a Nano-S Zetasizer in size measurement mode (Malvern Instruments, UK) at 30 °C, with an equilibration time of 60 s.

5.2.3.4 Zeta-potential

WPC samples were diluted 100 times in water (HPLC grade water) for ζ-potential measurements, which were performed using a Nano-S Zetasizer in zeta measurement mode (Malvern Instruments, UK) at 30 °C with an equilibration time of 60 s.

5.2.4 Statistical analysis

SENPAQ (version 5.01) was used to carry out analysis of variance (ANOVA) of QDA data. IBM SPSS Statistics (version 21) was used to carry out three-way repeated measures ANOVA (RM-ANOVA) on the sequential profiling data using sample (n = 6), assessors (n = 10), and

repeated consumption (n = 8) as explanatory variables; Tukey's HSD was used as a posthoc test. Rates of incline were calculated as the gradient of the linear trend line. Relative strength of aftereffects were calculated as a percentage of the initial intensity score:

$$Relative\ strength\ of\ aftereffect = \frac{x_{TN}}{x_{T0}} \times 100\%$$

where x = intensity score, and N = 30 or 60.

Analytical data were analysed by one-way ANOVA using IBM SPSS Statistics (version 24).

5.3 Results

5.3.1 Sensory data

5.3.1.1 QDA data

Of the 23 attributes selected through consensus vocabulary, 10 attributes showed significant differences ($p \leq 0.05$) between samples (flavour and mouthfeel attributes shown in Figure 5.1, mean intensities for all attributes shown in Appendix 5.1). It can be seen from Figure 5.1 that for most attributes the differences due to denaturation (WPC20 vs WPC00) were greater than the differences due to addition of polysaccharide. The WPC20 samples were more drying than the WPC00 samples ($p \leq 0.0211$) and, unfortunately, the polysaccharides had no effect on drying intensity. However, where WPC20 was significantly more chalky than WPC00 ($p < 0.0001$), this was significantly reduced by the addition of pullulan ($p = 0.0121$), with a similar but not significant trend on the addition of CMC to WPC20 ($p = 0.1836$). While WPC20 was higher in mouthcoating than WPC00 ($p < 0.0016$), WPC20 with pullulan was not significantly different to WPC00 ($p = 0.123$). WPC20 with pullulan was significantly lower in body intensity than WPC20 with CMC ($p = 0.0215$). WPC20 with CMC had a significantly higher creamy flavour intensity than WPC20 with pullulan ($p = 0.04$).

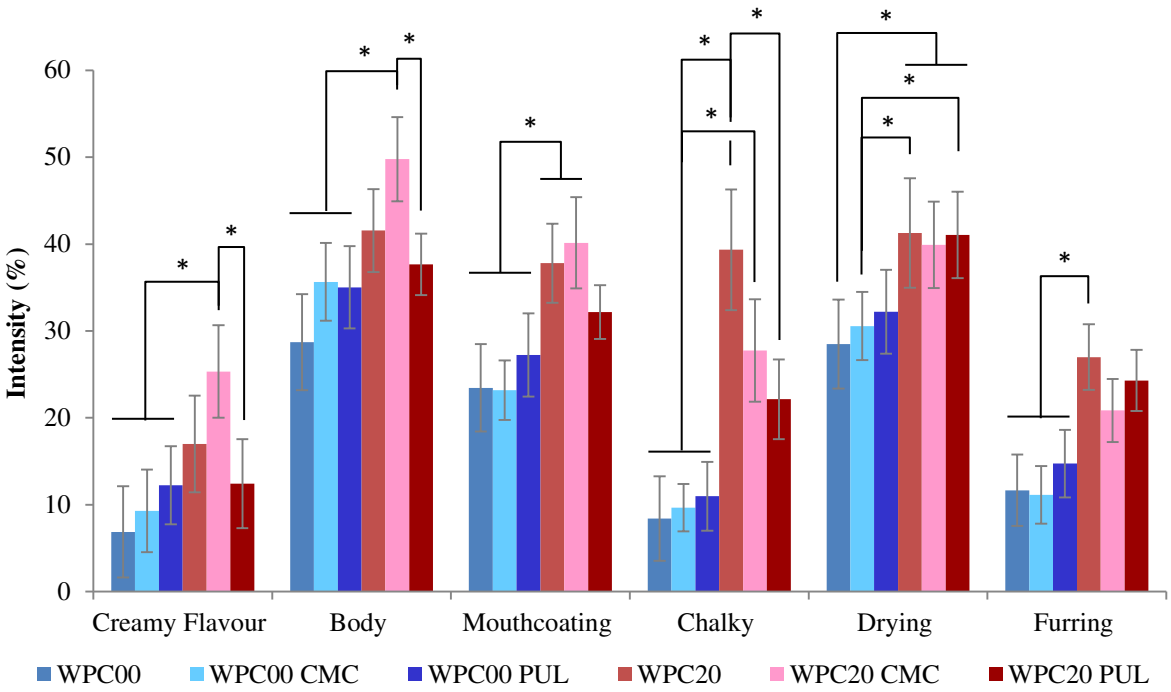

Figure 5.1: Mean QDA intensities for flavour and mouthfeel attributes with significant differences. Error bars represent standard error of the mean. * Significantly different scores between samples ($p = \leq 0.05$) calculated through ANOVA.

5.3.1.2 Sequential profiling data

Sequential profiling was used to measure the intensity of 6 attributes during repeated consumption. Chalky, creamy flavour, drying, metallic, mouthcoating and sour were measured for 8 consecutive aliquots during consumption, and as aftereffects at 30 and 60 s. Significant differences ($p \leq 0.05$) were observed between all WPC00 and all WPC20 samples for creamy flavour, mouthcoating, drying and chalky, with WPC20 samples scoring higher for these attributes (T0 scores for all 6 attributes shown in Figure 5.2). No significant differences were observed upon the addition of CMC or pullulan to WPC00 or WPC20; however trends were observed which require further investigation.

