The effect of pectin concentration and degree of methyl-esterification on the *in vitro* bioaccessibility of β-carotene-enriched emulsions

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Abstract

Soluble fibers, like pectin, are known to influence the physicochemical processes during the digestion of dietary fat and may therefore affect the absorption of lipophilic micronutrients such as carotenoids. The objective of the current work was to investigate whether the pectin concentration and degree of methyl-esterification (DM) influence the bioaccessibility of carotenoids loaded in the oil phase of oil-in-water emulsions. The *in vitro* β-carotene bioaccessibility was determined for different oil-in-water emulsions in which 1 or 2% citrus pectin with a DM of 99%, 66% and 14% was present. Results show that pectin concentration and DM influence the initial emulsion properties. The most stable emulsions with the smallest oil droplets (D(v,0.9) of 15-16 μm) were obtained when medium or high methyl-esterified pectin was present in a 2% concentration while gel-like pectin structures (D(v,0.9) of 114 μm), entrapping oil droplets, were observed in case low methyl-esterified pectin was present in the aqueous emulsion phase. During *in vitro* stomach digestion, these gel-like structures, entrapping β-carotene loaded oil droplets, significantly enlarged (D(v,0.9) of 738 μm),

whereas the emulsion structure could be preserved when medium or high methyl-esterified pectin was present. Initial emulsion viscosity differences, due to pectin concentration and especially due to pectin DM, largely disappeared during *in vitro* digestion, but were still significant after the stomach digestion phase. The observed differences in emulsion structure before and during *in vitro* digestion only resulted in a significant difference between emulsions containing low methyl-esterified pectin (β-carotene bioaccessibility of 33-37%) and medium/high methyl-esterified pectin (β-carotene bioaccessibility of 56-62%).

1 Introduction

Nowadays, consumers are very well aware of the importance of a balanced and healthy diet. In this context, fruits and vegetables play an important role. Several studies (including Furr and Clark, 1997; Maiani *et al.*, 2009; Shi and Le Maguer, 2011) relate the consumption of foods rich in carotenoids to a reduced risk of certain chronic diseases such as cardiovascular disease, cataract and cancer. Some carotenoids (like β-carotene) also have provitamin A-activity (Trumbo *et al.,* 2001). Nevertheless, the uptake of carotenoids from food can be limited because of poor carotenoid bioaccessibility and consequently limited bioavailability. The bioaccessibility of a nutrient is the fraction of a nutrient that is released from the food matrix during digestion and which is available for absorption in the small intestine (Hedrén *et al.*, 2002). However, the bioavailability of a nutrient is the fraction of an ingested nutrient that is actually absorbed in the small intestine and can be used for storage or metabolic processes in the body (Castenmiller and West, 1998; Kopsell and Kopsell, 2006; Maiani *et al.*, 2009). Literature shows that several factors (e.g. matrix properties) affect the absorption of carotenoids (Castenmiller and West, 1998; Borel, 2003). Many studies investigating bioaccessibility and bioavailability (for example Reboul *et al.,* 2006; Ryan *et al.,* 2008; Knockaert *et al.,* 2012 and Roman *et al.,* 2012), use real and thus complex food matrices implying that several factors at the same time affect the carotenoid absorption from that particular matrix. As a consequence, it is not easy to determine the effect of individual factors on the carotenoid bioaccessibility and/or bioavailability.

The influence of the structural build-up of foods on the bioaccessibility and bioavailability of carotenoids has hence not been fully clarified. The presence of oil and fibers seem to be two important factors in this context.

Several studies (Borel, 2003; McClements *et al.*, 2009; Salvia-Trujillo *et al.,* 2013) indicate a positive relationship between the presence of oil and the bioaccessibility of carotenoids, which might be explained by the fact that carotenoids are fat-soluble components and must be incorporated in micelles before they can be absorbed in the small intestine. Not only the presence of oil, but also the oil droplet size seems to be important for the lipid digestion (McClements *et al.,* 2009). Smaller oil droplets having a larger exchange surface area might promote oil digestion. The digestion of oil droplets may however not only be influenced by the oil droplet size but also by the viscosity of the continuous phase or the properties of interfacial layers surrounding the lipid droplets. For example surface active compounds at the oil droplet surface may hinder lipase activity (Michalski *et al.,* 2005; Kalantzi *et al.,* 2006; Mun *et al.,* 2007; McClements *et al.,* 2009). So, the exact role of oil and oil droplet sizes on lipid digestion, and thus also on the bioaccessibility of fat-soluble nutrients, is depending on several factors (Tyssandier *et al.*, 2001; Huo *et al.*, 2007; Salvia-Trujillo *et al.,* 2013).

