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Singhal, S., Swami Hulle, N. R., & Koidis, A. (2024). Bioaccessibility of iron in developed pectin iron complex using Citrus limon Burm F. peels subjected to in-vitro gastro-pancreatic digestion. *Food Chemistry*, 457, Article 140457. <https://doi.org/10.1016/j.foodchem.2024.140457>

Published in:
Food Chemistry

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
[Link to publication record in Queen's University Belfast Research Portal](#)

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**Bioaccessibility of iron in developed pectin iron complex using *Citrus limon*
Burm F. peels subjected to *in-vitro* gastro-pancreatic digestion**

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Abstract

Pectin from the citrus peel waste has novel applications in food and biomedical industries. The present work focused on addressing iron deficiency, which is a global health concern, by developing a functional ingredient using pectin extracted from Assam lemon (*Citrus limon* Burm. F) and supplementing iron via the pectin-iron complex (PIC). Extracted pectin was incubated with iron chloride hexahydrate (0.90-1.80 mM) for 180 h to optimize the complexation conditions, with the optimal concentration being 1.36 mM. The iron bioavailability and its absorption in the PIC was assessed using *in-vitro* simulation digestion and Caco-2 cell monolayers. The bioaccessible form of iron in the developed PIC during the intestinal phase was 5.34±0.16%, which was negligible in pectin. The absorption of bioaccessible iron in the PIC was found to be 2.93±0.03%. The results demonstrated that PIC could reduce iron deficiency and increase fibre intake, leading to several health benefits.

Keywords: pectin; bioavailability, *in-vitro* assay, Caco-2 cell model, pectin-iron complex

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1. INTRODUCTION

One of the major nutritional problems present globally is micronutrient malnutrition especially iron (Fe) deficiency affecting millions of people, especially in infants, children, and pregnant women. Iron deficiency is mostly prevalent in school-aged children in developing countries. The deficiency of the same induces anaemia, increased susceptibility to infection, and decreased cognitive abilities and motor activity among individuals (Kinyoki et al., 2021). The World Health Organization (WHO) has proposed three approaches to combat micronutrient malnutrition such as food diversification and education, fortification, and supplementation (Ritchie and Roser, 2024). Fe supplements, however, if taken orally could result in multiple gastrointestinal side effects due to the release of free Fe ions (Ma et al., 2021). Hence, alternative Fe supplements with limited or no side effects are highly desirable.

A study reported the effectiveness of a complex formed between a polysaccharide and Fe(III) as an oral Fe supplement. This complex demonstrated favourable characteristics such as water stability, chemical solubility, and minimal side effects (Saini et al., 2014). It was also found to be non-toxic even at high concentrations (Torino et al., 2014). However, there are certain issues linked with the utilization of polysaccharide-Fe(III) complexes such as the acidic pH in gastric fluid during the process of Fe digestion can lead to the degradation of the composition of a few polysaccharide-Fe(III) complexes, causing the release and dissolution of Fe ions (Cheng et al., 2019). Additionally, insoluble ferric compounds may develop in the intestinal juice, which may not be absorbed by the small intestine (Wang et al., 2015). The main challenge for polysaccharide-based carriers, therefore, lies in retaining Fe ions in the presence of gastric juice. Pectin, a substance with excellent gelling properties against gastric acid, is considered a promising solution. Importantly, pectin does not degrade when exposed to digestive enzymes present in the small intestine (Ma et al., 2021). Consequently, employing pectin to transport Fe ions seems to be a logical approach. **The development of iron-pectin beads from the ionic gelation method has resulted in a novel system for the delivery of iron to intestinal cells (Ghibaud et al., 2018). An innovative system for stabilizing and delivering lactic acid bacteria using pectin-iron complex beads showed that beads were stable in simulated digestive conditions and successfully released cultivable bacteria, iron, and pectin in the gut (Ghibaud et al., 2017).**

By definition, pectin is a type of anionic polysaccharide that, primarily contains of D-galacturonic acid residues linked together through α -(1→4)-glycosidic bonds along with

different neutral sugar residues. Pectin has gained much significance globally due to its diverse range of bioactivities and effectiveness. Pectin is one of the most widely used additives in food and beverage formulations for its ability to gel, thicken, replace fats, and stabilize colloids. Additionally, it has shown promise in biomedical research for drug delivery, antioxidative effects, and promoting wound healing. Pectin has also a significant role in lowering blood cholesterol among patients (Singhal et al., 2022). A patient must consume at least 6 g of pectin each day for their cholesterol to be significantly reduced (Abdulhakeem et al., 2023). Pectin has several advantages when added to food and drug formulations. One of them is the presence of divalent cations in pectin results in the formation of stable gels, making them suitable carriers for delivering bioactive substances (Tyagi et al., 2015) and actively managing obesity and weight loss issues (Abdulhakeem et al., 2023). Pectin from varying sources has been reported to be effective in health management, like lowering cholesterol, which lowers cardiovascular related risks (Naqash et al., 2017). Another reason is pectin has been regarded as harmless, easily accessible, and having low production costs (Abdulhakeem et al., 2023).

The present study focuses on extracting and applying pectin obtained from Assam lemon. This variety of Assam lemon is native to the state with a GI Tag and has a unique flavour and morphology, and the peel generally is discarded or pickled. There is no available literature that cites the extraction of pectin from Assam lemon. The present study is centered on the valorisation of this citrus by-product, which involves extracting pectin from the peel. This approach promotes sustainability and adds value to agricultural waste. In previously cited research works the commercial citrus pectin was used, while the present study is an extended work on the optimization of the valorisation process of Assam lemon using novel techniques. These differences could lead to novel applications or improvements in existing processes. There is limited scientific information available on the formation of pectin-iron complex (PIC) and their characterization for possible applications in food systems. The objective of this investigation is to create a citrus based pectin-iron complex (PIC) to develop a novel iron supplement. This involves complexing ferric ions with citrus pectin. The resultant complex was characterized using Spectrometric techniques, Scanning Electron Microscopy (SEM), Degree of esterification (DE), and galacturonic acid analysis. Moreover, the absorption of Fe from synthesized PIC was studied through *in vitro* assays and the Caco-2 cell model.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

The freshly harvested lemon samples were collected from the Citrus Garden, Tezpur University, Assam, India, during the period of June-July, 2021. The lemons were washed, peeled and were subjected to tray drying at 45°C/10 h and finely powdered. The lemon peel powder was then sifted via a sieve (40-mesh) and stored at ambient temperature. Caco-2 cells (P-35) were supplied by Prof. Lisa Connolly, School of Biological Sciences, Queen's University Belfast, UK. Pepsin sourced from porcine gastric mucosa ≥ 2500 units/mg protein (P7012), pancreatin sourced from porcine pancreas (P7545, 8X USP), and bile extract porcine (B8631) were purchased from Sigma-Aldrich (Dorset, UK). The rest of the reagents were analytical grade purchased from Sigma-Aldrich (Dorset, UK).

