Advanced insight into the emulsifying and emulsion stabilizing capacity of carrot pectin subdomains

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Abstract

Insight in the emulsifying and emulsion stabilizing potential of carrot pectin subdomains was established in the present study. For this purpose, (i) carrot pectin fractions enriched in linear homogalacturonan (HG) and branched rhamnogalacturonan (RG) regions were produced, (ii) specific structural and physicochemical properties of the isolated pectin fractions were analyzed and (iii) stability studies of emulsions where the different isolated pectin fractions were used as the sole emulsifier were performed.

Firstly, structural characterization confirmed the successful isolation of the subdomains from the original acid extracted carrot pectin generating three structurally diverse carrot pectin samples: acid extracted pectin (NA), HG rich pectin fraction (NA-HG) and RG rich pectin fraction (NA-RG). The main structural differences among these pectin samples were degree of methylesterification, linearity and molar mass. Analysis of the interfacial tension after pectin adsorption onto an oil droplet, demonstrated surface activity of all pectin samples, except for NA-RG at pH 2.5. Overall, carrot pectin created more stable emulsions at pH 2.5 compared to pH 6.0. Additionally, emulsions stabilized by the more complex NA are less prone to creaming than emulsions stabilized by NA-HG or NA-RG. Flocculation was identified as the main destabilization phenomenon in the emulsions stabilized by NA-HG and NA-RG at pH 6.0. NA stabilized emulsions remained relatively stable during 14 days of refrigerated storage. Therefore, it can be stated that the presence of both linear and branched regions in the pectin structure result in more stable emulsions as compared to the individual subdomains.

Keywords: Carrot pectin, Pectin subdomains, Emulsion, Stability

1 Introduction

Pectin, a group of complex plant cell wall polysaccharides containing galacturonic acid (GalA), can be used as a multifunctional ingredient in different food applications. The use of pectin as a thickening and gelling agent in for example sauces and jams is the best explored functionality of this polymer with applications on household and industrial scale (Willats, Knox, & Mikkelsen, 2006). Pharmaceutical applications include the use as a carrier in colon-specific drug delivery systems (Liu, Fishman, & Hicks, 2007; Wicker et al., 2014) and assisting in cholesterol reduction (Wicker et al., 2014). Recently, scientific interest in the emulsifying and emulsion stabilizing (further referred to as E_FEs) capacity of pectic polysaccharides is growing (Funami et al., 2011, 2007; Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015; Schmidt, Schmidt, Kurz, Endreß, & Schuchmann, 2015; Schmidt, Schütz, & Schuchmann, 2017).

The multifunctional nature of pectin is related to the structural diversity of these cell wall polymers. Structurally, pectin polymers are heteropolysaccharides mainly constituted of three different subdomains: homogalacturonan (HG), rhamnogalacturonan I (RG I) and rhamnogalacturonan II (RG II). The linear HG subdomain consists of α -1,4-linked GalA residues which can be methylesterified at the C6 carboxyl group. Both RG I and RG II are branched regions. RG I is composed of a backbone of repeating [$\rightarrow\alpha$ -D-GalpA-1,2- α -L-Rhap-1,4 $\rightarrow$] disaccharide units substituted at the rhamnose residues with heterogeneous side chains composed of galactose and arabinose residues. RG II has more complex branches consisting of up to 12 different types of glycosyl residues substituted on a backbone of seven to nine GalA residues (Caffall & Mohnen, 2009; Perez, Rodríguez-Carvajal, & Doco, 2003; Voragen, Coenen, Verhoef, & Schols, 2009; Willats et al., 2006). Moreover, structural characteristics of pectin vary between plant sources, physiological states of the plant and different plant tissues. Furthermore, structural properties can be influenced by extraction

conditions and purposively chemically or enzymatically modified (Müller-Maatsch et al., 2016; Ngouémazong et al., 2015; Voragen et al., 2009). Consequently, it is very challenging to elucidate the link between the structure of pectin polymers and its functionality.

Pectin is gaining interest as a potential food emulsifier of natural origin. However, a general and recurring conclusion in this research field is that the structural complexity of pectin polymers makes it extremely challenging to identify unambiguous relations between the structural properties of pectin and its functionality as E_FE_S agent (Alba & Kontogiorgos, 2017; Ngouémazong et al., 2015). Until recently, the potential of pectin to stabilize emulsions was mainly attributed to its ability to increase the viscosity of the aqueous, continuous phase (Ngouémazong et al., 2015). However, recently, increased evidence is being collected, showing the surface activity of pectin from various origins enabling adsorption to the oil-water interface (Akhtar, Dickinson, Mazoyer, & Langendorff, 2002; Funami et al., 2011; Leroux, Langendorff, Schick, Vaishnav, & Mazoyer, 2003; Schmidt, Schmidt, et al., 2015; Siew, Williams, Cui, & Wang, 2008; Verkempinck et al., 2018). Once adsorbed, pectin can also contribute to long term emulsion stability by means of steric and/or electrostatic effects. Adsorbed pectin is able to form thick hydrated layers at the oil-water interface causing steric hindrance increasing emulsion stability against droplet coalescence (Funami et al., 2011, 2007; Ngouémazong et al., 2015). These steric effects are mostly ascribed to the RG-I side chains of the pectin polymer (Funami et al., 2011, 2007). Next to steric effects, oil droplets stabilized by pectin can experience electrostatic interactions as well. Negatively charged GalA residues give pectin its polyanionic character. Adsorption of this negatively charged pectin at the oil-water interface creates a negatively charged layer around the oil droplets inducing electrostatic repulsive forces between emulsified oil droplets which increases emulsion stability against aggregation (Ngouémazong et al., 2015).

As mentioned above, complete elucidation of the structure-function relation of pectin acting as an E_{FEs} agent is very challenging. Structural properties of pectin polymers mentioned in literature to influence the E_{FEs} capacity include pectin related proteinaceous moieties (Akhtar et al., 2002; Funami et al., 2011, 2007; Leroux et al., 2003; Williams et al., 2005), degree of acetylation (Leroux et al., 2003; Schmidt, Koch, et al., 2015), degree of methylesterification (DM) (Schmidt, Koch, et al., 2015; Verkempinck et al., 2018) and molar mass (Akhtar et al., 2002; Funami et al., 2011; Leroux et al., 2003) and ferulic acid content (Chen, Fu, & Luo, 2016).