Next to oil, also fibers (abundantly present in plant-derived food systems) are assumed to influence the bioaccessibility and/or bioavailability of carotenoids because of a number of specific reasons (Parker, 1996; Löfgren *et al.,* 2005; Hur *et al.*, 2009; McClements *et al.*, 2009; Palafox-Carlos *et al.*, 2011). In fact, fibers are assumed to decrease the bioaccessibility and/or bioavailability because they (1) may hinder the contact between the micelles and the small intestine (2) may interact with bile salts and lipases, which are involved in the formation of micelles, and (3) may increase the viscosity thereby slowing down the transport of the digestive enzymes to their substrates. When present in plant-based emulsions, fibers however also affect the emulsion structural properties like for example the oil droplet size (distribution) and the rheological behavior. Moreover, particular fibers, like for example pectin (when applied under specified conditions), can act as an emulsifier (Akhtar *et al.,*

2002; Leroux *et al.,* 2003). Hence, next to their more direct influence on the bioaccessibility of β-carotene, fibers also influence the behavior of emulsions during digestion and consequently the digestibility of fat and the absorption of fat-soluble substances. In this context, it has been shown that emulsifiers, initially used to stabilize oil-in-water emulsions, significantly affect the oil droplets sizes and charges and the changes in emulsion microstructure during *in vitro* digestion (Liu *et al.,* 2012). In addition, soluble fibers, like pectin and chitosan, have been shown to respectively promote droplet aggregation and bind on droplet surfaces during *in vitro* digestion (Beysseriat *et al.,* 2006). Further detailed knowledge on the effect of specific fibers on fat digestion and fat-soluble micronutrients is currently missing. Therefore, the present work investigated the effect of pectin on the bioaccessibility of β-carotene that is loaded within the oil phase of oil-in-water emulsions. Next to the effect of the concentration of pectin, also the effect of pectin nanostructure in terms of degree of methyl-esterification was studied. In order to understand the effect of pectin on β-carotene bioaccessibility, the (micro)structural properties of the initial and the *in vitro* digested emulsions were determined, with particular focus on oil droplet size distribution and viscosity.

2 Material and Methods

2.1 Materials

Carrots (*Daucus carota* cv. Nerac) were purchased in a local shop and stored at 4°C. High methyl-esterified citrus pectin (CP) (Sigma Aldrich) was used as the starting material for the preparation of pectin with different degree of methyl-esterification (DM). Olive oil (extra virgin) was kindly donated by Vandemoortele (Ghent, Belgium). All chemicals and reagents were of analytical grade from Sigma Aldrich, except for NaCl, HCl, urea and ethanol (from VWR); $CaCl_2.2H_20$, NH_4Cl, $MgCl_2$ and $CaCl_2.2H_20$ (from Merck); hexane and acetone (from Chem Lab); glucose and $NaHCO_3$ (from Fisher Scientific); KCl (from MP Biomedicals).

2.2 Preparation of citrus pectin with different DM

CP with different DM was prepared by incubating high methyl-esterified CP with purified carrot pectin-methyl-esterase (PME). Hereto, carrot PME was extracted and purified (Jolie *et al.,* 2009), after which the activity was measured according to the procedure described by Jolie *et al.* (2009). The high methyl-esterified CP was de-esterified by incubating it with purified carrot PME at 30°C during 4 min or 30 hours as described in the work of Ngouémazong *et al.* (2011). The DM of the high methyl-esterified CP and of the resulting partially de-esterified pectin samples was measured using Fourier transform-infrared (FT-IR) spectroscopy (IRAffinity-1, Shimadzu) (100 interferograms per sample) (Manrique and Lajolo, 2002). The resulting values were 98.6% (± 1.5), 65.6% (± 5.8) and 14.1% (± 1.1). Therefore, the different pectin samples will be further called "CP99", "CP66" and "CP14".

2.3 Protein content of citrus pectin with different DM

The protein content of the pectin samples was measured by the Dumas method. This method is based on the AOAC-method (990.03) (1995). An automatic analysis-system (EAS Vario MAX CN, Elt, Gouda, The Netherlands) was used to measure the amount of molecular nitrogen (N_2). The conversion factor of 6.25 was used to calculate the amount of proteins.

2.4 Preparation of oil-in-water emulsions enriched with β-carotene

Carrot puree was prepared by mixing peeled carrot pieces and water (1:1) in a kitchen blender (Waring Commercial, Torrington, CT, USA) for 1 min. Olive oil was enriched with β-carotene by rotating olive oil end-over-end with carrot puree (1:5 w/w) for 5 h at room temperature. The enriched oil phase was collected after centrifugation at 4 °C for 15 min at 8739 g (J2-HS centrifuge, Beckman, CA, USA) (Colle *et al.*, 2010). Emulsions were prepared by blending 5% (w/w) of the enriched oil with demineralized water in which 1 or 2% (w/w) citrus pectin (CP99, CP66 or CP14) was dissolved. The pH was adjusted to 6.0 using a sodium hydroxide solution. An emulsion with 5% enriched oil and demineralized water in which a certain concentration of CP (c%) with a certain DM (dm%) was dissolved, is further indicated as a "c% CPdm emulsion". Emulsions were prepared in

duplicate to take into account the variability due to the preparation procedure. Each of them was independently submitted to the *in vitro* digestion procedure.

2.5 In vitro *digestion*

The digestion was simulated by using *in vitro* digestion juices described by Versantvoort *et al.* (2005). The composition of those digestion juices was validated by *in vivo* derived data and published in Versantvoort *et al.* (2004).

Stomach digestion was simulated by adding 12 ml stomach juice (mainly containing ions, glucose, urea, pepsin and mucin; pH 1.3) to 5 g emulsion. The samples were incubated by rotating end-over-end for 2 h at 37 °C. The small intestinal digestion was mimicked by adding 12 ml duodenal juice (mainly containing ions, urea, pancreatin and lipase; pH 8.1), 6 ml bile extract (mainly containing ions, urea and bile; pH 8.2) and 2 ml 1 M bicarbonate to the sample. The samples were again incubated for 2 hours (at 37 °C) while shaking end-over-end. To minimize the influence of light and oxygen, the samples were kept in the dark during the digestion procedure and the headspace of the tubes was flushed with nitrogen before each incubation step.

