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Extraction and Analysis of Pectin from Citrus Peels: Augmenting the Yield from *Citrus limon* Using Statistical Experimental Design

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Abstract: Pectin is a structural hetero polysaccharide, commonly obtained from the peels of citrus fruits and finds prime commercial use as a gelling agent and stabilizer in food industries. In the present study, pectin was extracted using alcohol precipitation method from the peels of orange (*Citrus sinensis*), sweet lime (*Citrus limetta*) and lemon (*Citrus limon*). When the extraction conditions were varied one-at-a-time, a maximum yield of 36.71% was obtained from *C. limon*, after which the yield was further enhanced using the Box-Behnken Design of Response Surface Methodology. Optimum conditions for the extraction process were established to be pH 3.5, temperature 65°C and time 67.5 min. The interaction effects of these variables were studied using 3-D and contour plots. A 1.5-fold increase in pectin yield was obtained as a result of this experimental design. Analysis of variance indicated the significance of the model. The pectin obtained was then subjected to qualitative and quantitative analyses and found to contain desirable methoxyl, hyaluronic acid contents and degree of esterification. Functional groups present in the pectin were investigated using FTIR spectroscopy. The overall results point towards the amenability of the extracted pectin for industrial applications.

Key words: Pectin · *Citrus limon* · Yield · Optimization · Response surface methodology · FTIR.

INTRODUCTION

Pectin, a complex mixture of polysaccharides occurring in the primary cell walls of terrestrial plants, is a high value functional food ingredient. It consists of a linear backbone of α-(1-4)-D-galacturonic acid residues partially esterified with methanol, with periodic interruptions to L-rhamnose residues that make the backbone irregular and with some other neutral sugars present as side chains. The general makeup of the pectin content varies with the ripening of the fruit [1].

Pectin is produced commercially in the form of white to light brown powder, mainly extracted from citrus fruits and is used in food as a gelling agent particularly in jams and jellies. It is also used in fillings, sweets, as a stabilizer in fruit juices and milk drinks and as a source of dietary fiber [2]. Several studies have reported novel pectin usages, like biodegradable water-soluble films, bulking agents, coating agents, chelators, emulsifiers and viscosity modifiers.

The amount, structure and chemical composition of the pectin differs between plants, within a plant over time and in different parts of a single plant [3]. Although pectin occurs commonly in most of the plant tissues, the number of sources that may be used for commercial manufacture of pectin is limited. This is because the ability of pectin to form a gel depends on molecular size and the degree of esterification (DE).

At present, commercial pectins are almost exclusively derived from citrus peel or apple pomace, both of which are by-products of juice manufacturing units. Apple pomace contains 10-15% of pectin on a dry matter basis. Citrus peel contains relatively higher, i.e. 20-30% of pectin as compared to that of apples [4]. Among the physical properties, citrus pectins are light cream or light tan in color, whereas apple pectins are often darker.

Commercially, pectin is extracted by treating the raw material with hot dilute mineral acid at pH 2, for 2-4 h duration and pectic substances are precipitated using ethanol or isopropyl alcohol [5]. The precipitated pectin

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is separated and washed with alcohol to remove impurities. It is dried, ground to a powder and blended with other additives, if necessary. The extracted pectin can be categorized into two major types depending on its degree of esterification (DE): high-methoxyl pectin (HMP, > 50% DE) and low-methoxyl pectin (LMP, < 50% DE).

The solubility and viscosity of pectin solution are related to the molecular weight, degree of esterification, concentration of the preparation, pH and presence of counter ions in the solution [6]. Viscosity, solubility and gelation are generally related to physical properties of the product. For example, factors that increase gel strength will increase the tendency to gel, decrease solubility and increase viscosity and vice versa. These properties of pectins are a function of their structure.

The present investigation aims to extract pectin from the peels of citrus fruits namely, *Citrus sinensis (*orange), *Citrus limon* (lemon) and *Citrus limetta* (sweet lime) using citric acid; to optimize the yield of pectin by varying one-factor-at-a-time (OFAT) and response surface methodology (RSM); and to characterize the extracted pectin by both qualitative and quantitative methods, thereby gauging its appropriateness for industrial usage.

MATERIALS AND METHODS

Chemicals: All reagents and chemicals used were of analytical grade. For the extraction process, citric acid was purchased from HiMedia, India and ethyl alcohol from SD Fine Chemicals, India.

