Paz-Yépez, C.; Peinado, I.; Heredia Gutiérrez, AB.; Andrés Grau, AM. (2019). Lipids digestibility and polyphenols release under in vitro digestion of dark, milk and white chocolate. Journal of Functional Foods. 52:196-203. https://doi.org/10.1016/j.jff.2018.10.028

Lipids digestibility and polyphenols release under *in vitro* digestion of dark, milk and white chocolate.

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ABSTRACT

This study evaluated the influence of intestinal conditions on lipolysis and polyphenols release and bioaccessibility in dark, milk and white chocolates. Chocolates were *in vitro* digested under different intestinal conditions of pH (6 and 7), bile concentration (1 and 10 mM) and pancreatic concentrations (1,000 - 3,000 LU / g Fat). The lipolysis varied from 300 to 500 mg FFA / g fat in dark chocolate and ranged between 600-1000 mg FFA / g fat in both, milk and white. Polyphenols release in dark chocolate (up to 12 mg GA/ g), seems to be related to the absence of dairy compounds. Finally, no effect of intestinal pH or biliary concentration was found on the lipolysis in digested dark and milk chocolates. The oral pancreatic supplementation, however, was crucial to lipolysis and polyphenols release in all chocolates, even if no differences were found on these parameters from 2,000 LU/ g fat.

Keywords: *in vitro* digestion; pancreatic insufficiency; chocolate; lipolysis; polyphenols.

1. Introduction

Chocolate is a fat continuous matrix within which there are particles of cocoa powder, sugar, and in the case of milk chocolate, milk powder. Besides the high fat content of chocolate, generally exceeding 30 g of fat per 100 g of food (United States Department of Agriculture, 2018), it has lately gained attention mainly due to its main component, cocoa. In fact, cocoa is well known for its lipid and antioxidant properties. Cocoa butter, has a lipid profile characterized mainly by oleic, stearic (≈34% w/w) and palmitic (≈27% w/w) acids, followed by minor fractions of linoleic, arachidic, palmitoleic, margaric, α-linoleic and myristic acids. In addition, cocoa is a polyphenols-rich product, being one of the largest known food sources of flavan-3-oles, mainly epicatechin, catechin and notably procyanidins (Beckett, 2008; Jalil & Ismail, 2008).

In the last years, many studies have reported a relation between the consumption of cocoa derivatives, especially dark chocolate, with beneficial health effects on cardiovascular and degenerative diseases, related to the antioxidant activity of procyanidins (Almoosawi, Tsang, Ostertag, Fyfe, & Al-Dujaili, 2012; Cooper, Donovan, Waterhouse, & Williamson, 2008; Jalil & Ismail, 2008; Keen, Holt, Oteiza, Fraga, & Schmitz, 2005; Ortega, Reguant, Romero, Macià, & Motilva, 2009). Most of these studies extensively reported not only the *in vitro* health benefits of cocoa consumption but also its effects in vivo (Jalil & Ismail, 2008; Kurosawa et al., 2005; Matsui et al., 2005; Ramiro et al., 2005; Vinson et al., 2006). However, while most polyphenols showed antioxidant action *in vitro*, they did not necessarily exert antioxidant potential in vivo (Cooper et al., 2008; Jalil & Ismail, 2008). Related to the health protective effects of cocoa polyphenols, it is important to point out the modifications that undergo the processing of cocoa such as seed fermentation, roasting, nib-grinding, alkalizing, tempering, molding or enrobing, cooling, and packing (Beckett, 2008; Ortega et al., 2009; Wollgast & Anklam, 2000) might modify the chemical features of polyphenols compounds and therefore, their effects on health. Besides, it is also important to consider the interaction that polyphenols might exert with other components within the food matrix, such as macromolecules, since these can have significant effects on their bioaccessibility and bioavailability (Jakobek, 2015). For instance, the interaction between lipids and polyphenols has been reported in literature. In a previous study of *in vitro* digestibility of cocoa polyphenols in a matrix containing fat, Ortega et al. (2009) suggested that the fat content in the cocoa liquor could have a protective effect on cocoa polyphenols because it enhances the micellarization and stability of polyphenols during digestion. Additionally, the access of free radicals to lipid molecules can be hindered by the presence of polyphenols in lipid molecules, reducing the harmful caused by lipid oxidation products (Jakobek, 2015). On the other hand, it has also been found that polyphenols affected the emulsification taking place during the gastrointestinal process by increasing the droplet size and decreasing specific surface area, causing in addition

a decrease in the activity of the gastric and/or pancreatic lipases (Shishikura, Khokhar, & Murray, 2006; Sugiyama et al., 2007; Uchiyama, Taniguchi, Saka, Yoshida, & Yajima, 2011).

