In Vitro Digestion of Lipids in Real Foods: Influence of Lipid Organization Within the Food Matrix and Interactions with Nonlipid Components

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Abstract: *In vitro* digestion research has scarcely addressed the assessment of the complexity of digestion in real food. The aim of the present study was to evaluate the influence of intestinal conditions, nonlipid components, and lipid organization within the food matrix on lipolysis extent. A selection of 52 foods was studied under different simulated intestinal conditions, including those related to patients with cystic fibrosis (pH6, bile salts 1 mM due to decreased pancreatic and biliary secretions) and to healthy subjects (pH7, bile salts 10 mM). Linear mixed regression models were applied to explain associations of food properties with lipolysis. Normal intestinal conditions allowed for optimal lipolysis in most of the foods in contrast to the altered intestinal scenario (30 compared with 1 food reaching > 90% lipolysis). Lipid-protein and lipid-starch interactions were evidenced to significantly affect lipolysis ($P < 0.001$) in all the digestion conditions, decreasing in those foods with low fat and high protein or high starch content. In addition, under decreased intestinal pH and bile concentration, lipolysis was lower in foods with complex solid structures and continuous lipid phase than in the oil-in-water continuous aqueous phase (global $P < 0.01$). However, in the normal conditions lipid organization within the food matrix did not show a significant effect on lipolysis (global $P = 0.08$). In conclusion, food properties play a crucial role in lipolysis, which should be considered when establishing dietary recommendations.

Keywords: food matrix, *in vitro* digestion, lipolysis, nutrition, pancreatic insufficiency

Practical Application: Food composition, lipid organization within the food matrix, and gastrointestinal conditions are key factors affecting lipolysis. Knowledge on that can be used to modulate lipolysis performance after food ingestion. Different applications are foreseen, as food design and nutritional recommendations for the general populations and specific target groups. The most immediate application is related to the scope of the research project that frames this work (www.mycyfapp.eu). These results have contributed to the development of a mobile app for cystic fibrosis patients, which includes an algorithm for enzyme dose prediction based on food properties. The app is currently being tested in a clinical trial setting.

Introduction

In vitro digestion methods rose in the past years as a powerful approach to study several aspects related to foods biotransformation within the gastrointestinal tract, especially those related to luminal digestion. They are a useful tool for reproducing the process in the laboratory, under controlled, accurate, and reproducible conditions. In this sense, the internationally harmonized protocol of Minekus et al. (2014) set up a common framework to conduct static *in vitro* digestion studies, indicating that the pertinent amendments have to be applied according to the nature of the research (Minekus et al., 2014).

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Then on, numerous studies have addressed the study of food properties on bioaccessibility of bioactive compounds, and the hydrolysis of macronutrients, among others. The vast majority of these studies have focused on monocomponent systems or ideal emulsions, in order to simplify the multifactor and complex nature of foods under digestion and to drive solid conclusions. Therefore, there are still few studies in the literature addressing the complexity of the real food and the influence of different variables in the gastrointestinal tract (Guo, Ye, Bellissimo, Singh, & Rousseau, 2017). Nonetheless, there is sufficient evidence to state that within the complexity of food structures, multiple matrix interactions occur among the components conforming it, which unequivocally alter the observed behavior of the components when assessed in isolation. However, today there is a poor systematic understanding of the impact of the food matrix on the processes that occur during nutrient digestion (Guo et al., 2017; Taylor et al., 2009).

One of the utility of studying the digestion of real foods is the potential application of the generated knowledge on the improvement of a food-related health condition. Exocrine Pancreatic Insufficiency (EPI) which is associated to some diseases, as for example cystic fibrosis (CF) (Woestenenk, van der Ent, & Houwen,

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2015), is related to altered intestinal conditions. Intestinal pH is lower due to the deficient secretion of bicarbonate as a consequence of the obstruction of the pancreatic duct, values being found between 5.5 and 6.5 (Gelfond, Ma, Semler, & Borowitz, 2013; Robinson, Smith, & Sly, 1990). There are also implications at the biliary level, including a decreased secretion of bile salts which can be up to 10 times lower as compared to healthy individuals. This defect has implications in lipid digestion. Bile salts are bio-surfactants that during lipid digestion are adsorbed onto fat droplets or remain as part of the continuous phase, and remove other compounds such as proteins or emulsifiers, and they also remove products from lipid digestion from the surface. Both mechanisms facilitate the access of lipases to the fat droplet and enhance lipolysis (Harries et al., 1979; Sarkar, Ye, & Singh, 2016).

Pancreatic insufficient patients have to adhere to pancreatic enzyme replacement therapy (PERT) to palliate this disorder, consisting on the exogenous administration of pancreatic enzymes in every meal. It enables digestion of nutrients, especially lipids, which is the most compromised. However, a PERT dosing criterion stills lacking of scientific evidence that considers the variety and complex nature of foods, being the general recommendation 2000 to 4000 Lipase Units/g lipid (LU/g) (Turck et al., 2016). In this sense, the current need of knowledge of lipolysis in real foods (Fieker, Philpott, & Armand, 2011; Li, Hu, & McClements, 2011) is an ideal target for applying the *in vitro* digestion methodology to adjust PERT.

Over the years, several studies in CF patients have been unable to establish any association between the dose of PERT and the coefficient of fat absorption, the method used to assess the PERT dose adjustment. Thus, we can find several authors claiming for an evidence-based method to adjust the dosing criterion in this therapy (Borowitz et al., 2005; Fieker et al., 2011; Schall, Bentley, & Stallings, 2006). In none of the studies assessing PERT dose on lipid digestion, composition of foods has been considered, so our hypothesis is that food properties could impact on PERT efficacy, thus explaining the historical lack of association between PERT dose and clinical outcomes.

