RELATIONSHIP BETWEEN CELLULOSE CHEMICAL SUBSTITUTION, STRUCTURE AND FAT DIGESTION IN O/W EMULSIONS.

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ABSTRACT

The effect of cellulose ether chemical substitution on the reduction of fat digestion in an o/w emulsion was investigated. Emulsions containing 47% sunflower oil and water were prepared with two types of hydroxypropyl methylcellulose (HPMC) and two types of methyl cellulose (MC), with different hydroxypropyl and methoxyl content. The changes in the emulsion structure were evaluated after mouth, stomach and small intestine in vitro digestion by Confocal laser microscopy and by small amplitude oscillatory shear (viscoelastic properties). The total amount of fat present in the supernatant after digesta centrifugation, serving as an indicator of fat bioaccessibility, and the free fatty acids, serving as an indicator of fat digestion, were determined at the end of the digestion. A relationship was found between cellulose ether chemical substitution, initial structure, structural changes during digestion, fat bioaccessibility and fat digestion. All the cellulose ether emulsions showed a lower level of fat digestion in comparison with a whey protein emulsion, the cellulose ether containing the highest amount of methoxyl being the most effective. The rise in the methoxyl content increases the emulsion viscoelastic properties before and after digestion and reduces fat bioaccessibility and the generation of free fatty acids. The decrease in the fat digestibility of the cellulose ether emulsions was mainly associated with a physical effect, which limits the emulsification of appropriate fats by bile salts, and the subsequent lipase digestion effect.

Keywords: Cellulose ethers, emulsion, fat digestion, rheology, viscoelasticity, microstructure, fat replacer
1. Introduction

Fat is an essential ingredient in the human diet; however, fat overconsumption is directly associated with overweight and obesity, which causes illnesses such as insulin resistance, dyslipidemia, pulmonary dysfunction, hypertension diabetes, among others. In addition, obese people may be subjected to unfair treatment in terms of employment opportunities, health-care facilities and educational positions and they may also be stigmatized by the media (Madadlou, Rakhsi, & Abbaspourrad, 2016).

A recent strategy for the purposes of controlling excessive fat intake is to decrease fat bioaccessibility through the employment of specifically designed emulsions.

The structure and stability of the emulsions play an important role in the digestion and absorption of the lipids (Golding & Wooster, 2010; McClements, Decker, & Park, 2009a; McClements, Decker, Park, & Weiss, 2009b). The initial properties of the oil-in-water emulsions affect both the rate and degree of lipid digestion (Armand et al., 1992; 1999; Mun, Decker, Park, Weiss, & McClements, 2006; Mun, Decker, & McClements, 2007). The flocculation and coalescence stability of the emulsions is heavily dependent on the nature of the emulsifiers. The use of emulsifiers with a great surfactant capacity can impede other substances with superficial properties that are present in the gastrointestinal tract (bile, lipase) from adhering to the interface of the fat droplets; therefore, the right modification to the oil-water interface can be used to inhibit lipid digestion. In addition, both the size and distribution of the fat globules affect the lipase activity (Mun et al., 2007). The rheological properties of the continuous phase are also of great importance. For example, fat globules which are small in size but covered with tightly bonded surfactants in a viscous medium are hydrolysed by the lipase more slowly than the bigger ones covered with surfactants that are more loosely bonded to the globule’s surface in a less viscous medium (McClements & Decker, 2009).

The presence of hydrocolloids in the continuous phase of the emulsion is also of significant influence. The effect of gelatin, colloidal casein and starch dispersion on an oil and water emulsion has been studied, using 20% oil and sodium caseinate and monoglyceride as surfactants (Wooster et al., 2014). The incorporation of low methoxyl pectin into caseinate emulsions with 2% corn oil increased the rate of digestion, which was attributed to the pectin exerting a suppressive effect on the flocculation process. Incorporating high levels of pectin in lactoferrin and Tween 80 emulsions, on the other hand, decreased the digestion rate; this was in all likelihood due to the calcium fixation
and the rise in viscosity that restricts lipase access to the surface of the fat (Zhang, Zhang, Xhang, Decker, & Mc Clements, 2015). The impact of carboxymethyl cellulose (CMC) on both lipid digestion and on the physiochemical properties of whey protein-stabilised emulsions during digestion was studied by Malinauskyte et al. (2014). 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. Methylcellulose, chitosan and pectin were also found to be effective at reducing lipid digestibility in a 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. Qiu, Zhao, Decker, & Mc Clements (2015) 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 (Qiu et al., 2015).

The ethers of cellulose methyl cellulose (MC) and hydroxypropyl methylcellulose (HPMC) are non-ionic cellulose derivatives with methyl and hydroxypropyl groups added to the anhydroglucose backbone. Despite the fact that methyl and hydroxypropyl moieties are hydrophobic groups, the polymer retains enough hydrophilicity to be highly water soluble. The introduction of these hydrophobic groups provides the polymer with surface activity and unique hydration-dehydration characteristics (Sarkar, 1979). Hydroxypropyl groups are more hydrophilic than methyl groups. HPMC and MC have been found to be suitable emulsifiers/thickeners of emulsions o/w with a high fat content (47% fat). These emulsions are considered low fat alternatives to conventional fat sources, such as butter and shortenings, having a lower fat content, fewer saturated fatty acids and no trans fatty acids. They are composed of a vegetable oil (for example, sunflower oil or olive oil), cellulose ether and water (Sanz, Falomir, & Salvador, 2015). In a previous study (Espert, Salvador, & Sanz, 2016), a MC stabilized emulsion was found to be effective at reducing fat digestion. It was speculated that the lower fat digestibility might be associated with the physical barrier exerted by the undigested...
continuous phase (hydrated cellulose ether), which impedes fat release and the appropriate contact between fat and the digestive fluids, reducing the effectivity of the fat digestion process.

