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Effect of Ca$^{2+}$ addition?
Effect of Calcium Ions and pH on the Structure and Rheology of Carrot-Derived Suspensions

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Abstract

In the present work, the role of calcium ions (Ca\(^{2+}\)) in the rheological behaviour of carrot-derived purées was investigated. Therefore, purées based on carrots containing pectin with different degree of methoxylation were prepared and the effects of Ca\(^{2+}\) addition in excess on the rheological properties of these purées were studied at different pH. More specifically, it was assessed if the purée stiffness and strength could be influenced by Ca\(^{2+}\) addition. Ion addition caused a decrease in both network stiffness and strength, in particular at pH values above 4.5. By separating the particle phase from the serum, and characterizing the rheology of both phases as a function of pectin degree of methoxylation, Ca\(^{2+}\) addition in excess and pH, it was concluded that the particle phase rather than the serum phase is affected by ion addition. Immunolabeling of the carrot-derived particles with anti-pectin antibodies showed the presence of non-methoxylated residues at the particle surfaces, which will be charged at specific pH. Hence, the calcium ions may compress the electrical double layer around the particles whereby they can approach each other more closely. The latter mechanism was confirmed by the relation between the phase volume and the rheological parameters. Rather than being involved in Ca\(^{2+}\) cross-link formation thus enhancing the pectin network in carrot-derived purées, it turned out that Ca\(^{2+}\) screens the negatively charged pectin at the surface of the particles whereby the rheological characteristics of these suspensions, such as the yield stress and storage modulus, are reduced and the flow is facilitated.

Keywords

Rheology, Carrot, Suspension, pH, Pectin, Calcium ion
**Abbreviations**

Ca$^{2+}$, calcium ions; DM, degree of methoxylolation; FITC, fluorescein isothiocyanate; GalA, galacturonic acid; HM, high-methoxylated; HT, heat treatment; LM, low-methoxylated; MM, molar mass; MPBS, phosphate-buffered saline containing 5% milk powder; PBS, phosphate-buffered saline; PME, pectin methylesterase

1. Introduction

Pectin is a collection of galacturonic acid (GalA) containing polysaccharides present in the cell wall of mainly dicotyledonous plants (Thakur et al., 1997). It is traditionally applied as gelling agent in jams and jellies, but nowadays its application is extended to a wide range of food products in e.g. dairy and soft-drink industry (May, 1990). GalA, present in the backbone of this macromolecule, can be methoxylated. The degree of methoxylation (DM) is defined by the amount of methylesters occurring on these GalA residues of the backbone. Pectins can be divided in high-methoxylated (HM) pectins with a DM > 50% and low-methoxylated (LM) pectins with a DM < 50%. HM pectin is able to from a network in the presence of high sugar concentrations and a pH below 4. LM pectin, on the other hand, can form a gel in the presence of divalent or trivalent ions, such as calcium ions (Ca$^{2+}$), and optimally at pH values well above the pK$_a$ of GalA since the carboxyl groups are dissociated under these conditions (Morris et al., 1982; Thakur et al., 1997; Thibault & Ralet, 2003). In Ca$^{2+}$ pectin gelation, adjacent pectin chains containing contiguous non-methoxylated GalA residues are cross-linked via Ca$^{2+}$ bridges as described by the egg-box model (Grant et al., 1973; Powell et al., 1982). Consequently, only non-methoxylated portions of the pectin backbone can be involved in Ca$^{2+}$ cross-link formation. It was already frequently demonstrated that gelling properties of pectins strongly depend on their DM (Fraeye et al., 2010). Beside DM, other pectin properties as e.g. the polymer average molar mass (MM) and the methyl ester distribution pattern were shown to play an important role in Ca$^{2+}$ pectin gelation (Ström et al., 2007; Fraeye et al., 2009; Ngouémazong et al., 2012a; Ngouémazong et al., 2012c). In addition to intrinsic parameters, also system conditions as e.g. pectin concentration, ion concentration or pH turned out to influence Ca$^{2+}$ pectin network formation (Fraeye et al., 2009; Fraeye et al., 2010; Ngouémazong et al., 2012a). Although the
effect of Ca$^{2+}$ addition is well investigated in pectin/H$_2$O systems, studies investigating the
effects of Ca$^{2+}$ addition in pectin containing plant-tissue-based food purées are currently missing.
These suspensions consist of plant-tissue-based particles in a continuous serum phase with
(among others) pectin, sugars and organic acids solubilised in it (Rao, 1987; Anthon et al., 2008).
Pectin at the particle surfaces or in the serum of plant-tissue-based food suspensions can be
charged, leading to electrostatic interactions between the plant-tissue-based particles. Besides the
possible effect of divalent ions on the gelling properties of pectin, the presence of ions may also
screen the electrostatic charges causing changes in the rheological properties of plant-tissue-
based food suspensions. In this context, Whittenberger and Nutting (1958) and Redgwell et al.
(2008) reported a decrease in the viscosity of cell-wall suspensions when electrolytes were
added.
In the present study, the effects of calcium ion addition in excess on the rheology of carrot-
derived suspensions were investigated. These suspensions were prepared from carrot tissue
containing especially HM pectin (cf. HT1) as well as from carrot tissue with especially LM
pectin (cf. HT2). The pH of the suspensions was adjusted to pH 3, 5 or 7, meaning beyond,
somewhat higher than and well above the pK$_a$ value of GalA, respectively (Ralet et al., 2001) to
influence among others conversion of pectin into pectinate and vice versa. More severe alkaline
or acidic conditions would lead to pectin changes (Van Buren, 1979; Voragen et al., 2009; Sila et
al., 2009) and would be irrelevant for food applications. Although carrots contain an intrinsic
amount of Ca$^{2+}$, extra Ca$^{2+}$ was added to a part of the samples, more than sufficient to saturate all
COO$^-$ groups (estimated from the GalA amount and the DM of the carrots) present on the carrot
pectin. Since pectin is present in both the serum and particle phases, Ca$^{2+}$ addition may affect
both particle and serum characteristics. Although many investigations on the effect of Ca$^{2+}$
addition to gelation of pectin solutions were performed, little is known about the role of Ca$^{2+}$ in
plant-tissue-based purées containing intrinsic pectin.
The obtained knowledge would allow industry to tailor the rheological properties (and
mouthfeel) of vegetable-based food suspensions by utilizing the structure determining role of
pectin intrinsically present in vegetables rather than adding commercial pectin.

