The effect of high pressure homogenization and endogenous pectin-related enzymes on tomato purée consistency and serum pectin structure

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[1]Abbreviations

[1] DAc, degree of acetylation; DM, degree of methyl-esterification; GalA, galacturonic acid; HG, homogalacturonan; HPH, high pressure homogenization; HP, high pressure treatment; HT, high temperature treatment; LT, low temperature treatment; M_w, molecular weight; MWCO, molecular weight cut off; PG, polygalacturonase; PLNS, pectin-linked neutral sugars; PME, pectin methyl-esterase; RG, rhamnogalacturonan; UA, uronic acid

Abstract

The influence of mechanical tissue disintegration techniques (i.e. blending and high pressure homogenization) and the stimulation of endogenous pectin-related enzymes (i.e. pectin methyl-esterase and polygalacturonase) on tomato purée consistency, serum composition and serum pectin structure were investigated. Serum pectin structure was characterized in terms of degree of methyl-esterification, acetylation, neutral sugar composition and molecular weight (M_w) distribution.

Endogenous pectin methyl-esterase and polygalacturonase stimulation resulted in the lowest purée consistency and highest serum yield. However, when such purée was homogenized, a higher purée consistency and a low serum yield were observed. Moreover, the M_w of serum pectin was exceptionally high for the homogenized purées. The low methyl-esterified, linear and remarkably high M_w tomato serum pectin of the homogenized purées partly explains their increased consistency. This work demonstrated that high pressure homogenization can at least partially restore the consistency of tomato purée despite an initial consistency loss ascribed to enzymatic pectin degradation.

Keywords

Tomato purée consistency, thermal treatment, mechanical treatment, high pressure homogenization, serum pectin structure, endogenous pectin-related enzymes

1. Introduction

Tomatoes, amongst fruits and vegetables, are commonly processed into dispersed food systems such as soups, sauces, juices and purées. The consumption of these tomato-based dispersions has been suggested due to the increased bioavailability of bioactive health promoting compounds (e.g. carotenoids) compared to the intake of raw tomatoes (Gartner, Stahl, & Sies, 1997; Porrini, Riso, & Testolin, 1998). These dispersions are produced involving a combination of processing unit operations such as the indispensable mechanical tissue disintegration by blending and high pressure homogenization that alters the microstructure of the tomato matrix and the widely used thermal treatments for preservation purposes (Gould, 1992; Moelants et al., 2014). These unit operations result in a complex food system composed of insoluble pulp/particles dispersed in a continuous liquid/serum phase (Barrett, Garcia, & Wayne, 1998). Both serum and particle phases influence the sensory, nutritional and/or rheological properties of these dispersions (Barrett, Garcia, & Wayne, 1998; Hayes, Smith, & Morris, 1998; Diaz, Anthon, & Barrett, 2009; Lopez-Sanchez et al., 2011; Moelants et al., 2014). Specifically, the consistency of tomato dispersions can be influenced by the presence of pectin in both serum and particle phases (Sánchez et al., 2003; Anthon, Diaz, & Barrett, 2008; Tibäck et al., 2009; Houben et al., 2014b).

Pectin is one of the most interesting cell wall polysaccharides due to its poly-anionic nature and solubility characteristics (Thakur et al., 1997). It largely comprises a linear chain of 1,4 linked α-D-galacturonic acid (GalA) residues and a branched chain with a repeating disaccharide $[-\alpha\text{-D-GalA-1,2-}\alpha\text{-L-Rha-1-4-}]_n$ backbone containing individual, linear, or branched oligosaccharide side chains attached to the rhamnose residues (Voragen et al., 2009). The most abundant building blocks of pectin is the linear homogalacturonan chain which can be methyl-esterified on the C-6 carboxyl groups up to 70% to 80% and O-acetylated at O-3 or O-2 depending on the plant source (Voragen et al., 2009). Changes on the

molecular structure of pectin due to biochemical reactions can influence the consistency of tomato dispersions (Sánchez et al., 2003; Anthon, Diaz, & Barrett, 2008; Tibäck et al., 2009; Houben et al., 2014b). The consistency loss of tomato dispersions has been related to enzymatic pectin degradation caused by the synergistic action of pectin methyl-esterase (PME) and polygalacturonase (PG) (Verlent et al., 2006; Tibäck et al., 2009; Houben et al., 2014). PME catalyzes the removal of the methyl groups in the linear galacturonic acid-rich domain and PG subsequently de-polymerizes the de-esterified pectic molecule (Duvetter et al., 2009). To circumvent the consistency loss due to pectin enzymatic conversion, hot break processing of tomatoes that typically refers to a chopping temperature of 85 to 90 °C is being commercially used (Goodman, Fawcett, & Barringer, 2002). However, the detrimental effect of high temperature on some sensory properties of tomatoes (e.g. color and flavor) is inevitable. Moreover, Sánchez et al. (2003) and Tibäck et al. (2009) inferred that prolonged heating also entails pectin breakdown resulting in consistency loss. The emergence of non-thermal technologies such as high pressure processing may provide additional alternatives to the food industry for the manufacture of tomato dispersions. High pressure treatment can selectively inactivate PG thereby preventing pectin de-polymerization (Duvetter et al., 2009; Houben et al., 2013). Therefore, the consistency and the overall quality (e.g. color) of tomato dispersions can be improved by high pressure pre-treatment of tomato pieces (Duvetter et al., 2009).

Recently, high pressure homogenization (HPH) of tomato dispersions presented higher consistency than non-homogenized dispersions (Thakur, Singh, & Handa, 1995; Bayod et al., 2007; Colle et al., 2010; Panozzo et al., 2013; Palmero et al., 2016). Different mechanisms, including turbulence, shear and cavitation, were proposed to cause cell wall disruption during HPH (Stang, Schuchmann, & Schubert, 2001; Floury et al, 2004). The influence of HPH on the particles and its consequent effect on the rheological properties of the dispersion were

extensively investigated. The concentration of the particles, their size, size distribution, morphology, deformability and surface properties largely influence the flow behavior of the high pressure homogenized dispersions, depending on the plant matrix (Bayod et al., 2007; Bayod & Tornberg, 2011; Lopez-Sanchez et al., 2011; Moelants et al., 2014). However, specific properties of the serum phase and the molecular characteristics of the solubilized biopolymers are scarcely known. Although it has been reported that the serum had less influence on the rheology of dispersions, the serum was suggested to be essential for the overall structure organization of the dispersion and its interaction with the particles (Moelants et al., 2012; Moelants et al., 2014). Therefore, profound investigation of the serum phase composition may provide a better insight on the solubilization of polymers (e.g. pectin) during processing. These solubilized pectic polymers may play a role in the interactions of (in)soluble constituents influencing the structural and physical stability of the dispersions (Christiaens et al., 2012; Moelants et al., 2014; Kyomugasho et al., 2015b). Moreover, pectin has been recognized as a ubiquitous component in the serum phase of plant-based dispersions due to its solubility. When pectin is extracted from commercial sources, it is an important ingredient owing to its functional properties (e.g. thickening, gelling, stabilizing, emulsifying) ascribed to its chemical structure. Therefore, it is interesting to investigate the structure of serum pectin that may offer the use of a naturally existing constituent in tomato dispersions as a functional component. In this view, changes on the chemical structure of serum pectin and its possible relation to consistency can be investigated. Furthermore, the likelihood of restoring the consistency loss of tomato dispersions, which is due to the uncontrolled enzymatic activities and/or prolonged heating, using HPH can be explored. Understanding the influence of a combination of unit operations on the chemical structure of serum pectin and its potential functional properties allows a targeted processing of tomato dispersions. In addition,

a holistic approach in processing tomato-based dispersed products considering the nature of the soluble serum components (e.g. pectin) could probably be considered.