The European Union's Horizon 2020 program for research and innovation has prioritized research for tackling societal challenges (European Commission, 2015). Following this practical approach, MyCyFAPP project pursues the establishment of a valid method to adjust PERT (Calvo-Lerma et al., 2017). As part of this project, the aim of the present study was to analyze lipolysis of a wide range of real foods under altered simulated gastrointestinal conditions in order to explain the influence of the inherent-to-food properties, such as nutrient composition and their interactions on lipolysis, and the lipid organization within the food matrix.

Methods

Materials

The selection of foods to be *in vitro* digested ($n = 52$) was made on the basis of a European multicenter study on CF nutritional habits and covering the whole range of food products (Calvo-Lerma et al., 2018): dairy, meat, fish, egg, nuts, fruit, oils, fats, potato, sweets, and cereal. Nutritional information of foods was collected from the official composition database of EuroFIR®. The lipid organization of the foods was established according to the criterion posed by Michalski et al. (Michalski et al., 2013). Information of the characteristics of the study foods can be found in Supplementary Table S1. In this table, the nutritional composition of the foods, including the estimation of the type of fat according to the free fatty acid (FFA) composition (saturated, SFA; monounsaturated, MUFA; and polyunsaturated, PUFA) is provided, on the basis of the official Spanish nutritional composition database (BEDCA, Base de datos Española de Composición de Alimentos; 2009. htpps://www.bedca.net. Updated August 2010. Accessed December 2017).

Pancreatic enzyme supplements (Kreon® 10000 LU) were kindly donated by Hospital Universitari i Politècnic La Fe (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme in the shape of gastro-resistant microspheres equivalent to 10000 lipase U, 8000 amylase U, and 600 protease U.

For the preparation of the simulated digestive fluids, the following chemicals were needed: pepsin from porcine gastric mucosa (≥2500 U/g protein), bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$ $(H2O)_6$, $(NH_4)_2CO_3$, and $CaCl_2$ all of them from Sigma-Aldrich Chemical Company (St Louis, MO, U.S.A.). NaOH (1 N) and HCl (1 N), were acquired from *AppliChem Panreac*. For the analytical determinations, Triton-X 100%, and the analytical standard palmitic acid were acquired from Sigma-Aldrich.

Experimental design

The experimental design consisted of a set of *in vitro* digestions with a dose of enzymes fixed at 2000 LU/g of lipid and different combinations of intestinal pH and bile salts concentration: pH6/10mM, pH7/1mM, and pH7/10mM, as study variables in order to analyze the impact of altered intestinal environments on lipolysis. Of note, the combination pH 6/1 mM would represents the worst possible case scenario in CF (Gelfond et al., 2013; Harries et al., 1979; Robinson et al., 1990) and the pH 7/10 mM approaches the regular intestinal conditions of a healthy adult (Minekus et al., 2014). All the experiments were conducted in triplicate, resulting in a total of 624 *in vitro* digestions.

In vitro digestion process

Food samples (5 g) were placed into 50 mL falcon tubes and *in vitro* digested according to the abovementioned experimental design. The static *in vitro* protocol applied consisted of 3 different stages, oral, gastric, and intestinal, established by the cost action-INFOGEST and published by Minekus et al. (Minekus et al., 2014). The digestion fluids were prepared fresh daily from stock solutions (Supplementary Table S2), and the enzymatic activity of the solutions was tested before each experiment following the protocol proposed by Carrière et al. (Carrière et al., 2000).

Oral stage: Simulated salivary fluid (5 mL) (SSF; pH 7) at 37 °C, was added to the food sample in a ratio 1:1 (v/w) and in case of solid foods, properly homogenized with a kitchen blender for 3 min (Vario Mixer, Ufesa 600 W).

Gastric stage: After the oral stage, simulated gastric fluid (SGF; pH 3) was added to each tube containing the oral bolus (1:1 v/w). Pepsin was added into the SGF to reach a concentration in the gastric mixture of (2000 U/mL). At this point, PERT dose 2000 LU/g lipid was added in order to simulate the oral administration of the enzymatic supplement, which is resistant to gastric digestion and the release of the enzymes occurs at the intestinal stage. The pH of the mixtures was adjusted with HCl (1N) to pH 2.8 ± 0.1 and samples were rotated head-over-heels at 55 rpm for 2 hr at 37 °C using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and an incubator chamber Selecta (JP Selecta SA, Barcelona). These mixing conditions provided constant mechanical energy to induce the breakdown of the food matrix during digestion.

Intestinal stage: Following the gastric stage, simulated intestinal fluid (SIF; pH 6 or 7) containing the bile salts (concentration 1 or 10 mM) was added in a proportion 1:1 (v/w) to each tube containing the gastric chime. The pH of the mixtures was adjusted to pH 6.0 ± 0.1 or 7 ± 0.1, depending on the experimental design, with NaOH (1N). Samples were then rotated head-over-heels at 55 rpm for another 2 h at 37 °C. pH was monitored during the digestion process and readjusted if necessary as lipase is not active at pH below 5.7 (Lesmes & McClements, 2012). After 2 hr of intestinal digestion, samples were placed in ice and pH adjusted to 9 to ionize all the FFAs and stop lipase activity (González-Bacerio, Rodríguez Hernández, & del Monte Martínez, 2010).