Food components, such as polyphenols or lipids, will need to be released from their food matrix in order to be bioavailable, so they can become then absorbable (bioaccessible), meaning they can be absorbed by intestinal cells and be metabolized (Asensio-Grau, Peinado, Heredia, & Andrés, 2018; Faulks & Southon, 2005; Nimalaratne, Savard, Gauthier, Schieber, & Wu, 2015). Nutrients bioaccessibility will depend on different factors related to the food itself such as food composition, its matrix, type of nutrients, and/or processing conditions (Granado-Lorencio et al., 2007; Nimalaratne et al., 2015; Pineda-Vadillo et al., 2017; Ryan, O'Connell, O'Sullivan, Aherne, & O'Brien, 2008). In addition, individual factors such as gastrointestinal conditions (pH, secretion and composition of the digestive fluids, transit time…) might affect the release of food bioactive compounds (Ryan et al., 2008; Whitcomb et al., 2010). All this leads to the importance of analysing to which extent not only food matrix, but other factors, can modify the stability and the bioaccessibility of bioactive compounds, in order to better understand the biological activity of food constituents (Rodríguez-Roque et al., 2015).

In fact, individual gastrointestinal conditions might be different among different individuals depending on their age, gender, diet, etc. (Shani-Levi et al., 2017). These differences will become even more relevant under specific digestive disorders. For instance, special attention has been addressed to rich-fat foods for dietary recommendations aimed at subjects with specific requirements of fat intake. This is the case of individuals suffering of exocrine pancreatic insufficiency (EPI). EPI is a physiological disorder characterized by a decrease of secretions of Cl^-, water and HCO^{-3}, with the consequent decrease of pancreatic and biliary secretions, causing dilation and obstruction of the pancreatic and bile ducts (Li & Somerset, 2014). Due to alterations at pancreatic level, a deficiency in the production of digestive enzymes at the duodenum might occur, leading this, to malnutrition and some associated problems. Currently, the treatment of EPI is carried out by enzymatic substitution therapy (EST), which consists on oral administration of porcine pancreatin formulated as gastro-resistant microcapsules. Actual guidelines of the supplement advise an enzyme amount of 2,000 Lipase Units LU/ g fat intake, based on the overall fat content of the meals or on patients body weight (Turck et al., 2016). However, these recommendations seem, nowadays, insufficient for the efficiency of the treatment since host-factors such as intestinal pH, bile concentration as well as food intrinsic ones (composition, structure and lipid organization in the food-matrix among others) might have a significant impact on the efficiency of the supplement, and therefore on macronutrients digestion (Rovner, Schall, Mondick, Zhuang, & Mascarenhas, 2013).

In this way *in vitro* digestion models are considered useful tools that can be used to study the digestive properties of different food forms, helping to understand the mechanisms behind the differences observed *in vivo.* To the authors knowledge, there are already some studies focusing on lipids absorption and polyphenols bioaccessibility of cocoa based products (Jalil & Ismail, 2008; Kurosawa et al., 2005; Matsui et al., 2005; Ortega et al., 2009; Ramiro et al., 2005; Vinson et al., 2006), however, in all of them, gastrointestinal conditions were simulated according to a standard healthy adult. Therefore, the aim of the present study was to evaluate the differences between dark, milk and white chocolate in terms of lipids digestibility and bioaccessibility of total polyphenols under different intestinal conditions (pH, concentration of bile and pancreatic enzymes). Besides, the enzymatic supplementation leading to the highest *in vitro* lipolysis extent under unfavourable intestinal conditions will be obtained for each chocolate.

2. Materials and Methods

2.1. Raw Material

Three types of chocolates, available in packets of 100 g each, were purchased from a local supermarket (Dark chocolate (D), Milk chocolate (M), White chocolate (W)), all of them form the same brand. Table 1 details the nutritional and compositional information available in the label.

