Pectin extraction from citron peel: optimization by Box–Behnken response surface design

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Abstract In this study, the effect of acidic extraction conditions (time of 30–90 min, temperature of 75–95 °C and pH of 1.5–3) on the yield and degree of esterification (DE) of citron peel pectin was investigated applying Box–Behnken design. The highest production yield of pectin ($28.31 \pm 0.11\%$) was achieved at extraction time of 90 min, temperature of 95 °C and pH of 1.5, as optimal extraction conditions, which was close to the predicted value (29.87%). Under optimum extraction conditions, the DE and the emulsifying activity were 51.33 and 46.2%, respectively. In addition, the emulsions were 93.9 and 93.5 stable at 4 °C, 93.7 and 93.1 at 23 °C after 1 and 30 days, respectively. The determination of flow behavior showed that the pectin solutions had a Newtonian behavior at low concentrations ($< 1.0\%$ w/v), while this behavior was changed to pseudoplastic with increasing concentration.

Keywords Pectin · Citron · Optimization · Response surface methodology

Introduction

Pectin is an exclusive structural polysaccharide that exists in the cell walls and middle lamella of terrestrial plants [25]. Pectin includes a backbone of $\alpha(1 \rightarrow 4)$ galacturonic acid residues which makes up at least 65% of total pectin [13]. The D-galacturonic acid includes carbonyl groups

(COO) and these are partially esterified with methyl alcohol or acetic acid [13]. The degree of esterification (DE) is an effective property in gel production by pectin. Based on DE, pectin is classified into two categories: The high methyl ester pectin (HMP) with the DE percentage above 50% and the low methyl ester pectin (LMP) with the DE less than 50% [9].

Pectin are widely used in the food industry as a gelling, stabilizing, thickening and emulsifying agent [15, 31]. Furthermore, pectin is employed to diverse pharmaceutical activities such as wound healing, lipase inhibition, apoptosis induction of human cancer cell, immunostimulating, antimetastasis and lowering cholesterol [17, 19, 31].

Citrus fruits (family *Rutaceae*) are cultivated widely around the world. Some citrus fruits are oranges, mandarins, lemons, grapefruits and citrons (*Citrus medica* L.) [23]. The citron belongs to the citrus species with properties similar to lemon fruit, which was observed for the first time in the near East and the Mediterranean [29]. Nowadays, this fruit cultivated in the south of France, Southern Italy (Calabria), Greece, North Africa, Puerto Rico, China, Vietnam and Japan [11].

Citron is mainly used for the production of candied peel, flavoring of liquors and medical purposes [40]. The large volume of the fruit is its peel, which is included the plenty of bioactive compounds and polysaccharides, such as pectin [26]. So, this by-product can be a suitable for extraction of pectin.

Pectin is most commonly extracted by a hot diluted solution of the strong mineral acids [32]. Strong mineral acids such as hydrochloric, sulfuric, or nitric acid have low price and capable to generate pectin enriched in homogalacturonic blocks [43]. However, they have disadvantages include toxicity and damaging the environment and also requiring high cost for treating the wastes [42].

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Therefore, the organic acids such as citric acids can be used to extract the pectin [19]. In addition to, organic acids have a lower hydrolyzing capacity and are expected to cause less de-polymerization of pectin [20].

In acidic extraction, the diverse factors such as extraction period, extraction temperature and pH can be influenced on the yield and quality of extracted pectin [36]. Therefore, the optimization of extraction conditions is necessary to reach the maximum production.

Response Surface Methodology (RSM), which indicates the behavior of experimental data with objective of making the statistical previsions by a polynomial equation, is a complex of mathematical and statistical techniques [3]. Also, it is necessary to mention that this methodology widely employed for processes optimization. In these processes the output usually is affected by many input variables [2].

According to the above-mentioned, in the current study, our goals were to investigate the effects of the diverse parameters including extraction period, extraction temperature, pH and liquid/solid ratio on the acidic extraction yield and DE of citron peel pectin and also optimization these extraction conditions to reach the maximum yield.

Materials and methods

Materials

Citron peels were purchased from local grocery in Mashhad, Khorasan razavi, Iran. The peels were washed, cut into small pieces and then dried in 50 °C for 24 h by a hot air oven. In the next step, the dried peels were eroded and passed through a 40-mesh sieve to obtain powdered sample. The prepared powders were kept in sealable bag inside desiccator until use. Citric acid, sodium hydroxide, hydrochloric acid, phenolphthalein reagent, sulfuric acid, sodium tetraborate and sodium azide were procured from Merck Chemical Co. (Darmstadt, Germany).

