Interfacial Aspects of *in vitro* Lipolysis Using Tensiometry and SAXS

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Abstract

Backgrounds/Aims: Lipolysis is an interfacial process in the conversion of dietary triglycerides to free fatty acids, and is affected by the interfacial compositions of the oil droplet. The aim of the study was to analyze any effects of co-dependency of lipase and protease enzymes on the hydrolysis of oil droplets stabilized by interfacial protein, and to determine the transitions in the internal structure of oil droplets during *in vitro* lipolytic digestion.

Methods: The changes of interfacial tension and dilatational rheology at the oil interface during gastric digestion was measured by pendent drop tensiometer, in which a droplet of olive oil was expressed in a 0.1% WPI solution at various pH levels (pH 3.5 and pH 4.5). Additionally, small angle x-ray spectrometry (SAXS) was used for identifying the differences of the self-assembled structure formed during gastrointestinal lipolysis of three different types of oils (coconut oil, olive oil and palm stearin).

Results: The interfacial tension of oil droplets at pH 3.5 was found to be higher than at pH 4.5 which indicated the interface was destabilized by acids. The emulsifier acted as a barrier at the oil interface and protected it from lipolysis by gastric lipase. The extent of pepsinolysis was enhanced with low pH because of the protein degradation by acids. At pH 3.5, the interfacial tension of WPI coated oil droplet was raised 35% whereas only 19% increased at pH 4.5. In the presence of both pepsin and gastric lipase, the protein layer was first weaken by pepsin and then lipase acted at the oil interface. From the SAXS data, there was no liquid crystals formed at the oil droplet during gastric digestion. The results showed the transitions of internal structure of oil droplets after intestinal digestion depended on the pre-digestion under gastric condition and the source of oil phase. After gastric digestion, lamellar phase was the dominate structure whereas various sub phases were formed after the intestinal digestion without the pretreatment, including bicontinuous cubic and hexagonal phase.

Conclusion: In vitro, the stability of oil droplet interface was affected by pH, emulsifier and interactions between enzymes. The study has proved gastric lipolysis and pepsionolysis were co-dependent at the oil interface. Gastric lipolysis was important for the following intestinal digestion as the self-assembled structure was weaken by gastric condition.

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Contents

List of Figures

List of abbreviations

ADSA	Axisymmetric drop shape analysis
BS	Bile salt
CARS	Coherent anti-stokes raman scattering microscopy
Cryo-TEM:	Cryogenic-transmission electron microscopy
CPP	Critical packing parameter
DLS	Polarized and depolarized dynamic scattering
EME	Emulsified microemulsion
GIT	Gastrointestinal tract
LCT	Long chain triglyceride
MCT	Medium chain triglyceride
NMR	Nuclear magnetic resonance spectroscopy
SANS	Small angle neutron scattering
SAXS	Small angle x-ray spectrometry
SGF	Simulated gastric fluid
SNEDDS	Self-nanoemulsifying drug delivery system
SIF	Simulated intestinal fluid
WPI	Whey protein isolate

Chapter 1. Introduction and objectives

The digestion and absorption of lipids by the human body is a critical aspect of nutritional wellbeing. In the case of infants, lipid digestion of fat droplets in milk represents the singular mechanism by which dietary lipids are assimilated, whereas for adults, lipids are consumed and digested in a considerably broader range of food materials. Understanding the digestion process under physiological conditions helps optimize lipid bioavailability, and has also been shown to enhance the absorption of other fat soluble nutrients and drugs. As indicated, foods are materials with varying degrees of structural a complexity, and usually that contains multiple ingredients, including macro and micronutrients. Most foods also contain varying degrees of moisture. Accordingly, many lipid-based foods that also contain water are encountered in the emulsified form, such as ice cream, infant formula and salad dressing. For many of these products, proteins are frequently added to increase the nutrient value, as well as providing an important sensory contribution, but equally they play a technical role as emulsifiers that bind at the oil interface and stabilize the emulsion. The emulsified state is also of considerable consequence during the digestion of dietary fats and oils.

The structure and stability of emulsions during the *in vitro* digestion process has been well studied (Lueamsaisuk, Lentle, MacGibbon, Matia-Merino, & Golding, 2014, 2015; Singh, Ye, & Horne, 2009), as well as the particular role of interfacial and colloidal compositions (Wilde & Chu, 2011). Interfacial analysis can be a useful indicator of fat digestion, being able to measure the conversion of apolar triglycerides via enzymatic hydrolysis into amphiphilic, surface active fatty acids and monoglycerides (Wilde & Chu, 2011). The rate and efficiency of lipolysis depends upon the ability of lipolytic enzymes to adsorb at the oil interface (Golding & Wooster, 2010), which in turn influences the relative rate of change of surface tension.

It is useful to note that the bulk of triglyceride hydrolysis takes place in the small intestine, and therefore many studies have focused on this region on the digestion process (Lueamsaisuk et al., 2015). Fewer studies have tended to cover gastric digestion, partly as the lower levels of hydrolysis were considered less consequential to overall lipid digestion, and (on a more practical basis) the limited availability of physiologically relevant gastric lipases. Lipid digestion itself is a complex process that is affected by various parameters, including temperature, enzyme activity, pH and ionic strength. For gastric digestion, most researches have analyzed the effects of the gastric environment on emulsion structure and stability rather than determining the extent of lipolysis (Beverung, Radke, & Blanch, 1999; Julia Maldonado-Valderrama, Holgado-Terriza, Torcello-Gómez, & A. Cabrerizo-Vílchez, 2013; Julia Maldonado-Valderrama, Wilde, Mulholland, & Morris, 2012). However, the role of gastric lipase has not been thoroughly explored in this context, and the particular dual effects of gastric lipase and pepsin on the

hydrolysis of protein stabilized emulsions even less considered. The first objective of the study was therefore to examine the effect of enzymes on the interface of protein coated oil droplets during *in vitro* gastric digestion at different pH conditions. The research will show whether lipolysis is influenced by both gastric lipase and pepsin and their relative activities at varying pH.

The second part of the research was to determine the transitions in the internal structure of oil droplets during gastrointestinal digestion. Previous studies have found self-assembled structures are formed during the lipid digestion as the result of combination of lipolytic products and endogenous fluids. These colloidal phases (liquid crystals, vesicles and micelles) are involved in the breakdown of emulsion droplets and their ultimate assembly into mixed micelles (J. S. Patton & Carey, 1979; Phan et al., 2013; Salentinig, Sagalowicz, Leser, Tedeschi, & Glatter, 2011). The structure of colloidal phase depends on the type of oil, pretreatment, enzymes and surrounding environment. Previous researches have studied lipolysis of long chain triglycerides and medium chain triglycerides under intestinal condition (Phan et al., 2013; Sek, Porter, Kaukonen, & Charman, 2002). In this study, the formation of self-assembled structure from the digestion of coconut oil, olive oil and palm stearin will be analyzed separately by small angle X-ray spectrometry. The emulsion will undergo gastric digestion and then intestinal digestion to observe the effect of gastric condition on the intestinal lipolysis.

Chapter 2. Literature review

2.1. Introduction

Digestive lipolysis is an interfacial process and depends on the binding of lipase enzymes during both gastric and small intestinal residency. The dynamic changes to the oil/water interface during different stages of digestion is presented in Figure 2.1. Oil-in-water (O/W) emulsions, such as milk, cream and yogurt, are dispersions of oil droplets in an aqueous continuous phase and for which the droplets are stabilized by surface-active emulsifiers. The adsorption of emulsifiers reduce the interfacial tension and prevents coalescence and aggregation between droplets.

Consumption and digestion of food emulsions passes through three sites, starting with the mouth, stomach and finally small intestine. During this transit the oil/water interface undergoes a series of pH changes, pH 1-3 up to 7.5 and hydrolysis by a range of enzymes (Singh et al., 2009). The cofactors such as electrolytes and bile salts also influence the changes occurred in the interfacial layer (Wilde & Chu, 2011).

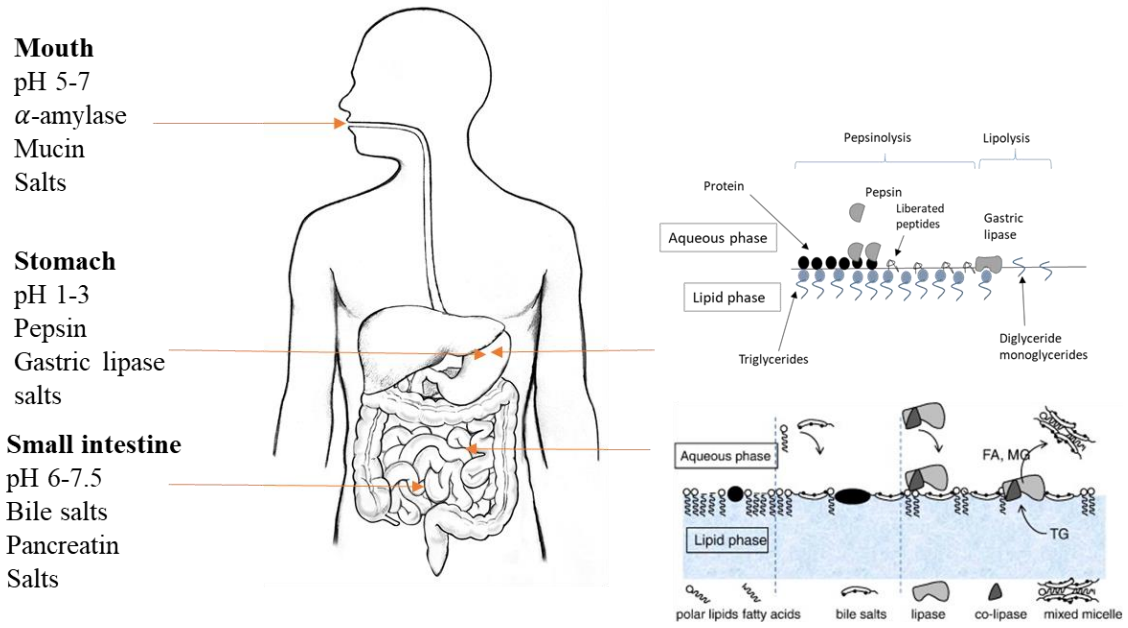


Figure 2.1. Schematic diagram of changes at oil/water interface in the different gastrointestinal tract (GIT) sites. Duodenal lipolysis is extracted from (Wilde & Chu, 2011).

The aims of the literature are 1) to review the current research on the lipid digestion in the colloidal and interfacial aspects, and 2) to review the changes of self-assembled structure of oil droplets in the different GIT sections.

2.2. Emulsions

2.2.1. Protein components used in emulsification

Whey protein

As the primary protein used in this study, it is worth outlining some of the key properties identified with whey protein that are consequential to its ability to stabilize emulsions and additionally influence their subsequent digestion. Whey protein is a mixture of predominantly globular proteins that make up 20% of the protein content in milk. Whey protein is isolated from milk after precipitating the casein with acid or rennet. It is then concentrated and separated from the serum by ultrafiltration, and spray drying. The main components of whey proteins are α -lactalbumin (19%), β -lactoglobulin (51%), immunoglobulins and bovine serum albumin. They are widely used in food industries as emulsifiers, gelling agents and foaming agents. α-lactalbumin is also used as the source of nitrogen in infant formula. Native β-lactoglobulin and α-lactalbumin have shown a certain resistance to gastric digestion by pepsin and the acidic environment (Malaki Nik, Wright, & Corredig, 2010; Sarkar, Goh, Singh, & Singh, 2009).

Whey protein isolate

Whey protein isolate is a type of whey protein product that contains 90% of proteins, 4-6% water and. 4-6% of fat, lactose and ash.

Before digestion, WPI molecules are packed at the oil interface under neutral pH, the post-acidification in the stomach reduce the dilatational modulus and increase the interfacial pressure (Mellema & Isenbart, 2004). The interface become coarsen and loss interfacial rigidity. The adsorption of proteins at the o/w interface will be discussed in detail in section 2.2.2.

β-lactoglobulin

β-lactoglobulin is produced from mammary gland with the secretion of milk, and represents 50% of the total whey proteins. It consist of 162 amino acids with a molecular weight around 18.3kDa (Griffin, Griffin, Martin, & Price, 1993). The conformational structure of protein is affected by temperature, pH and ionic strength in the surrounding environment. At room temperature and isoelectric point pH 5.2,

β-lactoglobulin is stabilized by the non-covalent intramolecular force. At pH 2-3 or pH 8, the S-S bonds are weaken and the proteins are dissociated into monomers. At pH 2.5 -6.8, β-lactoglobulin absorbed on to mica undergoes structure change and it is a reversible process. When the pH is increased back to pH 6.8, the morphology of the protein is similar to its original morphology at neutral pH (Kim, Weber, Shin, Huang, & Liu, 2007; J. Maldonado-Valderrama, Gunning, Ridout, Wilde, & Morris, 2009). At pH 4.6, it exists as an octamer. Previous researches on protein stabilized emulsions have shown that the β-lactoglobulin interface remains partly intact during gastric digestion, but is displaced by bile salts in the duodenum (Julia Maldonado-Valderrama et al., 2008).

α-lactalbumin

α-lactalbumin is a smaller globular whey protein with a molecular weight around 14.2kDa. In constrast with β-lactoglobulin, α-lactalbumin has four disulphide bonds and exists as a monomer at its isoelectric point pH 5.1. The structure of α-lactalbumin consists of α helices whereas β-lactoglobulin is mainly packed in the β-sheet. Therefore, α-lactalbumin unfolds at a lower temperature. As α-lactalbumin lacks of free thiol group, is resistant to the irreversible functional structural changes induced by high pressure (Schwenke, 1996).

2.2.2. Adsorption of protein at the o/w interface

The kinetics of protein adsorption at fluid/fluid interfaces form the basis of emulsion formation and stabilization. The state of the interfaces thus formed also has important consequences for their digestion. The adsorption mechanisms for (globular) proteins at o/w interface are shown in Figure 2.2. The adsorption process broadly covers three sequences. At the beginning, bulk proteins bind to the interface with their native globular conformation. The interfacial tension and surface mechanics are less affected at this stage because the inter-protein interactions for the native conformation of globular proteins are limited at this stage. As the process continuous, the interfacial concentration of protein increases and the conformational rearrangement of the adsorbed proteins is initiated. Tertiary structure is partially unfolded which leads the exposure and interaction of hydrophobic side of proteins to the oil phase. Also, inter-protein aggregation between neighboring protein molecules at the interface is able to commence. At this stage, the interface exhibits viscoelastic like behavior. Continuing inter-molecular association leads to the formation of an increasingly percolated protein network at the interface, characterized by an increase in surface elasticity over time (Freer, Yim, Fuller, & Radke, 2004).

Figure 2.2. Schematic presentation of protein adsorption at fluid/fluid interface a) initial conformational change b) interface aging c) aggregation of protein fragments (Freer et al., 2004).

As indicated, during adsorption to the interface, proteins undergo conformational change and rearrange within the interfacial region (Benjamins & van Voorst Vader, 1992; Beverung et al., 1999; H. Brockman, 2000). This can be affected by the stability of the native state of the protein and its resistance to unfold on adsorption. The adsorption and unfolding of tightly folded globular proteins, such as β-lactoglobulin can be slower in comparison with other forms of protein (such as the more disordered caseins) and thus their effect on interfacial tension and interfacial rheological properties could continue to change for days (Benjamins & van Voorst Vader, 1992; Beverung et al., 1999; Cascão Pereira, Théodoly, Blanch, & Radke, 2003). In comparison, more disordered and flexible proteins, such as β-casein that lack tertiary structures characteristic of globular protein, cause less effect on interfacial dilatational modulus during aging.

The adsorption of protein layers at o/w interfaces is affected by several factors, including the types and concentration of proteins, ionic strength, pH, temperature, oil hydrophobicity and protein pretreatments (Benjamins, Lyklema, & Lucassen-Reynders, 2006; Erni, Windhab, & Fischer, 2011; Mitropoulos, Mütze, & Fischer, 2014). At lower protein concentrations, proteins occupy a maximum of surface area and conformational rearrangement is not restricted. At higher protein concentrations, the diffusion and subsequent adsorption is faster and the interface become saturated rapidly. However, the protein unfolding can become limited under such situations, and the alignment of hydrophobic domains with oil phase is affected (Wüstneck, Moser, & Muschiolik, 1999). In terms of oil hydrophobicity, protein unfolding is decelerated at more polar oil interfaces which leads to a slower formation of viscoelastic networks. However, the hydrophilic bonding between polar oil interfaces and protein layer is strong and provides emulsion stability (Bergfreund, Bertsch, Kuster, & Fischer, 2018).

Previous research has shown that the conformational change of protein during adsorption greatly influences the gastric proteolysis by enhancing the flexibility for pepsin to cleave the proteins. The exposed and elongated active sites enable the accommodation of polypeptide chains in an extended conformation (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). This is particularly true for pepsinolysis, where protein adsorption exposes hydrophobic amino acids that provide preferential sites for hydrolytic cleavage. The consequences of interfacial proteolysis influenced by the structure of the adsorbed protein layer are also considered as affecting the relative ability of the gastric lipase to adsorb at the oil-water layer. This represents the hypothesis of this study, in that gastric lipolysis of protein stabilized emulsions is influenced by proteolysis of the interfacial layer by pepsin.

2.2.3. Oils

Fat type also plays an important role in the digestion behavior of food emulsions. In the current study, olive oil, coconut oil and palm stearin were chose because of their distinctive and diverse fatty acid compositions, and so a short summary of their main properties is highlighted in the sections below and in Table 2.1.

Table 2.1. Fatty acid composition in three types of fat (% weight) (Gunstone, 2011)

Fatty acid compositions (wt %)	**Olive oil**	**Coconut oil**	**Palm stearin**
6:0	-	0-0.6	-
8:0	-	4.6-9.4	-
10:0	-	5.5-7.8	-
12:0	-	45.1-50.3	0.1-0.6
14:0	0-0.1	16.8-20.6	1.1-1.9
16:0	7.5-20.0	7.7-10.2	47.2-73.8
16:1	0.3-3.5	-	0.05-0.2
17:0	0.0-0.5	-	-
17:1	0.0-0.6	-	-
18:0	0.5-5.0	2.5-3.5	4.4-5.6
18:1	55.0-83.0	5.4-8.1	15.6-37.0
18:2	3.5-21.0	1.0-2.1	3.2-9.8
18:3	0.0-1.0	-	0.1-0.6
20:0	0.0-0.8	0-0.2	0.1-0.6
22:0	0.0-0.3	-	-
24:0	0.0-1.0	-	-

Palm oil

Palm oil is one of the largest traded vegetable oils globally, being widely used in the food industries as a frying oil, in the replacement of butter (margarines), cocoa butter equivalents or shortenings. Palm oil is comparatively oxidatively stable because of the low content of polyunsaturated acids. Palm stearin is the hard fraction of palm oil which contains majority of saturated fatty acids and triacyglycerides. Palmitic acid is the main fatty acid in palm stearin that contributes to the high melting point and hardness of oil. Contrasting with palm stearin, palm olein is the liquid friction of palm oil that contains higher level of oleic and linoleic acids. Palm stearin is often used as a component for interesterification with liquid oils during the manufacture of margarines as an alternative to hydrogenation. Hydrogenation generates *trans*- acids that has adverse nutritional effect and can be avoided by the interesterification. The formulation of 60% palm stearin and 40% palm kernel olein is widely used in manufacture of margarines (Lin, 2011).

The melting point of palm stearin is at 50℃ which is related to the large amount of long chain saturated fatty acids. Fats that predominantly are composed of single chain length fatty acids are normally in β form which is stable under moderate heating (Lin, 2011).

Olive oil

Olive oil is one of the most widely consumed food oils, due to its nutritional effect against certain diseases, in particular heat disease. The phenolic compounds in olive oil have the function of antioxidant, anti-inflammation and antimicrobial activity. Therefore, olive oil is often characterized as a 'functional food' (Boskou, 2011). The application of olive oil in food includes salad dressing, cooking oil, margarines, mayonnaise and used as flavor component in many commercial foods.

Olive oil is classified into four different types based on the acidity in terms of oleic acid, including extra virgin, fine virgin, ordinary, mixture of refined with virgin. The main fatty acid compositions in olive oil are oleic acid, palmitic acid and linoleic acid. Oleic acid and palmitic acids are located at the *sn-2* position of the triacylglycerol molecule. Accordingly the oil is fully molten at ambient temperatures. Mono- and di-acyglycerols are present in olive oil due to the incomplete triacylglycerol synthesis and hydrolytic reaction. 1,2-diacylglycerols are isomerized to 1,3-diacylglycerol as the increase of shelf life (Boskou, 2011).

Coconut oil

Coconut oil contains 92% saturated fatty acids and is a solid at room temperature. Coconut oil is a notable example of medium chain triglyceride rich oil, with 62% of fatty acid being medium chain. Therefore, it has a relatively low melting point at 23-25℃. The sharp melting point of coconut oil is the result a high level of lauric acid (C12:0). It also contains reasonable amount of myristic acid (C14:0) located at *sn-1* or *sn-3* position. The location of lauric acid is randomly distributed in the triglyceride chain, but 78% of lauric acid is located at the *sn-2* position and 33% is located at the *sn-1* or *sn-3* position (Amri, 2011).

2.3. Gastrointestinal digestion

2.3.1. Gastric condition

The gastric fluid is a highly acidic environment that commences the breakdown of food structures and molecules as well as preventing the growth of microorganisms. The pH changes with the nature and amount of ingested foods. During the fasted state, the pH level is as low as pH 1-3, rising up to pH 5.5-7 after ingestion of foods. As the gastric digestion proceeds, the pH decreases to 4-5 after 60mins and then back to the pH level during fasting state. The acidic environment not only alters the food structures in bulk, it also contributes to enzyme activity for the hydrolysis of the food component.