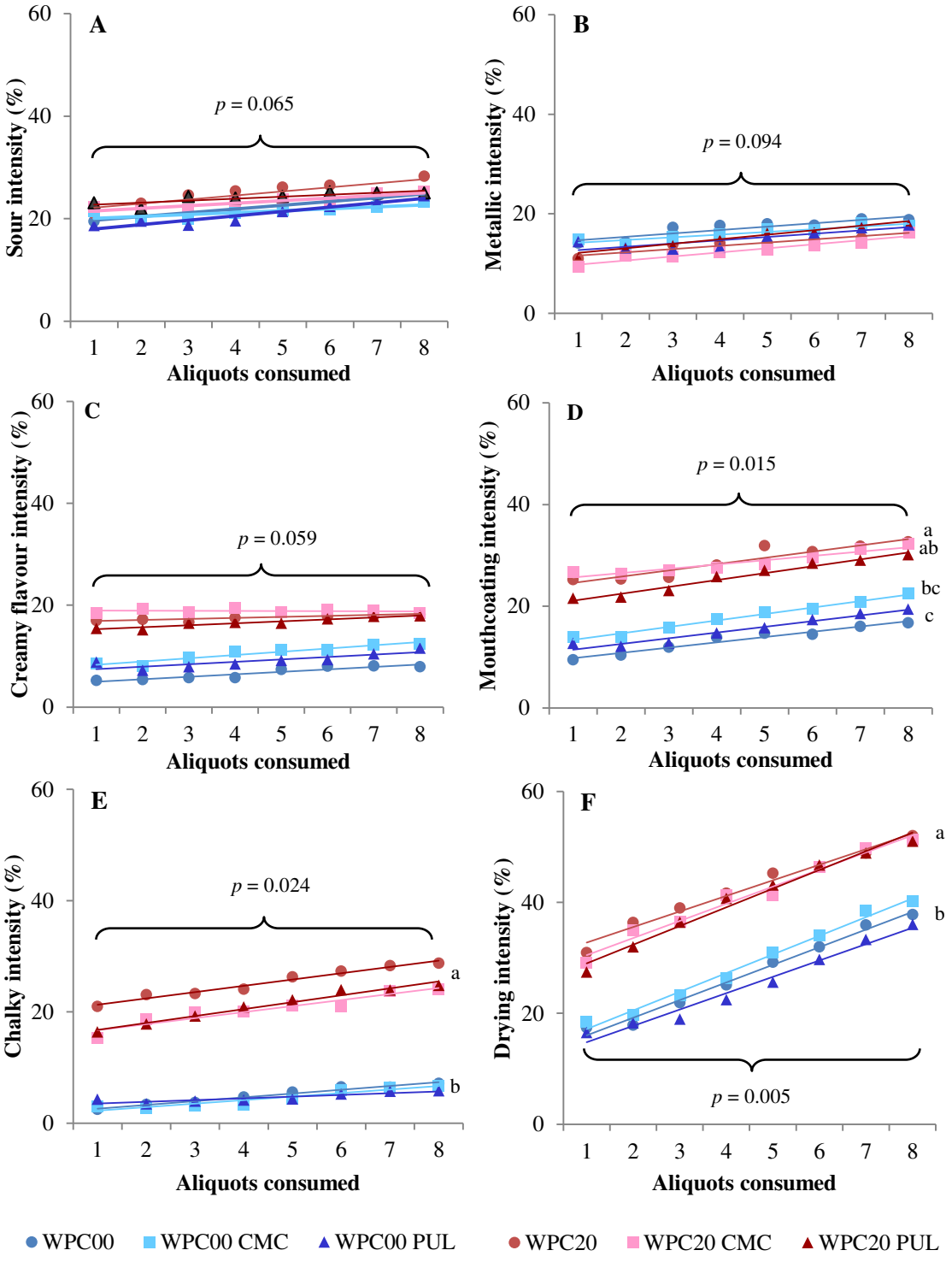

Figure 5.2: Mean intensities scored during consumption (T0) from sequential profiling over 8 repeated consumptions. Attributes shown are: A: Sour, B: Metallic, C: Creamy flavour; D: Mouthcoating; E: Chalky; F: Drying. Letters denote significantly different sample groupings as calculated by RM-ANOVA; *p*-values are shown for significant changes over repeated consumption.

Significant differences across consumption were seen for mouthcoating, chalky and drying, with an increase in intensity with repeated consumption; rates of incline are shown in Table 5.2. Rates of incline are shown for each attribute over 8 aliquots. Scores are separated by sample and by the scoring time in relation to the sip: immediately after consumption (T0), 30 s after swallowing (T30), and 60 s after swallowing (T60). Drying had the highest rates of incline, with gradients ranging from 2.94 to 3.73, whereas creamy flavour ranged from -0.03 to 0.71. Samples with CMC generally had lower rates of incline that samples without or with pullulan, particularly at T0 and T30, with the exception of drying. Samples with pullulan generally had similar or higher rates of incline in comparison to samples without. The rates of incline for chalky were initially lower at T0 for WPC00 PUL in comparison to WPC00; however for T30 and T60 they were similar. The rate of incline for T30 and T60 remained low for WPC20 CMC and WPC20 PUL in comparison to the values for WPC20, which increased from T0 to T30 and T60. Rates of incline for mouthcoating also increased less from T0 to T60 for WPC20 CMC and WPC20 PUL compared to WPC20.

Table 5.2: The average rates of incline (Δ intensity/aliquot) for tasting (T0) and aftereffects at 30 (T30) and 60 s (T60), from RM-ANOVA of sequential profiling data. Significant changes across aliquots are shown within a row for each sample at each scoring time, $p \leq 0.05$ (*).

Attribute	Time	WPC00	WPC00 CMC	WPC00 PUL	WPC20	WPC20 CMC	WPC20 PUL
Creamy Flavour	T0	0.5	0.6	0.5	0.2	-0.0	0.4
	T30	0.4	0.5	0.5	0.3	0.4	0.7
	T60	0.3	0.5	0.5	0.6	0.6	0.7
Sour	T0	0.7	0.4	0.9	0.8	0.5	0.4
	T30	1.0	0.5	1.2	1.0	0.7	0.7
	T60	1.3	0.8	1.3	1.1	1.2	0.7
Metallic	T0	0.6	0.5	0.6	0.5	0.6	0.8
	T30	0.9	0.7	1.2	0.6	0.7	0.6
	T60	1.1	0.9	1.2	0.9	0.9	0.8
Chalky	T0	0.7*	0.6*	0.3*	1.1*	1.1*	1.3*
	T30	0.8*	0.6*	0.7*	1.6*	1.2*	1.1*
	T60	0.6*	0.6*	0.6*	1.9*	1.4*	1.4*
Drying	T0	3.2*	3.4*	2.9*	2.8*	3.1*	3.4*
	T30	3.7*	3.3*	3.4*	3.0*	3.2*	3.2*
	T60	3.7*	3.6*	3.7*	3.0*	3.3*	3.5*
Mouthcoating	T0	1.0*	1.3*	1.1*	1.2*	0.9*	1.2*
	T30	1.3*	1.2*	1.4*	1.7*	1.4*	1.5*
	T60	1.5*	1.2*	1.4*	2.1*	1.9*	1.7*

Rates of incline are measured across eight aliquots, separated by sample and scoring time in relation to the sip: immediately after consumption (T0), 30 s after swallowing (T30), and 60 s after swallowing (T60).

