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Graphical abstract

“*In vitro* digestibility kinetics of oil-in-water emulsions structured by water-soluble pectin-protein mixtures from vegetable purées”

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Abstract

The potential use of water-soluble biopolymers from vegetable-origin in structuring oil-in-water emulsions and the influence on their digestion was investigated. The aqueous/serum phase, containing water-soluble biopolymers, of processed carrot and broccoli purées were isolated, lyophilized and then used either as the emulsifier or component in the continuous phase of carotenoid-loaded o/w emulsions. The kinetics of lipid digestion, micelle formation and carotenoid bioaccessibility of such emulsions were monitored during the *in vitro* small intestinal phase.

The vegetable sera were characterized by a mixture of pectin and protein, in which free proteins and/or proteins-linked to pectin were probably present. Such pectin-protein mixtures exhibited an emulsion stabilizing capacity that determined the rate and extent of lipolysis, micelle formation, and carotenoid bioaccessibility. Emulsion destabilization during the gastric phase caused larger droplets in the emulsion formulated with broccoli serum containing high protein content and low M_w pectin compared to carrot serum with low protein content and high M_w pectin. In turn, a broccoli serum-emulsion presented slower rate and lower extent of lipolysis, micelle formation and consequently carotenoid bioaccessibility. By contrast, broccoli serum as a component in the continuous phase had no effect on lipolysis, micelle formation and carotenoid bioaccessibility, while, carrot serum limited the extent of carotenoid bioaccessibility without affecting the lipolysis and micelle formation. Carrot serum in the continuous phase hindered the transfer of carotenoids into mixed micelles. Water-soluble vegetable-based biopolymers (which can be tuned by processing) can be potentially used as natural emulsifiers that might modulate lipolysis and bioaccessibility of lipophilic bioactive compounds.

Keywords

In vitro digestion, emulsion stability, carotenoid bioaccessibility, biopolymers, vegetable purée

1. Introduction

The growing consumer concern for health and environment is continuously driving the demand for clean label food products, which prompts the food industry to avoid the addition of ingredients perceived by consumers as artificial and replace the use of synthetic surfactants (e.g. Tweens, Spans) in structuring emulsion-based foods (Asioli et al., 2017; McClements & Gumus, 2016; Ozturk & McClements, 2016). Emulsifiers that are more natural, environmentally friendly and sustainable are currently being sought for the production of emulsion-based foods (McClements, Bai, & Chung, 2017). In this sense, surface active biopolymers (e.g. proteins, polysaccharide-protein complexes, polysaccharides with interfacial activity) from natural sources are considered as alternatives for the production of oil-in-water (o/w) emulsions (McClements et al., 2017). On the one hand, although certain proteins (e.g. whey protein isolate, casein, bovine serum albumin, soy) are already used as emulsifiers in industrial food applications, the search for more protein-based emulsifiers from sustainable sources is still ongoing (Lam & Nickerson, 2013). On the other hand, among the polysaccharides, pectin is gaining acceptance for its utilization as an emulsifier (Alba & Kontogiorgos, 2017; Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015). Recently, sugar beet pectin and whey protein isolate were reported to effectively produce fine o/w emulsions at low concentrations (Bai, Huan, Gu, & McClements, 2016; Bai, Huan, Li, & McClements, 2017). In particular, the interfacial tension of sugar beet pectin was comparable to gum arabic and since sugar beet pectin has an extended conformation and a semi-flexible chain, it can adsorb faster and cover the oil droplet surfaces at lower concentrations (Bai, Huan, Li, & McClements, 2017). For whey protein isolate, being a globular protein, it has high surface activity and it can adsorb fast to the droplet surface due to its small molecular weight (Bai, Huan, Gu, & McClements, 2016). The emulsifying properties of biopolymers can be generally dependent on intrinsic (e.g. molecular characteristics) and extrinsic (e.g. pH, ionic strength) factors (Dickinson, 2017; Lam et al., 2013; Xu, Luo, Liu, & McClements, 2017).

Controlling these factors is relevant for the production of more natural emulsion-based food products, while an insight into the digestibility of such emulsions formed by biopolymers might provide a better assessment of the role of biopolymers during digestion.

In recent years, the ingestion of oil together with lipophilic micronutrients such as carotenoids was found to enhance the bioaccessibility of carotenoids since lipids provide a hydrophobic pool in which carotenoids can dissolve (Hornero-Méndez & Mínguez-Mosquera, 2007; Lemmens et al., 2014). Besides the release of carotenoids from the plant matrix and its solubilization in the oil upon ingestion, oil emulsification into fine droplets and their digestibility can also influence carotenoid bioaccessibility (Goltz & Ferruzzi, 2013; McClements, Decker, Park, & Weiss, 2008). In fact, a strong correlation between micelle formation as a consequence of lipid digestion and the bioaccessibility of carotenoids was reported recently (Mutsokoti et al., 2017; Salvia-Trujillo et al., 2017). The fate of lipids during digestion and the consequent mixed micelle formation by lipid digestion products can be governed by the emulsion properties, including the droplet size, interfacial properties and the composition of the aqueous/continuous phase. In terms of droplet size, digestion of small individual droplets can be favorable due to its high surface area available for intestinal lipases to adsorb (Giang et al., 2015; Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013; Salvia-Trujillo et al., 2017). Regarding the interfacial composition, the properties of the adsorbed layer surrounding the lipid droplets can determine the behavior and stability of the emulsion during its passage to the gastrointestinal tract. For instance, hydrolysis of the interfacial layer by pepsin in protein-stabilized emulsions can lead to changes in the surface area of the droplets (Nik, Wright, & Corredig, 2011). In addition, the type of emulsifier surrounding the lipids can influence the adsorption of intestinal lipases onto the droplet interface as the emulsifier type determines the interfacial layer (Mun, Decker, & McClements, 2007). Similarly, the access of lipases into the oil-water interface can be dependent on the type of molecules present in the aqueous phase of the emulsion (McClements, Decker, & Park, 2009). For example, the

presence of dietary fiber such as pectin in the continuous phase of the emulsion was reported to hamper lipid digestion because of pectin's binding ability with key digestive components (such as calcium, free fatty acids, and bile), capacity to increase the viscosity of gastrointestinal fluids and/or due to droplet flocculation (Cervantes-Paz et al., 2017; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sánchez, & Narváez-Cuenca, 2014; Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014a, Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014b). Therefore, the stability of the emulsion as controlled by its characteristics can have a great influence on the sequential digestive events of lipid digestion, micelle formation and bioaccessibility of lipophilic bioactive compounds.

Moreover, the aqueous/serum phase of plant-based o/w emulsions such as vegetable soups and sauces, naturally contain water-soluble biopolymers. For instance, in such plant-derived food systems containing emulsified lipids, pectin and/or pectin-protein complexes solubilized from the plant matrix during processing can be inherently present as a component in the aqueous phase. In particular, as the most reactive cell wall polysaccharide during processing and owing to solubility characteristics, pectin is a ubiquitous biopolymer in plant-based foods (Sila et al., 2009). Nonetheless, it is still unclear whether these water-soluble biopolymers (e.g. pectin, protein, pectin-protein complexes) present in the aqueous phase can influence the stability and digestibility of the emulsified lipids in plant-based foods. Biopolymers present in the aqueous phase that are not digested in the stomach and small intestine (e.g. pectin) can be hypothesized to influence the emulsion stability and digestibility, depending on the biopolymer concentration and molecular properties. Similarly, regarding the use of biopolymers as natural emulsifiers, the water-soluble biopolymers (e.g. pectin, protein, pectin-protein complexes) inherently present in plant-based dispersions might be explored for their use in forming and protecting fine oil droplets from coalescence or flocculation given that such biopolymers contain both hydrophilic (e.g. neutral sugar side chains of pectin) and hydrophobic groups (e.g. protein, methyl and acetyl