This study aims to investigate the E_{FEs} capacity of two different pectin subdomains produced from the same pectin source: a more linear, HG rich pectin fraction as well as a branched, RG rich pectin fraction. Isolating these subdomains reduces the complexity of the pectin structure and possibly allows to more clearly elucidate the link between the pectin structure and its E_{FEs} potential. For this research challenge, carrot pectin was selected as its structure-function relation is not yet well known and as it is omnipresent in the industrial waste stream of β -carotene extraction and juice production (Bogacz-Radomska & Harasym, 2018; Jafari, Khodaiyan, Kiani, & Hosseini, 2017). A detailed and extensive research approach was designed integrating structural analysis of pectin at molecular scale, pectin physicochemical properties and an emulsion stability study performed at two different pH values. To the best of our knowledge, this study is the first to elucidate in-depth the relation between different carrot pectin subdomains and its E_{FEs} potential.

2 Materials and Methods

2.1 Materials

A batch of thermally blanched and frozen carrot dices produced at industrial scale was used as starting material for pectin extraction and was kindly donated by D'Arta Nv (Ardoorie, Belgium). Commercial sunflower oil was purchased at a local supermarket. Ultrapure water (organic free, 18.2MΩ cm resistance) was supplied by a Simplicity™ 150 water purification system (Millipore, Billerica, USA). All chemicals used were of analytical grade.

2.2 Preparation of pectin samples/structures

2.2.1 Production of Alcohol Insoluble Residue

Frozen carrot dices were grounded using a knife mill (Grindomix GM 200, Retch, Germany). Immediately hereafter, the milled carrot material was refrozen using liquid nitrogen and lyophilized (Christ Alpha 2–4 LSC, Germany). Cell wall material was isolated from the lyophilized carrot material as an alcohol insoluble residue (AIR) according to the method described by McFeeters & Armstrong (1984) with minor changes. Briefly, 15 g of lyophilized carrot material was moistened with 22.5 ml demineralized water, suspended in 192 ml 95% ethanol using a high speed mixer (3 times for 6 s) (Buchi mixer B-400, Flawil, Switzerland) and filtered over a paper filter (Macherey-Nagel MN 615 Ø 90 mm). Thereafter, the first residue was resuspended in 96 ml 95% ethanol, mixed and filtered. This resuspension step was instantly repeated a second time. Subsequently, the obtained third residue was resuspended in 96 ml acetone and filtered. This resuspension step was instantly repeated as well. The resulting residue is the Alcohol Insoluble Residue (AIR). The AIR was dried overnight at 40 °C, grounded with mortar and pestle and stored in a desiccator until use.

2.2.2 Nitric acid pectin extraction

Pectin was extracted from the AIR using a nitric acid extraction following the procedure described by Kaya, Sousa, Crépeau, Sørensen, & Ralet (2014). In particular, 2.5 g AIR was suspended in 200 ml demineralized water and incubated at 80 °C for 30 minutes while stirring to enhance suspension formation. After cooling to room temperature, the pH of the suspension was adjusted to 1.6 using 7 M HNO₃. The suspension was incubated at 80 °C for 30 minutes, cooled to room temperature and vacuum filtered over a filter paper (Macherey-Nagel MN 615 Ø 90 mm). The pH of the filtrate was adjusted to 6.1 using 1 M NaOH. The resulting pectin rich solution was extensively dialysed against demineralized water for 48h (Spectra/Por® Dialysis Membrane, 3.5 kDa MWCO), lyophilized (Alpha 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and stored in a desiccator until use. This sample is further referred to as the nitric acid extracted pectin (NA).

2.2.3 Production and isolation of a homogalacturonan rich fraction

In order to obtain a homogalacturonan (HG) rich fraction, the NA sample was first subjected to chemical demethylesterification followed by acid hydrolysis according to the work of Santiago, Kyomugasho, et al. (2018). First, 200 mg of NA sample was solubilized overnight at 4 °C in 60 ml demineralized water. The pH of the cooled pectin solution was slowly adjusted to a pH of 12.0 with cold 1 M NaOH while placed in an ice bath. The pectin solution was stirred for 6 hours in order to demethylesterify pectin, which was intended to increase the resistance of the linkages between GalA residues against acid hydrolysis under the given conditions (Thibault, Renard, Axelos, Roger, & Crépeau, 1993). After the chemical demethylesterification, the pH was reduced to 1.0 with 1 M HCl to start acid hydrolysis. For this, the pectin solution was incubated at 80 °C for 24 h. Consequently, the pectin solution was cooled to room temperature and ethanol was added to a final concentration of 80% (v/v). The suspension was subsequently filtered using a glass unit filter (Durapore® membrane

filters, 0.1 μm pore size, Merck Millipore Ltd., Ireland). The residue was solubilized in 40 ml demineralized water and 12 ml 0.1 M HCl was added whereafter the pH of the HG rich pectin solution was adjusted to 6.1 using 0.5 M LiOH. Finally, the pectin solution was extensively dialysed against demineralized water for 48h (Spectra/Por[®] Dialysis Membrane, 3.5 kDa MWCO), lyophilized (Alpha 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and stored in a desiccator until use. This HG rich pectin fraction is further referred to as NA-HG.

2.2.4 Production and isolation of a rhamnogalacturonan rich fraction

In order to obtain a rhamnogalacturonan (RG) rich fraction, the NA sample was submitted to a β -elimination reaction following the procedure of Santiago, Kyomugasho, et al. (2018). 150 mg of NA sample was solubilized overnight at 4 °C in 50 ml demineralized water. Subsequently, the solution was heated to 90 °C for 30 minutes, whereafter the pH was adjusted to 12 using warm 1 M NaOH and keeping the temperature of the total solution above 60 °C. Next, this solution was incubated for 2 hours at 90 °C under constant stirring. Immediately hereafter, the solution was cooled to room temperature and ethanol was added to a final concentration of 80% (v/v). The suspension was subsequently filtered using a glass unit filter (Porafil[®] membrane filters, 0.45 μm pore size, Macherey-Nagel, Germany) and the residue was solubilized in 30 ml demineralized water. The pH of the RG rich pectin solution was adjusted to 6.1 using 0.1 M HCl. Lastly, the pectin solution was extensively dialysed against demineralized water for 48h (Spectra/Por[®] Dialysis Membrane, 3.5 kDa MWCO), lyophilized (Alpha 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and stored in a desiccator until use. This RG rich pectin fraction is further referred to as NA-RG.

2.3 Structural characterization of the pectin samples

Three different pectin samples (NA, NA-HG and NA-RG) were structurally characterized quantifying the degree of methylesterification (DM), galacturonic acid (GalA) content, neutral monosaccharide profile and content, molar mass (MM) distribution and protein concentration.

