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A Box-Behnken experimental design for microwave assisted extraction optimization of pectin from citron peel

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ABSTRACT

Microwave assisted extraction technique was employed to extract pectin from citron peel. Box-Behnken design was applied to investigate the influence of irradiation time, microwave power and pH on the yield and DE of pectin. The finding indicated that the optimal conditions for the maximum yield of pectin (30.71%) were achieved at irradiation time of 3 min, microwave power of 700 W and pH of 1.5. DE of pectin ranged from 45.08 to 72.19% demonstrating that extracted pectin was classified as high methoxyl pectin. The emulsifier activity of extracted pectin under optimal conditions was 40.7%. Besides, the emulsions were 86.7 and 86.6% stable at 4°C and, 79.5 and 79.2 at 23°C after 1 and 30 days, respectively. The flow behavior of pectin solutions indicated that solutions in low (0.1, 0.5 and 1.0 %w/v) and high (2.0 %w/v) concentrations had Newtonian and pseudoplastic behaviors, respectively.

Keywords: Pectin, CPP, Optimization, Box-Behnken

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1. Introduction

Citron (*Citrus medica* L.) is a member of the family *Rutaceae* with features similar to lemon (Gabriele et al., 2009; Kim et al., 2013). Citron is cultivated in the warmth and moderate-warmth countries such as south of France, southern Italy (Calabria), Greece, North Africa, Puerto Rico, China, Vietnam and Japan (Essein et al., 2008). The cultivars of citron are classified into two categories: Sweet and acidic citron (Nicolosi et al., 2005). This fruit is mainly employed for the production of candies, flavouring of liquors and medical purpose (Verzera et al., 2005). Its peel is a proper source for the extraction of bioactive compounds and polysaccharides such as pectin (Lato et al., 1999; Menichini et al., 2011).

Pectin is a family of complex variables polysaccharides that is extensively distributed in the primary cell wall and middle lamella of all plant tissue (Ridley et al., 2001; Thakur et al., 1997). Three sequences are recognized in all pectins; homogalacturonan (HG), rhamnogalacturonans I (RG-I) and rhamnogalacturonans II (RG-II) (Morris et al., 2010). The homogalacturonan is formed from $\alpha(1 \rightarrow 4)$ galacturonic acid residues that is esterified partially with methyl groups (Mohnen, 2008). The presence of methoxyl groups in pectin characterize the pectin's degree of methoxylation (DE) which include high methyl ester pectin (DE > 50%) and low methyl ester (DE < 50%) (Joye & Luzio, 2000; Matia-Merino et al., 2004; Migliori et al., 2010). Pectin as gelling, emulsifiers and thickening agents is applied in the food and cosmetics industries (Saenza et al., 2004). Pectin is also demonstrates to possess diverse various pharmaceutical activities such as healing wound (Hokputsa et al., 2004), inhibiting lipase activity (Edashige et al., 2008; kumar & Chuhan, 2010), apoptosis induction of human cancer cell (Espinal-Ruiz et al., 2016), immunostimulating (Inngjerdingen et al., 2007), antimetastasis (Jackson et al., 2007; Nangia-Makker et al., 2002) and lowering cholesterol (Santos et al., 2013).

Generally, commercial pectins are obtained from citrus peels, sugar-beet pulp and apple pomace (Kurita et al., 2008; Mesbahi et al., 2005). Nowadays, pectin have been achieved from various non-traditional plant tissues, for example cacao pod husks (Vriesmann & Petkowicz, 2013), *Solanum lycocarpum* (Torralbo et al., 2012), pomegranate peel (Mooerthy et al., 2014), linseed (Diaz-Rojas et al., 2004), Carrot pomace (Jafari et al., 2016), melon peel (Raji et al., 2017), Peach pomace (Pagan et al., 2001) and sun flower (Shi et al., 1996).

