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In vitro digestion models to assess lipolysis: the impact of the simulated conditions for gastrointestinal pH, bile salts and digestion fluids

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ABSTRACT

In vitro digestion models are considered a valid methodology to study several mechanisms related to nutrient hydrolysis by simulating the standard physiological gastrointestinal conditions. However, there are pathologies in which some conditions are affected, and thus should be considered in the design of the *in vitro* digestion study. Our work aims at elucidating the role of different gastrointestinal conditions on lipolysis. In the context of exocrine pancreatic insufficiency, gastric pH, intestinal pH, bile salts composition, bile salts concentration, fat concentration in the digestion medium and volumetric ratio digestion fluid/food were the selected study parameters. The pH-stat method was applied to assess lipolysis extent and kinetics. Descriptive results were summarised in digestibility curves and beta regression models were used to explain the effect (odds ratio, OR) of the studied conditions on lipolysis. Results revealed that intestinal pH was the variable with the highest effect on lipolysis (OR 22.86, $p<0.001$), followed by fat concentration in the digestion medium (OR 6.76, $p<0.001$) and bile salts concentration (OR 1.56, $p<0.001$). We conclude that the assessment of lipolysis by means of *in vitro* digestion models is sensitive to the simulated gastrointestinal conditions, which should be adapted to the real physiological conditions occurring in altered health conditions.

KEYWORDS: *in vitro* digestion; gastrointestinal conditions; intestinal pH; bile salts; lipolysis, fat, pancreatic insufficiency

1. INTRODUCTION

When aiming to assess food digestion, available methodologies include *in vitro* digestion procedures (Ménard et al. 2014). Compared to human *in vivo* studies, *in vitro* methods are rapider, less expensive, and have no ethical restrictions. Besides, they allow for a large number of samples being measured in parallel for screening purposes. Reproducibility, choice of controlled and reproducible conditions and easy sampling at the site of interest make *in vitro* models very suitable for addressing the study of food digestion. Among other factors, *in vitro* digestion methods can mimic the physiological *in vivo* digestion by taking into account digestive enzymes, pH, digestion time and salts concentration of the digestive fluids (Minekus et al. 2014).

This way, it is possible to know the status of the digestion reactions at every specific point of the process, and to attribute the results only to the analysis conditions. In contrast, *in vivo* studies only allow for the evaluation of digestion at certain points, mainly at the end (e.g. measuring levels of a nutrient in plasma or faeces analysis, once digestion is finished), with no possibility to monitor the rest of the process (Ménard et al. 2014).

The application of *in vitro* digestion methodology can address such diverse scientific questions, like the digestibility and bioaccessibility of pharmaceuticals, and macronutrients such as proteins, carbohydrates and lipids. They have also been used to study matrix release of micronutrients such as minerals and trace elements, and bioactive compounds (Minekus et al. 2014). In particular, the study of lipid digestion has been targeted by several authors, given the important role of lipid in diets and their implication in health related conditions (Desnuelle & Savary 1963; Hunter 2001; Li et al. 2011; Fang et al. 2016).

In the light of the high application potential of *in vitro* methods, Minekus et al. (2014) published the harmonised international protocol to conduct this type of studies (Mineksu et al., 2014). This protocol describes a "smallest common denominator", i.e. a set of conditions that are close to the physiological situation, are practical, and can be seen as a basic suggestion to address various research questions. Authors indicate that further amendments to the suggested conditions may be needed, for example to simulate digestion in infants or the elderly, or pathologies that affect digestion such as inflammatory bowel disease or cystic fibrosis (Shani-Levi et al., 2017). In this sense, *in vitro* digestion studies could be used as a tool to shed light on the understanding of lipolysis in different physiological situations. Nevertheless, up to now, there are only a few known studies on lipid digestion of foods under digestion environments that are different to the standard ones (Asensio-Grau et al. 2018, Calvo-Lerma et al., 2018, Paz-Yépez et al. 2018). The scarcity of studies focused on gastrointestinal factors limit the translation of knowledge from *in vitro* digestion outcomes to the real life application.

Altered gastrointestinal conditions can be present in different health conditions and diseases, especially in the framework of exocrine pancreatic insufficiency (EPI), in which several affected parameters can be identified. Gastric and intestinal pH, characteristics of bile and the secretion of digestion fluids can be altered in different manners and to different extents, leading to a wide range of gastrointestinal scenarios, with subsequent implications on lipolysis (Clarke et al., 2001; Armand et al., 2004; Gelfond et al., 2013; Humbert et al., 2018).

Thereupon, the present study is aimed at elucidating the role of different simulated gastrointestinal conditions on lipolysis by means of an *in vitro* digestion model.

2. MATERIALS AND METHODS

2.1. Materials and equipment

A test-food, i.e. a nutritional supplement, was used to conduct the experiments (Resource®). The nutritional information of the product included: protein content solely from casein, lipid content from monounsaturated triglycerides and no phospholipids. Pancreatic enzyme supplements (Kreon® 10000 lipase units, LU) were used to simulate the intestinal digestion. Pepsin from porcine gastric mucosa (3200-4500 U/mg), bovine bile extract, porcine bile extract, taurocholic (TC), taurochenodeoxycholic (TCDC), glycocholic (GC) and glycodeoxycholic (GCDC) compounds were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). Chlorhydric acid 1N and sodium hydroxide 1N were used to adjust the pH at the different digestion stages.

The pH-stat method was applied to conduct all the experimental trials. A 718 STAT Titrino (Methrom) connected to the software Tiamo 1.3 was used. This equipment allows for automating acid-base reactions. The sample is introduced in the reaction vessel connected to a thermostated water bath. In the vessel, pH and temperature electrodes are placed, along with an automatic dosing tube pouring the titrant. Lipolysis during the intestinal stage was measured with the "stat pH" function, in which the equipment adds titrant automatically when a pH change is produced in the reaction vessel - due to the lipolysis reaction - in order to maintain the constant desired pH in the medium. As the digestion process occurs, the equipment registers every 10 seconds the volume of titrant consumed over time. Then, the added volume at any point of the process can be translated into the amount of free fatty acids released as the product of lipolysis.