In the fasted state, Cl^- ions and Na^+ ions are the dominate ions responsible for the ionic strength in the stomach with concentration of 100 ± 30 mM and 70 ± 30 mM, respectively. The ionic strength is positively affected after the injection of a meal, because of the addition of ions from food. The high ionic strengths change emulsion stability by weaken the interfacial layers (Kalantzi et al., 2006).

2.3.2. Gastric enzymes

Pepsin

Pepsin is a protease present in the stomach mucosa with a molecular weight of 40400 Da (Blanco & Blanco, 2017). Its major function is breaking down proteins into amino acids and smaller peptides. The proteolysis is facilitate and initiated by acid (Hur, Lim, Decker, & McClements, 2011). Its action at the interfacial layer of emulsions is discussed later in section 2.3.3.

Different types of proteins exhibit different resistance to pepsin. For the whey proteins, α-lactalbumins are relatively easily digested by pepsin whereas native β-lactoglobulin is highly resistant to pepsin and gastric condition in a bulk solution (S. B. Kim et al., 2007). Previous research has indicated pepsinolysis under gastric conditions can be enhanced for proteins adsorbed at oil-water interfaces. For example, the digestion rate of β-casein at oil interface was two times faster than in bulk solution and 10 times faster for β-lactoglobulin (Macierzanka et al., 2009). This was explained as the increase exposure of protein structure during adsorption at the interface, allowing pepsin to cleave them more efficiently. However, a recent study has proposed a different hypothesis, the hydrophobic patches of pepsin cleavage site could be covered by the oil interface and limited the digestion of protein by pepsin (Sams et al., 2018). Pepsin also could potentially degrade human gastric lipase at extreme acidic condition (pH <1.5) (Ville, Carrière, Renou, & Laugier, 2002).

Gastric lipase

Gastric lipase is the enzyme released from the gastric chief cells in the fundic mucosa in the stomach. Gastric lipase starts to appear from 10-11 weeks of gestation and develops to an adult level within the stomach by 3 months of life (Prozialeck & Wershil, 2017). Gastric lipases are acid stable enzymes and they hydrolyse 10-30% of dietary lipids in adult. They are only able to hydrolyse the triglyceride at one position, producing diglycerides and fatty acids (Hayes et al., 1994). The optimum enzyme activity of gastric lipase is around pH 4. The structure of gastric lipase consists of a α/β hydrolase fold and amphiphilic coated active site. The SerHis-Asp catalytic triad at the amphiphilic lid contributes to its extremophilic characteristics at low pH condition and physiological temperatures (Chahinian et al., 2006; Roussel et al., 1999). Gastric lipases are hydrophobic, and preferentially bind at the oil interface under gastric condition. In contrast with pancreatic lipase, gastric lipases do not require cofactors to hydrolyse lipids, and their digestion rate depends on their surface activity relative to the adsorbed layer (Chahinian et al., 2006). Previous researches believe gastric lipase may be a self-limiting reaction; as both fatty acid and diglycerides are polar and very active at the oil interface, their accumulation at the interface during the digestion will form a solid or crystalline layer that limits the access of lipase to the interface (Gargouri, Pieroni, Riviere, et al., 1986; J. S. Patton & Carey, 1979). Further investigation has

discovered long chain free fatty acids are the main contributors to the inhibitory effect, which may account for the limited overall extent of hydrolysis during the gastric stage of digestion (Pafumi et al., 2002).

Replacement of gastric lipase for in-vitro studies

The enzyme activity of human gastric lipase is highly sensitive to storage temperature and time, and it is not economic for long term research. Furthermore, sampling human gastric lipase from humans is realistically unviable, thus the replacement gastric lipase is a necessity for *in vitro* studies (Armand, 2007). To obtain the same effect in the digestion, the replacement of gastric lipase must have the same biochemical properties as human gastric lipase. In general, the biochemical properties of human gastric lipase can be summarized as: i) hydrolysis triglyceride specific at *sn-3* bond; ii) resistance to pepsin; iii) surface active; iv) remain active in acidic environment with an optimum around pH 5. The replacements of gastric lipase often comes from microbial, fungal, plant, rabbit and bacterial. Among those biochemical properties, sterospecificity appears to be the most important parameter and only a few lipases have been shown to display this characteristic. Plant lipase from *carica papaya* latex displays the *sn-3*sterospecificity, but its stability at strong acidic condition is low and its resistance to pepsin is unknown (Abdelkafi et al., 2011; Abdelkafi et al., 2009). Most microbial lipases exhibit *sn-1* sterospecificity and are reversely specific comparing with gastric lipase. The lipases often used *in vitro* studies are listed in Table 2.2 including the fungal lipase *rhizopus oryzae* that is used in the current study. *rhizopus oryzae and Aspergillus niger* are the most commonly used lipase for *in vitro* studies due to their stability in a wide range of pH conditions and similarities as gastric lipase. Although *Aspergillus niger* exhibit 1,3-*sn* regioselectivity, its steroespecificity is unknown (Sams, Paume, Giallo, & Carrière, 2016).

Table 2.2. Biochemical properties of gastric lipase and its fungal replacement (Sams et al., 2016)

Lipase	**Stereospecificity**	**Optimum pH**	**Resistance to pepsin**	**Inhibition by BS**
HGL	*sn-3*	4-5.4	Y	N
Rhizopus oryzae	*sn-1*	7-8	Y	Y
Aspergillus niger	-	5-6.5	Y	Y
Candida rugose	*sn-1*	5-8	N	Y
Pseudomonas fluorescens	*sn-1*	7-10	-	-
Rhizomucor miehei	*sn-1*	7-8.2	-	-
Burkholderia cepacia	*sn-1*	8	-	-

HGL: human gastric lipase; BS: bile salts

For *in vitro* studies, the extent of digestion and rate is affected by the type of lipase. SasseneSassene et al. (2016) compared the digestion of medium chain and long chain infant formulas with three types of gastric lipase replacement, *Rhizopus Oryzae* lipase (ROL), rabbit gastric lipase (RGL) and recombinant human gastric lipase (rHGL). Results show the digestion of emulsion by ROL is not affected by the chain length of lipid. However, RGL can only digest MCT, and rHGL favours releases of MC-FAs but also some LC-FAs. Nevertheless, ROL shows the highest digestion rate among three types of lipase.

2.3.3. Gastric digestion

During gastric digestion, protein coated emulsions can undergo interfacial proteolysis by pepsin under acidic conditions. The physical and chemical emulsification in the stomach enhances the process by increasing the surface area of the oil droplets and changes the conformational arrangement at the interface (Macierzanka et al., 2009).

The digestion of interfacial protein structures under gastric conditions has been studied by Julia and coworkers by pendent tensiometry (Julia Maldonado-Valderrama et al., 2013). The study has measured the interfacial tension of pepsin hydrolysis of β-lactoglobulin and β-casein, which indicates both protein layers are modified during digestion. However, their products of proteolysis have behaved differently at the interface. Proteolysis appears to increase the dilatational elasticity of oil/protein interface for β-casein but decreases for β-lactoglobulin. Although proteins are hydrolyzed,

predominantly hydrophobic domains will still remain at the interface (Julia Maldonado-Valderrama, Miller, B Fainerman, Wilde, & Morris, 2010; Julia Maldonado-Valderrama et al., 2012). Scheuble (2014) have used interfacial shear rheology to measure the effect of body temperature and acidic condition on the stability of the interfacial protein layer. The result has indicated the mechanical strength of the interfacial layer is weakened by the gastric conditions. The acidic environment for pH approaching fasting level (*i.e*. below the *pI* of the protein) affects the surface charge and charge density of the protein layer which induces interprotein repulsion. Thus, less binding sites between neighboring proteins (N. Scheuble et al., 2014; Nathalie Scheuble, Lussi, Geue, Carrière, & Fischer, 2016).

It can be speculated that pepsin represents one of the key factors that influence lipolysis, since lipase access to the oil-water interface may be hindered by the adsorbed protein layer (Kenmogne, Meynier, Viau, Llamas, & Genot, 2012; P. M. Reis et al., 2008). Interestingly, a recent study has simulate gastric lipolysis of β-lactoglobulin stabilized MCT droplet with recombinant dog gastric lipase (rDGL) at pH 5, 37℃. The interfacial tension of β-lactoglobulin increases because of the free fatty acid generated by lipolysis. Although proteolysis by pepsin has not been included in the study, rDG is still able to penetrate the protein layer and digest the oil Nathalie Scheuble et al. (2016). However, the efficacy of gastric lipase adsorption at the oil-water layer under physiological condition is still unclear in regards to the barrier properties of the adsorbed protein layer. In the current study, the effect of gastric lipase on the WPI interface will be studied by pendent drop tensiometry as well as investigating the potential dual role of gastric pepsin and lipase at the oil-water interface.

Gastric digestion and intestinal digestion are often analyzed separately (or the gastric lipolysis step is ignored), thus the complete gastrointestinal digestion on interfacial layers and lipolysis is less well studied. For example, prior research has analyzed the digestion of β-lactoglobulin and β-casein adsorbed layer at the oil-water interface under the entire GI conditions. The analysis exhibits the interfacial events during lipid digestion from gastric proteolysis to intestinal lipolysis. The interfacial tension changes underlying different process is shown is Figure 2.3 (Julia Maldonado-Valderrama et al., 2013). However, the study has not included the effect of gastric lipolysis. Accordingly, changes in interfacial tension correspond to changes in protein layer composition as a consequence of pH effects, gastric and intestinal proteolysis, intestinal lipolysis and competitive displacement by bile salts. Any potential contribution of gastric lipolysis (and generation of gastric fatty acids) is not evaluated. This is also a paucity in other research (Fernando A. Bellesi, Martinez, Pizones Ruiz-Henestrosa, & Pilosof, 2016; Fernando A Bellesi, Ruiz-Henestrosa, Maldonado-Valderrama, Santaella, & Pilosof, 2018).

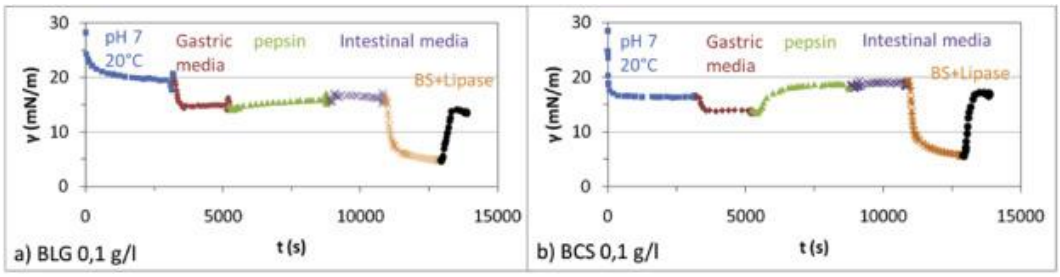


Figure 2.3. Interfacial changes during *in vitro* digestion of BLG and BCS adsorbed layers at o/w interfaces (Julia Maldonado-Valderrama et al., 2013).

2.3.4. Small intestinal conditions

The small intestine is the major region for lipid digestion and nutrient absorption. For the digestion of fats and oils, it comprises the majority of lipolysis by pancreatic enzymes, and their solubilization into mixed micelles through combination with bile salts, salts, and phospholipids. The generation of sodium bicarbonate in the duodenum result in a neutral pH 6-7.5. Therefore, the partially digested food undergoes a rapid pH change, including physiochemical changes at the interface of emulsion droplets. The increasing of pH induces negatively charged protein interface. The ionic strength and osmolality in the duodenum is related to the ingestion of food. The increasing of ions from food increase the osmolality and ionic strength. At the fasted state, the osmolality and ionic strength is around 180mOsm/kg and 140mM, respectively. After the meal, the osmolality has raised to 290mOsm/kg.

2.3.5. Intestinal enzymes

Pancreatic lipase

Pancreatic lipase, also known as pancreatic triacyglycerol lipase, is produced in the pancreas and is responsible for ~70% lipid hydrolysis. In contrast with gastric lipase, pancreatic lipase hydrolysis lipids at two positions, sn-1 and sn-3 yielding fatty acids and monoglyceride in the ratio of 2:1. Monoglycerides are considerably more amphiphilic than diglycerides, thus higher tendency for them to partition into the micellar phase at the o/w interface (J. S. Patton & Carey, 1979). The optimum active environment of pancreatic lipase is pH 7, and thereforeit can only be active under the intestinal environment. The binding of pancreatic lipase at the oil interface requires co-lipase. This is due to the additional presence of bile salt and phospholipid in the intestine which preferentially adsorb at the oil interface and limit the access of the pancreatic lipase to the surface (Erlanson-Albertsson, 1983). The kinetics of the binding of co-lipase to pancreatic lipase will not be discussed in the current study because the study only focus on the enzyme hydrolysis of lipids. It can be argued that the build-up of lipolysis products at the interface during intestinal lipolysis will ultimately inhibit lipase adsorption, as observed

for gastric lipolysis. However the presence of bile salts, assists in the formation of mixed micelles with hydrolyzed fatty acids, essentially solubilizing the interface and removing lipolysis products and allowing lipolysis to continue (Reis, Holmberg, Watzke, Leser, & Miller, 2009; Reis, Watzke, Leser, Holmberg, & Miller, 2010) (Wilde & Chu, 2011). Thus, the overall digestion of lipids in the intestine is a complex process, being interfacially mediated through the competitive adsorption process between bile salts, lipase, colipase and adsorbed components.

2.3.6. Adsorption of lipase at the interface

The adsorption kinetics of lipase at the interface are important for understanding the interfacial mechanisms of enzymatic lipolysis. An early exploration of this was provided by Verger-De Haa (Verger & de Haas, 1973) which adapts Michaelis-Menten-Henri's model on the interfacial hydrolysis of SCT and MCT. The model stated that the initial adsorption of enzyme at the o/w interface enhances the energy state of the enzyme, which allows the enzyme to bind to a substrate molecule to form an enzyme substrate complex. The enzymatic lipolysis is believed as a two-dimensional catalytic process and the generated product is soluble in aqueous phase. However, this model is only really applicable for lipids that generate appreciably soluble products (e.g. SCT) whereas digestion is a complex system that also include MCT and LCT, that are predominantly water insoluble products (Reis et al., 2010). The removal of insoluble products is essential for *in vitro* studies as bile salts are responsible for this *in vivo*. (Berg & Jain, 2002) has proposed a more complex model that includes the impact of different variables on interfacial enzyme kinetics, such as interfacial activity and kinetics of diffusion. Previous research has also concluded the rate of lipolysis is governed by the hydrophobic and electrostatic interactions between the lipase and ionized fatty acids (Petersen, Fojan, & Petersen, 2001). It can be argued that currently there is still no definitive model which integrates the interactions between substrate, enzyme and the products at the interface. In this study, the interactions between two enzymes, as well as their action at the protein stabilized o/w interface will be examined.

2.3.7. Factors affecting lipid digestion

Effect of oil composition and fatty acid location

Different sources of lipids have different physical and chemical properties which vary with the fatty acid compositions, saturation, and the location of fatty acids. In general, vegetable oils contain high levels of unsaturated fatty acids, whereas saturated fatty acids are the main constitute in animal fat. These fatty acid profile of any given lipid systems is believed to have a direct impact on the extent of

digestion and digestion rate. Saturated fats have a higher tendency to be digested than unsaturated fat. (Hur, Joo, Lim, Decker, & McClements, 2011) and (Zhu, Ye, Verrier, & Singh, 2013) have reported that the total fatty acid released from lard and milk fat is higher than olive oil, corn oil and soybean oil. Short chain triglycerides are the earliest fraction free fatty acids release group during gastric digestion followed by medium and long chain triglycerides. This is related to the location of fatty acid in the triglyceride. In milk fat, butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), stearic acid (C18:0) and linolenic acid (C18:3) are located at the *sn-1* and *sn-3* positions. Most lipases have sn-1 and sn-3 sterospecificity, therefore these fatty acids would be the fast release group (noting that short chain fatty acids can be solubilized to varying degrees in the aqueous digestive environment without need for assembly into mixed micelles). On the other hand, lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) located at the *sn-2* position and are not hydrolyzed by gastric lipase, and will form monoglycerides on hydrolysis via pancreatic lipase. For milk fat, oleic acid (C18:1) is located across all three positions, and can be relatively easily released (Angers, Tousignant, Boudreau, & Arul, 1998; Kalo, Kemppinen, Ollilainen, & Kuksis, 2004; Zhu et al., 2013). For vegetable fats, palmitic acid and stearic acid are predominantly located at *sn-1* and *sn-3* positions, and unsaturated fatty acids tend to be located at the *sn-2* position (Hunter, 2001; Mattson & Lutton, 1958). Therefore, palmitic acid and stearic acid are the main free fatty acid released during digestion of these oils.

During small intestinal lipolysis, the build-up of 2-monoglycerides and fatty acids at o/w interface can cause destabilization of the emulsion leading to coalescence, and accordingly reduces the surface area for lipase to adsorb at the surface (Golding & Wooster, 2010). Additionally, evidence shows short and medium chain fatty acids have lower interfacial activity than long chain fatty acids and their 2-monoglycerides and tend to disperse into aqueous phase (P. Reis, R. Miller, et al., 2008). Therefore, the rate and the extent of the digestion of LCT are slower than MCT and SCT.

Effect of calcium ion

Calcium is one of the main nutrients in dairy products, and it is also naturally present in small intestinal as a component in the digestion fluid. Calcium promotes the assembly of digestion products of LCT from the oil-water interface by forming insoluble calcium soaps. Both calcium ions and bile salts play a role as the removal of accumulated fatty acids at the interface, however their mechanisms are different. Bile salts remove the lipolytic products by forming BS mixed micelles in the dispersed medium. On the basis of le Chatelier's principle, the formation of calcium soap alter the equilibrium in the medium therefore equilibrium is altered toward the ionised fatty acid (Zangenberg, Müllertz, Kristensen, & Hovgaard, 2001). During digestion, the continuous peristalsis removes the accumulated insoluble crystalline phase from the oil droplets interface. Although calcium ions appear to increase the digestion rate in the digestion of medium and long chain triglycerides, the calcium concentration does not affect

the digestion rate for short chain triglycerides. This is explained as short chain fatty acid are more hydrophilic dispersible in water to varying degrees, compared to long chain fatty acid which are amphiphilic and have a higher tendency towards partitioning at the oil-water interface (Hu, Li, Decker, & McClements, 2010; MacGregor et al., 1997). Calcium ions are also considered as the cofactor of pancreatic lipase activation. The reduced digestion rates of triglycerides at low calcium levels has been attributed to a loss of activity of the enzyme (Hu et al., 2010).

On the aspect of interfacial properties, it has been demonstrated that calcium soaps form a strong viscoelastic network at the oil interface. Amelia and co-workers (Torcello-Gómez, Boudard, & Mackie, 2018) have measured the interfacial tension and dilatational modulus arising from the formation of calcium soaps. In the absence of bile salts, the formation of calcium soaps reduce the interfacial tension and increase the dilatational modulus of oil/water interface. As the concentration of calcium ions in bulk is sufficient to saturate the interface, the dilatational modulus is not further affected. The calcium ions also alter the interfacial behaviour of the adsorbed lipase via electrostatic interactions with the oppositely charged lipase molecules. Thus lipase are more compact organized at the interface (Torcello-Gómez et al., 2018). The removal of calcium soaps from the interface would induce the sudden decrease of the viscoelastic modulus and fluctuations (J. S. Patton & Carey, 1979).

Effect of gastric digestion on intestinal digestion

Bile salt and phosphatidylcholine are important for intestinal hydrolysis as disperse and remove lipolysis product from the interface. However, these two surface active components are considered as lipolysis inhibitors in many *in vitro* studies (H. Brockman, 2000). However, gastric lipolysis is not considered in these studies. *In vivo*, partial lipolysis by gastric lipase produces fatty acids and diacyglycerols, which shorten the ‘lag time’ for small intestinal lipolysis caused by non-substrate phosphatidylcholine in the intestine. Additionally, fatty acids generated by human gastric lipase have been considered responsible for the triggering of pancreatic lipase activity (Gargouri, Pieroni, Rivière, et al., 1986). Pro-colipase is secreted and activated in the stomach, assisting in the removal of bile salt micelles from the interface and thereby shortening the lag phase of lipolysis. Evidence shows colipase have the high tendency to actively bind to the interface (Sugar, Mizuno, Momsen, & Brockman, 2001), and thus the oil droplets are primed with these ‘lag phase inhibitor’ before entering the intestine.

2.4. Self-assembled structures during lipid digestion

2.4.1. Classification of polar lipids

While triglycerides are apolar and predominantly insoluble in solution, the byproducts of lipid digestion, such as fatty acids and monoglycerides are considerably more amphiphilic in nature and these polar lipids can undergo varying degrees of structuring in aqueous media. In the current study, polar lipids can be broadly classified based on their self-assembly structures and activity at the oil water interface. Accordingly, lipids are classified into three classes. According to Table 2.3, undigested triglycerides and extent of gastric lipolysis are Class I lipids and Class II lipids because diglyceride and long chain fatty acid are the main byproducts at the beginning of digestion. As the digestion proceeds, the class III bile salts form micelles which solubilize Class II monoglycerides and a small amount of Class I polar lipids (Reis et al., 2010). Thus the insoluble products are removed from the o/w interface.

Table 2.3. Classification of lipids (Phan, Salentinig, Prestidge, & Boyd, 2014; Reis et al., 2010)

Class	**Physical properties**	**interaction in aqueous system**		**Example**
		Dispersion in bulk	**Reaction at interface**	
I	Insoluble Non-swelling Amphiphiles	Crystals	Stable monolayer	di- and triglycerides, long-chain protonated fatty acids and alcohols, sterol esters, retinols
II	Insoluble Swelling Amphiphiles	Liquid crystals in water	Stable monolayer	Lecithin, monoglyceride and fatty acids
III	Soluble Amphiphiles	Liquid crystalline phase or micelles	Unstable film	Anionic, cationic and non-ionic detergents, bile salts, sodium or potassium salts of long chain fatty acids

A previous study has shown 2.5-4% of total triglycerides initially can be accessed by pancreatic lipase at the interface (Miller & Small, 1982). As the rate of lipolysis is accelerated during the initial stage of digestion, the large release of fatty acids and monoglycerides exceed the removal capacity by bile salt micelles (Carey, 1992). Thus the Class II lipids accumulate at the o/w interface and form a liquid crystalline phase (H. L. Brockman, Momsen, & Tsujita, 1988).

