Zinc bioaccessibility is affected by the presence of calcium ions and degree of methylesterification in pectin-based model systems

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Abstract

Minerals are required by the human body to perform physiological functions. Mineral deficiencies, often caused by low mineral bioaccessibility in plant-based foods, are a matter of great concern all over the world. Several antinutrients (e.g. pectin) may contribute to this reduced mineral bioaccessibility by formation of indigestible complexes due to mineral binding. Structural characteristics of the antinutrients, as for instance the degree of methylesterification (DM) in the case of pectin, may play a role in this mineral binding phenomenon and has been evaluated before, however, only in single mineral model systems. In natural food systems, several mineral types are present together which may affect each other's bioaccessibility. Therefore, this study investigated the influence of the presence of Ca^{2+} on Zn^{2+} binding capacity and bioaccessibility in mineral-pectin model systems with different DM. The results showed that increasing Ca^{2+} concentration and pectin DM reduces the Zn^{2+} binding capacity of pectin and consequently increases Zn^{2+} bioaccessibility in the *in vitro* small intestine. Moreover, the *in vitro* digestion procedure with adjustment of pH only, no addition of enzymes, bile salts nor digestive fluids during simulation of gastric and small intestinal phases, was found to be most appropriate to fundamentally study the influence of pectin DM and presence of Ca^{2+} on Zn^{2+} bioaccessibility in mineral-pectin model systems.

Keywords

Citrus pectin, degree of methylesterification, mineral (Ca^{2+} and Zn^{2+}) competition, Zn^{2+} binding capacity, Zn^{2+} bioaccessibility.

1. Introduction

Zinc deficiency, in addition to iodine, iron and vitamin A, is one of the four predominant micronutrient deficiencies worldwide due to its high prevalence and associated health consequences (Harding, Aguayo, & Webb, 2018). It is estimated that about 33% of the world's population, mainly located in developing countries, is at risk of zinc deficiency (Crook, 2011). Zinc has been shown to be essential for the structure and function of a large number of macromolecules and for more than 300 enzymatic reactions (Gharibzahedi & Jafari, 2017; Jackson & Lowe, 1992). Low zinc blood levels may lead to numerous clinical symptoms, such as growth retardation, impaired brain development and cognitive performance, poor wound healing, diarrhoea, infertility or increased risk of infections (Wapnir, 2000).

One of the major contributing factors towards mineral deficiencies is inadequate intake, as minerals cannot be synthesized by the human body and therefore must be obtained from the diet. Inadequate mineral intake can be attributed to low amounts in the ingested food or low mineral bioaccessibility (BAC) from the consumed food products (Platel & Srinivasan, 2015). The term bioaccessibility is defined as the fraction of a nutrient which is released from the food matrix into the gastrointestinal tract through the digestion process and that becomes available for intestinal absorption (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014). In plant-based foods, mineral BAC can be reduced due to the presence of several mineral antinutrients (Platel & Srinivasan, 2015), including dietary fibers, phytic acid or polyphenols. These mineral antinutrients can bind *in situ* minerals, minerals which are added to the food or minerals originating from other simultaneously ingested food ingredients, thereby potentially reducing the mineral release for intestinal absorption (Kumar, Sinha, Makkar, & Becker, 2010). The effect of these antinutrients on mineral bioaccessibility depends on their mineral binding capacity and digestibility of the chelate (antinutrient-

mineral complex) (Baye, Guyot, & Mouquet-Rivier, 2017; Bravo, 1998; Mandić, Sakač, & Mišan, 2013).

Particular dietary fibers, e.g. pectin, a cell wall polysaccharide in all higher plants, can act as mineral antinutrients. Pectin is a non-cellulosic and non-digestible hetero polysaccharide that may contain ionizable carboxylic groups which can show strong affinity for counter-ions such as mineral ions (Morris, Powell, Gidley, & Rees, 1982). Structurally, pectin is composed of three building blocks: homogalacturonan (HGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010). The major and most widespread domain of pectin, HGA, is linear and consists of α-(1,4)-linked galacturonic acid (GalA) residues. Some of the GalA units of HGA can be esterified with methanol defining the degree of methylesterification (DM) of pectin (Fraeye, Colle, et al., 2010; Voragen, Coenen, Verhoef, & Schols, 2009), which is an important property in determining its mineral binding capacity. Non-methylesterified GalA residues are ionised (COO^-) when the pH value of the system is above the pKa of pectin (3.8-4.1) (Sriamornsak, 2003). Consequently, it has been found that pectin can interact with several divalent cations (e.g. Ca^{2+}, Zn^{2+} and Fe^{2+}) through this COO^- groups which can result in the possible formation of a pectin-mineral network, the "egg-box" model (Morris et al., 1982). Therefore, pectin DM will determine its polyanionic nature and consequently influence its mineral binding capacity (Fraeye, Duvetter, et al., 2010; Kyomugasho et al., 2017). Furthermore, other pectin properties including the degree of blockiness (DB), the presence of neutral sugars as well as the degree of branching have been reported to influence the cation binding capacity of pectin (Kyomugasho et al., 2017). Although pectin-cation complexations can be desirable in the food industry in gelation and anti-oxidant applications (Celus, Salvia-Trujillo, et al., 2018; Sila, Van Buggenhout, Duvetter, Van Loey, & Hendrickx, 2009), it may be undesirable from a nutritional point of view (Bosscher, Van Caillie-Bertrand, Van

Cauwenbergh, & Deelstra, 2003; Celus, Kyomugasho, et al., 2018; Kyomugasho et al., 2017). Since pectin has the affinity to bind divalent cations (e.g. Ca^{2+}, Zn^{2+} and Fe^{2+}), which are considered as important minerals for human nutrition, pectin may reduce the availability of these essential minerals for absorption in the small intestine.

Towards exploring the role and extent to which pectin influences mineral bioaccessibility, understanding the mineral binding capacity as well as the interaction energy is important. To this extent, researchers including Celus et al. (2017; 2018) investigated the influence of DM and DB on the maximum binding capacity and interaction energy of pectin with Zn^{2+} or Ca^{2+} by establishing adsorption isotherms of these individual minerals. However, in real food systems, different minerals are present together and they may compete for the same binding sites of the mineral antinutrient. Therefore, understanding the pectin binding capacity of individual minerals as influenced by presence of other minerals is important and has not been investigated yet, to the best of our knowledge. Consequently, in the current study, ion binding capacity of pectin in competing mineral-pectin model systems with different DMs were explored. Since Ca^{2+} is more abundantly present in plant-based food systems than Zn^{2+} (Ekholm et al., 2007; Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014; Marles, 2017), the objective was to investigate the influence of the presence of Ca^{2+} on Zn^{2+} binding capacity and bioaccessibility in mineral-pectin model systems with different DMs. Therefore, citrus pectin with a high DM was enzymatically demethylesterified with carrot pectin methylesterase (PME) to obtain pectin with intermediate and low DMs. Based on the results of Celus et al. (2018), that a blockwise pattern of DM promotes a higher mineral binding capacity compared to pectin with a random pattern of DM, in the current study, pectin with a blockwise distribution of methylesters (generated by action of plant PME) was used. It can be hypothesised that a mineral-pectin model system, that consists of pectin with a blockwise methylester distribution, would provide greater insights into the influence of the presence of

Ca^{2+} ions and pectin DM on Zn^{2+} binding capacity and Zn^{2+} bioaccessibility in contrast to mineral-pectin model systems, containing pectin with a random methylester distribution. The obtained pectin samples were used to prepare single mineral- (Zn^{2+} or Ca^{2+}) and competing mineral- (Zn^{2+} and Ca^{2+}) pectin model systems. On the one hand, the mineral binding capacity of these model systems was evaluated through an equilibrium dialysis experiment. In addition, although some compounds present during digestion (such as enzymes and bile salts) are hypothesised to bind and interact with minerals (Bonar-law & Sanders, 1993; Celus, Kyomugasho, et al., 2018; Mukhopadhyay & Maitra, 2004), their potential role in mineral binding during digestion have not been explored. Therefore, on the other hand, the competing mineral-pectin model systems were subjected to simulated gastric and small intestinal phases through distinct *in vitro* digestion procedures. This allowed for evaluation of the Zn^{2+} BAC as influenced by (i) the presence of Ca^{2+} ions and (ii) pectin DM as well as (iii) digestive compounds, given that the complexity of the *in vitro* digestion procedure was gradually increased. Since the bulk of absorption for most minerals takes place in the small intestine (Goff, 2018), mineral BAC in this paper is defined as the fraction of mineral that is available for absorption in the in vitro simulated small intestine. The possible effect of fermentation in the large intestine is therefore not considered in this work.