Therefore, the present work was aimed to characterize the structure of serum pectin as influenced by mechanical tissue disintegration techniques (i.e. blending and high pressure homogenization) and the stimulation of endogenous pectin-related enzymes (i.e. PME and PG) during purée preparation. The serum phase composition, the chemical structure of serum pectin and the physico-chemical properties of the differently prepared tomato purées were examined.

2. Materials and methods

2.1 Raw material

A batch of red-ripe tomatoes (*Solanum lycopersicum* cv. Prince) was purchased from a local shop in Belgium, stored at 4 °C for maximum 3 days and utilized in preparing six different purées. These tomatoes were washed, dried, and then cut into slices or quarters. Except for the tomato quarters which were subjected to high pressure pre-treatment, the tomato slices (±1 cm) were immediately vacuum-packed in a single layer using polyethylene bags (DaklaPack® Lamigrip Stand-up Pouch Transparent; 220 mm × 300 mm + 65 mm bottom fold), frozen in liquid nitrogen and then stored at -40 °C. Upon use and to facilitate the blending, the frozen tomatoes were thawed in a temperature-controlled water bath at 25 °C for 5 min.

2.2 Preparation of tomato purées

A schematic overview of the purée preparation is presented in Figure 1. The various purée processing conditions were generally selected based on the aim of each specific treatment. High pressure pre-treatment was performed to selectively inactivate polygalacturonase (PG), while maintain the pectin methyl-esterase (PME) activity (Christiaens et al., 2012). For the heat treatment at 95 °C for 30 min, enzyme inactivation is reportedly achieved at this temperature at a shorter time (Moelants et al., 2012; Houben et al., 2013). Since we aimed to

obtain high amounts of pectin in the serum, 30 min heat treatment was chosen that could result in higher pectin solubilization (Moelants et al., 2012). For the same reason, we chose 100 MPa for high pressure homogenization to enhance the amount of pectin in the serum (Moelants et al., 2012). In the case of low temperature treatment at 40 °C for 30 min, this was previously reported as an optimum condition for the activities of tomato PME and PG enzymes (Houben et al., 2014).

2.2.1 High pressure pre-treatment

To selectively inactivate the endogenous PG enzyme, tomato quarters were pre-treated at 550 MPa for 10 min in a single-vessel, laboratory-scale high pressure equipment with the cryostat pre-set at 4 °C (Engineered Pressure Systems International, EPSI, Temse, Belgium). Tomato quarters were vacuum-packed in a double-film polyethylene bag and then placed into the vessel. After the treatment and instantaneous decompression, samples were immediately cooled in an ice-water bath, frozen in liquid nitrogen and then stored at −40 °C (Christiaens et al., 2012). The HP-treated tomatoes were blended and then these were either subjected or not into further mechanical tissue disintegration by HPH (Figure 1).

2.2.2 High temperature treatment

Sliced tomato pieces (250-300 g) were vacuum-packed and then thermally treated at 95 °C for 30 min to inactivate the enzymes and to solubilize pectin. The thermal treatment was sufficient to inactivate the endogenous pectin methylesterase and endo-polygalacturonase enzymes (Houben et al., 2013). Afterwards, the tomato pieces were blended and either subjected to further mechanical tissue disintegration by HPH or not (Figure 1).

2.2.3 Low temperature treatment

High pressure (HP) pre-treated and untreated tomato pieces were blended and then vacuum-packed (200-250 g). These samples were then exposed to low temperature treatment (LT) at 40 °C for 30 min (Figure 1). LT was aimed to stimulate the enzyme activity,

particularly the endogenous pectin-related enzymes PME or both PME and PG, for HP and untreated blended tomatoes, respectively. After LT, the samples were further treated at 95 °C for 30 min in a temperature-controlled water bath to inactivate the enzymes and enhance pectin solubilization in the serum phase (Moelants et al., 2012; Houben et al., 2014).

2.2.4 Mechanical treatment

Untreated tomato pieces, HP pre-treated and heat treated tomatoes were blended using a Buchi mixer (B-400, Flawil, Switzerland) for three times at 5 s (Figure 1). Afterwards, the homogenates were thoroughly sieved (1.0 mm pore size) to remove the skin and seeds (Christiaens et al., 2012). Half of the blended samples, after the high temperature treatment, was more intensely disrupted using a Panda 2K high pressure homogenizer (Gea Niro Soavi, Parma, Italy) at 100 MPa for a single pass (Moelants et al., 2012). The homogenizer inlet and outlet were connected to a cryostat (Haake, Karlsruhe, Germany) pre-set at 4 °C. The other half was not homogenized and used as control samples (Figure 1).

2.3 Isolation of tomato sera

Serum and particle phases of the purées were separated according to the work of Moelants et al. (2012). Briefly, purées were centrifuged at 12,400 x *g* for 30 min at 20 °C (J2-HS centrifuge, Beckman, CA, US). The supernatants (serum phases) were initially filtered using a double-layer cheesecloth to exclude remaining pulp fragments and then vacuum filtered (Machery-Nagel MN 615 Ø 90 mm) to remove residual particles. The different sera were dialyzed (3.5 kDa, MWCO) against demineralized water for 48 h to remove ions and monomeric sugars. Prior to dialysis, pH of each serum was adjusted to 6.0 to ensure the ionization of all the carboxylic groups of pectin which is required for the determination of pectin degree of methyl-esterification using FT-IR (Kyomugasho et al., 2015a). Furthermore, to concentrate the sera and obtain its solubilized components, lyophilization was achieved

using a Christ alpha 2-4 (Osterode, Germany) freeze-dryer. Lyophilized sera were stored over P_2O_5 in a desiccator until further analysis.

2.4 Determination of the macroscopic and mesoscopic properties of the purées

2.4.1 Bostwick consistency

To evaluate the flow behavior of the six differently prepared tomato purées, the empirical Bostwick consistency test was used. About 100 ml of purée was placed into the Bostwick consistometer reservoir (CSC Scientific Company, VA, USA) and was allowed to flow under its own weight along a level surface for 30 s at room temperature (22 ± 1 °C). The Bostwick consistency index is the distance in centimeter covered by the purée. For each sample, the measurement was performed in triplicate.

2.4.2 Particle size distribution

The particle size distribution of the purées, reported as the volumetric fraction (%), was measured using a laser diffraction instrument (Beckman Coulter Inc., LS 13 320, Miami, Florida) as described elsewhere (Santiago et al., 2016).

2.5 Determination and characterization of sera components

2.5.1 Determination of protein content

The total nitrogen content of the sera was measured using an EA 1110 CHNS-O elemental analyzer (CE-Instruments/Thermo Fisher Scientific). About 2 mg of lyophilized serum was placed in crimped tin capsules (8 mm x 5 mm) prior combustion in the elemental analyzer. To calculate the amount of proteins in the sample, a conversion factor of 6.25 was used (Immerzeel et al., 2006; Shpigelman et al., 2014). The analysis was conducted in duplicate.

2.5.2 Determination of ash content

About 0.1 g of lyophilized serum was dried in a convection oven at 103 °C for 16 h. Subsequently, to determine the ash content, the dried samples were incinerated in a muffle furnace (Nabertherm GmbH, Controller P330, Lilienthal, Germany) operating for 3 h at 350 °C and 21 h at 550 °C (Santiago et al., 2016).

213 *2.5.3 Determination of pectin content*

214 The sum of uronic acid and pectin-related neutral sugars was used to estimate the total
215 amount of pectin (Jamsazzadeh Kermani et al., 2014).

216 *2.5.3.1 Uronic acid content*

217 The uronic acid content of the sera was determined using the method of Ahmed & Labavitch
218 (1977). Hydrolysis of 10 mg of lyophilized serum in 8 ml concentrated sulphuric acid was
219 performed in duplicate. Afterwards, a spectrophotometric measurement for each hydrolysate
220 was performed at 520 nm at 25 °C according to the method of Blumenkrantz & Asboe-
221 Hansen (1973) (in triplicate).

