Enzymatic and chemical conversions taking place during *in vitro* gastric lipid digestion: the effect of emulsion droplet size behavior

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20 **Journal:** Food Chemistry

21 **Submitted:** November 2019

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Accepted manuscript

Abstract

This investigation reports the effect of droplet size behavior on the overall lipolysis profile and molecular lipolysis mechanisms under *in vitro* gastric conditions. O/W emulsions (5% triolein, 1% sodium taurodeoxycholate) with different initial droplet sizes (fine: 0.58 μm ; medium: 1.82 μm ; and large: 4.00 μm) were subjected to static *in vitro* digestion. For the first time, multiple lipolysis products including diolein and monoolein regioisomers were quantified within a single HPLC run. An inverse relation was found between the droplet size and the initial rate and final extent of lipolysis based on the digested triolein. Furthermore, a mechanistic gastric lipolysis model was established based on a reaction scheme including enzymatic and chemical isomerization conversions. The estimated rate of the *sn*-1/3 hydrolysis was around two- to thirty-fold faster compared to the rates of *sn*-2 cleavage and isomerization, respectively. These findings resulted in a profound insight in *in vitro* gastric molecular lipolysis mechanisms.

Keywords

Gastric lipid digestion; emulsion; *in vitro*; droplet size; gastric lipase; molecular mechanisms

1. Introduction

Lipid consumption has diverse health-related implications. On the one hand, these compounds provide energy and essential nutrients important for conservation of a good health status. On the other hand, lipid overconsumption may lead to obesity, cardiovascular diseases, diabetes and other metabolic-related disorders, being and more and more becoming a public health issue (Yaqoob, 2013). Therefore, a better understanding of the interrelation between the composition of lipid-based foods, microstructure and digestion is imperative (Dupont *et al.*, 2018). This interrelation is a crucial input to rationally design foods with tailored nutritional functionality, e.g. lipid-based products with prolonged satiety effect (Maljaars *et al.*, 2008).

Lipid digestion is an interfacial process which mainly occurs in the stomach and small intestine. Most researchers have focused on small intestinal lipolysis of emulsions as recently reviewed by McClements (2018). Nowadays, lipid digestion in the stomach is receiving more attention because several physical and (bio)chemical changes occur in this compartment (Bornhorst & Singh, 2014). *In vivo* studies have demonstrated that gastric lipolysis accounts for 5 to 30% of lipid hydrolysis, while the remaining lipids are further cleaved and absorbed in the small intestine (Armand *et al.*, 1999; Carrière *et al.*, 1993). The final extent of gastric lipolysis is relatively low, but important for people with pancreatic disorders who do not produce pancreatic lipase (Layzer & Keller, 2005). Additionally, human gastric lipase (HGL) is an essential enzyme for infant lipid digestion since their pancreas is not yet fully developed at birth. This results in a low secretion of pancreatic lipase, while on the contrary, HGL expression is already fully mature (Bourlieu *et al.*, 2014). Besides, gastric lipolysis stimulates pancreatic lipid digestion by inducing droplet disruption, increasing the solubilization of digestion products, promoting hormone release and augmenting colipase binding capacity of pancreatic lipase (McClements *et al.*, 2008). *In vitro* lipolysis studies in the gastric compartment are scarce until now, due to the unavailability of a relevant and convenient replacement for HGL. However, recently, rabbit gastric lipase (RGL) has shown similarities compared to HGL in terms of

activity level and stereospecificity (Sams *et al.*, 2016). Therefore, RGL appears to be a good alternative for usage in *in vitro* gastric lipolysis experiments.

During the last decades, the influence of emulsion design characteristics on lipid digestion profiles have been studied by means of *in vivo* and *in vitro* methods (Grassby *et al.*, 2017; Guo *et al.*, 2017). However, limited information is available on how gastric lipolysis can be influenced by emulsion design. The size of the emulsified oil droplets is an important emulsion characteristic, affecting lipolysis kinetics as it determines the surface available for lipase adsorption. This emulsion property has been widely studied under *in vitro* small intestinal conditions. Recent studies have confirmed the fact that an inverse relation between droplet size and the rate and extent of small intestinal lipid digestion exist (Liang *et al.*, 2018; Salvia-Trujillo *et al.*, 2017). The effect of emulsion droplet size on gastric lipolysis was demonstrated *in vivo* (Armand *et al.*, 1999) and *in vitro* (Bourlieu *et al.*, 2015), in which gastric digestion of a coarse and fine emulsion resulted in a higher final extent of lipolysis for the smaller droplet-sized emulsion. Nevertheless, a kinetic digestion approach and the quantification of multiple lipolysis products were not considered in these studies. The quantification of different lipolysis products during digestion may be the starting point to propose reactions of (bio)chemical conversions at a molecular level. Such a mechanistic approach could lead to the development of mathematical models describing lipolysis as influenced by emulsion design characteristics. One strategy is known as empirical, single-response modeling because one representative response is selected to explain the overall kinetic behavior through the estimation of kinetic parameters, e.g. rate and final extent of digestion. A more advance strategy considers multiple interrelated responses which may be modeled simultaneously to gain insight into the enzyme-substrate interactions of lipolysis at a molecular level under standardized conditions (Verkempinck *et al.*, 2019). To best of our knowledge, there is an open field of research regarding the mechanistic insight of emulsified lipids digestion under *in vitro* gastric conditions.

For this reason, the present investigation aimed (i) to evaluate the overall kinetic profile of gastric lipid digestion as affected by emulsion droplet size behavior; and (ii) to gain mechanistic insight into the

bio(chemical) conversions based on the quantification of multiple lipolysis products under specific simulated gastric conditions. To this end, emulsions were prepared consisting of pure triolein (5%) and sodium taurodeoxycholate (NaTDC) (1%) and were homogenized at different pressures to obtain distinct initial oil droplet sizes. These emulsions were subjected to static *in vitro* gastric digestion during which independent samples were taken at different time moments to quantify multiple lipolysis products and monitor the microstructure and droplet size behavior.

2. Materials and methods

2.1 Materials

Triolein (>99%) was purchased from Acros Organics (Geel, Belgium) and stored at -20°C with a nitrogen headspace until use. Sodium taurodeoxycholate (NaTDC) (>95%) was acquired from Cayman Chemical Company (MI, USA). Rabbit gastric extract was obtained from Lipolytech (Marseille, France) (measured lipase activity 19.9 ± 1.3 U/mg, using tributyrin as substrate). All other HPLC or analytical grade reagents were purchased from Sigma-Aldrich (Diegem, Belgium), except for NaHCO_3 , NaCl , H_2SO_4 , KH_2PO_4 , ethanol and trimethylamine (Fisher Scientific, Merelbeke, Belgium); KCl , $\text{MgCl}_2(\text{H}_2\text{O})_6$, acetone, heptane and ethyl acetate (Acros Organics, Geel, Belgium); HCl , diethylether and iso-propanol (VWR, Leuven, Belgium); and lipid standards (Larodan, Solna, Sweden).

