Food-based multiple emulsions as carriers of bioactive compounds: interactions at the interface and physical and chemical changes during \textit{in vitro} digestion

by

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ABSTRACT

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The intent of this study was to evaluate changes on interfacial properties of W₁/O/W₂ systems containing different emulsifiers and bioactive compounds. Interactions of Vitamin D₃ (VitD₃) and Phytosterols at interfaces containing mixtures of PGPR and β-lactoglobulin or sodium caseinate were evaluated. The oil-water interfacial properties were dominated by PGPR, even with the synergistic effect of the proteins to decrease the interfacial tension. These bioactives altered the interfacial properties. Double emulsions supplemented with these compounds resulted in increased short term stability, suggesting an interaction between bioactives with the emulsifiers at the interface. The in vitro digestion of emulsions loaded with multiple bioactives were evaluated in relation to the physico-chemical changes as well as the bioactive release behavior. Gastric digestion did not affect the size distribution of the emulsions, however, most of the Vitamin B₁₂ (VitB₁₂) entrapped in the inner aqueous phase was released during this stage. Phytosterols transfer was well correlated to lipid digestion, while VitD₃ was found to be significantly lower. Different hydrophobic compounds resulted in different transfer behavior being dependant in their partitioning towards the core or the interface of the oil droplet. Changes in the physical state of the oil and water internal phases of W₁/O/W₂ emulsions were evaluated by structuring them with gelation techniques. Similarly, the emulsions were stable during the gastric stage. When oil-fat gel was used, elongated structures (fat crystals) were visible and the droplets were not spherical. Coalescence of the liquid W₁ was observed with no relation to the physical state of the oil phase. W₁/O/W₂ droplets were found up to early stages of duodenal stage. Between 5 and 25 min of duodenal stage, single non spherical oil droplets with crystals present were observed. Emulsions with oil-fat gel lipid phase resulted in higher levels of lipid digestion and bioactive transfer. The presence of medium chain fatty acids and the surface-to-core distribution of the hydrophobic bioactive influenced the lipid digestibility and bioaccessibility of the hydrophobic compounds. Gelling the inner aqueous phase revealed lower values of VitB₁₂ released in the gastric stage and it was driven by the aqueous gel properties.
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Chapter 1: General Introduction and Objectives

Consumers are increasingly aware of the relationship between diet and health. As a result, there has been an intensified demand for health enhancing food products, which may contain natural bioactive molecules linked to the prevention or treatment of diseases such as vitamins, phytochemicals, pigments, antioxidants, lipids, peptides, minerals, etc. Unfortunately, these compounds are usually prone to rapid degradation or deactivation during processing, storage, pH changes and enzyme actions in the digestive tract among other reasons. Therefore, to ensure that they remain intact and active, encapsulation processes are often suggested as a solution to prevent degradation and unwanted reactions (De Vos et al. 2010).

One of the solutions to the delivery of bioactive compounds through the diet is their encapsulation in double emulsion matrices. Double emulsions consist of a water-in-oil (W/O) emulsion droplets dispersed in a secondary aqueous phase (Garti 1997). They have been successfully applied to wastewater treatment (Boyadzhiev et al. 1984), as filtration systems (Palencia & Rivas 2011), and as delivery systems in pharmaceutical and cosmetic products over the years (Marti-Mestres & Nielloud 2002). Also, an interest has risen in food applications, and studies have been published in relation to fortification of cheese with vitamins (Giroux et al. 2013), encapsulation of microorganisms (Pimentel-González et al. 2009), peptides (Giroux et al. 2016), vitamins and minerals (Benichou et al. 2007; Bonnet et al. 2009; O’Regan & Mulvihill 2010), modulation of sensory properties and foods with reduced fat content (Cofrades et al. 2013; Rodri et al. 2008; Muschiolik 2007). However, despite the promising studies, the use of double emulsion in food products is not widespread, in part because of instability issues, especially during processing stages.
Water-in-oil-in-water (W1/O/W2) emulsions are produced with a two-step procedure. In the first step, a simple W1/O emulsion is prepared, for example, by high pressure homogenization, to obtain small water droplets in a continuous oil phase. Subsequently, this emulsion is homogenized with a second aqueous phase using milder conditions, to obtain larger droplets containing droplets within. A primary W1/O stable emulsion is essential to avoid inner droplet coalescence and uncontrolled release of the encapsulated material, this being one of the worst problems faced by researchers. The presence of two aqueous phases separated by an oil layer make the system suitable to carry simultaneously, hydrophilic and hydrophobic substances. Also, it is important to mention that W1/O and O/W2 interfacial properties (charge, permeability, thickness, rheology and response to pH, ionic strength, temperature, etc.) play a significant role in the stability of the system, and can be controlled by the selection of the emulsifiers to be used (Sapei et al. 2012; Dickinson 2011; McClements & Li 2010b). For many years milk proteins have been used to stabilize emulsions for food applications. They are known to adsorb to the oil-water interfaces rapidly, producing both electrostatic and steric stabilization effects (Dickinson 2006b). Efforts have been carried out to improve steric repulsion at the interface of the droplets and this is particularly relevant when designing double emulsion systems (Bouyer et al. 2012; Lutz et al. 2009; Su et al. 2006; Fechner et al. 2007; Dickinson 2006a).

For stabilization of the inner aqueous droplets, especially for food applications, polyglycerol polyricinoleate (PGPR) is still the best choice of lipophilic emulsifier. PGPR is an extremely efficient synthetic emulsifier that is recognized as safe for human consumption (Márquez et al. 2010; Food and Drug Administration 2006). However, the Food and Drug Administration (FDA) (2006) clearly states that the use of PGPR is allowed only in certain food products (i.e. chocolate) and its presence has to be declared on the
label. Therefore, strategies to reduce the use of PGPR while keeping the desired stability of emulsions have been the subject of study of research groups around the globe.

Studies have shown that there are interactions between PGPR, other emulsifiers and biopolymers that may improve the stability of the inner phase and enhance the entrapment efficiency of the double emulsion, making possible a reduction in the amount of PGPR used (Gülseren & Corredig 2014; Gülseren & Corredig 2012; Rodriguez Patino & Pilosof 2011; Gromer et al. 2009; Muschiolik et al. 2006; Su et al. 2006; Garti & Benichou 2004; Dickinson 2003).

Structuring of the internal layers may also be an alternative for improvement of stability (Mao & Miao 2015). As an example, gelation of the inner aqueous phase has shown good results in improving the effects of swelling/shrinking of the inner aqueous droplets due to differences in the osmotic balance also improving their resistance to coalescence (Oppermann et al. 2015; Iqbal et al. 2013; Sapei et al. 2012; O’Regan & Mulvihill 2010; Surh et al. 2007). Furthermore, a common way to structure the oil phase is using solid fats and heat treatment, being possible to obtain W/O emulsion gels for example (Patel 2015).

Although advances have been done in achieving formulations resulting in more stable, food grade, double emulsions, reports on stability and breakdown during gastrointestinal transit are still scarce. Recently, there have been studies showing the in vitro digestion behavior of single O/W emulsions (Eldemnawy et al. 2015; Zhang et al. 2015; Malaki Nik, Corredig & Wright 2011) while little work has been reported on digestion of multiple emulsions (Kaimainen et al. 2015; Frank et al. 2012). The methodologies used tried to mimic physiological conditions; however, the broad range of research questions can lead to drastic differences in enzyme activities, ion concentrations, digestion time and other parameters and methodologies that lead to answers that are not
often comparable between research groups. Recently, an international network of experts published an international protocol with the objective of harmonizing an *in vitro* static digestion methodology (Minekus et al. 2014). This protocol describes parameters and guidelines to improve the quality of results amongst the research groups. Based on these guidelines, we are now able to obtain data comparable between research groups.

The present research was intended to evaluate the changes in properties of interfaces adsorbed by different emulsifiers and bioactive compounds, the emulsion itself, the microstructure breakdown, the bioactive release profile and *in vitro* digestion behavior of formulations of double emulsions, as well as the influence of the physical state of the internal phases of the double emulsion on the digestion behavior. Many variables can affect the stability of the system and also its digestion behavior such as, particle size, osmotic pressure difference between the aqueous phases, interactions at the interfaces, enzymatic activities employed, etc.

It was hypothesized that, in multiple emulsions, the interactions between the molecules present at the interface and the hydrophobic bioactives, as well as the physical state of the interface, may be important to the interfacial properties and to the controlled release of the loaded compounds. These aspects may have an impact on bioaccessibility of the bioactives during digestion, depending on the digestibility of the emulsion and the incorporation of the bioactive into mixed micelles during digestion. Very little is known about such interactions and their influence on the release of fatty acids in the duodenum and the aspects involving the transfer of bioactives.

The first part of this research consisted of an in depth study of oil-water interfaces containing different emulsifiers using drop tensiometry. In addition to that, Vitamin D3 and Phytosterols were added to the oil phase, to observe possible interactions with the
emulsifiers tested. The interfacial viscoelastic properties were evaluated, and related to the stability and encapsulation efficiency of a W\textsubscript{1}/O/W\textsubscript{2} formulation.

The results from the first part led to the study of interfacial composition on the \textit{in vitro} digestion and bioaccessibility of model bioactive compounds. The structure of the double emulsion was characterized, as well as the short term stability, encapsulation efficiency, microstructure breakdown, and the \textit{in vitro} digestion behavior was compared to that of single emulsion. In particular, the lipid digestion and release kinetics of the hydrophobic bioactives were measured for double emulsions and compared to single emulsions containing the same emulsifiers.

In the last part of this research, the focus was the effect of structuring double emulsions by means of gelation of the inner aqueous and the oil phases on their digestion behaviour. The breakdown of formulations with gelled oil-water internal phases was followed during \textit{in vitro} digestion. Liquid and gelled physical states were compared, and the differences in the release of model bioactive molecules, namely Vitamin B12, Vitamin D3 and Phytosterols were evaluated.

Overall, this research allowed a better understanding of features regarding the formulation, structuring, breakdown and transfer of entrapped compounds with different formulations. It was possible to determine the aspects involving the interfacial properties and the effects of the physical state of the internal phases in a complex system with presence of a synthetic emulsifier. The findings obtained provide fundamental information to research scientists and product developers regarding a food grade delivery system intended for oral administration. By fine tuning aspects related to the structure of water-in-oil-in-water emulsion, it might be possible to create a novel food based delivery system and even control lipolysis and bioactive transfer rates of entrapped compounds over time.
Therefore, this thesis had the following objectives:

- To study the interactions of selected hydrophobic compounds at interfaces containing mixtures of PGPR and milk proteins as emulsifiers and evaluate the physico-chemical properties of \( W_1/O \) primary emulsions and \( W_1/O/W_2 \) emulsions stabilized with PGPR and sodium caseinate, loaded with Vitamin D\(_3\) and Phytosterols at the oil phase.

- To formulate a \( W_1/O/W_2 \) emulsion encapsulating Vitamin B\(_{12}\) as the hydrophilic, with Vitamin D\(_3\) and Phytosterols as hydrophobic model bioactives molecules and to investigate their \textit{in vitro} digestion, bioactive release behaviour, and structural changes during the process.

- To evaluate if the physical state of the internal phases of the \( W_1/O/W_2 \) emulsion would affect the lipid digestion, the release and bioaccessibility of bioactives and the microstructural breakdown during \textit{in vitro} digestion.
Chapter 2: Literature Review

2.1. Emulsions as delivery systems

A great number of people are suffering from chronic diseases such as diabetes, cardiovascular diseases and obesity, while specialists look for different ways to target those problems by diet-based approaches. Natural substances with biological activity, such as vitamins, pigments, sterols and fatty acids are very susceptible to degradation when exposed to light, heat, shear, oxygen, pH changes and enzymes. These molecules can provide health benefits against heart disease, improve bone health and immune response, contribute to lower cholesterol levels, but due to their particular nature, the incorporation into food matrices is a challenge due to low water-solubility, poor chemical stability and low bioavailability (that is, low concentration of the compound reaches the systemic circulation after cell absorption). In addition, the processing of the food product and its digestion within the body can easily degrade these components. Therefore, it is imperative to protect these substances to avoid unwanted reactions while formulating and processing a food product to ensure the delivery of the molecule in its active form after degradation of the matrix in the digestive tract (Mao & Miao 2015; McClements & Li 2010b).

In this regard, emulsion-based delivery systems are suitable for encapsulation and protection of active ingredients. Emulsions have been used as delivery systems for a long time, especially in health care products and pharmaceuticals. The different available formulations have been used to carry drugs, vaccines, diagnostic agents, etc., however, there are still gaps-in-knowledge that challenge researchers working with these systems intended for oral administration (McClements 2010).
Double emulsions, in particular, have a significant potential in many food applications. The active components dissolved in the internal aqueous phase are entrapped within a lipid environment. It was previously reported that the release of the loaded active compound should occur slowly and could possibly work as a targetable delivery system, providing a sustained-release system with controlled transport mechanism (Garti & Benichou 2004; Davis & Walker 1987). In addition, double emulsions for food applications have been reported to aid in the reduction of the oil content of food products, assist with fortification with nutrients, probiotics and modulate sensory aspects of a food (Giroux et al. 2016; Giroux et al. 2013; Cofrades et al. 2013; O’Regan & Mulvihill 2010; Bonnet et al. 2009; Pimentel-González et al. 2009; Benichou et al. 2007; Muschiolik 2007). However, there are some drawbacks to formulating stable double emulsions for food and there are not many reports in the literature in relation to the microstructure breakdown and kinetics of release when the system is subjected to gastro-intestinal simulated conditions.

2.2. Emulsions and interfaces

Emulsions consist of two immiscible liquids (i.e. oil, water) in form of droplets of one liquid dispersed in a continuous phase of the other (McClements 2016). Usually, devices such as high pressure valve homogenizers are used to produce high energy disruptive forces, creating small droplets. When a new droplet is formed, an interfacial layer between the phases is created, however, if only oil and water are homogenized, the droplets tend to collide with each other, merge and eventually separate the system into a heterogeneous aspect. The contact of oil and water molecules is thermodynamically unfavorable and to keep the droplets apart from each other, the addition of substances capable of dissipating the energy excess at the interface is necessary (Walstra & Vliet 2008).
A surfactant or emulsifier is a surface-active stabilizer molecule of amphiphilic nature that adsorbs to the oil-water boundary layer at the surface of the droplets, lowering the interfacial tension and possibly preventing aggregation of the droplets. Therefore, different properties of the interfacial layer, such as charge and thickness, are changed after the absorption of the surfactant. These changes influence the stability of the newly formed droplet due to electrostatic and steric interactions, respectively (McClements 2016).

There are different types of emulsifiers and their hydrophile-lipophile balance (HLB) is an important indicator of their solubility in oil or water. A more hydrophilic emulsifier has HLB value higher than 7, while an HLB lower than 7 indicates the emulsifier is more lipophilic. (Walstra & Vliet 2008; Weiss & Muschiolik 2007).

Emulsifiers can be small molecules that are classified according to the nature of the hydrophilic part. They can be non-ionic (i.e. monoacylglycerols, sorbitan monostearate, hexadecanol), anionic (i.e. sodium oleate, lactic acid esters, phospholipids - lecithin, sodium dodecyl sulphate) or cationic (detergents, not common in foods). Large molecules such as proteins and other polymers can also be emulsifiers (Walstra & Vliet 2008).

Depending on the type of emulsifier molecule used, the distribution of the oil and aqueous phases can be altered and emulsions classified as oil-in-water (O/W) or water-in-oil (W/O). In the first case, when the oil droplets are dispersed in the aqueous phase as in food products such mayonnaise, milk, cream, salad dressings, a more hydrophilic emulsifier is used, while the latter refers to the opposite. Water droplets dispersed in the oil phase using an hydrophobic emulsifier as in margarine, butter and dairy spreads for example (Walstra & Vliet 2008; McClements 2016). In addition, it is also possible to prepare multiple or double emulsions. They consist of two emulsions, one inside the other, that is, a large globule of a single emulsion dispersed in a secondary phase, for example, oil-in-water-in-oil (O₁/W/O₂) or water-in-oil-in-water (W₁/O/W₂) emulsions. Double
emulsions can be prepared using two emulsification steps, the first to homogenize the first emulsion while the second step, with mild conditions, to homogenize large droplets of the primary emulsion into the secondary phase.

Milk proteins are often employed to stabilize oil droplets in O/W emulsions. In particular, the major caseins present in sodium caseinate, $\alpha_s$- and $\beta$- caseins, tend to adsorb at oil-water interfaces very quickly and their hydrophilic portions contribute to steric and electric stabilization of the oil droplets, preventing coalescence (Dickinson 2006a; Singh 2011). Milk whey proteins are also often used in food emulsions. For example, $\beta$-lactoglobulin (constituting about 80% of the total whey proteins) is known for its capacity of decreasing the interfacial tension and stabilizing oil droplets by electrostatic repulsion (Bouyer et al. 2012; Bouyer et al. 2011).

Regarding hydrophobic emulsifier, PGPR has been shown to be the most effective food-grade synthetic emulsifier (Wilson et al. 1998). It contains a long hydrophilic polyglycerol head group with high water bonding capacity. It is interesterified with of ricinoleic acid providing good steric repulsion properties. It has been reported that as more of this emulsifier is added in an emulsion preparation, droplets will be smaller and the emulsion more stable (Márquez et al. 2010; Mun et al. 2010; Fechner et al. 2007; Su et al. 2006). The structure of PGPR is represented in Figure 2.1.
Figure 2.1. Structure of the low HLB emulsifier polyglycerol-polyricinoleate (Food and Drugs Administration 2008).

In a hypothetical O/W system containing a mixture of anionic surfactant and protein, if the concentration of anionic surfactant is large enough and is capable of decreasing the interfacial tension more than the protein, competitive adsorption can happen. Ultimately, it can result in displacement of the protein from the interface, which would then be fully adsorbed by the anionic surfactant. This happens in many foods that naturally contain fatty acids, monoacylglycerols, phospholipids and other small surface active molecules that can modify the interfacial properties (Walstra & Vliet 2008).

