Pectin-chitosan hydrogels with modified properties for the encapsulation of strawberry phenolic compounds

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Abstract: Pectin-chitosan hydrogels with blends of low (50-190 kDa) and medium (310-395 KDa) molecular weight (MW) chitosan (LC and MC, respectively) were developed, and their characteristics were investigated before and after the encapsulation of an aqueous strawberry extract. The pectin to total chitosan mass ratio, the composition of the strawberry extract and the MW of chitosan greatly affected the interactions between pectin and chitosan at different pH values. More specifically, blends of low and medium MW chitosan improved the stability of the strawberry-gels in acidic conditions compared to their corresponding MC-gels, showed better flow and texture profiles, as well as slower release of phenolic compounds during *in vitro* digestion compared to the only stable LC-gel. Therefore, by manipulating the length range of chitosan chains would allow the formation of pectin-chitosan hydrogels with improved properties for the development of functional food products.

Keywords: Chitosan; Molecular weight; Hydrogels; Phenolic compounds; *In vitro* digestion

1. Introduction

Worldwide, there is a great interest in the development of stable physical hydrogels with specific properties for the delivery of bioactive compounds. Hydrogels may be formed by a single polymer, but may suffer from poor mechanical properties. Their replacement by hybrid hydrogels – developed by the combination of two or more polymers – has drawn attention to solve stability issues, and pectin-chitosan gels are one example of them. Such matrices have been used for drug delivery or wound healing (Neufeld and Bianco-Peled, 2017; Long et al., 2019), as well as in the food industry, for the development of functional food products (Yu et al., 2024). Several production processes of these systems have been suggested, depending, among others, on the pH of the blends during hydrogel formation. At pH between the pKa of pectin and chitosan (2.9-3.2 and 6.1-7.1, respectively) (Neufeld and Bianco-Peled, 2017; Capitain et al., 2020), the polymers form stable polyelectrolyte complexes through ionic interactions. On the other hand, at pH below 2, pectin-chitosan gels can be formed via a thermally induced procedure which, during the cooling of the samples to room temperature, leads to the development of hydrogen bonds between pectin and chitosan (Neufeld and Bianco-Peled, 2017; Ventura and Bianco-Peled, 2015). For the development of these binary systems, low-methoxyl pectin from citrus peels has been mainly used. On the other hand, a large variety in the characteristics of the selected chitosan has been reported. The MW of chitosan affects significantly its physicochemical and biological properties (Roman-Doval et al., 2023), due to differences in the interactions between chitosan and functional groups (e.g. $-COO^-$) of other compounds (Aranaz et al., 2021). As a consequence, chitosan-based hydrogels prepared with chitosan of different MW, and depending on the interacting compound, can have considerably different characteristics.

Instability and/or fast release of the encapsulated compounds from pectin-chitosan hydrogels make their use challenging. The formation of several encapsulation layers and the addition of extra components (e.g. salts, proteins) lead to pectin-chitosan hydrogels with improved stability and better release of the encapsulated compounds under specific conditions (Dziadek et al., 2022; Birch et al., 2015; Jiang et al., 2023). However, the development of hybrid pectin-chitosan hydrogels containing pectin and a combination of low and medium MW chitosan – possessing a wide range of physicochemical properties – in order to improve their stability is still to be investigated.

In the present study, we report the development of pectin-chitosan hydrogels – without the use of salts – with modified properties, which contain blends of low and medium MW chitosan, and thus varied length range of chitosan chains. Firstly, the characteristics and properties of the developed gels were investigated. Considering that the incorporation of more compounds in the gels may greatly affect their properties, the same hydrogels were developed in the presence of an encapsulated aqueous strawberry extract rich in phenolic compounds. Finally, the effect of the Mw of chitosan on

the pore size, rheological properties, texture, and swelling-shrinkage and release behavior of the strawberry-gels was investigated.

2. Materials and methods

2.1 Materials

Strawberries were purchased from a local market in Belgium. Medium MW chitosan (75% deacetylation degree (DD) and 310-395 KDa) (419419-50G), low MW chitosan (77% DD and 50-190 kDa) (448869-50G), pectin from citrus peel (84.1% galacturonic acid; 7.8% methylated) (P9135-100G), Folin & Ciocalteu's phenol reagent (47641-500ML-F) and sodium hydroxide pellets were purchased from Sigma-Aldrich (Belgium). Sodium acetate, acetic acid 99-100% and hydrochloride acid 37% were purchased from Chem-Lab. Sodium chloride, potassium chloride, and potassium phosphate monobasic were purchased from VWR (Belgium), and sodium phosphate dibasic from Supelco (Belgium). All chemicals were used without further purification.

2.2 Extraction of phenolic compounds from strawberries

The extraction of phenolic compounds from strawberries was performed according to the method of Li et al. (2019) with some modifications. Fresh strawberries with green leaves were smashed using a blender to produce a paste. Then, strawberry paste and HCl-acidified water (pH3) were mixed at 1:5 ratio (w/v) and shaken in a water bath at 60^{o}C for 1h. Afterwards, the extracts were filtered using 5-13 µm filter papers, and the filtrates were stored at -20 ^{o}C until further use.

2.2.1 Assessment of the total phenolic content (TPC) of the strawberry extracts

The total phenolic content was assessed with the Folin-Ciocalteu assay (Huynh et al., 2014). In brief, dilutions of the strawberry extracts were mixed with distilled water and Folin-Ciocalteu reagent, and incubated for 6 minutes in the dark. Then, 20% sodium carbonate solution and distilled water were added to each tube, vortexed, and incubated for 2h at room temperature in the dark. Finally, the absorbance of the samples was measured at 760 nm using a spectrophotometer (Shimadzu SBN-SPEC-94). The results were calculated from the calibration curve, which was prepared using gallic acid as a standard and expressed as mg/L gallic acid equivalents (GAE). A LOD and LOQ of 4.9 mg/L and 14.9 mg/L, respectively, were obtained. Strawberry extracts with 199±6 mg/L GAE were further used for the preparation of pectin-enriched strawberry extracts (SEP) and the physical pectin-chitosan hydrogels.

2.2.2 Total monomeric anthocyanin content

The total anthocyanin content was assessed via the pH differential method (Lee et al., 2005). Firstly, 0.025M potassium chloride of pH 1 and 0.4M sodium acetate of pH 4.5 buffer solutions were prepared. Afterwards, the strawberry extract was mixed with the buffers at 1:5, and let stand in the dark for 20 min. Finally, the absorbance was measured at 520 nm, as well as at 700 nm for haze correction, and the total anthocyanin content was calculated using the formula:

$$Total\ anthocyanin\ content = \frac{[(A520 - A700)pH1.0 - (A520 - A700)pH4.5] * MW * DF}{\varepsilon * l}$$

where MW = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor; l = path length in cm; ε = 26900 molar extinction coefficient, in L mol^{-1} cm^{-1}, for cyanidin-3-glucoside. The results were expressed as cyaniding-3-glucoside equivalents in mg/L.

2.2.3 Total protein content

The total protein content of the strawberry extract was investigated to understand if there are more charged compounds, apart from pectin and chitosan, which could affect the formation of gels and their further performance at different pH values. The protein content was assessed via the Bradford method (Bradford, 1976). Briefly, appropriate volumes of strawberry extract and the Bradford reagent were added to test tubes and vortexed. After 2 minutes, the absorbance was measured at 595nm. The results were calculated based on a standard curve prepared using bovine serum albumin (BSA) as a standard. A LOD and LOQ of 0.5 μg/mL and 1.5 μg/mL, respectively, were obtained.

