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Lipolysis kinetics of milk-fat catalyzed by an enzymatic supplement under simulated gastrointestinal conditions.

How gastrointestinal conditions may affect milk-fat digestibility.

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Abstract

Pancreatic insufficiency is a clinical manifestation characterized by the in-ability of the pancreas to release enough pancreatic enzyme into the small intestine, necessary to digest intraluminal nutrients. The lack of digestive enzymes leads to the difficulty to absorb nutrients, which drives in infants, to malnutrition and lack of growth and development, due to the loss of calories. These patients generally need oral administration of enzymes to favor lipolysis and absorption of lipids from foods. However, there are a number of food related factors (matrix, type of fat, etc.) and digestive environment (intestinal pH, bile concentration, among others), which will influence the digestibility of nutrients.

In this study, an *"*in vitro*"* digestion model was used to characterize the kinetics of the lipolysis of milk-fat catalyzed by an enzymatic supplement. Different intestinal conditions (pH (6, 7 and 8) and bile concentrations (1, 5 and 10 $mml \cdot L^{-1}$)) were simulated, using a fixed concentration of supplement. Gastro-Intestinal conditions, significantly affected lipolysis. High pH and bile concentrations were translated into low values of the Michaelis-Menten constant and high values of the catalytic constant. The kinetic parameters obtained from this work allowed estimating the dose of enzymatic supplement required to optimize the lipolysis of milk-fat under different intestinal environments, sufficient and insufficient pancreatic conditions.

Keywords: milk *"*in vitro*"* digestion; milk-fat lipolysis; kinetics; pancreatic insufficiency; supplementation therapy

1. Introduction

Exocrine Pancreatic insufficiency (EPI) is an associated disorder, which occurs in several diseases including pancreatic cancer, chronic pancreatitis (CP), cystic fibrosis (CF) and because of pancreatic surgery. EPI may occur due to loss of functional parenchyma (atrophy), blockage of the pancreatic duct, or postprandial asynchrony (Sikkens et al., 2010). In EPI, the obstruction of the pancreatic duct produces an insufficient secretion of sodium bicarbonate and pancreatic juice, containing digestive enzymes. Besides this lack of digestive enzymes, the decrease of pancreatic juice may also cause variations within the intestinal pH, this leading to nutrients mal-digestion and mal-absorption (Layer & Keller, 2003; Naikwade et al., 2009; Whitcomb et al., 2010). In this scenario, the hydrolysis and absorption of lipids are the most jeopardized, due to pancreatic lipase is the main responsible of lipolysis (Sikkens et al., 2010). Pancreatic Enzyme Replacement Therapy (PERT) consists on the oral administration of an enzymatic supplement of exocrine pancreatin to promote nutrients digestion and absorption (Armand et al., 2011). Even tough PERT has led to a large improvement of fats digestion and absorption, satisfactory levels of fat absorption are not often achieved. While the current guidelines for CF recommend an enzyme dose of 2000-4000 Lipase Units (LU)/ g fat (Turck et al., 2016), the optimal doses are still uncertain since they depend on food factors as well as on gastrointestinal (GI) conditions. Nowadays, the only available parameters to guide health professionals on adjusting the prescribed doses are based on the overall fat content of the meals or on patients body weight (Turck et al., 2016).

Individual factors such as gastric empting time, intestinal pH, intestinal transit, etc., may affect fat digestibility (Borowitz et al., 2013; Rovner et al., 2013). The decrease of pancreatic and/or bile secretion into the small intestine is frequently observed in some GI diseases (Layer & Keller, 2003; Whitcomb et al., 2010). As a consequence, the duodenal pH becomes more acidic (around pH 6) than in healthy person (around pH 7), while bile concentration might decrease

even 10 fold (1 mM) compared to a healthy adult (10 mM) (Aseeri et al., 2012; Borowitz et al., 2013).

On the other hand, factors related to foods such as fat content, type of fat, or food matrix can influence the enzyme activity. Therefore, the rate and extent of lipolysis will depend on the kinetic parameters of the enzyme for each substrate and medium characteristics. The pH-stat titration method is, in association with static systems, a classical approach that allows for monitoring the intestinal stage of "in vitro" digestions by directly providing the dynamics of the reaction (Li et al., 2011; Mat et al., 2016).

Lipolysis is an interfacial reaction and where the rate of the reaction depends on the emulsion characteristics (i.e. droplet size, concentration of fat…). In the majority of studies published until this date, the amount of free fatty acids (FFAs) released under simulated intestinal conditions has been monitored using formulated emulsions. That means that the characteristics of these emulsions were known and pre-designed (i.e. fat concentration, droplet size, concentration of surfactant…) (Charoen et al., 2012; Lesmes & McClements, 2012; Mat et al., 2016; Waraho et al., 2011). This approach makes sense since, in fact, lipids are most often consumed in the form of oil in water emulsions (milk, sauces…) characterized by their formulation and process conditions. However, one has to take into account that transformations during digestion may lead to changes in the oil/water interface area (Giang et al., 2015; Mat et al., 2016). Those changes will lead to unknown and maybe less favorable characteristics of the emulsions that will have different consequences on lipolysis, thus the importance of monitoring the reaction using food systems instead of predesigned model systems.