Interfaces containing mixed emulsifiers can also enhance the stability of emulsions, especially the double emulsions (Dickinson 2011). Mixed interfaces containing biopolymers (proteins, polysaccharides) and other emulsifiers have been reported to improve the film formation over the water and oil phase (Garti 1997) and the steric repulsion at the interface of the droplets, which is extremely important when designing double emulsion systems (Bouyer et al. 2012; Lutz et al. 2009; Su et al. 2006; Fechner et al. 2007; Dickinson 2006a).

The behaviour of protein-surfactant mixtures at fluid interfaces has been reviewed with respect to the type of protein, the surfactant, the type of interface, the interfacial
composition and the interactions between them, among other factors (Maldonado-Valderrama & Patino 2010). The study of interactions of emulsifiers and biopolymers has been carried out to understand the viscoelastic properties of those interfaces (Bouyer et al. 2012; Murray 2002), kinetics of adsorption, desorption and the molecular changes of the surface active compounds (Ravera et al. 2010; Benjamins et al. 1996).

Protein and polysaccharide interactions were investigated recently. A complex between sodium caseinate and carboxymethylcellulose formed by electrostatic interactions revealed significant changes in the characteristics of the oil-water interface. Carboxymethylcellulose was found to form a stronger viscoelastic layer at the interface, which is believed to improve the stabilization of emulsions containing these ingredients in their formulation due to increase the viscosity of the continuous phase and enhanced steric and electrostatic repulsion between emulsion droplets (Liu et al. 2016).

The interactions between milk proteins and synthetic emulsifier PGPR was also studied. Gülseren and Corredig (2012) revealed that both β-lactoglobulin and sodium caseinate have synergistic effect on reducing the interfacial tension when present simultaneously with PGPR at the interface. Moreover, the elastic properties of PGPR were minimally affected by addition of high levels of protein, suggesting that PGPR properties dominates the interface. The same authors published about the interactions between PGPR and polysaccharides. They found that pectin extracted from sugar beet was surface active and observed a further decrease in the interfacial tension of the water-oil interface when PGPR was present. In addition, PGPR together with sugar beet pectin or high methoxyl pectin formed a less elastic interfacial film. These results reflected an improved stability of W/O emulsions containing low levels of polysaccharide solubilized in the aqueous phase (Gülseren & Corredig 2014).
There are few studies in the literature relating interactions between bioactive molecules and proteins. The tea polyphenol epigallocatechin-3-gallate (EGCG) for example, was shown to not significantly change the interfacial tension of air-water interface when complexed with human saliva proteins, although it increased the elasticity of the interface (Rossetti et al. 2013; Rossetti et al. 2008). EGCG at the oil-water interface also did not significantly affect interfacial tension, but the association of EGCG with β-lactoglobulin was found to decrease the extent of protein-protein interactions at the interface, therefore impairing the creation of a more rigid interface (Lestringant et al. 2014). Although the molecule does not seem to be present at the interface, it seems to influence the physical and rheological characteristics of the interface.

The presence of the different compounds mentioned above in the bulk liquids interfere on physicochemical characteristics of the double emulsion system which may lead to improvements in the stability of the inner phase and enhancement of the entrapment efficiency (Massel et al. 2015; Gülseren & Corredig 2014; Massel 2011; Rodriguez Patino & Pilosof 2011; Muschiolik et al. 2006; Su et al. 2006; Dickinson 2003). Therefore, characteristics of oil-water interfaces containing PGPR, milk proteins (and their mixtures) and the changes on viscoelastic properties caused by addition of model hydrophobic bioactives (Vitamin D3 and Phytosterols) are reported further in this thesis, and the changes observed were related with stability, encapsulation efficiency, and digestion of $W_1/O/W_2$ emulsion system.

### 2.3. Emulsion stability issues

Physical or chemical aspects influence the way emulsions remain homogeneous. The ability of an emulsion to resist changes over time is what defines its stability, and
understanding the correct mechanism of droplet disruption in a particular formulation is necessary prior to determining the most effective strategy to improve the emulsion integrity (McClements 2016).

Differences in density between the oil and aqueous phases can lead to gravitational separation, that is, creaming or sedimentation, which is very noticeable and undesirable. The velocity of creaming of spherical shaped droplets dispersed in liquids is dependent on the radius of the droplet and the density difference between the dispersed and continuous phases, and it is described by Stoke’s Law:

\[ \nu = \frac{\alpha \Delta \rho d^2}{18 \eta} \]

where:
- \( \nu \) = velocity of creaming;
- \( \alpha \) = acceleration;
- \( \Delta \rho \) = difference in density between the aqueous and oil phases;
- \( \eta \) = viscosity of the continuous phase;
- \( d \) = particle diameter.

To avoid creaming or sedimentation, smaller droplets are desirable. During homogenization, by re-submitting the coarse emulsion to several homogenization steps, it is possible to decrease the size of the droplets and influence the rate of creaming or sedimentation of the system. Another option is the addition of ingredients that contribute to the density contrast among the liquids, such as weighting agents, thereby increasing the viscosity of the continuous phase (McClements 2016; Walstra & Vliet 2008).

A schematic diagram of the various mechanisms which would lead to destabilization and creaming of an emulsion is shown in Figure 2.2.
Droplet aggregation processes such as flocculation and coalescence can also increase the rate of creaming. Aggregation happens due to constant motion of the droplets and as they move, they frequently collide with their neighbours. If two or more droplets merge together, coalescence occur, forming a single larger droplet (McClements 2016).

On the other hand, after a collision, if the droplets stick together but retain their structure integrity, they form flocs. Flocculation may or may not be reversible. In the first instance, taking as an example an oil-water interface adsorbed with protein, if there are not
sufficient protein to fully cover the interface, the protein layers that are long enough to crosslink multiple droplets together can produce reversible adsorption, leading to a bridge between the droplets, characterizing bridging flocculation. If there are macromolecules such as nanoparticles, carbohydrates, protein aggregates or other non-adsorbed molecules present in the dispersed phase, a high osmotic effect between the droplets can happen, inducing an irreversible aggregation, process called depletion flocculation (Blijdenstein et al. 2004; Jenkins & Snowden 1996). In sodium caseinate stabilized O/W emulsions for example, factors such as the presence of ethanol, calcium ions, surfactant micelles, changes on pH and ionic strength are factors that are known to induce depletion flocculation of the droplets. These factors reduce the net charge on the adsorbed protein and induce loss of electrostatic stabilization and collapse of the charged protein tails that contribute to steric stabilization, leading to the formation of large sized flocs (Dickinson 2006b).

If the system is unstable due to flocculation, the longer time that the droplets are in close proximity, the greater the chance of coalescence occurring (McClements 2016).

Another type of aggregation that leads to an increase in droplet size is the Ostwald ripening. It is more common in W/O emulsions and it happens when the droplets are polydisperse. The difference in chemical potential between the droplets due to their different sizes leads the dispersed phase molecules to diffuse from the interface of the smaller droplets to the continuous phase. Subsequently these molecules are adsorbed at the interface of the larger droplets. As a result, smaller droplets population starts to decrease, while the large droplets become larger, reducing their interfacial area (Jiao & Burgess 2003; Taylor 1995). It has been shown that the addition of electrolytes into the aqueous phase of W/O has a major effect on increasing resistance of the oil film to coalesce by increasing the adsorption density and lowering the interfacial tension of the film (Márquez et al. 2010; Aronson & Petko 1993).
2.3.1. **Double emulsion design and structuring**

The stability issues mentioned for single emulsions can also be applied to double emulsion systems. Double emulsions have significant potential in many food applications, as the internal droplets can work as a reservoir for active ingredients that can be protected from the outer layer. However, there have been barriers to their utilization in foods, mostly because of their limited physical stability. In fact, the problems are even a larger issue in $W_1/O/W_2$ emulsions, where both primary and secondary phases are susceptible to physical instability. The inner aqueous droplet can coalesce within the oil globule without changing the outer interface. The droplet can leak the entrapped material to the external aqueous phase. It can also shrink or swell due to diffusion of entrapped material to the external phase; furthermore the oil droplets can coalesce just the same way as regular $O/W$ emulsions (Dickinson 2011; Garti & Benichou 2004).

It has been reported that the transfer of electrolytes entrapped in the inner aqueous phase to the outer phase is inevitable while using monomeric surfactants at the inner interface even with stable double emulsions with balanced osmotic pressure (Benichou et al. 2004). On the other hand, there is an agreement that the addition of electrolytes to the inner phase of PGPR stabilized $W_1/O/W_2$ emulsions controls the osmotic balance between the aqueous phases, which also can control the shrinkage or swelling of the inner droplet (Leal-Calderon et al. 2012; Muschiolik 2007).

Another alternative to control the stability of double emulsion is to structure the system, for example, by incorporating gelling agents to the emulsion formulation. Efforts have been carried out to better design double emulsion structures and to understand the physicochemical changes over environmental, processing and digestion conditions to
improve the delivery of the entrapped compound (Mao & Miao 2015). In the case of food emulsions, the design is influenced by several different factors including the type of bioactive to be delivered, type of lipid, salt concentration, type of emulsifiers, physical state of the bulk liquid, environment conditions where the emulsion will be submitted to, etc.

With regard to the inner aqueous phase, structuring it by means of gelation is a widely known technique that has been applied with improvements on emulsion stability, water holding capacity (also named as yield) and entrapment efficiency of loaded compounds. Different agents have been used, such as gelatin, whey proteins, starch, pectin, among others (Balcaen et al. 2016; Oppermann et al. 2015; Iqbal et al. 2013; Massel 2011; O’Regan & Mulvihill 2010; Iancu et al. 2009; Surh et al. 2007; Fechner et al. 2007).

An in-situ gelation method of the inner aqueous phase was proposed by Massel (2011). It is widely known that the plant structural polysaccharide pectin can be used to form hydrogels. In the method proposed, the author used pectin with a high degree of methyl groups esterified to the galacturonic acid chain with the presence of calcium ions and pectin methyl esterase solubilized in the internal phase of double emulsions. The enzymatic reaction at room temperature promotes the cleavage of the methyl groups, and the resulting low methoxyl pectin, in the presence of calcium ions, promote the gelation of the inner phase during the emulsion homogenization process. In contrast to gelation using whey proteins for example, this methodology do not require the addition of high concentrations of the biopolymer or heating processes. With this novel methodology, Massel was able to obtain significant improvement on encapsulation efficiency of the \( W_1/O/W_2 \) emulsion, and found out that the different loaded molecules influenced the encapsulation improvement.

Gelation of the oil phase has been applied to create W/O emulsion gels and \( O_1/W/O_2 \) emulsions. A classical and simple method to structure oils in foods is through mixture of
the oil phase with solid fats. By heating the oil phase at high temperatures (above melting point of the fat), and dispersing the solid fat into it, the solid fat is melted and after cooling to lower temperatures, a fat crystal network is formed which provides structure to the liquid oil, contributing to the functionality and texture of a food product (Patel & Dewettinck 2015; Patel et al. 2014; Pernetti et al. 2007).

Depending on the cooling rate of the system, the size and form of the crystals can vary. The presence of fat crystals within the oil droplets can form a network and also irregular clumps. When a crystal is large enough, it can cause a rupture of the interface of one droplet and can pierce a neighbouring droplet leading to partial coalescence of solidified droplets (Figure 2.2). This causes an increase in the apparent volume fraction of the dispersed material. Such a situation can happen during processing conditions such as flow or agitation (Walstra & Vliet 2008). To act as an emulsion stabilizer at the interface, the crystals have to be considerably smaller. Large crystals cannot adsorb at the interface and can flocculate as free crystals in the continuous phase (Garti et al. 1999).

It has been shown that in W/O food emulsions like butter and margarine, stabilization with fat crystals is ruled by synergistic effects of a solid fat network and crystals at the interface (Frasch-Melnik et al. 2010; Rousseau et al. 2003; Garti et al. 1998). In W\textsubscript{1}/O/W\textsubscript{2} emulsion with an oil phase containing a mixture of soybean oil and solid fat, the presence of PGPR was suggested to stabilize the dispersion of oil droplets and serve as bridge between the crystalline fat particles and the water, facilitating the anchoring of the fat particles in the oil phase in one direction. Thus, it was possible to obtain W\textsubscript{1}/O/W\textsubscript{2} emulsions stable to coalescence for 3 weeks of storage (Garti et al. 1999).
2.4. **In vitro digestion**

The digestive tract is the largest set of organs that functions as a barrier between our body and the environment. It promotes the first immune impact in our body and the digestion of foods, providing nutrients and energy to be used for cell maintenance and growth (Verhoeckx et al. 2015; Brandtzaeg 2011). During digestion, mechanical transformations reduce the size of the food particles and the enzymatic reactions break down the macromolecules into constituents of smaller sizes, able to be absorbed by the cells and reach the bloodstream (Guerra et al. 2012).

Because of its complexity, the study of the human digestion is difficult, expensive and time consuming. Although it can provide accurate results, it involves limitations regarding ethical constraints, individual variation in physiological responses and even how the food structure interacts with the body. Depending on the amount of constituents (i.e. fats, proteins or carbohydrates) or processing history of the food (raw or baked), the body will be behave differently, controlling the hormonal responses and releasing specific quantities of enzymatic fluids or controlling the dynamics of the peristaltic movements in the gastrointestinal tract (Guerra et al. 2012; Hur et al. 2011). *In vivo* is still considered the “gold standard” in diet related questions while for an *in vitro* method some conditions or effects will end up being compromised (Hur et al. 2011). On the other hand, *in vitro* methods allows the analysis of a large number of samples and function as a tool for screening foods or delivery systems with different structures (Coles et al. 2005).

In the past decades efforts have been made to develop and simulate physicochemical and physiological events. To do so, it is mandatory to submit the sample to conditions that are the closest to reality as possible, in relation to transit time, pH and enzymatic conditions (Guerra et al. 2012). The different research questions might require small modifications to well established methods. Physical and chemical alterations,
specific, mechanistic and structural questions, are particularly relevant to delivery systems
designed for oral administration, as well as the study of the fraction of the nutrients or
bioactives that are transferred to the aqueous phase of the digestate, prior to cell absorption
(bioaccessibility). These questions can be answered with in vitro methodologies, which is
a challenge while working with complex living beings (Arranz et al. 2015; Shani-Levi et
al. 2013; Hur et al. 2011; Hur et al. 2009). In vitro methods, particularly the static ones,
have advantages of being more rapid, reproducible, less costly and less labour intensive
and definitely is not affected by ethical hindrance (Verhoeckx et al. 2015; Minekus et al.
2014).

In dynamic digestion models, the conditions within the different stages change over
time. These models have a number of different digestive compartments to which the
digestion secretions are added at a particular rate or follow a programmed pattern, allowing
the rate to be changed over time, or the response to other parameters according to volume
reached in a compartment to be changed (Thuenemann 2015). The different methodologies
available such as TNO Gastro-Intestinal Model (TIM), Dynamic Gastric Model (DGM)
and the Simulator of the Human Intestinal Microbial ecosystem (SHIME®), allow the
study of different parts of the gastro-intestinal tract and even simulate different species of
animals, age and pathological conditions, however, there are disadvantages. Some methods
may not reproduce the in vivo peristaltic movements or may simulate it producing shear
forces that may represent a problem to the type of sample or microorganisms present in the
system (Minekus 2015; Guerra et al. 2012). Other methods may not consider the
contribution of microorganisms (Van de Wiele et al. 2015). Despite their achievements,
these systems still lack hormonal activities, mechanical feedback mechanisms, immunity
etc., and are more time consuming when compared to static methodologies (Arranz et al.
2016).
On the other hand, static methods take a more simplistic approach in this respect and include two or three digestion steps (oral, gastric, and intestinal) and the reaction products remain in a single static bioreactor or jar. The static models of human digestion have been used to study digestibility and bioaccessibility of a variety of products in a more simple way, with the advantage of a well-controlled environment. This allows for rapidly answering specific questions such as interfacial lipolysis, microstructure breakdown of emulsion based foods and measurement of bioaccessible fractions employing Caco-2 cells (Arranz et al. 2016; Arranz et al. 2015; Eldemnawy et al. 2015).

A common static digestion model used for studies of intestinal lipolysis is the pH-stat titration model. The method is well described in the literature based on titration of free fatty acids produced from hydrolysis of the lipids after lipase addition at pH values close to neutral (Li et al. 2011; McClements & Li 2010a). The pH Stat titration is a very simple method and enables quantitative comparison between different lipid formulations under the same conditions. This technique is useful when physicochemical factors that affect the kinetics of lipid digestion are studied such as calcium concentration and physical state of the emulsified lipid (Eldemnawy et al. 2015; Ye et al. 2013; Bonnaire et al. 2008).

Lipid digestion can also be followed by batch digestion in jars or bioreactors. These methods allows for more complex simulated gastric and intestinal fluids, which might bring more relevant answers regarding microstructure breakdown and bioaccessibility (Versantvoort et al. 2005; Versantvoort, van de Kamp, and Rompelberg 2004; Oomen et al. 2002).

In both cases, the pH Stat or the batch digestion, peristalsis is simulated by stirring at a constant speed (Kaimainen et al. 2015; Frank et al. 2012) or by a shaking water bath (Minekus et al. 2014; Malaki Nik et al. 2010), or by rotation (Versantvoort et al. 2005).
The alternative for using the pH Stat for quantifying free fatty acids is to use a colorimetric assay originally used to determine free fatty acids in the blood sera (non-esterified fatty acid (NEFA) kit) (Malaki Nik et al. 2012; Malaki Nik, Corredig & Wright 2011; Malaki Nik et al. 2010). Recently, Eldemnawy, Wright and Corredig (2015) compared lipid digestion with pH-stat and with an NEFA-kit with the batch digestion model. The authors found that the concentration of calcium ions in the digestion media affects the measurement of free fatty acid using the NEFA kit assay, which may result in an underestimated value of total free fatty acid released after digestion. However, the jar digestion model might be preferred to studies of bioaccessibility due to the possibility of using more complex and physiological simulated fluids and may answer fundamental questions of the digestion process.