2.2 Chemicals

α-amylase from human saliva (1000-3000 U/ mg protein) and pepsin from porcine gastric mucosa (≥ 2,500 U/ g protein), were obtained from Sigma-Aldrich. Pancreatin from swine pancreas (Kreon® 10,000 lipase units (LU), Abbot), was kindly donated by “Hospital Universitari Politècnic La Fe” (Valencia, Spain). Each capsule contains 150 mg of porcine pancreatic enzyme equivalent to 10,000 lipase U., 8,000 amylase U. and 600 protease U.

The following chemicals were needed for preparation of the simulated digestive fluids: bovine bile extract, KCl, KH_2PO_4, $NaHCO_3$, NaCl, MgCl2 $(H2O)_6$, $(NH_4)_2CO_3$ and $CaCl_2$ all of them from Sigma-Aldrich Chemical Company (St Louis, MO, USA). NaOH (1 N) and HCl (1 N), were acquired from AppliChem Panreac. For the analytical determinations, all solvents were analytical grade; Triton-X 100%, ethanol 96% (Labkem), enzymatic kits for the analysis of free fatty acids (Roche Diagnostics, Indianapolis, IN, USA). The analytical standards oleic acid and for the content of total polyphenols, methanol, Folin-Ciocalteu reagent, Na_2CO_3, gallic acid (GA) were all from Sigma-Aldrich).

2.3 In vitro simulation of gastrointestinal digestion

2.3.1 *In vitro* Digestion process

The digestion procedure used was based on the standardized static *in vitro* digestion method for food published by Minekus et al. (2014) with some modifications in order to simulate EPI conditions as detailed in previous studies (Asensio-Grau et al., 2018; Peinado, Larrea, Heredia, & Andrés, 2018). The digestion fluids were prepared fresh from stock solutions, salivary (SSS), gastric (SGS) and intestinal (SIS) according to Minekus et al. (2014). Table 2 summarizes composition and amounts of the fluids used in each of the stages of the digestion process. The enzymatic activity was tested before each experiment following the protocol proposed by Carrière et al., (2000). Each experimental condition was performed in triplicate. The *in vitro* digestion was performed as follows:

Oral stage: chocolates samples were mechanically broken down with a mortar, and the amount of sample to be digested was weighted in order to have 0.35 g fat in each tube (50 mL falcon tubes). Simulated salivary fluid (5 mL) (SSF; pH 8) at 37 ºC, was added to the falcon tubes containing chocolate samples in a ratio 1:1 (v/w), and then they were properly homogenized and incubated for 3 minutes at 37 ºC in an incubator chamber Selecta (JP Selecta SA, Barcelona). Human α-amylase was added as a part of SSF to reach a concentration in the saliva mixture of 75 U/ mL.

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 2,000 U/mL. The pH of the mixtures was adjusted with HCl (1N) to pH 3 ± 0.1 and samples were flipped from top to bottom at 55 rpm for 120 minutes at 37 ºC using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia) and incubated in a chamber Selecta (JP Selecta SA, Barcelona). These conditions provide mechanical energy to cause the disintegration of the food matrix during gastric digestion, in a similar way as it happens physiologically.

Intestinal stage: following the gastric stage, simulated intestinal fluid (SIF; pH 7) containing bile salts (1 or 10 mM) and pancreatin (0; 1,000; 2,000; 3,000 LU/g of fat) was added in a proportion 1:1 (v/w) to each tube containing the gastric chime. The pH of the mixtures was adjusted with NaOH (1N) to pH 6.0 ± 0.1 or 7.0 ± 0.1, depending on the experimental design. Samples were then flipped from top to bottom at 55 rpm for another 120 minutes at 37 ºC. pH was monitored during the digestion process and readjusted if necessary as pH below 5.7 might inactivate lipase activity (González-Bacerio, Rodríguez Hernández, & del Monte Martínez, 2010; Prazeres, Garcia, & Cabral, 1994).

144 *2.3.2. Experimental design*

145 The experimental design for each type of chocolate consisted of two sets of experiments. In the first set, the
146 amount of pancreatic enzymes was fixed at 2,000 LU/g of lipid, and the study variables consisted on different
147 combinations of intestinal pH/bile concentration (pH6/1mM, pH6/10mM, pH7/1mM and pH7/10mM) in order to
148 analyse the impact of different intestinal conditions on the lipolysis and bioaccessibility of total polyphenols. In
149 this scenario, pH 7 and 10 mM of bile salts would correspond to the standard intestinal conditions of a healthy
150 adult (Minekus et al., 2014); while pH 6 and 1 mM would correspond to the most disadvantageous scenario in
151 EPI individuals (Gelfond, Ma, Semler, & Borowitz, 2013). In the second experimental set, intestinal conditions
152 were fixed at pH 6 and bile salts concentration 1 mM, and different pancreatic enzymes amounts (0; 1,000;
153 2,000 and 3,000 LU/g of fat) were tested in order to assess the influence of enzyme concentration. All the
154 experiments were designed to analyse the impact of different intestinal scenarios on matrix degradation index,
155 lipolysis extent, and total polyphenols release.

