Digestion of dietary fat

Anne Helbig

2013

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Gastrointestinal behaviour of emulsions and human physiological responses

Anne Helbig

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Digestion of dietary fat

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To be held

on Friday, 21st June 2013,

at 13:30

in the Aula of Wageningen University
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Anne Helbig

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology and Health Sciences).

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Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 21 June 2013
at 1.30 p.m. in the Aula.

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Digestion of dietary fat. Gastrointestinal behaviour of emulsions and human physiological responses
166 pages

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summary in English, Dutch and German

ISBN: 978-94-6173-560-7

Abstract

Two *in vitro* models were used to understand emulsion behavior and the subsequent formation of free fatty acids (FFA), monoglycerides (MG) and diglycerides (DG). Emulsions stabilized by whey protein isolate (WPI) or gum arabic (GA), varying in droplet size, were digested under intestinal conditions. Concentrations of FFA, MG and DG, assessed by gas chromatography, decreased with increasing droplet size. FFA release from gum arabic-stabilized emulsions was higher compared to WPI-stabilized emulsions showing an influence of the interface. Next, lipolysis of protein stabilized emulsions (i.e. WPI or lysozyme) and the influence of flocculation at the isoelectric point (p*I*) were investigated in a dynamic gastrointestinal model. The stomach properties including gradual acidification caused WPI-stabilized emulsions to cream during transition through the p*I* of the protein. This resulted in delayed intestinal lipolysis compared to the lysozyme-stabilized emulsion. Thus, since gastric passage affects emulsion behavior and intestinal lipolysis, the gastric passage should be part of digestion models. Next, in a human study emulsion behavior and resulting lipolytic products were related to the release of satiety hormones, satiety perception and *ad libitum* intake. Also, gallbladder volume and oral processing were studied. A delayed entry into the duodenum and lipolysis for the un-homogenized sample resulted in lower CCK, delayed GLP-1/PYY responses and barely gallbladder contraction compared to the homogenized emulsion. No difference was found between treatments on ghrelin, only the perception `desire to eat´ was elevated for homogenized emulsions. Oral processing induced prolonged gallbladder contraction, but had no additive effect on other measures. A homogenous system as such is possibly not effective to induce pronounced satiety perceptions compared to phase separated or creamed systems using the same emulsifier. Moreover, the release of gastrointestinal hormones cannot directly be related to the satiating effect of food.

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Chapter 1

General Introduction

Lipids are omnipresent in foods. Lipids exist in many forms, but from a human nutritional point of view triglycerides are most important as nutrient part and energy source. In the context of this thesis the term lipids refers triglycerides. Their recommended total daily intake is 15-30% (or up to 35 % when eating diets rich in vegetables, legumes, fruits and wholegrain cereals) of total daily energy intake to prevent diet-related chronic diseases, i.e. diabetes mellitus, cardiovascular diseases, hypertension and stroke, some type of cancers and obesity (WHO/FAO report, 2003). Besides its role as energy source, triglycerides are necessary to enhance the intestinal absorption of lipophilic nutrients (Brown et al., 2004). In addition, triglycerides are contributing to the characteristic texture, flavor and mouthfeel of various foods (Drewnowski, 1995; Drewnowski 2010). The problem is, however, that we consume too much. According to the WHO, however, in 2008 worldwide more than 1.4 billion adults were overweight of which approx. one third was obese. Although there is evidence that prevalence of obesity is leveling off in western countries, there is a rapid rise in obesity in, for example, Asia (Rokholm et al., 2010).

In view of the current obesity epidemic an in-depth insight in digestion of lipids may offer leads to help control food intake. Already for a long time, triglycerides are known to play a role in the regulation of metabolic intake by activating satiety responses. In western diets, most food products are highly processed and contain lipids mostly in form of emulsions, for example, as a dispersion of oil in water. The question arises, if specifically designed emulsions could be a tool to influence satiety responses and thus help prevent overconsumption. The regulation of these satiety responses by using such emulsions is, however, not fully understood. This is related to the complexity of food intake regulation, where food properties, physiological responses and behaviour play a role. Hence, this thesis aims to provide knowledge on how changes in emulsion parameters can influence the digestion of triglycerides, specifically the release of free fatty acids, and how this can be linked to factors regulating satiety responses. Therefore, the research reported in this thesis starts with the influence of the emulsion parameters droplet size and interfacial composition, two key factors affecting the digestion of emulsions. The digestion of emulsions is studied under simulated static intestinal conditions and dynamic gastrointestinal conditions. Finally, in a human intervention study the oral administration and intragastric distribution of lipids is investigated to reveal influences on intestinal lipolysis and resulting satiety responses.

The following sections provide information on the current understanding of the regulation of satiety in response to lipid intake as observed from human studies. Moreover, the *in vitro* models used to gain mechanistic understandings of lipid digestion of emulsions are summarized.

1.1 The impact of fat digestion on satiety responses

1.1.1 General aspects of lipid digestion

Gastric lipolysis

It is considered that human gastric lipase (HGL) contributes to 10-40 % of overall lipolysis (Carriere et al., 1993; Armand et al., 1999). It was found that HGL is inhibited by triglyceride degradation products (Pafumi et al., 2002). These products (Fig. 1.1C) are mainly free fatty acids (FFA) and diglycerides (DG) as HGL has a preference for the sn-3 position of the triglyceride. For example, the FFA concentration to inhibit HGL was found to be 107 - 122 µmol per surface area (m^2) (Pafumi et al., 2002). In addition, gastric lipolysis is essential for an optimal digestion process in the intestine (for a review see Armand, 2007). For example, gastric lipolysis and the formation of FFA promote optimal human pancreatic lipase (HPL) activity. The released long-chain FFA reduce the lag-time of the HPL-colipase complex to bind to the lipid interface (Gargouri et al., 1986b; Bernback et al., 1989). Due to the activity of HGL over a broad pH range (Hamosh et al., 1990), it is still active in the duodenum. As a consequence, it is estimated that up to 7.5 % of intestinal lipid digestion derives from HGL-activity (Carriere et al., 1993).

Intestinal lipolysis

The major intestinal lipolytic enzyme is HPL, which contributes to 40 -70 % of overall lipid digestion (Carriere et al., 1993; Armand et al., 1996; Armand et al., 1999) resulting in FFA and 2-MG (Fig. 1.1C). Co-lipase, a cofactor secreted from the exocrine pancreas, binds to triglyceride in the presence of bile salts (Borgstrom, 1975). HPL forms a complex with co-lipase (Van Tilbeurgh et al., 1993; Pignol et al., 2000) and, by including micelles, consisting of for example, long-chain FFA, bile salts or lysolecithin, it forms a 'ternary' complex (Pignol et al., 2000). As this enables the anchoring of pancreatic lipase to the oil-water interface, it is clear that the composition of the lipid interface plays an important factor for this interaction.

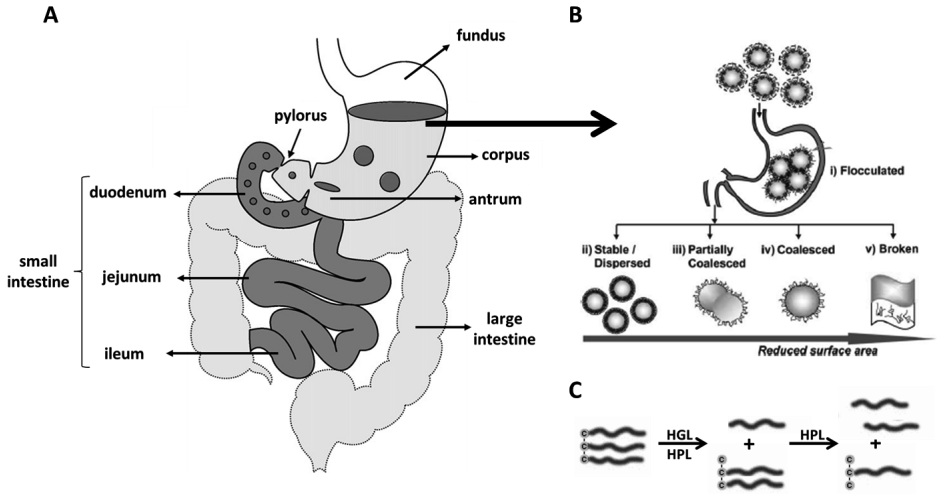

Fig. 1.1 Gastrointestinal tract (A) and behaviour of emulsions upon consumption (B): i-v indicate emulsion behaviour which can occur in the intestine as well as in the stomach. Emulsions can flocculate (i), be stable or, in the intestine, being redispersed (ii), partially coalesced (iii), coalesced (iv) or broken (phase separated, v). Triglycerides present in emulsions are hydrolysed by human gastric lipase (HGL) into 1 FFA and 1 DG and by human pancreatic lipase (HPL) into 2 FFA + 1 MG during their passage through the gastrointestinal tract (C). Figures A and B are adapted from Golding et al. (2010; 2011).

1.1.2 Lipid digestion in relation to satiety responses

Satiation and satiety

Satiation is a term representing a number of processes that terminate an eating period while satiety refers to processes, which inhibit further eating and thus, affect the interval between meals (Fig. 1.2) (Blundell & Halford, 1994; Blundell & Tremblay, 1995). Both processes are regulated (Fig. 1.2) by a cascade of events (Blundell et al., 1994; Blundell et al., 1995). Metabolic satiation and satiety, for example, include all neural and hormonal signals between the gastrointestinal tract and the brain (Blundell et al., 2010). These signals refer to stomach fullness as sensed by mechanoreceptors as well as hormones, such as Cholecystokinin (CCK), Glucagon-like peptide 1 (GLP-1), Peptide YY (PYY) and ghrelin (Blundell et al., 2010).

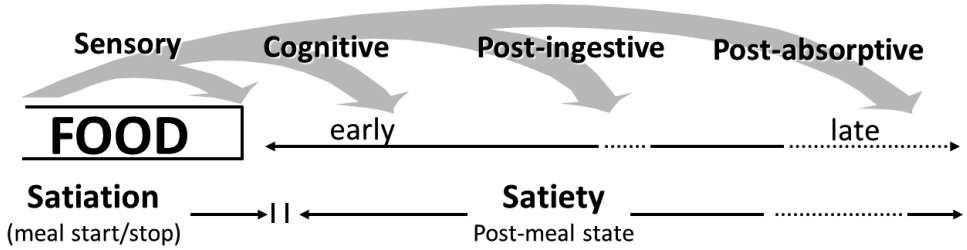

Fig. 1.2 Satiety cascade indicating the satiety and satiation response upon food intake (adapted from Blundell et. al., 2010).

Hormonal regulations

Fullness is a result of stomach distension. Thus, it is likely that prolonging gastric distension enhances satiety, which in turn could be realized by inhibition of gastric emptying. Different hormones are involved in the regulation of gastric emptying, such as CCK, GLP-1, PYY and ghrelin. First of all, CCK is released from I-cells which are most prominent in the proximal part of the intestine (Fig. 1.1A). With respect to lipid digestion, the release of CCK requires a fatty acid chain length of at least 12-carbons (McLaughlin et al., 1999). Only FFA of this chain length or longer were reported to delay gastric emptying (Hunt & Knox, 1968). CCK stimulates gallbladder contraction and pancreatic secretion (Wank, 1998), while it is also involved in the inhibition of gastric emptying (Cummings & Overduin, 2007). It was shown that lipolysis of triglycerides by pancreatic lipase triggered CCK release (Hildebrand et al., 1998), but the CCK secretion diminishes again after approximately 60 min, despite continuous delivery of lipids to the duodenum (Fried et al., 1988). Another hormone, which is secreted upon lipid ingestion, is GLP-1. GLP-1 is released from L-cells in the distal part of the small intestine and of the colon. Also, PYY is released from L-cells in the ileum and colon. Both GLP-1 and PYY are considered to be mediators of the `ileal brake´. This is a mechanism, which regulates gut transit time and reduces hunger and food intake (Maljaars et al., 2008). Ghrelin is released from endocrine cells in the stomach and the proximal part of the small intestine. Its release increases food intake and gastrointestinal motility and its circulating plasma levels are suppressed upon food intake (Cummings et al., 2007). Also, there is indication that the effects of ghrelin might be inhibited by CCK, but the mechanisms are not clear yet (Chandra & Liddle, 2007).

Lipid induced satiety responses

Responses to oral fat intake (`cephalic phase´) are considered to induce metabolic responses and, hence, to induce and regulate the gastrointestinal digestion of foods as well as food intake. These cephalic responses are mediated by vagal stimulation. With respect to lipid digestion, oral stimulation in the form of modified sham feeding (MSF), for example, stimulates gallbladder contraction (Witteman et al., 1993), increased plasma triglycerides (Robertson et al., 2002; Heath et al., 2004; Smeets & Westerterp-Plantenga, 2006) and hormones, such as insulin (Robertson et al., 2002; Smeets et al., 2006) and ghrelin (Heath et al., 2004). Also, MSF was found to reduce appetite (Heath et al., 2004) and increase satiety (Smeets & Westerterp-Plantenga, 2006). However, data on oral fat exposure, its contribution to intestinal lipid digestion and resulting effects on appetite in human is preliminary and limited (Mattes, 2002).

Including the gastric passage in *in vivo* studies, it was shown, that intragastric infused FFA were five times more potent to induce the release of CCK and PYY as well as the perception of fullness than TG per gram of the respective lipid (Little et al., 2007). This points towards the importance of gastric lipolysis. The emulsifier used in that study to either emulsify oleic acid or macadamia oil was milk protein which will be affected by the gastric environment and lead to, for example, flocculation of the droplets, The fundus and the corpus part of the stomach (Fig. 1.1A) store and cause a slow mixing of food with salivary and gastric secretions (Marciani et al., 2001). Most of the mixing is restricted to the antral part. Thus, physical instabilities of emulsions will result in creaming and phase separation (Fig. 1.1B), but also in precipitation (van Aken et al., 2011) (Table 1.1). As a consequence, these instabilities influence the lipid digestion. Moreover, as the release of FFA from small droplets is higher than that of large droplets (Armand et al., 1992, Li & McClements, 2010) (Table 1.1), the surface area should correlate with hormonal responses and satiety feelings. In fact, an emulsion, which retained a small droplet size in the stomach led to higher CCK release and gallbladder contractions compared to an emulsion that phase separated (Marciani et al., 2007; Marciani et al., 2009). In those studies, also the perception of fullness was higher, while hunger and appetite were reduced for the stable emulsion compared to the phase separated emulsion. Nevertheless, the relation of gastric behaviour of emulsions to satiety responses is still not fully understood. For example, a low amount of fat (i.e. 4 g) that was layered on an orally consumed liquid meal resulted in a fast emptying of the aqueous phase, whereas the fat layer was emptied delayed into the duodenum (Foltz et al., 2009). In that study, the increase in plasma CCK and lipid

absorption was not consistent with changes in hunger, satiety and fullness perception. A more recent study showed that when emulsions containing partially hydrogenated fat were administered, gastric emptying was prolonged compared to emulsions with oil (Keogh et al., 2011). Although in that study differences in plasma triglyceride, CCK, PYY and GLP-1 concentrations were observed between the applied emulsions, there was no difference with respect to satiety and hunger perception.

From duodenal intubation studies it is known that the release of long-chain FFA into the duodenum stimulates CCK secretion (McLaughlin et al, 1999), which is involved in the regulation of several gastrointestinal function as described above. It has been shown, that continuous intraduodenal infusion of emulsified fat stimulates CCK more than non-emulsified fat (Ledeboer et al., 1999). Later on, it was confirmed that the emulsion droplet size affects the release of satiety hormones, such as CCK (Seimon et al., 2009). In that study, the impact was larger for small droplets than for large droplets and the release correlated with the appetite sensation and antropyloroduodenal motility when presented intraduodenally. Inhibition of pancreatic lipase by using tetrahydrolipstatin (THL) eliminated the release of hormones, i.e. CCK (Hildebrand et al., 1998; Feinle et al., 2003), GLP-1 (Feinle et al., 2003), PYY and ghrelin (Feinle-Bisset et al., 2005), activated antropyloroduodenal motility (APD), increased food intake (Feinle et al., 2003) and reduced gallbladder emptying (Hildebrand et al., 1998). Nevertheless, it was also shown that the reduction of APD and food intake were more potent from fatty acids with a chain length of C12 (i.e. lauric acid) compared to C18 (i.e. oleic acid). CCK was stimulated likewise, while the PYY concentrations were higher for C18 (Feltrin et al., 2008) fatty acids than for C12. Thus, the direct application of emulsions intraduodenal influences to a large extent metabolic and satiety responses.

1.2 Properties of emulsions to influence lipid digestion

In vivo studies demonstrate that lipid digestion products like free fatty acids can trigger physiological responses that may lead to satiation. However, it remains unclear how lipid composition and the physicochemical state of lipids can be used to control digestion in the first place.

Lipids are present in processed food products as, for example, structural fats (e.g. in pastry products), bulk fats (e.g. in margarine) and emulsified fats (e.g. in dressings) (McClements, 2005; Mun et al., 2007). In the emulsified form, droplets are stabilized by

proteins or low molecular weight surfactants, such as Tween. In addition, lipids will be emulsified during their passage through the gastrointestinal tract due to, for example, surface active compounds, such as bile salts, which in turn can cause structural changes of the emulsions (McClements, 2008) (Fig. 1.1B). For example, WPI-stabilized emulsions were less stable against flocculation and coalescence compared to Tween 20-stabilized emulsions (Mun et al., 2007). Changes at the interface were demonstrated to cause coalescence, flocculation or phase separation of the emulsions (Fig. 1.1B). For example, proteolysis of the protein-covered interface of emulsions by pepsin resulted in weakening the interface (Sarkar et al., 2009; Golding et al., 2011). Consequently, droplets were more prone to coalescence. In combination with salts and acidic pH this resulted in flocculation (Sarkar et al., 2009; Golding et al., 2011). When a fungal lipase was introduced in such a system the flocculation of protein-stabilized emulsions was enhanced (Golding et al., 2011). Since a smaller droplet size, and hence a larger surface area, results in more lipolysis by both human gastric lipase (HGL) and human pancreatic lipase (HPL), as shown *in vitro* (Armand et al., 1992; Borel et al., 1994; Li & McClements, 2010) and *in vivo* (Armand et al., 1999), the above mentioned changes at the interface are likely to influence the release of FFA. This of importance as *in vivo* studies showed that FFA are the key lipolysis products to cause various satiety responses as introduced above (section 1.1.2) (Schwizer et al., 1997; Feinle et al., 2001; Feinle-Bisset et al., 2005; Little et al., 2007).

The digestion of these emulsified lipids is also influenced by the ability of lipases to anchor to the interface. For example, lipolysis by pancreatic lipase is inhibited in the presence of low molecular weight surfactants, such as Tween 80 (Gargouri et al., 1983; Pieroni et al., 1990), phospholipids (Wickham et al., 1998; Wickham et al., 2002) and by protein emulsifiers (Bläckberg et al., 1979). Co-lipase, in the presence of bile salt is able to restore these inhibitory effects of surface active compounds (Gargouri et al., 1983). Under simulated intestinal conditions, for example, the extent of lipid digestion of emulsions stabilized by surfactants, such as Tween 20 and lecithin, was lower compared to the extent of hydrolysis of emulsions stabilized by proteins, such as casein and whey protein isolate (WPI), (Mun et al., 2007) (Table 1.1).

1.2.1 *In vitro* models to reveal the release of FFA

As the small intestine is the major site for lipid digestion and absorption, most of previous *in vitro* research is conducted by simulating this part of the digestion to determine the concentration of FFA released. For example, test samples are exposed to enzyme mixtures, such as pancreatin, and bile salts at neutral pH (Table 1.1). In addition, various salts, such as NaCl and $CaCl_2$, and surface active compounds, such as phospholipids, can be added to try and better simulate the *in vivo* situation, as reviewed recently (McClements & Li, 2010). The lipolysis in those models is typically followed by pH-stat titration (Table 1.1) at a pH's between 6.2 and 8.0. The rate of digestion of lipids from the investigated emulsions is increasing with increasing calcium, bile or lipase concentration and with the presence of co-lipase. The pH-stat method will give relative rates of lipid digestion in the form of FFA release rather than an absolute measure of total FFA formed (Mun et al., 2006) or the formation of monoglycerides or diglycerides. *In vitro* models help to reveal phenomena, that are likely to be of importance *in vivo*. Using a dynamic model (Table 1.1), the formation of monoglycerides (MG) and DG were shown to play a role during lipid digestion. They act as emulsifiers at the oil droplet interface and participate in micelle formation in the intestine (Reis et al., 2009).

As can be seen in Table 1.1, only a few models simulate gastric digestion or include it, especially with respect to lipid digestion. In these models, samples are mostly subjected to an acidic pH, pepsin and salts, such as NaCl. As mentioned above, the addition of pepsin and salts will enhance coalescence and flocculation of protein-stabilized emulsions. Only a few recent studies include biopolymers, such as mucus (van Aken et al., 2011; Kenmogne-Domguia et al., 2012; Marze et al., 2013) or lipolytic enzymes, such as fungal lipase (Golding et al., 2011; van Aken et al., 2011). In the study of van Aken et al., (2011) physical changes in the emulsion were also observed: creaming of the protein-stabilized emulsion as well as precipitation of full fat milk. Although these changes are quite substantial, no effect was observed on the extent of lipolysis (Table 1.1) as revealed with gas chromatography. The pH-stat method is not suitable to follow lipolysis under gastric conditions. To assess the extent of lipid digestion under such acidic conditions, a back-titration technique can be used. This has been applied after incubation of lipid systems with e.g. HGL (Gargouri et al., 1986a; Gargouri et al., 1986b). This technique provides the total concentration of FFA at pH 9. But again, this method will not allow to studying MG and DG formation and is in our opinion very time consuming.

Overall, there are still many questions related to the way lipids are digested and how this contributes in controlling food intake. For the study of lipid digestion *in vitro* models are important. However, *in vitro* models are limited in their ability to mimic the digestive process and lack the ability to mimic physiological responses, such as hormonal induced feedback mechanisms. Therefore, combining *in vitro* models with *in vivo* studies is considered to be of relevance.

1.3 Aim and outline of the thesis

From the above it is clear, that it is yet not fully understood how emulsions can regulate satiety responses.. Hence, the aim of this thesis is to provide knowledge on mechanisms affecting the digestion of lipids, specifically the release of free fatty acids, and subsequent satiety responses. As a starting point within this project the lipolytic breakdown of emulsions under intestinal conditions is simulated with a focus on the effect of two well-known influencing factors: The type of emulsifier used and the droplet size of the emulsion (**Chapter 2**). Whey protein isolate (WPI) is used as emulsifier as it is a common proteinaceous emulsifier in food applications. Also, gum arabic is used as an emulsifier as it mainly consists of polysaccharides and these have been shown to decrease the release of free fatty acids. A gas chromatographic method is described, which is further used to determine FFA, monoglyceride , diglyceride and triglyceride (TG). The results for all lipolytic products (i.e. FFA, MG, DG) in relation to droplet size and emulsifiers used are presented. In case of FFA, they are compared with the pH-stat method. In **Chapter 3**, the effects of gastric and small intestinal processing of two differently charged protein stabilized emulsions on gastric and intestinal lipolysis are investigated under dynamic *in vitro* conditions. WPI and lysozyme are used as emulsifiers in order to reveal the impact of flocculation at the isoelectric point under gastric conditions. To reveal the effect of gastric behaviour on intestinal lipid digestion, a custom designed gastric compartment, simulating the motility of the different regions of the stomach, and gradual acidification in the gastric compartment is used. In **Chapter 4** the role of *in vivo* intragastric distribution of lipids on gastric and intestinal lipolysis in healthy subjects is investigated. For this purpose, a homogenized emulsion and an un-homogenized sample are perfused intragastrically. Furthermore, the additive effect of oral application of the homogenized emulsion on gastric lipolysis is studied. Gastric and duodenal aspirates are assessed for emulsions structures, pH and lipid compositions (i.e. FFA, MG, DG and TG) and plasma paracetamol concentrations are evaluated as measure

for gastric emptying. In **Chapter 5** the impact of gastric and intestinal lipolysis as well as the oral application on physiological responses related to lipid digestion and satiety were investigated. To this end, gallbladder contraction and changes in plasma concentrations of CCK, GLP-1, PYY and ghrelin are studied. These findings are further related to satiety perceptions and *ad libitum* intake. In addition, the additive effect of oral application on the same measures is investigated. In the last chapter (**Chapter 6**) it is discussed how all these results obtained help to improving our understanding of lipid digestion in relation to satiety with focus on the impact of the models used and results from a pig study. Finally, the impact of the different emulsion parameters on satiety are discussed.

Table 1.1 Overview of *in vitro* experiments conducted on the behavior of emulsion based systems with focus on the analysis of released FFA (adapted from McClements & Li (2010) and test samples 1-20 are depicted from this overview (McClements & Li, 2010)).

Test sample	Variable	Characterisation	Method for FFA/ lipid analysis	Digestion step	Result	Reference
O/W emulsion O = tuna oil E = Lec, Lec/Chit	Bile Lipase Polysaccharides	Microscopy ζ-potential Particle size Appearance FFA, Glucosamine	pH-stat	***M***: pH 7 (1h) ***S***: pH 2 (1h) ***SI***: pH 5.3, pancreatic lipase, bile (2h) ***SI***: pH 7.5 (2h)	Digestion rate increased with [bile] and dependent on PS.	Klinkesorn et al., 2010
O/W emulsion O = tuna oil E = Lec, Lec/Chit	Polysaccharides	Microscopy ζ-potential Particle size Appearance FFA, Glucosamine	pH-stat	***M***: pH 7 (1h) ***S***: pH 2 (1h) ***SI***: pH 5.3, pancreatic lipase, bile (2h) ***SI***: pH 7.5 (2h)	Increasing amounts of chitosan reduced the amount of FFA produced. Chitosan was degraded by lipase.	Klinkesorn et al., 2009
O/W emulsion O = corn oil E = Cas, WPI, Lec, T20	Emulsifier	Microscopy ζ-potential Particle size FFA	pH-stat	***SI***: pancreatic lipase, bile salts	Digestion rate and extend depends on emulsifier type: Cas, WPI > Lec > T20.	Mun et al., 2007
O/W emulsion O = corn oil E = BLG, Lec	Emulsifier BLG cross-linking	Microscopy ζ-potential Particle size FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Protein interfacial cross-linking did not have a big impact on lipid digestion.	Sandra et al., 2008
O/W emulsion O = tripalmitin E = SDS	Lipid physical state	ζ-potential Particle size, DSC FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Solid fat particles are digested more slowly than lipid fat droplets.	Bonnaire et al., 2008
O/W emulsion O = corn oil E = Cas, LF, Cas/LF	Emulsifier Multilayer	ζ-potential Particle size, DSC FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Lipid droplets with different initial protein compositions are equally digested.	Lesmes et al., 2010
O/W emulsion O = corn oil, MCT E = BLG, T20, Lec	Oil Particle size Lipid content Emulsifier type	ζ-potential Particle size Microscopy FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Digestion rate increases with decreasing droplets size and decreasing oil molecular weight, but does not depend strongly on the emulsifier type.	Li et al., 2010
O/W emulsion O = fish oil E = Citrem, Chit, Alg	Polysaccharide Multilayer formation	ζ-potential Creaming Particle size Microscopy FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	The rate and extend of lipid digestion was decreased when chitosan and chitosan/alginate were present around droplets.	Gudipati et al., 2010
O/W emulsion O = corn oil E = lyso-Lec, lyso-Lec/Chit	Polysaccharide [Bile] [Calcium] [pancreatic lipase]	ζ-potential Particle size FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Coating droplets with non-cross linked or cross-linked chitosan decreased the digestion rate.	Hu et al., 2010a

Table 1.1 continued

O/W emulsion O = corn oil E = Lec, Lec/Chit, Lec/Chit/Pec	Interfacial composition	ζ-potential Particle size Microscopy FFA	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Coating droplets with chitosan decreased lipid digestion, but having an additional pectin coating increased digestion again.	Mun et al., 2006
O/W emulsion O = olive oil E = PC	Bile [Bile] [Calcium]	ζ-potential Particle size FFA	pH-stat	***SI***: pH 7.5, pancreatic lipase, bile salts	FFA release increased with increasing calcium concentration and the amount depended on bile type.	Wickham et al., 1998
SEDDS O = soybean oil E = Tweens, Spans	Emulsifier Salts	FFA	pH-stat	***SI***: pH 6.5, pancreatic lipase, bile salts	Digestion rate increases with calcium addition and depends on surfactant type.	Ali et al., 2010
O/W emulsion O = olive oil E = galactolipids	Emulsifier type	ζ-potential Particle size FFA Interfacial tension	pH-stat	***SI***: pH 7, pancreatic lipase, bile salts	Digestion rate depends on surfactant type.	Hu et al., 2010b
Oil bodies & O/W emulsion O = sunflower seed oil E = Natural, WPI, T20	Emulsifier Microstructure	Particle size FFA Protein analysis	GC-MS	***SI***: pH 7, pancreatic lipase, bile salts	Digestion of oil bodies is slower than emulsion droplets.	White et al., 2009
O/W emulsion O = fish oil E = MG, BLG, lyso-Lec	Emulsifier	FFA Interfacial tension	GC-FID	TIM Model ***S***: pepsin, fungal lipase ***SI***: bile, pancreatin, trypsin	The digestion rate depends on initial emulsifier type and MG inhibits digestion.	Reis et al., 2008
O/W emulsion O = fish oil E = Cas, Cas-CHO complex	Emulsifier	Particle size Viscosity FFA Microstructure	pH-stat	***SI***: pH 6.8, pancreatin, bile salts	Interfacial covalent (Maillard) caseinate-carbohydrate complexes reduced lipid digestion.	Chung et al., 2008
O/W emulsion O = SCT, MCT, LCT E = MG	Oil [Lipase]	Microstructure FFA	pH-stat	***SI***: pH 6.5, pancreatin	Initial digestion rate increased with [Lipase] and depends on oil type.	Brogard et al., 2007
SEDDS O = SCT, MCT, LCT E = surfactants	Oil	FFA	pH-stat	***SI***: pH 7.5, pancreatin, bile salts	Formulation affects the lipid digestion rate.	Porter et al., 2004
O/W emulsion O = SCT, LCT E = Lec	Tea extract	Particle size FFA	TLC	***S***: pH 5.4, gastric juice (0.5h) ***SI***: pH 7.5, pancreatin, bile (1h)	A green tea extract inhibits lipid digestion.	Juhel, 2000
O/W emulsion O = triolein E = Lec	Polysaccharide (guar gum, gum arabic, pectin)	Particle size Viscosity FFA in ***S***	pH-stat	***S***: human gastric juice	Dietary fibers can inhibit lipid digestion by an amount depending on their structure.	Pasquier et al., 1996
O/W emulsion O = triolein E = WPI, GA	Emulsifier Particle size	Microscopy FFA Lipolysis products	pH-stat, GC-FID	***SI***: pH 7.5, bile salts, pancreatin	Digestion rate increases with decreasing droplets size. Ratio and concentration of FFA, MG and DG depend on emulsifier type.	this thesis, Chapter 2

Table 1.1 continued

O/W emulsion O = olive oil E = WPI, Lys	Emulsifier	Microscopy Particle size Appearance Lipolysis products from ***S+SI***	GC-FID	Adapted TIM Model ***S***: pepsin, pregastric lipase ***SI***: bile, pancreatin, trypsin	Transition through the p*I* of the protein results in creaming and this delays intestinal lipid digestion.	this thesis, Chapter 3
O/W emulsion O = sunflower oil E = T80	Intestinal pH [Calcium] [Bicarbonate]	Microstructure Lipolysis products Cholesterol	GC-FID TLC	***S***: pH 1.3, pepsin (0.5h) ***SI***: pH 6.2-7.5, pancreatin, bile salts (4h)	Bicarbonate increases the degree of lipid digestion. Cholesterol and FFA co-solubilize in bile micelles.	Vinarov et al., 2012
oil body O = walnut oil E = walnut		Microstructure ζ-potential Particle size Protein analysis FFA	pH-stat	***S***: pH 1.5, pepsin (1h) ***SI***: pH 7, pancreatin, bile salts (2h)	Digestion of oil bodies, spontanoues formation of multiple phase emulsion.	Gallier et al., 2013
O/W emulsion O = fish oil, MCT E = BLG	Oil	Particle size ζ-potential Interfacial tension FFA	pH-stat	***M***: pH 6.8, mucus, amylase (5 min) ***S***: pH 2, mucus, pepsin (2h) ***SI***: pH 8, pancreatin, pancreatic lipase, bile (4h)	Digestion of fish oil is incomplete.	Marze et al., 2013
O/W emulsion O = tricaprylin, triolein E = BLG, NaO	Oil [Emulsifier]	Particle size Interfacial tension FFA Lipolytic products	pH- stat HPLC-ELSD	***SI***: pH 7.5, bile salt, pancreatic lipase	Digestion rate depends mainly on oil type, no effect of [emulsifier].	Marze et al., 2012
O/W emulsion O = rapseed oil E = BSA	pH Pepsin Intestinal Incubation time	Microscopy Particle size Lipolysis products	HPLC-ELSD	***S***: pH 2.5 or 4, mucus, pepsin (1h) ***SI***: pH 6.5, pancreatin, pancreatic lipase, bile (0, 2, 5, 20h)	Pre-incubation with pepsin delays intestinal lipid digestion but not the final extent.	Kenmogne-Domguia et al., 2012
O/W emulsion and milk O = sunflower oil E = WPI, T80	Emulsifier Cas pH [Enyzme]	Microscopy Particle size Appearance FFA Nitrogen and fat content	GC-FID	***S***: pepsin, lipase, mucus (1h)	Emulsifier type did not affect extent of lipid digestion but creaming or sedimentation behavior.	Van Aken et al., 2011
O/W emulsion and Ivelip^{TM} O = canola oil (CO), CO/hydrogenated fat E = SSL, T80, WPI	Emulsifier Oil	Microscopy ζ-potential Particle size FFA	pH-stat	***S***: pH 1.9, pepsin, fungal lipase (2h) ***SI***: pH 6.8, pancreatin, bile salts (3h)	Incorporation of solid fat delays initial lipid digestion but hardly the extent.	Golding et al., 2011
O/W emulsion O = soybean oil E = WPI, SPI	Phospholipids Colipase (COL) Phospholipase A_2	ζ-potential Particle size Protein analysis FFA	Spectro-photometry	***S***: pH 2, pepsin (1h) ***SI***: pH 6.5, pancreatin, bile salts (2h)	Addition of COL increases lipase activity. Lipid digestion rate depends on emulsifier.	Malaki Nik et al., 2011

Table 1.1 continued

O/W emulsion O = soybean oil E = Cas	Gastric and intestinal incubation time	Microscopy Particle size Protein analysis FFA	pH-stat	***S***: pH 2, pepsin (1, 10, 30 min, 1h, 2h) ***SI***: pH 7.5, pancreatin, bile salts (1, 10, 30 min, 1h, 2h)	Variation in gastric and intestinal incubation time led to markedly differences in flocculation and coalescence but did not alter intestinal lipid digestion.	Li et al., 2012
milk raw or recombined	Interfacial composition Bile salts Incubation time	Microscopy ζ-potential Particle size FFA	pH-stat	***SI***: pH 7, pancreatin, bile salts, pancreatic lipase	Lipid digestion was influenced by the interfacial composition. Milk fat globules flocculated and addition of bile increased lipid digestion.	Ye et al., 2010
O/W emulsion O = corn oil E = LF, LF-Alg, LF-LMP, LF-HMP	Polysaccharide type Pepsin	Microscopy ζ-potential Particle size Appearance FFA	pH-stat	***S***: pH 2.5, pepsin (2h) ***SI***: pH 7, pancreatic lipase, bile salts (2h)	All were stable in gastric but aggregated in intestinal conditions except the presence of Alginate caused aggregation in both conditions. This did not affect the lipid digestion rate.	Tokle et al., 2012
O/W emulsion O = corn oil E = BLG, BLG-dextran	Dextran (MW) [Bile]	ζ-potential Particle size FFA	pH-stat	***S***: pH 1.2, pepsin (2h) ***SI***: pH 7, pancreatic lipase, bile salts (2h)	Increasing MW of Dextran moieties increase stability of emulsion under gastric and intestinal conditions. The extent of lipid digestion is reduced in the presence of dextran.	Lesmes et al., 2012
O/W emulsion O = olive oil E = lecithin, poloxamer (Pluronic®)	Emulsifier Particle size	Microscopy ζ-potential Particle size Interfacial tension Appearance FFA	pH-stat	***SI***: pH 7, bile salts, pancreatic lipase	Non-ionic surfactant is more resistant to displacement by bile at the interface than lecithin, thus lipid digestion was reduced.	Torcello-Gomez et al., 2011
O/W emulsion O = olive oil E = BLG, LF	Interfacial composition	Microscopy ζ-potential Particle size FFA	pH-stat	***SI***: pH 7, bile salts, pancreatic lipase	Mixed protein interfaces had enhanced stability to intestinal conditions, but did not affect the lipid digestion.	Schmelz et al., 2011

The following keys were used:
Digestion step M, mouth; S, stomach; SI, small intestine
Test samples O, oil; E, emulsifier; SEDDS, self-emulsifying drug delivery system
Emulsifiers Alg, Alginate; BLG, β-lactoglobulin; BSA, bovine serum albumin; Cas, caseinate; Chit, Chitosan; Citrem, citric acid ester of Mono- and diglycerides; GA, gum arabic; HMP, high methoxyl pectin; LF, Lactoferrin; Lec, Lecithin; LMP, low methoxyl pectin; Lys, Lysozyme; MG, monoglyceride; NaO, sodium oleate; PC, Phosphatidycholine; Pec, Pectin; SDS, sodium dodecyl sulfat; SSL, sodium stearoyl lactylate; T20, Tween 20; T80, Tween 80; WPI, whey protein isolate

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Chapter 2

In vitro study of intestinal lipolysis using pH-stat and gas chromatography

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In vitro study of intestinal lipolysis using pH-stat and gas chromatography
Food Hydrocolloids, 28(1), 10-19

Abstract

Developing healthy products requires in-depth knowledge of digestion. This study focuses on lipid digestion in relation to emulsion properties typically followed by pH-stat. Although this is a fast and easy method to follow the overall digestion, it provides no details on lipid digestion products. Thus, the aims of the present study were to use gas chromatography (GC) to determine all products present during lipolysis, i.e. monoglycerides (MG), diglycerides (DG) and triglycerides (TG), and to compare this method with the pH-stat method for free fatty acids (FFA). Fine, medium and coarse emulsions stabilized with two different emulsifiers whey protein isolate (WPI) or gum arabic were digested under in vitro intestinal conditions. Although the amount of FFA increased for both methods for WPI stabilized emulsions, the amount of FFA was 2–3 times higher when determined by GC compared with pH-stat. GC analysis showed decreasing amounts of MG and DG with increasing droplet size for both emulsions. Molar ratios of FFA/DG and MG/DG were twofold higher for WPI than for gum arabic stabilized emulsions. This indicates that the total production of lipolytic products (i.e. FFA+MG+DG) depends on the droplet size and the emulsifier but their proportions only depend on the emulsifier. Although pH-stat provides a fast measure of FFA release, it is influenced by the emulsifier type at the oil–water interface and therefore care should be taken when interpreting pH-stat results. We suggest combining this method with GC for accurate FFA determination and further evaluation of all lipolytic products.

