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University College Cork, Ireland
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Formulation, processing and functionality of plant-based alternatives to cheese

Thesis presented by

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for the degree of

Doctor of Philosophy

in

Food Science and Technology

July 2023

Declaration

I hereby declare that the work submitted is entirely my own and has not been submitted to any other university or higher education institute, or for any other academic award in this university.

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Nadia Grasso

Date: 19/07/2023

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Abstract

Numerous plant-based cheese alternatives developed using different raw materials, formulations, and processes are currently commercially-available. However, most of these products differ significantly from their dairy counterparts, and a lack of knowledge of the science underpinning development of plant-based cheese alternatives is evident in the scientific literature. This thesis investigates the physicochemical characteristics of commercially-available products against dairy benchmarks and studies the influence of different protein concentrations and profiles, ingredients, and calcium fortification strategies, to achieve the development of plant-based cheese alternative prototypes with enhanced nutritional profile and functionality than these types of products currently available. Chickpea protein ingredients were used to formulate samples with high protein contents and different texture and microstructure; however, such samples did not have acceptable melting behaviour. Binary blends of zein and chickpea protein ingredients allowed development of plant-based cheese alternatives with improved meltability and stretching properties, due to the unique rheological characteristics of zein. Different calcium fortification approaches were proposed to improve the nutritional profile of plant-based cheese alternatives; however, fortification resulted in changes in the physicochemical properties of the samples. The learnings obtained were used to develop a plant-based cheese alternative prototype with similar texture to processed cheese, and enhanced nutritional and physicochemical properties compared to the commercial plant-based cheese alternatives. However, further improvements of protein digestibility and sensory properties of the prototype are needed. The findings presented in this thesis represent significant advancements in our understanding of the ingredient, formulation and processing science required to develop plant-based cheese alternatives.

Publications

Grasso, N., Alonso-Miravalles, L., O'Mahony, J. A. (2020). Composition, physicochemical and sensorial properties of commercial plant-based yogurts. *Foods*, *9*, 252.

Grasso, N., Roos, Y. H., Crowley, S. V., Arendt, E. K., & O'Mahony, J. A. (2021). Composition and physicochemical properties of commercial plant-based block-style products as alternatives to cheese. *Future Foods*, *4*, 100048.

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Grasso, N., Roos, Y. H., Crowley, S. V. & O'Mahony, J. A. Physicochemical properties of plant-based cheese alternatives fortified with calcium [in preparation].

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Grasso, N., Alonso-Miravalles, L., & O'Mahony, J. A. (2019). Composition, physicochemical and sensorial properties of commercial plant-based yogurts. *Institute of Food Science and Technology of Ireland, 48th Annual Food Science and Technology Conference*, Limerick, Ireland.

Grasso, N., Bot, F., Roos, Y. H., Crowley, S. V., Arendt, E. K., & O'Mahony, J. A. (2021). The influence of protein concentration on key quality attributes of chickpea-based alternatives to cheese. *AOCS Plant Protein Science and Technology Forum*, Chicago, US (Online).

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Introduction and research objectives

The offering of commercially-available plant-based food is expanding, with numerous new products emerging on the shelves of supermarkets. The plant-based foods sector represented an $8 billion market in 2022 in the United States alone, with a 3-year growth in sales of 44% recorded between 2019 and 2022, and, in the same time-frame, the plant-based cheese sector showed 51% growth in sales in the US, with a sales growth of 23% in 2022 in Europe (Good Food Institute, 2023). For consumers who are limiting their consumption of animal-based food, health-related factors represent the main drivers for choosing plant-based food. However, certain consumer groups (e.g., flexitarians and younger consumers) consider drivers such as the environmental and animal welfare benefits of plant-based foods more frequently than general consumers (Good Food Institute, 2023).

Plant-based cheese alternatives can be developed using different raw materials, formulations, and processes. However, most of the plant-based cheese alternatives currently available commercially have low protein and high saturated fat contents, being largely starch and coconut oil-based. The main reason for the use of non-protein ingredients in the development of plant-based cheeses is the attempt to mimic the physicochemical characteristics of the principal structure forming protein in milk (i.e., casein) and milk fat. Dairy proteins, in addition to their important nutritional contribution, provide cheese products with unique structure, textural and sensory properties, and the replication of such properties using plant-based ingredients is challenging and poorly understood (Mattice & Marangoni, 2020; Short *et al*., 2021). However, a lack of knowledge of the science underpinning development of plant-based cheese alternatives is evident from reviewing the scientific literature (Grossmann & McClements, 2021). Given this context, in recent years, the scientific community have initiated investigating the suitability of plant protein ingredients for

the formulation of plant-based cheese alternative products (Mattice & Marangoni, 2020; Ferawati *et al*., 2021; Grossmann & McClements, 2021; Mefleh *et al*., 2021). Plant protein ingredients show great potential in the development of plant-based cheese with improved nutritional profile and functionality, having functional properties that are critical in developing cheese alternative products (e.g., water holding properties, heat-induced melt/flow and stretchability). Moreover, over the last few years, the range of plant protein ingredients (i.e., origins, formats and protein purity) and in turn, their functional properties have advanced considerably. However, although such properties are well understood for dairy proteins, to better understand, predict and control the functionality of plant protein, more research is needed. Functional properties that are relevant in plant-based cheese alternative applications include water and/or oil holding capacity, emulsifying and gelling properties. These properties are necessary to form protein networks that can entrap water and oil during processing. In particular, gelling properties (effectively controlled aggregation) are important during heating, and are related to the molecular weight of plant proteins, their solubility, reactive amino acid side chains, and denaturation temperature, which influence intra- and inter-molecular bonds (Grossmann & Weiss, 2021).

The main plant sources used for extracting plant protein, and employed as plant protein ingredients in plant-based food formulations, include cereals, legumes, pseudocereals, oilseeds, and root vegetables. Among these sources, legumes represent particularly interesting and promising crops. Indeed, legumes are rich in nutrients, and protein concentrates and isolates from legumes are generally milder in colour, flavour, odour, and have lower contents of antinutritional components compared to whole seeds, presenting suitability for application in plant-based cheese alternative production (Mefleh *et al*., 2021). In particular, due to their nutritional value and

functional properties, chickpea protein ingredients show strong potential in the development of new and reformulated food products (Boye *et al*., 2010; Hall *et al*., 2017; Grasso *et al*., 2022). Another plant protein ingredient that has shown properties of relevance in plant-based cheese alternative applications is zein, the prolamin extracted from maize. Zein displays unique plastic behaviour in aqueous environments, and softening and increased viscous properties with increasing temperature (Mattice & Marangoni, 2020).

The use of plant protein blends derived from different sources (i.e., cereals and legumes) in formulating plant-based foods allows the development of food products that provide all the essential amino acids, in blended format closely reflecting the nutritional profile of animal proteins (Gorissen *et al*., 2018). Indeed, for example, legumes have low sulphur amino acid (i.e., cysteine and methionine) and high lysine content, while cereals have high sulphur containing amino acids and low lysine content. Moreover, from a technological perspective, due to the variety of plant protein sources available and their diverse functionality, plant protein blends can be exploited in many food formulation applications (Jiménez-Munoz *et al*., 2021).

Although numerous studies have investigated the potential of plant protein ingredients, a knowledge gap on the role of plant protein blends in the development of plant-based cheese alternatives is evident in the literature.

Research objectives

The overall objective of this thesis was to advance our understanding of the ingredient, formulation and processing science and technology required to support the development of plant-based cheese alternatives with improved physicochemical and nutritional properties compared to current commercially-available products. These scientific advancements will help close the gap between plant-based cheese alternatives and their dairy counterparts.

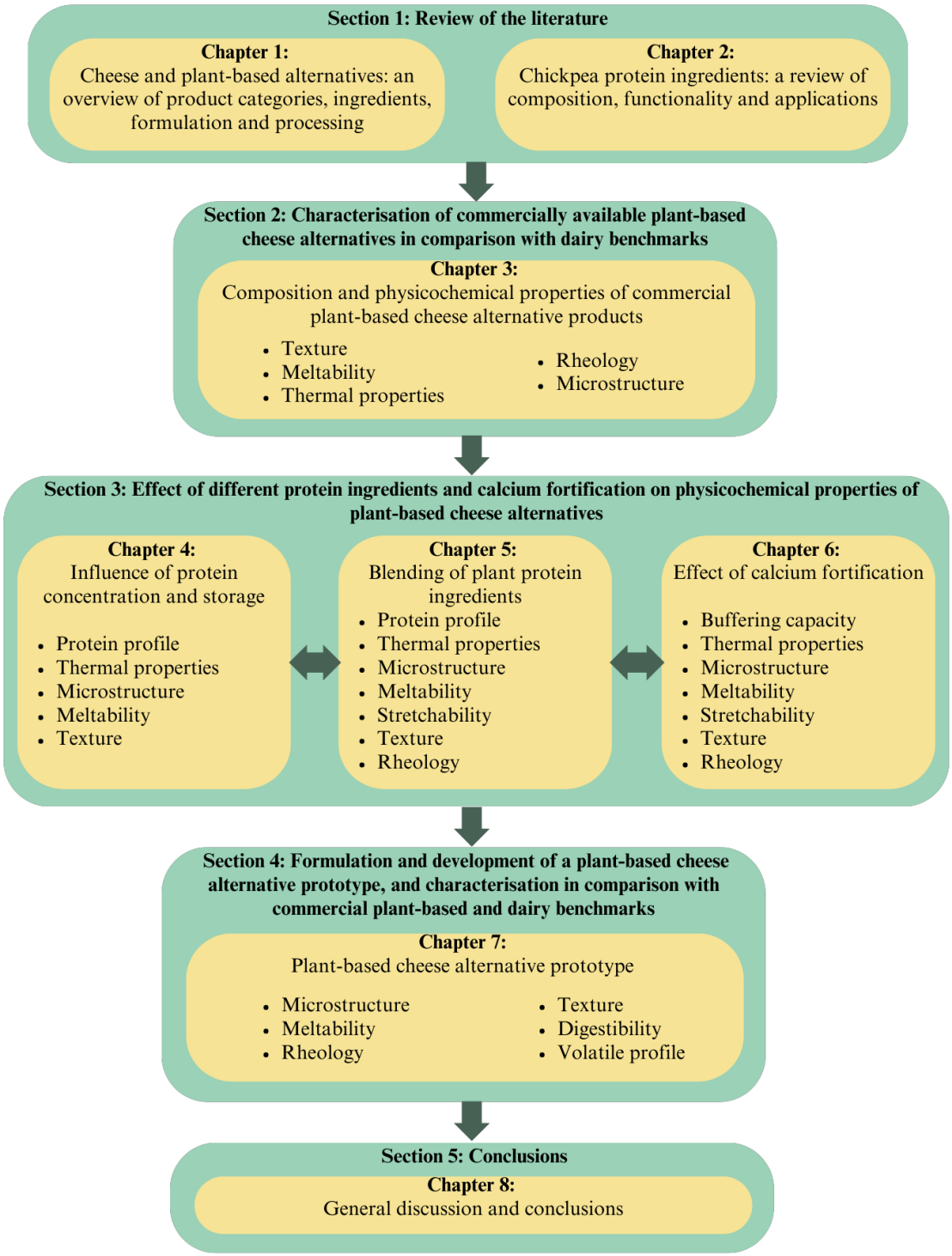
The specific objectives of the research conducted in this thesis are:

- To review the state of the art regarding the different product categories, ingredients, formulation and processing of cheese and plant-based cheese alternatives.
- To review the state of the art with reference to the composition, functionality and applications of chickpea protein ingredients, as well as methods to enhance protein quality and applications of the co-products resulting from protein extraction and processing.
- To evaluate the composition, structure and physicochemical properties of a number of representative commercially-available plant-based block-style cheese alternative products, and to compare these properties with those of Cheddar and processed cheeses as representative benchmarks.
- To determine the influence of protein concentration on chickpea-based alternatives to cheese, in terms of key quality attributes, such as structure and texture. Moreover, the age-induced changes in such attributes after 1 month of storage were assessed.
- To investigate the combined effects of zein and chickpea protein ingredients,

formulated in binary blends, on the development and physicochemical properties of plant-based cheese alternatives.

- To develop calcium fortification strategies, through the use of different calcium salts, for plant-based cheese alternatives formulated with a binary blend of chickpea protein concentrate and zein protein isolate.
- To formulate a plant-based cheese alternative prototype using a blend of zein and chickpea protein ingredients, and to investigate the physicochemical properties, protein digestibility and volatile profile of same in comparison with two commercially-available plant-based cheese alternatives, dairy processed and Cheddar cheeses.

A schematic representation of the chapters within this thesis, and their interlinkages is presented below.


Section 1: Review of the literature
Chapter 1:
Cheese and plant-based alternatives: an overview of product categories, ingredients, formulation and processing
Chapter 2:
Chickpea protein ingredients: a review of composition, functionality and applications
Section 2: Characterisation of commercially available plant-based cheese alternatives in comparison with dairy benchmarks
Chapter 3:
Composition and physicochemical properties of commercial plant-based cheese alternative products
Texture
Meltability
Thermal properties
Rheology
Microstructure
Section 3: Effect of different protein ingredients and calcium fortification on physicochemical properties of plant-based cheese alternatives
Chapter 4:
Influence of protein concentration and storage
Protein profile
Thermal properties
Microstructure
Meltability
Texture
Chapter 5:
Blending of plant protein ingredients
Protein profile
Thermal properties
Microstructure
Meltability
Stretchability
Texture
Rheology
Chapter 6:
Effect of calcium fortification
Buffering capacity
Thermal properties
Microstructure
Meltability
Stretchability
Texture
Rheology
Section 4: Formulation and development of a plant-based cheese alternative prototype, and characterisation in comparison with commercial plant-based and dairy benchmarks
Chapter 7:
Plant-based cheese alternative prototype
Microstructure
Meltability
Rheology
Texture
Digestibility
Volatile profile
Section 5: Conclusions
Chapter 8:
General discussion and conclusions

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Chapter 1

Cheese and plant-based alternatives: An overview of product categories, ingredients, formulation and processing

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Declaration: This chapter was written by author Nadia Grasso and reviewed by all co-authors.

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1.1 Introduction

"Each sort of cheese reveals a pasture of a different green, under a different sky." (Italo Calvino)

Cheese represents an important food product in many cultures, with a long history of production and consumption. Historically, the primary objective of cheesemaking was to conserve the principal constituents of milk; a great variety of cheeses are produced around the world from the same types of raw materials (usually milk, lactic acid bacteria, coagulant and salt) (Fox & McSweeney, 2004; McSweeney *et al*., 2004). Dairy production systems, such as the cheese sector, have rapidly intensified over the past several decades, leading to high productivity, which enhances overall economic performance, but is also associated with social and environmental challenges (Clay *et al*., 2020). Furthermore, the offer of food products is expanding, leading consumers to consider numerous, some relatively new, factors when purchasing their food (Tso *et al*., 2020). Thus, drivers such as food intolerances, environmental sustainability, social trends, health and animal welfare considerations, have been reported in the literature as enhancing consumer interest in plant-based foods (Aschemann-Witzel *et al*., 2020; Grossmann & McClements, 2021). As a result, the plant-based cheese sector is growing and household penetration of this category is increasing as more consumers try plant-based cheese; the number of households purchasing plant-based cheese in the US increased by 20% between 2020 and 2021 (Good Food Institute & SPINS, 2021). Different raw materials and formulations can be employed in the production of plant-based cheese alternatives; soy is used worldwide to produce various plant-based products and modern soy-based cheese alternatives are generally made from soymilk. Other raw materials include nuts, such

as cashews, macadamias and almonds (Tabanelli *et al*., 2018). Most commercially-available plant-based cheese alternatives rely on starch and coconut oil as their principal raw materials to confer selected functionality, often attempting to match the physicochemical characteristics of milk protein and fat (Mattice & Marangoni, 2020a; Grasso *et al*., 2021). However, these products often have low protein and high saturated fat contents and may not represent a healthy choice compared to dairy-based products (Clegg *et al*., 2021). To formulate plant-based alternatives to cheese with improved nutritional profiles and low environmental impact, researchers are currently investigating the suitability of plant protein ingredients (Mattice & Marangoni, 2020b; Ferawati *et al*., 2021; Grossmann & McClements, 2021; Mefleh *et al*., 2021). However, in addition to its important nutritional contributions, dairy protein provides cheese products with unique structural, textural and sensory properties and the replication of such properties using plant proteins is challenging and poorly understood (Grossmann & McClements, 2021; Short *et al*., 2021). An overview of the different product categories, ingredients, formulation and processing of cheese and plant-based cheese alternatives is presented in this review.

1.2 Cheese

Cheese is the name used to identify a group of fermented milk-based food products, produced around the world in a great variety of flavours, textures and forms (Fox *et al*., 2017b). The term "cheese", as well as milk, butter or yoghurt, is exclusively used for dairy products and cannot be used commercially to identify alternatives to such products (Leialohilani & de Boer, 2020). It is commonly believed that cheese evolved about 8000 years ago in the *"Fertile Crescent"* region, i.e., between the Tigris and Euphrates rivers, through what is now southern Turkey to the Mediterranean coast (Fox & McSweeney, 2004). Cheese manufacture accompanied the spread of civilization throughout the Middle East, Egypt, Greece and Rome. There are several references in the Old Testament to cheese, on the walls of tombs of Ancient Egypt and in classical Greek literature (Fox *et al*., 2017b). Cheese is a versatile and convenient food product, being consumed as it is, without preparation, as a condiment or as a component of various cooked dishes; an estimated 40% of cheese is used as an ingredient or component of other foods (Fox *et al*., 2017a).

Many dairy products are biologically, biochemically, chemically and physically stable; on the other hand, cheese is the most diverse group of dairy products and is biologically and biochemically dynamic, and, consequently, intrinsically unstable, and therefore, of particular scientific interest (Fox & McSweeney, 2004). Cheese quality is defined by several aspects, such as its physicochemical properties, sensory attributes, characteristics of use, and nutritional properties (Lamichhane *et al*., 2018). Many factors can influence cheese quality, with the study of such involving a wide range of scientific disciplines (e.g., chemistry and biochemistry, rheology, nutrition) (Fox & McSweeney, 2004).

1.2.1 Production volumes and value

Cheese consumption varies widely between countries, and is driven by its positive dietary image, convenience and flexibility in use, in addition to the great diversity of flavours and textures available to consumers (Fox *et al*., 2017b). While cheese manufacture is practiced worldwide, Europe displays the greatest consumption, production, and export of cheese (Fox *et al*., 2017b; USDA, 2021). In particular, Europe and North America have the highest levels of cheese consumption globally, with per capita consumption expected to increase further in the next few years. Moreover, cheese consumption will continue to grow where it was not traditionally part of the national diet (e.g., South East Asian countries) and where urbanisation and increasing income have resulted in more away-from-home eating (OECD/FAO, 2021). Between 2000 and 2020, production of cheese made from whole cow's milk increased worldwide, from ~13 to almost 20 million tons (FAO, 2020). In Europe, in 2023 cheese production is expected to grow by 1.5% to reach 10.6 million tons, while in the US, production is expected to reach about 6.5 million tons in 2023 (USDA, 2022). Over the next 10 years, cheese is estimated to account for almost two-thirds of the increase in dairy protein availability in high-income countries, which mainly consume processed dairy products (OECD/FAO, 2021). Furthermore, it is expected that the European Union's share in world cheese exports will reach ~46% by 2030, while the United Kingdom, Russia, Japan, the European Union, and Saudi Arabia are projected to be the top five cheese importers in 2030 (OECD/FAO, 2021). While production of cheese has increased in Asia over the last 20 years, production during this period amounted to just 3.2% of global cheese production (FAO, 2020). However, it is projected that between 2021 and 2030, cheese production in Asia will increase further by 2.12% (OECD/FAO, 2021). This scenario of increasing production and

consumption of cheese, together with a rapidly expanding global population, represents a sustainable supply-demand challenge for future generations and provides the plant-based cheese sector with an opportunity to meet the growing demand.

1.2.2 Manufacturing approaches and cheese products

Over the years, several schemes for classification of cheese have been proposed and used, to help consumers, retailers and cheese technologists. Unfortunately, there is no universally approved classification scheme, and the criteria normally used to categorise the large varieties of cheeses are based on the coagulating agent (i.e., rennet or acid), on the texture/moisture content (i.e., very hard, hard, semi-hard, semi-soft, soft), on the degree of freshness/maturation, or on the microflora (i.e., internal bacterial, surface/smear bacterial, internal or surface mould, propionic acid bacteria) (Fox *et al*., 2017c). Manufacturing approaches can vary according to the characteristics of the final product; however, the processes used for producing cheese can be summarised in a few steps: selection and standardisation of milk, acidification, coagulation, dehydration of the coagulum, curd forming and ripening (for most varieties) (Fox & McSweeney, 2004). In the following sections a description of the major families of cheeses is reported, following the classification scheme previously reported by Fox *et al*. (2017c) (Fig. 1.1).

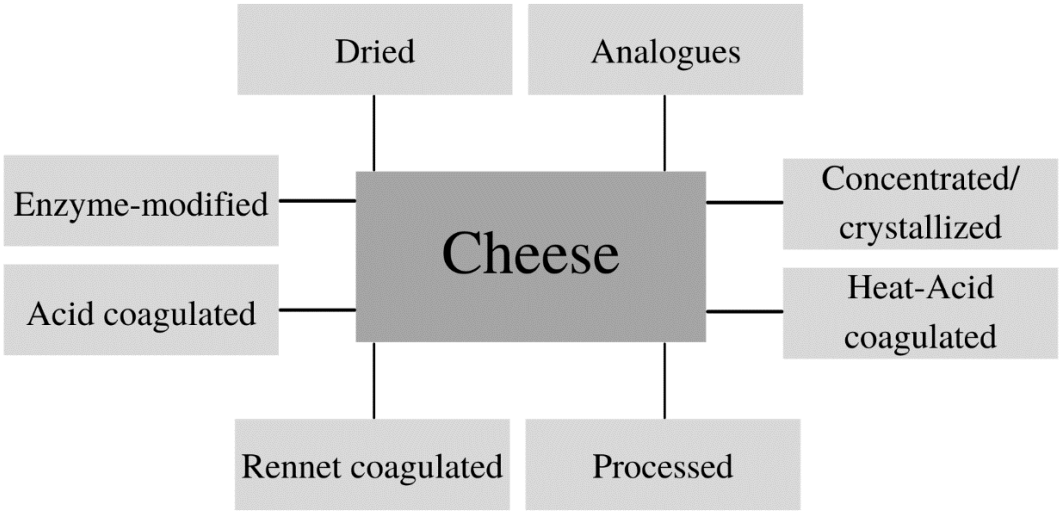


Figure 1.1. Classification of the major families of cheeses.

1.2.2.1 Rennet coagulated cheeses

Rennet coagulated cheeses account for ~75% of total world cheese production and need to be further classified due to the great diversity of products developed using rennet (Fox *et al*., 2017c). These cheeses are usually defined as natural cheeses, to differentiate them from processed cheese. The addition of the enzyme chymosin, or rennet, to prewarmed milk, leads to a fast clotting reaction, due to destabilisation of the colloidal casein-calcium phosphate particles (i.e., micelles) in milk. Indeed, casein micelles are stable in milk due to steric effects provided by their surface polyelectrolyte layer formed by the C-terminal regions of κ-casein. The proteolytic action of chymosin specifically hydrolyses the peptide bond in κ-casein that removes these C-terminal regions. The *para*-casein micelles come within close proximity of one another and, in the presence of ionic calcium, begin to aggregate via hydrophobic interactions, to eventually form a particulate gel with entrapped serum and fat globules (Kethireddipalli & Hill, 2015).

Depending on the manufacturing technology, the rennet-coagulated cheeses category has been divided by Fox *et al*. (2017c), in internal bacterially-ripened, which includes the extra hard, hard and semi hard varieties, but also cheeses with eyes (i.e., Swiss and Dutch types), high salt varieties and pasta filata cheeses; another group is the mould-ripened cheeses, which includes the surface mould and internal mould varieties; finally, the last group is the surface-ripened cheeses (Fig. 1.2). The internal bacterially-ripened group is vast and very diverse, and includes many varieties produced on a large industrial scale (e.g., Parmigiano Reggiano, Cheddar, Emmental, Mozzarella). For the cheeses of this group, the ripening process depends on agents originating from the milk (i.e., plasmin and other indigenous milk enzymes), the rennet (i.e., chymosin and/or other proteinases and, in certain cases, lipases), and the internal bacterial microflora (starter and non-starter bacteria) (Fox *et al*., 2017c).

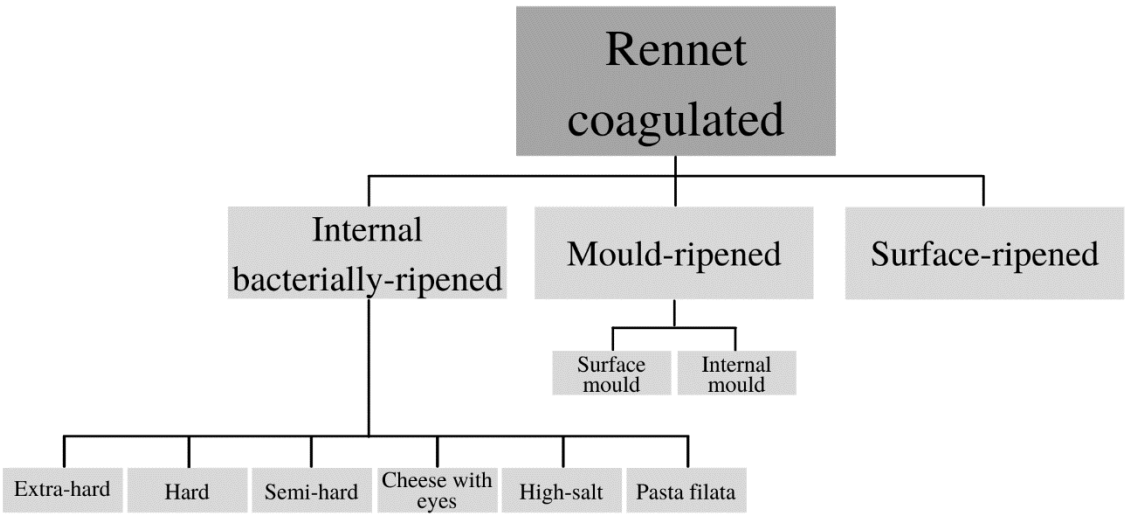


Figure 1.2. Rennet coagulated cheese types and subcategories.

Within this category, Cheddar cheese, originated from England, is one of the most important cheese varieties; it is made from pasteurized cow's milk and the typical steps for production are shown in Figure 1.3.

The mould-ripened group is composed of cheeses on which moulds grow during ripening; for the surface mould-ripened cheeses, the mould (*Penicillium camemberti*) grows on the surface, while for the internal mould-ripened (also known as blue-veined), *Penicillium roqueforti* grows in fractures within the cheese matrix. Surface ripened cheeses are ripened with a mixed surface microflora which often forms a red-orange smear, with this group representing the most heterogeneous sub-group of rennet-coagulated cheeses. Manufacture of these cheeses involves the use of mesophilic starter cultures for most varieties or a thermophilic starter for Gruyère and similar cheeses, and usually incorporates a brine-salting step (Fox *et al*., 2017c).

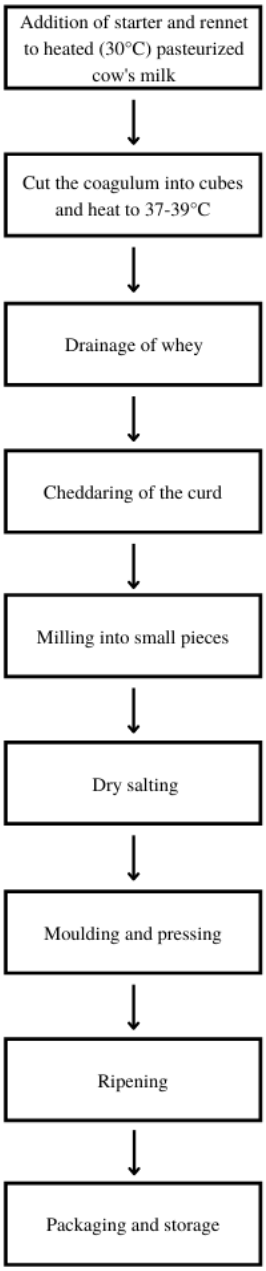


Figure 1.3. Typical process flow diagram for Cheddar cheese production.

1.2.2.2 Acid coagulated cheeses

The acid-coagulated cheeses are produced by acidification of milk or cream, generally using mesophilic starter cultures or direct acidification, with addition of food-grade acid (e.g., lactic, citric) or an acidogen, such as glucono-δ-lactone (GDL). Coagulation in the production of rennet coagulated cheeses is induced by the action of

rennet at pH 6.4–6.6, while for acid coagulated cheeses, coagulation occurs close to the isoelectric pH of casein (i.e., pH 4.6). These cheeses have high-moisture content and are usually consumed soon after manufacture (Fox *et al*., 2017c). The acid coagulated cheese category includes numerous varieties, with examples including Quark and Cream cheese, and for some varieties (e.g., Cottage cheese) rennet is added in small quantities to increase firmness. Acidification promotes two main physicochemical changes: a reduction of the negative surface charge on the casein micelles and solubilisation of micellar calcium phosphate, leading to a new stable state of casein in the form of a gel network (Schulz-Collins & Senge, 2004).

1.2.2.3 Heat-acid coagulated cheeses

A combination of heat and acid coagulation can be used to obtain a group of cheeses. One of the more well known varieties in this category is Ricotta, an Italian cheese originally produced from whey, with a small addition of milk, by heat-induced coagulation and addition of acidifying agents, such as lemon juice or vinegar. Other examples of heat-acid coagulated cheeses are Mascarpone, Queso Blanco and Paneer (Farkye, 2004). Nowadays, Ricotta is produced from full-fat or skimmed milk and acidification is achieved by addition of large amounts of starter or citric or acetic acid, followed by heat treatment by direct steam injection, salt addition and then shaping in moulds and cooling with ice (Fox *et al*., 2017c).

1.2.2.4 Concentrated/crystallized cheeses

Sweet whey is the main starting material used for production of concentrated/crystallized cheeses, while acid whey may be used for some varieties (Fox *et al*., 2017c). Skim milk or cream is sometimes added to the whey to give a

lighter-coloured product. Several Scandinavian cheeses, with a smooth creamy body and a sweet caramel-like flavour, are grouped in this cheese category (e.g., Mysost, Primost, Mysuostur). Two concentration steps bring the whey/cream mixture to >80% total solids and the resulting product is heated at high temperature (~95°C) to encourage the Maillard reaction. The concentrate is then kneaded and cooled rapidly to obtain crystal nucleation of the poorly soluble lactose and, thus, ensure a smooth texture in the final product (Jelen, 1992).

1.2.2.5 Enzyme-modified cheeses

Enzyme-modified cheeses are mainly used as flavouring ingredients in cheese products (e.g., pasteurized processed cheese products, imitation cheeses, cheese powders, ready-prepared meals), and variants of many natural cheeses, such as Cheddar, Blue cheese or Emmental are available on the market (Guinee & Kilcawley, 2004). Manufacture of these cheeses involves the addition of exogenous enzymes (i.e., proteases, peptidases, lipases and/or esterases) and/or lactic acid bacterial cultures to cheese curd, and incubation under controlled conditions to achieve a paste with a predictable flavour profile and intensity; the paste can be dried to obtain an enzyme-modified cheese powder (Section 1.2.2.6) with longer shelf-life, more suitable for dry blending with other ingredients (Guinee & Kilcawley, 2004).

1.2.2.6 Dried cheeses

Dried cheese is an ingredient used for an extensive range of food preparations (e.g., bakery products, ready-to-eat meals, soups, cheese dips, processed and analogue cheeses) as flavouring agent and/or nutritional supplement (Guinee & Kilcawley, 2004). The same authors classified dehydrated cheeses in four main sub-categories

depending on the ingredient used: (I) dried grated cheese (e.g., Parmesan); (II) natural cheese powders, made using natural cheeses, emulsifying salts and, sometimes, natural cheese flavours; (III) extended cheese powders, which incorporate natural cheese and other ingredients such as dairy ingredients, starches, maltodextrins, flavours, flavour enhancers and/or colours; (IV) dried enzyme-modified cheeses. Depending on the category, different approaches can be used for the production of dried cheeses; for example, dried grated cheeses are normally produced by grating and drying the cheese, usually using a fluidised bed drier and exposure to low humidity air (15–20% relative humidity) at an inlet temperature <30°C. Another example is the manufacture of cheese powders, which involves production of a pasteurized processed cheese slurry (40–45% dry matter) followed by spray drying (Guinee & Kilcawley, 2004). Moreover, the structure and hydration properties of cheese powders are affected by raw material, emulsifying salts, and drying technology employed (da Silva *et al*., 2017). Due to its convenience and flexibility in use, dried cheese allows development of numerous cheese-based snacks, which are available on the market in different formats.

1.2.2.7 Processed cheeses

Processed cheese originated from a desire to extend the shelf-life of natural cheese and/or to develop a cheese with milder taste or where greater stability was required. There are various types of processed cheeses, which differ depending on national legislation. The core, common steps for manufacture of processed cheeses include melting and heating blends of natural cheeses (e.g., different types, varying degrees of maturity, and cheese 're-work'), addition of emulsifying salts (e.g., sodium citrates, sodium orthophosphates, sodium pyrophosphates), agitation to produce a

homogeneous mixture, packaging and cooling (Tamime, 2011). The salts are not emulsifiers *per se*; however, they promote, with the support of heat and shear, numerous physicochemical changes within the cheese blend, resulting in hydration of the insoluble aggregated *para*-casein and its conversion to an active emulsifying agent (Guinee *et al*., 2004). Specifically, the emulsifying salts, added at a level of 1–3% (w/w), solubilise the protein of the cheese blend, which binds the free water and emulsifies the free fat released during processing (heating and shearing). The manufacture of processed cheese involves deconstruction of the casein network of natural cheese, representing a structural transformation from a concentrated fat-filled gel network to a concentrated oil-in-water emulsion (Fox *et al*., 2017d). Processed cheese products can be used in many applications, in raw or heated form, with the suitability for particular applications being dependent on textural and flavour characteristics of the unheated cheese and the cooking properties of the heated cheese (Guinee *et al*., 2004).

1.2.2.8 Cheese analogues

Cheese analogues are made from mixtures of dairy and/or non-dairy proteins and fat/oils, and are defined as products which are intended to partly or wholly substitute or imitate cheese products (Fox *et al*., 2017d). These imitation products have been developed to meet demand in fast food outlets (e.g., pizza), by the catering trade, ready cooked foods, in formulated foods and in school lunch programmes (Tamime, 2011). Depending on the source of fat and/or protein components (e.g., dairy or vegetable), cheese analogues can be categorized as dairy, partial-dairy or non-dairy, and, similar to processed cheeses, can be manufactured by blending various edible oils/fats, proteins, other ingredients and water into a smooth homogeneous blend with

the aid of heat, mechanical shear and emulsifying salts (Guinee *et al*., 2004; Fox *et al*., 2017d). The most common cheese analogues are partial-dairy analogues, in which the fat is mainly vegetable oil (e.g., soy oil, palm oil, rapeseed or their hydrogenated equivalents), and the protein is dairy-based, usually rennet casein and/or caseinate (Fox *et al*., 2017d). While it is possible to formulate cheese analogues using mixtures of dairy and plant protein ingredients (i.e., hybrid cheese analogue products) (Tamime, 2011), the complete substitution of milk fat and protein with plant materials leads to the development of plant-based cheeses, which are discussed in the following sections.

1.3 Plant-based cheeses

1.3.1 Introduction to plant-based cheeses

The global population is growing and is expected to reach almost 10 billion people by 2050; however, food systems are responsible for between 21 and 37% of all net anthropogenic greenhouse gas emissions, thus, the need to expand food supply for the increasing global population using current production systems represents a challenge (IPCC, 2019). The demand for meat (e.g., beef, pork and poultry) and dairy products (e.g., cheese, milk and butter) is growing; however, these products represent the most burdensome foodstuffs in most of the environmental impact categories (Notarnicola *et al*., 2017). The need to meet the demand for such products provides opportunities to formulate new foods. With a variety of food products available on the market, an increasing consumption of plant-based food is observed globally, with a growth in sales of 27% in the US in 2020 (SPINS & Good Food Institute, 2020). Even with the interest in plant-based cheese expanding for both the food industry and scientific researchers, there is still no legal definition for these products; however, Grossmann & McClements (2021) have recently provided an exhaustive definition that aligns with common perception of such products: "Plant-based cheese is an edible material prepared from plant ingredients that is designed to have a similar appearance, texture, and flavour to animal-based cheeses."

A lack of studies on the science underpinning development of plant-based cheese products is observed in the scientific literature; however, as shown in the next sections, in more recent years researchers have begun to investigate the potential of plant-derived ingredients for the formulation of such products. Nutritional properties of plant-based cheese, environmental impacts, structure, texture, melting properties and flavour are key aspects that need to be deepened in future research to develop high-

quality nutritionally balanced and viable alternatives to traditional cheese.

Plant-based cheeses can be made from different raw materials and using different processes; in this review, these products are classified, according to the main raw materials employed for their development, as formulated using polysaccharides (I), whole plant materials (II) or plant protein ingredients (III) (Fig. 1.4). Major differences in macronutrient composition can be observed depending on the approach used, even when starting with similar raw materials (Fig. 1.4). Fat source is also an important aspect of plant-based cheeses, since it strongly influences their texture, meltability, flavour and nutritional characteristics. In existing benchmark products, coconut oil is the most widely used fat source, because of its solid physical state at room temperature, the ability to melt with increasing temperatures and low cost (Mattice & Marangoni, 2020b; Grossmann & McClements, 2021). In some commercial formulations, softer oils (e.g., sunflower, canola, palm) are blended with coconut oil. Furthermore, thickeners, such as gums (guar, arabic, xanthan) and carrageenan, are often used in plant-based cheese applications in combination with the other ingredients, due to their ability to influence texture of such products (Foster & Wolf, 2011). Regarding the structure of plant-based cheeses (mostly gel-type), differences are observed according to the processing approaches and the ingredients employed for formulation. Indeed, these products can be considered composite gels, which can be further divided into filled and bicontinuous gels. Specifically, when particles are dispersed in a continuous gel matrix the result is a filled gel, with the filler being either bound or unbound to the gel matrix, while two interconnected phases with no dispersed phase are known as bicontinuous gels (Lyu *et al*., 2022). In the next sections, a description of the raw materials employed for formulation, processing approaches and characteristics of plant-based cheeses are presented.

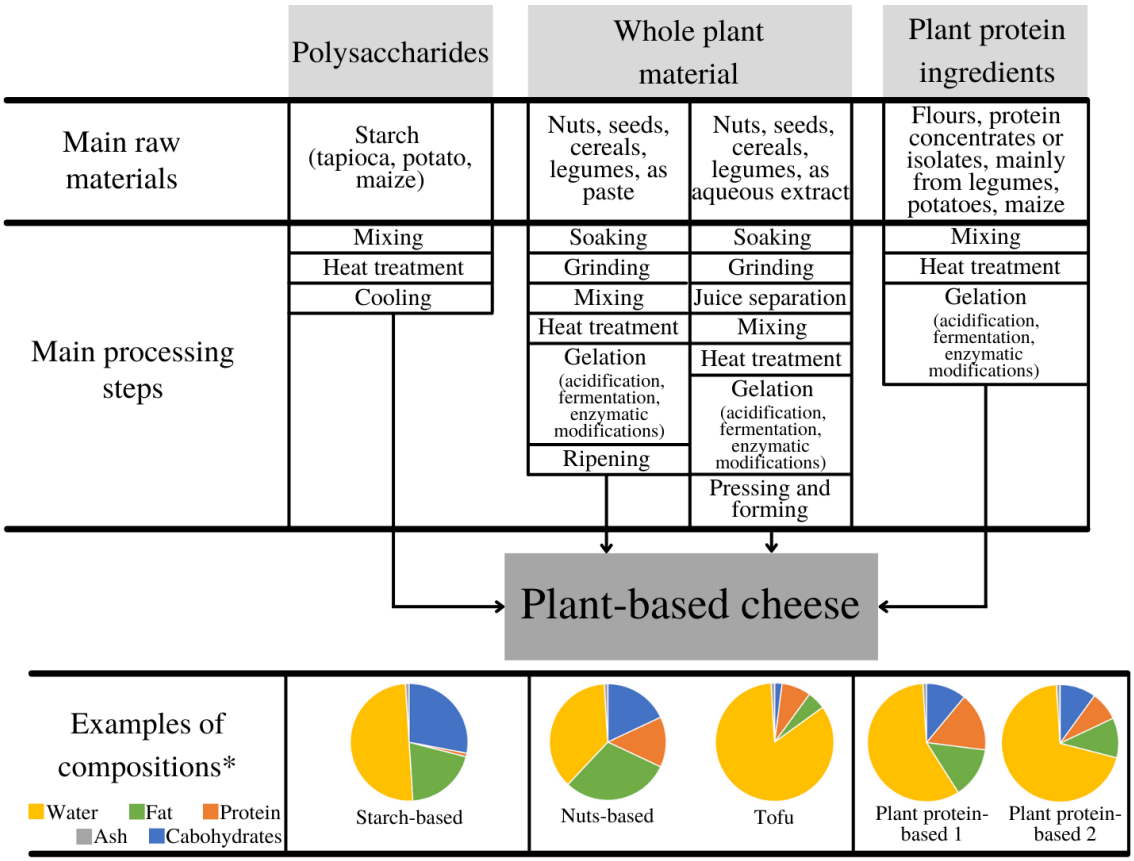


Figure 1.4. Types, ingredients, and technological approaches used in the formulation and processing of plant-based cheeses, along with examples of macronutrient composition of products derived from such raw materials and processing approaches. *Composition of represent examples of plant-based cheeses for each category.

1.3.2 Polysaccharide-based cheeses

Most of the commercially-available plant-based cheese products are based on starch and coconut oil (Grasso *et al.*, 2021). Starches are used in plant-based cheeses because of their ability to form gels upon heating and/or cooling, entrapping other ingredients within the polysaccharide network formed and, thus, building structure. Starch is often used in combination with other hydrocolloids, which are added to enhance the textural properties of the products. The plant-based cheeses produced using starch as main ingredient can have similar texture to some processed cheeses, as

the starch enables formation of a solid network; however, their texture differs significantly to traditional cheeses such as Cheddar (Grasso *et al*., 2021). Indeed, as shown in Figures 1.3 and 1.5, other than the ingredients, the process used to produce starch-based cheeses is very different to that of traditional cheese. Moreover, these products generally have low protein and high saturated fat, salt and carbohydrate contents, often being considered nutritionally inferior to dairy cheese (Clegg *et al*., 2021). Some functional properties of starch-based cheeses limit their application performance; for example, their poor ability to melt at high temperature represents a limitation for certain applications of these products (Grasso *et al*., 2021; Lyu *et al*., 2022).

Starch can be extracted from a variety of plants (e.g., potato, maize, cassava, rice, pea), and many differences in the starch characteristics and functional properties can be observed according to the source (e.g., amylose-to-amylopectin ratio, granule size). Gelatinisation is the process that occurs when a suspension of starch granules is heated in excess water, during which the starch granules swell and the amylopectin double helical structures are lost. On heating to greater than a particular temperature, the starch granules are ruptured, resulting in the release of amylose (Taggart & Mitchell, 2009). Upon cooling, the amylose forms a structured gel due to the formation of hydrogen bonds, which is known as retrogradation or setback; retrogradation of amylose is rapid whereas retrogradation of amylopectin is a slow process (Taggart & Mitchell, 2009). Depending on the processing conditions during starch gelatinisation (i.e., temperature >100°C and/or high shearing) the structure of the gel formed can change from a particle-type gel, with starch granules dispersed in an amylose network, to a polymer gel, with a complete polymer network and no starch granules (Keetels *et al*., 1996).

The production of this type of plant-based cheese product is generally based on a multi-step process (Fig. 1.5), initiated by mixing the starch with fat, other hydrocolloids (e.g., carrageenan, guar gum), water, flavourings, lactic acid and salts. This is normally followed by a heating step, which leads to gelatinisation of the starch, and followed by retrogradation during cooling, with formation of an emulsion gel (also known as emulsion-filled gel), that entraps the other ingredients (Grossmann & McClements, 2021). In these types of products, the amylose-to-amylopectin ratio of starch plays an important role as it influences the gelatinisation temperature (which can vary between 60-80°C), solubility, viscosity, gelation and retrogradation properties (Schirmer *et al*., 2013). Moreover, amylose is responsible for formation of strong irreversible gels, while amylopectin forms weak reversible gels, as a result of its low retrogradation rate (Schirmer *et al*., 2015). Due to their low tendency for retrogradation and high amylopectin content, which enables the formation of a softer texture, waxy starches (e.g., waxy potato or rice starches) are often used in plant-based cheese formulations (Grossmann & McClements, 2021). Due to its ability to form a clear, stretchy and cohesive paste with mild flavour, tapioca starch is used in a number of food industry applications, especially as a thickening and gelling agent (Singh *et al*., 2017). Furthermore, similar to waxy starches, tapioca starch has a low tendency to retrograde which influences the texture of the final product (Jackson, 2003). These characteristics make tapioca starch very suitable for use in plant-based cheese applications; indeed, it can be found as an ingredient in many commercially-available products, usually combined with other starches (e.g., potato, maize), and has been successfully employed in previous research to develop plant-based cheese prototypes (Mattice & Marangoni, 2020b). Often, the starches used in plant-based cheeses are modified to improve specific functional properties, with examples of such

modifications including cross-linking, heat treatment and enzymatic modifications. The required functional characteristics (e.g., heat tolerance, texture, adhesion, solubility) can be acquired using different modification approaches or combinations of same, offering the opportunity to tailor the functionality of plant-based cheese for selected applications (Obadi & Xu, 2021).

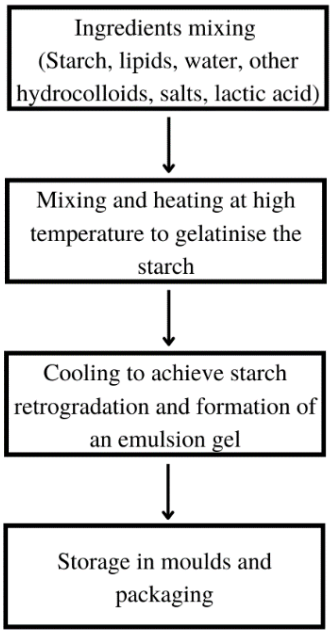


Figure 1.5. Diagrammatic representation of a typical process flow for polysaccharide-based cheese production

1.3.3 Whole plant material-based cheeses

Whole plant materials, such as nuts, seeds, cereals, legumes or aqueous extracts derived from same, can be used as starting materials to produce plant-based cheeses with fundamental structures of emulsion-filled gels. Depending on the chosen starting material, different approaches can be used for production, such as, heat treatments, fermentation, acidification, enzymatic treatments, or combinations thereof. The resultant products can differ significantly in terms of composition depending on the choices made for formulation (Fig. 1.4). Soy milk is a commonly used starting material for plant-based cheese production; due to its long history of consumption, soy is probably the best known source material to produce cheese-like products (e.g., tofu). Soft, hard or semi-hard soy-based cheeses produced from soy milk or soybeans, have been extensively studied and reported in the scientific literature; however, there are just a few soy-based products available commercially, probably due to the beany flavour associated with soybeans (Short *et al*., 2021). On the other hand, many fermented nut-based cheeses from different manufactures are available on the market, and coconut oil and hydrocolloids (e.g., carrageenan, guar gum) are often added to some of these formulations to improve texture. The nut-based products generally have higher protein content than starch-based cheeses or tofu (Fig. 1.4), but lower than traditional cheese; moreover, nuts are good sources of micronutrients and have low saturated fat contents, generally having a positive impact on health (Oyeyinka *et al*., 2019; Clegg *et al*., 2021). Legumes, as well as cereals, can also be employed for development of plant-based cheese, and are considered an important staple food and a source of plant protein, fat and carbohydrate, as well as bioactive components; furthermore, legumes are cheaper than nuts and seeds, and show strong potential for development of dairy alternatives (Mefleh *et al*., 2021).

Starting with pre-treatments (e.g., soaking, grinding) of nuts, seeds, cereals (e.g., oat and rice) or legumes, plant-based milk or a paste can be prepared, and either can be used to produce plant-based cheese. The plant-based milk is obtained by the removal of insoluble particles (e.g., larger protein fragments or insoluble polysaccharides), whereas the paste is produced from the whole nut, seed, legume or cereal, without separating any compounds, and thus insoluble materials and particles remain within the plant-based cheese, usually without whey separation (Grossmann & McClements, 2021). Protein denaturation is commonly achieved using heating to induce gel formation through aggregation of globular proteins. Moreover, such heat treatments contribute to inactivation of anti-nutritional factors such as enzyme inhibitors, and influence other relevant functional properties of protein (e.g., increase water and oil holding capacity) (Boye *et al*., 2010). Addition of salts or acidification to the isoelectric point of proteins further enhance the formation of a gel. Such approaches influence relevant physicochemical properties of gels (e.g., texture and water holding capacity), by modulating not only the net charge of the proteins, but also the interactions between protein molecules, stability of protein structure and dissociation of subunit polypeptides (Day, 2013). Curd formation starting from legumes (e.g., soy, chickpea, mung bean, cowpea, peanuts, winged bean) and with the use of thermal processing, followed by calcium or other salt addition and/or lowering the pH have been extensively studied in the literature (Lu *et al*., 1980; Kantha *et al*., 1983; Mohamed *et al*., 1989; Chung *et al*., 2011). The approaches used to develop legume-based curds are broadly similar to the process used for tofu production; briefly, the legume seeds are soaked and homogenised with water, after which the slurry is filtered to obtain the legume milk. After the heat treatment step (typically 15 min at 95°C), a coagulant (e.g., calcium sulphate, calcium chloride or magnesium chloride)

is added to the legume milk and pH adjusted, and the curds are typically pressed in cheese/tofu moulds to obtain the final product (Mohamed *et al*., 1989). The characteristics of the curds can vary significantly based on the legume used, unit operations, process parameters, and type of coagulant, as well as coagulant and protein concentrations.

Cereal-based milk can also be used for production of plant-based cheeses, with a few examples of oat and rice-based cheeses available on the market. The production of such products is normally based on formation of the gel matrix achieved by mixing the juice extract with gelling ingredients, similar to the approaches used for polysaccharide-based cheeses (e.g., starch or other thickeners), and using heat treatments, acidification, enzymatic crosslinking, or combinations of these approaches (Grossmann & McClements, 2021). Cashew nuts are one of the most used sources for production of fermented plant-based cheeses; after soaking, the nuts are blended with other ingredients and a starter culture, generally lactic acid bacteria (LAB), is added to the mixture. Fermentation is usually carried out for several hours to achieve desirable textural characteristics and flavour profile (Tabanelli *et al*., 2018). This approach allows production of different types of cheeses (e.g., soft, brie, blue, hard-mature types) depending on the processing conditions, fermentation time, and bacteria used (Chen *et al*., 2020). Indeed, the choice of the starter culture can vary according to the preferred texture of the final product, with mainly combinations of different mesophilic LAB or, sometimes, fungi being used (Pua *et al*., 2022). However, currently, in the scientific literature, very little is known about the effects of microbial biodiversity in plant-based cheeses (Harper *et al*., 2022). Soy milk can also be fermented with addition of LAB (e.g., *Streptococcus thermophilus*, *Lactobacillus fermentum*) to obtain a curd, usually following a heat treatment, as for the curd

formation approach previously described (Chumchuere *et al*., 2000). Other seeds, legumes and cereal milks can also be fermented to produce beverages with improved nutritional and sensory profiles (Tangyu *et al*., 2019); however, further studies on fermented plant-based beverages from such starting materials are necessary to understand their potential in plant-based cheese applications.