Extraction of pectin from dried citron peel

Acidic extraction of pectin was carried out according to the methods described by Canteri-Schemin et al. [4] with some modification. The dried peel powder (liquid–solid ratio (LSR) of 30 (v/w), Fig. 1) was soaked into citric acid aqueous solution adjusted to the desirable pH values (1.5, 2.25, 3), and then stirred. The solution was extracted with three temperature of 75, 85, 95 °C for three times of 30, 60, 90 min. After the extraction process, the mixture was centrifuged (10,000 g, 20 min) to isolate impurities. Then, the same volume of ethanol (98%) was added to solution and stored at 7 °C for 12 h. After this period, pectin was

separated from solution using centrifugation (10,000 g, 15 min) and dried in oven at 50 °C until constant weight was achieved. The yield of pectin (YP) was calculated from following equation [38]:

$$YP(\%) = \frac{\text{Mass of extracted pectin}}{\text{Mass of initial dried powder}} \times 100 \quad (1)$$

Determination of the degree of esterification

The degree of esterification was characterized by titration method, that explained by Santos et al. [38] with some modification. 20 mg pectin was soaked with 3 ml ethanol and dissolved in 20 ml of deionized water. The samples were stirred until the pectin dissolution completely. Thereafter, five drops of phenolphthalein reagent were added to solution and then with 0.1 N sodium hydroxide was titrated (V_1). After that, 10 ml of 0.1 N sodium hydroxide was added to samples and shaken 15 min for hydrolysis. Subsequently, 10 ml of 0.1 N hydrochloric acid was added to solution and stirred until the pink color disappeared. The solution was titrated with 0.1 N sodium hydroxide until the reappearance of pink color (V_2). Finally, the DE was determined according to the following equation:

$$DE(\%) = \frac{V_2}{V_1 + V_2} \times 100 \quad (2)$$

Emulsifying properties

Generally, the ability to form an emulsion is one of the most important properties of some food hydrocolloids such as pectin and the emulsifying properties of this polysaccharide can be very important in using it in food products. It should also be noted that these properties are probably due to the presence of protein parts and acetyl groups in structure of this polysaccharide because the pectin itself is predominantly hydrophilic. In this part, emulsifying activity (EA) and emulsion stability (ES) of pectin emulsions were evaluated based on the procedure described by Hosseini et al. [17]. Generally, oil-in-water (O/W) emulsions were prepared by adding 5 ml sun flower oil to 5 ml pectin solutions (0.5% w/v) containing 0.02% sodium azide as a bactericide. Solutions were homogenized in the ultraturax T-25 homogenizer (IKAT25 Digital Ultra-Turax, Staufen, Germany) at 10,000 g for 4 min, and then the emulsions were centrifuged for 5 min at 4000 g. The EA was determined as follows:

$$EA(\%) = \frac{\text{Volume of the emulsion phase}}{\text{Total volume of system}} \times 100 \quad (3)$$

Fig. 1 3-D response surface plots indicate the influence of time (min), temperature (°C) and pH on the extraction yield (%) and DE (%)

To investigate the emulsion stability (ES) as prepared above, the different tubes were kept at 4 and 23 °C for 1 and 30 days. The emulsion stability was characterized by following equation:

$$ES (\%) = \frac{\text{The remaining emulsified layer volume}}{\text{The initial emulsified layer volume}} \times 100 \quad (4)$$

Determination of the viscosity

A rotational programmable viscometer (LVDV-II Pro, Brookfield Engineering Inc., Middleborough, MA, USA) by a LV spindle were employed to investigate of flow behavior of pectin solutions in diverse concentrations (0.1,

0.5, 1.0 and 2.0% w/v). Thus, the cylinder of the rotational viscometer was filled with 25 ml of sample and shear rate was adjusted from 1.22 to 79.50 s⁻¹ within 5 s intervals.

Fourier transform infrared spectroscopy (FTIR)

FTIR is a structure spectroscopic technique that shows the bonding structure of atoms. This spectrum obtained from interaction of infrared radiation with matter. Analysis of FTIR spectrum was made to confirm presence of pectin in supernatant obtained from citron peel [36]. FTIR spectrum of citron peel pectin was registered by a device called Perkin Elmer FTIR spectrometer (Perkin Elmer Co., Waltham, MA, USA) applying the potassium bromide disk method with resolution of 4 cm⁻¹. It should also be noted that the FTIR measurement was achieved over the range of 4000–450 cm⁻¹.

