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Influence of formulation and processing strategies on the physicochemical properties of lentil protein stabilised emulsions for use in infant nutritional applications

Thesis presented by

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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.

Signature:

Nicolas Malterre 26/09/2024

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“If you put your mind to it, you can accomplish anything.”

Michael J. Fox as Marty Mcfly

This thesis is dedicated to my Grandparents

Mamie, Papou, c'est pour vous.

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Abstract

The interest and research for plant-based infant nutritional products is growing together with the attention for a sustainable food system. However, while the formulation of plant-based food products is widely studied, the investigations of processing innovations for the manufacture of sustainable, nutritional plant-based food systems are more limited. This thesis investigates the effect of processing techniques to formulate lentil protein-stabilised emulsions at high total solids for young child nutritional products, with enhanced techno-functional properties and physical stability. Formulation of high-solid lentil protein emulsions (lentil protein, sunflower oil and maltodextrin representing 15.85, 27.43 and 56.72% of total solids, respectively), showed high physical and heat stability in the range between 23 and 26% total solids. On the other hand, emulsions at higher total solid concentrations (i.e., 29% and above) had poor physical stability and showed extensive oil-droplet flocculation. The low protein solubility in the high total solid emulsions was identified as one of the main limiting factors in the techno-functional properties of the emulsified systems. Therefore, different physical pre-treatments, including high-shear mixing and high-pressure homogenisation (HPH), were selected to improve the techno-functional properties of the protein ingredients and the emulsions made therefrom. High-shear mixing showed an increase in protein solubility (as measured through nitrogen content in the soluble fraction) (from 46.87 to 68.42% after 0 min and 15 min at 15,000 rpm, respectively), and overall enhanced physical stability of the dispersions, with a reduction of the separation rate from 71.23 (0 min) to 24.16%·h^{-1} (15 min). HPH allowed an enhanced solubility compared to the high-shear mixing process and at 150 MPa the lentil protein isolate dispersions had a solubility of 96.4%. Moreover, the pre-treated lentil protein dispersions (5%, w/v, homogenised at 150 MPa), had higher hydrophobicity (from 607 ± 29 to 1271 ± 50), as well as smaller particle size (from 10.7 to 0.27 μm) and higher physical stability (from 8.12 to 4.97 %·h^{-1}) compared to untreated samples.

To further understand the impact of the pre-treatments on the techno-functional properties of lentil protein emulsions, HPH was selected as pre-treatment due to its high performance compared to high shear mixing and the effects of 0, 15, 50 and 150 MPa on the techno-functional properties of lentil protein dispersions and on the emulsions made therefrom were investigated. The results showed that increasing the solubility of the protein ingredient by HPH pre-treatment led to a significant improvement of the techno-functional properties of the subsequent emulsions. Particularly, emulsions formulated with HPH pre-treated lentil protein ingredient at 50 MPa displayed small oil globules (from 1.40 to 1.19 µm), enhanced heat stability with less flocculation and overall higher physical stability compared to the untreated sample (from 16.75 to 2.05%·h^{-1}). Furthermore, to better understand the colloidal stability of the emulsions at the processing conditions generally used in infant formula manufacture, the effect of combined HPH (50 MPa) and heat-treatment (120°C for 60 s) was investigated. A lentil protein dispersion subjected to HPH (50 MPa) and heat (120°C for 60 s) treatment was produced, which enabled the subsequent formulation of a high total solids (29% w/v) lentil protein-stabilised emulsion, with enhanced techno-functional properties as compared to the untreated sample, such as lower viscosity (from 19.18 to 7.43 mPa·s) and reduced flocculation, with high physical stability (from 15.11 to 1.85 %·h^{-1}). These improvements in the physico-chemical properties can be linked to higher protein solubility, as well as reduced particle size of the protein dispersion, induced by HPH and heat treatment. Furthermore, these results highlighted that combined HPH and heat treatment can support the development of physically stable emulsions at high total solids.

The novel scientific findings presented in this thesis contribute to a better understanding of the impact of processing techniques and subsequent improvement of protein solubility, for enhancing the techno-functional properties of plant protein-stabilised emulsions for use in the manufacture of sustainable plant-based young child infant formula.

Publications

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Malterre N., Bot F., Arendt E.K., Zannini E., O'Mahony J.A. Improving the functional properties of lentil protein isolate using high pressure homogenisation. *University College Cork, School of food and nutritional sciences internal conference*, 2023. Cork, Ireland. 8th June 2023.

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Malterre N., Bot F., O'Mahony J.A. Enhancing thermal and colloidal stability of lentil protein stabilised emulsions using HPH pre-treatment. *EU Horizon 2020 Smart Protein Final Conference*, Berlin, Germany, 23rd May 2024

Research context and objectives

The increasing demand for sustainable food systems has driven a significant interest in plant-based nutritional products (Springmann et al., 2016). Plant proteins are being increasingly recognised as viable alternatives to animal proteins due to their environmental benefits, nutritional value, and functional properties (Day et al., 2022; Day, 2013; Norton et al., 1978). The shift towards plant-based diets is motivated by the need to reduce the environmental impact of food production, enhance food security, and cater to the dietary preferences of an ever-growing population that seeks health-conscious and ethical food choices (Henchion et al., 2017). Furthermore, it is important to note that most infant nutritional formulae available in the market are dairy based, highlighting the need for research focusing on alternative plant-based products. Among the various plant proteins available, lentil protein stands out as a rich source of essential amino acids, vitamins and minerals, making it a highly nutritious option (Boye et al., 2010). Additionally, lentil protein displays interesting techno-functional properties such as emulsification, foaming and water-binding capacities, which are critical for the formulation of colloidally stable food products (Jarpa-Parra., 2018). These properties make lentil protein a promising ingredient for a wide range of food applications, including dairy alternatives and meat substitutes (Vogelsang-O'Dwyer et al., 2021; Shrestha et al., 2023).

However, one of the challenges hindering the wide use of plant proteins, including lentil protein, is their low solubility, particularly when used in high total solid liquid food systems. More specifically, in the manufacture of young child nutritional products, high total solid emulsions are formulated as they reduce the need for water removal before spray drying, which is an energy-intensive and costly process (Ramirez

et al., 2006). On the other hand, formulating stable high solid emulsions is challenging due to low protein solubility and physical stability. Various techniques have been explored to enhance protein solubility, including enzymatic hydrolysis, chemical modification, and physical treatments. Among the physical treatments, high-shear mixing and high-pressure homogenisation (HPH) have shown promising results in improving protein solubility and functional properties (Gharibzahedi et al., 2023, Sá et al., 2022). High-shear mixing has been explored for its ability to improve solubility and stabilise emulsions. On the other hand, HPH has been shown to be effective in reducing particle size, increasing protein solubility, and enhancing the stability of emulsions. These techniques can potentially address the challenges associated with formulating high solid lentil protein emulsions, making them more functional for food applications.

The primary objective of this thesis is to advance the fundamental understanding of the interactions and stabilisation mechanisms of high solid (>29% TS) lentil protein-stabilised emulsions. This research aims to explore the factors influencing the techno-functional properties of lentil protein in emulsion systems, with a focus on enhancing their application potential in young child nutritional products. The specific objectives are:

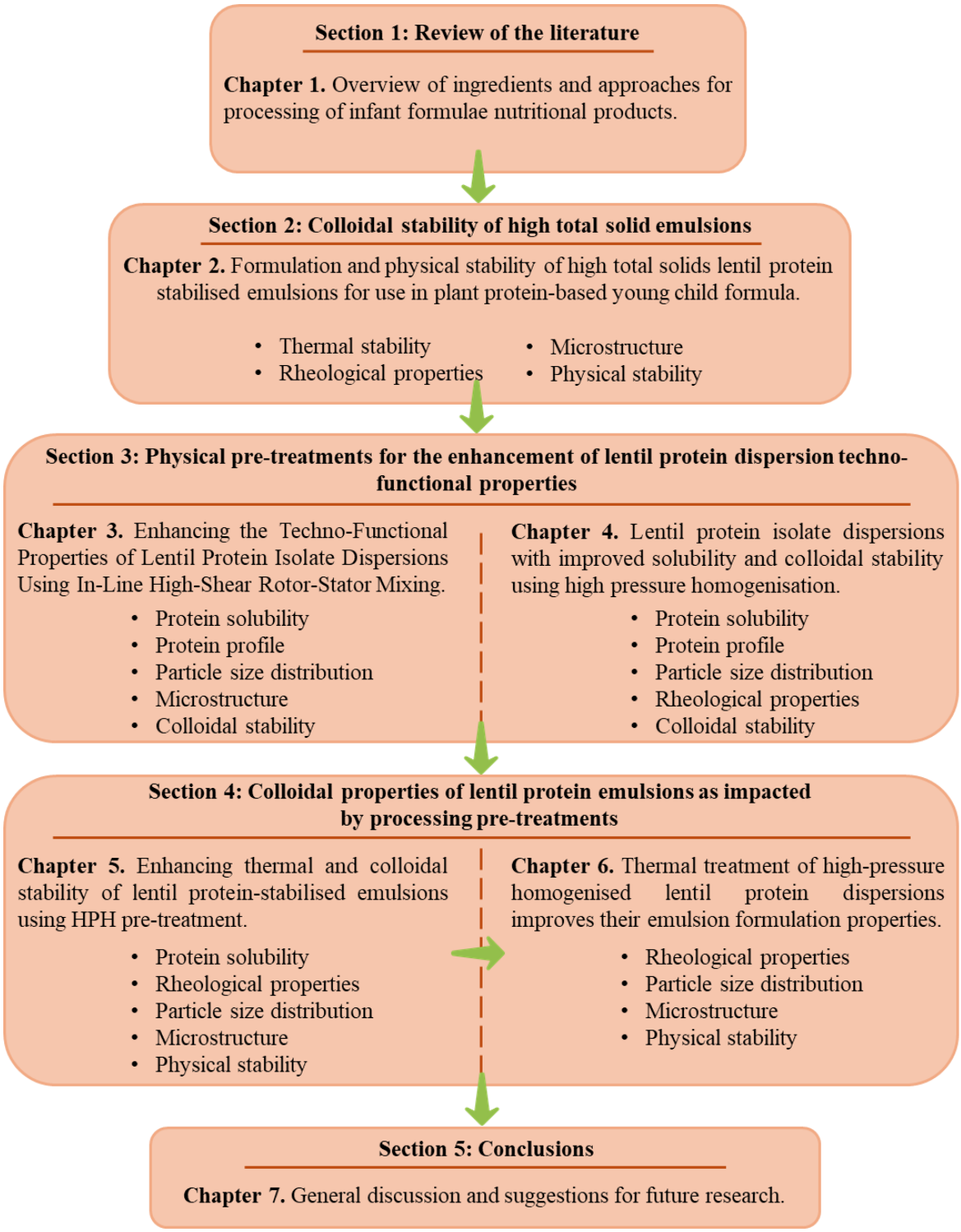
- To provide a thorough review of the current state of infant formula production, with a particular focus on the ingredients and processing techniques used to develop these nutritional products.
- To establish a foundational understanding of the composition, nutritional requirements, and technological processes involved in creating infant formulas, both dairy-based and plant-based. This will be achieved by examining recent

developments and emerging trends in the field, and highlighting the challenges and opportunities in formulating stable, nutritious, sustainable products.

- To investigate the functional properties and physical stability of lentil protein emulsions at solid concentrations between 23 and 35% (range identified through comparison with the literature and preliminary results).
- To investigate the effect of high-shear mixing and high-pressure homogenisation (HPH) pre-treatments on the functional properties of lentil protein dispersions.
- To understand the effect of HPH on the colloidal properties of lentil protein dispersions and the impact on subsequently formulated emulsions, in identifying the optimal processing parameters for such HPH.
- To study the combined effects of HPH and heat treatment on lentil protein dispersions and their effects on the formulation and stability of subsequent emulsions, while closely replicating the processing conditions used in industrial infant formula manufacture.

By addressing these objectives, this research aims to deepen our understanding of how processing techniques impact the properties of lentil protein-stabilised emulsions and to contribute to the development of more sustainable and efficient food manufacturing processes for the development of young child nutritional products.

A schematic representation of the work performed in this thesis is presented below.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Chapter 1

Overview of ingredients and approaches for processing of infant nutritional products

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Abstract

Infant formula includes a wide range of formulated nutritional products based on an evolving knowledge of human milk to satisfy the nutritional requirements of infants up to 3 years of age. Such products are accepted to be safe, nutritionally complete alternatives to mother's milk when a mother cannot, or chooses not to, breastfeed their infant, and are available in two principal physical forms: powders and liquids. Infant nutritional products are formulated with high quality ingredients from dairy and non-dairy sources to best match the composition of human milk and the nutritional requirements of infants. This chapter provides an overview of the different types of infant formulae, the key drivers in their formulation, processing technology options and finished product formats, with an emphasis on recent developments and innovation. The understanding developed on dairy based products is then used as a base to build up knowledge on the latest innovations on plant-based proteins as sustainable alternatives to traditional ingredients. The techno-functional properties of plant proteins essential for the formulation of infant nutritional products, such as solubility and emulsifying properties, are reviewed. The chapter concludes by addressing the potential of novel processing methods to enhance the techno-functional properties of these products, aiming to allow for the production of more sustainable, functional infant nutritional products.

1.1. Introduction

1.1.1. Context for formula feeding

The Food and Agriculture Organization/World Health Organization (FAO/WHO) definition of infant formula is: "Infant formula means a breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life, up to the introduction of appropriate complementary feeding" (FAO/WHO, 2007). In response to this, infant formulae are primarily designed to mimic the nutrient profile of human milk. While breastfeeding is exclusively recommended for the first 6 months of life, infant formulae provide a suitable alternative for specific situations when the mother cannot, or does not want to breast feed (Guo, 2014). Indeed, the WHO advises that mothers of new-born babies should be informed of the benefits and superiority of breastfeeding. However, formula-feeding presents advantages, such as meeting specific nutritional requirements, convenience, and flexibility. If formula feeding is chosen over breastfeeding, recommendations are given regarding their compositions (Section 1.3), based on composition of human milk.

1.1.2. Human and bovine milk

The raw material most extensively used for decades for production of infant formulae is bovine milk (Table 1.1; Blanchard et al., 2013). Consequently, a deep understanding of the composition of human milk is essential to be able to most closely match the chemical composition, and increasingly the nutrition and health outcomes, of breast-fed babies (O'Mahony and Fox, 2013; Crowley et al, 2016). At a macro level, the composition of human milk and infant formula can be considered as the macronutrients, proteins, lipids, carbohydrates, and minor nutrients. The protein content and quality (i.e., digestibility and bioavailability) of infant formula is critical

in influencing the growth and development of the infant (Blanchard et al, 2013). Protein in the formula will supply nitrogen and amino acids that are essential for the development of the infant. Bovine milk contains three times more protein than human milk, signifying that bovine milk-derived protein ingredients need to be recombined in ratios that will best match the protein content and profile of human milk, also considering the higher proportion of casein to whey (casein represents 80% of total protein for bovine milk compared to 40% for human milk), making raw bovine milk unsuitable for infants.

Table 1.1. Comparison of the composition of human milk and bovine milk[1]

Macro Ingredients (per 100 ml)	**Human milk**	**Bovine milk**
Total protein (g)	**1.55**	**3.5**
Casein (g)	0.85	2.8
Whey protein (g)	0.7	0.7
α-Lactalbumin (g)	0.35	0.2
β-Lactoglobulin (g)	0	0.35
Immunoglobulin (g)	0.15	0.05
Total Fat (g)	**3.5**	**3.6**
Linoleic acid (% fat)	10	3
Cholesterol (mg)	20	13
Total Carbohydrates (g)	**7.5**	**4.5**
Lactose (g)	6.5	4.5
Oligosaccharides (g)	1.0	Trace

[1] Data from Blanchard et al. (2013)

Beyond total protein content, another important aspect to be considered is the quantity of each essential and semi-essential amino acid. In this regard, the FAO/WHO declare that, for an equal energy value, the formula must contain an available quantity of each essential and semi-essential amino acid at least equal to that contained in the reference protein (human milk as defined in Table 1.2). Individual free amino acids may be added to infant formula only to improve its nutritional value for infants. Essential and semi-essential amino acids may be added to improve protein quality, only in amounts necessary for that purpose (Codex Standard 72, 1981).

Table 1.2. Amino acid content of human and bovine milk protein[1]

	% Amino Acid in protein	
	Human milk	**Bovine milk**
Alanine	4.0	3.2
Arginine	4.0	3.4
Aspartic acid	8.3	7.2
Cystine	1.7	0.8
Glutamic acid	17.8	19.8
Glycine	2.6	1.9
Histidine	2.3	2.6
Isoleucine	5.8	5.8
Leucine	10.1	9.5
Lysine	6.2	7.6
Methionine	1.8	2.5
Phenylalanine	4.4	4.7
Proline	8.6	9.2

Serine	5.1	5.1
Threonine	4.6	4.6
Tryptophan	1.8	1.3
Tyrosine	4.7	4.8
Valine	6.0	6.2

[1] Data from Heine et al. (1991)

Other major macronutrients to consider in the formulation of infant nutritional products are lipids, which provide 40-50% of the daily energy intake. The lipid fraction of infant formula is usually incorporated in the form of blends of different vegetable oils to best match the fatty acid profile of human milk (Blanchard et al., 2013). Concerning carbohydrates, the main constituents of human milk and infant formula are oligosaccharides and lactose, and to best match human milk, significant fortification with lactose is needed in bovine milk-based formulae. For detailed information on the composition of infant formulae and the ingredients used, please see Section 1.3. It is important also to appreciate that while infant formulae are based on human milk composition, this knowledge base is continually developing and evolving with new scientific information constantly becoming available (Hemmingway et al., 2020; Caldeo et al., 2021).

1.2. Types of infant formula and respective regulations

Infant formula comes in various types, each tailored to meet the specific needs of infants at different stages of development or with particular health conditions. The primary categories include infant or first-age formula, follow-on or second-age formula, and formulae for special medical purposes. Additionally, these formulas are available in multiple formats, including powder, liquid concentrate, and ready-to-feed, each with its own set of benefits and preparation requirements. Understanding the types of infant formula and their respective regulations is essential for caregivers, healthcare providers, and manufacturers. It ensures that infants receive appropriate nutrition, supports their healthy development, and aligns with stringent safety standards. This section describes the different types of infant formula, the regulations that govern their composition and labelling, and the various formats in which these products are available.

1.2.1. Nutritional and regulatory standards

The formulation and marketing of infant formulas are subject to strict regulations to ensure they meet the nutritional needs of infants. The European Commission has established specific guidelines for the nutrient content of infant formulas, including minimum and maximum levels of proteins, carbohydrates, fats, vitamins, and minerals (European Commission, 2016). These regulations are designed to ensure that infant formulas provide appropriate growth and maintain good health during the first months of life until complementary feeding is introduced. Regulatory standards also mandate the inclusion of certain essential nutrients and the exclusion of potentially harmful substances. For instance, the protein content must fall within specified ranges to ensure sufficient intake of essential amino acids (Table 1.3). Additionally, the levels of vitamins and minerals must be balanced to support the developing immune system and

overall health of the infant (European Commission, 2016). These regulations ensure that all infant formulas on the market meet high safety and quality standards, providing reliable nutrition for infants who rely on these products.

Table 1.3. Types of infant nutritional formulations, sources of protein and macronutrient composition thereof.

Age	0-6 months		>6 months	1-3 years
Stage	First-age infant formulae		Second age or follow-on formulae	Young child formulae
Source of Protein	Cow or goat	Soya	Protein hydrolysates	Young child formulae are governed by specific legislation
Energy (kcal/100 mL)	60 - 70	60 - 70	60 - 70	
Protein (g/100 kcal)	1.80 – 2.50	2.25 – 2.80	1.86 – 2.80	
Carbohydrates (g/100 kcal)	9.0 – 14.0	9.0 – 14.0	9.0 – 14.0	
Lipids (g/100 kcal)	4.40 – 6.00	4.40 – 6.00	4.40 – 6.00	
Regulation	EU 2016/127			

1.2.2. Birth up to to twelve months

Infant formula, or first-age infant formula, is specifically formulated to serve as a nutritionally complete substitute for human milk. These formulas are suitable for infants from birth up to twelve months of age. Typically, they are based on bovine or caprine milk and enriched with various ingredients such as lipids, carbohydrates, and minerals, ensuring they meet the specific nutritional requirements of infants (Guo & Ahmad, 2014). The primary objective of these formulas is to mimic the nutrient profile

of human breast milk, making them an appropriate alternative when breastfeeding is not possible due to medical, personal, or allergy-related reasons (Guo & Ahmad, 2014). The protein composition of infant formula can vary, with allowable sources including bovine and caprine milk proteins (whey and caseins), soy proteins, and their hydrolysates. The protein content typically ranges from 13 to 15 g/L, depending on the type and quality of the protein used. For bovine and caprine milk-based formulas, the minimum protein requirement is 1.8 g per 100 kcal, with a maximum of 2.5 g per 100 kcal (Codex Alimentarius, 1981). In contrast, soy protein-based formulas require a higher minimum protein content due to the lower digestibility and bioavailability of soy protein compared to bovine milk protein (Agostoni et al., 2006). This adjustment is crucial as plant proteins generally have lower amino acid quality and digestibility, necessitating a higher concentration to meet the nutritional needs of infants (Jiang & Xu, 2014). These formulations are strictly regulated to ensure they provide all essential nutrients required for infant growth and development.

1.2.3. Six months to one year

Follow-on formula, also known as second-age formula, is intended for infants aged six to twelve months as they transition from exclusive milk feeding to a diet that includes solid foods. This formula serves as a complementary food source, providing additional nutrients that may be lacking in an infant's diet. Follow-on formulas are usually based on cow’s milk and are designed to meet the changing nutritional needs of older infants, supporting their growth and development during this critical period (Codex Alimentarius, 1987; European Commission, 2006). The protein content in follow-on formulas is tailored to support the increased nutritional demands of infants at this stage. For bovine and caprine milk proteins, the protein content should range from 1.8 to 2.5 g per 100 kcal. For formulas based on protein hydrolysates and soy protein isolate, the

minimum protein content should be 2.25 g per 100 kcal. These formulations are also enriched with essential vitamins and minerals to ensure they meet the comprehensive nutritional needs of infants (Codex Alimentarius, 1987; European Commission, 2006).

1.2.4. One year to three years

Young child formulae (YCF), also known as growing-up milk or toddler milk, are designed to cater to the nutritional needs of children aged one to three years. These formulas serve as supplementary nutrition to a child's diet, providing essential nutrients that might be lacking in their regular food intake. The European Food Safety Authority (EFSA) recommends the use of the term "young child formula" to describe these products, ensuring a clear definition that encompasses bovine milk-based drinks or plant protein-based formulas intended for young children (EFSA, 2016). YCFs have been available in Europe for more than two decades, and their use has been increasing. However, the composition of these formulas is not strictly regulated, and their nutritional benefits have not been systematically studied (Hojsak et al., 2017). The composition of YCFs available on the European market varies significantly, with some products containing high levels of protein and carbohydrates, and even high amounts of added sugars.

The ESPGHAN Committee on Nutrition (CoN) has performed a systematic review to evaluate the composition and role of YCFs in the diet of young children. They recommend that the nutrient composition of YCFs should be similar to that of follow-on formulas, with an emphasis on providing nutrients that are often deficient in the diets of young European children, such as iron, vitamin D, and polyunsaturated fatty acids (n-3 PUFAs). Indeed, one of the main benefits of YCFs is their ability to provide essential nutrients that might be missing from a child's diet. For instance, studies have shown that YCFs can significantly increase the intake of vitamin D and iron, reducing

the risk of deficiencies (Akkermans et al., 2017; Houghton et al., 2011). However, there are also concerns about the high protein content and added sugars in some YCFs, which could contribute to obesity and other health issues (Hojsak et al., 2017).

Furthermore, these nutrients can also be provided through regular or fortified foods and supplements, making YCFs one of several strategies to enhance nutritional intake. Despite their benefits, the necessity of YCFs is debated. The EFSA and other nutritional bodies have concluded that while YCFs can contribute to better nutrient intake, they are not essential for all children. Alternative strategies, such as promoting a healthy and varied diet, using fortified foods, and providing supplements, can also effectively meet the nutritional needs of young children (EFSA, 2013; Przyrembel & Agostoni, 2013).

In terms of regulation, there is no international legal definition or compositional criteria for YCFs, and their regulation differs between European countries. This lack of regulation can lead to significant differences in the nutritional quality of YCFs available on the market (EFSA, 2013). Recently, efforts have been made to provide recommendations for the composition of YCFs. An International Expert Group coordinated by the Nutrition Association of Thailand and the Early Nutrition Academy has suggested specific guidelines to ensure that YCFs meet the nutritional needs of young children (Suthutvoravut et al., 2015). Furthermore, the European commission mandated the European Food Safety Authority to provide advice on the nutritional composition of all available YCFs on the market (Table 1.4).

Table 1.4. Average nutrient composition of young-child formulae

Nutrient	Minimum (g/100 Kcal)	Maximum (g/100 Kcal)	Median (g/100 Kcal)
Protein	2	6.7	2.6
Casein	0.1	2.4	1.7
Whey Protein	0.4	1.2	0.7
Whey/Casein ratio	0.5	6.3	1.5
Carbohydrates	7.3	15.4	12.6
Sugars	3.1	13.7	9.9
Lactose	0.1	13.5	9
Sucrose	0.6	10.4	2.1
Glucose	0	1.8	0.5
Maltose	0.1	5	0.2
Maltodextrin	1.4	11.2	4.1
Fat	3	5.7	4.3
Saturated Fat	0.2	4.3	1.4

Data from Perez *et al*. (2013)

1.2.5. Formulae for special medical purposes

Special medical formulae are tailored to meet the specific nutritional needs of infants with particular medical conditions, such as low birth weight, lactose intolerance, or prematurity. These formulas are designed to be nutritionally complete and may be modified to address specific dietary requirements. For instance, they may have altered protein compositions or be formulated to be high-caloric or nutrient-dense to support the unique needs of these infants (Codex Alimentarius, 1981; O'Callaghan et al., 2011).

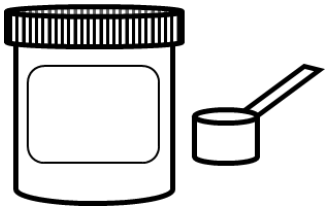
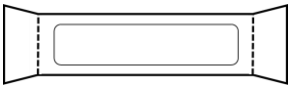
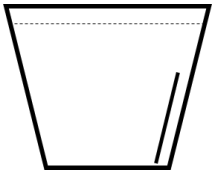
For infants with low birth weight or prematurity, specialised formulas provide higher concentrations of essential nutrients to support rapid growth and development. For those with lactose intolerance, lactose-free formulas or those with hydrolysed proteins are used to ensure proper digestion and absorption of nutrients (Guo, 2014). The development and regulation of these formulas involve careful consideration of the specific nutritional needs and potential health challenges faced by these infants, ensuring they receive the appropriate support for healthy growth.

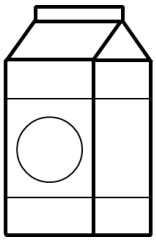
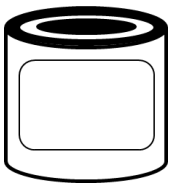
1.2.6. Available formats of infant and follow-on formula

Infant and follow-on formulas are available in three primary forms: powder, liquid concentrate, and ready-to-feed. Powdered formulas are the most economical option and must be mixed with water before feeding. Liquid concentrates also require mixing with an equal amount of water but offer the convenience of quicker preparation compared to powders. Ready-to-feed formulas are the most convenient, requiring no additional preparation, but they are also the most expensive option (Crowley et al., 2017). These formulas are packaged in various formats to meet different practical and medical needs. Common packaging options include cans, tablets, stick packs, pouches, plastic bottles, glass bottles, tetra briks, and concentrate forms (Table 1.5). The choice

of packaging often depends on factors such as storage stability, ease of use, and the specific requirements of the infant and caregiver (Guo, 2014). Each packaging format is designed to ensure the safety, nutritional integrity, and convenience of the formula.

Table 1.5. Overview of the different formats of infant formulae commercially available.

Format	Features of the format
 Powder in can	- Free-flowing bulk powder in multi-use can - Consumer scoops designated quantity for correct nutrient density - Dissolved in sterilised water/container prior to consumption - Reusable can
 Tablet	- Compressed powder in single-serve tablet - Correct nutrient density contained within tablet - Dissolved in sterilised water/container prior to consumption
 Stick pack	- Free-flowing powder in single-serve sachet - Correct nutrient density contained within sachet - Dissolved in sterilised water/container prior to consumption
 Pouch	- Free-flowing bulk powder in plastic pouch - Used to refill cleanable, reusable tubs/cans

 Plastic Bottle	- Ready-to-Feed/pre-sterilised liquid - Re-sealable container - Multi-use (within 48 h after opening)
 Glass Bottle	- Ready-to-Feed/pre-sterilised liquid - Heavier than other ready-to-feed formats - Single-use (within 48 h after opening)
 Tetra Brik	- Ready-to-Feed/pre-sterilised liquid - Occupies less transit space than other ready-to-feed formats - Single-use (within 48 h after opening)
 Concentrate	- Concentrated liquid infant formula - Mixed with designated quantity of sterilised water to achieve correct nutrient density - Ease-of-preparation is intermediate between can format (intensive) and ready-to-feed formats (easy)

Adapted from Crowley et al. 2017

1.3. Ingredients

The first step involved in manufacture of infant formula is the preparation of the bulk ingredients as a mixture in either water or liquid skimmed milk. All the major and minor nutrient contents are adjusted to closely match human milk composition (see Section 1.1), with some of the more heat-labile components (e.g., water-soluble vitamins) typically added later in the manufacturing process, post heat treatment. In the case of bovine milk-based formula, the concentration and profile of the major proteins is typically adjusted by adding demineralised whey or whey protein concentrate to a skim milk base. Also, to best reflect the fatty acid profile of human milk, vegetable oil blends are generally used as the fat source, with the carbohydrate component principally made up of lactose, contributed innately by skim milk and whey ingredients. Soluble fibre ingredients and oligosaccharides are also added in certain formulations (Kelly and Fox, 2016).

1.3.1. Proteins

The protein content and profile of human milk is very different from bovine milk, with human milk having considerably lower protein content and a higher proportion of whey protein than bovine milk (de Wit, 1998). As a consequence, first age infant nutritional products are specifically formulated using high quality ingredients to achieve the target protein content and deliver the essential amino acids required. These differences in protein content and profile have important implications for kinetics of protein digestion and curd-forming properties under gastric conditions – human milk generally forms softer and more flocculent curds, contributing to easier digestion (Thompkinson and Kharb, 2007) and also to differences in kinetics of release of amino acids and other nutrients (Crowley et al., 2017). The regulations state that the protein content of such products should not be less than 1.3 g/100 g, and not more than 1.5

g/100 g. This is higher than what is usually found in human milk (0.8-1.0 g/100 g), to compensate for the low levels of certain amino acids (tryptophan, tyrosine and cysteine) (European Commission, 2006). Thus, to satisfy the protein content and amino acid profile requirements, demineralised whey or whey protein concentrate are the most extensively used ingredients in first age formulae (European Commission, 2016). In addition, several other types of whey-based ingredients are also suitable for infant formula applications, with final selection being based on factors including nutritional, technological, marketing, compositional specification, and price/functionality interrelationships (Fenelon et al., 2019). Conversely, follow-on formulas are generally formulated to be casein dominant as they are mostly used as substitutes for bovine milk (O'Callaghan and O'Mahony, 2011).

In addition to satisfying the compositional and regulatory requirements in formulating infant nutritional products, it is also important to consider that the protein profiles of human and bovine milk are considerably different. In fact, human milk does not contain β-lactoglobulin (β-lg), and has higher level of α-lactalbulmin (α-lac) and lactoferrin (lf). In recent decades, the development of fractionation techniques has been a key driver in the development and commercialisation of infant formulae with protein profiles more tailored to match that of human milk. In recent work, Fenelon et al. (2019) and Barone et al. (2020) reviewed the major whey proteins and their possible benefits in infant formula applications.

It has been shown that the amino acid sequences of α-lac in human and bovine milk are 72% homologous, and with this high homology, bovine milk-derived α-lac is a suitable substitute for α-lac from breast milk in infant formulae. α-lac is rich in tryptophan and cysteine, hence using higher levels of α-lac allows formulators to meet amino acid requirements with reduced total protein levels; Davis et al. (2008) showed

that infants fed formulae containing a lower level of total protein and a higher level of α-lac presented a closer gastrointestinal (GI) tolerance to that of breast fed infants, with fewer GI events (constipation, gastroesophageal reflux, abdominal pain, vomiting, diarrhoea and regurgitation) than infants fed with non-enriched formulae.

In the same way, human lf has 77% homology with bovine lf, suggesting that bovine lf would be suitable for addition to infant formulae as it has a number of important biological activities in infants (Goulding et al., 2021, Tomita et al., 2009). For more information about bioactive proteins, the reader is referred to Section 1.3.5.

β-lactoglobulin is the major whey protein in bovine milk but is not present in human milk. Apart from the provision of essential amino acids, the biological function of β-lg is unclear. Most commercial dairy-based infant formulae contain β-lg, where it importantly provides amino acids during digestion, while work like that of Sanchon et al. (2018) has shown intact β-lg in human jejunal effluent after digestion, which has the potential to contribute to cow's milk allergy in some infants. Infants that are subject to intolerances and/or allergies such as cow's milk intolerance/allergy (CMI/CMA) (El-Agamy, 2007; Koletzko et al, 2012) require special medical formulations, with the protein platform of such formulae based on hydrolysates of whey and/or casein. Somewhat related to this are formulations designed for the treatment of IgE-mediated CMA, which are based on free amino acids. In addition, anti-regurgitation products containing starch, or other thickeners, as well as often being casein-dominant to limit the incidence and severity of gastroesophageal reflux in infants.

1.3.2. Lipids

Lipids provide the main energy source in infant feeding, with 40-55% of total energy typically contributed by lipids (Koletzko et al., 2001), and they are also involved in the regulation of a number of cell functions. Hence, the profile of lipids in infant

formula directly influences gastrointestinal function, lipoprotein metabolism, membrane composition and function, thereby affecting infant growth, development, and health (Mazzocchi et al., 2018). The fat profile of human milk is mainly composed of triacylglycerols (TAG) which consist of a glycerol backbone with three fatty acids (FA) attached at sn-1, sn-2, and sn-3 positions, with properties varying depending on the specific FA composition. Some of those FA are indispensable nutrients; polyunsaturated fatty acids (PUFAs) and long-chain PUFAs (LC-PUFA) such as docosahexaenoic acid (DHA) and arachidonic acid (ARA) must be supplemented to ensure appropriate development of the infant brain, retina acuity, electrophysiologic response, improved immune status, and problem-solving skills (Koletzko and Braun, 1991; Makrides et al, 1994, 2009; EFSA Journal, 2006; O'Callaghan et al., 2011). Many studies have shown the importance of those LC-PUFA's as well as the importance of achieving a ratio of at least 2:1 ARA and DHA (Manley et al, 2011; Campoy et al., 2012; Sabel et al., 2012).

The fat component of most infant formulae is constituted by a mixture of vegetable oils (e.g., palm oil, coconut oil, sunflower oil). The TAG structure of vegetable oil and human milk fat differ by the distribution of palmitic acid at the sn-2 position, which is only 10-20% of all palmitic acid for vegetable oil and 70-88% in human milk fat (Sun et al., 2018). During digestion, hydrolysis of vegetable oil TAG releases free-FA from the sn-1 and sn-3 position, which combine with calcium, forming indigestible calcium soaps, and have been shown to result in reduced calcium absorption, energy loss, constipation, and digestive discomfort (Mohan et al., 2020). Therefore, inter-esterification of vegetable oil TAG, which allows altered distribution of FA at the sn-2 position with the FA at the sn-1/sn-3 position, is implemented to produce TAG enriched in sn-2 palmitic acid (Hageman et al., 2019).

Dairy lipids were historically used in infant formula, and are still used in some parts of the world, but their use has diminished (Delplanque et al., 2015). However, the incorporation of dairy fats along with vegetable oils in infant nutrition is receiving renewed attention with recent work showing that adding dairy lipids presents an alternative approach for improving omega 3 FA status in infants (Giani et al., 2018). Lipids can be supplemented from different sources with the addition of single cell oils, fractionated lipids, and various polar lipids, re-esterified structured lipids, egg phospholipids, and fish oils (Delplanque et al., 2015).

1.3.3. Carbohydrates

The main carbohydrate (about 85% of total carbohydrates, Blanchard et al., 2013) present in human milk, and therefore in infant formulae, is lactose, which provides about 40% of total energy and has beneficial effects on infant gut physiology (Koletzko et al., 2005). However, other digestible carbohydrates are permitted by the FAO/WHO, such as maltodextrin, to improve the bulk handling and physicochemical properties of the infant formula powders (Masum et al., 2019), or sucrose and glucose to enhance the organoleptic properties. Cooked starch can also be added for specific medical formulae to confer increased satiety and less post feeding regurgitation (Salvatore et al., 2017). In addition, other oligomers such as galacto-oligosaccharide (GOS) and/or fructo-oligosaccharide (FOS) can be added to infant formula. The non-digestible carbohydrates, GOS and FOS, are now commonly used in infant formulae for their prebiotic activity. They are not naturally present in human or bovine milk but GOS is produced by enzymatic fermentation of lactose and sucrose while FOS is extracted from chicory and both are added to infant formulae to modify the infant gut microbiota towards one that is more similar to breast-fed infants by promoting the growth of *Bifidobacteria* (Bakker-Zierikzee et al., 2005).

1.3.4. Minor nutrients

Vitamins are present in human milk in the form of fat-soluble and water-soluble ingredients. Retinol (vitamin A), tocopherol (vitamin E), calciferol (vitamin D) and phylloquinone (vitamin K) are the fat-soluble vitamins, whereas the water-soluble vitamins are thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pantothenic acid (vitamin B5), pyridoxine (vitamin B6), cyanocobalamin (vitamin B12), ascorbic acid (vitamin C), folic acid (vitamin B9) and biotin (vitamin H or B8) (Montagne et al. 2009). The European Commission provides minimum and maximum requirements for each of these micronutrients to support infant growth and development (European Commission, 2006). Indeed, a lack of vitamins would result in deficiencies for the infant, while a high intake of water-soluble vitamins has no negative impact as they are easily eliminated by the body. Conversely, an excess of fat-soluble vitamins has the potential to result in their accumulation in the tissues and may induce unwanted effects (Koletzko et al., 2005). Adequate mineral intake is essential to support infant development, as calcium, iron and zinc deficiency can result in sub-optimal skeletal mineralization, cognitive decrement, and failure to thrive, respectively. Other minerals, such as phosphorus, magnesium, sodium, chloride, and potassium are nutritionally relevant for infant development and may be added to infant formulae as well as trace elements (manganese, iodine, selenium and copper). It should be emphasised that the exact chemical form of each mineral can significatively influence their bioavailability. In addition, other ingredients (e.g., choline, taurine and nucleotides) may be added to ensure that the formulation is suitable as the sole source of nutrition for the infant or to provide other benefits that are similar to human milk (FAO/WHO, 2007).

1.3.5. Other ingredients

Research and innovation in ingredients for infant formula is greatly influenced by our evolving understanding of the chemical composition of the gold standard, human milk, and also by our desire to better match the physiological and health outcomes of human milk-fed infants. As a consequence, this will be very dependent on developments in analytical science and the application of novel analytical tools in the study of human milk to match its nutritional/functional properties.

1.3.5.1. Innovation in dairy proteins

Bioactive proteins, such as α-lac and lf, are of considerable interest in infant formula, and it has been demonstrated that, beyond their basic nutritional contribution, they also contribute certain bioactivities such as mineral binding, anti-infective and immune modulation (Garcia-Montoya et al., 2012; Lyons et al., 2020). More specifically, it has been found that supplementation with α-lac promotes a plasma amino acid pattern similar to that of breast-fed infants (Kelleher et al., 2018), in addition to other similarities such as growth parameters, gastrointestinal tolerance and hormonal regulation (Lonnerdal, 2014; Barone et al., 2020). In addition to these important physiological and health outcomes, α-lac enriched formulas have greater heat stability, and reduced protein-protein interactions (Davis et al, 2007; Crowley et al, 2016; Buggy et al, 2017), all of which are desirable in formulation and processing of infant formulae. Concerning lf, recent studies have focused on the impact of bovine lf (blf) enriched infant formulae on iron bioavailibity, infections and intestinal microbiota of infants. However, while lf is essential to the development of a newborn's immune system, Grzywacz et al. (2020) showed that blf supplementation had minimal impact on the intestinal microbiota of infants and Asztalos et al. (2020) showed that blf supplemented formulas do not significantly reduce the mortality of low-birth-weight

infants. This limited impact of blf in infant formula might be due to its affinity with contaminants (e.g., lipopolysaccharide) present in commercial blf that could have blocked blf bioactivity (Lönnerdal, 2014). Therefore, future studies on blf enriched formulae should give particular attention to the detailed chemical composition of the commercial blf used. Heat treatment during processing of infant formulae has the potential to impact the bioactivity of lactoferrin (Goulding et al., 2021), therefore, developing enhanced understanding of the interrelationships between ingredient chemistry, processing and bioactivity are important (for more information on processing-mediated impacts on nutritional and functional properties, please see Section 1.4).

