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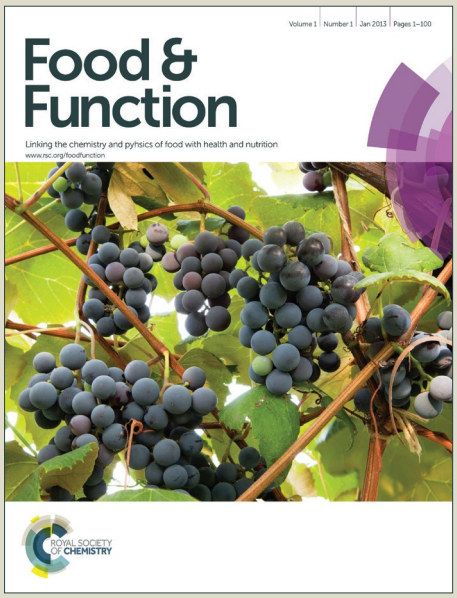

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In vitro digestibility of highly concentrated methylcellulose O/W emulsions. Rheological and structural changes.

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ABSTRACT

The changes in structure during digestion of highly concentrated methyl cellulose (MC) o/w emulsions, and of hydrated MC were investigated.

The effect of human saliva and in vitro stomach digestion was attributed to a dilution effect, rather than to pH or pepsin activity. After in vitro intestine incubation, a decrease in viscoelasticity and an increase in fat globule size were observed. Fat released after digestion the MC emulsion was 49.8% of the initial fat, indicating the existence of a big physical impediment. In comparison to an o/w whey protein emulsion with fat content equal to the fat released during the MC emulsion digestion, a 12% reduction in free fatty acid formation was found, which indicates that the decrease in fat bioaccesibility in the MC emulsion should be attributed to a physical effect against fat release but also to a further impediment related to the fat digestion process.

Fat released quantification informs about the physical retention of fat in the emulsion matrix structure. Enzymes may not act, if fat is not released and solubilized. Free fatty acid quantification is the real indicator of fat digestion, but contrary to total fat released, is affected but a wide variety of enzymatic factors, which should be considered for the correct comparison of systems of different properties, for example systems where the amount of fat release during digestion may be different or initially unknown.

Keywords: methylcellulse, emulsion, in vitro digestion, lipolysis, rheology, fat reduction

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1. Introduction

The design of colloid delivery systems to control the rate and extent of lipid digestion within the gastro-intestinal tract has been the subject of extensive attention lately. Inhibiting or slowing down lipid digestion is considered to be an effective means with which to reduce appetite and promote satiety, leading to a reduction in obesity and a more balanced energy intake.[1,2]

After ingestion, the emulsions undergo a complex series of physical and chemical changes as they pass through the mouth, stomach and small and large intestine (mechanical strength, presence of enzymes, changes in pH, etc) [3,4] which affect their capacity to be ingested. When designing low absorption emulsions, it is vital to bear these parameters in mind so as to achieve a level of structural stability which limits the enzymatic attack.

More attention has been paid to the effect of the interfacial layer surrounding the lipid droplets on the emulsion structuring/breakdown during digestion than anything else. Depending on the physicochemical properties of the interfacial layer, the lipid droplets may break up or coalesce as the emulsion passes through the mouth into the stomach and then the intestines, while at the same time altering the surface area of lipid exposed to enzymes. Emulsions stabilised by non-ionic surfactants tend to remain stable during the transit through the stomach because of the highly stable nature of the emulsion.[5,6] The emulsions stabilised by proteins tend to flocculate [7] while those stabilised by ionic surfactants can undergo coalescence.[8] The efffect of HPMC, B-lactoglobuline and soy protein as emulsifiers in the control of lipid digestion was studied by Bellesi, Martinez, Pizones Ruiz-Henestrosa & Pilosof.[9] Soy protein was found as resistant to digestion as HPMC, which is a non digestible emulsifier. Likewise, the physico-chemical composition of the fat and the size and the distribution of the fat globules noticeably regulate lipase activity.[10,11,12]

Polysaccharides can act as emulsion stabilisers by increasing the viscosity or gel strength of the continuous phase, as well as by inducing the flocculation of emulsion droplets through bridging or depletion mechanisms, depending on the adsorbing properties of the polysaccharides.[13] The role of emulsion stabilizers is gaining increasing importance in the area of lipid digestion control. It has been shown that the presence of certain hydrocolloids potentially influences lipid digestion control.[11,14,15] It

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has even been demonstrated that emulsions which remain stable in the stomach and/or have a delayed digestion in the small intestine may stimulate the release of intestinal hormones that induce a sensation of satiety and, therefore, reduce the quantity of foodstuffs ingested [16]. The impact of carboxymethylcellulose (CMC) on lipid digestion and the physicochemical properties of whey protein-stabilised emulsions during digestion was studied by Malinauskyte et al.[13] The thickening network formed in the continuous phase by CMC limits the interaction of fat droplets with gastrointestinal fluids, slowing down the rate of lipid digestion.[13] Methylcellulose, chitosan and pectin were also found to be effective at reducing lipid digestibility in 2% corn oil in water emulsion stabilized by Tween 80. This behaviour was attributed to the ability of the polysaccharides to induce droplet flocculation due to their interaction with molecular species.[17] Qiu et al.[15] studied the influence of xanthan gum and pectin on the lipid digestibility of fish oil emulsions stabilized by wheat proteins. In this case, surprisingly, the polysaccharides were found to promote the lipid digestion process. The increase in the lipid digestion rate in the presence of dietary fibres was attributed to their ability to alter the aggregation state of the oil droplets, thereby increasing the amount of lipid phase exposed to the lipase. Other mechanisms associated with the presence of dietary fibres, such as binding to calcium ions, bile salts, free fatty acids, and lipase were not able to explain the observed increase in lipid digestion.[15]

Highly fat concentrated (50% fat) oil in water (O/W) cellulose ether emulsion have been recently studied due to the fact that they may act as healthy replacers of conventional sources of solid fat in the diet, such as butter or margarine. Biscuits, in which conventional shortening was totally replaced by the cellulose ether emulsion, exhibited good sensory acceptability, having 33% less fat and no trans fatty acids.[18,19] This oil in water cellulose emulsion also showed reversible thermal gelation ability [20,21], which makes it a suitable option for applications where thermal stability is required, such as in cream fillers for bakery products. In these emulsions, the cellulose ether exhibits surface active properties and confers stability to the continuous phase due to the thickening effect.

