University of Reading

Development of a novel nanoemulsion with enhanced nutritional profile to reduce saturated fatty acids in bakery products

Thesis submitted for the requirement for the degree of Doctor
of Philosophy in Food and Nutritional Science

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Declaration

I confirm that this is my own work and the use of all materials from other sources have been properly and fully acknowledged.

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Abstract

The development of a saturated fat replacer for bakery products by formulating a novel nanoemulsion with high content of unsaturated fatty acids can play an important role in addressing the issue of adverse health effects due to a high consumption of saturated fat. However, the formation, physical and chemical stability of nanoemulsions can be influenced by several factors regarding the composition and processing parameters. Therefore, the aims of this research were (i) to investigate the effect of vegetable oils from different origins and the type of stabiliser on the formation and stability of nanoemulsion, (ii) to develop an optimum high-pressure homogenization (HPH) treatment for producing nanoemulsions with lecithin as stabiliser, (iii) to investigate the effect of mixed emulsifiers (lecithin and hydroxypropyl methylcellulose (HPMC)) on the formation, physical and chemical properties of nanoemulsions, and (iv) to evaluate the technological functionality of nanoemulsion made of extra virgin olive oil and stabilised with lecithin and HPMC as a saturated fat replacer on short dough biscuits.

The effect of vegetable oil type on the physical and chemical characterisation and stability of nanoemulsion (**Chapter 2**) showed that the nanoemulsion with higher stability were formulated with oils with lower fraction of unsaturated fatty acids, lower content of free fatty acids and higher value of total phenolic content. The results suggested that extra virgin olive oil (EVOO) could be the most suitable oil to formulate nanoemulsion as a saturated fat replacer because EVOO presented the highest radical scavenging activity and total phenolic content among the oils. The formation and stability of nanoemulsions were affected by the pressure and number of cycles of HPH treatments (**Chapter 3**). Furthermore, stabilisers' properties (Tween 20 or soy lecithin) such as interfacial tension, viscoelasticity and molecular structure had also a significant effect on nanoemulsion formation and stability. Although MDD and PDI of Tween 20 stabilised nanoemulsions were influenced by homogenisation pressure and cycles, there was not a significant effect on soy lecithin stabilised nanoemulsions. The most efficient HPH process was at pressures

of at least 400 bars and 1 cycle; these conditions produced nanoemulsions with great physical stability when using either Tween 20 or soy lecithin as emulsifiers. Soy lecithin is an interesting surfactant to formulate nanoemulsion for application in clean label food products. In order to improve the technological properties of nanoemulsions as a saturated fat replacer, a stabiliser and structing agent, such as HPMC was studied first in isolation (**Chapter 4**) and then when incorporated in nanoemulsions (**Chapter 5**). The molecular structure and concentration of HPMC played a major role in the viscoelastic behaviour, gelation temperature and strength of the gel formed during heating (**Chapter 4**). The firmness and work of shear of HPMC solutions increased significantly ($p < 0.05$) with increasing concentration, indicating lower spreadability. HPMC-L, the hydrocolloids with the lowest content of methyl and hydroxypropyl groups of the ones studied, was selected as the most suitable one to formulate fat mimetics because of its higher surface activity and more stable gel structure after heating. Furthermore, the combination of lecithin and HPMC in the formation and stability of emulsions showed an improvement of nanoemulsion physical and oxidative stability in comparison to using lecithin alone (**Chapter 5**). Addition of HPMC formed a stronger emulsion structure, showed higher lipid oxidative stability and similar physical properties, in terms of firmness and spreadability to butter indicating its potential to be used as a saturated fat replacer in food systems.

To formulate short dough biscuits with a healthier fatty acid profile, the nanoemulsion was used to replace 33% of the butter in the dough (**Chapter 6**). Biscuits made with the novel nanoemulsion presented less oil migration than the control biscuits made with butter. A trained sensory panel did not find significant differences ($p > 0.05$) in crumb density and hardness on the first bite among the biscuits made with the nanoemulsion and the control. The developed nanoemulsion worked as a saturated fat replacer in short dough biscuits, resulting in a reduction of 30% of saturated fat and 25% of total fat while maintaining high quality physical and sensory attributes. Overall, the development of this nanoemulsion provides a novel strategy for the reduction of saturated fat and total fat content in bakery products. This study will provide useful information about the basis to formulate saturated fat replacers with specific technological functionalities for application in food products.

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Abbreviations

Abbreviation	Meaning
EVOO	extra virgin olive oil
CPRO	cold pressed rapeseed oil
OO	olive oil
RO	rapeseed oil
SO	Sunflower oil
-E	conventional emulsion
-NE	nanoemulsion
SFA	saturated fatty acids
MUFA	monounsaturated fatty acids
PUFA	polyunsaturated fatty acids
FFA	free fatty acids
TPC	total phenolic content
CI	creaming index
TBARs	thiobarbituric acid reactive substances
CMC	critical micelle concentration
MDD	mean droplet diameter
PDI	polydispersity index
TS	thermal stability
HPMC	hydroxypropyl methyl cellulose
D-	dough
B-	biscuit
CNE	complex nanoemulsion
INE	indevidual ingredient of nanoemulsion
a_w	water activity
MC	moisture content
SI	spreadability index
WL	weight loss
3PB test	three-point bending test

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Chapter 1

Literature Review

1.1 Introduction

High consumption of saturated fatty acids has adverse health effects, promoting cardiovascular diseases (CVDs) (Buckland *et al.*, 2012; Ebbesson *et al.*, 2015), which are the leading cause of global death; an estimated 17.9 million people died in 2019 (World Health Organization, 2021). Nevertheless, saturated fat replacement with monounsaturated or polyunsaturated fatty acids have shown to have a beneficial impact on cardiovascular diseases and coronary heart disease events (SACN, 2019).

One interesting strategy to decrease saturated fat in food products is through food reformulation, by replacing saturated fat with unsaturated fat. However, the replacement of saturated fat by unsaturated fat in food products such as baked goods is very challenging due to the specific technological functionality of saturated fat in the food product. The fatty acid composition influences the lipid oxidation rate (Gunstone, 2009; Hernandez and Kamal-Eldin, 2013): oils with high content of unsaturated fatty acids are more susceptible to lipid oxidation, which can lead to undesirable flavour profile, shorter shelf life and loss of nutritional quality in food products (McClements and Decker, 2000; McClements, 2016c). Therefore, novel strategies for food reformulation and processing are being investigated and developed to for incorporating unsaturated fats into food products with the least impact on product processability, quality and shelf life. In recent years, there has been increasing interest in formulating nanoemulsion as lipid-based delivery systems with improved physicochemical stability of functional compounds, texture and fatty acid profile (Santhanam *et al.*, 2015; Joung *et al.*, 2016; Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Nanoemulsions with oil droplets particle size of less than 200 nm (Komaiko and McClements, 2016) could provide several potential benefits in food products such as improving physical stability to gravitational separation of the fat phase in the matrix (Kim *et al.*, 2014), changing the physical properties and sensory perception of the product (Mason *et al.*, 2006; Morley, 2011), and improving physicochemical stability of functional compounds and unsaturated oils (Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Thus, the use of nanoemulsions as delivery systems of oils could be a strategy to improve the stability of unsaturated oils in complex food products.

The formulation of the nanoemulsion depends not just on the fat phase used but also on the selection of emulsifiers. Combining emulsifiers can improve the formation, stability and functional properties of emulsions (McClements and Jafari, 2018). The addition of certain polysaccharides in emulsions can increase the thickness of the interface around the droplets and are able to improve the oxidative stability of the emulsion (Jacobsen *et al.*, 2008; Kargar *et al.*, 2011). Hydroxypropyl methyl cellulose (HPMC) is a carbohydrate polymer, with surface activity, high swellability, and thermal gelation properties (Yoo and Um, 2013; Li *et al.*, 2013b). In addition, the addition of HPMC into emulsions can improve the stability of the emulsion by inhibiting droplet aggregation; HPMC form a three-dimensional network in the continuous phase limiting droplet coalescence (McClements and Jafari, 2018; Sanz *et al.*, 2015b). Soy lecithin is an emulsifier widely used in the food industry (Klang and Valenta, 2011) to produce clean label food products. It is an amphiphile molecule derived from sn-glycero-3 phosphate (Goindi *et al.*, 2016), so it can be adsorbed to oil-water interfaces and can work as a surfactant. Therefore, the incorporation of two emulsifiers such as lecithin and HPMC into nanoemulsions could improve the technological functionality of nanoemulsions when used as a saturated fat replacer in food products.

Biscuits are very popular bakery products among consumers due to their convenient on-the-go format and palatability (Forker *et al.*, 2011; Singh *et al.*, 2015). Short dough biscuits specifically contain higher fat and sugar content than other biscuits such as hard developed biscuit (Edwards, 2007). This makes short dough biscuits an excellent target for reformulation. In terms of the reformulation strategies of saturated fat reduction in bakery product specially in biscuits, there have been a number of studies focusing on using vegetable oils with high content of PUFA and MUFA such as sunflower oil, olive oil, canola oil, and rapeseed oil as a saturated fat replacers (Curti *et al.*, 2018; Jang *et al.*, 2015; Mert and Demirkesen, 2016; Onacik-Gür and Żbikowska, 2020; Devi and Khatkar, 2017; Jacob and Leelavathi, 2007). The reformulation strategies studied include the replacement by bulk using sunflower oil (Devi and Khatkar, 2017; Jacob and Leelavathi, 2007); adding the oil in structured oleogels (Jang *et al.*, 2015; Mert and Demirkesen, 2016; Onacik-Gür and Żbikowska, 2020); and mixing the oils with solid fats such as cocoa and shea butter, and fibres to form structured emulsion (Curti *et al.*, 2018). Although these studies showed a reduction of saturated fat, there was a lower reduction of the total amount of fat in the final biscuits. In general, biscuits formulated with the saturated fat replacer showed higher spreadability and harder texture than the control (Devi and Khatkar, 2017; Jacob and Leelavathi, 2007). Other studies have investigated the incorporation of polysaccharides into oil in water emulsions to produced fat replacers that could be used to reduce both total fat and saturated fat in biscuits (Onacik-Gür *et al.*, 2015; Sanz *et al.*, 2017; Tarancón *et al.*, 2013; Giarnetti *et al.*, 2015). Giarnetti *et al.* (2015) reported that 50% butter replacement in shortbread cookies with an emulsion filled gel based on inulin and extra virgin olive oil provided a good texture and consumer acceptance. On another study, the fat reduced biscuits presented higher spreadability and required more force to break than control biscuits (Tarancón *et al.*, 2013; Sanz *et al.*, 2017). Furthermore, the increasing level of unsaturated fatty acids in a fat replacer can lead to greater lipid oxidation due to autoxidation of the oil (Choe and Min, 2006) causing problems of shelf life and rancidity in the end product (Patrignani *et al.*, 2014). Therefore, the incorporation of lecithin and HPMC into nanoemulsions is an interesting strategy to improve the stability and physical properties of a nanoemulsion when used as a saturated fat replacer.

To our knowledge, there is no studies formulating nanoemulsion stabilised with a combination of emulsifiers (lecithin and HPMC) as strategy to produce a saturated fat replacer for bakery products. Therefore, the hypothesis of this study was that nanoemulsions formulated with vegetables oils with high content of unsaturated fatty acids could work as saturated fat replacers improving the nutritional fatty acid profile of bakery products. By formulating a complex structured nanoemulsion with HPMC, lecithin and a vegetable oil, the physical properties and oxidation stability limitations of unsaturated fatty acids can be overcome in bakery product applications.

The specific objectives of the study were as follows:

- To investigate the effect of the oil type on nanoemulsion properties and stability;

  Chapter 2: Physical and chemical characterisation of conventional and nano/emulsions: influence of vegetable oils from different origin

- To optimise the nanoemulsion processing parameters of high-pressure homogenisation;

  Chapter 3: Optimisation of the physicochemical stability of extra virgin olive oil-in-water nanoemulsion: processing parameters and stabiliser type

- To develop a nanoemulsion formulation that could replace the technological properties of butter

  Chapter 4: Influence of hydroxypropyl methylcellulose (HPMC) hydrophobicity and concentration on its rheological, textural and surface activity properties

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- To apply the nanoemulsion as a saturated fat replacer into short dough biscuits.

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Chapter 7: General discussion and recommendations for future studies

1.2 Lipids in food products

1.2.1 Overview

1.2.1.1 Fatty acids

Fatty acids are the primary components of lipids in food. The chemical structure of a fatty acid is a hydrocarbon chain of between 4 and 24 atoms with a methyl (CH_3) group and a carboxylic acid (COOH) group at the end of the chain. Regarding the presence and number of carbon-carbon double bonds in the fatty acid chains, fatty acids can be divided into three main groups including saturated, monounsaturated and polyunsaturated fatty acids (O'brien, 2008). Regarding saturated fatty acids, saturated fatty acids contain no double bonds between the carbon atoms because they are saturated with hydrogen. The structure of saturated fatty acids is straight and linear (Figure 1.1). Some saturated fatty acids are: lauric (C-12:0), myristic (C-14:0), palmitic (C-16:0) and stearic (C-18:0) (O'brien, 2008).

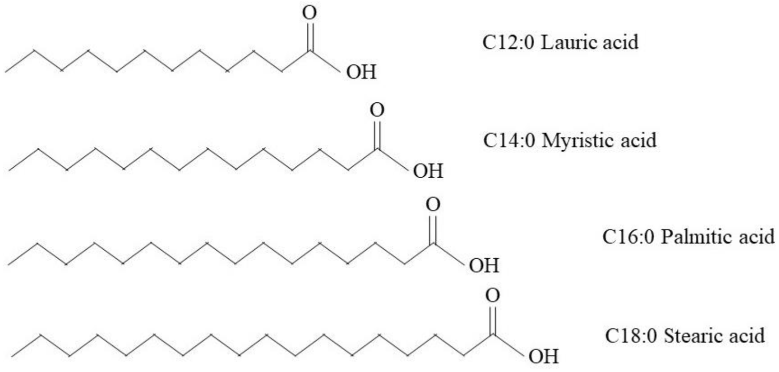


Figure 1.1. Saturated fatty acid structures

In terms of unsaturated fatty acids, the structure of unsaturated fatty acids contains one or more double bonds which can be arranged in either the cis or trans configuration (Talbot, 2011). Unsaturated fatty acids can be classified into monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). MUFAs contain only one double bond whereas PUFAs contain two or more double bonds. The cis conformation containing 18 carbon atoms are mainly occur in nature (Talbot, 2011) including oleic acid (C18:1 ω9) and linoleic acid (C18:2 ω6) (Figure 1.2).

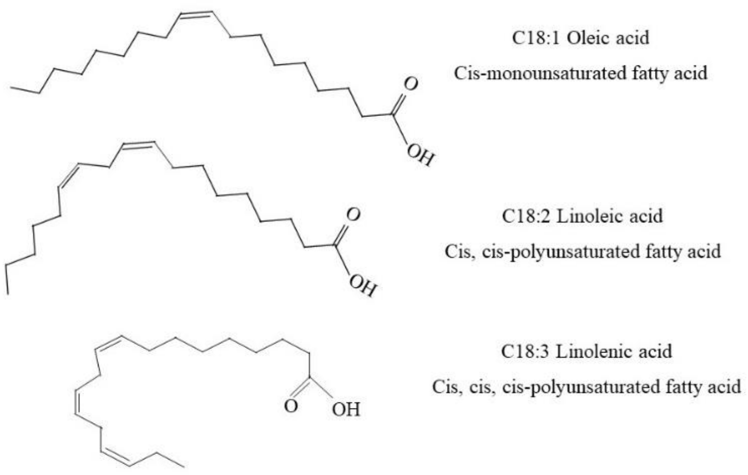


Figure 1.2. Unsaturated fatty acid structures

The structure of the fatty acids defines the physical and chemical properties of lipids depending on: chain length, the number and position of the double bonds, and the position of the fatty acids combined with glycerol and stearic (Gunstone, 2009; O'brien, 2008).

1.2.1.2 The physical and chemical properties of fatty acids

(i) Melting point

The melting point of fatty acids depend on the number of double bonds and carbon atoms in chain (Gunstone, 2009; Hernandez and Kamal-Eldin, 2013). There is a higher melting point of saturated fatty acids because of the straight and linear structure compared to unsaturated fatty acid (Gunstone, 2009). The melting points of saturated fatty acids is higher when the number of carbon atoms increased. Moreover, the melting points of fatty acid will decrease if the number of double bonds increase. Therefore, unsaturated fatty acids have a lower melting point than saturated fatty acids.

(ii) Lipid oxidation

The autoxidation is a free radical chain process which there are three main steps including initiation, propagation and termination (Figure 1.3) (Gunstone, 2009). The double bonds of fatty acids play an important role in the initiation step because the hydrogen atom attached to the carbon between double bond is easily removed and provide alkyl radicals (Choe and Min, 2006). This process needs energy such as heat, light and metals to remove the hydrogen atom. As a result, the higher the number of double bonds the greater oxidation, thus PUFAs are more sensitive to oxidise than MUFAs (Gunstone, 2009). The further stage is a propagation, the lipid alkyl radicals react with oxygen and produce lipid peroxy radicals before producing the primary lipid oxidation products as lipid hydroperoxides and secondary lipid oxidation as the core aldehyde and the volatile component. The propagation stage might continue several cycles until generate of stable products in termination process (Schaich, 2017).

Initiation	RH	$\longrightarrow$	$R\cdot + H\cdot$	alkyl radical
Propagation	$R\cdot + {}^3O_2$	$\longrightarrow$	$ROO\cdot$	peroxy radical
	$ROO\cdot + RH$	$\longrightarrow$	$ROOH + R\cdot$	hydroperoxide/ volatile
Termination	$ROO\cdot + R\cdot$	$\longrightarrow$	$ROOR$	stable products
	$R\cdot + R\cdot$	$\longrightarrow$	RR	stable products

R: lipid alkyl

Figure 1.3. Mechanisms of autoxidation

1.2.1.3 Fat sources and extraction process

Fats with higher proportion of saturated fatty acids can be from animal sources such as dairy butter fat (Bobe *et al.*, 2007; Talbot, 2011); or from vegetable sources such as palm oil and coconut oil (Bhatnagar *et al.*, 2009). On the other hand, there are several vegetable oils containing high level of unsaturated fatty acid. MUFAs are mainly found in olive oil (Szydłowska-Czerniak *et al.*, 2008; Poyato *et al.*, 2014b) and rapeseed oil (Szydłowska-Czerniak *et al.*, 2008). In addition, the major sources of PUFAs are found in sunflower oil and flaxseed oil (Bhatnagar *et al.*, 2009; Poyato *et al.*, 2014b; Elkin *et al.*, 2018).

The extraction of fats from their food matrix can lead to changes in their chemical composition; this process includes extraction and refining (Figure 1.4). Fat is generally extracted by pressing or by solvent extraction followed by refining of crude fats (Stauffer, 1996; O'brien, 2008). Cold press extraction is environmental friendly extraction methods that does not use chemical and heat (Cakaloglu *et al.*, 2018). The refining process involves either chemical or physical refining which includes several stages: degumming, neutralisation, bleaching and deodorization (Gunstone, 2009). These stages are responsible for eliminating the impurities such as phospholipids (Pal *et al.*, 2015), free fatty acids (FFA) (Costa *et al.*, 2011), colour compounds (Karabulut *et al.*, 2005) and moisture (Zhu *et al.*, 2016). The objective of the refining process is to provide the desirable organoleptic properties: a bland taste and odourless product for human consumption and better storage stability. The unrefined oils have higher free acidity, acid value and peroxide value than refined oils (Codex Alimentarius Commission, 1999; Codex Alimentarius Commission, 2003). Therefore, fatty acid profile and production of fats play an important role in the physicochemical properties of these compounds.

Regarding stability of fats, fats from different extraction process could lead to a difference in stability of oil. A higher content of free fatty acid in unrefined oils promote lipid oxidation in both bulk oils and emulsions (Yi *et al.*, 2013; Waraho *et al.*, 2011). Free fatty acids can work as prooxidant to accelerate the decomposition of lipid hydroperoxides, giving free radicals to form a secondary lipid oxidation product (Frega *et al.*, 1999). However, free fatty acids are removed in refining stages such as degumming, bleaching and deodorization at 91.4% of initial total free fatty acids (Ortega-García *et al.*, 2006). In addition, the differences in the TPC may be attributed to the different extraction and refining conditions used. According to the literature, the refining process causes the reduction of total phenolic content in oils (Janu *et al.*, 2014; Szydłowska-Czerniak *et al.*, 2008). Moreover, micronutrients including tocopherols, sterols and polyphenols are destroyed by refining processes, especially in deodorization (Fine *et al.*, 2016; Zacchi and Eggers, 2008). Regarding the extraction method, solvent extraction can provide a higher content of phenolics due to ability of solvents to extract phenolic compounds (Lee *et al.*, 2015). According to the literature, the refining process caused the reduction of total phenolic and tocopherols content in oils and the decreasing of scavenging activity (Janu *et al.*, 2014; Szydłowska-Czerniak *et al.*, 2008).

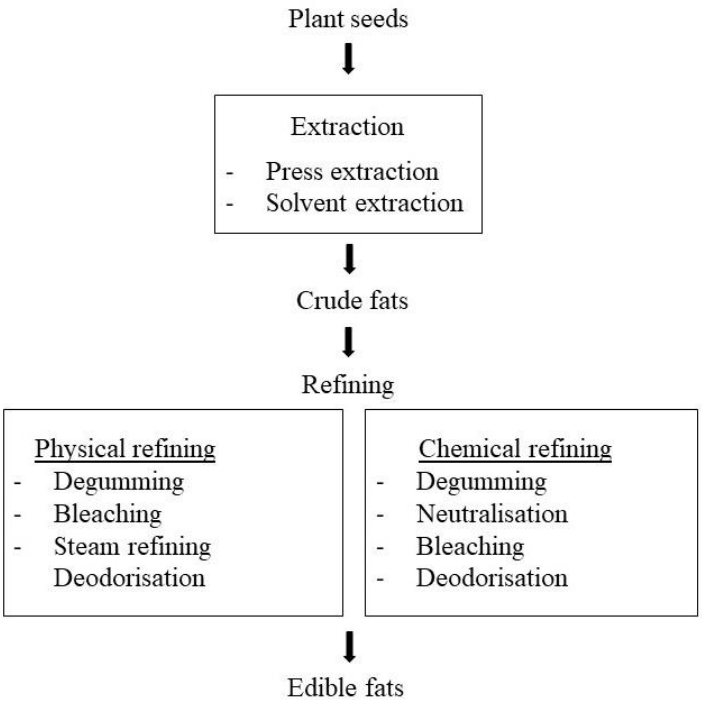


Figure 1.4. Edible fat processing

Source: Adapted from O'brien (2008) and Chen *et al.* (2011)

1.2.1.4 Fat ingredients in food products

Fats are typically found in food products including butter, margarine and shortening (Gunstone, 2009); however, each of them have a different total fat content and fatty acid composition. the requirement of fat content in butter and margarine are not less than 80 per cent (United States Department of Agriculture, 1989; United States Department of Agriculture, 1996). Butter has a significant higher level of SFAs (Bobe *et al.*, 2007; Esmaeilifard *et al.*, 2016; Roe *et al.*, 2013) than margarine (Esmaeilifard *et al.*, 2016; Kuhnt *et al.*, 2011) and shortening (Kuhnt *et al.*, 2011) with a high proportion of palmitic (C16:0) in all of them.

Butter is a water-in-oil emulsion made from milk; the cream is separated by centrifugation, and then churning and kneading operations are performed (Gunstone, 2009). On the other hand, margarine and shortening are made from vegetable oils or blending vegetable with animal oils using hydrogenation process (Ghotra *et al.*, 2002). The main difference between margarine and shortening is that margarine contains some water whereas shortening is anhydrous fat (Stauffer, 1996). Through a partial hydrogenation process fat crystals are developed; in the hydrogenated vegetable oils they are generally present as β crystals form. This provides a higher melting point compared to fats with α and β´crystals (O'brien, 2008). The greater crystallisation also can improve texture and oxidative stability (O'brien, 2008). However, the partial hydrogenation can lead to the formation of trans fatty acids.

Regarding the physicochemical properties of fat containing high level of saturated fatty acids and partial hydrogenated fats, they are more thermodynamically stable and present higher crystallization due to their linear structure of fatty acids. These properties have a positive impact on the oxidative stability and texture of some processed foods, such as bakery, dairy, and confectionary products (Wisconsin, 1991; Wilderjans *et al.*, 2013). However, when using fat with higher proportion of unsaturated fatty acids to replace saturated fats in food products reformulation it has been observed that there is a faster oxidative degradation during food processing and storage of the products (Silva *et al.*, 2010; Caponio *et al.*, 2013; Li *et al.*, 2013a; Li *et al.*, 2018). Moreover, due to the double bonds in cis configuration these structures have a lower melting point than saturated fatty acids (Gunstone, 2009; Hernandez and Kamal-Eldin, 2013), giving place to changes in texture and mouthfeel in the final product (Himawan *et al.*, 2006).

1.2.1.5 The role of fat in biscuits

Biscuits are a cereal based bakery product with a moisture content of less than 5 % (Manley, 2011a). There are different types which can be classified in many ways; each type of biscuit has a different amount of fat and sugar and can be classified into ten types (Figure 1.5) (Manley, 2011a) with the wire cut types presenting the highest fat content.

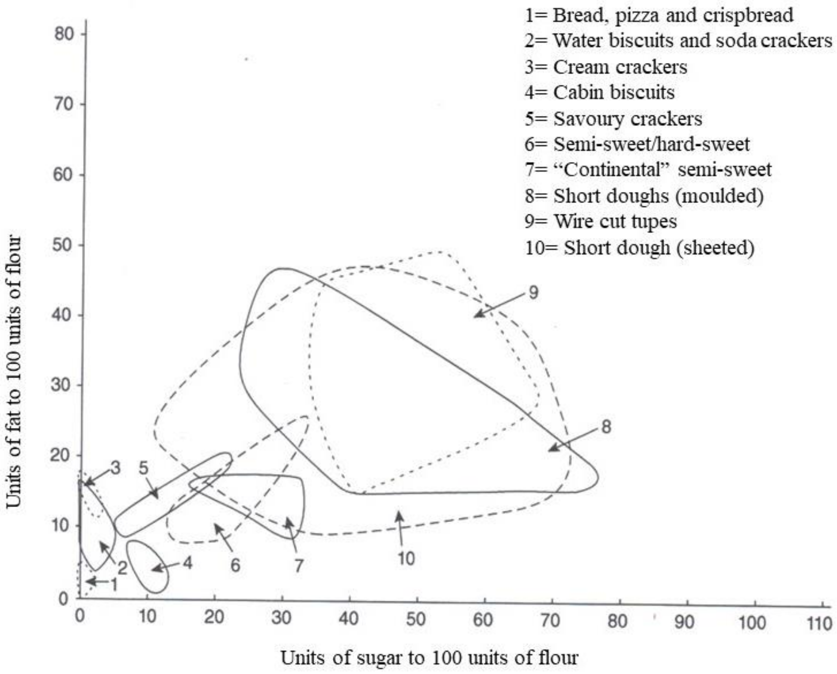


Figure 1.5. Relationship of fat and sugar content in biscuit recipes

Source: Manley (2011)

According to Edwards (2007), the dough of biscuits can be divided into three types; hard developed, semi-sweet and short doughs. Hard developed doughs are low in fat and sugar, which they are suitable for making crackers whereas semi-sweet doughs and short doughs content higher fat and sugar content. In terms of short doughs, there are two stages mixing the doughs regarding producing the fat-in-water emulsion before adding the flour (Edwards, 2007). The main functionality of fat in biscuit doughs is coating the flour during the mixing process in order to prevent the formation of gluten network, giving place to a more plastic and cohesive that gives place to a brittle texture after baking. Fat also provided stability to the gas bubbles in the dough; the fat coats the air dough interface during mixing and maintain their stability during the baking process. This leads to a more aerated structure and higher expansion in products (Stauffer, 1996; Atkinson, 2011).

1.2.2 Fat replacers in bakery products

1.2.2.1 Carbohydrate-based fat replacers

Carbohydrate-based fat replacer is the largest group of fat replacers (Swanson, 2006) (Table 1.1); they include starches, gums, fibres, maltodextrin and cellulose (Jones, 1996; Morley, 2011; Atkinson, 2011). Recently, carbohydrate-based fat replacers have been used in bakery products such as in muffin, biscuit and cake (Table 1.2). Carbohydrate-based fat replacers can contribute to the strength of the crumb structure through hydrogen bonds among their molecules and with starch molecules (Psimouli and Oreopoulou, 2013). The reduction of the fat content in the system may have hindered some of the main functionalities of fat in a dough system, lubrication and aeration (Pareyt and Delcour, 2008). Common adverse effects of using carbohydrate-based fat replacers in bakery products have been the poor volume and higher hardness texture of product (Rodriguez-Sandoval *et al.*, 2017; Chung *et al.*, 2010; Psimouli and Oreopoulou, 2013). These results could be due to a lower incorporation of air bubbles during mixing and weaker stability of the air phase during baking when fat was replaced, as fat replacers did not entrap and stabilise air cells effectively.

Table 1.1. Classification of carbohydrate-based fat replacers

Fat replacers		Energy density (Cal/g)	Reference
Starch	Native starches (such as tapioca, wheat, oat, corn, pea, rice, potato, pea, waxy corn)	4	Ognean *et al.* (2006); Peng and Yao (2017)
	Modified starches (Modified by acid, enzymatic hydrolysis, crosslinking, mono-substisution; pregelatinisation, instant forms)	1- 4	
Gums	Guar, Xanthan, Locust bean, Carrageenan, Gum Arabic, Pectins	noncaloric	
Fibre	Microcrystalline cellulose and powered cellulose	noncaloric	
	Methylcellulose, carboxymethyl cellulose, and hydroxypropyl methylcellulose		
	β-glucan	1-4	
	Inulin	1.5	
Maltodextrins		4	
Polydextrose		1	

Table 1.2. Examples of carbohydrate based-fat replacers in bakery products

Fat replacement ingredient	application	Per cent replacement	references
Modified cassava starch	Replacement of butter and margarine in muffins	8	Rodriguez-Sandoval *et al.* (2017)
Waxy corn starch	Replacement of butter in muffins	40	Chung *et al.* (2010)
Guar gum and polydextrose	Replacement of hydrogenated fat in soft dough biscuit	70	Chugh *et al.* (2015)
maltodextrin, inulin, oligofructose, citrus pectin, and microparticulated protein	Low fat cakes	65	Psimouli and Oreopoulou (2013)
Inulin	Replacement of sunflower oil in sponge cake	20	Rodríguez-García *et al.* (2012)
Inulin	Replacement of margarine in muffins	50	Zahn *et al.* (2010)
Inulin and hydroxypropyl methylcellulose (HPMC)	Replacement of shortening in biscuits	15	Laguna *et al.* (2014)

Hydroxypropyl methyl cellulose (HPMC) is a non-ionic cellulose ether, which contains hydrophobic methoxy and hydrophilic hydroxypropyl groups on the cellulose chains added to the anhydro glucose backbone (Camino *et al.*, 2011; Wollenweber *et al.*, 2000) (Figure 1.6). HPMC is an hydrocolloid that could work as fat replacers because of its surface activity, mechanical, and thermo-reversible gelation properties of HPMC (Yoo and Um, 2013; Li *et al.*, 2013b). When HPMC is dispersed in water, hydrogen bonding between the hydroxyl groups in HPMC and water are formed (Guarda *et al.*, 2004; Poonnakasem *et al.*, 2018). It has been reported that HPMC could impact some stability to the air phase by increasing the viscosity of the continuous phase of the system, e.g. cake batter (Bousquières *et al.*, 2017). During baking, there is an increase of strong hydrophobic interactions between HPMC chains that gave place to a sol-gel transition (Lim *et al.*, 2021; Bodvik *et al.*, 2010). When incorporating HPMC to bakery products such as biscuit, a three-dimensional network of HPMC chain has been observed, where fat globules were stabilised and dispersed (Sanz *et al.*, 2017). However, from previous study (Laguna *et al.*, 2014), there was some negative effect when using HPMC as a fat replacer in bakery product at higher replacement. The results showed that biscuits had a harder texture and a decrease in the overall acceptability.

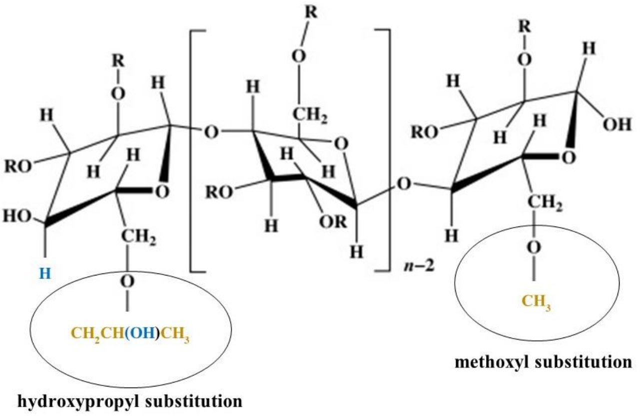


Figure 1.6 Chemical structure of HPMC. Hydrophilic groups and atoms in blue (-CH_3 or -H) and hydrophobic groups in yellow (-$CH_2CH(OH)$ CH_3) group. Source: Adapted from Ford (2014), Deshmukh *et al.* (2017) and Lim *et al.* (2021)

1.2.2.2 Lipid based fat replacers

(i) Structured emulsion as fat replacers

There are different ways to categorise oil structured technology such as based on chemical type of gelators, the molecular characteristics of gelators and number of gelators. Regarding the chemical type of gelators, there are two major types of gelators or structuring agents to incorporate liquid oils in food emulsions including lipid-based gelators such as waxes and monoglycerides (MGs), and non-lipid-based gelators such as ethyl celluloses (EC) (Patel and Dewettinck, 2016; Hwang *et al.*, 2016). Structured emulsion can be defined as oil-in-water systems where oil droplets are encapsulated in the layers of hydrated gelators (Patel and Dewettinck, 2016)(Figure 1.7). The interfaces around the dispersed oil droplets of structured emulsions are structured with crystallized

MAG layers. Structured emulsions provide a semi-solid structure with high viscosity and the desirable physical and organoleptic properties (Morley, 2011; Patel and Dewettinck, 2016).

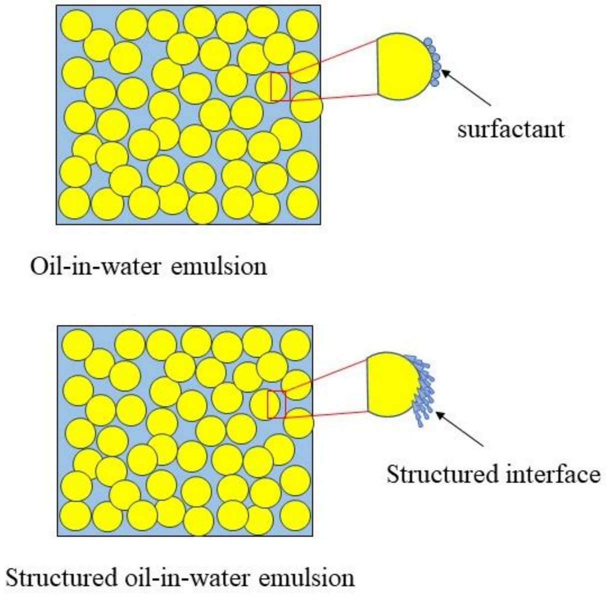


Figure 1.7. Oil-in-water emulsion and structured emulsion

Source: Adapted from Patel and Dewettinck (2016)

Wax structured emulsions are formed by a three-dimensional network of wax esters or n-alkanes arranged into microcrystalline platelets where oils can be entrapped and absorbed onto the surface of the network (Hwang *et al.*, 2016; Blake *et al.*, 2014). Various natural waxes such as candelilla wax, sunflower wax and rice bran wax have been successfully used as fat replacers in bakery product, such as muffins and cookies at concentration 2-10 % wax in oil (Hwang *et al.*, 2016; Lim *et al.*, 2017). Monoglycerides are commercial food emulsifiers, their structured emulsions have been reported to be an effective fat replacement in bakery products at a concentration 4 to 10 % of component (Manzocco *et al.*, 2012; Calligaris *et al.*, 2013; Wright *et al.*, 2014). In the case of non-lipid-based gelators, the most popular is ethyl cellulose and HPMC. They are hydrophobic derivative celluloses, that can be directly dispersed in oil and used to form structure emulsions that have been used as fat replacers in bakery products such as muffins and biscuits (Lim *et al.*, 2017; Giacomozzi *et al.*, 2018; Sanz *et al.*, 2017; Tarancón *et al.*, 2013). Structured emulsions made from non-lipid-based gelators have greater nutritional benefits as they can be used to reduce both saturated fat and total fat content in food products.

Table 1.3. Examples of structured fat replacers in bakery products.

structured fat replacers		Ingredient	Food products	References
lipid-based gelators	plant-based waxes, wax-based oleogels	Natural wax-vegetable oil organogels	cookies	Hwang *et al.* (2016)
		Candelilla wax -oleogels	muffins	Lim *et al.* (2017)
	Monoglyceride based-emulsion	Sunflower oil Monoglyceride based-emulsion	muffin	Giacomozzi *et al.* (2018)
		Palm oil with a monoglyceride-oil-water gel	short dough pastries	Manzocco *et al.* (2012)
Non-lipid-based gelators	polymer oleogels (Ethyl cellulose)	Sunflower oil-water-cellulose emulsion	biscuits	Sanz *et al.* (2017)
		Sunflower oil-water-cellulose ether emulsions	biscuits	Tarancón *et al.* (2013)

(ii) Complex structured emulsion as fat replacers

More recently, complex structures of emulsions have been developed (Figure 1.8 and Table 1.4) to be used as effective fat replacers in different food products by providing the desirable texture and organoleptic properties. Complex structured emulsions can be formulated by adding emulsifiers into emulsion, which the dispersed oil droplets can be structured with in the matrix of water phase gelled using polymer network (Patel and Dewettinck, 2016).

The addition of certain polysaccharides in emulsions can increase the thickness of the interface around the droplets, are able to improve the oxidative and physical stability of the emulsion (Jacobsen *et al.*, 2008; Kargar *et al.*, 2011) and can provide a desirable texture and sensory attributes such as smoothness and creaminess due to the characteristics (McClements, 2016b; Karimi *et al.*, 2015; Sala *et al.*, 2008). As shown in Table 1.4, polysaccharides have been used in combination with emulsions as fat replacers in food products (Poyato *et al.*, 2014a; Giarnetti *et al.*, 2015; Serdaroğlu *et al.*, 2017; Paglarini *et al.*, 2018).

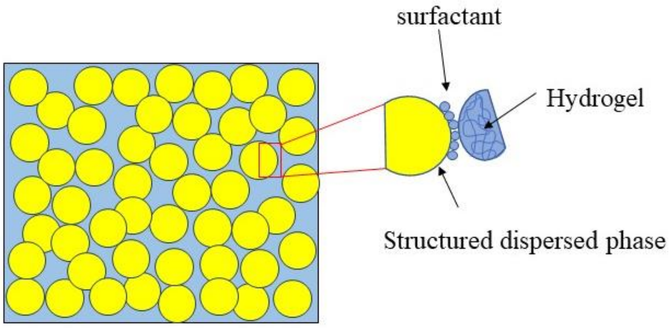


Figure 1.8. Complex-structures of emulsions and hydrogel

Source: Adapted from Patel and Dewettinck (2016)

As can be seen from Table 1.4, saturated fat replacers are mainly formulated with oils with high content of unsaturated fatty acid, which can lead to negative effects on the texture, lipid oxidative stability and sensory properties of the final product. The majority of these studies investigated the properties and applications of conventional emulsions. However, in order to improve physicochemical stability of unsaturated oils, there has been an increasing interest in formulating nanoemulsion as lipid-based delivery systems with improved physicochemical stability of functional compounds, texture and fatty acid profile (Santhanam *et al.*, 2015; Joung *et al.*, 2016; Komaiko and McClements, 2016; Pathakoti *et al.*, 2017).

Table 1.4. An overview of recent research on complex structured emulsions as fat replacer in food matrices.