groups of pectin) (Ngouémazong et al., 2015). In this sense, when used as the emulsifying agent, the solubilized biopolymers might have an impact on lipid digestibility and carotenoid bioaccessibility, depending on their emulsification ability which can be related to the molecular features of the biopolymer. Therefore, to understand the influence of solubilized biopolymers (i.e. pectin-protein) on emulsion stability and digestibility, the use of such water-soluble biopolymers either as component in the aqueous phase of the emulsion or as the emulsifier was explored in the present study. Since pectin is the most reactive among the cell wall biopolymers during processing, the solubilization of pectin with distinct molecular structures in terms of degree of methyl-esterification, molecular weight and neutral sugar composition, was induced by thermal and mechanical treatments of carrot or broccoli purée. The serum phase of the processed vegetable purées containing the water-soluble biopolymers was isolated, dialyzed and then lyophilized. The obtained pectin-rich lyophilized serum was characterized and used in stabilizing/structuring carotenoid-loaded olive o/w emulsions that were subjected to simulated gastrointestinal conditions. Specifically, the pectin-rich lyophilized serum was re-dissolved and then used either as the emulsifier or as the constituent of the continuous phase of o/w emulsions formulated with a non-ionic surfactant (Tween 80) as the emulsifier. The different emulsions were individually submitted to static *in vitro* digestion consisting of gastric and small intestinal phases. The physicochemical properties of the emulsions including the particle size, particle size distribution, microstructure and zeta (ζ -) potential were monitored during the *in vitro* digestion. During the small intestinal phase, lipid digestion and the consequent release of lipid digestion products and their formation into mixed micelles as well as the bioaccessibility of carotenoids were determined at different time points.

2. Materials and methods

2.1 Materials

All chemicals and reagents used were of analytical or HPLC-grade. Lipid standards, pepsin (2500 U/mg protein), pancreatin (from porcine pancreas, batch #SLBJ8147V), porcine bile extract (B8631), pancreatic lipase (from porcine pancreas Type II, batch #SLBN3801V), tocopherol (T3251) and pyrogallol (P0381) were obtained from Sigma-Aldrich. A single batch of fresh carrots (*Daucus carota* cv. Nerac) and broccoli (*Brassica oleracea* cv. Italica) were obtained from a local supplier and then stored at 4 °C for 3 days. Carrots were washed, peeled and then cut into 0.5 cm thick, while broccoli florets were cut around 5 cm from the head and sliced into 1 cm thick. Extra virgin olive oil was bought from a local supermarket.

2.2 Methods

2.2.1 Serum isolation and characterization

Water-soluble biopolymers were isolated from the aqueous (serum) phase of thermally and mechanically treated carrot or broccoli purée. Briefly, carrot pieces or broccoli florets were independently vacuum-packed in a single layer followed by heat treatment at 95 °C for 30 min in a temperature-controlled water bath to inactivate the enzymes and solubilize pectin. The heat-treated vegetables were cooled, added with de-mineralized water in a ratio of 1:1 (w/w) for carrots or 3:4 (w/w) for broccoli and then blended for 20 s at low speed and 40 s at high speed using a kitchen blender (Waring Commercial, Torrington, Connecticut, USA). To intensely disrupt the tissues, the blended carrot or broccoli was subjected to high pressure homogenization at 100 MPa for a single cycle (Panda 2 K, Gea Niro, Parma, Italy). Furthermore, to obtain the serum phase, the purées were centrifuged at 12 400 g for 30 min at 20 °C (J2-HS centrifuge, Beckman, CA, USA). The supernatant (serum) was filtered using a cheesecloth and then vacuum-filtered (Machery-Nagel MN 615 Ø 90 mm) to exclude remaining pulp fragments. The sera obtained were dialyzed (3.5 kDa, MWCO) against demineralized water for 48 h to remove small molecules (e.g. ions and monomeric sugars) and then lyophilized using Christ alpha 2-4 freeze dryer (Osterode, Germany) to obtain the polymeric material.

The polymeric (i.e. protein and pectin) composition of the lyophilized serum and consequently the pectin molecular properties, in terms of degree of methyl-esterification (DM), degree of acetylation (DAc), molecular weight (M_w) and neutral sugars, were characterized based on the methods described in our previous study (Santiago, Christiaens, Van Loey, & Hendrickx, 2016).

2.2.3 Carotenoid-enrichment of olive oil

Olive oil was enriched with carotenoids from high pressure homogenized carrot purée. Carrot pieces were blended with de-mineralized water (1:1 w/w) for 1 min followed by high pressure homogenization at 100 MPa for a single cycle (Pony NS2006L, GEA, NH, USA). Next, olive oil (20% w/w) was added and mixed with the homogenized carrot purée at 12 500 rpm for 10 min using a dispersing instrument (Ultra-turrax, Ika T-25, Janke and Kunkel, Germany). The oil-carrot purée mix was then subjected to high pressure homogenization at 100 MPa, single pass. To facilitate the phase separation, the homogenized oil-carrot purée mix was frozen at -40 °C overnight and subsequently thawed at 25 °C for 2 h. To obtain the carotenoid-rich upper oil layer, the oil-carrot purée mix was centrifuged at 165 000 g at 4 °C for 15 min (Optima XPN-80, Beckman Coulter, USA). The carotenoid-rich oil phase was recovered and stored at -80 °C until use. The α - and β -carotene concentrations of the enriched olive oil were 90.6 ± 1.5 and 199.8 ± 2.9 $\mu\text{g/g}$ oil, respectively.

2.2.4 Carotenoid enriched oil-in-water (o/w) emulsion preparation

Emulsions were formed using different emulsifiers, namely broccoli sera, carrot sera and Tween 80 (a non-ionic surfactant as the control). For the emulsion formulated with broccoli or carrot sera as the emulsifier, the lyophilized serum (1% w/w) was dissolved overnight in acidified Milli-Q water (94% w/w) at pH 3.5. Subsequently, carotenoid-enriched olive oil (5% w/w) was added and mixed at 8 000 rpm for 2 min. The coarse emulsion was further subjected to high pressure homogenization at 100 MPa for a single cycle (Stansted Fluid Power, Pressure cell homogenizer, UK). Similarly, an emulsion using Tween 80 as the emulsifier (0.5% w/w) was also prepared with carotenoid-enriched

olive oil (5% w/w) and acidified Milli-Q water (94.5% w/w) at pH 3.5, which served as a control. Furthermore, to investigate the influence of the sera when present in the continuous phase of the emulsion, emulsions formulated with Tween 80 (1% w/w), carotenoid-enriched olive oil (10% w/w) and acidified Milli-Q water (89% w/w) at pH 3.5 were prepared as previously described. The obtained emulsion was subsequently added with pre-dissolved broccoli or carrot sera (2% w/w) in a 1:1 ratio, which allows the serum biopolymers to constitute the aqueous (continuous) phase of the Tween 80-stabilized emulsion.

2.2.5 *In vitro* digestion

To investigate the stability and digestibility of the emulsions during gastrointestinal conditions, they were subjected to a static *in vitro* digestion based on the international consensus method (Minekus, et al., 2014) with minor modifications as described by Mutsokoti et al. (2017). First, the gastric phase (pH 3.0) was simulated by mixing 5 mL aliquot of emulsion with 5 mL Milli-Q water, 7.5 mL simulated gastric fluid (SGF), 5 μ L CaCl_2 (0.3 M), 1.6 mL porcine pepsin solution (200 U/mL) dissolved in SGF, HCl (1 M) to adjust the pH and Milli-Q water to adjust the volume of the simulated gastric phase to 20 mL. Prior to incubation at 37 °C for 2 h in an end-over-end rotator, the headspace of the tube was flushed with N_2 gas for 10 s.