By using non standardized guidelines and very simple salt solutions and buffers, the effect of osmotic balance or ionic strength of the media on the structure of the food or delivery system for example, cannot be taken in consideration. Therefore, in the case of food-grade double emulsions, if studying the bioaccessibility of bioactives, the conditions need to be changed and well characterized. Recently, Kaimainen et al. (2015) studied structural changes and bioaccessibility of red beet extract encapsulated in W₁/O/W₂ emulsion, using a very simplistic approach of the in vitro intestinal digestion. The effects of gastric digestion were not taken in consideration. In addition, the osmotic changes induced by the addition of simulated intestinal fluids were not discussed. Despite these facts, the authors suggested that the oil phase of the double emulsion system might act as a protective barrier to hydrophilic bioactives during the digestion. In another study with W₁/O/W₂ emulsion, Frank et al. (2012) analyzed the bioaccessibility of anthocyanins and found a small contribution of the osmotic balance to the release of the contents of the internal water droplets of the double emulsion, however, the influence of the environment.
was not clear as the osmolalities of the inner and outer aqueous phases of the emulsion were not specified.

With the variability in the conditions of the static methods used, the results obtained cannot be comparable between research groups in several occasions (Verhoeckx et al. 2015). In an attempt to standardize a static method, an international network of scientists involved in the “Food and Agriculture European Cooperation in Science and Technology (COST) Action FA1005 ‘Improving health properties of food by sharing our knowledge on the digestive process (INFOGEST)’”, outlined conditions for simulated digestion that is expected to aid the production of more reliable and comparable data in the future. The method was thought to be the “smallest common denominator” and according to the research question, involves oral, gastric and intestinal phases of human digestion, with very complex and well described conditions of pH values, mixture of ions, bile salts and enzymes, as well as the standardized methodologies for determination of enzyme activities (Arranz et al. 2016; Minekus et al. 2014). This is a recently developed consensus methodology, therefore few studies have been published with different types of food products and some modifications have been made. For example, in a study with dietary fiber obtained from lemon, grapefruit and pomegranate added to pork patties and submitted to *in vitro* digestion, the authors tried to employ the standardized protocol with few changes as possible, however, the exact enzyme activities were not reported (López-Marcos et al. 2015). In another example, a study regarding the determination of bioaccessibility of carotenoids (β-cryptoxanthin) from fruits included the oral, gastric and duodenal intestinal phases but one more enzyme was added on the duodenal phase, cholesterol esterase. (Estévez-Santiago et al. 2016). In a study of lycopene bioaccessibility of tomato pulp added with differently processed corn extrudates, it was also included three phases of digestion and some ions concentrations and enzymes activities modified (Tonyali et al. 2015).
Finally, Sarkar et al. (2016) studied the *in vitro* digestion of O/W emulsion containing whey protein microgel particles at the interface and compared the barrier properties of differently processed gel particles. In this work, the authors used the gastric and duodenal stages of the intestinal digestion and justified the changes on pH and digestion times employed.

Regarding the lipid digestibility, the lipid phase of double emulsions have to be carefully selected when designing double emulsions. The different lipids used can drive the physical state of the lipid phase, and when subjected to *in vitro* digestion studies, the lipids present in the oil phase can influence the rate and extent of lipid digestion and the bioaccessibility of the bioactive dissolved into it.

Several studies with different lipid types are available in the literature. Taking into consideration the level of unsaturation, fish oils (which contain high levels of polyunsaturated fatty acids) were found to be hydrolyzed more slowly by porcine pancreatic lipase than those containing monounsaturated fatty acids (Marze et al. 2013; Mu & Høy 2004). Zhang et al. (2015) did not observe an appreciable difference between the digestion of the fish oil and the digestion of the corn oil and medium chain triglyceride oil, but revealed a lower rate and extent of lipid digestion for the emulsions containing krill oil (reaching a total of approximately 43% of oil digested) than for the emulsions containing digestible triacylglycerol oils (about 90% digested). The size of the triglyceride chain also matters. It has been reported that the rate and extent of lipid digestion in the emulsions were higher for medium chain triglycerides (MCT) than for long chain (LCT) (Ozturk et al. 2015; Yu et al. 2012; McClements & Li 2010b; Porter et al. 2007).

The size of the lipid chain affects the sterol solubilization in mixed micelles. This directly influences bioaccessibility and bioavailability of compounds. Von Bonsdorff-Nikander et al. (2005) evaluated different sterol molecules and observed that in mixed micelles containing products of *in vitro* lipid digestion of formulations containing MCT,
Phytosterols showed lower solubilization (13%) when compared with the digestate of long chain based formulations (25%).

Vitamin D₃ showed better solubility in soybean oil compared to Phytosterols (Malaki Nik, Corredig & Wright 2011). Vitamin D₃ bioaccessibility was also investigated for O/W nanoemulsion formulations containing different oil types. The concentration of Vitamin D₃ in the micellar phase after in vitro digestion was quantified and results showed a relation between the bioaccessibility and the type of oil present in the nanoemulsion. Fish oil and corn oil, which contains more LCT, showed higher values when compared with oils containing MCTs, orange and mineral oils (Ozturk et al. 2015). On the other hand, Day et al. (Day et al. 2010) reported that during digestion of glycercyl trioleate (trygliceride comprising three oleic acid chains (C18 - LCT, monounsaturated)) O/W emulsion, Vitamin D₃ had less affinity for the micellar phase and remained concentrated within the lipid while digestion was taken place on the oil droplet. With a different bioactive but related results, Yu et al. (Yu et al. 2012) showed that oleogels containing MCT had high digestibility as expected, and promoted high bioaccessibility of curcumin. A recent in vivo study carried by Dawson-Hughes et al. (2015) revealed that plasma levels of Vitamin D₃ were 32% higher in patients that consumed meals containing fat than those who ate fat free meals, in addition, the ratio between monounsaturated and polyunsaturated fatty acid content in the oil did not seem to be relevant to Vitamin D₃ absorption.

Therefore in the current research, formulations of W₁/O/W₂ emulsion stabilized by PGPR and sodium caseinate, containing different oil phases and encapsulating multiple model bioactive molecules (Vitamin B₁₂ as hydrophilic and Vitamin D₃ and Phytosterols as hydrophobic models) were subjected to a standardized in vitro digestion protocol. Microstructural changes during the digestion process, the physical state of the internal
phases of the double emulsions, the lipid digestion and the bioaccessibility of the bioactives were investigated
Chapter 3: Vitamin D₃ and Phytosterols affect the properties of polyglycerol polyricinoleate (PGPR) and protein interfaces

3.1. Abstract

The present work tested the effect of addition of hydrophobic compounds at interfaces containing polyglycerol polyricinoleate (PGPR) and β-lactoglobulin or sodium caseinate. The effect of Vitamin D₃ or Phytosterols on a model double emulsion stabilized with PGPR and sodium caseinate was also studied. Water-soybean oil interfaces were studied using drop shape tensiometry. Emulsions were prepared using a high pressure homogenizer, and changes in particle size were followed using light scattering. The encapsulation efficiency of the double emulsions was estimated by measuring the release of Mg²⁺ from the inner droplet. PGPR dominated the oil-water interfacial properties. In the presence of proteins there was a decrease of the interfacial tension, with little changes in the viscoelastic properties of the emulsion. The presence of Vitamin D₃ and Phytosterols further affected the interfacial properties. Double emulsions were then prepared with 2% PGPR. While control emulsions showed limited stability with an increase in the particle size after one week of storage, emulsions containing 0.05% (w/w) of Vitamin D₃ or Phytosterol resulted in increased stability. Results suggested that Vitamin D₃ and Phytosterol molecules may interact with the emulsifiers at the interface, affecting the physico-chemical properties, and possibly their release during digestion.

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3.2. Introduction

Double emulsions have gained interest in the past years, because of their increased applications in cosmetic, pharmaceutical and food industries. A water-in-oil-in-water emulsion ($W_1/O/W_2$) consists of water-in-oil ($W_1/O$) droplets dispersed in a secondary aqueous phase ($W_2$). It is possible to carry, within this system, both hydrophilic and hydrophobic substances. Different phases can protect bioactives or nutrients from unwanted reactions (Dickinson 2011; McClements 2011). Double emulsions continue to be focus of basic research, as the interactions occurring at the interface between the various components in the mixtures are yet to be fully understood.

A stable primary $W_1/O$ emulsion is essential to obtain a double emulsion system applicable to the food industry. Efforts have been carried out to improve steric repulsion at the interface of the inner droplets (Lutz et al. 2009; Garti et al. 1999; Muschiolik et al. 2006; Su et al. 2006; Bouyer et al. 2011). Polyglycerol polyricinoleate (PGPR), a synthetic emulsifier, is the most common molecule employed to stabilize primary $W_1/O$ emulsion droplets. Albeit it has gained GRAS status (generally recognized as safe) from FDA (Food and Drug Administration 2006), the facts of being synthetic and having labeling requirements make its reduction or removal from the formulations desirable (Márquez et al. 2010). Mixing emulsifiers has been shown as a good strategy to decrease PGPR in $W_1/O/W_2$ emulsions, and synergistic effects have been found, for example, between sodium caseinate and PGPR (Su et al. 2006). The interactions between PGPR with other emulsifiers may improve stability of the inner phase and enhance the entrapment efficiency of the emulsion (Gülseren & Corredig 2012; Gülseren & Corredig 2014).

Other hydrophobic compounds present in the oil may also affect the interface. When bioactive compounds are added, their release during digestion may depend on their interaction with the interface. A recent *in vitro* digestion study of oil-in-water emulsions...
loaded with hydrophobic molecules showed differences in the transfer of the bioactives to the aqueous phase in the absence of lipolysis, suggesting partitioning of the molecules at the interface depending on their structure. For example, Vitamin D₃ and Phytosterols were transferred into mixed micelles in absence of lipolysis to a higher extent (around 30%) compared to β-carotene and coenzyme Q10 (only about 2%) (Malaki Nik, Corredig & Wright 2011). The authors suggested that Phytosterols and Vitamin D₃ would be preferentially closer to the interface of the oil droplet, while β-carotene and coenzyme Q10 would place towards the core.

Interactions between hydrophobic bioactives and emulsifiers may occur, and affect the properties of the oil-water interface. In this study, it was hypothesized that if interactions occur, interfacial properties will change, as well as the physico-chemical properties of W₁/O as well as W₁/O/W₂ emulsions. To test this hypothesis Vitamin D₃ and Phytosterols were used as model bioactives, and interactions at the interfaces were tested using mixed interfaces containing PGPR and milk proteins. Furthermore, the physico-chemical properties of W₁/O and W₁/O/W₂ emulsions stabilized with PGPR and sodium caseinate, and containing Vitamin D₃ and Phytosterols were studied.

### 3.3. Material and Methods

#### 3.3.1. Materials

Soybean oil (S7381, Sigma-Aldrich, St. Louis, MO, USA), was used as oil phase. In the case of tensiometry measurements, the oil was pre-treated with Florisil® (46385, Sigma-Aldrich) (Gülseren & Corredig 2012). In brief, oil (45g) was mixed with 4.5 g Florisil using a shaking plate (60 rpm for 2 h). The adsorbent was then filtered (Whatman
No:1, cellulose filter, Fisher Scientific, Fair Lawn, NY, USA). This process was carried out three times and during the final step, two filter papers were used. Emulsions were prepared using MilliQ water, while tensiometry experiments were carried out using HPLC grade water (Fisher Scientific). Synthetic emulsifier polyglycerol polyricinoleate (PGPR 4150) was obtained by Palsgaard (Juelsminde, Denmark) which stated the content of minimum 75% di-, tri- and tetruglycerols with a maximum of 10% of heptaglycerol or higher. Phytosterols (β-sitosterol (β-sit) as main compound – 85451) and Vitamin D3 (VitD3) (Cholecalciferol – C9756) as well β-lactoglobulin (β-lg) (L0130), were all obtained by Sigma-Aldrich, while sodium caseinate (NaCas) (NaCas 180) was purchased from Fonterra Inc. (Rosemont, IL, USA). Magnesium chloride hexahydrate (MgCl$_2$·6H$_2$O – BP214) and sodium chloride (NaCl – S271) were obtained by Fisher Scientific.

3.3.2. Drop shape tensiometry

The interfacial tension and dilational elasticity modulus of a soybean oil-water interface was measured using drop shape tensiometry (Tracker, IT Concept, Longessaigne, France) at room temperature. The aqueous drop (6 µl), was delivered by a syringe into an optical glass cuvette containing the oil phase. A video image was obtained with a CCD camera and processed using the Young-Laplace equation, while monitoring its shape. The interfacial tension was recorded over time (Benjamins et al. 1996; Fainerman et al. 2004; Wang & Narsimhan 2005). After an equilibration period of at least 3 h, the equilibrium interfacial tension ($\gamma$) was extrapolated, with results not significantly different from overnight equilibration experiments (Dopierala et al. 2011). After 3 h oscillatory changes of volume/surface area of the drop were performed with the strain amplitude kept constant at 10% ($\Delta A/A=0.1$, where $A$ is the droplet surface area) and with harmonic expansion and
dilation cycles ranging 5 to 100 mHz of frequency. The surface dilational modulus was obtained based on the following equation:

\[ E_{SD} = \frac{d\gamma}{d \ln A} \]  

(Eq. 1)

3.3.3. Emulsions Preparation

Water-in-oil emulsions (primary emulsions for the W1/O/W2) were prepared by mixing 30% (w/w) of aqueous and 70% (w/w) oil phases. The internal aqueous phase consisted of 0.5% (w/w) sodium caseinate and 0.1 M NaCl. MgCl₂ (0.1 M) was also added to the internal aqueous phase in selected systems to follow its encapsulation efficiency in W1/O/W2. The oil phase contained 2% (w/w) PGPR with or without 0.05% (w/w) bioactives (Vitamin D₃ or Phytosterols) in soybean oil. The oil and the water were pre-mixed using an ultra-turrax (PowerGen 125, Fisher Scientific) for 1 min, and this emulsion was then circulated through a microfluidizer (Microfluidics, Newton, MA, USA) at 500 KPa for 5 min to form the final W1/O emulsion.

A dispersion of 10% (w/w) primary emulsion (W1/O emulsion) was then mixed in 2% (w/w) sodium caseinate (NaCas) solution (prepared with MilliQ water) to obtain a W1/O/W2 emulsion with final ratio of 3:7:90. This second emulsification step was carried by passing the formulation once through the homogenizer (EmulsiFlex C5, Avestin, Ottawa, Canada) at approximately 5 MPa.

3.3.4. Emulsion Characterization

Diffusing wave spectroscopy (DWS) measurements were carried out to obtain the average droplet radius of primary W1/O emulsions. The sample was transferred to a 10 mm optical glass cuvette (Hellma Canada Limited, Concord, Canada) and placed in a 25°C
water bath, which was illuminated by a 350 mW solid-state laser (532 nm) (Coherent, Santa Clara, Ca, USA). Transmitted scattered light was collected by a single fibre optic that was then bifurcated and fed to two matched photomultipliers (HC120-03, Hamamatsu, Loveland, OH) and a correlator (FLEX2K- 12x2, Bridgewater, NJ). Correlation functions and transmitted light intensity was collected for 2 min. Data was analyzed using DWS-Fit (Mediavention Inc., Guelph, ON, Canada). The principles of DWS have been published elsewhere (Horne & Davidson 1993; Weitz et al. 1993).

Droplet sizes distributions of the double emulsions were measured using integrated light scattering (Malvern Mastersizer 2000S, Malvern Instruments Inc, Westborough, MA). A small volume of sample was diluted in distilled water present in the measuring cell and kept under stirring at room temperature. Refractive indices used were 1.33 for the dispersant (water) and 1.473 for the soybean oil phase. We assumed that the refractive index of the oil droplet was not affected by the presence of the internal aqueous droplets (Pawlik et al. 2010; Bonnet et al. 2010).

3.3.5. W/O/W Encapsulation efficiency

To determine the integrity of the primary emulsion in the double emulsion, the encapsulation efficiency of MgCl$_2$ was measured. Freshly made double emulsions were loaded with 100 mM MgCl$_2$ and then the amount of magnesium released was measured in the outer phase. The double emulsions were centrifuged at 16000 g for 30 min at room temperature (Eppendorf Centrifuge 5415 D). After separation of cream phase, the subnatant aqueous phase was filtered with 0.22 µm Millex-GV syringe filter (Millipore, Bedford, MA, USA) and samples were subjected to ion chromatography analysis (861 Advanced Compact IC – Metrom Ion Analysis, Metrohm Ltd., Herisau, Switzerland) after diluting 20
times in acceptor solution (2mM HNO₃). The eluent solution used was 0.7 mM dipicolinic acid and 1.7 mM HNO₃. The unit consists of an injection valve, high pressure pump, peristaltic pump and conductivity detector, all controlled by the 838-advanced sample processor (IC Net 2.3, Metrohm Ltd.). The 833 IC liquid handling dialysis unit with a 2-channel peristaltic pump and dialysis cell containing a 0.2 µm cellulose acetate membrane (Fisher Scientific) prepared the samples in-line. A standard curve was prepared with magnesium standard for IC TraceCERT (1,000 mg/L of Mg²⁺ in HNO₃, Fluka, Sigma, Steinheim, Germany) by diluting it in range between 1 to 10 mg/L and injected before the samples. The percentage of encapsulated material on the inner water droplet was calculated as follows.

\[
E_{\text{efficiency}} = \left[ \frac{(\text{Initial} - \text{Detected}_{\text{external}})}{\text{Initial}} \right] \times 100 \quad \text{(Eq. 2)}
\]

Where \text{Initial} indicates the original concentration added in the inner phase, while \text{Detected}_{\text{external}} indicates the amount measured in the external phase after centrifugation.

### 3.3.6 Statistical analysis

When analysed pairwise, data were tested with Student’s T-test. The differences in groups mean values were submitted to analysis of variance (ANOVA). TukeyHSD test was carried out when significant differences among groups were obtained. All statistical tests were done with 95% of significance. The statistical analysis was performed using the free software [R] (www.r-project.org) and Addinsoft XLStat extension for MS Excel.
3.4. Results and Discussion

The properties of a water-soybean oil interface were measured using drop shape tensiometry. Figure 3.1 illustrates the interfacial tension near equilibrium, after 3 h, for PGPR, sodium caseinate and β-lactoglobulin (β-lg) interfaces. In the absence of surfactants, the interfacial tension at equilibrium of the oil water interface was 31 ± 1 mN.m⁻¹, in agreement with previous studies (Gülseren & Corredig 2012; Gaonkar 1989). Solutions containing 0.008% (w/v) of synthetic emulsifier solubilised in soybean oil and aqueous solutions of 0.001% (w/v) of each protein were used. These concentrations were chosen based on previous literature data (Gülseren & Corredig 2012) to be below complete saturation of the interface by a single emulsifier and to observe possible changes promoted by the hydrophobic compounds.

