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**Utilization of Bitter orange seeds as a novel source for recovery of pectin:
Compositional and rheological characterization**

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20

21 **Abstract**

22 The seeds from bitter orange, a by-product from the juice making step, hold the potential to
23 facilitate novel, easy yet high quality pectin extraction. To test this hypothesis, the pectin from
24 orange seeds (OSP) were extracted by distilled water and its compositional parameters and
25 rheological behavior then evaluated. Results showed that galacturonic acid was the major
26 component of OSP (~ 425 mg/g) confirming the purity of extracted pectin, followed by glucose
27 and some minor neutral sugars. M_w , R_n and, R_z for the OSP were 4511.8 (kDa), 61 (nm), and 61.1
28 (nm), respectively. Rheological measurements showed shear-thinning behavior for OSP that by
29 increasing temperature from 5 to 45 °C, the viscosity of the gum decreased. Power law fitted as
30 the best rheological model describing the flow behavior of OSP. Strain sweep dynamic rheological
31 measurements confirmed an entangled network structure for OSP and the addition of NaCl to the
32 gum dispersion, decreased the consistency coefficient from 35.6 to 23.18 Pa.sⁿ, while the flow
33 behavior index remained unchanged. These results demonstrate for the first time that the OSP can
34 be used as a new source of pectin, with likely a wide range of applications in food industry.

35

36 **Keywords:** Pectin; Orange seeds; Sugar composition; Molecular weight; Rheological properties.

1. Introduction

Hydrocolloids are water-soluble biopolymers with a wide application in food industry. They are commonly used to improve textural properties of food as a gelation and viscosity improving agents (Nejatian et al., 2020). Pectin one of the major hydrocolloids in food processing industry is a polysaccharide with 1, 4-galacturonic acid units and some of its carboxyl groups can be substituted with methyl esters or amide groups (Löfgren and Hermansson, 2007). It is widely used as an ingredient for providing specific textural and rheological properties to processed food as a gelling/thickening agent or as an emulsion stabilizer for acidified dairy drinks (Christiaens et al., 2016).

The source of pectin, the extraction procedures, the particle size distribution, the patterns of acylation, the degree of esterification (DE) and the nature and position of the neutral sugars have a great impact on the specifications of pectin from the various origins (Maxwell et al., 2012). Pectin is considered as an invaluable by-product of the citrus processing industry. About 85% of the globally commercial pectin is sourced from citrus waste which can be produced from fresh or processed peels of lemon, grapefruit, and oranges (Berk, 2016).

Disadvantages associated with pectin extraction from citrus peels include the use of corrosion mineral acid, high temperature and extended extraction time. Together these may affect pectin quality and moreover damage equipment. Furthermore, the steps of filtration, discoloration and concentration of pectin solution before alcohol precipitation are associated with both high cost and use of time (May, 1990). Therefore, new sources of pectin with both lower extraction time and production costs is both economically and technologically appealing (Shan, 2016). Previous studies examining pectin extraction have been varied in their approach. The extraction and physiochemical properties of pectin from the heads of sunflowers was studied by Peng et al. (Peng

et al., 2020). Asgari and co-workers (Asgari et al., 2020) studied the walnut processing waste as a novel source of pectin. Gharibzahedi et al. (Gharibzahedi et al., 2019) evaluated the pectin extracted from Fig (*Ficus carica* L.) skin. Chaliha and co-authors (Chaliha et al., 2018) extracted pectin from *Terminalia ferdinandiana*- a native Australian fruit. Finally, the possibility of using Palmyra Palm (*Borassus aethiopum* Mart.) fruit was evaluated by Assoi et al. (Assoi et al., 2017). The bitter orange (*Citrus aurantium*) contains many seeds and differs from the orange by several characters including the acidic pulp and bitterer albedo (Moufida and Marzouk, 2003). It is commonly used for essential oils, in the perfume industry and for the production the marmalade. In the Middle East, the juice of the ripen fruit can be used as a salad dressing or as a flavoring (Zibae et al., 2020).

It is necessary to understand the rheological properties and determination of sugar composition of pectin from new sources to evaluate their thickener potential. The flow behavior of hydrocolloids solutions is important to assess processing parameters, textural properties of formulated foods, design of unit operations and development of product engineering (Balaghi et al., 2011; Rincón et al., 2014). To date there is no report on the extraction of pectin from orange seeds and hence an evaluation of its compositional and functional properties. Therefore, the main objective of this research was to extract and characterize the sugar composition, molecular weight, and rheological properties of pectin from bitter orange seeds as a novel source of pectin.