Immediately after the gastric phase, the small intestinal phase was simulated by adding 11 mL of simulated intestinal fluid (SIF), 2.5 mL bile solution (160 mM), 40 μ L CaCl_2 (0.3 M) and 5 mL pancreatin solution containing pancreatic lipase (200 U/mL), 1.4% α -tocopherol and 0.6% pyrogallol, into the gastric chyme. The pH was adjusted to 7.0 with NaOH (1 M) and the final volume of the sample was increased to 40 mL by adding Milli-Q water. After being flushed with N_2 gas, the samples were immediately incubated at 37 °C for 2 h in an end-over-end rotator. During the small intestinal phase, a kinetic study was carried out after the addition of pancreatin solution. Under subdued light, digestion was performed in individual tubes at 6 time moments: 7.5, 15, 30, 45, 60 and 120 min. After

each digestion, the digest was transferred into glass tubes and treated at 85 °C for 10 min to stop the lipid digestion. The samples were subsequently cooled in an ice-water bath followed by centrifugation at 165 000 g and 4 °C for 68 min (Optima XPN-80, Beckman Coulter, IN, USA) to harvest the micelle fraction.

2.2.6 Physicochemical characterization of initial and digested emulsions

2.2.6.1 Particle size and particle size distribution

The average particle size and particle size distribution of the initial emulsions as well as the digested emulsions after the gastric and small intestinal phases were determined using a laser diffraction instrument (Beckman Coulter Inc., LS 13 320, FL, USA). A few drops of the samples were poured into a stirring-tank filled with de-mineralized water until it reached a polarization intensity differential scattering (PIDS) of 45% to 55%. The diluted sample was pumped into a measuring cell wherein the laser light, with a wavelength main illumination source at 750 nm and wavelengths halogen light for PIDS at 462 nm, 604 nm and 900 nm, was scattered by the particles. Based on the intensity profile of the scattered light, the volumetric particle size distribution was automatically calculated with the instrument's software by use of the Mie theory. The refractive indices of the oil and aqueous phase used in the calculations were 1.468 and 1.333, respectively. The average particle size, expressed in micrometer (μm), was reported as the volume-weighted mean diameter (d_{43}).

2.2.6.2 Microstructure

The microstructure of the emulsions before and after subjecting in each simulated gastrointestinal condition was examined using an optical microscope (Olympus BX-41) equipped with an Olympus XC-50 digital camera (Olympus, Optical Co. Ltd., Tokyo, Japan) by applying differential interference contrast mode (DIC).

2.2.6.3 Zeta potential

The electrical charge of the particles before and after each simulated gastrointestinal condition was measured using a phase-analysis light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Emulsions were diluted 1:100 with either acidified Milli-Q water (pH 3.5), SGF (pH 3.0) or SIF (pH 7.0) for the initial emulsion, after the gastric phase or small intestinal phase, respectively. Afterwards, an aliquot of the diluted samples was placed in a capillary cell equipped with two electrodes to determine the electrophoretic mobility of the particles at 25 °C.

2.2.7 Investigation of lipid digestibility and carotenoid bioaccessibility

2.2.7.1 Lipid extraction and analysis

Lipid digestion was monitored by analyzing the amount of residual (undigested) triacylglycerols (TAG) and the concentrations of diacylglycerol (DAG), monoacylglycerol (MAG) and free fatty acid (FFA) during the small intestinal phase. The lipid species in the digested emulsions at different time moments (7.5, 15, 30, 45, 60 and 120 min) during the small intestinal phase and their respective micelle fraction were extracted and quantified as described by Salvia-Trujillo et al. (2017) with minor changes. Briefly, 1 mL sample (initial emulsion, digest after the small intestinal phase or micelle fraction) was mixed with 2 mL ethanol, 3 mL diethylether-heptane (1:1 v/v) and 0.2 mL sulfuric acid (2.5 M). The solution was vortexed for 2 min and then subjected to end-over-end rotation for 30 min at ambient condition. Afterwards, the lipid-rich upper layer was collected and transferred into a 5-mL volumetric flask. The remaining layer (bottom part) was added with 1 mL of diethylether-heptane solution (1:1 v/v), vortexed for 2 min and then submitted to end-over-end rotation for 15 min. Finally, the upper organic layer was again collected and transferred into the 5-mL volumetric flask containing the extract. The lipid extract was adjusted to a final volume of 5 mL with diethylether-heptane (1:1 v/v), filtered (Chromafil PET filters, 0.20 µm pore size, 25 mm diameter) and then stored at -40 °C until analysis.

The analysis of TAG, DAG, MAG and FFA was carried out using an HPLC system (Agilent Technologies 1200 Series, Diegem, Belgium), equipped with a silica column (5 μm x 150 x 4.6 mm ID, Spherisorb, Waters), protected with a guard column in an oven (5310, VWR, Hitachi Ltd., Tokyo, Japan) at 40 °C and coupled to evaporative light scattering detector (Agilent 1260 Infinity G4260B, Agilent Corporation, USA). The separation of the lipid species was achieved using a quaternary gradient consisting of iso-octane (solvent A), acetone:ethylacetate (2:1 v/v) containing 70 mM acetic acid (solvent B), propan-2-ol:Milli-Q water (85:15 v/v) containing 7.5 mM acetic acid and 7.5 mM triethylamine (solvent C), and propan-2-ol (solvent D). The elution gradient at a flow rate of 1 mL/min was carried out as described by Mutsokoti et al. (2017). The concentration of TAGs, DAGs, MAGs, and FFAs were analytically determined using calibration curves of equivalent standards, namely trilinolein (879.4 g/mol), dilinolein (616.97 g/mol), monolinolein (354.5 g/mol) and linoleic acid (280.4 g/mol), respectively.

The molar concentration of glycerol (GLY) and water released at each time point during the small intestinal phase was calculated from the analytical concentrations of DAGs, MAGs and FFAs, according to the following stoichiometric reactions (Moquin & Temelli, 2008):

Furthermore, the TAG concentration of each digestion time point was calculated as the sum of the analytically determined TAGs, DAGs, MAGs and the amount of completely digested TAG based on the analytical and stoichiometric FFA concentrations. Subsequently, the average of TAG calculated for the six (6) digestion time points served as the initial TAG concentration (time = 0 min). The amount of TAGs in each time point was calculated based on the mass balance considering the difference between the calculated (initial) TAG concentration at the start of the small intestinal phase and the sum of the lipid digestion products (DAG + MAG + FFA + GLY - H₂O). To normalize the TAG concentration for

each sample, amount of undigested TAG was expressed in percentage (%). Similarly, the concentration of lipid digestion products (mg/mg TAG₀) in the total digest and micelle fraction was normalized based on the respective initial TAG concentration for each sample.

2.2.7.2 Carotenoid extraction and analysis

α - and β - carotene were extracted and quantified based on the method described by Salvia-Trujillo et al. (2017) with minor modifications. To prevent carotenoid degradation, the extraction was performed under subdued light. In a dark brown Erlenmeyer flask, the initial emulsion (2 mL), digest (5 mL) or micelle fraction (5 mL) was mixed with 1 g NaCl and 25 mL (or 12.5 mL for micelle fraction) hexane:acetone:ethanol (50:25:25) containing 0.1% butylated hydroxytoluene. The mixture was stirred for 20 min at 4 °C and then added with 7.5 mL Milli-Q water or 3.8 mL for the micelle fraction. On the one hand, the initial emulsion and digest were transferred into a transparent glass tube and then the upper organic layer was collected and filtered (Chromafil PET filters, 0.20 μ m pore size, 25 mm diameter) into dark brown vials. On the other hand, to facilitate the separation of the organic phase, the micelle fraction was centrifuged at 500 g and 4 °C for 5 min (J2-HS centrifuge, Beckman, CA, USA) followed by the collection and filtration of the organic layer.