222 *2.5.3.2 Neutral sugar content*

223 The neutral sugar profile of the sera was determined using the method of Houben et al.
224 (2011). First, acid hydrolysis of the polysaccharides to monosaccharides was carried out.
225 Briefly, 5 mg of the lyophilized serum was hydrolyzed in 4 M trifluoroacetic acid (TFA) at
226 110 °C for 1.5 h. These were cooled, dried under N_2 at 45 °C, washed with 1 M ammonium
227 hydroxide, and then dried again under N_2 to remove and neutralize TFA. Afterwards, the
228 hydrolyzed and dried samples were dissolved in demineralized water (organic free, 18 MΩ
229 cm resistance) and then diluted to a final concentration of 0.1% (w/v). The hydrolysis was
230 performed in duplicate. Before chromatographic analysis, the samples were diluted and
231 filtered through a 0.45 µm syringe filter (Chromafil A-45/25, Macherey-Nagel, Duren,
232 Germany). The monosaccharides were analyzed using high performance anion exchange
233 chromatography (HPAEC) combined with pulsed amperometric detection (PAD). A Dionex
234 HPLC system (DX600), equipped with a GS50 gradient pump, a CarboPac™ PA20 column
235 (150 × 3 mm, pH range = 0–14), a CarboPac™ PA20 guard column (30 × 3 mm), and an
236 ED50 electrochemical detector (Dionex, Sunnyvale, USA), was used. The detector was
237 equipped with a reference pH electrode (Ag/AgCl) and a gold electrode. This was used in the

PAD mode performing a quadruple potential waveform. Ten microliters of samples were injected and eluted at 0.5 ml/min with 4 mM NaOH at 30 °C. Mixtures of sugar standards (L-Fuc, L-Rha, L-Ara, D-Gal, D-Glc, D-Xyl and D-Man) at varying concentrations (1–10 ppm) were used as standards for identification and quantification. Acid hydrolysis of these standards was also performed to correct for the degradation of the monosaccharides during the hydrolysis step. Peak areas of unhydrolyzed and hydrolyzed sugar standards were compared and the recovery values were considered in the quantification of the monosaccharides.

2.5.4 Characterization of pectin chemical structure

As pectin is one of the predominant components in tomato sera that may significantly contribute to the functionality of the dispersions, apart from its content, its chemical structure in terms of degree of methyl-esterification, degree of acetylation and molar mass was characterized.

2.5.4.1 Degree of methyl-esterification

The degree of methyl-esterification (DM) of pectin in the tomato sera was measured using Fourier transform infra-red (FT-IR) spectroscopy. Briefly, the lyophilized serum was compacted and placed on the sample holder of the attenuated total reflectance Fourier transform infrared spectrometer (ATR-FTIR, Shimadzu FTIR-8400S, Japan). A 100 scans were taken and the transmittance was recorded at wavenumbers from 4000 cm^{-1} to 400 cm^{-1} at resolution of 4 cm^{-1}. The spectra were converted into absorbance mode before baseline correction and reading of the absorption at the maxima of peaks at 1740 cm^{-1} (due to ester carbonyl group (C=O) stretching) and at 1630-1600 cm^{-1} (due to carboxylate group (COO^-)). Since peak intensities at 1530 and 1650 cm^{-1} were detected due to the presence of proteins, peak splitting was performed. After peak splitting, the obtained ratio (*R*) between the peak intensity at 1740 cm^{-1} to the sum of the peak intensities at 1740 cm^{-1} and 1630-1600 cm^{-1} was

used to predict the DM of the samples based on the calibration line: DM (%) = 123.45 x R + 6.59 (Kyomugasho et al., 2015a).

2.5.4.2 Degree of acetylation

The degree of acetylation (DAc) was measured using a Megazyme kit (K-ACETRM, Ireland) as described by Santiago et al. (2016). DAc is defined as the molar ratio of the acetyl groups to galacturonic acid.

2.5.4.3 Molecular weight distribution and intrinsic viscosity

The molecular weight and intrinsic viscosity of pectic polymers in the sera were analyzed using size exclusion chromatography (SEC) with 4 detectors: multi-angle laser light scattering (MALLS) (Postnova analytics, Germany); viscometer (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) at 280 nm to detect the presence of UV absorbing compounds such as proteins (Shpigelman et al. 2014; Shpigelman et al. 2015). Lyophilized serum of 0.5% w/v was dissolved in 0.1 M acetate buffer with 0.1 M $NaNO_3$, stirred overnight and then filtered through 0.45 µm filter (Millex-HV). Exactly 100 µl was injected to a series of three Waters columns (Waters, Milford, MA), namely, Ultrahydrogel 250, 1000 and 2000 with exclusion limits of 8 x 10^4, 4 x 10^6, and 1 x 10^7 g/mol, respectively. The columns were kept at 35 °C and the eluent (0.1 M acetic acid buffer with 0.1 M $NaNO_3$) flow rate was 0.5 ml/min. A dn/dc value of 0.146 ml/g was used to calculate the molar mass distribution. Considering the detected concentration of the polymers and the LS signals recorded at 21 different angles, the molecular weight was calculated using the Debye fitting method (second order) by the software provided by the manufacturer of the MALLS detector (NovaMals, version 1.2.0.0, Postnova analytics, Germany). Samples were analyzed in duplicate.

3. Results and discussion

3.1 Influence of mechanical treatment and endogenous pectin-related enzymes on the macroscopic and mesoscopic properties of the purées

3.1.1 Bostwick consistency

The Bostwick consistency is often used to describe the flow behavior of semi-solid foods. It commonly represents the apparent viscosity and the ability to hold the solid fraction in suspension thereby attaining a homogenous product (Porretta, 1996; Barret, Garcia and Wayne, 1998). In this context, the interaction of charged molecules as influenced by the pH is important (Moelants et al., 2014a; Jamsazzadeh Kermani et al., 2016). The pH of the differently prepared tomato purées was 4.34 ± 0.05, which is comparable with the reported pH of tomato dispersions (Anthon, Lestrange, & Barrett, 2011; Houben et al., 2014). Figure 2 shows the Bostwick consistency indices of the different tomato purées. There was no observed separation of the serum and particle phases (syneresis) in all the differently prepared purées indicating the homogeneity of the dispersed particles in the continuous serum. Negligible syneresis in blanched (95 °C for 8 min) tomato purée was also observed in the work of Houben et al. (2014). In terms of consistency, it can be observed that the non-homogenized (blended only) and the high pressure homogenized tomato purées showed disparate flow behaviors. Generally, on the one hand, purées that were only blended showed higher Bostwick consistency indices indicating less resistance to flow. On the other hand, the high pressure homogenized purées exhibited lower Bostwick consistency indices signifying more resistance to flow. This result confirms that HPH significantly increases the consistency of tomato dispersions (Thakur, Singh & Handa 1995; Colle et al., 2010; Augusto, Ibarz & Cristianini, 2013; Panozzo et al., 2013). Several researchers inferred that a fiber network ascribed to polymer-polymer interaction is formed upon HPH of tomato purée (Thakur, Singh & Handa 1995; Colle et al., 2010; Panozzo et al., 2013). Gallaher et al. (1999) proposed that

HPH reduces the length of tomato fibers and fibrillate the ends that could absorb and hold greater amounts of liquid in the product, thus increasing the consistency.

Furthermore, the purée treated at 40 °C for 30 min (LT) and non-high pressure homogenized had the lowest consistency. This supports previous observations that the consistency loss of tomato dispersions is due to the synergistic action of the endogenous PME and PG on pectin (Thakur et al., 1996; Lopez et al., 1997; Crelier et al., 2001; Verlent et al., 2007; Tibäck et al., 2009; Houben et al., 2014). Nonetheless, in the current work, we showed that the consistency loss due to the enzymatic pectin degradation was largely restored by HPH (Figure 2). The changes on pectin chemical structure (i.e. de-methyl esterification and de-polymerization) and the consequent effect on the interaction of polymers and formation of a fiber network upon HPH possibly resulted in the consistency increment of homogenized tomato purées. However, this fiber or fiber network whether it only involves particle-particle interactions, soluble polymer interactions in the serum or both still remains to be elucidated. The presence of a particle phase characterized by intact cells and cell fragments, the pectic substances on particle surfaces as well as the soluble pectin in the serum phase may influence the consistency of dispersions (Barrett, Garcia & Wayne, 1998; Moelants et al., 2014). An attempt to relate the consistency of the tomato dispersions to the particle and serum properties are presented in the succeeding sections.