2. Material and methods

2.1 Materials and extraction method

The bitter orange fruit (*Citrus aurantium* L.) was used in this study, collected from trees growing in Mazandaran province of Iran during October and November 2020 and according to the permission and the national guideline of Agricultural Research Education and Extension Organization of Iran. Phenotypic identification of the specimen was done by Dr. Saeid Hazrati, academic member of Shahid Madani University of Tabriz, Iran.

The seeds were collected from bitter oranges during the juice extraction. Seeds were washed with tap water to remove the fruit pulp and drained completely to remove the excess water. Afterward, the seeds soaked in distilled water (pH= 7) in a seed ratio of 6:1 at 70 ± 1 °C and stirred for 30 min (solid/liquid ratio selected based on preliminary test). Thereafter, pectin coats were removed from the seeds by passing through a 600 µm sieve and collected in a flask. The extracted solution mixed with three volumes of 96% v/v ethanol and placed in the fridge (4 °C). After 24 h, the flocculated pectin collected and dried in an oven with circulating air at 30 °C. The dehydrated pectin (OSP) was pulverized with a miller, packed, and kept in cool and dry condition prior test.

Standards of monosaccharides (galacturonic acid, glucose, arabinose, galactose, rhamnose, and fucose) and trifluoroacetic acid (TFA) with purity of $\geq 99\%$ were purchased from Merck (Darmstadt, Germany).

2.2 Compositional analysis

AOAC method (AOAC, 2016) was used for determination of moisture content and total ash content. Kjeldal method was used for the determination of total protein content with the nitrogen value of 6.25 (Razavi et al., 2014).

After hydrolysis 4 g/l of OSP by TFA (2 M) for 2h at 121 °C, the monosaccharide composition analyzed by high-performance anion-exchange chromatography with a pulsed amperometric

detector (DECADE Elite). Separations carried out in a CarboPac PA1(4×250mm) column (Dionex Corp., Sunnyvale, CA). Samples were passed through 0.22 µm filter prior injections into the column. The monosaccharides separation through the column was carried out according to the method of (Gavlighi et al., 2013).

2.3 Degree of esterification (DE)

The titration method proposed by Chaharbaghi et al. (Chaharbaghi et al., 2017) was used for determination of DE of pectin sample. To do this, 100 mg of dried powder of sample was added to 2 ml of ethanol and dissolved in 20 ml deionized water at 40 °C. Afterward, 5 ml of phenolphthalein reagent was added to the solution and titrated with NaOH (0.1 M). The amount of NaOH used for titration recorded as V_i . Afterward, 10 ml of NaOH was added to the solution and mixed for 30 min for complete hydrolysis. 10 ml of HCl (0.1 M) was introduced to the solution and mixed vigorously to completely disappear the pink color. After the addition of drops of phenolphthalein, the excessive amount of HCl was titrated with NaOH to obtain a pale pink color and the volume of NaOH was recorded as the V_f . The DE of pectin was calculated according the following equation (Hosseini et al., 2016):

$$DE = (V_f / V_i + V_f) \times 100$$

2.4 Determination of molecular parameters

Molecular weight averages (M_n , M_w , M_z) of the OSP were determined by using a size exclusion chromatography system (flow rate of 0.4 mL/min with 0.15 M NaNO_3 and 0.02% NaN_3) and equipped with TSK G5000 PW column (7.5×600 mm; Toso Biosep, Montgomeryville, PA, USA) joint to a UV detector (Waters, 2487), multi-angle laser light scattering (HELEOS; Wyatt Technology Corp, Santa Barbara, CA, USA) and a refractive index detector (Waters, 2414)

(HPSEC-UV-MALLS-RI). Bovine serum albumin (BSA) was used as a standard for determination of the volume delays among the US, MALLS, and RI detectors. ASTRA 5.3 software (Wyatt Technology Corp.) applied for data acquisition and to calculate the M_w average, M_n , and M_z .

2.5 Rheological Measurements

In order to determine the rheological characteristics of gum, OSP dispersions at concentrations 0.1, 0.3, 0.5, 0.7 and 1 % (w/v) were first prepared by dissolving of the required amount of dry powdered gum in distilled water and gently stirred at room temperature for 2 h. The gum dispersions were stored overnight at 5 °C to assure that the hydration of the polysaccharide was complete.