This study focuses on the structural changes during the in vitro mouth, stomach and small intestine digestion of a highly concentrated methylcellulose O/W emulsion and

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their relationship to lipid digestion. The emulsion's rheological properties, microstructure and droplet size distribution are investigated. The total fat release and the free fatty acids generated after the end of the digestion were calculated.

Likewise, in addition to the behaviour of the emulsion, the effect of the in vitro digestion of an aqueous solution of methylcellulose is researched in isolation. It is estimated that the behaviour of the hydrated hydrocolloid will be a determining factor in the structural changes undergone by the emulsion, as it constitutes the first barrier to come into contact with the digestion solutions. The research into the structural changes undergone during the in vitro digestion in the mouth, stomach and intestine will allow a better understanding of factors affecting fat release and fat digestibility. This will permit the rational design of fat emulsions which have low digestibility and that may substitute conventional fats.

2. Materials and methods

2.1. Materials

Methylcellulose (A4M type: 30.0% methoxyl, viscosity of 4000 mPa s at 2 % aqueous 130 solution at 20 °C measured by The Dow Chemical Company following reference methods ASTM D1347 and ASTM D2363) was supplied by The Dow Chemical Company. Sunflower oil "Koipe Sol" was purchased from Deoleo S.A. (Madrid, Spain). Whey protein was supplied by Best Protein (Barcelona, Spain).

Hydrochloric acid 6N, ammonia solution 25% and ethanol 96% were purchased from Scharlab S.L. (Spain) and sodium hydroxide 0.1N was provided by Panreac Química S.L.U. (Spain). Phenolphthalein solution, pepsin from porcine gastric mucosa (P7000), bile extract porcine (B8631) and pancreatin from porcine pancreas (P1750) were supplied by Sigma-Aldrich Chemical Company (St. Louis, MO).

2.2. Emulsion preparation

Oil-water-cellulose ether emulsions were composed of sunflower oil (51%), methylcellulose (2%) and water (47%).

The cellulose ether was first dispersed in the oil using a Heidolph stirrer at the lowest speed for five minutes. The mixture was then hydrated by gradually adding the water at 10ºC while continuing to stir. The 200 g mixture contained in a 600 ml baker (10 cm diameter) was then homogeneized with an IKA T18 basic (Ultra-Turrax) with the

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dispersion tool S18N-19G (stator diameter 19 mm and rotor diameter 12.7 mm) at 6500 (1/min) during 15 s and subsequently at 24000 (1/min) during 30 s.

2.3. Methylcellulose water dilution

Two hundred grams of a solution of methylcellulose (2% w/w) was prepared according to the hot/cold technique (The Dow Chemical Company). The powder was previously dispersed by gentle mixing with 1/3 of the total water at 80ºC for approximately 3 min (Heidolph stirrer at speed 3). Subsequently the beaker with the dispersed MC was quickly transferred to a refrigerated water bath at 10ºC and the rest of the water was added at 1ºC and stirred continuously for 10 min allowing a correct MC hydration.

2.4 In vitro digestion

An in vitro digestion model that simulated the mouth, stomach and small intestine was used.

2.4.1 Mouth phase

25g of emulsion sample were gently mixed for 5 s with 0.5ml of fresh stimulated human saliva (corresponding to a ratio saliva/emulsion of 1ml saliva/50 g emulsion), inside a water bath at 37°C.[22,23] The ratio human saliva/emulsion was selected according to the saliva flow data provided by Humphrey & Williamson [24], considering a short retention time of the emulsion in the mouth. The stimulated human saliva was obtained as described in Engelen et al. [25] The mouth of the donor was rinsed three times with water; subsequently, saliva stimulation was performed by chewing on a 5 cm square sheet of tasteless parafilm (Parafilm American National Can, Greenwich, CT, USA). Informed consent was obtained for the donor. Saliva was always employed within 1 h and was stored at 4ºC.

2.4.2 Gastric phase

The sample from the mouth phase was mixed with 1.5ml of a simulated gastric fluid (SGF), 6ml of distilled water and 100µl of HCl 6N, so a final pH of 2.0 was obtained. The ratio gastric volume/emulsion weight was 1/3. This ratio was selected so as to be able to evaluate the changes in the rheological properties associated to the digestion process. The SGF consisted of a pepsin solution containing 1.6 g of pepsin in 10 ml HCl

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0.1N. The mixture was incubated at 37°C for 1 hour with continuous agitation in a shaking water bath (speed 70 U/min).

2.4.3 Small intestine phase

After the gastric phase, 2 ml of simulated intestinal fluid (SIF) was added and the pH was adjusted to 7 with NH_3 (25% w/w). The ratio intestine volume/emulsion weight was 1/2.5. This ratio was selected so as to be able to evaluate the changes in the rheological properties associated to the digestion process, as a higher dilution in the system would have reduced measurement sensitivity. The SIF consisted of 0.1 g of pancreatin, 0.625 g of bile extract and 0.21 g of Ammonium hydrogen Carbonate in 25 ml milli-Q water. The pH of the SIF was adjusted to 7 with NH3 (25% w/w). The final oil concentration in the small intestine phase was 35.2%. However, it should be considered that not all the initial oil in the emulsion sample will be bioaccessible or available for digestion. In this article the amount of oil available for digestion is referred as "released fat" and it is considered as the oil isolated in the supernatant after centrifugation of the small intestine digesta (see section 2.9). The oil that remains in the pellet after centrifugation is considered not to be available for digestion.

The mixture was incubated for two additional hours in the shaking water bath under the same conditions as described in the gastric phase.

2.4.4 Effect of water dilution

Samples were also incubated in the stomach and in the small intestine model but only with the addition of distilled water at the dilution level of the stomach and small intestine. The incubation process (time, temperature and shaking conditions) was the same as in the samples with enzymes.

2.5. Rheological behaviour

The rheological behavior was evaluated by small amplitude oscillatory shear in a controlled stress rheometer (AR-G2, TA Instruments (Crawley, England)) with a Peltier heating system. A 40 mm diameter plate–plate sensor geometry with a serrated surface and a 1 mm gap was employed. In every case, the sample was protected with vaseline oil (Panreac, Barcelona, Spain) in order to prevent the sample from drying, as a result of either the time or temperature used.

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Stress sweeps were carried out at a frequency of 1 Hz to measure the extent of the linear viscoelastic response. Frequency sweeps from 10 to 0.01 Hz at a stress wave amplitude inside the linear region were performed. In some samples data value at the lower frequencies (from 0.1 to 0.01) are not shown due to a lack of sensitivity during measurements. Storage modulus (G′), loss modulus (G″) and loss tangent (tan δ = G″/G′) values were recorded. Test temperature was always 37ºC.