Sample Preparation: Lemon, sweet lime and orange were purchased from the local market. They were split into four parts and the peels were removed, which were then cut into smaller pieces, shade dried, ground to a consistency intermediate to coarse and fine (for avoiding clumping during solvent extraction) and stored at ambient temperature for further use.

Pectin Extraction from Citrus Peels

Effects of pH and Temperature on the Extraction Process: The effects of these factors on the yield of pectin from different citrus peels were studied by varying one-factor-at-a-time, while keeping the other one constant. The optimum conditions giving a good yield from each source were ascertained in this preliminary study.

For the extraction process, a dry mass of 5 g was subjected to extraction by adding 90 mL of distilled water followed by 10 mL of citric acid of different pH values ranging from 1.2 - 4.2. The mixture was then heated at different temperatures of 40 - 90°C and continuously stirred for 1 hour. The hot acid extract was filtered through a Whatman No. 1 filter paper. The filtrate was coagulated using an equal volume of 95% ethanol and left for 2 h to allow the pectin to float on the surface. The gelatinous pectin flocculants were then skimmed off. The extracted pectin was then filtered and washed 2 - 3 times with ethyl alcohol to remove any remaining impurities [7]. Finally, the precipitate was dried at 35 - 40°C in hot air oven and percentage yield was calculated.

$$Ypec(\%) = \frac{P}{Bi} * 100 \quad (1)$$

where, Y_{pec} is the yield of pectin in (%), P is the amount of extracted pectin in g and B_i is the initial amount of fruit peel powder.

Optimization of Pectin Yield

Statistical Design Using Response Surface Methodology: Statistical process optimizations using RSM have been widely employed by a number of researchers [8, 9]. This statistical optimization was limited to the pectin from *C. limon*. Box-Behnken design of RSM was used to investigate the effects of different independent variables - pH, temperature (T) and extraction time (ET) on the response, pectin yield (Y_{pec}). The levels of these variables were selected based on preliminary experiments [10]. The experiments were performed in random order. All analyses were done using the software Design Expert 8.0 (trial version). The experimental design consisted of a set of points lying at the midpoint of each edge and the replicated center point of a multidimensional cube. The polynomial equation generated by the software is as follows:

$$Yi = b0 + b1X1 + b2X2 + b3X3 + b12X1X2 + b13X2X3 + b11X11 + b22X22 + b33X33 \quad (2)$$

where, Y_i is the dependent variable, b_0 is the intercept, b_1 to b_{33} are the regression coefficients and X_1 to X_{33} are the independent variables. The experimental design set up is summarized in Table 1.

Physicochemical Characterization of the Pectin Samples: The dried pectin samples obtained from all three fruit peels were subjected to the following qualitative and quantitative tests in order to characterize them.

Qualitative tests

Color

This was done by visual observation.

Table 1: Experimental design set up of RSM and responses obtained

	Independent Variables			Response
Run no.	pH	*T (°C)	*ET (min)	Y_{pec} (%)
1	3.5 (0)	90 (+1)	15 (-1)	21.45
2	3.5 (0)	65 (0)	67.5 (0)	55.76
3	5.5 (0)	40 (-1)	67.5 (0)	18.73
4	1.5 (-1)	65 (0)	15 (-1)	34.79
5	5.5 (+1)	65 (0)	15 (-1)	9.59
6	3.5 (0)	40 (-1)	15 (-1)	18.98
7	1.5 (-1)	90 (+1)	67.5 (0)	11.62
8	5.5 (+1)	90 (+1)	67.5 (0)	10.65
9	3.5 (0)	65 (0)	67.5 (0)	55.98
10	3.5 (0)	65 (0)	67.5 (0)	56.71
11	3.5 (0)	40 (-1)	120 (+1)	9.82
12	1.5 (-1)	65 (0)	120 (+1)	11.92
13	5.5 (+1)	65 (0)	120 (+1)	8.92
14	1.5 (-1)	40 (-1)	67.5 (0)	23.93
15	3.5 (0)	65 (0)	67.5 (0)	53.88
16	3.5 (0)	65 (0)	67.5 (0)	54.91
17	3.5 (0)	90 (+1)	120 (+1)	22.75

*T – temperature, ET – extraction time. Each one of the variables was studied at 3 different levels: -1, 0 and +1. Extraction was performed using citric acid in all cases

Solubility of Dry Pectin in Cold and Hot Water: A 0.25g of the pectin samples were separately placed in two conical flasks, followed by addition of 10 mL of 95% ethanol and 50 mL of distilled water. The mixture in the second flask was shaken vigorously to form a suspension which was then heated at 85-95°C for 15 min [11].