Enzymes can be used to achieve coagulation of plant-based milk in combination with the approaches described earlier in this section (e.g., thermal treatments, acidification), and soy milk has been studied as starting material (Murata *et al*., 1987; Sánchez-Muñoz *et al*., 2017). Through the use of cross-linking enzymes as well as proteases, sol-gel transition can be achieved starting with a legume milk; however, such approaches have only been investigated using soy as raw material. Additional research with different substrates is necessary to determine the potential of enzyme modifications of whole plant materials for the development of plant-based cheese.

1.3.4 Plant protein ingredient-based cheeses

Plant protein ingredients (e.g., flours, protein concentrates and protein isolates) show strong potential in the development of plant-based cheese with improved nutritional profile and functionality compared to the approaches described in the previous sections. However, the use of plant protein ingredients for this application is challenging and more research is needed to achieve plant-based cheeses that are comparable to traditional cheese (Mattice & Marangoni, 2020b). Functional properties of plant proteins, such as water or oil holding capacity, emulsifying and gelling properties, are very important in developing cheese-like products, meaning that selection of the ingredients, as well as approaches to modify same, are critical.

Legumes are a common source material used for extraction of protein, and it is

possible to formulate plant-based cheeses therefrom with higher protein contents compared to the starch or whole plant material-based cheese making approaches; however, within the same group, variations in composition are observed based on the approach and starting materials selected for the development of these products (Fig. 1.4). Legumes are rich in nutrients and protein concentrates and isolates from legumes are generally milder in colour, flavour, odour, and have lower contents of antinutritional components compared to whole seeds, presenting potential for application in plant-based cheese production (Mefleh *et al*., 2021). Cereal, pseudocereal or potato protein fractions can also be used for development of plant-based cheeses; a combination of different protein sources in the formulation of such products could lead to improved nutritional characteristics in terms of amino acid profile. However, more research on the use and potential of combinations of protein ingredients enriched from different sources is needed (Jiménez-Munoz *et al*., 2021).

Protein flours, concentrates or isolates can be extracted from plant material using different approaches (i.e., wet or dry fractionation methods). Depending on the source, botanical characteristics, extraction method, and pre-treatments, the protein ingredient can behave very differently in plant-based cheese applications and result in products with different characteristics; indeed, all these factors significantly influence the functional properties of the protein ingredients. The main sources of plant protein ingredients employed for production of plant-based cheeses are legumes, potatoes and maize. Legume proteins, mainly globulins, are storage proteins and represent the major fraction of legume proteins, which can be extracted from soy, pea, chickpea, fava bean, lupin, lentil, and some differences in their physicochemical characteristics can be observed depending on the legume type (Day, 2013; Alonso-Miravalles & O'Mahony, 2018; Vogelsang-O'Dwyer *et al*., 2021). Potato proteins have shown

interesting properties, such as emulsifying and gelling properties, and are used to a limited extent in commercial plant-based cheeses. Potato proteins are extracted from the potato waste juice of potato starch manufacture, where the principal protein is patatin, a storage glycoprotein (Løkra & Strætkvern, 2009). Zein is the prolamin extracted from maize, with α-zein being the main fraction. Zein is soluble only in ethanol solutions and recent studies showed softening and increased viscous behaviour of zein with increasing temperature, a rare behaviour for plant-based proteins (Mattice & Marangoni, 2020a). Plant-based cheese made using plant proteins is usually obtained by mixing the protein ingredients with water and fat and heating to a target temperature, with starch and other hydrocolloids often added to build structure and improve texture of such products. Furthermore, enzymatic treatments and/or pH adjustments can also be performed in addition to thermal treatment (Grossmann & McClements, 2021). Enzymatic cross-linking is a promising technique used to obtain a continuous protein network and a solid-like texture starting from plant protein ingredients, such as zein, pea, soy and potato (Zeeb *et al*., 2017; Glusac *et al*., 2018, 2019; Mattice & Marangoni, 2021). Depending on the plant protein source and the processing parameters used for protein extraction, different enzymes can be employed to achieve a desired texture; for example, transglutaminase is commonly used on soy, pea and lupin proteins, while tyrosinase has been shown to be more suitable for zein (Yasir *et al*., 2007; Sun & Arntfield, 2011; Ceresino *et al*., 2021; Mattice & Marangoni, 2021). As for whole legumes or juice extracts from same (Section 1.3.3), plant protein ingredients can also be used for curd production, with the difference of using a protein enriched ingredient as starting material. Peas, soybeans, chickpeas, fava beans, mung beans and lentils, have all been employed for protein extraction and curd preparation; in such applications, protein dispersions are typically heat treated and a coagulant is

added to achieve the desired texture (Gebre-Egziabher & Sumner, 1983; Cai *et al*., 2001, 2002). Plant protein ingredients are promising starting materials for plant-based cheese production, presenting many advantages and potential, and with the support of future research, formulations with improved characteristics can be achieved.

1.4 Conclusion

Cheese is one of the most nutrient dense and globally-accepted food products of all time. The almost infinite number of varieties and its versatility of use drive the diffusion of cheese globally. However, the global population is growing, leading to the need to increase food supplies, representing a concern for the current and future generations and providing the plant-based sector with opportunities to meet the growing food demand. Consumer interest in plant-based food is increasing and, in particular, the plant-based cheese sector is growing, with an increasing household penetration of this category. Over the last few years, researchers have investigated the potential of plant-derived ingredients for the formulation of such products. However, a lack of scientific knowledge underpinning the development of plant-based cheese is evident in the scientific literature.

Plant-based cheese can be made from different raw materials and processes and in this review the most significant innovations in the formulation of these products have been presented. Nutritional characteristics of plant-based cheese, environmental impacts, structure, texture, melting properties and flavour have been identified as key aspects for future research to develop nutrient dense, functional, acceptable alternatives to traditional cheese.

1.5 References

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Chapter 2

Chickpea protein ingredients: a review of composition, functionality and applications

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2.1 Introduction

Chickpea, *Cicer arietinum* L., is an annual pulse of the legume family, grown from herbaceous, pod-producing plants, in areas with a semiarid or temperate climate (Knights & Hobson, 2016; Wallace *et al*., 2016). The types of chickpeas that are produced nowadays are the only domesticated legumes of the *Cicer* genus and are thought to have been cultivated from the wild species, *Cicer reticulatum* (Ladizinsky & Adler, 1976; Sharma *et al*., 2013). The origin of chickpeas can be traced to the Middle East, or the *"Fertile Crescent"* as it was known thousands of years ago. The geographic spread of this legume coincides with trading and emigration from this region to the rest of the world (Tanno & Willcox, 2006; Redden & Berger, 2007). Based on the seed shape, colour and size, chickpeas can be divided into two main categories, *'desi'* and *'kabuli'*; the *'desi'* chickpeas (from the Hindi and Urdu word "native") are small, dark brown and furrowed seeds, produced mainly in semiarid climates, such as Australia, Central America, East Africa and the Indian region (Knights & Hobson, 2016). They are grown as a winter crop, requiring about 16 inches of rain per annum and following the growth of crops that prefer monsoon seasons (Nasir & Sidhu, 2013; Knights & Hobson, 2016). Differentiation of the *'kabuli'* type is thought to have occurred in the Mediterranean region in more recent times compared to the *'desi'* chickpea. The *'kabuli'* chickpeas are characterised by their large seed size, smooth surface and cream colour, and are grown in more temperate climates (Gil *et al*., 1996). In the sixteenth century, the *'kabuli'* variety reached South and Central America, carried by Spanish and Portuguese travellers, where they came to be known by their Spanish name 'garbanzo'. The Mediterranean, Middle East, North Africa and North America are major producers of this type of chickpea (Knights & Hobson, 2016).

Pulses are well known for their soil fertility restoration value due to their ability to fix nitrogen, contributing to productive and sustainable agricultural systems. Indeed, legumes are included in crop rotations as a sustainable approach to reduce nitrogen fertilizer requirements and increase subsequent crop yields; chickpeas have been shown to be particularly effective in improving yields of wheat and other cereals (Danga *et al*., 2009).

Globally, the volumes of *'desi'* and *'kabuli'* types of chickpea now traded are similar; however, there is a generally higher demand for *'kabuli'* chickpeas and particularly for those with large seeds, preferred for direct human consumption (Yadav *et al*., 2007). Historically, Asia is a major producer of chickpeas, followed by Australia, Africa, America and Europe. According to the FAOSTAT data, chickpea production has increased considerably in the period 1980-2018, with global values of about 17.2 million tonnes in 2018. Of these, India produced the largest proportion of chickpeas, with over 11 million tonnes produced in 2018 (FAO, 2020).

Chickpeas are highly nutritious, having high protein, fibre and fat contents, and carbohydrate content lower than that of wheat, as well as containing many bioactive compounds, such as phenolic acid and isoflavones (Rachwa-Rosiak *et al*., 2015). Chickpeas are considered a good source of dietary protein due to their high protein bioavailability, biological value and well-balanced amino acid profile, while being deficient in the sulphur-containing amino acids methionine and cystine (Jukanti *et al*., 2012). Moreover, chickpea proteins show good functional properties, such as solubility, water and oil absorption capacity, emulsifying, foaming and gelling properties, which are strongly dependent on protein profile (e.g., amino acid composition and protein structure) as well as choice of extraction approach and processing parameters (e.g., pH and temperature) (Boye *et al*., 2010b; Day, 2013).

Therefore, chickpeas represent an interesting source of protein for the development of protein-enriched ingredients.

This review will describe the composition, functionality and applications of chickpea protein ingredients, as well as methods to enhance protein quality and applications of the co-products resulting from protein extraction and processing, informing researchers and industries on the potential applications of chickpea protein ingredients in the formulation and manufacture of novel food products.

2.2 Composition of chickpeas

Other than protein, chickpeas contain carbohydrates, fat, minerals, bioactive compounds and antinutrients, which all influence the efficiency of recovery and key quality attributes of chickpea protein ingredients. The nutritional profile of chickpea seeds, and pulses more generally, varies according to the environment, climate, soil nutrition and biology, agronomic practices and stress factors (Shevkani *et al*., 2019). Compositional differences, mainly in the protein content, can be observed between *'desi'* and *'kabuli'* chickpeas (Table 2.1) (Khan *et al*., 1995).

Starch is the main component of the carbohydrate fraction (47.4-66.9%) and accounts for 41.0-50.8% of total carbohydrate in chickpeas (Singh, 1985), with the remaining portion of carbohydrate composed of soluble sugars, crude fibre and dietary fibre. The granular starch structure of chickpeas is referred to as type C, due to its crystalline structure, typical of legumes. *'Kabuli'* chickpeas have slightly higher amylose content compared to *'desi'* chickpeas, both containing more amylopectin than amylose, while cereals generally have less amylose and more amylopectin (Singh *et al*., 2004). Chickpea starch has low glycaemic index, due to its relatively high amylose content and consequent high rate of retrogradation (Kaur & Prasad, 2021). During the

extraction process of protein components in the production of chickpea protein ingredients, starch is partially removed, and the amount of residual starch in the protein ingredient strongly influences its functional properties. Dietary fibre, the portion of carbohydrate that cannot be digested in the small intestine of humans, is an important constituent of chickpeas (18-22 g/100 g), with 10-18 and 4-8 g/100 g of insoluble and soluble fibre, respectively (Tosh & Yada, 2010).

Dietary fibre plays many important roles in gut health; for example, in the large intestine it increases the growth of bacteria, thus having a prebiotic effect, and it reduces the colon transit time and, consequently, the contact time of toxic compounds with colon mucosa (Capuano, 2017). Dehulled chickpeas have lower dietary fibre content than other pulses, due to the dehulling of the chickpea seed coat. *'Desi'* chickpeas have been reported to have higher levels of insoluble dietary fibre compared to *'kabuli'* chickpeas, due to the thickness and fibre content of the seed coat (Rincon *et al*., 1998). Similar to the starch fraction, the residual fibre in chickpea protein ingredients influences the functional properties.

The fat content of chickpeas is higher than other pulses and some cereals, but lower than other oilseed legumes, such as soybeans and groundnut, with values ranging from 3.10 to 5.67% depending on the chickpea type (Table 2.1). The fat in chickpeas is composed of approximately 66% polyunsaturated fatty acids, 19% monounsaturated fatty acids and 15% saturated fatty acids. The most dominant fatty acids include linoleic and oleic acid, and palmitic acid in smaller amounts (51.2 and 61.6%, 32.6 and 22.3% and 9.4 and 9.1% of total fat, for *'kabuli'* and *'desi'*, respectively) (Singh, 1985; Jukanti *et al*., 2012). To enhance protein purity and yield, fat is usually removed during the production of chickpea protein ingredients, normally using solvent extraction.

Other important compounds present in chickpeas are minerals and vitamins. Chickpeas have higher contents of minerals, such as zinc and phosphorous, than other legumes, in addition to B-complex vitamins and vitamins C, A, E and K. Furthermore, chickpeas contain several phenolic compounds (e.g., isoflavones biochanin A and formononetin) as well as carotenoids, which are present at higher concentrations in brown and black chickpea varieties (Jukanti *et al*., 2012; Hall *et al*., 2017; Serrano *et al*., 2017; Summo *et al*., 2019).

Chickpeas, as for other legumes, also contain antinutritional compounds (i.e., molecules that disrupt the digestion process); examples of these compounds are inhibitors of trypsin and chymotrypsin, as well as phytic acid, which inhibits the absorption of Ca, Zn and Fe by the body. Treatment of chickpea seeds or flour, using technological approaches such as thermal processing and extrusion, have been shown to reduce the levels of such antinutritional compounds, in addition to modifying the content of phenolic compounds; for example, cooking chickpeas under pressure reduces phytic acid content by 20% (Xu & Chang, 2009).

Table 2.1. Composition of *'desi'* and *'kabuli'* chickpeas.

	Protein (%)	**Carbohydrates (%)**	**Fat (%)**	**Ash (%)**	**Reference**
Chickpea *'desi'*	16.1-26.7	47.4-66.9	3.10-4.93	2.70-3.60	Khan *et al*. (1995) ; Rincon *et al*. (1998); Singh *et al*. (2004)
Chickpea *'kabuli'*	19.9-25.5	47.6-66.9	4.60-5.67	2.80-3.42	Khan *et al*. (1995) ; Rincon *et al*. (1998); Singh *et al*. (2004)

2.3 Biochemical features of the major protein fractions in chickpea proteins

Protein represents an important component of chickpea seeds. Indeed, chickpeas have a high content of protein, typically ~20-25% and, according to the Osborne fractions classification (Osborne, 1924), the principal proteins in chickpeas are albumin, globulin, prolamin, and glutelin, representing 8-12%, 53-60%, 3-7% and 19-25% of total protein, respectively (Day, 2013). Some variation can be seen in the protein content of the two seed types, *'desi'* and *'kabuli'* which may arise from differences in the environment in which they are grown, agronomic techniques used, or storage conditions (Khan *et al*., 1995). Differences in amino acid patterns between *'desi'* and *'kabuli'* chickpeas have been reported, for example methionine content was 1.4 and 1.1 g/100 g of protein for *'desi'* and *'kabuli'* flours, respectively, while more significant differences were observed in leucine, lysine and serine content between the two seeds (4.2 and 2.5, 7.2 and 7.6, 5.4 and 7.3 g/100 g of protein, respectively) (Ghribi *et al*., 2015b).

2.3.1 Albumins

Albumins represent 8-12% of total protein in chickpeas and are water-soluble proteins. They provide a good supply of essential amino acids and contain a higher proportion of sulphur-containing amino acids than globulins (Bhatty, 1982). They are constituted of many enzymatic and metabolic proteins, indicating they are essential for chickpea growth (Clemente *et al*., 2000). Despite providing the most nutritive value in chickpeas, albumins also contain many antinutritional components, such as amylase and trypsin inhibitors (Boye *et al*., 2010b). Because of their solubility in water, albumins are capable of enhancing the foaming properties of pulses, and they

can easily interact with the starch component, having important implications for chickpea protein ingredient functionality (Day, 2013; Ghumman *et al*., 2016).

2.3.2 Globulins

Globulins are salt-soluble proteins (Osborne, 1924) and the main globulins found in pulses are the storage proteins (11S) legumin and (7S) vicilin, classified based on their sedimentation coefficients (Boye *et al*., 2010b; Day, 2013). Legumin is the major globulin in chickpeas and is a hexameric protein. Legumin subunits are linked by disulphide bridges, with acidic chains sited at the surface of the molecule and the hydrophobic basic units sections located inside, limiting contact with water (Gueguen & Cerletti, 1994). In general, legumins have higher levels of the sulphur-containing amino acids methionine and cysteine than vicilins, while vicilins contain no cysteine residues and therefore cannot form disulphide bonds. Instead, vicilin is a trimeric protein and its monomers are linked together by non-covalent hydrophobic bonds (Shevkani *et al*., 2019). Because of their structure, and thus, reduced ability to unfold and entrap air, globulins have lower foaming capacity compared to albumins. On the other hand, the structure of globulins has important implications for other functional properties, such as emulsifying activity and water absorption capacity (Ghumman *et al*., 2016). Moreover, globular proteins form gels because of physical interactions (i.e., hydrophobic interactions and hydrogen bonding) caused by heating above a minimum unfolding temperature (Gosal & Ross-Murphy, 2000; Papalamprou *et al*., 2009). These gelling properties and thermal behaviours have been extensively studied for soy globulins (i.e., 11S glycinin and 7S β-conglycinin) which are comparable to chickpea globulins in terms of molecular characteristics (Chang *et al*., 2012; Chen *et al*., 2016). However, differences between heat-induced gels of the 11S glycinin and those

obtained from the 7S β-conglycinin were reported by Kinsella (1979), with the first being firmer than the latter. Due mainly to its well-studied gelling properties soy globulin is often considered an appropriate reference gelling protein in many semi-solid food products (Bessada *et al*., 2019).

2.3.3 Prolamins

Prolamins are alcohol-soluble proteins, typical of cereal proteins, representing a small fraction (3-7%) of total proteins in chickpeas. They are characterised by a high proportion of proline and glutamine (Osborne, 1924; Rachwa-Rosiak *et al*., 2015). Prolamins are thought to be responsible for the poorer foaming and emulsifying properties of cereal flours, compared to legume flours which are rich in albumins and globulins (Stone *et al*., 2019). However, chickpea prolamins have not been well characterised (Chang *et al*., 2012).

2.3.4 Glutelins

Glutelins are soluble in dilute acid or alkali detergents, and in the presence of chaotropic or reducing agents (Osborne, 1924). The relatively high glutelin content of chickpea (19-25%) is unique for a legume seed. Glutelins are of nutritional interest, containing higher levels of methionine and cysteine than globulins (Singh & Jambunathan, 1982). Like prolamins, chickpea glutelins have not been well characterised (Chang *et al*., 2012).

2.4 Chickpea protein ingredients

Chickpea protein ingredients are of major interest for both research and commercial purposes. Chickpeas, as well as other pulses (e.g., pea, lentil and/or

cowpea), represent important dietary components in countries where animal protein is too expensive or where they have a long history of consumption. In many parts of the world, traditional dishes are composed of chickpeas combined with wheat, rice or other cereal (Knights & Hobson, 2016). As such, producing formulated products that incorporate chickpea proteins, and more in general pulse proteins, can aid in meeting recommended daily protein requirements and improve the nutritional characteristics of selected food products (Day, 2013; Millar *et al*., 2017). Moreover, for individuals suffering from allergic reactions to gluten, eggs, milk, fish, shellfish and/or sesame and who do not have cross-reactivities to peanut and soybean, chickpea, and pulses in general, represent alternative sources of nutritional and functional proteins (Boye *et al*., 2010b). Indeed, pulses are not classified as major allergens; however, allergy to chickpea was reported in specific geographic areas with high consumption of chickpea-based products (e.g., India and the Mediterranean countries) (Bar-El Dadon *et al*., 2014; Wangorsch *et al*., 2020). Chickpea protein allergy is usually associated with cross-reactivity with other legumes (e.g., lentil or soybean) and peanut (Bar-El Dadon *et al*., 2014; Wangorsch *et al*., 2020). Furthermore, arsenic and chromium are the only heavy metals that have been reported in pulses, but in very low amounts compared to other staple foods (e.g., rice) (Bessada *et al*., 2019).

Chickpea proteins can be found in high proportions in chickpea flour, which can be further processed into chickpea protein concentrates and isolates.

2.4.1 Chickpea flour

Chickpea flour contains approximately 17-21% protein, 5-7% fat, 61-62% carbohydrate, 3% ash and 9-12% water (Boye *et al*., 2010a). Most raw legumes are subjected to one or more processing procedures that allow them to be edible, palatable

and often also to enhance their nutritional value. Such primary processes include milling, grinding, dehulling, sieving, germination, boiling or soaking. Although chickpeas can be consumed whole, they often undergo a number of primary processes to convert them into flour, which can have different effects on the content, functionality and nutritional quality of the proteins and, in turn, on the protein ingredients produced therefrom (Oghbaei & Prakash, 2016). Moreover, the chemical composition, structure and physical characteristics (e.g., size, hardness and density) of the chickpea seed strongly influence the performance of the milling process and, consequently, the physical properties of the resultant flour and its application in food products (Thakur *et al*., 2019). Compared to wheat flour, chickpea flour has higher protein and fibre contents and is a good source of polyunsaturated fats. Much research into the supplementation or substitution of wheat flour with chickpea flour has been performed over the past decade. In recent years, chickpea flour has been incorporated into different food products, such as bread, pasta and cakes, along with other cereal flours, and has been reported to enhance the quality of cereal-based products, especially in terms of protein content, nutritional values, as well as sensory properties, for example when added at substitution percentages of 30 or 50% in Lebanese pastry typically made of wheat flour (Dandachy *et al*., 2019). A study performed by Garcia-Valle *et al*. (2021) investigated the structural characteristics and digestibility of pasta made with semolina and chickpea flour. Results from this study indicated that incorporation of chickpea flour in pasta formulations increased protein and dietary fibre contents; however the protein from chickpea flour weakened the structure of dough and pasta, which led to unacceptable cooking characteristics, including reduced hardness and elasticity. Summo *et al*. (2019) examined the effect of supplementation of wheat flour with Apulian black chickpea flour in bakery products and reported a

decrease in bread-making performance, due to the absence of gluten and high fibre content of the chickpea flour, in spite of the nutritional improvement of the bakery products achieved by inclusion of chickpea flour.

Chickpea flour is almost invariably the starting point for the production of protein-enriched ingredients with acceptable yield, purity and functional properties (Boye *et al*., 2010b; Day, 2013; Schutyser *et al*., 2015). Consequently, the production of chickpea flour is reflective of the commercial opportunities that have led to industrial production of chickpea protein concentrates and isolates from the primary processing of chickpeas into flour, hence the importance of having a good baseline of relevant scientific information on chickpea flour.

2.4.2 Chickpea protein concentrates

Protein is a valuable part of chickpeas and it is of high, and growing, technological and nutritional interest globally. Chickpeas have a protein content of ~20-25%, and this protein can be extracted using dry and wet fractionation methods and enhanced to produce protein concentrates and isolates (Fig. 2.1) (Mondor *et al*., 2009 ; Boye *et al*., 2010b; Schutyser *et al*., 2015). Chickpea protein concentrates are characterised by having a dry weight of at least around 65% protein content (Boye *et al*., 2010b). However, currently there is no common universal classification for concentrates and isolates for any of the legumes (Singhal *et al*., 2016). Numerous approaches and technologies have been identified and developed in the extraction of protein from plant sources, particularly pulses. Selecting the most appropriate approaches for extraction and purification is essential as the choice of approach influences the functional, sensorial and nutritional properties of the concentrates (Aluko, 2004). Indeed, extraction of the proteins can be influenced by a number of

factors, which include pH, temperature, solubility of the flour, the ratio of solvent to flour (Singhal *et al*., 2016). Moreover, the chickpea variety used to obtain protein concentrates influences the characteristics of the ingredient; for example, Milán-Noris *et al*. (2018) reported higher soluble protein content for flour and concentrate produced from *'desi'* than *'kabuli'* chickpeas. Dry fractionation involves processes such as dehulling, milling and air classification (Schutyser *et al*., 2015). Air classification is based on separating flour particles based on their size and density, where air is fed into a classifier chamber, which causes centrifugal and gravitational forces to separate the light, fine fraction (typically protein) from the heavy, coarse fraction (typically starch) (Boye *et al*., 2010b; Singhal *et al*., 2016). This approach has been reported to result in high retention of functional properties of the proteins and is considered a more sustainable, energy efficient and effluent-free approach in extracting proteins from legumes. However, the use of dry extraction approaches results in significantly lower protein yield and purity compared with wet extraction approaches (Boye *et al*., 2010b; Schutyser & Van Der Goot, 2011; Singhal *et al*., 2016; Assatory *et al*., 2019; De Angelis *et al*., 2021). New approaches are currently being investigated to enhance protein yield and purity during dry fractionation extraction; for example, air classification can be followed by a triboelectrostatic separation step, during which protein and fibre are oppositely charged and thereby separated in an electrostatic field (Xing, *et al*., 2020a).

According to Schutyser *et al*. (2015), dry extraction approaches lead to higher oil, fibres and antinutritional components such as tannins, trypsin inhibitors and phytic acid, in the protein fractions compared to the wet methods. However, primary processes, such as dehulling, soaking or germination, can reduce the content of these components; in addition, solid state fermentation has been shown to be effective in

reducing such compounds after dry extraction (Xing, *et al*., 2020b). Moreover, dry extraction methods influence the mineral concentrations in the protein ingredients, with the fine fraction having higher contents of these components compared to the coarse fraction; however, this is not yet well investigated in the literature (De Angelis *et al*., 2021). Pelgrom *et al*. (2015a) investigated approaches to improve the dry fractionation of legume proteins, with optimal results reported after changing the milling settings to obtain finer particles, leading to detachment of starch granules from protein and fibre. Another possible approach would involve selection in plant breeding, where cultivars with large starch particles or low seed hardness are selected to breed legumes that have characteristics that enable protein extraction. Pre-treatments of the legumes, such as changing of the moisture content, removal of the hull and/or defatting, and post-treatments, such as electrostatic separation, can be performed to enhance protein purity and yield using air classification (Pelgrom *et al*., 2015b).

Wet fractionation, which includes alkaline/acid or salt extraction and an isoelectric precipitation or filtration step, usually leads to high protein concentrations (Fig. 2.1) (Rui & Boye, 2013). This type of process consumes large quantities of water and energy and the resulting proteins often display diminished functional properties due to temperature and pH changes that occur during the process (Schutyser *et al*., 2015). Such protein concentrates have higher purity than those produced using dry fractionation, and generally, have protein content greater than 70%. Therefore, it is the most commonly used approach for protein extraction (Pelgrom *et al*., 2015b). Preparation of protein concentrates commences with milling chickpeas into a flour which is usually defatted using petroleum ether, as described by Papalamprou *et al*. (2009). Different approaches can be combined to extract protein from chickpea flour,

which typically involve a type of solvent extraction, followed by a separation technique. Indeed, after the milling step, precipitation is performed by adjusting the pH to the isoelectric point (pI) of the protein (i.e., pH ~4 for legume proteins), followed by centrifugation. This type of extraction allows for the removal of insoluble material that includes carbohydrate, fibre and prolamins (Singhal *et al*., 2016). The combination of aqueous alkaline/acid extraction, followed by isoelectric precipitation, is the basis of a broadly used process; however, acid conditions are generally less extensively used than alkaline conditions (Boye *et al*., 2010b).

Ultrafiltration/diafiltration is another method that can be used as an alternative to isoelectric precipitation, after proteins have been solubilised in an alkaline/acid or salt solution. It is a type of filtration that utilises membranes with specific molecular weight cut-offs, selected to retain the proteins of interest while permeating much of the non-protein constituents (Boye *et al*., 2010b). Boye *et al*. (2010a) investigated the functional properties of different legume protein concentrates, including chickpeas, produced using isoelectric precipitation and ultrafiltration approaches. Using either isoelectric precipitation and ultrafiltration/diafiltration resulted in a four-fold increase of protein for the legume flours, and the results also showed that the method chosen affected the functional properties of the concentrates. For example, the protein obtained using ultrafiltration had better gelling properties than that produced using isoelectric precipitation (Boye *et al*., 2010a).

Salt extraction, or micellization, is a technique used to solubilise proteins in aqueous solution. This method is based on the "salting-in"/"salting-out" phenomena of proteins in foods, in which globulins and albumins are separated based on their solubility (Boye *et al*., 2010b; Singhal *et al*., 2016). "Salting-in" occurs at low ionic strength whereby protein-water interactions increase due to the presence of low salt

level. The protein solubility increases to a certain ionic strength, beyond which it decreases (i.e., "salting-out"), causing disruptions to the protein hydration layers (Maurer *et al*., 2011; Singhal *et al*., 2016). As a consequence, interactions between the ions and water are favoured over protein-water interactions, leading to increased levels of protein-protein interactions. The proteins self-aggregate and precipitate out of solution, where an appropriate methodology (e.g., centrifugation) can be used to enrich the aggregated protein (Maurer *et al*., 2011). Using the salt-extraction method, Paredes-Lopez *et al*. (1991) obtained a chickpea protein isolate containing 87.8% protein from defatted chickpea flour treated with NaCl. Unlike dry fractionation techniques, wet extraction approaches are suitable also for the production of chickpea protein isolates, as they can yield over 90% purity (Assatory *et al*., 2019).

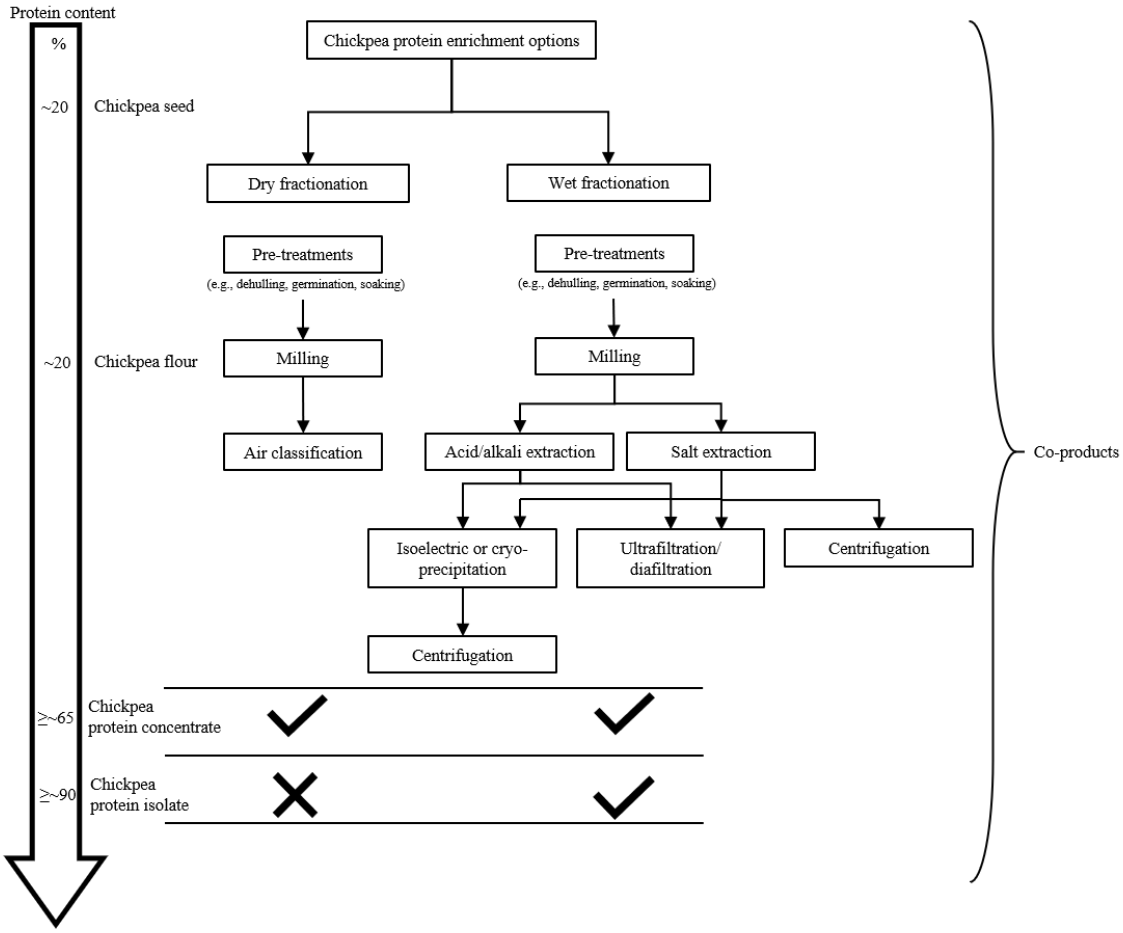


Figure 2.1. Different options available for the production of chickpea protein ingredients.

2.4.3 Chickpea protein isolates

As chickpeas are a good source of protein, there has been much investigation into the production of protein isolates for use as functional ingredients in food products and to improve the nutritional value of foods. Chickpea protein isolates are typically characterised by containing ≥90% protein on a dry basis (Singhal *et al*., 2016). The approaches used for preparation of chickpea protein isolates are similar to those used for preparation of chickpea protein concentrates, with the key difference being the extent of protein enrichment. Papalamprou *et al*. (2009) investigated the impact of processing technique on the physicochemical characteristics of protein isolates prepared from chickpea flour. Isolates were prepared using alkaline extraction with either isoelectric precipitation methods, or ultrafiltration technology, and another isolate was prepared using acidic extraction in combination with ultrafiltration. Electrophoretic analysis demonstrated that the main protein fractions in the chickpea protein isolates obtained with isoelectric precipitation were globulins, more specifically, legumin and vicilin, while albumins were not detected. Both globulin and albumin proteins were detected in the ultra-filtered isolate, implying that ultrafiltration of the protein extracts results in protein concentrates/isolates with more heterogeneous protein profile (Papalamprou *et al*., 2009). Dehulling prior to processing of the chickpeas can result in enhanced protein recovery and functional properties of the extracted protein ingredients (Ghavidel & Prakash, 2006). Moreover, Withana-Gamage *et al*. (2011) identified that separation of the seed coat of both *'desi'* and *'kabuli'* through manual dehulling resulted in chickpea protein isolates with high protein contents (72.8–85.3%), similar to those of soy or pea protein isolates. Chang *et al*. (2012) prepared and characterised chickpea protein isolates using alkaline extraction and cryoprecipitation, a technique used to produce a homogenous globulin

protein extract. This work involved alkaline extraction and centrifugation, with the extract filtered and refrigerated for 18 h to facilitate precipitation of protein from solution, with this protein retrieved using centrifugation and freeze dried to achieve a cryoprecipitate. Two proteins, legumin and vicilin, were identified in the cryoprecipitate using electrophoresis. A more homogenous protein isolate was extracted via cryoprecipitation compared to isoelectric precipitation (Chang *et al*., 2012).

2.5 Functional properties of chickpea protein ingredients

The functional properties are the physicochemical characteristics that determine the behaviour of proteins in a food product when processed for storage and consumption purposes (Kinsella, 1979). Therefore, these behaviours are considered extremely important in new product development. The functional properties of protein ingredients are influenced by amino acid profile, which in turn affect the structure and conformation of proteins, as well as processing conditions (e.g., pH, temperature and interactions with other compounds) (Zayas, 1997). The properties that are most significant in food applications include solubility, water and oil absorption capacity, emulsifying, foaming and gelling properties (Day, 2013) (Table 2.2).

Table 2.2. Functional properties of chickpea protein ingredients.

Functional property	Protein ingredient type	Reference
Solubility	CPI	Sánchez-Vioque *et al*. (1999) ; Kaur & Singh (2007) ; Boye *et al*. (2010b) ; Withana-Gamage *et al*. (2011)
Water absorption capacity	CF, CPC, CPI	Kaur & Singh (2007); Withana-Gamage *et al*. (2011); Boye *et al*. (2010a); Toews & Wang (2013)
Oil absorption capacity	CF, CPI	Kaur & Singh (2007); Withana-Gamage *et al*. (2011)
Emulsifying properties	CPC, CPI	Boye *et al*. (2010a) ; Withana-Gamage *et al*. (2011)
Foaming properties	CF, CPC, CPI	Kaur & Singh (2005); Kaur & Singh (2007); Toews & Wang (2013)
Gelling properties	CPC, CPI	Kaur & Singh (2005); Kaur & Singh (2007); Papalamprou *et al*. (2009)

CF= chickpea flour; CPC= chickpea protein concentrate; CPI= chickpea protein isolate

2.5.1 Solubility

Solubility is defined as the amount of protein in a sample that dissolves into solution and it is a centrally important functional property, often being a prerequisite for expression of other functional properties (Zayas, 1997). Factors that influence protein solubility include pH, ionic strength, type of solvent and temperature (Day, 2013). Much research has been carried out into the solubility of chickpea proteins, all of which have reported the solubility to be high between pH 1 to 3 and pH 7 to 10, and lowest at the isoelectric point at approximately pH 4, similar to other legume proteins, due to the net zero charge of the protein which reduces the inter-molecular electrostatic repulsion and ionic hydration, causing precipitation of the protein (Sánchez-Vioque *et*

al., 1999; Kaur & Singh, 2007; Boye *et al*., 2010b; Withana-Gamage *et al*., 2011; Bessada *et al*., 2019). At pH values higher, or lower than the isoelectric point, protein-water interactions increase due to the positive or negative charge of proteins (Zayas, 1997).

2.5.2 Water absorption capacity

Water absorption capacity (WAC) of proteins is the ability to retain water against gravity through physicochemical interactions, with the ability to bind water molecules being dependent on protein structure and conformation. Hydrogen bonding-mediated interactions occur between water molecules and hydrophilic groups of the protein side chains (e.g., imino, amino, carboxyl, hydroxyl, carbonyl and sulfhydryl groups) (Zayas, 1997). Potential food applications can be affected by WAC as it can determine the structure and organoleptic characteristics of any food formulations containing such protein ingredients (Singhal *et al*., 2016). While low WAC in food products is associated with inefficiency in holding water, high WAC leads to brittle and dry food products, especially during storage (Boye *et al*., 2010b).

Kaur & Singh (2007) compared the WAC, expressed as grams of water bound per gram of the sample on a dry basis, of chickpea flours and chickpea protein isolates, reporting higher levels for the isolates (approximately 1.5 g/g and 2.3-3.4 g/g, respectively), relating this to the greater ability of the isolates to swell, dissociate and unfold, exposing additional binding sites. Toews & Wang (2013) reported that defatted chickpea protein concentrates had higher WAC than the corresponding non-defatted chickpea protein concentrates (3.0-3.4 and 2.3-2.9 g/g DM, respectively), suggesting a considerable effect of choice of process on the functional properties of the protein ingredients. WAC of *'desi'* and *'kabuli'* chickpea protein isolates extracted via

isoelectric precipitation was investigated by Withana-Gamage *et al*. (2011), with the values reported exhibiting slightly higher WAC for *'desi'* than for *'kabuli'*. Boye *et al*. (2010a) reported lower results for the chickpea protein concentrates compared to pea and lentil concentrates. In contrast, significant differences between pea, soy and chickpea protein isolates (approximately 2.5, 4.3, and 2.3-4.3 g/g, respectively) were observed by Withana-Gamage *et al*. (2011), with pea isolates having lower WAC than the soy and almost all the chickpea protein isolates. Only one of the *'desi'* chickpea cultivars had lower WAC than the pea protein isolate. This could be attributed to the differences in protein conformation and composition between different varieties of chickpea, the environment in which they are cultivated, or the amount of protein extracted in the approaches applied in each study.

2.5.3 Oil absorption capacity

Oil absorption capacity (OAC) is important in food formulation as, in addition to WAC, it can influence organoleptic properties and structure of food (Singhal *et al*., 2016). Lipid-protein interactions occur on the nonpolar side chains of proteins and hydrophobic, electrostatic, hydrogen and non-covalent bonds are responsible for such interactions. The size of powder particles influences the OAC of protein powder ingredients; low-density and small particle size of the powder ingredients lead to higher absorption and entrapment of oil compared to high-density powders (Zayas, 1997).

Kaur & Singh (2007) reported OAC for chickpea protein isolates to range from 2.1 to 4.0 g/g, comparable to soy and bean protein isolates. Detailed examination of the chickpea isolates indicated that the use of *'kabuli'* chickpeas conferred a higher OAC than *'desi'* cultivars (approximately 4.0 and 2.1-3.7 g/g, respectively). This was

confirmed by Withana-Gamage *et al*. (2011), suggesting that the differences in OAC were based on the higher proportion of non-polar amino acids in *'kabuli'* chickpeas. The same authors also reported higher OAC for two cultivars of chickpeas compared to soy and pea protein isolates. Furthermore, Kaur & Singh (2007) reported higher values of OAC for isolates compared to their corresponding flours, indicating that extraction of the protein can result in improved fat retention when incorporated into a food in protein concentrate or isolate forms.

2.5.4 Emulsifying properties

The emulsifying performance of food proteins are determined by their emulsifying capacity and activity, with many plant proteins, soy in particular, considered to be good emulsifiers and finding many applications in the food industry. The capability of proteins to act as emulsifiers depends on their intrinsic properties including amino acid profile (i.e., charge and polarity), molecular weight, structure, conformational stability, water solubility and also environmental factors such as temperature, pH and ionic strength (Bessada *et al*., 2019). As an example, differences in such intrinsic properties between the 7S and 11S globulin protein fractions contribute directly to the superior emulsifying properties of the former (Sharif *et al*., 2018). In emulsion systems, proteins form films around oil droplets dispersed in an aqueous medium, serving to retard/prevent undesirable outcomes such as coalescence, creaming, flocculation and sedimentation (Day, 2013; Singhal *et al*., 2016). Emulsifying capacity is defined as the amount of oil emulsified by 1 g of protein, whereas emulsifying activity index determines how well a protein can emulsify an oil and measures the maximal interfacial area per gram of protein of a stabilized emulsion. Emulsifying stability index determines how well the emulsion can withstand structural

changes over a period of time (Zayas, 1997; Boye *et al*., 2010b).

Withana-Gamage *et al*. (2011) reported varying emulsification properties for the proteins isolated from different cultivars of chickpea, and for soy and pea protein isolates. Soy protein isolates showed the highest activity and stability indices (approximately 1.3 and 29, respectively), followed closely by *'kabuli'* protein isolates (1.1-1.3 and 20.3-26.6, respectively), and then *'desi'* protein isolates (0.9-1.1 and 19.2-21.3, respectively). The *'kabuli'* protein isolates had better ability to emulsify the oil/fat droplets in an aqueous medium and provided stability to the emulsion without any structural changes compared to *'desi'* protein isolates. Furthermore, Boye *et al*. (2010a) reported higher emulsifying stability and ability indices values for chickpea protein concentrate compared to pea or lentil protein concentrates, with slightly higher values for the *'kabuli'* type compared to the *'desi'*.

2.5.5 Foaming properties

Food proteins can stabilise foams due to their predisposition to be adsorbed onto air/water interfaces and their ability to reduce surface tension and to form strong membranes via protein-protein interactions (Day, 2013). Foaming properties are described by the foam capacity and foam stability indices. Foam capacity is usually expressed as the volume increase achieved with whipping of protein dispersions. Foam stability is determined by measuring the volume of the foam as it changes over a defined period of time (Bessada *et al*., 2019). The foaming properties differ among the protein fractions, with albumin generally having better foaming capacity and stability than globulin.

Kaur & Singh (2007) reported the foaming capacity for different cultivars of chickpea protein isolates ranged from 30 to 44%. Similar results were obtained by

Toews & Wang (2013) who reported foaming capacity of chickpea protein concentrates, expressed as the foam volume at 1 min per liquid volume before whipping in percentages, ranging from 26 to 48%. Defatted chickpea protein concentrates displayed foaming capacity of 201-228%; however, these values were significantly lower than those for other pulses also tested. According to the results reported by Kaur & Singh (2005), chickpea flours produced foam with low volume; however, the stability of the foam produced was high (over 90% after 2 h of storage), suggesting that the native proteins in chickpea flour, being reasonably soluble in water, are very surface-active.

2.5.6 Gelling properties

Food proteins, especially globular proteins, form gels in aqueous solutions when they are denatured by heat and the interactions between proteins and protein-solvent are balanced (Day, 2013). During heating, globulins dissociate and reassociate in different structural conformations, forming gels with various properties (Bessada *et al*., 2019). Indeed, proteins form gels when they are partially unfolded and develop uncoiled polypeptide segments that interact to form cross-linked networks (Zayas, 1997). Gelling capacity of proteins is measured by determining the least gelling concentration, that is, the lowest concentration of protein required to form a gel structure. According to Kaur & Singh (2007), chickpea protein isolates had a least gelling concentration ranging from 14 to 18%, higher than the values for the corresponding flours (10-14%). These differences were attributed to differences in protein profile of the ingredients, along with the profile of non-protein constituents. Previously, the same authors reported that *'kabuli'* flour formed a firmer gel, at lower concentration (10%) compared to *'desi'* chickpea flours, attributing this to the

variation of protein and non-protein components in the two flours (Kaur & Singh, 2005). Indeed, factors strongly influencing the gelation of chickpea protein, and indeed proteins generally, include protein concentration, other non-protein components, pH, ionic/reducing agents, and heat treatment conditions (Schmidt, 1981). Papalamprou *et al*. (2009) reported gel formation at concentrations in the range 4.5-11.5% for chickpea protein isolates extracted using different approaches (i.e., wet extraction followed by isoelectric precipitation or ultrafiltration), with the results demonstrating that the choice of the extraction technique had a significant impact on the gelation behaviour of protein isolates.

2.6 Modification and enhancement of chickpea protein quality

The term *protein quality* in relation to chickpea protein is herein intended to relate to both nutritional and techno-functional properties. Nutritional quality includes, for example, protein digestibility, bioavailability, antioxidant and antimicrobial properties, while the techno-functional quality consists of all the functional properties of proteins (e.g., solubility, oil and water absorption capacity, emulsifying properties), essential to support its use in food formulations and applications (Nasrabadi *et al*., 2021). Approaches that have been shown to enhance the quality of chickpea proteins, include germination of the chickpeas prior to processing, dehulling, fermentation, hydrolysis or other chemical modifications, extrusion and high hydrostatic pressure (Table 2.3). Due to the interdependence of the two qualities, these methods can be used for their effects on the techno-functionality of protein and, in turn, influence the nutritional quality of same. Germination is carried out following the basic steps of sterilisation, soaking and sprouting and initiated when the dry seed commences to take up water and is completed when the embryonic axis elongates (Gan *et al*., 2017;

López-Martínez *et al*., 2017). The functional properties of chickpea flour after germination and dehulling were studied by Ghavidel & Prakash (2006). The authors reported that after germination of the chickpeas and subsequent production of flour, solubility, emulsification properties and foaming capacity were enhanced compared with the un-germinated samples, with further increases evident with the inclusion of an initial dehulling step. In another study, the same authors investigated the effects of germination and dehulling on the protein digestibility of legume seeds, including chickpeas, whereby protein content after germination was significantly higher and increased more after dehulling, conferring higher protein digestibility (Ghavidel & Prakash, 2007). Furthermore, germination was shown to influence other important nutritional attributes, for example, enhancement of the anti-inflammatory effect in the lower gut of chickpea protein concentrates from *'desi'* and *'kabuli'* varieties (Milán-Noris *et al*., 2018). Fermentation of the chickpea flour and its application in food formulations has been extensively investigated in the literature (Angulo-Bejarano *et al*., 2008; Rizzello *et al*., 2014; Chandra-Hioe *et al*., 2016; Xiao *et al*., 2016; Shrivastava & Chakraborty, 2018). Fermentation is a process that can be used to enhance the nutritional value of chickpea flour and reduce the presence of undesirable compounds (e.g., protease inhibitors, phytates and tannins). Lactic acid bacteria are widely used in such fermentation processes due to the desirable organoleptic properties they can provide. As reported by Chandra-Hioe *et al*. (2016), after both natural and cultured (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) fermentations, improvements in OAC were observed for both the *'desi'* and *'kabuli'* varieties, while WAC was higher for the *'kabuli'* chickpeas compared to the *'desi'* and foaming capacity and stability decreased after fermentation for both the chickpea types. Xiao *et al*. (2016) compared the inclusion of fermented and non-

fermented chickpea flour in wheat bread and the effects thereof on quality and antioxidant activity. The study demonstrated that replacing a portion of the wheat flour with fermented chickpea flour improved the antioxidant properties of the bread, as well as physical and sensorial characteristics. Enzymatic hydrolysis of chickpea protein isolates using Alcalase was investigated by Ghribi *et al*. (2015a), reporting that protein solubility increased proportionally with degree of hydrolysis. Emulsifying activity initially increased with hydrolysis; however, as the degree of hydrolysis increased, the emulsion activity decreased. Emulsion stability also decreased with increasing hydrolysis due to the reduced ability of the small peptides, resulting from the hydrolysis, to interact, resulting in lower viscosity of the interfacial layer. Chickpea protein hydrolysates, obtained using enzymes, have shown to be promising sources of peptides for many food and pharmaceutical applications, due to their gastrointestinal absorption, bioactive compounds and hypoallergenic effects, in addition to improved solubility compared to chickpea flour, protein concentrates and isolates (Real Hernandez & Gonzalez de Mejia, 2019). Chickpea flour, as well as other pulse and cereal flours, often undergo extrusion processing in the preparation of snack foods. Extrusion is a thermal processing technique that involves the use of high temperatures and short times, and employs pressure and mechanical shear produced by heated barrels and rotating screws (Harper & Clark, 1979). During extrusion, major biochemical transformations occur, with potential to enhance digestibility of chickpea components (i.e., starch and protein) and inactivate anti-nutritional compounds (Kaur & Prasad, 2021). In addition to influencing the nutritional profile of chickpea protein ingredients, it is reported that extrusion has a major impact on the physicochemical properties of the extrudates and flours obtain therefrom (Yovchev *et al*., 2017; Wang *et al*., 2019). The effect of high hydrostatic pressure on chickpea flour was studied by

Angioloni & Collar (2013). Hydrated chickpea flour was treated at pressures of 0.1, 200, 350 and 450 MPa and improvements were reported in the rheological properties of the chickpea batters, thought to be attributed to the effect of high hydrostatic pressure on the formation of protein networks through protein aggregation. The use of selected pre-treatments, often in combination with manipulation of protein extraction processing parameters strongly influence the protein quality of chickpeas, especially in terms of nutritional characteristics and techno-functionality.

Table 2.3. Effect of different treatments on chickpea protein ingredients.