2.3 Preparation of SEP solutions

Strawberry extracts from Section 2.2 were adjusted to pH 1.5 with 1 mol/L HCl. Then, pectin was dissolved in the extracts to prepare SEP solutions with two different concentrations. Dissolution took place under stirring at room temperature for 2h and protected from light to prevent the degradation of the phenolic compounds. The final pectin content in SEP solutions was 0.5 and 1% (w/v). Finally, the solutions were stored at -20 °C until further use for the preparation of the physical hydrogels.

2.4 Preparation of chitosan solutions

For the preparation of the hydrogels, 1% chitosan solutions were freshly prepared by dissolving appropriate amounts of medium and low MW chitosan in 1% acetic acid solution (MC and LC, respectively). Chitosan solutions were freshly prepared before use to prevent changes in the MW of chitosan due to storage conditions.

2.5 Preparation of physical hydrogels

131 The preparation of pectin-chitosan hydrogels was performed based on the method of Neufeld and
132 Bianco-Peled (2017), with modifications. Firstly, the pH of the freshly prepared LC and MC was adjusted
133 to 2 with 1 mol/L HCl. Directly after the pH adjustment, LC, MC, or 1:1 (v/v) combination of MC and LC
134 solutions (LMC) were mixed with the 0.5 or 1% SEP solutions (pH1.5) at a 1:2 or 1:3 ratio (w/v) using a
135 vortex. The pH of the final mixtures was approximately 1.7 (pH<2). Afterwards, the mixtures were
136 placed in a static water bath at 60 ^{o}C for 1h. Subsequently, they were let to cool down at room
137 temperature for 1h to form a gel through hydrogen bonding. The resulting gels were coded as follows:
138 for the gels with LC, 0.5SL2 and 1SL2 corresponded to 0.5 and 1 %SEP, respectively, with a 1:2 w/v
139 chitosan solution to SEP solution, whereas 0.5SL3 and 1SL3 corresponded to 0.5 and 1 %SEP,
140 respectively, with a 1:3 w/v chitosan solution to SEP solution. Gels with MC and MLC were coded in a
141 similar way by replacing 'L' with 'M' and 'ML', respectively.
142 To evaluate the effect of the strawberry extract on gel formation, pectin solutions at pH1.5 without
143 strawberry extract (PEC solutions) were used as controls. The resulting control gels were coded in the
144 same way as the SEP-containing gels with the difference that 'S' was replaced by 'P' in all codes,
145 representing the corresponding PEC solutions.
146 Gel formation was assessed by the inversion tube test (Morello et al., 2021). After sample preparation,
147 the tubes were inverted and remained at a vertical position for 1 minute. Afterwards, gel formation
148 was evaluated based on the flowability of the sample. More specifically, if a sample did not flow, but
149 remained at the bottom of the inverted tube, it was considered as a gel. In contrast, if a sample flowed,
150 it was considered a liquid.
151
152 2.6 Characterization of hydrogels
153
154 2.6.1 Swelling-shrinkage behavior
155 The swelling-shrinkage behavior of the gels was tested at different pH values to investigate their
156 behavior in different environmental conditions. The selection of the pH values was done based on the
157 pH of the oral, gastric and intestinal phases according to the INFOGEST method (pH7, 3 and 7,
158 respectively) (Minekus et al., 2014), the pH of blood plasma (pH7.4), as well as the pH of dairy products
159 used mainly for the incorporation of hydrogels, ranging from quite acidic (~ 4) for fermented products
160 to neutral (~ 7) for milk. Finally, the behavior of the gels at pH1.7 was also tested. That pH corresponds
161 to the pH of the gels after preparation where the interactions between pectin and chitosan are weaker
162 than those formed at higher pHs (e.g. ionic). Therefore, the effect of weaker interactions on the
163 stability of the gels was tested.
164 The swelling-shrinkage behavior of the gels was evaluated by recording their weight variations over
165 time. Briefly, fresh hydrogels with and without SEP were immersed in phosphate-buffered saline (PBS)

(pH7.4) or diluted HCl solution of pH1.7, 3 and 5, at 37 °C. At specific time points during a 24h period, the gels were withdrawn from the solutions, wiped carefully to remove excess of the solutions, and weighed on watch glasses. Afterwards, the weight variations of the samples from their original weight were calculated. When the weight of a gel was higher than the starting weight, it was considered as a swelling (SW), whereas when the weight was lower, it was considered as a shrinkage (SH). Finally, the corresponding ratios were calculated using the following formula (Neufeld and Bianco-Peled, 2017):

$$\%\, SW = \%(-SH) = \frac{(w1 - w0)}{w0} * 100$$

where w0 is the starting weight and w1 the final weight of the fresh gels.

2.6.2 *In vitro* degradation

The degradation of the gels was evaluated according to Baniasadi et al. (2021) with some modifications. Fresh gels were immersed in PBS (pH7.4) and acidified water (pH3) at room temperature. The pH values were selected as the extremes from section 2.6.1 where the gels containing the strawberry extract were stable. The weight loss of the gels was followed over time, and calculated via the following formula:

$$\%\, WL = \frac{(w0 - w1)}{w0} * 100$$

where w0 is the starting weight and w1 the final weight of the fresh gels.

2.6.3 Encapsulation efficiency

The encapsulation efficiency of phenolic compounds of strawberry extract in the gels was investigated according to Sarkar et al. (2021), with some modifications. Briefly, fresh hydrogels of 1 g were placed in 50 mL falcon tubes with 5 mL of 0.1 mol/L HCl in methanol. The samples were placed on a shaker for 1h at 300 rpm. Afterwards, the supernatant was used to assess the total phenolic content using the Folin-Ciocalteu method, as described in Section 2.2.2. Encapsulation efficiency of the phenolic compounds was determined using the following equation:

$$Encapsulation\ efficiency\ (\%) = \frac{Mf}{Mi} * 100$$

where Mf=final concentration of total phenolic content in the gel, and Mi=initial concentration of total phenolic content in the gel.

2.6.4 Porosity

The pore size of the hydrogels was evaluated based on a method described by Morello et al. (2020) using an optical microscope (CARL ZEISS) equipped with a camera. Briefly, after gel formation, the fresh hydrogels were placed at -40 °C overnight, and then freeze-dried. The porosity of the freeze-dried gels

was assessed via the measurement of the pore size at magnification 5x. For each gel, the size of at least 100 pores was measured.

2.6.5 FT-IR analysis

To investigate the interactions within the gels, FT-IR spectra were recorded using a Nicolet FTIR spectrometer (Thermo Scientific) with a diamond accessory, in ATR mode. After gel formation, the hydrogels were freeze-dried, and the spectra of the dried gels were assessed through an average of 32 scans at a resolution of 4 cm^{-1} in a wavenumber range of 4000–600 cm^{-1} by triplicate.

2.6.6 Steady shear test

Rheological analysis of fresh pectin-chitosan hydrogel was performed using a Rheometer (MCR302, Anton Paar) according to Zarandona et al. (2021). In brief, fresh hydrogels were put on the bottom plate of the rheometer and choose 40 mm parallel plate for the experiment with a gap of 1 mm at 4 and 20 °C. After 1 min of equilibrium, the steady shear experiment was executed with a shear rate range from 0.1 to 100 s^{-1}.