2. Material and methods

2.1. Materials

Citrus pectin with a high degree of methylesterification (DM of 82.2 ± 1.2%) was purchased from Sigma-Aldrich (Diegem, Belgium) and used as a starting material to prepare pectin samples with low and intermediate degrees of methylesterification by enzymatic demethylesterification.

144 Fresh carrots (*Daucus carota* cv Nerac) were purchased from a local supermarket and stored
145 at 4 °C until use. For isolation of carrot pectin methylesterase (PME), the carrots were peeled,
146 cut into 1 cm^3 cubes and PME was extracted and purified with PME inhibitor (PMEI) from
147 kiwi fruit as described by Jolie et al. (2009). PME activity was determined prior to use in
148 demethylesterification of pectin (Ly-Nguyen et al., 2002).

149 All chemicals and reagents used were of analytical grade and were purchased from Sigma
150 Aldrich (Diegem, Belgium) except for KCl, $MgCl_2.6H_2O$, NaOH, methanol (Acros Organics,
151 Geel, Belgium); KH_2PO_4; $NaHCO_3$, NaCl, H_2SO_4 (Fisher Scientific, Merelbeke, Belgium);
152 HCl (VWR, Leuven, Belgium); $CaCl_2.2H_2O$ (Chem-Lab, Zedelgem, Belgium). Pancreatin
153 was kindly donated by Nordmark (Saeby, Denmark). Ultrapure water (organic free, 18.2 MΩ
154 cm resistance) was supplied by a Simplicitiy™ water purification system (Millipore,
155 Billerica, USA) and was used for all experiments.

156 **2.2. Preparation of pectin samples**

157 To obtain pectin samples with low and intermediate degrees of methylesterification, high
158 methylesterified citrus pectin (DM of 82.2 ± 1.2%) was enzymatically demethylesterified.
159 Therefore, this pectin was incubated with purified carrot PME at 30 °C for predetermined
160 time periods as described by Ngouémazong et al. (2011). The resulting pectin solutions were
161 adjusted to pH 6 with NaOH (0.1 M), dialyzed (Spectra/Por®, MWCO = 12-14 kDa) for 48 h
162 against demineralized water, lyophilized and stored in a desiccator at room temperature until
163 further use.

164 **2.3. Characterization of pectin samples**

165 All pectin samples obtained were characterized for their degree of methylesterification (DM),
166 GalA content, molar mass distribution and intrinsic mineral concentrations.

Degree of methylesterification; Measurement of DM was done by Fourier transform infra-red (FT-IR) (Shimadzu FTIR-8400S, Japan) spectroscopy according to the method described by Kyomugasho et al. (2015). Measurement was performed in triplicate.

GalA content; In order to determine the GalA content, pectin samples were first hydrolysed (in duplicate) with concentrated sulphuric acid as described by Ahmed & Labavitch (1978). Subsequently, GalA content of the hydrolysed samples was quantified in triplicate by a spectrophotometric method (Blumenkrantz & Asboe-Hansen, 1973).

Molar mass distribution; To ensure that no depolymerization occurred during the demethylesterification procedure, the molar mass distribution of the pectin samples obtained was determined (in duplicate) using high-performance size exclusion chromatography (HPSEC) coupled to a refractive index detector (Shodex RI-101, Showa Denko K.K., Kawasaki, Japan) and a multi-angle laser light scattering detector (PN3621, Postnova Analytics, Landsberg am Lech, Germany) as described by Shpigelman et al. (2014).

Intrinsic mineral concentrations; The pectin samples were incinerated (in duplicate) in a muffle furnace at 550 °C for 22 h and the intrinsic mineral content (^{44}Ca and ^{66}Zn) was determined by inductively coupled plasma mass spectrometry (ICP-MS), according to the method described by Kyomugasho et al. (2015).

2.4. Determination of adsorption isotherms of pectin samples for single minerals: Zn^{2+} or Ca^{2+}

Zn^{2+} and Ca^{2+} adsorption isotherms of pectin with low, high and intermediate DM were determined through an adsorption equilibrium study as described by Celus et al. (2018). First, a 10 mL mineral-pectin solution was obtained with a 0.1% (w/v) pectin concentration and the Zn^{2+} or Ca^{2+} concentration varied from 0 to 1000 mg mineral/L by using $ZnSO_4.7H_2O$ and

190 $CaCl_2.2H_2O$ solutions. The maximum achieved mol ion/mol GalA ratio depended on the
191 obtained pectin sample and ranged between 6.06 and 6.30 mol Ca^{2+}/mol GalA and between
192 3.73 and 3.87 mol Zn^{2+}/mol GalA. The pH of each solution was above 5.8, which exceeds the
193 pKa of pectin (3.8-4.1). The obtained mineral-pectin solutions were transferred into rinsed
194 dialysis membranes (Spectra/Por®, MWCO = 3.5 kDa) and then dialyzed against 50 mL of
195 ultrapure water at 15 °C for 48 h (to achieve equilibrium). Afterwards, the concentration of
196 unbound (free) Zn^{2+}- or Ca^{2+}- ions present in the solution outside the membrane (dialysis
197 water) was measured spectrophotometrically. Zn^{2+} concentration was measured (in triplicate)
198 as described by Platte and Marcy (1959). An aliquot of 0.2 mL of the (dialysis water) solution
199 outside the membrane at equilibrium was transferred into a cuvette along with 0.1 mL of
200 borate buffer (0.5 M, pH 9) and 0.06 mL zincon solution (0. 0028 M). Subsequently, the
201 volume was adjusted with ultrapure water to 1 mL. After 5 minutes, the absorbance was
202 measured at 620 nm (1800 UV spectrophotometer, Shimadzu, Kyoto, Japan). Ca^{2+}
203 concentration was measured (in triplicate) using a Spectroquant® Calcium kit (Merck KGaA,
204 Darmstadt, Germany). To a 1 mL aliquot of the (dialysis water) solution outside the
205 membrane at equilibrium, 0.1 mL of 8-hydroxyquinoline solution was added in order to limit
206 interference with other minerals (e.g. Mg^{2+} and Fe^{2+}) and subsequently 0.1 mL of colour
207 reagent (a phthalein derivative) was added. After 5 minutes, the absorbance was measured at
208 565 nm (1800 UV spectrophotometer, Shimadzu, Kyoto, Japan). The Zn^{2+} and Ca^{2+}
209 concentration were quantified using standard curves of Zn^{2+} (0-20 mg/L) and Ca^{2+} (0-4
210 mg/L), respectively. The binding capacity (q_e) of pectin samples for Zn^{2+} and Ca^{2+} was
211 estimated by the following equation (Khotimchenko, Kolenchenko, & Khotimchenko, 2008):

212 $$q_e = \frac{[(C_0.V_{in} - C_e.(V_{in}+V_{out})]}{M_w.n_{GalA}}$$ ..... Equation 1

with, q_e, the adsorption capacity of pectin (mol cation/mol GalA) at ion equilibrium concentration; C_0, the initial mineral concentration (mg/L); C_e, the mineral concentration at equilibrium (mg/L); V_{in} and V_{out}, the volume inside (10 mL) and outside (50 mL) the dialysis membrane, respectively; M_w, atomic weight of Zn^{2+} and Ca^{2+} (65.38 g/mol and 40.078 g/mol, respectively) and n_{GalA}, the absolute amount of GalA units present in the pectin sample (mol).