β-casein is another protein of interest in infant nutritional product formulation, and together with κ-casein, these two proteins are the most prevalent caseins in human milk. However, it is important to note that bovine β-casein is most frequently found in two variants, A1 β-casein and A2 β-casein, differing slightly by their amino acid profile; A2 β-casein being closer to that of human milk, which is of interest for humanisation of infant formula. In fact, A1 β-casein may be associated with issues such as digestive disorders, immune disorders, type 1 diabetes and respiratory dysfunction, while A2 β-casein is claimed to be more similar to that in human milk, and consequently reduce the risk of such disorders, as well as helping to maintain optimal growth and development (Sadler and Smith, 2013).

1.4. Technological options for manufacture of infant nutritional products

1.4.1. Powders *vs* liquid

Infant nutritional products can be classified into two physical forms, powders, and liquids, with each format prepared using different processing options, sharing some unit operations.

1.4.1.1. Powdered infant formula

Powdered infant formula can be produced using two core manufacturing approaches, "dry-blending" which involves the mixing of dry ingredients without heat treatment, and "wet-mixing" (Figure 1.1) (Montagne et al. 2009), which involves the recombination of water-soluble ingredients in milk or water to meet the desired macro chemical composition, followed by pre-heating and addition of oils and emulsifiers (if used). A stabilised emulsion is normally obtained by homogenisation and heat treatment to inactivate all potential pathogenic organisms, followed by evaporation and spray drying (Westergaard, 2004; Pisecky et al., 2012).

Choosing one approach over the other is influenced by multiple considerations; however, in general, it is important to consider that the dry processing approach (although much less widely practiced) requires less energy and involves lower capital expenditure, while wet processing generally involves higher capital and operating costs. Furthermore, through the dry processing approach, the microbiological and physical quality depends on the raw material and integrity of the actual dry blending process, and, without agglomeration, bulk handling properties of the blended product are determined largely by the properties of individual ingredient powders, with the possibility of segregation of ingredients occurring during transportation (Cuq et al., 2013)

Even if they can be used separately and present specific advantages, or disadvantages, the two approaches are often combined, as minor ingredients, such as dried vitamins and minerals, can be added directly after spray drying for dry blending. In such a case, special attention is given to the microbiological quality and safety of those ingredients. In any case, exact formulations, as well as specific technological approaches used for production of infant formula, are commercially sensitive and vary considerably between companies. (Montagne et al., 2009)

1.4.1.1.1. Recombination

Recombination takes place in two different ways where water and oil-soluble ingredients are hydrated separately before combining them. The water-soluble powders are added into the milk or water to achieve rehydration in several stages: wetting, sinking, dispersion and dissolution (McSweeney and Fox 2009), very often supported by the use of an in-line high shear mixing device to aid complete hydration of powder constituents (O'Sullivan et al. 2017) as well as increasing the mixing temperature (i.e., ≤50°C) (Bylund, 1995, Guo, 2014). Minerals are then added to the water-soluble ingredients mix and the pH of the solution can be adjusted to ensure its stability to subsequent heat treatment (~pH 6.8) (Montagne et al. 2009). Finally, oil-soluble ingredients are dispersed in the oils and blended with the wet-mix. Concerning water soluble vitamins, they can be added as a cold premix to the homogenous mix before spray drying or as a dry premix to the dried powder.

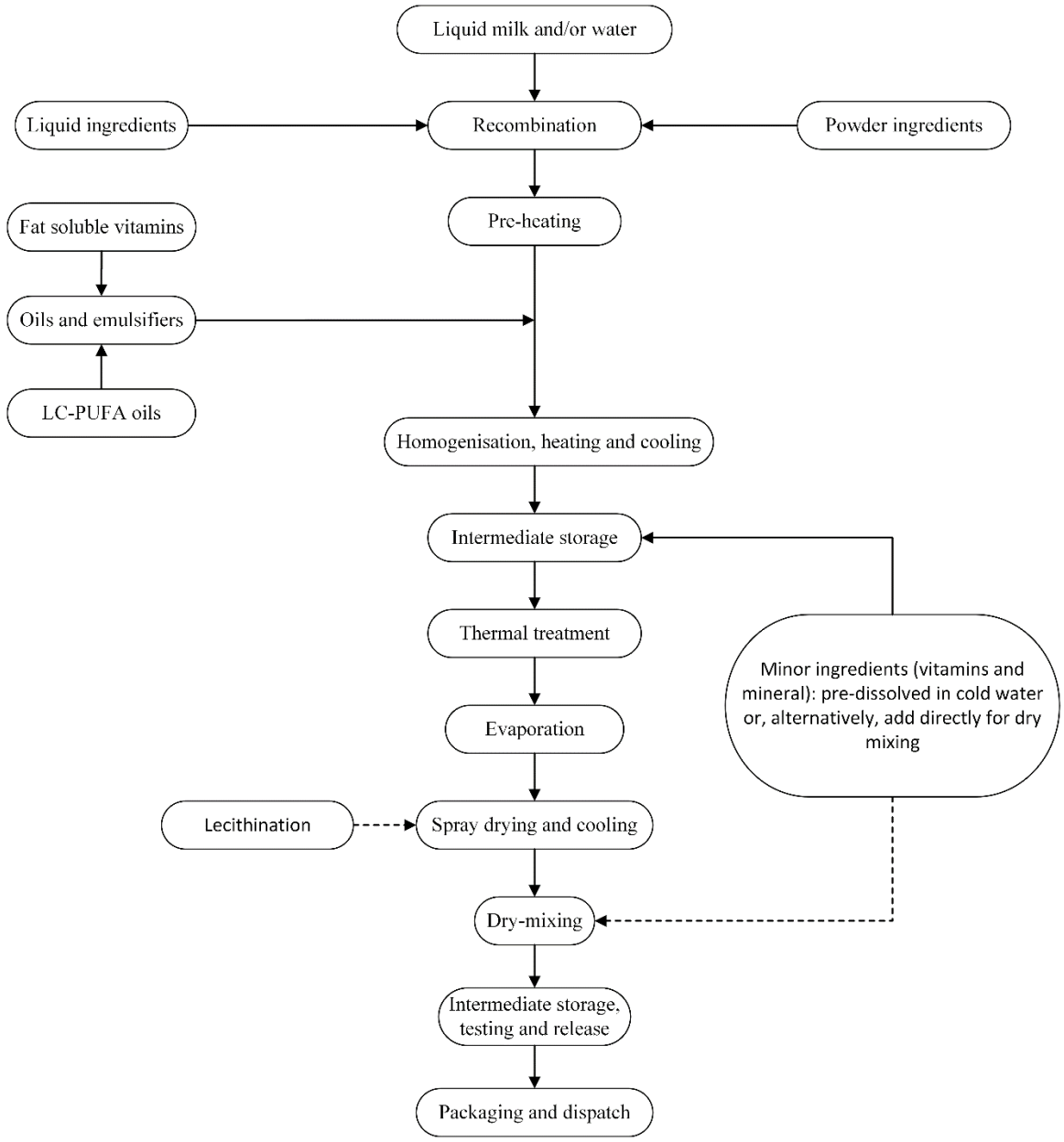


Figure 1.1. Schematic representation of the manufacture of dried infant formulae. Note: dotted lines represent alternative processing routes (Montagne et al., 2009)

1.4.1.1.2. Homogenisation

A stable emulsion is essential to ensure adequate functional properties of the powder after spray-drying, and to achieve same, homogenisation is normally performed using two-stage valve homogeniser technology, applying first and second stage pressures of ~14 and ~3 MPa, respectively (McCarthy et al., 2012; Murphy et al., 2015; Masum et al., 2019). A higher pressure at the first stage would lead to smaller fat globules and possibly result in instability and cavitation. The second stage pressure ensures the disruption of any fat clusters formed during the first stage (Robin et al., 1993; Olson et al., 2004). The pressures used in first and second stage have a significant impact on the oil droplet size and therefore on the stability of the emulsion. To have an emulsion as stable as possible, it has been suggested that the size of the droplets should be less than 1µm after homogenisation (McSweeney, 2008). The proteins present in the wet mix are amphiphilic and are the principal surfactants in most infant nutritional products. Notwithstanding this, non-protein emulsifiers (e.g., lecithin) are often added to support proteins/peptides in forming and stabilising emulsions (McSweeney et al., 2008; Singh 2011). The whey:casein protein ratio in the wet mix has been shown to influence emulsification, with previous work demonstrating that a higher proportion of casein (60:40) will result in a greater concentration of protein adsorbed at the interface compared to an emulsion made with whey protein isolate (WPI) alone (100:0), with important implications for emulsion stability (Sourdet et al., 2002; McCarthy et al., 2012). Furthermore, Dalgleish et al. (2002) demonstrated that casein is preferentially adsorbed over whey proteins at the oil-water interface and Buggy et al. (2017) reported that increasing the proportion of α-lac in IMF emulsions retards the aggregation of β-lg, conferring greater stability on resultant emulsions.

1.4.1.1.3. Heat treatment

Thermal processing may be performed on liquid infant formula mixes before or after homogenisation to ensure microbial quality in the finished product, as well as achieving targeted physiochemical properties (McSweeney et al. 2004, 2008; McCarthy et al. 2012, 2013). Buggy et al. (2017), suggested that heating before homogenisation would be preferred, allowing for physical disruption of any aggregated fat globules/fat-protein complexes created during heating. Direct heating, which consists of direct contact between the heating medium and the product (steam injection or infusion) and indirect heating, which involves using a (tubular or plate) heat exchanger are generally applied. Direct treatment is preferred for the rapidity of treatment as well as the limited denaturation of β-lg and α-Lac or to avoid the evaporation stage, with the added advantage of not having a physical heat exchange surface serving to limit fouling, while indirect treatment is preferred due to lower energy consumption. On the other hand, direct treatment may cause damage arising from cavitation and demands more maintenance, while indirect treatment may cause instability of the solution and induce more intensive Maillard reaction (Lewis and Deeth, 2009; Tran et al., 2008; Datta et al., 2002; McSweeney et al., 2004).

When using these technologies, the protein profile of the product needs to be considered, as for example, whey proteins are heat labile ingredients (Fenelon et al. 2019), hence their physicochemical properties are impacted by heat-induced denaturation (Mulcahy et al., 2017). In addition, as they are the dominant protein in IMF (≈60% of total proteins), the denaturation of whey proteins will impact the thermal sensitivity of the wet mix. β-lg is heat sensitive due to the presence of a free sulphydryl group which unfolds on heating and interacts with itself, as well as other whey proteins and casein micelles, leading to the formation of protein aggregates

(Fenelon et al., 2019). However, it is important to note that hydrophobic interactions also contribute to the creation of aggregates (Manderson et al. 1998). α-Lac is more stable than β-lg to thermal processing; indeed, the former protein does not contain free thiol groups. However, self-aggregation within α-lac monomers has been shown to be reversible (McGuffey et al. 2007). Caseins, representing 40% of total protein in infant formulae, are significantly more heat stable than whey proteins, but do have the ability to interact with whey proteins (Singh et al. 2019, Guyomarc'h et al. 2009, Kehoe and Foegeding, 2014, Murphy et al. 2015).

1.4.1.1.4. Evaporation

Evaporation is used to achieve a more cost-effective removal of water than spray drying, as well as increasing the total solids before spray drying, which directly influences the physical properties of the resultant powders (Fox et al., 2010). It is a process in which the homogenised wet mix flows by gravity through a falling film evaporator and forms a thin film inside of each tube that composed it, in which steam is applied on the outer surface of the tubes, causing the evaporation of water from the wet mix (Morison et al., 2006). The process is performed under vacuum in order to keep the wet mix at a relatively low temperature (50-70°C). The liquid viscosity is the limiting factor during evaporation, as the concentrated mix needs to be fed to the spray dryer afterwards; indeed, it is generally recommended that the viscosity of the wet mix after evaporation should be in the range of 60 to 100 mPa·s (Westergaard, 2004).

1.4.1.1.5. Spray Drying

After evaporation, the concentrated mixture is dehydrated by spray drying to produce an infant formula powder as it is an efficient and gentle drying technology (O'Callaghan and Wallingford, 2002). Multi-stage spray dryers are generally used to allow the production of a high-quality powder with good bulk-handling properties such

as agglomeration, solubility, dispersibility and wettability (Blanchard et al. 2013). The stabilised mixture is fed on top of the spray dryer into a nozzle or wheel atomiser that atomises it into fine droplets (10-400 µm) in the main chamber (180-200°C) to increase the contact surface with the hot air (Westergaard, 2004, Montagne et al. 2009). At this stage, the majority of water is removed, and the formed particles fall by gravity into an internal fluidised bed (50-60°C) at the bottom of the spray dryer main chamber where supplementary drying happens. An additional external fluidised bed (20-30°C) can be added to complement or replace the internal one (where relevant), with such external fluidised beds also facilitating cooling of the produced powder and their agglomeration (Hazlett et al. 2021). Full control of the parameters while spray-drying is essential as the rapid evaporation of water leads to the migration of milk components to or from the surface of the drying droplet and thus, influence the powder particle structure and their rehydration properties (Kim et al, 2003). Therefore, spray-drying, as described above, may change and will require specific optimisation for each product due to the variety and complexity of the ingredients involved.

1.4.1.1.6. Infant formula powder composition

The high temperatures used in spray drying have little effect on the denaturation of whey proteins as the particles only ever reach approximately 70°C due to the evaporative cooling effect, hence, limiting the denaturation of whey protein (Guyomarc'h et al., 2000; Haque et al., 2015). However, casein micelles are highly hydrated structures and through drying, the micelle structure changes leading to higher hydrophobicity and possible insolubility (Baldwin, 2010). Concerning fat, it has been widely shown (Fäldt and Bergenståhl, 1994; Foerster et al., 2016, Kim et al., 2009), that when dairy powder contains a high level of fat (such as infant formula powders) the particle surface is mainly composed of fat, leading to poor powder wettability and

immiscibility of the particles in water. The most commonly applied solution to this problem is instantanisation with lecithin (Hammes et al., 2015), known as lecithination. Lactose is highly impacted by the spray-drying process, with lactose forming an amorphous glass which reduces molecular mobility, restricting the movement of encapsulated fat (Baldwin et al., 2010; Zhou and Roos, 2011). However, certain conditions (reaching glass transition temperature, T_g) while spray-drying could lead to a transition to a rubbery state of lactose, leading to greater mobility of molecules and a higher level of surface free fat. A high concentration of lactose in its rubbery state could also cause stickiness and caking, causing damage to the spray-dryer as well as decreasing powder quality (Chuy and Labuza, 1994; Vuataz, 2002; Roos, 2002; Nasipour et al., 2006). Reaching the glass transition of lactose will also increase the crystallisation of lactose during storage and decrease powder flowing ability (Fitzpatrick et al., 2004).

1.4.1.2. Liquid infant formula

Liquid infant formulae are available in two states, ready-to-feed that can be used as it is and concentrates that require mixing with water. Those products represent a small minority of the infant formula market as they are relatively expensive formats and are available mainly for convenience reasons or to support specific medical conditions, such as preterm or low birth weight infant formula (Happe and Gambelli, 2015), as the manufacturing of liquid infant formula (Figure 1.2) results in a commercially sterile product. The early steps, from recombination to homogenisation, are similar to production of powdered infant formula with a few differences as follows. The recombination can be achieved in a batch or continuous mixing system, often under vacuum, to avoid possible foaming that can occur and induce issues with subsequent steps. Homogenisation is achieved as it is for infant formula powder manufacture, and is normally accompanied by additional flash cooling. The resulting stabilised mixture is then standardised, sterilised, and undergoes intermediate aseptic storage, allowing more control of the flow as well as traceability before the final aseptic storing (Montagne et al., 2009).

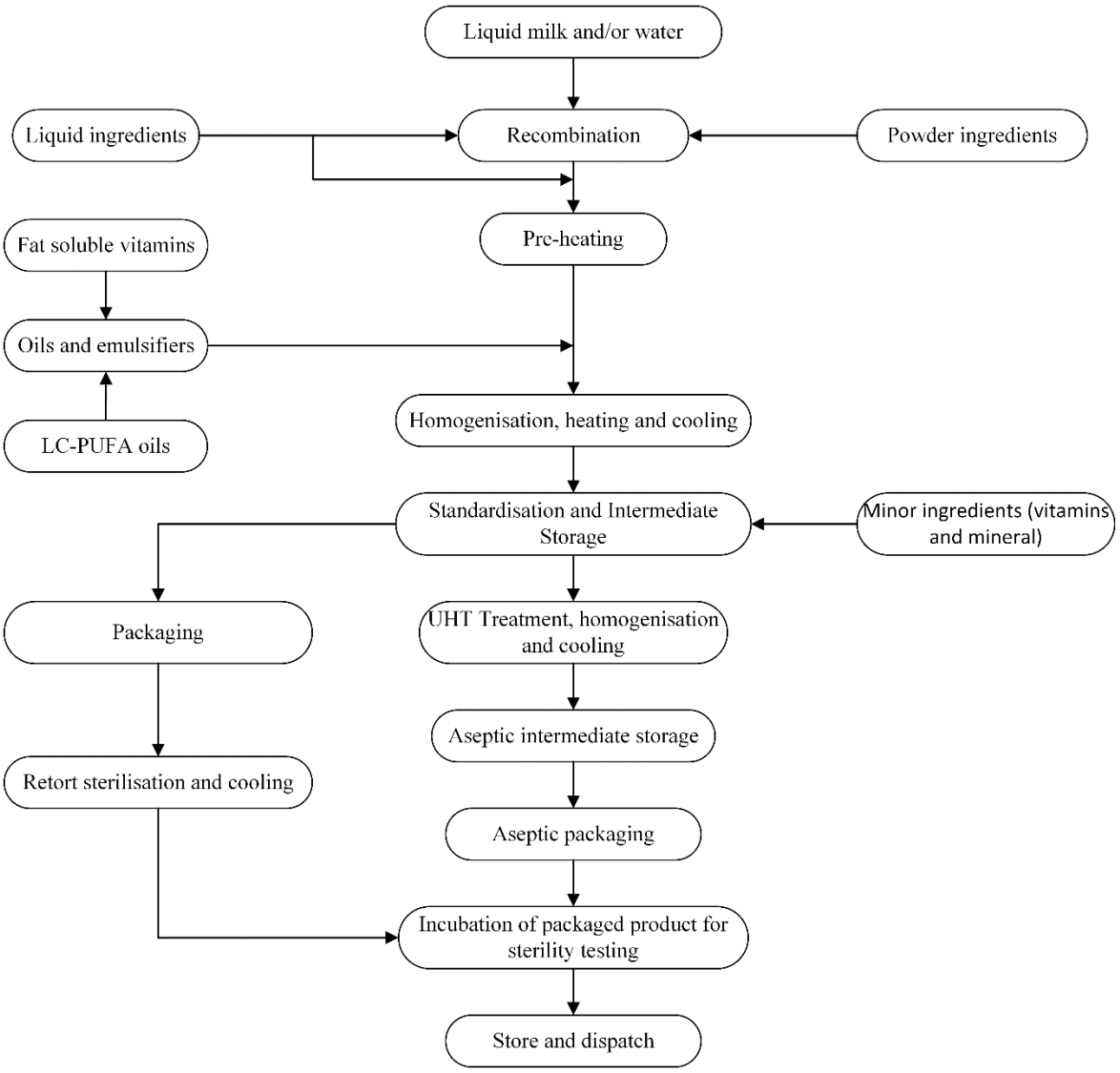


Figure 1.2. Schematic representation of the manufacture of liquid infant formulae (Montagne et al., 2009).

1.4.2. Innovation in processing technologies for infant formula

Several innovations in infant formula have come through to market in the areas of ingredient and formulation innovation, and it is true that in recent years, important innovations have been realised through the adoption of new processing technology and powder treatments. One important unit processing operation which is being applied in infant formula manufacture is microfiltration. More specifically, microfiltration has demonstrated ability to enhance the microbiological quality of raw materials for use in

infant formulae (e.g., reduction of microbiological spore counts in skim milk powder), and also for use in the chemical-free, sustainable fractionation of whey proteins from caseins in skim milk (McCarthy et al., 2017; France et al., 2021) for use in formulation of next-generation infant nutritional products. Following the treatment of raw materials, innovations have been investigated in the manufacture and processing of infant formulae to enhance the quality of the product but also to develop more sustainable processing practices. Indeed, the development of more energy efficient and sustainable processing practices has been under increasing investigation in the last decade. Furthermore, due to the relatively low heat stability of a number of bioactive and nutritional ingredients, interest in low heat load processes has increased. High-pressure processing (HPP), ultraviolet irradiation, ultrasound and microwave treatments are all promising technologies that present an alternative to conventional thermal treatment (O'Sullivan, 2015; Aceti et al., 2020; Harouna et al., 2020; Zhang et al., 2020). Other technologies such as pulsed electric field (PEF) processing and ohmic heating (OH) aim to ensure the microbial stability of the formulae with electrical energy to inactive pathogens and enzymes. The latter's have shown promising results as they allow a better stability of thermosensitive ingredients as well as better organoleptic properties due to their low-heat treatment (PEF) or rapid and uniform heating (OH) (Vanga et al., 2018; Pires et al., 2020). Reducing energy consumption and costs can also be achieved through process innovation to reduce the number of steps necessary for the production of infant formula; for example, adding certain lipids and fat-soluble heat-labile vitamins just before drying using in-line injection technology to reduce homogenisation costs while preserving the nutritional and functional properties of the ingredients (Murphy et al., 2013). In an effort to address some of these challenges, Murphy et al. (2011) developed a direct heat-

treatment shock-wave technology using high velocity steam injection, which allows for increased colloidal stability of high solids emulsions and lower whey protein denaturation (Murphy et al., 2013), suitable for spray drying while bypassing the evaporation step by recombination at high total solids.

Another approach is to investigate the inter-relationships between processing parameters and ingredients on the processing stability of formulae. As for example, Drapala et al. (2016), studied the effect of protein hydrolysate-maltodextrin conjugates as emulsifiers in model infant formula emulsions, showing that thermal and storage stability could be enhanced. Other studies have shown successes in addressing the high-level challenge of improving the processing stability and functional properties of infant formula through formulation such as calcium fortification, addition on metal ions polyunsaturated fatty acids, and vitamins, lactose pre-crystallization, or high solid formulation (France et al., 2020; Wang et al, 2020; Rodriguez Arzuaga et al., 2021; Saxena et al., 2021).

Other research areas are focused on improving the user experience with the end product through the investigation of new automated options for infant formula reconstitution. Such as the use of a collaborative robot (CoBoT) to better understand the human biomechanical movements used during the preparation of an infant's bottle (O'Shea et al., 2021) or by providing new physical product formats such as tablets, which combine some of the benefits of powder and liquid product format in a single physical format (Ndife et al., 2004). Another example of an interesting innovation was the development by Nestlé of the BabyNes® beverage dispensing apparatus which provide parents with an infant formulae preparation method with single-use capsules, allowing for a more convenient and time-effective preparation (Kutcher and Kutcher, 2016).

1.5.Plant proteins

The world faces a major challenge in food production and environmental sustainability over the next 30 years, coinciding with an expected growth of the global population to more than nine billion people by 2050 (Aiking, 2011; Day, 2013). Therefore, a significant increase in the demand for and consumption of food is anticipated. Being conscious of the environmental impact of such a demand (Heller et al., 2013), it is important to conduct research into new, innovative and more sustainable food products. It is widely recognised that diet choice is strongly related to environmental impact and, in this regard, plant-based diets are believed to have a lower environmental impact than animal-based diets (Springmann al., 2016). In addition to these considerations, allergies and intolerances to bovine milk (Novembre and Vierucci, 2001) are other factors that promote research activities to focus on plant-based products. In this effort to develop more sustainable food products through the investigation of plant proteins, it is important to highlight that the knowledge developed over the last few decades on dairy products and processing (as shown in previous sections) was used as the fundamental base for the development on plant based infant formulae.

The most common plant-based infant formulae on the market use soy proteins, as according to the FAO/WHO, only soy protein should be used as a plant protein source for infant formula. However, other plant-based infant formulae, such as rice, are available commercially (Bocquet et al., 2019) and prototypes have also been developed using other plant protein sources, such as lentil (Alonso-Miravalles et al., 2020). Despite the availability and use of soy protein-based infant nutritional products as alternatives for infants who cannot tolerate bovine milk due to conditions like cow's milk intolerance (CMI) or cow's milk allergy (CMA), as well as for other dietary

considerations, there are concerns that consuming soy-based products at an early age may affect hormonal development and potentially lead to certain disorders (Upson et al., 2019; Mvondo et al., 2019). Also, there is no evidence supporting the use of soy-protein formulae for the treatment of infantile colic, regurgitation or prolonged crying (Agostoni et al., 2006). In line with health and environmental controversies, recent studies have questioned the use of soy in Europe (Rekow, 2019; Rausch et al., 2019; Gollnow et al., 2018). Hence, these health and environmental considerations have led to innovation in infant formulae using new plant-based protein products classified into five sources (Norton et al., 1978; Alonso-Miravalles and O'Mahony, 2018):

- Cereals (e.g., barley, wheat, maize, rice, oats)
- Legumes (e.g., chickpea, soya bean, pea, lentils)
- Oil seeds (e.g., rapeseed, sunflower, cotton)
- Roots and tubers (e.g., potato, yam, cassava, sweet potato)
- Pseudocereals (e.g., amaranth, buckwheat, quinoa)

In addition to environmental and sustainability criteria, there are other important considerations when using vegetable-based proteins in infant formula. These proteins generally have lower digestibility compared to dairy, meat, and fish-based proteins due to their fibre content, presence of anti-nutritional factors, and different protein molecular structures (Moughan, 2017). Specifically, seed proteins have a lower overall nutritional quality than animal proteins, which can be attributed to their low levels of sulfur-containing amino acids, the compact proteolysis-resistant structure of native seed proteins, and the presence of antinutritional compounds. These factors can affect not only the digestibility of the proteins themselves but also that of other components (Duranti, 2006). Antinutritional factors can reduce the availability, digestibility, or absorption of essential nutrients, thus impacting the overall nutritional quality and

health outcomes of the diet (Francis et al., 2001). Antinutritional factors may occur naturally or may be formed during heat processing of protein products, yielding Maillard compounds, oxidized forms of sulphur-containing amino acids, D-amino acids, and lysinoalanine (an amino acid derivative) (Gilani et al., 2012). There are various antinutritional compounds that can impact the utilisation of proteins and minerals. For example, trypsin inhibitors and hemagglutinins found in legumes, tannins present in legumes and cereals, and phytates in cereals and oilseeds can all interfere with nutrient absorption (Alting and Van De Velde, 2012; Francis et al., 2001). Specifically, compounds like lectins, protease inhibitors, tannins, and phytates are known to hinder protein digestion and utilisation (Gilani et al., 2012).

The interest in plant protein is growing, however, and recent research has focused on new and emerging proteins for use in formulation of infant nutritional products. Potato protein has been investigated for its application in infant nutritional formulations. This hypo-allergenic protein has very good emulsifying properties and heat stability, particularly on modification of its properties (e.g., microparticulation) (Nestec, 2019). Other alternative protein sources that are being investigated are legumes (e.g., pea, lentil, fava beans and chickpea), oil seeds (e.g., sunflower and canola) and pseudocereals (e.g., quinoa, amaranth and buckwheat) that can provide nutritional and functional properties similar to soy. For example, lentil proteins have excellent techno-functional properties, such as emulsifying abilities, due to their amphiphilic characteristics. These properties enable lentil proteins to form a thick interfacial protein layer around oil droplets, enhancing emulsion stability and showing promising results for use in infant formulae (Can-Karaca et al., 2011, 2015). Given the importance of these functional properties in determining the suitability of plant proteins in infant formula, it becomes crucial to study and optimize them. Among the

most critical properties are solubility, emulsifying capacity, and thermal stability, which directly influence the product's texture, stability, and nutrient delivery. Understanding these functional characteristics is essential for tailoring plant-based ingredients to meet the specific demands of infant nutrition products.

1.5.1. Plant protein functionality

In the context of infant formula and emulsions, the functional properties of plant proteins are essential for product quality and stability. One of the most critical properties is protein solubility, which affects the ease of incorporation into the formula and the homogeneity of the final product. High solubility ensures that proteins are evenly distributed, contributing to the overall texture and nutritional profile (McClements & Grossmann, 2022). Emulsifying properties are another vital aspect, as they determine the protein's ability to stabilize fat droplets in the emulsion. This is crucial for infant formula, where a stable emulsion ensures uniform fat distribution, which is important for consistent nutrient delivery and proper digestion (Gumus et al., 2017). The thermal stability of plant proteins is also important as it influences their behaviour during processing, such as pasteurization or sterilization, which are necessary for ensuring the safety and shelf-life of the product. Proteins with good thermal stability maintain their functional properties and prevent phase separation or denaturation, preserving the intended texture and nutritional quality of the formula (McClements and Grossmann, 2022). Understanding and optimising these functional properties are key to developing high-quality plant-based infant nutrition products.

1.5.1.1. Protein solubility

Protein solubility is a fundamental property that significantly influences the functionality of proteins in various food applications. Solubility affects other functional properties such as emulsification, foaming, and gelation, which are critical

for the production of many food products (McClements & Grossmann, 2022). Generally, plant-based proteins have lower solubility than animal-based proteins, which could be attributed to multiple factors such as protein structure and physico-chemical properties, fibre content, and the presence of anti-nutritional factors (Sim et al., 2021; Zayas et al., 1997).

A widely used method for determining protein solubility involves dispersing the protein in a buffer solution, adjusting the pH, and then centrifuging to separate the soluble fraction from the insoluble residue. The protein concentration in the supernatant is then measured using methods such as Kjeldahl, Dumas, Bradford assay, or Lowry methods (Morr et al., 1985; McClements & Grossmann, 2022).

The solubility of plant proteins varies widely depending on the processing conditions used to isolate the proteins and the sources. For example, Karaca et al. (2011) compared isoelectric precipitation with salt extraction methods and showed that protein isolates (chickpea, faba bean, lentil and pea) obtained by isoelectric precipitation yield ingredients with better solubility than with salt extraction. Alonso-Miravalles et al. (2019) has also worked on ultrafiltration and isoelectric precipitation and showed that ultrafiltration allowed to obtain lentil protein isolates with higher solubility. The protein source also has a significant impact on the solubility of the protein ingredients. Proteins from soy, pea, lentil, and other legumes exhibit different solubility profiles, which can impact their use in food formulations (Grossmann and McClements, 2023). The solubility of these proteins is typically lowest near their isoelectric points, where electrostatic repulsion is minimized, leading to aggregation and precipitation. For most legume proteins including lentil, soy, pea, chickpea and faba beans, the isoelectric point lies between pH 4 and 5 (Ma et al., 2022). This study shows that the solubility of soy, chickpea, faba bean, pea, and lentil proteins is

relatively high in water (>80%) at pH 8, and moderate at pH 3 (40-60%), showing that solubility increases at pH levels further from the isoelectric point.

Furthermore, other environmental conditions such as temperature, and ionic strength play critical roles in influencing protein solubility. The effect of temperature on protein solubility is also significant; higher temperatures can increase solubility by enhancing molecular motion and the entropy of mixing, but excessive heating can lead to protein denaturation and aggregation, reducing solubility (Grossmann & McClements, 2022). The ionic strength of the solution also affects protein solubility. At lower ionic strengths, proteins can aggregate due to the lack of sufficient electrostatic repulsion. Conversely, at high ionic strengths, solubility can increase due to the shielding effect of ions that reduce protein-protein interactions (Grossmann & McClements, 2022).

The solubility of plant proteins is not just a fundamental property on its own but also significantly influences other functional attributes such as emulsification, thermal stability, and overall physical stability of the emulsions. Higher solubility often leads to better performance in these areas, which is critical for their application in various food products. Protein solubility plays a crucial role in emulsification as soluble proteins can adsorb more rapidly to the oil-water interface during emulsification, reducing interfacial tension and forming a more cohesive and stable barrier around each oil droplet. This barrier helps prevent aggregation and direct contact of oil droplets by maintaining electrostatic repulsion between them, thereby inhibiting flocculation and coalescence (Phillips & Williams, 2011; Maphosa and Jideani, 2018).

1.5.1.2. Emulsifying properties

Emulsifying properties are a critical functional attribute of proteins, influencing their ability to stabilize emulsions. These properties are essential for many food products,

including infant formulae, where stable emulsions are required to ensure the required consistency, texture, and nutritional delivery. The emulsifying properties of proteins are influenced by several factors, including the size, shape, flexibility, charge, hydrophobicity, and aggregation state of the protein molecules (Gumus et al., 2017).

To quantify the emulsifying properties of proteins, various methods are used. Two traditional approaches are the emulsion activity (EA), the emulsion activity index (EAI) and the emulsion stability index (ESI) (Ma et al., 2022). The EA method involves dispersing a protein in a buffer solution, blending it with oil using a high-shear mixer, and then measuring the volume of the emulsified layer after centrifugation (Yasumatsu et al., 1971). The EAI method also involves creating an oil-in-water emulsion, but it measures the turbidity of the emulsion to calculate the emulsifying activity index, which is based on the relationship between emulsion turbidity and droplet size (Pearce and Kinsella., 1978). The Emulsion Stability Index (ESI) assesses an emulsion's capacity to maintain its structure over a specified period. This is determined by observing the reduction in turbidity of a diluted emulsion over a short-term storage period (Kiosseoglou & Paraskevopoulou, 2011).

The emulsifying properties of plant proteins are affected by their solubility as discussed previously, but also by their hydrophobicity and structural constraint (molecular flexibility, molecular weight and length). All these parameters will dictate the kinetics of adsorption and unfolding of the protein at the oil-water interphase and therefore interfere in the stabilisation of the formed emulsion (Amagliani and Schmitt, 2017).

Ma et al. (2022) reviewed multiple studies in the literature and found that the emulsifying capacity of plant proteins varies significantly depending on their origin

and concentration. Their analysis, highlighted that pea protein isolates exhibited emulsifying activities ranging from 21% to 76%, demonstrating significant variability within the same protein source as a result of variable extracting methods. Soybean protein isolate, consistently showed high emulsifying activity, making it particularly suitable for applications where effective emulsification is crucial. More specifically to lentil protein, research reveals significant variability due to differing testing conditions and protein extraction methods, making comparisons challenging. Overall, lentil protein exhibits emulsifying properties comparable to other pulses, with values ranging from 5 to 90 m^2/g (Boye et al., 2010; Can Karaca et al., 2011; Joshi et al., 2012).

Furthermore, the emulsifying properties of plant protein are also highly dependant of the extraction and isolation techniques used, as shown by Alonso-Miravalles et al (2019) who compared ultrafiltration and isoelectric precipitation processing for the isolation of lentil protein isolates. The authors found that the emulsifying stability was significantly high from emulsions formulated using lentil protein obtained by ultrafiltration (63.8 min) as compared to isoelectric precipitation (51.0 min).

Various techniques can enhance the emulsifying properties of plant proteins. High-pressure homogenisation (HPH) and ultrasonication are effective methods for improving protein solubility and functionality, leading to better emulsion stability. HPH can reduce the particle size of protein aggregates, increasing their surface area and ability to adsorb to the oil-water interface (Sim et al., 2021; Mirmoghtadaie et al., 2016). Combining different processing techniques, such as HPH with heat treatment or ultrasonication, can further enhance the emulsifying properties of plant proteins. These combined methods can lead to smaller droplet sizes, increased stability against coalescence, and improved overall emulsion quality (Zhao et al., 2022). Further

information regarding the improvement of plant protein functionalities and emulsifying properties are available in section 1.5.2.

1.5.1.3. Thermal stability

Thermal stability is a crucial property of proteins, particularly for applications in food products that require heating during processing or cooking. The thermal stability of a protein refers to its ability to maintain its structure and functional properties when exposed to elevated temperatures. This property is vital for ensuring that proteins can withstand food processing conditions without undergoing denaturation or losing their functionality. The thermal denaturation of plant proteins is commonly assessed using techniques such as differential scanning calorimetry (DSC) (Durowoju et al., 2017), thermogravimetric analysis (TGA), and circular dichroism (CD) spectroscopy. DSC measures the heat flow associated with protein denaturation, providing insights into the temperature at which the protein denatures (denaturation temperature, Tm) and the enthalpy change (ΔH) during the process. TGA evaluates the thermal degradation profile of proteins by measuring the weight loss as a function of temperature (Ricci et al., 2018). CD spectroscopy assesses changes in the secondary structure of proteins upon heating, offering information about the unfolding process (Greenfield, 2006). The thermal stability of plant proteins directly impacts their functionality in various food applications. Proteins that maintain their structure and functionality at high temperatures are desirable for products such as baked goods, meat analogues, and dairy alternatives, where heating is involved in the processing (McClements and Grossmann, 2022). For instance, proteins with high thermal stability can help retain the texture, appearance, and nutritional value of plant-based meat products during cooking (Ma et al., 2020). Additionally, thermally stable proteins are essential for

infant formulae, ensuring that the nutritional quality and safety of the product are maintained throughout processing and storage (Alonso-Miravalles et al., 2020).

Mession et al. (2013) observed thermal denaturation of pea protein at 82.4°C associated to the denaturation of 7 S vicilin and found that the main interaction governing the protein denaturation was noncovalent interactions. Similarly, Rui et al (2011) observed a denaturation temperature in the range 85 to 90°C which was associated to the thermal denaturation of 7 S vicilin, working on nine varieties of Phaseolus vulgaris bean protein isolates. Furthermore, in the case of lentil protein, Lefèvre et al. (2022) observed a denaturation temperature in the range 80-90°C. Going deeper in the chemistry of pulses thermal denaturation, vicilin features a trimeric structure that is stabilised through hydrophobic interactions, as well as electrostatic and hydrogen bonds. In contrast, legumin possesses a quaternary structure composed of three dimers, which are held together by non-covalent bonds. Within each dimer, acidic and basic subunits are connected via disulfide bridges. These covalent bonds provide legumin with greater thermal stability compared to vicilin, as breaking these bonds requires more energy (Sirtori et al., 2012; Carbonaro et al., 2015; Lefèvre et al., 2022).

Protein ingredients are often found containing different protein content and concentration of other components. On top of the source choice, the purity of the ingredient used for the formulation of food products will also have a significant effect on the thermal stability. In a study conducted by Ricci et al. (2018), the thermal behaviour of proteins isolated from different legumes, including pea, lentil, faba bean, chickpea, and bean, was investigated using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The study found that the thermal stability of these proteins varies significantly depending on their source and purity. The range of

plant proteins exhibited similar denaturation temperature for all purities analysed but a wide range of degradation temperatures depending on their purity. At lower purity levels, the presence of polysaccharide co-products reduced their thermal stability, making them less suitable for high-temperature applications.

1.5.1.4. Foaming properties

In the formulation of infant formulae, it is essential to consider a range of functional properties beyond solubility, emulsification, and thermal stability. These additional properties include foaming and gelation which significantly impact the overall quality of the final product. Understanding and optimising these properties can enhance the performance and acceptance of plant-based infant formulae.

Foams are systems composed of gas bubbles dispersed within a continuous liquid phase, crucial for the texture of many food products such as ice cream, cakes, and mousses, but can also be undesirable in the manufacture of other food products (Ma et al., 2022). Gas bubbles in these foams are thermodynamically unstable, requiring the use of surface-active agents like proteins to stabilize the gas bubbles (Moll et al., 2022; Schmitt et al., 2018). Foaming properties are generally evaluated using two key parameters: foam capacity and foam stability. Foam capacity measures the volume of foam generated from a protein solution, whereas foam stability assesses how well the foam resists collapse over time. These properties are typically measured by aerating a protein solution and then quantifying the volume of foam produced (foam capacity) and observing the foam's durability over time (foam stability) (Schmitt et al., 2018). The foaming properties of various plant proteins vary significantly, influenced by their molecular characteristics and environmental conditions. For example, lentil protein (protein content: 87-95%) exhibit a foaming capacity of 34.8% and a foaming stability of 96.7%, making them highly effective in forming and maintaining foams. Similarly,

pea protein (protein content: 84.9%) have demonstrated a foaming capacity of 15%, and a foaming stability of 94%. The highest foaming stability was observed in soy (protein content: 82.2%) with a foam stability of 93%. Even though those foaming capacities were low, these proteins demonstrated high foaming stability. Conversely, other plant proteins like faba bean and mung bean at respectively 81.2 and 81.5% protein, exhibit lower foaming stabilities of 77 and 78.3% respectively (Ma et al., 2022). However, it is important to note that the results for foaming capacities are highly impacted by the blending methods used for the formation of the foams.

1.5.2. Physical treatments for enhancement of plant proteins functionalities

Physical treatments refer to non-chemical, mechanical, or thermal processes applied to plant materials to modify the structure and techno-functional properties of plant proteins (Mirmoghtadaie et al., 2016). These treatments can significantly enhance the solubility, emulsifying properties, foaming, and other functional characteristics of plant proteins, making them more suitable for various food applications, including infant formulae (Nikbakht Nasrabadi et al., 2021). The primary objective of applying physical treatments to plant proteins is to overcome the inherent limitations such as low solubility, and poor emulsifying properties. By modifying the ingredient structure and subsequent interactions, these treatments can lead to improved functional properties, thereby broadening the application scope of plant proteins in the food industry.

One of the main advantages of physical treatments is that they do not involve chemical additives, which aligns with the clean label trends in food manufacturing. These methods can also be more easily scaled-up for industrial applications, making them highly relevant for large-scale food production. Additionally, physical treatments can

be combined to achieve synergistic effects and further enhance the functional properties of plant proteins (Sá et al., 2022).

