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Master thesis

Encapsulation of probiotic bacteria in an oleogel

by

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Preface

This is my master thesis report, the last step in the master's program in Food technology and nutrition at Lund university, faculty of Engineering.

This project involving probiotics within a food grade oleogel was a great opportunity as the master thesis project for my master´s in food technology and nutrition. I have an interest in food technology and biotechnology, which is relevant for this project. I began this final project a few years after I studied at LTH, with just the degree project left, which shows that it is never too late to finish what you started.

Best regards, Philip Bergsell august 10th, 2022

Acknowledgement

I would not be able to finish this project alone, and I received support and help from many people around me.

Firstly, I would like to thank my examiner, Marie Wahlgren, for reading and reviewing my report and asking questions during my presentation.

Both my supervisors, Björn Bergenståhl and Lingping Zhang have helped me during the entire project. Plans have been changed and supplies have been delayed but with their help, I was able to finish the project. Special thanks to Lingping Zhang who have helped me with the literature research, laboratory practical work and proof-reading of the report.

I would also like to thank Elin Oscarsson for helping me with everything regarding preparation and execution of qPCR analysis.

Thank you!

Abstract

Probiotic bacteria administered orally that aim to reach the intestine alive need protection to be able to survive the human gastrointestinal tract. Ethyl cellulose is one food-grade material that can be used with oil to form an oleogel. This oleogel can encapsulate probiotics and provide physical protection. This project focuses on analysing different oleogel formulations, which are made with ethyl cellulose and maltodextrin. This project also focuses on evaluating the methods used to analyse the oleogel formulations.

An in vitro model simulating the small intestine was used in combination with qPCR to analyse the releasing of the encapsulated oil and probiotic bacteria.

Oleogel formulations with different ethyl cellulose molecular weight (22 cP and 46 cP) were significantly different regarding both in vitro and qPCR results. No significant difference was seen when altering the oleogels maltodextrin concentration. The in vitro method needs small improvements regarding sample position fixing to allow for more reproducible results. qPCR may provide uncertain results because the in vitro solution may destroy probiotic DNA. Another method-pair approach, for more accurately analysing oleogel release of probiotics, using microbiological water followed by qPCR and/or cultivating methods have been suggested.

Sammanfattning

Probiotiska bakterier som administreras oralt syftar oftast att nå tarmen levande och behöver skydd för att kunna överleva den mänskliga mag-tarmkanalen. Etylcellulosa är ett livsmedelsklassat material som kan användas med olja för att bilda en oleogel. En oleogel kan kapsla in probiotika och ge fysiskt skydd. Det här projektet fokuserar på att analysera olika oleogelformuleringar, som är gjorda med etylcellulosa och maltodextrin. Projektet fokuserar också på att utvärdera de metoder som används för att analysera oleogelformuleringarna.

En in vitro-modell som simulerar tunntarmen användes i kombination med qPCR för att analysera frisättningen av den inkapslade oljan och probiotiska bakterier.

Oleogelformuleringar med olika molekylvikt för etylcellulosa (22 cP och 46 cP) var signifikant olika gällande både in vitro- och qPCR-resultat. Ingen signifikant skillnad sågs vid förändring av oleogel-maltodextrinkoncentrationen. In vitro-metoden behöver små förbättringar gällande prov-positionsfixering för att möjliggöra mer reproducerbara resultat. qPCR kan ge osäkra resultat eftersom in vitro-lösningen kan förstöra probiotiskt DNA. Ett annat metod-par, för mer exakt analys av oleogelfrisättning av probiotika, med användning av mikrobiologiskt vatten följt av qPCR och/eller odlingsmetoder har föreslagits.

Contents

1. Introduction

Many probiotics are aimed to be delivered and reach the target location alive where they can benefit the host. Oral administration of probiotics faces many challenges along the gastrointestinal tract due to the unhospitable environment, such as acids and enzymes. To counteract the harmful environment, probiotics have been delivered within different delivery systems that aim to offer, amongst other features, a physical barrier. However, this barrier creates another challenge that the probiotic bacteria need a way of being released from its delivery system once it reaches the target location. Many advanced solutions to solve these challenges have been proposed. However, many of them include no food grade material. Ethyl cellulose is one food grade material that is easily made in to an oleogel simply by mixing with oil and heating. An oleogel system, where the liquid phase is made of oil and the network structure made of a structuring agent, such as ethyl cellulose. An oleogel system can encapsulate different active pharmaceutical ingredients, such as probiotics, within the formed gel network and can function as a delivery system. However, which exact oleogel formulation, and if any additives such as maltodextrin, may improve the delivery systems ability to release the probiotics is still unknown.

1.1 Aims:

The aims of this project are:

- To analyse and evaluate the influence of different oleogel formulations on releasing of oil and API (probiotics in this case) by varying different ethyl cellulose molecular weight and ethyl cellulose concentration.

- To explore if there are other ways (e.g. adding different excipients) of modifying an oleogel formulation, which could alter the release of API or oil. In this case, different maltodextrin concentrations were added.

- To discuss potential changes that can be made to the simplified in vitro model, and its connection to qPCR.

1.2 Hypothesis

Two hypotheses were used in this project:

- The first hypothesis was that the rate of releasing API from the oleogel system would be similar to the rate of lipolysis seen when oil is released from the oleogel system.

- The second hypothesis was that by including maltodextrin within an oleogel formulation would allow maltodextrin to act as a filling agent. Therefore, the added maltodextrin will create a capillary-effect, which allows for more release of the oleogels oil and API.

2.0 Background

This chapter contains background information for the report, including information on lipid digestion, ethyl cellulose (EC), oleogels, in vitro methods for lipolysis and background information on the thesis. The bacteria (API) used within the project has been donated by a company.

2.1 Probiotics

The human gut microbiota interacts with many aspects of health, for example aiding metabolism, digestion, and immunological reactions (Kvakova *et al.*, 2021). A disrupted gut microbiota, dysbiosis, have seen to be associated with various human diseases. The gut microbiota is possible to modulate through a few methods, one example being oral administration of probiotics which have shown health benefits (Kvakova *et al.*, 2021). The definition of probiotics (derived by the United Nations Food, World health Organization and Agriculture Organization in 2001) "…are live microorganisms which upon ingestion in sufficient concentrations can exert health benefits to the host" (Shi *et al.*, 2016).

2.2 Industrial way of administrating probiotics

Oral administration of probiotics is the most preferred route but due to the harsh environment in the gastro-intestinal (GI) tract alternative routes have been actively pursued (Baral *et al.*, 2021). Other administration routes are for example, intranasal and vaginal administration. As it seems, each alternative administration route has its own limitation. For example, intranasal administration of probiotics can be affected due to short retention time, enzymatic degradation, competitive nasal microbial community, and small surface area.
For an effective delivery of probiotics, the formulation strategy should vary depending on the biological barriers within each administration route. Each route encounters its own anatomical limitations, physiological conditions, and microclimate. Therefore, should the administration route vary depending on formulation strategy and therapeutic areas (Baral *et al.*, 2021).

2.3 Oral delivery for probiotics, benefits, and challenges

Oral delivery is the most cost-effective, ease of mass production and has the highest patient compliance regarding all dosage forms of probiotics (Baral *et al.*, 2021). There are different oral delivery dosage forms such as capsules, hydrogels, tablets, and oral films. Surface coating and microencapsulation are two of the techniques that can be applied to these dosage forms to enhance the probiotic stability throughout the GI tract (Baral *et al.*, 2021).

<u>Bile and bacteria</u>

The liver secrets about a liter of bile each day to the intestinal tract (Wahlgren *et al.*, 2019). Bile takes an important role in emulsification and solubilization in lipid digestion. The surfactant effect of bile has also been seen to affect proteins and phospholipids of cell

membranes and even disrupt cellular homeostasis. The commensal bacteria, pathogens, and probiotics, that target the intestinal tract, ability to withstand bile is therefore important for them to be able to colonize and survive. Bile includes mainly bile salts but also phospholipids like phosphatidylcholine, cholesterol and the biliverdin pigment.

Bile acids are made up of a perhydrocyclopentanophenanthrene steroid nucleus (Wahlgren *et al.*, 2019). All bile acids are conjugated as N-acyl amidates with either taurine or glycine before secretion. The ratio of taurine -to glycyine-conjuates in human bile is 1:3 but can vary depending on world location. The conjugation of bile acids lowers the pK_a value of a terminal acidic group which enables the conjugated bile acids to be soluble over a range of pH values, ionic strengths, and calcium concentrations. The unconjugated forms are not often soluble in physiological pH. Because the conjugates are completely ionized in physiological pH they are often called bile salts while the non-conjugated bile acids are not and thereby called bile acids. Bile acids have one hydrophilic side due to having substituents on the α side of the bile acid molecule while the β side is hydrophobic due to not having any substituents. The bile acid having both sides make the molecule amphipathic which enables it, above a certain concentration named the critical micellar concentration (CMC) to self-orient in water to form micelles, that are polymolecule aggregates. Micelles can solubilize lipids and turn the micelles into mixed micelles (Wahlgren *et al.*, 2019).

Antimicrobial activity of bile

Bile salts have been seen to have deleterious effect on cell membrane (Wahlgren *et al.*, 2019). Bile cause haemolysis in erythrocytes, which do not have organelles or metabolism mechanisms for bile salts, which indicate that lysis occurs due to membrane-damaging effects (Wahlgren *et al.*, 2019).

Cells have also been seen, through electron microscopy, to shrink and emptying after exposure to bile as well as confirming intracellular leakage through enzyme assays. Bile therefore seems to have direct effect on cell membranes permeability and integrity. The concentration of bile seem to be of most importance to dissolve lipids in the cell membranes and dissociation of membrane proteins. Low concentrations of bile can increase transmembrane flux of divalent cations and alter membrane-bound enzymes which can affect the permeability, fluidity, and integrity of the membrane (Wahlgren *et al.*, 2019).

The result of cell content leakage is cell death. Any change of cell membrane characteristics such as lipid fluidity, hydrophobicity and charge can have significant impact on the membrane's composition and architecture and thereby also its ability to resist bile. One example is that structural and conformational damages has been seen in membrane LPS due to freezing which decreases resistance to bile (Wahlgren *et al.*, 2019). Bile's detergent effect can also denature bacteria's membrane proteins rendering them inactive. Bile has also been seen to induce oxidative stress. Bacterial cells that express certain porins in the cell membranes, that are wide enough for bile acids to traverse, show more detrimental effects

from the bile acids compared with membranes with narrower porins that does not allow traverse of bile acids (Wahlgren *et al.*, 2019).

It is clear that bile impose a threat on many levels towards bacterial cells (Wahlgren *et al.*, 2019).