2.6.7 Texture analysis

Texture profile analysis (TPA) was performed using a Texture analyzer (LLOYD Instruments LF plus) according to Zarandona et al. (2021) with some modifications. Briefly, a probe disk (40 mm) was compressed into 2 g hydrogel at a speed of 1 mm/s at a distance of 3 mm. The texture properties (cohesiveness, adhesiveness and hardness) of the hydrogels were determined.

2.6.8 Release of phenolic compounds during *in vitro* digestion

For the release of phenolic compounds, a method was used based on the standardized INFOGEST static *in vitro* simulation of food digestion (Minekus et al., 2014). Simulated digestion fluids were prepared according to Table 1, and the pH was adjusted with 1 mol/L NaOH or 3 mol/L HCl. For the treatments, 3 g of gels were mixed with 4 mL simulated saline fluid (SSF), 25 µL 0.3 mol/L $CaCl_2$ and 225 µL water, and incubated at 37 °C for 2 min under mild shaking. Afterwards, 8 mL simulated gastric fluid (SGF), 5 µL 0.3 mol/L $CaCl_2$, 1.145 mL water, and pepsin (2,000 U/mL in digestion mixture) were added, and the incubation took place at 37 °C for 2 h, under mild shaking. Finally, 8 mL simulated intestinal fluid (SIF), 5 µL 0.3 mol/L $CaCl_2$, 3.16 mL water, pancreatin (100 U/mL in the digestion mixture) and 0.6 g bile were added and incubated at 37 °C for an additional 2 h, under mild shaking.

Samples were withdrawn at the beginning (IN) and the end (OUT) of the gastric and intestinal phases. Afterwards, the liquid phase was centrifuged at 4000 rpm for 15 min at 4 °C, and the supernatant was collected and stored at -20 °C till further analysis.

Finally, TPC was assessed in the supernatants as described in Section 2.2.1. The release was calculated using the following formula:

$$\%\ Release = \frac{C * V}{m} * 100$$

where m represents the mass of TPC (in g GAE) in the gel before treatment, C represents the concentration of TPC in the supernatant, and V is the volume of the medium corrected based on the swelling-shrinkage of the gels after treatment.

2.7 Statistical analysis

The results are presented as the mean ± standard deviation of three biological replicates for each experiment. Statistical analysis was carried out using one-way Analysis of variance (ANOVA) and Pearson Correlation from the Data Analysis Tools in Microsoft Excel 2016, and one- and two-way ANOVA in SPSS, with Tukey as a post hoc test.

3. Results & Discussion

3.1 Gel formation

Pectin-chitosan hydrogels are generated at pH below 2 through a thermally-induced procedure. Hydrogen bonds formed during the cooling of the samples create a polymeric network which leads to a gel-like material. The composition of the pectin-chitosan mixtures, as well as the presence of other compounds (e.g. phenolic), play an important role in the development of the aforementioned interactions. An increase in the concentration of chitosan or pectin may increase the strength of the interactions (Neufeld and Bianco-Peled, 2017; Zarandona et al., 2021), making the network more stable. However, the interactions that are formed also depend on the characteristics of the present polymers, such as the methoxylation of pectin and the DD and MW of chitosan.

In the current study, low and medium MW chitosan (LC and MC, respectively), as well as blends of 1:1 LC to MC (LMC) were applied in combination with low-methoxyl citrus pectin, at various ratios, and gel formation was assessed via the inversion tube test. Pectin-MC mixtures formed a gel in all tested cases – with 0.333, 0.375, 0.667 and 0.75% pectin – whereas LC mixtures started to gel when the pectin content was at least 0.375% (Table S1). Hence, an increase in the concentration of pectin allowed the formation of more interactions with LC. However, these interactions can be modified by the presence of other compounds, as can be seen by the gelation results after the incorporation of an aqueous strawberry extract. More specifically, SEP reduced the crosslinking between pectin and chitosan at pH below 2, as a gel was formed only in the sample with the highest pectin content (0.75%) (Table S1). On the other hand, LMC enhanced the gelation of pectin-chitosan-strawberry systems, when compared

to the LC samples. As a result, gel formation was affected by the MW of chitosan, the PEC or SEP to total chitosan volume ratio, as well as the presence of the strawberry extract in the pectin-chitosan systems.

3.2 Decomposition and swelling-shrinkage behavior of fresh pectin-chitosan hydrogels

Changes in the crosslinking between chitosan and pectin can result in SW or SH of the gels, leading to a less or more compact structure that can retain a specific amount of water or biological fluids (Feng and Wang, 2023; Ahsan et al., 2020). Variations in the SW or SH among different gels could be explained by a higher or lower crosslinking, which is dependent on their composition (pectin to chitosan ratio) and environmental conditions (such as pH) (Sarkar et al., 2020). Chitosan-based hydrogels suffer from instability in acidic solutions. Re-enforcement of its interactions with other compounds is needed to build up a more stable network which will be resistant to harsh acidic conditions, such as the gastric environment during digestion. In the present study, the weight of the gels at various pH values was measured over time until weight equilibrium was reached at 37 ^{o}C, and the results are illustrated in Fig. 1.

Regarding the PEC-gels, at pH1.7, 0.5PM2 – with 1:1 pectin to total chitosan mass ratio – swelled fast to an extreme extent and, as a result, it decomposed before weight equilibrium was reached. By increasing the ratio to 1.5:1 and 2:1, gels 0.5PM3, 1PM2 and 0.5PLM3 considerably swelled (52.1 ± 3.0%, 40.6 ± 2.3% and 50.1 ± 3.6%, respectively), but did not decompose, whereas 1PLM2 had no considerable increase in its weight (4.4 ± 2.5%). The main difference in the composition between 0.5PM2 and that of 0.5PM3 was the chitosan content (0.333 and 0.250%, respectively). This difference could explain the decomposition of the first gel and the high swelling for the second gel, but without causing a collapse of its structure. At pH1.7, pectin has no charge, whereas chitosan exists in its protonated form. Therefore, an increase in the chitosan content may lead to an electrostatic repulsion – because of the dense positively charged environment – increasing the distance between the polymeric chains. As a consequence, weak interactions between the polymers (e.g. hydrogen bonding) may break and lead to decomposition. Regarding the gels with MC and their corresponding gels with LMC, 0.5PM3 and 0.5PLM3 exhibited the same swelling behavior. In these gels, the total polymer content was low (0.625%). Furthermore, the availability of chitosan chains within the gel was low and, therefore, their length range did not affect the performance of the structure. On the other hand, in the case of 1PM2 and 1PLM2, a considerable increase in the pectin content (0.667%) increased the distance between the polymers to a lower extent. In this case, it was noticed that the length of chitosan chains had a considerable effect. More specifically, the distribution of the shorter chains of LC in 1PLM2 may have been located far from each other and between the pectin molecules, provoking a less intense electrostatic repulsion. Increasing the pectin to total chitosan mass ratio even more to 3:1, gels 1PM3,

1PL3 and 1PLM3 showed only a small deviation from their original weight. In this case, the chitosan concentration decreased (0.25%), whereas that of pectin increased (0.75%), allowing the spread of chitosan molecules far from each other, regardless of the length of their chains. This prevented the extensive repulsion between the positively charged groups. Therefore, gels with LMC had a different swelling-shrinkage behavior only at a 2:1 pectin to total chitosan mass ratio when compared to LC- or MC-gels. On the other hand, all PEC-gels shrank in PBS (pH7.4), and an adverse effect of the neutral pH on the SH was observed compared to the SW at low pH. More specifically, gels with a 1:1 ratio – which showed the highest SW at pH1.7 – displayed the highest SH at pH7.4. The SH of the gels at pH7.4 is in accordance with Neufeld and Bianco-Peled (2017) where 1% w/v pectin–chitosan hydrogels, prepared at pH below 2, shrank in PBS (pH7.4). This behavior is related to the reduced electrostatic repulsion followed by the deprotonation of chitosan.