2. Materials and methods
2.1 Plant material and preparation of carrot purée from carrot tissue with low and high degree of methoxylation

Fresh carrots (*Daucus carota* cv. Nerac) were purchased from a local supplier in Belgium and stored at 4 °C until further use. Carrots were peeled, cut into pieces and vacuum-packed in plastic bags to avoid loss of solubilised pectin in the heating medium. A part of the vacuum-packed pieces was heated in a temperature-controlled water bath at 95 °C for 5 min (to inactivate intrinsic enzymes) (referred to as HT1). The other part was first pretreated at 60 °C for 24 h (to stimulate pectin methylesterase (PME) activity) whereafter intrinsic enzymes were inactivated at 95 °C for 5 min (referred to as HT2). After the heat treatment, samples were immediately cooled in an ice water bath. HT1 and HT2 were designed to obtain pectin with a different DM. Subsequently, heat-treated carrot pieces were mixed with deionised water in a 1:1 ratio to facilitate the blending process, which was performed during 1 min using a kitchen blender (Waring blender 7010G, Torrington, CT, USA), resulting in a pulp percentage of 50 wt.%. The two different carrot blends were further disintegrated with a high-pressure homogeniser (Panda 2K, Gea Niro Soavi, Mechelen, Belgium) at 100 MPa to obtain two carrot purées differing only in the applied heat treatment, and consequently in degree of methoxylation.

The preparation of both carrot purées with different DM was repeated twice using different batches of carrots from the same variety. For the carrot purées prepared after receiving HT2, batch differences turned out to be more outspoken. Hence, for HT2 a third preparation was carried out.

2.2 Preparation of carrot purées with different pH and amount of Ca\(^{2+}\)

Carrot purées with different DM were prepared as described in Section 2.1. Extrinsic calcium ions (6.7 mL of 5.1 M CaCl\(_2\cdot2H_2O\) solution for 100 mL purée) were added to a part of both purées. Samples were well mixed to assure a homogeneous Ca\(^{2+}\) distribution. Subsequently, the pH of the resulting carrot purées was adjusted to pH 3, 5 and 7 with 1 M HCl or 1 M NaOH. The 12 original carrot purées with different DM of pectin, pH and amount of Ca\(^{2+}\) were stored at 4 °C until further analysis (maximally one week). Fig. 1 gives a schematic overview of this preparation process.
To study the effect of ionic strength on the rheological properties of carrot-derived purées (only for HT2), the ionic strength of carrot-derived purées with a pH of 5 was adjusted by the addition of CaCl₂ or NaCl solutions with different ion concentration.

2.3 Preparation of reconstituted carrot-derived suspensions with different pH and amount of Ca²⁺

Carrot purées with two different DM were prepared as described in Section 2.1. Subsequently, using the carrot purées of the first batch, the carrot pulp was separated in particle fractions with different particle sizes by using the technique of wet sieving (Retsch, Aartselaar, Belgium) with a set of sieves with pore sizes of 40, 80, 125, 250, 500 and 1000 µm. Pulp was drained over a filter (Macherey-Nagel 615 ¼, pore size of 8 µm) to remove excess water until constant weight was reached. Part of each sieve fraction, intended for microscopic investigation, was preserved in 70% ethanol. The remaining pulp was reassembled such that the mass percentage of each particle fraction was the same as in the particle phase of the original purées. Subsequently, the pulp was reconstituted with deionised water (50 wt.%, as in the original carrot purée). Extrinsic Ca²⁺ ions (6.7 mL of 5.1 M CaCl₂·2H₂O solution for 100 mL purée) were added to one part of both reconstituted suspensions with different DM. Samples were well mixed to assure a homogeneous Ca²⁺ distribution. Subsequently, the pH of the resulting reconstituted suspensions was adjusted to pH 3, 5 and 7 with 1 M HCl or 1 M NaOH. This resulted in 12 reconstituted suspensions with different pectin DM, pH and amount of Ca²⁺. In Fig. 1, this preparation process is schematically shown.

2.4 Preparation of carrot sera with different pH and amount of Ca²⁺

Part of the carrot purées with pectin of different DM, prepared as described in Section 2.1, were centrifuged for 30 min at 12400 × g at 20 °C and the obtained supernatant was vacuum-filtered (MN 615, pore size of 8 µm) to separate the pulp from the serum phase, based on the procedure of Caradec et al. (1985). Extrinsic Ca²⁺ ions (1 g CaCl₂·2H₂O for 10 mL serum) were added to half of the samples. Ca²⁺ ions were added in excess to assure saturation of all COO⁻ groups present on the serum pectin. Subsequently, the samples were well mixed to obtain a homogeneous Ca²⁺ distribution. The pH of the resulting sera was adjusted to pH 3, 5 and 7 with
1 M HCl or 1 M NaOH. This resulted in 12 sera with different pectin DM, pH and amount of Ca$^{2+}$. The preparation of different carrot sera starting from carrot purée is shown in Fig. 1.

2.5 Determination of the degree of methoxylation of carrot purée

The DM of pectin in each purée (prepared as described in section 5.2.1) was estimated as the ratio of the molar amount of methoxyl groups to the molar amount of GalA. First, the cell wall material was isolated as an alcohol-insoluble residue (AIR), based on the method described by McFeeters and Armstrong (1984). Approximately 30 g of carrot purée was homogenised in 192 mL 95% (v/v) ethanol using a mixer (Buchi mixer B-400, Flawil, Switzerland). The suspension was filtered (MN 615, pore size of 8 µm) and the residue was rehomogenised in 96 mL 95% (v/v) ethanol. After another filtration step, the residue was homogenised in 96 mL acetone. A final filtration resulted in the AIR which was dried overnight at 40 °C, ground using a mortar and pestle and stored in a desiccator until further use.

An estimate of the GalA content was obtained through hydrolysis of the pectin in AIR with concentrated sulphuric acid (Ahmed & Labavitch, 1978) followed by quantification of the GalA in solution according to the spectrophotometric method described by Blumenkrantz and Asboe-Hansen (1973). To estimate the methoxyl content, the ester-bonds of pectin in AIR were hydrolysed by an alkaline treatment using 2.0 M NaOH (Ng & Waldron, 1997) followed by quantification of the released methanol according to the spectrophotometric method of Klavons and Bennett (1986). The respective hydrolyses were performed in duplicate for both procedures and three colorimetrical analyses were carried out for each hydrolysate.