In order to discard possible titration effects derived from proteolysis during the intestinal stage, a complementary experiment was conducted without enzymatic

supplement and with pancreatic proteases and no pH changes were detected (Mat et al., 2016). Thus, in our setting, changes in pH along the intestinal stage can be attributed to the sole effect of lipolysis since complete proteolysis of the casein in the test food occurs during gastric stage by the action of pepsin (Mandalari et al., 2009).

2.2. Selection of the Study variables / study gastrointestinal conditions

A thorough literature research was conducted to elucidate the most relevant gastrointestinal conditions affecting lipolysis, and the standard value for each condition was established. Then, the common possible physiological alterations were explored, and the simulated values for each condition were determined. These alterations occur mainly in the context of exocrine pancreatic insufficiency. A total of six parameters were selected: gastric pH (3, 4 and 5), intestinal pH (6 and 7), bile salts composition (different glycocholic and taurocholic salts), bile salts concentration (1 mM and 10 mM), volume of digestion fluids secretion (expressed as the ratio with the test food, as 0.5/1, 1/1 and 2/1) and fat concentration in the digestion medium (obtained from the fat composition of the test food, as 5.5% of fat and 35% of fat, resulting in 0.7 and 4.8 g/mL digestion fluid respectively). **Table 1** summarises the selection and the rationale for the gastrointestinal conditions to be studied and the simulated values for each in the different experiments. **Table 2** presents the formulation of the four bile salts composition assessed.

2.3. Study design

The experimental design included three sets of experiments aimed at elucidating the role of the selected gastrointestinal conditions by combining them: gastrointestinal pH, bile salts and digestion fluids secretion and concentration of fat (**Table 3**). For all

the experiments, the enzyme to substrate ratio of lipase was 1000 LU/g of fat. All the experiments were done in triplicate, resulting in a total of 48 *in vitro* digestion experiments.

2.3. *In vitro* digestion process

The digestion process was simulated according to the static standardized method proposed by Minekus (2014) and thereafter amendments were applied according to the scope of this research (Minekus et al., 2014), which was the elucidation of the role of the altered conditions (**Table 3**). The static digestion process was simulated in three stages.

Oral stage: The test food was formulated with water (5 ml) and was mixed in the study volumetric ratio (0.5/1, 1/1 or 2/1, v/v) with simulated salivary fluid (SSF) in the digestion vessel for 2 minutes at 37 ºC.

Gastric stage: Then, simulated gastric fluid (SGF) (pH 3) was added in the study proportion (0.5/1, 1/1 or 2/1, v/v) to the digestion vessel containing the oral bolus. The pH of the mixture was readjusted according to the experimental set with HCl (1N) to pH 3, 4 or 5. Pepsin solution was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). The sample was stirred at 55 rpm for 2 h at 37 ºC, simulating the physiological process. Gastric lipase was not added because it is not commercially available, only fungal lipases can be obtained which exhibit different activity and specificity. Moreover, in physiological conditions lipase activity is much lower in the gastric than in the intestinal stage, because optimal gastric lipase pH is around 5; thus, its contribution to total lipolysis can be neglected (Minekus et al., 2014).

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF) (pH 6), was added to the vessel containing the gastric chyme in the volumetric ratio

according to the experimental design (0.5/1, 1/1 or 2/1, v/v). Bile salts solution (formulated with different proportions of bile salts, depending on the experimental set) was added to the SIF in order to reach the desired final concentration in the intestinal mix (to 1 mM or 10 mM). The pH of the mixtures was adjusted with NaOH (1N) to reach final pH6 or pH7. At this point lipase was added to reach a concentration of 1000 LU/g fat. The samples were then stirred at 55 rpm for other 2 h at 37 ºC. Intestinal pH was maintained during the process by the automatic addition of NaOH 0.5 N.

The composition of fluids required for each digestion stage, were described by Minekus et al. They were prepared fresh daily and kept at 37 ºC before their use.

2.4. Lipolysis extent and kinetics calculation

The percentage of free fatty acids released, as referred to the initial amount of lipids of the sample, was used to express the extent of lipolysis. It was calculated on the basis of the NaOH consumed during the intestinal stage (during pH-stat) as referred to the molecular weight of oleic acid (**Equation 1**).

$$\%\ lipolysis = \frac{(V\ NaOH)(N\ NaOH)(MW\ oleic\ acid)(100)}{m\ substrate} \qquad \textbf{(Equation 1)}$$

Where: V NaOH = titrant volume at any point (L); N NaOH = concentration of the titrant (N); MW oleic acid = molecular weight of the oleic acid; m substrate = mass of lipids in the food sample (g).

The curves of the progress of lipolysis for all the assessed conditions in the study were obtained by calculating the % of lipolysis every 10 seconds along 120 minutes.

To analyse the kinetics of lipolysis, log-logistic dose-response models were adjusted to estimate the parameters that describe the time-effect on the lipolysis extent

(f(x)) as an asymptotic curve. Several models were fitted for each condition in each experiment and each of them provided the three-parameter log-logistic function (**Equation 2**) where the lower limit is equal to 0. The numerator "d" refers to the estimated lipolysis extent asymptote while the parameter "e" represents the saturation rate. The saturation rate indicates the digestion time from which lipolysis does not increase. Finally, "b" represents the activation time. The parameters describing the kinetics of lipolysis for all the sets of experiments are summarised in a **supplementary table**.

$$f(x) = \frac{d}{1 + \exp\left(b(\log(x) - \log(e))\right)} \qquad \textbf{(Equation 2)}$$

Where: d = estimated lipolysis extent asymptote; b = activation time; e = activity saturation rate

2.5. Statistical analysis

For the descriptive analysis, the data were summarized using mean (standard deviation) or median (1^{st}, 3^{rd} Q.) in the case of continuous variables and with relative and absolute frequencies in the case of categorical variables.