The incorporation of the strawberry extract decreased the stability of the gels, as all SEP-gels decomposed quickly at pH 1.7, and, as a result, their weight variations could not be followed over time. At pH below 2, chitosan and pectin form interactions through hydrogen bonding, and the strawberry extract could eliminate their crosslinking by interacting with one or both polymers. An important class of strawberry compounds are anthocyanins (Lopes da silva et al., 2007) which, at pH below 2, exist in the form of a flavylium cation (Yang et al., 2023). At that pH, cationic amine groups of strawberry proteins are also present. Hence, the existence of both classes of compounds could develop intense electrostatic repulsion with the positively charged amine groups ($-NH_3^+$) of chitosan. This repulsion could increase the distance between pectin and chitosan weakening and/or breaking their interactions, leading finally to the collapse of the structure (Fig. 2). At pH3, only 3 SEP-gels appeared to be stable until weight equilibrium was reached (-3.8 ± 3.5%, 27.9 ± 0.9% and 1.5 ± 2.3% SW for 1SL3, 1SLM2 and 1SLM3, respectively). At this pH of 3, pectin possessed a weak negative charge and could form partial electrostatic interactions with the positively charged chitosan and proteins. Furthermore, at a pH-value of 3, the structure of anthocyanins changed to uncharged or anionic forms (Yang et al., 2023; León-Carmona et al., 2016), and could therefore interact with both polymers via the formation of hydrogen bonds (Zeng et al., 2023), as well as via electrostatic attraction with chitosan. Furthermore, strawberry amino acids still possessed a positive charge at that pH, which could interact with pectin too. Therefore, the modifications in the structure of anthocyanins and the interference of strawberry proteins could suppress the interactions between chitosan and pectin, contributing to the decomposition of some gels at a pH value of 3. Decomposition took place in all gels containing MC, as well as in 0.5SLM3 where the pectin content was low. On the other hand, gels with LC or LMC in combination with a high pectin content did not show any decomposition at that pH. This result may be related to the distribution of shorter chitosan chains within a pectin environment, preventing the extensive repulsion between positively charged groups. Hence, LMC helped the gels to remain stable

at low acidic pH, when compared to gels with MC alone. At pH5, 2 more gels did not decompose (0.5SM3 and 1SM3) showing a considerable SW (67.5 ± 9.1% and 47.9 ± 23.7%, respectively), but with high standard deviation, indicating their unstable nature. Instability may be the result of various reasons, probably mainly related to the composition of the strawberry extract. Apart from anthocyanins, other phenolic compounds, such as phenolic acids, are present in SEP (Wu et al., 2023), which, above their pKa, possess a negative charge due to the existence of carboxylic groups ($-COO^-$) in their structure (Seczyk et al., 2019). However, the pKa range of these compounds may be wide. For example, ellagic acid has a pKa of 5.6, whereas gallic acid of 4.4 (Agrawal and Kulkarni, 2020; Fernandes and Salgado, 2016). Differences in the composition, as well as in the concentration of specific compounds, could affect the interactions within the gel. In the present study, extracts were used from three different batches. Therefore, the composition of each replicate may slightly differ but cause a considerable standard deviation for some gels at pH5. As a result, further investigation would be needed to understand the way that modification of the phenolic profile of the extract could affect the subsequent behavior of the systems. At pH7.4, chitosan deprotonated, while phenolic acids and carboxyl side chains of amino acids possessed a negative charge, leading to a reinforcement of the electrostatic repulsion with the negatively charged pectin. As a result, this repulsion could have caused the collapse of 1SM3 at pH7.4. At that pH, LC could still possess some positive charges, permitting some electrostatic attraction between chitosan and pectin and/or compounds having carboxylic groups. Therefore, the presence of LC in the LC- and LMC-gels, in combination with the high pectin content (0.75%), could increase their stability. On the other hand, decomposition took place in a gel containing only MC (1SLH3).

3.3 *In vitro* degradation of the gels

The degradation of hydrogels under storage can give important information about the degree of crosslinking between pectin and chitosan, which further affects the resistance of the gels under specific conditions. In the current study, the degradation of 1SL3, 1SLM2 and 1SLM3 – stable gels till weight equilibrium at various pHs – was assessed via their weight loss in PBS over time (Fig. 1C2). Gel 1SL3 was stable for 15 days, and further storage caused considerable instability, as can be seen by the high standard deviation after that day, whereas its corresponding gel with LMC (1SLM3) just decomposed completely after 25 days of incubation. Therefore, no significant differences were observed in storage stability between 1SL3 and 1SLH3. However, 1SLM2 was found to be a more stable gel, as its weight remained constant for 28 days of storage. As a result, the presence of LMC in combination with the higher total chitosan content (0.333%) showed higher crosslinking between pectin and chitosan in 1SLM2 at pH7.4.

372 Opposite observations were noticed at pH3, where 1SLM2 decomposed faster than the other gels.
373 Crosslinking between pectin and chitosan decreased in the gel with the higher total chitosan content
374 at that pH, which was also verified by the higher swelling ratio of 1LSM2 at low pH. In contrast, high
375 pectin content (0.75%) improved the interactions and thus the stability of the gels in acidic conditions.
376
377 3.4 Porosity of the gels
378 The porosity of the gels with or without the strawberry extract was investigated under a light
379 microscope, and their pore size distribution is illustrated in Fig. 3. Regarding the PEC-hydrogels, the
380 use of LMC in the PEC gels led to a difference in the pore size distribution only for 1PLH3 whose mean
381 pore size shifted to lower values compared to 1PL3 and 1PM3. Furthermore, no correlation between
382 the mean pore size and swelling ratio could be observed, which could be due to the existence of small
383 and big pores within the same gel, as can be seen by the high standard deviation for the pore size
384 (Birch et al., 2015). Comparing the PEC- with the SEP-gels, the pore size distribution of 1PL3 versus
385 1SL3 was not affected by the presence of the strawberry extract. However, the mean pore size of
386 1SLM2 was shifted to lower values. A decrease in the pore size of pectin-chitosan hydrogels after the
387 incorporation of drugs or natural bioactive compounds has been previously reported as the result of a
388 more compact structure which could be related to the encapsulation of the bioactives (Long et al.,
389 2019; Sarkar et al., 2020). Furthermore, smaller pores have been linked to more stable structures (Lin
390 and Yeh, 2010), which, for 1SLM2, could be related to its enhanced storage stability in PBS. On the
391 other hand, the mean pore size of 1SLM3 shifted to higher values, causing faster degradation under
392 storage compared to 1SLM2.
393
394 3.5 Encapsulation efficiency
395 The encapsulation efficiency of strawberry phenolic compounds was 50.0 ± 3.6% for 1SL3, 58.8 ± 1.7%
396 for 1SLM2 and 55.2 ± 1.5% for 1SLM3. When comparing 1SL3 and 1SLH3, the incorporation of LMC in
397 pectin-chitosan gels did not have a significant effect on encapsulation. Hence, LMC did not influence
398 significantly the interactions between phenolic compounds and the polymeric network at a 3:1 pectin
399 to total chitosan mass ratio. In contrast, the higher total chitosan content (0.333%) in combination
400 with LMC led to a higher encapsulation efficiency when compared to the gel with LC and lower chitosan
401 concentration (0.25%) ($p < 0.05$). The improved encapsulation of 1LSM2 can be also confirmed by the
402 shift of its pore size to lower values after the incorporation of the strawberry extract to the gel, which
403 further led to a better storage stability in PBS.
404
405 3.6 FT-IR analysis