Over the diverse studies have been investigated quality and efficiency of various extraction techniques such as microwave-assisted extraction (MAE), super critical fluid extraction (SFE), pressurized solvent extraction (PSE), hot water and hot diluted mineral acids. Among the diverse extraction techniques, MAE has been adopted as a potential and powerful alternative to conventional extraction, in order to its particular heating mechanism, average consumption cost and its professional

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performance under atmospheric conditions (Spar Eskilsson & Bjorklund, 2000), shorter time, less solvent, high extraction rate and better production with moderate cost (Maran et al., 2013).

Subsequently, the optimization of extraction process is essential to reach maximum production. The response surface methodology (RSM) is an effective statistical technique for optimizing complex process (Lee et al., 2006). The behavior of independent factors was predicted by this methodology through fitting a polynomial equation to the experimental data (Bezerra et al., 2008). Also, RSM decreases the number of experimental trials need to investigate multiple parameters and their interactions. These characteristics successfully have been indicated that it can employ in variables optimization (Jackson et al., 1996; Mastrocola et al., 1997).

Accordingly, the purpose of this study is to investigate the effect of variables of MAE method including microwave power, irradiation time and pH on the extraction yield and DE of CPP (citron peel pectin) and also, the optimization of these factors to achieve maximum extraction yield.

2. Material and Methods

2.1. Materials

Dried citron peel was procured from local store in Mashhad, Khorasan razavi, Iran. The dried peel were powdered and passed through a 40-mesh sieve. The powder was stored in impervious bags to experimental analysis. Citric acids, sodium hydroxide, hydrochloric acid, phenolphthalein reagent, sulfuric acid, sodium tetraborate and sodium azide were purchase from Merck chemical Co.

2.2. Microwave assisted extraction of pectin and its recovery

Extraction of pectin was carried out according to the procedures explained by Li et al. (2012) and Wang et al. (2007). The dried citron peel powder (Liquid-Solid ratio (LSR) of 20 v/w. Fig.1) was soaked into citric acid solution adjusted to the desired pH values (1.5, 2 and 3) and were exposed to three microwave power (300, 500 and 700 w) and irradiation time (1, 2 and 3 min). In the next step, the solution was allowed to cool down to room temperature, centrifuged (10000 g, 20 min) and then, the supernatant was precipitated with an equal volume of ethyl alcohol (96%). Also, three times the coagulated pectin mass was washed with ethanol (95%) to remove the mono and disaccharides (Minkov et al., 1996). Eventually, the wet pectin was dried at 50°C in the hot air oven until a constant weight was achieved. The pectin yield (PY) was calculated by following equation:

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

2.3. Specification of pectin

2.3.1. Degree of esterification of CPP

The degree of esterification was specified according to the methods described by Pinheiro et al. (2008), Santos et al. (2013) and Wai et al. (2010). 20 mg pectin was poured in 3 ml of ethanol and dissolved in 20 ml of distilled water. Then, five drops of phenolphthalein reagent were added to sample and titrated with sodium hydroxide until the appearance of pink color (V_1). Afterwards, 10 ml of 0.1 N sodium hydroxide was added and stirred 15 min for hydrolysis. Then, 10 ml of 0.1 N hydrochloric acid was added to solution and stirred until disappearance of pink color. Finally, the sample was again titrated with 0.1 N sodium hydroxide until the appearance of pink color (V_2). The DE was determined by the following equation:

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

2.3.2. Emulsifying characteristics of CPP

Emulsifying activity (EA) and emulsion stability (ES) were investigated using the procedure described by Dalev and Simeonova (1995) with a slight modification. Briefly, oil-in-water (O/W) emulsions were provided by adding 5 ml sunflower oil to 5 ml of pectin solution (0.5% w/v) including 0.02% sodium azide as a bacteriocide. Mixture were homogenized by ultra-turax T-25 homogeniser (IKAT 25 Digital Ultra-Turax, Staufen, Germany) at 10000 g for 4 min. In the end, the emulsions were centrifuged for 5 min at 4000 g. The EA value was obtained by following equation:

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

The emulsions for 1 and 30 days were stored at 4 and 23°C to investigate the emulsion stability. The ES value was achieved by following equation:

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

2.3.3. Viscosity measurement of CPP

In this test, the flow behavior of pectin solutions in diverse concentrations (0.1, 0.5, 1.0, and 2.0% w/v) were characterized by a rotational programmable viscometer (LVDV--II Pro, Brookfield Engineering Inc., USA) employing a LV spindle at room temperature (~25°C). The samples were poured into the cylinder of the rotational viscometer and shear rate was programmed from 10 to 140 s^{-1} at an interval of 5 s.