2.3.1 Degree of methylesterification

The DM was determined exactly following the procedure described by Kyomugasho, Christiaens, Shpigelman, Van Loey, & Hendrickx (2015) using Fourier transform infra-red (FT-IR) spectroscopy (ATR-FTIR, Shimadzu FTIR-8400S, Japan). The calibration curve as described by Kyomugasho et al. (2015) was used to calculate the DM of the three pectin samples.

2.3.2 Galacturonic acid content

The GalA content of acid hydrolyzed pectin samples (Ahmed & Labavitch, 1978) was measured using the spectrophotometric method of Blumenkrantz & Asboe-Hansen (1973). The analysis was performed in triplicate.

2.3.3 Monosaccharide composition

The monosaccharide profile of each pectin sample was determined using high performance anion exchange chromatography (HPAEC) combined with pulsed amperometric detection (PAD). First, 5 mg of lyophilized sample was hydrolyzed in 4 M trifluoroacetic acid (TFA) at 110 °C for 1.5 h (De Roeck, Sila, Duvetter, Van Loey, & Hendrickx, 2008). After cooling the samples to room temperature, they were dried under nitrogen at 45 °C, washed with 1 M ammonium hydroxide and dried again under nitrogen at 45 °C to remove and neutralize TFA (Stolle-smits, Beekhuizen, Recourt, Voragen, & Van Dijk, 1997). The dried samples were

dissolved in ultrapure water and filtered before injection (Chromafil® A-45/25, 0.45 µm pore size, Macherey-Nagel, Duren, Germany). The hydrolysis was performed in triplicate.

The monosaccharide composition of the hydrolysates was quantified by HPAEC-PAD, similarly to the method described by Houben, Jolie, Fraeye, Van Loey, & Hendrickx (2011). A Dionex system (ICS-6000) equipped with a Dionex ICS-6000 Single Pump, a CarboPac™ PA20 guard column (30×3 mm) and column (150×3 mm) and an ED50 electrochemical detector (Dionex, Sunnyvale, CA, USA) was used. The detector, used in PAD mode performing a quadruple waveform, was equipped with a reference pH electrode (Ag/AgCl) and a gold electrode. The gradient used (40 min) started with a two stage equilibration performed with 100 mM NaOH for 5 min and 4 mM NaOH for 5 min, after which 10 µL of hydrolysate was injected and eluted (0.5 ml/min) by isocratic elution with 4 mM NaOH for 20 min at 30 °C. Lastly, column regeneration was performed with 500 mM NaOH for 10 min. Identification and quantification was done using corresponding calibration curves (1 to 10 ppm) of pure standards (L-fucose, L-rhamnose, L-arabinose, D-galactose, D-glucose, D-xylose and D-mannose). These standards were subjected as well to the above-mentioned TFA hydrolysis in order to correct for possible monosaccharide degradation.

2.3.4 Protein content

The total nitrogen content was determined using the Dumas method and an elemental analyzer (EA 1108 CHNS-O elemental analyzer, CE-Instruments/Thermo Scientific, Waltham, MA, USA). The protein amount was calculated from the total nitrogen content using 6.25 as conversion factor. Analysis was carried out in duplicate.

2.3.5 Molar mass distribution

The molar mass distribution of the pectin samples was analyzed according to the procedure described by Shpigelman, Kyomugasho, Christiaens, Van Loey, & Hendrickx (2014) using

high performance size exclusion chromatography (HPSEC) coupled to multiangle laser light scattering (MALLS) (PN3621, Postnova analytics, Germany), refractive index (RI) (Shodex RI-101, Showa Denko K.K., Kawazaki, Japan) and a diode array detector (DAD) (G1316A, Agilent technologies, Diegem, Belgium). Samples (0.2% w/v) were dissolved overnight in ultrapure water, filtered (Chromafil A-45/25, 0.45 mm pore size, Macherey-Nagel GmbH, Duren, Germany) and injected (100 µl). A series of three Waters columns were used, Ultrahydrogel 250, 1000 and 2000 with exclusion limits of 8×10^4 , 4×10^6 and 1×10^7 g/mol, respectively (Waters, Milford, MA), which were kept at 35 °C in an external oven. Elution of the pectin polymers was achieved at 0.5 ml/min with 0.1 M acetic acid buffer (pH 4.4) containing 0.1 M NaNO₃ in 103 min. The dn/dc value used to calculate the concentration was 0.146 ml/g. The molar mass (MM) was calculated using the Debye fitting method (up to second order) by the software provided (NovaMals, version 1.2.0.0, Postnova analytics, Germany).

2.4 Physicochemical properties of pectin samples

2.4.1 ζ-potential

To evaluate the charge density of the different pectin samples, the ζ-potential was measured as a function of pH using an automated capillary electrophoresis equipment (Zetasizer NanoZS, Malvern Instruments, Worcester, UK). Pectin samples were dissolved overnight (1% w/v) in a broad pH range (pH 1.5 to 7). Prior to the measurement, the samples were diluted 1:100 (v/v) with ultrapure water set at the respective pH and transferred into a capillary cell having two electrodes. An electric potential results in particle movement depending on particle charge towards the electrode with opposite charge. The velocity of the hydrocolloids in solution was monitored illuminating the sample with a laser beam and was used to determine the electrophoretic mobility. Finally, the equation of Henry was applied by

the instrument's software to obtain the ζ -potential of the measured hydrocolloid. All measurements were performed in duplicate with a minimum of 12 runs.

2.4.2 Hydrodynamic diameter

To investigate the behavior of the different pectin samples in solution, the hydrodynamic diameter (D_h) was measured based on the procedure described by Schmidt et al. (2017). As the conformation of pectin is pH dependent, measurements were executed at two particular pH values: pH 2.5 and 6.0. Pectin solutions with different concentrations (0.05 to 0.25% w/v) were prepared diluting a 0.25% (w/v) stock solution with ultrapure water set at the respective pH. Pectin solutions were transferred into macro UV-cuvettes and analyzed in terms of particle size using dynamic light scattering (DLS) (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). The detected Z-average diameter was plotted against the corresponding pectin concentration and extrapolated to zero to obtain the D_h of each pectin sample at both pH values. The measurement was performed in duplicate with a minimum of 12 runs.