In this study, the application of different cellulose ethers as a means of reducing emulsion fat digestibility is further investigated. Cellulose ethers with different substitution types are investigated and compared. The objective is to identify the effect of methoxyl and hydroxypropyl methylcellulose substitution on the structure of emulsions before and after in vitro digestion (mouth, stomach and small intestine), and to investigate the existing relationship between cellulose ether substitution type, structure (small amplitude oscillatory shear and confocal laser scanning microscopy), amount of fat released after digestion and the degree of lipid digestion. Two types of methylcellulose and two types of hydroxypropyl methylcellulose are employed. The rheological properties of the solely hydrated cellulose ethers after digestion are also investigated, as one of the hypotheses is that their structural changes will be related to the emulsion’s structural changes during digestion.

2. Materials and Methods

2.1 Materials and reagents

Oil-water-cellulose ether emulsions were prepared with sunflower oil with high levels of oleic acid (Carrefour, Madrid, Spain), water and four different cellulose ethers with thermogelling ability supplied by The Dow Chemical Co: two hydroxypropyl methylcelluloses (“K4M” (HPMC(M/HP:2.9)), “F4M” (HPMC(M/HP:4.3)) and two methylcelluloses (“A4M” (MC(30%M)) and “MX” (MC(>30%M)). The four types have different degrees of methoxyl content. Their percentage of chemical substitution is: HPMC(M/HP:2.9) (22.5% methoxyl, 7.7% hydroxypropyl), HPMC(M/HP:4.3) (29% methoxyl, 6.8% hydroxypropyl), MC(30%M) (30% methoxyl) and MC(>30%M) (methoxyl> 30%).

HPMC(M/HP:2.9), HPMC(M/HP:4.3) and MC(30%M) have approximately the same molecular weight (MW) and a viscosity of 4000 mPa s (2 % aqueous solution at 20 °C measured by The Dow Chemical Company following reference methods ASTM D1347 and ASTM D2363), type MC(>30%M) has a higher MW with a viscosity of 50000 mPa s (2 % aqueous solution at 20 °C measured by The Dow Chemical Company following
reference methods ASTM D1347 and ASTM D2363). Whey protein was provided from Best Protein (Barcelona, Spain).

Hydrochloric acid (6 N), ammonia solution (25%), ethanol (96%), calcium chloride anhydrous, di-potassium hydrogen phosphate trihydrate and di-sodium hydrogen phosphate dihydrate were purchased from Scharlab S.L. (Spain) and sodium hydroxide (0.1 N), sodium carbonate anhydrous, sodium chloride, sodium hydrogen carbonate, potassium chloride pure and calcium chloride 2-hydrate were provided by Panreac Química S.L.U. (Spain). Sodium phosphate monobasic dehydrate was provided by Acros Organics (Belgium).

Phenolphthalein solution, α-amylase from porcine pancreas (A3176-1MU), mucin from porcine stomach (M2378), 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).

Simulated Saliva Fluid (SSF) was composed of 5.2g of NaHCO₃, 1.37g of K₂HPO₄·3H₂O, 0.88g of NaCl, 0.48g of KCl and 0.44g of CaCl₂·2H₂O, dissolved in 1L of bidestilled water. 8.70g of α-amylase and 2.16g of mucin were added to this solution.

Simulated Gastric Fluid (SGF) was prepared by dissolving 3.10g of NaCl, 0.11g of CaCl₂, 1.10g of KCl and 5.68ml of Na₂CO₃ (1M) in 1L of bidestilled water. The solution was adjusted to pH 2. 0.15g of pepsin was dissolved in 1L of SGF.

Simulated Intestinal Fluid (SIF) was composed of an electrolyte solution and bile and pancreatin solutions. The electrolyte solution was prepared by dissolving 1.25g of NaCl, 0.15g of KCl and 0.055g of CaCl₂ in 1L of distilled water. Phosphate buffer solution was prepared (103.5mg NaH₂PO₄·2H₂O and 44.5mg Na₂HPO₄·2H₂O in 100ml of destilled water) setting the pH to 7.0 if necessary to prepare bile and pancreatin freshly suspensions.

2.2 Emulsion preparation

The different cellulose o/w emulsions were prepared using the following proportions: sunflower oil 47% (w/w), cellulose ether 2% (w/w) and water 51% (w/w), for a total final mass of 200g. 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 1°C while continuing to stir for 30 s. A water temperature of 1 °C was selected according to the specific hydration requirement of the cellulose with the highest methoxyl content (MC(>30%M)), and was also used for the other cellulose types. Stirring continued using a homogenizer (Ultraturrax T18, IKA, Germany) at 6500 (1 min⁻¹) for 15 s and subsequently at 17500 (1 min⁻¹) for 60 s until the emulsion was obtained.

2.3 Cellulose ether water dilution

Two hundred grams of a solution of the different cellulose ethers (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 80°C for approximately 3 min (Heidolph stirrer at speed 3). Subsequently, the beaker with the dispersed cellulose ether was quickly transferred to a refrigerated water bath at 10°C and the rest of the water was added at 1°C and stirred continuously for 10 min, allowing a correct cellulose ether hydration.

2.4 In vitro digestion

An in vitro digestion model that simulated the mouth, stomach and small intestine was used. The digestion process in the gastrointestinal tract in humans was simulated through an in vitro digestion model which was a modified version of that described by Qiu et al. (2015) and López-Pena et al. (2016).