The novel approach proposed in this work is based on enzyme kinetics methodology. As stated above, health and nutritional status of patients with pancreatic insufficiency strongly depends on the precise doses of enzyme supplements. In this sense, a complex food such as milk, a processed o/w emulsion where the fat globules are dispersed within the aqueous phase, has

been used to estimate the saturation substrate concentration for a certain amount of enzyme under "in vitro" simulated conditions.

The aim of the present work was to explore the above-explained approach to analyze the influence of some GI factors (pH and bile concentration) on the pancreatic lipase's affinity for milk-fat. The parameters obtained from this approach will describe the enzymatic supplement performance on milk-fat lipolysis and will contribute to better adjust the required dose for an optimal digestion.

2. Materials and methods

2.1. Materials

Pancreatic enzyme supplement (Kreon® 10,000 LU), was kindly donated by "Hospital Universitari Politècnic La Fe" (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme as gastro-resistant microspheres equivalent to 10,000 lipase units, 8,000 amylase units, and 600 protease units. The other chemicals used for the *"*in vitro*"* digestion: pepsin from porcine gastric mucosa, bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$ y $CaCl_2$ and Triton X-100 were obtained from Sigma-Aldrich Chemical Company (St Louis, MO, USA). NaOH (1, 0.1 and 0.05 N) and HCl 1 N, were acquired from *AppliChem Panreac*. Full fat milk (3.6 % f.m.) was purchased at a local supermarket.

2.2. Experimental Design

The digestion of emulsified lipids depends on different parameters such as their compositional and structural properties (Armand, 2007; Li et al., 2011; Li & McClements, 2010; Zhu et al., 2013), the composition and the surface area of the interface surrounding, or the droplet size (Borel et al., 1994; Li et al., 2011; Li & McClements, 2010), as well as the enzyme's affinity for the interfacial layer (Giang et al., 2015; Hur et al., 2009). The velocity of the reaction might

be correlated with the concentrations of substrate [S] and enzyme [E] as follows (equation 1):

$$r = \frac{k_{cat} \cdot [E]_o \cdot [S]}{km + [S]} \quad \text{(equation 1)}$$

where, r is the reaction velocity (μmol/ mL · min); k_{cat} is the catalytic constant (s^{-1}); k_m is the Michaelis-Menten constant (mM), $[S]$ is the substrate concentration (mM), and $[E]_0$, is the initial enzyme concentration (mM).

The kinetic parameters of the lipolysis reaction of milk-fat during the duodenal digestion processes were estimated. At this purpose, milk samples representing ten different amounts of fat substrate (**table I)** were digested for each one of the experimental conditions, intestinal pH-bile concentration. Full fat milk (3.6 % f.m.) was used for the experiments (1L package for each experiment), so the amount of fat for each experiment was calculated according to the initial amount of fat in the mil package. Furthermore, this experimental design allowed for assessing the influence of intestinal conditions (pH and bile concentration) on the lipolysis reaction. Every experimental condition was assayed at least in triplicate.

Table I: Volume of milk samples (mL); Mass of milk samples (g); fat concentration on the final digestion mixture (g/L) and substrate concentration (mmol of milk-fat/L) used for each one of the pH-bile combinations.

Sample	Milk volume (mL)	Milk weight (g)	Fat concentration ($g \cdot L^{-1}$)	[S] ($mmol \cdot L^{-1}$*)
1	1.33	1.37	1.40	1.66
2	2.70	2.78	2.66	3.24
3	4.07	4.19	3.87	4.71
4	5.38	5.54	5.00	6.02
5	6.76	6.96	6.01	7.32
6	8.07	8.31	6.95	8.46
7	10.78	11.11	8.80	10.64
8	13.50	13.91	10.32	12.58
9	16.18	16.67	11.73	14.28
10	18.86	19.43	13.00	15.82

*To simplify palmitic acid molar weight was used to calculate the molar concentration of milk-fat.

2.3. "in vitro" digestion process

The methodology used for the present study was based on the harmonized static *"*in vitro*"* protocol published by Minekus et al. (2014) with some modifications. Summarizing, for the oral stage, the milk sample was mixed with Simulated Salival Fluid (SSF) in a ratio 1:1 (v/v) at 37º C. After that, for the gastric stage, oral bolus was mixed with the Simulated Gastric Fluid (SGF) in an oral bolus: SGF ratio of 1:1 (v/v), the pH was adjusted to 3 with HCl, and the mix was shaken at 37º C for 2h in an incubator chamber Selecta (JP Selecta SA, Barcelona), using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia), to agitate the samples head- over-heels at 55 rpm. Finally, for the intestinal stage, the gastric chime was mixed with the Simulated Intestinal Fluid (SIF) to obtain a final ratio of gastric chime to SIF of 1:1 (v/v). The pH was then adjusted to 7 with NaOH and the mix continued to be shaken at 37º C for 2h. However, in the present study, in order to analyze the influence of the intestinal pH and bile concentration on milk-fat lipolysis, some modifications were made. Porcine pancreatin was replaced by the enzymatic supplement Kreon® (0.21 g/L, 8.27 LU/ mL in the final digestion mixture). The experimental design consisted of two different variables (pH and bile concentration) at different levels. Three different levels were used for intestinal pHs (6, 7, 8); these values were chosen as they belong within the optimum working pH for the enzyme (Kreon®), as well as for being close to the physiological duodenum conditions (Etienne-mesmin & Denis, 2012). Moreover, two levels were used for the duodenal bile concentrations (1, and 10 mM). From the different possibilities, the combination pH 7/ bile 10 mM, was considered as the standard, while pH 6/ bile 1 mM, could correspond to an extreme case in EPI disorder (Aseeri et al., 2012; Minekus et al., 2014). In addition, a central point consisting on an extra combination (pH 7 and bile concentration 5 mM) was added to the experimental design in order to strengthen it and make it more robust. The amounts and composition of the simulated digestive fluids are summarized in **table II**. They were made up from stock solutions: salival (SSS), gastric (SGS)