The liquid crystalline phase varies with the changing of temperature, water content and pH during digestion. At room temperature, the addition of water induces the existence of lamellar crystalline (L_c), lamellar liquid crystalline phase ($L\alpha$) and the inverted bicontinuous cubic phase. When heating is

applied, the bicontinuous cubic phase transits into the reversed hexagonal phase (H_2) (Reis et al., 2010). The transition between these mesophases depends on molecular shape which can be defined by the dimensionless packing parameter (Israelachvili, Mitchell, & Ninham, 1976; Tanford, 1980),

$$P = v/al$$

Where P is the packing parameter, v is the volume of surfactant, a is the area per molecule at the interface and l is the surfactant length. Figure 2.4. shows the schematics of a list of self-assembly structures with the Cryo-Tem pictures from previous studies. The packing parameter equals to one, indicates the formation of the lamellar phase which is a flat bilayer. The structure changes to vesicles as it disperses and the internal water content increases. Hydrophilic components tend to form oil in water self-assembly structures ($P < 1$), including micelles and hexagonal phase whereas lipophilic components ($P > 1$) induce the formation of reversed hexagonal structures or reversed micelles. *Fd3m* is a micellar cubic phase. For the reversed hexagonal phase, rod shaped inverse micelles hexagonal packed with closed water channels. For the reversed bicontinuous cubic phase, water channels are separated by the lipid bilayer (Phan et al., 2014).The bicontinuous cubic phase is further divided into three different space groups, including *Ia*3*d* (*gyroid)*, *Pn*3*m* (*double diamond*) and *Im*3*m* (*primitive*). The formation of bicontinuous cubic phases often induced by the present of unsaturated monoglycerides, such as monoolein (C18:1) and monolinolein (C18:2)(Sagalowicz, Leser, Watzke, & Michel, 2006). The transformation of the structures depends on the lipid structure, concentration, thermodynamic parameters and pressures, and generally follows the order as lamellar, cubic and hexagonal (Phan et al., 2014).

Figure 2.4. Schematic of self-assembly structures and Cryo-TEM for b) a dispersed H_2 phase (Yaghmur, De Campo, Sagalowicz, Leser, & Glatter, 2005); c) a dispersed Im3m phase *(L Sagalowicz et al., 2006); d) a lamellar liquid crystalline phase (obtained from mixture of Dimodan U and sodium stearoyl lactylate); e) a micelle phase (obtained from a polysorbat 80 solution). Figure reproduced from (L. Sagalowicz et al., 2006)*

2.4.2. Effect of chain length on the self-assembling structure

The chain length of the lipid has a great influence on the self-assembling structure of oil droplet. A diverse range of monoglycerides and triglycerides have been studied (Figure 2.5). The assembly of medium chain fatty with bile salts tends to induce the formation of mixed micelles and, as well as dispersed lamellar phases in combination with calcium. The solution is optically clear compared with the digestion extent of long chain triglycerides (Kossena, Charman, Boyd, Dunstan, & Porter, 2004).

Although previous research has indicated the molar ratio of lipid and bile salts affects the structure of colloidal phase, the digestion extent of medium chain triglycerides appears independent of this (Embleton & Pouton, 1997; Sek et al., 2002). For long chain fatty acids, the increasing of chain length or degree of unsaturation reduces the production of polar monoglycerides and fatty acids. As long chain triglycerides are digested slower than medium chain triglycerides, the product of digestion tends to locate at the oil interface forming a liquid crystal layer. The structure of colloidal phases for long chain triglycerides is generally more diverse, including lamellar, bicontinuous cubic and hexagonal depending on fatty acid type (Phan et al., 2013; Sek et al., 2002).

Medium chain triglycerides has shown effect as carriers for lipophilic and poorly soluble drugs by retaining lipophilic bioactives in a solubilized state over the course of digestion (Sek et al., 2002). As mentioned earlier, the reason for the different self-assembled structure formation between LCT and MCT is the composition of fatty acids and additionally the characteristics of the sn-2 monoglyceride. Early evidence has stated the monoglycerides system with carbons less than twelve would not generate liquid crystalline structures (Lutton, 1965). The chain length of the fatty acids affects its level of deprotonation. Triglycerides with shorter chain length fatty acids are more polar and has lower pKa values compared with long chain triglycerides (Heider, Hause, & Mäder, 2016; Kanicky, Poniatowski, Mehta, & Shah, 2000).

As lamellar phase is observed in the digestion of all types of lipids, Clulow and co-authors(Clulow, Salim, Hawley, & Boyd, 2018) have analyzed the calcium soaps of a range of fatty acids by SAXS, including oleic, stearic, palmitic, myristic lauric and capric. As shown in Figure 2.5. The average d-spacing between all lamellar peaks in long chain fatty acids is larger than between short chain fatty acids. The study also indicates the mixture of different types of fatty acids in the food system affect the colloidal phase in the individual fatty acids system (Clulow et al., 2018).

Figure 2.5. SAXS profiles of calcium soaps of different fatty acids(Clulow et al., 2018)

2.5. Tools for the study of structures during digestion

On exposure to gastrointestinal environment, hydrolysis of lipids induces the formation of self-assembled structures during the digestion process. The transformation from unstructured oil to highly geometrically packed liquid crystalline structures is a complex process. The present and the composition of liquid crystalline structures can be related to the digestion rate based on the appearance of interfacial assemblies. A range of tools have been applied to visualize these complex structures and monitor their appearance during digestion. Table 2.4 has summarized a list of tools applied for the study of structures during digestion. As SAXS is the only tool used in the current study, this technique will be discussed in detail.

Table 2.4. Summary of techniques for structure analysis during digestion (Phan et al., 2014)

Techniques	Function	Advantage and limitation
Microscopy techniques		
Light microscopy	Observe the size and interface texture of oil droplets during digestion	Cannot determine compositional or structural detail
Cross polarized light microscopy	Enable to distinguish anisotropic liquid crystalline phase and isotropic liquid crystalline phase	Simple Difficult to obtain compositional details Limited application for *in vivo* study
Freeze fracture electron microscopy	Frozen the sample at an extreme temperature in a fast speed and provide a snapshot view of dynamic lipolysis	Preserve water-dependent lipid phases Enable to terminate lipolysis precisely
Cryogenic-transmission electron microscopy (cryo-TEM)	Direct viewing of the sample and determine the morphology and internal structure of the sample, with nano- resolution	Limit visualization Avoids human error that often happen in other microscopy techniques
Scattering techniques		
Polarized and depolarized dynamic light scattering (DLS)	Determine particle size and size distribution of micelles, vesicles and emulsions	Particular suitable for optical study on anisotropic particles Ease to determine correlation functions
Small angle neutron scattering (SANS)	Using neutrons for scattering contrast relative to the bulk average	Not readily accessible Not suitable for kinetic studies
Spectroscopic techniques		
Nuclear magnetic resonance spectroscopy (NMR)	Analyze the physical state and chemical compositions of structures formed in aqueous phase during digestion	Eligible to determine single lipid components
Raman spectroscopy and multiplex coherent anti-stokes raman scattering (CARS) microspectroscopy	Lipid chain conformation during digestion	Cannot determine the total structure of lipid Chemically sensitive

Small angle X-ray scattering (SAXS) is a spectroscopic technique used for analyzing liquid crystals and colloidal structures in solutions (Lyon, 2002). It is able to provide morphological information for large particles in a range between 1nm to several hundreds of nm, normally with a scattering angle ϑ <10°. For dispersed systems, the electron density of the dispersed phase is different to that of the bulk average. The scattering intensity measures the inhomogeneity and reflects the size, shape and distribution of dispersed phase colloids in the sample. SAXS is used for study the structure during digestion because it can identify the structure type depending on the spacing between peaks, and follows the 'structure pattern recognition' approach.

The principle of SAXS is based on Bragg's law (Figure 2.6.),

$$n\lambda = 2d\sin(\theta)$$

where n is a positive integer, λ is the wavelength of the X-ray, d is the lattice spacing and θ is the scattering angle (Bragg & Bragg, 1913). The location of Bragg peaks is the identification of lattice planes in the sample and the spacing between peaks the indication of different types of crystal structure (Seddon et al., 2008). As shown in Table 2.5, SAXS has been widely used in the study the colloids of lipid or lipid based-drugs during digestion. It often used with cryo-TEM for directly observation of the sample and pH titration profile to record the extent released from the digestion.

Figure 2.6. Schematic presentation of SAXS setup (Kikhney & Svergun, 2015)

Table 2.5. Structures formed during dynamic in *vitro* digestion studies

Lipid system	Aqueous system	Technique	Results	Ref.
LCT				
Olive oil + gum arabic	human pancreatic lipase/colipase + BS+$CaCl_2$	Light microscopy	The calcium and ionized fatty acid induced the formation of a lamellar liquid crystalline phase. A 'viscous isotropic' phase formed by monoglycerides and protonated fatty acids	(J. S. Patton & Carey, 1979)
Trioleylglycerol + gum arabic	Porcine pancreatic lipase	Freeze-fracture electron microscopy	Without BS and calcium ion: pH=7: an amorphous layered 'crust' formed at the interface pH=8.3: a monogeneous spherical vesicles → a 'crust' crystalline and a swollen true lamellar phase. Possible formation of hexagonal phase composed of tubular-lamellar elements	(Rigler & Patton, 1983)
Olive oil	Human and porcine pancreatic lipase/colipase + NaTDC or NaTC + $CaCl_2$	Light microscopy	pH=6.5: crystalline phase (calcium fatty acid soap) pH=7.5: tubular projections of lamellar phase and hexagonal phase The crystalline structures are stable in the absence of BS	(John S Patton et al., 1985)
LCT based SNEDDS: Sesame oil	Pancreatic lipase + BS + phosphatidylcholine + $CaCl_2$	SAXS Cryo-TEM	Lamellar phase→ hexagonal phase	(Fatouros et al., 2007)
β-lactoglobulin stabilized triolein	Porcine pancreatic lipase + BS	SAXS Cryo-TEM	EME→Fd3m→H_2→vesicles CPP inversely dependent on the concentration of BS and pH	(Salentinig et al., 2011)
Long chain monoglyceride				
Monooelin	TLL lipase + sodium oleate/oleic acid/diolein	SAXS CPLM	MO+OA+water: cubic phase → H_2 → C_{mic} →L_2 MO+NaO+water: Lα → H_1 MO+DO+water: H_2 → C_{mic} →L_2	(Borné, Nylander, & Khan, 2002)
MCT				
Captex 355	Pancreatin extract + Phospholipid + BS + $CaCl_2$	SAXS Cryo-TEM	Uni and bilamellar vesicular phase The vesicle formation is affected by the concentration of BS and lipid	(Phan et al., 2013)

CPP: critical packing parameter; EME: emulsified microemulsion; LCT: long chain triglyceride; MCT: medium chain triglyceride; Lα: lamellar phase; C_{mic}: micellar cubic; L_2: reversed micellar phase; H_1: hexagonal phase; H_2: reversed hexagonal phase; TLL lipase : *Thermomyces lanuginose* lipase; SNEDDS: self-nanoemulsifying drug delivery system

Chapter 3. Materials and methods

3.1. Materials

The protein used in this study for stabilizing the emulsion is whey protein isolate, which was supplied by Fonterra Co-operative Group Ltd. (Palmerston North, New Zealand). The oils used in this study were coconut oil, olive oil and palm stearin. Coconut oil and olive oil were purchased from Woolworths, Australia and palm stearin was purchased from Davis Trading, New Zealand.

The fungal lipase (gastric lipase analogue) (Fungal Lipase 8000) derived from Rhizopus oryzae was purchased from Connell Brothers Company Australasia Pty Ltd. (Victoria, Australia), with an enzyme activity around 80000LU/g as stated by the manufacture. The pepsin (EC 232-629-3, P6887) from porcine gastric mucosa derived from hog stomach was in lyophilized powder form. It was purchased from Sigma-Aldrich Chemical Co. (Castle Hill, Australia) with an activity of 3200-4500 units/mg protein. Pancreatin (EC 232-468-9, MC23.261M) was purchased from MP Biomedicals Australia Pty Ltd. (New South Wales, Australia). It contained amylase, lipase and protease. Trizma maleate (EC 276-455-6, T3128) was used as the buffer salt and was purchased from Sigma-Aldrich Chemical Co. (Castle Hill, Australia).

Milli-Q water which was purified by a Milli-Q apparatus (Millipore Corp., Bedford, MA, USA) was used in all the experiments for sample preparation. All other chemicals such as sodium chloride, calcium chloride and sodium hydroxide pellets were of analytical grade obtained from Sigma-Aldrich chemical Co.

3.2. Methods

3.2.1 Preparation of digestion buffer

SAXS

The preparation of digestion buffer for SAXS follows the procedure applied in Monash University. The component concentration is shown in Table 3.1.

Table 3.1. Components in the digestion buffer

Component	**Quantities for 1L stock solution**
50mM tris maleate	11.86g
5mM $CaCl_2 \cdot 2H_2O$	0.735g
150mM NaCl	8.766g
NaOH pellets	1.5g
6mM NaN_3	0.39g

The pH of the stock buffer solution was adjusted to pH 6.5 with HCl. NaN_3 was used as the antimicrobial agent. After dissolving all the components, the stock solution was filtered with 0.45um filter paper.

Interfacial tension and dilatational rheology measurement

The procedure for preparing the digestion buffer for interfacial tension and dilatational rheology measurement was based on a previously reported methodology (Julia Maldonado-Valderrama et al., 2012). A 2mM Tris maleate buffer was made by mixing 0.2372g Tris maleate and 4.383g NaCl in water. The pH of the buffer was adjusted to the target pH (pH 3.4, 4.5 and 6.5) with HCl and the final volume was made to 500ml with Milli-Q water. The buffer was used within one week to avoid any microbiological growth.

3.2.2. Preparation of emulsions

The oil-in-water (O/W) emulsions were prepared with 4 wt% oil and 1 wt% WPI. Olive oil, coconut oil and palm stearin were used for investigating the digestion behavior of different types of oil under gastrointestinal conditions. Solid fat, coconut oil and palm stearin were melted under moderate heat before dispersing into aqueous phase. The aqueous phase was prepared by dispersing WPI powder in Tris buffer, and stirring on a hot plate at 50℃ for 10 mins to ensure complete dissolution. The emulsion was then hand shaken for 1 min for primary mixing. Emulsions were homogenized with an ultrasonic liquid processor (Misonix, S-4000, 600 watt) at 20A for 2 minutes. All the emulsions were prepared one hour before the experiment to reduce coalescence and flocculation.

3.2.3. "Flow through" digestion model

The SAXS experiment included two sections 1) *In vitro* digestion and 2) SAXS beamline measurement.

In vitro digestion studies were carried out according to the flow-through setup with a quartz capillary to observe the time dependent small-angle X-ray scattering (Salentinig, Phan, Khan, Hawley, & Boyd, 2013; Warren, Anby, Hawley, & Boyd, 2011). A schematic representation of the setup is shown in Figure 3.1. and the flow chart of the experiment is presented in Figure 3.2.

Figure 3.1. Schematic presentation of the in vitro digestion set up (Warren et al., 2011).

Simulated gastric fluid was prepared by dropping the pH of emulsion to pH 3.5 with 2M of HCl. 17.5ml of gastric fluid was added in the thermostated glass vessel under constant magnetic stirring. The temperature was controlled at 37℃ during the digestion. The pH was controlled at pH 3.5 with a pH-stat auto titration (Radiometer, Copenhagen, Denmark) using 0.2M NaOH. The concentration of enzyme in the gastric digestion was based on the literature from Lueamsaisuk (Lueamsaisuk et al., 2014). 0.1026g Pepsin and 0.00456g fungal lipase were prepared in the Tris buffer at pH 3.5 with total volume of 2.5ml, and was injected into the sample vessel using a remotely activated syringe pump. The enzyme solutions were prepared immediately before the experiment and kept in the fridge for storage.

Figure 3.2 summarises the flow chart of *in vitro* digestion process, both complete digestion (gastric and intestinal digestion) and incomplete digestion (intestinal digestion only) were conducted in the study. After 1h of gastric digestion, the pH of the sample in the glass vessel was immediately increased to pH 6.5 with 2M NaOH and was kept constant by auto titration with 0.2M NaOH during intestinal digestion. Pancreatin (1000 TBU in 1 ml of digestion buffer) was prepared and injected using the same method as pepsin and fungal lipase. Differently, pancreatin was prepared at pH 6.5. For the experiments without pre-digestion under gastric condition, pancreatin was the only enzyme mixture present in the sample.

The amount of fatty acid released during digestion was calculated from the volume of NaOH required to maintain the pH 6.5, with the following equation,

$$\text{Extent of digestion (\%)} = \frac{\text{C x V1(ml) x MW of triglyceride(g/mol)}}{\text{Theoretical free FA in oil x 0.7x0.91}}$$

Where, C is the molar concentration of NaOH (0.2M) used, V_1 is the volume of NaOH titrated in the emulsion during digestion. The molecular weight of triglyceride was calculated with the theoretical amount of fatty acids present in different types of oil (Amri, 2011; Boskou, 2011; Lin, 2011). The theoretical free fatty acid in oils was estimated based on the theory (Clulow et al., 2018) which assume 2 mols of free fatty acids were generated from 1 mol of triglyceride. The intestinal digestion was carried on for 1h.

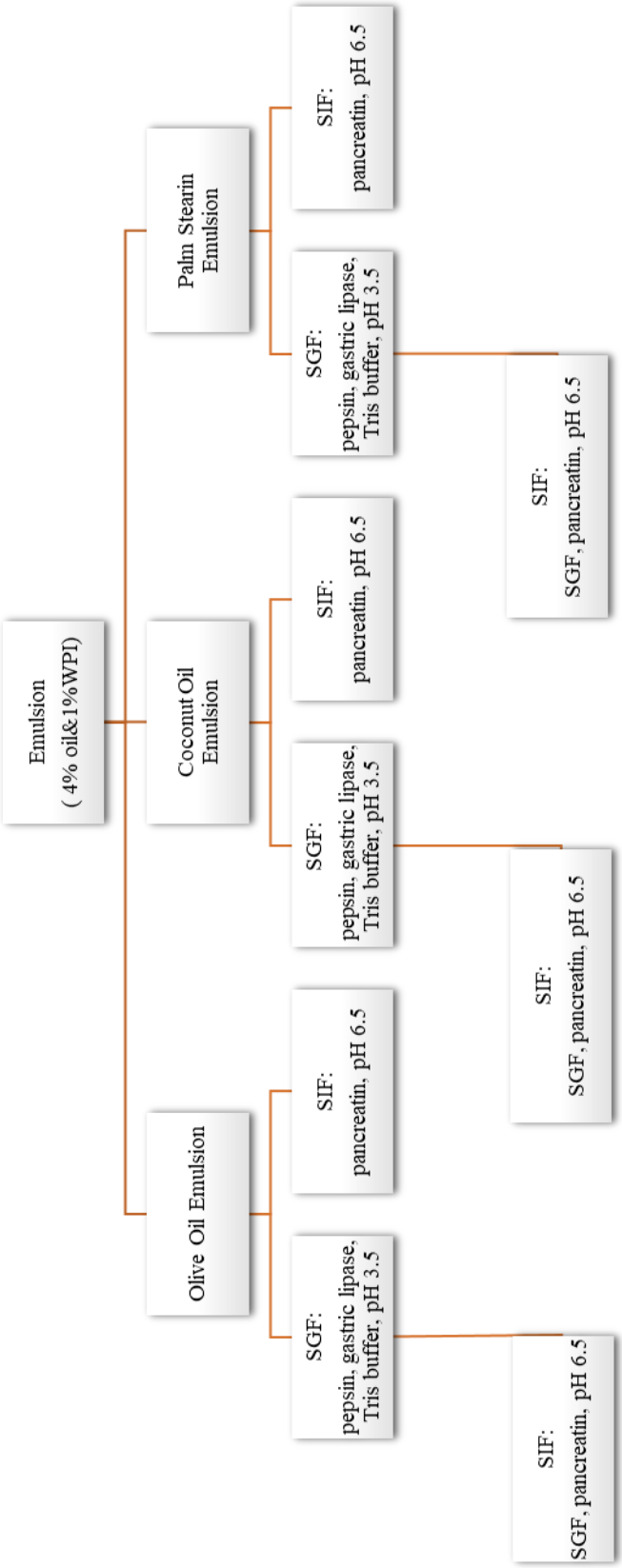


Figure 3.2. Flow chart of in vitro digestion

Characterization of self-assembled lipid structures during gastrointestinal digestion

Self-assembled lipid structures were observed *in vitro* by SAXS, using the beamline at the Australian Synchrotron (Clayton, Victoria). The measurements were taken during the *in vitro* digestion process. Emulsion samples were pumped by a peristaltic pump, through a 1.5 mm diameter quartz capillary mounted in the x-ray beam with silicone tubing (total volume < 1 ml), at approximately 10ml/min. The wavelength (λ) of the x-ray beam was set as 1.1271A (11 keV). The sample to detector distance was approximately 1.6 m, this gives a q range of $0.01 < q < 0.7$ A^{-1} whereq is the length of the scattering vector and its relationship with λ is expressed as:

$$q=4\pi/\lambda \sin(\theta/2)$$

where θ is the scattering angle. The 2D SAXS patterns were recorded at intervals of 17s (5s exposure time and 12s delay between frame) by a Pilatus 1 M detector (active area 169 x 179mm^2 with a pixel size of 172um). The 2D SAXS patterns were normalized to a 1D plot by an in-house developed software, ScatterBrain. The identification of liquid crystal phase (lamellar, cubic and hexagonal) were based on the relative positions of the Bragg peaks in the scattering curves.