2.2. Pectin extraction and its purification

Citrus pectin from Assam lemon peels was separated using the conventional method described in Singhal et al. (2024). Briefly, 5 g dried peel powder was suspended in 150 mL of citric acid solution (pH 2). The solution was heated at 90°C using a water bath shaker for 60 min and cooled down to room temperature (RT). It was filtered using a cheesecloth to separate any solid particles. The resulting filtrate, containing crude pectin, was collected and then subjected to precipitation with ethanol (99%) in 1:2 (v/v) ratio undisturbed at RT/3 h. Following this, the precipitated pectin underwent centrifugation (6500 rpm/15 min/room temperature), leading to the separation of the wet pectin fraction. Following that, the wet pectin fraction underwent a washing process twice with 150 mL of ethanol to eliminate any remaining monosaccharides and disaccharides. The acquired moist pectin was dried at 40°C in a hot air oven until constant weight was achieved. The obtained dried pectin was finely powdered using a mortar pestle and preserved in a sealed container at RT for further use.

2.3. Formulation and experimental design of the PIC

The pectin extracted from Assam lemon peels (refer to section 2.2.) was complexed with Fe by modifying the method mentioned in Chirug et al. (2018). Based on the preliminary results, the pectin (0.5% w/v) was suspended in the iron III chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), in a concentration ranging from 0.90 mM to 1.80 mM for 180 h at RT. After incubation, the solution underwent centrifugation at 6000 rpm for 10 min to eliminate unbound Fe from PIC. The PIC was subjected to dialysis against de-ionized water for 48 h/RT, freeze-dried (6 h) and then transferred to a sealed container at RT for further analysis.

The formulation of PIC was modelled and optimized using a three level of one-factor response surface methodology design (7 experimental runs). The details of the experimental design have been mentioned in Supplementary Table 1. The parameters of the process and their corresponding range were selected according to initial observations aimed at examining their influence on the response variable, Fe content (ppm) within the PIC. The general linear equation involving process variables was presented in the equation Eq. (1).

$$Y = \beta_o + \beta_1x_1 + \beta_2x_2 \dots + \beta_kx_k \quad \dots(1)$$

Where, Y is the response variable, β_o is the intercept term, β_1 , β_2 , and β_k represent the coefficient associated with the independent variables, and x_1 , x_2 , and x_k represent levels of the independent variables considered in the study. All experimental runs were done in triplicates. The effect of independent variables was analysed by employing Design Expert (Version 12) to do regression analysis and ANOVA test. The level of significance was analysed on the basis of F-value at 95% confidence interval.

2.4. Determination of Fe content in PIC

The Fe content in PIC was measured by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrophotometry) (model: 5100 ICP-OES, Make: Agilent Technologies, USA). The PIC powder (0.100 g) was transferred in labelled 50 mL centrifuge tubes and 2 mL nitric acid (69%) was added to all tubes. After incubating the tubes overnight, 2 mL of hydrogen peroxide was added to the respective tubes and were allowed to release gas for 15 min in a Class II safety cabinet. The tubes were then placed in a microwave digester's carousel, and a digestion program consisting of three stages was initiated, which could take a total of 65 min, including than that of cooling process. The chosen program gradually heated the samples for 35 min, reaching a temperature of 95°C followed by 30 min digestion. The heating stages were as follows: a 5-min ramp from RT to 55°C, a 10-min hold at 55°C, a 5-min ramp to 75°C, a 10-min hold at 75°C, a final 5-min ramp to 95°C, and a 30-min hold at 95°C. Subsequently, the volumes were adjusted to the final weight of approximately 30 g using deionized water, and the precise masses were recorded. The iron (Fe) content in PIC was then run into the ICP-OES instrument. The determination was conducted in triplicates (Wahengbam et al., 2019).

2.5. Physicochemical properties of PIC

2.5.1. Proximate analysis

Proximate analysis of moisture content, ash content, crude lipid content and crude protein content of pectin and PIC were determined according to AOAC method (2010).

2.5.2. Degree of Esterification (DE)

An FTIR analysis method was used to determine DE. The term DE denotes the proportion of carboxyl groups (COOH) that have undergone methylation compared with the total number of COOH in a compound. The DE (%) was calculated according to Eq. (2) (Liew et al., 2016):

$$DE(\%) = \frac{A_{1745}}{A_{1745} + A_{1630}} \times 100 \quad \dots(2)$$

where, A_{1745} and A_{1630} represent the absorbance intensity at wavelengths 1745 cm^{-1} and 1630 cm^{-1} , respectively.

2.5.3. Methoxyl group content

The methoxyl group content of pectin and PIC was determined using the method of Ranganna (1995) by equation 3.

$$\text{Methoxyl group content (\%)} = \frac{\text{Volume of alkali (mL)} \times \text{Normality of alkali} \times 3.1}{\text{Weight of sample (g)}} \quad \dots(3)$$

2.5.4. Galacturonic acid

The content of galacturonic acid in the PIC was analysed using the 3,5-dimethyl phenol method described by Kumar and Kumar (2017). In a test tube, 0.25 mL PIC solution (1 mg/mL in distilled water) was mixed with 0.25 mL of sodium chloride solution (2% w/v) and 4 mL of concentrated sulfuric acid (37% w/v). The solution was then heated at 70°C for 10 min followed by cooling to RT. Then, 0.1 mL of a 3,5-dimethyl phenol solution (10 mg of 3,5-dimethyl phenol in 10 mL glacial acetic acid) was added and mixed. Immediately afterwards, the absorbance difference at 450 nm and 400 nm was measured using a UV-Vis spectrophotometer. D-galacturonic acid solutions (0.2-2.0 mg/mL) were used as a reference standard for the calibration curve. Using this calibration curve, the galacturonic acid content of the PIC was calculated. The absorbance difference of the standard was multiplied by a correction factor of 1.1.

2.6. Structural characterization of PIC

2.6.1. Fourier transform infrared spectroscopy (FTIR)

About 1 mg of PIC was mixed with 10 mg of potassium bromate and subjected to hydraulic pressing to form a pellet. Subsequently, the scanning of pellet was done using an FTIR spectrophotometer (Model: Nicolet Instruments 410 FTIR, Make: Thermo Scientific, USA) across the frequency (400-4000 cm^{-1}) with a resolution of 4 cm^{-1} (Ma et al., 2021).

2.6.2. *X-Ray diffraction (XRD)*

The PIC's crystal structures and atomic spacing were examined using XRD. The diffraction patterns of crystals of the PIC were recorded using an XRD instrument (AXS SMART APEX-I, Bruker, Germany). The radiation settings were maintained at 40 kV and 40 mA. The scanning range extended from 2θ of 5° to 80° , and the scanning rate was set at 4 degrees per minute (4 deg min^{-1}) (Singhal et al., 2024).

2.6.3. *Scanning electron microscopy (SEM)*

Images of the PIC were taken following the application of gold sputter coating to evaluate the surface morphology of the PIC, particularly focusing on single crystals. This investigation was conducted utilizing a dual-beam Focused Ion Beam (FIB) TESCAN LYRA3 instrument (Wang et al., 2020). SEM image of pectin and PIC was captured at a view field from 1.38-3.85 mm with a scale bar indicating a length of 200 μm -1mm and in 75x to 200x magnification range.