2.6 Determination of the calcium concentration in carrot purée

The amount of calcium intrinsically present in carrot purée was determined by titration with the chelating agent ethylenediaminetetraacetic acid (EDTA). For both carrot purées (prepared as described in section 5.2.1), 1 g of purée was added to a flask of 100 ml and diluted with deionised water. 90 mL of the diluted purée was mixed with 10 mL 1 M NaOH and a pinch of NaCl-murexide. Subsequently, the calcium concentration in the purée was determined by EDTA titration (0.001 M).
2.7 Rheological characterisation of the carrot-derived suspensions

The rheological properties of the suspensions were measured with a stress-controlled rheometer (MCR 501, Anton Paar, Graz, Austria) at 25 °C. A six-bladed vane geometry with a diameter of 22 mm and a height of 16 mm was used to avoid wall slip. Approximately 50 mL of sample was loaded into a cup with 28.92 mm diameter. As it is known that a shear rate distribution occurs by the use of a vane geometry, all the shear rates indicated are representative shear rates. Because of the relatively short duration of each measurement (i.e. 20-30 min), evaporation was considered negligible and no significant sedimentation was noticeable. To avoid the effect of loading history on the structure, samples were presheared for 1 min at a shear rate ($\dot{\gamma}$) of 100 s$^{-1}$ followed by 2 min of rest ($\dot{\gamma} = 0$ s$^{-1}$) before all measurements. Heating (10 min at 80 °C) the samples prior to or immediately after Ca$^{2+}$ addition before measuring rheology and during the preshear step did not result in the expected increase in gel strength of the samples investigated in this study.

The rheological characteristics of the suspensions were studied by the execution of steady-shear tests (for the estimation of viscosity and yield stress) and small amplitude oscillatory tests (to study the viscoelastic behaviour). The shear rate ramp, stress ramp, strain and frequency sweep were performed as described by Moelants et al. (2013a). The flow curves (shear stress versus shear rate: $\sigma = f(\dot{\gamma})$) and the viscosity curves (viscosity versus shear rate: $\eta = f(\dot{\gamma})$) were measured by decreasing shear rate linearly from 100 to 0.1 s$^{-1}$. Each shear rate was applied to the sample for 20 s and it was verified that steady-state viscosities were obtained in this way. The static yield stress was determined by the conduction of a stress ramp test starting from 0.1 Pa until the yield stress was reached. In total, 40 measuring points per decade of the shear stress were obtained and each shear stress was applied for 10 s. For each sample, an oscillatory strain sweep test was carried out at an angular frequency of 10 rad/s to establish the range of linear viscoelastic response. A frequency sweep test was performed at a constant strain amplitude of 0.1% (within the linear viscoelastic region) from 100 to 0.1 rad/s to determine the frequency dependence of $G'$ and $G''$ of the suspensions.

All measurements were performed in duplicate. For each measurement, a fresh sample was loaded into the cup.

2.8 Determination of the phase volume of the carrot-derived suspensions
Samples (20 g) were centrifuged (Beckman J2-HS, Analis, Namen, Belgium) at 12900×g for 30 min at 20 °C. The phase volume (φ) of the particles in each suspension was determined as:

$$\varphi (\%) = \left[ \frac{M_p}{M_t} \right] \cdot 100\%$$  

with $M_p$ the mass of the precipitate (g wet weight) and $M_t$ the total mass of the carrot-derived suspension before centrifugation (g wet weight), assuming that the density of the carrot-derived particles was almost equal to that of water. All determinations were carried out in duplicate.

2.9 Determination of the degree of methoxylation of serum pectin

The DM of the serum pectins was calculated for each serum sample as the ratio of the molar amount of methoxyl esters on the serum pectins to the molar amount of GalA residues. The determinations of the amounts of methoxyl esters and GalA residues were performed as described by Moelants et al. (2013b). In short, pectin was hydrolysed with concentrated sulfuric acid and the concentration of GalA was subsequently determined spectrophotometrically. For the determination of the amounts of methoxyl esters, pectin was saponified to pectate and MeOH whereafter the released amount of MeOH was measured using GC-MS.

2.10 Analysis of the molar mass distribution of serum pectin

The MM distribution of the polysaccharides present in the sera was assessed by high-performance size exclusion chromatography as described by Moelants et al. (2013b). In short, the dialysed serum samples were analysed on an Äkta Purifier HPLC system, equipped with a mixed-bed column of Bio-Gel TSK using 0.05 M NaNO$_3$ as eluens.

2.11 Determination of kinematic viscosity of carrot sera

The kinematic viscosity of the serum samples (and of several dilutions thereof) was measured using an Ubbelohde capillary viscometer (Ubbelohde, SI Analytics, Mainz, Germany) at 25 °C. Serum was brought into a reservoir and sucked through the capillary into the measuring bulb marked with two calibration lines. Subsequently, the serum was allowed to flow under gravity from the measuring bulb through the capillary to the reservoir and the time required for the serum to pass along two calibrated marks was used to calculate the kinematic viscosity. The
Hagenbach correction was used. All sera samples were analysed in triplicate with capillaries with different diameters. For all sera, the obtained viscosities were independent of the diameter of the capillary, which confirmed the absence of shear rate dependency and slip at the studied shear rates. The intrinsic viscosity $[\eta]$ (L/g) was obtained as explained by Moelants et al. (2013b).

2.12 Determination of particle surface characteristics related to pectin composition: Immunolabeling of pectic epitopes

The sieve fraction 80-125 µm of both purées containing pectin with different DM, obtained after wet sieving (cf. Section 2.3) and fixed in 70% (v/v) ethanol, was selected for this microscopic investigation. Prior to immunolabeling, the ethanol was removed by centrifugation for 5 min at 22 °C and 3000 g (Microfuge 22R Centrifuge, Beckman Coulter, Germany) and the pellet was washed two times with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8.0 mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, pH 7.4). Immunolabeling of the carrot-derived particles was performed as described by Christiaens et al. (2011). The washed carrot-derived particles were incubated with primary antibody (diluted in PBS containing 5% milk powder (MPBS)) for 1 h and 30 min at room temperature. The primary antibodies JIM7 and 2F4 were used as 5-fold dilutions of hybridoma supernatant. PAM1 (PlantProbes, Leeds, United Kingdom) was used at a concentration of 20 µg/mL. Afterwards, a washing step in PBS was carried out. For the visualisation of JIM7 and 2F4, secondary labelling with an anti-rat Ig antibody and anti-mouse IgG antibody, respectively, coupled to fluorescein isothiocyanate (FITC) (Nordic Immunology, Tilburg, The Netherlands) was used. The secondary anti-rat antibody was diluted 1/20 in 3% MPBS, whereas the anti-mouse IgG antibody was diluted 1/50 in 3% MPBS. PAM1, on the other hand, was visualised using a three-stage labelling. After primary labelling, carrot-derived particles were subsequently incubated with an anti-polyhistidine antibody (Sigma–Aldrich, St. Louis, Missouri) and an anti-mouse IgG antibody coupled to FITC. Dilutions in MPBS of, respectively, 1/1000 and 1/50 were used. As control, samples without primary labelling also passed this entire labelling procedure.

