Incorporation of Whey Protein Isolate (WPI) into paneer: Impact on yield, texture and Microstructure

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Dedication

Thank you, God.

My father Pradeep Muraleedharan, my mother Kala Ramakrishnan Nair, and my brother Midhun Krishna for their constant support. I thank all my family and friends for their love and for helping me to become who I am today.

Abstract

Paneer is a traditional Indian dairy product rich in protein, fat, calcium, phosphorous, and vitamins though relatively low in whey protein content due to the conventional heat and acid coagulation processes, highlighting the innovative approach for increasing the nutritional content in paneer. This study investigates the incorporation of Whey Protein Isolate (WPI) into paneer to increase the protein content and nutritional value, while evaluating the effects on yield, texture and microstructure of the paneer. Different formulation methods were tried, including the addition of WPI with and without oil incorporation, also by varying the sequence of mixing with skim milk.

A 3% WPI concentration was selected based on preliminary study, as increase in concentration led to premature gelation of whey protein while heating and due to processing difficulties. The effect of WPI addition on the yield of the paneer and whey protein retention was studied. A significant improvement in yield of paneer was observed for WPI fortified samples with an increase of 12 to 26% compared to the control. A significant increase in weight from 100 grams to 350 grams was also observed in the paneer samples while fortified with WPI. SDS-Page was used to confirm the incorporation of whey protein into the paneer system.

To understand the structure and other functional changes occurred during WPI incorporation, particle size distribution analysis was conducted before and after the heat treatment to the milk. Confocal laser scanning microscopy (CLSM) was employed to understand the protein network and to confirm the incorporation of oil into the system. Texture profile analysis (TPA) helped in further understanding the hardness, cohesiveness, springiness and chewiness of the fortified sample and compare it with the control. TPA was also used for the pressing of the curd into paneer and the rheological behavior during the pressing was studied. Modelling of the TPA data demonstrated that the curd compression profile was primarily influenced by the total curd mass rather than the formulation method of the paneer.

Overall, the study proves that WPI can be successfully incorporated in the paneer to improve its overall protein content and yield with notable effects on its microstructure and texture. The texture of the samples with oil emulsions (SOH-WP, WOH-SP, SWOH-P) showed an increase in hardness compared to the control and SWP showing that the texture of the paneer is highly dependent on method of whey addition. Further studies can be done on improving the texture, sensory properties and formulation of the paneer. The impact of different coagulating agents

and detailed nutritional analysis, like i*n vitro* digestibility studies could be studied to assess the nutritional quality and protein bioavailability.

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Finally, I would like to thank my parents for their constant support and love. More than anything I am grateful to God who is my strength, my guide and blessing this work.

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WPI – Whey Protein Isolate

TPA – Texture Profile Analyzer

TS – Total Solids

WPC – Whey Protein Concentrate

CCP – Colloidal Calcium Phosphate

GDL – Glucono Delta Lactone

CLSM – Confocal Laser Scanning Microscopy

TPA – Texture Profile Analysis

SDS-PAGE – Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

BSA – Bovine Serum Albumin

GMP – Glycomacropeptides

SMP – Skim Milk Powder

SWP – Skim Milk Whey Paneer

WOH-SP – Whey Oil Homogenized – Skim Milk Paneer

SOH-WP – Skim Milk Oil Homogenized – Whey Paneer

SWOH-P – Skim Milk Whey Oil Homogenized – Paneer

1 Chapter 1 Introduction

1.1 Introduction

Paneer is a fresh, unripened cheese which is a mainstay in South Asian dishes and snacks. It is rich in protein, fat, calcium, phosphorous, and vitamins. Due to its high protein content, paneer is a valuable dietary component particularly due to the growing demand for non-meat based protein and for vegetarians and vegan diets(Khan & Pal, 2011; Kumar et al., 2014). The Global paneer market size reached USD 10.8 Billion in 2024 and is estimated to reach USD 18.5 billion by 2033, growing at a rate of 6.2% (IMARC., 2025). People include paneer in their daily diets because it strengthens bones and teeth, increases immunity, controls blood sugar, improves digestion, aids in weight loss, increases metabolism, and lowers the risk of a number of illnesses. Accordingly, the demand for the product has increased due to the growing vegetarian population and health and fitness conscious consumers(Jeske et al., 2018). Additionally, a large number of western fast foods, like pizzas, sandwiches, wraps, and burgers, have adopted the product. In addition, growing consumer preferences for quick-to-prepare foods like paneer as a result of busy lifestyles, work schedules, and dietary changes have fuelled market expansion and improved industry prospects(Boland et al., 2013).

As people become more health-conscious, there is an increasing demand for nutrient-rich foods, with protein being one of the most desired nutrients. In recent years, the focus on protein has increased due to the growing awareness on the role of protein has in our body such as muscle growth, recovery, and overall health and also the increase in popularity of plant-based diet(Carbonaro et al., 2015; Cheng et al., 2019). Foods like paneer, which is high in quality protein, have become popular as a result of this change. Paneer contains casein and a minor amount of whey protein, both which are complete proteins and contains all essential amino acids(Chandan, 2015). As the demand for protein rich food continues to grow, there is an opportunity to further increase the nutrient content of paneer. One potential opportunity, for instance, is incorporating more whey into paneer, which historically has been a byproduct of paneer manufacture. Whey protein is high in branched chain amino acids which if incorporated at higher levels into paneer would improve the protein profile(O'mahony & Fox, 2012).

During paneer production, the whey is separated from the curd, and it is either discarded or is used for animal feeds and beverages(Macwan et al., 2016). However, by incorporating this whey into the paneer, the overall protein content of the final product can be increased. It improves the amino acid balance of the paneer and makes it a complete protein source. Reusing whey in the manufacturing of paneer also addresses a major sustainability concern in the dairy industry. Large volumes of whey are discarded contributing to the waste in dairy industry. By reusing this whey, we are able to decrease this waste and also enhance the protein profile of the product(Pandey et al., 2019). By doing so, it aligns with circular food systems, where waste products are reused to create value, and sustainability food practices that minimize environmental impact(Macwan et al., 2016). Whey also contains a variety of bioactive compounds, such as antioxidants, that offer additional health benefits. These compounds can help support immune function and reduce oxidative stress in the body(Ha & Zemel, 2003; Solak & Akin, 2012).

Whey protein is also rich in amino acids, especially the branched- chain amino acids (BCAAs) like leucine, which play a key role in muscle protein synthesis and recovery (Ha & Zemel, 2003). Whey protein is considered as one of the efficient proteins sources for human nutrition due to its high absorption rate. It allows quick delivery of amino acids to muscle tissues helping in muscle growth and repair. In addition to its advantages for muscle health, whey protein offers a range of physiological benefits attributed to its bioactive peptides, including lactoferrin (Pal & Ellis, 2010). These components contribute to immune system support, inflammation reduction, and improved gut health (Keri Marshall, 2004; Smithers, 2008). Research indicates that whey protein may also benefit cardiovascular health by decreasing blood pressure and lowering the risk of heart disease(Phillips et al., 2009). Furthermore, it enhances insulin sensitivity and increases feelings of fullness, which can be beneficial for weight management and the prevention of diabetes(Veldhorst et al., 2009).

Whey protein, from rennet cheese manufacture, is extensively utilized in the food industry because of its functional characteristics, including gelling, emulsifying, and foaming properties(De Wit, 1998). It serves as a crucial component in sports nutrition, functional foods, and dairy products, contributing to both the protein content and the texture of these items. Recent studies have investigated its application in cheese alternatives and hybrid dairy products, where whey is combined with plant-based proteins to develop innovative, high-protein foods that promote enhanced sustainability(Saubenova et al., 2024).

This study was done by completing these objectives.

1.2 **Objectives**

1) To study the potential of developing paneer cheese with increased whey protein content using Whey protein isolate (WPI).

2) To study the different approaches for adding WPI to the paneer system and study the yield and amount of whey protein attained in the final product.

3) To study the microstructural properties, rheology and texture of the paneer enriched in whey protein.

2 Chapter 2 Literature review

2.1 Functional role of paneer in the dairy sector

2.1.1 Cultural and nutritional relevance of paneer

Paneer is a fresh cheese common in South Asian cuisine. Because of its high protein and nutrient content (including fat, calcium, phosphorus and vitamins), it occupies a special place in vegetarian diets (Satpute & Swami, 2023). Paneer traditionally, was made from buffalo milk which is rich in fat and protein and is known to offer a dense mass of nutrients(Kumar et al., 2014). Since it retains its shape when cooked because of structural stability to heat, it can be used in a fried, grilled or curry-based preparation (Khan & Pal, 2011).

International profile of paneer has grown over the years with many vegetarians and health-conscious people trying to find ways to incorporate the paneer into their diet in more ways than just the typical gravy-based curry such as shahi paneer. Its gentle, subtly sweet flavour and tender yet creamy texture mean that it is suitable for a variety of culinary uses. Paneer is also nutritionally looked upon as highly digestible so as beneficial for all age groups from toddlers to old age and for pregnant women as well (Karadbhajne & Bhoyarkar, 2010). Despite being perishable, with technological development it can be stored for a longer duration by way of freezing and vacuum packing (Agrawal & Sinha, 2018). There are a number of cheese analogs in different regions of the world, such as paneer (India) and Queso Blanco (Latin America) cheeses in terms of principle of manufacture; that have textures and flavours not too dissimilar to cheese (Farkye, 2017).

2.1.2 Composition of Paneer

The composition of paneer is generally reported as 53-55% moisture, 17-18% protein, 23-26% fat, 2-2.5% lactose and around 1.5-2% ash, and depends on milk quality and the process conditions (Chandan, 2007). Product structure mainly consists of caseins that create the coagulated network which traps milk fat globules and other solids (Ahmed & Bajwa, 2019). It is strongly influenced by milk sources and coagulation conditions as well as whey drainage (Kumar et al., 2014). Differences in the composition of raw milk sources typically employed for paneer manufacture are listed in Table 2-1.

Table 2-1 Chemical composition of paneer manufactured using different types of milk

Type of Milk	Constituents (%)					Reference
	Moisture	Fat	Protein	Lactose	Ash	
Buffalo milk (3.5% fat)	56.99	18.10	18.43	-	-	(Chawla et al., 1987)
Buffalo milk (4% fat)	54.05	23.27	16.78	2.69	2.20	(Chawla et al., 1987)
Buffalo milk (5% fat)	56.77	22.30	-	-	-	(Bhattacharya et al., 1971)
Buffalo milk (5% fat)	52.75	25.64	15.62	2.68	2.14	(Sunil Kumar et al., 2008)
Buffalo milk (6% fat)	54.76	25.98	-	-	-	(Bhattacharya et al., 1971)
Buffalo milk (5.8% fat)	50.72	27.13	17.99	2.29	1.87	(Rajorhia et al., 1984)
Buffalo milk (5.5% fat)	55.19	23.80	17.99	-	-	(Chawla et al., 1987)
Buffalo milk (5.8% fat)	54.10	23.50	18.20	2.40	1.80	(Sachdeva & Singh, 1987)
Buffalo milk (6% fat)	50.98	27.97	14.89	2.63	2.08	(Sunil Kumar et al., 2008)
Whole buffalo milk	51.52	27.49	17.48	2.28	2.18	(Dash & Ghatak, 1999)
Cow milk (3.5% fat)	55.97	18.98	20.93	2.01	1.45	(Mistry et al., 1992)
Cow milk (5% fat)	53.90	24.80	17.60	-	-	(Singh & Kanawjia, 1988)
Cow milk (4.5% fat)	55.26	24.15	18.43	-	-	(Syed et al., 1992)
Cow milk (2.65 % fat)	52.68	21.63	-	-	1.23	(Shanaziya et al., 2018)
Cow milk and soya milk (50:50)	64.33	15.42	17.29	-	1.41	(Jadhavar et al., 2009)
Cow Milk	56.9	19.2	18.3	-	1.9	(Khan et al., 2014)

Standardised milk	58.31	20.98	17.39	-	1.30	(Ahmed & Bajwa, 2019)

2.1.3 Manufacture of paneer

Good quality buffalo milk

↓

Filtration

↓

Standardization (Fat: SNF 1:1.65 using buffalo skim milk)

↓

Heating (82^0C, 5 min)

↓

Cooling (70^0C)

↓

Addition of coagulant (1 % Citric acid solution at 70^0C)

↓

Continuous stirring till clear whey separates out

↓

Settling for 10 min

↓

Draining of whey

↓

Pressing the coagulum after filling in muslin cloth lined hoops

↓

Removal of blocks and cutting into desired size

↓

Immersion of *paneer* blocks in chilled water (4^0C)

↓

Draining of water and wiping surface

↓

Packaging and storage at 4^0C

Figure 2-1 Flow diagram for the manufacture of pilot scale paneer

A commercially viable process for manufacture of paneer on a pilot plant was developed by (Bhattacharya et al., 1971). In this process, initially buffalo milk of 6% fat is taken and pasteurized for 5 minutes at 82°C in cheese vat and is then cooled to 70°C, then 1 % citric acid solution is added slowly in the milk with constant stirring for the coagulation of milk. Once the curd is removed from the whey, the mass is cooled to 63°C, at which point the whey is removed from the curd, in this case a muslin cloth, connected to a rectangular frame hoop (35×28×10 cm), which has holes top and bottom. A plank made of wood, with a weight of 45 kg is placed on the curd resulting in a pressure of 4.5kPa. This pressure results in additional whey drainage of the curd. The paneer is cut into pieces and soaked in cold water (4-6°C) for 2-3 hr. The paneer is then spread over a wooden floor in order to drain the excess water out of it and this is further packed and kept at 4 ± 1°C (Bhattacharya et al., 1971).

2.1.4 Protein enriched innovations in paneer manufacturing

Various nutritional intervention strategies have focused on improving the protein content of paneer, particularly for supplementation of dietary protein deficiencies among vegetarians and poor populations. Enrichment with dairy proteins like whey protein concentrate (WPC) or whey solids, has yielded positive results. (Singh et al., 1991) reported that whey solids markedly increased the total solids yield (41.6%) with no negative effects on milk solids recovery. (Salve, 2005) also observed an improvement in protein content when 2% WPC was mixed in low fat paneer made from buffalo milk (4% fat).

In addition to dairy proteins, plant proteins, such as soy protein, have also been studied. Research by (Babje et al., 1992) reported that soymilk replacement up to 20% in buffalo milk was acceptable for paneer, in respect of sensory evaluation as well as structural characteristics. Furthermore, addition of sodium caseinate has been reported to improve the textural properties and the retention of protein in soy-fortified paneer (Jaspreet Kaur et al., 2003; Usha Bajwa et al., 2005). These studies offer very valuable preliminary work for the use of WPI in paneer, clearly showing that protein fortification of the product is possible and, indeed, beneficial in some scenarios with respect to yield, nutritional content—although the formulation and process conditions must be optimized.

2.1.5 Development of reduced fat and functional paneer variants

Increasing awareness about health among consumers is the driving force for low-fat paneer format. Traditional paneer is made from whole buffalo milk which can be high in calories and efforts have been made in creating lower calorie versions using skim milk or partially defatted milk. It has been shown that low-fat paneer prepared from milk with fat content of about 3% could be prepared without seriously compromising the sensory and textural properties (Kanawjia & Khurana, 2006). Furthermore, the low-fat products also have a better rheological property making it more versatile with regard to use in broader application without compromising quality (Kumar et al., 2014).

2.2 Role and Composition of milk in paneer manufacturing

2.2.1 Nutritional importance of milk

Milk is a nutrient-rich secretion, synthesized by female mammals in order to nourish the babies and promote its well-being. It provides an all-inclusive origin of necessary nutrients such as proteins, fats and carbohydrates, vitamins and minerals (Bhat et al., 2016). Over the years, milk consumption has been important in various human diets, drawing from a range of cultural contexts as different as New Zealand, India, the United States, and several European countries (Evershed et al., 2008; Wijesinha-Bettoni & Burlingame, 2013).

Milk, which not only is a stand-alone drink but challenges the raw ingredient product in contributing towards the elaboration of a wide range of dairy products including but not limited to butter, cheese, yoghurt, and paneer (Chandan, 2015). Indeed, dairy contributes around one third of protein intake per day in several developed countries (McSweeney & Fox, 2013). This emphasizes the importance of milk in satisfying human proteins demand, especially in situations of low availability of animal-source proteins.

In such protein enhancement approaches for paneer, the basic nutritional composition and functional characteristics of milk are vital to identify the chances of enrichment via additives like WPI.

2.2.2 Milk constituents and their functional roles in paneer

Milk is a highly complex colloid, consisting mainly of water, fat, protein, lactose and minerals, and also minor constituents, such as enzymes, hormones and immunoglobulins (Boland & Singh, 2019). Water is the principal component of milk, and the medium in which nutrients are dissolved or suspended. As whey is the byproduct of paneer making a large amount of water is expelled. Nevertheless, the remaining moisture is also important in the textural and microbial stability of the product (Chandan, 2007). The composition of milk proteins is shown in Figure 2-2.