Lipolysis extent determination

The FFAs released after the *in vitro* gastrointestinal digestion process were measured. The products of the physiological triglyceride molecule hydrolysis are two fatty acids and one monoglyceride, so lipolysis extent was estimated assuming the release of 2 moles of fatty acids per 1 mole of the initial amount triglycerides (fat) in the food sample (Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014). Micellar phase from digested samples (100 μL) was separated by decantation using a 1.4 mm sieve and mixed with 10 mL of a solution made of 5.6 % Triton X-100 and 6% ethanol in water, to solubilize the FFAs. The release of FFAs after digestion was measured on the diluted samples using a FFA spectrophotometric assay kit (Roche Diagnostics, Indianapolis, IN, U.S.A.) in a spectrophotometer (UV/vis, Beckman Coulter) (Lamothe et al., 2014). Palmitic acid standard was used for quantitative determination of FFA.

Statistical analysis

The variables included for the statistical analysis were the nutritional information of food products: energy, protein, total carbohydrates, starch, sugar, total lipids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), fiber, calcium, iron, and sodium; and the lipid structure in the food matrix (Michalski et al., 2013): complex solid structure (lipid inclusion in CH and protein matrix and lipid inclusion in protein matrix), continuous aqueous phase (intracellular lipid droplets and tissues and oil-in-water emulsions), and continuous lipid phase (free fat, particles in solid fat, and water-in-oil emulsions). The response variable was lipolysis extent (%).

Data were summarized using mean, standard deviation, median, and first and third quartile in the case of continuous variables and with absolute ant relative frequencies in the case of categorical variables.

A heatmap diagram was performed to represent the similarity between all the assessed foods at the different experimental conditions. The distance measure used for heatmap clustering was Euclidean and the clustering method was Complete.

Linear mixed regression models were performed to assess the effect of the food composition on the lipolysis extent and other factors such as lipid organization in the food matrix were included as covariates. Additionally, because observations of the same food are more likely to have similar lipolysis extent due to their nutritional characteristics, the linear regression models were extended with the "Food" variable as random effect with random intercept to correct for the no independence of the data.

All analyses were performed using software R (version 3.4.2) using packages betareg (version 3.1-0), lme4 (version 1.1-14), and NMF (version 0.20.6). A *P*-value lower than 0.05 was considered statistically significant.

Results and Discussion

Effect of the intestinal conditions on lipolysis extent

The selection of 52 foods was *in vitro* digested at different intestinal conditions. When representing lipolysis extent of all the assessed foods as a function of the intestinal conditions (Figure 1), the condition of intestinal pH7 and bile 10 mM resulted to be the most related with a higher lipolysis extent, finding 30 foods with lipolysis extent >90%. In contrast, the combination of pH 6 and bile 1 mM depicted the lowest lipolysis results with only 1 food reaching >90% lipolysis. This is in accordance to previous research by our group (Calvo-Lerma et al., 2018). The mechanisms underpinning this result are that pancreatic lipase is well known to have a higher activity at pH 7 than 6 (Robinson et al., 1990). In addition, bile salts contribute to the emulsification process of lipids in the digestive fluids and consequently increasing the interfacial surface of the lipids available for being hydrolyzed (Verger & De Haas, 1976); therefore, the higher the bile concentration, the higher the lipolysis enabled.

However, the intermediate conditions, that is, pH 6 bile 10 mM and pH 7 bile 1 mM, showed different responses to the lipolysis (10 and 11 foods with >90% lipolysis, respectively), due to the complexity of the matrices being disintegrated at different rates and their interactions with the gastrointestinal environment. The heatmap brings a picture where it is easy to observe that the extension of lipolysis is more pH dependent for some foods than for others, as it is the case of cured cheese, spreadable chocolate, or crunchy biscuit bars; while in others, bile concentration is the main factor affecting fat hydrolysis (butter, drumstick, boiled egg, or bread). Additionally, there are some foods for which lipolysis depends on both factors, the pH and bile concentration (that is, Frankfurt sausage). Maldonado-Valderrama, Wilde, Macierzanka, & Mackie (2011) reported that there is a specific interaction between bile salts and colipase to promote lipase activity (Maldonado-Valderrama et al., 2011). Bile salts play a very important role in emulsifying fats entering the small intestine in chyme but also the interfacial composition of proteins and polysaccharides of emulsified fat. The consequence or the response to all these coupled factors is evidenced by differences on percentage of fat digested at the end of the intestinal stage. Therefore, for some foods the bile concentration showed a more favoring role than the pH such as in the hard egg or spreadable chocolate, while in others the opposite response was found, like in milk. Besides the influence of interfacial composition, digestion depends on the size of the emulsion droplets in the small intestine, since this influences the amount of lipid surface area exposed to the lipase, and the droplet size is inherent to the rheological properties of the surrounding medium (Taylor et al., 2009). Recently, the specific role of bile salts in the intestinal fluid and its pH have been described as related to fat droplets size in oil-in-water emulsions, this being a determinant of the lipolysis fate. Bile salts have shown to remove protein possibly present at the interface, this being the cause of enhanced lipase potential to act on the core of the hydrophobic lipid core (Sarkar, Horne, & Singh, 2010a). The pH of the intestinal environment is directly related to the isoelectric point of the protein that may be present at the lipid droplet interface, this causing possible electrostatic effect. When the protein present in the assessed foods shifts to a cationic form, these may bind to the anionic bile salts (Sarkar, Horne, & Singh, 2010b). As a consequence, different foods, with different types and amounts of protein, can result in a wide variety of intestinal environments, where the presence of bile salts may either promote or inhibit the activity of pancreatic