and intestinal (SIS), according to Minekus et al. (2014). Pepsin was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). Bile was added to the SIF in order to reach a final concentration in the intestinal mix of 1, 5 or 10 mM depending on the experimental design.

Table II: Composition of simulated digestion fluids.

Constituent	SSF mmol · L^{-1}	SGF mmol · L^{-1}	SIF mmol · L^{-1}
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl_2(H_2O)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2$	1.5	0.15	0.6

SSF (Simulated Salival Fluid), SGF (Simulated Gastric Fluid), SIF (Simulated Intestinal Fluid). The addition of pepsin, Ca^{2+} solution and water will result in the correct electrolyte concentration in the final digestion mixture.

2.4. Lipolysis kinetics assessment

The progress of lipolysis during the intestinal stage was monitored by two different methods:

a) pH-STAT method (the intestinal stage took place in a 902 STAT titrino (Metrohm)); this method, commonly used to characterize the *"*in vitro*"* digestibility of lipids under simulated small intestine conditions, measures the amount of free fatty acids (FFAs) released from lipids, usually triacylglcyerols (TAGs), after lipase addition at pH values close to neutral (Li & McClements, 2010). In our case, the total of gastric chime in a 50 mL falcon tube was poured into the reaction vessel and the SIF and Kreon® were added. This reaction was then monitored during 30 minutes, which allowed getting the linear slope at the beginning of the lipolysis reaction. Of notice, $NaHCO_3$ was replaced by NaCl in all the simulated fluids (SSF, SGF and

SIF) at the same molar ratio, in order to maintain the same ionic strength and avoid its buffering effect (Mat et al., 2016).

b) Rapid enzymatic kit (spectrophotometric method). The intestinal stage took place in the same tubes and incubator chamber as the gastric stage; this methodology was applied to the standard experimental conditions pH 7/ 10 mM, in order to test whether the pH changes registered by the pH-stat were the result of the FFA produced by lipid digestion. For this purpose, a substrate concentration of 6.9 mg fat / mL (8.09 mL of milk) was used. Immediately after the gastric stage, the SIF and Kreon® were added to a 50 mL falcon tube containing the gastric chime, pH was adjusted to 7, and samples were rotated head- over-heels at 55 rpm for 30 min at 37 ºC, with the same agitator and chamber used for the gastric stage. To analyze the FFAs release, aliquots of the digestion fluids (100 uL) were removed at different interval times during the intestinal stage (0, 5, 10, 25 and 30) and mixed with 10 mL of a solution made of 5.6 % Triton X-100 and 6 % ethanol in water (to solubilize the free fatty acids and ensure to stop lipase activity). The free fatty acids release was measured using a free fatty acid spectrophotometric assay kit (Roche Diagnostics, Indianapolis, IN, USA) in a spectrophotometer (UV/vis, *Beckman Coulter*) (Lamothe et al., 2014). Palmitic acid standard was used for quantitative determination of FFA. Digested fat was estimated assuming the maximum release of 2 moles of fatty acids per 1 mole of triglycerides (Hunter, 2001).

2.5. Optical microscopy

Aliquots (100 μL) from the samples digested in the incubator chamber were taken at the same time intervals (0, 5, 10, 25 and 30), mixed with a solution of Red Oil (5 % in isopropanol) in a 4:1 volume ratio, vortexed for 10 s and incubated for 10 min at 37º C to stain the fat. A drop of this mixture was then placed on a microscope slide, covered by a cover slip, on an optical