To demonstrate the decreasing intensity of each attribute after swallowing, the relative strength of the intensity scores for aftereffects results were calculated as percentages using the mean intensity scores at T30 and T60 and the equivalent T0 score (Table 5.3). Drying had the highest retention of intensity at both T30 and T60 aftereffect scores, with percentages ranging from 94% to 105%. WPC00 CMC and WPC00 PUL both had higher prevailing scores for creamy mouthfeel in comparison to WPC00 at T60.

Table 5.3: Relative strength of aftereffect, expressed as a percentage of the T0 score, for T30 and T60. Mean values are shown for all eight aliquots for each attribute. Errors are ± 2 standard error of the mean.

		Relative strength of aftereffect (%)					
Time	**Attribute**	**WPC00**	**WPC00 CMC**	**WPC00 PUL**	**WPC20**	**WPC20 CMC**	**WPC20 PUL**
T30	Creamy	55 ± 8	56 ± 4	63 ± 5	55 ± 3	61 ± 4	54 ± 4
	Sour	61 ± 5	64 ± 3	64 ± 5	75 ± 3	63 ± 3	66 ± 3
	Metallic	67 ± 5	72 ± 4	72 ± 9	84 ± 3	80 ± 3	80 ± 4
	Chalky	66 ± 13	70 ± 7	64 ± 19	77 ± 5	77 ± 4	73 ± 3
	Drying	103 ± 4	102 ± 2	105 ± 6	99 ± 1	101 ± 2	104 ± 2
	Mouthcoating	83 ± 6	77 ± 4	79 ± 7	79 ± 5	76 ± 5	79 ± 3
T60	Creamy	38 ± 5	50 ± 5	49 ± 6	45 ± 5	52 ± 6	43 ± 6
	Sour	56 ± 8	59 ± 5	60 ± 7	66 ± 5	54 ± 7	56 ± 4
	Metallic	65 ± 8	66 ± 7	66 ± 10	75 ± 7	75 ± 5	72 ± 3
	Chalky	51 ± 9	49 ± 14	50 ± 19	60 ± 10	60 ± 7	60 ± 6
	Drying	104 ± 7	99 ± 4	103 ± 7	94 ± 2	98 ± 3	102 ± 3
	Mouthcoating	75 ± 9	70 ± 5	69 ± 8	69 ± 8	66 ± 8	72 ± 6

5.3.2 Instrumental analysis

A frequency sweep of all samples at a strain of 1% is displayed in Figure 5.3. WPC20 with and without CMC were found to have the highest viscosity; all other samples had lower viscosities with no significant differences. At frequencies above 4 Hz, no significant differences were observed between sample complex viscosities.

Accessible thiol concentrations, particle size and ζ-potentials are summarised in Table 5.4. Accessible thiol concentration was found to be substantially higher for all WPC20 samples than WPC00 samples. The addition of CMC and pullulan to WPC00 did not significantly alter free accessible thiol concentrations; however the addition of pullulan to WPC20 caused a significant but small increase in free accessible thiol concentration. An increase in particle size was observed upon the addition of CMC and pullulan to WPC20, but not to WPC00. WPC20 with

CMC had a significantly larger Z-average than WPC20 with pullulan. WPC20 pullulan had a larger variation in z-averages between sample replicates, due to two overlapping peaks in the size distribution. No significant differences in ζ-potential were found upon addition of CMC or pullulan to WPC00, or to WPC20.

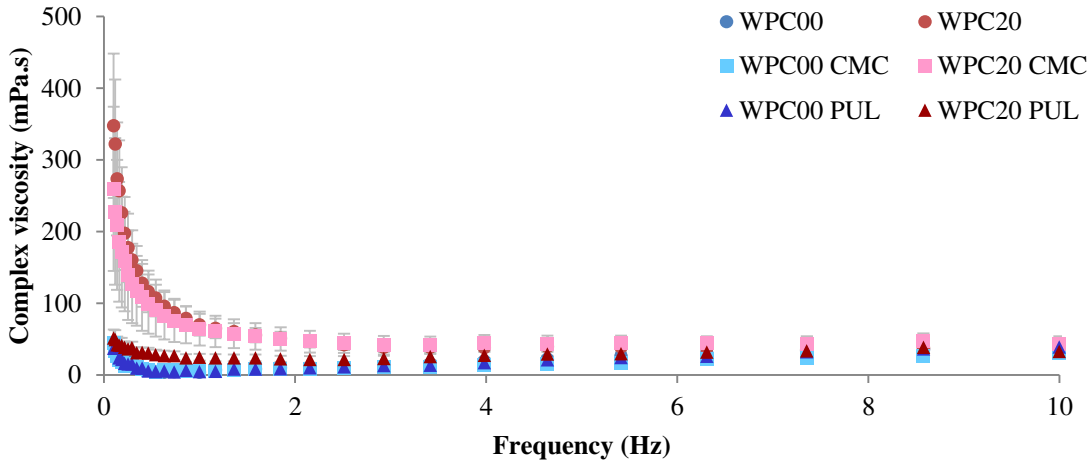

Figure 5.3: A frequency sweep at a strain of 1%, showing rheological behaviour across a frequency range of 0.1–10 Hz. Error bars represent ±2 SEM.

Table 5.4: Free accessible thiol concentrations, Z-averages, and ζ-potentials.Errors represent ± 2SEM (95% C.I.). Superscript letters in a column indicate significantly different groupings ($p \leq 0.05$).

Sample	Free thiol concentration (mM)	Z-ave (d.nm)	ζ-potential (mV)
WPC00	8.04 ± 0.25^{a}	220 ± 16^{a}	-27.7 ± 3.1^{a}
WPC00 CMC	7.91 ± 0.16^{a}	274 ± 33^{a}	-25.8 ± 2.4^{ab}
WPC00 Pul	7.77 ± 0.12^{a}	245 ± 11^{a}	-25.8 ± 0.9^{ab}
WPC20	13.60 ± 0.19^{b}	317 ± 71^{a}	-26.2 ± 4.0^{ab}
WPC20 CMC	14.26 ± 0.72^{bc}	881 ±45^{c}	-25.6 ± 0.5^{ab}
WPC20 Pul	15.69 ± 1.55^{c}	681 ± 174^{b}	-24.1 ± 1.4^{b}

TEM images showed smaller more spherical particles present in WPC00 samples, whereas larger aggregates were present in WPC20. The addition of CMC created larger particles, especially in WPC20 CMC, where large aggregates with bridging features were observed. Samples with pullulan increased in size slightly, with longer shapes observed (Figure 5.4 shows digitally enlarged TEM images with the same scale, for original images see Appendix 5.2).