156 ***2.4. Analytical determinations***

157 Immediately after digestion, samples were placed in ice for 10 minutes in order to stop the enzymatic reactions
158 before performing the analytical determinations. After that, tubes were centrifuged (4,000 x g-force during 20
159 minutes, 10 ºC) to separate the solid fraction from the liquid phase. After centrifugation, the total content of
160 each tube was filtered through a metallic sieve (1.6 mm x 1.6 mm mesh) to separate out solid food particles.
161 These solid particles were rinsed twice with 5 mL of appropriate juice to remove any digested material. Blotting
162 paper was placed around the metallic sieve for 10 min to drain residual digestion juice. The undigested
163 chocolate particles (solid particles) were then transferred to an aluminum dish (previously weighed). The
164 drained liquid, called from now on “liquid phase”, was collected and 0.1 mL was used for free fatty acids
165 analyses; the remaining liquid phase was freeze-dried (-40°C and 1.25 mbar, Telstar, Terrassa, Spain) and
166 stored for polyphenols determination.

167 *2.4.1 Matrix Degradation Index (MDI %)*

168 Matrix degradation Index was determined in all samples after in the vitro digestion process. This parameter
169 represents the proportion of solids dispersed in the digestion fluids after duodenal digestion. The aluminum
170 dish with the undigested particles was placed in a forced air oven at 60º C for 48 h until constant weight. The
171 matrix degradation index (MDI) corresponding to the proportion of chocolate particles passing the metallic
172 sieve and was calculated according to Lamothe, Corbeil, Turgeon, & Britten (2012).

173 *2.4.2 Lipolysis extent (mg FFA/ g fat)*

174 0.1 mL of the liquid phase of each sample was diluted in 10 mL of an aqueous solution (5.6 % Triton X-100
175 and 6 % ethanol in water) to solubilize the free fatty acids and inactivate any remaining activity of pancreatic

enzyme. Fatty acids release during digestion was estimated using a spectrophotometric assay kit (Roche Diagnostics, Indianapolis, IN, USA) in a spectrophotometer (UV/vis, Beckman Coulter) at 564 nm (Lamothe et al., 2012; Asensio- Grau et al., 2018; Peinado et al., 2018). Oleic acid standard was used for quantitative determination of FFA (Free Fatty acids), since it is the major fatty acid in chocolate fat (Beckett & Stephen, 2008). Digested fat was estimated assuming the maximum release of 2 mol of fatty acids per 1 mol of triglycerides (Hunter, 2001), and results were expressed as mg of FFA, considering the average of molecular weight of oleic acid 282.47 g mol^{-1}.

2.4.3 Total Polyphenols content

Total polyphenols in the digested fluids were determined by the modified protocol of the Folin–Ciocalteu assay (Singleton, Orthofer, & Lamuela-Raventós, 1998). Freeze dried powder (digested chocolate liquid phase (50 mg), and solid undigested phase (50 mg)) was extracted using methanol (1 mL, 30:70 methanol-water solution (v: v)) by means of agitation at 55 rpm, for 120 min at 25 ºC using an Intell-Mixer RM-2 (Elmi Ltd, Riga, LV-1006, Latvia). The mixture was then centrifuged at 14.1 x g-force for 20 minutes (Eppendorf® Minispin®). After centrifugation, methanolic extracts (125 µL) were added into a 4 mL plastic cuvette with distilled water (0.5 mL) and Folin-Ciocalteu reagent (125 µL). After 5 min, 1.25 mL of Na_2CO_3 solution (7% [w/v]) and distilled water (1 mL) were added and measurements recorded after 30 min, at a wavelength of 660 nm. Gallic acid (0–700 µmol of gallic acid / L) was used as standard. Results were expressed as mg of gallic acid (GA) equivalent per gram of food.