After a final washing step with PBS, particles were mounted in an anti-fade agent (Citifluor, Agar Scientific, Stansted, United Kingdom). Micrographs were taken using an Olympus BX-41 microscope (Olympus, Optical Co. Ltd., Tokyo, Japan) equipped with epifluorescence illumination (X-CiteR Fluorescence Illumination, Series 120Q, EXFO Europe, Hants, United
Kingdom). Two droplets of the stained sample were placed on a glass slide and studied using an objective of 10× or 40× magnification.

2.13 Statistical analysis of data

Statistical analysis was performed using one way ANOVA (SAS, version 9.3, Cary, USA). To evaluate significant differences among the results of carrot purées with different thermal pretreatment, amount of Ca²⁺ and pH, a post-hoc Tukey test was used. The level of significance was set at $P < 0.05$.

3. Results and Discussion

3.1 Influence of pH and Ca²⁺ on the rheological properties of original carrot purées

In a first set of experiments, it was investigated if the rheology of carrot purées (derived from low- and high-methoxylated tissue) can be influenced by Ca²⁺ addition in excess. To start, the Ca²⁺ concentration intrinsically present in carrot purée was measured and compared to the amount of non-methoxylated carboxyl groups. Results are represented in Table 1. The amount of Ca²⁺ intrinsically present in carrot purée prepared from carrots containing especially HM pectin was similar to the amount of non-methoxylated carboxyl groups, meaning enough Ca²⁺ ions are intrinsically present to make Ca²⁺ cross-linking possible in these systems (under appropriate pH conditions). Since the amount of non-methoxylated carboxyl groups was somewhat higher in carrot purée prepared from carrots containing especially LM pectin, it can be expected that the addition of extra Ca²⁺ to this purée will result in additional Ca²⁺ cross-link formation and strengthening of the pectin network in the suspension.

Subsequently, the effects of Ca²⁺ on the storage modulus ($G'$) and on the ratio of the loss modulus to the storage modulus ($G''/G'$ or tan$\delta$) of carrot purées at different pH were investigated. $G'$ at low angular frequencies can be used as a measure for the network stiffness. Tan$\delta$, on the other hand, gives insight into the network type (Steffe, 1996). A low-frequency plateau for $G'$ and $G''$ was noticeable for all samples. In Fig. 2A, the effect of pH and Ca²⁺ on the low-frequency $G'$ value can be seen. Large error bars on the average $G'$ values of different
repetitions of the purée preparation are visible. These error bars originate from a rather limited reproducibility of purée preparation at different time instants possibly caused by large batch-to-batch variations. Therefore, little significant differences between these values could be detected if results were compared as averages of the different repetitions of the purée preparation at different time instants. However, when the effect of pectin DM present in the carrot-derived purée, pH and Ca\textsuperscript{2+} addition on the value of $G'$ was investigated for each repetition of the sample preparation separately (data not shown), similar effects of these parameters were observed for all repetitions. Furthermore, the observed effects of pectin DM present in the carrot-derived purée, pH and Ca\textsuperscript{2+} addition were significant in some repetitions and non-significant for others. From Figure 2, it turned out that, for all purées, irrespective of pH and ion addition, $G'$ was affected by the heat treatment that was used during the preparation of the carrot purées (however, only for the purée at pH 5 without Ca\textsuperscript{2+} addition, the average $G'$ was significantly different for purée derived from high-methoxylated carrot tissue and purée derived from low-methoxylated carrot tissue). The different heat treatments resulted in a different DM of the carrot pectin (cf. Table 1). Since the characteristics of particles derived from carrot tissue containing LM pectin are expected to be more suitable for Ca\textsuperscript{2+} cross-link formation, the higher value of $G'$ can be possibly explained by an increase in Ca\textsuperscript{2+} cross-links in these purées as compared to the high-methoxylated carrot purées. Also previous studies investigating the effect of DM on the value of $G'$ in calcium-pectin gels with pH 6, observed a decrease in $G'$ in function of DM (Ström et al., 2007; Fraeye et al., 2009; Ngouémazong et al., 2012a). As demethoxylated carboxylic acid groups of pectin are not charged at pH 3, no Ca\textsuperscript{2+} cross-link formation was expected. However, Gilsenan et al. (2000) reported network formation under acidic conditions (pH < 3) for LM pectins with a DM < 30\% in the absence of sugar. It was suggested that intermolecular hydrogen bonds between among others protonated and unprotonated carboxyl groups of different pectin chains gave rise to this network (Walkinshaw & Arnott, 1981). Secondly, it can be observed from Fig. 2A that whereas the effect of Ca\textsuperscript{2+} addition on $G'$ of high-methoxylated systems was rather limited, $G'$ of purées prepared from carrots containing LM pectin was reduced by adding Ca\textsuperscript{2+}, especially at pH values above the pK\textsubscript{a} of GalA (valid for pH 5 or pH 7) (however, for a particular heat treatment and pH, the reduction in $G'$ induced by Ca\textsuperscript{2+} addition was not significant). The latter observation is not supporting the hypothesis that Ca\textsuperscript{2+} addition could enhance Ca\textsuperscript{2+} cross-link formation in plant-tissue-based suspensions. Since $G'$ can be used as a
measure for gel stiffness of the purée, this parameter was expected to be the highest for purées derived from carrot tissue containing LM pectin with a pH value above the pKₐ of GalA (pH 5 or pH 7) in the presence of sufficient Ca²⁺. Under the given conditions, negatively charged pectin is present possibly resulting in Ca²⁺ cross-link formation whereby the gel stiffness should be increased. However, at pH 5 and pH 7 \( G' \) turned out to be the largest for purées derived from low-methoxylated carrot tissue without extra Ca²⁺ addition. By adding extra Ca²⁺ to these systems \( G' \) was rather diminished instead of increased which is in contrast with the behaviour of pure Ca²⁺-pectin gels (Fraeye et al., 2009; Ngouémazong et al., 2012a; Ngouémazong et al., 2012c). The effect of pH and Ca²⁺ on tanδ is shown in Fig. 2B. For all purées, tanδ is between 0.09 and 0.2, which indicates that all purées are predominantly elastic. Remarkably, the value of tanδ was the largest for systems derived from tissue containing LM pectin at pH 5 or 7 with Ca²⁺ addition. Again, the assumption that Ca²⁺ addition could enhance pectin gel formation is disputed by this latter observation. The latter observations suggest that Ca²⁺ addition results in a mechanism decreasing the network stiffness. Possibly, calcium ions screen the charges of the pectin polymers at the particle surface instead of acting as cross-linking agents (between particles or polymers in the serum) whereby the electrostatic repulsion between these polymers (and the particles) is lowered. When two particles approach each other their electrical double layers will overlap and they will repel each other. However, when the ionic strength of the dispersing medium increases (e.g. by adding calcium ions) there is a greater compression of the ionic atmosphere toward the particle surface whereby particles can approach each other more closely (Derjaguin & Landau, 1941). Shomer et al. (1991) already described the existence of such an electrical double layer in parenchymatous plant cell walls. Previously, Whittenberger and Nutting (1958) stated that insoluble pectin associated with the cellulose fibrils in the cell walls contributes to the electrostatic charge. They reported a decrease in viscosity of cell-wall suspensions when electrolytes, as e.g. NaCl or CaCl₂, were added. Also Redgwell et al. (2008) observed a reduced viscosity of kiwifruit and tomato cell-wall-material dispersions in the presence of electrolytes indicating an important role of the electrical double layer in the rheological properties of these systems. In that view, the higher values of \( G' \) for purées derived from low-methoxylated carrot tissue at pH 5 or 7 as compared to these of purées derived from high-methoxylated carrot tissue at the
same pH may be explained by the larger negative particle charge in the latter, causing repulsion between particles whereby \( G' \) turns out to increase.