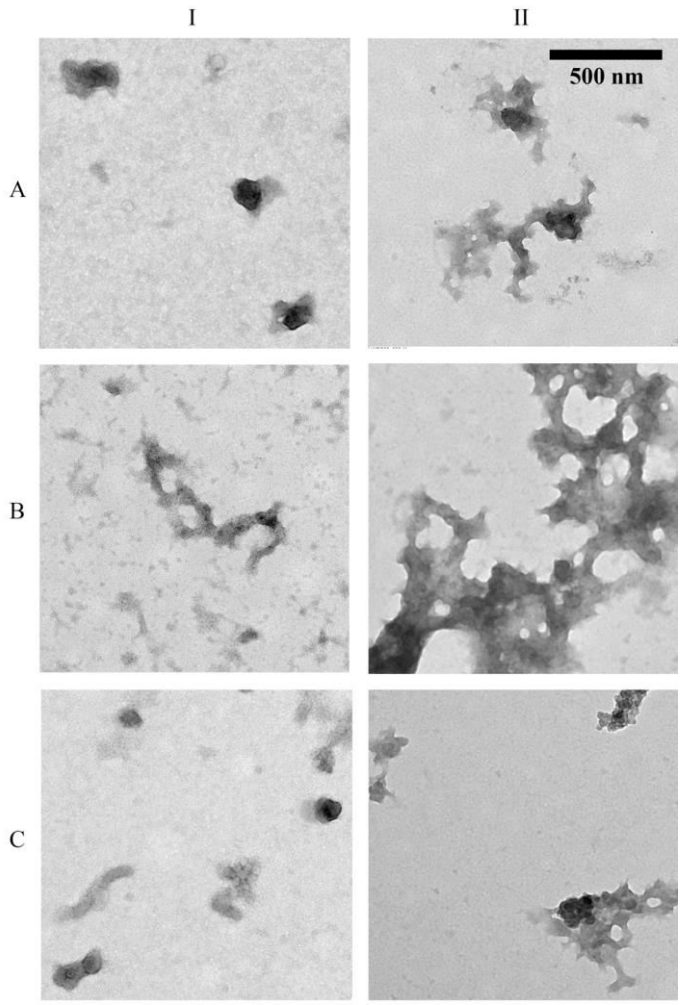

Figure 5.4: TEM images of WPC samples with CMC and pullulan. I: WPC00, II: WPC20. A: no polysaccharide, B: CMC, C: pullulan. Dark regions show protein stained with uranyl acetate, grey regions show unstained areas. Scale bar shown in upper right is 500 nm; images have been digitally enlarged to achieve the same scale.

5.4 Discussion

As the addition of polysaccharides to WPC could impart negative sensory attributes, QDA was used to assess the overall sensory profile of the samples. The results from QDA were used to inform the 6 attributes chosen for sequential profiling.

Higher mouthfeel attributes were observed by QDA in WPC20 samples in comparison to WPC00 samples, corresponding to findings in Chapter 2 Part 2. Sequential profiling found that with repeat consumption, WPC20 samples were significantly higher in intensity for creamy flavour, chalky, drying and mouthcoating; however the addition of CMC and pullulan did not significantly lower any of the six attributes. The differences observed between heated and unheated samples were greater than any differences observed upon the addition of CMC or pullulan, therefore the treatments of these samples by the addition of these polysaccharides are

not sufficient to counteract the development of negative sensory attributes associated with thermal processing.

There were, however, some changes in attributes, which could be further explored to uncover a more successful mitigation strategy. The decrease in chalky upon addition of pullulan to WPC20 demonstrates that mouthfeel can be modified by the addition of a polysaccharide, and that the type of polysaccharide used can elicit different modifications.

A significant increase in particle size was seen upon addition of CMC and pullulan to WPC20, with larger particles observed in WPC20 CMC. While particle size generally correlates positively with grainy or chalky attributes, the characteristics of the particles also influence the sensory perception. Softer particles decrease in grittiness with increasing particle size (Tyle, Kuenn, Geier, & Jarosz, 1990), and increasing viscosity can counteract grittiness (Engelen, Van der Bilt, Schipper, & Bosman, 2005). The difference in chalky perception observed between WPC20 CMC and WPC20 PUL could be due to the difference in particle characteristics; for example, the lack of large aggregates forming, as seen in WPC20 CMC TEM images. While both formed large particles as observed by DLS, TEM showed a difference in the appearance of particle structures, with large bridged structures present in WPC20 CMC. DLS measures the hydrodynamic radius of particles, assuming a spherical shape, and therefore will vary in comparison to the non-spherical aggregates observed using TEM. The hydration properties of the particles will also influence the particle size as observed by DLS, as the structure of water around the hydrated particle will also be measured. Pullulan is extremely water soluble due to a high degree of motional freedom (Okada, Matsukawa, & Watanabe, 2002) and, as such, a large number of water molecules will be bound to polysaccharide particles, giving a larger discrepancy in particle size observed using DLS and TEM. Although high hydration is also observed in CMC (Kumsah, Pass, & Phillips, 1976), the particles observed in TEM correlate more closely to the particle sizes observed by TEM. These larger particles are observed as chalkier than the WPC20 PUL, despite a higher viscosity in WPC20 CMC, this could be due to less hydrated particles, larger aggregates, or different structural properties. An increase in accessible thiol concentration was observed between all WPC00 samples and WPC20; indicating that shielded thiol groups were made more accessible upon denaturation of the protein. A small but significant increase was observed upon addition of pullulan to WPC20, which was not observed in WPC00. An increase in accessible thiol groups should increase mucoadhesive strength (Bernkop-Schnurch, 2005); however the accessibility to thiol by the Ellman's reagent does not necessarily indicate exposure to mucosal tissues. Large pullulan macromolecules could physically shield the thiol groups from forming sulfur bridges with the mucosa, while still allowing the Ellman's reagent to react with the free thiol. This shielding

effect could be the reason that neither a higher drying nor chalky effect was observed with WPC20 PUL.

5.5 Conclusions

Whey beverages often contain thickeners such as polysaccharides to increase stability and modify thickness of the product. This research shows that the type of polysaccharide used can affect both physical and sensory properties, including negative attributes such as chalky, which may contribute to non-compliance in clinical uses of whey protein beverages. Further differences are observed between the two polysaccharides with WPC which has undergone different heat treatments. While this study is limited to two polysaccharides, further research could be carried out on the effect of a variety of gums and polysaccharides as thickeners in WPC, with thermal processing as a consideration.