The Langmuir adsorption model is most often used to describe an equilibrium sorption isotherm, which assumes monolayer adsorption of a ligand (minerals) at homogenous and finite binding sites within an adsorbent (pectin chain) (Celus et al., 2017; Foo & Hameed, 2010; Khotimchenko et al., 2008). Therefore, Langmuir adsorption equation was used to model the adsorption isotherm by plotting q_e as a function of C_e:

$$q_e = \frac{q_{max}.K_L.C_e}{1+K_L.C_e} \quad \text{Equation 2}$$

with, q_{max}, the maximum binding capacity at the monolayer (mol cation/mol GalA); q_e, the adsorption capacity of pectin (mol cation/mol GalA) at equilibrium concentration; K_L, the Langmuir constant (L/mmol cation), which is a measure of the interaction energy and C_e, the mineral equilibrium concentration (mmol cation/L). By using non-linear one step regression (SAS version 9.4, Cary, North Carolina), modelling of the experimental results by Langmuir adsorption isotherm was performed.

2.5. Determination of Zn^{2+} binding capacity of pectin samples in presence of Ca^{2+}

In order to determine the influence of DM and the presence of Ca^{2+} on the Zn^{2+} binding capacity of pectin, an adsorption equilibrium study was performed as described in Section 2.4. However, in this experiment, the pectin-mineral solutions contained both Zn^{2+}- and Ca^{2+}-ions. The solutions consisted of 0.1% (w/v) pectin, a constant Zn^{2+} concentration (100 mg/L) and a varying Ca^{2+} concentration (0-1000 mg/L). These solutions were then dialyzed as

described in Section 2.4 and Zn^{2+} binding capacity (q_e) was estimated using Equation 3. Specificity of zincon for Zn^{2+} in presence of Ca^{2+} was tested and no interference of Ca^{2+} on the spectrophotometric determination of Zn^{2+} was detected (Table A, Supplementary material).

The fractional conversion equation was used as an empirical model to plot binding capacity of pectin samples for Zn^{2+}, q_e, as a function of the Ca^{2+} to Zn^{2+} ratio, defined as R (Van Boekel, 1996):

$$q_e = q_{min} + (q_0 - q_{min}).e^{-xR} \quad \text{Equation 3}$$

with, q_e, the binding capacity of pectin (mol Zn^{2+}/mol GalA) at equilibrium concentration; q_{min}, the minimal amount of Zn^{2+} that is bound to pectin (mol Zn^{2+}/mol GalA); q_0, the binding capacity of pectin samples for Zn^{2+} (mol Zn^{2+}/mol GalA) without added Ca^{2+}; x, change in amount of bound Zn^{2+} to pectin depending on the Ca^{2+} concentration (mol GalA/mol Ca^{2+}); and R, Ca^{2+} to Zn^{2+} ratio. Using non-linear one step regression (SAS version 9.4, Cary, North Carolina), modelling of the experimental results by the fractional conversion equation was performed.

2.6. *In vitro* simulated digestion of Zn^{2+} and Ca^{2+}- enriched pectin samples

In order to investigate the influence of the presence of Ca^{2+} as well as pectin DM on Zn^{2+} bioaccessibility (BAC), competing mineral-pectin model systems were subjected to a static *in vitro* simulation of the gastric and small intestinal phases of human digestion. To selectively examine the influence of these two factors, the competing mineral-pectin model system was subjected to the most simple *in vitro* simulation of gastric and small intestinal phases. This means that only the pH in these digestive phases was adjusted and influence of other factors could be excluded. Moreover, to evaluate the effect of digestive compounds on mineral

bioaccessibility, the complexity of the applied *in vitro* digestion model was gradually increased by adding enzymes, bile salts and simulated digestive fluids.

Competing mineral-pectin model systems with a constant Zn^{2+} concentration and different Ca^{2+} concentrations were prepared, ensuring that the final digestion mixture contained 1000 mg/L pectin, 100 mg/L Zn^{2+} and 0, 50, 100 or 1000 mg/L Ca^{2+}. Each model system was then subjected to the *in vitro* digestion procedures described below. Experiments were performed in duplicate.

***In vitro* digestion procedure 1 (pH);** In the simplest *in vitro* digestion procedure, only the pH of the mineral-pectin model systems was adjusted to simulate the gastric and small intestinal phase since the pH has been found to be an important factor influencing the interaction between cations and pectin (Kyomugasho, Willemsen, et al., 2015). The pH of the pectin-Zn^{2+} solution, with our without Ca^{2+}, was adjusted to 3 by using HCl (2 M), followed by addition of ultrapure water until 5 mL. This solution was incubated at 37 °C with end-over-end rotation (40 rpm) for 2 h in order to simulate the gastric phase. The pH was then adjusted to 7 with NaOH (1 M) and the solution adjusted to 10 mL with ultrapure water. Thereafter, the solution was incubated at 37 °C with end-over-end rotation (40 rpm) during 2 h to simulate the small intestinal phase.

***In vitro* digestion procedure 2 (pH, enzymes and bile salts);** The same procedure as described above was followed, however, enzymes and bile salts were added as well, according to the method described by Minekus et al. (2014). During the gastric phase, after adjusting the pH to 3, a pepsin solution was added (ensuring an enzymatic activity of 2000 U/mL of digest) followed by incubation for 2 h at 37 °C. During the intestinal phase, bile salts were added in a concentration of 10 mM in the final digestion mixture before adjusting the pH to 7. After adjusting the pH, pancreatin was added (ensuring α-amylase activity of 200

U/mL digest) followed by the addition of pure trypsin and chymotrypsin solutions to reach an enzymatic activity of 100 U/mL for trypsin and 25 U/mL for chymotrypsin in the final digestion mixture.

***In vitro* digestion procedure 3 (pH, enzymes, bile salts and electrolyte solutions);** In order to further increase the complexity of the procedure, gastric and small intestinal phase simulations were supplemented with simulated digestive fluid electrolyte solutions. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared based on the method of Minekus et al. (2014) (Table 1). SGF stock solution was added to the gastric phase before adjusting the pH and SIF stock solution was added at the start of the small intestinal phase.

Each final digestion mixture, after simulation of the small intestinal phase, was subjected to an equilibrium dialysis experiment as described in Section 2.4. Subsequently, the free Zn^{2+} concentration in the dialysis water was measured spectrophotometrically as explained in Section 2.4. The Zn^{2+} bioaccessibility was determined using the following equation:

$$Zn^{2+}\ \text{bioaccessibility}\ (\%) = \frac{\text{Free } Zn^{2+} \text{ at equilibrium}}{\text{Total amount of added } Zn^{2+}} . 100 \quad \text{.....Equation 4}$$

2.7. Statistical analysis

Significant differences ($p < 0.05$) with 95% confidence interval among the mean values were analyzed by applying ANOVA (one-way analysis of variance) and Tukey HSD tests, which were performed using the statistical software JMP (JMP 13, SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Characterization of pectin samples

An overview of the investigated characteristics of the enzymatically demethylesterified pectin samples is given in Table 2. Through performing enzymatic demethylesterification of citrus pectin with high (DM80) degree of methylesterification, pectin samples with low (DM10) and intermediate (DM45) degree of methylesterification were obtained (Table 2). The GalA content of these samples was comparable and their weight-average molar masses were not significantly different (Table 2). Consequently, it can be concluded that PME was specific for removal of methyl groups from pectin without changing other structural parameters. In other words, the pectin samples obtained only differed in DM. Cations bound to pectin are not removed during dialysis and therefore the pectin samples obtained contain intrinsic cations (Table B, Supplementary material). Furthermore, Ca^{2+} was naturally present in substantially higher amounts than Zn^{2+} and the concentration of both minerals were shown to increase with decreasing DM (Table 2). This increase can be explained since the electrostatic cation-pectin interaction is higher for pectin with a low DM, as explained further. Nonetheless, intrinsic Zn^{2+} and Ca^{2+} concentrations of the pectin samples obtained were found to be negligible in comparison to the mineral amounts that were added externally during the following experimental set-up.