2.1 Introduction

Understanding lipid digestion is of crucial importance to the food industry when designing food products that not only taste good but also regulate food intake. In the regulation of metabolic intake, free fatty acids (FFA) trigger the release of hormones, such as cholecystokinin, leading to satiety and a delay in stomach emptying (for a review see Cummings & Overduin, 2007). FFA are generated from triglycerides (TG) and diglycerides (DG) by gastric and pancreatic lipase. TG and DG can be present in food products as bulk fats (e.g. in margarine), structural fats (e.g. in bread) and emulsified fats (e.g. in dressings) (McClements, 2005; Mun et al., 2007). Moreover, TGs and DGs are emulsified during their passage through the gastrointestinal tract (GIT) (McClements, 2008). The behaviour of these emulsified TGs and DGs in the GIT, and particularly their lipolysis, is dependent on parameters such as fat composition, continuous phase of the emulsion and droplet size. The influence of the droplet size on lipase activity has been investigated *in vitro* (Armand et al., 1992; Borel et al., 1994) and *in vivo* (Armand et al., 1999) for both gastric and pancreatic lipase showing that a smaller droplet size leads to a higher lipolysis rate. Other important key features are the interfacial properties of the fat droplets and a considerable number of *in vitro* studies have been carried out in the past on this topic. They show that bile salts, surfactants (Gargouri et al., 1983; Pieroni et al., 1990), phospholipids (Wickham et al.,, 1998; Wickham et al., 2002) and protein emulsifiers (Bläckberg et al., 1979) have an inhibitory effect on the pancreatic lipase activity. Moreover, it has been shown that the addition of bile salts and co-lipase to those systems is able to restore such inhibitory effect (Gargouri et al., 1983). Recent studies also investigate the effect of droplet interfacial properties, as affected by the type of emulsifiers used, to further explore the impact on lipolysis. For example, it has been shown that pancreatic lipase accessibility on emulsions stabilized by surfactants (e.g. Tween 20 and lecithin) is much lower than for emulsions stabilized by proteins (e.g. casein and whey protein) (Mun et al., 2007). However, β-lactoglobulin (β-lg) was more effective in inhibiting the initial lipolysis than lysolecithin (Hu et al., 2010). Polysaccharides such as gum arabic, which has been used as emulsifier during lipase activity assays (Rathelot et al., 1975; Gargouri et al., 1984; Salah et al., 2006), seem to represent a suitable interface component for lipases to adsorb as well.

Besides the type of emulsifier (surfactants vs. proteins), the role of the charge of the interfacial layer on the lipase activity has been also investigated but it has not been fully elucidated yet. In fact, Wickham et al. (1998) showed that there is no specific correlation between the charge of the emulsion and the lag phase of enzymatic action. However, the

release of FFA has been found to be slightly higher using positively charged emulsifiers (e.g. lactoferrin) than negatively charged emulsifiers (e.g. β-lg) (Sarkar, Horne & Singh, 2010a).

In vitro models of the GIT are frequently used to understand the biochemical and physicochemical mechanisms that occur during lipid digestion (McClements et. al, 2009). In those models, gastric and intestinal lipolysis of samples are simulated either separate or in combination and different compositions of gastric and intestinal fluids to simulate the in vivo conditions are used (for review see McClements & Li, 2010). For example, samples in simulated gastric fluids are exposed to an acidic pH, salts (such as NaCl), biopolymers (such as porcine gastric mucosa) as well as proteolytic and/or lipolytic enzymes (such as pepsin and fungal lipase) (Sarkar et al., 2009; van Aken et al, 2011). During simulated intestinal digestion, samples are subjected to neutral pH, mixtures of enzymes (such as pancreatin), salts, bile salts and phospholipids (Hu et al., 2010; Hur et al., 2009; Sarkar et al., 2010a; Sarkar, Horne & Singh, 2010b; Porter & Charman, 2001). In vitro lipolysis is typically monitored by pH-stat titration to obtain relative rates of lipolysis, by measuring the amount of FFA (Mun et al., 2006), rather than to get an absolute measure of the extent of digestion. The pH-stat titration can be carried out either as a direct assay by titrating ionized FFA with titrant (e.g. NaOH) to maintain a certain constant pH or using a back-titration. In the latter case, the emulsified lipids are first incubated at a given pH for a certain time. Then, the pH is rapidly increased with NaOH to pH 9 to stop the reaction and to favour the release of FFA. Subsequently, the total amount of FFA is assessed by titration. This technique was used under simulated gastric conditions after incubation of lipid systems with e.g. human gastric lipase (Gargouri et al., 1986a; Gargouri et al., 1986b).

However, both direct and back titration using pH-stat do not provide any details on the formation of monoglycerides (MG) and DG which play a role during lipid digestion acting as emulsifiers at the oil droplet interface and participating in micelle formation in the intestine (Reis et al., 2009). Moreover, they are probably involved in inhibiting human gastric lipase activity (Pafumi et al., 2002). Therefore, it is important to monitor the formation of FFA, MG and DG during digestion.

To our knowledge, only few publications are available in literature reporting the lipase activity in relation to the simultaneous formation of FFA, MG and DG from emulsions (Carriere et al., 1991; Armand et al., 1996a, 1996b, 1999; Janssen et al., 2006; Persson et al. 2007). Hydrolysis profiles (i.e. formation of glycerol, MG and DG from Intralipide) of

different gastric lipases in vitro (i.e. human, dog and rabbit gastric lipase) were monitored using high-performance liquid chromatography (Carriere et al., 1991). Using the same technique, Persson et al. (2007) could separate and analyse phospholipids, bile acids and neutral lipids from in vivo fed-state human small intestinal aspirates. Thin-layer chromatography has also been used to analyse and quantify the TG content and its digestion products in gastric and duodenal aspirates (Armand et al., 1996a, 1996b, 1999). Janssen and coworkers (2006) used gas chromatography (GC) to analyse FFA, MG, DG and TG in oil samples. Although estimations of the hydrolysis rates can be obtained from the above-mentioned studies, no in-depth research has been conducted so far on the relation between each single lipid digestion product and the interfacial composition and emulsion droplet size.

As a starting point for the lipolysis research within our project, we decide to use a rather simple system with focus on pancreatic lipase activity under specific biochemical conditions, ignoring the oral or gastric digestion phase both having an important role on lipid digestion. Moreover, we wanted to establish a GC procedure to determine FFA, MG and DG to be further used during our whole research and, in the future, to analyse *in vivo* digested samples. Therefore, we simulated the lipolytic breakdown of emulsions under intestinal conditions and focused on the effect of two well-known influencing factors: the type of emulsifier and droplet size. In this paper, we describe the GC results obtained for all lipolytic products (i.e. FFA, MG, DG) in relation to droplet size and emulsifiers used and, in the case of FFA, we compare them with the pH-stat method.

2.2 Materials and methods

2.2.1 Materials

Whey protein isolate (WPI) was purchased from Davisco Foods International (BiPro®, lot JE216-6-440, Le Sueur, MN, USA, 93% (w/w) protein determined by the Dumas method) and gum arabic from Caldic Ingredients BV (spray-dried gum acacia 368A, lot 0T060521p, Oudewater, The Netherlands, 2% (w/w) protein content determined by the Dumas method). Triolein, used as the oil phase, was obtained from Sigma–Aldrich (T7752, lot 016k0715, approx. 65% practical grade which corresponds to approx. 65% oleic acid according to the manufacturer, Sigma-Aldrich, St. Louis, MO, USA). The fatty acid profile of this triolein was determined after conversion into fatty acid methyl esters (FAMEs) following the AOCS Official method (AOCS Method Ce 1f-96), in the Laboratory of Food

Technology and Engineering, Ghent University, Belgium. It contains the following fatty acids: myristic acid (C14:0, 2.1%, M), palmitic acid (C16:0, 4.1%, P), palmitoleic acid (C16:1, 5.7%, Pi), oleic acid (C18:1, 74%, O) and linoleic acid (C18:2, 8.6%, L). Furthermore, triglyceride composition expressed as the percentage of the total triglyceride present was determined with high-performance liquid chromatography according to Rombaut, De Clercq, Foubert, and Dewettinck (2009) also in the Laboratory of Food Technology and Engineering, Ghent University. Pancreatin from porcine pancreas was purchased from Sigma–Aldrich (P3292, lot 117k1343, 4x USP). Porcine bile extract (BE), containing 49 % (w/w) bile salts, 5% (w/w) phosphatidyl choline and less than 0.06% (w/w) calcium according to Zangenberg, Müllertz, Kristensen and Hovgaard (2001b), was obtained from Sigma–Aldrich. FFA standards, i.e. C14:0, C16:0, heptanoic acid (C17:0), C18:1, C18:2 as well as monoolein, diolein, and TG standards triolein and trilaurin (TG36) were obtained from Sigma–Aldrich (>99 % pure). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich. Solutions were prepared in demineralized water.

2.2.2 Preparation and characterization of emulsions

WPI and gum arabic solutions were prepared by dissolving powder in 10 mM NaCl solution and stirred overnight. Triolein, used as the oil phase, was kept at −20 °C and thawed before use. WPI stabilized emulsions contained 20% (w/w) oil and 1% (w/w) emulsifier. Gum arabic stabilized emulsions contained 5% (w/w) oil and 7.5% (w/w) emulsifier. Coarse WPI and gum arabic stabilized emulsions with a surface weighted mean diameter (d_{32}) of 36 and 21 μm, respectively, were prepared using an Ultra-Turrax S25 KR 18 G (IKA-Werke, Staufen, Germany) at 6500 rpm for 1 min. Medium-sized emulsions, WPI (d_{32}=4 μm) and gum arabic (d_{32}=7 μm), were prepared using the Ultra-Turrax at 21,500 and 13,500 rpm, respectively, for 1 min. Fine emulsions, WPI (d_{32}=0.3 μm) and gum arabic (d_{32}=1.6 μm), were made from pre-emulsions (Ultra-Turrax at 9500 rpm for 1 min) and further passed 10 times through a laboratory homogenizer (LH-scope HU-3.0, Delta Instruments, Drachten, The Netherlands) at a pressure of 180 bar.

Droplet size distributions, d_{32} and surface area of the emulsion droplets were determined directly after preparation by dynamic light scattering (Mastersizer Hydro 2000SM, Malvern Instruments, Malvern, UK) at room temperature. Dilution in demineralized water was applied during the measurements according to other studies (Vingerhoeds et al., 2005; Silletti et al., 2007; Sarkar et al., 2010b). It has been shown so far

that only in flocculated systems dilution upon water might break aggregates leading to changes in particle sizes (Silletti et al., 2007).

2.2.3 pH-stat measurements of *in vitro* intestinal digestion of emulsions

The digestion of freshly prepared emulsions was monitored using a pH-stat automatic titration unit (Metrohm, Herisau, Switzerland) at pH 7.5. This pH 7.5 was chosen in order to be able to compare our data with the results already available in literature (e.g. Wickham et al., 1998) and moreover because the used emulsions are known to be stable at this pH.

The emulsions were diluted in a solution consisting of 150 mM NaCl and 5 mM KCl to obtain a final oil concentration of 10% (w/w) and 2.5% (w/w) for WPI and gum arabic emulsions, respectively. BE were dissolved in 5 mM Tris buffer (pH 6.9), containing 20 mM $CaCl_2$ and 40 mM NaCl, and subsequently added to the emulsions. The pH of the BE/emulsion mixture was adjusted to 7.5 with 0.5 M NaOH solution. The reaction was started by adding pancreatin dissolved in a solution containing 150 mM NaCl and 5 mM KCl. Final concentrations were therefore 6.4 mg/mL BE, 0.8 mg/mL pancreatin and 110 mM NaCl. The pH was kept constant at 7.5 by addition of 0.5 M NaOH solution while gentle stirring. Each measurement was done in duplicate. The BE/emulsion mixture without pancreatin was taken as reference and processed in the same way as the emulsions with pancreatin. The amount of FFA (mol) was calculated from the number of moles of NaOH used to keep the pH at 7.5 during digestion.

2.2.4 Extraction and GC measurements of *in vitro* intestinal digestion of emulsions

Samples from duplicate measurements under the pH-stat conditions were taken for GC analysis. A 0.5-mL sample was taken with no enzyme present (t_0), and 10 min (t_{10}), 20 min (t_{20}), and 60 min (t_{60}) after addition of enzyme. Each sample was poured into an extraction mix consisting of 1 mL ethanol, 1.5 mL diethylether/heptane (DEE/Hep, 1:1 v/v), and 0.1 mL of 2.5 M sulphuric acid in a glass Kimax tube. Sulphuric acid is only used to protonate FFA and did not lead to additional FFA formation (data not shown). The tube was closed with a cap, shaken vigorously for 1 min and centrifuged for 5 min at 500×*g*

and 20 °C. The upper layer was removed with a glass pipette and transferred into a new glass tube. This extraction was repeated twice with 1 mL of DEE/Hep. The final supernatant was collected and its volume was brought to 4 mL. Anhydrous sodium sulphate (100 mg) was added as drying agent. It was necessary to further apply a 1:20 dilution to quantify the TG for WPI stabilized emulsions. After extraction, samples were stored at −20 °C until further analysis. An internal standard mix (50 µL), consisting of C17:0 and TG36, was added to obtain a final concentration of 0.4 mg/mL. The reference mixture of BE/emulsion without pancreatin was subjected to the same procedure. The values obtained were used as a baseline for the actual digestion process. Hence, lipolysis at any time point is expressed as the additional amount of product formed.

GC was performed using a Trace GC Ultra (Thermoscientific, Milan, Italy) as follows. Samples (1 µL) were injected splitless into a liner using a programmed temperature vaporizing injector with an autosampler (CombiPAL, CTC Analytics, Zwingen, Switzerland) and analyzed by flame ionization detector. The column used was a FactorFour capillary column (30 m × 0.32 mm inner diameter (ID), film thickness (d_F) = 0.10 µm, VF-5ht UltiMetal, Varian, Middelburg, The Netherlands), connected to an UltiMetal retention gap (2 m × 0.53 mm ID, d_F = 0.10 µm, uncoated, methyl deactivated, outer diameter 0.8 mm, Varian). Helium was used as carrier gas at a constant flow rate of 2 mL/min. The program started at an initial temperature of 80 °C (1 min) and increased to 400 °C (2 min) at a rate of 10 °C/min over a total oven run time of 35 min.

Peaks were identified by comparing the relative retention times with those of standards. Quantification of each digestion product was based on standard curves (R^2 = 0.99). The GC data was processed using Xcalibur® software (version 2.0, Thermoscientific, Milan, Italy) and FFA, MG, DG and TG were calculated using the following molecular masses of the main components: 282.5 g/mol for FFA, 320.4 g/mol for MG, 602.9 g/mol for DG, and 885.4 g/mol for TG. The hydrolysis rate was calculated according to the following equation (Rodriguez et al., 2008):

$$\text{Hydrolysis (\%)} = 100\times FFA/(3\times TG_0)=100\times FFA/(3\times TG+2\times DG+MG+FFA)$$

where TG_0 is the initial amount of TG (mol) present. The concentration of free glycerol (G) was calculated based on the following equation (Rodriguez et al., 2008):

$$G = (FFA - DG - 2\times MG)/3$$

For each time point, mass balances were calculated in % from TG_0:

$FFA\ (\%) = (FFA/3\times TG_0)\times 100$
$MG\ (\%) = (MG/3\times TG_0)\times 100$
$DG\ (\%) = ((2\times DG)/3\times TG_0)\times 100$
$TG\ (\%) = (TG/TG_0)\times 100$

The formation of lipolysis products was further expressed in μM (Table 2.1).

In order to compare the effect of emulsion properties on lipolysis, we calculated the apparent lipase activity. This activity is defined as the amount of FFA released per minute (μmol/min) at a concentration of pancreatin of 0.8 mg/mL (1 U is 1 μmol FFA released/min). It was calculated from the initial slope of the FFA released as a function of time fitted by SigmaPlot software (SigmaPlot® 8.0, Systat Software Inc., San Jose, CA, USA).

2.3 Results

2.3.1 GC emulsion characterization

When we analyzed the TG composition of the practical grade triolein used to prepare the emulsions, only 53.7% was identified as triolein (OOO). The remaining 46.3% is a mixture of TG consisting of LOO (13.8%), PiOO (11.9%), MOO (3.6%), PLO (2.1%), PiOP (2%), POO (6.5%), LLO (1.2%), PiOL (1.3%), OLM (0.7%) and 3% not identified TG. Although the TG composition of the oil is known, TG with the same equivalent carbon number could not be discriminated by GC because they co-eluted in one peak (Fig. 2.1A, insert). Besides TG, in the initial triolein, we find less than 1% FFA, 6% MG and 21% DG which might be due to the esterification during its production. Looking at the chromatograms of the emulsions alone we find about 5% FFA, 3% MG and 16% DG. Probably also during the emulsification some MG and/or DG were converted in FFA. After mixing the emulsions with the digestion fluid in the absence of pancreatin (t_0), still small amounts of FFA, MG and DG (Fig. 2.1A) were observed giving an initial amount of TG at t_0 of about 80% of the total oil volume weighed in and present at the beginning of the experiment. In small-sized WPI stabilized emulsion, for example, FFA concentration at t_0 was 175.1 μM; MG and DG concentrations were 104.0 μM and 133.4 μM, respectively. As expected, these contents did not change during incubation of the emulsion without enzyme for 60 min. Moreover, the

initial amount of FFA, MG and DG was similar at all time points for medium and coarse emulsions, for both WPI and gum arabic.

2.3.2 GC identification of lipolytic products

A typical GC chromatogram of the digested fine WPI stabilized emulsion at t_{60} is shown in Fig. 2.1B. Chromatograms from gum arabic stabilized emulsions gave comparable chromatograms and are not shown. The peak indicated as C16:1 in Fig. 2.1A and B, with elution time of 12.1–12.4 min, contains C16:0 and C16:1; the peak indicated as C18:1 (elution time 12.8–13.6 min) contains C18:1 and C18:2. Those two major peaks were used to calculate the total FFA content in the samples. C14:0, present in amounts less than 1%, was not taken into account. The peaks indicated as MG and DG were identified as monoolein and diolein and eluted between 17.0 and 17.5 min and between 25.8 and 26.4 min, respectively. To calculate the total TG content, all peaks within the elution time of 29.9–32.4 min were considered together. During the digestion, no differences in the ratios of the TG peaks were found. As indicated in Section 2.2.2, the WPI emulsion concentration in the mixture was 10% (w/w). Because of this we had to dilute the samples 1:20 to quantify the TG during the digestion process. A chromatogram of this dilution, also at t_{60}, is shown in Fig. 2.1C.

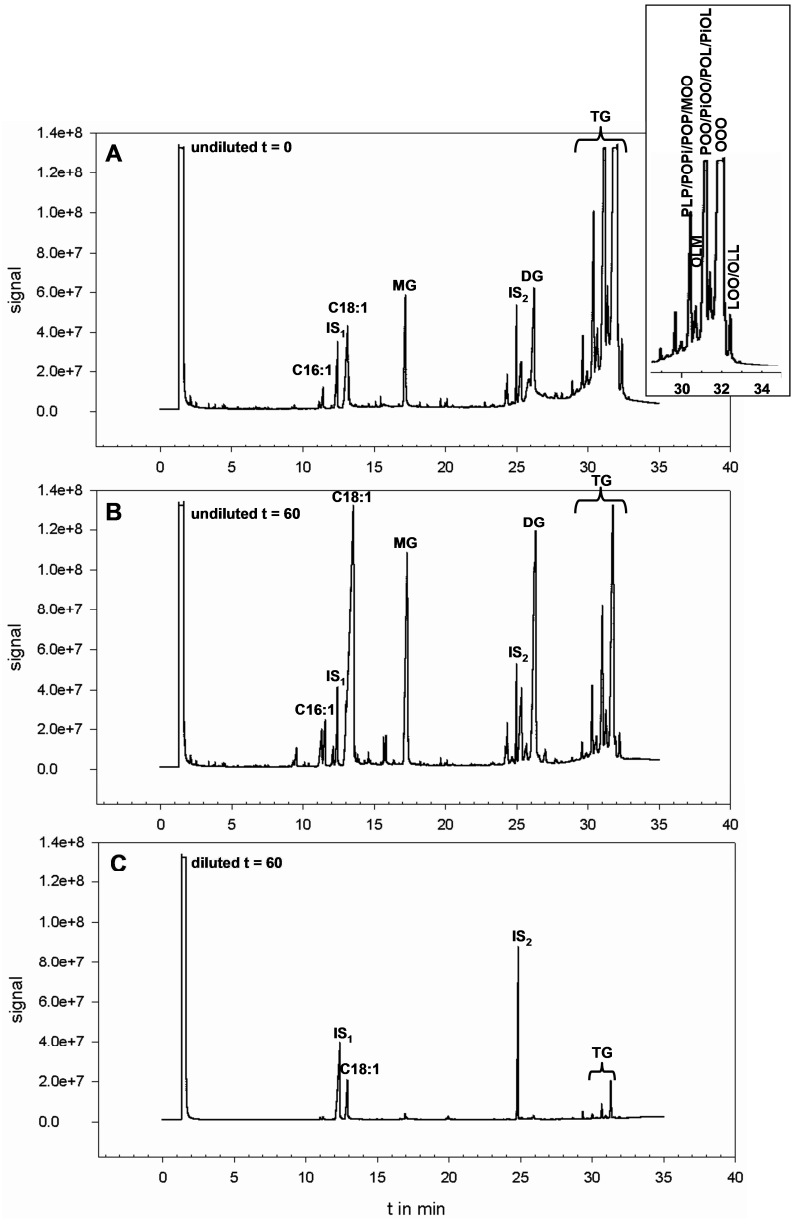

Fig. 2.1 Chromatograms of digestion mixtures of WPI stabilized emulsions, undiluted 0 min (A), 60 min undiluted (B) and 60 min diluted 1:20 (C) after digestion. C16:1, palmitoleic acid; C18:1, oleic acid; MG, monoglyceride; DG, diglyceride; TG, triglyceride; IS_1, internal standard C17:0; IS_2, internal standard TG36. M, myristic; P, palmitic, Pi, palmitoleic; O, oleic; L, linoleic acid.

2.3.3 Release of fatty acids: pH-stat versus GC

The production of FFA from emulsions was measured using two methods: pH-stat and GC at a constant pH of 7.5. In general, duplicate measurements of emulsions had a maximum variation of 11% for both methods. FFA measured with pH-stat showed a variation of 24% within the duplicates only in the case of coarse WPI emulsions.

The release of FFA was first investigated for fine WPI stabilized emulsion. In general, both methods showed a continuous increase in FFA over the digestion time of 60 min (Fig. 2.2A). However, the amount of FFA measured by GC gives 2–3 times higher values compared with the pH-stat data (Fig. 2.2A). To confirm the consistency of those results, we further explored this difference by investigating the influence of droplet size and type of emulsifier. We found that independently of the droplet size, both methods give essentially the same results for FFA released from gum arabic stabilized emulsions with an average correlation factor between the FFA amount obtained with the two methods of 0.9±0.1 (Figs. 2.2A and 2.2B). In contrary, the average correlation factor for WPI stabilized emulsions, considering the studied emulsions altogether, is 0.3±0.1. This indicates that in the case of WPI stabilized emulsions, the pH-stat actually underestimates the FFA release for all droplet sizes under the used conditions.

2.3.4 Lipase activity and formation of lipolytic products

Due to the underestimation of FFA release by pH-stat in case of WPI stabilized emulsions the lipolytic activity of pancreatin on both emulsions was calculated using only the GC data. In general, lipolytic activity increases with higher available surface area as illustrated in Fig. 2.3. Furthermore, comparing both emulsifiers, lipase activity was equal (i.e. 4.5 U/mg for fine WPI and gum arabic emulsions), although the initial total surface areas per volume oil in the assays were 75.0 m^2 and 3.5 m^2, respectively. This demonstrates that the emulsifier has a larger effect on the hydrolysis than the surface area.

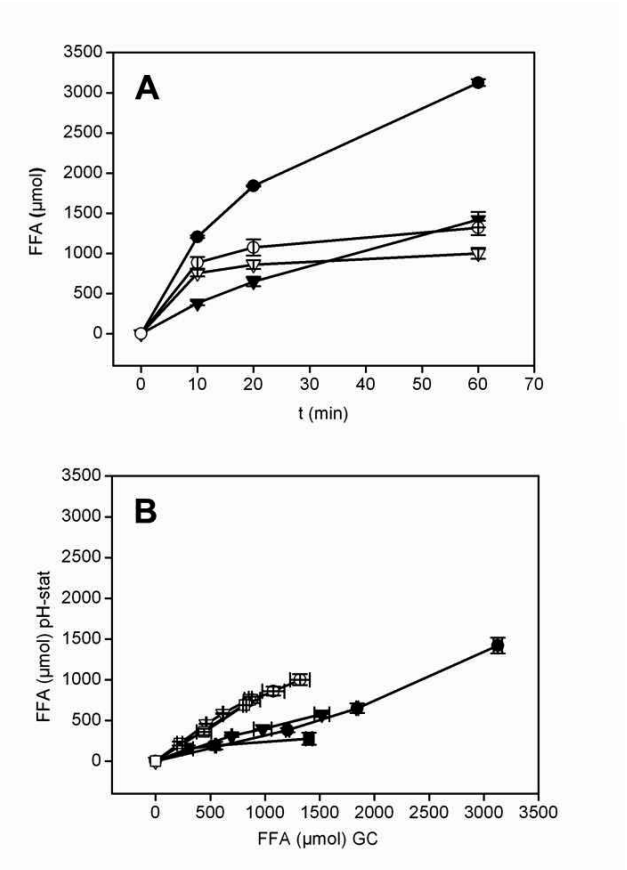

Fig. 2.2 Comparison of the release of FFA (µmol) during 60 min digestion (A) of fine WPI stabilized emulsions with closed symbols determined by GC (●) or by pH-stat (▼) and of fine gum arabic stabilized emulsions with open symbols determined by GC (○) or by pH-stat (∇). Correlation between the release of FFA at t_0, t_{10}, t_{20} and t_{60} determined by pH-stat and GC (B). WPI stabilized emulsions are indicated with closed symbols (fine, ●; medium, ▼; coarse, ■), and gum arabic stabilized emulsions with open symbols (fine, ○; medium, ∇; coarse, □). Error bars indicate the standard deviations.

In addition to FFA, the formation of MG and DG was calculated as a percentage of glycerol present in TG_0 for fine emulsions, made with both WPI and gum arabic (Fig. 2.4A and B) and the end point at 100% was assumed. Here, DG seem to reach a maximum in the gum arabic stabilized emulsion at 47% hydrolysis, whereas for WPI stabilized emulsions the maximum was calculated to be at 32% hydrolysis. Table 2.1 reports the quantities of lipolytic products for the different droplet sizes and emulsifiers used. The

amount of FFA increases with time for all emulsions. At each time point, the FFA amount released decreases with increasing droplet size. MG patterns follow the same trend as DG when comparing the same time points for the different sizes. As expected, the amount of TG decreases with time for all droplet sizes, except for medium WPI emulsions and coarse gum arabic emulsions, where the TG amount remains unchanged with time. Overall, independent of the droplet size, gum arabic stabilized emulsions have a higher hydrolysis rate compared with WPI stabilized emulsions.

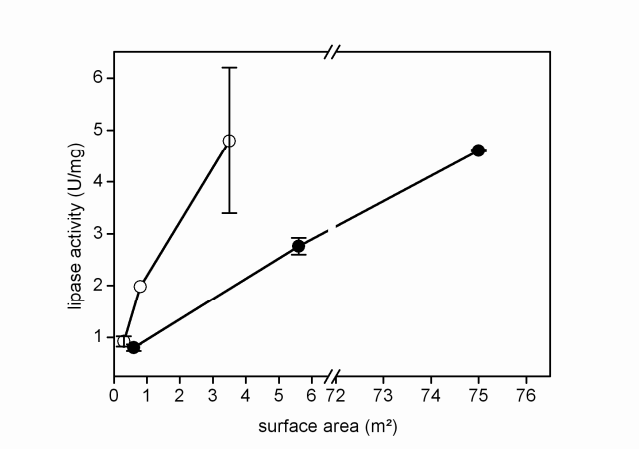

Fig. 2.3 Lipolytic activity of pancreatin on WPI (●) or gum arabic (▼) stabilized emulsions obtained by GC as a function of the surface area. Error bars indicate the standard deviations.

Furthermore, we analyzed the ratios between the different digestion products (Table 2.1). The molar ratios of FFA/MG, FFA/DG and MG/DG from digestion of the WPI stabilized emulsions are similar for all droplet sizes (3.8 ± 0.9, 3.7 ± 0.5 and 1.0 ± 0.3, respectively). We found similar behaviour in gum arabic stabilized emulsions. Our data, therefore, indicate that the size of the droplets influences the total production of the reaction products but not the proportions. In contrast, the ratios of FFA/DG and MG/DG were affected by the emulsifiers. For gum arabic stabilized emulsions their ratios are twofold higher than for WPI stabilized emulsions, independent of the droplet sizes. The ratios stay constant during digestion for 60 min under all conditions tested indicating a constant lipolytic reaction.

Table 2.1 Overview of lipolytic products formed during 60 min digestion from WPI and gum arabic stabilized emulsions with different droplet sizes (fine, medium and coarse) analyzed by GC. (*) Mass balance calculated as sum of the mass balances according to equations in material and method session.

		t (min)	FA (μM)	MG (μM)	DG (μM)	TG (μM)	Mass balance (%)	Molar ratio: FFA/MG	Molar ratio: FFA/DG	Molar ratio: MG/DG
WPI	**Fine**	0	0	0	0	902.7	100	-	-	-
		10	321.7	57.4	78.4	656.3	92.5	5.6	4.1	0.7
		20	491.3	108.6	127.1	577.2	95.5	4.5	3.9	0.9
		60	833.9	237.1	188.7	396.6	97.4	3.5	4.4	1.3
	Medium	0	0	0	0	605.3	100	-	-	-
		10	185.1	59.3	52.6	543.4	109.0	3.1	3.5	1.1
		20	260.7	91.3	69.9	562.6	120.0	2.9	3.7	1.3
		60	405.4	131.1	96.8	559.0	132.5	3.1	4.2	1.4
	Coarse	0	0	0	0	787.9	100	-	-	-
		10	78.5	20.7	25.7	610.9	83.9	3.8	3.0	0.8
		20	146.1	35.0	50.4	508.8	76.5	4.2	2.9	0.7
		60	373.7	105.4	102.5	372.6	76.2	3.5	3.6	1.0
Gum arabic	**Fine**	0	0	0	0	862.7	100	-	-	-
		10	943.1	271.0	110.1	481.2	111.2	3.5	8.6	2.5
		20	1147.3	391.7	159.6	341.2	111.4	2.9	7.2	2.5
		60	1407.1	431.7	167.7	274.1	115.9	3.3	8.4	2.6
	Medium	0	0	0	0	1012.0	100	-	-	-
		10	493.2	169.9	66.0	686.8	94.1	2.9	7.5	2.6
		20	656.4	214.3	83.1	667.7	100.1	3.1	7.9	2.6
		60	922.9	219.7	80.0	600.6	102.2	4.2	11.5	2.7
	Coarse	0	0	0	0	671.9	100	-	-	-
		10	232.8	53.5	11.9	530.9	94.4	4.4	19.6	4.5
		20	471.5	105.6	69.2	657.4	133.3	4.5	6.8	1.5
		60	867.3	174.5	90.9	541.1	141.2	5.0	9.5	1.9

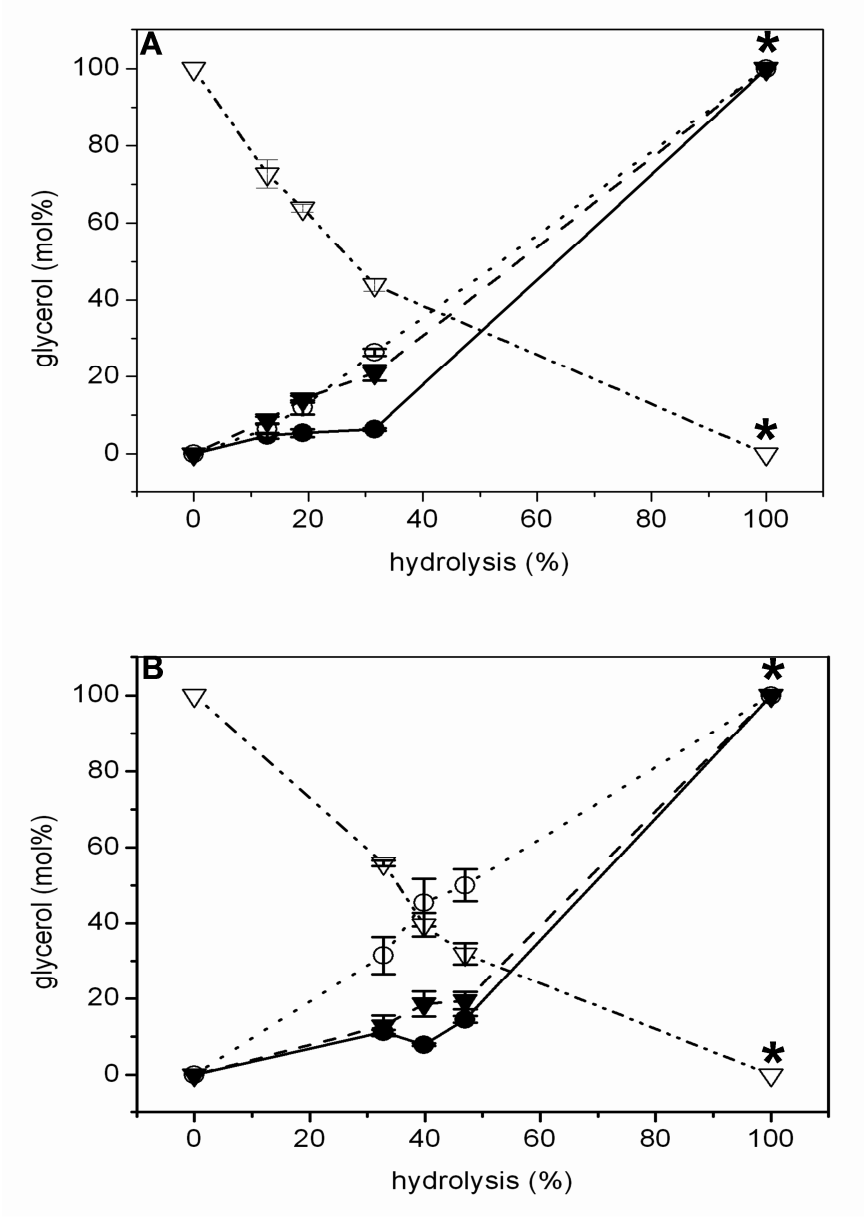

Fig. 2.4 Formation of glycerol (●), MG (○), DG (▾) and decrease of TG (∇) depending on the hydrolysis level for fine WPI (A) and gum arabic (B) stabilized emulsions. The data are based on glycerol in mol % and 100% corresponds to the initial moles of TG used within the assays. 100% hydrolysis is assumed as the end point and indicated with *. Error bars indicate the standard deviations.

