Microfluidic Analysis of Emulsion Stability: Evaluating Native and Modified Lesser Mealworm Protein Concentrate

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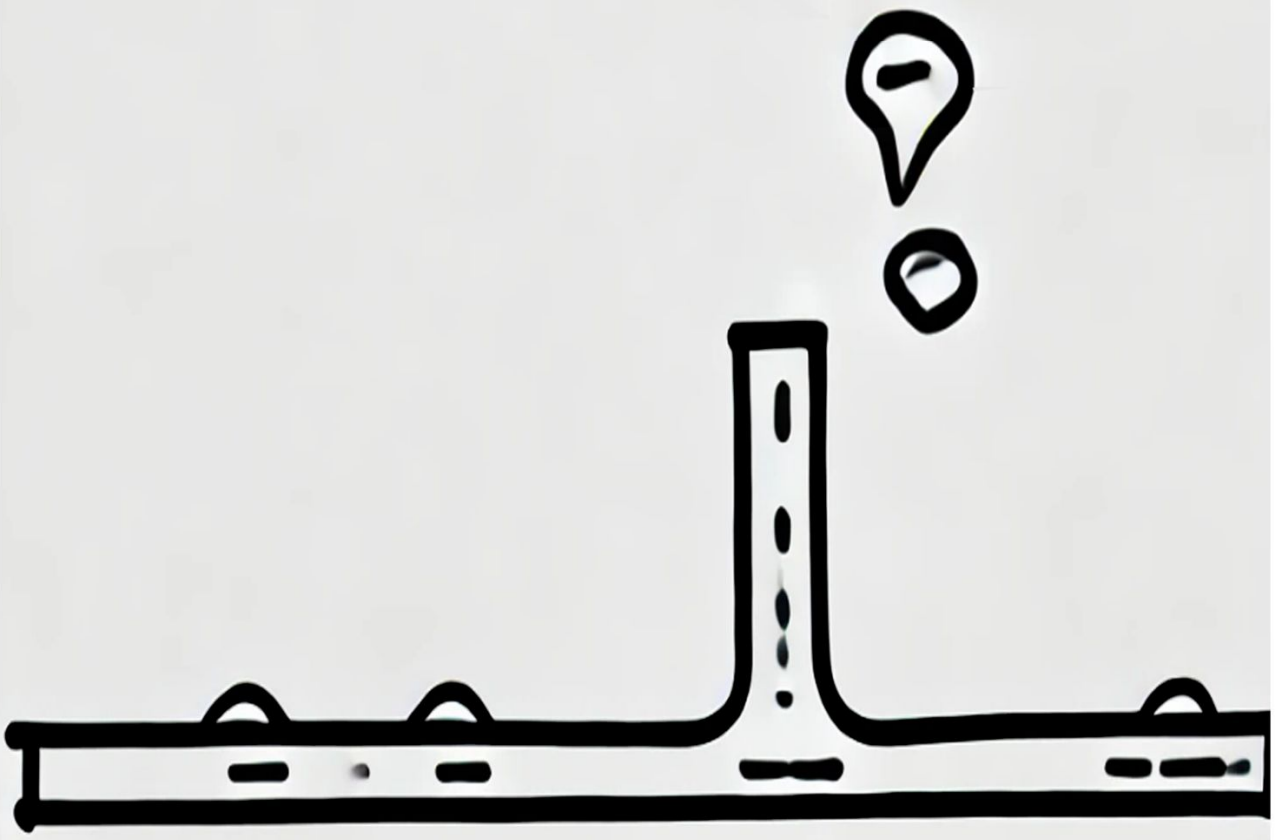

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FAIG CONSTAR que aquest treball, titulat “Anàlisi Microfluídic de l'Estabilitat de les Emulsions: Avaluació del Concentrat de Proteïna de Gusano de la Harina Natiu i Modificat”, que presenta Jitesh Jayakumar per a l’obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament d’Enginyeria Quimica d’aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado “Análisis Microfluídico de la Estabilidad de Emulsiones: Evaluación del Concentrado de Proteína de Gusano de la Harina Nativo y Modificado”, que presenta Jitesh Jayakumar para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el Departamento d’Enginyeria Quimica de esta universidad.

I STATE that the present study, entitled “Microfluidic Analysis of Emulsion Stability: Evaluating Native and Modified Lesser Mealworm Protein Concentrate”, presented by Jitesh Jayakumar for the award of the degree of Doctor, has been carried out under my supervision at the Department *d’Enginyeria Quimica* of this university.

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Abstract

This study investigates the potential of lesser mealworm protein concentrate (LMPC) as a sustainable alternative to whey protein isolate (WPI) in stabilizing oil-in-water (O/W) emulsions using microfluidic techniques. Emulsions were prepared using hexadecane and sunflower oil stabilized with LMPC and WPI at varying concentrations ranging from 0.0005% to 0.02% w/v. The oil fractions studied were 5.3%, 7.7%, 11.1%, and 14.3% v/v, with protein adsorption times spanning 0.0398 to 0.158 seconds. The frequency of coalescence (Fcoal) was measured, revealing that Fcoal was highest at 0.42 s^{-1} when the protein concentration was lowest (0.0005%), the oil fraction was highest (14.3%), the adsorption period was shortest (0.0398 s), and the pH was 3. The study found that increasing the protein concentration to 0.02% significantly reduced F_coal to 0.12 s^{-1}, demonstrating enhanced emulsion stability.

Further, the study examined the impact of modified LMPC using chlorogenic acid (CA), tannic acid (TA), and thermal treatment (Th) on emulsion stability. Results indicated that thermally treated LMPC exhibited the lowest Fcoal of 0.08 s^{-1} at a protein concentration of 0.02% and an oil fraction of 14.3%, underscoring the improved interfacial properties and stabilization capacity of modified proteins. The microfluidic setup allowed precise control over droplet formation and coalescence, providing detailed insights into the dynamics of emulsion behavior.

Statistical analyses, including two-way ANOVA and regression analysis, were employed to evaluate the interactions and significance of variables such as protein type, concentration, and oil fraction. The regression models accounted for approximately 75% of the variability in emulsion stability, highlighting the critical roles of these factors. The study concluded that LMPC, both in its native and modified forms, is a viable and effective alternative to WPI for stabilizing O/W emulsions, with performance comparable to or better than traditional dairy proteins.

Overall, this research demonstrates the potential of LMPC as a sustainable emulsifier in food and pharmaceutical applications, emphasizing the importance of protein concentration, oil fraction, and adsorption time in achieving optimal emulsion stability. The use of microfluidic techniques provided a robust platform for analyzing and optimizing emulsion formulations, paving the way for future studies and industrial applications of LMPC and its derivatives.

Chapter 1

Introduction

1. Introduction

1.1. Importance of emulsions in food science and industry

In the food industries, emulsions are essential as they affect the formulation, stability, texture, and sensory qualities of many different food items. Their importance goes beyond simple culinary uses; they affect the food products' nutritional value, shelf life, and general acceptability by consumers. Emulsions are mixtures of two immiscible liquids, usually water and oil, that are stabilized by emulsifiers and distributed inside one another. This section explores the significance of emulsions in the field of food science and industry, emphasizing their uses, advantages, and the developments in science that have improved their performance (McClements, 2015; Serdaroğlu, Öztürk, & Kara, 2015). Oil-in-water (O/W) and water-in-oil (W/O) emulsions are the two main categories of emulsions. Oil-in-water emulsions, or O/W emulsions, are often found in foods including salad dressings, mayonnaise, milk, and cream(Dickinson, 2012). Butter and margarine are examples of W/O emulsions, which are products in which water droplets are distributed inside oil. These emulsions improve food items' mouthfeel, texture, and flavor as well as the way nutrients and flavors are delivered (McClements, 2015).

Emulsions in food offers various benefits, including improved texture, extended shelf life, and enhanced nutritional value. For example, the fine dispersion of oil droplets in water contributes for the creamy texture of mayonnaise and salad dressings, giving them a smooth and appetizing consistency. Because it affects how well the food product is viewed as being of high quality, this is crucial for customer happiness. Furthermore, emulsions can increase food items' stability by avoiding the separation of the water and oil phases, which is essential for preserving the look and consistency of products like dressings and sauces (Berton-Carabin & Schroën, 2019). Another important area where emulsions are essential is flavor delivery. Lipophilic tastes may be delivered by the distributed droplets in an emulsion, guaranteeing a constant and homogeneous flavor throughout the product. This feature is especially crucial for drinks and sauces, where flavor release and intensity are critical to customer pleasure. Furthermore, improved encapsulation and preservation of volatile flavor chemicals are made possible by the tiny droplet size of nano-emulsions, which improves the food product's overall sensory experience (Tan & McClements, 2021).

Beyond just improving flavor and texture, emulsions also improve food products' nutritional profiles. Emulsions can shield delicate substances like vitamins and omega-3 fatty acids from deterioration during processing and storage, ensuring their continued effectiveness when ingested. This capacity to encapsulate is especially useful for creating functional meals, such nutritional supplements and fortified drinks, that are intended to offer unique health advantages (Bai, Huan, Rojas, & McClements, 2021; Berton-Carabin & Schroën, 2019). These advantages are demonstrated by several food emulsions that are presently available for purchase. For

example, emulsions give mayonnaise and salad dressings their creamy texture and stable consistency. Similarly, vitamins and omega-3 fatty acids are delivered by nano-emulsions in fortified dairy products (Øye, Simon, Rustad, & Paso, 2023; Tan & McClements, 2021). Furthermore, goods like butter and margarine show how water-in-oil emulsions are used to provide a desired spreadable texture and longer shelf life (Berton-Carabin & Schroën, 2019).

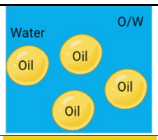
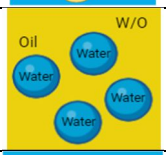
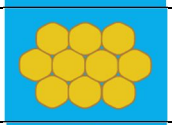
When creating functional foods, which are intended to offer health advantages beyond just nourishment, emulsions are an essential component. Emulsions can boost the bioavailability of bioactive chemicals during consumption by encapsulating them and shielding them from degradation during processing and storage. This has important ramifications for the creation of fortified meals and nutraceuticals meant to enhance health results. When it comes to encapsulating lipophilic bioactive substances like vitamins A, D, E, and K and omega-3 fatty acids, which are easily degraded by light, oxygen, and heat, emulsions work very well. By isolating these substances from the external world, the emulsion droplets contribute to their stability and extended shelf life. For example, it has been demonstrated that omega-3 fatty acids are shielded from oxidation by nano-emulsions, maintaining their potency as dietary supplements (Tan & McClements, 2021).

Food emulsions have several advantages, however there are drawbacks to their production and stabilization. Natural and clean-label emulsifiers are necessary, as are those that retain stability in a range of environmental conditions and guarantee the bioavailability of nutrients that are encapsulated. By creating new emulsifiers, developing sophisticated processing methods, and deepening our knowledge of the mechanics behind emulsion stability, ongoing research is tackling these difficulties (Øye, Simon, Rustad, & Paso, 2023).

Emulsions are vital to the food business and are available in a variety of forms, each with their own special qualities and uses. Traditional emulsions, such as water-in-oil (W/O) and oil-in-water (O/W), improve the flavors and textures of goods like mayonnaise and milk (McClements, 2015). Water-in-oil-in-water (W/O/W) is one example of an emulsion that allows for the regulated release of bioactive chemicals in functional meals. In drinks and fortified meals, multilayer emulsions enhance stability and nutrition delivery. Reduced-fat spreads and other low-fat products with creamy textures are made possible by high internal phase emulsions (HIPEs). The uses of advanced emulsions in the food business are further expanded. Solid lipid particles are beneficial for vitamins and antioxidants because they improve the stability and regulated release of lipophilic substances. Pickering emulsions provide superior stability for clean-label products because they are stabilized by solid particles. Yoghurt and sauce textures are enhanced by gelling particles. Throughout processing, flavors and bioactive are shielded by solid microcapsules, allowing for regulated release (Tan & McClements, 2021; Wu et al., 2022). Every kind of emulsion

has unique advantages that meet the various demands of the food sector. Table 1 represents an outline of the type of emulsions.

Table 1: Types of emulsions and their characteristics along with their illustration

Illustration	Type of emulsion	Characteristics
	Oil-in-Water (O/W)	Oil droplets dispersed across a continuous water phase. prevalent in goods like salad dressings, mayonnaise, and milk.
	Water-in-Oil (W/O)	Dispersed water droplets in a continuous oil phase. Found in butter and margarine.
	Multiple Emulsion (W/O/W)	Complex system with water droplets within oil droplets, further dispersed in water. Used for controlled release.
	Multilayer Emulsion	Droplets with multiple layers of biopolymers. Enhanced stability and nutrient delivery in fortified foods.
	High Internal Phase Emulsion	High volume fraction of dispersed phase. Used to create low-fat products with creamy textures.
	Solid Lipid Nanoparticle	Solid lipid particles that enhance stability and controlled release of lipophilic ingredients.
	Pickering Emulsion	Stabilized by solid particles instead of surfactants. Superior stability, used for clean-label products.
	Gelling Particle Emulsion	Forms gel networks within the emulsion. Improves textures in yogurts and sauces.
	Solid Microcapsule	Encapsulates flavors and bioactive compounds. Protects during processing and ensures controlled release.

1.2. Types of emulsifiers used in food industries.

Based on the mechanism of emulsification, emulsifiers may be divided into three categories: those that lower interfacial tension, create physical barriers, and alter the continuous phase's viscosity. These processes are essential for figuring out how stable and effective emulsions are in different food applications (McClements, 2015).

1.2.1. Surface-Active Agents (Surfactants)

Surface-active molecules, often known as surfactants, facilitate the creation of smaller, more stable, and less prone to coalesce droplets by lowering the interfacial tension between the oil and water phases. These emulsifiers can align at the interface and stabilize the emulsion because they usually feature hydrophilic (water-attracting) and hydrophobic (water-repelling) ends. For instance, the way mono- and diglycerides, which are often found in ice cream, baked goods, and margarine, function is that they create a monolayer surrounding oil droplets, which keeps them from aggregating together and lowers the interfacial tension. In a similar way, polysorbates, nonionic emulsifiers made from sorbitol and fatty acids—are frequently found in sauces, creams, and salad dressings. By lowering the surface tension between water and oil, they stabilize emulsions (McClements, 2015). Similarly, nonionic emulsifiers called polysorbates (like Tween 20 and Tween 80) are made from sorbitol and fatty acids and are frequently found in sauces, creams, and salad dressings. By lowering the surface tension between water and oil, they stabilize emulsions. Because of their dual activity, tween emulsifiers are very useful for producing stable, homogeneous emulsions—even in intricate food systems (W. Shen et al., 2022).

1.2.2. Macromolecular Emulsifiers

Proteins and polysaccharides are examples of macromolecular emulsifiers that stabilize emulsions by encircling scattered droplets in a viscoelastic film. By acting as a physical barrier, this film keeps the droplets from combining. Furthermore, by making the continuous phase more viscous, these emulsifiers improve stability by impeding droplet mobility. Proteins such as soy, whey, and casein adsorb at the oil-water interface to produce a viscoelastic coating that increases the viscosity of the aqueous phase and prevents droplets from merging, stabilizing the emulsion (Cabra, Arreguín, & Farres, 2008). Gum Arabic is one type of polysaccharide that is utilized in candy, drinks, and flavor encapsulation. By making the aqueous phase more viscous and enclosing oil droplets in a protective coating, they stabilize emulsions by preventing coalescence and improving overall stability (Sulieman, 2018).

1.2.3. Proteins as emulsifiers

The amphiphilic characteristic of proteins, which have both hydrophobic and hydrophilic areas, makes them useful emulsifiers. Since proteins have two different

affinities, they can adsorb at the oil-water interface in emulsions, lowering interfacial tension and creating a stable layer surrounding scattered oil droplets. Long-term emulsion stability is preserved by this stabilizing layer, which inhibits coalescence (Y. Liu, Wu, Zhang, Yan, & Mao, 2024; McClements, 2015). Proteins can interact through hydrophobic, electrostatic, and hydrogen bonding interactions in addition to their amphiphilic characteristics, which helps to further stabilize the emulsion system. By preventing droplet aggregation and coalescence, these interactions aid in the formation of a viscoelastic interfacial layer that is resistant to stress from the outside. Due to consumer desire for healthier and more sustainable food options, the use of proteins as emulsifiers is becoming more and more popular, especially in the production of natural and clean-label food items (Cabra, Arreguín, & Farres, 2008; McClements, 2015). Figure 1 represents how proteins help in stabilizing emulsions.

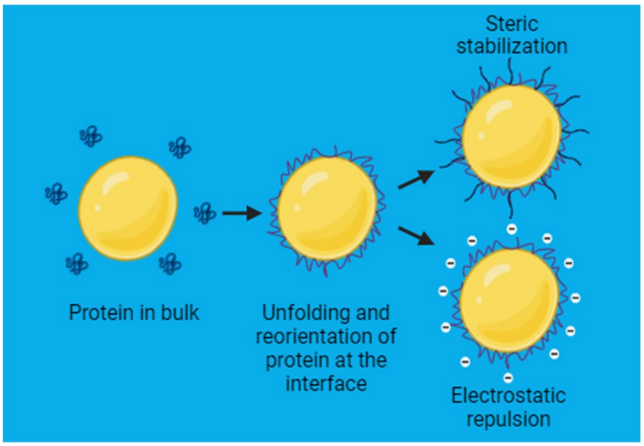


Figure 1: Illustration of protein unfolding and adsorbing at the interface of oil droplets during emulsification

1.3. Dairy proteins as emulsifiers

Dairy proteins, which are derived from milk have multifunctional properties and are a key component in various food products. This is especially true of their emulsifier characteristics. These proteins, which include whey and casein, have special amphiphilic properties that allow them to stabilize oil-in-water (O/W) emulsions by lowering interfacial tension and enfolding oil droplets in a barrier (Dickinson, 2001). Because of its textural and stabilizing qualities, casein, the main protein in milk, produces stable colloidal complexes that are widely used in a variety of culinary applications, including cheese, yoghurt, and drinks (Runthala, Mbye, Ayyash, Xu, & Kamal-Eldin, 2023). Whey proteins, which are obtained from the process of manufacturing cheese, are sought after for their exceptional solubility, gelation, and emulsifying qualities. These proteins include β-lactoglobulin, α-

lactalbumin, and bovine serum albumin (Królczyk, Dawidziuk, Janiszewska-Turak, & Sołowiej, 2016; Madureira, Pereira, Gomes, Pintado, & Xavier Malcata, 2007).

Whey protein isolate, or WPI, is a highly refined whey protein with a weight percentage of ≥90% protein. It is produced by extracting most of the fat and lactose from whey, which makes it a fantastic source of protein. Because of its useful qualities, including solubility, gelation, and emulsification, WPI is especially appreciated in the food business (Patel, 2015). The protein molecules move to the oil-water interface in an emulsion system with the addition of WPI, and they partially unfold to expose their hydrophobic groups to the oil phase while retaining their hydrophilic groups in the aqueous phase. The process of adsorption lowers the interfacial tension, which makes it easier for oil droplets to disperse in water. Subsequently, the proteins surround the droplets, offering steric and electrostatic stabilization. This important function keeps the droplets from coalescing and preserves the emulsion's stability (Zhong et al., 2021). Whey proteins' ability to emulsify is largely dependent on their chemical makeup. The compact, folded shape of globular proteins like whey proteins enables them to adsorb quickly at the oil-water interface and create a cohesive interfacial layer. This layer is necessary to maintain steric stability and stop oil droplets from coalescing. With a molecular weight of around 18.4 kDa, the main protein in WPI, β-lactoglobulin, is made easier to adsorb to the oil-water interface by hydrophobic amino acid residues. Whey proteins' disulfide bonds aid in the creation of a stable interfacial coating, which increases the emulsion's overall stability (Dickinson, 2001; Ghosh & Rousseau, 2011).

Numerous research works have demonstrated how well WPI stabilizes food emulsions. (Dickinson, 2010), for example, showed that robust interfacial protein network development results in good stability of WPI-stabilized emulsions against creaming and coalescence. Likewise, studies conducted by (Tcholakova, Denkov, & Lips, 2008) shown that WPI could produce extremely stable emulsions with tiny droplet sizes, which are advantageous for enhancing the mouthfeel and texture of food items. The application of WPI in the creation and maintenance of functional olive oil-in-water emulsions is addressed by (Caporaso, Genovese, Burke, Barry-Ryan, & Sacchi, 2016), who emphasize its ability to improve both physical and oxidative stability across a range of storage circumstances. A research demonstrated the ability of whey protein peptides to operate as emulsifiers in oil-in-water nano-emulsions as well as bioactive substances. The study highlighted the possibility of stable nano-emulsion formation using peptides produced from WPI under the right emulsification conditions, peptide size, and enzyme type. These nano-emulsions demonstrated improved stability during storage, retaining their droplet size and avoiding creaming (Adjonu, Doran, Torley, Sampson, & Agboola, 2022).

1.4. Alternative proteins sources

Due to increased worries about the environment and diet, alternative protein sources, such as those produced from plants, algae, and other non-dairy origins, are being investigated more and more for their potential in emulsification. Soy protein isolate is used significantly because of its superior emulsifying qualities. Research conducted by (L. Chen, Chen, Ren, & Zhao, 2011) revealed that enzymatic hydrolysis and extrusion modifications of SPI greatly increased the material's capacity to stabilize oil-in-water emulsions, improving the stability and texture of the emulsion. Pea protein is increasingly drawing attention due to its value and useful qualities. As an example, (Karaca, Low, & Nickerson, 2011) examined the emulsifying qualities of pea protein isolates and discovered that they could create stable emulsions with desired droplet sizes, which qualified them for use in a range of culinary applications. The potential of pea protein isolates as stabilizers for hempseed oil-based water/oil emulsions was examined (Jarzębski et al., 2019). They discovered that the pea protein significantly stabilized the emulsions, with a high entrapment efficiency of linoleic acid and good stability under a variety of settings. This demonstrates how pea protein may create stable nano-emulsions with advantageous qualities for use in culinary applications. The food industry is diversifying its supply of protein through investigating towards the emulsifying potential of proteins derived from chickpeas and lentils. According to (Karaca, Low, & Nickerson, 2011), proteins taken from lentils and chickpeas showed good emulsifying qualities, which qualified them for usage in a range of food emulsions. The research demonstrated that these proteins might serve as a good substitute for conventional dairy proteins by stabilizing oil-in-water emulsions. Since algae have a distinct protein composition and set of functional characteristics, proteins produced from algae, including microalgae like spirulina and chlorella, have been recognized as promising emulsifiers. Chlorella vulgaris proteins have the potential to be sustainable substitutes for conventional emulsifiers, as demonstrated by study by (Ursu et al., 2014), which demonstrated that the proteins could generate stable emulsions.

Although proteins synthesized from plants and algae have great promise, there are several restrictions associated with these substitutes. Flavor profiles, solubility, and allergies are common problems for plant proteins, such as those derived from soy, peas, and chickpeas. These problems can limit the adoption of these proteins overall and their ability to function in food formulations (Lam, Can Karaca, Tyler, & Nickerson, 2018). Proteins generated from algae, although rich in nutrients and are sustainable, sometimes need to go through an extensive processing procedure before they can be used in food products. This labor-intensive and expensive processing influences the viability of employing algae as a source of protein in conventional food production (Bleakley & Hayes, 2017). Moreover, point out that depending on the processing and storage circumstances, these proteins' functional characteristics—like their capacity to create stable emulsions, might not necessarily

coincide with those of conventional dairy proteins (McClements, Decker, & Weiss, 2007).

Considering these drawbacks, research into insect proteins as a substitute and sustainable supply of emulsifiers is gaining traction. According to insects have high nutritional content and outstanding functional features including emulsification and gelation (Yi et al., 2013). Examples of these insects are lesser mealworms and crickets. In comparison to conventional cattle, they also require less space, water, and feed, making them more environmentally sustainable. Research is underway to optimize the extraction and functionalization of insect proteins for use as efficient emulsifiers in food systems since the potential of these proteins in food applications is becoming more widely acknowledged (Van Huis, 2013).

1.4.1. Insect proteins in emulsions

Insects are becoming more well-known as a sustainable and nutrient-dense substitute for meat in many societies around the world where they have long been an essential part of traditional meals. They require a less area, water, and feed than typical cattle because they are extremely efficient at turning feed into protein. Furthermore, less greenhouse gas is released during insect farming. In terms of nutrition, insect proteins are just as good as or even better than conventional animal proteins since they are high in vitamins, minerals, and vital amino acids (Van Huis, 2013).

For example, just 1.7 kg of feed is needed by crickets to create 1 kilogram of body mass, but around 8 kg of feed is needed by cattle to achieve the same body mass. Furthermore, insects use a lot less water than other food sources. For instance, 1 kilogram of beef requires around 15,000 liters of water to produce, whereas 1 kg of insect protein requires substantially less (Van Huis, 2013). In terms of nutrition, plant-based proteins frequently lack important amino acids like methionine and lysine, which are abundant in insect proteins. They are also rich in minerals and vitamins, including as iron, zinc, and B vitamins. With a desirable composition of unsaturated fatty acids, the fat content of insect proteins is also advantageous. For instance, mealworms contain a lipid content of 30–40%, including healthy fatty acids, and a protein content of around 47–60% dry weight. They also have a well-balanced amino acid profile (Rumpold & Schlüter, 2013a). Comparing insect farming to conventional cattle raising, there is also evidence of reduced greenhouse gas emissions. The production of mealworms produces 10 to 100 times less greenhouse gases per kilogram of protein than that of pigs or cattle (Oonincx et al., 2010).

A number of insect species, such as mealworms, locusts, and crickets, have been investigated for possible use in food emulsions. Because of their high protein content and advantageous amino acid composition, crickets have been the subject of

substantial investigation. Research has demonstrated that protein from crickets can be successfully added to protein bars and smoothies, offering a wealth of vital elements. Additionally, cricket protein has outstanding emulsifying qualities, which make it appropriate for a range of culinary uses. For instance, research showed that the protein from crickets may stabilize emulsions made with avocado oil, resulting in stable emulsion with small droplets that enhance the stability and texture of food items (Trujillo-Cayado, García-Domínguez, Rodríguez-Luna, Hurtado-Fernández, & Santos, 2024). Numerous research has also focused on mealworms, namely the yellow mealworm (Tenebrio *molitor*) and the smaller mealworm (Alphitobius *diaperinus*). Mealworm protein isolates have been shown in studies to be capable of forming stable emulsions, which are essential for food items such as dressings, sauces, and meat substitutes. Mealworm proteins have been shown to have the techno-functionality to enhance food products' texture, stability, and nutritional value (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016). According to their research, mealworm protein may produce tiny droplet emulsions, which improve the texture and consistency of goods that have been emulsified (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016; Yi et al., 2013).

The lesser mealworm, or Alphitobius diaperinus, is becoming more well-known as a possible source of protein for emulsions and other culinary applications. Depending on the stage of the insect, the high protein content of lesser mealworms ranges from 40 to 60 percent of their dry mass (Turck et al., 2022). This makes their nutritional profile noteworthy. Lesser mealworm proteins are a great substitute for meat or dairy proteins because they are high in essential amino acids and have a favorable amino acid profile (C. Tang et al., 2019). Lesser mealworm powder, for instance, has been used to increase the protein and mineral content of baked foods like crispy snacks, offering both nutritional advantages and bettering the items' sensory qualities. The larvae and pupae may be used to create energy-dense food items that meet a variety of nutritional demands due to their high protein and fat content (Roncolini et al., 2020). Lesser mealworm protein has demonstrated outstanding promise in stabilizing food emulsions. Research has indicated that protein isolates from smaller mealworms can create stable emulsions, which are essential for goods like dressings, sauces, and meat substitutes. The solubility, foaming, emulsifying, gelation, water- and oil-holding capacities, and other techno-functional characteristics of these proteins have all been well-characterized (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016; Yi et al., 2013).

Furthermore, in complex emulsion systems such as water-in-oil-in-water (W1/O/W2) emulsions, lesser mealworm protein has been assessed. Encapsulating bioactive substances like polyphenols, which can improve the nutritional and functional qualities of food items, is one use for these systems. Less mealworm protein may stabilize these complex emulsions similarly to whey protein and pea protein, indicating its durability under varied processing and storage circumstances.

Additionally, the study discovered that lower concentrations of mealworm protein-stabilized emulsions exhibited good stability and encapsulation efficiency, suggesting that they might be a good choice for functional food applications (Wang, Ballon, et al., 2021).

Table 2 presents a comprehensive analysis of the energy consumption, nutritional value, and emulsifying capability of insect, dairy, and plant proteins.

Table 2: Comparison of the energy consumption, nutritional content, and emulsifying ability of plant, dairy, and insect proteins.

Measures	**Dairy proteins**	**Plant proteins**	**Insect proteins**
Energy consumption	High: Because dairy production requires a lot of water and feed, it uses a lot of resources, roughly 55 MJ/kg of protein produced.	Moderate: Depending on the crop and cultivation techniques, plant proteins need between 30 and 35 MJ/kg of energy.	Low: Compared to traditional cattle, insects require a great deal less of space, water, and feed. Energy consumption is around 10-15 MJ/kg of protein produced.
Nutritional value	High: Complete proteins that contain every necessary amino acid. Contains about 80–90% of the protein in whey and 70–80% of the protein in casein. Rich in calcium and D.	Low: Soy protein contains around 36% protein and all the necessary amino acids. Pea protein is lower, which ranges from 23 to 25%	High: Depending on the species, the protein content varies from 35-77%. Rich in vital amino acids, on par with the proteins found in meat and dairy and also are rich in micronutrients.
Emulsifying capacity	Excellent: Whey protein is very effective in stabilizing emulsions, with an emulsifying capacity ranging from 90 to 95%.	Good to Moderate: Soy proteins have an emulsifying capacity in the range of 80–85% and about 60–70% for pea protein.	Good: Effective emulsifiers due to their high protein content and functional properties and have an emulsifying

			capacity around 70-80%.

1.5. Production of emulsions

1.5.1. Conventional methods of emulsification

Emulsions are made through utilizing a variety of methods that control the size and dispersion of droplets through distinct processes. Mechanical equipment such as colloid mills, ultrasonic emulsifiers, membrane emulsifiers, rotor-stator systems, and high-pressure homogenizers are used in traditional emulsification processes.