Beta regression models were applied in order to explain the association of the study variables (gastric pH, intestinal pH, bile salts composition, bile salts concentration, volumetric ratio of digestive fluids, fat concentration in the digestion medium) with the response variable, i.e. the lipolysis extension (%). The results of the model can be interpreted with the estimated effect (i.e., the odds ratio) and the 95% confidence interval (95% CI). If the estimated effect is >1 the variable is positively associated with the response variable, i.e. lipolysis extent, and if <1 the effect is diminishing of the response variable. The higher the value is, the higher the effect is. Complementarily, the confidence intervals that do not contain 1 are those significantly associated with the response variable.

The descriptive results for all the experimental sets (i.e., lipolysis extent at all digestion times) are displayed in the figures, while the parameters of the beta regression models explaining the association of the study variables are presented in tables.

All the analyses were performed using R software (version 3.3.3), and betareg (version 3.1-0), drc (version 3.0-1) packages. A p-value below 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effect of gastric pH and intestinal pH on lipolysis

The intestinal pH was the most significant variable affecting lipid digestibility. As shown in **Figure 1**, lipolysis kinetics curves were characterized by the following pattern: when the longer was the saturation rate, the lowest was lipolysis extent reached. This was the tendency found at intestinal pH 6 (saturation rates from 30.2 to 37.5 min; lipolysis extent asymptotes from 38 to 43%). In contrast, at pH 7, lipolysis kinetics curves described a short saturation rate (10.2 to 12.8 min) at which lipolysis asymptotes were in the range of 92 to 102%. Therefore, at the final point of the intestinal stage, lipolysis extent was significantly higher at pH 7 than at pH 6 ($p < 0.001$, 95% CI [16.4, 31.9]) (**Table 4**).

On the other hand, the gastric pH also showed a significant effect. Compared to pH 3, the highest lipolysis extents were found at pH 4 ($p = 0.027$, 95% CI [1.0, 2.1]), while at pH 5 no significant differences were detected. However, the parameters defining lipolysis kinetics did not show differences between gastric pH 3, 4 and 5.

According to our findings, the intestinal pH is the condition determining lipolysis the most, both in terms of lipid digestion rate before saturation and extent, as increasing pH from 6 to 7 led to an improvement of 54 % of lipolysis in the test food.

Intestinal pH is known to increase progressively from an initial value around 4 and up to 7 at the end of the stage (Aburb et al., 2018). However, some physiological conditions, as in the case of exocrine pancreatic insufficiency (Robinson et al., 1990; Gelfond et al., 2013), impede that progressive alcalinisation of the digestion fluid occurs allowing for a maximum of 6. In that scenario, the activity of lipases decreases, being their optimal at around pH 7-8 (Desnuelle et al., 1963). Another previous study showed that lipolysis extent of foods in an *in vitro* digestion setting was significantly higher when simulated conditions included pH 7 versus pH 6 (Calvo-Lerma et al. 2018). This fact is also supported by an *in vivo* study conducted in nasoduodenal intubated children, which showed that intestinal pH was unequivocally associated with the percentage of lipids hydrolyzed (Robinson et al., 1990). Thus, increasing intestinal pH in patients suffering from pancreatic insufficiency should be considered a therapeutic priority. This challenge was previously addressed by Kalnins et al. (2006), by using sodium bicarbonate supplements in patients, although results showed neutral effects. Possibly the coating system used in the encapsulation of the compound was not the optimal.

In some kinds of pathology, such as gastroesophagic reflux, the use of proton pump inhibitors (PPI) is clinically advised to decrease the acidity of the gastric compartment (Tran et al., 1998). The resulting augmented pH value, up to 4 or 5, causes a change in the activity of pepsin and other proteases that have an optimal activity at pH 2-3. In addition, an aggregation phenomenon could be occurring, provided that pH 4.5 is the isoelectric point of pepsin. The decrease in proteolysis at this stage can have implications in further nutrient hydrolysis in the intestinal stage: a lower gastric proteolysis might compromise matrix degradation and the subsequent nutrient release for interaction with enzymes. On the other hand, the increase of the pH of the gastric content may enhance lipolysis further on intestinal stage: a less acid gastric content so

when the chyme passes into the small intestine the pH increase at this stage is higher than when starting from a gastric chyme around pH 3. Then a higher intestinal pH can increase lipases activity (Proesmans et al., 2003). This is a crucial point in the case of pancreatic insufficiency. In this context intestinal lipolysis is highly compromised because it completely relies on the efficacy of the exogenous pancreatic enzymes administration, which certainly requires a high enough pH value (Fieker et al. 2011).

3.2. Effect of bile composition and concentration on lipolysis extent

As shown in **Figure 2**, at intestinal pH 6, all the bile formulations at 1 mM concentration described similar lipid digestibility curves, characterized by saturation rates ranging from 39.7 min (F4) to 53.7 min (F3) and relatively low lipolysis asymptotes that were found between 30.2 % (F1) and 36.6 % (F4). In terms of final lipolysis extents, the porcine (F2) and low-taurocholic (F3) bile formulas, lipolysis reached values of 39 and 40% respectively, while the bovine (F1), and the high-taurocholic (F4) allowed for mean values of 32% and 30% respectively. As compared to bovine formula, there were significant differences in the porcine formula and the high-glycocholic formula (**Table 5**), in which lipolysis was higher ($p = 0.017$, 95% CI [1.03, 1.38], and $p < 0.001$ 95% CI [1.12, 1.50] respectively).