2.4 Discussion

2.4.1 Using GC to characterize lipid degradation of emulsions

In literature several *in vitro* models have been used to study the lipolysis of fats and oils. The pH-stat is a popular analysis technique in this kind of research because it allows continuous monitoring of FFA formed. However, the titration method has some limitations, as highlighted by numerous reviews (see for example Beisson et al., 2000), due to the need of certain reaction conditions such as pH, ionic strength, BS and calcium concentration. The pH of the assay must be, for instance, higher or equal of the apparent p*K*a value of the FFA and the p*K*a is, in turn, highly influenced by the ionic strength (Mattson & Volpenhein, 1966), BS and calcium concentration (Benzonana & Desnuelle, 1968) present within the reaction medium. From a model point of view, that is still far from the actual situation *in vivo* as the concentration of BS and calcium are known to be subjected to physiological oscillation related to the fed state in the human body. Also, the technique does not provide any information about the different products (i.e. MG and DG) and their rate of formation or further digestion. This information is important from the physiological and physical points of view as lipolysis products can affect stomach emptying (Cummings & Overdiun, 2007). Moreover, they play a role in the emulsification process and, under stomach conditions, limit fat digestion (Golding & Wooster, 2010).

Considering the above-mentioned reasons, we developed a GC method that allowed us to analyze lipolysis irrespective of digestion conditions. The method is presented in this article and, for FFA release, compared, under the same assay conditions, with pH-stat, as this is a widely used method in lipolysis studies. To evaluate the data obtained by GC, we determined mass balances (%) based on total FFA present (Table 2.1). Values less than 100% could indicate that, under the given conditions, a further degradation of MG to glycerol takes place. Although pancreatic lipases are known to be strictly specific to the hydrolysis of primary ester bonds, sn-2 MG can be hydrolyzed after isomerization to sn-1 MG (Carriere et al., 1991, 1997; Constantin et al., 1960; Desnuelle & Savary, 1963; Rodriguez et al., 2008). Moreover, glycerol formation is possible under certain *in vitro* conditions without the need for isomerization (Borgström, 1964). When we calculated the amount of glycerol present, a slight increase in glycerol was seen for fine gum arabic and WPI emulsions (Fig. 2.4). This is in contrast with a previous study (Rodriguez et al., 2008) in which no glycerol formation of emulsions digested by porcine pancreatic lipase was

observed. However, some cases show mass balances greater than 100%, for which we do not have any plausible explanation at the present moment.

Other factors that can influence the lipid digestion are the initial amounts of FFA, MG, and DG present in the oil phase after preparation of the emulsions. The initial amount of FFA per lipid surface area found in our study was, for example, 8.8 μmol/m^2 for fine WPI emulsions, which is about 13 times lower than the threshold concentration for inhibiting lipase (Pafumi et al., 2002). In addition, as the amount of FFA increased until t_{60} and did not reach a plateau, we can assume that there was no product inhibition. Also, bile salts present in our digestion medium are known to incorporate FFA and MG and thus prevent product inhibition as occurs with human gastric lipase (Pafumi et al., 2002).

2.4.2 Difference in FFA release for methods used

As shown in Fig. 2.2A and B, the amount of FFA liberated during digestion and measured with GC is higher than that observed with pH-stat. Being aware of the limitations of the pH-stat method as discussed before, it was surprising to find, under the same assay conditions, differences as those observed with the emulsifiers used. This is, to our opinion, of interest to the reader as it makes him more aware that relying only on one technique might not be always the proper choice. We have several suggestions for the differences found when comparing both methods. In our study, a discrepancy of 60% between pH-stat and GC was only observed in the case of WPI stabilized emulsions. This discrepancy has also previously been found by others. For example, Patton and Carey (1981) reported 50–70% less FFA released at pH <9 when using pH-stat compared with radioactive labelled TG. It is possible to avoid this issue by titrating to pH 9, a pH at which all fatty acids are released into solution (Mun et al., 2006, 2007; Sandra et al., 2008; Sarkar et al., 2010a). However, when investigating the FFA release at pH 6.5–7.5, as also done in other studies (Hu et al., 2010; Li & McClements, 2010; Wickham et al., 1998; Zangenberg et al., 2001a), the various p*K*a values for fatty acids should be taken into account as they depend on the surrounding media. For example, in the case of oleic acid (p*K*a of 9 in pure water), the p*K*a shifted to 7.8 in rat pancreatic juice with 1 M NaCl (Mattson & Volpenhein, 1966). Similarly, a shift to p*K*a 6.4 was caused by medium consisting of 0.1 M NaCl and 0.5 mM Ca2+ (Benzonana & Desnuelle, 1968). However, when BS, Ca2+ and NaCl were present in physiological concentrations, 100% of FFA could be titrated at pH 6.5 (Zangenberg et al., 2001a). The used BS concentration in our study was 8.2 mM, which is above the critical micelle concentration and close to the fed state

(Wickham et al., 1998; Porter & Charman, 2001). This is important, as recently demonstrated by Golding et al. (2011), to minimize the difference in FFA release with the pH-stat method. Additionally, Ca^{2+} enhances the pancreatic lipase activity as it precipitates FFA from the droplets surface by formation of calcium-soaps with long chain fatty acids and can be added in excess in order to control the rate of lipolysis (Zangenberg et al., 2001a; Zangenberg et al., 2001b). In our study the calcium concentration was 2.7 mM. In relation to the *in vivo* situation it is known that the calcium-concentration in the fasted state of the jejunum is 0.5 ± 0.3 mM (Lindahl et al., 1997). However, in the fed state the calcium content in the intestine is highly determined by the calcium concentration in the ingested meal (Fordtran & Locklear, 1966; Mansbach et al., 1975; Sheikh et al., 1988) whereas the basal secretion of calcium is considered to be negligible (Sheikh et al., 1988). The free calcium concentration depends further on other substances present in the GIT such as bile salts (Hofmann & Mysels, 1992), mucus or calcium binding proteins as well as on substances present in the meal (e.g. oxalate or phytin). Therefore, the rather complex digestion fluid in terms of BS and ions might explain the amount of FFA released from gum arabic stabilized emulsions in our pH-stat measurements and the comparable results obtained with GC. However, it does not explain the differences we found for WPI as the reaction medium for both emulsions was the same.

The difference in values obtained by GC and pH-stat for WPI stabilized emulsions could also be due to binding of FFA to β-lg. β-Lg is the main component in WPI, a globular protein of 18 kDa, which might have two potential binding sites for small hydrophobic molecules such as fatty acids (Frapin et al., 1993). It is possible, that this binding is disrupted during the extraction step prior GC analysis. However, the β-lg amount is 10 µmol at the concentration of WPI used, which is too small to be able to bind 1.7 mmol of FFA, which is the difference found between GC and pH-stat.

Another explanation could be that positively charged amino acid residues in WPI enable more interactions of FFA at the interface than those of gum arabic. Consequently, the FFA in WPI BE/emulsion mixture are not available for titration but can be extracted using the GC method. Again, calcium might influence that interaction by precipitating FFA, which on the other hand does not influence gum arabic stabilized emulsions as the negatively charged carboxyl groups in gum arabic do not interact with the FFA. In contrast to our findings, studies on interactions of FFA and gum arabic showed reduced lipolysis as FFA possibly bind to the protein moiety of gum arabic and prevent FFA release in the aqueous phase (Fang et al., 2010; Pasquier et al., 1996).

Another factor to try to explain our findings could be the hydrolysis of the protein by pancreatin, which could contribute to the titration. However, this can be neglected as a WPI solution, similar in concentration to that used for the emulsions, subjected to the same assay conditions showed an increase of only 5 μmol per assay. Furthermore, Sarkar et al. (2010b) suggested a residual lipase activity of bile salts. However, when both emulsions were incubated without pancreatin, we did not find any production of FFA. Finally, we excluded a possible buffering capacity of protein in the pH-stat as the amount of HCl needed to decrease the pH from 7.5 to 6.5 was the same for both emulsions (data not shown).

2.4.3 Effect of droplet size on FFA, MG and DG formation

Our data clearly showed the effect of droplet size on the rate of FFA release. The higher lipolytic activity of gastric as well as pancreatic lipase on smaller droplets compared with larger droplets has been reported by others (Armand et al., 1992; Armand et al., 1999; Borel et al., 1994; Pafumi et al., 2002). In agreement with our results, Armand et al. (1999) observed during digestion of emulsions for both gastric and duodenal lipase, not only higher FFA release from fine emulsions compared with coarse emulsions, but also higher MG and DG concentrations. In contrast, we found the same ratios of MG/DG for all droplet sizes for the respective emulsifier, whereas ratios obtained by Armand et al. (1999) were almost twofold higher for fine compared with coarse emulsions for both gastric and duodenal digested emulsions. This means that the reaction kinetics in terms of MG and DG formation in our study under the specific biochemical conditions is unexpectedly independent from the droplet size.

2.4.4 Effect of emulsifier on FFA, MG and DG formation

The influence of interfacial composition on the formation of FFA, which was previously found in another study (Mun et al., 2007) as well as the lower influence of droplet size on gastric lipase activity (Borel et al., 1994) is in agreement with our findings. In our study, a higher lipase activity was observed during the digestion of gum arabic stabilized emulsions compared with WPI stabilized emulsions. It is likely that WPI is more strongly shielding the interface than gum arabic, hindering the pancreatic lipase reaching the interface. However, the effect of BS, which weakens the protein layer and displaces it from the oil–water interface (Maldonado-Valderrama et al., 2008), should be more

pronounced with WPI interfaces than gum arabic allowing higher FFA release in this case.

It is known that C18:1, for example, has a higher affinity to the surface compared with diolein or triolein (Pafumi et al., 2002), which might cause an increase in FFA concentration at the oil–water interface. This in turn could cause product inhibition at the surface of the droplets, which might affect the digestion process once again. However, under intestinal conditions they are normally removed by BS and phospholipids in the aqueous phase due to micelle formation. Moreover, β-lg present in WPI could form a covalent cross-linked interfacial layer and, therefore, make complete displacement by BS and phospholipids present in the digestion fluid more difficult (Hur et al., 2009). Similar to β-lg, it is possible that the WPI layer prevents the release of FFA into the aqueous solution more than gum arabic, causing the discrepancies observed between pH-stat and GC.

Similar to the higher FFA release, the formation of MG occurred much faster from gum arabic stabilized emulsions compared with the WPI emulsion. To our knowledge, differences in DG and/or MG formation for different emulsifiers have not been reported in the literature. An increase in MG up to 66% hydrolysis was previously reported for a longer digestion time (Rodriguez et al., 2008). In the case of DG, the maximum value present in fine WPI stabilized emulsions at 32% hydrolysis is in line with the maximum DG value reported in the literature (Rodriguez et al., 2008). In addition, the slight increase in the ratios of MG/DG obtained from WPI stabilized emulsion indicates that an incubation time longer than 60 min might lead to a higher degradation of DG into MG. So far it has been shown for various lipase species that the rate of DG production is much higher than its hydrolysis rate (Carriere et al., 1991; Rodriguez et al., 2008). However, it seems that the type of emulsifier at the interface also influences DG and MG formation; for gum arabic stabilized emulsion we found hydrolysis of DG was faster than its production rate.

At the present stadium of our research, we are not able to clarify the reasons of the different breakdown of TG into DG, MG and FFA as that one observed in our systems. One might speculate that the mode of action of the lipase might be influenced by the location of components (i.e. FFA, MG, DG and TG) caused by different partitioning coefficient, as has been shown by Pafumi et al. (2002). In our opinion this is rather hypothetical as this might be influenced by the different properties of oil/water interfaces

in terms of different interfacial viscosity and/or surface tension. In addition, the different interaction between interface and enzyme, could also lead to a better accessibility/presence of DG for the lipase in gum arabic stabilized emulsions resulting in a faster degradation of DG. On the contrary, TG present in the core of the oil phase would lead to a slower production of DG. Although we have no clear indication for it and furthermore it was out of the scope of this study, we are still convinced that the interaction between lipase and the emulsifier at the oil/water interfaces of emulsion droplets might play a greater role in lipase digestion.

2.5 Conclusions

This study has shown that GC and pH-stat analysis do not always give the same results in terms of FFA production. In particular, the interface influenced the amount of FFA obtained by GC or pH-stat. This should be considered when comparing FFA release from emulsions stabilized by different emulsifiers. The fact that, although several hypotheses have been tested, a satisfactory solution was not found yet shows that further research in this area needs to be undertaken. Our results also show a clear effect of the surface area; the larger the area the higher the rate of digestion. With the GC method, we showed that gum arabic stabilized emulsions are more easily digested than WPI stabilized emulsions resulting in a higher release of FFA. In addition, the emulsifiers influenced the hydrolysis rate of DG into MG, which was faster for gum arabic compared with WPI stabilized emulsions. The specific conclusions of lipase digestion presented in this paper are related to the *in vitro* experimental conditions used during the study. Nevertheless, the application of the GC method, presented here, to *in vivo* samples will be of great relevance since, beside the FFA, also MGs and DGs are simultaneously measurable. Therefore, the information obtained with the GC method will surely add to the understanding of the digestion process of emulsion systems not only *in vitro* but also *in vivo*.

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Chapter 3

Lipid digestion of protein stabilized emulsions investigated in a dynamic *in vitro* gastro-intestinal model system

Helbig A., Silletti, E., van Aken, G.A., Oosterveld, A., Minekus, M., Hamer, R.J., Gruppen H. (2012)
Lipid digestion of protein stabilized emulsions investigated in a dynamic *in vitro* gastro-intestinal model system
Food Digestion, published online

Abstract

This study investigated the effect of gastric passage of protein stabilized emulsions, i.e. whey protein isolate (WPI) and lysozyme, under dynamic *in vitro* conditions on both the gastric and intestinal lipolysis. Emulsions were prepared at neutral pH to enable an opposite surface charge. Experiments were performed in a multi-compartmental digestion model (TNO Gastro-Intestinal Model) including a gastric compartment simulating *in vivo* conditions, i.e. gradual acidification, mixing, lipolysis and proteolysis. Under gastric conditions lysozyme-stabilized emulsions remained macroscopically homogenous, whereas WPI-stabilized emulsions separated into a cream and serum layer. Microscopy revealed flocculation of both emulsions but larger particles were found for the initial negatively charged WPI-stabilized emulsions compared to the positively charged lysozyme-stabilized emulsions. This suggested that creaming was due to larger flocs formation caused by a change from net negative to net neutral charge as an effect of the gradual decreasing pH. Analysis of lipid composition, i.e. free fatty acids (FFA), monoglycerides, diglycerides (DG) and triglycerides revealed mainly FFA and DG in the gastric compartment. As a result of creaming, the entry of lipids into the small intestinal part was delayed for WPI-stabilized emulsions. However, the total amount of FFA released at the end of the experiment was similar for both emulsions. Our results show, that the charge differences affected the creaming behavior but not the lipase activity on the two studied emulsions.

3.1 Introduction

Triglycerides (TG) are important and necessary dietary components to enhance the intestinal absorption of lipophilic nutrients, such as essential fatty acids, fat-soluble vitamins, and pharmaceuticals. In addition, TG have a clear function as texture and flavor components in foods. TG have for long been known to play a role in the regulation of metabolic intake by activating satiety responses such as the release of cholecystokinin (CCK), the delay in gastric emptying (Lal et al., 2004; Cummings & Overduin, 2007) or the increase in small intestinal transit time (Maljaars et al., 2008; van Aken, 2010). These responses are induced in the small intestine by the detection of free fatty acids (FFA), which are generated by gastric lipase and small intestinal lipolytic enzymes such as pancreatic lipase, phospholipases and carboxyl ester lipase. A more in depth insight in TG digestion can lead to a better control of food intake, which is highly desired in view of the current obesity epidemic.

In the present study, we investigated the gastric and small intestinal processing of two differently charged emulsions under dynamic *in vitro* conditions. To this end, we prepared two protein stabilized emulsions, i.e. whey protein isolate (WPI) and lysozyme-stabilized emulsions, having opposite net droplets surface charges at neutral pH. It is known that oppositely charged emulsions as, for instance β-lactoglobulin and lysozyme, exhibit a different physico-chemical behaviour when mixed with oral fluid, i.e. saliva (Silletti et al., 2007). Furthermore, the differences in surface charge changes the sensory behaviour of WPI and lysozyme-stabilized emulsions (Vingerhoeds et al., 2009). Therefore, it is thought that these two differently charged emulsions might also induce different gastric-intestinal behaviour and consequently satiety responses.

Gastric conditions can lead to major structural changes of the protein stabilized emulsions. The fundus and the corpus part of the gastric compartment contribute to a slow mixing of food with mucus, electrolytes and enzymes (Marciani et al., 2001) and act more like a storage, whereas mixing takes place mainly in the antral part. This implies that creaming and phase separation, but also precipitation (van Aken et al., 2011) as a result of physical instabilities of the emulsions can take place and is likely to influence the lipid digestion. Accordingly, it has been shown *in vivo* that fat from emulsions which break under acid conditions, tends to float to the surface of the gastric lumen (Marciani et

al., 2007; Marciani et al., 2008). As a consequence, the release of fat into the small intestine is delayed (Marciani et al., 2007; Marciani et al., 2008; Foltz et al., 2009).

Moreover, gastric lipase activity contributes to 10-40 % of overall lipid digestion (Carriere et al., 1993; Armand et al., 1999) and up to 7.5 % of lipid hydrolysis in the duodenum (Carriere et al., 1993). Furthermore, gastric lipolysis and the formation of FFA promote optimal pancreatic lipase action in the small intestine (for a review see Armand, 2007). For example, the released FFA reduce the lag-time for activation of the pancreatic-colipase complex (Gargouri et al., 1986b; Bernback et al., 1989) by the formation of a ternary complex (Van Tilbeurgh et al., 1993; Pignol et al., 2000) involving FFA such as oleic acid (Pignol et al., 2000). Additionally, the released FFA (e.g. C12 and other longer chain FFA) are detected in the small intestine and lead to a decrease of gastric motility and emptying, and can signal satiety. These responses, however, might be delayed if an emulsion creams in the stomach.

Several studies have been conducted using *in vitro* and *in vivo* approaches, to tackle from one side the fundamental understanding of how emulsions are digested and which parameters are playing a major role in a "simplified" environment (*in vitro*), as well as under more complicated conditions such as the human physiological situation. Our choice was to combine both approaches insuring from one the reproducibility of the *in vitro* studies and the dynamic component of the human digestions. To this extent the study was performed by means of a dynamic model of the gastro-intestinal tract (GIT), which mimics the major events occurring in the lumen of the GIT (Minekus, 1995). The system used was based on a simplified version (called "Tiny TIM") of the TNO gastro-Intestinal Model (TIM) consisting of a single instead of three small intestinal compartments (Schaafsma, 2005). The system was provided with a purposely designed gastric compartment that simulated the motility of the different regions of the stomach. Further, we used a gradual acidification in the gastric compartment, and, in order to reveal the impact of flocculation at the isoelectric point (p*I*), we compared emulsions stabilized by WPI (p*I* ~4.9) and lysozyme (p*I* ~10.5).

3.2 Materials and methods

3.2.1 Materials

Whey protein isolate (WPI) was purchased from Davisco Foods International (BiPro®, lot JE216-6-440, Le Sueur, MN, USA) and lysozyme from chicken egg-white was from Belovo SA (lot 1395, Bastogne, Belgium). Extra virgin olive oil was purchased from Oleificio cooperativo Goccia di Sole Molfetta S.r.l. (Molfetta, Italy). Pepsin from porcine gastric mucosa (P7012, 600 U/mL) and trypsin from bovine pancreas (T4665, ≥7,500 U/mg) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Lipase was extracted from lamb pharyngeal tissue (Lamb Pre Gastric Lipase, Renco, Eltham, New Zealand). Lamb pharyngeal lipase was selected as an alternative to human gastric lipase, which is not commercially available. It has a specificity for the sn-3 position of triglycerides (Villeneuve et al., 1996) and an activity as a function of pH that is similar to that of human gastric lipase (Moreau et al., 1988). Pancreatin from porcine pancreas (Pancrex V powder) was bought from Paines & Byrne Limited (Greenford, UK). Bile was obtained from fresh porcine bile bladders obtained from an abattoir. The bile was pooled on ice immediately after collection and stored below -18^{o}C. Fatty acid standards, i.e. C14:0, C15:0, C16:0, C18:1, C18:2 as well as monoolein, diolein, and triglyceride standards triolein and trilaurin (TG36) were obtained from Sigma–Aldrich (>99 % pure) as well as MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide, lot BCBD5976V). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma–Aldrich. Solutions were prepared in demineralized water.

3.2.2 Preparation of emulsions

WPI and lysozyme solutions were prepared by dissolving the proteins in a 10 mM NaCl solution and gently stirring for 1 hour at room temperature. WPI-stabilized stock emulsions where prepared, which contained 40% (w/w) oil and 1% (w/w) emulsifier. Lysozyme-stabilized stock emulsions contained 20% (w/w) oil and 1% (w/w) emulsifier. The stock emulsions were made from pre-emulsions using an Ultra-Turrax, (Polytron®, Kinematica AG, Luzern, Switzerland, at 1000 rpm for 1 min), which were further passed through a two-stage laboratory homogenizer (Ariete, Model NS1001L 2K-Panda, GEA Niro Soavi, Parma, Italy) at a pressure of 250 bar for the first stage and 20 bar for the

second stage, respectively. The resulting WPI and lysozyme stock emulsions were diluted to 8% (w/w) oil with a solution containing 1% (w/w) WPI or lysozyme, respectively.

3.2.3 *In vitro* digestion

The Tiny Tim model used in this study is schematically presented in Fig. 3.1. The gastric compartment (Fig. 3.1A) consisted of a main body, simulating the fundus and corpus, and a peristaltic section in order to simulate antral mixing. Like all other compartments, it was composed of a glass jacket with flexible inner walls. Water was pumped in between the jacket and the flexible wall to control the temperature (37 °) and to mix and move the contents by changing the pressure on the flexible walls. The pumping action in the region of the lower antral part and the pyloric sphyncter was simulated by peristaltic actions of three valves.

To imitate the pre-prandial state, 30 g of simulated gastric fluid (SGF) was initially present in the gastric compartment. SGF consisted of Pepsin (600 U/mL) and lamb pharyngeal lipase (160 U/mL), dissolved in an electrolyte solution consisting of 106 mM NaCl, 30 mM KCl, 2 mM $CaCl_2$ and 18 mM $NaHCO_3$.

Each experiment was started by introducing 230 g of freshly prepared emulsions into the gastric compartment. In order to mimic the gastric *in vivo* situation, the pH was controlled to follow a pre-set curve (Fig. 3.1C) obtained from physiological measurements conducted for a high fat meal study (Dressman et al., 1990). A changing mixture of 1 M HCl solution and water were added at a rate of 0.5 mL/min. The SGF was secreted at a flow rate of 0.5 mL/min for 180 min.

The digest was gradually released from the gastric compartment to the small intestinal part (Fig. 3.1C). The transit profile of the chyme followed a pre-set curve (Elashoff et al., 1982), with a halftime of gastric emptying of 80 min. The small intestinal compartment (Fig. 3.1B) consisted of two mixing segments. In order to simulate the absorption of nutrients and water by the small intestine, the lumen of this compartment was filtrated using a filtration unit with a cut-off of 0.05 μm (MiniKros®Plus, M20S-300-01P, Spectrum®Laboratories, Rancho Dominguez, CA, USA). The simulated duodenal secretion fluid (SDSF) consisted of 50 mM bile salts and 35 g/L pancreatin in a small intestinal electrolyte solution (SIES) containing 86 mM NaCl, 8 mM KCl and 2.3 mM $CaCl_2$. The solutions were separately introduced in the compartment each at a flow rate of 0.5

mL/min for 180 min to follow a pre-set curve (Marteau et al., 1990; Minekus, 1995). Prior to the experiment, the small intestinal compartment was filled with 60 g of secretion fluid consisting of 15 g SIES, 15 g of a 7% (w/w) pancreatin solution, 30 g of porcine bile solution and 2 mg trypsin to simulate the initial intestinal content. The pH of the digest was kept constant at a set pH 6.5 by automatic titration with 1 M $NaHCO_3$ solution (Table 3.1).

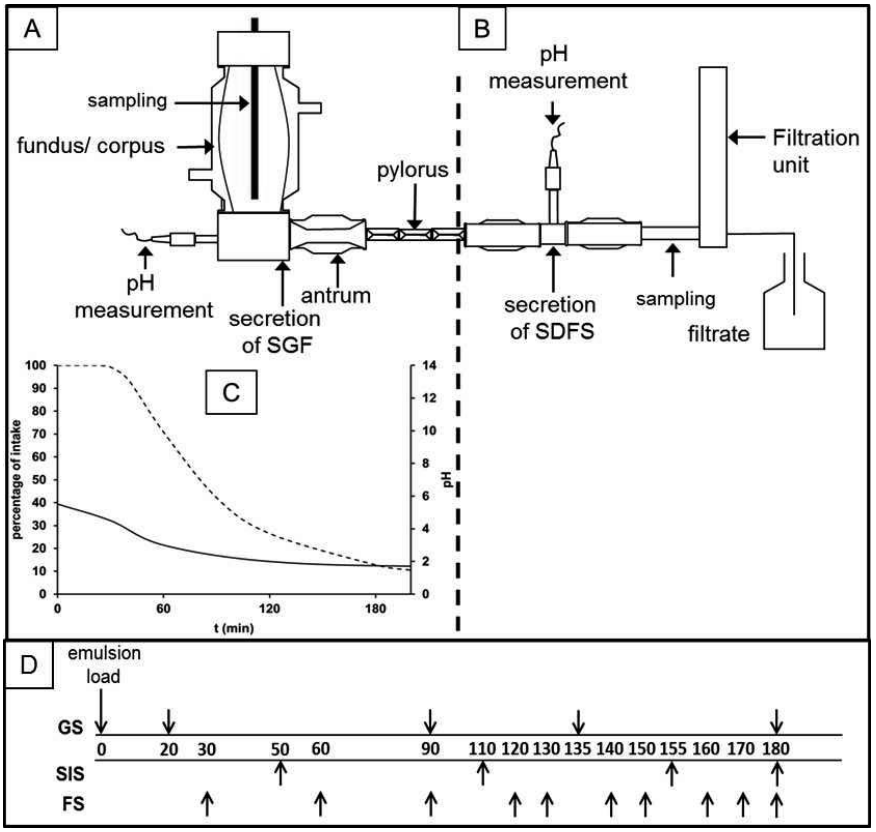

Fig. 3.1 Schematic diagram of the *in vitro* gastro-intestinal model (TIM) including the gastric (A) and small intestinal (B) compartment, and the filtration unit. SGF: simulated gastric fluid, SDFS: simulated duodenal secretion fluid. C: Gastric emptying curve (dotted line) and gastric pH profile (continuous line) used to control the gastric emptying and pH. D: Schematic schedule of sampling during 180 min of experiment. Samples were subjected to particle size measurements, microscopic imaging and lipid composition analysis as described in the methods. GS: gastric samples, SIS: small intestinal samples, FS: filtrate samples.

The pH profile and the addition of all reagents were recorded. Each digestion process lasted 180 min and data values are the average of two independent experiments.

Table 3.1 Fluid addition.

	Gastric compartment		**Small intestinal compartment**	
salts	NaCl	106 mM	NaCl	86 mM
	KCl	30 mM	KCl	8 mM
	$CaCl_2$	2 mM	$CaCl_2$	2.25 mM
	$NaHCO_3$	18 mM	bile salt	~50 mM
enzymes	pepsin	600 U/mL	pancreatin	35 g/L
	lipase	160 U/mL	trypsin	≥7,500 U/mg
pH control	1 M HCl water		1 M $NaHCO_3$	
flow rates	SGF: 0.5 mL/min		SDSF: 0.5 mL/ min	
Pre-prandial state	30 g SGF		15 g SIES	

SGF, simulated gastric fluid; SDSF, simulated duodenal secretion fluid; SIES, small intestinal electrolyte solution

3.2.4 Physicochemical characterisation of *in vitro* gastric and duodenal digestion of emulsions

The behaviour of the emulsions in the gastric compartment was visually monitored and recorded on digital photo-camera. Samples were taken directly from the lumen of the different compartments according to the time line (Fig. 3.1D). In particular, samples from the gastric compartment were taken at the bottom of the compartment at specific time points and, additionally, after 90 min from the top part of the compartment. The filtrates were collected by a fraction collector. From each experiment, also the residual material from the gastric and small intestinal compartment and the samples after rinsing the system with alcohol were collected to calculate the total recovery of lipids. Each time point presents the time from the start of the experiment, i.e. when the emulsions were introduced in the stomach compartment.

Droplet size distributions, surface-weighted-mean diameter (d_{32}) and volume-weighted-mean diameter (d_{43}) of the emulsion droplets were determined directly after preparation and at various time points by dynamic light scattering (Mastersizer Hydro 2000S, Malvern Instruments Ltd, Malvern, UK) at room temperature. Microscopic images of the initial emulsions and of the emulsions in different parts of the model at different

time points during the digestion period were taken using a BYO-503T laboratory microscope equipped with a DCM 500 5.0 Mp ocular camera (Byomic).

Samples were analyzed for free fatty acids (FFA), mono-, di- and triglycerides (MG, DG, TG) composition by gas chromatography (GC) as previously described (Helbig et al., 2012) with minor modifications. Briefly, 2 mL samples were taken from each compartment and filtrates at intermediate time points, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. After extraction, the final supernatant was collected and its volume was brought to 4 mL with Diethylether/Heptane (1:1, w/w). An internal standard mix (50 µL), consisting of C15:0 and TG36, was added to obtain a final concentration of 0.4 mg/mL. Prior to analysis, 400 µL of each sample was derivatized with 150 µL MSTFA, gently mixed and left at least 3h for reaction.

Since the digestion model, used in the present study, can be considered as an in-between situation of the *in vitro* and *in vivo* digestion process two levels of hydrolysis (L_1% and L_2%, respectively) were calculated according to Capolino et al. (Capolino et al., 2011). The level of hydrolysis (L_1%), occurring in *in vitro* experiments, was calculated according to the following equation:

$$L_1\% = 100 * \frac{FFA}{3 * TG + 2 * DG + MG + FFA}$$

As the absorption of lipolytic products in the intestine requires only the hydrolysis of 1 TG molecule into 1 MG and 2 FFA molecules, the physiological relevant level of hydrolysis (L_2%) was calculated based on the equation (Carrière et al., 2005):

$$L_2\% = 100 * \frac{FFA + MG}{3 * TG + 2 * DG + MG + FFA}$$

3.3 Results

3.3.1 Behaviour of emulsions in the gastric and intestinal compartment

In the gastric compartment, the positively charged lysozyme-stabilized emulsions appeared homogenous on a macroscopic level throughout the experiment (Fig. 3.2A). In contrast, the negatively charged WPI-stabilized emulsions showed an upper cream layer and a lower serum layer after approximately 35 min (Fig. 3.2 B-D). This creamed appearance remained until the gastric compartment was completely emptied. As only in the case of WPI-stabilized emulsions, a lower concentration of flocs was found in the bottom part, we define it as serum layer throughout the manuscript, as the presence of low particle concentrations is typical for such a droplet-depleted layer. Likewise, we define the layer in the top part in case of the WPI-stabilized emulsion as cream layer as it is a droplet-enriched layer. Both emulsions flocculated in the gastric compartment as shown in the microscopic pictures (Fig. 3.3).

The freshly prepared lysozyme and WPI-stabilized emulsion droplets had similar droplet sizes with a d_{32} of 2.8 µm and 2.7 µm, respectively (Table 3.2). During the first 20 min of gastric residence, both lysozyme and WPI-stabilized emulsions exhibited monomodal particle size distributions (Fig. 3.4). The d_{32} and d_{43} of lysozyme-stabilized emulsions increased during the first 20 min (Table 3.2). After 20 min, the particle size distributions became narrower to exhibit one sharp peak between 1-10 µm (Fig. 3.4). At t=90 min, a broadening in particle size distribution occurred in the WPI-stabilized emulsions (1-100 µm) which is reflected in the increase of the d_{32} and d_{43} (Table 3.2). To characterize the creamed WPI-stabilized emulsions, particle size distributions and microscopic pictures were analyzed from the bottom and top part of the gastric compartment. As shown in Figure 3.4 (insert), particle size distributions of both parts were found to be similar for both emulsions. Comparing the microscopic pictures obtained from the bottom and top part it can be seen that both fractions showed flocculation of the emulsions (Fig. 3.4, insert).

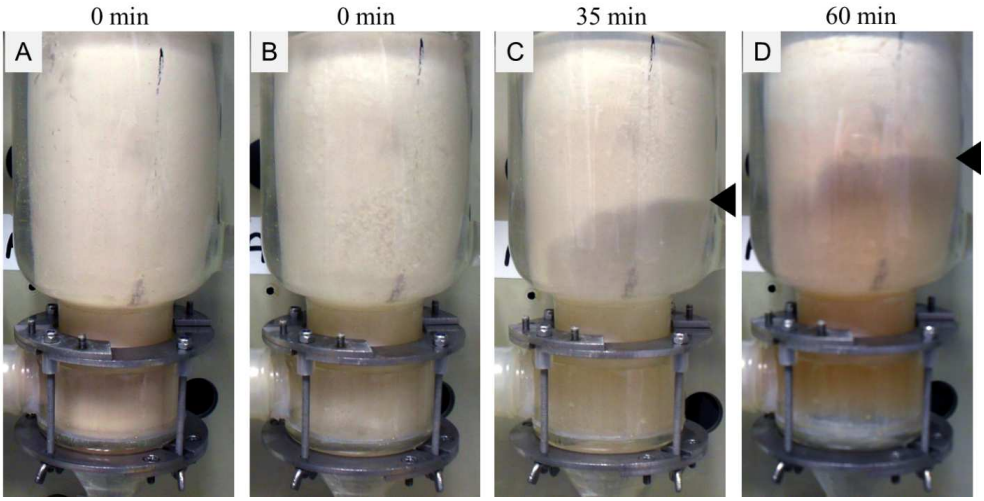

Fig. 3.2 Behavior of lysozyme- (A) and WPI- (B-D) stabilized emulsions in the gastric compartment after adding the emulsion to the gastric compartment (0 min) and at=35 min and t=60 min of digestion. The arrow indicates the separation of the WPI-stabilized emulsion into cream and serum layer.

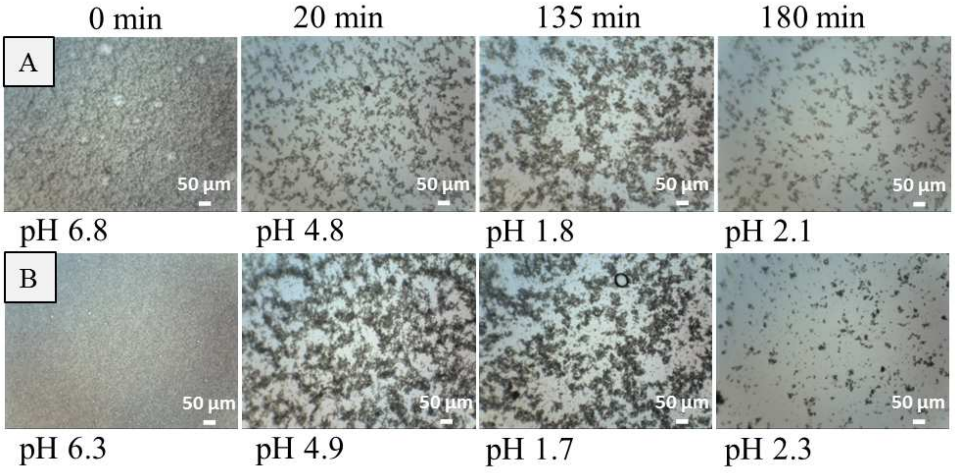

Fig. 3.3 Light microscopic characterization of lysozyme- (A) and WPI- (B) stabilized emulsions (0 min) in the gastric compartment at t=20, 135 and 180 min of digestion. Below each picture is the corresponding pH, scale bar is 50 µm.

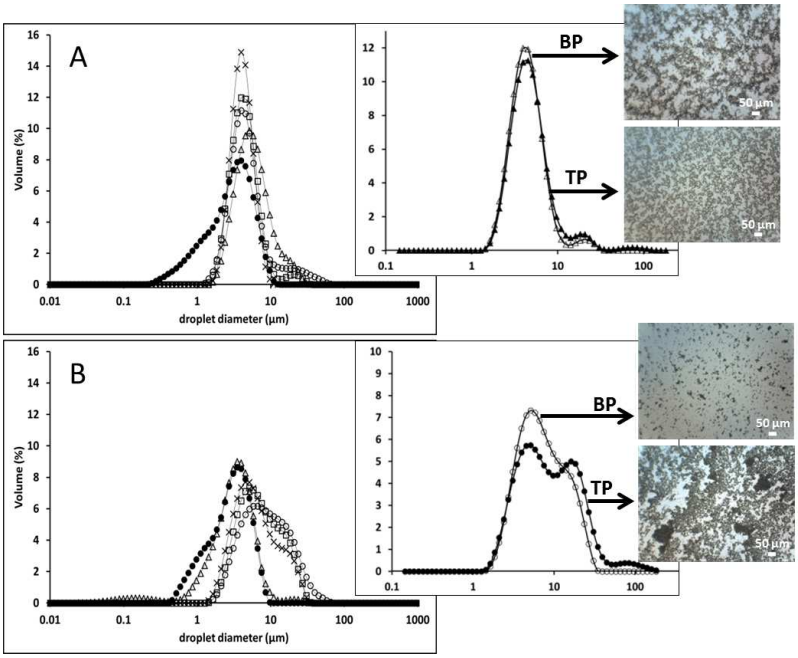

Fig. 3.4 Particle size distribution of lysozyme- (A) and WPI- (B) stabilized emulsions at t=20 (Δ), 90 (□), 135 (×) and 180 (o) min of digestion in the gastric compartment compared to freshly prepared emulsions (●). Inserts show the comparison of particle size distributions between the top part (TP, closed symbols) and bottom part (BP, open symbols) of the gastric compartment at t=90 min of digestion.