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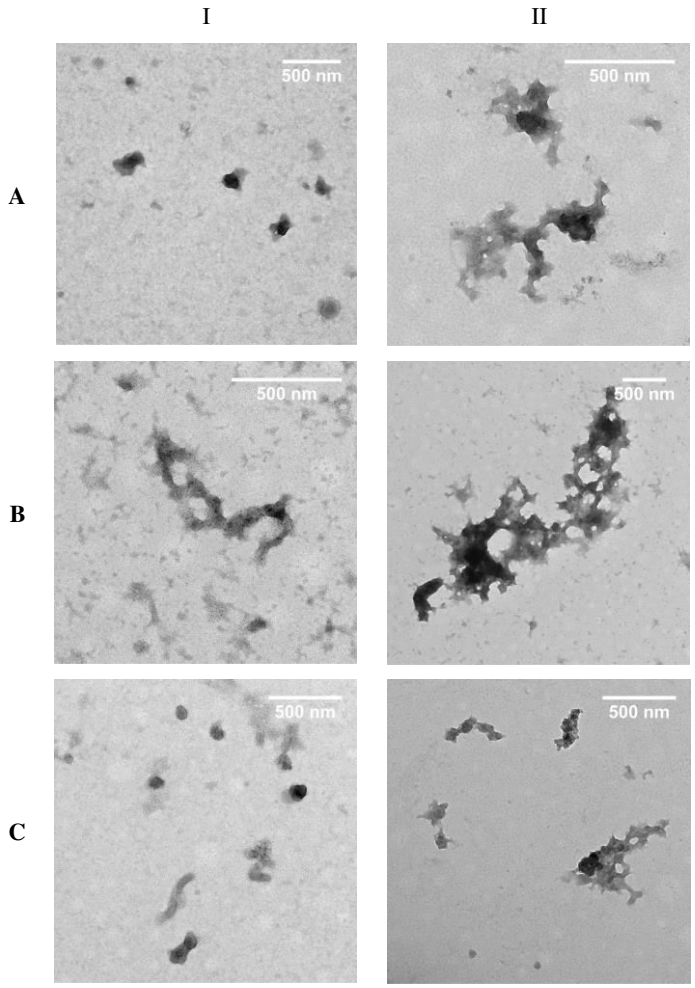
Appendix

Appendix 5.1: QDA attributes and reference descriptions or standard, with results for WPC samples with CMC and Pullulan. Superscript letters in a column indicate significantly different groupings ($p \leq 0.05$).

Attribute	Reference description	WPC00	WPC00 CMC	WPC00 PUL	WPC20	WPC20 CMC	WPC00 PUL
Sour	Citric acid (0.76 g/L)	20.3	19.5	18.5	14.3	17.6	22.6
Bitter	Quinine (0.04 g/L)	8.6	8.3	9.5	6.2	6.3	8.3
Metallic	Iron (II) sulfate (0.0036 g/L)	22.4	18.1	19.0	19.2	15.3	20.1
Salty	Sodium chloride (1.19 g/L)	1.5	1.2	2.4	1.5	2.6	2.2
Sweet	Sucrose (5.76 g/L)	12.6	17.0	17.5	17.3	17.7	18.8
Umami	Monosodium glutamate (0.29 g/L)	4.8	6.9	3.9	2.9	0.8	2.8
Cooked butter flavour	Unsalted butter fully melted	4.4	5.5	4.3	4.7	5.1	7.5
Cooked milk flavour	Semi-skimmed milk heated for 3 min	22.8	23.6	23.5	32.5	34.5	27.3
Powdered milk (wet) flavour	10% skim powdered milk in deionised water	16.9	15.2	19.5	16.0	14.1	19.3
Creamy flavour	Single cream	6.9^{b}	9.3^{b}	12.2^{b}	17.0^{ab}	25.3^{a}	12.4^{b}
Salivating	Degree of salivation	17.6	19.1	17.3	18.6	17.3	16.2
Body	Fullness of sample	28.7^{c}	35.7^{bc}	35.0^{bc}	41.6^{ab}	49.8^{a}	37.7^{bc}
Chalky	Dry fine insoluble powder	8.4^{c}	9.7^{c}	11.0^{c}	39.4^{a}	27.8^{ab}	22.1^{bc}
Drying	The absorbance of moisture from the mouth	28.5^{c}	30.6^{bc}	32.2^{abc}	41.3^{a}	39.9^{ab}	41.1^{a}
Furring	Rough 'furry' texture on tongue and mouth	11.7^{c}	11.1^{c}	14.7^{bc}	27.0^{a}	20.9^{abc}	24.3^{ab}
Mouthcoating	Degree of coating of the mouth	23.5^{b}	23.2^{b}	27.2^{b}	37.8^{a}	40.1^{a}	32.2^{ab}
Mouthcoating aftereffect	Degree of coating of the mouth	14.9^{b}	16.6^{b}	17.1^{b}	28.9^{a}	28.5^{a}	26.0^{a}
Salivating aftereffect	Degree of salivation	19.1	19.2	18.5	18.3	16.5	16.7
Chalky aftereffect	Dry fine insoluble powder	5.3^{d}	7.0^{cd}	8.7^{bcd}	32.1^{a}	21.4^{ab}	18.3^{bc}
Drying aftereffect	The absorbance of moisture from the mouth	30.5^{b}	29.1^{b}	31.0^{b}	45.2^{a}	38.7^{ab}	42.3^{a}
Furring aftereffect	Rough 'furry' texture on tongue and mouth	10.9^{b}	10.8^{b}	12.9^{b}	25.2^{a}	17.0^{ab}	19.5^{ab}
Metallic aftereffect	Iron (II) sulfate (0.0036 g/L)	15.6	14.2	16.0	13.2	11.6	14.0
Sour aftereffect	Citric acid (0.76 g/L)	12.4	10.2	10.2	7.5	9.9	13.3

Appendix 5.2: Original TEM images of WPC samples with CMC and pullulan. I: WPC00, II: WPC20. A: without polysaccharide, B: CMC, C: pullulan. All scale bars are 500 nm, scale varies to show a variety of structure sizes.