Differences in lipolysis extents depending on the composition must be taken into account when planning *in vitro* digestion experiments. The standardised protocol (Minekus et al. 2014) recommends the bovine bile, but the porcine is also commercially available, and the application of it would lead to higher lipolysis.

Results point that bile enriched in taurocholic salts (F4) does not lead to improved lipid digestion as compared to the standard formulation (bovine formula). Studies conducted several years ago, aimed at supplementing patients with decreased

bile with taurine to achieve better digestion of fat. Literature, however, gathers controversial conclusions on this topic, some studies pointing out no effects (Thompson et al., 1987; Merli et al., 1994) while others confirming its beneficial role (Colombo et al., 1988; Belli et al., 1986). The discrepancy may be related to different experimental designs and assessed outcomes.

The effect of the bile salts concentration on lipid digestibility was also assessed in the bovine (F1) and porcine (F2) origin simulated bile. Lipolysis curves showed the same tendency as in the case of digestion simulated at 1 mM concentration (**Figure 2**), with slightly shorter saturation rates and higher lipolysis asymptotes. In both formulas, the concentration of 10 mM reached statistically higher lipolysis extents after 2h of intestinal digestion ($p<0.001$, CI 95% [1.37, 1.78]) (**Table 6**). Furthermore, there was a significant interaction between the composition and the concentration of the bile ($p=0.017$), provided that the effect of the concentration 10 mM was higher in bovine bile (F1), than in porcine bile (F2) (CI 95% [0.66, 0.96]). This is another relevant finding of this study, which concerns the positive effect of the bile salts concentration in the digestion medium on lipolysis. Pathologies coursing with decreased bile salts secretion, as the case of cystic fibrosis, could be slightly benefited if this compound (F1) was encapsulated by means of a delivery-controlled and administrated as a therapeutic routine. However, for the moment there is no other available research supporting this evidence.

3.3. Effect of the volume of fluid secretion and lipid concentration in the digestion medium

Experiments conducted with a high concentration of lipid in the digestion medium led to statistically higher lipolysis extent than when the lipid concentration was

low ($p < 0.001$), with mean lipolysis extent values of 80% and 40% respectively in the normal proportion of fluid secretion (1/1). In fact, lipid concentration in the digestion medium was the study variable with the second highest effect on lipolysis (95% CI [5.62, 8.13], being the variable with the overall highest effect the intestinal pH (95% CI [16.35, 31.94], **Table 4**). The difference between the two concentrations of lipid was also noticed in terms of the kinetics of lipolysis (**Figure 3**). In this experiment, the high-lipid concentration of the digestion medium led to high saturation rate (93.1 s) and high lipolysis extent asymptote (125.2 %), while the low-lipid concentration described a low saturation rate (30.2 min) together with a low lipolysis extent asymptote (43.3%). In the context of EPI, recommendations include a high dietary fat intake, as it has showed improved fat absorption result in patients. Our findings suggest that this improvement starts at the digestive enzymes level, which are more effective when the concentration of fat in the medium is higher (Desnuelle and Savary, 1963).

The study of the ratio between the volume of the digestion fluid and the food sample, showed that lipolysis extent was lower in the proportions 0.5/1 and 2/1 than in the standard physiological volumetric proportion of 1/1. However, unlike the concentration of lipid in the digestion medium, the volume of simulated digestion fluids did not have a significant effect on total lipolysis extent. As shown in **Table 7**, in the experimental scenario with low fat concentration in the medium, the volumetric ratio of digestion fluids / food 0.5/1 and 1/1 described similar tendencies in kinetics parameters; while the 2/1 proportion was present with a highest lipolysis extent asymptote.

3.4. Summary of the relative role of the gastrointestinal conditions on lipolysis extent and their implications in *in vitro* digestion models

To sum up, through the present study we assessed the influence of several gastrointestinal conditions on lipolysis extent and kinetics by means of an *in vitro* digestion methodology. The intestinal pH was the condition showing the greatest incremental effect in lipolysis extent by far when comparing 6 *vs*. 7. The other conditions showing improved lipolysis were the high concentration of fat in the digestion medium and the bile salts concentration 10 mM. With lower effects, the bile formulation with high glycocholic salts, and the ratio of digestive fluids/food 1/1 played also an enhancing role. **Table 8** compiles, in decreasing order, the estimated effect of the assessed gastrointestinal conditions on lipolysis, and provides a short practical application of the finding. Complementarily, the study of lipolysis kinetics reinforced the effects described by the statistical models developed on the basis of final lipolysis: the highest asymptotes were found in the conditions intestinal pH 7, high fat concentration in the digestion medium and bile 10 mM, along with shortest time before reaching saturation. The kinetics study may be also useful for future experiments, guiding in the duration of the intestinal stage which could be reduced according to the moment from which lipolysis extent does not increase.

4. CONCLUSION

In conclusion, our results evidence that there are gastrointestinal conditions that could be modulated and strongly affect lipase activity during dietary lipid digestion. Consequently, the main findings of the present study encourage the modification of the simulated gastrointestinal conditions when applying *in vitro* digestion methodologies; or can be used as supporting references to address future clinical treatments.

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COMPETING INTERESTS STATEMENT

None of the authors have any competing interest to declare.

AUTHOR CONTRIBUTIONS

J. Calvo-Lerma and A. Andrés designed the study. J. Calvo-Lerma performed the experiments and collected the data. V. Fornés-Ferrer and J. Calvo-Lerma performed the statistical analysis. J. Calvo-Lerma, A. Andrés and A. Heredia interpreted the results and drafted the manuscript. All the authors reviewed the manuscript and approved its final version for submission.