In exploring the physical treatment methods for enhancing plant protein functionality, several techniques such as ultrasonication, ohmic heating, microwaves, and extrusion have been investigated in the literature. However, this review specifically focuses on high-pressure homogenisation (HPH) and high shear mixing and microfluidisation. These methods were chosen due to their scalability, widespread use in the food processing industry, and the promising results they have demonstrated in improving plant protein characteristics. By concentrating on these techniques, we aim to provide an understanding of their potential in industrial applications and their advantages over other available methods.

1.5.2.1. High-pressure homogenisation

High-pressure homogenisation (HPH) is a mechanical method commonly used for the formation of stable emulsions. It has been used to enhance the functional properties of various food ingredients, including plant proteins. This technique involves pushing a liquid through a narrow gap under high pressure, which induces the disruption of the processed sample (Levy et al., 2021).

In an HPH system, a high-pressure pump drives the fluid through a homogenizing valve at pressures between 50 and 300 MPa. As the fluid navigates the narrow gap of the valve, its velocity rapidly increases while its pressure simultaneously drops, causing cavitation. Cavitation entails the formation and subsequent collapse of vapor bubbles, producing intense localised forces that can disrupt particle structures (Dumay et al., 2013; Tobin et al., 2015). The primary mechanisms for particle disruption in HPH are shear forces, cavitation, and turbulent forces. Together, these mechanisms

reduce particle size and ensure uniform particle distribution, which can lead to better functional properties. The effectiveness of HPH is influenced by several factors, including the homogenisation pressure, the design of the homogenising valve, and the physical properties of the material being processed (Tobin et al., 2015; Levy et al., 2021; D'Alessio et al., 2023).

Through these mechanisms, HPH can enhance the solubility of plant proteins by breaking down protein aggregates and exposing more hydrophilic sites, which enhances their interaction with water (Yang et al., 2018). This is particularly beneficial for proteins that are naturally less soluble, such as those from legumes and grains. Additionally, this increase in solubility can lead to faster adsorption of the protein at the oil/water interface in the case of emulsification. Faster adsorption will then result in a thicker and stronger layer of protein around the oil droplets, preventing coalescence and flocculation, leading to more stable emulsions (Navare et al., 2023).

Keuleyan et al. (2023) studied effects of high-pressure homogenisation (HPH) on the dispersibility and functional properties of commercial pea and lupin protein isolates. The application of HPH at 30 MPa decreased the particle size from 67 to 20 µm for pea protein isolates and from 13 to 1.1 µm for lupin protein isolate dispersions. This reduction in particle size allowed for an improvement of protein solubility, with pea protein isolate solubility increasing from 14.9% to 52.4%, and lupin protein isolate solubility increasing from 49 to 60%. Further on pea protein, Melchior et al (2022) used HPH at 70 MPa, reporting an increase in solubility from 22.3 to 32% along with an increase of oil holding capacity from 149.3 to 301.2%. Moll et al (2022) also observed similar results on pea proteins with a reduction in particle size from 87.2 to 24.3 µm using HPH at 50 MPa and an improvement in solubility from 19.8 to 83.8%. These improvements reported by all authors on pea proteins were associated with the

particle breakage induced by HPH which allowed for better dispersibility and protein rehydration which then led to higher solubility and stronger interfacial properties. On soybean protein, similar results were reported by Liang et al (2022) who showed that HPH at 60 MPa decreased the particle size from 4.5 to 2.5 µm, increased the solubility from 74 to 80.5%, water and oil holding capacities from 5.0 to 7.7 g/g and from 4.6 to 6.5 g/g, respectively. Emulsifying activity and emulsifying stability were also improved from 32.0 to 43.5 m2/g and from 160 to 190.4 min. Song et al (2013) also observed a reduction in particle size from 3.3 to 0.1 µm with treatment at 207 MPa yielding increased mechanical, transparent and water absorption properties of the subsequently formed soy protein isolate film. Furthermore, in the case of lentil protein, Saricaoglu (2020) used a pressure range of 0-150 MPa to assess the solubility, emulsifying and foaming properties of lentil protein isolate (LPI) dispersions (4% protein w/v). Their findings indicated that HPH treatment effectively reduced the particle size from 98.7 µm to 12.6 µm, with the smallest particle size observed at 150 MPa. Additionally, HPH treatment enhanced the solubility of LPI from 32% to 46%, with the highest solubility recorded at 75 MPa, and increased the emulsifying activity index and emulsifying stability index from 100.2 to 125.2 m^2/g and from 39.1 to 226.3 min respectively, at 100 MPa. Moreover, the foaming capacity also increased from 10 to 85.3% along with an increase in foam stability from 4.3 to 66.6%.

It is also important, however, to note that overly processing at higher pressures (>100 MPa) led to an increase in particle size as well as lower solubility. This was attributed by several authors to opening of the protein and overexposure of hydrophobics groups which led to protein aggregation and decrease in solubility along with decrease in techno-functional properties. (Liang et al., 2022; Melchior et al.; 2022; Saricaoglu et al., 2020).

1.5.2.2. Microfluidisation

Microfluidisation is a high-pressure homogenisation technique widely employed for creating emulsions with extremely narrow particle size distributions. This process involves forcing a fluid through a narrow gap or channels at high pressures, typically ranging from 20 to 300 MPa. The technology uses a double-acting intensifier pump and an interaction chamber designed to split the fluid into two streams. These streams then collide at high velocities within the chamber, inducing intense turbulence, shear forces and cavitation, causing particle disruption. The interaction chamber's design, including the narrowest cross-section, facilitates the precise control of particle disruption and ensures a uniform particle size distribution (Huppertz, 2022).

Microfluidisers are particularly valued in the food, cosmetic, and pharmaceutical industries for their ability to produce fine, stable emulsions with narrow particle size distributions. Unlike traditional homogenisers, microfluidisers do not contain moving parts within the interaction chamber, reducing maintenance needs and increasing operational efficiency. Although this technology is commonly used for the formulation of stable emulsions in the food industry with small and narrow particle size distribution, it has gained interest for the improvement of plant protein functional properties through the disruption of powder particles. Moll et al. (2021) studied the microfluidisation processing of pea protein in the range 25-150 MPa for 1 to 15 cycles, resulting in a decrease in particle size from 180.0 to 0.2 μm (at≥ 125 MPa) which reduced the turbidity of the dispersion but also increased the protein solubility from 23 to 86%. The authors attributed these changes to the disruption of disulphide bonds but also disruption of hydrogen bonds and hydrophobic interaction caused by the high-pressure of microfluidisation. Similarly, Hu et al. (2011) used microfluidisation at pressure from 30 to 120 MPa to enhance the techno-functionalities of pea protein.

Protein solubility was increased by 3.78 folds at 120 MPa from 16.99 to 64.28%, along with an increased surface hydrophobicity and free sulfhydryl groups content indicating protein unfolding cause by the high-pressure processing. Shen and Tang (2012) investigated the effect of microfluidisation at 120 MPa on the functional properties of soy protein isolate, which led to an increase in protein solubility (63% to 80%), surface hydrophobicity (2000 to 2300), free sulfhydryl groups (6.13 to 5.19) and emulsifying ability index (110 to 135 m2/g). Furthermore, the microfluidisation treatment also improved the stability against creaming of subsequent emulsions formulated using improved soy protein. The significant increases in solubility, surface hydrophobicity, and emulsifying ability, along with the reduction in particle size, shown in these studies highlight the potential of microfluidisation to enhance the performance of plant proteins in various applications. These findings underscore the versatility and efficiency of microfluidisation as a processing method, making it a valuable tool for the development of plant-based food products with superior functional properties. As the demand for plant-based ingredients continues to grow, microfluidization stands out as a promising technique for optimizing the quality and functionality of plant protein ingredients.

1.5.2.3. High-shear rotor-stator mixing

High-shear rotor–stator mixers are highly efficient devices used for intensive mixing applications due to their ability to deliver energy and shear forces within a small fluid volume. These mixers are cost-effective, versatile, and efficient in terms of energy usage. They feature a high-speed rotor encased within a stator, with gaps ranging from 100 to 3000 μm, typically operating at speeds between 10 and 50 m/s resulting in very high shear rates. Although they require a considerable amount of power input for a brief period, the energy is uniformly delivered and dissipated within a small volume,

ensuring consistent processing intensity across the fluid. This uniform energy distribution often makes rotor–stator mixers more effective than other mixing devices, especially in applications where product quality depends heavily on structure and ingredient distribution (Pacek et al., 2013). Rotor-stators are often used to blend immiscible liquids to create a coarse emulsion prior to homogenisation, or in some applications, they can be used as the main emulsifying device (Ashar et al., 2018). Another usage of rotor stators is the blending of miscible products often used in industrial context to mix several products but can also be used for the deagglomeration and dispersion of powders and, more specifically, protein powders (Baldyga et al., 2008a; Ding et al., 2009).

Baldyga et al. (2008b) studied the dispersion of nanoparticle clusters in a rotor-stator and found that high shear mixing at 9000rpm for 180 min allowed for a reduction in particle size of clusters from a monomodal distribution of 50 μm to a bimodal distribution with a peak at 9μm and a peak at 0.5 μm. Pacek et al. (2007) found that using an in-line high-shear mixer at 8000 rpm reduced silicon particles from 90 μm to 9 μm after 20 minutes and from 100 μm to 15 μm after 30 minutes of shearing. However, they noted that within shorter durations (<15 minutes), the high-shear mixing did not break down aggregates below 1–2 μm. Furthermore, going towards applications in food systems, O'Sullivan et al. (2017) studied the effects of in-line high-shear mixing on dairy powders, specifically skim milk powder and milk protein isolate, at a concentration of 7.2% (w/w). They observed a significant reduction in particle size from 100 μm to 20 μm after 15 minutes of mixing, with smaller particles stabilising in the range of 0.1–1 μm. On plant-based proteins, Kong et al. (2019) observed a continuous reduction in particle size as well as an increase in solubility

from 500 to 100 μm and from 5 to 20% (pH 7), respectively when using a high-shear mixer at 15,000 rpm for 10 min for the treatment of walnut protein.

These results are promising for the use of high-shear mixers in the processing of food products. Although direct studies on plant proteins are limited, the successful application of high-shear mixing in reducing particle size and improving the dispersion of dairy and nanoparticle systems suggests that similar benefits could be achieved with plant proteins. By reducing particle size and breaking down aggregates, high-shear mixers could enhance the solubility and functional properties of plant proteins, making them more effective as emulsifiers and stabilisers in various food applications. Future research should focus on exploring these potential benefits in plant protein systems to confirm these promising indications. As the demand for plant-based ingredients continues to grow, high-shear mixing stands out as a promising technique for optimising the quality and functionality of plant protein ingredients.

1.6. Conclusion

The development of infant nutritional products has traditionally been centred on mimicking the composition of human milk as closely as possible. Over time, this focus has expanded to not only replicate the nutritional profile but also to achieve the health outcomes associated with breastfeeding. This evolution in infant formula design is underpinned by our deepening understanding of the composition and functional properties of both human and bovine milk, leading to more refined dairy-based formulations. As sustainability and dietary diversity become increasingly important, the shift towards plant-based infant formulae has gained momentum. These alternatives, however, present unique challenges, particularly in terms of matching the functional properties essential for effective formulation, such as solubility, emulsification, and thermal stability.

This literature review highlights the critical role of these functional properties in determining the quality and efficacy of the final product, whether dairy- or plant-based. Techniques to enhance these properties, including high-pressure homogenisation, microfluidisation, and other innovative processing methods, are pivotal in overcoming the inherent limitations of plant proteins. Additionally, the emergence of new product formats, such as tablets and other convenient delivery forms, signifies the ongoing innovation in the field, driven by both consumer demands and scientific advancements.

Looking forward, the integration of ingredient innovations, advanced processing technologies, and an increased focus on sustainability will continue to shape the future of infant nutritional products. This comprehensive approach, bridging traditional dairy-based formulations with cutting-edge plant-based alternatives, ensures that the industry will continue to provide nutritionally adequate and safe products that meet the diverse needs of infants around the globe.

1.7.References

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Chapter 2

Formulation and physical stability of high total solids lentil protein-stabilised emulsions for use in plant protein-based young child formulae

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Abstract

The demand for high-quality plant protein products is increasing and the aim of this work was to evaluate the impact of increasing the total solids content on the formation and stability of lentil protein stabilised oil-in-water emulsions. A series of emulsions were formulated using different proportions of total solids: 23, 26, 29, 32, and 35% (w/v). The emulsions were formulated using three ingredients—lentil protein, sunflower oil, and maltodextrin—which made up 15.85, 27.43, and 56.72% (w/w) of the total solids, respectively. The changes in apparent viscosity, particle size distribution, and colour during thermal processing were evaluated, with the physical stability investigated using an analytical centrifuge. The apparent viscosity of the solutions in-creased with total solids content (25.6 to 130 mPa·s), as did redness colour intensity (a* value increased from 5.82 ± 0.12 to 7.70 ± 0.09). Thermal processing resulted in greater destabilisation for higher total solids samples, as evidenced by greater changes in particle size, along with decreased redness colour. These results bring a better understanding of high total solids plant protein emulsions and factors affecting their stability, which could be used for the development of cost-effective and sustainable processing solutions in the production of plant protein young child formulae.

2.1.Introduction

The European plant-based food market has experienced a significant sales growth of 49% between 2018 and 2020 ((Smart Protein Project, European Union's Horizon 2020 research and innovation programme (No 862957, 2021)). Indeed, diets have evolved throughout the years with increasing awareness of the environmental impact of the food system (Springmann et al., 2016) as well as allergies and intolerances associated with animal-based diets (Høst, 1994; Novembre and Vierucci, 2001). Collectively, these considerations have stimulated research activities into plant proteins. Even though those proteins also carry some limitations such as allergens, multiple studies have been performed in recent years in response to these needs of consumers, such as Vogelsang-O'Dwyer et al. (2021) who investigated the suitability of pulse proteins in the production of high nutritional value plant-based milk alternatives, or Alonso-Miravalles et al. (2022), who investigated the suitability of lentil protein for the processing and preparation of an infant nutritional product. Concerning infant nutritional products specifically, the protein ingredients used must meet specific requirements such as a high protein concentration to meet the growth and maintenance requirements of the infant, as well as low fibre and antinutritional compound concentrations to not impair the digestibility and overall nutritional quality of the formulated product. To meet these requirements, a number of different plant protein sources have been studied in recent years, with the most widely commercially available being soy-based formulae (FAO and WHO, 2007) or rice-based formulae for specific medical requirements (Bocquet et al., 2019).

Although there is a lack of characterisation in the scientific literature and very limited commercial availability of alternative proteins, some protein sources, such as pulses, represent a good alternative to animal-based proteins for infant nutritional products as

they contain a relatively high concentration of protein (20–30%) and amphiphilic protein with good ability to form thick interfacial layers around oil droplets, conferring good emulsification properties on such proteins (Can Karaca et al., 2015, 2011; Jarzębski et al., 2019; Joshi et al., 2012). More specifically, lentil proteins have shown good emulsifying properties as well as good heat and pH stability (Funke et al., 2022; Gumus et al., 2017). On the other hand, the presence of antinutritional factors (e.g., trypsin inhibitors, condensed tannins, and phytic acid) has made the use of lentil protein for infant formula manufacture challenging (Joshi et al., 2017). Therefore, recent studies have investigated sustainable membrane filtration and isoelectric precipitation approaches to produce lentil protein isolate with high protein content (>90%) and low levels of antinutritional compound (Alonso-Miravalles et al., 2019). Other studies have also recently focused on the formulation and functionalities of lentil protein matrices; (Jeske et al., 2019)studied the suitability of lentil protein to produce milk substitutes with high protein content (3.3% w/w), whereas Alonso-Miravalles et al. (2022) studied the suitability of lentil–quinoa protein blends for the formulation of stable oil-in-water emulsions, showing that mixtures of plant proteins including lentils have a positive effect on the physical stability and nutritional profile of the resultant emulsions.

Although the formulation of lentil protein-stabilised emulsions is being studied and the interest is increasing, studies investigating technological and processing innovations to enable the use of lentil protein in the formulation of sustainable, nutrient dense, colloidally stable plant protein-based infant formulas are extremely limited. Indeed, re-search has been conducted to improve the processing of dairy infant formulae, including the work of Vanga (2018) and Pires et al. (2020), which investigated new heating technology to improve the thermal stability of heat-labile

ingredients. Other innovations have focused on reducing the energy consumption of infant formula manufacture processes, including the work of Ramirez et al. (2006) that showed that almost 90% of the energy consumption is the result of the energy intensive water removal to obtain a powder. Additionally, it has been shown that an increase of 2% total solids in the feed concentrate prior to spray-drying could lead to a reduction of 6% of energy consumption (Patil et al., 2021). Therefore, some researchers have investigated technologies to increase the total solid concentration of the feed and/or improve the evaporation step. Chamberland et al. (2020) investigated the suitability of pre-concentration technologies to decrease the amount of drying necessary such as pre-concentration using membrane technology involving the separation of the water from the product. Others have investigated novel technology to recombine the ingredients at higher solids and reduce the necessity for water removal downstream, such as Murphy et al. (2011), who developed a high-solids steam injection technology to increase the colloidal stability (reducing protein denaturation) of high-solids emulsions, allowing for spray-drying while by-passing the evaporation step, which significantly reduced the cost of infant formula production. These studies have shown that reducing the water removal pre-drying through different technologies considerably reduced energy consumption and improved the energy efficiency of the existing processing lines. However, these studies have been mainly performed on dairy and animal-based protein systems. To the authors knowledge, in the literature, no information is available on plant-based emulsions at high levels of total solids (25–35% total solids). Indeed, in the area of plant proteins and more particularly lentil protein, most of the research is focused on diluted systems (Alonso-Miravalles et al., 2019; Alonso-Miravalles et al., 2022; Funke et al., 2022; Jeske et al., 2019) that do not

allow prediction of the performance of the protein ingredients at high levels of total solids.

The aim of this study was to investigate the effect of increasing the total solids content (in the range 23 to 35%) on the thermal and physical stability of lentil-protein stabilised oil-in-water emulsions. This is necessary to predict the quality and stability of high total solids content emulsions during evaporation and spray-drying, underpinning more energy efficient conversion of concentrated emulsions into young child formula powders with tailored functional properties.

2.2.Materials and methods

2.2.1. Materials

Red lentil protein isolates with a protein content of 79.63% (w/w) were acquired from the Fraunhofer Institute (Munich, Germany). Sunflower oil was obtained from a local commercial outlet (Tesco, Welwyn Garden City, Hertfordshire, UK), whereas maltodextrin with a dextrose equivalent value of 17 (Glucidex® 17) was acquired from Roquette Frères (Lestrem, France). All reagents used in the study were of analytical grade and sourced from Sigma-Aldrich (St Louis, MO, USA) unless explicitly specified.

2.2.2. Preparation of oil-in water emulsions

Model young child formula emulsions containing 15.85, 27.43, and 56.72% red lentil protein, sunflower oil, and maltodextrin, respectively, on a total solid basis, were prepared as follows. The red lentil protein was dispersed in pre-heated deionised water (70 °C) and stirred using magnetic stirring at 300 rpm for 1 h at 22 °C, followed by the addition of maltodextrin and stirring magnetically for an additional 2 h at 300 rpm and 22 °C. The solution was adjusted to pH 6.8 and allowed to rehydrate fully at 4 °C

for 18 h, after which the temperature of the aqueous solution was adjusted to 22 °C and the pH was adjusted to 6.8 before adding sunflower oil. The mixture was heated to 50 °C in a water bath and an Ultra-Turrax (T25 Ultra-Turrax, Staufen, Germany) was used (12000 rpm for 3 min) to obtain a coarse emulsion. The coarse emulsion was then passed through a two-stage valve homogeniser (APV 1000, SPX flow, North Carolina, United States) in two passes at 18 MPa total pressure, with first and second stage pressures of 15 and 3 MPa, respectively. The homogenised emulsions were cooled to room temperature and analysed on the same day. In all formulations, the lentil protein, sunflower oil, and maltodextrin ratio was maintained constant, whereas five different total solids (TS) contents of 23, 26, 29, 32, and 35% TS were prepared as described above.

2.2.3. Colour of lentil-protein-stabilised emulsions

The colour of the emulsions before and after heating at 90 °C for 120 s (see Section 2.2.4) was assessed using a Chroma Meter CR-400 (Konica Minolta Sensing, Inc, Osaka, Japan) equipped with a liquid cell, using the CIELAB coordinates L*, a*, and b*, corresponding respectively to the brightness (ranging from 0, black, to 100, white), green to red (negative values being green, positive values being red), and blue to yellow (negative values being blue, positive values being yellow). Before the analysis, a calibrated white tile was used for calibration.

2.2.4. Thermal stability and heat-induced changes in viscosity

An AR-G2 controlled-stress rheometer (TA Instruments Ltd., Waters LLC, Leatherhead, Surrey, UK) equipped with a starch pasting cell geometry was used to determine the changes in viscosity during the heat treatment of emulsions with increasing TS. The rheometer cell had an internal diameter of 36.0 mm, whereas the rotor diameter was 32.4 mm and the gap between the two elements at the base was 5.5

mm. The sample (28 g) was weighed into the cell before being successively conditioned and held at 30 ℃ for 120 and 300 s, respectively, after which the temperature was raised to 90 ℃ at a rate of 10 ℃/min and held at 90 ℃ for 120 s. The temperature was then decreased to 30 ℃ at a rate of 10 ℃/min and held at 30 ℃ for 300 s. Throughout the analysis, a fixed shear rate of 10 rad/s was maintained, and viscosity was measured continuously.

2.2.5. Particle size distribution

The particle size distribution (PSD) of the emulsions was measured before and after heat treatment at 90 ℃ for 120 s using a Mastersizer 3000 static light scattering instrument equipped with an automated Hydro MV wet cell disperser (Malvern Instruments, Worcestershire, UK). The refractive index of protein was set at 1.45, the absorption refractive index used was 0.001, and the dispersant refractive index was 1.33. The emulsions were measured at 20 ℃ and dispersed into ultrapure water until a laser obscuration of 10% was reached. Data were presented as volume-distribution-based particle size Dv(50) (particle size below which 50% of sample volume is found) and peak range (width of the particle size distribution for each peak). Each emulsion was sampled 3 times and measured 3 times by the instrument for a total of 9 measurements.

2.2.6. Confocal laser scanning microscopy

Microstructural analysis of the emulsions was performed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan). The emulsions before and after heat treatment at 90 ℃ for 120 s were prepared as described by (Alonso-Miravalles et al., 2020) with the following modifications: 1 mL of the emulsion was mixed in 4 mL of low-gelling-temperature agarose (Sigma-Aldrich, St. Louis, MO, United States) solution (1.5%, w/v) at 37 ℃ to fix the oil

globules and allow for higher-quality imaging. As described by (Grasso et al., 2021), a mixture of the two stains, 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 added to 1 mL of the emulsion–agarose solution, which was then incubated at 20 °C until gelation. Fast Green FCF and Nile Red were excited at 633 and 488 nm, respectively (Auty et al., 2001), and representative images, performed using a 40× objective lens, were reported where fat and protein were stained green and red, respectively.

2.2.7. Accelerated physical stability analysis

The stability of the emulsions was measured using an analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). The samples where centrifuged at 3 successive speeds: 117 g for 60 min, 1057 g for 60 min, and 1878 g for 16.7 min (Jeske et al., 2017). Near-infrared light illuminated the samples throughout the analysis and the intensity of the transmitted light over the entire length of the sample was measured every 10 s. The separation rate was determined by plotting the integral of the intensity of the transmitted light over time and extracting the slope (%/h). Additionally, the thickness (i.e., height) of the cream layer (mm) as well as the thickness (i.e., height) of the sediment layer (mm) were measured on the last profile for each sample.s.

2.2.8. Statistical data analysis

All samples were formulated in independent triplicate trials and all analyses were conducted in triplicate, unless otherwise stated. The data generated was subject to one-way analysis of variance (ANOVA) using SPSS (IBM SPSS statistics for Windows, version 28, IBMCorp, Armonk, NY, USA). To determine whether there were statistically significant differences ($p < 0.05$) between the mean values of different samples at a 95% confidence level, a Tukey's paired comparison test was used.

2.3.Results and discussion

2.3.1. Colour of lentil protein-stabilised emulsions

The colour parameters of the lentil protein-stabilised emulsions at 23% TS were 78.37, 5.82, and 10.85 for L*, a*, and b*, respectively (Table 2.1). The brightness (L*) values significantly ($p < 0.05$) decreased, whereas the a* values, which are generally associated with red colour, significantly ($p < 0.05$) increased with increasing TS from 23 to 35%. This is expected for food systems formulated with high proportions of red lentil, which contains polyphenolic compounds such as tannins, phenolic acids, and anthocyanins (Giusti et al., 2017). When the lentil protein-stabilised emulsions were subjected to a thermal treatment of 90 °C for 120 s, the brightness values (L*) increased in all the samples, whereas a significant ($p < 0.05$) decrease in a* values was observed in all samples. The magnitude of changes was significantly ($p<0.05$) higher for the higher total solid concentration with higher ΔE values calculated for the concentration of 29, 32 and 35% TS. This is likely due to degradation of polyphenolic compounds (i.e., tannins, phenolic acids, and anthocyanins) upon thermal treatment (Sadilova et al., 2007; Volf et al., 2014). Overall, these findings confirm that both total solids and thermal treatment play critical roles in the colour stability of lentil protein-based emulsions. Increasing TS levels intensify red coloration and reduce brightness, while heat treatment reverses these effects by promoting polyphenol degradation, leading to lighter and less red emulsions. These changes are important for the visual and sensory attributes of emulsions in food applications.

Table 2.1. Colour space values of lentil protein-stabilised emulsions before and after heat treatment at 90 °C for 120 s.

TS (%)	Before Heat Treatment			After Heat Treatment			ΔE
	L*	a*	b*	L*	a*	b*	
23	78.37 ± 0.56^{a}	5.82 ± 0.12^{a}	10.85 ± 0.25^{a}	80.00 ± 0.18^{a}	1.69 ± 0.03^{a}	9.60 ± 0.11^{a}	4.64±0.11^{a}
26	78.22 ± 0.52^{a}	6.30 ± 0.27^{b}	11.55 ± 0.01^{b}	79.46 ± 0.21^{a}	2.04 ± 0.07^{b}	10.25 ± 0.08^{c}	4.63±0.25^{a}
29	77.29 ± 0.17^{b}	6.93 ± 0.02^{b}	12.57 ± 0.04^{c}	78.41 ± 0.31^{b}	2.11 ± 0.02^{b}	11.21 ± 0.24^{c}	5.14±0.19^{b}
32	76.44 ± 0.36^{c}	7.20 ± 0.03^{c}	12.93 ± 0.14^{c}	77.36 ± 0.18^{b}	2.40 ± 0.10^{c}	11.64 ± 0.02^{c}	5.07±0.12^{b}
35	75.78 ± 0.13^{c}	7.70 ± 0.09^{c}	13.67 ± 0.13^{d}	76.42 ± 0.13^{c}	2.59 ± 0.08^{c}	12.47 ± 0.06^{d}	5.29±0.19^{b}

Values within a column that share a common superscript are not significantly different from one another ($p<0.05$).

2.3.2. Apparent viscosity of the solutions during thermal processing

The initial apparent viscosity of the emulsions before heating (30 °C) increased with increasing TS, with the 23, 26, 29, 32, and 35% TS samples having mean apparent viscosity values of 25.6, 42.8, 66.6, 113, and 130 mPa·s, respectively (Figure 2.1). During heating from 30 to 75 °C, the viscosity decreased, as expected for emulsions (Drapala et al., 2016) or protein suspensions; at 75 °C, which corresponds to the denaturation temperature of lentil proteins as reported by (Barbana and Boye, 2013), a sharp increase in viscosity was observed. Similar viscosity profiles have been reported for oil-in-water emulsions stabilised by lentil protein in the range 25–30% TS (Alonso-Miravalles et al., 2020) and in emulsions stabilised by hydrolysed whey proteins (Drapala et al., 2016). An increase in the viscosity of protein-stabilised emulsions at a relatively high temperature (70 °C) is attributed to the unfolding of polypeptide chains, disruption of hydrophobic interactions, and aggregation of unfolded protein molecules through covalent and non-covalent bonding (Considine et al., 2007; Drapala et al., 2016; Goetz and Koehler, 2005; Joshi et al., 2012). The

greatest heat-induced changes in viscosity were observed for the emulsion with 35% TS, with the viscosity increasing from 56.2 mPa·s at 75 °C to 89.6 mPa·s at 90 °C. The larger increase in viscosity at 35% TS is likely due to the higher concentration of solids, which leads to stronger interactions between the components. These interactions become much stronger at higher concentrations, which explains the bigger jump between 32% and 35%. During cooling, the viscosity reached final values of 31.5, 53.6, 84.0, 105, and 155 mPa·s for the emulsions at 23, 26, 29, 32, and 35% TS, respectively. Similar viscosity values have also been observed in oil-in-water emulsions stabilised using lentil protein isolate in the range 25–30% TS (Alonso-Miravalles et al., 2020).

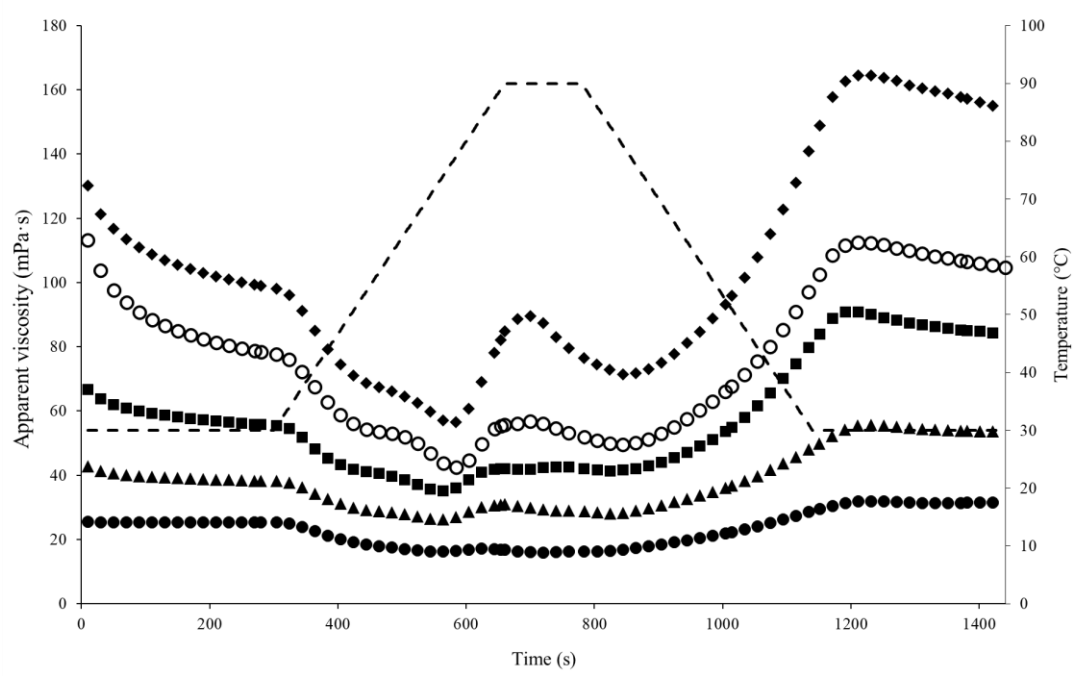

Figure 2.1. Apparent viscosity profiles of lentil protein-stabilised emulsions prepared at total solids contents of 23 (●), 26 (▲), 29 (■), 32 (○), and 35% (◆) during heat treatment with a peak hold at 90 °C for 120 s using a starch pasting cell geometry on a controlled-stress rheometer. Dashed line (- -) represents the temperature profile.

Although the focus of the present study is on formation and stability of protein-stabilised emulsions, protein–protein interactions (e.g., unfolding and aggregation) between unadsorbed proteins in the continuous phase would also be expected to contribute strongly to the measured changes in viscosity during heating (Aranberri et al., 2004).

2.3.3. Particle size distribution

Bimodal particle size distributions were observed in all emulsion samples (Figure 2.2). The population of smaller particles (Peak 1, corresponding to particles in the range 0.1–1 µm) can be associated with discreet oil droplets formed through homogenisation and stabilised by the lentil proteins, with Dv(50) (particle size below which 50% of sample volume is found) values of 0.34, 0.36, 0.36, 0.38, and 0.35 µm for the emulsions at 23, 26, 29, 32, and 35% TS, respectively. The results are in accordance with those of (Alonso-Miravalles et al., 2020), while formulating lentil protein-stabilised emulsions with similar protein-to-oil ratios (1:1.71). The population of larger particles (Peak 2, corresponding to particles in the range 2 to 80 µm) had Dv(50) values of 12.3, 17.9, 18.6, 20.9, and 17.0 µm, for emulsions with 23, 26, 29, 32, and 35% TS, respectively. This second population is associated with insoluble plant material from the lentil protein ingredient that was not fully solubilised during the powder rehydration process and homogenisation used in this study (Figure 2a); indeed, particles in the size range 2–80 µm were present in both the lentil protein isolate powder and lentil protein isolate suspensions. In addition, the emulsion sample at 23% TS displayed a lower particle size range (Table 2.2), with a peak 1 range of 0.69 µm and a peak 2 range of 40.1 µm before heating, whereas the other samples displayed peak 1 ranges of 0.98, 0.99, 0.94, and 0.90 µm and peak 2 ranges of 64.3, 84.0, 117, and 92.6 µm for the samples with 26, 29, 32, and 35%TS, respectively. Overall, the

samples showed a wider range of particle sizes at higher TS contents, which is indicative of lower-quality emulsions due to more heterogeneous oil globule size distributions.

		TS (%)				
		23	**26**	**29**	**32**	**35**
Dv(50) (μm)	**Peak 1 BH**	0.34 ± 0.01^{a}	0.36 ± 0.01^{a}	0.36 ± 0.02^{a}	0.38 ± 0.02^{a}	0.35 ± 0.02^{a}
	Peak 2 BH	12.3 ± 0.11^{b}	17.9 ± 3.05^{b}	18.6 ± 1.65^{b}	20.9 ± 3.27^{b}	17.0 ± 3.22^{b}
	Peak 1 AH	0.34 ± 0.01^{a}	0.34 ± 0.01^{a}	0.38 ± 0.03^{c}	0.43 ± 0.02^{c}	0.42 ± 0.01^{c}
	Peak 2 AH	14.1 ± 0.5^{b}	21.3 ± 2.98^{c}	28.8 ± 1.98^{d}	34.2 ± 3.14^{d}	29.9 ± 2.97^{d}
Range (μm)	**Peak 1 BH**	0.69 ± 0.03^{a}	0.98 ± 0.01^{a}	0.99 ± 0.05^{a}	0.94 ± 0.08^{a}	0.90 ± 0.07^{a}
	Peak 2 BH	40.1 ± 3.32^{b}	64.3 ± 11.5^{b}	84.0 ± 9.81^{b}	117 ± 24.21^{b}	92.6 ± 40.3^{b}
	Peak 1 AH	0.70 ± 0.01^{a}	0.95 ± 0.05^{a}	1.03 ± 0.07^{a}	1.04 ± 0.07^{c}	1.04 ± 0.08^{c}
	Peak 2 AH	51.4 ± 2.93^{c}	80.9 ± 11.5^{c}	158 ± 28.2^{c}	251 ± 57.3^{d}	232 ± 42.3^{d}
% Change in range on heat treatment	**Peak 1**	1.13	2.60	3.53	10.5	15.3
	Peak 2	28.1	25.9	89.0	112	181

Table 2.2. Particle size distribution parameters of lentil protein-stabilised emulsions before (BH) and after heat treatment (AH) at 90 °C for 120 s.

Dv(50) = particle size below which 50% of sample volume is found. Range = width of the particle distribution. Values within a column that share a common superscript are not significantly different from one another ($p<0.05$).

The particle size distribution of the emulsions was also measured after heat treatment at 90 °C for 120 s (Figure 2.2). The results showed an increase in particle size for both populations of particles, with the particles having Dv(50) values of 0.34, 0.34, 0.38, 0.43, and 0.42 μm for Peak 1 and 14.1, 21.3, 28.8, 34.2, and 29.9 μm for Peak 2 for the emulsions with 23, 26, 29, 32, and 35% TS, respectively, indicating that heating caused some emulsion destabilisation. Although the size of the emulsified particles (i.e., Peak 1) increased for all samples, a greater increase in particle size was recorded for the higher TS samples (Table 2.2), as evidenced by the wider range in particle size.

For example, the 35% TS emulsion displayed a Peak 1 range increase of 15.3%, whereas the 23% TS emulsion displayed a Peak 1 range increase of just 1.13%.

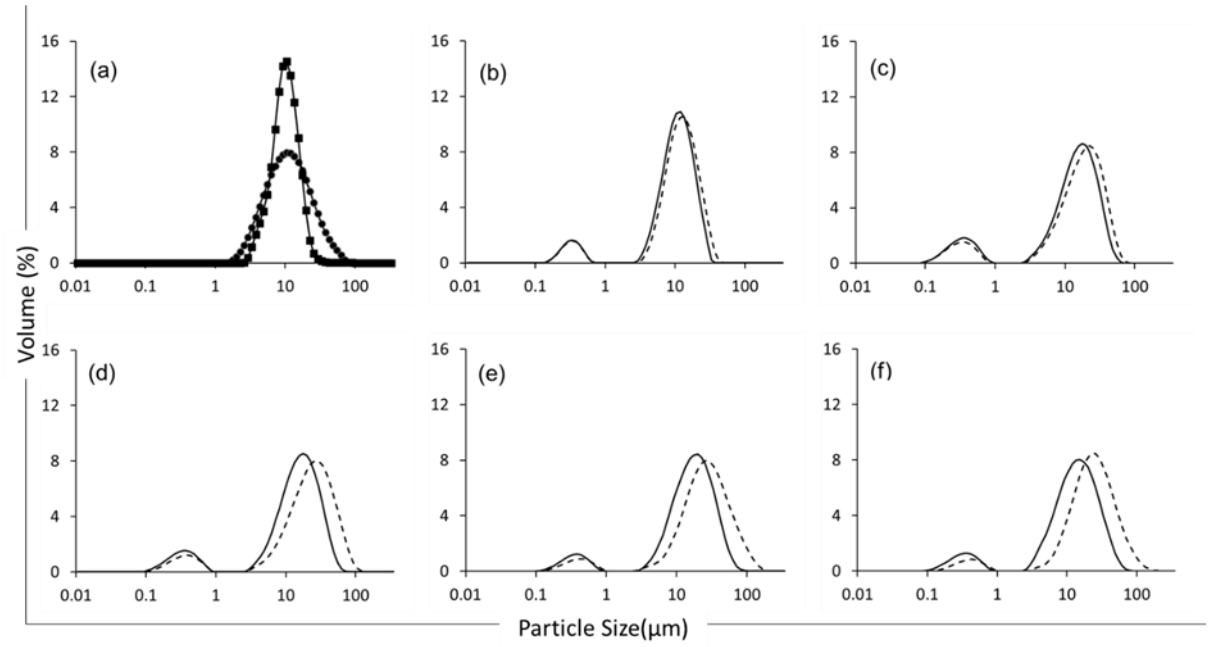

Figure 2.2 Particle size distribution of lentil protein isolate powder (-●-), lentil protein isolate dispersion (-■-) (a) and lentil protein-stabilised emulsions at total solids contents of 23% (b), 26% (c), 29% (d), 32% (e) and 35% (f), before (solid line) and after (dashed line) heat treatment at 90 °C for 120 s.

Similarly, the second population of larger particles (i.e., Peak 2) underwent an increase in particle size that was more extensive for the higher TS emulsions, with range increases of 181% for the 35% TS emulsion and 28% for the 23% TS emulsion. In this regard, Euston et al. (2000) found that an increase in TS in whey protein-based oil-in-water emulsions at protein contents ranging between 0.2 and 2% could lead to a decrease in the inter-droplet space, leading to increased flocculation upon heat treatment. These results are in agreement with those of Alonso-Miravalles et al. (2022), who found similar results for lentil–quinoa protein blends thermally treated at 95 °C for 30 s.

2.3.4. Microstructure and fat distribution

Using confocal laser scanning microscopy analysis, the emulsions at 23% TS before heat treatment displayed small, uniformly distributed oil droplets (Figure 2.3). With increasing the TS content up to 35%, the concentration of emulsified oil droplets increased, as shown by the higher density of green particles on the micrographs. This is in agreement with the observations of Jeske et al. (2019), who investigated the microstructure of lentil protein-stabilised emulsions homogenised at a similar pressure to that used in this study (i.e., 18 MPa). In addition to green-labelled oil droplets, red-labelled protein-dense particles were also evident on the confocal micrographs in Table 2.3; these increased in relative proportion with increasing the TS content from 23 to 35%. It is likely that these were mostly undissolved lentil protein powder particles. After heat treatment at 90 °C for 120 s, larger clusters of flocculated oil droplets were observed, in addition to protein-dense aggregates; this was in agreement with the particle size distribution (Figure 2.2) and viscosity (Figure 2.1) results. The greatest abundance of flocculated oil droplets and aggregated protein particles were evident in the high TS content samples (29 (c), 32 (d), and 35 (e) % TS). These results are in agreement with Primozic et al. (2017), who investigated the stability of lentil protein isolate-stabilised oil-in-water nanoemulsions (0.1–5%, w/w, protein) and reported that higher concentrations of protein in the continuous phase led to stronger interactions between proteins at the interface and in the continuous phase, which caused the formation of protein networks and greater flocculation of oil droplets. Similarly, Tang and Ghosh (2021), who worked with oil-in-water emulsions stabilised with canola protein isolate (1–4%, w/w, protein), found evidence that unadsorbed protein in the continuous phase allowed for the formation of protein networks that promoted aggregation of protein-coated droplets.