Carotenoids were analyzed using an RP-HPLC-DAD system (Agilent Technologies 1200 Series, Diegem, Belgium) equipped with a C30-column (3 μ m x 150 mm x 4.6 mm, YMC Europe, Diegem, Belgium) coupled with a guard column and kept at 25 °C. An elution gradient consisting of Milli-Q water, methanol and methyl-t-butyl-ether was applied at a flow rate of 1 mL/min. To quantify α and β carotene, peak responses at 450 nm were measured and the peak areas were compared to a calibration curve with purified standards (CaroteNAture, Lupsingen, Switzerland). The bioaccessibility of carotenoids (BAC, %) was reported as the concentration of carotenoids in the micelle fraction versus the concentration of carotenoids in the initial emulsions multiplied by 100.

2.2.8 Statistical analysis and kinetic modelling

Statistical analysis and data modelling were performed using JMP statistical software (JMP Pro 13.0.0, SAS Institute Inc.). For each sample, the physicochemical characteristics (i.e. particle size, zeta potential) of the emulsions under simulated gastrointestinal conditions were analyzed in duplicate. To determine the statistical significance, sample means were analyzed by one-way analysis of variance and the Student's *t* test was performed to determine significant differences at a 5% significance level (*p*-value < 0.05).

A nonlinear regression approach, assuming an empirical kinetic model, was applied to simultaneously estimate the model parameters at constant temperature (JMP Pro 13.0.0, SAS Institute Inc.). The decrease of TAG as a function of digestion time (Eq. 1) and the consequent formation of MAG, FFA and GLY in the small intestinal digest as well as in the micelle fraction (Eq. 2) were modelled by a fractional conversion kinetic model. Similarly, the evolution of carotenoid BAc during small intestinal phase digestion was also modelled assuming fractional conversion kinetics (Eq. 2). The goodness of fit between the experimental and predicted data was assessed by visual analysis of the residual plots and based on the adjusted correlation coefficient (R^2_{adj}), considering that the higher the R^2_{adj} , the better the fit.

$$C = C_f + (C_0 - C_f) \cdot e^{(-kt)} \quad (\text{Eq. 1})$$

$$C = C_f \cdot (1 - e^{(-kt)}) \quad (\text{Eq. 2})$$

The initial concentration of MAG or FFA in the digest and micelle fraction, as well as the carotenoid BAc at the start of the small intestinal phase was assumed to be zero. *C* is the parameter value at intestinal digestion time *t* (min); *C_f* is the final value of the parameter when the constant or plateau phase is reached that represents final concentration in the case of MAGs or FFAs (mg/mg TAG₀) and percentage (%) in the case of undigested TAG and carotenoid BAc; *C₀* is the initial parameter value at time 0 (start of intestinal phase); and *k* (min⁻¹) is the reaction rate constant. To

determine significant differences, the jointly estimated parameters C_f and k were evaluated based on their joint confidence regions (90%) obtained using SAS 9.3 software.

3. Results and discussion

3.1 Serum composition and pectin molecular characteristics

To obtain the water-soluble biopolymers, the serum phase of thermally and mechanically-treated broccoli or carrot purée was isolated, dialyzed and then lyophilized. Both vegetable (lyophilized) sera contained substantial quantities of pectin and protein, as shown in Table 1. Broccoli serum contained higher protein content, being 27%, while carrot serum had only 8.9% protein. A fraction of the detected proteins can be free proteins, particularly for broccoli serum as broccoli florets inherently contain high amount of water-soluble proteins (Masih et al., 2002), while a part of these proteins might be associated to pectin. In this context, pectin was reported to be covalently-linked with (arabinogalactan) protein in carrots (Immerzeel, Eppink, de Vries, Schols, & Voragen, 2006), while pectin-protein complexes were also reported in extracted sugar beet and tomato pectins (Kirby, Macdougall, & Morris, 2008; Kirby, MacDougall, & Morris, 2006). Nevertheless, until now, the association and linkage between pectin and protein is yet to be elucidated.

Furthermore, both vegetable sera predominantly contained pectin ($\geq 49\%$). In this sense, the molecular structures of serum pectin were characterized in terms of DM (%), DAc (%), sugar composition and molecular weight (M_w). Although the emulsifying activity of pectin is often ascribed to the protein moieties, the complex molecular structure of pectin might contribute to oil emulsification and stabilization given that sufficient hydrophobic (e.g. methyl and acetyl groups) and hydrophilic (e.g. neutral sugar side chains) residues are present. Broccoli serum was characterized by an intermediate DM (52.7%) and more acetylated (19.1%) pectin, while carrot serum contained a high DM (66%) and was less acetylated (11%) pectin. Both methyl and acetyl groups have hydrophobic characters (Ngouémazong et al., 2015), and hence, pectin with high DM and DAc can be expected to have a

higher emulsification capacity. Besides the hydrophobic groups, the capacity of forming small oil droplets and preventing their destabilization can be dependent on the linear homogalacturonan and branched rhamnogalacturonan I subdomains of pectin (Ngouémazong et al., 2015). In this regard, the molar ratios representing the linearity and the extent of RGI branching of pectin were calculated based on the sugar composition (Houben, Jolie, Fraeye, Van Loey, & Hendrickx, 2011; Kaya, Sousa, Crepeau, Sorensen, & Ralet, 2014). Both vegetable sera were characterized by less linear and more branched pectin populations, which is in agreement with the results on the water-soluble pectin fractions of carrot root and broccoli florets by Houben et al. (2011).

In terms of M_w , remarkable differences on the M_w of broccoli and carrot serum pectins were observed for both low and high M_w pectin populations. Higher average M_w was observed for carrot serum pectin (population I = 6220 kDa; population II = 527 kDa) compared to broccoli serum pectin (population I = 338 kDa, population II = 84 kDa), which can be related to the number of sugar units in the molecular structure as well as to the hydrodynamic volume of the polymers in solution (Vilaplana & Gilbert, 2010). In this regard, carrot serum pectin exhibited a more linear structure which possibly led to its higher hydrodynamic volume, while broccoli serum pectin was less linear and more branched probably resulting in a more coiled structure in solution. In fact, the vegetable sera (1% w/w) imparted different apparent viscosities at the same shear rate ($5 \cdot s^{-1}$) when dissolved in acidified MilliQ water at pH 3.5 (results not shown). As expected, carrot serum exhibited higher apparent viscosity (5.50 mPa·s) compared to broccoli serum (1.85 mPa·s), given its remarkably high M_w pectin. In general, these molecular properties and the presence of different amounts of protein might present differences on the emulsifying and/or stabilizing capacity of carrot and broccoli sera under simulated gastrointestinal conditions.

3.2 Physicochemical properties of o/w emulsions during *in vitro* digestion

Physicochemical properties, including particle size, microstructure and ζ -potential, were measured after 2 h incubation in the simulated gastric and small intestinal phases, respectively. The stability of o/w emulsions, containing water-soluble (serum) biopolymers either as emulsifiers or as constituents of the aqueous phase, under simulated gastrointestinal conditions will be discussed in this section.