2.4.1. Mouth phase

0.5 mL of Simulated Saliva Fluid (SSF) was added to 25 g of the emulsion to obtain a final ratio of 1:45 (v/v). The blend was gently mixed for 5 seconds in a water bath at 37°C, to mimic the time the food material spends within the mouth prior to swallowing. The ratio saliva/emulsion was selected according to the saliva flow data provided by Humphrey & Williamson (2001), considering a short retention time of the emulsion in the mouth.

2.4.2. Gastric phase

The “bolus” sample from the mouth phase was mixed with 8 mL of Simulated Gastric Fluid (SGF) to obtain a final enzyme-sample ratio of 1:250 (v/v). The pH of the mixture
was adjusted to 2.0 and incubated for 1 hour under continuous agitation in a shaking water bath at 37°C (speed 70 U/min).

2.4.3. Small intestinal phase

A total volume of 10mL of Simulated Intestinal Fluid (SIF) was added to the digested sample mix. Firstly, 5.3 mL of bile extract (46.87mg/mL) solution dissolved in phosphate buffer and 2 mL of electrolyte solution was added to the sample, and the pH was adjusted to 7.0 using NH₃ (25% w/w). Then, 2.67mL of pancreatin dissolved in phosphate buffer was added to the mix (1:14 (v/v) ratio). The resulting mixture was incubated for two additional hours in the shaking water bath under the same conditions as described in the gastric phase.

2.5. Rheological behaviour

The rheological behaviour 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. The samples were allowed to rest for a 10 min equilibration time after reaching the measurement position, as equilibration time. 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. 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.

In the hydrated cellulose, temperature sweeps were also carried out at 1Hz, 1ºC/min, in the linear viscoelastic region (strain 1.00E-3) from 20ºC to 37ºC, followed by a time sweep at 37ºC for 20 minutes.

2.6. Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy was carried out according to Rodriguez-García, Laguna, Puig, Salvador, & Hernando (2013). A Nikon confocal microscope C1 unit that
was fitted on a Nikon Eclipse E800 V-PS100E microscope (Nikon, Tokyo, Japan) was used. An argon laser line (488 nm) was employed as the light source to excite fluorescent dyes, Rhodamine B and Nile Red. Rhodamine B (Fluka, Sigma-Aldrich, Missouri, USA) was solubilised in distilled water at 0.2%. This dye was used to stain proteins and carbohydrates. Nile Red (Fluka, Sigma-Aldrich, Missouri, USA) was solubilized in PEG 200 at 0.1 g/L. This dye was used to stain fat. The detection wavelengths were 515 nm and 570 nm for Nile Red and Rhodamine B, respectively. A 60X/1.0/oil/Plan Apo VC Nikon objective lens was used. Twenty microliters of the sample were placed in the central microscope slide. Rhodamine B solution and Nile Red solution were added and the cover slide was carefully positioned to exclude air pockets. The observations were performed 10 min after the diffusion of the dyes into the sample. The images were observed and stored at 1024X1024 pixel resolution using the microscope software (EZ-C1 v.3.40, Nikon, Tokyo, Japan).

The droplet size of the emulsions was determined from the CLSM images. The diameter of at least 95 droplets was measured with the software NIS-Elements F, Version 4.0 (Nikon, Tokyo, Japan).

2.7. 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 (bioaccessible), the amount of fat released after centrifugation both from the cellulose ether emulsions and from a whey protein (2% w/w) emulsion containing the same oil content 47% (w/w), considered as control, was calculated. It is necessary to consider that the release of fat from the hydrocolloid/emulsion structure is the first requirement if the digestive enzymes are to act correctly.

After small intestine in vitro digestion, the digesta was mixed with 15 mL ethanol and centrifuged (10 minutes, 10,000rpm) (Sorvall® RC-5B Refrigerated Superspeed centrifuge). The total supernatant was quantified and the mixture of water and ethanol was 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 remaining liquid is considered to be the amount of fat released. The amount of fat that remains in the pellet after centrifugation will not be bioaccessible fat.

2.8. 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 cellulose ether emulsions and in a whey protein (2% w/w) emulsion, containing 25% oil (w/w), considered as control. The 25% oil concentration in the control emulsion (without cellulose) was selected on the basics of the fat released results, as the amount of fat released was significantly higher in the control than in the cellulose emulsion. 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 min at 10,000 rpm in a 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 (colored pink).

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 as “g oleic acid/g fat released”.

### 2.9. 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. Linear viscoelastic properties

##### 3.1.1. Hydrated cellulose ethers

This work focuses on the investigation into the structural changes during the *in vitro* digestion of o/w emulsions prepared with different types of cellulose ethers. The cellulose ethers will act as emulsifiers and/or thickeners of the aqueous continuous phase, this ability being dependent on the specific cellulose chemical substitution.

As a first step, the properties of the solely hydrated cellulose ethers during digestion were analysed, as they are expected to be of help in the understanding of the mechanisms governing the emulsion behaviour.
The effect of mouth, stomach and small intestine in vitro digestion on the frequency dependence at 37°C before and after each digestion step is shown in Figure 1. A big difference was found between the type MC(>30%M) fresh methylcellulose and all the other fresh cellulose ether types. In type MC(>30%M) cellulose, the plateau zone was visualized in the available frequency window, with values of G’ higher than G”’. In the hydroxypropyl methylcellulose types (HPMC(M/HP:4.3) and HPMC(M/HP:2.9)) and the methylcellulose, type MC(30%M), the terminal zone of the mechanical spectra was visualized, denoting their lower viscoelasticity in comparison to type MC(>30%M). The mean values of G’, G”’ and tan δ at 1 Hz corresponding to the fresh and digested cellulose ethers are shown in Table 1. The viscoelastic behaviour is related to the degree of methoxyl content. The highest values of G’ and G”’ and the highest viscoelasticity (the lowest tan δ value) was found in type MC(>30%M) methylcellulose, which contains the highest methoxyl content.