After ingestion of a fatty meal triggers a high level of bile secretion (Wahlgren *et al.*, 2019). Ingestion of food also enables bacteria additional protection against bile, in the form of microenvironments, created by the food matrix and constituents that bind the bile acids. Such protective results have been seen by ingestion of bacteria and meat at the same time. Encapsulating bacteria in artificial sesame oil emulsions have also seen great increase in bacteria viability when being exposed to bile (Wahlgren *et al.*, 2019).

2.4 Character of ethyl cellulose, structure & properties

Polymers is an important group of excipients that can be used within pharmaceutical technology to create drug dosage forms that enable site specific drug delivery (Wasilewska and Winnicka, 2019). Polymers also enables modifying of drug release and masks unpleasant drug tastes.

Cellulose derivatives are polymers which has been widely utilized as biocompatible templates for designing novel drug delivery systems. Cellulose is a natural polymer which is linear and composed of glucopyranose residues, connected by 1,4-β-glycosidic bonds. The structure is fibrous, practically insoluble in cold and hot water, and tasteless/odourless. Cellulose derivatives often share the same physiochemical characteristics and properties, such as, viscosity, biodegradability, pH dependency, mucoadhesion and swell ability (Wasilewska and Winnicka, 2019).

Ethyl cellulose

Ethyl cellulose (EC) is one derivative that have widespread applications, for example within pharmaceutical technology as flavouring fixative, binder, film-former, filler, coating agent, stabilizer, or drug carrier (Wasilewska and Winnicka, 2019). EC is considered non-allergenic, non-irritant, and biocompatible. EC is also considered non-ionic and thereby being non-reactive. EC is generally regarded as safe and is included in US Food and Drug Administration (FDA) inactive Ingredients database of Acceptable Non-medicinal Ingredients to can be used in tablets, oral capsules, topical emulsions, suspensions and vaginal or ocular preparations (Wasilewska and Winnicka, 2019). EC is prepared from cotton or wood pulp (Wasilewska and Winnicka, 2019). The molecular form of EC with ethoxyl substitution is $C_{12}H_{23}O_6(C_{12}H_{22}O_5)nC_{12}H_{23}O_5$, where n can vary in order to provide a wide variety of molecular weights, which affect its properties. EC is obtained through synthesis (etherification) by substitution of the cellulose hydroxyl groups with ethoxyl groups. The chemical reaction occurs through cellulose dissolution in sodium hydroxide which degrades the cellulose molecular structure, which ends up forming an alkali cellulose and exposes the

cellulose hydroxyl group for reaction. Ethyl chloride gas is added to the reaction which enables bonding with the alkalized cellulose, se **Figure 1**, (Wasilewska and Winnicka, 2019).

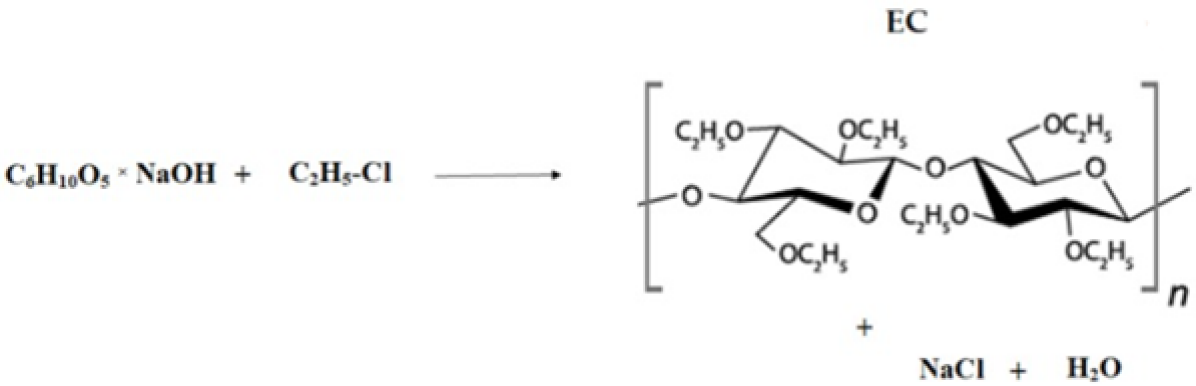

Figure 1. The chemical reaction of forming Ethyl cellulose (Wasilewska and Winnicka, 2019).

EC is hydrophobic and not soluble in glycerol, propylene glycol or water (Wasilewska and Winnicka, 2019). EC is soluble in a variety of organic solvents (ketones, alcohols, and polycyclic hydrocarbons). EC is insoluble at all pH that occurs within organisms. However, EC swells in presence of gastric juice which makes it permeable for water and thereby permits extended modified drug release in the human stomach. EC consists of between 44-51% ethoxyl groups ($-OC_2H_5$). EC derivates that have less than 46.5% ethoxyl groups is freely soluble in ethanol, chloroform, ethyl acetate, toluene, and methanol.
EC is composed of β-anhydroglucose units that are linked together via acetal linkage. EC show high stability at pH 3-11 which enables efficient usage in both alkaline and acidic mixtures. EC is tough, forms tensile films, have good thermal stability, and maintains flexibility at low temperatures. EC's physical characteristics are highly dependent on the degree of etherification and substitution (ethoxyl content), the molecular weight and molecular uniformity. EC's molecular weight is often expressed indirectly as viscosity. Increasing the polymeric chain length increases EC's viscosity (Wasilewska and Winnicka, 2019).
EC can also be used as a matrix in the preparation of water soluble/moderately soluble drugs using solid dispersion technique. The mechanical strength EC has enables usage as a coating material in sustained-release preparations due to its film-forming properties (Wasilewska and Winnicka, 2019).

Ethyl cellulose commercial usage

EC's ability of modified drug release enables its usage as a coating agent (Wasilewska and Winnicka, 2019). There are a few commercially available types of EC that utilize this modified release, for example, Ethocel™ 4, Ethocel™ 10, and Ethocel™ 45. These EC differ in polymer chain length, molecular weights, rate of dissolution and therefore their solution viscosities.
A study used a double layer coated colon-specific drug delivery system that was made using a

sub coating of chitosan-based polymer followed by an EC coating. The drug, in tablet form, was released in a controlled manner gradually in the colon. The release-retarding coating usage of EC preparation is commercially under the name of Micro-K®. Micro-K® essentially contains crystalline particles of potassium chloride that have been microencapsulated with EC coating, which function as a semi-permeable membrane that enables release in a controlled way of the ions. EC also exists commercially as coating material in modified release solid dosage forms, for example; Geomatrix®, Diffucaps, SODAS®, DiffCORE™ systems) (Wasilewska and Winnicka, 2019).

2.5 Oleogel gelation using ethyl cellulose

The definition of a gel is that it is a solid-like or solid material that is soft and consists of two or more components (Davidovich-Pinhas, Barbut and Marangoni, 2015). One component, present at a much larger extent regarding weight, is liquid whilst the other is a solid. The solid makes a three-dimensional cross-linked network within the liquid component that causes the gel to acquire solid like properties and its structure. Another definition is that the gel state is when the liquid components loss modulus (G´´) is a lot smaller compared to the solid component's storage modulus (G´).

Structuring liquid oil using oleogels have been examined broadly during the past century. There have been a few macromolecules, acting as gelator of oil and creating an oleogel, for example, modified polymers, synthetic polymers, proteins, and dendrimers, but most of these does not live up to a food grade level. EC is the only known food grade polymer gel system and is also unique because it can gel the liquid by solely direct addition and heating (CM *et al.*, 2017).
The EC based oleogel can thereby acquire solid-like gel characteristics that can easily be used within countless food applications (Davidovich-Pinhas, Barbut and Marangoni, 2015). The oleogel that is formed can have several beneficial advantages within the food sector, such as improving shelf life and stability (CM *et al.*, 2017). The applications for EC oleogels also venture into the ability of encapsulating lipophilic molecules within the oleogel which would perhaps allow a physical barrier against lipid digestion, inhibition or slowing the oil release due to the polymer network structure within the gel and/or preventing access of lipase to the oil within the oleogel (CM *et al.*, 2017).

The gelation of liquid oil using EC occurs by increasing the temperature of the EC & oil mixture above 140°C, where the polymer has its glass transition, followed by cooling to room temperature (Davidovich-Pinhas, Barbut and Marangoni, 2015). At temperatures above EC glass transition the polymers become viscous and flexible which ends up with the polymer interacting with the entire surrounding liquid component (Laredo, Barbut and Marangoni, 2011). It is believed that ECs semi-crystalline characteristics, hydrophobic nature, and other factors including hydrogen bonding between the EC strands, allow for the gelation of liquid oils (Davidovich-Pinhas, Barbut and Marangoni, 2015), (CM *et al.*, 2017). The properties of

the formed oleogel have shown to be strongly related to the polymer molecular weight, polymer concentration and the cooling rate etc. The polymer molecular weight does seem to share a relationship regarding the cross-over behaviour, the final gel strength, and the gel point temperature. Increasing the polymer weight increases all three before mentioned attributes. The gelation mechanism of EC in oil is not believed to involve secondary order structure formation during thermal transitions (Davidovich-Pinhas, Barbut and Marangoni, 2015). The EC oleogel gelation mechanism has been called "unique" in one study due to the oleogel not displaying the same thermo reversible systems that other polymers display (Davidovich-Pinhas, Barbut and Marangoni, 2015).

Oleogelators, like EC, self-assemble and form a network with filled voids in-between, trapping the solvent or molecule added of interest within the encapsulating compartments of the oleogel (CM *et al.*, 2017). The pockets formed are 3-4.5 um in diameter and filled with oil. The size of these pockets seems to be affected based on the gel composition, as one study showed a decrease in pocket dimeter size, from 4.5 to 3.0 um, when increasing the concentration of 45 cP EC from 10% to 14%. However, no significant difference in pocket size were seen when increase the EC molecular weight (CM *et al.*, 2017).

2.6 Maltodextrin and the microenvironment inside an oleogel

Maltodextrin is commonly used within the food industry within many food applications, such as for coating material, neutral fillers or increasing the viscosity of a food product (Saavedra-Leos *et al.*, 2015). Maltodextrin is a polysaccharide that is produced during enzymatic or acidic hydrolysis of starch and can be produced from many starch types. Maltodextrin is considered a polymer based of D-glucose chains that are linked by glycosidic bonds. Maltodextrin is highly soluble in water (Saavedra-Leos *et al.*, 2015). However, there is no information on the solubility of maltodextrin in oil.

The microenvironment within an oleogel containing oil, EC, maltodextrin, and bacteria can be roughly estimated based on the particles sizes and if they are hydrophobic or hydrophilic, see **Figure 2**. Individual maltodextrin particles can vary in size depending on origin and type, but have roughly the size around 100-200 µm (Ogrodowska *et al.*, 2022). Bacteria also vary in size depending on type but generally range between 0.5-2.0 µm (Osiro *et al.*, 2012). However, most bacteria are generally hydrophilic and will therefore aggregate and create "bacteria-lumps" when put in oil and the aggregates are estimated to be around 100 µm (Hanpanich *et al.*, 2017). Ethyl cellulose strands are small and have the size of less than 1 µm (Wu *et al.*, 2017).