Food matrices	Emulsion system	oils	surfactant	Polysaccharide	% Replacement	Reference
Pork sausages	Conventional emulsion	ω-3 PUFA from Linseed oil 40%	polysorbate 80 (surfactant-oil ratio of 0.003)	carrageenan 1.5%	8% (fat reduction 10.20%)	Poyato *et al.* (2014a)
Chicken patties	Conventional emulsion	Olive oil 46%	Polyglycerol polyricinoleate (PGPR) (6.4g/100 g oil)	inulin 9% and gelatin 3%	50% beef fat replacement	Serdaroğlu *et al.* (2017)
Meat product	Conventional emulsion	Soybean oil 50%	Soy lecithin 1%	carrageenan, inulin 1%	-	Paglarini *et al.* (2018)
Meat product	Conventional emulsion	Olive oil 5-40%	-	alginate and gelatin	-	Pintado *et al.* (2016)
Confectionery filling	Conventional emulsion	-	-	Maltodextrin 15 and 20%	15% hydrogenated soybean and palm oil blends	Hadnađev *et al.* (2014)
Shortbread cookies	Conventional emulsion	Extra virgin olive oil 37%	Soy lecithin 2%	Inulin 19%	50% butter replacement	Giarnetti *et al.* (2015)
Cookies	Conventional emulsion	Cold pressed sunflower oil 50 and 70%	-	Starch sodium octenyl succinate (OSA starch)	100% shortening replacement	Dapčević Hadnađev *et al.* (2015)

(iii) Nanoemulsions (NEs) as fat replacers

Emulsions are defined as colloidal dispersions of two immiscible phases (disperse and continuous phase). The diameter of the droplets in food emulsions generally is bigger than 200 nm, while the mean droplets diameter of NEs is smaller than 200 nm (Komaiko and McClements, 2016) (Figure 1.9). NEs could provide several potential benefits when used to formulate food products such as improving physical stability to gravitational separation of the fat phase (Kim *et al.*, 2014), modifying the physical properties and sensory perception of the product (Mason *et al.*, 2006; Morley, 2011), and improving physicochemical stability of functional compounds and unsaturated oils (Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Thus, the use of nanoemulsions as delivery systems of oils could be a strategy to improve the stability of unsaturated oils when incorporated in fat replacers systems. This research will develop and characterise the functionality of a fat replacer formulated by combining a NE, an emulsifier and a polysaccharide hydrogel.

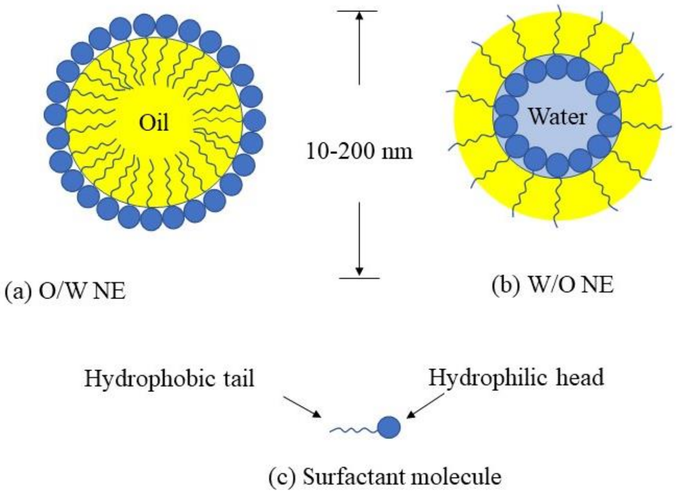


Figure 1.9. NE droplet and surfactant molecule

According to droplet particle size, emulsions can be classified into three types including conventional emulsion, nanoemulsion and microemulsion (Table 1.5).

Table 1.5. Classification of emulsion type based on droplet particle size and physicochemical properties.

Emulsion type	Droplet diameter range	Thermodynamic stability	Surface-to-Mass Ratio (m^2/g particles)	Optical properties	Method of preparation
Conventional emulsion	> 200 nm	Unstable	0.07 – 70	Turbid/ Opaque	High and low energy methods
Nanoemulsion	10 – 200 nm	Unstable	70 – 330	Clear/ Turbid	High and low energy methods
Microemulsion	< 100 nm	Stable	330 - 1300	Clear/ Turbid	low energy methods

Source: McClements and Rao (2011); Komaiko and McClements (2016)

1.3 Composition of nanoemulsions

1.3.1 Oil component

Oils consist of different triacylglycerol molecules depending on the type of fatty acids and the different positions of fatty acids attached to the molecules of glycerol. Both the molecular structure of oil and molecular interaction play a major role in properties of oil including melting point, rheology and density, which lead to an important influence on the formation and characteristics of NEs (McClements, 2016b).

The selection of the appropriated oil depends on the desirable nutritional, physicochemical and organoleptic characteristics of the final emulsion (Gunstone, 2009; McClements, 2016b). Due to the physical and chemical properties of unsaturated fatty acids (section 1.2.1.2), unsaturated fatty acids can lead to problems of oxidative stability and sensory properties during processing and storage (Choe and Min, 2006). This can lead to an undesirable flavour profile including off-flavours and off-odours (McClements, 2016b). Therefore, in recent years, NE technology has been used to improved oxidative stability of emulsion with unsaturated fatty acids profile (Polychniatou and Tzia, 2014; Mehmood, 2015). Oil in structured oleogelation technology has been also researched to convert liquid oil to solid-like gel structures with the functionality of fats, but with the nutritional profile of lipid oils oleogels (Jang *et al.*, 2015; Mert and Demirkesen, 2016; Onacik-Gür and Żbikowska, 2020). Oleogels showed both positive and negative effects on properties of bakery products. Although oleogels from high oleic sunflower oil showed an improvement of the nutritional profile (without trans-fat and low saturated fat) in muffin (Giacomozzi *et al.*, 2018), cookies prepared with carnauba wax or candelilla wax oleogels from sunflower oil had slightly harder texture and higher spread than samples prepared with shortening (Mert and Demirkesen, 2016).

1.3.2 Aqueous component

A water molecule consists of two hydrogen atoms attached to an oxygen atom. Each hydrogen atom has a single positive charge (δ^+) while oxygen atom has a single negative charge (δ^-). Due to these charges, water molecules are able to form hydrogen bonds with other polar molecules, such as proteins and carbohydrates, which allow to produce emulsion with different characteristics (McClements, 2016b). Water-soluble molecule such as surfactants and biopolymers are usually mixed to the aqueous phase in order to contribute to the physical, chemical and sensory properties of emulsion such as viscosity (Guerra-Rosas *et al.*, 2016; Alfaro *et al.*, 2015), physical stability (Arancibia *et al.*, 2016) and oxidative stability (Mehmood, 2015). The solubility of these molecules is influenced by the interaction with water (McClements, 2016b). They are dissolved in water before mixing with oil phase in order to develop oil-in-water NE. Therefore, the ratio of water to surfactants has an effect on developing NEs. In addition, water molecules interact with ionic molecules, namely salts, acids, proteins and polysaccharides. The positive charge (δ^+) of hydrogen atom of the water molecules interacts with a negative charge (δ^-) of the ion. The pH of aqueous phase also has an impact on the ionic degree of solutes and this interaction. As a consequence, ionic solutes play a vital role in the interaction between droplets, which lead to an effect on stability of oil-in-water emulsions, particular in the incorporation with ionic surfactants and biopolymer.

1.3.3 Emulsifiers

An emulsifier is a substance that is able to reduce the oil-water interfacial tension. Emulsifiers play a major role in protecting droplets coalescence, which lead to providing emulsification and improving emulsion stability (Kronberg *et al.*, 2014). There are various types of emulsifier used in food industry such as surfactants, phospholipids and amphiphilic biopolymers (McClements, 2016b). According to Tadros *et al.* (2004b) and McClements (2016b) small-molecule surfactants (Table 1.6) are effective emulsifiers to develop emulsions to incorporate in food products. Surfactants are amphiphilic molecules that consist of a hydrophilic head group and at least one hydrophobic tail as shown in Figure 1.9. A hydrophilic group is ionic and can be divided into several types including anionic, cationic, zwitterionic, and nonionic (McClements, 2016b) as shown in Table 1.6. The Hydrophile-Lipophile Balance (HLB) is a ratio of hydrophilic to lipophilic groups, which depend on the chemical structure of the surfactant and usually used to select the surfactants for specific application (Table 1.7).

Table 1.6. The general small molecules surfactants used in food emulsion.

Chemical Name	Abbreviation or common name	Solubility	HLB Number	Charge
Ionic				
Lecithin	-	Oil and water	2-8	Zwitterionic
Lysolecithin	-	water	8-11	Zwitterionic
Fatty acid salts	FA	Oil and water	1-3	anionic
Lauroyl arginate	LAE	Water		Cationic
Nonionic				
Polyglycerol polyricinoleate	PGPR	Oil	1.5	
Sorbitan monooleate	Span 80	Oil	4.3	
Sorbitan monostearate	Span 60	Oil	4.7	
Polyoxyethylene sorbitan monooleate	Tween 80	water	15	
Polyoxyethylene sorbitan monostearate	Tween 60	Water	14.9	
Polyoxyethylene sorbitan monopalmitate	Tween 40	Water	15.6	
Polyoxyethylene sorbitan monolaurate	Tween 20	Water	16.7	

Source: McClements (2016b)

Table 1.7. HLB number ranges of surfactant and the applications.

HLB value	Field of application
3-6	W/O emulsion
7-9	Wetting agent
8-18	O/W emulsion
13-15	Detergent
15-18	Solubiliser

Source: Tadros *et al.* (2004b); McClements (2016b)

Nowadays, consumers are increasingly demanding cleaner labels on food and beverage products; therefore, there is an increasing interest in using surfactants that are obtained from natural sources (Goindi *et al.*, 2016) that could be effective in creating stable nanoemulsions with minimal impact on their organoleptic properties. Regarding the clean label characteristic, Asioli *et al.* (2017) stated that there are three categories of clean label: organic, natural and 'free from' artificial additives/ingredients. Lecithin can be extracted from natural sources such as soybeans, egg and milk. The molecules of lecithin are derived from *sn*-glycero-3 phosphate. As shown in Figure 1.10, the lipophilic groups are fatty acids that attached to carbon atom position 1 and 2 while the hydrophilic groups generally are zwitterionic of amino alcohol (phosphatidylcholine; PC and phosphatidylethanolamine; PE), and anionic of polyvalent alcohol (phosphatidylinositol; PI) (Bueschelberger, 2004; McClements, 2016b). The HLB value of lecithin is typically 2-8. Thus, lecithins are capable of producing W/O emulsions. Regarding O/W emulsion formation, lecithins are a potential surfactant to combine with others such as nonionic surfactants in order to improve stability of emulsion (Paradiso *et al.*, 2015; Mehmood, 2015; Sahafi *et al.*, 2018).

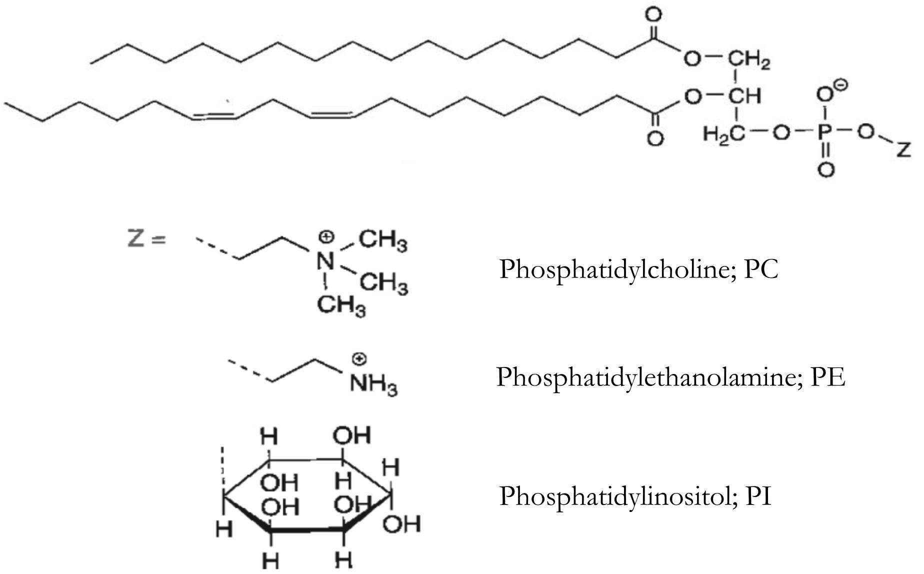


Figure 1.10. 1,2-diacyl-sn-glycero-3-phospholipids

Source: Bueschelberger (2004)

1.4 Formation of nanoemulsions

1.4.1 High energy homogenization

The formation of emulsions is mainly affected by ingredient composition (oil, surfactant, water) and process parameters (Öztürk, 2017). An emulsion can be formulated with either a single step or many consecutive steps according to the initial material and the method used to develop it.

High-energy homogenization is a two-step formation process which includes the creation of a macro-emulsion in first stage (primary homogenization) and the reduction of droplet size from macroparticles to nanoparticles in a second stage (secondary homogenization) (Figure 1.11).

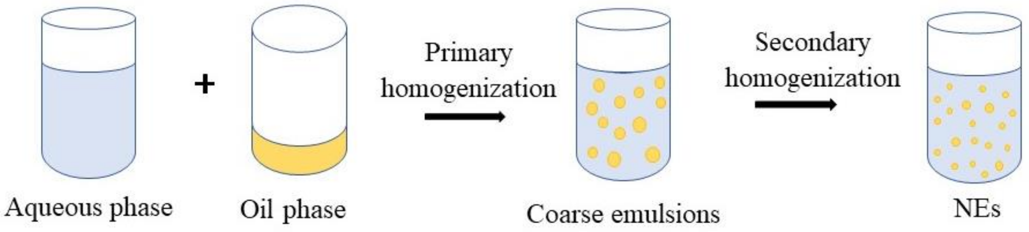
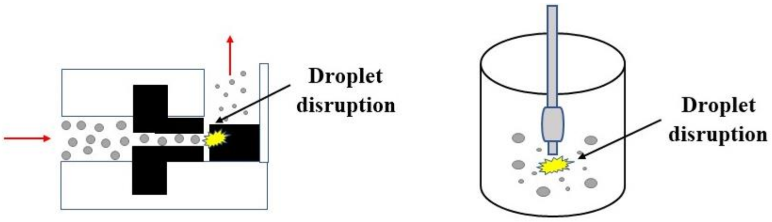


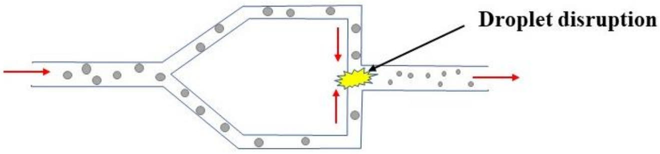
Figure 1.11. Scheme of nanoemulsions

In the primary stage, a coarse emulsion is usually produced by using a high-speed homogenizer whereas the secondary stage, the droplet size is normally reduced by using a homogenizer: ultrasonication, high pressure homogenization or microfluidizers (McClements, 2012) (Figure 1.12). A high-pressure valve homogenizer is a common device to produce fine emulsion. A coarse emulsion could be fed into a chamber and pass through a narrow channel of a high-pressure valve homogenizer (McClements and Rao, 2011; Öztürk, 2017). The second stage could be also performed with a microfluidizer, which the process generates in an interaction chamber. A coarse emulsion is pumped to flow through microchannels before they interact with each other at an impingement area (Maa and Hsu, 1999; McClements, 2016a). The second stage could also take place in an ultrasonic processor, where the NE droplets are created by cavitation with high-energy sound waves (frequency of sound waves at >20 kHz) (Jafari *et al.*, 2007).

High-energy methods could be used with various oils and emulsifiers, and they are high yield processes (Öztürk, 2017; Goindi *et al.*, 2016). This approach was selected for emulsion formulation in this study.



(a) High-pressure valve homogeniser (b) Ultrasonic probe homogenizer



(c) Microfluidizer

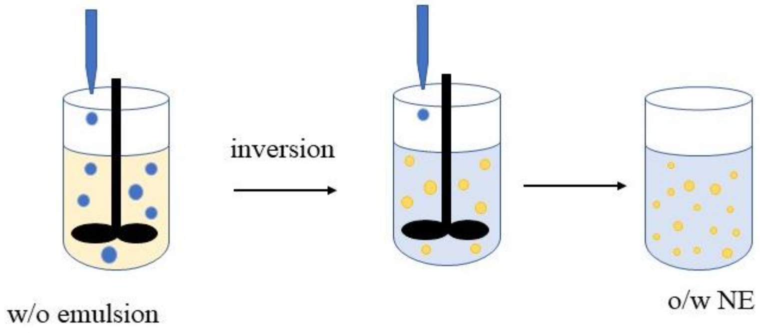
Figure 1.12. High-energy methods

Source: Adapted from McClements and Rao (2011) and Öztürk (2017)

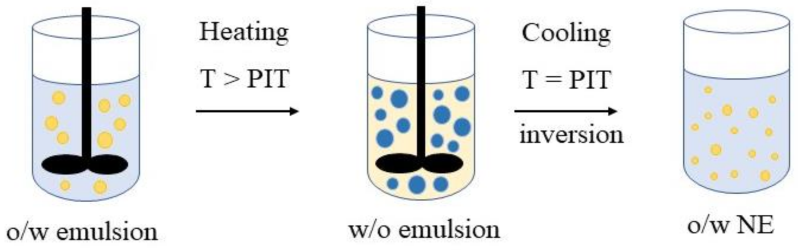
1.4.2 Low-energy methods

Through this approach the small droplets are created because the system changes in composition or temperature. There are two main methods: emulsion inversion point (EIP) and phase inversion temperature (PIT). As regards the EIP, the system which usually consist of oil and surfactant, is titrated with water while temperature is increased gradually until the system reaches an inversion point in which the emulsion changes from a w/o to an o/w/o before forming an o/w emulsion or NE (Komaiko and McClements, 2016). In contrast, The PIT method is related to the effect of temperature changes on the molecular geometry and solubility of non-ionic surfactants. First of all, a coarse o/w emulsion is formulated at room temperature before heating up to slightly above the PIT which allow non-ionic surfactant is more stable in oil phase and forming w/o emulsion. Following this step, the emulsion is rapidly cooled, then an o/w NE is produced because surfactant is more hydrophilic (Komaiko and McClements, 2016; McClements, 2016a) (Figure 1.13).

The advantages of the low-energy methods are lower operating costs, simple approaches with an effective formation of small particle sizes. However, they require specific types of oils and higher concentration of synthetic surfactant compared to high-energy methods (Komaiko and McClements, 2016; Goindi *et al.*, 2016).



(a) Emulsion inversion point (EIP)



(b) Phase inversion temperature (PIT)

Figure 1.13. Low-energy methods

Source: Adapted from Gupta *et al.* (2016)

1.5 Emulsion stability

Emulsion stability can be defined as the ability of an emulsion to keep its physicochemical properties unchanged over a period of time. The mechanisms driving physical instability in emulsions are gravitational separation (creaming or sedimentation), flocculation, coalescence, phase inversion, and Ostwald ripening; whereas lipid oxidation and hydrolysis are notable examples of chemical instability (McClements, 2016c)(Figure 1.14).

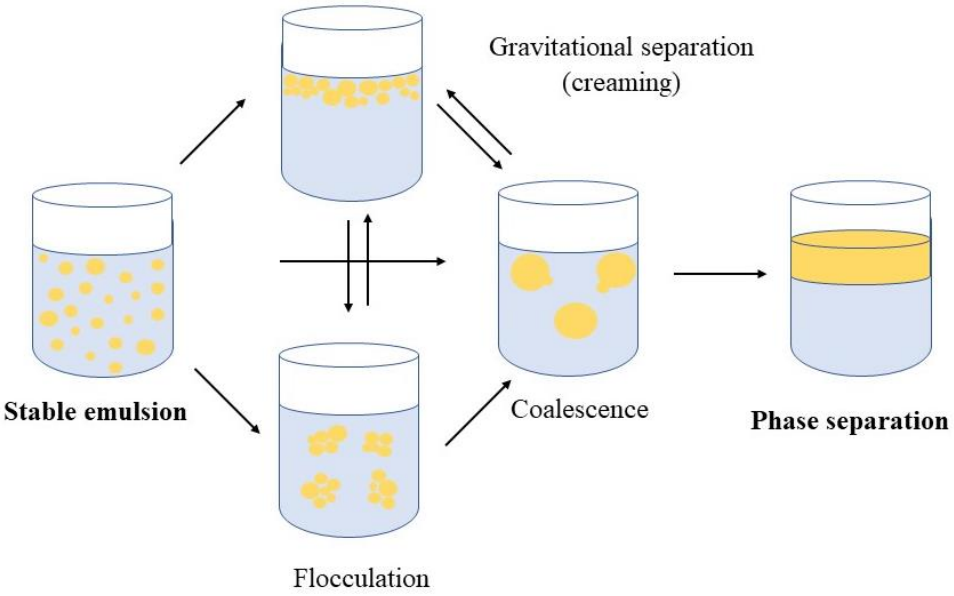


Figure 1.14. Schematic representation of nanoemulsion breakdown process.

Source: Adapted from McClements and Jafari (2018)

1.5.1 Gravitational separation

The gravitational separation is a typical physical instability in emulsions due to a different density of the droplets and continuous phase. There are two main forms of gravitational separation including creaming and sedimentation. If droplets have a lower density than continuous phase, they tend to move upward and form as creaming at the top of the emulsion. On the other hand, if droplets have a higher density than continuous phase, they tend to move downward and form as sedimentation at the bottom of the emulsion. The rate of gravitational separation can be calculated using Stokes' law (McClements, 2016c):

$$V_{stokes} = -\frac{2gr^2(P_2 - P_1)}{9\eta 1} \qquad (1.1)$$

where V is the velocity of dispersed phase, r is the radius of the particle, P_2 is the density of dispersed phase, P_1 is the density of continuous phase, $\eta 1$ is the viscosity of continuous phase, and g is gravity force. In terms of particle size, smaller droplets in NE provide a greater stability to gravitational separation due to the lower attractive forces between the droplets (Mason *et al.*, 2006; Liang *et al.*, 2007).

1.5.2 Droplet aggregation

A collision between the droplets can lead to aggregation, which mainly include flocculation and coalescence. Collisions can be induced by Brownian motion, gravitational separation, or even mechanical forces (McClements, 2016c). Flocculation is the process by which at least two droplets associate with each other, but their particle size remains unchanged, they maintain their individual integrity. Coalescence is the process by which two droplets merge together into one larger droplet. The aggregation of droplets in emulsions depends on colloidal interactions, mainly on attractive and repulsive forces between the droplets. Regarding a first approximation of colloidal interactions, the overall interactions between the droplets can be described by the sum of various attractive interaction including van der Waals and hydrophobic, and repulsive interaction including steric and electrostatic (McClements, 2016c; Tadros *et al.*, 2004b).

1.6 Thesis layout

In order to guide the reader, a brief explanation of the Thesis structure is provided here. The core structure of this work comprises of an introduction, five experimental chapters, and concluding remarks including considerations for further work (Figure 1.15). The first experimental chapter (**Chapter 2**) focus upon the effect of the type of oil on physical and chemical characterisation of nano/emulsions in order to select suitable oils for developing nano/emulsions. The second experiment chapter (**Chapter 3**) focuses on the optimisation of the emulsion processing parameters to formulate a clean label nanoemulsion. The following two chapters (**Chapter 4** and **Chapter 5**) focus on the formulation of a complex nanoemulsion, initially by understanding the properties of structuring hydrocolloids (HMPCs) and then evaluating the properties of nanoemulsions made with mixed emulsifiers (lecithin and HPMC) to stabilise and improve the technological functionalities of the nanoemulsion as a saturated fat replacer. The last experimental chapter (**Chapter 6**) focus on the application of the developed complex nanoemulsion on shorth dough biscuits by studying the effect of fat replacement on the physical characteristics and sensory attributes of the final product.

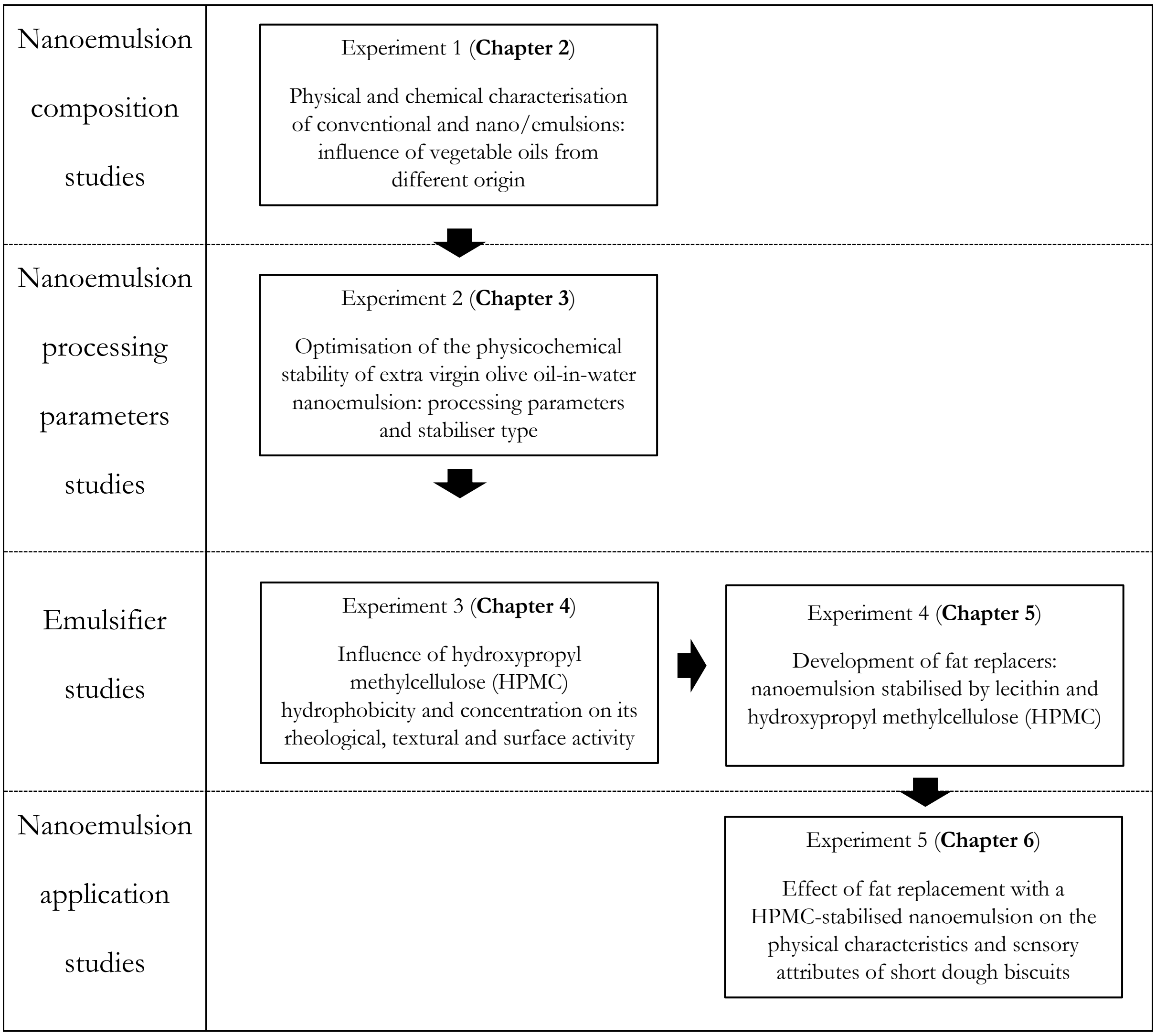


Figure 1.15. Illustrative plan mapping the order of experimental works.

Chapter 2

Physical and chemical characterisation of conventional and nano/emulsions: influence of vegetable oils from different origin

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Abstract

The processes of oil production play an important role in defining the final physical and chemical properties of vegetable oils (Stauffer, 1996; O'brien, 2008), which have an influence on the formation and characteristics of emulsions (Gunstone, 2009; McClements, 2016b). Therefore, the objective of this study was to investigate the correlations between oil physical and chemical properties with the stability of conventional and nanoemulsions. Five vegetable oils obtained from different production processes and with high proportion of unsaturated fatty acids were studied: extra virgin olive oil (EVOO), cold pressed rapeseed oil (CPRO), refined olive oil (OO), refined rapeseed oil (RO) and refined sunflower oil (SO) were used in this study. The results showed that the physicochemical stability of emulsion was affected by fatty acid composition, the presence of antioxidants and free fatty acids in oils. There was a significant positive correlation ($p < 0.05$) between the fraction of unsaturated fatty acids and emulsion oxidative stability, which SO, OO and EVOO showed a significantly higher lipid oxidative stability compared to RO and CPRO emulsions. Moreover, MDD and PDI of conventional and nano/emulsions with different type of oils showed a significant negative correlation ($p < 0.01$) with thermal stability, whereas there were insignificant correlations with TBARs. Nanoemulsions showed better physical stability than conventional emulsions. This study suggests that EVOO, OO and SO could be a suitable oil to formulate emulsion with greater physical and chemical stability, particularly EVOO due to presenting a higher radical scavenging activity.

Keywords: Refined vegetable oil, Virgin vegetable oil, Fatty acids, Antioxidants, Creaming index, Lipid oxidation, Emulsion

2.1 Introduction

In recent decades, there has been an increasing interest from consumers, food industry and health organisations in vegetable oils with high content of unsaturated fatty acids due to their effect on human health compared to saturated fatty acids. The consumption of saturated fatty acids increases the risk of cardiovascular diseases and increases low-density lipoprotein (LDL) and cholesterol in blood (Ebbesson *et al.*, 2015; Sun *et al.*, 2015). World Health Organization and other governmental organisations have consistently highlighted the importance of controlling saturated fat and trans-fat content in food products in order to decrease disease burden in the population (Regulation, 2011; World Health Organization, 2015). However, the replacement of saturated fat by vegetable oils with high content of unsaturated fatty acids is very challenging due to the specific technological functionality of saturated fat in food products. The fatty acid composition influences their lipid oxidation rate (Gunstone, 2009; Hernandez and Kamal-Eldin, 2013); oils with high content of unsaturated fatty acids are more susceptible to lipid oxidation, which can lead to undesirable flavour profile, texture, shelf life and loss of nutritional quality in food products (McClements and Decker, 2000; McClements, 2016c).

There are several vegetable oils containing high level of unsaturated fatty acids; monounsaturated fatty acids are mainly found in olive oil and rapeseed oil (Szydłowska-Czerniak *et al.*, 2008; Poyato *et al.*, 2014b); the major sources of polyunsaturated fatty acids are found in sunflower oil and flaxseed oil (Bhatnagar *et al.*, 2009; Poyato *et al.*, 2014b; Elkin *et al.*, 2018). However, not only fatty acid composition of oil, but also oil production process has an influence on physical and chemical properties of oils. The difference in extraction and refining process of oil production can lead to a difference in the content of free fatty acids, colour compounds, total phenolic compounds and antioxidant capacity between refined and unrefined oils. Oil is generally extracted by pressing or by solvent extraction followed by refining of crude fats (Stauffer, 1996; O'brien, 2008). The refining process involves either chemical or physical refining which includes several stages:

degumming, neutralisation, bleaching and deodorization (Gunstone, 2009). The content of inherent antioxidants including total phenolic, and tocopherols are a major influence on free radical scavenging activity (Tuberoso *et al.*, 2007; Janu *et al.*, 2014) and oxidative stability in oils (Psomiadou and Tsimidou, 1998). Pigments, natural antioxidants and free fatty acids are removed during degumming, bleaching and deodorisation stages of oil refining processes (Karabulut *et al.*, 2005; Szydłowska-Czerniak *et al.*, 2008; Costa *et al.*, 2011). Therefore, the compositional differences in the oils such as higher phenolic and free fatty content in unrefined oils or lower level of antioxidants and phenolic content in refined oils could have an effect on the final properties and stability of emulsions elaborated with these oils to be used as saturated fat replacers.

Research has been carried out to develop strategies to replace saturated fat in food products using vegetable oils with high content of unsaturated fatty acids (Gutiérrez-Luna *et al.*, 2020; Rogers, 2009; Espert *et al.*, 2021; Nieto and Lorenzo, 2021). Several studies have focused on the effect of fatty acid chain length (long chain triglycerides, medium chain triglycerides and short chain triglycerides) on emulsion stability and bioaccessibility of lipophilic bioactives (Qian *et al.*, 2012; Hategekimana *et al.*, 2015; Öztürk, 2017); other work has been carried out on the effect of fatty acid profile of vegetable oils on characterisation and stability of emulsions (Chan *et al.*, 2013; Sahafi *et al.*, 2018; Vasilean *et al.*, 2018). Research has shown that the particle size of oil droplets has an effect on the physical and chemical stability of emulsions. Droplet diameter was influenced by the chain length of triglicerides: smaller droplets were obtained when using medium chain triglyceride than long and short chain triglycerides (Hategekimana *et al.*, 2015). Sahafi *et al.* (2018) reported that nanoemulsions prepared from cold-pressed pomegranate seed oil had a bigger droplets and lower emulsion stability because of its higher viscosity and proportion of unsaturated fatty acids in comparison to other oils. Nanoemulsion with oil droplets particle size of less than 200 nm (Komaiko and McClements, 2016) could show better physical stability to gravitational separation

(Kim *et al.*, 2014). However, the smaller droplet size could accelerate reactions at the surface of the droplets promoting lipid oxidation (McClements and Decker, 2000).

Although there have been a great number of studies dealing with the effect of fatty acids profile of oils on emulsion properties such as oil droplet size and lipid oxidation, there are no studies that have studied the effect of the oil production processes on stability of conventional and nano/emulsions. The evaluation of correlations between oil composition due to origin and processing emulsion structure and stability will help elucidating the importance of oil selection for the production of emulsions with specific applications in the food industry. It is hypothesised that the type of oils with different fatty acids profile and production process have effects on physical and chemical properties and stabilities of emulsion, and the stability of nanoemulsions could be improved when selecting an oil with higher content of unsaturated fatty acids. The objective of this study was to assess the physicochemical properties (colour, pH, mean droplet diameter, polydispersity index) and stability (creaming index and lipid oxidation) of emulsions made of five vegetable oils from different botanical origins (olive, rapeseed, sunflower) and different production process (refined and unrefined). The effect of droplet size and distribution on conventional and nanoemulsion stability was also investigated to elucidate which oil should be selected for this type of colloidal structures.

2.2 Materials and methods

2.2.1 Oil samples

Five oils were selected for the study due to their high content on unsaturated fatty acids, their different unsaturation profile and extraction process: extra virgin olive oil (EVOO) (14.26% of saturated fat, 77.69% of monosaturated fat and 8.04% of polyunsaturated fat; Napolina brand, UK retail market), cold pressed rapeseed oil (CPRO) (4.88% of saturated fat, 73.39% of monosaturated fat and 20.73% of polyunsaturated fat; Farrington's mellow yellow brand, UK retail market), Olive oil (OO) (composed of refined olive oil and virgin olive oil, 13.28% of saturated fat, 79.73% of monosaturated fat and 6.98% of polyunsaturated fat; Brakes Bros Co., Ltd., UK), rapeseed oil (RO) (refined rapeseed oil, 4.99% of saturated fat, 74.11% of monosaturated fat and 20.90% of polyunsaturated fat; Mazola brand, UK retail market), and sunflower oil (SO) (refined sunflower oil, 6.52% of saturated fat, 31.95% of monosaturated fat and 61.53% of polyunsaturated fat; Brakes Bros Co., Ltd. UK). The appearance of oils was showed in Figure 2.1A.

2.2.2 Reagents and standards

Tween 20 (Polyoxyethylene sorbitan monolaurate) with hydrophilic-lipophilic balance (HLB) value of 16.7, was used as food-grade nonionic surfactant. Fatty acid methyl esters (the standard reference material FAMEs included C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C18:1, C18:2, C18:3), boron trifluoride reagent (BF_3) (13 – 15% w/w in methanol), methanol, heptane, sodium chloride, folin-Ciocalteu's reagent, gallic acid, sodium carbonate, diethyl ether, ethanol (95% v/v), potassium hydroxide, phenolphthalein, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), trichloroacetic acid, and thiobarbituric acid were purchased from Sigma-Aldrich Co., Ltd (Dorset, UK). Sodium hydroxide, anhydrous sodium sulphate, and hydrochloric acid were purchased from Fisher scientific Co., Ltd (Loughborough, UK). High purity water was used for the preparation and dilution of reagents and standards.

2.2.3 Emulsion preparation

The conventional emulsion preparation procedure was based on the method described by Arancibia *et al.* (2016) and Taha *et al.* (2018) with some modifications. Emulsions were prepared in three steps. Firstly, a magnetic stirrer (ChemLab, Model SS3H) was used to disperse Tween 20 (5% w/w) in water (85% w/w) at 200 rpm for 30 min at ambient temperature for complete dispersion. Then the oil was added (10% w/w) to the aqueous phase during continuous stirring. Secondly, the emulsions were homogenized with a high-speed homogenizer (Silverson, Model L4RT) at 10,000 rpm for 10 min. Thirdly, the emulsions were further processed in an ultrasound processor (with 363.7 mm diameter titanium probe, Model P100/6-20, Sonic Systems Limited, UK.) at 100 watt, 20 kHz frequency, 5 microns amplitude at ambient temperature for 15 min. Then the emulsions were left to cool down at ambient temperature and kept at 25 °C and 40 °C for 1, 7, 14, 21 and 28 days before measurements. The appearance of conventional emulsions was showed in Figure 2.1B. All conventional emulsions were prepared in triplicate.

Nanoemulsions were produce after selecting three of the oils (EVOO, RO and SO). For nanoemulsion preparation, the coarse emulsions were processes in a high-pressure homogenizer (8.30H, Rannie, APV, Denmark) at 500 bars for 1 cycle to produce droplet sizes < 200 μm. Then the nanoemulsions were left to cool down at ambient temperature before measurements for 24 h. All nanoemulsions were prepared in triplicate.

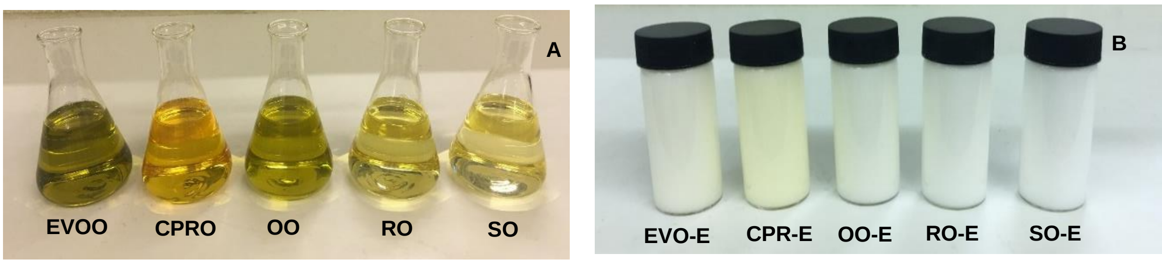

Figure 2.1. Visual appearance of A) vegetable oils, B) conventional emulsions (-E) prepared with the different oils: extra virgin olive oil (EVO-E), cold pressed rapeseed oil (CPR-E), olive oil (OO-E), rapeseed oil (RO-E), sunflower oil (SO-E).

The nomenclature used for the oil samples was as follows: extra virgin olive oil (EVOO), cold pressed rapeseed oil (CPRO), olive oil (OO), rapeseed oil (RO), sunflower oil (SO). Conventional emulsion samples were named by adding a '-E' after the oil name; and nanoemulsion samples were named by addition a '-NE' after the oil name. When using the term 'emulsions' the authors refer to both conventional and nano/emulsions.

2.2.4 Physical and chemical properties of oils

2.2.4.1 Fatty acid composition

The fatty acid composition of oils was determined according to Association of Analytical Communities method 969.33 (AOAC, 2000). Fatty acid methyl esters (FAMEs) were prepared by adding oils (200 mg) to a 0.5 M methanolic sodium hydroxide solution (4 mL). Then, the solutions were attached to a condenser and refluxed for 10 min until fat globules disappeared. Boron trifluoride solution (5 mL) was added and the mixture continued boiling for 2 min. Heptane (5 mL) was added through the condenser and the mixture was boiled a further minute. After boiling, the mixture was allowed to be tepid by keeping it at room temperature for 2 min and saturated sodium chloride solution was added. Subsequently, the heptane layer was transferred into the test tube and anhydrous sodium sulphate was added in order to remove the water. For the heptane phase, the solution was diluted with heptane to a 10% concentration and 1μl was injected in a gas chromatographer (GC; Agilent 7890B gas chromatograph (Agilent Technologies Ltd., UK) equipped with a flame ionization detector (FID) detector and an HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness) (Agilent Technologies, USA) for analysis.

GC conditions were set up following the method described by Nhu-Trang *et al.* (2006). The column temperature was programmed from 70 °C (held for 3 min), then increased up to 166 °C at 3 °C/min rate, and to 285 °C at 15 °C/min. Injector and detector temperatures were 250 °C and 300 °C, respectively. Split ratio injection was 1:50. Helium was used as carrier gas at a flow rate of 1.5 mL/min. The relative percentage compositions of fatty acids were computed by normalization method from the GC peak areas and calculated as the mean value of three injections.