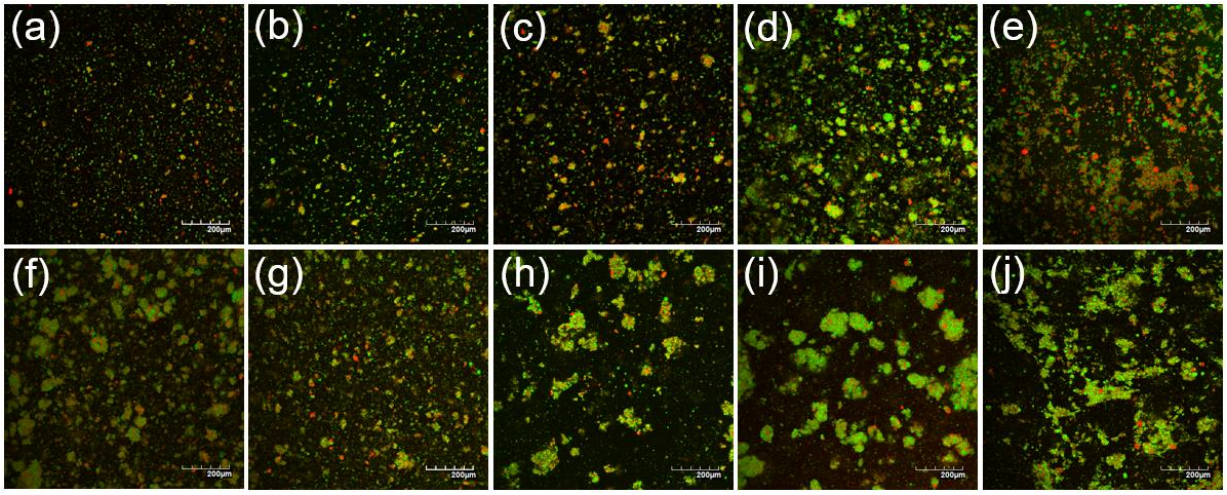

Figure 2.3. Confocal laser micrographs of lentil protein-stabilised emulsions before (a–e) and after (f–j) heat treatment at 90 °C for 120 s for the solutions at 23 (a,f), 26 (b,g), 29 (c,h), 32 (d,i)), and 35% (e,j) total solid content; fat and protein were stained green and red, respectively.

2.3.5. Accelerated physical stability

Representative transmission profiles during centrifugation for lentil protein-stabilised emulsions with TS in the range 23–35% are shown in Figure 2.4. The initial transmission profiles of the emulsions were similar among all samples, with integral transmission values ranging from 3.2 to 3.4%. All the emulsions showed a rapid increase in transmission on centrifugation, which was greater for emulsions with higher TS contents. Specifically, the separation rates were 2.93 ± 0.19, 9.84 ± 0.51, 10.46 ± 0.46, 12.03 ± 1.01, and 11.46 ± 0.85%/h for the emulsions with 23, 26, 29, 32, and 35% TS (Figure 2.5), respectively. Jeske et al. (2019) found similar profiles and separation rates for emulsions stabilised with lentil protein formulated at 3.3% protein (w/w) and homogenised at 18 MPa. No significant ($p > 0.05$) differences were observed in the sediment thickness between the samples, with values ranging between 1.54 ± 0.20 and 1.90 ± 0.15 mm. Furthermore, it was possible to observe differences among the samples in the thickness of the cream layer, which is the result of the upward

motion of the oil phase. The thickness of the cream layer of the emulsions was 3.99 ± 0.07, 4.99 ± 0.09, 5.49 ± 0.34, 6.02 ± 0.33, and 7.17 ± 0.38 mm for the emulsions with 23, 26, 29, 32, and 35% TS, respectively, indicating that with increasing TS content the emulsions were significantly ($p < 0.05$) less physically stable.

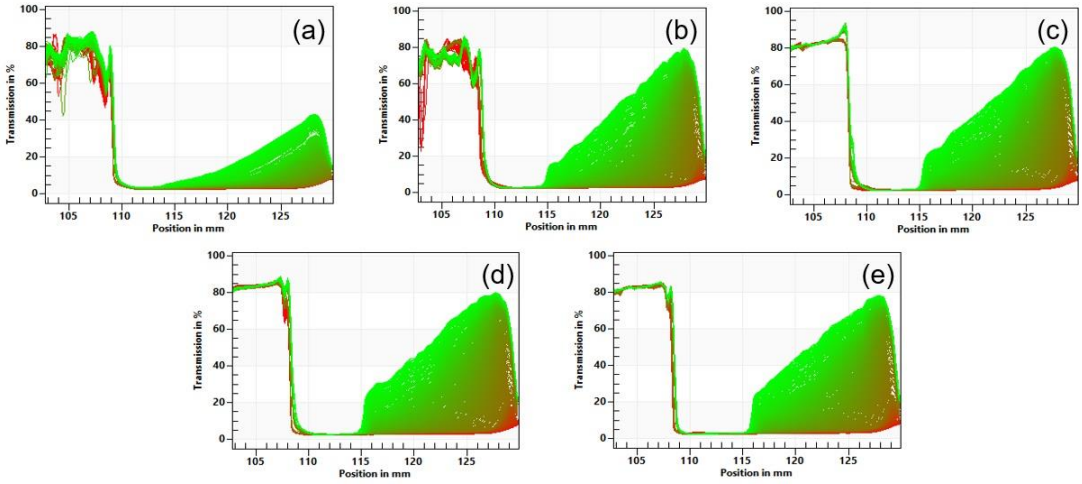

Figure 2.4. Representative accelerated stability analysis transmission profiles for the lentil protein-stabilised emulsions at 23 (a), 26 (b), 29 (c), 32 (d), and 35% (e) total solids content.

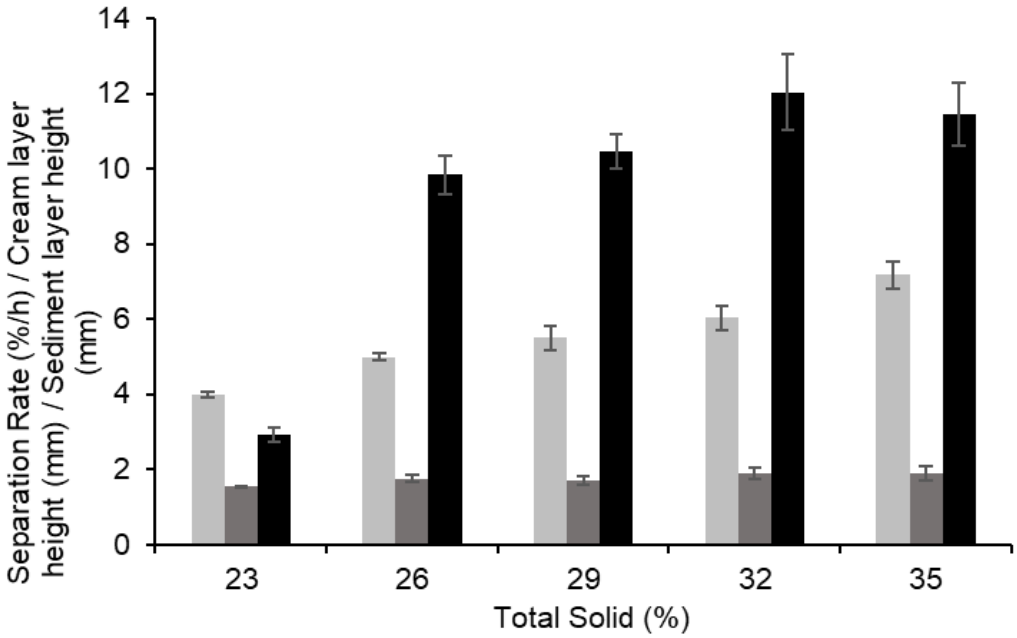

Figure 2.5. Separation rate (black), cream layer height (light grey), and sediment layer height (dark grey) of lentil-protein-stabilised emulsions at total solid contents of 23, 26, 29, 32, and 35% from accelerated stability analysis.

2.4. Conclusion

The physicochemical properties and heat stability of high total solids lentil-protein-stabilised oil-in-water emulsions were studied. The emulsions, ranging from 23 to 35% TS, showed bimodal particle size distributions in addition to increasing viscosity with increasing TS content. On heating at 90 °C for 120 s, the emulsions at low TS concentrations (i.e., 23–29%) showed better physical stability than emulsions at high TS concentrations. Microstructural analysis confirmed that these physical changes can be attributed to strong protein–protein interactions, which resulted in protein aggregation and ultimately oil droplet flocculation. These findings have several important implications for the development and processing of plant-protein-based emulsions. First, they highlight the challenges associated with stabilising high-solid emulsions, particularly in the context of plant proteins that exhibit strong aggregation tendencies due to their inherent structural properties. Secondly, the study underscores the importance of optimising both formulation (e.g., protein concentration) and processing conditions (e.g., thermal treatment) to achieve desirable stability. Adjusting the protein-to-solid ratio and refining heat treatments could mitigate aggregation and enhance functionality in plant-protein systems.

From a practical standpoint, this research provides valuable insights for the development of plant-based young child formulae and similar high-nutrient emulsions. By better understanding the mechanisms underlying emulsion stability at different TS levels, food scientists and manufacturers can design more sustainable and cost-effective formulations. This is particularly relevant as consumer demand grows for plant-based alternatives to traditional dairy-based formulas.

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Chapter 3

Enhancing the techno-functional properties of lentil protein isolate dispersions using in-line high-shear rotor-stator mixing

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Abstract

In response to global challenges such as climate change and food insecurity, plant proteins have gained interest. Among these, lentils have emerged as a promising source of proteins due to their good nutritional profile and high sustainability degree. However, their widespread use in food products has been impeded by limited solubility. This study aimed to investigate the potential of high-shear mixing, a resource-efficient technique, to enhance lentil protein solubility and their functional properties. Red lentil protein isolate powders were rehydrated and subjected to a semi-continuous in-line high-shear treatment at 10,200 rpm for a timespan ranging from 0 to 15 min. The results highlighted a significant ($p<0.05$) increase in solubility from 46.87% to 68.42% after 15 min of shearing and a reduction in particle size as a result of the intense shearing and disruption provided by the rotor and forced passage through the perforation of the stator. The volume-weighted mean diameter decreased from 5.13 µm to 1.72 µm after 15 minutes of shearing, also highlighted by the confocal micrograph which confirmed the breakdown of larger particles into smaller and more uniform particles. Rheological analysis indicated consistent Newtonian behaviour across all dispersions, with apparent viscosities ranging from 1.69 mPa·s to 1.78 mPa·s. Surface hydrophobicity increased significantly ($p<0.05$), from 830 to 1245, indicating exposure of otherwise buried hydrophobic groups. Furthermore, colloidal stability of the dispersion was improved, with separation rates decreasing from 71.23 $\%\cdot h^{-1}$ to 24.16 $\%\cdot h^{-1}$. The significant enhancements in solubility, particle size reduction, and colloidal stability highlight the potential of in-line high-shear mixing in improving the functional properties of lentil protein isolates for formulating sustainable food products with enhanced techno-functional properties.

Keywords: lentil proteins, plant proteins, in-line high-shear mixer, solubility

3.1.Introduction

The need to tackle global challenges, ranging from climate change to food insecurity and chronic diseases as well as the growing demand for healthy and sustainable food protein ingredients have recently driven interest in plant proteins (Day et al., 2022; Henchion et al., 2017; World Population Prospects, 2022) Multiple protein sources including legumes, pseudocereals, cereals, tubers, grains, nuts, and seeds offer diverse nutritional profiles and health benefits (Day et al., 2022; Shaghaghian et al., 2022). Among these, pulses and more particularly lentils emerge as an interesting animal protein alternative, as they are rich in essential amino acids, dietary fibres, vitamins, and minerals as well as being affordable, sustainable and abundant raw material (Can Karaca et al., 2011). Despite their positive attributes, the widespread utilization of lentil proteins in food products has been impeded by their limited solubility (Can Karaca et al., 2011; Grossmann and McClements, 2023). Indeed, solubility is a fundamental functional property of proteins that plays a crucial role in processes such as gelation, foaming and emulsification where soluble proteins allow for the stabilisation of oil globules by adsorption at the oil/water interface (Day, 2013; Ebert et al., 2020; Zhao et al., 2020). On the other hand, the low solubility of protein-based ingredients, impacted by extraction processing parameters (e.g., pH changes, drying temperatures),can lead to processing challenges such as clumping, phase separation, sedimentation, and overall poor physical stability (Grossmann and McClements, 2023; Grossmann and Weiss, 2021). To enhance the functional properties of plant-based proteins, a range of strategies have been investigated, including chemical and enzymatic modifications, as well as physical treatments (Bou et al., 2022; Mirmoghtadaie et al., 2016). High-pressure homogenisation (HPH), a well-regarded technique known for its ability to modify protein structures and enhance their

functional properties, has been widely used in recent years (Avelar et al., 2021; Gharibzahedi et al., 2023; Sá et al., 2022; Tobin et al., 2015). Several studies have shown that HPH could be used to improve the functional properties of plant-protein ingredients and particularly their solubility. In particular, HPH treatment in the range 70-150 MPa could lead to a 20 to 30% increase in solubility (Keuleyan et al., 2023; Moll et al., 2022; Zhao et al., 2022). Similarly, Saricaoglu (2020) has shown that HPH treatment in the pressure range 0-150 MPa could increase the solubility of the protein ingredient from 32% to 46%. Other studies have investigated the effect of HPH on protein ingredient for the formulation of lentil protein stabilised emulsions, such as Jeske et al.(2019) and Primozic et al. (2018) who studied the suitability of HPH (34 and 103 MPa) to improve lentil protein isolate (LPI) for use in nanoemulsions. The results showed that HPH treatment allowed for a reduction in particle size and hydrophobicity. However, while effective, HPH comes with challenges as it is resource-intensive, both in terms of cost and energy consumption (Calligaris et al., 2016; Fayaz et al., 2022).

On the other hand, high-shear mixers, which are composed of a rotor-stator allowing for shearing rate in the range 20,000 – 100,000 s^{-1}, are highly efficient in increasing powder solubility and more cost-effective (Hall et al., 2011; O'Sullivan et al., 2018). This physical treatment has been used extensively in the dairy industry for the disintegration of powder particles. O'Sullivan et al. (2017) compared different method of powder rehydration and found that the use of an in-line high-shear mixer allowed for the best reduction in particle size of the dispersion from 20 µm to 10 µm. Furthermore, extensive literature is available on the use on in-line shear mixers for the formulation of stable emulsions (Pacek et al., 2013; Rueger and Calabrese, 2013; Schuch et al., 2013; Van Der Schaaf and Karbstein, 2018). Scholz and Keck (2015)

have shown that an in-line high-shear rotor-stator mixer could allow for the formulation of stable emulsions with droplet size slightly larger than the one obtained by high pressure homogenisation, however no evidence on differences in the functionalities of the emulsions were found.

Even though the use of in-line high-shear mixers in the dairy industry for the disruption of dairy powder particles and formulation of stable emulsions has been well documented, to the author's knowledge, there is no information available on the influence of in-line high-shear mixers on plant proteins solubility and functional properties.

This study aims to investigate the potential of high-shear mixing in enhancing the functional properties of lentil protein dispersions. The results of this study will support the development of cost-effective, sustainable processing solutions for the use of plant-based protein ingredients in food systems including infant formulae and milk alternatives.

3.2.Materials and methods

3.2.1. Materials

The lentil protein dispersions were prepared by rehydrating red lentil protein isolate powder (72.15% protein, w/w), provided by Döhler GmbH (Darmstadt, Germany), at a concentration of 5% powder (w/v) (corresponding to 3.61% protein (w/v)) in preheated deionised water (70°C), stirred magnetically at 300 rpm for 1 h at 20°C. The dispersions were then adjusted to pH 6.8 and allowed to rehydrate fully at 4°C for 18 h, after which the samples were equilibrated at 20°C and pH was readjusted, if necessary, to pH 6.8.

3.2.2. High-shear mixing treatment

The lentil protein dispersion was subjected to a semi-continuous in-line high-shear treatment (figure 3.1) using a Silverson laboratory high-shear mixer (L5MA; Silverson, Massachusetts, United states), equipped with an in-line mixing assembly to allow for recirculation. The in-line mixing assembly was chosen to reduce foaming and allow for a homogenous feeding in the shearing head. A peristaltic pump (Watson Marlow, Marlow, UK) was used to feed the shearing head. A valve was set on the outlet of the shearing head to apply 0.5 bar of back pressure, and the outlet was redirected to the feeding tank. Samples were collected from the outlet after 0 (no shearing), 1, 3, 5 and 15 min of shearing at 10,200 rpm. A control sample (control), which did not pass through the shearing system was included in the analysis.

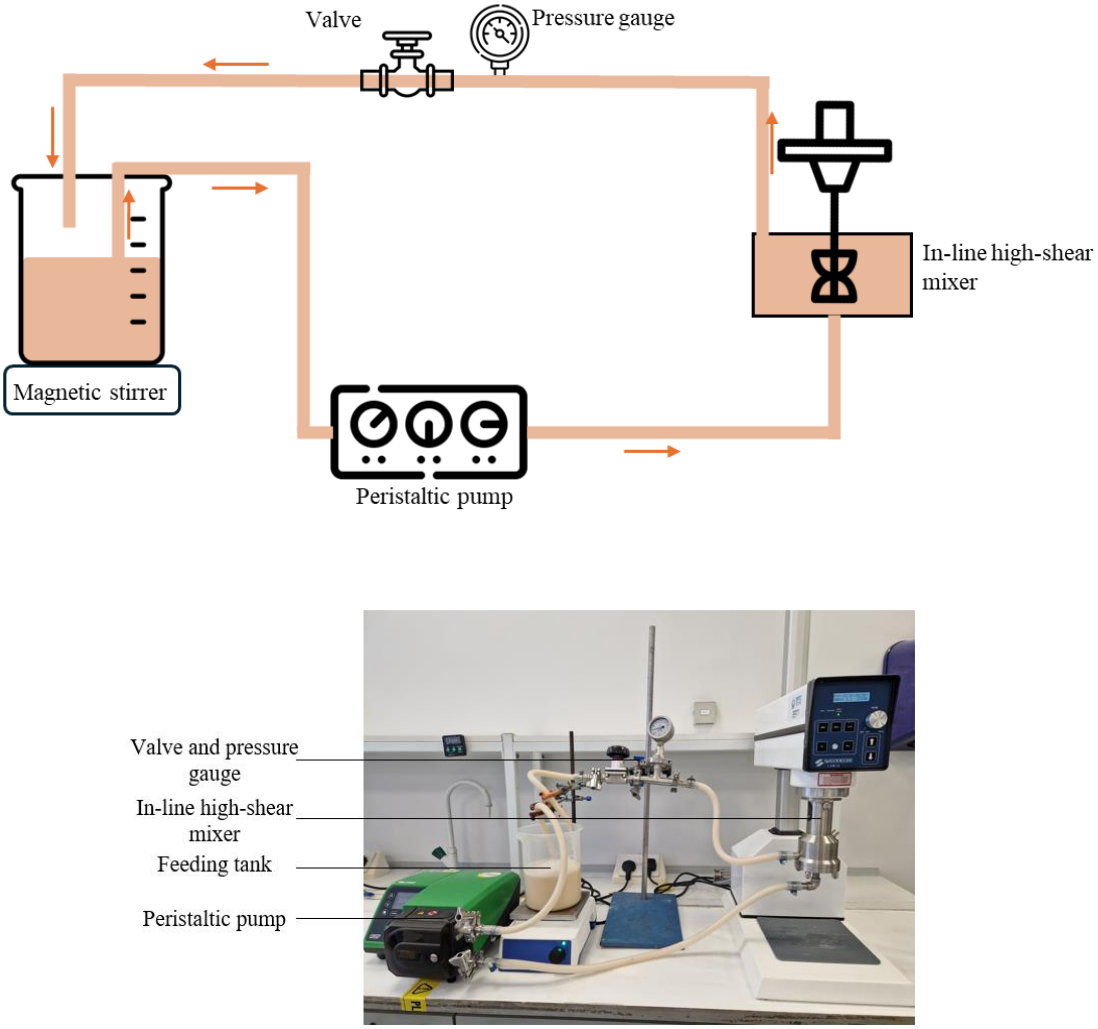

Figure 3.1. Schematic representation and representative picture of the in-line high-shear mixer assembly.

3.2.3. Particle size distribution

A Mastersizer 3000 (Malvern Instruments, Worcestershire, UK) static light scattering device fitted with an automated Hydro MV wet and dry cell disperser was used to evaluate the particle size distribution (PSD) of the dispersion and powder sample forms respectively. The protein's refractive index was set at 1.338, the dispersant's refractive index at 1.33, and the adsorption index was 0.001. The dispersions were diluted in ultrapure water until 10% laser obscuration was achieved (at 20°C). The volume-

weighted mean particle size (D[4,3]), specific surface area, and the particle sizes below which 10% and 90% of the sample volume are detected (Dv(10) and Dv(90) are presented as the results. Each dispersion and the powder were sampled 3 times and measured 3 times by the instrument for a total of 9 measurements.

3.2.4. Confocal laser scanning microscopy

Microstructural analysis of the dispersions was performed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan). The dispersions were prepared as described by Malterre et al. (2023) with the following modifications, whereby 1 ml of the dispersion was mixed in 4 ml of low gelling temperature agarose (Sigma-Aldrich, St. Louis, Missouri, United States) solution (1.5%, w/v) at 37°C to fix the dispersion and allow for higher quality imaging. As described by Grasso et al. (2021), 20 µl of Fast Green FCF aqueous solution (200 µL of 0.1 g/L) was added to 1 ml of the dispersion-agarose sample, which was then incubated at 20°C until gelation. Fast Green FCF was excited at 633 nm, and representative images, performed using a 20x objective lens, were reported whereby protein was stained red.

3.2.5. Rheological analysis

A modular compact rheometer MCR 102e (Anton Paar, Austria) fitted with a concentric cylinder geometry was used to assess the rheological characteristics of the dispersions. The rotor's diameter was 26.7 mm, the cell's internal diameter was 28.9 mm, and the distance between the two parts at the geometrical base was 2 mm. After loading the sample (20 ml) into the cylinder and preheating it to 20°C, the shear rate was increased to 100 s^{-1} over 30 s and kept there for another 30 s. A constant temperature of 20°C was kept throughout the measurement, and viscosity was

continuously recorded. The Ostwald-de Waele model (Eq.1) was used to describe the rheological properties of the protein dispersions, while ensuring that $R2 \geq 0.95$.

$$\tau = K\dot{\gamma}^n \quad (1)$$

Where τ is the shear stress (mPa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the flow consistency coefficient ($mPa \cdot s^n$) and n is the flow behaviour index (dimensionless).

3.2.6. Protein solubility

An RC5C plus centrifuge (Thermo ScientificTM SorvallTM, Massachusetts, USA) with a GSA rotor was used to determine the protein solubility of the dispersions (Alonso-Miravalles et al., 2019). The protein concentration in the supernatant of the dispersions, obtained after centrifugation at 1,000 g for 20 min at 20°C, was measured using the Kjeldahl method, with a nitrogen-to-protein conversion factor of 6.25 (IDF, 2001); this conversion factor was selected since a specific ones for lentil is not available in the literature and to be consistent with the work of Vogelsang-O'Dwyer et al., 2023; Alonso-Miravalles et al., 2019. The results are expressed as percentage of protein in the supernatant (soluble protein) as compared to the total protein content of the dispersions (3.61% (w/v)).

3.2.7. Protein profile analysis

The protein profile of the dispersions, and their fractions, was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with precast gels (Mini-PROTEAN TGX, Bio-Rad Laboratories, CA, USA), following the method developed by Laemmli (1970), with minor modifications. The samples were centrifugated at 1000 g for 20 min at 20°C using an RC5C plus centrifuge (Thermo Scientific™ Sorvall™, Massachussets, USA) with a GSA rotor to isolate the soluble protein in the supernatant, and diluted to a protein concentration of 2 mg/ml. In method

I (non-reducing conditions), 100 µl of the diluted supernatant was mixed with 100 µl of sample loading buffer (2X Laemmli sample buffer, Bio-Rad Laboratories, CA, USA) whereas in method II (reducing conditions), 100 µl of the diluted dispersion was mixed with 95 µl of sample loading buffer and 5 µl of β-mercaptoethanol and incubated at 95 °C for 10 min. Each well was loaded with 7 µl of sample solution and 5 µL of SDS-PAGE broad range protein standard (BioRad, Hercules, CA) was loaded as a protein molecular weight standard. The gels were run in a running buffer (10x Tris/Glycine/SDS, Bio-Rad Laboratories, CA, USA) at 160 V for 1 h, then fixed for 15 min using a solution of water:methanol:acetic acid (40:50:10), stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, CA, USA) and destained using a solution of water:methanol:acetic acid (50:40:10) until a clear background was achieved. The gels were then stored at 4°C in a 5% (v/v) acetic acid solution overnight to reverse the shrinkage induced during the fixing and destaining steps.

3.2.8. Surface hydrophobicity

Hydrophobicity of proteins in the supernatant (dispersion centrifugated at 1,000 g for 20 min as described in Section 3.2.6) was measured using the method previously described by Nakai (2003) (Nakai, 2003) and Goulding et al. (2021) (Goulding et al., 2021), with minor modifications, using the hydrophobic probe 8-Anilino-1-naphthalenesulfonic acid (ANS). Samples were diluted to each of the following concentrations, 0.003, 0.006, 0.009, 0.012 and 0.015% (v/v) in sample buffer (0.01 M phosphate buffer, pH 7) and 100 µL of the diluted solutions were transferred to a 96 black microwell plate, after which 50 µL of ANS solution (8 mM in 0.1 M phosphate buffer) was added. The well plate was then covered with foil and gently rocked for 15 min on a plate shaker. After this, the fluorescence intensity of the samples was measured using a Varioskan Flash microplate reader (Fisher Scientific, Ireland) with

excitation wavelength of 390 nm and emission wavelength of 470 nm. The fluorescence intensity was plotted as a function of protein concentration (%, v/v) and only linear regression with an R^2 higher than 0.98 were considered. The slope (S0) value was used to compare the surface hydrophobicity of proteins in the different samples.

3.2.9. Accelerated physical stability

The colloidal stability of the dispersions was measured using an analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). The samples were centrifugated through two successive cycles at 800 and 3,000 rpm for 10 min each, with near-infrared light illuminating the samples throughout the analysis and the intensity of the transmitted light over the entire length of the sample was measured every 10 s. The separation rate was determined by plotting the integral of the intensity of the transmitted light over time and extracting the slope ($\%.h^{-1}$). The light transmission profile before centrifugation, after the first cycle and after the second cycle were extracted and plotted.

3.3.Results and discussion

3.3.1. Particle size distribution and microstructure

The particle size distribution parameter, volume-weighted mean particle diameter (D[4,3]) of the lentil powders, control sample and processed through the in-line high-shear mixer are shown in Table 3.1. The red lentil isolate powder ingredient displayed a D[4,3] value of 30.7 ± 0.06 μm in line with the literature (Malterre et al., 2023; Vogelsang-O'Dwyer et al., 2023; Alonso-Miravalles et al., 2019). The control sample underwent no treatment, whereas the 0 min sample passed through the rig without applied pressure to isolate the effects of the pump and rig on the samples. The control red lentil dispersion and the sample processed through the in-line high-shear mixer for 0 min displayed a similar ($p>0.05$) D[4,3], with values of 5.13 ± 0.58 μm and 5.00 ± 0.04 μm, respectively, indicating that the pumping through the semi-continuous system and application of back pressure did not affect the particle size distribution parameters. Interestingly, increasing the shearing time, caused a significant ($p<0.05$) decrease on the PDS parameters, with a decrement in D[4,3] to 2.81 ± 0.06, 2.17 ± 0.04 and 1.72 ± 0.01 μm after 1, 5 and 15 min of processing, respectively. This progressive reduction in particle size can be attributed to the high-shearing forces induced by the rotor of the mixing head, which causes the breakage of the powder particles with the forced passage of the particles through the perforated small gap of the stator (Ding et al., 2009; Pacek et al., 2013, 2007).

Changes in particle size are also visible on the confocal micrographs (Figure 3.2), as the control and 0 min dispersions displayed large particles in the range 40-50 μm. Furthermore, micrographs of the 1 min dispersion, showed heterogeneous particles in the range of 1-5 μm and 15-25 μm. Longer processing up to 15 min showed further reduction of the population of large particles and an increase in small particles as

illustrated by an increase in the intensity of red particles on the micrographs (Figure 3.2).

Table 3.1. Particle size distribution parameters, protein solubility, surface hydrophobicity and separation rate for lentil protein isolate powder and protein dispersions (5% powder, w/v) of untreated (control) and treated with high-shear mixing for 0, 1, 3, 5 and 15 min.

	D[4,3] (µm)	**Span (µm)**	**Protein solubility (%)**	**Surface hydrophobicity (-)**	**Separation rate (%.h^{-1})**
Powder	30.7 ± 0.06^{a}	1.84 ± 0.02^{a}	n/a	n/a	n/a
Control	5.13 ± 0.58^{b}	7.25 ± 2.98^{b}	46.87 ± 1.55^{a}	830 ± 123^{a}	71.23 ± 18.15^{a}
0 min	5.00 ± 0.04^{b}	6.96 ± 0.16^{b}	48.04 ± 4.03^{a}	852 ± 73^{a}	69.67 ± 18.85^{a}
1 min	2.81 ± 0.06^{c}	4.28 ± 0.22^{c}	57.18 ± 5.65^{b}	1044 ± 26^{b}	36.57 ± 5.19^{b}
3 min	2.49 ± 0.18^{d}	3.99 ± 0.22^{c}	63.69 ± 7.14^{c}	1116 ± 70^{bc}	30.81 ± 2.63^{bc}
5 min	2.17 ± 0.04^{e}	3.72 ± 0.06^{d}	63.94 ± 5.70^{c}	1245 ± 43^{c}	28.89 ± 3.01^{cd}
15 min	1.72 ± 0.01^{f}	3.14 ± 0.02^{e}	68.42 ± 8.04^{d}	1236 ± 41^{c}	24.16 ± 2.58^{d}

Values within a column that share a superscript are not significantly different from one another($p<0.05$).

D[4,3] = volume-weighted mean particle diameter.

Span = measurement of the width of the distribution calculated as: (Dv(90) – Dv(10)) / Dv(50).

n/a: not applicable

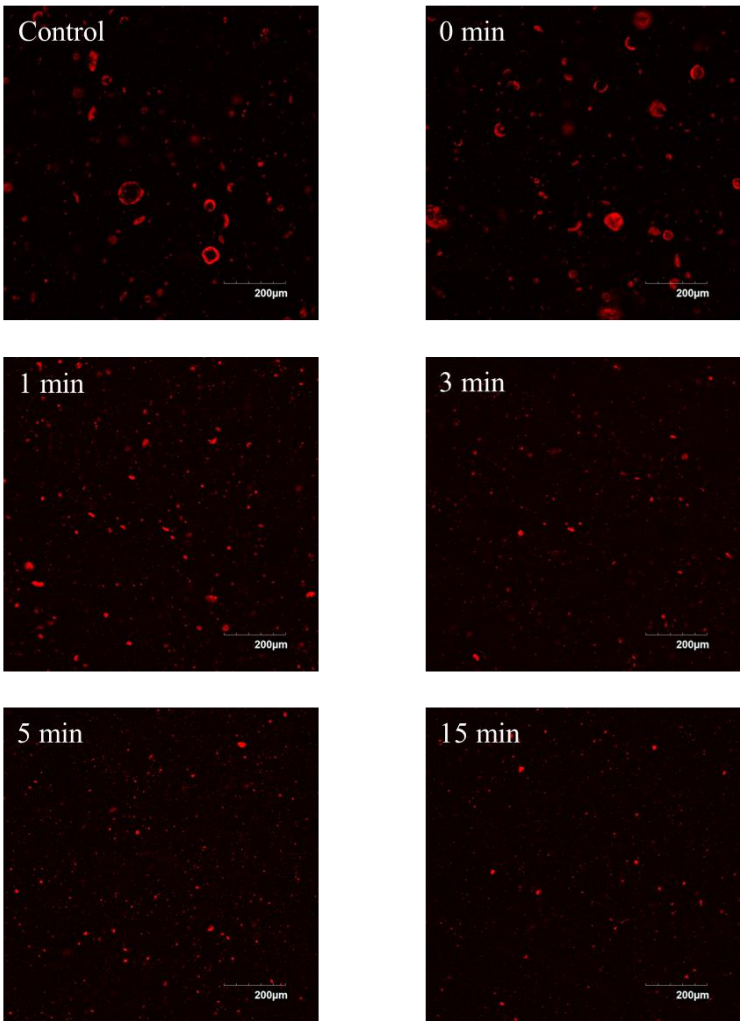

Figure 3.2. Confocal laser micrographs of lentil protein isolate dispersions (5% powder, w/v) of untreated (control) and treated with high-shear mixing for 0, 1, 3, 5 and 15 min.

The reduction in number of bigger particles can also be shown through the particle size parameters D(90) (particle size below which 90% of sample volume is found), which progressively decreased throughout the process from 12.71 ± 3.33 µm for the control dispersion to 6.83 ± 0.35 and 3.76 ± 0.07 µm after 1 and 15 min of shearing (Figure 3.3). On the other hand, the D(10), which represents the particle size below which 10% of sample volume is found, had minimal reduction ($p>0.05$) through shearing from 0.46±0.05 µm for the control dispersion and 0.42 ± 0.01 µm for the dispersion treated for 1 min. This parameter then plateaued ($p>0.05$) with increasing shearing time to

reach a D(10) value of 0.35 ± 0.01 µm after 1 min. Furthermore, the particle disruption induced by the high-shearing caused a significant ($p<0.05$) increase in specific surface area from 4.14 ± 0.46 $m^2 \cdot g^{-1}$ for the control dispersion to 5.87 ± 10.0 $m^2 \cdot g^{-1}$ after 15 min of shearing (Figure 3.3). Higher specific surface area could allow for higher exposure of each particle to water and possibly better dissolution of the powder as indicated by the Noyes Whitney equation that indicate a link between the exposed surface and the dissolution rate of the powder (Dokoumetzidis and Macheras, 2006; Noyes and Whitney, 1897). The results are in agreement with the literature as similar results on the effect of high-shear mixing on particle disruption have been found in the literature for powders. In particular, Pacek et al. (2007) and Padron and Özcan-Taşkın (2018), found that in-line high-shear mixer at 8000 rpm, caused the reduction of particles of silicon from 90 µm to 9 µm after 20 min and from 100 µm to 15 µm after 30 min of shearing. The authors have also shown that in the timespan used in the present study (<15 min), in-line high-shearing did not allow for the disruption of aggregates below 1-2 µm. Similarly, O'Sullivan et al. (2017) have found that the use of in-line high-shear mixer for the rehydration and dispersion of dairy powders (skim milk powder and milk protein isolate) at concentration of 7.2% (w/w) caused a significant reduction of particles size from 100 µm to 20 µm after 15 min, while the size of the smaller particles plateaued in the range 0.1-1 µm. Overall, these results show that in-line high-shear mixing has the potential to effectively reduce the particle size on lentil protein isolate from large aggregates to small particles, which could have potential benefit on the increase in the dissolution rate and rehydration improvement.

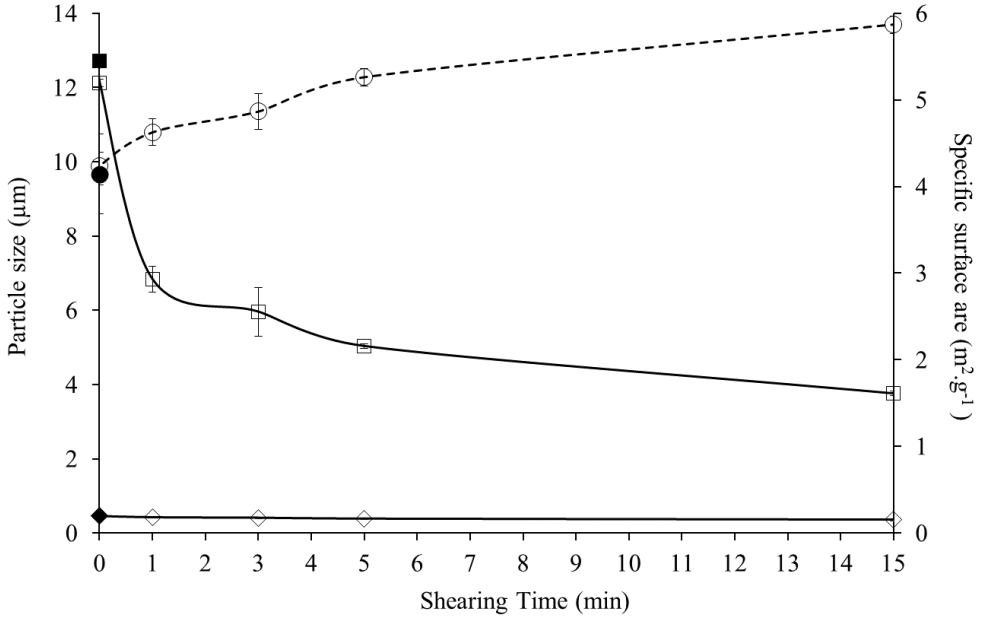

Figure 3.3. Particle size distribution parameters (-◇-, Dv(10), particle size below which 10% of sample volume is found), (-□- Dv(90), particle size below which 90% of sample volume is found), and (--○--, specific surface area) of protein dispersions (5% powder w/v) untreated (control) and treated with high-shear treatment for at 0, 1, 3, 5 and 15 min. Filled shapes (-■-, -◆- and -●-) correspond to the control dispersion.

3.3.2. Rheological properties

The apparent viscosity of the dispersions as well as their flow properties n (-) and k (mPa·s^n) as defined by fitting the Ostwald de Waele model, are given in Table 3.2. The apparent viscosity at 100 s^{-1} and 20°C ranged between 1.69±0.3 and 1.78±0.34 mPa·s with no significant differences ($p>0.05$) between the dispersions. Similar apparent viscosity values were measured for lentil protein dispersions with protein concentrations of 4% (w/w) (Saricaoglu, 2020). The Ostwald de Waele model allowed for the accurate description of the rheological properties of the dispersions ($R^2 > 0.98$), and similar ($p>0.05$) consistency coefficient ranging between $1.57\times10^{-3} \pm 0.81\times10^{-3}$ and $1.87\times10^{-3} \pm 0.72\times10^{-3}$ were observed over the range of treatments. The flow

behaviour index of all dispersions ranged between the values of 1.00 ± 0.06 and 1.03 ± 0.09 mPa·s^n, indicating a Newtonian behaviour of the dispersion. Similar results have been observed by Jeske et al. (2019) who studied the HPH treatment of lentil protein isolate at a concentration of 3% (w/w).

Table 3.2. Apparent viscosity (η at 100 s^{-1}, 20°C), flow behaviour (n) and consistency coefficient (K) of protein dispersions (5%, w/v, powder) untreated (control) and treated with high-shear treatment for 0, 1, 3, 5 and 15 min.

Treatment	η (mPa·s)	n (-)	K (mPa·s^n)
Control	1.74 ± 0.35^a	1.02 ± 0.06^a	1.73×10^{-3} ± 0.78×10^{-3} ^{a}
0 min	1.75 ± 0.33^a	1.02 ± 0.07^a	1.77×10^{-3} ± 0.76×10^{-3} ^{a}
1 min	1.69 ± 0.30^a	1.02 ± 0.07^a	1.57×10^{-3} ± 0.81×10^{-3} ^{a}
3 min	1.73 ± 0.26^a	1.00 ± 0.06^a	1.70×10^{-3} ± 0.60×10^{-3} ^{a}
5 min	1.72 ± 0.32^a	1.03 ± 0.09^a	1.67×10^{-3} ± 0.75×10^{-3} ^{a}
15 min	1.78 ± 0.34^a	1.00 ± 0.05^a	1.87×10^{-3} ± 0.72×10^{-3} ^{a}

Values within a column that share a superscript are not significantly different from one another($p<0.05$).

3.3.3. Protein solubility

Protein solubility is an essential functional property which strongly influences other protein properties, such as emulsifying, foaming or gelling properties (Day, 2013; Zhao et al., 2020). The control dispersion had a protein solubility of 46.87 ± 1.55%, similarly ($p>0.05$) to the dispersion treated for 0 min displaying a solubility of 48.04 ± 4.03% (Table 3.1), as expected for plant protein ingredients which have low solubility as compared to dairy isolates (>90% in similar conditions) (Grossmann and McClements, 2023). The high-shearing generated through the treatment allowed for a 20% increase in solubility when treated for 1 min (57.18 ± 5.65%), and continuously increased up to a value of 68.42 ± 8.04% for the dispersion treated for 15 min, allowing for 42% increase in solubility. The increase in solubility can be attributed to the particle breakage caused by the intense shearing as observed in Table 3.2. Similar

results have been observed by other authors that showed that, mechanical shearing up to 8,000 rpm is able to increase the solubility of protein ingredients by breaking down the protein aggregates and facilitating their dispersion and dissolution (O'Sullivan et al., 2017; Pereira et al., 2016).