2.7. Rheological and thermal study of pectin and PIC

Pectin and PIC powder were dissolved in distilled water to prepare pectin and PIC solutions with a 1%, 2%, and 3% (w/v) concentration. The rheometer (MCR72, Anton Paar, Austria) equipped with parallel plate geometry (60 mm diameter) and a 1 mm gap between plates was used to conduct dynamic frequency sweeps. The strain (1%) was kept within the linear viscoelastic zone during tests, which were performed at a constant temperature of 25°C over an angular frequency range of 0.1 to 100 rad/s producing storage modulus (G') and loss modulus (G'')

The thermal characteristics of the pectin and PIC in a nitrogen environment were measured using a differential scanning calorimeter (DSC 214 Polyma, Netzsch, Germany). A sample of around 10 mg was weighed and sealed with the lid inverted in an aluminium concave. A blank sample was used as a control. The samples were heated from 30 - 300°C at a rate of 10°C/min . The enthalpy (H), conclusion temperature (T_c), peak temperature (T_p), and onset temperature (T_o) were obtained from the thermogram.

The degrading parameters of pectin and PIC were examined using thermogravimetric (TGA) processing. To evaluate each sample's thermal stability, a thermogravimetric analyzer (TG 209 F1 Libra, Netzsch, Germany) was employed. Samples were heated at a rate of 10°C/min to temperatures between 25°C and 600°C in a nitrogen environment.

2.8. *In vitro* Fe release from PIC for assessment of bioaccessible Fe

Bioaccessibility of Fe in the pectin and PIC was carried out by the INFOGEST *in vitro* digestion protocol (Brodkorb et al., 2019). In order to replicate the human digestive system, *in-vitro* digestion was performed comprising three main steps: oral, gastric, and intestinal digestion. In the oral phase, 1 g of PIC powder was combined with a 30 mL solution containing 140 mM NaCl and 5 mM KCl in a 1:1 (v:v) ratio. Then, added 25 µL CaCl₂ (0.3M) and 500 µL of 100 mM ascorbic acid solution to the oral bolus. The oral bolus was placed in a water bath shaker at a rate of 200 rpm maintained at 37°C for 2 min. For the gastric phase, 0.5 mL of pepsin solution with an 11000 U/mL concentration was introduced to the previously orally digested sample. The pH of 3 was adjusted using 1 M HCl. The oral bolus was thereafter placed in a shaking water bath for 2 h at 200 rpm and 37°C. For the intestinal phase, the gastric chime after incubation was adjusted to pH 5 using 1 M NaHCO₃. Following pH adjustment, a 2.5 mL solution of the pancreatin-bile mixture (0.45 g bile salts and 0.075 g pancreatin in 37.5 mL of 0.1 M NaHCO₃) was introduced to the gastric chime. After adding 40 µL CaCl₂ (0.3 M), the pH was adjusted to 7.0 using 1 M NaOH. The mixture was then incubated for 2 h in a shaking water bath set at 37°C/200 rpm. Following incubation, the samples that had undergone digestion were cooled in ice for 10 min and centrifuged at 3500 g⁻¹ for 1 h at the temperature of 4°C. The resulting supernatants (soluble mineral fraction) were carefully isolated and stored at -80°C for subsequent analysis.

2.9. Quantification of bioaccessible Fe

The supernatants from the preceding step (Section 2.7.) were analysed for bioaccessible Fe by ICP-OES (Section 2.4.). The % bioaccessibility of Fe in PIC was calculated by using an equation.

$$\%bioaccessibility = \frac{bioaccessible\ m.f.}{total\ m.c.} \times 100 \quad \dots(4)$$

where: the bioaccessible mineral fraction (bioaccessible m.f.) and the total micronutrient content (m.c.) represents the soluble Fe fraction obtained post simulated digestion and the overall Fe content found in dried PIC (ppm), respectively (Wahengbam et al., 2019).

2.10. Fe release study from PIC using Caco-2 cells

As mentioned in section 2.7, the soluble m.f. from the *in-vitro* simulated digestion was used to examine the bioavailability of Fe transfer across Caco-2 cells. The cells were cultured in flasks (75 cm²) and nurtured in a nutrient medium comprising Dulbecco's minimum essential media (MEM). Fetal bovine serum (10% v/v), sodium pyruvate (1% v/v), 4 mM L-glutamine, and 1% (v/v) penicillin-streptomycin were added to the solution. The cells were incubated (5% CO₂ and 95% relative humidity) at 37°C under standard conditions (Wahengbam et al., 2019).

For testing the bioavailability, 0.1 mL of the cell solution, having 2×10⁵ cells/well for each 6.5 mm diameter filter, was seeded in the inserts (apical compartment) of 24 well plates, while 0.6 mL of nutrient medium was added to the basolateral compartment. The plate was then incubated with the filter supports at 37°C in an incubator with 5% CO₂, and 95% relative humidity for 6 h. After incubation, the apical medium was removed without disturbing the inserts and replaced with 0.1 mL of nutrient medium to remove non-adherent cells thus, reducing the risk of multilayer formation. The nutrient medium was refreshed every 48 hours during the initial 21-29 days after seeding. After this time frame, the growing media was removed. Then, 0.6 mL and 0.1 mL of Ca²⁺ and Mg²⁺ free Hank's balance salt solution (HBSS) were poured into the basolateral and apical compartments, respectively, to rinse the cell monolayer. Iron can compete with calcium and magnesium ions for binding sites on transport proteins and chelators. The competition for these binding sites is reduced by utilizing a Ca²⁺ and Mg²⁺ free solution, which results in more precise studies of iron binding and absorption. After 20 min of incubation at 37°C and 100 rpm, the plates were removed and the HBSS was poured out of both compartments. For the transport experiment, 0.6 mL of HBSS and 0.1 mL of soluble mineral fraction (section 2.4.) were included in the basolateral and apical compartments, respectively. The plates were thereafter subjected to incubation at 37°C for 2 h at 500 rpm in an incubator shaker. The sample was obtained from the basolateral compartment and preserved at -80°C to assess the absorption of Fe and the transportation of bioavailable Fe across the cell monolayer using ICP-OES (section 2.4.). The viabilities of the cell were evaluated using trypan blue exclusion, with typical rates ranging between 92-97% following a 2-h exposure period.

$$\% \text{ absorption of bioavailable iron} = \frac{\text{Transport } x_{\text{basolateral}}}{\text{Bioaccessible } x_{\text{apical}}} \times 100 \quad \dots(5)$$

where "transport $x_{\text{basolateral}}$ " represents the quantity of Fe (ppm) that has been carried out via the cell monolayer to reach the basolateral compartment. On the other hand, "bioaccessible x_{apical} "

in the apical compartment signifies the quantity of bioaccessible Fe (ppm) (Wahengbam et al., 2019).