2.2.4.2 Total phenolic content

The total phenolic content (TPC) of oils was determined by using Folin-Ciocalteau colourimetric method according to Lee *et al.* (2015) with some modifications. The oil samples (0.5 g) were extracted with methanol (10 mL) using a vortex mixer for 1 min. Then 5 mL of Folin's reagent (previously diluted 10-fold with water) was added to 2 mL of the methanolic extract. The solution was incubated at room temperature for 5 min, followed by adding 7% (w/v) sodium carbonate

(1mL) and incubated for 90 min at room temperature. The absorbance was read at 725 nm using a spectrophotometer (CECIL, CE 1021, 1000 SERIES, UK). A calibration curve was constructed using gallic acid as standard and the results were expressed as gallic acid equivalents (mg GAE/ kg oil). A stock solution of gallic acid (0.5 mg/ mL) was dissolved in 1 mL of methanol before diluting with distilled water to prepare a calibration curve of concentrations of gallic acid standards between 0.001 to 0.250 mg/ mL. Measurements were done in triplicate for each oil sample.

2.2.4.3 Free fatty acids

The free fatty acids were determined using the official cold volumetric titration method (Commission Regulation (EEC), 1991; Martins *et al.*, 2006) with potassium hydroxide. The samples (2.5 g) were dissolved in 50 mL of neutralized mixture of diethyl ether and ethanol 95% (v/v). The mixture was titrated with potassium solution (0.1 mol/L) and using 0.3 mL of phenolphthalein solution (10g/L solution in 95% ethanol (v/v)) per 100 mL of mixture as an indicator. Measurements were done in triplicate.

Results were expressed as percentage of oleic acid (C18:1), and calculated using the following equation (Eq. (1)):

$$FFA\ (\%) = \frac{V \times c \times M}{10 \times m} \qquad (1)$$

where:

V = the volume of titrate potassium hydroxide solution used, in millilitres,

c = the exact concentration in moles per litre of the titrated solution of potassium hydroxide used,

M = the molar weight in grams per mole of the acid used to express the result (oleic acid = 282), and

m = the weight in grams of the sample.

2.2.4.4 Determination of radical scavenging activity

The antioxidant property of oils was evaluated by the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) essay. The DPPH radical from the odd electron of nitrogen atom can be scavenged by receiving a hydrogen atom from antioxidant compounds (Kedare and Singh, 2011). When the DPPH stable free radical is reduced a change colour from violet to yellow was observed. The oil samples were analysed for their radical scavenging activity following the method described by Mraihi *et al.* (2013) with some modifications. As a reagent, a 0.1 mM solution of 1, 1- diophenyl-2-picry-hydrazyl (DPPH) was prepared in 80% methanol and 5 mL of the solution was mixed with 0.25 mL of oil. This sample was incubated in the dark for 30 min. Then the absorbance was measured at 517 nm against a control sample (5 mL of 0.1 mM DPPH in methanol with 0.25 mL of blank solution). Measurements were done in triplicate. The radical scavenging activity was expressed as the inhibition percentage of free radical DPPH, calculated using the following equation (Eq. (2)):

$$\text{DPPH} \cdot \text{scavenging activity (\%)} = \left(1 - \frac{As}{Ac}\right) \times 100 \qquad (2)$$

Where: Ac was the absorbance of the control and As was the absorbance of the sample

2.2.4.5 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to the method of Qiu *et al.* (2015) and Sharif *et al.* (2017) with some modifications. Briefly, 0.1 ml of the oil sample was added to 5 ml of thiobarbituric acid (TBA) solution, which was prepared by mixing 15 g of trichloroacetic acid (TCA), 0.375 g of TBA and 2.1 g hydrochloric acid (37% w/w). Samples were heated in a water bath at 95 °C for 10 min, then the samples were allowed to cool down to room temperature for 10 min, followed by centrifugation (Heraeus Multifuge 3SR Plus Centrifuge, Thermo Scientific Ltd., UK) at 10,000 g for 15 min. The absorbance of the supernatant was measured at 532 nm using a UV spectrophotometer (CECIL CE 1021 1000 Series, Cecil Instruments Ltd., UK). The concentrations of TBARS values were determined by using a standard curve prepared using 1,1,3,3-tetraethoxypropane (TEP) standard curve (coefficient correlation (R^2) = 0.9994). TEP standards between 0.01 to 0.20 μg/ mL were prepared with trichloroacetic acid 7.5%. Three analytical repetitions of each measurement were done for each emulsion batch.

2.2.4.6 Viscosity

The viscosity of oils was measured with a rheometer (MCR 102, Anton Paar, UK) with a concentric cylinder (CC27, Anton Paar, UK). The shear rate range used was from 0.1 s-1 to 200 s-1 and the temperature was maintained at 25 °C. Measurement were done in triplicate. The viscosity values at 100 s-1 were taken for data analysis and comparison.

2.2.4.7 Density

The apparent density (weight-by-volume) of oil samples was determined by the method followed by Gunstone (2009). Oils weight and volume were measured at the room temperature and apparent density was calculated using the following equation (Eq. (1)):

$$\text{Apparent density}\ (g/ml) = \frac{Mass\ of\ oil\ (g)}{Volume\ of\ oil\ (ml)} \qquad (1)$$

2.2.5 Physical and chemical properties of conventional and nanoemulsions

2.2.5.1 Measurement of emulsion mean droplet diameter (MDD) and polydispersity index (PDI)

Particle size and polydispersity index of emulsions were determined in a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) by following the method of Guerra-Rosas *et al.* (2016) and Sharif *et al.* (2017). Emulsions were diluted 100-fold with deionized water and agitated in order to avoid multiple light scattering effects. The dispersion was decanted into polystyrene cuvettes and the measurement was done at wavelength of 633 nm at 25 °C. Measurements were done in triplicate.

2.2.5.2 Creaming index (CI) and thermal stability (TS)

Creaming index (%) was evaluated based on the method reported by Arancibia et al. (2016) with some modifications. 10 mL of each emulsion was poured into a glass tube and stored at 25 °C and

40 ˚C in order to accelerate destabilisation mechanisms during storage. The total height (mm) of emulsion and cream layer were measured with a digital calliper after 1, 7, 14, 21 and 28 days. The Cl (%) was calculated using the following equation (Eq. (3)):

$$Creaming\ index\ \% = \left(\frac{Hc}{Ht}\right) \times 100 \qquad (3)$$

where Ht is the total height of the emulsion (mm) and Hc is the height of cream layer (mm).

Thermal stability was determined as described by Sahafi *et al.* (2018). Each emulsion (10 mL) was heated in a water bath at 80 ˚C for 30 min followed by centrifugation at 1200 x g for 10 min. The height (mm) of initial emulsion, cream layer and sedimentation phase were measured with a Digital Vernier Caliper. Emulsion thermal stability will be calculated according to the Eq. (4):

$$Thermal\ stability\ \% = \left(\frac{HE - (HS + HC)}{HE}\right) \times 100 \qquad (4)$$

where HE is the height of initial emulsion (mm), HS is the height of sedimentation phase (mm) and HC is the height of cream layer (mm).

2.2.5.3 Determination of thiobarbituric acid reactive substances (TBARS)

The measurement of TBARS was done as explained in section 2.2.4.5 with a different sample/reagent proportion (Qiu *et al.*, 2015): 1 mL of emulsion was added to 5 ml thiobarbituric acid (TBA) solution and measured after 1, 7, 14, 21 and 28 days at stored temperature of 25 ˚C and 40 ˚C. Measurements were done in triplicate.

2.2.6 Statistical analysis

Statistical analysis of experimental data was performed using IBM SPSS 25 (Armonk, NY: IBM Corp, USA). One-way analysis of variance (ANOVA) and Tukey's test at 95% confidence level ($p < 0.05$) were used to compare the mean values of viscosity, density, total phenolic components, free fatty acids, radical scavenging activity and TBARS of oil samples; and MDD, PDI, ζ-potential, creaming index and TBARs of emulsion samples. Moreover, to evaluate the effect of storage time in conventional emulsions a two-way analysis of variance was conducted. The interaction of the two independent factors: oil type (EVOO, CPRO, OO, RO, SO) and storage time (1, 7, 14, 21 and 28 days) at two different storage temperatures (25 °C and 40 °C) were evaluated for the creaming index and TBARS values of conventional emulsion samples. Pearson correlation was calculated for the physical and chemical properties of vegetable oils, conventional and nanoemulsions. The correlation coefficient (r) was obtained: very weak correlation ($0.01 \leq r < \pm 0.10$), weak ($\pm 0.10 \leq r < \pm 0.50$), moderate ($\pm 0.50 \leq r < \pm 0.80$), strong ($\pm 0.80 \leq r < \leq 1.00$), and perfect ($r = \pm 1.00$) (Granato *et al.*, 2010).

2.3 Results and discussion

2.3.1 Effect of oil type on the stability of conventional emulsions

2.3.1.1 Mean droplet diameter (MDD) and polydispersity index (PDI)

The droplet size influences the physicochemical stability of emulsions (Tadros *et al.*, 2004b; McClements and Decker, 2000). A smaller droplet size could lead to better stability against droplet coalescence and flocculation because of the reduction in Brownian motion and gravitation forces (Tadros *et al.*, 2004b), which could lead to a slower rate of creaming compared to larger droplet size. Regarding polydispersity index (PDI), it describes the particle size distribution of droplets, which is closely related to the stability of the emulsion (McClements, 2016c). Low PDI values represent more narrow distribution of particle size, so a small difference in droplet size. Emulsions with narrower PDI values showed to be more stable than emulsions with wider PDI (Sabliov *et al.*, 2015) due to a lower Ostwald ripening (Liu *et al.*, 2006; Tadros *et al.*, 2004a); Laplace pressure inside smaller droplets is higher than in larger droplets resulting in smaller droplets merging with larger droplets (Lochhead, 2017). The results of the MDD and PDI of conventional emulsions are presented in Table 2.1. The results showed that there were not significant differences on droplets size and PDI ($p \geq 0.05$) among emulsions. The formation of emulsions could be mainly affected by ingredient composition (oil, surfactant, water) (Öztürk, 2017), ratio of components (Joung *et* *al.*, 2016), and significantly by process parameters (Öztürk, 2017), such as energy intensity and sonication duration (Mehmood *et al.*, 2017; Nourbehesht *et al.*, 2018). In the present study the only variable factor was oil type with constant oil and emulsifier concentration, which did not have a significant effect on droplet size and PDI. This observation is similar to the findings of Sharif *et al.* (2017) and Sahafi *et al.* (2018), who reported that PDI was not influenced by oil type on nano-, conventional emulsions formulated with constant emulsifier concentration. Therefore, this result indicated that the type of oils with high content of unsaturated fatty acids have a negligible effect on MDD and PDI.

Table 2.1. Mean droplet diameter (MDD), polydispersity index (PDI) and ζ-potential of conventional emulsions (-E) prepared with the different oils: extra virgin olive oil (EVO-E), cold pressed rapeseed oil (CPR-E), olive oil (OO-E), rapeseed oil (RO-E), sunflower oil (SO-E).

Conventional emulsions	MDD (nm)	PDI	ζ-potential (mV)
EVO-E	247.57 ^{a} (9.32)	0.448 ^{a} (0.038)	-32.42 ^{a} (0.48)
CPR-E	249.60 ^{a} (4.60)	0.472 ^{a} (0.050)	-31.69 ^{a} (2.35)
OO-E	238.23 ^{a} (8.75)	0.449 ^{a} (0.040)	-29.79 ^{a} (1.55)
RO-E	251.94 ^{a} (7.69)	0.495 ^{a} (0.049)	-31.30 ^{a} (0.67)
SO-E	239.41 ^{a} (8.20)	0.474 ^{a} (0.040)	-31.81 ^{a} (1.12)

Indicated values are reported as means (standard deviation). Values with the different superscript letters (within same column) are significantly different ($p < 0.05$).

2.3.1.2 Creaming index (CI)

Creaming index (CI) is a method of measuring gravitational separation in emulsions (McClements, 2016c); low CI (%) values are related with more stable emulsions. In this study, the emulsions were stored at 25 °C and 40 °C, during 28 days to evaluate their physical stability over time. No significant interaction ($p > 0.05$) between oil type and storage time was observed at both storage temperatures (25 °C and 40 °C). However, both factors (oil type and storage time) showed a significant effect on CI values (Figure 2.2A and 2.2B). EVO-E, CPR-E and SO-E showed a significantly ($p < 0.05$) lower CI value than RO-E, which showed the highest CI value (Figure 2.2A). The CI values of emulsions can be influenced by various factors including droplet size and density of dispersed (oil) phase. It can be supported by Stokes' law that rate of gravitational separation can be decreased by a decrease in droplet size, a decrease in density difference between dispersed phase and continuous phase, and an increase in viscosity of continuous phase (McClements, 2016c). As a consequence, the lower CI value of SO-E than RO-E at both 25 °C and 40 °C could be due to a significant lower of density difference between oil phase and water phase (Table 2.S2) and a largest difference in the droplet size between SO-E and RO-E compared to other oils, which the size of SO-E was the smallest whereas a biggest size was found in RO-E (Table 2.1). On the other hand, an increase in storage time showed a significant increase ($p < 0.05$) of CI values at both temperatures over the period of time investigated (Figure 2.2B). Oil droplet aggregation including flocculation and coalescence take place over time and lead to creaming layer formation in emulsions (McClements and Jafari, 2018). This was in agreement with previous studies that reported a higher level of creaming index of emulsion when storage period increased (Zarena *et al.*, 2012; Sobhaninia *et al.*, 2017). Although there was a similar trend of Cl values at 25 °C and 40 °C, the emulsions stored at 40 °C showed faster destabilisation than at 25 °C, indicating that the aggregation rate of oil droplet particles was greater at higher temperature, giving place to a higher value of creaming index. This result could be due to the density of the dispersed phase (oil) is decreasing with increasing with higher temperature (Esteban *et al.*, 2012), which could lead to an increase in density difference between dispersed phase and continuous phase, resulted in higher rate of CI value.

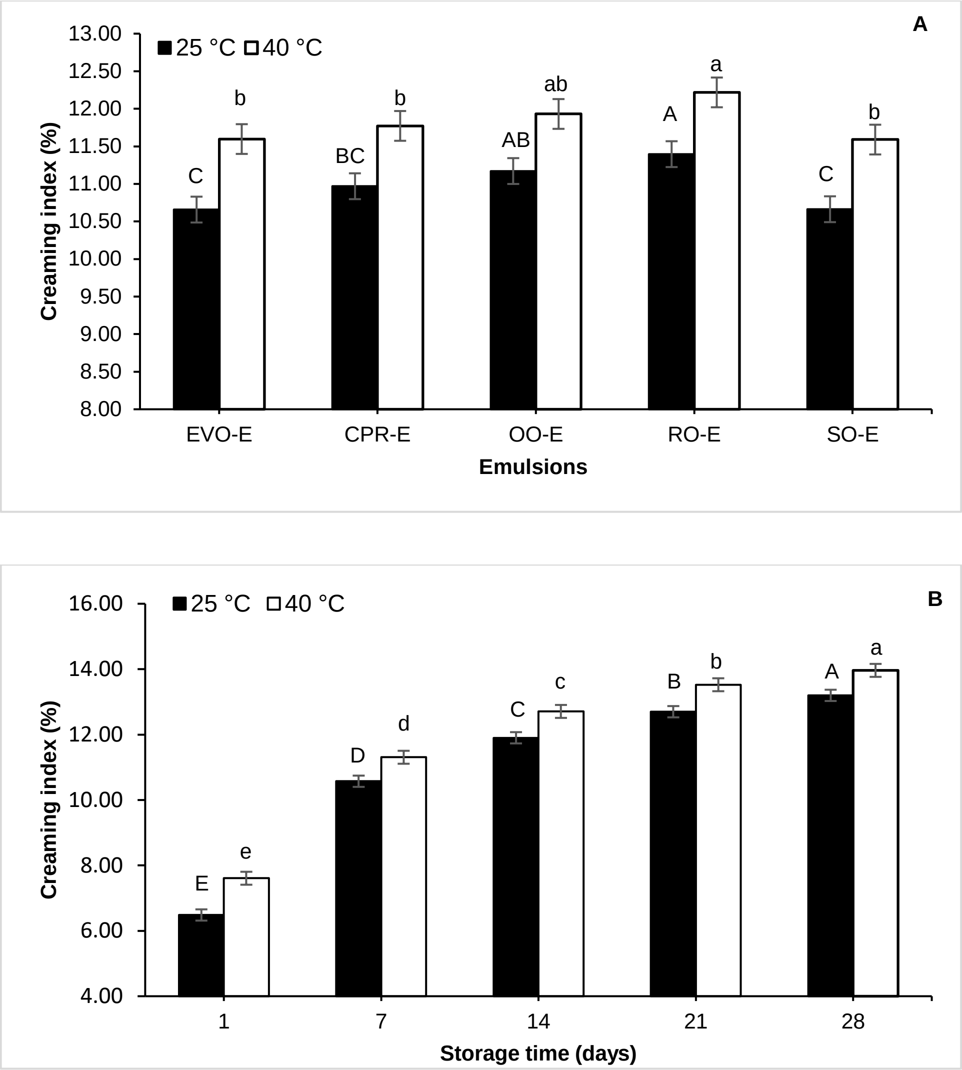

Figure 2.2. Creaming index (%) means values and 95% confidence intervals of the conventional emulsions stored at 25 °C and 40 °C. A: Effect of oil type; B: effect of storage time. Different capital letters indicate significant differences between mean at 25 °C and different lower-case letters indicate significant differences between means at 40 °C. Conventional emulsions (-E) prepared with the different oils: extra virgin olive oil (EVO-E), cold pressed rapeseed oil (CPR-E), olive oil (OO-E), rapeseed oil (RO-E), sunflower oil (SO-E).

2.3.1.3 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS was used to measure the secondary products of lipid oxidation in emulsions in order to investigate the effect of oil types in the lipid oxidative stability over time. In the present study, the emulsions were kept at 25 °C and 40 °C after formation and TBARS lipid oxidation value was measured at 1, 7, 14, 21 and 28-days storage time. At 25 °C storage temperature, there was a significant interaction ($p < 0.05$) between oil type and storage time when evaluating TBARS (Figure 2.3A). EVO-E, OO-E and SO-E presented significant lower ($p < 0.05$) TBARS values than CPR-E and RO-E during the storage time. In contrast CPR-E and RO-E, showed a significant increase in TBARS values at 1, 7, 14 and 21 days. The highest TBARS values were found in CPR-E at 28 days storage. At 40 °C storage temperature, there was a significant interaction between oil type and storage time for the TBARS values (Figure 2.3B). At 40 °C, TBARS values were higher and increased more rapidly than at 25 °C. Storage at 40 °C accelerated lipid oxidation, giving place to higher TBARS values in all emulsions during storage. The lipid oxidative stability of the emulsions presented a similar trend as at 25 °C; EVO-E, OO-E and SO-E showed significantly lower TBARS values than CPR-E and RO-E. These three oils showed a greater stability during storage with no significant differences in TBARS values after 21 days of storage. On other hand, as storage time increased TBARS values significantly increased ($p < 0.05$) for RO-E and CPR-E. The highest TBARS value was shown in RO-E at 28 days. The significantly lower TBARS values in SO-E, OO-E and EVO-E than in CPR-E and RO-E could be related to differences in the fatty acid composition of the oils (Table 2.S1). Emulsions formulated with oils with higher fraction of unsaturated fatty acids (CPRO, RO) lead to higher oxidation rates because unsaturated fatty acids are more susceptible to oxidation than saturated fatty acids. The unsaturated fatty acids have a double bond in the carbon chain; the hydrogen atom attached to the carbon between the double bond is easily removed and provide alkyl radicals (Choe and Min, 2006). Similar results were also reported by Kiokias *et al.* (2006) in a study where they observed that olive kernel oil emulsion was oxidized less than corn oil, cottonseed oil and sunflower oil emulsions because of olive kernel oil contained less polyunsaturated fatty acids than the other oils.

The correlations between emulsion stability and the type of oil were further assessed. The Pearson's correlation coefficients (r) between the physical and chemical properties of oils (USFA, TPC, antioxidant capacity and FAA) and the stability of emulsions (creaming index and TBARs) are presented in Table 2.2. USFA exhibited significant positive correlations with creaming index (p < 0.05) and TBARs (p < 0.01) of emulsions. The moderate positive correlation for creaming index and TBARs suggested that vegetable oils with higher content of unsaturated fatty acids exhibited higher values of creaming index and TBARs, indicating that fatty acid composition of oils affects the stability of emulsions. The higher proportion of unsaturated fatty acids in oils lead to higher rates of lipid oxidation (Choe and Min, 2006). Refined oils such as SO and RO showed lower values of FFA than unrefined oils such as EVOO and CPRO. In terms of SO, the crude sunflower oil is processed through the conventional alkali refining process, which consist of degumming, alkali neutralisation, dewaxing, bleaching and deodorization stages (Gunstone, 2011). Therefore, the lower TBARS value of SO-E among the emulsions could be due to a lower content of free fatty acids in the oil (Table 2.S2), which could be due to the refining process of oil, especially in neutralisation and deodorization stages (Costa *et al.*, 2011). Another factor influencing the emulsion oxidative stability is the presence of antioxidant components in oils such as in EVOO and OO (Table 2.S2) (Jimenez-Alvarez *et al.*, 2008), which could lead to lower TBARS values in EVO-E and OO-E (Figure 2.3). As a consequence, EVO-E, OO-E and SO-E had more stable than CPR-E and RO-E. Therefore, in order to evaluate the effect of oils on the properties of conventional and nanoemulsions oils that have different fatty acid profile, content of free fatty acids, total phenolic compounds and a high oxidative stability where selected (EVOO, RO and SO).

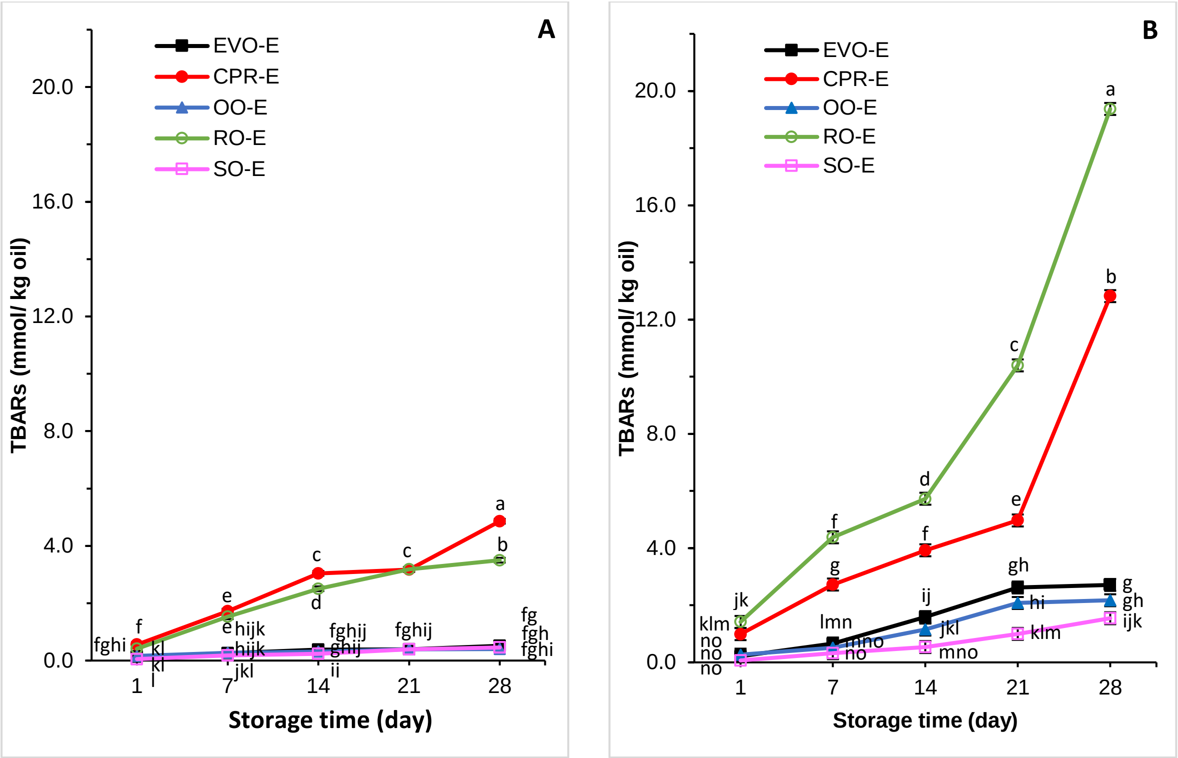

Figure 2.3. TBARS of conventional emulsions during storage time at A) 25 ºC and B) 40 ºC. Conventional emulsions (-E) prepared with the different oils: extra virgin olive oil (EVO-E), cold pressed rapeseed oil (CPR-E), olive oil (OO-E), rapeseed oil (RO-E), sunflower oil (SO-E).

Table 2.2. Pearson's correlation coefficients between the unsaturated fatty acids content (USFA), total phenolic content (TPC), radical scavenging activity, free fatty acids (FFA, of oils and the creaming index (CI) and thiobarbituric acid reactive substances (TBARS) conventional emulsions at 25 °C

Variable	USFA (%)	TPC (mg GAE/ kg oil)	radical scavenging activity (%)	FFA (% oleic acid)	CI (%)	TBARs (mmol/kg oil)
USFA	1	-	-	-	-	-
TPC	-0.749**	1	-	-	-	-
Antioxidant	-0.277	0.745**	1	-	-	-
FAA	-0.632*	0.759**	0.287	1	-	-
Creaming index	0.592*	-0.208	0.232	-0.255	1	-
TBARs	0.741**	-0.356	-0.063	-0.103	0.789**	1

*, ** indicates that the correlation is significant at $p < 0.05$ and 0.01, respectively.

2.3.2 Comparison between conventional and nanoemulsions

Following the results of the effect of oil type on the stability of conventional emulsions (section 2.3.1.2 and 2.3.1.3), this part of the study focused on the evaluation of the effect of oil droplet size and the influence of attractive forces between the droplets on conventional and nanoemulsions stability. Conventional and nanoemulsions were formulated with EVOO, RO and SO. Correlations between MDD, PDI, thermal stability and TBARs were evaluated for those samples. As shown in Table 2.3, MDD and PDI of nanoemulsions presented significant lower values ($p <$ 0.05) than conventional emulsions. When using high pressure homogenisation to produce the nanoemulsions there is an increase in shear forces and cavitation that results in a reduction of particle size and polydispersity values (Qian and McClements, 2011; Floury *et al.*, 2000). A smaller droplets size could lead to greater physical stability (Tadros *et al.*, 2004b) and higher lipid oxidation of nanoemulsions due to an increased droplet surface area (McClements and Decker, 2000; Gohtani *et al.*, 1999).

Correlation coefficients between MDD, PDI and stability of conventional and nanoemulsions are presented in Table 2.4. MDD and PDI showed a significant strong negative correlation ($p < 0.01$) with CI, whereas there were insignificant correlations with TBARs. The negative correlation suggested that emulsions with smaller droplet size and narrower size distribution values exhibited the higher thermal stability. This correlation could be explained by Stokes' law: the rate of gravitational separation can be decreased by a decrease in droplet size (McClements, 2016c). A decrease in particle droplet size gave place to better stability against droplet coalescence and flocculation due to the reduction in Brownian motion and gravitation forces (Tadros *et al.*, 2004a), and a decrease of the attractive forces between the droplets (Tadros *et al.*, 2004a; Qian and McClements, 2011). Moreover, the narrower PDI values of nanoemulsions lead to more stable colloids (Table 2.3) due to a lower Ostwald ripening; there is diffusive migration of smaller droplets with higher Laplace pressure to larger droplets (Liu *et al.*, 2006; Tadros *et al.*, 2004a; Lochhead, 2017). In summary, MDD and PDI played an important influence on thermal stability of conventional and nanoemulsions.

Table 2.3. Mean droplet diameter (MDD), polydispersity index (PDI), creaming index (CI), and thiobarbituric acid reactive substances (TBARs) of conventional and nanoemulsions at 25 °C.

Emulsion type	Vegetable oils	MDD (nm)	PDI	TS (%)	TBARs (mmol/kg oil)
Conventional emulsions	EVOO	467.60^{a} (66.23)	0.928^{ab} (0.122)	89.49^{b} (0.25)	0.31^{c} (0.02)
	RO	452.02^{ab} (69.76)	0.890^{b} (0.134)	89.00^{b} (0.29)	2.33^{b} (0.07)
	SO	401.09^{b} (14.66)	1.000^{a} (0.000)	89.42^{c} (0.36)	0.20^{d} (0.03)
Nanoemulsions	EVOO	192.19^{c} (3.28)	0.261^{c} (0.011)	100.00^{a} (0.00)	0.39^{c} (0.07)
	RO	192.86^{c} (4.08)	0.249^{c} (0.017)	100.00^{a} (0.00)	2.85^{a} (0.10)
	SO	195.02^{c} (3.37)	0.258^{c} (0.017)	100.00^{a} (0.00)	0.20^{d} (0.06)

Indicated values are reported as means (standard deviation). Values with the different superscript letters are significantly different ($p < 0.05$) between samples in the same column.

Table 2.4. Pearson's correlation coefficients between Mean droplet diameter (MDD), polydispersity index (PDI), of and the creaming index (CI), and thiobarbituric acid reactive substances (TBARs) of conventional and nanoemulsions

Variable	MDD (nm)	PDI	CI (%)	TBARs (mmol/kg oil)
MDD	1	-	-	-
PDI	0.899**	1	-	-
Thermal stability	-0.938**	-0.978**	1	-
TBARs	-0.054	-0.138	0.097	1

** indicates that the correlation is significant at $p < 0.01$.

2.4 Conclusion

This study investigated the influence of vegetable oils from different natural origins and production processes on the physicochemical properties and stability of emulsions. The selected vegetable oils used in this study showed differences in the level of unsaturation total phenolic content, free fatty acids and antioxidant activity, which could be due to the difference in refining and extraction processes used for oil production. This study showed that SO-E, OO-E and EVO-E showed significantly higher lipid oxidative stability compared to RO-E and CPR-E after 28 days storage time due to a higher fraction of unsaturated fatty acids on CPRO and RO. The content of free fatty acids and total phenolic compound in the oil could be also an important factor influencing emulsions' TBARs values. Nanoemulsions showed greater physical stability than conventional emulsions, mainly when formulated with EVOO due to its higher radical scavenging activity and oxidation stability. Overall, this study provides valuable insights into the characteristics of refined and unrefined oils with high content of unsaturated fatty acids and their effects on the physicochemical properties of both conventional emulsions and nanoemulsions. This information is important for the selection of suitable oils to develop emulsion with enhance nutritional fatty acid profile and desirable characteristics for application in food products.

Chapter 3

Optimisation of the physicochemical stability of extra virgin olive oil-in-water nanoemulsion:

processing parameters and stabiliser type

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Abstract

Extra virgin olive oil in water nanoemulsions stabilised with synthetic or clean label surfactants (Tween 20 or lecithin) were prepared using high-pressure homogenisation (HPH). The effect of HPH pressure and the number of cycles were assessed through response surface methodology to optimise homogenisation processing parameter. Mean droplet diameter (MDD), polydispersity index (PDI) and thermal stability of the resulting emulsions were evaluated. The effect of surfactant types on thermal stability and lipid oxidative stability of the emulsions were also measured. The results showed that the formation and stability of nanoemulsions can be affected by the homogenisation processing parameters (pressure and cycles) and the properties of surfactants (interfacial tension, viscoelasticity and molecule structure). Although MDD and PDI of Tween 20 stabilised nanoemulsions were influenced by homogenisation pressure and cycles, there was not a significant effect on soy lecithin stabilised nanoemulsions. A homogenisation pressure of at least 400 bars produced Tween 20 stabilised nanoemulsion (MDD < 200 nm) whereas soy lecithin stabilized nanoemulsion were obtained just after high-speed homogenisation without using HPH. HPH at 400 bars for 1 cycle produced nanoemulsions with greater physical stability when using either Tween 20 or soy lecithin as emulsifiers. Tween 20 stabilised nanoemulsion showed significantly higher ($p < 0.05$) thermal stability and lipid oxidative stability than lecithin stabilized nanoemulsion. Following an optimisation study using regression modelling, the optimal homogenisation parameter for MDD of Tween 20 stabilised emulsion was found at pressure of 764 bars with 1 cycle, while soy lecithin stabilised emulsion was found at pressure of 3 bars with 2 cycles. Overall, this study has important implications for optimizing nanoemulsion production for potential application in the food industry.

Keywords: Soy lecithin, Tween 20, Extra virgin olive oil, O/W nanoemulsion, High-pressure homogenisation, Optimisation

3.1 Introduction

Nanoemulsions are increasingly used in the food industry because of their unique properties and potential applications such as improving physicochemical stability of functional compounds, texture modification and nutrient enrichment (Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Previous studies have established that the characteristics and stability of nanoemulsions depend on several factors such as the composition and processing conditions (Calligaris *et al.*, 2016; Mehmood, 2015; Qian and McClements, 2011). Extra virgin olive oil contains high level of monounsaturated fatty acids (oleic acid) (Szydłowska-Czerniak *et al.*, 2008) and minor bioactive compounds such as phenolics and tocopherols that could scavenge the free radicals and contribute to the antioxidant activity (Szydłowska-Czerniak *et al.*, 2008; Poyato *et al.*, 2014b). There has been a positive correlation between the phenolic and tocopherol content and radical scavenging activity (Tuberoso *et al.*, 2007; Bhatnagar *et al.*, 2009).

Polyoxyethylene sorbitan esters (polysorbates) are small molecular and non-ionic surfactants that are of interest in this study; they have been reported as effective surfactants to formulate stable nanoemulsions (Mehmood, 2015; Guerra-Rosas *et al.*, 2016; Sharif *et al.*, 2017) due to their ability to rapidly absorb to the droplet surfaces and reduce interfacial tension (Karbstein and Schubert, 1995; Donsì *et al.*, 2012). However, polysorbates can give a bitter taste when used at high concentrations (Cottrell and Peij, 2014), limiting their food applications. On the other hand, consumers are increasingly demanding clean labels on food and beverage products; therefore, there is an increasing interest in using surfactants that are obtained from natural sources (Goindi *et al.*, 2016) that could be effective in creating stable nanoemulsions with minimal impact on their organoleptic properties. Soy lecithin has been widely used as emulsifying agent in the food industry (Klang and Valenta, 2011); it is commonly obtained from soybeans (Goindi *et al.*, 2016). Soy lecithin is an amphiphile molecule, which is derived from *sn*-glycero-3 phosphate. The lipophilic groups are fatty acids that attached to carbon atom position 1 and 2 while the hydrophilic groups generally are zwitterionic of amino and anionic of polyvalent alcohol (Bueschelberger, 2004; McClements, 2016b).

High energy mechanical processes are performed at industrial scale due to an easy control of the homogenization machine (Öztürk, 2017). High-pressure homogenisation (HPH) is a high energy method used to produce nanoemulsions (Goindi *et al.*, 2016); a coarse emulsion passes through a narrow channel of the high-pressure valve homogenizer (McClements, 2011; Öztürk, 2017). Several studies have focused on the effect of high-pressure homogenisation processing parameters including pressure and number of cycles on nanoemulsion's particle size, droplet size distribution and physical stability (Mehmood, 2015; Calligaris *et al.*, 2016; Qian and McClements, 2011; Galvão *et al.*, 2018; El Kinawy *et al.*, 2012). In general, when the pressure and number of homogenisation cycles increases, the droplets size and particle size distribution decreases (Calligaris *et al.*, 2016; Yuan *et al.*, 2008; El Kinawy *et al.*, 2012; Qian *et al.*, 2012), and it has been reported that the physical stability of a nanoemulsion increases initially when the pressure or number of cycles are increased (Yuan *et al.*, 2008; Mehmood, 2015). However, there is limited research on the effect of high-pressure homogenisation parameters on nanoemulsion stabilised by surfactants from natural sources such as soy lecithin in comparison to synthetic surfactants such as Tween 20. Kim *et al.* (2016) indicated that the droplet size of nanoemulsion decreased when partially replacing Tween 20 with soy lecithin or sorbitan monooleate (prepared at 1034 bars), which was attributed to a considerable improvement of Ostwald ripening stability of curcumin loaded medium chain triglycerides (MCT) nanoemulsion. Mehmood (2015) studied the optimisation of canola oil-based vitamin E nanoemulsions when using mixed surfactants (Tween 80 and soy lecithin), and the results on particle size and emulsion stability showed that the optimum processing parameters were homogenisation pressure (135 MPa), oil concentration (6.18%), surfactant concentration (6.39%) and vitamin E acetate contents (1%). Therefore, not only processing parameters but also the type of surfactant could influence the physicochemical properties of nanoemulsions.

Nanoemulsion droplet size could promote lipid oxidation by accelerating reactions at the surface of the droplets; a high rate of lipid oxidation is attributed to a high droplet surface area as the droplet size decreases (McClements, 2011; McClements and Decker, 2000). Surfactant type also played a major role in lipid oxidation, particularly when prooxidants such as transition metals (Fe, Cu) are present into the aqueous phase; therefore, the emulsion droplet interface can influence the interaction between prooxidant and lipids (Fomuso *et al.*, 2002; Uluata *et al.*, 2015; Arancibia *et al.*, 2017). When formulating nanoemulsions, lipid oxidation is a parameter to control because it can lead to an undesirable flavour including off-flavours and off-odours (McClements, 2016b). The objective of the present study was therefore to evaluate the effect of homogenisation processing conditions and surfactant type (soy lecithin or Tween 20) on physical stability and lipid oxidation of nanoemulsions. This research work initially focused on the characterisation of surfactants (Tween 20 and soy lecithin) by measuring interfacial tension and viscoelastic properties. Additionally, the influence of homogenisation conditions (pressure and number of cycles) on physicochemical properties (mean droplet diameter and polydispersity index) and stability of emulsions (thermal stability and lipid oxidation) were also assessed. The results of this study will provide useful information about the emulsifying capacities soy lecithin and the design of an optimum HPH process to produce stable nanoemulsions for application in food and beverage products.

3.2 Materials and methods

3.2.1 Materials

Nanoemulsions were elaborated with extra virgin olive oil (EVOO) (14.26% of saturated fat, 77.69% of monosaturated fat and 8.04% of polyunsaturated fat, Napolina brand, UK retail market) and two types of emulsifiers: Tween 20 (Polyoxyethylene) with hydrophilic-lipophilic balance (HLB) value of 16.7 was used as synthetic nonionic surfactant (Sigma-Aldrich Co., Ltd, UK) or soy lecithin (HLB value ranges between 2-8) was used as zwitterionic surfactant, (Louis

Francois Co, France). The molecular weight of soy lecithin and Tween 20 are 643.9 and 522.g/mol respectively (National Center for Biotechnology Information, 2021a; National Center for Biotechnology Information, 2021b). Trichloroacetic acid, thiobarbituric acid, and malondialdehyde (MDA) were purchased from Sigma-Aldrich Co., Ltd (UK), and hydrochloric acid purchased from Fisher scientific Co., Ltd (UK). High purity water was used for the preparation and dilution of reagents.

3.2.2 Nanoemulsion preparation

The emulsion preparation procedure was based on the methods described by Arancibia *et al.* (2016) and Taha *et al.* (2018) with some modifications. Emulsions were prepared in three steps. Firstly, a magnetic stirrer (Model SS3H, ChemLab, UK) was used to prepare the aqueous phase dispersing Tween 20 and soy lecithin (5% w/w) in water (85% w/w) at 200 rpm for 30 min at ambient temperature to ensure complete dispersion. Then the oil was added (10% w/w) to the aqueous phase during continuous stirring. Secondly, the emulsions were homogenized with a high-speed homogenizer (Model L4RT, Silverson, Chesham, UK) at 10,000 rpm for 10 min. Thirdly, the coarse emulsions were passed through a high-pressure homogenizer (8.30H, Rannie APV, Denmark). For the evaluation of the effect of the homogenisation pressure on emulsion properties, the coarse emulsions were homogenised at 1, 200, 400, 600 and 800 bars for 1 cycle. For the study of the effect of cycle number on emulsion properties, the coarse emulsions were passed through the high-pressure homogeniser for 1, 2 and 3 cycles at different homogenisation pressures (200 and 400 bars). Then the emulsions were left to cool down for 2 hours at ambient temperature before measurements. All emulsions were prepared in triplicates.

3.2.3 Characterisation of surfactants: interfacial tension and critical micelle concentration

The interfacial tension was determined according to the method of Bai *et al.* (2016) and Luo *et al.* (2017). The interfacial tension between oil-water interface, with different emulsifiers (Tween 20 at 5% w/w or soy lecithin at 5% w/w) was determined using a pendant drop analyser (DS4270, Krüss

GmbH, Hamburg, Germany) at 20 °C. An axisymmetric drop (20 μL) of surfactant dispersion was delivered and allowed to stand at the tip of the needle inside a quartz container of extra virgin olive oil (9 mL) for 15 min, to achieve emulsifier adsorption at oil-water interface. Three analytical repetitions of each measurement were done for each emulsion batch.