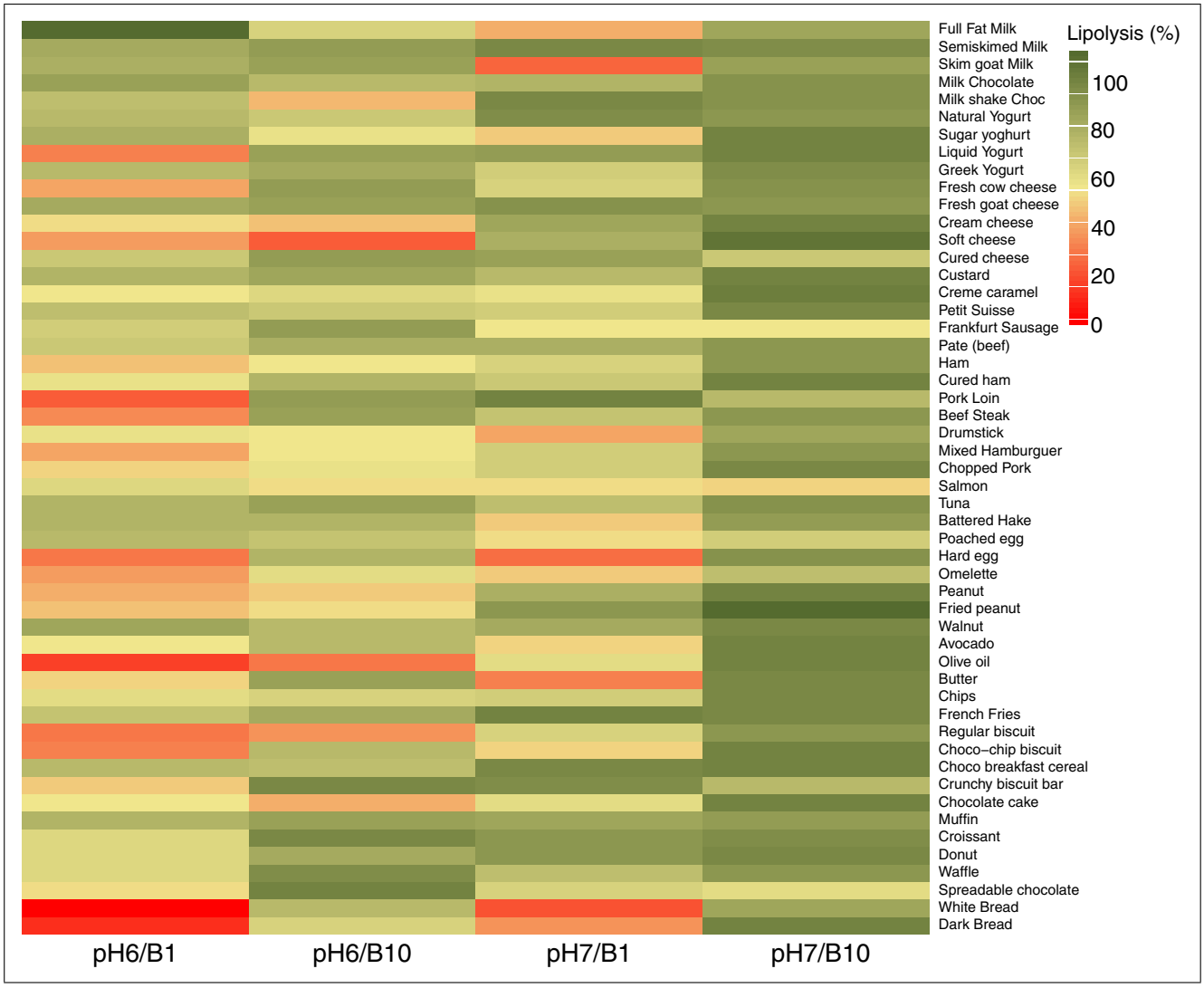

Figure 1–Heatmap representing lipolysis extent of the 52 assessed foods under different combinations of intestinal conditions pH/bile concentration at the PERT dose of 2000 LU/g fat.

lipase depending on their concentration (Bauer, Jakob, & Mosenthin, 2005; Lowe, 2002). In addition, bile salts can promote or inhibit lipase activity, depending to their ability to solubilize lipid digestion products and remove them from the oil-water interface, favoring the accessibility of lipases and preventing their inhibition by the products of lipolysis (Sarkar et al., 2016). This variable effect will depend on the type of lipids in the digestion medium (lipid organization and capacity of the lipids to be released from the matrix) and then, on the capacity of the bile salts to compete for the oil-water interface with the lipase (Gargouri, Julien, Bois, Verger, & Sarda, 1983).

The result is that lipids accessibility in food matrix and the interactions of the nonlipid components of the matrix remarkably contribute to modulate lipids digestion. These interactions were analyzed from the experimental data obtained in this work and the results are shown in the next section.

Interactions of lipids with the nonlipid components of the same food matrix

When analyzing the effect of nutrient composition on lipolysis in a multivariable setting, no clear effects were obtained, that is, the amount of none of the nutrients was significantly associated with lipolysis extent. This can be interpreted as the presence of a specific nutrient could favor lipid digestion in some foods but diminish it in some others (Guo et al., 2017), being the nutrient concentrations and the interactions among nutrients the determinant factor on lipolysis extent. Foods are complex structures in which lipids can be present at different levels of interaction with the other components of the matrix. In this sense, lipids can be embedded within hydrogel or protein structures in solid matrices, like in cheese, meat, fish, or nuts. In contrast, in liquid matrices like milk, lipids are less bounded to the structure what make them easy accessible to the enzyme action. Thus, digestion of lipids may depend on the breakdown of these matrices before they can be exposed to lipases (Chen, Remondetto, & Subirade, 2006). Besides, many of these nonlipid components may interfere with lipid digestion by altering the viscosity of the digestion media, by competing with lipase for the oil-water interface of the emulsified lipids, or by interfering with enzyme activity(Taylor et al., 2009). Therefore, the statistical models were readjusted considering these interactions among macronutrients (Table 1).