Subsequently, the effects of \( \text{Ca}^{2+} \) addition in excess on the static (\( \sigma_{0S} \)) and dynamic (\( \sigma_{0D} \)) yield stress of carrot systems containing pectin with different DM and different pH were investigated and results are presented in Fig. 3. Whereas the former characterises the strength of the undisrupted structure in the purée, the latter is determined for a sample with a completely broken-down structure, often from extrapolation of flow curves to zero shear rate (Cheng, 1986).

In Fig. 3A, trends in \( \sigma_{0S} \) with changing pH and \( \text{Ca}^{2+} \) were similar as observed for the storage modulus (cf. Fig. 2). \( \sigma_{0S} \) turned out to be larger for purées derived from low-methoxylated carrot tissue to which no extra \( \text{Ca}^{2+} \) was added as compared to \( \sigma_{0S} \) of the purées derived from high-methoxylated carrot tissue without extra \( \text{Ca}^{2+} \) addition (although, at a particular pH, no significant differences between the average \( \sigma_{0S} \) of purées containing HM or LM pectin without extra \( \text{Ca}^{2+} \) could be detected). Again, the effect of \( \text{Ca}^{2+} \) addition on \( \sigma_{0S} \) of high-methoxylated systems was rather limited and no significant effect of \( \text{Ca}^{2+} \) addition could be detected for these systems. Ion addition to purées prepared from carrots containing LM pectin resulted in a non-significant reduction of the average \( \sigma_{0S} \), especially at pH 7. Results suggest that \( \text{Ca}^{2+} \) is possibly screening the negative charge of LM pectin, especially at pH values above the pK\(_a\) of GalA, reducing the flow resistance of the carrot-derived suspensions. This LM pectin can be present at the particle surface as well as in the serum phase. Changes in the rheological properties with pH and \( \text{Ca}^{2+} \) could not be explained by the formation of \( \text{Ca}^{2+} \) cross-links between carrot pectin chains (located at the particle surface or in the serum phase). As demonstrated in a previous study on carrot-derived suspensions (Moelants et al., 2013a), \( \sigma_{0S} \) is always larger than \( \sigma_{0D} \) of a particular sample, meaning that the shear stress that is required to initiate flow is larger than the shear stress required to maintain flow at low shear rates. Fig. 3B clearly shows that trends in \( \sigma_{0D} \) with changing pH and \( \text{Ca}^{2+} \) were similar but less outspoken as observed for \( \sigma_{0S} \).

Previously, it was shown for carrot-derived suspensions that the ratios of \( \sigma_{0S} \) to \( G' \) and \( \sigma_{0S} \) to \( \sigma_{0D} \) were independent of the particle size (Moelants et al., 2013a). Here, these ratios turned out to be similar for the different heat treatments meaning that they were not affected by the DM of the pectin in the purées investigated here. Also \( \text{Ca}^{2+} \) addition was not affecting these ratios.

From the results discussed so far, it turned out that \( \text{Ca}^{2+} \) is possibly screening negatively charged pectin, present at the particle surface or in the serum phase of carrot-derived suspensions.
containing LM pectin. By increasing the ionic strength of the suspension, repulsions between suspension compounds can be reduced. In addition, reduction of internal repulsions in the plant cells could cause particle shrinkage. Hence, ion addition can result in a reduction of the volume that is occupied by the particles whereby the rheological parameters can be reduced.

3.2 The influence of pH and Ca$^{2+}$ on the rheological properties of carrot serum

To obtain a better insight in the role of Ca$^{2+}$ ions on the rheology of carrot-derived suspensions, the effect of calcium addition was investigated in both phases separately. It is known that the rheology of suspensions is determined both by the particle phase and the serum characteristics (cf. Einstein relation represented in Eq. 2) (Tanglertpaibul & Rao, 1987; Genovese & Lozano, 2000),

$$\eta = \eta_m (1 + [\eta] \cdot \varphi)$$ (2)

with $\eta$ = the viscosity of the suspension, $\eta_m$ = the viscosity of the continuous phase, $[\eta]$ = the intrinsic viscosity and $\varphi$ = the phase volume.