3.2. Adsorption isotherms of pectin samples for single minerals: Zn^{2+} or Ca^{2+}

In order to study the effect of pectin DM and cation type on mineral binding capacity of pectin, adsorption isotherms of single minerals (Zn^{2+} or Ca^{2+}) to pectin with different DMs were established. Figure 1 represents these adsorption isotherms in which the binding capacity of pectin samples for Zn^{2+} (Figure 1A) and Ca^{2+} (Figure 1B) at equilibrium, i.e. q_e (mol cation/mol GalA) is plotted as a function of the cation concentration at equilibrium, i.e. C_e (mmol cation/L). The experimental values could be well fitted with the Langmuir adsorption model ($R^2_{adjusted} > 0.98$, data not shown), which implies that the pectin samples can

be considered to contain a certain amount of homogeneous binding sites and cation interactions are occurring via monolayer adsorption with a constant adsorption energy (Foo & Hameed, 2010). In addition, when these binding sites are saturated with ions, i.e. maximum binding capacity of pectin samples is reached, no additional pectin-cation interaction can occur which is indicated as a plateau condition (Foo & Hameed, 2010). Two main parameters of the Langmuir adsorption model, q_{max}, which indicates maximum ion binding capacity at the monolayer (mol cation/mol GalA) and K_L, which is the Langmuir constant (L/mmol cation) representing the pectin-cation interaction energy, were estimated and are enlisted in Table 3.

From these results, it can be concluded that with a decreasing pectin DM, the maximum binding capacity (q_{max}) for Zn^{2+} and Ca^{2+} increases. For instance, a pectin sample with DM of 80% bound 0.159 ± 0.003 mol Zn^{2+}/mol GalA, while decreasing DM to 10% resulted in 0.494 ± 0.014 mol Zn^{2+} bound per mol GalA. This can be explained by a higher number of negatively charged free carboxyl groups (COO^-) with decreasing pectin DM. These groups act as bindings site for cations and are present when pH is above the pKa of pectin (3.8-4.1) (Sriamornsak, 2003). Consequently, for a lower DM, there are a higher number of negatively charged free COO^- groups which results in more cation binding. These results are in line with the study of Celus et al. (2018), who concluded as well that the maximum Zn^{2+} and Ca^{2+} binding capacity of pectin was largely directed by the DM. The observed q_{max} of Zn^{2+} was significantly higher ($p < 0.05$) than q_{max} of Ca^{2+} for pectin of a given DM. This can be attributed to the potential of Zn^{2+} to interact with both carboxyl groups (COO^-) and hydroxyl groups (OH^-) while Ca^{2+} only has the ability to bind with COO^- groups (Assifaoui et al., (2015). Moreover, due to the higher electronegativity of Zn^{2+} (1.6-1.81) in comparison to Ca^{2+} (1-1.36), the former probably has more potential to interact with pectin in comparison to Ca^{2+} (Kyomugasho et al., 2017). In contrast to what is seen for q_{max} values, this research

provided different findings for interaction energy (K_L values) than what has been reported in other studies. From the current study, it can be concluded that no significant differences are observed between pectin with different DM, particularly for high and intermediate DM, and between Zn^{2+} and Ca^{2+}. The difference between this study and others could be explained since in this study more conditions with low cation concentrations (before the plateau value is reached) were experimentally determined, which allows a more precisely estimation of the Langmuir constant (K_L).

3.3. Influence of pectin degree of methylesterification and presence of Ca^{2+} on the Zn^{2+} binding capacity of pectin

Given that a real food system is more complex than the single mineral-pectin model explored in Section 3.2, with several minerals being present at the same time, the complexity of the mineral-pectin model system was increased to a competing mineral model system in which both Zn^{2+} and Ca^{2+} were simultaneously present. Since plant-based foods mostly contain higher levels of Ca^{2+} than Zn^{2+}(Ekholm et al., 2007; Marles, 2017), it was the objective to investigate the effect of increasing Ca^{2+} concentration on Zn^{2+} binding capacity of pectin with different DMs. Therefore, competing mineral-pectin model systems with a constant Zn^{2+} concentration and increasing Ca^{2+} concentrations until a Ca^{2+} to Zn^{2+} ratio of 10:1, which can be a relevant in a real plant-based food system (e.g. in legumes) (Hayat et al., 2014), were established. The results obtained are shown in Figure 2, in which the amount of Zn^{2+} bound to pectin at equilibrium (mol Zn^{2+}/mol GalA), q_e, is plotted as a function of the Ca^{2+} to Zn^{2+} ratio. The experimental results were well described by an empirical model, the fractional conversion equation (Equation 3) ($R^2_{adjusted} > 0.99$, data not shown). Two parameters of the fractional conversion equation, i.e. q_{min}, the minimal amount of Zn^{2+} that is bound to pectin (mol Zn^{2+}/mol GalA) and x, the change in amount of bound Zn^{2+} to pectin depending on the Ca^{2+} concentration (mol GalA/mol Ca^{2+}) were estimated and enlisted in Table 4.

Degree of methylesterification; The effect of DM on the Zn^{2+} binding capacity of pectin in presence of Ca^{2+} is comparable to what was observed for single mineral-pectin model systems. Pectin with a lower DM exhibits a higher Zn^{2+} binding capacity (q_e) since it possesses more potential binding sites for cations (COO^- groups) than pectin with intermediate or high DM. In addition, the minimal amount of Zn^{2+} that is bound to pectin, q_{min}, significantly increases with decreasing pectin DM (Table 4). On the contrary, there was no significant difference in the x values for pectin samples with different DM, which indicates that the dependency of the change in amount of bound Zn^{2+} to pectin on the Ca^{2+} concentration is not influenced by DM.

Presence of Ca^{2+}; In Figure 2 it can be seen that the amount of Zn^{2+} that is bound to pectin at equilibrium condition decreases when the Ca^{2+} to Zn^{2+} ratio increases. This suggests that Zn^{2+} and Ca^{2+} are competing for the same binding sites of the pectin molecule (i.e. COO^- groups). This could be confirmed by the fact that interaction energy (K_L) for Zn^{2+} and Ca^{2+} with pectin, was not significantly different (Table 3). However, since q_{min} was found significantly different for pectin samples with different DM, not all COO^- binding sites can be occupied with Ca^{2+} ions if Zn^{2+} is present (Table 4). Moreover, Zn^{2+} has the potential to interact with both carboxyl groups (COO^-) and hydroxyl groups (OH^-) while Ca^{2+} only has the ability to bind with COO^- groups (Assifaoui et al., 2015). Consequently, despite the presence of Ca^{2+}, there always is a certain amount Zn^{2+} attached to pectin.

3.4. *In vitro* Zn^{2+} bioaccessibility

In order to determine the influence of the presence of Ca^{2+} as well as pectin DM on Zn^{2+} bioaccessibility in the small intestine (BAC), pectin-Zn^{2+} model systems with different pectin DMs and different Ca^{2+} concentrations were subjected to distinct *in vitro* digestion procedures with simulation of gastric and small intestinal phases. Since the oral phase is only

of major relevance in starch-containing and/or solid foods, both of which do not apply to the pectin-mineral solution, it was chosen to only simulate gastric and small intestinal phases of human digestion for the simple pectin-mineral model systems studied in this work (Minekus et al., 2014). Moreover, it was opted not to simulate large intestinal phase since Zn^{2+} adsorption primarily takes place in the small intestine and although evidence has been found for colonic absorption of other minerals, such as Ca^{2+}, there is limited data to suggest a similar capacity for Zn^{2+} (Carbonell-Capella et al., 2014; Gopalsamy et al., 2015; Halsted, 2003). Since pectin is a non-digestible hetero polysaccharide, digestive enzymes do not act on it (Baye et al., 2017). Therefore, a simple *in vitro* procedure was initially selected in which gastric and small intestinal phases (pH conditions only) were simulated without addition of enzymes, bile salts nor electrolyte solutions. Since digestive compounds (such as enzymes and bile salts) might be able to interact with minerals as well, the complexity of the model was the gradually increased by including enzymes and bile salts. In a third step, the complexity of the *in vitro* digestion procedure was further increased by the addition of simulated digestive fluids since they contain electrolytes, which might interact as well with the pectin molecule and thereby might influence the Zn^{2+} BAC. In Section 3.4.1, the effect of pectin DM and Ca^{2+} on Zn^{2+} BAC will be discussed based on the results of the simplest digestion procedure. Differences between the explored *in vitro* digestion procedures will be discussed in Section 3.4.2.