The critical micelle concentration (CMC) of each emulsifier was determined according to the method of Mukherjee *et al.* (2013) and El-Sukkary *et al.* (2008). Several concentrations of Tween 20 and soy lecithin were prepared: 0.0, 0.0001, 0.001, 0.01, 0.1, 1.0, 5.0 and 10.0 %w/w. CMC was determined at the intersection points of the interfacial tension values versus the surfactant concentration (logarithm) plot. Three analytical repetitions of each measurement were done for each emulsion batch.

3.2.4 Physical and chemical properties of nanoemulsions

3.2.4.1 Rheological properties of emulsions

The viscoelastic behaviour of surfactant solutions was determined using a rheometer (Anton Paar MCR 302, Anton Paar, Graz, Austria) equipped a Peltier temperature control device. A serrated parallel plate geometry was used; the diameter of lower stationary plate (PPTD 200/56/1) and superior plate (PP50/ P2) was 50 mm and the gap between the plates was 1 mm. The samples were allowed to rest in the measurement position for 5 min for relaxation and temperature equilibration (20 °C). Strain sweeps were carried out at strain amplitude range of 0.001 to 1000 % at a constant frequency of 1 Hz in order to determine the linear viscoelasticity region (LVR). The LVR was identified where the storage modulus (G') and loss modulus (G") were not influenced by applied strain from this region, constant strain amplitude of 10% was selected. Frequency sweeps bewteen 0.1 to 100 Hz were performed at constant stress amplitude (10%). The G' and G" moduli values were recorded; G' characterizes of the elastic nature or solid-like behaviour of a substance, while G" is indicative of viscous nature or liquid-like behaviour of a substance. Measurements were performed in duplicate in two emulsion batches.

3.2.4.2 Measurement of emulsion mean droplet diameter (MDD) and polydispersity index (PDI)

Particle size and polydispersity index of emulsions were determined in a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) following the method of Guerra-Rosas *et al.* (2016) and Sharif *et al.* (2017). Emulsions were diluted 100-fold with deionized water and agitated to avoid multiple light scattering effects. The dispersion was decanted into polystyrene cuvettes for measuring MDD and PDI at wavelength of 633 nm at 25 °C. Three analytical repetitions of each measurement were done for each emulsion batch.

3.2.4.3 Thermal stability (TS)

Emulsion stability at high temperature was determined as described by (Sahafi *et al.*, 2018). Each emulsion (10 mL) was heated in a water bath at 80°C for 30 min followed by centrifugation at 1200 g for 10 min. The height (mm) of initial emulsion, cream layer and sedimentation phase were measured with a Digital Vernier Caliper. Emulsion thermal stability was calculated according to the Eq. (1):

$$Thermal\ stability\ \% = \left(\frac{HE-(HS+HC)}{HE}\right)\times 100 \qquad (1)$$

where HE was the height of initial emulsion (mm), HS was the height of sedimentation phase (mm) and HC was the height of cream layer (mm).

3.2.4.4 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to the method of (Sharif *et al.*, 2017; Qiu *et al.*, 2015) with some modifications. Briefly, 1 ml of the emulsions was added to 5 ml of thiobarbituric acid (TBA) solution, which was prepared by mixing 15 g of trichloroacetic acid (TCA), 0.375 g of TBA and 2.1 g hydrochloric acid (37% w/w). Samples were heated in a water bath at 95 °C for 10 min, then the samples were allowed to cool down to room temperature for 10 mins, followed by centrifugation (Heraeus Multifuge 3SR Plus Centrifuge, Thermo Scientific Ltd., UK) at 10,000 g for 15 mins. The absorbance of the supernatant was measured at 532 nm using a UV spectrophotometer (CECIL CE 1021 1000 Series, Cecil Instruments Ltd., UK). The absorbance of the samples was measured against a blank solution (7.5% w/v TCA). The concentrations of TBARS values were determined by using a standard curve prepared using malondialdehyde MDA standard (4.17 M). A concentration of MDA standards between 0.02 to 0.10 mM were prepared where linear response was observed coefficient correlation (R^2) = 0.9987. Three analytical repetitions of each measurement were done for each emulsion batch.

3.2.5 Statistical analysis

Statistical analysis of the data was performed using IBM SPSS 25 (Armonk, NY: IBM Corp, USA). To assess the effect of the pressure (1, 200, 400, 600 and 800 bars) on the properties of nanoemulsions one-way analysis of variance (ANOVA) and Tukey's HSD test were used to evaluate the mean values' difference ($p < 0.05$). Then, a two-way ANOVA was conducted to assess the effect of the processing conditions: pressure (200 bars and 400 bars) and number of cycles (1, 2, 3 cycles). Furthermore, a two-tail paired t-test was used to compare the effect of the two-surfactant studied (tween 20 and soy lecithin) on emulsion properties. The experimental data was analysed and reported as means and standard deviations.

The regression ANOVA was applied to determine the best HPH conditions to produce stable nanoemulsions. This regression ANOVA was employed using the Statistical Analysis System (SAS) software (SAS Institute Inc., USA). The regression model of the influence of high-pressure homogenisation processing parameters were analysed; homogenisation pressure at 1, 200, 400, 600 and 800 bars and the cycle number of 1, 2 and 3 were optimized in order to minimize the response variables under study of MDD, PDI, thermal stability (TS) and TBARs, and to maximize the response variables under study of TS using response surface regression. The empirical second-order polynomial model used to fit the measured responses was:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2 \qquad (2)$$

where y is the predicted response, β_0 is the model constant, β_1 and β_2 are the linear coefficient, $\beta_{11}$and β_{22} are the quadratic coefficient, β_{12} is the coefficient for the interaction effect, and x_1 and x_2 are independent variables. The goodness of the fit model was evaluated by the lack of fit test, the determination coefficient (R^2), and the analysis of variance (ANOVA) using the Response Surface Regression (RSREG) procedure of SAS. Statistical fit of the model was determined by Fisher's statistical test. The robustness of the model was assessed by the determination coefficient (R^2) and Fisher's F test at 95% confidence level.

3.3 Results and discussion

3.3.1 Effect of HPH pressure on the physical and chemical properties of nano/emulsions

The effect of homogenisation pressure on MDD and PDI of emulsions formulated with Tween 20 or soy lecithin are shown in Table 3.1. Tween 20 stabilised emulsions showed a significant decreased ($p < 0.05$) in droplet diameter and polydispersity values when the homogenisation pressure increased up to 400 bars; above this pressure there was no significant decrease in both parameters. The decrease in droplet size and polydispersity index of emulsions with increasing pressure can be attributed to an increase in shear forces and cavitation during HPH, which resulted in reduction of particle size and a more homogeneous particle size distribution (Qian and McClements, 2011; Floury *et al.*, 2000). The decrease in droplet size in the Tween 20 stabilised emulsions correlated to significantly higher ($p < 0.05$) lipid oxidation values in these emulsions (Table 3.1). An increase in droplet surface area as the droplet size decreases has been related to higher lipid oxidation rates (McClements and Decker, 2000; Gohtani *et al.*, 1999). In the lecithin stabilised emulsion, the homogenisation pressure did not have a significant ($p > 0.05$) effect on MDD, but PDI values significantly decreased ($p < 0.05$) with increasing homogenisation pressure up to 400 bars; higher pressures, did not have a significant effect on emulsions' PDI ($p \geq 0.05$). The limited effect of HPH on the MDD values of lecithin emulsions could be attributed to its molecular weight (lecithin, 643.9 g/mol; Tween 20, 522 g/mol); larger molecules need more time to be absorbed at interfaces (Karbstein and Schubert, 1995; Jafari *et al.*, 2008), thus time during homogenization did not allow sufficient time to the surfactant to relocate at the interface. Moreover, as no further effects on nanoemulsions' droplet size and polydispersity were observed above 400 bar, 200 and 400 bars were selected to study the effect of the two different surfactants and the interaction between pressure and number of cycles on nanoemulsion properties.

Table 3.1. Effect of homogenisation pressure on mean droplet diameter, polydispersity index and lipid oxidation of emulsions formulated with Tween 20 (5%) or soy lecithin (5%).

Pressure (bars)	MDD (nm)		PDI		TBARS (μM MDA)	
	Tween 20	Soy lecithin	Tween 20	Soy lecithin	Tween 20	Soy lecithin
0	594.00^{a} (81.85)	185.43^{a} (2.38)	0.872^{a} (0.094)	0.360^{a} (0.039)	5.07^{c} (0.20)	9.51^{a} (0.59)
200	277.03^{b} (2.83)	182.98^{a} (2.24)	0.413^{b} (0.017)	0.312^{b} (0.054)	5.17^{bc} (0.24)	8.77^{a} (0.50)
400	188.72^{bc} (1.45)	183.59^{a} (2.17)	0.285^{c} (0.024)	0.273^{c} (0.013)	5.40^{ab} (0.20)	9.07^{a} (0.86)
600	170.53^{c} (1.68)	185.06^{a} (1.59)	0.252^{c} (0.006)	0.260^{c} (0.010)	5.56^{a} (0.22)	9.42^{a} (0.81)
800	170.39^{c} (3.05)	185.23^{a} (1.07)	0.250^{c} (0.009)	0.252^{c} (0.016)	5.40^{ab} (0.10)	8.80^{a} (0.67)

Indicated values are reported as means (standard deviation). Values with the different superscript letters are significantly different ($p < 0.05$) between samples in the same column.

3.3.2 Effect of surfactant type on the physical and chemical properties of nanoemulsions

The interfacial activity of surfactants plays an important role providing emulsification and reducing the oil-water interfacial tension, protecting droplets against coalescence, and improving emulsion stability (Kronberg *et al.*, 2014). In this section, the effect of a non-ionic synthetic surfactant (Tween 20) and a zwitterionic surfactant extracted from a natural source (soy lecithin) on physicochemical properties and stability of nanoemulsions produced using high-pressure homogenisation at 400 bars and 1 cycle was investigated (Table 3.2). The HPH process was selected as the shortest and most efficient process that can produce stable nanoemulsions as shown in section 3.3.1.

Regarding the particle size and polydispersity index values, lecithin gave place to nanoemulsions with significantly ($p < 0.05$) smaller droplet size than Tween 20. The effect of surfactant on particle size can be attributed to its concentration (Arancibia *et al.*, 2017; Bai *et al.*, 2016) and viscoelastic properties (Nash and Erk, 2017). When the viscoelasticity of the surfactant solutions was evaluated, both samples showed higher G' than G" in the whole frequency range studied, which indicated solid-like viscoelastic structure (Figure 3.1). The lecithin solution presented greater G' and G" moduli values than Tween 20 solution. The molecules of lecithin have ability to form a thick viscoelastic film, which is strengthened by hydrogen bonding between phosphate groups on neighbouring molecules; whereas Tween 20 molecules, form a weaker network with neighbouring molecules, as they are non-ionic surfactants (Shchipunov, 2001; Nash and Erk, 2017). Similar results were observed when evaluating the viscoelasticity of oil-in-water interfaces when using lecithin or Tween 20 as surfactants at their minimum concentration to form nanoemulsions (Nash and Erk (2017).

In terms of the thermal stability, soy lecithin nanoemulsions showed a significantly lower ($p < 0.05$) stability than Tween 20 nanoemulsion. It has been reported that lecithin molecules can transit from a solid-like to a liquid-like structure when increasing temperature (Pichot *et al.*, 2013). This change in the viscoelastic properties of lecithin could lead to oil droplets aggregation and lower emulsion stability during storage and temperature changes.

Soy lecithin nanoemulsion presented a significantly higher ($p < 0.05$) TBARs value than Tween 20 nanoemulsions. These results could be explained by two mechanisms; the first one relates to the structure of lecithin. The hydrophobic groups of soy lecithin are fatty acids (Bueschelberger, 2004; McClements, 2016b), the double bonds of these fatty acids play an important role in the initiation step of lipid oxidation because the hydrogen atom attached to the carbon between double bond is easily removed and provide alkyl radicals (Choe and Min, 2006). For this reason, soy lecithin stabilised nanoemulison could experience higher lipid oxidation rates compared to Tween 20 samples. The second mechanism is related to the rate of lipid oxidation at the surface of the oil droplets; a high rate of lipid oxidation is attributed to an increased surface area as the droplet size decreases (McClements, 2011; McClements and Decker, 2000). Therefore, the smaller droplet size of soy lecithin stabilised nanoemulsion may promote a higher lipid oxidation rate than in Tween 20 nanoemulsion. These results are in agreement with previous studies in which it was found that emulsions stabilised by xanthan gum with smaller droplets (3.4 μm) showed higher lipid oxidation values than emulsions with larger droplets (6.4 μm) (Gohtani *et al.*, 1999); lipid oxidation of nanoemulsions (66 nm) stabilised by whey protein isolate was higher than in emulsions (325 nm) (Lee *et al.*, 2011), indicating that emulsions with smaller droplet size were contributed to higher values of lipid oxidation.

Table 3.2. Effect of Tween 20 or soy lecithin on the physicochemical properties and stability of nanoemulsions.

Properties	Surfactant type	
	Tween 20	Soy lecithin
MDD (nm)	188.72^a (1.45)	183.59^b (2.17)
PDI	0.285^a (0.024)	0.272^a (0.013)
Thermal stability (%)	100.00^a (0.00)	96.78^b (0.43)
TBARS (μM MDA)	5.40^b (0.20)	8.72^a (0.69)

Indicated values are reported as means (standard deviation). Values with the different superscript letters (within same row) are significantly different ($p < 0.05$).

Table 3.3. Characterisation interfacial tension

Aqueous phase	Interfacial tension (mN m^{-1}) at concentration 5% w/w	critical micelle concentration (% w/w)
Water	14.15^{a} (0.17)	-
Tween 20	3.03^{c} (0.08)	0.001
Soy lecithin	5.83^{b} (0.74)	5.000

Indicated values are reported as means (standard deviation). Values with the different superscript letters (within same column) are significantly different ($p < 0.05$).

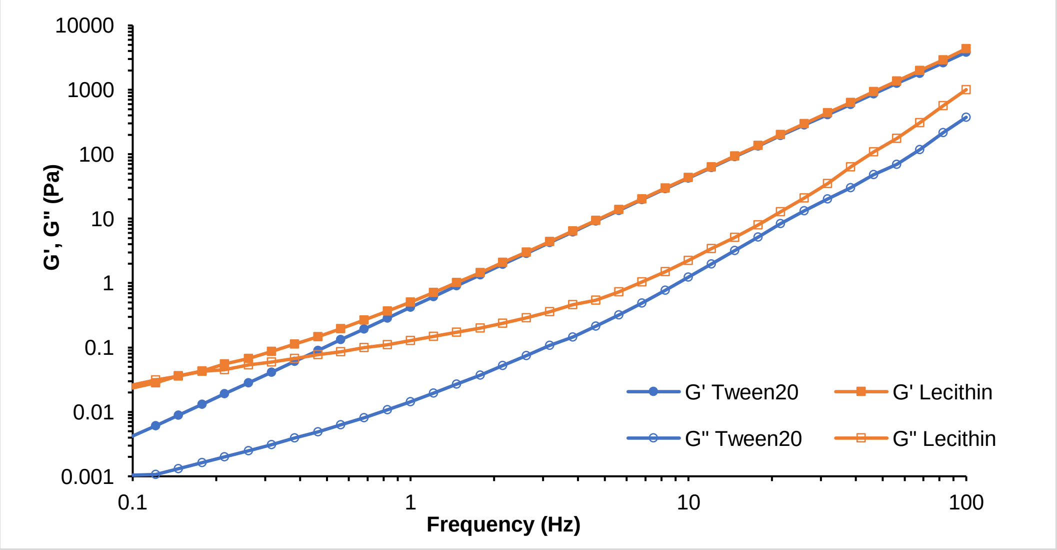


Figure 3.1. Storage modulus (G') and loss modulus (G") of Tween 20 and soy lecithin solutions.

3.3.3 Effect of HPH pressure and number of cycles interaction on the physical and chemical properties of nano/emulsions stabilised with Tween 20 and lecithin

The interaction between HPH pressure and cycle number on nano/emulsion properties was assessed. The pressures applied were 200 and 400 bars because at these pressures the MDD values of both emulsions were around 200 nm, as it has been shown in previous sections (3.3.2). The number of cycles selected were 1, 2 and 3 in order to produce nanoemulsions with oil droplets particle size of less than 200 nm. There was a significant interaction ($p < 0.05$) between homogenisation pressure and number of cycles for the MDD of nano/emulsions formulated with Tween 20 (Figure 3.2A). At both homogenisation pressures (200 and 400 bars) when the number of cycles increased the droplet size significantly decreased ($p < 0.05$). However, there was no significant interaction between factors ($p > 0.05$) for the PDI of nano/emulsions formulated with Tween 20. The PDI significantly decreased ($p < 0.05$) when the homogenisation pressure and number of cycles increased (Figure 3.2C). When the number of cycles increased there were more events prompting to oil droplet break-up (Lee *et al.*, 2014) due to the following cycles of homogenization allowed an increase in energy input for emulsification (Floury *et al.*, 2000), and an more time was available for the surfactant to be absorbed on the oil-in-water interface (Karbstein and Schubert, 1995; Jafari *et al.*, 2008). Thus, there was more efficient surfactant absorption onto the new oil-water interface after several cycles of HPH. These results were in agreement with previous studies, in which increasing the number of homogenisation cycles resulted in a significant reduction in the droplet size and size distribution in o/w emulsions stabilised with Tweens surfactants (Galvão *et al.*, 2018; Yuan *et al.*, 2008). Interestingly, in terms of nanoemulsions formulated with soy lecithin, there was no significant interaction between homogenisation pressure and number of cycles ($p > 0.05$) for the MDD and PDI (Figure 3.2B and D, respectively). In general, the increase in pressure or cycle number did not have a significant effect on the lecithin emulsions' properties. This result could be due to the viscoelastic property of lecithin at the emulsion interface that formed a thick viscoelastic film stabilising the oil/water interface (Shchipunov, 2001; Nash and Erk, 2017), but the forces generated in the HPH and time timescale weren't enough to promote a significant change in droplet particle size.

Regarding the thermal stability, there was a significant interaction ($p < 0.05$) between homogenisation pressure and number of cycles for the thermal stability of both Tween 20 and lecithin stabilised nano/emulsions (Figure 3.2E and F), an increase in the homogenisation pressure and number of cycles resulted in significantly ($p < 0.05$) higher stability. When applying 200 bars, the effect of the number of cycles was greater than at 400 bars. When evaluating the implications of scaling up energy intensive processes, such as HPH other factors a part of product properties should be taking into account, such as time and costs of the process. For nanoemulsions formulated with Tween 20, the shorter and less intensive homogenisation process that gave the emulsions with the greatest stability was 1 cycle at 400 bars (Figure 3.2E), due to the smaller droplets size and narrower size distribution values than when processed at 200 bars (Figure 3.2A and C). A decrease in particle droplet size gives place to a decreases of the attractive forces between the droplets (Tadros *et al.*, 2004a; Qian and McClements, 2011); smaller droplets have better stability against droplet coalescence and flocculation because of the reduction in Brownian motion and gravitation forces (Tadros *et al.*, 2004a), allowing nanoemulsions to be protected against flocculation phenomena. Regarding the nanoemulsions formulated with soy lecithin the shorter and less intensive homogenisation process was 1 cycle at 400 bars (Figure 3.2F).

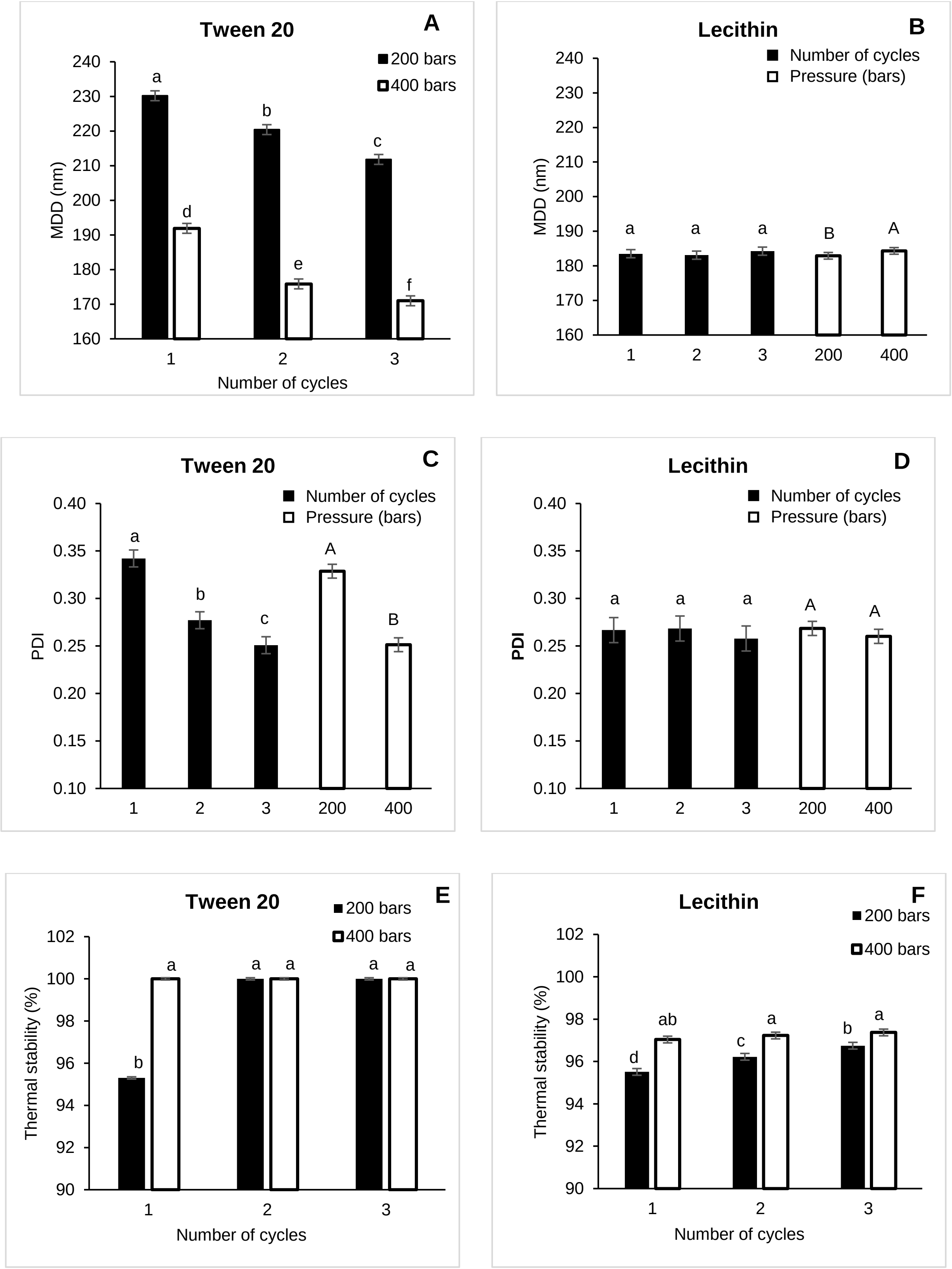


Figure 3.2. Interaction plots (mean ± 95% confidence interval). A and B: interactions between the pressure and number of cycles for the MDD of nanoemulsions formulated with Tween 20 and lecithin, respectively. C and D: interactions between the pressure and number of cycles for

the PDI of nanoemulsions formulated with Tween 20 and lecithin, respectively. E and F: interactions between the pressure and number of cycles for the thermal stability of nanoemulsions formulated with Tween 20 and lecithin, respectively. Different capital or lower-case letters above bars indicate significant differences between samples ($p < 0.05$).

3.3.4 Optimisation of high-pressure homogenisation processing parameters

3.3.4.1 Model fitting

MDD, PDI, TS and TBARs are most important properties of a nanoemulsion used to optimize homogenisation processing parameters (Jafari *et al.*, 2006; Ruiz-Montañez *et al.*, 2017; Sadeghpour Galooyak *et al.*, 2015). The experimental conditions including homogenisation pressure and number of cycles with their corresponding response values were subjected to regression analysis. The regression coefficients for the second-order polynomial equations and results for the linear, quadratic, and interaction terms as well as the coefficient of determination (R^2) are presented in Table 3.4 and 3.5.

The *P*-values were used as a tool to check the significance of the interactions among the variables. According to the ANOVA of the regression models (Table 3.4), there was a high coefficient of determination (R^2) obtained for dependent variables such as MDD and PDI without significant lack of fit, indicating a satisfactory adjustment of the polynomial model to the experimental data. The polynomial model of MDD and PDI was calculated to be between 0.737 and 0.982, indicating that 73.7% and 98.2% of the variability in the response could be explained by the second-order polynomial prediction equation. However, there was a low R^2 for TBARs and a significant lack of fit test for TS of Tween 20 stabilised emulsion, indicating that TS and TBARs were not a good fit for the model. Therefore, the models of MDD and PDI can be used to describe the optimisation of homogenisation parameters.

Table 3.4. *P*-values for Regression ANOVA hypothesis testing to determine the effect on processing on the properties of the emulsions using a quadratic fit.

Regression	ANOVA							
	MDD		PDI		TS		TBARS	
	Tween 20	Soy lecithin	Tween 20	Soy lecithin	Tween 20*	Soy lecithin	Tween 20	Soy lecithin
Linear	<.0001	0.136	<.0001	0.001	0.001	0.002	0.167	0.137
Quadratic	0.001	0.043	0.002	0.157	0.179	0.489	0.969	0.544
Cross product	0.134	0.861	0.190	0.480	0.288	0.219	0.451	0.939
Total model	<.0001	0.086	<.0001	0.005	0.003	0.008	0.438	0.343
R-Square	0.979	0.737	0.982	0.905	0.918	0.889	0.483	0.539
Lack of Fit	0.076	0.970	0.952	0.939	0.011	0.889	0.860	0.735

* indicated significant lack of fit.

The regression coefficients along with corresponding *P*-values for the model of MDD and PDI are shown in Table 3.5. Analysis of significance of each term of polynomial model obtained for MDD of Tween 20 stabilised emulsions indicated that linear terms of pressure (x_1) and cycle (x_2) and the quadratic term of pressure (x_2^2) resulted significant for the response, whereas the polynomial model for MDD of soy lecithin stabilised emulsions indicated that linear term of cycle (x_2) and quadratic term of pressure (x_2^2) resulted significant for the response. The second-order polynomial prediction model of MDD can be expressed as follows:

$$y_{MDD\ of\ Tween\ 20} = 568.306 - 0.947x_1 - 212.810x_2 + 0.001x_1^2 + 38.203x_2^2 + 0.161x_1x_2$$

$$y_{MDD\ of\ Lecithin} = 184.275 + 0.009x_1 - 3.977x_2 + 1.126x_2^2 - 0.0004x_1x_2$$

where y is the predicted response of MDD, and x_1 and x_2 are homogenisation pressure (bars) and number of cycles, respectively.

Analysis of significance of each term of polynomial model obtained for PDI of Tween 20 stabilised emulsions indicated that linear terms of pressure (x_1) and cycle (x_2), and the quadratic term of pressure (x_2^2) resulted significant for the response; the PDI of soy lecithin stabilised emulsions indicated that all terms of linear, quadratic and the interaction resulted non-significant for the response. The second-order polynomial prediction model of PDI can be expressed as follows:

$$y_{PDI\ of\ Tween\ 20} = 0.809 - 0.001x_1 - 0.244x_2 + 0.035x_2^2 + 0.0002x_1x_2$$

$$y_{PDI\ of\ Lecithin} = 0.365 - 0.0001x_1 - 0.079x_2 + 0.011x_2^2$$

where:

y is the predicted response of PDI, and

x_1 and x_2 are homogenisation pressure and number of cycles, respectively.

The R^2 of the quadratic model remained very high as seen in the Table 3.4, which indicates a very good statistical fit. This suggests that we can use the quadratic models to navigate the response space.

Table 3.5. Results of regression coefficients and analysis of the quadratic model for MDD, PDI, TS and TBARS of Tween 20 and soy lecithin stabilised emulsions.

Terms	Coefficient estimate							
	MDD		PDI		TS		TBARS	
	Tween 20	Soy lecithin	Tween 20	Soy lecithin	Tween 20	Soy lecithin	Tween 20	Soy lecithin
Intercept β_0	568.306***	184.275***	0.809***	0.365***	90.694***	93.950***	5.477***	9.257***
Linear β_1(pressure)	-0.947*	0.009	-0.001*	-0.0001	0.027	0.023*	-0.001	-0.001
β_2 (cycle)	-212.810*	-3.977*	-0.244*	-0.079	2.126	-1.946	0.311	-1.165
Quadratic β_{11} (pressure × pressure)	0.001*	<0.0001	<0.0001 *	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

β_{22} (cycle× cycle)	38.203	1.126*	0.035	0.011	0.165	0.784	-0.082	0.204
Cross product β_{12} (pressure × cycle)	0.161	-0.0004	0.0002	<0.0001	-0.005	-0.004	0.001	0.0002

*Significant at the 0.05 level
**Significant at the 0.01 level
***Significant at the 0.001 level

3.3.4.2 Canonical and ridge analysis

The canonical analysis of the response surface was performed to determine the shape of the fitted response and the estimated stationary point. The critical value of the canonical analysis represents the value of the factors (processing) with the highest sensitivity to the measured emulsion parameters with sensitivity. Regarding the variables of pressure and number of cycles, please note that both variables can be outside the design space in the canonical analysis output. For example, the number of cycles cannot be lower than 1 cycle and must be an integer. Therefore, the column next to calculated critical value for cycle represents the more meaningful output. As shown in Table 3.6, the canonical and stationary point analysis of MDD and PDI of Tween 20 indicated that the stationary point was a minimum point. Therefore, the estimated surface of Tween 20 stabilised emulsions has a unique optimum, and a ridge analysis was performed to determine the optimum. On the other hand, the stationary point of MDD and PDI of soy lecithin was saddle point (mixed signs of all eigen values), suggesting that movement away from these points would cause an increased or decreased response, depending upon direction of movement.

Table 3.6. Canonical analysis of MDD and PDI

Parameter	Emulsifier	Critical value			Stationary point	
		Pressure (bars)	cycle	c.c.	Predicted value	type
MDD	Tween 20	519.55	1.69	2	142.69	minimum
	Soy lecithin	929.26	1.94	2	184.45	saddle
PDI	Tween 20	539.18	2.09	2	0.21	minimum
	Soy lecithin	6226.16	-10.47	0	0.33	Saddle

c.c correction for cycle: cycle must be an integer.

Optimal homogenisation parameters were determined by ridge minimum analysis. The method of ridge minimum analysis computes the estimated ridge of the minimum response by decreasing radius from the centre of the original design. The results of ridge analysis (Table 3.7 and 3.8) indicated that homogenisation pressure and cycle were positively related to the response. Due to the fact that both variables of pressure and number of cycles must be an integer, the ridge minimum analysis of Tween 20 stabilised emulsion indicated that the minimum value of MDD was 174.64 nm at the optimal pressure at 764 bars and 1cycle, and the minimum value of PDI was 0.23 at the optimal pressure of 465 bars and 3 cycles. In terms of the soy lecithin stabilised emulsions, the minimum value of MDD was 180.80 nm at the optimal pressure of 3 bars and 2 cycles, and the minimum value of PDI was 0.23 at optimal pressure of 223 bars and 3 cycles.

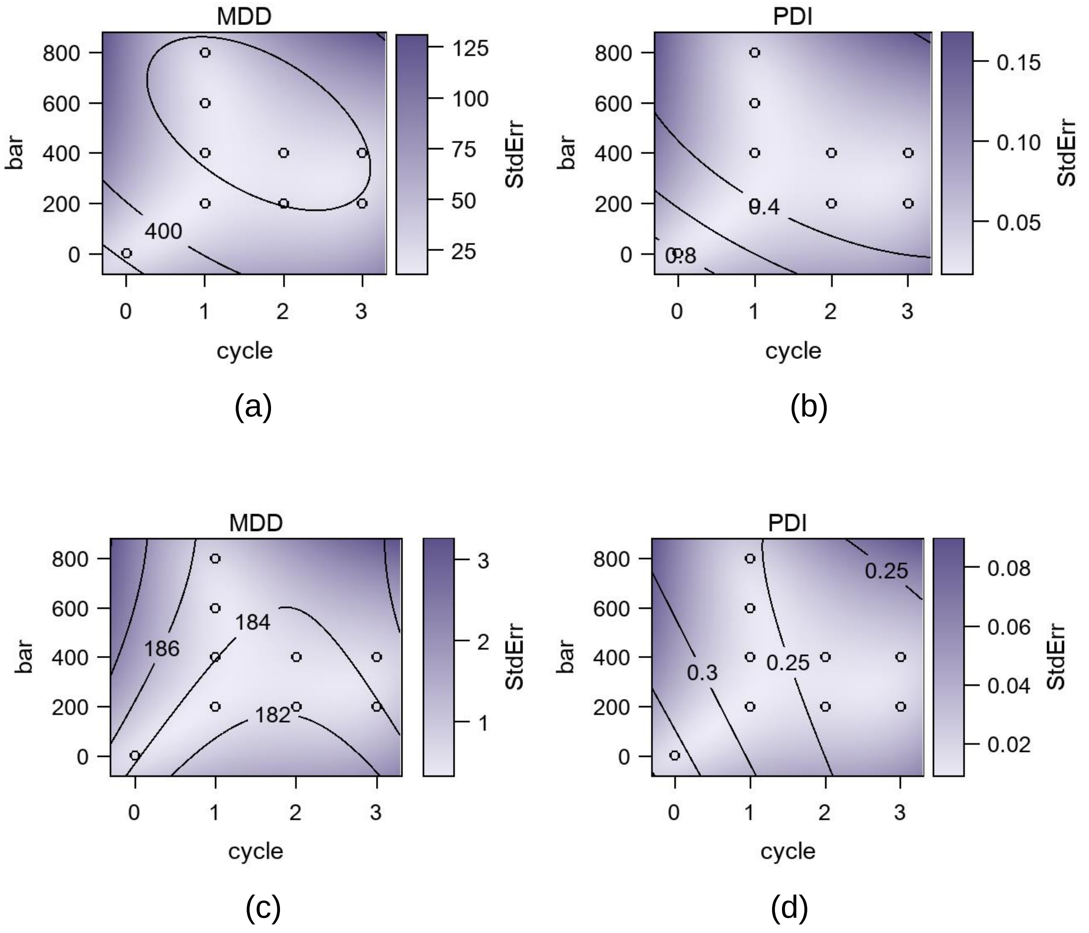
Table 3.7. Estimated Ridge of Minimum Response for Variable MDD of Tween 20 and soy lecithin stabilised emulsions.

Coded Radius	Estimated Response (MDD)	Standard Error	Pressure (bars); X1	Cycle; X2
Tween 20				
0.0	156.88	15.23	400.50	1.50
0.2	144.68	19.19	469.59	1.65
0.4	143.35	23.74	555.26	1.65
0.6	149.54	21.54	638.52	1.39
0.8	160.16	19.49	704.47	1.13
1.0	174.64	25.66	763.92	0.88
Soy lecithin				
0.0	183.39	0.38	400.50	1.50
0.2	182.95	0.39	325.39	1.60
0.4	182.48	0.44	245.24	1.64
0.6	181.97	0.60	164.53	1.66
0.8	181.41	0.86	83.88	1.66
1.0	180.80	1.21	3.38	1.66

Table 3.8. Estimated Ridge of Minimum Response for Variable PDI of Tween 20 and soy lecithin stabilised emulsions.

Coded Radius	Estimated Response (PDI)	Standard Error	Pressure (bars); X1	Cycle; X2
Tween 20				
0.0	0.25	0.02	400.50	1.50
0.2	0.22	0.03	463.22	1.69
0.4	0.21	0.03	517.94	1.91
0.6	0.21	0.05	540.91	2.23
0.8	0.21	0.05	510.12	2.63
1.0	0.23	0.04	465.15	2.98
Soy lecithin				
0.0	0.25	0.01	400.50	1.50
0.2	0.24	0.01	415.65	1.79
0.4	0.24	0.01	405.03	2.10
0.6	0.23	0.01	358.94	2.39
0.8	0.23	0.01	293.27	2.63
1.0	0.23	0.01	222.55	2.84

To visualise the impact of the processing parameters to the properties of the emulsions, two-dimensional contour plot between homogenisation pressure and cycle were constructed as shown in Figure 3.3a-d. The prominent interaction effect is shown in Figure 3.3A and B. The significant effect of pressure and cycle could be observed in the graphs, which indicated that MDD and PDI of Tween 20 stabilised emulsions decreased when increasing the pressure and cycle of homogenisation. Regressing analysis (Table 3.5) also confirmed that MDD and PDI of Tween 20 stabilised emulsions were significantly ($p < 0.05$) affected by pressure and cycle, which is also in agreement with the classical statistical analysis (Table 3.2 and Figure 3.2). On the other hand, the number of cycles was a main variable affecting on MDD of soy lecithin stabilised emulsion, which MDD decreased when increasing cycle number. As the results of optimisation analysis and model fitting, although there is a good fit with the model for the MDD and PDI of both nanoemulsions, the experimental validation of the predicted model should be carried out as further work. Moreover, this optimisation analysis would be a more effective result if there was a larger size of samples for a more robust calibration.



(a) (b)

(c) (d)

Figure 3.3. Contour plot for the impact of the processing parameters to the properties of the emulsions for Tween 20 stabilised emulsion (a-b) and soy lecithin stabilised emulsion (c-d).

3.4 Conclusions

This study has demonstrated the effect of high-pressure homogenisation processing parameters including pressure and number of cycles on the formation and physicochemical stability of extra virgin olive oil-in-water nanoemulsions stabilised by Tween 20 or soy lecithin. Although the particle size and size distribution of Tween 20 nanoemulsions were influenced by homogenisation pressure and cycles, there was not an effect on soy lecithin nanoemulsions. This showed that the molecular weight and viscoelastic properties of Tween 20 and soy lecithin play a major role in the formation and properties of emulsion. In addition, high-pressure homogenisation at pressure at least 400 bars and 1 cycle showed the better results in terms of nanoemulsion stability. Although Tween 20 nanoemulsion showed significantly higher values ($p < 0.05$) of thermal stability and lipid oxidative stability than soy lecithin nanoemulion, soy lecithin is an interesting surfactant for application in food industry as it is obtained from natural sources and provides more desirable taste compared to Tween 20. The optimal homogenisation parameters for both emulsions were successfully modelled by RSM and provided information to support the production of nanoemulsions at industrial scale. Overall, HPH proved to be an effective process to obtain nanoemulsions stabilised by soy lecithin for application in food and beverage products and the data of optimisation of processing parameters. For more complete the results, future work could include the validation of the model.

Chapter 4

Influence of hydroxypropyl methylcellulose (HPMC) hydrophobicity and concentration on its rheological, textural and surface activity properties

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Abstract

Hydroxypropyl methyl cellulose (HPMC) is a hydrocolloid with surface activity, rheology, and thermo-reversible gelation properties. Thus, there is an interest in applying HMPC solutions as fat mimetics in food matrices. The objective of this study was to investigate the properties of HPMC samples with different degree of substitution of hydroxyl groups by methoxy and hydroxypropyl groups. Two different types of HPMC with low and high content of methyl and hydroxypropyl groups (HPMC-L and HPMC-H) at different concentrations (2-10% w/w) were evaluated. Interfacial tension, viscoelastic properties, thermal gelation, and spreadability were examined. Scanning electron microscopy was used to observe the network structures formed. The results showed that HPMC-L had a higher surface activity leading to a lower interfacial tension value than HPMC-H. The molecular structure and concentration of HPMC play a major role in the viscoelastic behaviour, the gelation temperature and the strength of gel formed. HPMC-H presented higher storage and loss moduli values than HPMC-L, and the moduli values of HPMC solutions increased with increasing the concentration. Butter showed viscoelastic solid behaviour, whereas all HPMC solutions behaved as viscoelastic fluid and showed a crossover point at high frequency values. The firmness and work of shear of HPMC solutions increased significantly ($p < 0.05$) with increasing concentration, indicating lower spreadability. HPMC-L illustrated a more stable gel structure than HPMC-H solution. In conclusion, HPMC-L could be more suitable hydrocolloid to formulate fat mimetics because of its higher surface activity and more stable gel structure.