The tests were carried out in the fresh systems (emulsion and hydrated cellulose) and after incubation in each of the in vitro digestion steps (oral, gastric and intestinal), with and without enzymes.

2.6. Extrusion properties

The extrusion properties of the samples were determined by using a TA-XT plus Texture Analyzer equipped with the Texture Exponent software (Stable Microsystems, Godalming, UK). A back extrusion assay was carried out using a bucket of 5 cm in diameter and 7.5 cm in height and a compression probe of 4.9 cm in diameter. The distance force was 15mm, the compression rate 1mm/s, and the trigger force 10g. From the force time profiles obtained, the area under the curve and the maximum force achieved were recorded.

2.7. Particle Size measurements

The measurements were taken with a particle size analyzer by laser diffraction (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The particle size calculations are based on Mie theory or Mie scattering theory and were performed with the software provided with the equipment (Mastersizer 2000 V5.40). A refractive index of 1.330 and 1.472 was used for the aqueous phase and the fat phase respectively. The volume-weighted mean particle diameter D[4,3] was calculated.

2.8. Microstructure

The microstructure of the emulsions was evaluated using optical microscopy (Nikon Eclipse 90i, Kanagawa, Japan). A small aliquot of each sample was placed on a microscope slide and observed using a magnification of 20x. The emulsions were observed after 24 h of preparation and after digestion in the in vitro models of the mouth, stomach and intestine.

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2.9. Amount of fat released after in vitro digestion

Before fat can be digested, one necessary step is the release from the initial matrix and solubilisation. In order to determine the real amount of fat that will be available for digestion, the amount of fat release from the emulsion after centrifugation was calculated. It is necessary to consider that fat release from the hydrocolloid/emulsion structure is a first necessary requirement for a correct action of digestive enzymes.

After small intestine in vitro digestion the sample was mixed with 15 ml Ethanol and centrifugated (10 minutes, 10,000rpm) (Sorvall® RC-5B Refrigerated Superspeed centrifuge). The total supernatant was quantified and the mixture of water and ethanol evaporated in a boiling water bath. After evaporation, the container was dried in an oven at 100ºC for 30 minutes to completely eliminate residual water or ethanol. The remained liquid is considered the amount of fat released. The amount of fat that remains in the pellet after centrifugation will not be bioaccesible fat.

2.9. Free fatty acid (FFA) content

Fat digestion was determined by measuring the amount of FFA before and at the end of the in vitro digestion. FFA were determined in the MC emulsion an in an o/w whey protein (2% w/w)) emulsion, considered as control. The percentage of oil in the control emulsion (25% w/w) was selected according to the total fat released from the MC emulsion (section 2.9), so the total amount of fat available for the digestive enzymes is kept constant.

After incubating in the intestine model, 15 ml of ethanol were added to the digestion mixture (6.25g) in order to stop the enzyme action of pancreatic lipase. The sample mixed with ethanol was centrifuged for 10 minutes at 10,000 rpm in a centrifuge (Sorvall® RC-5B Refrigerated Superspeed centrifuge). The total supernatant was quantified and the free fatty acids were determined in 10 ml of supernatant by titration with 0.05M NaOH and phenolphthalein as an indicator to end point (pink color). A ph-stat automatoc tritation unit was not employed due to the inhomogeneous consistency of the emulsion digested sample, as many of the FFAs released will not be soluble enough to be detected.[26]

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A standard curve was prepared using oleic acid (0, 50, 100, 150, 200 and 250 mM), and this was used to calculate the free fatty acid concentration of the samples. The results are expressed as "g oleic acid/g fat" and in the MC emulsion also as “g oleic acid/g fat released”. The determination of free fatty acids was performed on the emulsion sample and in a control consisting of a 2% whey protein o/w emulsion taken as example of digestible emulsifier, without hydrocolloid barrier.

2.10. Statistical analysis

For each test, three replicates were performed with samples prepared on different days. An analysis of variance (ANOVA) was applied to study the differences between the samples. The least significant differences were calculated by the Tukey test and the significance at $p < 0.05$ was determined. These analyses were performed using XLSTAT 2009.4.03 statistical software (Addinsoft, Barcelona, Spain).

3. Results and discussion

3.1. Hydrated cellulose ether

3.1.1. Linear viscoelastic properties

Before the evaluation of the changes in the viscoelastic properties of the cellulose ether emulsion during digestion, the behaviour of the solely hydrated methylcellulose ether was investigated. In the emulsion the hydrated cellulose constitutes the continuous phase of the emulsion, being the oil the dispersed phase. Therefore the hydrated cellulose is the first barrier that will come into contact with the digestion fluids during the digestion of emulsions. For this reason, it is expected that the changes in the solely hydrated cellulose ether would be closely related to the emulsion stability during digestion and may help to understand the general behaviour of the emulsion.

The study of the linear viscoelastic properties of the fresh and the digested samples was carried out by applying small amplitude oscillatory shear. These small forces do not simulate the peristaltic movement along the digestive tract but are considered an effective tool for the purposes of evaluating the resulting inner structure after incubation in the shaking water bath.

The effect of mouth, stomach and small intestine in vitro digestion on the linear viscoelastic properties of the hydrated cellulose at 37ºC is shown in Figure 1. The spectra reveal the very low elasticity of the hydrated methylcellulose at this

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temperature. In the fresh sample, the terminal zone of the mechanical spectra was observed, with values of G'' higher than G' in the available frequency window, implying the predominance of the viscous properties of the sample (Figure 1A). It is interesting to compare this mechanical spectra obtained at 37ºC with a previously reported one measured at 15ºC.[27] In comparison with 15ºC, at 37ºC the distance between G' and G'' is shorter and the slope of the increase in G' and G'' with frequency is also gentler. These differences between the shape of the mechanical spectra at 15ºC and 37ºC are explained by the thermal changes associated with the methylcellulose ether. As previously reported [27], the increase in temperature reduced the differences in G'' and G' until the gelation temperature is reached (around 49ºC) and a crossover of G'' and G' occurs. This explains the higher viscoelasticity at 37ºC in comparison with the previous results at 15ºC.