Treatment	Effect on chickpea ingredients	Reference
Germination and dehulling	• Improvements of flour solubility and emulsifying properties • Reduce levels of phytate and tannin, increasing protein digestibility	Ghavidel & Prakash (2006, 2007)
Fermentation	• Reduce antinutritional compounds and increase digestibility • Enhancement of oil absorption capacity, foaming capacity and stability	Chandra-Hioe *et al*. (2016); Xiao *et al*. (2016)
Enzymatic hydrolysis	• Increase of protein solubility • Enhancement of emulsifying properties at low levels of hydrolysis	Ghribi *et al*. (2015a)
Extrusion	• Nutrients' retainment and inactivation of antinutritional components • Improvements of textural properties	Kaur and Prasad (2021)
High hydrostatic pressure	• Improvements of rheological properties of batters through enhancement of protein network formation	Angioloni & Collar (2013)

2.7 Applications of chickpea protein ingredients

Chickpea protein ingredients are applied mainly in food products (e.g., cereal-based and bakery products, infant foods and meat products); however, they have the potential to be also used in nutraceutical applications (Boye *et al*., 2010b; Shevkani *et al*., 2019). Incorporation of chickpea protein ingredients in cereal-based food products improves protein content and quality, along with enhancing nutritional value and some organoleptic characteristics of food products. In particular, partial substitution of

wheat flour with chickpea flour improves the protein content and nutritional value of food products made therefrom (e.g., pasta, bread and other baked goods) and in some cases can enhance the rheological, functional and sensory properties of such products (Rachwa-Rosiak *et al*., 2015; Ouazib *et al*., 2016; Dandachy *et al*., 2019; Summo *et al*., 2019; Garcia-Valle *et al*., 2021). Chickpea flour has been used in the production and formulation of pasta products with low glycaemic index, which may be suitable for diabetic patients. Chickpea pasta is commercially-available and it is produced by many food companies worldwide. Incorporation of chickpea flour in pasta has been reported to significantly slow sugar release into blood (Goñi & Valentín-Gamazo, 2003). As recently reported, incorporation of legume flours in bakery products (e.g., pasta, biscuits and bread), reduces the *in vitro* glycaemic response of such products, providing the potential for new product development for those who require low glycaemic index type foods (Monnet *et al*., 2019). Moreover, chickpea flour is used, in combination with other ingredients, to produce puff snacks and crisps available in the retail sector. Chickpea protein concentrate was used in the improvement of organoleptic properties of "Merguez" sausage by Ghribi *et al*. (2018), with chickpea protein concentrate added at 1.5, 2.5 or 5% protein into cooked sausages. The results suggested that addition of chickpea protein concentrates in meat products provides satisfactory organoleptic characteristics, reduces the level of lipid oxidation, improves the stability of colour during storage and provides antioxidant properties. Aider *et al*., (2012) investigated the effect of partial substitution of wheat flour with different levels of pulse protein concentrates (i.e., lentil, pea and chickpea) in bread-making. This study demonstrated that the mass volume of the bread supplemented with 6% and 9% chickpea protein was the highest of the experimental breads, albeit lower than the control with wheat flour alone. The authors attributed this to the high WAC of the plant

proteins that may have lowered water vapour formation in the dough during the baking process. A study conducted by Malunga *et al*. (2014) investigated the use of chickpea protein ingredients, from *'desi'* and *'kabuli'* varieties, in the formulation of follow-on infant formulae. The formula developed was found to meet nutritional requirements set by the World Health Organisation (WHO) in terms of protein and carbohydrate contents, amino acid profile and most micronutrients with minimal addition of oils, minerals and vitamins. Preliminary treatments such as germination, dehulling, boiling and enzymatic hydrolysis were applied to reduce the amount of anti-nutritional components. More recently, Kyriakopoulou *et al*., (2021) described chickpea protein ingredients as good alternatives to soy for sausage-type meat analogues, due to their good gelling properties, in addition to good emulsifying and foam stability. Furthermore, new chickpea-based yogurt alternatives are emerging in the market, with chickpea protein concentrate often used in the formulation of such products. Applications of chickpea protein isolates can also be found in the nutraceutical sector as a capsule for micronutrient supplementation; for example, Ariyarathna & Nedra Karunaratne (2015) reported the application of chickpea protein isolates in the microencapsulation of folate. The use of proteins in the encapsulation of micronutrients is an emerging technology showing great potential due to the biocompatibility of proteins and their nutritional value (Ariyarathna & Nedra Karunaratne, 2015). The limited amount of work published on encapsulation efficiency of chickpea protein isolates has indicated they are suitable for the encapsulation of bioactive compounds; however, its use is not fully explored in the literature.

2.8 Applications of the co-products of chickpea protein ingredient processing

Generation of co-products during food processing is unavoidable and, in the past, these co-products were often considered waste. However, in recent years, novel applications of such co-products have been identified for use in many industries. The most common co-product of chickpeas that results from the cooking or canning of chickpeas in water is aquafaba, which has been shown to exhibit similar foaming ability to egg white. Aquafaba has high moisture content (92-95%), while the dry matter consists mainly of soluble and insoluble fibre, protein (0.9-1.5%), ash, saponin, and phenolic compounds. Due to the heating treatments of chickpeas, the protein antinutritional components in aquafaba is lower compared to the chickpea seeds; however, other compounds, such as saponins, are leached into the cooking water (Mustafa & Reaney, 2020). Buhl *et al*. (2019) investigated the use of aquafaba as an egg white substitute in foams and emulsions and it was concluded that aquafaba had the potential to be used as an ingredient in foods where foaming properties are required. Emulsion properties were determined to be superior to those exhibited by the egg white and none of the properties of aquafaba were disrupted by increasing salt levels in selected food products. Factors such as chickpea composition, processing methods (e.g., heat treatments, extrusion and soaking) and auxiliary agents employed in processing (e.g., enzymes, salts, acids or bases), protein and carbohydrate types and concentrations, influence aquafaba functional properties; investigation of the impact of these factors on the functional properties aquafaba can help in its further application in food products (Mustafa & Reaney, 2020). Production of chickpea protein concentrates and isolates often results in the removal of the seed hull and the fractionation of lipid, starch and fibre during the process, all of which can provide

economic, environmental and nutritional benefits (Tassoni *et al*., 2020). New emerging extraction technologies will help in removing the non-protein portions of chickpeas during extraction without causing undesirable changes; these fractions can be further processed for applications in food products as functional components (Tassoni *et al*., 2020).

Starch is the main carbohydrate constituent found in chickpeas and is one of the residues removed during protein enrichment, with starch granules usually removed following isoelectric precipitation of the proteins and separated via sieving and washing of the residue (Emami *et al*., 2007). Applications of chickpea starch are dependent on the physicochemical properties that it exhibits, which include gelatinisation, solubility and swelling. For example, chickpea starch has low swelling power, probably due to the presence of numerous crystallites formed by the association between amylopectin chains, that increase granular stability, which makes it suitable for applications where restricted swelling is required (e.g., sauces and salad dressings) (Singh *et al*., 2004; Miao *et al*., 2009). Chickpea starch can be included into products that require gluten-free ingredients, such as pasta or noodles, due to their suitable pasting properties (Jagannadham & Parimalavalli, 2015).

Numerous co-products of chickpea cultivation and processing, such as bran from de-hulling, crop residues (e.g., husks and straw) and chickpea hay, are used for animal feed. These co-products are considered good sources of nutrients due to the presence of bioactive compounds, such as fibre and polyphenols (Tassoni *et al*., 2020). Chickpea hulls are a major co-product of protein extraction as most studies utilise dehulled chickpea flour as a raw material. From these hulls, dietary fibre can be extracted and used in different industries, which has been studied by Niño-Medina *et al*. (2017, 2019). The authors reported results on the potential use of chickpea hulls as

a source of fibre and phenolics with antioxidant capacity for development of new high-value products. Addition of chickpea dietary fibre in formulation of white bread resulted in improved sensory characteristics during storage, with the colour remaining unaffected by the chickpea fibre and improvements were observed in calcium content and antioxidant activity due to the phenolic compounds. Both Kanatt *et al*. (2011) and Kumar *et al*. (2015) reported the potential of phenolic compounds extracted from chickpea hulls for use as antioxidants to prevent lipid oxidation of meat. Application of natural antioxidants, for example, phenolic compounds extracted from legume hulls, has become more common in the meat industry due to higher consumer acceptability than synthetic antioxidants (Kumar *et al*., 2015).

Another application of chickpea hulls is as a textile dye for clothing items. Jose *et al*. (2019) extracted textile grade dye, composed of phenols, tannins and flavonoid, from the hulls of chickpeas and used it for the colouration of cotton, wool and silk clothing. The process did not require the use of solvents and therefore, was more environmentally friendly compared to traditional processes of dyeing clothes.

Use of chickpea straw obtained post-harvesting and the potential for use in animal feed has been investigated by Bampidis & Christodoulou (2011), who reported that addition of chickpea straw improves the nutritional value of the feed provided for ruminant animals or can be used as an alternative forage instead of hay or silage in the diet of ruminants. Other than applications in food products, animal feed, food additives and textiles industries, a number of emerging technological applications, such as production of biodegradable packaging and cosmetics, have been identified for chickpea protein co-products by Tassoni *et al*. (2020).

2.9 Conclusion

Global trends show that chickpea production has increased significantly to meet the needs of populations worldwide. Chickpeas provide high nutritional value, being a good source of protein, fibre, fat and carbohydrate. Much research has been carried out on chickpea protein ingredients and the potential uses that they may have in the development of new and reformulated food products, most especially on chickpea flour to date. Several different approaches and analytical techniques/unit operations have been used to extract proteins from chickpea flours, based largely either on dry and wet fractionation approaches. Use of these techniques is dependent on various factors, including nature of starting material, yield and purity of protein required and the desired functional properties. Numerous applications of chickpea protein ingredients have been documented, highlighting the potential of these ingredients for novel product development and improvement of the nutritional profile of existing food products. The number of investigations on the possible uses of chickpea protein ingredients is increasing, in particular in recent years, filling the gap with respect to potential applications of such ingredients in the food industry. Although most of the published work was performed using chickpea flour, more research on chickpea protein concentrates and isolates is required to support the needs of increasingly discerning customers for high quality protein. Furthermore, applications of co-products (e.g., uses in the food, nutraceutical and textile industries) resulting from chickpea processing, allow for the conversion of low value waste products into new high value products. Future research may be useful to improve applications of such co-products that result from the extraction of chickpea proteins, thereby leading to even more sustainable processes.

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Chapter 3

Composition and physicochemical properties of commercial plant-based block-style products as alternatives to cheese

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Abstract

The global market for plant-based foods intended as alternatives to cheese products is increasing and will reach almost $4 billion by 2024. In this study, an evaluation of the composition, structure and physicochemical properties of four commercial plant-based block-style products was conducted, with results compared with those for Cheddar and processed cheeses. The plant-based products had considerably lower protein contents (0.11–3.00%) compared to the Cheddar and processed cheeses (25.0 and 18.5%, respectively). Analysis of microstructure demonstrated that the plant-based products did not have a continuous protein network, with the fat globules being stabilised by starch and other hydrocolloids. The Cheddar cheese had the highest hardness, firmness and Young's modulus values (126.8, 98.8 N and 953.3 KPa, respectively), with some of the plant-based products showing similar textural properties to the Cheddar cheese. Furthermore, rheological analysis showed that the meltability profiles of the plant-based products differed to those of Cheddar cheese. The differential scanning calorimetry thermograms showed a similar peak at ~20 °C for all plant-based products, being different from the two peaks displayed by the dairy-based products. This study shows the complexity of the mechanisms behind the physicochemical properties of plant-based block-style alternatives to cheese and the challenges related to them.

3.1 Introduction

The interest in plant-based foods designed to provide an alternative to cheese products is increasing, with the global market for such products growing at a compound annual growth rate (CAGR) of 7.6% from 2016 to 2024, and is expected to reach a value of almost $4 billion by 2024 (Bharat Book Bureau, 2017). US retail sales of these plant-based products are worth $189 million per annum, having grown by 50.8% between 2017 and 2019 (SPINS & Good Food Institute, 2019). Furthermore, in Europe the demand for plant-based food is increasing, with the principal drivers for purchasing these products being concerns about the impact of dietary choice on climate change and personal health (Proveg International, 2019). The need to develop more sustainable and nutritious food products is compounded by a rapidly increasing global population that needs to be adequately nourished without exacerbating the negative environmental impact of the respective food production systems, which are responsible for between 19 and 29% of the total global anthropogenic greenhouse gas (GHG) emissions (FAO, 2009; Vermeulen *et al*., 2012). Moreover, dietary shifts from consumption of meat and dairy, towards more plant-based diets offer potential for reducing GHG emissions, in addition to benefits for human health (Stehfest *et al*., 2009; Popp *et al*., 2010; Springmann *et al*., 2016). Indeed, in most of the environmental impact categories (e.g., climate change, eutrophication, acidification, land use) meat and dairy products (i.e., cheese, milk and butter) are the most burdensome foodstuffs (Notarnicola *et al*., 2017) and, more specifically, the environmental impact of cheese is mostly related to primary production of raw milk followed by processing of milk into cheese (Finnegan *et al*., 2018).

Cheese represents an important food product in many cultures and is produced globally in a wide diversity of flavours, textures and consumption patterns. In most

countries, cheese consumption is increasing, at an average annual rate of ~3%, with the key reasons for same being a positive dietary image of the product, its variety, convenience and versatility in use (Fox & McSweeney, 2004; Fox *et al*., 2017a). Amongst the different types of cheese currently available, Cheddar cheese, which originated in England, is one of the most important varieties worldwide; it is considered a hard cheese and is made from pasteurized cows' milk, coagulated with calf rennet or a rennet substitute (McSweeney *et al*., 2004). Processed cheese emerged from the need to develop cheese products that were stable at temperatures ≤ 40°C and could be stored for long time periods with minimal changes in physicochemical characteristics (Fox *et al*., 2017b). Processed cheese is generally prepared by melting and heating blends of natural cheeses, with addition of emulsifying salts and other ingredients (e.g., vegetable oils, starch and colourants), shearing to produce a homogeneous mixture, packaging and cooling (Tamime, 2011).

Some plant-based products have been consumed for centuries as traditional foods in many cultures (e.g., tofu and sufu), and have been studied extensively (Jeske *et al*., 2018). Soy is used worldwide to produce various plant-based products and modern soy-based block-style products are generally made from soymilk, and factors including coagulant type and concentration, temperature and time have been investigated to improve the structural development of soy-based products (Jeewanthi & Paik, 2018). Other raw materials employed for the production of plant-based foods intended as alternatives to cheese products are nuts, such as cashews, macadamias and almonds, generally being soaked and ground with water and fermented to obtain the final product (Tabanelli *et al*., 2018). Moreover, as non-allergenic sources, coconut oil and starch are important ingredients in such product formulations. Indeed, researchers have investigated the use of non-protein ingredients (e.g., starch, gums and other

hydrocolloids) to confer selected functionality to plant-based foods, often aiming to replace the physicochemical characteristics of animal proteins (Mattice & Marangoni, 2020a). However, these plant-based block-style products often have low protein content and high levels of saturated fat and carbohydrates and may not represent a healthy choice compared to dairy-based products. In addition, one of the major limitations associated with commercially-available plant-based block-style products is being able to simulate the functional properties of cheese products (Mattice & Marangoni, 2020b). These properties, such as the textural characteristics of the unheated cheese and the heat-induced functional properties (e.g., meltability and flowability), are particularly important quality attributes of cheese products and depend on initial milk composition, choice of manufacturing process and maturation (Lucey *et al*., 2003; McCarthy *et al*., 2016). In particular, the interactions between, and within, the casein particles and the extent and pattern of proteolysis play key roles in influencing the physicochemical characteristics of cheese (Lucey *et al*., 2003).

The aim of this study was to evaluate the composition, structure and physicochemical properties of a number of representative commercially-available plant-based block-style products, and to compare these properties with those for Cheddar and processed cheeses as benchmarks. The results will aid in the design of plant-based block-style products by improving our understanding of the inter-relationship between composition, structure and function and increasing knowledge about the complexity of the mechanisms behind the physicochemical properties of plant-based block-style alternatives to cheese and the challenges related to them.

3.2 Materials and methods

3.2.1 Commercial products

The products analysed were four commercial plant-based block-style products, all considered as alternatives to Cheddar (plant A, B, C and D), with Cheddar and processed cheeses also analysed as benchmarks. The products were purchased from Sainsbury (London, UK), the Quay Co-op and Tesco (Cork, Ireland). The ingredients used in formulating the products were as described on the packaging material for the products and are given in Table 3.1, along with the pictures indicating the surface on the left and the cross-section on the right for each product. The products were stored at 4°C and used within 7 d of opening.

Table 3.1. Ingredient list of dairy and plant-based products.

Product	Ingredients
Cheddar 	Milk, rennet, salt
Processed 	Cheese (60%), water, vegetable oils (coconut, palm), milk protein, emulsifying salts (sodium phosphates, sodium polyphosphate), modified maize starch, whey powder (milk), tri calcium phosphate, acidity regulator (citric acid), colour (annatto), vitamins E, A and D3
Plant A 	Water, coconut oil (21%), starch, modified starch, sea salt, flavourings, olive extract, colour: β-carotene, vitamin B12
Plant B 	Water, coconut oil (21%), modified potato starch, maize starch, gluten free oat fibre, modified maize starch, thickeners (carrageenan, guar gum), salt, yeast extract, tricalcium citrate, natural flavourings, acidity regulators (lactic acid, sodium lactate), colour (mixed carotenes)
Plant C 	Water, stabilisers: modified potato starch, coconut oil (21%) coconut cream (8%), salt, vegetable glycerine, tri-calcium phosphate, acetic acid, natural flavouring, colour: carrot juice concentrate, lactic acid, vitamin D2, vitamin B12
Plant D 	Filtered water, tapioca starch, coconut oil, vegan natural flavours, pea protein isolate, non-GMO expeller pressed: canola and/or safflower oil, chicory root extract, sea salt, xanthan gum, lactic acid (vegan), tricalcium phosphate, pea starch, potato protein, vegan enzyme, cane sugar, annatto (colour), coconut cream

3.2.2 Analysis of composition, pH and water activity measurements

The moisture content was measured using oven drying at 103°C for 5 h, according to AOAC Official Methods of Analysis 926.08 (AOAC, 1990c), moisture-in-nonfat solids (MNFS) was also calculated. Ash was analysed by incineration in a muffle furnace at 800°C for 5 h after pre-ashing in crucibles for 10 min, according to

AOAC Official Methods of Analysis 935.42 (AOAC, 1990a). The protein content was measured using the Kjeldahl AOAC Official Methods of Analysis 2001.14 (AOAC, 2002) using a nitrogen-to-protein conversion factor of 6.38 for the two dairy-based products and 6.25 for the four plant-based products (Jones, 1931). Fat content was determined using the Gerber AOAC Official Methods of Analysis 933.05 (AOAC, 1990b). Total carbohydrate was calculated by difference (i.e., 100 – sum of protein, fat, ash and moisture). The pH of the products was determined by measuring the pH of homogenized slurries prepared from 10 g of product and 10 mL of water at room temperature and blended in a Stomacher (Seward Ltd., Worthing, West Sussex, UK) for 5 min. The water activity (a_w) of the products was measured at 20°C using a water activity meter (Aqua Lab, Decagon Devices, Inc., Pullman, WA, US). Cylinders of height 5 mm and diameter 40 mm were prepared using a meat slicer (Scharfen G330F, Hermann Scharfen GmbH & Co. Maschinenfabrik KG, Witten, Germany) and a circular cutter. After the calibration, water activity of the products was measured.

3.2.3 Colour

The colour of all products was measured using a chromameter CR-400 (Konica Minolta Sensing, Inc., Osaka, Japan) as described previously by Li *et al*. (2020). Colour was expressed as Hunter CIELAB coordinates (L*, a*, b*). The chromameter was calibrated before the measurement using a white tile.

3.2.4 Microstructural analysis

3.2.4.1 Confocal laser scanning microscopy

Microstructural analysis of the products was performed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation,

Japan). Samples were prepared as described by Le Tohic *et al*. (2018), whereby fat and protein were stained with Nile Red and Fast Green FCF, respectively. A mixture of Fast Green FCF aqueous solution (200 µL of 0.1 g/L) and Nile Red in 1,2-propanediol (600 µL of 0.1 g/L) was prepared and ~50 µL of the mixture was applied onto the sample, which was held at 4°C for 10 min before imaging. Fast Green FCF and Nile Red were excited at 633 and 488 nm, respectively (Auty *et al*., 2001) and representative images, performed using a 40x objective lens, were reported.

3.2.4.2 Cryogenic scanning electron microscopy

Cryo-scanning electron microscopy (cryo-SEM) was conducted following the method described by Ong *et al*. (2011). Small pieces of the products, cut manually using a blade, were mounted on copper holders using Tissue-Tek (OCT Compound, Sakura Finetek, Alphen aan den Rijn, the Netherlands) to fix them, and immersed in liquid nitrogen slush for 15 s using an Alto 2500 cryo sample preparation system (Gatan, UK). The frozen samples were immediately transferred to the cryo preparation chamber of the Alto 2500 system, previously equilibrated at -140ºC, through its vacuum transfer device. Specimens were then fractured inside the cryo preparation chamber using a scalpel blade and etched for 30 min at -95ºC. After cooling the chamber again to -140ºC, samples were sputter coated with a gold/palladium alloy at 10 mA for 120 s and finally transferred under vacuum into the microscope. Microscopy analysis was conducted on a Gemini field emission scanning electron microscope (ZEISS, Germany) at an accelerating voltage of 2 kV. Two detectors, an in-lens detector and a secondary electron detector, were used to acquire the images.

3.2.5 Rheological properties

3.2.5.1 Texture profile analysis

Texture profile analysis (TPA) was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK) according to the method of O'Mahony *et al*. (2005). Cylinders of 20 mm diameter and 10 mm height were prepared using a stainless steel wire cutter and a circular cutter and stored overnight at 4°C. Immediately after removal from storage, samples were compressed to 25% of their original height in a double compression at a rate of 1.0 mm/s. For each sample, hardness, which is defined as the force required to compress a food between the molars, adhesiveness, the work required to separate the food from another material, springiness, intended as the propensity of food to recover from large deformation after removal of deforming stress, and cohesiveness, which is the strength of the internal bonds making up the food, were measured as described by Kasapis and Bannikova (2017), corresponding to the sensory parameters of the same name (Fox *et al*., 2017c)

3.2.5.2 Uniaxial compression testing

Uniaxial compression testing was performed using a Texture Analyser TAXT2i (Stable Micro Systems Ltd., Godalming, Surrey, UK), equipped with a 25 kg load cell and a compressing plate (Ø = 75 mm), operating at a fixed test speed of 1.0 mm/s, to a depth of 7 mm. Cylinders were prepared as described in Section 3.2.5.1 and stored overnight at 4°C. The firmness (i.e., maximum positive force in compression), was measured from the stress-strain curves. Fracture stress (σ_f), the stress at fracture, as indicated by the inflection point in the compression curve, and fracture strain (γ_f), the fractional displacement at fracture were calculated from the resultant stress-strain curves (McCarthy *et al*., 2016). Youngs modulus (E), the resistance of the cheese

structure to reversibly deform without fracturing, was also calculated by estimation of the slope of the early linear region of the stress-strain curves (Noël *et al*., 1996). The work of fracture (G_c) was measured as the area under the stress-strain curve.

3.2.5.3 Dynamic low amplitude oscillatory shear rheology

Rheological properties of the products were measured using an AR-G2 controlled-stress rheometer (TA Instruments Ltd., Waters LLC, Leatherhead, UK) equipped with crosshatched surface stainless steel parallel plates. Samples of height 2 mm and diameter 41 mm were obtained using a meat slicer (Scharfen G330F, Hermann Scharfen GmbH & Co. Maschinenfabrik KG, Witten, Germany) and a circular cutter and stored overnight at 4°C. As the Cheddar cheese was more brittle and mature than the other products, it was sliced using a cheese slicer board and a wire, from which the discs were obtained using a circular cutter. Samples were equilibrated at room temperature before analysis, which was performed by applying force at a constant frequency of 1 Hz, with a temperature ramp from 20 to 80°C at a ramp rate of 2°C/min. The viscoelastic behaviour of the system at a strain oscillation frequency is characterised by the storage modulus (G′), and by the loss modulus (G″) (i.e., solid-like and liquid-like contributions to the measured stress response, respectively), moreover the ratios between the two moduli is defined as the loss tangent (Tan δ) (Fox *et al*., 2017c). The values for such parameters were reported, as well as the values at 20 and 80°C, and the minimum storage (G′min) and loss (G″min) moduli, maximum loss tangent (Tan δ_{max}) and temperature of maximum loss tangent (T at Tan δ_{max}).

3.2.6 Differential scanning calorimetry

Thermograms of products were obtained using a Mettler DSC821 (Mettler-

Toledo, Schwerzenbach, Switzerland) differential scanning calorimeter (DSC) equipped with liquid nitrogen cooling. Samples (16–29 mg) were cut, prepared and weighed into standard aluminium pans (Mettler, 40 µl) and pans were hermetically sealed. The calorimeter was calibrated for temperature and heat flow using indium and the thermal behaviour of the products was recorded first from -50 to 100°C at a heating rate of 5°C/min, then from 100 to -50°C at a cooling rate of 10°C/min and from -50 to 100°C, again at a heating rate of 5°C/min. The DSC curves were analysed using Mettler-Toledo STARe system version 8.10 for thermal analysis. Only the data from the first heating and cooling ramps from -10 to 60°C and from 60 to -10°C were reported for the thermal properties of the products, as no differences were observed from the second heating ramp.

3.2.7 Meltability

Meltability of the products was assessed using the Schreiber test as described by Altan *et al*. (2005), with minor modifications. Cylinders, of height 5 mm and diameter 41 mm, were prepared using a meat slicer (Scharfen G330F, Hermann Scharfen GmbH & Co. Maschinenfabrik KG, Witten, Germany) and a circular cutter and stored at 4°C until testing. The samples were placed in a covered glass Petri dish and heated at 232°C for 5 min in an oven (Memmert, Schwabach, Germany). Afterwards, the samples were removed from the oven and cooled at room temperature for 30 min. Pictures of the products were taken and specimen expansion was measured with a ruler along six lines marked on a set of concentric circles. Meltability was given as the mean of the six readings and expressed as percentage specimen expansion (Ramel & Marangoni, 2018).

3.2.8 Statistical data analysis

All analyses were performed in at least triplicate with samples taken from the same commercial product, the texture profile analysis was performed with four samples and the uniaxial compression analysis performed with six samples. Levene's test was used to check the homogeneity of variance and one-way analysis of variance (ANOVA) was carried out using SPSS version 25 (SPSS Inc., Chicago, IL, USA). A Tukey's paired comparison post-hoc test was used to determine statistically significant differences ($p < 0.05$) between mean values for different samples, at a 95% confidence level. Results are expressed as mean ± standard deviation with statistically significant differences identified using superscript letters.

3.3 Results and discussion

3.3.1 Formulation, chemical composition, pH and water activity of products

The chemical composition and pH of the products is provided in Table 3.2. Variations in the composition of commercial cheeses might be observed depending on the product chosen and this can consequently affect the physicochemical properties of such products; however, the measured values for chemical composition and pH for the Cheddar and processed cheeses were comparable to the data for retail Cheddar and processed cheeses previously reported in the literature by McCarthy *et al*. (2017) and Trivedi *et al*. (2008), respectively. Visual differences between the products were also evident from the pictures provided in Table 3.1. The Cheddar cheese had the highest protein and fat contents (25.0 and 30.3%, respectively), followed by the processed cheese (18.5 and 24.7%, respectively). The Cheddar cheese had the simplest (i.e., least number of ingredients) formulation, followed by the plant A product. All four plant-based products had significantly lower protein content than the dairy-based products, with values ranging from 0.11 to 3.00%. Analysis of the ingredient listings indicated that all the plant-based products were combinations of coconut oil and mixtures of starch from different sources, with flavourings also included in all plant-based products. The plant D product had the highest protein content among these products, attributed to the pea protein isolate and potato protein components of the formulation. Plant A had the highest moisture and MNFS contents (53.7 and 67.8%, respectively) and the Cheddar cheese the lowest moisture and MNFS contents, at 36.9% and 52.9%, respectively. The processed cheese had 61.2% MNFS and showed the highest ash content of 3.85%. According to the nutritional information on the packaging of the products, the Cheddar and the processed cheese had salt content of 1.70 and 3.00%, respectively, while the salt content of the plant-based products ranged from 0.71 to

2.50%. The processed cheese formulation contained 60% cheese, in addition to emulsifying salts, coconut and palm oils, and modified maize starch. The pH of the plant-based products ranged from 4.19 to 4.31, while the Cheddar and processed cheeses had significantly higher pH values (5.21 and 5.87, respectively). The a_w of the products ranged from 0.95 to 0.99 (Table 3.2). The a_w of the Cheddar cheese was the lowest (0.95), being significantly different from the other products and comparable to results for mature Cheddar reported by Hickey *et al*. (2013) and to the results for retail Cheddar cheese products reported by Marcos *et al*. (1981) and McCarthy *et al*. (2017). The concentration and distribution of salt in Cheddar cheese is one of the main factors affecting the a_w, in addition to conferring a preservative effect (Guinee & Fox, 2017). The a_w of the plant B product (0.99) was the highest, while the a_w of the processed cheese (0.97) was similar to the results for the processed cheeses with ~50% moisture (0.97-0.98) reported by Duggan *et al*. (2008).

Table 3.2. Chemical composition, pH, colour space values, water activity and meltability using Schreiber test, of dairy and plant-based products.

	Cheddar	**Processed**	**Plant A**	**Plant B**	**Plant C**	**Plant D**
Protein (%)	25.0 ± 0.05^{e}	18.5 ± 0.12^{d}	0.11 ± 0.02^{a}	0.59 ± 0.01^{b}	0.64 ± 0.01^{b}	3.00 ± 0.04^{c}
Fat (%)	30.3 ± 0.58^{e}	24.7 ± 0.58^{d}	20.7 ± 0.58^{b}	19.3 ± 0.58^{b}	22.3 ± 0.58^{c}	16.3 ± 0.58^{a}
Moisture (%)	36.9 ± 0.02^{a}	46.1 ± 0.09^{b}	53.7 ± 0.09^{f}	50.5 ± 0.04^{e}	48.3 ± 0.28^{d}	47.2 ± 0.11^{c}
MNFS (%)*	52.9 ± 0.03^{a}	61.2 ± 0.12^{c}	67.8 ± 0.12^{f}	62.6 ± 0.05^{e}	62.1 ± 0.36^{d}	56.4 ± 0.13^{b}
Ash (%)	2.37 ± 0.06^{d}	3.85 ± 0.15^{f}	0.41 ± 0.04^{a}	1.12 ± 0.12^{b}	2.86 ± 0.16^{e}	1.56 ± 0.20^{c}
Carbohydrates (%)	5.53	6.85	25.1	28.5	25.9	31.9
pH	5.21 ± 0.02^{c}	5.87 ± 0.03^{d}	4.19 ± 0.04^{a}	4.31 ± 0.01^{b}	4.26 ± 0.04^{b}	4.31 ± 0.02^{b}
L*	81.6 ± 0.18^{c}	79.5 ± 0.36^{b}	87.8 ± 0.15^{e}	86.4 ± 0.43^{d}	88.3 ± 0.36^{e}	77.4 ± 0.29^{a}
a*	-3.00 ± 0.08^{c}	0.21 ± 0.06^{d}	-4.30 ± 0.08^{a}	-3.31 ± 0.07^{b}	-3.07 ± 0.06^{bc}	6.83 ± 0.15^{e}
b*	26.2 ± 0.27^{a}	30.1 ± 0.60^{c}	38.2 ± 0.68^{e}	34.1 ± 0.66^{d}	28.4 ± 0.35^{b}	46.1 ± 0.61^{f}
a_w	0.95 ± 0.00^{a}	0.97 ± 0.00^{b}	0.98 ± 0.00^{bc}	0.99 ± 0.00^{c}	0.97 ± 0.01^{b}	0.98 ± 0.01^{bc}
Meltability (%)	49.3 ± 6.37^{c}	1.69 ± 0.59^{a}	21.0 ± 1.78^{b}	17.3 ± 0.58^{b}	6.10 ± 2.33^{a}	5.59 ± 0.88^{a}

Values followed by different superscript letters (a–f) in the same row are significantly different ($p < 0.05$).

*MNFS, moisture-in-nonfat solids

3.3.2 Colour

The CIELAB coordinates of the products are shown in Table 3.2. All products, except the Cheddar cheese, contained added colourants such as annatto, which represents the main colourant used in the dairy industry, with applications in cheese production, and β-carotene (Kang *et al*., 2010; Sharma *et al*., 2020). The L* value, representing the brightness, was the highest for the plant C product, being significantly different from the other products, and the lowest for the plant D (88.3 and 77.4, respectively). The a* values, representing the degree of redness to greenness, were all negative except for the processed cheese and the plant D product (0.21 and 6.83, respectively), with the latter having the highest a* value (i.e., towards red colour), as also visible from the pictures in Table 3.1. The b* values, representing the degree of yellowness to blueness, were all positive (i.e., towards yellow colour) with the Cheddar cheese having the lowest value (26.2). As reported by Póltorak *et al*. (2015), the type of fat (e.g., milk fat *vs* vegetable oils) used in the formulation of cheese products strongly influences the colour of the product as well as other factors, such as the presence of natural pigments in milk (e.g., β-carotene) and the manufacturing process. Furthermore, the fat content of cheese is related to the number of light-scattering centres (i.e., cheese products with low fat content have lower numbers of light-scattering centres), as well as the protein and moisture content (i.e., high values lead to a translucent appearance of the cheese) (Johnson *et al*., 2009; Ibáñez *et al*., 2016).

3.3.3 Microstructure

3.3.3.1 Confocal laser scanning microscopy and cryogenic scanning electron microscopy

The analysis of the microstructure of Cheddar cheese (Fig. 3.1a and 3.2a) showed non-spherical shaped coalesced pockets/pools of fat and a continuous protein phase, confirming previous microstructural observations for this type of cheese as reported by Guinee *et al*. (1999, 2000) and Rogers *et al*. (2010). The shape of the milk fat globules in Cheddar cheese is affected by the shearing of their membrane during milk treatment and by coalescence of the fat globules during the cheesemaking process, when the curd is warm (Guinee *et al*., 2000). The CLSM analysis of the processed cheese (Fig. 3.1b) showed the milk and vegetable fat globules distributed throughout the protein network, as well as the starch particles (black areas on the image). Similar microstructural properties were evident from the cryo-SEM analysis (Fig. 3.2b), where the fat globules had a smooth surface with spherical and non-spherical shapes. The microstructural properties of the processed cheese were comparable to those of commercial processed cheeses reported by Ramel and Marangoni (2017) and to processed cheese at pH 5.07 containing potato starch reported by Talbot-Walsh *et al*. (2019). Differently from the dairy products, the plant-based products showed a distribution of fat globules, which had a generally spheric shape, within a matrix of starch and other hydrocolloids. The plant C product showed very small fat globules (Fig. 3.1e and 3.2e) and in colour measurement had the highest L* value and lower b* value compared to all the other products, except the Cheddar. On the other hand, the plant D had the lowest L* and highest b* values, with larger fat globules than the other plant-based products (Fig. 3.1f and 3.2f).

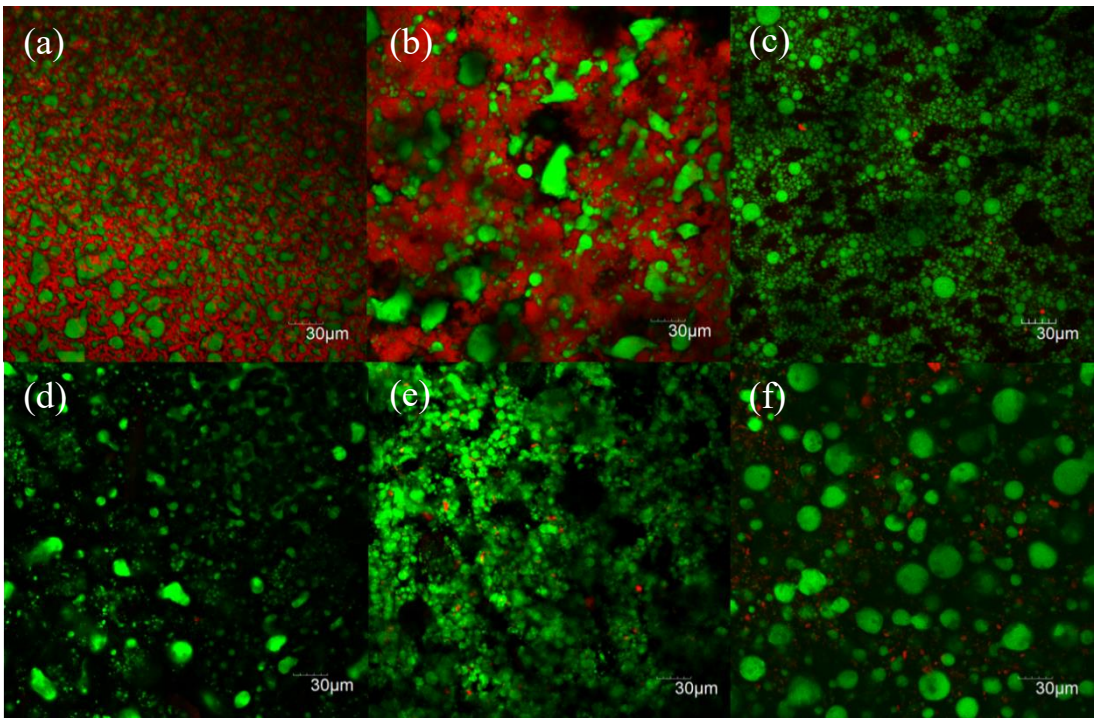
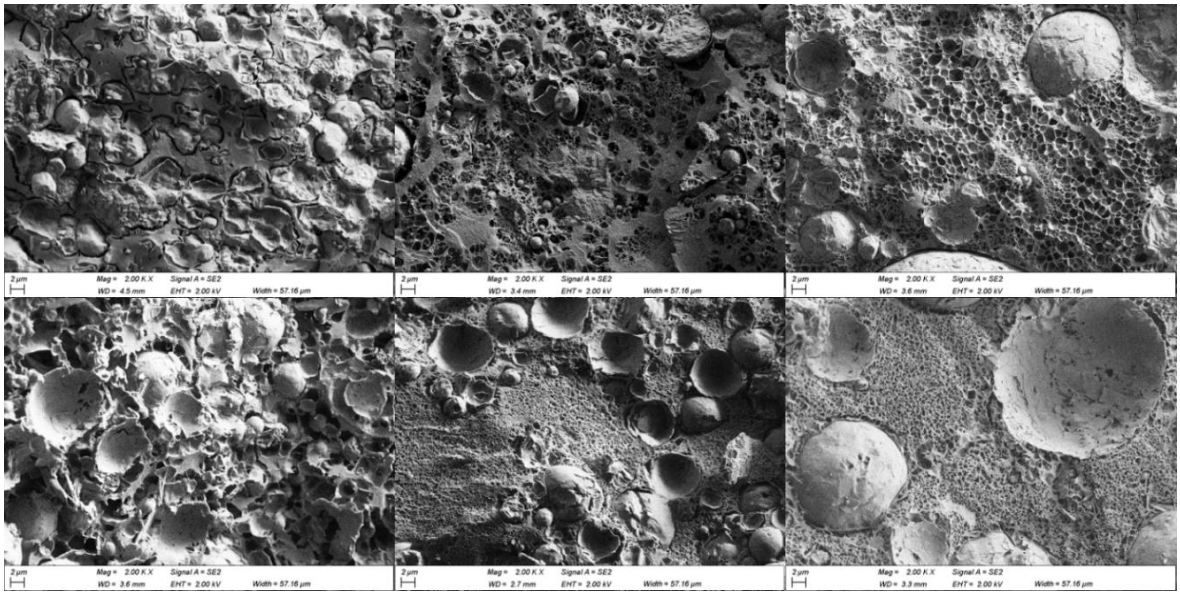


Figure 3.1. Confocal laser scanning microscopy images (x40) of Cheddar (a), processed (b), plant A (c), plant B (d), plant C (e) and plant D (f) products. The images show distribution of fat globules (green) and protein (red).

Smaller fat particles are associated to whiter and less yellow colour, due to the higher light scattering (Rudan *et al*., 1998). Moreover, the plant D product, with slightly higher protein content (3.00%) than the other plant-based products, showed a low occurrence of protein aggregates/clusters which were non-homogeneously distributed within the cheese network and not visible in the other plant-based products, which had protein content ranging from 0.11 to 0.64%. As reported previously by Liu *et al*. (2019), the structure of starch gels and their rheological behaviour are more strongly influenced by the amylose content, rather than source/type of the starch. Moreover, pre-gelatinisation generates a more dense starch matrix, with significant implications for the internal structure of resultant foods in which the gelatinisation of starch is exploited.

(a) (b) (c)



(d) (e) (f)

Figure 3.2. Cryo-scanning electron microscopy images (x2000) of Cheddar (a), processed (b), plant A (c), plant B (d), plant C (e) and plant D (f) products.

3.3.4 Rheological properties

3.3.4.1 Texture profile analysis

The texture parameters hardness, adhesiveness, springiness and cohesiveness for the products are reported in Table 3.3. TPA measures the response of food products to double-bite deformation and involves assessment of key parameters of relevance during consumer mastication, simulating the several compressions of food between the molar teeth (Fox *et al*., 2017c). Hardness, springiness and cohesiveness values for the Cheddar cheese were comparable with the results for mature (90-120 d) Cheddar cheese reported by O'Mahony *et al*. (2005). According to Everard *et al*. (2006), the moisture content, as well as pH of the product, and more generally its chemical composition, strongly influence cheese texture, therefore variations might be observed in cheese products of different nature. In particular, MNFS content strongly impacts cheese texture, and it is considered a key quality attribute of cheese (Lawrence *et al*.,

2004). The processed cheese showed the highest adhesiveness and cohesiveness, with values significantly different from the other products, but the lowest hardness and springiness. The high values of cohesiveness for processed cheese indicate that the structure is not easily disintegrated (So *et al*., 2014). The hardness of the plant-based products ranged from 80.5 to 98.0 N, all being significantly different from the dairy-based products. The plant C product had the lowest adhesiveness and cohesiveness and the highest springiness, as well as a very different shape force-time profile compared to the other products, with the hardness (second peak) being lower than the first fracture peak (profiles not shown). On the other hand, springiness of the plant-based products was comparable to the Cheddar cheese. Consideration of these data in combination with formulation details suggest that the textural parameters of the plant-based products were more strongly influenced by the use of hydrocolloids than the protein component, which was extremely low in these products. The synergistic interactions between starch and other hydrocolloids affected the texture of the products; indeed, these ingredients are often used in the food industry as emulsifiers, thickeners, stabilisers or gelling agents (Mahmood *et al*., 2017). Hydrocolloids, due to their influence on food texture, find many applications in the formulation of cheese products, such as processed and imitation cheeses, low fat or fat free cheeses and cream cheese (Tamime, 2011; Fox *et al*., 2017b; Masotti *et al*., 2018).

Table 3.3. Texture profile analysis parameters, hardness, adhesiveness, springiness and cohesiveness, and uniaxial compression testing parameters, firmness, fracture stress (σ_f) and strain (γ_f), Youngs Modulus (E) and work of fracture (G_c) and rheological parameters storage modulus (G′), loss modulus (G″) at 20°C and 80°C, minimum G′ and G″, maximum loss tangent (Tan δ_{max}) and temperature of maximum loss tangent (T @ Tan δ_{max}), of dairy and plant-based products.

	Cheddar	Processed	Plant A	Plant B	Plant C	Plant D
Hardness (N)	126.8 ± 13.9^{d}	62.2 ± 3.5^{a}	94.2 ± 4.2^{bc}	96.8 ± 6.9^{bc}	80.5 ± 5.4^{b}	98.0 ± 4.4^{c}
Adhesiveness (N·s)	6.13 ± 2.19^{b}	20.0 ± 3.36^{a}	2.32 ± 1.09^{bc}	5.54 ± 1.20^{b}	1.51 ± 0.24^{c}	2.22 ± 0.25^{bc}
Springiness (-)	0.47 ± 0.11^{bc}	0.26 ± 0.04^{a}	0.43 ± 0.06^{ac}	0.32 ± 0.05^{ab}	0.57 ± 0.09^{c}	0.47 ± 0.11^{bc}
Cohesiveness (-)	0.17 ± 0.01^{c}	0.20 ± 0.01^{d}	0.17 ± 0.01^{c}	0.15 ± 0.00^{b}	0.10 ± 0.00^{a}	0.13 ± 0.01^{b}
Firmness (N)	98.8 ± 3.09^{e}	57.5 ± 3.82^{a}	84.2 ± 3.92^{c}	95.4 ± 10.53^{de}	87.4 ± 6.33^{cd}	73.3 ± 5.71^{b}
Fracture stress (σ_f) (kPa)	212.5 ± 16.6^{b}	93.6 ± 3.5^{a}	239.5 ± 13.3^{c}	202.8 ± 7.5^{b}	282.0 ± 22.1^{d}	193.2 ± 12.6^{b}
Fracture strain (γ_f) (-)	0.33 ± 0.03^{b}	0.30 ± 0.02^{ab}	0.38 ± 0.02^{c}	0.31 ± 0.02^{b}	0.33 ± 0.02^{b}	0.27 ± 0.03^{a}
Youngs Modulus (E) (kPa)	953.3 ± 73.5^{c}	460.9 ± 73.1^{a}	709.6 ± 62.8^{b}	771.5 ± 69.8^{bc}	876.0 ± 186.7^{be}	815.6 ± 205.5^{bc}
Work of fracture (G_c) (kJ/m^3)	42.6 ± 6.43^{d}	16.9 ± 1.42^{a}	47.9 ± 4.72^{d}	34.4 ± 2.09^{c}	46.1 ± 5.36^{d}	26.0 ± 3.15^{b}
G′ 20°C (kPa)	118.5 ± 21.8^{d}	78.4 ± 5.7^{c}	24.8 ± 4.9^{a}	68.4 ± 6.4^{bc}	46.7 ± 12.1^{ac}	45.5 ± 11.5^{ab}
G″20°C (kPa)	48.2 ± 9.6^{b}	18.7 ± 2.0^{a}	8.80 ± 1.1^{a}	21.9 ± 1.3^{a}	20.2 ± 4.9^{a}	21.3 ± 5.9^{a}
G′ 80°C (kPa)	0.83 ± 0.18^{a}	25.0 ± 3.23^{c}	7.93 ± 0.67^{b}	0.70 ± 0.11^{a}	3.11 ± 0.15^{a}	3.18 ± 0.10^{a}
G″80°C (kPa)	0.75 ± 0.10^{a}	7.56 ± 1.13^{c}	3.23 ± 0.37^{b}	0.21 ± 0.03^{a}	0.37 ± 0.04^{a}	0.75 ± 0.11^{a}
G′$_{min}$ (kPa)	0.83 ± 0.18^{a}	23.7 ± 1.74^{c}	6.13 ± 1.47^{b}	0.70 ± 0.11^{a}	3.11 ± 0.15^{a}	3.18 ± 0.10^{a}
G″$_{min}$ (kPa)	0.74 ± 0.11^{ab}	7.51 ± 1.07^{c}	1.44 ± 0.07^{b}	0.21 ± 0.03^{a}	0.37 ± 0.04^{ab}	0.75 ± 0.11^{ab}
Tan δ_{max} (-)	1.09 ± 0.10^{b}	0.36 ± 0.01^{a}	0.41 ± 0.06^{a}	0.32 ± 0.05^{a}	0.44 ± 0.11^{a}	0.48 ± 0.14^{a}
T @ Tan δ_{max} (°C)	73.3 ± 0.1^{d}	61.8 ± 0.1^{c}	79.0 ± 0.0^{e}	21.2 ± 0.0^{b}	21.3 ± 0.0^{b}	21.0 ± 0.0^{a}

Values followed by different superscript letters (a–e) in the same row are significantly different ($p < 0.05$).

3.3.4.2 Uniaxial compression testing

Uniaxial compression testing demonstrated that the Cheddar cheese had the highest firmness (98.8 N) (Table 3.3.). The fracture stress (σ_f) and stain (γ_f) values for the Cheddar cheese (212.5 kPa and 0.33, respectively) were similar to the results for mature retail Cheddar cheese (211 kPa and 0.27, respectively) reported by McCarthy *et al*. (2017). According to Guinee (2011), the firmness of Cheddar-type cheese is positively correlated to the content of intact casein. The processed cheese showed the lowest values for firmness, fracture stress (σ_f), Young's modulus (E) and work of fracture (G_c), with values significantly different from the other products. Guinee and O'Callaghan (2013) reported a correlation between firmness and protein-to-fat ratio in processed cheeses (i.e., high fat content leads to low firmness), hence, depending on the composition of processed cheese differences in the texture can be observed. The plant-based products showed firmness values ranging from 73.3 to 95.4 N. The fracture stress (σ_f) values for the plant B and D products were not significantly different from that of the Cheddar cheese, while the plant C product showed the highest value (282.0 kPa). The plant A product showed the highest work of fracture (Gc) (47.9 kJ/m^3), followed by the plant C product (46.07 kJ/m^3), which were both significantly different from the other plant-based products and statistically similar to that for Cheddar cheese. The plant D had the lowest firmness, fracture stress (σ_f), fracture strain (γ_f) and work of fracture (G_c) values among the plant-based products.

3.3.4.3 Dynamic low amplitude oscillatory shear rheology

Rheological profiles of the products, as determined using dynamic low amplitude oscillatory shear testing, are shown in Figure 3.3, with the relevant rheological parameters reported in Table 3.3. The Cheddar cheese showed storage

modulus (G′), loss modulus (G″) and loss tangent (Tan δ) profiles comparable to those for 3 and 9 mo ripened cheese reported by Lucey *et al*. (2005), who reported a change in the rheological parameters of Cheddar cheese as a consequence of increasing proportion of soluble Ca during maturation, other than the more widely known effect of proteolysis. The Cheddar cheese was the only product which showed a transformation from a viscoelastic solid to liquid rheological behaviour, with G′ equal to G″ (2.11 kPa) at the cross-over temperature of 68°C. Such transformation is due to the shrinkage of the para-casein network, which occurs between 60-90°C, and the consequent expulsion of moisture from the protein network (Fox *et al*., 2017c). The cross-over temperature is particularly relevant as it indicates the temperature required for the cheese to become more fluid-like and start to flow (Fox *et al*., 2017c). This finding was in agreement with the melting behaviour of the products observed during the meltability test, where only the Cheddar cheese melted under the conditions tested (Section 3.3.6). The Cheddar cheese also showed the highest G′ and G″ values at 20°C (118.5 and 48.2 kPa, respectively), being significantly different from the other products, as well as the highest maximum loss tangent (Tan δ_{max}) (1.09), parameter that is indicative of the fluidity of melted cheese and the degree to which it flows (Fox *et al*., 2017c). The loss tangent (Tan δ) slightly decreased after reaching a peak at 73.3°C, due to the increase in hydrophobic-induced protein interactions (Bryant and McClements, 1998). The processed cheese had maximum loss tangent (Tan δ_{max}) value of 0.36, being significantly different from the Cheddar cheese and statistically comparable to that of the plant-based products. According to Mounsey and O'Riordan (1999), the addition of starch to processed cheese retards meltability, with elastic properties of the cheese being stable during heating. Moreover, the type and level of emulsifying salts and the composition of the natural cheese employed in the

formulation of processed cheese, as well as the processing conditions, have a major impact on its melting performance (Guinee, 2011). The plant-based products showed very different behaviour to the Cheddar and processed cheeses. The Tan δ_{max} of the plant-based products ranged between 0.32 and 0.48, while the temperature at Tan δ_{max}, representing the temperature of maximum fluidity (Fox *et al*., 2017c), ranged from 21.0 to 21.3°C for the plant B, C and D products, and 79.0°C for the plant A product. These products, as well as the processed cheese, did not show a melting profile, due to the starch and hydrocolloid interactions and their rheological characteristics, as also reported previously by Mattice and Marangoni (2020b). As shown by the DSC analysis (Section 3.3.5), the melting temperature of the coconut oil was approximately 20°C and from the rheological analysis it appeared that, even at higher temperature, highly elastic behaviours were observed, attributing this to the high starch content of the plant-based products with just a slight softening displayed at temperature >50°C, likely due to starch gelatinisation. Furthermore, pre-treatments of the starch, such as gelatinisation and consequent re-association of the starch components, are responsible for the increased starch gel hardness at higher heating temperatures (Liu *et al*., 2019). However, information about the process steps and conditions employed to produce these plant-based products is necessary to understand the rheological mechanisms for same.