2.11. Statistical analysis

The analysis of data was performed utilizing the one-way analysis of variance (ANOVA) method, and Duncan's mean comparison test was conducted at a significance level of $p = 0.05$ to identify any variations among treatments. The statistical analysis was carried out utilizing IBM SPSS Statistics 20. The experiment is conducted in triplicates and data is represented in mean $\pm$ standard deviation.

3. RESULTS AND DISCUSSION

3.1. Formulation and optimization of PIC

Based on preliminary results, a one-factor design was implemented keeping 180 h as the constant incubation time and FeCl₃.6H₂O concentration varying from 0.90 mM to 1.80 mM as an independent variable for optimizing PIC.

The Fe concentration (dependent variable) in PIC at different concentrations of FeCl₃.6H₂O (independent variable) varied between 1238.41 to 3776.74 ppm. According to the employed design, a linear model was evaluated. ANOVA depicted the significance of the model variations compared to the variation of the experimental results. The results of the ANOVA are mentioned in Supplementary Table 2. The F -value of 70.34 indicates that the linear model was statistically significant.

The R^2 and adjusted R^2 values for the obtained Fe concentration were found to be 0.93 and 0.92, respectively. These values indicate a strong correlation between the predicted and actual Fe concentrations, with no significant difference noted between them. The coefficient of variation was found to be 10.95%. This lower coefficient indicated minimal deviation between experimental and predicted values, underscoring the accuracy of the model. Furthermore, the adequate precision, which gauges the signal-to-noise ratio, was calculated to be 14.793 for the Fe concentration in PIC. This observation suggests that the model can predict data with more precision. The linear equation, presented in coded form as Eq. (6), includes significant parameters that contribute to the model's predictive capability.

$$I = +2517.89 + 1089.61 \times A \quad \dots(6)$$

In Equation (5), the coefficient of parameter 'A' was determined to be 1089.61. This value indicates a positive effect of parameter A on the response variable. In other words, increasing the value of parameter 'A' contributes positively to the response variable as described by the model. The positive effect of the independent variable on a dependent variable indicates that with an increase in the independent variable value ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) the value of a dependent variable increases. So, the increase of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ concentration increases the Fe content in PIC. This might be due to a combination of factors related to the availability of Fe ions and the binding capacity of pectin molecules. As the concentration of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ increases, more Fe ions are available in the solution (Persson, 2018). Maksudova et al. (2010) stated that this higher concentration provides more opportunities for Fe ions to bind to the pectin molecules, leading to an increase in Fe content in the PIC. Pectin molecules possess binding sites that can be complex with metal ions such as Fe. However, Maksudova et al. (2010) did not report the limiting concentration of Fe. With a higher concentration of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, more Fe ions are available to bind to these sites, resulting in an increased Fe content in the PIC.

The linear equation mentioned in Equation (5) was used to study the optimal conditions of process parameters aiming to achieve a target Fe content of 3000 ppm in PIC. According to the model, an Fe content of 2542 ppm was predicted at 1.36 mM of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ with a desirability of 1.000. For validation purposes, experiments were conducted at the optimal conditions, resulting in an observed Fe content in PIC of 2657 ± 23.61 ppm. Under optimum conditions, there was a relative deviation between the experimental and predicted response values under optimal conditions was 1.06%, between the predicted and observed response values. This concludes that the developed linear model accurately predicts the Fe content in PIC under the specified conditions.

3.2. Physicochemical properties of PIC

3.2.1. Proximate composition

This section covers the PIC's physicochemical characteristics. It was found that the moisture content of PIC ($19.10 \pm 1.12\%$ w/w) and pectin ($21.10 \pm 1.43\%$ w/w) was non-significant. Likewise, there was no statistical significance found in the protein content of pectin ($2.62 \pm 0.21\%$) and PIC ($2.34 \pm 0.17\%$). On the other hand, Koh et al. (2014) reported that the protein and moisture contents of the pectin derived from jackfruit rinds were 1.53–1.74% and 5.19–9.98% w/w, respectively. Additionally, both PIC and pectin have no fat content. The ash content of PIC ($2.785 \pm 0.10\%$) was found to be lower than that of pectin ($3.873 \pm 0.10\%$).

3.2.2. Degree of Esterification (DE)

The term DE stands for the esterified COOH groups inside galacturonic acid chains with methyl or acetyl groups and is essential for determining the quality of pectin. On the basis of DE value, pectin is categorized as either high methoxyl (HM) pectin ($DE \geq 50\%$), or low methoxyl (LM) pectin ($DE \leq 50\%$) (Liew et al., 2016). The DE values for the pectin (51.87%) and PIC (50.35%) exceed 50%, indicating that it falls into the category of HM pectin. This type of pectin is commonly utilized in the production of jams and jellies.

3.2.3. Galacturonic acid

The concentration of galacturonic acid was higher than 65% for pectin and PIC however, pectin had higher galacturonic acid content ($84.33 \pm 1.44\%$) compared to PIC ($67.39 \pm 4.92\%$), which might be because pectin, after forming PIC, is not solely composed of galacturonic acid molecules. Due to the presence of negatively charged carboxylic groups, galacturonic acid exhibits a strong propensity to bind with Fe ions, leading to the formation of a highly stable complex (Cheng et al., 2019).

3.2.4 Methoxyl group content

Methoxyl group content has a key role in regulating pectin's gel-forming ability and setting time. The methoxyl group content of pectin and PIC was found to be $2.02 \pm 0.01\%$ and $13.52 \pm 0.04\%$, respectively. These results, however, were less than those reported for banana (7.03%) and mango (7.33%) peels (Apsara & Pushpalatha, 2002), but they were comparable to dragon fruit pectin (Zaidel et al., 2017), which had 2.98 to 4.34%. This could be due to the partial degradation of pectin. According to Aina et al. (2012), depending on the source and extraction conditions, pectin's methoxyl group concentration can range from 0.20 to 12%. The amount of methoxyl groups in fruits is dependent on their maturity because, as they mature, their sugar content rises while their methoxyl group content falls. A high amount of methoxyl groups improves pectin's capacity to bind sugar and its dispersion quality (Apsara & Pushpalatha, 2002).

3.3 Structural characterization of PIC

3.3.1 FTIR Spectroscopy

The FTIR spectra of PIC and extracted pectin are represented in Fig. 1a. The O-H stretching band ($3100\text{--}3600\text{ cm}^{-1}$), the C-H stretching bands ($2800\text{--}3000\text{ cm}^{-1}$), and the fingerprint region (below 2000 cm^{-1}) were among the distinct sections that were the focus of the spectral

investigation. Specifically, bands that contribute to the resonant absorption energy of the pyranose cycle vibrations ($950\text{--}1200\text{ cm}^{-1}$) and the area between 1200 and 1800 cm^{-1} indicating the condition of carboxylic groups, were highlighted (Wang et al., 2014). Table 1 represents the FTIR frequency range and functional groups identified in pectin and PIC.

Hydrogen bonding and complexation inside the molecule determine the position of the $\text{C}=\text{O}$ stretching wave number within these ranges. Delocalization of the $\text{C}=\text{O}$ group following complexation with a $\text{C}=\text{C}$ band causes the absorption to shift to a lower wave number (Stuart, 2004). Pectin and the PIC lacked lignin and proteins, as indicated by the absence of the bands at about $1600\text{--}1500\text{ cm}^{-1}$ that are attributed to aromatic rings and protein amide (Liu et al., 2010).