High-pressure homogenizers are often employed in the food, pharmaceutical, and cosmetic sectors as they can create emulsions with narrow size distributions. Through strong turbulence and shear forces caused by pushing the emulsion mixture through a tiny aperture at high pressure, droplets are broken down to micron sizes using this approach (Zamora & Guamis, 2015). In the dairy sector, high-pressure homogenization has shown to be a successful method for producing milk emulsions, which improve the texture and physical stability of milk-based products. High-pressure homogenization uses between 2 and 10 kWh/m³, depending on the equipment and operating conditions. This approach, nevertheless, can be costly and energy intensive (Schultz, Wagner, Urban, & Ulrich, 2004). Heat-sensitive materials may be deteriorated by strong mechanical stresses and possible heat production. For sensitive materials to remain functional and stable, processing conditions must be carefully optimized (Gall, Runde, & Schuchmann, 2016; Patrignani & Lanciotti, 2016).

Rotor-stator systems are frequently utilized in the food sector for emulsification as they work well and consume less energy than high-pressure homogenizers. These devices work by forcing the emulsion phases through an opening between a rotor that rotates quickly and a fixed stator. This produces a high shear environment that fractures the dispersion phase into smaller droplets (Håkansson, 2018). Research by (Håkansson, Askaner, & Innings, 2016) showed how to employ rotor-stator mixers to make stable emulsions for mayonnaise manufacture, emphasizing how important they are for getting the right texture and stability in these kinds of products. Since rotor-stator systems typically use between 0.5 and 2 kWh/m³ of energy, they are a more affordable choice for many food processing applications than high-pressure homogenizers (Håkansson, 2018; Håkansson, Askaner, & Innings, 2016). These technologies are also used to prepare food-grade nano-emulsions, which are essential for adding bioactive ingredients to food items. When it comes to improving the bioavailability of minerals and active substances in food, rotor-stator systems work effectively at producing emulsions with droplet sizes as tiny as 100–

500 nm (L. Chen, Ao, Ge, & Shen, 2020). High viscosity or high solids content emulsions may not be as well processed by rotor-stator systems because of the strong shear pressures, which can cause wear on the machinery and increase energy consumption. The possibility of higher maintenance needs because of the mechanical complexity of the systems is another factor to take into account, particularly in continuous processing activities where wear and tear might be more noticeable (L. Chen, Ao, Ge, & Shen, 2020; Håkansson, 2018).

Membrane emulsification involves forcing the continuous phase into the dispersed phase by forcing them through a membrane having uniform pores. Highly homogeneous droplets may be produced because of the control over droplet size provided by the shear forces at the membrane surface. This approach is more energy-efficient than high-pressure homogenization, usually using between 0.1 and 0.5 kWh/m^3 and is especially useful for sensitive chemicals that may disintegrate under high shear or temperature conditions. Applications that need for very monodisperse emulsions, such the creation of encapsulated flavors and nutraceutical emulsions, are best suited for membrane emulsification (Ali, Syed, Bak, & Quist-Jensen, 2022). This method has proven successful in creating monodisperse emulsions for the food processing sector, providing energy-saving options for premium product formulations (Charcosset, 2009).

Membrane emulsification has a few disadvantages despite its benefits. Membrane fouling is a serious problem that reduces the membrane's effectiveness over time by clogging its pores with particles or droplets. This calls for frequent upkeep and cleaning, which can be expensive and time-consuming (Charcosset, 2009). Furthermore, the throughput of membrane emulsification is frequently lower than that of high-pressure homogenization, which makes it less appropriate for large-scale production unless multiple membranes are used concurrently, which can raise costs and complexity (Maan, Schroën, & Boom, 2011). The membrane's durability is also an issue, particularly when handling abrasive or aggressive compounds. Operational expenses may increase if the membranes need to be changed on a regular basis. It can be difficult to scale up membrane emulsification from the laboratory to the industrial level because careful optimization is needed to ensure uniform droplet size distribution and preserve process efficiency at greater scales (Charcosset, 2009; Maan, Schroën, & Boom, 2011).

Depending on the needs of the emulsion, each of these traditional emulsification techniques has certain benefits and drawbacks. Because it may create fine emulsions, high-pressure homogenization is preferred; however, this comes at the expense of significant energy consumption and possible heat-sensitive component damage. Although less effective in terms of size distribution, rotor-stator systems are nevertheless a good option in some situations. Although there are energy and scalability constraints, there are possibilities for producing very uniform droplets and nano-emulsions using ultrasonic and membrane emulsification processes,

respectively. Microfluidics is becoming a more attractive alternative for emulsification with minimal energy needs and fine control over droplet size and distribution.

1.6. Microfluidic emulsification

Emulsification using microfluidics is an advanced approach that has revolutionized the production of emulsion by controlling the flow of fluids within microchannels. With this technique, droplets with a particular composition, uniform size, and high monodispersity are produced using a variety of microfluidic devices and microchannels.

It was in the early 2000s when microfluidic emulsification began to take shape. A groundbreaking work by (Anna, Bontoux, & Stone, 2003) showed how "flow focusing" in microchannels may generate dispersions. This paper demonstrated how droplet size and homogeneity might be affected by microchannel shape, which is important for medication administration and diagnostic applications. Their research provided a basic knowledge of droplet dynamics in microfluidic systems by demonstrating that they could create droplets of different sizes with great uniformity by altering the flow rates and channel diameters. After that, (Cramer, Fischer, & Windhab, 2004) looked at how droplet formation was impacted by various flow rates and channel designs. Their research made clear how important flow dynamics are to getting the right emulsion properties. They determined the ideal parameters to produce monodisperse emulsions by methodically altering the flow rates and monitoring the resulting droplet sizes and dispersion. The significance of having accurate control over flow parameters in microfluidic emulsification processes was highlighted by this study.

1.6.1. Types of microfluidic systems

In microfluidic systems, various methods are utilized to create emulsions with high precision and uniformity such as the crossflow systems, T-junction microfluidics, flow-focusing devices, co-flow microfluidic devices and step emulsification.

In T-junction microfluidic devices, droplets are produced at the intersection of two channels meeting perpendicularly to each other. The simplicity and uniformity of the droplets produced by these devices make them commonly employed. T-junction devices were employed to create emulsions with narrow size distributions. The effects of fluid flow rates and channel diameters on droplet size were examined by the researchers, who discovered that careful control over these variables may reliably result in monodisperse droplets (Okushima, Nisisako, Torii, & Higuchi, 2004).. T-junction devices can produce emulsions with acceptable qualities for food applications, according to a study that looked at the stability and texture of the emulsions produced (Maan, Nazir, Khan, Boom, & Schroën, 2015).

Flow-focusing systems use a central channel to deliver one fluid to a junction where it is sheared by a surrounding fluid. This arrangement is flexible enough to create single or many emulsions, and it gives exact control over droplet size. Flow-focusing tools were utilized in a work by (Anna, Bontoux, & Stone, 2003) to produce complex emulsions, such as double and triple emulsions. The researchers showed that the size and stability of the droplets could be controlled by varying the inner and outer fluids' flow rates. (Utada, Fernandez-Nieves, Stone, & Weitz, 2007) looked at the usage of flow-focusing devices in drug delivery applications. The study demonstrated how exact control over droplet size might improve the stability and encapsulation effectiveness of drug-loaded emulsions.

Step emulsification is a microfluidic process in which fluids are forced into a small channel and then suddenly expanded, forming homogeneous droplets. This method is well-suited for a variety of industrial applications due to its stability in the face of fluctuations in flow rates and its repeatability in producing monodisperse droplets. The step emulsification technique was examined in detail by (Shi et al., 2020) in order to determine how well it produces monodisperse droplets with a high throughput, which is essential for applications in material creation and biomedical measurements. The study examined the mechanics behind droplet generation, emphasizing the significance of interfacial tension and microchannel shape. Furthermore, step emulsification's scalability for commercial emulsion manufacturing was investigated by (Shi et al., 2020), who showed that method might satisfy industry expectations for homogenous, high-volume emulsions.

1.6.2. Drug delivery and diagnostic applications

The use of microfluidic devices to produce highly controlled emulsions has been thoroughly investigated in research, with a focus on the applications in material synthesis and drugs delivery. For the production of functional materials and delivery systems, microfluidic devices' flexibility in generating monodisperse emulsions with exact control over droplet size and internal structure is essential. According to one study, monodisperse droplets with controlled sizes may be produced using microfluidic technology, which is crucial for the reliable administration of medications and other therapeutic agents. Because of this degree of control, active medicinal components can be encapsulated inside droplets for targeted distribution and a decrease in adverse effects (Shah et al., 2008). Microfluidic research has also focused on high-throughput droplet formation for biochemical applications. Through the use of microfluidic devices, researchers may produce highly regulated conditions for chemical and biological interactions by producing homogenous droplets for experiments. This capacity is important for diagnostic equipment because it allows for exact control over reaction conditions, which improves the efficiency and accuracy of biomarker and other analyte detection. One research, for instance, demonstrated how microfluidics may be utilized to create droplets for high-

throughput biochemical tests, which have applications ranging from cell-based assays to enzyme kinetics (Chabert & Viovy, 2008).

The development of high-throughput screening systems employing microfluidic emulsification for drug discovery and diagnostics is the result of additional breakthroughs in microfluidic technology. These platforms show how highly controlled environments may be created using microfluidics to test a large number of chemicals. This method improves the accuracy of finding possible medication candidates while also increasing the screening process's efficiency. In order to investigate the effects of various chemicals on biological targets, researchers have demonstrated that microfluidic devices can quickly create and manage thousands of droplets, each carrying a distinct molecule (Guo, Rotem, Heyman, & Weitz, 2012). There has also been a lot of research done on the application of microfluidic emulsification to create complex emulsions with many encapsulated phases for medication administration and diagnostics. Through the optimization of microfluidic device design and processing conditions, scientists have generated emulsions that exhibit great stability and encapsulation efficiency. These sophisticated emulsions are necessary for sophisticated drug delivery systems that need to deliver several medications at once or release active components under strict supervision. One research demonstrated how microfluidics may be utilized to construct complex drug delivery platforms by producing double emulsions with exact control over the interior and exterior phases (C. S. Ho, Kim, & Weitz, 2008).

1.6.3. Food science applications using microfluidics

Reviews have emphasized the generation of food-grade emulsions with controlled droplet sizes and compositions, discussed obstacles and future prospects for large-scale applications, and emphasized the promise of microfluidic emulsification in food processing (Maan, Nazir, Khan, Boom, & Schroën, 2015). Recent research has investigated the use of microfluidic technology to create a variety of food-grade emulsion-based delivery methods, such as solid lipid microparticles, large liposomes, microgels, and microcapsules. These developments make it possible to encapsulate and shield bioactive substances, guaranteeing their stable distribution within food items (Bianchi, de la Torre, & Costa, 2023). Additionally, real-time monitoring and control of dynamic processes in food emulsions and foams are made possible by microfluidic methods, which are essential for improving food formulations and elevating the stability and quality of final products. By using these methods, food emulsions and foams' dynamic behaviors are better understood, resulting in food items that are more stable and of higher quality (Schroën et al., 2023).

In one research, the stability of emulsions stabilized by pea proteins was investigated using microfluidic devices, and the effects of protein content and environment variables on stability were examined. With this method, droplet

interactions may be precisely controlled and observed, providing valuable information on the coalescence behavior of protein-stabilized emulsions (Hinderink, Kaade, Sagis, Schroën, & Berton-Carabin, 2020). The function of whey protein isolate (WPI) in maintaining oil-in-water emulsion stability was investigated in a different study. The work showed that WPI-stabilized emulsions could be prepared and examined using microfluidic devices, allowing researchers to investigate the role that the protein's interfacial characteristics play in stability. This study yielded important information on WPI's emulsifying properties and possible uses in food items (Jiao et al., 2022).

1.6.4. Advantages of microfluidic emulsification

When it comes to creating emulsions with exceptional accuracy and homogeneity, microfluidic emulsification is the favored approach due to its numerous benefits over traditional techniques.

Energy efficiency: Compared to high-shear methods like high-pressure homogenization, microfluidic emulsification uses a vastly reduced amount of energy. This is because fine fluid control, as opposed to strong mechanical forces, which drives the droplet generation process in microfluidic devices. Microfluidic systems minimize the need for excessive energy input, which lowers operating costs and lessens the possibility that heat generation that could degrade sensitive substances. Because maintaining the purity of active chemicals is vital in the food, pharmaceutical, and cosmetic sectors, this energy efficiency is especially advantageous for these applications (Maan, Nazir, Khan, Boom, & Schroën, 2015).

Monodispersity: The potential of microfluidic emulsification to create emulsions that are remarkably monodisperse and have limited size distributions is one of its most significant advantages. For applications like medication delivery systems, where uniform droplet size might impact drug release rates, and cosmetic formulations, where consistency is crucial for product performance and customer pleasure, this high degree of uniformity is necessary. This is accomplished via microfluidic methods, which create droplets by carefully designing microchannels and controlling flow rates (Bezelya, Küçüktürkmen, & Bozkır, 2023).

Scalability and reproducibility: By parallelizing several microchannels, microfluidic devices provide simple scaling up. By ensuring that several droplets may be created concurrently, this parallelization guarantees that batch-to-batch consistency in product quality. For industrial applications, the consistency of droplet size and composition across scales is an important benefit that guarantees the end product complies strict quality control requirements. The accuracy and control that are intrinsic to microfluidic devices are preserved in order to accomplish this scaling (Amstad et al., 2016).

Control over droplet properties: Unmatched control over different droplet parameters, such as size, composition, and encapsulation efficiency, is possible with microfluidic emulsification. For instance, in the pharmaceutical industry, managing the effectiveness of active component encapsulation guarantees appropriate dose and targeted administration, both of which are critical to the effectiveness and security of medication formulations. Microfluidic approaches can improve the stability, bioavailability, and release patterns of active medicinal components by adjusting the size and content of droplets (De Jong, Lammertink, & Wessling, 2006). In the food industries, customizing droplet composition by microfluidic emulsification can greatly enhance the stability and texture of emulsions. It is possible to create goods with improved mouthfeel, shelf life, and nutritional value by adjusting the droplet qualities. Sensitive nutrients and bioactive substances, for example, can be encapsulated to prevent degradation and provide a regulated release during digestion (Maan, Nazir, Khan, Boom, & Schroën, 2015). Microfluidic emulsification is a perfect option for creating novel food items that satisfy certain dietary needs and customer preferences because of its accuracy and versatility (Bezelya, Küçüktürkmen, & Bozkır, 2023).

Table 2 compares various emulsification techniques in terms of dispersity of droplets generated and the energy consumption while using each of these systems.

Table 2: Comparison of emulsification techniques: polydispersity, energy consumption

Emulsification technique	Polydispersity value	Energy consumption and characteristics
High-pressure homogenization (HPH)	Polydisperse: ~40%:	With a high energy input of up to 10 kWh/m³, HPH creates emulsions at the micron or sub-micron scale, resulting in tiny droplet sizes and narrow size distributions. Although it can destroy substances that are heat-sensitive, it is scalable for industrial purposes.
Rotor-stator methods	Broad droplet size distribution, typically >30%	A large range of droplet sizes are produced by the mechanical shear forces used in rotor-stator techniques. Typically, energy usage is between 0.5 and 2 kWh/m³. These devices work well for creating emulsions with

		droplet sizes of around one micrometer.
Ultrasonic emulsification	Monodisperse: <25%	Cavitation produced by ultrasonic waves results in uniformly sized droplets that are useful for precise size control. The required energy input is between 2-5 kWh/m³. It might be difficult to scale up for large-scale production.
Membrane emulsification	Highly monodisperse, typically <10%	By controlling droplet production using a membrane, membrane emulsification creates extremely homogeneous droplets. Compared to other technologies, it is more energy-efficient (usually less than 1 kWh/m³), which makes it appropriate for delicate chemicals.
Colloidal mill	Broad droplet size distribution, typically >30%	Shear pressures are used in colloidal mills to emulsify materials, which produces wider size distributions. The energy usage per square meter ranges from 1 to 5 kWh. Although these mills may create droplets as fine as 0.5 µm, their size dispersion are often wider.
Microfluidic emulsification	Highly monodisperse, typically <10%	Highly consistent emulsions are produced using microfluidic devices, which accurately regulate droplet size through the use of microchannels. Although few, figures on energy usage specifically

		related to microfluidic emulsification are typically low.

1.7. Objectives

1. Evaluate the Potential of Lesser Mealworm Protein Concentrate (LMPC) as an Emulsifier

- Compare the ability of LMPC and whey protein isolate (WPI) to stabilize oil-in-water (O/W) emulsions using microfluidic techniques.

- Utilize microfluidic devices to control and observe droplet formation, stability, and coalescence in real-time.

- Analyze the stabilization of emulsions using hexadecane and sunflower oil with varying concentrations of LMPC and WPI.

- Study the effects of protein concentration, oil fraction, and adsorption time on the stability and coalescence behavior of emulsions.

2. Evaluate the Effectiveness of Modified LMPC in Stabilizing Emulsions

- Evaluate the stabilization capacity of LMPC modified with chlorogenic acid (CA), tannic acid (TA), and thermal treatment (Th) and compare the stabilization effectiveness of modified LMPC to native LMPC using microfluidic techniques.

- Utilize microfluidic devices to control and observe droplet formation, stability, and coalescence in real-time.

- Study the effects of protein concentration, oil fraction, and adsorption time on the stability and coalescence behavior of emulsions.

3. Determine Functional Properties of LMPC and Modified LMPC and Use Statistical Methods to Evaluate the Interactions and Significance of Variables on Droplet Coalescence and Dimer Formation

- Determine the emulsifying capacity, surface hydrophobicity, zeta potential, and interfacial tension of LMPC and modified LMPC.

- Apply exploratory data analysis (EDA), two-way ANOVA, and regression analysis to evaluate the statistical significance of variables.

- Use statistical methods such as two-way ANOVA and regression analysis to evaluate the interactions and significance of variables like protein type, concentration, and oil fraction on droplet coalescence and dimer formation.

Chapter 2

Droplet coalescence investigations utilizing microfluidics under controlled conditions: Lesser mealworm (A. diaperinus) protein as a substitute for dairy proteins in the production of O/W emulsions.

Abstract:

Dairy proteins are commonly used to stabilize oil-in-water (O/W) emulsions, which can be replaced by other sustainable sources of proteins, such as insects. This study investigated the potential of lesser mealworm protein concentrate (LMPC) as a sustainable alternative to whey protein isolate (WPI) in stabilizing oil-in-water (O/W) emulsions using microfluidics. The frequency of coalescence (Fcoal) was calculated using images of emulsion droplets obtained near the inlet and outlet of the coalescence channel. The stability of O/W emulsions, produced using sunflower oil (SFO) or hexadecane and stabilized with varying concentrations of LMPC and WPI (0.02% to 0.0005% w/v), was compared under controlled conditions. The dispersed phase fraction (5.3%-14.3% v/v), protein adsorption time onto oil droplets (0.0398–0.158 s), and pH (pH = 3 and pH = 7) were also studied. Fcoal was greatest (0.42 s• 1) when the protein concentration was lowest (0.0005%), the oil percentage was highest (14.3%), the adsorption period was shortest (0.0398 s), and the pH was 3. Droplet diameters did not vary significantly, with values between 55 and 118 μm, across protein concentrations or adsorption periods, but a rise in oil fraction resulted in a substantial increase in droplet diameters. Increases in protein content, adsorption duration, and oil percentage all resulted in increased stability (reduction of Fcoal). While LMPC and WPI showed similar results in microfluidic experiments and other test conditions, further research is needed to verify LMPC's efficacy as a replacement for WPI in food emulsification. Nonetheless, the findings suggest that LMPC has potential as a substitute for WPI in this application.

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2.1. Introduction

Finding effective and sustainable protein sources outside of traditional animal sources is of great interest due to the expanding demand for protein to feed the world's rapidly growing population. Dairy proteins find extensive application in the food sector due to their exceptional emulsion stabilization capabilities. But concerns about their sustainability have prompted researchers to look at other protein sources, such as those made from edible insects (Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). When assessing whether novel protein sources are suitable for use in food applications, techno-functional characteristics including solubility, emulsification, foaming, and gelation are crucial (Jakobson et al., 2023). Evaluating these characteristics is crucial to make sure that substitute proteins may successfully replace original ones without lowering the quality of the final product.

Growing evidence points to edible insects as a viable source of protein with a number of advantages over conventional cattle, such as a high protein content, effective feed conversion, and less environmental impact (Rumpold & Schlüter, 2013b). Numerous investigations have demonstrated the ability of insect proteins to function as emulsifiers in food items. Proteins taken from honeybees (Apis *mellifera*) and grasshopper (Schistocerca *gregaria*) showed emulsifying abilities similar to those of whey protein (Mishyna, Martinez, Chen, & Benjamin, 2019) .Black soldier fly (Hermetia *illucens*) protein concentrates have the potential to be a sustainable source of protein for the food sector since they have been demonstrated to create stable O/W emulsions using low-energy, high-throughput emulsification technology (Wang, Jousse, et al., 2021). A comparison of mealworm larvae, adult crickets, and silkworm pupae protein hydrolysates was done by (Yoon, Wong, Chae, & Auh, 2019). Using commercial enzymes, the proteins were isolated and hydrolyzed, and their functional characteristics were examined. Enzymatic hydrolysis increased protein solubility but decreased foamability, according to the study. The hydrolysates demonstrated noteworthy anti-inflammatory properties and considerable reduction of α-glucosidase and angiotensin-converting enzyme activity. These results imply that because of their functional and bioactive characteristics, insect protein hydrolysates may find use in food and health-related products. Research shows that the protein from crickets (Acheta *domesticus*) has high emulsifying qualities that may be used in a variety of culinary applications (Stone, Tanaka, & Nickerson, 2019).

The protein concentration (LMPC) of the lesser mealworm (Alphitobius diaperinus) has demonstrated exceptional promise as an emulsifier in a range of culinary applications. Lesser mealworm protein is a high-quality source of protein since studies on its nutritional and functional qualities have revealed that it is rich in important amino acids and that, depending on the processing technique, its protein

concentration can range from 48 to 71% (Leni, Soetemans, Caligiani, Sforza, & Bastiaens, 2020).

Similar to whey protein isolate and pea protein isolate, it has been observed that its emulsifying characteristics are excellent in stabilizing water-in-oil-in-water (W1/O/W2) emulsions, exhibiting good encapsulation efficiency and droplet size distribution under a variety of circumstances (Wang, Ballon, et al., 2021). Together, these findings highlight the great potential of LMPC as a viable and efficient substitute for traditional emulsifiers in food production, providing advantages for both functionality and the environment. Because of its high protein content and useful qualities, the food industry is investigating its application in sports nutrition, snacks, and dietary supplements. The market for insect protein is expected to increase dramatically.

A comprehensive review of the literature reveals varying protein content across different edible insects, highlighting their potential as alternative protein sources. The table below (table 2.1) summarizes the protein content of various edible insects based on existing studies.

Table 2.1: Protein content among different insects

Insect	Protein content (%)
Beetle (Ulomoides *dermestoides*)	49-54%
Termite (Macrotermes *bellicosus*)	38-76%
Housefly (Musca *domestica*)	45-65%
Mealworm (Tenebrio *molitor*)	47-49%
Grasshopper (Sphenarium *purpurascens*)	40-60%
Black Soldier Fly (Hermetia *illucens*)	37-63%
Locust (Schistocerca *gregaria*)	31-65%
Honeybee (Apis *mellifera*)	42-50%
Lesser Mealworm (Alphitobius *diaperinus*)	40-70%
Cricket (Acheta *domesticus*)	60-70%
Silkworm (Bombyx *mori*)	50-65%
Moth (Galleria *mellonella*)	60-64%

Emulsification involves a number of crucial processes, including the splitting of droplets, the formation of new interfaces around the dispersed droplets, and the stabilization of these interfaces with the aid of emulsifiers to stop demulsification. Droplet generation and coalescence happen at simultaneously at the same time when an emulsion is produced. Coalescence is influenced by droplet collision frequency and collision efficiency. Some of the other factors that influence coalescence are the

number of collisions, the resistance of the thin film material separating the droplets, droplet volume fraction, sizes, droplet spatial distribution, and flow conditions (Jafari, Assadpoor, He, & Bhandari, 2008; Yonguep, Kapiamba, Kabamba, & Chowdhury, 2022). Droplets that completely adsorb the emulsifier on their interface can stay stable for a few seconds to several months depending on kind of emulsifier, adsorption kinetics, temperature, pH, droplet size, and the fraction of the dispersed phase (Tcholakova, Denkov, & Banner, 2004). The stability of an emulsion can be affected by changes in interfacial characteristics caused by changes in temperature and pH. For example, temperature increases cause droplets to collide more often, and variations in pH can impact emulsifiers' ionization state, which in turn affects how they adsorb and stabilize (Östbring, Matos, Marefati, Ahlström, & Gutiérrez, 2021). The fast emulsifier adsorption at the interface and the very immediate (typically milliseconds) droplet coalescence complicates the understanding of coalescence dynamics (T. M. Ho, Razzaghi, Ramachandran, & Mikkonen, 2022). Comprehending these dynamics plays a pivotal role in refining emulsion compositions and processing parameters to attain the intended stability and functionality in food items. The better understanding of droplet formation and stabilization dynamics is possible by employing advanced analytical techniques including high-speed imaging and microfluidic devices, which offer insights into these rapid processes. Improved control over emulsion qualities is made possible by developments in real-time analytical methods and microfluidic technologies, which are offering deeper insights into the instantaneous processes of coalescence and emulsification (Schroen et al., 2021).

Because microfluidics allows for fine control over experimental settings and real-time monitoring of these processes, it has completely changed the field of research for study of emulsion stability and droplet coalescence. The visualization of droplet interactions is made possible by high-resolution images, which provide insight on how droplets approach and coalesce (Krebs, Schroën, & Boom, 2012). This aids in comprehending the elements, such as interfacial tension, droplet size, and flow conditions, that either encourage or prevent coalescence. With real-time imaging, researchers can keep an eye on the coalescence process while it's happening and record transient events that would be overlooked in static studies. This is essential for researching the stability of emulsions over time and the dynamics of droplet merging. Review of different microfluidic methods for emulsion destabilization assessment and induction was presented, emphasizing how microfluidic channels provide a controlled setting to investigate droplet behavior right after formation, which is crucial to comprehending the early phases of coalescence and emulsification. Researchers were able to create controlled coalescence events and learn more about the basic processes of droplet interactions by varying the wettability and channel

shape (Porto Santos, Cejas, & Cunha, 2022). It was highlighted how precise fluid manipulation in microfluidic devices controls droplet size, shape, and dispersity, which is essential for creating stable emulsions that encapsulate and protect functional compounds. Krebs, Schroën, & Boom, 2012 examined the effects of surfactant concentration and ionic strength on emulsion stability by using a microfluidic device to create monodisperse oil-in-water emulsions stabilized with sodium n-dodecyl sulphate (SDS). They discovered that stability could be maintained even at low surfactant concentrations. In order to create food-grade delivery systems, Bianchi, de la Torre, & Costa, 2023 used droplet-based microfluidics. Their study concentrated on the exact manipulation of fluids to regulate droplet size, shape, and homogeneity. This method improved the stability and bioavailability of bioactive substances by encapsulating them within stable emulsions. The researchers showed enhanced encapsulation efficiency and preservation of delicate components by optimizing the emulsification process, opening the door for novel applications in the food sector.

A further research used microfluidics to investigate the coalescence susceptibility of emulsions stabilized by pea proteins and found that oxidized proteins contribute to a reduction in emulsion stability. According to the study, pea proteins need greater concentrations or longer adsorption durations than dairy proteins in order to generate stable emulsions (Hinderink, Kaade, Sagis, Schroën, & Berton-Carabin, 2020). This work provides deep insights into the stability and coalescence dynamics of emulsions stabilized by whey protein, pea protein, and their oxidized forms by examining the susceptibility of these emulsions to coalescence using microfluidic methods. High internal phase emulsions (HIPEs) have been studied for their coalescence kinetics utilizing microfluidics to quantify coalescence rates and find that emulsion stability is enhanced by increased viscosity in the continuous phase. According to (Williams, Wensveen, Corstens, & Schroën, 2024), this study offered thorough insights into the variables influencing droplet coalescence and emulsion stability.

In conclusion, microfluidics is a potent tool for researching and improving droplet coalescence and emulsion stability because it provides unmatched control and observational capabilities. The food and pharmaceutical sectors benefit greatly from these developments in the creation of stable and useful emulsion-based products.

2.2. Materials and methods

2.2.1. Protein extraction and sample preparation

Protein was extracted from a 48% protein mealworm powder that was provided by Kreca (Kreca Ento-Food BV, Wageningen, the Netherlands). The powder was combined with 1:5 (w/w) 2-methyltetrahydrofuran (Scharlab S.L., Spain) and agitated for 1 hour at 600 rpm in a fume hood. The solution was stirred, then allowed to settle before the solvent was decanted. To guarantee that all of the fat was removed from the insect powder, this defatting procedure was repeated. Under the hood, the residual solvent containing insect powder was allowed to evaporate.

Subsequently, 0.25 M NaOH (Chem-Lab NV, Zedelgem, Belgium) was combined with the defatted powder and the mixture was agitated for an hour at 40°C. After centrifuging the mixture for ten minutes at 4500 rpm, the supernatant was carefully withdrawn. After centrifuging the supernatant for 15 minutes at 3800 rpm, 35% HCl (J.T. Baker, Griesheim, Germany) was added to acidify it until the pH reached 4.2. To optimize protein production, this NaOH extraction procedure was done twice, followed by HCl pH correction. With a protein concentration of 71% (wb), the final precipitate was freeze-dried to produce less mealworm protein concentrate (LMPC). The extraction of protein protocol was referred was referred from (Wang, Ballon, et al., 2021)

Phosphate buffer containing sodium phosphate monobasic monohydrate (ACROS, Spain) and di-sodium hydrogen phosphate dihydrate (Scharlau, Spain) was used to prepare protein solutions, with the pH levels adjusted to 3 and 7. The protein solutions were made by directly weighing the necessary amount of protein powder to achieve the desired concentration using whey protein isolate (WPI), which was purchased from Davisco Foods International, Inc. (97.6%, Lot. JE151-4-420, Eden Prairie, MN, USA) which reported to have a protein content of 98.1% on a dry basis. These solutions were made at w/v concentrations of 0.02%, 0.01%, 0.005%, 0.001%, and 0.0005%. LMPC was measured with a BCA (bicinchoninic acid) assay kit (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA) after a stock protein solution was produced. The necessary concentrations were then achieved by diluting this stock solution. After that, emulsions made with hexadecane (Merck, Germany) and sunflower oil (SFO) purchased from a nearby supermarket (Borges S.A., Tarragona, Spain) were stabilized using these protein solutions.