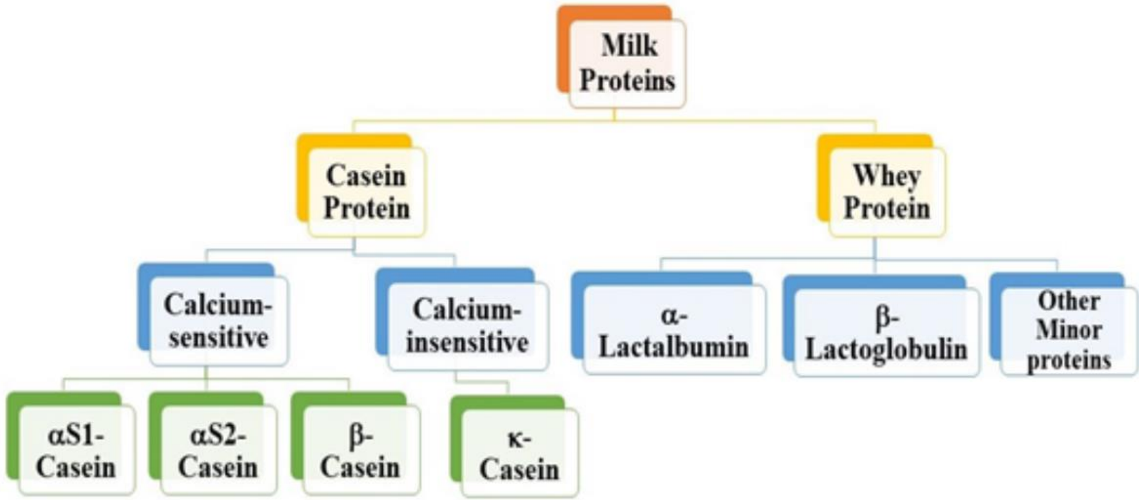


Figure 2-2 Composition of milk proteins(Dhasmana et al., 2022)

Lactose is the predominant carbohydrate in milk, consisting of glucose and galactose linked by β-1,4 glycosidic bonds. It serves as a fuel, and this is particularly vital during early growth stages (Boland & Singh, 2019). Lipids are the most abundant lipid class in milk and consist of approximately 98–99% of milk fat, with the remaining corresponding to other classes such as phospholipids, carotenoids, sterols, and liposoluble vitamins (A, D, E, K). Those fat globules are surrounded by a protein membrane, which is responsible to stabilize dispersion stability and for texture in milk-based food.

While fat levels and moisture in milk impact paneer texture, mouthfeel and appearance, it also impacts how well external proteins such as WPI can be incorporated into the paneer matrix without loss in quality.

2.2.3 Influence of milk type and quality on paneer characteristics

The variety and quality of milk used largely affect the yield and organoleptic appeal of paneer. Buffalo milk is conventionally selected for its higher fat and casein contents, combined with greater size of casein micelles and fat globules than those of cow milk (Bhattacharya et al., 1971). The firm and elastic texture combined with a richer flavour are believed to be due to these properties. Differential solvation activity of buffalo milk casein micelles, improves moisture retention and curd texture (Sindhu, 1996).

Cow milk is abundant and cheaper, but its fat and casein content is lower than goat milk, and this leads to a weaker, less firm interface, which is reflected in the sensory scores of paneers

(Khan & Pal, 2011). Advances in the process modifications and use of functional additives have, however, enabled the production of acceptable paneer from cow milk, thus for example in New Zealand where cow milk is the major source of milk, the making of acceptable cow milk paneer is now a reality.

Studies showed that though fat-enhancing cow-milk supplementation or blending it with buffaloes' milk resulted in better quality paneer. For example, milk mixture containing cow and buffalo milk at a 1:1 ratio with fat content adjusted to 5% may be found optimal for production of desirable textural and flavour attributes of paneer (Kumar et al., 2014; Sachdeva et al., 1985). It has been demonstrated in some studies that the usage of goat milk and sheep milk is also possible for making of paneer but with their strong flavour and unique textural nature has limited their use on large scale (Supekar, 2013).

The comprehensive perspective about the quality of milk is crucial during manipulation of the paneer formulations for protein enrichment and so on. Protein composition and fat composition determine coagulating behaviour, where choosing appropriate type of milk becomes critical in case of incorporation of high-functionality proteins such as WPI into the paneer matrix.

2.3 Protein behavior during coagulation and heat processing

The changes in functional and structural properties of milk protein during heat treatment and acidification are important for curd development in dairy products such as paneer. Caseins and whey proteins exhibit distinct behaviours regarding heat treatments and pH changes, which affect the texture, moisture retention and yield of the end product. In this section, I discuss the separate but related behaviours between casein micelles and whey proteins, and on how these interactions make up the cohesive curd matrix.

2.3.1 Rearrangement and destabilization of casein micelles

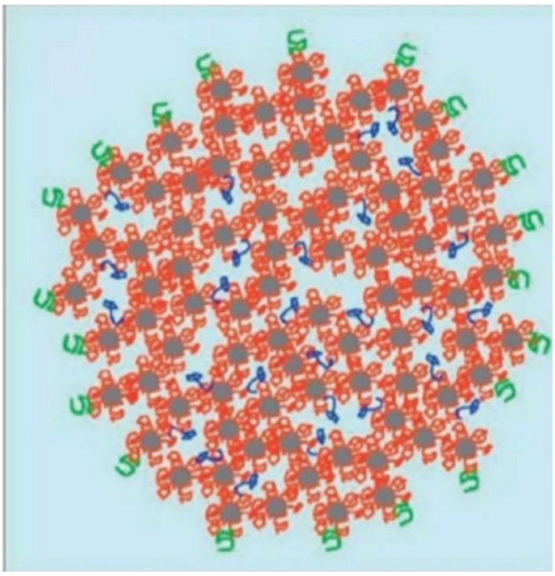


Figure 2-3The chemical structure of casein protein

Casein micelles are highly entangled, complex colloidal structures consisting mostly of αs1-, αs2-, β-, and κ-casein and stabilized by colloidal calcium phosphate (CCP) and steric repulsion from the κ-casein "hairy layer" on their surface (Lucey & Singh, 2003; Lucey & Horne, 2018). At neutral pH of milk (~6.7) the micelle is stable - electrostatic repulsion and hydration force. However, in the course of acidification, they both are reduced.

As pH is dropped from 6.7 to 6.0, the negative surface charge of the micelle decreases, which weakens electrostatic repulsion. At a pH of around 5.0-6.0 colloidal calcium phosphate starts to dissolve and the κ-casein layer collapses resulting in the beginning of micellar disintegration (De Kruif, 1997; Horne, 1998). At pH<5.0 micellar distance becomes closer because of reduced repulsion and the aggregation is mainly governed by van der Waals and hydrophobic factors. These modifications proceed to the close isoelectric point of casein (~4.6), at which micelles consolidate irreversibly into a network (Lucey, 2017). The properties of casein micelles seen in milk is shown in Table 2-2

The acid-induced instability is important in paneer, because it allows for coagulation to be accomplished when milk is acidified by the use of an acidifier such as citric acid, or glucono delta-lactone (GDL). This casein network is the basis of the curd, since it is responsible for its microstructure and the initial gel strength.

Table 2-2 Properties of casein micelles (O'mahony & Fox, 2012)

Characteristic	Value
Diameter	130-160 nm
Surface	8×10^{10} cm2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g/ cm^3
Mass	2.2×10^{-15} g
Water content	63 %
Hydration	3.7 g H_2O/g protein
Voluminosity	4.4 cm^3/g
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5 × 104 Da
Number of peptide chains (MW: 30,000 Da)	10^4
Number of particles per mL milk	10^{14}–10^{16}
Whole surface of particle	5×10^4 cm^2/mL milk
Mean free distance	240 nm

2.3.2 Heat induced denaturation and interactions of whey proteins

In contrast to caseins, whey proteins (β-lactoglobulin and α-lactalbumin) are compact spherical proteins with disulfide bonds (Figure 2-4). They are heat labile and denature at temperatures above 70°C, when heated β-lactoglobulin unfolds and frees reactive thiol groups, which later can form disulfide-linked aggregates (Donato & Guyomarc'h, 2009).

A) **B)**

Figure 2-4 Three-dimensional structure of A) β-lactoglobulin and B) α-lactalbumin(Kilara & Vaghela, 2018)

The denatured whey proteins are capable of interaction with the casein micelles via sulfhydryl-disulfide interchange, as well as hydrophobic interactions. This binding is facilitated when pH is decreased, as 30% of whey protein (WPs) interact with micelles at pH 6.7 and 75% at pH 6.3 (Anema & Li, 2003; Raikos, 2010). At the same time, at higher temperature (>65–70°C), milk experiences mineralisation, where soluble calcium and phosphate returns back to the colloidal phase (Blecker et al., 2012).

These activities affect gelation and water binding. Whey protein aggregates increase proportion of protein in curd matrix and leads to firmer and denser texture. In heat-acid type of coagulated systems like paneer, the effect is even more pronounced as the curd formation involves both the casein as well as whey proteins.

2.3.3 Formation of gels and curd matrix

Paneer is produced by a twofold coagulation, acid and heat, induced denaturation of proteins. The interplay between re-arranged casein micelles and bonded whey proteins creates a composite gel structure conferring a distinctive texture and sensory properties (Lucey, 2020).

Acidification involving organic acids or followed by heat treatment, mainly under the action of GDL, lowers the pH and can lead to the destabilization of casein micelles, whereas whey protein denaturation is promoted by previous heat treatment. These denatured whey proteins act as filler or binder of the casein network, strengthening the gel network and increasing the strength of the curd (Donato & Dalgleish, 2006).

Quick acidification, which occurs in chemical coagulation, forms a gel which is generally more compact and firmer compared to the one formed by microbial acidification (Renan et al., 2008). As the system cools following coagulation, the firmness of the curd continues to grow as the gel swells and further hydrophobic interactions occur (Lucey, 2017). Gel properties are also influenced by ionic components in milk; higher salt contents will decrease surface charge on protein and decrease the strength of inter-particle interactions (Lucey, 2020).

Finally, the paneer matrix is formed by a compromise between heat induced whey protein incorporation and acid induced casein association. This relationship controls not only the texture but also the water binding capacity and the microstructure of the finished product.

2.4 Paneer Whey

2.4.1 Composition and property of paneer whey

When paneer is made, about 80 % of the initial milk mass become acid whey after the curd has been discarded. This whey comprises large quantity of lactose, minerals, water-soluble vitamins and whey proteins that remain soluble under acidic conditions. Only a small fraction of the original whey protein is retained in the acid coagulated paneer. Paneer whey possesses an acid pH (≈5.25) and 6.05–6.23% (w/w) total solids, respectively at 20°C (Hussain et al., 2025). Lactose, normally 4.11–4.40 % (w/w), is the major dissolved solid and residual proteins, such as β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins, lactoferrin, and glycomacropeptides represents 0.65–0.72 % (w/w) (Gawande et al., 2023; Hussain et al., 2025). Fat content is low (< 1% w/w) as most of the fat is bound to the curd. Mineral composition analysis has revealed that, although certain amounts of Ca and P are co-precipitated with casein the amount of Ca and P which remain soluble in whey is appreciable and hence affects the electrolyte composition of whey (Hussain et al., 2025). Slight variations in these values will naturally be dependent on the initial milk composition and exact heating/coagulation regime but the above ranges are typical of paneer whey under industrial or small-scale circumstances. These compositional characteristics are indicative of both the behaviour of acid coagulation and the processing conditions applied (e.g., heating to 75–90 °C prior to acid addition), determining how much whey proteins are co-precipitated with the caseins, or how much of them were retained in the liquid fraction (Gawande et al., 2023; Hussain et al., 2025).

2.4.2 Characterization, losses and utilization potential

This is due to caseins being in their isoelectric point (pH ≈ 4.6–5.3) during acid coagulation and therefore lack of complete incorporation of whey proteins into the curd network. Consequently, 0.65%–0.85% (w/w) high-value whey protein fractions end up in the whey stream(Gawande et al., 2023; Hussain et al., 2025). At the same time, most of the lactose is held in the whey (4.1 % – 4.4 % w/w), because lactose is not precipitated under acidic conditions. The retention of these nutrients in the whey as opposed to the paneer curd is a loss not only for the nutrient versatility offered by whey proteins high in branched chain amino acids (BCAA), immunoglobulins, and bioactive peptides, but also for potential economic value because these components are marketable products themselves(Hussain et al., 2025). Higher coagulation temperatures combined with minimal hold times (e.g., 90 °C heating, 10 min holding, before acid addition,) result in whey protein retention in curd form that is increased relative to lower temperature coagulation (≈ 0.65 % w/w versus ≈ 0.72 % w/w) but even under these optimized conditions, a significant quantity appears in the liquid fraction(Hussain et al., 2025).

Instead of wasting the ~80 % of milk that is left over as whey, its nutrients—0.65 %–0.85 % (w/w) protein and 4.11 %–4.40 % (w/w) lactose—can be recycled for value-added applications. The acid whey concentrates were further processed by ultrafiltration (UF) which denatured the whey proteins (β-lactoglobulin, α-lactalbumin, immunoglobulins, lactoferrin, glycomacropeptides) and results in whey protein concentrates (WPCs) with functional emulsifying and gelation properties. This WPC can be purified further to a whey protein isolate (WPI) which is used in high protein formulations(Hussain et al., 2025).

2.5 Advanced fortification of protein in paneer

2.5.1 Nutritional enhancement in paneer

Given the increasing demand by the consumer for protein-rich diets as well as the requirement for wide availability of vegan sources of high-quality protein, the fortification of dairy products has become increasingly popular. Paneer, a fresh cheese and often consumed item in South Asia, is an excellent base for enhancing its nutritional quality with a neutral taste and adaptable structure. Researchers have investigated several approaches to increase its protein quality by using both animal and plant sources focusing on nutritional imbalances and cost.

Protein enrichment increases the nutritional quality of paneer as well as the functional properties such as moisture retention, yield and firmness. It is well-established that enrichment of milk with protein sources before coagulation may promote curd formation and improve textural characteristics of cheese. (Singh et al., 1991) found that the inclusion of whey solids increased the total solids output by more than 40% a reflection of effective solid recovery and curd volume enhancement. These interventions also add to production efficiency as they help improve the paneer yield at the same level of milk input.

Whey protein isolate (WPI) is a high-quality protein obtained from dairy streams and is especially appropriate for fortifying soft dairy systems, such as paneer on account of its high solubility, gelling, and nutritional properties. Similarly, (Salve, 2005) reported a significant increase in protein content of low fat paneer by fortifying buffalo milk (4% fat) with whey protein concentrate 2%, with no undesirable changes in organoleptic properties. Heat denaturation of whey proteins which occurs during preparation of paneer, promotes their incorporation into the curd matrix leading to better water holding and textural properties, thereby.

2.5.2 Plant based protein fortification

Apart from the dairy proteins, plant-based proteins gained importance as environmentally friendly and low-cost panacea for paneer fortification. Soy milk is high in essential amino acids and is commercially available, and research on its use as a partial milk replacer. (Babje et al., 1992) reported that incorporation of about 20% soy milk into buffalo milk did not affect the sensory and structural quality of the resultant paneer. The paneer produced was found to maintain elasticity, colour and flavour characteristics similar to that of the conventional milk-based paneer. Better texture and stability have also been reported with the inclusion of functional proteins such as sodium caseinate (Jaspreet Kaur et al., 2003; Usha Bajwa et al., 2005).

With the growing trend towards plant-based nutrition, other non-dairy proteins like pea, chickpea, and rice protein are also being investigated for their use in the manufacture of paneer. These technological breakthroughs allow for the expansion of nutritional inclusivity and protein supply in vegetarian diets, in-line with the contemporary trend of innovative functional food development.

2.6 Whey protein

2.6.1 Composition and structural characteristics of whey protein

Whey proteins are about 20 % of the whole proteins comprised in milk and represent a valuable by-product of the cheese industry (Cheison & Kulozik, 2017; O'mahony & Fox, 2012). Whey proteins, also called serum proteins as they are soluble at the natural pH of milk (~4.6), are mostly globular proteins including b-lactoglobulin, α-lactalbumin and a number of minor proteins. These proteins fold in complex 3D structures (Figure 2-4) that maintain noncovalent interactions stabilized by disulfide bonds between cysteines, with amino acid hydrophobic residues kept inside so that the protein does not unfold at native condition (Chandan, 2015).

The major whey proteins vary in concentration and properties: β-lactoglobulin comprises almost 50% of the whey protein (approximately 12% of the total milk protein) and α-lactalbumin comprises approximately 20% of the whey protein (O'mahony & Fox, 2012, 2014). The structure of the β-Lactoglobulin with an extended chain of 162 amino acids is overall more flexible than α-lactalbumin which contains 123 amino acids held together by four disulfide bridges and has a higher resistance (Cheison & Kulozik, 2017). Interestingly, α-lactalbumin also possesses a calcium binding potential that gives it stability; it has the ability to renature when thermally denatured, unlike β-lactoglobulin (O'Mahony & Fox, 2014).