![Figure 3.1](image)

**Figure 3.1.** Effect of the presence of different emulsifiers on interfacial tension, as measured by drop tensiometry. Solutions contained 0.008% (w/v) of PGPR in soybean oil, and/or 0.001% (w/v) of sodium caseinate or β-lactoglobulin. Values were taken after 3 h equilibration and are the average of at least two experiments ± standard deviation. The dotted line indicates the interfacial tension for soybean oil.
Both proteins and PGPR decreased the equilibrium interfacial tension when compared to the oil-water interface (Figure 3.1). Milk proteins are widely known for their ability to adsorb at oil water interfaces and decrease interfacial tension. When comparing to water-soybean oil values, NaCas and β-lg caused a decrease in the interfacial tension. PGPR dispersed in the oil promoted a slightly lower decrease in equilibrium interfacial tension compared to the proteins (Figure 3.1). These findings were in full agreement with previous studies of β-lg and NaCas at sunflower oil interfaces (Wüstneck et al. 1999; Ogden & Rosenthal 1997). The presence of PGPR and proteins caused further decrease of the interfacial tension, suggesting that both molecules (protein and PGPR) were present at the interface.

Dilational viscoelasticity was also measured applying a strain amplitude of 10% (ΔA/A = 0.1) and oscillatory frequencies between 5 to 100 mHz (Table 3.1). In all systems studied, a similar pattern was observed: elasticity values were nearly constant at low frequency, reaching a maximum at 50 mHz. The interface containing β-lg showed higher elastic modulus than those obtained with the synthetic emulsifier. Nonetheless, in all cases, in the presence of PGPR, elasticity measurements revealed that the mixed interfaces were dominated by PGPR, as reported in the literature (Gülseren & Corredig 2012). These results further explained the observation that the presence of NaCas in the inner phase of W1/O/W2 emulsions causes an improvement in the overall stability of the emulsions (Su et al. 2006). It was then suggested that proteins and other macromolecules adsorbed at the interface may act as a barrier to the release of bioactives entrapped within the emulsion droplets (Benichou et al. 2004).
Table 3.1. Elastic modulus obtained at strain amplitude of 10% of water-soybean oil interfaces containing PGPR and/or milk proteins. Results are the average of minimum two different experiments ± standard deviation. Different superscript letters indicate significant differences among each frequency studied.

<table>
<thead>
<tr>
<th>Frequency (mHz)</th>
<th>PGPR</th>
<th>β-lg</th>
<th>NaCas</th>
<th>PGPR / β-lg</th>
<th>PGPR / NaCas</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>25 ± 5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 ± 3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25 ± 3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>24 ± 2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>36 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 ± 4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>28 ± 4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>35 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>39 ± 22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100</td>
<td>21 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

3.4.1. Influence of bioactives at W/O interfaces

Figure 3.2 illustrates the effect of 0.005% (w/v) Vitamin D<sub>3</sub> or β-sitosterol to the oil phase. When these compounds were added in isolation or in the case of an interface dominated by β-lg, no significant changes in interfacial tension were observed.

Figure 3.2. Interfacial tension of different water-oil interfaces without (black bars) or with either β-sitosterol (white bars) or Vitamin D<sub>3</sub> (gray bars). Hydrophobic compounds were solubilised in soybean oil at 0.005% (w/v) concentration, with PGPR at 0.008% (w/v). NaCas and β-lactoglobulin concentrations in the water phase were 0.001% (w/v). Results are the average of a minimum of two independent experiments and bars indicate standard deviations.
While the presence of PGPR in the oil showed a value of interfacial tension of the water droplet at equilibrium of about $20 \pm 1 \text{ mN.m}^{-1}$, the addition of the bioactive molecules $\beta$-sitosterol and Vitamin D$_3$, caused a smaller decrease in the interfacial tension. The highest value of interfacial tension was observed when Vitamin D$_3$ was present ($28 \pm 1 \text{ mN.m}^{-1}$). A similar pattern of significant changes were observed with interfaces containing mixtures of PGPR and sodium caseinate and sodium caseinate alone. These results would suggest an effect of Phytosterols and Vitamin D$_3$ on the adsorption behaviour of the emulsifiers.

The viscoelastic properties of the mixed interfaces were also measured, and the presence of the bioactive molecules did not show statistically significant differences, apart from when only PGPR and Vitamin D$_3$ were present at the interface. In this case, the elastic modulus measured at 10 and 50 mHz showed an increase (with values of 38 and 60 mN.m$^{-1}$, respectively) compared to when PGPR was measured in isolation.

### 3.4.2. Effect of bioactive molecules on W$_1$/O and W$_1$/O/W$_2$

High pressure homogenization was used to prepare 30% water-in-oil emulsions. The oil phase contained 2% PGPR and 0.05% Vitamin D$_3$ or $\beta$-sitosterol when was the case. The internal water phase contained 0.1 M NaCl, and 0.1 M MgCl$_2$. The double emulsions were stabilized by 2% NaCas. Light scattering measurements (DWS) were carried out in fresh made emulsions and after 1 week of storage at 4°C (Table 3.2). All primary emulsions were < 300 nm in radius, when measured by dynamic light scattering under concentrated conditions (DWS).
Table 3.2. Apparent radius of W₁/O emulsions containing or not bioactives loaded in function of storage time at 4°C obtained through DWS apparatus. Results are average of triplicates ± standard deviation, and Student’s T-test with 95% significance. Different superscript letters indicate significant differences within each row.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fresh (nm)</th>
<th>1 week (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - without bioactive</td>
<td>292 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>394 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>200 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>249 ± 37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MgCl₂ / β-sit</td>
<td>240 ± 43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>259 ± 16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MgCl₂ / VitD₃</td>
<td>276 ± 22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>268 ± 52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Statistical analysis revealed that control samples and those carrying only the hydrophilic bioactive had a significant (p<0.05) increase in the apparent radius. PGPR is known for its capacity of producing interfaces with very strong steric repulsion between the droplets, reducing droplet coalescence (Knoth et al. 2005; Dedinaite & Campbell 2000). The amount of emulsifier used in this work was sufficient to create small emulsion droplets, but not to ensure long term stability of the emulsion droplets. Using 2% or less PGPR, Su et al. (2006) reported complete phase separation of the W/O emulsions. In the presence of either Vitamin D₃ or β-sitosterol, the emulsions showed constant apparent radius after 1 week of storage, suggesting their effect on improving stability of the W₁/O emulsion droplets. It is important to note that among all samples studied, no phase separation was observed after 1 week of storage.

The freshly made primary emulsions were re-dispersed in 2% NaCas, and re-homogenized to obtain W₁/O/W₂ (3:7:90) double emulsions. Particle size distribution, was measured by integrated light scattering, after extensive dilution in water. Figure 3.3 summarizes the changes in average diameter after 1 week storage at 4°C. All fresh samples showed a monomodal distribution of droplet sizes. Due to their large particle size, the double emulsion systems showed rapid creaming, resulting in a heterogeneous system in
approximately 30 min. However, creaming was fully reversible after mixing. After 1 week of storage, control emulsions and those containing 0.1M MgCl$_2$ showed extensive coalescence, while those containing β-sitosterol and Vitamin D$_3$ still showed a monomodal distribution of sizes, consistent with the improved stability of the inner droplets shown in Table 3.2.

Fresh formulations resulted in high values of encapsulation of MgCl$_2$ with statistical changes observed (p<0.05): the highest value was obtained on samples without hydrophobic compounds, 96.3 ± 0.3%; samples containing β-sitosterol or Vitamin D$_3$ showed smaller values 92.3 ± 1.5% and 90.9 ± 2.6% respectively.
Figure 3.3. Particle size distribution of W₁/O/W₂ emulsions measured by integrated light scattering. Measurements were carried out after preparation (filled symbols) and after one week of storage at 4°C (open symbols). Internal aqueous phase consisted of 0.5% (w/w) sodium caseinate, 0.1 M NaCl with or without 0.1 M MgCl₂. Emulsions were stabilized with 2% (w/w) PGPR on the oil phase and 2% NaCas on external aqueous phase. (A) emulsion control; (B) controls containing 0.1 M MgCl₂; (C) emulsions containing MgCl₂ and 0.05% β-sitosterol; (D) emulsions containing MgCl₂ and 0.05% Vitamin D₃.

3.5. Conclusions

This research demonstrated that when hydrophobic molecules such as Vitamin D₃ or β-sitosterol are added to an emulsion, they affect the physico-chemical properties of the interface. These hydrophobic molecules caused an increase in the interfacial tension at mixed interfaces, demonstrating that they affected the adsorption properties of the emulsifiers used in this study. Furthermore, their addition to a water-in-oil or water-in-oil-in-water emulsion showed no changes in particle size after 1 week of storage, in contrast
to control emulsions. These effects on physical properties of the emulsion may indicate interactions of the bioactive molecules with the interface and/or with the emulsifiers, ultimately affecting their release behavior during digestion.
Chapter 4: Bioactive release in double emulsions during *in vitro* digestion

4.1. Abstract

The objective of this work was to evaluate the *in vitro* digestion behaviour of double emulsions carrying model bioactive molecules, namely Vitamin B<sub>12</sub>, Vitamin D<sub>3</sub> and Phytosterols. The physical chemical changes as well as the extent of the release of bioactives were studied. It was hypothesized that the interactions of emulsifiers and bioactives occurring at the oil-water interfaces would influence the release behaviour of the hydrophobic bioactives during digestion. W<sub>1</sub>/O/W<sub>2</sub> emulsions were prepared using a high pressure homogenizer, with PGPR and sodium caseinate as emulsifiers. Emulsion characterization and changes during *in vitro* digestion were followed using light scattering and confocal microscopy (CSLM). The digestion protocol consisted of a 30 min gastric phase followed by a 2 h duodenal stage. The release of free fatty acids and the amount of bioactive transferred to the aqueous micellar phase were measured during digestion. In all cases, the emulsions were stable during gastric digestion; however, 87% of entrapped Vitamin B<sub>12</sub> was released during this stage. After the gastric phase, the particle size distribution showed a significant increase over time. The free fatty acid release was well correlated with Phytosterols transfer (approximately 30%). In the case of Vitamin D<sub>3</sub> the maximum release was 16% at 28% fatty acid release, and this delay was caused by the surface-to-core distribution of the bioactive molecules within the oil phase. This work clearly demonstrated that different bioactives will show different release behaviour in water-in-oil-in-water emulsions, depending on their distribution in the oil phase and the interactions with the components present at the interface.
4.2. Introduction

The growing appeal for functional foods has driven the food industry to develop food matrices that not only deliver nutrition, but can bring additional health benefits. Water-in-oil-in-water ($\text{W}_1/\text{O}/\text{W}_2$) emulsions are complex systems that have been extensively studied for applications in pharmaceutical and cosmetic industries. In this regard, the different compartments within the double emulsion system make it a solution to simultaneously carry hydrophilic and hydrophobic substances (McClements 2012). Their potential use in the food industry involves, amongst others, foods with reduced fat content, modulation of the sensory properties of a food matrix, encapsulation of probiotics, enzymes, and other bioactive molecules (Dickinson 2011; Pimentel-González et al. 2009; Muschiolik 2007). However, in a double emulsion system, there are two interfaces which require different emulsifiers and their stability during processing and storage is often a challenge.

The most efficient lipophilic emulsifier used to stabilize the primary water-in-oil emulsion is polyglycerol polyricinoleate (PGPR). This molecule, adsorbed at the interface provides very good steric stabilization of water droplets (Su et al. 2006; Mun et al. 2010). The external phase of the double emulsion requires instead a hydrophilic emulsifier, such as sodium caseinate (NaCas). Emulsifiers, proteins and other biopolymers present in the matrix may interact at the interfaces, affecting the physical and chemical properties of the double emulsions. These interactions may improve the stability of the inner phase of the emulsion and even enhance the entrapment efficiency of the emulsion (Su et al. 2006; Rodriguez Patino & Pilosof 2011; Gülseren & Corredig 2014; Gülseren & Corredig 2012). The presence of hydrophobic bioactives in the oil phase may also interfere with the ability of PGPR and milk proteins to reduce the interfacial tension; it was shown that bioactive
molecules such as Vitamin D₃ (VitD₃) and Phytosterols affected the physicochemical properties of emulsions (Andrade & Corredig 2016).

Up to date, there are not many reports available regarding the structure breakdown and release of entrapped compounds of food grade double emulsions during static \textit{in vitro} digestion. Recently, Kaimainen et al. (2015) used a very simple \textit{in vitro} intestinal digestion model to follow the structural changes and release behavior of the entrapped red beet extract in the inner aqueous phase. The authors concluded that the double emulsion system might act as a protective barrier to hydrophilic bioactives during the digestion, however, the effects of the gastric environment or osmotic balances were not taken into consideration.

In the present work it was hypothesized that the interactions between hydrophobic bioactive molecules and emulsifiers present in the oil/water interface would decrease the release behavior of these compounds during \textit{in vitro} digestion. The objective of this work was to formulate a W₁/O/W₂ emulsion encapsulating hydrophilic and hydrophobic bioactives molecules and to investigate their physical and chemical changes during digestion and release behaviour.

4.3. Material and Methods

4.3.1. Materials

Soybean oil (S7381), Phytosterols (β-sitosterol (β-sit) as main compound – 85451), Vitamin D₃ (VitD₃) (Cholecalciferol – C9756), Vitamin B₁₂ (VitB₁₂) (Cyanocobalamin - V2876), Nile Red (72485), Butylated hydroxytoluene (BHT) (W218405), Pepsin from porcine pancreas mucosa (P7000, 1064 U/mg of powder), Pancreatin from porcine
pancreas (4xUSP – P1750 – lipase activity: 18.5 U/mg of powder), Phospholipase A2 from porcine pancreas (P6534 – 902U/mL) were obtained from Sigma Aldrich (St. Louis, MO, USA). Sodium caseinate (NaCas) (NaCas 180) was purchased from Fonterra Inc. (Rosemont, IL, USA). Calcium chloride dehydrate (CaCl₂(H₂O)₂ – C79), Potassium chloride (KCl – P217), Monopotassium phosphate (KH₂PO₄ – P285), Sodium bicarbonate (NaHCO₃ – S233), Sodium chloride (NaCl – S671), Magnesium chloride hexahydrate (MgCl₂.6H₂O – BP214) and Ammonium carbonate ((NH₄)₂CO₃ – A656), and the HPLC grade solvents hexane, methanol, anhydrous ethanol and acetone were obtained by Fisher Scientific (Ottawa, ON, Canada). Fat free soybean phospholipids with 75% Phosphatidylcholine (ALCOLEC PC75), were provided by American Lecithin Company (Oxford, CT, USA). Polyglycerol polyricinoleate (PGPR 4150) was obtained by Palsgaard (Juelsminde, Denmark) which stated the content of minimum 75% di-, tri- and tetraglycerols with a maximum of 10% of heptaglycerol or higher. Ultrapure MilliQ water was used in all reagents and buffers, unless stated differently.

4.3.2. Emulsions Preparation

Water-in-oil emulsions were prepared by mixing 30% (w/w) of aqueous phase and 70% (w/w) oil phases. A solution containing 0.5% (w/w) NaCas, 100 mM NaCl, 100 mM MgCl₂ as well the hydrophilic bioactive model VitB₁₂ (1% (w/w) when necessary), was used as internal aqueous phase (W₁). It is known that excess salt entrapped in W₁ plays an important role in balancing the effect of osmotic and Laplace pressures between the two aqueous phases of W₁/O/W₂, keeping the internal aqueous droplets stable (Rosano et al. 1998; Sapei et al. 2012). Incorporation of Mg²⁺ in the formulation contributed to the rise of osmotic pressure in the internal phase. The release of Mg²⁺ was followed during storage, to further understand the integrity of the inner water droplets.
Vitamin B₁₂ was also added to the inner water phase, and its release was observed during digestion. The oil phase formulation was obtained by mixing 2% (w/w) PGPR with or without 0.5% (w/w) of hydrophobic bioactive (Phytosterols and Vitamin D₃) in soybean oil. An ultra-turrax (PowerGen 125, Fisher Scientific) was used to mix the two phases for 1 min at 12000 rpm, forming a coarse emulsion which was passed through a high pressure homogenizer (EmulsiFlex C5, Avestin, Ottawa, Canada) 10 times at 70 MPa to obtain the final W₁/O emulsion.

A dispersion of 10% (w/w) W₁/O emulsion into 2% (w/w) NaCas solution (prepared with MilliQ water) was prepared to obtain a W₁/O/W₂ emulsion with a final ratio of 3:7:90. This second emulsification step was carried out by passing the formulation once through the high pressure homogenizer at minimal pressure, approximately 3.5 MPa, to obtain a monomodal distribution of sizes, without disrupting the inner aqueous droplets.

4.3.3. Light Scattering measurements

The average radius of W₁/O emulsions was obtained using diffusing wave spectroscopy (DWS) (Horne & Davidson 1993; Weitz et al. 1993), which relies on multiple scattering of the light and without depending on dilution of the samples. Conditions applied as described by Massel, Alexander and Corredig (2015) and summarized in brief in section 3.3.4.

The double emulsions and the digestate samples droplet sizes distribution were measured using integrated light scattering equipment as described on section 3.3.4.
4.3.4. Microscopy

Confocal Scanning Laser Microscopy (CSLM) was used to capture images of the emulsions and the digestate during different time points of digestion. In this case, emulsions without VitB\textsubscript{12} and hydrophobic bioactive supplementation were prepared by adding 0.01% (w/w) of Nile Red (Sigma Aldrich, St. Louis, MO) to the oil phase, following methodology described above (Emulsion Preparation section). The samples were placed on concave glass slide and covered with a cover slip. The Upright Leica TCS SP2 microscope (Leica Microsystems, Heidelberg, Germany) was used with oil-immersion 100x magnification lenses and a 50 mW 488 nm Ar laser. Images shown are representative of 5 images from 2 replicates.