Design of experiment

One factor at the time design was applied to investigate the most appropriate LSR. In this design, one factor variable and other factors are constant. For this purpose, different levels of LSR (20:1, 30:1, 40:1 and 50:1 v/w) were considered and other factors (time: 90 min, temp: 95 °C, pH: 1.5) considered to be constant. To optimize the effect of extraction conditions on the yield, a Box–Behnken response surface experimental design was applied. The levels of independent variables are shown in Table 1. It should also be noted that the all computation and graphics in this research were performed employing the statistical software Design Expert (version 8.0.0, Stat-Ease Inc.,

Minneapolis, MN, USA) and Excel (version 2010, Microsoft Corporation, Redmond, WA, USA).

Results and discussion

One factor at the time for evaluation of LSR effect on the yield of pectin

Liquid–solid ratio (LSR) is a extremely influential factor on the yield of pectin. One factor at the time was employed to evaluate the most appropriate LSR. As above-mentioned, the different levels of LSR (20:1, 30:1, 40:1, 50:1 v/w) were selected and other factors (time of 90 min, temperature of 95 °C and pH of 1.5) were considered to be constant. The results showed that the extraction yield increased by a increase in LSR up to 30:1 v/w, which this is probably due to increasing the content surface area between peel particles and solvent by increasing solvent volume and thereby, increase in extraction of pectin [34]. However, when the LSR continued to increase, the yield of pectin showed no significant change. Therefore, in the current study, in order to reduce alcohol consumption and therefore the cost and volume of work, the LSR of 30:1 v/w was selected for all experiments [37].

Model fitting and statistical analysis

In this paper, three factors, three levels Box–Behnken response surface design (BBD) was applied to evaluate and optimize the effect of process variables such as time (30–90 min), temperature (75–95 °C) and pH (1.5–3) on

Table 1 Coded levels and actual values (in parentheses) of the variables in Box–Behnken design: extraction time (X₁, min); temperature (X₂, °C); pH (X₃); pectin yield (PY, %); degree of esterification (DE, %)

Run	Independent variables			Measured responses		Predicted responses	
	X1	X2	X3	PY	DE	PY	DE
1	− 1 (30)	− 1 (75)	0 (2.25)	9.20	61.23	9.09	60.15
2	+ 1 (90)	− 1 (75)	0 (2.25)	12.76	54.92	12.67	54.39
3	− 1 (30)	+ 1 (95)	0 (2.25)	13.75	50.05	13.83	50.58
4	+ 1 (90)	+ 1 (95)	0 (2.25)	14.34	48.27	14.45	49.35
5	− 1 (30)	0 (85)	− 1 (1.50)	22.26	45.83	22.14	45.93
6	+ 1 (90)	0 (85)	− 1 (1.50)	25.98	45.88	25.84	45.43
7	− 1 (30)	0 (85)	+ 1 (3.00)	7.03	69.48	7.17	69.92
8	+ 1 (90)	0 (85)	+ 1 (3.00)	7.55	63.54	7.67	63.43
9	0 (60)	− 1 (75)	− 1 (1.50)	20.41	45.11	20.64	46.08
10	0 (60)	+ 1 (95)	− 1 (1.50)	27.95	44.10	28.00	43.47
11	0 (60)	− 1 (75)	+ 1 (3.00)	8.20	71.15	8.16	71.78
12	0 (60)	+ 1 (95)	+ 1 (3.00)	7.56	60.76	7.33	59.78
13	0 (60)	0 (85)	0 (2.25)	11.02	58.57	10.62	58.54
14	0 (60)	0 (85)	0 (2.25)	10.65	58.03	10.62	58.54
15	0 (60)	0 (85)	0 (2.25)	10.20	59.01	10.62	58.54

the extraction yield of pectin from citron peel. The predicted values and experimental results demonstrated in Table 1.

Second order polynomial equation includes linear, interactive and quadratic terms was employed to organize mathematical models to detect the optimum conditions and express the relationship between process variables and the responses [27, 35]. The second order equations related to the extraction yield and DE are given below:

$$\begin{aligned} \text{PY (\%)} = & 71.8 + 0.2211X_1 - 0.957X_2 - 20.13X_3 \\ & + 0.000869X_1^2 + 0.01107X_2^2 + 7.644X_3^2 \\ & - 0.002475X_1X_2 - 0.03556X_1X_3 \\ & - 0.2727X_2X_3 \end{aligned} \quad (5)$$

$$\begin{aligned} \text{DE (\%)} = & -209.7 + 0.039X_1 + 5.06X_2 + 47.38X_3 \\ & - 0.002237X_1^2 - 0.02913X_2^2 - 0.62X_3^2 \\ & + 0.00378X_1X_2 - 0.0666X_1X_3 - 0.3130X_2X_3 \end{aligned} \quad (6)$$

where X_i is coded independent factor (X_1 = time, X_2 = temperature, X_3 = pH).