2.2 Emulsion preparation

Triolein (5% w/w), sodium taurodeoxycholate (1% w/w) and Milli-Q water (94% w/w) were mixed in a high-shear mixer (Silverson L5M-A, Silverson Machines, Inc. Massachusetts, USA) at 8000 rpm for 5 min to obtain a coarse emulsion. This coarse emulsion was further processed in a high-pressure homogenizer (Stansted Fluid Power, Pressure cell homogenizer, U.K.) for one cycle at 15, 40 or 200 MPa, to create a fine emulsion ($d(4,3) 0.58 \pm 0.03 \mu\text{m}$), medium emulsion ($d(4,3) 1.82 \pm 0.02 \mu\text{m}$) and large emulsion ($d(4,3) 4.00 \pm 0.39 \mu\text{m}$), respectively. Triolein was chosen as a substrate because it is a prochiral molecule. Additionally, triolein and its hydrolysis products can be reliably quantified by HPLC because one peak

represents one pure compound in the chromatogram. Moreover, triolein is found in high concentrations in commercial oils such as olive, canola and high oleic sunflower oil (Karupaiah & Sundram, 2007). The criteria for selecting NaTDC as the emulsifier relies on preliminary experiments in which a significant final extent of lipolysis was reached for NaTDC in comparison to other emulsifiers.

2.3 *In vitro* digestion of emulsions

The effect of digestion time on gastric lipolysis during *in vitro* digestion was investigated. The standardized protocol of the international network INFOGEST (Minekus *et al.*, 2014) was employed with minor modifications. This protocol relies on the application of static conditions for each digestive reactor which means that physiological conditions were adjusted solely at the beginning of the gastric phase.

In a brown falcon tube (to avoid lipid oxidation), 5 mL of emulsion was mixed with 5 mL of Milli-Q water to simulate the typical dilution effect of saliva in the oral compartment, 8 mL of simulated gastric fluid set at pH 5.5, and 5 μ L of CaCl₂. Afterwards, Milli-Q water and HCl (2M) were added to reach a pH value of 5.5. Finally, 1480 μ L rabbit gastric extract (RGE) solution containing RGL was added to reach a final chyme volume of 20 mL (RGE was dissolved in Milli-Q water). The use of RGL has been recommended for *in vitro* digestion experiments (Capolino *et al.*, 2011; Sams *et al.*, 2016). The *in vitro* gastric digestion was performed at a static pH value of 5.5 and gastric lipase activity of 20 U/mL of chyme. These values are reported as the most physiological relevant for lipid digestion studies in the gastric phase, since they represent the conditions in a half-emptied stomach of a healthy adult (Carrière *et al.*, 2000; Sams *et al.*, 2016). The pH value of 5.5 was also employed by other authors (Capolino *et al.*, 2011; Couëdelo *et al.*, 2015).

The falcon tube was flushed with nitrogen for 20 s and incubated at 37°C in an end-over-end rotator (40 rpm). A kinetic study was conducted for the *in vitro* gastric phase. In total, seven independent digestion moments (5; 10; 15; 30; 60; 90; 120 min after addition of the gastric lipase) were chosen to study the

influence of initial droplet size on the kinetics of gastric lipolysis. The enzymatic reaction was stopped via chemical inhibition, adding 200 μ L of Orlistat (100 mM in ethanol).

2.4 Particle structural characterization

Initial emulsions and their respective chyme samples taken at 4 gastric digestion moments (after 15; 30; 60 and 120 min in the gastric phase) were characterized in terms of microstructure and particle size analysis. The combination of these two techniques gives a complementary overview of the emulsion stability behavior (Salvia-Trujillo *et al.*, 2017; Verkempinck *et al.*, 2018a).

Microstructure: Samples without dilution were observed with an optical microscope (Olympus BX-41) equipped with an Olympus XC-50 digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan) at 40x of magnification.

Particle size: The particle size distribution was determined using a laser diffraction equipment (Beckman Coulter Inc., LS 13 320, FL, USA). Prior to particle size analysis, a vortex step was applied to homogenize samples. Some drops of sample were added into a stirring tank filled with deionized water. The diluted sample was pumped into the measurement cell where particles scattered the laser light (wavelength main illumination source: 750 nm; wavelengths halogen light for Polarization Intensity Differential Scattering (PIDS): 450 nm, 600 nm, 900 nm). The Mie model was employed to analyze the data (refractive index of triolein is 1.470). The volume-weighted mean particle size $d(4,3)$ of all samples was reported and it was determined in duplicate.

2.5 Quantification of lipolysis species

The kinetics of gastric lipolysis were evaluated based on the release of triolein (TAG) hydrolysis products: *sn*-1,2/2,3-diolein (*sn*-1,2/2,3-DAG); *sn*-1,3-diolein (*sn*-1,3-DAG); *sn*-2-monoolein (*sn*-2-MAG); *sn*-1/3-monoolein (*sn*-1/3-MAG) and oleic acid (FFA) were generated. Therefore, these hydrolysis products were first extracted and then quantified as described below.

For the lipid extraction, the procedure indicated by Verkempinck *et al.* (2018a) was followed. Briefly, a chyme aliquot of 1 mL was mixed with 2 mL of ethanol, 3 mL of diethylether:heptane (1:1) and 0.2 mL of sulphuric acid (2.5 M), subsequently vortexed for 2 min and then subjected to an end-over-end rotation (15 rpm) for 30 min at room temperature. Afterwards, the top layer was collected in a 5 mL volumetric flask. The extraction was repeated adding 1 mL of diethylether:heptane (1:1) to the bottom layer. This was mixed again for 2 min using a vortex and end-over-end rotated for 15 min at 15 rpm. The top layer was collected and added as well to the same volumetric flask, which volume was adjusted to 5 mL with diethylether:heptane (1:1). This extract was immediately filtered (Chromafil PET filters, 0.20 μ L pore size, 25 mm diameter). The lipid extracts were kept at -80°C for a maximum period of one week until analysis.

Regarding the quantification, lipolysis products were separated based on the HPLC method proposed by Graeve & Janssen (2009) with some modifications. The separation was performed in a HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium) equipped with a silica column (Chromolith Performance Si, 100-4.6 mm, Merck, Darmstadt, Germany) which was protected with a guard column (Merck, Darmstadt, Germany) in an external oven (Chromaster 5310, VWR, Hitachi Ltd., Tokyo, Japan) set at 40°C. The running time of the gradient program was 20 min. Lipolysis products were eluted using a quaternary gradient consisting of isooctane (solvent A), acetone:ethyl acetate (2:1 v/v) containing 0.02% (v/v) of acetic acid (solvent B), propan-2-ol:water (85:15 v/v) containing 7.5 % (v/v) of acetic acid and triethylamine (solvent C) and propan-2-ol (solvent D). Analytes were detected in a charged aerosol detector (Corona Veo, Thermo Fisher Scientific, Geel, Belgium) set at 50°C of evaporator temperature and 5.5 bar of gas pressure. The identification of the lipolysis products was performed by aligning their retention times with the ones of individual, high-purity lipid standards (exactly the same compounds as the lipolysis products). For quantification, a standard mixture was injected with different amounts on column to construct calibration curves for each analyte.