However, the main hypothesis justifying such a large difference between the viscoelastic behaviour of the type MC(>30%M) methylcellulose and all the other celluloses is the fact that, at 37°C, the sol gel transition associated with thermogelling must have already occurred in this cellulose type. This lower gelation temperature will be associated with the higher methyl content, which increases the hydrophobicity of the system, reducing the gelation temperature.

Methylcellulose (MC) and hydroxypropyl methylcellulose (HPMC) ethers possess the unique property of reversible thermogelation. In solution form these polymers are completely hydrated and there is little polymer–polymer interaction other than simple entanglement. Upon heating, these structures distort and break to expose the hydrophobic regions, inducing the formation of aggregates. When lower critical solution temperature (LCST) is reached, sufficient dehydration occurs to promote polymer–polymer interactions instead of polymer–solvent interactions. As a consequence, these cellulose ether solutions start to gel. Thus, gelation is a manifestation of the hydrophobic effect. The specific temperature at which bulk thermal gelation occurs (incipient gel temperature) (IGT) and the strength of the gel formed depends on the type and degree of substitution of the cellulose, molecular weight and concentration and presence of electrolytes (Nishinari, Hofmann, Moritaka, Kohyama, & Nishinari, 1997; Sarkar, 1979).

To investigate whether the greater viscoelasticity of the fresh MC(>30%M) cellulose ethers could be associated with gelation, the changes in G’ and G”’ upon increasing the
temperature from 20 to 37°C were analysed. In Figure 2, the evolution of $G'$ and $G''$ versus temperature at 1 Hz for cellulose types MC(>30%M), MC(30%M) and HPMC(M/HP:4.3) (as an example of hydroxypropyl methylcellulose) is shown. In type MC(>30%M), the values of $G'$ were equal to $G''$ in this temperature range. In types HPMC(M/HP:4.3) and MC(30%M), the values of $G'$ and $G''$ and the viscoelasticity were lower than for type MC(>30%M) and a predominance of $G''$ versus $G'$ is observed. In consequence, the predominance of $G'$ versus $G''$ found in the mechanical spectra (Figure 1) of MC(>30%M) cellulose cannot be explained. To obtain further information, the evolution of $G'$ and $G''$ at 37°C over time just after the application of the temperature sweep was also evaluated (Figure 3). Figure 3 shows that a change in structure occurs at 37°C over time in cellulose MC(>30%M). In type MC(>30%M), an increase in the elastic modulus $G'$ over time was observed, revealing an increase in viscoelasticity and the fact that 37°C is the temperature at which gelation starts. In types MC(30%M) and HPMC(M/HP:4.3), the stability of the viscoelastic functions $G'$ and $G''$ is found. This result confirms the hypothesis that the greater viscoelasticity shown by MC(>30%M) can be associated with the fact that gelation is already under way at this lower temperature, because of the higher degree of hydrophobicity in the system. It should be emphasized that the correct measurement of the frequency dependence of type MC(>30%M) at 37°C should consider the instability of the polymer at this temperature; therefore, appropriate equilibration time is required in the measurement position of the rheometer, otherwise the structure of the system will not be stable during the frequency sweep. This increase in the viscoelastic properties of type MC(>30%M) at 37°C should be considered an interesting point to take into consideration for further new food applications, for example as a satiety enhancer. An appropriately designed emulsion for the treatment of obesity should not only decrease lipid digestibility/absorbability in the upper intestine but may also promote a feeling of fullness in the consumer, leading to a reduction in the size of the portions. A delayed emptying of the stomach and a prolonged transit of the food through the small intestine would mean that the obese would reduce their food intake (Maljaars, Peters, Mela, & Masclee, 2008).

During digestion, the behaviour of the different cellulose ethers was very similar (Figure 1 and Table 1). Mixing with saliva did not affect the viscoelastic properties in any of the cellulose types. After stomach incubation, whereas a significant decrease was
found in the values of $G'$ and $G''$, tan $\delta$ was observed to increase, implying the sample was more viscous in character. To differentiate whether the effect found after stomach incubation is associated with the specific stomach conditions (pH, electrolytes, enzymes) or only with the effect of dilution, samples were incubated in the stomach conditions (time, temperature, shaking), but only in the presence of water. In general, no significant differences were found between the viscoelastic parameters of the stomach samples and the water diluted samples, which implies that the changes in the viscoelastic properties occurring in the cellulose ethers are not specific to the stomach environment but associated with a dilution effect. After intestine incubation, a slight decrease, not enough to be significant, was found in the viscoelastic functions, whereas tan $\delta$ was seen to increase. Similarly to the stomach, the effects found in the intestine were mainly associated with a dilution effect, rather than with a specific effect of the intestinal fluids in the structure of the hydrated cellulose ether.