Solubility of Pectin Solution in Cold and Hot Alkali: To 1 mL of 0.1 N NaOH in two different conical flasks, 5ml of pectin solution was added and the second flask was heated at 85- 90°C for 15 min [12].

Quantitative Tests

Equivalent Weight (Titration A): Pectin sample (0.5 g) was weighed into a 250 mL conical flask and moistened with 5 mL ethanol. A 1.0 g NaCl was added to the mixture followed by 100 mL distilled water and few drops of phenol red indicator. Care was taken to ensure that all the pectin had dissolved and that no clumping occurred. The solution was then slowly titrated with 0.1 M NaOH to an end point of pale permanent pink color [13]. Equivalent weight was calculated using equation (3):

$$Equivalent\ Weight = \frac{(weight\ of\ pectin\ sample * Molarity\ of\ alkali)}{Volume\ of\ alkali} * 100 \quad (3)$$

Methoxyl Content (MeO) (Titration B): This was done using the neutralized solution obtained from equivalent weight determination, by the saponification of pectin followed by titration of the liberated acid. 25 mL of 0.25 M NaOH was added to the neutralized solution and the mixture was stirred thoroughly and allowed to stand for 30 min at ambient temperature. A 25 ml of 0.25N HCl was added and titrated with 0.1N NaOH to the same end point as earlier [14]. The percentage methoxyl content was calculated using equation (4):

$$Methoxyl\ content\% = \frac{Volume\ of\ alkali * weight}{Weight\ of\ pectin\ sample} * 100 \quad (4)$$

Moisture Content: An empty crucible was dried in an oven, cooled in a desiccator and weighed. A 5 g of pectin sample was transferred to it and placed in a hot air oven set at 100°C for 1 h. Thereafter the petri dish was removed, cooled in a desiccator and weighed. This process was repeated once. The moisture content was calculated using equation (5):

$$Moisture\ content\% = \frac{Weight\ of\ the\ Reside}{Weight\ of\ the\ sample} * 100 \quad (5)$$

Anhydrouronic Acid (AUA) Content: The AUA content was calculated using the values of equivalent weight and methoxyl content previously determined, according to equation (6) [13]:

$$AUA\% = \frac{176 * 100}{Z} \quad (6)$$

where, 176 is the molecular weight of AUA and

$$Z = \frac{Weight\ of\ sample\ (mg)}{meq\ of\ Titration\ A + meq\ of\ Titration\ B}$$

Degree of Esterification (DE): The DE of extracted pectin was calculated using equation (7), applying the data from methoxyl and anhydrouronic acid content determinations [15]:

$$DE(\%) = \frac{176 * MeO\% * 100}{31 * AUA\%} \quad (7)$$

Spectral Analysis: Subsequent to the above mentioned tests, the pectin from *C. limon* was further subjected to FTIR analysis (Shimadzu, IRAffinity-1) and the resulting spectrum was studied in order to understand the functional groups present.

RESULTS AND DISCUSSION

Pectin Extraction from Citrus Peels

Effects of pH and Temperature on Extraction Process: When the effects of these factors on pectin yield were

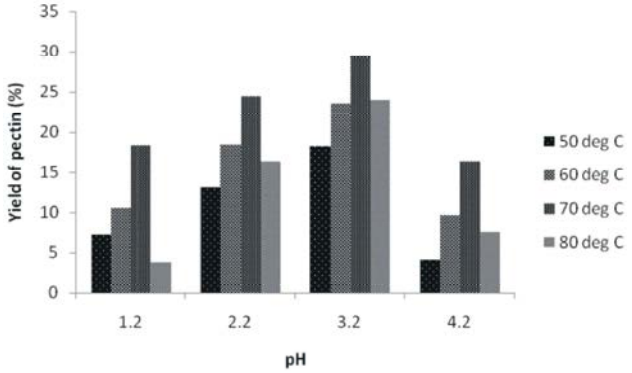

Fig. 1: Yield of pectin from *Citrus sinensis*
*The extractions were performed using citric acid under varying conditions of pH and temperature