2.5 Statistical analyses

Analysis of variance (simple and multivariate ANOVA) followed by Fisher LSD post-hoc tests were performed to find out the statistical significance of the type of chocolate and the intestinal variables (pH, bile salts concentration (mM) and pancreatin amount (UL/ g fat)) on MDI (%), lipolysis extent (%), and polyphenols release (mg GA/ g) by means of Statgraphics Centurion. Differences were considered statistically significant when $p<0.05$.

3. Results and discussion

3.1. Effect of intestinal conditions on matrix degradation Index (%) of the digested chocolates

The matrix degradation index (MDI) corresponds to the percentage of digested particles and it provides global information about the extent of different processes (solubilisation, mechanical disruption, chemical and enzymatic reactions) undergone by the food matrix during oral, gastric and intestinal stages. During digestion, food absorbs a significant amount of water, and together with the action of digestive enzymes it promotes the

softening of the matrix and the reduction of cohesive forces; depending on the food matrix composition and structure it will result in different degradation extent (Kong & Singh, 2009). Additionally, in fat continuous products as chocolate, softening and matrix disruption is mainly due to melting of fat as oral temperature. Table 3 shows the results of MDI of the different chocolate types after the *in vitro* digestion process at different intestinal pH and bile concentrations, as well as for 2,000 LU / g fat and different pancreatin amounts. MDI ranged from 50 to 75 % (average value) in dark chocolate, while it reached 87 to 94 % (average value) in the other two types of chocolates (milk and white chocolate). The statistical analyses revealed that neither the intestinal pH, the bile concentration nor the amount of pancreatin had a significant effect on MDI. The significant differences in this parameter were found to depend on the type of chocolate ($p < 0.001$), being the dark chocolate the one showing the lowest values of MDI.

It is important to consider how the different ingredients in chocolates (fat, lecithin and crystalline lactose) might affect their structure and therefore, their matrix degradation when they undergo digestion. Chocolate microstructure is the result of many factors including particle size, amount and distribution of fat, type and amount of emulsifier and solid particles (Afoakwa, Paterson, Fowler, & Vieira, 2009). Among the main components of chocolate, the most soluble is sugar, which will explain the major degradation of chocolates containing high content of this ingredient. In addition, the amount of fat will affect particle interactions, related to their distances and distribution within the food matrix (Glicerina, Balestra, Dalla Rosa, & Romani, 2016). In their study Glicerina et al., (2016), compared the microstructural and rheological properties of dark, milk and white chocolate, revealing that dark chocolate showed a more aggregated structure, with the lowest minimum distance between particles and less open spaces (filled with fats) than milk chocolate samples. These microstructural properties would explain the lower MDI obtained in digested dark samples (Table 3) compared to white and milk samples. The MDI values of white chocolate were the highest, although very similar to the values obtained for milk chocolate samples, probably due to the similar amount of fat in both samples (Table 1). However, the more aggregated microstructure due to the presence of cocoa particles between the sugar ones in milk chocolate would explain the lower values of MDI compared to the white chocolate.

3.2. Effect of intestinal conditions on total polyphenols release from the digested chocolates and on their final bioaccessibility

Figure 1 shows the polyphenols content (mg equivalent of gallic acid / g of chocolate) of dark, milk and white chocolate for both, solid undigested chocolate as well as digestion fluids, after oral, gastric and intestinal digestion. The effect of intestinal pH (6 or 7) and bile concentration (1 and 10 mM) on these antioxidants is also gathered in this figure. As it can be observed, the initial quantity of polyphenols seemed to be directly related to the cocoa content in the product. Of note, dark chocolate presented the highest polyphenols content,

five-fold superior to the content found in milk chocolate and seven-fold than in white chocolate. As it can be observed, the oral stage characterised by simulated mastication and primary hydrolysis of glucids by α-amilase, resulted in a low release of polyphenols mainly due to the short duration of this stage. Therefore, most polyphenols remain in the solid phase, i.e. in the food matrix, after simulated mastication. The acidic pH, together with the enzymatic proteolysis carried out by pepsin on chocolate-protein (6, 7 and 10.4% in white, milk and dark chocolate, respectively) and the mechanical disruption acting during 120 min, were the main responsible of the increase of total polyphenols content during this gastric digestion.