406 FTIR analysis was conducted to investigate the interactions between pectin, chitosan and the
407 strawberry extract, in the PEC- and SEP-gels. Changes were observed in the IR spectra of the PEC-gels
408 – without the strawberry extract– compared to that of pectin and chitosan alone (Fig. 4A). At 3000-
409 3600 cm^{-1}, fluctuations in the intensity of the peaks were observed among different gels,
410 corresponding to the –OH stretching coming from all polymers and to the N–H stretching of the
411 primary amine of chitosan. However, the presence of LMC did not seem to have a significant effect. C-
412 H stretching vibrations for MC appeared at 2915 and 2872 cm^{-1}, and for LC at 2920 and 2867 cm^{-1} (Long
413 et al., 2019), and a C-H stretching for pectin was detected at 2933 cm^{-1} (Mishra et al., 2008), whereas,
414 in the spectra of the gels, broader peaks were observed in the range 2889-2928 cm^{-1}. The peak
415 corresponding to the C=O stretching of amide I of LC was found at 1648 cm^{-1}, whereas at 1609 cm^{-1},
416 approximately, a broad peak did appear for MC, and the peak of C=O stretching for pectin was detected
417 at 1607 cm^{-1} (Zarandona et al., 2021). The same peak for all gels was detected in the range 1624-1629
418 cm^{-1}, indicating that physicochemical interactions took place between chitosan and pectin, probably
419 through hydrogen bonding (Zarandona et al., 2021). Finally, two bands at 1413 and 1377 cm^{-1} for MC,
420 and at 1419 and 1375 cm^{-1} for LC, were attributed to the CH_3 deformation (Zarandona et al., 2021),
421 whereas in all gels, apart from 0.5PM2, a shifted peak was observed between 1363 and 1370 cm^{-1}.
422 However, for 1PL3, 1PM3 and 1PLM3, this peak was barely detected, which could be linked to their
423 lowest chitosan content. Again, LMC did not seem to have any effect on the spectra of the gels,
424 compared to LC or MC alone.
425 Higher bands between 3000 and 3600 cm^{-1} were observed in the IR spectra of the SEP-gels after the
426 incorporation of the strawberry extract (Fig. 4B). The peak corresponding to the stretching of the C=O
427 of the ester groups of pectin was observed at 1735 cm^{-1}, whereas for the strawberry extract it was
428 found at 1719 cm^{-1} (Zarandona et al., 2021; Espinosa-Acosta et al., 2018). In the PEC-gels, the same
429 peak was noticed at the same wavenumber as pectin (1735 cm^{-1}), whereas for the SEP-gels, the peak
430 shifted between that of pectin and the strawberry extract (1730, 1727 and 1730 cm^{-1} for 1SL3, 1SLH2
431 and 1SLH3, respectively), indicating the presence of strawberry compounds in the gels (Safitri et al.,
432 2021). The IR spectra of the strawberry extract showed a peak at 1400 cm^{-1} which was attributed to
433 the symmetric stretching vibration of COO- (Long et al., 2019; Zhao et al., 2021), but the same peak in
434 the SEP-gels was not observed at that wavelength.
435
436 3.7 Flow curves and texture profiles
437 All gels showed a shear-thinning behavior at 4 and 20 ^{o}C, which was in accordance with previously
438 reported results (Zarandona et al., 2021) (Fig. 5A). The viscosity corresponding to the zero-shear rate
439 of 1SL3 at 20 ^{o}C was 35.9 ± 6.6 Pa*s. On the other hand, it was higher for 1SLM2 and 1SLM3 (74.6±2.5
440 and 74.3±25.0 Pa*s, respectively). At 4 ^{o}C, the viscosity increased significantly for all gels, being 159.7

± 31.5, 179.8 ± 15.3 and 254.5 ± 4.8 Pa*s for 1SL3, 1SLM2 and 1SLM3, respectively, indicating a better stability of the gels under low-temperature conditions Furthermore, the presence of LMC in the gels improved the viscosity of the pectin-chitosan systems by increasing the shear rate.

The textural properties of the gels, such as hardness, cohesiveness and chewiness, were also investigated. Hardness corresponds to the maximum force needed for the deformation of the gel during compression, cohesiveness is the capacity of the gel to retain its form after compression, and chewiness is the energy required to turn the solid food suitable to swallow, and is positively correlated to the previous two properties (Zarandona et al., 2021; Calvarro et al., 2016). Enhancement of the texture profiles of the pectin-chitosan gels after the incorporation of LMC was in accordance with their improved flow properties. More specifically, 1SL3 showed a hardness of 117.0 ± 1.4 g, cohesiveness of 0.141 ± 0.046 and chewiness of 0.21 ± 0.03 N*mm whereas these values increased similarly for 1SLM2 and 1SLM3 (262.0 ± 8.2 g and 281.7 ± 60.5 g for hardness, 0.465 ± 0.147 and 0.435 ± 0.084 for cohesiveness, and 2.36 ± 0.50 and 2.30 ± 0.17 N*mm for chewiness, respectively) (Fig. 5B).

3.8 Release of phenolic compounds in the gastric and intestinal fluids during *in vitro* digestion

Encapsulation of sensitive bioactive compounds prevents or delays their degradation during their passage by the oral and gastric phases during digestion and helps them reach the intestine. There, they should be slowly released from the matrix to achieve their maximum possible absorption by the human body. Encapsulation should eliminate the release of sensitive compounds in the oral and gastric fluids, and successfully transfer them to the intestine. The most challenging conditions for the stability of the pectin-chitosan hydrogels in the gastrointestinal tract are those found in the stomach where the environment is quite acidic and the composition is complex (e.g. presence of salts, enzymes). Hydrogels should be developed which could offer stability of the gel structure and the lowest possible release of the encapsulated compounds in the harsh environment of the stomach.

In the present study, the release of phenolic compounds during *in vitro* digestion was tested from the three most stable pectin-chitosan gel formulations, and the non-encapsulated extract was used as a control (Fig. 6).