The breakages and disruptions induced by the in-line high-shear treatment, reducing the particle size, could have allowed for a better dispersion of the agglomerated powder particles (Schuck et al., 2012), increasing the rehydration and solubility of the ingredients as shown by other authors who worked on the improvement of protein ingredients through mechanical processing such as the well documented use of HPH for the improvement of plant protein functionalities (Bader et al., 2011; Melchior et al., 2022; Song et al., 2013; Yang et al., 2018; Yu et al., 2018; Zhao et al., 2022).

A better understanding of the protein solubilisation as affected by processing time was obtained by analysing the protein profile of the soluble fractions of the dispersions (Figure 3.4a & 3.4b). The supernatant of lentil protein isolate dispersions had proteins with a molecular weight of 50 kDa, which may correspond to vicilin subunits, and the bands at 37 and 25 kDa may correspond to the acidic and basic subunits of legumin. Under reducing conditions, legumin dissociated into its acidic and basic subunits due to the dissociation of disulphide bonds, resulting in a decrease in the intensity of the bands at 50 and 37 kDa and an increase in the intensity of the bands at 20-25 kDa. Similar results have been observed by Jarpa-Parra et al. (2015), Barbana and Boye (2013), Joshi et al. (2012), and Alonso-Miravalles et al. (2019). The protein profile of all the samples were similar to the one of the control sample, indicating that in-line high-shear treatment did not have an effect on the profile of solubilised proteins.

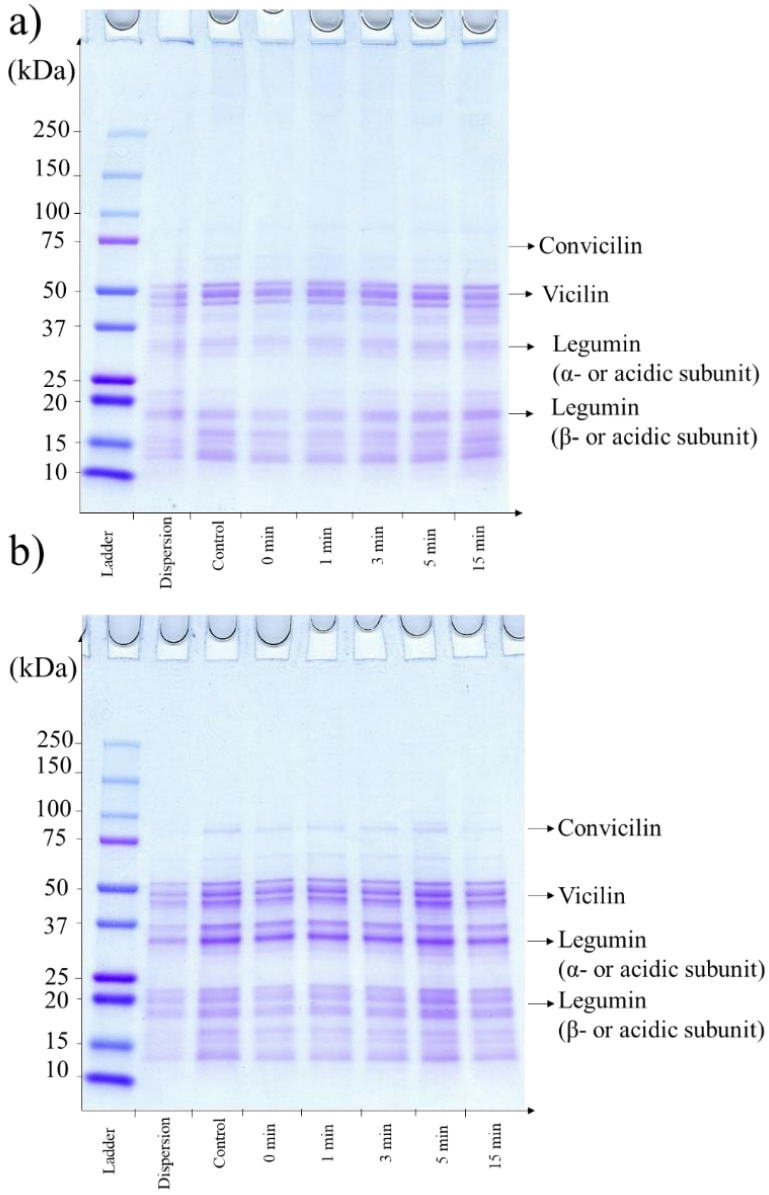

Figure 3.4 Representative sodium dodecyl sulphate-polyacrylamide gel electrophoretograms (SDS-PAGE) of lentil protein isolate dispersions (5% w/v) untreated, control supernatant untreated and supernatant treated with high-shear-mixing for 0, 1, 3, 5 and 15 min under non reducing (a) and reducing (b) conditions.

3.3.4. Surface hydrophobicity

Understanding hydrophobic interactions is crucial for the development of food products containing high concentrations of protein ingredients, as they have a strong contribution to the stability and overall interfacial properties. The control sample had the lowest value of 830 ± 123 similarly ($p>0.05$) to the sample treated for 0 min, revealing a neglectable effect of the pumping in the semi-continuous system studied (Table 3.1). Treating the sample at 10,200 rpm for 1 min allowed for a significant ($p<0.05$) increase of the surface hydrophobicity to 1044 ± 26, which reached the maximum value of 1245 ± 43 for the sample treated for 5 min. This increase in surface hydrophobicity could be attributed to exposure of hydrophobic residues, otherwise buried, as a result of mechanical disruption (Goulding et al., 2021; Yu et al., 2018). The results are in agreement with the literature, where the application of mechanical forces (i.e., shear, cavitation, turbulences) caused an increase in surface hydrophobicity and thus an improvement of plant protein functionalities (i.e., emulsifying properties) (Xu et al., 2018; Yu et al., 2018, 2018). Enhanced surface hydrophobicity could lead to improvement of the emulsifying properties of proteins by promoting stronger adsorption at the oil-water interface. This effect is particularly beneficial in high-solid emulsions, where protein aggregation can otherwise reduce stability.

3.3.5. Colloidal stability

The physical stability of the dispersions was analysed by measuring the transmission of light through the samples during centrifugation. In Figure 3.5, representative light transmission profiles for each of the dispersions treated at 10,200 rpm from 0 to 15 min are shown. The control and the sample treated for 0 min, showed a low physical stability with a separation rate of 71.23 ± 18.15 $\%\cdot.h^{-1}$ and 69.67 ± 18.85 $\%\cdot h^{-1}$, after

the second centrifugating cycle, respectively. In addition, at low centrifugation speed (i.e., 800 rpm), it can be observed that the treated dispersions (1, 3, 5 and 15 min) did not undergo to any changes through the first cycle, indicating relative physical stability of the large, dispersed particle as also underlined by Crowley et al. (2019). When increasing the speed to 3000 rpm during the second cycle, a complete sedimentation and destabilisation of the samples can be measured. In particular, the separation rate of the dispersion decreased sharply ($p<0.05$) with the treatment of 1 min (36.57 ± 5.19 $\%\cdot h^{-1}$) indicating a slower movement of particles in the samples as a result of particle disruption and particle size reduction, which resulted in more colloidally stable dispersions. The separation rate decreased progressively to reach a value of 24.16 ± 2.58 $\%\cdot h^{-1}$ for the dispersion treated for 15 min. The results are in agreement with the literature and particularly Moll et al. (2021) who found that the reduction in particle size caused by physical treatment with microfluidisation allowed for an increase in colloidal stability and Jeske et al. (2019) using HPH at 18 MPa observed a reduction in separation rate from $48\%\cdot h^{-1}$ to $22\%\cdot h^{-1}$.

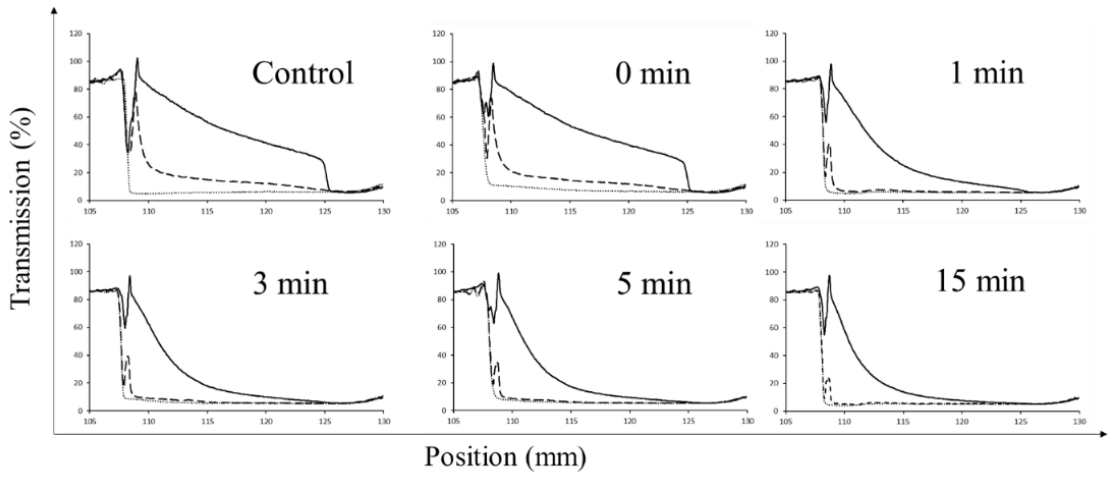

Figure 3.5. Accelerated physical stability profiles of lentil protein isolate dispersions (5% w/v powder) untreated (control) and treated with high-shear treatment for 0 min, 1 min, 3 min, 5 min and 15 min. Original light transmission of the dispersions (time 0min) (….) together with the profile after the first cycle (10 min at 800 rpm) (- -) and the profile after the second cycle (10 min at 3000 rpm) (-) are shown.

3.4. Conclusion

The effect of in-line high-shearing at 10,200 rpm on the techno-functional properties of lentil protein isolates was studied during treatment times ranging between 0 and 15 min. It was shown that the high-shear and long time provided by the high-shear mixer allowed for a 42% improvement in solubility of the protein ingredients from 46.87 ± 1.55 % of soluble protein at 0 min up to 68.42 ± 8.04 when treated for 15 min. This increase in solubility has been attributed to the high-shearing induced at the rotor and the passage through the small perforation of the stator, which also a significant ($p<0.05$) reduction in particle size from 5.13 ± 0.58 µm (at 0 min) to 1.72 ± 0.01 µm after 15 min. Furthermore, the particle disruption caused an increase in hydrophobicity due to the exposition of hydrophobic groups, which can be associated to an improvement of the emulsifying properties of the lentil protein isolate. The lentil protein isolate dispersions after in line high-shear treatment had an improved colloidal stability with values of 69.67%·h^{-1} at 0 min, down to 35.77%·h^{-1} after 1 min of treatment and further treatment for 15 min reduced it to 24.16%·h^{-1}. These results show that in-line high-shear mixers have the potential to improve the functional properties of lentil protein isolate for the formulation of more sustainable food products, including infant formulae with enhanced techno-functional properties.

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3.5.References

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Chapter 4

Lentil protein isolate dispersions with improved solubility and colloidal stability using high pressure homogenisation

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Declaration: N.M., J.A.O.M. and F.B. conceived and designed the experiments; N.M. performed the experiments and collated the data, N.M analysed the data and prepared the manuscript; N.M, F.B., E.K.A., E.Z. and J.A.O.M reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

Abstract

Lentil protein is a good alternative to animal protein due to its high protein content and good techno-functional properties. However, the limited solubility may be a hindrance to its use in food matrices. This study aimed to evaluate the impact of high-pressure homogenisation (HPH) in the range 0 to 180 MPa on the techno-functional properties of lentil protein isolate dispersions. Their solubility increased from 62.8 (control) to 86.5% (15 MPa), with a further increase in pressure up to 180 MPa resulting in a solubility of 95.3%. The weighted mean volume diameter decreased from 10.7 ± 1.1 (control) to 0.27 ± 0.01 µm (150 MPa). This reduction in particle size was attributed to disruptions and breakages induced during the homogenisation process, leading to the dissociation of insoluble protein aggregates. Surface hydrophobicity increased from 614 to 1312, due to the exposure of previously buried hydrophobic groups. The physical stability was improved with increased pressure as indicated by the separation rate from 8.55%/h (control) to 4.92%/h (180 MPa). These results indicate that HPH is a promising processing strategy to develop colloidally stable lentil protein dispersions with enhanced solubility and improved techno-functional properties for the further use of lentil protein ingredients in sustainable food products.

4.1. Introduction

In recent years, the urgent need to address global challenges, such as climate change, food insecurity, and chronic diseases, have highlighted the increasingly important contribution of plant proteins in securing a sustainable future food system (United Nations, 2022; McClements, 2020; Henchion et al., 2017). Protein concentrate and isolate ingredients extracted from plant sources such as legumes, pseudocereals, cereals, tubers, grains, nuts, and seeds possess diverse nutritional profiles and exhibit numerous health benefits (Day et al., 2022; Ma et al., 2022; Shaghaghian et al., 2022; Stone et al., 2019; Boye et al., 2010). Pulses, such as lentils, chickpeas, and peas, are leguminous crops that have been cultivated for centuries as valuable sources of protein, dietary fibre, vitamins and minerals. Among these, lentils have gained significant attention in the development of innovative food products for their high protein content, typically ranging from 20 to 30%, as well as being a rich source of essential amino acids (Boye et al., 2010). Lentil proteins possess good techno-functional properties, including emulsifying properties, thanks to their amphiphilic features and ability to form a thick interfacial protein layer around the oil droplet (Can-Karaca et al., 2011, 2015). However, their limited solubility hinders their more widespread use in food products (Grossman and McClements, 2023; Can-Karaca et al., 2011). To improve the functional properties of plant-based protein ingredients, the potential of chemical and enzymatic modifications, in addition to physical treatments, have been explored (Gharibzahedi et al., 2023; Bou et al., 2022; Mirmoghtadaie et al., 2016). Among those, high-pressure homogenisation (HPH), a continuous, cost-effective, non-thermal processing technique, commonly used in the formation and stabilisation of emulsions, has the potential to modify the protein structure, and thereby improve the techno-functional properties of protein dispersions (Gharibzahedi et al., 2023, Sá et al., 2022;

Avelar et al., 2021; Tobin et al., 2015). During such HPH treatment, the fluid sample is forced through a narrow gap which induces a sudden drop in pressure, resulting in the formation of high velocity jets and turbulence, in addition to cavitation, involving the formation and subsequent collapse of tiny bubbles within the fluid. Together, turbulence and cavitation generate intense shear forces that contribute to particle disruption and size reduction (Levy et al., 2021; Dumay et al., 2013; Diels et Michiels, 2006). It has been shown that HPH, applied in the pressure range 70-150 MPa can significantly modify the physical properties of pea protein isolate dispersions, with a decrease in particle size together with an increase in solubility and emulsifying properties (D'Alessio et al., 2023; Keuleyan et al., 2023; Zhao et al. 2022; Melchior et al., 2022; Moll et al., 2022; Ong et al., 2022). Similar effects of HPH on other plant proteins such as soy protein (Liang et al., 2022; Xu et al., 2018; Song et al., 2013) and faba beans (Yang et al., 2018) have also been observed.

Even though the effects of HPH on pea protein and some other plant protein sources (e.g., soy and faba beans) have been documented, very little information can be found on the impact of HPH on lentil protein isolate. To the authors' knowledge, only Saricaoglu et al. (2020) have focused on understanding the impact of HPH on lentil protein isolate (LPI) dispersions. The authors used a pressure range of 0-150 MPa and evaluated solubility, emulsifying, foaming and rheological properties of the LPI dispersions obtained (4% protein w/v). The results showed that HPH treatment decreased the particle size from 98.73 μm to 12.62, with the lowest value reached for the treatment at 150 MPa, and increased the solubility of LPI from 32% to 46%, with the highest solubility also achieved at a 75 MPa. In this study, the emulsifying and foaming properties of LPI also improved with HPH treatment in the pressure range 50-100 MPa. Their study demonstrated that HPH reduced particle size and increased

solubility, as well as improved emulsifying and foaming properties. Yet, this work was limited to a protein concentration of 4% (w/v) and a maximum pressure of 150 MPa. There remains a need to further explore the effects of HPH on LPI across a broader range of pressures, higher protein concentrations, and additional functional properties.

Therefore, the aim of this study was to investigate the effect of HPH, in the range 0-180 MPa, on the particle size distribution, solubility, hydrophobicity, rheological properties and colloidal stability of lentil protein dispersions. This work is essential to improve the understanding of the impact of HPH on plant protein-based ingredients and will support their greater use in food structures and matrices, such as emulsions, with enhanced techno-functional properties and physical stability.

4.2.Materials and methods

4.2.1. Preparation of lentil protein isolate dispersions

The lentil protein dispersions were prepared by rehydrating red lentil protein isolate powder (79.63% protein, w/w), provided by the Fraunhofer Institute (Munich, Germany), at a concentration of 5% (w/v) in pre-heated deionised water (70°C), stirred magnetically at 300 rpm for 1 h at 20°C. The dispersions were then adjusted to pH 6.8 and allowed to rehydrate fully at 4°C for 18 h, after which the samples were equilibrated at 20°C and pH was readjusted, if necessary, to pH 6.8.

4.2.2. High pressure homogenisation treatment

Lentil protein dispersions were first subjected to a brief high shear mixing regime at 12000 rpm for 2 min at 20°C, to ensure homogenous feed dispersion. The dispersions were then subjected to homogenisation using a two-stage high pressure valve homogeniser (APV 2000, SPX flow, North Carolina, United States), in one pass, at total pressures of 0, 15, 35, 50, 100, 150 and 180 MPa, with first to second stage

pressure ratio of 83:17 for each treatment. A corresponding control sample (i.e., no homogenisation) was also included in analysis.

4.2.3. Particle size distribution

The particle size distribution (PSD) of the emulsions was measured using a Mastersizer 3000 static light scattering instrument equipped with an automated Hydro MV wet and dry cell disperser (Malvern Instruments, Worcestershire, UK), for the dispersion and powder sample formats, respectively. The refractive index of protein was set at 1.45, the adsorption index used was 0.001 and the dispersant index was 1.33. The dispersions were measured at 20°C, and dispersed into ultrapure water until a laser obscuration of 10% was reached. The results are reported as the volume-weighted mean particle size (D[4,3]), as well as particle size below which 10% of sample volume is found (Dv(10)), particle size below which 50% of sample volume is found (Dv(50)), particle size below which 90% of sample volume is found (Dv(90)) and specific surface area. Each dispersion was sampled 3 times and measured 3 times by the instrument for a total of 9 measurements.

4.2.4. Rheological analysis

The rheological properties of the dispersions (5% (w/v)) were determined using a modular compact rheometer MCR 102e equipped with a concentric cylinder geometry (Anton Paar, Austria). The cell had an internal diameter of 28.9 mm, the diameter of the rotor was 26.7 mm, and the gap between the two elements at the geometry base was 2 mm. The sample (20 ml) was loaded into the cylinder, pre-heated at 20°C, after which the shear rate was increased to 100 s^{-1} over 30 s and maintained at 100 s^{-1} for 30 s. Throughout the analysis, a fixed temperature of 20°C was maintained, and viscosity was measured continuously. The Ostwald-de Waele model (Eq.1) was used

to describe the rheological properties of the protein dispersions, while ensuring that $R^2 \geq 0.95$

$$\tau = K\dot{\gamma}^n \quad (1)$$

Where τ is the shear stress (mPa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the flow consistency index ($mPa \cdot s^n$) and n is the flow behaviour index (dimensionless).

4.2.5. Protein solubility

The protein solubility of the dispersions was determined by measuring the concentration of protein in the supernatant obtained after centrifugation at 1000 g for 20 min at 20°C using an RC5C plus centrifuge (Thermo Scientific™ Sorvall™, Massachussets, USA) with a GSA rotor (Alonso-Miravalles et al., 2019). The protein content of the supernatant was measured using the Kjeldahl method with a nitrogen-to-protein conversion factor of 6.25 (IDF, 2001); this conversion factor was selected since a specific ones for lentil is not available in the literature and to be consistent with the work of Vogelsang-O'Dwyer et al. (2023) and Alonso-Miravalles et al. (2019).

4.2.6. Protein profile analysis

The protein profile of the dispersions, and their fractions, was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with precast gels (Mini-PROTEAN TGX, Bio-Rad Laboratories, CA, USA), following the method developed by Laemmli (1970), with minor modifications. The samples were centrifugated at 1000g for 20 min at 20°C using an RC5C plus centrifuge (Thermo Scientific™ Sorvall™, Massachussets, USA) with a GSA rotor to isolate the soluble protein in the supernatant, and diluted to a protein concentration of 2 mg/ml. In method I (non-reducing conditions), 100 µl of the diluted supernatant was mixed with 100 µl of sample loading buffer (2X Laemmli sample buffer, Bio-Rad Laboratories, CA, USA) whereas in method II (reducing conditions), 100 µl of the diluted dispersion was

mixed with 95 µl of sample loading buffer and 5 µl of β-mercaptoethanol and incubated at 95 °C for 10 min. Each well was loaded with 7 µl of sample solution and 5 µL of SDS-PAGE broad range protein standard (BioRad, Hercules, CA) was loaded as a protein molecular weight standard. The gels were run in a running buffer (10x Tris/Glycine/SDS, Bio-Rad Laboratories, CA, USA) at 160 V for 1 h, then fixed for 15 min using a solution of water:methanol:acetic acid (40:50:10), stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, CA, USA) and destained using a solution of water:methanol:acetic acid (50:40:10) until a clear background was achieved. The gels were then stored at 4°C in a 5% (v/v) acetic acid solution overnight to reverse the shrinkage induced during the fixing and destaining steps.

4.2.7. Surface hydrophobicity

Hydrophobicity of proteins in the supernatant (dispersion centrifugated at 1000 g for 20 min as described in Section 4.2.6) was measured using the method previously described by Nakai (2003) and Goulding et al. (2021), with minor modifications, using the hydrophobic probe 8-Anilino-1-naphthalenesulfonic acid (ANS). Samples were diluted to each of the following concentrations, 0.003, 0.006, 0.009, 0.012 and 0.015% (v/v) in sample buffer (0.01 M phosphate buffer, pH 7) and 100 µL of the diluted solutions were transferred to a 96 black microwell plate, after which 50 µL of ANS solution (8 mM in 0.1 M phosphate buffer) was added. The well plate was then covered with foil and gently rocked for 15 min on a plate shaker. After this, the fluorescence intensity of the samples was measured using a Varioskan Flash microplate reader (Fisher Scientific, Ireland) with excitation wavelength of 390 nm and emission wavelength of 470 nm. The fluorescence intensity was plotted as a function of protein concentration (%, v/v) and only linear regression with an R^2 higher than 0.98 were

considered. The slope (S_0) value was used to compare the surface hydrophobicity of proteins in the different samples.

4.2.8. Accelerated physical stability

The colloidal stability of the dispersions was measured using an analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). The samples were centrifugated at 117 g for 8 h with near-infrared light illuminating the samples throughout the analysis and the intensity of the transmitted light over the entire length of the sample was measured every 10 s. The separation rate was determined by plotting the integral of the intensity of the transmitted light over time and extracting the slope (%/h). In addition, the thickness (i.e., height) of the sediment layer (mm) was measured on the last profile for each sample.

4.2.9. Statistical data analysis

All samples were formulated in independent triplicate trials and all analyses were conducted in triplicate, unless otherwise stated. The data generated was subject to one-way analysis of variance (ANOVA) using R version 4.3.1 (R foundation for statistical computing, Vienna, Austria). A paired comparison test was used to determine statistically significant differences ($p < 0.05$) between mean values for different samples, at a 95% confidence level.

4.3.Results and discussion

4.3.1. Particle size distribution

The volume-weighted mean particle diameter (D[4,3]) and span of the lentil protein powder, as well as untreated (control) and high pressure homogenised lentil protein dispersions, are shown in Table 4.1. The control sample had a D[4,3] value of 10.70 ± 1.07 µm, similar to the lentil protein isolate powder (14.2 ± 0.0 µm), and span (1.12 ± 0.06). This was smaller than the powder particle span (2.23 ± 0.04), indicating a narrower size distribution of particles after rehydration, while undissolved powder particles were still present. Similar overall PSD's for lentil protein dispersions at 5% (w/v) produced under similar conditions have been reported by Alonso-Miravalles et al. (2019) and Vogelsang-O'Dwyer et al. (2023). The sample processed at 0 MPa had similar PSD parameters (D[4,3] of 10.87 ± 0.49 µm and Span of 1.11 ± 0.04) to that of the control sample. This analysis confirmed that the pumping and liquid transfer during homogenisation had negligible effects on PSD. On increasing homogenisation pressure, the particle size of lentil protein dispersions decreased significantly ($p<0.05$), with D[4,3] of 1.15 ± 0.10 µm at the lowest pressure (15 MPa), decreasing to 0.27 ± 0.01 µm at 150 MPa. Dv(10) and Dv(50), which correspond to the particle size below which 10 and 50% of the sample volume are found, respectively, decreased sharply with the application of pressure, before reaching a plateau at homogenisation pressures of 15 MPa and above (Figure 4.1). On the other hand, the Dv(90) (corresponding to the particle size below which 90% of the sample volume is found) decreased progressively with increasing homogenisation pressure. The dispersion treated at 15 MPa showed a large span of 15.1 ± 3.22, while on increasing homogenisation pressure up to 180 MPa, the span progressively decreased to reach 3.96 ± 0.08, indicating a narrower distribution centred around the mean for samples treated at higher pressures.

Furthermore, as a result of the particle breakage induced by HPH, the specific surface area increased from 0.50 $m^2.g^{-1}$ for the control sample to 67.26 $m^2.g^{-1}$ for the sample treated at 180 MPa (Figure 4.1). This increase allowed for the individual powder particles to have greater exposure to, and possibility for interactions with, water, which would be expected to enable increased dissolution of the powder. This is in agreement with the Noyes Whitney equation, which expresses the dissolution rate of a powder over time as a function of saturation solubility and powder concentration in the dispersion at a given time (Dokoumetzidis and Macheras, 2006; Noyes and Whitney, 1897).

Table 4.1. Particle size distribution parameters, protein solubility and surface hydrophobicity for lentil protein isolate powder and protein dispersions (5% protein powder, w/v) untreated (control) and treated with high pressure homogenisation at 0, 15, 35, 50, 100, 150 and 180 MPa.

	D[4,3] (µm)	**Span (µm)**	**Protein solubility (%)**	**Surface hydrophobicity (-)**
Powder	14.2 ± 0.00^{a}	2.23 ± 0.04^{a}	n/a	n/a
Control	10.7 ± 1.07^{b}	1.12 ± 0.06^{b}	62.5 ± 2.63^{a}	614 ± 66^{a}
0 MPa	10.9 ± 0.49^{b}	1.11 ± 0.04^{b}	62.8 ± 1.01^{a}	607 ± 29^{a}
15 MPa	1.15 ± 0.10^{c}	15.1 ± 3.22^{c}	86.5 ± 1.73^{b}	804 ± 84^{b}
35 MPa	0.52 ± 0.08^{c}	4.88 ± 0.59^{d}	89.8 ± 0.53^{c}	948 ± 87^{c}
50 MPa	0.39 ± 0.03^{d}	4.10 ± 0.06^{d}	92.7 ± 0.91^{d}	1075 ± 65^{cd}
100 MPa	0.28 ± 0.02^{ef}	3.88 ± 0.12^{e}	94.3 ± 0.92^{e}	1147 ± 119^{de}
150 MPa	0.27 ± 0.01^{f}	3.86 ± 0.07^{e}	96.4 ± 0.55^{f}	1271 ± 50^{ef}
180 MPa	0.33 ± 0.06^{de}	3.96 ± 0.08^{e}	95.3 ± 0.68^{e}	1312 ± 82^{f}

Values within a column that share a superscript are not significantly different from one another ($p<0.05$).

D[4,3] = volume-weighted mean particle diameter.

Span = measurement of the width of the distribution calculated as: (Dv(90) – Dv(10)) / Dv(50).

n/a: not applicable

Considered collectively, these results demonstrate that HPH is very effective in reducing the particle size of lentil protein dispersions. The reduction in particle size can be attributed to the breakage induced by intense mechanical forces, cavitation, shear, and turbulence, generated by the forced passage of protein dispersions through the small orifices of the valve gaps in the homogeniser, which allowed the disruption of protein aggregates into supramolecular aggregates, with mean particle size <1 µm (Moll et al., 2021; Yang et al., 2018; Dumay et al., 2013). These results are in agreement with the literature, in particular, Saricaoglu (2020) who reported similar results following the HPH treatment of lentil protein isolate dispersions, with processing at 20 MPa allowing for a reduction of D[4,3] from 98.73 ± 3.32 µm to 22.50 ± 0.40 µm. A limited number of previous studies (Song et al., 2013; Ong et al., 2022; Keuleyan et al., 2023) have investigated the influence of HPH on selected functional properties of plant proteins other than lentil, including pea and lupin protein isolate. For example, Keuleyan et al. (2023) demonstrated that homogenisation at 30 MPa was effective in bringing about a considerable shift in mean particle size from 13 to 1.1 µm for lupin protein isolate dispersions. Similarly, Ong et al. (2022) reported a reduction in mean particle size of pea protein isolate dispersions from 31.7 ± 0.5 µm to 9.4 ± 0.2 µm on HPH treatment at 60 MPa.

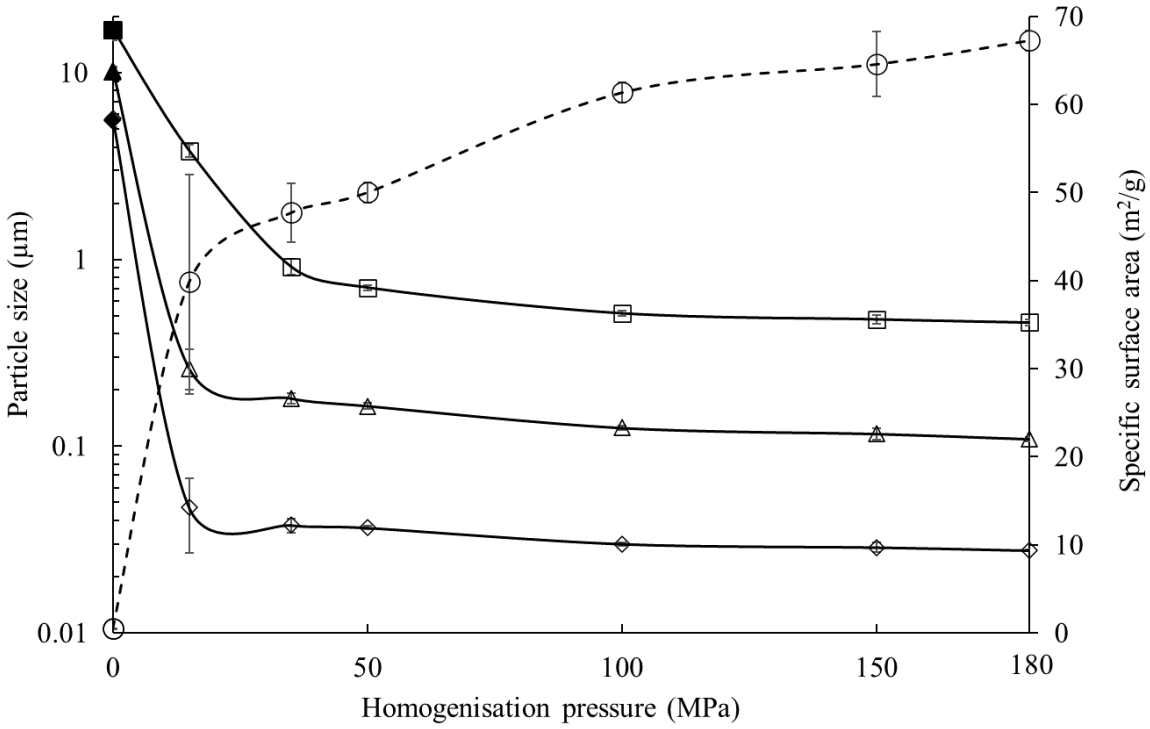

Figure 4.1. Particle size distribution parameters (-◇-, Dv(10), particle size below which 10% of sample volume is found), (-△-, Dv(50), particle size below which 50% of sample volume is found), (-□- Dv(90), particle size below which 90% of sample volume is found), (--○--, specific surface area) of protein dispersions (5% w/v) untreated (control) and treated with high pressure homogenisation at 0, 15 35, 50, 100, 150 and 180 MPa. Filled shapes (-■-,-▲- , -◆- and -●-) correspond to the control dispersion.

4.3.2. Rheological properties

The rheological properties of protein dispersions are primarily governed by their composition, concentration, and intrinsic properties of the protein constituents including, size, shape, charge, and interactions. In addition, other environmental and external factors such as temperature, pH, ionic strength, and pre-treatments play significant roles in influencing these properties (Ma et al., 2022; Nikbakht Nasrabadi et al., 2021; Jeske et al. 2019; Tung, 1978). Table 4.2 presents the apparent viscosity

of the dispersions measured at 100 s^{-1} and 20°C, as well as the flow consistency index (K) and flow behaviour index (n). The apparent viscosity of the control dispersion showed a relatively low value of 1.75 ± 0.12 mPa·s, which was expected as the dispersion has a relatively low protein concentration (5% w/v); these results were similar to those of Saricaoglu (2020) using lentil protein isolate dispersions (4.76% w/v). The apparent viscosity of the homogenised dispersions ranged between 1.70-1.82 mPa·s, showing that on increasing the homogenisation pressure no significant changes ($p>0.05$) were observed in either apparent viscosity or consistency index. All the lentil protein dispersions displayed non-Newtonian pseudoplastic behaviour as shown by the flow behaviour index (n) with values ranging from 0.58 ± 0.07 to 0.71 ± 0.16, typical of shear-thinning fluids. Similar values have been reported previously for lentil, soy and pea protein dispersions (Melchior et al, 2022;

Saricaoglu, 2020; Song et al., 2013).

Table 4.2. Apparent viscosity and flow behaviour rheological parameters of protein dispersions (5%, w/v, protein powder) untreated (control) and treated with high pressure homogenisation at 0, 15, 35, 50, 100, 150 and 180 MPa.

Treatment	η (mPa·s)	*n* (-)	K (mPa·s^n)
Control	1.75 ± 0.12^a	0.61 ± 0.02^a	$9.30\times10^{-3} \pm 1.25\times10^{-3\ a}$
0 MPa	1.83 ± 0.13^a	0.58 ± 0.07^a	$9.47\times10^{-3} \pm 5.69\times10^{-4\ a}$
15 MPa	1.87 ± 0.04^a	0.68 ± 0.10^a	$8.57\times10^{-3} \pm 9.71\times10^{-4\ a}$
35 MPa	1.70 ± 0.11^a	0.65 ± 0.08^a	$9.43\times10^{-3} \pm 1.39\times10^{-3\ a}$
50 MPa	1.73 ± 0.14^a	0.68 ± 0.13^a	$7.50\times10^{-3} \pm 1.11\times10^{-3\ a}$
100 MPa	1.82 ± 0.12^a	0.71 ± 0.06^a	$6.83\times10^{-3} \pm 1.59\times10^{-3\ a}$
150 MPa	1.74 ± 0.13^a	0.71 ± 0.16^a	$8.13\times10^{-3} \pm 1.53\times10^{-3\ a}$
180 MPa	1.74 ± 0.13^a	0.61 ± 0.02^a	$8.03\times10^{-3} \pm 2.00\times10^{-3\ a}$

Values within a column that share a superscript are not significantly different from one another ($p<0.05$).

η = Apparent viscosity at 100 s^{-1}

K = Flow consistency index (mPa·s^n), *n* = Flow behaviour index (-)

4.3.3. Protein solubility and protein profile

Solubility of protein ingredients in water is an essential parameter to understand and control, as it greatly influences the colloidal stability and functional properties (e.g., emulsification) of proteins. The solubility of the control dispersion was 62.50 ± 2.63% (Table 4.1), which was similar to the sample passed through the homogeniser with no pressure applied (62.8 ± 1.01%). Homogenisation of the lentil protein dispersions increased considerably the solubility, with the lowest pressure applied, 15 MPa, increasing solubility to 86.5 ± 1.73%, with solubility increasing progressively to 96.4 ± 0.55% with increasing homogenisation pressure up to 150 MPa. A pressure of 50 MPa was identified as the most optimal condition, resulting in improved solubility without the adverse effects associated with higher pressures. This increase in solubility is in agreement with findings for other plant protein dispersions, such as pea protein isolate and concentrate treated at 80 and 150 MPa, respectively (Zhao et al., 2022; Melchior et al., 2022), soy protein isolate treated at 207 MPa (Song et al., 2013), and faba bean protein concentrate treated at 103 MPa (Yang et al., 2018). The increase in solubility could be due to the reduction of particle size due to the disruption of large protein aggregates, favouring the dispersion and hydration of proteins (Yang et al., 2018; Yu et al., 2018; Bader et al., 2011). Furthermore, the shearing induced by intense mechanical forces, cavitation, shear, and turbulence may enable unfolding of the protein, leading to greater protein-solvent interactions, as reported by Yu et al. (2018), Chen et al. (2016) and Song et al. (2013).

To obtain a deeper understanding of the mechanisms of protein solubilisation in this study, the protein profile of the soluble fractions of the samples, obtained by centrifugation, were analysed (Figure 4.2a & b). The supernatant contained proteins with molecular weight (MW) of ~50 kDa which may correspond to vicilin subunits,

whereas the bands at 37 and 25 kDa may denote the presence of the acidic and basic subunits of legumin (Jarpa-Parra et al., 2015; Barbana and Boye, 2013; Joshi et al., 2012) similar to that reported by Alonso-Miravalles et al. (2019) studying lentil protein isolate. Under reducing conditions, the intensity of the bands at ~50 and ~37 kDa decreased, while intensity of the bands at ~20-25 kDa increased, due to the dissociation of legumin into its acidic and basic subunits following dissociation of disulphide bonds, as reported by Alonso-Miravalles et al. (2019). It can be observed that on increasing homogenisation pressure up to 180 MPa, the protein profiles of all samples were very similar to those of the control samples, indicating that the profile of proteins solubilised by HPH treatment was not influenced by homogenisation pressure.

4.3.4. Surface hydrophobicity

Hydrophobic interactions contribute very important roles in the stability, conformation and function of proteins and also influence interfacial properties thereof. The supernatant fractions of the control and 0 MPa treated samples showed the lowest surface hydrophobicity of 614 ± 66 and 607 ± 29 respectively, with no significant differences ($p>0.05$) between the two samples (Table 4.1). HPH treatment caused a significant increase ($p<0.05$) in the surface hydrophobicity of the soluble fractions of the lentil proteins, with, for example, a value of 1075.68 ± 65.1 for the sample treated at 50 MPa. Increasing homogenisation pressure further caused only slight increases in surface hydrophobicity, with a maximum value of 1312.44 ± 82.89 for the samples treated at 180 MPa. These results are in agreement with the increases in hydrophobicity reported in other studies investigating the impact of HPH on protein dispersions (Guo et al., 2021; Yang et al., 2018; Yu et al., 2018; Xu et al., 2018). HPH has the potential to cause the unfolding of protein and exposure of hydrophobic groups, otherwise buried, increasing the surface hydrophobicity of the proteins, thereby modifying

interfacial properties of the ingredients. For example, Yang et al. (2018), demonstrated that HPH treatment at 150-300 MPa of faba bean resulted in increased surface hydrophobicity which allowed enhanced subsequent protein diffusion at air-water interfaces.