3.2.1 Particle size and particle size distribution

To monitor the changes in the emulsion particle size and assess their stability during *in vitro* digestion, the volume weighted mean particle size (d_{43}) and particle size distribution of initial emulsions as well as the emulsions after the gastric and small intestinal phase were examined, as presented in Figure 1. The initial emulsions presented significantly different d_{43} values, in which the one formulated with Tween 80 (T80) showed the smallest particle size, being 0.90 μm (Figure 1A). The initial emulsions formulated with broccoli serum biopolymers (BS) or carrot serum biopolymers (CS) presented the same d_{43} values, being 1.19 μm and 1.23 μm , respectively. This relatively small particle size suggests the capacity of serum biopolymers in forming fine o/w emulsions, although the d_{43} values of emulsions formulated with BS or CS were significantly larger than T80. The emulsion forming and stabilizing capacity of BS and CS under the given quiescent condition (1% sera, pH 3.5, storage for 24 h at 4 °C) can be explained by the presence of pectin and protein, which are considered surface active molecules (Dickinson, 2017). Since pectin can achieve a compact molecular conformation below or around the pKa of its GalA residues thereby allowing the hydrophobic groups to get close to the oil interface (Alba & Kontogiorgos, 2017), the hydrophobic molecules associated to pectin (e.g. methyl, acetyl, protein) were probably able to adsorb at the droplet surface especially in carrot serum, while hydrophilic molecules such as the neutral sugar side chains possibly provided steric stabilization (Ngouémazong et al., 2015).

However, after the gastric phase, the particle size of BS and CS emulsions significantly increased which is clearly complemented with the shift of the particle size distribution to the right, towards 10 μm particle size (Figure 1C, D). The d_{43} value of BS emulsion, being 5.2 μm , was significantly larger than 3.4 μm of CS emulsion (Figure 1A). This indicates that BS-stabilized emulsions were more unstable after the gastric phase than the emulsion formulated with CS. Nevertheless, the increased and widened particle size distribution for both sera-stabilized emulsions implies that the serum biopolymers were less effective in preventing the destabilization under simulated gastric conditions compared to T80, which could be ascribed to different factors (*cf.* Supplementary data, Figure 1). For the gastric phase, gastrointestinal components (e.g. simulated gastric fluid, pepsin solution) were added to the initial emulsions leading to its dilution followed with mechanical stirring of the mixture by end-over-end rotation. Under these conditions, desorption of weakly adsorbed molecules from the interface and/or breakage of the interfacial layer consequently leading to the exposure of the oil surface (McClements, 2004) possibly occurred which further contributed to droplet coalescence as evidenced by the increased particle size distribution of the modified gastric phase (GP4) exclusively formulated with the emulsion and milliQ-water at pH 3.0 (Supplementary data, Figure 1). This implies that the addition of MilliQ water to the emulsion and the submission of this mixture to an end-over-end rotation were sufficient to shift the particle size distribution from 10 μm towards the 100 μm particle size. Moreover, especially for BS-stabilized emulsion, the action of pepsin on the proteins also contributed to the destabilization of the emulsion based on the shift of the particle size distribution of the emulsion formulated with pepsin and milliQ water (pH 3.0) towards the 100 μm particle size (modified GP3 in Figure 1, Supplementary data). Enzymatic degradation of free proteins that adsorbed at the droplet surface could lead to the exposure of the oil surface, while the degradation of protein-associated to pectin could result in partial desorption of the adsorbed pectin, given that these proteins were accessible to proteolytic enzymes (Singh, Ye &

Horne, 2009). Since CS contained significantly lower amount of protein compared to BS, the stability of the emulsions formulated with CS could be less dependent on the presence of proteins than the BS-stabilized emulsion. Besides the action of pepsin, the emulsion destabilization after the gastric phase can be ascribed to bridging interactions and/or screening of electric charges by the ions in the simulated gastric fluid as droplet flocculation was observed in both BS- and CS-stabilized emulsions (*cf.* Figure 2E, H). Since droplet bridging flocculation could lead to coalescence in the absence of an adequately thick and strong steric barrier, an increase in the particle size distribution of the emulsion formulated with SGF as well as CaCl_2 was observed (modified GP 1 & 2 in Figure 1, Supplementary data). The lower extent of coalescence observed in CS-stabilized emulsion compared to the BS-stabilized emulsion might be due to the lower capacity of CS pectin to interact with divalent ions, being a high DM pectin (~66%), and it possibly formed thicker interfacial layers. Given the sensitivity of polyanionic pectin to cations, desorption of pectin from the droplet surface was possibly induced with the presence of divalent ions (e.g. Ca^{2+}) in the simulated gastric fluid. Pectin-stabilized emulsions can be dependent on the ionic strength and pH of the emulsion continuous phase (Alba & Kontogiorgos, 2017). By contrast, the initial particle size of T80-stabilized emulsions including T80+BS and T80+CS was maintained after the gastric phase indicating its high stability under acidic condition, which is in agreement with the results of Salvia-Trujillo et al. (2017).

Interestingly, at the end of the small intestinal phase, no significant differences (p -value < 0.05) were observed on the d_{43} values of the emulsions formulated with T80 without and with BS (T80+BS) or CS (T80+CS) in the aqueous phase of the emulsion, suggesting that the serum polymers present in the aqueous phase had no influence on the particle size. After the small intestinal phase, the d_{43} value of the emulsion formulated with T80 was significantly larger, being 6.5 μm , compared to the initial emulsion and after the gastric phase. Similarly, the d_{43} values of T80+BS and T80+CS emulsions presented a significant increase after the small intestinal phase, being 5.5 μm and 4.5 μm , respectively.

Such emulsions also exhibited multimodal particle size distribution profiles ranging from 0.05 μm to 100 μm with the highest peak at around 0.28 μm (Figure 1B, E, F), which can be explained by the presence of submicron colloidal structures (e.g. mixed micelles, vesicles) and the coalescence of undigested oil (Salvia-Trujillo et al., 2017). The particle size of colloidal structures generated during lipid digestion can be between 0.003 μm to 0.01 μm for mixed micelles (Armand et al., 1999; Christensen, Schultz, Mollgaard, Kristensen, & Mullertz, 2004; Kossena, Boyd, Porter, & Charman, 2002), while larger particle size (>0.01 μm to 1 μm) in the micellar fraction has been found and attributed to the formation of vesicles (Ahmed, Li, McClements, & Xiao, 2012; Kossena, et al., 2002). In this context, the predominant peak from 0.05 μm to 0.86 μm with the maximum intensity at around 0.28 μm can possibly be ascribed to the formation of colloidal structures, in which T80 emulsions without and with serum polymers in the aqueous phase (T80+BS and T80+CS) showed comparable amounts of particles at this size. This probably suggests that they had the same extent of lipid digestion and consequently the same size of colloidal structures were formed (Salvia-Trujillo et al., 2017). Moreover, the d_{43} values of the emulsions formulated with BS or CS remained relatively large after the small intestinal phase as emulsion destabilization already occurred during the gastric phase. In particular, BS-stabilized emulsion showed a larger d_{43} value of 8.5 μm after the small intestinal phase, indicating a greater degree of coalescence of undigested oil. In terms of particle size distribution, multimodal particle size distribution profiles ranging from 0.05 μm to 100 μm were observed for both BS and CS emulsions after the small intestinal phase (Figure 1C, D). Specifically, CS emulsion exhibited a higher peak at particle size of around 0.28 μm compared to BS emulsion, which possibly indicates that more colloidal (e.g. mixtures of mixed micelle and vesicles) structures were formed for CS emulsion. Nevertheless, the particle size distribution of both BS and CS emulsions clearly showed lower peaks at particle size of around 0.28 μm compared to T80-stabilized emulsions (T80, T80+BS

and T80+CS). This might indicate that T80-stabilized emulsions were highly digestible and consequently formed more submicron colloidal structures.