2.5.1 Particle size distribution during *in vitro* digestion

The particle/oil droplet size distributions of the initial emulsions and the digested emulsions (after the stomach phase and after the small intestinal phase) were measured by laser diffraction (Malvern Instrument Ltd., Worcestershire, UK) and visualized by a microscope (Olympus BX-41) equipped with an Olympus XC-50 digital camera (Olympus, Opticel Co. Ltd., Tokyo, Japan).

2.5.1.1 Laser diffraction

A few droplets of each sample were poured into a stirring tank, filled with deionized water. The sample was pumped into a cell wherein the laser light (H-Ne laser, wavelength 633 nm) was scattered by the particles. The parameters D(v,0.1), D(v,0.5) and D(v,0.9) are calculated from the intensity profile of the scattered light using the instrument's software (Mie theory) and reported accordingly. The relative width of the particle size distribution (spread) was calculated as:

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149 $$\text{spread} = \frac{(D[v,0.9]-D[v,0.1])}{D[3,2]} \quad (1)$$

150 All analyses were carried out in duplicate.

151 2.5.1.2 Microscopic analysis

152 CP99, CP66 and CP14 were covalently labeled using a non-ionic fluorescent dye, i.e. BODIPY FL
153 hydrazide (4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionylhydrazide) (Nordmark
154 and Ziegler, 2000). The labeling resulted in approximately 2.6-4.4 labels per 100 000 galacturonic acid
155 monomers in the emulsions. Microscopic pictures were taken with an Olympus BX-41 microscope
156 (Olympus, Optical Co. Ltd, Tokyo, Japan) equipped with epifluorescence illumination (X-Cite
157 Fluorescence Illumination, Series 120Q EXFO Europe, Hants, United Kingdom) using Image-analysis
158 software (cell*, Soft Imaging System, Münster, Germany). To avoid fluorescent interfering of β-
159 carotene on the pictures, the CP emulsions were made with olive oil instead of with β-carotene
160 enriched olive oil.

161 2.5.2 Viscosity

162 The viscosity of the initial emulsions and of the digested (after stomach phase and after small
163 intestinal phase) emulsions was measured using a stress-controlled rheometer (MCR 501, Anton
164 Paar, Graz, Austria) at 25 °C. A concentric cylinder (double wall couette cell) was used as geometry.
165 To neglect the loading history of the emulsion, a constant shear rate of 100 s^{-1} was applied for 60
166 seconds, followed by a rest-period (shear rate of 0 s^{-1}) of 300 seconds. The viscosity was measured by
167 decreasing the shear rate linearly from 100 to 0.1 s^{-1}. Each shear rate was applied for 40 sec and it
168 was verified that steady state viscosities were obtained in this way. Evaporation was considered
169 negligible due to the short duration of the tests. All analyses were carried out in duplicate.

170 2.5.3 *In vitro* β-carotene bioaccessibility

171 The *in vitro* β-carotene bioaccessibility was measured after digesting the emulsions by the *in vitro*
172 digestion model described above. After the small intestinal phase, the micelle fraction was collected

by ultracentrifugation (165 000 g, 1 h and 5 min, 4 °C) and the concentration of β-carotene in this fraction was determined according to the procedure described by Colle *et al.* (2013) with some small modifications. To this micelle fraction, 50 ml extraction solvent, containing hexane, aceton, ethanol (50:25:25) and 0.1% buthylated hydroxytoluene (BHT), was added to extract the β-carotene fraction. Besides this solvent, also 1 g sodium chloride and 15 ml ultrapure water were added to facilitate the separation of the organic (containing β-carotene) and the aqueous phase. The organic phase was taken and the amount of β-carotene was measured by spectrophotometric analysis at 450 nm (=λ_{max} for β-carotene in hexane) and calculated as:

$$\text{amount of } \beta-\text{carotene } \left(\frac{\mu g}{\text{g emulsion}}\right) = \frac{\text{A .V (ml)}.10^4}{\text{E}_{1\text{ cm}}^{1\%}.\text{m (g)}} \quad (2)$$

where A is the measured absorbance (at 450 nm), V is the volume of the extract (25 ml hexane), $E_{1\ cm}^{1\%}$ is the extinction coefficient (2560 $\frac{100\ ml}{g\ cm}$) and m is the mass of the emulsion (in g) (Hart & Scott, 1995).

The obtained valuerepresents the amount of β-carotene in the micelles after digestion. Also the initial amount of β-carotene in each emulsion was measured. The *in vitro* β-carotene bioaccessibility ($\frac{B}{C}$) is defined as the amount of β-carotene in the micelles after digestion (B) relatively to the initial amount of β-carotene in the emulsion (C). All analyses were carried out in triplicate.

2.5.4 Statistical analysis

Differences in mean relative β-carotene bioaccessibility were analyzed using one-way anova and the Tukey's Studentized Range Post-hoc Test (Statistical Software Package SAS, version 9.2., Cary, N.C., U.S.A.). The level of significance was 95% (P<0.05).