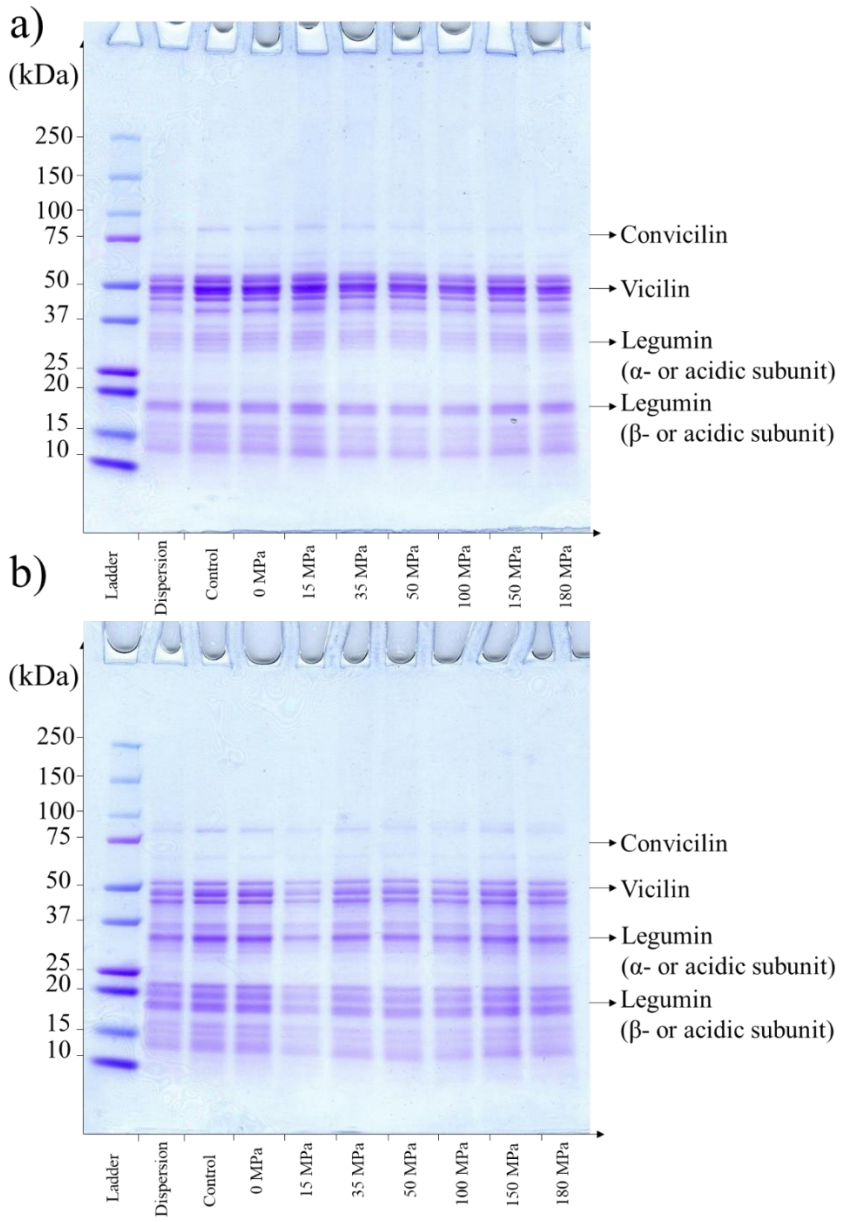

Figure 4.2. Representative sodium dodecyl sulphate-polyacrylamide gel electrophoretograms (SDS-PAGE) of lentil protein isolate dispersions (5% w/v) untreated, control supernatant untreated and supernatant treated with high pressure

homogenisation at 0, 15, 35, 50, 100, 150 and 180 MPa under non reducing (a) and reducing (b) conditions.

4.3.5. Colloidal stability

The stability of the protein dispersions was evaluated by measuring the transmission of light through the samples during centrifugation, with Figure 4.3 showing examples of transmission profiles for each dispersion. The initial transmission of light through the samples increased with homogenisation pressure, with the control and 180 MPa samples displaying transmission values of 8 and 20%, respectively. This is in agreement with the results for particle size analysis presented earlier (Table 4.1), as smaller particles scatter less light and allow for higher light transmission through the dispersions (Kleizen et al., 1995). Similarly, Moll et al. (2021) studied microfluidisation of pea protein and reported that samples treated at 150 MPa had higher light transmission.

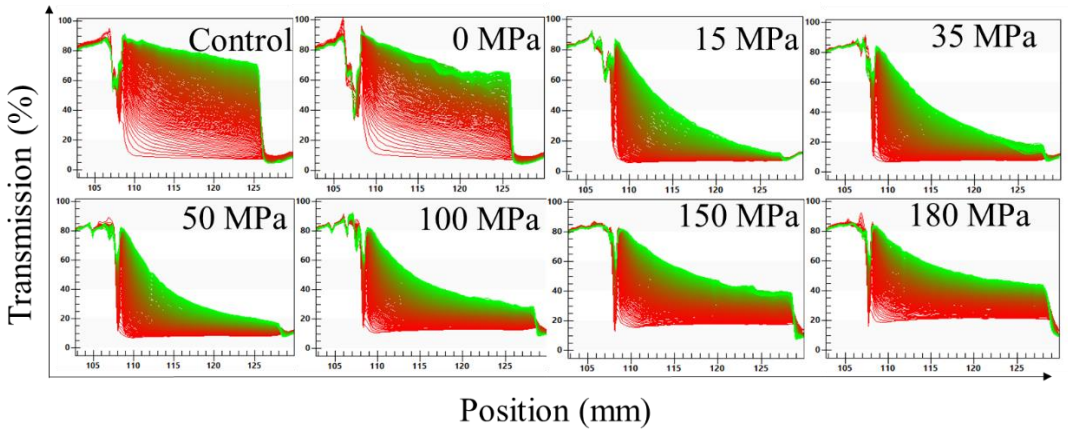

Figure 4.3. Accelerated physical stability profiles of lentil protein isolate dispersions (5% w/v protein powder) untreated (control) and treated with high-pressure homogenisation at 0, 15 35, 50, 100, 150 and 180 MPa, where the first and last transmission profiles are red and green, respectively.

The control dispersion and that treated at 0 MPa were similarly unstable with a rapid increase of transmission (red and green being the first and last profiles, respectively), and extensive sedimentation. More particularly, the control and 0 MPa treated samples displayed separation rates of 8.55 ± 1.40 and 8.12 ± 0.65 %/h, respectively, in addition to sediment height of 4.31 ± 0.09 and 4.20 ± 0.09 mm, respectively (Figure 4.4). The separation rate decreased sharply with increasing homogenisation pressure and plateaued at 4.51 ± 0.66 %/h with the pressure of 15 MPa.

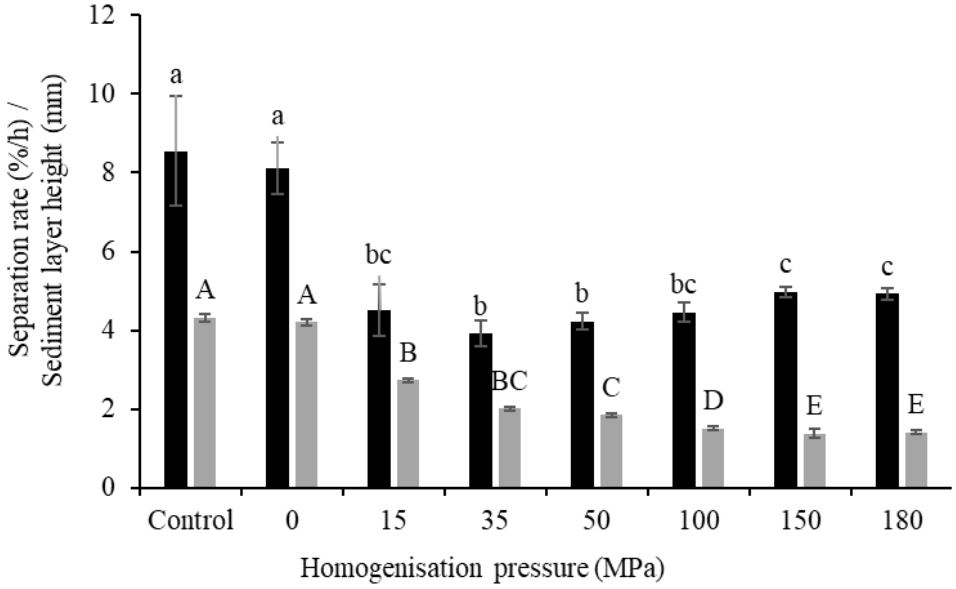

Figure 4.4 Separation rate (black) and sediment layer height (light grey) of lentil protein isolate dispersions (5% w/v protein powder) untreated (control) and treated with high pressure homogenisation at 0, 15 35, 50, 100, 150 and 180 MPa. Labels over the bars, in the same case (i.e., upper- or lower-case) sharing a superscript, are not significantly different from one another ($p<0.05$).

Conversely, the height of the sediment layers decreased progressively with increasing pressure from 4.31 ± 0.09 mm for the control dispersion, down to 2.74 ± 0.05 mm for the dispersion treated at 15 MPa, and 1.41 ± 0.04 mm for the dispersion treated at 180 MPa. The results are in agreement with the literature, as Moll et al. (2021) found that physical treatment at 150 MPa increased the physical stability of pea protein.

4.4. Conclusion

The effect of high-pressure homogenisation on the techno-functional properties of lentil protein isolate dispersions was studied across the pressure range from 0 to 180 MPa. HPH treatment strongly effected the solubility of the dispersions, which was influenced by the significant ($p<0.05$) reduction in particle size from volume-weighted mean diameter of 10.7 ± 1.1 μm for the control dispersion to 0.27 ± 0.01 μm for the dispersion treated at 150 MPa, due to the intense mechanical forces, cavitation, shear, and turbulence. Hydrophobicity was increased as the unfolding of protein allowed for buried hydrophobic groups to be exposed. Furthermore, the accelerated physical stability analysis showed that HPH could produce more colloidally stable protein dispersions, with reduced rate and extent of sedimentation. The application of 50 MPa emerged as the most suitable condition, optimising functionality without overprocessing the sample. These results indicate that HPH can be used as a processing strategy for developing colloidally stable lentil protein dispersions with high solubility and enhanced techno-functional properties.

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Chapter 5

Enhancing the thermal and colloidal stability of lentil protein-stabilised emulsions using high pressure homogenisation pre-treatment

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Declaration: This chapter was written by author Nicolas Malterre (NM) and reviewed by co-authors James A. O'Mahony (JAOM) and Francesca Bot (FB). NM co-designed the study with co-author JAOM and FB and performed the experimental work.

Abstract

This study examines the enhancement of thermal and colloidal stability of lentil protein-stabilised emulsions through high-pressure homogenisation (HPH) pre-treatments. Lentil protein dispersions were homogenised at pressures ranging from 0 to 150 MPa and subsequently used to formulate emulsions at high total solid concentrations (29% w/w). The impact of HPH pre-treatments on protein solubility, particle size distribution, rheological properties and colloidal stability was evaluated. Results indicated that HPH significantly improved protein solubility, with an increase from 55.7% at 0 MPa to 93.2% at 50 MPa. Emulsions prepared from HPH pre-treated dispersions exhibited improved physical stability, with separation rates decreasing from 16.75%/h (0 MPa) to 2.05%/h (150 MPa). Rheological analysis demonstrated that HPH pre-treatment led to lower initial apparent viscosities (28.30 and 22.56 mPa·s at 0 and 150 MPa, respectively) as well as a lower viscosity increase upon heat treatment, resulting in lower final viscosity (60.52 and 34.88 mPa·s at 0 and 150 MPa, respectively) than the untreated samples. The smaller changes in final viscosity indicate an enhancement in the thermal stability of the treated samples. Confocal laser scanning microscopy confirmed a more homogeneous distribution of oil globules and reduced flocculation in emulsions prepared from HPH-treated dispersions. These findings highlight the potential of HPH as an effective pre-treatment to enhance the techno-functional properties of lentil protein-stabilised emulsions, supporting the development of stable and sustainable plant-based food products.

5.1. Introduction

As the global population grows towards an estimated 9 billion by 2050, the need for sustainable nutritious food has never been more pronounced (Springmann, 2016). Concurrently, the requirements of climate change, food security, and the prevalence of chronic diseases require a shift towards more sustainable and healthful food production systems. In this context, plant-based food products offer a promising alternative in terms of environmental sustainability, health benefits, and economic viability (Vogelsang-O'Dwyer et al., 2021; McClements, 2020; Willet et al., 2019). Plant proteins, sourced from legumes, pseudocereals, cereals, and other botanical origins, not only offer a reduced environmental impact relative to animal protein-based food systems, but also cater to the nutritional requirements of a diverse global population (Alonso-Miravalles et al., 2020; Sim et al., 2021).

Among the range of plant proteins available commercially, and in the advanced stages of development, lentil protein emerges as a particularly interesting one. Lentils, with their rich content of essential amino acids, dietary fibres, vitamins, and minerals, offer a promising nutritional profile (Berghout et al., 2014; Day, 2013; Boye et al., 2010). Furthermore, their relatively good techno-functional properties, such as emulsification (Geerts et al., 2017; Can-Karaca et al., 2011, 2015), make them ideal for a wide range of food applications, from dairy alternatives to infant nutrition. However, the wider adoption of lentil proteins in food products is impaired by its limited solubility, a pivotal techno-functional attribute that influences processing and product quality. Indeed, solubility affects not only the sensory attributes, but also the colloidal stability of food formulations and the bioavailability of nutrients (Grossman and McClements, 2023; Grossman and Weiss, 2021; Akharume et al., 2021).

To improve the techno-functional properties of plant protein dispersions, multiple physical treatments involving high shear forces have been investigated over the years (Gharibzahedi et al., 2023; Avelar et al., 2021). Among these, high-pressure homogenisation (HPH) has been shown to be a promising processing technique which can alter protein structure (Levy et al., 2021; Bouaouina et al., 2006), consequently enhancing the techno-functional attributes of protein dispersions. In this regard, several authors have investigated the effect of HPH on the techno-functional properties of plant protein dispersions, such as Melchior et al. (2022) and Keuleyan et al. (2023) for pea protein, Xu et al. (2018) and Liang et al. (2022) for soy protein and Yang et al. (2018) for faba bean proteins. However, only a few studies have been found in the literature regarding the effects of HPH on lentil protein dispersions (Navare et al., 2023; Saricaoglu et al., 2018). Specifically, Navare et al. (2023) reported that the use of HPH, at pressures up to 350 MPa, allowed for an improvement of the solubility of the lentil protein concentrate dispersions, while having minor effects on emulsifying and foaming properties. The authors also found that the use of higher pressure, up to 550 MPa, induced a deterioration of the functional properties, which was attributed to the increase in surface hydrophobicity of the proteins.

Taking this further in the direction of food formulation and design, to the author's knowledge, only Primozic et al. (2018) has investigated how specific techno-functional properties of lentil protein-stabilised emulsions may be modified by treatment with HPH. The authors demonstrated that homogenisation of lentil protein dispersions (1%, w/w) at 34.5 and 103.4 MPa allowed for a reduction in particle size. Additionally, when formulating emulsions using HPH pre-treated dispersions, the authors observed decreases in surface average oil droplet diameter from 0.248 ± 0.028 μm in the untreated lentil protein emulsions, to 0.199 ± 0.032 and 0.196 ± 0.020 μm

in the lentil protein emulsions HPH pre-treated at 34.5 and 103.4 MPa, respectively. These changes were attributed to HPH-induced differences in interfacial viscoelasticity, due to particle size reduction of the dispersion, and suggested that a less strong protein network at the oil-water interface could allow for better droplet disruption during emulsification and therefore lead to smaller oil droplets. However, when using a higher concentration (2%, w/w) of protein in the dispersion during pre-treatment, the subsequent emulsions did not indicate changes in oil droplet size. They also found that after 28 days of storage, the emulsions formulated using HPH pre-treated protein showed less changes in particle size and less clarification, indicating greater physical stability induced by HPH pre-treatment. Although previous studies have shown the impact of HPH pre-treatment for enhancing plant protein-stabilised emulsification properties (Navare et al., 2023; Zhang et al., 2022; Primozic et al., 2018), limited information is available on the techno-functional properties and heat stability of emulsions at high total solids, similar to those found in young child infant formulae.

Therefore, this study aims to investigate the techno-functional properties of lentil protein-stabilised emulsions formulated with lentil protein isolate (LPI) subjected to HPH pre-treatment at 0-150 MPa. The impact of HPH pre-treatments on the protein solubility of the dispersion, as well as on the particle size distribution, rheological properties, and colloidal stability of lentil protein-stabilised emulsions formulated at high solid concentration (29%) will be evaluated. This work will help to tailor the processing of plant protein ingredients, by achieving an optimal treatment that enables the enhancement of techno-functional properties of oil-in-water emulsions, and support the further development of novel, functional and sustainable plant-based food products.

5.2.Materials and methods

5.2.1. Materials

Red lentil protein isolate (LPI) (72.15% protein, w/w) was provided by Döhler GmbH (Darmstadt, Germany). Sunflower oil was obtained from a local commercial outlet (Tesco, Welwyn Garden City, Hertfordshire, United Kingdom), and maltodextrin with a dextrose equivalent value of 17 (Glucidex® 17) was obtained from Roquette Frères (Lestrem, France). All the reagents used in this study were of analytical grade and supplied by Sigma-Aldrich (St Louis, MO, USA), unless otherwise stated.

5.2.2. High-pressure homogenisation pre-treatment of protein dispersions

Lentil protein dispersions were prepared (Figure 5.1) by rehydrating red LPI powder at a concentration of 6.5% (w/v) in pre-heated deionised water (50°C), stirred magnetically at 300 rpm for 1 h at 20°C. The dispersions were then adjusted to pH 6.8 and subjected to a brief high shear mixing regime at 12000 rpm for 2 min at 20°C, to ensure a homogenous feed dispersion for subsequent homogenisation. The dispersions were homogenised using a two-stage high pressure valve homogeniser (APV 2000, SPX flow, North Carolina, United States), operating in a single pass configuration, at total pressures of 0, 15, 50, and 150 MPa, with a first to second stage pressure ratio of 83:17 for each treatment.

5.2.3. Protein solubility

An RC5C plus centrifuge (Thermo ScientificTM SorvallTM, Massachusetts, USA) with a GSA rotor was used to determine the protein solubility of the dispersions before and after HPH pretreatment, as described previously by Alonso-Miravalles et al. (2019). The protein concentration in the supernatant of the dispersions, obtained after centrifugation at 1000 g for 20 min at 20°C, was measured using the Kjeldahl method,

with a nitrogen-to-protein conversion factor of 6.25 (Morr et al., 1985); this conversion factor was selected since a specific ones for lentil is not available in the literature and to be consistent with the work of Vogelsang-O'Dwyer et al., 2023; Alonso-Miravalles et al., 2019.

5.2.4. Preparation of lentil protein isolate emulsions

Model young child formula emulsions containing 15.85, 27.43 and 56.72% (w/w) lentil protein isolate, sunflower oil, and maltodextrin, respectively, on a total solid basis, were prepared as follows (Figure 5.1). Maltodextrin was added to the red lentil protein dispersion and stirred magnetically for 2 h at 300 rpm and 22°C. The samples were adjusted to pH 6.8 and allowed to rehydrate fully at 4°C for 18 h, after which the temperature of the aqueous solution was adjusted to 22°C and pH was adjusted to 6.8, before adding sunflower oil to reach final concentrations of 5, 8.65 and 17.89% (w/v) of lentil protein isolate, sunflower oil, and maltodextrin, respectively. The mixture was heated to 50°C in a water bath and an ultraturrax (T25 Ultra-Turrax, Staufen, Germany) was used (12,000 rpm for 2 min) to obtain a coarse emulsion. The coarse emulsion was then passed through a two-stage valve homogeniser (APV 1000, SPX flow, North Carolina, United States), in two passes, at a total pressure of 18 MPa, with first and second stage pressures of 15 and 3 MPa, respectively. The homogenised emulsions were cooled to room temperature and analysed on the same day.

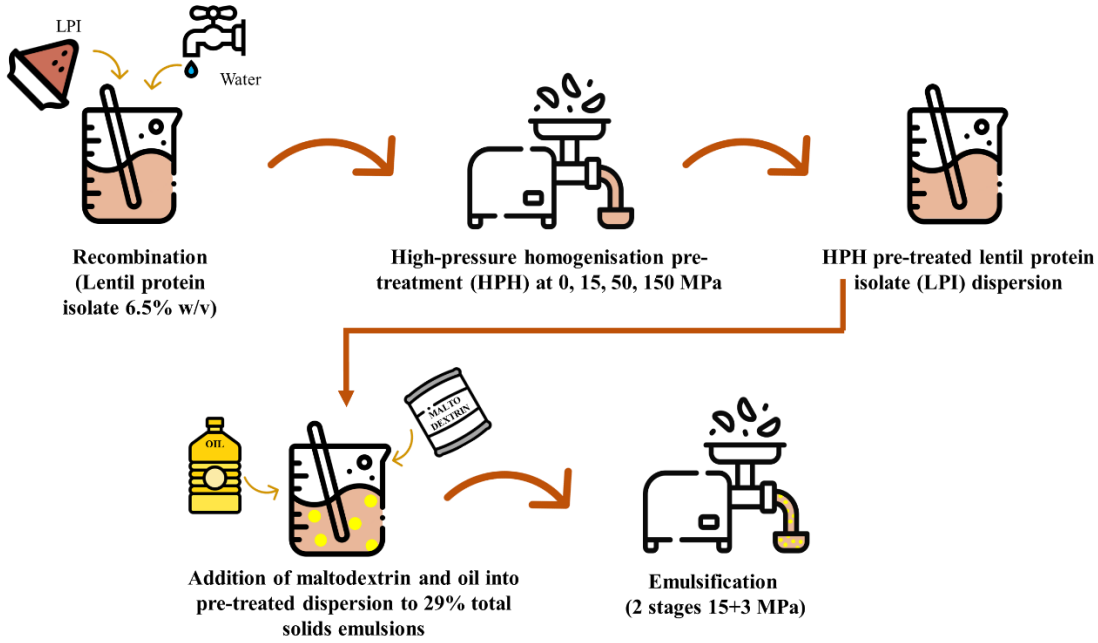

Figure 5.1. Schematic representation of the sample's preparation, where LPI = Lentil protein isolate.

5.2.5. Rheological analysis

A modular compact rheometer MCR 102e (Anton Paar, Dublin, Ireland) fitted with a starch pasting cell was used to assess the rheological characteristics of the emulsions upon heat treatment. The cell had an internal diameter of 28.92 mm, the diameter of the rotor was 24 mm, and the gap between the two elements at the geometry base was 1.0 mm. The sample (20 ml) was introduced into the cell before being successively conditioned and held at 30°C for 120 and 300 s, respectively, after which the temperature was increased to 90°C at a rate of 10°C/min and held at 90°C for 120 s. The temperature was then decreased to 30°C at a rate of 10°C/min and held at 30°C for 300 s. Throughout the analysis, a fixed shear rate of 10 rad/s was maintained, and apparent viscosity was measured continuously. The results are reported as apparent viscosities at the start and end of the heat-treatment.

5.2.6. Particle size distribution

A Mastersizer 3000 (Malvern Instruments, Worcestershire, UK) static light scattering device fitted with an automated Hydro MV wet disperser was used to evaluate the particle size distribution (PSD) of the emulsions before (BHT) and after (AHT) heat treatment, with and without sodium dodecyl sulphate (SDS) (i.e., SDS-BHT, SDS-AHT, respectively). The refractive index for protein was set at 1.338, the refractive index for the dispersant was set at 1.33, and the adsorption index was 0.001. The dispersions were diluted in ultrapure water until 10% laser obscuration was achieved (at 20°). The volume-weighted mean particle size (D[4,3]), specific surface area, and the particle sizes below which 10 and 90% of the sample volume are detected (Dv(10) and Dv(90), respectively) are presented as the results. Each emulsion was sampled 3 times and measured 3 times by the instrument for a total of 9 measurements.

5.2.7. Confocal laser scanning microscopy

Microstructural analysis of the emulsions was performed using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Japan). The emulsions were prepared as described by Malterre et al. (2023) using 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). An aliquot (50 μl) of this mixture was added to 1 ml of the emulsion-agarose solution, which was then incubated at 20°C until gelation. Fast Green FCF and Nile Red in 1,2-propanediol were excited at 633 and 488 nm, respectively. Representative images performed using a 40× objective lens were reported, where fat and protein were stained green and red, respectively.

5.2.8. Accelerated physical stability

The colloidal stability of the emulsions was measured using an analytical centrifuge (LUMiSiz-er®, LUM GmbH, Berlin, Germany). The samples were centrifuged at 3

successive speeds: 117 g for 60 min, 1057 g for 60 min and 1878 g for 16.7 min (Malterre et al. 2023). Near-infrared light illuminated the samples throughout the analysis, and the intensity of the transmitted light over the entire length of the sample was measured every 10 s. The separation rate was determined by plotting the integral of the intensity of the transmitted light over time and extracting the slope (%/h). Additionally, the thickness (i.e., height) of the cream layer (mm) as well as the thickness (i.e., height) of the sediment layer (mm) were measured on the last profile. Representative transmission profiles of the samples along with pictures at the end of the centrifugation are reported.

5.2.9. Statistical data analysis

All samples were formulated in independent triplicate trials and all analyses were conducted in triplicate, unless otherwise stated. The data generated was subject to one-way analysis of variance (ANOVA) using R version 4.3.1 (R foundation for statistical computing, Vienna, Austria).

5.3.Results and discussion

5.3.1. Protein solubility of lentil protein dispersions

Protein solubility is a critical factor in determining the ability of protein ingredients to form and stabilise oil-in-water emulsions when used in nutritional beverage applications. The solubility of lentil protein pre-treated at 0 MPa was 55.7 ± 0.7%, in agreement with previous studies (Malterre et al., 2024; Jeske et al., 2019) (Table 5.1). Upon HPH pre-treatment, there was a significant ($p<0.05$) increase in solubility, with 84.0 ± 0.7% solubility achieved at the lowest pressure of 15 MPa, with solubility increasing to 93.2 ± 0.5% at 50 MPa and 97.6 ± 0.6% at 150 MPa. These results are generally attributed to the mechanical forces, cavitation, shear, and turbulence occurring during HPH treatment, with the combined ability to break down powder particles and protein aggregates into smaller, more soluble particles (Yang et al., 2018; Yu et al., 2018; Chen et al., 2016; Bader et al., 2011).

Table 5.1. Solubility of the protein dispersions, apparent viscosity (η) before and after heat treatment (90°C for 2 minutes), protein solubility, separation rate, creaming height and sediment height for lentil protein stabilised emulsions pre-treated with high pressure homogenisation at 0, 15, 50, 150 and MPa.

Pre-treatment	**Protein dispersion**	**Emulsion**				
HPH Pressure (MPa)	**Protein solubility (%)**	**η before treatment at 90°C for 120 s (mPa·s)**	**η after treatment at 90°C for 120 s (mPa·s)**	**Separation rate (%/h)**	**Cream layer height (mm)**	**Sediment layer height (mm)**
0	55.7 ± 0.7^{a}	28.00 ± 2.17^{a}	60.52 ± 1.97^{a}	16.75 ± 3.00^{a}	9.18 ± 1.05^{a}	1.92 ± 0.32^{a}
15	84.6 ± 0.7^{b}	25.34 ± 1.83^{ab}	52.53 ± 4.07^{b}	13.63 ± 2.48^{a}	9.03 ± 0.92^{a}	1.65 ± 0.19^{a}
50	93.2 ± 0.5^{c}	24.01 ± 2.90^{b}	47.63 ± 2.32^{b}	5.66 ± 2.84^{b}	9.06 ± 0.20^{a}	1.61 ± 0.11^{a}
150	97.6 ± 0.6^{d}	22.56 ± 2.78^{b}	34.88 ± 0.92^{c}	2.05 ± 0.27^{b}	8.01 ± 0.63^{a}	1.60 ± 0.26^{a}

Values within a column that share a common superscript are not significantly different from one another ($p<0.05$).

The ability of physical pre-treatment to effectively improve the solubility of protein ingredients in plant protein dispersions has been shown to be linked to particle size reduction of the ingredients by several authors (D'Alessio et al., 2023; Keuleyan et al., 2023; Zhao et al. 2022; Melchior et al., 2022; Moll et al., 2022; Ong et al., 2022; Liang et al., 2022; Yang et al., 2018; Xu et al., 2018; Song et al., 2013). Specifically for lentil protein isolate, Saricaoglu (2020) reported that HPH pre-treatment decreased the particle size of lentil protein isolate dispersion (4.0% w/v) from 98.73 μm in the untreated sample to 18.00 μm with a pre-treatment at 75 MPa, which then allowed a solubility increase from 32.0% to 46.0%. Similarly, in faba bean protein concentrate (1.0% w/v) treated at 103 MPa (Yang et al., 2018) and pea protein isolate (4.3% w/v) treated at 70 MPa (Melchior et al., 2022), an increase in solubility from 35.0 to 98.0% and from 22.3 to 32.0%, were respectively reported.

Increasing solubility of such protein dispersions could be beneficial for use in emulsions, as poorly soluble proteins would be expected to have a greater tendency to aggregate in the continuous phase, thus increasing phase separation and decreasing emulsion physical stability (Hinderink et al., 2021; Tang et al., 2020). Additionally, soluble proteins can effectively adsorb at the newly created oil-water interface during homogenisation, therefore reducing interfacial tension and forming a protective layer around oil droplets, consequently creating a stable barrier that prevents coalescence and phase separation, allowing for better physical and heat stabilisation of emulsions (Navare et al, 2023; Xie et al., 2018; Maphosa and Jideani, 2018). Furthermore, Ma et al. (2020) found that HPH pre-treatments (20 to 100 MPa) could lead to an increase in solubility of fish proteins from 69.63 to 80.15%, which corresponded to an increase from 34 to 45% in the amount of adsorbed protein at the oil-water interface of emulsions made therefrom; this was attributed to disruption of protein aggregates

leading to smaller particles, better solubility and faster adsorption of the protein at the oil-water interface.

5.3.2. Rheological properties of lentil protein stabilised emulsions

Rheological properties are fundamental in understanding, predicting and controlling emulsion systems, as they influence the formation, processability, physical stability, texture, and consumer acceptability of emulsion-based systems. The LPI dispersions pretreated at different HPH pressures were used to formulate emulsion systems. The initial apparent viscosity of the emulsions formulated with LPI pre-treated at 0 MPa was 28.00 ± 2.17 mPa·s (Table 5.1), while the emulsion prepared using LPI pre-treated at 15 MPa, had a significant ($p<0.05$) lower viscosity (25.34 ± 1.83 mPa·s). Further increases in HPH pre-treatment pressure led to additional decreases in viscosity, down to 24.01 ± 2.90 and 22.56 ± 2.78 mPa·s at 50 and 150 MPa, respectively. The significantly ($p<0.05$) lower apparent viscosities recorded for the emulsions formulated with HPH pre-treated lentil protein dispersions could be attributed to the increased protein solubility (Table 5.1). Similar results have been reported for protein emulsions prepared with *Cyperus esculentus L.* by Zhang et al. (2022), who measured a decrease in emulsion viscosity from 30.0 to 8.0 mPa·s with application of HPH at 100 MPa. Indeed, it has also been shown that the application of intense mechanical forces, including shear stress, turbulence and cavitation to protein dispersions by HPH prior to emulsification facilitates the dispersion of protein aggregates (Sherman et al., 2024; Gharibzahedi et al., 2023; Karki et al., 2009), thereby leading to a more homogeneous continuous phase, less prone to protein-protein interactions and formation of protein aggregates.

On increasing temperature, the viscosity of the emulsions decreased (Figure 5.2) due to the combined effects of increased molecular motion, and changes in intermolecular

interactions (e.g., hydration, swelling) (Benoit et al., 2013). This decrease persisted until the solutions reached approximately 55°C, where physicochemical changes such as protein denaturation and unfolding occurred (Ma et al., 2011). At temperatures greater than 55°C, the apparent viscosity increased for all the emulsions, consistent with the results of other studies (Malterre et al., 2023; Alonso-Miravalles et al., 2019). It is also possible to observe that this viscosity increase was more pronounced for the emulsions formulated with the protein dispersions treated at lower pressures and thus having lower protein solubility (Table 5.1). In particular, at 0 MPa the peak viscosity was 49.42 mPa·s, while at 150 MPa the viscosity was significantly ($p<0.05$) lower (17.91 mPa·s) than in the untreated samples. Upon cooling, the viscosity of the emulsions increased to reach significantly ($p<0.05$) higher final viscosity than before heat treatment, with significant ($p<0.05$) differences observed for the emulsions prepared with protein dispersions pre-treated at the lower pressures. The final viscosity reached 60.52 ± 1.97 mPa·s and 52.53 ± 4.07 mPa·s, for the emulsions formulated with pre-treated protein ingredient at 0 and 15 MPa, respectively. Increasing the pressure of pre-treatment resulted in lower final viscosities of 47.63 ± 2.32 mPa·s and 34.88 ± 0.92 mPa·s for the emulsions prepared with pre-treated protein dispersions at 50 and 150 MPa, respectively. The high final viscosity observed in the samples treated at low pressure could be attributed to protein aggregation in the continuous phase, similar to what highlighted in high concentration protein formulations by Piedmonte et al (2018). Alonso-Miravalles et al. (2020) also observed similar behaviour for the thermal stability of lentil protein emulsions when studying the effect of mineral addition (i.e. $CaCl_2$); on physicochemical and colloidal stability of emulsions, due to calcium-mediated protein aggregation, with the formation of large flecks due to decreased electrostatic repulsion, as well as a decrease in solubility of lentil proteins.

Upon heating, proteins can undergo significant changes, such as protein denaturation, where the protein structure unfolds, resulting in the exposure of hydrophobic and other functional groups that were previously buried within the folded protein structure. These hydrophobic groups tend to aggregate with each other to minimise their exposure to the aqueous phase, causing the proteins to aggregate, often with diminished emulsification performance (Gumus al., 2017; Drapala et al., 2016; Keerati-u-rai and Corredig, 2009).

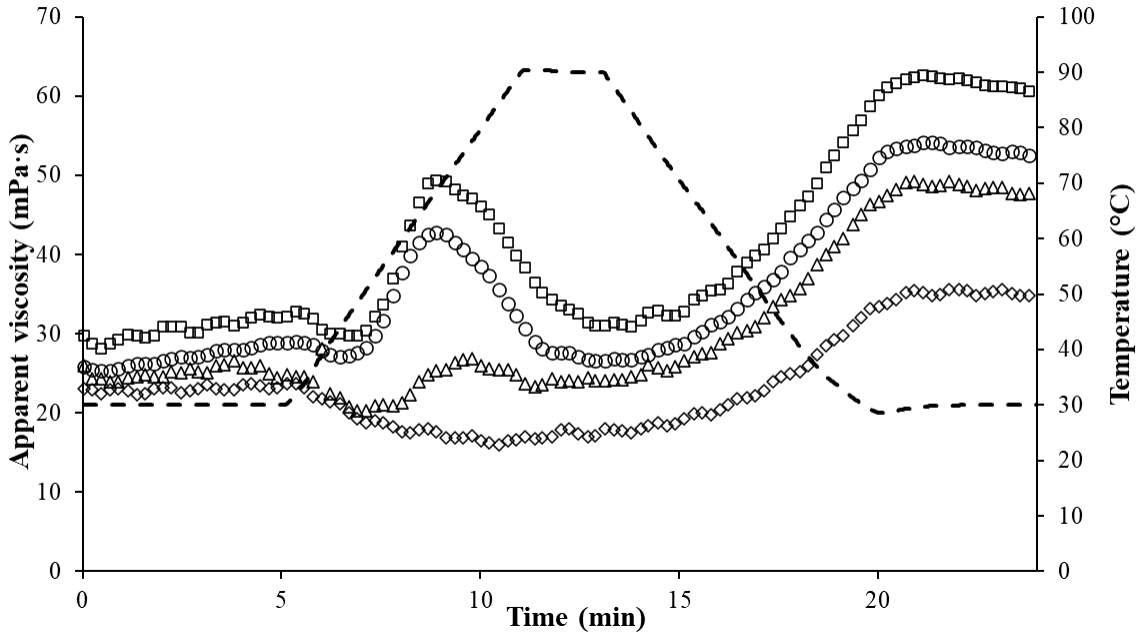

Figure 5.2. Apparent viscosity profiles of lentil protein-stabilised emulsions prepared using pre-treated lentil protein dispersion with high pressure homogenisation pressured of 0 (□), 15 (○), 50 (△) and 150 MPa (◇), during heat treatment with a peak hold at 90°C for 120 s using a starch pasting cell geometry on a controlled-stress rheometer. Dashed line (- -) represents the temperature profile.

5.3.3. Particle size distribution of lentil protein stabilised emulsions

For the sample pre-treated at 0 MPa, the volume-weighted mean particle size (D[4,3]) was 1.4 ± 0.17 µm (Figure 5.3), which is consistent with observations from other studies on lentil protein-stabilised emulsions, using similar homogenisation conditions (i.e. 18 MPa) (Malterre et al., 2023; Jeske et al., 2019; Primozic et al., 2018). Increasing the pre-treatment homogenisation pressure up to 150 MPa, D[4,3] remained largely unchanged, with values of 1.19 ± 0.17 µm observed at 150 MPa. On the other hand, the Dv(90) of the emulsions formulated with protein pre-treated at 150 MPa showed a slight reduction from 2.82 ± 0.34 µm (0 MPa) to 2.40 ± 0.22 µm (150 MPa). The results, in agreement with the literature, highlighted that HPH pre-treatment of plant protein dispersions could lead to a reduction of particle size of the emulsions made therefrom (Primozic et al., 2018).

To identify if the presence of flocculation of emulsions had occurred, and to enable the measurement of discrete accurately measure protein-stabilised individual oil droplets, SDS was added to the emulsions before the heat treatment (BHT-SDS). The observed decrease in particle size across all PSD parameters on addition of SDS indicated the occurrence of flocculation in all emulsions. However, at high pre-treatment pressure the magnitude of changes in PSD parameters was reduced, thus indicating a decrease in flocculation. The small particle size of the emulsions formulated with HPH pre-treated LPI could be attributed to the higher solubility (Table 5.1) of the protein ingredient, which allowed for enhanced interfacial properties (Faria-Silva et al., 2020; Mohan et al., 2016; Given et al., 2009). The HPH pre-treatment resulted in increased solubilisation of protein, which would be expected to lead to enhanced adsorption of the protein at the oil-water interface during homogenisation,

thus creating a more cohesive and stable barrier around each oil droplet (Navare et al, 2023; Xie et al., 2018; Maphosa and Jideani, 2018).

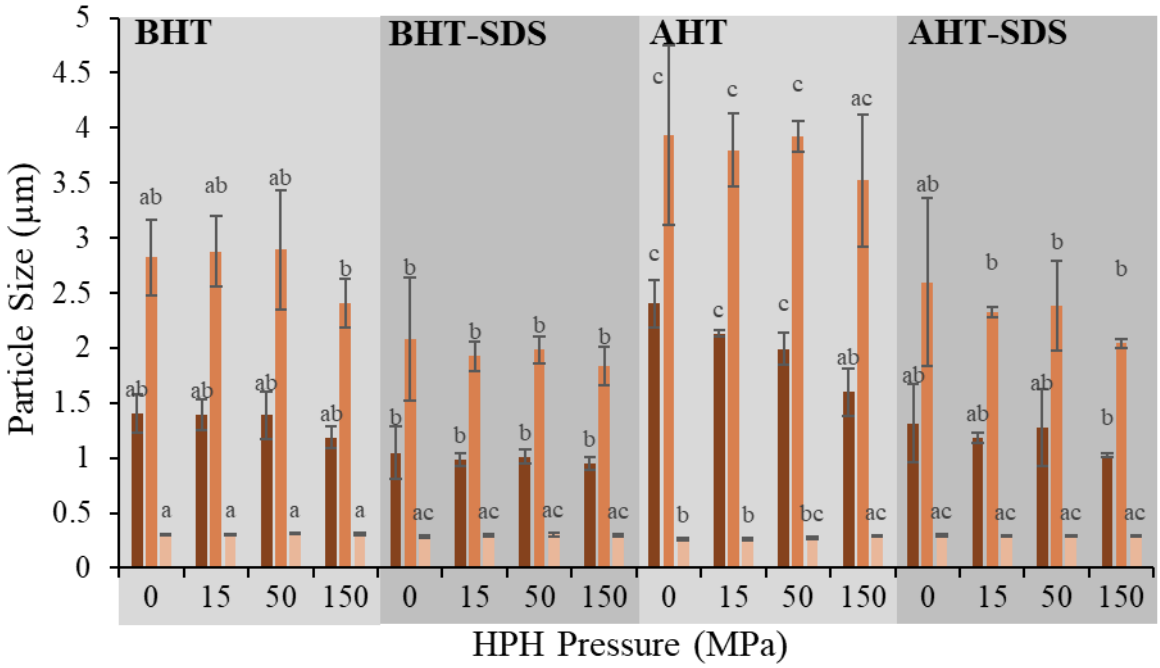

Figure 5.3. Particle size distribution parameters (D[4,3], Dv(10), Dv(90)) for lentil protein stabilised emulsions pre-treated with high pressure homogenisation at 0, 15, 50, 150 and MPa, Before heat treatment at 90°C (BHT) after heat treatment at 90°C (AHT), without and with Sodium dodecyl sulphate (BHT-SDS; AHT-SDS). Bright orange = particle size below which 10% of sample volume is found (Dv(10)), dark orange = volume-weighted mean particle diameter (D[4,3]), brown = particle size below which 90% of sample volume is found (Dv(90)). Values on same colour bars that share a common superscript are not significantly different from one another ($p<0.05$).

In addition, Wu et al. (2023) found that the HPH pre-treatment (60 MPa) of soy protein isolates and whey protein isolates mixture could lead to an increase of interfacial

protein adsorption rate and greater emulsion stability. Therefore, the stronger protein interfacial layer achieved as a result of the HPH pre-treatment could help retain a cohesive protein layer preventing oil droplets from direct contact and aggregation by maintaining electrostatic repulsion between droplets, thus inhibiting aggregation and flocculation (Zhang et al., 2022).

Upon heat treatment, all the emulsions (AHT) exhibited increases in particle size (Figure 3). In particular, emulsions formulated with pre-treated protein dispersions at higher pressures showed smaller changes in particle size distribution and ultimately had smaller particle sizes post-heat treatment (90°C for 120 s), with D[4,3] at 0 MPa of 2.40 ± 0.22 μm and 1.6 ± 0.21 at 150 MPa. Indeed, the magnitude of particle size increase was greater in the emulsion formulated with protein pre-treated with the lowest pressure, showing 71.68% higher values for D[4,3] as compared to emulsions before heat treatment (Figure 5.3), whereas the sample treated at 150 MPa showed D[4,3] values to be 34.53% higher. In addition, Dv(90) also increased significantly ($p<0.05$) for all emulsions, with values of 3.93 ± 0.82 and 3.52 ± 0.60 μm for emulsions pre-treated at 0 and 150 MPa, respectively, while no changes were evident on Dv(10), corresponding to the smaller particles (values ranging from 0.31 ± 0.01 to 0.26 ± 0.01 μm).