3.2.5. Interfacial measurement

Enzyme and protein solution preparation

0.0044g pepsin and 0.00196g fungal lipase were prepared in bulk and dispersed into 2ml of 2M pre-adjusted Tris buffer (pH 3.5 or 4.5), respectively. The enzyme solution was prepared immediately before the experiment and placed on ice for storage.

WPI was dispersed into the neutral Tris buffer (pH 6.5) at least 1 hour before the experiment for fully dispersion. All the protein solutions were prepared daily to avoid microbial growth.

Interfacial measurements

The interfacial tension was measured by a pendant drop tensiometer, PAT-1 (SINTETFACE Technologies, Germany) and the schematic diagram of thetensiometer is shown in Figure 3.3.

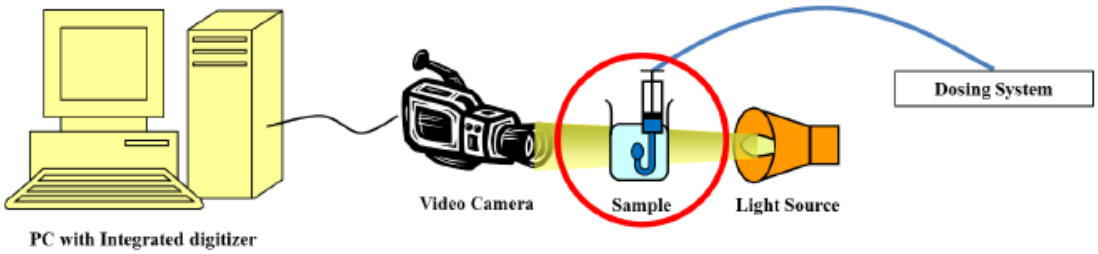


Figure 3.3. Schematic diagram of the pendant drop tensiometer (Won, 2016)

The profile of an axisymmetric shape droplet was extracted from the image taken by an electronic camera. An oil droplet was formed from the J-shaped needle which had the diameter 2.95 mm. The detection and calculation of interfacial tension was based on Axisymmetric Drop Shape Analysis (ADSA) which uses Young-Laplace equation and it is expressed as the volume (V), surface tension (γ) and the surface area (A) of the oil droplet. The Young-Laplace equation is,

$$\Delta P = \gamma \left(\frac{1}{R1} + \frac{1}{R2}\right)$$

where ΔP is the pressure difference at the interface, γ is the interfacial tension, and R is the principal radii of interface curvature. The calculation was automatically conducted by the tensiometer software.

The adsorption process was recorded at a constant volume, 30µl. The equipment was calibrated with air bubble in Milli-Q water before experiments. Olive oil was used as the oil phase with the theoretical density of 0.9077 g/cm^3. The oil was used directly from the original package without further purification. The aqueous phase was either 2mM Tris buffer or 0.1 wt% WPI solution which was dispersed into 2mM Tris buffer. The aqueous phase was placed in an approximate 25ml glass cuvette which was kept on an externally thermostaticed cell. The thermostaticed cell was connect to a water bath which maintained the sample in the cuvette at

37℃. The aqueous phases were prepared with Tris buffer at pH 6.5 and then reduced to desired pH with HCl. Later, they were pre-warmed in the water bath at 38℃ for 10 mins before the experiment. The experiment set is summarized in Figure 3.4. The adsorption of WPI or buffer to the oil interface was carried for 1h and dilatational oscillation was applied immediately after this step. 0.5ml enzymes were injected by a micro pipette, the final concentration of enzyme in the system was 0.0011g pepsin or 0.0005g lipase. The adsorption of the enzyme to the interface and digestion was carried for 1h, and the second run of dilatational oscillation was applied with the same setup as the first run. All the experiment were done in duplicate for precision.

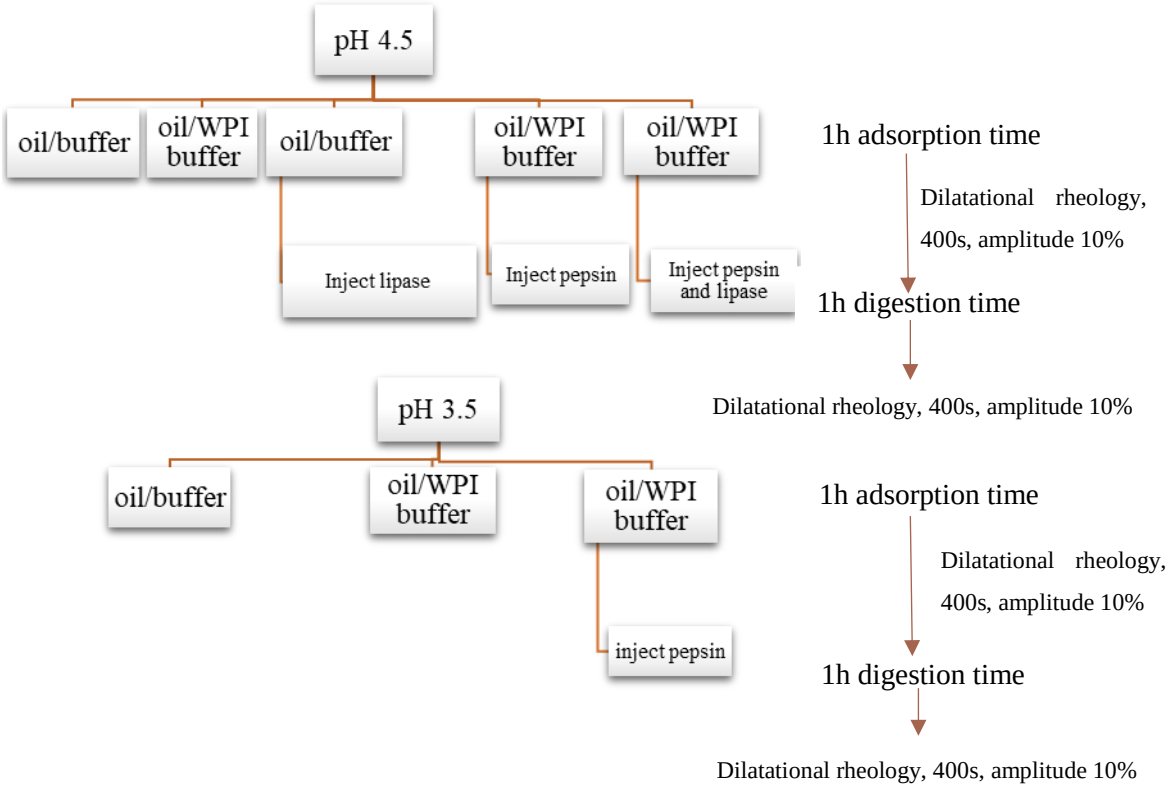


Figure 3.4. Schematic diagram of interfacial measurement setup

The dilatational rheology of the interfacial layers was measured by applying a 400s oscillatory perturbation to the interface at the end of each experiment step. The injecting and extracting volume were automatically controlled by the computer with the glass syringe. The computer recorded the response of the interfacial tension to the volume change and provides as outputs dilatational elasticity (E_0) and dilatational viscosity (K_s). The applied oscillations at the

interface were maintained at amplitude 10% and frequency was set to 0.2 Hz. The high frequency applied at WPI interface resulted in a small viscous component of the dilatational modulus and an elastic adsorbed layer (Julia Maldonado-Valderrama et al., 2013).

Chapter 4. Interfacial analysis of oil/water interface during gastric digestion

4.1. Crystal formation during gastric digestion

Hydrolysis of triglycerides under gastric conditions yields a combination of fatty acids and diglycerides. Given that the reaction occurs at the oil-water interface, and based on the fact that fatty acids display amphiphilic characteristics, it is reasonable to assume that these fatty acids will partition at the interface.

It can also be understood that interfacially located fatty acids will form liquid crystal structures in combination with calcium ions at the o/w interface, thus providing a marker that hydrolysis is occurring. The growth kinetics of liquid crystals in different oils were monitored by SAXS under gastric conditions and the results are presented in Figure 4.1. Findings appear to indicate that no mesophase structures had formed during the gastric digestion of coconut oil and olive oil, only micellar phase was observed. In terms of palm stearin, a peak at q=0.1518 $Å^{-1}$ was present, later another two peaks (q= 0.449 $Å^{-1}$ and q=0.464 $Å^{-1}$) appeared at 13 min after enzyme injection. However, the spacing between these peaks did not correspond to any sub phase structure. The lack of observed mesophase structuring under purely gastric conditions could have been a consequence of lack of interaction between Ca ions and the protonated fatty acid at low pH, but equally may have been indicative of a lack of hydrolysis of the protein-stabilized droplets. To probe this further, pendant drop tensiometry was used to determine the dynamics of interfacial structure during digestion.

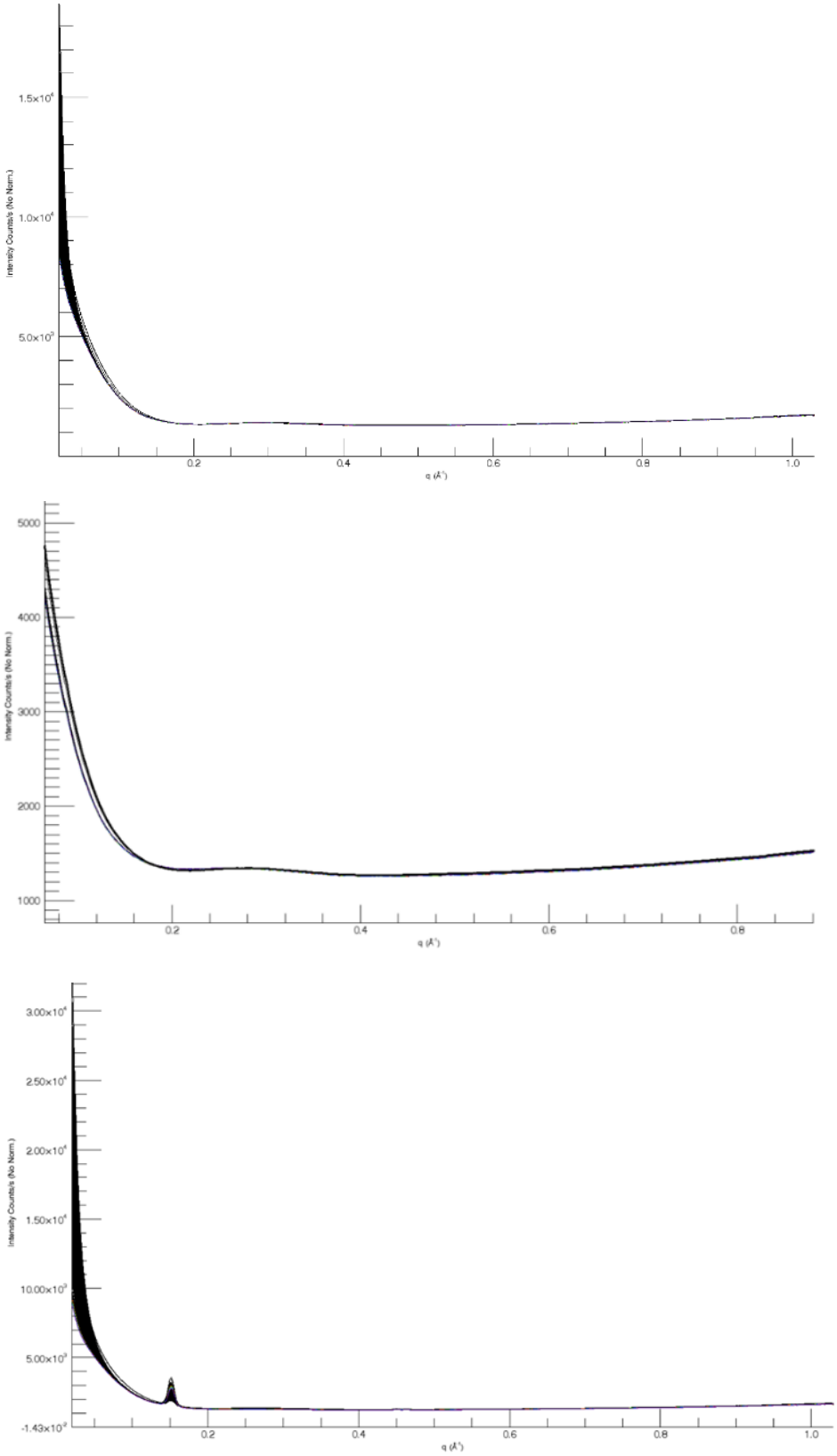


Figure 4.1. SAXS profile for the digestion of a) coconut oil b) olive oil c) palm stearin under gastric condition pH 3.5, 37℃

4.2. Enzyme action at the o/w interface

As there was no clear observation for the gastric digestion in the SAXS analysis. The study of o/w interface was later carried with the tensiometer, which is able to monitor changes in interfacial tension change under various gastric condition. Two pH levels were measured (pH 3.5 and pH 4.5) and gastric lipolysis was conducted at pH 4.5 only.

The changes of interfacial tension of o/w interface during proteolysis at different pH levels are exhibited in Figure 4.2 and Figure 4.3. The enzyme was added immediately after the first dilational rheology measurement finishes (4100s). In general, the interfacial tension of the oil surface dispersed in buffer was higher at pH 3.5 compared to at pH 4.5. The initial drop in interfacial tension at the oil-buffer interface (in the absence of WPI) is most likely due to the presence of polar lipid components (monoglycerides and fatty acids) naturally present in the oil that are able to adsorb to the interface. When 0.1% WPI was added to the water phase, the interfacial tension decreased dramatically, from 17.614mN/m to 7.509mN/m. As the o/w interface became stabilized after one hour, 0.0011g of pepsin was added into the WPI solution which raised the interfacial tension from 8.460mN/m to 9.882mM/m at pH 3.5, and a slight decrease from 6.706mN/m to 6.652mN.m at pH 4.5. The gastric lipolysis was carried out at pH 4.5 only because the interface at pH 3.5 was too weak to be measured using the pendant drop method; in spite of multiple attempts at analysis the oil droplet drifted away with the injection of enzymes. The interfacial tension of oil droplet in buffer was reduced from 14.7mN/m to 12.7mN/m when fungal lipase was added. This could be attributed to lipolysis generating fatty acids which would lower surface tension on accumulation at the o/w interface. However, it cannot be discounted that adsorption of the enzyme itself (being amphiphilic) would result in a reduction of interfacial tension, regardless of whether subsequent triglyceride hydrolysis was taking place. Certainly, where the o/w interface initially comprised WPI, the system was not affected by the addition of lipase as there was no obvious change in the interfacial tension. However, the presence of both lipase and pepsin in the WPI, the interfacial tension raised from 5.3mN/m to 5.9mN/m, noting that this value was appreciably different from the change when pepsin alone was added. Comparing with previous studies (Julia Maldonado-Valderrama et al.,

2013; Julia Maldonado-Valderrama, Miller, Fainerman, Wilde, & Morris, 2010), the increase was small, the result was reasonable with consideration of enzyme activity at pH 4.5.