Other bioactive substances found in whey are bovine serum albumin (BSA), immunoglobulins (IgG, IgA, IgM), lactoferrin, and glycomacropeptides (GMP). BSA (5–10% of whey protein) supports the transport of fatty acids, and immunoglobulins (10–15%) are closely related with a defensive function against infections (Madureira et al., 2010; Smithers, 2008). Lactoferrin though present in minor quantity (~2%) has antimicrobial and anti-inflammatory actions. GMP, which originates from κ-casein in the cheese making process, is associated with health of the gut and regulation of the appetite (Madureira et al., 2010).

2.6.2 Functional properties and processing behavior

Whey proteins have a variety of functional properties that are important in food applications. With their high water solubility, they could be used in beverages and nutraceuticals, and as they gel, act as emulsifiers, foaming, and binding agents, could be used to improve texture and stability in yogurts and other desserts, as well as adding firmness when used in baked goods and meat systems (Foegeding et al., 2002). These activities are, however, largely dependent on processing parameters like temperature and pH.

Heat treatment causes the unfolding of whey proteins with the exposure of reactive groups and thus can lead to new type of intermolecular bonding such as disulfide bond formation. This denaturation changes protein solubility and the tendency to aggregation and forms gels or textural characteristics used in different food applications (Havea et al., 2001; Phillips et al., 2009). Enzymatic modifications, such as transglutaminase, can also improve gelation and emulsification through crosslinking of protein molecules, which assists in texture and stability in high protein food systems (Ju & Kilara, 1998).

2.6.3 Nutritional and health benefits

Whey proteins are high in branched-chain amino acids (BCAAs), particularly leucine, which activates muscle protein synthesis and maintains muscle repair and growth (Ha & Zemel, 2003). As such, whey protein is the supplement of choice of athletes and those suffering with muscle wasting-associated conditions (Phillips et al., 2009). Furthermore, eating whey protein may lead to a greater feeling of fullness, helping you eat fewer calories overall.

In addition to the nutritional properties, whey proteins also have bioactive peptides with antioxidant, immuno- modulatory and antimicrobial potentials. Among those, immunoglobulins and lactoferrin are responsible for improving immune, while others play an antioxidant role together providing the health benefits (Ha & Zemel, 2003).

2.7 Key processing variables in paneer

Paneer making, especially enriched paneer with functional ingredients like whey protein isolate (WPI) and oil is extremely susceptible to processing variables. Important variables like coagulation temperature and pH, coagulant type and concentration, and pressing parameters can affect the physicochemical characteristics, texture, microstructure, and quality of the end product. In fortified and modified products, they influence the extent to which additional proteins and lipids are entrapped within the paneer matrix. The order of mixing the milk, WPI, and oil also impacts curd formation and integrity. In this section, these five key variables are further discussed.

2.7.1 Coagulation pH and temperature

Heat processing forms the basis for the manufacture of paneer, and it has microbiological as well as functional objectives. Heating causes denaturation of whey proteins and lowers the solubility of calcium phosphate, that order the co-precipitation of whey proteins with casein through acid coagulation (Jenness et al., 1984). Usually, the milk is heated to 90°C, briefly stored and then cooled back to the coagulation temperature. Table 2-3 shows different types of milk used at different time and temperature before coagulation.

Table 2-3 Types of time-temperature combinations with respect to different milk before coagulation

Type of milk	Temperature-time	References
Standardised buffalo milk (6 % fat)	82 °C/ 5 min	(Sunil Kumar et al., 2008)
Crossbred cow milk	85 °C/ no hold	(Usha Bajwa et al., 2005)
Standardised buffalo milk (6 % fat)	85 °C/ 5 min	(Masud et al., 2007)
Skim milk	80 - 85 °C/ 5 min	(Mendiratta et al., 2007)
Buffalo milk	95 °C/ 10 min	(Chawla et al., 1985)

The coagulation temperature greatly affects the process of curd formation and final texture of paneer. It has been reported that when coagulation temperatures in the range of 60-90 °C are used, strikingly different textural properties are obtained. At more elevated coagulation temperatures (greater than 80°C), paneer is firm and drier because of the increased denaturation of proteins and the formation of firmer curd. On the other hand, lower coagulation temperatures (approximately 60°C) produce moisture surface paneer with soft textural qualities (Dwivedi et al., 2010; Jenness et al., 1984). Thus, it is necessary to optimize the coagulation temperature in cheese making with WPI, as the protein, WPI in particular, is more heat sensitive than casein and could unfold and aggregate easily at higher temperatures (Chandan, 2015).

The coagulation pH value is yet another important factor which affects the yield, moisture and organoleptic attributes of PHE. The paneer produced at pH 5.0 has better organoleptic properties but has poor yield and low recovery of total solids (Vishweshwaraiah & Anantakrishnan, 1985). Textural quality has been traded for a higher moisture content and yield; the latter being maximized at pH 5.1–5.4 of coagulation. Several researchers have advocated that pH of 5.2–5.25 is optimum for cow milk paneer in terms of yield, moisture retention and consumer acceptance (Sachdeva et al., 1991). The pH under which WPI exists when processed is responsible for whether protein can still be soluble or not precipitation, which affects the final curd structure and texture.

2.7.2 Type and strength of coagulant used

Paneer is formed by coagulating milk proteins, casein, by acidifying it to the isoelectric point. The selection and the amount of coagulant are largely responsible for the rate and extent of curd formation, for the protein matrix and hence for the ultimate yield and texture of paneer.

Several acids have been employed for paneer production such as citric, lactic, ascorbic, malic, phosphoric, lemon juice and hydrochloric acid (Khan & Pal, 2011). Of these, citric acid is the most popular because of its availability and effectiveness. Though 1% citric acid is used for this purpose, but some researchers are investigated the concentration variation of citric acid in order to maximize curd quality. The optimum level of 2% citric acid in case of cow milk was determined from the point of view of firmer body and better yield in paneer (Khan et al., 2014; Singh & Kanawjia, 1988; Vishweshwaraiah & Anantakrishnan, 1985). If the acid content is high, then paneer will be firm and compact and if it is low then it will soft and little bit delicate (Khan et al., 2014).

The final texture is also influenced by the buffering capacity of the acid, the protein content of the milk and the temperature at which the coagulation occurs. This becomes ever more important particularly in high protein formulations containing WPI where the excess of acidity may enhance denaturation and aggregation processes, with potential detrimental effects on microstructure and texture stability.

2.7.3 Pressing, pressure and whey drainage conditions

Whey drainage and pressing are required to define the ultimate moisture content, texture, and microstructure of paneer after coagulation and curd formation. The curd, when rested five minutes under zero shear at temperature above 63°C allows the protein network to stabilize, and it initiates the whey separation naturally and also it improves the texture and structure of the final paneer during pressing (Bhattacharya et al., 1971), it is then transferred into a hoop with muslin cloth for whey draining.

Pressing parameters—time, pressure and the pressing mold geometry—with a wide variation is a significant factor affecting the paneer quality. Conventional methods suggest use of a pressure of 40–45 kg for 10–15 min after placing inside a 35×28×10 cm frame which result in retention of paneer with about 56 % moisture (Sachdeva et al., 1991). Newer methods have standardized the input of pressure using mechanical pressing techniques, e.g., Texture Profile Analyses (TPA). (Tellabati et al., 2023) stated that pressing of paneer in TPA was found to be the best at 2.71 kg/cm² for 15 min giving uniform texture and the extent of moisture content.

These parameters are of special significance for the modified formulations. For example, the water-binding properties of WPI could influence syneresis, oil addition likely hinders curd structure development.

2.7.4 Texture of Paneer

Texture is the aggregation and interaction between particles in food matrix structure and is firmly involved in the final sensory description and acceptability of the product. It is very sensitive to multiple processing factors including cooking, freezing, and storage conditions. Texture properties also play a significant role in consumer acceptability and the shelf life of food (Jain & Mhatre, 2009). Generally, sensory attributes such as soft, chewy, course, fragile, mealy, rubbery, hard and pasty are used to describe texture with specific tactile sensations experienced during consumption (Arora & Mital, 1991).

In terms of paneer, texture is heavily influenced by the types of ingredients and their ratio used. It has been reported by a number of researchers that the forming, processing method have an effect on the textural characteristics of paneer (Paril & Gupta, 1986; Sachdeva & Singh, 1987). For example, comparison of paneer from milk of various sources has shown that hardness, gumminess and chewiness vary significantly whereas cohesiveness and springiness are produced consistently (Desai et al., 1991). An increase in water content has also been correlated with a decrease in hardness, suggesting that the water content is directly linked to a soft body.

Milk source also adds to texture differences. Buffalo milk paneer has higher levels of hardness and springiness than cow milk paneer. Nevertheless, cow milk paneer is reported to possess superior cohesiveness, chewiness and gumminess over the buffalo milk paneer (Kumari & Singh, 1992). These differences may be critical in achieving the desired texture with different consumer preferences or product needs.

The category and content of coagulant applied in paneer manufacture also play an important role towards its texture. For instance, the higher concentration (4%) of ascorbic acid led to higher yield, colour and flavour as compared with 2% concentration. However, for firmness and springiness values, the most effective concentration for textural quality were 2% (Ahmed & Bajwa, 2019). Although 1% citric acid is most commonly used in the manufacturing of paneer, some studies have shown that 2% citric acid improves textural properties of the paneer from cow milk (Khan et al., 2014).

Some fruit juices have been used as a coagulant in the preparation of paneer. Though good functional properties were developed in these, the texture of the product did not provide the desired firmness and cohesiveness, hence perhaps limiting its commercial applications (Ahmed & Bajwa, 2019).

The process conditions such as coagulation temperature, time and acid type have equivalent control over rheological and textural attributes of paneer. A number of studies have been conducted on the optimum conditions of those parameters to improve the quality of the final product (Khan et al., 2012). And they have also tried mixing different types of milk to make paneer. In their study, addition of soy milk to cow or buffalo milk has been reported to adversely affect texture properties for instance. But the texture of the resultant paneer was not changed significantly when the proportion of soy milk was restricted to 20% along with buffalo milk (Babje et al., 1992; Jain & Mhatre, 2009; Shere et al., 1991).

Texture profile values of paneer for different investigators are involved in Table 2-4, and the impact of these differences on major characteristics namely hardness, cohesiveness, and springiness are depicted.

Table 2-4 Texture profile of different paneer reported in various studies

Product		Hardness	Cohesiveness	Chewiness	Springiness	Reference
Cow milk (4.5% fat)	**Citric acid**	34.98	0.53	15.76	0.86	(Kumar et al., 2019)
	Malic acid	25.80	0.51	10.97	0.83	(Kumar et al., 2019)
Buffalo milk 3% fat		12.272	0.678	5.525	0.66	(Uprit & Mishra, 2004)
Cow milk 3% fat		16.18	1.067	1.349	0.99	(Jain & Mhatre, 2009)
Cow milk (4% fat)		10.11	1.53	94.69	-	(Kapoor et al., 2021)

2.8 Instrumental analysis techniques

The instrumental methods are necessary in paneer research to measure/quantify its different quality parameters (e.g., colour, texture, microstructure, protein composition, and particle size) objectively. Such approaches offer crucial information for product development, process optimisation, and quality control.

2.8.1 Colour and appearance measurement

For food products, colour is one of the most important properties used to qualitatively assess their goodness, given its strong impact on the perception and purchase of food by consumers(Pathare et al., 2013). The colour of food is commonly associated with its flavour, sensory quality and nutritional value by consumers(Diezhandino et al., 2016). Colour can be done by visual (subjective) or instrumental (objective) evaluation(Pathare et al., 2013).

Figure 2-5 CIELAB colour space(Pathare et al., 2013)

A spectrophotometer or a colorimeter is used for objective colour measurements. The most common system for the purpose of the present description is that referred to as the CIE Lab* system, produced by the Commission Internationale de lEclairage (CIE) in 1976 (Pathare et al., 2013). Colour in such a system is described in three co-ordinates(Granato & Masson, 2010):

L*: Psychometric index of lightness, from black to white.

a*: Red-green coordinate; positive a* values indicate red, negative a* values indicate green.

b*: Yellow-blue coordinate; positive values correspond to yellow, negative values to blue.

The CIELAB model gives a more consistent measurement of colour differences as perceived by humans. It has been extensively used in the literature of dairy products, such as cheese (El-Nimr et al., 2010; Ibáñez et al., 2016; Okpala et al., 2010) and paneer (Chandravanshi et al., 2018; Sharma et al., 2018; Tojan et al., 2024).

2.8.2 Texture profile analysis (TPA)

Texture is a key characteristic of paneer and directly contributes to consumer acceptability and eating experience. Texture as defined by the International Organization for Standardization (ISO, 1992) encompasses all rheological and structural properties of a product that may be perceived through the mechanical, tactile, visual, or sound sensors(Gunasekaran & Ak, 2002).

Texture Profile Analysis (TPA) is a popular technique for texture measurement of food. Hardness, cohesiveness, springiness and chewiness were calculated by doubling the compression on the sample in TPA to mimic the act of chewing(Gunasekaran & Ak, 2002).

Hardness represents the maximum force of the first compression of the firmness of the paneer. It represents the energy required to deform the sample (Jain & Mhatre, 2009).

Cohesion is the internal bonding strength of the sample (ratio of the area under the second compression curve to the area under the first) (Jain & Mhatre, 2009).

Springiness is the capacity of the sample to recover its former shape after compression. This is defined by the amount of height recovered during the second compression as a percentage of the height during the first. Springiness is affected by variables such as protein interaction, heat treatment and protein unfolding (Supekar et al., 2014).

Chewiness is the energy necessary to break down food to swallow. This dimension applies only to solid foods (Jain & Mhatre, 2009).

Some researchers have used TPA in paneer like products. For instance, (Tojan et al., 2024) studied the effect of the incorporation of mung bean protein into paneer cheese while (Uprit & Mishra, 2004) investigated the effect of soy addition.

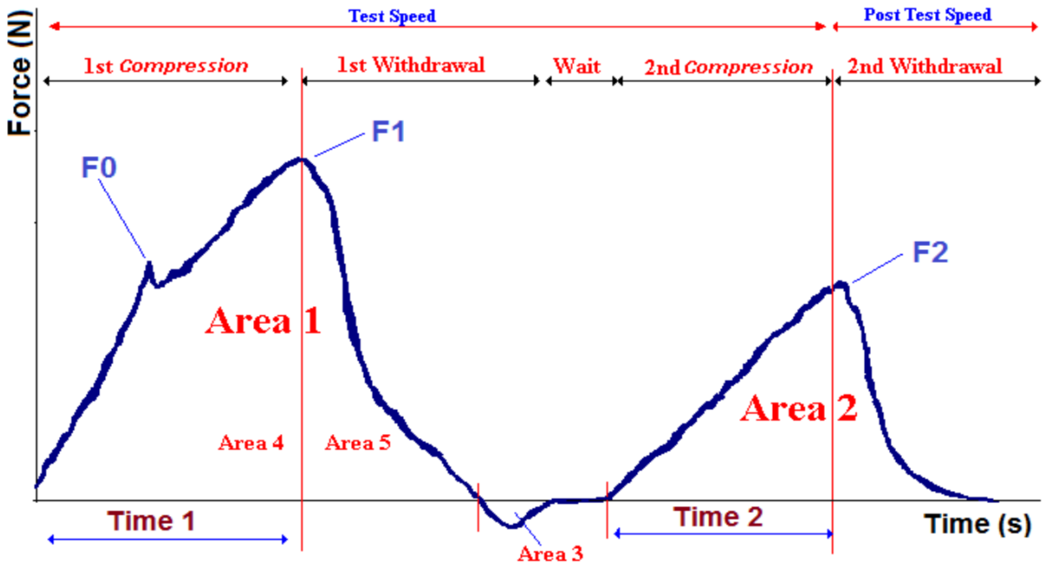


Figure 2-6 Texture profile analysis curve(Guiné et al., 2016)

2.8.3 Confocal Microscopy for Visualizing Microstructure

Confocal Laser Scanning Microscopy (CLSM) appears to be one of the promising techniques for studying the microstructure of dairy products like paneer. In fact, this technique enables three-dimensional, high-resolution images to be formed by the co-registering of two-dimensional (2-D) micrographs, thereby recording structures in depth in a manner which implicate the surface of the sample and are non-destructive of the internal structures (Everett & Auty, 2008; Lopez et al., 2007). A narrow beam, usually derived from an argon laser, is scanned across and along the optical axis of the sample; these thin optical sections image the deep planes at high resolution. This enables the visualization of structures several hundreds of microns deep and their spatial orientation (El-Bakry & Sheehan, 2014).

Fat globules and proteins in the paneer matrix are differentiated by using appropriate staining agents in CLSM. Fluorescein isothiocyanate, rhodamine, and fast green are popular dyes to stain proteins, whereas oil red O and Nile red are established stains for fat (Auty et al., 2001). These stains result in a sharp chemical differentiation of components and the investigator can consider both their isolated radio-autographic distribution and also their interaction within the matrix. The resolution of CLSM (0.2 µm) is better than that of conventional microscopy methods, which leads to a much clearer view of structural features (Everett & Auty, 2008; Ong et al., 2011).