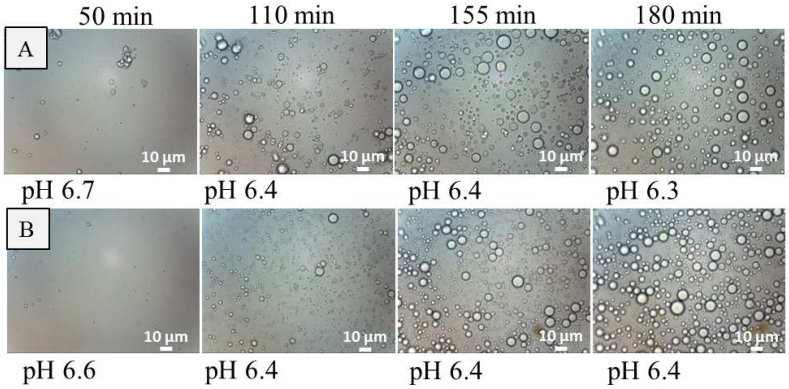

Fig. 3.5 Light microscopic characterization of lysozyme- (A) and WPI- (B) stabilized emulsions in the small intestinal compartment at t=50, 110, 155 and 180 min of digestion. Below each picture is the corresponding pH, scale bar is 10 μm.

When the digest reached the small intestinal part, the emulsions completely deflocculated. In contrast to flocculation behaviour exhibited in the gastric compartment, coalescence was observed for both emulsions in the small intestinal compartment (Fig. 3.5).

Within 110 min of intestinal digestion of lysozyme-stabilized emulsions, a shift to larger particle sizes was found as well as a broadening of the peaks (Fig. 3.6). Particles in the range of 50-500 µm were found only at t=50 and t=110 min and likewise the d_{43} was 6-fold and 5-fold higher, respectively, compared to the d_{43} at t=0 (Table 3.2). At t=155 min, the particle size distribution of lysozyme-stabilized emulsions reduced in size again. In contrast, at t=50 min of intestinal digestion, the distribution of WPI-stabilized emulsions became multimodal with very small droplets appearing (0.02-0.4 µm). While the particle size of lysozyme-stabilized emulsions decreased with time, a broadening and a shift to larger particles occurred during 180 min of small intestinal digestion of WPI-stabilized emulsions.

Table 3.2 Surface-weighted-mean diameter (d_{32}) and volume-weighted-mean diameter (d_{43}) of lysozyme and WPI-stabilized emulsions at different time points of digestion in the gastric and small intestinal compartment compared to freshly prepared emulsions (t=0 min).

sample	Time point (min)	lysozyme-stabilized emulsions		WPI-stabilized emulsions	
		d_{32} (µm)	d_{43} (µm)	d_{32} (µm)	d_{43} (µm)
emulsion	0	2.83	5.82	2.74	4.66
GS	20	8.94	12.60	3.34	6.63
	90	7.17/7.65*	11.82/12.17*	10.47/11.49*	16.85/26.70*
	135	6.74	7.63	9.19	15.11
	180	7.40	12.29	12.18	21.64
SIS	50	8.67	34.66	0.59	6.88
	110	9.65	26.99	3.11	5.03
	155	9.07	15.99	4.27	10.36
	185	6.66	11.27	7.59	16.48

GS: gastric samples, SIS: small intestinal samples,
* indicates the value obtained from the cream phase

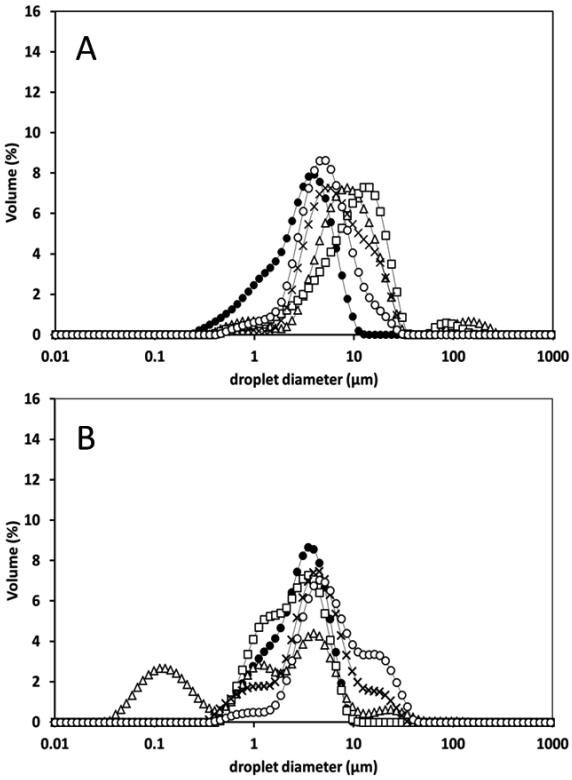

Fig. 3.6 Particle size distribution of lysozyme- (A) and WPI- (B) stabilized emulsions at t=50 (Δ), 110 (□), 155 (×) and 180 (o) min of digestion in the small intestinal compartment compared to freshly prepared emulsions (●).

3.3.2 Formation of lipolytic products in the gastric and duodenal compartment

The quantitative analysis of TG and lipolytic products, i.e. FFA, MG and DG, was conducted by means of GC on the same samples characterized by microscopy and particle size analysis. When the emulsions were subjected to the gastric environment, the amount of TG decreased continuously in time (Fig. 3.7) with only residual amounts of TG present in the stomach after 180 min. This is partly due to the emptying of the compartment and partly to the TG hydrolysis. The amount of FFA and DG slightly increased within the first 20 min (Fig. 3.7). The gastric hydrolysis levels L_1 and L_2 of TG for both emulsions and for each time point did not exceed 12 and 15%, respectively.

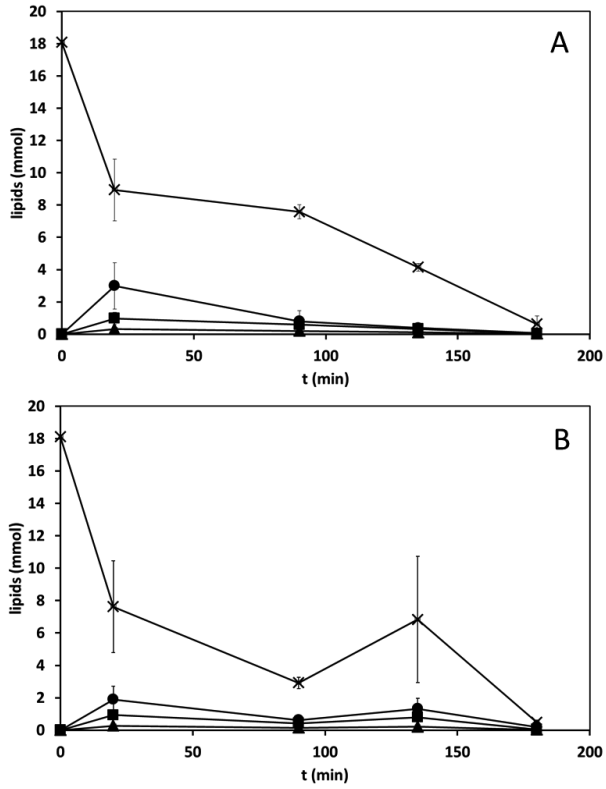

Fig. 3.7 Comparison of lipid compositions, i.e. FFA (●), MG (▲), DG (■) and TG (×), in the gastric compartment from lysozyme- (A) and WPI- (B) stabilized emulsions taken at the bottom. Error bars indicate standard deviations.

As we have observed creaming for one of the two emulsions, we have performed GC measurements at t=90 min to compare the distribution of fat components between the bottom and the top of the stomach compartment. In the case of lysozyme-stabilized emulsions, values of FFA, MG, DG and TG of the bottom part were found to be 0.8, 0.2, 0.6 and 7.6 mmol, respectively and those ones of the top part were 1.1, 0.3, 0.8 and 11.7 mmol, respectively.

In contrast, the difference between the bottom and top part was more pronounced in WPI-stabilized emulsions. The amount of FFA, MG, DG and TG of the bottom part were found to be 0.6, 0.1, 0.4 and 2.9 mmol, respectively, whereas values of the top part were 2.5, 0.4, 1.7 and 13.9 mmol, respectively. The results indicate that during digestion, when

creaming is occurring, WPI-stabilized emulsions have higher levels of TG and lipolytic products on top of the stomach compartment. Nevertheless, the digestion rate (L_1) of the top part compared to the bottom part was similar, i.e. 6.4 ± 1.3 and 7.4 ± 0.0%, respectively. The higher amount of FFA, DG and TG present at t=90 min in the top part of the stomach from WPI-stabilized emulsions will be released at a later stage into the intestine.

In the overall intestinal digestion of the emulsions the amount of FFA increased rapidly up to a final concentration of about 13 to 14 mmol (Fig. 3.8), which is about 4.6 times higher than the maximum amount produced in the stomach.

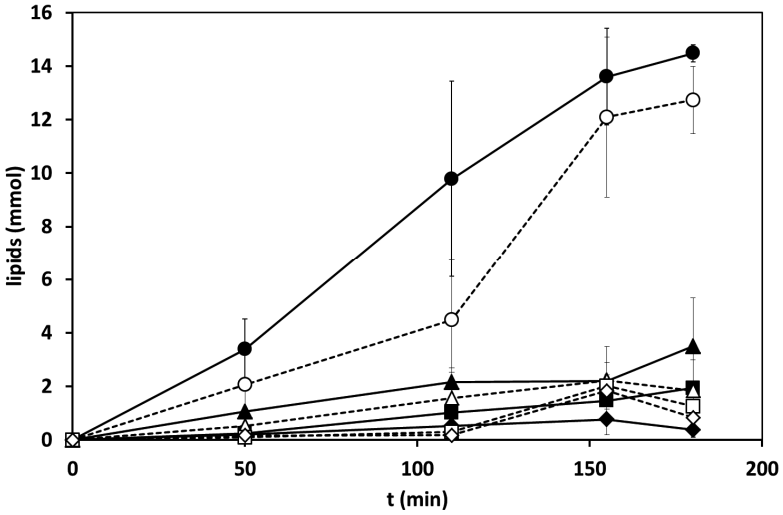

Fig. 3.8 Comparison of lipid compositions, i.e. FFA (●), MG (▲), DG (■) and TG (◆), in the intestinal compartment from lysozyme- (closed symbols) and WPI- (open symbols) stabilized emulsions. Error bars indicate standard deviations.

At the first sampling point (i.e. t=50 min) the FFA in the small intestine from both emulsions were equal to the amount present at t=20 min in the stomach (Figs. 3.7, 3.8). Higher amounts of FFA were obtained from lysozyme-stabilized emulsions than from WPI-stabilized emulsions within the first 110 min (Fig. 3.8). Additionally, TG from lysozyme-stabilized emulsions were present in rather constant amount during the experiment, whereas the TG of WPI-stabilized emulsions increased after 155 min. The

hydrolysis levels L_1 and L_2 in the small intestine were about 62% and 76%, respectively for both emulsions.

3.4 Discussion

3.4.1 Influence of gastric conditions on the emulsion behaviour

As observed in our experiments, the prepared negatively charged WPI emulsion and positively charged lysozyme-stabilized emulsions flocculated in the gastric compartment but only the WPI-stabilized emulsions exhibit a pronounced cream layer formation. In our opinion, the cream layer is produced by differences in flocculation of the two emulsions as clearly illustrated from the observed particle size distribution (Fig. 3.4). Despite the applied dilution, inevitable during measurement and inherent to the used instrument, the WPI-stabilized emulsions contained mainly larger particles indicating the presence of larger aggregates with a high fat content as possible causes for the cream layer. It is known that in emulsions systems, in the absence of polymers, flocculation can occur if the electrostatic repulsion between droplets decrease and cannot contrast the Van der Waals interaction between droplets. This occurs, for example, when the pH of the emulsions approaches values close to the isoelectric point (p*I*) of proteins at the oil/water interface and when the net charge on the droplets is almost zero. Flocculation of WPI-stabilized emulsions at pH 4.9 is known as the pH is close to p*I* of β-lactoglobulin (β-lg), which is the main component in WPI. Besides the effect of pH, flocculation is also induced by salts from the gastric secretion. Typically, salts decrease electrostatic repulsion between droplets by screening the droplets surface charges. The combination of salts and transitions through the p*I* has been shown recently to cause flocculation of WPI-stabilized emulsions (Golding et al., 2011). Lysozyme-stabilized emulsions are instead always positively charged in the applied pH range as the p*I* of lysozyme is about 11. Possibly, these two charge related effects had a larger impact on the flocculation and the consequent creaming behavior of WPI-stabilized emulsions compared to lysozyme-stabilized emulsions under gastric conditions (Fig. 3.2). Therefore, the presence of salt could be a major factor causing the observed less pronounced flocculation of lysozyme-stabilized emulsions as shown from the particle size analysis, since they screen the positively charged emulsions droplets. However, it has been shown recently, that the most important factor which determines emulsion stability is the hydrolysis of the protein interface by pepsin (Singh & Sarkar, 2011). In fact, the addition of pepsin to β-lg

or WPI-stabilized emulsions, in the presence of salts and at acidic pH, accentuated flocculation and caused slight coalescence of the emulsions droplets (Golding et al., 2011; Sarkar et al., 2009) since pepsin hydrolyses the β-lg interfacial layer at the oil-water interface resulting in a loss in positive charge, peptide formation and a reduction of the interfacial thickness (Sarkar et al., 2009; Malaki Nik et al., 2010; Nik et al., 2010). The formed peptides, for example of ~ 10-15 kDa (Sarkar et al., 2009), are less sufficient to provide steric and electrostatic hindrance thus leading to flocculation of the droplets. When evaluating the peptides formation by pepsin using the Peptide Cutter tool available on Expasy platform (http://web.expasy.org/cgi-bin/peptide_cutter/peptidecutter.pl) β-lg originates 36 peptides with molecular masses varying from 131,17 Da of the ammino acid leucin up to 2613,07 Da of the longest peptide QKWENGECAQKKIIAEKTKIPAV.

Pepsin digestion of lysozyme originates, instead, 15 peptides, presenting in general higher molecular masses compared to those formed for β-lg reaching a maximum of the 4939 Da for the sequence SSDITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDVQ AWIRGCRL. Therefore, due to the differences in type, length and amount of peptides produced by pepsin digestion, lysozyme stabilized interfaces might be less susceptible to hydrolysis. This in turn causes less flocculation and consequently less creaming. Additionally, as hydrolysis of the protein by pepsin weakens the viscoelastic interface (Golding et al., 2011; Sarkar et al., 2009) the increase in droplets size for both emulsions found in the present study will be to some extend due to slight coalescence.

Further, the addition of lipase and the resulting formation of FFA will contribute to an accentuate flocculation of the emulsions as recently shown for WPI-stabilized emulsions upon addition of a fungal lipase (Golding et al., 2011). Surface active components such as FFA, MG and DG are thought to displace the stabilizing protein layer and change therefore the interfacial properties of the emulsions. For example, 2-MG have been found to be highly surface active and able to dominate the interfacial layer (Reis et al., 2008). As we found only traces of MG we do not expect them to cause major changes in the interfaces. However, the amount of FFA and DG of lysozyme-stabilized emulsions at t=20 min, i.e. 441.25 and 144.0 μmol/m^2, respectively, might be sufficient to change the interfacial properties. Since the values of FFA and DG are lower in WPI-stabilized emulsions at t=20 min, i.e. 123.1 and 60.9 μmol/m^2, respectively, their ability to influence the interface might be less compared to lysozyme-stabilized emulsions.

During the digestion in the small intestine, we found an increase in droplet size for WPI-stabilized emulsions as a result of coalescence. Coalescence of WPI-stabilized emulsions under *in vitro* intestinal conditions was previously attributed to the gastric proteolysis of the interface by pepsin and the resulting peptide formation when the emulsions were incubated before under simulated gastric conditions (Malaki Nik et al., 2011). The authors suggested, that a further proteolysis of WPI (and its peptides) by trypsin and chymotrypsin, both present in pancreatin, together with a displacement of the interfacial layer by bile salts (BS) and phospholipids (PL) caused the increase in the sensitivity to coalescence under duodenal conditions (Malaki Nik et al., 2011). Therefore, both the addition of pepsin and porcine bile in our study will have contributed to the occurred coalescence.

Beside coalescence, we observed smaller particle with d_{32} < 1 µm at t=50 min for WPI stabilized emulsion. This finding is also in line with Malaki et al. (Malaki Nik et al., 2011) where addition of phospholipids was hold to be responsible for the appearance of particles <1 µm.

3.4.2 Influence of creaming on intestinal lipid digestion

The separation into a cream and serum layer of WPI-stabilized emulsions within the gastric compartment, as reported in Figure 3.2, was observed to cause a delay of their intestinal hydrolysis.

Firstly, the higher amount of FFA and DG present at t=90 min in the cream layer from WPI-stabilized emulsions will be released at a later stage into the small intestine. Probably, gastric lipolysis will not proceed further in this layer, due to the low pH and possible product inhibition of the enzyme (Pafumi et al., 2002). In fact, only within the first 20 min FFA and DG increased. This time span corresponded to the time necessary for the digestion process to reach a pH between 4 and 5, which is the optimal pH for the lamb pharyngeal lipase (Moreau et al., 1988). Also, the amount of FFA per surface area (m^2) as shown in section 3.4.1., was above the FFA concentration to inhibit HGL, i.e. 107 - 122 µmol/m^2 (Pafumi et al., 2002).

Secondly, the lower amounts of lipid products in the intestine at t=110 min (Fig. 3.8) reflected the later entry of fat when WPI-stabilized emulsions are compared to lysozyme-stabilized emulsions. This is due to the creaming in the stomach compartment since the

activation of pancreatic lipase by FFA requires only very small concentration in the oil, i.e. less than 1 % (Gargouri et al., 1986b).

Since satiety and related physiological responses such as hormone secretion or gastric emptying are related to the concentration and rate of delivery of FFA in the small intestine, it could be expected that those responses will be different for the two types of emulsions. It is known that layering of fat in the stomach has implications *in vivo*, i.e. the fat layer empties deferred into the duodenum (Foltz et al., 2009). In such case, both the release of FFA into the small intestine as well as the release of satiety hormones, such as cholecystokinin (CCK), will be delayed. Since such a behavior cannot be explored with the digestion model used in our study, we could only speculate that the digestion of lysozyme-stabilized emulsions would have an earlier influence on satiety than WPI-stabilized emulsions. Nevertheless, the presence of FFA, found in our study in the serum layer of WPI-stabilized emulsions, might still trigger the release of CCK and delay gastric emptying *in vivo*.

Although to our knowledge, concentration related studies to clearly evaluate the FFA threshold for hormone secretion have not been performed yet, it has been reported that such low amount of FFA as 0.025 mM in the stomach were able to trigger a CCK response (McLaughlin et al., 1999). This FFA concentration induced the release of about 4 pM of CCK. Little and coworkers (Little et al., 2007) obtained the same amount of CCK released with FFA concentrations of 40 mM present in the small intestinal lumen. In the study of Little et al., this amount of CCK was related to a higher feeling for fullness, a decreased gastric emptying and a suppressed energy intake. In our case, the amount of FFA present at all different time points and for both emulsions was largely above 0.025 mM. Therefore, although creaming occurs we would not expect different effects on the CCK release *in vivo* between the two emulsions.

Finally, it must be noted, that the rate of gastric emptying in the Tiny TIM is programmed and not influenced by the nature of the emulsions. Hence, feedback mechanisms due to FFA detection in the small intestine occurring *in vivo* were not taken into account. Those mechanisms must be studied *in vivo*. In addition, the used Tiny TIM model was built to remove the lipolytic products by filtration in order to avoid product inhibition of the pancreatic lipase. Thus, in theory an over-efficient removal of FFA could unrealistically reduce the rate of intestinal lipolysis, since pancreatic lipase needs a very low concentration of FFA. Additionally, the model might not be realistic in the selectivity

and speed of transport of the FFA incorporated in bile micelles through the mucus lining and enterocytes as it occurs *in vivo*.

3.5 Conclusions

It can be concluded that the difference in creaming behavior during gastric digestion delays the intestinal lipid digestion. This is due to the separation into a cream and serum layer caused by pronounced flocculation of WPI-stabilized emulsions. The total amount of FFA produced in the small intestinal compartment at the end of the experiment is the same for both emulsions. This seems to imply that the charge differences are only affecting the creaming behavior but not the enzymatic activity of the lipase on the two studied emulsions. From the detected amount of FFA we would not expect any difference in CCK induced satiety responses *in vivo* despite the creaming of the WPI-stabilized emulsion.

Acknowledgements

The authors gratefully acknowledge Esther Bomhof, Franklin D. Zoet (NIZO food research) and Hans Kooijman (TNO Triskelion) for their technical assistance.

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Chapter 4

Effect of oral administration and intragastric distribution of lipids on lipolysis in gastric and duodenal human fluids

To be submitted

Abstract

The present study examined, how gastric and duodenal lipolysis is affected by the intragastric colloidal state of the emulsions and how the administration route, i.e. oral or intragastric, affects gastric lipolysis. In a randomized, single blind crossover study, 15 healthy subjects were intubated nasogastric on three separate occasions and of this a subset of 6 healthy subjects was intubated nasoduodenal on two separate occasions. Volunteers received a homogenized emulsion orally or by gastric infusion or a gastric infusion of water followed by the oil phase (un-homogenized sample). The subset of 6 volunteers received a gastric infusion of a homogenized emulsion or of an un-homogenized sample. Emulsion structure, pH and lipid composition was assessed in both gastric and duodenal fluids. Results showed a gastric lipolysis of approx. 3% for all treatments. Free fatty acid (FFA) concentrations in duodenal aspirates at 30 min were higher after ingestion of the homogenized emulsion than after ingestion of the un-homogenized samples ($p<0.01$). Gastric emptying was faster for the un-homogenized sample than for the homogenized emulsion. Thus, overall, there was no effect observed of oral processing and homogenization had no effect on gastric lipolysis. Nevertheless, differences in inhibition of gastric emptying were observed: duodenal release of FFA and inhibition of gastric emptying occurred earlier for the homogenized emulsion than for un-homogenized samples.

4.1 Introduction

The intake of fat and its passage through the gastrointestinal tract triggers physiological responses thus impacting gut transit time (Heddle et al., 1988; Feinle et al., 1996; Little et al., 2007; Maljaars et al., 2008). It is expected that such responses are able to influence and contribute towards regulation of food intake and satiety, a goal to combat obesity. Several studies (see for review Golding & Wooster, 2010) have investigated the *in vitro* behaviour of fat present in the form of emulsions to predict their possible *in vivo* digestion. Recently, we showed that by using a dynamic digestion model, gradual acidification in combination with a custom designed gastric compartment imitating the functions of the stomach, caused protein stabilized emulsions to cream (Helbig et al., 2013). We have shown that this behaviour delayed intestinal lipolysis (Helbig et al., 2013). Although this model represents a significant step towards better prediction of *in vivo* digestion, there are still many factors unaccounted for.

In vivo digestion is very complex due to many simultaneously occurring processes, such as pH lowering, salt secretions, enzymatic hydrolysis, change in the emulsion structure and physiological feedback mechanisms like gastric, pylorus and intestinal activation. Therefore, it is challenging to investigate how the physico-chemical properties of foods affect the digestion and absorption of lipids *in vivo* and how this is related to satiety and -related physiological responses, such as hormone secretion or gastric emptying. There are increasing indications in literature, that free fatty acids (FFA) are primarily responsible for triggering gastrointestinal effects of lipids when delivered to the small intestine (Schwizer et al., 1997; Feinle et al., 2001; Feinle-Bisset et al., 2005; Little et al., 2007). For example, Little et al. (Little et al., 2007) showed that FFA were more effective to delay gastric emptying, stimulate hormone release and suppress appetite compared to triglycerides. In this study, the FFA were directly administrated intragastrically. In a normal meal, however, the concentration and delivery of FFA will depend on the colloidal behaviour of the emulsion which changes progressively due to variation in pH and due to enzyme action when reaching the stomach and the intestine. Also, the release of FFA in the gastrointestinal tract will be affected by factors like the continuous phase of the emulsion, type of emulsifier, fat composition and droplet size (see for review McClements et al., 2009). For example, both *in vitro* (Armand et al., 1992; Borel et al., 1994; Helbig et al., 2012) and *in vivo* (Armand et al., 1999) studies revealed that a smaller droplet size leads to a higher rate of FFA release for both gastric and pancreatic

lipase. However, the changes in droplets size in the stomach are not well understood as, for example, the droplet size of a coarse emulsion, with a diameter of 10 μm, did not change after ingestion while an increase in droplet size was observed with a fine emulsion (Armand et al., 1999). Though, in this study a system with complex interfaces was used as they are made out of different emulsifiers, such as lecithin and milk proteins. As proteolysis and decrease in pH are both occurring in the stomach, the smaller droplets are likely to aggregate and cream. Thus, it is difficult to interpret the changes in the colloidal state of the emulsion, i.e. the intragastric distribution of lipids, and to pin point which variables are inducing satiety and related physiological responses. Indeed, little research has been conducted on the influence of the gastric lipolysis as affected by the intragastric lipid distribution in relation to satiety perception. Although it has been reported that emulsions that are intragastric stable reduce hunger and appetite (Marciani et al., 2009), the effect of gastric hydrolysis and the subsequent release of FFA in relation to physiological human responses is not clear.

In addition to the effect of gastric hydrolysis, a number of studies point at the role of oral stimulation. The role of orally administrated fat and its cephalic responses is thought to be of importance in regulating food intake as it has been shown that vagal stimulation upon modified sham feeding accentuated effects on metabolites, hormones and satiety (Robertson et al., 2002; Heath et al., 2004; Smeets & Westerterp-Plantenga, 2006). Thus, investigating the role of oral stimulation might reveal the contribution of the mouth on gastric lipolysis, as measured by the release of FFA, and its influence on gastric emptying. The role of oral stimulation and its contribution to hormone release and satiety perceptions will be discussed in Chapter 5.

The focus of this chapter is to describe how gastric and duodenal lipolysis are affected by the intragastric colloidal state of the emulsions. Furthermore, the effect of the administration route, i.e. oral or intragastric, on gastric lipolysis was explored.

4.2 Materials and methods

4.2.1 Emulsion preparation

Emulsions were produced following good manufacturing practice in a food grade pilot plant of NIZO food research (Ede, The Netherlands). Tween 80 (Lamesorb©SMO20, Cognis, Monheim am Rhein, Germany) solutions were prepared by dissolving powder in

0.01 M NaCl solution. The final emulsions contained 8% (w/w) sunflower oil (Reddy sunflower oil, Vandermoortele, Izegem, Belgium) and 0.4% (w/w) Tween 80. All ingredients used were food grade. In total 3 batches of each solution were prepared. Each batch consisted of 10 L (20 x 500 mL) of each solution. Emulsions were homogenized at 80°C at a pressure of 70 to 180 bar followed by homogenization at 0 to 20 bar (Rannie Lab12-16.5, Leuze, Waardenburg, The Netherlands). The solutions for the un-homogenized samples, i.e. Tween 80-solution and sunflower oil, were heated separately at 80°C. These samples were not homogenized and applied separately but for the ease of the reading we refer to them as emulsions. Homogenized and un-homogenized emulsions were then sterilized in a Combitherm sterilizer (Minister 1987, AlfaLaval, Lund, Sweden) for 7 seconds at 141°C. After cooling to room temperature, solutions were filled aseptically in sterilized plastic containers and stored <7°C in a dark place until the start of the experiments in order to prevent off-flavor formation. Of every batch 2 samples were tested for sterility. During the course of the experiment fresh emulsions were prepared every 6-10 weeks in order to prevent excessive off-flavor formation or formation of physical instabilities. The surface-weighted-mean- diameter (d_{32}) and the volume-weighted-mean diameter (d_{43}) of the freshly prepared homogenized emulsions were 2.33 and 8.75 μm, respectively, as determined by dynamic light scattering (Mastersizer Hydro 2000S, Malvern Instruments, Malvern, UK) at room temperature.

During the production of the emulsions, 0.034% (w/w) and 0.012% (w/w) of vanilla powder and saccharine, respectively, were added to the Tween 80 solutions. As a result, the emulsions tasted pleasantly sweet with a sweet vanilla flavor having a somewhat bitter off-note. On the days of intervention, 1 g of paracetamol (Apotheek van Thoor, Maastricht, the Netherlands) was manually added to the homogenized emulsions or, in the case of the un-homogenized emulsions, to the Tween 80 solution to measure gastric emptying of the water phase.

4.2.2 Subjects

15 healthy volunteers participated in the study (11 male and 4 female, age 28±8 y, body mass index of 22.7±2.3 kg/m^2). A short physical examination and a cognitive restraint eating behaviour using a Dutch translation of the Three Factor Eating Questionnaire (TFEQ) were performed. Participants did not have gastrointestinal or hepatic disorders or previous major abdominal surgery interfering with gastrointestinal function. They were non-smokers, moderate alcohol consumers (≤ 10 alcoholic consumptions per week), non-

medicated except of using contraceptives and non-restraint eaters according to the Three Factor Eating Questionnaire (TFEQ Factor 1 score ≤ 9). The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center, conducted according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO). All participants signed informed consent. Volunteers were informed that they receive a lipid-containing drink and that its behaviour in the stomach and duodenum as well as the resulting physiological responses were investigated.

4.2.3 Study design

The study was a randomized crossover design and divided in two experiments. All experiments took place on separate days. Subjects were requested to refrain from any food or beverage intake with the exception of water from 2200h the evening before the test day. Subjects received a total load of 500 mL of samples with a total energy content of 360 kcal.

Experiment 1: Gastric intubation study

On all 3 test days of the gastric intubation study, subjects arrived in the morning at 0830h in fasted state. After local anesthesia of the nasal mucosa using lidocaine 10% spray, each subject was intubated with a double-lumen nasogastric feeding catheter (Bengmark® Nutricia, Amsterdam, The Netherlands). One lumen was used to inject emulsions into the stomach and the other lumen was used to collect stomach fluid by aspiration using a standard 10-mL syringe. The position of the nasogastric catheter was controlled by determination of the pH of the aspirate. After intubation, a cannula was placed in a superficial antecubital vein of the forearm for blood sampling. At 0900h, subjects received the emulsions infused into the stomach either homogenized (hs) and in case of the un-homogenized emulsions (uhs), the infusion of the water phase was followed by the oil phase within 2 min. In the case of the oral administration, the emulsions were homogenized (ho) and drunk within 2 min.

Experiment 2: Duodenal intubation study

To investigate the intestinal behaviour of the emulsions, 6 volunteers, who participated in the gastric intubation study, joined on two further occasions following the same procedure as described above with minor modification. On the study days, subjects were intubated with a double-lumen naso-duodenal catheter (Bengmark® Nutricia, Amsterdam, The Netherlands) after anaesthesia of the nasal mucosa. The intraduodenal part of the catheter was placed under radiological control and placed with the tube tip located of about 10 cm in the proximal small intestine directly distal to the stomach. The position of the sample port in the stomach was controlled by aspiration. The emulsions were identical in their composition to the ones used in the gastric intubation study and applied to the stomach either un-homogenized (uhs) or homogenized (hs).

4.2.4 Measurements

Physicochemical characterisation of in vitro gastric and duodenal digestion of emulsions

Samples (10-14mL) of stomach contents were withdrawn within 2 min at baseline (t=-10 min) and at 10, 15, 30, 45, 60, 90, 120 and 180 min after application of the emulsions. Samples of the duodenal contents were collected continuously by aspiration over 15 min at baseline (t=-10 min) and at 15, 30, 45, 60, 90, 120 and 180 min after application of the emulsions. The pH of these gastric or duodenal aspirates was directly measured using pH-indicator strips (Merck, Darmstadt, Germany). On the day of administration, the behaviour of the emulsions and of the emulsions in the aspirates was monitored visually and recorded on camera. Microscopic images were obtained by putting one drop of the samples on a hemocytometer (Bürker-Türk, Hofheim, Germany), covered and immediately analysed using a Bresser® Trino Researcher microscope (40-1000x, Meade instruments, Rhede, Germany) equipped with a moticam 2300 camera (MC2300, 3.0Mp, USB2.0, Motic Instruments, Richmond, BC, Canada).

Samples were analyzed for FFA, mono-, di- and triglycerides (MG, DG, TG), respectively, composition by gas chromatography (GC) as previously described (Helbig et al., 2012) with minor modifications. Briefly, 2x0.5 mL samples were taken from the fresh aspirates, immediately poured into extraction fluid and stored at -40 °C until further analysis. After extraction, the final supernatant was collected and its volume was brought

to 8 mL with diethylether/heptane (1:1, w/w). An internal standard mix (50 µL), consisting of C15:0 and TG36, was added to obtain a final concentration of 0.4 mg/mL. Prior to analysis, 200 µL of each sample was diluted 1:2 with diethylether/heptane (1:1, w/w), derivatized with 150 µL MSTFA, gently mixed and left at least 3h for reaction. Variations between duplicates from 5 test persons and different application forms, i.e. 3 times uhs, 4 times ho and 3 times hs, were <3 %. Therefore, all further measurements with GC were carried out once. Fatty acid standards, i.e. C14:0, C15:0, C16:0, C18:1, C18:2 as well as monoolein, diolein, and triglyceride standards triolein and trilaurin (TG36) were obtained from Sigma–Aldrich (>99 % pure, Sigma–Aldrich, St.Louis, MO, USA) as well as MSTFA (N-methyl-N-(trimethylsilyl)trifluoroacetamide, lot BCBD5976V). All other chemicals were of analytical grade and purchased from Merck or Sigma–Aldrich. Solutions were prepared in demineralized water.

Levels of hydrolysis (L_1% and L_2%, respectively) were calculated according to Capolino et al (Capolino et al., 2011) and as shown in Chapter 3.

Pancreatic lipase activity was measured in 0.5 mL aliquots of duodenal samples. Samples were stabilized by adding 100 µL of protease inhibitors (Protease Inhibitor Cocktail, Sigma) after which they were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Lipase activity was analysed using a fluorometric assay (Cortner et al., 1976). Quantification was carried out against a standard curve of 4-methylumbelliferone and 1 nmol hydrolysed substrate per minute is defined as 1 unit.

Blood samples

During the test days, blood samples were taken at baseline (t=-10 min) and at 10, 15, 30, 45, 60, 90, 120 and 180 min after application of the emulsions. After sampling, physiological salt solution (NaCl 0.9 % w/v) and heparine were injected to prevent coagulation and occlusion of the catheter. Blood samples for paracetamol analysis were drawn in EDTA tubes. All samples were kept on ice and all tubes were centrifuged at 1500 × *g* for 10 min at 4°C within one hour. Aliquots of the supernatant plasma samples were stored at -80°C until further analysis. Paracetamol was analysed by reversed phase HPLC with UV detection using an in-house method at the department of Toxicology, Maastricht University. After taking the last sample at t=180 min, subjects were immediately extubated and received 30 min thereafter a lunch.

4.2.5 Data analysis

Data transformation

Data analyses were performed on the measured pH of gastric (GA) and duodenal (DA) aspirates, the concentrations of free fatty acids (FFA) and mono- (MG), di- (DG) and triglycerides (TG) in gastric and duodenal aspirates, lipase activity, and blood concentrations of paracetamol. First, the normality of dependent variable distributions was assessed by the Kolmogorov-Smirnov test. These tests revealed that the normality assumption was violated for most dependent variables. Therefore, data transformations were performed prior to ANOVA testing. The nature of data transformation depended on skew and range constraints of dependent variables: $y' = {}^{10}log(y)$ transformations were used for right-skewed distributions with lower-bound delimiters (i.e. lipid concentrations, lipase activity, plasma paracetamol). Dependent variable distributions that were delimited on two sides were transformed by $y' = ln(y/(max-y))$, with *max* being the maximum allowable value (pH).

Dose-response functions

In addition, pharmaco-kinetic dose-response curves were fit to the timed observations of the dependent variables used. For this, the mathematical Weibull model was used, which describes physiological responses to the dissolution of drugs (Thelen et al., 2012) by the following equation:

$$y = c * (x/a)^{(b-1)} * \exp(-(x/a)^b)$$

where the dose-response function (y) is described as a function of time (x) and a time scaling factor (*a*), a shape factor (*b*) and an amplitude scaling factor (*c*). The amplitude scaling factor includes negative values for downward going pulse responses. Weibull functions were fit to individual dose-response observations by minimizing the summed squared distances of observed data points to the function using the MS Excel solver function with the GRG non-linear fit engine and the restraints $1.05 < a < 6$ and $0.5 < b < 10$. This procedure was repetitively performed for all dependent variables.