The variations observed in the bands within specific regions, such as the ($\text{C}\text{--}\text{H}$) bands at 2929 cm^{-1} , 1340 cm^{-1} , and 1240 cm^{-1} , as well as the complex band ($\text{C}\text{--}\text{H}$) spanning $1000\text{--}700\text{ cm}^{-1}$, were attributed to alterations in the interactions between pectin and Fe (III) . Furthermore, in the $1153\text{--}1019\text{ cm}^{-1}$ region, both the spectra of pectin and the PIC exhibited several highly fused bands. These changes likely reflect the complex interplay and molecular interactions between pectin and Fe (III) in the synthesized PIC. The loss of these bands may suggest that following Fe (III) chelation, changes occurred to the pectin's organized structures and glycosidic bridge (Wang et al., 2015).

3.3.2 X-Ray diffraction (XRD)

To discern the amorphous or crystalline nature of pectin, XRD is a valuable technique utilized to study its physical and functional characteristics. In Fig. 2b, the XRD graphs depict the extracted pectin and the developed PIC. The diffraction patterns of the pectin exhibited a single peak around 2θ of 9.4° . However, upon complexing pectin with ferric ions, freshly diffraction patterns emerged, revealing two peaks approximately 2θ of 6.15° and 10.65° (as shown in Fig. 2b). These alterations in the diffraction patterns suggest changes in the crystalline or amorphous properties of pectin induced by the complexation process with ferric ions. The diffraction peak of PIC was sharper and more intense than that of pectin, resulting in a larger crystallite grain size. The broad peaks observed in pectin disappeared following the coordination reaction, suggesting a modification in the crystal morphology. Similar results were observed in the soybean polysaccharide- Fe (III) complex by Gao et al. (2018). The observed higher intensity of pectin compared to the PIC suggests a higher degree of crystallinity in pectin. The esterified $\text{C}=\text{O}$ related peaks' reduced intensity in the FT-IR spectrum of pectin implies a correlation

between the crystallinity of pectin and its degree of esterification. Jiang et al. (2019) also noted that pectin with a higher DE tends to exhibit a higher degree of crystallinity. The crystallization process is attributed to hydrophobic interactions among methyl ester groups during pectin dehydration.

The evolution of the diffraction pattern indicates that the PIC is relatively more amorphous compared to pectin, despite having a larger crystallite grain size. This suggests that the complexation with ferric ions influences the crystalline structure of pectin, potentially altering its physical properties and functionality. Therefore, PIC can be used as a functional ingredient in food products such as jams, jellies, fruit preserves, and confectionery.

3.3.3 Scanning Electron Microscopy (SEM)

The surface morphologies of extracted pectin and developed PIC are illustrated in Fig. 3. The overall texture of PIC appears relatively smooth and flat compared to that of pectin. This could be due to the coordination bonding present among pectin and Fe. The size of PIC grains is around 1.08-1.59 μm and is relatively well defined than compared to that of pectin. The PIC grains seem to be more cylindrical in shape, while the pectin grains are more rock like shape. Overall, SEM micrographs conclude that PIC exhibited more disordered structures or less well-defined crystal lattices along with a large grain size, which is in agreement with the diffraction patterns of PIC in XRD.

3.4 Rheological and thermal study of pectin and PIC

The dynamic frequency test was done to study the viscoelastic properties of pectin and PIC solutions at different concentrations (1-3% w/v). The storage modulus (G') and loss modulus (G'') were measured as a function of angular frequency. In all concentrations of pectin and PIC, the G'' was higher than G' at low frequencies, signifying dominant viscous behaviour. With the increase in angular frequency, G' increased compared to G'' causing crossover points. In both pectin and PIC solutions, the crossover point was shifted to a lower angular frequency with an increase in pectin and PIC concentration, as seen in Fig. 2. This shifting of crossover points suggests that the solution behaves more elastic at lower frequencies. The stronger elastic characteristics of the solution are caused by increased network formation and molecular interactions like ionic and hydrogen bonding. As a result, G' becomes more prominent over a wider frequency range, indicating a better organized viscoelastic network and stronger gel-like activity in the solution. This behaviour of pectin and PIC solutions is necessary for food

industry such as jam and jellies processing and pharmaceutical industry for controlled-release drug delivery systems.

The TGA analysis also showed three main regions at 50-200, 200-400 and 400-600°C (Fig. 1c). A little weight loss of around 10% to 15% was seen in the first area, most likely as a result of free or absorbed water evaporating in the space between chains. Due to the pyrolytic breakdown of the PIC and pectin chains, there was a significant mass loss (roughly 50%) during the second stage (200–400°C) (Ezzati et al., 2020). First, the galacturonic acid chains underwent extensive thermal degradation. Consequently, the acid side group and carbon in the ring underwent decarboxylation, resulting in the development of various gaseous products and the production of solid char (Wang et al., 2016). According to Ezzati et al. (2020), the third stage result revealed an additional little mass loss associated with the thermal breakdown of char. The solid char containing polyaromatic structures bonded by aliphatic and ketonic groups would partially collapse and stack compactly when the pyrolytic temperature rose (Wang et al., 2016). PIC was shown to have a lower mass residue (13.50%) than pectin (16.59%), most likely as a result of PIC exhibiting a lower DE value (Ezzati et al., 2020). According to Einhorn-Stoll et al. (2007), pectin with a lower DE value has more free carboxylic groups accessible, which can create more hydrogen bonds and accelerate the breakdown of pectin chains.

The Fig. 1d shows the DSC thermogram of pectin and PIC. The thermal curve of pectin shows two broad endothermic peaks and one exothermic peak. One of the endothermic peaks was found to be at 79.2°C having an onset at 35.8°C and ending at 152.7°C with the enthalpy of fusion of 0.9047 J/g, and another at 197.9°C, having an onset at 183.7°C and ending at 216.6 °C with the enthalpy of fusion of 0.7932 J/g. The thermal curve of pectin also exhibits one sharp exothermic peak at 249.2°C with the onset at 236°C and end at 263.1°C with the enthalpy of fusion of 0.4782 J/g. Whereas, the thermal curve of PIC exhibits one endothermic peak at 66.2°C with an onset at 38°C and end at 107.3°C and exothermic peak at 256.0°C having an onset at 236.4°C and an end at 275.7°C with the enthalpy of fusion of 1.527 J/g and 0.2635 J/g, respectively.

The endothermic peaks could be caused by hydrogen bonding between galacturonic acid units, the presence of water, or a conformational shift that resulted in the galacturonan ring changing from its more stable ⁴C₁ chair conformation to its inverse ¹C₄ chair conformation (Einhorn-Stoll & Kunzek, 2009). The peak of PIC shifted somewhat toward a lower temperature, most likely as a result of its altered structure and increased water content. According to Wang et al.