3.1.2. Emulsions

The effect of incubation in the mouth, stomach and intestine models on the frequency-dependence of the viscoelastic functions is shown in Figure 4. Similarly to the results found in the case of hydrated cellulose ethers, the MC(>30%M) demonstrated completely different viscoelastic behaviour. The viscoelastic properties of the fresh MC(30%M), HPMC(M/HP:4.3) and HPMC(M/HP:2.9) showed a marked dependence on frequency. The end of the plateau zones of the mechanical spectra were visualized, with a cross over between $G'$ and $G''$ occurring in the available frequency window. These mechanical spectra were defined in previous studies (Sanz et al, 2015 and Espert et al, 2016). Saliva only produced a slight decrease in the values of both $G'$ and $G''$, and did not significantly affect tan $\delta$ values at 1 Hz in any of the cellulose emulsions studied (Table 2). After incubation in the stomach, although a significant decrease was found in the values of $G'$ and $G''$ in emulsions MC(30%M), HPMC(M/HP:4.3) and HPMC(M/HP:2.9), the tan $\delta$ values were observed to increase. The shape of the mechanical spectra also changed after stomach incubation with a displacement of the cross over point towards higher frequencies, revealing a decrease in viscoelasticity, and the range of frequencies wherein $G''$ predominates over $G'$ grows. In comparison, a further decrease in viscoelasticity was found after intestine incubation (a decrease in the moduli, and a displacement of the cross over point to higher frequencies). When
incubated in the stomach and intestine models in the presence of water only, there were no significant differences if compared with the standard incubation using MC(30%M), HPMC(M/HP:4.3) and MC(>30%M) emulsions, revealing that the observed change must be mainly associated with the contribution of water dilution, more than with a specific effect of pH or enzymes. It was type HPMC(M/HP:2.9) which was the most affected by intestine incubation, with a significant increase in tan δ values in comparison to the water dilution, implying that the structure of the HPMC(M/HP:2.9) emulsion was specifically affected by the intestine fluids (Figure 5).

The MC(>30%M) emulsion demonstrated different viscoelastic properties, both in terms of the behaviour of the fresh sample, and of the changes during digestion. The changes observed in the MC(>30%M) emulsion during digestion were smaller than in the other cellulose emulsions. The fresh MC(>30%M) emulsion followed a viscoelastic behaviour which was characterized by values of G’ higher than G’’ in the entire available frequency window, with practically no frequency dependence. Although incubation in the mouth, stomach and intestine produced a mild, progressive decrease in the values of G’ and G’’, no significant differences in the viscoelastic functions were found.

3.2. Confocal Laser Scanning Microscopy of the emulsions

The emulsions made with cellulose were generally compact and stable in appearance, in all likelihood due to the formation of a network of hydrated cellulose in the aqueous phase which covers the fat globules (Lin, Wang, & Xu, 2003). This network prevents possible flocculation and coalescence phenomena thanks to the more limited mobility of the globules inside the matrix (Aranberri, Binks, Clint, & Fletcher, 2006). The original emulsions formulated with the hydroxypropyl methylcelluloses, HPMC(M/HP:4.3) and HPMC(M/HP:2.9) (Figure 6, A and E), made up of globules which are semi-rounded in appearance and of differing sizes (from 2.14 to 21.34 µm), looked compact and thick. The mean diameters of F4M and K4M emulsions were 9.84 and 9.35 µm, respectively. No flocculation or coalescence phenomena could be observed. The in vitro mouth digestion phase (Figure 6, B and F) did not seem to affect the structure of these emulsions as they were very similar in appearance to the undigested or original one (Figure 6, A and E). The mean diameters of F4M and K4M emulsions at the mouth digestion phase were 8.67 and 9.55 µm, respectively. Neither did the in vitro gastric digestion phase (Figure 6, C and G) affect either the size (8.62
µm for F4M and 9.00 µm for K4M) or the shape of the globules which were still semi-rounded; it did, however, influence the overall appearance of the emulsion which lost density and became more fluid due to the addition of the digestion fluids. The *in vitro* intestine digestion phase (Figure 6, D and H) had a marked effect on the two emulsions, HPMC(M/HP:4.3) and HPMC(M/HP:2.9); the globules became significantly smaller (3.28 and 2.51 µm, respectively) if compared with the original emulsions that are to say, undigested. In accordance, Bellesi, Martinez, Ruiz-Henestrosa, & Pilosof (2016) observed that HPMC-coated droplets only underwent a slight change in size throughout gastric digestion, which remained almost constant, but sizeable changes when passing from gastric to intestinal conditions, where the oil droplets became smaller. Furthermore, flocculation and coalescence phenomena were observed, which gave rise to large, irregular structures; these were more significant in the case of emulsion HPMC(M/HP:2.9). Some authors (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2014; López-Pena, et al., 2016) also observed an appreciable aggregation of lipid droplets after the intestinal phase leading to an increase in mean particle size, which suggests that the droplets had become coalesced or flocculated. Droplet aggregation may have occurred because of the presence of bile salts and mineral ions in the simulated intestinal fluid (Bellesi et al., 2016; Li & McClements, 2010). Therefore, these results indicate that bile salts are able to displace some of the cellulose ether molecules from the droplet surface, facilitating the accessibility of lipase to the lipid core (Mun et al., 2007), thereby resulting in emulsion destabilization (Singh & Sarkar, 2011). Mineral ions may also have promoted droplet flocculation through charge neutralization and bridging effects (Li & McClements, 2010). The fact that the droplets were flocculated or coalesced in the emulsions might alter their rate of lipid digestion. Firstly, droplet flocculation reduces the surface area of lipids available for lipase adsorption (Bellesi et al., 2016; López-Pena et al., 2016). Secondly, it would be more difficult for lipase molecules to penetrate through a floc and reach the lipid droplets in the interior (Espinal-Ruiz et al., 2014; Li & McClements, 2010). Therefore, what the micrographs show is that, in all likelihood, the HPMC(M/HP:4.3) and HPMC(M/HP:2.9) emulsions have been digested to a similar degree. Furthermore, emulsified fat globules may still be seen in both cases, which would indicate that neither of the emulsions has been fully digested.