197 microscope (Leica DM 5000 B). The images were acquired using a camera (Leica DFC, 550)
198 connected to a digital image processing system.
199
200 ***2.6. Statistical analyses***
201 Statistical analysis of variance (simple ANOVA) was performed using Statgraphics Centurion.
202 All the experiments were performed at least in duplicate and the results presented are always
203 the mean values ± standard deviation.
204 Principal Component Analysis, PCA, (SPSS) was also applied to assess whether the velocities
205 of lipolysis reaction could be grouped depending on the gastrointestinal conditions or the
206 substrate concentration.
207
208 **3. Results and discussion**
209 The pH-stat method, has been commonly used to characterize the amount of free fatty acids
210 (FFAs) released under simulated intestinal conditions. Generally, this methodology has been
211 proved to successfully work with o/w emulsions, when fat digestion is catalyzed by lipases
212 (Charoen et al., 2012; McClements et al., 2008; Waraho et al., 2011). However, in the present
213 work, a more complex system, milk, was digested using an enzymatic supplement (Kreon®).
214 The enzymatic activity of this enzyme (amylase, protease and lipase activities) might have
215 produced digestion products other than FFA such as peptides and amino acids that would have
216 an alkalinisation effect on the digestion mixture. The pH changes registered by the pH-stat
217 might therefore, not only occur as a result of the FFA produced by lipid digestion. In order to
218 assess whether the data obtained by the pH-stat method were those corresponding to the FFA
219 release during the intestinal stage, an extra experiment was performed. This was undertaken at
220 pH 7/ bile 10 mM with a substrate concentration of 6.9 mg fat /mL using both, an enzymatic
221 kit (Lamothe et al., 2012, 2014), and the pH-stat method (Li et al., 2011; Li & McClements,

2010; Mat et al., 2016). **Figure 1** illustrates the linear correlation between the two sets of data, confirming that the pH variations registered by the pH-stat method can be used to estimate the FFAs released during milk-fat lipolysis, and therefore, to analyze the influence of intestinal pH and bile concentration on lipolysis kinetics.

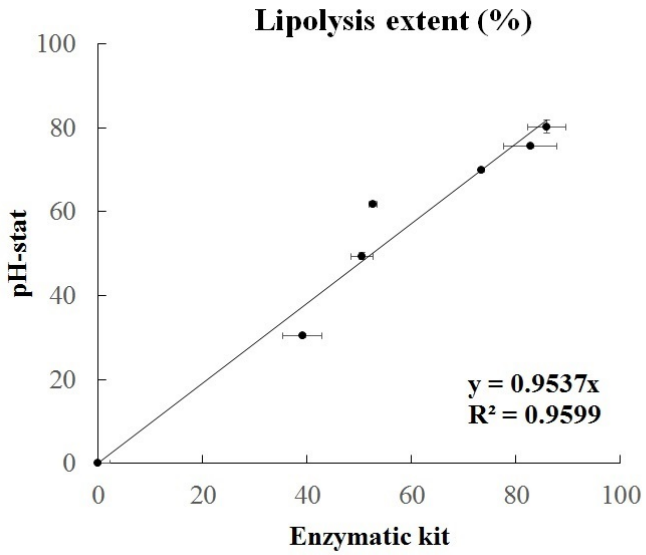
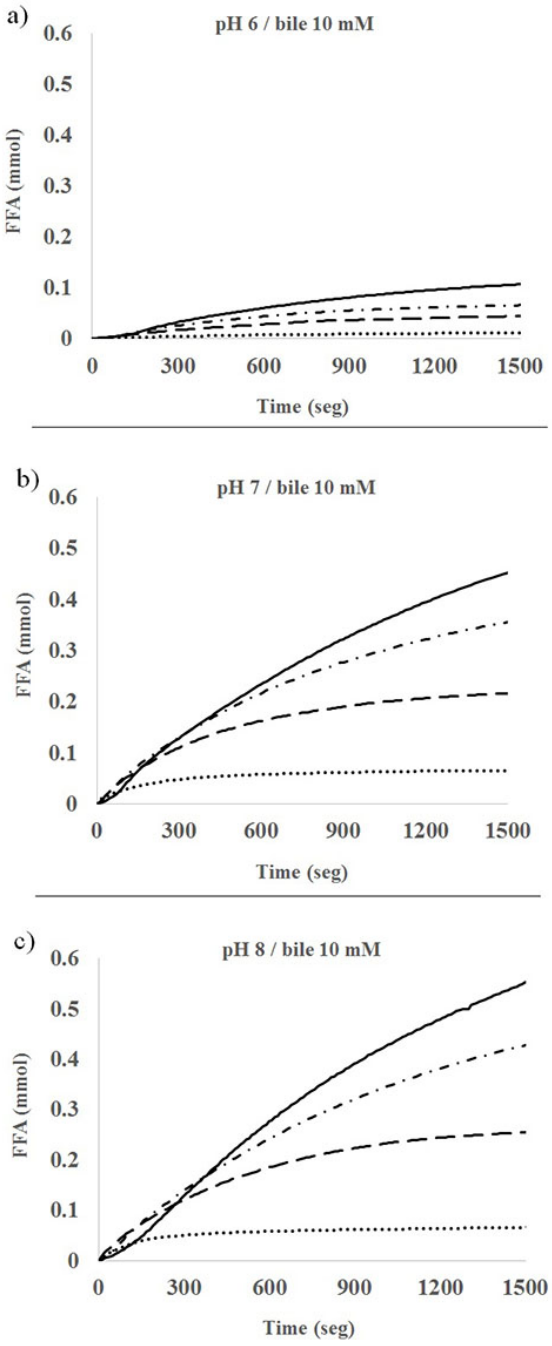


Figure 1: linear correlation between the two sets of data for the lipolysis (%), obtained by the enzymatic kit *vs* the pH-stat data for the simulated intestinal conditions pH 7 / bile 10 mM. The dots correlate the amount of digitised TAG (%) measured at different interval points (0, 5, 10, 25 and 30 min) of intestinal stage.