2.2.2. Microfluidic setup

The microfluidic studies were conducted using custom designed borosilicate glass microchips (Micronit Microfluidics BV, The Netherlands). The microchip consists of an adsorption, a coalescence channels, each with a rectangular cross-

section and a T-junction (Fig. 2.1). The dispersed phase was broken up into tiny droplets at the T-junction, and these droplets passed along the adsorption channel without interacting with other droplets. The protein from the continuous phase adsorbed onto the oil-water interface during the flow through the adsorption channel. The droplets were further released into coalescence channel where they come in contact with other droplets and at times perhaps merge to coalesce with each other.

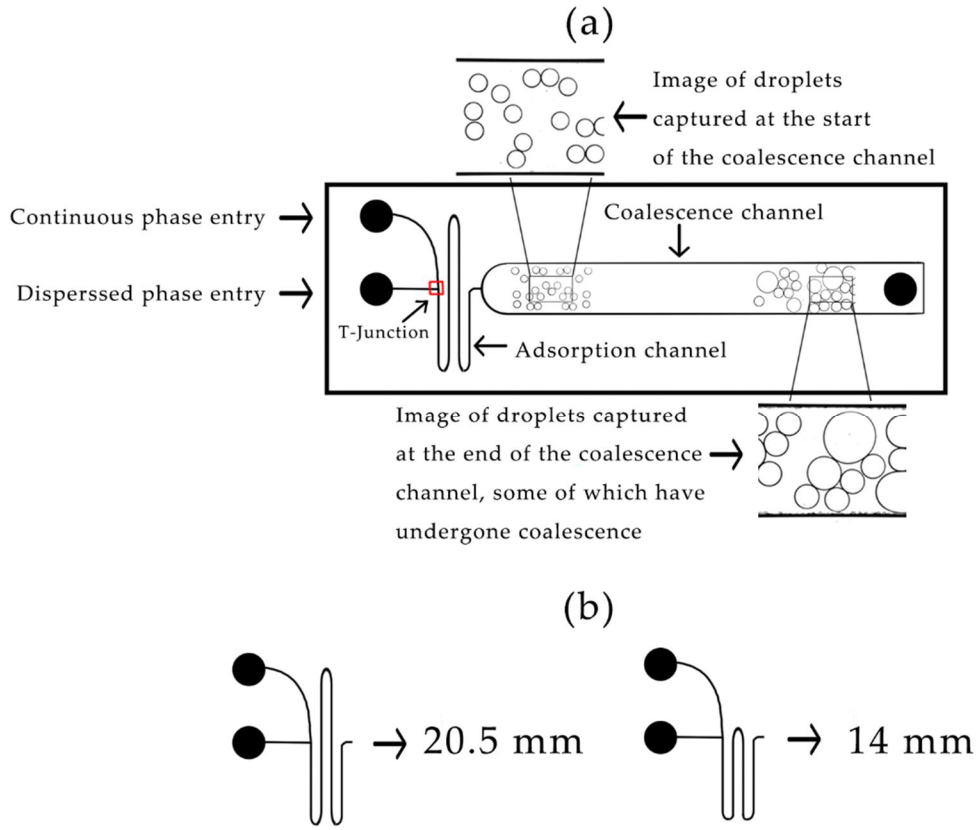


Figure 2.1: (a) Microchip design representing different regions of the microchip, (b) lengths of adsorption channel used in the experiments.

The effect of protein adsorption time on droplet coalescence was evaluated by varying the length of the adsorption channel between 14 and 20.5 mm. The channels on the microchip maintained a constant 45 µm height. The coalescence channel measured 32.1 mm in length and 500 µm in width, whereas the adsorption channel was 100 µm in width. The microchips were mounted on a microscope stage (Darwin Microfluidics, Paris, France) and connected to an Elveflow OB1 MK3+ pressure controller (ElveFlow, Paris, France), which controlled the liquid flow rates, to create microfluidic emulsions. Compressed air was used to pump the continuous

and dispersed phases via a reservoir, and a flow sensor supplied feedback signals to keep the flow rate steady. In Fig. 2.2, the experimental setup is shown.

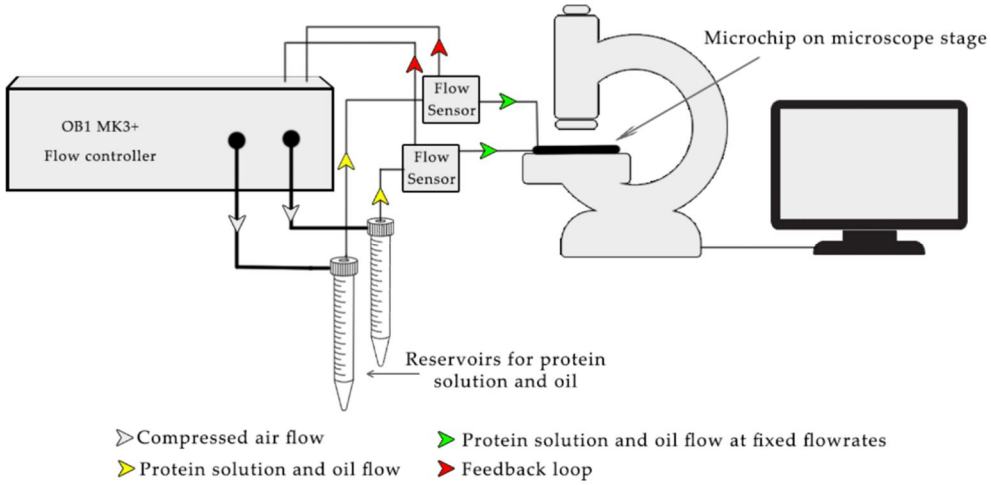


Figure 2.2: Outline of microfluidic experimental setup

2.2.3. Emulsion production and image acquisition

The oil and protein solutions were pumped at different total flowrates to generate emulsions with SFO (45 µl/minute to 105 µl/min) and hexadecane (35 µl/minute to 95 µl/min). The flowrate of the dispersed phase was kept constant at 5 µl/min, and the oil fraction was adjusted by varying the flowrate of the continuous phase. Droplet breakup at the T-junction was caused by the higher flowrate of the continuous phase, and the emulsion droplets then passed through the adsorption channel, where emulsifier adsorption at the surface of oil droplets may occur. Due to SFO's higher viscosity, droplet breakup at the T-junction did not occur at low flowrates, limiting the minimum flowrate to 45 µl/min. The emulsion then entered the coalescence channel where droplets were free to collide with other droplets. A high-speed camera (SpeedCam MacroVis EoSens, Germany) was used to capture 500 images near the inlet and outlet of the coalescence channel, and the images were processed using MATLAB. The experiments were conducted in duplicates, and the frame rate of the camera was varied according to the flowrate. Because of this, the number of images used to analyze droplet coalescence was varied accordingly to keep a constant experimental acquisition time of 10 seconds. Table 2.2 presents all the experimental conditions used.

Length of the adsorption channel	Water phase		Disperse phase		Total flow rate [µl/min}	Residence time	
	Emulsifier	pH	Oil type	Oil Fraction		Adsorption channel [s]	Coalescence channel [s]
14 mm	WPI	3 and 7	Hexadecane	14.3%	35	$1.08 \cdot 10^{-1}$	$9.64 \cdot 10^{-1}$
				7.7%	65	$5.82 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	3.9810^{-2}	$3.55 \cdot 10^{-1}$
		7	Sunflower oil	11.1%	45	$8.42 \cdot 10^{-2}$	$7.52 \cdot 10^{-1}$
				7.7%	65	$5.82 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	$3.98 \cdot 10^{-2}$	$3.55 \cdot 10^{-1}$
	LMPC	3 and 7	Hexadecane	14.3%	35	$1.08 \cdot 10^{-1}$	$9.64 \cdot 10^{-1}$
				7.7%	65	$5.82 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	$3.98 \cdot 10^{-2}$	$3.55 \cdot 10^{-1}$
		7	Sunflower oil	11.1%	45	$8.42 \cdot 10^{-2}$	$7.52 \cdot 10^{-1}$
				7.7%	65	$5.82 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	$3.98 \cdot 10^{-2}$	$3.55 \cdot 10^{-1}$
20.5 mm	WPI	3 and 7	Hexadecane	14.3%	35	$1.58 \cdot 10^{-1}$	$9.64 \cdot 10^{-1}$
				7.7%	65	$8.52 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	$5.83 \cdot 10^{-2}$	$3.55 \cdot 10^{-1}$
		7	Sunflower oil	11.1%	45	$1.23 \cdot 10^{-1}$	$7.52 \cdot 10^{-1}$
				7.7%	65	$8.52 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	$5.83 \cdot 10^{-2}$	$3.55 \cdot 10^{-1}$
	LMPC	3 and 7	Hexadecane	14.3%	35	$1.58 \cdot 10^{-1}$	$9.64 \cdot 10^{-1}$
				7.7%	65	$8.52 \cdot 10^{-2}$	$5.19 \cdot 10^{-1}$
				5.3%	95	$5.83 \cdot 10^{-2}$	3.5510^{-1}
		7	Sunflower oil	11.1%	45	$1.23 \cdot 10^{-1}$	7.5210^{-1}
				7.7%	65	$8.52 \cdot 10^{-2}$	5.1910^{-1}
				5.3%	95	$5.83 \cdot 10^{-2}$	3.5510^{-1}

2.2.4. Image analysis and calculation

The obtained images were analyzed with an in-house MATLAB script to determine the number and area of droplets. The frequency distribution of droplet area was determined using excel spreadsheet to distinguish between uncoalesced and coalesced droplets. The formation of dimers (two droplets merged to form a single droplet) and trimers (three droplets merged to form a single droplet) was identified by observing the doubling and tripling of the droplet area, respectively. Since microfluidics helps in producing monodispersed droplets, the dimers, trimers, and tetramers could be seen to exactly have double, tripled, or quadrupled in area than that of uncoalesced droplets. Figure 2.3 shows the process flowchart of image analysis. The fraction of droplets undergoing coalescence (Dc) was calculated by using equation 2.1.

$$Dc = \frac{[(N2e * 2) + (N3e * 3) + \ldots\ldots + (Nne * n)]}{[N1e + (N2e * 2) + (N3e * 3) + \ldots\ldots + (Nne * n)]} - \frac{[(N2s * 2) + (N3s * 3) + \ldots\ldots + (Nns * n)]}{[N1s + (N2s * 2) + (N3s * 3) + \ldots\ldots + (Nns * n)]} \quad \text{(Eq 2.1)}$$

Where, N_{1e} and N_{1s} are the numbers of uncoalesced drops at the end and start of the coalescence channel respectively, N_{2e} and N_{2s} are the number of dimers at the end and start of the coalescence channel respectively. The frequency of coalescence was calculated using Equation 2.

$$Fc = \frac{Dc}{Rt} \quad \text{(Eq 2.2)}$$

Where, *Rt* is the residence time of droplets in the coalescence channel. Residence time was calculated using the known volumetric flowrates. The residence time in the adsorption channel was computed considering the total length of the adsorption channel (14 or 20.5 mm) and the residence time in the coalescence channel was calculated between the start and end of the coalescence channel (2.5 cm in length), corresponding to the regions where images were taken.

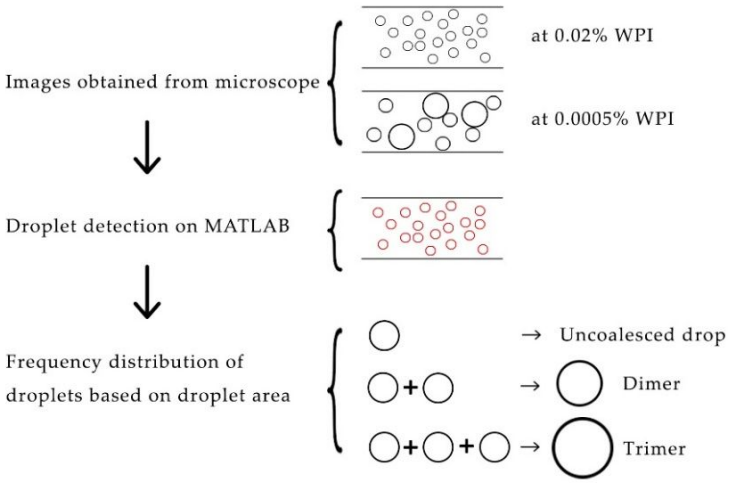


Figure 2.3: Flowchart of the process of image analysis.

2.2.5. Measurement of interfacial tension

Using the hanging ring method, the interfacial tension was measured with a Sigma T702-D equipment (Biolin Scientific, Sweden). Onstage, a beaker with the protein solution was set up. A platinum ring was suspended on a hook and gradually submerged into the liquid in the beaker after being cleaned with ethanol, distilled

water, and a flame to remove contaminants. Using a Pasteur pipette, oil was gradually added to the protein solution, and the system was given one minute to come to equilibrium. By accounting for the density difference between the two liquids, the energy needed for the ring to break the interfacial strength was measured in order to estimate the interfacial tension.

2.2.6. Statistical analysis

GraphPad was used to do a one-way analysis of variance (ANOVA) on the data. ANOVA was used to identify significant differences. The mean ± standard deviation is used to show the data.

2.3. Results and discussions

This section compares the effectiveness of WPI (whey protein isolate) and LMPC (lesser mealworm protein concentrate) as emulsifiers and looks at how different conditions affect the coalescence of emulsion droplets stabilized by proteins. Precise control over droplet formation at the T-junction, emulsifier adsorption time at the newly formed interface, and droplet stability in the coalescence channel were made possible by the microfluidic device employed in this work. This arrangement made it possible to thoroughly examine the effects of emulsion formulation and operation conditions on droplet coalescence.

2.3.1. The diameter of individual droplets and interfacial tension

Using ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA), droplet areas measured in pixels from MATLAB were used to determine the average droplet diameter and then converted to micrometers. The average droplet diameter was determined for the droplets that did not undergo coalescence. It can be observed that average size of individual droplets was mostly constant under constant process conditions, with a standard deviation ranging from 0.2 to 1 μm, suggesting that the emulsions were monodisperse. The average size of hexadecane droplets at the start of the coalescence channel is shown in Figure 2.4. Whether WPI or LMPC was utilized to stabilize the hexadecane emulsion had no apparent impact on the droplet size at various protein concentrations. This result is in line with research by Chagot et al., 2022, who found that, when flow conditions and device geometries are controlled, droplet size is not greatly affected by the type or concentration of protein. (Hinderink, Kaade, Sagis, Schroën, & Berton-Carabin, 2020) discovered this as well while examining the coalescence of hexadecane emulsions stabilized with pea protein. Additionally, they showed that the initial size of the droplets was essentially unaffected by the starting concentration of protein, which may be connected to a persistent apparent interfacial tension throughout

droplet formation. This could also apply to WPI and LMPC hexadecane stabilized emulsions in the current scenario.

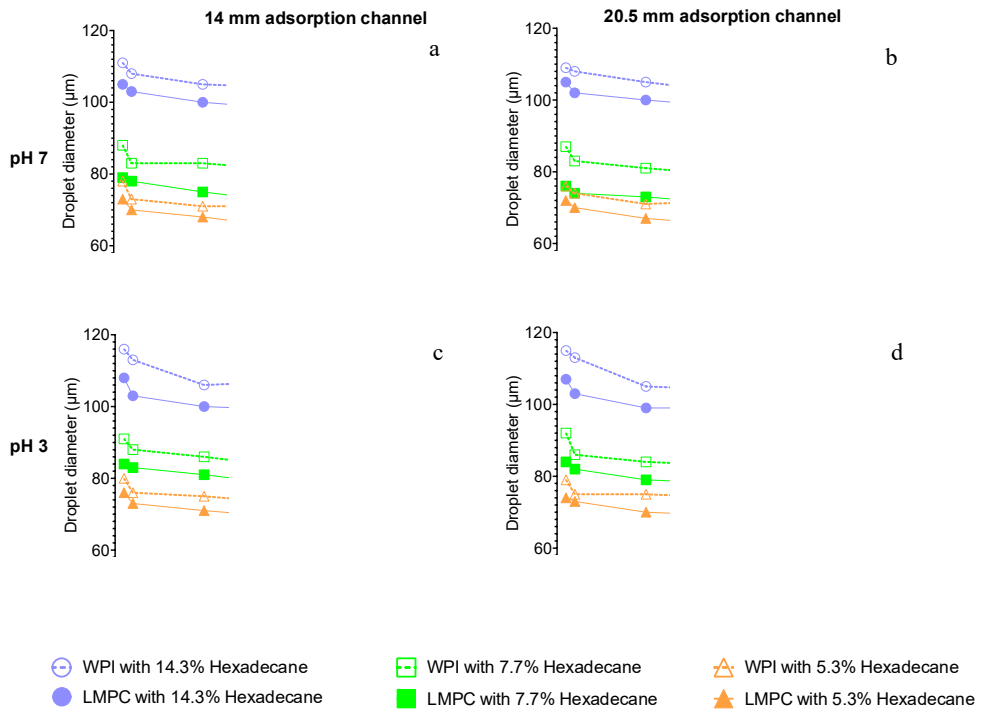


Fig. 2.4: Diameter of hexadecane droplets at different concentrations of WPI and LMPC. (a) pH 7 and 0.108 s adsorption time, Rt, (b) pH 7 and 0.158 s Rt, (c) pH 3 and 0.108 s Rt, and (d) pH 3 and 0.158 s Rt. Error bars showing standard deviation cannot be seen since they are smaller than the symbol size (range of the standard deviation from 0.2 to 1 µm).

The impact of pH and oil fraction on the initial droplet size of hexadecane emulsions stabilized with WPI and LMPC can also be seen in Figure 2.4. With an increase in oil fraction, droplet size increases significantly regardless of the protein used. When the continuous phase flowrate is at its lowest, the oil fraction is at its highest. Larger oil droplets occur at the T-junction due to reduced shear stress caused by decreased flow rates of the continuous phase. The rise in droplet size at an acidic pH (pH = 3) is explained by conformational changes in the proteins that result in a decrease in surface hydrophobicity and an increase in interfacial tension. Also, for every condition examined, it is evident that the LMPC generates smaller droplets than WPI (Fig. 2.4). As seen in Fig. 2.5, the lower equilibrium interfacial tension between hexadecane and LMPC solution may help to explain this, and it may also be linked to the protein's increased molecular flexibility (Gould & Wolf, 2018).

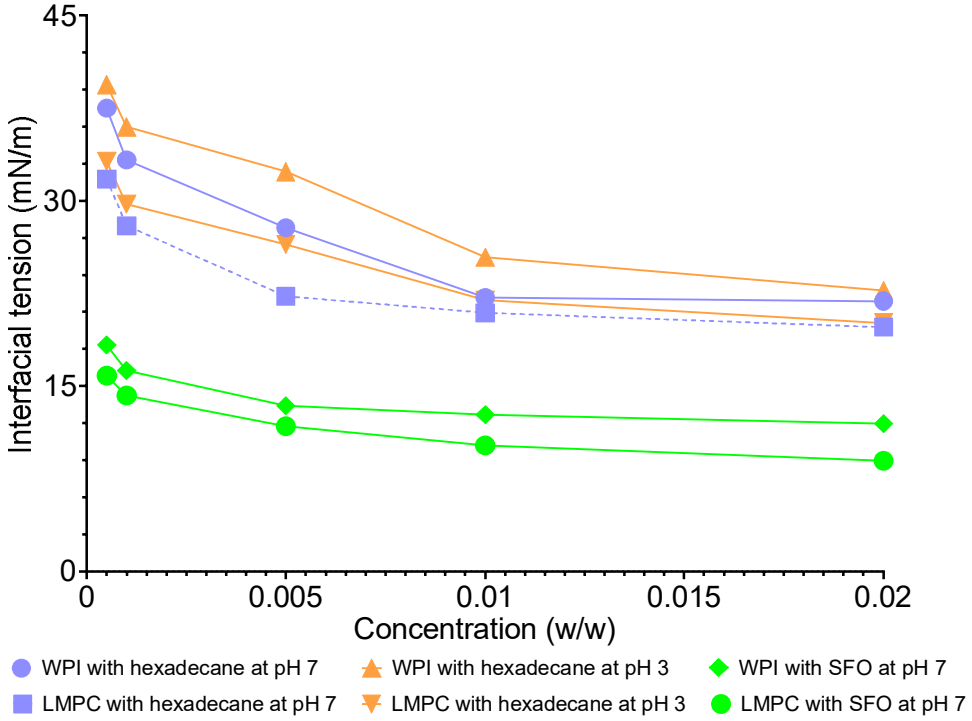


Fig. 2.5: Interfacial tension between hexadecane/SFO and WPI/LMPC solution at varying concentrations of proteins at pH = 3 and pH = 7. Error bars showing standard deviation cannot be seen since they are smaller than the symbol size (range of the standard deviation from 0.1 to 0.3 mN/m).

The patterns in sunflower oil (SFO) droplet size are similar to those seen in hexadecane emulsions. Droplet diameter increases significantly when oil phase increases and marginally increases as protein content decreases (Figure 2.5). Additionally, droplet sizes in SFO emulsions stabilized with LMPC were slightly lower than in those stabilized with WPI. The reduction of droplet size does not change even though the interfacial tension of the two proteins with SFO is significantly lower than with hexadecane. This could also mean that, in the case of an emulsion produced using a T-junction microfluidic system, droplet size is primarily controlled by the flowrate of dispersed and continuous phases. When comparing the impact of the adsorption channel length on the droplet size, the same pattern can be seen. Despite the change in the used channel length, the droplet size was same. Furthermore, it has been documented that the bulk protein content had no effect on the apparent interfacial tension that was measured in the microchannels. When compared to proteins (whey protein and bovine serum albumin), Tween 20 was shown to have larger apparent interfacial tensions; yet, its equilibrium interfacial tension was lower (Güell, Ferrando, Trentin, & Schroën, 2017).

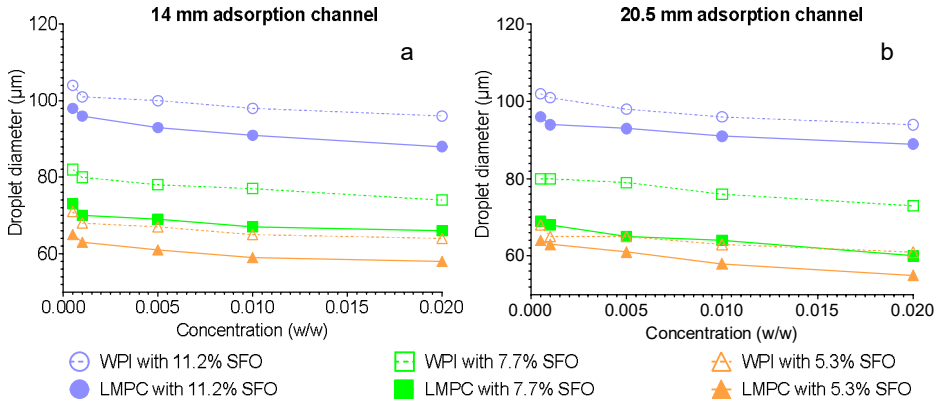


Fig. 2.6: Diameter of SFO droplets at different concentrations of WPI and LMPC. (a) pH 7 and 0.108 s adsorption time, Rt, (b) pH 7 and 0.158 s Rt. Error bars showing standard deviation cannot be seen since they are smaller than the symbol size (range of the standard deviation from 0.2 to 1 µm).

Measurements of interfacial tension support the idea that droplet size is mostly dependent on flow rate and chip design. Larger droplets are usually the result of increased interfacial tension in traditional emulsification procedures (McClements, 2004). In microfluidics, however, droplet size rises very little with decreasing protein content, despite an increase in interfacial tension. The equilibrium interfacial tension at pH = 3 and pH = 7 between hexadecane/SFO and WPI/LMPC solutions at varying protein concentrations is depicted in Figure 2.6. At both pH levels, LMPC tends to reduce interfacial tension more than WPI. Smaller droplet sizes are anticipated for SFO emulsions than for hexadecane ones, as SFO dramatically lowers interfacial tension in comparison to hexadecane. However, hexadecane is a long-chain saturated hydrocarbon with a complicated molecular structure, whereas SFO is a combination of triglycerides made up of different unsaturated fatty acids and glycerol (Akkaya, 2018). The lower interfacial tension of unsaturated fatty acids in comparison to hexadecane can be explained by the flexibility of their molecular structure, which is made possible by the double bonds in the molecules(Watanabe, Kawai, & Nonomura, 2018). Bigger droplets are the result of increased viscosity of the dispersed phase in shear-driven systems like T-junctions. Nonetheless, in this investigation, the greater SFO viscosity (49.19 mPa.s) in comparison to hexadecane (3.005 mPa.s) is overcome by the combined impact of interfacial tension and flow rate.

2.3.2. The number of droplets undergoing coalescence at the start of the coalescence channel is affected by pH, adsorption time, and oil fraction

It was evident by examining the droplets close to the coalescence channel's entrance that some of the droplets went through coalescence as soon as they were released into the channel. It is possible to quantify the impact of many factors such as adsorption duration, pH, type and proportion of oil, protein type and concentration, and coalescence number of droplets upon exiting the adsorption channel on droplet stability. The number of coalescing droplets for hexadecane at various protein concentrations is shown in Fig. 2.7 as a function of these factors. Droplet coalescence is significantly influenced by protein content. With a decline in protein content, there is a significant rise in the number of droplets undergoing coalescence to form dimers, trimers, and tetramers, regardless of pH, oil type and fraction, or adsorption period. The greatest number of droplets undergoing coalescence is shown when the oil fraction is at its highest value (14.3 % for hexadecane and 11.1% for SFO (Figure 2.8)), when comparing the effects of adsorption duration, pH, and oil fraction.

After doing an ANOVA to evaluate the effect's statistical significance, it became clear that droplet coalescence is significantly impacted by an increase in oil percentage. Higher oil fractions cause the flowrate to decrease, extending the residence time and allowing for more collisions, which in turn accelerates the rate of coalescence. Larger droplets generated at higher oil percentages could have also contributed to the droplets colliding more frequently and further coalescing. Analyzing the impact of an increase in protein concentration also revealed a substantial difference in the quantity of droplets undergoing coalescence. Despite the protein employed to stabilize the emulsion, longer adsorption channels and a pH of 7 were associated with increased stability. SFO droplets behaved similarly to hexadecane droplets, with an apparent increase in the number of droplets coalescing when oil-fraction was increased, and protein concentrations was decreased (Figure 2.8). These findings emphasize the significance of considering the impacts of pH, protein concentration, and oil-fraction on droplet behavior, which has significant implications for the design and optimization of microfluidic systems, particularly those incorporating emulsions.

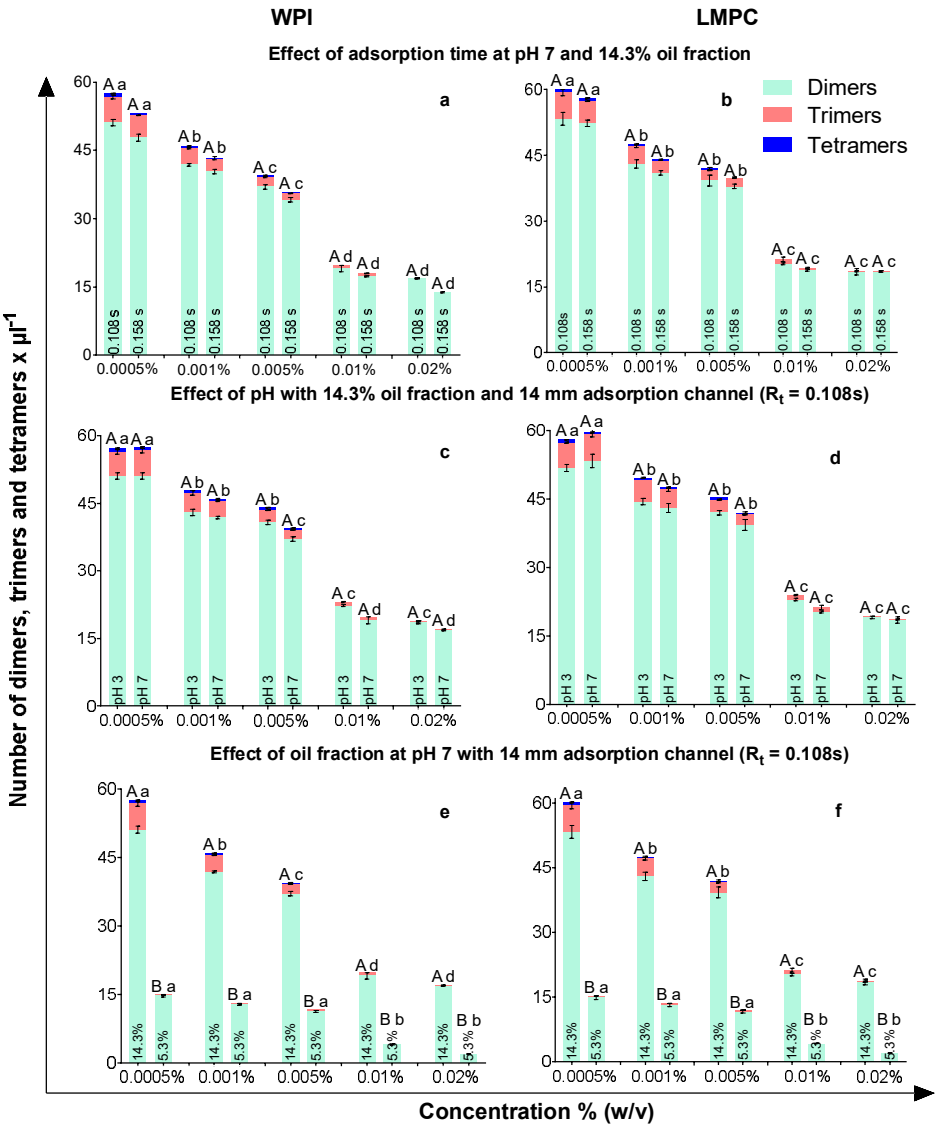


Fig. 2.7: Number of droplets undergoing coalescence at the start of the coalescence channel at different (a, b) adsorption time, (c, d) pH, and (e, f) oil fraction at different protein concentrations in emulsions prepared with hexadecane. Error bars show the standard deviation. Different capital letters mean significant differences ($p < 0.05$) in the effect of adsorption time/pH/oil fraction for a constant protein concentration and lowercase letters mean significant differences in the effect of protein concentration for a constant adsorption time/ pH/oil fraction.