Original carrot-derived purées were unravelled into particles and serum allowing the evaluation of the effect of Ca$^{2+}$ addition on the rheology of both phases separately. In this way, it can be assessed if pH and Ca$^{2+}$ are affecting the particle phase or the serum. Moreover, it was attempted to elucidate why ion addition resulted in a reduction of rheological parameters such as $G'$ and the yield stress instead of enhancing the network strength by Ca$^{2+}$ cross-link formation between pectin chains. To start, the serum gelling ability and the effect of Ca$^{2+}$ addition on the rheology of the serum phase were studied in more detail. If the serum pectin properties favour gelation, a Ca$^{2+}$ cross-linked serum phase could be formed by Ca$^{2+}$ addition, leading to a gelled continuous phase filled with plant-tissue-based particles. The pectin characteristics important for the gelling ability of the serum pectin are represented in Table 2. The GalA concentration in the serum phase (as measure for serum pectin concentration) of the investigated carrot purées was low due to limited pectin solubilisation. Fraeye et al. (2009) and Ngouémazong et al. (2012a) used a more than 10 times higher pectin concentration to obtain a calcium pectin gel. However, apple or citrus pectin, having deviating pectin properties from carrot, was used in these studies. Nevertheless, the low serum pectin concentration can be responsible for the fact that also between the chains of solubilised serum pectin no pectin network was formed. Furthermore, the DM of the serum pectin was lower for serum obtained from carrots that received HT2 as
compared to carrots that were only blanched. Fraeye et al. (2009) observed that DM has to be low enough (<30%) for pectin to exhibit gel formation. To examine whether interactions between polymer chains are possible, a coil overlap parameter was calculated by multiplying \( C_{\text{polymer}} \) (g/L) with \( [\eta] \). Since the value of this latter parameter turned out to be smaller than one for the sera, irrespective of the heat treatment, no pectin entanglement and overlap is expected (Macosko, 1994). The results in Table 2 show that whereas HT2 produces pectin with an increased gelling capacity by lowering DM, it suppresses gelation in plant-tissue-based suspensions by reducing the coil overlap parameter. Thus, observations from Table 2 can explain the lack of stiffening of the purees after \( \text{Ca}^{2+} \) addition, as serum gelation is not expected. Previously, Peters et al. (1954) concluded that the effect of added calcium on the serum viscosity of tomato purée was dependant on the processing conditions (influencing pectin content in the serum) and Calgon addition (liberating especially LM pectin in the serum). An increase in serum viscosity was only observed for hot break tomato purée containing Calgon, explained by the rather high serum pectin concentrations and presence of LM serum pectin.

Furthermore, the effect of pH and \( \text{Ca}^{2+} \) on the serum viscosity is presented in Fig. 4. The observed increase in serum viscosity after the addition of \( \text{Ca}^{2+} \) was most probably caused by an increase in serum solid content rather than by pectin gelation, as could be expected based on the low values of the coil overlap parameter (cf. Table 2). Moreover, the serum viscosity was low, especially for HT2 serum. The low serum viscosity is caused by a combination of the low pectin content \( C_{\text{polymer}} \), measured as GalA content, and the small MM of the serum pectin, represented in Table 2. Since the observed effects of \( \text{Ca}^{2+} \) addition and pH on the serum viscosity were rather small, it can be concluded that the observed changes in rheological properties of carrot purée induced by changing the pH and the amount of \( \text{Ca}^{2+} \) (cf. section 3.1) are most likely dominated by effects of those system conditions on the particle phase and not by changes on the level of the serum phase.

3.3 The influence of pH and \( \text{Ca}^{2+} \) on the rheological properties of reconstituted carrot-derived suspensions

Besides the effect of pH and \( \text{Ca}^{2+} \) on the rheological properties of the serum phase, the effect of these parameters on the structural properties of the particle phase was assessed. The surface characteristics of the particles were first investigated. Negatively charged pectin at the surface of
the particles is a prerequisite for both electrostatic particle interactions and Ca$^{2+}$ cross-link formation. Both types of interactions can occur between the pectin on the surface of the carrot-derived particles and pectin polymers solubilised in the serum phase or at the surface of other carrot-derived particles. Insight in the carrot-tissue particle surface characteristics was obtained by the use of a specific staining procedure. Particles originating from carrot purées with different DM obtained after HT1 and HT2 were labelled with antibodies towards pectin with very diverse degrees and patterns of methoxylation. Anti-pectin antibodies were proven to be suitable to visualise various in situ phenomena in processed plant tissue systems according to their binding specificities (Christiaens et al., 2011; Christiaens et al., 2012). In this study, cell clusters originating from the 80 to 125 µm particle fraction of the carrot purées with different DM (and pH 7) were selected for immunofluorescence labelling with JIM7, PAM1 and 2F4. Whereas JIM7 can be used as a general anti-pectin probe, PAM1 is specific for LM pectin. Localisation of Ca$^{2+}$ cross-linked pectin is possible with the monoclonal antibody 2F4. Micrographs of the carrot-derived particles labelled with JIM7, PAM1 and 2F4 are presented in Fig. 5. JIM7 clearly labelled the cell wall of carrot-tissue particles obtained both after HT2 and HT1, meaning pectin is present at the surface of both types of particles. Labelling of carrot-tissue particles with PAM1 was only successful in particles obtained after HT2. This indicates that only HT2 resulted in long blocks of non-methyl-esterified GalA residues at the surface of carrot-tissue particles. Consequently, in the low-methoxylated carrot-tissue particles Ca$^{2+}$ cross-link formation will be more likely. The latter was confirmed by labelling with 2F4. Micrographs clearly show that whereas 2F4 labelling was rather rare in particles derived from high-methoxylated carrot tissue, Ca$^{2+}$ cross-linked pectins were abundantly present in particles derived from low-methoxylated carrot tissue. Results from the performed immunofluorescence labelling demonstrate that surface characteristics of particles derived from low-methoxylated carrot tissue are suitable for Ca$^{2+}$ cross-link formation. This leaves open the question if ‘Ca$^{2+}$ ions will merely engage in the formation of intraparticle cross-links and screening of particle charges’ or if ‘Ca$^{2+}$ cross-links between different particles or between pectin on the particles and serum pectin can be effectively formed and will consequently affect the rheology of carrot purées’.