Chapter 6 General discussion

Oral nutritional supplements (ONS) containing whey protein are commonly consumed by older adults in hospitals. Withers, Gosney, and Methven (2013) found older adults scored mouthdrying higher than younger adults in milk beverages, and therefore the negative attribute of drying could be of more importance when considering compliance in this population. While ONS contain both whey protein and casein, Withers, Lewis, Gosney, and Methven (2014) observed a higher correlation between whey protein concentration and mouthdrying than for casein. The mouthdrying sensation was found to build up over repeated consumption, which correlated with findings that whey proteins were capable of binding to the oral mucosa (Withers, Cook, Methven, Gosney, & Khutoryanskiy, 2013).

The mechanism of action of mouthdrying in whey protein is an area currently under investigation. The original proposed mechanism of action was linked to the acidity of many whey protein beverages (Lee & Vickers, 2008), with theories linking the low pH to interactions between whey protein and saliva (Kelly et al., 2010; Vardhanabhuti & Foegeding, 2010; Vardhanabhuti, Kelly, Luck, Drake, & Foegeding, 2010; Ye, Streicher, & Singh, 2011). The use of neutral pH whey protein beverages has also been found to elicit a drying sensation (Withers et al., 2014). Recent research has observed the ability of whey protein to adhere to the oral mucosa (Withers, Cook, et al., 2013; Ye, Zheng, Ye, & Singh, 2012), and interact with mucin (Celebioglu, Gudjonsdottir, Chronakis, & Lee, 2016; Celebioglu et al., 2015). A further factor, which may affect the perception of mouthdrying, is the disruption of salivary structure leading to a reduction its lubricating ability (Celebioglu, Kmiecik-Palczewska, Lee, & Chronakis, 2017; Vardhanabhuti, Cox, Norton, & Foegeding, 2011).

This study aimed to investigate the relationship between protein structure and the perception of mouthdrying, specifically relating to thermal denaturation and interactions with the oral mucosa. The hypothesis of this study was that thermal denaturation would increase the perception of drying in whey protein beverages, through increased interactions with the oral mucosa.

Initial sensory experiments investigated the correlation between heating time and sensory attributes. Four samples were assessed: WPC00, WPC05, WPC10 and WPC20 (heated for 0, 5, 10 and 20 min respectively). Quantitative descriptive analysis (QDA) found 15 out of 34 attributes as significantly different between samples. Significant increases in chalky, drying, furring and mouthcoating were seen with increasing heating time. Bitter, sour, metallic, cooked milk flavour, mouthcoating, chalky and drying were selected to be assessed using a sequential profiling method adapted from Methven et al. (2010). Mouthcoating, chalky and drying were selected as important mouthfeel attributes linked to mucoadhesion, with significant increases in

intensity correlating to increasing heating time. Bitter, sour and metallic were not significantly different between samples in QDA and are taste attributes associated with whey protein beverages (Martini & Walsh, 2012; Whetstine, Croissant, & Drake, 2005). Cooked milk flavour was selected as this attribute showed significant differences between samples in the QDA data as both an odour and flavour attribute, and could therefore be used to compare to mouthfeel attributes with repeated consumption. Drying, mouthcoating and chalky were found to significantly increase over repeated consumption, with higher scores observed for samples with higher heating times. Drying was found to have the steepest rate of incline over repeat consumption. Drying, chalky and mouthcoating all elicited scores which remained as high during aftereffect as during consumption, with drying generally increasing in intensity as an aftereffect both at 30 and 60 s. This persistence indicates the physical presence of whey protein in the oral cavity, which builds up over repeated consumption, and remains at least 60 s after consumption. The use of heat processing in the production of products from WPC will increase denaturation and mouthfeel attributes including drying, mouthcoating and chalky. These findings show that the reduction of heating time could reduce the intensity of these mouthfeel attributes in products containing WPC. Further analysis of samples aimed to establish the chemical and physical differences between the samples, which could lead to a mucoadhesive mechanism of mouthdrying.

No differences in ζ-potential were seen between thermally treated samples; therefore charge based mechanisms of interactions between oppositely charged whey protein and salivary protein cannot account for the differences seen between WPC samples in both QDA and sequential profiling methods. Samples were found to have similar viscosities across a range of frequencies. As viscosity can influence mouthfeel attributes (Courregelongue, Schlich, & Noble, 1999; Withers et al., 2014), this removes the viscosity as a factor influencing differences in mouthdrying. Particle size was measured using dynamic light scattering (DLS) and shown to increase with heating time, as would be expected during aggregation of proteins, which has been shown to relate to an increase in drying (Ye et al., 2011). This could also be a factor behind the increase in chalky upon heating, with larger particles building up in the mouth to contribute to a build-up of chalky across repeated consumption. As no significant difference in free ionic calcium concentration was seen between samples, differences in perception of chalky are likely to be due to protein mucoadhesion rather than the presence of calcium.

An *in vivo* retention method was designed to assess the physical presence of whey protein in the mouth after consumption. Following consumption of WPC samples, an initial increase in protein concentration in expectorated saliva was seen, with a peak between 10 and 30 s, before a steady decrease in protein concentration was observed. WPC20 had a significantly higher

protein concentration than WPC00, with a trend of having a higher oral retention up to 60 s. This demonstrates a higher presence of whey protein in the oral cavity with samples with higher heating time, which could explain the high aftereffect scores observed for mouthcoating, chalky and drying in sequential profiling. The increase in mucoadhesive strength observed for increasing thermal treatment has been observed in the utilisation of whey protein as a mucoadhesive coating in drug delivery (Hsein, Garrait, Beyssac, & Hoffart, 2015). As the presence of whey protein from one 5 mL sip was present in the mouth for up to one minute, a build-up could be expected over repeated sips, which would account for the increase observed in mouthcoating, drying and chalky in sequential profiling in Chapter 2.

An increase in accessible thiol concentration was observed with increasing heating time in WPC samples using Ellman's assay. The increase in free thiol concentration is a result of denaturation, as the buried thiol group in β-lactoglobulin (β-LG) is exposed as the α-helix which shields the thiol from solvent is melted (Zeiler & Bolhuis, 2015). Structural changes were observed upon heating the WPC using circular dichroism (CD): in the far-UV, a peak at 208 nm increased with heating time from WPC00 to WPC10, indicating a tendency to develop a random coil conformation over an α-helix (Greenfield, 2006). This supports the finding that the buried thiol group is exposed, as the shielding α-helix is lost. The near-UV aromatic region (260 – 310 nm) showed a decrease in signal strength with heating time, indicating a change in the tertiary structure of the proteins affecting residues in the β-barrel of the protein (Matsuura & Manning, 1994). The structural changes in WPC upon heating may result in stronger mucoadhesive forces, leading to stronger oral retention and negative mouthfeel sensations.