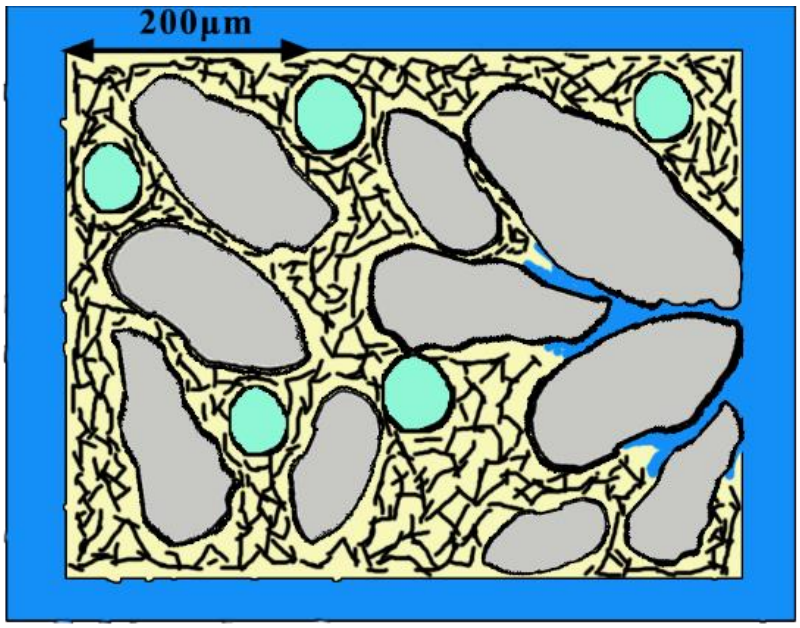

Figure 2. Two-dimensional illustration of the microenvironment in an oleogel containing oil (yellow) as the continuous phase, maltodextrin (gray), aggregated "bacterial lumps" (green) and ethyl cellulose strands (black lines) forming a cross linked network. Outside the oleogel is water (blue). The illustration depictates a capillary-effect where the water is penetrating in to the oleogel in-between maltodextrin particles on the pictures right side.

2.7 Lipid digestion (adsorption and hydrolysis)

Lipid digestion starts in the mouth and stomach but mainly occurs in the small intestine. The majority of dietary lipids are consumed as triglycerides (TAG) (Wilde, 2014). The process in humans is mainly carried out by the two endogenous lipases, human gastric lipase (HGL), and human pancreatic lipase (HPL) (Engelking, 2015).

Both HGL and HPL have hydrophobic surface active regions designed to adsorb to a lipid structure where the enzymes can perform hydrolyzation catalyzation's (Wilde, 2014). The adsorption to the insoluble lipid droplets is essential for lipolysis.

HGL, which is secreted by the chief cells in the stomach mucosa, has a working pH between 3-6 (Wilde, 2014). The surface-active site on the lipase is a hydrophobic region which is covered, like a lid, during transport and once the enzyme is near the lipid surface there are conformational changes which enables the hydrophobic region to bind to the active site of the TAG substrate. This then allows the hydrolyze catalyzation of the fatty acid (FA) ester bonds. HGL prefers to hydrolyze the sn3 positioned FA which mainly produces sn1,2-DAG and small amounts of sn2-MAG. The adsorption is only dependent on the enzymes own surface-active ability to adsorb to the lipid surface. The hydrolyzation creates the product molecules, FA, mono- di-glycerides, which are not entirely water-soluble and therefore accumulates on the lipid surfaces and competes with the enzyme for space. The HGL enzyme therefore eventually becomes product inhibited and is forced away from the lipid surface. This enzyme inhibition restricts the amount of TAG that can be hydrolyzed, which together with the HGL

specific hydrolyzation preference, are reasons why only 10-30% of TAG is hydrolyzed in the stomach (Wilde, 2014).

The presence of lipids in the lumen of the duodenum triggers secretion of two hormones, cholecystokinin (CCK) and secretin, by endocrine cells of the duodenum (Engelking, 2015). The pancreas releases digestive enzymes to the duodenum as a response to CCK and the gallbladder release bile. Bile contains the emulsifying agent's bile salt and lecithin. Secretin enables release of a solution with $NaHCO_3$ from biliary and pancreatic ductular cells which aids the neutralization of acid contents from the stomach (Engelking, 2015).

The fat globules entering the duodenum are covered by the lipolysis products that were released in the stomach (Wilde, 2014). The bile salts, which are synthesized in the liver from cholesterol and stored in the gallbladder, are highly surface-active compounds (cholic acid and taurocholic acid) that adhere and displace the lipolysis products from the lipid surface. The displaced products are solubilized and form mixed micelles. The lipid surface is now covered by the even more surface-active bile salts. The bile salts reduce the surface tension between the lipids and water which enables emulsification and the breakdown of the fat globules into smaller lipid droplets. The increase in lipid surface area is now more available for hydrolysis by the HPL. However, the lipid surface is covered in surface active bile salts which makes it difficult for HPL to adhere to the lipid surface. For lipolysis to continue in the presence of bile salts, HPL needs to interact directly with the bile salts which are already adsorbed to bring lipase close enough to the lipid surface. This is enabled through the cofactor colipase, another enzyme secreted from the pancreas, which assists in facilitating adsorption to the adhered bile salts on the lipid surfaces. HPL can then continue hydrolyze the FA ester bonds. The entire process of displacing lipolysis products and HPL adsorption occurs passively and is a highly efficient process which results in the body being able to absorb 95% of digestible lipids (Wilde, 2014).

HPL provides the majority of all hydrolyzation activity regarding dietary TGA (Wilde, 2014). HPL has an active working pH around pH 8.0 and the enzyme is released in an active form directly from the pancreas to the duodenum (Engelking, 2015). Once the HPL has adsorbed to a lipid surface the catalytic site, being exposed to the substrate (TAG or DAG), begins lipolytic activity (Wilde, 2014). HPL have active hydrolysis of the sn1 or sn3 ester bonds of TAG and DAG and thus completes the remaining 70-80% of lipid hydrolyzation. The hydrolyzation results in FFA and sn2-monoglycerol. It has also been shown that the FA in Sn2-MAG can migrate to either the sn1 or sn3 position, and if that is hydrolysed it would have resulted in complete degradation of the TAG (Wilde, 2014).

2.8 In vitro lipolysis model

The difference between a simulated in vitro lipolysis model and the real in vivo process is mainly the dynamic ever-changing processes of fluids and components altering the lipolysis in the human body (Zangenberg *et al.*, 2001). A dynamic dissolution model of lipolysis needs to fulfil two requirements; The first is that the lipolysis products that are produced must be continuously removed to not inhibit further lipolysis of TG. The second is that to get reproducible results the rate of lipolysis must be controllable (Zangenberg *et al.*, 2001).

To control the pH in a lipolysis model a pH-stat (Titrino 718) can be used to maintain pH at 6.5 that resemble the pH range within the small intestine (pH 5.5 – 7.5) (Wahlgren *et al.*, 2019). The amount of FA generated from lipolysis reflects the volume of added 1 M NaOH. The titrated amount of FA can be used as a measure of TG hydrolysis by lipase by having it as a function of time (Zangenberg *et al.*, 2001).

In vivo, the solubilization of the lipolysis products (FA, monoglycerides) in bile salt-mixed micelles is believed to be an important way to remove the products from the lipid surface to enable further lipolysis (Zangenberg *et al.*, 2001). Studies indicate that this mechanism can be accomplished in vitro using Ca^{2+}. The Ca^{2+} enables $Ca(FA)_2$ precipitation which removes FA from the lipid surfaces and enables HPL to continue lipolysis. It is therefore important when performing in vitro simulation, including TAG lipolysis, to include either bile salts or Ca^{2+} to remove the inhibiting effect lipolysis products have on HPL to allow for unimpeded lipolysis. Continuous addition of Ca^{2+} in an in vitro lipolysis model enables control over the lipolysis rate. Increased addition of Ca^{2+} show increased rate of lipolysis. After a while of Ca^{2+} addition the hydrolysis rate becomes constant and shows approximately 2 mmol FA/mmol Ca^{2+}, which indicates Ca^{2+} must be in excess to keep hydrolysis rate constant over time (Zangenberg *et al.*, 2001).

FA are titratable with NaOH at pH 6.5 at physiological NaCl concentrations and in the presence of bile salts and Ca^{2+} (Zangenberg *et al.*, 2001).

Porcin pancreatin have been used instead of HPL for in vitro lipolysis studies (Jannin *et al.*, 2015). The amount pancreatin used should be in excess, the same as in the human duodenum (Zangenberg *et al.*, 2001).
The digestibility of the enzyme is measured as USP (activity/gram) or in enzymatic units (TBU). Pancreatin contain lipase, trypsin, protease, amylase, and ribonuclease which are all enzymatic components produced in the pancreas of the porcine by the exocrine cells. The enzyme combination allows hydrolyzation of fats, proteins and starch (Sigma-Aldrich).

2.9 qPCR

Real time polymerase chain reaction (quantitative PCR or qPCR) is a method for quantifying, detecting and typing microbial agents such as DNA, RNA, specific genes etc within the world of biotechnological use cases (Kralik and Ricchi, 2017). The basic concept of PCR starts with a mix of nucleotides, DNA polymerase, primers, a DNA template and ions which together undergo cycles of DNA denaturation, primer annealing and lastly extension. The result is the amplification of the DNA target of interest which can be monitored in real time using fluorescence in qPCR. After each cycle the fluorescence intensity signal is measured and quantifies the amount of target amplifications in the sample at that given time. The amount of target sample can be detected once the signal surpasses the detection level. Above the detection level, the intensity of the fluorescence increases proportionally to the initial number of target molecule template in the sample. This data is then compared to a standard curve of the sample DNA molecule of interest with known amounts which helps determine the DNA molecules in the sample (Kralik and Ricchi, 2017).

One way of identifying and quantifying bacteria through qPCR is by amplifying the 16S rRNA (16S ribosomal RNA) genes using an universal primers (Oscarsson *et al.*, 2020).

3.0 Materials and methods

3.1 Materials

Materials used for In vitro method

Pancreatin (porcine, P-1625-100G, lot# SLCF5908, SLBM0006V, SLCD7175, SLBT0854), bile extract (porcine, B-8631-100G, Lot# SLBX1760), Trizma Maleate ((mono[tris(hydroxymethyl)aminomethane]maleate), T-3128, LOT: 55H5740), ethyl cellulose (viscosity 46 cP, 5% in toluene/ethanol 80:20(lit.), extent of labelling: 48% ethoxyl, 433837-250G, LOT# MKCL2418) were purchased from Signa-Aldrich (St. Louis, MO, USA). Phosphatidylcholine (soybean (L-a-Lecithin), EMD Millipore Corp, 429415-100GM, LOT: 3761811), sodium hydroxide (1 mol/L (1N)), VWR, aqueous solution, CAS: 1310-73-2, LOT: 20C274006), sodium chloride (VWR, 27810.295, LOT: 19A034143), calcium chloride dihydrate (VWR, 22322.295, Batch: 11K020017), maltodextrin (Glucidex 12D, Roquette/Barentz, Batch: E509E), MCT oil (fractionated coconut oil, Miglyol 812, (fatty acid composition: C6 0.1%, C8 60.9%, C10 38.5%, C12 0,4%, certificate of analysis, Condea, supplied by Condea, Germany), Gelatin capsules (Lonza capsugel, size: 00, batch: 7181524) was also used.