3 Results and discussions

3.1 Protein content of citrus pectin with different DM

It is known that proteins may have emulsifying capacities (Singh *et al.,* 2009). Therefore it is needed to quantify the amount of proteins in our pectin samples to ensure that results might be explained by

either the present of the proteins or by the pectin itself. Like expected, the protein content of the pectin samples slightly increased by adding PME during the de-esterification procedure. The CP99 contained 1.68 (± 0.11) mg protein per 100 mg CP compared to 4.14 (± 0.66) and 3.52 (± 0.19) mg protein per 100 mg CP66 and CP14 respectively. Nevertheless, the protein content is very low. The protein content agrees well with the protein content found by Kravtchenko *et al.* (1992) (3.0-3.3 wt%) for citrus pectin with a DM of approximately 72%.

3.2 Particle size (distributions) of emulsions during in vitro *digestion*

3.2.1 Initial emulsions

The results on the particle size distribution analysis of the different emulsions (Table 1) show a clear decrease of oil droplet size with increasing pectin concentration added to the aqueous phase of the emulsions. For example, the median oil droplet size (D(v,0.5)) decreases from 8.1 µm to 4.6 µm when the concentration of CP99 is increased from 1% to 2%. Furthermore, 90% of the oil droplets (indicated as D(v,0.9)) in the 1% CP99 emulsion is smaller than 23.7 µm, whereas 90% of the oil droplets in the 2% CP99 emulsion is smaller than 15.8 µm. These results were confirmed by means of microscopy (results not shown) and agree well with those of Leroux *et al.* (2003) who concluded that high methyl-esterified citrus pectin is able to reduce the interfacial tension between the water and the oil phase. It is probably because of its hydrophobicity (due to $COOCH_3$-groups) that pectin has some emulsifying properties. Besides the pectin concentration, also the DM has an influence on the oil droplet size (distribution). When 1% of de-esterified pectin (CP66 or CP14) was added to the aqueous phase of the emulsions, the median oil droplet size decreased to 5.8 and 3.6 µm respectively compared to 8.1 µm for the 1% CP99 emulsion. On the other hand, the spread of the oil droplet size was larger for emulsions containing pectin with a lower DM (spread of 18.9 and 54.5 for CP66 and CP14 respectively instead of 5.9 for 1% CP99 emulsion), suggesting that CP66 as well as CP14, when added in a 1% concentration, cannot stabilize the interface of the oil droplets as efficient as the CP99 does. In case 2% of citrus pectin was added to the emulsions, the CP66 seems to stabilize the emulsion equally well as the CP99. Because of the slightly higher protein content of the low

methyl-esterified pectin samples, it might be expected that especially those types of pectin sample (CP66 and CP14) has more emulsion stabilizing properties because the proteins can act as emulsifiers as well (Leroux *et al.,* 2003). It seems however that the proteins have a negligible influence compared to the influence of (the DM of) the pectin. The results of Table 1 can be compared with Figs. 1a and 1d in which the 1% CP99 and 1% CP14 emulsions are visualized. The 2% CP emulsions had the same trend (results not shown). In Fig. 1, the greenish color is the result of the fluorescent BODIPY FL-molecules which were attached to the pectin molecules in order to label them. The pictures clearly show only small individual oil droplets in the 1% CP99 emulsion (Fig. 1a) whereas a mix of single oil droplets and large green-colored structures in the 1% CP14 emulsion were observed (Fig. 1d). These larger structures represent gel-like pectin structures in which oil droplets are embedded and can explain the high D(v,0.9)-values observed in the CP14 emulsions. The apparent green coloring of the oil droplets suggests that pectin molecules are concentrated at the oil droplet surface and that they indeed can function as an emulsifier.

3.2.2 Digested emulsions

Figs. 2a and 2b show that the oil droplet size and the oil droplet size distribution of the CP99 and CP66 emulsions remain approximately constant during the stomach phase. Only the results of 1% CP are presented, but similar trends were observed for the 2% CP emulsions. Also the microscopy pictures (Figs. 1a and 1b), clearly show that oil droplets within the 1% CP99 emulsion are approximately the same before digestion and after the stomach phase. Similar observations were done for the 2% CP99, 1% CP66 and 2% CP66 emulsions. This means that both high and medium methyl-esterified pectin present in emulsions apparently allows preserving the initial emulsion structure during *in vitro* digestion in the stomach. This type of pectin thus seem to prevent oil droplet clustering which could occur because of the presence of mucin within the stomach juice (McClements and Li, 2010) and prevents coalescence of oil droplets which could be expected for oil droplets with an average size smaller than 10-20 μm (McClements *et al.*, 2009).

In contrast, the particle size distribution of the CP14 emulsions significantly changed during the stomach phase. Also here, only the results of a 1% CP14 emulsion are given, but the 2% CP14 emulsion gave similar results. In order to better understand these changes, the samples were also visualized using microscopy (Fig 1). From the microscopic analysis, it became clear that the larger particles measured by laser diffraction (for example D(v,0.1) of 105 µm, D(v,0.5) of 393 µm and D(v,0.9) of 693 µm for the 1% CP14 emulsion), are gel-like pectin clusters in which oil droplets are embedded. These clusters were already present in the initial CP14 emulsions but significantly enlarged during *in vitro* stomach digestion. Probably, these large gel-like pectin clusters are formed because ions (including Ca^{+2}) and proteins are added during the stomach phase of the *in vitro* digestion procedure, a phenomenon which is an issue especially in samples where pectin with a high level of free carboxyl groups is present (Löfgren *et al.,* 2005). The block wise distribution of the non-methyl-esterified galacturonic acids (degree of blockiness, DB), as a result of the de-esterification action of carrotPME (plantPME), will probably contribute to the formation of a strong pectin gel (so called 'egg-box models') because consecutive non-methyl-esterified galacturonic acids are needed to cross-link with Ca^{+2} (Fraeye *et al.,* 2010; Ngouémazong *et al.,* 2011: Ngouémazong *et al.,* 2012).