A total of ten curves of lipolysis progress for the different amounts of substrate (1.4 to 13 mg fat / mL) were experimentally obtained for each of the combinations pH-bile concentration. As example, **Figure 2** shows the curves of lipolysis progress for a selection of substrate amounts (1.4, 5.0, 8.8 and 13.0 mg fat / mL), in this case at 10 mM bile concentration for pH 6-7-8. The progress curves of lipolysis illustrate that the rate of the enzymatic reaction and the extent of fat digestion, are strongly dependent on both the pH of the medium and the biliary concentration used, as one would have expected. The results show that the enzymatic activity of the enzymatic supplement was much more effective at pH 7 and 8, and 10 mM concentration, both from the kinetic point of view (velocity of the reaction) and from the point of view of the lipolysis extent (% of Free Fatty Acids (FFA) released). The influence of these variables is also

241 evident in the pattern or shape of the curves, in which, depending on the conditions, the
242 interfacial activation period can be observed with greater or less clarity. The activation period
243 corresponds to the initial stretch in which the enzyme adsorbs to the surface of the fat droplets.
244 When the enzyme comes into contact with the interface, the dielectric environment on the
245 protein surface is modified so that electrostatic interactions are enhanced. This allows the
246 displacement of the active site cover (Anthonsenl et al., 1995; Foresti & Ferreira, 2004) and
247 the restructuring of the conformation of the molecule (Aloulou et al., 2006; Jensen et al., 2002;
248 Lin et al., 2007). As a result, the amino groups are exposed in a suitable orientation, allowing
249 access to the active center, until that moment inaccessible (González-Bacerio et al., 2010). Its
250 duration (activation time) depends on the enzyme concentration and the release of fatty acids.
251 During this activation process, a competition is established for the oil / water interface between
252 the lipases and other emulsifying compounds present in the system surrounding the fat droplets.
253 In the case of the system used in this work (milk), there could be a competition between the
254 enzyme and the caseins to be positioned at the oil / water interface. At very low enzyme
255 concentrations, there may not be sufficient enzyme to displace the caseins and therefore an
256 inadequate contact between the enzyme and the substrate would be established (Macierzanka
257 et al., 2009). At higher concentrations of fat, depending on the pH conditions of the medium
258 and biliary concentration, the interfacial stage could be clearly observed, with an initial rate of
259 very low FFA release, which can be explained based on the activation time required so that the
260 enzyme moves the caseins and reaches the interface of the substrate. However, under certain
261 conditions (pH 7-8, 10 mM), these displacement and adsorption processes occurred very
262 rapidly and lipid digestion begun almost immediately. As the fat concentration increased and
263 therefore the enzyme: substrate (UL / g fat) ratio decreased, the interfacial activation time was
264 increasingly longer. In contrast, for the lower substrate concentrations (0.05 g and 0.25 g), this
265 activation time was practically non-existent since the enzyme rapidly bind to the interface of

266 fat micelles.



267

268 **Figure 2**: Curves of lipolysis progress during the intestinal stage obtained by the pH-stat (NaOH 0.05 N) with
269 enzymatic supplement Kreon® (0.21 g/L, 8.27 LU/ mL in the final digestion mixture), for the experimental
270 conditions pH 7 /10 mM.
271

272 Once the activation time finished, the constant reaction rate period begun, step in which the
273 rate was dependent on the pH and the biliary concentration in the reaction media. This can be

observed in **Figure 3**, where for each of the pH and biliary concentration conditions tested, the velocity in the linear section of the progress curve was calculated (V_{max}). This allowed the correlation curves to be plotted between the reaction velocity (V_{max}) in the linear section (μmol / min · mL), and substrate concentration [S], for the enzyme concentrations assayed. As expected, the rate of lipid digestion increased with pH and biliary concentration, the latter related to the emulsifying power of bile salts, increasing the contact surface between the enzyme and fat droplets, so that the reaction happens at a faster rate (Maldonado-Valderrama et al., 2011).