The original emulsions formulated using methylcellulose, MC(30%M) y MC(>30%M) (Figure 6, I and M), were more compact and denser in appearance than those formulated
using hydroxypropyl methylcellulose (HPMC(M/HP:4.3) and HPMC(M/HP:2.9)) (Figure 6, A and E). This can be attributed to the fact that there is a greater presence of hydrophobic substituent groups in the methylcellulose compared with the hydroxypropyl methylcellulose. This favours intermolecular interactions between the hydrophobic groups and leads to the emulsion looking denser (Sanz et al., 2015). The MC(30%M) emulsion (Figure 6 I) had differently sized globules (from 2.18 to 31.18 µm), polyhedric in shape, whereas the MC(>30%M) emulsion was made up of globules which were generally bigger in size (17.74 µm) than those in the other emulsions (10.41 µm in MC(30%M), 9.84 µm in HPMC(M/HP:4.3) and 9.35 µm in HPMC(M/HP:2.9)) and were also polyhedric in shape (Figure 6 M). As in the case of the HPMC(M/HP:4.3) and HPMC(M/HP:2.9) emulsions, the in vitro mouth digestion phase (Figure 6, J and N) did not seem to affect the structure of the MC(30%M) and MC(>30%M) emulsions, since they were similar in appearance to the undigested ones. Moreover, the droplet size remained almost unchanged (10.38 and 16.07 µm in A4M and MX emulsions at mouth level). The in vitro gastric digestion phase, however, (Figure 6, K and O) did modify the appearance of the globules as their structure was less polyhedric. The in vitro intestine digestion (Figure 6, L and P) markedly affected the structure of both emulsions, as has previously been seen in the case of the emulsions formulated using hydroxypropyl methylcellulose. The MC(30%M) and MC(>30%M) digested emulsions were of irregular appearance and different from the rest of the emulsions. Not only was the structure of the fat in these two emulsions different in shape and size, there being both small round globules and large polyhedric ones, but there were also major flocculation and/or coalescence phenomena. In the case of the MC(30%M) emulsion (Figure 6 L), the globules were seen to be generally smaller (4.94 µm) than in the MC(>30%M) (6.42 µm), which could point to the fact that the former emulsion was more digested than the latter. Nevertheless, as occurred with the emulsions formulated using hydroxypropyl methylcellulose, the MC(30%M) and MC(>30%M) emulsions still partly preserved a compact appearance prior to digestion because the globules remained embedded within the emulsions.

If the emulsions are compared and the results analysed, it can be seen that it is the emulsions containing methylcellulose which have been digested the least, particularly the MC(>30%M). The reason for this is probably twofold: it is due both to the original dense appearance of these emulsions, made up of large, closely bonded globules which
means that there is a limited surface area upon which the enzymes may act and also to
the fact that they preserve this very density after in vitro digestion. As mentioned
before, this is especially so in the case of the MC(>30%M) emulsion, which has the
largest globules both before and after digestion. Espinal-Ruiz et al. (2014) observed that
methylcellulose promoted depletion flocculation when it is present in sufficiently high
concentrations and, therefore, this highly flocculated appearance resulted in appreciably
reduced digestion rates. So, according to the results, it is important to take into account
the size of the lipid droplets reaching the small intestine. As described previously, the
initial size of the lipid droplets affects their digestion rate because the surface area of
lipid exposed to the surrounding aqueous environment is inversely related to the mean
droplet diameter. Emulsions with smaller droplets have a bigger specific surface area,
which provides more sites for lipase molecules to bind to and, therefore, the release rate
of free fatty acids (FFA) was appreciably higher (Li & McClements, 2010).

3.3. Fat release and free fatty acid generation after intestine digestion

In this study, the quantification of the fat released from the structure is considered a
method by which to simulate the amount of fat which will be accessible to bile and the
digestive enzymes during in vitro digestion (Espert et al., 2016). It is to be expected that
the higher fat that is released, the greater is the possibility that the digestive fluids can
exert their functionality, leading to a higher degree of hydrolysis of the triglycerides
into monoglycerides and free fatty acids.

Table 3 shows the amount of fat released after emulsion centrifugation. When compared
with all the cellulose ether emulsions, the whey protein emulsion showed the highest
percentage of fat released (87.8%). Significant differences were found among the
difference cellulose ether emulsions. The two HPMC types studied showed a
significantly higher amount of fat released than the MC(>30%M) type. No differences
were found between HPMC types HPMC(M/HP:4.3) and HPMC(M/HP:2.9). Although
no significant differences were found between MC(30%M) and MC(>30%M) types, the
MC(>30%M) emulsion was the one that released the lowest amount of fat (only 10.5%).
This result is associated with its highest hydrophobicity and viscoelasticity. After
digestion the changes in the structure of emulsion MC(>30%M) remained little,
reducing the accessibility of the enzymes and other digestive fluids to the fat.

In Table 4 the amount of oleic acid values generated in the different cellulose emulsions
and in a whey protein control with 25% oil content are shown. The percentage of oil in
the control sample (25%) differed from the one employed in the cellulose ether emulsions (47%) because of their big differences in fat released. As the percentage of fat released was significantly higher in the control without cellulose, the initial amount of oil was reduced, so the real amount of fat which becomes available for the digestion enzymes will be closer to the fat released in the cellulose emulsion. When considering the amount of oleic acid generated after the emulsion digestion, differences were also found between the whey protein and the cellulose ether emulsions (Table 4). The amount of oleic acid (in relation to the total initial fat) present in the emulsion was significantly higher (p<0.05) in the whey protein emulsion than in all the cellulose ether emulsions. The lowest amount was found in the MC(>30%M) emulsion, which indicates that this type of cellulose is the most effective in terms of reducing fat digestion. No significant differences (p>0.05) were found among the cellulose types HPMC(M/HP:4.3), HPMC(M/HP:2.9) and MC(30%M).

If the results are expressed as g of oleic acid in relation to g of fat released, the differences associated with a mere physical effect are eliminated (Espert et al., 2016). In this case, the MC(>30%M) emulsion showed higher values than the other cellulose emulsions, which indicates that the big inhibition of fat digestion in this system can be mainly related to the physical effect. The other cellulose types also show lower values than the whey protein emulsion.