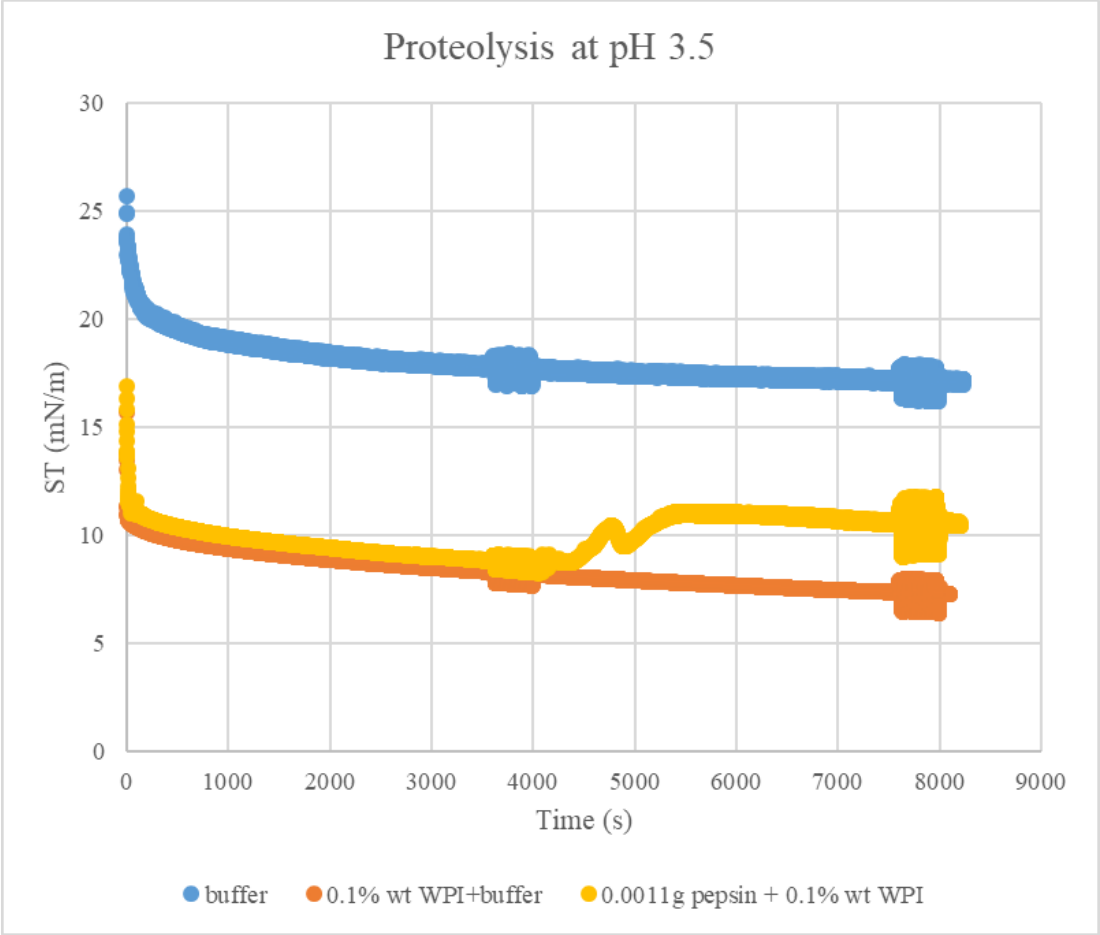


Figure 4.2 Interfacial tension of olive oil/water during proteolysis at pH 3.5

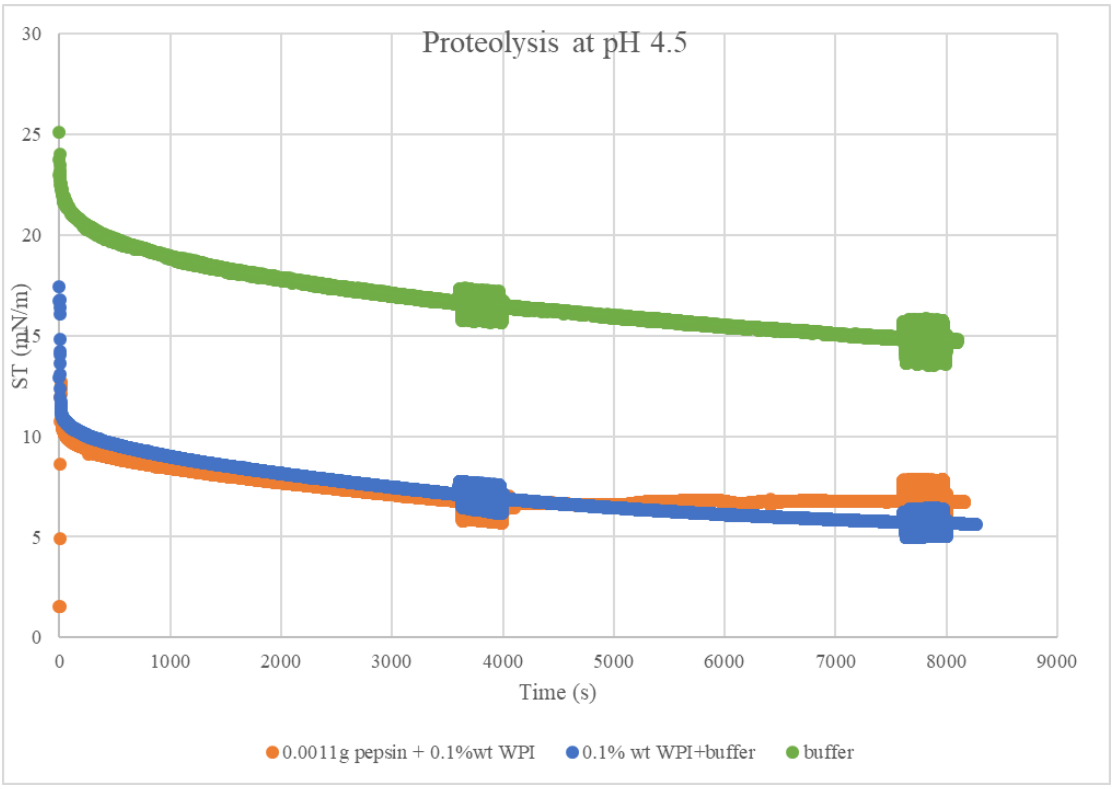


Figure 4.3. Interfacial tension of olive oil/water during proteolysis at pH 4.5

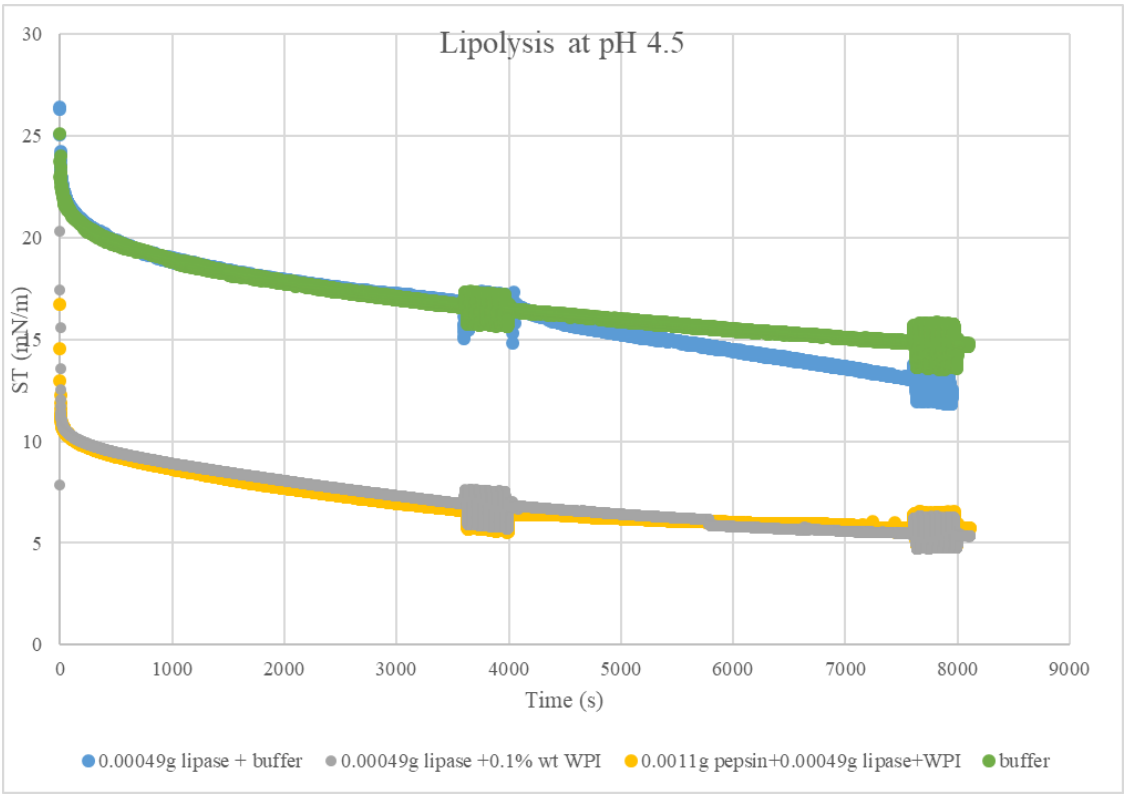


Figure 4.4. Interfacial tension of olive oil/water during lipolysis at pH 4.5

The dilatational rheology results are presented in Figure 4.5 and Figure 4.6, and the other relevant data was summarized in Appendix I. The dilatational rheology of the o/w interface was conducted after 3600s and 7200s (noting that enzymes were added after the dilational elasticity measurement at 3600s). The results from the first hour measurement were different at different pH level. At pH 3.5, the rheological parameters for WPI system were smaller than the buffer system whereas the opposite was observed at pH 4.5. However, the results from the second hour measurement were concordant, the rheological parameters for WPI system were larger than for the buffer system. The pepsin has greater effect on the rheological parameters at pH 3.5 than at pH 4.5. More specifically, only the dilatational viscosity was affected by the action of pepsin at pH 4.5 and the elasticity was constant with the non-enzyme WPI system. On the side of gastric lipolysis, lipase had a negligible effect on the WPI coated oil interface, the differences between rheological parameters were minor. However, with both the action of pepsin and lipase, the rheological parameters were reduced compared with the non-enzyme WPI system.

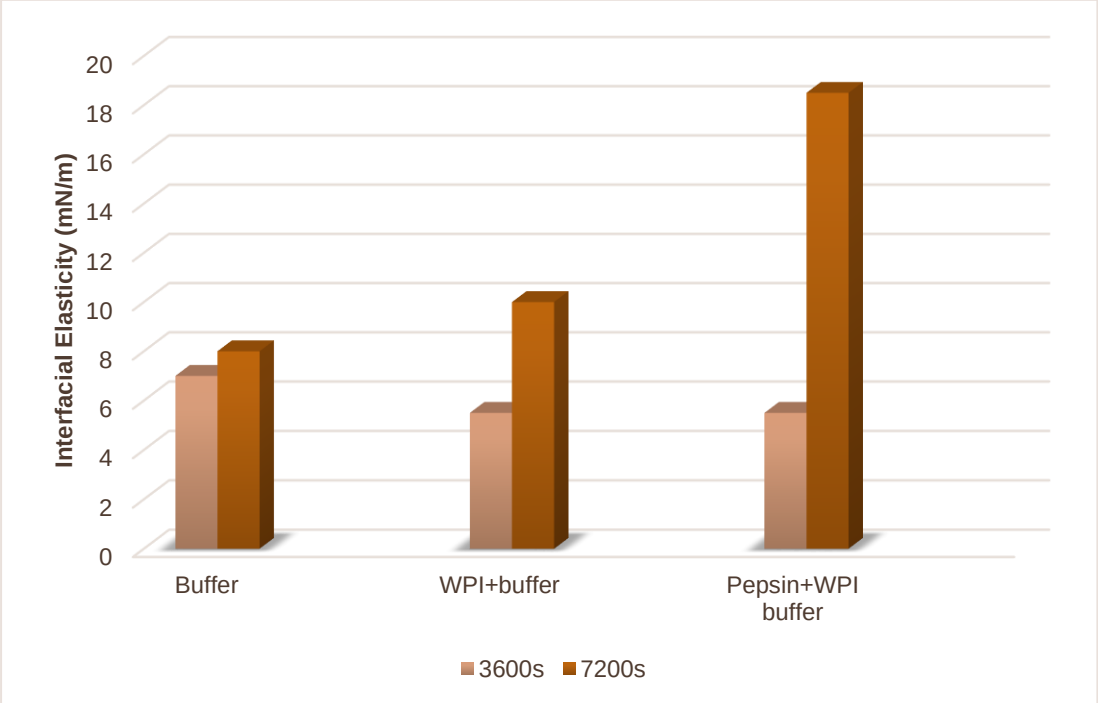


Figure 4.5. Dilatational Elasticity of o/w interface at pH 3.5

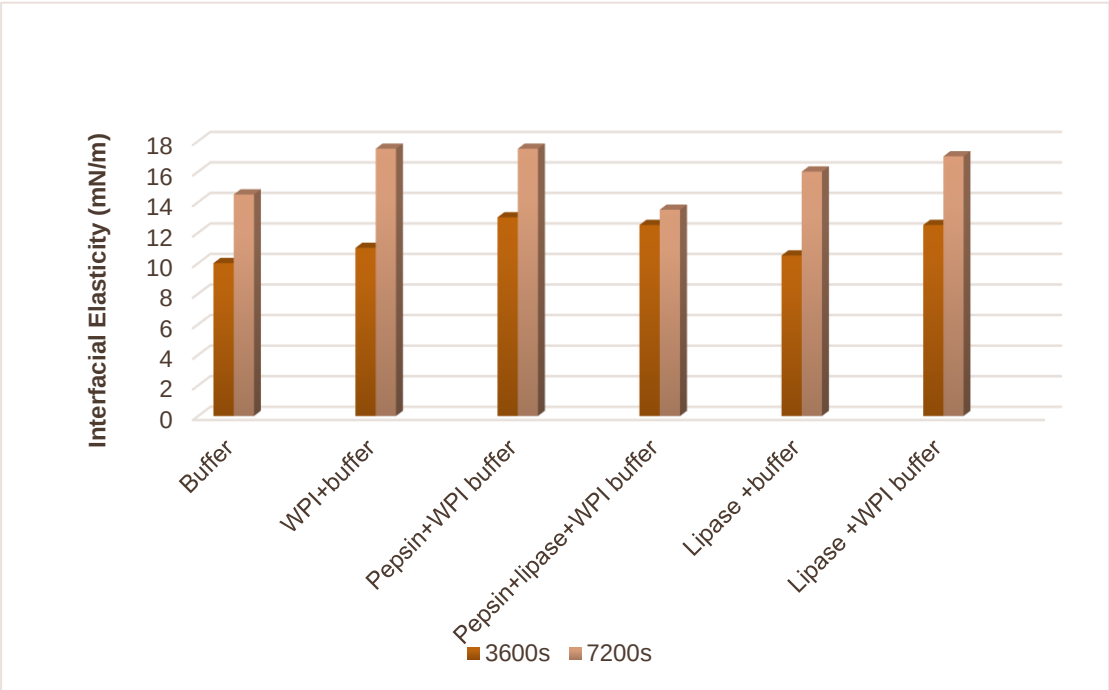


Figure 4.6. Dilatational Elasticity of o/w interface at pH 4.5

4.3. Discussion

Food is a complex system and often contains multiple nutrients and they tend to interact with each other during digestion. WPI is a globular protein that often is used as an ingredient in food. The surface activity and interfacial mechanics of WPI makes it an excellent emulsifier, providing stability against aggregation and coalescence of droplets over a broad range of conditions. Considering the interfacial measurements, addition of WPI cause the interfacial tension to decrease and dilatational elasticity increase slightly. This agrees with the characteristic of a globular protein at the o/w interface and the interface is enhanced (Julia Maldonado-Valderrama et al., 2013; Julia Maldonado-Valderrama, Miller, Fainerman, et al., 2010). The adsorption of WPI is mainly promoted by salt screening of charges (Julia Maldonado-Valderrama, Miller, Fainerman, et al., 2010). Our result showed that pepsin plays an important role on o/w interface coverage and mechanics. At pH 3.5 (Figure 4.2), the pepsin activity is maximized, and hydrolysis of the protein layer is clearly observed based on the change in interfacial tension. The increase of the interfacial tension can be attributed to the detachment of hydrophilic peptides leading to reduced occupancy of the interface. The increase of the dilatational elasticity and viscosity is in contrast with previous works at the olive o/w

interface where the exposure to the pepsin leads to a decrease of dilatational elastic modulus (Julia Maldonado-Valderrama et al., 2013; Julia Maldonado-Valderrama et al., 2012). As the frequency is constant, dilatational elastic modulus changes depend on the dilatational elasticity and viscosity. In theory, pepsin cleave the polypeptide and reduce the adhesivity of the protein layer, which leads to a decrease of dilatational elastic modulus. Our results only explained the removal of interfacial protein. However, our study has confirmed earlier research, visualized by atomic force microscopy, that pepsin partially hydrolyses the adsorbed protein molecules, but does not break the interconnected interface network (Julia Maldonado-Valderrama, Gunning, Wilde, & Morris, 2010), and may in fact enhance inter-peptide interactions having removed charged domains from the interface that may have contributed to intermolecular repulsion. Our result shows the interfacial tension is not steady during proteolysis which is different to previous research on β-lactoglobulin and β-casein stabilized oil droplets (Macierzanka et al., 2009; Julia Maldonado-Valderrama, Miller, Fainerman, et al., 2010). WPI is a complex protein which contains various surface active components, including β-lactoglobulin, α-lactabumin, serum albumin, immunoglobulin and lactoferrin (Tsutsumi & Tsutsumi, 2014). The digestion of the mixture of peptides is different to the digestion of individual components.

The addition of lipase, while showing some effect on interfacial tension when added to the aqueous phase in the absence of protein (and thus possibly indicating lipolysis) (Figure 4.4), appears to have no effect on interfacial tension when introduced to the aqueous phase comprising the pre-adsorbed WPI. Here it might be assumed that in the absence of pepsin, the lipase cannot penetrate the protein layer to access the oil layer as the primary substrate (Kenmogne et al., 2012). Alternatively, lipolysis may be occurring but the synthesis of fatty acids does not provide any additional contribution to surface tension. Alternatively, the enzyme concentration may be insufficient to generate lipolysis, noting that a number of additional experiments were carried out to explore the effects of enzyme concentration, and that findings were generally consistent with those observed in Figure 4.3. The dilatational parameters and the interfacial tension in lipase WPI system shows no difference with the WPI only system (Figure 4.4 and Figure 4.6). In contrast, the decrease of the interfacial tension and the increase

of the dilatational rheology in the lipase buffer system may further indicate the formation of fatty acid and monoglyceride. Most interestingly, when both lipase and pepsin are present (Figure 4.4), pepsin breaks down the protein layer first which allows lipase to penetrate and reach the oil interface. The slight increase of interfacial tension (below that observed for proteolysis in the absence of lipolysis) and decrease of dilatational parameters may indicate the depletion of interfacial protein by fatty acids, although without further evidence this cannot be confirmed. The formation of fatty acids, diglycerides and monoglycerides at the interface enhanced the network and therefore the growth of interfacial tension was eased. It also indicates that at pH 4.5, lipolysis plays a dominate role on dynamic interfacial stability, while for pH 3.5, pepsinolysis appears to have greater consequence. Findings are broadly consistent with the divergence of gastric enzyme activities across this pH range noting that lipase activity is maximized at pH 6.5-7 whilst pepsin is most active at pH~2. Certainly higher and lower pH levels are worth further study in the future.

In the stomach, gastric lipase initiates the lipid digestion and approximately 10-30% of lipids hydrolysed. Although there is no research monitoring the gastric lipolysis by SAXS, the results obtained by light and electron microscopy has also showed no visible product phases (John S. Patton, Rigler, Liao, Hamosh, & Hamosh, 1982). This indeed agreed with our result by SAXS, no crystal formation during gastric condition. During gastric lipolysis, the protonated fatty acid and diacylglycerol are still dissolved in the oil phase (M C Carey, D M Small, & Bliss, 1983; John S. Patton et al., 1982). As they are not exposed to aqueous phase and calcium ions, no crystals are formed. Previously one research has observed lamellar phase under gastric condition by freeze fracture with bovine milk, which contains short chain fatty acid. The oil samples used in our experiments are mainly composed of medium or long chain fatty acid which cannot be digested easily. Short chain triglyceride can be used for future SAXS research.

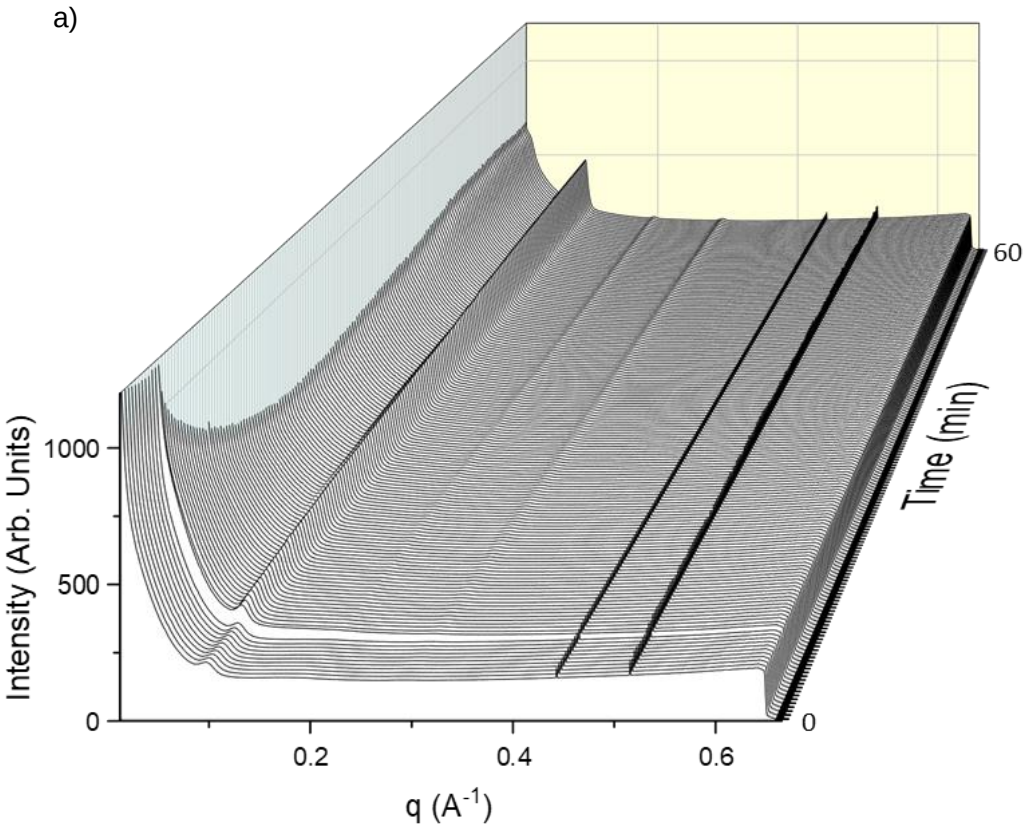
As noted, there is an initial decreasing of interfacial tension for the buffer-oil system, even in the absence of protein. Impurities in the oil samples can exist due to aging or oxidation. These naturally presented fatty acids and monoglycerides have lower molecular weight and adsorb faster than WPI to the interface (De Feijter, Benjamins, & Tamboer, 1987). Thus, the accuracy of the result might be affected. Interestingly, there is another theory suggesting that the

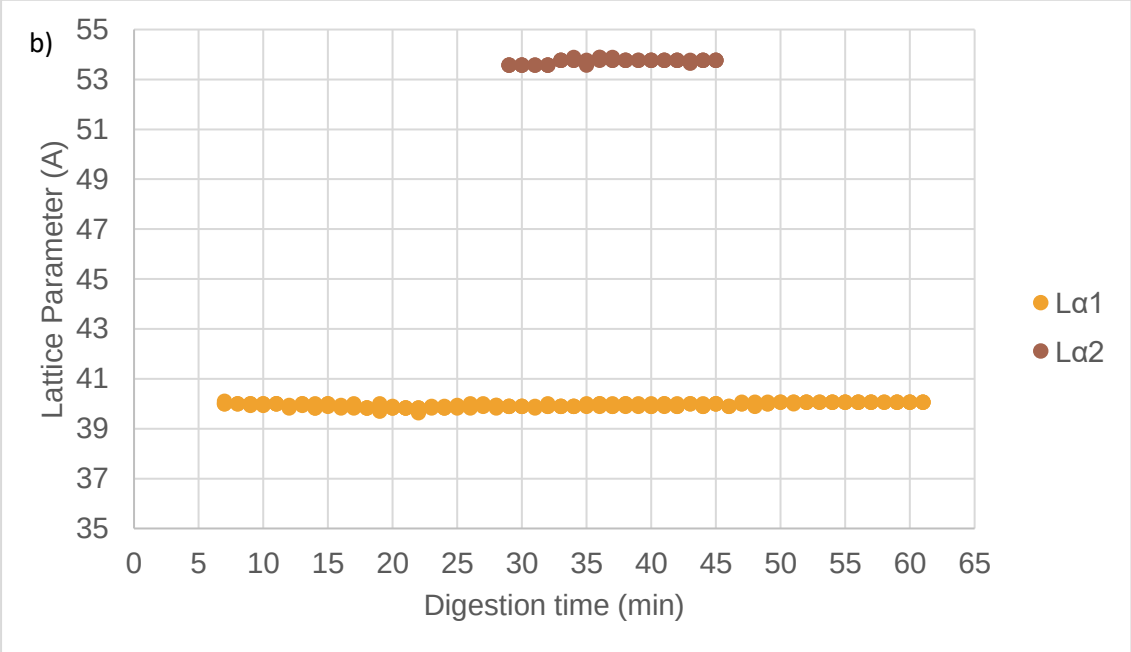
impurities in the triglyceride oil has no effect on the adsorption/reconfirmation process during the first 5h (Roth, Murray, & Dickinson, 2000). Either way, future experiments should consider the use of “clean” oils for which any polar lipid fractions are removed prior to analysis.