Moreover, CLSM also avoids sample preparation related artefacts which frequently take place in common microscopical imaging, e.g. fat migration or loss of globules during slicing. This makes CLSM especially suitable to high fat food products such as paneer (El-Bakry & Sheehan, 2014; Ong et al., 2011). Another important advantage is the possibility of fluorescently tagging various elements of the specimen, which makes it possible to study the position and interaction of proteins and lipids in the food matrix (Auty et al., 2001).

Other Studies CLSM has also been widely used as an investigative tool to study the dairy product structure such as texture of lactic acid gels and its correlation with sensory texture (Pereira et al., 2003), microstructure of rennet gels (Ong et al., 2010), product melting behaviour of cheese (Auty et al., 1999), permeability of rennet casein gels (Mellema et al., 2000), location of probiotic bacteria or exopolysaccharides (Auty et al., 2001; Hassan et al., 2002). In addition, CLSM has been employed for multimodal fluorescent labelling for combination imaging of proteins, lipids, and whey proteins in complex dairy systems (Herbert et al., 1999). While informative, the approach is costly and requires specialized instrumentation and maintenance, perhaps restricting its use in routine laboratory testing (El-Bakry & Sheehan, 2014).

2.8.4 SDS-PAGE

The analysis of the protein composition of protein-based dairy products such as paneer is often performed with the help of Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). It is especially suitable for the separation and weight determination of polypeptides. Deciding between an array of PAGE techniques, SDS-PAGE is most popular due to its simplicity, accessibility and reproducibility(Chandel & Pahadiya, 2005; Nur Azira et al., 2012).

By SDS-PAGE, proteins are denatured with the aid of SDS, which predominantly binds to proteins through non-covalent hydrophobic interactions. This process maintains an even charge-to-mass ratio so that resolution of proteins by size alone is possible. The electrophoresis is performed with a discontinuous gel system composed of a stacking gel and a resolving gel. The proteins are focused as a distinct band in the stacking gel before entering the resolving gel, which separates the proteins by molecular weight. The smaller the proteins in size the further they go into the gel, the larger they are the slower they travel (Chandel & Pahadiya, 2005).

The extent of protein separation in SDS-PAGE is affected by the pH and composition of the electrophoresis buffer in addition to the acrylamide concentration of the gels. Each protein binds SDS at a theoretical ratio of 1.4 g of SDS to 1 g of protein, resulting in predictable migration distances (Dupont & Tomé, 2020). Protein quantification by SDS-PAGE can be reported with more variability then on techniques such as urea-PAGE, because of band shape and resolution differences. Nevertheless, this constraint can be overcome with densitometry software that increases the accuracy of band protein analysis (Chandel & Pahadiya, 2005; Nur Azira et al., 2012).

SDS-PAGE is not simply used to predict molecular weights but also to provide indication of the relative amounts of proteins present, detect hydrolysates and impurities and to check the effects of processing on protein composition. Being able to gain qualitative and semi-quantitative information is valuable in paneer research concerning protein profiling and quality control as well (Dupont & Tomé, 2020).

2.8.5 Particle size analysis

Particle size is an important factor in influencing the textural characteristics and keeping quality of paneer. The extent of dispersion of casein micelles and fat globules contribute to rheological and sensory traits, including smoothness, hardness, chewiness, and consumer preference. Finer particles will give a smoother, more cohesive texture whereas larger particles will yield graininess(Smoczyński, 2020).

The stability of emulsions in milk drink depends on the particle size. The larger fat globules tend to coagulate and separate, which is detrimental to keeping quality. Homogenizing down to a smaller particle size increases the stability of the emulsion and enables a more uniform product. Finer microparticles also improve the enzymatic degradation and nutrient uptake, something very important in the case of the functional food industries (Smoczyński, 2020). Similarly, viscosity and flow behaviour are also dependent on particle size (Zebeli et al., 2012).

The Mastersizer 2000 and 3000 are popular laser diffraction instruments used in wet and dry particle size analysis. These offer the advantages of rapid, repeatable and reliable measurements. The Mastersizer 2000 is suitable for different types of dairy matrices such as milk solids, fat droplets and casein aggregates with size range 0.02 µm-2000 µm. Helps in the development of processing steps such as coagulation, homogenization, drying (Malvern, 2016; Smoczyński, 2020).

3 Chapter 3 Materials and Methods

3.1 Introduction

The main objective of this study was to increase protein concentration across paneer using whey protein isolate (WPI). The preliminary step was to study the different approaches for adding WPI to the paneer system and study the yield and amount of whey protein attained in the final product.

The effect on paneer by adding WPI to the system on the microstructure, colour, rheology and texture was studied. The pressing of the paneer from the milk was done by texture profile analyzer.

3.2 Materials

Skim milk powder (SMP) was sourced from NZMP and stored under refrigerated conditions to maintain its quality. The contents of the skim milk powder are described as per the packaging table label given in Table 3-1. The water used in the experiment is RO water and Milli-Q water. The oil used for the experiment is premium clarified butter ghee from Eclipse Dairy. The samples were homogenized twice using an M-110P Microfluidiser (Microfluidics, USA) at a pressure of 300 bar. The citric acid (LAbServ) used was procured from Thermo fisher Scientific Ltd. The pressing equipment for the paneer used was a Texture Profile Analyzer. The curd was pressed using a Texture Profile Analyzer (TPA) from TA.XT. Plus Texture Analyser, UK. Whey protein isolate (WPI) 895 was obtained from NZMP, New Zealand. The Mastersizer used here is a Mastersizer 2000 from Malvern, UK. Rheometer used here is a controlled stress rheometer (Anton paar, MCR 302, Germany). All the chemicals and reagents used for the study were of analytical grade.

Table 3-1 Composition of the skim milk powder from cow procured from NZMP

Composition	Percentage composition (%)
Lactose	54.5
Protein	32.9
Minerals	7.9
Moisture	3.8
Fat	0.9

3.3 Methods

3.3.1 Preliminary study

The first phase of the study involves the making of the paneer using different homogenized solutions involving oil, whey protein, skim milk protein and water. This mixture was made up to 1000 grams. WPI was selected considering its availability and that whey proteins are currently lost from paneer during the pressing stage. Milk fat was used as the source of oil here. Oil is used to maintain the fat: protein ratio of 2:1 in the paneer. Different concentrations of WPI to SMP milk were looked to find the potential ratio to make the paneer. WPI was incorporated into skim milk at varying concentrations (1%, 2%, 3%, 4%, 5%, 6%) and the resulting denaturation and gelation of WPI while heating was observed. The optimal concentration of WPI to be added to skim milk was selected for further experiments.

3.3.2 Preparation of different types of paneer

Skim milk powder 100g (SMP) was made into milk with an appropriate ratio of 1:10 with RO water 900g and were made up to a total of 1000 grams. This solution was stored overnight for hydration. The 1000ml solution was heated up to 82°C to 90°C with constant stirring and was held there for 5 minutes. Then the solution was cooled to 70°C. Once the solution was cooled 2% citric acid was added to the solution until the pH was within 5.2-5.25 under constant slow stirring with temperature not going below 60°C. When the pH range was achieved, the solution was allowed to settle for 10 minutes and was cooled to 31°C. The texture pressure analyzer (TPA) was used to press the curdled solution and separate the whey and paneer cheese. The manufacture of the different paneers is shown in Figure 3-1.

Similarly different paneer samples were made with different ratio of mixture (Table 3-2).

Sample 2 (SWP) is WPI along with SMP (100g) is made up 1000g using RO water.

Sample 3 (WOH-SP) is WPI (30g) made into solution is completely emulsified with oil. Once emulsified, SMP (100g) solution is added making to 1000g

Sample 4 (SOH-WP) is SMP milk completely emulsified with oil. Once emulsified, WPI (30g) powder made to solution is added and then is made up to 1000g.

Sample 5 (SWOH-P) is SMP milk along with WPI solution completely emulsified with oil making up to 1000g.

All the five samples were duplicated, and studies were conducted on all the samples.

Table 3-2 Summary of different type of samples prepared.

Type of Sample	Content (1000g)
Sample 1 (Control)	SMP (100g) + Water (900g)
Sample 2 (SWP)	WPI (30g) + SMP (100g) + Water (870g)
Sample 3 (WHO-SP)	WPI (30g) + Oil (24g) + SMP (100g) + Water (846g)
Sample 4 (SOH-WP)	SMP (100g) + Oil (24g) + WPI (30g) + Water (846g)
Sample 5 (SWOH-P)	SMP (100g) + Oil (24g) + WPI (30g) + Water (846g)

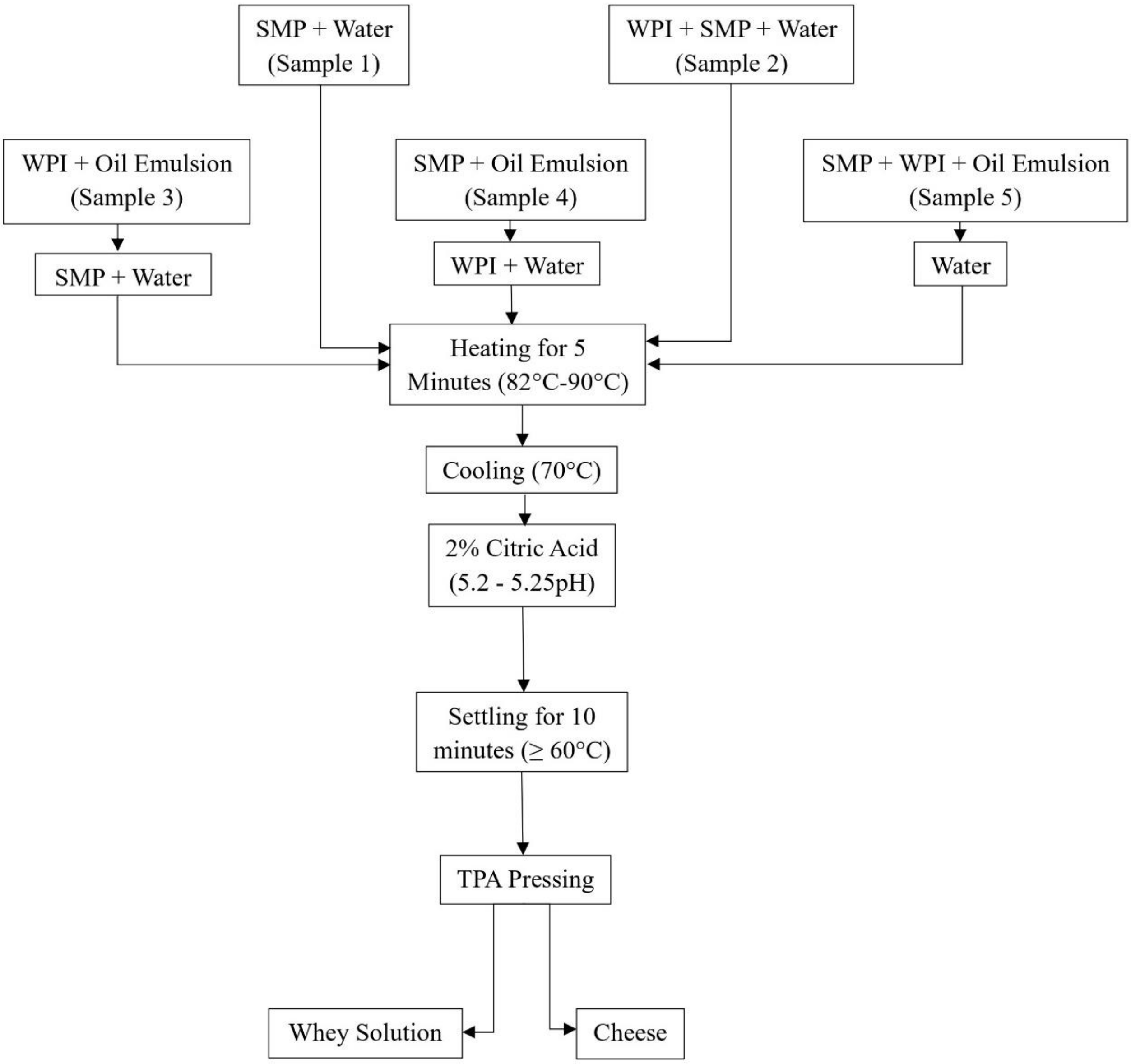


Figure 3-1 Flow chart describing the manufacture of different paneer samples.

3.3.3 Composition analysis

Hot air oven method was used to analyse the moisture content of the paneer samples. A known amount of sample (w1) was heated to 105°C and maintained for 24 hours. The final weight of the sample was recorded after the cooling in a desiccator for 2 hours (w2). The formula used for calculation of moisture content is shown in equation (1).

$$Moisture\ content\ (\%) = \frac{(w1 - w2) \times 100}{w1} \tag{1}$$

A sample from the whey and the paneer, acquired after the pressing done by the TPA were taken for the protein and fat content analysis. The samples were given to nutrition lab to analyse and determine the content of protein and fat. The crude protein check was done using Dumas method (AOAC 968.06). The conversion factor for nitrogen used for paneer was 6.38 and whey solution was 6.41 and the fat content analysis for paneer and whey was done using Mojonnier method.

3.3.4 Colour analysis

Minolta Chroma meter CR 400 was used to analyse the colour profile of the paneer. The L*(Light range between 0 and 100), a*(Positive a* value means redness and negative a* means greenness) and b* (Positive b* value means yellowness and negative b* value means blueness) parameters were determined using CIELAB colour space. The equipment was calibrated using a standard white tile (Y=86.6, X=0.3162, Y=0.3232). The average value obtained from the duplicated samples were taken (Six measurements per each sample).

3.3.5 Particle size distribution

The particle size distribution of the samples before and after heat treatment were measured by laser light scattering, using a Mastersizer 2000 (Malvern, UK). The refractive index used for the control was 1.52, for SWP was 1.45 And for SWOH-P, SOH-WP and WOH-SP were 1.37 The absorption coefficient was 0.001 For all the samples. All the experiments on the samples were performed under room temperature. 50–150 μL of the samples before heat treatment and 20–40 μL after the heat treatment were introduced into the measurement cell of the system containing 100 mL of water for it to reach 10% obscuration (Optimal condition for the measurement). All the measurements were done in triplicates. All standard parameters were calculated by the software.

3.3.6 Rheology

A controlled stress rheometer (Anton paar, MCR 302, Germany) was used to measure viscosity measurement on the paneer samples. The rheology of the milk after the addition of whey and oil when heated was measured and studied. The rheometer was used to simulate the paneer making process and to understand difference in viscosity of the milk when preparing the paneer. Five different milk samples were analysed to study the viscosity using a rheometer attached with a C25 cup and bob. The water jacket was bought to the desired holding temperature of 5˚C. 20 ml of sample was taken and slowly poured into the cup and after a holding period of 5 minutes, the bob was slowly immersed into the sample. A thin layer of mineral oil was added to the top of the sample to prevent evaporation. The sheer rate was set at 100^{-s} and held constant throughout the experiment. The temperature is set to increase from 5˚C to 85˚C at 5˚C/min and kept constant for 5 mins. It is then cooled to 70˚C at 1˚C/min. The apparent viscosity was measured and recorded. The graph obtained was interpreted to understand the behaviour of different milk samples.

3.3.7 Confocal microscopy

The microstructure of the five paneer samples was examined using Confocal laser scanning microscopy (CLSM) (Leica SP5 DM6000B scanning confocal microscope). Thin slices of paneer obtained by cutting from the middle portion of the fresh paneer. These slices were then put on a glass slide and stained using a fluorescent dye (Nile red) for identifying fat phase and fast green for staining the protein phase. A cover slip was kept on top of cheese slide for 30 min to make sure enough time is given for the dye to be diffused through the sample. Any excess dyes were carefully wiped of using kimwipes (KIMTECH). The stained sample was then observed under the light microscope under different oil immersion objective lens and emission filters was set to 488 nm for Nile red and 633 nm for fast green. The Zeiss Zen 3.11 lite software was used to analyse the micrographs obtained from the CLSM.

3.3.8 Texture profile analysis (TPA)

Texture profile analyser (TA.XT. Plus Texture Analyser, UK) was used for determining the texture profile analysis of all the paneer sample. A double compression test was used to perform the experiment which generated a force-force time graph, which was used to calculate the hardness, springiness, cohesiveness and chewiness of the samples. The samples were cut into cubes of 10x10x10 mm^3. The sample was compressed to 70% of its original length using a cylindrical probe were the pre-test speed, test speed and post-test speed of the probe was set to

0.5 mm/s, 1 mm/s and 5 mm/s respectively. The data reported is an average of 5 measurements for each type of paneer.