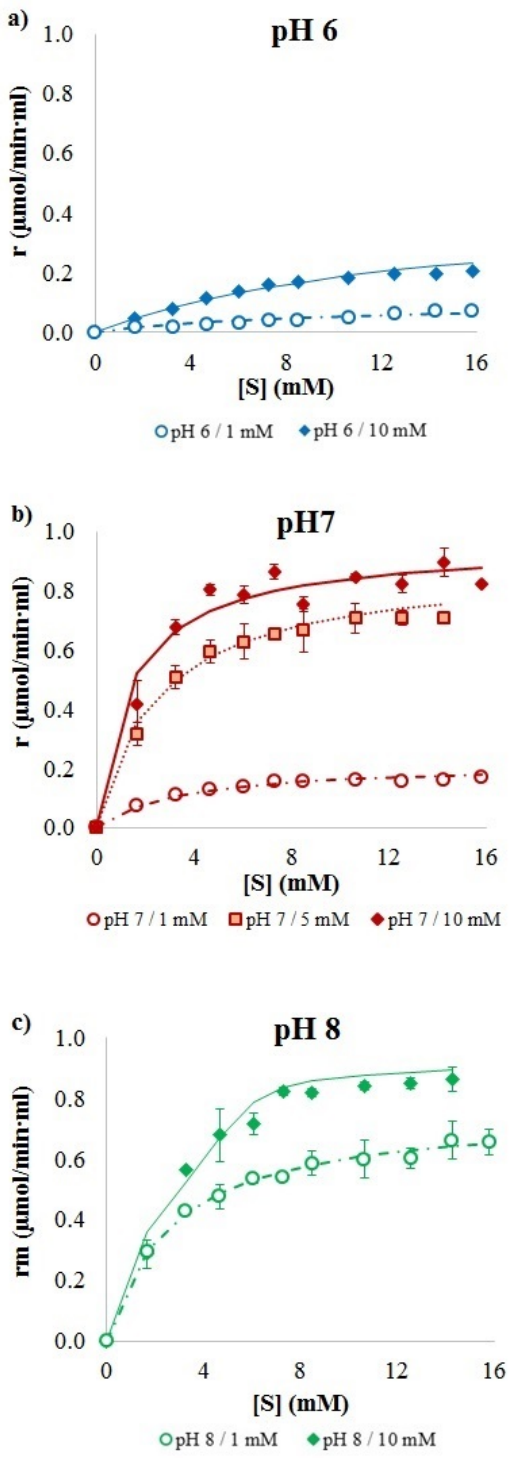


Figure 3: Velocity of the reaction (r) (µmol/min·mL) *vs* substrate concentration [S] (mM) for the different combinations pH (6, 7 and 8) and bile concentrations (1, 5 and 10 mM). Michaelis Menten fitting models. The dots are the average of at least two replicates.

A PCA was conducted in order to better understand the influence of the gastrointestinal conditions on the velocity of the lipolysis reaction. **Figure 4** illustrates the two-dimensional

plots of the sample scores (slopes of the curves for each experimental condition (V_{max})) and compound loadings (pH, Bile concentration and substrate concentration) obtained by the PCA. The first three dimensions explained 96.4 % of the total variance (PC1, 43.3 %; PC2, 25.6% and PC3, 25.5 %).

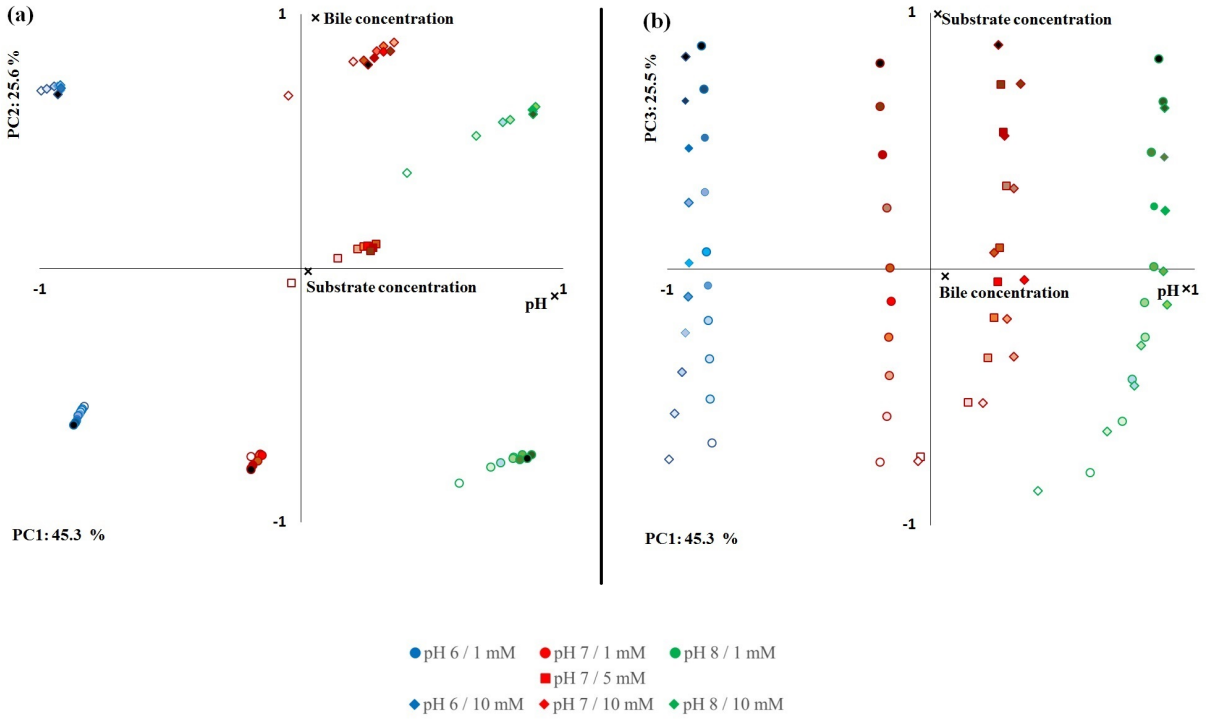


Figure 4: Biplots for the different velocities of reaction (r) (light markers correspond with the lower fat concentration (1.4 mg/mL) and dark markers with the highest fat concentration (13 mg / mL)), depending on the different parameters (Substrate concentration, pH and bile concentration) obtained by the PCA. (a) PC1 *vs* PC2 and (b) PC1 *vs* PC3 (PC1: 43.6 %, PC2: 25.6 % and PC3: 25.5 %).