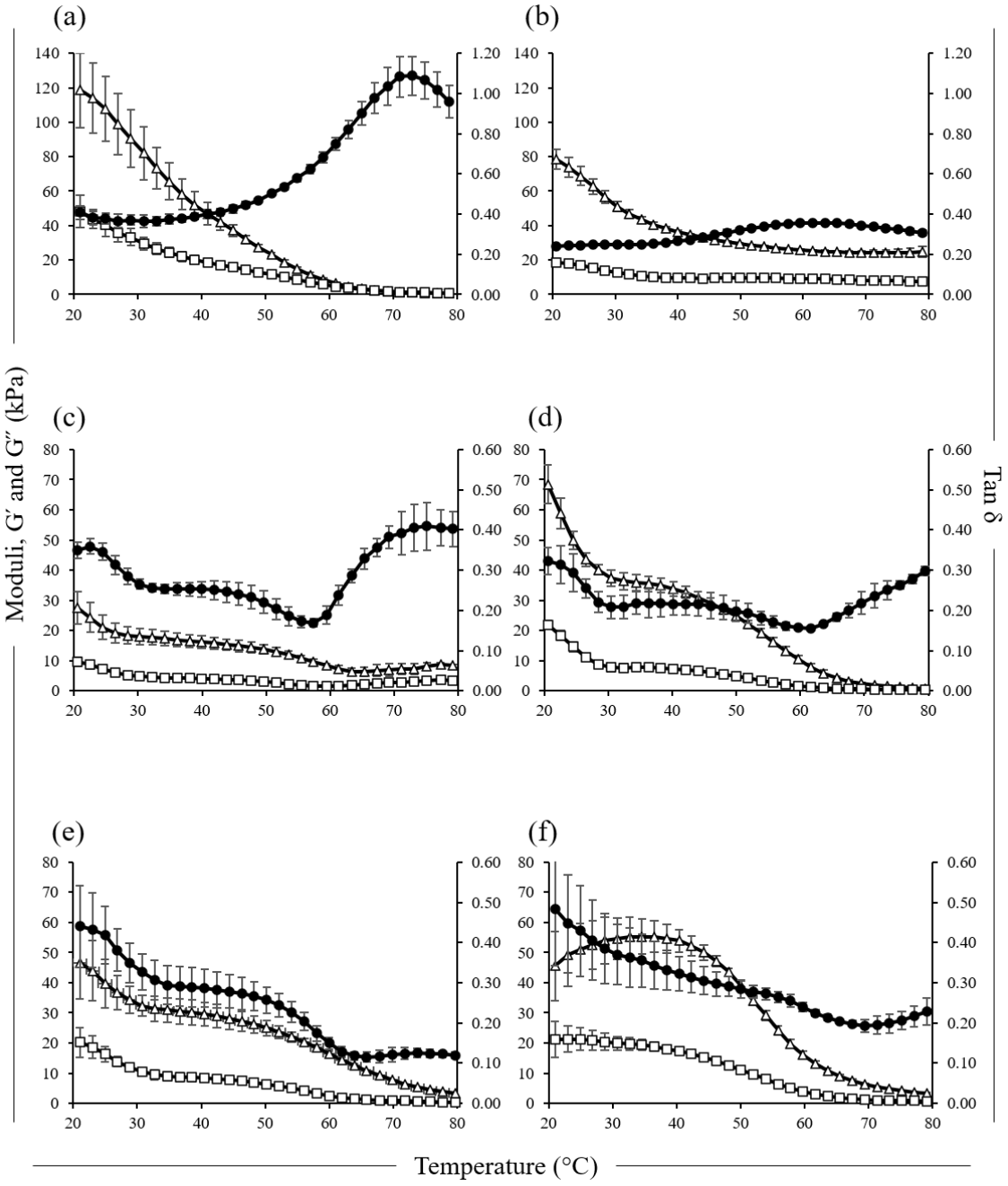


Figure 3.3. Rheological profiles showing storage modulus (G′) (*open triangle*), loss modulus (G″) (*open square*) and loss tangent (Tan δ) (*filled circle*) as a function of temperature in the range 20-80°C for Cheddar (a), processed (b), plant A (c), plant B (d), plant C (e) and plant D (f) products.

3.3.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) analysis was used to study thermal properties of dairy and plant-based products (Fig. 3.4). Multiple transitions due to fat crystallisation, recrystallisation and melting were observed from the thermograms, differing in response to the fat source used in the samples. The presence of multiple peaks during heating after cooling to -50°C, particularly in the thermograms for the dairy products, indicated the melting or crystallization of different crystalline species or different polymorphic forms of fat (Mattice & Marangoni, 2018). The dairy-based products showed two separate endotherms, the first with a peak at ~10°C and the second with a peak at ~30°C, corresponding to the melting of the three milk fractions, as also shown by the two crystallisation peaks during cooling, i.e., low and middle melting fraction (LMF and MMF) melting together at the first peak and high melting fraction (HMF) at the second peak (Ramel and Marangoni, 2017). The shape of the Cheddar cheese thermogram was similar to the results reported previously by Mulet *et al*. (1999) and Chen *et al*. (2020), showing a consistent melting pattern. The thermograms for all the plant-based products showed a similar peak at ~20°C, representing the melting of the coconut oil as also previously reported by Tan & Che Man (2002).

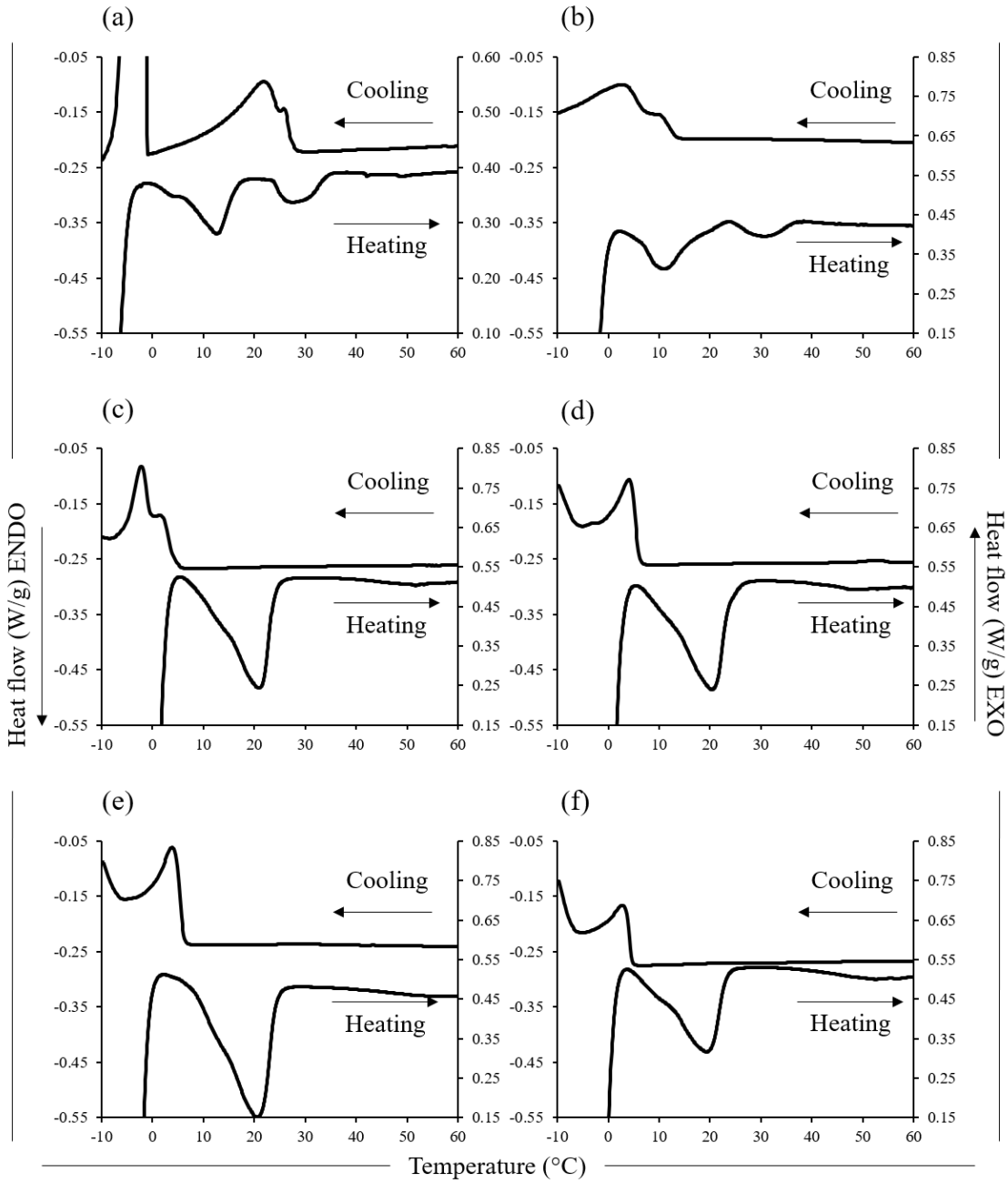


Figure 3.4. Differential scanning calorimetry thermograms of Cheddar (a), processed (b), plant A (c), plant B (d), plant C (e) and plant D (f) products. Heating ramp from -10 to 60°C and cooling ramp from 60 to -10°C are reported.

3.3.6. Meltability

Meltability represents an important characteristic of cheese products and can be defined as the ease and extent to which cheese will melt and spread upon heating (Gunasekaran & Mehmet Ak, 2002). The results of the Schreiber test for meltability are reported in Table 3.2. The Cheddar cheese showed the greatest extent of diameter expansion (49.3%), being significantly higher than the other products (1.69-21.0%), which displayed only modest diameter expansion, as evident from the pictures of the products after 5 min at 232°C (Fig. 3.5). The protein network strongly influences the meltability of cheese, in particular the moisture to protein ratio, the MNFS, and to a lesser extent the fat content (Lucey *et al*., 2003). The lowest meltability was reported for the processed cheese (1.69%), which was statistically comparable to the plant C and D products. This result might be explained by the use of starch in the formulation which generally leads to reduced meltability of processed cheese, arising from physical disruption of the protein matrix with swollen starch granules (Mounsey & O'Riordan, 2001). In addition, emulsifying salts have a considerable effect on the meltability of processed cheese, promoting hydration and solubilisation of proteins by causing physicochemical changes in the cheese matrix (Masotti *et al*., 2018). The plant-based products showed diameter expansions ranging from 5.59 to 21.0%. These products displayed no evidence of a continuous protein network, and the structure was strongly influenced by the starch and/or other hydrocolloids used in the formulation (e.g., carrageenan or gums). Indeed, when starch builds a continuous network in food systems, especially in gelatinised form, the properties of the product are strongly related to the properties of the starch, usually leading to poor melting characteristics (Ye *et al*., 2009).

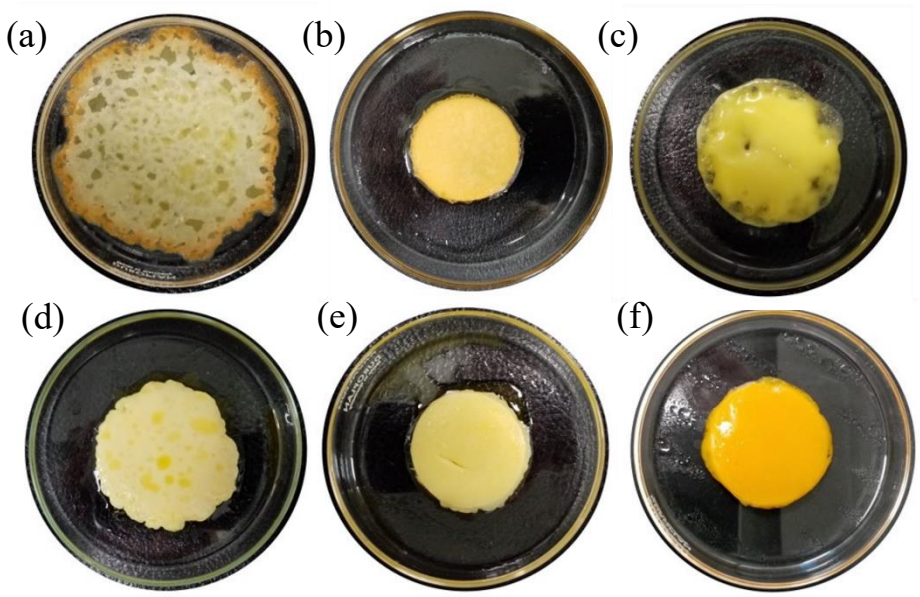


Figure 3.5. Meltability after 5 min in the oven at 232°C of Cheddar (a), processed (b), plant A (c), plant B (d), plant C (e) and plant D (f) products

3.4 Conclusion

The composition, structure and physicochemical properties of four commercial plant-based block-style products intended as alternatives to traditional cheese were studied, comparing the results with those for Cheddar and processed cheeses as benchmarks. The results showed that the plant-based products differed considerably from the dairy-based products, especially in terms of composition, microstructure and selected physicochemical properties. The protein content of the plant-based products was significantly lower than the dairy-based products and the microstructural analysis showed an absence of any continuous protein network in the plant-based products. Some of the physicochemical properties were comparable between the plant- and dairy-based products; for example, selected plant-based products had similar fracture stress, fracture strain and springiness to the Cheddar cheese. Furthermore, the Cheddar cheese was the only product which showed a transformation from a viscoelastic solid

to liquid rheological behaviour. The processed cheese, as well as the plant-based products, displayed only modest diameter expansion under the meltability test conditions, related to the use of starch in the formulation of such products. Indeed, the composition, structure and physicochemical properties of the plant-based products was shown to be strongly influenced by their formulation; however, further details on the production of such products are needed to provide more understanding of these inter-relationships. This study shows the complexity of the mechanisms behind the physicochemical properties of plant-based block-style products designed to provide an alternative to traditional dairy-based cheese products and the challenges therein.

3.5 Acknowledgements

The authors would like to thank Dr Laura Gómez-Mascaraque for providing cryo-SEM images.

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Chapter 4

The influence of protein concentration on key quality attributes of chickpea-based alternatives to cheese

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Abstract

In response to consumer demands, plant protein ingredients are increasingly being used in the formulation of plant-based alternatives to cheese. The aim of this study was to determine the influence of protein concentration on key quality attributes of chickpea-based alternatives to cheese. Moreover, the age-induced changes in such attributes were assessed, analysing the samples after 1 month of storage. After characterisation of the ingredients, the chickpea-based formulations were prepared by blending chickpea flour and protein concentrate in different proportions to obtain four samples of increasing protein content (i.e., 8.68-21.5%). Formulations were developed at pH ~4.5, at a moisture of 50% and shea butter was used to obtain 15% fat content. The differential scanning calorimetry thermograms of the samples showed a main peak around 30°C, corresponding to transition of the shea butter, and a smaller peak around 70°C related to starch gelatinisation. Analysis of microstructure showed formation of a protein matrix with more extensive protein structure at high protein concentration. Furthermore, none of the chickpea-based samples melted under the testing conditions and all samples showed increasing values for adhesiveness, springiness and cohesiveness with increasing protein content. However, hardness was the highest for the sample with the lowest protein content, likely due to starch retrogradation. After storage, hardness increased further for all samples. This work improves our understanding of the role of chickpea protein in developing plant-based alternatives to cheese and the challenges therein.

4.1 Introduction

Food systems (i.e., production, processing, distribution, preparation and consumption of food) are responsible for between 21 and 37% of all net anthropogenic greenhouse gas emissions (IPCC, 2019). In particular, due to their impact on global emissions, animal-based systems are currently major contributors to climate change and, in turn, biodiversity loss (Notarnicola *et al*., 2017; Benton *et al*., 2021). The growing global population and the corresponding need to increase food supply, combined with the high environmental impact of animal food production, are driving growth in the development of plant-based alternatives to animal-based products, such as cheese.

Due to increasing consumer awareness of the impact of food production on the environment, animal welfare and human health, consumption of plant-based food is increasing globally, with a growth in sales of 27% in the US in 2020 (SPINS & Good Food Institute, 2020). In particular, the US dollar sales for plant-based alternatives to cheese grew by 42% in 2020, and the sector is projected to reach almost $4 billion by 2024 (Bharat Book Bureau, 2017; SPINS & Good Food Institute, 2020). However, most commercially-available products currently rely on starch and solid fats (e.g., coconut oil) as their principal components, and have low protein and high saturated fat contents, making them nutritionally inferior to traditional cheese. Furthermore, from a consumers perspective, the taste and price of such commercial plant-based alternatives to cheese do not meet consumer expectations, and in fact represent the plant-based product category with the highest potential demand (i.e., product type that consumers would like to see more of in supermarkets) (Proveg International, 2020). To formulate plant-based alternatives to cheese with improved nutritional profiles and low environmental impact, a number of research groups are currently investigating the

suitability of plant protein ingredients (Mattice & Marangoni, 2020; Ferawati *et al*, 2021; Grossmann & McClements, 2021; Mefleh *et al*., 2021). The aim of several of these recent studies is mainly to develop alternatives to non-protein ingredients (e.g., polysaccharides) often used to build structure and mimic dairy proteins and fat in such applications. These are frequently used in cheese analogue formulations as inexpensive alternatives to protein, to partially replace casein (Bachmann, 2001). However, in addition to nutritional quality, dairy proteins provide cheese products with unique sensory and textural properties and the replication of such properties using plant proteins is challenging (Grossmann & McClements, 2021; Short *et al*., 2021).

Among the plant protein sources available, soy has been extensively used in plant-based cheese alternative applications for its ability to form a curd under specific processing conditions; more recently new ingredients such as zein have also been studied, showing promising results for such applications (Mattice & Marangoni, 2020).

Pulses are part of traditional diets in many countries and represent important sources of dietary proteins; thus, pulse flours, protein concentrates and isolates offer opportunities for novel food product development and can contribute to achieving recommended daily protein requirements (Boye *et al*., 2010). In particular, chickpeas are considered highly nutritious, with a protein content of 20-25% and high levels of fat, starch and fibre, as well as significant concentrations of minerals, vitamins and bioactive compounds (e.g., phenolic acid and isoflavones) (Hall *et al*., 2017). Due to their nutritional value and functional properties, chickpea protein ingredients (i.e., flour, protein concentrate and isolate) show great potential in the development of new and reformulated food products. Previous studies investigated functional properties of chickpea proteins of relevance in plant-based alternatives to cheese applications, such

as oil absorption capacity, emulsifying and gelling properties (Kaur & Singh, 2007; Papalamprou *et al*., 2009; Withana-Gamage *et al*., 2011). Chickpea protein ingredients showed good performance in such functional properties, probably due to the high levels of globulins (53-60% of total chickpea proteins), which, because of their highly structured nature due to disulphide bonds and hydrophobic interactions, strongly influence functionality (Ghumman *et al*., 2016). In addition, as for other pulses, chickpea protein ingredients have been employed in the development of plant-based milk alternatives (Wang *et al*., 2018; Lopes *et al*., 2020).

To the authors' knowledge, there are no published scientific studies available that investigated the use of chickpea protein ingredients in the development of plant-based alternatives to cheese. In this work, chickpea-based alternatives to cheese were formulated using chickpea flour and chickpea protein concentrate in different ratios.

The aim of this study was to determine the influence of protein concentration on chickpea-based alternatives to cheese, in terms of key quality attributes, such as structure and texture. Moreover, the age-induced changes in such attributes were assessed after 1 mo of storage. The results of this work will enhance our understanding of the role, and potential, of chickpea protein ingredients in formulating and developing high protein content chickpea-based alternatives to cheese, and the challenges therein.

4.2 Materials and methods

4.2.1 Ingredients

Commercially-available chickpea flour (CF) (Müller's Mühle GmbH, Gelsenkirchen, Germany), with 20% protein, 10.4% moisture, 60.8% carbohydrate, 37.8% starch, 6.15% fat and 2.67% ash, and chickpea protein concentrate (CPC) (Artesa, PLT Health Solutions, Morristown, NJ, US), with 53.1% protein, 7.73% moisture, 33.3% carbohydrate, 2.86% starch, 1.37% fat and 4.47% ash, were used in this study to formulate the chickpea-based alternatives to cheese. The composition of the CF and CPC were typical of pulse flours and concentrates. A shea butter ingredient (Zenitex M 50 G) kindly provided by Fuji Oil Europe (Gent, Belgium), was used as fat source, and was composed of 99.9% fat, with 49% of the fatty acids being saturated and 45% mono-unsaturated. The shea butter ingredient was chosen in this study due to its solid nature at room temperature and high ratio of unsaturated to saturated fatty acids and the lower content of saturated fats compared to coconut oil, which represents the most used source of fat in commercially-available plant-based cheeses. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, US), unless stated otherwise.

4.2.2 Formulation of cheese alternatives

The protein components of chickpea-based alternatives to cheese, hereafter referred to as chickpea-based samples, were formulated by blending CF and CPC in different proportions. Selected additions of CPC were used to obtain four chickpea-based samples of increasing protein, and consequently decreasing carbohydrate, contents (Table 4.1). An ingredient ratio based on protein contribution of 0:100, 50:50, 75:25 and 100:0 from CPC and CF was used to obtain the 4 samples, 0CPC-100CF,

50CPC-50CF, 75CPC-25CF and 100CPC-0CF, respectively. Lactic acid was added to water to achieve a pH of ~4.5 and 50% moisture content in the chickpea-based samples. Shea butter was added to obtain 15% fat content for all the samples, which was set as a target to align with the typical fat content of commercially-available "reduced-fat" cheese products. The final formulation and processing conditions described here were confirmed after numerous preliminary and optimisation trials. Samples were prepared by mixing the CF and CPC with water in a Thermomix (TM 5, Vorwerk, Wuppertal, Germany) at speed 1 (100 rpm). The temperature was set to 85°C and when 45°C was reached, shea butter was added to the mixture at speed 2 (200 rpm) for 5 min. After 2.5 min, the speed was increased to 3.5 (800 rpm) for 10 s to ensure that all ingredients were uniformly dispersed and incorporated in the mixture. Following this, samples were poured into moulds and stored for 24 h at 4°C before analysis and for 1 mo at 4°C to assess the influence of storage on selected properties of the chickpea-based samples.

Table 4.1. Formulation (%) of the chickpea-based samples made using chickpea flour (CF) and chickpea protein concentrate (CPC).

	CF	CPC	Shea butter	Lactic acid	Water
0CPC-100CF	43.3	0	12.3	5.10	40.4
50CPC-50CF	30.9	11.6	12.9	7.25	38.6
75CPC-25CF	19.6	22.2	13.5	9.20	37.0
100CPC-0CF	0	40.5	14.4	12.5	34.3

4.2.3 Compositional analysis of chickpea flour and protein concentrate ingredients and cheese alternatives

The composition of the CF and CPC was analysed prior to formulating the chickpea-based samples. Protein content of chickpea ingredients and chickpea-based cheese alternatives was measured using the Kjeldahl method and a nitrogen-to-protein conversion factor of 6.25, according to method 930.29 (AOAC, 1930) and 2001.14 (AOAC, 2002), respectively. Moisture of protein ingredients and samples was determined using oven drying at 103°C for 5 h, according to method 925.10 (AOAC, 1925) and 926.08 (AOAC, 1990), respectively. Ash content of CF and CPC was measured by incineration in a muffle furnace to 700°C for 5 h, according to method 923.03 (AOAC, 1923); for chickpea-based samples, ash content was analysed by incineration at 800°C for 5 h after pre-ashing in crucibles for 10 min, according to method 935.42 (AOAC, 1990). Fat content of protein ingredients and samples was assessed using the Soxhlet method with SoxCap and Soxtec (Foss UK Ltd, UK) according to the AACC method 30-25.01 (AACC, 2009) and AACC method 30-25.01 (AACC, 2009), respectively; activated silica was used to absorb moisture in the chickpea-based samples. Moreover, total starch content of CF and CPC was analysed using the enzyme kit K-TSTA (Megazyme, Bray, Ireland) according to method 996.11 (AOAC, 2005). Total carbohydrate of protein ingredients and chickpea-based samples was calculated by difference (i.e., 100 – sum of protein, fat, ash and moisture). Moreover, the pH of the chickpea-based samples was measured using a pH meter equipped with a FC200B Foodcare pH electrode for semi-solid foods (Hanna Instruments, Woonsocket, RI, US) and the water activity (a_w) was measured at 20°C using a water activity meter after calibration (Aqua Lab, Decagon Devices, Inc., Pullman, WA, US).

4.2.4 Colour assessment

The colour of the chickpea-based samples was assessed by measuring the CIE LAB coordinates (L*, a* and b*) with a Chroma Meter CR-400 (Konica Minolta Sensing, Inc., Osaka, Japan), calibrated using a white tile. The colour assessment was performed after 24 h of storage at 4°C and after meltability measurement (Section 4.2.8) and repeated on 1 mo old samples before and after meltability measurement.

4.2.5 Electrophoretic protein profile analysis of chickpea flour and protein concentrate ingredients

The protein profile of CF and CPC was measured using a Capillary Electrophoresis instrument (PA 800 plus Pharmaceutical Analysis System, Sciex, Kildare, Ireland) equipped with a photo diode array (PDA) detector. The powder samples were mixed directly with the sodium dodecyl sulphate (SDS) molecular weight (MW) sample buffer (Sciex, Kildare, Ireland) containing 100 mM Tris-HCl, pH 9.0, 1% SDS at a protein concentration of 2 mg/mL and mixed over 14 h at 4°C and over 6 h at 20°C. After rehydration, 95 µL of sample was mixed with 2 µL of 10 kD internal marker, and 5 µL 2-iodoacetamide (IAM) and heated at 70°C for 3 min for non-reducing conditions. While under reducing conditions, samples (95 µL) were mixed with 2 µL of 10 kDa internal marker, and 5 µL 2-mercaptoethanol (2ME) and heated at 100°C for 3 min. After heating, samples were cooled at room temperature and transferred into micro sample tubes.

Separation was obtained using a 50-µm bare fused-silica capillary of 30 cm with a 20.2 cm effective length from the inlet to the detection window. All CE-grade reagents were obtained as part of the ProteomeLab™ SDS-MW Analysis Kit (Beckman Coulter, CA, US), designed for the separation of protein-SDS complexes

using a replaceable gel matrix. The separating gel was formulated to provide an effective sieving range of approximately 10 to 225 kDa. The SDS-MW size standard (from 10 to 225 kDa, Beckman Coulter, CA, US) was used to estimate the protein MW distribution of the sample, with a 10 kDa protein (Beckman Coulter, CA, US) used as a mobility marker. A capillary conditioning method was run before analysing each sample, which consisted of a basic rinse (0.1 N NaOH, 10 min, 20 psi), followed by an acidic rinse (0.1 N HCl, 5 min, 20 psi), a water rinse (CE-grade H2O, 2 min, 20 psi) and finally an SDS gel separation buffer rinse (10 min, 70 psi). The voltage equilibration (15 kV for 10 min, with 5 min ramping time) was then applied to the filled SDS gel. The total protein concentration of each sample was 2 mg/mL after the addition of the SDS-MW sample buffer (Beckman Coulter, CA, US). Each sample was injected into the gel-filled capillary by pressure injection in reverse polarity at -5 kV for 20 s. The separation was performed at 15 kV for 30 min with reverse polarity in filled SDS gel. All CE steps were carried out at room temperature. UV detection of migrating proteins was monitored at 220 nm. Data were analysed using 32 Karat™ software (version 8.0, Beckman Coulter, CA, US).

4.2.6 Differential scanning calorimetry analysis of the ingredients and cheese alternatives

Thermograms of the CF, CPC, shea butter and chickpea-based samples were obtained using a Mettler DSC821 (Mettler-Toledo, Schwerzenbach, Switzerland) differential scanning calorimeter (DSC) equipped with liquid nitrogen cooling. The shea butter ingredient was weighed (12.5-18.1 mg) into standard aluminium pans (Mettler, 40 µl) which were hermetically sealed. The powder ingredients (i.e., CF and CPC) were weighed (5.2-8.6 mg) into aluminium pans and ~10 mg of water was added

to hydrate the powders. Chickpea-based samples were also weighted (17.2-21.1 mg) into aluminium pans. The calorimeter was calibrated for temperature and heat flow using indium. The thermal behaviour of the ingredients and chickpea-based samples was recorded from 0 to 100°C at a heating rate of 5°C/min. The DSC curves were analysed using Mettler-Toledo STARe system version 8.10 for thermal analysis. Samples were analysed after 24 h at 4°C and after 1 mo of storage at 4°C.

4.2.7 Confocal laser scanning microscopy

The microstructural observations of the chickpea-based samples were performed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan) with a 40x objective lens. The chickpea-based samples were placed onto a glass slide and fat and protein were stained as previously described by Le Tohic *et al*. (2018) with ~50 µL of a mixture of Nile Red in 1,2-propanediol (600 µL of 0.1 g/L) and Fast Green FCF aqueous solution (200 µL of 0.1 g/L), respectively. Images were obtained after exciting the Nile Red and Fast Green FCF at 488 and 633 nm, using Ar and He-Ne lasers, respectively (Auty *et al*., 2001). Representative images of the chickpea-based samples after ~5 d at 4°C and after 1 mo at 4°C were reported.

4.2.8 Schreiber meltability test

Meltability of the chickpea-based samples was measured after 24 h at 4°C and after 1 mo, using the Schreiber test (Altan *et al*., 2005). Cylinders, of height 5 mm and diameter 41 mm, were prepared by pouring the chickpea-based mixture into stainless steel moulds after preparation in the Thermomix and stored. After storage, the samples were placed in a covered glass Petri dish, pictures were taken, and the samples were

heated at 232°C for 5 min in an oven (Memmert, Schwabach, Germany). After cooling the samples at room temperature for 30 min, pictures were taken again, and specimen expansion was measured with a ruler along six lines marked on a set of concentric circles.

4.2.9 Texture profile analysis

Texture profile analysis (TPA) of the chickpea-based samples, defined as the compression of a bite-size piece of food, two times in a reciprocating motion, imitating the action of the human jaw (Bourne, 2002a), was performed using a Texture Analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK), as previously described by Grasso *et al*. (2021), with minor modifications. Cylinders of 12 mm height and 20 mm diameter were prepared by pouring the chickpea-based mixture, after the Thermomix step, in glass moulds precoated with siliconizing reagent for glass (Sigmacote®, Sigma-Aldrich, MO, US). The samples were kept at room temperature in the moulds for at least 4 h, after which they were removed from the moulds and stored at 4°C for 24 h and for 1 mo. After removal from storage, samples were compressed to 30% of their original height in a double compression at a rate of 1.0 mm/s. Hardness, adhesiveness, springiness and cohesiveness, as previously defined by Fox *et al*. (2017) and Kasapis & Bannikova (2017), were measured for each sample.

4.2.10 Statistical data analysis

Compositional analysis of the CF and CPC ingredients, and of chickpea-based samples, was performed in triplicate, as well as DSC analysis of the ingredients (i.e., CF, CPC and shea butter). Electrophoretic protein profile analysis of the powder ingredients was performed in duplicate. Two independent trials were conducted to

develop the chickpea-based samples and three independent replicates from each trial were used for all the analyses, except for the DSC analysis of the chickpea-based samples which was performed with two independent replicates from each of the two trials. Results are expressed as mean ± standard deviation, unless otherwise stated. Levene's test was used to check the homogeneity of variance and one-way analysis of variance (ANOVA) was carried out using SPSS version 25 (SPSS Inc., Chicago, IL, USA). A Tukey's paired comparison post-hoc test was used to determine statistically significant differences ($p < 0.05$) between mean values for samples with different formulations, at a 95% confidence level. The paired t-test was used to identify statistically significant differences ($p < 0.05$) between fresh and aged (1 mo) samples, at a 95% confidence level.

4.3 Results and discussion

4.3.1 Composition of chickpea flour and protein concentrate ingredients and cheese alternatives, and physical appearance of cheese alternatives

The chickpea-based samples were formulated as described in Section 4.2.2, using the compositional information available for the CF and CPC ingredients, with the target compositional parameters provided in Table 4.1. In good agreement with the predicted formulation, measured protein content of the chickpea-based samples ranged from 8.68 to 21.5% (Table 4.2). Measured moisture contents were slightly lower than those values from formulation prediction (50%), probably due to water evaporation during the thermo-mechanical processing. Consequently, the carbohydrate content of the samples, calculated by difference, was higher than the predicted values. Fat contents, as expected from the formulations, were not significantly different among the chickpea-based samples. Ash values were in agreement with the ash content found in the powder ingredients, with total ash content increasing with increasing addition level of CPC. The addition of lactic acid led to pH values ranging from 4.39 to 4.50, similar to commercial plant-based cheeses (Grasso *et al*., 2021). To achieve these pH values, higher amounts of acid were added with increasing protein contents, probably due to the buffering capacity of the globulin fractions of chickpea protein (Martínez-Villaluenga *et al*., 2007). After 1 mo of storage at 4°C, pH values ranged from 4.42 to 4.50. The pH value for the 0CPC-100CF sample (4.50) did not differ from the value measured after 24 h at 4°C, again, likely due to the higher buffering capacity of the chickpea-based samples at higher protein contents; indeed, for the 100CPC-0CF sample, the pH increased very slightly from 4.39 to 4.42. The a_w values for chickpea-based samples ranged from 0.974 to 0.981, with the values being similar to those for plant-based cheeses available commercially (Grasso *et al*., 2021).

Table 4.2. Composition of the chickpea-based samples made using chickpea flour (CF) and chickpea protein concentrate (CPC).

	Protein (%)	**Fat (%)**	**Carbohydrates (%)**	**Ash (%)**	**Moisture (%)**	**pH (-)**	**a_w (-)**
0CPC-100CF	8.68 ± 0.10^{d}	15.8 ± 0.30^{a}	27.8	1.07 ± 0.07^{d}	46.7 ± 0.40^{ab}	4.50 ± 0.00^{a}	0.981 ± 0.001^{a}
50CPC-50CF	12.2 ± 0.20^{c}	15.8 ± 0.25^{a}	23.2	1.33 ± 0.11^{c}	47.4 ± 0.94^{a}	4.42 ± 0.00^{c}	0.979 ± 0.001^{a}
75CPC-25CF	15.7 ± 0.01^{b}	15.6 ± 0.84^{a}	21.4	1.64 ± 0.02^{b}	45.6 ± 0.24^{b}	4.45 ± 0.01^{b}	0.976 ± 0.00^{b}
100CPC-0CF	21.5 ± 0.65^{a}	15.0 ± 0.91^{a}	16.0	2.03 ± 0.07^{a}	45.4 ± 0.39^{b}	4.39 ± 0.00^{d}	0.974 ± 0.001^{b}

Values followed by different superscript letters in a column (a-d) are significantly different ($p < 0.05$).

The colour space values of the chickpea-based samples after 24 h and 1 mo of storage at 4°C are reported in Figure 4.1. The L* value, representing brightness with values ranging from 0 to 100, was significantly higher for the 0CPC-100CF sample compared to the other samples, with values for L* decreasing with increasing protein content. The a* value measures the degree of redness (associated with positive values) or greenness (associated with negative values), and increased with increasing protein content in samples stored for 24 h at 4°C. The b* value, representing the degree of yellowness (associated with positive values) or blueness (associated with negative values), was significantly lower for the 0CPC-100CF sample than the other chickpea-based samples. After 1 mo of storage, all samples showed lower L* and b* values compared to fresh samples stored for 24 h.

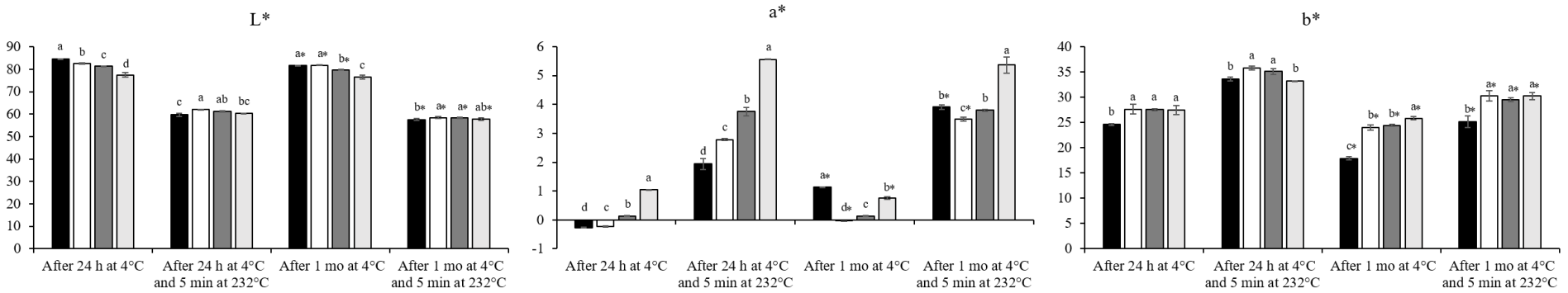


Figure 4.1. Colour space values before and after 5 min at 232°C of chickpea-based samples after 24 h at 4°C and after 1 mo of storage at 4°C are shown. Bars represent 0CPC-100CF (■), 50CPC-50CF (□), 75CPC-25CF (▩) and 100CPC-0CF (▨) samples. Different letters on bars of the group (a-d) indicate significant differences between samples ($p < 0.05$), with significance of differences between samples after 24 h at 4°C and after 1 mo of storage at 4°C identified with independent t-test and ∗ indicates significant differences ($p < 0.05$).

4.3.2 Protein profile of chickpea flour and protein concentrate ingredients

The protein profiles of the CF and CPC under reducing and non-reducing conditions are shown in Figure 4.2. Chickpea protein fractions are classified as globulins, 53-60% of total protein, glutelins, 19-25%, albumins, 8-12%, and prolamins, 3-7% (Osborne, 1924; Day, 2013). The peaks around 35-40 and 20 kDa of the CF and CPC electropherograms under reducing conditions (Fig. 4.2), corresponded to the 11S legumin (the main globulin in chickpeas) acidic (α-legumin) and basic (β-legumin) chains, respectively, probably due to the dissociation of legumin into its acidic and basic subunits under reducing conditions, in agreement with previous studies (Sánchez-Vioque *et al*., 1999; Papalamprou *et al*., 2009). Indeed, under non-reducing conditions, such peaks were smaller and a peak at higher MW (i.e., around 60 kDa) was visible (Papalamprou *et al*., 2009; Vogelsang-O'Dwyer *et al*., 2020). Other than legumin, another globulin found in chickpeas is 7S vicilin, a trimeric protein, and its subunits corresponded to the peaks around 50 kDa (i.e., major fraction) and around 15, 32 and 70 kDa (i.e., several minor subunits) of the CF and CPC electropherograms, particularly visible under non-reducing conditions, as also reported by Chang *et al*. (2012). Peaks around 20 and 55 kDa might be associated with glutelin fractions, as observed by Chang *et al*. (2011); indeed, the same authors reported similarities between these MWs of chickpea protein fractions and those for rice glutelins. While generally similar protein profiles were evident for both the CF and CPC ingredients, two peaks situated between 60 and 100 kDa, were more intense for the CF than the CPC ingredient, under both reducing and non-reducing conditions. The first of the two peaks, with lower MW, may be attributed to convicilin, a globular protein with MW ~70 kDa. The proportion of convicilin, and more generally the protein profile of chickpea, may vary according to the agronomic practices used for

chickpea seed production (e.g., conventional *vs* organic) and to the exact chickpea genotype (De Santis *et al*., 2021). The higher MW protein is possibly lipoxygenase, which normally has MW of 92-94 kDa. The lipoxygenase enzyme, an albumin protein, might be partially lost during protein enrichment, which is why its peak is less intense on the CPC electropherograms, in agreement with results from previous research (Sánchez-Vioque *et al*., 1999).

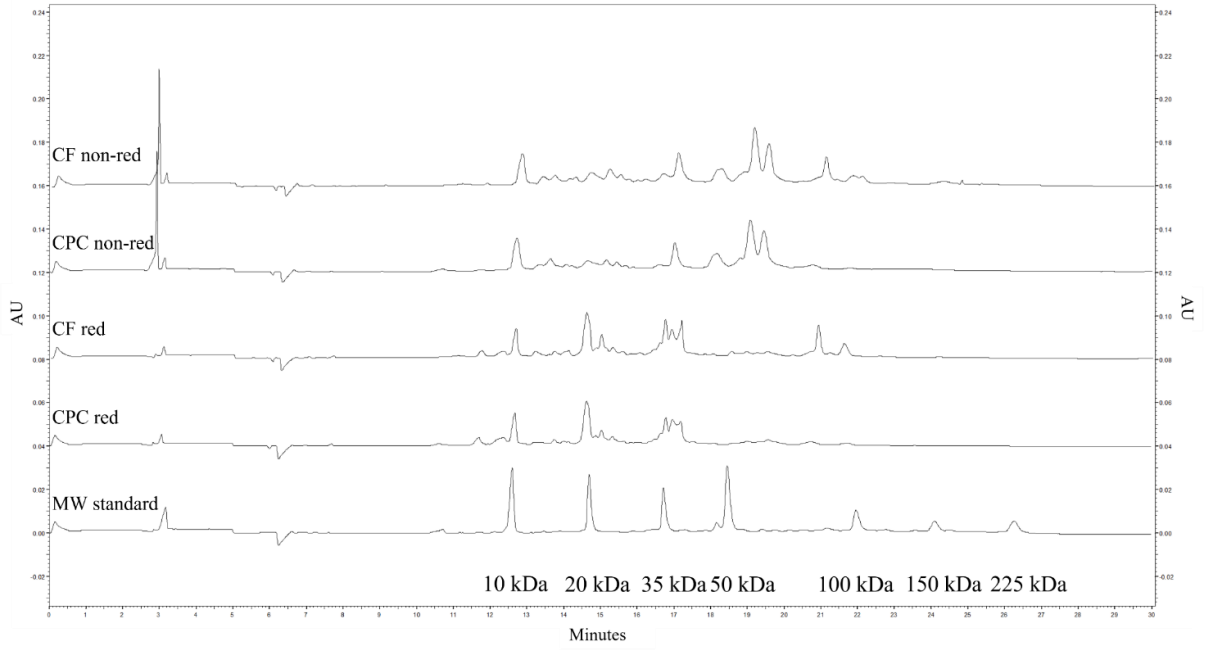


Figure 4.2. Protein profile of chickpea flour (CF) and chickpea protein concentrate (CPC) under reducing and non-reducing conditions. From top to bottom, the first two electropherograms represent CF and CPC under non-reducing conditions, respectively, third and fourth electropherograms represent CF and CPC under reducing conditions, respectively. The bottom electropherogram represents the MW standard.

4.3.3 Thermal behaviour of the ingredients and cheese alternatives

Differential scanning calorimetry (DSC) analysis was performed on the ingredients to develop an understanding, and ultimately support prediction, of the behaviour of these ingredients during the thermal processing involved in the manufacture of the chickpea-based samples, with the results presented in Figure 4.3. The shea butter ingredient (Fig. 4.3a) showed a main peak at 35°C and smaller peaks at lower temperatures (around 5, 15 and 25°C), due to the polymorphic nature of shea butter, in agreement with the thermograms previously reported by Lawer-Yolar *et al*. (2019). The main peak of CF was at 68.1°C (Fig. 4.3b), corresponding to starch gelatinisation, with starch representing 37.8% of the CF ingredient. This temperature was comparable to the peak temperatures for desi and kabuli chickpea starches reported by Miao *et al*. (2009). According to the same authors, some of the main factors influencing gelatinisation temperature of chickpea starch are amylose content, size, form and distribution of starch granules, as well as distribution of amylopectin short chains. The CPC showed 2 peaks, the first at 77.1°C, which was smaller compared to the second peak at 93.7°C (Fig. 4.3c). The presence of a shoulder peak at 77.1°C was probably associated with denaturation of the (7S) vicilin, while the major peak (93.7°C) corresponded to denaturation of the (11S) legumin fraction, as also previously reported by Withana-Gamage *et al*. (2011). Denaturation temperatures reported in the literature for chickpea protein ingredients range between 78.7 and 99.8°C, with protein structure and composition, chickpea variety (i.e., desi or kabuli) and the processing conditions used to concentrate the proteins, influencing the thermal properties of the ingredient (Paredes-Lopez *et al*., 1991; Kaur & Singh, 2007; Mousazadeh *et al*., 2018).

The thermograms of the chickpea-based samples are shown in Figure 4.4. All

four samples displayed a main peak around 30°C, corresponding to transition of the shea butter ingredient, with a smaller peak around 70°C related to starch gelatinisation. This second peak decreased with decreasing carbohydrate content (i.e., mainly starch) in the chickpea-based samples, according to the formulations (Table 4.1). The starch component was gelatinised during the thermal process, due to the processing temperature of 85°C. However, the samples were stored for 24 h at 4°C before analysis (Section 4.2.6), leading to starch retrogradation and consequent re-gelatinisation during the heating ramp of the DSC analysis, as previously observed for native potato starch analysed before and after 5 d of storage (Morikawa & Nishinari, 2000). The profile of the shea butter transition peak in the chickpea-based samples was narrower for the 0CPC-100CF sample (Fig. 4.4a), comparable to the thermogram of the shea butter ingredient (Fig. 4.3a), with the respective component showing wider profiles for the samples with higher protein contents. This was probably due to the different distribution of protein and fat among the samples; indeed, for the 100CPC-0CF sample, the protein formed a matrix surrounding the fat globules, as evident from the microstructural analysis (Fig. 4.5g). No differences were observed between the thermograms before and after 1 mo of storage at 4°C (data not shown).

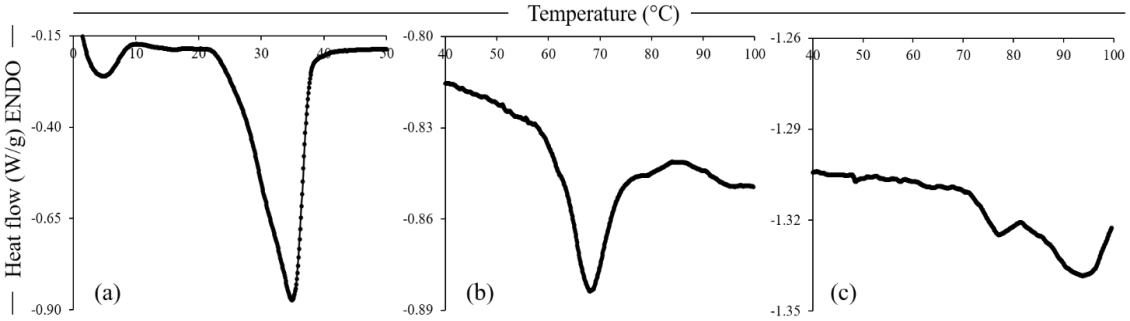


Figure 4.3. Differential scanning calorimetry thermograms of shea butter (a), chickpea flour (CF) (b) and chickpea protein concentrate (CPC) (c).

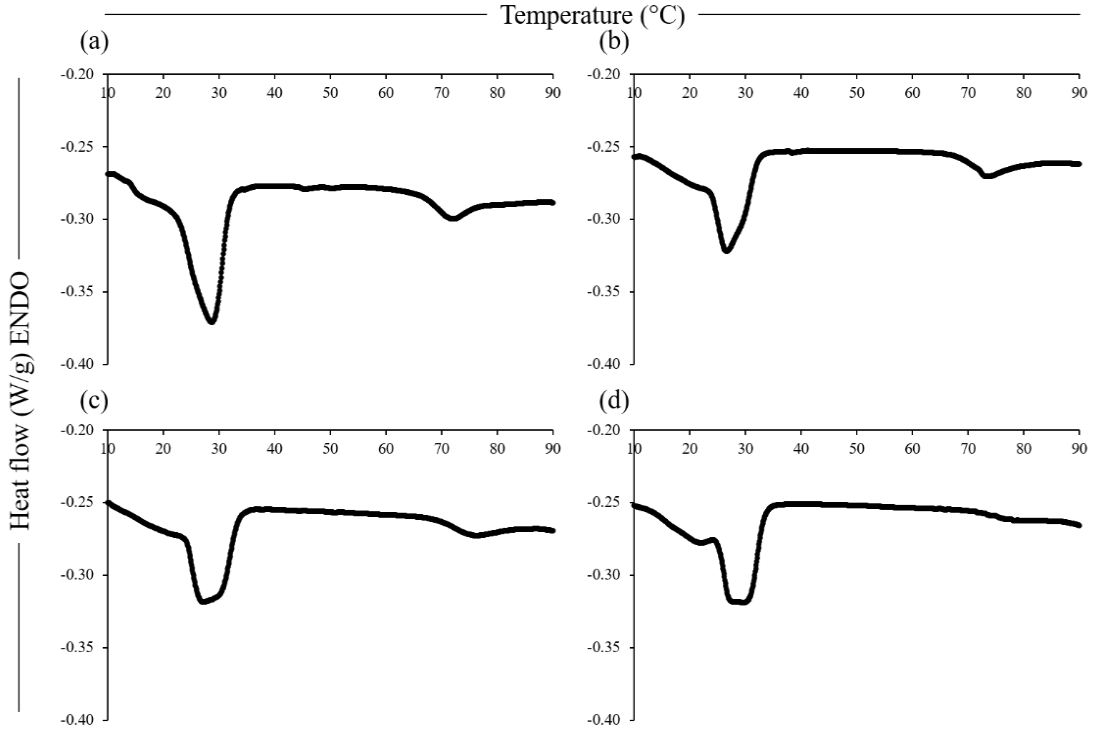


Figure 4.4. Differential scanning calorimetry thermograms of the chickpea-based samples, 0CPC-100CF (a), 50CPC-50CF (b), 75CPC-25CF (c) and 100CPC-0CF (d).