Both steady shear viscosity and oscillatory shear tests were performed by Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria) so that a concentric cylinder geometry (radius ratio of 1.035) and a parallel plate geometry (25-mm diameter; 0.5-mm gap) were used for dilute samples and concentrated dispersions, respectively. The temperature was adjusted to 25 °C with a Viscotherm VT2 circulating bath and a controlled Peltier system (Anton Paar, GmbH) with an accuracy of ± 0.01 °C.

Shear sweeps were conducted at 25 °C between 0.001 to 1000 s^{-1} so as to obtain flow curves. Flow behavior of the dispersions were assessed by fitting the shear rate versus shear stress values to five usual models, i.e., Newtonian ($\tau = m\dot{\gamma}$), Power-law ($\tau = m\dot{\gamma}^n$), Herschel-Bulkley ($\tau = m\dot{\gamma}^n + \tau_0$), Bingham ($\tau = m\dot{\gamma} + \tau_0$), and Casson ($\sqrt{\tau} = m\sqrt{\dot{\gamma}} + \sqrt{\tau_0}$). Where τ is the shear stress (Pa), m is the consistency coefficient (Pa.sn), $\dot{\gamma}$ is the shear rate ($1 s^{-1}$), the exponent n is flow behavior index (dimensionless) and τ_0 is the yield stress (Pa) (Nejatian and Abbasi, 2019; Nejatian et al., 2018).

The effect of temperature on the flow properties was measured by performing shear sweeps for 1 % (w/v) OSP dispersion at 5 °C, 25 °C and 45 °C. Also, the temperature dependency of apparent viscosity was evaluated by fitting the Arrhenius model ($\eta = \eta_0 \exp \frac{E_a}{RT}$) in which η_0 is the pre-exponential factor (Pa.), E_a the activation energy for viscous flow (J/mol), R the universal gas constant (8.314 J/mol K), and T the absolute temperature (K).

The 1 % (w/v) OSP gum dispersion was prepared for oscillatory shear measurements. Strain sweep test was performed over the range 0.05–100 % at a fixed frequency (1 Hz) to determine the linear viscoelastic region (LVR). Frequency sweep tests were also carried out at a wide range of frequencies (0.01–20 Hz) and a constant strain (<LVR, ~ 0.5 %) to evaluate the dynamic rheological properties (G' and G'').

In addition, the rheological behavior in response to the salt concentration (0.2 M) and type (NaCl and CaCl₂) were determined just at a certain gum concentration, 1 % (w/v) and temperature, 25 °C.

2.6 Statistical analysis

All the measurements were made in triplicate and data were presented as mean $\pm$ standard deviation. Microsoft Excel Software (Microsoft Office, Package 2012) used for plotting the rheological curves.

3. Results and discussions

3.1. Compositional analysis

The physicochemical and molecular parameters of the OSP are presented in Table 1. The results showed the OSP contained 9.17% moisture, 1.88% ash, 2.14% of protein, 86.8% of total

carbohydrates, and DE of 79.68%. Plant's variety and growing conditions, extraction and purification process are important factors affecting the chemical composition of hydrocolloids (Razavi et al., 2014). Similar chemical composition for pectin extracted from sour oranges and the total ash, moisture content, and protein content were 1.89, 8.81, and 1.45, respectively (Hosseini et al., 2019).

Monosaccharides analysis using liquid chromatography (HPLC) showed that galacturonic acid was the major component in OSP (~ 425 mg/g, about 85% of sugar composition). This was followed by glucose (54 mg/g, about 10% of sugar composition). The wide diversity of composition by acid and glucose indicates that OSP has rich pectin content but also some cellulose or starch-like glucans. Such sugar composition was previously observed in the commercial low methoxyl pectin (Peng et al., 2020) and some pectin from different food waste streams (Müller-Maatsch et al., 2016). Also, minor quantities of fucose, galactose, arabinose, and rhamnose (about 4% of sugar composition) were also identified which can explain the complex polysaccharide composition of OSP (Razavi et al., 2014). It has been reported that galactose, rhamnose, arabinose, xylose and fucose are the principal neutral sugars found in pectin side chains (Hosseini et al., 2019). Hydrocolloids with a higher amount of fucose, xylose, galacturonic acid, methoxyl groups, and lower amounts of arabinose and nitrogenous fractions are reported to exhibit high viscosity (Anderson and Grant, 1988). Similar observations were also reported by Balaghi et al. (2011) and showed that tragacanth gums with a greater quantity of galacturonic acid and fucose exhibited higher consistency coefficients (Balaghi et al., 2011). Similarly, Hosseini et al. (2019) observed that galacturonic acid (65.3%) was the main monosaccharide of pectin extracted from sour orange peels (Hosseini et al., 2019).