The molar balance of the two isomers of diolein (DAG, 620.99 g/mol), two isomers of monoolein (MAG, 656.55 g/mol) and oleic acid (FFA, 282.47 g/mol) allowed the calculation of glycerol (GLY, 92.09 g/mol)

and residual triolein (TAG, 885.43 g/mol) at each digestion moment. Glycerol was calculated based on the following stoichiometric reactions:

The residual amount of TAG (undigested) per digestion moment ($\text{TAG}_{\text{residual}}$) was calculated using the equation below:

$$\text{TAG}_{\text{residual}} = \text{TAG}_{\text{initial}} - (\text{DAG} + \text{MAG} + \text{FFA} + \text{GLY} - \text{H}_2\text{O}) \quad (4)$$

In equation (4), $\text{TAG}_{\text{initial}}$ corresponds to the concentration of triolein in the initial emulsion while the sum of DAG, MAG and GLY refers to all the lipolysis products corrected by the amount of water at each digestion moment. The % of TAG digested was calculated from the concentration of $\text{TAG}_{\text{residual}}$ with respect to the $\text{TAG}_{\text{initial}}$.

2.6 Statistical analysis and single-response kinetic modeling

The change of the volume-weighted mean droplet size along gastric digestion was evaluated. For this, the statistical software JMP (JMP pro14, SAS Institute Inc., Cary, NC, USA) was used to carry out one-way ANOVA and Tukey HSD comparison analyses to determine significant differences at a 5% significance level ($P < 0.05$).

Single-response modeling was performed to evaluate the overall kinetic behavior of gastric lipolysis as affected by droplet size. The software utilized was JMP (JMP pro14, SAS Institute Inc., Cary, NC, USA).

The response selected for modeling was the % of TAG digested during the gastric phase with respect to the initial emulsion. For this purpose, an empirical, fractional conversion model was chosen to model the data and compare the digestion behavior as affected by droplet size. The parameters to be estimated are indicated

in equation (5), where C (%) represents the predicted response at time t during the gastric phase; C_f (%) is the maximum or plateau value of the parameter;; and k (min^{-1}) is the reaction rate constant of the evaluated process.

$$C = C_f (1 - e^{-kt}) \quad (5)$$

The estimated kinetic parameters (C_f (%) and k (min^{-1})) were compared by calculating confidence intervals (95%).

2.7 Multi-response kinetic modeling

Multi-response modeling was applied to the generated data sets to gain insight into the reaction mechanisms of gastric lipolysis. This advanced statistical technique was utilized based on the work of Verkempinck *et al.* (2019). Multiple lipid digestion species, including positional isomers, were quantified using a kinetic digestion approach (indicated in section 2.5). These interrelated experimental data set allowed the establishment of diverse (bio)chemical reaction schemes based on the state-of-the-art knowledge about gastric lipase stereospecificity and isomerization conversions during lipid digestion. Each reaction scheme was transformed into associated differential equations which included the molar concentrations of multiple lipid digestion species (experimental data) and reaction rate constants (parameters to be estimated). Rate constants were estimated using the ‘proc model’ command of the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). Integration of differential equations was done with a variable order, variable step-size backward difference scheme. The method for estimating the parameters was full information maximum likelihood (FIML) while the minimization of the objective function was performed with the Gauss-Newton method. Molar concentrations of all lipolysis products (i.e. DAG, MAG, FFA and GLY) at the starting point of digestion were set equal to zero, except for triolein that was considered equal to the concentration in the initial emulsion ($\text{TAG}_{\text{initial}}$). In the ‘proc model’ command, the ‘fit’ statement was used with standard options and explicitly indicating the ‘dynamic’ option as well as a convergence criterion of 0.01, and the maximum number of iterations equal to 500.

3. Results and discussion

3.1 Changes in structural characteristics along gastric digestion

The volume-weighted average particle size $d(4,3)$ and particle size distribution were assessed for the initial emulsions and chyme samples along gastric digestion (Figure 1). These measurements aimed to evaluate the stability of the generated emulsions during gastric lipolysis. The $d(4,3)$ value for initial emulsions was $0.58 \pm 0.03 \mu\text{m}$ for the fine, $1.82 \pm 0.02 \mu\text{m}$ for the medium and $4.00 \pm 0.39 \mu\text{m}$ for the large emulsion. These particle sizes were significantly different among the initial emulsions. In addition, flocculation is visually observed in the micrographs of all initial emulsions (Figure 1). This phenomenon may have occurred due to the depletion flocculation mechanism which is reversible since the flocs are usually broken when the emulsion is diluted (McClements, 2015). This flocculation reversibility is supported by (i) the particle size measurements of the initial emulsions whereby these emulsions were largely diluted and only low $d(4,3)$ values were measured, not indicating flocculation (Figure 1); (ii) the micrographs of the diluted emulsion during gastric digestion in which only limited flocculation is observed (Figure 1).

It is widely acknowledged that the droplet size of initial emulsions affects not only the rate but also the final extent of lipid digestion during *in vivo* (Armand *et al.*, 1999) and *in vitro* (Bourlieu *et al.*, 2015) experiments. Nevertheless, emulsion stability, or in other words the droplet size created along the digestive tract, also plays an important role on lipid digestion kinetics because the available surface area for lipase adsorption may change drastically (Verkempinck *et al.*, 2018b). For this reason, the structural changes were monitored during the gastric phase. For the three emulsions, it was observed that the smaller the initial particle size, the longer period the droplet size was stable. For instance, the large, medium and fine emulsion mean particle size already significantly changed from the beginning: to be more specific, at the beginning, after 15, and after 30 min of gastric digestion, respectively. As observed in the micrographs, droplet coalescence was the predominant phenomenon causing particle polydispersity (Figure 1). Despite the droplet size behavior during digestion being different, all emulsions reached a large mean particle diameter of 42-63 μm at the end of the gastric phase (Figure 1). These results are in alignment with previous studies

reported by other authors who employed acid-unstable emulsifiers during the gastric phase (e.g. sucrose esters (Verkempinck *et al.*, 2018a); CITREM (Lamothe *et al.*, 2019)). Emulsions formed with NaTDC seemed to be affected by the acidic pH value (5.5) leading to destabilization after 2 hours of simulated gastric digestion only. However, the initial droplet sizes generated in these experiments determined different stability behaviors during gastric digestion which could result in variable lipolysis patterns which was studied and discussed on following sections.