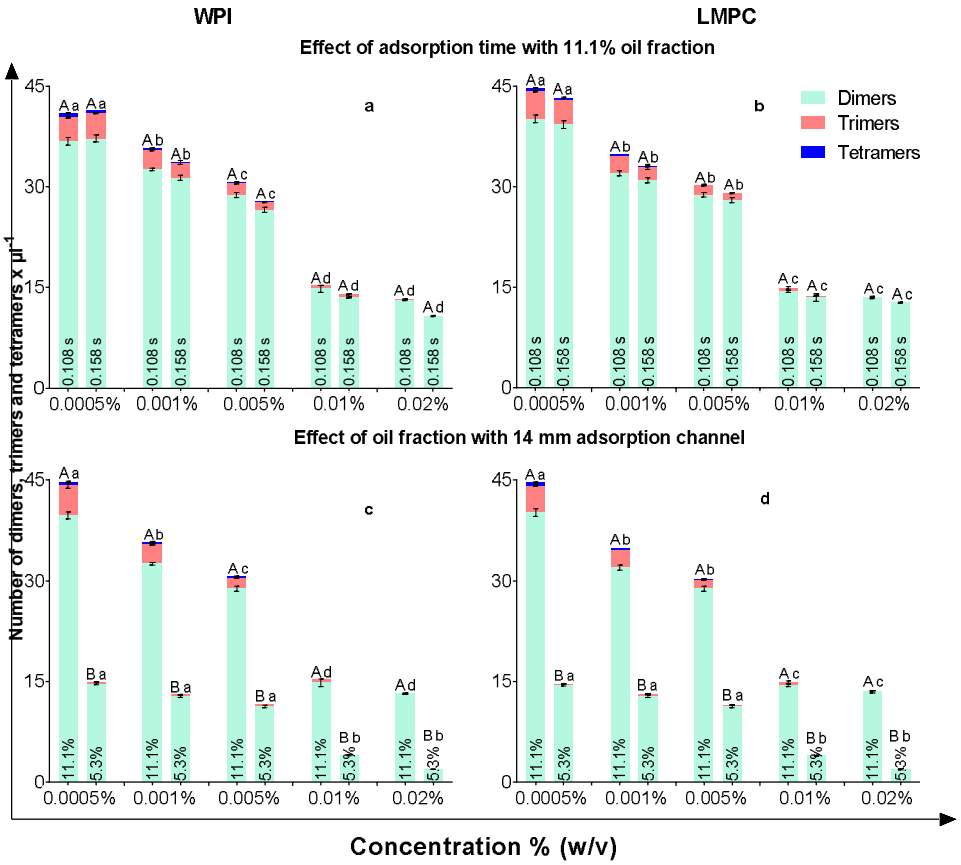


Fig. 2.8: Number of droplets undergoing coalescence at the start of the coalescence channel at different (a, b) adsorption time, and (c, fd) oil fraction at different protein concentrations in emulsions prepared with hexadecane. Error bars show the standard deviation. Different capital letters mean significant differences ($p < 0.05$) in the effect of adsorption time/oil fraction for a constant protein concentration and lowercase letters mean significant differences in the effect of protein concentration for a constant adsorption time/oil fraction.

2.3.3. Frequency of Coalescence

Figure 2.9 plots the frequency of coalescence was calculated using equation 2.2 for hexadecane and SFO emulsions as a function of various WPI and LMPC concentrations at varied oil fractions, pH levels, and adsorption durations. It is evident that as the concentration of protein decreased, so did the frequency of coalescence. The low amount of protein present to stabilize the oil droplets at the interface, which promotes droplet coalescence when they come in contact, favors the increase in the frequency of coalescence, as the protein concentration is decreased. It should be noted that a number of factors influence the emulsion's stability. It is possible to see that the frequency of coalescence rises as pH drops from 7 to 3 while looking at a protein

concentration of 0.02%. This must be related to the conformational changes that impact LMPC and WPI, which happen to be greater for the largest oil fraction under investigation. In addition to the pH and protein content, the oil percentage is also very important. As a result, when the system's hexadecane percentage increasing, the pH was lowered, and the adsorption duration was brief, the frequency of coalescence increased. Once more, as shown in figure 2.9, the values of the frequency of coalescence for both proteins are quite close, and at the maximum protein concentration, there is no noticeable distinction between them.

Upon producing the emulsions with SFO, it was noted that these systems have a comparable pattern to those made with hexadecane; however, the frequency of coalescence was found to be marginally reduced, irrespective of the protein, oil fraction, and adsorption duration. One of the factors influencing how the protein interacts with the oil at the interface is the type of oil (Kalaydzhiev et al., 2019). The thinning of the layer between merging droplets is one of the processes that leads to droplet coalescence (Narayan, Metaxas, Bachnak, Neumiller, & Dutcher, 2020). The type of film produced between droplets is determined by several characteristics, one of which is the viscosity ratio (μ_d/μ_c) between the discontinuous and continuous phases. Less coalescence occurs when the droplet's deformability approaches that of an immobile thin film, which is correlated with a larger viscosity ratio. The frequency of coalescence at pH 7 and low oil fractions, 5.3% and 7.7%, was substantially greater for hexadecane in this investigation during the longest adsorption duration when protein concentrations were below 0.01%. In our instance, the viscosity ratios for hexadecane and SFO are around 3.5 and 32, respectively. This may help to explain why, in the case of SFO, the frequency of coalescence is somewhat lower when insufficient protein is present to stabilize the oil droplets. It was demonstrated that both proteins showed comparable results when LMPC and WPI were compared in terms of the frequency of coalescence, especially at pH 7, with only slight variations at pH 3. This behavior may be due to the effects of pH 3 on surface charge and protein structure.

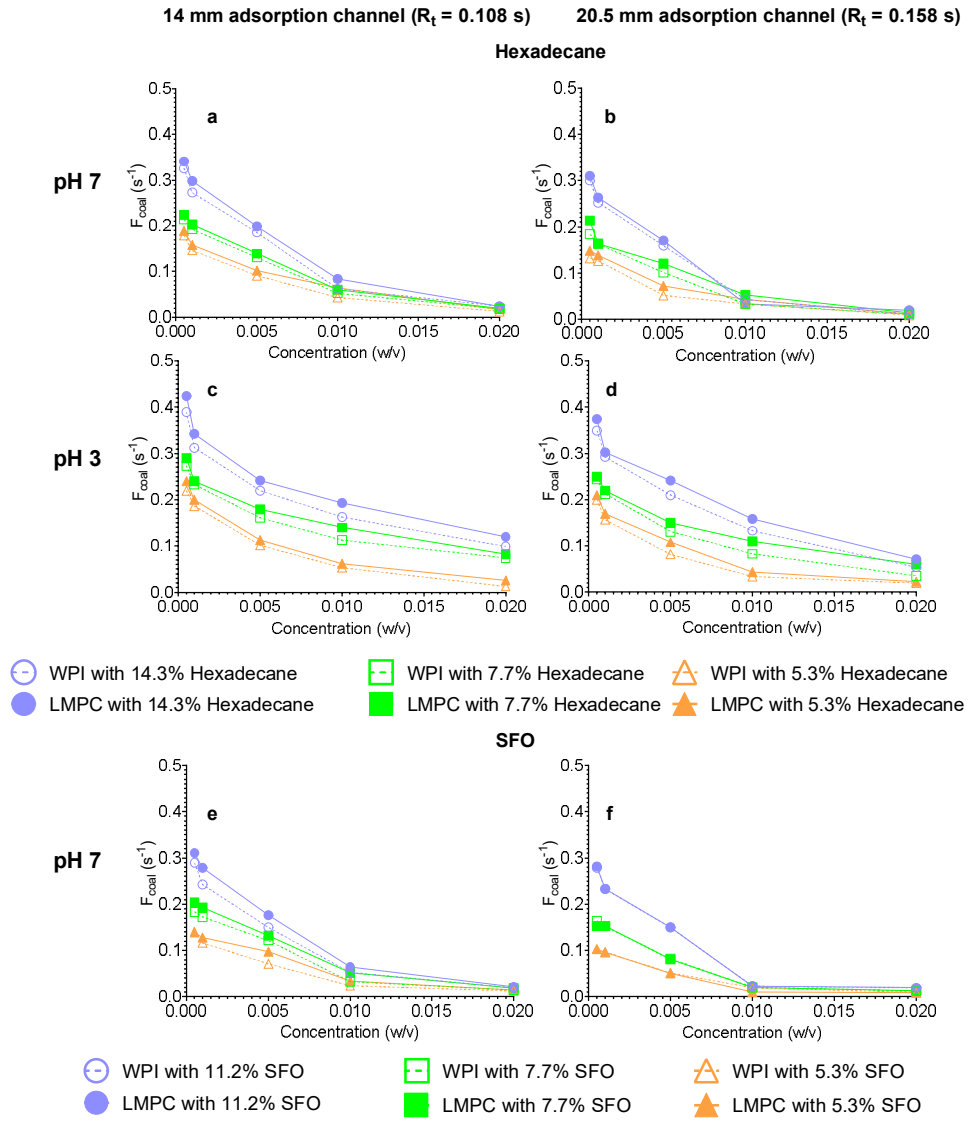


Fig. 2.9. Frequency of coalescence of emulsions stabilized with WPI and LMPC at different oils fractions and protein concentrations at (a) hexadecane, pH 7, and shorter adsorption time, 0.108 s; (b) hexadecane, pH 7, and longest adsorption time, 0.158 s; (c) hexadecane, pH 3, and shorter adsorption time, 0.108 s; (d) hexadecane, pH 3, and longest adsorption time, 0.158 s; (e) sunflower oil, pH 7, and shorter adsorption time, 0.108 s; (f) sunflower oil, pH 7, and longest adsorption time, 0.158 s. Error bars showing standard deviation cannot be seen since they are smaller than the symbol size.

2.3.4. Effect of Protein Concentration, Adsorption Time, and Oil Fraction on Droplet Coalescence

It was possible to compare the production of these droplets at the coalescence channel's outlet and outlet and understand coalescence by counting the number of dimers, trimers, and tetramers. It is evident from Fig. 2.10 that for hexadecane emulsions with pH = 7 and 14.3% oil fraction, the quantity of trimers and tetramers generated decreases as the protein concentration rises, irrespective of the type of protein. At the maximum protein concentration, essentially no trimers and tetramers occur. In addition to trimers and tetramers, the number of dimers generated rises significantly near the channel exit compared to the channel inlet at the lowest protein concentration levels. However, there is a decrease in the differential between the dimers generated at the channel's intake and output when the protein concentration rises. Since it is reasonable to assume that oil droplets are more prone to coalescence when there is insufficient protein in the system to fully adsorb on to their surface (lowest protein concentrations), an increase in protein concentration helps prevent the droplets from going through coalescence. When it comes to stabilizing these emulsions, LMPC and WPI exhibit a similar tendency. At lower protein concentrations, LMPC exhibits a slightly larger number of droplets undergoing coalescence, but at higher protein concentrations, WPI and LMPC exhibit identical behavior.

For the hexadecane and SFO emulsions, the generation of dimers decreases as the protein adsorption period increases (figure 2.10 and figure 2.11). This suggests that extending the time that oil droplets take in contact with the protein in the adsorption channel aids in better stabilizing the droplets because longer adsorption times give proteins more time to travel to the oil-water interface, which in turn stabilizes the droplet more thoroughly. Therefore, when a longer adsorption channel was utilized for both proteins, the number of droplets undergoing coalescence was smaller at the beginning and end of the channel. However, as the protein concentration rises, the number of coalesced droplets as a function of the adsorption time are very similar (Fig.2.10), suggesting that, at least for the values examined in this work, the adsorption time has very little effect when the system contains enough protein to stabilize the formed interfaces.

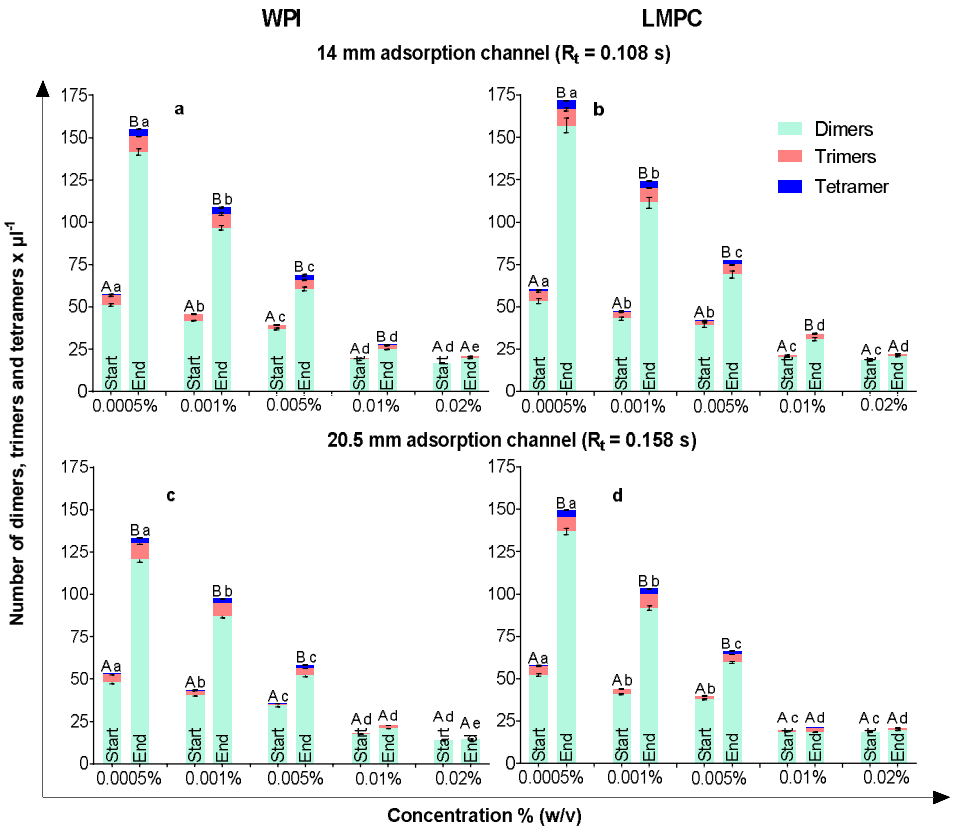


Fig. 2.10: Effect of protein concentration on the number of hexadecane droplets undergoing coalescence at the start and end of coalescence channel with 14.3% oil fraction for (a, b) adsorption times of 0.108 s (c, d) adsorption times of 0.158 s. Error bars show the standard deviation. Different capital letters mean significant differences ($p < 0.05$) in the number of droplets undergoing coalescence at the start and end of coalescence channel for a constant protein concentration and lowercase letters mean significant differences in the effect of protein concentration at the start/end of coalescence channel.

The behavior of SFO emulsions with pH = 7 and 14.3% oil fraction is similar to that of hexadecane emulsions (figure 2.11). The formation of trimers and tetramers declines with increasing protein concentration, with nearly no trimers or tetramers present at the highest protein concentrations. Dimers are more prevalent close to the channel's outlet than at its inlet when protein concentrations are lower. Higher protein concentrations, however, appear to provide better protection against coalescence as the difference in the number of dimers between the outlet and intake decreases. This suggests that the droplets are more prone to coalescence when the protein levels are too low for the droplets to completely adsorb onto the surfaces.

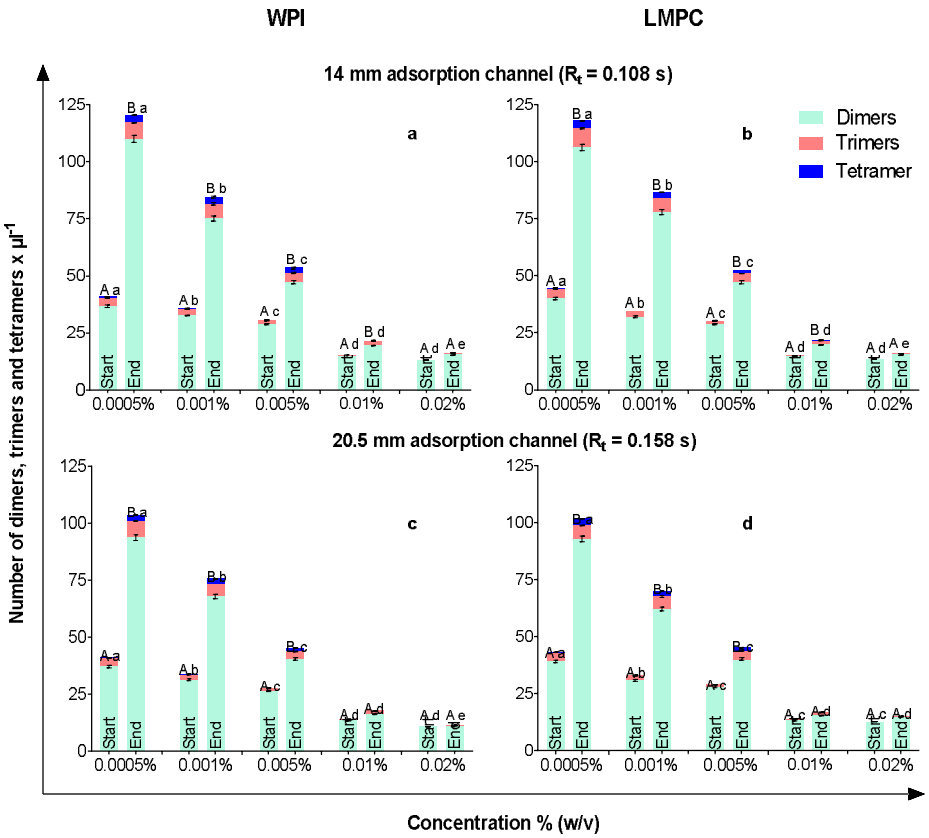


Fig. 2.11: Effect of protein concentration on the number of SFO droplets undergoing coalescence at the start and end of coalescence channel with 11.2% oil fraction for (a, b) adsorption times of 0.108 s (c, d) adsorption times of 0.158 s. Error bars show the standard deviation. Different capital letters mean significant differences ($p < 0.05$) in the number of droplets undergoing coalescence at the start and end of coalescence channel for a constant protein concentration and lowercase letters mean significant differences in the effect of protein concentration at the start/end of coalescence channel.

It appears that oil fractions have a significant influence on how frequently the droplets coalesce. Figure 2.12 shows the number of droplets undergoing coalescence for hexadecane emulsions at pH 7 stabilized with WPI and LMPC at the beginning and end of the coalescence channel for each of the three oil fractions under investigation (5.3, 7.7, and 14.3%). Regardless of the protein and even for the lowest protein concentration tested (0.0005%), it is evident that the total number of droplets undergoing coalescence is more than 5 times at 14% oil fraction as compared to the emulsion with 5% oil fraction.

According to (Muijlwijk et al., 2017), a longer residence time of the emulsion in the microchip increases the likelihood of oil droplets colliding with one another,

hence creating more space for droplets to undergo coalescence. Notably, the size of the droplets formed at a high oil percentage was bigger than that of the droplets at a low oil fraction. Another element that increases the likelihood of droplets coalescing is their bigger size, which increases the likelihood of their colliding. Emulsions made with SFO in the dispersed phase had comparable outcomes (figure 2.13).

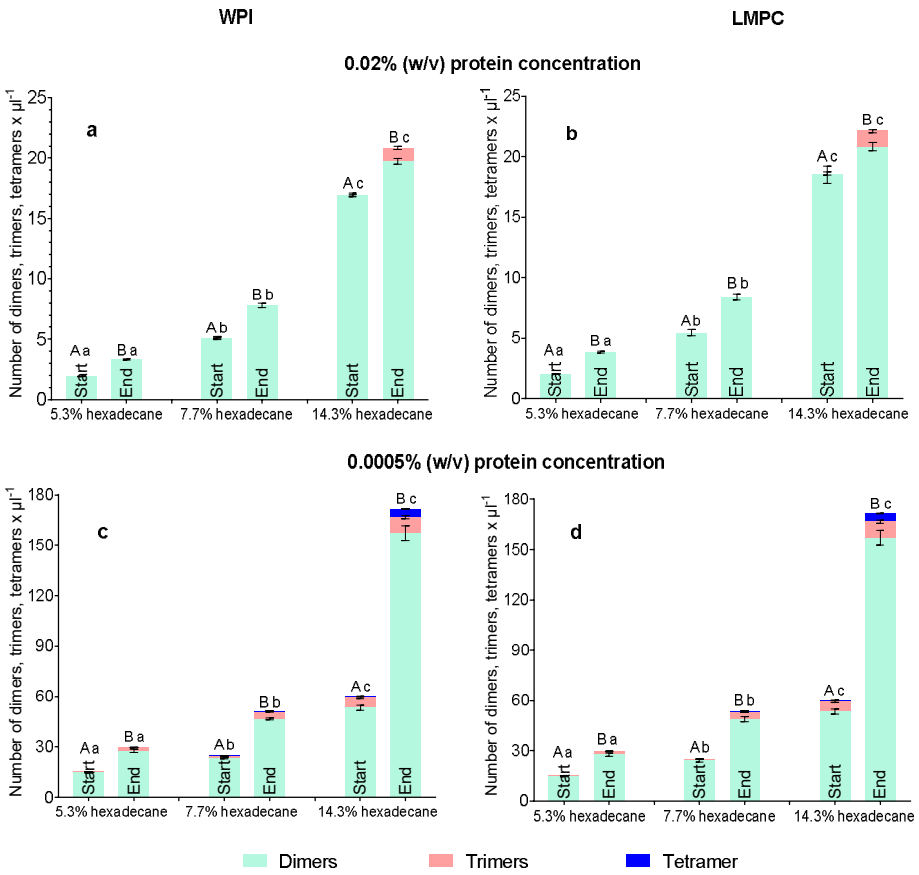


Fig. 2.12: Effect of hexadecane fraction on the number of droplets undergoing coalescence in the emulsions stabilized with (a, b) 0.02% and (c, d) 0.0005% WPI and LMPC using the chip of 0.108 s adsorption time. Error bars show the standard deviation. Different capital letters mean significant differences ($p < 0.05$) in the number of droplets undergoing coalescence at the start and end of coalescence channel at a constant oil fraction and lowercase letters mean significant differences in the number of droplets undergoing coalescence at different oil fractions at the start/end of coalescence channel.

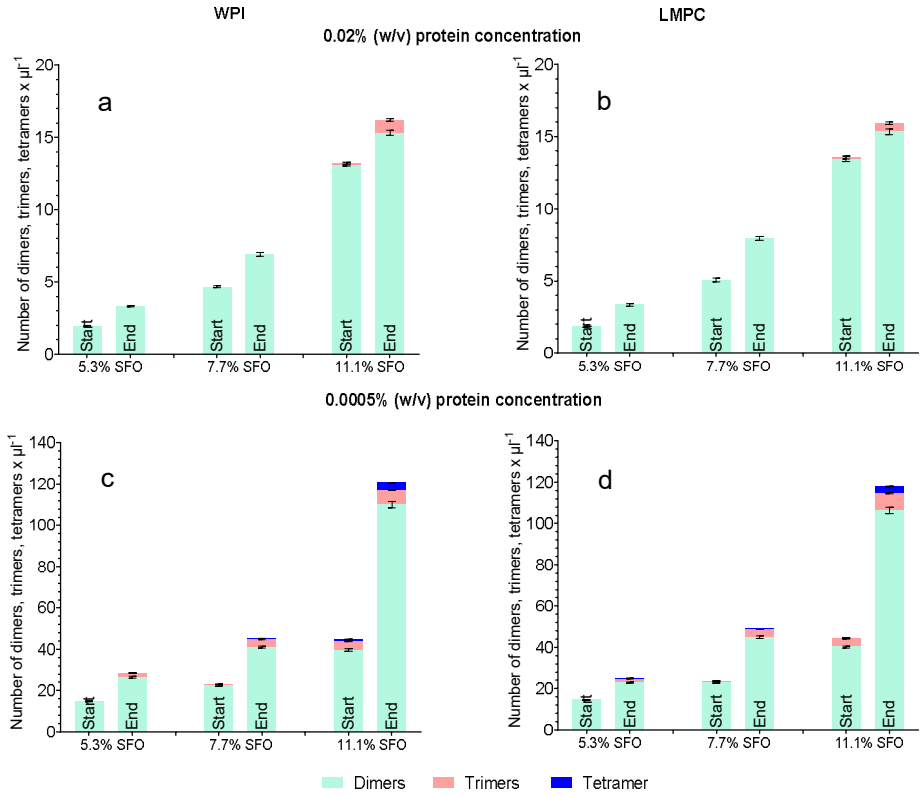


Fig. 2.13: Effect of SFO fraction on the number of droplets undergoing coalescence in the emulsions stabilized with (a, b) 0.02% and (c, d) 0.0005% WPI and LMPC using the chip of 0.108 s adsorption time. Error bars show the standard deviation. Different capital letters mean significant differences ($p < 0.05$) in the number of droplets undergoing coalescence at the start and end of coalescence channel at a constant oil fraction and lowercase letters mean significant differences in the number of droplets undergoing coalescence at different oil fractions at the start/end of coalescence channel.

2.3.5. Rate of Formation of Dimers and Trimers

The rate of formation of dimers and trimers gives the number of droplets undergoing coalescence per unit of volume and time equation 2.3.

$$\text{Rate of formation of dimers and trimers} = \frac{\text{Nie} - \text{Nis}}{\text{Rt}} \qquad \text{Eq (2.3)}$$

Where, Nie, and Nis are the number of dimers and trimers near the end and start of the coalescence channel respectively.

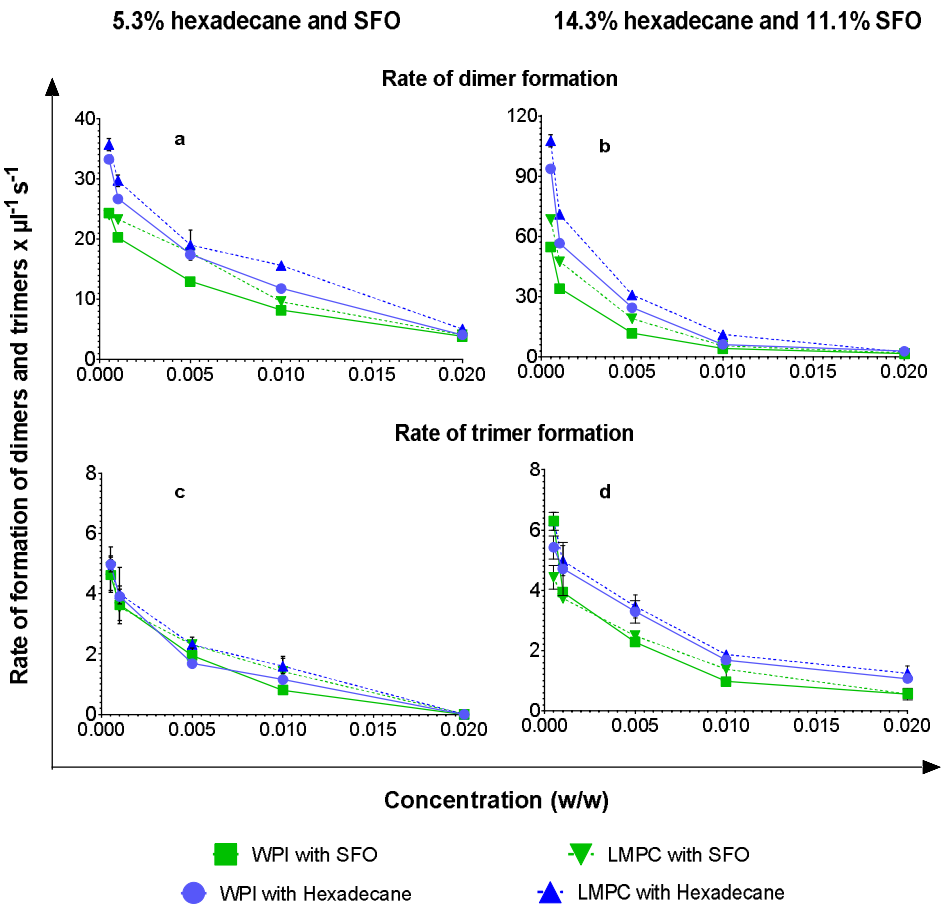


Fig. 2.12: Rate of formation of dimers and trimers at two different oil fractions in emulsions prepared with hexadecane and SFO with the shortest adsorption time (0.108 s), (a, c) 5.3% hexadecane and sunflower oil and (b, d) 14.3% hexadecane and 11.1% sunflower oil.

The rate at which dimers and trimers develop while using the shortest adsorption period for the hexadecane and SFO at two oil fractions is shown in Fig.2.12. The prior studies have shown that an increase in oil fraction, particularly in the case of dimer formation, has a significant effect on the rate of coalescence. While the oil fraction and the protein concentration have an effect on the rate of dimer formation, the rate of trimer formation is not greatly affected by the increase in oil fraction. At the lowest protein concentration, the rate of trimer formation is in between 4 and 6 $\mu L^{-1}s^{-1}$ for both 5.3% and 14.3%/11.2% oil fraction, whereas the rate of dimer formation varies between 20-35 $\mu L^{-1}s^{-1}$ and 30-100 $\mu L^{-1}s^{-1}$ for 5.3% and 14.3/11.2% oil fraction respectively. This shows a clear influence of oil fraction on the rate of dimer formation.

The variation in the physical characteristics of SFO and hexadecane, such as viscosity and interfacial tension, may account for the difference in the rate of dimer and trimer formation at the same oil percentage. In comparison to hexadecane, SFO has a higher viscosity and a lower interfacial tension, which may result in a more stable contact between the water and oil phases. As a result, the type of oil employed can impact both the rate of coalescence and the stability of the emulsion. Regarding how the kind of protein affects the rate of dimer and trimer formation, WPI and LMPC appear to function similarly.

2.3.6. Capillary Number and Rate of Dimer Formation

The properties of droplets in a multiphase system are reliant upon the many forces operating against them. These forces may be quantified by dimensionless numerical representations such as the Reynolds number (Re), Weber number (We), Bond number (Bo), and capillary number (Ca). Re, We, and Bo are shown to be tiny for a microdroplet flow; this results in a laminar flow in which surface tension and viscous forces are important (F. Shen, Li, Liu, Cao, & Wang, 2015). Surface tension to viscous force ratio, Ca, is computed using Equation (2.4).

$$\mathrm{Ca} = \frac{\mu U}{\sigma} \qquad \text{Eq (2.4)}$$

where σ is the interfacial tension, U is the velocity, and μ is the dynamic viscosity. The relationship between the capillary number and the rate of dimer production is seen in Fig. 2.13.

Ha, Yoon, & Leal, 2003 state that capillary number has a role in predicting how two droplets will collide. The stability of droplets increases as the number of capillary increases as the viscous forces that shear a droplet cause the interfacial forces that keep it circular to weaken. Droplets become deformable when they are brought together in a shear flow because their interfaces flatten. The fluid layer that develops between the droplets must drain before the surfaces can get close enough for intermolecular interactions to become dominant for the interfaces to merge. The droplets slide over one another and if the film does not thin enough during contact, they merge and coalesce. As a result, droplets only coalesce when the capillary number is low enough, and they cannot merge when it is high enough. The critical capillary number is the value (Cac) below which droplet coalescence occurs. For the hexadecane and SFO emulsions stabilized with whey protein or LMPC, we can see an exponential decrease rising Ca when we plot the rate of dimer formation with the most significant coalescence seen in the current study versus the capillary number (Fig. 2.13). For the hexadecane emulsions, the rate of dimer formation increases dramatically for $\mathrm{Ca} < 0.02$; the coalescence values decline and approach the minimum values for $\mathrm{Ca} > 0.02$. This may imply that this is the value of the Cac for hexadecane.