4.3.5. Encapsulation efficiency of hydrophilic bioactives

The double emulsions were loaded with 100 mM MgCl\textsubscript{2} and 1% VitB\textsubscript{12} when necessary. The secondary emulsion droplets were separated using centrifugation at 16000 g for 30 min at room temperature (Eppendorf Centrifuge 5415 D). The amount of Mg\textsuperscript{2+} or VitB\textsubscript{12} present in the outer aqueous phase was then measured. The subnatant aqueous phase was filtered with 0.22 μm Millex-GV syringe filter (Millipore, Bedford, MA, USA). For detection of Mg\textsuperscript{2+}, the samples were subjected to ion chromatography analysis as previously described on section 3.3.5.

When loaded with VitB\textsubscript{12}, the level of the bioactive transferred to the external aqueous phase was measured using RP-HPLC (Sami et al. 2014). The unit (1200 series HPLC, Agilent Technologies, Santa Clara, CA, USA) consisted of a quaternary pump, auto degasser, diode array detector, auto-injector, and ChemStation software. A constant column temperature of 25ºC and flow rate of 1 mL/min were maintained under isocratic
conditions and using a ZORBAX Eclipse XDB-C18 (4.6 x 150mm, 5 mm) column (Agilent Technologies). To analyse VitB$_{12}$ present in the subnatant sample, a volume of 20 µL was injected. The mobile phase of 33:67 (v/v) methanol:H$_3$PO$_4$ solution (23mM, pH 2) was used with flow rate of 1 mL/min and the corresponding VitB$_{12}$ peak was detected at wavelength of 360 nm. VitB$_{12}$ standard curve was prepared by dilution in 2% NaCas solution at concentrations ranging from 10–400 µg/mL. The calculation of the percentage of encapsulated material was based on Eq. 2, section 3.3.5.

4.3.6. In vitro digestion

The in vitro digestion process was carried out following the protocol described by Minekus et al. (2014) with few modifications in order to observe the changes in the early stages of duodenal digestion.

It is known that negatively charged emulsion droplets may be subjected to reversible bridging flocculation in the oral phase (Sarkar & Singh 2012; Vingerhoeds et al. 2005; Silletti et al. 2007). Hence, preliminary experiments were carried out by incubating human saliva and a sample of double emulsion (50:50) for 6 min under 37°C temperature in shaking water bath, and there were no differences in the particle size distribution of the fresh emulsion and the sample processed with saliva. In addition, the osmolality of the human saliva revealed values below the lower limit of the equipment (data not shown). Therefore, the oral phase was not used in this study.

A transit time of 30 min was chosen for the gastric stage of digestion as under these conditions, most of the sodium caseinate was hydrolyzed (data not shown). This digestion time is consistent with literature data, where it has been shown that approximately 40 min
was the approximate gastric emptying time observed in adults after ingestion of 250 ml of apple juice (Hellmig et al. 2006).

For each time point studied (end of gastric phase at 30 min as starting point, and after 5, 12.5, 25, 60, 120 min of duodenal phase) one digestion jar was used.

The simulated gastric fluid was a solution containing 6.9 mM KCl, 0.9 mM KH₂PO₄, 25 mM NaHCO₃, 47.2 mM NaCl, 0.1 mM MgCl₂(H₂O)₆, 0.5 mM (NH₄)₂CO₃ and pH adjusted to 3 with HCl. Pepsin was dispersed in a volume of gastric fluid to reach the final concentration of 2000 U/mL each jar. To avoid precipitation, 0.15 mM CaCl₂(H₂O)₂ and 2 mg of BHT (as antioxidant) was added right after the emulsion sample. The mixture of 10 mL of emulsion sample and 10 mL of gastric fluid were placed in a shaking water bath (model 89032-226, VWR International, USA) at 37ºC and 220 rpm for 30 min to simulate the gastric environment. Concentrations set to 20 mL final mixture. After 30 min gastric digestion, the constituents of the duodenal phase were added, however, with the absence of duodenal enzymes, in order to stop proteolysis and keep the same final volume between all the samples. The digestate sample was cooled to 0-4ºC in freezer, with a cooling rate of approximately -3ºC/min.

To study the duodenal stage, 20 mL of simulated duodenal fluid was added and kept for 2h under the same temperature and shaking conditions. The duodenal fluids consisted of a salt solution containing 6.8 mM KCl, 0.8 mM KH₂PO₄, 85 mM NaHCO₃, 38.4 mM NaCl, 0.33 mM MgCl₂(H₂O)₆, 0.6 mM CaCl₂(H₂O)₂ and pH adjusted to 7 with HCl, 10 mM of bile salts, 5mM phospholipids, 0.8 pg (5µL) Phospholipase A₂ and pancreatin from porcine pancreas at 92 U/mL of pancreatic lipase (Malaki Nik, Corredig & Wright 2011), with concentrations described set to 40 mL final volume. The same temperature and cooling rate described in the gastric stage were used to stop duodenal digestion process at the different time points.
4.3.7. Determination Free Fatty Acids (FFA)

The amount of fatty acids released after the hydrolysis of the oil droplets during the duodenal stage was monitored by organic extraction under acidic conditions using a non-esterified fatty acid kit (NEFA-HR2) obtained from Wako Pure Chemical Industries (Wako diagnostics, VA, USA) as previously described (Malaki Nik, Corredig & Wright 2011). Briefly, a sample of 100 µL of the digestate was extracted by adding 900 µL of hexane and 100 µL of 0.1 M HCl, vortexed and centrifuged at 10000 g for 30 min. The supernatant was collected in a clean glass vial and diluted to 3 mL with hexane. 5 µL of each sample extract was added to 225 µL of reagent A in a 96-well plate and incubated at 37 °C for 10 min. 75 µL of Reagent B was then added to each sample well and the mixture incubated for an additional 15 min at 37 °C. A UV-VIS microplate spectrophotometer (Synergy HT, Biotek Instruments Inc., Winooski, VT, USA) was used to quantify the FFA content by measuring absorbance at \( \lambda_{\text{max}} \) of 550 nm and by reference to a standard curve prepared using oleic acid ranging from 0.1 to 1 mM. The percentage of fatty acids released was calculated based on the moles of hydrolyzed fatty acids present for each time point with respect to the dilution factor and total moles present initially.

4.3.8. Isolation of aqueous micellar phase and quantification of bioactives

Following exposure to the digestive conditions, ultracentrifugation at 114,000 g at 4 °C for 1 h using a Sorvall WX Ultra 80 ultracentrifuge (Mandel Scientific, ON, Canada) was promoted to separate the aqueous micellar fraction from the undigested oil droplets and solid particles. The aqueous fraction was collected with a 10 mL syringe and filtered.
(0.22 μm nylon filters, Fisher Scientific) to remove any insoluble particles. The aqueous phase was stored in plastic vials at -20 °C until quantification (within 3 days).

To quantify the amount of bioactive present in the emulsion oil phase and at each time point after digestion, a solvent extraction was used as previously described (Wright et al. 2008). An aliquot of 1 mL of aqueous phase of the digestate (or 1 mL of the emulsion sample) was mixed with 1.0, 6.0 and 2.0 mL of ethanol anhydrous, acetone and water respectively, with vortexing for 10 s between each solvent. The addition of 2 mL of hexane was promoted and vortexed for 10 s, in order to extract the hydrophobic bioactive to the organic layer. The mixture was left alone for 10 minutes and the organic layer collected to a clean glass vial with a fine glass transfer pipette. The addition of hexane was promoted 3 times, pooling approximately 6 mL of organic phase, which was submitted to evaporation under nitrogen at 35ºC (Zanntek Analytical Evaporator, Z1800, Glas-Col LLC, Terre Haute, IN, USA). The dried sample was then solubilised in 1 mL of methanol by ultrasonication for 30 seconds to assure solubilisation of crystals and filtered (0.22 nylon filters) prior to chromatography analysis using RP-HPLC. The detections were carried out under isocratic conditions using the same equipment and column described for encapsulation efficiency. The column temperature was kept constant at 25ºC and flow rate was set to 1 mL/min. To analyse Phytosterols present in the sample, a volume of 50 µL was injected, while VitD₃ 25 µL. The mobile phase of methanol:MilliQ water (99:1 v/v) for VitD₃, and acetonitrile:methanol (60:40 v/v) for Phytosterols were utilized and peaks corresponding to VitD₃ and Phytosterols were detected at wavelengths of 265, and 208 nm, respectively. Standard curves were prepared by dilution in methanol at concentrations ranging from 12.5–250 μg/mL and 25–500 μg/mL for VitD₃ and Phytosterols, respectively.

For quantification of VitB₁₂, the collected aqueous phase of the digestate was submitted to the same conditions of RP-HPLC as applied for encapsulation efficiency,
while standard curve prepared by diluting VitB\textsubscript{12} on a digestate sample of water at concentrations between 10–400 µg/mL.

All data are expressed as a percentage of transferred bioactive and calculated based on the initial amount of each molecule present in the respective emulsion samples.

4.3.9. Determination of osmolality of the aqueous solutions

The osmolalities of internal and external aqueous phases of emulsions, as well the simulated digestion fluids and stock solutions, were measured using a vapour pressure osmometer (VAPRO 5520, Wescor Inc, Logan, Utah, USA) and standard calibration solutions of 100, 290 and 1000 mmol/kg, to estimate the osmotic pressure gradient between the double emulsion sample and the digestion environment.

4.3.10. Statistical analysis

The differences in group’s mean values were submitted to analysis of variance (ANOVA). TukeyHSD test was carried out when significant differences among groups were obtained. When analysed pairwise, data were tested with Student’s T-test. All statistical tests were done with 95% of significance. The statistical analysis was performed using the free software [R] (www.r-project.org) and Statistica 7.1 for Windows (StatsoftInc, Tulsa, OK, USA).
4.4. Results and Discussion

4.4.1. Characterization of the emulsions

A primary emulsion was prepared with 0.5% NaCas in the inner aqueous phase and 2% PGPR in the oil phase. This primary W<sub>1</sub>/O emulsion was then stabilized with 2% NaCas in the outer phase. The use of both emulsifiers during the homogenization of the primary W<sub>1</sub>/O emulsion contributed to maintenance of the average size of the inner aqueous droplets and resulted in good encapsulation efficiency of the double emulsions (Su et al. 2006; Andrade & Corredig 2016; Gülseren & Corredig 2012). After the first homogenization step, the apparent radius of the primary W<sub>1</sub>/O emulsions was measured by DWS. The same samples were also measured after 7 days storage at 4ºC. As shown in Table 4.1, all primary emulsions showed an apparent radius of about 200 nm. The presence of bioactive molecules did not affect the average radius of the droplets. Furthermore, there was no difference in the average size of the water droplets after 1 week of storage.

Table 4.1. Apparent radius of W<sub>1</sub>/O emulsions with or without hydrophobic bioactive (β-sitosterol or VitD<sub>3</sub>) measured immediately or after 1 week storage time at 4ºC. Results are average of at least three independent experiments ± standard deviation. Within a row, letters indicate significant differences (p<0.05).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fresh (nm)</th>
<th>1 week (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>187 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>196 ± 21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-sit</td>
<td>206 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>230 ± 31&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VitD&lt;sub&gt;3&lt;/sub&gt;</td>
<td>208 ± 46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>221 ± 40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Figure 4.1 shows representative confocal image of the double emulsion (final ratio of 3:7:90 W<sub>1</sub>/O/W<sub>2</sub>). Small water droplets were quite visible inside larger oil droplets, in
turn dispersed in the external aqueous phase. When measured by integrated light scattering, all double emulsions showed a monomodal distribution of sizes with an average diameter around 6 µm. Table 4.2 shows the average surface mean diameter of the outer droplets ($d_{32}$) for all treatments, with and without model bioactive molecules. There were no differences between treatments, and no significant changes in the average size of the secondary emulsion droplets after 1 week of storage.

![Confocal Scanning Laser Microscopy image of a double emulsion (W₁/O/W₂). Arrows indicate oil phase (O), internal aqueous phase (W₁) and the dispersed phase (W₂).](image)

**Figure 4.1.** Representative Confocal Scanning Laser Microscopy image of a double emulsion ($W_1/O/W_2$). Arrows indicate oil phase (O), internal aqueous phase ($W_1$) and the dispersed phase ($W_2$).
Table 4.2. Apparent diameter (surface mean diameter, \(d_{32}\)), measured by integrated light scattering, for double emulsions (W₁/O/W₂) prepared with or without hydrophobic bioactives. Values are also shown after storage at 4°C for 7 days. The results are the average of three independent experiments ± standard deviation. Within a row, letters indicate significant differences (\(p<0.05\)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>(d_{32}) (µm)</th>
<th>Fresh</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>5.9 ± 0.0ᵃ</td>
<td>6.0 ± 0.2ᵃ</td>
</tr>
<tr>
<td>β-sit</td>
<td></td>
<td>6.0 ± 0.4ᵃ</td>
<td>6.0 ± 0.4ᵃ</td>
</tr>
<tr>
<td>MgCl₂ / VitD3</td>
<td></td>
<td>5.8 ± 0.7ᵃ</td>
<td>6.0 ± 0.6ᵃ</td>
</tr>
</tbody>
</table>

Although the primary W₁/O emulsion did not show any visible sign of instability during the study (Table 4.1), and there were no changes in the particle size of the secondary emulsion (Table 4.2), the final double emulsions showed fast creaming (approximately 30 min), because of the large particle size of the double emulsion. The creaming was fully reversible upon mixing. Controlling the creaming rate was out of the scope of this work, as other stabilizers would need to be included in the research study.

To follow possible changes in the integrity of the inner aqueous phase, the amount of Mg\(^{2+}\) recovered in the inner aqueous phase was quantified, by measuring the concentration released in the subnatant (Table 4.3). All double emulsion samples showed Mg\(^{2+}\) encapsulation efficiency of approximately 95% even after 1 week of storage at 4°C, in agreement with previous reports (Andrade & Corredig 2016; Herzi et al. 2014).
Table 4.3. Encapsulation efficiency of Mg$^{2+}$ ions present in the internal aqueous phase of double emulsions measured fresh and after storage at 4ºC for 7 days. Results are average of two independent experiments ± standard deviation. Within a row, letters indicate significant differences ($p<0.05$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fresh (%)</th>
<th>1 week (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>95.5 ± 0.1$^a$</td>
<td>94.7 ± 0.0$^b$</td>
</tr>
<tr>
<td>β-sit</td>
<td>95.2 ± 0.2$^a$</td>
<td>94.6 ± 0.2$^b$</td>
</tr>
<tr>
<td>VitD3</td>
<td>95.1 ± 0.5$^a$</td>
<td>95.0 ± 0.5$^a$</td>
</tr>
</tbody>
</table>

Similar encapsulation values were also obtained for emulsions containing VitB$_{12}$ in the inner phase. Emulsions showed about 97.1 ± 0.2% encapsulation in the freshly made emulsions and 93.8 ± 5.7% for the samples stored for one week at 4ºC. The differences during storage were not statistically significant after a week. It is important to note that the high value of encapsulation obtained for both Mg$^{2+}$ and VitB$_{12}$ demonstrated that the centrifugation method used to separate the double emulsions from the outer water phase did not cause internal droplet disruption, and the values obtained were not influenced by the nature of the hydrophilic molecule used in the study.

4.4.2. *In vitro* digestion

The fresh emulsions were subjected to *in vitro* digestion experiments, as described in the literature (Minekus et al. 2014). After the addition of the simulated gastric fluids and pepsin to the emulsion samples, after 30 min incubation, the pH reached 3.5 in all cases. When duodenal fluids (pH 7.0) containing bile salts, pancreatin, phospholipids and PLA$_2$, were added, the pH increased from 3.5 to 6.7. This value was then maintained throughout the processing time. It is important to note that these pH values are within the range of maximum activity of the digestion enzymes (Minekus et al. 2014).
To explore changes in the interfacial area of the $W_1/O/W_2$ emulsions, the evolution of the droplets through the digestion process was investigated. Figure 4.2 shows CSLM images of control emulsions at different times of \textit{in vitro} digestion. At the end of the gastric stage, the images revealed that little structural changes had occurred (Figure 4.2A). After the gastric phase, during the early stages of the duodenal phase, coalescence of the inner water droplets was observed (Figure 4.2B-F). As digestion progressed, a lower number of double emulsion droplets was observed and after 1 h, only single oil droplets were visible by confocal imaging, indicating that most of the internal water droplets were transferred to the digestate aqueous media.

![Figure 4.2](image)

**Figure 4.2.** Representative CSLM images of double emulsion controls (no bioactive added) during \textit{in vitro} digestion progression of $W_1/O/W_2$. (A) after 30 min under gastric environment, taken as starting point and followed by 5 min (B), 12.5 min (C), 25 min (D), 1 h (E) and 2 h (F) of duodenal phase.
The evolution of the droplet size distribution during digestion for control emulsions (containing no hydrophobic bioactive) or emulsions loaded with Phytosterols or VitD₃ was followed by light scattering. Figure 4.3 compares the size distribution before and after the gastric stage. All the emulsion samples showed similar monomodal distributions. When low protein levels are used and the sample is submitted to a gastric environment, protein-stabilized emulsions suffer major changes due to proteolysis of the interfacial layer and low pH (Singh & Sarkar 2011). In this work, the amount of NaCas used provided an interface sufficiently thick to promote steric stabilization, causing the oil droplets to remain stable under gastric conditions (Sarkar et al. 2009).

Figure 4.3. Particle size distribution of fresh double emulsions (filled circles) and after 30 min of the gastric stage of in vitro digestion. Results are representative of at least 3 experiments. A: control samples; B: Phytosterols supplemented samples; C: Vitamin D₃ loaded samples.

There was a reduction of approximately 1 µm in the average apparent diameter (d₃₂) of the oil droplets. This shrinkage of the oil globules was due to proteolysis, which reduced the thickness of the caseinate layer at the interface, and more importantly, to the change in osmotic balance, which resulted in a release of the inner aqueous phase.
After the gastric phase, during the in vitro duodenal phase, there was a significant increase in the particle size, as shown in Figure 4.4. By the end of the gastric phase, the aggregation of oil droplets started as result of the action of the enzymes and bile salts. Bile salts are very surface active, and together with phospholipids present, they favour the displacement of protein over the external interface, causing coalescence of the oil droplets.