3.2 Evolution of lipolysis species during *in vitro* gastric digestion

In this research, multiple lipid digestion products from the prochiral substrate triolein, including DAG and MAG regioisomers, were detected and quantified within a single HPLC-CAD run. The enantiomers *sn*-1,2 and *sn*-2,3-DAG as well as *sn*-1 and *sn*-3 MAG were detected together in a single peak since the utilized silica column is not able to resolve optical isomers. These compounds will be referred to as *sn*-1,2/2,3-DAG and *sn*-1/3-MAG. On the contrary, their correspondent regioisomers, namely *sn*-1,3-DAG and *sn*-2-MAG, could be separated and quantified. In the case of the large emulsion, MAG regioisomers were identified, but not quantified because their concentration was below the quantification limit. To the best of our knowledge, this is the first study evaluating multiple gastric lipolysis products as function of gastric digestion time using a relevant substitute of human gastric lipase.

Regardless the initial droplet size, a fast hydrolysis of triolein in the first 30 min of digestion can be observed in Figure 2A which resulted in the formation of oleic acid, a small amount of glycerol, and the two regioisomers of DAG and MAG in case of the fine and medium emulsion. It is generally accepted that gastric lipase has a preference for the *sn*-3 position. This means that the *sn*-3 position is preferentially cleaved but activity over the *sn*-1 position has been also reported, however, occurring in a lesser extent. This selectivity has been reported for gastric lipases of different sources, such as human, dog and rabbit (Carrière *et al.*, 1997; Rogalskas, Ransac, & Vergers, 1990). In our study, rabbit gastric lipase was employed, which hydrolyses the *sn*-1/3 positions generating a racemic mixture of *sn*-1,2 and *sn*-2,3-DAG. We link the reduced formation of the latter compound to rabbit gastric lipase preference for *sn*-1 position.

Furthermore, a considerable amount of *sn*-2-MAG, generated from *sn*-1,2/2,3-DAG, was quantified in the first minutes of digestion. The presence of this analyte indicates the preference of gastric lipase towards the *sn*-1 position of *sn*-1,2-DAG. This change in preference of gastric lipase (*sn*-1 for triglycerides and *sn*-3 for diglycerides) is called reverse stereospecificity (Carrière *et al.*, 1997). With respect to *sn*-1,3-DAG; *sn*-1/3-MAG and GLY, their early production may indicate that rabbit gastric lipase has certain affinity for the *sn*-2 position as also suggested by Carrière *et al.*, (1991) based on early glycerol formation.

We hypothesize that due to the generation of oleic acid clusters, which restrict the access of gastric lipase to the TAG core of the oil droplet, the enzymatic conversion of triolein was limited from 30 min of simulated gastric digestion onwards (Pafumi *et al.*, 2002) (Figure 2A). Consequently, gastric lipase might have only been able to approach substrates in the neighborhood of the interface which was mainly composed of accumulated lipolysis products. These substrates were primarily the intermediate products DAG and MAG isomers, which could be further converted from 30 min gastric phase onwards to produce oleic acid and glycerol. The above explained conversions only occurred in case of the emulsions with the smaller initial particle sizes (i.e. fine and medium emulsion), while the large emulsion seemed to have no further conversion of DAG, probably due to saturation of FFA at the interface. DAG and MAG hydrolysis by rabbit and human gastric lipase was also observed during *in vitro* experiments at pH 5.0 (Carrière *et al.*, 1991).

A clear effect of the droplet size on the time dependent evolution of multiple lipid digestion products was observed during *in vitro* gastric digestion (Figure 2). This effect on each lipolysis product is explained as follows: (i) In the case of *sn*-1,2/2,3-DAG; this was generated to a larger extent for the emulsions with the smaller initial droplet sizes. After its formation, DAG was probably cleaved into other lipolysis products for the fine and medium, but not for the large emulsion. This might be explained by the higher entrapment extent of gastric lipase in oleic acid clusters reached by the larger droplet size in the large emulsion stopping all biochemical conversions (Figure 2B). (ii) For *sn*-1,3-DAG; a similar trend compared to *sn*-1,2/2,3-DAG is observed. However, in case of large emulsion, the *sn*-1,3-DAG concentration was considerably higher compared to the concentration levels detected for smaller droplet sizes after 30 min of digestion (Figure

2C). As mentioned before for the large emulsion, it seems that no enzymatic conversions occurred after 30 min of gastric lipid digestion. Nonetheless, production of *sn*-1,3-DAG was observed after 30 min and might be explained by the non-enzymatic isomerization of *sn*-1,2/2,3-DAG into *sn*-1,3-DAG. This isomerization reaction could probably occur for the fine and medium emulsion as well but was not explicitly observed from the experimental data as the gastric lipase was still active in latter cases. Isomerization of fatty acids attached to the *sn*-2 position towards the outer positions was reported before as FFA at the *sn*-2 position are unstable (Serdarevich, 1967). (iii) With regard to *sn*-2 and *sn*-1/3-MAG, they reached higher concentrations for the fine compared to medium emulsion which can be related to the available surface area for lipase adsorption during digestion. After 15 min of digestion, the MAG isomers started to be hydrolyzed but the conversion was limited for medium emulsion samples due to product inhibition (Figure 2D and E). (iv) In the case of oleic acid, an inverse relation between rate/final extent of their synthesis and the initial droplet size of the emulsions was evidenced (Figure 2F). Additionally, it is interesting to notice that oleic acid production for the fine and medium emulsion samples did not reach a plateau when TAG hydrolysis seems to level off (i.e. after 30 min gastric digestion). This is attributed to the continued hydrolysis of DAG and MAG by gastric lipase. A slower hydrolysis was observed for the medium emulsion samples due to the smaller available surface area compared to the fine emulsion. Regarding oleic acid formation for the large emulsion, its production seems to reach an asymptotic value from 30 min of digestion probably because gastric lipase was inhibited. (v) Finally, glycerol was produced in relatively similar concentrations for the three droplet sizes during the first 30 min of simulated gastric digestion. Hereafter, glycerol generation seemed negligible for the large emulsion, while for the medium and fine emulsion, an increase was observed even after 30 min of gastric lipolysis (Figure 2G). Due to a larger available surface area for lipase adsorption, leading to a retarded product inhibition, this increase was more pronounced for the fine emulsion.

The data presented in this section shows interesting trends for lipolysis products derived from *in vitro* gastric digestion experiments. The application of the kinetic approach used in this study and the

quantification of multiple lipolysis species resulted in a data set with interrelated responses, part of a common reaction network. Therefore, we used different strategies to integrate the data coming from these different responses. In Section 3.3, this is done at the level of calculating the % of digested TAG including the quantified responses of the different lipid digestion products. This calculated % of digested TAG was modelled with single-response kinetic modelling. In Section 3.4, since the different lipolysis products quantified can be interlinked in a common reaction network, multi-response kinetic modelling techniques were applied to integrate the information and to build a mechanistic understanding on the (bio)chemical reactions taking place.