To more fully understand the mechanisms underlying these increases in particle size upon heat-treatment, particle size was measured after the addition of SDS. With these experimental conditions, all PSD parameters decreased significantly ($p<0.05$) for all emulsions, indicating the presence of reversible flocculation of oil globules upon heat treatment. As previously discussed, pre-treatment of the protein ingredient with HPH could lead to better adsorption of the protein at the oil-water interface, creating a more cohesive and stable barrier around each oil droplet in the subsequent formulated

emulsions. This helped to prevent aggregation and protected oil droplets from direct contact by maintaining electrostatic repulsion between droplets and then inhibiting flocculation in the emulsions before, but also during, heat treatment, thereby minimising any increases in particle size (Navare et al, 2023; Wu et al., 2023; Xie et al., 2018; Maphosa and Jideani, 2018).

5.3.4. Confocal microscopy of lentil protein stabilised emulsions

Confocal laser micrographs of the emulsions formulated with lentil protein dispersion pre-treated with HPH at respective pressures of 0, 15, 50 and 150 MPa are reported in Figure 5.4. All emulsions presented homogeneous dispersion of oil globules (green) stabilised by lentil protein (red). Furthermore, the micrographs of the emulsions prepared with dispersions treated at 0 and 15 MPa (a and b) showed the presence of larger protein aggregates (red aggregates/clusters). After heat treatment at 90°C for 120 s, large aggregates were evident in the microstructure of the emulsion (Figure 5.4), being consistent with the results from particle size distribution analysis (Figure 5.3).

Furthermore, emulsions formulated with dispersions having higher solubility (f, g, h) resulted in less visible flocculation and more homogeneous particle distribution in the sample after the thermal treatment. Indeed, increasing the solubility of the protein dispersion before emulsification allowed for better stabilisation of the oil globules which led to greater thermal stability as highlighted in the PSD results (Figure 5.3).

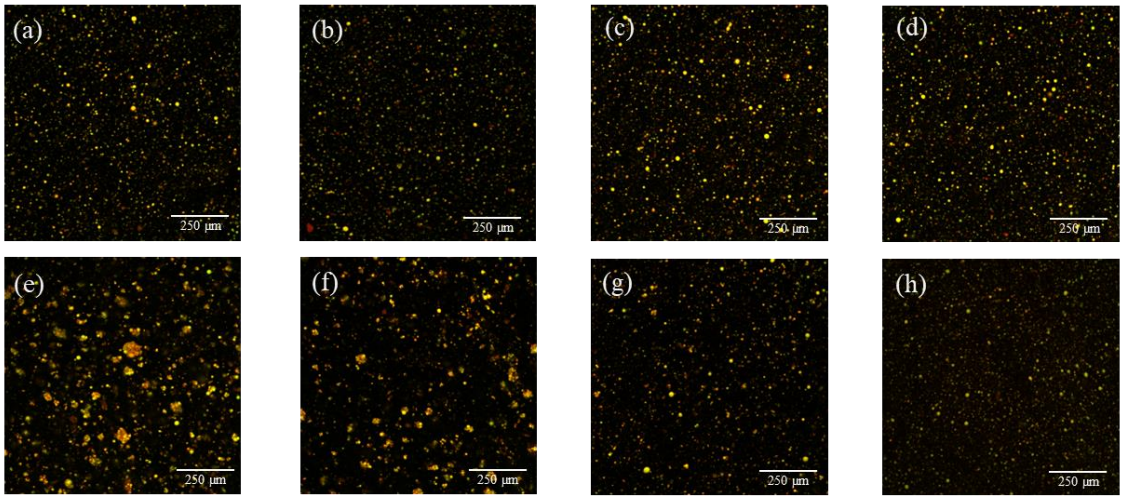

Figure 5.4. Confocal laser micrographs of lentil protein-stabilised emulsions before (a to d) and after (e to h) heat treatment at 90°C for 120 s for the emulsions prepared using pre-treated lentil protein dispersion with high pressure homogenisation pressures of at 0 (a & e); 15 (b & f); 50 (c & g) and 150 MPa (d & h)).

5.3.5. Colloidal stability of lentil protein stabilised emulsions

Figure 5.5 shows representative transmission profiles during centrifugation for lentil protein-stabilised emulsions. The initial transmission values of all emulsions ranged between 3.0 and 3.5% (Figure 5.5). During the centrifugation, the transmission increased rapidly for the sample treated at the lowest pressure, as indicated by the highest separation rate measured of 16.75±3.00 %/h. This high separation rate can be attributed to larger particles in the emulsion, as shown in Figure 5.4. Furthermore, the final transmission of the emulsions pre-treated at 0 and 15 MPa where significantly ($p<0.05$) higher than the corresponding values for the emulsions pre-treated at 50 and 150 MPa. In particular, the separation rate decreased with increasing pre-treatment pressure, reaching a value of 2.05±0.27%/h for the highest pressure of 150 MPa. Similarly, Levy et al. (2022) studied the effect of HPH pre-treatment on the stability of potato protein stabilised emulsions and noted a reduction in separation rate with creaming velocity reducing from 10.70 ± 0.11 (0.1 MPa) to 0.59 ± 0.04 after treatment

at 200 MPa. Furthermore, after centrifugation, the emulsions displayed similar cream height (9.18 ± 1.05 mm and 8.01 ± 0.63 mm at 0 and 150 MPa, respectively) and sediment height (1.92 ± 0.32 mm and 1.60 ± 0.26 mm at 0 and 150 MPa, respectively) (Table 5.1). The stability of a protein-stabilised emulsion is influenced by several factors, including particle size distribution, protein solubility and viscosity. These results clearly indicate that lentil protein dispersions pre-treated with HPH, and with consequently higher protein solubility, have a greater ability to produce emulsions with higher colloidal stability.

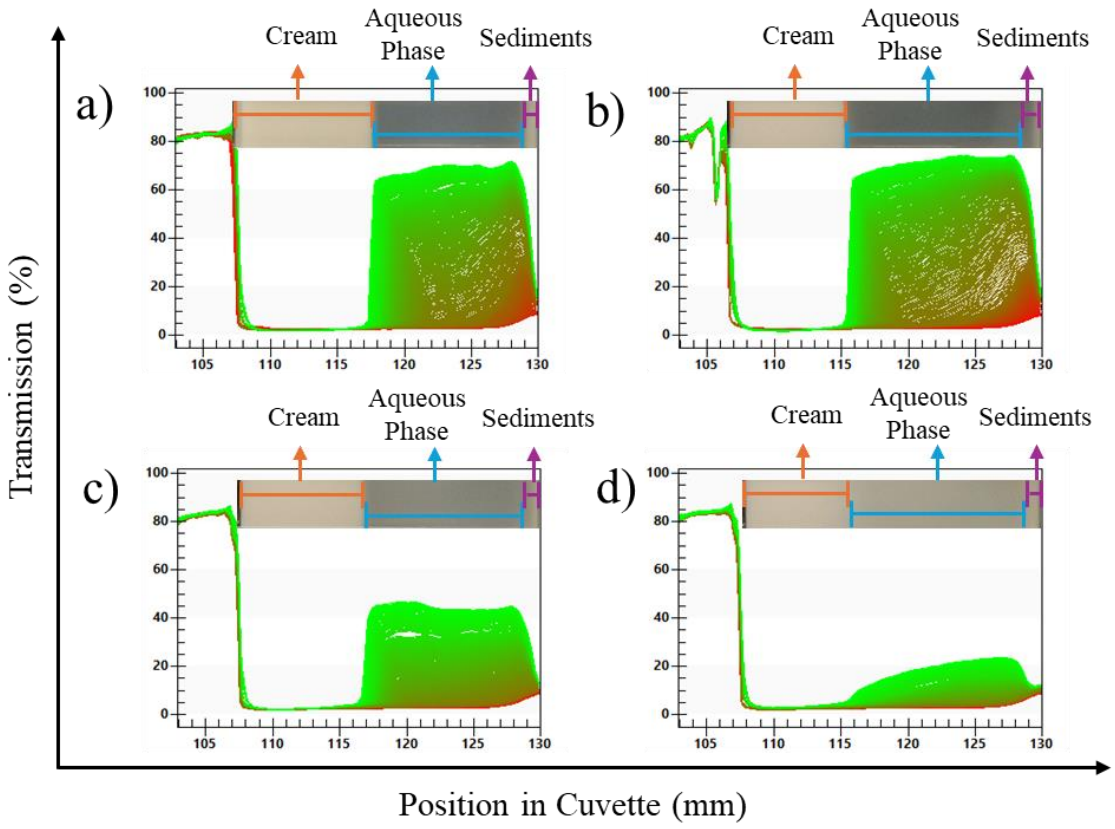

Figure 5.5. Representative transmission profiles and pictures (with phases indicated i.e. cream, aqueous phase and sediments) for the emulsions prepared using pre-treated lentil protein dispersion with high pressure homogenisation pressures of at 0 (a); 15 (b); 50 (c) and 150 MPa (d).

5.4. Conclusion

This study demonstrates that increasing protein solubility through high-pressure homogenisation (HPH) significantly enhances the physical stability and functional properties of lentil protein-stabilised emulsions. The application of HPH as a pre-treatment, at pressures ranging between 0 and 150 MPa, caused a significant increase in protein solubility, and consequently, enhanced heat stability. Specifically, protein solubility in the LPI dispersions increased from 55.74 to 97.6% at 150 MPa due to the mechanical breakage of the protein powder particles through HPH. This increase allowed for a reduction in particle size distribution of the emulsions, specifically a reduction in oil-globule size, as well as a reduction in the extent of flocculation, as confirmed using confocal microscopy with an optimal pressure found at 50 MPa as it provides the best improvement in techno-functional properties while avoiding excessive pressures.

These findings underscore the effectiveness of HPH as a non-thermal processing technique for improving the techno-functional properties of lentil protein isolate (LPI). By enhancing both solubility and stability, this approach addresses a major limitation of plant proteins—limited solubility—which often restricts their use in complex food formulations. The improved stability and dispersibility of HPH-treated LPI emulsions facilitate for their application in high-solid, shelf-stable products, including plant-based infant formula, dairy alternatives, and high-protein beverages. This work not only contributes to the scientific understanding of protein functionality under mechanical processing but also supports the practical development of plant-based emulsions with improved performance characteristics.

Moreover, these findings have significant implications for sustainability and the global food system. As the demand for environmentally friendly, plant-based food

alternatives continues to grow, techniques such as HPH offer viable solutions for enhancing the functionality of sustainable protein ingredients without the need for chemical additives or high-energy thermal processing. By enabling the use of lentil protein this study contributes to efforts aimed at reducing, water usage, and land conversion associated with conventional protein production.

Future research should focus on scaling up the HPH process to validate its industrial feasibility across a range of food applications. Additional studies could explore how other factors, such as pH, ionic strength, and ingredient interactions, influence the stability and sensory properties of HPH-treated lentil protein. These advancements will further strengthen the use for lentil protein as a core ingredient in the development of nutritionally rich, sustainable food products capable of addressing both consumer needs and global environmental challenges.

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Chapter 6

Thermal treatment of high-pressure homogenised lentil protein dispersions improves their emulsion formulation properties

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Declaration: this chapter was written by author Nicolas Malterre (NM) and reviewed by co-authors James A. O'Mahony (JAOM) and Francesca Bot (FB). NM co-designed the study with co-author JAOM and FB and performed the experimental work.

Abstract

This study investigated the combined effects of high-pressure homogenisation (HPH) and thermal treatments of lentil protein dispersions on the techno-functional properties of emulsions made therefrom. Protein-carbohydrate dispersions untreated and pre-treated with HPH at 50 MPa, were subjected (except for the control) to thermal treatment at 120^{o}C for 60 s. The technological functionality of the suspensions and of the emulsions made therefrom were evaluated. The particle size distribution of the dispersions was significantly reduced by HPH, with the volume-weighted mean diameter (D[4,3]) decreasing from 3.63 µm (control) to 1.48 µm (HPH). However, subsequent heat treatment caused aggregation, with a D[4,3] reaching 3.63 µm. Focusing on the emulsions subsequently formulated, those made with the HPH and heat-treated dispersions exhibited smaller oil droplets (D[4,3] of 1.07 µm) as compared to the control (D[4,3] of 1.61 µm) and improved physical stability, with separation rate decreasing from 15.11 to 1.85 %/h and cream height decreasing from 10.37 to 3.84 mm). The improvement in emulsion stability was attributed to the reduction in particle size (due to HPH) and the increased protein denaturation (due to heat treatment), which led to higher functionality of the protein ingredients at the oil/water interface resulting in better stabilisation of the oil-globules. These findings demonstrate that the combined effects of HPH and thermal treatment can significantly improve the stability and functionality of high-solids lentil protein-stabilised emulsions, offering potential applications in the development of sustainable plant-based food products.

6.1.Introduction

The demand for sustainable and nutritious food products is driving significant innovation in food science and technology. Plant-based proteins, such as those derived from lentils, offer a promising alternative to animal proteins due to their environmental sustainability, health benefits, and economic viability (Goldstein and Reifen, 2022). In particular, lentil proteins are rich in essential amino acids, dietary fibers, vitamins, and minerals, making them an attractive ingredient for various food applications, including dairy alternatives and young child nutritional products (Joshi et al., 2017).

However, the use of lentil protein in food formulations is often limited by its functional properties, such as low solubility and low emulsifying properties, which are crucial for the development of stable and highly nutritious food products (Grossmann and McClements, 2023). Improving the techno-functional attributes of lentil proteins can enhance their applicability and performance in food systems. High-pressure homogenisation (HPH) has emerged as a promising non-thermal processing technique to enhance the functional properties of plant proteins (Ma et al., 2022; Gharibzahedi et al., 2023). HPH involves forcing a liquid through a narrow gap at high pressure, which induces intense mechanical forces such as cavitation, shear, and turbulence (Dumay et al., 2013). These forces can alter the protein structure, leading to improved solubility, emulsifying properties and overall technological functionality (Sá et al., 2022; Avelar et al., 2021). Previous studies have demonstrated that HPH can significantly improve the solubility and functional properties of various plant proteins, including several pulses such as lentil, pea, and faba bean proteins. In their study on soybean protein, Liang et al. (2022) observed that high-pressure homogenization (HPH) at 60 MPa significantly reduced the powder particle size from 4.5 to 2.5 μm, enhanced solubility from 74 to 80.5%, and increased water and oil holding capacities

from 5.0 to 7.7 g/g and from 4.6 to 6.5 g/g, respectively. Similarly, Saricaoglu (2020) investigated the effects of HPH in the range of 0-150 MPa on the solubility and emulsifying properties of lentil protein isolate (LPI) dispersions (4% protein w/v). Their results demonstrated that HPH treatment reduced the particle size from 98.7 µm to 12.6 µm, with the smallest size at 150 MPa and increased the solubility of LPI from 32% on treatment at 0 MPa to reach a maximum of 46% at 75 MPa. The emulsifying activity index and emulsifying stability index increased from 100.2 to 125.2 m^2/g and from 39.1 to 226.3 min, respectively, at 100 MPa. Other researchers, including Song et al. (2013), Keuleyan et al. (2023), Melchior et al. (2022), Moll et al. (2022), Xu et al. (2018), Yang et al. (2018), Navare et al. (2023), Zhang et al. (2022) and Primozic et al. (2018) have reported similar findings on the effects of HPH on plant proteins. These enhancements are attributed to the physical disruption of particles caused by HPH, which promotes better water interaction and increases solubility, leading to stronger interfacial properties and improved emulsifying activity.

Other processing techniques are used in the context of food formulations, one of them being thermal treatment. In the food industry, thermal treatments such as pasteurization or sterilization are crucial steps for ensuring the safety and shelf-life of products. However, these processes can also significantly impact the functional properties of food ingredients, particularly proteins. Understanding the effects of thermal treatment on protein functionality during formulation is especially important when developing products like young child formulae, where both nutritional quality and physical stability are critical. Indeed, thermal processes apply heat to the sample, inducing thermal mobility of the peptide chain, which leads to the disruption of intermolecular and intramolecular hydrophobic interactions, as well as electrostatic, hydrogen, and disulfide bonds, particularly when applied at temperatures greater than

the protein denaturation temperature (Akharume et al., 2021). As a result, the hydrophobic core of the protein is exposed, facilitating crosslinking through hydrophobic interactions, hydrogen bonding, and disulfide/sulfhydryl bonding, causing protein aggregation (Drapala et al., 2016). Controlled heat application can thus be employed to modify the structure of food protein ingredients, enhancing certain functional properties (Davis & Williams, 1998).

The effect of thermal processing on plant protein functionality has been investigated throughout the years on multiple protein sources. These studies have shown that the aggregation caused by heat treatment has the potential to cause a decrease in protein solubility. For instance, Ma et al. (2011) reported a decrease in protein solubility for thermally treated (80°C for 60 s) dehulled green lentil protein of 27 to 30%, with similar results presented by other authors for chickpea and pea proteins (Ghribi et al., 2015; Shand et al., 2007). Despite the reduction in solubility, thermal treatment can actually enhance other functional properties of proteins. Significant increases in fat binding capacity, water holding capacity, and emulsifying activity index (EAI) have been reported for pulse flours following thermal treatment (Ma et al., 2011). Similarly, the thermal treatment of pea protein isolates at 90°C for 30 min showed higher protein adsorbed at the oil-water interface (from 66.40 to 96.20%) which led to greater stability against creaming (Peng et al., 2016). Soybean protein isolate treated at 95°C for 15 min displayed higher concentration of protein at the interface (from 19.7% for untreated to 31.3% after treatment) as well as higher physical stability, as indicated by lower creaming index (i.e., reduced from 70 to 30%) (Shao and Tang., 2014). These improvements were attributed to hydrophobic interactions leading to aggregation, which enhanced diffusion of protein molecules at the oil–water interface, ultimately resulting in emulsions with improved physical stability.

Although numerous studies have examined the individual effects of HPH and thermal treatment on plant proteins, there is a notable lack of research exploring the combined impact of these processes on protein dispersions and emulsions made using these selectively pretreated protein systems. This gap in the literature underscores the need for comprehensive investigations that evaluate how these combined treatments can enhance the functional properties of plant protein ingredients, potentially leading to more stable and effective emulsions for various food applications. Therefore, this study aims to explore the impact of HPH (50 MPa) and thermal (120°C for 60 s) pre-treatment of LPI dispersion on the techno-functional properties of subsequent emulsions. Specifically, rheological properties, particle size distribution, and overall stability of pre-treated dispersions and formulated emulsions were investigated. This study builds on previous work by providing a detailed analysis of the interplay between dispersion characteristics and emulsion stability, thereby enhancing our fundamental understanding of the mechanisms underlying the thermal behaviour of HPH-treated LPI emulsions. The findings of this research will contribute to the development of more physically stable and functional high-solid plant-based emulsions, supporting the broader goal of creating sustainable and nutritious food products.

6.2.Materials and methods

6.2.1. Materials

Red lentil protein isolate, containing 72.15% protein (w/w), was sourced from Döhler GmbH (Darmstadt, Germany). Sunflower oil was procured from a local commercial outlet (Tesco, Welwyn Garden City, Hertfordshire, United Kingdom). Maltodextrin, with a dextrose equivalent (DE) value of 17 (Glucidex® 17), was obtained from Roquette Frères (Lestrem, France). All reagents utilised in this research were of analytical grade and were supplied by Sigma-Aldrich, based in St. Louis, MO, USA, unless specified otherwise.

6.2.2. Pre-treatment of lentil protein isolate dispersions

Lentil protein dispersions were formulated by rehydrating red lentil protein isolate powder to a protein concentration of 6.5% (w/v) in pre-heated deionized water at 50°C. This dispersion was then magnetically stirred at 300 rpm for 1 h at 20°C. Following this, maltodextrin was incorporated at a concentration of 23.3% (w/v) and stirred for an additional 2 h at 20°C, after which the dispersions were adjusted to a pH of 6.8 and subjected to high shear mixing at 12,000 rpm for 1 min at 20°C to achieve a homogeneous feed dispersion. Subsequently, the dispersions were subjected to homogenisation using a two-stage, high-pressure valve homogeniser (APV 2000, SPX Flow, North Carolina, United States) in a single pass, at total pressures of 0 (Control) and 50 MPa (HPH), with a first to second stage pressure ratio of 83:17. Following this, the HPH and control dispersions were heat treated at 120°C for 60 s using a pilot-scale HTST/UHT thermal processing unit (Microthermics, Raleigh, North Carolina, United States) to simulate an industrial process for young child formula product manufacturing where thermal processing is used to ensure microbial safety. The obtained dispersions were identified as HT from the control dispersion heat treated and

HPH-HT for the pre-treated sample at 50 MPa heat treated. The samples were collected for further analysis and used to formulate the emulsions $Control_{Emu}$, HT_{Emu}, HPH_{Emu} and $HPHHT_{Emu}$. Figure 6.1 provides a schematic of sample preparations, with corresponding sample codes.

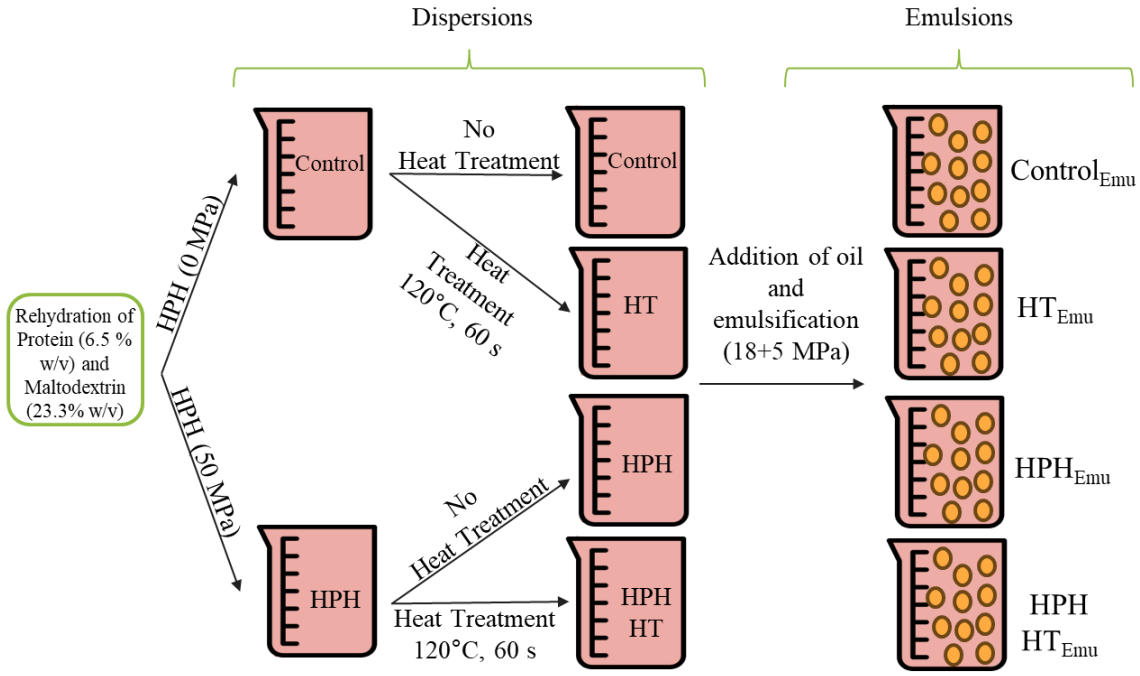

Figure 6.1. Schematic representation of the sample preparation, including corresponding sample codes.

6.2.3. Preparation of lentil protein isolate emulsions

The emulsions were prepared containing 15.85, 27.43, and 56.72% lentil protein isolate, sunflower oil, and maltodextrin, respectively, on a total solid basis to simulate a model young child formula (Alonso-Miravalles et al., 2022). To achieve the final concentrations of 5% (w/v) lentil protein isolate, 8.65% (w/v) sunflower oil, and 17.89% (w/v) maltodextrin, sunflower oil was added to the dispersion (Figure 6.1) similarly to the emulsions formulated at 29% TS in chapter 2 which corresponded to the first highly unstable emulsion. The mixture was then heated to 50°C in a water bath and a coarse emulsion was produced using an ultra-turrax (T25 Ultra-Turrax, Staufen, Germany) at 12,000 rpm for 2 min. This coarse emulsion was subsequently homogenised using a two-stage valve homogeniser (APV 1000, SPX Flow, North Carolina, United States) in two passes at a total pressure of 18 MPa, with first and second stage pressures set at 15 and 3 MPa, respectively. The homogenised solutions were cooled to room temperature and analysed on the same day.

6.2.4. Physicochemical characterisation of lentil protein dispersions and emulsions

6.2.4.1. Protein content

An RC5C plus centrifuge (Thermo Scientific™ Sorvall™, Waltham, MA, USA), equipped with a GSA rotor, was used to determine the protein content of the dispersions, as described by Malterre et al. (2023). The protein concentration in the supernatant, obtained after centrifugation at 1000 × g for 20 min at 20°C, was measured using the Kjeldahl method with a nitrogen-to-protein conversion factor of 6.25 (Morr et al., 1985); this conversion factor was selected since a specific ones for lentil is not available in the literature and to be consistent with the work of Vogelsang-O'Dwyer et al., 2023; Alonso-Miravalles et al., 2019. The results are expressed as the

percentage of soluble protein in the supernatant relative to the total protein content of the dispersions.

6.2.4.2. Protein profile analysis

The protein profile of the dispersions was analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with precast gels (Mini-PROTEAN TGX, Bio-Rad Laboratories, Hercules, CA, USA), based on the method developed by Laemmli (1970), with minor modifications. For non-reducing conditions, 100 μL of the diluted dispersion (2 mg/ml) was mixed with 100 μL of sample loading buffer (2× Laemmli sample buffer, Bio-Rad Laboratories, Hercules, CA, USA). In reducing conditions, 100 μL of the diluted dispersion was combined with 95 μL of sample loading buffer and 5 μL of β-mercaptoethanol and incubated at 95 °C for 10 min. Each well was loaded with 7 μL of sample solution, and 5 μL of SDS-PAGE broad range protein standard (Bio-Rad Laboratories, Hercules, CA, USA) was used as a protein molecular weight standard. The gels were run in a running buffer (10× Tris/Glycine/SDS, Bio-Rad Laboratories, Hercules, CA, USA) at 160 V for 1 h, after which they were fixed for 15 min in a solution of water:methanol acid (40:50:10), stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA), and destained in a solution of water:methanol acid (50:40:10) until a clear background was achieved. Finally, the gels were stored at 4°C in a 5% (v/v) acetic acid solution overnight to reverse any shrinkage caused during the fixing and destaining steps.

6.2.4.3. Particle size distribution analysis

A Mastersizer 3000 (Malvern Instruments, Worcestershire, UK) static light scattering device, equipped with an automated Hydro MV wet disperser, was used to measure the particle size distribution (PSD) of the dispersions and emulsions. The refractive

indices were set at 1.338 for the sample and 1.33 for the dispersant, with an adsorption index of 0.001. The dispersions or the emulsions were diluted in ultrapure water to achieve 10% laser obscuration at 20°C. The results are presented in terms of the volume-weighted mean particle size (D[4,3]), specific surface area, and the particle sizes at which 10 and 90% of the sample volume are detected (Dv(10) and Dv(90), respectively). Each emulsion was sampled 3 times and measured 3 times by the instrument for a total of 9 measurements.

6.2.4.4. Rheological analysis

The rheological properties of the dispersions and emulsions were determined using a modular compact rheometer MCR 102e equipped with a concentric cylinder geometry (Anton Paar, Austria). The cell had an internal diameter of 28.9 mm, the diameter of the rotor was 26.7 mm, and the gap between the two elements at the geometry base was 2 mm. The sample (20 ml) was loaded into the cylinder, pre-heated at 70°C, after which the shear rate was increased to 100 s^{-1} over 120 s and maintained at 100 s^{-1} for 10 s, then the shear rate was increased to 1000 s^{-1} over 120 s and maintained at 1000 s^{-1} for 10 s. Throughout the analysis, a fixed temperature of 70°C was maintained, and viscosity was measured continuously. The Ostwald-de Waele model (Eq.1) was used to describe the rheological properties of the protein dispersions, while ensuring that $R^2 \geq 0.95$.

$$\tau = K\dot{\gamma}^n \quad (1)$$

Where τ is the shear stress (mPa), $\dot{\gamma}$ is the shear rate (s^{-1}), and n is the flow behaviour index (dimensionless). The flow index behaviour was measured in 2 regions, before and after the flow behaviour evolved as measured using Eq. 1.

6.2.4.5. Confocal laser scanning microscopy

Microstructural analysis of the dispersions and emulsions were conducted using an OLYMPUS FV1000 confocal laser scanning biological microscope (Olympus Corporation, Tokyo, Japan). The dispersions were prepared following the method described by Malterre et al. (2023), with modifications. Specifically, 1 mL of the samples were mixed with 4 mL of low gelling temperature agarose solution (1.5%, w/v) at 37°C to fix the samples, facilitating higher quality imaging. As outlined by Malterre et al. (2024), for dispersions, 20 μL of Fast Green FCF aqueous solution (200 μL of 0.1 g/L) was added to 1 mL of the dispersion-agarose mixture, which was then incubated at 20°C until gelation. For emulsions, 50 μL of 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 added to 1 mL of the emulsion-agarose sample. Fast Green FCF and Nile Red were excited at 633 and 488 nm, respectively. Representative images were acquired using a 60× objective lens. The images provided detailed insights into the microstructure, aiding in the evaluation of the stability and homogeneity of the dispersions and emulsions.

6.2.4.6. Accelerated physical stability analysis

The colloidal stability of the dispersions and emulsions were assessed using an analytical centrifuge (LUMiSizer®, LUM GmbH, Berlin, Germany). The dispersions underwent centrifugation in two successive cycles at 800 and 3000 rpm for 10 min each (Malterre et al., 2024). The samples were centrifuged at three successive speeds: 117 g for 60 min, 1057 g for 60 min, and 1878 g for 16.7 min, following the method described by Malterre et al. (2023). During the analysis, near-infrared light illuminated the samples, and the intensity of the transmitted light across the entire length of the sample was recorded every 10 s. The separation rate was calculated by plotting the

integral of the transmitted light intensity over time and determining the slope (%.h^{-1}). Additionally, the quantity of sediment material and the extent of creaming was measured at the end of the cycles from the final profile and reported as sediment height (mm) and creaming height (mm), respectively.

6.2.5. Statistical data analysis

All samples were formulated in independent triplicate trials and all analyses were conducted in triplicate, unless otherwise stated. The data generated was subject to one-way analysis of variance (ANOVA) using R version 4.3.1 (R foundation for statistical computing, Vienna, Austria).

6.3.Results and discussion

6.3.1. Physicochemical properties of lentil protein dispersions

6.3.1.1. Particle size distribution

The particle size distribution of lentil protein dispersions treated under various conditions, including control, high-pressure homogenization (HPH), heat treatment (HT), and the combination of HPH and HT, was thoroughly investigated (Table 6.1). The control dispersion exhibited a D[4,3] value of 3.63 ± 0.71 µm, Dv(90) of 7.56 ± 1.52 µm and Dv(10) of 0.41±0.03 µm, with large particles visible on the confocal micrographs (Figure 6.2a). The application of HPH significantly ($p<0.05$) reduced all particle size distribution (PSD) parameters (D[4,3] = 1.48 ± 0.10 µm, , Dv(90) = 3.25 ± 0.25 µm and Dv(10) = 0.32 ± 0.02 µm), which aligns with previous studies that demonstrate the efficacy of HPH in reducing particle size and disrupting protein aggregates into smaller particles (Saricaoglu et al., 2020; Moll et al., 2021; Yang et al., 2018). It was possible to observe the changes in particle size also on the confocal micrographs (Figure 6.2b), where homogeneous small red particles were present in the HPH treated dispersion, while in the control dispersions, bigger unbroken particles were visible. This reduction in particle size is due to the forced passage of the dispersions through a narrow gap inducing intense mechanical forces, cavitation, shear and turbulence (Dumay et al., 2013; Levy et al., 2021). Similar results were found using a variety of plant protein dispersions such as lentil protein (Navare et al., 2023; Saricaoglu et al., 2020), pea protein (D'Alessio et al., 2023; Keuleyan et al., 2023), soy protein (Liang et al., 2022; Xu et al., 2018) and faba beans (Yang et al., 2018).

Table 6.1. Particle size distribution parameters of lentil protein dispersion pretreated at 0 (Control) and 50 MPa (HPH), with heat treatment at 120°C for 60 s (HT) and combined HPH followed by heat treatment (HPH-HT) and emulsions made thereof with and without sodium dodecyl sulphate (SDS).

Sample		***D[4,3] μm***	***Dv(90) μm***	***Dv(10) μm***
Dispersion	Control	3.63±0.71^{a}	7.56±1.52^{a}	0.41±0.03^{a}
	HPH	1.48±0.10^{b}	3.25±0.25^{b}	0.32±0.02^{b}
	HT	3.75±0.34^{a}	8.77±0.71^{a}	0.43±0.02^{a}
	HPH-HT	3.63±0.18^{a}	9.36±0.47^{a}	0.61±0.26^{c}
Emulsion	$Control_{emu}$	1.61±0.20^{a}	3.46±0.59^{a}	0.32±0.01^{a}
	HPH_{emu}	1.38±0.30^{ab}	2.85±0.81^{abc}	0.32±0.04^{a}
	HT_{emu}	1.36±0.33^{ab}	2.58±0.51^{ab}	0.31±0.01^{a}
	$HPH\text{-}HT_{emu}$	1.07±0.07^{b}	2.11±0.13^{c}	0.30±0.03^{a}
	$Control_{emuSDS}$	1.13±0.06^{b}	2.17±0.05^{abc}	0.29±0.01^{a}
	HPH_{emuSDS}	1.08±0.05^{b}	2.13±0.12^{bc}	0.30±0.02^{a}
	HT_{emuSDS}	1.21±0.08^{ab}	2.30±0.16^{abc}	0.30±0.03^{a}
	$HPH\text{-}HT_{emuSDS}$	1.14±0.15^{b}	2.22±0.32^{bc}	0.29±0.03^{a}

Values in same column for individual treatments (dispersion or emulsion) that share a common superscript are not significantly different from one another ($p<0.05$).

D[4,3] = volume-weighted mean particle diameter.

Dv(90) = particle size below which 90% of sample volume is found.

Dv(10) = particle size below which 10% of sample volume is found.

Confocal laser scanning microscopy, revealed the presence of large protein aggregates in the HT samples due to the temperature and time conditions (120°C for 60 s) applied during the treatments, indicating denaturation and aggregation of proteins in the heat-treated samples. This is in agreement with observations made by Tang et al. (2008) who studied the thermal denaturation of legumes (red bean, kidney bean and mung bean isolates) and soybean isolates and found that peak temperature of thermal denaturation of proteins in those systems occurred at respective temperatures of 87.1, 90.2, 84.6 and 94.9°C. Although the temperature used in this study (120°C) was higher than the denaturation peaks reported by Tang et al. (2008), the reference is included to support the concept that legume proteins denature and aggregate upon thermal

treatment. The comparison highlights that denaturation in plant proteins generally occurs at elevated temperatures, which aligns with the aggregation observed in our samples. The combination of HPH and HT (HPH-HT) resulted in a D[4,3] value of 3.63 ± 0.18 µm, Dv(90) of 9.36±0.47 µm and Dv(10) of 0.61±0.26 µm, showing a further increase in particle size, especially in the larger particles (Dv(90)) as also highlighted by the confocal laser scanning microscopy revealing the presence of large aggregates.

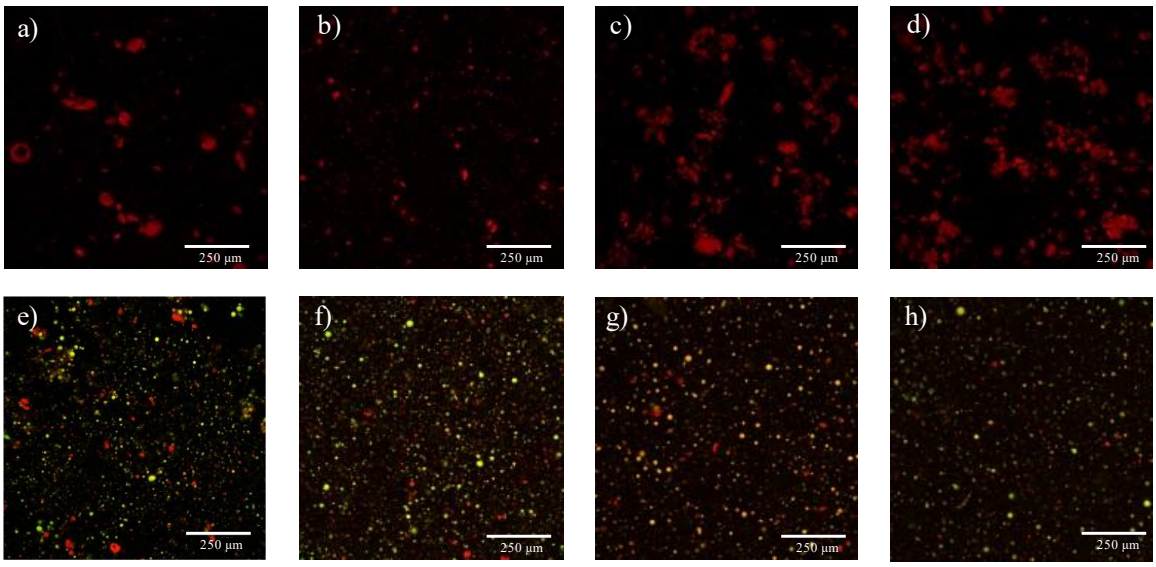

Figure 6.2. Confocal laser micrographs of lentil protein dispersions (a to d) and lentil protein stabilised emulsions (e to h): Control samples (a and e), samples pre-treated with high-pressure homogenisation (HPH) (b and f), samples pre-treated with heat treatment (HT) (120°C for 60 s) (c and g), samples pre-treated with high-pressure homogenisation ;and heat treated (120°C for 60 s) (HPH-HT) (d and h).

In comparison with the HPH pre-treated samples, the particle size of the heat treated (HPH-HT) samples was larger, suggesting that while HPH effectively reduces particle size, the subsequent heat treatment causes the formation of larger protein aggregates. Furthermore, this treatment (HPH-HT) led to significantly ($p<0.05$) higher (as

compared to HPH) Dv(90) and Dv(10) values of 9.36 ± 0.47 µm and 0.61 ± 0.26 µm, respectively, indicating a broader particle size distribution, likely attributed to denaturation and aggregation of the proteins. Similarly, Li et al. (2023) observed an increase in particle size from 300 to 900 nm when *Phaseolus vulgaris* lentil protein was treated for 9 min at 80°C, which was attributed to the unfolding of the lentil protein structure and subsequent aggregation. Upon heating, the protein structure unfolds, exposing hydrophobic sites which interact with each other, leading to the formation of larger aggregates by covalent and non-covalent bonding (Wang and Zhao, 2023; Drapala et al., 2016; Wang et al., 2010). The formation of protein aggregates can have both positive and negative effects on emulsification, depending on their size and structure. Moderate unfolding and partial aggregation can improve emulsifying properties by increasing surface activity and flexibility. However, extensive aggregation may reduce solubility and interfacial adsorption, which could negatively impact emulsion stability. In this study, the observed aggregation was not excessive, suggesting a potentially beneficial effect, though further analysis (e.g., oil droplet size) would be needed to confirm this.

6.3.1.2. Protein content and profile

During the heat treatment of protein dispersions, the formation of fouling due to protein denaturation/aggregation can occur and lead to reduction in protein content of the thermally processed protein solutions, especially in continuous industrial processes (Huppertz and Nieuwenhuijse, 2022; Wang et al., 2018; Nema and Datta, 2006). The protein content and profile in the lentil protein dispersions and emulsions were analysed to investigate the effect of the pre-treatments on the protein quality. The control sample had a protein content of 4.5 ± 0.10%, which was consistent across all the other samples; HPH (4.56 ± 0.08%), HT (4.55 ± 0.04%), and HPH-HT (4.56 ±

0.10%). These results indicated that the pre-treatments did not cause any significant loss of protein and formation of fouling in contrast with what found by Huppertz and Nieuwenhuijse, 2022.

Although no changes in protein content were observed, there might be differences in the protein profile of the dispersions, as influenced by the processing treatment. Therefore, a better understanding of the protein profile, as affected by processing conditions, was obtained by analysing the protein profile of the dispersions (Figure 6.3, a). The control lentil protein isolate dispersions displayed proteins with molecular weights corresponding to 70, 50, 37 and 25 kDa, which would correspond to convicillin, vicilin subunits and acidic and basic subunits of legumin, respectively.

Similar protein profile analyses were previously reported by Jarpa-Parra et al. (2015) and Alonso-Miravalles et al. (2019) on lentil protein isolates. Under non-reducing conditions (left side of Figure 6.3, a), these proteins are seen as intact complexes, while under reducing conditions (right side of Figure 6.3, a), legumin dissociates into its acidic and basic subunits due to the reduction of disulfide bonds. This results in slightly less intense bands at 50 and 37 kDa and slightly more intense bands at 20–25 kDa, as observed on comparing lanes for the control sample under non-reducing and reducing conditions. These observations are consistent with previous studies by Alonso-Miravalles et al. (2019) who showed similar behaviour under reducing conditions on the investigation of lentil protein isolates protein profile.