After 5 min of incubation with duodenal fluids, the emulsions showed a polydistributed size, with an average diameter (d$_{32}$) of 7.8±0.8 µm (Figure 4.4). A small population of particles with a diameter of about 1 µm was evident, which may be related to the aqueous micellar phase. After about 12 min the diameter (d$_{32}$) of control double emulsions was 12.0±2.0 µm, with no further changes to the size, until the end of the duodenal incubation. Similar trends were also shown for the emulsions containing the model bioactive molecules, as shown in Table 4.4.

**Figure 4.4.** Particle size evolution of double emulsions submitted to in vitro digestion. A: control samples; B: Phytosterols supplemented samples; C: Vitamin D$_3$ loaded samples. Symbols: ● starting point after 30 minutes after gastric phase, ○ after 5 min, ▼ 12.5 min, ▲ 25 min, □ 1 h and ◆ 2 h duodenal phase. Results are representative of minimum three distinct experiments.
Table 4.4. Average apparent surface mean diameter (d_{32}) of W₁/O/W₂ emulsions samples during *in vitro* digestion. Results are average of three independent experiments ± standard deviation.

<table>
<thead>
<tr>
<th>Digestion Stage</th>
<th>Time (min)</th>
<th>Control (µm)</th>
<th>β-sit (µm)</th>
<th>VitD₃ (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric</td>
<td>30</td>
<td>4.8 ± 0.1</td>
<td>4.9 ± 0.6</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Duodenal</td>
<td>5</td>
<td>7 ± 3</td>
<td>9 ± 3</td>
<td>8 ± 2</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>11 ± 3</td>
<td>14 ± 4</td>
<td>11 ± 1</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>12 ± 1</td>
<td>14 ± 3</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>11 ± 2</td>
<td>13 ± 3</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>8 ± 1</td>
<td>12 ± 4</td>
<td>9 ± 2</td>
</tr>
</tbody>
</table>

4.4.2.1 Lipid hydrolysis and transfer of bioactives

During the *in vitro* gastric stage, low levels of free fatty acids were measured (data not shown). In this work, the concentration of porcine pancreatin in the *in vitro* simulated duodenal juice was optimized to obtain a rate of hydrolysis sufficiently slow to follow the release of the bioactive compounds (see methods). In all double emulsion samples, approximately 30% of the oil phase was digested after 2.5 h experiment (Figure 4.5A). There were no significant differences between treatments.
Figure 4.5. Percentage of free fatty acid released (A) and hydrophobic bioactive transferred to the aqueous micellar phase (B) during the duodenal stage of in vitro digestion. Results are representative of minimum three experiments. Bars indicate the standard deviation.

The percentage of transfer of hydrophobic bioactives into the aqueous micellar phase as a function of time of digestion is shown in Figure 4.5B and it was not always in line with the free fatty acid release shown in Figure 4.5A, but it was dependent on the type of hydrophobic bioactive present (Figure 4.5B). In a study with different types of carotenoids (Borel et al. 1996) it was found that depending on the polarity of the molecules, there would be a preferential location towards the droplet surface (more polar) or the core (more apolar), causing a difference in the release during lipolysis.

Control samples contained a small percentage of Phytosterols (naturally present in the soybean oil); these samples showed a similar trend in the release to that of the Phytosterols supplemented double emulsions. These samples showed about 30% of the total bioactive released by the end of the duodenal phase, and the release was well correlated with the release of the free fatty acids ($R^2=0.989$). The correlation value was similar to the one previously reported in a work with O/W emulsions (Malaki Nik, Corredig & Wright 2011). These molecules may be well distributed within the oil droplets.
On the other hand, this was not the case for the release of VitD₃. The release of VitD₃ was significantly lower, at 16% - with a plateau reached after 5 minutes of duodenal exposure. These findings are in full agreement with Day et al. (Day et al. 2010), where VitD₃ was found to concentrate within the oil droplet during digestion. The presence of lyso-phospholipids, product of the hydrolysis of phospholipids by PLA₂, was shown to play an important role on micellization and transfer of carotenoids (Sugawara et al. 2001; Malaki Nik et al. 2010). Even with these components present, the release was limited, and it was concluded that the molecules are concentrated within the oil droplet.

Bile salts together with other surface active molecules will aid the bioactive transfer of hydrophobic molecules to mixed micelles. Interfacial composition may affect the transfer of VitD₃. To test this hypothesis, the effect of the presence of PGPR at the interface was evaluated in respect to bioactive release. Simple O/W emulsions (with no PGPR) with a similar particle size distribution to W₁/O/W₂ emulsions (with PGPR) were submitted to in vitro digestion to evaluate the transfer of VitD₃. The same trend of distribution of sizes of the particles during the digestion was obtained (data not shown); however the O/W emulsions showed a statistically significant lower concentration of lipid hydrolysis products (about 23%). In contrast, there was no significant difference in VitD₃ release profile, regardless of the type of emulsion. After 2 h of duodenal digestion, 15% of VitD₃ present in the single oil droplets were transferred to mixed micelles. The results suggested that the interfacial composition does not play a role in the release and transfer of VitD₃ to mixed micelles and supports the surface-to-core argument for the release of hydrophobic compounds such as VitD₃.

The release of water soluble compounds was also studied, by using VitB₁₂ in the inner aqueous phase of the double emulsions. Figure 4.6 illustrates the amount of transfer of the model hydrophilic molecule during the in vitro duodenal stage. Contrary to what was
recently suggested (Kaimainen et al. 2015), the results indicated that the oil layer does not protect the inner aqueous phase from the release. Right at the initial stages of the duodenal digestion about 87% of the VitB\textsubscript{12} entrapped in the inner aqueous phase was released.

![Figure 4.6](image)

**Figure 4.6.** Percentage of Vitamin B\textsubscript{12} transferred from the inner aqueous phase of double emulsion during *in vitro* duodenal stage. Results are average of three independent experiments ± standard deviation.

Using a simpler *in vitro* model (Frank et al. 2012), with simulated gastric fluids at a lower ionic strength, it was suggested that there may be a contribution of the osmotic balance to the release of the internal water droplets during double emulsion digestion. In this study, the contribution of the gastric and duodenal fluids to the osmolality of the environment is evident. The osmolalities found for the internal and external aqueous phases of VitB\textsubscript{12} emulsions were 947±4 mmol/kg and 19±1 mmol/kg, respectively. The osmolality of the simulated gastric fluids (corrected for dilutions) was 113±2 mmol/kg, and after mixing gastric and duodenal fluids, the osmolality was 185±2 mmol/kg. This increase in osmolality of the environment where the oil globules are dispersed was insufficient to protect the inner droplets from the release during digestion, and that the high release of
VitB$_{12}$ was promoted by the gastric stage. After 5 min, a plateau seemed to be reached, where approximately 93% of VitB$_{12}$ was transferred (Figure 4.6).

4.5. Conclusion

This work described the formulation of a food grade double emulsion system carrying hydrophilic and hydrophobic bioactives and the changes promoted by an *in vitro* digestion process. Our findings demonstrated that the double emulsion structure was kept stable during 30 min of gastric simulated digestion. The transfer of hydrophilic bioactive was not delayed by the presence of surrounding oil layer, with 87% of VitB$_{12}$ released at the gastric stage. VitB$_{12}$ release was influenced by the osmolality of the environment. In the case of hydrophobic bioactive molecules, the transfer depended on their surface-to-core distribution. Furthermore, the release did not seem to be affected by possible interactions between the bioactive molecules and emulsifiers as observed in the previous chapter of this thesis. Indeed, VitD$_{3}$ solubilisation within the digestate was not affected by interfacial composition, that is, the type of emulsion (O/W or W$_1$/O/W$_2$).
Chapter 5: *In vitro* digestion behaviour of Water-in-Oil-in-Water emulsions with gelled oil-water inner phases

**Abstract**

Double emulsions may be able to protect and release in a controlled manner bioactive compounds during digestion of food matrices. It was hypothesized that the physical state and solid content in the inner phases of water-in-oil-in-water (W₁/O/W₂) emulsions may affect the overall stability and the release behaviour of bioactives during *in vitro* digestion. Therefore, hydrophobic (Phytosterols or Vitamin D₃) and hydrophilic (Vitamin B₁₂) molecules were incorporated in double emulsions prepared either with a liquid (soybean oil – SO) or oil-fat gel (soybean oil + trimyristin – STO) lipid phase and liquid internal aqueous phase. In addition, the impact of a gelled inner aqueous phase was studied, using high methoxyl pectin. W₁/O/W₂ emulsions were prepared with PGPR and sodium caseinate as emulsifiers. After the 30 min *in vitro* gastric stage, all double emulsions showed no significant change in size. Lipid crystals were visible in the STO emulsions. Fat crystallization, and the formation of an oil fat gel, led to coalescence of the inner aqueous droplets. The inner aqueous droplets were no longer visible by confocal microscopy after the initial stages of 2 h *in vitro* duodenal digestion. Fat crystals and droplets of non-spherical shape were also noted in the STO double emulsions up to 25 min of *in vitro* duodenal stage. Overall, the STO emulsions had a higher extent of free fatty acid release and consequent bioactive transfer compared to the SO emulsions. The presence of the medium chain fatty acids (from trimyristin), in addition to the surface-to-core distribution of the hydrophobic bioactives within the oil droplet were key factors in lipid digestibility and bioactive release. The STO and SO samples did not differ in terms of the release of the hydrophilic molecule, Vitamin B₁₂, over time. On the other hand, there was a significant increase in the stability of the inner water phase, after gastric digestion, when this phase was gelled with high methoxyl pectin. This work demonstrated that the physical properties of the different internal phases of W₁/O/W₂ influenced lipid digestion and bioactive transfer kinetics during *in vitro* digestion.
5.1. Introduction

Double emulsions, such as water-in-oil-in-water (W₁/O/W₂), are multi-compartmentalized systems that consist of a water-in-oil emulsion (W₁/O) dispersed in a secondary aqueous phase (W₂). They are interesting as delivery systems with some advantages over simple emulsion. For many compounds, a simple emulsion delivery system does not offer the desired properties in terms of solubilisation, protection against chemical degradation or controlled release of nutritional compounds. In principle, in a W₁/O/W₂, the water droplets inside the oil droplets can be used to deliver hydrophilic molecules, when they need to be separated from the outer aqueous phase. The oil phase can alternatively be used to deliver a hydrophobic compound (Sagalowicz & Leser 2010; McClements et al. 2007).

Double emulsions have been studied over the years in a wide variety of applications in the environmental (Palencia & Rivas 2011), pharmaceutical and cosmetic areas (Martí-Mestres & Nielloud 2002). There has been an increased interest in the use of double emulsions in foods, especially for applications involving the encapsulation of microorganisms (Pimentel-González et al. 2009), fortification of foods with vitamins (Giroux et al. 2013), modulation of sensory properties of a food matrix and to reduce fat content of food products (Cofrades et al. 2013; Muschiolik 2007; Rodrì et al. 2008). In spite of their potential value, these systems are difficult to stabilize. The main instability issues are swelling or shrinkage of the inner droplets, flocculation, coalescence, all leading to the release of the inner droplets in the outer phase, and phase separation (Dickinson 2011). Due to the presence of two interfaces, at least two different emulsifiers have to be used.
The interfaces are stabilized by various emulsifiers, and it has been shown that molecules such as vitamins, sterols, carotenoids, or biopolymers often used in the formulation of such complex emulsions, can interfere with the emulsifiers, affecting the properties of the interfaces (Andrade & Corredig, 2016; Aditya et al., 2015; Gülseren & Corredig, 2012, 2014; Rodríguez Patino & Pilosof, 2011).

It is also known that the phases utilized in a double emulsion need to be well designed to ensure long term shelf life of the system. For example, it has been reported that the stability and encapsulation efficiency of double emulsions may be improved by causing gelation of the inner water phase, by addition of whey protein isolate, starch or gelatin under heat treatment (Oppermann et al. 2015; O’Regan & Mulvihill 2010; Iancu et al. 2009; Surh et al. 2007). In situ gelation of the inner droplets can be achieved using enzymes specific for biopolymers. In particular, it was shown for water in oil droplets containing high methoxyl pectin (HMP) in presence of calcium ions (Massel 2011).

Furthermore, structuring of the oil phase in an emulsion can be obtained by the addition of solid fats; these high melting triacylglycerol molecules can be dispersed into the oil phase at temperatures above their melting points, and subsequent cooling causes the formation of W/O emulsion gels (Patel 2015; Patel & Dewettinck 2015; Wright & Marangoni 2006; Irmscher et al. 2015). In W/O emulsions containing solid lipids, the presence of a fat crystal network in the oil phase can arrest water droplet movement, preventing drop coalescence (Rousseau & Hodge 2005). However, very little is known about the properties of such complex systems when used to design multiple emulsions.

Few studies are available reporting the stability and breakdown of double emulsions under gastrointestinal environment. Recently, an in vitro intestinal digestion was applied to investigate a W₁/O/W₂ and the release of entrapped beet extract was followed. It was suggested by the authors that the double emulsion system can act as a barrier to protect
hydrophilic bioactives, of which a gradual release can be obtained during oil digestion (Kaimainen et al. 2015). In another study, it was proposed that the emulsifier present at O/W interface plays a role on the stability of the system in the gastro intestinal tract (Frank et al. 2012). In both studies, the release of the internal aqueous phase contents seemed to be triggered by the presence of bile salts and the action of intestinal enzymes.

In the present work, it was hypothesized that by changing the physical properties of the inner droplets and/or the lipid phase, it would be possible to influence the overall stability of the double emulsions, and additionally, the release behavior of bioactive molecules during in vitro digestion.

The objective of this study was to evaluate if the physical state of the internal phases of a double emulsion would affect their in vitro digestion behaviour and the release and transfer of model bioactive molecules, namely, Phytosterols (β-sitosterol as main compound), Vitamin D3 and Vitamin B12. Emulsions were prepared with a liquid or gelled internal water phase, and with an oil phase containing either of soybean oil (SO) or soybean oil and 15 % trimyristin (STO). The inner aqueous phase contained HMP, and was gelled in-situ with pectin methyl esterase, an enzyme specific for HMP.

5.2. Material and Methods

5.2.1. Materials

The following materials were obtained from Sigma Aldrich (St. Louis, MO): soybean oil (S7381), Phytosterols (β-sitosterol (β-sit) as main compound – 85451), Vitamin D3 (VitD3) (cholecalciferol – C9756), Vitamin B12 (VitB12) (cyanocobalamin - V2876), Nile Red (72485), butylated hydroxytoluene (BHT) (W218405), pepsin from porcine
pancreas mucosa (P7000, 434 U/mg of powder), pancreatin from porcine pancreas (4xUSP – P1750 – lipase activity: 18.5 U/mg of powder), phospholipase A2 from porcine pancreas (P6534 – 902U/mL). Sodium caseinate (NaCas) (NaCas 180) was purchased from Fonterra (Rosemont, IL). Calcium chloride dehydrate (CaCl2(H2O)2 – C79), potassium chloride (KCl – P217), monopotassium phosphate (KH2PO4 – P285), sodium bicarbonate (NaHCO3 – S233), sodium chloride (NaCl – S671), magnesium chloride hexahydrate (MgCl2.6H2O – BP214) and ammonium carbonate ((NH4)2CO3 – A656), trimyristin 90% (Acros Organics – Geel, Belgium) and the HPLC grade solvents hexane, methanol, anhydrous ethanol and acetone were obtained from Fisher Scientific (Ottawa, ON, Canada). High methoxyl pectin (HMP) was obtained from CP Kelco (unstandardized, DE 72.8, Lille Skensved, Denmark). Pectin methyl esterase (Fructozym Flot® – Erbslöh Geisenheim AG, Germany) was generously provided by IDL Process Solutions (White Rock, BC, Canada). Fat free soybean phospholipids with 75% phosphatidylcholine (ALCOLEC PC75), were provided by American Lecithin Company (Oxford, CT, USA). Polyglycerol polyricinoleate (PGPR 4150) containing minimum 75% di-, tri- and tetragelycerols with a maximum of 10% of heptaglycerol or higher, was obtained by Palsgaard (Juelsminde, Denmark). Solutions were prepared with ultrapure MilliQ water, unless stated differently.

5.2.2. Differential Scanning Calorimetry (DSC) Measurements

For each blend of trimyristin and soybean oil supplemented or not with hydrophobic bioactives, aliquots (5 to 10 mg) were hermetically sealed in aluminum pans and analyzed by calorimetry (TA Q2000 DSC, TA Instruments, New Castle, DE, USA). After equilibrating at 5°C for 1 min, the samples were heated to 70°C at a rate of 5 °C/min, following isothermal condition for 15 min then cooled to 5°C at -5° C/min. Each formulation was prepared and measured at least twice. The melting onset temperature
(T_{onset}), melting temperature (T_m) and the melting enthalpy (\Delta H) of the bulk oil-fat mixtures were analysed using Universal Analysis 2000 Software (v.4.5A build 4505 – TA Instruments, New Castle, DE, USA). Enthalpy changes involved during the melting event were measured by integrating the area under the curve of the thermograms with the same software.

5.2.3. Emulsion Preparation

A solution containing 0.5% (w/w) NaCas, 100 mM NaCl, 100 mM MgCl$_2$ and VitB$_{12}$ 1% (w/w) (when necessary), was used as primary aqueous phase (W$_1$). The ions entrapped within the W$_1$ phase contributed to maintaining the stability of the inner aqueous phase, by balancing the osmotic pressure gradient with the external aqueous phase (Rosano et al. 1998; Sapei et al. 2012). The concentrations of NaCas and PGPR chosen were previously found to be effective in stabilizing the primary W$_1$/O emulsions (Gülseren & Corredig 2012; Su et al. 2006). The liquid oil phase consisted of mixing at room temperature 2% (w/w) PGPR with or without 0.5% (w/w) of the hydrophobic bioactive in soybean oil.

Partial crystallization of the oil phase was obtained by adding 15% (w/w) trimyristin in the oil mixture. The mixture of soybean oil and solid fat was heated under stirring to reach 65°C and held for 5 min. This ensured that the fat crystals were completely melted (trimyristin melting point: 58°C). The mixture was then cooled down at -3°C/min to 4°C to form an oil-fat gel that was stored overnight at room temperature.