3.3 Single-response kinetic modeling of triolein hydrolysis

A single-response approach was utilized to evaluate the impact of emulsion droplet size on the overall kinetic behavior of gastric lipolysis under *in vitro* conditions. The percentage of digested TAG was selected as the most representative response for this purpose because TAGs constitute the main substrate for gastric lipase. This response was modelled using an empirical two-parameter fractional conversion model (Equation 5). It is important to consider that particle sizes of the three emulsions did not remain constant during digestion, but different profiles were created depending on the initial particle size (Figure 1). Specifically, extended stability periods were observed when the initial droplet size was smaller. This particle size behavior affected the rate and final extent of digestion as discussed below.

As observed in Figure 2H, the droplet size behavior during digestion impacted the reaction rate r of TAG hydrolysis. The initial rate of hydrolysis r (slope of model curve tangent at $t=0$) was considerably higher for smaller droplet sizes (i.e. fine and medium emulsion) in comparison to larger droplet sizes (i.e. large emulsion) because the droplet size remained stable for a longer period and was lower during the whole digestion phase (Table A, Supplementary material; Figure 1). The fact that the magnitude of r was higher for smaller droplet sizes may be related to the larger surface available for lipase adsorption during the first period of lipid digestion. Similar observations were reported when pancreatic lipase was utilized to digest emulsions with different initial droplet sizes to study kinetics of small intestinal lipid digestion (Majeed *et*

al., 2016). Although the k value for the medium emulsion was significantly higher compared to other droplet sizes, this parameter was comparable for the three cases in practical terms.

A clear impact of the initial droplet size on the final extent of gastric lipolysis (asymptotic value) could be observed. In this sense, a higher final extent of gastric lipolysis was observed for emulsions with the smaller initial oil droplet size (FE > ME > LE). Different digestion final extent values among the three emulsions may be explained by the combined effect of product inhibition and available surface at the interface. Product inhibition occurred in the gastric phase as bile salts are not present in this digestive compartment, therefore lipolysis products cannot be removed and accumulate at the interface entrapping gastric lipase (mainly by oleic acid). (Pafumi *et al.*, 2002). This effect is reached faster for larger droplets since a lower amount of FFA is needed to cover the droplet surface area with these clusters (FE > ME > LE). In other words, if the available surface area is decreased due to instability phenomena, the critical concentration of oleic acid at the interface to inhibit gastric lipase is reached faster. Similar trends with regard to the impact of initial droplet size on *in vitro* (Pafumi *et al.*, 2002) and *in vivo* (Armand *et al.*, 1999) gastric lipolysis final extent have been reported in literature. Despite both studies quantified lipids during digestion, they did not employ independent reactors. Moreover, kinetic modeling was not applied in these studies which limits the possibilities to compare kinetic parameters for different samples. Even though product inhibition occurred, the final extent of digestion reached values between 5.1 and 12.4% for the three generated emulsions (Table A, Supplementary material). This final extent of gastric digestion is physiological relevant compared to values obtained in *in vivo* studies (Armand, 2007; Carriere, *et al.*, 1993).

It is widely acknowledged that lipid digestion consists of a complex set of sequential and parallel (bio)chemical conversions. The empirical, single-response modeling applied in this section or other basic statistical techniques do not allow to gain mechanistic insight into the interconversions of the lipolysis products. For this reason, a more advanced technique, multi-response modeling, was employed. This statistical analysis was recently utilized by our research unit for the first time in the context of small intestinal lipolysis as impacted by emulsion design properties (Verkempinck *et al.*, 2019). In our current

study, we aim to elucidate the gastric digestion molecular mechanisms under *in vitro* conditions by the used of multi-response modeling. In Section 3.4, detailed information is given about the process of multi-response modeling of gastric lipolysis data as affected by droplet size.

3.4 Multi-response kinetic modeling to elucidate gastric lipolysis mechanisms

As explained in section 3.2, multiple lipolysis products were identified and quantified showing conversion patterns which can be simultaneously modelled as they are part of a common reaction network. Multi-response modeling allowed the elucidation of reaction mechanisms occurring during lipid hydrolysis via the estimation of kinetic parameters of a set of (bio)chemical reactions. (Verkempinck *et al.*, 2019). For this purpose, reaction schemes were proposed based on the information available in literature about gastric lipase stereospecificity, other relevant chemical reactions and our experimental data. After proposing a reaction scheme, all chemical equations were transformed into differential equations including kinetic parameters (reaction rate constants and final extent of digestion). These parameters were estimated by the numerical solution of the differential equations. In the following sub-sections, reaction schemes will be step-wise adapted based on how adequate the kinetic model fits the experimental data. These adaptations are made considering (i) the model convergence, (ii) the adjusted determination coefficient (R^2_{adj}) from the experimental and predicted values (parity plots), (iii) the residual plots and (iv) the errors of the parameter estimates. If one of these conditions are not fulfilled, a new reaction scheme will be proposed and evaluated again. This process in data modeling is known as an iterative approach and indispensable in multi-response modeling. The three data sets (fine, medium and large emulsion) were initially considered in the modeling process.

3.4.1 Hypothesis 1: Gastric lipase can hydrolyze *sn*-3 and *sn*-1 position of the glycerol moiety, and isomerization reactions also occur

This first hypothesis was proposed relying on (i) the generally accepted theory about gastric lipase ability to hydrolyze fatty acids at the *sn*-3 and *sn*-1 positions (Carrière *et al.*, 1997); (ii) the glyceride isomerization

reaction from *sn*-2 to *sn*-1/3 positions (Serdarevich, 1967); and (iii) the experimental data obtained in this study.

Scheme 1: (Bio)chemical reactions postulated from hypothesis 1.

Reaction scheme 1 considers biochemical conversions postulated based on the gastric lipase stereospecificity for the outer positions of the glycerol moiety. If we assumed that only enzymatic hydrolysis reactions take place, this first scheme would only include the first two reactions. In other words, the final products would be *sn*-2-MAG and FFA, which cannot be further hydrolyzed. However, the products formed in the first two reactions do not completely match the ones identified in our experiments. Therefore, two chemical isomerization reactions involving the rate constants k_3 and k_4 were included to form *sn*-1,3-DAG and *sn*-1/3-MAG from *sn*-1,2/2,3-DAG and *sn*-2-MAG, respectively. These isomerization reactions form intermediate products that can be further hydrolyzed to eventually generate glycerol. After these considerations, all products detected experimentally could be included in a relevant (bio)chemical reaction scheme.