Regarding the unprotected extract, only 50.0±3.1% of the original TPC content was available at the beginning of the gastric phase (GP). This observed decrease emphasizes the need for the encapsulation of the strawberry extract. A significant decrease in the TPC of berries after the passage from the OP has been previously reported (Sánchez-Velázquez et al., 2021). After 2h of incubation in a gastric environment, TPC remained constant at 51.7±3.6%. This result can be explained by the good stability of phenolic compounds, such as anthocyanins and phenolic acids, in acidic conditions. However, when the compounds entered the intestinal phase (IP), a further decrease in the TPC was observed to 21.4±11.9% which remained constant (23.3±9.2%) till the end of the treatment, presenting high

476 standard deviation values which suggested the instability of the non-encapsulated phenolic
477 compounds.
478 Regarding the behavior of the encapsulated extracts in the gastric phase, the release of TPC from 1SL3
479 was 23.8±3.1 and 45.9±4.3% in the beginning and end of the gastric phase, respectively, which was
480 significantly higher than the release from 1SLM3 (11.8±2.9 and 30.3±2.2% at GP-0h and GP-2h,
481 respectively) ($p < 0.05$) (Fig. 6). Furthermore, higher release from 1SL3 was also observed at IP-0h
482 compared to 1SLM3 (50.2±7.9 and 26.6±5.4%, respectively) ($p < 0.05$), whereas there was no
483 difference at IP-2h (66.5±8.2 and 70.2±9.2% for 1SL3 and 1SLM3, respectively). These results indicate
484 the slower release of TPC when a combination of LMC is used instead of LC within the same gel,
485 suggesting a better protection of the strawberry phenolic compounds under gastrointestinal
486 conditions.
487 Comparing the two gel formulations which contain LMC, no differences were observed in the released
488 TPC at GP-0h, GP-2h and IP-2h. However, a decrease was observed in the release of TPC from 1SLM2
489 at IP-0h compared to that at GP-2h (16±5.3 and 32.7±5.1%, respectively) ($p < 0.05$). This decrease could
490 be due to the degradation of some compounds due to a shift in pH between the GP and IP. On the
491 other hand, no difference was observed between GP-2h and IP-0h for 1SL3 and 1SLM3, probably due
492 to the faster release of TPC compared to that of 1SLH2 which could mask the degradation of some
493 compounds. All the above results indicate that LMC helped more phenolic compounds to remain
494 within the polymeric network during their passage from the GP and till their insertion into the IP, while
495 being released more slowly in the IP, making them more bioavailable for absorption.
496
497 3.8 Conclusions
498 Pectin-chitosan hydrogels suffer from instability and/or fast release of the encapsulated compounds
499 in an acidic environment. In the present study, to improve the stability and properties of those gels,
500 blends of low and medium MW chitosan were applied within the pectin-chitosan systems, in the
501 presence of an encapsulated aqueous strawberry extract. The characteristics of the gels were
502 investigated through the pore size distribution, FTIR and rheological analysis, as well as their swelling-
503 shrinkage and release behaviour. It was found that the pectin to total chitosan mass ratio, the
504 composition of the strawberry extract and the MW of chitosan greatly affected the interactions
505 between pectin and chitosan at different pHs. As a result, a considerable effect was observed on the
506 properties of the gels, such as stability and release behaviour. More specifically, blends of low and
507 medium MW chitosan improved the stability of the strawberry-gels in acidic conditions compared to
508 their corresponding MC-gels, and showed a higher viscosity during a steady shear test, improved
509 texture profile and a lower release of phenolic compounds during *in vitro* digestion compared to the
510 only stable LC-gel. Therefore, by manipulating the length range of chitosan chains in pectin-chitosan

systems would allow the development of more stable hydrogels with improved properties, for the evolution of functional food products.

Finally, further investigation is needed to understand how the composition of the digestion fluids could affect the interactions between the polymeric network and the phenolic compounds. Furthermore, further study is necessary to understand the way that modification of the phenolic profile of berry extracts could affect the subsequent behavior of the pectin-chitosan-berry systems.

CRediT author statement

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Declaration of Competing Interest

Katleen Raes reports financial support was provided by ERA-NET BlueBio Cofund. Johan Robbens reports financial support was provided by ERA-NET BlueBio Cofund. Katleen Raes reports financial support was provided by Research Foundation Flanders. Johan Robbens reports financial support was provided by Research Foundation Flanders. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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652

Table 1

Composition of pectin-chitosan-strawberry hydrogels per 100g of fresh gels (n=3).

Gel code	pectin to total chitosan mass ratio	LC (mg)	MC (mg)	P (mg)	SE (mL)	TPC (mg GAE)	TAC (mg cyd-3-glu E)	TPrC (mg)
0.5SM2	1:1	NA	335±2 [a]	333±1 [a]	66.6±0.2	13.3±0.2 [a]	2.5±0.1 [a]	247±10 [a]
0.5SM3	1.5:1	NA	252±2 [b]	376±2 [b]	75.1±0.2	15.0±0.3 [b]	2.9±0.1 [b]	278±12 [b]
1SM2	2:1	NA	335±1 [a]	667±3 [c]	66.7±0.3	13.3±0.2 [a]	2.5±0.1 [a]	248±11 [a]
1SM3	3:1	NA	252±1 [b]	751±2 [d]	75.1±0.1	15.0±0.3 [b]	2.9±0.1 [b]	278±12 [b]
1SL3	3:1	251±1 [a]	NA	750±2 [d]	75.0±0.2	15.0±0.3 [b]	2.9±0.1 [b]	278±12 [b]
0.5SLM3	1.5:1	126±1 [b]	125±1 [c]	377±3 [b]	75.2±0.3	15.0±0.3 [b]	2.9±0.1 [b]	279±11 [b]
1SLM2	2:1	168±1 [c]	168±1 [d]	668±4 [c]	66.8±0.3	13.3±0.2 [a]	2.5±0.1 [a]	248±10 [a]
1SLM3	3:1	125±3 [b]	127±3 [c]	752±3 [d]	75.1±0.2	14.9±0.2 [b]	2.8±0.1 [b]	278±12 [b]

Abbreviations: LC=low MW chitosan; MC=medium MW chitosan; P=pectin; SE=strawberry extract (SE); TPC=total phenolic content; TAC=total anthocyanin content; TPrC=total protein content; GAE=gallic acid equivalents; cy-3-glu E=cyanidin-3-glucoside equivalents; NA=not applicable. Values with a different letter are different at a 0.05 level of significance in a one-way ANOVA performed per column.

660

661 **Table 2** Composition of SSF, SGF and SIF

	Simulated saline fluid (SSF) (mg/L)	Simulated gastric fluid (SGF) (mg/L)	Simulated intestinal fluid (SIF) (mg/L)
KCl	1408	643	634
KH_2PO_4	629	153	136
$NaHCO_3$	1428	2625	8925
NaCl	-	3452	2808
$MgCl_2$	38.13	30.5	83.88
$(NH_4)_2CO_3$	7.2	60	-
pH	7	3	7