Keywords: Hydroxypropyl methylcellulose (HPMC), Butter, Rheology, Viscoelasticity, Temperature ramp test, Texture properties

4.1 Introduction

Hydrocolloids have a wide range of functionalities in food products; they could be use as thickener, gelling, emulsifier, stabilizer and coating agents (Milani and Maleki, 2012). HPMC is a hydrocolloid associated with surface activity, mechanical structure, and thermo-reversible gelation properties (Sanz *et al.*, 2015a; Li *et al.*, 2013b). HPMC is a non-ionic cellulose ether, which contains hydrophobic methoxy and hydrophilic hydroxypropyl groups on the cellulose chains added to the anhydro glucose backbone (Camino *et al.*, 2011; Wollenweber *et al.*, 2000)(Figure 1.6 in chapter 1). Due to the presence of these substituents on the polymer chains, it has surface activity and reduces the surface tension of colloidal systems (Li *et al.*, 2013b). Moreover, when the temperature increases a HPMC gel network is formed due to an exposure of the hydrophobic groups and consequent association between HPMC chains (Li *et al.*, 2001; Liu *et al.*, 2008). There are several studies conducted on the thermo-reversible gelation of HPMC aqueous solutions (Seyedlar *et al.*, 2014; Hussain *et al.*, 2002; Silva *et al.*, 2008; Yoo and Um, 2013) that showed that the molecular weight with different content of methoxy and hydroxypropyl substitution and concentration of HPMC played an important role in the behaviour of solutions, strength of gels and the gelation temperature. Due to these functional properties, HPMC has been used in strategies to reduce saturated fat in many food applications (Sanz *et al.*, 2015c; Meng *et al.*, 2018a; Meng *et al.*, 2018b); HPMC improved crumb texture, and sensory properties of biscuits and muffins when shortening was reduced by HPMC or inulin (Laguna *et al.*, 2014). Moreover, HPMC controlled the viscosity of cake batter increased viscosity of cake batter, which increased the viscosity of the continuous phase of the system and limited bubble loss during batter preparation when starch addition (Bousquières *et al.*, 2017).

Butter is a water in oil emulsion made from milk, which is the separated cream with centrifuging before churning and kneading (Gunstone, 2009). The structure of butter consists of crystallized fat and non-globular fat in a continuous phase, fat globules and partially crystalline fat globules are dispersed (Martini and Marangoni, 2007; Rønholt *et al.*, 2013) as seen in Figure 4.1. The fat crystals formed will aggregate and grow into a three-dimensional network in the continuous fat phase and has a major influence on the rheological attributes of butter (Fuquay *et al.*, 2011). Due to the difference in structure between butter and HPMC solutions, an understanding of their rheological and texture properties is essential for formulating fat replacers low in saturated fatty acids.

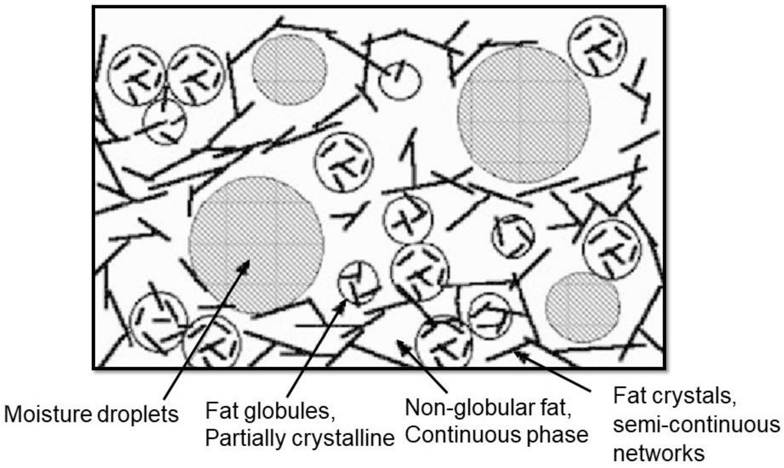


Figure 4.1. Schematic structure of butter.

Source: Martini and Marangoni (2007)

Therefore, the objective of this study was to examine the influence of degrees of substitution and concentration of HPMC on rheological and texture properties of its aqueous solutions compared to butter. In addition, the surface activity and microstructure of HPMC solution and gelation analyzed. This study was to obtain a better understanding of characteristic and physical properties of HPMC and butter, which was very important to select a suitable type of HPMC and concentration for incorporation with nanoemulsion used as fat replacers in food products.

4.2 Materials and methods

4.2.1 **Materials**

Two different types of commercial hydroxypropyl methylcellulose (HPMC) were used (Biosynth carbosynth Limited, UK); one with low methoxy and hydroxypropyl content (HPMC-L) and another one with higher content of methoxyl and hydroxypropyl (HPMC-H) (Table 4.1). Butter was used as a reference material (salted butter with 82.0% of fat and 1.5% of salt; Co-operative Group., Ltd, UK).

Table 4.1. Properties of the HPMC used, as provided by the manufacturer.

Sample	Viscosity (mPa·s)	Methoxy (%)	Hydroxypropyl (%)	Molecular weight (g/mol)
HPMC-L	80 – 120 (low viscosity)	21.4	7.2	26,000
HPMC-H	3000 – 5600 (high viscosity)	28.9	8.1	86,000

4.2.2 Preparation of HPMC solutions

HPMC solutions (4, 6, 8 and 10% w/w of HPMC-L and 2, 3, 4 and 5% w/w of HPMC-H) were prepared following the method described by Liu *et al.* (2008) and Ding *et al.* (2014) with some modifications. HPMC powder was dispersed into distilled water using a high-speed homogeniser (Silverson, Model L4RT, UK) at 200 rpm for 30 min at room temperature, then left at 4 °C for at least 24 h in order to allow the HPMC to be completely hydrated before the measurements. All samples were prepared in triplicate.

4.2.3 Interfacial tension measurement

The interfacial tension between HPMC solutions and extra virgin olive oil was determined using a pendant drop analyser (DS4270, Krüss GmbH, Hamburg, Germany) at 20 °C. An axisymmetric drop (20 μL) of HPMC dispersion was delivered and allowed to stand at the tip of the needle inside a quartz container of extra virgin olive oil (9 mL) for 15 min, to achieve emulsifier adsorption at oil-water interface (Bai *et al.*, 2016; Luo *et al.*, 2017). Three analytical repetitions of each measurement were done for each emulsion batch.

Following the interfacial tension, different concentrations of HPMC solutions at 0.00, 0.001, 0.05, 0.1, 0.1, 0.3, 0.5, 0.8 and 1.0 %w/w were prepared to measure critical micelle concentration (CMC).

CMC was determined at the intersection points of the interfacial tension values versus the HPMC concentration (logarithm) plot (El-Sukkary *et al.*, 2008; Mukherjee *et al.*, 2013). All measurements were performed in triplicate.

4.2.4 Rheological measurements

The rheological measurements were performed using a rheometer (Anton Paar MCR 302, Anton Paar, Graz, Austria) equipped a Peltier temperature control device. A serrated parallel plate geometry was used; the diameter of lower stationary plate (PPTD 200/56/1) and superior plate (PP50/ P2) was 50 mm, and the gap between the plates was 1 mm. The samples were allowed to rest in the measurement position for 5 min for sample relaxation and temperature equilibration before measurements. Amplitude sweep, frequency sweep and temperature ramp measurements were performed at 20 °C for HPMC solutions and at 30 °C for butter. In bakery products, especially in short dough biscuits the fat surrounds the flour particles forming a continuous coat to avoid hydration of protein, starch and other components (Manley, 2011a); in order to achieve this functionality, the shortening to be used has to behave as a plastic and are usually incorporated to the mixing process at 25 °C, when they become more easily spreadable. All the measurements were done in duplicates in two batches of each HPMC solution. The storage modulus (G') and loss modulus (G'') values were recorded.

4.2.4.1 Amplitude sweep

The strain sweeps were carried out at strain amplitude range of 0.001 to 1000% and at a constant frequency of 1 Hz in order to determine the linear viscoelasticity region (LVR). The LVR was identified where G' and G'' were not influenced by applied strain.

4.2.4.2 Frequency sweep

Frequency sweeps were conducted from 0.1 to 100 Hz at a constant strain amplitude within the linear viscoelastic region, in order to determine G' and G'' values.

4.2.4.3 Temperature ramp viscoelasticity

Temperature ramp viscoelasticity was determined according to the method of Sanz *et al.* (2015a) and Yoo and Um (2013). Temperature ramp analyses were conducted in HPMC solutions with a constant strain amplitude of 1.0 % and a frequency of 1.0 Hz. Two temperature ramps were performed from 20 °C to 90 °C and 90 °C to 20 °C with a heating and cooling rate of 1 °C/min and no waiting time between the two ramps. The storage modulus (G'), the loss modulus (G'') and the complex shear modulus (G*) were recorded. The complex shear modulus was calculated as $G^* = G' + i\,G''$.

4.2.5 Scanning electron microscopy (SEM)

SEM was used to analyse the microstructure of HPMC-L and HPMC-H (4 %w/w) gel samples, which were lyophilized in a vacuum freeze dryer for three days and stored in a desiccator. All the samples were cut into small slices and fixed on a brass holder. Then, the surface of samples was coating with gold-palladium and observed in the microscope (FEI Quanta 600 FEG SEM version 2.4, UK) at an accelerating voltage of 20 kV and a magnification of 500x.

4.2.6 Textural analysis

Spreadability was determined according to the method of Stefan and Kocevski (2013) and Chetana *et al.* (2019). Spreadability of samples was measured in a texture analyser (TA.XT Plus, Stable Micro systems Ltd., England) using a TTC spreadability rig (HDP/SR) attachment and a 5 kg load cell. Approximately 10 g of sample were filled into a female cone (90° angle) avoiding bubble occlusion and levelling up the surface. The male cone (90° angle) penetrated the samples 23 mm at a speed of 3 mm/s and the samples were allowed to flow outward at 45° angle between the male and female cone surfaces. Spreadability was measured at 20 °C for HPMC solutions and at 30 °C for butter. The parameter recorded was the work of shear calculated based on the area under the force curve as a measurement of spreadability. The tests were performed in triplicate for each solution batch.

4.2.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed using SPSS 25 (Armonk, NY: IBM Corp, USA). Multiple pairwise comparisons using Tukey's HSD test were used to compare the mean values ($p < 0.05$).

4.3 Results and discussion

4.3.1 Interfacial tension of HMPC solutions

The results of the interfacial tension are shown on Figure 4.2. Both HPMC-L and HPMC-H could reduce the interfacial tension. The balance between hydrophilic hydroxypropyl groups and hydrophobic methoxy groups (hydrophilic lipophilic balance: HLB) provides HPMC amphiphilic properties (Lim *et al.*, 2021; Camino *et al.*, 2011). When HPMC is adsorbed at the oil/water interface acts as an emulsifier reducing the interfacial tension of the system (Wollenweber *et al.*, 2000; Camino *et al.*, 2011; Li *et al.*, 2013b). HPMC-L presented a higher surface activity giving place to lower interfacial tension values across the range of concentrations studied, and a lower value of CMC compared to HPMC-H. Differences in the emulsifying properties of both types of HPMC could be explained due to their molecular weight, HLB values, surface activity; HPMC-L had a lower molecular weight that HPMC-H (Table 4.1), which could be more rapidly adsorbed onto the oil/water interface (Li *et al.*, 2013b); HMPC-L also showed a higher reduction of interfacial tension compared to HPMC-H. The interfacial tension values at their CMC were 10.27 mN/m and 12.54 mN/m for HPMC-L and HPMC-H, respectively. The CMC values of HPMC-L and HPMC-H were obtained at 0.3 %w/w and 0.5 % w/w, respectively. This is consistent with the formation of smaller emulsion droplets at 0.5 %w/w HPMC (22% methoxy; 8% hydroxypropoxy) in comparison with 0.1%, reported in previous studies (Lim *et al.*, 2021).

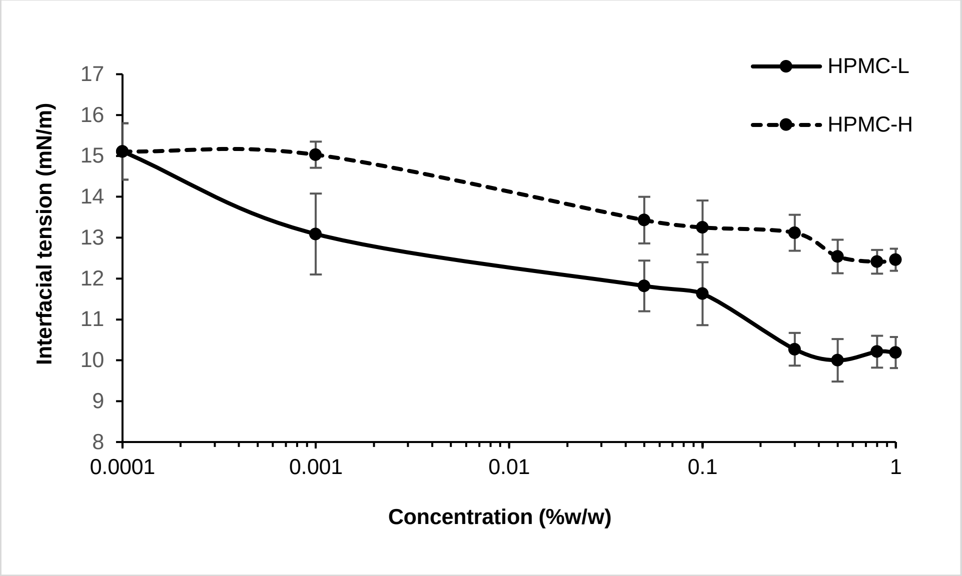


Figure 4.2. Characterisation interfacial tension of HPMC-L and HPMC-H.

4.3.2 Viscoelastic properties of HMPC solutions and butter

The dynamic spectra of HPMC-L and HPMC-H solutions compared with butter is shown in Figure 4.3A and B. The mechanical strength of butter was greater than that of HPMC-L and HPMC-H solutions as illustrated by higher moduli values and lower dependence on frequency. Butter showed a gel-like structure of a viscoelastic solid material as the G' modulus was higher than G" modulus in the whole frequency range studied. In contrast, HPMC-L and HPMC-H showed a high frequency dependence and a predominant viscous over elastic behaviour at low frequencies (0.1-5 Hz) as expected from viscous fluids. At low temperatures (~20 °C) HPMC forms a solution in water as the water molecules form hydrogen bonds with the hydroxyl groups and form enclosed structures to surround the hydrophobic groups of the HPMC chain (Liu *et al.*, 2008; Li *et al.*, 2013b) as shown in Figure 4.3A. As frequency increased G' values increased more rapidly than G", giving place to a moduli crossover point (5-100 Hz) that indicated a change from a liquid-like to a solid-like viscoelastic structure. Other authors have also observed similar moduli cross over point when assessing the microstructure of HMPC concentration in mixed solutions with collagen (Ding *et al.*, 2014). At high frequencies the polymer chains did not have enough time to relax and detangle, so a fixed network was formed that stored the energy increased and behaved more like an elastic structure (Ding *et al.*, 2014). It could hypothesis that in the HMPC solutions under study higher number of hydrophilic-hydrophilic and hydrophobic-hydrophobic interactions formed remained fixed at higher frequencies given place to an elastic HPMC network. In particular, at low temperatures, it has been reported that hydroxypropyl groups domine the aggregation process (Bodvik *et al.*, 2010).

Higher concentration of both HPMCs gave place to an increase in G' and G" moduli values and lower frequency dependence compared with lower concentrations of the polymers (Figure 4.3). These results could be due to the formation of a more structured solution as concentration of the polymer increased. Other authors also found an increase in moduli values for the more concentrated HMPC solutions (Silva *et al.*, 2008; Hussain *et al.*, 2002).

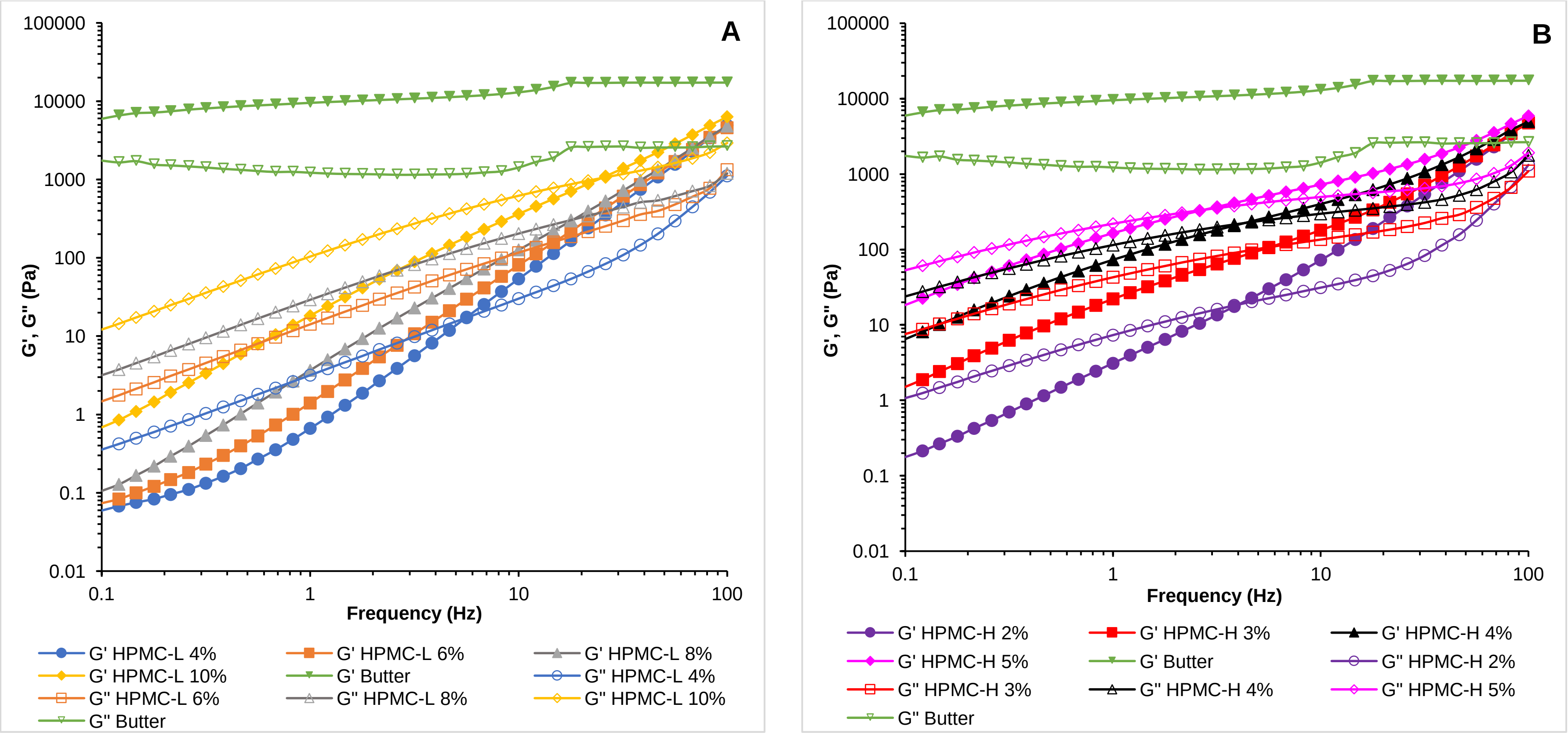


Figure 4.3. G' and G'' moduli as a function of frequency of HPMC solutions and butter. A) HPMC-L solutions at different concentrations (4% blue circles; 6% orange squares; 8% grey triangles; 10% yellow diamond) and butter (green down-pointing triangle). B) HPMC-H solutions at different concentrations (2% purple circles; 3% red squares; 4% pink triangles; 5% black diamond) and butter (green down-pointing triangle). Filled symbols correspond to storage modulus (G') and open symbols to loss modulus (G'').

When comparing both types of HMPC polymers (-L and -H) at the same concentration of 4% w/w, HPMC-L solution exhibited lower G' and G" moduli values, a stronger dependence on frequency than HPMC-H solution (Figure 4.4). These results indicate that HPMC-L had a weaker mechanical strength compared to in HPMC-H solution, and it could be explained by the lower amount of hydroxypropyl (7.2%) and methoxyl (21.4%) substitution level in HPMC-L in comparison to HMPC-H (8.1% and 28.9%, respectively). When hydroxyl groups in methylcellulose (MC) are substituted by hydrophobic groups, some hydrogen bond within MC are prevented and the polymer derivate interacts more with the surrounding water (Li *et al.*, 2001). Moreover, HPMC-H with higher molecular weight range and high ratio of methyl groups results in longer polymer chains with high density of physical cross-linking sites as shown in Figure 4.6, indicating the stronger network and more stable shape (Joshi, 2011). These properties could lead to a stronger and more stable HPMC-H solution.

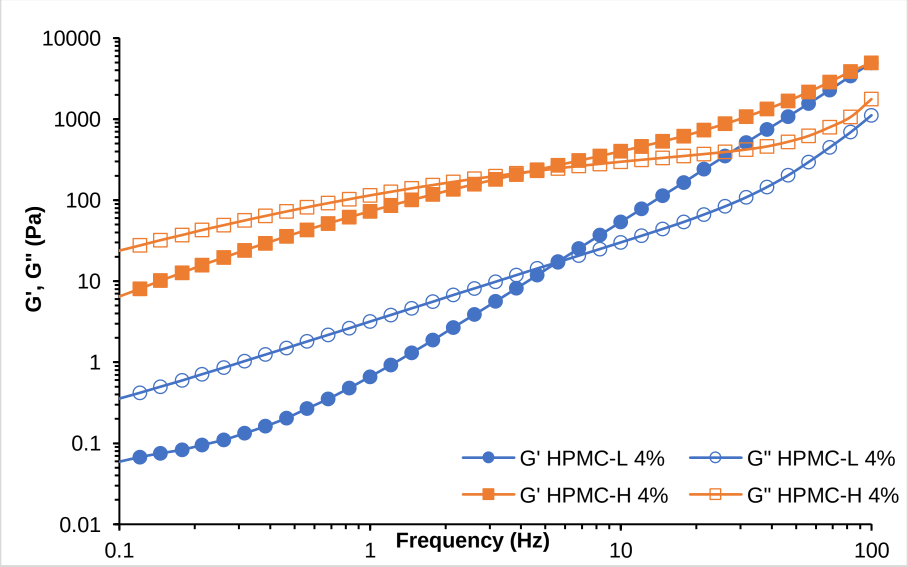


Figure 4.4. G' and G" moduli versus frequency sweep of HPMC-L and HPMC-H solutions at 4% w/w. Filled symbols correspond to storage modulus (G') and open symbols to loss modulus (G").

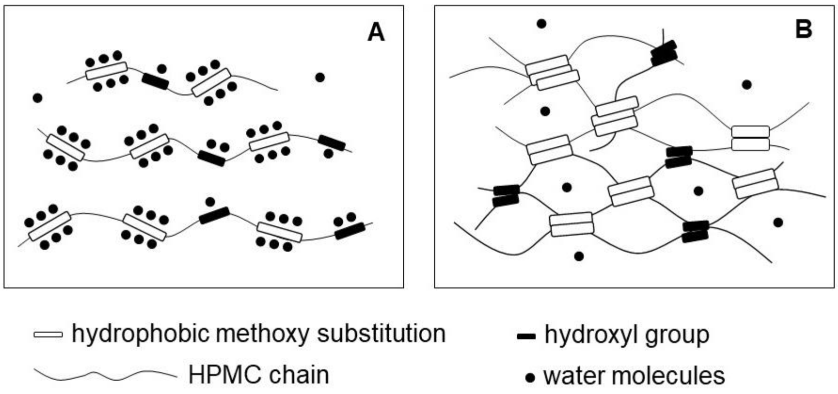


Figure 4.5. Physical structured of the HPMC aqueous solutions: A) at lower temperature; B) at higher temperature Source: Modified from Liu *et al.* (2008) and Joshi (2011).

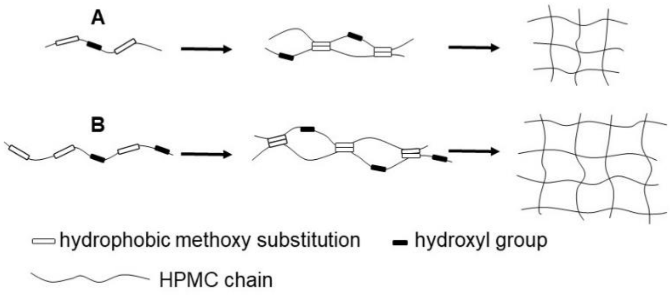


Figure 4.6. Aggregation pattern for different types of HPMC having different content of hydrophobic methoxy substitution and molecular weight as: A) network of HPMC-L; B) network of HPMC-H.

4.3.3 Temperature ramp viscoelasticity

Figure 4.7 shows the gelation pattern as a function of temperature for HPMC-L and HPMC-H solutions. The viscoelastic behaviour of the HPMCs solutions during the heating process (Figure 4.7A and C) can be divided into three regions. At low and mild temperatures, from 20 °C to 30-40 °C, both HPMCs solutions showed a liquid-like viscoelastic behaviour, as G" values were higher than G' values. As already discussed in section 4.3.2., both HMPC polymers form an entangle polymer solution in water at low temperatures. In general, at this temperature range G' and G" moduli values showed a smooth decrease as temperature increased. Then as temperature increased G' and G" moduli increased dramatically and a moduli cross over point was observed. Similar temperature effect on HPMC solutions was reported by other authors (Silva *et al.*, 2008; Liu *et al.*, 2008; Lim *et al.*, 2021; Hussain *et al.*, 2002). The initial decrease on G' and G" moduli values has been related to the disentanglement of cellulose chains, giving place to a loss of viscous and elastic consistency as temperature increased (Liu *et al.*, 2008). Then HMPC backbone flexibility and the dynamics of the water increased with temperature, unveiling the hydrophobic moieties of HPMC and giving place primarily to the formation of strong hydrophobic interactions in the formation of a three-dimensional network, with a contribution from interchain hydrogen bond formation (Liu *et al.*, 2008; Lim *et al.*, 2021; Bodvik *et al.*, 2010) as shown in (Figure 4.5B). This phase transition from a polymer solution to a gel is referred as sol-gel transition and the temperature at which G' increase abruptly is considered the starting point of the gelation temperature (Li *et al.*, 2001; Desbrières *et al.*, 2000; Silva *et al.*, 2008; Liu *et al.*, 2008). The gelation temperature of the different HPMC solutions is shown in Table 4.2. As the HPMC concentration of both HPMC types increased a decrease in the gelation temperature was observed. Lower onset temperature of aggregation and gelation of HPMC solutions has been attributed to higher hydrophobicity due to a higher concentration of methoxy and hydroxypropyl in the solution (Yoo and Um, 2013). As explained before, the hydrophobic interactions among HMPC chains are the principal forces for the formation of a three-dimensional network. However, when comparing the thermogelation of both types of HMPC at the same concentration (Table 4.2), the hydrophobicity did not define the changes in gelation temperatures. The lower gelation temperature of HPMC-L could be explained due to its lower molecular weight that allowed easier interactions between hydrophobic groups for the formation of the gel. These results agreed with previous observations on the effect of temperature on gelation of HPMC with different molecular weight (Seyedlar *et al.*, 2014); low molecular weight HPMC polymers presented a rod-like structure, which allowed a parallel arrangement along the chain leading to an easier aggregation in comparison with higher molecular weight HPLC which had a random coil configuration (Sarkar, cited in Seyedlar *et al.*, 2014).

On cooling, a two-stage process was observed (Figure 4.7B and D): first a gradual decrease, and second a dramatic decrease of G' and G" moduli values. A similar degelation process has been described previously where initially, hydrophobic associations were gradually weakened, and the network dissociated; and then intermolecular hydrogen bonding and water cages reformed around the hydrophobic moieties (Liu *et al.*, 2008). HPLC-H gels showed a more pronounce decreased in the G' and G" values than HPMC-L. These results indicated that HPMC-L gels presented higher mechanical strength during the cooling process. HPMC-L could have higher potential to be applied as structuring agent in complex emulsions, as HPMC-L gel could retain a liquid phase and provide a better stability do to its strength after heating. These results are crucial for the development of complex systems, such as emulsions, in which liquid components (oils) must be retained in the gel network over a series of thermal cycles (e.g. baking).

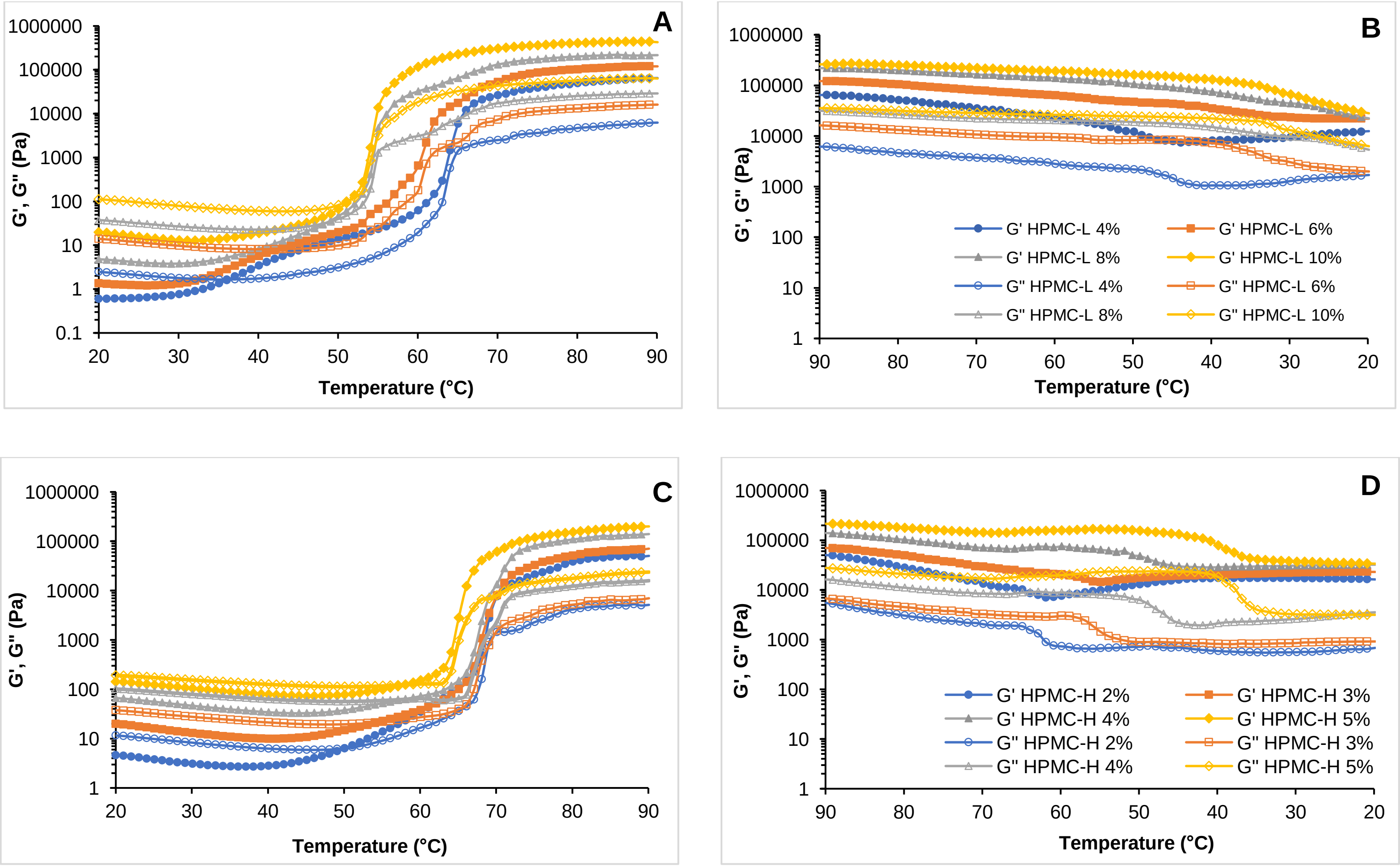


Figure 4.7. G' and G" moduli of HPMC solutions as a function of temperature. A) temperature ramp up for HMPC-L; B) temperature ramp down bot HPLC-L; C) temperature ramp up for HMPC-H; D) temperature ramp down bot HPLC-H. Filled symbols correspond to elastic modulus (G') and open symbols to viscous modulus (G"). Frequency = 1 Hz, heating rate = 1 °C/min.

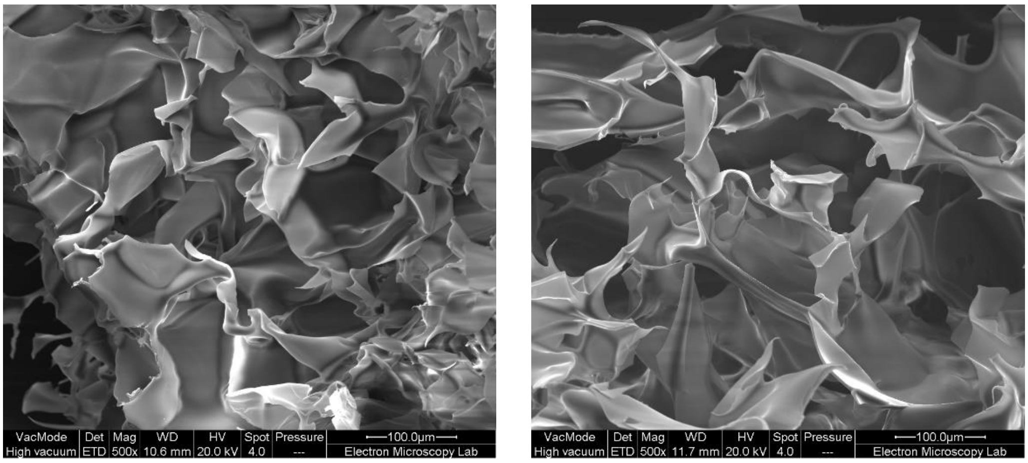
Table 4.2. The gelation temperature and the temperature of HPMC-L and HPMC-H solutions.

Samples	Gelation temperature (°C)
HPMC-L 4%	63.42^{bc} (0.58)
HPMC-L 6%	60.76^{cd} (1.53)
HPMC-L 8%	56.42^{de} (1.53)
HPMC-L 10%	55.09^{e} (1.00)
HPMC-H 2%	70.75^{a} (1.52)
HPMC-H 3%	68.09^{a} (1.00)
HPMC-H 4%	66.42^{ab} (2.08)
HPMC-H 5%	62.75^{bc} (2.51)

Indicated values are reported as means (standard deviation). Values with the different superscript letters are significantly different ($p < 0.05$) between samples in the same column.

4.3.4 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to further understand the microstructure of HPMC gels at 4% w/w as shown in Figure 4.8. The pores of HPMC-L gel were smaller than those of HPMC-H. These results could be related to differences in the polymer configuration. As Sarkar proposed (cited in Seyedlar *et al.* (2014)), higher molecular weight HMPC presents a random-coil configuration leading to a slower gel network formation. As can be seen from Table 4.2, HPMC-H solution showed a significantly higher gelation temperature, leading to a slower gel network formation than HPMC-L solution; this could potentially explain the formation of larger cells, creating a more open in the structure of hydrophobic association between HPMC-H chains. In comparison, HMPC-L with lower molecular weight and a rod-like structure (Sarkar, cited in Seyedlar *et al.*, 2014) could from more easily the hydrophobic interactions giving place to a more regular and compact network.



(A) (B)

Figure 4.8. SEM images of: A) HPMC-L gel, B) HPMC-H gel at 500x.

4.3.5 Firmness and spreadability of HPMC solutions

The firmness and spreadability properties of a colloid define how it will behave during certain unit operations (mixing, sheeting, cutting); thus, these parameters will give us an indication of the technological functionalities of the HPMC solutions. The firmness and spreadability of HPMC solutions were analyzed compared with butter in order to select the suitable type and concentration of HPMC that could be used to formulate emulsions that could be used as saturated fat replacer. As shown in Table 4.3, there were no significant differences in both firmness and work of shear between butter and 4%HPMC-L solution. These results suggest that HPMC-L 4% solution will behave more like butter than the other solutions during food production operations such as mixing or dough sheeting. The concentration of HPMC had a significant effect ($p < 0.05$) on firmness and spreadability of HPMC solutions. It was found that firmness and work of shear of HPMC solutions increased significantly ($p < 0.05$) with increasing concentration: high values of work of shear suggest higher force is needed to induce shearing deformation, which indicate low spreadability. An increase in HPMC concentrations could lead to an increase in cellulose hydrophobicity and the formation of transient three-dimensional network (Sanz *et al.*, 2015a; Sun *et al.*, 2007). Regarding the effect of HPMC type, at the same concentration of 4% w/w, HPMC-

H solution presented significantly ($p < 0.05$) higher values of firmness and work of shear than HPMC-L solution. These results could be explained by the higher mechanical strength of the HPMC-H solution as explained in section 4.3.2.

Table 4.3 Firmness and work of shear of different concentration of HPMC solutions and butter.

Samples	Firmness (N)	Work of shear (N.s)
Butter	0.35^{f} (0.06)	0.26^{de} (0.04)
HPMC-L 4%	0.24^{f} (0.02)	0.10^{e} (0.01)
HPMC-L 6%	0.92^{e} (0.02)	0.48^{d} (0.02)
HPMC-L 8%	2.39^{d} (0.06)	1.31^{c} (0.10)
HPMC-L 10%	5.17^{b} (0.46)	2.86^{b} (0.26)
HPMC-H 2%	0.87^{e} (0.06)	0.49^{d} (0.03)
HPMC-H 3%	2.23^{d} (0.14)	1.39^{c} (0.09)
HPMC-H 4%	3.44^{c} (0.22)	2.94^{b} (0.22)
HPMC-H 5%	8.05^{a} (0.26)	5.77^{a} (0.19)

Indicated values are reported as means (standard deviation). Values with the different superscript letters are significantly different ($p < 0.05$) between samples in the same column.

4.4 Conclusion

This study showed that HPMC-L had a higher surface activity with a lower interfacial tension than HPMC-H. The molecular structure of HPMC and its concentration in solution were determinant factors for the behavior of the polymer in solution during heating, specifically in the mechanical strength of the structures and the onset of the gelation temperature. Higher concentration of hydrophobic groups gave place to HPMC solutions with higher mechanical strength. However, lower molecular weight HPMC formed thermo-reversible gels that presented higher stability during cooling and a higher final strength. Thus, HPMC-L (low molecular weight) could be more suitable hydrocolloid for incorporation in complex emulsions because of its better surface properties and stable gel network that could trap the oil into a more compact gel structure through processing.

Chapter 5

Development of fat replacers:

Nanoemulsion stabilised by lecithin and hydroxypropyl methylcellulose (HPMC)

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This chapter will be combined with chapter 4 to submit to *Food Hydrocolloids* for publication.

Abstract

In this study, the combination of two emulsifiers, lecithin and hydroxypropyl methyl cellulose (HPMC), into emulsions was used as a strategy to design fat replacers high in unsaturated fatty acids with improved physical and chemical stability than emulsion with a single emulsifier. The effect of HPMC concentration (0, 2 and 4% w/w) on the formation, thermal reversibility, viscoelastic properties, textural properties, creaming index and chemical stability (thiobarbituric acid reactive substances (TBARs) of conventional emulsions and nanoemulsions (10% (w/w) of extra virgin olive oil and 5% (w/w) of lecithin) was investigated. The results showed that addition of HPMC increased storage and loss moduli values, indicating a stronger emulsions structure. Moreover, the firmness and work of shear of emulsions increased significantly ($p < 0.05$) with increasing concentration of HMPC, but there were no significant differences between butter and nanoemulsion stabilised with HPMC at 2 % w/w. Nanoemulsions showed lower moduli values, firmness and work of shear than conventional emulsions due to the influence of high-pressure homogenization on their microstructure. A combination of lecithin and HPMC improved the physical and lipid oxidative stability of the emulsions, presenting a lower in creaming index (CI) and TBARs values after 14 days of storage at 4 °C and 20°C. In conclusion, HPMC at 2% w/w could be a suitable concentration to formulate a saturated fat replacer that could mimic butter technological performance during food manufacturing operations.

Keywords: Hydroxypropyl methyl cellulose (HPMC), Lecithin, Nanoemulsion, Rheology, Stability

5.1 Introduction

The consumption of saturated fatty acids has adverse health effects, promoting cardiovascular disease (CVD) (Buckland *et al.*, 2012; Ebbesson *et al.*, 2015). One interesting strategy to decrease saturated fat in food products is through food reformulation, by replacing saturated fat with unsaturated fat; however, this is very challenging due to the specific technological functionality of saturated fat and the higher susceptibility of unsaturated fat to lipid oxidation, which can lead to undesirable flavour profile, texture, shelf life and loss of nutritional quality in food products (McClements and Decker, 2000; McClements, 2016c).