It is important to point out that the polyphenols method quantifies only soluble polyphenols. According to these results, it seems that polyphenols take part in complex molecules before digestion, being released from these structures under both, gastric and intestinal conditions. In food, polyphenols are found mainly as esters, glycosides and polymers usually not absorbable in those forms, and therefore they need to be hydrolysed by digestive enzymes or intestinal microflora. Due to the low pH in the stomach, the flavonoid oligomers are transformed into smaller units. The flavon-3-ols pass intact to the duodenum in the form of aglycones. In the small intestine, deglycosylation, glucuronidation, methylation, sulphonation and hydroxylation of flavonoids occurs. Under alkaline pH conditions in the small intestine, the absorption of free phenolic acids occurs. Undigested polyphenols then pass into the large intestine, where colonic microflora degraded them into phenolic acids (Tarko, Duda-Chodak, & Zajac, 2013). Different factors such as mechanical destruction, nature of the food matrix, residence time under different gastrointestinal conditions and the enzymatic action contribute positively to the physic-chemical release of the phenolic compounds, resulting in an augmentation of the total free polyphenols content. Moreover, most polyphenols are present in the liquid phase compared to those present in the solid phase after gastric and intestinal stages, and therefore they would be more bioaccessible, and hence available to be absorbed. Of note, the solubilisation and release of polyphenols in digested white chocolate reached its maximum value during the intestinal stage, being comparable to milk chocolate, despite its low initial content of polyphenols in the undigested product. Even if polyphenols content was higher at pH 7 and 1 mM than under the other studied conditions, a slight increase in polyphenols released within the liquid phase was observed for the highest bile concentration (10 mM) at pH 6. A clear effect was not found, however, depending on bile concentration for intestinal pH 7. This could be explained, due to the fact that the optimum pH for pancreatic enzymes ranges from 7 to 8 (González-Bacerio et al., 2010; Prazeres et al., 1994). In fact, in a previous study on milk lipolysis, Peinado et al., (2018) reported that the relevance of bile salts concentration was higher at low intestinal pHs than at higher (7 and 8). In this scenario, bile salts motivate lipolysis-products transportation (mainly free fatty acid and monoglycerides) from the lipid surface to the intestinal fluid and their further micellation, facilitating thus, solubilisation of digestion products such

antioxidants (Maldonado-Valderrama, Wilde, MacIerzanka, & MacKie, 2011). Regarding the type of chocolate, white and milk chocolate are mainly made from cocoa butter, an extract from cocoa liquor, containing phenolic compounds in less proportion. In contrast, dark chocolate is mainly made with cocoa liquor what is reflected in the results regarding to the polyphenols content and final bioaccessibility. Figure 2 details the release and solubilisation of phenolic compounds (mg equivalent GA / g of chocolate), depending on the amount of pancreatin (0-3,000 LU/ g fat), at constant conditions of pH (6) and biliary concentration (1 mM). A positive effect of the presence of pancreatin on the soluble content of polyphenols was observed. According to these results, the oral supplementation with encapsulated pancreatin seems to be crucial for polyphenols release under EPI conditions. The amount of pancreatin, however, was only relevant for the polyphenols release with digested dark chocolate. The absence of dairy compounds in dark chocolate might be favourable, given that a negative effect of caseins on polyphenols release has been reported (Keogh, McInerney, & Clifton, 2007). Their antioxidant activity seems to be also compromised in presence of α-casein (Bourassa, Côté, Hutchandani, Samson, & Tajmir-Riahi, 2013) and milk β-lactoglobulin by the formation of weak bonds between these proteins and polyphenols (Kanakis et al., 2011), which is the case for milk and white chocolate. Previous studies have demonstrated the reduction of the antioxidant potential by the union of phenolic compounds with milk proteins, reducing their accessibility (Kilmartin & Hsu, 2003; O'Connell, Fox, Tan-Kintia, & Fox, 1998). Some previous studies have highlighted a positive correlation between the percentage of cocoa solids identified in the labelling and the concentration of polyphenols (Vinson & Motisi, 2015). This information could allow consumers identifying those chocolates with greater content of pure cocoa which directly affects the bioavailability of its antioxidants. The above mentioned relation has been highlighted in previous studies (Ortega et al., 2009) in which it is emphasized that a higher content of fat in the digested fluids, favours the formation of emulsified fat drops and with it, the incorporation of cocoa phenols in the lipid phase, protecting them during the duodenal stage. It is also important to emphasize that the consumption of polyphenols in pieces of dark chocolate produces a post-prandial anti-oxidant effect in vivo human despite the presence of fats and sugars in the product (Keen et al., 2005; Ortega et al., 2009).