2.3.4. Viscosity measurement of CPP

FT-IR spectrum of sample was registered by Perkin Elmer FTIR spectrometer (Perkin Elmer Co., MA, USA). FT-IR spectrum was collected at the absorbance mode in the frequency range of 4000 to 400 cm^{-1} employing KBr disk at 4 cm^{-1} resolution.

2.4. Experimental design

One factor at the time (one factor is variable at the time and other factor are constant (irradiation time of 2 min, microwave power of 500 w, pH of 2.25)) was used to obtained the most suitable LSR for the extraction of pectin. In the next step, A Box-Behnken design was employed to optimize the influence of extraction conditions. The levels of independent variables are present in Table 1. The all calculations and graphics were accomplished using the statistical software Design Expert 8.0 and Excel.

Table 1. Levels of independent variables employed in the Box-Behnken design.

Factors	Coded symbols	Levels		
		-1	0	1
Irradiation time (min)	X_1	1	2	3
Microwave power (W)	X_2	300	500	700
pH	X_3	1.5	2.25	3

3. Results and Discussion

3.1. Influence of various LSR on extraction yield of CPP

LSR is an exceedingly influential parameter on the extraction yield of pectin. In this study, the influence of various LSR (10:1, 15:1, 20:1, 25:1, 30:1, 35:1 and 40:1 v/w) was investigated on the extraction yield of pectin using one factor at the time design. Other parameters (irradiation time of 2 min, microwave power of 500 W, pH of 2.25) were constant. As illustrated in Fig. 1, the extraction yield of pectin augmented remarkably from 7.2% to 11.4% with an enhance in LSR from 10:1 to 20:1 (v/w), which is probably due to an increase in the contact surface area between solvent and particles by an increase in solvent volume. On the other hand, with further increase in LSR, the yield of the CPP was decreased, which may be duo to reduction of microwave absorption by solid particles and thereby reduction of mass transfer rate of pectin (Li et al., 2012; Maran et al., 2013). Therefore, in this study, LSR 1:20 v/w was used for the all experiments.

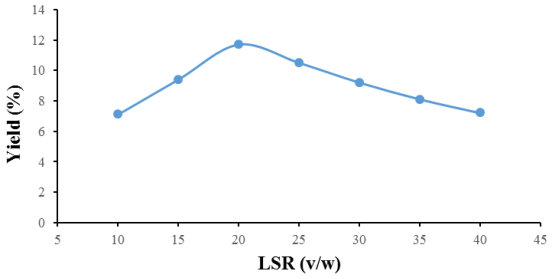

Fig. 1. Influences of various LSR on extraction yield of pectin with irradiation time of 2 min, microwave power of 500 W and pH of 2.25.

3.2. Optimization of extraction factors and validation of optimized conditions

The aim of optimization was to evaluate the MAE parameters which give the maximum extraction yield of pectin. Table 2 reveals that the extraction yield of pectin was between 8.12 and 28.81. The optimum extraction conditions which calculated by solving Eq. (5), were found to be: irradiation time of 3 min, microwave power of 700 W and pH of 1.5, and the maximum extraction yield of pectin in these conditions was 30.71% with a desirability value of 0.946. Triplicate experiments were carried out under the optimum conditions and the mean values (28.82 ± 0.26%) achieved from real experiments, indicated the validation of the optimized conditions.