(2016), the exothermic peak, on the other hand, represents the thermal degradation of the PIC and pectin samples, which is connected to their chemical ingredient profiles. The exothermic peak of PIC (256.0°C) was higher than that of pectin (249.2°C) indicating that PIC pectin has better thermal stability (Ezzati et al., 2020). Typically, pectin is a food ingredient that may be added to baked goods like cakes, bread, and pastries that are exposed to high temperatures (Combo et al., 2013). Furthermore, the thermal analysis in the present study revealed that PIC had greater thermal stability than pectin, suggesting that PIC can be preferable to pectin when thermal food processing is involved.

3.5 *In-vitro* Fe release from PIC

The proportion of Fe remaining in the supernatant of extracted pectin and PIC after *in vitro* simulated digestion indicates the bioavailable condition of soluble Fe. This bioaccessible Fe has the potential to be absorbed by intestinal cells, increasing its bioavailability and physiological usage inside the body. It can be deduced from Fig. 4b that the PIC's % bioaccessibility was much greater than that of extracted pectin. The total Fe content of the pectin and PIC is 23 ppm and 2657 ppm, respectively. Fe concentration in pectin is negligible after exposure to *in vitro* conditions. While, after exposure to simulated oral, gastric, and intestinal conditions, the Fe concentration in PIC is 143.978 ± 4.20 , 144.235 ± 0.85 , and 141.913 ± 4.20 ppm, respectively and is statistically insignificant ($p < 0.05$) (Fig. 4a). The concentration of Fe remained unchanged under simulated *in vitro* conditions, indicating that the structure of the PIC stayed intact even in the acidic environment of the gastric fluid. This stability might be attributed to the existence of a Fe core encased by pectin ligands (Ma et al., 2021). The bioavailability of Fe released from the selected PIC was determined to be $5.34 \pm 0.16\%$, indicating effective absorption potential. Similar findings were seen in Gupta et al. (2006). The iron bioavailability in green leafy vegetables such as *Digera arvensis* Forsk. and *Trianthema portulacastrum* L. were 7 and 6%, respectively. While in other green leafy vegetables in the study, iron bioavailability was found to be in the range of 16-43%, which is 3 to 8-fold of the result found in the present study (Gupta et al., 2006). Additionally, the structure of the PIC shielded the Fe ions during passage through the stomach phase, thereby mitigating potential stomach discomfort induced by the Fe ions and preserving their activity. Consequently, a higher concentration of Fe was transported to and accumulated in the intestinal fluid. These results highlight the possibility of PIC as a novel Fe supplement for addressing Fe deficiency in anaemic patients (Ma et al., 2021). Utilizing Fe-deficient fruits and vegetables

such as cucumbers and pineapple to develop functional foods holds tremendous promise for PIC towards better nutrition.

3.6 Fe release study from PIC using Caco-2 cells

Caco-2 cells may differentiate to become tiny intestinal enterocytes with all the necessary Fe absorption and transport proteins therefore, they are a well-established model for assessing non-heme Fe intake through ferritin synthesis (Eagling et al., 2014). In this study, specialized Caco-2 cells were exposed to pectin and PIC supernatants. The conversion of Fe(III) to Fe(II) has the potential to enhance Fe absorption, as Fe(II) can be efficiently conveyed through enterocytes via the divalent metal-ion transporter 1. This process facilitates the uptake of Fe by the cells, thereby contributing to its bioavailability and physiological utilization (Wang et al., 2019). The average Fe absorption was 77.807 ± 0.697 ppm, representing 2.93% (Fig. 4c) of the total Fe present in PIC while 54.82% of the overall Fe was introduced to the differentiated cells. This result is comparable with Bryszewska et al. (2019), conducting an *in-vitro* study of iron transport using Caco-2 cells from breads fortified with microencapsulated iron. In their case, the iron transport of four breads with the addition of microencapsulated iron by sourdough fermentation was in the range of 3.23-5.25% (Bryszewska et al., 2019). However, a negligible amount of Fe was present in pectin in the intestinal fluid therefore, there was no chance of Fe uptake by the differentiated Caco-2 cells. The Fe absorption values observed after 2 h of incubation remained consistent throughout the study, with no significant differences observed over time. It is well-established that under saturation conditions, Fe accumulation occurs rapidly, typically showing a sharp increase in Fe uptake within the initial 40-60 min of exposure. This phenomenon indicates the efficient uptake and accumulation of Fe by the cells under the experimental conditions. This causes the Fe concentration within the cells to plateau (Ghibaudo et al., 2018). In the brush border of enterocytes, soluble free inorganic Fe is absorbed at the gut level. While Fe(III) is absorbed through divalent metal transporter 1 (DMT1), Fe(II) is absorbed directly. During the first reduction of Fe(III) to Fe(II), DMT1 can then transport the latter over the brush border membrane (Ghibaudo et al., 2018).

4 Conclusion

This study aimed to utilize pectin extracted from *Citrus limon* Burm f. peels for the formation of a pectin iron complex. The optimal concentration of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was determined and the bioavailability and absorption of iron were evaluated. During the intestinal phase, an increase in the bioaccessible iron was identified. The findings suggest that PIC could serve as a

promising source to address iron deficiency while augmenting dietary fibre intake, potentially offering numerous health advantages to human health. Future work could be increasing the bioavailability of Fe in PIC high enough to meet its recommended daily allowance in the human population and study the form of iron (Fe(III) or Fe(II)) in the PIC reduced by Caco-2 cells. Moreover, the experimental results from this investigation establish the foundation for the potential utilization of PIC along with the *in vivo* evaluation of its efficacy in addressing Fe deficiency.

Declaration of Competing Interest

The authors assert that they have no conflicting interest.

CRediT authorship contribution statement

Somya Singhal: Investigation, methodology, writing original draft. **Nishant Rachayya Swami Hulle:** Conceptualization, review and editing, supervision. **Anastasios Koidis:** methodology review, editing, supervision.

Data availability

Data will be made available on request.

Acknowledgements

The authors are thankful to the Commonwealth Scholarship Commission (CSC) for funding the study and Queen's University, Belfast (QUB), UK for providing all necessary facilities for the successful completion of the study. Moreover, we are thankful to Prof Lisa Connolly for supplying Caco-2 cells and cell culture facilities. **The first author is thankful to Dr Shagufta Rizwana for her constant support.**