3.4.1. Influence of pectin degree of methylesterification and presence of Ca^{2+} on *in vitro* Zn^{2+} bioaccessibility

Figure 3 represents the Zn^{2+} BAC as influenced by pectin DM and the Ca^{2+} to Zn^{2+} ratio, evaluated by the simplest *in vitro* digestion procedure (only pH; no enzymes, bile salts nor simulated digestive fluids).

Degree of methylesterification; It can be seen from Figure 3 that for every Ca^{2+} to Zn^{2+} ratio, Zn^{2+} BAC increased significantly ($p < 0.05$) with increasing pectin DM except for the Ca^{2+} to Zn^{2+} ratio of 10. For example, when no Ca^{2+} was added (Ca^{2+} to Zn^{2+} ratio of 0), the maximum Zn^{2+} BAC (56.4 ± 0.1%) was exhibited by the pectin sample with DM of 80%, while the minimum Zn^{2+} BAC (5.1 ± 0.1%) was observed for pectin samples with a DM of 10%. These results are in agreement with Kyomugasho et al. (2017) and Celus et al. (2018) who reported that Zn^{2+} BAC decreases with decreasing DM. According to Sections 3.2 and 3.3, representing the influence of DM on mineral (Ca^{2+} and Zn^{2+}) binding capacity, a decrease in DM increases the mineral binding capacity. Furthermore, the maximum mineral binding capacity of pectin, expressed as q_{max} (mol cation/mol GalA), was also dependent on the DM i.e. lowest DM resulted in the highest q_{max} (Table 3). In combination with results of ion binding, it can be assumed that lowering DM is associated more binding sites for minerals and therefore, makes the mineral less free, i.e. less bioaccessible.

Presence of Ca^{2+}; The effect of Ca^{2+} on Zn^{2+} BAC can be observed from Figure 3. For all evaluated pectin samples, the Zn^{2+} BAC significantly ($p < 0.05$) increased with an increasing Ca^{2+} to Zn^{2+} ratio (i.e. with increasing Ca^{2+} concentration). For each pectin sample, the minimum Zn^{2+} BAC, was associated with the lowest Ca^{2+} to Zn^{2+} ratio (when no Ca^{2+} was added) while maximum Zn^{2+} BAC was obtained for the highest Ca^{2+} to Zn^{2+} ratio. According to Section 3.3, adding Ca^{2+} ions decreased the amount of Zn^{2+} bound to pectin until a certain level was reached upon which addition of more Ca^{2+} had no effect on the Zn^{2+} binding capacity of pectin. These results can be related to the observations from Figure 3. When the Ca^{2+} concentration increases, Zn^{2+} becomes more bioaccessible because less Zn^{2+} is bound to pectin. Moreover, from Figure 2, it can be hypothesised that a Ca^{2+} to Zn^{2+} ratio of 10 is not necessary to reach the high Zn^{2+} BAC and that probably with a Ca^{2+} to Zn^{2+} ratio of 4 the same high Zn^{2+} BAC would hypothetically be established. In addition, Zn^{2+} BAC never

increased to 100% in spite of increasing Ca^{2+} concentration. This could be explained by the observed plateau value (q_{min}) in Figure 2, which did not reach 0, implying a certain amount of Zn^{2+} attached despite an increasing Ca^{2+} concentration. Hence, when the Ca^{2+} concentration keeps increasing, Zn^{2+} BAC is increasing until a certain level because Zn^{2+} ions, attached to COO^- groups of pectin samples, can be replaced by Ca^{2+} ions but Zn^{2+} ions, attached to OH^- groups, are probably not replaced by Ca^{2+} ions (Section 3.3).

During digestion, reorganisation of cations, bound to the pectin molecule, is hypothesized to occur. The pH during the gastric phase (2-3) is lower than the pKa of pectin (3.8-4.1), therefore, cations are released at this stage of digestion. The pH of the food entering the small intestine increases again (pH 6-7) and there cations (which might also be coming from other simultaneous ingested food sources) can possibly bind the pectin molecule. The cation type binding to pectin at this stage of digestion will mainly be dependent on the cation type concentration and affinity for pectin.

After passing through the small intestine, pectin, as soluble dietary fiber, will be fermented by microbiota. At the large intestinal phase, pectin can be depolymerized through fermentation, reducing its binding capacity. Consequently, minerals can be released. However, Zn^{2+} absorption is described to not or only limitedly occur in the colon (Goff, 2018; Gopalsamy et al., 2015). Moreover, if pectin would be immediately fermented at the beginning of the large intestine, the released minerals might still be absorbed at the end of the small intestine, however, mineral absorption is decreasing as the cation goes through the intestine: from duodenum over jejunum to ileum and eventually the large intestine (Lopez & Leenhardt, 2002).

3.4.2. Differences between *in vitro* digestion procedures

To cope with a more real human digestive system, the complexity of the *in vitro* model was increased by adding bile salts, enzymes and simulated digestive fluids along with adjusting pH when simulating the gastric and/or intestinal phase (Minekus et al., 2014). The possible effect of digestive compounds on Zn^{2+} BAC is represented in Figure 4 for each DM.

According to Figure 4, Zn^{2+} BAC generally decreases with addition of enzymes and bile salts in comparison to the digestion method with only pH adjustment. In addition, almost no further changes in Zn^{2+} BAC are observed after addition of simulated digestive fluids (electrolyte solutions). This could probably be explained by the fact that the simulated digestive fluids mainly contain monovalent ions, which are less likely to be bound to pectin in presence of divalent ions. Because of the reduction in Zn^{2+} BAC after addition of digestive enzymes and bile salts, it can be assumed that presence of enzymes and/or bile salts can decrease Zn^{2+} BAC. In order to investigate the individual effect of these compounds on Zn^{2+} BAC, an extra experimental set-up was designed (only pectin with DM 45 was used). On the one hand, *in vitro* digestion was performed with adjustment of pH and addition of digestive enzymes. On the other hand, *in vitro* digestion was performed with adjustment of pH and addition of bile salts. The results obtained are presented in Figure 5.

After enzyme addition, values for Zn^{2+} BAC were comparable to those after only adjustment of pH. After bile salt addition, values for Zn^{2+} BAC were significantly reduced. From this comparison, it can be concluded that bile salts may interact with Zn^{2+} and Ca^{2+}, thereby reducing the Zn^{2+} BAC, in contrast to addition of enzymes which had no effect.

Bile salts are bio-surfactants that can play an essential role in digestion and absorption of nutrients (e.g. lipids) and also help in the excretion of several blood waste products (e.g. bilirubin) (Bauer, Jakob, & Mosenthin, 2005; Maldonado-Valderrama, Wilde, MacIerzanka, & MacKie, 2011; Mukhopadhyay & Maitra, 2004). Bile salts are amphiphilic in nature and

consist of two connecting units: a rigid steroid nucleus (with a hydrophobic and hydrophilic face) and a short aliphatic side chain (Maldonado-Valderrama et al., 2011; Mukhopadhyay & Maitra, 2004). Cholate, chenodeoxicholate and deoxicholate are the most abundant bile salts found in human and they contain both carboxylic (COO^-) and hydroxyl groups (OH^-) in their chemical structure. Therefore, it can be deduced that when bile salts are added in the small intestine, Zn^{2+} can bind with bile salts through the COO^- and/or OH^- groups. This is in accordance with both Bonar-Law & Sanders (1993) and Mukhopadhyay & Maitra, (2004), who have reported that bile salts and their analogous can be used as supramolecular receptors for several guest ions and molecules. Furthermore, 95% of the bile salts which are separated from the dietary lipid in the ileum (at the lower end of the small intestine) are reabsorbed and returned to the liver for recirculation (Maldonado-Valderrama et al., 2011; Mukhopadhyay & Maitra, 2004). Therefore, it can be possible that, although bile salts may bind Zn^{2+}, in the small intestine, Zn^{2+} can be released and become bioaccessible after the reabsorption of bile salts in the lower end of the small intestine. However, the release of cations from bile salts, after reabsorption has not been investigated yet.