Mixing the hydrated hydrocolloid with human saliva did not affect the characteristic shape of the mechanical spectra or the values of the viscoelastic functions, implying that saliva did not affect the fresh methylcellulose structure. After digestion in the stomach, only a mild increase in tan δ (lower viscoelasticity) was observed (Figure 1B), although a significant decrease in the values of G' and G'' was observed. Finally, the intestine digestion induced a greater decrease in the viscoelastic functions with a significant increase in tan δ. In order to establish whether the observed changes in the viscoelastic properties are associated merely with a dilution effect or more specifically with the enzymatic effect and the changes in pH, the hydrated methylcellulose dispersion were also incubated only in the presence of water, at the corresponding dilution level. The effect of water dilution is shown in Table 1, where values of G', G'', and tan δ at 1 Hz of the digestion systems and water diluted systems are shown. The dilution of water at the level of the stomach showed a similar profile to the stomach incubation with no significant differences in tan δ, indicating that the observed changes after stomach incubation could be mainly associated with the dilution effect. The slight effect of the acidic pH on the methylcellulose structure was expected do to its non-ionic nature. On the contrary, dilution at the level of the intestine did not affect tan δ values while intestine digestion did, indicating that, in this case, the observed changes in the structure can be associated with the effect of the bile salts and the pancreatic enzymes.

3.1.2. Extrusion properties

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The effect of greater forces on the structure was studied by applying a back extrusion test. The extrusion forces versus time were recorded in the fresh sample and after digestion in the mouth and stomach models, also analysing the isolated effect of water dilution. The area under the curve and mean force values are shown (Table 2). After stomach incubation, the values of force and area were significantly lower than in both the fresh sample and the saliva sample. No significant differences were found between the stomach sample and the diluted sample, which, similarly to the viscoelastic results, indicates that the decrease in the force values should be attributed to water dilution more than to the structural change associated with stomach conditions (acid pH and pepsin). Although saliva incubation only produced a small decrease in force values, the differences found were significant.

3.2. Methylcellulose O/W emulsion

The visual appearance of the emulsions after incubation in the different in vitro models is shown in Figure 2. No phase separation can be distinguished in the emulsion and, after the different phases of digestion, the structure adopted by the emulsion is homogeneous.

3.2.1. Linear viscoelastic properties

The effect of saliva, stomach and intestine digestion on the emulsion viscoelastic properties are shown in Figure 3. The fresh emulsion was highly dependent on frequency. In comparison with the hydrated hydrocolloid at the same temperature, the emulsion is characterized by a higher elasticity. The values of the viscoelastic function in the emulsions were more than one order of magnitude higher than in the hydrated hydrocolloid (Figure 3A). In the fresh emulsion, the end of the plateau zone was observed in the available frequency window with the crossover between G' and G'', the values of G'' being higher than those of G' at lower frequencies. Similarly to the behaviour found in the hydrated methylcellulose, the behaviour of the undigested emulsion at 37ºC differs slightly from the behaviour of the emulsion at 20ºC, as previously described.[21] At both temperatures, the crossover of G' and G'' was observed in the available frequency window, but the distance between G' and G'' was shorter in the emulsion at 37ºC, which is associated with the decrease in viscoelasticity occurring in this temperature range.[21] Mixing with saliva did not exert any effect on the emulsion

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viscoelastic properties, and the values of tan δ (Figure 3B) and G' and G'' (Figure 3A) were unaltered. After incubation in the stomach, a change in viscoelasticity occurred: the values of G' and G'' fell and the cross over point between G' and G'' moved towards higher frequencies, implying a decrease in viscoelasticity. After digestion in the intestine, the system's viscoelasticity continues to decrease: the G' and G'' values fell, tan δ rose and the crossover point shifted towards higher frequencies.

The effect of water dilution was also studied. The increase in water dilution led to a progressive decrease in the values of G' and G'' and moved the cross over towards higher frequencies. The comparison of the mechanical spectra of the water dilution and the stomach digestion reveals very similar spectra, indicating that the observed effect could simply be associated with dilution. Values of G', G'' and tan δ are shown in Table 3. After digestion in the intestine, the values of G' and G'' were slightly lower than those corresponding to the dilution, although the differences were not significant.

3.2.2. Extrusion properties

The area under the curve and medium force values are shown in Table 4. Similarly to the viscoelastic results, the extrusion profile (data not shown) revealed the greater consistency of the methylcellulose emulsions in comparison with the hydrated methylcellulose (mean extrusion force around 0.5 N for the hydrated cellulose and around 8 N for the emulsions). After digestion in the stomach, the extrusion force decreased significantly and no differences were found between the stomach digestion samples and the water diluted samples; similarly to the linear viscoelastic results, this implies that the effect of stomach digestion should be mainly associated with the dilution effect rather than with the specific effect of the stomach conditions.

3.2.3. Emulsion microstructure

The microstructure of both the fresh emulsion and also that of the emulsion after digestion in the in vitro model of the mouth, stomach and intestine is shown in Figure 4. The initial microstructure corresponds to the existence of a dense matrix composed of the fat globules that are immersed in the continuous phase of the emulsion, made up of water and hydrated cellulose.

No significant changes in the microstructure can be appreciated after it is mixed with saliva. After incubation in the stomach, it can be seen that the cellulose still retains its

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emulsifying effect on the acidity of the stomach and no flocculation or coalescence phenomena can be observed. As a result of the existing dilution, the fat droplets in the emulsion can be seen to be farther apart after incubation in the stomach.

Finally, the microstructure reveals there has been a significant increase in the size of the fat globules after incubation in the intestine. These results indicate that the bile salts and pancreatic lipase have been to some extent capable of accessing the interface of the fat globules and displacing the methylcellulose of the interfacial surface. Li, Hu & Mc Clements [28] found an increase in the mean particle diameter when bile was added to the emulsions. Mc Clements [29] and Mun et al. [10] associated the phenomenon of coalescence with fat digestion in the following manner: the formation of free fatty acids and monoacylglycerides on the surface of the droplets during lipase digestion boosts coalescence as the surfactant effect of these substances is not strong enough to stabilise the oil emulsions in water when coalescence occurs. The degree to which this happens will depend both on the ability of the lipase to come into contact with the emulsified lipids and on the composition and properties of the interfacial films that surround the water droplets.

3.2.4. Particle size distribution

The changes in the oil droplet size distribution during mouth, stomach and intestine digestion are shown in Figure 5. Prior to digestion, the emulsion exhibited a bimodal distribution with a minority population of a smaller size, around 1 μm, and a majority, larger-sized population (around 10 μm).