References

- Abdulhakeem, Z. R., Odda, A. H., & Abdulsattar, S. A. (2023). Pectin-based nanomaterials as a universal polymer for type 2 diabetes management. *Medical Journal of Babylon*, 20(1), 7-12. <https://doi.org/10.4103/MJBL.MJBL.242.22>
- Aina, V.O., Barau, M.M., Mamman, O.A., Zakari, A., Haruna, H., Umar, M.H., & Abba, Y.B. (2012). Extraction and characterization of pectin from peels of lemon (*Citrus limon*), grape fruit (*Citrus paradisi*) and sweet orange (*Citrus sinensis*). *British Journal of Pharmacology and Toxicology*, 3(6), 259-262. <http://maxwellsci.com/print/bjpt/v3-259-262.pdf>
- AOAC (2010) Official Methods of Analysis of Association of Official Analytical Chemists 18th Edition, Washington, DC.
- Apsara, A.M. & Pushpalatha, P.B. (2002). Characterization of pectin extracted from different fruit wastes. *Journal of Tropical Agriculture*, 40 (1/2), 53-55. <https://jtropag.kau.in/index.php/ojs2/article/view/78>
- Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., ... & Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature protocols*, 14(4), 991-1014. <https://doi.org/10.1038/s41596-018-0119-1>
- Bryszewska, M.A., Tomás-Cobos, L., Gallego, E., Villalba, M., Rivera, D., Saa, D.L.T. & Gianotti, A. (2019). In vitro bioaccessibility and bioavailability of iron from breads fortified with microencapsulated iron. *LWT*, 99, 431-437. <https://doi.org/10.1016/j.lwt.2018.09.071>
- Cheng, C., Huang, D. C., Zhao, L. Y., Cao, C. J., & Chen, G. T. (2019). Preparation and in vitro absorption studies of a novel polysaccharide-Fe (III) complex from *Flammulina velutipes*. *International journal of biological macromolecules*, 132, 801-810. <https://doi.org/10.1016/j.ijbiomac.2019.04.015>
- Chirug, L., Okun, Z., Ramon, O., & Shpigelman, A. (2018). Fe ions as mediators in pectin-flavonols interactions. *Food Hydrocolloids*, 84, 441-449. <https://doi.org/10.1016/j.foodhyd.2018.06.039>
- Combo, A.M.M., Aguedo, M., Quiévy, N., Danthine, S., Goffin, D., Jacquet, N., Blecker, C., Devaux, J. & Paquot, M. (2013). Characterization of sugar beet pectic-derived

584 oligosaccharides obtained by enzymatic hydrolysis. *International journal of biological*
585 *macromolecules*, 52, 148-156. <https://doi.org/10.1016/j.ijbiomac.2012.09.006>

586 Cui, J., Li, Y., Yu, P., Zhan, Q., Wang, J., Chi, Y., & Wang, P. (2018). A novel low molecular
587 weight Enteromorpha polysaccharide-Fe (III) complex and its effect on rats with Fe
588 deficiency anemia (IDA). *International journal of biological macromolecules*, 108,
589 412-418. <https://doi.org/10.1016/j.ijbiomac.2017.12.033>

590 Eagling, T., Wawer, A. A., Shewry, P. R., Zhao, F. J., & Fairweather-Tait, S. J. (2014). Fe
591 bioavailability in two commercial cultivars of wheat: comparison between wholegrain
592 and white flour and the effects of nicotianamine and 2'-deoxymugineic acid on Fe
593 uptake into Caco-2 cells. *Journal of agricultural and food chemistry*, 62(42), 10320-
594 10325. <https://doi.org/10.1021/jf5026295>

595 Einhorn-Stoll, U., & Kunzek, H. (2009). Thermoanalytical characterisation of processing-
596 dependent structural changes and state transitions of citrus pectin. *Food Hydrocolloids*,
597 23(1), 40-52. <https://doi.org/10.1016/j.foodhyd.2007.11.009>

598 Einhorn-Stoll, U., Kunzek, H. & Dongowski, G. (2007). Thermal analysis of chemically and
599 mechanically modified pectins. *Food hydrocolloids*, 21(7), 1101-1112.
600 <https://doi.org/10.1016/j.foodhyd.2006.08.004>

601 Ezzati, S., Ayaseh, A., Ghanbarzadeh, B., & Heshmati, M.K. (2020). Pectin from sunflower
602 by-product: Optimization of ultrasound-assisted extraction, characterization, and
603 functional analysis. *International Journal of Biological Macromolecules*, 165, 776-786.
604 <https://doi.org/10.1016/j.ijbiomac.2020.09.205>

605 Gao, W., Huang, Y., He, R., & Zeng, X. A. (2018). Synthesis and characterization of a new
606 soluble soybean polysaccharide-Fe (III) complex using ion exchange
607 column. *International journal of biological macromolecules*, 108, 1242-1247.
608 <https://doi.org/10.1016/j.ijbiomac.2017.11.038>

609 Ghibaud, F., Gerbino, E., Hugo, A. A., Simões, M. G., Alves, P., Costa, B. F., **Campo Dall'**
610 **Orto, V., Gómez-Zavaglia, A. & Simões, P. N.** (2018). Development and
611 characterization of Fe-pectin beads as a novel system for Fe delivery to intestinal
612 cells. *Colloids and Surfaces B: Biointerfaces*, 170, 538-543.
613 <https://doi.org/10.1016/j.colsurfb.2018.06.052>

- Ghibaud, F., Gerbino, E., Dall'Orto, V. C., & Gómez-Zavaglia, A. (2017). Pectin-iron capsules: Novel system to stabilise and deliver lactic acid bacteria. *Journal of Functional Foods*, 39, 299-305. <https://doi.org/10.1016/j.jff.2017.10.028>
- Gupta, S., Lakshmi A, J., & Prakash, J. (2006). In vitro bioavailability of calcium and iron from selected green leafy vegetables. *Journal of the Science of Food and Agriculture*, 86(13), 2147-2152. <https://doi.org/10.1002/jsfa.2589>
- Hosseini, S. S., Khodaiyan, F., & Yarmand, M. S. (2016). Optimization of microwave assisted extraction of pectin from sour orange peel and its physicochemical properties. *Carbohydrate polymers*, 140, 59-65. <https://doi.org/10.1016/j.carbpol.2015.12.051>Get rights and content
- Jiang, Y., Zhang, C., Yuan, J., Wu, Y., Li, F., Li, D., & Huang, Q. (2019). Effects of pectin polydispersity on zein/pectin composite nanoparticles (ZAPs) as high internal-phase Pickering emulsion stabilizers. *Carbohydrate Polymers*, 219, 77-86. <https://doi.org/10.1016/j.carbpol.2019.05.025>
- Kinyoki, D., Osgood-Zimmerman, A.E., Bhattacharjee, N.V., Kassebaum, N.J. & Hay, S.I. (2021). Anemia prevalence in women of reproductive age in low-and middle-income countries between 2000 and 2018. *Nature medicine*, 27(10), 1761-1782. <https://doi.org/10.1038/s41591-021-01498-0>
- Koh, P.C., Leong, C.M., & Noranizan, M.A. (2014). Microwave-assisted extraction of pectin from jackfruit rinds using different power levels. *International Food Research Journal*, 21(5), 2091.
- Kumar, P., & Kumar, V. (2017). Estimation of uronic acids using diverse approaches and monosaccharide composition of alkali soluble polysaccharide from *Vitex negundo* Linn. *Carbohydrate polymers*, 165, 205-212. <https://doi.org/10.1016/j.carbpol.2017.02.034>
- Liang, R. H., Chen, J., Liu, W., Liu, C. M., Yu, W., Yuan, M., & Zhou, X. Q. (2012). Extraction, characterization and spontaneous gel-forming property of pectin from creeping fig (*Ficus pumila* Linn.) seeds. *Carbohydrate polymers*, 87(1), 76-83. <https://doi.org/10.1016/j.carbpol.2011.07.013>