Hardness (n) = Maximum force of the first compression

Cohesiveness = Area under 1st compression (Area 2) / Area under 2nd compression (Area 1)

Springiness = Length 1 / Length 2

Chewiness (N) = Hardness x Cohesiveness X Springiness

Gumminess (N) = Hardness x Cohesiveness

3.3.9 SDS-PAGE

Reduced-Tricine-SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to understand the breakdown of proteins and to identify the variety of proteins present in the paneer sample.

Triscine SDS page was done using 16.5 Criterion^{TM} Tris-tricine gel (Bio-Rad Laboratories Pty Ltd., Auckland, New Zealand) to evaluate the protein profiles.

The paneer obtained from TPA pressing is freeze dried into powder format, this powder was used by diluting it in a specific ratio 100mg:1ml using Milli-Q water. The Milli-Q water was added to the prepared sample to achieve 100µL solution with the desired concentration.

The 100mL running buffer is made mixing 10x buffer and 900mL Milli-Q water. The sample was mixed with sample buffer without β-mercaptoethanol. And another sample was mixed with 100µL sample buffer with 2% β-mercaptoethanol. The sample was then heated for 10 minutes up to 60°C in the incubator. 100mL running buffer is mixed with 900mL Millipore water. The sample was loaded to a 20 µL well with a molecular weight ladder of 10µL along with 10 µL of protein standard (Precision Plus Protein TM All Blue Prestained Protein Standard) as reference onto the gel. The gel was then run for 125 V for 2 hrs using 1×Tricine running buffer (Bio-Rad Laboratories Pty. Ltd, New Zealand) in Criterion cell (Bio-Rad Laboratories Pty. Ltd, New Zealand). Gel was then run with RO water. The gel is then stained with using Bio-safe^{TM} Coomassie blue stain (Bio-Rad Laboratories Pty. Ltd, New Zealand) for at least 2 hours to overnight. The gel is then rinsed again and incubated in RO water until the bands are clear. The gels were then stored in Milli-Q water at 4˚C.

The quantification of protein bands is done by gel scanning densitometer (Molecular Imager Gel Doc XR, Bio-Rad Laboratories Pty Ltd.)

3.3.10 Statistical analysis

Microsoft Excel (Microsoft Corporation, U.S.A.) was used for calculating mean ± SD (standard deviation) of all the obtained values. Originpro 9.1 was used to graphically represent the results. The means were compared to determine the significant difference between the paneer samples by one-way analysis of variance (ANOVA) at $P < 0.05$ significant level.

4 Chapter 4 Result and Discussion

The analysis and results obtained for paneer samples using different methods are discussed in this chapter. Different research articles about paneer and cheese are used to compare the results obtained in this study.

4.1 Initial standardization of WPI addition

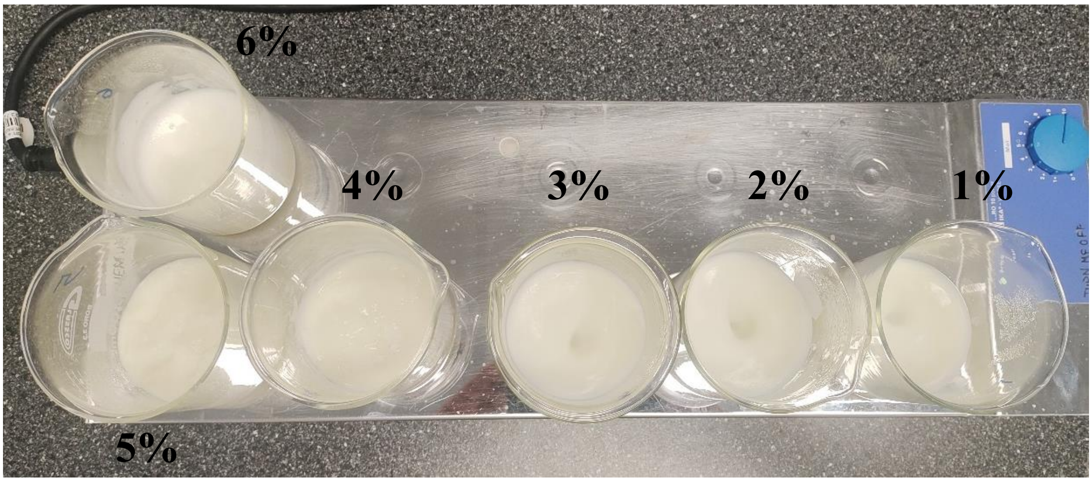


Figure 4-1 Skim milk samples fortified with different concentrations (1 - 6%) of the WPI prior to acidification

For the preliminary study, different concentrations (1 – 6%) of whey protein isolate (WPI) were evaluated to determine the optimal level of WPI to be added for the fortification of the paneer. The objective was to incorporate the maximum amount of WPI into the milk without compromising the processability and the quality of the final product.

For concentrations above 3%, It was observed that the milk began to form thick curd like gel while heating (80 ˚C), even before the acidification (Figure 4-1). This could possibly cause processing challenges while manufacturing the paneer. Such as the viscosity of the solution may hinder the even distribution of the acid and could interfere with proper coagulation and cause incomplete and uneven curd formation during acidification.

The addition of WPI at a 3% concentration into milk resulted in a thick but flowable solution which was suitable for acid- induced coagulation with the highest concentration of WPI. The 3% WPI milk did not form a gel-like structure while heating and was easier to handle during the manufacturing process of the paneer samples. Based on these observations during the initial study, 3% of WPI was selected for the further studies due to it maintaining the highest concentration without gel thickening.

4.2 Composition of paneer

Table 4-1 gives the moisture, MNFS, protein, and fat, weight of the paneer, and the yield of protein in the paneer composition of five cheese formulations/processes manufactured: Control, SWP, SOH-WP, WOH-SP, SWOH-P. The total moisture content of the paneers remained relatively comparable with the control sample (69.53%) ranging 67 – 71% except SWP (74.83%) but there is meaningful difference in the Moisture Non-Fat Substance (MNFS). The MNFS of the control was 69.83%, whereas the rest of the samples with WPI fortified exhibited higher MNFS values: 74.92% in SWP, 71.08% in SOH-WP, 72.66% in WOH-SP, and the highest at 75.78% in SWOH-P. The increased water-binding capacity of the non-fat solid in the paneer matrix is seen from this data. From the increase in MNFS, it can be seen that the WPI incorporation, combined with oil through homogenization can increase the water retention capacity and it may be likely due to the enhanced hydrophilicity and gel-forming properties of whey proteins. The whey proteins have the ability to form interconnected matrix with casein during heat and acid coagulation, which may explain the increased moisture retention that is observed here.

Furthermore, the control sample shows a protein content of 23.02% and a protein yield of 71.73% and an average paneer weight of 101.67g. Even with the addition of the WPI into the paneer, there is a decrease in the protein % observed, which ranged from 16.75% to 19.17% where the lowest is seen in the one where skim milk, whey and oil were homogenized together (SWOH-P). However, this reduction in percentage is counterbalanced by a significant increase in paneer weight, reaching up to 348.1 g in the SWOH-P sample. This suggests that while the relative protein concentration in paneer (on a mass of protein per cheese mass basis) decreased due to increased moisture and fat incorporation, the absolute amount of protein retained in the paneer relative to the amount of protein in the starting milk (i.e. protein yield) increased across all WPI-fortified samples. The fat free paneer sample with only WPI addition (SWP) showed a yield of 83.05%, while fat containing samples involving various homogenization methods, such as SOH-WP, WOH-SP, and SWOH-P, displayed progressively higher yields, with the SWOH-P treatment reaching the maximum at 97.13%. The paneer produced from emulsions that included whey (WOH-SP, and SWOH-P) were higher than when the whey protein was added after the emulsion was already formed (SOH-WP)

These findings suggest that homogenization, particularly when combining milk, whey, and oil, enhances the integration of whey proteins into the paneer matrix, thereby improving protein retention during coagulation. This implies that the WPI was successfully incorporated into the matrix, even if diluted by higher moisture or fat.

Table 4-1 Composition of different paneer samples (mean ± standard deviation)

	Moisture %	***MNFS***	***Protein %***	***Fat %***	***Weight of paneer (g)***	***Protein yield %***
Control	69.53 ± 0.60	69.83 ± 0.67	23.02 ± 1.96	0.42 ± 0.09	101.67 ± 9.67	71.73 ± 12.84
SWP	74.83 ± 3.75	74.92 ± 3.76	19.17 ± 1.54	0.11 ± 0.01	261 ± 18.5	83.05 ± 0.79
SOH-WP	66.87 ± 1.12	71.08 ± 0.1	17.72 ± 1.93	5.91 ± 1.43	288.35 ± 0.35	85.29 ± 9.19
WOH-SP	69.80 ± 1.50	72.66 ± 2.78	17.42 ± 0.71	3.87 ± 1.61	307.5 ± 21.5	89.17 ± 2.61
SWOH-P	71.11 ± 0.61	75.78 ± 0.22	16.75 ± 1.87	6.16 ± 0.53	348.1 ± 5.7	97.13 ± 9.30

The protein and fat of the whey solution, ex-pressing of the curd, was also measured to study the protein retention of the paneer samples. The protein % and fat % of the whey is shown in Table 4-2, in which the control whey exhibits a protein content of 0.54 %. Among the homogenized samples SOH-WP shows a slight reduction in protein content (0.48 %), this suggests more efficient incorporation of the whey protein into the paneer matrix of SOH-WP (17.72 %), which is higher than other homogenized paneer samples. The whey content in SWOH-P (0.80%) is higher than the control which indicates that the whey protein was not fully incorporated in the paneer sample. The highest protein content whey solution, for the samples fortified with WPI, is from the non-fat SWP sample (0.86%), which indicates that the inclusion of fat in a formulation is more likely to increase protein retention in the curd.

Table 4-2 The protein and fat composition in paneer whey of different samples (mean ± standard deviation).

	Protein %	***Fat %***	***Total whey volume (g)***
Control	0.54 ± 0.10	0.07 ± 0.03	837.40 ± 21.5
SWP	0.86 ± 0.05	0.04 ± 0.01	674.45 ± 22.95
SOH-WP	0.48 ± 0.09	0.06 ± 0.005	628.05 ± 11.15
WOH-SP	0.57 ±0.14	0.25 ± 0.21	624.6 ± 12.5

SWOH-P	0.80 ± 0.21	0.07 ± 0.01	614.10 ± 19.8

4.3 Colour analysis

The colour of a food is the basic parameter to assess the quality of food by a customer (Ramos et al., 2013). The colour profile of paneer samples according to CIELAB colour space is shown in Table 4-3 The control shows the lowest L* value of 81.09. It indicates that the control has relatively darker appearance than all the WPI-fortified samples. All the WPI-fortified samples have higher L* values, with highest observed in SWOH-P (91.50) followed by SWP (90.37) and SOH_WP (89.41). Similar results were obtained by (Kumar et al., 2011) using soy protein isolate as a fat replacer in low-fat paneer. The increase in colour can be due to increase in its fat content (Kumar et al., 2011) and WPI addition, as L* value is directly proportional to fat content because of the scattering of light by fat molecules. An exception to this however is observed in the SWP cheese which has a significantly higher L* value than the control despite having the same fat content. In this case it is likely that the higher L* value is due to the formation of whey casein complexes that are in the same size range as fat globules. There is evidence for this in the particle size data (refer section 4.8) where the SWP sample has its largest peak, post heating, in the range of 10 to 100 µm similar to the fat containing milks. In contrast the largest peak for the control is in the range of 100 to 1000 µm. Also, the homogenisation of the cheese milk could have contributed to a smoother microstructure and enhanced light scattering due to more uniform distribution of fat and protein.

Table 4-3 The colour analysis of different samples (mean ± standard deviation).

Paneer sample	***L****	***a****	***b****
Control	81.09 ± 0.62^{a}	-6.22 ± 0.11^{a}	16.49 ± 0.46^{a}
SWP	90.37 ± 0.93^{b}	-5.56 ± 0.42^{a}	24.95 ± 0.41^{a}
SOH-WP	89.41 ± 0.34^{b}	-4.47 ± 0.13^{a}	19.39 ± 0.44^{a}
WOH-SP	88.02 ± 0.67^{c}	-4.56 ± 0.24^{b}	20.61 ± 0.46^{a}
SWOH-P	91.5 ± 0.58^{b}	-4.62 ± 0.21^{c}	20.31 ± 0.66^{b}

Means (±SD) with different superscript letters among the paneer samples are significantly different ($p < 0.05$).

The a* values, which indicate the red-green axis, were negative for all samples, reflecting a shift toward greenness (Sant'Ana et al., 2013). The control sample exhibited the most negative a* value (-6.22), while the a* values of the fortified samples were less negative, particularly the samples containing oil (SOH-WP (-4.47), *SWOH-P (*-4.62) and WOH-SP (-4.56). The whey fortified sample with no added fat had an intermediate a* value of -5.56. This shift suggests a reduction in the green tone and an overall warming colour, possibly due to the influence of whey protein complexes and oil emulsions on the optical properties of the paneer surface.

The b* values, representing the yellow-blue axis, increased considerably in WPI-fortified samples. The control had a b* value of 16.49, while the highest yellowness was recorded in the SWP sample (24.95), followed by WOH-SP (20.61) and SWOH-P (20.31). This may be due to the increase in amine compounds that react with animo acids (Maillard reaction) to build up dark pigments (Akesowan, 2009). The difference in colour between the WPI-fortified paneers and control, which was lighter in colour as compared to the fortified paneers, can be observed under naked eye. Similar b* values were observed in (Tojan et al., 2024) (17.52) which is close to the b* value of the control in this work. The paneer samples obtained are shown in Figure 4-2.

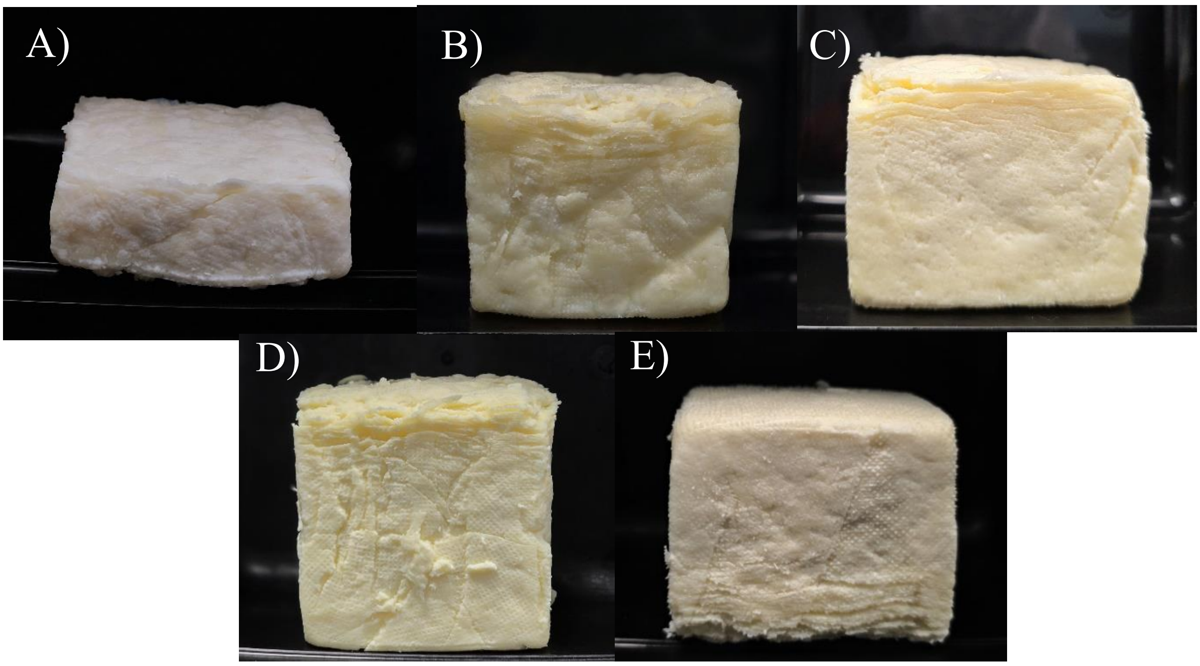


Figure 4-2 The different paneer samples developed. (A) Control. B) SWP. C)WOH-SP D) SOH-WP E) SWOH-P)

4.4 Texture profile analysis

The texture profile of the paneer sample is shown in Table 4-4 .The control exhibits hardness, cohesiveness, chewiness, springiness and gumminess of 15.92 N, 0.37, 3.16 N.mm, 5.69 N, respectively. (Ahmed & Bajwa, 2019) received similar results of hardness (12.64 N), cohesiveness (0.49), springiness (0.77) and gumminess (6.36 N) when manufacturing paneer using homogenised full fat milk and citric acid as the coagulant. The results obtained by (Kapoor et al., 2021) revealed that the hardness of paneer was 10.11 ± 0.29 N even though they used full fat homogenized milk whereas the lower value of hardness in this research when using skim milk may be attributed to the pressing of the paneer. In this research, the pressing of the paneer was done using TPA and it had significant contribution to the texture of the paneer (Tellabati et al., 2023). When compared to the control, the SWP sample, which included added whey protein without homogenization or oil, showed increased hardness (19.27 N), chewiness (6.04 N·mm), and gumminess (7.02 N), while springiness rose significantly to 0.86 mm. However, cohesiveness remained similar at 0.36. The enhanced textural values suggest that whey protein alone can influence the protein matrix, contributing to a firmer and more elastic structure.