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TABLES

Table 1. Selected simulated gastrointestinal conditions to study their impact on lipolysis: standard condition, rationale for the possible physiological alterations and values used to simulate *in vitro* digestion

Simulated condition (Standard value)	Possible physiological alterations	Simulated values
Gastric pH (pH 3)	• pH is maintained at an average 3 during gastric digestion (Armand et al., 2004; Youngberg et al., 1987). • Proton pump inhibitors (PPI) avoid secretion of HCl and gastric pH remains at higher values (Kalantzi et al., 2006).	pH 3 pH 4 pH 5
Intestinal pH (pH 7)	• Intestinal pH starts at around 4 and progressively increases, up to 7 in the last part of the small intestine (Aburb et al., 2018). • In EPI the decreased secretion of sodium bicarbonate to the intestine makes the pH at this point cannot reach values higher than 6 (Robinson et al., 1990; Gelfond et al., 2013).	pH 6 pH 7
Bile salts composition (Bovine-like)	• The bovine bile has the most similar composition to the human bile in the proportion taurocholic/glycocholic salts. The porcine is also similar (Minekus et al. 2014).	Bovine bile (F1) Porcine bile (F2) High-glycocholic bile (F3)

	• A reduced in taurocholic salts bile can be found in patients with cystic fibrosis (Harries 1979). • In some pathologies associated with EPI, such as cystic fibrosis, supplementation with taurine lead to increased in taurocholic salts bile (Belli et al. 1987)	High- taurocholic bile (F4)
Bile salts concentration (10 mM)	• The formulation of bile salts in an *in vitro* setting should allow for a concentration of 10 mM in the intestinal digestion medium) (Minekus et al. 2014) • Biliary duct obstruction, biliary lithiasis and cystic fibrosis can cause reduced bile salts concentration of up to 10 times lower (Harries, 1979). A recent study has found that patients with EPI have a 1 mM concentration of bile salts (Humbert et al., 2018)	1 mM 10 mM
Volume of digestive fluids. Volumetric proportion with food sample (1/1)	• The physiological proportion of digestive fluids/food in the medium is 1/1 (Minekus et al., 2014). • Some pathologies (exocrine pancreatic insufficiency) cause the reduction of the volume of fluids secreted, up to half (Couper et al., 1992). • The co-intake of water and food, causes a dilution effect of enzymes and fat in the digestion medium	0.5/1 1/1 2/1
Fat concentration in the digestion medium	• Foods have a wide range of fat intake. When fat is released to the digestion medium, it is diluted with the digestion fluids. Considering a wide range of foods from all categories (dairy, meat, bakery…) two groups could be differenciated: with low content of fat	Food 5.5% fat (0.7g fat/mL digestion fluid) Food 35% fat (4.8g fat/mL

(Variable)	(around 5%) and with high content (around 40%) (Calvo-Lerma et al. 2018)	digestion fluid)

Table 2. Formulation of the four bile compositions and concentrations

	Bovine	Porcine	Taurocholic (TC)	Taurochenodeoxicholic (TCDC)	Glycocholic (GC)	Glycocholideoxicholic (GCDC)
% (w/w)	100	-	-	-	-	-
g/mol	440	-	-	-	-	-
% (w/w)	-	100	-	-	-	-
g/mol	-	440	-	-	-	-
% (w/w)	50	-	5	5	20	20
g/mol	220	-	26,85	24,98	97,4	94,32
% (w/w)	50	-	20	20	5	5
g/mol	220	-	107,4	99,94	24,35	23,58

w/w, weight/weight; F1, formula 1, bovine bile; F2, formula 2, porcine bile; F3, formula 3, high-glycocholic bile; F4, formula 4, high-taurocholic bile

Table 3. Experimental design: combination of the gastrointestinal conditions as study variables and resulting fat and enzyme concentrations in the digestion media

	Gastrointestinal pH		**Bile salts**		**Digestion fluids**	
	Gastric pH	**Intestinal pH**	**Bile concentration (mM)**	**Bile composition (Formula)**	**Volume ratio digestion fluid/food (v/v)**	**Fat concentration in digestion medium (g/mL)**
Gastric and	pH 3	pH 6	10	F1	1/1	0.7

intestinal pH	pH 4	pH 7 pH 6 pH 7				
	pH 5	pH 6 pH 7				
Bile salts composition and concentration	pH 3	pH 6	1 10 1 10 1 1	F1 F1 F2 F2 F3 F4	1/1	0.7
Volume of digestion fluid secretion	pH3	pH7	10	F1	0.5/1 1/1 1/2	0.7 4.8 0.7 4.8 0.7 4.8

mM, mili molar; F1, formula 1, bovine bile; F2, formula 2, porcine bile; F3, formula 3, high in glycocholic salts bile; F4, formula 4, high in taurocholic salts bile; mL, milliliter; LU, lipase units.

Table 4. Linear mixed regression models to assess the effect of gastric and intestinal pH on lipolysis extent.

Variable	Estimated effect	95% Confidence interval		p-value
Gastric pH 4 vs. Gastric pH 3	1.482	[1.045,	2.101]	0.027
Gastric pH5 vs. Gastric pH 3	1.366	[0.964,	1.937]	0.08
Intestinal pH7 vs. Intestinal pH 6	22.858	[16.357,	31.942]	<0.001
R-squared 0.934				

Table 5. Linear mixed regression models to assess the effect of the bile salts composition on lipolysis compared to the Bile formula 1 (bovine)

Variable	Estimated effect	95% Confidence Interval	p-value
Bile formula 2 (porcine)	1.19	[1.03, 1.38]	0.017
Bile formula 3 (high-glycocholic)	1.30	[1.12, 1.50]	<0.001
Bile formula 4 (high-taurocholic)	0.88	[0.76, 1.02]	0.086
R-squared 0.737			

F1, formula 1, bovine bile; F2, formula 2, porcine bile; F3, formula 3, high-glycocholic bile; F4, formula 4, high-taurocholic bile

Table 6. Linear mixed regression models to assess the effect of the bovine and porcine bile salts concentration on lipolysis

Variable	Estimated effect	95% Confidence Interval	p-value
Bile formula 2 vs. Bile formula 1	1.19	[1.04,1.36]	0.01
10 mM vs. 1 mM concentration	1.56	[1.37,1.78]	<0.001
Interaction bile formula 2 and 10 mM concentration	0.79	[0.66,0.96]	0.017
R-squared 0.823			

Table 7. Linear mixed regression models to assess the effect of the volume of digestion fluids and fat concentration in the digestion medium.