Table 1–Linear mixed regression models showing the influence of nutritional composition on lipolysis: interaction between protein and lipids, interaction of type of fatty acid with the protein content and the interaction between starch content and lipids.

Interaction	Estimate	95% Confidence Interval	*P*-value
Protein : lipids	3.431	[1.491, 5.371]	<0.001
Protein : SFA	6.141	[1.48, 10.825]	0.027
Protein : MUFA	−2.972	[−7.781, 2.043]	0.295
Protein : PUFA	5.09	[−2.222, 12.03]	0.217
Starch : lipids	4.031	[2.195, 5.868]	<0.001

The statistical analysis revealed that the interaction of protein content with lipids content (CI 95% [1.49, 5.37] $P < 0.001$) and with saturated fatty acids (SFA) (CI 95% [1.50, 10.85] P 0.027) had a significant effect on lipolysis extent, and also the interaction between starch content and lipids concentration (CI 95% [2.20, 5.87] $P < 0.001$).

The interaction protein-lipids (Figure 2A) did not represent a change in lipolysis extent in those foods with a high amount of fat (around 36 g/100 g) regardless the content of protein. According to this, cured cheese and chips, which both contain 34 g fat/100 g product but very different content of protein (24 and 6 g/100 g, respectively) showed a similar lipolysis extent. However, foods with a medium (12 g/100 g) and low (4 g/100 g) content of lipids resulted in a decreased lipolysis according to the increasing protein content. For example, tuna, which is low in fat (4 g/100 g) but has as much protein content as cheese (around 25 g/100 g), showed a much lower lipolysis extent, that is, 60% compared with 80%, when digested under the conditions of pH 6 bile 1 mM.

The interaction protein-SFA (Figure 2B) showed a similar effect than the protein-lipid one. Foods with medium or low SFA content tended to decrease lipolysis extent as long as the protein content increases; whereas a high SFA content led to a mild increase lipolysis with the increasing protein content. The physical state of the food matrix and lipids process have proved to play a crucial role during digestion, and the composition in fatty acids is an indirect measure of the fat melting point (Knothe & Dunn, 2009). In front of the difficulty of assigning a specific melting temperature to all the foods/fats, the saturated/unsaturated fatty acids ratio was considered. The statistical analysis revealed a significant association between this ratio and lipolysis extent ($P = 0.002$, CI = [2.526, 10.185]), what meant that the higher the SFA content and the lower MUFA and PUFA, the higher lipolysis. This finding suggests that in those foods with a saturated fatty acid profile, and so a higher lipids melting point, lipolysis is more favorable.

The observed interaction between the content of proteins and lipids, and its effect on lipolysis, has not been explicitly reported in the literature, although 1 study attributed the amount of protein forming a gel mesh to the lipolysis achieved of a lipid emulsion. In this study, the higher gel structure forming capacity of the protein was related to the lower lipase diffusion capacity to the interface of fat droplets. As proteases facilitated the breakdown of the gel network, digestion of fat occurred (Sarkar et al., 2015). Therefore, the lipid-protein interaction of our study could be explained in a physicochemical basis similarly.

None of the rest of the nutrients (total carbohydrates, sugar, fiber, calcium, iron, and sodium), or their interactions, presented any effect on lipolysis extent with the exception of starch (Figure 2B). The higher the content of starch, the lower the lipolysis in foods with a low content of lipids, while the inverse association was obtained for foods with a high content of lipids. According to the model, a high-fat low-starch product like pate, would show a higher lipolysis than a food with a similar content of starch but with a low content of lipids like bread. These results are in accordance with those reported by other authors: Nakamura et al. concluded that polysaccharides protect the lipid emulsion droplet surface by forming a thick hydrated layer (Nakamura, Yoshida, Maeda, & M. Corredig, 2006). It has been proposed that carbohydrates can affect lipid digestion in the small intestine through a variety of physicochemical mechanisms, such as increasing viscosity of the digestion medium, alteration of droplet disruption or coalescence kinetics, binding of bile salts, phospholipids, or enzymes (Guo et al., 2017; Lairon, 1997; Lairon, Play, & Jourdheuil-Rahmani, 2007). This evidence suggests that in the case of the assessed breads which had 40 and 50 g starch/100g of product, lipolysis could be low due to the viscosity these high amounts may have conferred to the digestion medium. In addition, as compared to other high-starch products such as biscuits ranging in starch content from 35