3.2.2 Microstructure

Similar to observations for particle size and particle size distribution, differences in microstructure of initial emulsions and changes during *in vitro* digestion were detected, as presented in Figure 2. Emulsions formulated with T80, BS or CS as the emulsifier initially showed small and less visible oil droplets, which confirms the results on particle size distribution (Figure 2A, D, G). By contrast, the microstructure of emulsions formulated with T80 containing BS or CS in the aqueous phase were characterized by highly dense and aggregated droplets most likely due to depletion flocculation mechanism (Figure 2J, M). The high concentration of serum biopolymers in the aqueous phase of the emulsion caused an osmotic pressure difference that subsequently led to particle aggregation, which is in agreement with previous findings (Beysseriat, Decker, & McClements, 2006; Espinal-Ruiz, Restrepo-Sánchez, Narváez-Cuenca, & McClements, 2016). For T80+BS and T80+CS initial emulsions, the presence of flocculated oil droplets was not detected during the particle size measurement due to the dilution and mechanical stirring of the samples that resulted in the disintegration of aggregated droplets and release of individual small droplets. This confirms the relevance of examining the microstructure of emulsions together with their particle size distribution obtained by static light scattering measurements (McClements, 2007).

Moreover, after the gastric phase, the depletion flocculation observed for T80+BS and T80+CS emulsions largely diminished, which suggests that the droplet interaction and the flocs formed were weak as the dilution and stirring during the gastric phase resulted in floc breakdown (Figure 2K, N). For BS and CS emulsions, their microstructures presented large oil droplets that indicated the prevalence of droplet coalescence after the gastric phase (Figure 2E, H). This result supports the observed particle size increase, as previously discussed in Section 3.2.1. The relative changes in the

microstructure and overall stability of the emulsions during gastric phase are subject to the gastric environment itself having a high electrolyte, acidic pH and mechanical shear (Golding & Wooster, 2010).

After the small intestinal phase, depending on the emulsion type, large oil droplets were still observed which can be ascribed to the coalescence of undigested oil droplets. In particular, the microstructure of the emulsion stabilized by BS showed several large droplets of undigested oil (Figure 2F), while relatively smaller and less undigested oil were observed for CS emulsion (Figure 2I). By contrast, T80 emulsions without or with serum biopolymers in the aqueous phase exhibited very few undigested oil (Figure 2C, L, O). This is in agreement with the result of Salvia-Trujillo et al. (2017), in which few large droplets remained after small intestinal *in vitro* digestion that corresponded to less undigested oil.

3.2.3 Particle charge

The ζ -potential represents the electrical characteristics of emulsion droplets at the oil-water interface and it inherently accounts for the adsorption of any charged counter ions at the oil droplet surface (McClements, 2007). In the present study, changes in the electrical characteristics of the emulsions were observed after subjecting them to different stages of *in vitro* digestion, as shown in Figure 3. Emulsions were characterized by negatively charged droplets that differed depending on the emulsion composition. The emulsion formulated with Tween 80 as the emulsifier and without serum polymers in the continuous phase had the least negative particle charge (-15 mV). This is in agreement with several studies that emulsions formulated with non-ionic surfactant (Tween 80) unexpectedly resulted in negatively charged particles under certain pH conditions (Beysseriat et al., 2006; Espinal-Ruiz et al., 2016; Salvia-Trujillo et al., 2017; Zhang, Zhang, Zhang, Decker, & McClements, 2015). This can probably be attributed to the presence of anionic impurities from the oil (e.g. free fatty acids) or surfactant (e.g. free oleic fatty acid), the possible ionization of the hydroxyl groups of Tween 80 and

the preferential adsorption of its hydroxyl ions (OH^-) from the aqueous phase to the oil droplet surface (Espinal-Ruiz et al., 2016; Nikiforidis & Kiosseoglou, 2011; Zhang et al., 2015). Furthermore, differences on the initial particle charge of emulsions formulated with BS and CS were observed. CS emulsion presented a significantly lower ζ -potential value (-22 mV) compared to BS emulsion (-19 mV), which can be due to the higher concentration of polyanionic pectin in carrot serum. For the same reason, the emulsion containing CS in the aqueous phase of the emulsion (T80+CS) exhibited a significant more negative ζ -potential value (-35 mV) than the emulsion containing BS in the aqueous phase (T80+BS).

However, after the gastric phase, the ζ -potential values of the emulsions significantly increased. For all the emulsions studied, a substantial decrease in the magnitude of the negative charge resulted in ζ -potential values between -4 mV to -6 mV. Such increased ζ -potential values can be mainly attributed to the presence of electrolytes at low pH in the simulated gastric fluid (SGF) as evidenced by the comparable ζ -potential values of modified gastric phase formulations containing SGF (Supplementary data, Figure 2). In fact, the ζ -potential increase during gastric phase is widely observed, which is frequently related to the high ionic strength and low pH of simulated gastric fluid that possibly induced electrostatic screening effects (Beysseriat, Decker, & McClements, 2006; Zhang et al., 2015; Espinal-Ruiz et al., 2016; Salvia-Trujillo et al., 2017).

After the small intestinal phase, for all the emulsions, the ζ -potential values became more negative compared to the initial ζ -potential values, which can be due to various negatively charged species such as free fatty acids released during lipolysis, complex colloidal structures, undigested oil and anionic polymers (Espinal-Ruiz et al., 2016; Zhang et al., 2015). No significant differences were observed on the ζ -potential values of emulsions formulated with Tween 80 without (T80) and with serum polymers in the aqueous phase (T80+BS and T80+CS) after the *in vitro* small intestinal phase. This might suggest that such emulsions resulted in the same net charge possibly due to the same extent

of lipolysis, and hence, lipid digestion products and undigested oil. These emulsions (T80, T80+BS and T80+CS) also showed similar particle size distribution profiles after the small intestinal phase (*cf.* section 3.2.1), suggesting that the extent of their digestibility and droplet changes could be comparable. Furthermore, for emulsions stabilized by BS or CS, no significant differences were observed on their ζ -potential values. However, such emulsions exhibited significantly lower ζ -potential values, being -39.7 mV, compared to T80 emulsions with -30.8 mV. The lower ζ -potential values observed for BS and CS emulsions could indicate that more pectin or lipid digestion products (e.g. FFA) were present at the interface of undigested oil droplets compared to that of the continuous phase of the emulsion, as the ζ -potential represents the potential difference between the surface of the particles and the bulk of the dispersing liquid. The presence of pectin as well as other anionic species and their localization in the emulsion can influence the overall electrical charge of the particles (Espinal-Ruiz et al., 2016). Therefore, ζ -potential values cannot be univocally explained by higher concentrations of lipid digestion products in the digest, when pectin is present or other charged species.

3.3 Lipid digestibility and mixed micelle formation

3.3.1 Triacylglycerol hydrolysis and the concentration of its products in the digest

TAG digestion by lipases can simultaneously yield DAGs, MAGs, FFAs and GLYs (Mu, 2004). In the present study, very low concentrations of DAGs were detected. Therefore, during the *in vitro* small intestinal phase, the lipid digestibility of emulsions formulated with T80, BS or CS as the emulsifier and the T80 emulsions containing BS or CS in the aqueous phase was determined based on the amount of residual (undigested) TAG and the concentrations of MAGs, FFAs and GLYs in the digest at different time points. To predict the rate and extent of lipolysis, the experimental (MAG and FFA) and calculated (TAG and GLY) values obtained were fitted using a fractional conversion model.