Nuclear magnetic resonance (NMR) was used to compare the structure of the heated WPC samples, finding differences between WPC00 and heated samples in the ^{1}H spectra, and trends across heating time. Differences were found in the aromatic region of the ^{1}H spectra, supporting the CD data that the tertiary structure changes upon heating, affecting aromatic residues. Other differences were observed in H^{β} regions of the spectra, corresponding to side chains. Due to the large amount of overlap in this region it is not possible to determine the residues responsible; however speculations can be made based on the full ^{1}H assignment of β-LG by Uhrinova et al. (1998).

To study the interactions of WPC samples and mucin, bovine submaxillary mucin (BSM) was added to WPC samples at a 1:1 ratio. As a comparison, isolated whey proteins were also mixed with BSM including β-LG, the most abundant whey protein. Celebioglu et al. (2015) studied the interactions between β-LG and BSM at different pH using spectroscopic methods. Evidence of interactions were observed through a change in particle size distribution, and differences in ^{1}H NMR spectra for summed spectra and the spectra of mixtures of β-LG and BSM. CD data

showed little evidence for interaction between β-LG and BSM at any pH. The study in Chapter 4 aimed to investigate the interactions between BSM mixed with WPC and individual proteins from WPC: β-LG, α-lactalbumin (α-LA), bovine serum albumin (BSA), immunoglobulin (IG), lactoferrin (LF) and lactoperoxidase (LP). By comparing the differences in CD spectra for these mixtures, the complex nature of interactions within WPC could be taken into account. The effect of heating of WPC on interactions with BSM was also investigated, by measuring the CD spectra for WPC00, WPC05, WPC10 and WPC20.

Upon mixing WPC and BSM, the aromatic peak in CD significantly reduced, indicating an interaction between BSM and WPC altering the structure of WPC. In contrast, this was not observed in the CD spectra of BSM mixed with individual proteins. The reduced negative peak shifted towards 290 nm upon mixing with BSM, which corresponds to a tryptophan residue, located in the β-barrel of β-LG (Matsuura & Manning, 1994). This change in CD spectra for WPC samples shows that the interactions between WPC and BSM are not dependent on one protein, but are a complex interaction between the mixture of proteins and mucin; and therefore, when studying the effects of whey protein in the oral cavity, using a model of one isolated protein such as β-LG is not sufficient.

The particle size of BSM measured by DLS decreased upon mixing with WPC samples, which would be expected due to the presence of calcium ions in all WPC samples, which has the ability to bind to mucin, leading to a contraction in the mucin particle size (Su et al., 2009). All WPC samples were found to have similar amounts of free ionic calcium present, therefore this effect should be equal on all sample mixtures with BSM. The interaction between β-LG and BSM has been shown to lead to an increase in particle size (Celebioglu et al., 2015; Ye et al., 2011). The particle size distribution increases for all samples upon mixing with BSM, increasing in amount with WPC heating time. This increase in particle size indicates a higher degree of interaction with WPC samples with longer heating times due to factors such as an increase in free thiol concentration.

Differences were also found for β-LG and WPC when mixed with BSM using ^{1}H NMR, in regions corresponding to methyl groups, H^{β}, and H^{α} in α-helices and β-sheets in WPC, and glycosylated side chains of BSM. These results agree with those presented by Celebioglu et al. (2015). This indicates that interactions occur between the hydrophilic side chains of BSM and the non-aromatic moieties of WPC. The lack of differences seen in the ^{1}H spectra around the aromatic region may indicate the changes evidenced by CD in the aromatic region are not caused by the aromatic residues themselves; however interactions causing the secondary structure to change affect the CD spectra in the aromatic region.

The interactions between WPC and BSM seem to be hydrophilic, as suggested by Celebioglu et al. (2015), due to the regions differing between summed and mixture ^{1}H NMR spectra. As the ζ-potential of WPC remains negative, and no significant change is observed upon mixing with BSM, the interactions are unlikely to be driven by electrostatic attractions, as seen for low pH whey protein (Beecher, Drake, Luck, & Foegeding, 2008; Vardhanabhuti & Foegeding, 2010). An increase in thiol concentration with heating time may contribute to the increase in retention of WPC in the oral cavity, by increasing interactions with BSM. Another factor affecting interactions between WPC and BSM is the increasing particle size of WPC with heating time, which may cause more interactions due to increasing electrical double layer stabilisation via aggregation with other particles, and Van der Waals interactions.

The interactions between WPC and BSM indicate only one aspect of mucoadhesion, as mucoadhesive interactions may occur in mucins in free saliva, pellicle bound mucins (Nayak & Carpenter, 2008), or the buccal cells themselves (Ye et al., 2012). It has been suggested that interactions between β-LG and BSM occur through hydrophilic forces in the bulk saliva, with hydrophobic interactions dominating at the air/water interface (Celebioglu et al., 2017).

Sensory analysis was carried out on WPC00 and WPC20 with carboxymethyl cellulose (CMC) (0.25%) and pullulan (1%) added to assess the potential of these to mitigate drying in whey protein beverages. 10 of 24 attributes were found to be significantly different ($p \leq 0.05$) by QDA. WPC00 was lower in intensity for mouthfeel attributes (drying, furring, chalky, body and mouthcoating) than WPC20, consistent with findings in Chapter 2 Part 2. The addition of polysaccharides did not affect the intensity of drying; however, other attributes were affected. WPC20 had significantly higher chalky and mouthcoating intensities than WPC00, whereas WPC20 with pullulan did not. WPC20 with CMC had significantly higher body and creamy flavour intensities than WPC20 with pullulan. This demonstrates that in choosing a thickener to add to a whey protein based beverage, the addition of CMC could be used to increase body and creamy flavour, while mouthcoating and chalky would also be increased; whereas pullulan could be selected to maintain lower chalky and mouthcoating intensities, while decreasing body and creamy flavour.

Sequential profiling found that all WPC20 samples were significantly higher in intensity than WPC00 ($p \leq 0.05$) across creamy flavour, mouthcoating, drying and chalky. Unfortunately no significant differences were observed by sequential profiling upon the addition of CMC or pullulan to either WPC00 or WPC20.

The sequential profiling method developed and validated by Methven et al. (2010) and utilised by Withers et al. (2014) was used here to establish the build-up of attributes over repeated

consumption with controlled time intervals between sips, and measure of the persistence of sensations after consumption. The sequential profiling method is more suitable for these purposes than other temporal designs such as time intensity or temporal dominance of sensations; it allows more than two attributes to be scored, and does not focus on the dominant attribute, which may not be the main contributor to the sensation of drying. Due to the experienced nature of the trained panel, monadic presentation of the different aliquots in order to conceal the sequential nature of the profiling was not possible, as the lack of palate cleansing between aliquots alerted the panelists to the sequential nature, which was familiar to them.