Response curves of dependent measures over time were compared to ascertain the temporal sequence of responses of different modalities (i.e. pH, paracetamol uptake,

lipolysis, lipid synthesis) in relation to different treatments. For this purpose, peak onset times (t-onset) and peak optimum times (t-peak) were estimated from the modelled response curves. Individual t-onsets were defined as the time at which the response estimates deviated 20 % from the baseline towards the optimum value. The t-peak was defined as the time point at which Weibull functions showed the maximum value for upward going response curves or the minimum value for downward going response curves. Both t-onset and t-peak were estimated from the Weibull model using the excel solver function with the restraints t-peak > 0 and 0 < t-onset < t-peak.

Statistical testing

Gastric intubation study Effects by the factor Time (fixed factor, "t=-10", "t=10", "t=15", "t=30", "t=45", "t=60", "t=90", "t=120", "t=180"), Treatment (fixed factor, "ho", "hs", "uhs") and Subjects as a random factor were tested by multifactor ANOVA. This analysis also included all two-factor interactions. In addition, multifactor ANOVA was used to test effects on t-peak and t-onset by the factor Measures (fixed factor, "pH-GA", "plasma paracetamol", "FFA-GA", "MG-GA", "DG-GA", "TG-GA"), Treatment (fixed factor, "ho", "hs", "uhs"), and Subject (random factor) and all two-factor interactions of these.

Duodenal intubation study Effects on the selected measures, i.e. pH, FFA, MG; DG, TG, lipase activity, by Time (fixed factor, "t=-10", "t=15", "t=30", "t=45", "t=60", "t=90", "t=120", "t=180"), Treatment (fixed factor, "hs", "uhs"), and Subjects (random-factor) and all two-factor interactions were tested by multifactor ANOVA. In addition, effects on t-peak and t-onset by the factors Measure (fixed factor, "pH-DA", "lipase activity", FFA-DA", "MG-DA", "DG-DA", "TG-DA), Treatment (fixed factor, "hs", "uhs"), and Subject (random factor) and their two-factor interactions were tested by multifactor ANOVA. In case of significant ANOVA test results, pairwise post-hoc comparisons were performed with Tukey HSD correction for multiple comparisons.

All data were analysed using Statistica (version 10, StatSoft,Tulsa, OK, USA). All results are expressed as means ± standard error (SE). The significance level was 0.05.

4.3 Results

The study procedure was well tolerated by the subjects. Only two subjects were sensitive to the tubing and felt nauseas on two occasions during the gastric intubation

study. Therefore, we stopped those investigations after 90 and 120 min, respectively. Nevertheless, none of the 15 enrolled subjects withdrew from the study.

4.3.1 Gastric intubation study

Appearance of the emulsions

In the stomach, the homogenized Tween 80-stabilized emulsions appeared visually homogenous on a macroscopic level throughout the experiment with a white cream upper layer on top and a turbid lower phase. There was no difference in the appearance of the homogenized emulsions between the oral or gastric application. In contrast, the un-homogenized emulsions were clearly watery, but became more turbid or had an oil upper layer after 90 min. Droplets of the homogenized emulsions tended to flocculate and coalesce (Fig. 4.1A, B). As expected, un-homogenized emulsions revealed either no or very large droplets (data not shown).

Table 4.1 summarizes ANOVA results of the all independent variables, i.e. pH, plasma paracetamol concentration, lipids (such as FFA, MG, DG and TG) in gastric or duodenal aspirates as well as lipase activity.

pH profile and paracetamol plasma levels

The gastric pH profiles are shown in Fig. 4.2A and the initial pH was 3.3 ± 0.3. After ingestion of the emulsions the pH was 3.1 ± 0.2 at t=10 min and as revealed post hoc decreased significantly to 2.1 ± 0.1 at t=30 min. After this interval the gastric pH remained constant until the end of the experiment. There was no difference between the treatments ho, hs or uhs as well as no Treatment × Time interaction (Table 4.1).

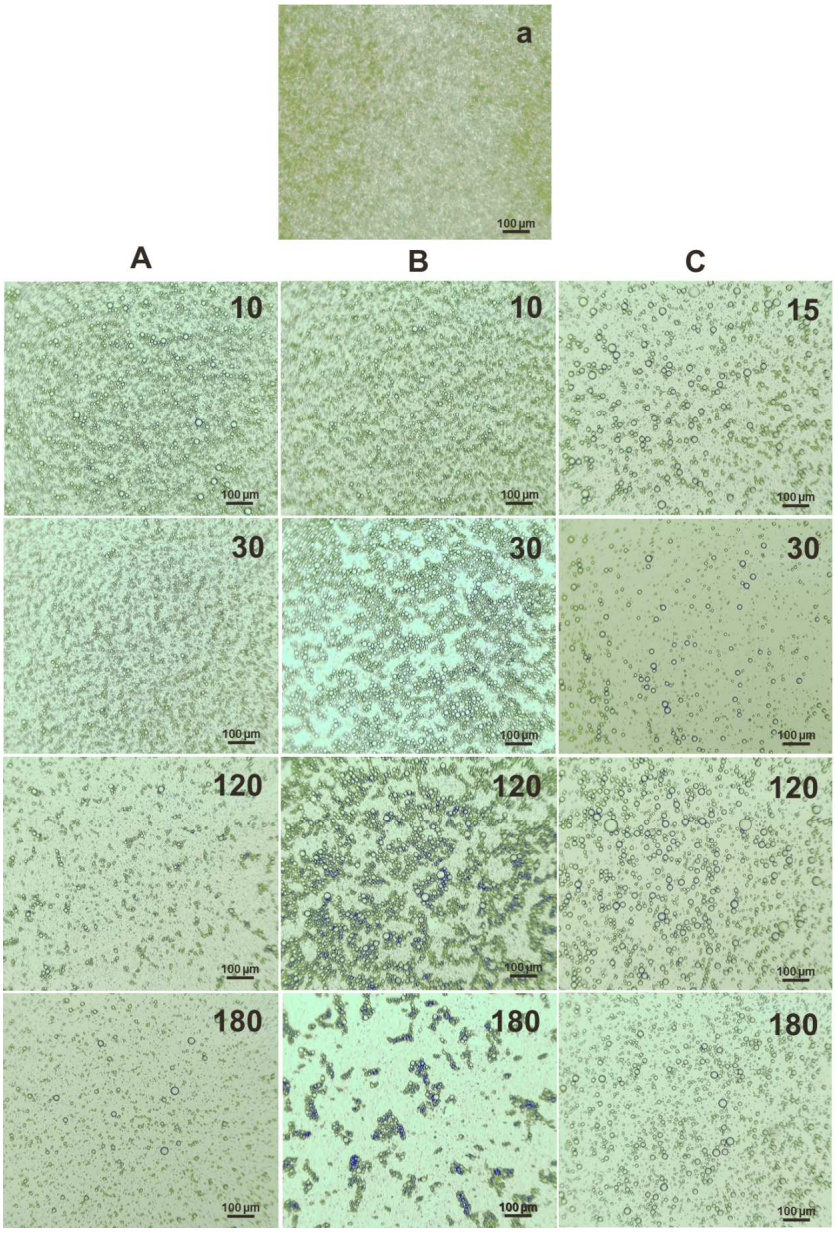

Fig. 4.1 Light microscopic appearance of the homogenized Tween 80-stabilized emulsion (a) and of gastric samples after the treatment hs (A) and ho (B), Experiment 1 at 10, 30, 120 and 180 min and of duodenal samples after the treatment hs (C), Experiment 2 at 15, 30, 120 and 180 min; Magnification is 100x.

Table 4.1 Effect of Time of aspirate or plasma collection, emulsions used as Treatment, and Time × Treatment interaction on pH, FFA, MG, DG, TG in gastric or duodenal aspirates and indicated with GA or DA, respectively, on lipase activity or plasma paracetamol concentration.

dependent variable	Time		Treatment		Time x Treatment	
	$F (df_1,df_2)$	Sig	$F (df_1,df_2)$	Sig	$F (df_1,df_2)$	Sig
pH-GA	11.61 (8,224)	***	1.11 (2,224)	Ns	1.19 (16,224)	Ns
plasma paracetamol	69.18 (8,224)	***	9.80 (2,224)	***	9.72 (16,224)	***
FFA-GA	4.71 (8,224)	***	0.08 (2,224)	Ns	0.72 (16,224)	Ns
MG-GA	1.39 (8,224)	Ns	0.58 (2,224)	Ns	0.83 (16,224)	Ns
DG-GA	18.43 (8,224)	***	4.65 (2,224)	*	1.54 (16,224)	Ns
TG-GA	35.55 (8,224)	***	14.26 (2,224)	***	3.03 (16,224)	***
FFA-DA	10.97 (7,35)	***	12.63 (1,35)	*	2.02 (7,35)	Ns
MG-DA	6.39 (7,35)	***	1.33 (1,35)	Ns	2.42 (7,35)	*
DG-DA	2.06 (7,35)	Ns	10.93 (1,35)	**	3.72 (7,35)	**
TG-DA	3.29 (7,35)	**	2.98 (1,35)	Ns	2.46 (7,35)	*
lipase activity	2.58 (7,35)	*	0.34 (1,35)	Ns	2.82 (7,35)	*
pH-DA	0.64 (7,35)	Ns	0.05 (1,35)	Ns	1.42 (7,35)	Ns

Significant differences are indicated with asterisks: *p<0.05, **p< 0.01, ***p < 0.001, based on ANOVA analysis. Ns is not significant.

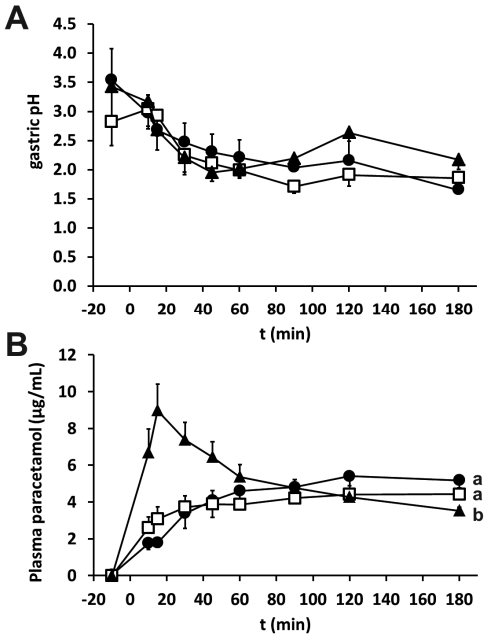

Fig. 4.2 Gastric pH-profile (A) and plasma paracetamol concentrations (B) during 180 min of Experiment 1 after the treatments ho (●), hs (□) and uhs (▲).Values are expressed as mean ± SE. Different low letters indicate a significant difference at p<0.001.

Based on ANOVA, plasma paracetamol concentration showed an effect of Treatment, Time and Treatment × Time interaction (Table 4.1). Post hoc analysis further revealed that the concentration increased significantly after application of all emulsions and did not reach baseline values at the end of the experiment. There was no difference between ho and hs. For the treatments ho and hs plasma concentration curves were significantly different from uhs (Fig. 4.2B) indicating a faster gastric emptying of the water phase for the treatment uhs compared to hs and ho. Also, a specific concentration pattern was found which is depending on the type of treatment and time of sampling (supplementary, Table S4.1). In the case of plasma paracetamol, concentrations were highest between t=10 and 45 min for treatment uhs compared to hs and ho.

Lipid composition

The lipid compositions of gastric aspirates for the different treatments are presented in Figure 4.3. ANOVA (Table 4.1) revealed a significant Time effect on the release of FFA, but no Treatment effect or Treatment × Time interaction. In case of the main effect Time, post hoc testing showed that the concentration of FFA increased significantly within the first 10 min to 1.8±0.2, 2.5±0.4 and 2.6±1.2 mM for the treatments ho, hs and uhs, respectively and stayed constant during the rest of the experiment. Furthermore, when considering the other lipolytic products, only traces of MG production were found with no significant difference between treatments and time of collection. Also Treatment × Time interaction was not significant. When considering the formation of DG, we found significant effects of Treatment and Time, but no Treatment × Time interaction ($p=0.09$). Post hoc, the concentration of DG for uhs was significantly lower compared to ho and hs, but for all treatments the concentration increased significantly after application compared to baseline. There was a Treatment and Time effect and a Treatment × Time interaction on the concentration of triglycerides (TG). Post hoc analysis revealed that for all treatments the concentration of TG was significant higher at all time points compared to baseline and decreased with the increase of time. Furthermore, when evaluating the Treatment × Time interaction uhs had, for example, similar TG concentration compared to ho at 30 min but not at 45 min, where in fact the TG were significantly lower (supplementary, Table S4.2). The results of the lipolytic products indicate similar behavior observed between ho and hs.

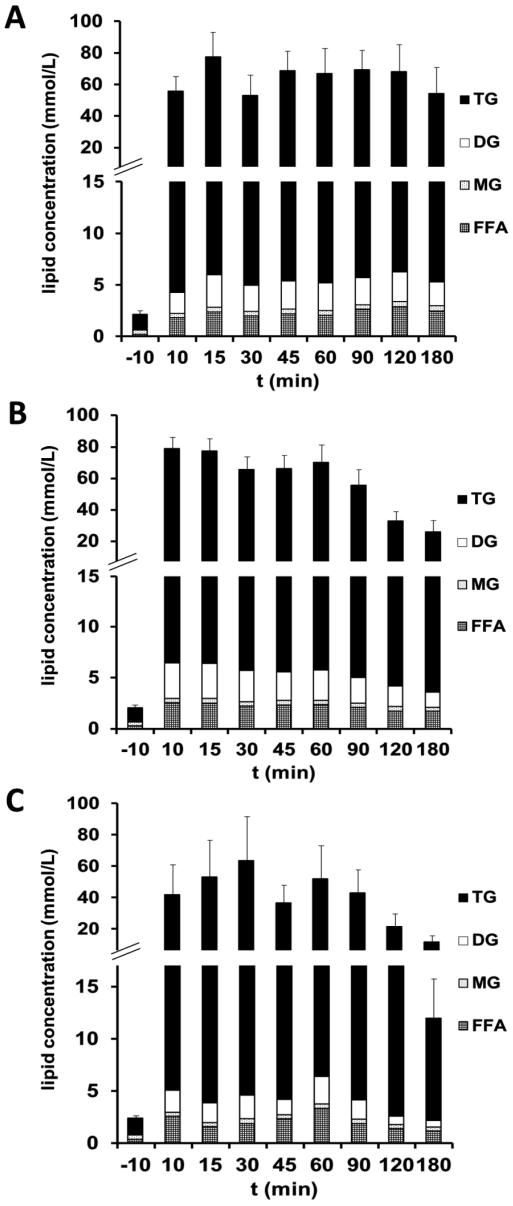

Fig. 4.3 Lipid composition, i.e. TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid, of gastric aspirates as determined by gas chromatography during 180 min of Experiment 1after the treatments ho (A), hs (B) and uhs (C). Values are expressed as mean and error bars indicate the respective standard errors for TG.

The level of lipolysis were calculated by using data in Figure 4.3. The level of gastric lipolysis (L_1%) did not exceed 2.8 % for all treatments and time points (data not shown). Also, the molar ratios of FFA/DG were found to be about 1, which is typical for the hydrolysis by human gastric lipase.

4.3.2 Duodenal intubation study

Appearance of the emulsion

For two persons it was not possible to draw samples between t=15 and t=60 min for the treatment hs. All other samples obtained from the duodenum were transparent before ingestion and became turbid after ingestion of the homogenized emulsions (no further data shown). During digestion of the un-homogenized emulsions, the samples stayed clear. Homogenized emulsions coalesced immediately at t=15min (Fig. 4.1C). For uhs no droplets were found (no further data shown).

The initial pH in the duodenum was 6.4 ± 0.3 (Fig. 4.5B) and ANOVA revealed no Time, Treatment effect or Treatment × Time interaction on duodenal pH (Table 4.1). Similarly to the gastric intubation study, the water phase emptied faster from the stomach for uhs compared to hs as determined from plasma paracetamol concentrations (no further data shown).

Lipid composition

Figure 4.4 shows the lipid composition determined in duodenal aspirates. ANOVA revealed main effects of Treatment and Time on the concentration of FFA, but no Treatment × Time interaction (p=0.08) (Table 4.1). Thus, the concentrations of FFA were higher for hs compared to uhs. Furthermore, there was a Time effect and a Treatment × Time interaction on MG, but no Treatment effect. There was a significant Treatment effect and a Treatment × Time interaction on the concentration of DG but no Time effect (p=0.07). There was an effect of Time and a Treatment × Time interaction on TG, but no Treatment effect (p=0.15). Comparing hs with uhs, post hoc analysis revealed higher DG concentration in the duodenal aspirates at 15 min for hs than for uhs (Supplementary, Table S4.3). In particular, for hs there were significant concentrations of FFA at 30, 60 and 120 min, of MG at 45 and 60 min, of DG at 15 min and of TG at 120 min present compared to baseline. The higher TG concentration found at t=120 min compared to baseline

suggests the entry of a cream layer formed in the stomach. For uhs, there were significant amounts of FFA and MG at 60 min present in the duodenal aspirates compared to baseline but there were no significant amounts of DG or TG for all time points.

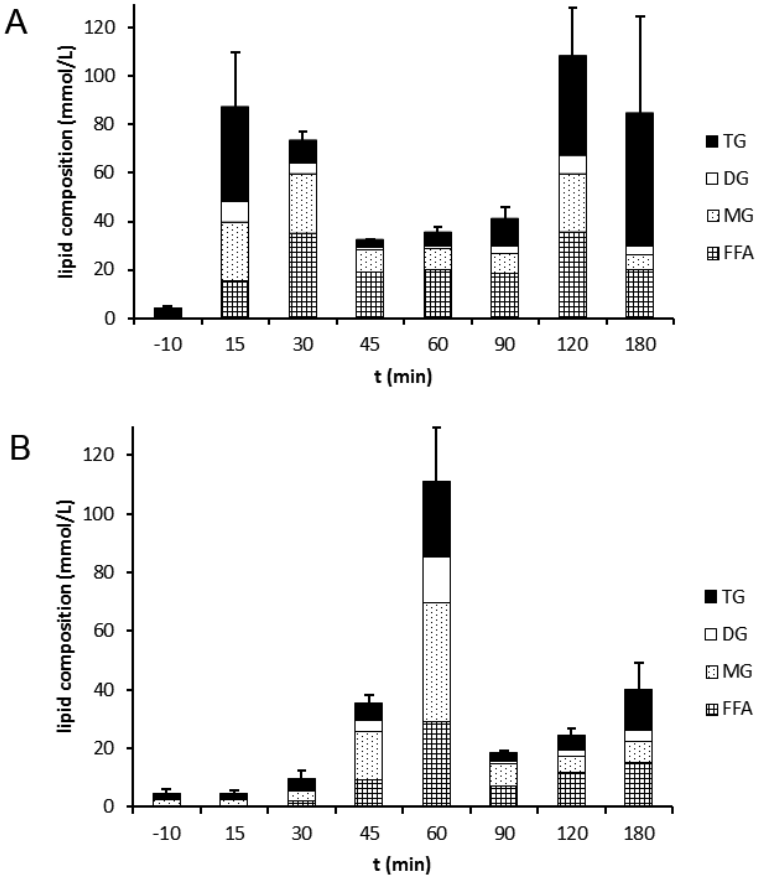

Fig. 4.4 Lipid composition, i.e. TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid, of duodenal aspirates as determined by gas chromatography during 180 min of Experiment 2 after the treatments hs (A) and uhs (B). Values are expressed as mean and error bars indicate the respective standard errors for TG.

The lipase activity is shown in Fig. 4.5A. There was a Time effect and Treatment × Time interaction on lipase activity (Table 4.1). Post hoc analysis revealed no significant increase of lipase activity for both treatments compared to baseline. For uhs, there was a

significant increase at t=60 and t=180 min compared to 15 min indicating a later response to the treatment uhs compared to hs.

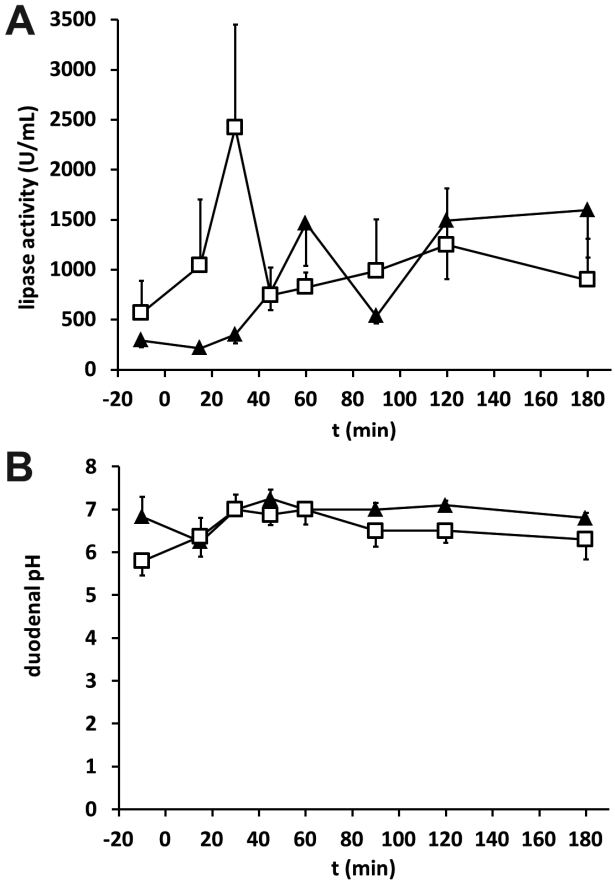

Fig. 4.5 Lipase activity obtained in duodenal aspirates (A) and pH-profile (B) during 180 min of Experiment 2 after the treatments hs (□) and uhs (▲).Values are expressed as mean ± SE.

4.3.3 T-peak and t-onset

To ascertain if we could observe difference in timed responses of measures in relation to the treatments, peak optimum times (t-peak) and peak onset times (t-onset) were extracted from fitted curves. A typical example for such a Weibull fit (and the indications for t-peak and t-onset) is shown in Fig. 4.6. ANOVA results are shown in Table 4.2.

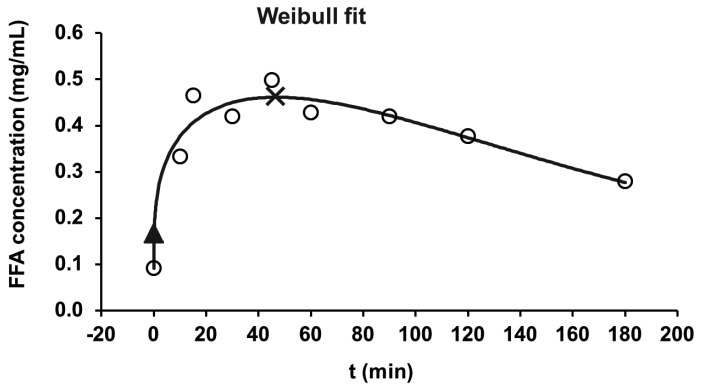

Fig. 4.6: Example of a typical Weibull fit of released free fatty acids (FFA) during 180 min for an individual subject, with peak onset time (▲), peak time (×) and the line which indicates the model fit.

For example, there was a significant Measure effect and a Measure× Treatment interaction on t-peak and a significant Measure effect on t-onset. Post hoc analysis revealed that in the gastric intubation study t-peaks were early for FFA, DG, TG and pH at approx. 50 min and this was not different between the treatments (Fig. 4.7A). Compared to this, t-peak for MG was significantly later at approx. 170 min. Only for plasma paracetamol concentration t-peak for uhs was earlier than for ho and hs. There was no difference for t-onset. In the duodenal intubation study, t-peaks for lipids were earlier for uhs than for hs (Fig. 4.7B).

Table 4.2 Effect of Measure on t-peak and t-onset, emulsions used as Treatment, and Measure × Treatment interaction on t-peak and t-onset.

dependent variables	Measure		Treatment		Measure x Treatment	
	F (df_1,df_2)	Sig	F (df_1,df_2)	Sig	F (df_1,df_2)	Sig
t-peak§ gastric	7.35 (5,140)	***	3.13 (2,140)	Ns	2.14 (10,140)	***
t-onset§ gastric	1.52 (5,140)	Ns	1.22 (2,140)	Ns	0.83 (10,140)	Ns
t-peak# duodenal	1.45 (5,25)	Ns	1.25 (1,25)	Ns	1.06 (5,25)	Ns
t-onset# duodenal	1.32 (5,25)	Ns	1.14 (1,25)	Ns	0.66 (5,25)	Ns

Significant differences are indicated with asterisks: *p<0.05, **p< 0.01, ***p < 0.001, based on ANOVA analysis. Ns is not significant.
§ includes measures pH-GA, FFA-GA, MG-GA, DG-GA, TG-GA and plasma paracetamol concentration; GA, gastric aspirate
# includes measures pH-DA, FFA-DA, MG-DA, DG-DA, TG-DA and lipase activity; DA, duodenal aspirate

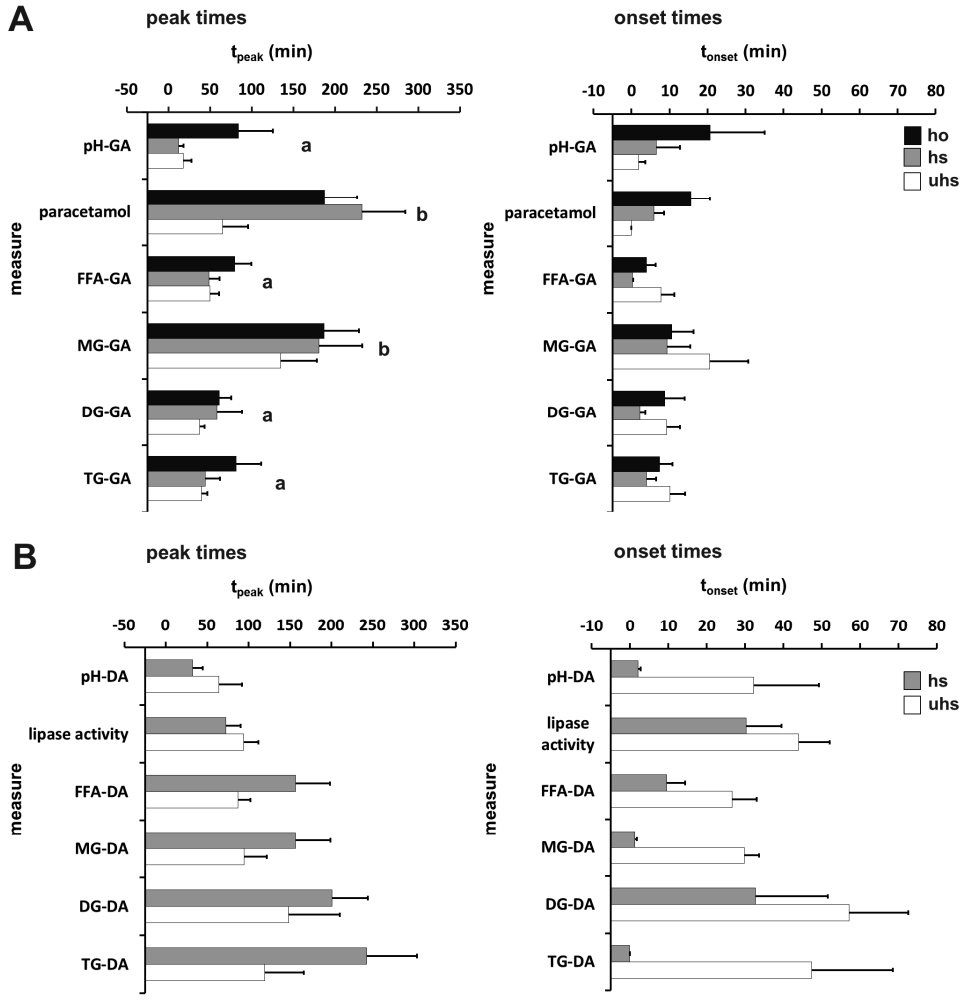

Fig. 4.7 Comparison of peak times and onset times of timed responses (A, gastric intubation study) for gastric pH, plasma paracetamol concentration, lipids in gastric aspirates (GA), i.e. TG, triglyceride; DG, diglyceride; MG, monoglyceride; FFA, free fatty acid; for the treatments ho, hs and uhs and (B, duodenal intubation study) for lipase activity and lipids in duodenal aspirates (DA), i.e. TG, DG, MG, FFA for the treatments hs and uhs. Values are expressed as mean ± SE from 15 (A) or 6 (B) people, respectively. Different low letters indicate a significant difference at $p<0.001$.

4.4 Discussion

The small intestine is the most important site for fat digestion and absorption. Thus, fat-related physiological responses are often investigated by local perfusion of fat in the small intestine (Little et al., 2007). However, such mechanistic studies neglect the effects of fat in the oral cavity and digestion in the gastric compartment on overall fat digestion and fat-related physiological responses. Therefore, in the present study, the oral administration of an emulsion load was included and compared to the intragastric infusion of the same emulsion.

4.4.1 Effect of oral processing on emulsion behavior

When comparing the oral processing with the intragastric infusion of the homogenized emulsions, the results showed that oral exposure had no additional effect on the gastric lipolysis, the structure of the emulsions or the pH-profile. In addition, we did not observe differences in the rate of emptying of the homogenized emulsions between the oral and the intragastric administration as determined by plasma paracetamol concentrations. To our knowledge this has not been reported before. The effect of oral exposure in relation to hormonal responses and satiety feelings will be discussed in Chapter 5.

4.4.2 Effect of intragastric distribution on gastric and duodenal lipolysis

To investigate the effect of intragastric distribution of lipids, i.e. present as fine emulsion droplets or as oil layer in the stomach, on lipolysis a homogenized (hs) and an un-homogenized emulsion (uhs) were used, respectively, both having the same composition and amount of calories. Although gastric lipolysis was similar for both treatments and gastric lipolysis was not influenced by the distribution of the lipids there were overall significant higher concentrations of FFA and for DG within the first 15 min present in duodenal aspirates for hs compared to uhs. It is known that gastric lipolysis and the subsequent release of FFA enhance pancreatic lipase activity (Gargouri et al., 1986; Borel et al., 1994) and the formed DG will be hydrolysed faster than TG (Phan & Tso, 2001). This means that the low concentration of FFA released from the stomach for hs was sufficient to increase pancreatic lipase activity and, therefore, led to a fast

duodenal hydrolysis with subsequent FFA release. Probably, this fast hydrolysis contributed to a delay in gastric emptying for hs as indicated by the plasma paracetamol concentration (Fig. 4.2B). In contrast, plasma paracetamol concentrations indicated a fast gastric emptying of the water phase from uhs within 45 min (Fig. 4.2B, Fig. 4.7A). Thus, the lower concentrations of FFA in uhs compared to hs were not sufficient to reduce gastric emptying within the first 45 minutes. However, for uhs we observed highest FFA concentrations in duodenal aspirates at t=60 min (Fig. 4.4B) and the modeled peak time was at approx. 85 min (Fig. 4.7B). This suggests the start of emptying of the lipid layer within this time frame for uhs and resulting lipolysis, despite the fact, that the surface area of the lipid layer is small.

4.4.3 Effect of creaming or phase separation on gastric lipid emptying

The emulsions infused in the case of hs were Tween-stabilized emulsions. They are considered to be stable emulsions in the acidic gastric environment (Marciani et al., 2007; Marciani et al., 2008) which do not cream and remain its initial droplet size. Surprisingly, we found significantly high concentrations of TG in the duodenal aspirates at t=120 min (Fig. 4.4A) whereas for all other time points the lipolytic products FFA, MG and DG were dominant. This observation could be explained by a cream layer formed in the stomach, which is composed of a higher TG concentration and will enter the duodenum at this time range. Recently we have observed an increase in TG concentration after the digestion of a Tween 80-stabilized emulsion at t=150 min in a dynamic digestion model (Oosterveld et al., unpublished data). In this model Tween 80-stabilized emulsions were found to cream in the stomach compartment. Similar to our study, slight coalescence has been observed for Tween 80-stabilized emulsions under simulated gastric conditions (van Aken et al., 2011). Although a high concentration of substrate is present at t=120 min, the action of lipase is not increasing and the ratio of TG/FFA is 1.1 at t=120 min. It has been described that pancreatic lipase secretion decreases despite on-going nutrient delivery to the duodenum within 180 min after meal intake (Fried et al., 1988; Keller et al., 1997; Schwizer et al., 1997). Since lipase action will also be reduced by a smaller surface area of the droplets in the cream phase, these factors together lead to less formation of FFA. As no further lipolytic products might be formed, un-hydrolysed TG present in the cream layer will enter the duodenum. Such an effect might also be seen for the treatment uhs after the 180 min of experiment, since we did not find high TG concentration in duodenal aspirates

following the rapid emptying of the water phase. It is likely that most of the oil phase of the un-homogenized emulsion is still present in the stomach and thus will enter the duodenum at a later stage. Both a cream and a remaining oil phase might affect responses to the intake of a second meal. For instance, it has been observed that lipids ingested at breakfast contributed to an increase in TG plasma concentration after intake of a lunch (Maillot et al., 2008). Additionally, CCK plasma concentration was higher after the preload of a water-oil-layered meal compared to an emulsified meal followed by intake of a second meal (Foltz et al., 2009). However, it is not clear yet if this effect derives from stored lipids in the enterocytes or from that present in the gut. Our study supports the possibility that the second meal stimulates the remaining oil layer to empty from the stomach (Foltz et al., 2009).

4.5 Conclusions

The study showed no effect of oral processing but a clear effect of the degree of emulsification on emulsions behavior. In detail, we observed initial high intraduodenal lipid concentrations for the homogenized emulsion. This in turn resulted in faster release of FFA and delayed gastric emptying compared to the un-homogenized emulsion. Furthermore, none of the treatments had a pronounced effect on gastric lipolysis. Despite a low gastric lipolysis and the fact, that in case of the un-homogenized emulsion only a small surface area was available, hydrolysis was high when TG emptied into the duodenum.

4.6 Supplementary data

Table S4.1 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on plasma paracetamol concentration (µg/mL) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment		
	ho	hs	uhs
-10	0.10 ± 0.07^{a}	0.09 ± 0.09^{a}	0.06 ± 0.06^{a}
10	1.61 ± 0.28^{b}	2.53 ± 0.52^{bcd}	6.82 ± 1.13^{fg}
15	1.74 ± 0.21^{bc}	2.94 ± 0.61^{bcd}	8.52 ± 1.27^{g}
30	3.19 ± 0.66^{bcd}	3.46 ± 0.56^{cde}	7.08 ± 0.83^{fg}
45	3.81 ± 0.74^{de}	3.62 ± 0.66^{cde}	6.30 ± 0.73^{fg}
60	4.38 ± 0.75^{def}	3.67 ± 0.63^{de}	5.31 ± 0.57^{efg}
90	4.74 ± 0.68^{def}	4.27 ± 0.50^{def}	4.40 ± 0.49^{def}
120	5.47 ± 0.56^{efg}	4.18 ± 0.44^{def}	4.47 ± 0.40^{defg}
180	5.23 ± 0.42^{fg}	4.21 ± 0.35^{def}	3.72 ± 0.20^{def}

Table S4.2 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on TG concentration in gastric aspirates (mg/mL) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment		
	ho	hs	uhs
-10	1.31 ± 0.22^{a}	1.16 ± 0.22^{a}	1.33 ± 0.19^{a}
10	45.38 ± 8.25^{efghi}	64.11 ± 6.46^{i}	32.66 ± 16.74^{bc}
15	63.24 ± 13.88^{ghi}	62.73 ± 6.95^{i}	43.72 ± 20.66^{bc}
30	42.49 ± 11.43^{defghi}	53.05 ± 7.04^{hi}	52.29 ± 24.62^{bcde}
45	55.87 ± 10.95^{fghi}	53.64 ± 7.55^{ghi}	28.85 ± 9.72^{bc}
60	54.50 ± 14.22^{fghi}	57.02 ± 9.90^{ghi}	40.65 ± 18.26^{bcdef}
90	56.31 ± 10.79^{ghi}	44.82 ± 8.84^{fghi}	34.68 ± 12.81^{bc}
120	54.67 ± 15.26^{efghi}	25.51 ± 5.29^{cdefgh}	16.96 ± 7.02^{bc}
180	43.15 ± 14.76^{cdefg}	19.79 ± 6.48^{bcd}	8.67 ± 3.35^{ab}

Table S4.3 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on DG concentration in duodenal aspirates (mg/mL) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment	
	hs	uhs
-10	0.07 ± 0.01^{ab}	0.07 ± 0.02^{a}
15	7.45 ± 2.85^{b}	0.10 ± 0.01^{a}
30	4.06 ± 0.83^{ab}	0.25 ± 0.08^{a}
45	0.78 ± 0.23^{ab}	3.41 ± 2.29^{ab}
60	1.21 ± 0.38^{ab}	13.67 ± 10.10^{ab}
90	2.67 ± 0.92^{ab}	0.70 ± 0.39^{ab}
120	6.64 ± 2.65^{ab}	1.76 ± 1.53^{ab}
180	3.48 ± 1.25^{ab}	3.64 ± 1.36^{ab}

Table S4.4 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on TG concentration in duodenal aspirates (mg/mL) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment	
	hs	uhs
-10	2.16 ± 0.69^{a}	1.96 ± 0.81^{a}
15	34.21 ± 19.97^{ab}	1.66 ± 0.59^{a}
30	7.94 ± 3.41^{ab}	3.51 ± 2.10^{a}
45	2.60 ± 0.33^{ab}	5.20 ± 2.43^{ab}
60	4.94 ± 1.73^{ab}	23.06 ± 15.73^{ab}
90	10.00 ± 4.01^{ab}	2.49 ± 0.63^{ab}
120	36.15 ± 17.67^{b}	4.46 ± 2.01^{ab}
180	48.11 ± 35.63^{ab}	12.31 ± 7.71^{ab}

Table S4.5 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on lipase activity in duodenal aspirates (U/mL) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment	
	hs	uhs
-10	567.30 ± 324.15^{ab}	293.90 ± 66.31^{ab}
15	1043.99 ± 659.79^{ab}	215.75 ± 19.06^{a}
30	2424.38 ± 1028.31^{ab}	351.31 ± 89.19^{b}
45	745.66 ± 275.89^{ab}	780.54 ± 183.41^{ab}
60	825.91 ± 148.81^{ab}	1464.06 ± 425.38^{b}
90	988.95 ± 513.35^{ab}	537.17 ± 71.24^{ab}
120	1248.61 ± 563.65^{ab}	1492.93 ± 584.66^{ab}
180	895.98 ± 412.17^{ab}	1597.76 ± 477.19^{b}

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Chapter 5

Effect of oral administration and intragastric distribution of lipids on satiety responses in humans

To be submitted

Abstract

Significantly higher FFA concentration were present in duodenal aspirates in humans after the intragastric infusion of homogenized emulsion compared to a sample where water infusion was followed by oil within the first 30 min after the application (Chapter 4). In this Chapter, we aimed to investigate if such imposed effects would influence satiety responses. In short, 15 volunteers received a homogenized emulsion orally or by gastric infusion or gastric infusion of water followed by the oil phase (un-homogenized sample). In a subset 6 volunteers received gastric infusion of a homogenized emulsion or of an un-homogenized sample. Gallbladder volume, plasma CCK, PYY, GLP-1 and ghrelin concentration, satiety ratings and *ad libitum* intake were analysed. Gallbladder contraction was strongest for the oral application of the homogenized emulsion and lowest for the gastric infusion of the un-homogenized sample. Plasma CCK and PYY concentrations were significantly higher for homogenized emulsions compared to the un-homogenized sample. The response of PYY and GLP-1 to the un-homogenized sample was delayed compared to the homogenized emulsion. However, unexpectedly, there were no differences in ghrelin responses, satiety ratings and *ad libitum* intake between the treatments. In conclusion, intragastric distribution of lipids influenced plasma hormone concentrations and gallbladder motility, but had no effect on satiety as assessed via a VAS. Also, there was no correlation between duodenal concentration of FFA and CCK plasma concentration. As a tentative explanation, we postulate that hormonal induced satiety feelings might have been overruled by other mechanisms such as gastric distention.