From the experiments in this study, it can be assumed that Zn^{2+} bound to the added bile salts is not detected given that the method is based on quantification of free ions. Consequently, in the case that minerals are released after reabsorption of the bile salts, Zn^{2+} BAC could be underestimated in this experiment. In addition, if lipids are present in a real food matrix, bile salts will interact with these compounds and resulting in possibly less interaction with minerals.

From Figure 5, it is clear that the influence of DM and presence of Ca^{2+} on Zn^{2+} BAC is less pronounced in the complex *in vitro* digestion procedures (with enzymes, bile salts and/or simulated digestive fluids) than in the simple *in vitro* digestion procedure (in which only the pH adjusted). However, the influence of DM and presence of Ca^{2+} on Zn^{2+} BAC can best be

evaluated by the most simple digestion model (with pH adjustment only). Because, on the one hand, it is reported that most of the bile salts usually reabsorb and return to the liver for recirculation (reference) which may result in the possible release of bound minerals. On the other hand, a very simple pectin-mineral model system (without for example lipids) is considered.

4. Conclusion

The general objective of this study was to investigate the influence of the presence of Ca^{2+} on Zn^{2+} binding capacity and bioaccessibility (BAC) in a competing mineral-pectin model system with different degrees of pectin methylesterification. It could be concluded that with increasing Ca^{2+} concentration, the Zn^{2+} binding capacity of pectin decreases due to the competition between Zn^{2+} and Ca^{2+} for the available binding sites (COO^- groups). However, even if the Ca^{2+} to Zn^{2+} ratio reaches 10:1, a minimal amount of Zn^{2+} remains bound to pectin. A plateau value is reached from which the Zn^{2+} binding capacity is not further decreasing despite a further addition of Ca^{2+} ions. In addition, pectin DM has an influence on the Zn^{2+} binding capacity of pectin, with lower DM pectin exhibiting a higher Zn^{2+} binding capacity since it possesses more binding sites (COO^- groups). Furthermore, in order to determine the influence of the presence of Ca^{2+} as well as pectin DM on Zn^{2+} BAC, it was a challenge to find the most appropriate *in vitro* digestion procedure that could simulate the upper digestive tract, to fundamentally study the influence of these specific factors only. Therefore, pectin-Zn^{2+}-(Ca^{2+}) model systems with constant Zn^{2+} concentration and different DMs as well as Ca^{2+} concentrations were subjected to different *in vitro* digestion procedures. Upon addition of bile salts, Zn^{2+} BAC reduces, as Zn^{2+} probably interacts with bile salts. However, no significant changes in Zn^{2+} BAC were found either by the addition of enzymes or simulated digestive fluids. Since in this work, a very simple model system was used (only containing pectin and minerals), it was recommended to evaluate the influence of Ca^{2+}

549 concentration and pectin DM on Zn^{2+} BAC based on the results of the simplest *in vitro*
550 digestion procedure. Furthermore, when it is assumed that minerals, bound to bile salts, can
551 again be released after reabsorption, the addition of bile salts would lead to an
552 underestimation of Zn^{2+} BAC. Based on the results of the simplest procedure (only
553 adjustment of pH) it can be concluded that with increasing Ca^{2+} concentration as well as DM,
554 Zn^{2+} BAC increases. However, Zn^{2+} BAC never increased to 100% since there always is a
555 certain amount of Zn^{2+} attached to pectin, independently from a further increase in Ca^{2+}
556 concentration.

557 Since this study shows for the first time that Zn^{2+} BAC can increase when Ca^{2+} is added
558 without taking into account, there is potential in the addition of Ca^{2+} to foods (in which pectin
559 is believed to be the major antinutrient) in order to increase Zn^{2+} BAC. However, this should
560 be confirmed by *in vivo* studies. Besides, in several food applications, a certain (low) pectin
561 DM is necessary to meet desired functionalities (e.g. pectin as a thickening and gelling
562 agent). However, from a nutritional point of view it is best to opt for the case in which the
563 functionality is met for the highest DM since in this condition mineral BAC will be less
564 reduced.

565 **5. Future perspectives**

566 As mentioned in the conclusion, these results show *in vitro* proof for the potential of addition
567 of Ca^{2+} to increase Zn^{2+} BAC in a simple pectin model system in which pectin is the major
568 antinutrient. In addition, recently, a lot of research is done on the relevance and importance of
569 the large intestinal phase and the gut microbiome. If researchers can prove *in vivo* that Zn^{2+} is
570 absorbed at this stage of digestion, it might be interesting to include this digestive phase in
571 future experiments as well since pectin is fermented in the large intestine and the
572 fermentation process is supposed to influence the pectin mineral binding capacity. Moreover,

573 further research on the potential role of bile salts as mineral chelator is needed. In general,
574 further *in vivo* validation is required starting from the fundamental understanding on the
575 influence of a large range of food product intrinsic factors (e.g. pectin structure condition,
576 mineral concentrations) on digestibility phenomena that can be obtained through *in vitro*
577 studies.

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704

705 **Table 1.** Recommended concentrations of electrolyte solutions in simulated gastric fluid and simulated intestinal
706 fluid (Minekus et al., 2014).

Constituent	Simulated gastric fluid (SGF) mmol/L	Simulated intestinal fluid (SIF) mmol/L
K^+	7.8	7.6
Na^+	72.2	123.4
Cl^-	70.2	55.5
$H_2PO_4^-$	0.9	0.8
HCO_3^-, CO_3^{2-}	25.5	85
Mg^{2+}	0.1	0.33
NH_4^+	1. 0	-

707

708 **Table 2.** The investigated characteristics of enzymatic demethylesterified pectin samples. DM (%) is the degree
709 of methylesterification, GalA content is the concentration of galacturonic acid and M_w (kDa) is the weight-average
710 molar mass. All average values are listed with their standard deviations. Different letters (a, b and c) indicate
711 significant ($p < 0.05$) differences between pectin samples for a characteristic.

Sample code	DM (%)	GalA content (mg GalA/g pectin)	M_w (kDa)	Intrinsic Zn^{2+} content (µg/g pectin sample)	Intrinsic Ca^{2+} content (µg/g pectin sample)
DM10	9.4 ± 0.1^{c}	767.1 ± 52.2^{b}	54.5 ± 2.5^{a}	17.3 ± 5.4^{a}	839.4 ± 87.3^{a}
DM45	44.2 ± 1.8^{b}	796.6 ± 20.7^{a}	60.7 ± 2.8^{a}	11.8 ± 4.9^{a}	731.0 ± 292.3^{ab}
DM80	82.2 ± 1.2^{a}	768.5 ± 56.3^{b}	52.5 ± 1.4^{a}	7.7 ± 6.9^{a}	516.6 ± 41.7^{b}

712

713

Table 3. Parameter estimates for Langmuir adsorption model-based isotherms of Zn^{2+} and Ca^{2+} to pectin with different degrees of methylesterification (DM). q_{max} is the maximum cation binding capacity, expressed as mol cation/mol GalA and K_L(L/mmol cation) represents the adsorption energy. All average values are listed with their standard deviations. Different capital letters (A-B) indicate significant differences ($p < 0.05$) in q_{max} or $K_L$values between Zn^{2+} and Ca^{2+} of the same pectin sample. Different lower case letters (a-c) indicate significant differences ($p < 0.05$) in q_{max} or $K_L$values between different pectin samples for a particular cation (Zn^{2+} or Ca^{2+} adsorption).