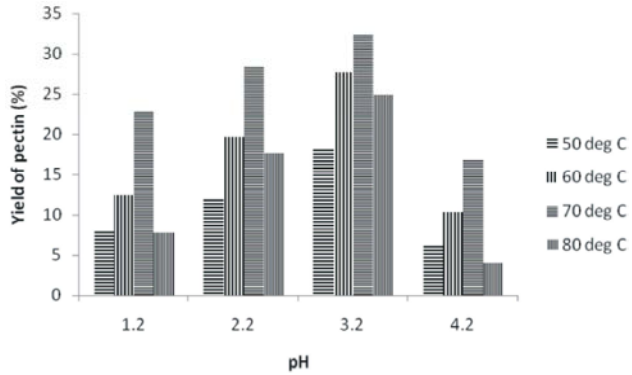

Fig. 2: Yield of pectin from *Citrus limetta*
*The extractions were performed using citric acid under varying conditions of pH and temperature

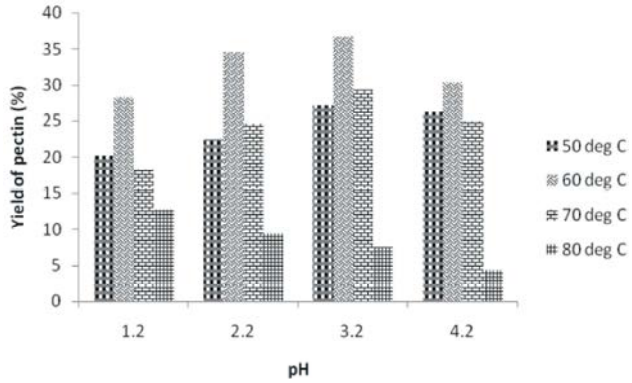

Fig. 3: Yield of pectin from *Citrus limon*
*The extractions were performed using citric acid under varying conditions of pH and temperature

monitored, the maximum yield from *C. sinensis* was found to be 29.41% at pH 3.2 and temperature of 70°C (Fig. 1). A 46.46% yield from orange peel residue after simple distillation of the orange oil has been reported in literature. Therefore, in the process of orange oil and pectin extraction, it has been recommended to first extract oil using simple distillation and then isolate pectin with acid hydrolysis technique [16]. In contrast to this, a very low yield of pectin obtained from dried orange peel using zeocarb as extractant at 85-90°C has also been reported [17].

When pectin present in *C. limetta* peel was extracted by citric acid based method, it showed a maximum yield of 32.42% at pH 3.2 and a temperature of 70°C (Fig. 2). Aina *et al*. have documented that the extraction from *C. limetta* resulted in a yield of 15.92% [18].

The extraction from *C. limon* resulted in a maximum yield of 36.71% at pH 3.2 and a temperature of 60°C (Fig. 3). In other studies, the percentage yield of pectin in wet weight basis from lemon has been observed to be 16.71% at pH 4.1 and a temperature of 60°C [18].

Thus, a pH of 3.2 appears to be optimum for the extraction of pectin from all the citrus peels studied. The optimum temperature for pectin extraction was observed to be 70°C for *C. sinensis* as well as *C. limetta*, except for *C. limon*, in which case, a lower temperature of 60°C was preferred. Further, on comparison of the above mentioned sources of pectin, it could be inferred that *C. limon* provided the highest yield and it was hence chosen for statistical optimization of the extraction process using design of experiments (DoE).

Optimization of Pectin Yield

Statistical Design Using Response Surface Methodology: The variables of pH, temperature (T) and extraction time (ET) were fitted for the Box-Behnken design of RSM. Yield of pectin for each individual run was determined by carrying out the acid based extraction method using citric acid. A maximum yield of 36.71% had been achieved prior to the process optimization. As a result of applying statistical optimization using RSM, a maximum yield of 56.81% was recorded in run 10. The desired conditions: pH, extraction time and extraction temperature were 3.5, 67.5 min and 65°C, respectively (Table 1). This represents a 1.5-fold increase in the yield of pectin. Kliemann *et al*. has obtained a yield of 61.32% at a pH of 1.9, temperature of 40°C and extraction time of 40 min [19].

The results obtained after the experimentation were fed into the Design Expert software, which generated the following regression equation:

$$Y = 55.58 - 4.25*A - 0.62*B - 3.97 * C + 1.06 * AB + 5.65 + AC + 2.61 * BC - 20.70 * A2 - 18.65 * B2 - 18.6 * C2$$

where, A- pH, B-temperature (°C) and C- extraction time (min).