After the small intestinal phase, the oil droplet size (distribution) of the CP99 (Fig. 2a) and CP14 (Fig. 2c) emulsions was similar to the one after the stomach phase (results only shown for the 1% emulsions). In the CP66 emulsion, the formation of gel-like pectin clusters entrapping oil droplets during the small intestinal phase was however observed. This suggests that the conditions in the small intestinal phase are more favorable to form a gel-like structure which is stable enough to entrap oil droplets, because the amount of ions and the pH increased compared to the stomach phase. The microscopic pictures of the emulsions after the small intestinal phase (Figs. 1c and 1f) show that pectin within the CP99 emulsions is no longer present at (the surface) of the oil droplets but is present in the continuous phase, while large gel-like particles keep existing in case of the CP14 emulsions. The reason can be that in case of CP99 emulsions, the oil droplets are (partially) digested.

3.3 Viscosity of emulsions during in vitro digestion

3.3.1 Initial emulsions

Fig. 3 shows the viscosity in function of the shear rate for all emulsions tested, indicating that all emulsions, except the 2% CP14 emulsion, behave Newtonian before digestion. The 2% CP14 emulsion showed a shear thinning behavior. The viscosity of the latter emulsion varies between 0.06 and 0.10 Pa.s (for shear rates from 100 to 0.1 s^{-1}). The viscosity increases with the pectin concentration for all tested DM values. Upon comparing the viscosity of the CP99 emulsion with the viscosity of the CP66 or CP14 emulsions, it becomes clear that the emulsion viscosity increases with decreasing DM. The viscosity increased for example with a factor 2 when the DM of the pectin in the aqueous phase decreased from 99% to 66% in a 1% CP emulsion and with a factor 3 when the DM decreased from 99% to 14% in a 1% CP emulsion.

3.3.2 Digested emulsions

After stomach phase, the emulsion still behave Newtonian which means that the viscosity of the CP99 emulsions is still independent of the shear rate. The viscosity decreases however when stomach juice is added. For example, the viscosity of the 1% CP99 emulsion decreases from 0.0050 Pa.s (before digestion) to 0.0023 Pa.s after stomach digestion. This decrease is probably due to the addition of aqueous stomach juice and it seems that if reactions took place between the emulsion and the added ions or proteins, they were negligible compared to the diluting effect. On the other hand, the CP66 emulsions behave Newtonian before digestion but became pseudoplastic when stomach juice was added. This shear thinning behavior is probably a consequence of the formation of gel-like pectin structures because of the addition of ions (like $CaCl_2$) or proteins (Löfgren *et al.,* 2005) present in the stomach juice. Due to high shear rates, those structures can be broken down so that the viscosity decreases in function of the shear rate (Steffe, 1996). These structures were however not visible in the particle size distribution, possibly because of the mixing applied during this type of measurement. In contrast to the CP99 and CP66 emulsions, the viscosity of the CP14 emulsions

increased when stomach juice was added. This can be explained by the addition of ions at the start of the stomach phase causing interaction between the pectin molecules with a low DM and ions like Ca^{+2} as was shown by the presence of large pectin containing gel-like structures on the microscopic pictures of the CP14 emulsions.

After the small intestinal phase, the viscosity decreases for all emulsions compared to the viscosity of the emulsions after the stomach phase. The reason for this observation is probably that the small intestinal juice and the bile extract dilute the system. As plenty of ions were already added in the stomach phase, it is possible that adding more ions to the emulsions did not resulted in more pectin gel-formation, although the pH changed. Fig. 3 shows that the concentration dependency of the viscosity decreased after the small intestinal phase since the viscosity of 1% emulsions of a certain DM are very similar to those of the corresponding 2% emulsions. In addition, the differences in viscosity between the emulsions with a different DM decreased after the small intestinal phase.

3.4 In vitro *β-carotene bioaccessibility*

The results in Fig. 4 show that there is no significant effect of the pectin concentration (1% versus 2%) on the β-carotene bioaccessibility in the CP99 emulsions (bioaccessibility of 62% versus 57%), the CP66 emulsions (bioaccessibility of 56% and 60%) and the CP14 emulsions (bioaccessibility of 37% versus 33%). The (small) observed differences in oil droplet size and viscosity before and during *in vitro* digestion due to the concentration of pectin with a certain DM were apparently too small to result in differences in β-carotene bioaccessibility. The relatively long *in vitro* digestion time might have contributed to rule out these differences.