The statistical meaningful of the models were investigated applying analysis of variance (ANOVA). ANOVA, with the aim of testing hypothesis on the parameters of the model, is a statistical method that subdivides the total variation in a collection of data into component parts associated with especial sources of variation (Table 2) [34]. As can be seen, the model p values of lower than 0.001 (significant) for both the yield and DE, and also lack of fit higher than 0.05 (insignificant) for these parameters demonstrated that the models were well adapted to the responses [16]. In the other hand, the determination coefficient of yield and DE (99.92 and 99.41%, respectively), adjusted determination coefficient (99.77 and 98.34%, respectively) and predicted determination coefficient (99.37% and 91.12%, respectively) are calculated to examine the adequacy of the models [1, 5]. The high values of R^2 , Adj- R^2 values clearly demonstrated a very high degree of precision and good reliability of the conducted experiments [33].

Optimization conditions and influence of process variables on the yield

Table 1, demonstrates that the yield of pectin was varied between 7.03 to 27.95%. The optimum conditions were calculated by solving Eq. (5), and the results showed that the highest yield (29.87%) were obtained under the extraction time of 90 min, temperature of 95 °C and pH of 1.5. The validation experiments were carried out in triplicate and indicated that the yield of pectin in optimum conditions was $28.31 \pm 0.11\%$. As can be seen, the

obtained yield was close to the predicted yield and showed that there is a high compatibility between measured and predicted values.

According to the experiments that were conducted at diverse pH levels (1.5–3) and the results were depicted in (Fig. 1B and C). The results clearly represent that the yield of pectin increased with decreasing pH value. The acidic solvent with low pH has the ability to hydrolysis of the insoluble pectin and converts to its soluble form, and thereby increasing the extraction yield of pectin from citron peel [10]. On the other hand, the low pH could diminish the molecular weight of pectin and so, increase its release from plant tissue without any degradation [12]. It should also be noted that at high pH levels, the yield of pectin considerably reduced thereby pectin accumulates that prevents its release [27]. This observation was also similar to the results published from apple pomace, sugar beet pulp, mango peel, and pomegranate peel by Canteri-Schemin et al. [4], Yapo et al. [43], Prakash Maran et al. [35] and Moorthy et al. [27] respectively.

Temperature is considered as one of the more crucial parameters affecting the amount of extraction yield of pectin. The results indicated that the yield of pectin was increased with increasing temperature (Fig. 1A and C). Increase in temperature can hasten the solubility and diffusivity of solvent into the plant tissue and increases the extraction yield of pectin [41]. This result agreed with pagan et al. [30] and Raji et al. [36], who extracted pectin from peach pomace and melon peel, respectively. Also, in the case of the effect of extraction time, it must be said that the yield of pectin was increased with increasing the time (Fig. 1A and B) because longer time provide more reaction time opportunity. The similar results were reported by Chen et al. [6] and Zheng et al. [44].

Influence of process variables on the DE

Table 1, indicated that DE under diverse extraction conditions was ranged from 44.10–69.48%. Also, the DE of pectin produced under optimal extraction conditions (Extraction time of 90 min, temperature of 95 °C and pH of 1.5) was 51.33%, which represented the citron peel pectin could be categorized as HMP. It should be said that this type of pectin ($\text{DE} > 50\%$) is desirable for preparing high sugar products [4]. As Fig. 1(D), (E) and (F) depicted, under increased pH, decreased time and temperature of extraction, the DE was increased. The reason for this phenomenon is due to de-esterification of polygalacturonic chains, when use from harsh conditions such as very high temperature, long time and low pH [18, 28]. The similar results were observed by Raji et al. [36] for melon peel pectin and Tang et al. [39] for dragon fruit peel pectin.