The reactions established in Scheme 1 were transformed into differential equations (Appendix, Differential equation set 1). These equations were solved by means of the ‘proc model’ from the SAS software. Parameter estimates k_{1-6} are the reaction rate constants, while TAG_f refers to the final extent of lipid digestion in the gastric compartment. The differential equations derived from the first reaction scheme

converged for the data set corresponding to the fine, but not in the case of the medium and large emulsion. Specifically for the large emulsion, the lack of convergence was attributed to the two regioisomers of MAG which were not included as experimental data because they were below the quantification limit. Consequently, given the lack of convergence, the modelled data for the medium and large emulsion could not be calculated and these cases are absent in Figure 3. Regarding the responses of the fine emulsion, a relatively good fit for triolein, *sn*-1,2/2,3-DAG, oleic acid and glycerol was obtained, but an inappropriate fit for the other responses when representing the experimental values and modeling lines in the same graph (Figure 3A-F). Either a significant under- or overestimation of the experimental values were observed for *sn*-2-MAG and *sn*-1/3-MAG, respectively. Furthermore, the low R^2_{adj} values reported in Figure 3 also confirm the lack of fit for these responses. As observed in Figure 3, the response of *sn*-1,3-DAG was not included in the modeling process. This decision was taken based on the very low concentration quantified which did not allow an adequate goodness of fit (Figure 3G). In addition, the estimated parameters k_2 ; k_3 ; k_5 and k_6 show a high standard error and even parameter k_5 was estimated as a negative value (Table 1). Therefore, it is very unlikely that this model represents the mechanisms of gastric lipolysis under the conditions of our experiments.

This first attempt in proposing a mechanistic model for gastric lipolysis gave a valuable input to target specific responses which the first model failed to adequately fit. In a next step, some adaptations were applied to improve the first reaction scheme proposed (i.e. iterative approach). Consequently, two, characteristic cases will be presented in the following sections.

3.4.2 Hypothesis 2: Gastric lipase can hydrolyze the three positions of the glycerol moiety, and isomerization reactions also occur

This second hypothesis is adapted starting from hypothesis 1, but additionally considers the capacity of gastric lipase to hydrolyze the *sn*-2 position. The affinity of gastric lipase towards *sn*-2 position has also been suggested by Carriere *et al.* (1991) and (Rodriguez *et al.*, 2008). Some reactions were reconsidered to improve the goodness of fit of two specific responses in comparison to Scheme 1: *sn*-1,2/2,3-DAG and *sn*-

1/3-MAG. From Figure 3B, the model curve of *sn*-1,2/2,3-DAG shows its rapid degradation after being formed in the first minutes of digestion. This probably occurred because *sn*-1,2/2,3-DAG was the substrate to generate two other products in Scheme 1, namely *sn*-2-MAG and *sn*-1,3-DAG. Therefore, the substrate to produce *sn*-1,3-DAG was changed to triolein by enzymatic conversion in Scheme 2 instead of *sn*-1,2/2,3-DAG by chemical isomerization in Scheme 1. This change should allow the gradual hydrolysis of *sn*-1,2/2,3-DAG. In addition, *sn*-1/3-MAG production was largely underestimated in the early stage of gastric digestion as shown by the model line in Figure 3D. The reaction adaptation previously explained may also increase the early generation of *sn*-1/3-MAG. Now the compound *sn*-1,3-DAG, precursor of *sn*-1/3-MAG, will be produced from the enzymatic conversion of TAG. This biochemical reaction normally occurs faster than the chemical isomerization stated in Scheme 1 (Carriere *et al.*, 1991). Next to this adaptation, an additional biochemical reaction was included: direct formation of GLY and FFA from TAG. This extra reaction was anticipated to contribute to the early formation of glycerol because the model curve in Figure 3F shows an underestimation of this compound in the first min of gastric digestion. In view of this extra information, the following reaction scheme was proposed:

Scheme 2: (Bio)chemical reactions postulated from hypothesis 2.

The proposed reactions in Scheme 2 were transformed into differential equations and solved using the SAS software (Appendix, Differential equation set 2). These differential equations converged for the data sets corresponding to the medium and fine emulsion. The data set corresponding to the large emulsion could not be successfully included in this data analysis because the regioisomers of MAG could not be quantified. In general, the response *sn*-1,3-DAG was not fitted because of its very low concentration during gastric digestion. A good fit was obtained for TAG, FFA and GLY (Figure 4A, E and F). In addition, an improved fit was achieved for *sn*-1,2/2,3-DAG and *sn*-1/3-MAG compared to Scheme 1 (Figure 4B and D). Nevertheless, the model did not adequately fit the experimental data of *sn*-2-MAG which resulted in a R^2_{adj} value of 0.09 and 0.41 for fine and medium emulsion, respectively. From Figure 4C, a large underestimation was observed in the first half of gastric digestion phase for both the fine and medium emulsion. Additionally, the parameters k_5 , k_6 and k_7 presented high standard errors compared to the estimated values (Table 1). Based on the observations previously indicated, the model derived from Scheme 2 is not expected to be a good representation of the lipid digestion mechanisms occurring under simulated *in vitro* gastric conditions. Further adaptations of this model to the final multi-response model are presented in the next section.

3.4.3 The final multi-response model: adapted version of hypothesis 2

The major drawback found in the model derived from Scheme 2 was the lack of fit for *sn*-2-MAG, while all other responses had an acceptable fit. Therefore, a new biochemical reaction was suggested to overcome the initial underestimation of *sn*-2-MAG. This reaction consists of the direct conversion of one molecule of triolein into one *sn*-2-MAG molecule. From these considerations, a final reaction scheme was proposed:

503 Scheme 3: Final (bio)chemical reactions postulated to describe the gastric lipolysis mechanism.

504 Each reaction in Scheme 3 was translated into differential equations (Appendix, Differential equation set
505 3) and solved through a numerical method. This resulted in a successful convergence of the differential
506 equations for the data sets corresponding to fine and medium emulsion. Modelling of the large emulsion
507 data was infeasible for the same reason as discussed in the above. A very good fit was obtained for all
508 responses as depicted in Figure 5A-F. Furthermore, only a small error was observed for the parameters
509 estimates of both FE and ME, except for k_5 (Table 1). The high standard error of the parameter k_5 can be
510 explained by the compounds that participate in the reaction related to that parameter. Specifically, *sn*-1,3-
511 DAG was found in very low concentrations and did not present a defined time dependent pattern during
512 digestion.