- Liu, T., Liu, T., Liu, H., Fan, H., Chen, B., Wang, D., Zhang, Y. & Sun, F. (2019). Preparation and characterization of a novel polysaccharide-Fe (III) complex in *Auricularia auricula* potentially used as an Fe supplement. *BioMed Research International*, 2019. <https://doi.org/10.1155/2019/6416941>
- Ma, X., Jing, J., Yu, J., Wang, J., Zhu, H., & Hu, Z. (2021). Synthesis and characterization of a novel apple pectin-Fe (III) complex. *ACS omega*, 6(2), 1391-1399. <https://doi.org/10.1021/acsomega.0c05029>
- Maksudova, S. D., Milusheva, R. Y., Kholmuminov, A. A., & Rashidova, S. S. (2010). Nanostructures of pectin and its metal complexes. *Chemistry of natural compounds*, 46, 677-681. <https://doi.org/10.1007/s10600-010-9714-2>
- Naqash, F., Masoodi, F. A., Rather, S. A., Wani, S. M., & Gani, A. (2017). Emerging concepts in the nutraceutical and functional properties of pectin—A Review. *Carbohydrate polymers*, 168, 227-239. <https://doi.org/10.1016/j.carbpol.2017.03.058>
- Noqta, O. A., Sodipo, B. K., & Aziz, A. A. (2020). One-pot synthesis of highly magnetic and stable citrate coated superparamagnetic Fe oxide nanoparticles by modified coprecipitation method. *Functional Composites and Structures*, 2(4), 045005. <http://doi.org/10.1088/2631-6331/abdcdb>
- Ognyanov, M., Remoroza, C., Schols, H. A., Georgiev, Y. N., Petkova, N. T., & Krystijan, M. (2020). Structural, rheological and functional properties of galactose-rich pectic polysaccharide fraction from leek. *Carbohydrate Polymers*, 229, 115549. <https://doi.org/10.1016/j.carbpol.2019.115549>
- Persson, I. (2018). Ferric chloride complexes in aqueous solution: an EXAFS study. *Journal of solution chemistry*, 47(5), 797-805. <https://doi.org/10.1007/s10953-018-0756-6>
- Qi, T., Ren, J., Li, X., An, Q., Zhang, N., Jia, X., Pan, S., Fan, G., Zhang, Z. & Wu, K. (2023). Structural characteristics and gel properties of pectin from citrus physiological premature fruit drop. *Carbohydrate Polymers*, 309, 120682. <https://doi.org/10.1016/j.carbpol.2023.120682>
- Ritchie, H. & Roser, M. (2024). *Micronutrient deficiency*. *Our World in data*. Retrieved from <https://ourworldindata.org/micronutrient-deficiency>. Accessed June 9, 2024

672 Saini, R. K., Manoj, P., Shetty, N. P., Srinivasan, K., & Giridhar, P. (2014). Dietary Fe
673 supplements and Moringa oleifera leaves influence the liver hepcidin messenger RNA
674 expression and biochemical indices of Fe status in rats. *Nutrition Research*, 34(7), 630-
675 638. <https://doi.org/10.1016/j.nutres.2014.07.003>

676 Singhal, S., & Hulle, N. R. S. (2022). Citrus pectins: Structural properties, extraction methods,
677 modifications and applications in food systems—A review. *Applied Food*
678 *Research*, 2(2), 100215. <https://doi.org/10.1016/j.afres.2022.100215>

679 Singhal, S., Deka, S. C., Koidis, A., & Hulle, N. R. S. (2024). Standardization of extraction of
680 pectin from Assam lemon (Citrus limon Burm f.) peels using novel technologies and
681 quality characterization. *Biomass Conversion and Biorefinery*, 1-12.
682 <https://doi.org/10.1007/s13399-024-05367-x>

683 Stuart, B. H. (2004). *Infrared spectroscopy: fundamentals and applications*. John Wiley &
684 Sons.

685 Torino, A. B. B., Gilberti, M. D. F. P., Costa, E. D., Lima, G. A. F. D., & Grotto, H. Z. W.
686 (2014). Evaluation of red cell and reticulocyte parameters as indicative of Fe deficiency
687 in patients with anemia of chronic disease. *Revista brasileira de hematologia e*
688 *hemoterapia*, 36, 424-429. <https://doi.org/10.1016/j.bjhh.2014.09.004>

689 Tyagi, V., Sharma, P., & Malviya, R. (2015). Pectins and their role in food and pharmaceutical
690 industry: A review. *Journal of Chronotherapy and Drug Delivery*, 6(3), 65-77. ISSN:
691 2249–6785

692 Wahengbam, E. D., Das, A. J., Green, B. D., Shooter, J., & Hazarika, M. K. (2019). Effect of
693 Fe and folic acid fortification on in vitro bioavailability and starch hydrolysis in ready-
694 to-eat parboiled rice. *Food chemistry*, 292, 39-46.
695 <https://doi.org/10.1016/j.foodchem.2019.04.044>

696 Wang, J., Chen, H., Wang, Y., & Xing, L. (2015). Synthesis and characterization of a new
697 Inonotus obliquus polysaccharide-Fe (III) complex. *International Journal of Biological*
698 *Macromolecules*, 75, 210-217. <https://doi.org/10.1016/j.ijbiomac.2015.01.041>

699 Wang, L., Song, S., Zhang, B., Ai, C., Wen, C., Gong, Y., Jiang, L., Sun, Z., Han, Y. & Xiao,
700 H. (2019). A sulfated polysaccharide from abalone influences Fe uptake by the contrary

impacts of its chelating and reducing activities. *International journal of biological macromolecules*, 138, 49-56. <https://doi.org/10.1016/j.ijbiomac.2019.07.072>

Wang, L., Wang, L., Su, C., Wen, C., Gong, Y., You, Y., Zhao, J., Han, Y., Sing, S. & Xiao, H. (2020). Characterization and digestion features of a novel polysaccharide-Fe (III) complex as an Fe supplement. *Carbohydrate Polymers*, 249, 116812. <https://doi.org/10.1016/j.carbpol.2020.116812>

Wang, S., Zhang, X., Wang, S., & Copeland, L. (2016). Changes of multi-scale structure during mimicked DSC heating reveal the nature of starch gelatinization. *Scientific reports*, 6(1), 28271. <https://doi.org/10.1038/srep28271>

Wang, X., Chen, Q., & Lü, X. (2014). Pectin extracted from apple pomace and citrus peel by subcritical water. *Food Hydrocolloids*, 38, 129-137. <https://doi.org/10.1016/j.foodhyd.2013.12.003>

Zaidel, D.N. A., Md Rashid, J., Hamidon, N.H., Md Salleh, L. & Mohd Kassim, A.S. (2017). Extraction and characterisation of pectin from dragon fruit (*Hylocereus polyrhizus*) peels. *Chemical Engineering Transactions*, 56, 805-810. <https://doi.org/10.3303/CET1756135>