First, it can be noted that a relatively high β-carotene bioaccessibility for CP99 and CP66 emulsions was measured (Fig. 4). The reason for this, is that natural barriers of β-carotene are removed by transferring the β-carotene from carrots to the oil in the emulsions (Verrijssen *et al.,* 2013). Decreasing the citrus pectin DM from 99% to 66% did not substantially affect the β-carotene bioaccessibility, whereas a significant decrease of the bioaccessibility was noticed by further

decreasing the citrus pectin DM to 14% (from approximately 56-62% for the CP99 and CP66 emulsions to 33-37% for the CP14 emulsions). This decrease (compared to the other emulsions) is in line with the fact that the oil droplets in the CP14 emulsions were embedded in gel-like pectin clusters during digestion which might have inhibited the lipase activity, due to the high visocisty, steric hindering or by decreasing the available surface area (Bauer *et al.,* 2005; McClements *et al.,* 2009), at its turn limiting fat digestion and absorption of fat-soluble components like β-carotene. Also the higher viscosity of the CP14 emulsions compared to the other emulsions might have contributed to the observed differences in β-carotene bioaccessibility, as the digest viscosity is known to be very important in the context of the transport of digestive enzymes to their substrates.It should be mentioned that a health related consequence of a lower β-carotene bioaccessibility is that less β-carotene can be converted to vitamin A, which is important for normal vision, immune function, gene expression, reproduction and embryonic development (Trumbo *et al.,* 2001).

4 Conclusions

In this work, we have shown that citrus pectin concentration (1 and 2%) and DM (99%, 66% and 14%) in simple β-carotene enriched model emulsions are influencing the oil droplet size distribution and the viscosity during digestion leading to β-carotene bioaccessibility changes for those cases where large differences of these factors exist. For example relatively small differences in oil droplet size distributions did not result in different β-carotene bioaccessibility, whereas the formation of large pectin gel-like clusters in the CP14 emulsions resulted in a decrease of β-carotene bioaccessibility. The large pectin gel-like structures probably result from Ca^{+2}-induced gelation of CP14 in the emulsions and Ca^{+2} being present in stomach and small intestinal juices. These structures seem to embed oil droplets leading to oil droplets which are less accessible for lipase to be digested due to higher viscosity and the gel-like structures.

In addition, this manuscript shows that pectin can be used as an emulsifier. This knowledge is important for the food industry because pectin is a compound naturally present in plants and may be

preferred over artificial emulsifiers by consumers. Furthermore, the manuscript shows the interactions in terms of bioaccessibility when two important compounds normally present in fruit and vegetables, i.e. β-carotene and pectin, are considered, as well as when an oil phase, which is often present in fruit and vegetable based food such as soups or sauces, is taken into account. The next step in this research could be to design more complex model emulsions, to end up with realistic food systems.

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Table 1: Particle size distribution of the different initial emulsions

% CP in emulsion		D(v,0.1) (µm)	D(v,0.5) (µm)	D(v,0.9) (µm)	Spread
Concentration CP99					
	1%	1.22 ± 0.13	8.08 ± 0.95	23.68 ± 3.51	5.91 ± 1.74
	2%	1.05 ± 0.15	4.58 ± 0.85	15.81 ± 2.14	5.67 ± 0.74
Concentration CP66					
	1%	1.18 ± 0.13	5.82 ± 3.01	62.45 ± 39.70	18.86 ± 8.15
	2%	1.13 ± 0.18	4.82 ± 2.97	15.37 ± 3.98	5.26 ± 0.17
Concentration CP14					
	1%	1.10 ± 0.03	3.55 ± 0.36	143.28 ± 54.57	54.53 ± 18.96
	2%	1.18 ± 0.04	4.53 ± 0.75	114.10 ± 18.92	44.44 ± 4.06