Table 4-4The texture profile of the different paneer samples (mean ± standard deviation).

	Hardness (N)	**Cohesiveness**	**Chewiness (N.mm)**	**Springiness (mm)**	**Gumminess (N)**
Control	15.92 ± 3.09	0.37 ± 0.03	3.16 ± 0.52	0.56 ± 0.01	5.69 ± 1.04
SWP	19.27 ± 0.75	0.36 ± 0.01	6.04 ± 0.27	0.86 ± 0.01	7.02 ± 0.34
SOH-WP	27.56 ± 10.69	0.35 ± 0.003	8.67 ± 3.44	0.88 ± 0.01	9.95 ± 4.05
WOH-SP	22.59 ± 8.03	0.38 ± 0.02	7.30 ± 2.31	0.85 ± 0.01	8.55 ± 2.72
SWOH-P	26.11 ± 2.68	0.35 ± 0.01	7.90 ± 1.03	0.87 ± 0.004	9.08 ± 1.16

Further increases in hardness were observed in the SOH-WP sample, where milk was homogenized with oil and whey was added later prior to acid coagulation. This sample recorded the highest hardness (27.56 N), chewiness (8.67 N·mm), and gumminess (9.95 N), alongside a springiness of 0.88 mm. The cohesiveness (0.35) almost remained the same, but the overall texture increased compared to the control. The increase in hardness, chewiness, and gumminess indicates that the homogenization of the milk was able to improve the textural property of the paneer. Enhanced textural properties were also seen in WHO-SP, with a hardness 22.59 N, chewiness of 7.30 N·mm, cohesiveness of 0.38 and gumminess of 8.55 N. A hardness of 26.11 N, chewiness of 7.90 N·mm, springiness of 0.87 mm, cohesiveness of 0.35 and gumminess of 9.08 N was observed in SWOH-P similar to SOH-WP, where the milk, oil and whey were homogenized together in SWOH-P. Compared to the control all the homogenized samples demonstrated increased textural properties. Although all of the homogenized samples had exactly the same formulation it is evident that the two paneers that were manufactured from emulsions that contained casein proteins (SWOH-P and SOH-WP) had firmer textures than the fat containing paneer that was manufactured from an emulsion formed from WPI and fat (WHO-SP). This suggests that the fat droplets formed with significant amounts of casein on the fat globule interface are able to reinforce the continuous phase of the paneer cheese to a greater extent than fat globules with whey proteins as the dominant interfacial protein species.

It has been observed that the hardness value decreases with an increase in fat content in some researches (Uprit & Mishra, 2004) but here when the fat content was increased, the hardness of the paneer samples also increased. They are, however, subjective, because they depend on different milk composition, kind, and concentration of coagulant to be used and difference in provision and parameters established in the texture analysis. Thus, such values can give an approximate comparison only (Kapoor et al., 2021).

4.5 Confocal microscopy of the Paneer

The microstructure of food describes the way food elements behave and fit with each other at a very small-scale length. Cheese microstructure has milk fats surrounded and trapped by a matrix made of casein. The manufacturers should understand how cheese is structured, since different parts of the cheese interact and affect the functions and taste of the cheese. The microstructure of these constituents can be studied during processing and storage.

The Confocal Laser Scanning Microscopy (CLSM) was used here to study the microstructures of the paneer samples. The control (A) made by only using skim milk (refer Figure 4-3.) shows a dense, continuous and compact protein matrix. The green indicating the protein matrix appears to be uniform and the fat globules marked in red in the control are scattered and finely dispersed across the matrix. It reflects the low-fat content and the homogeneous nature of the skim milk paneer (Control). Whereas the SWOH-P (B) prepared by homogenizing skim milk, WPI and oil showed the protein network with fat dispersed across the matrix and some clusters of fat molecules. The matrix of SWOH-P appeared to be less compact compared to the control which could be due to the homogenization and the partial integration of fat and WPI within the protein matrix.

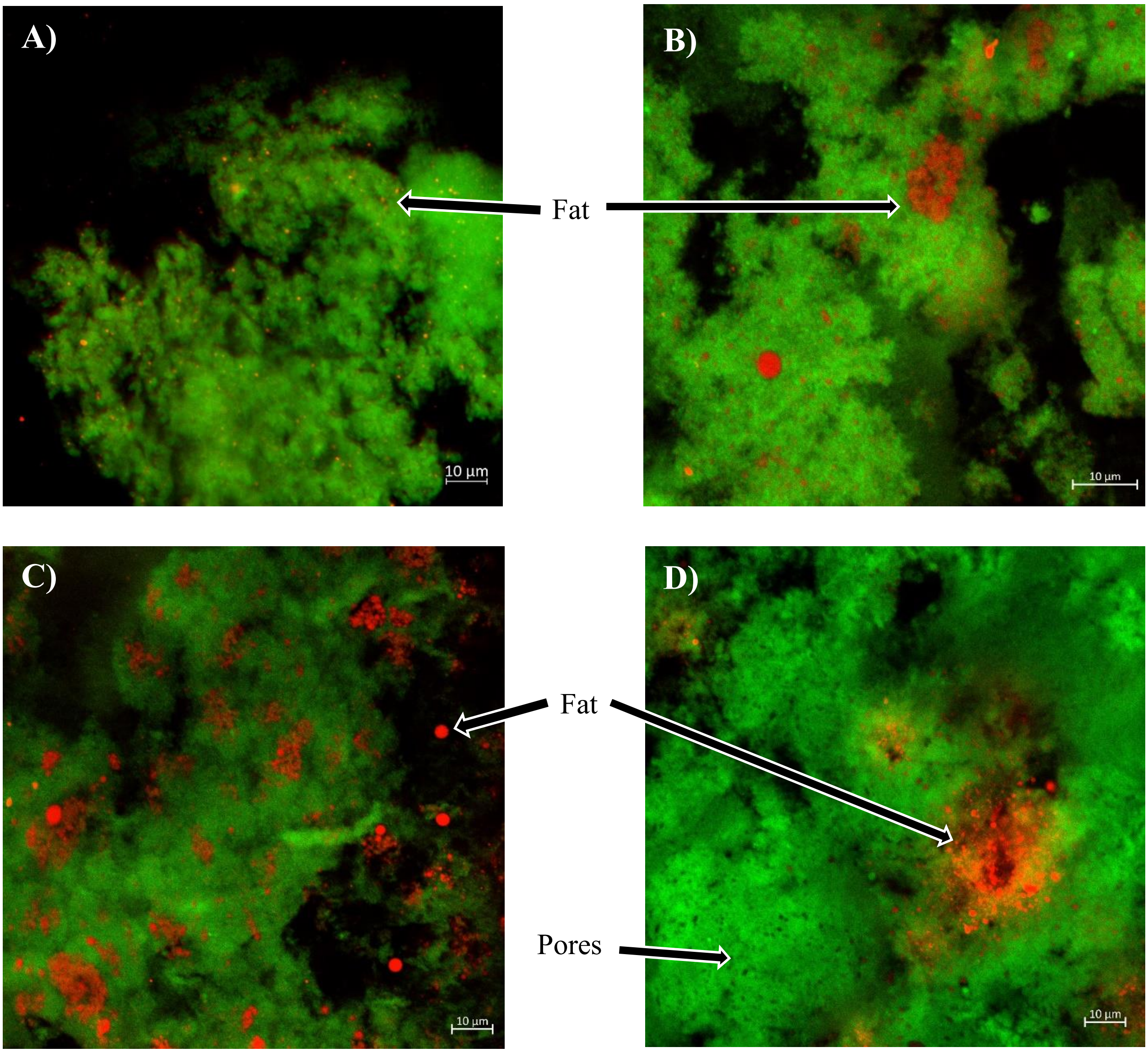


Figure 4-3The Confocal Laser Scanning Microscopy (CLSM) images of A) Control B) SWOH-P C) SOH-WP D) WOH-SP

In SOH-WP (C), in which Skim milk and oil was homogenized exhibits more prominent porosity and aggregation of fat globules. More aggregates of fat globules can be seen in SOH-WP than SWOH-P.

The WOH-SP (D) displayed the most heterogeneous structure, with pores devoid of both fat and protein and intense fat–protein co-localization. It can be seen that the oil was not distributed across the protein matrix in WHO-SP. The specific porous structures were only seen in WOH-SP and appear to be the same size as fat globules. A potential explanation of the fat globule sized pores is that the fat globules could have been entrapped uniformly in the initial acid coagulated protein matrix curd particles but on tightening and contraction of the curd particles into a large cohesive cheese the fat globules may have been squeezed out of the matrix to pool in larger voids. That is the pores may be ghost remnants of the original placement of whey protein stabilised fat globules in the system. For this theory to be possible the whey stabilised fat globules must not have been able to interact, at least initially, with the casein matrix. It is worth noting that whey proteins, in their native state, are not precipitated by at the pH of paneer manufacture.

Due to time constraints and last-minute decision to make SWP, the CLSM was not analysed but it is included in other physicochemical analyses.

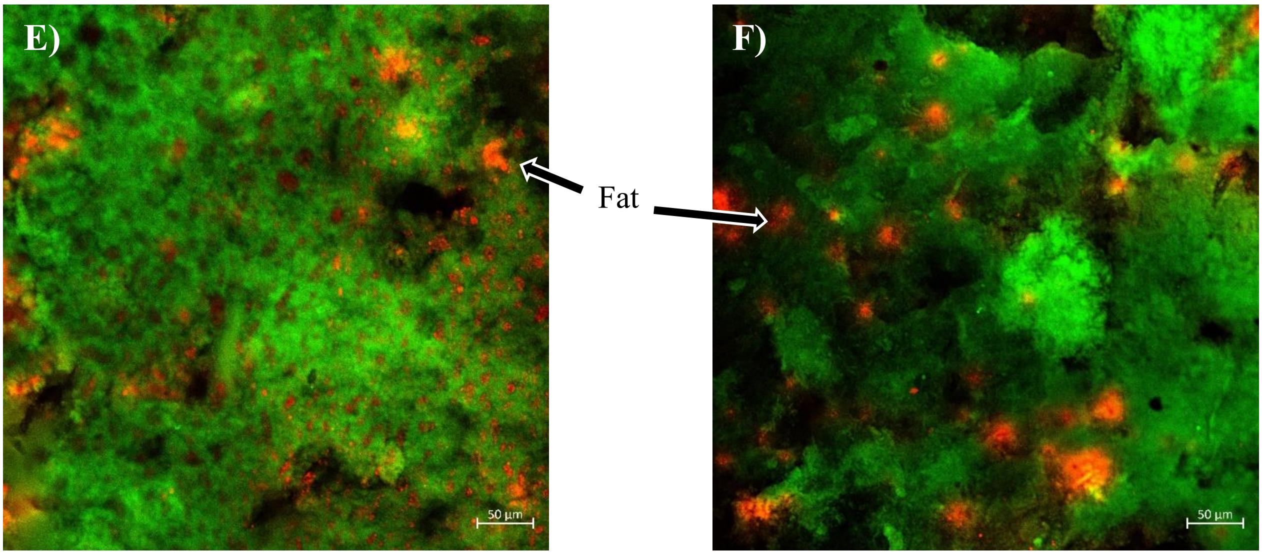


Figure 4-4 Low magnitude confocal of E) SOH-WP. F) WOH-SP

From the low magnification of SOH-WP (E) in Figure 4-4 the protein network appears to be broadly organized, with numerous fat globules distributed homogeneously across the protein matrix. The structure of SOH-WP shows uneven protein matrix and small voids. But in WOH-SP (F) exhibits large fat clusters and more intense localization of fat globules resulting in irregular and disrupted structure. Since the WPI was homogenized with oil, the protein-fat interaction may have interfered with the proper formation of casein network. The low magnification of SOH-WP and WOH-SP gives clearer picture of the oil and protein interaction in the paneer samples.

From the confocal analysis, it is evident that the sequence in which the whey and oil is added has significant influence in the microstructure of the paneers, protein network integrity, fat distribution and pores formation. These have high impact on the final texture, moisture retention and overall quality of the paneer.

4.6 Rheology of the cheese milks during heating

This analysis maps textural changes in the cheese milks through the heating cycle prior to acid addition and thus gives an indication of the impact of formulation/processing prior to curd formation. The viscosity trend of the five different samples (refer Figure 4-5) during a simulated paneer making thermal cycle (5 → 85 °C hold → 70 °C) shows the liquid phase textural contributions resulting from potential protein agrregation: i.e. casein micelle gelation and/or whey protein denaturation and aggregation. .

The control sample with just the skim milk shows no rise in viscosity at 85 °C. All the samples show a drop in viscosity as the temperature is rising from 5 °C to 85 °C. In SWP, where the WPI is added to the milk without the presence oil, viscosity increases substantially (peak ≈ 12 mPa$^{\cdot s}$) compared to the control. It indicates that the whey protein concentration is at a high enough level to effect a viscosity change likely through heat denaturation and the formation of aggregates. However, without the oil presence for the fat-protein interaction, the viscosity remains lower than the oil containing systems.

The oil added systems showed higher viscosity depending on the different homogenized solutions. In SWOH-P, where skim milk, WPI and oil are homogenized together showed the highest viscosity peak (~34 mPa·s). The whey denaturation occurred around 75–85 °C for all the samples but the highest viscosity was shown by SWOH-P, which may be due to the skim milk and oil emulsion with whey. In SOH-WP, the milk and oil, homogenization increased the viscosity of the sample (~27 mPa·s). This lower value compared to SWOH-P suggests that while whey denaturation still contributes significantly, adding WPI into an existing oil–casein matrix yields fewer protein–fat binding sites. The WOH-SP only reached ~10 mPa·s which may be due to the whey oil emulsion that may have limited whey/casein and whey/whey aggregation during heating.

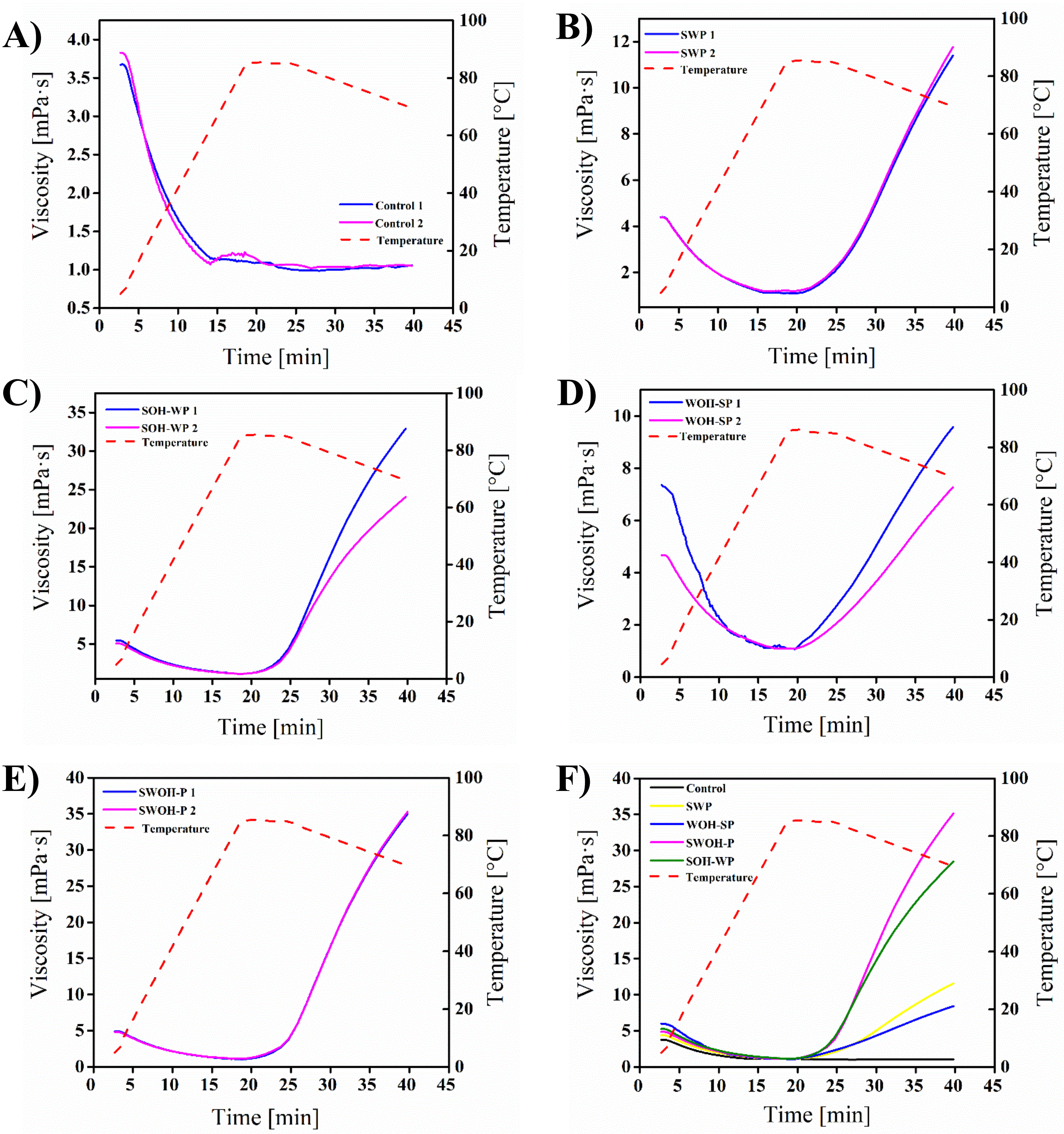


Figure 4-5 The viscosity trend of the five different samples (A) Control B) SWP C) SOH-WP D) WOH-SP E) SWOH-P F) overlay of average of all samples) during the simulated paneer making thermal cycle (5 → 85 ° C hold → 70 ° C)

In the overlaid graph (F), it can be seen that, SWOH-P > SOH-WP ≫ SWP > WOH-SP > Control. Since caseins are inherently stable at the temperatures at which these experiments were conducted viscosity changes are therefore likely to result from the product of whey denaturation: that is, when whey proteins denature what macro-sized objects are formed that will impact on viscosity. Simultaneous homogenization (SWOH-P) increases whey denaturation and showed highest viscosity. Addition of WPI after oil–milk homogenization (SOH-WP) showed a modest reduction in peak viscosity and a slightly delayed denaturation of whey compared to SWOH-P. Without the presence of oil (SWP), the viscosity was low compared to SOH-WP, but was still able to be higher than WOH-SP. The whey oil emulsification resulted in a significantly lower viscosity, which means it was able to reduce the impact of any whey protein denaturation. The low viscosity of the control at 40 min compared to 5 min shows that there was no significant aggregation of protein happening in the system without the addition of whey protein. This viscosity of the samples aligns with confocal microscopy observations. The conclusion is not just that the addition of fat via homogenization increases the viscosity of heated solutions but also the nature of the proteins that are at the oil water interface is also important. Specifically structuring whey proteins in the form of an emulsion enables the manufacture of heat-treated whey fortified milks that have low viscosities compared to when emulsions are formed in the presence of casein proteins.