Variable	Estimated effect	95% Confidence Interval	p-value
High fat vs. low fat media	6.764	[5.626, 8.132]	<0.001
Low fluid V vs. normal fluid V	0.914	[0.769, 1.087]	0.309

High fluid V vs. normal fluid V	0.966	[0.813, 1.147]	0.69
R-squared 0.982			

Table 8. Summary of the estimated effects of the study variables on lipolysis extents and comments on practical applications of the findings.

Gastrointestinal condition	Statistical estimated effect on lipolysis extent (OR)	Practical application
Intestinal pH	22.86 *	Intestinal pH value can drastically change the result when assessing lipolysis *in vitro*. In the clinical practice, therapies aimed at increasing intestinal pH should be implemented in the treatment of exocrine pancreatic insufficiency.
Fat concentration in digestion medium	6.76 *	The assessment of lipid digestibility with an *in vitro* methodology must consider the fat composition of the sample food, as it drastically affects the result.
Bile salts concentration	1.56 *	Altered bile secretion occurring in EPI must be considered in *in vitro* digestion models. For the clinical practice, supplementation with bile salts is encouraged in EPI patients to enhance lipolysis
Gastric pH	1.48 *	Models simulating the application of PPIs should consider the gastric pH change. In the

		clinical practice changes in lipolysis should be expected in patients taking PPIs.
Bile salts formulation	1.19 * 1.30 * 0.88	To understand lipolysis in the context of altered biliary functions, the proportion of glyco- and taurocholic salts must be considered. *In vitro* digestion models using porcine bile will obtain higher lipolysis than with the regular bovine bile.
Volume of digestion fluids	0.91 0.97	Simulating digestion with a higher or lower volume of fluids does not affect lipolysis in the case of low fat foods, but it is significant in the case of high fat foods

* Statistically significant; OR, odds ratio; SD, standard deviation

FIGURE LEGENDS

Figure 1. Progress of lipolysis curves showing the effect of the gastric and the intestinal pH on lipolysis extent over time

Figure 2. Progress of lipolysis curves showing the effect of bile salts composition and concentration on lipolysis extent over time. F1, bovine bile; F2, porcine bile; F3, high-glycocholic salts bile; F4, high-taurocholic salts bile

Figure 3. Progress of lipolysis curves showing the effect of the volumetric ratio digestion fluid/food and fat concentration in the digestion medium on lipolysis extent over time