In the experiments where a gelled internal aqueous phase was required, the inner water phase consisted of a solution containing 1.9% (w/w) HMP, 0.1 M CaCl$_2$ and 1% (w/w) VitB$_{12}$, mixed with pectin methyl esterase (Fructozym Flot®). The inner water phase was then added to the oil phase, and in situ gelation occurred after homogenization of the
W₁/O (Massel 2011). The enzyme promotes the cleavage of the methyl ester groups from the galacturonic acid chain and increases the number of free carboxylic groups, which are sensitive to calcium ions. The reaction causes the formation of a gel like network by forming ionic bridges between adjacent pectin chains (Braccini & Pérez 2001).

Water-in-oil primary emulsions were prepared by mixing 30% (w/w) of aqueous phase and 70% (w/w) oil phases. The coarse W₁/O emulsion was formed by premixing with an ultra turrax (PowerGen 125, Fisher Scientific), for 1 min at 12000 rpm. This coarse emulsion was then passed through a high pressure homogenizer (EmulsiFlex C5, Avestin, Ottawa, Canada) 10 times at 70 MPa to obtain the final W₁/O emulsion.

To obtain the final W₁/O/W₂ emulsion, 10% (w/w) W₁/O emulsion was mixed with 2% (w/w) NaCas solution (prepared with MilliQ water). The final ratio of the W₁/O/W₂ emulsion was 3:7:90 (based on volume fraction). This second emulsification step was carried out by passing the formulation once through the high pressure homogenizer at approximately 3.5 MPa, to obtain oil droplets large enough to incorporate the inner aqueous droplets. For emulsions containing the partially crystalline lipid phase, the oil-fat gel (O) was first heated to 65°C in shaking water bath and the correspondent aqueous phase (W₁) added. In these samples, the emulsion preparation was carried out at 65°C water bath and the final emulsion was then cooled down at a rate of -3°C/min, to room temperature.

5.2.4. Light scattering measurements

Droplet size distribution of the double emulsions before and during digestion were determined using integrated light scattering (Malvern Mastersizer 2000S, Malvern Instruments Inc, Westborough, MA) with distilled water as the dispersant material (refractive index 1.33) at room temperature. It was assumed that the refractive index of the
oil globules (1.473 – soybean oil) did not change in the presence of the internal aqueous droplets (Pawlik et al. 2010; Bonnet et al. 2010). Both water and NaCas solution showed osmolality values were below the measurement uncertainty of the equipment (9±1 and 19±1 mmol/kg, respectively).

5.2.5. Microscopy

The emulsions microstructure before and during digestion was observed using Confocal Scanning Laser Microscopy (CSLM). Only control emulsions, with no bioactive molecules were measured by microscopy, as 0.01% of Nile Red (Sigma Aldrich, St. Louis, MO) was added in the oil phase. The samples were placed on concave glass slide and covered with a cover slip. The Upright Leica TCS SP2 microscope (Leica Microsystems, Heidelberg, Germany) was used with oil-immersion 100 x magnification lenses and a 50 mW 488 nm Ar laser. Images shown are representative of at least 5 images from 2 independent experiments.

The images of primary W₁/O emulsion samples were used for droplet diameter calculations. Droplets were randomly chosen from 5 images obtained from 2 slide preparations for each sample. The diameter of minimum 20 droplets were measured in pixels using GNU Image Manipulation Program (GIMP 2.8.14) and the calculations were promoted accordingly to the number of pixels of the scale bar at the corresponding image.

5.2.6. Encapsulation efficiency of Vitamin B₁₂

The encapsulation efficiency of the inner water phase was evaluated measuring the residual amount of VitB₁₂ present in the subnatant aqueous phase after centrifugation as
described previously in section 4.3.5. Similarly, the calculation of the percentage of encapsulated material was based on Eq. 2, section 3.3.5.

5.2.7. *In-vitro* digestion

The *in vitro* digestion experiments were performed following the international consensus protocol (Minekus et al. 2014) without the initial oral digestion. The description of the method was presented in section 4.3.6. In this Chapter, however, the pancreatin from porcine pancreas used in the duodenal fluids was added in order to reach the lipase activity of 300 U/mL.

2.8. Determination Free Fatty Acids (FFA)

Hydrolysis of the oil droplets was quantified by colorimetric method after extraction under acidic conditions as described on section 4.3.7 of this thesis.

5.2.9. Isolation of aqueous micellar phase and quantification of bioactives

To separate the micellar fraction from the undigested oil droplets and other solids present in the digestate, the digestate was submitted to the conditions described on section 4.3.8. In the same section, it was described the methodology used for measuring the amount of bioactive molecules.

The data obtained are expressed as a percentage of transferred bioactive, calculated based on the initial amount of each molecule present in the respective sample.
5.2.10 Statistical analysis

The data was submitted to analysis of variance (ANOVA/factorial ANOVA). When analysed pairwise, Student’s T-test was used. All statistical tests were done with 95% of significance and using the Statistica 7.1 for Windows (Statsoft Inc, Tulsa, OK, USA).

5.3. Results and Discussion

5.3.1 Characterization of oil-fat gel lipid phase

The melting profiles of the bulk lipid phases containing 15% trimyristin were measured by DSC. The melting onset temperature ($T_{onset}$), melting temperature ($T_m$) and the melting enthalpy ($\Delta H$) of the oil-fat gels are shown in Table 5.1. Both temperature and enthalpy values were not statistically different amongst the samples, indicating that the presence of the bioactive molecules did not affect the melting behavior of the fat mixture. The melting temperature was approximately 44.5 ºC and with melting onset temperatures in the range of 38-41ºC. Therefore, during the *in vitro* digestion experiments carried out at 37ºC, the fat crystals would be very close to their melting temperature. The properties of trimyristin-soybean oil emulsions (STO) may be different from those of soybean oil emulsions (SO), not only during storage, but also during digestion.
Table 5.1. Melting properties (melting temperature, $T_m$; melting enthalphy, $\Delta H$; temperature of onset $T_{onset}$) of the bulk mixtures of partially crystalline lipid containing 15% trimyristin and 85% soybean oil. Values are the average of at least two independent runs with standard deviation. Control mixtures, and mixtures containing β-sitosterol or Vitamin D$_3$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$T_{onset}$ (ºC)</th>
<th>$T_m$ (ºC)</th>
<th>$\Delta H$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40.8 ± 0.7</td>
<td>44.6 ± 0.4</td>
<td>25.5 ± 1.8</td>
</tr>
<tr>
<td>β-sit</td>
<td>40.9 ± 2.2</td>
<td>44.4 ± 1.1</td>
<td>27.8 ± 0.3</td>
</tr>
<tr>
<td>VitD3</td>
<td>38.8 ± 0.7</td>
<td>45.1 ± 0.2</td>
<td>24.2 ± 0.4</td>
</tr>
</tbody>
</table>

5.3.2 Emulsions characterization

The average diameter of primary W$_1$/O emulsions prepared with soybean oil (SO) was 401 ± 23 nm as estimated using CSLM images. These values were in agreement with previous research results presented on Chapters 3 and 4 of this thesis. In the case of primary W$_1$/O emulsions containing trimyristin and soybean oil (STO), after homogenization and cooling, the diameter of the inner water droplets was 477±76 nm. In this case there was no visible phase separation instability for over a month of storage at 4ºC No significant differences between the treatments were found ($p=0.14$), between STO and SO oil phases, regardless of the presence of hydrophobic bioactives.

After approximately 30 min from manufacture of the double emulsions, creaming was observed in all cases. Creaming was reversible after mixing, with no difference in the particle size distribution of the double emulsions, as measured by integrated light scattering. Therefore creaming was not a concern in this work, particularly as the emulsions were used fresh. No other stabilizer was introduced, in order to reduce the complexity of the experimental design.
Figure 5.1 shows the particle size distribution of the double emulsion samples without hydrophobic bioactive supplementation, freshly made and after 7 days of storage. A monomodal size distribution was shown for all samples, including the case of emulsions containing the hydrophobic bioactives, and containing SO or STO (data not shown). Table 5.2 summarizes the average surface mean diameter ($d_{32}$) of fresh emulsion droplets containing VitB$_{12}$ and the hydrophobic bioactives, prepared either with SO or STO. The fresh emulsions had a smaller average size when STO was employed, especially in the presence of β-sit. In this case, a small population if droplets was present in the range of $<$ 3.5 µm.
Figure 5.1. Particle size distribution of control W₁/O/W₂ emulsions (without hydrophobic bioactive). Emulsions were prepared with (A) liquid inner aqueous phase and SO as lipid phase; (B) liquid inner aqueous phase and oil-fat gel (STO); (C) inner aqueous phase gelled with HMP and SO as lipid phase; (D) inner aqueous phase gelled with HMP and oil-fat gel (STO). Results are representative of minimum two distinct experiments. Measurements carried out by integrated light scattering on (●) fresh sample; (○) after 7 days of storage at 4°C.
Table 5.2. Surface mean diameter (d_{32}) of W_{1}/O/W_{2} emulsions prepared with soybean oil (SO) or soybean oil with trimyristin (85/15 w/w, STO) and containing entrapped Vitamin B_{12} in the inner aqueous phase (control) or with addition of β-sitosterol or Vitamin D_{3}. Values shown are for freshly prepared emulsions. Results are the average of two independent experiments ± standard deviation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>d_{32} (µm)</th>
<th>SO</th>
<th>STO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8 ± 0.1</td>
<td>4.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>β-sit</td>
<td>6.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>VitD_{3}</td>
<td>5.6 ± 0.1</td>
<td>3.5 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

After 1 week of storage, control emulsions prepared with SO (Figure 5.1A) and samples loaded with VitD_{3} or β-sit showed no significant difference in size distribution over 7 days of storage at 4°C (Table 5.2). Emulsions containing crystalline fat (STO) and liquid inner aqueous phase had monomodal distribution as well (Figure 5.1B). On the other hand, with storage, there was evidence of instability, with aggregates appearing within 3-4 days.

Emulsions with a gelled internal water phase (containing HMP) and SO showed a monomodal distribution of droplets (Figure 5.1C), but with a smaller average diameter when compared to control emulsions (made with SO and without HMP). The d_{32} for these emulsions was 3.1 ± 0.1 µm with no significant differences observed after 7 days of storage at 4°C. The smaller sizes obtained for the emulsions with a liquid lipid phase and gelled with HMP might be an effect of the homogenization process. Emulsions with HMP that were formulated with STO lipid phase (Figure 5.1D) had a particle size distribution with the main peak in the same range as the others, but with a larger base, with average diameter of 4.1 ± 0.4 µm, similar to control SO and STO (Table 5.2). Similarly to control STO, sample HMP-STO showed aggregates within 3-4 days of storage.
These results confirmed what has been reported in the literature. Gelation of the inner aqueous phase of double emulsions does not affect the average size or physical stability of the final emulsion (Surh et al. 2007). In addition, gelation may increase the stability of double emulsions during storage, or during exposure to processing conditions such as shear and heat (Oppermann et al. 2015). Inner droplets coalescence may also be avoided by gelling the internal phase of double emulsions (Massel 2011).

In conclusion, the freshly prepared double emulsions prepared with HMP (inner aqueous phase) or STO (oil-fat phase), i.e. where one of the phases was in a solid-liquid state, showed the same particle size distribution behaviour as those containing liquid inner phases.

The microstructure of the emulsions was observed using confocal microscopy. In emulsions containing liquid oil (SO) or oil-fat gel (STO) as lipid phase, it was possible to clearly observe the presence of the small water droplets within the oil droplets (Figure 5.2). In the STO emulsion images obtained at higher magnification, droplets with non-spherical shape were observed, in contrast to SO sample. Previous research suggested that presence of solid lipids could create a fat crystal network within the lipid environment that could impair the movement of the water droplets of W/O emulsions (Rousseau & Hodge 2005). The non-spherical shapes observed in some of the inner droplets of STO emulsions was an indication of the formation of a fat crystal network. In addition, elongated structures were visible in STO sample which may be associated to solid fat crystals (Figure 5.3). Similar to the other samples, emulsions with a gelled internal water phase (containing HMP) and SO also showed small water droplets within the oil droplets (Figure 5.4).
**Figure 5.2.** $W_1/O/W_2$ emulsion samples obtained by Confocal Scanning Laser Microscopy. Oil phase was loaded with 0.01% Nile red. (A) Control SO emulsion. (B) Control STO.

**Figure 5.3.** $W_1/O/W_2$ emulsion sample containing oil-fat gel (STO) as lipid phase. Droplets with non-spherical shape were visible in addition to elongated structures (fat crystals) within the lipid phase.
Emulsions were also prepared containing VitB\textsubscript{12} in the inner water phase, in order to follow the fate of the aqueous bioactive in gelled systems. The fresh emulsions showed, in all cases, high encapsulation efficiency for VitB\textsubscript{12} (i.e. 95 ± 1%), regardless of the presence of STO or SO, or with a gelled inner water core. In particular, in the case of the emulsions prepared by gelling HMP in the core with liquid SO, the average values found for encapsulation of VitB\textsubscript{12} were 98 ± 2% of fresh and 98 ± 2% after one week of storage. The emulsion with a gelled water core as well as a partially crystalline (oil-fat gel) lipid phase resulted in 96 ± 2% encapsulation of VitB\textsubscript{12}. The samples prepared with STO were not analyzed after one week of storage due to destabilization of the oil phase, as mentioned previously.

5.3.3. \textit{In vitro} digestion

Freshly made emulsions were subjected to \textit{in vitro} digestion, starting with a gastric stage for 30 min. SDS-PAGE electrophoresis analysis of the emulsions after 30 min
demonstrated that this time was sufficient to hydrolyze the proteins, as only traces of small molecular weight peptides were evident (data not shown).

Overall, all emulsions, regardless of their composition, showed the same pattern of size distribution during the *in vitro* digestion process as the controls (without hydrophobic bioactive supplementation), which are shown in Figure 5.5. After 30 min of *in vitro* gastric digestion, the samples still had a size distribution similar to that of the fresh, undigested emulsions. After the gastric stage, the oil droplets had a monomodal size distribution, but with reduction in the average size ($d_{32}$), of about $<0.5$ µm. The shrinkage was due to a reduction of the protein interfacial layer and the release of the content of the inner aqueous droplets. This release was confirmed by the dramatic decrease in the amount of encapsulated VitB$_{12}$ (see below).
**Figure 5.5.** Particle size distribution of control W/I/O/W emulsions (without bioactive) during *in vitro* digestion. (A) liquid inner aqueous phase and SO as lipid phase; (B) liquid inner aqueous phase and oil-fat gel (STO); (C) inner aqueous phase gelled with HMP and SO as lipid phase; (D) inner aqueous phase gelled with HMP and oil-fat gel (STO). Results are representative of minimum two distinct experiments. Measurements carried out by integrated light scattering on fresh emulsion, after 30 min *in vitro* gastric, and after duodenal stage (5, 12.5, 25, 60 and 120 min).

Table 5.3 summarizes the average apparent droplet diameter measured by light scattering during digestion, for emulsions with a liquid aqueous phase surrounded by either SO or STO fat phase. There were no significant differences between them. During the duodenal stage, the surface active species such as bile salts, phospholipids and enzymes
present in the duodenal fluids caused significant aggregation and coalescence. The changes occurred at the initial stages (10 min) of duodenal in vitro digestion (Figure 5.5A and 5.5B). After 5 min of simulated duodenal digestion, for all emulsions, the average $d_{32}$ increased to about 10 µm (Table 5.3). After the first 10 min of duodenal digestion, the sizes further increased to an average diameter ($d_{32}$) between 12-19 µm. These significant differences in droplet diameter between gastric and duodenal stages were in agreement with the confocal microscopy observations. Emulsions containing gelled pectin in the internal aqueous phase (regardless the lipid phase) revealed the same behavior (Table 5.4 – Figures 5.5C and 5.5D), with an increase in size during the duodenal stage.

Table 5.3. Surface mean diameter ($d_{32}$) measured by light scattering of emulsions at various stages of in vitro digestion. W₁/O/W₂ emulsions were prepared with either soybean oil (SO) or soybean oil and trimyristin (85/15 w/w, STO), and contained also β-sitosterol or Vitamin D₃. Measurements were carried out after 30 min of in vitro gastric and duodenal (5, 12.5, 25, 60 and 120 min) digestion. Results include the standard deviation based on a minimum two independent experiments.

<table>
<thead>
<tr>
<th>Digestion Stage</th>
<th>Time (min)</th>
<th>Control $d_{32}$ (µm)</th>
<th>β-sit $d_{32}$ (µm)</th>
<th>VitD₃ $d_{32}$ (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SO</td>
<td>STO</td>
<td>SO</td>
</tr>
<tr>
<td>Gastric</td>
<td>30</td>
<td>4.6 ± 0.2</td>
<td>3.3 ± 0.3</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>10.4 ± 1.5</td>
<td>11.1 ± 1.0</td>
<td>8.4 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>17.6 ± 1.1</td>
<td>15.4 ± 4.2</td>
<td>11.3 ± 2.7</td>
</tr>
<tr>
<td>Duodenal</td>
<td>25</td>
<td>16.8 ± 1.2</td>
<td>16.5 ± 1.1</td>
<td>14.3 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>16.4 ± 2.9</td>
<td>12.8 ± 0.3</td>
<td>14.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>11.3 ± 1.5</td>
<td>8.5 ± 1.2</td>
<td>9.6 ± 1.5</td>
</tr>
</tbody>
</table>
Table 5.4. Surface mean diameter ($d_{32}$) for W/O/W emulsions containing a gelled inner aqueous phase (containing HMP and two different oil phases (soybean oil, SO or soybean oil and trimyristin, 85/15 w/w, STO). Measurements were taken at various times during *in vitro* digestion. Results described with respective standard deviation of minimum two independent experiments.