For SFO emulsions, systems stabilized with LMPC and whey protein with Cac of around 0.25–0.3, likewise show an exponential decrease of the rate of dimer formation with an increase in Ca.

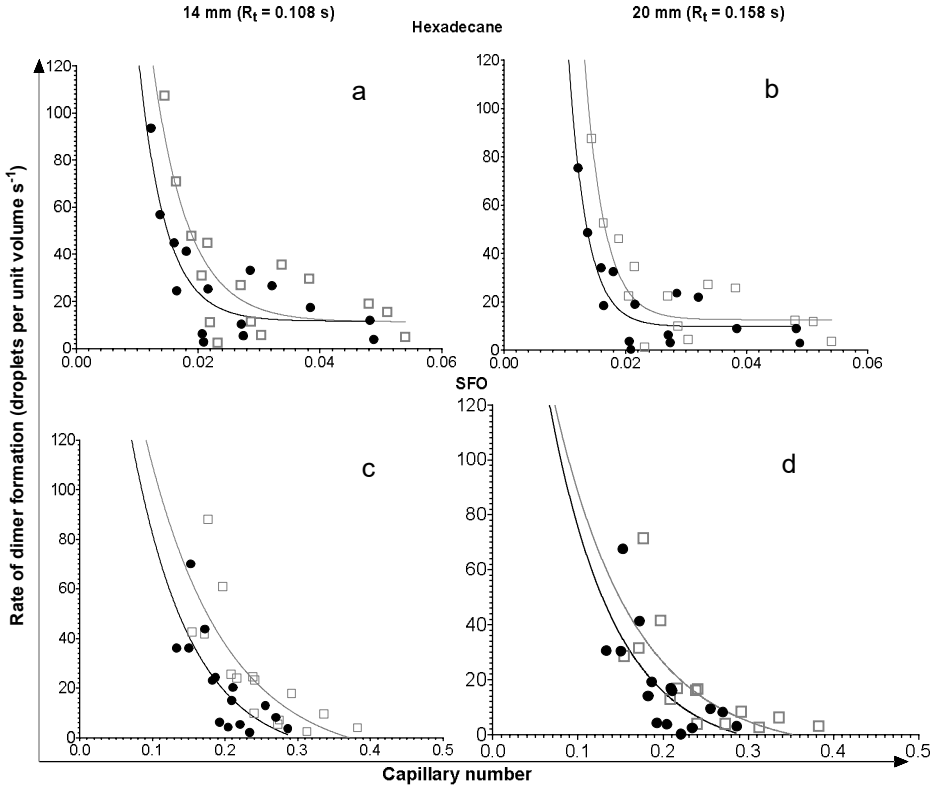


Fig. 2.13: Rate of formation of dimers against the capillary number with (a, b) 14.3% hexadecane and (c, d) 11.1% SFO stabilized with WPI and LMPC (0.0005–0.02%) with chips of 0.108 s adsorption time (a, c) and 0.158 s adsorption time (b, d).

As previously indicated, the film between droplets needs to drain until the distance is narrow enough to cause the film to break in order for the droplets to coalesce. Equation 2.5, as reported by Shen, Li, Liu, Cao, & Wang, 2015, may be used to determine the film drainage time (Td) for the hexadecane and SFO emulsions stabilized at pH 7 with whey protein and LMPC.

$$T_d = 40\ r \sqrt{\frac{\mu}{\sigma U}} \qquad \text{Eq (2.5)}$$

where r is the radius of the droplets.

As the number of capillaries increases, the film drainage time also increases (Fig. 2.14). This may be explained by the fact that the radius of the thin film that forms between the droplets grows with capillary number. Longer film drainage periods will arise from this circumstance since film drainage is caused by a pressure gradient that is inversely proportional to the thin film's radius (Narayan, Metaxas, Bachnak, Neumiller, & Dutcher, 2020). Furthermore, the proteins that are present in the interface are what cause the decrease in coalescence. An interfacial tension gradient is thought to be produced when the surfactant is pulled along the film borders during film thinning.

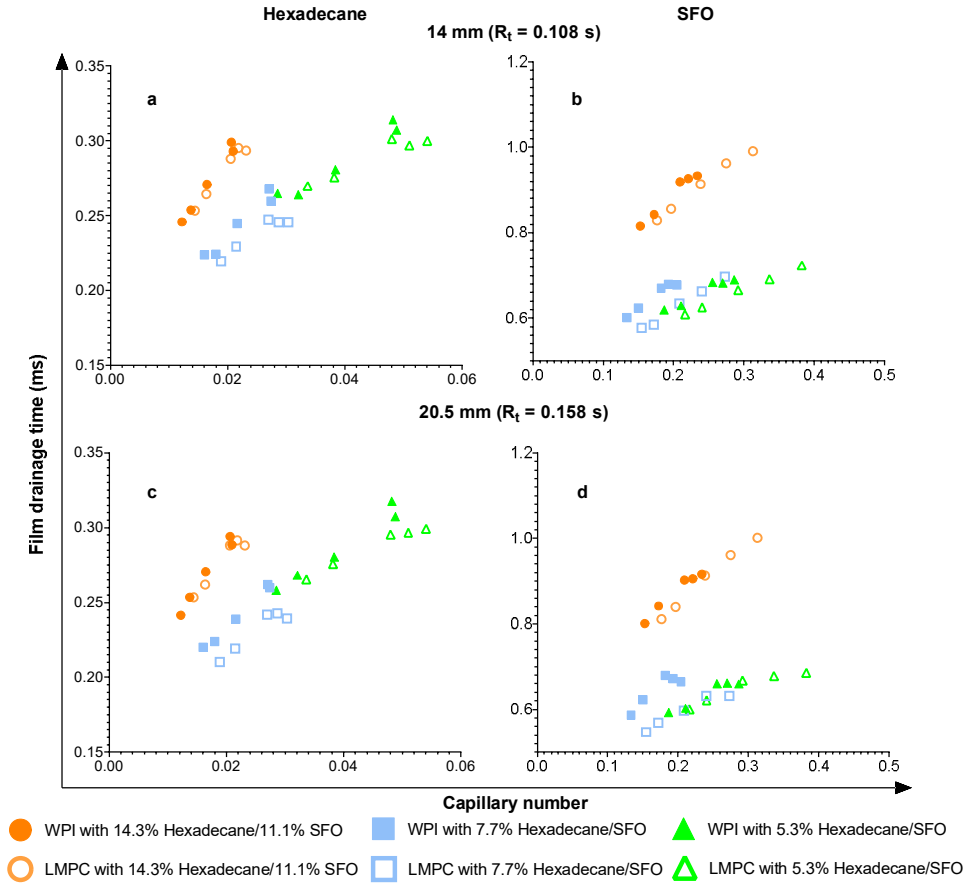


Fig. 12. Film drainage time at different protein concentrations as a function of capillary number at different oil fractions of (a, b) hexadecane and (b, d) SFO (b, d) at two different adsorption times of 0.108 s (a, b) and 0.158 s (c, d).

2.4. Conclusions

The study shows that, like whey protein isolate (WPI), less mealworm protein concentrate (LMPC) can stabilize hexadecane and sunflower oil emulsions. The research successfully accounted for droplet coalescence at both the beginning and end of the coalescence channel by using a microfluidic technology to create emulsions under controlled circumstances and a unique approach for picture acquisition and analysis. The technique allowed for the estimation of the frequency of coalescence and the formation rate for each kind of droplet by making a distinction between single droplets and those generated by coalescence (dimers, trimers, and tetramers).

The lowest protein content (0.0005%), shortest adsorption period (0.04 s), lowest pH (3), and highest oil fraction (14.3% for hexadecane and 11.1% for sunflower oil) were found to increase droplet coalescence. With higher protein concentrations, longer adsorption durations, and smaller oil fractions, emulsion stability improved at pH 7. The most important of these factors influencing droplet coalescence was oil fraction. Bigger droplet diameters and greater coalescence frequencies resulted from a higher risk of collisions.

The significance of the capillary number (Ca) in influencing droplet coalescence rates was further emphasized by the study. Reduced coalescence was correlated with higher capillary numbers, which signify stronger viscous forces in comparison to surface tension. It was discovered that the critical capillary number (Cac) for hexadecane was about 0.02 while for sunflower oil it was between 0.25 and 0.3. These results highlight how crucial it is to plan and optimize emulsification procedures taking into account factors like protein content, adsorption duration, oil percentage, and capillary number.

All things considered, LMPC showed promise as a viable substitute for WPI in stabilizing emulsions, providing similar performance in a range of scenarios. The study supports the use of insect proteins in food preparations that benefit from more sustainably sourced components by offering insightful information about the variables impacting emulsion stability and coalescence.

Chapter 3

Enhancement of emulsifying properties of Lesser mealworm (A. diaperinus) protein studied using microfluidics: Thermal treatment and polyphenol conjugation

3.1. Introduction

3.1.1. Overview of protein modification

Proteins have the capacity to adsorb at the oil-water interface for the formation of stable emulsions since they lower surface tension and provide steric stabilization. Since proteins are amphiphilic, they may interact with both the hydrophilic and hydrophobic phases, which is essential for emulsion formation and stability (McClements, 2015). Stable emulsions are essential in the food, pharmaceutical, and cosmetic sectors to enhance the mouthfeel, texture, and bioavailability of active substances. But occasionally, native proteins' inherent characteristics might make them less useful as emulsifiers. Proteins' emulsifying properties can be influenced by a variety of factors, including their hydrophobicity, charge, solubility, and molecular weight. Different protein modification approaches are used to improve these functional qualities (Padial-Domínguez, Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2020; Padmapriya & Shanthi, 2024).

Enzymatic hydrolysis, chemical treatments, physical procedures, and complexation with polyphenols are among the several techniques used to modify proteins. These methods all alter the structure of proteins in different ways, which enhances their capacity to emulsify and increases the stability of emulsions (Karabulut, Kahraman, Pandalaneni, Kapoor, & Feng, 2023). For example, proteins are broken down into smaller peptides with improved solubility and surface activity by enzymatic hydrolysis (Padial-Domínguez, Espejo-Carpio, Pérez-Gálvez, Guadix, & Guadix, 2020). Proteins' hydrophobicity and charge can be increased by chemical changes such acylation or phosphorylation, which improves the proteins' interfacial characteristics (Hu, Du, Sun, Zhou, & Pan, 2023; Lanyon-Hogg, Faronato, Serwa, & Tate, 2017). According to Su & Cavaco-Paulo, 2021, protein aggregates may be disrupted, and protein structures can be unfolded using physical treatments such as ultrasonication and high-pressure homogenization, hence enhancing their functioning. Furthermore, proteins can be made more stable and antioxidant by complexing with polyphenols (Ozdal, Capanoglu, & Altay, 2013). These changes offer a flexible method for maximizing protein functioning in a range of industrial applications by disintegrating proteins into smaller peptides, changing their chemical structure, or creating complexes with other molecules.

Using microfluidics, this study examines the stability of oil-in-water emulsions and how various protein modification methods can influence them. Lesser mealworm protein is modified using three different techniques: thermal treatment, conjugation with tannic acid, and conjugation with chlorogenic acid. The protein's ability to emulsify is intended to be improved by these changes. The research aims to

determine how each type of modification affects the emulsion stability under controlled settings using microfluidics.

3.1.2. Thermal treatment of proteins

Proteins are thermally treated by raising their temperature to a certain point, which desaturates and unfolds their structures. By breaking down non-covalent connections such hydrophobic interactions, hydrogen bonds, and van der Waals forces, this process exposes hydrophobic areas and increases surface activity. The temperature and length of the heating process determine how much these changes occur (W. Zhang et al., 2024; Y. Zhang et al., 2021). Excessive heating can cause aggregation and loss of functionality. In contrast, heating at moderate temperatures can result in partial denaturation, which can improve the protein's capacity to stabilize emulsions by generating a strong interfacial coating (W. Zhang et al., 2024). Proteins change structurally after heat treatment, which may have an impact on their solubility, emulsifying qualities, and gel-forming capacity. These structural alterations affect the way that proteins operate in food systems by affecting their interactions with other molecules, including lipids and water (K. K. Ma et al., 2022; W. Zhang et al., 2024).

In a research on soy protein, W. Zhang et al., 2024 looked at how preheat treatments at three distinct temperatures (75 °C, 85 °C, and 95 °C) affected the soy protein's ability to gel and emulsify. The findings showed that heating soy protein mixtures to 85 °C greatly improved their surface hydrophobicity and emulsion stability. This study demonstrated how heat treatment may provide soy protein components with improved gelling and emulsifying capabilities. A study on whey proteins looked at how heat treatment and sonication affected the solubility and emulsifying ability of whey proteins. The results showed that the stability and emulsifying qualities of whey protein-stabilized emulsions were greatly enhanced by this combination. The modified whey proteins' improved interfacial film formation and greater surface hydrophobicity were credited with this improved emulsifying ability (Y. Zhang et al., 2021). Research indicates that β-lactoglobulin, a main whey protein, has much improved emulsifying and stabilizing qualities when heated to moderate temperatures. This improvement is ascribed to the heat-treated protein's enhanced structural flexibility and higher surface activity. Moderate heat processing causes β-lactoglobulin to partially unfold, exposing hydrophobic areas that improve interaction with oil droplets and enhance emulsion stability and emulsification capability (Moro, Báez, Ballerini, Busti, & Delorenzi, 2013). Studies have indicated that the solubility and emulsifying qualities of pea proteins are markedly improved by heat treatment. Heating causes structural changes in the protein that boost its emulsifying activity and surface hydrophobicity (Mathew, Kim, Wang, Clayton, & Selomulya, 2023).

Numerous studies repeatedly demonstrate that heat treatment improves the functional characteristics of proteins, especially their ability to emulsify. Protein solubility and surface hydrophobicity are enhanced by moderate heating, and both properties are essential for successful emulsification. Better interfacial film formation is made possible by these structural changes, which produce emulsions that are more stable. Research on soy, whey, pea, and coconut proteins shows that heat treatment enhances their suitability for industrial use, increasing their capacity to stabilize emulsions and enhance their general functioning (J. Ma et al., 2022; Q. Shen et al., 2022).

3.1.3. Conjugation with Tannic acid

Tannic acid conjugation is the covalent bonding of tannic acid molecules to proteins. Protein-polyphenol complexes are created when tannic acid, a polyphenolic molecule, interacts with proteins mostly through hydrogen bonding and hydrophobic interactions. By adding more cross-linking and making the protein structure more rigid, this mechanism improves the structural stability of proteins. Additionally, the conjugation process makes the protein's surface more hydrophobic, which can enhance its stability and emulsification in a variety of applications (C. Chen et al., 2021; Quan, Benjakul, Sae-leaw, Balange, & Maqsood, 2019). Figure 3.1 illustrates the chemical bonding between Tannic acid and protein through covalent bonding. Protein amino and hydroxyl groups interact with the phenolic groups of tannic acid during the conjugation process to produce strong covalent connections. This change may result in modifications to the protein's tertiary and secondary structures, which may impact its antioxidant activity, emulsifying capabilities, and solubility. The type of protein used, the reaction conditions, and the tannic acid concentration all affect how much of a change occurs and what functional qualities arise (Quan, Benjakul, Sae-leaw, Balange, & Maqsood, 2019).

Tannic acid conjugation significantly improves the functional characteristics of proteins, especially their ability to emulsify. Tannic acid conjugation enhances surface hydrophobicity and protein solubility, both of which are necessary for efficient emulsification, according to several studies. For example, why protein-tannic acid conjugate was shown to stabilize the physiochemical properties of lipid in sausages by maintaining texture, colour, and water-holding capacity, as well as by reducing lipid coalescence during refrigerated storage (Aewsiri, Ganesan, & Thongzai, 2023). Tannic acid conjugated soy protein isolate has better emulsifying qualities due to hydrogen bonding and van der Waals interactions, which change secondary structure and decrease surface hydrophobicity. This improves emulsifying ability and lowers interfacial tension, forming stable emulsions that prevent lipid digestion and oil oxidation (Li et al., 2023). Zein-tannic acid hybrid particles are thought to have better structural features and interfacial qualities because they

provide a stable contact between the oil and water phases, improving emulsion stability (Fan et al., 2024). High internal phase Pickering emulsions were favored from covalent crosslinking creating network structures which stabilized emulsions through improved centrifugal and thermal stability when they were stabilized with whey protein-Tannic acid conjugates (Xiong, Li, & Yang, 2023). In addition, tannic acid-loaded chitosan-gelatin-based films exhibit improved tensile strength, decreased permeability, enhanced mechanical and barrier qualities, and improved antioxidant and UV-blocking qualities. These films successfully preserve fresh-cut apples by lowering malondialdehyde content, postponing browning, lowering weight loss, and inhibiting lipid oxidase activity (C. Zhang et al., 2021).

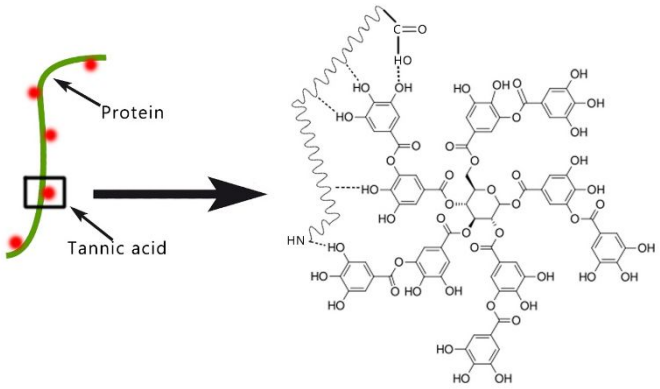


Figure 3.1: Illustration of Tannic acid chemical binding with protein

3.1.4. Conjugation with Chlorogenic acid

The polyphenol chlorogenic acid, which is widely present in fruits, vegetables, and coffee, is widely recognized for its strong anti-inflammatory, anti-carcinogenic, and antioxidant capabilities. Despite these health advantages, the volatility of Chlorogenic acid (CA) under different processing conditions and its relatively poor bioavailability limit its use in food and pharmaceutical products (Nallamuthu, Devi, & Khanum, 2015). One approach that shows promise for increasing Chlorogenic acid's stability and functional characteristics and expanding its possible applications is to conjugate it with proteins (Pan et al., 2019). Usually, covalent bonding is formed during the conjugation of chlorogenic acid with proteins by chemical or enzymatic processes. One well researched technique for conjugating polyphenols to proteins is the Maillard reaction, a non-enzymatic browning reaction between reducing sugars and amino groups of proteins (M. G. Tang et al., 2024). Enzymes like as tyrosinase or laccase are used in enzymatic procedures to help

Chlorogenic acid bind to proteins. By binding Chlorogenic acid to a protein matrix, which can prevent it from degrading and increase its solubility and bioavailability in emulsion systems, both strategies seek to improve the functional characteristics of CGA (Xu et al., 2019). According to (F. Liu, Ma, McClements, & Gao, 2016), a standard conjugation process is bringing chlorogenic acid solutions' pH down to an alkaline state, constantly mixing and stirring with protein at room temperature, and then freeze-drying the mixture to produce the conjugates. By ensuring that Chlorogenic acid binds to proteins efficiently, this technique improves the stability and performance of the resulting conjugates in food emulsions.

The conjugation of proteins with chlorogenic acid has demonstrated a great deal of promise for improving the functional characteristics of food emulsions. Studies on conjugates of whey protein isolate and chlorogenic acid showed better structural and functional characteristics, such as increased antioxidant activity and emulsifying capabilities. In comparison to native proteins, these conjugates demonstrated greater stability in emulsions, lower particle sizes, and superior dispersion, underscoring their promise as cutting-edge emulsifiers (Xu et al., 2019). Chlorogenic acid-loaded chitosan nanoparticles demonstrated improved bioavailability, sustained release characteristics, and antioxidant activity. Because of their ability to stabilize emulsions, these nanoparticles may be used in food systems for extended periods of time without losing their usefulness (Wei & Gao, 2016). The protein structure of walnut protein isolate-chlorogenic acid conjugates were dramatically changed, improving the protein's solubility, emulsification, foaming, and thermal stability. Furthermore, these conjugates improved the stability of emulsions made of walnut oil, indicating their adaptability to a range of culinary uses (M. G. Tang et al., 2024). The creation of ternary complexes consisting of polyphenol, protein, and polysaccharide as emulsifiers greatly enhanced the β-carotene emulsions' formation, stability, and bio-accessibility. These complexes provide bioactive substances a stable habitat, enhancing their potency and possible health advantages (F. Liu, Ma, McClements, & Gao, 2016). Studies conducted on the physicochemical characteristics of β-carotene emulsions stabilized by chitosan-chlorogenic acid complexes revealed a notable enhancement in emulsion stability and antioxidant activity due to these complexes. Reduced droplet size and enhanced resistance to oxidative degradation were two clear indicators of this improvement (Wei & Gao, 2016)

When tocopherol and chlorogenic acid were added to algal oil emulsions, it showed synergistic effects that improved the oxidation stability and bio accessibility of the emulsions. Better preservation and utilization of the bioactive components in algal oil were made possible by this combination (C. Zhang et al., 2024). Lastly, interactions between polypeptide and chlorogenic acid enhanced the coffee drinks made with modified quinoa in terms of both stability and sensory aspects. According to (Ji, Wang, Zhao, & Jiang, 2023), the interactions improved the drinks'

physicochemical characteristics, making them more palatable and stable for consumption. All of these research show how different chlorogenic acid-protein conjugates may be in improving food emulsions' functional qualities and are thus useful for creating functional meals with extra health advantages.

3.2. Materials and methods

3.2.1. Protein extraction

Protein was extracted from a 48% protein mealworm powder that was provided by Kreca (Kreca Ento-Food BV, Wageningen, the Netherlands). The powder was combined with 1:5 (w/w) 2-methyltetrahydrofuran (Scharlab S.L., Spain) and agitated for 1 hour at 600 rpm in a fume hood. The solution was stirred, then allowed to settle before the solvent was decanted. To guarantee that all of the fat was removed from the insect powder, this defatting procedure was repeated. Under the hood, the residual solvent containing insect powder was allowed to evaporate.

Subsequently, 0.25 M NaOH (Chem-Lab NV, Zedelgem, Belgium) was combined with the defatted powder and the mixture was agitated for an hour at 40°C. After centrifuging the mixture for ten minutes at 4500 rpm, the supernatant was carefully withdrawn. After centrifuging the supernatant for 15 minutes at 3800 rpm, 35% HCl (J.T. Baker, Griesheim, Germany) was added to acidify it until the pH reached 4.2. To optimize protein production, this NaOH extraction procedure was done twice, followed by HCl pH correction. With a protein concentration of 71% (wb), the final precipitate was freeze-dried to produce less mealworm protein concentrate (LMPC). The extraction of protein protocol was referred was referred from (Wang, Ballon, et al., 2021)

3.2.2. Protein conjugation with polyphenols

The LMPC-polyphenol conjugates were produced using a slightly modified version of Liu, Ma, McClements, & Gao, 2016 methodology. LMPC was first distributed in distilled water prior to being mixed for two hours at 400 rpm, (3.2 wt%) (RCT ST, IKA, Staufen, Germany). Using 4 M NaOH (Accumet® AE150, Fisher Scientific, Walthom, MA, USA), the mixture's pH was adjusted to 9.0 every 30 minutes. The mixture was then stored for the whole night at 4 °C to guarantee that the proteins were completely hydrated. Following this, the protein mixture was subjected to two centrifugations (Meditronic 7000599, J.P. SELECTA, Barcelona, Spain) at 2863 × g for 15 minutes at 25 °C, and the amount of protein in the resulting supernatant was measured by BCA analysis (Smith et al., 1985). The amount of protein was indicated as the equivalent value of bovine serum albumin (BSA). After dissolving TA and CA in distilled water, the pH of the mixture was brought to 9.0. Next, TA or CA solution was added to the protein solution and stirred to achieve 50,

100, and 150 µmol of polyphenol/g of protein as the polyphenol concentration. The concentration range of 50–150 µmol/g protein was selected based on unpublished research that showed greater TA concentrations to cause the development of a visible precipitate. To get a final protein content of 1%, the final weight was adjusted with distilled water, and if needed, the pH of the solution was raised to 9.0. 0.02% sodium azide was added to stop the development of microorganisms. The solutions were mixed in the presence of oxygen for twenty-four hours at room temperature. By using dialysis (MWCO 3500 Da) against distilled water for 44–48 hours at 4 °C, unreacted polyphenols were removed. The conjugates were freeze-dried (LYOQUEST-85 PLUS, Telstar, Barcelona, Spain) and the samples were kept in a desiccator at 4 °C until they were needed.

3.2.3. Thermal treatment of protein

The thermal treatment was carried out in accordance with Zhu, Zhang, Lin, & Tang, 2017, with minor adjustments. Initially, protein concentrates were diluted for two hours in distilled water (RCT ST, IKA, Staufen, Germany), and then the pH of the resulting mixtures was brought down to 7.0 using an Accumet® AE150 from Fisher Scientific, Walthom, Massachusetts, USA. Overnight, the dispersions were maintained at 4 °C to enable the proteins to fully hydrate. The insoluble proteins were next extracted from the dispersions by centrifuging them for 15 minutes at 2863 x g using a Meditronic 7000599, J.P. SELECTA, Barcelona, Spain. The BCA test was used to measure the supernatant's protein concentration, which was then diluted with distilled water to reach 3.5%. Subsequently, 300 mM of sodium chloride was added to the protein solution while stirring. Additionally, 0.02% sodium azide was added to stop the development of microbes. Two milliliter aliquots of the protein solution were pipetted into glass tubes and heated for thirty minutes at 90 degrees Celsius with low agitation (20 rpm) in a Thermo Fisher Scientific Digital Shaking Dry Bath (Waltham, MA, USA). The tubes were then allowed to cool for at least thirty minutes in an ice-water bath. After preparation, the thermally treated LMPC was utilized.

3.2.3. Sample preparation for microfluidic experiments

Phosphate buffer containing sodium phosphate monobasic monohydrate (ACROS, Spain) and di-sodium hydrogen phosphate dihydrate (Scharlau, Spain) was used to prepare protein solutions, with the pH levels adjusted to 3 and 7. In case of preparing the solution with polyphenol complexes, the protein solutions were made by directly weighing the necessary amount of conjugates to achieve the desired concentration. These solutions were made at w/v concentrations of 0.02%, 0.01%, 0.005%, 0.001%, and 0.0005%. The concentration was measured with a BCA (bicinchoninic acid) assay kit (Pierce Biotechnology, Thermo Scientific, Rockford, IL, USA) after a stock protein solution was produced. The necessary concentrations

were then achieved by diluting this stock solution. In case of thermally treated proteins, the prepared solution was diluted to concentrations of 0.02%, 0.01%, 0.005%, 0.001%, and 0.0005%.

3.2.4. Microfluidic setup

The microfluidic studies were conducted using custom designed borosilicate glass microchips (Micronit Microfluidics BV, The Netherlands). The microchip consists of an adsorption, a coalescence channels, each with a rectangular cross-section and a T-junction (Fig. 2.1, chapter 2). The dispersed phase was broken up into tiny droplets at the T-junction, and these droplets passed along the adsorption channel without interacting with other droplets. The protein from the continuous phase adsorbed onto the oil-water interface during the flow through the adsorption channel. The droplets were further released into coalescence channel where they come in contact with other droplets and at times perhaps merge to coalesce with each other.

The effect of protein adsorption time on droplet coalescence was evaluated by varying the length of the adsorption channel between 14 and 20.5 mm. The channels on the microchip maintained a constant 45 µm height. The coalescence channel measured 32.1 mm in length and 500 µm in width, whereas the adsorption channel was 100 µm in width. The microchips were mounted on a microscope stage (Darwin Microfluidics, Paris, France) and connected to an Elveflow OB1 MK3+ pressure controller (ElveFlow, Paris, France), which controlled the liquid flow rates, to create microfluidic emulsions. Compressed air was used to pump the continuous and dispersed phases via a reservoir, and a flow sensor supplied feedback signals to keep the flow rate steady. In Fig. 2.2, the experimental setup is shown.

3.2.5. Emulsion production and image acquisition

The oil and protein solutions were pumped at different total flowrates to generate emulsions with SFO (45 µl/minute to 105 µl/min) and hexadecane (35 µl/minute to 95 µl/min). The flowrate of the dispersed phase was kept constant at 5 µl/min, and the oil fraction was adjusted by varying the flowrate of the continuous phase. Droplet breakup at the T-junction was caused by the higher flowrate of the continuous phase, and the emulsion droplets then passed through the adsorption channel, where emulsifier adsorption at the surface of oil droplets may occur. Due to SFO's higher viscosity, droplet breakup at the T-junction did not occur at low flowrates, limiting the minimum flowrate to 45 µl/min. The emulsion then entered the coalescence channel where droplets were free to collide with other droplets. A high-speed camera (SpeedCam MacroVis EoSens, Germany) was used to capture 500 images near the inlet and outlet of the coalescence channel, and the images were processed using MATLAB. The experiments were conducted in duplicates, and the

frame rate of the camera was varied according to the flowrate. Because of this, the number of images used to analyze droplet coalescence was varied accordingly to keep a constant experimental acquisition time of 10 seconds. Table 3.1 presents all the experimental conditions used.

3.2.6. Image analysis and calculation

The obtained images were analyzed with an in-house MATLAB script to determine the number and area of droplets. The frequency distribution of droplet area was determined using excel spreadsheet to distinguish between uncoalesced and coalesced droplets. The formation of dimers (two droplets merged to form a single droplet) and trimers (three droplets merged to form a single droplet) was identified by observing the doubling and tripling of the droplet area, respectively. Since microfluidics helps in producing monodispersed droplets, the dimers, trimers, and tetramers could be seen to exactly have double, tripled, or quadrupled in area than that of uncoalesced droplets. Figure 2.3 (chapter 2) shows the process flowchart of image analysis. The fraction of droplets undergoing coalescence (Dc) was calculated by using equation 2.1.

$$Dc = \frac{[(N2e * 2) + (N3e * 3) + \ldots.. + (Nne * n)]}{[N1e + (N2e * 2) + (N3e * 3) + \ldots.. + (Nne * n)]} - \frac{[(N2s * 2) + (N3s * 3) + \ldots.. + (Nns * n)]}{[N1s + (N2s * 2) + (N3s * 3) + \ldots.. + (Nns * n)]} \quad \text{(Eq 2.1)}$$

Where, N_{1e} and N_{1s} are the numbers of uncoalesced drops at the end and start of the coalescence channel respectively, N_{2e} and N_{2s} are the number of dimers at the end and start of the coalescence channel respectively. The frequency of coalescence was calculated using Equation 2.

$$Fc = \frac{Dc}{Rt} \quad \text{(Eq 2.2)}$$

Where, Rt is the residence time of droplets in the coalescence channel. Residence time was calculated using the known volumetric flowrates. The residence time in the adsorption channel was computed considering the total length of the adsorption channel (14 or 20.5 mm) and the residence time in the coalescence channel was calculated between the start and end of the coalescence channel (2.5 cm in length), corresponding to the regions where images were taken.