2.4.3 Dynamic interfacial tension

The dynamic interfacial tension was determined using a pendant drop tensiometer (CAM 200, KSV Instruments, Finland) following the procedure described by Verkempinck et al. (2018). The main principle of this measurement depends on Profile Analysis Tensiometry (PAT) of an oil droplet formed in an aqueous, continuous phase (Loglio, Pandolfini, Liggieri, Makievski, & Ravera, 2011). Fitting this profile with the Laplace equation results in the surface tension to be the only free parameter as function of analysis time. As the analysis started immediately after droplet formation, the analysis time can be considered as the contact time between droplet surface and pectin solution. Pectin solutions (0.2% w/v set at pH 2.5 or 6.0) were prepared in ultrapure water. Purified sunflower oil (pSO) was used to form a droplet at the tip of a U-shaped needle placed in the pectin solution. The optical determination of the profile of the droplet started immediately after droplet formation.

Measurements for all samples at both pH values were performed during a period of 1 h and in duplicate.

Purification of the sunflower oil was executed aiming to remove surface-active components inherently present in dietary oils (e.g. polyphenols). Briefly, 500 mL of commercial sunflower oil was rotated with 100 g Florisil® (i.e. an inorganic adsorbent used for lipid separation and purification purposes) overnight, whereafter the oil was filtered and stored in dark at -40 °C until use (Verkempinck et al., 2018). It was opted to use pSO for the experiment to be able to study solely the surface activity of the pectin samples on the change in interfacial tension. Furthermore, this pSO was used for emulsion preparation as well (described in section 2.5).

2.5 Emulsion preparation

First, a coarse oil-in-water (o/w) emulsion was prepared by mixing 5% (w/v) pSO and 1% (w/v) pectin solution for 10 min at 9500 rpm using an high speed mixer (Ultra-Turrax T25, IKA, Staufen, Germany). Next, the coarse emulsion was further stabilized by high pressure homogenization at 100 MPa (Pressure Cell Homogenizer, Stansted Fluid Power Ltd., UK) for one cycle. The resulting emulsion was divided in several test tubes (two for each analysis day) which were stored independently at 4 °C. Emulsions were prepared using pectin solutions of all three aforementioned pectin samples (i.e. NA, NA-HG and NA-RG) at both pH 2.5 and 6.0.

2.6 Evaluation of physical stability of emulsions

Emulsion stability was evaluated during refrigerated storage for 14 days. During this period, analyses were performed on the day of emulsion preparation (cfr. day 0), day 1, day 4, day 8 and day 14 of storage. The particle size distribution and the volume-weighted mean particle size of each emulsion were determined by laser diffraction. Single Particle Optical Sizing

(SPOS) was used to study droplet size at the level of individual oil droplets. The microstructure of each emulsion was evaluated by light microscopy. Gravitational destabilization (i.e. creaming) was visually investigated taking macroscopic pictures each analysis day as well as by performing an accelerated and direct stability analysis using freshly prepared emulsions.

2.6.1 Volume-weighted particle size

All emulsions were analyzed using a laser diffraction equipment (Beckman Coulter Inc, LS 13 320, Miami, Florida, USA) to determine the particle size distribution and the mean volume-weighted particle size (d_{43}). Prior to analysis, emulsions were shaken, brought into a stirring tank filled with demineralized water and pumped to the measurement cells. The laser light (wavelength main illumination source: 750 nm; wavelengths halogen light for Polarization Intensity Differential Scattering (PIDS): 450 nm; 600 nm; 900 nm) was scattered by the oil droplets. The intensity of the scattered laser light was detected and transformed to particle sizes by the Fraunhofer model. Reporting the d_{43} was preferred as this value is more prone to the presence of small amounts of large particles and thus is considered a good indicator for emulsion stability against coalescence. Each analysis day, emulsions stored in two different test tubes were injected twice in the laser diffraction equipment. Particle size distributions and d_{43} are averages of these four measurements.

2.6.2 Individual oil droplet size

The SPOS technique (AccuSizer 780 APS, Particle Sizing Systems, Santa Barbara, California, USA) was used to determine the volume % of oil droplets larger than 1 μm . In order to analyze one oil droplet at a time, the emulsion needed to be diluted. First, the emulsion was manually diluted (1:50) with ultrapure water at the pH of the analyzed emulsion. Secondly, the diluted emulsion was injected and further diluted in a two-stage automated dilution system of the equipment to a concentration below the preset limit of 3500

particles/ml to avoid signal coincidence of multiple particles in the detector optical-sensing zone. Hereafter, the system started counting and sizing all particles that pass through the optical-sensing zone of the sensor (LE400, Particle Sizing Systems, Santa Barbara, California, USA). The sensor was used in ‘summation’ mode, collecting data based on both light extinction and light scattering. Each analysis day, emulsions stored in two different test tubes were analyzed twice using the SPOS technique. Reported volume % of oil droplets larger than 1 μm are averages of these four measurements.

2.6.3 Microstructure

The microstructure of all pectin containing emulsions was visualized by light microscopy (Olympus BX-41 microscope, Olympus, Optical Co. Ltd., Tokyo, Japan) coupled to image-analysis software (cell^F, Soft Imaging Systems, Münster, Germany).

2.6.4 Accelerated physical stability test

The physical stability of all emulsions to creaming was evaluated using an analytical centrifuge (LUMiFuge, LUM GmbH, Berlin, Germany). Designated cuvettes were filled with a fixed amount of emulsion and centrifuged at 20 °C at 3000 RPM (1308 g). A transmission profile was recorded each minute and used to calculate the instability index with both the SEPView[®] 6 software (LUM GmbH, Berlin, Germany) and the Space- and Time-resolved Extinction Profiles (STEP[®]) Technology[®] (Detloff, Sobisch, & Lerche, 2014). A total of 800 consecutive transmission profiles were recorded for each emulsion and the analysis was carried out in duplicate.

2.7 Statistical analysis

All statistical analyzes were performed using the statistical software JMP (JMP Pro14, SAS Institute Inc., Cary, NC, USA). One way ANOVA and Tukey's Studentized Range Post-hoc test with a 95% level of significance ($p < 0.05$) were used to evaluate significant differences

359 in structural characteristics of all samples. Significant differences in hydrodynamic diameter
360 were determined calculating 95% confidence intervals.

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3 Results and discussion

In order to understand the role of the different pectin subdomains in emulsification and emulsion stabilization, the results obtained will be presented in three parts. First, the structural properties of the pectin samples will be discussed (section 3.1). Secondly, different physicochemical properties giving insight in the emulsifying capacity of the pectin samples were studied and the results are presented in section 3.2. Finally, in section 3.3, the storage stability of o/w emulsions produced with each pectin sample under consideration as sole E_FE_S agent was investigated.