513 With respect to the parameters, it can be observed that the estimated reaction rate constant values are similar
514 for fine and medium emulsion. This is alignment with the estimation of the overall reaction rate constants
515 performed with the single-response modeling (Section 3.2). Regarding the reaction involving k_1 , it
516 represents a single enzymatic conversion with a stereospecificity towards the outer positions (*sn*-1/3) of the
517 glycerol moiety. For this reason, the magnitude of this parameter was the highest of all parameter estimates
518 for both the fine and medium emulsion. The preference of gastric lipase to hydrolyze the outer positions,
519 and especially the *sn*-3 position, was suggested before by Rogalskas *et al.* (1990). The reaction related to
520 k_2 expresses the activity of gastric lipase towards the *sn*-2 position. The magnitude of k_2 is around half
521 compared to k_1 for both fine and medium emulsion. This proportion is logic since gastric lipase has
522 preference towards the outer position of the glycerol backbone. Tirupathi & Balasubramania (1982) also
523 reported a higher preference of HGL for the *sn*-1/3 compared to the *sn*-2 position based on the release of

fatty acids of different structures attached to the glycerol moiety (proportion between fatty acids released from *sn*-1/3 compared to *sn*-2 was around 3). However, these authors only quantified the generated fatty acids at two digestion moments via GC analysis and a prochiral substrate was not employed. The reaction involving k_3 is a fast two-step conversion that represents the hydrolysis of both fatty acids bound to the outer positions. This reaction occurred faster than the hydrolysis of the *sn*-2 position, but slower than the cleavage of one of the outer positions (e.g. reaction related to k_1), which is a coherent finding. About k_4 and k_6 , these are rate constants related to the chemical isomerization reactions. Their magnitude was 30-fold slower to the magnitude of the reactions rate constants related to the enzymatic conversions (specifically the *sn*-1 position hydrolysis). Nonetheless, these reactions contributed to the early formation of glycerol. With respect to k_7 , this parameter represents the production of glycerol and oleic acid from *sn*-1/3-MAG. The magnitude of this reaction rate constant was lower compared to the ones representing the hydrolysis of the same positions (e.g. k_1). This discrepancy might have occurred because MAG was less accessible for gastric lipase. The final extent of triolein hydrolysis (TAG_f) was 49.7 and 51.4 $\mu\text{M/mL}$ for the fine and medium emulsions, respectively. A significant difference between them exists, which is hypothesized to be due to the lipase entrapment, potentially reached faster for the medium emulsion due to the smaller available surface area. This effect did not allow gastric lipase to approach the triolein located in the core of oil droplets.

4. Conclusions

In this study, the *in vitro* procedure selected and combined modelling approaches allowed (i) a quantitative insight in the effect of food design and (ii) a mechanistic insight in the enzymatic and chemical reactions taking place during the gastric lipolysis phenomenon. For this purpose, the empirical single-response modeling showed that the final extent and initial rate of lipid digestion had an inverse relation with respect to the droplet size of emulsions during digestion. This was mainly ascribed to the higher specific surface area available for lipase adsorption when the droplet size was smaller. Additionally, the quantification of multiple responses during digestion allowed the utilization of multi-response modeling to obtain insight

into the gastric lipolysis mechanisms. This advanced technique permitted the estimation of kinetic parameters at a molecular level. The final multi-response model converged for the fine and medium emulsion and an appropriate fit was observed for the evaluated responses. This model consisted of a set of seven interrelated reactions of which five correspond to enzymatic hydrolysis and two are chemical isomerization reactions. The magnitude of the reaction rate constants indicated that the rate of hydrolysis of the *sn*-1/3 positions of the glycerol backbone was almost double compared to the rate of hydrolysis of the *sn*-2 position. The rate constants corresponding to the isomerization reactions resulted in around 30-fold times lower value compared to the enzymatic conversions. Even if these experiments were performed under *in vitro* static conditions, the relevance of the final mechanistic model remains because: (i) dynamic gastric conditions could probably lead to different magnitudes in the reaction rate constants but it is unlikely that variable lipase activity would result in different reaction types, and (ii) there is no uptake of lipolysis products in the *in vivo* gastric compartment which does not interfere with the enzyme-substrate interaction phenomena. To conclude, this work proposes a physiological relevant reaction scheme giving advanced insight into the lipolysis mechanisms under simulated gastric digestion conditions. This proposed model may be the starting point to develop *in silico* models to predict the gastric lipid digestion profile based on emulsion design properties.

Acknowledgement

M.R. Infantes-Garcia is a Doctoral Researcher funded by the Research Foundation Flanders (FWO - Grant No. 1S03318N). S.H.E. Verkempinck is a Postdoctoral Researcher funded by Onderzoeksfonds KU Leuven (Grant No. PDM/18/156). The authors also acknowledge the financial support of the KU Leuven Research Fund.

Declaration of interests

The authors of this work declare no conflict of interests.

572 Appendix

573 Differential equation set 1 (derived from Scheme 1):

$$574 \frac{d(\text{TAG})}{dt} = -k_1(\text{TAG} - \text{TAG}_f)$$

$$575 \frac{d(\text{sn-1,2/2,3-DAG})}{dt} = k_1(\text{TAG} - \text{TAG}_f) - k_2(\text{sn-1,2/2,3-DAG}) - k_3(\text{sn-1,2/2,3-DAG})$$

$$576 \frac{d(\text{sn-1,3-DAG})}{dt} = k_3(\text{sn-1,2/2,3-DAG}) - k_5(\text{sn-1,3-DAG})$$

$$577 \frac{d(\text{sn-2-MAG})}{dt} = k_2(\text{sn-1,2/2,3-DAG}) - k_4(\text{sn-2-MAG})$$

$$578 \frac{d(\text{sn-1/3-MAG})}{dt} = k_3(\text{sn-1,3-DAG}) - k_6(\text{sn-1/3-MAG})$$

$$579 \frac{d(\text{FFA})}{dt} = k_1(\text{TAG} - \text{TAG}_f) + k_2(\text{sn-1,2/2,3-DAG}) + k_5(\text{sn-1,3-DAG}) + k_6(\text{sn-1/3-MAG})$$

$$580 \frac{d(\text{GLY})}{dt} = k_6(\text{sn-1/3-MAG})$$

581 Differential equation set 2 (derived from Scheme 2):

$$582 \frac{d(\text{TAG})}{dt} = -(k_1 + k_2 + k_3)(\text{TAG} - \text{TAG}_f)$$

$$583 \frac{d(\text{sn-1,2/2,3-DAG})}{dt} = k_1(\text{TAG} - \text{TAG}_f) - k_4(\text{sn-1,2/2,3-DAG})$$

$$584 \frac{d(\text{sn-1,3-DAG})}{dt} = k_2(\text{TAG} - \text{TAG}_f) - k_5(\text{sn-1,3-DAG})$$

$$585 \frac{d(\text{sn-2-MAG})}{dt} = k_4(\text{sn-1,2/2,3-DAG}) - k_6(\text{sn-2-MAG})$$

$$586 \frac{d(\text{sn-1/3-MAG})}{dt} = k_5(\text{sn-1,3-DAG}) + k_6(\text{sn-2-MAG}) - k_7(\text{sn-1/3-MAG})$$

$$587 \frac{d(\text{FFA})}{dt} = k_1(\text{TAG} - \text{TAG}_f) + k_2(\text{TAG} - \text{TAG}_f) + 3k_3(\text{TAG} - \text{TAG}_f) + k_4(\text{sn-1,2/2,3-DAG})$$
$$588 \quad \quad \quad + k_5(\text{sn-1,3-DAG}) + k_7(\text{sn-1/3-MAG})$$

$$589 \frac{d(\text{GLY})}{dt} = k_3(\text{TAG} - \text{TAG}_f) + k_7(\text{sn-1/3-MAG})$$