The digestion with saliva leads to the appearance of a small tail on the right-hand side and a slight reduction in the size of the majority population. This means that the presence of saliva does have an effect on the emulsion's structure that could be associated with a gentle displacement of the interface cellulose caused by the saliva's glycoproteins (mucines), which would lead to a slight coagulation or coalescence. Digestion in the stomach produced no noticeable changes compared with in-mouth digestion. Lastly, after digestion in the intestine there was a widening of the curve and a clear displacement to the right, which coincides with a growth in the size of the fat globules as observed in the microstructural analysis.

The values of the average droplet diameter D[4,3] are shown in Table 5. A slight, but significant, change can be observed during digestion. This change in the size

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distribution of the particles reflects the fact that, although only to a limited extent, the bile salts and pancreatic lipase do gain access to the dispersed phase of the emulsion, leading to coagulation or coalescence phenomena.

3.2.5. Total fat released after emulsion digestion

Two main requirements are needed for the fat in the cellulose emulsion to be digested. Firstly, the fat should be released from the hydrocolloid matrix and solubilized; secondly, the lipase must be in close contact with the fat surface. Undoubtedly, if the digestive fluids have limited access with the oil phase, fat digestion will be reduced.

The amount of fat which becomes released from the initial emulsion structure was calculated quantifying the amount of fat present in the total supernatant after emulsion centrifugation. It is considered that the fat remaining in the pellet after emulsion centrifugation will not be available for digestion. A photo of the appearance of the digested emulsion is shown in Figure 6, the pellet and the supernatant are clearly observed. In a fresh, no digested MC emulsion, the amount of fat quantified in the supernatant was very low (3,76%). After being digested in the intestine model 49.8 (±6.3)% of the fat was quantified in the supernatant (Figure 6), implying that 50.2% of the initial fat present in the emulsion will not be released from the semisolid structure. This result indicates that independently of a possible additional inhibition of methylcellulose during the fat digestion process, there exists a first effective physical barrier that limits fat release to the aqueous phase. Therefore the employ of highly concentrated o/w methylcellulose emulsions is an effective physical strategy to reduce fat bioaccesibility.

It should be keep in mind that the total fat released will include non-digested fat and digested fat products, so it is not an index of fat digestion. Fat released is an index of the available fat to be solubilized and subsequently digested.

3.2.6 *Free fatty acids generated after emulsion digestion*

In addition to fat released quantification, the content of free fatty acids at the end of the small intestine digestion was quantified, measured as oleic acid. Free fatty acids are the product of fat digestion, so they are a real indicator of the amount of fat which is digested. However, contrary to total fat released, free fatty acids generation is the result of an enzymatic reaction, which is influenced by a wide variety of factors. It is well

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known that lipid digestibility is influenced by a great variety of factors, which makes the quantitative comparison of systems of a different nature not appropriate. Li et al.[28] studied some of the factors that affect emulsion lipid digestion. These authors found that the rate and extent of lipid digestion increase as the lipase concentration rises, decreasing the bile extract, droplet size and droplet concentration.

Special attention has to be taken to employ free fatty acids as an indicator of fat digestion or fat release. In systems where the amount of fat available for digestion may be different (amount, particle size), or initially unknown, it is necessary to guarantee that the enzyme concentration, and other reaction factors, such as bile content are suitable. This is of extreme importance especially in highly concentrated oil systems, where if a high release of fat occurs, the amount of lipase in the in vitro system may not be enough to hydrolyse all the fat in the system, or an inactivation of the enzyme may occur due to an increase in the reaction products.

In this work the free fatty acids generated after digestion the cellulose ether emulsion were compared with a whey protein stabilized emulsion (without hydrocolloid), taking as example of a digestible emulsion with no hydrocolloid barrier. The oil content in the control emulsion was selected considering the total fat released after digestion of the cellulose ether emulsion (section 3.2.5). In this way the amount of fat available for digestion will be the same in both systems, making simpler a comparison of the results.
The amount of free fatty acid (g oleic acid/g fat) was very low in every system before digestion: 0.0018 (cellulose ether emulsion), and 0.0031 (whey protein emulsion), corresponding to the very small amount of free fatty acids present in the fresh oil before digestion. After digestion in the intestine, values of the free fatty acids expressed as g oleic acid/g total fat were 0.105 (±0.033) g/g in the MC emulsion and 0.236 (±0.017) g/g in the control emulsion, which implies a total reduction of 55.51%. If the results in the MC emulsion are expressed relative to fat released (instead of relative to total fat), the origin of the differences (physical or associated to digestion) can be isolated. The value of g oleic acid/g fat released was 0.207 (0.073) g/g, which implies a 12.28% reduction associated solely to the oil digestion process.
Undoubtedly, a more realistic approach will be obtained performing in vivo analysis. Also, the evaluation of the effect of specific conditions in the in vitro digestion process

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and the employ of more realistic in vitro models, such as the one proposed by Minekus et al.[30] will be a future line of research. Somehow, with the results obtained in this paper we can affirm that the cellulose emulsion tested in this study is a promising structure with which to protect fat from being digested and deserves further investigation.

4. Conclusions

This study represents an important contribution regarding the role of methylcellulose as physical barrier in the control of the digestion of highly concentrated oil/water emulsions. The results reveal that the methylcellulose emulsion structure is highly resistant during digestion, reducing in 49.8% the release of the initial fat due to a physical effect, therefore preventing an effective action of the enzymes.

In comparison to a whey protein control emulsion with equal amount of fat available for digestion, a 12% reduction in the free fatty acid content was also found, which supposes 55% reduction in free fatty acids per gram of initial fat.

The effect of human saliva and in vitro stomach digestion on the emulsion's rheological properties and microstructure was attributed to a dilution effect, rather than to pH or pepsin activity. After in vitro intestine incubation, a decrease in viscoelasticity and an increase in fat globule size were observed, indicating that the intestine digestive fluids were able to come into contact, at least partially, with the oil phase of the emulsion.

Special attention should be taken in the correct interpretation of free fatty acid generation as indicator of digestion in highly concentrated emulsions and in the establishment of relationships among free fatty acid generation and total fat release from a specific matrix structure. If the amount of fat released is very high, fat digestion could not be completed.

A definitive answer about the real effectivity of methylcellulose emulsions to reduce fat bioaccesibility will be obtained performing in vivo studies.

In this study it is concluded that the highly concentrated methylcellulose emulsion studied is a good candidate to perform in vivo studies.

A further point of interest of this study is the application of these emulsions in the development of food with low fat bioaccessibility, which will help in obesity control and fat related illnesses.

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627

628 **FIGURE CAPTIONS**

629

630 Figure 1. A (G' and G'') and B (tan δ) as a function of frequency of the fresh hydrated
631 methylcellulose ether and after in vitro digestion at 37ºC.