Chapter 5. SAXS characterisation of liquid crystal formation and phase changes during intestinal digestion

5.1. Hydrolysis of coconut oil

Coconut oil represents a particular class of lauric-dominated fats (including palm kernal oil) that comprise predominantly medium chain triglycerides. The hydrolysis of coconut oil in intestinal digestion after gastric digestion is depicted in Figure 5.1 using SAXS, and which aims to show the development of polar lipid mesophase stuctures accumulating at the interface. The system contains the partialy hydrolysed triglyceride and their product (from gastric lipolysis, as shown previously in Figure 4.1), as well as residual pepsin, lipase and added pancreatin. Before commencement of the small intestinal stage of SAXS analysis, the pH of the system is adjusted from gastric to small intestinal conditions followed by the addition of pancreatin into the system. Figure 5.1c shows that the peak (q= 0.154 $Å^{-1}$) in the co-existing micellar phase continuously shifted to higher values after adjusment of pH and across the time period prior to pancreatin addition. After less than 1 min of pancreatin action, the intensity of the peak was markedly increased and the width of the peak was narrowed, indicating the formation of emulsified microemulsion or emulsified L_2 phase. As the digestion proceeded, the first lamellar phase was formed at 3min, EME peak at q= 0.154 $Å^{-1}$ with higher order of reflections at q=0.314 $Å^{-1}$ and q=0.473 $Å^{-1}$. The lattice parameter of the lamellar phase was 40 Å. After 25 min, the second lamellar phase appeared at peak q = 0.117 $Å^{-1}$, q = 0.232 $Å^{-1}$, and q = 0.359 $Å^{-1}$ with lattice parameter 54 Å. However, and somewhat curiously, this lamellar phase only lasted for 16 mins. The intensity of the peak q = 0.232 $Å^{-1}$ and q = 0.359 $Å^{-1}$ was very small and disappeared after 16 mins whereas the peak q = 0.117 $Å^{-1}$ disappeared 4mins later. The formation of the second lamellar phase was unclear and further research is required in the future.


a)
Intensity (Arb. Units)
1000
500
0
0.2
0.4
0.6
q (A-1)
Time (min)
60
0


b)
Lattice Parameter (A)
55
53
51
49
47
45
43
41
39
37
35
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Digestion time (min)
Lα1
Lα2

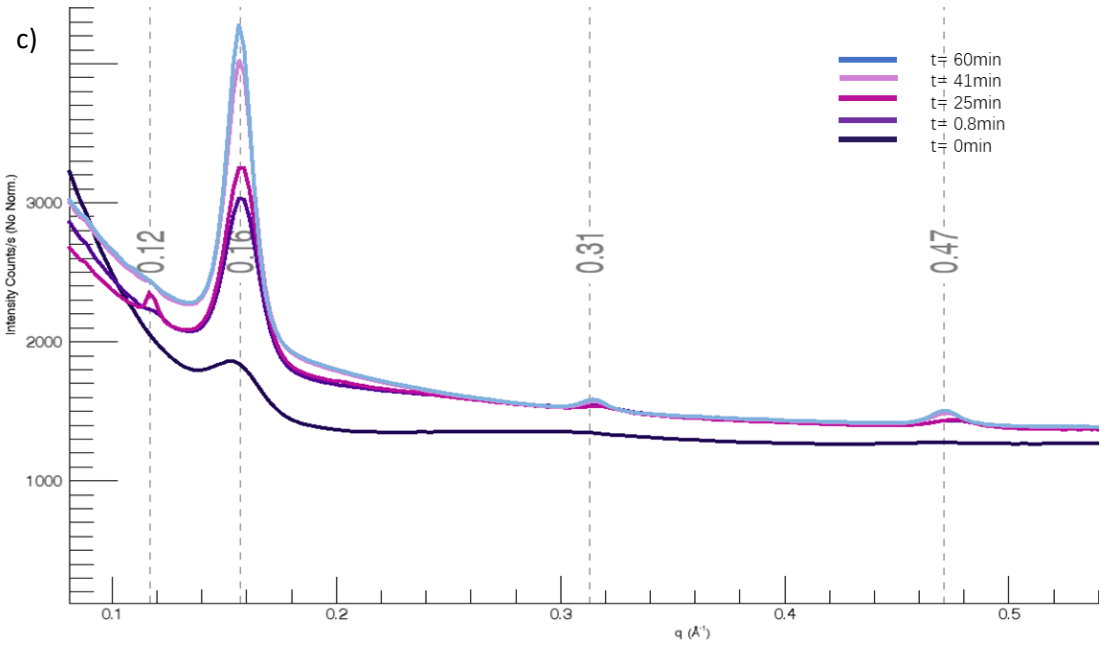
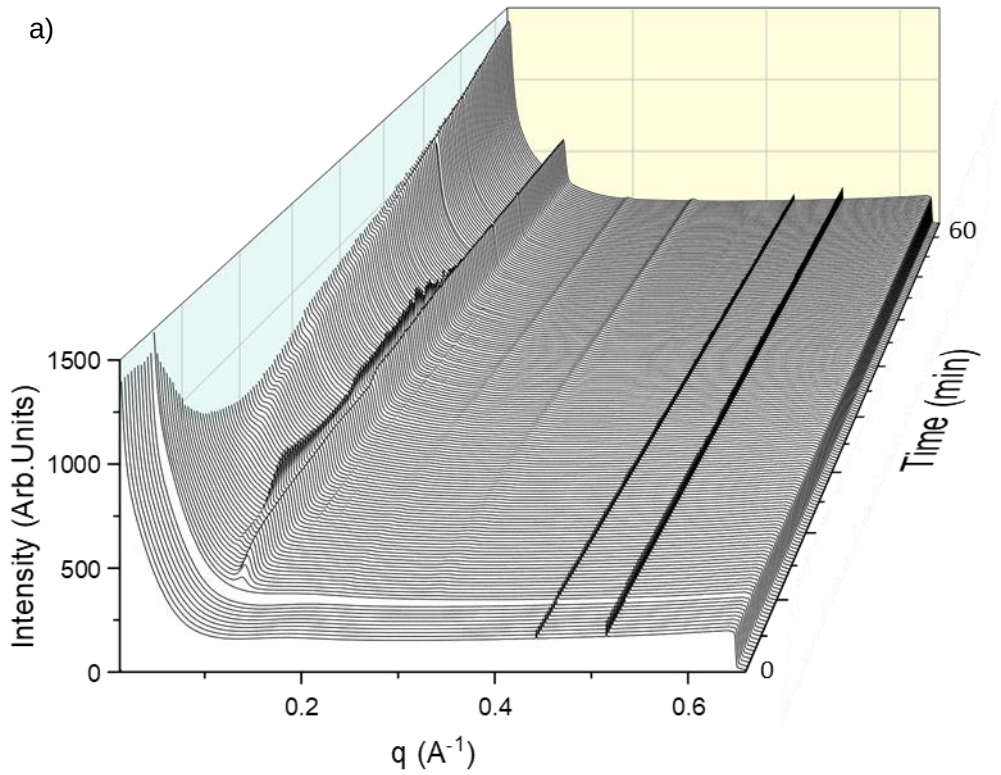


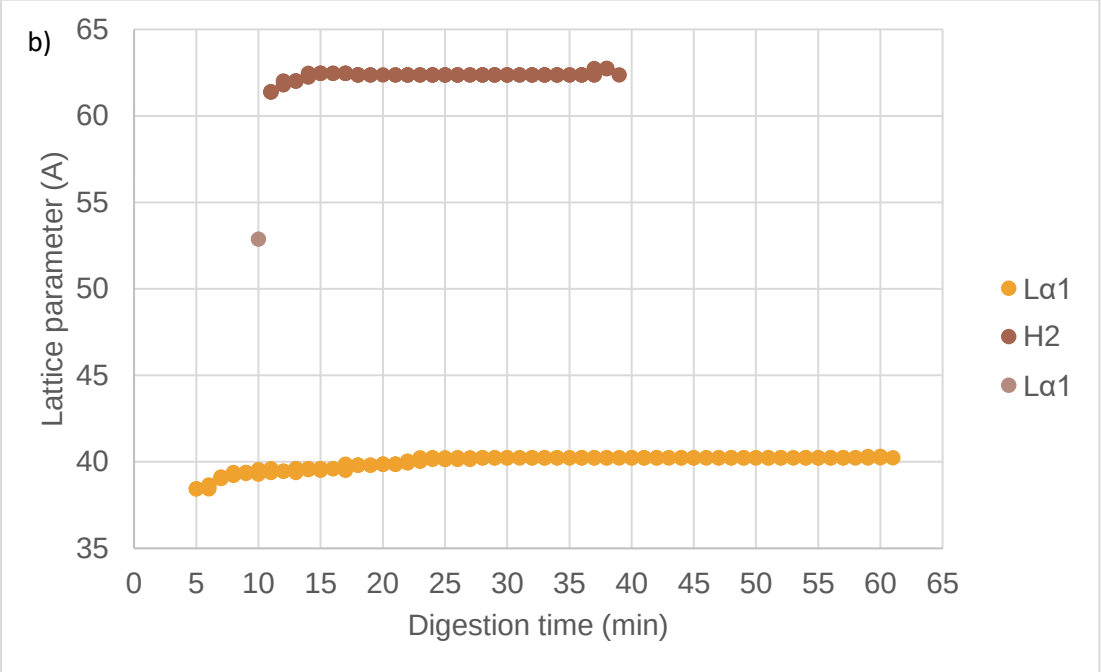
Figure 5.1. SAXS profiles for the digestion of coconut oil under intestinal digestion after gastric digestion over 60min at T=37℃ and pH=6.5: a) transition of structures with time of pancreatin action on coconut oil b) Trends in lattice parameters and kinetics of formation of mesophases during intestinal digestion of coconut oil and c) Transition of peaks during the digestion

When the experiment was repeated in the absence of the gastric stage of digestion (Figure 5.2), it became apparent the no particular mesophase structures were observed before the addition of pancreatin to the system, which indicated there was no pre-existing liquid crystalline structures. This was clearly different to the intestinal system after gastric digestion indicating the prior formation of lamellar structures during gastric lipolysis. The absence of any mesophase structures during SAXS analysis during gastric digestion (Figure 4.1) may be indicative that assembly only took place after the pH change from gastric conditions to small intestinal conditions, leading to a deprotonation of the fatty acids and subsequent self-assembly.

After addition of pancreatin, the intestinal digestion of coconut oil followed a similar pattern to the sample with prior gastric digestion, noting that a lamellar phase (q=0.1612 $Å^{-1}$, 0.3140$Å^{-1}$ and 0.4757 $Å^{-1}$) was formed 3mins after the injection of pancreatin, with the lattice parameter 40 Å. The lamellar phase was consistent during the digestion and no discernible change in peak

positions was observed, noting that a slight delay in appearance of the phase was observed after pancreatin addition. An inverse hexagonal phase (q = 0.1165 Å $^{-1}$, 0.2012 Å $^{-1}$and 0.2341 Å $^{-1}$) had formed 7mins after the injection of pancreatin, however, it disappeared 40mins after the digestion start. Lamellar phase is the final structure of the liquid crystal.





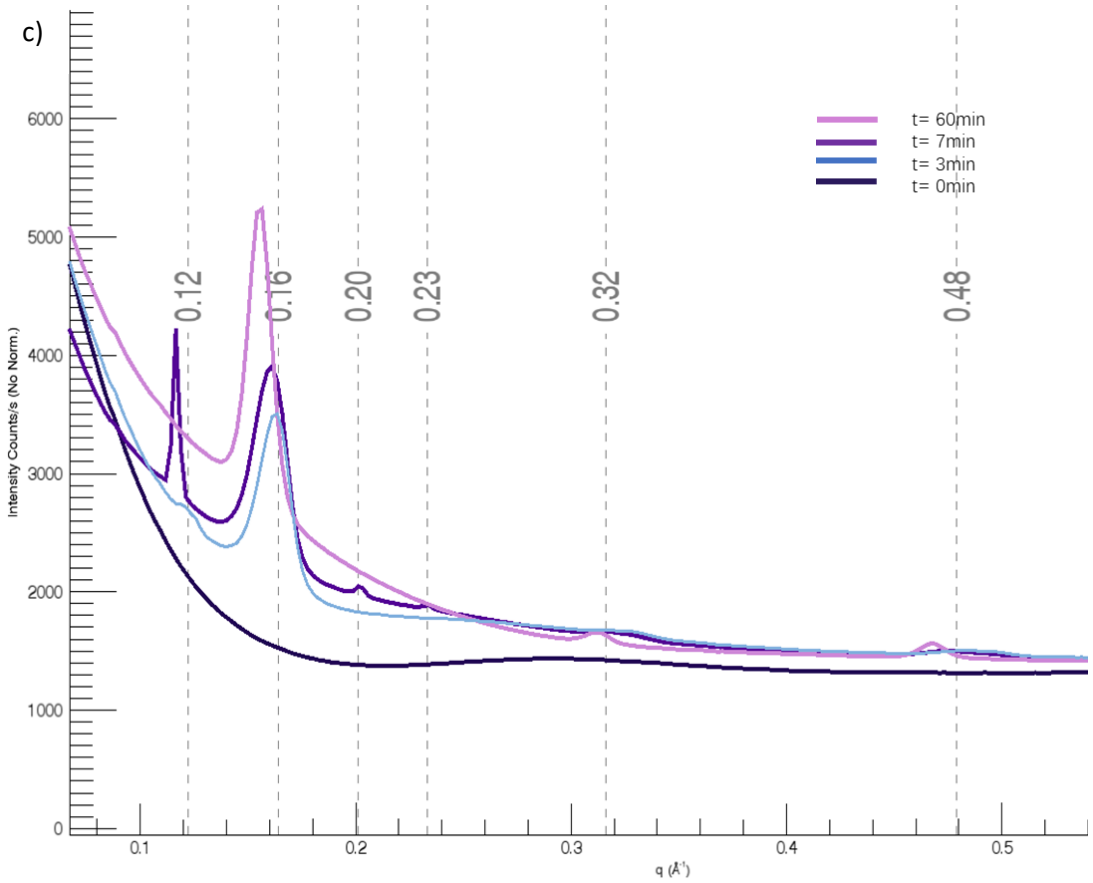


Figure 5.2. SAXS profiles for the digestion of coconut oil under intestinal condition with pancreatin only over 60min at T=37℃ and pH=6.5: a) transition of structures with time of pancreatin action on coconut oil b) Trends in lattice parameters and kinetics of formation of mesophases during intestinal digestion of coconut oil and c) Transition of peaks during the digestion

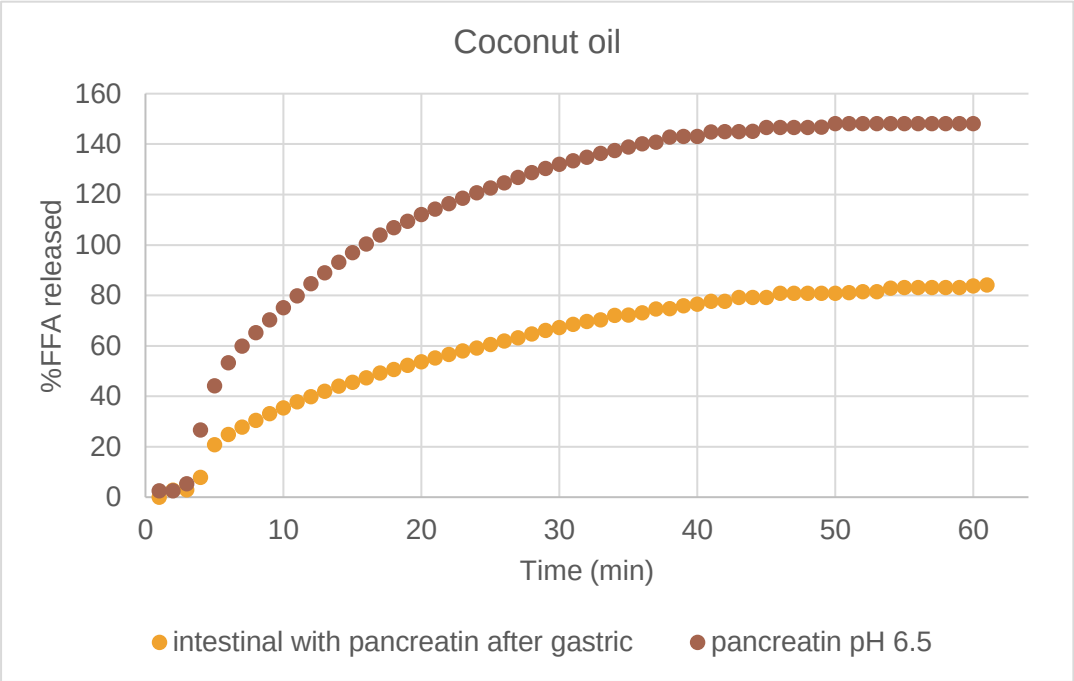


Figure 5.3. pH titration profiles of free fatty acids during the digestion of coconut oil.

Comparing Figure 5.1 and 5.2, the structures presented before the addition of enzyme (t=0min) are same, both of them were in EME phase and no co-existing lamellar phase. Differently, the intensity of EME peak in the emulsion after gastric digestion (Figure 5.1) was higher than the undigested sample (Figure 5.2). This was also observed in other types of oil system. Previous research has studied the nature of the persistent lamellar phase formed during digestion of fat. Calcium ions are naturally present in the digestion solution and are the component in many foods as nutrient, such as dairy product and infant formula. The formation of lamellar phase was identified as the formation of calcium soap. Clulow and co-authors (Clulow et al., 2018) listed the lattice parameter of specific fatty acid calcium soap and suggested the lamellar phase with lattice parameter around 40 Å could be myristic acid (C14:0). The lamellar phase in our results are in agreement with observations made in the literature. Interestingly, the main fatty acid component in coconut oil, lauric acid (C12:0), was absent. This is somewhat in disagreement with the theory that short chain fatty acids tend to be digested faster than long chain fatty acids. The overshooting of free fatty acid released (Figure 5.3) is the evidence of the large amount of short and medium fatty acids naturally in coconut oil. As the digestion proceed,

these fatty acids were quickly released. Also, the oil sample was used without further purification. The impurities in the sample might slightly affect the actual results.

5.2. Hydrolysis of olive oil

The same protocol for SAXS digestion of coconut oil emulsions was applied to samples comprising olive oil. After the gastric digestion of olive oil emulsion, the pH was immediately raised from pH 3.5 to intestinal condition pH 6.5. The intestinal enzyme, pancreatin was then added into the system. As shown in Figure 5.4, there was a peak observed before the addition of enzyme located at q=0.1330 $Å^{-1}$ (again noting that, as with coconut oil samples, this peak was not observed during gastric digestion). The lipolysis progressed steadily after the injection of enzyme shown with continued growth of this pre-existing peak. After 3mins, another two peaks q=0.2552 $Å^{-1}$and q=0.3984 $Å^{-1}$were formed and formed the lamella phase with the first peak. The collapse of lamellar phase started with the disappearing of the last two peak from 42 mins and the intensity of the first peak continuously decreased until the experiment finished. The lamellar phase was the only liquid crystal phase observed in olive oil under intestinal condition. The lattice parameter was around 47.3 Å. Comparing with the literature value of the fatty acid calcium soap, the lamellar phase is more likely be the palmitic acid calcium soap which is one of the main constitutes in olive oil. However, our lattice parameter was slightly higher than the standard.

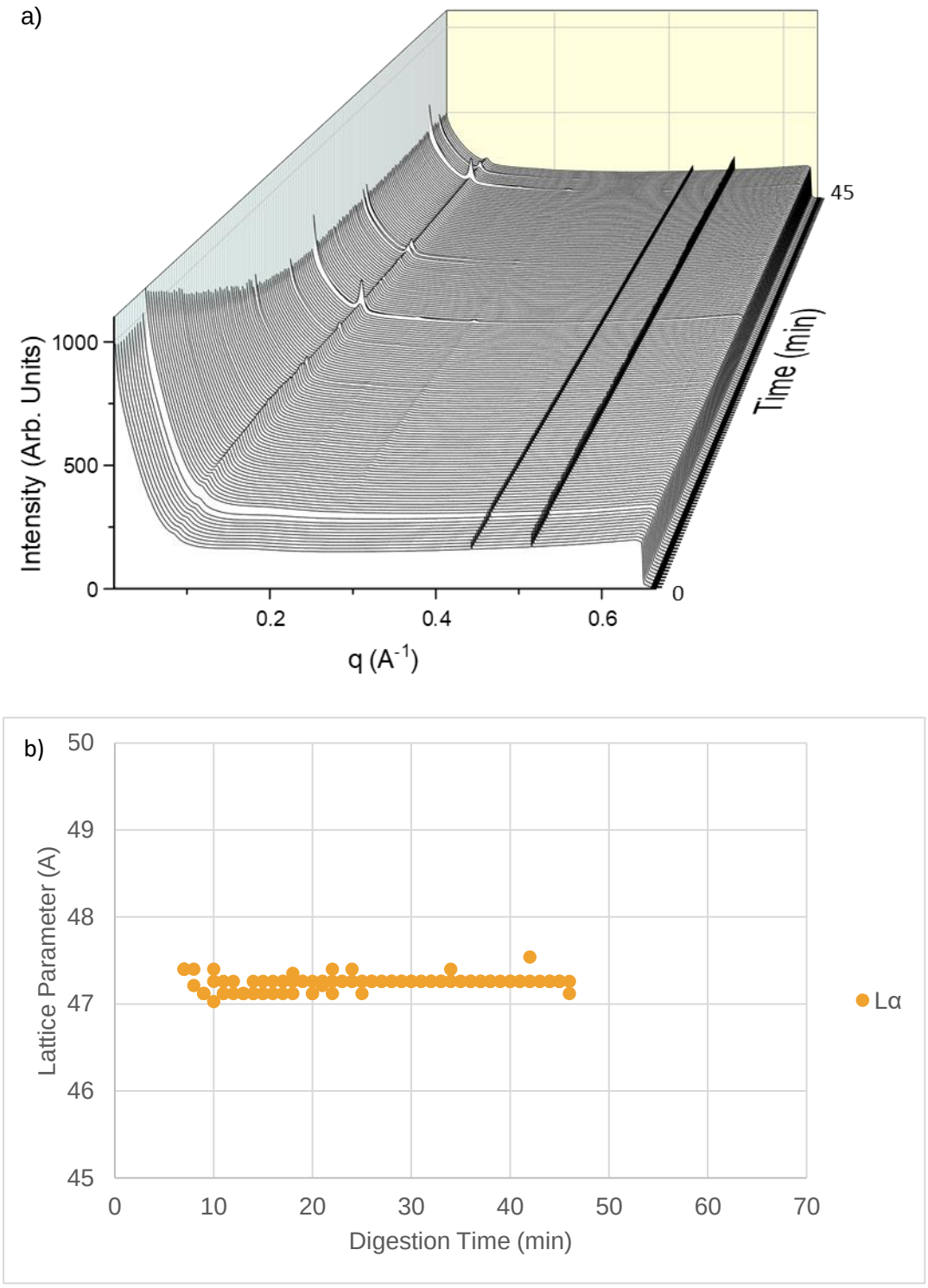


Figure 5.4. SAXS profiles for the digestion of olive oil under intestinal digestion after gastric digestion over 60min at T=37℃ and pH=6.5: a) transition of structures with time of pancreatin action on olive oil and b) Trends in lattice parameters and kinetics of formation of mesophases during intestinal digestion of olive oil.

The intestinal lipolysis of olive oil without pre-gastric digestion is presented in Figure 5.5. The first brag peak q = 0.1353 $Å^{-1}$was formed 3 mins after injection of pancreatin and the other two peaks q= 0.2693 $Å^{-1}$and q= 0.4054 $Å^{-1}$was formed 2 mins after the appearance of the first peak. Thus, the first lamellar phase was formed with lattice parameter 46.53 Å. The lattice parameter suggested this lamellar phase was a palmitic acid calcium soap. The second lamella phase was formed 17 mins after the injection of pancreatin with lattice parameter 52.67 Å, and the three peaks were q= 0.1188 $Å^{-1}$, q=0.3585 $Å^{-1}$ and q= 0.4780 $Å^{-1}$. The fatty acids released under the two digestion conditions was shown in Figure 5.6. The digestion rate was maximized during the first 10mins and 20-30% of fatty acid was released. The results showed more fatty acids were released from the sample without pre-gastric digestion treatment.