3.2. Molecular weight parameters

The results of molecular weights parameters are presented in Table 1. M_w (weight-average molar mass), R_n (number average molar mass) and, R_z (z-average molar mass) for the OSP were 4511.8 (kDa), 61 (nm), and 61.1 (nm), respectively. The M_w measurement indicates a large M_w for OSP confirming that such polysaccharides have a tendency to exhibit a higher viscous and pseudoplastic properties when dissolved in water (Hosseini-Parvar et al., 2010). The high molecular weight of OSP is similar to other hydrocolloids such as xanthan (4200 kDa), locust bean gum (50-3000 kDa), Basil seed gum (1045-5980 kDa), guar (50-8000 kDa), psyllium (1500 kDa), and Karaya (10,000 kDa) (Faria et al., 2011; Harding et al., 2017; Imeson, 2011; Milani and Maleki, 2012; Naji-Tabasi et al., 2016). Different studies have shown that the pH of extraction may have an effect on the molecular weight of pectin and pectin extracted in higher pH shows the higher M_w due to the higher DE value of the pectin (Cho et al., 2019; Yapo et al., 2007). Using distilled water (pH=7) to extract pectin from OSP, may explain the high M_w observed for the extracted pectin in this research. Gavligi and co-workers (2018) reported that the M_w of pectin extracted from pomegranate peels depended on the extraction condition and the highest M_w and R_g observed for the buffer extracted pectin ($18,631.85 \times 10^3$ g/mol and 102.80 nm) (Gavligi et al., 2018).

3.3. Rheological properties

3.3.1. Flow behavior

Figure 1a compares the flow behavior of OSP dispersions within a concentration range of 0.1-1 wt%. On the basis of the highest determination coefficient (R^2) and the lowest root mean standard error (RMSE), the flow behavior of all dispersions of OSP with different concentrations were particularly nonlinear and best fitted to Power-law model ($\tau = m\dot{\gamma}^n$). The coefficients of evaluated rheological models are shown in Table 2. The power law model has a wide application in food

industry and many studies have proved this model as the most appropriate model for flow behavior study of the majority of food hydrocolloids (Khodaei et al., 2014).

The dependency of the apparent viscosity on the shear rate for the OSP dispersions in various concentrations is shown in Figure 1b. As evident, the OSP dispersions indicated the typical viscosity vs shear rate relationship of a colloidal food system including the polysaccharides solutions. At lower shear rate (~ 0.1 1/s) and especially at higher gum concentrations (0.5-1 wt%), the apparent viscosity was nearly independent of the shear rate. Indeed, when the shear rate is low, the Brownian motion dominates the structural forces and favors the alignment of the elongated coil along the flow direction. However, the viscosity reduced with increasing shear rate (shear thinning behavior) arose from the equilibrium between the hydrodynamic forces and the structural forces (Barnes, 2000; Windhab, 1995). In fact, above a critical shear rate, the deformation rate of the gum chain entanglement due to the application of external forces becomes greater than the formation rate of the new entanglement (Lapasin and Pricl, 1995).

As seen (Fig. 1b), the apparent viscosity decreased with reducing the gum concentration to 0.1%, throughout the shear rate study. In addition, as the concentration of dispersion increases, the intermolecular interactions increase and improve the viscosity. The positive effect of gum concentration on the apparent viscosity can be also followed in Power law model parameters (Table 1), so that with increasing in OSP concentration, the consistency coefficient (k) and flow behavior index (n) increased and decreased, respectively. Generally, it seems that the apparent viscosity of OSP is similar or even better than that of some other polysaccharides such as some species of gum tragacanth (Balaghi et al., 2010), pectin (Marcotte et al., 2001), Persian gum (Fadavi et al., 2014), guar gum (Kayacier and Dogan, 2006), carboxymethyl cellulose (Yasar et al., 2007), etc.

Strain sweep dynamic rheological measurements showed that for OSP dispersion (1wt%) storage modulus (G') was somewhat higher than loss modulus (G'') (Fig. 2a). Nevertheless, the superiority of each of these modules over the other in the frequency sweep test depended on the frequency. As is evident in Fig. 2b, there was the transition from a predominantly viscous response at longer time scales ($G'' > G'$) to a predominantly elastic response at shorter time scales ($G' > G''$), indicating the dispersion has an entangled network structure. Such rheological behavior can be compared to some gum tragacanth species (*Astragalus parrowianus* and *fluccosus*) (Balaghi et al., 2011) and deacetylated *Sterculia striata* polysaccharide (De Brito et al., 2005).