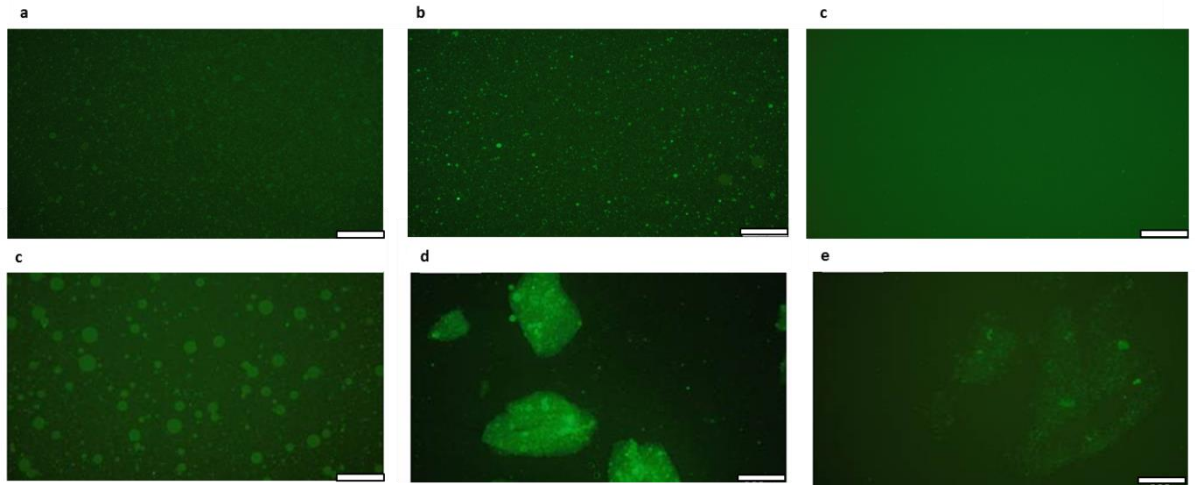
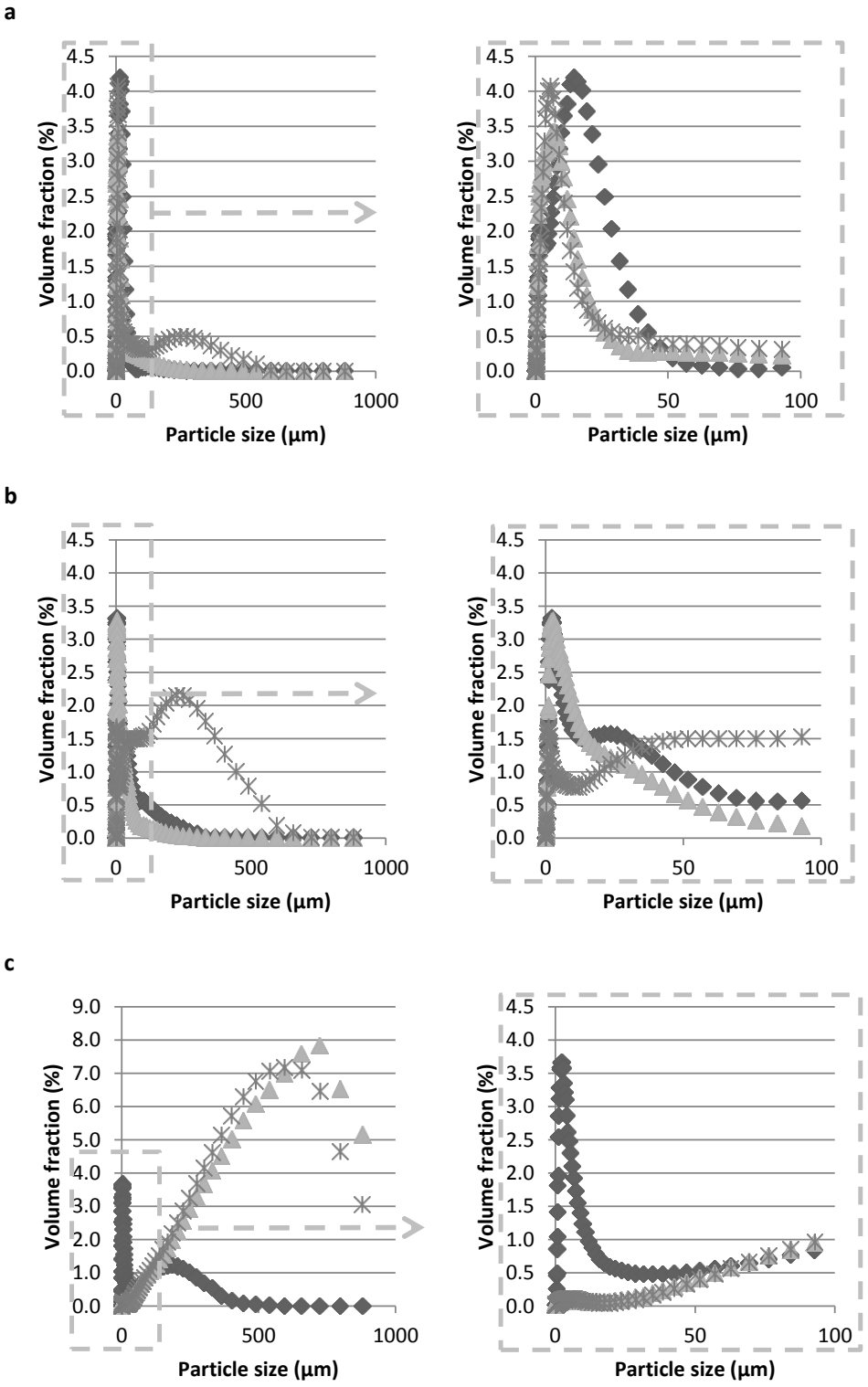