<table>
<thead>
<tr>
<th>Digestion Stage</th>
<th>Time (min)</th>
<th>HMP / SO $d_{32} (\mu m)$</th>
<th>HMP / STO $d_{32} (\mu m)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric</td>
<td>30</td>
<td>3.6 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.8 ± 0.1</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Duodenal</td>
<td>12.5</td>
<td>14.9 ± 1.5</td>
<td>11.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>19.8 ± 1.4</td>
<td>10.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>14.0 ± 0.7</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>10.2 ± 1.8</td>
<td>11.6 ± 1.1</td>
</tr>
</tbody>
</table>

Figure 5.6 shows CSLM images of emulsions prepared with a liquid internal water phase, and the oil-fat gel containing STO. The population of oil-fat gel double emulsion droplets after 30 min of gastric stage was smaller when compared with fresh emulsions (Figures 5.2B and 5.6A) and double emulsion droplets still observed after 5 min of duodenal stage (Figures 5.6A and 5.6B). Thereafter, only single oil droplets were observed. Droplets of non-spherical shape could be seen up to 25 min of the duodenal stage (Figure 5.6A-D). It is possible that the fat crystal network inside the lipid droplet melted because the digestion temperature (37°C) was so close to the melting point of the trimyristin-soybean oil mixture. It is also possible to hypothesize that, although a fast melting of the crystalline fat lipid droplets was not promoted, the warm environment allowed movement and coalescence of the inner aqueous droplets. In Chapter 4 of this thesis, with a lower lipase activity, it was shown that double emulsions with formulation similar to SO were still present after 1 h of *in vitro* duodenal digestion.
Figure 5.6. CSLM images of *in-vitro* digestion progression of W1/O/W2 with liquid internal aqueous phase and soybean oil and trimesristion (STO) as lipid phase. (A) after 30 min under gastric environment, followed by 5 min (B), 12.5 min (C), 25 min (D), 60 min (E) and 120 min (F) of duodenal phase. Note difference in the scale bar.

5.3.3.1. Lipid digestion and hydrophobic transfer

To understand the effects of the physical state of the oil phase on the *in vitro* lipid digestion and the transfer of hydrophobic bioactive, emulsions prepared with a liquid internal aqueous phase and SO or STO were studied (Figure 5.7).
Figure 5.7. Percentage of free fatty acid released (A, B) and hydrophobic bioactive transferred to the aqueous phase (C, D) during the duodenal stage of \textit{in vitro} digestion, after being submitted to 30 min gastric conditions. Emulsions were prepared with SO (A, C) or STO (B, D). Control emulsions (black bars); \(^\beta\)-sitosterol (white bars); Vitamin D\(_3\) (gray bars). Note that for control, the amount of bioactive measured is the naturally occurring Phytosterols. Results presented as mean ± standard deviations, obtained from at least two distinct experiments.

As expected, after 30 min of \textit{in vitro} gastric digestion, the amount of FFA was minimal, i.e. below 4%. This low level was due to the absence of gastric lipase and the exposure of lipid domains at the interface of droplets to the acidic environment after proteolysis of the thick layer of caseinate. Overall, levels of FFA from SO samples (Figure 5.7A) were significantly lower than those for the emulsions containing STO (Figure 5.7B). Therefore, the presence of a different fatty acid composition and/or a partially crystalline lipid phase was associated with differences in lipid digestibility. In both cases, a plateau
was reached in the amount of FFA released within the first 15 min of in vitro duodenal digestion. Control and samples supplemented with Phytosterols revealed comparable plateau values within each group: in SO samples, 31 ± 5% and 32 ± 7% ($p = 0.74$) of the lipid were digested in control and β-sit samples respectively, while for the emulsions containing STO, the amount of FFA was approximately 42± 7% and 38± 3% ($p = 0.08$). Interestingly, samples containing VitD$_3$ reached lower plateau levels of FFA (Figures 5.7A and 5.7B) compared to control or β-sit emulsions: 23 ± 5% in SO and 34 ± 4% in STO ($p = 0.0003$), suggesting an effect of VitD$_3$ on the lipid digestion. VitD$_3$ was reported to modulate the activity of different enzymes (Sardar et al. 1996) and also to aid the loss of body fat and treatment of obesity (Ofner et al. 2013).

These results confirm those of Chapter 4 with emulsions containing SO. In this study, the plateau was obtained earlier, as the amount of lipase used was higher than in the previous work. However, even with the higher lipase activity in the simulated duodenal juice, the levels obtained for FFA released in the emulsion control were similar to the earlier study, at about 30%. The amount of FFA released in multiple emulsions containing SO and VitD$_3$ in this study was even lower (23%) than that reported previously (27%). In this study, the quantity of pancreatin added to reach the 300 U/mL of lipase activity resulted into a considerable amount of solids in suspension; this may impact the enzymatic activity (Segura et al. 2004).

Figures 5.7C and 5.7D show the amount of bioactive solubilized in the aqueous phase during the duodenal stage. There were significantly ($p<0.05$) lower transfers of bioactive from emulsions prepared with SO compared to STO. In the case of control emulsion, the amount of Phytosterols naturally present and transferred from the oil phase was measured.
Similar transfer levels of Phytosterols were obtained in the control samples between the groups, with an average of approximately 22% of naturally present Phytosterols transferred once a plateau was reached. When β-sit was added to the emulsion, the % of the bioactive transferred from emulsions prepared with SO was comparable to that of the same emulsion, without additional β-sit (Figure 5.7C). On the other hand, when compared to the amount of β-sit released from the emulsions prepared with STO (Figure 5.7D), the transfer for the SO emulsions was significantly lower. The higher values observed for bioactive transfer for the STO emulsions were in full agreement with the higher levels of FFA released (Figures 5.7B and 5.7D). At plateau, the emulsion containing SO had 32% of the lipid content digested, with a Phytosterol transfer of about 20%, while the emulsion containing STO had had 38% and 36% of FFA released and Phytosterol transferred, respectively. The values of FFA released and total Phytosterols transferred were well correlated ($R^2 = 0.98$). Such correlation was very similar to what previously reported with O/W (Malaki Nik, Corredig & Wright 2011) and with W₁/O/W₂ emulsions (Chapter 4 of this thesis).

In the case of VitD₃ transfer, the relative transfer to the aqueous phase was higher for the STO compared to SO emulsions. In the SO double emulsions, while 23% of the lipid was digested, only 11% of the loaded VitD₃ was transferred to the mixed micelles (Figure 5.7C). On the other hand, in STO double emulsions, with 34% FFA released, 23% of the total VitD₃ was transferred to the aqueous micellar phase.

The higher digestion of the STO emulsion may be due to the higher digestibility of the medium chain triglycerides, compared to long chain triglycerides (Yu et al. 2012). The products of their digestion were also found to have better dispersibility in aqueous media, while the long chain fatty acids tend to accumulate at the oil-water interface until they are removed and solubilised in micelles or precipitated by calcium ions (Li et al. 2011). In
addition, the presence of medium chain fatty acids (generated by hydrolysis during the gastric stage) in the duodenal environment was found to increase the overall activity of pancreatic lipase, while long chain fatty acid can even reduce it (Borel et al. 1994).

The results obtained in this study also are supported by previous results obtained on curcumin release (Yu et al. 2012), reporting better bioaccessibility when solubilized in oleogels containing medium chain triglycerides. Day et al. (Day et al. 2010) observed that during lipid digestion, VitD$_3$ had less affinity with the micellar phase and concentrated within the lipid droplet as the interface was digested. The higher transfer values for Phytosterols compared to VitD$_3$ also seemed to reinforce the idea that depending on the polarity of the solubilized molecule, its surface-to-core distribution within the lipid droplet is a key factor, i.e. more polar towards the surface, less polar, towards the core. According to the data obtained, Phytosterols could partition towards the interface or more evenly within the oil droplet, while VitD$_3$ towards the core of the lipid droplet.

5.3.3.2. Inner aqueous phase release behavior

To better understand the stability of the inner water phase during digestion, the release of VitB$_{12}$ entrapped within the inner aqueous phase of the emulsions was studied. Figure 5.8A shows the influence of supplementation of VitD$_3$ or Phytosterols on the release of VitB$_{12}$ of emulsions with liquid internal aqueous phase. Most of the release occurred at the gastric stage. A plateau was reached after the 5 first minutes of duodenal stage, with 88-90% of VitB$_{12}$ released. There was a significantly lower level of VitB$_{12}$ released in control samples, compared to the emulsions containing β-sit or VitD$_3$. However, the amounts of VitB$_{12}$ released at plateau was within the same order of magnitude.
Figure 5.8. Vitamin B₁₂ transferred to the aqueous media during the duodenal stage of *in-vitro* digestion. (A) Emulsions with liquid internal aqueous phase and soybean oil supplemented or not with hydrophobic bioactives. (B) Emulsions with inner phases with different physical states. SO: liquid W₁ and soybean oil; SOT: liquid W₁ and oil-fat lipid phase; HMP-SO: gelled W₁ and soybean oil; HMP-STO: gelled W₁ and oil-fat lipid phase. Results are representative of two individual experiments and vertical bars denote $p<0.05$ confidence intervals.

Regarding the physical state of the different phases, the release of VitB₁₂ was compared between samples with a liquid inner water phase, and a gelled inner phase (containing HMP) as shown in Figure 5.8B. Regardless of the type of oil present, or physical state of the water phase, all emulsions demonstrated that most of the release occurred in the gastric stage (Figure 5.8B) and following the duodenal stage, the plateau was reached within the first 5 min. These results confirmed the microscopy observations in Figure 5.6 (STO sample), showing relatively few double emulsion droplets remaining at 5 min of the duodenal processing.

Gelation of the inner aqueous phase with HMP resulted in lower levels of VitB₁₂ released within the gastric stage. At the end of the gastric stage, the amount of VitB₁₂ was about 75% in these emulsions, regardless of the type of oil used. At the initial stages of
duodenal digestion, a plateau was reached with values similar to those for the non gelled W1 emulsions. It was suggested that the action of bile salts and intestinal enzymes may trigger the release of the contents of the inner aqueous phase (Kaimainen et al. 2015; Frank et al. 2012). The data obtained is not in agreement to that once the release of VitB12 started prior to the addition of simulated duodenal fluids.

Double emulsions with hydrophilic gelling agents were found to be less sensitive towards an imbalance in osmotic pressure compared to their counterparts without gelling agents (Oppermann et al. 2015). It is known that osmotic pressure balance between the inner phase and external phase play a role on the stability of the emulsion (Mezzenga et al. 2004), therefore, the environment where the emulsion is subjected to (simulated digestion fluids) will influence the release of the entrapped compound. The VitB12 more controlled values obtained when the inner aqueous phase was gelled during gastric and the high one at the end of digestion experiment might have been an effect of a more controlled osmotic pressure balance between the environment and the gelled emulsion.

The results of this study suggest that, overall, the use of double emulsion system as a vehicle designed for oral administration, does not serve as an effective protective barrier to entrapped hydrophilic compounds, in contrast of what exposed by Kaimainen et al. (2015), unless the inner aqueous phase is gelled. The in situ gelation of the inner aqueous phase with HMP in this work resulted in less VitB12 being released in the first 30 min of digestion. When submitted to intestinal tract simulated conditions, the aqueous gel properties such as water holding capacity and the lower sensibility to the osmotic imbalance induced by the gel, seem to be important factors regarding the release behavior of the entrapped compound.
5.4. Conclusions

This work clearly demonstrated that the physical properties of the phases in a complex emulsion can affect the kinetics of digestion as well as the release of bioactive compounds during \textit{in vitro} digestion. These properties may be of interest when designing nutritional food and beverages. The presence of medium chain fatty acids, in addition to the surface-to-core distribution of the hydrophobic bioactive within the oil droplet are factors influencing the lipid digestibility and bioaccessibility of the hydrophobic compounds used. The VitB\textsubscript{12} release from the samples with liquid internal aqueous and lipid phases were not affected by the presence of hydrophobic bioactives solubilized in the soybean oil. Similar data was obtained when the oil phase was liquid or gelled. In contrast, when compared with samples with gelled inner aqueous phase, it was observed a significant lower release of VitB\textsubscript{12} at the end of the gastric stage, demonstrating that the physical properties of the inner aqueous phase can affect the release of this hydrophilic compound.
Chapter 6: General Conclusions

The design of double emulsion matrices for a preliminary evaluation of the system for a potential use as an alternative to reduce fat content of a food product, as a functional food product itself or as a delivery mechanism of microorganisms, multiple drugs, nutrients or bioactive molecules is a challenge. The study of the microstructural breakdown of the emulsion and the relation with lipid digestion and bioactive transfer kinetics, provides valuable knowledge when considering formulations intended for oral administration.

The present work aimed to study the characteristics of interfaces adsorbed by different emulsifiers and the effects of the presence of loaded molecules in double emulsions. The effects of bioactive compounds interacting with emulsifiers at the interface, the stability and encapsulation efficiency of double emulsions loaded with these bioactives, the in vitro digestion behavior, the effects of interactions at the interface on the bioactive release profile, and the influence of the physical state of the inner water and lipid phases during the digestion of emulsions were evaluated.

In the first part of this research, the oil-water interfaces were studied to observe possible interactions between the synthetic emulsifier PGPR, milk proteins and mixtures. The fact that it was known that other molecules (e.g. pectin) can change interfacial characteristics lead us to hypothesize that when hydrophobic bioactives are dispersed in the oil phase, interactions at the oil-water interface could happen and they would alter the physico-chemical and viscoelastic properties of the interface and ultimately, influence the stability of W\textsubscript{1}/O and W\textsubscript{1}/O/W\textsubscript{2} emulsions.

When PGPR and milk proteins were present at the interface, the viscoelastic properties were very similar to those observed with PGPR alone, even with the observation
of a synergistic effect on the decrease of interfacial tension after the addition of proteins. This suggested a PGPR domination over the proteins. The model bioactives Vitamin D₃ and Phytosterols (β-sitosterol) were solubilized in the oil phase and increased the interfacial tension of interfaces adsorbed by mixed emulsifiers, revealing an ability to influence the adsorption properties of the emulsifiers. Sodium caseinate and PGPR was the emulsifier mixture most affected by the presence of hydrophobic bioactives. W₁/O and W₁/O/W₂ containing PGPR and sodium caseinate as emulsifiers were successfully produced, with high encapsulation efficiency of water soluble molecules in the inner phase. It was also observed that interactions of emulsifiers and hydrophobic bioactives may influence the short term storage stability of the emulsions.

These changes in the physico-chemical properties of emulsions brought to the hypothesis that these type of interactions at the interface could impair the transfer behavior of the loaded hydrophobic compounds during in vitro digestion. Therefore, in the second part of the research, hydrophilic and hydrophobic bioactives were loaded in the double emulsions stabilized by sodium caseinate and PGPR. The bioaccessibility and the structural changes were studied over six different time points during in vitro digestion, simulating the gastric and the early stages of the duodenal digestion.

Vitamin B₁₂ loaded in the inner aqueous phase of the double emulsions was used to track the release of hydrophilic component. Under the present conditions, even with a low rate of lipolysis (controlled by decreasing the enzyme activity of pancreatin), 87% of the total hydrophilic bioactive was released after the gastric stage. Double emulsion droplets were observed up to 1.5 hour of the in vitro digestion process. These results demonstrated that the surrounding oil phase of the double emulsion did not act as a protective barrier to the inner aqueous phase.
In relation to bioaccessibility of Phytosterols and Vitamin D$_3$, a higher level of transfer was obtained with Phytosterols and a significantly lower level of Vitamin D$_3$ was released from the double emulsion samples. To clarify if the lower transfer was an effect of interfacial interactions observed in the first part, a comparison between an O/W (without PGPR) and W$_1$/O/W$_2$ (with PGPR) was carried out. Levels of Vitamin D$_3$ were similar, therefore, the emulsifier composition at a liquid interface did not affect the release of the bioactive. The differences obtained between the two hydrophobic bioactives in the double emulsion system seemed to be related to their partitioning within the lipid phase, that is, their surface-to-core distribution as suggested by previous studies (Malaki Nik, Corredig & Wright 2011; Day et al. 2010).

This work also demonstrated that by changing the physical state of the interface, it was possible to affect the release of bioactives during digestion. The internal oil phase contained 15% of trimyristin, a triglyceride of the medium chain fatty acid myristic acid, and the solid-lipid content was shown to have an impact on the overall stability and release behavior of bioactives during the digestion. This is of particular importance, as medium chain triglycerides were found to have better digestibility than the long chain ones (Yu et al. 2012), and increase the activity of pancreatic lipase (Borel et al. 1994). Significantly higher lipid digestion was observed in the compared double emulsions that contained trimyristin. Emulsions containing a higher solid fat content in the oil phase were not stable over 7 days of storage at room temperature. The extent of transfer of hydrophobic bioactives to the aqueous micellar phase at the duodenal stage was higher in these double emulsions. Levels of Phytosterols were very well correlated with lipid digestion, while Vitamin D$_3$ showed a smaller transfer, again confirming the hypothesis that surface-to-core distribution of the hydrophobic molecules play an important role in the kinetics of transfer of bioactive molecules.
Structuring of the inner aqueous phase was also evaluated to determine if the release could be modulated during digestion. In this case, the release of Vitamin B\textsubscript{12} was measured. While samples containing liquid internal aqueous phase and liquid or gelled oil phase did not have a significant effect on the release of Vitamin B\textsubscript{12}. When the inner aqueous phase was gelled with pectin, significantly lower levels of the bioactive were measured at the gastric stage and at the end point. It was clearly shown that gelation of the inner aqueous phase was more effective in protecting the release of the entrapped compound than structuring the oil or using a liquid interface.

This research provided an in depth study of fundamental aspects of the interfaces of double emulsion systems and their relation to digestion and transfer of encapsulated bioactives. It was demonstrated that different ingredients added to a system affect the viscoelastic properties of the interfaces (e.g. interfacial tension and elasticity). It was possible to obtain a double emulsion system with high encapsulation efficiencies of hydrophilic substances (Mg\textsuperscript{2+} and VitB\textsubscript{12}) and also to structure the internal water-oil phases. The static in vitro digestion methodology was successfully applied and demonstrated as a useful tool to study aspects related to how structuring of emulsions can affect the lipid digestion and bioaccessibility of hydrophobic bioactives. Results obtained in this research revealed that double emulsions as food-grade delivery systems intended for oral administration do not seem to have the oil phase as a protective barrier to the release of the hydrophilic compound as previously suggested, however, it may be a promising alternative when structuring the inner water droplets.
Chapter 7: References


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