3.3. Data fitting and ANOVA analysis

An empirical second order polynomial model was applied to understand optimum conditions which can express interactive correlation between process variables and the responses related to extraction yield and DE of CPP (Maran et al., 2014). Final models are given below:

$$\begin{aligned} \text{PY}\ (\%) = {} & 55.63 + 2.03X_1 + 0.01809X_2 - 36.64X_3 \\ & + 0.745X_1^2 + 0.000027X_2^2 + 7.657X_3^2 \\ & - 0.003300X_1X_2 - 1.040X_1X_3 \\ & - 0.01345X_2X_3 \end{aligned} \quad (5)$$

$$\begin{aligned} \text{DE}\ (\%) = {} & -86.94 + 25.82X_1 + 0.1896X_2 + 64.83X_3 - 6.530X_1^2 \\ & - 0.000185X_2^2 - 8.67X_3^2 + 0.00600X_1X_2 \\ & - 1.997X_1X_3 \\ & - 0.01573X_2X_3 \end{aligned} \quad (6)$$

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

Pareto analysis of variance (ANOVA) was employed to analyze the experimental data, for extraction yield and DE of CPP (Table 3). The high models *F*-value for pectin yield and DE (545.83 and 113.97, respectively) and very low models *P*-value ($p < 0.001$) indicated that the regression model was exceedingly meaningful (Maran & Manikandan, 2012). Determination co-efficient (R^2), adjusted determination co-efficient (adj-R^2), predicted determination co-efficient (pred-R^2) and co-efficient of variance (CV) confirm the goodness of fit of the model (Table 3). The high R^2 values for extraction yield and DE of CPP (99.90% and 99.51%, respectively) indicated that the models are significant (Maran et al., 2013). The values of pred-R^2 (99.38% and 92.83%. respectively) and adj-R^2 (99.72% and 98.64%, respectively) demonstrated that the form of chosen to describe the relationship between variables and the responses is well-correlated (Maran et al., 2013). Lower CV% values (2.47 and 2.02, respectively) intelligibly explained that, the deviations between observed and predicted values are low and also indicated a high degree of precision and reliability of experiments (Maran & Priya, 2014).

Table 2. Box-Behnken design with measured and predicted values for extraction yield (PY) and degree of esterification (DE) of CPP.

Run	Independent variables			Measured responses		Predicted responses	
	X_1	X_2	X_3	PY	DE	PY	DE
1	1	300	2.25	10.21	62.44	10.16	61.29
2	3	300	2.25	13.67	55.80	13.52	55.32
3	1	700	2.25	14.50	51.04	14.64	51.51
4	3	700	2.25	15.32	49.20	15.36	50.34
5	1	500	1.50	23.26	46.80	23.10	46.94
6	3	500	1.50	26.77	46.89	26.70	46.36
7	1	500	3.00	8.12	70.37	8.18	70.89
8	3	500	3.00	8.51	64.47	8.66	64.32
9	2	300	1.50	21.42	46.13	21.62	47.12
10	2	700	1.50	28.81	45.08	28.82	44.46
11	2	300	3.00	9.20	72.19	9.18	72.80
12	2	700	3.00	8.52	61.70	8.31	60.70
13	2	500	2.25	12.08	68.50	11.61	68.53
14	2	500	2.25	11.66	68.00	11.61	68.53
15	2	500	2.25	11.10	69.11	11.61	68.53

Table 3. Analysis of variance (ANOVA) for regression model of CPP yield and DE.

Source	Sum of squares	DF	Mean square	*F*-Value	*p*-Value
(A) Yield					
Regression	662.832	9	73.648	545.83	0.000
Linear	571.383	3	190.461	1411.57	0.000
Square	70.992	3	23.664	175.38	0.000
Interaction	20.457	3	6.819	50.54	0.000
Residual error	0.675	5	0.135		
Lack-of-fit	0.191	3	0.064	0.26	0.849
Pure error	0.483	2	0.242		
Total	663.507	14			
CV%			2.47		
R^2			0.9990		
Adj R^2			0.9972		
Pred R^2			0.9938		
(B) DE					
Regression	1439.75	9	159.972	113.97	0.000
Linear	1013.04	3	337.679	240.57	0.000
Square	389.70	3	129.900	92.54	0.000
Interaction	37.01	3	12.336	8.79	0.019
Residual error	7.02	5	1.404		
Lack-of-fit	6.40	3	2.133	6.90	0.129
Pure error	0.62	2	0.309		
Total	1446.76	14			
CV%			2.02		
R^2			0.9951		
Adj R^2			0.9864		
Pred R^2			0.928		

3.4. Influence of process variables on the extraction yield of CPP

Fig. 2(a) demonstrates the perturbation plot for extraction yield of CPP. This graph could be employed to realize variables that have greatest influence on the response (Chaharbaghi et al., 2017). A slope or a relatively flat line for a variable indicates that the response is sensitive or insensitive to it variable, respectively (Maran et al., 2014). Fig. 2(a) expressed that irradiation time has the highest influence on yield of extraction.