4.7 Texture profile analysis of curd pressing and dewheying operation

The behaviour of the paneer curd during pressing was studied using Texture Profile Analyser (TPA) under a constant force, and the rate of compression (dd/dt) was plotted against log time for all the five samples (Figure 4-6). This analysis explains the mechanical behaviour of the paneer curd mass during compression over time.

For each sample, a duplicate run was made. Figure 4-6(F) is the overlay of all the sample averages for comparison. The rate of compression (dd/dt) declines as the log time increases, pointing that the curd rapidly compresses at the beginning of the pressing and over time it gradually stabilizes as the moisture is expelled from the curd and the structure resistance of the curd increases. This curve trend is almost consistent with all the samples.

In Figure 4-6(E), the pressing of SWOH-P is shown and it can be seen that the 2 replicates are different: modelling of the 2 replicates below reinforced this observation. It was noted during compression that the TPA suffered from a malfunction and had to be restarted for the sample SWOH-P 2. Therefore, it is likely that the data for SWOH-P 2 is an artifact and as such this data was omitted from further analysis. From the graph, it is observed that the rate of compression slows done substantially at 0.1 and around 90% of the compression is completed at 2.25 (log time). The time to reach this threshold is same for all the samples except the control which is 1.75 (log time). So, it can be concluded that the mass alone is responsible for the pressing behaviour of the curd, not the formulation.

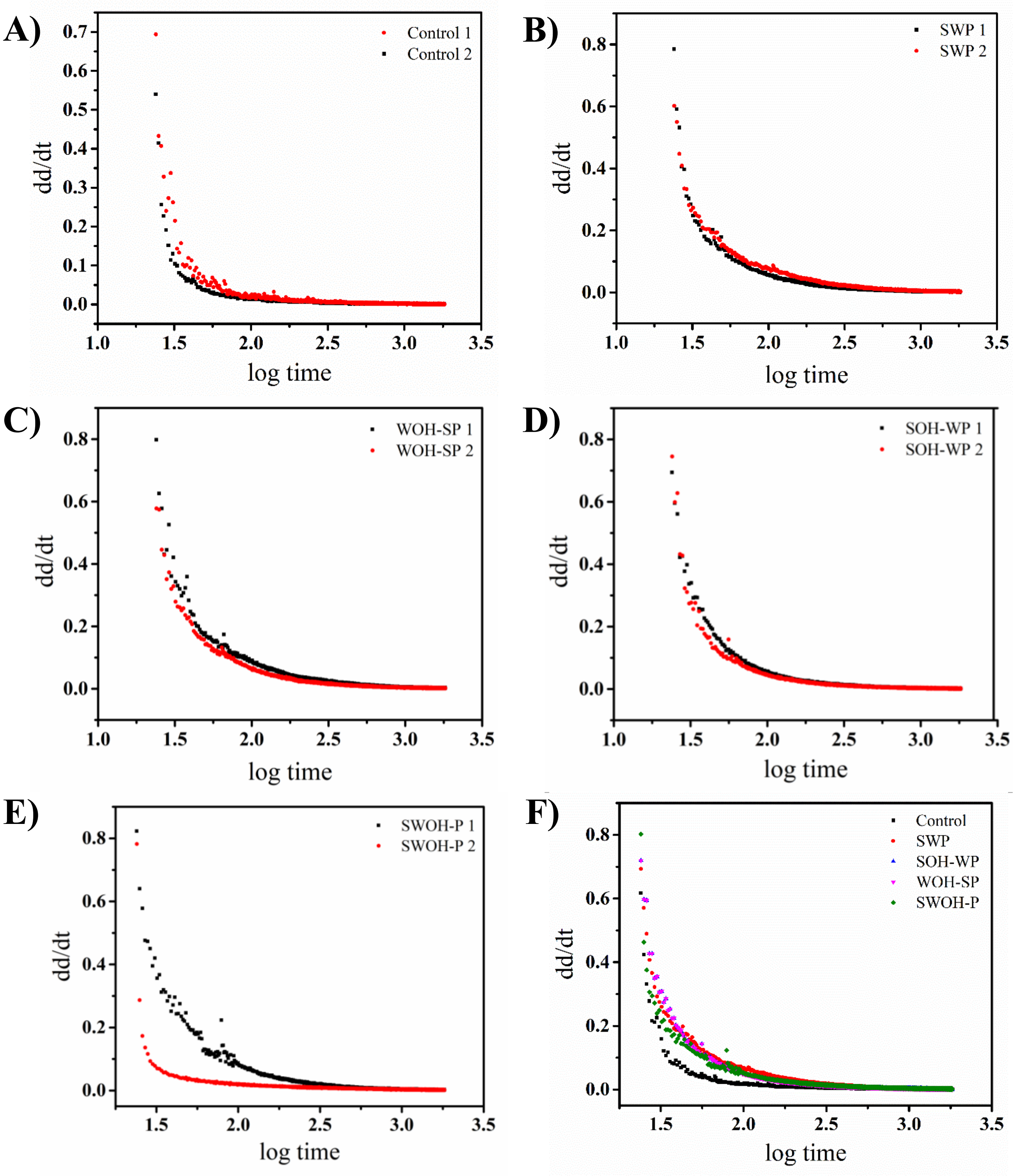


Figure 4-6 The behaviour of the paneer curd during pressing via TPA by formulation and processing conditions: A to E show replicates for each condition denoted by 1 or 2 and F shows all the average compression profile for each condition in a single plot.

To further understand the mechanical response of the paneer during pressing under constant pressure, a power – law model was fitted to the rate of compression data (dd/dt) for each sample. The model was under the form:

$$\frac{dd}{dt} = a.(\log t)^b$$

$$\frac{dd}{dt} = rate\ of\ compresssion\ (R_c); \log t = pressing\ time\ (t_p)$$

Where *a* represents the initial compression rate and *b* is the rate at which this value decays over time. The fitting parameters *a, b,* R^2 for each of the sample along with the corresponding final weight of the paneer after pressing is shown in Table 4-5. A potential variable that might impact curd compression is the initial mass of curd placed in the TPA: this mass is also included in Table 4-5.

Table 4-5 The fitting parameters a, b, R^2 and weight of the paneer for each of the sample

Sample	***a***	***b***	R^2	***Weight of paneer***
Control 1	0.72	-5.71	0.74	92.00
Control 2	2.24	-6.53	0.87	111.35
SWP 1	9.03	-7.24	0.96	279.50
SWP 2	11.32	-7.04	0.94	242.50
SOH-WP 1	9.60	-7.32	0.99	288.70
SOH-WP 2	6.92	-7.11	0.97	288.00
WOH-SP 1	15.54	-7.31	0.95	286.00
WOH-SP 2	11.00	-7.30	0.97	329.00
SWOH-P 1	15.41	-7.48	0.95	353.80
SWOH-P 2	0.64	-4.78	0.48	342.40

There is a notable increase in the initial rate of compression, parameter *a*, with increase in paneer mass. The control samples with relatively low mass (92 – 111.35 g) shows a low value of 0.72 – 2.24 for *a* parameter, while other samples such as WOH-SP, SWOH-P, SWP, SOH-WP with higher paneer mass, shows an elevated *a* value. This suggests that the larger curd mass undergo higher initial compression rates, that may be due to the increased internal resistance and water load at the start of the pressing.

One hypothesis is that as the mass of the paneer increases, the mechanical resistance of the water to express out increases. This would lead to a longer time required for pressing of the paneer. To test the hypothesis, the power-law model fitting results were plotted as a function of paneer mass (Figure 4-7) and a clear correlation was observed. The increase in mass corresponded to a higher in a value and more negative b value consistent with intense initial compression.

These findings support the earlier observation that the mass of the curd is responsible for the pressing behaviour of the curd, which indicated that the pressing time can be optimized by accounting for the total curd weight rather.

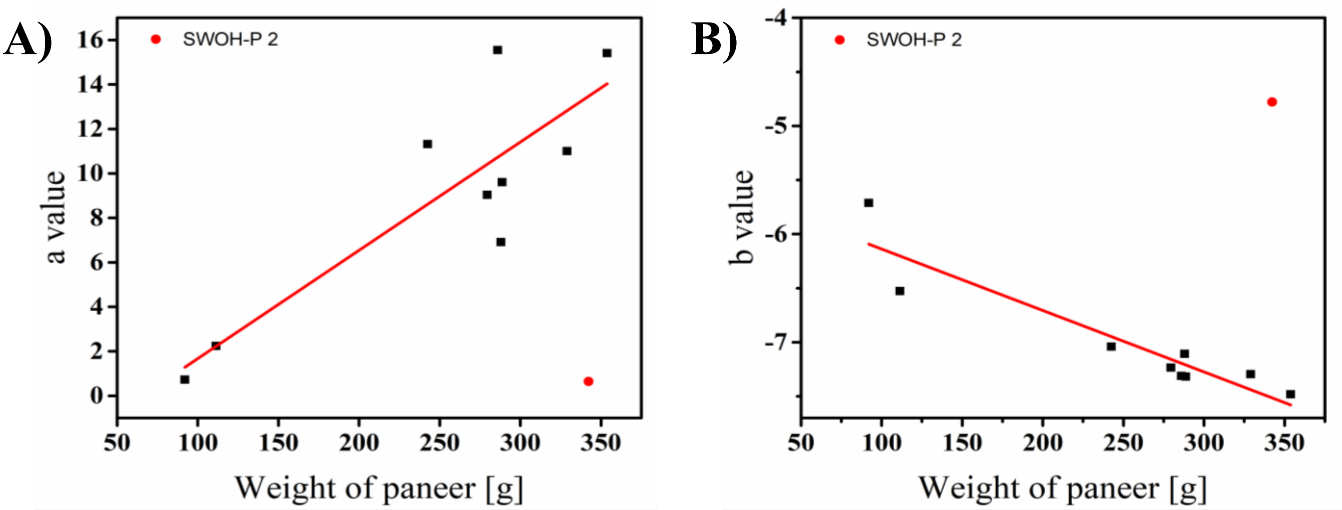


Figure 4-7 Corresponding (A) a value and (B) b value of power-law model with weight of paneer

The red data point (SWOH-P 2) in the graph is an outlier, as discussed earlier, caused by a malfunction during texture profile pressing and was not considered in the regression. Both the graph was fitted with a linear regression model.

$$a\ value = -3.19919 + 0.0487.\mathrm{M}_p;\ \ R^2 = 0.70467; \rightarrow \text{ Figure 4-7 (A)}$$

$$b\ value = -5.57019 - 0.00568.\mathrm{M}_p;\ \ R^2 = 0.84745; \rightarrow \text{ Figure 4-7}(B)$$

$M_p = Mass\ of\ the\ pressed\ paneer$

Substituting this into;

$$\frac{dd}{dt} = a.(\log t)^b$$

$$\frac{dd}{dt} = rate\ of\ compresssion\ (R_c);\ \ \log t = pressing\ time\ (t_p)$$

Therefore:

$$R_c = (-3.19919 + 0.0487.\mathrm{M}_p).t_p{}^{-5.57019-0.00568.\mathrm{M}_p}$$

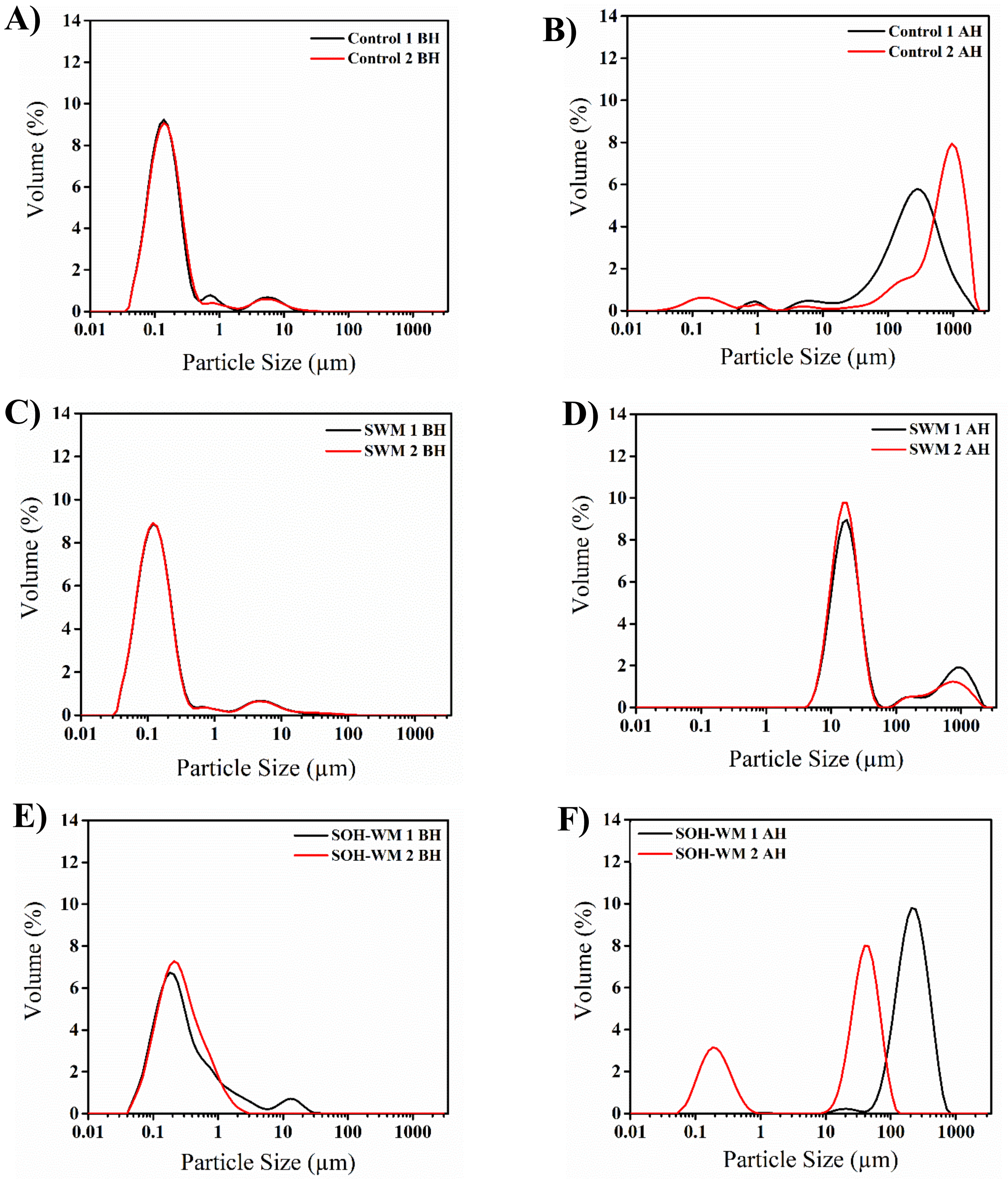
This equation is derived from Figure 4-7, that can be used to find the rate of compression (R_c) or the time required for pressing (t_p) regardless of the processing method.