4.3.4 Microstructure

Microstructural images of the chickpea-based samples are reported in Figure 4.5. Samples after ~5 d of storage (Fig. 4.5a, b, c, d) showed formation of a protein matrix and a low occurrence of the carbohydrate components, associated with increasing

protein contents. Similar observations were recorded for samples stored for 1 mo (Fig. 4.5e, f, g, h). Fat globules showed both spherical and non-spherical coalesced pools in all samples. However, the size of fat globules, as well as coalescence of same, decreased with increasing protein contents and a homogeneous distribution throughout the protein matrix was observed in the 75CPC-25CF and 100CPC-0CF samples. The 0CPC-100CF sample showed many black areas, indicating that the carbohydrate constituents gave structure to the sample and, in turn, influenced its physicochemical properties. No major differences in the microstructure were observed between samples before and after storage.

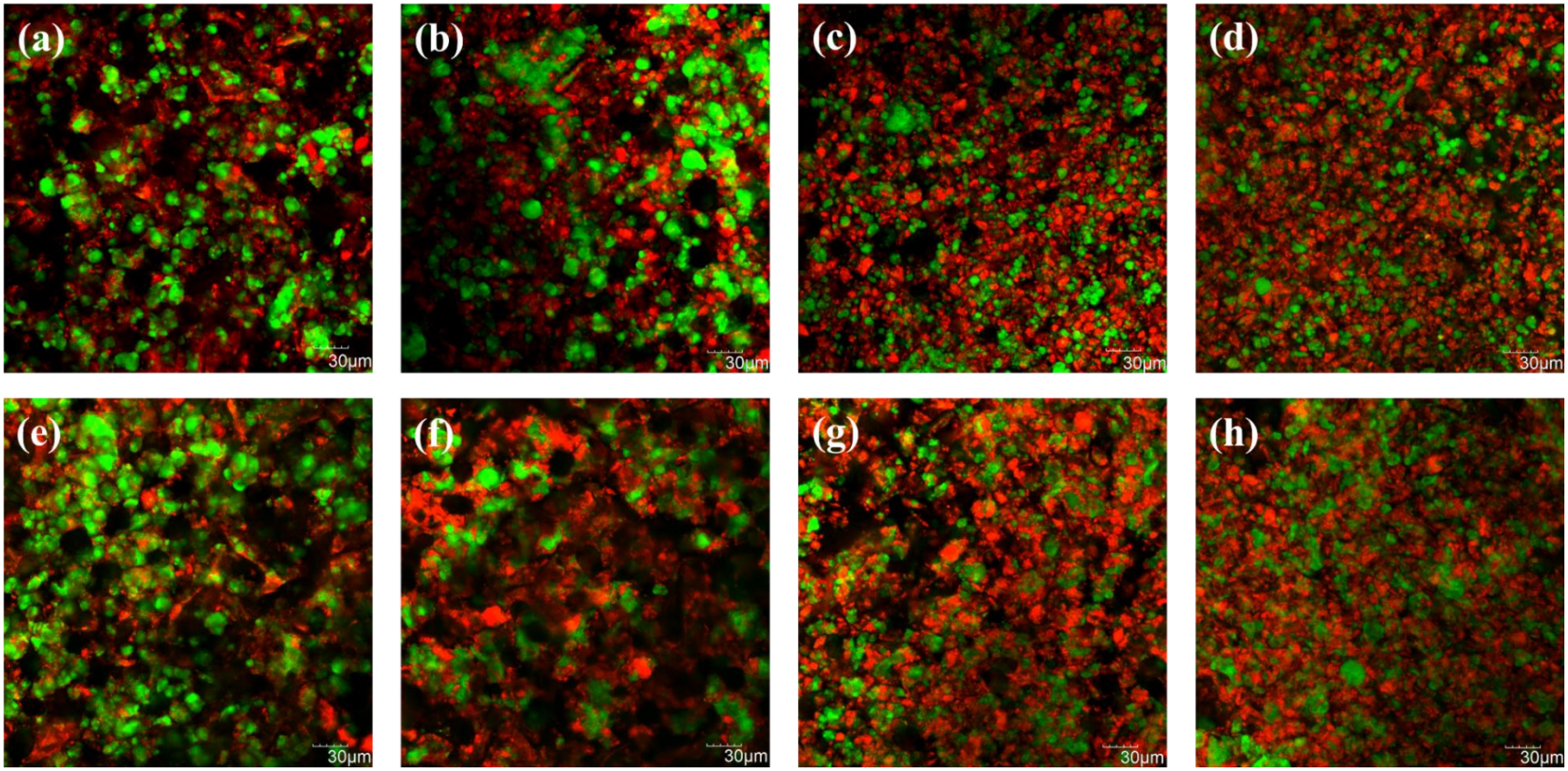


Figure 4.5. Confocal laser scanning microscopy images of chickpea-based samples, after ~5 d at 4°C 0CPC-100CF (a), 50CPC-50CF (b), 75CPC-25CF (c) and 100CPC-0CF (d) and samples after 1 mo of storage at 4°C 0CPC-100CF (e), 50CPC-50CF (f), 75CPC-25CF (g) and 100CPC-0CF (h) are shown. Fat and protein are represented in green and red, respectively.

4.3.5 Meltability

As evident in Figure 4.6 (a, c, e, g), none of the chickpea-based samples melted under the testing conditions, since no differences in diameter were observed between the samples before and after the test. The same behaviour was noted for the samples after 1 mo of storage (Fig. 4.6b, d, f, h). A dry surface of the samples after oven heating was visually observed, and this increased with increasing protein content. On heating, samples stored for 1 mo were dryer compared to samples stored for 24 h at 4°C, with sample 100CPC-0CF (Fig. 4.6h) showing fractures on the surface. During oven heating, in the lower protein content samples water was probably absorbed by the starch granules to gelatinise, while the high protein samples showed more dehydration. Furthermore, with temperature increasing over the gelatinisation temperature, water continues to be absorbed by starch, leading to disorganisation of the crystalline structure and more solid-like texture, affecting meltability, and this is probably due to the high levels of amylose in chickpea starch (Lertphanich *et al*., 2013; Zhang *et al*., 2016). Poor melting characteristics were previously observed for commercial plant-based cheese products (Grasso *et al*., 2021). Improvements of the melting behaviour of chickpea-based systems will be necessary for application of same in the formulation of alternatives to cheese products. Moreover, as evident from the data for colour analysis reported in Figure 4.1, thermal processing greatly affected the colour of the samples, which had lower L* and higher a* values (i.e., more intense red colour), and higher b* values (i.e., more intense yellow colour), with L* values of heated samples decreasing after 1 mo of storage.

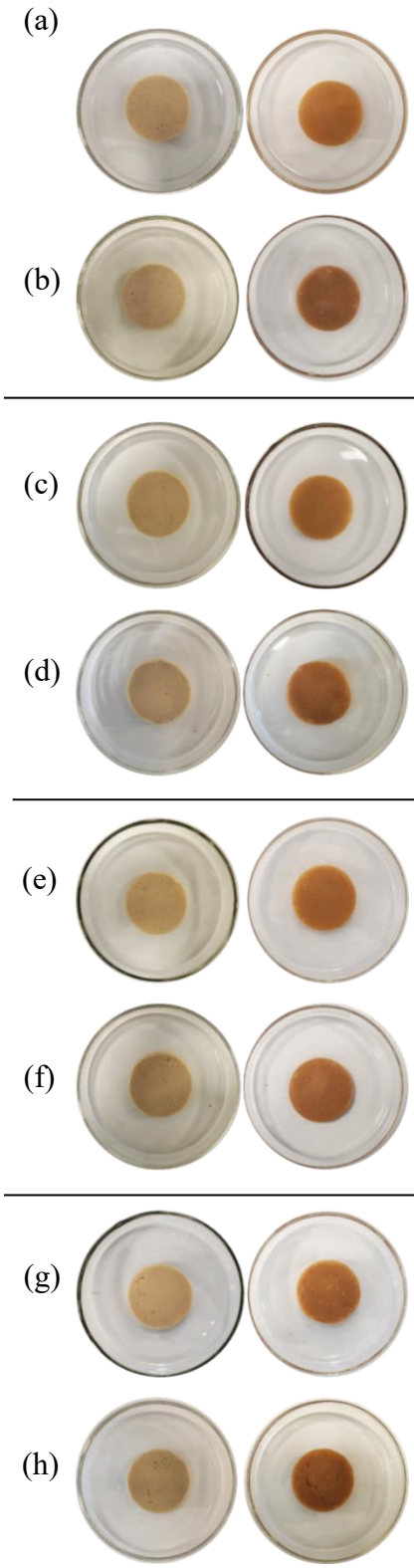


Figure 4.6. Photographs of chickpea-based samples before (left) and after 5 min at 232°C (right), samples after 24 h at 4°C 0CPC-100CF (a), 50CPC-50CF (c), 75CPC-25CF (e) and 100CPC-0CF (g) and samples after 1 mo of storage at 4°C 0CPC-100CF (b), 50CPC-50CF (d), 75CPC-25CF (f) and 100CPC-0CF (h) are shown.

4.3.6 Textural properties

Texture is one of the principal quality features of food and is defined as the response of the tactile sense to physical stimuli, resulting from contact between the food and some part of the body (Bourne, 2002b). The texture parameters hardness, adhesiveness, springiness and cohesiveness, derived from TPA analysis, of the chickpea-based samples after 24 h at 4°C and after 1 mo of storage, are shown in Figure 4.7. All the samples showed increasing values of adhesiveness, springiness and cohesiveness with increasing protein contents, with the same general trend evident after storage. Adhesiveness is related to the structure of the protein matrix and to the interactions between fat and protein, which influence the adherence between the product and the contact surface (Cunha *et al*., 2010); increasing adhesiveness with increasing protein content was previously observed in processed cheese (Sołowiej *et al*., 2015). The values for adhesiveness of chickpea-based samples were higher than those observed previously for commercial plant-based cheeses and Cheddar, being more similar to commercial processed cheese, with the same observed for the cohesiveness results (Grasso *et al*., 2021). The high cohesiveness for the 100CPC-0CF sample, which is a measure of the strength of the internal bonds within the product, was attributed to the strong protein matrix, as observed from microstructural analysis (Section 4.3.4). Hardness was highest for the 0CPC-100CF sample, being significantly different from the other samples; this is possibly due to retrogradation of the starch component of the 0CPC-100CF sample. After 1 mo at 4°C, a slight increase in hardness was observed for all samples, in particular for the 0CPC-100CF sample, likely due to rearrangement of starch (e.g., retrogradation) and protein fractions during storage. This is in agreement with the results reported by Zhang *et al*. (2016), where chickpea starch gels showed increasing firmness over time. Indeed, in combination

with the high proportion of amylose in chickpea starch, the authors related this firm texture to the crystallisation of amylopectin within the starch paste. Indeed, amylose can form junction zones quickly, re-associate, and then re-create intermolecular hydrogen bonds (Zhang *et al*., 2016). In general, the hardness values reported in the current study for chickpea-based samples were lower compared to commercial plant-based and dairy cheese products previously studied, with only the 0CPC-100CF sample being similar to processed cheese (Grasso *et al*., 2021), with adhesiveness, springiness and cohesiveness decreasing during storage for all samples.

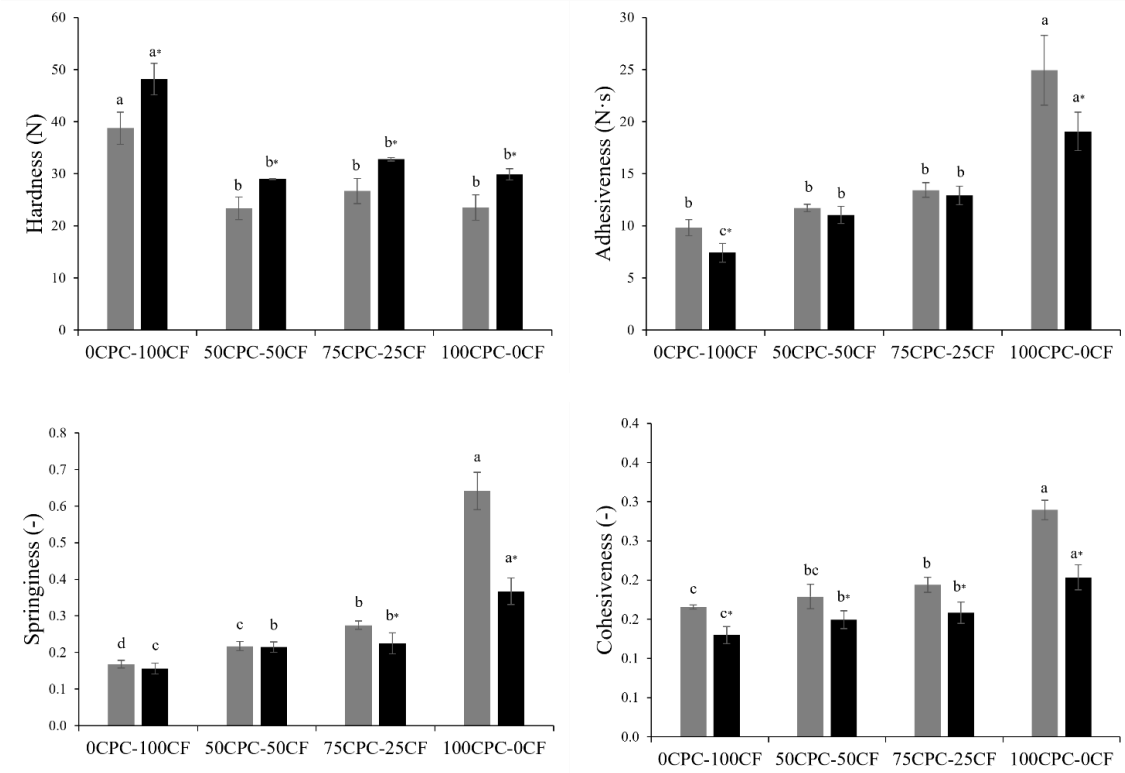


Figure 4.7. Texture profile analysis parameters hardness, adhesiveness, springiness and cohesiveness of chickpea-based samples, after 24 h at 4°C (▣) and after 1 mo of storage at 4°C (■) are shown. Different letters on bars of the same colour (a-d) indicate significantly different samples ($p < 0.05$), with significance of differences between samples after 24 h at 4°C and after 1 mo of storage at 4°C identified with independent t-test and ∗ indicates significant differences ($p < 0.05$).

4.4 Conclusion

The influence of protein concentration on key quality attributes of chickpea-based alternatives to cheese was studied. The samples showed differences based on protein content, particularly in terms of microstructure and textural analyses. Microstructural analysis of the samples demonstrated that formation of a stronger protein matrix, with the ability to surround fat globules and reduce coalescence, was intensified with increasing protein content. The samples showed higher values for adhesiveness, springiness and cohesiveness with increasing protein content, while hardness was highest for the sample with lowest protein content, associated with the high starch content of that sample. None of the samples melted under the testing conditions; further research should focus on improving the melting behaviour of such formulations for application as alternatives to cheese. The effect of storage for 1 mo was mainly only evident in terms of colour and texture analyses, with lower brightness and higher hardness observed after storage. The results of this work showed the effect of chickpea protein concentration on quality attributes in the development of chickpea-based alternatives to cheese and improved the understanding of the challenges related to such applications. Furthermore, these new insights will help inform future research questions in this area.

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Chapter 5

Plant-based alternatives to cheese formulated using blends of zein and chickpea protein ingredients

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Abstract

In this study, zein protein isolate (ZPI) and chickpea protein concentrate (CPC) ingredients were used to formulate five plant-based cheese alternatives. Ingredient ratios based on protein contributions of 0:100, 25:75, 50:50, 75:25 and 100:0 from ZPI and CPC, respectively, were used. Formulations were developed at pH ~4.5, with a moisture target of 59%. Shea butter was used to target 15% fat, while tapioca starch was added to target the same carbohydrate content for all samples. Microstructural analysis showed differences among samples, with samples containing ZPI displaying a protein-rich layer surrounding the fat globules. Schreiber meltability and dynamic low amplitude oscillatory shear rheological analyses showed that increasing the proportion of ZPI was associated with increasing meltability and greater ability to flow at high temperatures. In addition, the sample containing only CPC showed the highest adhesiveness, springiness and cohesiveness values from the texture profile analysis, while the sample containing only ZPI exhibited the highest hardness. Furthermore, stretchability increased with increasing ZPI proportions. This work will help understanding of the role and potential of promising plant-protein ingredient blends in formulating plant-based alternatives to cheese with desirable functional properties.

5.1 Introduction

Cheese represents an important food product in many cultures, with a long history of production and consumption (McSweeney *et al*., 2004). Dairy production sectors have rapidly intensified over the past several decades, leading to high productivity, which, although increasing overall economic profits has been accompanied by some undesirable social and environmental consequences (Clay *et al*., 2020). On the other hand, the availability of new food products is expanding, leading consumers to consider numerous factors when purchasing their food (Tso *et al*., 2020). In particular, drivers known to enhance consumer interest in plant-based food include food intolerances, social trends, and environmental sustainability, health and animal welfare considerations (Aschemann-Witzel *et al*., 2020; Grossmann & McClements, 2021). As a result, the plant-based cheese alternative sector is growing, and household penetration of this category is on the rise as more consumers experiment with plant-based cheese. Indeed, in the US between 2020 and 2021, the percentage of households purchasing plant-based cheese increased by 20% (Good Food Institute & SPINS, 2021).

Plant-protein ingredients are currently being studied for their potential in the development of plant-based alternatives to cheese. However, designing plant-based products with composition and functionality that closely match the properties of traditional cheese is challenging (Grossmann & McClements, 2021). Indeed, dairy proteins are largely responsible for the unique nutritional, physicochemical and sensorial attributes of cheese products. At present, commercially-available plant-based cheese alternatives rely strongly on non-protein ingredients (i.e., starch and coconut oil) to deliver functionality to the final product, resulting in low protein and high saturated fat contents. Moreover, the composition, microstructure and functional

properties (e.g., meltability) of such products differ considerably from those of cheese (Grasso *et al*., 2021). Plant-protein ingredients can be employed in the development of new and reformulated foods with improved nutritional profiles, while also providing specific desirable functional attributes (Boye *et al*., 2010).

Among the plant-protein ingredients studied for application in alternative cheese products, pulses are considered to be a valuable source of macronutrients, with functionality of relevance in such applications (Mefleh *et al*., 2021). In particular, chickpeas are very nutritious and represent an important source of dietary protein, with a content of 20–25%, as well as having high contents of fat, starch and fibre, minerals and vitamins (Hall *et al*., 2017). Furthermore, due to the predominance of globulin, chickpea protein ingredients show good oil absorption capacity and emulsification and gelling properties (Kaur & Singh, 2007; Withana-Gamage *et al*., 2011). Chickpea protein ingredients have been studied by our group in relation to the development of plant-based cheese alternatives, and have shown both promising results and challenges, the latter mainly related to poor meltability of the final product (Grasso *et al*., 2022). Besides pulses, another plant protein that displays strong potential in plant-based cheese alternative applications is zein, due to its unique plastic behaviour in aqueous environments (Mattice & Marangoni, 2020b). Zein is the hydrophobic major prolamin fraction extracted from maize, in which it represents 45–50% of the total protein (Shukla & Cheryan, 2001); it is composed of α, β, γ and δ fractions and is soluble in aqueous ethanol solutions. However, the production process for commercial zein results primarily in the α fraction (Anderson & Lamsa, 2011). Due to its structure and high proportion of non-polar amino acids, zein is self-aggregating and hydrophobic, and non-covalent interactions are largely responsible for its ability to form viscoelastic networks (Argos *et al*., 1982; Smith *et al*., 2014). Given these unique

physicochemical characteristics, zein has been extensively used to entrap other substances, such as vitamins, and to stabilise oil-in-water emulsions (Fathi *et al*., 2018). From a nutritional point of view, based on the Protein Digestibility-Corrected Amino Acid Score (PDCAAS), zein can be compared to wheat proteins, which are usually combined with complementary proteins to improve their nutritional value without losing functionality (Mattice & Marangoni, 2020a).

The use of blends of different plant proteins in food products is increasing (e.g., in commercial plant-based milk alternatives), providing improvements in the physicochemical, nutritional and sensory properties of the final product (Jiménez-Munoz *et al*., 2021; Reyes-Jurado *et al*., 2021). In this study, zein and chickpea protein ingredients were formulated in binary blends and their combined effect on the development and physicochemical properties of plant-based cheese alternatives was investigated. The results of this work will help in furthering understanding of the role and potential of blends of promising plant-protein ingredients in the formulation of plant-based alternatives to cheese with desirable functional properties.

5.2 Materials and methods

5.2.1 Ingredients

The cheese alternative samples were formulated using commercially-available zein protein isolate (ZPI) (Flo Chemical Corporation, Ashburnham, MA, USA), with 81.5% protein, 5.62% moisture, 3.92% carbohydrate, 7.68% fat and 1.28% ash, and chickpea protein concentrate (CPC) (Artesa, PLT Health Solutions, Morristown, NJ, USA), with 53.1% protein, 7.73% moisture, 33.3% carbohydrate, 1.37% fat and 4.47% ash. Tapioca starch, with 0.11% protein, 11.3% moisture, 88.3% carbohydrate, 0.25% fat and 0.03% ash, was purchased from a local retail outlet (Quay-coop, Cork, Ireland). Shea butter was kindly provided by Fuji Oil (Zenitex M 50 G, Fuji Oil Europe, Ghent, Belgium), and was employed as a source of solid fat and as an alternative to the more commonly used coconut oil, which has a higher saturated-fat content. Sunflower lecithin powder (Bungemaxx®) was obtained from Bunge-Loders Croklaan (Rotterdam, The Netherlands).

5.2.2 Formulation of cheese alternative samples

Blends of CPC and ZPI were used to formulate the cheese alternatives (Table 5.1). Increasing proportions of ZPI were used to obtain five different formulations, 0Z-100C, 25Z-75C, 50Z-50C, 75Z-25C and 100Z-0C, with ingredient ratios based on protein contribution of 0:100, 25:75, 50:50, 75:25 and 100:0 from ZPI and CPC, respectively. Shea butter was included to achieve a fat content of 15%, and a 2.3 M lactic acid solution was mixed with the water to obtain a pH of ~4.5 and 59% moisture for all the samples. Moreover, tapioca starch was added at increasing concentrations, from 0.10 to 10.6% (Table 5.1), to target a carbohydrate content of 10.1% for all samples. Samples were prepared as described by Grasso *et al*. (2022), with slight

differences. In brief, the powder ingredients were mixed with water (previously combined with the lactic acid solution) in a Thermomix (TM 5, Vorwerk, Wuppertal, Germany) at speed 1 (100 rpm) for 1 min; then, temperature was set to 100°C; and finally, when a temperature of 40°C was reached (after ~30 s), shea butter was added at speed 2.5 (350 rpm) for 5.5 min. After heating, samples were transferred into moulds and analysed after 24 h of storage at 4°C.

Table 5.1. Formulation (%) of the cheese alternative samples.

	ZPI	**CPC**	**Starch**	**Shea butter**	**Lecithin**	**Lactic acid**	**Water**
0Z-100C	0	29.9	0.10	14.4	0.20	8.50	48.2
25Z-75C	4.90	22.5	2.80	14.1	0.20	6.30	50.4
50Z-50C	9.80	15.0	5.40	13.8	0.20	4.30	52.4
75Z-25C	14.6	7.50	8.00	13.6	0.20	2.10	54.6
100Z-0C	19.5	0	10.6	13.3	0.20	0.10	56.6

ZPI=Zein protein isolate, CPC=Chickpea protein concentrate

5.2.3 Compositional and colour analyses of powder ingredients and cheese alternatives

Composition of the ZPI and tapioca starch was analysed, while composition of the CPC ingredient was previously determined by Grasso *et al*. (2022). Total nitrogen content of the ingredients and cheese alternative samples was measured using Kjeldahl methods 930.29 (AOAC, 1930) and 2001.14 (AOAC, 2002), respectively, with a nitrogen-to-protein conversion factor of 6.25. Moisture content of the ingredients and cheese alternative samples was determined by oven drying, according to method 925.10 (AOAC, 1925) and method 926.08 (AOAC, 1990b), respectively. Ash content was measured by incineration in a muffle furnace according to method 923.03 (AOAC, 1923) for the ingredients and method 935.42 (AOAC, 1990a) for the cheese alternative samples. Fat content was assessed using the Soxhlet AACC method 30-25.01 (AACC, 2009). Total carbohydrate was calculated by difference (i.e., 100 minus the sum of protein, fat, ash and moisture). The pH, water activity (a_w) and colour of the samples were measured as previously described by Grasso *et al*. (2022).

5.2.4 Protein profile analysis of protein ingredients

Protein profile of CPC was previously assessed by Grasso *et al*. (2022), and protein profile of ZPI was measured following the same method, using a capillary electrophoresis instrument (PA 800 plus Pharmaceutical Analysis System, Sciex, Kildare, Ireland).

5.2.5 Differential scanning calorimetry analysis of the ingredients and cheese alternatives

Thermograms of the tapioca starch and cheese alternative samples were obtained

using a Mettler DSC821 (Mettler-Toledo, Schwerzenbach, Switzerland) differential scanning calorimeter (DSC). Shea butter and CPC thermograms were previously measured by Grasso *et al*. (2022), while the tapioca starch was weighed (5.5–7.1 mg) into standard aluminium pans and water (~10 mg) was added to hydrate the powder. The cheese alternatives were weighed (2.8–4.9 mg) into aluminium pans which were hermetically sealed. The thermal behaviour of the ingredients and cheese alternative samples was assessed using the method reported by Grasso *et al*. (2022).

5.2.6 Confocal laser scanning microscopy

Microstructural analysis of the cheese alternatives was carried out using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan). Fat and protein of samples were stained as previously described by Le Tohic *et al*. (2018). Nile Red and Fast Green FCF were excited at 488 and 633 nm, using Ar and He-Ne lasers, respectively (Auty *et al*., 2001). Images of the cheese alternative samples, obtained using a 40X objective lens, were reported. Images of the 100% zein sample obtained using a 60X objective lens were also reported.

5.2.7 Dynamic low amplitude oscillatory shear rheology

Rheological properties of the products were measured using an AR-G2 controlled-stress rheometer (TA Instruments Ltd., Waters LLC, Leatherhead, UK) equipped with crosshatched-surface stainless-steel parallel plates. Samples were prepared by pouring the ingredient mixture in moulds of 40 mm diameter after processing and stored at 4°C overnight for 24 h. Samples were analysed according to the method described by Grasso *et al*. (2021). The exposed edges of the cheese alternative samples were coated with liquid paraffin to prevent drying. The viscoelastic

behaviour of the system is characterised by the storage (G′) and loss (G″) moduli, while the ratios between the two moduli is defined as the loss tangent (tan δ) (Fox *et al*., 2017). The melting index was calculated from G′ at 20 and 80°C according to the following equation (5.1):

$$\text{Melting index (\%)} = \frac{G'_{20} - G'_{80}}{G'_{20}} \cdot 100 \quad (5.1)$$

5.2.8 Schreiber meltability test

Meltability of the cheese alternatives was assessed using the Schreiber test as previously described by Altan *et al*. (2005). Samples were prepared as described by Grasso *et al*. (2022) and heated at 232°C for 5 min in an oven (Memmert, Schwabach, Germany). After 30 min at room temperature, specimen expansion was measured using a ruler along six lines marked on a series of concentric circles. Meltability was taken as the mean of the six readings and expressed as percentage sample expansion (Ramel & Marangoni, 2018). Photographs of the samples were taken before and after heating.

5.2.9 Extensibility analysis

A Texture Analyser TA-XTPlus (Stable Micro Systems, Godalming, Surrey, UK) with a Cheese Extensibility Rig (model A/CE) attachment equipped with a PT100 temperature probe was used to assess the extensibility of the samples. The cheese alternative samples were weighted (60 g) and distributed evenly on the fork in the sample pot. The sample pot was heated for 12 min in the oven (Memmert, Schwabach, Germany) at 220°C. The sample-pot assembly was then inserted into the slotted base, and the PT100 probe was placed into the cheese. Once the temperature reached 55°C the test started, pulling the fork out of the melted sample. The test speed and distance

were set to 10 mm/s and 220 mm, respectively. From the raw data obtained from the Exponent Connect (version 8,0,7,0) computer software, the area under the curve from 0 to 10 s was calculated.

5.2.10 Texture profile analysis

Texture profile analysis (TPA) was performed using a texture analyser TA-XT2i (Stable Micro Systems, Godalming, Surrey, UK), using the method previously described by Grasso *et al*. (2022), with the cheese alternative samples being stored at 4°C for 24 h in the moulds before being removed and analysed. Textural parameters, hardness, adhesiveness, springiness and cohesiveness were measured.

5.2.11 Statistical data analysis

Compositional analysis of the ingredients, and of the cheese alternative samples, was performed in triplicate, as well as DSC analysis of the ingredients. Protein profile analysis of ZPI was performed in duplicate. Three independent trials were conducted to develop the cheese alternative samples, and three independent replicates from each trial were used for all analyses. The homogeneity of variance was assessed using Levene's test, and one-way analysis of variance (ANOVA) was performed using SPSS version 25 (SPSS Inc., Chicago, IL, USA). A Tukey's paired-comparison post hoc test was carried out to identify significant differences ($p < 0.05$) between mean values of samples, at a 95% confidence level.

5.3 Results and discussion

5.3.1 Composition of powder ingredients and cheese alternatives

5.3.1.1 Chemical composition

Composition of the ingredients was used to formulate the plant-based cheese alternative samples (Table 5.1). As expected from the formulations, the samples had similar macronutrient contents; importantly, protein content was not significantly different among the plant-based cheese alternatives, and the same was observed for moisture and pH (Table 5.2). Furthermore, slight differences were observed in the fat contents of the cheese alternatives, with a target value of 15% for all formulations. To achieve a pH of ~4.5, increasing amounts of lactic acid were used with increasing CPC content (Table 5.1), as previously observed by Grasso *et al*. (2022), probably due to the buffering capacity of the globulin fractions of chickpea protein (Martínez-Villaluenga *et al*., 2008). The a_w of the cheese alternatives ranged between 0.985 and 0.997.

Table 5.2. Composition of plant-based cheese alternative samples.

	Protein (%)	Moisture (%)	Carbohydrate (%)	Fat (%)	Ash (%)	pH (-)	a_w
0Z-100C	16.3 ± 0.26^{a}	56.2 ± 0.01^{a}	11.1	15.3 ± 0.33^{ab}	1.40 ± 0.04^{a}	4.55 ± 0.01^{a}	0.985 ± 0.000^{e}
25Z-75C	16.2 ± 0.17^{a}	56.8 ± 0.52^{a}	10.9	15.0 ± 0.13^{b}	1.01 ± 0.01^{b}	4.54 ± 0.02^{a}	0.988 ± 0.001^{d}
50Z-50C	16.1 ± 0.06^{a}	56.7 ± 0.04^{a}	11.4	15.0 ± 0.37^{b}	0.73 ± 0.03^{c}	4.55 ± 0.01^{a}	0.992 ± 0.002^{c}
75Z-25C	16.3 ± 0.17^{a}	56.3 ± 0.20^{a}	11.6	15.5 ± 0.11^{ab}	0.49 ± 0.02^{d}	4.53 ± 0.02^{a}	0.994 ± 0.001^{b}
100Z-0C	16.3 ± 0.30^{a}	56.2 ± 0.34^{a}	10.7	15.9 ± 0.34^{a}	0.22 ± 0.01^{e}	4.53 ± 0.01^{a}	0.997 ± 0.001^{a}

Values followed by different superscript letters in a column (a-e) are significantly different ($p < 0.05$).

5.3.1.2 Colour

The CIELAB coordinate values of the cheese alternatives are reported in Figure 5.1. The b* coordinate values indicate the degree of yellowness (or blueness for negative values), and these values increased with the ZPI addition level, showing the highest value for the 100Z-0C sample, due to the characteristic strong yellow colour of ZPI. This value was higher also when compared with commercially-available plant-based and dairy cheeses previously studied, which ranged between 26.2 and 46.1 (Grasso *et al*., 2021). Moreover, the a* coordinate values, with the colour green representing negative values and the colour red representing positive values, were the highest for the sample containing no CPC (towards the colour red) and negative for the 25Z-75C and 50Z-50C samples. The opposite trend was observed for the L* coordinate (which represents the lightness), where the lowest value was observed for the 100Z-0C sample; this value was lower than commercially-available plant-based and dairy cheeses (Grasso *et al*., 2021).

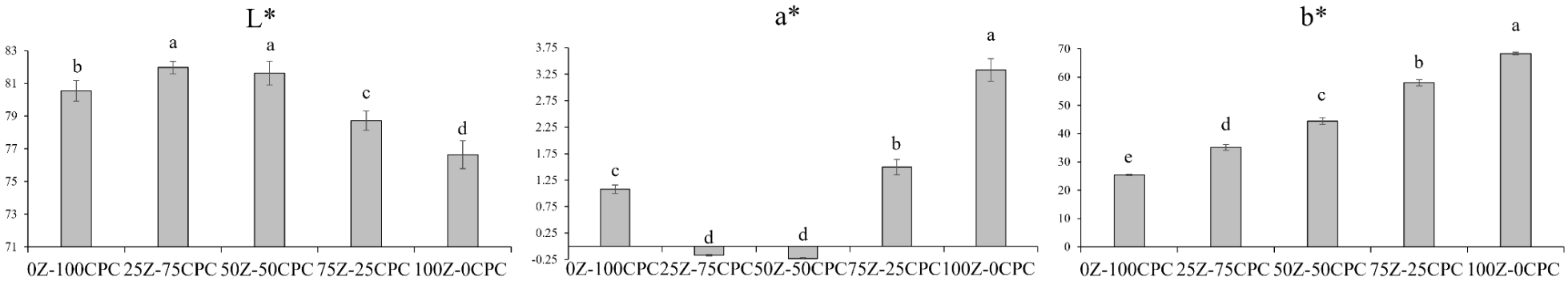


Figure 5.1. Colour space values (L* a* b*) of plant-based cheese alternative samples. Different letters on bars of the figure (a-e) indicate significant differences between samples ($p < 0.05$).

5.3.2 Protein profile of powder ingredients

Protein profile of ZPI under non-reducing and reducing conditions is reported in Figure 5.2. As expected, the electropherogram showed the typical zein profile, with two main peaks at 19 and 22 kDa corresponding to α-zein, the major fraction found in commercial zein, as previously reported (Argos *et al*., 1982; Wallace *et al*., 1990; Hamaker *et al*., 1995; Anderson & Lamsa, 2011). Moreover, smaller peaks around ~48 kDa were evident, in particular under non-reducing conditions. These might correspond to a dimer of γ-zein, which is also visible from the lower MW peak around 18 kDa (Anderson & Lamsa, 2011). Esen (1987) proposed the classification of zein fractions into α-, γ-, β-, and δ-zein, which correspond to ~71–85%, 10–20%, 1–5%, and 1–5%, respectively, of total protein (Wilson, 1991; Anderson & Lamsa, 2011). The protein profile of CPC as previously studied showed mainly the 11S legumin and 7S vicilin globulin fractions (Grasso *et al*., 2022), which represent 53-60% of total protein in chickpeas (Day, 2013). Under reducing conditions, the CPC electropherogram showed peaks around 35-40 and 20 kDa, corresponding to the 11S legumin acidic (α-legumin) and basic (β-legumin) chains, respectively. Subunits of 7S vicilin corresponded to the peaks around 50 kDa (i.e., major fraction) and around 15, 32 and 70 kDa (i.e., minor subunits) of the CPC electropherograms.

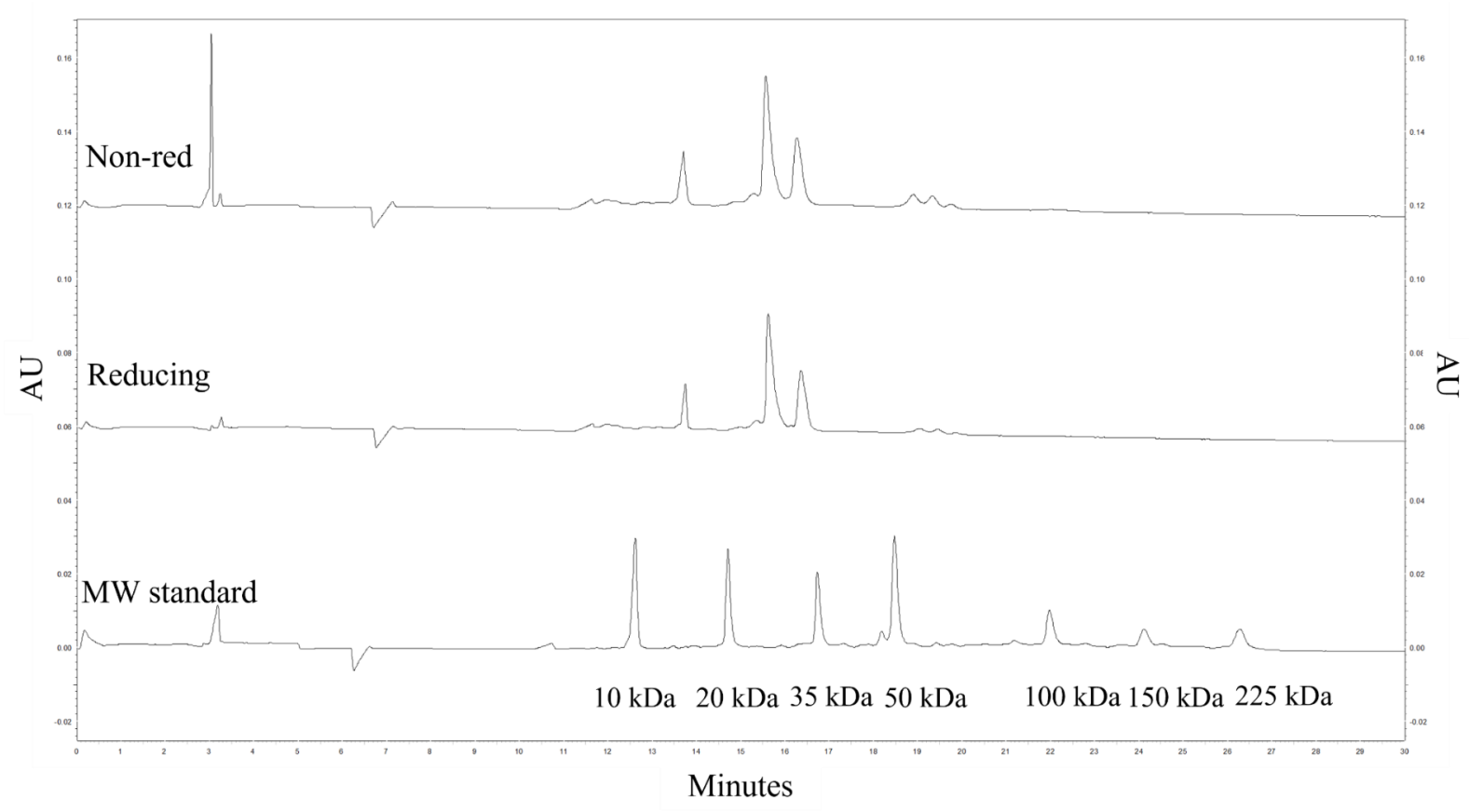


Figure 5.2. Protein profile of zein protein isolate (ZPI) under non reducing and reducing conditions. The bottom electropherogram represents the molecular weight (MW) standard.

5.3.3 Thermal behaviour of the powder ingredients and cheese alternatives

The DSC thermograms of the cheese alternatives are reported in Figure 5.3. A main peak was observed for all samples around 30°C, corresponding to melting of the shea butter (Grasso *et al*., 2022). However, the samples with higher ZPI contents showed a narrow peak shape, probably linked to the differences in structure of the samples and the distribution of fat and protein within them. Two small peaks around 75°C and 90°C were shown by the thermograms of the samples containing CPC, corresponding to denaturation of globulin fractions, in agreement with the thermogram of the CPC ingredient, which previously displayed a main peak at 93.7°C and a smaller peak at 77.1°C, corresponding to denaturation of the (7S) vicilin and (11S) legumin fractions, respectively (Grasso *et al*., 2022).

The gelatinisation of the tapioca starch occurred at 61.4°C (thermogram not reported), in agreement with values reported in the literature (Breuninger *et al*., 2009). However, the transition of the tapioca starch, used in the formulations in different proportions, was not visible from the thermograms of the samples; an explanation for this might be the reduction in the gelatinisation enthalpy due to the effect of ZPI, as previously observed for mixtures of proso millet starch and zein (Zheng *et al*., 2020). This decrease might be attributed to the competition between starch and protein for available water, or due to the distribution of the zein on the surface of the starch granules, which protects the structure and prevents swelling, similar to the behaviour reported for mixtures α-casein and waxy maize starch (Kett *et al*., 2012). Furthermore, the denaturation of zein was not visible from the sample thermograms, with the denaturation temperature of dry zein powder reported in the literature to be ~139°C; however, zein is significantly plasticised by water, and transition temperatures can vary according to the a_w of zein (Madeka & Kokinii, 1996).

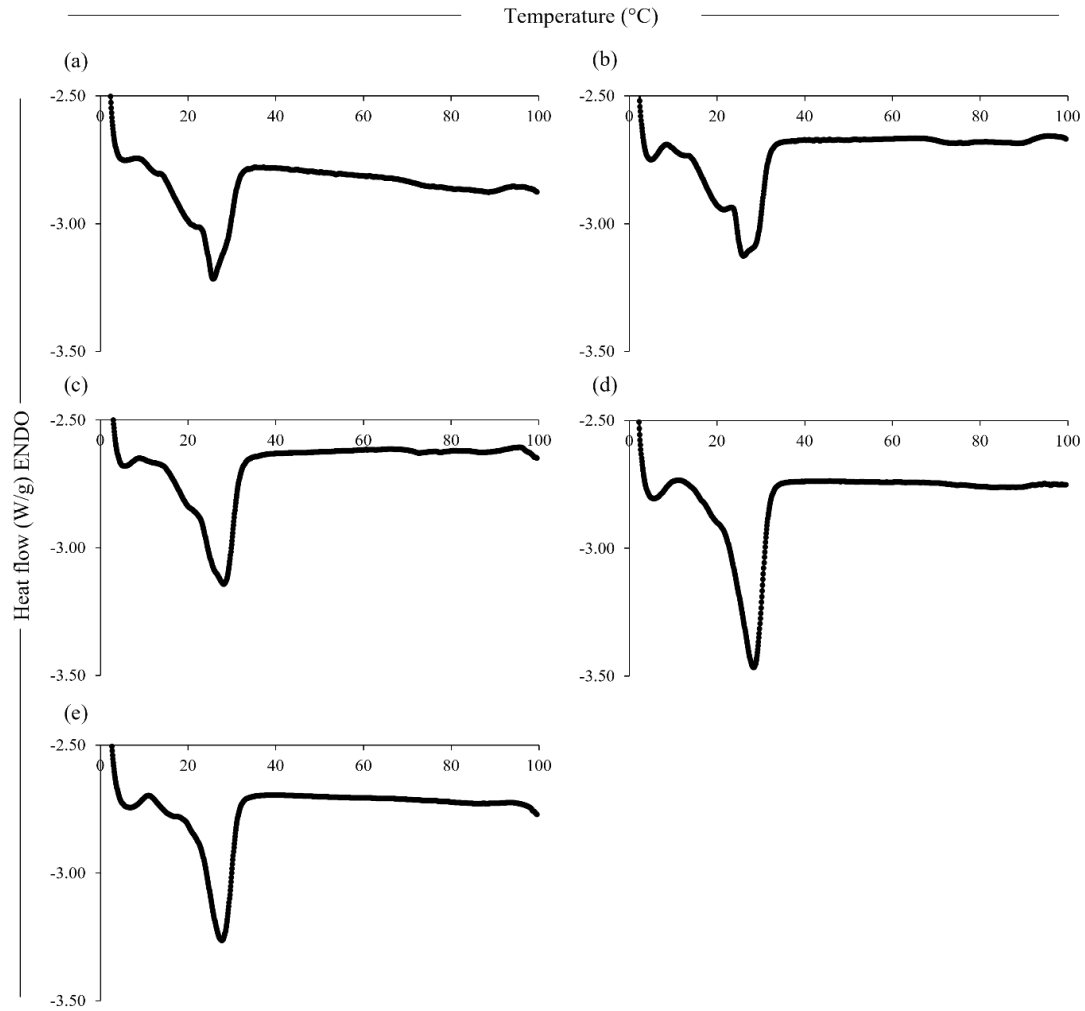


Figure 5.3. Differential scanning calorimetry thermograms of the plant-based cheese alternative samples, 0Z-100C (a), 25Z-75C (b), 50Z-50C (c), 75Z-25C (d) and 100Z-0C (e).

5.3.4 Microstructure

Analysis of the microstructural images showed considerable differences between samples (Fig. 5.4). Samples containing ZPI (Fig. 5.4b-f) showed evidence of a protein layer surrounding the fat globules, while, possibly, chickpea proteins were more homogeneously distributed. The hydrophobicity of zein, and, therefore, its ability to orientate at the interface of fat droplets, has been previously used to stabilise Pickering emulsions (Dai *et al*., 2018). Pools of fat globules were observed for all

samples; however, the size of these pools increased with increasing ZPI content, with the largest non-spherical coalesced pools of fat globules evident in the 100Z-0C sample (Fig. 5.4e,f). Some aggregation, possibly representing chickpea protein, was observed in all samples containing the CPC ingredient (Fig. 5.4a-d). This was in agreement with a previous study, in which the aggregation of pea proteins was observed in formulations of zein and pea protein dough (Ozturk *et al*., 2023). The black areas in the images represent carbohydrate, which was present at the same concentration for all samples. These differences in microstructure are reflected in the differences in physicochemical properties between the cheese alternative samples.

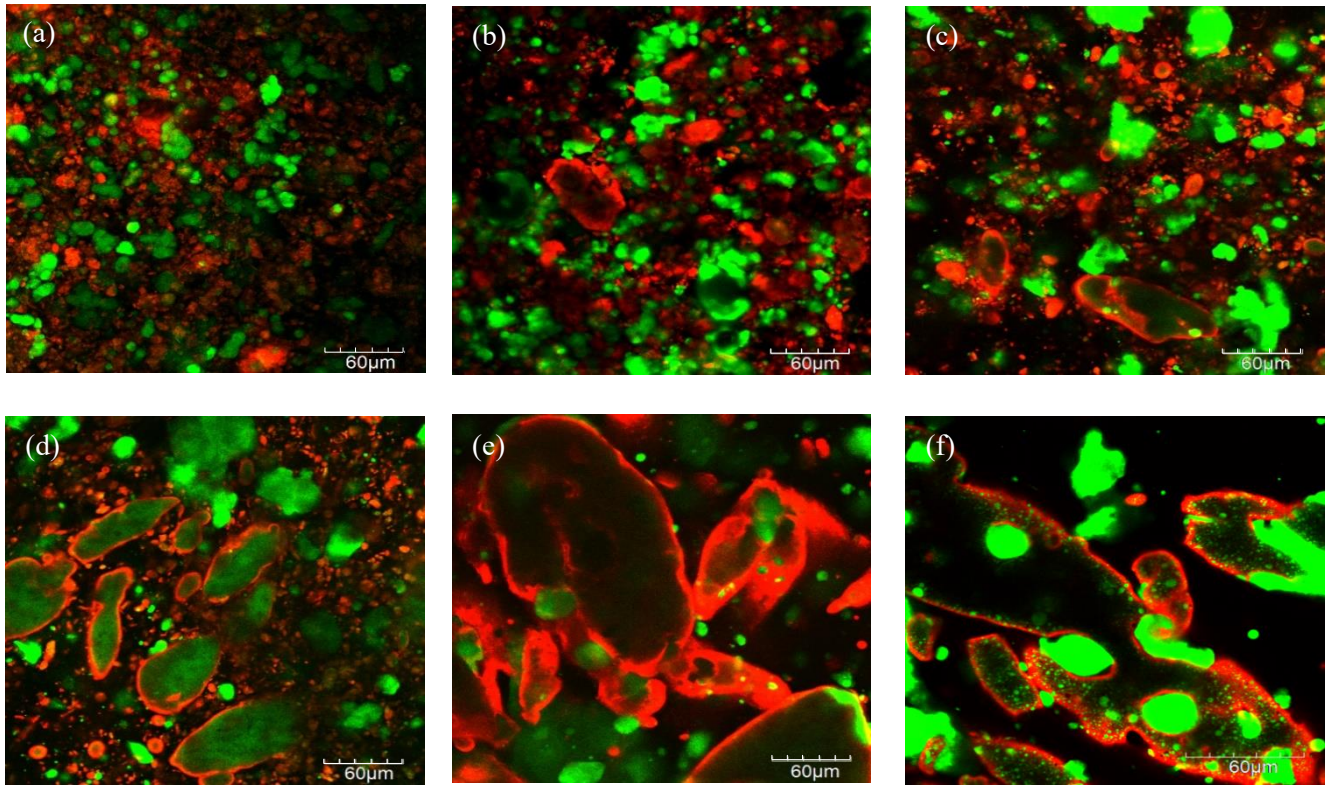


Figure 5.4. Confocal laser scanning microscopy images of the plant-based cheese alternative samples, 0Z-100C (a), 25Z-75C (b), 50Z-50C (c), 75Z-25C (d) and 100Z-0C (e), at 40X. Microstructure of 100Z-100C (f) at 60X is also shown. Fat and protein are represented in green and red, respectively.

5.3.5 Rheological properties

The melting index and the rheological profiles of the cheese alternative samples are reported in Figure 5.5 and Figure 5.6, respectively. The rheological behaviour of cheese on heating is linked to shrinkage of the para-casein network, which occurs between 60 and 90°C, and the consequent expulsion of moisture, leading to complete transformation from viscoelastic solid to liquid (Fox *et al*., 2017). As shown in Figure 5.5, increased proportions of ZPI resulted in more extensive melting behaviour of the cheese alternatives. Particularly at the high temperatures, the increasing melting index, as well as the decreasing distance between G′ and G″ (Fig. 5.6), with increasing ZPI content, indicated greater viscous behaviour and ability to flow at high temperatures for samples with higher ZPI contents, compared with samples having lower ZPI contents. This behaviour was in agreement with previous observations on zein-based products (Mattice & Marangoni, 2020b), and is likely to be linked to the weakening of non-covalent bonds in zein at high temperatures, which is strongly responsible for its viscoelastic behaviour (Smith *et al*., 2014).