632

633 Figure 2. Visual appearance of the fresh methylcellulose O/W emulsion and after in
634 vitro digestion at 37ºC.

635 Figure 3. A (G' and G'') and B (tan δ) as a function of frequency of the fresh
636 methylcellulose O/W emulsion and after in vitro digestion at 37ºC.

637 Figure 4. Microstructure of the fresh methylcellulose O/W emulsion and after in vitro
638 digestion at 37ºC.

639

640 Figure 5. Particle size distribution of the fresh methylcellulose O/W emulsion (red) and
641 after in vitro digestion at 37ºC (saliva: green; stomach: blue; intestine: black).

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643 Figure 6. Appearance of the digested intestine sample after centrifugation.

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Table 1. Viscoelastic rheological parameters of the fresh hydrated methylcellulose ether and after in vitro digestion.

Sample	G' (Pa)	G'' (Pa)	Tan δ
Fresh	5.6a	12.4a	2.2c
Saliva	5.6a	11.9a	2.1c
Stomach	1.0b	3.7b	3.9bc
Stomach dilution	1.1b	3.5b	3.3bc
Intestine	0.3b	2.1b	6.5a
Intestine dilution	0.5b	2.6b	5.3ab

[abcd]Means in the same column without a common letter differ ($P < 0.05$) according to the Tukey test.

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681 **Table 2. Extrusion parameters of the fresh hydrated methylcellulose ether and**
682 **after in vitro digestion.**

Sample	Area under the curve (N x mm)	Maximum Force (N)
Fresh	7.4a	0.5a
Saliva	5.5b	0.4b
Stomach	3.0c	0.2c
Stomach dilution	2.4c	0.2c

683 [abcd]Means in the same column without a common letter differ ($P < 0.05$) according to the Tukey test.

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686 **Table 3. Viscoelastic rheological parameters of the fresh methylcellulose O/W**
687 **emulsion and after in vitro digestion.**

Sample	G' (Pa)	G'' (Pa)	Tan δ
Fresh	418.1a	319.0a	0.8c
Saliva	389.1a	322.7a	0.8bc
Stomach	82.7b	126.9b	1.5a
Stomach dilution	89.9b	133.5b	1.5ab
Intestine	10.6c	20.1c	1.9a
Intestine dilution	31.2c	55.1c	1.8a

688 [abcd]Means in the same column without a common letter differ ($P < 0.05$) according to the Tukey test.

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Table 4. Extrusion parameters of the fresh methylcellulose O/W emulsion and after in vitro digestion.

Sample	Area under the curve (N x mm)	Maximum Force (N)
Fresh	107.4a	8.0a
Saliva	83.2b	6.3b
Stomach	18.3c	1.4c
Stomach dilution	14.9c	1.1d

[abcd]Means in the same column without a common letter differ ($P < 0.05$) according to the Tukey test.

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697 **Table 5**. **D[4,3] values of the fresh methylcellulose O/W emulsion and after in vitro**
698 **digestion.**

699

Sample	D4,3 (μm)
Fresh	10.5d
Saliva	20.1b
Stomach	17.4c
Intestine	23.9a

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701 [abcd]Means in the same column without a common letter differ ($P < 0.05$) according to the Tukey test.

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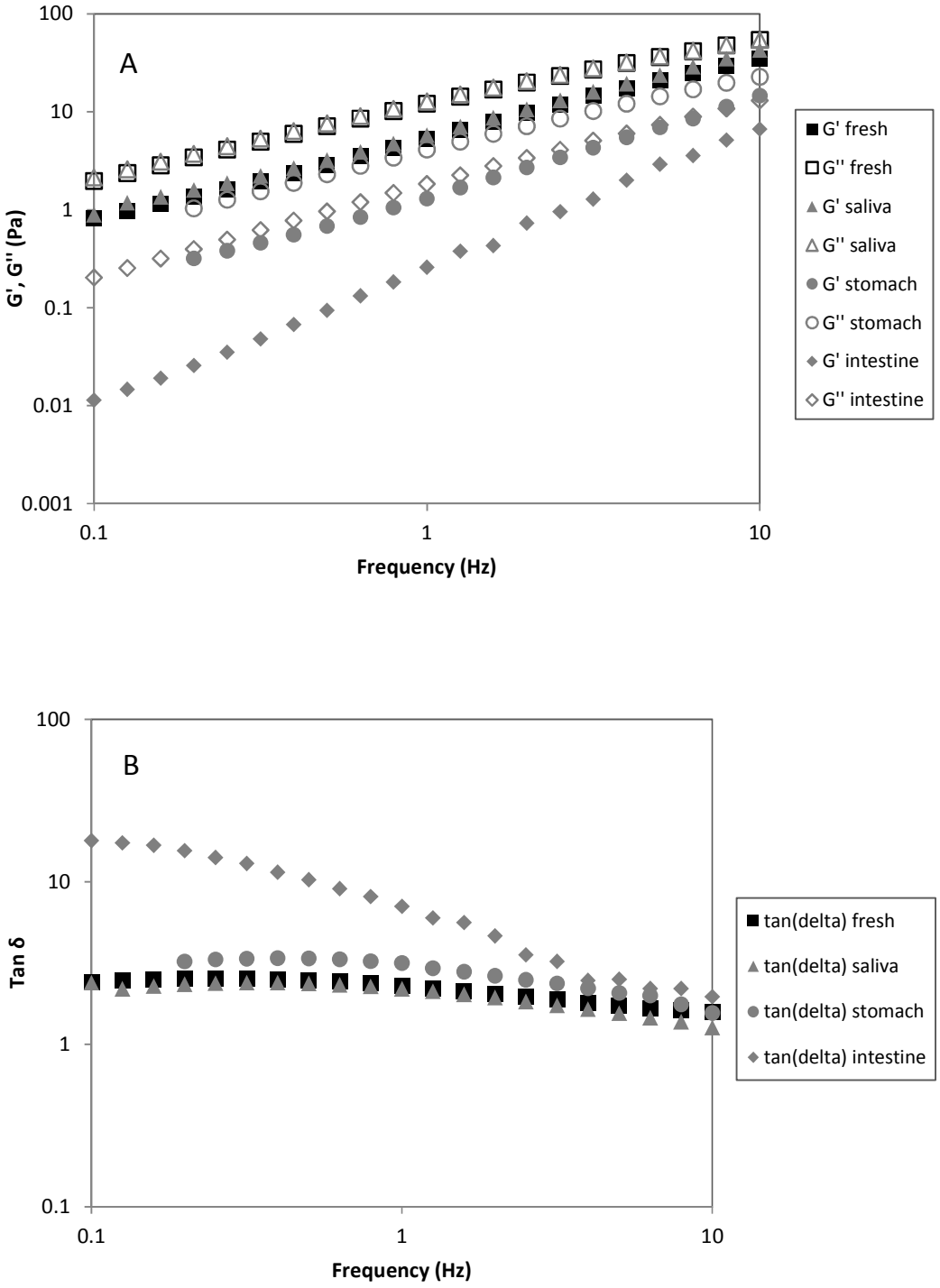

Figure 1. A (G' and G'') and B (tan δ) as a function of frequency of the fresh hydrated methylcellulose ether and after in vitro digestion at 37ºC.