3.1 Structural characterization

The composition of all pectin samples based on the structural characterization is represented in **Table 1**. The acid extraction resulted in a pectin rich sample with a GalA content of 572.86 ± 20.85 mg per g sample and considerable amounts of neutral monosaccharides, such as rhamnose, arabinose and galactose, related to the RG-I side chains of pectin polysaccharides. Furthermore, the NA sample showed a relatively high DM ($63.02 \pm 2.26\%$) and average MM (429.50 ± 2.12 kDa). Santiago, Kyomugasho, et al. (2018) reported similar DM values for pectin rich carrot samples extracted after heat treatment while reporting a range of MM values of the same order of magnitude for AIR extracted from carrot tissue. The protein content was measured to be around 5%, which can play a role in the emulsification process (Alba & Kontogiorgos, 2017). This is comparable to the protein content of sugar beet pectin and okra pectin (Funami et al., 2011; Kpodo et al., 2017) but much lower compared to tomato and broccoli pectin (Santiago, Salvia-trujillo, et al., 2018). Using this NA sample as starting material, NA-HG and NA-RG were produced with yields of $53.58 \pm 7.26\%$ and $29.45 \pm 5.83\%$ respectively. During the targeted modification procedures to produce NA-HG and NA-RG, the structural properties of the pectin clearly changed due to the conditions used.

Based on the GalA content and neutral monosaccharide concentrations, it can be stated that the procedures used resulted in the enrichment of NA with the respective subdomains, homogalacturonan and rhamnogalacturonan. In more detail, both NA-HG and NA-RG experienced alkaline demethylesterification and depolymerization indicated by the lower DM and MM values in comparison to the original acid extracted NA sample. In **Figure 1**, the MM distributions of the three pectin samples are represented. Looking at the overall characterization results of all pectin samples, it can be stated that the NA sample is composed of large polymer structures with both linear and branched regions. NA-HG is composed of small and highly linear pectin fragments evidenced by the low MM, the high GalA content and the lower contents of the main neutral sugars related to the branches of pectin like rhamnose, arabinose and galactose (**Table 1**). For NA-RG, the lower MM and relatively higher contents of rhamnose, arabinose and galactose signify that this sample is composed of small and highly branched pectin fragments. This proves that the targeted modification procedures used in this study were effective and allowed to further investigate the E_FE_S potential of the pectin subdomains. Based on the obtained results, the molecular structure of the pectin samples is visualized as hypothetical model structures presented in **Figure 2**.

3.2 Physicochemical properties

Emulsifiers and emulsion stabilizing compounds can exhibit specific properties that enhance their functionality. Consequently, some physicochemical properties relevant to evaluate E_FE_S agents were analyzed for the three pectin samples. More specifically, the following sections discuss the results obtained from the measurements of the ζ -potential, the hydrodynamic diameter and the dynamic interfacial tension.

3.2.1 ζ -potential as function of pH

Pectin polymers can bear negative charges at the GalA residues with free C-6 carboxyl groups, affecting its functional properties. Verkempinck et al. (2018) clearly showed that the

negative charge density of pectin polymers depends both on the DM and the pH of the surrounding, continuous phase. Consequently, the ζ -potential of the pectin samples (NA, NA-HG and NA-RG) was measured as function of pH (**Figure 3**). All pectin samples show a sigmoidal relation between ζ -potential and pH with an inflection point around the pK_a value of the GalA residues in the pectin structure ($pH = 3.38$ to 4.10) (Kyomugasho et al., 2017; Manrique & Lajolo, 2002). From **Figure 3**, it is clear that NA-HG has at all pH-values tested a more negative charge density than NA and NA-RG. This can be explained by the molecular composition of NA-HG which has a high GalA content and a low DM. In other words, compared to the other two samples, NA-HG contains more GalA residues of which only 18% is methylated (**Table 1**) yielding a higher negative charge density in its molecular structure and hence a lower ζ -potential. Especially at high pH values, this high charge density can have a significant impact on the E_{FES} capacity of this pectin sample due to electrostatic interactions. NA and NA-RG carry similar charge densities in the considered pH range. Based on the results obtained, two pH values were selected at which the different pectin samples will be further studied: pH 2.5 and 6.0. It was opted to select two pH conditions situated at both sides of the pK_a value of the GalA residues as the ζ -potential measurements show clear differences in charge density at those pH values for all pectin samples.

3.2.2 Hydrodynamic diameter

Using DLS measurements, the hydrodynamic diameter of the pectin samples at pH 2.5 and 6.0 was determined. The results are presented in **Table 2**.

For the NA sample, the D_h increased with increasing pH, indicating intramolecular repulsion at neutral pH values. Besides, ζ -potential measurements showed that the pectin polymers carry a higher charge density at pH 6.0 than at pH 2.5 (section 3.2.1), most probably causing intramolecular repulsion which eventually resulted in a larger hydrodynamic diameter at this

higher pH. This trend was observed as well by Verkempinck et al. (2018) for citrus pectin samples with varying DM. However, the D_h values obtained by Verkempinck et al. (2018) were smaller compared to the values for NA. This was expected since NA has a higher MM than citrus pectin (around 55 kDa). By contrast, this increasing trend was not observed for both NA-HG and NA-RG as these pectin samples manifest larger D_h values at pH 2.5 than at pH 6.0 (**Table 2**). A possible explanation for this behavior is the existence of intermolecular attractive interactions between different polymer chains leading to the formation of aggregates at pH 2.5. Since these pectin polymers carry more negative charges at high pH (**Figure 3**), the increased intramolecular repulsion under these conditions might become stronger than the attractive forces and consequently hamper aggregate formation for these particular isolated pectin samples, as previously observed by Sawayama, Kawabata, Nakahara, & Kamata (1988).

In conclusion, it could be observed that due to structural differences the NA sample behaved differently when increasing the pH from 2.5 to 6.0 in comparison with the isolated pectin fractions, NA-HG and NA-RG.

3.2.3 Dynamic interfacial tension

A very important characteristic of emulsifiers is their ability to adsorb at the oil-water interface and consequently lowering the interfacial tension. A lower interfacial tension facilitates droplet breakup during emulsification and hampers droplet recoalescence during storage (Stang, Karbstein, & Schubert, 1994). Therefore, the interfacial tension of a pSO droplet in 0.2% pectin solutions was measured as function of time (**Figure 4**). Adsorption at the interface supports the statement that pectin polymers exhibit surface activity. In case the analysis was executed in ultrapure water, the interfacial tension remained constant at both pH conditions as no surface-active compounds were added to this continuous phase, this was to

be expected. Furthermore, these results indicate that no self-emulsifying surface-active molecules were present in the sunflower oil after the executed purification procedure.