3.1.2 Particle size distribution

The particle characteristics (e.g. size, shape, type) of plant-based food dispersions are primarily dependent on the extent of mechanical tissue disintegration that further contributes to the flow behavior of the product (Colle et al., 2010; Panozzo et al., 2013; Moelants et al., 2014). As shown in Figure 3, the different mechanical disintegration techniques resulted in distinct particle size and particle size distribution. It can be generally observed that all non-high pressure homogenized purées were composed of a broad particle size distribution

characterized by relatively large particles. These non-high pressure homogenized tomato purées had a mean particle diameter of 390 to 490 μm, which is in agreement with the reported mean cell diameter of non-homogenized tomato purées (Lopez-Sanchez et al., 2011; Houben et al. 2014; Panozzo et al., 2013). By contrast, all high pressure homogenized purées were composed of a narrow particle size distribution of smaller particles. These high pressure homogenized purées had mean particle diameter of 70 to 100 μm which is comparable to the results of Lopez-Sanchez et al. (2011) and Panozzo et al. (2013) at similar pressure conditions (100 MPa, single pass). Intense HPH decreases the particle size due to the intense cell breakage resulting in highly disintegrated cells and cell walls that comprise the insoluble tomato pulp (Diaz et al., 2009; Colle et al., 2010; Panozzo et al., 2013; Moelants et al., 2014b). Particle size and its distribution are contributing factors to the consistency of plant-based food dispersions. The non-negligible contribution of the serum phase in the consistency of tomato dispersions has also been recognized (Moelants et al., 2014b). In this context, the combined effects of thermal and mechanical treatments, specifically the influence of HPH, on the serum phase and the structure of serum pectin in tomato purées were not yet investigated and therefore is being focused on in this study.

3.2 Influence of mechanical treatment and endogenous pectin-related enzymes on serum yield and characteristics

3.2.1 Serum phase yields of differently treated purées

The differently prepared tomato purées were centrifuged to isolate the serum phases from the particles. These serum phases were dialyzed (MWCO, 3.5 kDa) and lyophilized to concentrate and obtain the high molecular weight soluble components. The yield of the serum phase after centrifugation as well as the yield of the soluble components after lyophilizing the serum phase expressed as weight percentage of each corresponding purée are presented in Table 1. The control purée (HT+Blending), whereby the tomato pieces were high temperature treated and then blended, showed a serum yield of 79.5%. As shown in Table 1, it can be

observed that when the activity of the endogenous pectin-related enzymes were stimulated and no HPH was applied (HP+Blending+LT+HT and Blending+LT+HT), a higher yield for both serum and soluble components was obtained. This increase in the relative yield of the serum phase due to the endogenous PME (HP+Blending+LT+HT) or both PME and PG (Blending+LT+HT) activities can probably be ascribed on the release of water from the particles into the serum phase (Jamsazzadeh Kermani et al., 2015). As enzymatically induced pectin changes occurred, the release of trapped water by the cell wall network was possibly facilitated. Moreover, it can be generally noticed that the serum phase yields of the high pressure homogenized purées were lower compared to the ones that were only blended. Prior to HPH, blending was initially performed which means that during blending water has been released from the matrix, but was most likely entrapped in the more homogenous small particles and serum upon HPH. This observation seems to correspond with the previously observed high purée consistencies due to HPH as shown in Figure 2. It has been proposed that HPH causes the insoluble tomato fibers to fibrillate thereby resulting in a higher water binding capacity and subsequently increases the product consistency (Gallaher et al., 1999). Recently, Palmero et al. (2016) suggested that the insoluble pectin possibly released during HPH induces the formation of a gel structure that accounts the consistency increment.

In terms of the yield of the high molecular weight components (yield of lyophilized serum) that leached into the serum phase of the purée and remained solubilized after dialysis and centrifugation, it can be observed that high pressure homogenized purées generally contained lower yields. This suggests that as the particle size decreases upon HPH, the interaction between particles and the serum containing soluble polymeric compounds such as pectin possibly increased. This further resulted in higher tendency of the high molecular weight compounds to remain in the particles after centrifugation of the purée. This possible

388 interaction together with water entrapment upon HPH can be the reason for the low serum
389 phase yields of high pressure homogenized purées compared to non-HPH purées.
390 *3.2.2 Sera composition of differently treated purées*
391 The high molecular weight serum components may contribute to the consistency and stability
392 of the purée. Leaching and solubilization of cell wall polysaccharides and cell contents are
393 common during processing (Rickman, Bruhn & Barrett, 2007). To be able to gain an insight
394 into the extent of leaching and the nature of high molecular weight components, the
395 composition of the lyophilized serum was determined. As shown in Table 2, the serum phase
396 contains proteins, sugars and inorganic compounds. The different sugars were identified and
397 quantified after subjecting the dialyzed and lyophilized tomato serum to acid hydrolysis. This
398 means that these different sugars originated from polysaccharides. It can be noticed that
399 uronic acid is the predominant monosaccharide in all serum samples (Table 2), which is in
400 agreement with previous results for tomato serum samples (Moelants et al., 2012;
401 Kyomugasho et al., 2015b). Galacturonic acid (GalA), as the major sugar in tomato serum,
402 usually represents pectin specifically for highly linear and less branched pectin as it is the
403 building block of the homogalacturonan pectic ploysaccharide. A generally lower GalA
404 content in the serum of high pressure homogenized purées compared to their corresponding
405 non-high pressure homogenized purées can be observed. This suggests that a fraction of the
406 previously solubilized pectin (high GalA content) probably remained in the particle phase as
407 gelation of the purée was observed upon HPH. Moreover, besides GalA, lyophilized serum
408 contained major neutral sugars associated to pectin. Arabinose and galactose were found in
409 higher quantities, while rhamnose to a lower extent. Fucose and xylose were also found in
410 minor concentrations. This is in agreement with the reported neutral sugar content in tomato
411 (Kyomugasho et al., 2015b) and carrot (Santiago et al., 2016) sera. These monosaccharides
412 have been reported as the constituents of the rhamnogalacturonan pectic polysaccharide

(Voragen, Beldman & Schols, 2001). In this regard, the total concentrations of fucose, rhamnose, arabinose, galactose and xylose which is referred as pectin-linked neutral sugars (PLNS) was assessed. The sera of HPH purées had generally lower PLNS compared to the non-homogenized samples except for "HT+Blending+HPH". Besides the PLNS, glucose and mannose were also present in considerable amounts. Glucose possibly originated from starch remnants and from fragments of glucose-containing hemicelluloses such as glucomannan together with mannose. The amount of proteins as well as the ash content of the sera were also investigated (Table 2). It can be observed that the sera contained appreciable amounts of proteins as well as inorganic compounds. Generally, both the protein and ash contents of all the high pressure homogenized samples were lower compared to the non-homogenized samples. This corresponds to the previous observations that less quantity of high molecular weight components was obtained in the serum as they were possibly forming a fiber network and therefore were not isolated as part of the serum phase (Table 1). As presented in Table 2, pectin appeared to be the major constituent of the serum phase of the differently prepared tomato purées, thus, our study focuses on the characterization of the chemical structure of serum pectin.