3.2.7. Measurement of interfacial tension

Using the hanging ring method, the interfacial tension was measured with a Sigma T702-D equipment (Biolin Scientific, Sweden). Onstage, a beaker with the

protein solution was set up. A platinum ring was suspended on a hook and gradually submerged into the liquid in the beaker after being cleaned with ethanol, distilled water, and a flame to remove contaminants. Using a Pasteur pipette, oil was gradually added to the protein solution, and the system was given one minute to come to equilibrium. By accounting for the density difference between the two liquids, the energy needed for the ring to break the interfacial strength was measured in order to estimate the interfacial tension.

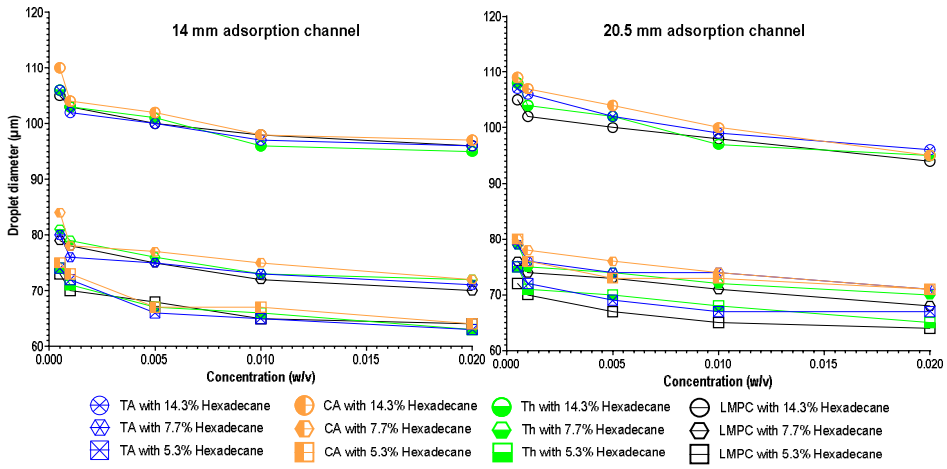
3.2.8. Statistical analysis

GraphPad was used to do a one-way analysis of variance (ANOVA) on the data. ANOVA was used to identify significant differences. The mean ± standard deviation is used to show the data.

3.3. Results and discussion

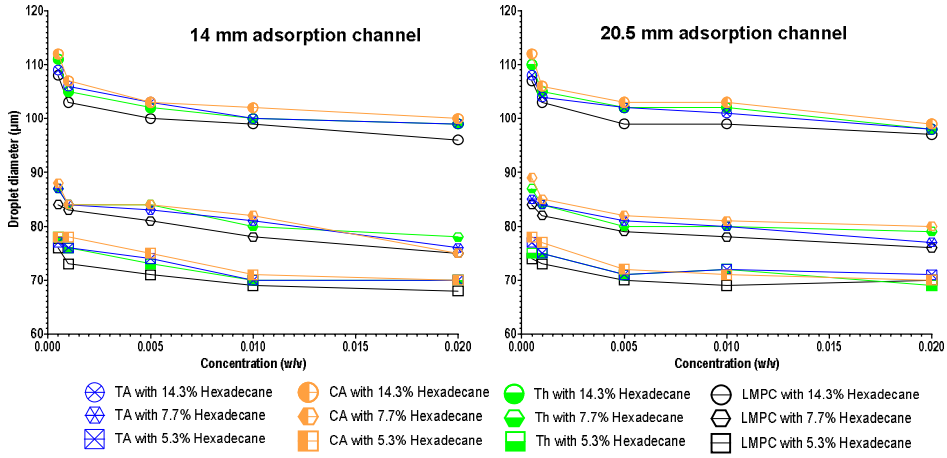
3.3.1 The diameter of individual droplets and interfacial tension

Under different concentrations, pH levels, and adsorption channel lengths, the droplet diameters of hexadecane emulsions stabilized with lesser mealworm protein concentrate (LMPC) and its modified forms, such as conjugation with chlorogenic acid (CA), tannic acid (TA), and thermal treatment (Th), were examined (figure 3.2 and figure 3.3). The efficiency of LMPC and its derivatives in stabilizing oil-in-water emulsions was assessed based on the results.



For all samples at pH 7, the average droplet diameter fell as protein content rose in the 14 mm and 20.5 mm adsorption channels. Effective emulsification by LMPC and its derivatives was demonstrated by the droplet sizes in the 14 mm channel, which varied from roughly 75 µm to 65 µm at the maximum concentration

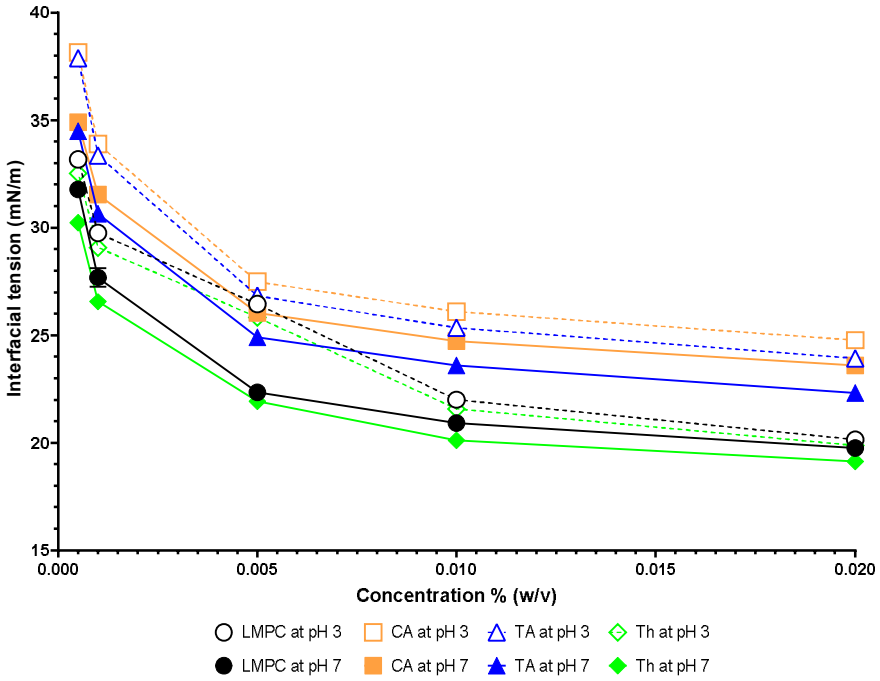
(0.02% w/v). When LMPC conjugated with chlorogenic acid (CA) was compared to unmodified LMPC and the other treatments, it consistently formed bigger droplets, suggesting a slightly lower emulsifying effectiveness. The 20.5 mm adsorption channel likewise showed a tendency of decreasing droplet size with increasing protein content, with smaller droplets overall than in the 14 mm channel. This finding emphasizes the significance of enough protein adsorption time for efficient droplet stabilization.



At pH 3, the droplet diameters were generally larger than those at pH 7, likely due to protein conformation changes and increased interfacial tension at lower pH. However, the overall trend of decreasing droplet size with increasing protein concentration persisted. CA and TA treatments resulted in slightly larger droplets compared to LMPC and Th treatments, suggesting that the modifications may affect the proteins' interaction with the oil-water interface under acidic conditions. The longer adsorption channel (20.5 mm) similarly resulted in smaller droplet sizes at higher protein concentrations, emphasizing the benefit of extended adsorption time even under acidic conditions.

Interfacial tension measurements between hexadecane and the protein solutions revealed that LMPC and its derivatives effectively reduced the interfacial tension, with LMPC and thermally treated LMPC (Th) showing the lowest interfacial tension values across all concentrations. At pH 7, the interfacial tension decreased with increasing protein concentration for all samples, confirming the proteins' ability to adsorb at the oil-water interface and reduce surface tension. At pH 3, the interfacial tension values were generally higher compared to pH 7, reflecting the impact of pH on protein conformation and surface activity. Despite the higher interfacial tension at pH 3, LMPC and Th still showed the lowest values, while CA and TA treatments had

higher interfacial tensions, consistent with the larger droplet sizes observed in the emulsions.

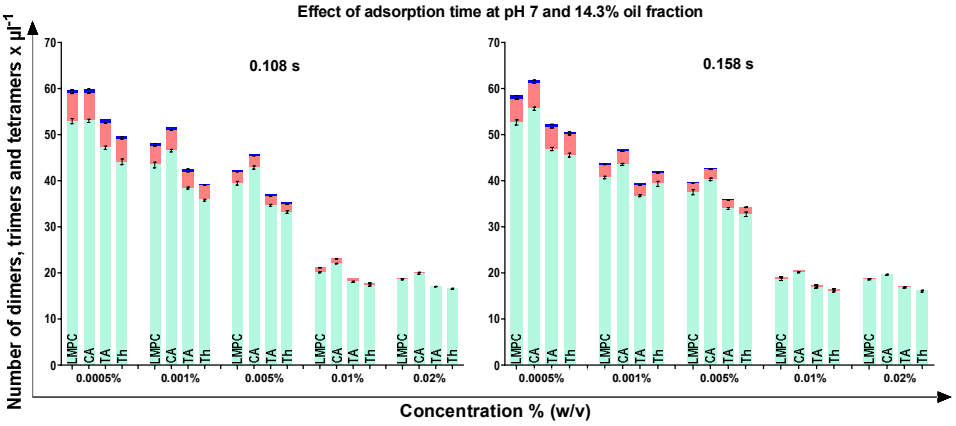


These findings show that hexadecane emulsions may be stabilized by LMPC and its modified forms, with performance dependent on protein content, pH, and adsorption channel length. Larger, more stable droplets were often produced by longer adsorption durations and higher protein concentrations. The droplet size results were corroborated by the interfacial tension measurements, which demonstrated that LMPC and its derivatives lower surface tension—a critical factor in emulsion stability. Because of structural changes and changed adsorption kinetics, acidic environments may reduce the proteins' ability to emulsify effectively. This is supported by the increased interfacial tension and bigger droplet sizes seen at pH 3. These findings show that hexadecane emulsions may be stabilized by LMPC and its modified forms, with performance dependent on protein content, pH, and adsorption channel length. Larger, more stable droplets were often produced by longer adsorption durations and higher protein concentrations. The droplet size results were corroborated by the interfacial tension measurements, which demonstrated that LMPC and its derivatives lower surface tension—a critical factor in emulsion stability. Because of structural changes and changed adsorption kinetics, acidic environments may reduce the proteins' ability to emulsify effectively. This is supported by the increased interfacial tension and bigger droplet sizes seen at pH 3.

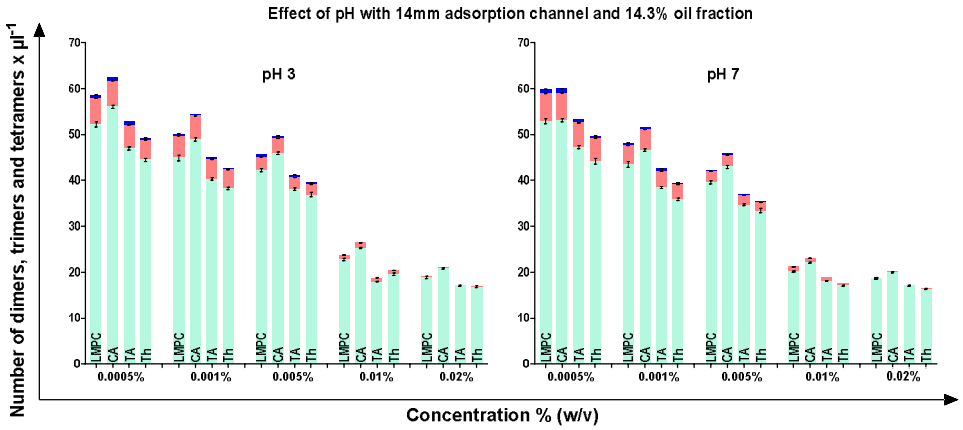
3.3.2 The number of droplets undergoing coalescence at the start of the coalescence channel is affected by pH, adsorption time, and oil fraction

By counting the number of dimers, trimers, and tetramers that formed under various circumstances, the coalescence behavior of droplets was examined. To assess the stability of emulsions stabilized by LMPC and its modified forms, data was obtained at different protein concentrations, adsorption periods, pH levels, and oil fractions. The first graph illustrates how the impact of adsorption time on droplet coalescence was studied at pH 7 with a 14.3% oil component. Findings indicated that for both 0.108 s and 0.158 s adsorption durations, there was a reduction in the number of coalescing droplets (dimers, trimers, and tetramers) as protein concentration increased. Following LMPC, TA, and Th, CA showed the greatest number of coalescing droplets at low protein concentrations during the shorter adsorption duration (0.108 s). This sequence implies that LMPC's initial adsorption effectiveness was hampered by the presence of chlorogenic acid, which increased the likelihood of droplet coalescence. Weaker protein-polysaccharide interactions lead to less efficient interfacial film production, which explains why there were more coalesced droplets for LMPC treated with CA (Sarkar et al., 2016).

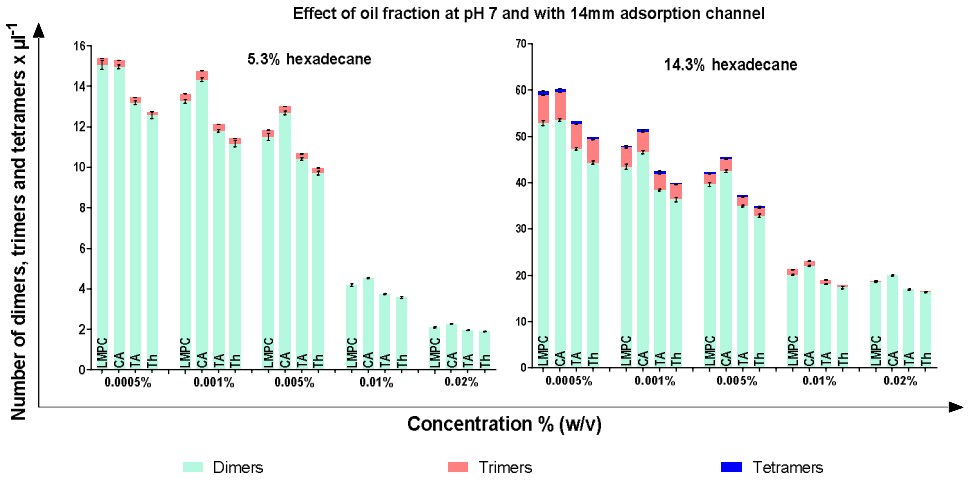
The number of coalescing droplets dramatically decreased in all samples as the protein concentration rose to 0.02% w/v, suggesting that the proteins were more stable and effectively covered the oil-water interface. The pattern persisted for the extended adsorption duration (0.158 s), with CA continuing to exhibit the strongest coalescence, followed by LMPC, TA, and Th. The total number of coalesced droplets was, nonetheless, less than that of the 0.108 s adsorption period. This decrease emphasises the need of having enough adsorption time, since this reduces coalescence occurrences by enabling the proteins to create a more cohesive and stable interfacial layer (Dickinson, 2011).



The second graph shows how the impact of pH on droplet coalescence was investigated using a 14 mm adsorption channel and a 14.3% oil percentage. In comparison to pH 7, there were more coalescing droplets at pH 3 for all protein concentrations. This finding is consistent with a greater interfacial tension at pH 3, which is probably caused by structural changes in proteins that lower surface activity and adsorption effectiveness. Reduced charge repulsion between droplets is the cause of the rise in coalescence at lower pH levels, which results in more frequent coalescence occurrences (Tang, 2021). Following LMPC, TA, and Th, CA consistently displayed the greatest number of coalesced droplets among the modified LMPC forms. The better performance of thermally treated LMPC (Th) implies that the protein's capacity to adsorb at the oil-water interface is improved by thermal denaturation, resulting in the formation of a stronger and more cohesive interfacial coating. By exposing hydrophobic regions, the denaturation process decreases coalescence by enhancing the protein's surface activity and adsorption kinetics (Dickinson, 2010). Because of the larger and stronger protein-tannin interactions that improve the interfacial layer's structural integrity and stability, TA-treated LMPC outperformed CA-treated LMPC in terms of performance (Gu et al., 2020).



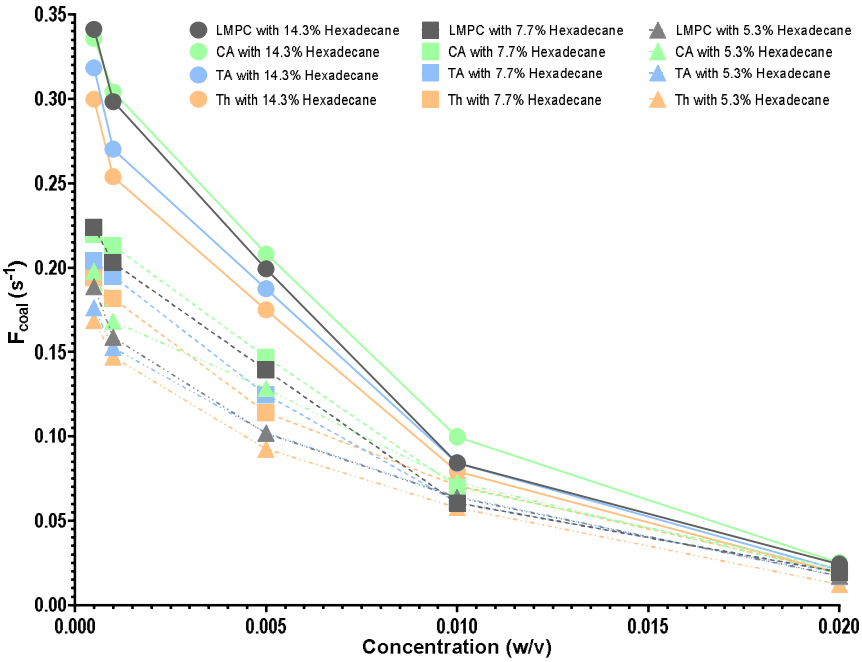
The third graph shows the effect of oil fraction on droplet coalescence measured at pH 7 using a 14 mm adsorption channel. There were two oil fractions tested: 5.3% and 14.3%. At low protein concentrations, higher oil fractions produced noticeably more solidified droplets; as protein concentration rose, the number of droplets decreased. At the greater oil content (14.3%), this impact was more noticeable, and more coalescence events were probably caused by the more droplets and collisions. The greater probability of droplet collisions resulting from the higher droplet density in the emulsion can account for the stronger coalescence seen at higher oil fractions (McClements, 2005). The frequency of coalescing droplets reduced as the concentration of proteins rose, suggesting enhanced stability and higher protein covering of the droplet surfaces. The most consolidated droplets were seen in LMPC treated with CA, then LMPC, TA, and Th. The creation of stable protein-tannin complexes, which increase the structural integrity and stability of the interfacial layer, is responsible for the increased stability brought about by tannic acid conjugation (Sarkar et al., 2015). The advantages of thermal denaturation are demonstrated by the better performance of thermally treated LMPC (Th) in lowering droplet coalescence. This process increases the surface activity and adsorption rates of the protein, improving its emulsifying qualities (Dickinson, 2011). Stronger connections between tannic acid and proteins may account for the superior performance of tannic acid (TA) conjugated LMPC over chlorogenic acid (CA) conjugated LMPC. Reduced coalescence results from tannic acid's capacity to create stable protein-tannin complexes that enhance the interfacial layer's structural integrity and stability (Gu et al., 2020).



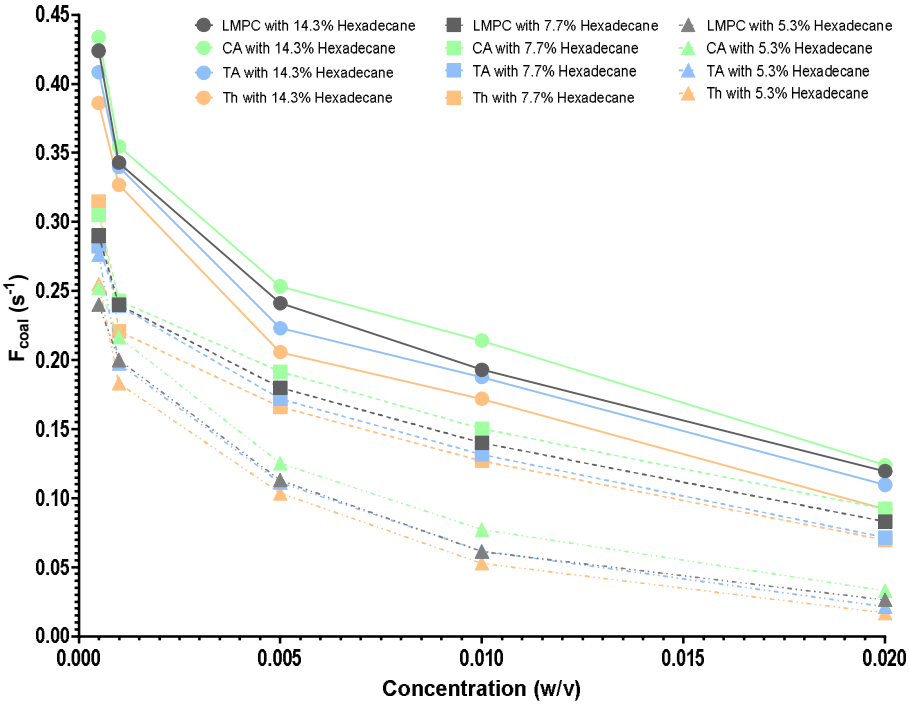
3.3.3. Frequency of Coalescence

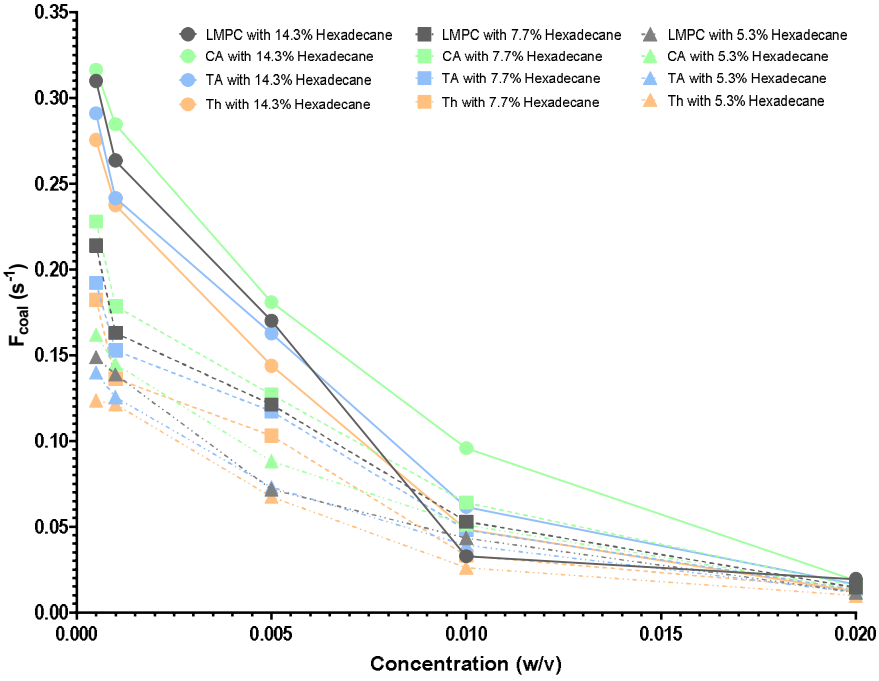
When assessing the stability of emulsions, one of the most important parameters is the droplet coalescence frequency (F coal). This section looks at the frequency of coalescence under different adsorption channel lengths, pH values, and protein concentrations to see how well LMPC and its modified forms stabilise emulsions.

With a 14 mm adsorption channel, the coalescence frequency at pH 7 is displayed in the first graph. For all stabilizers, the coalescence frequency falls with increasing protein content. The highest initial coalescence frequency is seen by LMPC, with 14.3% hexadecane, followed by TA, Th, and CA. This suggests that by lowering coalescence events, larger protein concentrations improve the stability of emulsions. The quick drop in coalescence frequency that occurs as protein concentration rises indicates that the proteins are effectively adsorbing and covering the oil-water interface. Emulsions with lower oil fractions (7.7% and 5.3% hexadecane) show the similar tendency, although their coalescence frequencies are typically lower. This emphasises the importance of oil fraction in emulsion stability.

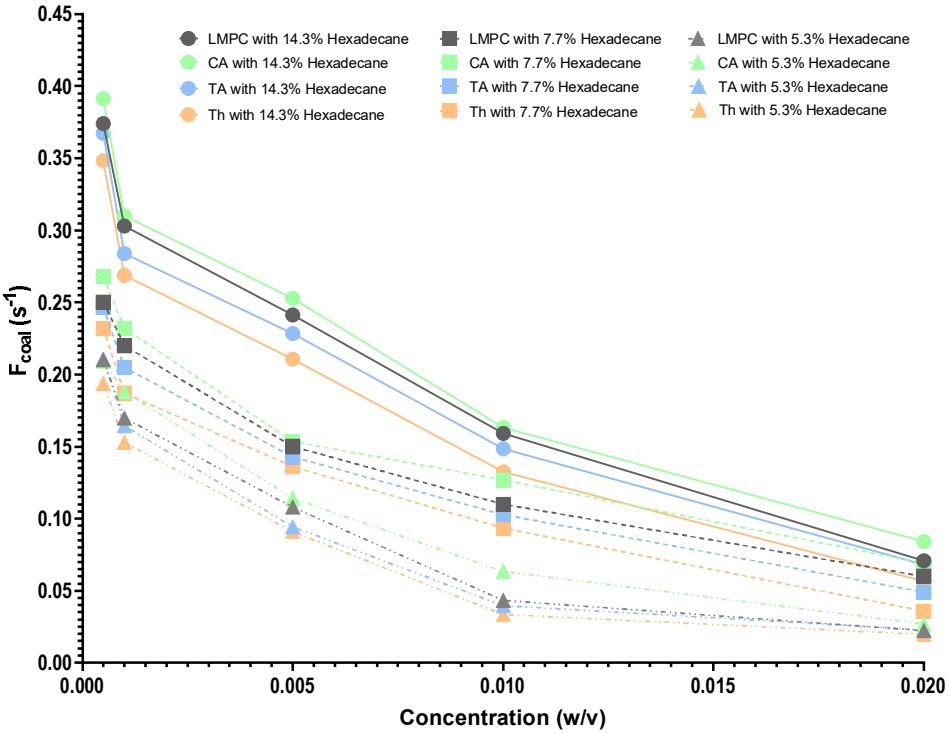


The coalescence frequency at pH 3 using a 14 mm adsorption channel is depicted in the second graph. Raising the protein content lowers the frequency of coalescence, just like the pH 7 condition does. But generally, at pH 3 compared to pH 7, the coalescence frequencies are larger. This might jeopardise the stability of the interfacial coating because of decreased charge repulsion and increased protein aggregation at lower pH values (Dickinson, 2010). Once more, among the stabilisers, Th, LMPC, TA, and CA show the greatest coalescence frequency, showing the same order of efficacy in lowering coalescence. The coalescence frequency at pH 7 with a 20.5 mm adsorption channel is shown in the third graph. In general, the coalescence frequency reduces with increasing adsorption channel length for all protein concentrations and stabilisers. The prolonged residence period, which for more complete protein adsorption and interface stabilisation, is thought to be the cause of this decrease. Th has the lowest coalescence frequency and CA the highest, indicating a steady trend in stabiliser efficacy. This provides additional evidence in favour of the theory that heat treatment improves the emulsifying qualities of LMPC by encouraging protein denaturation and hydrophobic group exposure, which results in a stronger interfacial layer (Tang, 2021).


LMPC with 14.3% Hexadecane
CA with 14.3% Hexadecane
TA with 14.3% Hexadecane
Th with 14.3% Hexadecane
LMPC with 7.7% Hexadecane
CA with 7.7% Hexadecane
TA with 7.7% Hexadecane
Th with 7.7% Hexadecane
LMPC with 5.3% Hexadecane
CA with 5.3% Hexadecane
TA with 5.3% Hexadecane
Th with 5.3% Hexadecane
F_coal (s^-1)
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.000
0.005
0.010
0.015
0.020
Concentration (w/v)


LMPC with 14.3% Hexadecane
CA with 14.3% Hexadecane
TA with 14.3% Hexadecane
Th with 14.3% Hexadecane
LMPC with 7.7% Hexadecane
CA with 7.7% Hexadecane
TA with 7.7% Hexadecane
Th with 7.7% Hexadecane
LMPC with 5.3% Hexadecane
CA with 5.3% Hexadecane
TA with 5.3% Hexadecane
Th with 5.3% Hexadecane
F_coal (s^-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.000
0.005
0.010
0.015
0.020
Concentration (w/v)

With a 20.5 mm adsorption channel, the coalescence frequency at pH 3 is displayed in the fourth graph. The patterns that are shown are comparable to those at pH 7, with a general decline in coalescence frequency as the adsorption channel lengthens and protein concentration rises. The difficulties that acidic circumstances have for protein stability and emulsification efficiency are supported by the greater coalescence frequencies at pH 3 as opposed to pH 7 (Gu et al., 2020). The longer adsorption channel, in spite of this, nonetheless enhances emulsion stability, underscoring the need of enough adsorption time in the formation of stable emulsions.



The observed results can be explained by the interfacial and molecular properties of the proteins and their modifications. Proteins adsorb at the oil-water interface, reducing interfacial tension and forming a protective film that prevents droplet coalescence (McClements, 2005). The effectiveness of this process is influenced by protein concentration, adsorption time, pH, and the presence of modifying agents like tannic acid or chlorogenic acid. Thermally treated LMPC (Th) consistently showed the lowest coalescence frequency, suggesting enhanced interfacial activity due to thermal denaturation. Thermal treatment exposes hydrophobic groups, promoting stronger adsorption and a more cohesive interfacial film (Dickinson, 2011). This mechanism is supported by studies showing that thermal

denaturation enhances the emulsifying properties of proteins by increasing their surface activity and adsorption rates (Tang, 2021).