Therefore, the effect of Ca$^{2+}$ addition in excess on the rheological properties of reconstituted carrot-derived suspensions (representative for the particle phase) was investigated. By using reconstituted carrot-derived suspensions instead of carrot purée, the effect of pH and Ca$^{2+}$ on the
serum phase is eliminated and changes in rheological properties will be caused by the particle behaviour only. The effect of those system conditions on the rheological properties of reconstituted carrot-derived suspensions was studied and a selection of the data is shown in Fig. 6. For the reconstituted purées, the trends in rheological parameters with changing pH and Ca\textsuperscript{2+} were comparable as observed for the original carrot purées (cf. Section 3.1), meaning that also in reconstituted carrot-derived suspensions, Ca\textsuperscript{2+} rather neutralises negative charges (reducing the hydrodynamic volume of an individual particle) than being involved in Ca\textsuperscript{2+} cross-link formation between pectin chains on different particles. For the formation of calcium-pectin networks, described by the egg-box model in which Ca\textsuperscript{2+} is chelated between the carboxyl groups of different pectin chains (Powell et al., 1982), pectin chains have to approach each other rather closely. Consequently, steric hindrance of large particles can possibly impede Ca\textsuperscript{2+} cross-link formation between pectin chains present on the particle surfaces.

Changing pH and Ca\textsuperscript{2+} of the carrot purées investigated here turned out to affect the particle phase rather than leading to pectin gelation. The latter hypothesis is supported by the fact that trends in phase volume with changing pH and Ca\textsuperscript{2+} were similar as observed for the rheological properties of the different carrot purées and reconstituted suspensions. As example, the effect of pH and Ca\textsuperscript{2+} is shown for reconstituted carrot purée in Fig. 7. Previously, Husband et al. (1993) among others already demonstrated the dependency of the yield stress on particle volume fraction. Since stacking of particles is more compact in systems to which Ca\textsuperscript{2+} was added, it can be concluded that repulsion between charged particles can be partially removed by addition of Ca\textsuperscript{2+}. These ions compress the electrical double layer whereby the hydrodynamic volume of a particle is reduced. As consequence of the reduction of this volume, also the phase volume of the purée will decrease, resulting in a decrease in rheological parameters.

3.4 Influence of ionic strength on the rheological properties of carrot purée

As the previous results clearly show that the rheology of carrot purées as a function of pH and Ca\textsuperscript{2+} addition is dominated by screening of the charges on the particle surfaces due to the presence of ions, a correlation between purée rheology and ionic strength may be present. To assess the effects of ionic strength, Fig. 8 presents the value of $G'$ for carrot purées containing especially LM pectin with different ionic strengths at similar pH (pH = 5). The ionic strength was adjusted by the addition of NaCl or CaCl\textsubscript{2} solutions with different ion concentrations. Since
the intrinsic amount of ions in carrot purée turned out to be negligible as compared to the amount of ions added to the suspension, the ionic strength of the purée without ion addition is represented as 0. When Na\(^+\) is added, \(G'\) decreases monotonously with increasing ionic strength. In contrast to Na\(^+\) addition, Ca\(^{2+}\) addition did not result in an unambiguous decrease of \(G'\) with increasing ionic strength. After an initial pronounced decrease of \(G'\) at low ionic strength, \(G'\) slightly increased and verged towards a constant value at higher values of ionic strength. However, the value of \(G'\) for the puree without Ca\(^{2+}\) addition was not reached. Observed trends were similar in reconstituted carrot purées with different ionic strengths (data not shown), indicating once more that especially the particle phase, rather than the serum phase, is affected by ion addition.

Although negatively charged pectin was present at the particle surface and in the serum (under appropriate conditions), increasing the stiffness and strength of the purée by Ca\(^{2+}\) cross-link formation after Ca\(^{2+}\) addition turned out to be not possible in these carrot purées. Ca\(^{2+}\) appeared to neutralise negative charges rather than being involved in Ca\(^{2+}\) cross-link formation. Consequently, the rheological behaviour of carrot-derived suspensions was not dominated by the effects of pectin Ca\(^{2+}\) cross-link formation, but this behaviour turned out to be mainly affected by ions screening the negative charged particle surfaces. However, the different trend with ionic strength, depending on the type of added ions (Fig. 8), clearly shows that in case of Ca\(^{2+}\), other phenomena, such as Ca\(^{2+}\) cross-link formation, also contribute slightly to the rheology. Although the properties of pectin-calcium gels can be fine-tuned by changing pectin chemical structure (degree of branching, DM), system properties (e.g. pH, pectin or Ca\(^{2+}\) content) and environmental conditions (e.g. temperature) (Fraeye et al., 2010; Ngouémazong et al., 2012a; Ngouémazong et al., 2012b; Ngouémazong et al., 2012d), the complex suspensions investigated here turned out to behave rather different than pectin-calcium gels. Previously, it was demonstrated that particles present in particulate food suspensions may weaken a gel. In that context, Nussinovitch et al. (1991) and Fiszman and Durán (1992) reported a decrease in gel strength or firmness in different polysaccharides gel (tests did not include pectin) systems when pulp was added due to interference with the network formation. Genovese et al. (2010), on the other hand, concluded that gel strength of high-methoxylated pectin gels could be retained when small apple particles were added, depending on the particle concentration.
4. Conclusion

In this work, the effect of calcium ions (Ca$^{2+}$) in excess and pH on the rheological properties of carrot purées without extra added pectin was investigated. With a suitable heat treatment, pectin demethoxylation was accomplished, leading to low-methoxylated pectin in the serum and at the particle surfaces. The addition of Ca$^{2+}$ in excess turned out to affect the rheological properties of carrot-derived suspensions prepared by blending and high-pressure homogenisation of the heat-treated carrot tissue. The rheological characterisation showed that the rheological parameters of carrot-derived suspensions were not dominated by effects of Ca$^{2+}$ cross-link formation of the intrinsic pectin. Results suggested that Ca$^{2+}$ is possibly screening the negative charge of low-methoxylated pectin present at the particle surface, especially at pH values above the pK$_a$ of GalA, reducing the rheological parameters of carrot-derived suspensions. Besides steric hindrance of particles, low serum pectin concentrations, due to insufficient pectin solubilisation, can be responsible for the fact that also between the chains of solubilised serum pectin no pectin network was formed. In the present work, separate investigations of the effects of pH and Ca$^{2+}$ addition on the serum viscosity and the rheology of carrot-derived suspensions reconstituted in water, showed that pH and Ca$^{2+}$ ions were affecting the particle phase rather than the serum. Future work focussing on the possibility to form Ca$^{2+}$ cross-links in plant-tissue-based suspensions with higher serum pectin concentrations can therefore be recommended. By using targeted processing, the food composition can be tailored with the goal to obtain plant-tissue-based systems with high serum pectin content, possibly altering the gelling properties of these systems.