Water used was obtained from Milli-Q-water purification system (Millipore, MAMA, USA). Titration program (Tiamo 2.5 (including stirring, pH measurement data collection)), Titrator (Metrohm, 902 Titrando), stirrer (Metrohm, 801 Stirrer), water bath (Lauda, Ecoline 006, E100), centrifuge (Beckman coulter, Allegra X-15R centrifuge), heat plate (Heidolph MR 3001 K), thermocouple data logger ((Pico TC-08, Pico technology limited) and PicoLog 6 software).

Materials used for DNA extraction

For DNA extraction Nucleospin Soil (MACHEREY-NAGEL, 740780.250, LOT: 2201-003) and centrifuge (Heraeus Pico 21 centrifuge, Thermo scientific) was used.

Materials used for qPCR

Material for qPCR were SYBR Green PCR kit (QuantiNova, 208054, LOT: 172018539), premade *L.reuteri* DNA fragments standard samples, primer Lact-F (AGC AGT AGG GAA TCT TCC A), primer Lact-R (CAC CGC TAC ACA TGG AG), qPCR analysing software (Rotor-Gene Q Series Software, version 2.0.2), qPCR instrument (Rotor-Gene Q model: 2-Plex HRM, Qiagen).

3.2 Experimental design

Oleogels were made through heating a mix of MCT oil and EC before adding maltodextrin and API. Certain amount of oleogel formulation were put in gelatin capsules and run through in vitro trials to view the lipolysis amount. Samples were taken during the in vitro trials for DNA extraction and qPCR analysis to see if the different oleogel recipe formulations had an impact on the amount of released API. The qPCR data was then compared with in vitro results and analyzed if those results correlated with the in vitro lipolysis conversion. The workflow can be seen in **Figure 3**.

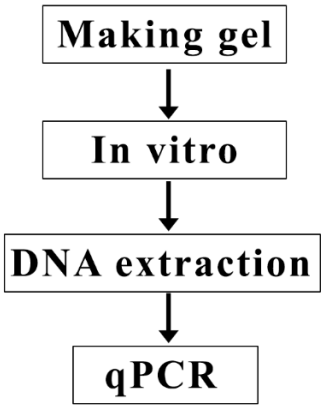

Figure 3. The workflow through the project, starting with the creation of gel samples (with and without API) that were used in an in-vitro model, which is a model that simulates the smaller intestine to analyse the conversion of TGA to FFAs. Samples were taken during the in vitro trial, from oleogels including API, which were run through DNA extraction and qPCR to analyse if the amount of converted TGA to FFAs correlated with the release of API from the oleogel matrix.

3.3 Oleogel sample preparations

The oleogel was made according to a previous study (Zhang, Wahlgren and Bergenståhl, 2022). EC and MCT oil were put in a beaker and heated to 160° C and held at 160°C for 5 minutes to allow for complete dissolution of the ethyl cellulose. The beaker was then allowed to cool in the fridge overnight where it formed the gel network. When adding maltodextrin to the gel, it was added after the EC and oil mix had already been heated to 160°C and cooled down to 100°C before adding the maltodextrin. The combined mix was then stirred for 30 seconds with a spatula before the beaker were put in the fridge for cooling overnight. When adding API to the formulation, the initial EC and oil mixture was first heated, cooled, added eventual maltodextrin, and allowing the mixture to cool down to 40°C before adding the API and mixing for 30 seconds. The gel was then cooled in the fridge overnight. The different oleogel formulation recipes can be seen in **Table 1**.

Table 1. The oleogel formulation recipes separated by trial batch number.

In vitro trial batch 1 and 2			
Sample		20%	40%
46 cP EC (g)		0.58	0.81
MCT oil (g)		3.50	3.50
API suspension (1 gram)		yes	yes
Bacteria (g)		0.05	0.05
Maltodextrin (g)		0.45	0.45
MCT oil (g)		0.50	0.50
Maltodextrin (g)		0.71	2.79
Total weight (g)		5.79	8.10
EC in oil phase		12.64%	16.85%
maltodextrin in total weight		20%	40%
Oleogel added to in vitro (g)		0.72	1.01
Bacteria added to in vitro (g)		0.006	0.006

In vitro trial batch 4			
Sample	No malto	20%	40%
46 cP EC (g)	0.44	0.50	0.50
MCT oil (g)	4.00	4.00	4.00
Maltodextrin (g)		1.13	3.00
Total weight (g)	4.44	5.63	7.50
% EC in oil phase	10.00%	11.11%	11.11%
% maltodextrin in total weight		20%	40%
Added to in vitro (g)	0.56	0.70	0.94

In vitro trial batch 5 & 6			
Sample	No malto	20%	40%
22 cP EC (g)	0.21	0.21	0.21
MCT oil (g)	4.00	4.00	4.00
Maltodextrin (g)		1.05	2.81
Total weight (g)	4.21	5.26	7.02

% EC in oil phase	5.00%	5.00%	5.00%
% maltodextrin in total weight		20%	40%
Added to in vitro (g)	0.52	0.66	0.88

In vitro trial batch 7			
Sample	No malto	20%	40%
22 cP EC (g)	0.21	0.21	0.21
MCT oil (g)	3.50	3.50	3.50
API suspension (1 gram)	yes	yes	yes
Bacteria (g)	0.05	0.05	0.05
Maltodextrin (g)	0.45	0.45	0.45
MCT oil (g)	0.50	0.50	0.50
Maltodextrin (g)		0.62	2.39
Total weight (g)	4.71	5.33	7.10
EC in oil phase	5%	5%	5%
Maltodextrin in total weight	9.6%	20%	40%
Oleogel added to in vitro (g)	0.59	0.67	0.89
Bacteria added to in vitro (g)	0.006	0.006	0.006

3.4 Preparation of pancreatin solution for in vitro

The pancreatin solution was prepared by taking 675 TBU porcin pancreatin USP 8 into a centrifuge tube, add 30 mL of deionized water, dissolve the pancreatin by vortex for 10 seconds and add deionized water to a final volume of 35 mL. The tube was centrifuged at 4000 RPM, 7 minutes at 20°C. 30 mL of the supernatant was then transferred to another tube. Before adding to the in vitro simulation solution, the pH was adjusted to 7 before starting the experiment.

3.5 Method descriptions

3.5.1 In vitro lipolysis model

To evaluate the release of MCT oil and API from the oleogel, an in vitro system which mimic the fasted state of the small intestine was used. This in vitro model is a two-step titration procedure (Zhang, Wahlgren and Bergenståhl, 2022). The model resembles the small intestine by being a titration system that measures pH, using a thermostatic pH-stat equipment, and maintain the trial solution at pH 7.0 by titrating NaOH (1 mol/L). The in vitro solution was

made with porcine bile extract, porcine pancreatin and a buffer, and was used to resemble the environment within the small intestine. The concentrations of the created bile solution, buffer solution and pancreatin solution was determined in a former study, se recipes in **Table 2** (Zhang, Wahlgren and Bergenståhl, 2022). The porcine based additives were used to resemble the complexity of the liquids within the small intestine without including pure enzymes and mixed bile salts. To remove the released FFAs, as a lipolysis product, from the lipid surfaces during lipolysis a concentration of 15 mmol/L of calcium ions was used (Zhang, Wahlgren and Bergenståhl, 2022). Both porcin pancreatin USP 3 and USP 8 was used but amounts were accounted for regarding TBU.

The in vitro trial was first prepared by mixing bile solution and buffer solution in a thermostatic beaker. The mix was then homogenized using an autotorrax (ultra Turrax, speed 3) for 30 seconds. The thermostatic beaker was then connected to a water bath, holding 42°C, which maintained the mixed solution at 37°C. The solution mix was then pH adjusted to pH 7 using NaOH (1 mol/L). The titration was then initiated by adding both the prepared pancreatin solution and the sample of interest to the thermostatic beaker. During the first two hours the system run the titration to a pH-stat kept at pH 7 followed by an endpoint titration to pH 9. The endpoint titration was used to avoid underestimating the extend of lipolysis during the first two hours.

Table 2. In vitro solution recipe.

Name	Final concentration
Bile solution	
Bile extract	8 mmol/L
Phosphatidulcholine (soybean origin)	2 mmol/L
	2 mmol/L
Buffer solution	
NaCl	150 mmol/L
Ca^{2+}	15 mmol/L
Trizma-maleate	2 mmol/L
Pancreatin solution	
Porcin pancreatin	675 TBU
Total volume	100 mL

When the in vitro method was started a sample containing 0.5 oil was added as well as pancreatin solution to facilitate the lipolysis reaction to occur. As the pancreatin performs lipolysis with the help of the bile salts, that creates FFAs that lower the solution pH. The pH-meter sense the decrease in pH and adds titrant, NaOH. The total amount of added NaOH from the two-hour in vitro trial is a direct correlation to how much lipolysis that had occurred in the trial.

All samples run through the in vitro trial had the same amount of hydrolysable oil (MCT oil). By administrating the same amount of oil for each trial, the other variable formulation parameter concentrations could be observed and their effect on the release of oil and lipolysis.

Background samples containing only in vitro solution were run several times. The mean background sample result was then subtracted from all following in vitro trial results.

A mean result of MCT oil samples was acquired through in vitro trials and the mean value was assumed as 100% conversion from TAG to FFAs. All remaining in vitro samples were then calculated into a relative conversion to this mean MCT oil value, to avoid limitations and unknown errors with the in vitro method.

3.5.2 DNA extraction

DNA extraction was performed on samples including API taken at 10, 30 and 120 minutes during the in vitro trials. The extraction was performed accordingly to the manufacturer's instructions.

3.5.3 qPCR analysis

DNA samples were quantified using qPCR.
Quantitative real-time PCR was used to quantify the rRNA (16S ribosomal RNA) genes from *Lactobacillus* using the qPCR instrument (Rotor-Gene Q and the Rotor-Gene Q Series Software) as previously performed in another study (Oscarsson *et al.*, 2020). Each 20µL PCR sample reaction contained 2 µL DNA sample, 10 µL SYBR-Green, 6.5 µL nuclease-free water, and 0.75 µL of primer Lact-R and Lact-F respectively. Each sample was mostly run in triplicates. Each run included in vitro samples, background samples, negative control (nuclease-free water) and standard solutions. The program for the thermal cycling settings were the following; 95°C 5 minutes hold time, 40 cycles including 95°C denaturation for 5 seconds followed by annealing at 60°C for 20 seconds and elongation at 72°C for 20 seconds (Oscarsson *et al.*, 2020). The qPCR results were calculated into the unit "relative copies amount" by using another qPCR result of just API in water that had been predetermined, named "Actual" in **Table 3**.