662

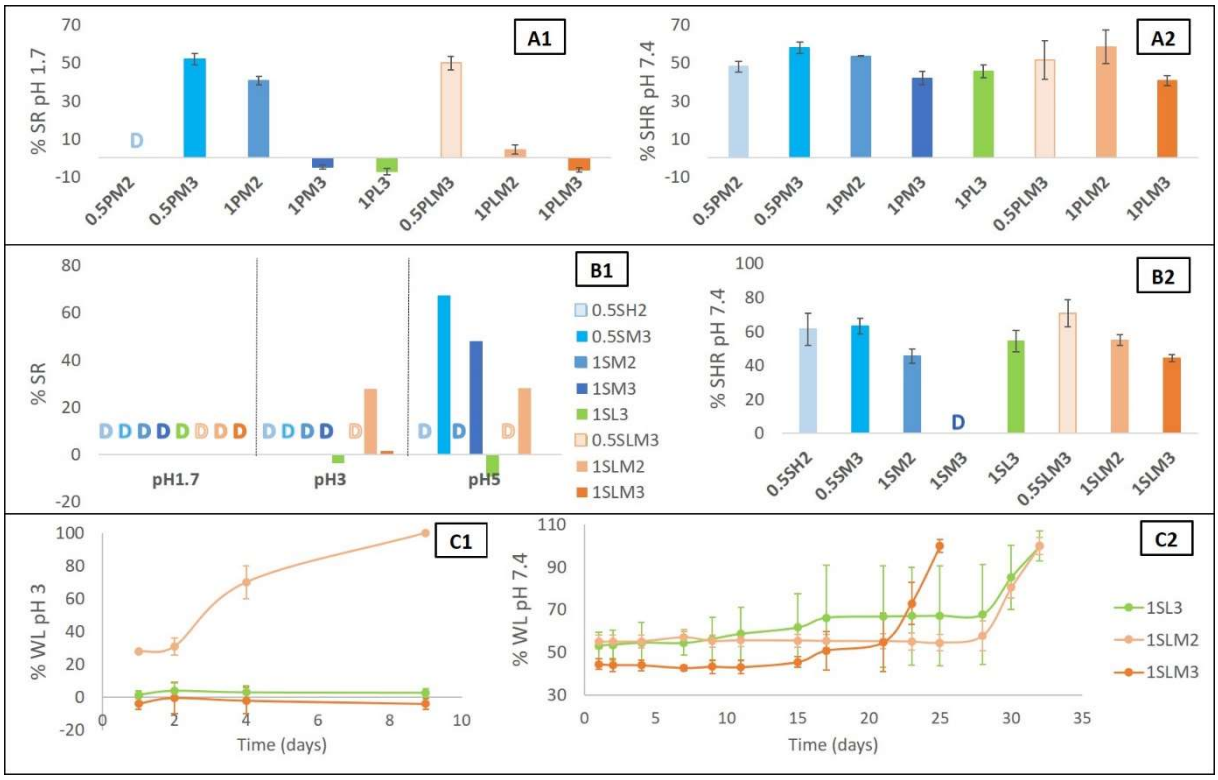
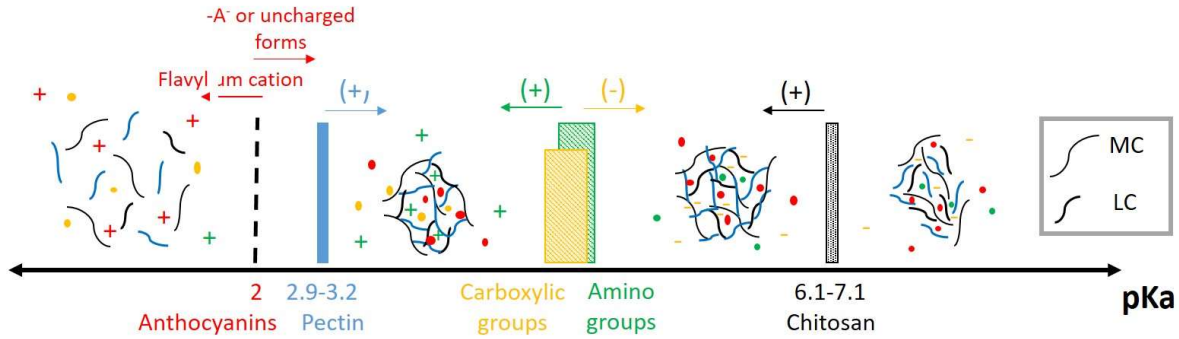


Fig. 1. Swelling ratio (SR) (%) and shrinkage ratio (SHR) (%) of A) PEC-gels, and B) SEP-gels, in equilibrium at 37 °C at different pH values where D = Decomposition (n=3); Degradation of SEP hydrogels, expressed as weight loss (WL) (%), at C1) pH3, and C2) pH7.4, at room temperature at different timepoints (n=3)

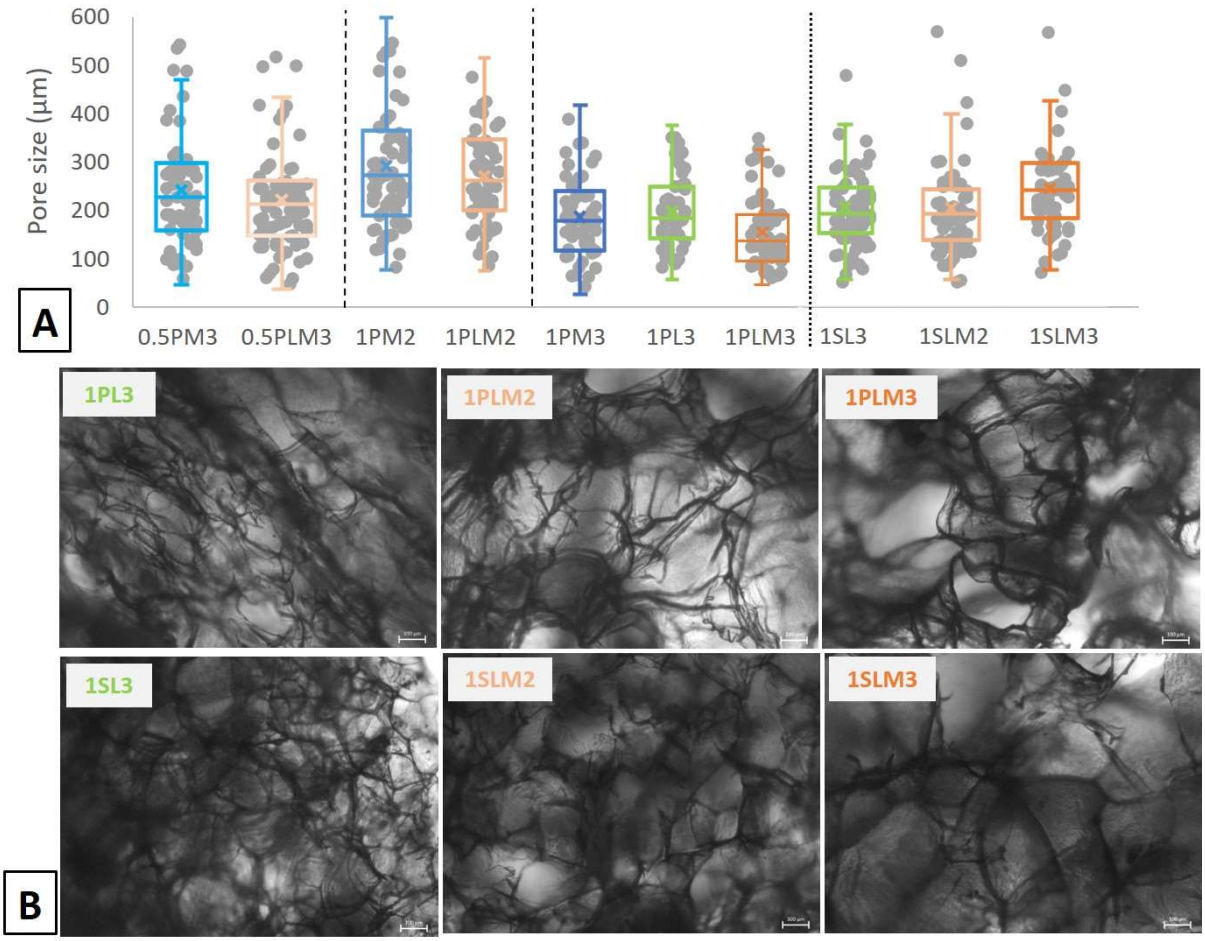


669

670 Fig. 2. Potential interactions within pectin-chitosan-strawberry hydrogels at different pHs (Capitain et

671 al., 2020; Neufeld and Bianco-Peled, 2017; Yang et al., 2023; León-Carmona et al., 2016).