Analysis of variance (ANOVA) indicated that the model F-value is 17.49, which implies that the model is significant. The model suggested for the yield of pectin from *C. limon* was a 'quadratic model'. The R^2 value of 0.9574 validates the accuracy of the model. This value provides a measure of how much variability in the observed response can be explained by the experimental factors and their interactions. It always lies between 0 and 1. The closer that the R^2 value is to 1.0, the stronger the model is and the better it predicts the response. The adjusted R^2 value was found to be a close 0.9027. The statistical analysis also determines which experimental factors generate signals which are large in comparison to the noise. This is measured as 'adequate precision' and a value of 9.791 means a good signal.

Three-dimensional response surface curves were plotted in order to understand the interactions between the variables and the optimum levels of each variable for maximum yield of pectin. The interaction between two variables, viz. pH and temperature is shown in Fig. 4. Significance of interaction between the corresponding variables is indicated by saddle nature of the contour plots. At lower and higher levels of both pH and temperature, decreased yield of pectin was observed. At intermediate concentrations, higher yield was obtained. Fig. 5 represents the interaction between pH and extraction time and its effect on the yield of pectin. In this case too, at intermediate levels of the variables, the yield was maximal. Fig. 6 depicts the interaction between extraction time and temperature. The yield was observed to be minimal at both lower and higher levels, whereas at intermediate levels, maximum yield was observed.

Physicochemical Characterization of the Pectin Samples

Qualitative and Quantitative Tests: The qualitative and quantitative characteristics of pectin are summarized in Table 2. The colour of pectin obtained from the orange peel sample was brown, whereas samples extracted from the other two sources were yellowish in colour. While pectins are usually light in colour, factors such as surface contamination or environmental factors might have contributed to the discrepancy in colour. This could also be due to the amount of alcohol used for precipitation or purification during the experiment not being enough [20]. In cold alkali (NaOH), the pectin suspensions formed a yellow precipitate, which dissolved when heated at 85-90°C for 15 min. Fishman *et al.* have stated that pectins are unstable in alkaline solutions, which agrees with the finding from our research [21].

The equivalent weight was found to be the highest for *C. sinensis* pectin and least for *C. limon* pectin. The methoxyl content of pectin usually varies from 0.2 - 12% depending on the source and mode of extraction. Among pectins from the three different sources studied, the methoxyl content varied from 6.8% (*C. sinensis*) to 2.3% (*C. limon*), the values thus falling within the range. Since all the values obtained experimentally were below 7%, the pectins are of low ester characteristic, indicating that they are desirable in terms of quality [12]. Anhydrouronic acid content of *C. limon* pectin was above 65%, indicating its purity.

Table 2: Qualitative and quantitative tests for pectin

	Source of Pectin		
Parameter	*C. sinensis*	*C. limetta*	*C. limon*
Qualitative tests:			
Color	Brown	Yellow	Yellow
Solubility of dry pectin in cold water	Insoluble, forms suspension	Insoluble	Soluble
Solubility of dry pectin at 85-90°C	Mixture dissolves	Mixture dissolves	Mixture dissolves
Solubility of pectin in cold alkali	Pectin forms a yellow precipitate	Pectin forms a yellow precipitate	Pectin forms a yellow precipitate
Solubility of pectin in hot alkali	Dissolved and turned milky	Dissolves	Dissolves
Quantitative tests:			
Equivalent weight	594.86	386.45	253.70
Methoxyl content (%)	6.840	4.460	2.348
Moisture content (%)	58.72	75.80	82.70
AUA (%)	68.74	42.80	39.48
DE (%)	3.50	2.98	1.50

*The samples were extracted using citric acid under optimum conditions of temperature and pH

Table 3: Functional groups present in *C. limon* pectin

Frequency (cm^{-1})	Bond	Functional group
3595.31 (s, sh)	O-H stretch, H-bonded	Alcohols, phenols
2931.80 (m)	C-H stretch	Alkanes
2862.36 (m)	C-H stretch	Alkanes
2222.00 (w)	C = C stretch	Alkynes
1728.22 (s)	C=O stretch	α,β-unsaturated ester
1319.31 (s)	C-O stretch	Alcohols, carboxylic acid, esters
1242.16 (s)	C-N stretch	Aliphatic amines
1149.57 (m)	C-H wag ($-CH_2X$)	Alkyl halides
1095.57 (m)	C-N stretch	Aliphatic amines
1056.99 (m)	C-N stretch	Aliphatic amines
1026.13 (m)	C-N stretch	Aliphatic amines
804.97 (m)	C-Cl stretch	Alkyl halides
840.98 (m)	C-Cl stretch	Alkyl halides