The use of sequential profiling allows the controlled use of time intervals for each aliquot, with timed breaks in between each sip for scoring after effects. While this can be balanced for sample presentation, all of one sample must be consumed in a row, and thus it is biased towards finding differences across repeated consumption. Ideally, each sample would be repeated with scores being recorded after a certain number of sips, which could be balanced across the sample and between samples. This would increase profiling time 10-fold and is not suitable for the number of samples and attributes studied in this research project. This bias may contribute to the lack of differences observed from sequential profiling in Chapter 5 in comparison to the QDA data for the same samples.

DLS showed a significant increase of particle size upon addition of CMC and pullulan to WPC20. DLS uses light scattering to predict particle size distribution based on Brownian motion. The water surrounding the particles will also be measured, giving a hydrodynamic radius of the particle. DLS assumes the particles are spherical, and therefore a wide distribution can be observed when this is not the case. Transmission electron microscopy (TEM) showed smaller particles for WPC00 with and without pullulan, which were more spherical in shape; in comparison to larger irregularly shaped aggregates seen for WPC00 with CMC, and WPC20 with and without polysaccharides. The particle sizes seen for WPC00 and WPC20 are consistent with those measured by DLS, and are slightly smaller, as would be expected without the solvated water layer. Particle bridging can be seen upon addition of CMC, with much larger particles forming for WPC20 CMC (> 1000 nm). These larger particles correspond to the high body and chalky intensities seen for WPC20 CMC, as body relates to the sensation of thickness in the mouth, and chalkiness can be related to graininess, which generally increases with particle size (Tyle, Kuenn, Geier, & Jarosz, 1990). The exception to the increase in graininess with particle size is when the particles are soft, which could be the case for WPC20 with pullulan. Increasing viscosity can also counteract grittiness (Engelen, Van der Bilt, Schipper, & Bosman, 2005); however WPC20 pullulan is less viscous than WPC20 with or without CMC, and so this effect must be outweighed by another factor.

Although the effect of heating had larger effects on the sensory properties of WPC than the addition of polysaccharides, significant differences were observed for some attributes, and therefore the addition of different polysaccharides at different concentrations would be an area to explore in the future.

As the thiol concentration is a significant difference between thermally heated samples, which may contribute to mucoadhesion and drying, the use of thiol blockers would be a potential mitigation strategy. Thiol blockers, such as 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and N-ethylmaleimide (NEM) have been shown to successfully bind to β-LG (Wijayanti, Waanders, Bansal, & Deeth, 2015); however thiol blocking agents were not explored within these studies due to the lack of food grade thiol blocking agents available that would not impart negative sensory attributes themselves, for example sulphurous aroma.

This project aimed to investigate the effects of thermal denaturation on whey protein drying and other mouthfeel attributes, with a focus on the build-up of attributes over repeated consumption. This was to represent the consumption of large volumes (> 200 mL) of whey protein enriched beverages in clinical settings for the treatment and prevention of sarcopenia. The hypothesis was that the denaturation of whey protein would increase the sensation of drying, and that this would be due to an increase in oral mucoadhesion. This hypothesis was found to be correct, with thermally denatured whey protein scoring higher in drying intensity and increasing over repeated consumption and persisting after consumption. Particle size was found to increase with heating time, which was identified as a potential underlying factor contributing to the increased drying. The nature of oral retention was investigated using an *in vivo* retention method, measuring the total concentration of protein in expectorated saliva over time. It was hypothesised that thermally denatured whey protein would increase oral retention, and potential factors were theorised to be: an increase in accessible thiol groups, an increase in free ionic calcium, a change in secondary structure and a change in tertiary structure. The hypothesis that thermally denaturation of whey protein would increase oral retention was proven correct, and an increase in thiol groups and a change in structure were also found to correlate with increased heating times. This indicates that oral retention could be the underlying cause of whey protein derived drying, and that the structure of the protein, including the accessibility of thiol groups, could contribute to the retention, and therefore drying. Free ionic calcium was not found to significantly change upon heating, and therefore was not considered a cause of increased oral retention. The interactions between whey protein and salivary mucin was hypothesised to increase with heating time, and was investigated by studying the change in particle size, the change in secondary and tertiary structure, and change in accessible thiol groups upon mixing. A larger change in particle size distribution was observed for samples with longer heating times,

as hypothesised, and changes in structure were observed using CD and NMR. No change was observed in accessible thiol groups. Based on the evidence that mucoadhesive interactions formed the underlying mechanism of drying, a mitigation strategy was proposed. Two polysaccharides were selected to interrupt the effect of drying: CMC, a mucoadhesive polysaccharide, to competitively interact with the oral mucosa and reduce drying; and pullulan, a non-mucoadhesive polysaccharide. Although neither CMC nor pullulan reduced the intensity of drying, some attributes, including chalky and mouthcoating, were altered by the addition of CMC and pullulan to WPC20. This indicates that the use of polysaccharides to alter mouthfeel in whey protein beverages varies based on the polysaccharide used, and is an area for future investigation. The mixtures of whey protein and polysaccharides were analysed using DLS, with the expectation that particle size would increase with the addition of polysaccharides. Particle size was shown to increase slightly upon the addition of polysaccharides to WPC00, but significantly ($p \leq 0.05$) upon addition to WPC20. TEM was utilised to assess particle shape and size, as it was theorised that the larger particles were not spherical, due to their wide particle distribution. Larger particles were observed using TEM for all WPC20 samples than their WPC00 counterparts, with larger sizes and irregular shapes observed for CMC. Near-spherical shapes were seen for WPC00, and some WPC00 pullulan particles. Accessible thiol concentrations were measured, with the expectation that no changes would occur upon addition of polysaccharides. A small but significant increase in thiol concentration was seen for WPC20 pullulan in comparison to WPC20; however, this could be shielded from the mucosa by the pullulan and, therefore, not interact with the mucosa. The mitigation strategies used in this study were not successful; though they did demonstrate the affect polysaccharides could impart on mouthfeel and indicate an area requiring more research.

The above findings contribute towards a more full understanding of the mechanism of whey protein derived drying, in particular towards the implications of mouthdrying in whey protein beverages. The findings of this research could help inform thermal processing conditions and formulations of whey protein concentrate powder used to create end products, in order to reduce attributes such as drying, chalky and mouthcoating. Wider implications of this research could extend to the pharmaceutical industry, in which whey protein can be used as a mucoadhesive drug carrier, and as such, information on the oral retention of thermally treated whey protein could inform the use of whey protein in oral mucoadhesives.

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