3.6 Data analysis

All result data, that managed to complete entire in vitro-/ qPCR-trials, were gathered and evaluated using Excel. In vitro-/qPCR-trials that were stopped or cancelled any time during the trial were discarded. All data sets from in vitro trials and qPCR were first subtracted by the mean background sample result (except **Figure 6**, **7** and **8**), to remove background noise. All data was then correlated to relative sample results to make up for any limitations/errors due to the nature of the methods. The relative unit were "relative conversion" for in vitro results and "Relative copies amount" for qPCR results. All data sets that were used for

statistical analysis were first run through Grubbs test to remove any outliers, within that specific data set, according to Grubbs critical values based on observations at significance level of 0.05 (Grubbs and Beck, 1972). Grubbs test was repeated per data set until the entire data set cleared the Grubbs critical values. Comparing data sets between three different groups was performed by using ANOVA, (one factor, significance level of 0.05) and comparing two data set groups by using T-test (two samples assuming different variances, significance level of 0.05) to view if data sets mean results were significant or not.

4. Results

The results from in vitro trials and qPCR on different oleogel formulations.

4.1 In vitro results – background & MCT oil samples

The in vitro results from the different oleogel trial batches can be seen in **Table 3**. Four background samples were performed in trial batch 3 and one in trial batch 4. The result from "Background samples" containing no MCT oil and no sample had a mean result of 0.61 ± 0.03 mL NaOH. Results from samples only containing MCT oil was 2.78 ± 0.03 mL NaOH. The background samples and MCT oil samples data can be seen in **Figure 4**.

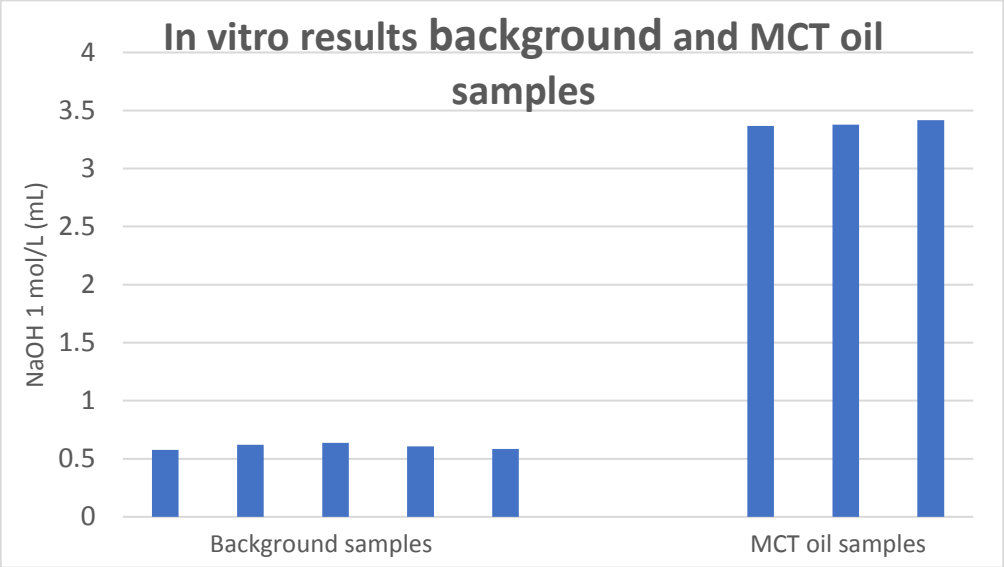

Figure 4. In vitro results of 5 background samples including no sample and 3 MCT oil samples including only 0.5 g MCT oil.

4.2 In vitro results

All in vitro results separated by trial batch can be found in **Table 3** and for graph format, see **Figure 5.**

Table 3. All in vitro and qPCR results for all trial batches. "Malto" is maltodextrin. "Actual" is a predetermined qPCR result of API in water, used to gain a relative copies amount percentage. Samples with "*" had large gel remnants left in the in vitro beaker after the in vitro trial, see 4.2.5 In vitro – other observations.

Trial batch information	Sample name	EC% in OP	In vitro relative conversion	qPCR copies Real/Actual at 10 minutes	qPCR copies Real/Actual at 30 minutes	qPCR copies Real/Actual at 120 minutes
Trial batch 1, 46 cP EC, no capsule, with API, pancreatin: USP 3, batch SLCF5908	20% malto	12.64%		0.56%	0.89%	1.05%
	20% malto	12.64%		1.49%	3.55%	4.55%
	40% malto	16.85%		1.68%	2.31%	5.26%
	40% malto*	16.85%	31.04%	1.05%	1.11%	1.59%
Trial batch 2, 46 cP EC, capsule, with API, pancreatin: USP 3, batch SLCF5908	20% malto*	12.64%	5.04%	0.05%	0.29%	0.84%
	20% malto	12.64%	7.20%	0.15%	0.49%	1.16%
	40% malto*	16.85%	7.49%	0.16%	0.40%	1.44%
	40% malto	16.85%	8.43%	0.29%	0.87%	0.69%
Trial batch 3, no EC, no capsule, no API, pancreatin: USP8, batch SLBT0854	MCT oil 1		99.23%			
	MCT oil 2		99.66%			
	MCT oil 3		101.10%			
Trial batch 4, 46 cP EC, capsule, no API, pancreatin: USP8, batch SLCF5908, SLBM0006V, SLCD7175, SLBT0854	20% malto	11.11%	1.44%			
	20% malto	11.11%	11.23%			
	40% malto	11.11%	0.00%			
	40% malto	11.11%	2.16%			
	No malto	10.00%	4.90%			
Trial batch 5, 22 cP EC, capsule, no API, pancreatin: USP 8, batch SLCD7175, SLBT0854	No malto	5.00%	47.02%			
	No malto	5.00%	21.32%			
	20% malto	5.00%	78.42%			
	20% malto	5.00%	50.70%			
	20% malto	5.00%	49.33%			
	40% malto*	5.00%	5.26%			

	40% malto	5.00%	7.71%			
Trial batch 6, 22 cP EC, capsule, no API, pancreatin: USP 8, batch SLCD7175, SLBT0854	No malto	5.00%	64.52%			
	No malto	5.00%	20.60%			
	No malto	5.00%	10.37%			
	20% malto	5.00%	103.84%			
	20% malto	5.00%	59.91%			
	40% malto*	5.00%	27.72%			
	40% malto	5.00%	59.55%			
Trial batch 7, 22 cP EC, capsule, with API, pancreatin: USP 8, batch SLCD7175, SLBT0854	No malto	5.00%	96.93%	124.95%	6.05%	4.60%
	No malto	5.00%	91.53%	2.97%	15.29%	4.03%
	20% malto	5.00%	82.96%	1.29%	33.13%	6.55%
	20% malto	5.00%	97.58%	24.64%	15.09%	1.49%
	40% malto	5.00%	101.46%	7.91%	22.16%	9.40%
	40% malto*	5.00%	3.67%	0.00%	0.00%	0.00%

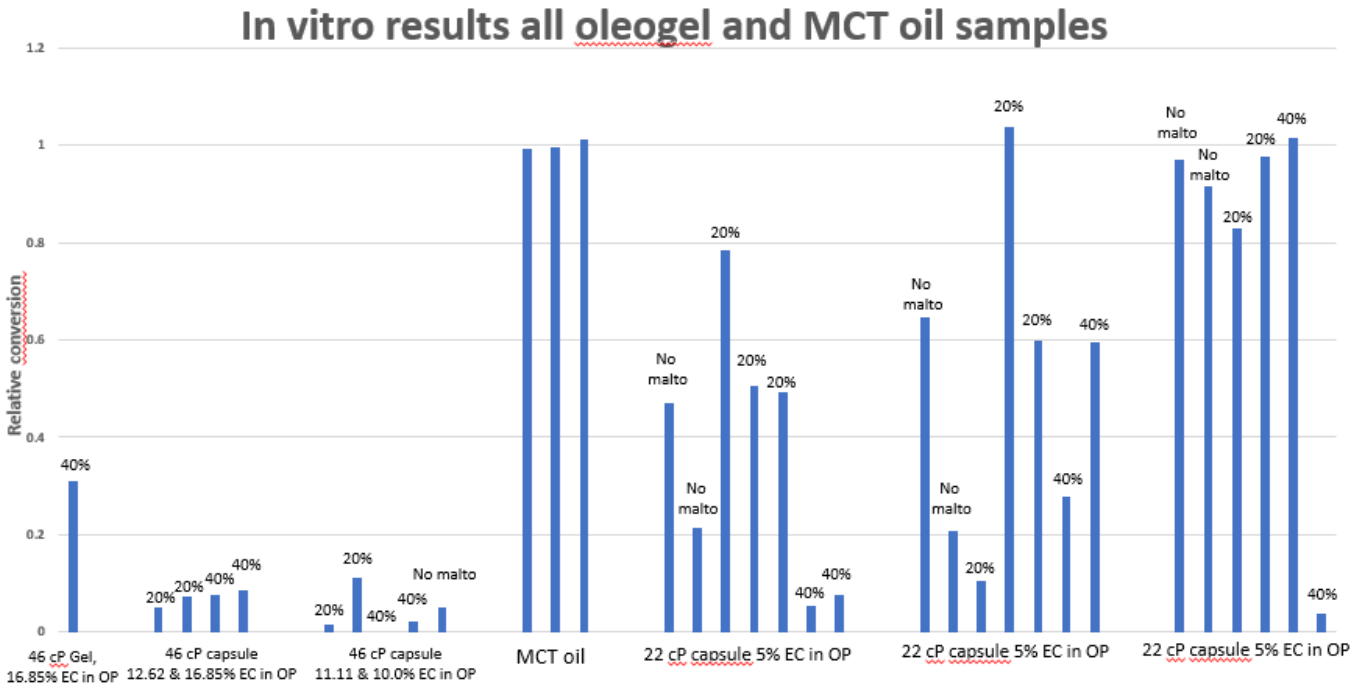

Figure 5. In vitro results from all trial batches. The samples "No malto" are No maltodextrin, "20%" are 20% maltodextrin and "40%" are 40% maltodextrin.

4.3 Oleogel – varying maltodextrin

When varying the maltodextrin concentration in the oleogel formulation for in vitro trials with the EC molecular weight 22 cP (trial batch 5, 6 and 7), there was no significant difference (P=0.11) between "no maltodextrin", "20% maltodextrin" and "40% maltodextrin", see **Figure 6**. When varying maltodextrin concentration for capsule in vitro trials with EC molecular weight 46 cP (trial batch 2 and 4) there was no significant difference (P=0.84), see **Figure 7**.