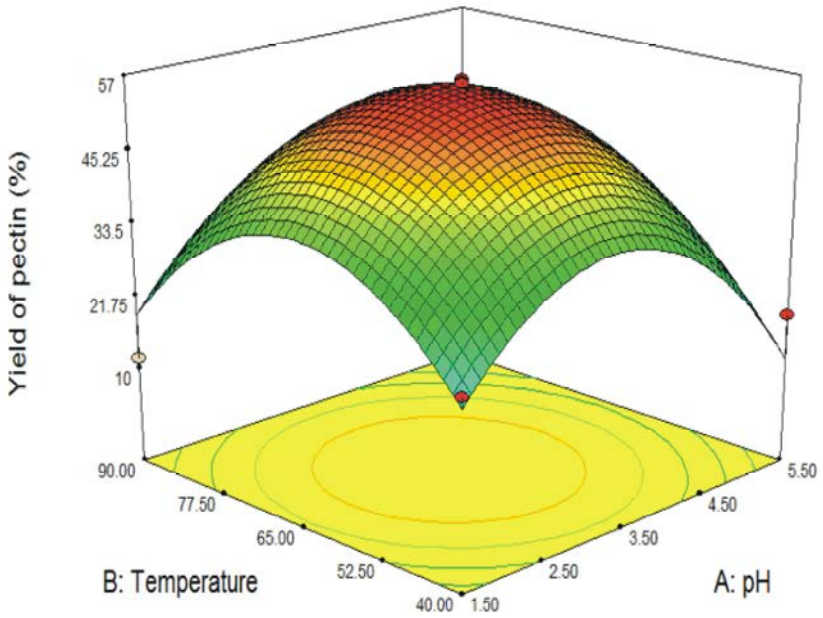

Fig. 4: Response surface curve showing interaction between pH and temperature

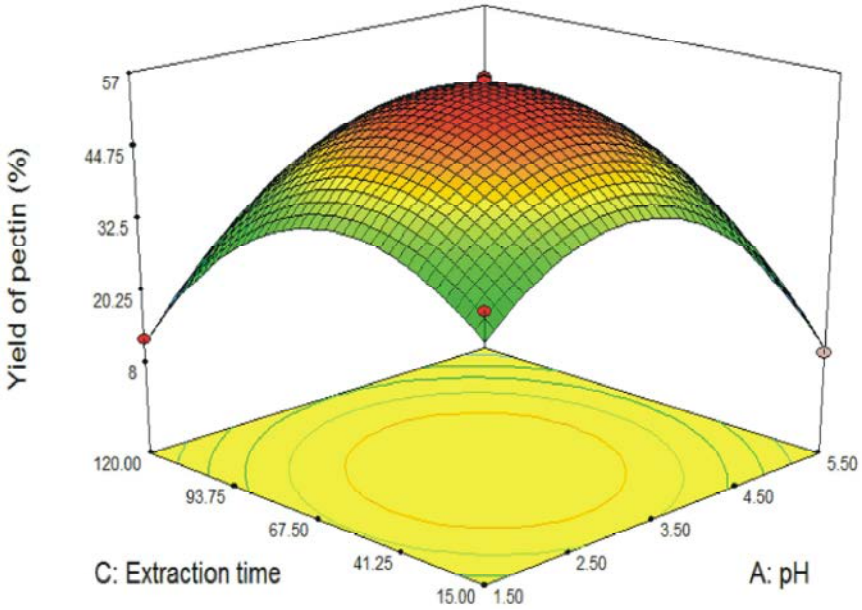

Fig. 5: Response surface curve showing interaction between pH and extraction time