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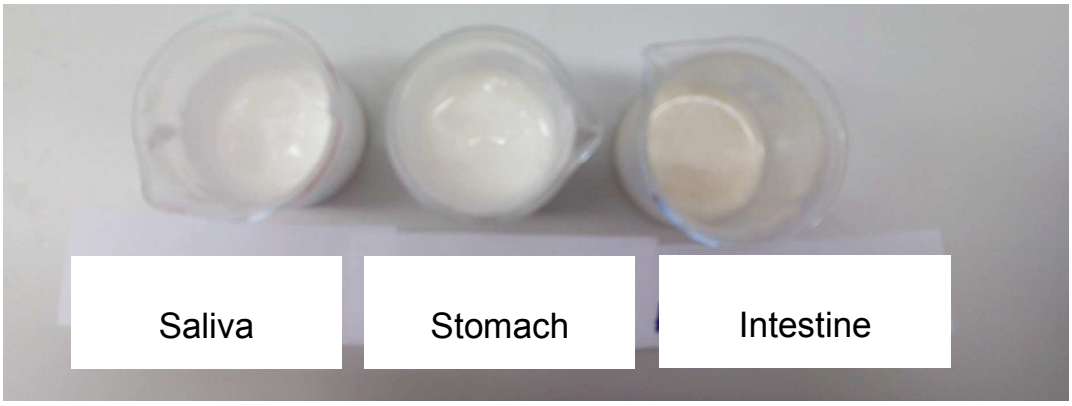

Figure 2.

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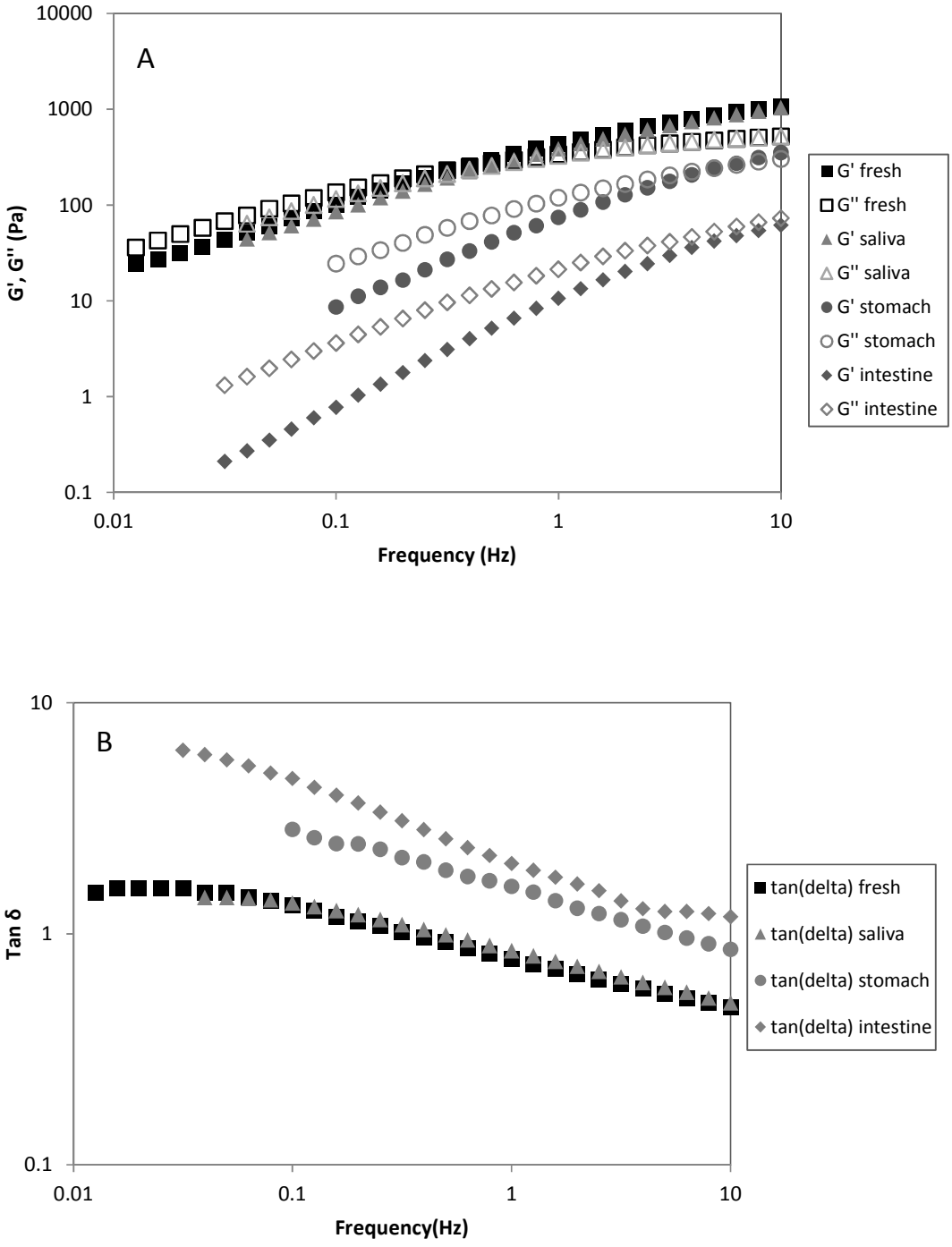

Figure 3. A (G' and G'') and B (tan δ) as a function of frequency of the fresh methylcellulose O/W emulsion and after in vitro digestion at 37ºC.