In recent years, structured emulsions have been developed to be used as effective fat replacers in different food products by providing the desirable texture and organoleptic properties. Structured emulsions are oil-in-water systems where oil droplets are encapsulated in the layers of hydrated gelators (Patel and Dewettinck, 2016). These structures are made of a blend of liquid oils and gelators providing a semi-solid structure with higher viscosity and leading to desirable physical and organoleptic properties (Morley, 2011; Patel and Dewettinck, 2016). Structured emulsions such as wax-based oleogels, monoglyceride based- emulsion and cellulose emulsion have been used as fat replacers in bakery products such as muffins and biscuits (Lim *et al.*, 2017; Giacomozzi *et al.*, 2018; Sanz *et al.*, 2017; Tarancón *et al.*, 2013). However, in order to improve physicochemical stability of unsaturated oils, there has been an increasing interest in formulating nanoemulsion as lipid-based delivery systems with improved physicochemical stability of functional compounds, texture and fatty acid profile (Santhanam *et al.*, 2015; Joung *et al.*, 2016; Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Nanoemulsions with oil droplets particle size of less than 200 nm (Komaiko and McClements, 2016), which have particle size smaller than conventional emulsion could provide several potential benefits in food processing such as improving physical stability to gravitational separation of the fat phase in the matrix (Kim *et al.*, 2014), changing the physical properties and sensory perception of the product (Mason *et al.*, 2006; Morley, 2011), and improving physicochemical stability of functional compounds and unsaturated oils (Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Thus, the use of nanoemulsions as delivery systems of oils is proposed in this study as a novel strategy to improve the stability of unsaturated oils saturated fat replacers.

The combination the oil component and the emulsifier play an important role in the formation of the nanoemulsion. Several studies reported on the effect of oil type, with different fatty acid chain length, and the effect of fatty acid profile emulsion stability and bioaccessibility of lipophilic bioactives (Qian *et al.*, 2012; Hategekimana *et al.*, 2015; Öztürk, 2017; Chan *et al.*, 2013; Sahafi *et al.*, 2018). Other works have been carried out on the effect of emulsifier type and concentration on the physical and chemical stability of bioactive compounds such as β-carotene and vitamin D of nanoemulsions (Qian *et al.*, 2012; Gahruie *et al.*, 2020; Sahafi *et al.*, 2018). Moreover, combining emulsifiers into emulsions can improve the formation, stability, and functional properties of emulsions (McClements and Jafari, 2018). The addition of certain polysaccharides in emulsions can increase the thickness of the interface around the droplets and are able to improve the oxidative stability of the emulsion (Jacobsen *et al.*, 2008; Kargar *et al.*, 2011). However, the combination of emulsifiers may also give place to challenges arising from interactions between molecules through hydrophobic, electrostatic, steric or hydrogen bonding, which can impact the formation and stability of emulsions (McClements and Jafari, 2018). Hydroxypropyl methyl cellulose (HPMC) is a hydrocolloid with surface activity, mechanical, and thermo-reversible gelation properties (Yoo and Um, 2013; Li *et al.*, 2013b). There were some studies focusing on using HPMC to incorporate with emulsion as for improving formation, physicochemical properties, and stabilities of emulsions, which could be used as saturated fat replacer (Sanz *et al.*, 2015c; Meng *et al.*, 2018a; Meng *et al.*, 2018b). The addition of HPMC into emulsions can improve stability of emulsion by inhibiting droplet aggregation; HPMC form a three-dimensional network in the continuous phase limiting droplet coalescence (McClements and Jafari, 2018; Sanz *et al.*, 2015a). Emulsion stabilised with HPMC showed greater thermal stability, showing no significant changes in the microstructure at heating and cooling cycles (Sanz *et al.*, 2015a). Lecithin is an amphiphile molecule, which is derived from *sn*-glycero-3 phosphate (Bueschelberger, 2004; McClements, 2016b). Lecithin has greater surface activity compared to HPMC (Chapter 4); therefore, it is assumed that it will absorb preferentially to an oil-water interface than other molecules with lower surface activity (McClements and Jafari, 2018). To our knowledge, there is no studies on a nanoemulsion formulation considering the use of an emulsifier mixture with lecithin and HPMC to produce a stable and functional fat replacer. Therefore, in this study we aim to understand the effect of the combination of HPMC and lecithin in the design of nanoemulsions as saturated fat replacers. The objective of this study was to investigate the effect of HPMC concentration on the formation, rheological properties, physical and chemical stabilities of nanoemulsions and conventional emulsions formulated with lecithin. The results of this study will provide useful information about the basis to formulate saturated fat replacers with specific technological functionalities.

5.2 Materials and methods

5.2.1 Materials

Emulsions were elaborated with extra virgin olive oil (EVOO) (14.26% of saturated fat, 77.69% of monosaturated fat and 8.04% of polyunsaturated fat; Napolina brand, UK retail market), soy lecithin (Louis Francois Co., France) and HPMC (Biosynth carbosynth Limited, UK) (methoxy 21.4% and hydroxypropyl content 7.2%). Butter (salted butter with 82.0% fat and 1.5% of salt) was purchased from Co-operative Group Ltd (UK), which used as reference material high in saturated fat.

5.2.2 Reagents and standards

Trichloroacetic acid, thiobarbituric acid and 1,1,3,3-tetraethoxypropane (TEP) were purchased from Sigma-Aldrich Co., Ltd (UK), and hydrochloric acid purchased from Fisher scientific Co., Ltd (UK). High purity water was used for the preparation and dilution of reagents.

5.2.3 Preparation of emulsions stabilised by lecithin and HPMC

Emulsions were stabilised with two emulsifiers including lecithin at 5% w/w and HPMC (0, 2 and 4% w/w). The concentration of lecithin selected (5% w/w) was its critical micelle concentration (CMC) (as shown in chapter 3, Table 3.3.), which provided enough emulsifying effect at the interphase generated during high-pressure homogenization. Following results gathered in Chapter 4, 2 and 4% w/w HPMC were selected as the concentrations for this study, as they presented similar technological properties as butter.

5.2.3.1 Conventional and nanoemulsion preparation

The emulsion preparation procedure was based on the methods described by Arancibia *et al.* (2016) and Taha *et al.* (2018) with some modifications. Emulsions were prepared in two steps. Firstly, a magnetic stirrer (ChemLab, Model SS3H) was used to prepare the aqueous phase dispersing soy lecithin (5% w/w) in water (85% w/w) at 200 rpm for 30 min at ambient temperature to ensure complete dispersion. Then, the oil was added (10% w/w) to the aqueous phase during continuous stirring. Secondly, the emulsions were homogenised with a high-speed homogeniser (Silverson, Model L4RT) at 10,000 rpm for 5 min to produce conventional emulsions (CE) or for 10 min to produce nanoemulsion (NE). Then, HPMC was added to the obtained CE and NE. Regarding conventional emulsions, HPMC was added at three different concentrations (0, 2 and 4% w/w; CE-0, CE-2, CE-4, respectively). The mixtures were stirred using a magnetic stirrer (ChemLab, Model SS3H) at 200 rpm for 3 hour at ambient temperature to ensure complete dispersion, then left at 4 °C for at least 24 h in order to allow the HPMC to be completely hydrated before the measurements (Liu *et al.*, 2008; Ding *et al.*, 2014). In terms of nanoemulsions, HPMC was added at two different levels (0% and 2%; NE-0 and NE-2, respectively). The NE was processed through a high-pressure homogeniser (HPH) (8.30H, Rannie, APV, Denmark) at 400 bars for 1 cycle. This step was carried out to obtain emulsions with a mean droplet diameter lower than 200 nm (Komaiko and McClements, 2016). It was not possible to produce a NE with 4% HMPC, as the emulsion viscosity was too high to be processed in the HPH. All samples were left to for 2 hours at 19 °C before measurements were performed. All emulsions were prepared in triplicate.

5.2.4 Measurement of emulsion mean droplet diameter (MDD), polydispersity index (PDI) and particle charge (ζ-potential)

Particle size and polydispersity index of emulsions were determined in a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., UK) following the method of Guerra-Rosas *et al.* (2016) and Sharif *et al.* (2017). Emulsions were diluted 100-fold with deionized water and agitated to avoid multiple light scattering effects. The dispersion was decanted into polystyrene cuvettes for measuring MDD and PDI, and placed in a disposable zeta cuvette (DTS 1070, Malvern Instruments) for measuring zeta potential at wavelength of 633 nm at 25 °C. All the measurements were performed in triplicate for each emulsion batch, resulting in a total of nine replicates per sample.

5.2.5 Rheological measurements

The rheological analyses were done using a rheometer (Anton Paar MCR 302, Austria) equipped a Peltier temperature control device. A serrated parallel plate geometry was used; the diameter of lower stationary plate (PPTD 200/56/1) and superior plate (PP50/ P2) was 50 mm and the gap between the plates was 1 mm. The samples were allowed to rest in the measurement position for 5 min equilibration time before measurements. The measurements including amplitude sweep, frequency sweep and temperature ramp measurements were performed at 20 °C for the emulsions and at 30 °C for butter. In bakery products, especially in short dough biscuits the fat surrounds the flour particles forming a continuous coat to avoid hydration of protein, starch and other components (Manley, 2011a); in order to achieve this functionality, the shortening to be used has to behave as a plastic and are usually incorporated to the mixing process at 25 or 30 °C, when they become more easily spreadable (Vithanage *et al.*, 2009). All the measurements were done in duplicates for each emulsion batch. The storage modulus (G') and loss modulus (G'') values were recorded; G' characterizes of the elastic nature or solid-like behaviour of a substance, while G" is indicative of viscous nature or liquid-like behaviour of a substance. The rheological measurements were determined according to the method of Haque *et al.* (1993), Hussain *et al.* (2002) and Sanz *et al.* (2015a).

5.2.5.1 Amplitude sweep

The strain sweep measurement was carried out at strain amplitude range of 0.001 to 1000% at a constant frequency of 1 Hz in order to determine the linear viscoelasticity region (LVR). The LVR was identified where G' and G" were not influenced by applied strain.

5.2.5.2 Frequency sweep

Frequency sweep was conducted from 0.1 to 100 Hz at a constant strain amplitude of 1.0 % for complex nano/emulsions and 0.01% for butter in order to determine the mechanical spectra of emulsion samples.

5.2.5.3 Temperature ramp viscoelasticity

Temperature ramp viscoelasticity was determined according to the method of Sanz *et al.* (2015a) and Yoo and Um (2013). Temperature ramp analysis of emulsions was conducted with a constant strain amplitude of 1.0 % and a frequency of 1.0 Hz. Two temperature ramps were performed from 20 °C to 90 °C and 90 °C to 20 °C with a heating and cooling rate of 1 °C/min, and from 20 °C to 4 °C and 4 °C to 20 °C with no waiting time between the two ramps. The storage modulus (G'), the loss modulus (G") was recorded. The complex shear modulus (G*) was calculated as $G^* = G' + i\,G''$.

5.2.6 Firmness and spreadability

A TA-XT2 texture analyser equipped with the Texture Exponent software (Stable Micro systems Ltd., UK) was used to determine the spreadability of the butter and emulsions. The measurement was determined according to the method described by Glibowski *et al.* (2008). Spreadability and firmness of samples were measured using a TTC spreadability rig (HDP/SR) and a 5 kg load cell. Approximately 10 g of sample were filled into a female cone (90° angle) avoiding bubbles occlusion and levelling up the surface. The male cone (90° angle) was placed in the centre of the base. The male cone penetrated the samples 23 mm at a speed of 3 mm/s and the samples were allowed to flow outward at 45° between the male and female cone surfaces. Spreadability was measured at 20 °C for emulsions stabilised by HPMC and lecithin and at 30 °C for butter. The parameters recorded were the maximum force as a measurement of firmness, and the area under the force curve as a measurement of spreadability. The tests were performed in triplicate for each emulsion batch.

5.2.7 Creaming index (CI)

Creaming index (%) was evaluated based on the method reported by Arancibia *et al.* (2016) with some modifications. 10 mL of each emulsion were poured into a glass tube and stored at 4 °C and 20 °C. The total height (mm) of the emulsion and the cream layer were measured with a digital calliper after 1, 7 and 14 days. The Cl (%) was calculated using the following equation (Eq. (1)):

$$Creaming\ index\,\% = \left(\frac{Hc}{Ht}\right) \times 100 \qquad (1)$$

where Ht is the total height of the emulsion (mm) and Hc is the height of cream layer (mm). Measurements were performed in triplicate.

5.2.8 Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were determined according to the method of Qiu *et al.* (2015) and Sharif *et al.* (2017) with some modifications. Briefly, 0.1 mL of the oil sample was added to 5 mL of thiobarbituric acid (TBA) solution, which was prepared by mixing 15 g of trichloroacetic acid (TCA), 0.375 g of TBA and 2.1 g hydrochloric acid (37% w/w). Samples were heated in a water bath at 95 °C for 10 min, then the samples were allowed to cool down to room temperature for 10 min, followed by centrifugation (Heraeus Multifuge 3SR Plus Centrifuge, Thermo Scientific Ltd., UK) at 10,000 g for 15 min. The absorbance of the supernatant was measured at 532 nm using a UV spectrophotometer (CECIL CE 1021 1000 Series, Cecil Instruments Ltd., UK). The concentrations of TBARS values were determined by using a standard curve prepared using 1,1,3,3-tetraethoxypropane (TEP) standard curve (coefficient correlation (R^2) = 0.9994). TEP standards between 0.01 to 0.20 μg/ mL were prepared with trichloroacetic acid 7.5%. Measurements were done in triplicates for each emulsion batch.

5.2.9 Scanning electron microscopy (SEM)

SEM observations were performed according to the method described by Ding *et al.* (2014) and Perone *et al.* (2014). SEM was used to analyse the microstructure of the emulsions, which were lyophilized by a vacuum freeze dryer (F.J. Stokes Corporation, USA) for three days and stored in a desiccator for 24 h, which was prepared for SEM. All the samples were cut into small slices and fixed on a brass holder. Then, the surface of samples was observed after coating with gold-palladium. SEM was performed using a microscope (FEI Quanta 600 FEG SEM version 2.4, UK) and analysed at an accelerating voltage of 20 kV and a magnification of 500x.

5.2.10 Statistical analysis

One-way analysis of variance (ANOVA) was performed using IBM SPSS 25 (Armonk, NY: IBM Corp, USA). Tukey's HSD test was used to compare the mean values ($p < 0.05$) of MDD, PDI, spreadability and back extrusion. A two-way ANOVA was conducted to evaluate the influence of two independent factors: the type of emulsion (CE-0, CE-2, CE-4, NE-0 and NE-2) and storage time (1, 7 and 14 days) on the creaming index and TBARS results.

5.3 Results and discussion

5.3.1 Mean droplet diameter (MDD) and polydispersity index (PDI) and particle charge of emulsions

The MDD of both CE and NE significantly increased ($p < 0.05$) with increasing concentration of HPMC (Table 5.1). However, the HPMC concentration had no effect on the PDI ($p \geq 0.05$). The increase in droplet size of emulsions could be due to changes in the electrostatic interaction between oil droplets. Lecithin is composed by a mixture of non-phospholipid components and phospholipids with electrical charge (Bueschelberger, 2004; McClements, 2016a). The presence of a charged interface that provides a high electrostatic repulsion stabilises the droplets, preventing them from aggregating (McClements and Jafari, 2018). When adding HPMC, a non-ionic amphiphilic polysaccharide, the negative charge of emulsion droplets surface decreased significantly ($p < 0.05$); the ζ-potential of CE-0 was -53.67 mV, while for CE-2 it was -47.65 mV. ζ-potential of emulsion droplets continued to decrease when increasing HMPC concentration, but not significantly (ζ-potential of CE-4 was -46.45 mV) when adding it to emulsions initially stabilised by lecithin it promoted changes in the interface oil-water, giving place to a decrease in electrostatic repulsion between oil droplets, which resulted in higher droplet aggregation. These results could be related to the fact that HPMC, as an amphiphilic biopolymer may have interacted with lecithin through hydrophobic attraction (McClements and Jafari, 2018), thus decreasing the electrostatic repulsion between droplets which resulted in higher droplet aggregation.

When comparing CE and NE stabilised with 2% HPMC (CE-2 and NE-2), the MDD and PDI of NE showed significant smaller particles and narrower particle size distribution ($p < 0.05$) than CE (Table 5.1). These results could be explained by the effect of high-pressure during emulsion formation; an increase in shear forces and cavitation during high pressure homogenisation, resulted in particle size and polydispersity reduction (Floury *et al.*, 2000; Qian and McClements, 2011). These results suggest that the mixture of emulsifiers used was effective in being adsorbed at the interface rapidly enough to reduce the surface tension between fragmentation events and inhibiting droplet coalescence during the HPH treatment (McClements and Jafari, 2018) because the molecule structure of HPMC and lecithin contain hydrophobic and hydrophilic groups, which allow these emulsifiers with surface activity to reduce the surface tension of colloidal system (Li *et al.*, 2013b).

Table 5.1. MDD and PDI of conventional (CE) and nanoemulsion (NE) stabilised with lecithin and HMPC (0%, 2% and 4%).

Emulsions	MDD (nm)	PDI
CE-0	220.13^{c} (2.22)	0.313^{a} (0.030)
CE-2	257.51^{b} (5.26)	0.356^{a} (0.063)
CE-4	275.01^{a} (6.78)	0.346^{a} (0.035)
NE-0	185.83^{e} (4.52)	0.230^{b} (0.018)
NE-2	192.08^{d} (3.93)	0.255^{b} (0.013)

Indicated values are reported as means (standard deviation). Values with the different superscript letters (within same column) are significantly different ($p < 0.05$).

5.3.2 Rheological properties of emulsion.

5.3.2.1 Viscoelastic properties of emulsions

The dynamic spectra of CE and NE stabilised with lecithin and HMPC in comparison to butter is shown in Figure 5.1. All samples showed a solid-like viscoelastic behaviour as the G' modulus was higher than G" modulus in the whole frequency range studied. The mechanical strength of butter was greater than that of CE and NE samples as illustrated by higher moduli values and lower dependence on frequency. Emulsions with higher concentration of HPMC (CE-4) showed higher G' and G" moduli values and lower frequency dependence compared to CE-2, CE-0, NE-0 and NE-2 (Figure 5.1). These results could be explained by the increase in the polymer concentration, which lead to the formation of a higher number of entanglements in the network and thus to a more structured solution. This finding was in agreement with other authors that found an increase in moduli values at higher concentrations of HPMC in solution (Silva *et al.*, 2008; Hussain *et al.*, 2002) and CMC in o/w emulsions (Arancibia *et al.*, 2016) . As frequency increased, moduli values tend to approach. This could be explained by a reduction in the strain response of the sample, as at higher frequencies the time of strain response is shorter and samples may not be able to follow the stress variation.

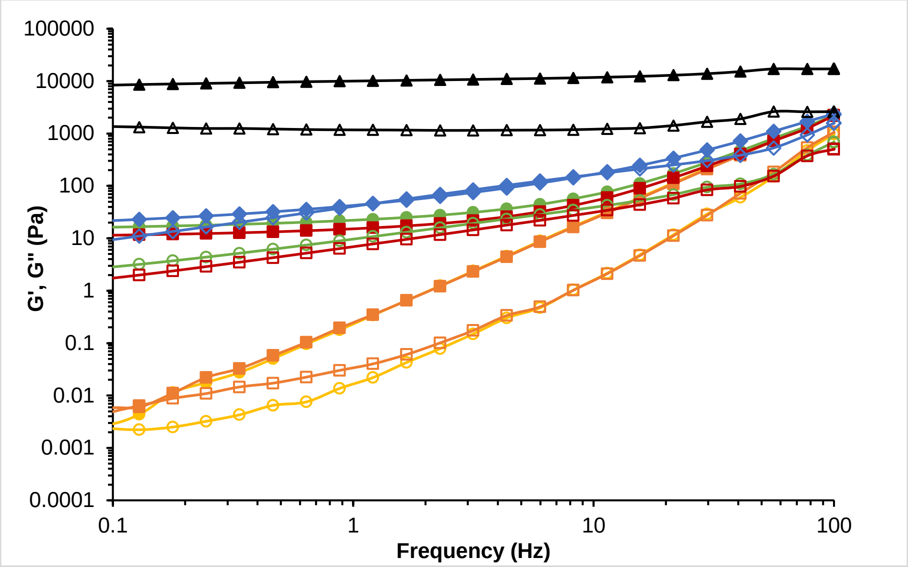


Figure 5.1. Dynamic moduli as a function of frequency of emulsions and butter ▲. Conventional emulsion (CE-0 ■; CE-2 ■; CE-4 ◆). Nanoemulsion (NE-0 ●; NE-2 ●). Filled symbols correspond to elastic modulus (G') and open symbols to viscous modulus (G").

5.3.2.2 Temperature dependent rheological behaviour of emulsions

As discussed in chapter 4, aqueous solutions of HPMC undergo thermoreversible sol-gel transitions (Liu *et al*., 2008). In this chapter, the gelation as a function of temperature for CE and NE stabilised by lecithin and HPMC was investigated to evaluate the effect of the o/w emulsion on the gelation behaviour of HMPC. Figure 5.2A shows that all emulsions showed a solid viscoelastic behaviour, with a predominance of elastic (G') over viscous (G") behaviour. In comparison, HMPC solutions studied in Chapter 4 showed a fluid viscoelastic behaviour at low temperatures because HPMC forms a solution in water as the water molecules form hydrogen bonds with the hydroxyl groups and form enclosed structures to surround the hydrophobic groups of the HPMC chain (Liu *et al*., 2008; Li *et al*., 2013b). Regarding the rheological behaviour of CE and NE stabilised by lecithin and HPMC, the differences in the microstructural behaviour of both type of samples could be due to interactions between HPMC and lecithin. Amphiphilic surfactants tend to aggregate around hydrophobic segments of water-soluble carbohydrate polymers, promoting integration between the polymer chain through alignment of polymer chains and physical cross-links (Joshi, 2011). At low temperatures (20 °C- 40 °C) both moduli values showed a plateau or a smooth decrease in moduli values. These results could be related to a sort of thermal softening as the intermolecular hydrogen bonds are gradually weakened by the increase in temperature (Liu *et al.*, 2008). As the temperature increased, G' and G" moduli increased abruptly, marking the starting point of the gelation temperature (Li *et al.*, 2001; Desbrières *et al.*, 2000; Silva *et al.*, 2008). The gelation temperature of the emulsions was influence by HPMC concentration following the same trend observed in Chapter 4 for HMPC solutions; as HPMC concentration increased, the emulsion gelation temperature decreased. As discussed in Chapter 4, these results could be due to a higher hydrophobicity from methoxy and hydroxypropyl groups when increasing HMPC content, leading to a lower onset temperature of aggregation and gelation of HPMC solutions (Yoo and Um, 2013). The thermorheogram shows a fluctuation of moduli values after the gelation temperature; G' and G" values decreased. This change in the viscoelastic behaviour during heating could be due to phase separation between HPMC gel and the liquid components after the start of the gelation process. Other authors observed that phase separation is caused by hydrophobic association between the HPMC chains when the temperature increased (Hussain *et al.*, 2002; Yoo and Um, 2013).

During the cooling cycle, from 90 °C – 50 °C (Figure 5.2B), the moduli values of the emulsions showed a smooth increase; the highest G' values were at around 50 °C for all samples, which was a similar temperature to the gelation temperature during the heating cycle. Then the G' values showed a smooth decreased until the end of the cooling cycle (20 °C). This behaviour could be related to the effect that high temperatures have on the formation and stability of hydrophobic interactions and stability of the gel network. Then as soon as the temperatures were lower to the gelation temperature (< 50 °C) emulsions' moduli values showed a gradual decrease. These observations differ to the degelation behaviour of HPMC solutions discussed in Chapter 4. In the case of HPMC solutions a two-stage process was observed: initially on cooling hydrophobic associations were gradually weakened and the network dissociated giving place to a smooth decrease on moduli values; then water caged around hydrophobic moieties and intermolecular hydrogen bonding were formed producing a dramatic decrease on moduli values. In this study, the interaction between the emulsifiers, as explained above, may have increased the moduli values at the start of the degelation process, until hydrophobic interactions are weakened and hydrophilic ones prevail.

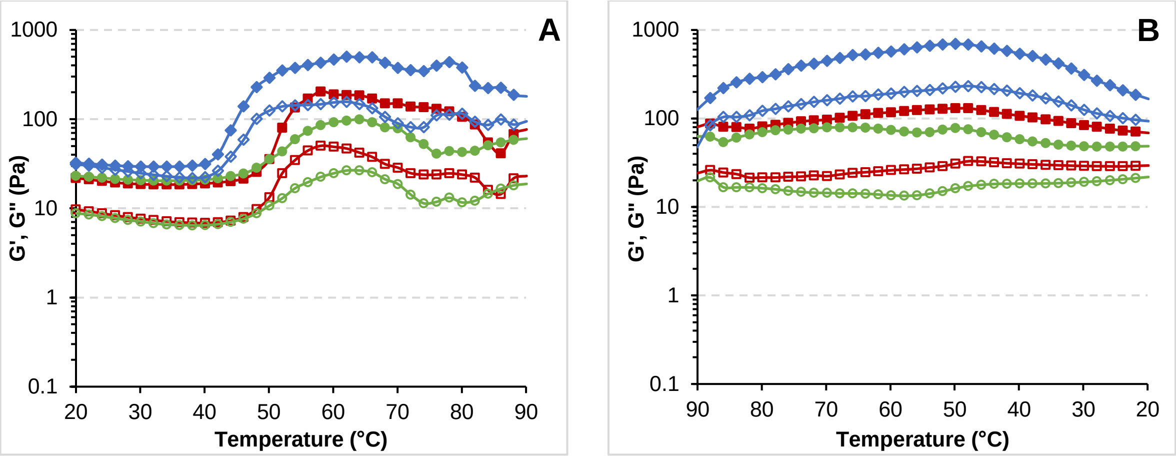


Figure 5.2. G' and G'' moduli of complex emulsions as a function of temperature. Conventional emulsion (CE-2 ■; CE-4 ◆). Nanoemulsion (NE-2 ●). Filled symbols correspond to elastic modulus (G') and open symbols to viscous modulus (G''). Frequency = 1 Hz, heating rate = 1 °C/min.

5.3.3 Firmness and spreadability of complex nano/emulsions

The evaluation of textural properties such as firmness and spreadability was designed to gather an indication of the behaviour of the samples in food processing operations such as scooping, mixing or sheeting where penetration and shear forces are applied in the food products. The firmness and spreadability values of butter and NE-2 were similar ($p>0.05$), suggesting that when NE-2 will be used in mixing or dough sheeting operations, it will respond to the forces in a similar way as butter does. This similarities in technological performance could help in the incorporation of newly developed ingredients to the food industry as minimal changes in the processing lines will be required to adapt it to new product formulations.

The concentration of HPMC had a significant effect ($p < 0.05$) on firmness and spreadability of emulsions (Table 5.2). Higher HPMC concentration in CE required significantly higher ($p > 0.05$) penetration and shear forces to induce a deformation in the samples, thus CE-4 was firmer and more difficult to spread than CE-2. These results agree with the viscoelastic behaviour of the samples, where it was also observed that higher HPMC concentration gave place to more structured samples (section 5.3.2.1.). As it was discussed previously an increase in HPMC concentration could lead to an increase in hydrophilic-hydrophilic interactions, that dominate the formation of the HPMC network structure at room temperature (Lim *et al.*, 2021; Liu *et al.*, 2008). In fact, polysaccharides are often added to oil-in water emulsions to increase the continuous phase viscosity giving place to advantageous textural properties and better physical stability of the emulsion (McClements, 2000).

When comparing CE-2 and NE-2 with the same HMPC concentration (2%), NE presented significantly ($p < 0.05$) lower values of firmness and shear (higher spreadability) than CE. Although this trend was not observed in the analysis of the viscoelastic behaviour of the samples (the dynamic spectra showed that CE-2 and NE-2 had similar moduli values (Figure 5.1), previous studies on the structure of nanocellulose materials reported that the network structure between cellulose chains formed by intermolecular and intra-molecular hydrogen bonds were destructed by the shearing forces during HPH (Wang *et al.* (2015). In contrast with results reported in previously (Wang *et al.*, 2015), no effect of the HPH process was observed in the emulsion microstructural behaviour. Higher deformation forces applied in the penetration and spreadability tests may have also contributed to the breakage of parts of the network that were affected by the HPH treatment and through these measurements the changes in the microstructure of the samples were detected.

Table 5.2. Firmness and work of shear of butter, conventional emulsions (CE) and nanoemulsion (NE) stabilised with HMPC (0%, 2% and 4%).

Samples	Firmness (N)	Work of shear (N·s)
Butter	0.33^{c} (0.03)	0.24^{bc} (0.06)
CE-2	0.44^{b} (0.02)	0.29^{b} (0.01)
NE-2	0.30^{c} (0.02)	0.18^{c} (0.02)
CE-4	1.87^{a} (0.11)	1.14^{a} (0.06)

Indicated values are reported as means (standard deviation). Values with the different superscript letters are significantly different ($p < 0.05$) between samples in the same column.

5.3.4 Physical and chemical stability of emulsion

5.3.4.1 Creaming index (CI)

There were significant interactions ($p < 0.05$) between the emulsion type and storage time when the CI of the emulsions was evaluated (Figure 5.3). The effect of a polysaccharide on an emulsion physical stability depends on the polysaccharide molecular structure and concentration (McClements, 2000). In our study the concentration of HPMC played an important role in CI values. At both storage temperatures, CE-0 and NE-0 had a significantly ($p < 0.05$) higher CI value than CE-2, CE-4 and NE-2, and thus a creaming layer was observed in these emulsions (Figure 5.3A). These results could be attributed to the effect of HPMC in the mechanical properties of the emulsions, as higher concentrations of HPMC gave place to a more structured and firmer viscoelastic gels (Figure 5.3 and Table 5.2) that could have prevented droplet aggregation more effectively than less structured emulsions. Some polysaccharides, at certain concentrations form entangled networks that trap the droplets and inhibits their movement (Dickinson, 1992). These results were in agreement with previous research where it was observed that the creaming index decreased with increasing viscosity of the continuous phase of o/w emulsions stabilised with HPMC and sodium dodecylsulfate (SDS) (Sovilj and Petrović, 2006). Borreani *et al.* (2017) also observed that o/w emulsions stabilised with cellulose ether did not present phase separation during a 3-day storage period. Moreover, the used of mixed emulsifiers could have a dual stabilisation mechanism: the aforementioned thickening effect of the continuous phase by HPMC and the formation of lecithin-HPMC complexes, forming cross-links around the hydrophobic sites of HMPC (Joshi, 2011), that may have improved the stability of the emulsion. Sun *et al.* (2007) observed that the stability of o/w emulsions prepared with hydrophobically modified hydroxyethyl cellulose (HMHEC) was based on (i) the thickening effect caused by the alkali chains of the HMHEC and (ii) the adsorption of the HMHEC at the interface forming a film that prevented coalescence. Further work on elucidating the adsorption mechanisms, competition and complex formation between lecithin and HMPC at oil droplets interface should be carried out in future projects.

In general, an increase in storage time from day 1 to day 7 gave place to a significant increase ($p < 0.05$) in CI values for CE-0 and NE-0. Oil droplet aggregation including flocculation and coalescence take place over time, leading to the formation of a creaming layer in emulsions (McClements and Jafari, 2018). When evaluating the effect of the droplet size on emulsion stability it was observed that NE-0 presented higher stability, with significantly lower CI values ($p < 0.05$) than CE-0, which could be due to the smaller droplets size (MDD) and narrower size distribution values (PDI) of the NE (Table 5.1). The CI values of emulsions can be influenced by various factors including droplet size and density of dispersed (oil) phase. The Stokes' law defines the rate of gravitational separation; it can be decreased by a decrease in droplet size, a decrease in density difference between dispersed phase and continuous phase, and an increase in the viscosity of the continuous phase (McClements and Jafari, 2018). A decrease in particle droplet size gave place to a decreases of the attractive forces between the droplets (Tadros *et al.*, 2004a; Qian and McClements, 2011); smaller droplets have better stability against droplet coalescence and flocculation because of the reduction in Brownian motion and gravitation forces (Tadros *et al.*, 2004a), allowing nanoemulsions to be protected against flocculation phenomena.

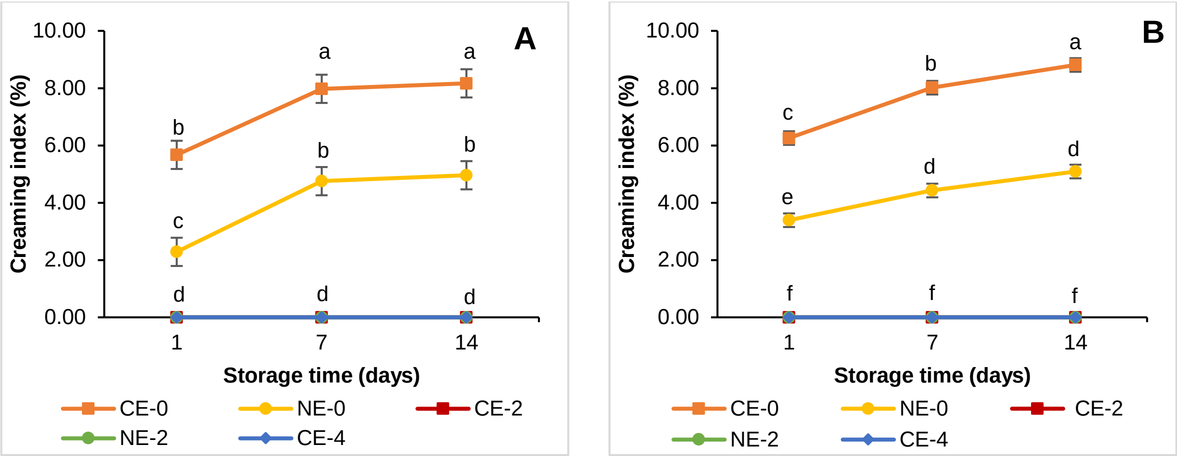
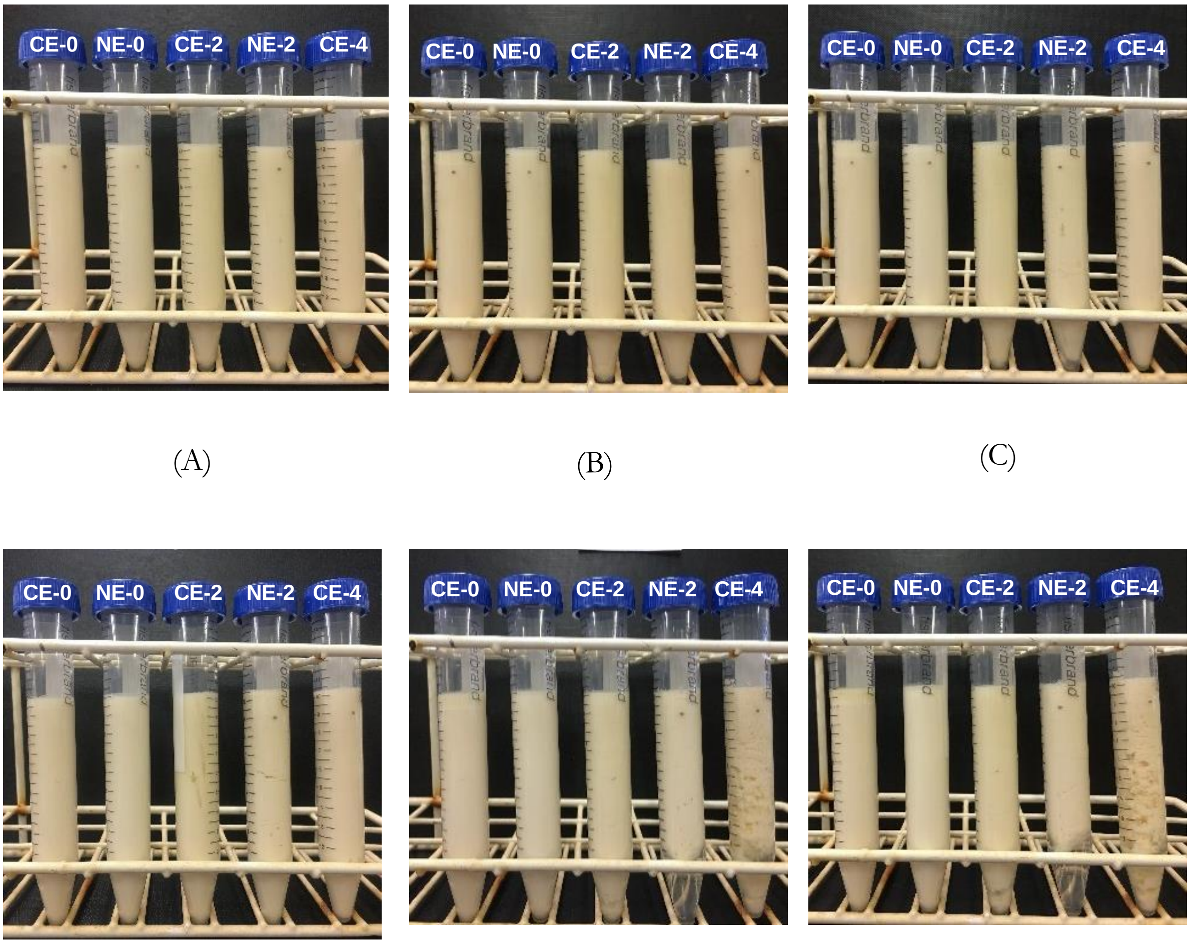


Figure 5.3. Creaming index of emulsions during storage (day 1, 7 and 14) at different storage times: A) 4 °C and B) 20 °C (mean values and 95% HSD confidence intervals). Conventional emulsion (CE-0 ■; CE-2 ■; CE-4 ◆). Nanoemulsion (NE-0 ●; NE-2 ●). Values with different superscript letter are significantly different ($p < 0.05$).

Phase separation was observed in CE-2, CE-4 and NE-2 at 20 °C after 7 and 14 days of storage as shown in Figure 5.4. At 4 °C, emulsions' G' and G" moduli values were higher than at 20 °C; for NE-2 G' modulus values at 4 °C and 20 °C of were 20.52 and 15.50, respectively (Table S1), indicating a higher level of intermolecular hydrogen bonds between entangled HPMC molecules were formed, giving place to a stronger emulsion structured. At higher temperatures, a slight decrease in moduli values was observed due to the weakening of the hydrogen bonding. Li *et al.* (2019) also found that an increasing temperature (4 °C to 35 °C) weaken the hydrogen bonding interaction between cellulose macromolecules, leading to a reduction of cellulose emulsifying capacity.



(A) (B) (C)

(D) (E) (F)

Figure 5.4. Pictures of emulsions stored at 4 °C at A) day 1; B) day7 and C) day 14, at stored at 20 °C at D) day 1; E) day7 and F) day 14 (D, E and F) of conventional (CE) and nanoemulsion (NE) stabilised with HMPC (0%, 2% and 4%).

5.3.4.2 Determination of thiobarbituric acid reactive substances (TBARS)

TBARs method was used to measure the secondary products of lipid oxidation in the emulsions over storage time (1, 7 and 14 days) at two temperatures (4 °C and 20 °C). There were no significant interactions ($p > 0.05$) between the type of emulsions and storage time at any of the two storage temperatures tested. The mean plots are shown in Figure 5.5 (A and B, respectively). HPMC concentration improved the lipid oxidative stability of the emulsions; CE-2, CE-4 and NE-2, showed significant lower ($p < 0.05$) TBARs values than CE-0 and NE-0 (Figure 5.5A). In o/w emulsions oxidation is usually initiated in the aqueous phase where the concentration of prooxidants is higher than in the oil droplets (Jacobsen *et al.*, 2008). HPMC may have a dual function in the improvement of the lipid oxidation stability of emulsions: (i) in the formation of a protective interfacial layer with lecithin, as HPMC adsorption in the interface of the oil droplets will increase the thickness of this physical layer thus, improving the protection of the lipids, avoiding prooxidant substances to come into contact with them (Jacobsen *et al.*, 2008; McClements and Jafari, 2018); (ii) as a thickener, limiting the diffusion of prooxidants (Borreani *et al.*, 2017).

Complex nanoemulsions did not shown significantly different TBARs values than conventional emulsions (Figure 5.5); these results meant that droplet size had no effect on TBARs values of emulsions stabilised with HPMC. In contrast, it has been reported in previous literature that smaller droplets size could lead to higher lipid oxidation of nanoemulsions due to an increased droplet surface area (McClements and Decker, 2000; Gohtani *et al.*, 1999). In this study, the stabilisation functionality exerted by HPMC had greater and limiting effect on the lipid oxidation rate than the increase in droplet surface area. The viscoelastic film of absorbed HPMC on the droplet surfaces protected oil droplets against prooxidant substances. This result was in agreement with another author (Kargar *et al.*, 2011), who reported that oil droplet size of oil-in-water emulsions stabilised by sodium caseinate (CAS) had no significant influence on the rate of lipid oxidation, whereas the ability of CAS to form a thick interfacial layer surrounding droplets and scavenge free radicals has been proven to be effective in inhibiting lipid oxidation (Kargar *et al.*, 2011).