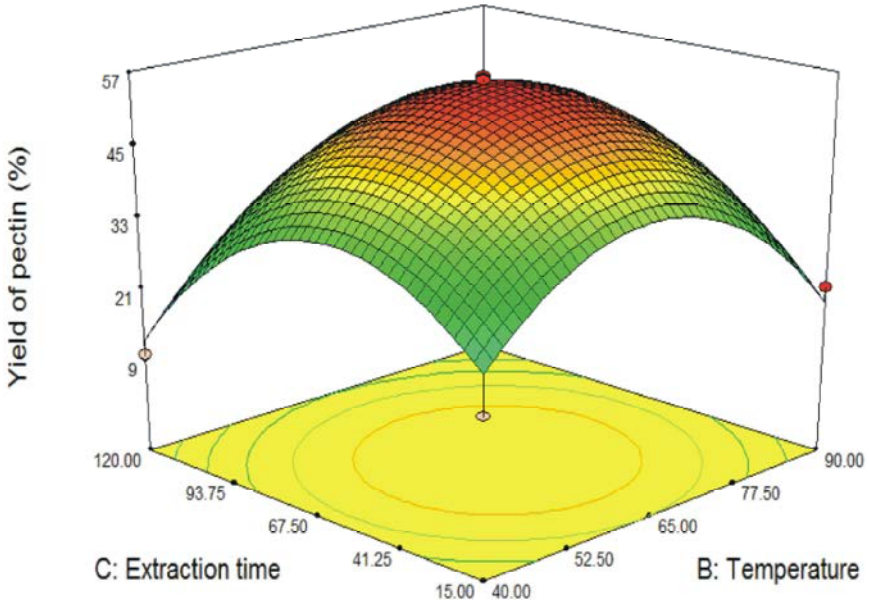

Fig. 6: Response surface curve showing interaction between temperature and extraction time

FTIR Spectral Analysis: The FTIR spectrum of *C. limon* pectin is presented in Fig. 7 and the corresponding functional groups are given in Table 3. From the results it could be inferred that the *C. limon* pectin exhibits sharp and strong peaks at 3595.31 cm^{-1} as O-H stretch, C-H stretch in the frequency 2830-2695 cm^{-1} shown as carbohydrate ring [22] and strong C=O stretch occurring at 1710-1665 cm^{-1}. The strong peak in the range of 1319.31 cm^{-1} suggests the stretching vibration of alcohols, carboxylic acid and esters [23]. Comparable study by Khule *et al*. has showed IR peaks at 4000-600 cm^{-1} for a sample of pectin present as a drug mixture [7]. Moreover, the presence of peaks at 1728.22 cm^{-1} and 1242.16 cm^{-1} indicate the existence of α, β-unsaturated esters and aliphatic amine functional groups.