5.1 Introduction

Food intake is normally regulated by signals indicating start, i.e. hunger, and stop, i.e. satiety. Hence, understanding these signals could help to understand how to regulate food intake. Lipids are a key component of the daily diet and they are mostly present in food in form of emulsions. The importance of the lipid phase on metabolic responses has been highlighted recently (Golding et al., 2011; Marze & Choimet, 2012; Keogh et al., 2011). For instance, the inclusion of solid fat has been used to introduce partial coalescence and these emulsions had a prolonged effect on gastric emptying *in vivo*, but no influence on satiety ratings (Keogh et al., 2011). Furthermore, it has been observed that the emulsion droplet size affects the release of satiety hormones, such as cholecystokinin (CCK), the appetite sensation and antropyloroduodenal motility when presented intraduodenal (Seimon et al., 2009). It was shown that this effect was larger for small droplets compared to large droplets. It is also known that the release of free fatty acids (FFA) from small droplets is higher compared to large droplets (Armand et al., 1992; Borel et al., 1994; Armand et al., 1999; Helbig et al., 2012). In addition, it has been reported that FFA presented in the stomach were able to trigger CCK responses (McLaughlin et al., 1999; Little et al., 2007). CCK was correlated to a higher feeling for fullness, a decreased gastric emptying and a suppressed energy intake (Little et al., 2007). Gastric emulsions stability, i.e. the persistence of small, homogenous distributed emulsions droplets in the gastric environment is, therefore, thought to enhance satiety signals, and thus reduce overconsumption. In fact, an intragastric stable emulsion led to higher CCK concentration and higher gallbladder contractions (Marciani et al., 2007a), and increased fullness feeling, reduced hunger and appetite feeling compared to an unstable emulsion (Marciani et al., 2009). In these studies, MRI was used to monitor intragastric emulsions behaviour and emptying of intragastric stable and unstable emulsions (Marciani et al., 2007a; Marciani et al., 2009). However, the contribution of gastric and intestinal lipolysis and the resulting gastric and duodenal lipolytic products could not be established. Although knowledge on the gastric behaviour of emulsions *in vitro* is increasing, the relations to satiety responses *in vivo* are still not fully understood. For example, layering of fat in the stomach resulted in a fast emptying of the aqueous phase and a delayed emptying of the fat layer into the duodenum (Foltz et al., 2009). However, in that study the increase in CCK and lipid absorption was not consistent with changes of the subjective feelings for hunger, satiety and fullness. In Chapter 4 we showed that significantly higher FFA concentrations were present in duodenal aspirates after the intragastric infusion of an emulsion compared to a

sample where water infusion was followed by oil. In the present chapter the objective is to link changes in, for example, the FFA concentration to metabolic and satiety responses, such as gallbladder contraction, CCK release, satiety perceptions and *ad libitum* intake.

Furthermore, as modified sham feeding has been shown to stimulate gallbladder contraction (Witteman et al. 1993), reduce plasma ghrelin (Heath et al., 2004) and to increase satiety (Smeets & Westerterp-Plantenga, 2006), we investigated the effect of oral stimulation of fat on gallbladder volume, hormone release, i.e. CCK, ghrelin, peptide tyrosine-tyrosine (PYY) and glukagon-like peptide-1 (GLP-1) and satiety perceptions.

5.2 Materials and methods

5.2.1 Emulsion preparation

Emulsions were produced following good manufacturing practice in a food grade pilot plant of NIZO food research (Ede, The Netherlands). Tween 80 (Lamesorb©SMO20, Cognis, Monheim am Rhein, Germany) solutions were prepared by dissolving powder in 0.01 M NaCl solution. The final emulsions contained 8% (w/w) sunflower oil (Reddy sunflower oil, Vandermoortele, Izegem, Belgium) and 0.4% (w/w) Tween 80. All ingredients used were food grade. In total 3 batches of each solution were prepared. Each batch consisted of 10 L (20 x 500 mL) of each solution. Emulsions were homogenized at 80°C at a pressure of 70 to 180 bar followed by homogenization at 0 to 20 bar (Rannie Lab12-16.5, Leuze, Waardenburg, The Netherlands). The solutions for the un-homogenized samples, i.e. Tween 80-solution and sunflower oil, were heated separately at 80°C. These samples were not homogenized and applied separately but for the ease of the reading we refer to them as emulsions. Homogenized and un-homogenized emulsions were then sterilized in a Combitherm sterilizer (Minister 1987, AlfaLaval, Lund, Sweden) for 7 seconds at 141°C. After cooling to room temperature, solutions were filled aseptically in sterilized plastic containers and stored <7°C in a dark place until the start of the experiments in order to prevent off-flavor formation. Of every batch 2 samples were tested for sterility. During the course of the experiment fresh emulsions were prepared every 6-10 weeks in order to prevent excessive off-flavor formation or formation of physical instabilities. The surface-weighted-mean- diameter (d_{32}) and the volume-weighted-mean diameter (d_{43}) of the freshly prepared homogenized emulsions were 2.33

and 8.75 μm, respectively, as determined by dynamic light scattering (Mastersizer Hydro 2000S, Malvern Instruments, Malvern, UK) at room temperature.

During the production of the emulsions, 0.034% (w/w) and 0.012% (w/w) of vanilla powder and saccharine, respectively, were added to the Tween 80 solutions. As a result, the test drinks tasted pleasantly sweet with a sweet vanilla flavor having a somewhat bitter off-note. On the days of intervention, 1g of paracetamol (Apotheek van Thoor, Maastricht, The Netherlands) was manually added to the homogenized emulsion or Tween 80 solution in the case of the un-homogenized emulsions to measure gastric emptying of the water phase.

5.2.2 Subjects

15 healthy volunteers participated in the study (11 male and 4 female, age 28±8 y, body mass index of 22.7±2.3 kg/m2). A short physical examination and a cognitive restraint eating behaviour using a Dutch translation of the Three Factor Eating Questionnaire (TFEQ) were performed. Participants did not have gastrointestinal or hepatic disorders or previous major abdominal surgery interfering with gastrointestinal function. They were non-smokers, moderate alcohol consumers (≤ 10 alcoholic consumptions per week), non-medicated except of using contraceptives and non-restraint eaters according to the Three Factor Eating Questionnaire (TFEQ Factor 1 score ≤ 9). The study was approved by the Medical Ethics Committee of the Maastricht University Medical Center, conducted according to the principles of the Declaration of Helsinki and in accordance with the Medical Research Involving Human Subjects Act (WMO). All participants signed informed consent. Volunteers were informed that they receive a lipid-containing drink and that its behaviour in the stomach and duodenum as well as the resulting physiological responses were investigated.

5.2.3 Study design

The study was a randomized crossover design and divided in two experiments. All experiments took place on separate days. Subjects were requested to refrain from any food or beverage intake with the exception of water from 2200h the evening before the test day. Subjects received a total load of 500 mL of samples with a total energy content of 360 kcal.

Experiment 1: Gastric intubation study

On all 3 test days of the gastric intubation study, subjects arrived in the morning at 0830h in fasted state. After local anesthesia of the nasal mucosa using lidocaine 10% spray, each subject was intubated with a double-lumen nasogastric feeding catheter (Bengmark® Nutricia, Amsterdam, The Netherlands). One lumen was used to inject emulsions into the stomach and the other lumen was used to collect stomach fluid by aspiration using a standard 10-mL syringe. The position of the nasogastric catheter was controlled by determination of the pH of the aspirate. After intubation, a cannula was placed in a superficial antecubital vein of the forearm for blood sampling. At 0900h, subjects received the emulsions infused into the stomach either homogenized (hs) and in case of the un-homogenized emulsions (uhs), the infusion of the water phase was followed by the oil phase within 2 min. In the case of the oral administration, the emulsions were homogenized (ho) and drunk within 2 min.

Experiment 2: Duodenal intubation study

As explained in Chapter 4, six volunteers, who participated in Experiment 1, joined on two further occasions (duodenal intubation study), were intubated with a double-lumen naso-duodenal catheter and received the emulsions un-homogenized (uhs) or homogenized (hs). Samples from duodenal aspirates were drawn (Chapter 4) and, as defined in the following section, samples from blood were collected and satiety ratings as well as *ad libitum* intake was determined. These data were only used to correlate Experiment 1 with Experiment 2 as described in 5.3.4. All other data were obtained from Experiment 1.

5.2.4 Measurements

Gallbladder volume

Gallbladder volumes were measured in volunteers by real-time ultrasonography (3.5 MHz transducer, Technos Scientific, Concord, ON, Canada) at baseline (t=-10 min) and at 10, 15 min and then every 15 min until 180 min after application of the emulsions. The assumptions and the mathematical formula used to calculate gallbladder volume have been described and validated by Hopman and coworkers (Hopman et al., 1985). In short, gall bladder volumes were calculated by the sum of cylinders method from real-time

sonograms. This was done automatically by the computer connected to the echoscope according to the following equation, where n is the number of cylinders, d_i is the diameter of individual cylinders and h_i is the height of individual cylinders:

$$V = \sum_{n=i}^{n} \pi * \frac{di^2 hi}{4}$$

In this method, the longitudinal image of the gallbladder was divided into series of equal height cylinders, which have a diameter perpendicular to the longitudinal axis of the gallbladder image. The sum of volumes of these separate cylinders was the uncorrected volume. A correction factor was calculated from the longitudinal and transversal scans of the gallbladder to correct for the displacement of the longitudinal image from the central axis. The gallbladder volume was then calculated by multiplication of the uncorrected volume with the square of the correction factor.

Blood samples

During the test day, blood samples were taken at baseline (t=-10 min) and at 10, 15, 30, 45, 60, 90, 120 and 180 min after application of the emulsions. After sampling, physiological salt solution (NaCl 0.9 % w/v) and heparine were injected to prevent coagulation and occlusion of the catheter. Blood samples for ghrelin analysis were drawn in EDTA tubes. EDTA tubes for cholecystokinin (CCK) and peptide YY (PYY) contained aprotinin (Trasylol, Bayer, Leverkusen, Germany) at a concentration of 500 kallikrein inhibitor units (KIU)/mL. Glucagon-like peptide 1 (GLP-1) was collected in special tubes containing dipeptidyl peptidase IV (BD™ P700 Blood Collection and Preservation System for GLP-1 Analysis, BD, Franklin Lakes, NJ, USA). All samples were kept on ice and all tubes were centrifuged at 1500 × *g* for 10 min at 4°C within one hour. Aliquots of the supernatant plasma samples were stored at -80°C until further analysis. RIA-kits were used to analyse ghrelin (i.e. Human ghrelin (Total) RIA), PYY (i.e. Human PYY (Total) RIA) and GLP-1 (i.e. Glucagon Like Peptide-1 (Total) RIA). These were obtained from Millipore (Millipore, St. Charles, Missouri, USA) and kits to analyse CCK were from Eurodiagnostica (EURIA-CCK, Eurodiagnostica, Malmö, Sweden). The hormones were analysed at TNO Triskelion (Zeist, The Netherlands) using the RIA-kits according to the manufacturer's instructions with minor modifications, optimized by TNO Triskelion, for better performance and lower detection limits (unpublished data).

Satiety ratings and ad libitum intake

Scores for satiety feelings (i.e. fullness, satiety, desire to eat, hunger) were measured using Visual Analogue Scales (VAS) anchored with 'not at all' and 'extremely' at baseline (t=-10 min), at t=30 min and then every 30 min until 180 min after application of the emulsions. Volunteers were asked to indicate on a line of 10 cm which place on the scale best reflected their feeling at that moment. The distance from 0 cm to the indication mark was measured in cm.

After taking the last sample at t=180 min, subjects were extubated and received 30 min thereafter a lunch, which was prepared according to Veldhorst (Veldhorst et al., 2009). The food provided for lunch was weighed before and after eating. The energy intake (EI) was calculated by multiplying the amount of food consumed by the energy value of the food as indicated by the product label (11.4 kJ/g).

Statistics

Data were transformed and statistical testing was carried out as described in Chapter4 with some minor modifications as follows. Response curves of dependent measures were modelled by fitting Weibull functions as described in Chapter 4 and onset times and peak times were determined for hormones, gallbladder volume and satiety ratings. Multi-factor ANOVA was used to test for significant effects of Time (fixed factor, t=-10", "t=10", "t=15", "t=30", "t=45", "t=60", "t=90", "t=120", "t=180" for hormones including "t=75", "t=105", "t=135", "t=150" "t=175" for gallbladder volume and "t=-10", "t=30", "t=60", "t=90", "t=120", "t=150", "t=180" for satiety ratings), Treatment (fixed factor, "ho", "hs", "uhs"), Time × Treatment interaction and, in the case of onset and peak times, of Measure (fixed factor, "hormones", "gallbladder volume" and "satiety rating"), Treatment (fixed factor,"ho", "hs", "uhs") and Measure × Treatment interaction. A significant effect was followed by a multiple pairwise post hoc test (Tukey HSD test) and in the case of onset and peak times Within-categories of Measure were compared. In addition, data of experiment 1 and 2 was correlated using Multi-factor ANOVA to test for significant effects of Experiment × Treatment, Experiment × Time and Experiment × Treatment × Time interaction on plasma paracetamol concentration, hormone release, satiety responses and *ad libitum* intake. These data were obtained from the 6 people who participated in both experiments for the treatments hs and uhs.

5.3 Results

5.3.1 Gallbladder motility

The ANOVA results are shown in Table 5.1. There was a significant effect of Treatment and Time on gallbladder volume, but no Treatment × Time interaction (p=0.07). Initial gallbladder volumes were 19.2±1.2 mL (Fig. 5.1). Post hoc analysis revealed that gallbladder volumes were lower in the following order ho<hs<uhs indicating a pronounced contraction of the gallbladder for treatment ho compared to hs, whereas gallbladder contraction was barely stimulated by the treatment uhs.

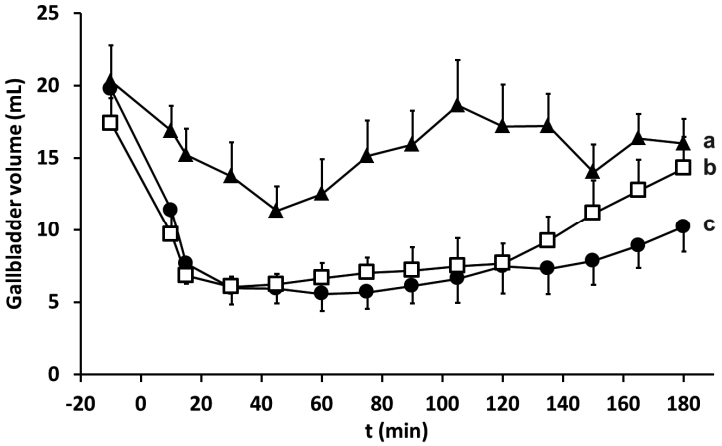

Fig. 5.1 Change of gallbladder volumes in function of time observed during Experiment 1 for the treatments ho (●), hs (□) and uhs (▲).Values are expressed as mean value ± Standard Error (SE). Different low letters indicate a significant difference at p<0.001.

5.3.2 Hormone release

In Figure 5.2 plasma concentrations of CCK, GLP-1, PYY and ghrelin dependent on time and treatment are shown. Initial values of plasma CCK concentration were 0.47±0.03 pmol/L (Fig. 5.2A). ANOVA revealed main effects of Treatment and Time, and a Treatment × Time interaction on plasma CCK (Table 5.1). Further post hoc analysis showed significantly higher plasma CCK concentrations for the treatment ho and hs, when both were compared to uhs (p<0.01). In detail, ho differed at t=10 and t=15 min and hs differed at t=15 min from uhs. In addition, ho induced the longest elevation of plasma

CCK concentrations between 10 and 90 min compared to baseline. For hs, CCK concentration was only elevated at t=10 and t=15 min and for uhs highest concentrations were found at t=45 min compared to baseline. For GLP-1 plasma concentrations, there was a main effect of Time and a Treatment × Time interaction (Table 5.1). For example, the initial GLP-1 plasma concentrations were 7.42±0.85 pmol/L (Fig. 5.2B) and increased significantly for all treatments after application compared to baseline as revealed with post hoc test. Further, there was a significant effect of Treatment and a Treatment × Time interaction on plasma PYY concentration (Table 5.1). Initial values were 82.7±3.2 pg/mL (Fig. 5.2C) and this concentration was significantly elevated between 15 and 180 min for all treatments as revealed post hoc. Also, PYY concentrations differed for uhs from ho and hs at t=30 min. There was no difference between hs and ho. There was a Time but no Treatment effect (p=0.17) or Treatment × Time interaction (p=0.13) on the plasma ghrelin concentration (Table 5.1). The initial amount of 883.4±26.2 pg/mL (Fig. 5.2D) was, as revealed post hoc, significantly decreased between t=30 and 180 min for all treatments. Post hoc results of main Treatment × Time interaction on CCK, GLP-1 and PYY are given in the supplementary (Tables S5.1-S5.3).

Table 5.1 Effect of Time of plasma collection, or on gallbladder contraction or satiety ratings, emulsions used as Treatment, and Time × Treatment interaction on gallbladder volume, plasma hormone concentrations of CCK, GLP-1, PYY and ghrelin, and satiety ratings of fullness, satiety, hunger and desire to eat.

dependent variable	Time		Treatment		Time x Treatment	
	$F (df_1,df_2)$	Sig	$F (df_1,df_2)$	Sig	$F (df_1,df_2)$	Sig
gallbladder volume	18.66 (13,364)	***	14.02 (2,364)	***	1.47 (26,364)	Ns
CCK	22.65 (8,224)	***	6.61 (2,224)	**	3.03 (16,224)	***
GLP-1	42.46 (8,224)	***	1.08 (2,224)	Ns	2.64 (16,224)	***
PYY	37.69 (8,224)	***	2.72 (2,224)	Ns	2.20 (16,224)	**
ghrelin	47.10 (8,224)	***	1.87 (2,224)	Ns	1.42 (16,224)	Ns
fullness	33.20 (6,168)	***	0.59 (2,168)	Ns	1.13 (12,168)	Ns
satiety	17.57 (6,168)	***	0.21 (2,168)	Ns	0.66 (12,168)	Ns
hunger	18.70 (6,168)	***	0.31 (2,168)	Ns	1.78 (12,168)	Ns
desire to eat	23.27 (6,168)	***	0.59 (2,168)	Ns	2.51 (12,168)	**

Significant differences are indicated with asterisks: *p<0.05, **p< 0.01, ***p < 0.001, based on ANOVA analysis. Ns is not significant.

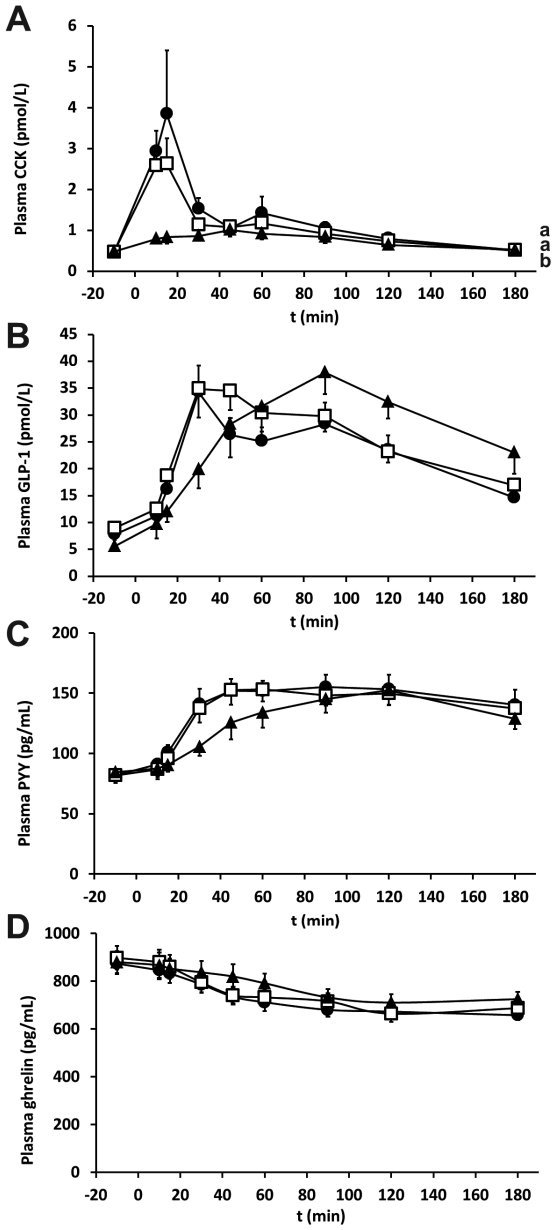

Fig. 5.2 Change of plasma CCK (A), GLP-1 (B), PYY (C) and ghrelin (D) concentration in function of time observed during Experiment 1 for the treatments ho (●), hs (□) and uhs (▲). Values are expressed as mean ± SE. Different low letters indicate a significant difference at $p<0.01$.

5.3.3 Satiety ratings and subsequent energy intake

There was a main effect of Time on all satiety ratings as evaluated with ANOVA (Table 5.1). Treatment × Time interaction was only observed on the rating "desire to eat" but not on "hunger" (p=0.06), "fullness" (p=0.34) and "satiety" (p=0.79). Post hoc analysis on Time revealed that the perception "desire to eat" was significantly decreased at t=30 min for all treatments (Fig. 5.3). Compared to t=30 min, it was significantly increased for hs between 120 and 180 min and for ho between 90 and 180 min, but not for uhs (Table S5.4). For all treatments, "hunger" was significantly decreased between 30 and 60 min compared to baseline. The perceptions "fullness" and "satiety" were significantly elevated between 30 and 90 min and 30 and 60 min, respectively, for all treatments.

Subsequent *ad libitum* energy intake was 2318.1±279.2, 2501.0±178.7 and 2123.3±291.1 kJ for ho, hs and uhs, respectively and there was no difference between the treatments as determined with ANOVA (p=0.32).

5.3.4 Correlation of gastric and duodenal intubation study

Data obtained in the gastric and duodenal intubation study was compared by means of ANOVA for the treatments hs and uhs and was from 6 people who participated in both experiments. In the case that this data, i.e. paracetamol plasma concentrations, hormone concentrations, satiety ratings and *ad libitum* intake, reveals similar outcomes, a correlation between both experiments is considered. There was no difference in plasma paracetamol concentrations obtained in the gastric and duodenal intubation study, i.e. Experiment × Treatment (p = 0.40), Experiment × Time, (p=0.54), and Experiment × Treatment × Time (p = 0.80). Further, there was no difference in hormone concentrations between the Experiments, i.e. Experiment × Treatment × Time interaction for CCK was p = 0.64, for GLP-1 was p = 0.96, for PYY was p = 0.64 and for ghrelin was p = 0.32, respectively. Additionally, satiety ratings and *ad libitum* intake did not differ between the experiments. Hence, conclusions can be drawn by correlating both experiments. We, therefore, correlated gallbladder volume with plasma CCK concentration (both obtained from the gastric intubation study), gallbladder with FFA concentration in duodenal aspirates (the latter was obtained from the duodenal intubation study) and plasma CCK concentration with FFA concentration in duodenal aspirates (Fig. 5.4). With increasing plasma CCK concentration the gallbladder volume was decreasing (Fig. 5.4A). Likewise, with increasing FFA concentration in duodenal aspirates, gallbladder volume was

decreasing (Fig. 5.4B). However, there is no correlation between FFA concentrations in duodenal aspirates and plasma CCK concentration (Fig. 5.4C).

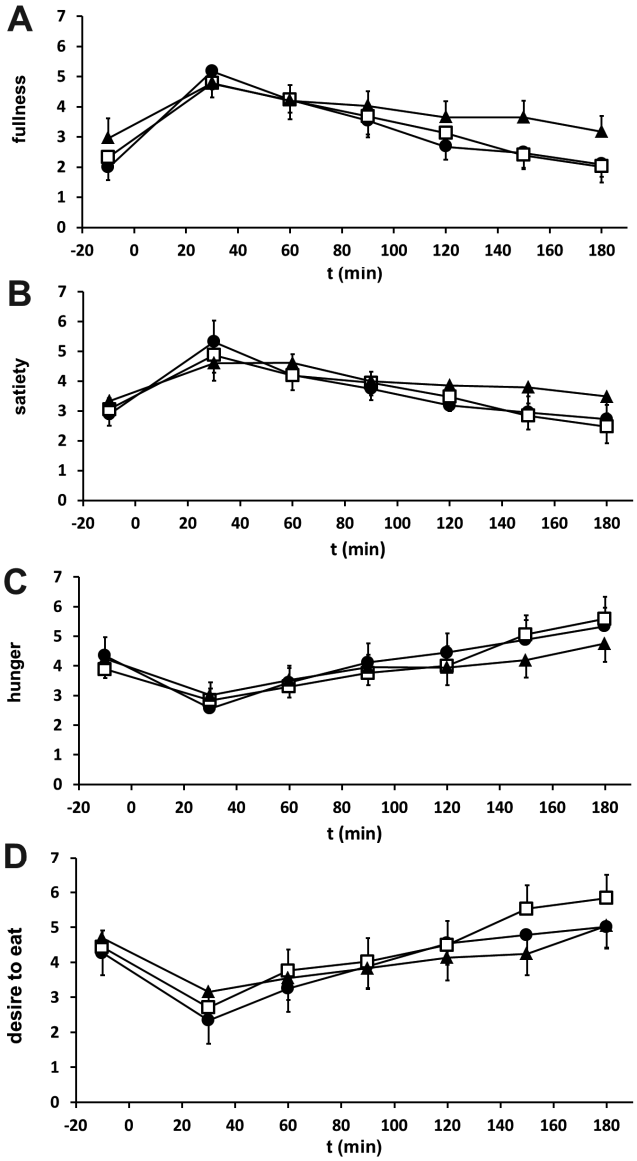

Fig. 5.3 Change of fullness (A), satiety (B), hunger (C) and desire to eat (D) ratings in function of time observed during Experiment 1 for the treatments ho (●), hs (□) and uhs (▲).Values are expressed as mean ± SE.

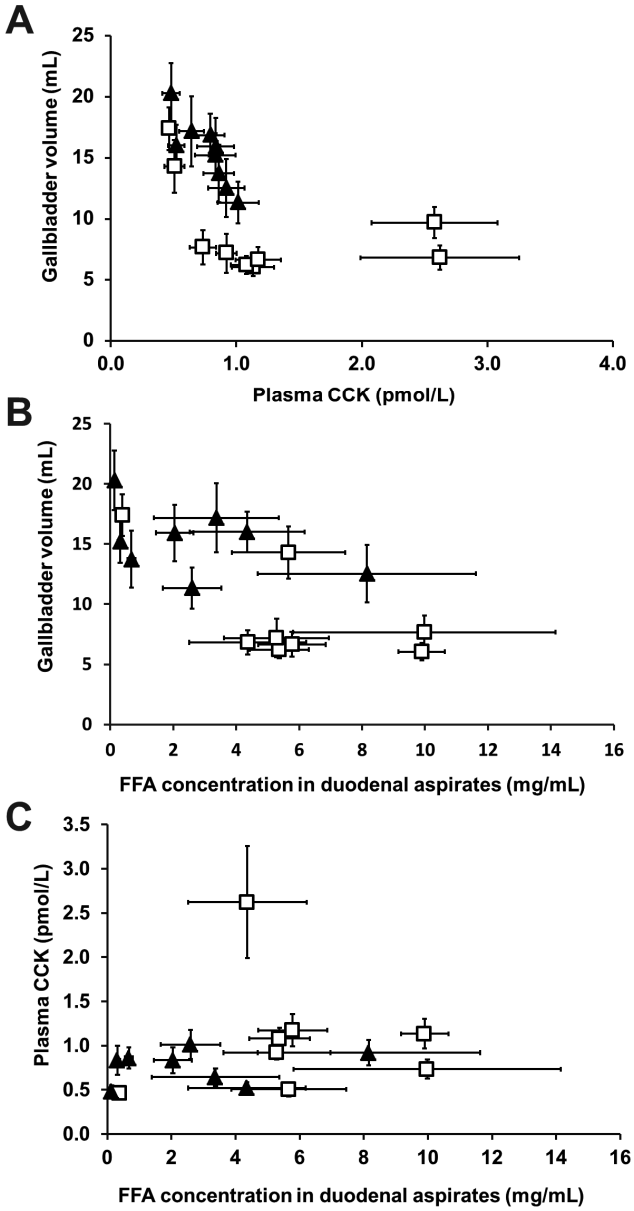

Fig. 5.4 Correlation of gallbladder volumes with plasma CCK concentrations (A) and with the FFA concentration as determined in duodenal aspirates (B), and correlation of plasma CCK concentration and the FFA concentration as determined in duodenal aspirates (C) at corresponding time points after the treatment hs (□) and uhs (▲). Values are expressed as mean ± SE.

5.3.5 T-peak and t-onset

As described in Chapter 4, the onset and peak times were extracted from Weibull fits to determine timed responses of Measures (Fig. 5.5). When comparing timed responses of hormones, ANOVA revealed main effects of Measure and Treatment but no Measure × Treatment interaction on t-peak (Table 5.2). As evaluated with post hoc analysis, hormones t-peak appeared significantly in the order of CCK<GLP-1<PYY<ghrelin and occurred significantly earlier for ho and hs compared to uhs. On t-onset the main effects of Measure, Treatment and a Measure × Treatment interaction were found by ANOVA. Post hoc analysis revealed that these effects were caused by a significantly earlier t-onset for CCK compared to the other hormones and significantly later responses for uhs compared to ho and hs. The Measure × Treatment interaction were caused by the late t-onset of GLP-1 and PYY in response to uhs.

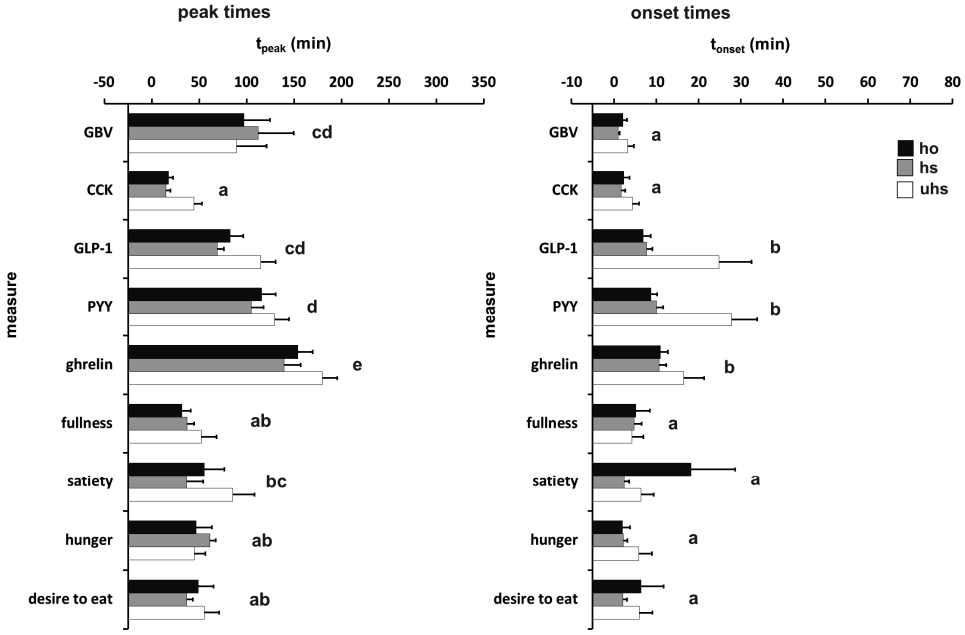

Fig. 5.5 Comparison of peak times and onset times of timed responses for gallbladder volume (GBV), plasma hormone concentrations, i.e CCK, GLP-1, PYY and ghrelin, and satiety ratings, i.e fullness, satiety, hunger and desire to eat, for the treatments ho, hs and uhs (gastric intubation study). Values are expressed as mean ± SE. Different low letters indicate a significant difference at $p<0.05$.

When comparing timed responses of satiety perceptions and hormone concentration main effects of Measure and Treatment but no Measure × Treatment interaction on t-peak were determined with ANOVA (Table 5.2). On t-onset, there were main effects of Measure and a Measure × Treatment interaction, but no Treatment effect. When evaluating Within-category effects of Measures post hoc it was found, for example, that t-peak were earlier for CCK and the perceptions fullness, hunger and desire to eat (Fig. 5.5). The perceived satiety had a later t-peak compared to CCK, and satiety was associated with GLP-1. T-peak of ghrelin was latest compared to all other, and PYY was similar to GLP-1. The interaction Measure × Treatment and the effect of Measure, as shown in Table 5.2 (ANOVA), on t-onset was entirely caused by the late t-onset of hormones for the treatment uhs as revealed post hoc.

Table 5.2 Effect of Categories including several Measures on t-peak and t-onset, emulsions used as Treatment, and Measure × Treatment interaction on t-peak and t-onset.

Category of Measures	**Measure**		**Treatment**		**Measure x Treatment**	
	F (df_1,df_2)	**Sig**	**F (df_1,df_2)**	**Sig**	**F (df_1,df_2)**	**Sig**
t-peak hormones[§]	102.30 (3,84)	***	3.49 (2,84)	*	0.24 (6,84)	Ns
t-onset hormones[§]	10.71 (3,84)	***	7.77 (2,84)	**	2.35 (6,84)	*
t-peak VAS[#] + lipids[†]-GA	13.05 (7,196)	***	0.41 (2,196)	Ns	0.53 (14,196)	Ns
t-onset VAS[#] + lipids[†]-GA	1.98 (7,196)	Ns	1.80 (2,196)	Ns	0.71 (14,196)	Ns
t-peak VAS[#] + lipids[†]-DA	3.50 (7,35)	**	15.0 (1,35)	*	0.77 (7,35)	Ns
t-onset VAS[#] + lipids[†]-DA	0.85 (7,35)	Ns	1.61 (1,35)	Ns	1.20 (7,35)	Ns
t-peak VAS[#] + hormones[§]	28.68 (7,196)	***	3.38 (2,196)	*	0.39 (14,196)	Ns
t-onset VAS[#] + hormones[§]	5.92 (7,196)	***	2.64 (2,196)	Ns	2.53 (14,196)	**
t-peak hormones[§] + lipids[†]-GA	31.11 (7,196)	***	0.56 (2,196)	Ns	0.75 (14,196)	Ns
t-onset hormones[§] + lipids[†]-GA	4.98 (7,196)	***	7.30 (2,196)	**	0.92 (14,196)	Ns
t-peak hormones[§] + lipids[†]-DA	1.15 (7,35)	Ns	1.63 (1,35)	Ns	1.0 (7,35)	Ns
t-onset hormones[§] + lipids[†]-DA	1.01 (7,35)	Ns	13.2 (1,35)	*	0.74 (7,35)	Ns
t-peak[$] GBV + CCK + FFA-DA	2.89 (2,10)	Ns	2.94 (1,10)	Ns	2.52 (2,10)	Ns
t-onset[$] GBV + CCK + FFA-DA	5.81 (2,10)	*	1.47 (1,10)	Ns	1.19 (2,10)	Ns

Significant differences are indicated with asterisks: *p<0.05, **p< 0.01, ***p < 0.001, based on ANOVA analysis. Ns is not significant.
[§] Category includes measures CCK, GLP-1, PYY and ghrelin
[#] Category includes measures fullness, satiety, hunger and desire to eat
[†] includes measures FFA, MG, DG and TG
[$] Measures GBV, CCK are depicted from gastric intubation study and compared to FFA-DA for treatments hs and uhs
GA, gastric aspirate; DA, duodenal aspirate; GBV, gallbladder volume

Including the results on lipids as described in Chapter 4, ANOVA revealed similar onset times for satiety ratings and lipids in gastric aspirates (Table 5.2). T-onset was significantly later for lipids in the duodenal aspirates compared to lipids in gastric aspirates and satiety ratings (post hoc, p<0.01).