The changes in the TBARs values of emulsions through storage time (Figure 5.5B) were significant ($p < 0.05$). TBARs values increased when storage time increased from day 7 to day 14 at 4°C and from day 1 to day 7 at 20 °C. There are many factors that could affect the oxidation reaction of emulsions such as the chemical structure of lipids, oxygen concentration, temperature, antioxidants, droplet characteristics, light (Sun *et al.*, 2011; McClements and Decker, 2000;

Gunstone, 2009). Therefore, longer storage time gave place to a prolong exposition to increased oxygen, light and temperature, increasing TBARs on emulsions.

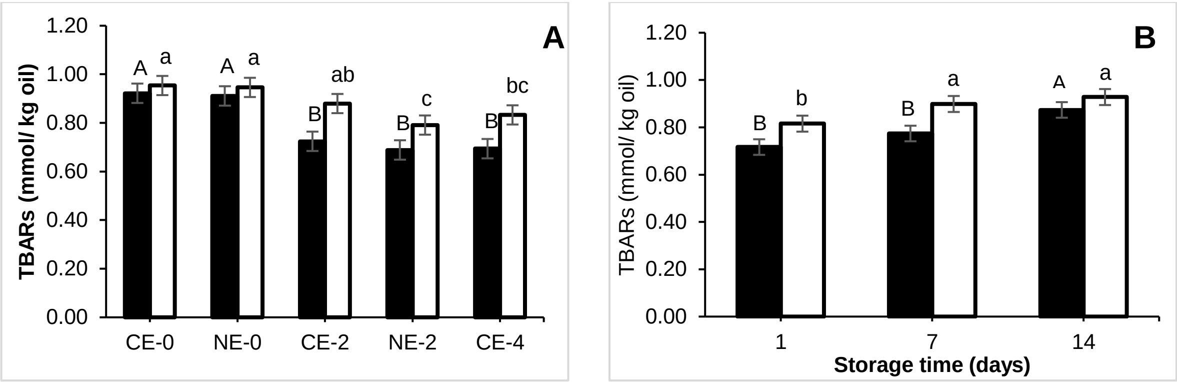


Figure 5.5. Mean values and 95% HSD confidence intervals. A: TBARs mean values according to the type of in emulsions at 4 °C (filled bars) and 20 °C (empty bars), conventional emulsions (CE-) and nanoemulsion (NE-) stabilised with lecithin and HMPC (-0, -2 and -4 %); B: TBARS mean values according to the storage day at 4 °C and 20 °C. Different capital letters indicate significant differences in the mean at 4 °C and different lower-case letters indicate significant differences in the mean at 20 °C ($p < 0.05$).

5.3.5 Scanning electron microscopy (SEM)

SEM images of emulsions are shown in Figure 5.6. The oil droplets (white arrows) were covered by an HPMC layer. As, expected nanoemulsions (NE-2) showed a distribution of smaller oil droplets than CE-2. Moreover, when comparing these two samples, at the same HPMC concentration (2%) it could be observed that HPMC network structure was discontinuous in NE-2 in comparison to continuous layer in CE-2. This result could be explained by the destruction of HPMC during the high-pressure homogenisation process. There was high shear forces and cavitation during high-pressure homogenization (Qian and McClements, 2011; Floury *et al.*, 2000), which the high intensity of the disruptive forces could break up the oil droplets into smaller size droplets and destroy HPMC network (Silva *et al.*, 2015; Mehmood *et al.*, 2019).

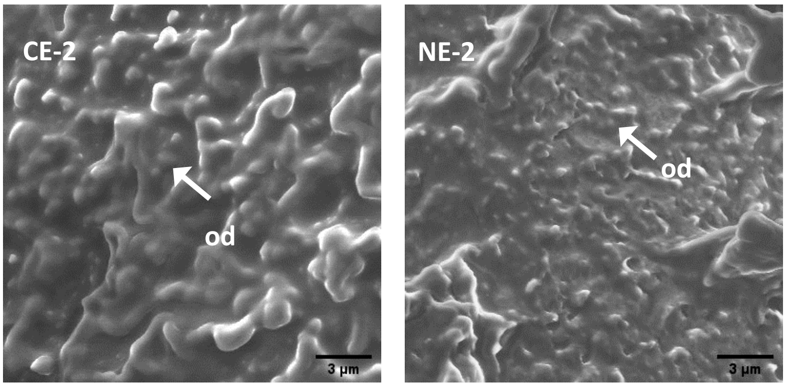


Figure 5.6. SEM images of conventional emulsions (CE) and nanoemulsions (NE) stabilised with lecithin (5%) and HMPC (2%). 500x. od: oil droplets.

5.4 Conclusion

This study revealed that the combination of emulsifiers (lecithin and HPMC) had a positive effect on the rheological and texture properties and on the physical and chemical stability of conventional and nano/emulsions. The results demonstrated that a combination of lecithin and HPMC improved the physical and lipid oxidative stability of the emulsions during storage, due to (i) the formation of a thicker protective layer of lecithin and HPMC adsorbed in the interface of the oil droplets and (ii) the formation of a stronger network in the continuous phase. Nanoemulsions showed higher spreadability than conventional emulsions due to the disruptive forces of high-pressure homogenization on the emulsion structure. A nanoemulsion formulated with 5% lecithin and 2% HPMC could be used as a suitable saturated fat replacer on bakery products, as it showed a similar firmness and spreadability to butter, indicating a similar deformation response in processing operations. These results will be the basis to formulate saturated fat replacers with specific technological functionalities to achieve the right performance on food manufacturing operations such as mixing, dough sheeting, baking and storage. Future work should focus on understanding the adsorption mechanisms, competition and complex formation between lecithin and HMPC at oil droplets interface.

Chapter 6

Effect of fat replacement with a HPMC-stabilised nanoemulsion on the physical characteristics and sensory attributes of short dough biscuits

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Abstract

Biscuits are widely consumed because their palatability and easy to eat format. However, biscuits contain high proportion of saturated fats and sugar, which could lead to an adverse health effect. The replacement of saturated fat in biscuits is very challenging due to its role in biscuit texture and mouthfeel, among other properties. The objective of this study was to investigate the functionality of a nanoemulsion made of extra virgin olive oil (EVOO) in water stabilised with lecithin and hydroxypropyl methylcellulose (HPMC) when used as saturated fat replacer on the physical characteristics and sensory attributes of short dough biscuits. Four biscuits formulations were studied including a control formulation (100% butter), three formulations where 33% of the butter was replaced with EVOO, with nanoemulsion (CNE), and with the individual ingredient of the nanoemulsion added separately (INE). The texture, spreadability, moisture, water activity, oil migration and colour of the doughs and biscuits were evaluated. The results showed that CNE doughs and biscuits were similar ($p > 0.05$) to the control formulation in terms of spreadability during baking, fracturability and colour. Moreover, CNE and INE showed less oil migration during the storage compared to control and EVOO formulations. Quantitative descriptive analysis was carried out with a trained sensory panel to describe the attributes of biscuits. The trained panel did not find significant differences in crumb density and hardness on the first bite among CNE, INE and the control formulation. In conclusion, biscuits with 30% less saturated fat content could be formulated with a novel nanoemulsion stabilised with lecithin and HPMC, while maintaining high quality physical and sensory attributes.

Keywords: short-dough biscuits, hydroxypropyl methyl cellulose (HPMC), nanoemulsion, fat replacer, texture, oil migration

6.1 Introduction

Biscuits are very popular bakery products among consumers due to their convenient on-the-go format and palatability (Forker *et al.*, 2011; Singh *et al.*, 2015). Biscuits can be defined as a cereal based product with a moisture content of less than 5 % (Manley, 2011a); short dough biscuits specifically contain higher fat and sugar content than other biscuits such as hard developed biscuit (Edwards, 2007). The main functionalities of fats in baked products are to impart shortening, richness, and to improve flavour and mouthfeel (Pareyt and Delcour, 2008). The typical fat products used in bakery goods include butter, margarine, shortenings, and palm oil (Edwards, 2007; Gisslen, 2012; Manley, 2011a), which lead to a high content of saturated fatty acids and trans fatty acids in the products. A high consumption of saturated fat increases cardiovascular diseases (CVD) and coronary heart disease (CHD) events, impacting overall health and quality of life (SACN, 2019; Buckland *et al.*, 2012; Ebbesson *et al.*, 2015). This set of evidence corroborates the link between diet and health and has urged governments to set strategies to reduce the average contribution of saturated fatty acids to total dietary energy; in UK a policy restricting the promotion of foods high in fat, sugar and salt was recently issued, aiming to favour healthier options (Department of Health & Social Care, 2021).

Fat replacers are mainly categorized into three groups including lipid, protein, and carbohydrate based-fat replacers according to their chemical composition (Akoh, 1998; Peng and Yao, 2017).Carbohydrate-based fat replacer is the largest group of fat replacers (Swanson, 2006); recently, guar gum, polydextrose, inulin, and HPMC have been used in biscuits (Chugh *et al.*, 2015; Laguna *et al.*, 2014). However, the evidence indicates that substitution of saturated fats with carbohydrates was associated with increase in CHD events; while substitution of saturated fats with polyunsaturated fatty acids (PUFA) and/or monounsaturated fatty acids (MUFA) has a beneficial impact on CVD and CHD events, levels of serum LDL cholesterol and markers of glycaemic control (SACN, 2019). In recent years, there have been a number of studies focusing on using vegetable oils with high content of PUFA and MUFA such as sunflower oil, olive oil, canola oil, and rapeseed oil as a saturated fat replacers in bakery products such as biscuits (Curti *et al.*, 2018; Jang *et al.*, 2015; Mert and Demirkesen, 2016; Onacik-Gür and Żbikowska, 2020; Devi and Khatkar, 2017; Jacob and Leelavathi, 2007). The reformulation strategies studied including replacement by bulk using sunflower oil (Devi and Khatkar, 2017; Jacob and Leelavathi, 2007); adding the oil in structured oleogels (Jang *et al.*, 2015; Mert and Demirkesen, 2016; Onacik-Gür and Żbikowska, 2020); and mixing the oils with solid fats such as cocoa butter and shea butter and fibres to form structured emulsion (Curti *et al.*, 2018). Although these studies showed a reduction of saturated fat, there was a less reduction of the total amount of fat; cookies containing the saturated fat replacer had higher spread value and harder texture than the control (Devi and Khatkar, 2017; Jacob and Leelavathi, 2007); cookies prepared with carnauba wax or candelilla wax oleogels had slightly harder texture and higher spread than samples prepared with shortening (Mert and Demirkesen, 2016). Other studies have investigated the incorporation of polysaccharides into oil in water emulsions as fat replacers in biscuits (Onacik-Gür *et al.*, 2015; Sanz *et al.*, 2017; Tarancón *et al.*, 2013; Giarnetti *et al.*, 2015) to enable both total fat and saturated fat reduction (Sanz *et al.*, 2017). Giarnetti *et al.* (2015) reported that 50% butter replacement in shortbread cookies with an emulsion filled gel based on inulin and extra virgin olive oil provided a good texture and consumer acceptance. However, the fat reduced biscuits presented higher spreadability and required more force to break than control biscuits (Tarancón *et al.*, 2013; Sanz *et al.*, 2017). Furthermore, the increasing level of unsaturated fatty acids in a fat replacer can lead to greater lipid oxidation due to autoxidation of the oil (Choe and Min, 2006) causing problems of shelf-life and rancidity in the end product (Patrignani *et al.*, 2014).

Nanoemulsions with oil droplets particle size of less than 200 nm (Komaiko and McClements, 2016) could provide several potential benefits in food processing such as improving physical stability to gravitational separation of the fat phase in the matrix (Kim *et al.*, 2014), changing the physical properties and sensory perception of the product (Mason *et al.*, 2006; Morley, 2011), improving physicochemical stability of functional compounds or of highly unsaturated oils (Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Thus, the use of nanoemulsions as a strategy to reduce saturated fat and total fat in food products could provide better stability of unsaturated oil. This strategy is a novelty because no research has been done before. Moreover, the formation, stability and functional properties of emulsions can be improved using combination of emulsifiers (McClements and Jafari, 2018), which may have different characteristics such as surface activity and thickening ability. Soy lecithin, has been widely used as emulsifying agent in the food industry (Klang and Valenta, 2011); it is an amphiphile molecule, which is derived from *sn*-glycero-3 phosphate (Bueschelberger, 2004; McClements, 2016b). Lecithin can be adsorbed to the oil-water interface and work as a surfactant. Onacik-Gür *et al.* (2015) reported that the biscuit made with high-oleic sunflower oil and lecithin presented a breaking strength similar to the control biscuit made with shortening, and also observed that the sensory quality significantly improved with the addition of lecithin to the oil formulation. Hydroxypropyl methyl cellulose (HPMC) is a carbohydrate polymer with surface activity, high swellability, and thermal gelation properties (Yoo and Um, 2013; Li *et al.*, 2013b). When the polymer is primarily in the solution phase it has shown to develop a three-dimensional network that compartmentalises the continuous aqueous phase and immobilises the oil globules, resulting in a dense matrix of stable fat globules (Sanz *et al.*, 2017). This structural functionality could help improving the stability of the fat phase in biscuits formulated with saturated fat replacements made of oils high in PUFA and MUFA. Therefore, in this study, it is proposed that the incorporation of two emulsifiers such as lecithin and HPMC into nanoemulsions could improve the functionality of nanoemulsions to work as a saturated fat replacers in short dough biscuits, specifically to improve the product nutritional fatty acids profile, texture and sensory properties. Therefore, the objective of this study was to investigate the application of a novel saturated fat replacer formulated as nanoemulsion made of extra virgin olive oil in water stabilised with lecithin and HPMC on the physical characteristics and sensory attributes of short dough biscuits.

6.2 Materials and methods

6.2.1 Preparation of the nanoemulsion stabilised by lecithin and HMPC

The emulsion preparation procedure was based on the methods described by Arancibia *et al.* (2016) and Taha *et al.* (2018) with some modifications. Firstly, a magnetic stirrer (ChemLab, Model SS3H) was used to prepare the aqueous phase dispersing soy lecithin (5% w/w) (Louis Francois, Marne La Vallée, France), in water (83% w/w) at 200 rpm for 30 min at ambient temperature to ensure complete dispersion. Then, extra-virgin olive oil (EVOO) (10% w/w) (Napolina, Liverpool, England) was added to the aqueous phase during continuous stirring. The emulsions were homogenised with a high-speed homogeniser (Silverson, Model L4RT) at 10,000 rpm for 15 min. HPMC powder (2% w/w) (Carbosynth Ltd, 8&9 Old Station Business Park, GB-COMPTON RG20 6NE) was added and the mixture was processed through a high-pressure homogeniser (HPH) (8.30H, Rannie, APV, Denmark) at 400 bars for 1 cycle.

6.2.2 Dough and biscuit preparation

Dough (D-) and biscuits (B-) were prepared according to Rodríguez-García *et al.* (2013) with specific adjustments. The ingredients used to produce the control biscuit doughs were (flour weight basis): soft wheat flour 100% (Heygates Ltd, Bugbrooke, England; composition data provided by the supplier: protein% 9.1; Carbohydrates% 80.9; moisture% 13.5; falling number S 262), butter 57% (fat% 82.0, unsalted butter coop, Manchester, England), 30% sugar (Silver Spoon White Granulated Sugar, Lynch Wood, Peterborough, English), tap water 11.7%, milk powder 0.58% (Nestlé, Vevey, Switzerland), salt 0.2%(Morrisons brand, UK retail market), sodium bicarbonate 0.2% (Hexeal brand, Norwich, UK), ammonium bicarbonate 0.12% (Atom Scientific Ltd, Manchester, England).

In addition to the control formulation (100% butter), three more dough formulations were developed using the same amount of all ingredients except for the fat component. 33% of the butter was replaced with extra virgin olive oil (EVOO), or the complex nanoemulsion (CNE), or the individual ingredient of the nanoemulsion (INE). The biscuits obtained from the CNE and INE had 30% less saturated fatty acids. The final water content in all formulations was 22% (in flour basis) and the total fat content was 46 % in D-Control, 50% in D-EVOO, 33% in D-CNE and 33% in D-INE (in flour basis). A 25% total fat reduction was achieved in C-CNE and C-INE. The ingredient and method to prepare CNE following the Chapter 5 in section 5.2.3.

For the dough preparation the butter and the saturated fat replacer were beaten in a mixer (Kenwood Chef XL 1200W, UK) at speed 1 for 4 min. Then the sugar was added and mixed at speed 3 for 2 min until results in a homogeneous cream. After this, milk powder was solubilised in the water and added to the mixer at speed 1 for 2 min. Finally, the flour, milk powder, sodium bicarbonate, ammonium bicarbonate and salt were added and mixed at speed 1 for 4 min. The resulting dough was allowed to rest in heat-sealed polyethylene bag at 21 °C for 10 min. The dough was then sheeted to a thickness of 10mm. using a sheeting machine (Rondo STM-503 table-top reversible sheeter; Burgdorf, Schweiz). Once sheeted the dough was allowed to rest at 4 °C covered between 2 layers of parchment paper. After resting the dough was shaped in circular dough pieces of 30 mm of diameter. Dough pieces were packed in polypropylene bags and analysed on the following 24 h. Each dough formulation was prepared in triplicate. Twenty dough pieces were placed onto a perforated tray and baked at 170 °C for 20 min with oven heating ratio at 90% top and 10% bottom (Polin 9 tray deck oven, Ing. Polin E C. S.p.A., Verona, Italy). After baking, biscuits were allowed to rest at 21 °C for 90 min. Biscuits were packed in polypropylene bags and analysed on the following 24 h. Each biscuit formulation was prepared in triplicate.

6.2.3 Dough characteristics

6.2.3.1 Sphere penetration measurement

The texture of the dough pieces was analysed using a TA-XT2 texture analyser equipped with the Texture Exponent software (Stable Micro systems Ltd., England). A sphere penetration test was performed following the method described by Rodríguez-García *et al.* (2013) with some modifications. A stainless-steel spherical probe (P/0.75) was used to compress the centre of the dough piece at 60% strain deformation, with a trigger force of 0.049 N, and test speed 1 mm/s. The maximum force (N), denoted as the peak of the curve was measured. The test was performed in ten dough pieces of each dough batch.

6.2.3.2 Oil migration

Oil migration was determined according to Onacik-Gür *et al.* (2015) with some modifications. Five dough pieces were placed in a Petri dish with pre-weighted seven layers of tempura paper (Daiso oil-absorbing cooking paper, Japan) on the bottom and two layers on top. Samples were stored in temperature-controlled cabinets at 18 °C and at 30 °C during 2 h. After this time, the dough pieces will be compress in the TA-XT2 texture analyser with a cylinder probe (diameter 50 mm, P/50) up to 60% strain deformation for 10 s, with a trigger force of 0.98 N. The fat/oil migration was calculated as difference in the mass (g) of the paper before and after the test and expressed in percentage, considering the initial fat content of the sample. The analysis was conducted in three dough pieces per dough batch per formulation.

6.2.4 Biscuit characteristics

6.2.4.1 Weight loss during baking, moisture and water activity

The weight loss (WL) of biscuits was recorded after resting at 21°C for 90 min, which calculated by using the following equation (Eq. (1)):

$$WL\ (\%) = W_{dough} - \ W_{biscuit}\ / W_{dough}) \ \times 100\% \quad (1)$$

where w indicates weight (g). From each formulation, ten samples were weighted before (W_{dough}) and after baking $(W_{biscuit})$.

For moisture content and water activity (a_w) measurements biscuits were crumbled. Around 3 g of crumbs were placed on an aluminium tray and heated at 105 °C until constant weight in a Sartorius balance (M-Pact Series, Sarotorius Lab Instruments, Germany). For water activity measurements a Rotronic HygroLab meter (Rotronic, Zurich, Switzerland) was used. Measurements were performed in duplicate per batch of biscuits per formulation.

6.2.4.2 Spreadability Index

The spreadability index (SI) was calculated following the method described by Onacik-Gür and Żbikowska (2020). The height and diameter of 5 biscuits per batch were measured using an electronic caliper (RS PRO 150mm Digital Caliper, RS PRO professional, Singapore). The spreadability index was calculated from the following equation (Eq. (2)):

$$SI = \frac{Diameter}{Height} \qquad (2)$$

6.2.4.3 Three-point Bending Test

The texture of the biscuits was analysed using the texture analyser described above. Biscuits were broken suing the three-point-bending rig probe (A/3PB) using the following conditions: supports 15 mm a part, the probe moved 5 mm with a test speed of 1 mm/s, and a trigger force of 0.05 N. The parameters measured were the force at break (N), and the distance at break (mm) as the fracture strength and the fracturability, respectively. The analysis was performed in ten biscuits per formulation batch.

6.2.4.4 Oil Migration in biscuits

Oil migration in biscuits was performed following the same procedure as described for doughs (section 6.2.3.2) with some modifications. The biscuits were stored at in temperature-controlled conditions at 18 °C and at 30 °C during 3 and 5 days. The analysis was done with three biscuits per batch, per formulation.

6.2.4.5 Colour of biscuit

The colour of the biscuits surface of was determined using a colorimeter (Chroma Meter CR-400, Konika Minolta, Inc., Chiyoda City, Tokyo, Japan). The parameters measured were L* (L* = 0 [black] and L* = 100 [white]), a* (–a* = greenness and +a* = redness), b* (–b* = blueness and

+b* = yellowness). The test was performed taking three measurements on 3 different points on the top-surface of 5 biscuits for each formulation batch. The total colour difference (ΔE) between the three formulations and the control was calculated (Onacik-Gür and Żbikowska, 2020) as below equation (Eq. (3)):

$$\Delta E = \sqrt{(L_c * - L_s *)^2 + (a_c * - a_s *)^2 + (b_c * - b_s *)^2} \quad (3)$$

Where the subscript "c" means control sample and the subscript "s" means the other sample that compared to control, thus B-EVOO or B-CNE or B-INE.

The values used to determine whether the total colour difference was visually obvious were the following:

ΔE* < 1 colour differences are not obvious for the human eye,

1 < ΔE* < 3 minor colour differences could be appreciated by the human eye depending on the hue, and Chroma,

ΔE* > 3 colour differences are obvious for the human eye (Bodart *et al.*, 2008).

6.2.5 Sensory analysis

Sensory profiling of biscuits was carried out using quantitative descriptive analysis (QDA). A trained sensory panel of ten people (n = 10; 8 female and 2 male) was used. All sensory evaluation was carried out in a temperature-controlled room (22 °C), in isolated booths, and under artificial daylight. The trained panel were provided with 10 g biscuit, which were prepared as described in Section 6.2.2. A panel of trained assessors developed a consensus vocabulary of 28 attributes, which were grouped in 5 modalities: appearance, aroma, flavour, mouthfeel and aftereffect as outlined in Table 6.S1 in the results section. The purpose of the sensory profiling was to describe and quantify changes in biscuit descriptors occurring with change in formulation. All panellists scored in duplicate for each sample on two consecutive days. Samples were blind coded with three-digit numbers, and were presented in a monadic balanced order, with sample sets randomly allocated to panellists. Warm filtered water (~40 °C) and peeled raw carrot were provided for palate cleaning between samples. Unstructured linescales (0-100) with suitable anchors for each attribute were used (Compusense Cloud Software, Guelph, ON, Canada).

6.2.6 Statistical analysis

One-way analysis of variance (ANOVA) was performed using IBM SPSS 25 (Armonk, NY: IBM Corp, USA). Tukey's HSD test was used to compare the mean values ($p < 0.05$) of weight loss during baking, water activity, textural properties, total count of crumb cells, cell size, spreadability index and colour parameters. A two-way analysis of variance was conducted on the influence of two independent factors: the formulation and storage time in the measurement of oil migration in biscuits. For sensory data, the QDA results were analysed using analysis of variance (ANOVA) in SenPAQ (version 5.01, Qi Statistics, Berkshire, UK). The panellists were fitted as random effects and the samples were fixed effects. The treatment effects (samples and assessors) were tested against the panellist by assessor interaction. An hoc tests for multiple means comparisons were carried out using Fisher's least significant difference test (LSD) ($p > 0.05$).

6.3 Results and discussion

6.3.1 Dough characteristics

6.3.1.1 Texture

Results from the sphere penetration test are shown in Table 6.1. All doughs were significantly different ($p < 0.05$) to one another. Some of the main factors to consider when evaluating the functionality of the fat in a biscuit dough are the ratio of the solid phase to the liquid phase and the crystal structure of the solid fats (Pareyt and Delcour, 2008); the effect of different fats in the overall dough texture is determined by the consistency of the fat and its content in solid fats (Jacob and Leelavathi, 2007). Among the fat systems used in this study, EVOO had the lowest solid fat content thus producing the softest ($p < 0.05$) dough. It has been observed previously that the use of highly unsaturated fats in biscuits gave place to doughs with softer texture (Culetu *et al.*, 2021; Mert and Demirkesen, 2016).

The replacement of butter with the CNE gave place to dough pieces (D-CNE) with significantly higher ($p < 0.05$) hardness values than the D-control. The reduction of the fat content in the system may have hindered some of the main functionalities of fat in a dough system, lubrication and aeration (Pareyt and Delcour, 2008). During mixing fat surrounds sugar and flour particles, breaking the continuity of the protein-starch matrix and reducing gluten development (Pareyt and Delcour, 2008; Lai and Lin, 2006). When less fat is present in the system water has easier access to flour proteins, hydrating them and creating a cohesive and extensive matrix (Pareyt and Delcour, 2008), thus giving place to harder doughs. When the ingredients of the emulsion were added individually during the mixing process the hardest ($p < 0.05$) dough was obtained (D-INE). When adding the HMPC as a dry ingredient it may have not formed an entangled polymer solution within the dough matrix due to limited time and shearing forces during mixing. The water added for the formation of the HMPC solution was freely available to interact with other ingredients, such as flour particles, thus higher dough hydration over dough shortening effect could have taken place in D-INE.

Table 6.1. Hardness of biscuit doughs (D-). Control is the dough made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

Formulations	Hardness (N)
D-Control	2.42 ^{d} (0.1)
D-EVOO	0.74 ^{c} (0.05)
D-CNE	2.88 ^{b} (0.15)
D-INE	3.44 ^{a}(0.52)

Values are reported as means. Values in parentheses are the standard deviation. Means in the same column without a common letter are significantly different ($p < 0.05$).

6.3.1.2 Oil migration in doughs

When the fat consistency is very soft or liquid at typical dough manufacturing temperatures, it can cause problems of oiling out (Manley, 2011b). Oil migration indicates that there is not strong interactions between the fat phase and all the other ingredients (Onacik-Gur and Zbikowska, 2020). The migration of the fat phase outside the surface of the dough was examined and results are shown in Table 6.2. D- Control, D-CNE and D-INE showed no significant differences ($p > 0.05$) in oil migration at 18 °C. However, D-EVOO showed the highest oil migration from its surface ($p < 0.05$); more than double the quantity of oil migrated from D-Control. These results were expected due to the use of liquid fat to replace of butter without using any other structuring strategy that could give more stability to the liquid fat in the dough. In fact, the addition of HPMC and lecithin in the dough formulation, as a pre-prepared emulsion or as individual ingredients, stabilised successfully the oil in the biscuit doughs. When adding the CNE, the oil was emulsified by the combination of lecithin and HMPC in nanodrops in a continuous aqueous phase of entangle

HMPC (Chapter 5). Confocal micrographs of biscuit doughs with emulsions made of sunflower oil and HMPC as shortening replacer showed that the oil was dispersed homogeneously in a continuous phase of proteins, carbohydrates and dispersed starch granules (Sanz *et al.*, 2017). However, the efficacy of the emulsifier mix in the stabilisation of the oil in the dough matrix was significantly ($p < 0.05$) decreased at higher storage temperature (30 °C). It has been observed in previous chapters (4 and 5) that when increasing the temperature above 20 °C there was a decrease in the viscoelasticity of the HMPC solutions or emulsions due to a sort of a thermal softening effect in the inter and intra-molecular hydrogen bonds between HPMC molecules (Liu *et al.*, 2008). It has also been reported that at temperatures from 30 °C to 90 °C the hydrophobic headgroup of lecithin molecules changed its shape inducing oil droplet coalescence (Ogawa *et al.*, 2003). These destabilisation mechanisms, together with the increase in volume and movement of the fat phase at higher temperatures could have led to oil droplet aggregation and diffusion to the surface of the biscuit dough.

Table 6.2. Oil migration in doughs (D-) at 18 °C and 30 °C. Control is the dough made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

Formulation	Oil migration (%)	
	18 °C	30 °C
D-Control	5.35^b (0.66)	9.11^c (0.73)
D-EVOO	12.69^a (1.18)	19.19^a (1.53)
D-CNE	6.51^b (1.58)	10.99^b (1.45)
D-INE	5.88^b (2.54)	11.43^b (2.17)

Values are reported as means. Values in parentheses are the standard deviation. Means in the same column without a common letter are significantly different ($p < 0.05$).

6.3.2 Biscuit characteristics

6.3.2.1 Weight loss during baking (WL), moisture and water activity

WL, moisture and water activity (a_w) values of the biscuits are presented in Table 6.3. B-EVOO lost significantly more weight during baking ($p < 0.05$) giving place to a final biscuit with lower moisture and a_w values. During baking water is available to solubilise sugars and gives place to a rich fatty sugary liquid viscous enough to contain the water vapour at initial stages of baking (Manley, 2011a). Then the viscosity of the continuous network increases and the protein film formed is set, defining the width of the biscuit; then gases are released (carbon dioxide and water vapor) and towards the end of the baking the height of the structure collapses (Pareyt and Delcour, 2008; Manley, 2011a). The effects of replacing butter by EVOO in WL and moisture could be explained by the distribution of the oil in the dough and its effects on water distribution and availability during baking. During mixing oil gets dispersed as fine droplets that are significantly less effective in imparting the shortening functionality than plastic fats do (Pyler, cited in Jacob and Leelavathi, 2007). When fat is badly distributed in the dough, flour particles are more readily available to be hydrated (Jacob and Leelavathi, 2007). It is then hypothesised that when water is interacting with flour particles in the dough instead of forming the syrupy phase the rate of evaporation during baking is higher, as it has been shown for B-EVOO.

The incorporation of emulsifiers, such as lecithin and HMPC helped in the retention of water during baking, giving place to final biscuits with similar WL, moisture and a_w than B-Control. When HPMC is dispersed in water hydrogen bonding between the hydroxyl groups in HPMC and water are formed (Guarda *et al.*, 2004; Poonnakasem *et al.*, 2018). Then during baking, there is an increase of strong hydrophobic interactions between HPMC chains that gave place to a sol-gel transition (Lim *et al.*, 2021; Bodvik *et al.*, 2010), which will have retained more water within the biscuit systems (B-CNE and B-INE).

Table 6.3. Weight loss during baking (WL), moisture, water activity (a_w), spreadability index (SI), hardness and fracturability of the biscuits (B-). Control is the biscuit made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

Biscuits	WL (%)	Moisture (%)	a_w	SI (mm)	Fracture strength (N)	Fracturability (mm)
B-Control	13.07^b (0.58)	4.14^{ab} (0.38)	0.49^a (0.04)	3.32^b (0.04)	27.31^c (8.83)	38.00^a (0.21)
B-EVOO	15.33^a (0.77)	3.48^b (0.53)	0.36^b (0.03)	3.73^a (0.16)	18.37^d (5.53)	37.24^b (0.32)
B-CNE	13.20^b (1.19)	4.78^a (1.01)	0.51^a (0.08)	3.31^b (0.14)	43.33^b (10.38)	38.32^a (0.31)
B-INE	13.23^b (0.97)	4.99^a (0.89)	0.50^a (0.06)	3.21^b (0.14)	50.86^a (16.47)	36.57^c (1.07)

Values are reported as means. Values in parentheses are the standard deviation. Means in the same column without a common letter are significantly different ($p < 0.05$).

6.3.2.2 Spreadability Index (SI)

Biscuit quality can be summarised in two general aspects: the size and the bite (Pareyt and Delcour, 2008). Pareyt and Delcour (2008) and Manley (2011a) explained that final biscuit dimensions depend on several factors; the dough spread onset which can be explained by an increase in the mobility of gluten proteins as temperature increases and is influence by the level of plasticiser (water) or anti-plasticiser (syrup); the dough spread rate during baking, which is defined by the levels of dissolved sugar and melted fat, or in other words it is defined by dough viscosity; and the set time which is defined by an ‘apparent’ glass transition of the protein network, increasing its viscosity and stopping cooking spreading. The height of the biscuit continues to increase due to gases formation until the structure collapses at the end of the baking process. The appearance of four formulation of biscuits was showed in Figure 6.1.

Results of the SI of biscuits are presented in Table 6.3. B-Control, B-CNE and B-INE showed no statistical differences ($p > 0.05$) in terms of spreadability. On one hand, it could have been hypothesised that the fat reduction would have decreased the spread rate and the addition of water would have decreased the anti-plasticiser effect giving place to smaller biscuits. Previous work where the shortening was replaced by hydrocolloids (polydextrose, maltodextrin, inulin or whey proteins) in biscuits showed that when replacing fat (and sugars) in biscuits the diameter of the final products decreases due to elastic shrinkage tendency of these samples during baking (Rodríguez-García *et al.*, 2013; Zoulias *et al.*, 2002). On the other hand, the results from this study showed that the functionality of the CNE and its ingredients had a different effect on biscuit SI than other emulsions with HPMC. Previous works in which oil in water emulsion with HPMC or ethyl cellulose were used to replace shortening in short dough biscuits observed a later set time for biscuits made with the emulsions (Sanz *et al.*, 2015c); and reported higher biscuit diameter, thickness and SI values due to a softer and more fluid dough behaviour during baking (Tarancón *et al.*, 2013). However, the emulsions from the aforementioned studies, although using HMPC with similar degree of substitutions than the HPMC used in this study, contained higher amounts of oil (47%-52%). The lower oil content (10%) and the combination of emulsifiers (HMPC and lecithin) used in this study to formulate the emulsions gave place to similar spreadability properties as butter (Chapter 5), As a consequence, the strategy used in this study has given place to a similar spreadability behaviour in biscuits made with the nanoemulsion (B-CNE) than the ones with butter (B-control).

As expected, B-EVOO biscuit showed the highest ($p < 0.05$) SI. The higher content of liquid fat gave place to a softer dough with higher spreadability rate, higher final diameter and lower height. These results were in agreement with other studies, which presented that higher fraction of unsaturated fats lead to a higher spreadability index (Sciarini *et al.*, 2013; Jacob and Leelavathi, 2007); the degree of saturation affects physical and thermal properties in fatty acids, specifically unsaturations decrease the fat melting point. This characteristic of the oil decreases the viscosity of the dough, increasing the spreadability on set and rate.

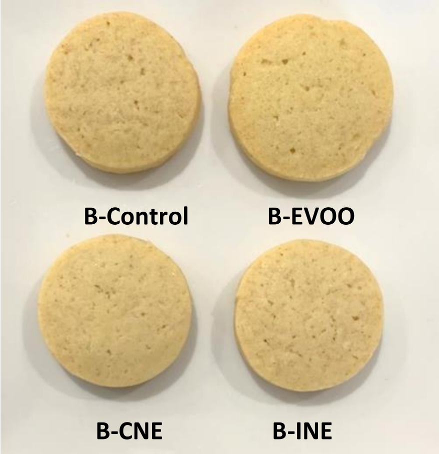


Figure 6.1. Visual appearance of biscuits

6.3.2.3 Texture

During the baking process the viscoelastic dough changes into a solid with an aerated cellular structure and a characteristic texture (Chevallier *et al.*, 2000). Fracture strength and fracturability values of the biscuits are presented in Table 6.3. The results suggested that dough mechanical properties (Table 6.1) defined biscuits' fracturability strength. B-CNE and B-INE showed significantly higher ($p < 0.05$) fracture strength than B-Control; and B-INE showed the highest fracturability ($p < 0.05$) among biscuit samples. These results could be explained by the fact that when less fat is present in a dough formulation less shortening effect is achieved, so flour particles are more accessible to water, giving place to harder and brittle biscuits (Manley, 2011a). When comparing B-CNE showed similar ($p > 0.05$) fracturability to B-control, suggesting that this fat replacer offers the potential to be used as butter replacer for specific bakery applications. Biscuits in which 33% of butter was replaced by EVOO presented the lowest ($p < 0.05$) fracture strength values due to higher content of total fat and the lowest consistency of the oil at ambient temperature in comparison to butter or the emulsion.

6.3.2.4 Oil migration

It is essential to evaluate the level of oil migration in short-dough biscuits to predict problems such as migration into any coating or filling that could lead to bigger issues such as fat bloom (Manley, 2011a). Oil migration to the surface of the dry product may undergo faster degradation leading off-flavours and off-odours that will alter the sensory properties of the product (Onacik-Gur and Zbikowska, 2020). Significant interactions between biscuit formulation and storage time were observed when the oil migration was analysed at 18 °C and 30 °C (Figure 6.2A and B). In general, all the biscuits showed a significant increase ($p < 0.05$) in oil migration from day 3 to 5, at both 18 °C and 30 °C; except for B-CNE at 18 °C that showed similar oil migration values ($p > 0.05$) on day 3 and 5 at 18 °C. Nanoemulsions with HPMC showed a high physical stability during storage (Figure 5.4 and 5.5 in Chapter 5). As it has been discussed in Chapter 5, when producing the nanoemulsion the oil droplets are stabilised by the emulsifier combination and the HMPC forms an entangled continues phase. It has been observed that HMPC forms a three-dimensional structure in the continuous phase that compartmentalises and immobilises the oil globules (Sanz *et al.*, 2017). During baking the HPMC went through the sol-gel transition increasing the strength of the matrix and after cooling down although some of the hydrophobic interactions forming the gel may have been broken, other inter and intramolecular interactions may remain, providing some of the structural strength and physical stability to the fat phase in the biscuit during storage. The addition of structural components, such as ethyl cellulose also gave place to a significant reduction in oil migration in biscuits in comparison to control samples with palm oil (Onacik-Gür and Żbikowska, 2020).

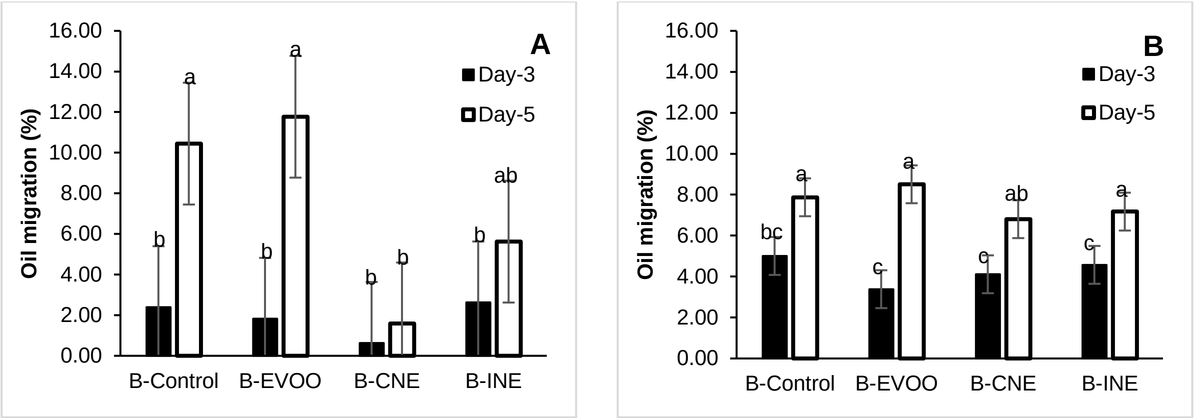


Figure 6.2. Oil migration on the biscuits (B-) after 3 and 5 days at A) 18 °C and B) 30 °C (mean values and 95% HSD confidence intervals). Control is the biscuit made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

6.3.2.5 Colour

The colour parameters of biscuits (*L**, *a** and *b**) and the total colour difference are presented in Table 6.4. During baking, colour development begins through non-enzymatic browning and the Maillard's reaction (Davidson, 2016). B-CNE showed similar colour values than control ($p > 0.05$) B-EVOO showed a significant lower *L** and were greener and yellower ($p < 0.05$) than all the other samples. These results could be explained by the presence of pigments such as chlorophyll and carotenoids on EVOO (Moyano *et al.*, 2010), which are responsible for greenness and yellowness, respectively.