3.3.2. Effect of salt and temperature on rheological properties

The Addition of salt (NaCl) to the OSP dispersion (1 wt%) decreased the apparent viscosity with no changes in the reduction pattern or flow behavior (Fig. 3a). Moreover, the consistency coefficient of 1 wt% OSP aqueous dispersion in the presence of 0.2 M NaCl decreased from 35.60 to 23.18 Pa.sⁿ while the flow behavior index remained almost unchanged (0.22 against 0.25). This effect can be assigned to charge screening effect of the salt on long-range electrostatic repulsion among the uronic acid residues of OSP (Balaghi et al., 2010). Based on strain sweep test, the salt addition reduced amount of both G' and G'' , but the G' values in LVR were still greater than G'' (Fig. 2a). On the contrary to the NaCl-free OSP dispersion, the dispersion containing NaCl (0.2 M) indicated a crossover point at a high frequency (Fig. 2b). Furthermore, in the presence of NaCl, the G' and G'' gap was larger. This suggests that NaCl caused a more drastic decrease in elastic component than the viscous component. Interestingly, as is evident (Fig. 3a), incorporation of CaCl_2 to OSP dispersion increased the viscosity which can be related to the bridging effect of Ca^{2+} forming a strong gel network of polysaccharide chains.

Figure 3b shows the temperature influence on the apparent viscosity of 1 wt% OSP dispersion as a function of shear rate. As seen, the viscosity significantly declined by increasing in temperature from 5 to 45 °C. This effect can be also observed in Power law model parameters so that by increasing the temperature, the consistency coefficient decreased and the flow behavior index increased (Table 3). However, the flow behavior index of the dispersion is still far from the typical value of a Newtonian fluid (i.e., $n = 1$). In addition, the shear rate had a considerable effect on the temperature dependence of OSP dispersion viscosity. Figure 3c shows the value of activation energy obtained at two shear rates, 0.1 and 10 1/s for 1 wt% OSP aqueous dispersions. High R^2 values suggested that the apparent viscosity of dispersion in relation to temperature follows the Arrhenius model. A higher flow E_a value at low rates of shear implicates more sensitivity of OSP viscosity to temperature changes. This trend was also reported for other hydrocolloids such as pectin (da Silva et al., 1994).

4. Conclusion

The compositional and rheological behavior of pectin extracted from bitter orange seeds (OSP) as a novel source of high-quality pectin was evaluated in this study. Monosaccharides compositions revealed that galacturonic acid was the major structure of pectin followed by glucose. Arabinose, galactose, rhamnose, and fucose were also observed in smaller amounts and confirm the side chain structure of the pectin. Molecular weight analysis of the pectin showed a high M_w of 4512 kDa. OSP dispersions exhibited a non-Newtonian shear thinning behavior. Strain sweep dynamic rheological measurements exhibited a higher storage modulus (G') than loss modulus (G'') and it confirms the entangled structure of OSP. In conclusion, the mucilage extracted from orange seeds

279 is rich in pectin and demonstrates potential use as gelling or emulsion stabilizer in food
280 applications. Further studies to evaluate the functional properties and characterization of this novel
281 source of pectin is needed but if fully elucidated could potentially revolutionize the use of pectin
282 in the food industry.

283 **Conflict of interest**

284 All the authors declare no conflict of interest.

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401

Figures

Figure 1. Comparison of the flow curves (a) and influence of shear rate on apparent viscosity of the OSP dispersions in different concentrations [0.1 wt% (◆), 0.3 wt% (■), 0.5 wt% (▲), 0.7 wt% (●), 1 wt% (✕)]

Figure 1

See figure for legend.

Figure 2. Comparison of the dependency of G' (filled) and G'' (hollow) on (a) strain and (b) frequency for 1 wt% OSP dispersions in the absence (□, ■) and presence (○, ●) of added NaCl (0.2 M).

Figure 2

See figure for legend.

Figure 3. Effect of salts (a; 0 wt% (●), 0.2 M NaCl (?) and 0.2 M CaCl₂ (■) and temperature (b: 5 °C (□), 25 °C (○) and 45 °C (△) on the apparent viscosity of 1 wt% OSP dispersion as a function of shear rate. Arrhenius plots (c) at shear rate of 0.1 1/s (■) and 10 1/s (□) for 1 wt% OSP dispersions.

Figure 3

See figure for legend.