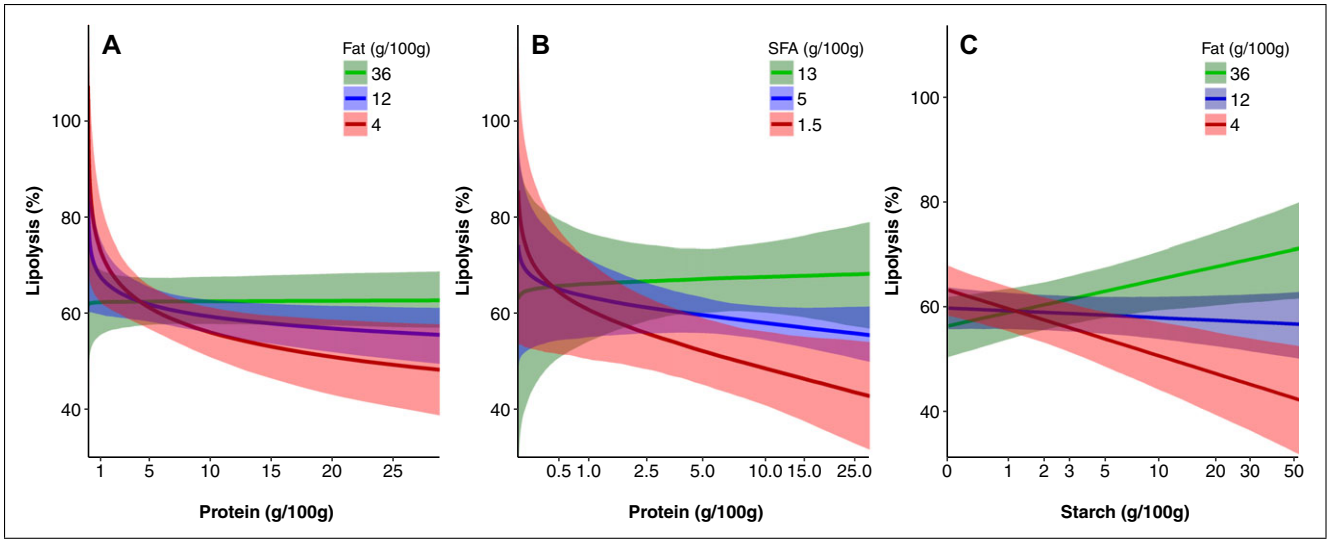

Figure 2–Nutrient interaction and the effect on lipolysis: protein-lipid interaction (A), protein-SFA interaction (B), and starch-lipid interaction (C). SFA, saturated fatty acids.

Table 2–Linear mixed regression model explaining the effect of the lipid structure in the food matrix on lipolysis at the digestion conditions of intestinal pH 6 and bile concentration 1 mM.

Structure	Substructure	Estimate	Std. Error	95% Confidence Interval	*P*-value
Complex solid structure	Lipid inclusion in protein matrix	−17.167	8.753	[−33.43 −1.21]	0.050
Complex solid structure	Lipid inclusion in CH and protein matrix	−24.468	8.081	[−39.48 −9.46]	0.004
Continuous aqueous phase	Intracellular lipid droplets and tissues	−21.482	7.649	[−35.69 −7.27]	0.007
Continuous lipid phase	Free fat	−57.281	20.947	[−96.19 −18.37]	0.009
Continuous lipid phase	Particles in solid fat	−20.027	20.947	[−58.94 18.88]	0.344
Continuous lipid phase	Water-in-oil emulsion	−23.157	20.947	[−62.07 15.75]	0.275
Global					0.01

Table 3–Linear mixed regression model explaining the effect of the lipid structure in the food matrix on lipolysis at the digestion conditions of intestinal pH 7 and bile concentration 10 mM.

Structure	Substructure	Estimate	Std. Error	95% Confidence Interval	*P*-value
Complex solid structure	Lipid inclusion in protein matrix	−6.976	5.16	[−16.56 2.60]	0.183
Complex solid structure	Lipid inclusion in CH and protein matrix	−2.692	4.76	[−11.54 6.15]	0.575
Continuous aqueous phase	Intracellular lipid droplets and tissues	−7.358	4.51	[−15.73 1.01]	0.11
Continuous lipid phase	Free fat	4.109	12.34	[−18.89 27.04]	0.741
Continuous lipid phase	Particles in solid fat	−35.66	12.34	[−58.59 −12.73]	0.006
Continuous lipid phase	Water-in-oil emulsion	2.861	12.34	[−20.07 25.79]	0.818
Global					0.08

to 52 g/100 g and in lipids from 11 to 22 g/100 g, lipolysis was much lower in breads due to their low content of lipids, 4.4 and 1.5 g/100 g.

Overall, the study of the interaction of lipids with protein and starch, which play a key role in food structure, revealed that lipolysis decreases in those foods with low lipid and high content of the other macronutrients, while this effect is not shown if the food presents a high lipid content too. Besides the discussed mechanisms supporting these results, Binks et al. (2002) pointed out that solid particles like carbohydrates and protein, can anchor irreversibly to the oil-water interface, limiting the ability of bile salts and enzymes to physically contact, thus decreasing lipolysis. The effect of the food structure, especially lipid structure, is further studied in the following section.

Organization of lipids in food matrices and their influence on lipolysis

Besides nutrient composition, the lipid organization in the food matrix was considered as a categorical variable to explore its influence on lipolysis. The 52 tested foods were classified in 3 groups: (1) foods with complex solid structure, (2) foods with a continuous aqueous phase, and (3) foods with a continuous lipid phase. Foods with complex solid structure were divided in 2 subgroups, those with lipids inclusion in a protein matrix (for example, cheese) and those with lipids inclusion in carbohydrate and protein matrix (for example, cookies). Lipids in foods with a continuous aqueous phase can be structure as oil in water emulsion (for example, milk) or as intracellular lipid droplets and membrane structures (tissues) (for example, meat, egg yolk, vegetables). Finally, different subgroups of foods with a continuous lipid phase were established: free fat (for example, oil, lard), foods with particles dispersed in solid fat (for example, chocolate), and water in oil emulsions (for example, butter or margarine). For the analysis of the results, the two intestinal digestion conditions were explored separately: intestinal pH7 and bile concentration 10 mM (corresponding to the standard conditions of a healthy adult), and pH 6 bile 1 mM corresponding to EPI. Table 2 and 3 show the results of the linear mixed regression model for each set of conditions, in which the differences in lipolysis for the lipid structures and substructures are reported as compared to the oil-in-water emulsion substructure as the reference.