Both at pH 2.5 and 6.0, the interfacial tension clearly decreases for the NA sample. A decrease of 54.2% and 58.3% was reached for the NA samples in comparison to ultrapure water at pH 2.5 and 6.0, respectively. Comparable final values were obtained for citrus pectin samples of varying DM (Verkempinck et al., 2018) and for whey protein isolate (Bai, Huan, Gu, & McClements, 2016) while gum arabic lowered the interfacial tension to a lower extent (Bai et al., 2016). Similarly to NA, NA-HG presented a decrease in interfacial tension at both pH conditions. In comparison to ultrapure water, the interfacial tension decreased with 27.87% and 34.22% at pH 2.5 and 6.0 respectively. However, the decrease was faster at pH 2.5 compared to pH 6. NA-RG at pH 6.0 lowered the interfacial tension as well (38.31% decrease with respect to ultrapure water). On the contrary, NA-RG at pH 2.5 did not substantially lower the interfacial tension as only a 9.32% decrease was achieved at the end of the measurement. Moreover, it can be noted from **Figure 4** that this sample did not show a decrease as fast as all other pectin samples in the early minutes of the measurement. Based on these results, it can be stated that NA-RG does not or only to a very minor extent exhibit surface-active behavior at pH 2.5. In conclusion, it can be expected based on these results that the NA sample has the most promising potential as an emulsifier at both pH-values tested.

3.3 Storage stability of pectin stabilized emulsions

The results discussed in previous sections suggest that the pectin samples display interesting properties to serve as E_FE_s agents. Almost all pectin samples studied at two pH conditions showed spontaneous adsorption at an oil-water interface lowering the interfacial tension (section 3.2.3), except NA-RG at pH 2.5. Furthermore, combining the structural characterization of the pectin samples (section 3.1) and the ζ -potential and hydrodynamic diameter measurements (sections 3.2.1 and 3.2.2, respectively) indicates that the adsorbed

pectin might promote emulsion stabilization by both electrostatic and steric interactions. To further investigate the E_{FEs} potential of the pectin subdomains, 5% o/w emulsions were prepared with 1% (w/v) pectin sample as the only E_{FEs} compound. These emulsions were produced for all pectin samples at both pH 2.5 and 6.0. The physical stability of these emulsions was evaluated during a refrigerated storage period of 14 days. More specifically, the particle size (d_{43} and distribution), volume % of oil droplets larger than 1 μm , microstructure and gravitational stability (accelerated stability analysis and macroscopic pictures) were evaluated.

3.3.1 Particle size characterization and microscopic evaluation

Particle size distributions of all emulsions were evaluated during 14 days of refrigerated storage. **Figure 5** shows the results obtained on the day of emulsion production (day 0) and after 14 days of refrigerated storage (day 14). Distributions of day 1, day 4 and day 8 were in line with the observed trends between day 0 and day 14 (data not shown). Corresponding d_{43} values are represented in **Figure 6** for all samples and all analysis days.

For all samples, the distributions reveal a possibly better emulsifying capacity of pectin at low pH than at higher pH values as smaller oil droplets are created at pH 2.5. Especially the wide distribution for NA at pH 6.0 supports this statement. This can be explained by a better adsorption of pectin and a denser organization of adsorbed pectin chains at the interface at lower pH better stabilizing newly formed interface during the emulsification process (Verkempinck et al., 2018). In case of NA, a small shift towards larger particle diameters can be noticed at both pH conditions during storage, indicating that instability phenomena such as coalescence or flocculation occur, increasing droplet size. However, considering the detailed particle size distributions, the extent of these phenomena seems limited for the emulsions stabilized with NA. NA-HG at pH 2.5 displays a narrow particle size distribution that remains similar during 14 days of storage. At high pH, a different observation can be made for NA-

HG. Starting from day 0 and for each analysis day during storage, a bimodal distribution can be observed, showing a peak around 1 μm and one around 20 μm . Coalescence and flocculation phenomena may both lead to the appearance of this second, larger peak. However it is important to note that the occurring destabilization mechanism(s) take place immediately after emulsion formulation and not during storage as the overall distribution remains similar as function of storage time. Similar observations can be made for the NA-RG emulsion at pH 6.0. On the contrary, at pH 2.5, NA-RG does not seem to be able to emulsify and stabilize oil droplets as efficient as the other two pectin samples at low pH. Already at the day of emulsion preparation, the particle size distribution comprises a wide size range suggesting an ineffective emulsification and/or fast destabilization. After 14 days of storage, the distribution becomes even broader and consequently coalescence can be expected to be the main destabilization mechanism for this particular sample. The d_{43} values display similar trends for all samples and do not substantially change over a 14 days storage period despite being sensitive to the presence of larger particles (e.g. flocculated droplets). In other words, the small changes of d_{43} as a function of storage time indicate no drastic changes in emulsion stability. Similarly as discussed above, it becomes more evident that both NA-HG and NA-RG stabilized emulsions at pH 6.0 present fast destabilization leading to increased oil droplet sizes already on the day of emulsion production.

Next to the analyses with laser diffraction, which is a so-called ensemble technique, the SPOS method was used to characterize oil droplet size at the level of individual droplets. By counting and sizing each particle that passes through the detector volume, this technique is able to calculate the volume fraction of oil droplets larger than a certain threshold value. Considering the characteristics of the created o/w emulsions, 1 μm was chosen as the threshold value for this study. The evolution of the volume % of oil droplets larger than 1 μm during storage is displayed in **Figure 7** for all emulsions.

When evaluating the effect of storage time on the volume % of oil droplets larger than 1 μm , a small increase is noticeable for the emulsions stabilized by NA and NA-HG at pH 2.5. This indicates a slow increase of the size of individual oil droplets over storage time, which is most probably caused by coalescence. However, it should be noted that this phenomenon of coalescence only occurs to a very limited extent. Flocculation can be ruled out for these emulsions since the SPOS method allows to characterize individual oil droplets and moreover, a unimodal distribution situated around 1 μm was observed using laser diffraction (**Figure 5**). For the NA-RG emulsion at pH 2.5 there is no gradual increase observed as for the other two emulsions at pH 2.5, but a steep increase at day 1 which remains stable during the continuation of the storage experiment. Examining the results at pH 6.0, two interesting conclusions can be made. Firstly, a clear impact of pH can be observed as the volume % larger than 1 μm for the emulsion stabilized by the NA sample confirms the statement above that at higher pH the emulsification process is unable to achieve droplet sizes as small as at pH 2.5 (**Figure 7A vs 7B**). In other words, emulsification is less efficient at high pH using NA as emulsifier. Secondly, when comparing **Figure 5** and **Figure 7**, the volume % larger than 1 μm for the emulsions stabilized by NA-HG and NA-RG at pH 6.0 are surprisingly low compared to the particle size distributions obtained for these pectin samples. This demonstrates that the SPOS technique is able to detect and determine the size of individual particles even if they appear in flocs as long as the forces causing flocculation are not too strong. Furthermore, the increase in volume % as function of storage time indicates the occurrence of coalescence as well in these samples. Consequently, it can be postulated that combining laser diffraction and SPOS is a promising and powerful approach for particle size characterization and emulsion stability studies.