The protein profile of the HPH sample was similar to the control, indicating that HPH did not have an effect on the protein profile or interactions therein in the dispersions, which is similar to that reported Saricaoglu et al. (2018) in the study of HPH treated hazelnut meal proteins as well as Melchior et al (2022) investigating HPH treated pea

protein. Furthermore, both heat-treated samples (HT and HPH-HT) displayed changes in protein profile with light bands between 40 and 75 kDa, as well as an increase in intensity of protein material retained at the well with molecular weight greater than 250 kDa, indicative of protein aggregation occurring throughout the heat treatment. This is a well-known behaviour of protein ingredients under heating conditions as highlighted by Galvão et al. (2023) who heat-treated lentil protein isolate at 80°C for 20 min. The authors observed an increase in the intensity of the unresolved material at high molecular weight after heat treatment, which was associated with protein aggregation. Upon heating, plant proteins undergo thermal denaturation, where the secondary, tertiary, and quaternary structures are disrupted, as also observed in most globular dairy proteins (Ho et al., 2018; Goetz and Koehler, 2005).

The unfolding exposes hydrophobic regions, reactive amino acid residues and other regions that were previously buried within the folded core of the protein structure, which then leads to protein aggregation and formation of larger aggregates (Bühler et al., 2020). This phenomenon is common to all protein ingredients and has been reasonably well studied on plant proteins, including pulses such as pea, lentil and faba bean proteins (Hall and Moraru., 2021; Najib et al., 2023). The observation of aggregation and presence of protein aggregates on heat treated samples was also supported by the protein profile obtained on reducing conditions as the high molecular weight aggregates band intensity decreases to result in an increase in intensity of the bands at 45-70 kDa.

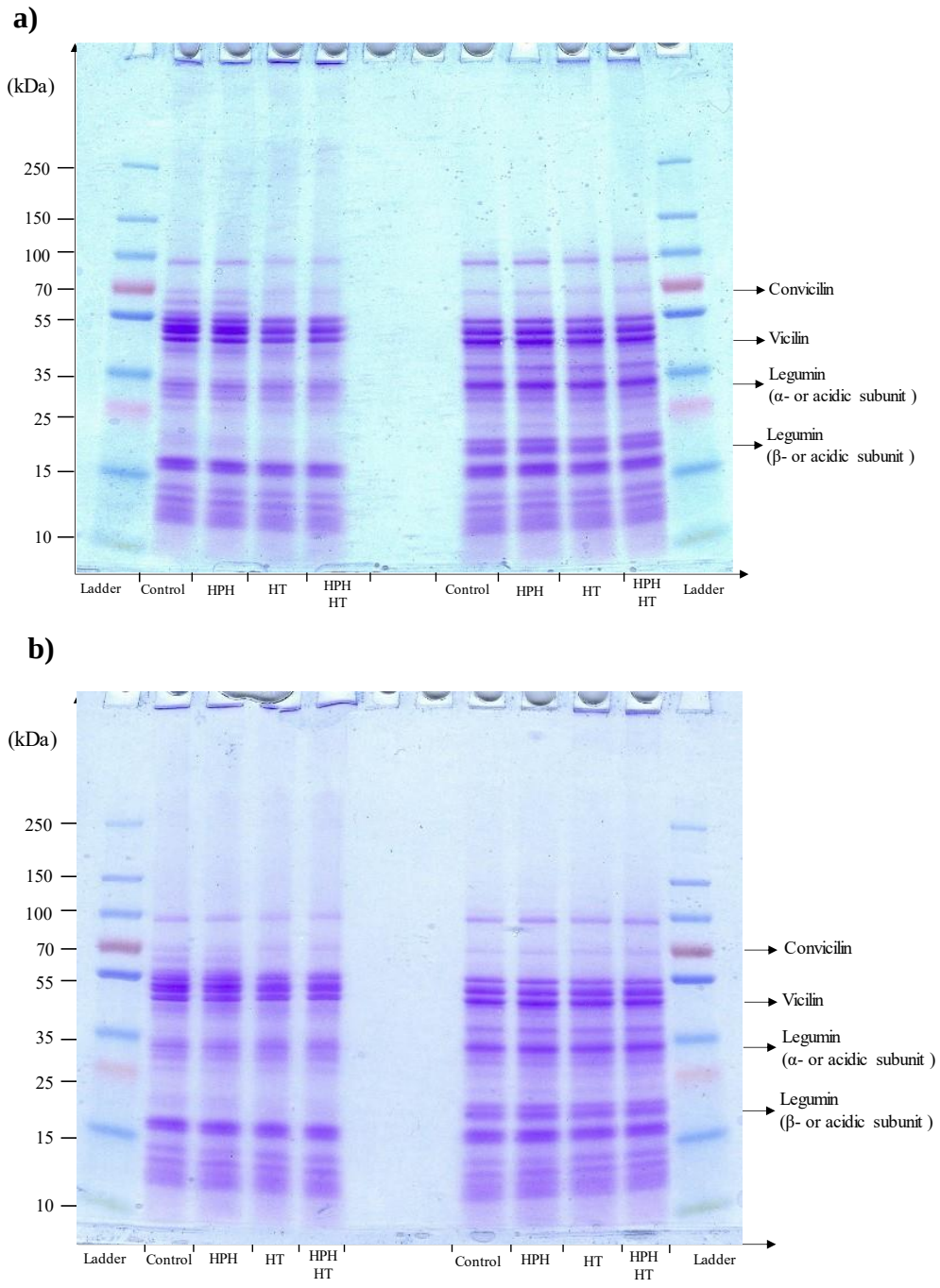

Figure 6.3. Representative sodium dodecyl sulphate-polyacrylamide gel electrophoretograms (SDS-PAGE) of lentil protein isolate dispersions (a) and emulsions (b) under non reducing (left) and reducing (right) conditions, for lentil protein dispersion and lentil protein stabilised emulsions pre-treated at 0 (Control) and 50 MPa (HPH). With heat treatment (HT and HPH-HT).

6.3.1.3. Rheological properties

The apparent viscosity (η_{app}) was measured across a wide range of 0-1000 s^{-1} for all samples, with the average η_{app} values at 100 and 1000 s^{-1} reported in Table 6.2. These values of shear rate were selected for direct comparison of the samples as they are relevant for pumping/liquid transfer and atomisation processes during spray drying, respectively. The control sample exhibited the highest apparent viscosity at 19.18 ± 3.38 mPa·s, and the application of high-pressure homogenisation (HPH) pre-treatments significantly ($p<0.05$) reduced the apparent viscosity of the samples to 11.37 ± 1.75 mPa·s ($p<0.05$). Similar behaviours were observed by Saricaoglu et al. (2020), showing that treatment at 150 MPa could disrupt protein aggregates, reducing the viscosity of lentil protein dispersions from 8.7 to 5 mPa·s. In the HT and HPH-HT pre-treated samples, the viscosity was the lowest, with values reaching 7.99 ± 2.68 mPa·s and 7.43 ± 0.82 mPa·s, respectively. The reduced viscosity observed in the heat-treated dispersions could be due to protein rearrangements caused by denaturation/aggregation. Similarly, Nicoud et al. (2015) found that, in protein solutions, aggregation causing polydispersity could significantly lower the viscosity as compared to non-aggregated samples.

Apparent viscosity at a shear rate of 1000 s^{-1} measured to evaluate the flow behaviour of the dispersions under high shear conditions to mimic conditions as experienced in the spray-drying nozzle. Furthermore, the n value extracted from the shearing profiles before and after the inflection point was used to determine the type of flow exhibited by the samples. The control dispersion exhibited a shear thinning behaviour common in plant protein dispersions as particles align (Jeske et al., 2017; Malterre et al., 2024; Wittek et al., 2021), causing less resistance to flow as highlighted by the n value in regions 1 and 2, leading to a smaller viscosity of 12.30 ± 1.08 mPa·s at 1000 s^{-1}.

HPH-treated samples exhibited a shear thinning behaviour as shown by the flow index behaviour (n) value in region 1 (0.76±0.05), leaning towards Newtonian in region 2 (21.12±0.05), and leading to a viscosity of 0.45 ± 0.59 mPa·s at 1000 s^{-1}. On the other hand, HT and HPH-HT dispersions showed a higher flow behaviour index in region 1 up to 0.94 ± 0.04 and 0.92 ± 0.05 respectively, indicating near-Newtonian behaviour and a significantly lower shear-thinning behaviour. In region 2, the HT dispersion showed an index of 1.45 ± 0.26, indicating shear-thickening behaviour at high shear rates with a higher apparent viscosity of 10.14 ± 1.14 mPa·s at 1000 s^{-1}. The combined treatment resulted in the highest n index at 1.66 ± 0.05, indicating pronounced shear-thickening behaviour resulting in apparent viscosity of 10.14 ± 0.18 mPa·s at 1000 s^{-1}. This shear-thickening behaviour observed in heat-treated samples at high shear rates can be explained by the increased tendency for polymers or particles to aggregate as the shear rate increases, due to the higher collision frequency between the particles at elevated shear rates (McClements 2023; Serra et al., 2024).

Table 6.2. Protein content, apparent viscosity (η_{app}) and flow behaviour rheological parameters for lentil protein dispersion and lentil protein stabilised emulsions pre-treated at 0 (Control) and 50 MPa (HPH) with heat treatment (HT and HPH-HT).

Sample		***Protein Content (%)***	**η_{app} at $100s^{-1}$ (mPa·s)**	**η_{app} at $1000s^{-1}$ (mPa·s)**	***n region 1* (-)**	***n region 2* (-)**	***n region total*** (-)
Dispersion	Control	4.50±0.10^{a}	19.18±3.38^{a}	12.30±1.08^{a}	0.52±0.16^{a}	0.9±0.18^{a}	n.a
	HPH	4.56±0.08^{a}	11.37±1.75^{b}	10.45±0.59^{b}	0.76±0.05^{a}	1.12±0.05^{a}	n.a
	HT	4.55±0.04^{a}	7.99±2.68^{c}	10.14±1.14^{b}	0.94±0.04^{b}	1.45±0.26^{b}	n.a
	HPH-HT	4.56±0.10^{a}	7.43±0.82^{c}	10.14±0.18^{b}	0.92±0.05^{b}	1.66±0.05^{b}	n.a
Emulsion	$Control_{emu}$	3.39±0.09^{a}	22.64±3.62^{a}	12.56±1.54^{a}	n.a	n.a	0.57±0.11^{a}
	HPH_{emu}	3.34±0.04^{a}	16.97±3.50^{a}	10.27±0.59^{b}	n.a	n.a	0.56±0.05^{a}
	HT_{emu}	3.37±0.08^{a}	3.33±0.37^{b}	7.62±0.49^{c}	1.01±0.01^{a}	1.62±0.02^{a}	n.a
	$HPH\text{-}HT_{emu}$	3.31±0.11^{a}	3.32±0.16^{b}	7.66±0.30^{c}	1.01±0.01^{a}	1.64±0.02^{a}	n.a

Values on same column for individual treatments (dispersion or emulsion) that share a common superscript are not significantly different from one another ($p<0.05$)

n = Flow behaviour index (-) n.a = not applicable

6.3.1.4. Accelerated physical stability of the dispersions

The control sample exhibited a separation rate of 13.67 ± 2.28 %/h (Table 6.3), similar to the results of Malterre et al. (2024) and Jeske et al. (2019) using lentil protein isolate dispersions at a concentration of 5 and 3% (w/w), respectively. HPH significantly reduced the separation rate to 2.31 ± 0.57%/h ($p < 0.05$), while the heat-treated samples (HT and HPH-HT) showed significantly higher separation rates of 55.00 ± 11.65%/h and 28.26 ± 3.32%/h, respectively (Figure 6.4).

The observed reduction in the separation rate with HPH is consistent with previous studies, which have shown that HPH can enhance the physical stability of protein dispersions by breaking down large aggregates and creating a more homogeneous system (Moll et al., 2021; Chen et al., 2016).

Table 6.3. Separation rate, sediment height and cream layer height for lentil protein dispersions and lentil protein stabilised emulsions pre-treated at 0 (Control) and 50 MPa (HPH) with heat treatment (HT and HPH-HT).

Sample		Separation Rate (%/h)	Sediment height (mm)	Cream layer height (mm)
Dispersion	Control	13.67 ± 2.28^{a}	13.18 ± 6.28^{a}	n.a
	HPH	2.31 ± 0.57^{b}	0.02 ± 0.00^{b}	n.a
	HT	55.00 ± 11.65^{c}	8.69 ± 0.09^{a}	n.a
	HPH-HT	28.26 ± 3.32^{d}	7.00 ± 0.92^{a}	n.a
Emulsion	$Control_{emu}$	15.11 ± 1.23^{a}	1.92 ± 0.10^{a}	10.37 ± 1.66^{a}
	HPH_{emu}	12.73 ± 2.47^{a}	1.60 ± 0.17^{a}	11.16 ± 1.57^{a}
	HT_{emu}	3.50 ± 1.33^{b}	4.16 ± 0.24^{b}	3.66 ± 0.50^{b}
	$HPH\text{-}HT_{emu}$	1.85 ± 0.42^{b}	4.45 ± 0.39^{b}	3.84 ± 0.43^{b}

Values on same column for individual treatments (dispersion or emulsion) that share a common superscript are not significantly different from one another ($p<0.05$).

n.a. = not applicable

Regarding the heat-treated dispersions, it was shown that heat treatment could lead to the formation of larger particles due to protein denaturation and aggregation (see

Section 6.3.1.1, Table 6.1); these larger particles settle more quickly under centrifugal forces, as also shown by Galvão et al. (2023), which then lead to faster sedimentation and higher separation rates.

Sediment height indicates the amount of settled material after centrifugation. The control sample showed a sediment height of 13.18 ± 6.28 mm, while the HPH-treated sample exhibited a significantly lower sediment height of 0.02 ± 0.00 mm, owing to the higher solubility of the suspensions after the HPH treatment. Similarly, Jeske et al. (2019) observed a decrease in separation rate from 48 to 22%/h when using HPH at 18 MPa. Furthermore, Moll et al. (2021) found that the reduction in particle size achieved through microfluidisation contributed to enhancement in colloidal stability.

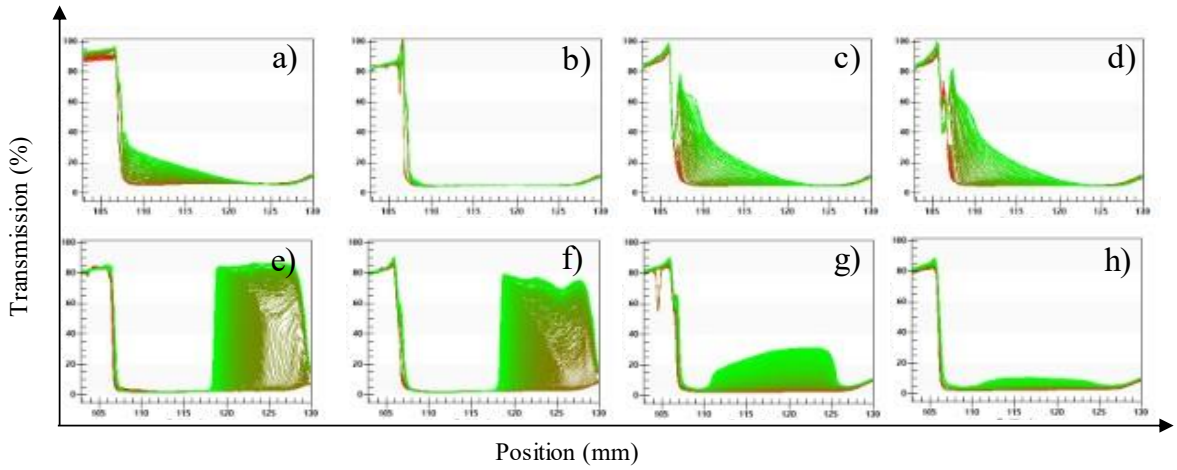

Figure 6.4. Representative transmission profiles of lentil protein dispersion (a to d) and lentil protein stabilised emulsions (e to h): Control samples (a and e), samples pre-treated with high-pressure homogenisation (HPH) (b and f), samples pre-treated with heat treatment (HT) (120°C for 60 s) (c and g), samples pre-treated with high-pressure homogenisation and heat treated (120°C for 60 s) (HPH-HT) (d and h).

The heat-treated samples (HT and HPH-HT) had sediment heights of 8.69 ± 0.09 mm and 7.00 ± 0.92 mm, respectively. These heights were lower than that for the control

but still indicate considerable sedimentation due to protein aggregation and formation of large aggregates

6.3.2. Physicochemical properties of lentil protein stabilised emulsions

6.3.2.1. Particle size distribution

The particle size of the emulsions was measured to evaluate the effect of different treatments on the ability to form physically stable oil-in-water emulsions (Table 6.1). The particle size distribution of the emulsions was analysed, showing that the control had a D[4,3] value of 1.61 ± 0.20 µm, Dv(90) of 3.46 ± 0.59 µm, and Dv(10) of 0.32 ± 0.01 µm, which are typical size ranges for lentil protein-stabilised oil globules as also observed by Alonso-Miravalles et al. (2020) (D[4,3] = 0.75 µm) working on lentil protein emulsions formulated under similar conditions. Additionally, confocal laser scanning micrographs (Figure 6.3e) showed a homogeneous distribution of oil droplets (green) stabilised by lentil protein (red). Emulsions formulated with HPH pre-treated ingredient (HPH_{emu}) exhibited a reduced D[4,3] value of 1.38 ± 0.30 µm, Dv(90) of 2.85 ± 0.81 µm, and Dv(10) of 0.32 ± 0.04 µm, which is consistent with findings by Primozic et al. (2018), who noted that HPH pre-treatment of lentil protein dispersions could lead to a reduction in subsequent emulsion particle size. Indeed, the particle size reduction induced by HPH allows for higher dispersion and protein solubility (Table 6.1), which then leads to improved emulsifying properties

For the emulsions formulated with heat-treated ingredient (HT_{emu}), the D[4,3] value was reduced 1.36 ± 0.33 µm, Dv(90) was 2.58 ± 0.51 µm, and Dv(10) was 0.31 ± 0.01 µm as compared to the control. Similarly, Galvao et al. (2018) found that heat treatment on lentil protein dispersion at 80°C could lead to a reduction in the oil droplet size of subsequent emulsions from 48.37 µm to 21.77 µm. This reduction in oil droplet particle size was attributed to an improvement in solubility, leading to faster adsorption

at the interface. Similarly, Li et al. (2023) observed that heat treatment of lentil protein ingredient at 80°C for a time period of 3 to 7 min led to a reduction of emulsions particle size from 0.30 µm to 0.05 µm. Furthermore, the combined HPH and heat treatment (HPH-HT_{emu}) resulted in the smallest particle size, with a D[4,3] value of 1.07 ± 0.07 µm, Dv(90) of 2.11 ± 0.13 µm, and Dv(10) of 0.30 ± 0.03 µm. Indeed, reduction in the dispersion particle size induced by HPH and the heat-treatment could both lead to improvement of interfacial properties and faster adsorption of the protein at the oil-water interface (Maphosa and Jideani, 2018). In addition, the comparison of the micrographs (Figure 6.3), shows the disappearance of the heat-induced protein aggregates suggesting that the emulsification step allowed for the disruption of these aggregates.

The addition of SDS was used to gain insights into the mechanisms responsible for instability of the emulsions and thus differentiating between flocculation and coalescence. The control emulsion with SDS ($Control_{emuSDS}$) had a D[4,3] value of 1.13 ± 0.06 µm, Dv(90) of 2.17 ± 0.05 µm, and Dv(10) of 0.29 ± 0.01 µm. The HPH-pretreated emulsion with SDS (HPH_{emuSDS}) showed similar values, with a D[4,3] of 1.08 ± 0.05 µm, Dv(90) of 2.13 ± 0.12 µm, and Dv(10) of 0.30 ± 0.02 µm. These results show a reduction in oil-droplet size for both samples with addition of SDS, indicating the presence of reversible flocculation. The magnitude of reduction using SDS was smaller for the HPH_{emu} sample, indicating less flocculation. In addition, the reduction in Dv(90) value for the control sample from 3.46 ± 0.59 µm to 2.17 ± 0.05 µm indicates the presence of large flocculated aggregates which were larger than for the other samples. This supports the previous results, indicating that HPH pre-treatment allows for better stabilisation of the emulsions through the disruption of

protein aggregates and improvement of protein solubility, which then led to more stable lentil-protein-stabilised emulsions.

The heat-treated control emulsion with SDS (HT_{emuSDS}) had a D[4,3] value of 1.21 ± 0.08 µm, Dv(90) of 2.30 ± 0.16 µm, and Dv(10) of 0.30 ± 0.03 µm. The combined HPH and heat-treated emulsion with SDS (HPH-HT_{emuSDS}) had a D[4,3] value of 1.14 ± 0.15 µm, Dv(90) of 2.22 ± 0.32 µm, and Dv(10) of 0.29 ± 0.03 µm. Similar to the $Control_{SDSemu}$ and HPH_{emuSDS} samples, the reduction in particle size highlights the presence of flocculation in the HT_{emu} sample but it was minimal as compared to the non-heat-treated ones. This result also indicates less flocculation and a stronger stabilisation of the oil globule in the emulsions. Furthermore, the combined HPH-HT_{emuSDS} samples displayed similar PSD parameters before and after the addition of SDS (D[4,3] from 1.07 ± 0.07 to 1.14 ± 0.15), indicating no flocculation and supporting that both HPH and heat-treatment have combined effects leading to stronger stabilisation of lentil protein stabilised emulsions.

6.3.2.2. Rheological properties

The $Control_{emu}$ sample exhibited an apparent viscosity (η_{app}) at 100 s^{-1} of 22.64 ± 3.62 mPa·s (Table 6.2), while HPH_{emu} showed a reduced apparent viscosity of 16.97 ± 3.50 mPa·s at 100 s^{-1} indicating that HPH pre-treatment of the dispersion allowed for a reduction in viscosity of the subsequent emulsion. In Section 6.3.1.3, it was highlighted that the disruption of protein aggregates allowed for a reduction in viscosity of the dispersion through the disruption of protein aggregates, which then translated in a reduction in viscosity in the subsequent emulsions. Heat-treated control emulsions (HT_{emu} and HPH-HT_{emu}) exhibited a significant reduction in apparent viscosity at 100 s^{-1}, with values of 3.33 ± 0.37 and 3.32 ± 0.16 mPa·s, respectively, aligned with the reduction in viscosity observed for the dispersion through heat

treatment. However, the viscosity of the heat-treated emulsions was smaller than the viscosity of the associated protein dispersion, suggesting that homogenisation used for emulsification allowed for disruption of protein aggregates formed during heat treatment, therefore reducing the viscosity further.

For all emulsions, similarly to the dispersions previously presented (Section 6.3.1.3), their apparent viscosity was measured at 1000 s^{-1} and the flow index behaviour was extracted before and after the inflection point. The $Control_{emu}$ exhibited an apparent viscosity (η_{app}) at 1000 s^{-1} of 12.56 ± 1.54 mPa·s and no inflection point was observed. The lower viscosity at high shear rates indicates shear thinning behaviour, as evidenced by the n value of 0.57±0.11. This is a typical behaviour of plant-based emulsions, where particles within the emulsion are disrupted during shearing, resulting in reduced turbulence and decreased viscosity (McClements, 2023). HPH_{emu} also displayed a shear-thinning behaviour similar to the control emulsion, with apparent viscosity of 10.27 ± 0.59 mPa·s at 1000 s^{-1}, and n value of 0.56±0.05, with no inflection point.

At 1000 s^{-1} the heat-treated emulsions HT and HPH-HT had viscosities of 7.62 ± 0.49 mPa·s and 7.66 ± 0.30 mPa·s, respectively. Both heat-treated samples displayed an inflection point splitting the viscosity-shear rate profile in a region displaying near-Newtonian behaviour at lower shear rates (n region 1 = 1.01 ± 0.01 and 1.01 ± 0.01 for HT_{emu} and HPH-HT_{emu},respectively) and a region displaying shear-thickening behaviour at higher shear rates, as indicated by the flow behaviour indices (n region 2 = 1.62 ± 0.02 and 1.64 ± 0.02 for HT_{emu} and HPH-HT_{emu}, respectively).

6.3.2.3. Accelerated physical stability

The separation rate measures the rate at which phase separation occurs in the emulsions. $Control_{emu}$ exhibited the highest separation rate at 15.11 ± 1.23%/h, indicating poor stability under accelerated conditions compared to the other samples (Table 6.3). HPH_{emu} showed a slightly lower separation rate of 12.73 ± 2.47%/h, suggesting an improvement in stability, similarly to what is shown by Levy et al. (2022) which observed a similar phenomenon with a slower separation rate for HPH treated (200 MPa) potato protein stabilised emulsions. The authors reported a stronger stabilisation of the oil globules and reduced creaming velocity from 9 µm/s to 1.25 µm/s with the use of HPH pre-treatment. As shown in previous sections, HPH treatment leads to better solubilisation of the protein ingredient which then led to faster adsorption of the protein at the interface and stronger stabilisation of the oil globules.

HT_{emu} displayed a significant reduction in separation rate to 3.50 ± 1.33%/h, indicating enhanced stability induced by heat treatment, and further, HPH-HT_{emu} exhibited the lowest separation rate at 1.85 ± 0.42%/h, indicating the highest stability among all samples. Similarly, Galvão et al. (2023) observed better physical stability of emulsions formulated with heat treated lentil protein over a period of 60 days where the backscattering measurement yielded Turbiscan Stability Index (TSI) values close to 2 as opposed to a value close to 4 for untreated emulsions (the lower the TSI value, the higher the stability). This higher physical stability of the heat-treated samples can be attributed to the lower oil droplet size (Table 6.1).

$Control_{emu}$ had a sediment height of 1.92 ± 0.10 mm, while the HPH_{emu} sample had a slightly lower height of 1.60 ± 0.17 mm due to the reduction in particle size observed in Section 6.3.1.1 which also contributed to the lower separation rate. Heat-treated emulsions showed a substantial increase in sediment height, with HT_{emu} at 4.16 ± 0.24

mm and HPH-HT_{emu} at 4.45 ± 0.39 mm. This increase was related to the protein aggregation seen during heat treatment (Section 6.3.1.1) of the protein dispersion which results in bigger aggregates in the continuous phase leading to a thicker sediment layer.

$Control_{emu}$ had a cream layer height (result of the upward motion of oil droplets) of 10.37 ± 1.66 mm, and HPH_{emu} had a similar height of 11.16 ± 1.57 mm, suggesting that HPH alone did not affect creaming of the emulsion. However, heat treated emulsions showed a marked reduction in cream layer height, with HT_{emu} at 3.66 ± 0.50 mm and HPH-HT_{emu} at 3.84 ± 0.43 mm, indicating that oil globules are stable in the emulsion and did not move to the upper layer of the sample. This can be attributed to the stronger interfacial properties of the protein ingredient induced by protein denaturation through heat treatment allowing for a stronger protein layer around the oil globule which results in more physically stable emulsions (Levy et al., 2022; Galvao et al., 2023). Likewise, as shown by Shao and Tang (2014) soybean protein isolate treated at 95°C for 15 min exhibited a higher concentration of protein at the interface, increasing from 19.7% in untreated samples to 31.3%, and improved physical stability, evidenced by a reduction in the creaming index from 70 to 30%.

6.4. Conclusion

This study demonstrated the combined effects of pre-treatment with high-pressure homogenisation (HPH) and heat treatment on the techno-functional properties of lentil protein stabilised emulsions. HPH significantly reduced the particle size of the lentil protein dispersions, while the heat treatment increased the particle size, as observed in confocal microscopy, indicative of denaturation and aggregation of proteins. The reduction in particle size of the dispersions as well as the protein denaturation allowed for a better stabilisation of the emulsions made therefrom, resulting in smaller oil droplets and a strong reduction in flocculation. Furthermore, the pre-treatments allowed for a reduction in apparent viscosity of the emulsions and changes in rheological properties, leading to shear-thickening behaviour at higher shear rates, likely due to the presence of protein aggregates interacting at higher shear rates. Physical stability analysis revealed that HPH and heat treatment improved the physical stability of the emulsions by stabilising the oil globules more efficiently, resulting in a slower separation and reduced creaming. This enhanced stability can be attributed to the HPH induced reduction in particle size of the dispersion as well as the heat-induced denaturation of the proteins, resulting in better functionality of the protein ingredient which translated to better stabilisation of the oil globules. These findings suggest that the combined application of HPH and heat treatment is a promising approach to improve the stability and functionality of high solid lentil protein-stabilised emulsions. Optimising these processing conditions, would lead to the development of more stable and functional sustainable plant-based emulsions suitable for various food applications.

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Chapter 7

General discussion and Suggestions for future research

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General discussion

Young child formulae are traditionally based on bovine milk, which provides proteins, fats, and carbohydrates essential for young child growth and development. These formulae are designed to mimic human breast milk by offering balanced nutrition and the required micronutrients (such as vitamins and minerals). Bovine milk proteins, such as casein and whey, are the primary proteins used in these formulae due to their high nutritional value and digestibility (Blanchard et al., 2013; O'Mahony and Fox, 2013; Crowley et al, 2016). However, there are certain limitations to the use of animal-based proteins in young child formulae, due to allergic reactions and intolerances (Koletzko et al., 2012. These health issues, combined with ethical and environmental considerations, have driven the search for alternative protein sources for young child nutrition (**Chapter 1**). In response to these concerns, there has been a growing interest in the development of plant-based food products (Alonso-Miravalles et al., 2020). Plant proteins, including those derived from soy, pea, and lentil, are being explored as viable alternatives to animal-based proteins (Grossmann and Weiss, 2021). These plant proteins are highly sustainable, aligning with the increasing consumer demand for environmentally friendly and ethically produced food products (Heller et al., 2013). Among these, lentil protein is particularly interesting due to its rich amino acid profile (DIAAS in the range 55 to 65; Cargo-Froom et al., 2023; Hang et al., 2022), and high protein content, making it a promising candidate for young child nutrition (Alonso-Miravalles et al., 2018; Boye et al., 2010).

Despite these advantages, plant proteins pose significant challenges in formulation, primarily due to their low solubility and low physical stability, particularly in high solid formulations (Primozic et al., 2018; Jeske et al., 2019). Solubility is a crucial

functional property for proteins in liquid foods and beverages, as it influences texture, mouthfeel, and overall stability of the product (Grossmann and McClements., 2023). In addition to these functional challenges, the presence of off-flavours in plant proteins can negatively affect sensory properties, making flavour optimisation critical for product development. Additionally, the stability of lentil protein-stabilised emulsions, which are crucial for delivering fats and other nutrients, is often compromised due to their poor functional properties (Vogelsang-O'Dwyer et al., 2023; Alonso-Miravalles et al., 2018). To address these challenges, various processing techniques have been investigated to improve the solubility and selected functional properties of plant proteins (Gharibzahedi et al., 2023). High-pressure homogenisation (HPH) and in-line high-shear mixing are two such technologies that have shown promising results in enhancing protein solubility and colloidal stability of protein ingredients (Moll et al., 2022; Saricaoglu., 2020; Pacek et al., 2013). These techniques apply mechanical forces to break down powder particles, protein aggregates, reduce particle size, and increase surface area, thereby improving solubility and functional properties (Dumay et al., 2013). In the context of young child formula, these processing techniques can play a crucial role in developing stable, high-solid plant-based formulations, which can be advantageous as it minimises the necessity for water removal through evaporation prior to spray drying, a process that is both energy demanding and expensive (Patil et al., 2021; Ramirez et al., 2006). Therefore, formulating stable high solid emulsions can improve production efficiency and lower costs, contributing to a more sustainable manufacturing approach.

This thesis investigates the techno-functional properties of lentil protein isolate for use in high total solid emulsions. **Chapter 1** highlighted the challenges and, the potential of plant-based young child formulas, together with the role of processing techniques

in overcoming some of these challenges. Building on this foundation, **Chapter 2** delved into the formulation of high-solid lentil protein emulsions aiming at reducing the need for water removal before spray drying (Patil et al., 2021; Chamberland et al., 2020). Emulsions were stable in the range of 23-26% total solids, while at 29%, the samples displayed poor heat and physical stability, which was mainly attributed to the higher concentration of protein and insoluble material in the continuous phase. Therefore, low protein solubility was identified as one of the limiting factors and led to the exploration of processing techniques to improve the functional properties of lentil protein dispersions (**Chapters 3 and 4)**.

Chapter 3 explored the use of in-line high-shear rotor-stator mixing to enhance the techno-functional properties of lentil protein isolate dispersions. Although high-shear mixing improved protein solubility and stability, it was less effective than HPH. In particular, high-shear mixing increased protein solubility and decreased particle size but to a lesser extent compared to HPH. For instance, high-shear mixing at 15,000 rpm for 15 min resulted in a protein solubility increase from 46.87 to 68.42% and a reduction in separation rate from 71.23 to 24.16%·h^{-1}. This suggested that while high-shear mixing could be a viable alternative for certain applications, it might require further optimisation for maximum efficacy for plant protein techno-functional properties enhancement. High-pressure homogenisation (HPH), which has been shown to be particularly effective in enhancing the solubility and stability of plant proteins was used in **Chapter 4**. The optimal pressure of 50 MPa resulted in significant improvements in solubility, particle size reduction, and physical stability of the dispersions due to its ability to disrupt powder particles and protein aggregates. Specifically, HPH used at 50 MPa increased protein solubility from 62.8 to 92.7%, reduced particle size from 10.7 µm to 0.39 µm, and improved physical stability, with

a reduction in the separation rate from 8.12 to 4.22%·h^{-1}. The higher performance observed using HPH led to a deeper investigation of its application for the formulation of young child formulae. For this reason, **Chapter 5** used the learnings from **Chapters 2, 3 and 4** to investigate the effect of HPH pre-treatment on the properties of lentil protein-stabilised high-solids emulsions. The study focused on how different HPH pressures (0, 15, 50 and 150 MPa) applied to lentil protein dispersions influenced the functional properties of the resulting emulsions. The findings showed that HPH pre-treatment improved the emulsification properties, leading to emulsions with smaller droplet sizes and enhanced stability. Specifically, emulsions formulated with lentil protein isolate pre-treated at 150 MPa had lower viscosity (from 28.00 to 22.56 mPa·s) and improved heat stability with significantly less flocculation, as underlined by particle size reduction after addition of SDS, compared to the untreated samples. Furthermore, the physical stability of the emulsions formulated with pre-treated lentil protein isolate displayed significantly reduced separation rates. These results highlighted the effect of HPH pre-treatment of the protein dispersion on the techno-functional properties of the lentil protein emulsions as affected by increasing protein solubility.

In **Chapter 6** the impact of thermal treatment on HPH-treated lentil protein dispersions and subsequent emulsions formulated at high solids (29%) were investigated. Emulsions formulated from HPH-treated lentil protein dispersions at 50 MPa, followed by thermal treatment at 120°C for 60 s, exhibited lower viscosity, reduced flocculation, and improved overall stability compared to those without thermal treatment. Specifically, the viscosity of these emulsions decreased from 19.18 mPa·s to 7.43 mPa·s, which is crucial for high solid emulsions, as it facilitates processing and handling.

Furthermore, when the emulsions were thermally treated after HPH, an improvement in the physical stability of the samples was observed. Emulsions made from dispersions treated with both HPH and thermal processing showed significantly less flocculation and higher resistance to creaming and sedimentation. The separation rate, an indicator of emulsion destabilisation, was significantly reduced from 15.11%·h^{-1} in the control sample, to 1.85%·h^{-1} in the treated sample, demonstrating the effectiveness of this combined treatment in maintaining emulsion integrity. These results showed that combined effects of HPH and thermal treatment are highly beneficial for the formulation of stable, high total solid lentil protein-stabilised emulsions (29%). These findings also suggest that applying both HPH and thermal treatment can overcome the inherent challenges associated with plant protein solubility and stability, making lentil protein a viable and effective ingredient for plant-based young child nutritional products.

In summary, this thesis investigated the formulation of high solid lentil protein-stabilised emulsions for plant-based young child nutrition through the combinations of processing techniques, including HPH and thermal treatments. The research presented here addressed the challenges associated with the low solubility and stability of plant proteins, particularly lentil protein, in high solid formulations. In particular, this thesis has demonstrated that HPH, used in combination with thermal treatment, can significantly improve the solubility, stability, and overall techno-functional properties of lentil protein dispersions and emulsions. These findings directly address key limitations in plant protein functionality, paving the way for wider applications in plant-based, high-nutrient food systems. The implications of these results extend beyond technical improvements. By enhancing the functional properties of lentil protein, this research supports cost-effective and sustainable manufacturing processes.

The reduced need for extensive water removal during production and the improved stability of high-solid emulsions can lower both energy consumption and production costs. This aligns with global sustainability goals and the growing consumer demand for plant-based, environmentally friendly food products and supports the development of sustainable, plant-based young child nutritional products.

Suggestions for future research

The findings from this thesis provide a robust foundation for the continued exploration and enhancement of plant-based protein ingredients for young child nutrition. While significant advancements have been made, several areas need further investigation to fully exploit the potential of lentil protein and other plant-based ingredients. Future research should consider the following directions:

Combined processing technologies for the improvement of plant-based protein formulations.

While high-pressure homogenisation (HPH) and thermal treatment (**Chapters 4, 5 and 6**) have shown substantial improvements in the functional properties of lentil protein, exploring the combined effects of these with other processing techniques, such as ultrasonication, could yield even greater enhancements. For example, the combined use of processing methods has shown promising results in terms of energy efficiency (Kamani et al., 2021). It was shown that combining HPH with ultrasonication (US) significantly enhances multiple functional properties of plant proteins (Zhao et al., 2022). Other research also indicates that the energy density level of nanoemulsions prepared using HPH-US combined technology is approximately half of that required by single HPH or US alone (Calligaris et al., 2016).

Sequence of unit operations in the manufacture of young child formulae, with a focus on evaporation

Future research should systematically study the effects of evaporation on the stability and functional properties of high solid emulsions, as well as the processing order (evaporation before or after emulsification). This will help in optimising the sequence of processing steps to achieve the best product quality and stability. Additionally, examining the molecular and structural changes that occur during these different sequences will provide insights into the mechanisms driving emulsion stability. This knowledge can then be applied to optimise the process, ensuring that the final product maintains its integrity during storage and usage. Moreover, these studies should consider the scalability of the optimised processes to ensure that they can be effectively implemented in industrial settings.

Effect of HPH pre-treatment on the spray drying of high solid emulsions

Chapter 6 allowed for the formulation of high-solid (29%) lentil protein stabilised emulsions. The next stage in the manufacture of powders is the investigation and optimisation of the spray drying process to develop shelf-stable powdered products. Spray drying of plant-based lentil protein stabilised emulsions can bring processing challenges due to their viscosity differences and presence of other components such as starch and fibres (Alonso-Miravalles et al., 2020). Spray-drying parameters will also have a significant impact on the properties of the resulting powders, therefore their optimisation for plant protein application is necessary (Kim et al., 2003). Future research should focus on optimising spray drying parameters to maintain the enhanced functional properties observed in the liquid emulsions.

Additionally, studying the reconstitution properties of the powdered products will be critical for ensuring consumer acceptance and product performance.

Bulk handling and rehydration properties of powdered young child formulae

Once high solid emulsions are spray dried, their bulk handling properties become crucial for industrial applications. Future studies should evaluate the flowability, dispersibility and stability of the dried powders. Furthermore, the drying of high fat emulsions has shown to yield powders with high surface free fat (Fäldt et al., 1994; 41 Foerster et al., 2016; Kim et al., 2009, 2003). Techniques such as lecithination, could be explored to improve these bulk handling properties and ensure ease of use in manufacturing processes. Ultimately, improving these properties will enhance the efficiency of manufacturing processes, ensure consistent product quality, and extend the shelf life of the final product, making it more appealing and reliable for consumers.

Digestibility of lentil protein young child formulae

Chapters 5 and 6 have shown significant improvements of techno-functional properties of lentil protein stabilised emulsions. These changes were mainly due to changes in the solubility and particle size of the protein ingredients. Protein digestibility is correlated to a certain extent with protein solubility and particle size of the ingredients (Pengbin et al., 2002; Bai et al., 2015). Furthermore, other studies have examined the effect of HPH on digestibility of different plant protein sources (D'Alessio et al., 2022), showing a reduction of β-sheets antiparallel (%) and β-sheets parallel, from 13.86 to 8.55% and 14.79 to 7.13%, respectively, which was correlated with enhanced digestibility. But the studies focusing on lentil protein are rare, and

more particularly, very few studies to date focus on the digestibility of food products formulated using plant protein ingredients with high solubility upon HPH process. Future studies should focus on how HPH affects the digestibility of lentil proteins and investigate how these changes in protein structure ensure that improved functional properties also bring nutritional benefits. These future studies should also establish that the proteins are bioavailable and beneficial for young child growth and development.

This thesis has made significant progress in improving the techno-functional properties of lentil protein for use in young child nutrition, and the suggested future research directions aim to build on the current knowledge, address remaining challenges, and ultimately support the development of innovative, sustainable, and high-quality plant-based nutritional products.

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