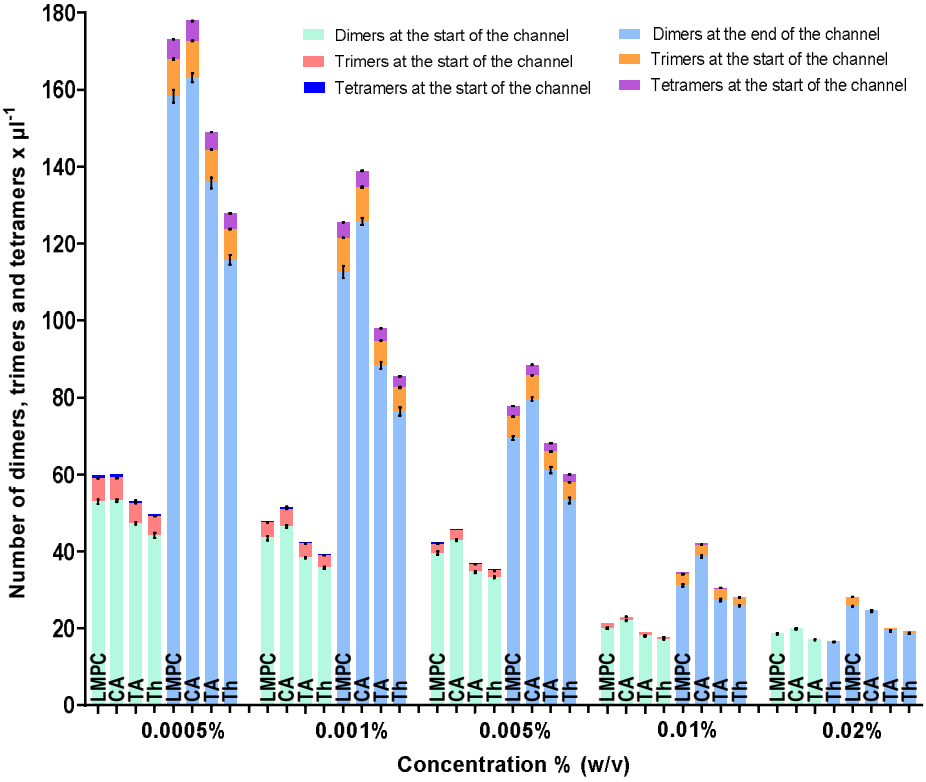
Tannic acid (TA) conjugated LMPC performs better than chlorogenic acid (CA) conjugated LMPC because of the larger and more robust interactions tannic acid has with proteins. According to Gu et al. (2020), tannic acid improves the structural integrity and stability of the interfacial layer by forming stable protein-tannin complexes. Compared to the weaker connections that are encouraged by chlorogenic acid, these complexes offer a more robust barrier against droplet coalescence (Sarkar et al., 2016). Lower pH levels are associated with greater coalescence rates, which align with the effects of acidic environments on protein structure and interfacial activity. Proteins can clump together and create less stable interfacial coatings at lower pH levels, which increases coalescence (Tang, 2021). The lower electrostatic repulsion between droplets, which promotes coalescence, intensifies this impact (Dickinson, 2010).

3.3.4. Effect of Protein Concentration, Adsorption Time, and Oil Fraction on Droplet Coalescence

The study focused on the generation of dimers, trimers, and tetramers along the microchannel and the coalescence rate ($Fcoal$) in order to get an understanding of the dynamics and stability of the emulsions stabilised by LMPC and its modified forms. The given graphs, which contrast the quantity of coalesced droplets at the beginning and end of the microchannel under various pH and protein concentration settings, serve as the basis for this investigation. The first graph shows that when protein concentration rises from 0.0005% to 0.02% w/v, the number of dimers, trimers, and tetramers dramatically reduces. This behaviour is seen at pH 7 with a 14 mm adsorption channel and 14.3% hexadecane. Emulsions stabilised with CA have the greatest number of coalesced droplets at the lowest concentration, followed by LMPC, TA, and Th, suggesting that CA had greater initial coalescence rates. This implies that droplet coalescence occurs more frequently at lower concentrations of CA-modified LMPC because it is less effective. Th-treated LMPC, on the other hand, has the fewest coalesced droplets, suggesting better stability. All stabilisers show decreased coalescence with increasing protein content, highlighting the significance of adequate protein coverage at the interface (Schmitt and Turgeon, 2011).

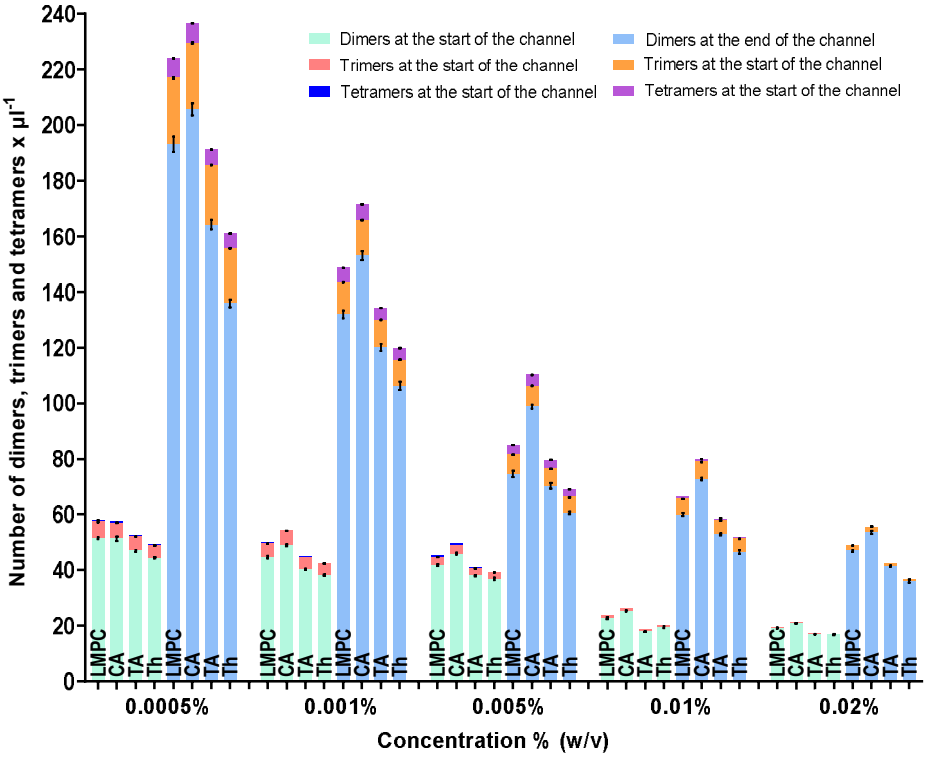
We find greater overall coalescence rates when we compare this to the second graph, which was created under the identical circumstances at pH 3. This is explained by the fact that droplets exhibit less electrostatic repulsion at lower pH values, which promotes closer proximity and coalescence (Alargova et al., 2004). The same stabiliser effectiveness hierarchy is still in place, with CA exhibiting the greatest coalescence with LMPC, TA, and Th following. Given that protein aggregation and

conformational changes can degrade the interfacial layer, this tendency emphasises the difficulties that acidic environments present for protein-stabilized emulsions (Tolstoguzov, 2003).



The two plots show that there is continuous coalescence during flow since there are substantially more coalesced droplets at the end of the channel than there were at the beginning. This rise is more evident at lower protein concentrations, suggesting larger rates of coalescence. The discrepancy between the start and end values decreases with increasing protein content, suggesting enhanced stability as a result of more efficient interfacial film production (Damodaran, 2005). This pattern of increased stability as protein concentration rises is consistent with other findings, underscoring the importance of having enough protein coverage. The results indicate that the protein's interfacial activity is enhanced by thermal denaturation, since thermally treated LMPC (Th) consistently exhibits the lowest rates of coalescence under all circumstances. According to Diftis and Kiosseoglou (2003), heat treatment unfolds proteins, revealing hydrophobic residues that enhance adsorption and film formation at the oil-water interface. As a result of the increased adsorption, interfacial coatings become stronger and the chance of coalescence is decreased (Gunning et al., 1999). The results reported in previous sections are consistent with the higher

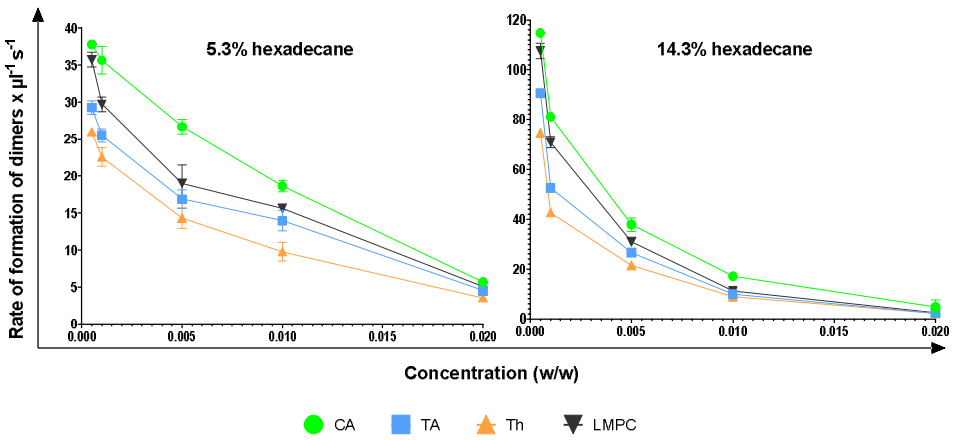
performance of Th-modified LMPC in minimising coalescence, highlighting the efficacy of thermal treatment in improving emulsion stability.



In comparison to conjugating chlorogenic acid (CA), tannic acid (TA) conjugation also improves emulsion stability. According to Joshi et al. (2012), tannic acid strengthens the interfacial layer and increases resistance to coalescence by forming more stable protein-tannin complexes. By strengthening the barrier at the interface, these complexes decrease droplet coalescence (Dickinson, 2008). The better interfacial characteristics mentioned in previous sections are consistent with the increased stability with TA over CA seen here. The greater rates of coalescence at pH 3 are consistent with the detrimental effects of acidic environments on the stability of proteins. Lower pH might jeopardise the integrity of the interfacial coating since it decreases electrostatic repulsion and increases the propensity for protein aggregation (Harnsilawat et al., 2006). The necessity for stabilisers that remain effective in acidic conditions is demonstrated by the higher number of coalescing droplets in the second graph as compared to the first (Murray et al., 2011). These outcomes support the conclusions from previous sections, which showed that emulsion stability was constantly hampered by acidic environments.

3.3.5. Rate of Formation of Dimers and Trimers

This section compares two distinct oil fractions, 5.3% and 14.3% hexadecane, to examine the rate of dimer formation in emulsions stabilised by LMPC and its modified forms. The above graph displays the rate of dimer formation for unmodified LMPC, CA, TA, and Th at different protein concentrations. Important new information on the stability and emulsifying effectiveness of these stabilisers is revealed by the analysis.



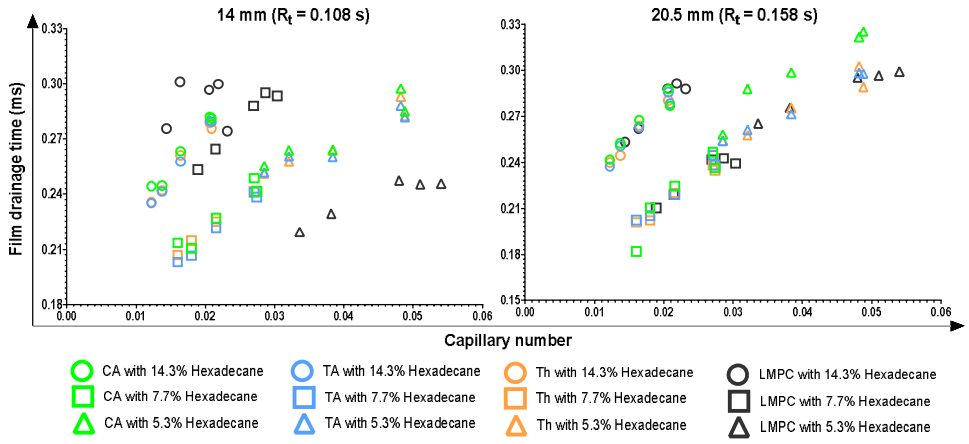
For both oil fractions, the rate of dimer formation falls with increasing protein content. CA has the greatest rate of dimer formation at the lowest concentration (0.0005% w/v), followed by LMPC, TA, and Th. This suggests that CA-modified LMPC is less successful in stopping droplet coalescence at low concentrations, which leads to increased dimer formation. Th-treated LMPC, on the other hand, has the lowest rate of dimer formation, demonstrating better emulsion stability. The observed trend is constant for both oil fractions, indicating that the LMPC changes have a consistent effect on emulsifying efficiency. The dimer formation rate for all stabilisers in the 5.3% hexadecane fraction is high at first but drastically drops as protein concentration rises. All stabilisers exhibit nearly nil dimer formation rates at higher doses (0.02% w/v), suggesting low coalescence and efficient stabilisation. Stronger and more cohesive interfacial films result from the proteins' enhanced covering of the oil-water interface, which is why dimer formation decreases as protein concentration rises (Dickinson, 2011). Th-treated LMPC exhibited consistently decreased dimer formation rates, indicating that thermal denaturation provides greater stability and improves adsorption and film formation (Gunning et al., 1999). The 14.3% hexadecane emulsions show greater initial dimer formation rates than the 5.3% fraction when comparing the two oil fractions. The larger droplet concentration in the emulsion, which causes more collisions and coalescence events, can be used to

explain this higher rate (McClements, 2005). Both fractions exhibit the same pattern, though, which is a decrease in dimer formation as protein concentration rises. The dimer formation rate is about the same for both fractions at the maximum concentration (0.02% w/v), suggesting that an adequate protein content may stabilise emulsions regardless of the oil component.

The findings in this part are consistent with those in other sections, where emulsion stability was consistently increased by tannic acid conjugation and heat treatment. The efficacy of these changes in promoting protein adsorption and interfacial film formation is demonstrated by the decreased dimer formation rates for LMPC that has been treated with Th and LMPC that has been changed with TA. These results are in line with studies published in the literature that highlight how protein changes might enhance emulsifying qualities (Damodaran, 2005; Tcholakova et al., 2008).

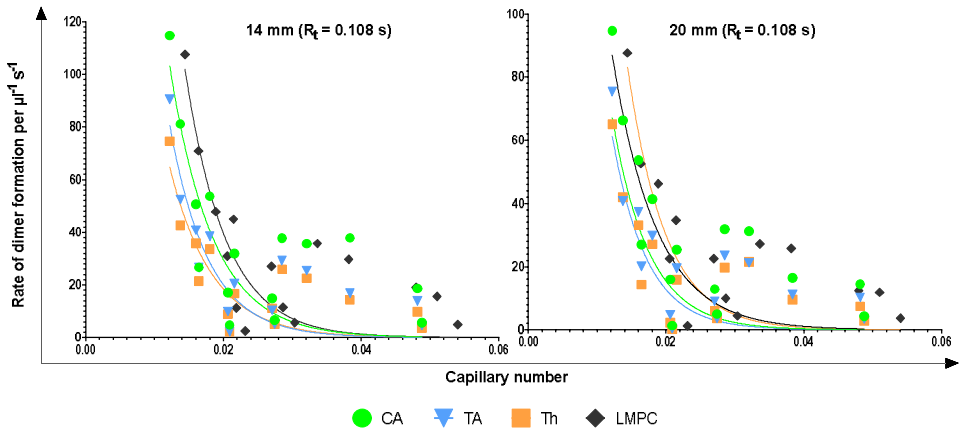
3.3.6. Capillary Number and Rate of Dimer Formation

This section investigates, for emulsions stabilised with LMPC and its modified forms, the link between the capillary number and the rate of dimer formation as well as the film drainage time under various circumstances. Essential information on droplet coalescence behaviour in microfluidic flows may be obtained from the capillary number (Ca), which is the ratio of surface tension forces to viscous forces ($Ca = \mu U/\sigma$).



One important factor that determines the stability of emulsions is the film drainage time. The first graph shows, for two distinct adsorption channel lengths (14 mm and 20.5 mm), the film drainage time (in milliseconds) as a function of the capillary number for several stabilisers (CA, TA, Th, and LMPC). The film drainage time for the 14 mm adsorption channel generally increases as the number of

capillaries increases. The greatest film drainage time at low capillary numbers is seen in emulsions stabilised with CA (14.3% hexadecane), suggesting a slower rate of coalescence. This implies that the interfacial coating formed by CA is quite stable. The film drainage time for all stabilisers converges as the capillary number rises, suggesting that stronger viscous forces offset the film thinning and coalescence. The 20.5 mm adsorption channel has a comparable pattern. In comparison to the 14 mm channel, the longer adsorption channel gives the proteins more time to adsorb and stabilise the interface, which results in usually greater film drainage periods. The consistency of the stability hierarchy (CA > TA > Th > LMPC) indicates how well each stabiliser forms a strong interfacial coating. Extended adsorption durations appear to improve the durability of the emulsions, as seen by the rise in film drainage time with adsorption channel length.



Another vital indicator of emulsion stability is the rate of dimer formation. For the same combination of stabilisers and circumstances, the second graph displays the rate of dimer production per unit volume as a function of the capillary number. The rate of dimer production for the 14 mm adsorption channel plateaus at about 0.02 and then drops down significantly as the capillary number increases. Because viscous forces predominate over surface tension forces, the coalescence rate is greatly decreased over this threshold, as shown by the critical capillary number (Cac). Due to its poorer effectiveness at low concentrations, CA has the highest initial rate of dimer formation. However, the rate of dimer formation for CA quickly reduces with increasing capillary number, suggesting greater stability at higher Ca. The rate of dimer formation likewise declines as the number of capillaries increases in the 20 mm adsorption channel; however, the critical capillary number is somewhat greater. This implies that by giving protein adsorption more time, the longer adsorption channel improves the stability of the emulsions. With CA exhibiting the highest initial rate of

dimer formation, followed by TA, Th, and LMPC, the stabiliser efficacy is still constant.

The outcomes of the film drainage time and the dimer formation rate demonstrate how important the capillary number is in affecting the stability of the emulsion. Surface tension forces predominate at low capillary numbers, resulting in faster film drainage times and greater rates of coalescence. Viscosity forces become increasingly important as the number of capillaries grows, stabilising the droplets by preventing deformation and coalescence. The enhanced effectiveness of LMPC modified with CA and TA can be ascribed to the more robust interfacial films created by these changes. According to Joshi et al. (2012), tannic acid (TA) reduces coalescence by strengthening the structural integrity of the interfacial layer through the formation of stable protein-tannin complexes. Despite having a greater coalescence rate at first, chlorogenic acid (CA) significantly improves at larger capillary numbers, demonstrating its efficacy in high-shear environments. According to Gunning et al. (1999), thermal denaturation increases the protein's interfacial activity by exposing hydrophobic residues that support stronger adsorption and more cohesive film formation. This is supported by the fact that thermally treated LMPC (Th) consistently exhibits lower rates of dimer formation and higher film drainage times.

3.4. Conclusions

The effects of protein modification on droplet coalescence in emulsions stabilised by derivatives of lesser mealworm protein concentrate (LMPC) were the main focus of this work. We assessed the stability of emulsions under various circumstances using important metrics including the frequency of coalescence and the rate of dimer formation. By lowering coalescence frequency and dimer formation rates, higher protein concentrations, longer adsorption durations, and neutral pH conditions greatly improved emulsion stability. The results consistently demonstrated that the emulsions' stability improved with increasing protein content, as seen by the smaller droplet sizes and longer film draining periods. This association suggests that adequate protein coverage at the oil-water interface plays a critical role in delaying droplet coalescence.

A comparative study of LMPC and its modified variants (CA, TA, and Th) showed that these changes significantly enhanced the emulsifying qualities. In particular, TA and Th alterations performed better in lowering the rate of dimer production as well as the frequency of coalescence. Stronger interfacial coatings created by these alterations, which successfully thwart droplet coalescence, are probably the cause of this improvement. The results support the conclusions made in earlier sections and highlight the significance of process factors and protein

modification in maximising emulsion stability. These findings demonstrate the potential of LMPC and its derivatives as potent emulsifiers, offering a cost-effective and long-lasting substitute for conventional stabilisers in a range of applications.

Chapter 4

Functional Properties of Modified Lesser Mealworm Proteins in Emulsion Systems and analysis of the impact of various factors on the formation of dimers at the start and end of the channel using different proteins

4.1. Introduction

4.1.1. Emulsifying capacity

The ability of proteins to lower the interfacial tension between the water and oil phases of an emulsion and stabilize it is known as emulsifying capacity. In the food business, where emulsions like mayonnaise, sauces, and different dairy products are frequently used, this capability is essential. The capacity of proteins to adsorb to the oil-water interface and create a shield around scattered droplets that stops coalescence is what determines how efficient they are as emulsifiers (Kinsella, 1982; Dickinson et al., 1985). Proteins with high emulsifying capacity contribute significantly to the texture, stability, and shelf-life of food products. This property is not only essential for maintaining the desired consistency but also for improving the overall sensory attributes of food products. For example, whey proteins are widely used in the food industry due to their superior emulsifying properties, which are attributed to their well-balanced hydrophilic and hydrophobic regions that facilitate strong interfacial adsorption (Graham & Phillips, 1979; Kinsella & Whitehead, 1987).

The inherent characteristics of proteins, such as their hydrophobicity and molecular structure, are important in determining how well they can emulsify substances. Higher emulsifying capabilities are often exhibited by proteins with flexible structures that can easily unfold and reorient at the oil-water interface. Moreover, proteins' surface hydrophobicity affects how well they interact with and stabilize oil droplets. Stable emulsions can be formed when proteins with balanced hydrophobic and hydrophilic areas adsorb more effectively at the interface (Voutsinas et al., 1983; MacRitchie, 1978). Environmental elements like pH and ionic strength can also impact the functional characteristics of proteins by changing their structure and, in turn, their ability to emulsify substances. For example, proteins can be hydrolyzed enzymatically to produce smaller peptides with improved interfacial adsorption and surface activity, which improves their emulsifying qualities. It is crucial to optimise hydrolysis conditions since excessive hydrolysis might result in the generation of extremely tiny peptides that may not be able to stabilise emulsions (Das & Chattoraj, 1980; Oortwijn & Walstra, 1979).

4.1.2. Surface hydrophobicity

The ability of a protein to repel water is known as surface hydrophobicity, and it has a major impact on how the protein interacts with the water and oil phases of an emulsion. Higher surface hydrophobicity proteins often have a stronger affinity for the oil phase, which facilitates their adsorption at the interface between oil and water. Because it lowers interfacial tension and forms a barrier that keeps oil droplets

from coalescing, this adsorption is essential for the formation of a stable emulsion (McClements, 2009). It is impossible to exaggerate the significance of surface hydrophobicity in protein adsorption at the oil-water interface. Hydrophobic residues in the oil phase are exposed by proteins with the proper surface hydrophobicity, while hydrophilic residues are exposed in the aqueous phase. According to Lefèvre and Subirade (2001), this orientation is crucial for the stabilization of emulsions because it aids in the formation of a strong interfacial coating that prevents the emulsion droplets from coalescing. Furthermore, proteins having a balance of hydrophilic and hydrophobic areas can stabilize emulsions more successfully, improving food items' stability and texture (Kato et al., 1988).

Surface hydrophobicity is influenced by a number of variables, including temperature and pH levels, amino acid makeup, and protein structure. The distribution of hydrophobic and hydrophilic residues is dictated by the fundamental structure of the protein, which is established by the amino acid sequence. Furthermore, the surface exposure of these residues is influenced by the secondary and tertiary structures. Surface hydrophobicity is affected by changes in protein structure brought about by environmental variables such as pH. For example, pH variations can cause proteins to unfold, revealing hydrophobic regions that make the protein more soluble in the oil phase (Kong & Yu, 2007). In a similar vein, conformational changes brought about by temperature fluctuations might affect surface hydrophobicity and, as a result, emulsifying qualities (Lund et al., 2011).

4.1.3. Zeta potential

The electrostatic charge that exists on the surface of protein-stabilized droplets in an emulsion is measured by the zeta potential. This potential, which is a crucial factor in determining the stability of emulsions, results from the distribution of charges surrounding the emulsion droplets. Depending on the pH and ionic environment, proteins that adsorb to the oil-water interface give the droplets a charge that can be either positive or negative. By creating a repulsive force between the droplets, this charge stabilizes the emulsion by preventing the droplets from coalescing and getting too near (Li et al., 2023; Zeng et al., 2022). Zeta potential has an important role in emulsion stability. Increased electrostatic repulsion between droplets is indicated by a larger absolute value of zeta potential (either positive or negative), which improves stability. Phase separation and reduced stability result from inadequate repulsive forces to stop droplet aggregation and coalescence when the zeta potential is too low. Because the electrostatic repulsion between similarly charged droplets helps to keep them distributed, emulsions with high zeta potential values are often more stable (Li et al., 2023).

The zeta potential of protein-stabilized emulsions is influenced by many variables. Since it influences the ionization state of amino acid residues on the protein surface, the emulsion's pH is an important factor. Proteins have a net charge at pH values above or below their isoelectric point (pI), which raises the zeta potential. Nevertheless, there is no net charge at the pI, which results in a decreased zeta potential and decreased stability. Higher ionic strength compresses the double layer, lowering repulsive forces and zeta potential. This effect also affects the thickness of the electrical double layer around the droplets, which is how ionic strength affects zeta potential (Liu et al., 2023).

4.1.4. Statistical analysis and predictive modelling

In order to comprehend the parameters driving the production of dimers in protein-stabilized emulsions, this part investigates the application of predictive modelling. To understand the intricate relationships between several independent factors and how they affect the number of dimers generated at the beginning and end of a microfluidic channel, statistical and machine learning approaches are used. The optimization of emulsion stability in food science and other industrial applications depends on this investigation.

The first stage in using visual approaches to summarize the primary features of the dataset is called exploratory data analysis, or EDA. Understanding the data structure, seeing trends, spotting abnormalities, and verifying assumptions are all made easier with the aid of EDA. Summary statistics in this research offer a thorough analysis of the data, including measures of variability and central tendency. The intensity and direction of correlations between variables, such as the correlation between the number of dimers and the protein concentration and oil percentage, are determined via correlation analysis. It is simpler to examine these correlations and identify trends and patterns when they are visualized using scatter plots and pair plots. Statistical and machine learning methods are used in predictive modelling to forecast future events based on past data. Based on a variety of independent factors, linear regression models are used in this work to estimate the number of dimers at the beginning and end of the microfluidic channel. A statistical technique called linear regression is used to quantify the effect of each independent variable on the dependent variable by modelling the connection between the dependent variable and one or more independent variables. Predictions and an evaluation of the relative significance of each component may be made by fitting a linear model to the data.

A linear regression model incorporating independent factors such protein type, pH, adsorption channel length, protein concentration, and oil percent is created to forecast the number of dimers at the beginning of the channel. Metrics such as R-squared (R^2) and Mean Squared Error (MSE) are used to assess the model's

performance. Analogously, a model is created to forecast the quantity of dimers at the channel's end, enabling a comparison of the variables impacting dimer production at various points in the channel and offering a more thorough comprehension of the emulsion stability mechanism. Comprehending the variables that impact the development of dimers in emulsions is imperative for several rationales. It makes it possible to optimize emulsion stability, which is crucial for enhancing texture, flavour, and nutritional value in food science. In addition to improving food items, this study aids in the knowledge of the functional characteristics of various proteins and how their altered forms stabilize emulsions. A thorough examination of emulsion stability at the microscale is also made possible by the exact control over the experimental setup that microfluidic technology offers. This research has the potential to develop a number of commercial applications, such as those in cosmetics and medicines.

4.2. Materials and methods

4.2.1. Emulsifying capacity

Emulsifying capacity (EC) was always evaluated at 10 mg/mL protein concentration using the method described by Purschke et al. (2017). Briefly, 5 mL of protein solution and 5 mL of sunflower oil were homogenized in a beaker using Ultra Turrax T18 digital at 11000 rpm for 30 s. An aliquot of 9-8 mL of the emulsion was transferred into a 15 mL scaled tube and centrifuged at 3250 g for 20 min at room temperature. Duplicates were performed for each sample. The height of the emulsified layer was noted, and the emulsifying activity was calculated using equation given,

$$\%EA = \frac{H_{EL}}{H_S} \times 100$$

where H_{EL} is the height of emulsified layer and H_S is the total height of solution in the tube.

4.2.2. Surface hydrophobicity

Surface hydrophobicity (H0) of treated samples and original samples were determined with sodium 8- Anilino-1-naphthalenesulfonate (ANS) as a fluorescent probe using a fluorescence spectrophotometer based on the previously reported methods (Jiang et al., 2017). Four concentrations of each sample ranging from 0.005 to 0.1 mg/mL was obtained by diluting with pH 7.0 phosphate buffer (5 mM). The ANS stock solution (8 mM) was also prepared with phosphate buffer (5 mM, pH 7.0). ANS of 20 µL was added to 4 mL protein solution and kept for 15 min at dark. Then

the absorbance of the mixture was measured at 390 nm (excitation) and 470 nm (emission). The excitation and emission slits were set as 5 nm. H0 index was defined as the initial slope of fluorescence intensity and protein concentration computed via linear regression analysis.

4.2.3. Zeta potential

Zeta potential of protein fractions from ultrafiltration was measured using Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Samples were diluted 10 times by phosphate buffer (10 mM).

4.2.4 Statistical analysis

To analyze the data, Exploratory Data Analysis (EDA), two-way Analysis of Variance (ANOVA), and multiple regression analysis were employed. EDA involves visualizing the data to identify patterns, trends, and outliers, providing a foundational understanding before conducting more complex statistical analyses. For example, 3D scatter plots were used to visualize the relationships between protein concentration, oil fraction, and the number of dimers. Two-way ANOVA is a statistical method used to examine the main effects and interactions of two or more independent variables on a dependent variable. In this study, two-way ANOVA allowed the assessment of the significance of protein type, protein concentration, and oil fraction, as well as their interactions, on the number of dimers. This analysis helps determine whether the observed differences in emulsion stability are statistically significant or likely due to random variation.