5.4 Discussion

The behaviour of emulsions in the stomach in relation to satiety responses is still not fully understood with respect to the contribution of intragastric and intraduodenal lipolytic products as a result of their hydrolysis. In the present study we aimed to associate the intragastric and intraduodenal lipid hydrolysis with metabolic and satiety responses, such as gallbladder contraction, hormone release, satiety perceptions and *ad libitum* intake. Moreover, the impact of oral processing on these responses was investigated.

5.4.1 Additive effect of oral processing on gallbladder contraction, hormonal responses, satiety ratings and ad libitum intake

It has been shown by Witteman et al. (1993), that for modified sham feeding fat stimulated the gallbladder contraction but not CCK release (Witteman et al., 1993). They associated gallbladder contraction with CCK independent vagal cholinergic stimulation mechanisms induced by olphactoric and gustatoric chemoreceptors in the mouth. In Figure 5.1 we have shown a significantly prolonged contraction of the gallbladder after oral application compared to intragastric application of the same emulsion. To our knowledge we are the first to report this. This finding points towards an additive effect of oral processing on responses relevant for lipid digestion. In agreement to Witteman and coworkers (Witteman et al., 1993), we found no significant difference in CCK release between the oral and intragastric application of the emulsions. There was also no additive effect of oral processing on plasma GLP-1, PYY and ghrelin concentrations. It has been described, though, that ghrelin was reduced upon modified sham feeding (Heath et al., 2004). This suppression in ghrelin release was related to reduction in appetite (Heath et al., 2004). In addition, modified sham feeding was shown to increase satiety (Smeets et al., 2006). In the present study, only the satiety rating "desire to eat" was significantly elevated earlier, i.e. at t=90 min, for the oral application compared to the intragastric application, which elevated at t=120 min. In addition, there was no additive effect of oral application on the *ad libitum* intake. Recently, it has been reported that the duration of oral exposure has a strong influence on the regulation of energy intake (Wijlens et al., 2012). It was shown, that eight minutes of oral exposure decreased *ad libitum* intake (Wijlens et al., 2012). However, this is surely not realistic for drinking an emulsion.

5.4.2 Effect of intragastric lipid distribution on gallbladder contraction, hormonal responses, satiety ratings and ad libitum intake

Correlation between gallbladder contraction, CCK and FFA

Our data clearly shows the correlation of gallbladder volumes with CCK plasma concentrations (Fig. 5.4A) and this is in agreement with previous observations (Marciani et al., 2007a). In addition, high CCK plasma concentrations were found after administration of the homogenized emulsions (Fig. 5.2). CCK is known to regulate gallbladder contraction and bile secretion after food intake by binding to G-protein coupled CCK-A-receptors on the smooth muscle of the gallbladder (Wank, 1998). The release of CCK and the subsequent contraction of the gallbladder has been attributed to the release of FFA (Hildebrand et al., 1998). Accordingly, we found significantly higher gallbladder volumes after the un-homogenized emulsion compared to the homogenized emulsion and gallbladder volumes correlated with FFA concentrations in the duodenal aspirates (Fig. 5.4B). However, as shown in Fig. 5.4C, we did not find a correlation between plasma CCK concentration and intraduodenal FFA as released during intraduodenal lipolysis and analysed according to the method previously described (Helbig et al. 2012). It is possible, as hypothesized before, that due to the high gallbladder contraction and the resulting bile secretion FFA are absorbed quickly, thereby removing CCK stimulating agents which results in a low response in CCK (Liddle, 1995). The response in CCK release was, as expected, low (Fig. 5.2A) since only low concentrations of FFA were present in the duodenum (Chapter 4).

Correlation between lipids, hormone release and satiety

Duodenal infusion of triglyceride has been shown not only to increase CCK but also GLP-1 (Feinle et al., 2003) and PYY and to decrease ghrelin (Feinle-Bisset et al., 2005). This effect was abolished when a lipase inhibitor, i.e. tetrahydrolipstatin, was applied indicating the necessity of intraduodenal lipolysis for the release of these hormones. Accordingly, GLP-1 and PYY concentrations increased for hs (Fig. 5.2B, C), which correlate with the presence of lipolytic products in duodenal aspirates (Chapter 4). Nevertheless, these hormone concentrations increased as well for uhs, although much later as shown by the later onset times. In contrast to CCK, GLP-1 and PYY, ghrelin

decreased independently from the intragastric distribution of the lipids. This finding might indicate that already low concentrations of lipids are sufficient to supress ghrelin or that its suppression is predominantly caused by, for example, intragastric distension.

In summary, a clear influence of the intragastric distribution of the lipid phase on the gallbladder contraction as well as on the release of CCK, GLP-1 and PYY was observed. Especially for gallbladder volume this can be associated with the intraduodenal FFA concentration. However, this was neither reflected in the perception of satiety nor in the *ad libitum* intake. Only on the perception "desire to eat" a stronger response was seen for hs compared to uhs. Whether the identical ghrelin release obtained for both treatments plays a role in the similar perceptions of satiety found in the present study is still speculative. Also for this hormone only a minor change of the satiety perception "prospective consumption" was found in earlier studies although plasma ghrelin concentration were differently suppressed after various duodenal fat infusions (Feinle et al., 2003; Feinle-Bisset et al., 2005). We therefore hypothesize, that the satiating effect of food can not only be predicted by changes in hormonal circulation levels. We might speculate that the similar effect of hs and uhs on satiety perceptions could be due to the initial volume of the meal. In fact, it has been reported that the sense of fullness is proportional to postprandial gastric volume (Marciani et al., 2001; Marciani et al., 2007b). As proposed earlier (Goetze et al., 2007; Foltz et al., 2009) and since we applied 500 mL of the emulsions having the same energy content and the same composition, the initial volume might have had a larger effect on satiety ratings compared to that of the lipids itself.

5.5 Conclusions

In conclusion, an additive effect of oral intake of an emulsion compared to the same emulsion applied intragastrical was found on gallbladder contraction which points toward the supporting influence of oral processing on metabolic responses. Furthermore, the differences in intragastric distribution of the lipid phase influenced the release of FFA into the duodenum and metabolic responses, such as plasma hormone concentrations and gallbladder motility, but had no major effect on satiety feelings. Unexpectedly, there was no correlation between duodenal concentration of FFA and CCK plasma concentration, which suggests that satiety feelings might be induced by other mechanisms such as

intragastric volume and hence gastric distention rather than by hormonal responses as such.

Acknowledgements

The authors gratefully thank Daniel Keszthelyi, Samefko Ludidi and Mark van Avesaat (Division of Gastroenterology-Hepatology, Department of Internal Medicine, University Hospital Maastricht) for their technical contribution to the clinical trial. George van Aken and Lex Oosterveld (NIZO food research) are acknowledged for their technical expertise and contribution to the experimental set up. We thank all participants in this study and Eefjan Timmermann (TI Food and Nutrition) for his technical assistance during the measurements.

5.6 Supplementary data

Table S5.1 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on plasma CCK concentration (pmol/L) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment		
	ho	**hs**	**uhs**
-10	0.45 ± 0.05^{ab}	0.46 ± 0.06^{abcd}	0.48 ± 0.07^{a}
10	2.92 ± 0.52^{j}	2.58 ± 0.50^{ghij}	0.79 ± 0.11^{abcdefg}
15	3.85 ± 1.55^{ij}	2.62 ± 0.63^{hij}	0.83 ± 0.16^{abcdef}
30	1.52 ± 0.27^{fghij}	1.13 ± 0.17^{defghi}	0.86 ± 0.12^{abcdef}
45	1.05 ± 0.08^{defghi}	1.08 ± 0.12^{defghi}	1.01 ± 0.16^{bcdefg}
60	1.42 ± 0.40^{efghij}	1.17 ± 0.18^{defghi}	0.92 ± 0.15^{abcdef}
90	1.05 ± 0.15^{cdefgh}	0.92 ± 0.08^{abcdef}	0.84 ± 0.15^{abcdef}
120	0.79 ± 0.10^{abcde}	0.73 ± 0.11^{abcdef}	0.64 ± 0.10^{abcdef}
180	0.52 ± 0.06^{abc}	0.51 ± 0.08^{ab}	0.52 ± 0.07^{abcde}

Table S5.2 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on plasma GLP-1 concentration (pmol/L) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment		
	ho	**hs**	**uhs**
-10	7.77 ± 0.87^{abcd}	8.93 ± 2.19^{ab}	5.60 ± 0.79^{a}
10	11.27 ± 1.97^{abcd}	12.50 ± 18.64^{abcd}	9.71 ± 2.67^{abc}
15	11.64 ± 2.49^{bcdefg}	18.64 ± 2.94^{cdefgh}	12.07 ± 2.02^{abcde}
30	34.27 ± 4.93^{hijk}	34.87 ± 5.37^{ijk}	19.87 ± 3.46^{defghij}
45	26.33 ± 3.09^{ghijk}	34.47 ± 3.52^{jk}	28.33 ± 6.24^{efghijk}
60	25.13 ± 2.59^{ghijk}	30.40 ± 3.47^{hijk}	31.60 ± 5.99^{hijk}
90	28.33 ± 3.98^{ghijk}	29.79 ± 2.88^{hijk}	37.93 ± 4.03^{k}
120	23.47 ± 2.73^{fghijk}	23.13 ± 2.00^{ghijk}	32.43 ± 3.09^{hijk}
180	14.62 ± 2.13^{bcdef}	16.93 ± 2.05^{defghi}	23.00 ± 3.94^{defghij}

Table S5.3 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on plasma PYY concentration (pg/mL) as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment		
	ho	**hs**	**uhs**
-10	81.67 ± 4.92^{a}	81.80 ± 6.28^{ab}	84.64 ± 5.82^{ab}
10	91.00 ± 4.87^{ab}	86.93 ± 8.35^{a}	87.50 ± 6.86^{ab}
15	101.13 ± 6.09^{abc}	95.93 ± 8.58^{abc}	90.67 ± 5.79^{ab}
30	140.93 ± 12.78^{ef}	137.40 ± 11.81^{ef}	105.80 ± 7.87^{bcd}
45	152.27 ± 9.65^{f}	152.53 ± 12.25^{ef}	125.80 ± 14.12^{cde}
60	151.73 ± 8.51^{f}	153.27 ± 10.25^{f}	134.20 ± 12.97^{def}
90	155.13 ± 10.12^{f}	148.14 ± 8.50^{f}	145.21 ± 11.23^{ef}
120	153.36 ± 11.83^{f}	149.93 ± 9.47^{ef}	152.57 ± 12.36^{f}
180	140.46 ± 12.61^{ef}	137.53 ± 10.58^{ef}	128.86 ± 8.66^{ef}

Table S5.4 Post hoc analysis (Tukey HSD) of the Time × Treatment interaction on the satiety perception `desire to eat´ as determined from ANOVA. Different letters indicate a significant difference at $p<0.05$.

Time	Treatment		
	ho	**hs**	**uhs**
-10	4.27 ± 0.63^{bdefg}	4.44 ± 0.48^{cfg}	4.70 ± 0.50^{dg}
30	2.34 ± 0.67^{ac}	2.71 ± 0.52^{abde}	3.16 ± 0.57^{abcef}
60	3.25 ± 0.67^{abcdf}	3.76 ± 0.60^{abcde}	3.55 ± 0.63^{abcdefg}
90	3.89 ± 0.66^{bdefg}	4.02 ± 0.68^{abcde}	3.83 ± 0.57^{abcdefg}
120	4.55 ± 0.62^{bdefg}	4.50 ± 0.69^{cfg}	4.14 ± 0.65^{abcdefg}
150	4.79 ± 0.58^{eg}	5.54 ± 0.67^{fg}	4.25 ± 0.63^{abcdefg}
180	5.02 ± 0.59^{bdefg}	5.84 ± 0.66^{g}	5.05 ± 0.66^{abcdefg}

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Chapter 6

General Discussion

As described in the Introduction section, a wide range of *in vitro* models is presently available. These models simulate gastric and intestinal lipolysis either separately or in combination, while containing gastric and intestinal fluids of different compositions. Until recently, most of the research reported in literature had been conducted to simulate intestinal lipolysis as the intestine is the major site of lipid digestion. Also, most of the published work focuses on the release of free fatty acid (FFA), while little is known on the formation of monoglyceride (MG) and diglyceride (DG). Furthermore, little is known on the gastric behaviour of emulsions and the resulting effects on the intestinal lipolysis. Most *in vitro* models used did not include the gastric compartment and when it was included, it was mimicked simple. At the same time limited data was available from human studies on the behavior of emulsions. With respect to human studies, mostly small intestinal perfusion studies have been used to gain mechanistic understanding of lipid digestion neglecting the impact of gastric digestion. Also, the intragastric release of FFA as a result of triglyceride lipolysis, the corresponding release of hormones, as detected by FFA, at the intestinal site as well as the resulting satiety perceptions were poorly understood. Hence, the many questions remained: What are possible mechanisms, which effect the digestion of triglycerides, what is the impact of gastric passage and what are the consequences for satiety perception? Therefore, the aim of this study was to build up knowledge concerning the influence of emulsion parameters on the release of FFA, MG, and DG. In addition, the impact of the gastric passage on lipolysis was investigated. Finally, in a human study the lipolysis of emulsions was correlated with the subsequent satiety perceptions.

6.1 Mechanisms that effect the digestion of triglycerides

6.1.1 Effect of emulsion properties on lipid digestion

In Chapters 2 and 3, two *in vitro* models, i.e. a static intestinal and a dynamic gastrointestinal, were used to investigate the release of FFA from emulsions. As both the oil-water interface of an emulsion and the droplet size are key factors influencing the release of FFA, the investigated emulsions were varied in type of emulsifier, i.e. gum arabic, WPI and lysozyme, and in droplet size. It was shown (Chapter 2) that in a simulated intestinal environment the release of FFA was higher from emulsions stabilized by gum arabic compared to WPI. Hence, a clear influence of the type of emulsifier on the release of FFA was shown, comparable to other studies (Mun et al., 2007; Hu et al., 2010).

It must be noted though, as shown recently, that changing the oil type reduces the impact of emulsifier type on lipid digestion (Marze & Choimet, 2012). This effect was not investigated in the present study. In addition, there is obviously an influence of the interface on the hydrolysis rate of DG into MG, which is, until now, only described for lipases depending on the origin (Carriere et al., 1991; Rodriguez et al., 2008). Furthermore, a clear effect of the surface area was observed. This showed that the larger the area the higher the rate of digestion, which is in agreement with previous studies (Li & McClements, 2010).

Previously, it was shown under simulated intestinal conditions that the initial charge of the interface did not specifically correlate with the lag phase of pancreatic lipase (Wickham et al., 1998). In the case of protein-stabilized emulsions, the FFA release under simulated intestinal digestion was only slightly higher for positively charged lactoferrin-stabilized emulsions compared to negatively charged β-lactoglobulin-stabilized emulsions (Sarkar et al., 2010). The opposite was found when the intestinal digestion of lysozyme- and WPI-stabilized emulsions, as used in Chapter 3, was investigated using the pH-stat method (Chapter 2). For WPI-stabilized emulsions 60 % of initial FFA present in the form of triglycerides were released after 30 min, whereas for lysozyme only 0.5 % of FFA were determined. As shown in Fig. 6.1, WPI-stabilized emulsions were homogenous, whereas lysozyme-stabilized emulsions flocculated immediately upon mixing with bile salts. This behaviour caused the difference in FFA release, which in turn could lead to wrong interpretation, as shown in Chapter 3 and discussed further in 6.1.3.

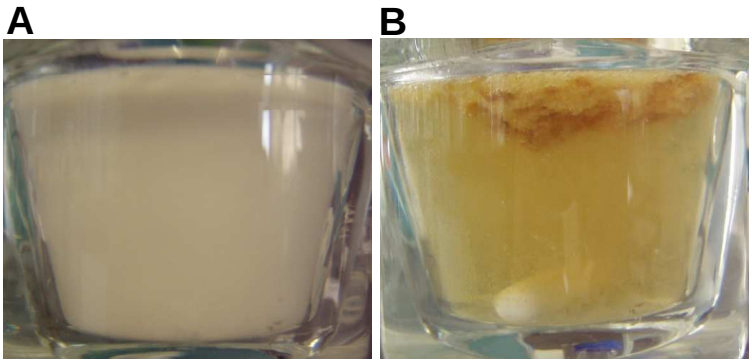

Fig. 6.1 Simulated intestinal digestion of WPI-stabilized emulsions (A) and lysozyme-stabilized emulsions (B) using the pH-stat method at pH7.5, as described in Chapter 2.

6.1.2 Effect of simulated digestion on lipid digestion - pH-stat method

The question remains if *in vitro* models can predict the behavior and therefore the release of FFA as occurring in humans. The pH-stat method is an easy and fast single-step model (McClements, 2010) to investigate underlying mechanisms of intestinal emulsion digestion, such as compositional changes, at the interface. When using this model, it was shown (Chapter 2) that the FFA release is strongly dependent on the emulsifier used, i.e. a discrepancy of 60% was found only for WPI-stabilized emulsions between pH-stat and gas chromatography. As mentioned in Chapter 2, specific conclusions on lipid digestion were related to the *in vitro* experimental conditions used during the study. For example, the FFA detection with pH-stat is very much dependent on the presence of calcium, which is not advantageous for determination of overall lipid digestion and needs to be taken into account when comparing experimental results. Nevertheless, the detectable FFA in the pH-stat as affected by the calcium concentration could still be of benefit. For example, it could provide knowledge on the available FFA which are in turn detectable at the intestinal site, when emulsions are present in the form of milk products containing high calcium concentrations.

Several hypotheses have been discussed (i.e. role of the digestion medium, binding of FFA to β-lactoglobulin, interaction of FFA with positively amino acid residues of WPI, hydrolysis of protein by proteolytic enzymes present in pancreatin) dealing with the question why the detection of FFA with pH-stat was different between the emulsifiers. No conclusive statement could be made, showing that further research in this area needs to be undertaken. Indeed, as shown recently, when β-lactoglobulin-stabilized emulsions were digested under simulated intestinal conditions and β-lactoglobulin was present in the bulk in excess a higher lipid digestion was shown with pH-stat, but not with HPLC compared to emulsions in which β-lactoglobulin completely covered the interface (Marze et al., 2012). In that study, the presence of vesicles and/or the accumulation of digestion products at the interface have been suggested to be responsible, but this hypothesis has not been confirmed yet.

Overall, the pH-stat model can be used to investigate the FFA release. However, the results should be interpreted with care and we suggested to combining it with other analytical tools, such as gas chromatography (Chapter 2). Also, in static *in vitro* models,

the digestion of emulsions and their behavior should be analyzed under different conditions such as varying calcium concentrations.

6.1.3 Effect of simulated digestion on lipid digestion - Including the stomach in *in vitro* models

In Chapters 3-5 it was shown that the behaviour of the emulsions was influenced by the gastric passage. By simulating the gastric and intestinal passage of protein stabilized emulsions through a dynamic model, a clear influence of gradual acidification and the shape of the gastric compartment was found (Chapter 3). Both led to creaming of the emulsions when the emulsifier, i.e. WPI, transitioned through the isoelectric point of the protein. This in turn caused a delay in intestinal lipid digestion. Nevertheless, the overall concentration of FFA at the end of the experiment was similar compared to emulsions stabilized by lysozyme. Thus, transition through the isoelectric point affects the creaming behavior, delays intestinal lipid digestion, but does not influence the overall pancreatic lipase activity. Using un-homogenized emulsions, which represents phase separation of oil and water (Chapter 4), a delayed entry of lipids in the duodenum was clearly observed compared to a homogenized emulsion. Both creaming and phase separation delayed the intestinal lipolysis. Nevertheless, in the model used in Chapter 3, the change in emulsion structure did not influence the emptying of the emulsion from the gastric compartment as this was computer controlled.

Furthermore, the model did not include the presence of mucin, which might change emulsion behaviour as well. For example, the absorption of bile salts to the interface enhanced the diffusion of lipid droplets in the intestinal mucin (Macierzanka et al., 2011) and this is pronounced with increasing negative surface charge (Macierzanka et al., 2012). During the human study (Chapters 4 and 5), gastric emptying was estimated based on plasma paracetamol concentration. However, this is solely a marker for emptying of the water phase. Moreover, we assumed layering of the oil phase for the un-homogenized emulsion based on studies using MRI (Marciani et al., 2007a; Marciani et al., 2007b; Marciani et al., 2009) or scintigraphy (Edelbroek et al., 1992).

In order to further explore the layering of the oil-phase and gastric emptying in a gastric environment a pig study was conducted. The experimental setup and the results of this study are given below.

Experimental set up Two male pigs (TOPIGS20, Van Beek SPF Lelystad, The Netherlands), provided with a gastric canula at the bottom of the stomach were used. The investigated emulsions were prepared as described in Chapter 3. Lipids were analyzed according to the method described in Chapter 3 and emptying of the water phase was determined by analyzing paracetamol concentration in gastric samples using UPLC-MS/MS (TNO, Zeist, The Netherlands). In total, 1 L of the respective emulsion was introduced in the stomach via the gastric canula and contained 1.2 g paracetamol. WPI and Tween-80 stabilized emulsions were followed over time, i.e. at t=30, 90 and 180 minutes on three separate occasions. Lysozyme-stabilized emulsion and an un-homogenized Tween-80 sample were studied after 90 minutes residence in the pig stomach. To study the local behavior of emulsions at different time points the complete gastric content was collected in fractions of 100 mL. The first fraction represents the bottom part of the stomach. The last fraction represents the top part.

The pig is similar to the human with respect to anatomy and physiology of the digestive tract (Pond & Houpt, 1978). Therefore, extracts of gastro-intestinal juices obtained from pigs are frequently used in *in vitro* models (for a review see McClements & Li, 2010) to investigate the digestion of emulsions. Nevertheless, it is still challenging to translate the dynamics of the *in vivo* digestion, such as dynamic secretion of digestive fluids and gastric emptying, into *in vitro* models. We hypothesized that results obtained from pigs on emulsion behavior will confirm observations as found in the dynamic model. In turn, they will support the need of advanced (i.e. complex) *in vitro* models to predict emulsions behaviour under mixing and digestive processes as occurring *in vivo*. Examples are given below to discuss the effects of intragastric distribution of lipids as determined by the emulsifier or the droplets size on gastric emptying in pig.

Protein stabilized emulsions - influence of mucin?

In Chapter 3 the importance of a gradual acidification in the gastric compartment on the behavior of protein stabilized emulsions, i.e. WPI and lysozyme, was shown. In combination with the custom designed gastric compartment, which simulated the motility of the different regions of the stomach, it was possible to follow separation of WPI-stabilized emulsions into a cream and serum layer, but not the gastric emptying. In fact, when monitoring the behavior of WPI-stabilized emulsions in the pig, it can be seen that the gastric volume after application of the WPI-stabilized emulsion decreased rapidly (Fig. 6.2A). Similarly as in the dynamic model (Chapter 3), the WPI-stabilized emulsion started to cream in the pig stomach at t=30 minutes, most likely as a result of the gradual acidification. When comparing WPI-stabilized emulsions with lysozyme, both emulsions creamed at 90 minutes after the start of the experiment as can be seen from the intragastric distribution of lipids (Fig. 6.3A). The fact that lysozyme-stabilized emulsions creamed to the same extent as WPI-stabilized emulsions (Fig 6.3A) is possibly related to the presence of mucin. As discussed in Chapter 3, slight flocculation of lysozyme-stabilized emulsions occurred upon addition of pepsin and salt. As mucin, similar as pepsin is negatively charged above pH 3 (Froehlich et al., 1995), it is likely to interact with the lysozyme stabilized interface resulting in flocculation. Still, the water phase emptied approximately 1.5 times faster in the case of WPI-stabilized emulsion compared to lysozyme-stabilized emulsions. However, the interaction of mucin with emulsions is barely investigated. One might speculate that such investigations are of importance, especially as protein stabilized interfaces or charged emulsifiers interact with mucin. This has been proven upon saliva mixing (Silletti et al., 2007). In the pig model, we investigated the distribution of mucin by collecting several fractions of emulsion-gastric fluid samples as described above. The mucin concentration was determined as the sum of the constituent galactosamine and glucosamine contents by the analysis of sugar composition (Verhoef et al., 2005). The results revealed an equal distribution of mucin over the fractions at a concentration of 26±8 mg/mL. Mucin was shown to promote the flocculation of β-lactoglobulin-stabilized emulsions (Sarkar et al., 2010) and to decrease the interfacial tension (Marze et al., 2013). It is not known yet to what extent the presence of mucin influences the FFA release from emulsions or the FFA sensing by receptors at the intestinal site. This clearly shows the effect of inducing complexity into models on emulsions behavior. Hence, digestion of lipids needs to be evaluated by using *in vivo* studies and adapted in dynamic models.

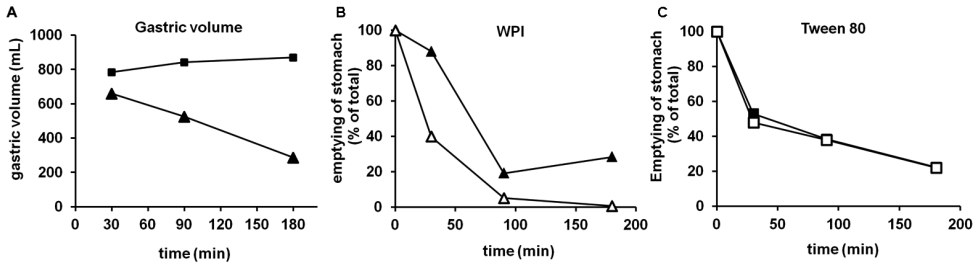

Fig. 6.2 Example of change in gastric volume (A) over time after administration of WPI (▲) or Tween 80- (■) stabilized emulsions for one pig. In B and C examples of emptying of lipid (closed symbols) and water (open symbols) phase is given. It is determined from the initial amount of lipids present in the emulsion (i.e. 8% (v/v) sunflower oil) or paracetamol in the water phase (i.e. 1.2 g), respectively of the WPI or Tween 80-stabilized emulsions for one pig.

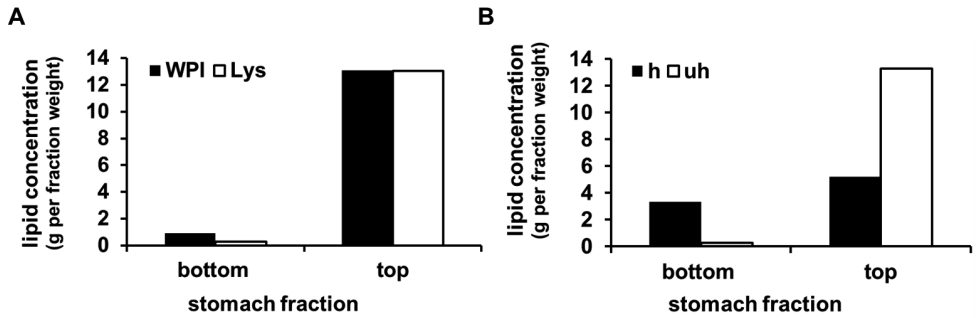

Fig. 6.3 Distribution of lipids in the stomach as determined from fractionated samples at 90 minutes after administration of the emulsions. The first fraction represents the lowest part of the stomach, i.e. bottom. The last fraction represents the highest part, i.e. top. In (A) an example of lipid distribution in the stomach after administration of WPI and Lysozyme (Lys)-stabilized emulsions and in (B) of a homogenized (h) and un-homogenized (uh) Tween 80-stabilized emulsion is given.

The distribution of fat in the stomach – homogenized and un-homogenized Tween 80-stabilized emulsions and creaming

In the human study, two emulsion systems were used to simulate both a homogenous system as well as a phase separated system of oil and water (Chapters 4 and 5). By measuring the plasma paracetamol concentrations, it was shown that the emptying of the homogenized Tween 80-stabilized emulsion was slower compared to the un-homogenized emulsion. This finding was also confirmed in the pig model. The gastric volume for the treatment with homogenized Tween 80-stabilized emulsions was constant over time (Fig 6.2A). One must note that the lipid and water phase continued to empty from the stomach (Fig 6.2C), which was possibly caused by gastric secretion exceeding gastric emptying. Compared to the homogenized emulsion, the gastric volume was 1.6 times less for the un-homogenized emulsion after 90 minutes of the start of experiment. As expected, the lipids of un-homogenized emulsions layered on top of the stomach (Fig 6.3B). Although Tween-stabilized emulsions are described as being stable, i.e. not aggregated or coalesced, in the acidic environment of the stomach (Marciani et al., 2007a; Marciani et al., 2009), we observed high concentrations of triglyceride at 120 minutes (Chapter 4). This was also found using a dynamic model as described in Chapter 3 (Oosterveld et al., unpublished data). This was interpreted as creaming of the homogenized emulsion. In agreement with this, fractions of Tween 80-stabilized emulsions revealed creaming at 90 minutes in the pig stomach. Therefore, the results of stable emulsions observed *in vitro* should be generally interpreted with care in the view of the effect of creaming *in vivo* on, for example, metabolic responses.

The presence of gastric lipase

Human gastric lipase is able to digest up to 40 % (Armand, 2007) or 25 % (Carriere et al., 1993) of lipid digestion and also helps to accelerate pancreatic lipase activity. Furthermore, as recently shown, the addition of a fungal lipase in simulated gastric conditions enhanced flocculation of WPI-stabilized emulsions (Golding et al., 2011) gastric environment. Next to the ethical consideration, a drawback in using the pig as a model is, that porcine gastric lipase is irreversibly inactivated at pH 4 (Moreau et al., 1988). Therefore, besides the information that can be gained on emulsion behavior, such as creaming or gastric emptying, it is not advantageous to study lipolysis of emulsions in a pig stomach. Several options are commercially available to be used as replacers for human gastric lipase *in vitro*. In the TIM (Chapter 3), a preduodenal lamb lipase was used as it is

similar to human gastric lipase in its pH profile (Moreau et al., 1988) and its activity towards the sn-1 and sn-3 position of a triglyceride. Recently, a 3.5 fold higher hydrolysis level (defined as L_1% as shown in Chapters 3 and 4) was observed for a gastric lipase extracted from rabbits compared to human gastric lipase (Capolino et al., 2011). This rabbit lipase was, therefore, considered as a good alternative to be used in *in vitro* assays.

Overall, dynamic models and the pig model revealed the importance of investigating the intragastric lipolysis and the change in emulsions structure, i.e. flocculation, as well as the creaming or phase separation behavior of emulsions *in vitro*. Furthermore, the effect of this behaviour on intestinal lipolysis needs to be investigated *in vitro* to eventually predict possible satiety perceptions, but also metabolic responses *in vivo*.

6.2 Influence of emulsion parameter on satiety

6.2.1 The role of lipid digestion products FFA, MG, DG and TG

In vitro studies clearly miss the ability to mimic complex physiological responses as, for example, induced by hormones. In case of lipid digestion, FFA are considered as the strongest trigger to induce these hormonal responses (McLaughlin et al., 1999; Feltrin et al., 2007). From the present study the effect of a single lipid digestion product cannot be related to hormonal responses. As shown, the duodenal aspirates consisted of approximately 16 mM FFA at t=15 and 36 mM FFA at t=30 minutes. Similar duodenal FFA concentrations were found after ingestion of TG containing meals (Borgström et al., 1957) or after ingestion of emulsions (Armand et al., 1996; Armand et al., 1999). For example, FFA concentrations ranged for a fine emulsion with a mean droplet size of 0.7 µm between 18-30 mM and for a coarse emulsion with a mean droplet diameter of 10 µm, between 7-22 mM (Armand et al., 1999). These data were based on emptying rates as determined from a water soluble marker. It has been shown that concentrations of FFA between 40 and 72 mM stimulated the release of CCK and PYY and suppressed energy intake (Feltrin et al., 2007). Unfortunately, the gastric emptying measured by means of plasma paracetamol reflect only the emptying of the water phase and not necessarily the oil phase. The emptying rate of nutrients to the duodenum is approximately 2-3 kcal/min (Horowitz et al., 1993). This corresponded well with Tween-stabilized emulsions, which had a similar TG content (i.e. 50 g oil; Marciani et al., 2009) as used in our study. Nevertheless, it is clear that in our study the initially emptying of the un-homogenized

was faster compared to the homogenized emulsion. Also in the study of Marciani et al., (2009) the half time emptying of the emulsion, which was homogenous in the gastric environment, was determined to be at approximately 185 min and for the emulsion, which phase separated at approx. 70 min. Thus, the gastric volume decreased more rapidly for the phase separated emulsion (Marciani et al., 2009). Despite these differences observed in this mentioned study, only modest variation in satiety perception were found, but unfortunately the CCK responses were not determined. Overall, we failed to induce marked differences in satiety perception despite the huge differences in emulsion behavior. This shows that other mechanisms are involved in satiety responses. Also, it could point to the influence of gastric lipolysis, which was in our study approx. 3%, as intragastric applied FFA were about 5 times more effective to induce satiety related responses than TG (Little et al., 2007).

In Chapter 2 it was mentioned that analysis of not only FFA, but also MG and DG would add to better understanding of the digestion process *in vivo*. Although it is known, that FFA when applied as such, have more potential to induce hormonal responses compared to TG (Little et al., 2007) the extent in which DG, as formed in the stomach as well as intraduodenal and MG, as formed during duodenal lipolysis, contributed to hormonal responses is not clear yet.

6.2.2 Hormones CCK, GLP-1, PYY and ghrelin

Based on literature, the release of CCK is considered as an important factor to mediate satiety responses upon digestion of fat. CCK release is provoked by the detection of FFA at the duodenal site (Schwizer et al., 1997; McLaughlin et al., 1999; Feltrin et al., 2007; Little et al., 2007). However, in Chapter 5 it was shown that no correlation was found between CCK circulating plasma levels and FFA concentration in duodenal aspirates (Fig 5.4C), whereas the CCK release for the homogenized emulsion was significantly higher than for the un-homogenized (Fig 5.2A). What is striking in Figure 5.4C, is the FFA concentration at 4.4 mg/mL (i.e. 16 mM) and the corresponding maximum CCK plasma concentration at 2.5 pmol/L. This data point corresponds to the time point at 15 minutes for the homogenized emulsion when both the duodenal aspirate and the plasma were collected. Since CCK is an early responding hormone, it is apparent that a certain concentration of FFA has to enter the duodenum within these 15 minutes in order to induce release of CCK. In fact, the same concentration of FFA in the aspirates was observed, for example, after 60 minutes for the un-homogenized emulsion (Chapter 4, Fig.

4.4B) without an elevation in plasma CCK concentration (Chapter 5, Fig. 5.2A). This is in line with results of other studies (Fried et al., 1988; Foltz et al., 2009), but in contrast to a previous duodenal intubation study, where emulsions infused at constant rates and caused elevated CCK plasma levels over 2h (Seimon et al., 2009). As discussed in Chapter 5 and suggested before (Liddle, 1995), bile secretion led to removal of the CCK stimulating agent, i.e. FFA. Whether this removal is caused by simple incorporation of these fatty acids into micelles or by removal of such complexes through the mucus layer at the intestinal site is not clear yet. With the analytical method (i.e. GC) used in this project, incorporated FFA in micelles will be measured as free fatty acids. This could in turn lead to an overestimation of the fatty acids which are available to stimulate the release of CCK.

Furthermore, based on our results, we hypothesized that hormones alone are not a predictor per se for satiety responses of foods (Chapter 5). Nevertheless, a relation was found between CCK and gallbladder volume. Also, the hormones GLP-1 and PYY responded differently to the emulsions (Chapter 5). In our study the suppression of ghrelin, however, was not different between the treatments. Intraduodenal infusion of protein-enriched meals suppressed ghrelin release after 60 min of protein load of 3 kcal/min compared to saline infusion (Ryan et al., 2012). Recently, it was shown in lean men that ghrelin concentrations was suppressed during intraduodenal infusion of test meals varying in macronutrients in the following order high protein>high fat>high carbohydrate (Brennan et al., 2012). In that study, the high protein meal was associated with the lowest *ad libitum* intake. Hence, as indicated previously (Keogh et al., 2011) and as discussed in Chapter 5, the ghrelin response in our study could be relevant for the similar satiety perceptions and the *ad libitum*.