3.2.3 Chemical structure of serum pectin of differently treated purées

3.2.3.1 Degree of methyl-esterification and acetylation

Because the gelling and stabilizing properties of pectin, considered as its prime functionality, are ascribed to the degree of methyl-esterification (DM), the DM of pectic polysaccharides has been recognized as an essential characteristic of pectin (Thakur et al., 1997). The DM is defined as the molar percentage of the methyl groups to galacturonic acid (Schols and Voragen, 2002). Pectin can be classified as low DM when less than 50% of the GalA units are methyl-esterified, while pectin is considered as high DM when more than 50% of GalA units are methyl-esterified. As shown in Table 3, all the serum pectins of the differently prepared

tomato purées are characterized as a low DM pectin. These tomato serum pectin DM values are similar to the result of Tibäck et al. (2009) on tomato pectin as alcohol insoluble residues of tomato purées, but comparably lower to the values reported by Houben et al. (2014) and Moelants et al. (2012). In the present work, initial PME action during the thawing of frozen tomato pieces for 5 min at 25 °C could have occurred that led to the low DM values of some of the samples, besides the possible differences on tomato ripeness and varieties used in the aforementioned studies. Although, all serum pectin samples are classified as low DM, it can be noticed that the DM of the high pressure homogenized samples were generally higher compared to the non-high pressure homogenized samples. It can be hypothesized that the low DM pectin, which can form a gel-network in the presence of divalent ions, contributed to the observed purée gelation upon HPH. In this regard, it can be suggested that upon centrifugation of the purées to isolate the serum phase from the particles, the serum pectin of lower DM was deposited with the particles resulting in the isolation of serum pectin of higher DM.

Besides the DM, the degree of acetylation (DAc) has also been recognized as an important characteristic and functional property determinant of pectin (Leroux et al., 2013). Generally, pectin DAc of fruits and vegetables is ranging from 1.4-1.6% in citrus pectin (Thibault, 1988) to 16-35% in sugar beet pectin (Axelos and Thibault, 1991; Levigne, Ralet, and Thibault, 2002). It can be observed that the tomato serum pectins were relatively mildly acetylated (6.6% to 9.5%).

3.2.3.2 Linearity and extent of branching

To assess the linearity/branching of serum pectin, molar ratios of the pectin associated sugars were defined (Houben et al., 2011). The structure of pectin is assumed with the backbones of RG -I and RG-II being continuous with the linear HG structure (Christiaens et al., 2015). The linearity of pectin was estimated from the molar ratio of the pectic (galact)uronic acid to

neutral sugars (Fuc, Rha, Ara, Gal and Xyl), while, the extent of branching of RG-I is estimated based on the molar ratio of Ara and Gal to Rha (Houben et al., 2011). As displayed in Table 3, the serum pectins of tomato generally exhibited high linearity in agreement with the result of Houben et al. (2011) on the water soluble pectin fraction of tomato purées and Kyomugasho et al. (2015b) on tomato serum pectin. "HT+Blending" and "HT+Blending+HPH" had the most linear pectin in the serum. The tomato serum pectin was generally more linear compared to carrot serum pectin (linearity=0.5-2.0) (Santiago et al., 2016) and water soluble pectin fraction of broccoli florets (linearity=3.1) (Houben et al. 2011). In terms of the extent of branching, it can be observed that the rhamnogalacturonan-I domain of all serum pectins contained similar proportion of arabinose and galactose containing polymers attached to rhamnose that were relatively lower compared with the water soluble pectin fraction of tomato (branching of RG-I=6.8) reported by Houben et al. (2011). The current study obtained a relatively lower degree of branching of RG-I possibly due to the different tomato varieties used and the different treatments applied during purée preparation.

3.2.3.3 Molecular weight distribution and intrinsic viscosity

The molecular weight of polysaccharides affects the flow/textural properties of fluid/semi-solid foods (Harte & Venegas, 2010). The absolute weight average molecular weight (M_w) of the serum polymers was determined based on the hydrodynamic volume of polymers eluted from the size exclusion column at a particular time. In this context, the large polymers eluted earlier from the column while the small polymers eluted later. The M_w distribution profile and the concentration chromatograms are presented in Figure 4a, while the corresponding light scattering profile superimposed with the UV absorbance chromatograms are shown in Figure 4b. It can be observed that the serum polymers had three polymer populations with high (35-48 min), medium (48-53 min) and low (53-62 min) M_w as depicted by the three peaks of the concentration chromatogram at ~35 min to ~62 min (Figure 4a,

488 Table 4). The elution time of pectic polymers is situated from ~30 to 62 min, hence the fourth
489 peak of the concentration chromatogram was excluded in calculating the M_w of the serum
490 polymers (Shpigelman et al., 2015). The varying hydrodynamic properties of the serum
491 polymers generally suggest the solubilization of different pectic populations from the particle
492 into the serum phase upon thermal treatment, enzyme activity stimulation (for HP pre-treated
493 and LT treated samples) and the mechanical tissue disruption (Houben et al., 2014).
494 "HP+Blending+LT+HT" purée, for which the stimulation of endogenous PME activity was
495 aimed, had generally lower M_w serum polymers compared to "HT+Blending" purée.
496 However, when the stimulation of both endogenous PME and PG activities was targeted
497 (Blending+LT+HT purée) serum polymers had the lowest M_w (Table 4). This shows that the
498 endogenous PG de-polymerized the enzymatically de-methylesterified pectin that
499 consequently resulted in a decreased M_w (Houben et al., 2014a; Moelants et al., 2014).
500 By contrast, a remarkable increase in the M_w of the enzymatically de-polymerized serum
501 pectic polymers was observed upon HPH. Specifically, serum polymers of
502 "HP+Blending+LT+HT+HPH" and "Blending+LT+HT+HPH" purées had extremely higher
503 M_w compared to the non-high pressure homogenized purées (Table 4). At the same elution
504 time, an extremely high M_w serum polymer was found in "HP+Blending+LT+HT+HPH"
505 purée that may suggest a change in pectin conformation. It can be hypothesized that the
506 intense shear induced by HPH promoted the interaction between serum pectic polymers
507 which, in turn, changed their structural conformation. Increased polymer-polymer interaction
508 has already been suggested to result in conformational changes of pectic polymers (Diaz et
509 al., 2009). Furthermore, as the serum pectins were low methyl-esterified, an increased
510 tendency for their aggregation as well as association with endogenous divalent ions upon
511 HPH is plausible. However, as the UV chromatogram does not clearly reflect an absorbance
512 at elution time of ~34.9 min to 53.8 min, it is unlikely that such pectins are associated with

513 UV absorbance charged polymers such as proteins (Figure 4b). Shpigelman et al. (2015b)
514 inferred that the effect of DM on the M_w of polymers is possibly due to changes in the
515 strength of intermolecular ionic repulsions and attractive forces between the chains.
516 As presented in Table 4, the intrinsic viscosities of the serum polymers under region I (highest
517 M_w) that accounts to 12 to 32% of the mass found were considerably higher compared to the
518 other two regions of medium and low M_w serum polymers. These intrinsic viscosity
519 (3.78-7.68 dL/g) values under region I is within the range of intrinsic viscosities of
520 differently extracted citrus pectin (4.05-7.73 dL/g) reported by Kaya et al. (2014). However,
521 the M_w of pectin in our study was higher than the (citrus) pectin in their study. Moreover, it is
522 generally noticeable that the intrinsic viscosities of the serum polymers of HPH purées were
523 rather lower compared to the non-homogenized samples (Table 4). As serum pectin of HPH
524 treated purées had higher M_w compared to non-HPH purées, it can be concluded that HPH of
525 tomato purées resulted in compact polymer conformation due to increased polymer-polymer
526 interactions.

527 **Conclusion**

528 Intense high pressure homogenization (HPH) and stimulation of endogenous pectin-related
529 enzymes resulted in different tomato purée properties (e.g. consistency) with distinct serum
530 pectin structures. It was shown that the consistency loss of tomato purées due to enzymatic
531 pectin degradation can be at least restored and improved by intense HPH. The stimulation of
532 endogenous PME and PG activities without consequent HPH treatment increased the serum
533 phase yield as well as total serum pectin. This indicated the release of entrapped water and
534 solubilization of pectin from the particles into the serum. However, intense HPH resulted in
535 low serum phase yield and lower amounts of pectin besides a reduction and uniformity of the
536 particle size.