When digested under the conditions intestinal pH 6 and bile concentration 1 mM (Table 2), lipolysis extent resulted significantly different among lipid structures as compared to the oil-in-water emulsion structure (global P 0.01), which showed the highest lipolysis extent (Figure 3). The reason for foods with lipids structured as oil-in-water emulsion showing the highest lipolysis extent, might be due to the presence of surfactant agents which are inherently present, what at the time increase the lipid droplets surface area (Verger & De Haas, 1976). In addition, liquid phase systems offer less resistance to diffusion, so enzymes accessibility to fat is facilitated (Guo et al., 2017). Food emulsions, such as milk, cream, and dairy-based deserts, may be stabilized by a wide variety of different natural or added emulsifiers, including small molecule surfactants, phospholipids, proteins, polysaccharide, and their mixtures (McClements, 2005), what make lipid droplets more accessible to the enzymes.

In this scenario, the free fat structure, that is, olive oil, showed the most negative effect on lipolysis extent (95% CI [-96.2, -18.4] P 0.009). Oil has 98% lipid composition and contains no other macronutrient such as protein or starch. This makes it a bulky substrate for the enzymes, with no other surfactant in the medium than a low concentration of bile salts (1 mM), what represent unfavorable conditions for lipase activity. The foods with complex solid structures did also achieve a significant lower lipolysis. The negative effect of these matrices was more pronounced in the case of solid matrices containing both protein and carbohydrates, such as bread, biscuits and pastries (95% CI [-39.5, -9.5] P 0.004) than in those containing mainly protein, including meat and fish (95% CI [-33.4, -1.2]. The reason for the difference between the 2 substructures may relay on the starch content of some foods such as bread, which, as above discussed, may increase the viscosity of the digestion medium, making difficult the accessibility of lipase to fat (Guo et al., 2017). The other lipid substructure included in the continuous aqueous phase category, that is, intracellular lipid droplets and membrane structures, was also negatively associated to

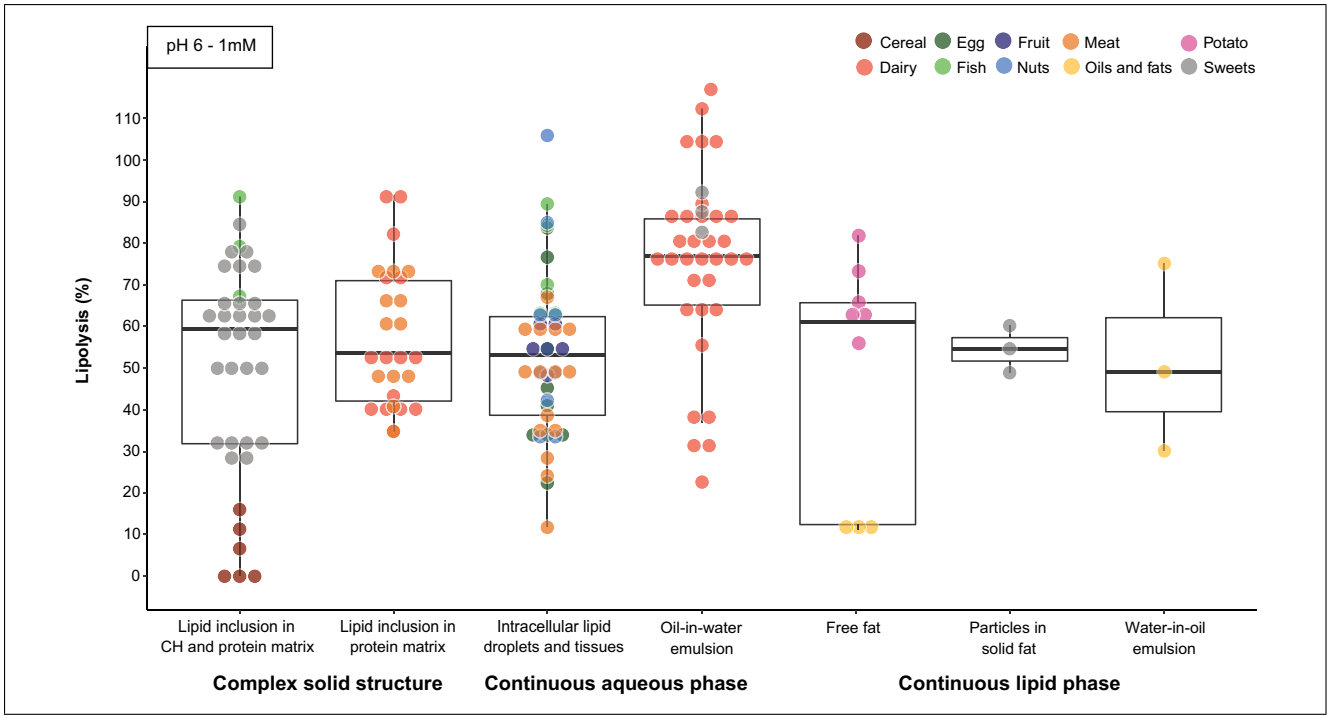

Figure 3–Boxplots representing lipolysis extent of the assessed food groups according to the lipid structure in the food matrix, under the digestion conditions of intestinal pH 6 and bile salts concentration 1 mM.

lipolysis (95% CI [-39.7, -7.3] *P* 0.007). In this type of structure, lipids are very embedded like in the case of nuts what makes difficult the matrix disruption during digestion and therefore the release of fat to be accessible to the enzymes (Grundy et al., 2016).