6.3 Concluding remarks

Structuring of the lipid phase is considered to be a tool to influence satiety. Recently, it was shown that changes in lipid structuring also results in changes of plasma lipids (Keogh et al., 2011). In this study, for example, hydrogenated fat as part of the oil phase did not have a beneficial effect on satiety compared to emulsion including only the oil. Nevertheless this structuring caused lower TG-plasma levels (Keogh et al., 2011). Also, phase separation of emulsions was shown to influence the postprandial lipemia and β-oxidation of lipids in humans (Vors et al., 2013). In that study, for example, chylomicron

plasma concentrations were delayed and chylomicrons were smaller in size in normal and also obese people. Thus, the way fat is structured may impact not only satiety, but also plasma lipid profiles that play a role in the metabolic syndrome, in diabetes and in obesity.

Lipids in the form of an emulsion are a relevant model to study satiety perception. Emulsified lipids are present in a wide range of processed foods. However, it is difficult to assess the impact of lipids or lipids structure in relation to other meal components like carbohydrates and proteins. Proteins, for example, have a high satiating effect. Also they could help inducing precipitation as shown for milk (van Aken et al., 2011) and with that changing the behavior of emulsified lipids. The dynamic model (see Chapter 3) would be a relevant model to investigate such structures.

It is possible, as shown in literature and in the human study presented in this thesis, to induce satiety perceptions in humans by various emulsions system. Possibly a combination of several aspects, such as emulsions droplets size, emulsifier and the structure of fat and especially their resulting behavior in the stomach play a major role. Also, which model to use clearly depends on the research question. For example, the role of emulsion stability can be investigated with simple model systems. However, the final link with satiety can only be made with *in vivo* studies.

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Summary

Samenvatting

Zusammenfassung

Lipids are key components in our diet and, specifically in the western diet, commonly eaten in the form of emulsions present in highly processed food. In view of the current obesity prevalence one strategy to prevent overconsumption of food could be the use of specifically designed emulsions to induce satiation and satiety.

In **Chapter 1** we introduce the regulation of satiety responses, which occur upon intake of lipids and their digestion. The focus is on the regulation of gastric emptying and the hormones involved as observed by several *in vivo* studies. It shows the current understanding that lipolytic products, i.e. free fatty acids (FFA), are most likely responsible for lipid induced release of hormones, such as CCK, and the resulting satiety responses when detected at the intestinal level. Nevertheless, it also shows that this knowledge is gained mostly upon intestinal intubation studies and little research involves the gastric passage. Including this passage has shown the importance of aspects like gastric lipolysis or colloidal state of lipids in the regulation of satiety responses. However, these aspects are not investigated in combination or show inconsistent results. A similar situation is found for *in vitro* models that have recently been used to gain mechanistic understanding of the behaviour of emulsions. At the start of this project the focus in literature was on the intestinal digestion of lipids and mainly on the release of FFA but not on monoglycerides (MG) or diglycerides (DG). In addition, only a few publications included the gastric digestion, but the specific shape or functions of the stomach are neglected or mimicked simply.

The study in **Chapter 2** focused on FFA release in relation to emulsion properties monitored by a commonly used static *in vitro* model, i.e. the pH-stat method. Emulsions differing in droplets size, i.e. fine, medium and coarse, stabilized by whey protein isolate (WPI) or gum arabic (GA) were digested under intestinal conditions. Gas chromatography (GC) was used to determine lipolysis products, i.e. FFA, MG, DG and triglycerides (TG). Overall, GC analysis revealed decreasing FFA, MG and DG concentrations with increasing droplet size. It was shown that digestion of emulsions stabilized by a protein-polysaccharide emulsifier (i.e. GA) resulted in higher FFA release than of emulsions stabilized with a proteinaceous emulsifier (i.e. WPI). In addition, GA-stabilized emulsions resulted in a faster hydrolysis rate of DG into MG compared to WPI-stabilized emulsions. The concentrations of FFA determined with GC were 2–3 times higher compared with pH-stat for WPI stabilized emulsions. It was concluded that since the detection of FFA with pH-stat was influenced by the type of emulsifier, pH-stat results should be interpreted with care and combined with other methods, such as GC.

In **Chapter 3** the effect of gastric passage of protein stabilized emulsions on lipolysis influenced by both gradual acidification and stomach properties was studied. For this purpose, a dynamic *in vitro* model (TNO Gastro-Intestinal Model) was introduced, which had a custom designed gastric compartment. To determine the effect of flocculation at the isoelectric point WPI or lysozyme were used as emulsifiers. Microscopy, particle size analysis and GC were used for characterization. Under gastric conditions WPI-stabilized emulsions separated into a cream and serum layer when the protein transitioned through its isoelectric point. Microscopy and particle size analysis revealed larger flocculated particles for the initial negatively charged WPI-stabilized emulsions compared to the positively charged lysozyme-stabilized emulsions. Mainly FFA and DG were present in the gastric compartment, which is typical for the hydrolysis of gastric lipase. In addition, analysis of the serum layer revealed the presence of FFA, which could induce the release of CCK *in vivo*. The creaming caused a delayed entry of lipids into the small intestinal part. Nevertheless, the total FFA concentration in the gastric compartment at the end of the experiment was similar for both emulsions. The effect of creaming or presence of FFA in the serum layer on gastric emptying, however, could not be assessed due to the nature of the model.

In a human study, described in **Chapters 4 and 5**, we investigated several aspects of lipid administration, lipid digestion and the corresponding physiological responses influenced by the physical properties of the lipids. In **Chapter 4**, we focused on the influence of the intragastric distribution of lipids on gastric and duodenal lipolysis. Also, the additive effect of oral application on gastric lipolysis was studied. In a randomized, single blind crossover study, 15 healthy subjects were intubated nasogastric on three separate occasions. In addition, a subset of 6 healthy subjects was intubated nasoduodenal on two separate occasions. Volunteers received a homogenized emulsion orally or by gastric infusion or a gastric infusion of water followed by the oil phase (un-homogenized sample). The subset of 6 volunteers received a gastric infusion of a homogenized emulsion or a gastric infusion of an un-homogenized sample. Emulsion structure, pH and lipid composition were assessed in both gastric and duodenal fluids. A gastric lipolysis of approx. 3% for all treatments was found and the main products were FFA and DG. Concentrations of FFA in duodenal aspirates were higher after homogenized emulsions than after un-homogenized samples ($p<0.05$). Gastric emptying was faster for the un-homogenized sample than for the homogenized emulsion as assessed by plasma paracetamol concentrations. Overall, there was no effect observed of oral processing. Also, homogenization had no effect on gastric lipolysis. Nevertheless, differences in

inhibition of gastric emptying were observed: Duodenal release of FFA and inhibition of gastric emptying occurred earlier for homogenized emulsion than for un-homogenized samples.

Subsequently, in **Chapter 5**, it was investigated if such implemented effects would influence responses relevant for lipid digestion and satiety perception. Thus, in the same setting as described in Chapter 4, gallbladder volume, plasma CCK, PYY, GLP-1 and ghrelin concentration, satiety ratings as assessed via a visual analogue scale, and *ad libitum* intake were analyzed. The results showed a significant influence of the treatments on gallbladder volume ($p<0.001$). In detail, the most prolonged contraction of the gallbladder was found when the homogenized emulsion was applied orally. In contrast, when the un-homogenized sample was administered intragastrically, the contraction of the gallbladder was lowest. In addition, we found significant effects of time and treatment on circulating hormone concentrations. In the case of plasma CCK, concentrations were significantly higher at 15 minutes after application of the homogenized emulsion compared to the application of the un-homogenized sample. For PYY, the concentrations were significantly higher at 30 minutes for the homogenized emulsion compared to the un-homogenized sample. The responses of PYY and GLP-1 to the un-homogenized sample were delayed by approximately 20 minutes compared to the responses to the homogenized emulsion. On the suppression of ghrelin, however, only an effect of time, but not of the treatments was found. Also, the perception of satiety and the *ad libitum* intake were not different for the treatments. Nevertheless, the feeling `desire to eat´ was stronger for the homogenized emulsions compared to the un-homogenized sample. It was concluded, that plasma hormone concentrations and gallbladder motility were affected by the intragastric distribution of the lipids. These effects were neither reflected in satiety perceptions nor in the *ad libitum* intake. Hence, the release of gastrointestinal hormones cannot directly be related to the satiating effect of food. When comparing the results of FFA release as observed in Chapter 4, a correlation between duodenal concentration of FFA and gallbladder volume was found, but not with CCK plasma concentration. Therefore, it was speculated that hormonal induced satiety feelings might have been overruled by other mechanisms, such as gastric distention.

In **Chapter 6,** the influence of emulsion properties and the *in vitro* digestion models used, with attention to their complexity, on lipolysis were discussed. In addition, the effect of gastric passage of emulsions with respect to the creaming behaviour was discussed with findings from a study in a pig model. Also, the impact of lipid digestion products on satiety and the influence of satiety hormones were discussed.

Summary

Samenvatting

Zusammenfassung

In een westers voedingspatroon vormen vetten een belangrijk bestanddeel. Ze worden voornamelijk geconsumeerd als emulsie; aanwezig in bewerkte voedingsmiddelen. Met het oog op de toegenomen incidentie van obesitas, zou overconsumptie van voedsel voorkomen kunnen worden door het gebruik van speciaal ontworpen emulsies, die het verzadigingsgevoel bevorderen en daarmee de eetlust helpen controleren.

In **hoofdstuk 1** introduceren we de regulatie van verzadigingsreacties, die optreden na inname en vertering van vetten. De focus ligt op de regulering van de maaglediging en de hormonen die hierbij betrokken zijn, zoals is waargenomen in verschillende *in vivo* studies. Huidig inzicht toont aan dat afbraakproducten van vet, d.w.z. vrije vetzuren (FFA), zeer waarschijnlijk verantwoordelijk zijn voor vet-geïnduceerde afgifte van hormonen zoals CCK en voor de verzadigingsreacties die gedetecteerd worden in het intestinale (darm) spijsverteringsstelsel. Niettemin, toont het ook aan dat deze kennis vooral wordt opgedaan bij de intestinale intubatie studies en dat weinig onderzoek is gedaan waarbij vertering in de maag is toegevoegd. Door het maag spijsverteringsstelsel mee te nemen blijken aspecten zoals maag-lipolyse en de colloïdale toestand van de vetten in de regulatie van verzadigingsreacties zeer belangrijk te zijn. De combinatie van deze aspecten is echter nooit onderzocht of tonen inconsistente resultaten. Een vergelijkbare situatie is waargenomen bij *in vitro* modellen die recentelijk zijn gebruikt om het mechanistische gedrag van emulsies te begrijpen. Bij aanvang van dit project lag de focus in de literatuur op de intestinale vertering van lipiden en vooral op het vrijkomen van FFA, maar niet op monoglyceriden (MG) of diglyceriden (DG). Bovendien waren er slechts een aantal publicaties waarbij de vertering in de maag werd meegenomen, maar waarbij de specifieke vorm of functie van de maag werden verwaarloosd of op een simpele manier nagebootst.

Hoofdstuk 2 is gericht op het vrijkomen van FFA in relatie tot de emulsie-eigenschappen, door middel van een veelgebruikte statisch *in vitro* model, de pH-stat-methode. Emulsies van verschillende druppelgrootte, (fijne, medium en grof), gestabiliseerd door wei-eiwit isolaat (WPI) of arabische gom (GA) werden verteerd onder intestinale condities. Gaschromatografie (GC) werd gebruikt om de afbraakproducten te weten: FFA, MG, DG en triglyceriden (TG) te bepalen. Uiteindelijk, bleek met GC analyse dat met toenemende druppelgrootte de concentraties van FFA, MG en DG af namen. Aangetoond werd dat de afbraak van emulsies, gestabiliseerd door een eiwit-polysaccharide emulgator (bijvoorbeeld GA), resulteerde in hogere FFA afgifte dan van emulsies gestabiliseerd met een eiwit (bijvoorbeeld WPI). Bovendien, resulteerde GA-gestabiliseerde emulsies in een snellere hydrolyse van DG naar MG in vergelijking met

WPI gestabiliseerde emulsies. De concentraties FFA, die bepaald zijn met GC, waren 2-3 maal hoger dan de concentraties bepaald met pH-stat voor WPI gestabiliseerde emulsies. Doordat de bepaling van FFA met de pH-stat werd beïnvloed door het type emulgator, moeten de resultaten zorgvuldig geïnterpreteerd worden en met andere methoden zoals GC gecombineerd worden.

In **hoofdstuk 3** wordt het effect bestudeerd van maag passage op eiwit gestabiliseerde emulsies en hun lipolyse, beïnvloed door zowel geleidelijke verzuring en maag eigenschappen. Hiervoor werd een dynamisch *in vitro* model (TNO gastrolintestinale Model) geïntroduceerd met een speciaal ontworpen maag compartiment. Om het effect van flocculatie op het isoelectrische punt te bepalen, werd WPI of lysozym gebruikt als emulgator. Voor de karakterisering werden microscopie, deeltjesgrootte analyse en GC gebruikt. Onder de in de maag aanwezige condities werden WPI-gestabiliseerde emulsies, op het moment dat het eiwit langs haar iso-electrisch punt komt, gescheiden in een crème en serum laag. Microscopie en deeltjesgrootte analyse laten grotere geflocculeerde deeltjes zien voor de aanvankelijk negatief geladen WPI-gestabiliseerde emulsies in vergelijking met de positief geladen lysozym-gestabiliseerde emulsies. Vooral FFA en DG waren aanwezig in het maag compartiment, wat typisch is voor de hydrolyse van maag lipase. Daarbij onthulde analyse van de serum laag de aanwezigheid van FFA, die mogelijk de afgifte van CCK *in vivo* kan induceren. Het opromen veroorzaakt een vertraagde toegang van vetten in de dunne darm. Niettemin, bleek de totale FFA concentratie in het maag compartiment aan het einde van het experiment gelijk te zijn voor beide emulsies. Het effect van opromen of de aanwezigheid van FFA in de serum laag op maaglediging kan echter niet door het model worden bepaald.

In humane studies, beschreven in **hoofdstuk 4 en 5**, onderzochten we verschillende aspecten van vetten zoals de toediening, de spijsvertering en de bijbehorende fysiologische reacties die beïnvloed zijn door de fysische eigenschappen van de vetten. In **hoofdstuk 4** hebben we ons gericht op de invloed van de intragastrische distributie van vetten op gastrische en duodenale lipolyse. Ook werd gekeken naar het effect van orale toediening op gastrische lipolyse. In een gerandomiseerde, single blind cross-over studie werden bij drie verschillende gelegenheden 15 gezonde proefpersonen nasogastrisch geïntubeerd. Daarnaast werd op twee verschillende gelegenheden een subset van 6 gezonde proefpersonen naso-duodenaal geïntubeerd. Vrijwilligers kregen oraal of door maag infusie een gehomogeniseerde emulsie toegediend, of een gastrische infusie van water gevolgd door de oliefase (niet-gehomogeniseerd monster). De subset van 6 vrijwilligers kregen een maag infusie van een gehomogeniseerde emulsie of een

gastrische infusie van een niet-gehomogeniseerde monster. De structuur van de emulsie, de pH en de samenstelling van de vetten werden gemeten in zowel gastrische als duodenale vloeistoffen. In ca. 3% van alle behandelingen werd vertering van vetten in de maag (maag lipolyse) gevonden en waren de belangrijkste producten FFA en DG. De concentraties van FFA in duodenale aspiraten waren hoger na toediening van gehomogeniseerde emulsies dan na niet-gehomogeniseerde monsters ($p < 0,05$). Maaglediging was sneller voor de niet-gehomogeniseerde monsters dan voor de gehomogeniseerde emulsies, zoals bepaald is door plasma paracetamol concentraties. In zijn algemeenheid, was er geen effect waargenomen van orale verwerking. Ook homogeniseren had geen effect op maag lipolyse. Wel zijn er verschillen in remming van de maaglediging waargenomen: Duodenal release van FFA en de remming van de maaglediging deden zich eerder voor bij gehomogeniseerde emulsies dan bij niet-gehomogeniseerde monsters.

Vervolgens werd in **hoofdstuk 5** onderzocht of de doorgevoerde effecten relevante reacties op de spijsvertering en verzadigingsperceptie van vetten zou beïnvloeden. In dezelfde opstelling als beschreven in hoofdstuk 4, werden galblaas volume, plasma CCK, PYY, GLP-1 en ghreline concentratie, verzadigingswaarderingen zoals vastgesteld d.m.v. een visuele analoge schaal, en *ad libitum* inname geanalyseerd. De resultaten toonden aan dat de behandelingen van grote invloed zijn op het volume van de galblaas ($p < 0,001$). Meer specifiek, de meest langdurige samentrekking van de galblaas werd gevonden wanneer de gehomogeniseerde emulsie oraal werd toegepast. Wanneer daarentegen, het niet-gehomogeniseerde monster intragastrisch werd toegediend, was de samentrekking van de galblaas het laagst. Daarnaast hebben we aanzienlijke effecten gevonden m.b.t. de tijd en de behandeling op circulerende hormoon concentraties. In het geval van plasma CCK, waren de concentraties 15 minuten na toevoeging van de gehomogeniseerde emulsie significant hoger in vergelijking met het niet-gehomogeniseerde monster. De concentraties voor PYY waren na 30 minuten significant hoger voor de gehomogeniseerde emulsie in vergelijking met het niet-gehomogeniseerde monster. De respons van PYY en GLP-1 op de niet-gehomogeniseerde monsters werden ongeveer 20 minuten vertraagd ten opzichte van de gehomogeniseerde emulsie. Over de onderdrukking van ghreline werd echter slechts een effect van tijd, maar niet van de behandelingen gevonden. Ook de perceptie van verzadiging en *ad libitum* inname waren niet verschillend voor de behandelingen. Toch was het gevoel om meer te eten sterker voor de gehomogeniseerde emulsies in vergelijking met de niet-gehomogeniseerde monsters. Er werd geconcludeerd, dat de intragastrische verdeling van vetten een effect heeft op plasma

hormoonconcentraties en galblaasmotoriek. Deze effecten werden niet gezien in de verzadigingspercepties en de *ad libitum* inname. Derhalve staat de afgifte van gastrointestinale hormonen niet in rechtstreeks verband met het verzadigende effect van voedsel. Bij vergelijking van de resultaten van de vrijgekomen FFA, zoals waargenomen in hoofdstuk 4, werd er wel een correlatie gezien tussen de FFA concentratie in de twaalfvingerige darm (duodenum) en het volume van de galblaas, maar niet met de CCK plasma concentratie. Daarom werd gespeculeerd dat hormonaal geïnduceerde verzadigingsperceptie overheerst kunnen worden door andere mechanismen, zoals maagdistentie.

In **hoofdstuk 6**, werd de invloed van emulsie eigenschappen en de gebruikte *in vitro* digestiemodellen, met aandacht voor hun complexiteit, op lipolyse besproken. Bovendien werd het effect van emulsies, in het maag verteringsstelsel, met betrekking tot hun romigheid besproken met behulp van de bevindingen van een studie in een varkensmodel. Daarnaast werden de effecten van vet digestieproducten op verzadiging en de invloed van verzadigings hormonen besproken.

Summary

Samenvatting

Zusammenfassung

Fette sind Schlüsselkomponenten unserer Nahrung. Da, insbesondere in der westlichen Ernährung, gewöhnlich hochverarbeitete Lebensmittel aufgenommen werden, sind diese Fette hauptsächlich in Form von Emulsionen vorzufinden. So können im Hinblick auf die aktuelle Prävalenz von Adipositas speziell erzeugte Emulsionen, die die Sättigung und das Sättigungsgefühl beeinflussen, eine Strategie darstellen, um einen übermäßigen Verzehr von Lebensmitteln vorzubeugen.

In **Kapitel 1** wird die Regulation einer Sättigungsreaktion während Fettaufnahme und –verdauung betrachtet. Der Focus liegt hierbei auf der Regulation der Magenentleerung und den daran beteiligten Hormonen, wie sie aus verschiedenen *in vivo* Untersuchungen bekannt sind. Nach derzeitigem Kenntnisstand sind lipolytische Produkte, wie z.B. freie Fettsäuren (FFS) hauptverantwortlich für die Ausschüttung von Hormonen, wie CCK, sowie für die resultierenden Sättigungsreaktionen, wenn diese FFS im Darm detektiert werden. Dennoch zeigt sich, dass diese Erkenntnisse vor allem auf intestinalen Intubationsstudien beruhen und bisher nur wenige Untersuchungen den Magenbereich einbeziehen. Diese wenigen Untersuchungen zeigen die Bedeutung der Lipolyse im Magen und des kolloidalen Zustands der Fette in der Regulation der Sättigung. Bisher sind diese Aspekte jedoch nicht in Kombination untersucht und es zeigen sich widersprüchliche Ergebnisse. Ähnlich verhält es sich mit *in vitro* Modellen, die derzeit verwendet werden, um ein grundsätzliches Verständnis des Verhaltens von Emulsionen zu erhalten. Als diese Untersuchungen begannen, lag der Fokus in der Literatur auf der intestinalen Verdauung von Fetten und hauptsächlich auf der Ausschüttung von FFS aber nicht auf weiteren Produkten, wie Monoglyceriden (MG) und Diglyceriden (DG). Weiterhin schlossen nur wenige Studien die Verdauung im Magen in ihre Überlegungen mit ein. Wenn doch, wurde die spezifische Form und die Funktion des Magens vernachlässigt oder vereinfacht nachgeahmt.

In der Studie in **Kapitel 2** wurde die Freisetzung von FFS in Abhängigkeit von den Eigenschaften der Emulsion mit Hilfe eines gängigen statischen *in vitro* Models, der pH-stat Methode, untersucht. Emulsionen, die sich hinsichtlich ihrer Tropfengröße unterschieden (fein, mittel und grob) und mittels Molkenproteinisolat (MPI) oder Gummi Arabicum (GA) stabilisiert waren, wurden unter intestinalen Bedingungen verdaut. Mittels Gaschromatographie (GC) wurden die Produkte der Lipolyse, wie FFS, MG, DG und Triglyceride (TG) untersucht. Insgesamt zeigten die Untersuchungen mittels GC

geringere Konzentrationen an FFS, MG und DG bei gleichzeitig ansteigender Tropfengröße. Es konnte gezeigt werden, dass die Verdauung von Emulsionen, die mittels eines Protein-Polysaccharid-Emulgators (GA) stabilisiert wurden, zu einer höheren Freisetzung von FFS führten, als Emulsionen die mittels eines proteinartigen Emulgators (MPI) stabilisiert wurden. Weiterhin konnte bei mittels GA stabilisierten Emulsionen eine schnellere Hydrolyse von DG zu MG im Vergleich zu MPI-stabilisierten Emulsionen beobachtet werden. Die Konzentration an FFS war bei MPI-stabilisierten Emulsionen bei der Messung mit Hilfe der GC zwei- bis dreimal höher im Vergleich mit den Ergebnissen der pH-stat-Methode. Da die Bestimmung von FFS mittels pH-stat durch die Art des Emulgators beeinflusst wird, lässt sich schlussfolgern, dass die Ergebnisse der pH-stat-Methode mit Vorsicht bewertet und stets mit anderen Methoden, wie der GC-Methode abgeglichen werden sollten.

In **Kapitel 3** werden die Effekte von Magenpassage und Proteinstabilisierung auf die Lipolyse, unter Berücksichtigung der schrittweisen Azidifikation und den Eigenschaften des Magens, betrachtet. Zu diesem Zweck wurde ein dynamisches *in vitro*-Modell (TNO Magen-Darm-Modell) mit einem speziell angefertigten Magenabschnitt eingesetzt. Um die Flockung am isoelektrischen Punkt zu bestimmen, wurden MPI oder Lysozym als Emulgatoren eingesetzt. Zur genaueren Charakterisierung wurden Mikroskopie, Partikelgrößen-Analyse und GC verwendet. Unter den Bedingungen im Magen trennten sich die MPI-stabilisierten Emulsionen am isoelektrischen Punkt des Proteins in Rahm und Serumschicht. Mikroskopie und Partikelgrößen-Analyse zeigten größere ausgeflockte Partikel bei den anfänglich negativ geladenen MPI-stabilisierten Emulsionen verglichen mit den positiv geladenen Lysozym-stabilisierten Emulsionen. Vor allem FFS und DG konnten im Magenabschnitt nachgewiesen werden, welche typische Spaltprodukte der Magenlipase sind. Weiterhin konnten bei der Untersuchung der Serumschicht FFS nachgewiesen werden, deren Vorhandensein *in vivo* möglicherweise zur Ausschüttung von CCK führen könnte. Das Aufrahmen hatte einen geringeren Übertritt von Fetten in das Duodenum zur Folge. Unabhängig davon unterschied sich die Gesamtkonzentration an FFS im Magenabschnitt am Ende der Untersuchung kaum für beide Emulsionstypen. Der Einfluss des Aufrahmens und des Vorhandenseins von FFS in der Serumschicht auf die Magenentleerung kann hier aufgrund der Art des Modells nicht beurteilt werden.

In einer Humanstudie, die in den **Kapiteln 4 und 5** vorgestellt wird, wurden verschiedene Aspekte der Fettverabreichung, Fettverdauung und der damit verbundenen physiologischen Antworten unter Einfluss der physikalischen Eigenschaften der Fette untersucht. Kapitel 4 konzentriert sich dabei besonders auf den Einfluss der Verteilung

des Fettes im Magen auf die Lipolyse in Magen und Duodenum. Weiterhin wurde der additive Effekt der oralen Verabreichung auf die Lipolyse im Magen untersucht. In einer randomisierten, doppel-blinden Cross-Over-Studie wurden 15 gesunde Probanden zu drei unterschiedlichen Zeitpunkten nasogastral intubiert. Zusätzlich wurden 6 Personen dieser Gruppe zu zwei weiteren Zeitpunkten nasoduodenal intubiert. Die Probanden erhielten oral oder direkt in den Magen eine homogenisierte Emulsion oder eine Infusion von Wasser gefolgt von einer Ölphase (unhomogenisierte Probe) direkt in den Magen. Die 6 Probanden erhielten eine Infusion einer homogenisierten Emulsion oder einer unhomogenisierten Probe direkt in den Magen. Die Zusammensetzung der Emulsion, pH-Wert und Fettzusammensetzung wurden in Magen- und Darmflüssigkeit analysiert. In allen Behandlungsgruppen wurde im Magen eine Lipolyserate von ca. 3 % festgestellt. Die Hauptprodukte waren FFS und DG. Die FFS-Konzentrationen waren in der Darmflüssigkeit nach Gabe der homogenisierten Emulsionen höher im Vergleich zu den unhomogenisierten Proben ($p<0,05$).

Anschließend, wie in **Kapitel 5** zusammengefasst, wurde untersucht, ob diese bereits gezeigten Effekte Vorgänge beeinflussen, die wichtig für die Fettverdauung und das Sättigungsempfinden sind. Dafür wurde unter den gleichen Versuchsbedingungen wie in Kapitel 4 bereits beschrieben, das Volumen der Gallenblase sowie die Plasmakonzentrationen an CCK, PYY, GLP-1 und Ghrelin gemessen. Weiterhin wurde der Grad der Sättigung, welcher mittels einer visualen, analogen Skala abgeschätzt wurde, sowie die *ad libitum* Nahrungsaufnahme bestimmt. Die Ergebnisse zeigen einen signifikanten Einfluss der unterschiedlichen Behandlungen auf das Gallenblasenvolumen ($p<0,001$). Die am längsten anhaltende Kontraktion der Gallenblase konnte nach der oralen Gabe der homogenisierten Emulsion beobachtet werden. Im Gegensatz dazu war die Kontraktion der Gallenblase nach der in den Magen verabreichten unhomogenisierten Probe am geringsten. Es konnte weiterhin gezeigt werden, dass die Konzentration der zirkulierenden Hormone in Abhängigkeit von Zeit und Behandlung signifikant beeinflusst wurden. Die Plasmakonzentration an CCK war 15 min nach der Gabe der homogenisierten Emulsion signifikant höher im Vergleich zur unhomogenisierten Probe. Nach 30 min war die Konzentration an PYY bei der homogenisierten Emulsion signifikant höher im Vergleich zur unhomogenisierten Probe. Die Hormonantwort von PYY und GLP-1 auf die Gabe der unhomogenisierten Probe erfolgte etwa 20 min später als die Antwort auf die Gabe der homogenisierten Emulsion. Der Effekt auf die Suppression von Ghrelin war nicht behandlungs- sondern nur zeitabhängig. Auch das Sättigungsempfinden und die *ad libitum* Nahrungsaufnahme unterschieden sich nicht in

Abhängigkeit von der Behandlung. Dennoch war das „Verlangen nach Essen“ nach Gabe der homogenisierten Emulsion stärker im Vergleich zur unhomogenisierten Probe. Schlussfolgernd kann festgestellt werden, dass die Plasmahormonkonzentration und die Motilität der Gallenblase durch die Verteilung der Lipide im Magen beeinflusst werden. Die Effekte spiegeln sich weder im Sättigungsempfinden noch in der *ad libitum* Aufnahme der Nahrung wider. Folglich besteht zwischen der Ausschüttung von gastrointestinalen Hormonen und dem Sättigungseffekt von Lebensmitteln kein direkter Zusammenhang. Betrachtet man gleichzeitig die Ergebnisse zur FFS-Ausschüttung aus Kapitel 4, zeigt sich eine Korrelation zwischen der duodenalen Konzentration an FFS und dem Gallenblasenvolumen. Die Konzentration an FFS korrelierte jedoch nicht mit der Plasmakonzentration an CCK. Demzufolge wird das hormonal induzierte Sättigungsgefühl vermutlich durch andere Mechanismen, wie die Magendehnung, überdeckt.

Kapitel 6 beschreibt den Einfluss der Emulsionseigenschaften und der eingesetzten *in vitro* Modelle auf die Lipolyse, mit besonderem Augenmerk auf deren Komplexität. Weiterhin wurde der Effekt des Magendurchflusses der Emulsionen hinsichtlich des Aufrahmverhaltens mit den Ergebnissen einer Studie an Schweinen diskutiert. Auch auf die Bedeutung der Produkte der Fettverdauung und den Einfluss der Sättigungshormone für das Sättigungsgefühl wurde in diesem Kapitel eingegangen.

Acknowledgements

The Netherlands, and in particular Wageningen, was not only a place where I did my PhD-work for the last 4 years but also felt like home thanks to a lot of special people. I gratefully acknowledge all your direct and indirect support throughout these last years.

Dear Harry and Rob, your advices and support helped me growing a lot in the past and I feel very honoured to have you both as my promotors. I admire your critical way of looking at my work and manuscripts, and your straightforward way of working. Above all, you supported not only my work but also my private life, for which I am deeply grateful.

Erika, I appreciate your help in every manner. You were not only a supervisor but became a very good friend to me. Especially our work in Maastricht challenged us a lot and brought us very close together. Thanks for all your good advices and endless patience, especially in how to get my thoughts on paper.

I would like to acknowledge all members of the B-one007 TIFN project for their contribution to this book. Raymond thank you for giving me this opportunity to start this great project, George, Lex, Eric, Franklin, Esther, Rob, Mans, Jeroen, Monique, Marijke – our meetings and discussions have been inspiring and I also very much appreciate all your practical contributions. Marijke, special thanks goes to you as being my FCH-buddy in the first year and now for being my paranymph. I loved the chats, sharing the lab and my leisure time with you! Thanks for your support met de samenvatting! Jolan, it was very nice to share the lab with you and thank you for giving me several rides to NIZO. Eefjan, Rob and Peter, thanks for going through the ups and downs of endless GC sample analysis with me. Harold, I would like to thank you again and again for your statistical expertise – we both got grey hairs from it.

My dear 509-roomies Rudy, Jun, Jianwei, Marijn, Uttara, Milou, Ya and Stéphanie – it was a pleasure to sit with you in an office and to share the life as a PhD in detail and in general. Thanks for all your supporting discussions! Stéphanie, I feel very honoured that you will go up the last stairs with me as my paranymph.

Martine, Amrah, Simone, Anja, Book and Ruud: organising such a great PhD trip to Switzerland/Italy was a wonderful experience and I am proud that I could do that with you together. Jolanda, I would like to thank you very much for helping with all your organising and managing support!

I am very grateful for all assistance I received at Maastricht University. Ad Masclee and Freddy Troost, thanks for giving us the opportunity to collaborate in the human study. Ronald, Daniel, Samefko and Mark thanks for your support and the great time during our study and for showing us around in Maastricht.

Meine lieben german buddies Anja, Anita, Stefan, Kerstin und Wibke, mit euch habe ich tolle Freunde gefunden auch über die PhD-Zeit hinaus. Susann, ohne dich wäre ich wahrscheinlich gar nicht nach Holland gegangen – danke für alles!! Simone, Yvonne, es war eine große Freude, mit euch zusammen Holland zu entdecken.

Vier Jahre in Holland waren eine lange Zeit und trotzdem sind unsere Freundschaften geblieben – ich drücke euch alle und danke euch dafür!! Jule – ohne dich wär alles doof – und danke, dass du dich auch mit meiner Zusammenfassung auseinandergesetzt hast. Nicole, Thomas, Steph, Steffi, Janni, Sandy, Matthias – danke für eure Besuche, damit habt ihr mir immer ein Stück Heimat vorbeigebracht.

Meine liebe Mutti und mein lieber Vati, danke für euer Vertrauen, eure Zuversicht und eure Unterstützung, und das nicht nur in den letzten Jahren – ihr seid großartige Eltern. Meine liebe Ina, ich bin stolz darauf, so eine tolle Schwester zu haben, und Pierre und Louis ich bin unendlich froh, dass es euch gibt. Liebe Margit, ich möchte dir für deine Unterstützung vor allem in der letzten Zeit danken, da du uns, auch zusammen mit Olaf, viele Dinge erleichtert hast.

Franky, die letzten Jahre wären ohne dich sehr leer gewesen und zusammen haben wir diese Zeit durch Höhen und Tiefen bewältigt. Danke für deine unendliche Geduld und deine Sicht der Dinge – es rückt alles wieder gerade. Meine kleine Laura, noch kannst du nicht lesen, aber du sollst wissen, dass du mein größter Schatz bist und ich sehr stolz auf dich bin.

About the author

Curriculum Vitae

Anne Helbig was born on the 12th of June 1980 in Halle/Saale, Germany. After graduating from high school (Torgymnasium, Halle/Saale) in 1999 she started training at St. Elisabeth and St. Barbara hospital (Halle/Saale). She obtained a degree as a nurse in 2002 and started studying Nutrition Science at Friedrich-Schiller-University in Jena in the same year while continuing working as a nurse at the Internal Medicine Department (St. Elisabeth- and St. Barbara hospital, Halle/Saale). Before leaving to the Netherlands, she went to the German Institute of Food Technology (Quakenbrück) for her Diploma thesis entitled "Development of sugar reduced sweets using double emulsions of the w/o/w type". In 2008 she graduated in Nutrition Science and started her PhD thesis at the Laboratory of Food Chemistry (Wageningen University, The Netherlands) and Top Institute Food and Nutrition (TIFN). Her research was part of the TIFN project "Engineered sensory and dietary functionality of dispersed fat". The results of her PhD project are described in this thesis.

List of publications

Helbig, A., Silletti, E., Timmerman, E., Hamer, R.J., Gruppen, H. (2012) *In vitro* study of intestinal lipolysis using pH-stat and gas chromatography. *Food Hydrocolloids*, 28(1), 10-19.

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Overview of completed training activities

Discipline specific activities

- International advanced course food intake regulation - Nutrient sensing, VLAG, Maastricht, The Netherlands, 2008
- Food hydrocolloids - Fundamentals and application, VLAG, Wageningen, The Netherlands, 2009
- Nutritional and lifestyle epidemiology, VLAG, Wageningen, The Netherlands, 2011
- Delivery of functionality in complex food systems, Wageningen, The Netherlands, 2009
- Food colloids - On the roads from interfaces to consumers, Granada, Spain, 2010
- Training period, Maastricht University, 2010/2011

General courses

- PhD week, VLAG, Bilthoven, The Netherlands, 2008
- Project & time management, WGS, Wageningen, The Netherlands, 2009
- Scientific writing, WGS, Wageningen, The Netherlands, 2009
- PhD competence assessment, WGS, Wageningen, The Netherlands, 2008
- Presentation course, TIFN, Wageningen, The Netherlands, 2009

Additional activities

- Preparation PhD research proposal, Wageningen, The Netherlands, 2008
- BSc/MSc students presentations and colloquia, Wageningen, The Netherlands, 2008-2012
- PhD presentations, Wageningen, The Netherlands, 2008-2012
- Food Chemistry study trip, Ghent, Belgium, 2009
- PhD study trip, China, 2008
- PhD study trip, Switzerland, Italy, 2010
- Member of Organization committee for the PhD study trip to Switzerland/Italy, 2010

The study presented in this thesis was performed within the framework of TI Food and Nutrition.

Cover design by Kerstin Kaminski, cover picture oil drop© Graphik 123 RF Michelangelus

This thesis was printed by GVO drukkers & vormgevers B.V. / Ponsen & Looijen, Ede, The Netherlands