Table 2 The results of analysis of variance (ANOVA) for regression model of pectin yield and DE

Source	Sum of squares	DF	Mean square	F value	p value
(A) Yield					
Regression	671.395	9	74.599	674.69	0.000
Linear	578.820	3	192.940	1744.98	0.000
Square	71.081	3	23.694	214.29	0.000
Interaction	21.493	3	7.164	64.80	0.000
Residual error	0.553	5	0.111		
Lack-of-fit	0.216	3	0.072	0.43	0.757
Pure error	0.337	2	0.169		
Total	671.948	14			
R^2			0.9992		
Adj R^2			0.9977		
Pred R^2			0.9937		
(B) DE					
Regression	1092.51	9	121.391	92.97	0.000
Linear	1013.16	3	337.719	258.66	0.000
Square	43.22	3	14.405	11.03	0.012
Interaction	36.14	3	12.048	9.23	0.018
Residual error	6.53	5	1.306		
Lack-of-fit	6.03	3	2.009	8.02	0.113
Pure error	0.50	2	0.251		
Total	1099.04	14			
R^2			0.9941		
Adj R^2			0.9834		
Pred R^2			0.9112		

Emulsifying features

The emulsifying features (emulsifying activity and emulsion stability) of pectin achieved under optimal extraction condition (Extraction time of 90 min, temperature of 95 °C and pH of 1.5) were assessed and the stability of emulsions was investigated during maintenance for 1 and 30 days at temperatures of 4 and 23 °C (Table 3). After centrifuging the emulsions, oil, emulsified layer and aqueous phase observed from top to bottom, respectively [24]. The emulsifying activity according to the Eq. 3 was 46.2%. This observation was similar to results obtained from sugar beet pulp (43–47%) and sour orange peel pectin (45%) by Yapo et al. [43] and Hosseini et al. [18], respectively. Also,

Table 3 Emulsifying features of oil/0.5% (w/v) pectin solutions

Storage time	Emulsion activity (%)	Emulsion stability (%)			
		1 day		30 day	
Temperature (°C)	23	4	23	4	23
Pectin ^a	46.2	93.9	93.7	93.5	93.1

^aCitron peel pectin extracted under optimal extraction conditions (temperature of 90 °C, extraction time of 180 min and LSR of 40 w/w)

the results illustrated that the emulsions were 93.9 and 93.5% stable at 4 °C, 93.7 and 93.1 at 23 °C after 1 and 30 days, respectively. These data express that the emulsions are more stable at low temperature (4 °C) that is similar to results published by Yapo et al. [43] and Cui et al. [8] for sugar beet pulp and pumpkin pectin, respectively.

Fig. 2 The apparent viscosity of different concentrations of pectin solutions versus shear rate

Fig. 3 Fourier transform infrared spectrum of extracted pectin under optimal extraction conditions

Measuring pectin viscosity

In this study, four solutions with various concentrations (0.1, 0.5, 1.0, and 2.0, % w/v) of citron peel pectin obtained under optimal extraction conditions (time: 90 min, temperature: 95 °C and pH: 1.5) were prepared to investigate the viscosity and flow behavior of pectin at room temperature (~ 25 °C). According to Fig. 2, the viscosity of pectin solutions was increased with increasing concentration. As can be seen, at low concentrations ($< 1.0\%$ w/v) of pectin solutions, all solutions exhibited Newtonian flow behavior. However, the pectin solution with high concentration (2.0% w/v) had a shear-thinning (pseudoplastic) behavior. These findings are in agreement with the results reported on *Abelmoschus esculentus* and sour orange peel pectin by Chen et al. [7] and Hosseini et al. [18], respectively. Pseudoplastic flow behavior in high concentrations is explained to arise from disentanglement of the polymer network and the partial chain orientation in the direction of the shear flow with a relative increase in shear rate [22].

FTIR spectroscopy

The FTIR spectrum of the pectin obtained under optimum extraction conditions (extraction time of 90 min, temperature of 95 °C and pH of 1.5) is depicted in Fig. 3. The peak between 3200 and 3600 cm^{-1} is referred to OH groups. The peak at around 2940 cm^{-1} is related to C–H of CH, CH₂ and CH₃ groups [7]. Besides, the peak about

1747 cm^{-1} is attributed to the vibrating of CO group of OCH₃. Carboxylate groups have two peaks: one peak is due to asymmetrical vibrating at 1631.05 cm^{-1} , and another peak is referred to weaker symmetric vibrating at 1444.06 cm^{-1} . The two intense absorption at 1015.09 and 1103.45 cm^{-1} are related to glycosidic linkage between sugar units [14]. Commonly, the total peak area between 800 and 1200 cm^{-1} is represented “Finger print” region which is exclusive and its interpretation also is difficult [21]. Based on the above statements, it can be express that the achieved precipitate is rich in polygalacturonic acid [38].

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