3.4.1. Influence of microwave power

The recovery of pectin was increased by enhancing microwave from 300 to 700 W (Fig. 3a and c). This observation agreed with Maran et al. (2013) and Maran et al. (2015). The accelerated extraction of pectin by raising microwave power is attributed to influence of microwave on the plant tissue. Electromagnetic radiation increased the softening of cell wall tissue and this will lead to enhanced interaction between extracting agent and plant

tissue in extraction process (Kratchanov et al., 2004). Microwave energy affected on biomolecules through ionic conduction and dipole rotations which lead to molecular movement and creation of heat through intermolecular friction and eventually, increasig the extraction yield (Gfrerer & Lankmayr, 2005).

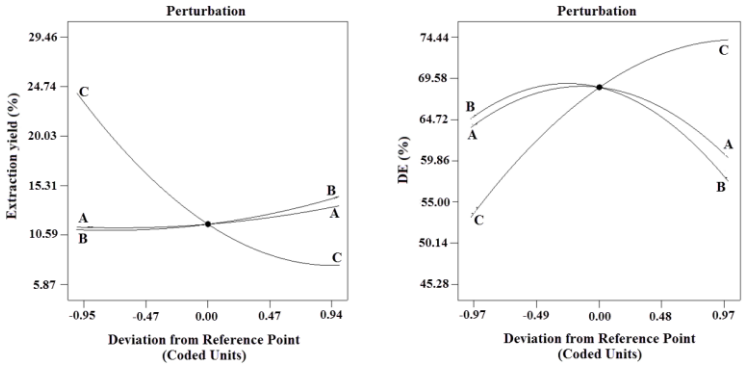

Fig. 2. Perturbation plot for extraction yield and DE of CPP (A= Irradiation time , B= Microwave power and C=pH).

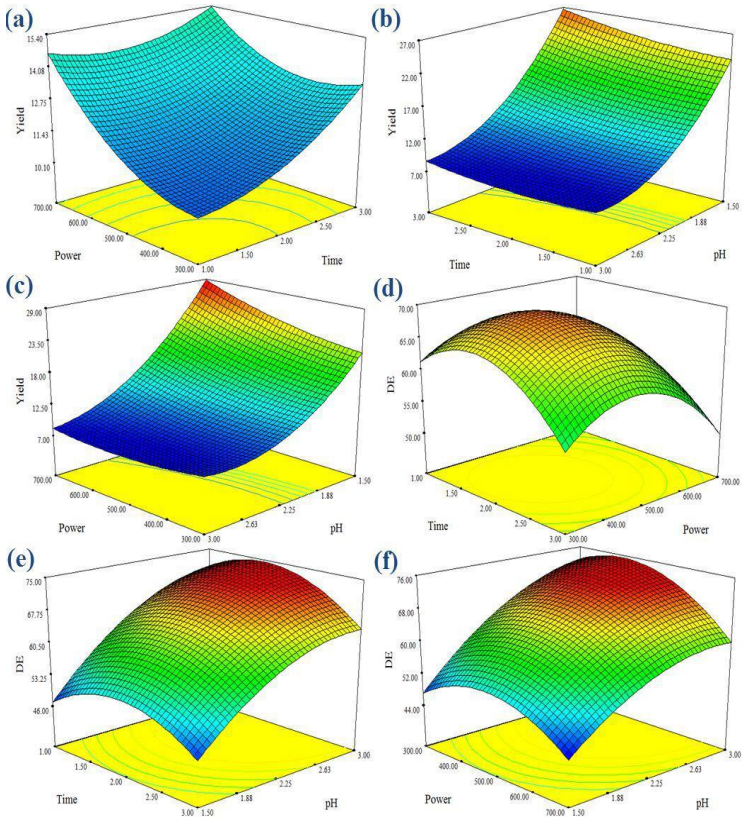

Fig. 3. 3-D response surface plots indicate the influence of irradiation time (min), power microwave (W) and pH on the extraction yield (%) and DE (%).