590 Differential equation set 3 (derived from Scheme 3):

$$\begin{aligned}
591 \quad & \frac{d(\text{TAG})}{dt} = -(k_1 + k_2 + k_3)(\text{TAG} - \text{TAG}_f) \\
592 \quad & \frac{d(\text{sn-1,2/2,3 DAG})}{dt} = k_1(\text{TAG} - \text{TAG}_f) - k_4(\text{sn-1,2/2,3 DAG}) \\
593 \quad & \frac{d(\text{sn-1,3 DAG})}{dt} = k_2(\text{TAG} - \text{TAG}_f) + k_4(\text{sn-1,2/2,3 DAG}) - k_5(\text{sn-1,3 DAG}) \\
594 \quad & \frac{d(\text{sn-2 MAG})}{dt} = k_3(\text{TAG} - \text{TAG}_f) - k_6(\text{sn-2 MAG}) \\
595 \quad & \frac{d(\text{sn-1/3 MAG})}{dt} = k_5(\text{sn-1,3 DAG}) + k_6(\text{sn-2 MAG}) - k_7(\text{sn-1/3 MAG}) \\
596 \quad & \frac{d(\text{FFA})}{dt} = k_1(\text{TAG} - \text{TAG}_f) + k_2(\text{TAG} - \text{TAG}_f) + 2k_3(\text{TAG} - \text{TAG}_f) + k_5(\text{sn-1,3 DAG}) \\
597 \quad & \quad \quad \quad + k_7(\text{sn-1/3 MAG}) \\
598 \quad & \frac{d(\text{GLY})}{dt} = k_7(\text{sn-1/3 MAG})
\end{aligned}$$

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Fig. 1. (A) Evolution of the average volume-based particle size $d(4,3)$, particle size distribution (PSD) and microstructure of the fine emulsion (FE), medium emulsion (ME) and large emulsion (LE) during *in vitro* gastric digestion. Scale bars in the micrographs represent 100 μm.

Fig. 2. Time dependency of triolein, different lipolysis products and digested triolein during simulated gastric digestion as affected by initial droplet size. Triolein, glycerol and digested triolein were calculated based on the quantified lipolysis products. Symbols represent the experimental value of the compound concentration for the (Δ) fine, (●) medium and (□) large emulsion. Solid, dotted and dashed lines in the first graph represent the

Fig. 3. (A, B, C, D, E, F) Representation of the multi-response model derived from reaction scheme 1 describing the evolution of different lipolysis products over simulated gastric digestion for the fine emulsion (FE). The symbol represents the experimental value of the compound concentration for the (Δ) fine emulsion. Solid lines are the predicted curves for each compound. (G) Residual plot of model derived from reaction scheme 1 for FE. Roman numbers indicate the standardized residual set of points for triolein (I); *sn*-1,2/3-diolein (II); *sn*-2-monoolein (III); *sn*-1/3-monoolein (IV); oleic acid (V) and glycerol (VI).

721 Fig. 4. (A, B, C, D, E, F) Representation of the multi-response model derived from reaction scheme 2 describing the evolution of different lipolysis products over simulated gastric digestion as affected by initial droplet size. Symbols represent the experimental value of the compound concentration for the (Δ) fine and ($\bullet$) medium emulsion. Solid and dotted lines are the predicted curves for each compound corresponding to fine or medium emulsion, respectively. (G, H) Residual plots of model derived from reaction scheme 2. Plots for medium (ME) and fine emulsions (FE) are presented on the left and right side, respectively. Roman numbers indicate the standardized residual set of points for triolein (I); *sn*-1,2/2,3-diolein; (II); *sn*-2-monoolein (III); *sn*-1/3-monoolein (IV); oleic acid (V) and glycerol (VI).

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Fig. 5. (A, B, C, D, E, F) Representation of the multi-response model derived from reaction scheme 3 describing the evolution of different lipolysis products over simulated gastric digestion as affected by initial droplet size. Symbols represent the experimental value of the compound concentration for the (Δ) fine and ($\bullet$) medium emulsion. Solid and dotted lines are the predicted curves for each compound corresponding to fine or medium emulsion, respectively. (G, H) Residual plots of model derived from reaction scheme 3. Plots for medium (ME) and fine emulsions (FE) are presented on the left and right side, respectively. Roman numerals indicate the standardized residual set of points for triolein (I); *sn*-1,2/2,3-diolein; (II); *sn*-2-monoolein (III); *sn*-1/3-monoolein (IV); oleic acid (V) and glycerol (VI).

Table 1. Kinetic parameters estimated by the multi-response model presented for the three proposed reactions schemes, where k_{number} represents the reaction rate constants for specific (bio)chemical conversions for the proposed reaction scheme (expressed in min^{-1}) and TAG_f is the asymptotic concentration of triolein during gastric digestion (expressed in $\mu\text{M/mL}$ of emulsion). The abbreviation “n.d.” refers to “not determined”.

	Scheme 1	Scheme 2		Scheme 3	
	FE	FE	ME	FE	ME
k_1	0.242 ± 0.043	0.110 ± 0.011	0.145 ± 0.014	0.081 ± 0.004	0.098 ± 0.005
k_2	0.066 ± 0.014	0.045 ± 0.007	0.050 ± 0.008	0.034 ± 0.003	0.051 ± 0.007
k_3	0.008 ± 0.005	0.024 ± 0.005	0.031 ± 0.004	0.058 ± 0.003	0.071 ± 0.004
k_4	0.018 ± 0.003	0.009 ± 0.002	0.009 ± 0.001	0.004 ± 0.000	0.002 ± 0.001
k_5	-0.006 ± 0.007	0.415 ± 0.171	0.171 ± 0.044	0.541 ± 0.285	0.097 ± 0.027
k_6	0.034 ± 0.008	0.006 ± 0.003	0.001 ± 0.002	0.004 ± 0.000	0.002 ± 0.001
k_7	n.d.	0.018 ± 0.004	0.008 ± 0.003	0.037 ± 0.003	0.024 ± 0.003
TAG_f	50.22 ± 0.19	49.84 ± 0.12	51.61 ± 0.08	49.68 ± 0.06	51.44 ± 0.05