3.3. Lipolysis extent affected by chocolate type and intestinal conditions

During digestion chocolate is mixed with digestive fluids and so the hydration of the matrix occurs, leading this to the solubilisation of some components such as sugars, together with the melting and emulsification of fat. Additionally, the digestive enzymes activity is responsible of macronutrients hydrolysis, and due to the high fat content of chocolates, lipolysis extent after the *in vitro* digestion process was analysed. Since fat digestion mainly takes place at the duodenum, and because it can be affected by pH, bile concentration and pancreatic enzyme activity in the case of exocrine pancreatic insufficiency, different conditions were analysed. Figure 3A

shows the concentration of FFA/ g of fat in the digested samples at different intestinal conditions. These results reveal that the influence of intestinal pH and bile concentration depends on the type of chocolate; while milk chocolate lipolysis does not depend on pH or bile concentration, a significant influence of both factors was found for both, dark and white chocolate lipolysis, being more significant in the case of white chocolate. These results are related with the above-mentioned factors affecting MDI and polyphenols release, which are also involved in lipolysis extent during digestion. Food lipids have to be accessible to the digestive enzymes and especially to pancreatic lipase, so it would be expected that those samples with lower MDI would be the ones presenting lower progress of lipolysis. This is the case of dark chocolate samples, which reached the lowest values of FFA/g of fat after digestion independently of the intestinal conditions applied during the *in vitro* digestion. The more aggregated structure of this type of chocolate makes difficult, not only the matrix disintegration, but also the fat digestion. On the contrary, for milk chocolate, which has a less aggregated structure with more open spaces (filled with fats) than dark chocolate, higher values of lipolysis were obtained. Additionally, the milk proteins that characterize milk chocolate are mainly 80 % of caseins and 20 % of whey proteins. The casein fraction acts as surfactant reducing the viscosity of the chocolate (Afoakwa, Paterson, & Fowler, 2007) and this decrease in viscosity improves lipid digestibility (Guo, Ye, Bellissimo, Singh, & Rousseau, 2017); and thus the effect of biliary salts and intestinal pH is not as important as in the case of the other two types of chocolate.

In order to analyse the influence of pancreatic enzyme concentration on lipolysis extent, chocolate samples were *in vitro* digested in presence of different LU per gram of fat. The most critical intestinal conditions were applied, mimicking the exocrine pancreatic insufficiency (pH6 and bile 1mM) (Figure 3B). No significant differences were found between the enzyme amount used above and below the recommendation (2,000 LU/g fat), but it draws attention that white chocolate reached lower values of lipolysis extent than the milk one, in spite of being the samples with the highest MDI. These results can be explained comparing the formulation of both types of chocolate, and especially those ingredients acting as emulsifiers since they usually play a key role during lipids digestion. The effect of interfacial molecules on lipolysis has been well described in the literature. Borgström, & Erlanson (1973) and Borgström & Erlanson (1978) found that high surfactant concentration inhibited lipolysis even in the presence of colipase, and postulated that the bile salts desorbed the surfactants allowing lipase adsorption in the presence of colipase. The low bile concentration used in this work (to mimic abnormal conditions in some chronic diseases such in Cystic Fibrosis) together with the presence of certain emulsifiers would explain the obtained results of FFA. E476 is a synthetic vegetable fat obtained by a combination of polyglycerol and castor oil, which has been reported in the literature to delay lipolysis (Shima, Tanaka, Kimura, Adachi, & Matsuno, 2004). It is worth noting, that polyglycerol

polyrincinoleate (E476) is found among the emulsifiers included in white chocolate formula, while it is not included in dark and milk chocolate. In the case of dark chocolate, it is also important to highlight the effect of antioxidants on lipid digestibility. The polyphenols affect the emulsion that occurs during the digestive transit, increasing the particle size and decreasing the specific surface area, causing a decrease in the activity of the lipase (Sugiyama et al., 2007; Uchiyama et al., 2011). Thus, small differences in the formulation of chocolates, especially those with emulsification properties, can have a significant impact on fat digestion, especially under abnormal conditions of bile secretion.

4. Conclusions

As the results have shown, we can conclude that it is the type of chocolate the main factor affecting matrix degradation index during digestion. The different ingredients and their proportion that conform the food matrix, like fat, emulsifiers, and crystalline lactose, have a significant impact on the structure and consequently their matrix degradation during digestion.