Sample code	q_{max} (mol Zn^{2+}/mol GalA)	K_L (L/mmol Zn^{2+})	q_{max} (mol Ca^{2+}/mol GalA)	K_L (L/mmol Ca^{2+})
DM10	0.494 ± 0.014^{Aa}	211.1 ± 25.2^{Aa}	0.424 ± 0.008^{Ba}	305.6 ± 30.5^{Aa}
DM45	0.334 ± 0.005^{Ab}	144.2 ± 10.3^{Aa}	0.270 ± 0.008^{Bb}	148.1 ± 11.5^{Ab}
DM80	0.159 ± 0.003^{Ac}	129.1 ± 12.2^{Aa}	0.118 ± 0.002^{Bc}	124.9 ± 19.9^{Ab}

Table 4. Parameter estimates for fractional conversion equation of the Zn^{2+} binding capacity (in presence of Ca^{2+}) of pectin with different degrees of methylesterification (DM). q_{min} is the minimal amount of Zn^{2+} that is bound to pectin (mol Zn^{2+}/mol GalA) and x is the change in amount of bound Zn^{2+} to pectin depending on the Ca^{2+} concentration (mol GaIA/ mol Ca^{2+}). All average values are listed with their standard deviations. Different lower case letters (a-c) indicate significant differences ($p < 0.05$) in q_{min} and x values between different pectin samples.

Sample code	q_{min} (mol Zn^{2+}/mol GalA)	x (mol GaIA/ mol Ca2+)
DM10	0.083 ± 0.002^{a}	0.71 ± 0.02^{d}
DM45	0.056 ± 0.002^{b}	0.75 ± 0.02^{d}
DM80	0.026 ± 0.002^{c}	0.76 ± 0.04^{d}

729 **Figure captions**

730 **Figure 1.** Adsorption isotherms representing q_e (mol cation/mol GalA) as a function of C_e, the
731 cation equilibrium concentration (mmol/L), for (A) Zn^{2+} and (B) Ca^{2+}. Symbols indicate the
732 experimental data and curves are corresponding modeled Langmuir adsorption isotherms.

733 **Figure 2.** q_e, the Zn^{2+} binding capacity (in presence of Ca^{2+}) of pectin with different degrees
734 of methylesterification (DM) (mol Zn^{2+}/mol GalA) as a function of the Ca^{2+} to Zn^{2+} ratio.
735 Symbols indicate the experimental data and curves are the experimental results modeled by the
736 fractional conversion equation. (Zn^{2+} concentration: 100 mg/L).

737 **Figure 3.** *In vitro* Zn^{2+} bioaccessibility (BAC) (%) ± standard deviation as a function of the
738 Ca^{2+} to Zn^{2+} ratio evaluated by the simplest *in vitro* digestion procedure (only pH; no enzymes,
739 bile salts nor simulated digestive fluids). Different capital letters (A-C) indicate significant
740 differences ($p < 0.05$) between pectin degree of methylesterification (DM) for a specific Ca^{2+}
741 to Zn^{2+} ratio. Different lower case letters (a-d) indicate significant differences ($p < 0.05$)
742 between Ca^{2+} to Zn^{2+} ratios for a specific DM.

743 **Figure 4.** *In vitro* Zn^{2+} bioaccessibility (BAC) (%) ± standard deviation as a function of the
744 Ca^{2+} to Zn^{2+} ratio for pectin with a degree of methylesterification (DM) of (A) 10%; (B) 45%
745 and (C) 80%. Procedure 1 is an *in vitro* digestion procedure with adjustment of pH; procedure
746 2 is an *in vitro* digestion procedure with adjustment of pH and addition of enzymes and bile
747 salts; and procedure 3 is an *in vitro* digestion procedure with adjustment of pH and addition of
748 enzymes, bile salts and simulated digestive fluids. Different capital letters (A-C) indicate
749 significant differences ($p < 0.05$) between the different procedures for a specific Ca^{2+} to Zn^{2+}
750 ratio. Different lower case letters (a-d) indicate significant differences ($p < 0.05$) between Ca^{2+}
751 tot Zn^{2+} ratios for a specific procedure.

752 **Figure 5.** *In vitro* Zn^{2+} bioaccessibility (BAC) (%) ± standard deviation as a function of the
753 Ca^{2+} to Zn^{2+} ratio for pectin with degree of methylesterification (DM) of 45%. Zn^{2+} BAC is
754 evaluated by an *in vitro* digestion procedure with adjustment of pH and addition of enzymes or
755 bile salts. Different capital letters (A-B) indicate significant differences ($p < 0.05$) between the
756 different procedures for a specific Ca^{2+} to Zn^{2+} ratio. Different lower case letters (a-d) indicate
757 significant differences ($p < 0.05$) between Ca^{2+} tot Zn^{2+} ratios for a specific procedure.

758

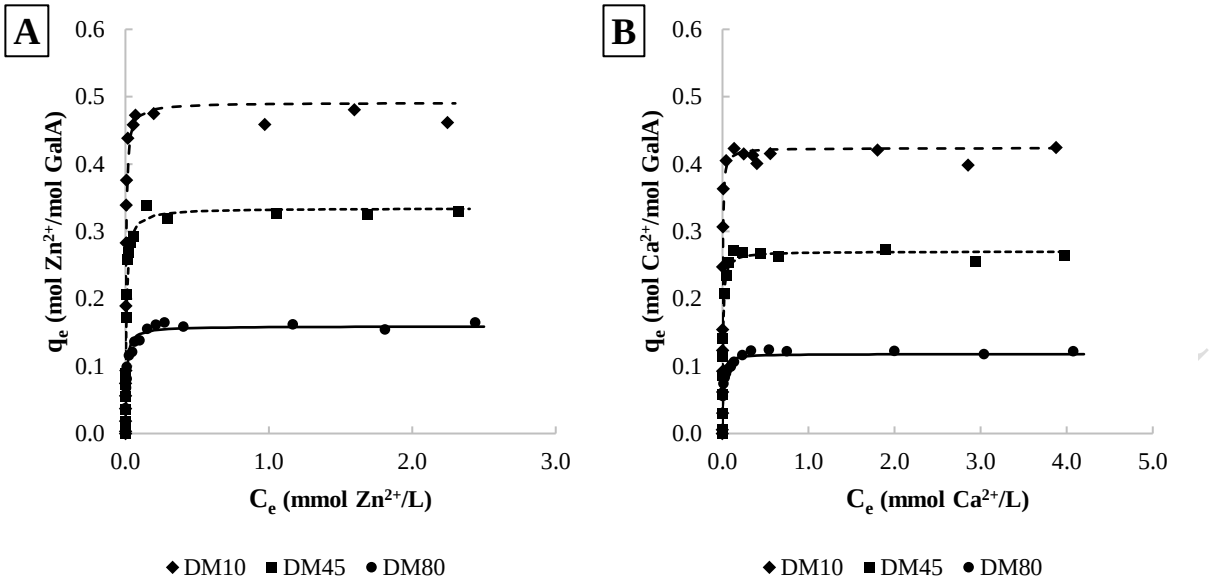
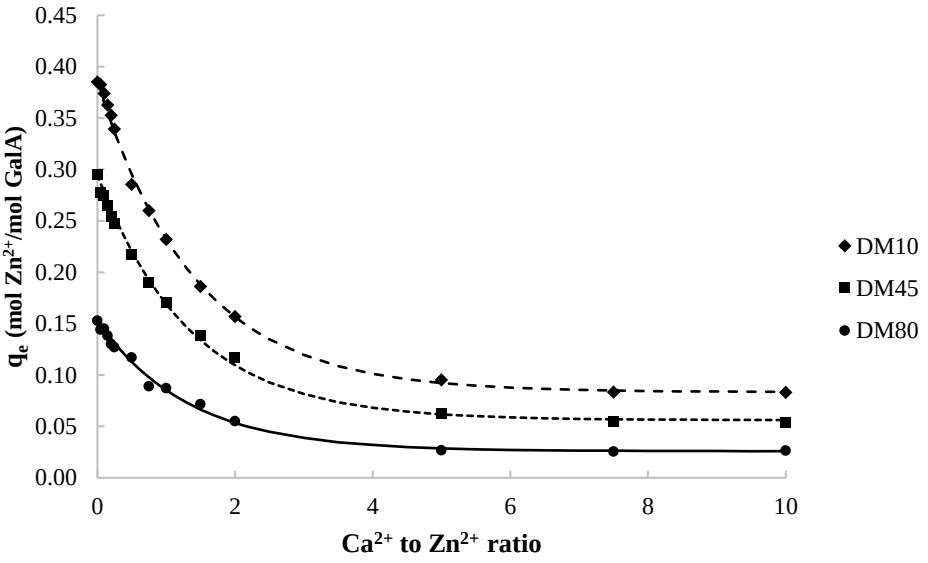