In contrast, when assessing lipid organization in the digestion conditions of intestinal pH 7 and bile concentration 10 mM, nonsignificant effect on lipolysis was observed except in the case of "particles in solid fat" (Table 3), represented by chocolate products,

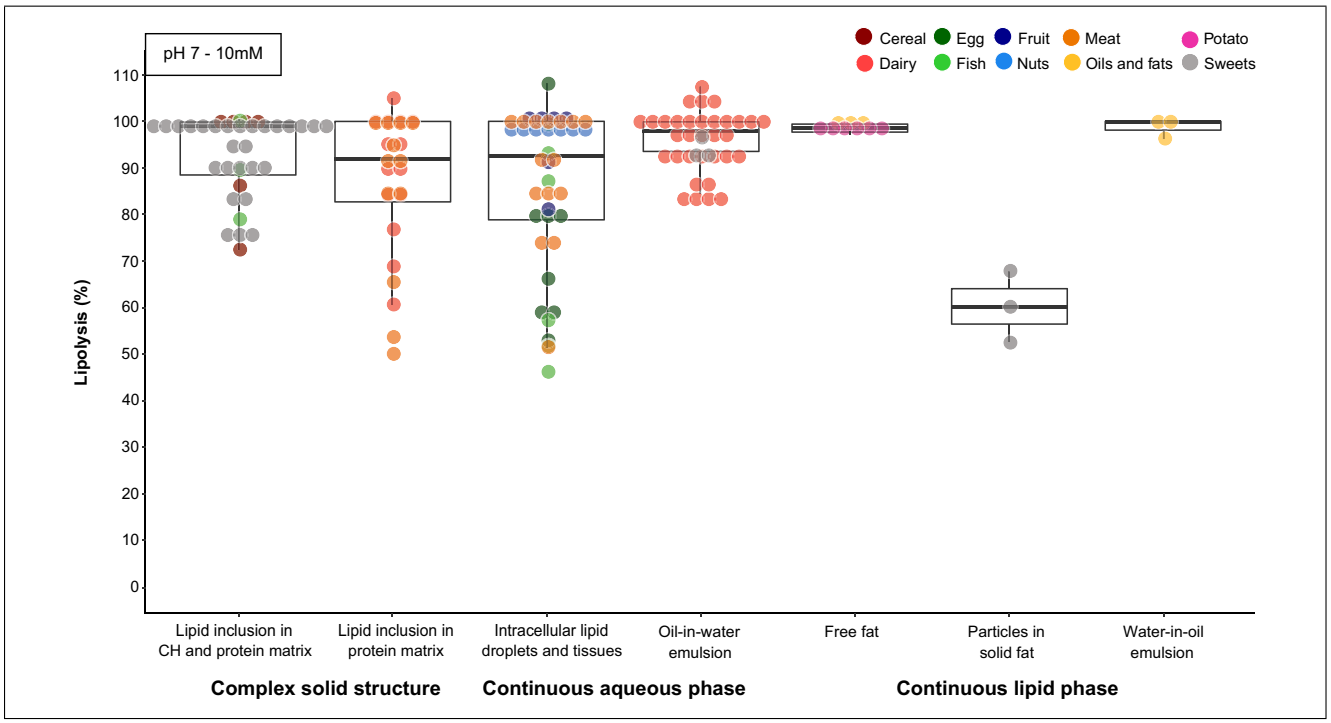

Figure 4–Boxplots representing lipolysis extent of the assessed food groups according to the lipid structure in the food matrix, under the digestion conditions of intestinal pH 7 and bile salts concentration 10 mM.

in which lipolysis was negatively associated to this structure (95% CI [-58.6, -12.7] P 0.006). Thus, lipids in chocolate products were those more difficultly hydrolyzed in both digestion environments scenarios assessed. Apart from this exception, all the categories obtained a similar median lipolysis extent between 90% and 100% (Figure 4).

This finding evidences that, when in normal digestion conditions (pH 7/10 mM), the lipid organization in the food matrix does not affect lipolysis, and satisfactory extents are achieved. It is of special relevance in the case of CF, in which pH and bile salts concentration are lower, and in which the dose of enzymatic supplements to be taken will depend on this factor. For example, according to our results, in the intestinal pH 6 and bile 1 mM scenario, for some dairy products the assessed dose of 2000 LU/g fat would enable for optimal lipolysis >90%. However, in the case of foods with other structures, higher doses of the supplements might be given in order to reach higher lipolysis extents.

To sum up, through the present study we have elucidated the role of different factors on lipolysis in real foods. First, the intestinal conditions play a key role, being the conditions pH 7 and bile concentration 10 mM those leading to lipolysis extents >90% in almost all the assessed foods. Second, the results reveal strong interactions among nutrients conditioning lipolysis: foods with medium and low lipid (and SFA) content show limited lipolysis extent when the content of protein or starch are high, but not those with high amount of fat. Finally, in the intestinal conditions pH 6 and bile 1 mM, the lipid organization in the food matrix is significantly associated with lower lipolysis extent, compared to the highest median value achieved in oil-in-water emulsions.

Conclusion

Besides simulated intestinal conditions, food properties such as nutrients composition and their interactions, and lipid organization in the food matrix determine lipolysis during *in vitro* digestion of real foods, leading to a wide range of lipolysis extents. Therefore, food characteristics should be considered for dietary recommendations whatever the objective is to maximize or minimize lipid extent, but especially for people suffering EPI. The results of this study will be used as key data in the development of a predictive enzyme dose calculator algorithm in the framework of MyCyFAPP Project, which will support CF patients self-adjusting of the dose based on evidence-based data.

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Author Contributions

J. Calvo-Lerma and A. Andrés designed the study. J. Calvo-Lerma 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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