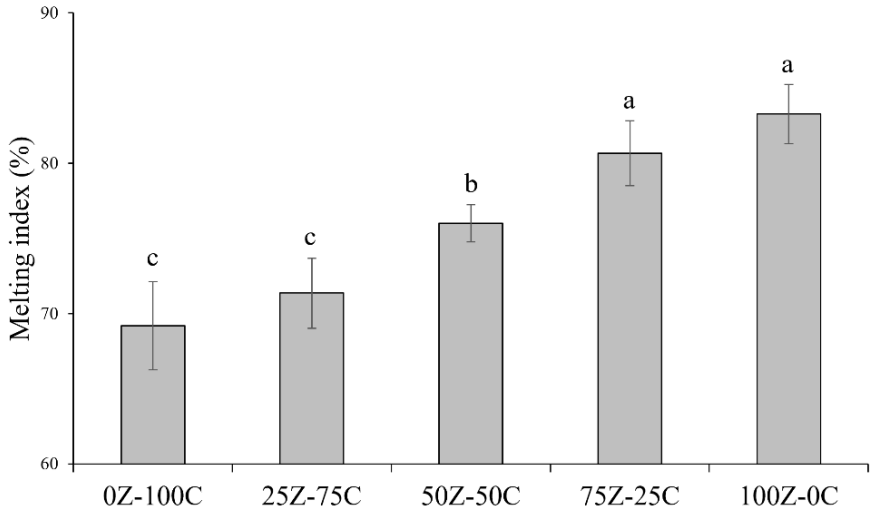


Figure 5.5. Melting index of plant-based cheese alternative samples. Different letters on bars of the figure (a-c) indicate significant differences between samples ($p < 0.05$)

From 20 to 35°C, all samples showed softening, with rapid decrease in G′ values; this is likely to be due to melting of the shea butter, which had a transition temperature of 35°C (Section 5.3.3). Furthermore, tan δ increased between 60 and 80°C with increasing ZPI content, suggesting increasing ability of the samples to flow with increasing proportions of zein, with only the 100Z-0C sample having a final tan δ value higher than the respective initial value.

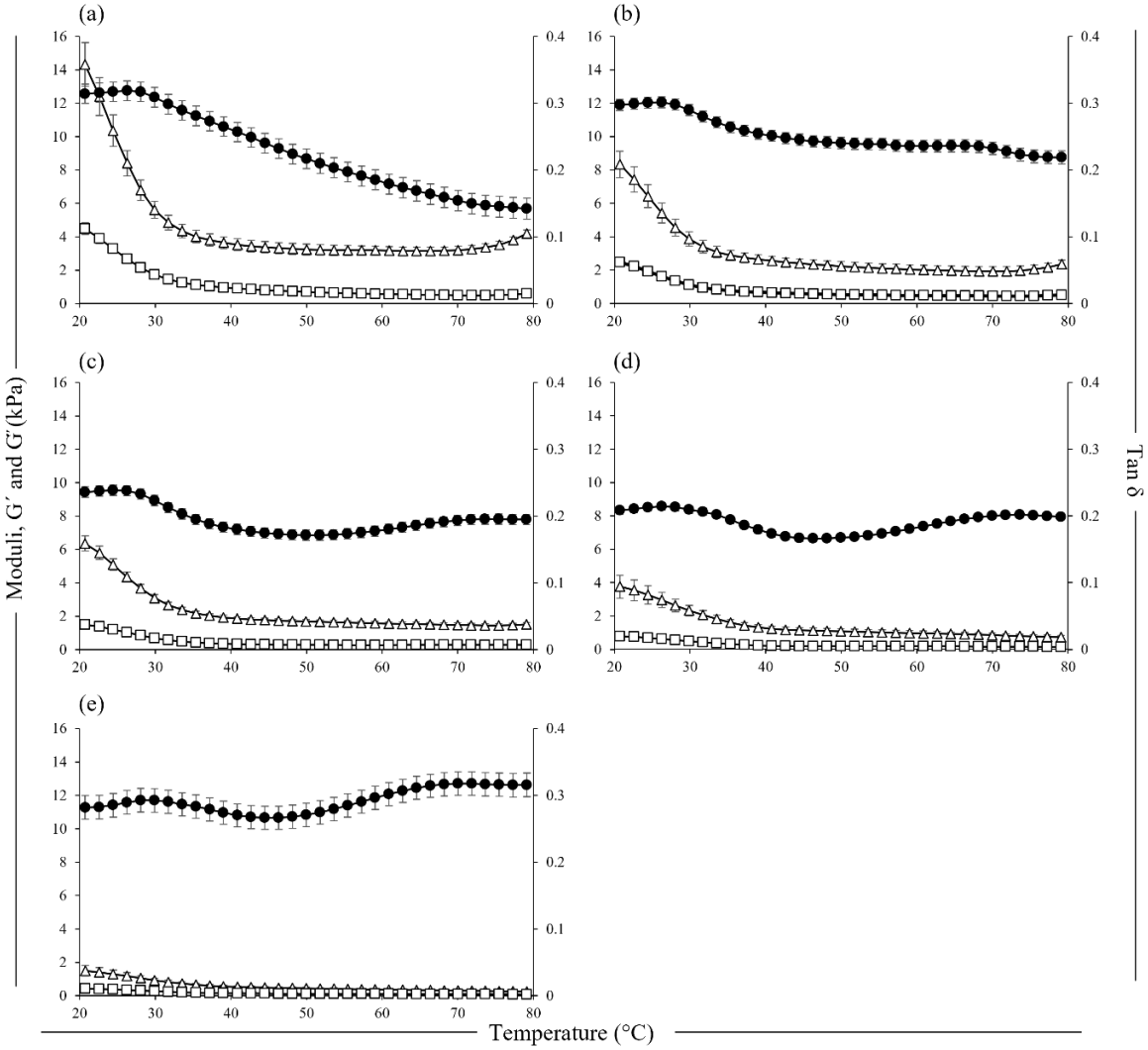


Figure 5.6. Rheological profiles showing storage modulus (G′) (open triangle), loss modulus (G″) (open square) and loss tangent (Tan *δ*) (filled circle) as a function of temperature in the range 20–80°C for 0Z-100C (a), 25Z-75C (b), 50Z-50C (c), 75Z-25C (d) and 100Z-100C (e) plant-based cheese alternative samples.

5.3.6 Meltability

Meltability has previously been defined as the ease with which cheese flows or spreads upon heating (Muthukumarappan *et al*., 1999) and is a key quality attribute of cheese products. The photographs of the samples before and after heating and the data for meltability of the cheese alternatives in this study are reported in Figure 5.7 and 5.8, respectively. Increasing proportions of ZPI were associated with increasing meltability, in agreement with the rheological data (Section 5.3.5). This was evident from the photos of the samples before and after heating (Fig. 5.7), with the 100Z-0C sample showing the highest meltability, with 25% diameter expansion (Fig. 5.8). This sample displayed better meltability than commercial plant-based cheese alternatives previously studied; in addition, the 75Z-25C sample showed greater extent of diameter expansion (17.6%) than most such products (Grasso *et al*., 2021).

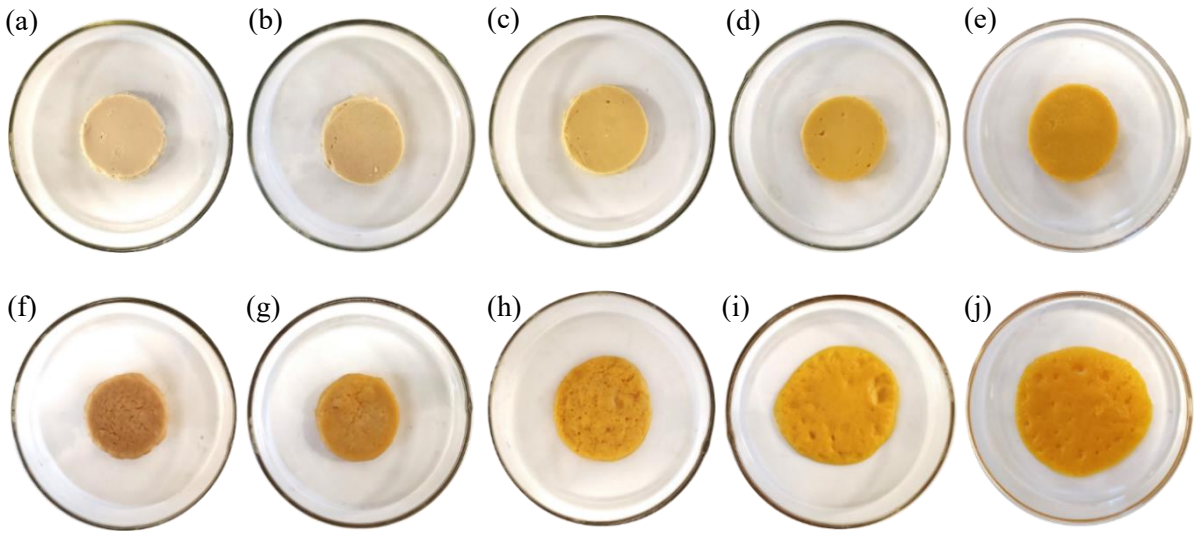


Figure 5.7. Photographs of plant-based cheese alternative samples, 0Z-100C, 25Z-75C, 50Z-50C, 75Z-25C and 100Z-0C before (a-e) and after (f-j) oven heating at 232°C for 5 min.

As for the rheology results, at high temperatures, samples containing zein showed ability to flow, probably due to the weakening of non-covalent bonds in zein, similar to casein networks in traditional cheese, as previously observed by Mattice & Marangoni (2020a, 2020b). Indeed, due to its water insolubility, when hydrated and heated above its glass transition temperature, zein self-assembles in a plastic and viscoelastic mass (Mattice & Marangoni, 2020a). The 0Z-100C did not show any diameter expansion, with similar results reported for the 25Z-75C sample; indeed this limited meltability of chickpea-based cheese alternatives was previously reported (Grasso *et al*., 2022).

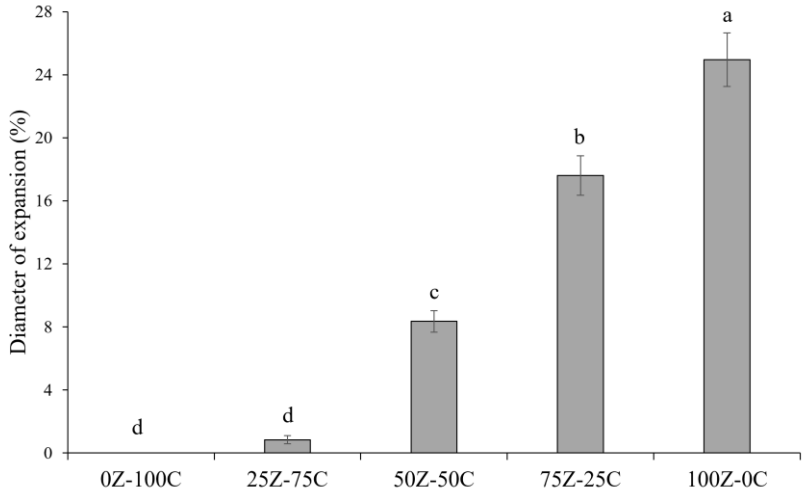


Figure 5.8. Schreiber meltability test of plant-based cheese alternative samples. Different letters on bars of the figure (a-e) indicate significant differences between samples ($p < 0.05$).

5.3.7 Stretchability

The stretchability of melted cheese represents an important quality attribute. In this study, the area under the force-time curve in the interval 0-10 s was calculated and used as a parameter to compare stretching of the samples. As reported in Figure 5.9, increasing proportions of ZPI were associated with increasing area values and, thus, increased stretching, as also evident from the photographs of the samples (Fig. 5.10).

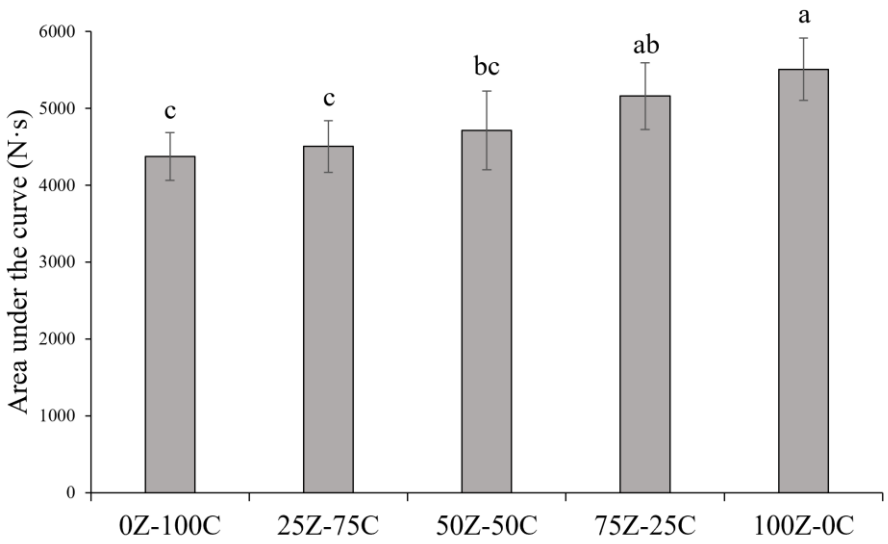


Figure 5.9. Area under the force-time curve from time 0 to 10 s of plant-based cheese alternative samples. Different letters on bars of the figure (a-c) indicate significant differences between samples ($p < 0.05$).

The sample containing only ZPI showed the highest area value, statistically comparable only to the 75Z-25C sample. Samples with high CPC contents maintained a rigid structure and did not stretch under the testing conditions; as reported in Section 5.3.6, such samples were unable to achieve a molten mass – a prerequisite for subsequent stretching of the sample. In cheese, stretching is defined as the ability of the casein network to maintain its integrity, without breaking, when a continuous stress is applied (Lucey *et al*., 2003). Similarly, due to weakening of non-covalent interactions, zein networks soften at high temperatures, leading to enhanced stretching properties, as previously observed by Mattice & Marangoni (2020a).

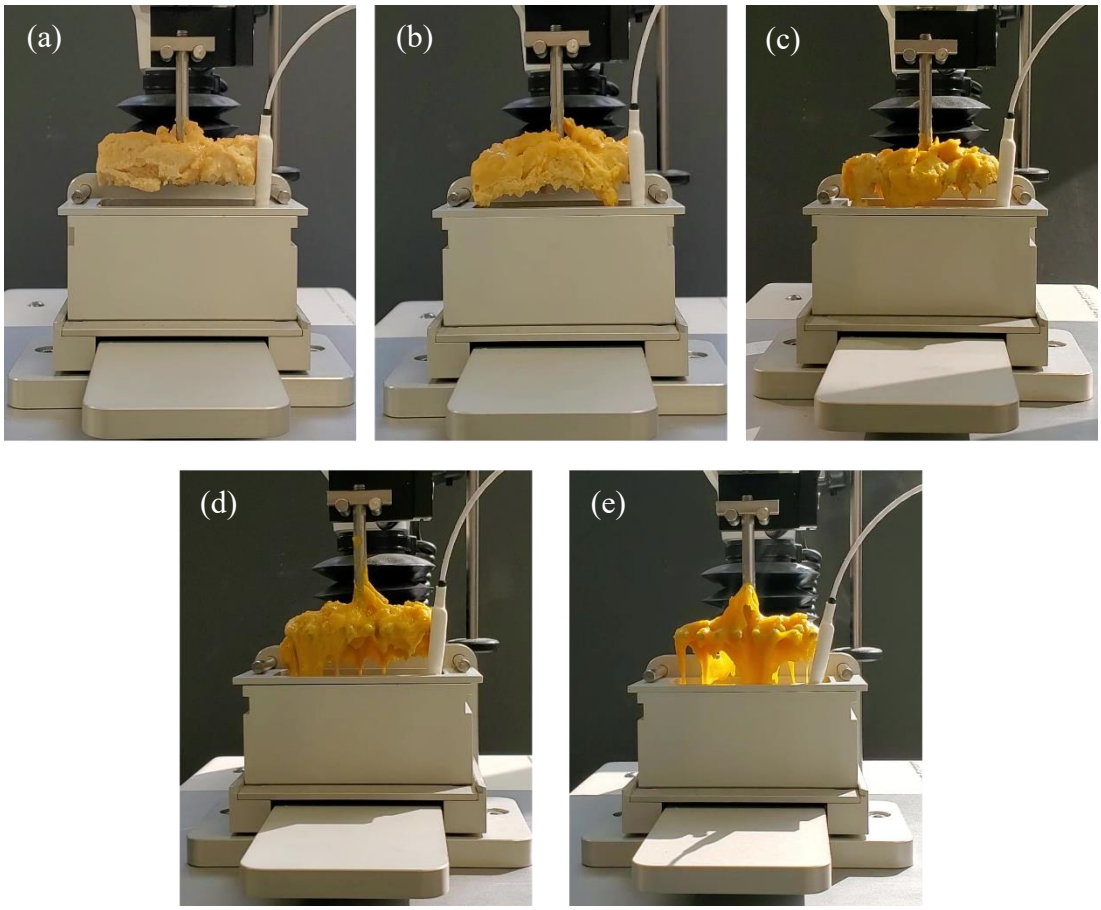


Figure 5.10. Photographs of melted plant-based cheese alternative samples, 0Z-100C (a), 25Z-75C (b), 50Z-50C (c), 75Z-25C (d) and 100Z-0C (e) during stretchability testing.

5.3.8 Textural properties

The texture parameters of plant-based cheese alternatives samples are shown in Figure 5.11. Hardness, defined as the height of the force peak on the first compression cycle (Bourne, 2002), was the highest for the 100Z-0C sample, significantly different from the other samples. Interestingly, the samples containing both ZPI and CPC had lower hardness than the ZPI-only and CPC-only samples. The 25Z-75C sample had the lowest values for all parameters, with the other two blends (i.e., 50Z-50C and 75Z-25C) showing higher values. These differences are likely to be linked to the interactions between the two protein ingredients (i.e., ZPI and CPC) and the structure of these samples, as previously reported in Section 5.3.4. The 0Z-100C sample showed the highest adhesiveness, springiness and cohesiveness values, with the latter being statistically comparable to the 100Z-0C and 75Z-25C samples. Adhesiveness is correlated with the interactions between fat and protein, which influence the adherence between the product and the contact surface, as well as the structure of the protein matrix (Cunha *et al*., 2010). The 0Z-100C sample had a protein matrix which differed considerably that of the samples containing ZPI, which may have been responsible for the significantly higher adhesiveness of this sample. Moreover, the different starch (i.e., chickpea starch *vs* tapioca starch) and the overall carbohydrate profiles of the samples are likely to have influenced texture; the final carbohydrate content (i.e., ~10.1%) was constant for all samples.

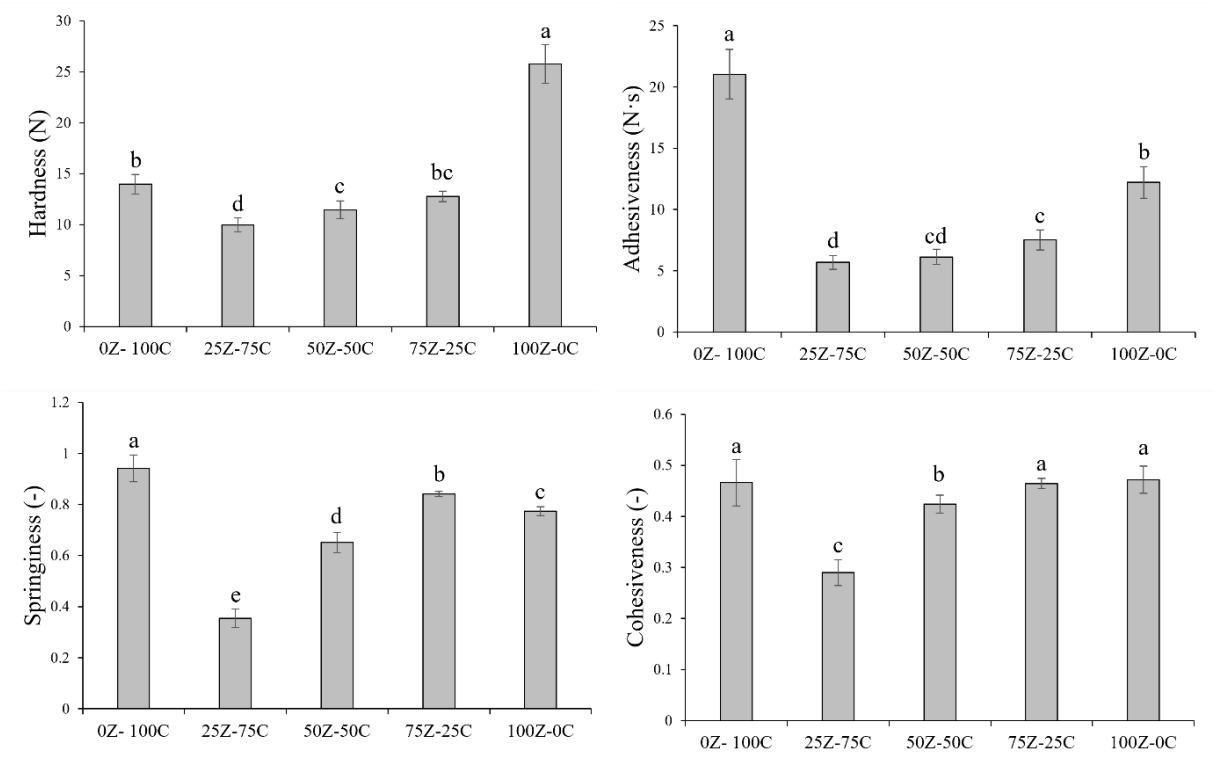


Figure 5.11. Texture profile analysis parameters, hardness, adhesiveness, springiness and cohesiveness of plant-based cheese alternative samples. Different letters (a-e) indicate significant differences between samples ($p<0.05$).

5.4 Conclusion

The effect of different ratios of ZPI and CPC on the development and physicochemical properties of plant-based cheese alternatives was investigated. Large differences were observed in the microstructure of the cheese alternative samples, with samples containing ZPI showing a protein layer surrounding the fat globules. Improvements in the meltability and stretching behaviour of the samples were associated with increasing ZPI content, due to its viscous and plastic nature in aqueous environments and the ability of zein to flow at high temperatures. Furthermore, samples showed different texture, with the 100Z-0C sample having the highest hardness value and the 0Z-100C sample the highest adhesiveness, springiness and cohesiveness. The results of this work show the potential of blending plant protein ingredients in the development of promising plant-based alternatives to cheese with desirable functional properties, and assisted in our understanding of their role in formulating such products.

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Chapter 6

Physicochemical properties of plant-based cheese alternatives fortified with calcium

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Declaration: This chapter was written by author Nadia Grasso (NG) and reviewed by all co-authors. NG co-designed the study and performed all the experimental work with the exception of mineral analysis, which was performed by an external laboratory (Teagasc, Moorepark, Fermoy, Co. Cork, Ireland).

Abstract

Fortification with calcium is proposed as an effective strategy to improve the nutritional profile of plant-based cheese alternatives (PBCAs). In this study, the effects of two calcium salts, individually or as a blend, on the physicochemical properties of PBCAs were investigated. Three different formulations were obtained based on the calcium (Ca) salts employed, CaChloride, CaCitrate, and CaChlo:CaCitr, with the latter having a 50:50 contribution of calcium from calcium chloride and calcium citrate; a control with no added calcium salt was also analysed. Zein protein isolate and chickpea protein concentrate were used in combination with other ingredients to formulate the PBCAs. Different additions of lactic acid were necessary to adjust the pH to a target of ~4.5 for all samples, due to the differing buffering capacity of calcium salts. Fortification with calcium led to decreased meltability, as shown by Schreiber meltability and dynamic low amplitude oscillatory shear rheology analyses. Stretching properties of calcium fortified samples were enhanced compared with that of the control. Texture profile analysis demonstrated that calcium fortification significantly affected the texture of samples. Microstructural analysis demonstrated that calcium fortification resulted in a matrix with generally smaller fat globules. The results of this work will help in understanding the role of different calcium salts in formulating PBCAs with improved nutritional profile and the impact thereof on relevant physicochemical properties.

6.1 Introduction

Calcium is an important micronutrient, involved in numerous vital functions, with recommended daily intakes tailored to the different stages of life (FAO/WHO, 2004). Dietary reference values for those over the age of 19 vary from 1000 to 1300 mg/d, depending on the reference guidelines (Cormick & Belizán, 2019). Cheese is considered a particularly good source of bioavailable calcium, with most hard cheeses having contents of approximately 800 mg/100 g; however, depending on the variety calcium contents can differ significantly, with values ranging from 73 to 1200 mg/100 g (O'Callaghan *et al*., 2017). In the human diet, other calcium-rich food products include some cereals, nuts and seeds, and some vegetables (e.g., kale and broccoli) (Cormick & Belizán, 2019).

Fortification of food with calcium or other nutrients is commonly used by the food industry to improve the nutritional profile of products. However, when fortifying a food product using calcium salts, there are many important processing and finished product qualities requiring consideration, based on the characteristics of such ingredients and their effects on the food matrix to which they are fortified. For instance, solubility is an important property of calcium salts, and examples of soluble salts include calcium lactate, chloride and gluconate, while insoluble or partially insoluble ones are calcium carbonate, phosphate, citrate and malate (Palacios *et al*., 2021). Sedimentation and sensory defects, pH and colour changes are some of the consequences of fortification with calcium. Over the years, numerous studies have reported the effects of calcium fortification on the physicochemical properties of different food products, such as milk, yogurt, infant formula and other infant nutritional products, and meat products (Crowley *et al*., 2014; Santillán-Urquiza *et al*., 2017; France *et al*., 2020; Barone *et al*., 2021; Yang *et al*., 2021).

Currently, the majority of the plant-based cheese alternatives (PBCAs) available on the market rely on starch and coconut oil to provide structure and functionality to the final product, resulting in inferior nutritional profiles compared with traditional cheeses; indeed, the majority of such products have no added calcium, with only a small number being fortified with calcium, using calcium phosphate, calcium citrate, or blends of different salts (Craig *et al*., 2022). On the other hand, the plant-based cheese alternatives sector displayed 51% growth in sales in the US between 2019 and 2022, with the market in Europe showing sales growth of 23% in 2022 (Good Food Institute, 2023). Different approaches and ingredients can be employed to design PBCAs and, at present, plant protein ingredients are being investigated for their potential in the development of new and reformulated alternatives to dairy food products with enhanced nutritional value (Jiménez-Munoz *et al*., 2021; Mefleh *et al*., 2021). Among the plant protein ingredients studied, pulses, and chickpeas in particular, are deemed to be valuable sources of macronutrients, with functionality of relevance in plant-based food applications (Mefleh *et al*., 2021; Grasso *et al*., 2022). Other than pulses, zein, the prolamin fraction extracted from maize, also displays strong potential in development of PBCAs, due to its unique plastic behaviour in aqueous environments (Mattice & Marangoni, 2020; Grasso *et al*., 2023).

Fortification using calcium salts is proposed as an effective strategy to improve the nutritional profile of PBCAs, with an ultimate target of matching the calcium content of traditional cheeses (Grossmann & McClements, 2021). In a similar way, many plant-based milk alternatives commercially-available are fortified with calcium (Sethi *et al*., 2016). Calcium carbonate and tricalcium phosphate are insoluble salts often employed in milk alternatives in a colloidal form to avoid charge neutralisation and cross-linking of proteins, due to ion binding and electrostatic screening effects

(McClements, 2020). However, these calcium salts are known to result in sedimentation. Furthermore, depending on processing approaches and raw materials employed, the bioavailability of calcium in plant-based milk alternative products has been shown to vary significantly (Aydar *et al*., 2020).

Specific studies on the effects of calcium fortification on the physicochemical properties of some plant-based food systems (e.g., beverages, emulsion gels, protein ingredients, bread) are reported in the scientific literature, showing promising results in certain applications (Charlton *et al*., 2007; Pathomrungsiyounggul *et al*., 2010; Alonso-Miravalles *et al*., 2020; Kaharso *et al*., 2021; Peng *et al*., 2022; Min *et al*., 2023). However, to the authors' knowledge, there is no information available in the scientific literature on the impact of mineral fortification on PBCAs with high protein content. Therefore, the aim of this study was to understand the effects of the addition of two different calcium salts and a combination thereof, on key physicochemical properties and quality attributes of PBCAs, formulated with a binary blend of chickpea protein concentrate and zein protein isolate.

6.2 Materials and methods

6.2.1 Ingredients

Zein protein isolate (Flo Chemical Corporation, Ashburnham, MA, US), with 81.5% protein, 5.62% moisture, 3.92% carbohydrate, 7.68% fat and 1.28% ash and chickpea protein concentrate (Artesa, PLT Health Solutions, Morristown, NJ, US), with 53.1% protein, 7.73% moisture, 33.3% carbohydrate, 1.37% fat and 4.47% ash were used to formulate the PBCA samples. Tapioca starch, with 0.11% protein, 11.3% moisture, 88.3% carbohydrate, 0.25% fat and 0.03% ash, was purchased from a local retail outlet (Quay-coop, Cork, Ireland), while shea butter, kindly provided by Fuji Oil (Zenitex M 50 G, Fuji Oil Europe, Gent, Belgium), was used as a source of fat. Sunflower lecithin powder (Bungemaxx®), was obtained from Bunge-Loders Croklaan (Rotterdam, the Netherlands). The calcium salts used for this study were calcium chloride dihydrate and calcium citrate tetrahydrate and were purchased from Sigma-Aldrich (St Louis, MO, US).

6.2.2 Formulation of plant-based cheese alternatives

The PBCAs were prepared essentially as described in our previous work by Grasso *et al*. (2023), with some modifications. Three different formulations were obtained based on the calcium salts employed, CaChloride, CaCitrate, and CaChlo:CaCitr, the latter representing a 50:50 combination of calcium from calcium chloride and citrate; a control with no added calcium was also analysed. The calcium salts were added to target calcium content of 0.6% in all samples and, according to their potency, they were added to a solution of water, NaCl (1%) and lactic acid in different amounts (Table 6.1). The target of 0.6% calcium was chosen based on the calcium content of processed cheese and other cheese varieties (O'Callaghan *et al*.,

2017). Ingredient addition levels of 7.5% chickpea protein concentrate and 14.7% zein protein isolate were mixed with the water and salt solution, tapioca starch (6.7%) and lecithin (0.2%) in a Thermomix (TM 5, Vorwerk, Wuppertal, Germany) at speed 1 (100 rpm) for 1 min. Immediately following this, temperature was set to 100°C and shea butter was added to the mixture at speed 2.5 (350 rpm) for 6 min, when 40°C was reached (after ~30 s). After the heating process, samples were poured into moulds and analysed after 24 h of storage at 4°C. The target formulation of PBCAs was 16% protein, 9% carbohydrate, 14.8% fat, and 59% moisture. The target pH was 4.5 and was achieved by addition of lactic acid to the batches, in varying quantities reflective of the differences in buffering capacity of the formulations (Table 6.1).

Table 6.1. Calcium salt additions required to achieve 0.6% calcium content in all samples, moisture content of samples, pH of samples containing 1.8% lactic acid, lactic acid additions needed to adjust the pH of samples to ~4.5, and adjusted pH of samples.

	Calcium salt addition (%)	**Moisture (%)**	**pH with 1.8% Lactic acid (-)**	**Lactic acid addition (%)**	**pH adjusted (-)**
Control	0	56.6 ± 0.19^{a}	4.54 ± 0.00^{b}	1.8	4.54 ± 0.00^{b}
Ca Chloride	2.20	56.5 ± 0.04^{a}	4.07 ± 0.01^{d}	0.7	4.55 ± 0.01^{a}
Ca Citrate	2.85	55.8 ± 0.14^{ab}	4.61 ± 0.01^{a}	2.1	4.53 ± 0.01^{bc}
CaChlo:CaCitr	1.1+1.4	55.4 ± 0.62^{b}	4.28 ± 0.01^{c}	0.8	4.53 ± 0.01^{c}

Values followed by different superscript letters in a column (a-d) are significantly different ($p < 0.05$).

6.2.3 pH, moisture content, mineral profile and colour analysis

The pH of the PBCAs was measured before and after pH adjustments using a pH meter equipped with a FC200B Foodcare pH electrode for semi-solid foods (Hanna Instruments, Woonsocket, RI, US) after calibration. Moisture content of samples was determined by oven drying, according to method 926.08 (AOAC, 1990). Mineral profile of the PBCA samples was measured for macro elements (Ca, K, Mg, Na, and P) using inductively coupled plasma mass spectrometry (ICP-MS) and was performed by Teagasc (Fermoy, Co. Cork, Ireland). The colour of the PBCAs was assessed with a Chroma Meter CR-400 (Konica Minolta Sensing, Inc., Osaka, Japan) and after calibration, by measuring the CIE LAB coordinates (L*, a* and b*).

6.2.4 Buffering capacity

Buffering capacity of samples was measured using a Titrando automated titrator with TIAMO v.2.2 software (Metrohm Ireland Ltd., Carlow, Ireland). The pH probe was calibrated using buffer solutions at pH 4, 7 and 9 and samples were prepared by mixing PBCA (8 g) with 40 mL of ultrapure water at 50°C for 5 min with an ultraturrax (T25 Ultra-Turrax, Staufen, Germany) at 5,500 rpm, as reported by O'Mahony *et al*. (2006). Samples were equilibrated at 25°C before the analysis. Based on the method described by Lucey & Fox (1993), samples were continuously stirred during analysis and starting at the natural pH of the solutions, acid titration to pH 3 with 0.5 N HCl, followed by base titration to pH 8 with 0.5 N NaOH, were performed. Titrants were added in 0.1 mL increments at 30 s intervals. The buffering index (dB/dpH) was calculated according to Van Slyke (1992) as reported in equation 6.1, and the acid and base buffering curves were obtained by plotting the buffering index as a function of pH.

$$\frac{\mathrm{dB}}{\mathrm{dpH}} = \frac{\text{mL of acid or base added} \times \text{normality of acid or base}}{\text{volume of sample} \times \text{change in pH}} \quad (6.1)$$

6.2.5 Confocal laser scanning microscopy

Microstructure of the PBCAs was analysed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan). Fat and protein were stained with Nile Red and Fast Green FCF, respectively. As previously described by Le Tohic *et al*. (2018), a few drops of a blend of Nile Red in 1,2-propanediol (600 µL of 0.1 g/L) and Fast Green FCF aqueous solution (200 µL of 0.1 g/L) was applied onto the sample. Nile Red and Fast Green FCF were excited at 488 and 633 nm, using Ar and He-Ne lasers, respectively (Auty *et al*., 2001). Images of the samples were obtained using a 40X objective lens.

6.2.6 Differential scanning calorimetry

Thermograms of the PBCAs were obtained using a Mettler DSC821 (Mettler-Toledo, Schwerzenbach, Switzerland) differential scanning calorimeter (DSC) equipped with liquid nitrogen cooling. After 24 h at 4°C, the samples were weighted (2.9-4.7 mg) into hermetically sealed aluminium pans. Indium was used to calibrate the calorimeter for temperature and heat flow. The thermal behaviour of the samples was recorded as previously described by Grasso *et al*. (2022).

6.2.7 Schreiber meltability test

The Schreiber test, as previously described by Altan *et al*. (2005), was used to assess meltability of the PBCAs. Cylinders were prepared as described in Section 6.2.2 and using moulds of height 5 mm and diameter 41 mm. After storage, the samples were placed in a covered glass Petri dish and were heated at 232°C for 5 min in an

oven (Memmert, Schwabach, Germany). The samples were cooled at room temperature for 30 min, after which, the specimen expansion was measured with a ruler along six lines marked on a set of concentric circles. Meltability was calculated as previously reported by Ramel & Marangoni (2018). In addition, photographs of the samples were taken before and after the oven heating step.

6.2.8 Extensibility analysis

Extensibility of the PBCAs was measured using a Texture Analyser TA-XTPlus (Stable Micro Systems, Godalming, Surrey, UK) with a Cheese Extensibility Rig (A/CE) attachment. The samples were prepared and analysis performed as previously described by Grasso *et al*. (2023); briefly, 60 g of the PBCA was placed on the fork in the sample pot and heated for 12 min at 220°C in the oven. After this, a PT100 temperature probe was inserted into the cheese and when temperature of 55°C was reached the fork was pulled out of the sample at a speed of 10 mm/s and to a distance of 220 mm. From the raw data the area under the curve from 0 to 10 s was calculated for all samples and used as a measure of extensibility.

6.2.9 Dynamic low amplitude oscillatory shear rheology

The rheological characteristics of the PBCAs were measured using an AR-G2 controlled-stress rheometer (TA Instruments Ltd., Waters LLC, Leatherhead, UK), equipped with stainless steel parallel plates with crosshatched surfaces. Samples were prepared as described in Section 6.2.7. After storage for 24 h at 4°C, samples were equilibrated at room temperature and the analysis was performed by applying a constant frequency of 1 Hz and 0.5% strain, with a temperature ramp from 20 to 110°C at a ramp rate of 2°C/min. Liquid paraffin was used to coat the exposed edges of the

samples to prevent drying during the test. The rheological profiles of the samples were presented as the storage (G′) and loss (G″) moduli were reported, as well as the ratios between the two moduli, defined as the loss tangent (tan δ) (Fox *et al*., 2017). The melting index was calculated from G′ at 20 and 100°C as previously reported (Grasso *et al*., 2023).

6.2.10 Texture profile analysis

Texture profile analysis (TPA) of PBCAs was performed using a Texture Analyser TA-XTPlus (Stable Micro Systems, Godalming, Surrey, UK), with samples compressed to 30% of their original height, at a fixed speed of 1.0 mm/s. Samples were prepared by pouring the formulations prepared as described in Section 6.2.2, into glass moulds, precoated with siliconizing reagent for glass (Sigmacote®, Sigma-Aldrich, MO, US). After 24 h at 4°C, cylinders of 12 mm height and 20 mm diameter were analysed immediately after removal from storage. For each sample, the textural parameters hardness, adhesiveness, springiness and cohesiveness, were measured.

6.2.11 Statistical data analysis

Moisture, pH and buffering capacity of PBCAs were performed in triplicate. The mineral analysis was performed on one replica for each sample. Three independent trials were conducted to develop the cheese alternative samples and three independent replicates from each trial were used for all analyses. One-way analysis of variance (ANOVA) was performed using R i386 version 3.3.1 (R foundation for statistical computing, Vienna, Austria). A Tukey's paired comparison post-hoc test was performed to determine statistically significant differences ($p < 0.05$) between mean values for samples with different formulations, at a confidence level of 95%.

6.3 Results and discussion

6.3.1 Formulation, pH, moisture, mineral profile, buffering capacity and colour of samples

The PBCA samples were formulated to achieve the macro- and micronutrient targets described in Section 6.2.2, in accordance with the composition of the ingredients described in Section 6.2.1, and, as expected, the compositional targets of the samples were met. The mineral profile of samples is reported in Table 6.2; the Na content was lower than most commercially-available cheese products and plant-based cheese alternatives, while the Mg and P contents were comparable to, and lower than, traditional cheeses, respectively (O'Callaghan *et al*., 2017; Craig *et al*., 2022). The high levels of Na in cheese products is associated with negative consequences on consumers' health, such as increased blood pressure and decreased Ca absorption (Cruz *et al*., 2011). Values for K were slightly higher than most commercially-available cheese products; however, these were comparable to values reported for cream cheese (O'Callaghan *et al*., 2017). Moreover, the innate Ca content, corresponding to the content measured in the Control sample, was 0.01%, while the measured Ca content of fortified samples was in the range 0.57-0.60%, in agreement with the target value. As reported in Section 6.2.2, the target value of 0.60% was based on the calcium content found in processed cheese and other cheese varieties, such as mozzarella and Cheshire cheese. Moreover, the aim was to achieve a calcium content in the PBCA which provided a significant proportion of the recommended daily intake for this mineral, which is between 1000 and 1300 mg/d for adults (i.e., between 60 and 46.2% for 100 g of product) (FAO/WHO, 2001). Different amounts of each of the individual calcium salts were added, as reported in Table 6.1, depending on the potency of the salt, and consequently, small differences in moisture content were

measured in the samples. Furthermore, 1.8% of lactic acid was added to the Control sample to achieve pH ~4.5, and, with the same lactic acid content, differences in pH were observed depending on the calcium salt added to the sample (Table 6.1). A slight increase in pH was observed in the CaCitrate sample, when 1.8% of lactic acid was added; on the other hand, a decrease in pH with $CaCl_2$ addition was measured in the CaChloride sample. This was previously observed in emulsions prepared using dairy or plant protein ingredients (Crowley *et al*., 2014; Keowmaneechai & McClements, 2002; Alonso-Miravalles *et al*., 2020). As reported in these studies, this effect was likely due to positively charged salt ions displacing H^+ ions from the acid groups on the proteins, decreasing the pH.

Table 6.2. Mineral profile of Control and Ca fortified plant-based cheese alternative samples.

	Calcium (Ca)	**Potassium (K)**	**Magnesium (Mg)**	**Sodium (Na)**	**Phosphorus (P)**
Control	0.01	0.15	0.02	0.49	0.06
Ca Chloride	0.58	0.19	0.02	0.52	0.06
Ca Citrate	0.57	0.16	0.02	0.47	0.06
CaChlo:CaCitr	0.60	0.16	0.02	0.50	0.06

The results of the buffering capacity analysis (Fig. 6.1) showed that the CaCitrate sample had the highest buffering capacity, hence, a slightly higher amount of lactic acid was needed to adjust the pH to ~4.5 (Table 6.1). The main buffering peak for this sample was observed at pH ~3.5, probably linked to solubilisation of calcium citrate, resulting in buffering against acidification by release of counterions, as previously observed by Crowley *et al*. (2014) for calcium fortified skim milk. The CaChloride sample had similar buffering behaviour to the Control and had lower buffering capacity compared to CaCitrate.

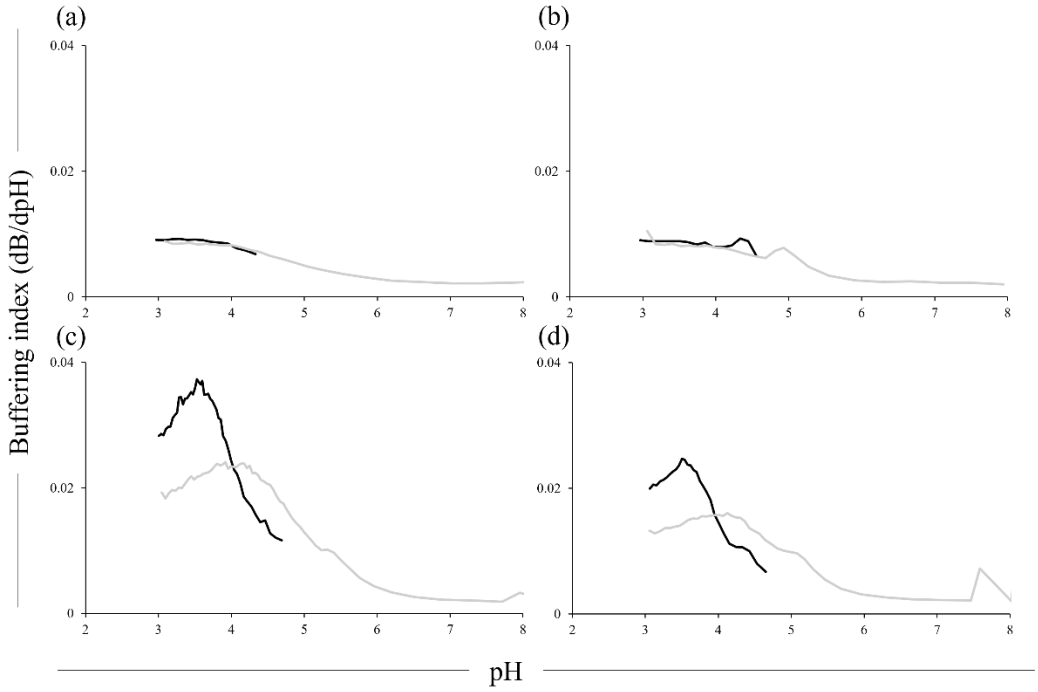


Figure 6.1. Acid (■) and base (□) buffering capacity curves of samples, Control (a), CaChloride (b), CaCitrate (c), and CaChlo:CaCitr (d).

Colour analysis showed that calcium fortification of samples considerably affected their colour characteristics (Figure 6.2). Calcium salt additions generally increased the greenness and brightness of the samples represented by the a* and L* coordinates, respectively. On the other hand, calcium fortification resulted in decreasing yellowness, represented by the b* coordinate. CaCitrate and CaChlo:CaCitr samples were statistically comparable for all the colour coordinates, whereas CaChloride was the most similar to the Control.

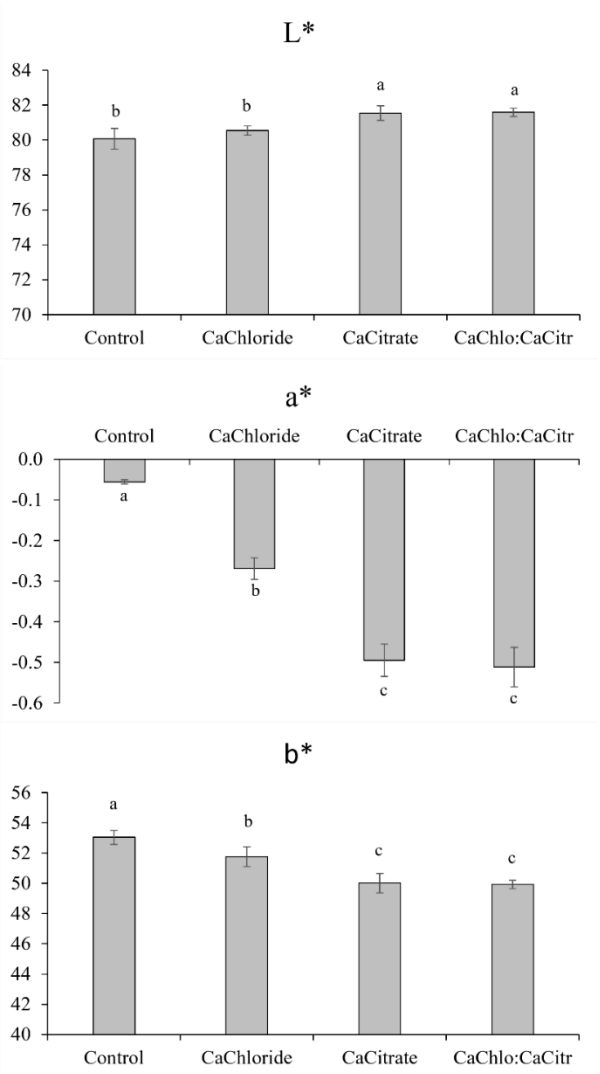


Figure 6.2. Colour space values (L* a* b*) of plant-based cheese alternative samples. Different letters on bars of the figure (a-c) indicate significant differences between samples ($p < 0.05$).

6.3.2 Microstructure

Differences in the microstructure of samples were shown by the images obtained with the confocal laser scanning microscope (Fig. 6.3). The fortification with calcium of the samples resulted in generally smaller fat globules; however, differences were observed depending on the calcium salts used. The CaChloride sample (Fig. 6.3b) showed smaller fat globules compared to the other fortified samples (Fig. 6.3c, d). Moreover, the majority of such fat globules appeared to be confined what was presumably a zein protein layer, with only a small proportion of total fat not entrapped. Indeed, as observed in a previous study, zein seemed to surround the fat globules with a protein layer, while the chickpea protein was more uniformly distributed in the matrix, with less evidence of protein aggregate formation (Grasso *et al*., 2023). In contrast, the CaCitrate sample showed larger fat globules, with numerous free non-spherical, coalesced pools of fat (Fig. 6.3c). The CaChlo:CaCitr sample had both free and entrapped globules of fat, reflecting the microstructure observed for the CaChloride and CaCitrate samples. In addition, all of the calcium fortified samples showed elongated shapes of protein containing the fat globules, which might correspond to zein, as previously observed by Ozturk *et al*. (2023) and defined by the same authors as zein fibrils. However, these were present to only a limited extent in the Control sample, suggesting contributions of calcium chloride and citrate in influencing the structure of zein. Calcium fortification resulted in some protein aggregation compared to the Control, with this aggregation possibly attributed to chickpea protein; indeed, a similar result was reported by Ozturk *et al*. (2023) for pea protein in zein-pea protein blends. The effect of calcium on the structural arrangement of zein was previously studied by the scientific community, and, although different testing conditions were used, the results showed that calcium ions led to changes in

secondary and tertiary structure of the protein, promoting protein-protein interactions and self-assembly of zein (Sun *et al*., 2017; Wang *et al*., 2022).

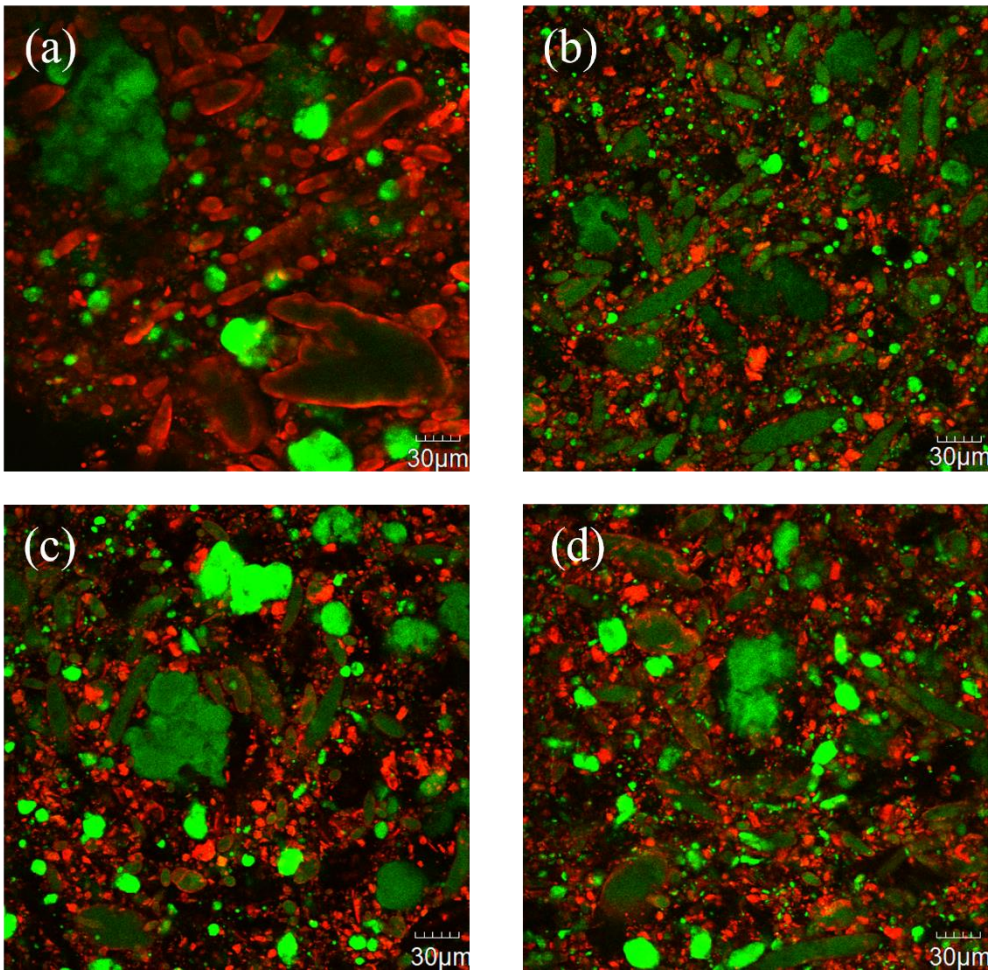


Figure 6.3. Confocal laser scanning microscopy images of the plant-based cheese alternative samples, Control (a), CaChloride (b), CaCitrate (c), and CaChlo:CaCitr (d), at 40X magnification. Fat and protein are represented in green and red, respectively.