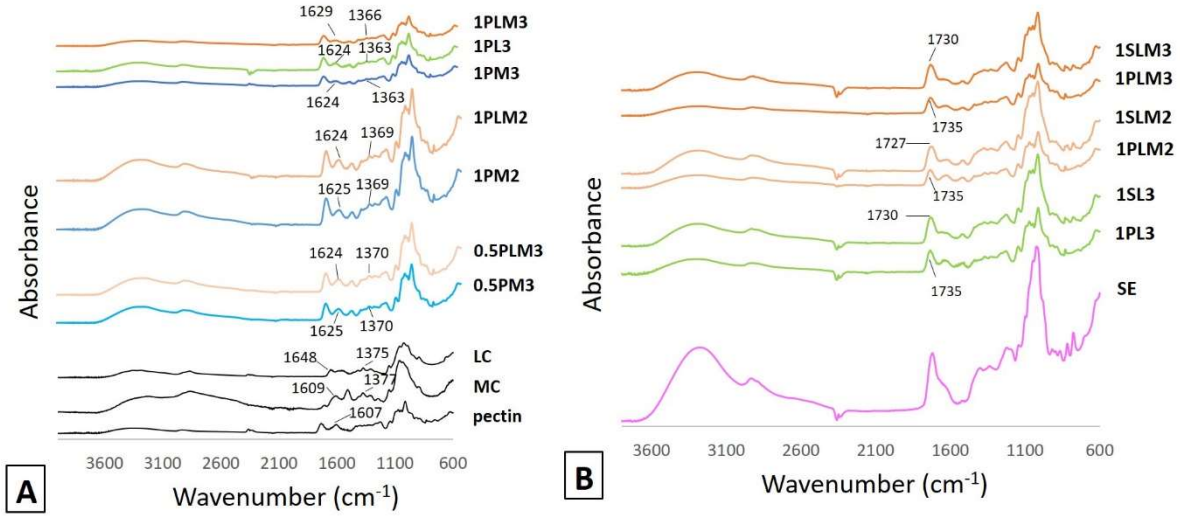
672



673

674 Fig. 3. A) Pore size distribution of PEC- and SEP gels. B) Pictures taken with an optical microscope at
675 magnification 5x, representing the porosity of PEC- and their corresponding SEP gels. The pore size was
676 evaluated by three biological replicates, and from each replicate, three different parts of the gel were
677 tested.

678

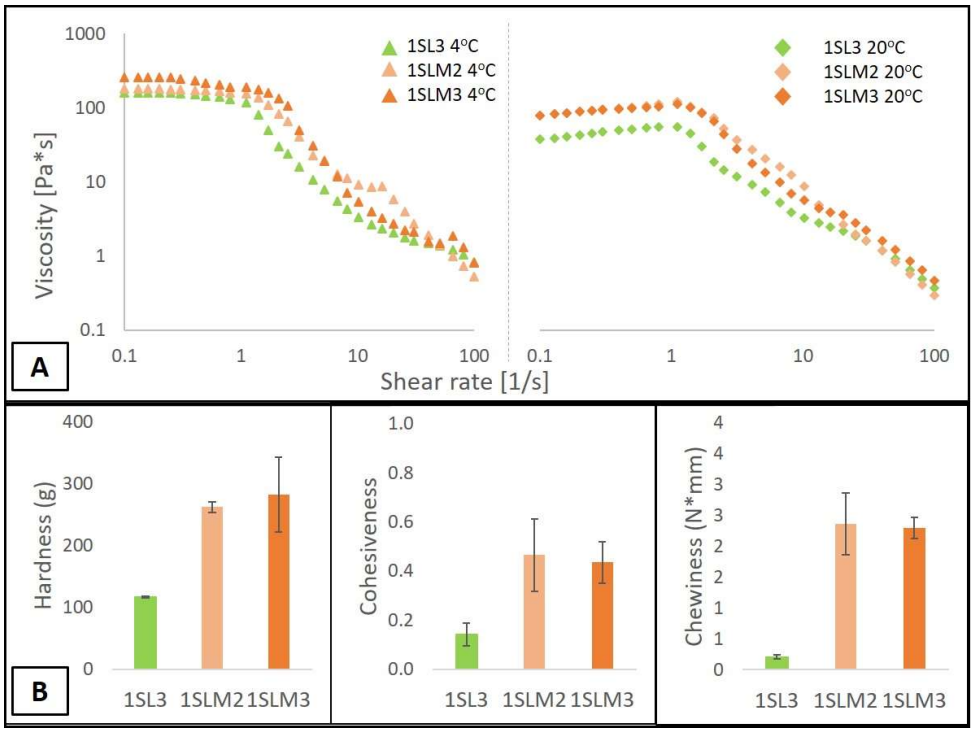


679

680 Fig. 4. IR spectra of A) pectin, medium molecular weight chitosan (MC), low molecular weight chitosan
681 (LC), and PEC gels, and B) the strawberry extract (SE), and of gels with and without the strawberry
682 extract (n=3)

683

684



685

686 Fig. 5. A) Flow curves during a steady shear test, and B) Textural properties (hardness, cohesiveness
687 and chewiness) of SEP-gels (n=3).

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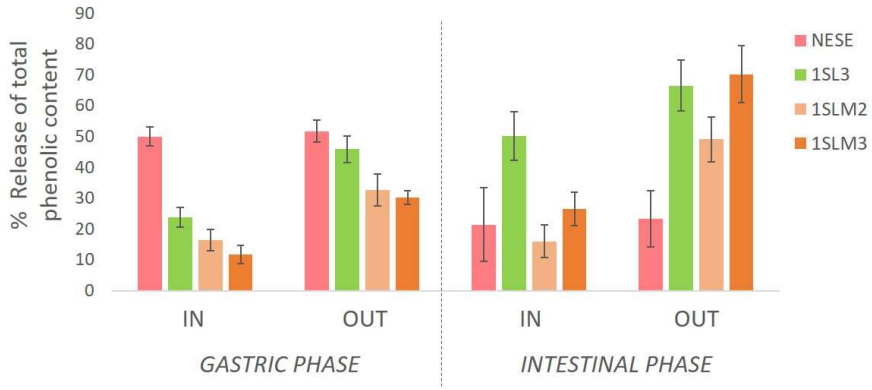

689

690 Fig. 6. Release (%) of phenolic compounds from the non-encapsulated strawberry extract (NESE) and
691 the SEP-gels at the beginning (IN) and end (OUT) of the gastric and intestinal phases, during *in vitro*
692 digestion (n=3).

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