Figure 1

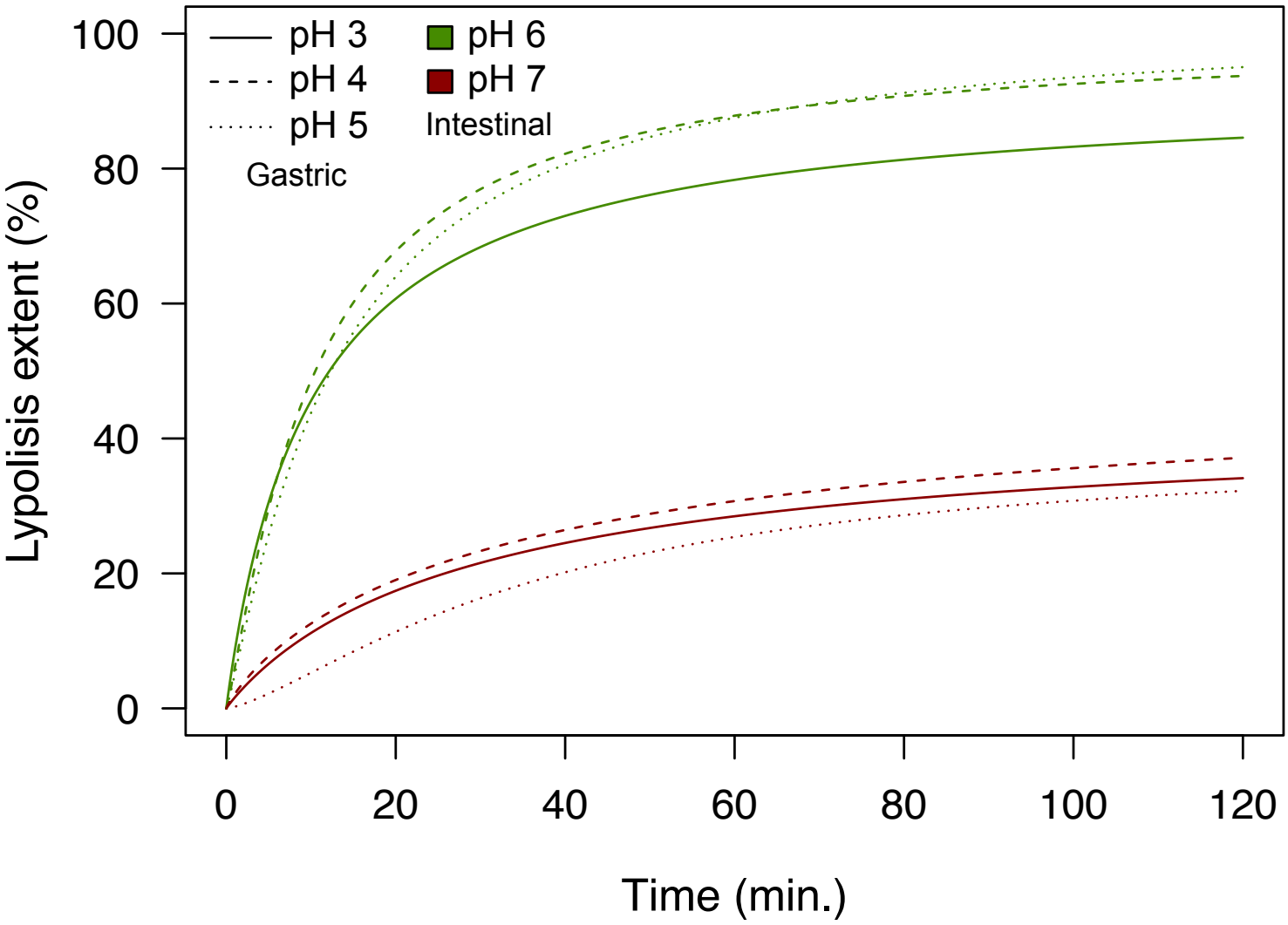

pH 3
pH 4
pH 5
Gastric
pH 6
pH 7
Intestinal
Lypolisis extent (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Time (min.)

Figure 2

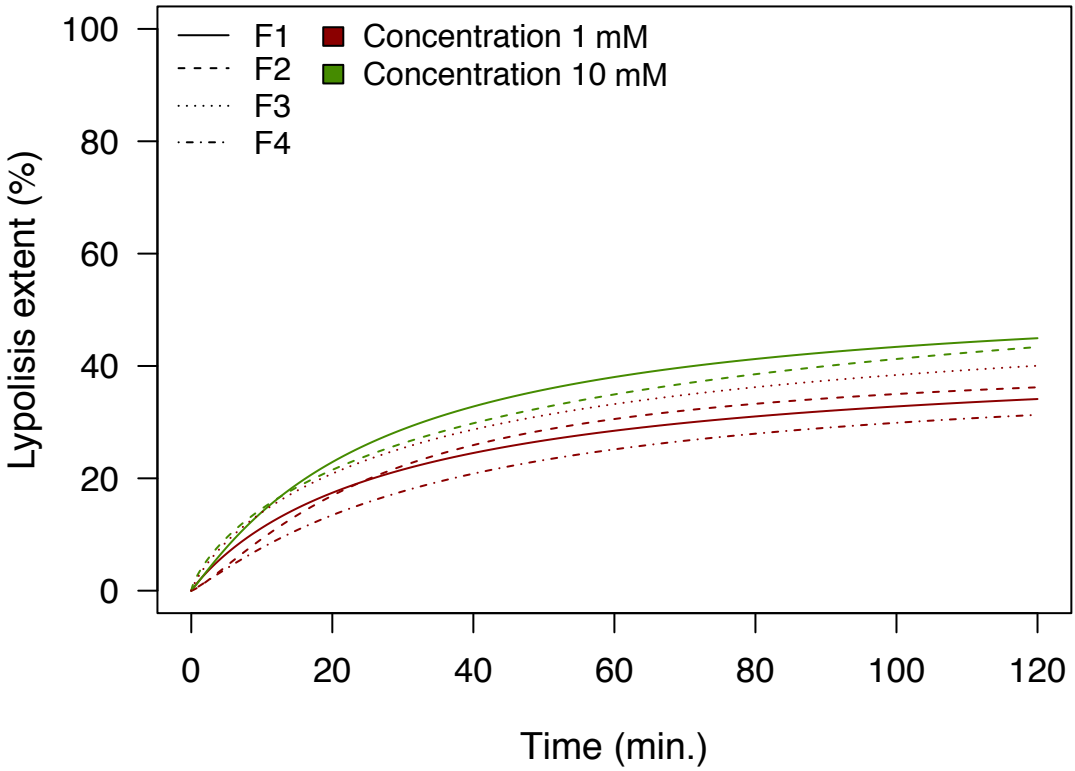
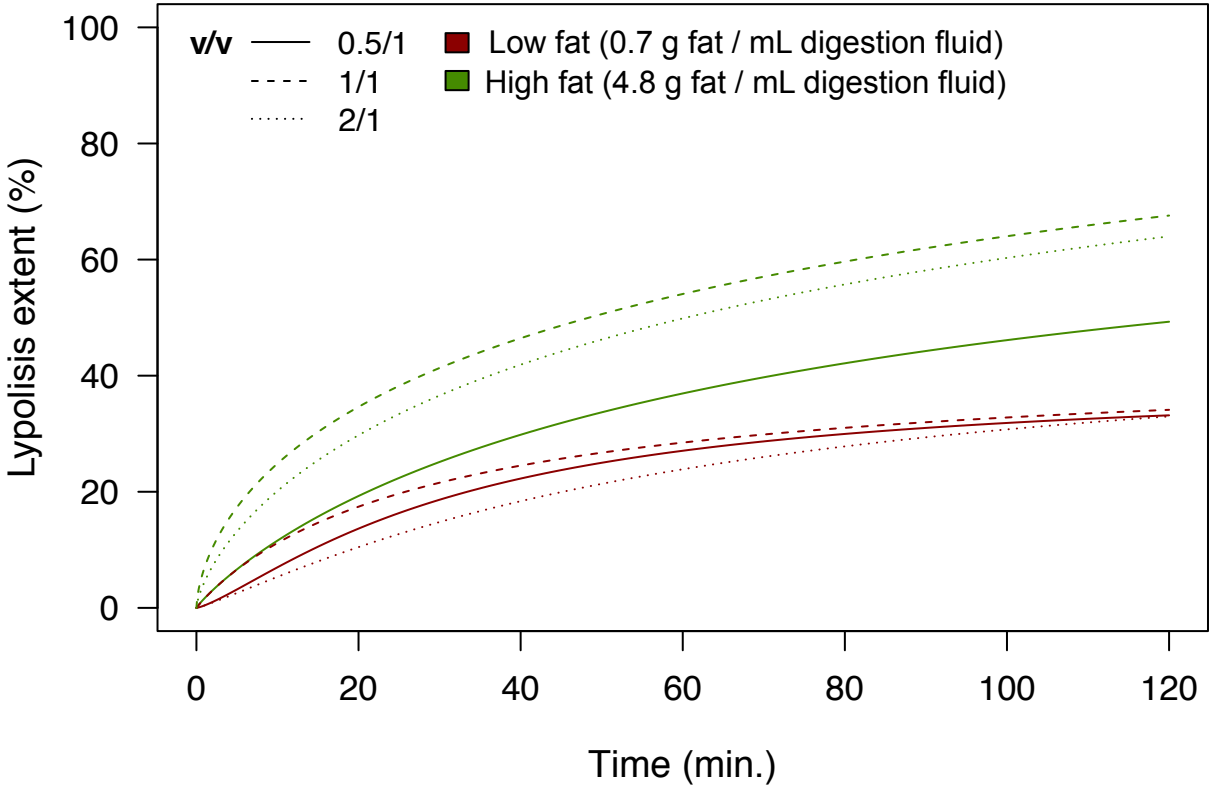
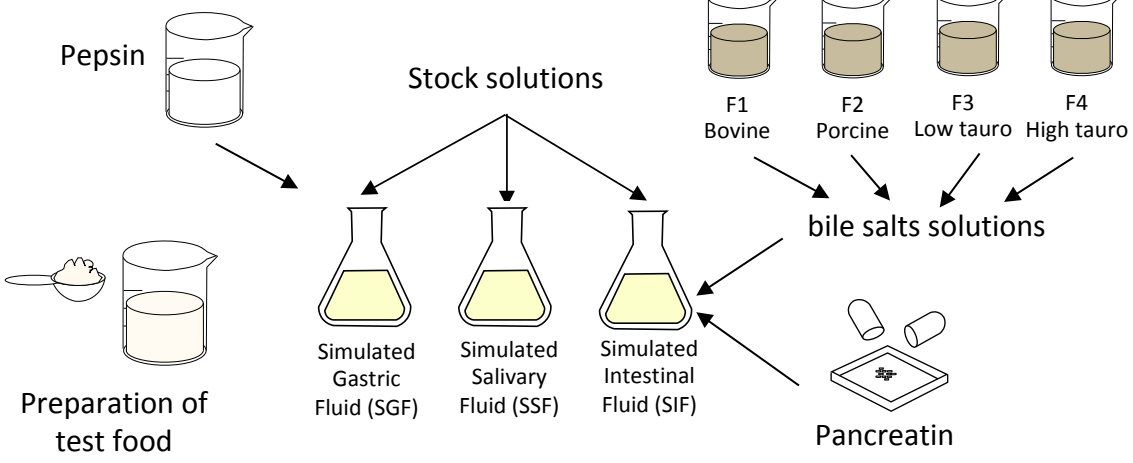