Figure 1



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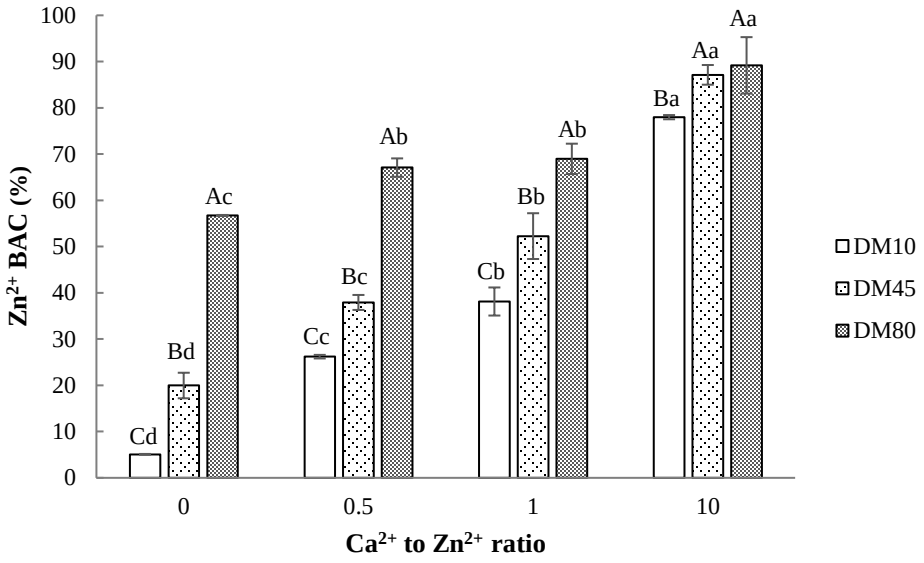

Figure 3

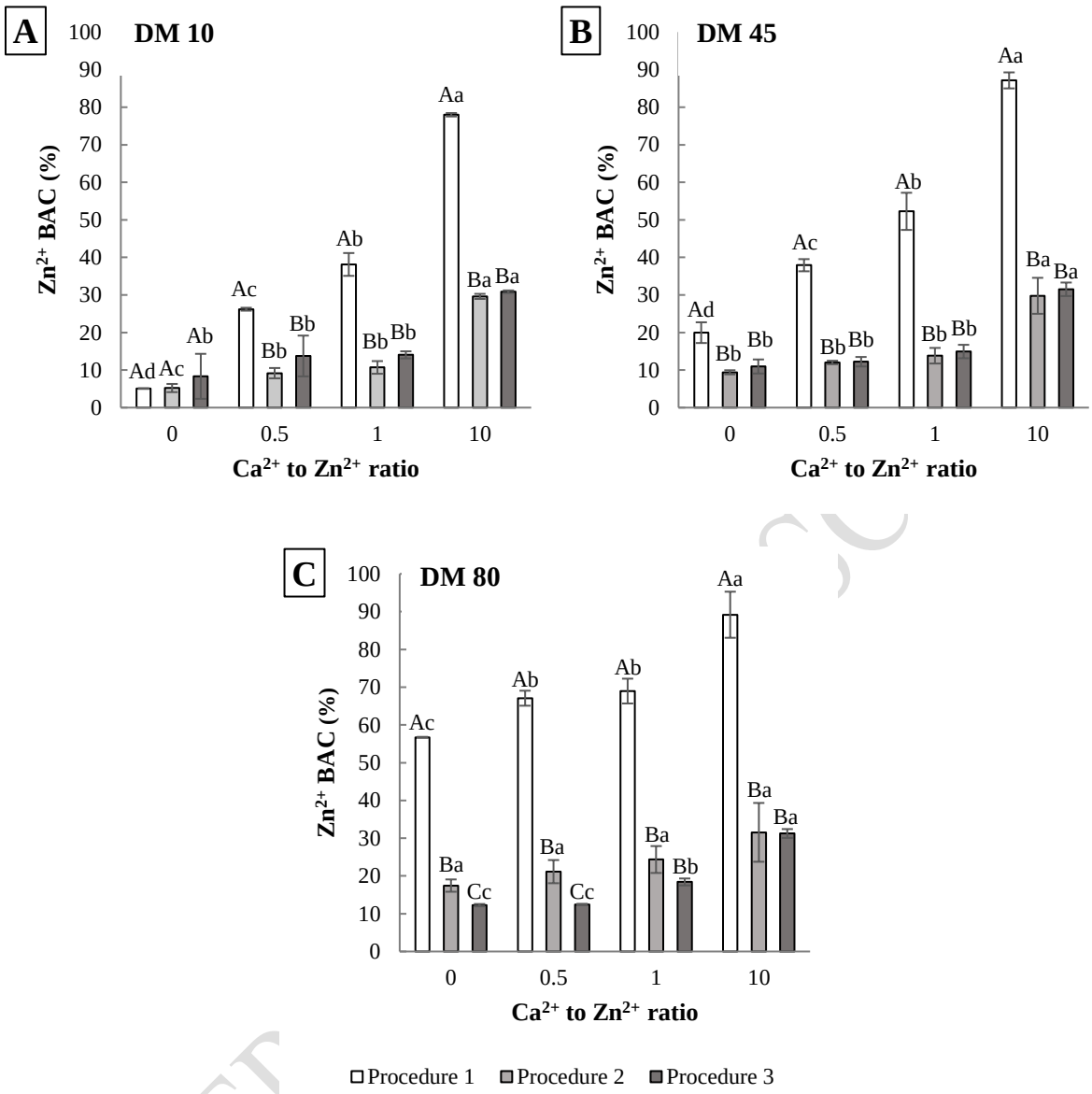
A
DM 10
B
DM 45
C
DM 80
Zn2+ BAC (%)
Ca2+ to Zn2+ ratio
0
0.5
1
10
Ad
Ac
Ab
Aa
Ba
Bb
Cc
Procedure 1
Procedure 2
Procedure 3

Figure 4

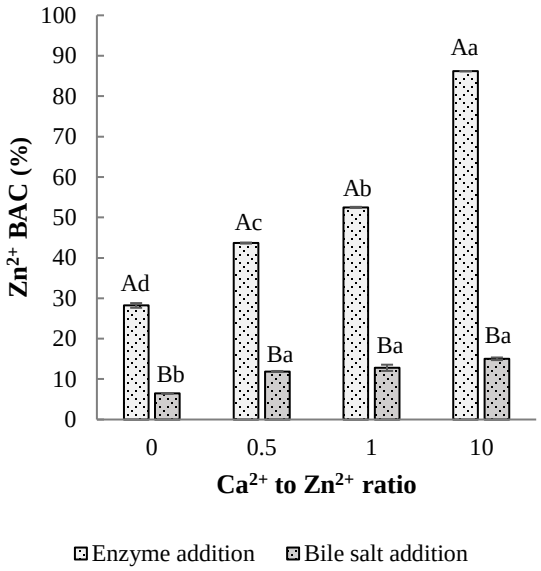

Figure 5