In terms of serum pectin structure, tomato serum pectin is characterized as linear/less branched. The endogenous pectin-related enzymes decreased the serum pectin DM and its M_w. By contrast, HPH induced the solubilization of higher DM and high apparent M_w serum pectin. It can be hypothesized that the exceptionally high apparent M_w serum pectins of the homogenized purées is possibly a result of soluble polymer-polymer interaction that formed a compact conformation upon HPH. However, the underlying mechanisms of these observed high M_w serum pectins and increased purée consistency due to HPH still needs to be further investigated conjointly with the particle phase. The serum phase characteristics and the high M_w serum pectins of the high pressure homogenized purées at least partly explain the purée consistency increment. Nonetheless, this work clearly demonstrates that intense HPH can partly restore and increase the consistency of tomato purées in spite of the initial consistency loss ascribed to enzymatic pectin degradation.

Acknowledgements

This research has been carried out with the financial support of the Research Foundation Flanders (FWO grant G.0D17.14N) and the KU Leuven Research Council (METH /14/03) through its long term structural funding program−Methusalem funding by the Flemish Government. Zahra Jamsazzadeh Kermani is a postdoctoral researcher funded by KU Leuven internal funds (PDM/15/159).

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Figure captions

Figure 1. Schematic overview of the different purée preparation conditions. (HT: high temperature treatment; HP: high pressure pre-treatment; LT: low temperature treatment; HPH: high pressure homogenization)

Figure 2. Bostwick consistency index (± standard deviation) of the differently prepared tomato purées. HT: high temperature treatment (95 °C for 30 min); HPH: high pressure homogenization (100 MPa); HP: high pressure pre-treatment (550 MPa for 10 min, 4 °C); LT: low temperature treatment (40 °C for 30 min)

Figure 3. Volumetric particle size distribution of the differently prepared tomato purées. HT: high temperature treatment (95 °C for 30 min); HPH: high pressure homogenization (100 MPa); HP: high pressure pre-treatment (550 MPa for 10 min, 4 °C); LT: low temperature treatment (40 °C for 30 min)

Figure 4. Size exclusion elution profile of serum polymers of the differently prepared tomato purées (**a**) log molar mass (thick solid line) against elution volume superimposed on concentration chromatogram (square dot curve) (**b**) Light scattering signal at 92° angle (solid curve) superimposed on UV absorbance chromatogram at 280 nm (round dot curve). HT+Blending (black); HT+Blending+HPH (grey); HP+Blending+LT+HT (dark blue); HP+Blending+LT+HT+HPH (light blue); Blending+LT+HT (dark red); Blending+LT+HT+HPH (light red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

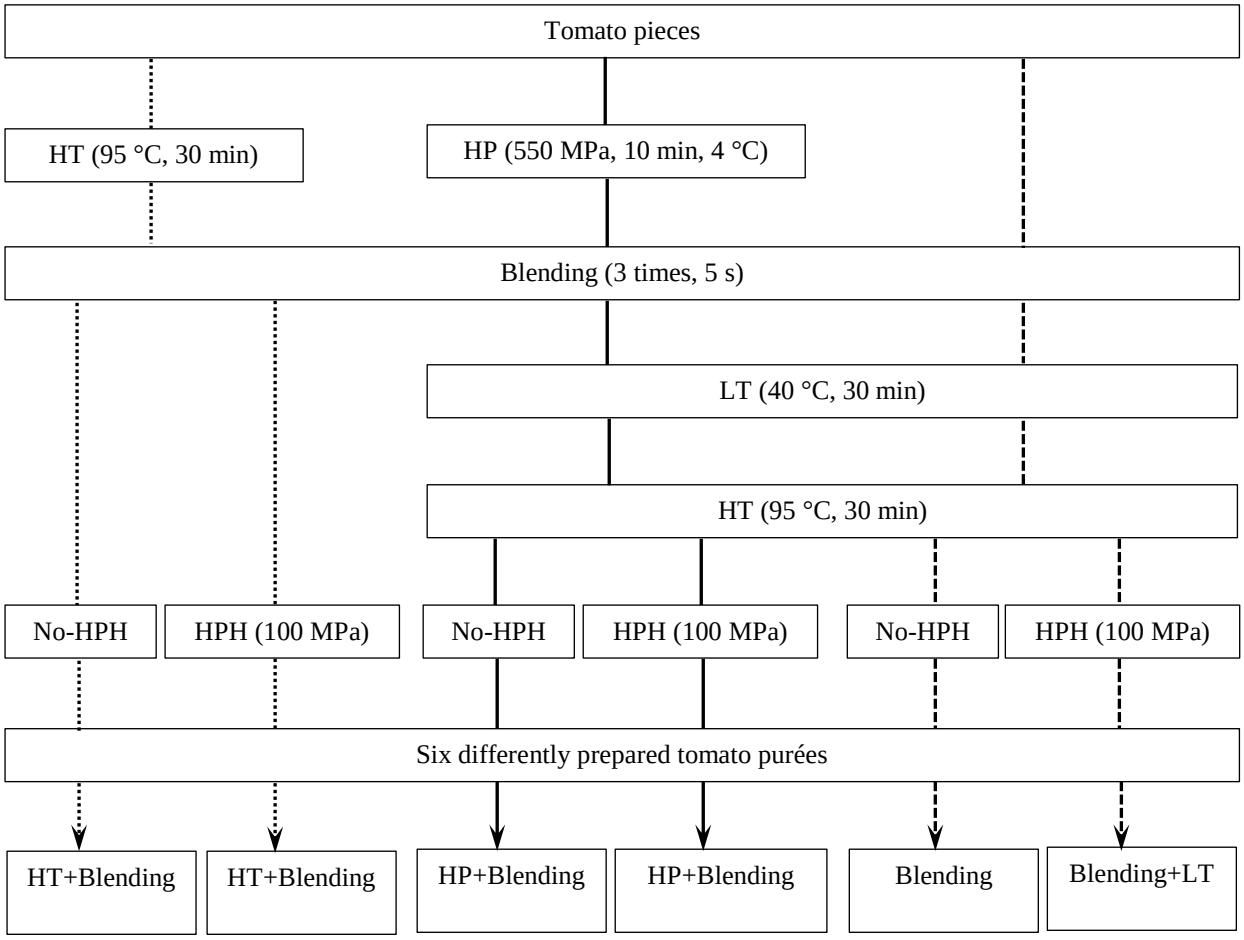
Tomato pieces
HT (95 °C, 30 min)
HP (550 MPa, 10 min, 4 °C)
Blending (3 times, 5 s)
LT (40 °C, 30 min)
HT (95 °C, 30 min)
No-HPH
HPH (100 MPa)
No-HPH
HPH (100 MPa)
No-HPH
HPH (100 MPa)
Six differently prepared tomato purées
HT+Blending
HT+Blending
HP+Blending
HP+Blending
Blending
Blending+LT

Figure 1.