F1
F2
F3
F4
Concentration 1 mM
Concentration 10 mM
Lypolisis extent (%)
0
20
40
60
80
100
0
20
40
60
80
100
120
Time (min.)

Figure 3

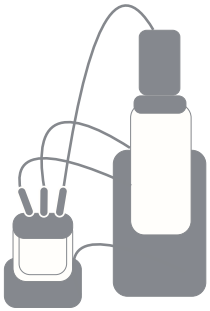

v/v
0.5/1
1/1
2/1
Low fat (0.7 g fat / mL digestion fluid)
High fat (4.8 g fat / mL digestion fluid)
Lypolisis extent (%)
0
20
40
60
80
100
0
20
40
60
80
100
120
Time (min.)

*Graphical Abstract

Preparation



In vitro digestion



pH-stat method
Automatic titration

Oral digestion
- 2.5/**5**/10 ml SSF
- 5 minutes
- pH 7

Gastric digestion
- 5/**10**/20 ml SGF
- 120 minutes
- pH **3**/4/5

Gastric digestion
- 10/**20**/40 ml SIF
- 120 minutes
- pH **6/7**
- [bile] 1mM/**10mM**
- Bile formula: **1**/2/3/4

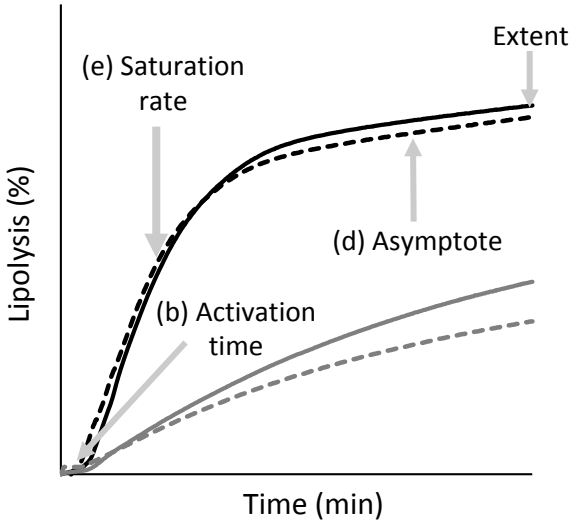
Lipolysis calculation

Lipolysis kinetics
(% lipolysis over time)

(b) activation time
(d) asymptote
(e) Saturation rate

$$f(x) = \frac{d}{1 + \exp\left(b(\log(x) - \log(e))\right)}$$

Lipolysis extent
(% lipolysis at end point)



% lipolysis = $\frac{\text{(V NaOH) (N NaOH) (MW oleic acid) (100)}}{\text{m substrate}}$