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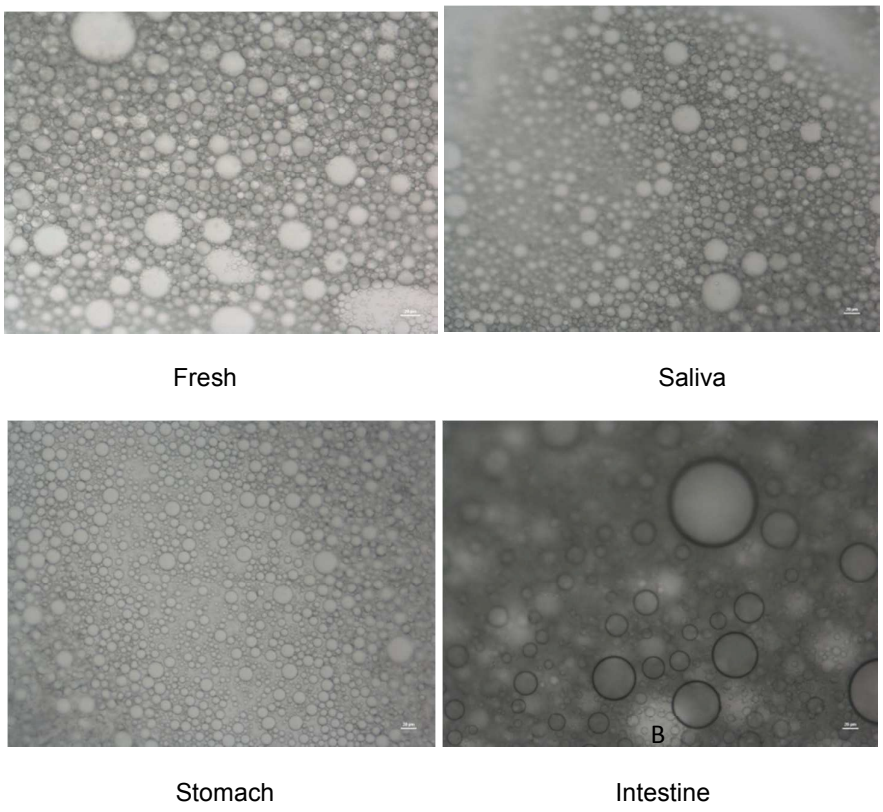

Figure 4.

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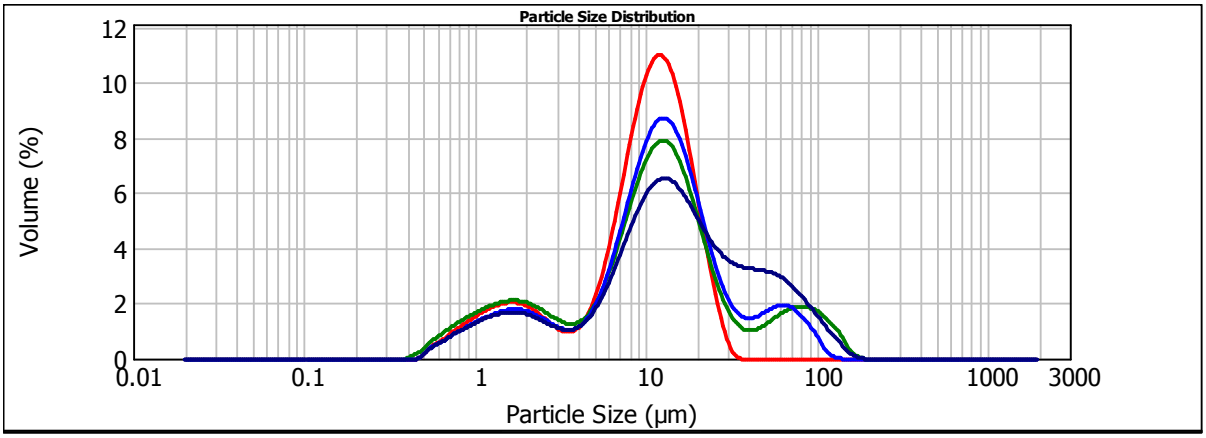

Figure 5.

B

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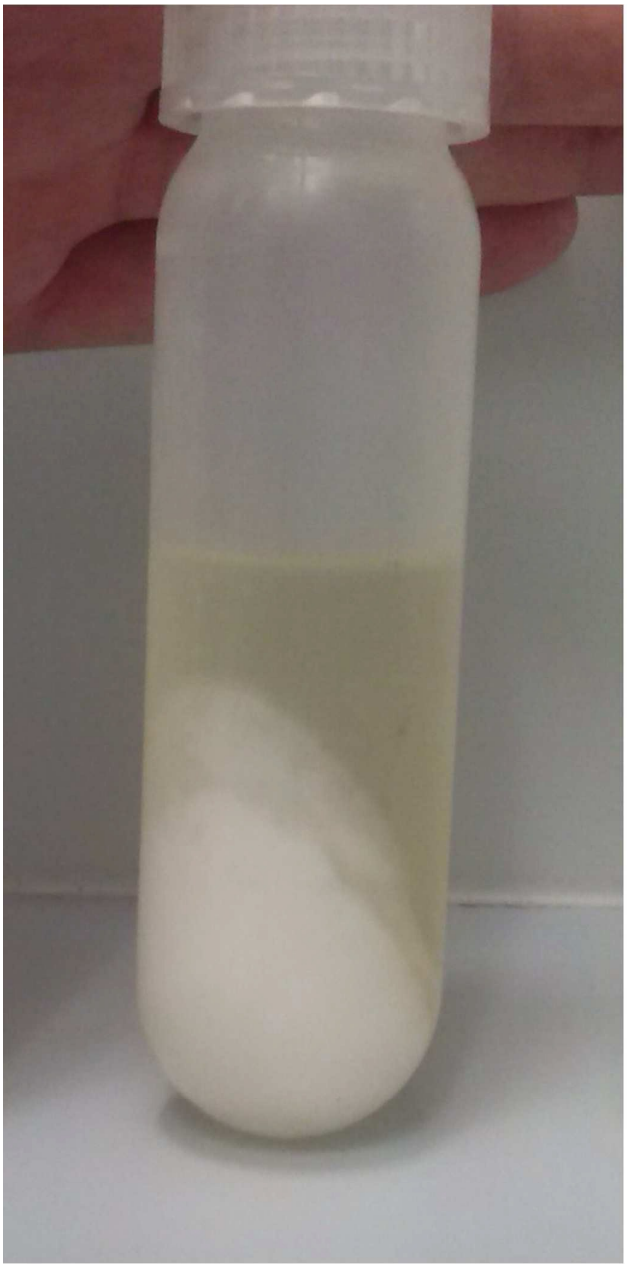

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Figure 6.

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