Under EPI conditions it is important the supplementation of pancreatin for polyphenols release, especially in the case of dark chocolate, which is the one with the highest polyphenols content. A slight increase of polyphenols on the liquid phase for the different chocolates was found by increasing the bile concentration at low pH (6); while no influence of biliary salts was found at higher intestinal pH (7). Additionally, a negative effect in the antioxidant activity due to the dairy compounds in milk and white chocolate was observed.

Regarding the influence of different intestinal conditions on lipolysis extent, pH and bile concentration do not determine the extent of lipolysis in milk chocolate, nevertheless, an influence of both factors was found in both, dark and white chocolate lipolysis. No significant differences were observed between the different amounts of pancreatin used below or above the recommended one (2,000 LU / g fat). If we speak about lipolysis, it is important to consider those factors that, due to the nature of the matrix, will affect the results. This is how the presence of emulsifiers in the case of white chocolate, or the presence of antioxidants in dark chocolate determined a decrease in lipolysis.

To sum up, the results obtained in the present study highlight the influence that not only food related factors such food composition, food matrix, processing conditions etc., but also individual physiological parameters (intestinal pH and bile concentration) might have on nutrients release and bioaccessibility. However, in vivo studies are advisable to be conducted in order to complement these findings and assist food industry in developing tailor-made foodstuffs and to help health professional and dieticians to adapt the existing nutritional guidelines addressed to specific target populations in which intestinal conditions could be altered.

Conflicts of interest

There are no conflicts of interest to declare.

5. Acknowledgements

Authors of this paper, on behalf of MyCyFAPP consortium, acknowledge the European Union and the Horizon 2020 Research and Innovation Framework Programme for funding the above-mentioned project under grant agreement number 643806. The authors would like to thank the Secretaría de Educación Superior, Ciencia, Tecnología e Innovación (Ecuador) for the PhD scholarship given to Carolina Alicia Paz Yépez.

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Figure Captions:

Figure 1. Polyphenols content (mg Gallic Acid eq/ gram of chocolate) along the *in vitro* digestion of different type of chocolates (dark, milk and white) at intestinal conditions of pH 6 or 7, bile concentration of 1 or 10 mM and at a fixed pancreatic enzyme amount of 2,000 LU/ g fat. Polyphenols content has been measured in chocolate before digestion (discontinuous line), and in both, liquid and solid phases, in digested chocolate.

Letters a-e refer to the homogenous groups obtained by the Fisher LSD post-hoc test after the ANOVA applied to data to each chocolate type, they provide information about the effect of the different digestion stages on the release of polyphenols available for absorption (p-value <0.05).

Figure 2. Polyphenols content (mg Gallic Acid eq/ gram of chocolate) after *in vitro* digestion of different type of chocolate**s** (dark, milk and white), digested under fixed intestinal conditions (pH 6 and bile concentration 1 mM) and different pancreatin concentrations (0-3,000 LU/ g fat). **(A)** Values from liquid phase **(B)** values from solid phase.

Letters a-c refer to the homogeneous groups obtained by the Fisher LSD post-hoc test after the ANOVA applied to data within each single type of chocolate and they provide information about the release of polyphenols under different pancreatin concentrations. Letters A-C refer to the homogenous groups obtained by the Fisher LSD post-hoc test after the ANOVA applied to data between all type of chocolates for each single pancreatin concentration; thus they provide information related to the effect of the type of chocolate on the release of polyphenols (p-value <0. 05).

Figure 3. A) Lipolysis (mg FFA/g fat) after the *in vitro* digestion with a fixed pancreatin concentration (2000 LU/ g fat) and different combinations of intestinal pH and bile concentrations (pH 6 or 7, bile salts concentration 1 or 10 mM); B) Lipolysis (mg FFA/g fat) after the *in vitro* digestion under fixed intestinal conditions (pH 6 and bile concentration 1 mM) and different pancreatin concentration**s** (0-3,000 LU/ g fat).

Letters (a-b “lower case”) refer to the homogenous groups obtained by the Fisher LSD post-hoc test after the ANOVA applied comparing between different lipase concentrations or intestinal conditions within the same type of chocolate, and letters (A-C "capital letters") refer to the homogenous group**s** obtained by the Fisher

LSD post-hoc test after the ANOVA applied comparing between different type of chocolates for each experimental condition (p-value <0.05).