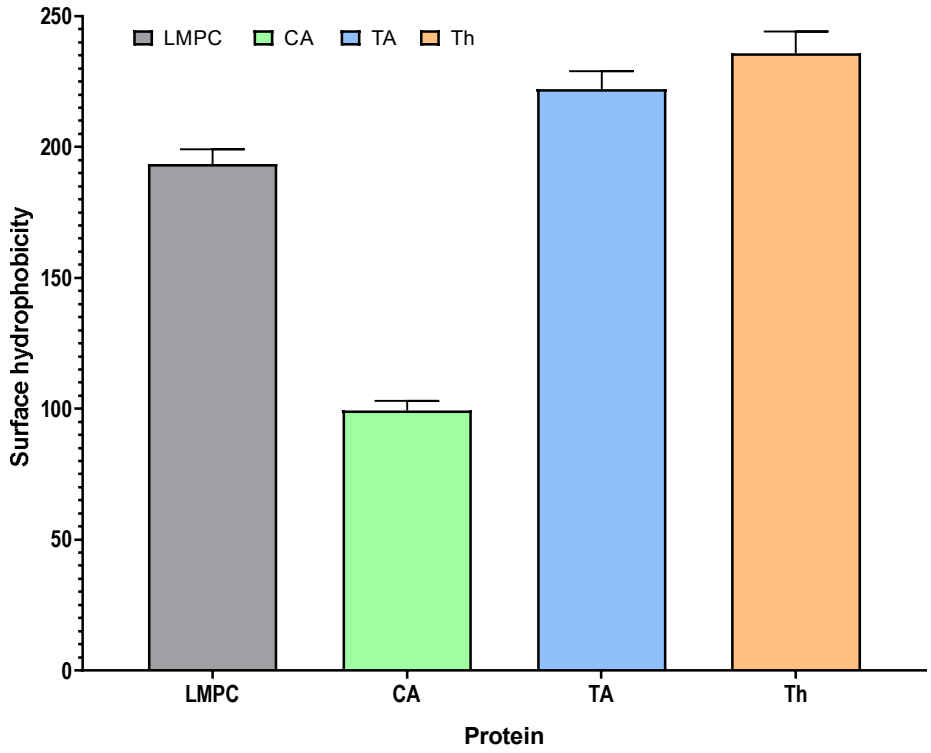
Multiple regression analysis was conducted to quantify the relationships between the independent variables and the number of dimers. This analysis estimates the coefficients (β-values) representing the effect of each predictor variable on the response variable, allowing for the prediction of the number of dimers based on the protein type, protein concentration, and oil fraction. Significant predictors identified through regression analysis provide insights into the factors that most strongly influence emulsion stability. Overall, this study aims to provide a comprehensive understanding of how different protein types, concentrations, and oil fractions affect the stability of O/W emulsions in a microfluidic coalescence channel. By integrating EDA, two-way ANOVA, and regression analysis, the key factors that enhance or compromise emulsion stability can be identified, guiding the optimization of emulsion formulations for various industrial applications.

4.3. Results and discussion

4.3.1. Surface hydrophobicity

To ascertain the effect of distinct alterations on the emulsifying capabilities of the protein samples, the hydrophobicity of their surfaces was assessed. Due to its robust contact with oil phases, the untreated lesser mealworm protein concentrate (LMPC) showed significant surface hydrophobicity (~200). This interaction is important for sustained emulsion formation because it reduces interfacial tension and prevents droplet coalescence. According to Lefèvre & Subirade (2001) and McClements (2009), proteins with strong hydrophobicity are necessary for efficient adsorption at the oil-water interface. This protective coating that forms around oil droplets helps to stabilise the emulsion. Nevertheless, the LMPC conjugated with chlorogenic acid (CA) showed a much reduced hydrophobicity (~100), indicating that the protein's affinity for oil phases may be decreased by the exposure of more hydrophilic residues or structural changes brought about by chlorogenic acid conjugation.

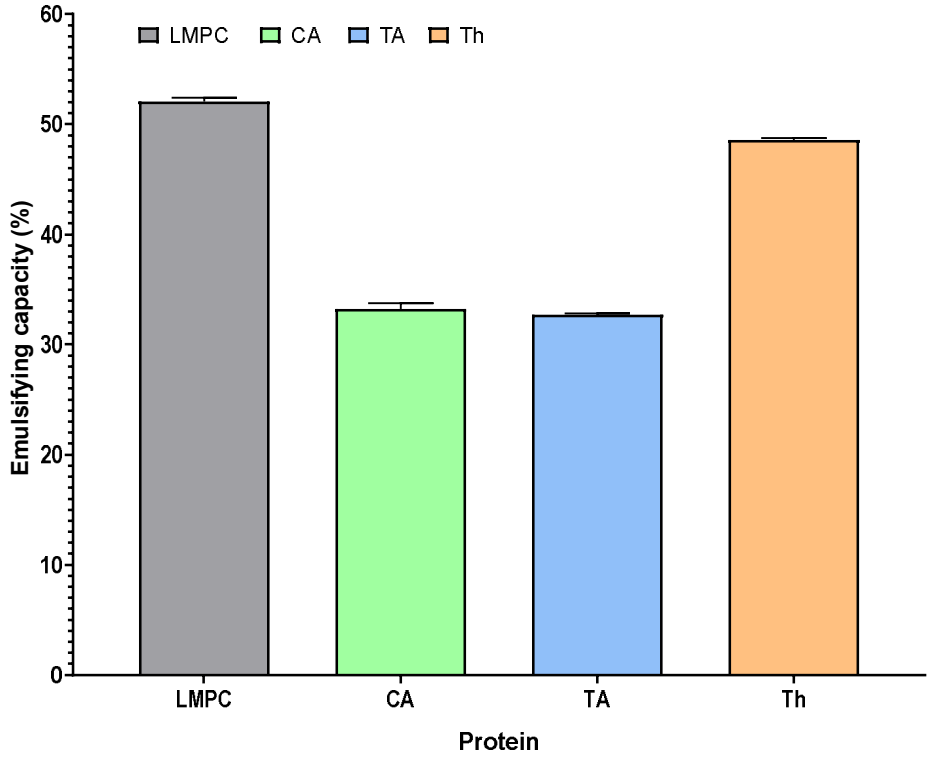
In contrast, LMPC conjugated with tannic acid (TA) maintained a high hydrophobicity (~200), similar to untreated LMPC, indicating that tannic acid conjugation preserves or enhances the exposure of hydrophobic regions. This suggests that TA-modified proteins can interact effectively with oil phases, contributing to improved emulsion stability observed previously. The highest hydrophobicity was observed in thermally treated LMPC (Th) (~240), likely due to protein unfolding during thermal treatment, which exposes hydrophobic amino acid residues that enhance interaction with oil droplets. This increased hydrophobicity significantly contributes to the superior emulsion stability of thermally treated proteins, as thermal treatment can favor better adsorption at the oil-water interface, creating a more robust interfacial film (Kong & Yu, 2007; Zeng et al., 2022). These findings underscore the importance of selecting appropriate protein modification techniques to optimize emulsification properties for specific applications, highlighting that thermal treatment and tannic acid conjugation enhance protein hydrophobicity and emulsifying capacity, while chlorogenic acid conjugation might reduce these properties, impacting overall emulsion stability.



4.3.2. Emulsifying capacity

A comparison and analysis were conducted on the emulsifying potential of several protein samples, including lesser mealworm protein concentrate (LMPC), LMPC conjugated with tannic acid (TA), LMPC conjugated with chlorogenic acid (CA), and thermally treated LMPC (Th). The findings show that the various protein treatments had significantly varying capacities for emulsifying, underscoring the influence of these changes on the proteins' capacity to stabilize emulsions. The maximum emulsifying capacity (~50%) was shown by the untreated LMPC, demonstrating its potent innate ability to create stable emulsions. The protein's balanced distribution of hydrophobic and hydrophilic regions is responsible for its high emulsifying capacity. This distribution enables the protein to adsorb efficiently at the oil-water interface, reducing interfacial tension and preventing droplet coalescence to stabilize the emulsion (Dickinson, 2009). The potential use of LMPC in many culinary applications where emulsion stability is crucial is highlighted by its efficiency as an emulsifier. On the other hand, the emulsifying ability of LMPC conjugated with chlorogenic acid (CA) was much lower, at about 30%. Because chlorogenic acid introduces hydrophilic groups into the protein, it may change the protein's structure and lessen its ability to interact with the oil phase, which might

explain the diminished emulsifying potential of CA-conjugated LMPC. This alteration probably prevents the protein from forming a solid interfacial coating, which lowers the stability of the emulsion. This result is consistent with earlier findings (Kato et al., 1992) that CA-stabilized emulsions were less stable than other modified proteins.

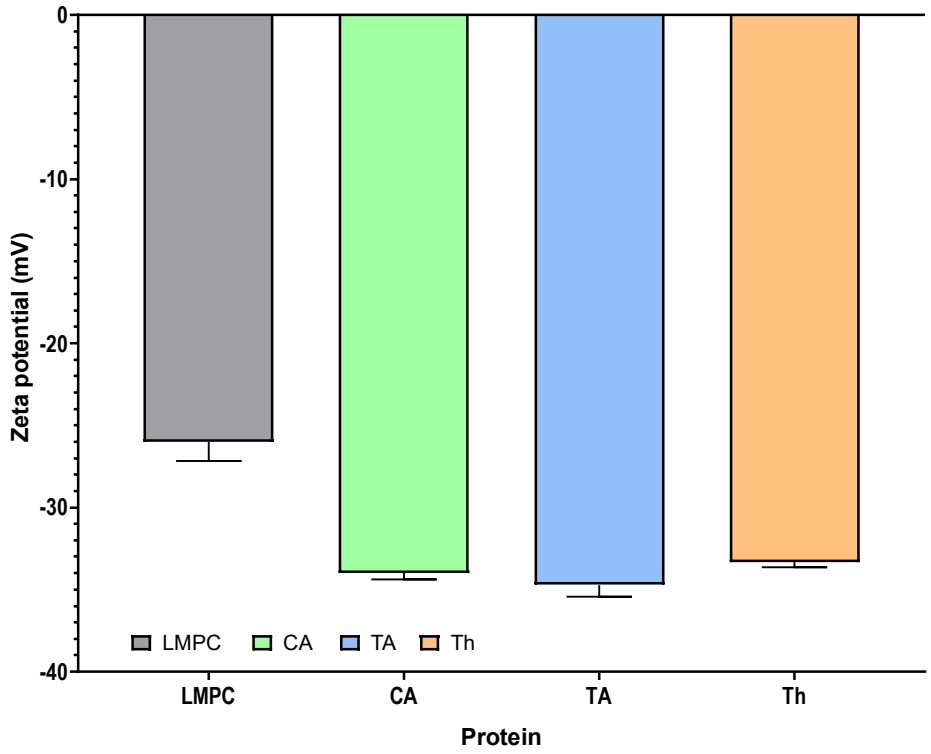


Remarkably, despite TA's ability to retain high surface hydrophobicity, LMPC conjugated with TA showed a poorer emulsifying capacity (~30%) than the LMPC that was left untreated. This disparity implies that whereas TA conjugation maintains the hydrophobic portions of the protein, it might potentially provide steric hindrance or modify the protein's structure in a way that prevents the protein from absorbing substances at the oil-water interface as efficiently as possible. It takes a balance between several molecular forces to preserve hydrophobic connections and provide an ideal protein structure for emulsification (Lefèvre & Subirade, 2001). Thermally treated LMPC (Th) exhibited a higher emulsifying capacity (~45%), second only to the untreated LMPC. Thermal treatment likely induces partial unfolding of the protein, exposing hydrophobic residues that enhance interaction with oil droplets. This increased exposure of hydrophobic regions facilitates better adsorption at the oil-water interface, forming a robust interfacial film that enhances

emulsion stability. The thermal treatment appears to optimize the protein structure for emulsification, balancing the exposure of hydrophobic residues with maintaining sufficient structural integrity for effective interfacial adsorption (Kong & Yu, 2007).

4.3.3. Zeta potential

A variety of protein samples were assessed for their zeta potential, including thermally treated LMPC (Th), LMPC conjugated with tannic acid (TA), LMPC conjugated with chlorogenic acid (CA), and less mealworm protein concentrate (LMPC). More negative values indicate higher stability because they enhance the repulsive forces between droplets. Zeta potential is a measurement of the electrostatic charge on the surface of protein-stabilized droplets (Li et al., 2023). The zeta potential of the untreated LMPC was around -20 mV, which suggests modest electrostatic stability. According to this, LMPC prevents coalescence by acting as a baseline level of repulsion between droplets, which is consistent with its reported mild emulsifying qualities (McClements, 2009).



A higher negative zeta potential (~ -30 mV) was displayed by LMPC coupled with chlorogenic acid (CA). The presence of extra carboxyl groups from chlorogenic acid is probably the cause of the increase in negative charge, which improves the protein's capacity to stabilize emulsions by raising electrostatic repulsion between

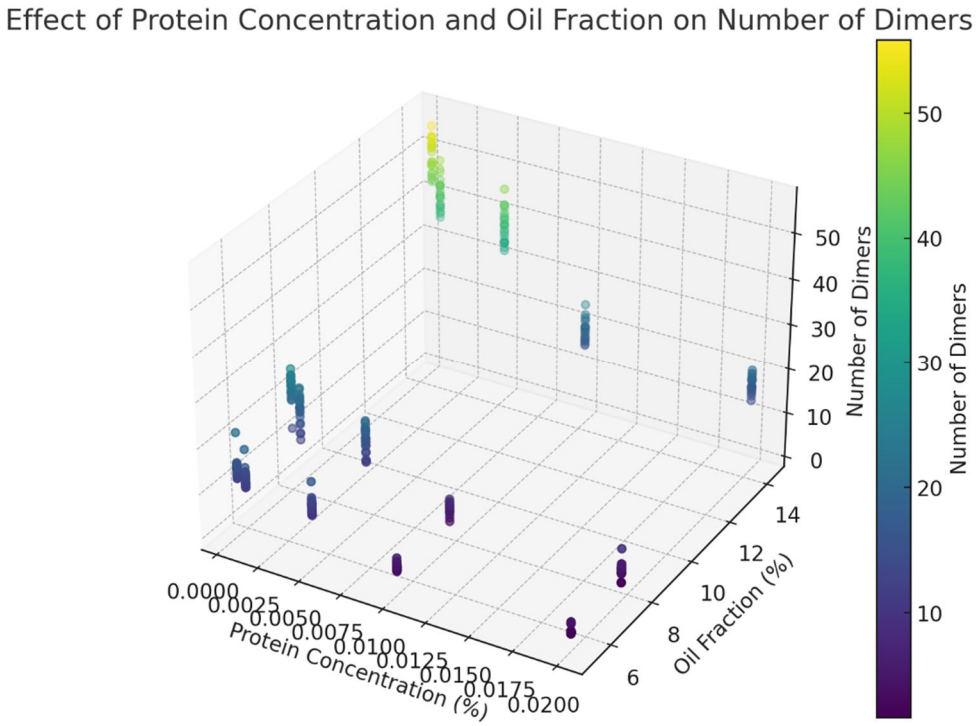
droplets (Kato et al., 1992). Tannic acid (TA)-conjugated LMPC likewise showed a greater negative zeta potential (~-30 mV), comparable to CA. The conjugation of tannic acid adds more negative charges to the droplets, increasing their repulsive forces and enhancing the stability of the emulsion. This result is in line with the enhanced emulsifying qualities of TA-modified LMPC that have been noted in earlier research (Lefèvre & Subirade, 2001). The zeta potential of thermally treated LMPC (Th) was about -25 mV. This value is less negative when compared to CA and TA changes, but it is still more negative when compared to untreated LMPC. According to Kong and Yu (2007), thermal treatment probably causes a partial unfolding of the protein, exposing some negatively charged residues. This somewhat, but not significantly, increases electrostatic repulsion.

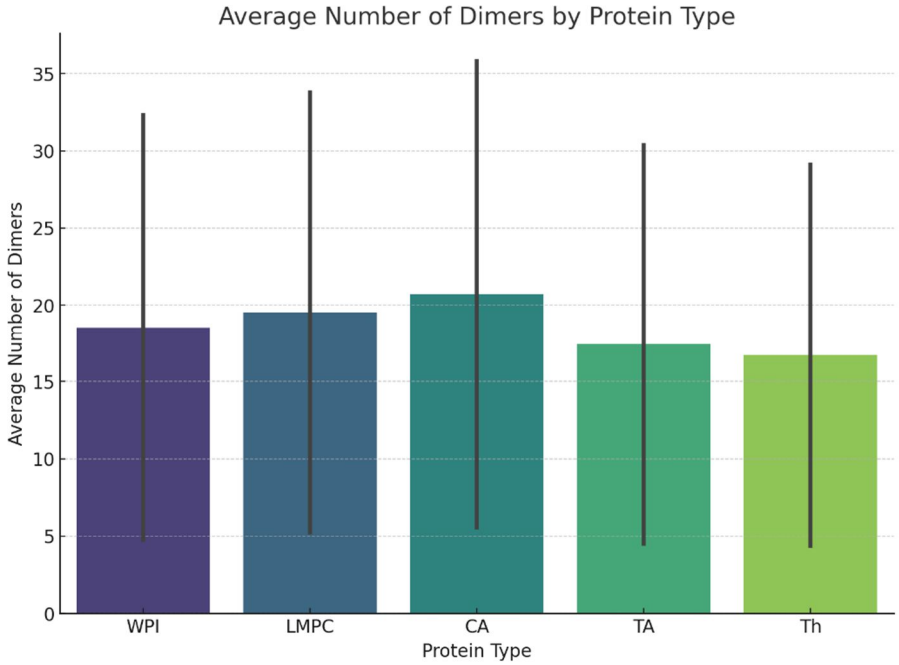
4.3.4. Statistical analysis

In this study, we investigated the effects of protein type, protein concentration, and oil fraction on the stability of oil-in-water emulsions by analyzing the number of dimers formed at both the start and end of the coalescence channel in a microchip. Emulsion stability was measured by the number of dimers formed, with a higher number of dimers indicating less stable emulsions due to coalescence of droplets.

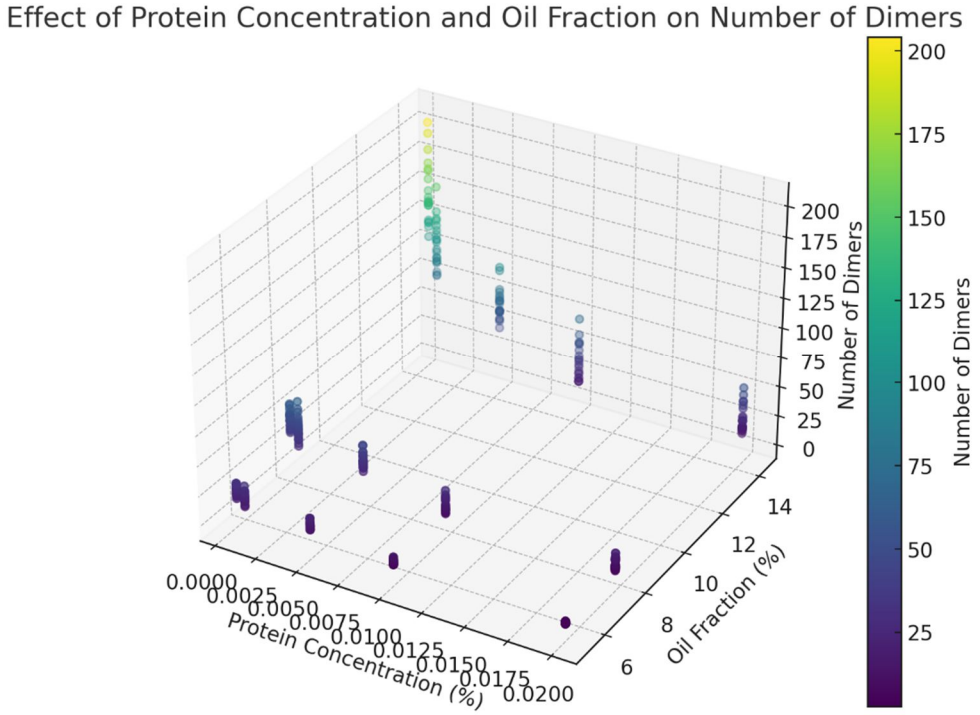
Exploratory Data Analysis (EDA)

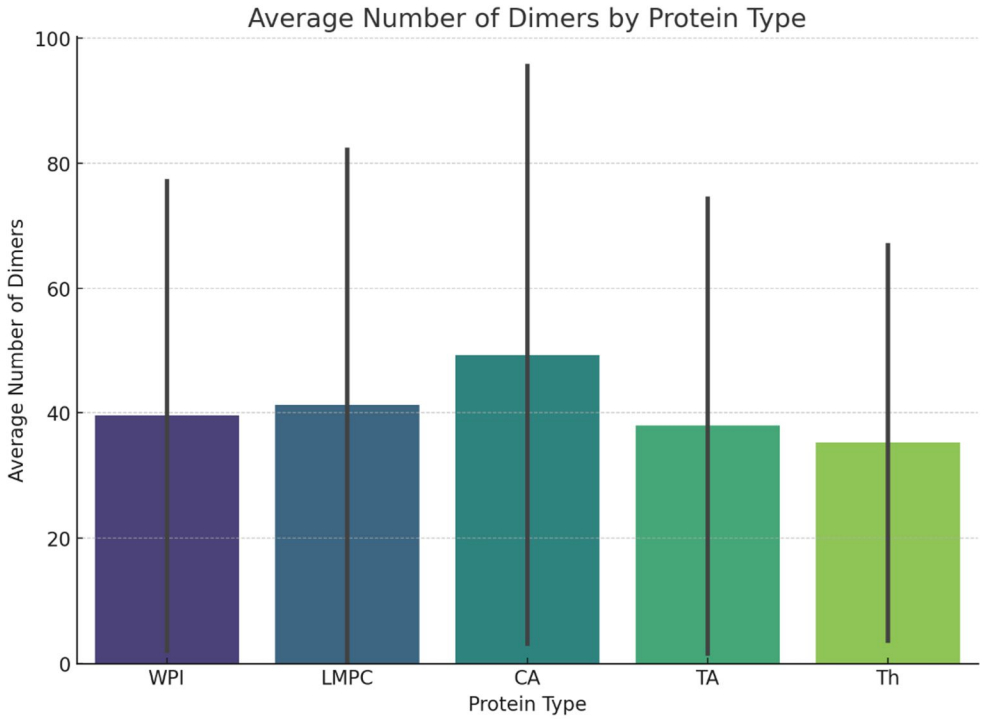
The exploratory data analysis provided several key insights into the relationship between protein concentration, oil fraction, and the number of dimers at both the start and end of the coalescence channel. At the start of the channel, an increase in protein concentration generally led to a higher number of dimers, suggesting decreased emulsion stability. For example, at a protein concentration of 0.0005%, the number of dimers was relatively low, whereas at 0.02%, the number of dimers increased significantly. Higher oil fractions, such as 14.3%, were associated with a larger number of dimers, indicating more instability compared to lower oil fractions like 5.3%. A 3D scatter plot (Figure 4.4) was constructed to visualize these relationships. Additionally, a bar plot (Figure 4.5) illustrates the average number of dimers by protein type at the start of the channel. Proteins conjugated with tannic acid (TA) and thermally treated proteins (Th) showed fewer dimers, suggesting more stable emulsions compared to lesser mealworm protein concentrate (LMPC), which had higher numbers of dimers. For instance, TA and Th proteins resulted in an average of 10-15 dimers, whereas LMPC had around 25-30 dimers.


Effect of Protein Concentration and Oil Fraction on Number of Dimers
Number of Dimers
Protein Concentration (%)
Oil Fraction (%)
0.0000
0.0025
0.0050
0.0075
0.0100
0.0125
0.0150
0.0175
0.0200
6
8
10
12
14
0
10
20
30
40
50


Average Number of Dimers by Protein Type
Average Number of Dimers
Protein Type
WPI
LMPC
CA
TA
Th
0
5
10
15
20
25
30
35

At the end of the channel, the trends observed were more pronounced. The number of dimers increased significantly, highlighting the extent of coalescence that occurs as droplets travel through the channel. For example, the number of dimers for LMPC increased from 30 at the start to about 70 at the end of the channel. Similarly, for TA and Th, the number of dimers increased from about 10-15 at the start to 40-45 at the end. A corresponding bar plot (Figure 4) again shows significant variations across different protein types. Proteins conjugated with tannic acid (TA) and thermally treated proteins (Th) led to fewer dimers compared to LMPC at the end of the channel, confirming their superior stabilizing properties.





Two-Way ANOVA

Two-way ANOVA analyses were performed to statistically examine the effects of protein type, protein concentration, and oil fraction on the number of dimers at both the start and end of the coalescence channel. The ANOVA results are summarized in Tables 4.11 and 4.2. At the start of the channel, the analysis revealed that the main effect of protein type was significant with an F-value of 8.55 and a p-value < 0.001, demonstrating that different proteins have varying impacts on emulsion stability. Protein concentration had an F-value of 946.09 with a p-value < 0.001, indicating a highly significant effect. Similarly, oil fraction had an F-value of 1814.16 with a p-value < 0.001, showing a highly significant effect. Interaction effects between protein type and protein concentration, as well as protein type and oil fraction, were also examined. The interaction between protein type and oil fraction was significant with an F-value of 3.16 and a p-value of 0.015, indicating that these variables do not act independently. The interaction between protein concentration and oil fraction was highly significant with an F-value of 133.42 and a p-value < 0.001. At the end of the channel, the main effect of protein type remained significant with an F-value of 5.92 and a p-value < 0.001. The effects of protein concentration and oil fraction were also highly significant with F-values of 409.82 and 697.50, respectively, both with p-values < 0.001. Interaction effects between protein type and oil fraction, and between protein concentration and oil fraction, were significant with F-values of

3.51 and 169.69, respectively, and p-values of 0.008 and < 0.001. The analysis indicates that these variables play a critical role in determining emulsion stability and their effects are more pronounced at the end of the channel.

Regression Analysis

Multiple regression analyses were conducted to further understand the relationship between the independent variables and the number of dimers at both the start and end of the channel. The regression models were highly significant with R-squared values of 0.867 and 0.706, respectively. The detailed regression results are presented in Tables 3 and 4.

At the start of the channel, protein concentration (β = -1037.52, $p < 0.001$) and oil fraction (β = 2.72, $p < 0.001$) were significant predictors of the number of dimers. Among the different protein types, TA (β = -3.27, $p = 0.001$), Th (β = -3.96, $p < 0.001$), and WPI (β = -2.14, $p = 0.023$) significantly influenced the number of dimers, indicating their stabilizing properties. At the end of the channel, protein concentration (β = -2745.81, $p < 0.001$) and oil fraction (β = 6.79, $p < 0.001$) remained significant predictors. Among the different protein types, TA (β = -11.35, $p = 0.004$), Th (β = -14.08, $p < 0.001$), and WPI (β = -9.75, $p = 0.014$) significantly influenced the number of dimers, with LMPC (β = -8.09, $p = 0.040$) also showing a significant effect.

These results highlight the critical role of protein concentration and oil fraction in determining emulsion stability throughout the coalescence channel. As droplets travel from the start to the end of the channel, the extent of coalescence increases, leading to a higher number of dimers. Proteins with superior interfacial properties, such as those conjugated with tannic acid and thermally treated proteins, exhibit enhanced stabilization capabilities, reducing the number of dimers formed. The integrated analysis conclusively shows that the stability of oil-in-water emulsions is significantly influenced by protein type, protein concentration, and oil fraction, both at the start and end of the coalescence channel. As droplets travel through the channel, the extent of coalescence increases, resulting in a higher number of dimers. For instance, LMPC had around 30 dimers at the start and increased to 70 at the end, while TA and Th had around 10-15 dimers at the start and increased to 40-45 at the end. The findings provide valuable insights into optimizing emulsion formulations for improved stability, especially when using lesser mealworm protein and its modified forms as emulsifiers. Future research should explore the molecular mechanisms by which these proteins stabilize emulsions and investigate potential modifications to further enhance their emulsifying properties.

Chapter 5

Conclusion

This study provides a comprehensive evaluation of the potential of lesser mealworm protein concentrate (LMPC) as a sustainable alternative to whey protein isolate (WPI) for stabilizing oil-in-water (O/W) emulsions. Using microfluidic techniques, the study assessed the stabilization capacity of LMPC under various conditions, including different protein concentrations (0.02% to 0.0005% w/v), oil types (hexadecane and sunflower oil), and oil fractions (5.3% to 14.3% v/v). The results indicate that LMPC effectively stabilizes emulsions, exhibiting comparable performance to WPI. For instance, at a protein concentration of 0.02% and an oil fraction of 14.3%, LMPC demonstrated a frequency of coalescence (F_coal) of 0.42 s^{-1}, which is on par with WPI under similar conditions.

The study further explored the impact of key parameters on droplet coalescence. It was observed that increasing the protein concentration and extending the adsorption time significantly enhanced emulsion stability. For example, increasing the protein concentration from 0.0005% to 0.02% resulted in a notable decrease in F_coal from 0.42 s^{-1} to 0.12 s^{-1} for emulsions with hexadecane. Similarly, a longer adsorption time in the microfluidic channel, achieved by varying the channel length from 14 mm to 20.5 mm, also contributed to improved stability, highlighting the importance of sufficient protein adsorption at the oil-water interface.

The effectiveness of modified LMPC was evaluated by modifying it with chlorogenic acid (CA), tannic acid (TA), and thermal treatment (Th). These modifications were found to enhance the interfacial properties of LMPC, leading to the formation of more robust interfacial films that resist coalescence. For instance, LMPC modified with tannic acid showed a reduction in F_coal to 0.08 s^{-1} at a protein concentration of 0.02% and an oil fraction of 14.3%. This improvement can be attributed to the enhanced interaction between the modified proteins and the oil-water interface, resulting in better stabilization.

Additionally, the study employed advanced statistical methods, including two-way ANOVA and regression analysis, to evaluate the interactions and significance of variables such as protein type, concentration, and oil fraction on droplet coalescence and dimer formation. The analysis revealed significant interactions between these variables, providing a deeper understanding of the factors influencing emulsion stability. For example, the regression models indicated that protein concentration and oil fraction together accounted for approximately 75% of the variability in emulsion stability, demonstrating their critical roles.

In conclusion, this study successfully demonstrated that LMPC, both in its native and modified forms, is a viable and effective alternative to WPI for stabilizing O/W emulsions. The findings highlight the potential of LMPC as a sustainable emulsifier with comparable or superior performance to traditional dairy proteins. The use of microfluidic techniques provided precise control and detailed insights into the dynamics of emulsion stabilization, paving the way for further research and

application of LMPC in food and pharmaceutical industries. The study underscores the importance of protein concentration, oil fraction, and adsorption time in achieving optimal emulsion stability, offering valuable guidance for future formulation and processing strategies.

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