6.3.3 Thermal behaviour

The thermograms of the PBCAs are shown in Figure 6.4. All samples showed a main peak around 30°C, corresponding to transition of the shea butter, as previously studied and reported (Grasso *et al*., 2022). The shape of the peak differed between the samples, with this peak in the CaChloride sample (Fig. 6.4b) being smaller and more similar to the Control, compared to the other calcium fortified samples (Fig. 6.4c, d). One explanation for the differences in peak shape, may be the higher amount of free fat in the CaCitrate and CaChlo:CaCitr samples (Section 6.3.2); moreover, the different distribution of fat and protein within the sample matrices likely influenced the thermal transition of the shea butter, as previously observed (Grasso *et al*., 2023). All samples contained 6.7% tapioca starch; however, transition of this ingredient was not visible from the thermograms. This was in agreement with previous studies (Zheng *et al*., 2020; Grasso *et al*., 2023), with a possible explanation being the competition between starch and protein for available water.

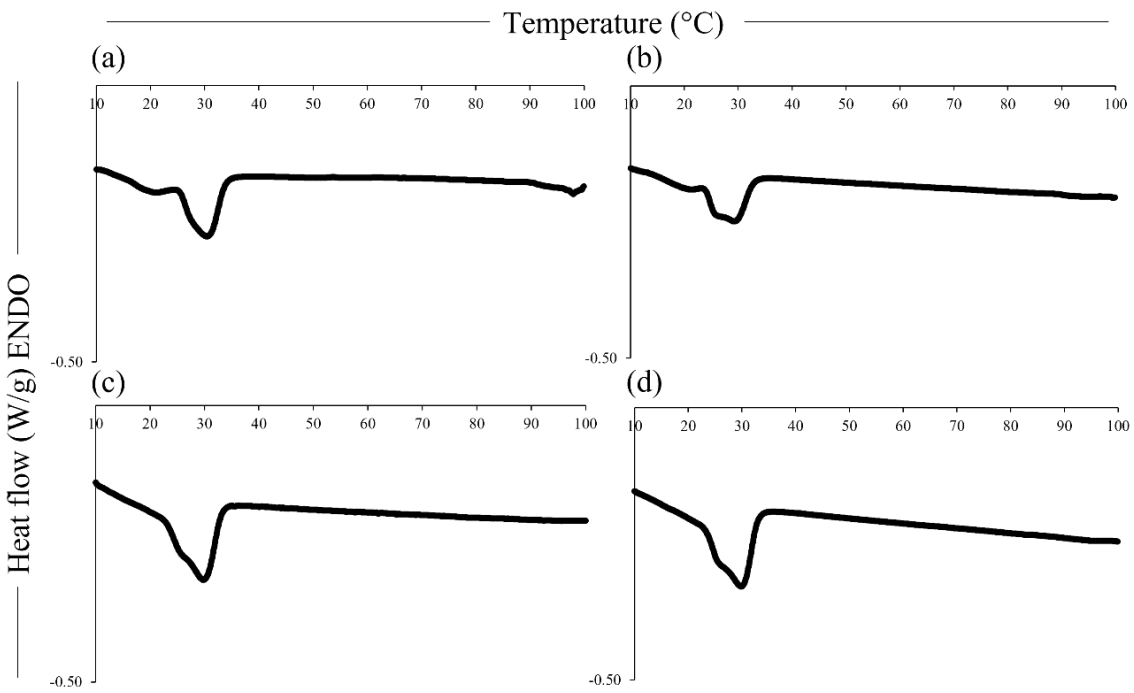


Figure 6.4. Differential scanning calorimetry thermograms of the plant-based cheese alternative samples, Control (a), CaChloride (b), CaCitrate (c), and CaChlo:CaCitr (d).

6.3.4 Meltability

Meltability of samples is reported in Table 6.3 and Figure 6.5. Calcium fortification resulted in decreasing meltability, with statistically comparable diameter expansions shown by CaChloride, CaCitate and CaChlo:CaCitr samples (Table 6.3), and also by the photographic images of the samples before and after oven heating (Fig. 6.5). Lower meltability of Cheddar and processed cheeses with high calcium contents was previously reported in the scientific literature (Chevanan *et al*., 2006; Biswas *et al*., 2015).

Table 6.3. Schreiber test meltability results expressed as diameter expansion, stretchability results expressed as area under the curve, melting index, and textural parameters of control and calcium fortified samples.

	Meltability – Diameter expansion (%)	**Stretchability – Area under the curve (kN·s)**	**Melting index (%)**	**Hardness (N)**	**Adhesiveness (N·s)**	**Springiness (-)**	**Cohesiveness (-)**
Control	25.7 ± 2.38^{a}	4.17 ± 0.45^{b}	91.4 ± 0.75^{a}	8.16 ± 0.97^{b}	3.97 ± 0.40^{c}	0.55 ± 0.04^{b}	0.33 ± 0.01^{c}
Ca Chloride	20.3 ± 2.10^{b}	5.06 ± 0.31^{a}	88.1 ± 0.99^{b}	11.4 ± 0.93^{a}	6.34 ± 0.51^{b}	0.65 ± 0.04^{a}	0.38 ± 0.03^{a}
Ca Citrate	20.7 ± 1.83^{b}	4.67 ± 0.28^{a}	90.2 ± 1.41^{a}	11.8 ± 0.89^{a}	7.36 ± 0.67^{a}	0.60 ± 0.03^{a}	0.34 ± 0.01^{bc}
CaChlo:CaCitr	21.7 ± 1.83^{b}	4.98 ± 0.38^{a}	90.5 ± 1.01^{a}	10.9 ± 0.93^{a}	7.48 ± 0.35^{a}	0.61 ± 0.05^{a}	0.36 ± 0.03^{ab}

Values followed by different superscript letters in a column (a-c) are significantly different ($p < 0.05$).

Indeed, in cheese the number and strength of interactions between casein molecules contribute strongly to the melting properties of the products, and calcium, in particular insoluble forms, plays an important role in enhancing such interactions and promoting cross-linking between casein molecules, having a negative impact on cheese melting (Lucey *et al*., 2003). The melting behaviour of zein, and its ability to flow at high temperature creating a viscoelastic mass, was previously studied (Mattice & Marangoni, 2020; Grasso *et al*., 2023). Similar to cheese, it is possible that calcium salts promoted interactions between proteins, and in turn, a more rigid structure in calcium fortified PBCAs, resulting in lower meltability compared to the Control.

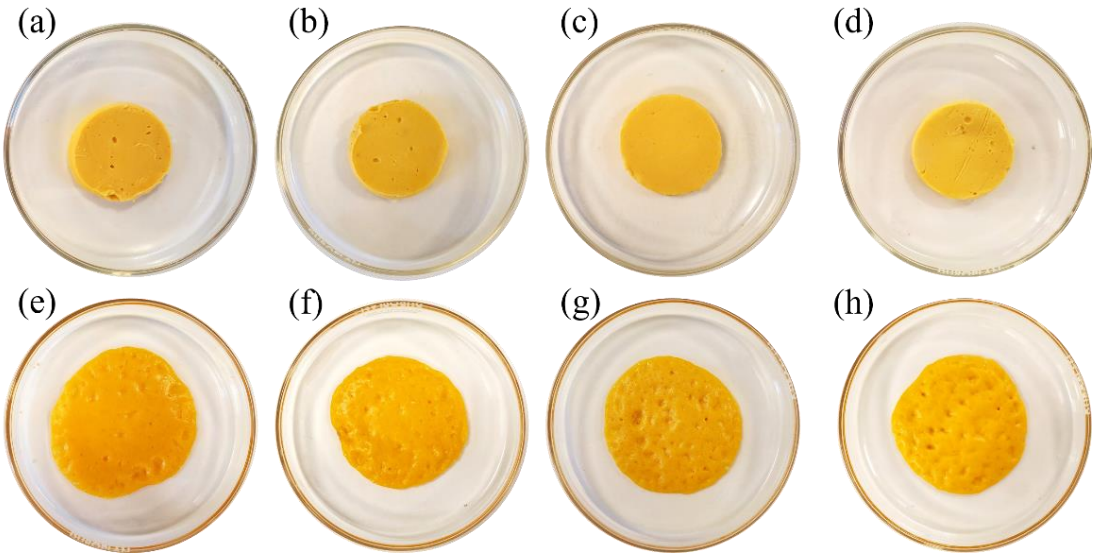


Figure 6.5. Photographs of plant-based cheese alternative samples, Control, CaChloride, CaCitrate, and CaChlo:CaCitr, before (a-d) and after (e-h) oven heating at 232°C for 5 min.

6.3.5 Stretchability

Interestingly, fortification of the PBCAs resulted in improved stretching properties for CaChloride, CaCitrate and CaChlo:CaCitr samples, as shown in Table 6.3. However, no significant differences were observed between the fortified samples. The stretchability results were in agreement with the results reported by Salgado *et al*. (2023). Indeed, the authors performed an extensional viscosity test on zein dough formulations containing calcium hydroxide, and observed higher extension for samples with high levels of calcium, attributing this to the influence of calcium ions on formation of structured networks due to calcium-protein and calcium-starch interactions. The stretching properties of zein in a model plant-based cheese alternative were previously studied by Mattice & Marangoni (2020), and the extensibility observed at high temperature was attributed to the weakening of non-covalent interactions within zein networks, without complete loss of same. Furthermore, the ability of zein networks to stretch in plant-based cheese alternatives was previously studied by our group and promising results were reported for samples containing zein protein isolate (Grasso *et al*., 2023).

6.3.6 Rheological properties

The rheological behaviour of the samples followed a similar trend to the meltability results, with higher melting index for the Control sample compared to the fortified samples, and with CaChloride having the lowest (Table 6.3). Moreover, the Control had lower storage modulus (G′) at 20°C and higher loss tangent (tan δ) at 110°C than the other samples (Fig. 6.6). The results of this study were in agreement with Salgado *et al*. (2023); in fact, the authors reported more solid-like behaviour of zein dough formulations with increasing calcium hydroxide contents, with a possible

explanation for this being the enhancement of interactions and consequent formation of a strong zein structure. As previously described by Grasso *et al*. (2023), all samples showed a rapid and extensive softening between 20 and 40°C, probably due to melting of shea butter. Furthermore, increasing tan δ values were observed for all samples at temperature greater than 90°C, representing further softening and suggesting the ability of zein to flow at high temperatures due to weakening of non-covalent bonds, as reported in Sections 6.3.4 and 6.3.5, and by Mattice & Marangoni (2020).

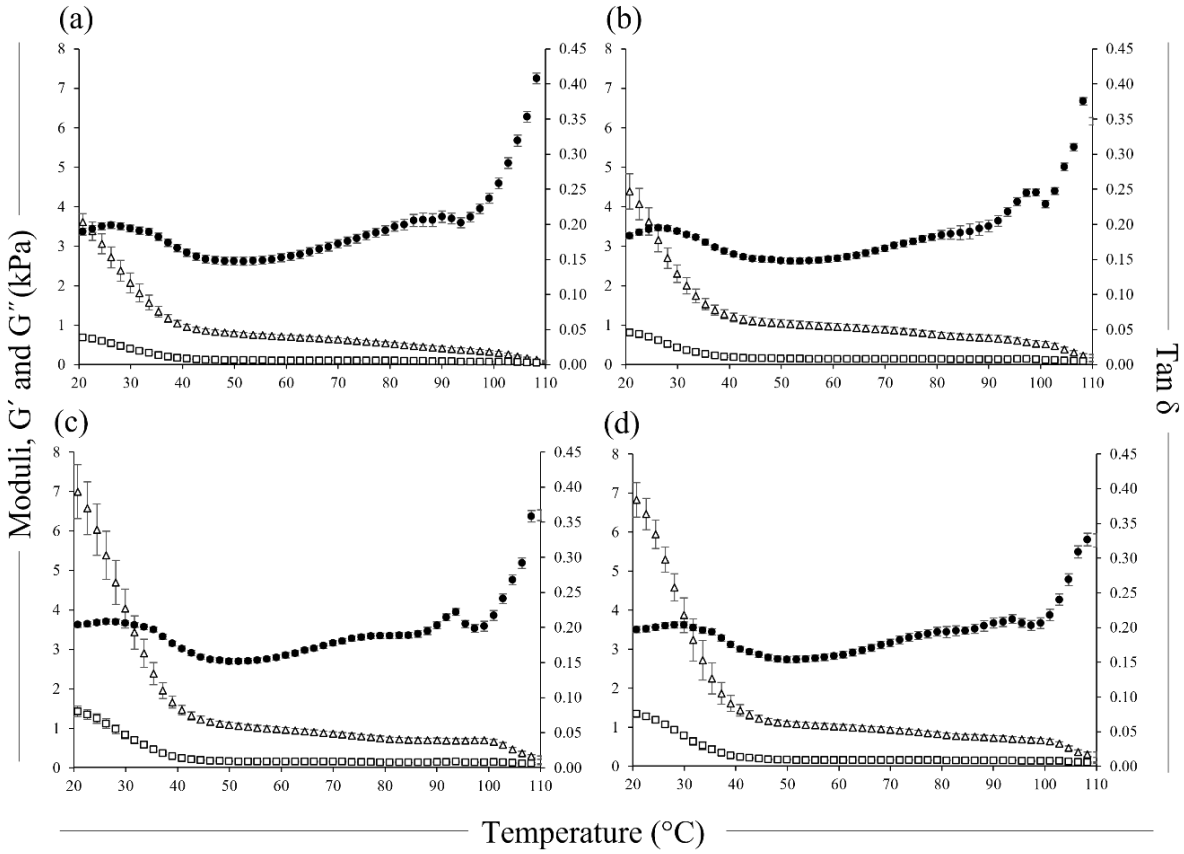


Figure 6.6. Rheological profiles showing storage modulus (G′) (open triangle), loss modulus (G″) (open square) and loss tangent (tan δ) (filled circle) as a function of temperature in the range 20–110°C for Control (a), CaChloride (b), CaCitrate (c), and CaChlo:CaCitr (d) plant-based cheese alternative samples.

6.3.7 Textural properties

The textural characteristics of the PBCA samples are reported in Table 6.3. Samples fortified with calcium showed higher hardness, adhesiveness, springiness and cohesiveness compared to the Control sample, in agreement with the results for zein dough formulations blended with calcium and other ingredients, reported by Salgado *et al*. (2023). Increased hardness was previously observed in cheese with high calcium contents, proving that calcium strongly affects the textural properties of cheese products (Lucey & Fox, 1993; Chevanan *et al*., 2006; O'Mahony *et al*., 2006; Biswas

et al., 2015) However, in this study, CaChloride, CaCitrate and CaChlo:CaCitr samples showed no significant differences in hardness values. The springiness and cohesiveness were the highest for the CaChloride sample, while the CaChlo:CaCitr sample showed the highest adhesiveness value, with the CaCitrate being statistically comparable. The results of the TPA analysis showed potential in modulating the textural properties of PBCAs using different calcium salts or combinations thereof.

6.4 Conclusion

In this study, the effects of two different calcium salts (i.e., calcium chloride and calcium citrate) and a combination thereof, on the physicochemical properties of plant-based cheese alternatives, were investigated. Calcium fortification of samples generally resulted in smaller fat globules; however, differences in the microstructure were shown depending on the calcium salt used. Decreasing meltability was observed with calcium addition, while stretching properties were enhanced for the fortified samples compared to the Control. Texture profile analysis showed that calcium strongly affected the texture of samples, with higher hardness, adhesiveness, springiness and cohesiveness values, showing potential in modulating texture using calcium salts. This work helps develop our understanding of the role of calcium fortification and the effect of different calcium salts in formulating plant-based cheese alternatives with improved nutritional profile and the impact on relevant physicochemical properties of same.

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Chapter 7

Formulation and key quality attributes of a plant-based alternative to cheese prototype prepared using zein and chickpea protein ingredients

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Declaration: This chapter was written by author Nadia Grasso (NG) and reviewed by all co-authors. NG co-designed the study and performed all the experimental work with the exception of the digestion analysis, which was performed by co-authors Linea H. Thulesen and Iben L. Petersen, and the volatile profile analysis, which was performed by co-authors Iwona Skibinska and Kieran N. Kilcawley.

Abstract

With the expanding global population, the growing demand for food provides opportunities to formulate new products. Plant-based cheese alternatives can be made from different raw materials and processes. In this study, a plant-based alternative to cheese prototype was formulated using a blend of zein and chickpea protein ingredients. The composition, physicochemical properties, protein digestibility and volatile profile of the prototype were investigated, using two commercially-available plant-based cheese alternatives, dairy processed cheese and Cheddar cheese, as benchmarks. Meltability of the prototype was comparable to Cheddar and to one of the commercial plant-based cheese alternatives, as shown by the Schreiber meltability test. Texture of the prototype was similar to the processed cheese sample, with comparable Young's Modulus results and similar hardness values. *In vitro* protein digestibility was not measured for the commercial plant-based cheese alternatives, due to the absence of protein in the products, while the prototype sample showed lower digestibility (7.9%) than the dairy benchmarks (20.5 and 21.5% for processed and Cheddar cheeses, respectively). Analysis of volatile profiles showed that the prototype sample had the highest number of compounds, with a profile that differed greatly from the other samples. The results of this study show that plant-based cheese alternatives can be formulated to closely match selected physicochemical characteristics of dairy products. In this work, a prototype with improved compositional and physicochemical properties compared with plant-based cheese alternatives available commercially was successfully formulated.

7.1 Introduction

The expanding global population, which reached 8 billion people in 2022, and is expected to reach almost 10 billion by 2050 (United Nations, 2022), and the resultant growing demand for food, provide opportunities to formulate new products. Indeed, the availability of food products on the market is already increasing, with consumers considering a greater number of factors when purchasing their food (Tso *et al*., 2020). In this scenario, the plant-based foods sector represented an $8 billion market in 2022 in the United States alone, with a 3-year growth in sales of 44% observed between 2019 and 2022, and, in the same time-frame, the plant-based cheese sector showed 51% growth in sales in the US, and in Europe a sales growth of 23% in 2022 (Good Food Institute, 2023). In parallel, global cheese production and consumption are also increasing and are expected to increase further in the next few years, with the highest values observed in Europe and North America (OECD/FAO, 2021).

Furthermore, the WHO/Europe (2021) and the most recent report on climate change (IPCC, 2023), recommended a transition towards more plant-based diets (with animal products not necessarily omitted) for nutritional and environmental reasons; however, in the scientific community a lack of knowledge of the science underpinning development of plant-based products, and in particular plant-based cheese alternatives, is observed (Grossmann & McClements, 2021).

Plant-based cheese alternatives can be made from different raw materials and using several different technological and processing options. Currently, most of the commercially-available plant-based cheese alternatives have low protein and high saturated fat contents, containing starch and coconut oil as the principal ingredients. Nuts, such as cashews and almonds, are also employed in some commercial products,

with many of these products being fermented. In recent years, researchers have investigated the suitability of new plant-derived ingredients for the formulation of plant-based cheese alternative products. Plant proteins show great potential in such applications; however, dairy protein provides cheese products with unique textural properties and the replication of such properties using plant proteins is challenging and poorly understood (Mattice & Marangoni, 2020; Grossmann & McClements, 2021). Moreover, another important aspect to consider when using plant protein ingredients is their sensorial contribution to the product in which they are included; indeed, plant proteins are often responsible for undesirable or off-flavours in the final product (Short *et al*., 2021).

Legumes are a common source material used for extraction of protein, and plant-based cheese alternatives produced therefrom could be formulated with higher protein contents compared to the products currently available commercially. Legumes are rich in nutrients, and protein concentrates and isolates from legumes are generally milder in colour, flavour, odour, and have lower contents of antinutritional components compared to whole seeds, presenting potential for application in production of plant-based cheese alternative production (Mefleh *et al*., 2021). Digestibility of legume proteins is generally lower than animal proteins; however, factors such as legume variety, processing and extraction methods play key roles in determining, and often enhancing, digestion of legume proteins (Sá *et al*., 2019).

Among legumes, chickpeas represent a significant source of dietary protein, with protein contents of 20–25%, as well as high contents of fat, starch and fibre, minerals and vitamins (Hall *et al*., 2017). Furthermore, chickpea protein ingredients show functional properties of relevance in cheese alternative applications, such as oil absorption capacity and emulsification and gelling properties, due to the

predominance of globulin protein fractions (Kaur & Singh, 2007; Withana-Gamage *et al*., 2011). Zein is another plant protein with considerable potential in plant-based cheese alternative applications; indeed, zein displays plastic behaviour in aqueous environments, along with softening and increased viscous properties on increasing temperature, a rare characteristic for plant proteins (Mattice & Marangoni, 2020a; Grasso *et al*., 2023). The hydrophobicity and self-aggregating behaviours of zein are associated with its high proportion of non-polar amino acids; this and non-covalent interactions result in formation of viscoelastic networks (Argos *et al*., 1982; Smith *et al*., 2014).

In a previous study, blends of zein and chickpea protein ingredients were evaluated for the development of plant-based cheese alternatives, with some of the formulations showing desirable functional properties (e.g., melting and stretching) in cheese applications (Grasso *et al*., 2023). The use of blends of different plant proteins in food products provides opportunities to improve the physicochemical, nutritional and sensory properties of the final product (Jiménez-Munoz *et al*., 2021).

The aim of this study was to formulate a plant-based cheese alternative prototype using a blend of zein and chickpea protein ingredients, which previously displayed uniquely promising properties in such applications, and to investigate the physicochemical properties, protein digestibility and volatile profile of same. Two commercially-available plant-based cheese alternatives, dairy processed cheese and Cheddar, were used as benchmarks.

7.2 Materials and methods

7.2.1 Materials

To formulate the plant-based cheese alternative prototype, commercially-available zein protein isolate (ZPI) (Flo Chemical Corporation, Ashburnham, MA, USA), with 81.5% protein, 5.62% moisture, 3.92% carbohydrate, 7.68% fat and 1.28% ash, and chickpea protein concentrate (CPC) (Artesa, PLT Health Solutions, Morristown, NJ, USA), with 53.1% protein, 7.73% moisture, 33.3% carbohydrate, 1.37% fat and 4.47% ash, were used. Moreover, tapioca starch, with 0.11% protein, 11.3% moisture, 88.3% carbohydrate, 0.25% fat and 0.03% ash, was purchased from a local retail outlet (Quay-coop, Cork, Ireland) and shea butter was kindly provided by Fuji Oil (Zenitex M 50 G, Fuji Oil Europe, Ghent, Belgium), and was employed as a source of solid fat. Calcium chloride dihydrate was purchased from Sigma-Aldrich (St Louis, MO, US) and dairy-free natural cheese flavourings (Natural Butter Cream Type Flavour #1413189, Natural Cheddar-Type Flavour #1411433, Natural Cheddar Cheese Mature-Type Flavour #1413366) were kindly provided by Edlong Europe Ltd. (Moorepark, Fermoy, Cork, Ireland). The commercial plant-based cheese alternatives, processed and Cheddar cheeses, in the format of slices, were purchased from a local retail outlet (Tesco, Cork, Ireland). The ingredient list of the products as reported on the labels is shown in Table 7.1 and their photographs are reported in Figure 7.1.

Table 7.1. Ingredient list of benchmarks products.

Product	Ingredients
Plant-based 1	Water, coconut oil (23%), modified starch, starch, sea salt, flavourings, olive extract, colour: β-carotene, vitamin B12
Plant-based 2	Water, coconut oil (25%), modified potato starch, salt, calcium lactate, preservative: sorbic acid, natural flavourings, natural colour: β-carotene, iron, vitamin D2, vitamin B6, vitamin B12
Processed	Cheese (60%) (Milk) (contains acidity regulator: lactic acid), palm oil, water, emulsifying salts (polyphosphate, calcium phosphate, sodium phosphate), modified potato starch, milk protein, natural cheese flavouring (milk), colours (β-carotene, paprika extract)
Cheddar	Milk, rennet, salt

7.2.2 Formulation of the plant-based cheese alternative prototype

An ingredient ratio of 1:1.2 (corresponding to a 35:65 ratio based on protein contribution) of CPC and ZPI, respectively, was used to formulate the cheese alternative prototype sample (Table 7.2), and to target 16% protein. Shea butter was included to target 14.1% fat content and a lactic acid solution was mixed with water, NaCl and $CaCl_2$ to obtain a pH of ~4.5 and 59% moisture. Tapioca starch was added to target 7.2% carbohydrate content and three different flavourings (i.e., natural butter cream, natural Cheddar and natural Cheddar mature) were added to a final concentration of 1.5%.

Table 7.2. Formulation of plant-based cheese alternative prototype.

Ingredients	(%)
Chickpea protein concentrate	10.5
Zein protein isolate	12.8
Tapioca starch	3.61
Shea butter	11.8
Water	55.7
Lactic acid solution	0.86
Sodium Chloride	0.85
Calcium Chloride	2.20
Flavourings	1.50

The method previously reported by Grasso *et al*. (2023) was used to formulate the prototype with some modifications. Briefly, ZPI, CPC and tapioca starch were mixed with the water solution (containing lactic acid, NaCl and $CaCl_2$) in a Thermomix (TM 5, Vorwerk, Wuppertal, Germany) at speed 1 (100 rpm) for 1 min. After that the flavourings were added, and the temperature was set to 100°C, when 40°C was reached (after ~30 s), pre-melted shea butter was also added to the mixture at speed 2.5 (350 rpm) for 6 min. The mixture was poured into moulds after heating and stored at 4°C for 24 h before analysis. The photograph of the prototype is reported in Figure 7.1.

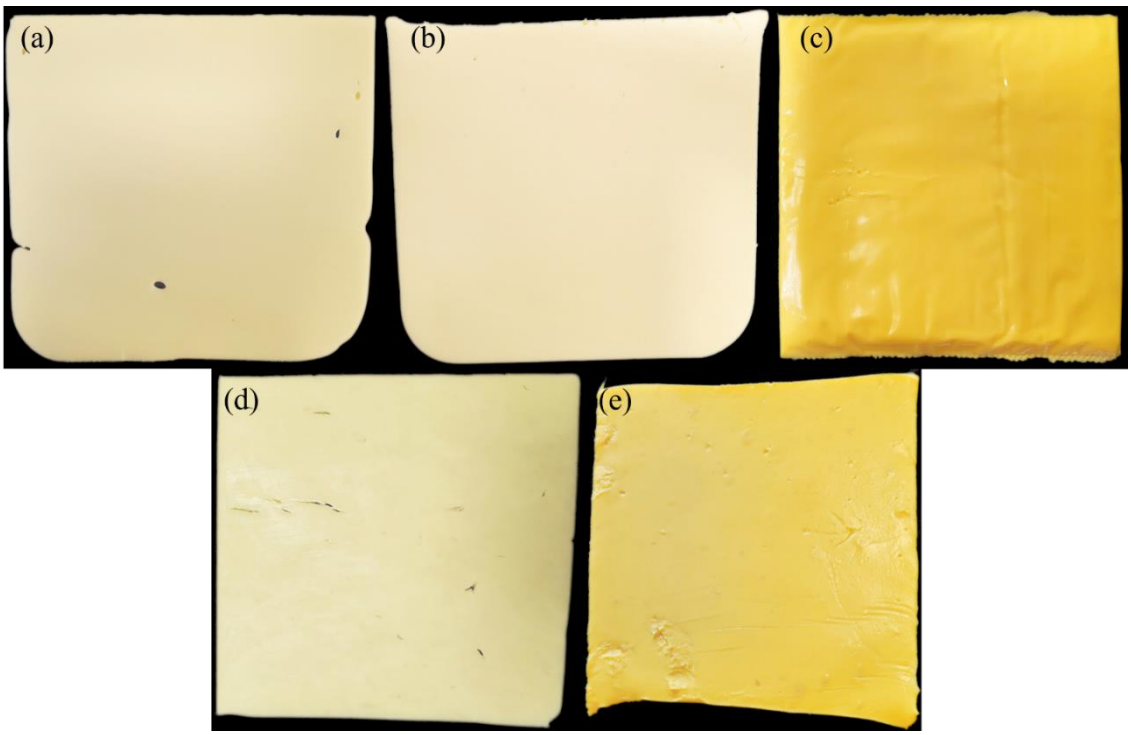


Figure 7.1. Photographs of the plant-based cheese alternative prototype and benchmark products, Plant-based 1 (a), Plant-based 2 (b), Processed (c), Cheddar (d) and Prototype (e)

7.2.3 Composition, pH and colour of cheese and plant-based cheese alternatives

Total nitrogen content of the prototype was measured using the Kjeldahl method 2001.14 (AOAC, 2002), with a nitrogen-to-protein conversion factor of 6.25. Moisture content was determined by oven drying, according to method 926.08 (AOAC, 1990b). Ash content was measured by incineration in a muffle furnace according to method 935.42 (AOAC, 1990a). Fat content of the prototype sample was assessed using the Soxhlet AACC method 30-25.01 (AACC, 2009). Total carbohydrate was calculated by difference (i.e., 100 minus the sum of protein, fat, ash and moisture). The colour and pH of the plant-based cheese alternative prototype and benchmark products were assessed as previously described by Grasso *et al*. (2022).

7.2.4 Confocal laser scanning microscopy

Microstructural analysis of the prototype and benchmark products was performed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan). Fat and protein in the samples were stained using Nile Red and Fast Green FCF, respectively, following the method previously described by Le Tohic *et al*. (2018). Images of the prototype and benchmark products, obtained using a 60X objective lens, were reported.

7.2.5 Dynamic low amplitude oscillatory shear rheology

Rheological properties of the prototype and commercial samples were measured with an MCR 102e rheometer (Anton Paar GmbH, Graz, Austria), equipped with crosshatched surface stainless steel parallel plates of diameter 50 mm. The prototype sample was prepared by pouring the mixture after processing in the Thermomix into moulds of diameter 50 mm and height 2 mm, and after 24 h at 4°C the samples were

analysed. The commercial samples were prepared from the slices using a manual circular cutter. Samples were equilibrated at room temperature before analysis and the force during sample loading did not exceed 0.5 N. Immediately post loading, the exposed edges of the samples were coated with liquid paraffin to prevent drying. The analysis was performed at a constant frequency of 0.5 Hz, strain of 0.08% and force of 0 N during the test, with a temperature ramp from 20 to 105°C. The rheological profiles, showing the storage (G′), and loss (G″) moduli and the loss tangent (tan δ), representing the ratio between the two moduli, were reported for all samples.

7.2.6 Schreiber meltability test

Meltability of the prototype sample and benchmark products was assessed using the Schreiber test (Altan *et al*., 2005). Discs of diameter 41 mm and height 2 mm were prepared from the slices using a manual circular cutter and heated at 232°C for 5 min in an oven (Memmert, Schwabach, Germany). After 30 min at room temperature, specimen expansion was measured and reported as previously described by Ramel & Marangoni (2018). Photographs of the samples were taken before and after heating.

7.2.7 Textural properties

To access the textural properties, a penetration test was performed on the prototype and benchmark products using a texture analyser TA-XTPlus (Stable Micro Systems, Godalming, Surrey, UK) equipped with a P/6 cylindrical probe of diameter 6 mm. Samples were prepared as described in Section 7.2.5. The test was performed for a distance of 12 mm and at a compression rate of 1.0 mm/s. Hardness, time to peak, and Young's modulus (E), as well as the force-time profiles, were reported for all samples.

7.2.8 In vitro protein digestibility

A static multi-step *in vitro* protein digestibility (IVPD) method was used according to Joehnke *et al*. (2018) to simulate the gastro-pancreatic stages of digestion. In brief, samples (standardised to 25 mg protein) were suspended in 10 mL of HCl (0.05 M) and homogenised using a ultraturrax mixer for 30 s. An equivalent amount of bovine serum albumin (BSA) and free alanine amino acid samples were included as reference protein with high protein digestibility and as internal standard representing 100% protein digestibility, respectively. The two steps of enzymatic digestion consisted of hydrolysis by pepsin (0.5 mg/mL) for 1 h at 37°C and pH 1-2, followed by pancreatin (1 mg/mL) for 1 h at 37°C and pH 7-8 at constant enzyme:substrate ratios of approximately 1:50 and 1:10 w/w, respectively. Before enzymatic digestion, sample aliquots were withdrawn, as well as after pepsin and pancreatin digestion. IVPD of samples before enzymatic digestion (untreated), after pepsin digestion and after pancreatin digestion were quantified using a trinitrobenzenesulfonic acid (TNBS)-based assay according to Joehnke *et al*. (2018). IVPD (%) was calculated as the ratio of the concentration of free α-amino groups in the samples and an alanine standard solution representing 100% protein digestibility. The enzymatic self-digestion was accounted for by subtracting the value of blank samples containing only buffer.

7.2.9 Analysis of volatile compounds

Samples were prepared for the analysis by weighing 4 g and adding 100 µL of internal standard at 5 ppm into a 20 ml screw capped solid-phase microextraction (SPME) vial, equilibrated to 40°C for 10 min with pulsed agitation of 5 s at 500 rpm. A single 50/30 µm CarboxenTM/divinylbenzene/polydimethylsiloxane

(DVB/CAR/PDMS) fibre was used to perform the analysis. The fibre was exposed to the headspace above the samples for 20 min at a depth of 1 cm at 40°C. The fibre was retracted and injected into the gas chromatography (GC) inlet and desorbed for 2 min at 250°C. Injections were made on a Shimadzu 2010 Plus GC with an Agilent DB-624 UI (60 m x 0.32 mm x 1.8 μm) column using a split/splitless injector with a 1/10 split, with a merlin microseal used as the septum. The temperature of the column oven was set at 40°C, held for 5 min, increased at 5°C/min to 230°C then increased at 15°C/min to 260°C, held for 5 min yielding total GC run time of 65 min. The carrier gas was helium, held at a constant flow of 1.2 ml/min. The detector was a Shimadzu TQ8030 mass spectrometer detector, run in single quad mode, with ion source temperature of 220°C and the interface temperature was set at 260°C. The mass spectrometry (MS) mode was electronic ionization (70 v) with the mass range scanned between 35 and 250 amu. Compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, a commercial flavour and fragrance library (FFNSC 2, Shimadzu Corporation, Japan) and an in-house library created using authentic compounds with target and qualifier ions and linear retention indices for each compound using Kovats index. Retention indices were matched against peer reviewed publications where possible to confirm compound identification. Spectral deconvolution was also performed to confirm identification of compounds using an Automated Mass Spectral Deconvolution and Identification System (AMDIS). Batch processing of samples was carried out using MetaMS, an open-source pipeline for GC-MS-based untargeted metabolomics (Wehrens *et al*., 2014). An auto-tune of the GC-MS was carried out prior to the analysis to ensure optimal GC-MS performance. A set of external standards was run at the start and end of the sample set and abundances

were compared to known amounts to ensure that both the SPME extraction and MS detection was performing within specification.

7.2.10 Statistical data analysis

Compositional analysis of the prototype, as well as *in vitro* protein digestion and volatile profile analysis of all samples were performed in triplicate. Three independent trials were conducted to develop the prototype, and three independent replicates from each trial were used for all analyses. For the benchmark products, three separate product samples were bought for each sample and three replicates from each product were used for analysis. One-way analysis of variance (ANOVA) was carried out using R i386 version 3.3.1 (R foundation for statistical computing, Vienna, Austria). A Tukey's paired comparison post-hoc test was used to determine statistically significant differences ($p < 0.05$) between mean values for different samples, at a 95% confidence level. Principal component analysis (PCA) of volatile compounds in the samples was also performed. Results are expressed as mean ± standard deviation with statistically significant differences identified using superscript letters.

7.3 Results and discussion

7.3.1 Composition, pH and colour of prototype and benchmark products

As expected from the formulation, the prototype sample had 16.6% protein (Table 7.3), with the target value being 16%. In contrast, the commercial plant-based cheese alternatives had no protein and were starch and coconut oil based (Table 7.1), with carbohydrate contents of 20 and 23% for the Plant-based 1 and 2 samples, respectively, as reported in the labels, being considerably higher than the other dairy and prototype samples (Table 7.3). These products were chosen due to their ingredients and composition being representative of most of the plant-based cheese alternatives commercially-available (Grasso *et al*., 2021). The fat content was the highest for the Cheddar and the lowest for the prototype sample, while pH was higher for the dairy samples compared to the plant-based cheese alternatives, both commercial and prototype. For the prototype sample, the pH was targeted at 4.5, with 0.86% lactic acid solution used to achieve a value of 4.51. Further studies on development of plant-based cheese alternatives with comparable pH to that of traditional cheese and on the effect of pH on sensory, are needed. The colour of the samples is reported in Table 7.3. The CIELAB coordinates for the Cheddar sample were comparable to previous results (Grasso *et al*., 2021). The L* value, representing the brightness, was higher for the commercial plant-based cheese alternatives compared to the other samples and the prototype was not significantly different from the Cheddar. The a* value was the highest for the Processed sample (i.e., towards red colour) and the Plant-based 1 was more negative (i.e., towards green colour). All samples, except for the Processed, had negative a* values. The b* coordinate, representing the degree of yellowness to blueness, was the highest for the prototype (i.e., towards yellow colour), due to the characteristic yellow colour of ZPI, as previously reported by Grasso *et al*. (2023).

Table 7.3. Composition of plant-based cheese alternative prototype and benchmark products.

	Protein (%)	Fat (%)	Moisture (%)	Ash (%)	Carbohydrate (%)	pH (-)	L* (-)	a* (-)	b* (-)
Plant-based 1	0*	23*	n.a.	n.a.	20*	4.94 ± 0.01^{c}	89.8 ± 0.66^{b}	-4.67 ± 0.15^{e}	37.2 ± 0.78^{c}
Plant-based 2	<0.1*	25*	n.a.	n.a.	23*	4.65 ± 0.01^{d}	90.7 ± 0.27^{a}	-2.40 ± 0.22^{c}	26.4 ± 0.51^{e}
Processed	13*	21*	n.a.	n.a.	7.9*	5.91 ± 0.01^{a}	83.7 ± 0.51^{c}	8.50 ± 0.26^{a}	44.0 ± 1.00^{b}
Cheddar	25.4*	34.9*	n.a.	n.a.	0.1*	5.40 ± 0.01^{b}	81.0 ± 0.33^{d}	-4.33 ± 0.21^{d}	33.6 ± 0.69^{d}
Prototype	16.6 ± 0.64	14.5 ± 0.30	57.0 ± 0.74	3.10 ± 0.17	8.85	4.51 ± 0.01^{e}	80.9 ± 0.77^{d}	-0.06 ± 0.01^{b}	47.5 ± 1.02^{a}

*Values as reported on the labels of the benchmark products.

n.a.= not available

Values followed by different superscript letters in a column (a-e) are significantly different ($p < 0.05$).

7.3.2 Microstructure

From analysis of the microstructure, it was evident that the commercial plant-based samples (Fig. 7.2a and b) showed mostly spherical fat globules, in particular the Plant-based 2 sample, within a matrix of starch and other hydrocolloids, with no protein evident, aligned with the information provided on the label of these samples (Table 7.2). The Cheddar sample showed typical structure (Fig. 7.2d), with a continuous protein phase and non-spherical shaped coalesced pools of fat, as previously reported by Guinee *et al*. (2000) and Grasso *et al*. (2021). The Processed sample had the smallest fat globules and few coalesced pools of fat (Fig. 7.2c), similar to previous microstructural images reported by Ramel & Marangoni (2017). The prototype sample (Fig. 7.2e) showed a protein layer (possibly zein) surrounding some of the fat globules, which were mostly spherical and/or elongated, with a number of free fat pools and globules. In addition, some aggregation of protein was observed in the prototype sample, in agreement with previous microstructural observations on plant-based cheese alternatives developed using ZPI and CPC ingredients (Grasso *et al*., 2023).

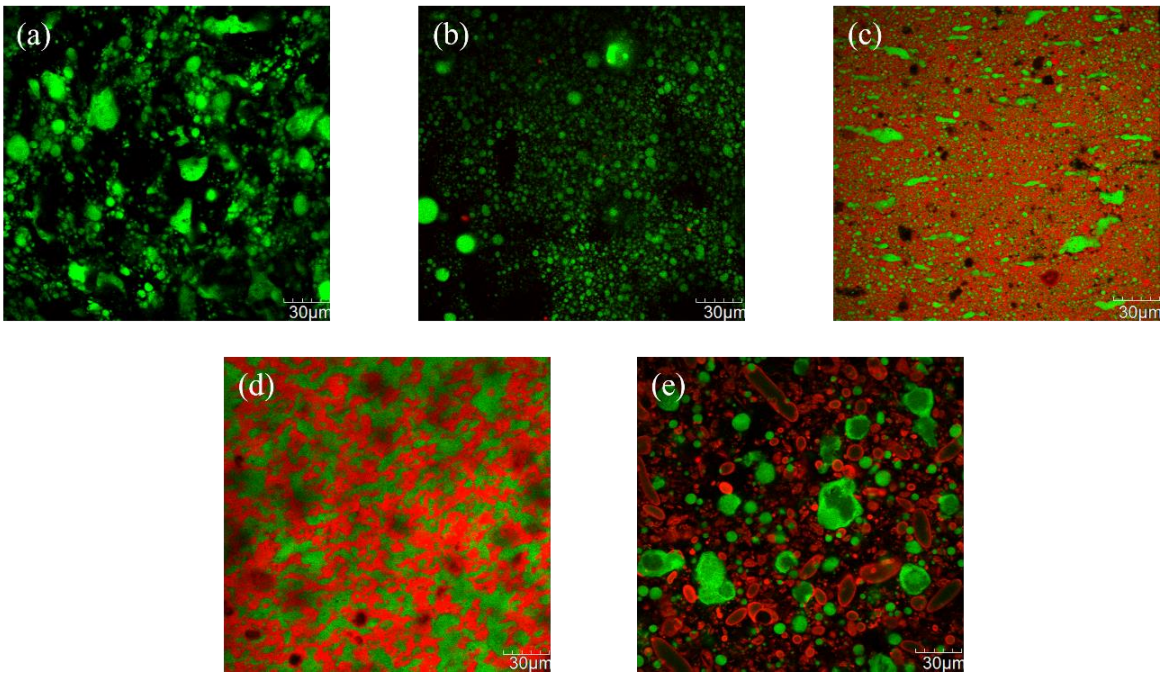


Figure 7.2. Confocal laser scanning microscopy images of the plant-based cheese alternative prototype and benchmark products, Plant-based 1 (a), Plant-based 2 (b), Processed (c), Cheddar (d) and Prototype (e), at 60X. Fat and protein are represented in green and red, respectively.

7.3.3 Rheological properties

The rheological profiles of the samples are reported in Figure 7.4. The profiles of the two dairy-based cheeses (Fig. 7.3c and d, representing Processed and Cheddar samples, respectively), showed a transformation from a viscoelastic solid to liquid rheological behaviour, with G′ equal to G″ at the cross-over temperatures of 45.2°C for the Processed sample and at 53.8°C for the Cheddar. However, the Processed sample showed a second cross-over with G′ equal to G″ at 72.4°C and maximum loss tangent (tan δ) value reached at 56.8°C, corresponding to the temperature of maximum fluidity (Fox *et al*., 2017). The tan δ value decreased at higher temperature, showing increased hardening of the sample. This behaviour was in agreement with a previous study on commercial processed cheese (Lu *et al*., 2007), where the authors reported

tan δ values lower than 0.5 at temperature lower than 30°C, and increasing values with increasing temperature, reaching a maximum between 55 and 65°C, with values decreasing at higher temperatures. Numerous factors can impact the rheological behaviour of processed cheese, for example the addition of starch in the formulation, the type and level of emulsifying salts and the composition of the natural cheese employed, as well as the processing conditions (Guinee, 2011).

On the other hand, the two commercial plant-based cheese alternatives, Plant-based 1 and 2, showed softening with increasing temperature, with the highest tan δ values (i.e., 0.25 and 0.40, respectively) reached at the end of the temperature ramp. The Plant-based 2 sample showed softer characteristics at high temperature compared to the Plant-based 1. The prototype sample showed a different profile than the other samples, with almost linear profiles for G′, G″ and tan δ between 20 and 100°C; however, G′ and G″ for this sample almost crossed over at the end of the ramp, with a tan δ value of 0.44, suggesting increasing ability of the sample to flow at high temperature, probably due to weakening of non-covalent bonds in zein, in agreement with previous observations on zein-based plant-based cheese alternatives (Mattice & Marangoni, 2020b; Grasso *et al*., 2023).

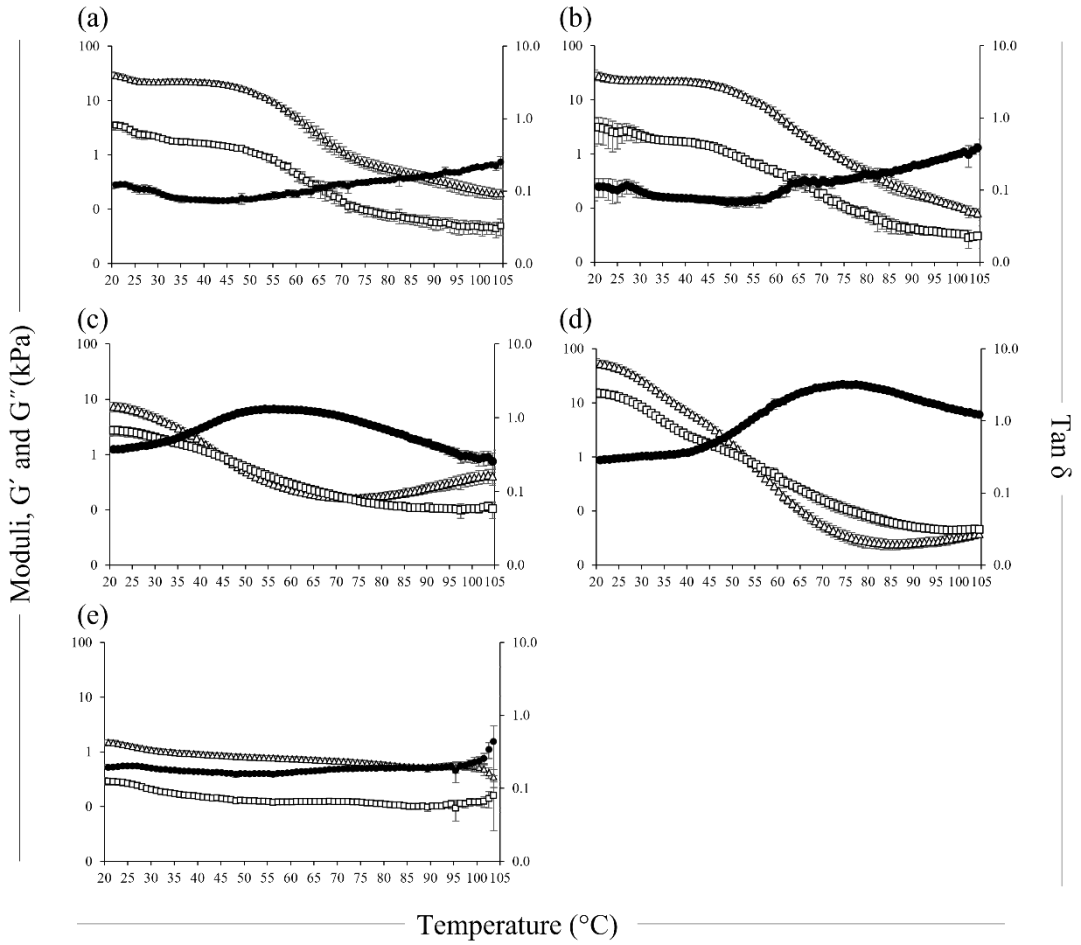


Figure 7.3. Rheological profiles showing storage modulus (G′) (*open triangle*), loss modulus (G″) (*open square*) and loss tangent (Tan *δ*) (*filled circle*) as a function of temperature in the range 20–105°C for Plant-based 1 (a), Plant-based 2 (b), Processed (c), Cheddar (d) and Prototype (e).

7.3.4 Meltability

The photographs of the samples before and after oven heating are reported in Figure 7.4 and the results of the Schreiber test are reported in Table 7.4. Although showing softening during the rheological analysis (Section 7.3.3), the Plant-based 1 sample did not melt under the conditions tested. Interestingly, the same was observed for the Processed sample; this sample showed no diameter expansion and slight crust formation at the edges, suggesting water evaporation.

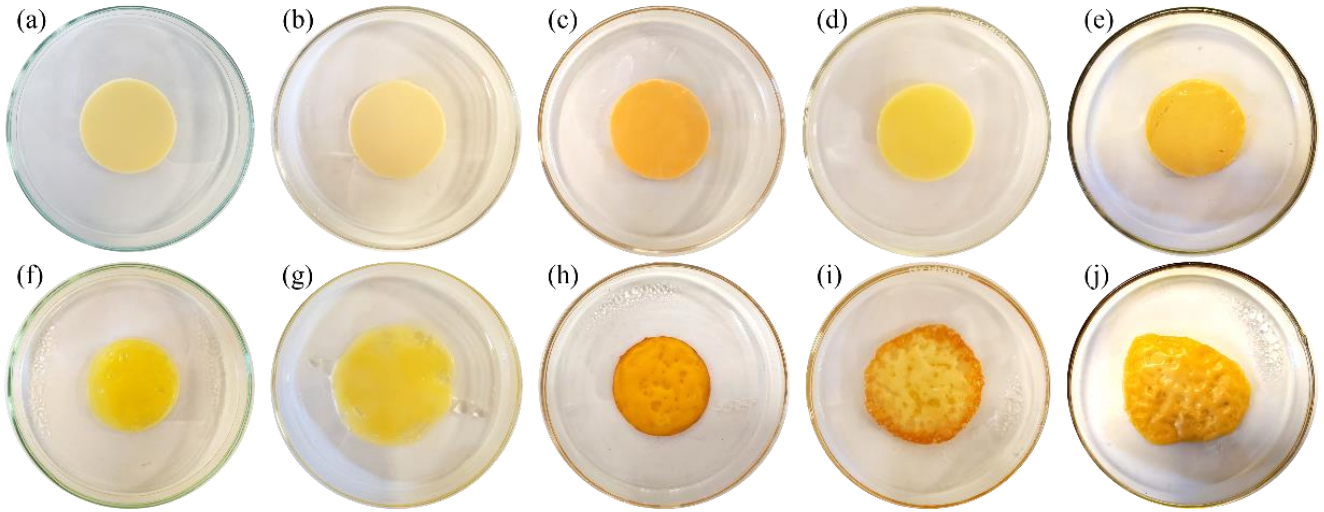


Figure 7.4. Photographs of the benchmark products and plant-based cheese alternative prototype, Plant-based 1, Plant-based 2, Processed, Cheddar and Prototype before (a-e) and after (f-j) oven heating at 232°C for 5 min.

This behaviour was in agreement with the rheological profile reported earlier, where the Processed sample showed higher hardness at higher temperatures, and also with meltability results from a previous study (Grasso *et al*., 2021). The Plant-based 2, Cheddar, and prototype samples showed statistically comparable results, with diameter expansions of 14.5, 14.0 and 14.8%, respectively. As observed from the rheological results (Section 7.3.3), the prototype sample started softening and melting at temperature greater than 100°C; in agreement with the rheological results, the meltability analysis showed diameter expansion of the sample at the testing temperature of 232°C, possibly associated with the ability of zein to flow and viscoelastic behaviours at high temperatures, as also previously reported by Mattice & Marangoni (2020a, 2020b). In contrast, the melting mechanisms of Cheddar cheese are related to numerous factors, such as concentration/activity of chymosin, moisture and fat contents, proteolysis, temperature, pH, sodium chloride and calcium concentrations, and casein-casein, casein-water or casein-fat interactions (Atik & Huppertz, 2023).