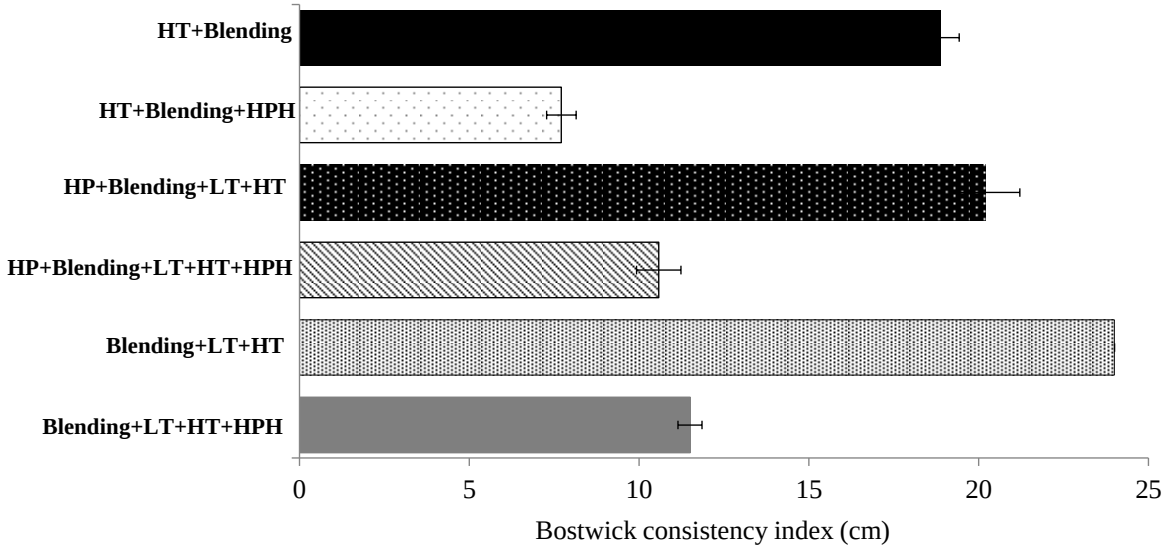
HT+Blending
HT+Blending+HPH
HP+Blending+LT+HT
HP+Blending+LT+HT+HPH
Blending+LT+HT
Blending+LT+HT+HPH
0
5
10
15
20
25
Bostwick consistency index (cm)

Figure 2.

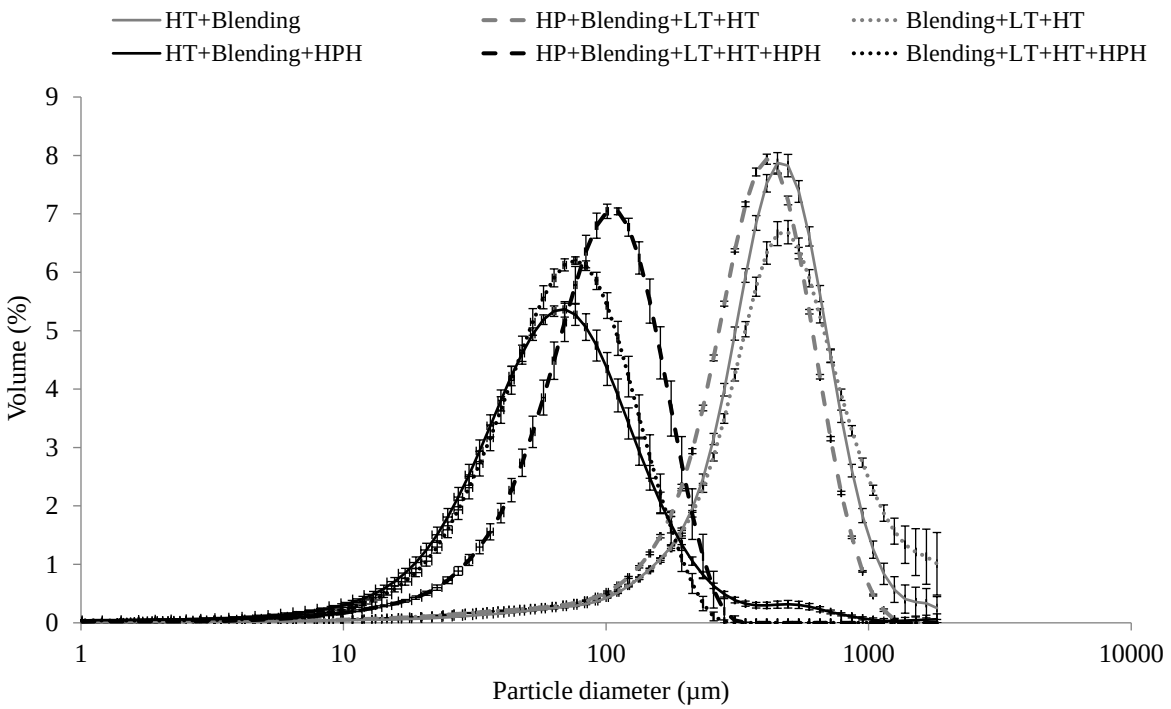
HT+Blending
HP+Blending+LT+HT
Blending+LT+HT
HT+Blending+HPH
HP+Blending+LT+HT+HPH
Blending+LT+HT+HPH
Volume (%)
0
1
2
3
4
5
6
7
8
9
1
10
100
1000
10000
Particle diameter (µm)

Figure 3.

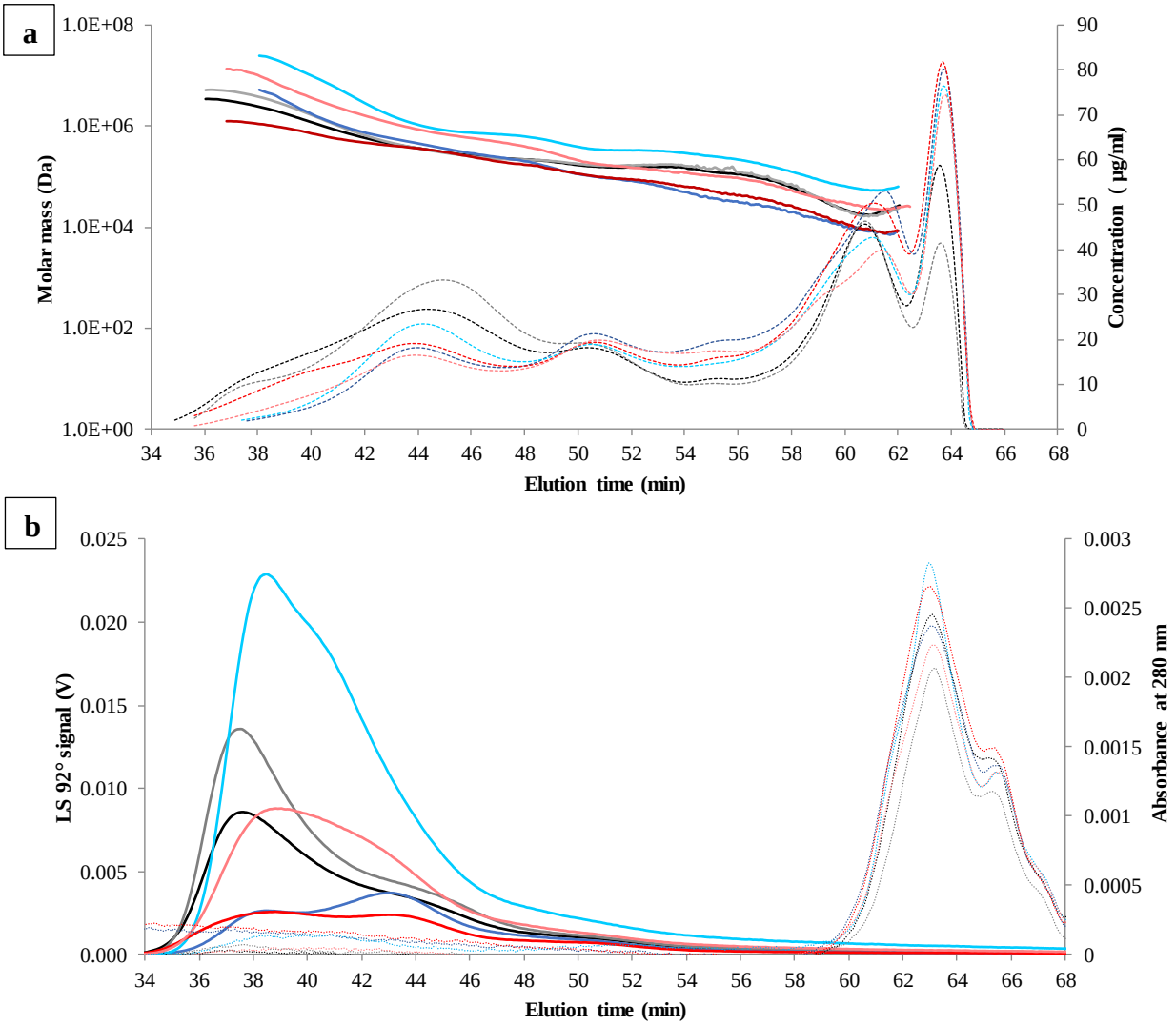

Figure 4. (**a**) and (**b**)