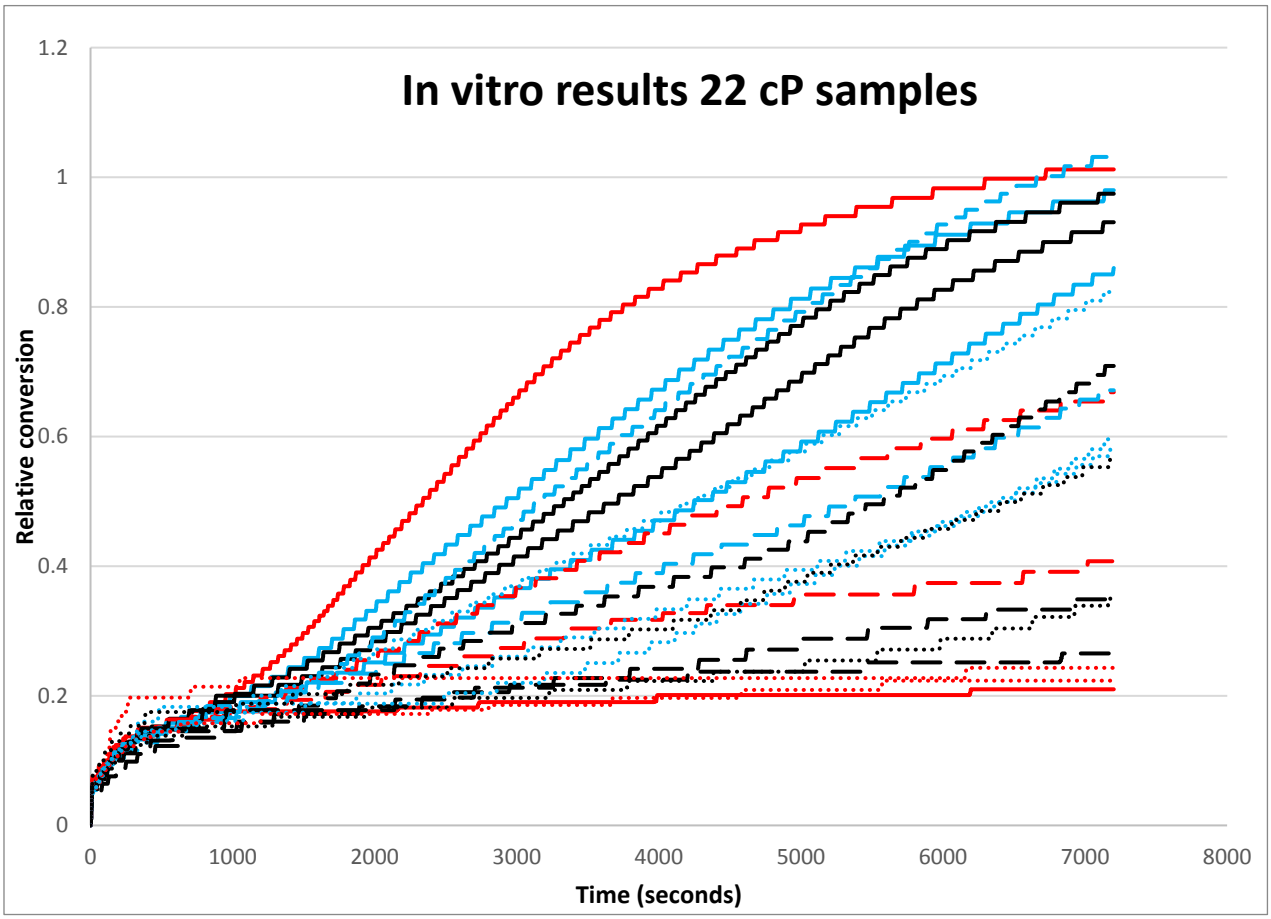

Figure 6. All in vitro results from trial batch 5, 6 and 7. Samples from trial batch 5 are dotted (…), trial batch 6 are dashed (- - -) and trial batch 7 are full lines. Samples containing "No maltodextrin" are black, "20% maltodextrin" are blue and "40% maltodextrin" are red. Data in this figure has not been subtracted with the mean background sample result to allow for greater comparison between sample release rates.

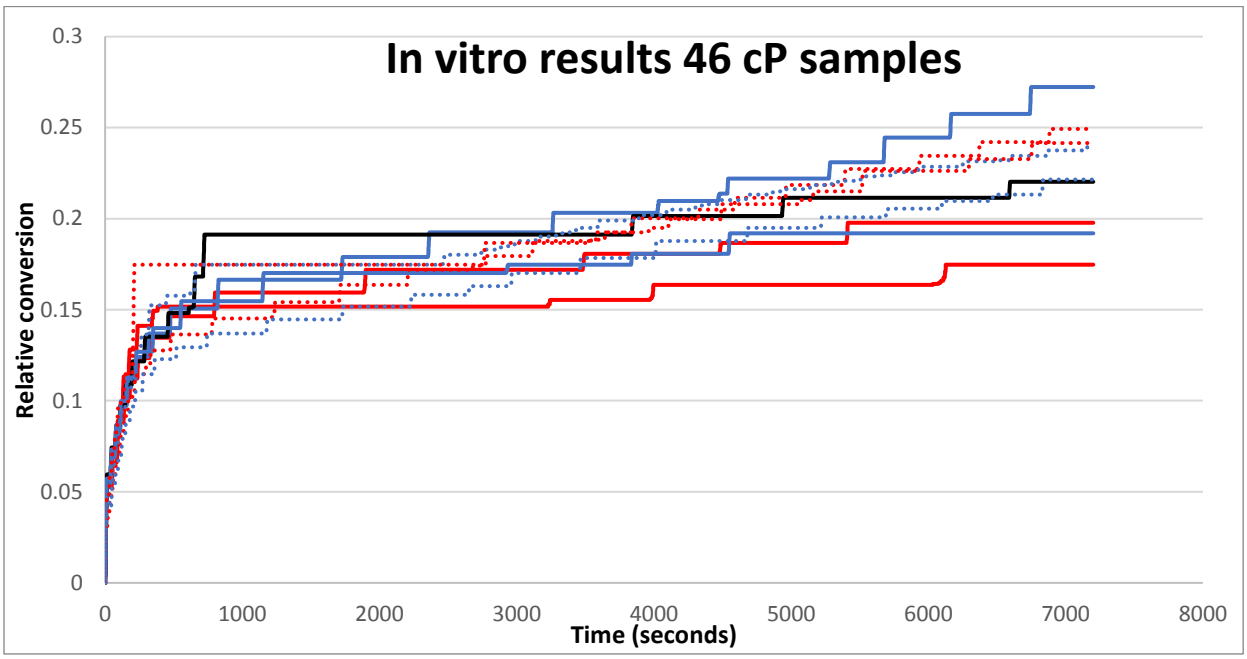

Figure 7. In vitro results from trial batch 2 and 4. Samples from trial batch 2 are dotted (…), trial batch 4 are full lines. Samples containing "No maltodextrin" are black, "20% maltodextrin" are blue and "40% maltodextrin" are red. Data in this figure has not been subtracted with the mean background sample result to allow for greater comparison between sample release rates.

4.4 Oleogel – varying ethyl cellulose molecular weight

When comparing all the results between the two different EC molecular weight formulations (22 cP and 46 cP) there was a significant difference (P=0.000005), see **Figure 8**.

When comparing the 22 cP sample trial batches (5, 6 and 7) against each other, one sample (40% maltodextrin from trial batch 7 (3.6% relative conversion)) was removed due to Grubbs test, it showed that there was a significant difference (P = 0.005) between trial batch 5, 6 and 7. Further t-tests showed it was trial batch 7 that was significantly different. When comparing the 46 cP sample trial batches (2 and 4) against each other it did not show a significant difference (P=0.20) between the two trial batches.

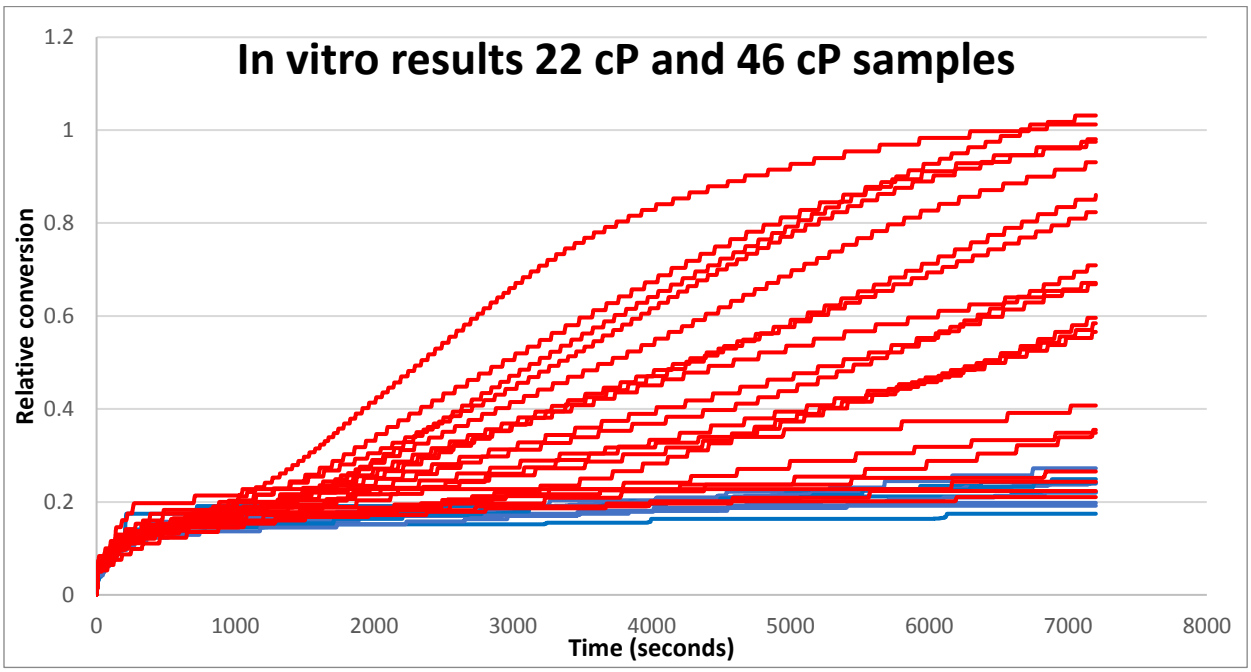

Figure 8. All in vitro results of 46 cP trial batch 2 and 4 (coloured blue) and of 22 cP trial batch 5, 6 and 7 (coloured red). Data in this figure has not been subtracted with the mean background sample result to allow for greater comparison between sample release rates.