Microscopy is a well-known technique to visualize destabilization mechanisms occurring in emulsions during storage. Therefore, the microstructure of all emulsions was evaluated using

light microscopy (**Figure 8**). Overall the microscopic images are in line with the previous results characterizing oil droplet size and stability in the emulsions.

The emulsions stabilized by NA do not show clear changes in microstructure during storage. This is in line with the results obtained by the laser diffraction and SPOS analyses. For the NA-HG stabilized emulsions, clear differences between both pH conditions can be observed. Whereas at pH 2.5 the micrographs look rather similar to what was observed for the NA stabilized emulsions, the micrographs taken of the emulsion with NA-HG at pH 6.0 clearly show flocculated oil droplets. As described above, the particle size distributions also show large differences between these two pH conditions. Based on the scale bar of the micrographs, an estimation of the size of the flocs present in the emulsion can be made. This leads to the conclusion that the particle size distributions at pH 6.0 are composed of a peak representing individual oil droplets (peak around 1 μm) and a peak of oil droplets organized in flocs (peak around 20 μm). As stated before, SPOS counts and sizes individual oil droplets and consequently it could be concluded that during this analysis, the flocs were disrupted. A closer look at some flocs presented in **Figure 8**, confirms that the structure of these flocs is rather loose than closely packed. Disruption of such flocs during the SPOS measurement is therefore a plausible explanation. For the emulsions stabilized by NA-RG, similar conclusions can be made as for the NA-HG stabilized emulsions. Combining the particle size distributions, the results from the SPOS technique and the microstructures shows that emulsions stabilized by NA-HG and NA-RG at pH 6.0 consist of both single and flocculated droplets. Consequently, flocculation is clearly identified as the main destabilization mechanism affecting the stability of these emulsions.

3.3.2 Creaming rate

Using an analytical centrifuge, an accelerated stability test was executed to evaluate the creaming stability of emulsions. Space and time resolved extinction profiles allow to

quantitatively evaluate the clarification of emulsions during the creaming process. The measurement is based on the direct extinction of laser light passing through the sample from bottom to top during centrifugation. The instability index is a quantitative parameter for the extent of clarification in the samples due to the centrifugal forces imposed on the samples. In **Figure 9**, the instability indices for all emulsions under consideration are presented as function of analysis time. Under the conditions used, the emulsions stabilized by NA clearly creamed slower or in other words showed more resistance against creaming than the emulsions stabilized by one of the pectin fractions enriched in HG or RG.

The fresh NA stabilized emulsion at pH 2.5 showed a narrow oil droplet distribution, while at pH 6.0 the main intensity peak of the particle size distribution was situated at higher droplet diameters (**Figure 5**). This could explain the difference in creaming rate between these two emulsions as the creaming of oil droplets can be described by Stokes' Law for particle diffusion in a viscous fluid, relating the terminal (creaming) velocity to the square of the particle radius. However, the results obtained from this accelerated stability study indicate not only the effect of particle size, but influence of other emulsion characteristics as well. For example, when comparing the emulsions stabilized by NA and NA-HG at pH 2.5, the stability indices diverge strongly while the oil droplet size distribution of their respective fresh emulsions does not differ much. In other words, for the NA and NA-HG emulsions at pH 2.5, the differences in creaming rate cannot be explained by different oil droplet dimensions only. Exhibiting distinct structural characteristics, both pectin samples most probably affect emulsion stability influencing the continuous phase as well. Possible differences in viscosity increasing capacity of the different pectin samples may lead to differences in oil droplet mobility, explaining the different creaming behavior. The NA sample exhibits a higher MM compared to NA-HG and NA-RG (**Table 1**). This probably leads to a higher viscosity increasing effect as the intrinsic viscosity of polymers in solution

608 is mainly dependent on the MM of the polymers according to the Mark-Houwink relation
609 (Picout & Ross-Murphy, 2007). Consequently, oil droplet mobility in the emulsions
610 stabilized by NA is reduced to a larger extent than in the emulsions stabilized by NA-HG and
611 NA-RG.

612 Next to the accelerated stability study, creaming of all emulsions was assessed on the shelf
613 during refrigerated storage taking macroscopic pictures. Pictures for all emulsions taken on
614 the different analysis days are displayed in **Figure 10** and confirm the observations of the
615 accelerated stability study. Briefly, both emulsions stabilized by NA presented no visual
616 cream layer during 14 days of storage, while cream layers were already visually observed
617 after one day of storage in all emulsions stabilized by NA-HG and NA-RG, at both pH
618 conditions.

4 Conclusion

In the work presented, a linear HG rich pectin fraction (NA-HG) and a more branched RG rich pectin fraction (NA-RG) were successfully produced from an acid extracted carrot pectin (NA). The structurally diverse pectin samples allowed an in-depth study of the emulsifying and emulsion stabilizing potential of extracted carrot pectin and its subdomains. Besides the structural differences, the pectin samples also displayed different charge density, hydrodynamic diameters and had a different capacity to lower the interfacial tension of an oil droplet in an aqueous pectin solution. NA-HG displayed a more negative charge density compared to the original acid extracted NA sample and NA-RG, attributed to its highly linear structure and low DM. The hydrodynamic diameter of NA increased at higher pH as a result of increased intermolecular repulsion. For both pectin subdomains, opposite behavior was observed possibly due to intramolecular attractive forces at low pH that are overcome by intermolecular electrostatic repulsion at higher pH. All pectin samples were able to decrease the interfacial tension of an oil droplet in an aqueous pectin solution, except for NA-RG at pH 2.5. At both pH values, the NA sample was able to decrease the interfacial tension faster and to a lower final value compared to NA-HG and NA-RG.