For all the emulsions studied, the amount of TAGs rapidly decreased while the concentrations of FFAs and MAGs in the digest consequently increased in the first 30 min until a constant (plateau)

value was reached (Figure 4). Significant differences on the rate and extent of TAG hydrolysis as well as the release of lipid digestion products were observed, depending on the emulsion composition (Supplementary material, Figure 3). In general, less than 10% of undigested TAGs were detected from 60 min to 120 min of small intestinal digestion, indicating a high extent of TAG hydrolysis except for the emulsion formulated with BS as the emulsifier (Figure 4). The digestibility of BS emulsion presented a significantly lower reaction rate constant (k -value) and hence a higher predicted final value of undigested TAG (26.6%) compared to CS (9.2%) and T80 (0.9%) emulsions (Supplementary material, Table 1 & Figure 3). This indicates a slow rate and low extent of BS emulsion (TAG) digestibility which can be explained by the higher extent of destabilization of the BS-stabilized emulsion compared to CS emulsion during the gastric phase that promoted larger droplets. In turn, with the increased droplet size of BS emulsion, the surface area available for lipase activity was limited that possibly led to the delay and incomplete TAG digestion. By contrast, the emulsions formulated with T80 or CS as the emulsifier had a faster and higher TAG digestibility given their relatively smaller particle size than the BS emulsion during the gastric phase. For the emulsions containing BS or CS as components in the aqueous phase, although they differed on the rate that TAG digestion reached a stationary phase, the final undigested TAG value was 5.1% for T80+BS and 8.7% for T80+CS that suggested their comparable high extent of digestibility (Figure 4D, E & Supplementary material, Figure 3). This high digestibility evidences that the presence of serum polymers in the aqueous phase of T80 emulsion had no influence on the extent of TAG digestion, as the depletion flocculation caused by the polymers was significantly diminished during the gastric phase and the small particle size remained. In the current study, TAG digestibility as influenced by the droplet size during the gastric phase was observed especially by comparing the T80+BS and BS emulsions, which is in agreement with the result of Salvia-Trujillo et al. (2017).

Moreover, for both MAG and FFA concentrations in the digest, significant differences were detected among the emulsions studied (Supplementary material, Figure 3B, C). On the one hand, BS emulsion presented a significantly low extent of both MAG and FFA concentrations in the digest which can be related to the incomplete TAG digestion, as previously discussed. On the other hand, emulsions that showed high digestibility also corresponded to high MAG and FFA concentrations in the digest. For instance, T80 or CS emulsion resulted in a high final MAG and FFA concentrations in the digest as they exhibited a high extent of TAG digestibility. In general, the relation of TAG hydrolysis and the concomitant release of MAGs and FFAs as well the dependence of these sequential reactions on emulsion stability was observed. In the case of emulsions formulated with T80, BS or CS as the emulsifier, lipid digestion was shown to be dependent on the capacity of the molecule to protect and prevent droplet changes thereby maintaining small droplet size that can be easily hydrolyzed by lipases during the small intestinal phase. In this sense, carrot and broccoli serum polymers presented different capacities in preventing emulsion destabilization during the gastric phase which might offer different possibilities in modulating lipolysis. Interestingly, when used as a component in the aqueous phase of the emulsion, BS or CS did not hamper the digestibility of the emulsion, suggesting that the presence of serum polymers in the aqueous phase of the emulsion allowed the lipases to access the droplet interface.

3.3.2 *Monoacylglycerol and free fatty acid concentrations in the micellar phase*

To gain an insight whether the use of serum polymers as emulsifier or as constituents in the aqueous phase of the emulsion could influence the formation of mixed micelles, FFA and MAG concentrations in the micellar phase were monitored during the small intestinal phase as a function of digestion time. In addition, the relation between lipid digestion and mixed micelle formation in the studied emulsions were assessed. The experimental values of MAG and FFA concentrations in the micellar phase was fitted by a fractional conversion kinetic model to predict the final extent and rate of

micelle formation (Figure 5 & Supplementary material, Table 2). Differences on the extent and rate of MAG and FFA concentrations in the micellar phase were observed among emulsions, which can be related to the extent of TAG digestibility and subsequent release of lipid digestion products. In this regard, BS emulsion presented a significantly lower extent and rate of MAG and FFA concentrations in the micellar phase than T80 or CS emulsion, which can be explained by its slow digestion that controls the amounts of MAGs and FFAs available to form micelles (Mutsokoti et al., 2017). By contrast, both the final concentrations of MAGs and FFAs in the micellar phase of T80 and CS emulsions did not show significant differences, as they had comparable extent of lipolysis. Similarly, such emulsions did not exhibit significant differences on the rate of transfer of both MAGs and FFAs into the mixed micelles, as depicted by their overlapped joint confidence regions (Supplementary material, Figure 4A, B).

Furthermore, the extent of FFA concentration in the micellar phase were comparable for emulsions stabilized by Tween 80 without (T80) and with serum polymers in the continuous phase (T80+BS and T80+CS). However, T80+BS and T80+CS emulsions presented significantly lower rate of FFA incorporation into mixed micelles than T80 (Supplementary material, Figure 4B). This indicates that the presence of either CS or BS in the aqueous phase slowed down the transfer of FFAs into the micelles, possibly due to slower diffusion as the polymers could act as physical barriers. Regardless of the small intestinal digestion time, the FFAs incorporated into the micelle fraction (relative to the amount of FFA in the digest) was constant with an average of 88% for the emulsions stabilized by T80 with and without serum polymers in the continuous phase as well as for CS (data not shown). This percentage of FFA incorporation into mixed micelles is in agreement with the result of Salvia-Trujillo et al. (2017). However, for BS emulsion, FFA incorporation into the micelle fraction was only about 58% (data not shown), suggesting that 42% of the released FFAs in the digest did not contribute to the micelle formation and possibly complexed with oppositely charged molecules. For BS

emulsion, less micelle formation could have occurred during the small intestinal phase, which can be linked to the previously observed low percentage of particles in the submicron size range (0.05 μm to 0.86 μm) that might correspond to the size of mixed micelles (Espinal-Ruiz et al., 2014a)

3.3.3 Carotenoid bioaccessibility

The incorporation of carotenoids into bile salt-lipid micelles (carotenoid bioaccessibility) is a prerequisite step for carotenoid absorption and utilization in the human body (Faulks & Southon, 2005). In the current work, carotenoid bioaccessibility (%) was monitored during the *in vitro* small intestinal phase at different digestion time point and defined as the carotenoid concentration in the micelle fraction relative to the carotenoid concentration of the initial emulsions. For all the emulsions studied, carotenoid BAc values slowly increased until it reached a plateau starting from 45 min to 120 min, which is in agreement with the results of Salvia-Trujillo et al. (2017) and Mutsokoti et al. (2017).

The bioaccessibility values of both α - and β -carotene generally showed the same behavior and trends for each emulsion (Figure 6). Significant differences on the predicted final carotenoid bioaccessibility values were observed, depending on the emulsion composition (Supplementary material, Figure 5). The emulsions formulated with T80, BS or CS presented significantly different carotenoid bioaccessibility values, in which BS emulsion had the lowest extent (32.7%) and rate (0.085 min^{-1}) of carotenoid incorporation into the mixed micelles. For BS emulsion, the low carotenoid BAc can be explained by the poor digestibility of coalesced oil droplets and in turn less micelle formation, as previously discussed. Moreover, the carotenoid BAc values of emulsions stabilized by Tween 80 without (T80) and with broccoli serum polymers in the continuous phase (T80+BS) did not show significant differences, as depicted by their overlapped joint confidence regions (Supplementary material, Figure 5). For such emulsions, the highest carotenoid BAc value (~74%) was observed that can be attributed to their high digestibility and mixed micelle formation, which is in agreement with the result of Salvia-Trujillo et al. (2017). However, in the current study, despite the good digestibility and

high mixed micelle formation of the emulsions containing carrot serum as the emulsifier (CS) or as component in the continuous phase (T80+CS), significantly lower extent of carotenoid BAc was observed for such emulsions (~46%) compared to T80 and T80+BS emulsions (Supplementary material, Figure 5). In particular, regardless of the high micelle formation, the presence of carrot serum in the aqueous phase of T80 emulsion (T80+CS) significantly hindered carotenoid incorporation in the micelles, which was not observed with the presence of broccoli serum in the aqueous phase of T80 emulsion (T80+BS). Similarly, a direct relationship between high micelle formation and carotenoid bioaccessibility was not observed for CS emulsion, although it showed significantly higher carotenoid bioaccessibility compared to BS emulsion. Therefore, it can be inferred that carrot serum limited the extent of carotenoid incorporation into mixed micelles, which can possibly be related to its pectin molecular characteristics. Due to the high M_w carrot serum pectin, which was presumably a much larger polymer than broccoli serum pectin, it can be hypothesized that non-adsorbed carrot serum pectin acted as a barrier and entrapped the carotenoids.