4.5 In vitro - other observations

After in vitro trials, remnants of not dissolved oleogel samples were still intact after a few trials, see **Figure 4**. This parameter of remaining remnants was not measured and cannot be stated to have been overserved at all trials. Trials that perhaps had smaller gel remnants may therefore have been missed. The observations that were noticed were a total of six observations. Two of the observations were “46 cP, 16% EC in OP, 40% maltodextrin”, three were “22 cP, 5% EC in OP, 40% maltodextrin” and one were “46 cP, 12% EC in OP, 20% maltodextrin”. All six observations in vitro results passed Grubbs test both when comparing within the same EC molecular weight formulation and within the same maltodextrin concentration.

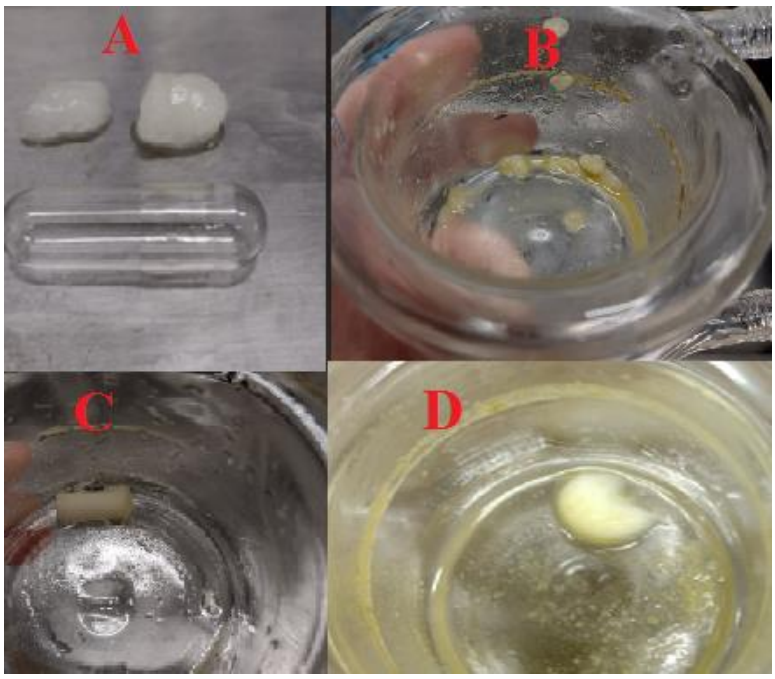

Figure 9. Oleogel remnants left in the in vitro trial beaker after in vitro trials. A: showing "46 cP, 12% EC in OP, 20% maltodextrin", B: showing gel without capsule "46 cP, 16% EC in OP, 40% maltodextrin", C: showing "46 cP, 16% EC in OP, 40% maltodextrin" and D: showing "22 cP, 5% EC in OP, 40% maltodextrin".

4.6 qPCR results

qPCR results from the different trial batches can be seen in **Table 3**. qPCR results from trial batches made with 46 cP molecular weight formulation can be seen in **Figure 10**, and 22 cP molecular weight formulation can be seen in **Figure 11**. qPCR negative control (nuclease free water) results showed 0 copies/µL which indicated no DNA contamination in the qPCR samples. Trial batch 1, 2 and 7 got standard curve results of R^2= 1.00, R^2= 1.00, and R^2=0.99 respectively.

qPCR result when comparing "20% maltodextrin" and "40% maltodextrin" within 46 cP oleogel formulation samples (trial batch 1 and 2), one outlier was removed from "40% maltodextrin" (5.2% relative copies amount), showed no significant difference (P=0.54).

The qPCR results when comparing "no maltodextrin", "20% maltodextrin" and "40% maltodextrin" within 22 cP oleogel formulation samples (trial batch 7), two outliers from "no maltodextrin" were removed (124% and 15% relative copies amount), there was no significant difference (P=0.30).

qPCR results when comparing all 22 cP oleogel formulation samples (trial batch 7) against all 46 cP oleogel formulation samples (trial batch 1 and 2), one outlier from 22 cP (124% relative copies amount) and three outliers from 46 cP (3.5%, 4.5% and 5.2% relative copies amount) was removed, there was a significant difference (P=0.003), see **Figure 12**.

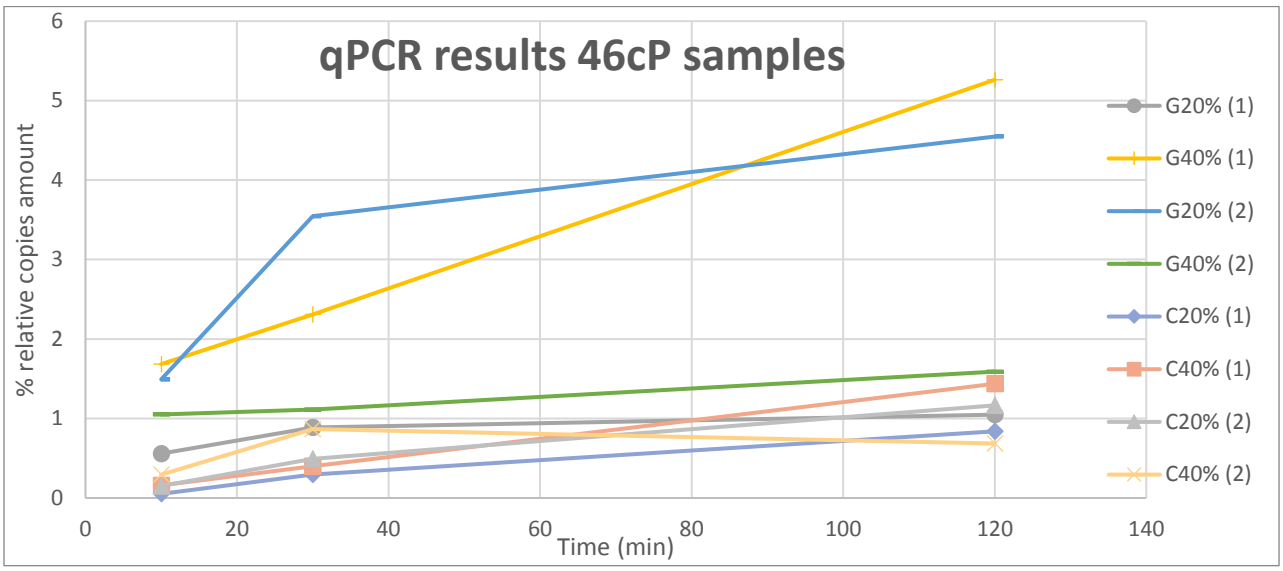

Figure 10. qPCR results from trial batch 1 "G" and trial batch 2 "C". The maltodextrin amount in the oleogel samples was varied between 20% "20%" and 40% "40%".

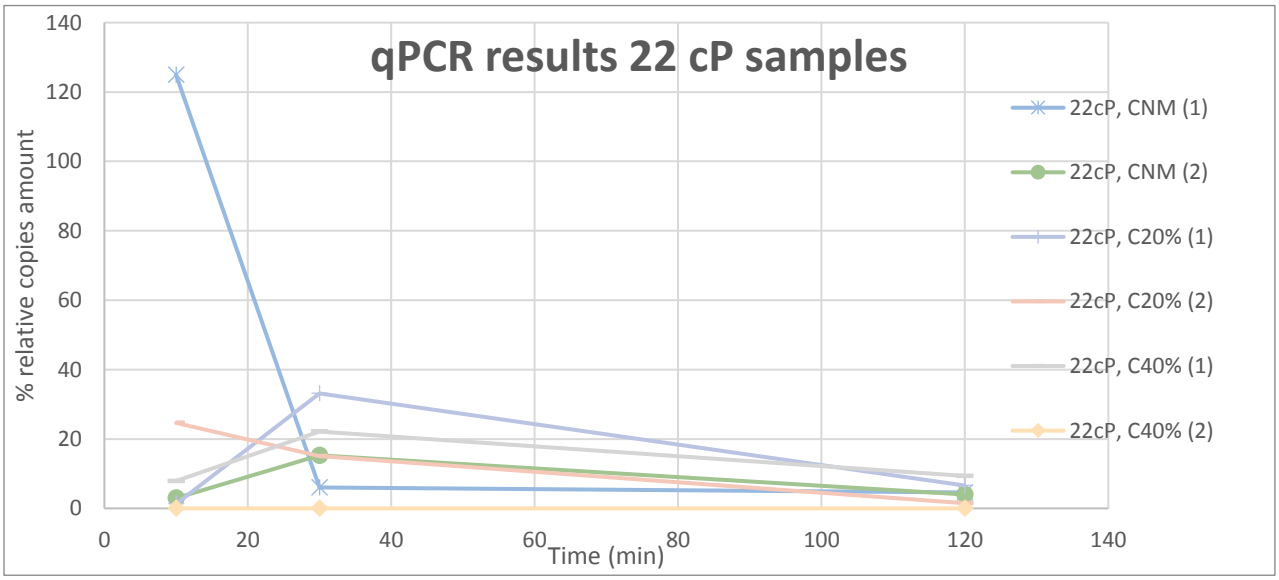

Figure 11. The qPCR results from trial batch 7. Samples "No malto" are no maltodextrin, "20%" are 20% maltodextrin and "40%" are 40% maltodextrin.

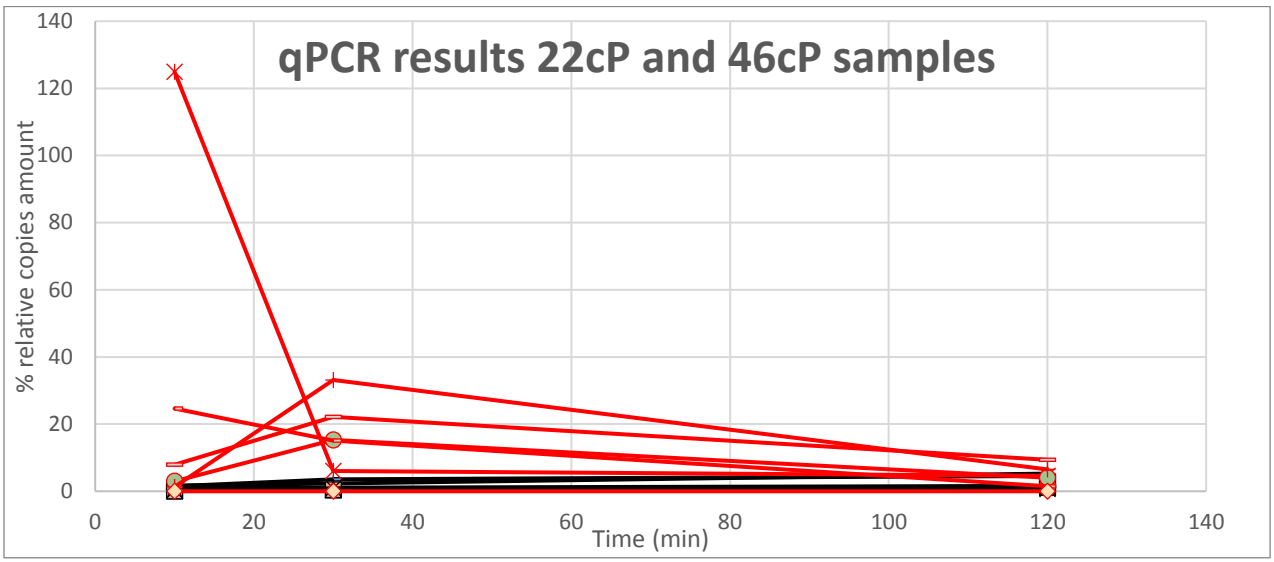

Figure 12. qPCR results for all samples in trial batch 1, 2 (black) and trial batch 7 (red).