Fat constitutes the dispersed phase in the emulsions and it is, therefore, involved inside the continuous phase structure. Fat digestion requires, as a first step, the “destructuration” or weakening of the continuous outer phase, so the activity of the digestive fluids (bile and enzymes) can be effective. In this study, it is speculated that the resistance of the continuous phase to digestion is associated with a decrease in the amount of fat that the digestive fluids have access to. Lipase is water soluble and can only work on the surface of fat globules, for which reason emulsification is a necessary prelude to its efficient activity. Bile acids play a critical role in lipid assimilation by promoting emulsification. On exposure to a large triglyceride aggregate, the hydrophobic portion of bile acids intercalate into the lipid, with the hydrophilic domains remaining on the surface.

4. Conclusions

Cellulose chemical substitution affects the emulsion structure, structural changes during digestion and fat bioaccessibility. Although all of the cellulose ethers were found to be
Effective at reducing emulsion fat bioaccessibility, differences were found. The highest methoxyl content (type MC(>30%M)) was associated with the highest viscoelasticity (before and after digestion), and the lowest fat bioaccessibility. The highest viscoelasticity of type MC(>30%M) was mainly associated with its greater hydrophobicity, which reduces the gelling temperature, so that the system gellified during incubation at 37ºC. The fact that the largest oil droplets are found in the initial MC(>30%M) emulsion and maintained during digestion, can also be related to the decrease in fat digestibility.

The total fat released from the emulsion structure after digestion is an indicator of the degree of structural resistance, and is positively related to the fat digestion process. It is speculated that the more resistant the structure is after digestion, the higher the barrier that will be exerted against an appropriate physical contact between the emulsified lipid and the digestive enzymes, with a subsequent decrease in fatty acid generation.

A physical barrier is recognized as being the main reason for the fact that the lowest fat digestion is to be found mainly in type MC (>30%M), the effectiveness of the barrier being dependent on the cellulose ether substitution type: the physical effect was more marked for type MC(>30%M) (a higher methoxyl content) and lower for type HPMC(M/HP:2.9) (a lower methoxyl content).

Animal testing will be required to validate the obtained in vitro results. Future research will also focus on using the cellulose emulsions as substitutes for conventional fat in different food applications.

**Acknowledgements**

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References


Table 1. G’, G’’ and tanδ for each hydrated cellulose ethers as a function of digestion step.

<table>
<thead>
<tr>
<th>Cellulose</th>
<th>Digestion step</th>
<th>G’ (Pa)</th>
<th>G’’ (Pa)</th>
<th>tan δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC(30%M)</td>
<td>Fresh</td>
<td>2.9a</td>
<td>9.6a</td>
<td>3.3a</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>2.4a</td>
<td>7.5b</td>
<td>3.1a</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>0.6b</td>
<td>3.0c</td>
<td>5.1ab</td>
</tr>
<tr>
<td></td>
<td>Stomach dilution</td>
<td>0.4b</td>
<td>2.3cd</td>
<td>5.5ab</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>0.2b</td>
<td>1.5de</td>
<td>6.2ab</td>
</tr>
<tr>
<td></td>
<td>Intestine dilution</td>
<td>0.1b</td>
<td>0.8e</td>
<td>7.8b</td>
</tr>
<tr>
<td>HPMC(M/HP:4.3)</td>
<td>Fresh</td>
<td>1.9a</td>
<td>5.1a</td>
<td>3.2bc</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>1.8a</td>
<td>5.2a</td>
<td>2.8c</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>0.4b</td>
<td>1.9ab</td>
<td>4.5b</td>
</tr>
<tr>
<td></td>
<td>Stomach dilution</td>
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<td>1.0b</td>
<td>3.7bc</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>0.2b</td>
<td>1.4b</td>
<td>6.4a</td>
</tr>
<tr>
<td></td>
<td>Intestine dilution</td>
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<td>0.7b</td>
<td>6.7a</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
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<td>8.4a</td>
<td>3.4c</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
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<td>7.0a</td>
<td>3.4c</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>0.3b</td>
<td>1.7b</td>
<td>5.2ac</td>
</tr>
<tr>
<td></td>
<td>Stomach dilution</td>
<td>0.4b</td>
<td>2.3b</td>
<td>5.2ac</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>0.2b</td>
<td>1.2b</td>
<td>6.2ab</td>
</tr>
<tr>
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<td>0.9b</td>
<td>7.2b</td>
</tr>
<tr>
<td></td>
<td>Fresh</td>
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<td>0.4c</td>
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<td></td>
<td>Saliva</td>
<td>143.9ab</td>
<td>51.2ab</td>
<td>0.4c</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
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<td>36.9ab</td>
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</tr>
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<td>27.4b</td>
<td>23.3ab</td>
<td>0.9a</td>
</tr>
<tr>
<td></td>
<td>Intestine</td>
<td>40.6ab</td>
<td>10.8b</td>
<td>0.3c</td>
</tr>
<tr>
<td></td>
<td>Intestine dilution</td>
<td>188.7a</td>
<td>58.5a</td>
<td>0.3c</td>
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abcMeans with different letter in columns for each parameter and each cellulose type indicate significant differences among the samples (p < 0.05) according to the Tukey test.
Table 2. G, G’’ and tanδ for each cellulose emulsion as a function of digestion step.