Table 7.4. Meltability, textural parameters and *in vitro* protein digestibility of benchmark products and prototype sample.

	Meltability (%)	Hardness (N)	Time to peak (s)	Young's Modulus (E) (kPa)	*In vitro* protein digestibility (%)	
					Pepsin (1 h)	Pepsin + Pancreatin (1+1 h)
Plant-based 1	0.00^{b}	2.86 ± 0.10^{b}	1.73 ± 0.09^{d}	169 ± 20.6^{b}	n.a.	n.a.
Plant-based 2	14.5 ± 1.59^{a}	3.66 ± 0.11^{a}	1.35 ± 0.05^{d}	217 ± 10.8^{a}	n.a.	n.a.
Processed	0.00^{b}	0.66 ± 0.06^{d}	4.24 ± 0.23^{b}	10.4 ± 0.69^{d}	3.10 ± 0.10^{b}	20.5 ± 0.44^{a}
Cheddar	14.0 ± 0.85^{a}	2.39 ± 0.20^{c}	2.35 ± 0.39^{c}	125 ± 11.1^{c}	3.90 ± 0.12^{a}	21.5 ± 0.62^{a}
Prototype	14.8 ± 0.90^{a}	0.21 ± 0.02^{e}	5.88 ± 0.79^{a}	2.89 ± 0.37^{d}	2.10 ± 0.08^{c}	7.90 ± 0.26^{b}

n.a.= not available

Values followed by different superscript letters in a column (a-e) are significantly different ($p < 0.05$).

7.3.5 Textural properties

Textural parameters of benchmark products and the plant-based cheese alternative prototype are reported in Table 7.4. In addition, the force-time profiles of the samples truncated to the respective maximum force value, are shown in Figure 7.5. The Plant-based 2 sample showed the highest hardness value, followed by the Plant-based 1 and Cheddar samples. On the other hand, the Processed and prototype samples had similar textural characteristics, with lower hardness compared to the other samples. As expected, the time to peak force, representing the time needed to reach maximum hardness, showed an opposite trend to the hardness results. The prototype sample had the highest time to peak value, followed by the Processed sample. The two Plant-based commercial samples had statistically comparable time to peak values. Youngs modulus (E), defined as the resistance of the cheese structure to reversibly deform without fracturing, was also calculated by estimation of the slope of the early linear region of the stress-strain curves (Noël *et al*., 1996). The prototype sample had the lowest Youngs modulus (E) value, not significantly different from the Processed sample. As reported in Tables 7.1, 7.2, and 7.3, the samples had different ingredients and compositions, strongly influencing their textural properties (Everard *et al*., 2006). Indeed, the high hardness and Youngs modulus (E) of the Plant-based 1 and 2 products compared to the other samples, can be related to the use of starch in their formulations, in agreement with previous observations on commercially-available plant-based cheese alternatives (Grasso *et al*., 2021).

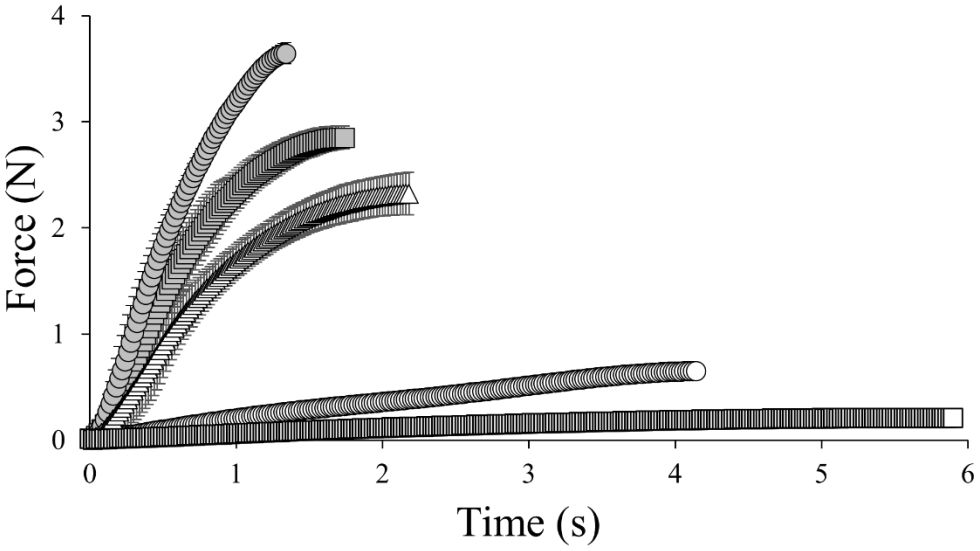


Figure 7.5. Force-time profiles to maximum force of Plant-based 1 (▣), Plant-based 2 (◉), Processed (○), Cheddar (△) and Prototype (□) samples.

7.3.6 In vitro protein digestibility

The *in vitro* protein digestibility of the commercial dairy products and the plant-based cheese alternative prototype sample is reported in Table 7.4. The Plant-based 1 and 2 samples were not analysed for protein digestibility due to the absence of protein in these products. The Processed and Cheddar samples had similar digestibility results, in particular during the pancreatin treatment, with overall digestibility values of 20.5 and 21.5%, respectively, similar to the 22.7% of bovine serum albumin (BSA), used as reference protein. As expected, and in agreement with a previous study (Fang *et al*., 2016), digestibility of these samples was higher during the pancreatin treatment than the pepsin step, probably due to caseins not being completely released from the cheese matrix during pepsin digestion. On the other hand, digestibility of the prototype sample was significantly lower (7.90%), probably due to the poor digestibility of zein, which contains a high proportion of non-polar amino acid residues (>50%) and a compact molecular structure (Zhao *et al*., 2022). The generally low digestibility of

plant proteins associated with the highly compact structure of their polypeptide chains and their tendency to form large aggregates, limiting access to digestive proteases (McClements & McClements, 2023). Zhao *et al*. (2022) suggested post-harvesting ripening approaches, such as storage for 28 days at constant temperature and relative humidity, to enhance digestibility of zein. Moreover, other methods that can be employed to improve digestibility of plant proteins include fermentation, enzymatic treatments, high hydrostatic pressure and heat treatments (Duodu *et al*., 2002; Mefleh *et al*., 2021).

7.3.5 Volatile profile

The distribution of the samples based on their volatile profiles is reported in the Principal Component Analysis (PCA) plot in Figures 7.6. In total, 121 volatile compounds were identified across the samples (Table A1), consisting of acids (9), alcohols (15), aldehydes (12), alkanes (10), benzenes (5), esters (22), ether (1), furans (3), ketones (14), lactone (6), pyrazine (7), phenol (2), sulphur (9), terpenes (4) and others (2). The plant-based samples and dairy cheeses had some volatile compounds in common, typical for dairy cheeses (e.g., butanoic acid, hexanal and acetoine); however, the proportions thereof were very different. On the other hand, many flavour compounds present in the plant-based samples, such as isovaleric acid or furfural, are rarely or never found in Cheddar cheese. The total variance was 73%, with PC-1 axis accounting for 54.2% of difference, while PC-2 axis accounting for 18.8% thereof. The Cheddar, Processed and Plant-based 1 samples clustered together in the lower, left quadrant of the PCA plot, sharing some compounds, and having, nevertheless, very distinct volatile profiles. The Plant-based 2 and prototype samples were located far from this cluster, with the latter being the only sample on the positive side of PC-1.

All samples except for the Cheddar contained flavourings in their formulation. Cheddar and Processed samples had the lowest numbers of compounds, at 51 and 45 respectively, typically found in cheese products, such as acids (butanoic, hexanoic acids), aldehydes (acetaldehyde, butanal, 3-methyl, hexanal, heptanal, nonanal, benzenacetaldehyde), esters (ethyl acetate), ketones (acetone, 2,3-butanedione, acetoin, 2-heptanone) and ether (ethyl ether) (Kilcawley, 2017; Xia *et al*., 2022). Some such compounds were also found in the Plant-based 1 sample, including butanoic acid, present in high amounts, hexanoic acid, acetaldehyde, butanal, 3-methyl, ethyl butanoate and ethyl acetate. The prototype sample had the highest number of compounds (i.e., 77) and most of them were either much lower or not detected in the other samples, in particular some acids, alcohols and esters (Figures A1 and A2). This large amount of different volatile components might be due to the presence of ingredients such as zein, chickpea protein concentrate and flavourings added to the formulation. Flavour of commercially-available plant-based cheese is often considered a barrier for consumption, indicating the need for further enhancement of the volatile profile of same in future product development (Falkeisen *et al*., 2022).

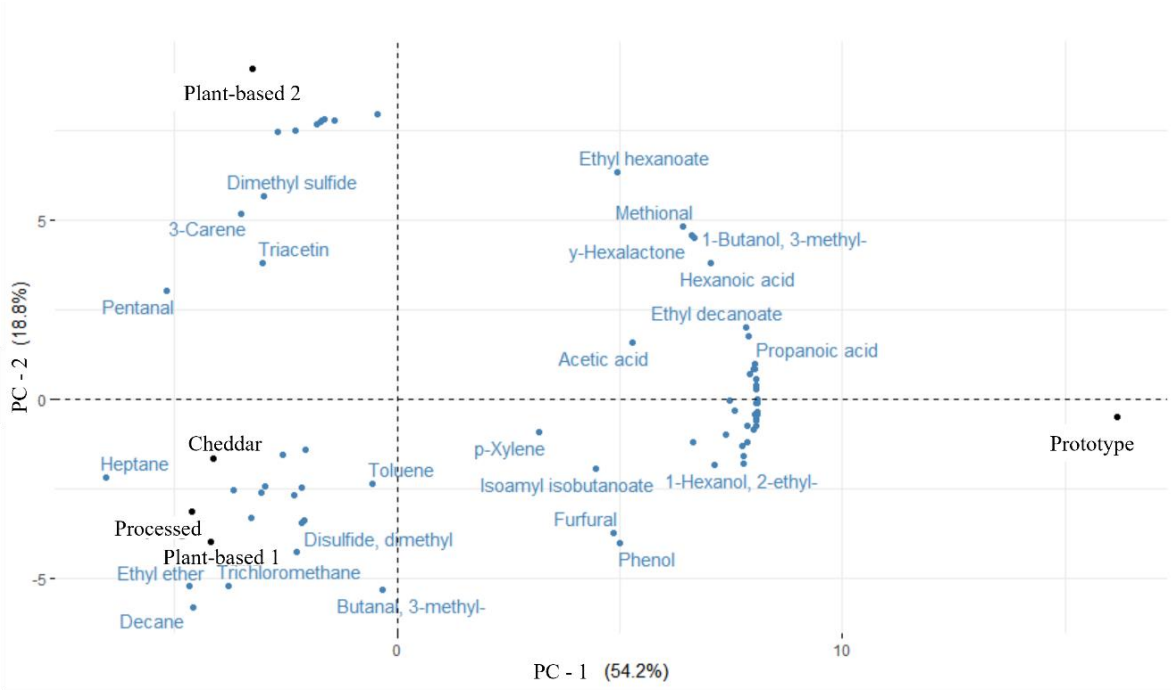


Figure 7.6. Principal component analysis plot of the volatile profiles of benchmark products and prototype sample.

7.4 Conclusion

A plant-based cheese alternative prototype was formulated and its physicochemical properties, protein digestibility and volatile profile were investigated, using two commercially-available plant-based cheese alternatives, dairy processed cheese and Cheddar, as benchmarks. Composition of the prototype was similar to the processed cheese, while the commercial plant-based cheese alternatives had very different formulations and macronutrient contents, generally having higher carbohydrate contents, with no protein. The prototype showed melting behaviour comparable to Cheddar and to one of the commercial plant-based cheese alternatives. Textural characteristics were similar between the prototype and processed cheese sample, while protein digestibility was lower than the dairy benchmarks, suggesting the need for improvements of protein digestion in plant-based cheese alternative matrices. All samples had very distinct volatile profiles, with the prototype having the highest number of compounds and differing considerably from the other samples. Although some improvements are needed, the results of this study show that plant-based cheese alternatives can be formulated with enhanced nutritional and quality attributes than plant-based cheese alternatives available commercially.

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Chapter 8

General discussion and conclusions

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Declaration: This chapter was written by author Nadia Grasso and reviewed by all co-authors.

General discussion

Numerous new plant-based cheese alternative products are emerging on the shelves of supermarkets, and such products can be developed using different raw materials, formulations, and processes (**Chapter 1**). However, a lack of knowledge of the science underpinning development of plant-based cheese alternatives is evident in the scientific literature (Grossmann & McClements, 2021). To formulate the next-generation of plant-based cheese alternatives, with improved physicochemical and nutritional properties compared to current commercially-available products, research regarding the ingredient, formulation and processing science and technology of same, is required.

As discussed in **Chapter 1** and **Chapter 3**, many differences are observed among the numerous product categories, ingredients, formulations and processing approaches of traditional cheese and plant-based cheese alternatives. Indeed, most of the plant-based cheese alternatives currently available commercially are largely starch and coconut oil-based. In terms of nutritional and physicochemical properties, these products differ significantly from their dairy counterparts (Grasso *et al*., 2021; **Chapter 3**). Non-protein plant-based ingredients (e.g., starch) are often used in an attempt to mimic the functionality of dairy proteins; however, dairy proteins provide cheese products with unique structure, textural and sensory properties (Mattice & Marangoni, 2020a; Short *et al*., 2021). The use of non-protein ingredients, such as starch combined with a fat source under specific processing conditions (i.e., high temperature followed by cooling), allows formation of composite gels (mainly emulsion-filled gels). Although having characteristics of solid foods, the physicochemical properties of these gels are, for the most part, not comparable to those of cheese products (**Chapter 1**).

Other ingredients used in the development of plant-based cheese alternatives include nuts, such as cashews, macadamias and almonds, generally resulting in products with higher protein content than starch-based cheeses; however, these represent allergenic and expensive raw materials (**Chapter 1**).

Plant protein ingredients have recently been studied for their potential in the development of plant-based cheese alternatives with improved nutritional profile and functionality (Mattice & Marangoni, 2020b; Ferawati *et al*., 2021; Mefleh *et al*., 2021). Among the sources available for protein extraction, due to their nutritional characteristics and techno-functionality, legumes represent particularly interesting and promising crops. In particular, chickpea protein ingredients show strong potential in new and reformulated food product applications, having functional properties of relevance in new product development, such as water and oil absorption capacity, emulsifying and gelling properties (Boye *et al*., 2010; Hall *et al*., 2017; Grasso *et al*., 2022b; **Chapter 2**). In **Chapter 2**, approaches to enhance protein quality of chickpea protein ingredients and applications of the co-products resulting from protein extraction and processing were also identified, with the aim of sustainably maximising the potential of such ingredients. Another plant protein ingredient that has shown properties of relevance in plant-based cheese alternative applications is zein, the prolamin protein fraction extracted from maize. In aqueous environments, zein shows plastic behaviour due to its hydrophobicity and non-covalent interactions that lead to formation of viscoelastic networks. Furthermore, zein shows unique softening and increased viscous properties with increasing temperature (Mattice & Marangoni, 2020a).

In this thesis (**Chapters 4**, **5**, and **6**), the effects of using chickpea and zein protein ingredients and calcium fortification on the physicochemical properties of

plant-based cheese alternatives, were investigated. Chickpea flour and protein concentrate can be used to formulate plant-based cheese alternatives with different protein contents and, in **Chapter 4**, products with different texture and microstructure were obtained from such ingredients. Such differences were associated mainly with the protein and carbohydrate content of the samples; however, none of the chickpea-based products melted under the testing conditions, highlighting the challenges associated with the use of chickpea protein ingredients (Grasso *et al*., 2022a).

Based on the results of **Chapter 4**, binary blends of zein and chickpea protein ingredients were used for the development of plant-based cheese alternatives in **Chapter 5**. Increasing the proportion of zein in the samples led to improved meltability and stretching properties, due to the unique plastic characteristics of zein and to the weakening of non-covalent bonds upon heating at high temperature, which strongly influenced its viscoelastic behaviour. The use of zein in plant-based cheese alternative applications showed great potential to enhance functionality of the final product. Moreover, the use of plant protein blends derived from different sources (i.e., cereals and legumes) allows the formulation of plant-based food with improved nutritional and physicochemical characteristics (Jiménez-Munoz *et al*., 2021; Grasso *et al*., 2023). However, a knowledge gap on the role of plant protein blends in the development of plant-based cheese alternatives is evident from an analysis of the literature.

The results from **Chapter 5** provided information on how combinations of zein and chickpea protein ingredients can be used to formulate plant-based cheese alternatives. As described in **Chapter 6**, calcium fortification strategies, using different calcium salts, were developed for plant-based cheese alternatives formulated with a blend of zein and chickpea protein ingredients. Fortification with calcium was

proposed as an effective approach to improve the nutritional profile of plant-based cheese alternatives and the results showed the impact of fortification on selected physicochemical properties. Indeed, calcium fortification increased hardness, decreased meltability and enhanced stretchability of samples, also impacting their microstructure, to an extent depending on the calcium salts used. This suggests that calcium salts can be added to plant-based cheese alternatives for nutritional purposes, as well as to modulate selected physicochemical properties.

In **Chapter 7** the learnings from **Chapters 4**, **5** and **6**, were used to develop a plant-based cheese alternative prototype. The physicochemical properties, protein digestibility and volatile profile of same, in comparison with two commercially-available plant-based cheese alternatives, dairy processed and Cheddar cheeses, were investigated. The prototype showed similarities to the processed cheese, in particular in terms of textural properties, and enhanced nutritional and physicochemical properties compared to the plant-based cheese alternatives available commercially. However, the study showed the need to improve protein digestion of the ingredients employed in the formulation of plant-based products.

In summary, the studies reported in this thesis enhanced and developed new understanding of the science required to formulate plant-based cheese alternatives with improved physicochemical properties and high protein content compared to commercially-available products. In particular, these studies provide greater understanding of the key considerations of relevance in selecting ingredients, formulation approaches and processing conditions to develop plant-based cheese alternatives. Furthermore, the knowledge developed through this thesis can be exploited by the food industry to formulate products that represent a better choice for consumers compared to the products currently available on the market.

Suggestions for future research

Follow-up studies that would be complementary to the work presented in this thesis include:

Environmental impact of plant-based cheese alternatives

Investigations on the environmental impact of specific plant-based cheese alternatives are necessary to properly assess the sustainability of these products (Carlsson Kanyama *et al*., 2021). Indeed, life cycle assessments (LCAs) of plant-based cheese alternatives will help in understanding the limits and potential of different ingredients and approaches. Some assumptions can be made based on the limited publications available on the ingredients employed for formulation; for example, nuts require large amounts of water to grow and the land use requirements are also high, on the other hand, the CO_2 emissions are generally low (Clune *et al*., 2017; Poore & Nemecek, 2018). Legumes represent sustainable protein sources and based on the studies available on the LCA of soy-based cheese alternatives and tofu, it can be concluded that these have a global warming potential lower than traditional cheese (Mejia *et al*., 2018). In general, plant proteins represent a sustainable alternative to animal based protein; nevertheless, the environmental impact of fractionated proteins depends on the approach chosen for extraction (i.e., wet *vs* dry methods) (Boye *et al*., 2010).

Modification and enhancement of quality of plant protein ingredients

As discussed in **Chapter 2**, the term *protein quality* in relation to plant protein is related to both nutritional and techno-functional properties. Nutritional quality

includes, for example, protein digestibility, bioavailability, antioxidant and antimicrobial properties, while the techno-functional quality consists of all the functional properties of proteins (e.g., solubility, oil and water absorption capacity, emulsifying properties), essential to support its use in food formulations and applications (Nasrabadi *et al*., 2021). Numerous approaches have been identified to enhance the quality of plant proteins; for example, germination prior to processing, dehulling, fermentation, hydrolysis or other chemical modifications, extrusion and high hydrostatic pressure. Enzymatic approaches reported in the literature have shown interesting results, suggesting that, depending on the plant protein source and the processing parameters used for protein extraction, different enzymes can be employed to achieve a desired texture (**Chapter 1**). Due to the interdependence of the nutritional and techno-functional qualities, all these methods can be used for their effects on the techno-functionality of protein and, in turn, influence the nutritional characteristics of same. In **Chapter 7**, protein digestion was studied and the results for the plant-based cheese prototype were low compared to the dairy commercial benchmarks; the approaches mentioned in this section could help in improving protein digestibility.

Effect of structure of the plant-based cheese alternatives on nutritional quality

Many differences in nutritional profiles can be observed depending on the raw materials and approaches used to develop plant-based cheese alternatives, highlighting the need for studies on the characteristics of selected products. Indeed, as extensively discussed in this thesis (**Chapters 1** and **3**), currently, most of the commercially-available products differ significantly to traditional cheese, often being nutritionally inferior. An important aspect that has been studied for cheese (Feeney *et al*., 2021), but needs to be further explored in plant-based cheese alternatives is the effect of the

matrix on the nutritional quality of the final product and, consequently, on human health, as well as the effect on absorption of micronutrients, and digestibility of plant protein when included in such structures. For cheese products, a matrix effect exists, whereby the various components interact with the overall structure, leading to health benefits (Feeney *et al*., 2021). Plant-based cheese alternatives can be developed in numerous structures (e.g., filled or bicontinuous gels), having different nutritional characteristics, hence, requiring further investigation.

Understanding of plant proteins-calcium interactions

In **Chapter 6**, calcium salts were used to fortify plant-based cheese alternative samples, impacting the physicochemical properties of same. Specific studies on calcium and protein systems (e.g., chickpea and zein protein ingredients) using a calcium-ion-selective electrode, would inform on interaction mechanisms between calcium and proteins and their impact on selected physicochemical properties. Moreover, as previously reported by Ozturk *et al*. (2023), FT-IR spectroscopy could be used to develop a better understanding of the molecular and structural characteristics of calcium-protein systems, specifically studying the changes in protein structure when calcium is added to the system.

Development of plant-based cheese alternatives through fermentation-based approaches

Currently, very little is known about the effects of microbial biodiversity in plant-based cheese alternatives, and, as reported in **Chapter 1**, cashew nuts are the main sources used for production of fermented plant-based cheese alternatives. The choice of the starter culture can vary according to the preferred texture and sensory

profile of the final product, with principally combinations of different mesophilic lactic acid bacteria (LAB) or, sometimes, fungi being added to the mixture of different ingredients during processing (Pua *et al*., 2022). Fermentation approaches might be studied to develop plant-based cheese alternative products with improved nutritional characteristics and sensory profiles.

Sensory/GC-MS studies for selection of flavourings for application in plant-based cheese alternatives

In **Chapter 7**, different flavourings that mimic dairy products, were added to the formulation of the prototype sample, resulting in a volatile profile that differed significantly from the other commercially-available plant-based and dairy products. In the future, it might be interesting to design studies on sensory or GC-MS approaches for selection of the flavourings that most resemble cheese products when added to a plant-based cheese alternative matrix.

Sensory and consumer acceptability of plant-based cheese alternative prototypes

As emerged from **Chapter 7**, many differences were observed between the volatile profile of the prototype and commercial samples. Sensory and consumer studies would help in defying the overall acceptability of the plant-based cheese prototypes in comparison to commercially available plant-based and traditional cheeses. Currently, sensory represents a limit for the plant-based cheese alternative sector and such studies would help in developing plant-based cheese alternatives that meet consumer preferences.

Effect of packaging, storage time and conditions on physicochemical characteristics of plant-based cheese alternatives

Storage time and conditions (e.g., relative humidity and temperature), as well as the material and environmental conditions (e.g., modified atmosphere) used for packaging, are known to largely impact the physicochemical, microbiological, and sensory characteristics of cheese, with different bacteria growing depending on these factors, as well as enzymatic activities being imported. However, the effect of packaging and storage time and conditions on plant-based cheese alternatives needs to be further investigated. In **Chapter 4**, samples were stored for one month and selected physicochemical properties (e.g., colour and texture) changed over time. Further studies on storage of final products would inform their microbiological stability, physicochemical and sensory changes over time, depending on storage conditions and packaging used.

Use of different fat sources for the development of plant-based cheese alternatives

Shea butter was used for the development of plant-based cheese alternatives in **Chapters 4**, **5**, **6** and **7** of this thesis, due to its solid nature at room temperature and high ratio of unsaturated to saturated fatty acids, and the lower content of saturated fats compared to coconut oil, which represents the most widely used source of fat in commercially-available plant-based cheese alternatives (Short *et al*., 2021). Although shea butter represents an alternative to coconut oil, especially when ethically sourced as for the ingredient used in this thesis, tropical oils in general have low productivity and high costs. Moreover, such oils are cultivated in geographic regions where increased intensity of production would result not only in higher carbon emissions, but also in negative impacts on biodiversity through land-clearing and deforestation, as

also observed for palm oil (Parsons *et al*., 2020). Single cell oils, edible oils produced from microalgae, yeasts, fungi or moulds, represent emerging sources of oil for food applications, and future studies should focus on their use in development of potentially more sustainable plant-based cheese alternatives.

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Appendix I

Analysis of volatile flavour compounds in dairy and plant based cheese alternatives

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Table A1: Compounds identified using Solid Phase Microextraction (SPME) in cheese and plant-based cheese alternatives (total abundance).

Class	Name	CAS	RI	Ref RI	Ref odour descriptor	Prototype	Processed	Plant-based 1	Plant-based 2	Cheddar
Acid	Acetic acid	64-19-7	690	690	pungent, vinegar, sour	1179254	0	0	496461	1037947
Acid	Propanoic acid	79-09-4	780	807	pungent, acidic, cheesy, vinegar	514902	0	0	138496	0
Acid	2-Methylpropanoic acid	79-31-2	835	836	sour, cheesy, dairy, buttery, rancid	0	0	1829602	0	0
Acid	Butanoic acid	107-92-6	867	864	rancid, cheesy, strong, sweaty	20334975	2471412	9530810	6328669	2266668
Acid	Isovaleric acid	503-74-2	917	917	cheesy, sweaty, rancid, goat, rotten fruit	7550860	40921	1639822	0	0
Acid	Hexanoic acid	142-62-1	1052	1049	acidic, sweaty, cheesy, sharp, goaty	12913043	1463420	475856	7474763	370256
Acid	Sorbic acid*	110-44-1	1153		acrid	0	0	47659	2392329	6334
Acid	Octanoic acid	124-07-2	1242	1242	goaty, waxy, soapy, cheesy, rancid, pungent, sweat	3086568	132079	34891	338927	33467
Acid	Decanoic acid	334-48-5	1438	1450	rancid, fatty	46642	0	0	0	0
Alcohol	Ethanol	64-17-5	508	506	sweet, alcoholic, medicinal	3104804	0	0	0	1247103
Alcohol	Isopropyl Alcohol	67-63-0	540	539	alcoholic, musty, woody	4118150	0	0	500688	0
Alcohol	1-Propanol	71-23-8	612	612	sweet (candy), fruity	0	0	64638	830208	0
Alcohol	1-Butanol	71-36-3	714	716	banana-like, fruity, green, medicinal	178624	0	0	0	0
Alcohol	1-Butanol, 3-methyl-	123-51-3	784	784	whiskey, fusel, alcoholic, fruity, banana	114673	0	0	76945	0
Alcohol	1-Butanol, 2-methyl-	137-32-6	784	789	malty, roasted, winey, fruity, fusel, alcoholic	0	30086	0	0	0
Alcohol	1-Pentanol	71-41-0	815	815	fermented, yeasty, balsamic, fusel, winey	114737	28059	0	0	13357
Alcohol	2,3-Butanediol	513-85-9	876	891	fruity, creamy, buttery	391014	0	0	0	234292
Alcohol	2-Propanol, 1-propoxy-*	1569-01-3	880			0	0	102348	0	0
Alcohol	4-Heptanol*	589-55-9	930			0	0	347361	0	0
Alcohol	1-Hexanol, 2-ethyl-	104-76-7	1071	1077	citrus, floral, green, fresh	358434	163163	12535	0	8648
Alcohol	Benzyl alcohol	100-51-6	1120	1119	floral, rose, phenolic, balsamic	16810	598	4527	0	987
Alcohol	Phenylethyl Alcohol	60-12-8	1199	1201	rose, violet-like, honey, floral, spicy	87874	0	0	0	0
Alcohol	Triacetin	102-76-1	1415		clean, tropical, fruity	0	0	1449111	1502680	0
Alcohol	Hexadecanol	36653-82-4	1931		waxy, greasy, floral, oily	0	0	0	0	1647
Aldehyde	Acetaldehyde	75-07-0	450	452	pungent, ethereal, fresh, fruit	3048522	28848	178943	627098	119731
Aldehyde	Butanal, 3-methyl-	590-86-3	691	692	malty, cheese, green, dark chocolate, cocoa	684145	1024068	552920	398968	740957
Aldehyde	Butanal, 2-methyl-	96-17-3	700	700	malty, dark chocolate, almond, cocoa, coffee	221491	0	0	0	0
Aldehyde	Pentanal	110-62-3	734	733	pungent, almond, malty	0	169348	79663	163914	30866
Aldehyde	Hexanal	66-25-1	838	839	green, grassy, herbal, lemon, tallow	1947156	78886	183532	406311	12608
Aldehyde	Furfural	98-01-1	898	899	sweet, woody, almond, caramellic, baked, bread	34446	7095	33732	0	0
Aldehyde	Heptanal	111-71-7	941	943	fatty/oily, green, citrus, rancid	213654	15893	16046	22132	5628
Aldehyde	Benzaldehyde	100-52-7	1028	1031	bitter almond, sweet cherry	650218	17570	13737	19405	9678
Aldehyde	Octanal	124-13-0	1045	1047	waxy, citrus, orange peel	79845	0	0	12708	0
Aldehyde	Benzeneacetaldehyde	122-78-1	1117	1120	green, sweet, floral, clover, honey, cocoa	1035555	18969	25769	0	29872

Aldehyde	Nonanal	124-19-6	1148	1150	green, citrus, fatty, floral	330873	37732	15632	22504	9399
Aldehyde	α-Ethylidenbenzeneacetaldehyde	4411-89-6	1366		metallic, green	32711	0	0	0	0
Alkane	1-Pentene, 2-methyl-*	763-29-1	595			0	22245	0	0	0
Alkane	n-Hexane	110-54-3	602	600	gasoline	0	15865	0	0	7276
Alkane	Heptane	142-82-5	700	700	gasoline	0	446610	312346	215559	184071
Alkane	Pentane, 2,3,3-trimethyl-*	560-21-4	762			0	45448	0	0	0
Alkane	1-Octene*	111-66-0	795	791		0	8587	0	0	0
Alkane	Octane	111-65-9	818	800	gasoline	0	450684	0	0	0
Alkane	2,4-Dimethyl-1-heptene*	19549-87-2	848	848		0	50837	0	0	0
Alkane	Nonane	111-84-2	876	900	gasoline, petroleum	0	11567	0	0	0
Alkane	Decane	124-18-5	1000	1000	gasoline	0	87495	84865	0	31620
Alkane	Hexane, 1,1-diethoxy-	3658-93-3	1100		gasoline	17195	0	0	0	0
Benzene	Benzene	71-43-2	686	682	aromatic	0	0	23765	0	0
Benzene	Toluene	108-88-3	792	794	nutty, bitter, almond, plastic	52728	12395	31446	0	211528
Benzene	Ethylbenzene	100-41-4	889	890	heavy, floral	0	0	9394	0	0
Benzene	p-Xylene	106-42-3	897	898	grainy, sweet	18044	6922	22980	10814	0
Benzene	o-xylene	95-47-6	926	929	geranium	0	0	9567	0	0
Ester	n-Propyl acetate	109-60-4	741	743	pineapple, banana	0	69947	2949216	0	0
Ester	Methyl butanoate	623-42-7	749	754	fruity	0	0	0	0	12287
Ester	Isobutyl acetate	110-19-0	799	800	fruity, floral	0	0	332374	0	0
Ester	Ethyl butanoate	105-54-4	824	826	fruity, pineapple	429741	0	634981	7265505	99844
Ester	Ethyl lactate	97-64-3	861	862	buttery, creamy, fruity, coconut	393753	0	0	0	0
Ester	Isoamyl acetate	123-92-2	901	902	banana, sweet, pear, apple peel	0	0	0	406151	0
Ester	Butyl, 2-propenoate	141-32-2	930	918	fruity	0	0	210590	0	0
Ester	Butyl propanoate	590-01-2	932	928	fruity	218366	0	0	0	0
Ester	Ethyl 3-methyl-2-butenoate*	638-10-8	952			41045	0	0	0	0
Ester	Isobutyl butyrate*	539-90-2	979		fruity	70968	0	0	0	0
Ester	Amyl propionate	105-68-0	993	994	fruity	0	0	0	28243	0
Ester	Butyl butanoate	109-21-7	1019	1019	pineapple, banana, sweet	794448	0	94086	0	0
Ester	Ethyl hexanoate	123-66-0	1022	1024	fruity, pineapple, waxy, fatty	947401	0	0	1028032	13905
Ester	Butyl lactate*	138-22-7	1058		mild, green	0	0	1680253	0	0
Ester	Isoamyl isobutanoate*	2050-01-3	1080		fruity, ethereal, tropical	28408	0	31235	6422	0
Ester	Methyl octanoate	111-11-5	1150	1155	waxy, green, orange	88218	0	0	0	0
Ester	Ethyl octanoate	106-32-1	1221	1222	fruity, apple-like, green, fatty, orange	198732	4925	0	0	2969
Ester	Diethyl succinate*	123-25-1	1226		fruity, winey, ethereal	26143	0	0	0	0
Ester	Ethyl benzoate	93-89-0	1230	1232	fruity, dry, musty	119570	0	0	0	0
Ester	Ethyl benzeneacetate	101-97-3	1303	1305	sweet, floral, honey, rose, balsamic, cocoa	596582	6149	0	0	0
Ester	Ethyl decanoate	110-38-3	1420	1422	fruity, grape, cognac, waxy	53681	0	0	16219	0
Ester	Ethyl acetate	141-78-6	642	642	fruity, pineapple, apples, weedy, green	76735	56004	1085716	182110	45613
Ether	Ethyl ether	60-29-7	518	515	pungent, ethereal	0	101571	44493	0	57693
Ether	n-Butyl ether	142-96-1	889	886	ether-like	292920	0	0	0	0
Furan	Furan, 2-methyl-	534-22-5	623	615	chocolate, ethereal, acetone,	0	0	1829	42182	0

Furan	2-n-Butyl furan	4466-24-4	910	909	fruity, winey, sweet, spicy	24333	0	0	0	0
Furan	Furan, 2-pentyl-	3777-69-3	1010	1012	green bean, vegetable, earthy, metallic	326290	0	7283	20782	0
Ketone	Acetone	67-64-1	535	533	solvent, ethereal, sour milk, apple	23746	176451	88775	22870	629469
Ketone	2,3-Butanedione	431-03-8	632	631	buttery, creamy, sweet, pungent	12872	27957	279271	1640951	395710
Ketone	2-Butanone	78-93-3	638	639	buttery, sour milk, etheric	0	0	0	0	907413
Ketone	2-Pentanone	107-87-9	729	730	sweet, fruity, ethereal, fermented ,winey	0	97209	0	0	268386
Ketone	2-Propanone, 1-hydroxy-	116-09-6	734	734	pungent, sweet, caramellic, ethereal	0	0	0	0	76373
Ketone	2,3-Pentanedione	600-14-6	735	736	creamy, cheesy, oily, sweet buttery, caramellic	6708294	0	0	267302	0
Ketone	Acetoin	513-86-0	797	778	buttery, creamy, dairy, milky, fatty	714739	2070828	1561261	1043123	5114742
Ketone	2-Heptanone	110-43-0	933	936	fruity, spicy, sweet, herbal, woody	9097195	226881	0	1073802	369621
Ketone	2-Octanone	111-13-7	1035	1035	earthy, woody, herbal, cheesy, parmesan	0	0	0	474667	0
Ketone	trans-3-Octen-2-one	18402-82-9	1094	1095	earthy, spicy, herbal, sweet, mushroom, hay	17866	0	0	0	0
Ketone	2-Nonanone	821-55-6	1137	1140	fresh, green, weedy, earthy, woody, herbal	1385919	0	0	0	0
Ketone	Acetophenone	98-86-2	1142	1030	must, flower, almond	48876	3633	4124	3182	0
Ketone	2-Undecanone	112-12-9	1342	1353	waxy, fruity, creamy, fatty, orris, floral	58844	8498	14587	0	1444
Lactone	γ-Hexalactone	695-06-7	1156	1170	herbal, coconut, coumarinic	9021	0	0	5954	0
Lactone	γ-Heptalactone*	105-21-5	1272		sweet, coconut, nutty, tonka	11070	0	0	74155	0
Lactone	γ-Nonalactone*	104-61-0	1490		coconut, creamy, waxy	57412	0	0	0	0
Lactone	δ-Decalactone*	705-86-2	1680		sweet, creamy, coconut	62153	0	0	10708	12676
Lactone	5-Hydroxy-2-decenoic acid δ-lactone*	54814-64-1	1657		creamy, coconut, peach	50545	0	0	0	0
Lactone	γ-Decalactone*	706-14-9	1623		peach, waxy	0	0	0	20244	0
Other	Trichloromethane	67-66-3	655	651		0	2778	4506	0	554
Other	Styrene	100-42-5	927	929	plastic	37231	0	2652	0	1630
Pyrazine	Pyrazine, 2,5-dimethyl-	123-32-0	949	951	nutty, musty, roasted, cocoa, woody	800666	0	0	0	183018
Pyrazine	Pyrazine, ethyl-*	13925-00-3	956			32756	0	0	0	0
Pyrazine	Pyrazine, 2,3-dimethyl-	5910-89-4	960	962	roasted nuts, coffee, peanut	16126	0	0	0	0
Pyrazine	Pyrazine, trimethyl-	14667-55-1	1039	1039	roasted, chocolate, earthy, musty	380075	0	0	0	25670
Pyrazine	Pyrazine, 3-ethyl-2,5-dimethyl-*	13360-65-1	1113			165891	0	0	0	0
Pyrazine	Pyrazine, tetramethyl-	1124-11-4	1121	1123	musty, nutty, chocolate, coffee, cocoa	0	0	0	20639	0
Pyrazine	2,3,5-Trimethyl-6-ethylpyrazine	17398-16-2	1192	1192	nutty	15527	0	0	0	0
Phenol	Phenol	108-95-2	1095	1112	sweet, tarry, chemical, phenolic	19110	6326	18345	4805	8715
Phenol	2-Methoxy-4-vinylphenol*	7786-61-0	1408		spicy, clove, peppery, smoky, woody	34176	0	0	0	0
Sulfur	Methanethiol	74-93-1	459	462	cabbage, garlic, eggy, vegetative, sulphur	7167	2927	3172	2751	1204
Sulfur	Dimethyl sulfide	75-18-3	538	538	sufurous, onion, cabbage, tomato, vegetable	0	0	0	9894	8779
Sulfur	Carbon disulfide	75-15-0	548	546	cabbage, sulphur, fruity, burnt	3165	0	17559	7393	71109
Sulfur	Disulfide, dimethyl	624-92-0	777	771	sulfurous, vegetable, cabbage, onion	2866	2977	13250	2006	4293
Sulfur	Methyl thiobutanoate*	2432-51-1	920		sulfurous, musty, onion, fruity	0	0	0	570183	0
Sulfur	Diethyl sulphite*	623-81-4	940		garlic	255098	0	0	0	0
Sulfur	Methional	3268-49-3	971	975	potato, tomato, veg, earthy, brothy, meaty	60445	0	0	43424	0
Sulfur	Dimethyl sulfone	67-71-0	1053	1056	sulfurous, hot milk, burnt	0	0	0	0	17570
Sulfur	5-Methylthiophene-2-	13679-70-4	1166		bready, woody, almond	15569	0	0	0	0

	aldehyde*									
Terpene	a-Pinene	80-56-8	953	950	pine, camphoreous, earthy, woody	0	1443	11777	68063	4661
Terpene	β-Myrcene	123-35-3	1003	1004	herbaceous, metallic	0	0	0	10483	0
Terpene	3-Carene	13466-78-9	1035	1027	sweet, citrus	0	0	4831	6981	1491
Terpene	D-Limonene	5989-27-5	1051	1055	fruity, citrus, orange, sweet, peely	0	0	5427	0	0

Compounds identification, chemical class, and average abundance values measured (n=3)

CAS: Chemical CAS (Chemical Abstract Service) (Blanks relate to isomers where we could not be 100% sure of identification and therefore could not provide full identification. **LRI:** Linear Retention Indices as determined using the method by Van Den Dool & Kratz, (1963)

REF LRI: These values were obtained from published papers or NIST 2014; NA: No published reference available to date (not many published as yet on a DB624 column); *tentative identification, might be isomer of this chemical compound.

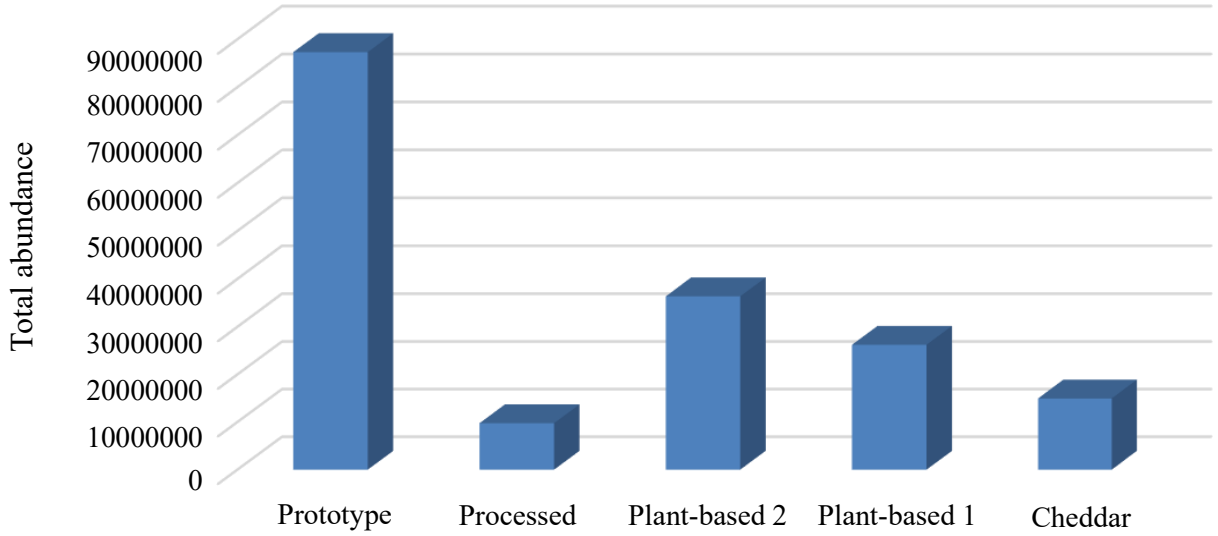


Figure A1. Total peak area of cheese and plant-based cheese alternatives.

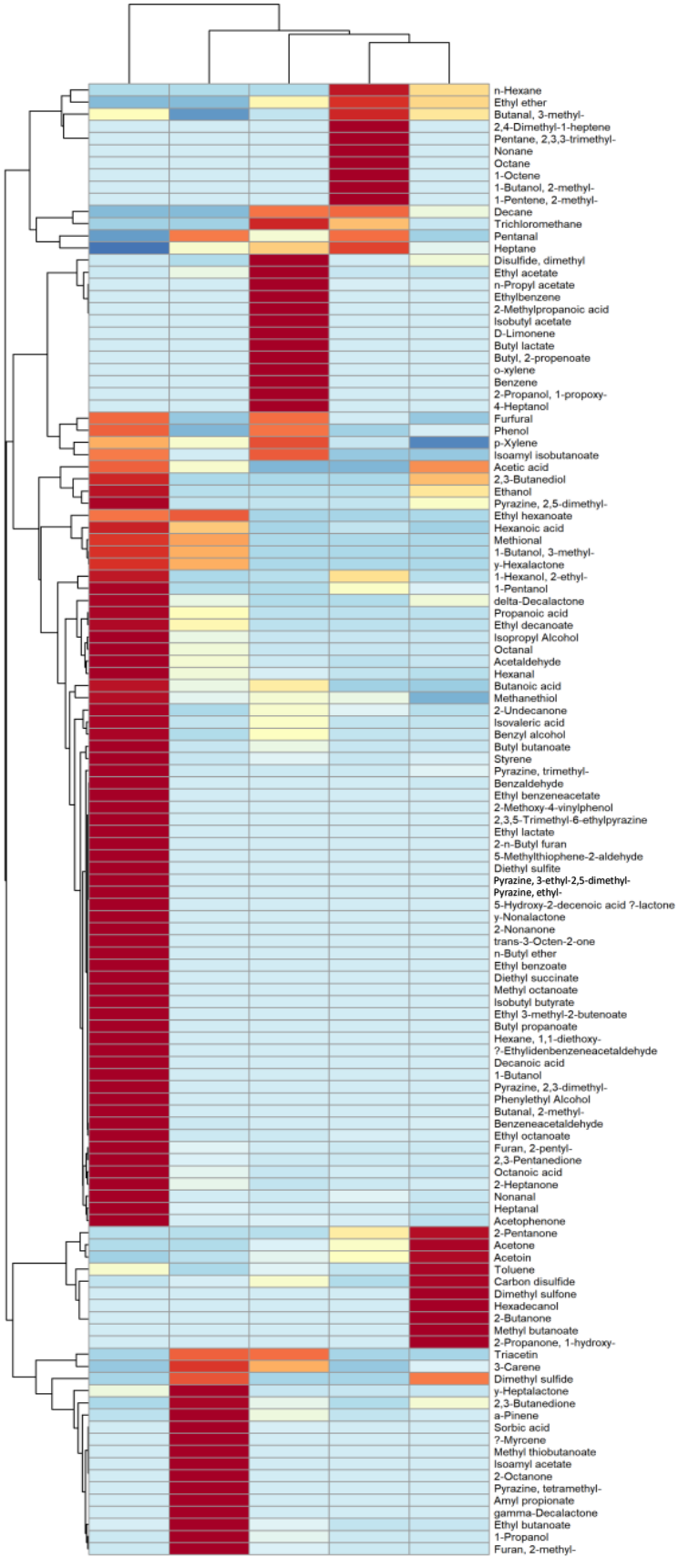


Figure A2. Heat map – visualisation of volatiles distribution across the sample set.

Appendix II

Carbohydrate profiling and scanning electron microscopy analysis of ingredients used in formulating plant-based cheese alternatives

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Materials and methods

Total starch content and swelling volume of powder ingredients

The total starch content of the tapioca starch, chickpea flour, chickpea protein concentrate and zein protein isolate powder ingredients was determined according to approved method 76-13.01 using an assay kit from Megazyme (Bray, Ireland). The swelling volume of the powder ingredients was determined according to the method described by Huang *et al*. (2007a, 2007b). The powder ingredients were weighed into aluminium cans and deionized water was added. Samples were equilibrated at 25°C for 30 min and then heated at 50, 60, 70, 80 or 90°C for 30 min using a Rapid Visco Analyzer. The samples were cooled to room temperature and centrifuged at 1000 rpm for 15 min after which the quantity of the supernatant was measured. Finally, the swelling volume was calculated from the gel volume of each sample and was reported in mL/g of sample (Simsek *et al*., 2012).

Scanning electron microscopy

Powder ingredients were mounted on aluminium mounts using colloidal silver or carbon adhesive tabs and coated with gold using a Balzers SCD 030 sputter coater (BAL-TEC RMC, Tucson, AZ). Images were obtained using a JEOL JSM-6300 Scanning Electron Microscope (JEOL USA, Peabody, MA) (Ovando-Martinez *et al*., 2017).

Results

Starch content and swelling volume of the powder ingredients is reported in Table A(II)1. Tapioca starch showed the highest swelling volume value at the temperature of 90°C, in agreement with previous results (Huang *et al*., 2017), followed by chickpea flour. The value for the zein protein isolate powder was higher than the chickpea protein concentrate at all temperatures.

The microstructure of the powder ingredients is reported in Figure A(II)1. The tapioca starch had typical shape of starch granules (Huang *et al*., 2017). The chickpea flour image showed smooth starch molecules surrounded by proteins or fragments of the protein matrix, in agreement with previous images of the flour (Guldiken *et al*., 2022). On the other hand, the chickpea protein concentrate had smaller particles and a few of the starch granules. The zein powder had very different microstructure compared to the other powder ingredients, with flakes and smooth surface, as previously reported by Rodríguez-Félix *et al*. (2020).

Table A(II)1. Starch content and swelling volume at different temperatures of powder ingredients.

	Starch (%)	**Swelling volume @ 50°C (ml/g)**	**Swelling volume @ 60°C (ml/g)**	**Swelling volume @ 70°C (ml/g)**	**Swelling volume @ 80°C (ml/g)**	**Swelling volume @ 90°C (ml/g)**
Tapioca starch	81.5 ± 0.83	1.63 ± 0.13	8.00 ± 0.50	13.0 ± 0.25	17.63 ± 1.12	21.25 ± 0.25
Chickpea flour	38.0 ± 0.97	1.90 ± 0.10	2.70 ± 0.10	6.10 ± 0.10	8.30 ± 0.10	11.40 ± 0.00
Chickpea protein concentrate	1.83 ± 0.57	2.29 ± 0.14	2.57 ± 0.14	3.57 ± 0.29	4.21 ± 0.07	6.43 ± 0.29
Zein protein isolate	0.10 ± 0.00	3.36 ± 0.50	3.57 ± 0.00	5.57 ± 0.43	6.14 ± 0.14	8.29 ± 0.14

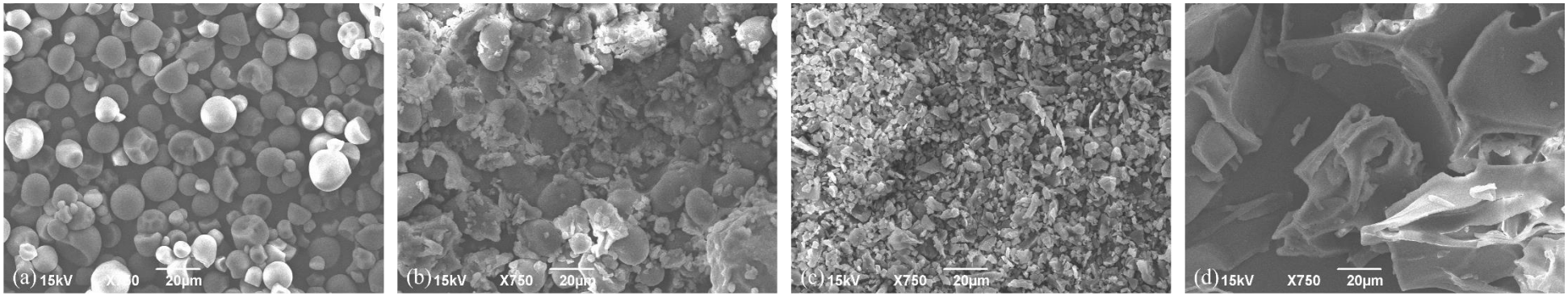


Figure A(II)1. Scanning electron micrographs of powder ingredients, tapioca starch (a), chickpea flour (b), chickpea protein concentrate (c) and zein protein isolate (d).

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