As it can be observed in **Figure 4a,** the velocities (V_{max}) of the different assays are grouped together in the plot according to the different intestinal conditions (pH and bile concentration). PC1 clearly differentiates between pHs, with the velocities (V_{max}) obtained under pH 6 at the left side of the plot and the ones obtained under pH 8 at the right side of the plot. On the other hand, PC2 differentiates between bile concentrations, with velocities (V_{max}) obtained with the lower concentration (1 mM) at the bottom of the plot and the ones obtained with the highest

309 concentration (10 mm) at the top of the plot. Furthermore, PC1 represented *vs* PC3 (**Figure**
310 **4b**) illustrates the influence of the amount of substrate on the velocity of the reaction. In this
311 case, velocities (V_{max}) obtained with the higher fat concentration can be found at the top of the
312 plot. Summarizing, the three parameters influenced the velocity of the reaction with the major
313 reaction rates reached at high intestinal pH (7 or 8). In addition, the effect of pH on this velocity
314 became more important at high bile concentrations (5 and 10 mM) and high substrate
315 concentrations.
316 The experimental results (the calculated slopes of the linear section of each curve, (V_{max})) were
317 fitted into Michaelis-Menten equations, in order to obtain the corresponding values of the
318 Michaelis-Menten constant (k_m) and the catalytic constant (k_{cat}) (**Table III)**. The statistical
319 analysis indicated that both pH and bile concentration, as well as their interaction significantly
320 affected both kinetic parameters ($p < 0.05$). It should be noted that the studied system was a
321 heterogeneous reaction in which the enzyme, being soluble, only had access to the molecules
322 of substrate on the surface of the drop of the dispersed phase (fat globules) (García, 2005). The
323 enzyme activity would then not depend on the molar concentration of the enzyme, but on the
324 concentration of substrates at the interfaces (Reis et al., 2009). Therefore, the specific
325 interfacial area of the emulsion would be a critical parameter in this type of systems, just as the
326 substrate concentration is in homogeneous systems. However, milk being a homogeneous
327 processed food, was considered for the calculations, to be a homogeneous system (where the
328 substrate is dispersed throughout the reaction volume) and not a heterogeneous system. The
329 increase of pH and bile concentration led to a higher affinity of the enzyme for the substrate
330 (decreasing values of the Michaelis-Menten constant (k_m)). In a similar way, the catalytic
331 constant, (k_{cat}), related with the velocity of the reaction, was dependent on the pH and bile
332 concentration (in this case k_{cat} increased as the pH and bile concentration did). Accordingly,
333 the extent of lipolysis will depend on both parameters. Bile salts are essential as they compete

to remove other compounds such as proteins and emulsifiers from the fat droplets surface and allow lipase and co-lipase to absorb onto the lipid surface and instigate lipolysis. Complementary, bile salts motivate lipolysis-products transportation (mainly free fatty acid and monoglycerides) from the lipid surface to the intestinal fluid and their further micellation (Maldonado-Valderrama et al., 2011). In this case, the effectiveness of the enzymatic supplement seemed to be highly dependent on the removal of caseins, precipitated after gastric digestion, from the fat droplets surface by bile salts. As expected, pH of the intestinal stages resulted crucial as well, because of the absence of pancreatic activity under 5.7 ranging the optimum pH for pancreatic enzymes from 7 to 8 (González-Bacerio et al., 2010; Prazeres et al., 1994). This allowed lipase and its cofactor co-lipase to adsorb onto the lipid surface and instigate lipolysis.

Table III: Michaelis-Menten constant (k_m) and catalytic constant (k_{cat}) for the different combinations: pH and bile concentration

pH / bile (mmol · L^{-1})	Km (mM)	Kcat (seg^{-1})
6/1	10.58 ± 0.07 $^{(e)}$	0.391 ± 0.002 $^{(a)}$
6/10	5.06 ± 0.14 $^{(d)}$	1.030 ± 0.073 $^{(c)}$
7/1	3.56 ± 0.18 $^{(c)}$	0.819 ± 0.022 $^{(b)}$
7/5	2.56 ± 0.05 $^{(b)}$	3.223 ± 0.082 $^{(e)}$
7/10	1.47 ± 0.23 $^{(a)}$	3.515 ± 0.045 $^{(f)}$
8/1	2.64 ± 0.08 $^{(b)}$	2.785 ± 0.007 $^{(d)}$
8/10	1.56 ± 0.13 $^{(a)}$	3.612 ± 0.009 $^{(f)}$