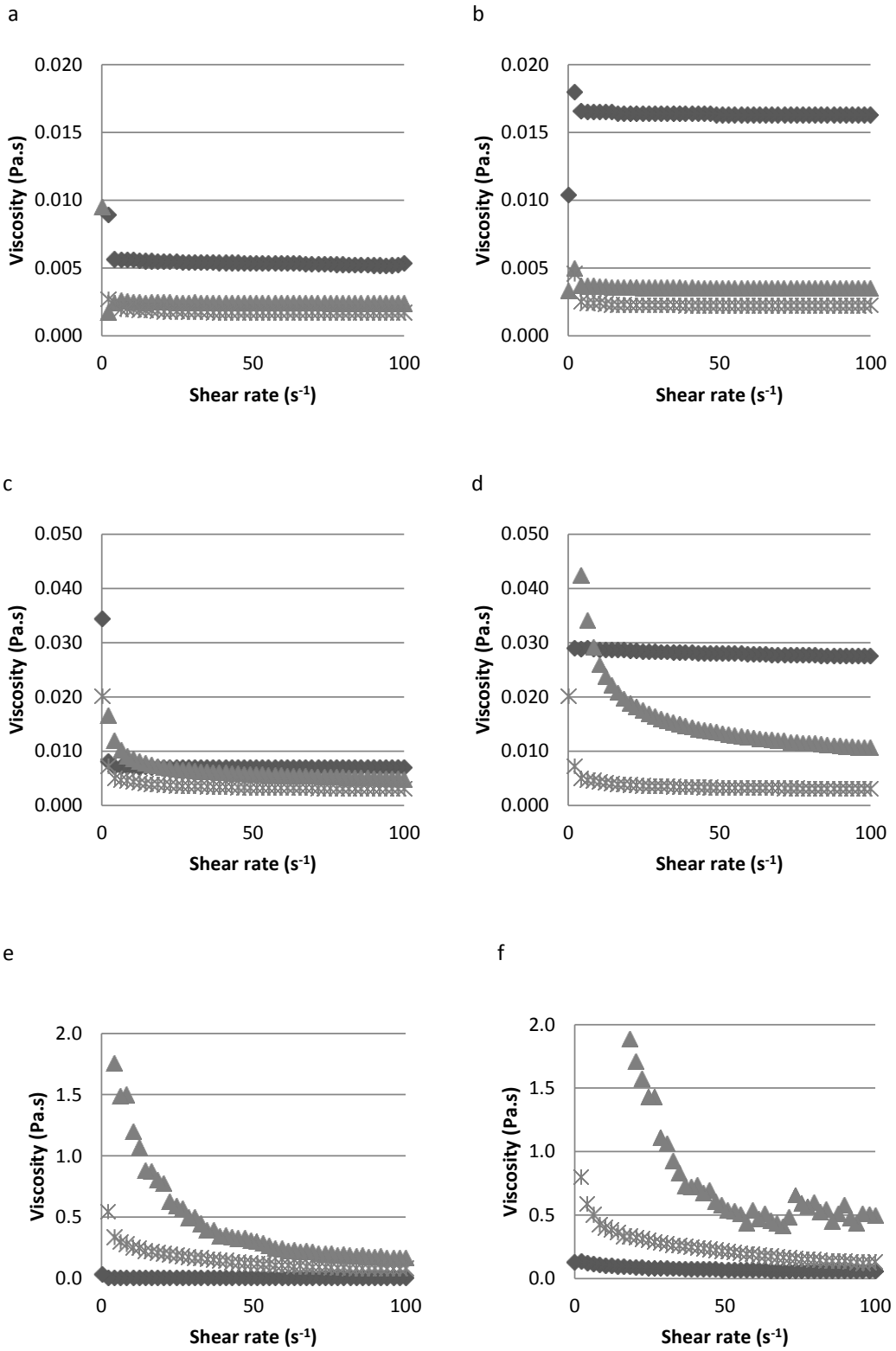
Figure 1: Representative microscopic images of the oil droplet distributions in a 1% CP99 emulsion ((a) before digestion, (b) after the stomach phase and (c) after the small intestinal phase) and a 1% CP14 emulsion ((d) before digestion, (e) after the stomach phase and (f) after the small intestinal phase). Scale bars (▭) represent a length of 200 µm.



488

489 Figure 2: Particle size distribution of 1% CP99 emulsion (a), 1% CP66 emulsion (b) and 1% CP14 emulsion (c) during digestion

490 (Before digestion (◆), after the stomach phase (▲) and after the small intestinal phase (✳)).



491

492 Figure 3: Viscosity of 1% CP99 emulsion (a), 2% CP99 emulsion (b), 1% CP66 emulsion (c), 2% CP66 emulsion (d), 1% CP14

493 emulsion (e) and 2% CP emulsion (f) during digestion (Before digestion (◆), after the stomach phase (▲) and after the

494 small intestinal phase (✳)).

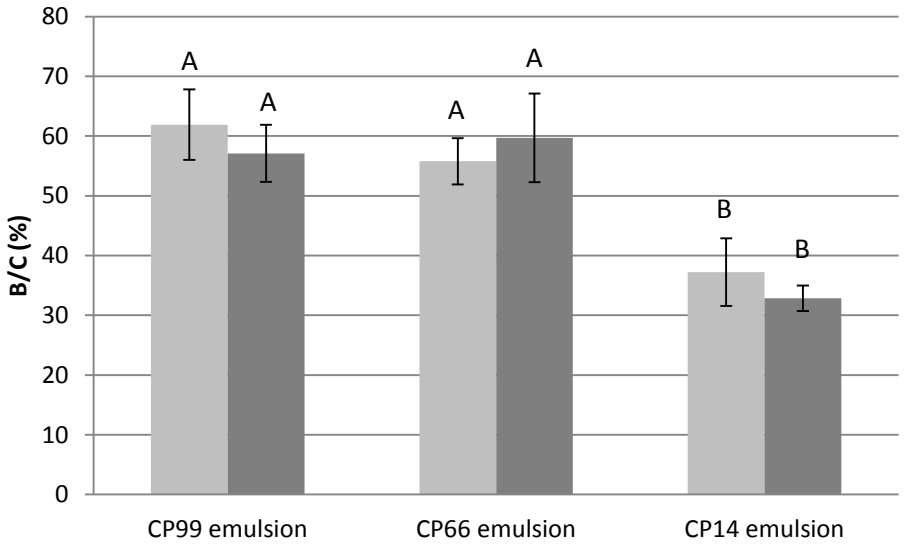


Figure 4: Percentage *in vitro* β-carotene bioaccessibility (calculated as the absolute β-carotene bioaccessibility (B) divided by the initial amount of β-carotene (C) of the sample) (mean ± standard deviation) in the 1% ( ) or 2% ( ) CP emulsions. Significant differences (Tukey test, $P<0.05$) are indicated with different letters (A,B).