Acknowledgements

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References


Whittenberger RT & Nutting GC (1958) High viscosity of cell wall suspensions prepared from tomato juice. Food Technology, 8(12), 420-424.
**Table 1** The intrinsic calcium (Ca$^{2+}$) concentration, the degree of methoxylation (DM) and the concentration of non-methoxylated carboxyl (COO$^-$) groups in carrot purée prepared from blanched carrot tissue without or with pretreatment at 60 °C for 24 h (HT1 and HT2, respectively).

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>Intrinsic Ca$^{2+}$ concentration (µmol/g)</th>
<th>DM (%)</th>
<th>COO$^-$ concentration (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1</td>
<td>4.2 ± 0.1</td>
<td>70.6 ± 2.7</td>
<td>4.8</td>
</tr>
<tr>
<td>HT2</td>
<td>3.8 ± 0.1</td>
<td>39.1 ± 1.7</td>
<td>11.4</td>
</tr>
</tbody>
</table>

*a st dev
Table 2 Pectin properties of the serum phase in carrot purée obtained from blanched carrot pieces, without or with pretreatment at 60°C for 24 h (HT1 and HT2, respectively) (GalA = galacturonic acid; DM = degree of methoxylation; MM = molar mass; \([\eta]\) = intrinsic viscosity; \(C_{polymer}\) = serum pectin concentration; \(C_{polymer}[\eta]\) = coil overlap parameter).

<table>
<thead>
<tr>
<th>Heat treatment</th>
<th>GalA content (µmol/ml)</th>
<th>DM (%)</th>
<th>MM (kDa)</th>
<th>([\eta]) (L/g)</th>
<th>(C_{polymer}) (g/L)</th>
<th>(C_{polymer}[\eta])</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT1</td>
<td>4.47 ± 1.54</td>
<td>49.98 ± 18.17</td>
<td>258.33</td>
<td>1.15</td>
<td>0.80</td>
<td>0.92</td>
</tr>
<tr>
<td>HT2</td>
<td>2.26 ± 0.47</td>
<td>22.28 ± 33.84</td>
<td>139.32</td>
<td>0.80</td>
<td>0.40</td>
<td>0.32</td>
</tr>
</tbody>
</table>

\(^{st}\) dev
Fig. 1. Schematic overview of the preparation of different carrot purées, carrot sera and reconstituted carrot-derived suspensions with different pH, different amount of Ca$^{2+}$ and containing pectin with a different degree of methoxylation (HT1 = heat treatment at 95 °C for 5 min; HT2 = heat treatment at 60 °C for 24 h followed by blanching step at 95 °C during 5 min; HPH = high-pressure homogenisation).

Carrot pieces
\[ \downarrow \text{HT1 / HT2} \]
\[ \downarrow \text{Blending} \]
Carrot blend
\[ \downarrow \text{HPH} \]
Carrot purée

Wet sieving

Reconstituted carrot-derived suspensions

\[ \downarrow + \text{H}_2\text{O} \]

Carrot-derived particles

Centrifugation + filtration

Carrot purée
\[ \text{No Ca}^{2+} \quad \text{pH 3, 5 and 7} \]
\[ \text{Ca}^{2+} \quad \text{pH 3, 5 and 7} \]

Carrot serum

\[ \text{No Ca}^{2+} \quad \text{pH 3, 5 and 7} \]
\[ \text{Ca}^{2+} \quad \text{pH 3, 5 and 7} \]
Fig. 2. Effect of pH and Ca$^{2+}$ on the storage modulus ($G'$) (A) and the ratio of the loss to the storage modulus (tanδ) (B) (at $\omega = 0.1$ rad/s) (± standard error, n=2-3) of carrot purée derived from high- (■) and low- (■) methoxylated carrot tissue. Averages of rheological parameters of different repetitions of the purée preparation, represented on the same curve, marked with the same letter are not significantly different.
**Fig. 3.** Effect of pH and Ca\(^{2+}\) on the static (\(\sigma_{0S}\)) (A) and dynamic yield stress (\(\sigma_{0D}\)) (B) (± standard error, n=2-3) of carrot purée derived from high- ( ■ ) and low- (■) methoxylated carrot tissue. Averages of rheological parameters of different repetitions of the purée preparation, represented on the same curve, marked with the same letter are not significantly different.
**Fig. 4.** Effect of pH and Ca$^{2+}$ on the serum kinematic viscosity (± standard deviation) from carrot purée derived from high- (■) and low- (▲) methoxylated carrot tissue.
Fig. 5. Immunolabeling of low- (HT1) or high- (HT2) methoxylated carrot-tissue particles with anti-pectin antibodies JIM7, PAM1 and 2F4.
Fig. 6. Effect of pH and Ca\textsuperscript{2+} on the static yield stress ($\sigma_{0S}$) (A) and storage modulus ($G'$) (at $\omega = 0.1$ rad/s) (B) (± standard deviation) of reconstituted carrot-derived suspensions derived from high- (■) and low- (●) methoxylated carrot tissue.
Fig. 7. Effect of pH and Ca^{2+} on the phase volume (± standard deviation) of reconstituted carrot-derived suspensions derived from high- (■) and low- (■) methoxylated carrot tissue.
Fig. 8. Effect of ionic strength on the value of $G'$ for carrot-derived purées with similar pH (pH = 5). The ionic strength was adjusted by the addition of CaCl$_2$ (■) or NaCl (■) solutions with different ion concentration. The intrinsic ion concentration of the purée was negligible as compared to the amount of ions added and was not included in the calculation of the ionic strength.
- Ca$^{2+}$ addition and changes in pH can change the rheology of carrot-derived suspensions
- At specific pH, Ca$^{2+}$ addition causes a decrease in network stiffness and strength
- Ca$^{2+}$ affects the properties of the particle phase rather than those of the serum
- Ca$^{2+}$ can screen the negative charge of low-methoxylated pectin