3.4.2. Influence of irradiation time

Irradiation time is a very effective factor on the pectin extraction efficiency and it is essential to determine appropriate irradiation time to discover the maximum extraction of pectin by MAE. Therefore, various times (1, 2, 3 min) were evaluated to select an appropriate time to increase the extraction yield of pectin. The results demonstrate the extraction yield was increased steadily and achieved the maximum at 3 min (Fig. 3(a, b)). This phenomenon is due to the absorption of microwave energy by the extraction solution and thermal accumulation in the solution, which causes dissolution of pectin into the solution. It should also be noted that the extreme time leads to disjoint the pectin and reduced extraction yield (Xianzhe et al., 2011). Similar results with this study were obtained by Chen et al. (2007) and Xianzhe et al. (2011).

3.4.3. Influence of pH

Observations show that pectin yield was increasing obviously as the decreasing pH values (Fig. 3(b, c)). The higher acidic extraction solvent lead to hydrolysis of the insoluble pectin into its soluble type and thereby increasing the pectin yield (El-Nawawi & Shehata, 1988). The low pH also diminished the molecular weight of pectin and leading to its release from plant tissue without any degradation (Fravash & Ashtiani, 2007).

3.5. *Influence of variables on the DE*

Table 2, reveals DE values was about 45.08 to 72.19% that similar range was reported by Happi et al. (2012). The DE of pectin obtained under optimum conditions (irradiation time of 3 min, microwave power of 700 W and pH of 1.5) was 63.51%. As shown in Fig. 2(b), pH has the highest effect on DE of extracted pectin. Fig. 3 demonstrated that DE of extracted pectin reduced with an enhancement in microwave power and irradiation time and decrease in pH. This phenomenon could duo to the use of harsh conditions (high irradiation time and microwave power, and also low pH), which these conditions could reduce the DE due to the increasing de-esterification of polygalacturonic chains (De Roeck et al., 2008; Jafari et al., 2017).

3.6. *Emulsifying attributes*

The emulsifying attributes (emulsifying activity and emulsion stability) of pectin extracted under optimal conditions (irradiation time of 3 min, microwave power of 700 W and pH of 1.5) were examined (Table 4). Furthermore, stability of emulsions was tested during preservation for 1 and 30 days at temperatures of 4 and 23°C. After centrifuging the emulsions revealed oil, emulsified layer and aqueous phase from up to down, respectively (Ma et al., 2003). Based on Eq. 3, the emulsifying activity was 40.7%. This evidence was conformed to achieved results from sugar beet pulp (43-47%) and sour orange peel pectin (45%) by Yapo et al. (2007) and Hosseini et al. (2016), respectively. On the other hand, Table 4 showed that emulsions are extremely stable at low temperature (4°C) that this result was also reported by Yapo et al. (2007) and Cui and Chang (2014) for sugar beet pulp and pumpkin pectin, respectively.

Table 4. Emulsifying attributes of oil/0.5% (w/v) pectin solutions: emulsifying activity (EA, %); emulsion stability (ES, %).

	EA	ES			
Storage time (day)		1		30	
Temperature (°C)	23	4	23	4	23
Pectin*	40.7	86.7	79.5	86.6	79.2

* Pectin obtained under optimal extraction conditions.