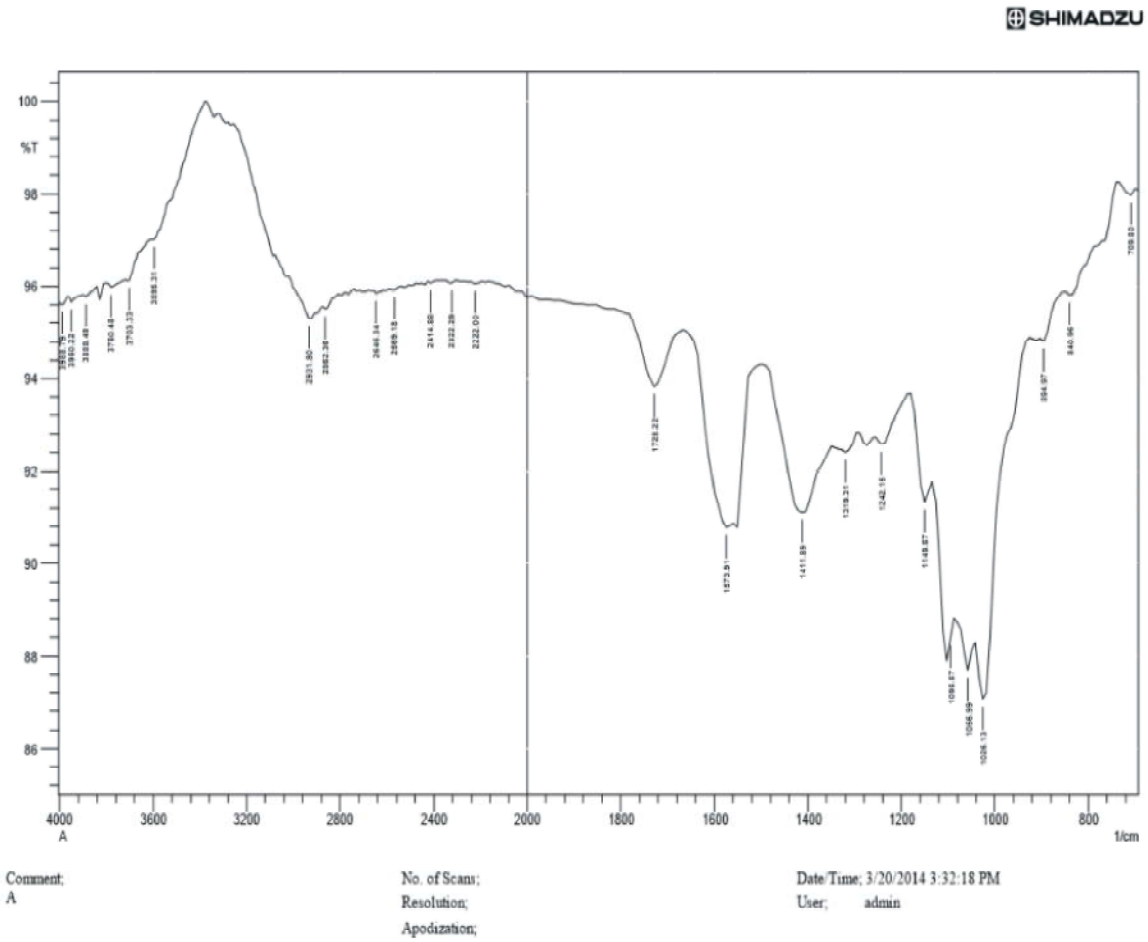

Fig. 7: FTIR spectrum of *C. limon* pectin

CONCLUSION

Our study has facilitated a detailed investigation on pectins from citrus peels, a product of enormous value for food-industry applications. Initially, the maximum yield of pectin was found to be 36.71% from *C. limon* at pH 3.2 and a temperature of 60°C. Process optimization for enhancing the yield of pectin was carried out using RSM statistical tool. pH, temperature and extraction time played a significant role in the yield of pectin and the levels of these factors were optimized. A 1.5-fold increase in pectin yield was achieved after optimization. Low p-value and high F-value indicated the significance of the model. The extracted pectins from all sources were characterized extensively in terms of solubility, equivalent weight, methoxyl and anhydrouronic acid contents and degree of esterification. Functional groups of *C. limon* pectin were analysed by FTIR spectroscopy. Thus, the work has facilitated the optimized production of pectins from different citrus peels and their characterization, with the pectins, especially the one from *C. limon*, exhibiting desirable properties for industrial applications.

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Persian Abstract

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چکیده

پکتین پلی ساکاریدی با ساختار غیریکنواخت است که معمولا از پوست مرکبات بدست می آید و در سطح تجاری به عنوان عامل ژل کننده و پایدارکننده به کار می رود. در این پژوهش پکتین با روش ته نشینی با الکل از پوست پرتقال، لیموی شیرین و لیمو استخراج شد. هنگامی که شرایط استخراج به صورت جداگانه تغییر داده شد، حد اکثر بازده در حدود ۳۶/۷۱ درصد از لیمو بدست آمد و سپس بازده با استفاده از روش□□□□□□□□□□ (در رابطه با روش شناسی پاسخ سطحی) بازده نیز افزایش داده شد. شرایط بهینه برای فرایند استخراج پکتین مربوط به ۳/۵ =□□ ، دمای ۶۵ درجه سانتی گراد و زمان ۶۷/۵ دقیقه می باشد. اثر متقابل این متغیرها بر یک دیگر با استفاده از منحنی□□3 و منحنی های کانتوری بررسی شد. استفاده از این طراحی تجربی سبب افزایش ۱/۵ برابری در بازده تولید پکتین شد. آنالیز واریانس ها معنی دار بودن مدل را نشان داد. پکتین به دست آمده مورد آزمایشات کمی و کیفی قرار گرفت و این نتیجه حاصل شد که پکتین بدست آمده محتوی متوکسیل، هیالورونیک اسید و درجه استریفیکاسیون مطلوبی دارد. گروه های چند عملکردی موجود در پکتین با استفاده از □□□ مورد بررسی قرار گرفت. نتایج کلی حاصله نشان دهنده قابل استفاده بودن پکتین استخراج شده برای استفاده های صنعتی می باشد□