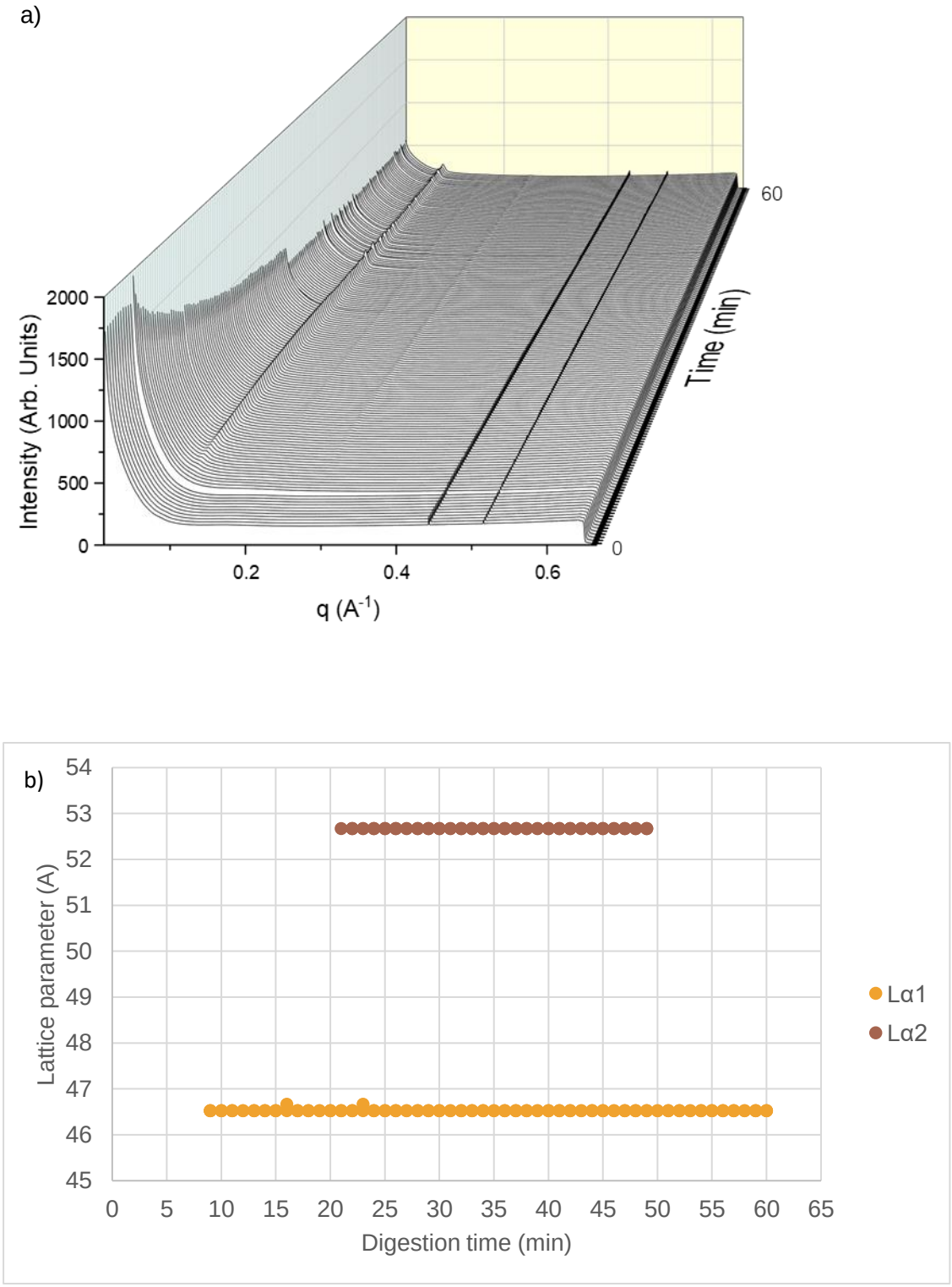


Figure 5.5. SAXS profiles for the digestion of olive oil under intestinal condition with pancreatin only over 60min at T=37℃ and pH=6.5: a) transition of structures with time of pancreatin action on olive oil and b) Trends in lattice parameters and kinetics of formation of mesophases during intestinal digestion of olive oil.

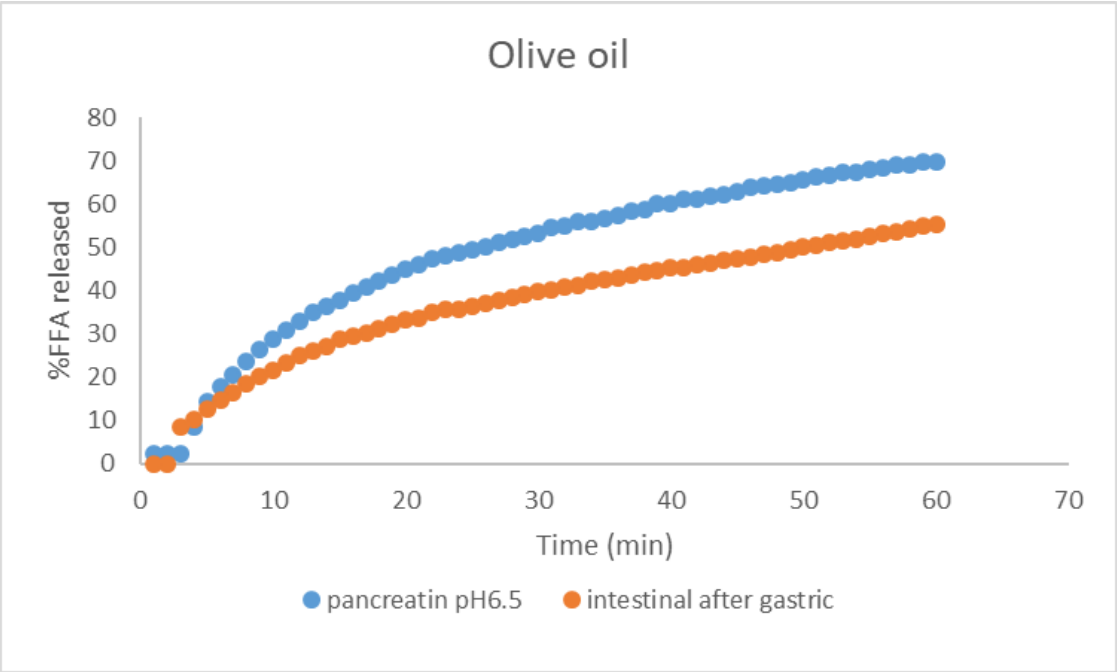


Figure 5.6. pH titration profiles of free fatty acids during the digestion of olive oil.

Figure 5.7 indicates the different observations between complete digestion and incomplete digestion. In contrast with the pre-digested emulsion, the intensity of the final liquid crystal in pancreatin treated emulsion is higher which indicated the crystal structure was more stable.

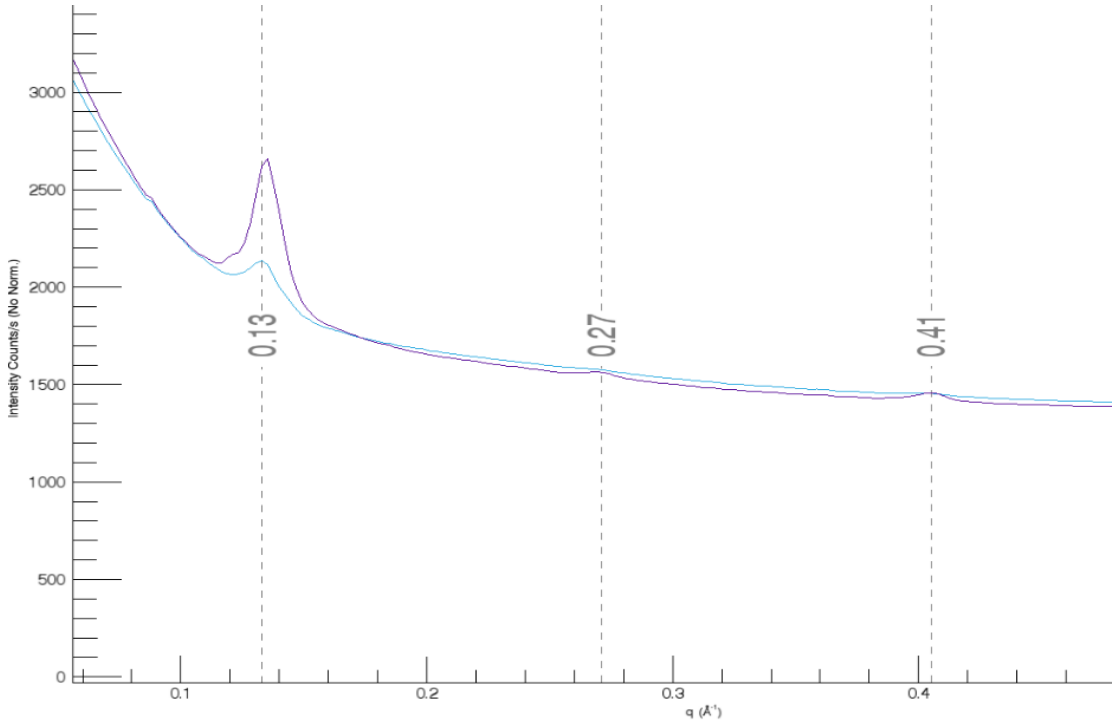
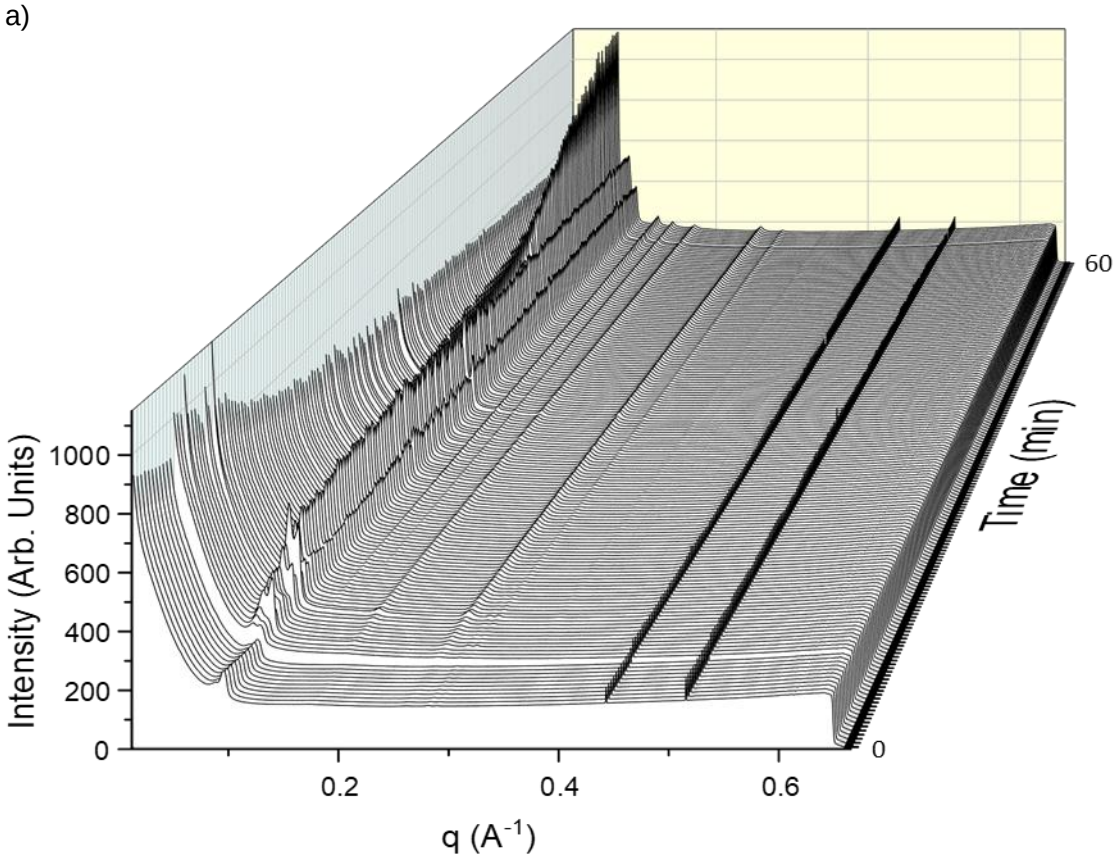


Figure 5.7. SAXS profiles of the final structure of the liquid crystal after digestion of olive oil under intestinal digestion after gastric digestion (blue) and intestinal condition with pancreatin only (purple)

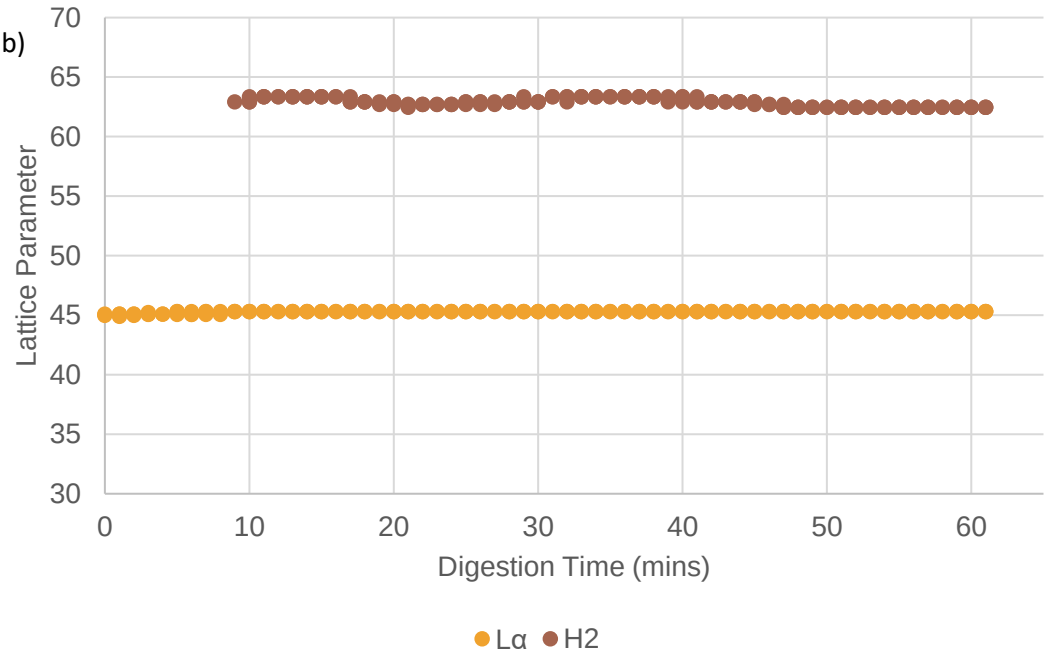
5.3. Hydrolysis of palm stearin

The SAXS profiles of the intestinal digestion of palm stearin are shown in Figure 5.8 and Figure 5.9. In contrasting with olive oil and coconut oil, a distinct lamella phase was presented in the pre-digested emulsion before the injection of pancreatin, brag peaks were located at q=0. $Å^{-1}$, q = 0.276 $Å^{-1}$and q = 0.419 $Å^{-1}$. This suggested liquid crystal was formed during the gastric digestion of palm stearin, as evidenced in Figure 4.1. The lattice parameter of the lamellar phase was 45.3 Å which indicate the formation of palmitic acid calcium soap. As the pancreatin was injected, the lamellar phase was rapidly transitioned into an inverse hexagonal phase with the lattice parameter 63.3 Å. The first peak of H_2 phase q = 0.1165 $Å^{-1}$was formed 3 mins after the injection of enzyme, the rest of the peaks q = 0.2012 $Å^{-1}$and q = 0.2317 $Å^{-1}$were formed 2 mins after the appearance of the first H_2 peak. The intensity of both lamellar and inverse hexagonal phase increased during digestion, however there was a change in the H_2 phase. From 29 mins, a small peak q = 0.124 $Å^{-1}$ was formed near the first H_2 peak q = 0.116 $Å^{-1}$. The intensity of the peak increased rapidly and merged with the q = 0.116 $Å^{-1}$13 mins after its appearance. This could be the internal structure transient of the H_2 phase. There were numbers of unknown peaks present in the SAXS profiles as the spacing between the peaks did not fit the peak distance of a specific crystal structure. Brag peaks q = 0.149 $Å^{-1}$, q = 0.359 $Å^{-1}$ and q = 0.419 $Å^{-1}$ were naturally presented in the emulsion before the injection of enzyme. These peaks could be the residual crystalline triglyceride from the solid frication of palm stearin. Palm stearin comprises predominantly LCT that contain fractions that melt above body temperature, so it is possible that some crystalline di- or triglyceride also remains during the digestion.

a)


Intensity (Arb. Units)
1000
800
600
400
200
0
0.2
0.4
0.6
q (A-1)
Time (min)
60
0

b)


Lattice Parameter
70
65
60
55
50
45
40
35
30
0
10
20
30
40
50
60
Digestion Time (mins)
Lα
H2

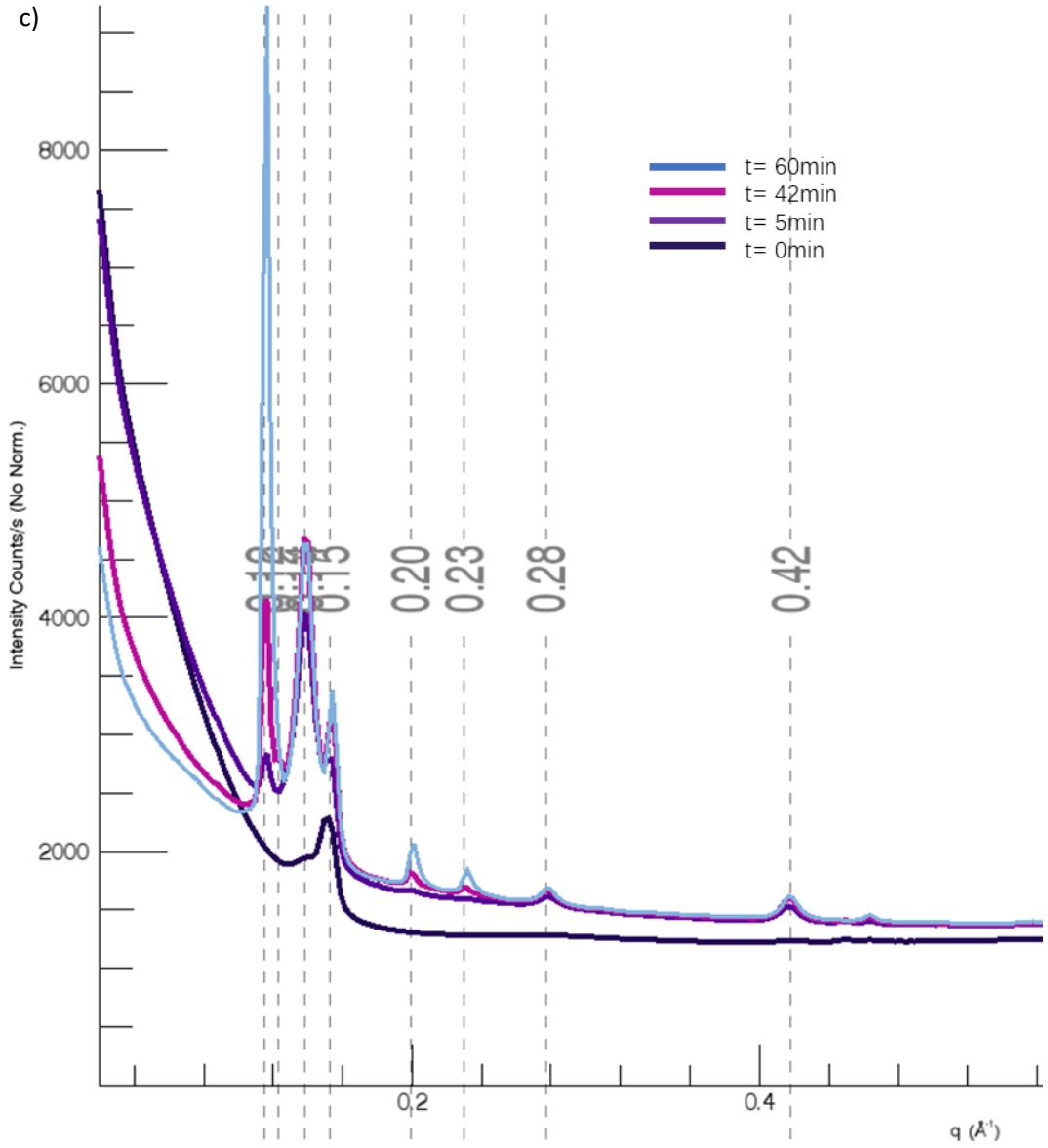
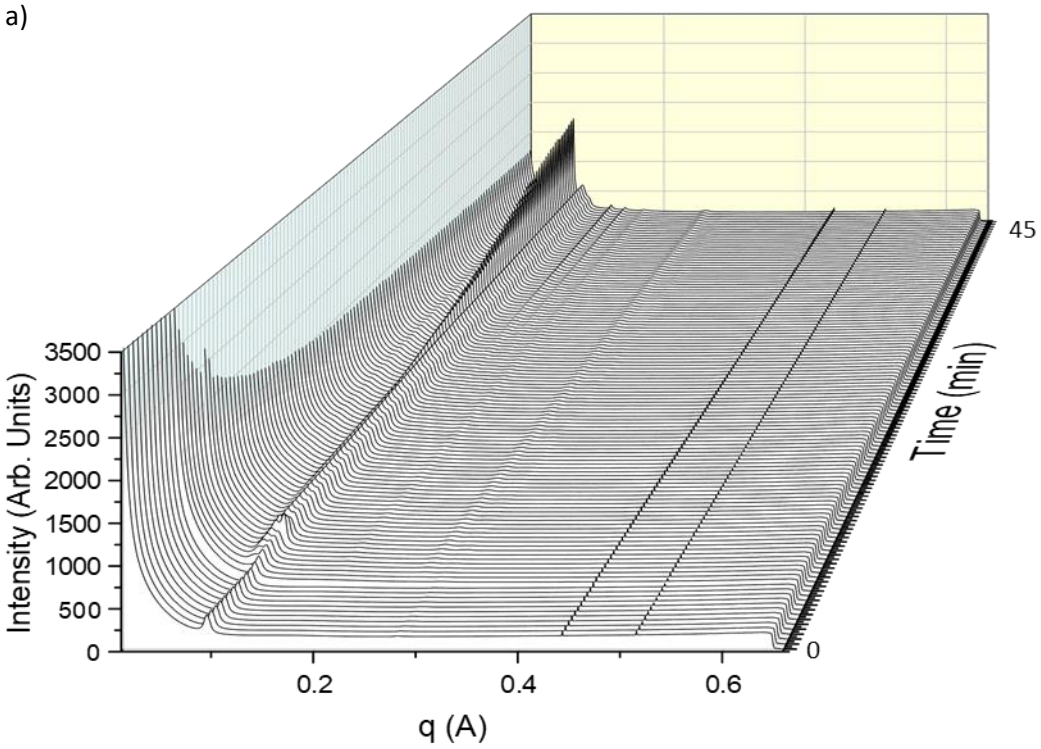


Figure 5.8. SAXS profiles for the digestion of palm stearin under intestinal digestion after gastric digestion over 60min at T=37℃ and pH=6.5: a) transition of structures with time of pancreatin action on palm stearin, b) Trends in lattice parameters and kinetics of formation of mesophases during intestinal digestion of palm stearin and c) Transition of peaks during the digestion.