3.7. *Investigating the flow behavior of CPP solutions*

For this purpose, four solutions with diverse concentrations (0.1, 0.5, 1.0 and 2.0, %w/v) of extracted pectin under optimal conditions (irradiation time of 3 min, microwave power of 700 W and pH of 1.5) were prepared to measure the viscosity and flow behavior of pectin at room temperature. In accordance with Fig. 4, the viscosity of pectin solutions was enhanced with increasing concentration. As can be seen, at low concentrations (< 1.0 %w/v) of pectin solutions, all solutions represented Newtonian flow behavior. However, the concentrated solution of pectin (2.0 %w/v) was revealed shear-thining (pseudoplastic) behavior. These achievements are in agreement with findings published about *Abelmoschus esclentus* and sour orange peel pectin by Chen et al. (2014) and Hosseini et al. (2016), respectively. Behavior of concentrated pectin solution is probably duo 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 (Kumbar et al., 2017).

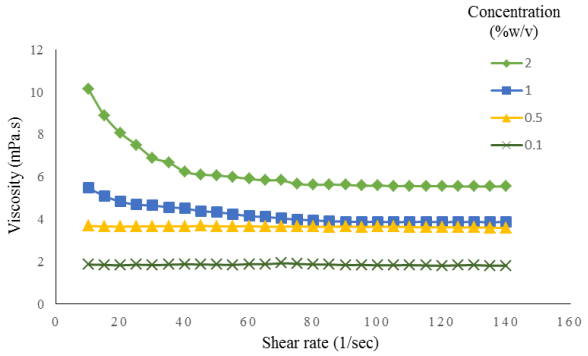

Fig. 4. The apparent viscosity of various concentrations of pectin solutions versus shear rate.

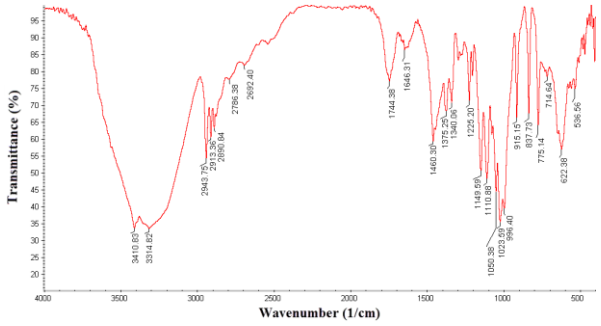

Fig. 5. FTIR spectrum of CPP under optimal extraction conditions.

3.8. *FTIR spectroscopy*

The FTIR spectrum of the extracted pectin under optimal conditions (irradiation time of 3 min, microwave power of 700 W and pH of 1.5) is exhibited in Fig. 5. The peak between 3000 and 3600 cm^{-1} is assigned to OH groups. The peak at around 2943.75 cm^{-1} is dedicated to C-H of CH, CH_2 and CH_3 groups (Chen et al., 2014). Moreover, the peak about 1744.38 cm^{-1} is related to the vibrating of CO group of OCH_3. Carboxylate groups have two peaks: one peak is attributed to asymmetrical vibrating at 1646.31 cm^{-1}, and another peak is due to weaker symmetric vibrating at 1460.30 cm^{-1}. The two absorptions at 1110.88 and 1149.59 cm^{-1} are referred to glycosidic linkages between sugar units (Gnanasambandham & Proctor, 2000). Overall, the peaks between 800 to 1200 cm^{-1} are due to "Finger print" region which is particular and thereby, its explanation also is laborious (Kozarski et al., 2011). According to the above explanation, it can be express that the achieved precipitate is rich in polygalacturonic acid (Santos et al., 2013).

4. Conclusion

Abstract Microwave assisted extraction technique was applied to obtain pectin from citron peel. Box-Behnken design was used to investigate the effect of irradiation time (min), microwave power (W) and pH on the yield and DE of pectin. The results showed that the maximum pectin yield (30.71%) was obtained at irradiation time of 3 min, microwave power of 700 W and pH of 1.5. DE of pectin ranged from 45.08 to 72.19% and indicated that the CPP could be classified as high methoxyl pectin. The emulsifier activity of extracted pectin under optimal conditions was 40.7%. Besides, the emulsions had suitable stability in both storage temperatures (4 and 23°C). Also, the results of viscosity measurement indicated that pectin solutions had different behavior in different concentrations (Newtonian and pseudoplastic behaviors in low and high concentrations, respectively).

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