4.8 Particle size analysis

The particle size distribution of the different milk samples used for the preparation of paneer was analysed both before heating (BH) and after heating (AH) (Figure 4-8). The heating procedure was accordance with the standard manufacturing process of paneer. The aim of the particle size analysis was to observe how the addition of oil, WPI and their respective addition sequence affected the milk matrix prior to acid induced coagulation.

The Before heated (BH) milk samples showed similar particle size distribution with predominant peaks in the range of (0.1 - 1µm). This range showed by the milk sample is characteristic to the peaks of casein micelles and globular whey protein present in milk, which is seen in Control (Figure 4-8 (A)). The similarity in the particle sizes among BH samples shows that initial mixing and incorporation of WPI and oil into the milk did not lead to aggregation or instability in the system before the thermal processing.

After heating (AH), Notable changes were observed in the samples. The control samples showed shift in peaks from (0.1 - 1µm) BH to (100 - 1000µm), that may be due to the thermal aggregation of casein and whey proteins in the milk. Similarly skim-whey added milk (SWM) and skim milk oil homogenized – whey added milk (SOH-WM) exhibited an increase in particle size, indicating that the heat treatment caused the proteins to denature and form aggregates. The WOH-SM AH showed its major peak in the range of (0.1 - 1µm) even after heating, which shows us that there was no big aggregate formation or increase in particle size, but the SOH-WM and WOH-SM samples showed a distinct bimodal distribution after heating, which may be due to partial phase separation or different aggregation of whey oil and casein matrix. It can be seen that the different addition method of whey and oil addition into the system have influenced the microstructure significantly. The stability and instability of the system is affected by the incorporation method of whey and oil.


A)
Control 1 BH
Control 2 BH
Volume (%)
Particle Size (µm)
B)
Control 1 AH
Control 2 AH
Volume (%)
Particle Size (µm)
C)
SWM 1 BH
SWM 2 BH
Volume (%)
Particle Size (µm)
D)
SWM 1 AH
SWM 2 AH
Volume (%)
Particle Size (µm)
E)
SOH-WM 1 BH
SOH-WM 2 BH
Volume (%)
Particle Size (µm)
F)
SOH-WM 1 AH
SOH-WM 2 AH
Volume (%)
Particle Size (µm)

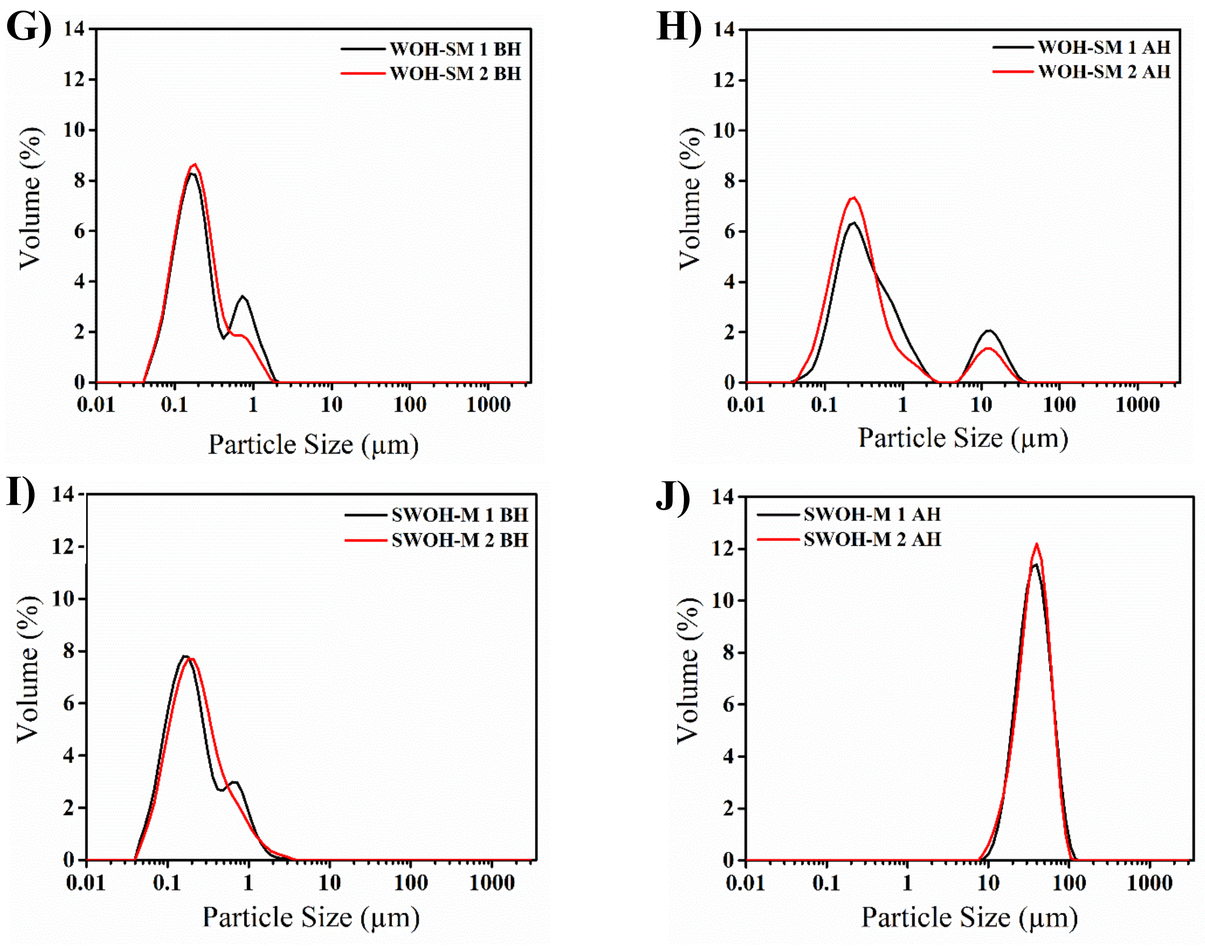


Figure 4-8 Particle size of different sample milks Before (BH) and After (AH) heating during the manufacturing process.

In SWOH-M AH, the samples remained narrowly distributed and stayed around (10 - 100µm). This sharp unimodal distribution after heating suggests that the homogenization of the milk, oil and WPI to together is able to stabilize the system during heating, that may be due to the formation of uniform protein network, which was able to resist large scale aggregation.

The heating process has a significant role in the processing of paneer. Heating altered the particle size in most samples and is likely caused due to denaturation and aggregate formation of the protein in the system. However, this change in the particle size distribution after heating was controlled by the formulation and ingredients used for making paneer, were SWOH-M showed least aggregation and controlled size distribution among the samples after heating. The processing order and formulation has a big role in maintaining the stable colloidal system before the acid-induced coagulation.

4.9 Protein profiling by SDS-PAGE

SDS-page analysis of paneer samples under reduced conditions (B-sample) and non-reduced conditions were examined to investigate the protein composition and protein complexes formed due to protein fortification of the paneer (Figure 4-9). During the manufacturing of traditional paneer, most of the whey proteins are lost during the acid inducted coagulation. From this analysis, the whey protein retention after the addition of WPI was determined. The reduced condition was examined to identify the presence of specific denaturable proteins such as β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA) and also to identify the proteins that were participating in disulfide-linked aggregated with other proteins such as casein.

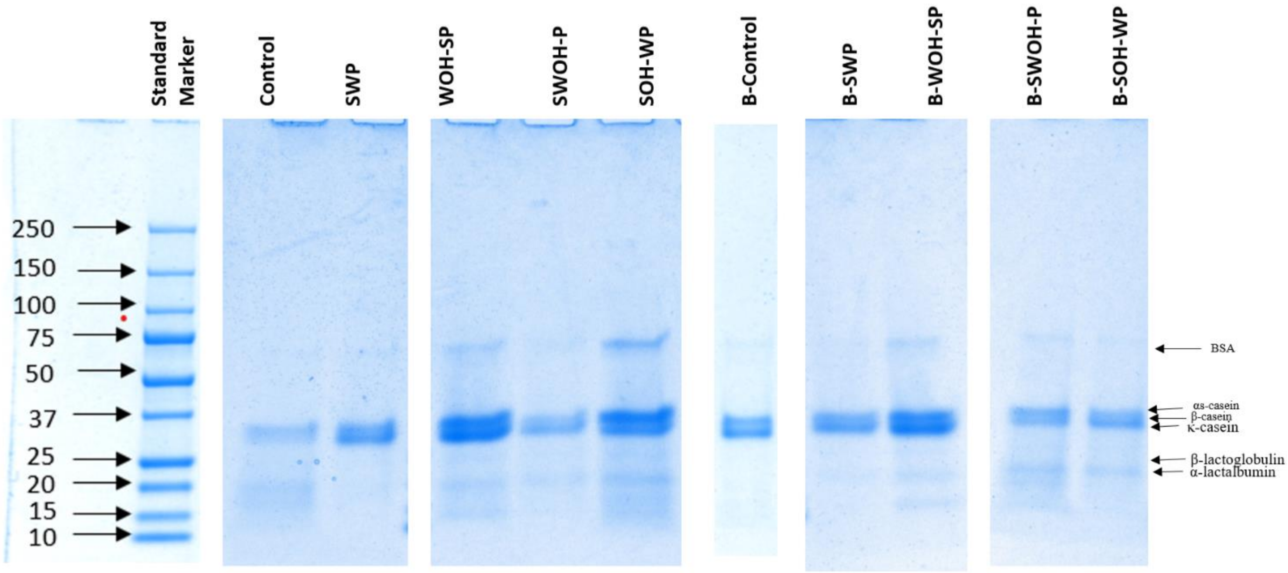


Figure 4-9 SDS-PAGE gels of different paneer sample under non-reduced and reduced (B- sample) conditions

The gel images were analysed using ImageJ software to understand the intensity of the bands. In the non-reduced paneer samples, the control showed relatively weak bands for β-lactoglobulin, α-lactalbumin (14-18 kDa) and there was no visible band for BSA (66 kDa). The protein bands were identified using the procedure described by (Hussain et al., 2025). The low intensity of the β-lactoglobulin, α-lactalbumin bands, in both the reduced and non-reduced gels, may be due to the low retention of the whey protein from milk during the making of paneer. When the paneers were fortified with whey protein, there is an increase in the β-lactoglobulin, α-lactalbumin band intensity compared to control across the samples with the exception of the fat free paneer - SWP. Among the WPI fortified samples, all the samples homogenized with oil showed increased band intensity showing the retention of whey protein. BSA band intensity is also high for these samples, compared to control. But for SWP, the band intensity is less than control, that may be because of the disulfide linkage of the proteins with casein.

The retention of whey proteins and BSA appear to have strong connection with the method of incorporation of whey protein. The homogenization of milk with oil and whey, showed high intensity, which suggests that homogenization promotes better interaction of WPI and milk proteins. The strength of the whey bands in the non-reduced gel indicates the the mechanism for whey protein inclusion is in part via physical entrapment in the curd matrix rather than through thermally induced whey aggregates or whey casein aggregates. This is able to increase the integration of whey into the paneer matrix.

Th reduces samples (treated with β-mercaptoethanol), showed a better intensity compared to non-reduced samples. This increase in intensity may be the effect of reduction and the breakage of the disulfide linkage. The β-lactoglobulin, α-lactalbumin band became sharper and the comparative intensity with casein protein has increased for β-lactoglobulin and α-lactalbumin. Upon reduction, the disulfide bonds were cleaved, and the individual proteins have appeared more distinctly in the gel. BSA band on the gels are also showing good intensity and, this increase in intensity suggests that a portion of the WPI has been fortified into the paneer matrix. Compared to non-reduced SWP, the reduced SWP is showing an increase in intensity for β-lactoglobulin, α-lactalbumin bands.

Overall, The SDS-page analysis confirms that the WPI fortification was able to successfully incorporate whey protein into the paneer system. The low intensity of the β-lactoglobulin, α-lactalbumin in total may be due to only 3% of WPI was added to the system and due to the increase in paneer weight obtained compared to the control and the whey protein is spread across the paneer. There is variation in the casein bands as well as the whey bands. The casein band intensity variation indicates that the concentration of protein in the samples was not optimised. Unfortunately due to time constraints it was not possible to optimise the PAGE analysis method for the paneers studied.

5 Chapter 5 Conclusion and Recommendations

This study was able to evaluate the potential of incorporating whey protein isolate (WPI) into the paneer with the objective of increasing the overall protein content, importantly the retention of whey protein which are lost during the traditional heat and acid coagulation process. Different formulation and processing approaches were used to determine the best effective way to incorporate whey protein. All the formulations gave better result. Though the highest yield was shown by SWOH-P. However, despite the increase in yield for all the samples, there were significant differences observed in the structural and functional properties of the paneer samples depending on the method of incorporation. Among the different concentrations of WPI trials, 3% WPI was selected as optimal, as higher concentrations formed thick gel like structures in the milk during heat treatment, which made it difficult to proceed with acid coagulation. In contrast 3% WPI showed thick fluid like structure that was able to be further processed into a paneer.

Addition of WPI into the paneer system increased the paneer yield and also the increase retention of whey protein, which was confirmed by SDS-PAGE. The confocal laser scanning microscopy which was used to study the microstructure of the paneer was able to detect more continuous protein network and the incorporation of oil into the system. Different methods to add WPI while homogenizing with oil had different outcomes which was seen in the microstructure of the protein fortified paneer samples. Specifically, WOH-SP showed large visible oil aggregates in the confocal images with oil pockets appearing concentrated, without coalescence, and poorly distributed. Visible uniformly distributed pores were seen in the WOH-SP matrix which would likely result in a weakened structure. It is postulated that the pores result from ghost holes in which whey stabilised fat globules may have been present at the time of initial curd particle formation before being squeezed out of position, due to contraction of the casein curd matrix, to pool in groups in the relatively large interstitial voids between aggregating curd particles. For this mechanism to be true the whey stabilised fat globules must be non-interacting with the casein matrix – in this case the presence of the fat globules, in addition to the ghost pores, would also weaken the structure. In contrast the SWOH-P and SOH-WP samples showed more uniform microstructure with better oil and protein distribution even though there were some aggregates visible in the SWOH-P sample. It is postulated that the more even fat distribution indicates that the fat globules are able to interact with the casein matrix to the extent that they are not squeezed out to pool in voids. The presence of casein on these fat globules makes the hypothesis that these fat globules interact with the main casein matrix more likely. The presence of interacting fat globules would be expected to reinforce the overall structure. The structural differences which was seen in the WPI fortified samples was supported by texture profile analysis due to the increase in hardness, chewiness, springiness and gumminess of the fortified samples. Among the oil emulsified treatments, WOH-SP showed lower hardness compared to the others, which further confirmed the weaker structural integrity that one would expect based on the hypothesis described above. The particle size analysis conducted on the milk samples before and after the heat treatment showed significant changes in the colloidal structure, showing the impact of WPI addition to the system. WOH-SP, in particular, showed smaller particle size after heating compared to the others. This particle size observation lend further support to the hypothesis that the whey emulsions are less likely to interact with the casein matrix.

The rate of compression during pressing of the paneer curd was analysed using Texture profile analyser (TPA), showed that the pressing behaviour of the curd was influenced by the mass of the curd being pressed rather than the method of formulation of paneer. With this finding we were able to support the hypothesis that as curd mass increases, the liquid expression becomes more difficult, and it is time dependant under constant force.

WOH-SP showed low viscosity than other samples. The presence of pores, low hardness, and low viscosity may be due to the absence of sufficient casein interaction, the oil was not properly bound within the protein matrix, and it may have migrated through the curd and formed aggregates. It shows the importance of the method for the incorporation of oil and whey. The SWOH-P sample, which was homogenized together with skim milk, oil and whey showed good results compared to all the samples in all the characterization, suggests that the processing step and the incorporation method have great influence on the integration of WPI into the paneer.

With the help of this findings, it is recommended that 3% WPI can enhance the protein content in paneer and improve the nutritional and functional property without compromising the processing methodology. Further work can be done on improving the processing conditions and to increase the shelf life, sensory characteristics, and consumer acceptance for the WPI fortified paneer. Further studies can also be done on improving the texture, sensory properties and formulation of the paneer. The impact of different coagulating agents and detailed nutritional analysis, like *in vitro* digestibility studies could be studied to assess the nutritional quality and protein bioavailability.

Overall, this study shows a pathway of increasing the nutritional and structural quality of the paneer through the addition of WPI.

6 Chapter 6 Reference

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Appendix

Declaration of usage of AI software throughout the thesis

I have utilized ChatGPT and Grammarly in various sections of my thesis while ensuring that all AI- assisted outputs are critically reviewed and ethically integrated into my thesis. As a responsible researcher, I acknowledge that I have used AI only to overcome writer's block and to identify grammar or vocabulary errors to polish the contents that was originally written by myself.