All values are expressed as mean ± SD of at least two replicates. Different superscripts within a same column means statistical differences by Tukey Kramer test ($p<0.05$).Total

Furthermore, a comparative analysis of the state of the digested milk sample was performed by microscopy. **Figure 5** illustrates the lipolysis extent after 5 and 30 minutes of intestinal stage, for the experimental conditions pH 6/1 mM (A and C) y pH 7/10 mM (B and D). Even after 30 minutes of intestinal digestion, a great amount of fat globules was still observed under pH 6/1 mM (7.5 % of lipolysis extent). However, for pH 7/10 mM, almost the totality of the fat appeared to be digested after 30 minutes of intestinal digestion (80.3 %). These results agree

with those obtained previously, since, for pH 6/1 mM and pH 6/10 mM, the smallest kinetic parameters were obtained. These kinetic parameters allowed for estimating the reaction velocity of fat hydrolysis under different intestinal conditions. In this sense, **table IV** gathers the calculated enzyme concentration ratios (R) needed under the different intestinal conditions to obtain the same reaction velocity than in the standard conditions (pH 7 and 10 mM bile concentration). As expected, the highest value of the ratio (R) corresponds to the conditions of pH 6/1 mM indicating that under these conditions the enzyme is in a very unfavorable environment. On the contrary, at pH 8 and bile concentration of 10 mM, the amount of enzyme needed was the same than for the standard conditions (pH 7 and 10 mM bile). All this confirmed the great influence of these physiological parameters (intestinal pH and bile concentration) on the lipolysis of fat.

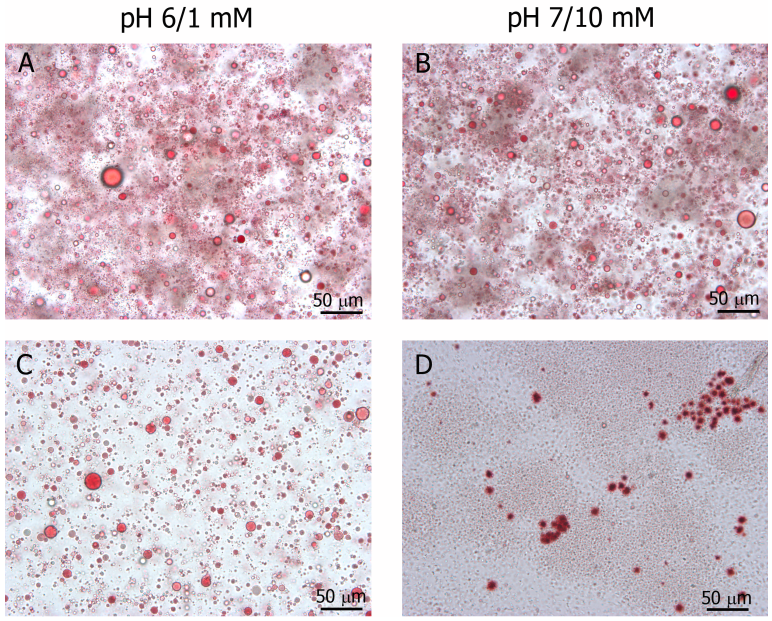


Figure 5: Optical microscopy images (20x) from the milkfat digested aliquots after 5 and 30 minutes of the intestinal stage for two experimental conditions: pH 6/1 mM (A and C (1.1 ± 0.2 % and 7.5 ± 0.8 % of lipolysis extent respectively)) and C) y pH 7/10 mM (B and D (30.4 ± 0.5 % and 80.3 ± 1.5 % of lipolysis extent respectively)). (Black bars correspond to 50 µm).

Table IV: R ratio between de enzyme concentrations needed at different pH/bile concentration to obtain the same velocity reaction that at pH 7 and 10 mM bile concentration.

pH / bile (mmol ·L^{-1})	R
6/1	42.04 ± 0.36 $^{(d)}$
6/10	8.37 ± 0.41 $^{(c)}$
7/1	7.92 ± 0.09 $^{(c)}$
7/5	1.57 ± 0.06 $^{(b)}$
8/1	1.85 ± 0.04 $^{(b)}$
8/10	1.00 ± 0.05 $^{(a)}$

4. Conclusions

The pH stat method was successfully used to monitor the free fatty acids release during the *"in vitro"* digestion process of a food sample (milk). The Michaelis-Menten constant (km) decreased and the velocity of the lipolysis reaction (r) increased as pH and bile concentrations did (pH 7/10 mM y pH 8/10 mM). The knowledge of kinetics behavior of fat-milk hydrolysis allowed estimating the amount of substrate to optimize the enzymatic supplement (Kreon®) activity required under different intestinal conditions of pH and bile concentration.

The results obtained in the present study point out the huge influence that the alteration of these physiological parameters (intestinal pH and bile concentration) might have on fat lipolysis. These GI alterations together with some inherent to food factors such as matrix characteristics, processing, interactions, etc. need to be better explored to assist food industry to develop tailor-made foodstuffs and to help health professional and dieticians to adapt the existing nutritional guidelines.

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