Conclusion

Different stabilities of emulsions formulated with water-soluble (serum) biopolymers from vegetable origin and their lipid digestibility under simulated gastrointestinal conditions were shown. Serum biopolymers, consisting of naturally solubilized pectin and protein from processed carrot or broccoli purée, were either used as emulsifier or component in the continuous phase of the emulsion. Serum biopolymers, used as emulsifiers, initially formed small oil droplets (1.19 to 1.23 μm) and they were unable to resist destabilization during *in vitro* gastric phase that subsequently determined the rate and extent of lipolysis, micelle formation and carotenoid bioaccessibility. Depending on serum composition, coalescence during the gastric phase led to larger droplets in emulsion formulated with broccoli serum with higher protein content and lower M_w pectin compared to carrot serum. The presence of free proteins and/or proteins complexed with pectin and their molecular characteristics

especially in broccoli serum probably influenced the mechanism of emulsification and stabilization as well as the digestion kinetics, but it still needs to be investigated. In turn, broccoli serum-emulsion presented slower rate and lower extent of lipolysis, micelle formation and consequently carotenoid bioaccessibility. By contrast, broccoli serum as component in the continuous phase of the emulsion had no effect on lipolysis, micelle formation and carotenoid bioaccessibility, while, carrot serum limited the extent of carotenoid bioaccessibility without affecting the lipolysis and micelle formation. Non-adsorbed carrot serum, presumably characterized by large pectin molecules, possibly entrapped the carotenoids and hence hampered their bioaccessibility. Overall, water-soluble biopolymers from vegetables can be potentially used as natural emulsifiers that might modulate lipolysis and bioaccessibility of lipophilic bioactive compounds.

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Figure captions:

Figure 1. Changes in the volume-weighted mean particle size (A) and particle size distribution of the emulsions formulated with Tween 80 (T80) (B), broccoli serum (BS) (C) or carrot serum (CS) (D) as the emulsifier and the emulsions stabilized by T80 containing BS (E) or CS (F) in the aqueous phase after the gastric and small intestinal *in vitro* digestions. Different upper case letters indicate significant differences (p -value < 0.05) between different phases of *in vitro* digestion of the same sample. Different lower case letters indicate significant differences (p -value < 0.05) between different samples and similar phase of *in vitro* digestion.

Figure 2. Changes in the microstructure of the emulsions formulated with Tween 80 (T80), broccoli serum (BS) or carrot serum (CS) as the emulsifier and the emulsions stabilized by T80 containing BS or CS in the aqueous phase after the gastric and small intestinal *in vitro* digestions. Scale bars = 100 μ m

Figure 3. Changes in the zeta potential of the emulsions formulated with Tween 80 (T80), broccoli serum (BS) or carrot serum (CS) as the emulsifier and the emulsions stabilized by T80 containing BS or CS in the aqueous phase after the gastric and small intestinal *in vitro* digestions. Different upper case letters indicate significant differences (p -value < 0.05) between similar samples and different phases of *in vitro* digestion. Different lower case letters indicate significant differences (p -value < 0.05) between different samples and similar phase of *in vitro* digestion.

Figure 4. Amount (%) of undigested triacylglycerol (TAG) and the concentrations (mg/mg TAG initial) of monoacylglycerol (MAG), free fatty acids (FFA) and glycerol (GLY) during *in vitro* small intestinal digestion at different time (min) points of the emulsions formulated with Tween 80 (T80) (A), broccoli serum (BS) (B), carrot serum (CS) (C), T80+BS (D) or T80+CS (E). MAG and FFA values during digestion were experimental results, while TAG and GLY were calculated based on

stoichiometric reactions and mass balance. Solid, square dot, long dash and round dot lines are the predicted curves by a fractional conversion model for TAGs, MAGs, FFAs or GLY, respectively.

Figure 5. Concentration (mg/mg initial TAG) of monoacylglycerols (MAGs) (A) and free fatty acids (FFA) (B) in the micelle fraction during *in vitro* small intestinal digestion time (min) of the emulsions formulated with broccoli serum (BS), carrot serum (CS) and Tween 80 with the absence (T80) or presence of serum polymers in the continuous phase (T80+BS or T80+CS). Solid, dash, long dash dot, round dot and long dash lines are the predicted curves by a fractional conversion model for T80, BS, CS, T80+BS or T80+CS, respectively.

Figure 6. Carotenoid bioaccessibility of the emulsions stabilized by broccoli serum (BS), carrot serum (CS) and Tween 80 with the absence (T80) or presence of serum polymers in the continuous phase (T80+BS and T80+CS) as a function of *in vitro* digestion time during the small intestinal phase. Experimental data points are represented by the different data markers at specific time point. The solid, dash, long dash dot, round dot and long dash lines are the predicted curves of (a) α - and (b) β -carotene by a fractional conversion model for T80, BS, CS, T80+BS and T80+CS emulsions.

Table 1. Serum composition and physicochemical properties of pectin (mean values $\pm$ standard deviation, n= 2)

Composition	Broccoli sera (BS)	Carrot sera (CS)
Protein content (% d.w.)	27.0 $\pm$ 0.1	8.9 $\pm$ 0.1
Total pectin content (% d.w.)	48.6 $\pm$ 3.6	61.1 $\pm$ 3.2
Galacturonic acid (% d.w.)	25.8 $\pm$ 1.4	44.2 $\pm$ 1.6
Pectin-related neutral sugars* (% d.w.)	22.8 $\pm$ 2.1	16.9 $\pm$ 1.6
Physicochemical characteristics of pectin		
DM (%)	52.7 $\pm$ 1.6	66.3 $\pm$ 0.6
DAC (%)	19.1 $\pm$ 0.5	11.2 $\pm$ 0.4
Sugar ratio: Linearity (moles GalA/ moles Fuc+Rha+Ara+Gal+Xyl)	0.9 $\pm$ 0.1	2.2 $\pm$ 0.1
Sugar ratio: Branching of RG-I (moles Ara+Gal/ moles Rha)	10.1 $\pm$ 0.1	6.2 $\pm$ 0.5
High molecular weight population (kDa)	338.0 $\pm$ 21.6	6220 $\pm$ 254
Low molecular weight population (kDa)	84.0 $\pm$ 7.9	527 $\pm$ 4.2

*sum of fucose (Fuc), rhamnose (Rha), arabinose (Ara), galactose (Gal), xylose (Xyl)

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Highlights

- Water-soluble (serum) pectin-protein mixtures can stabilize an o/w emulsion
- Emulsion destabilization during gastric phase influenced the digestibility kinetics
- Lipid digestibility kinetics governed the mixed micelle formation
- Serum biopolymers had limited influence on lipolysis and micelle formation kinetics
- Serum pectin molecular properties influenced carotenoid bioaccessibility