Table 1. Yield of the serum phase and high molecular weight components expressed per 100 g purée. HT: high temperature treatment (95 °C for 30 min); HPH: high pressure homogenization (100 MPa); HP: high pressure pre-treatment (550 MPa for 10 min, 4 °C); LT: low temperature treatment (40 °C for 30 min)

Sample	Yield serum phase (g /100 g purée)	Yield lyophilized serum (g/100 g purée)
HT+Blending	79.5 ± 3.1	0.194 ± 0.0
HT+Blending+HPH	71.0 ± 2.6	0.183 ± 0.0
HP+Blending+LT+HT	83.5 ± 1.4	0.263 ± 0.0
HP+Blending+LT+HT+HPH	61.4 ± 1.4	0.141 ± 0.0
Blending+LT+HT	86.9 ± 1.0	0.197 ± 0.0
Blending+LT+HT+HPH	63.6 ± 2.7	0.136 ± 0.0

Table 2. Solubilized serum components of the differently prepared tomato purées expressed in µg/g purée. HT: high temperature treatment (95 °C for 30 min); HPH: high pressure homogenization (100 MPa); HP: high pressure pre-treatment (550 MPa for 10 min, 4 °C); LT: low temperature treatment (40 °C for 30 min)

Sample	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Xylose	Mannose	Uronic acid	PLNS	Total serum pectin	Crude protein	Ash
HT+Blending	1.5 ± 0.0	27.7 ± 0.0	68.6 ± 0.0	63.1 ± 0.0	156.4 ± 0.1	1.0 ± 0.0	9.3 ± 0.0	841.3 ± 0.1	176.8 ± 0.1	1018.1 ± 0.2	212.4 ± 0.0	267.1 ± 0.0
HT+Blending+HPH	1.2 ± 0.0	30.9 ± 0.0	77.9 ± 0.0	70.9 ± 0.0	91.0 ± 0.0	1.9 ± 0.0	3.1 ± 0.0	827.3 ± 0.1	197.4 ± 0.0	1024.7 ± 0.2	196.7 ± 0.0	205.8 ± 0.0
HP+Blending+LT+ HT	2.0 ± 0.0	51.2 ± 0.0	131.2 ± 0.0	139.3 ± 0.0	197.9 ± 0.0	0.6 ± 0.0	2.3 ± 0.0	1071.2 ± 0.0	353.1 ± 0.0	1424.3 ± 0.0	309.2 ± 0.0	373.0 ± 0.0
HP+Blending+LT+ HT+HPH	1.0 ± 0.0	26.0 ± 0.0	66.9 ± 0.0	58.9 ± 0.0	99.4 ± 0.0	2.4 ± 0.0	0.4 ± 0.0	540.5 ± 0.1	175.0 ± 0.0	715.5 ± 0.1	150.6 ± 0.0	213.8 ± 0.0
Blending+LT+HT	1.7 ± 0.0	31.4 ± 0.0	82.6 ± 0.0	77.6 ± 0.0	156.4 ± 0.0	0.9 ± 0.0	4.5 ± 0.0	813.2 ± 0.0	211.7 ± 0.0	1024.9 ± 0.0	204.7 ± 0.0	335.0 ± 0.0
Blending+LT+HT+ HPH	1.1 ± 0.0	21.1 ± 0.0	56.2 ± 0.0	49.4 ± 0.0	101.0 ± 0.0	0.5 ± 0.0	1.4 ± 0.0	539.3 ± 0.1	140.5 ± 0.1	679.8 ± 0.1	150.2 ± 0.0	208.7 ± 0.0

Table 3. The chemical structure of tomato serum pectin as influenced by high pressure homogenization and endogenous pectin related enzymes. HT: high temperature treatment (95 °C for 30 min); HPH: high pressure homogenization (100 MPa); HP: high pressure pre-treatment (550 MPa for 10 min, 4 °C); LT: low temperature treatment (40 °C for 30 min)

Sample	% DM	% DAc	Linearity of pectin (UA/Fuc+Rha+Ara+Gal+Xyl)	Branching of RG-I (Ara+Gal/Rha)
HT+Blending	27.0 ± 0.8	6.6 ± 0.6	4.1 ± 1.0	4.8 ± 0.4
HT+Blending+HPH	35.9 ± 1.1	7.2 ± 0.4	4.2 ± 1.1	4.5 ± 0.1
HP+Blending+LT+HT	18.4 ± 0.6	8.6 ± 0.2	2.6 ± 0.4	5.4 ± 0.6
HP+Blending+LT+HT+HPH	21.3 ± 1.7	9.5 ± 0.3	2.6 ± 0.2	4.9 ± 0.4
Blending+LT+HT	13.5 ± 2.4	7.8 ± 0.2	3.2 ± 0.3	5.1 ± 0.3
Blending+LT+HT+HPH	20.0 ± 1.2	8.3 ± 0.3	3.3 ± 1.1	5.1 ± 0.3

Table 4. The weight average molecular weight of the regions with three clear peaks observed at the concentration chromatogram, the intrinsic viscosity of the serum polymers in each corresponding region and the fraction of the mass found relative to the mass of the sample prior to filtration as calculated from the SEC-MALS-RI-viscometer. HT: high temperature treatment (95 °C for 30 min); HPH: high pressure homogenization (100 MPa); HP: high pressure pre-treatment (550 MPa for 10 min, 4 °C); LT: low temperature treatment (40 °C for 30 min)

	Mw (kDa)			Intrinsic viscosity (dL/g)			Fraction of mass found		
Sample	Region I	Region II	Region III	Region I	Region II	Region III	Region I	Region II	Region III
HT+Blending	768.5 ± 297.7	166.5 ± 37.5	45.6 ± 14.9	7.68 ± 3.52	0.91 ± 0.15	0.06 ± 0.01	0.29 ± 0.03	0.10 ± 0.01	0.23 ± 0.00
HT+Blending+HPH	951.5 ± 54.4	182.0 ± 32.5	49.1 ± 13.4	5.36 ± 0.27	1.03 ± 0.03	0.08 ± 0.01	0.32 ± 0.01	0.11 ± 0.01	0.24 ± 0.01
HP+Blending+LT+HT	722.5 ± 299.1	121.5 ± 29.0	18.1 ± 8.9	4.37 ± 1.79	0.76 ± 0.06	0.08 ± 0.02	0.12 ± 0.02	0.13 ± 0.02	0.32 ± 0.04
HP+Blending+LT+HT+HPH	2710.0 ± 169.7	370.5 ± 41.7	110.9 ± 17.2	3.78 ± 0.04	0.71 ± 0.01	0.08 ± 0.01	0.18 ± 0.01	0.10 ± 0.00	0.27 ± 0.01
Blending+LT+HT	378.0 ± 22.6	77.1 ± 10.6	10.1 ± 0.5	6.73 ± 3.48	0.67 ± 0.29	0.06 ± 0.02	0.21 ± 0.08	0.11 ± 0.02	0.32 ± 0.10
Blending+LT+HT+HPH	1500.0 ± 410.1	126.4 ± 159.3	50.3 ± 27.9	5.20 ± 3.44	0.72 ± 0.04	0.10 ± 0.01	0.15 ± 0.00	0.11 ± 0.01	0.26 ± 0.01

Region I: ~36 min to 48.5 min

Region II: ~48.5 min to 53.8 min

Region III: ~53.8 min to 62.5 min