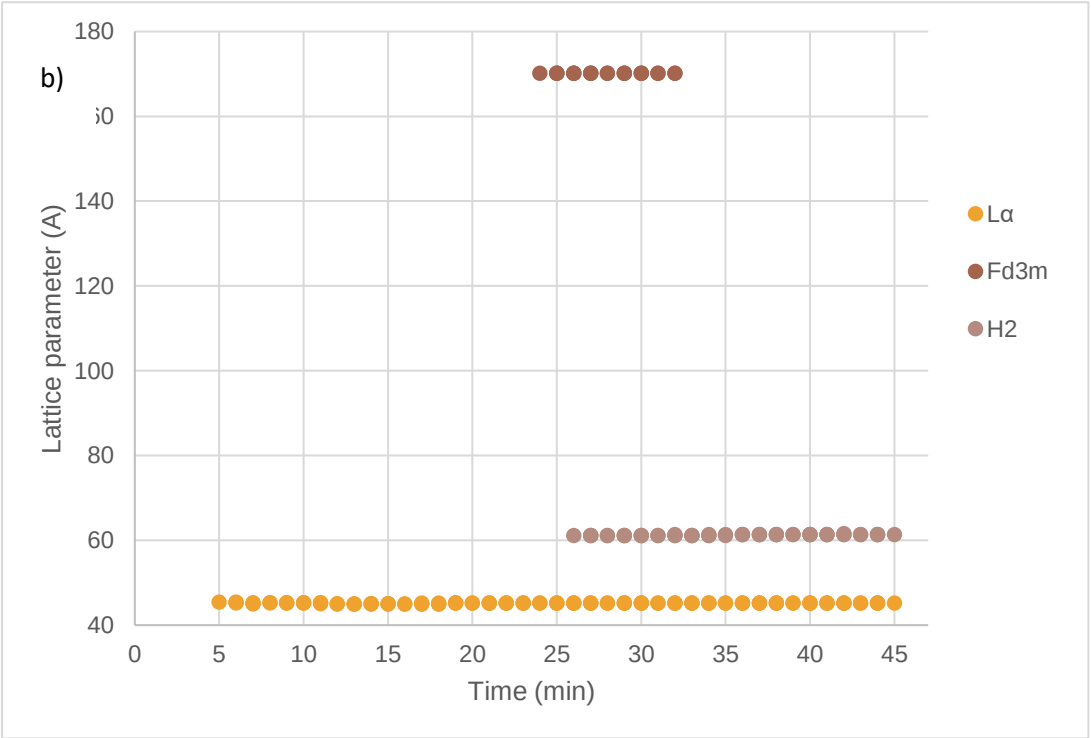
In the palm stearin emulsion directly treated with intestinal digestion (i.e. no prior gastric treatment), the emulsion started with an EME phase (Figure 5.9.). The enzyme action started immediately after the injection of the pancreatin, lamellar phase was formed with the lattice

parameter 45.2 Å, start with the peak q = 0.1377, 0.2787 and 0.4195 $Å^{-1}$. The spacing between the peaks corresponded to the formation of palmitic calcium soap. Further digestion increased the amount of calcium soap and the hydrophilicity of the amphiphilic constituents. 24mins after the digestion started, the lamellar phase was transferred into an ordered *Fd3m* (q = 0.0647, 0.1024 and 0.1236 $Å^{-1}$) type micellar cubic structure with a lattice parameter 170.1 Å. The *Fd3m* phase only lasted for 8mins. During the development of *Fd3m* phase, H_2 phase (q =0.1188, 0.2035 and 0.2364 $Å^{-1}$) was formed 2 mins after the appearance of *Fd3m* phase with the lattice parameter 61.39 $Å^{-1}$. The formation of the inverse hexagonal phase indicated large amount water and hydrophilic molecules were transferred into the lipid phase of the palm stearin, thus the lipid core became more hydrophilic (Salentinig et al., 2013). At the end of the digestion, lamellar and H_2 phase were present together in the emulsion.

a)


Intensity (Arb. Units)
3500
3000
2500
2000
1500
1000
500
0
0.2
0.4
0.6
q (A)
0
45
Time (min)

b)


Lattice parameter (A)
180
160
140
120
100
80
60
40
0
5
10
15
20
25
30
35
40
45
Time (min)
Lα
Fd3m
H2

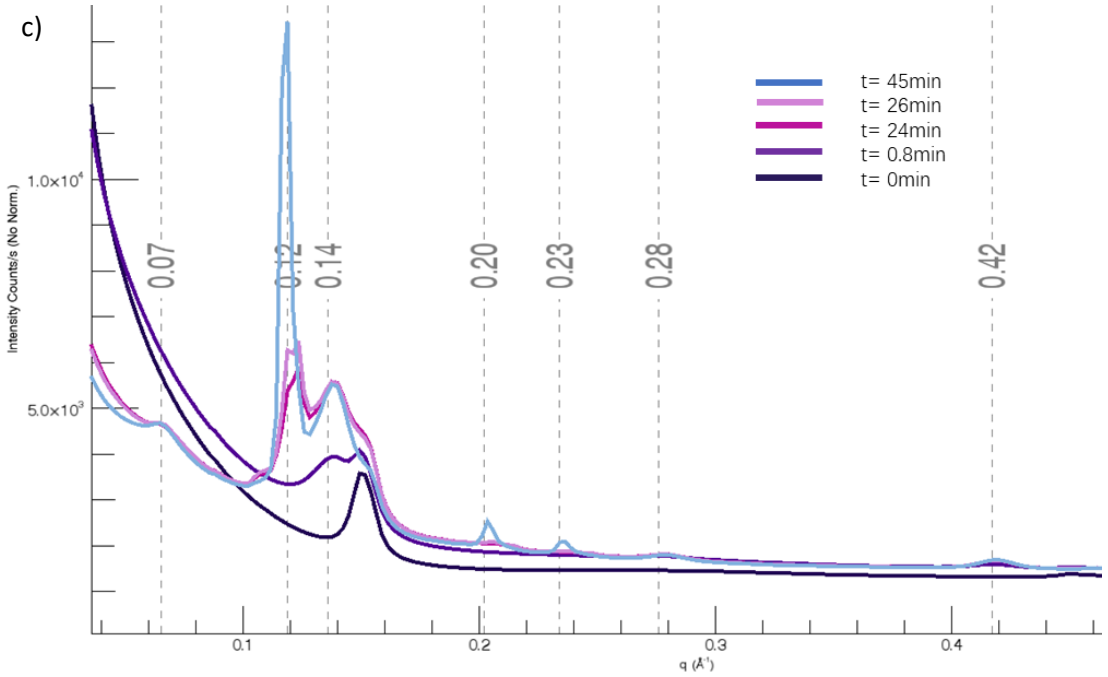


Figure 5.9. SAXS profiles for the digestion of palm stearin under intestinal condition with pancreatin only over 60min at T=37℃ and pH=6.5: a) transition of structures with time of pancreatin action on palm stearin and b) Trends in lattice parameters and kinetics of formation of mesophases during intestinal digestion of palm stearin and c) Transition of peaks during the digestion.

In contrast with olive oil and coconut oil, the digestion rate of palm stearin was maximized during the first 7mins. The digestion rate was closely related with the internal structure transition of oil. The formation of lamellar phase corresponded to the maximized digestion rate, the deceleration lead to the formation of ordered structure, *Fd3m* and H_2 phase. In comparison, the digestion rate of the emulsion pre-digested under gastric condition was stabilized earlier than the emulsion with intestinal digestion only. Thus gastric digestion appeared to enhance the digestion of lipid. As shown in Figure 5.10, there was a slight overshooting of fatty acid released from the incomplete digestion. Comparing with coconut oil, the fatty acid released from the complete digestion of palm stearin was similar to the coconut oil, but the extent level for the incomplete digestion was a lot lower.

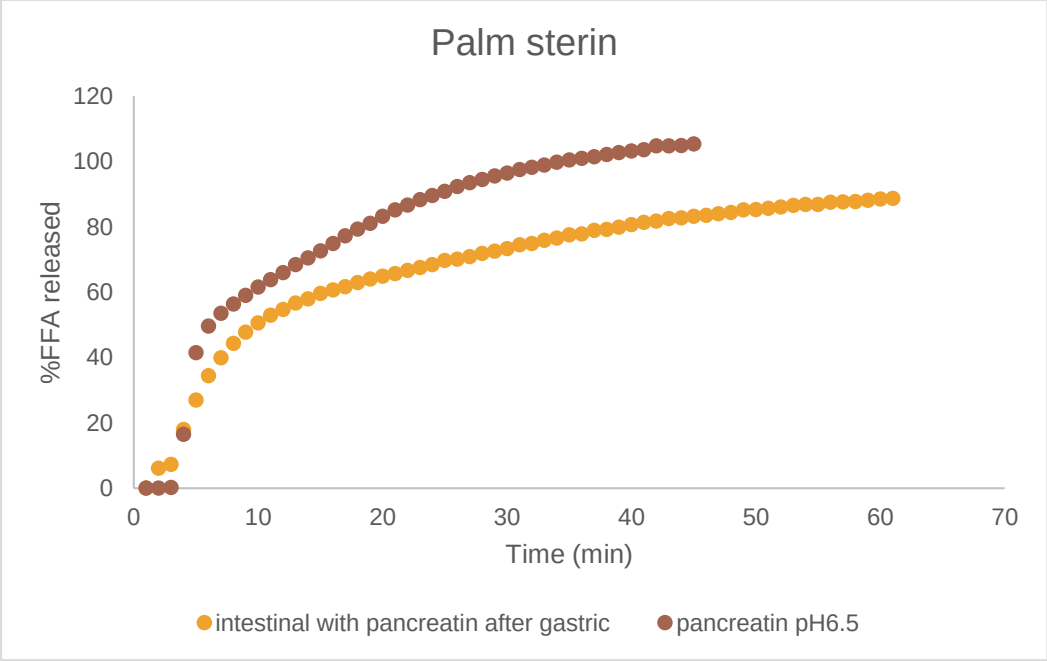


Figure 5.10. pH titration profiles of free fatty acids during the digestion of palm stearin.

4.4. Discussion

This study presents the intestinal lipolysis of different types of oil by pancreatin with SAXS. In all of experiments, lamellar phase remains at the end of the reaction. The final mesophase of liquid crystals after intestinal lipolysis is dependent on the type and chain length of the fat, the released of fatty acids and gastric lipolysis. Olive oil is a typical monounsaturated dominated vegetable oil that contains 55-83% of oleic acid (C18:1), whilst coconut oil is a medium chain triglyceride which is mainly consisted of lauric acid (45-50.3%) and myristic acid (16.8-20.6%)(Gunstone, 2011). Lamellar phase is the only liquid crystalline phase that has been observed in lipids containing predominantly lauric acids, such as coconut, leading to the formation of L2-phase (Lindström, Ljusberg-Wahren, Larsson, & Borgström, 1981; Mandell, Fontell, & Ekwall, 1967; Phan et al., 2013). Our findings also demonstrate the formation of a lamellar phase, but somewhat curiously also demonstrate the emergence of an H_2 phase upon digestion with pancreatin.

The SAXS profiles of lipolysis of olive oil and coconut oil show no big difference between their liquid crystal structures, with the lamellar phase dominant in both emulsion systems on digestion. This could be related to formation of lipolysis products in the absence of bile salts. During the digestion of MCT without bile salt, the lipolytic products are still able to partially disperse into the aqueous phase and create mesophase structures. For LCT, the lipolytic products tend to reside in the oil phase or partition at the interface. Previous studies have demonstrated that bile salts are required for assembly of these more hydrophilic components and to enable their detachment from the oil droplet (MacGregor et al., 1997; Sek et al., 2002). Here, we could make a hypothesis as the bile salt being more important for the digestion of LCT than MCT. To confirm this, the experiment could be repeated with bile salt for further research.

Palm stearin is the only LCT in the study that showed the formation of the cubic and H_2 phases. This is related to its solid fat content and higher levels of saturated triglycerides. Palm stearin is the fractionated solid fraction of palm oil, and which has a higher melting point than unrefined palm fat. This could explain the observation of unknown peaks in the palm stearin emulsions. The reflection of the crystal phase is weak and only partially observed with SAXS. The observation of limited peaks is insufficient to identify the composition of the subphase, however, the location of the observed peaks does indicate the potential of existing crystals, possibly crystalline solid fat material.

During the gastric digestion, the EME phase has been shown to form prior to the addition of pancreatin, and the emulsion with gastric pre-treatment appears to produce more simple crystals during intestinal digestion. The formation of EME phase is because of the formation of crystallizing diglycerides and fatty acids after gastric lipolysis (Salentinig et al., 2011). This is also observed in the interfacial tension experiments where gastric lipolysis destabilizes the o/w interface and decreases the interfacial tension.

Previous studies with light microscopy have shown that the formation of calcium soap during lipid digestion is a more-or-less instantaneous process (J. S. Patton & Carey, 1979). In our result, all the lamellar phases were formed within 5 mins of the injection of pancreatin. According to

Clulow and co-author's (Clulow et al., 2018) study on the lattice parameter of specific fatty acid calcium soaps, all the calcium soaps have been identified. Using this information, for emulsions comprising palm stearin triglycerides, palmitic acid appears as the primary calcium soap for this particular LCT.

In our study the amount of fatty acid released from the small intestinal digestion experiments without prior gastric treatment is typically higher than the small intestinal digestions combined with prior gastric digestion. This suggests that fatty acids have already been produced during the gastric lipolysis (thus depleting the concentration of measurable fatty acids during intestinal digestion). Specifically, in the digestion of olive oil and palm stearin, ~20% more fatty acids are generated during small intestinal digestion without gastric pre-treatment, which broadly fits the physiological observations that 10-30% of lipids are digested in stomach (Golding et al., 2011). It was noted that there was an overshooting of the fatty acid concentrations in the digestion of coconut oil (i.e. recorded FFA levels were seen to exceed 100%). This might be related to the appearance of the H_2 phase. The pH titration profiles have confirmed the theory that the liquid crystal structure is closely related with the amount of fatty acid be released from the digestion and the digestion rate (Phan et al., 2013). During the first 10-15 mins, the digestion rate has been maximized and induces the formation of lamellar phase. We have discovered non-lamellar phase only appears when an excess of fatty acid is released. This agrees with previous research on the digestion of milk, in which the H_2 phase appears after 90% of fatty acid has been released (Clulow et al., 2018). The theory behind the phenomenon is unclear and further experiments are required.

Previous research has shown gastric digestion can assist in the initiation of intestinal lipolysis but ultimately has a limited impact on the final extent of lipolysis (this argument is based on adult physiology, although it is acknowledged that the situation for neonates may be quite different) (Kenmogne et al., 2012). As shown in our interfacial tension measurements with pepsin and lipase during gastric digestion, both enzymes have different consequence for dynamic structures of the protein stabilized o/w interface, and their action on the interface is different. Accordingly, we might expect their effects on the subsequent intestinal lipolysis to

be different as well. In our research, the gastric lipolysis not only affects the onset, it also appears to affect the extent and rate of lipolysis. The SAXS profiles has shown the structure of liquid crystals during small intestinal digestion following gastric digestion formed faster than the for the emulsion systems that only underwent small intestinal digestion. This arbitrarily suggests that the rate of intestinal digestion is accelerated by the gastric digestion. Moreover, the formation of simple liquid crystals in the pre-digested oil emulsions indicates gastric digestion enhance the digestion and adsorption of lipids. Further work is recommended here to confirm this hypothesis.

In contrast to coconut oil, olive oil has slower digestion rate and lower extent of fatty acid synthesis. Here, long chain lipolytic products are more likely to form interfacial mesophase structures rather than aqueous dispersible mesophases that would be generated for the MCT in coconut oil (at least in the absence of bile salts). In addition, the region specificity of lipase also affects the extent of lipolysis. sn-2 monoglycerides are an interfacially active lipolytic products when sn-1,3 regiospecific lipase hydrolyses lipids, and it is more likely formed in LCT. It often expels the enzyme from the interface and limit the lipolysis (P. Reis, K. Holmberg, et al., 2008). Thus, the extent of lipolysis in olive oil is lower than in coconut oil.

Two lamellar phases were observed during small intestine digestion of the olive oil emulsions that had undergone prior gastric digestion. This was similar to the phases observed for the coconut oil emulsions that had undergone only small intestinal digestion. As mentioned before, the first lamellar phases are involved in the formation of calcium soaps. The formation of the second lamellar phase is more complex, and it has not been observed in other lipolysis research. There are two possible reasons for it :1) it could be the crystallization of different chain fatty acids or the conversion between lamellar forms, and 2) the remaining triglycerides are crystallizing during hydrolysis of lipid which result in sharper peaks than the Ca soap peaks. The lattice parameter of the second lamellar phase in olive oil is 52 Å whereas the lattice parameter for coconut oil is 53 Å. According to Andrew's research, lamellar phases with the lattice parameter higher than 50 Å are likely be the oleic acid (C18:1) soap. In our case, olive oil consists of large amount of oleic acid and the peaks of the second lamellar phase are broad

and flat. There is no significant oleic acid fraction naturally present in coconut oil, and the formation of sharp peaks have observed. Therefore, the formation of the second lamellar phase dependent on the nature of the lipid.

In our study, lamellar phase is the only liquid crystal phases has observed in olive oil digestion and is conflict with previous study. To study the structure difference between the liquid crystals in olive oil and coconut oil, cryogenic transmission electron microscopy could be used as the supporting method in the future.

Chapter 6. Conclusions

Insights into the interfacial aspects of lipolysis have been studied *in vitro* in real time by pendant drop tensiometry and SAXS. The effect of pH and emulsifier on the stability of oil interface was confirmed. The dependence between lipolysis and pepsinolysis under gastric conditions indicated that the interactions between enzymes was a major factor for the extent and rate of gastriointestinal digestion. Comparison of the self-assembled structures formed in different emulsion systems after digestion demonstrated the importance of gastric lipolysis to the following intestinal digestion. Vesicles are the dominating phases at the end of the lipid digestion, increasing the solid content of oil favors the transition from vesicles to bicontinuous cubic and hexagonal phase. Although lamellar phase is dominant in both olive oil (LCT) and coconut oil (MCT) emulsion systems on digestion, their responds to the absent of bile salts seems different. On the other hand, the SAXS profile of lipolysis of palm stearin is much more complex due to its extremely high level of solid fat content and saturated triglycerides.

While findings from this study show some interesting phenomena that can be related to the mechanisms responsible for lipid digestion, it is important to consider how this research could be developed further to determine the veracity of these effects. For the interfacial tension and dilatational rheology study, purified oils are suggested to be used for future studies to eliminate errors caused by impurities. The pH of gastric condition varies from pH 1-7 depends on the injection of food, more pH level should be covered for the complete observation of gastric lipolysis. Moreover, bile salts act as the stabilizer on the internal structure of the dispersed particles, and could be included in the future work. Finally, repeat studies using a more physiologically relevant gastric lipase, such as rabbit gastric lipase or recombinant human gastric lipase would be required to ensure that enzyme specific effects such as role of relative pKa and specificity can be taken into consideration.

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Chapter 8. Appendix

Appendix I. Dilatational rheology study of o/w interface at pH 3.5 and pH 4.5.

	Buffer		**WPI+buffer**		**Pepsin+WPI buffer**		**Pepsin+lipase+ WPI buffer**		**Lipase +buffer**		**Lipase +WPI buffer**	
	E_0	**K_s**	**E_0**	**K_s**	**E_0**	**K_s**	**E_0**	**K_s**	**E_0**	**K_s**	**E_0**	**K_s**
pH3.5	7	14.5	5.5	12	5.5	8.5	-	-	-	-	-	-
	8	16.5	10	17.5	18.5	21.5	-	-	-	-	-	-
pH4.5	10	17	11	18	13	20	12.5	20	10.5	17.5	12.5	20.5
	14.5	22.5	17.5	20	17.5	26	13.5	17.5	16	20.5	17	21

E_0= Dilatational elasticity; K_s= Dilatational viscosity