Several interesting lessons can be learnt from the emulsion stability study of model o/w emulsions stabilized by the different pectin samples. First, pectin displays better emulsifying capacity at lower pH evidenced by the formation of smaller oil droplets in the emulsions at pH 2.5 compared to pH 6.0 possibly explained by better and denser adsorption of pectin chains at low pH better stabilizing the newly formed interface during emulsification. Secondly, it can be stated that irreversible destabilization phenomena such as coalescence only take place to a limited extent during refrigerated storage. Specifically, for the emulsions stabilized by NA-HG and NA-RG at pH 6.0, flocculation was clearly identified as the main destabilization phenomenon. Finally, it was clear that the emulsions stabilized by NA-HG or

NA-RG are more susceptible to creaming than the NA pectin stabilized emulsion. This can possibly be explained by the larger viscosity increasing effect of NA in the continuous phase compared to the isolated pectin fractions which have a lower molar mass. Overall, the NA sample, having both linear and branched regions, showed the best emulsifying and emulsion stabilizing potential.

In conclusion the results of this research proved the potential of combining laser diffraction measurements and the SPOS technique as a promising and powerful methodological approach for particle size characterization and emulsion stability studies. Regarding the role of the pectin subdomains, this study showed that the presence of both linear and branched subdomains in the pectin structure is beneficial to create physically stable emulsions.

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Declaration of interests

The authors of the present work declare no conflict of interests.

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807 **Tables**

808 **Table 1. Structural properties of generated pectin samples expressed as average values**
 809 **± standard deviation. (GalA: galacturonic acid; DM: degree of methylesterification;**
 810 **MM: molar mass)**

	NA	NA-HG	NA-RG
DM (%)	63.02 ± 2.26 ^a	18.62 ± 3.31 ^b	5.89 ± 2.51 ^c
GalA content (% w/w)	57.29 ± 2.09 ^b	72.11 ± 3.21 ^a	42.00 ± 1.51 ^c
Neutral sugars (% w/w)			
Rhamnose	3.24 ± 0.16 ^b	0.77 ± 0.01 ^c	5.24 ± 0.39 ^a
Arabinose	5.34 ± 0.41 ^b	ND	6.81 ± 0.85 ^a
Galactose	6.69 ± 0.59 ^b	0.16 ± 0.01 ^c	10.68 ± 1.19 ^a
Fucose	0.23 ± 0.12 ^b	0.01 ± 0.01 ^c	0.46 ± 0.08 ^a
Glucose	0.89 ± 0.16 ^a	0.53 ± 0.01 ^b	0.51 ± 0.06 ^b
Xylose	0.23 ± 0.01 ^b	0.10 ± 0.02 ^c	0.30 ± 0.03 ^a
Mannose	0.44 ± 0.08 ^a	0.04 ± 0.03 ^c	0.17 ± 0.02 ^b
Protein content (% w/w)	5.28 ± 0.37 ^a	3.13 ± 0.04 ^c	4.33 ± 0.01 ^b
MM (kDa)	429.50 ± 2.12 ^a	20.50 ± 0.70 ^c	71.30 ± 0.50 ^b

811 ND: not detected

812 ^{a, b, c}: Different superscript letters indicate significant differences ($p < 0.05$) between the values
 813 of a structural property for the different pectin samples.

814

Table 2. Hydrodynamic diameter of generated pectin samples: NA, NA-HG and NA-RG at pH 2.5 and pH 6.0.

	NA	NA-HG	NA-RG
pH 2.5	555.4 ± 23.2 ^b	553.9 ± 36.6 ^a	739.3 ± 27.9 ^a
pH 6.0	834.8 ± 101.8 ^a	278.8 ± 24.3 ^b	213.8 ± 18.0 ^b

^{a, b}: Different superscript letters indicate significant differences between measurements of the same pectin sample at different pH values based on 95% confidence intervals.

821 Figures

822

823 **Figure 1. Molar mass distribution of different generated pectin samples (NA, NA-HG and**
 824 **NA-RG). Molar mass (thick lines) and concentration (thin lines) are presented as a**
 825 **function of elution time.**

826

Figure 2. Hypothetical representation of the molecular structures of the pectin samples. Grey lines represent the backbone of the structures while black lines represent the sidechains.

Figure 3. ζ -potential of 1% w/v solutions of different generated pectin samples ($\blacktriangle$ NA, $\blacksquare$ NA-HG and $\circ$ NA-RG) as function of pH.

Figure 4. Interfacial tension as function of time for a droplet of purified sunflower oil in 0.2% solutions of NA (dashed-dotted line), NA-HG (dashed line) and NA-RG (full line). To allow comparison, measurements of purified sunflower oil in ultrapure water are displayed as well (dotted line). Black lines represent measurements at pH 2.5, while grey lines represent measurements at pH 6.0.

Figure 5. Particle size distributions for 5% o/w emulsions stabilized by 1% (w/v) (A) NA, (B) NA-HG and (C) NA-RG for the day of emulsion preparation (full lines) and day 14 of refrigerated storage (dashed lines). Black lines represent emulsions at pH 2.5, while grey lines represent emulsions at pH 6.0.

Figure 6. Average particle size (μm) during refrigerated storage for 5% o/w emulsions stabilized by 1% (w/v) (A) NA with the insert showing a magnified view focused on d₄₃ values between 0 μm and 4 μm, (B) NA-HG and (C) NA-RG. Black lines represent emulsions at pH 2.5, while grey lines represent emulsions at pH 6.0.

Figure 7. Volume percentage of oil droplets larger than 1 μm for 5% o/w emulsions stabilized by 1% (w/v) NA, NA-HG and NA-RG as measured by the single particle optical sizing technique at (A) pH 2.5 and (B) pH 6.0. Increasing color intensity of bars indicates longer storage time: ■ day of emulsion production (day 0), ■ day 1, ■ day 4, ■ day 8 and ■ day 14.

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868 **Figure 8. Microscopic images of 5% o/w emulsions stabilized by 1% (w/v) NA, NA-HG and NA-RG at pH 2.5 and 6.0. Scale bars represent**
869 **a length of 100 μm.**

Figure 9. Instability index as function of analysis time for 5% o/w emulsions stabilized by 1% (w/v) NA (dashed-dotted line), NA-HG (dashed line) and NA-RG (full line). Black lines represent measurements at pH 2.5, while grey lines represent measurements at pH 6.0. Calculations were based on the STEP[®] technology according to Detloff et al. (2014).

Figure 10. Macroscopic pictures of 5% o/w emulsions stored at 4 °C stabilized by 1% (w/v) of different pectin samples. Arrows on the pictures indicate the visual cream layer. For each set of pictures from left to right: day of emulsion production (day 0), day 1, day 4, day 8 and day 14 of refrigerated storage.