<table>
<thead>
<tr>
<th>Cellulose</th>
<th>Digestion step</th>
<th>G' (Pa)</th>
<th>G'' (Pa)</th>
<th>tan δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC(30% M)</td>
<td>Fresh</td>
<td>418.2a</td>
<td>295.9a</td>
<td>0.7b</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>368.6a</td>
<td>293.5a</td>
<td>0.8b</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>42.4b</td>
<td>65.5bc</td>
<td>1.6ab</td>
</tr>
<tr>
<td></td>
<td>Stomach dilution</td>
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<td>103.7b</td>
<td>1.5ab</td>
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<td>Intestine</td>
<td>3.3b</td>
<td>7.0e</td>
<td>2.3a</td>
</tr>
<tr>
<td></td>
<td>Intestine dilution</td>
<td>8.4b</td>
<td>19.5bc</td>
<td>2.3a</td>
</tr>
<tr>
<td>HPMC( M/HP:4.3)</td>
<td>Fresh</td>
<td>412.6a</td>
<td>293.9a</td>
<td>0.7c</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>318.6b</td>
<td>232.4ab</td>
<td>0.7c</td>
</tr>
<tr>
<td></td>
<td>Stomach</td>
<td>183.1c</td>
<td>181.1bc</td>
<td>1.0bc</td>
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<tr>
<td></td>
<td>Stomach dilution</td>
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<td>144.9c</td>
<td>1.1b</td>
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<tr>
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<td>19.5d</td>
<td>1.5a</td>
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<td>119.0c</td>
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<tr>
<td>HPMC( M/HP:2.9)</td>
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<td>320.5a</td>
<td>261.2a</td>
<td>0.8b</td>
</tr>
<tr>
<td></td>
<td>Saliva</td>
<td>323.8a</td>
<td>259.8a</td>
<td>0.8b</td>
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<tr>
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<td>138.1b</td>
<td>155.2b</td>
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<td>82.3bc</td>
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<td>Intestine</td>
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<td>7.8c</td>
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<tr>
<td>MC(&gt;30 %M)</td>
<td>Fresh</td>
<td>1834.3a</td>
<td>339.8a</td>
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<td></td>
<td>Saliva</td>
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<td></td>
<td>Intestine</td>
<td>817.6a</td>
<td>127.0b</td>
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<td></td>
<td>Intestine dilution</td>
<td>1392.0a</td>
<td>210.8b</td>
<td>0.2b</td>
</tr>
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abcMeans with different letter in columns for each parameter and each cellulose type indicate significant differences among the samples (p < 0.05) according to the Tukey test.
Table 3. Fat released after intestine in vitro digestion of the whey protein and the cellulose emulsions. Oil concentration in all the emulsions was 47%.

<table>
<thead>
<tr>
<th>Emulsion type</th>
<th>Released fat (g)</th>
<th>Released fat/ initial fat (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein (control)</td>
<td>2.57a</td>
<td>87.80a</td>
</tr>
<tr>
<td>HPMC(M/HP:4.3)</td>
<td>2.01ab</td>
<td>68.61ab</td>
</tr>
<tr>
<td>HPMC(M/HP:2.9)</td>
<td>1.94ab</td>
<td>66.13ab</td>
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<tr>
<td>MC(30%M)</td>
<td>1.40bc</td>
<td>47.61bc</td>
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<tr>
<td>MC(&gt;30%M)</td>
<td>0.31c</td>
<td>10.50c</td>
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abc Means with different letter in columns for each parameter indicate significant differences among the samples (p < 0.05) according to the Tukey test.

Table 4. Oleic acid values after in vitro digestion of the cellulose emulsions (47% oil) and the whey protein with 25% oil.

<table>
<thead>
<tr>
<th>Emulsion type</th>
<th>Oleic acid (g)</th>
<th>Oleic acid/initial fat</th>
<th>Oleic acid/released fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein (control)</td>
<td>0.69a</td>
<td>0.44a</td>
<td>0.51a</td>
</tr>
<tr>
<td>HPMC(M/HP:4.3)</td>
<td>0.40b</td>
<td>0.14b</td>
<td>0.20a</td>
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<tr>
<td>HPMC(M/HP:2.9)</td>
<td>0.38b</td>
<td>0.13b</td>
<td>0.20a</td>
</tr>
<tr>
<td>MC(30%M)</td>
<td>0.30b</td>
<td>0.10b</td>
<td>0.22a</td>
</tr>
<tr>
<td>MC(&gt;30%M)</td>
<td>0.14c</td>
<td>0.05c</td>
<td>0.44b</td>
</tr>
</tbody>
</table>

abc Means with different letter in columns for each parameter indicate significant differences among the samples (p < 0.05) according to the Tukey test.
FIGURE LEGENDS

Figure 1. G’ and G’’ as a function of frequency of the fresh hydrated cellulose ethers and after in vitro digestion at 37 °C (A: MC(30%M), B: HPMC(M/HP:4.3), C: HPMC(M/HP:2.9), D: MC(>30%M)).

Figure 2. G’ and G’’ versus temperature in the linear viscoelastic region of the fresh hydrated cellulose ethers at 1Hz.

Figure 3. G’ and G’’ versus time in the linear viscoelastic region of the fresh hydrated cellulose ethers at 1Hz.

Figure 4. G’ and G’’ as a function of frequency of the fresh cellulose o/w emulsions and after in vitro digestion at 37 °C (A: MC(30%M), B: HPMC(M/HP:4.3), C: HPMC(M/HP:2.9), D: MC(>30%M)).

Figure 5. G’, G’’ (A) and tan δ (B) as a function of frequency of HPMC(M/HP:2.9) o/w emulsion after the intestine step versus the water-diluted sample (at 37ºC).

Figure 6. Confocal laser scanner microscopy (CLSM) of stained fresh cellulose o/w emulsions and after in vitro digestion at 37 °C with Rhodamine B and Nile Red (fat in green. Magnification 60x.