Table 6.4. Colour parameters values for biscuit (B-). Control is the biscuit made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

Formulation	L^*	a^*	b^*	ΔE
Control	75.31^{a} (0.26)	$-0.64^{b,c}$ (0.26)	27.55^{b} (0.55)	0.00
B-EVOO	74.53^{b} (0.6)	-0.73^{c} (0.07)	30.29^{a} (0.56)	2.86
B-CNE	75.18^{a} (1.00)	-0.52^{b} (0.22)	27.72^{b} (1.39)	0.25
B-INE	75.12^{a} (0.46)	-0.29^{a} (0.25)	27.32^{b} (1.03)	0.46

Values are reported as means. Values in parentheses are the standard deviation. Means in the same column without a common letter are significantly different ($p < 0.05$).

6.3.3 Sensory profile of biscuits

The results showed that 21 out of 28 attributes were found to differ significantly ($p > 0.05$) between samples.

The trained panel developed a consensus vocabulary of 28 attributes in 5 main modalities including appearance, aroma, flavour, mouthfeel and aftereffect as shown in (Table 6.S1). The sensory profiles of biscuits are presented in Figure 6.3. As expected, QDA evaluation identified that B-CNE and B-INE had more similar sensory attributes to B-Control than B-EVOO. There were no significant ($p > 0.05$) differences among the samples in terms of floury (aroma and taste), savoury (taste), dryness (mouthfeel), sweet (taste), greasy (aftereffect) and numbing/ cooling (aftereffect).

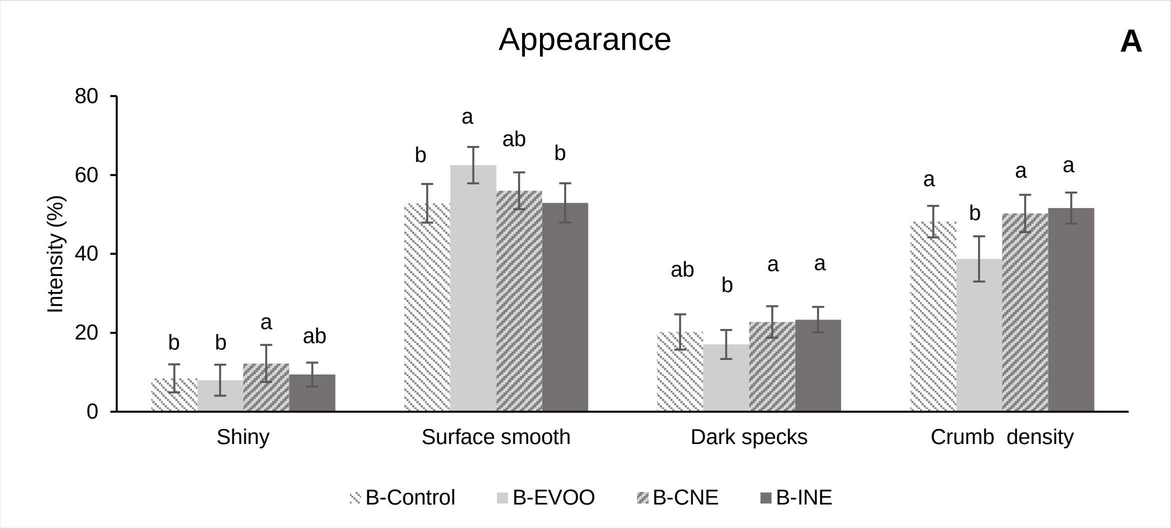
In terms of the appearance (Figure 6.3A), B-EVOO presented a significantly lower crumb density that the other three formulations and a significantly higher surface smoothness than B-Control. On the other hand, B-CNE and B-INE showed a similarly surface smooth, dark specks and crumb density as B-Control. The presence of HPMC in the formular could be a factor to improve crumb density of B-CNE and B-INE because HPMC could develop a more stable system by keeping air bubbles during dough preparation and baking. This is in agreement with Bousquières *et al.* (2017), who stated that HPMC controlled the viscosity of cake batter and limited bubble loss during batter preparation. This paper also reported that during baking, methylcellulose (MC) and HPMC governed the sol-gel transition by forming hydrophobic bonds and at the same time there was a starch gelatinization, which affected on a wide range of cellular homogeneities in crumb. Moreover, B-CNE exhibited a significantly higher shiny appearance than B-Control and B-EVOO, indicating that B-CNE had more tendency of a surface reflect light. This could be due to a coating ability of HPMC layer. HPMC was a widely used edible coating component and showed an enhancement of glossy appearance of fruit skins (Pastor *et al.*, 2010; Choi *et al.*, 2016). As a result of oil migration (Figure 6.2), B-CNE showed a lower level of oil migration than other biscuits, indicating that the oil at the surface of biscuit was not a cause of shiny appearance of B-CNE.

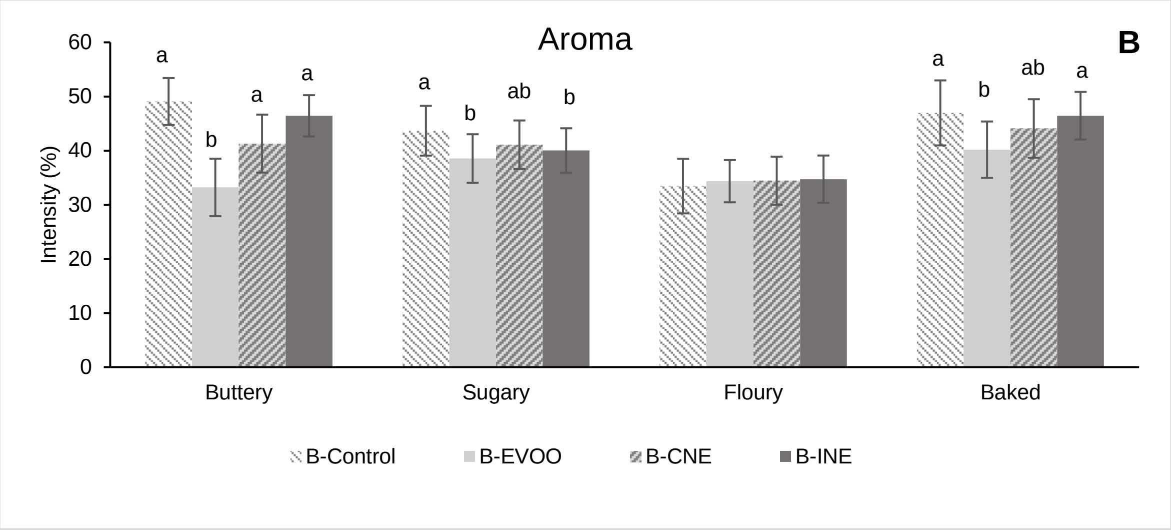
With regards to the aroma (Figure 6.3B), a decrease in buttery, sugary and baked aroma was found in B-EVOO compared to B-Control, which may be expected due to the reduction aroma compounds associated with butter and others derived from the Maillard reaction linked to baking and sugary aromas. Mallia *et al.* (2007) reported that there were more than 230 volatile compounds identified in butter, which the aroma of butter oil was characterised by aldehydes, such as (E)- and (Z)-2-nonenal and (E, E)-2,4 decadienal. However, there were no significant differences across aroma attributes between B-Control and B-CNE, indicating that aroma perception of B-CNE matched the same levels of B-Control. This suggested that although there was a reduction of butter in biscuits, CNE could allow consumer to perceive the same aroma of buttery, sugary, floury and baked.

Regarding the taste and flavour (Figure 6.3C), B-EVOO and B-CNE were found to have significantly lower buttery flavour than B-control. This suggests that a reduction of butter in biscuits by replacing with EVOO and CNE leads to a lower perception of buttery taste and flavour. The previous study (Dadalı and Elmacı, 2020) also showed that the perception of butter aroma and taste showed less intense in low-fat margarine samples. In addition, B-EVOO was significantly more fatty/oily than the other three formulations. This attribute relates specifically to the flavour components of a fatty/oily taste, and not to the mouthfeel of fattiness. The perception of fatty/oily could be due to higher amounts of oil in the composition. B-Control was also found to be significantly more salty than B-EVOO. This result was agree with previous findings of Paneras *et al.* (1996), Romeih *et al.* (2002) and Chabanet *et al.* (2013), who observed greater saltiness intensity with a lower fat content in food products such as sausages, frankfurters and cheese. However, there was no significant difference in sweet, floury, fatty/oily, salty and savoury among B-control, B-CNE and B-INE. This indicated that assessors perceived a similar intensity of these taste and flavour attributes to the control when replacing butter with CNE and INE.

Significant differences between samples were found across almost all mouthfeel attributes (Figure 6.3D). Many of these differences found B-CNE and B-INE to be similar, with largest differences to B-EVOO. A significant decrease ($p < 0.05$) in firmness at first bite was found in the B- EVOO, which was in accordance with the instrumental texture results on fracture strength (Table 6.3). B-EVOO scored significantly higher for crumbly attribute, followed by B-Control; B-CNE and B-INE scored significantly lower crumbliness. This indicates that B-CNE and B-INE were more compact and with a stronger continues phase broking into fewer crumbs during first bite. The fact that B-CNE and B-INE scored similarly to B-Control in bite but lower in the crumbly attribute than B-control could be explained by the presence of HPMC in the formula. This is supported by previous literature (Sanz *et al.*, 2017), which reported that the microstructure of biscuits made with cellulose showed a dense matrix of stable fat globules, corresponding to the dispersed phase or oil globules, immersed in a continuous phase made up of water hydrated cellulose (a three-dimensional network of HPMC chains). Moreover, pastiness of biscuits significantly increased in B-CNE and B-INE, compared to B-Control. These results could be due to the tendency of HPMC to interact with water; when breaking the biscuits in smaller pieces the continuous HPMC network is exposed to saliva more, giving place to a higher water absorption rate on the crumb pieces. This hypothesis could also explain the significant higher tooth packing aftereffect (Figure 6.3E) of B-CNE samples in comparison to other biscuits. The PCA result (Figure 6.4) also showed that the pasty attribute was negatively correlated with rate of clearance and crumbly mouthfeel. B-INE presented significantly lower gritty mouthfeel than other formulations. However, there were no significant differences in any other aftereffect attribute (Figure 6.2E).

Overall, the sensory data showed that both B-CNE and B-INE were similar to B-control in many attributes. However, there were significant differences in shiny appearance, butter taste and flavour, and tooth packing between B-CNE and B-control, and significant differences in sugary aroma and gritty mouthfeel between B-INE and B-Control. Moreover, the finding of this study suggests that HPMC plays an important role on improving crumb density (appearance and mouthfeel) and bite of the biscuits. However, pasty and tooth packing of biscuits could be affected by HPMC, whereas EVOO could be a cause of fatty/ oils in biscuits.




Aroma
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Floury
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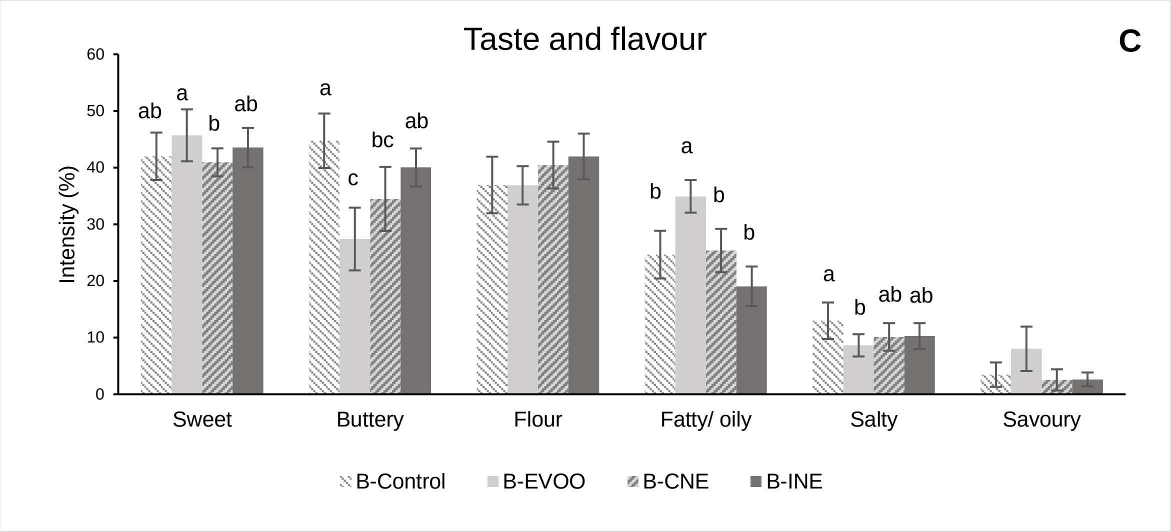
B-EVOO
B-CNE
B-INE


Taste and flavour
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Intensity (%)
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a
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b
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Sweet
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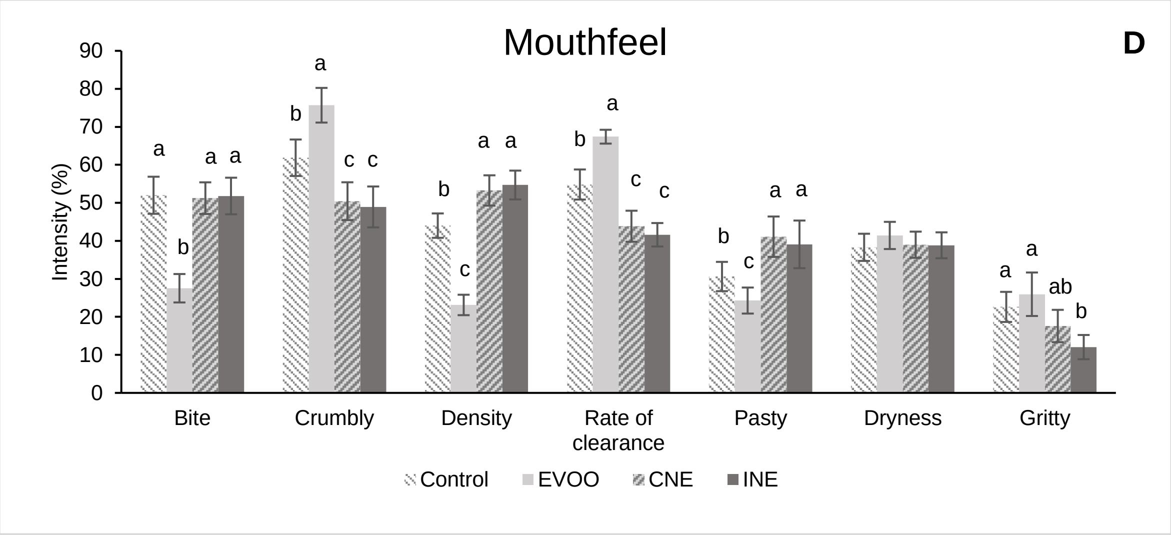
Fatty/ oily
Salty
Savoury
B-Control
B-EVOO
B-CNE
B-INE


Mouthfeel
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60
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10
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Intensity (%)
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c
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c
a
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Crumbly
Density
Rate of clearance
Pasty
Dryness
Gritty
Control
EVOO
CNE
INE

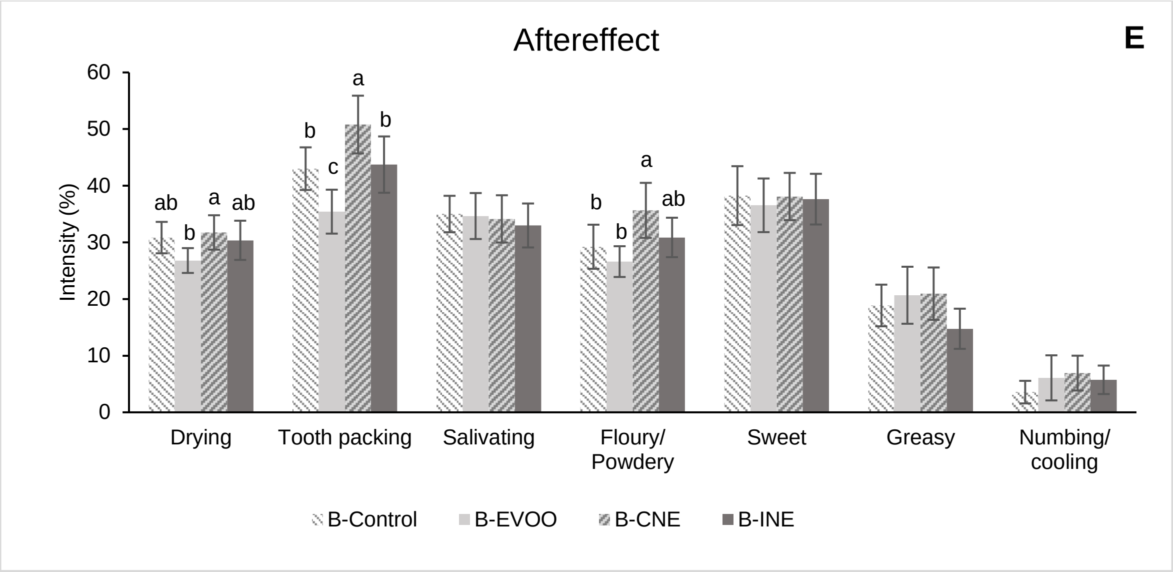

Figure 6.3. QDA intensities of the evaluated biscuits for A) Appearance; B) Aroma; C) Taste and flavour; D) Mouthfeel and E) Aftereffect. Error bars represent ± standard error of the mean. Different letters indicate significant differences in the mean ($p < 0.05$) and no letter reflects no significant difference. Control is the biscuit made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

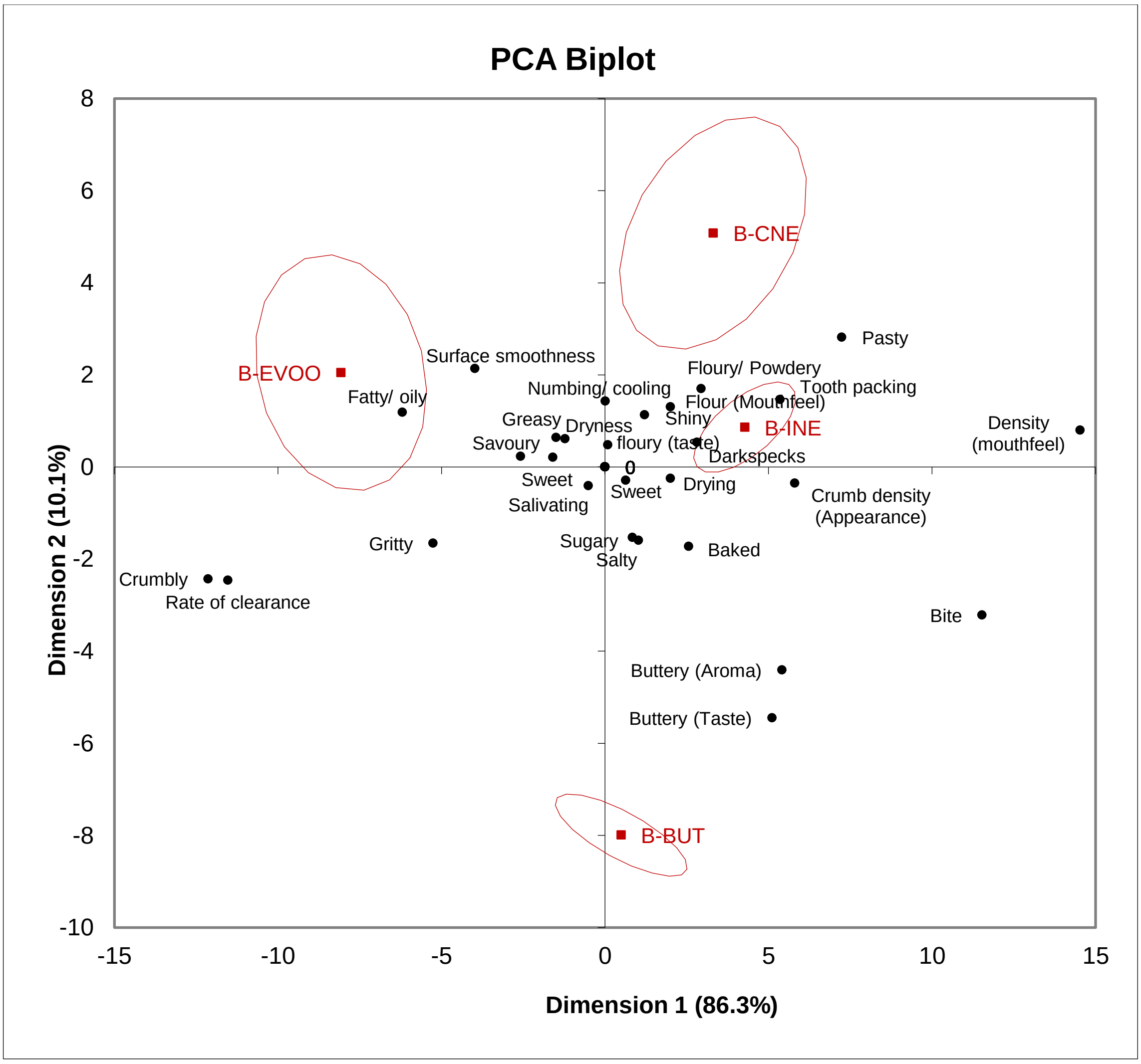

Figure 6.4. Principal components analysis from QDA data. Control is the biscuit made with butter; in EVOO 33% of the butter was replaced with Extra Virgin Olive Oil; D-CNE has 30% less saturated fat by replacing butter with a Complex Nano-Emulsion; INE has 30% less saturated fat by replacing butter with the individual ingredients of the complex nanoemulsion.

6.4 Conclusion

CNE doughs and biscuits were similar (p>0.05) to the control formulation (butter) in terms of spreadability during baking, water activity, fracturability, and colour. Moreover, CNE biscuits showed less oil migration during storage compared the other biscuits. Although CNE biscuits showed a higher fracture strength, the trained panel did not find significant differences in crumb density and hardness on the first bite among CNE and the control biscuit. HPMC plays a major role on improving oil migration and crumb density of biscuits. The use of the emulsion in the biscuit production was more effective and presented better results than the addition of the individual ingredients (B-INE). Quantitative descriptive analysis identified that B-CNE and B-INE had more similar sensory attributes to B-Control than B-EVOO. The trained panel did not find significant differences in crumb density and hardness on the first bite among CNE, INE and the control formulation. Overall, CNE which is the incorporation of a EVOO nanoemulsion stabilised with lecithin and HMPC has shown the potential to be used to reduce by 30% saturated fat and 25% total fat in short dough biscuits by replacing 33% of the butter with CNE. Therefore, future work should focus on the effect of higher level of fat replacement on the biscuit properties and the shelf-life analysis of short dough biscuits.

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Chapter 7

General discussion and recommendations for future studies

7.1 General discussion

The consumption of saturated fatty acids has adverse health effects (Buckland *et al.*, 2012; Ebbesson *et al.*, 2015), which motivates their replacement with monounsaturated or polyunsaturated fatty acids for their more beneficial impact on cardiovascular diseases and coronary heart disease events (SACN, 2019). However, the replacement of saturated fat by unsaturated fat in food products such as baked products is very challenging due to the importance of the physical and chemical stability of the fat in the system both technologically and for consumer perception, and the higher susceptibility of unsaturated fat to lipid oxidation, which can lead to undesirable flavour profile, texture, shelf life and loss of nutritional quality in food products (McClements and Decker, 2000; McClements, 2016a). In recent years, there has been an increasing interest in formulating emulsion as lipid-based delivery systems with improved physicochemical stability of functional compounds, texture and nutritional fatty acid profile (Santhanam *et al.*, 2015; Joung *et al.*, 2016; Komaiko and McClements, 2016; Pathakoti *et al.*, 2017). Therefore, the overarching purpose of this research project was to develop a system that could mimic the technological properties of saturated fats and deliver an improved the overall fatty acid profile and fat content in food products, specifically bakery products. To this end the main aim of this study was to develop a nanoemulsion stabilised by lecithin and HPMC as a novel strategy to produce a saturated fat replacer. To achieve this the objectives of this work were to investigate the effect of the nanoemulsion composition including the type of oil and emulsifiers; the optimisation of processing parameters; and the application of the nanoemulsion as a saturated fat replacer into short dough biscuits.

The first experimental chapter (**Chapter 2**) investigated the influence of vegetable oils from different natural origins and extraction processes on the physical and chemical properties and stability of conventional and nanoemulsions. Five vegetable oils with high proportions of unsaturated fatty acids were studied: extra virgin olive oil (EVOO), cold pressed rapeseed oil (CPRO), composed virgin and refined olive oil (OO), refined rapeseed oil (RO) and refined sunflower oil (SO) were used in this study. The results showed that emulsions formulated with oils with higher fraction of unsaturated fatty acids (CPRO and RO) had higher lipid oxidation values. Not only the type of oils, but also the particles of oil droplets had influence on stability of emulsions. Nanoemulsions with smaller droplet size and narrower size distribution values showed greater physical stability than conventional emulsions. Moreover, oils with a lower content of free fatty acids and higher values of total phenolic content could lead to lower TBARS values of emulsions. This result suggested that EVOO (14.26% of saturated fat, 77.69% of monosaturated fat and 8.04% of polyunsaturated fat) could be a suitable oil to formulate nanoemulsion for the next experiment study due to presenting a higher radical scavenging activity and greater stability.

In **Chapter 3**, the optimisation of high-pressure homogenisation (HPH) conditions including pressure and number of cycles on physicochemical properties and stability of emulsions stabilised with soy lecithin or Tween 20 were assessed. The aim of this study was to produce clean labels on nanoemulsions stabilised by soy lecithin with greater stability and minimal impact on its organoleptic properties for application in food products. The effect of the homogenisation pressure (1, 200, 400, 600 and 800 bars) and the number of cycles (1, 2 and 3) was studied. The shortest and most efficient HPH process was at pressures of at least 400 bars and 1 cycle; these conditions produced nanoemulsions with high physical stability when using either Tween 20 or soy lecithin as emulsifiers. Moreover, the results revealed that the molecular weight and viscoelastic properties of the surfactant play an important role on the formation and stability of emulsions. The higher viscoelastic moduli values of lecithin facilitated formation of nanoemulsions with significantly smaller droplet size than Tween 20 due to the formation of a stronger film at the oil-water interface. Moreover, this emulsion was less affected by an increased intensity of the HPH process; in fact, no significant effect ($p > 0.05$) of homogenisation pressure and cycles on soy lecithin stabilized nanoemulsions was observed and a lower MDD than nanoemulsions stabilised with Tween 20.

In order to improve the technological properties of nanoemulsions as a saturated fat replacer, a combination of emulsifiers was studied. Initially, the focus of **Chapter 4** was on the study of the functionality of HPMC, the incorporation process and concentration in aqueous solutions. The influence of the degree of substitution (hydroxyl and methoxyl) and concentration of HPMC on rheological and textural properties of aqueous solutions was assessed using butter as a comparator. HPMC solutions (2-10% w/w) with two different types of HPMC with low and high content of methyl and hydroxypropyl groups (HPMC-L and HPMC-H, respectively) were prepared. The result showed that HPMC-L had a higher surface activity with a lower interfacial tension value than HPMC-H. The molecular structure and concentration of HPMCs were important factors for the mechanical strength of the aqueous solutions of the polymer and the onset temperature of the gelation process. It was concluded that HPMC with low molecular weight (HPMC-L) could be a more suitable hydrocolloid for incorporation in nanoemulsion because of its better surface properties and it being able to form a stable gel network that could trap the oil into a more compact gel structure through processing. This study suggested that HPMC-L solution at 4% w/w will behave more similarly to butter during food production operations such as mixing or dough sheeting.

The use of a combination of emulsifiers (lecithin and HPMC) on the formation, rheological properties, physical and chemical stability of nanoemulsions vs conventional emulsions was investigated in **Chapter 5**. The properties of the emulsion stabilised with HPMC and lecithin were compared with butter's technological properties to examine its potential as a saturated fat replacer. The results revealed that a combination of lecithin and HPMC improved the physical and lipid oxidative stability of emulsions in comparison to using lecithin alone. There results were explained by the two main reasons including the protective interfacial layer that lecithin creates at the oil-water interface and the effect of HPMC molecules adsorbed at the interface of the oil droplets increasing the thickness of the interfacial layer and forming a continuous network in the continuous phase. A nanoemulsion stabilised with lecithin (5% w/w) and HPMC (2% w/w) showed a greater lipid oxidative stability and similar physical properties, in terms of firmness and spreadability to butter indicating its potential to be used as a saturated fat replacer in food systems.

The developed nanoemulsion made of extra virgin olive oil (10% w/w) in water stabilised with lecithin (5% w/w) and HPMC (2% w/w) was used as saturated fat replacer in short dough biscuits (**Chapter 6**); the physical characteristics and sensory attributes of the dough and final biscuits were investigated. Four biscuits formulations were studied including a control formulation (100% butter) and three formulations where 33% of the butter was replaced with EVOO, with nanoemulsion (CNE), and with the individual ingredients of the nanoemulsion added separately (INE). The results revealed that CNE biscuits presented less oil migration than the other biscuits due to a three-dimensional structure formed by HPMC in the continuous phase and its sol-gel transition during heating and cooling operations. A trained sensory panel did not find significant differences in crumb density and hardness on the first bite among CNE and the control biscuit. The incorporation of a EVOO nanoemulsion stabilised with lecithin and HMPC has shown the potential to reduce 30% of saturated fat and 25% of total fat in short dough biscuits.

In conclusion, the development of this nanoemulsion provides a novel strategy for the reduction of saturated fat and total fat content in bakery products. It also represents a new and exciting avenue for researchers to explore the development of healthier bakery products with enhanced nutritional fatty acids profile.

7.2 Contribution to knowledge

Whilst there is abundant literature regarding the use of nanoemulsions as lipid-based delivery systems with improved physicochemical stability of functional compounds, there have been limited studies exploring the use of nanoemulsion as a saturated fat replacer in bakery products.

The results from this research provide useful knowledge to the community, food scientists and food industry which can be used to further develop CNE as a saturated fat replacer. The contribution to the scientific knowledge is summarised as follows:

(i) A higher stability of nanoemulsion could be achieve by formulating nanoemulsion with vegetable oils that have a lower proportion of unsaturated fatty acids, lower content of free fatty acids and higher value of total phenolic content.

(ii) The development of clean labels on nanoemulsions can be achieved with soy lecithin through an effective high-pressure homogenization as a low as possible energy intensive process, within which the larger molecular weight and higher viscoelastic properties of soy lecithin play a major role on the formation and stability of nanoemulsions.

(iii) A combination of emulsifiers between lecithin and HPMC with a suitable molecule structure (low content of methyl and hydroxypropyl groups) and concentration can improve the stability of extra virgin olive oil in water nanoemulsion and yield a texture similar to butter.

(iv) A fat replacement with a mixed lecithin and HPMC stabilised nanoemulsion can work as a saturated fat replacer in short dough biscuits due to a functionality of interfacial layer and a continuous network forming of HPMC, providing satisfactory physical characteristics and sensory attributes of biscuits similar to those made with butter.

7.3 Considerations for future work

Upon completion of this research, there were several interesting findings; however not all were fully explored due to the limited time frame and scope of the study. Further investigations can be explored to fill the gap of knowledge and provide further information regarding the interaction of mixed emulsifiers between lecithin and HMPC at the oil droplets interface, the optimum formulation of biscuit made with CNE, the shelf-life of reduced fat biscuits and consumer perceptions, or the addition of lipophilic compounds into nanoemulsion, the stability and application of this technology in food products and its efficiency as delivery systems of nutrients in to the human body.

For the formulation of nanoemulsions stabilised by a combination of emulsifiers, although the interaction between HPMC and lecithin in nanoemulsion in terms of concentration, rheological properties, physical and chemical stabilities of the nanoemulsions were studied in **Chapter 5**, further understanding on the competitive adsorption between these emulsifiers or their interaction in the interface could be worth exploring in depth. The competitive adsorption of mixed emulsifiers at droplet surfaces plays an important role on the functionality and stability of emulsions (McClements and Jafari, 2018; Li *et al.*, 2013b). The emulsifier that adsorbs to the droplet surfaces more rapidly will be present initially, but due to competitive adsorption with other emulsifiers in the system may displaced the first one during storage (McClements and Jafari, 2018).

Regarding the results of fat replacement in biscuit (**Chapter 6**), biscuit with 30% reduction of saturated fat by replacing 33% of the butter with CNE presented no significant differences in crumb density and hardness on the first bite in comparison to the control biscuit. However, further reformulation work could be explored to investigate the effect of higher level of fat replacement on the biscuit properties. The main functionality of fat in biscuit doughs is coating the flour during the mixing process and providing the stability of gas bubbles in dough and maintain them in the baking process, resulted in a great structure and an expansion in products (Stauffer, 1996; Atkinson, 2011). Moreover, fats in baked products are also to impart shortening, richness, and to improve flavour and mouthfeel (Pareyt and Delcour, 2008). Therefore, an evaluation of consumer perception and acceptability of short dough biscuit with increasing levels of butter replaced by the CNE could be another area to explore in more detail in future works.

Another aspect worth investigating is the shelf-life analysis of short dough biscuits made with this novel nanoemulsions (B-CNE). A reduction of saturated fat and total fat content in biscuits may also have an effect on biscuits' shelf life. Although in **Chapter 6** the level of oil migration in short-dough biscuits at 18 °C and 30 °C for 5 days was already evaluated, a shelf-life study could design to assess the effect of storage conditions on fat oxidation, textural changes and flavour development and mouthfeel perception. A storage study of 2-3 months or using under accelerated conditions such as 25, 35 and 45 °C (Romani *et al.*, 2015) would be necessary to assess changes in moisture, texture, colour stability and lipid oxidation in the biscuits.

The nanoemulsions developed in this project could be used as delivery systems for lipophilic compounds that are essential for human health, in reduced fat food products. Nanoemulsion can provide a potential benefit to improve physicochemical stability of lipophilic compounds such as β-carotene (Guan *et al.*, 2016; Liang *et al.*, 2013) and lipophilic vitamins A, D and E (Öztürk, 2017). Therefore, it would be of interest to study on the addition of bioactive compounds or antioxidant compounds into the complex nanoemulsions and the effect on the physical and chemical properties of the biscuits.

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Appendix

Table 2.S1. Fatty acid composition of oils (% of total fatty acids)

Fatty acid	Oil type				
	EVOO	CPRO	OO	RO	SO
C8:0	ND	ND	ND	ND	ND
C10:0	ND	ND	ND	ND	ND
C12:0	ND	ND	ND	ND	ND
C14:0	ND	ND	ND	ND	ND
C16:0	1.10 (0.16)	ND	0.88 (0.01)	0.20 (0.01)	ND
C18:0	13.16 (0.47)	4.88 (0.03)	12.40 (0.02)	4.79 (0.02)	6.52 (0.13)
C18:1	77.69 (1.43)	74.39 (0.10)	79.73 (0.08)	74.11 (0.10)	31.95 (1.75)
C18:2	8.04 (0.86)	20.73 (0.11)	6.98 (0.08)	20.57 (0.10)	61.53 (1.63)
C18:3	ND	ND	ND	0.33 (0.01)	ND
SFA%	14.26 (0.59)	4.88 (0.03)	13.28 (0.02)	4.79 (0.02)	6.52 (0.13)
MUFA%	77.69 (1.43)	73.39 (0.10)	79.73 (0.08)	74.11 (0.10)	31.95 (1.75)
PUFA%	8.04 (0.86)	20.73 (0.11)	6.98 (0.08)	20.90 (0.09)	61.53 (1.63)
Total	100.00	100.00	100.00	100.00	100.00

Indicated values are reported as means (standard deviation).

ND – not detectable

Table 2.S2. Total phenolic content (TPC), free fatty acids (FFA), radical scavenging activity and thiobarbituric acid reactive substances (TBARS) of vegetable oils. Extra virgin olive oil (EVOO), cold pressed rapeseed oil (CPRO), olive oil (OO), rapeseed oil (RO), sunflower oil (SO).

Oils	TPC (mg GAE/ kg oil)	FFA (% oleic acid)	Radical scavenging activity (%)	TBARS (mmol/kg oil)	Viscosity (mPa·s)	Density (g/ml)
EVOO	202.64 ^{a} (13.96)	0.312 ^{a} (0.073)	34.15^{a} (2.64)	0.158 ^{c} (0.007)	63.82 ^{d} (2.10)	0.9112 ^{d} (0.0002)
CPRO	50.43 ^{c} (11.71)	0.241 ^{b} (0.025)	3.97 ^{b} (0.23)	0.278 ^{a} (0.020)	58.45 ^{b} (2.90)	0.9143 ^{b} (0.0004)
OO	91.98 ^{b} (5.88)	0.221 ^{b} (0.012)	8.31 ^{b} (2.75)	0.200 ^{b} (0.013)	65.84 ^{cd} (1.60)	0.9122 ^{cd} (0.0016)
RO	85.14 ^{b} (11.71)	0.159 ^{c} (0.036)	31.79 ^{a} (3.46)	0.092 ^{d} (0.018)	57.72 ^{bc} (2.23)	0.9140 ^{bc} (0.0004)
SO	31.90 ^{d} (1.75)	0.153 ^{c} (0.023)	4.91 ^{b} (1.00)	0.011 ^{e} (0.002)	53.97 ^{a} (3.67)	0.9187 ^{a} (0.0013)

Indicated values are reported as means (standard deviation). Values with the different superscript letters (within same column) are significantly different ($P < 0.05$).

Table 5.S1. G' and G" moduli of HPMC stabilised nano/emulsions at 4 °C and 20 °C at temperate ramp down.

Samples	4 °C		20 °C	
	G'	G"	G'	G"
HPMC 0% CE	0.22^{c} (0.01)	0.02^{c} (0.00)	0.22^{c} (0.01)	0.02^{c} (0.00)
HPMC 0% NE	0.23^{c} (0.02)	0.02^{c} (0.00)	0.23^{c} (0.02)	0.02^{c} (0.00)
HPMC 2% CE	19.88^{b} (0.71)	11.74^{b} (0.50)	14.14^{b} (0.62)	7.01^{b} (0.30)
HPMC 2% NE	20.52^{b} (0.58)	10.93^{b} (0.08)	15.50^{a} (0.77)	6.83^{b} (0.05)
HPMC 4% CE	23.86^{a} (0.98)	40.39^{a} (0.95)	14.49^{ab} (0.51)	23.64^{a} (0.43)

Indicated values are reported as means (standard deviation). Values with the different superscript letters are significantly different ($P < 0.05$) between samples in the same column.

Table 6.S1. Attributes, scale extremes and definitions used in the descriptive sensory analysis of the biscuits by a trained panel for the QDA.

Attribute	Scale extremes	Definitions
Appearance		
Shiny	low - high	Degree of shininess, which is the tendency of a surface to reflect light
Surface smoothness	none - extremely	Overall impression of the texture without roughness of the top surface of the biscuit.
Dark specks	none - lots	The number of dark specks within the biscuit.
Crumb density	low - high	The amount of air incorporated into the interior of the biscuit, observed visually upon breaking the biscuit in two.
Aroma		
Buttery	none - extremely	Intensity of a distinct butter aroma
Sugary	none - extremely	Intensity of sweet aroma, similar to table sugar.
Floury	none - extremely	Intensity of a distinct white wheat flour aroma
Baked	none - extremely	Intensity of bake aroma, similar to baked bakery
Taste and Flavour		
Sweet	none - extremely	Intensity of sweet taste (Sucrose)

Buttery	none - extremely	Intensity of a distinct butter flavour
Flour	none - extremely	Intensity of a distinct white wheat flour flavour
Fatty/ Oily	none - extremely	Intensity of a distinct vegetable oil flavour
Salty	none - extremely	Intensity of salty taste (sodium chloride)
Savoury	none - extremely	Intensity of umami taste (monosodium glutamate)
Mouthfeel		
Bite	soft – firm	Degree of force perceived at first bite
Crumbly	not – extremely	Degree of which the biscuit breaks into crumbs upon first bite
Density	low – high	Degree of how compact the inside of biscuit was upon chewing
Rate of clearance	slow- fast	Speed at which sample was able to be cleared from the mouth
Pasty	none - extremely	Degree to which the crumbs of biscuit begin to absorb saliva and form a paste
Dryness	none - extremely	Lack of lubrication in the mouth
Gritty	none - extremely	Degree of grainy or sandy texture
Aftereffect		
Drying	none - extremely	Degree to which 30 s after the biscuit was swallowed, your mouth still feels dry.
Tooth packing	none - extremely	Degree to which biscuit sticks to the surface of teeth
Salivating	none - extremely	Amount of saliva produced in the mouth
Floury/ powdery	none - extremely	Intensity of a powdery aftertaste
Sweet	none - extremely	Intensity of sweet aftertaste
Greasy	none - extremely	Degree to which the biscuit leaves an oily residue
Numbing/ cooling	none - extremely	Intensity of a numb sensation associated with a coolness in the mouth