5.0 Discussion

5.1 In vitro results

Throughout the in vitro trials, different batches of pancreatin and their USP number (USP 3 and USP 8) were used, however the same amount of TBU were used for each trial batch and thereby provided the same lipolysis capacity from the pancreatin in all trial batches. The different pancreatin batches used could however influence the in vitro results due to containing different amount of fat.

The different oleogel formulations also varied between trial batches. Trial batch 1 were the only trial batch that used samples without an enclosing gelatin capsule. Trial batch 1, 2 and 4 had the same EC molecular weight (46 cP) however, trial batch 1 and 2 had the same EC concentration in the oil phase (OP) whilst trial batch 4 had a different EC concentration in the OP. Trial batch 5, 6 and 7 had the same EC molecular weight (22 cP) and the same EC concentration in the OP. These oleogel formulation differences can have had an impact on the results.

A few figures, (**Figure 6**, **7** and **8**) displaying the in vitro results over time did not have the mean background result subtracted which made each data point have a 0-7% error from its actual value. This was done because a few sample results were lower than the mean background result. By not subtracting the background mean result it allowed for an overall sample comparison. The inherent error within the in vitro model and trial batch variations, that impacts all data, most likely exceeds that of the applied (0-7%) error and so the benefits was believed to outweigh the drawback.

5.1.2 Oleogel – varying maltodextrin

None of the oleogel samples with different maltodextrin concentrations showed any significant difference when being compared within the same EC molecular weight formulation. This result is also supported by the release rates, see **Figure 6** and **7**, which visually indicates that there is no difference between the release rates and the different maltodextrin concentrations. These results indicate that maltodextrin have no impact on the release of oil from an EC oleogel during in vitro analysis. The second hypothesis, in this project, that maltodextrin would act as a filling agent and allow for a capillary-effect to arise (as illustrated in **Figure 2**), making water enter into the gel in-between maltodextrin particles and aid the breakdown of oleogel/release of oil, shows no support. The reason could be that the maltodextrin concentration was not enough to enable a capillary-effect to arise. It could also be that a capillary-effect was present but not enough to have an impact on the breakdown of the oleogel. The addition of maltodextrin could also perhaps have affected the rheology of the oleogel impacting the breakdown of the gel/or release of oil.

5.1.3 Oleogel – varying ethyl cellulose molecular weight

When comparing all 22 cP molecular weight formulation samples with all 46 cP molecular weight formulation samples in vitro end result there was a significant difference. This is also visually indicated in **Figure 8** regarding the different oleogel release rates during in vitro analysis. The 22 cP samples release rates in **Figure 8** are overall more visually linear over the entire in vitro trial whilst the 46 cP samples seem to plateau after about 500 seconds into the trial. Another study on EC oleogels investigating lipolysis, using an in vitro method simulating digestion conditions, showed that 45 cP oleogel samples had significantly lower lipolysis and that the release rate plateaued compared to other oleogel formulations with lower EC molecular weight (CM *et al.*, 2017). The same study also showed that an increase of EC molecular weight up to 45 cP had a significant impact on creating an increased gel strength. The increased gel strength could have been the reason for the difference in that the in vitro solution had a more difficult time penetrating in to the higher strengthen gel (Davidovich-Pinhas, Barbut and Marangoni, 2015). Another study showed that increasing the EC concentration formed harder gels and was thought to occur due to it providing more polymer-polymer associations within the gel (Laredo, Barbut and Marangoni, 2011). However, another study showed that when comparing a 45 cP 10% EC gel with a 20 cP 15.5% EC gel, with about the same mechanical strengths (17 N and 18 N respectively), the lipolysis was still significantly lower in the 45 cP sample (CM *et al.*, 2017). This was hypothesized, by the same study, to occur because of the 45 cP oleogel to form thicker more ridged network walls compared to the 20 cP oleogel. The thicker walls were thought to give the 45 cP 10% EC oleogel a higher resistance to breakdown, lowered oil release and ultimately lowered lipolysis. These results are in line with this thesis findings and indicates that it was mainly the EC molecular weight that caused the higher EC molecular weight formulation oleogel to have lower lipolysis during in vitro analysis.

5.1.4 In vitro - other observations

The in vitro results are overall scattering to some extent even though 24 out of the 30 oleogel samples were fully dissolved during the trial. Since the same amount of oil were included in each sample, if the entire oleogel sample was dissolved during the trial, the same amount of lipolysis should have occurred no matter the oleogel formulation. One thought is that the sediment that appears during in vitro trial might still include small pieces of oleogel that still have encapsulated oil. This thought is supported by another study where their results indicated that the oil did not completely release from the oleogel system but was thought to be still retained within the gel network (CM *et al.*, 2017). This would imply that small gel remnants managed to keep both perhaps oil and API encapsulated during the entire in vitro trials. Further research could analyse this by collecting sediment samples and check for oil- and API-content.

Observations of intact oleogel remnants in the in vitro beaker after the in vitro trials was seen throughout this project. There were three observations of 22 cP samples and two of 46 cP samples. None of the observation sample results were differing enough to be removed as outliers. However, five out of the six observations were of samples with 40% maltodextrin which indicates that the maltodextrin improved the likelihood of the oleogel being able to stay intact during the entire in vitro trial. The reason for this could be due to the 40% maltodextrin samples weighing more causing them to sink and getting stuck at the bottom of the in vitro trial beaker. All other samples float on top of the in vitro solution. The samples that got stuck to the bottom cause these samples to have less exposed oil/water interfacial area available for lipolysis, which could affect the oil release rate and overall lipolysis amount. These samples also avoided being destroyed by the magnetic stirrer, which was seen destroying samples into small pieces which can also have had an impact on the oil/water interfacial area and overall lipolysis amount.

5.2 qPCR

The qPCR results, in % relative copies amount, were expected to increase over time, as more API was thought to be released from the oleogel system over time during the in vitro trials. The 46 cP molecular weight formulation trials (trial batch 1 and 2) seemed to have a small overall increasing amount over time. This was not seen for the 22 cP molecular weight formulation trial (trial batch 7) and the overall results indicates that more API is not released into the solution over time. The first hypothesis, in this project, that the release of API would be similar to the rate of lipolysis/or release of oil from the oleogel system shows no support. The reason why the qPCR results were not increasing over time could have been because of the oleogel not being completely broken down during the in vitro trial, with gel remnants forming a sediment, and still retained encapsulated API, as formerly discussed in 5.1.4 In vitro – other observations. Another thought, why more API was not released over time, is that it might be due to the bile, destroying the bacteria’s membrane, and pancreatin enzymes

destroying the DNA during the in vitro trial and during storage of sample for 1-3 days before DNA extraction and qPCR was performed.

The qPCR results, when using different maltodextrin concentrations, was not significant within any of the oleogel formulations which correlates with the in vitro trial results. When comparing the qPCR results between the different oleogel formulations (22 cP and 46 cP) there was a significant difference which is also in line with in vitro results. The reason to these results, and similarities to the in vitro results, are thought to be because of the same reasons, of the EC molecular weight causing the significant difference, explained in "5.1.3 Oleogel – varying ethyl cellulose molecular weight".

5.3 Drawbacks and improvement to analysing oleogel release

In vitro background sample results and MCT oil sample results did both get high reproducible results. However, when comparing three identical in vitro trial batches (trial batch 5, 6 and 7) that were performed at three separated occasions the results were significantly different. This indicates that the low reproducibility was shown when running capsule sample trials because of either sample irregularities or trial irregularities. One of these trial irregularities might be the observed intact oleogel remnants, due to samples sinking to the bottom, that were observed in six in vitro trials. To avoid these complications of the sample having a risk of ending up in various positions throughout the in vitro beaker, a fixed position, by putting the sample in an enclosed tea-strainer for example, would allow for more uniform solution exposure and to avoid the sample getting destroyed by the magnetic stirrer. This would allow for a more reproducible result and easier way of comparing different oleogel formulations.

Because the possibility of bacteria being destroyed by bile salts, and DNA by pancreatic lipase, the in vitro method followed by qPCR might not be an optimal method-pair to analyse API release from an oleogel.
The method-pair could swap the in vitro method and instead have the oleogel sample in microbiological water that is being stirred for two hours. Sample would be taken from the water at 10, 30 and 120 minutes like the in vitro procedure. Using this simple setup, instead of the in vitro method, the release rate from the oleogel is only affected by the parameters of the oleogel formulation. The samples taken could then be used for qPCR but also for different cultivation methods since the bacteria would still be alive. Cultivating bacteria would be cheaper and less labour intensive in comparison with qPCR.

5.4 Conclusion

Certain oleogel formulations showed less lipolysis and less release of API. The main reason for this difference is thought to be due to the higher ethyl cellulose molecular weight in these formulations, which is in line with other studies results.

Adding maltodextrin to the oleogel formulation showed no effect on lipolysis amount or release of API. The hypothesis of maltodextrin, within an oleogel, causing a capillary-effect and ultimately increased lipolysis during in vitro trial showed no support.

Further analysis of in vitro trial sediment is needed to investigate if oleogel remnants are able to stay intact, and encapsulate oil and API, even after a two hour in vitro trial. The low reproducibility of capsule samples during in vitro analysis could been due to the oleogel sediment still encapsulating oil and API. It could also have been due to samples varying in positioning throughout the in vitro trial and/or getting destroyed by the magnetic stirrer, thus affecting the sample surface area and lipolysis amount. The in vitro method could therefore acquire a fixed sample holder within the in vitro beaker to allow for more reproducible results.

The hypothesis that the rate of lipolysis, by oleogel samples during in vitro trials, would be similar to the release of API showed no support. The overall trend showed no increase in API release from the oleogels during the in vitro trials.

Using qPCR on in vitro samples might not have been an optimal approach to analysing API release due to the uncertainty if the bile and pancreatin, in the in vitro solution, destroy the API's DNA. A separate method-pair approach, without the in vitro method, including samples in stirred microbiological water, followed by qPCR and/or cultivating methods have been suggested. This method-pair setup would limit the parameters affecting API release to that of only the oleogel formulation and avoid any DNA getting destroyed.

6. References

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