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Effect of Extraction Conditions on Purity and Structure of Pectin in Tobacco by CP/MAS ^{13}C NMR Spectroscopy

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The structure and composition of pectins depend on their source, the method used to extract from other soluble material, the analytical procedures and so on. This study was to investigate the influence of extraction conditions (pH, temperature and extraction time) on the yield, purity and structure of pectin in tobacco by CP/MAS ^{13}C NMR spectroscopy. The results showed that the highest yield of pectin (10.87 %) was obtained at pH 1.5, but the highest purity of pectin (galacturonic acid content, GalA) was obtained at pH 2, which was 78.4 %. The degrees of methylation and acetylation decreased with increasing of acid strength (at pH 1.5-2.5), temperature and extraction time. With stronger acid, higher temperature and longer time, the degrees of methylation and degrees of acetylation of pectin decrease. These data provides a prerequisite for controlling and manipulating pectins processing properties.

Keywords: Pectin, Extraction conditions, Purity, Structure, CP/MAS ^{13}C NMR.

INTRODUCTION

Pectin is a complex and structurally diverse group of heterogeneous polysaccharides found in the primary cell wall and middle lamellae of most plants[1]. These polysaccharides provide them with mechanical strength, flexibility and important effects on their industrial applications due to their interaction with other cell wall components[2]. Pectins and hemicelluloses constitute the amorphous matrix of the cell wall, whereas cellulose microfibrils provide the crystalline core. Pectin constitutes about 8-15 % of dry weight of cured bright or burley tobacco. It is a component of carbohydrate fraction of biomass-derived materials and represents water-soluble galacturono-glycans[3]. The main structural element of pectins is galacturonan a linear polymer of $\alpha(1 \rightarrow 4)$ linked, partially methyl esterified-galacturonic acid. Pectins with more than 50 % methyl ester groups are classified as high-methoxyl (HM) and those with less than 50 % methyl ester groups as low-methoxyl (LM). During smoking process, pectin, as one of the most important part in tobacco, undergoes pyrolysis and combustion and forms many pyrolysis gaseous products such as acetic acid, formic acid, methanol and formaldehyde, which greatly influence the characters of tobacco smoke and increase its insecurity[4].

The structure and composition of pectins depend on their source, the tissue preparation method, the extraction protocol, the method used to separate the extracted pectins from other soluble material, the type and extent of purification before analysis and the analytical procedures used for assay and characterization[5,6]. The extraction process is the most important operation for obtaining pectin from plant tissue. Pectin may be extracted from the cell-wall material by cold and/or hot water or buffer solutions, cold and/ or hot solutions of chelating agents, hot diluted acids and cold diluted sodium hydroxide[7-9]. Extraction with chelating agents has the disadvantage that it is difficult to remove residual chelators. Alkaline extraction would decrease the degree of methylation (DM), degree of acetylation (DA) and the length of the galacturonic acid main chain by β-elimination[10]. The highest amount of pectin is generally obtained by hot acid extraction. It is also the most convenient approach for industrial extraction. Most investigators have selected conditions for extraction that yield the highest quantity of the pectin with desired properties[11,12]. Compared to other sources of pectins such as citrus[13], soy hull[14], apple[15] and peach pomace[11]. There are few scientific publications about the influence of different acid extraction conditions on the chemical characteristics of tobacco. Consequently, the research on the influence of extraction conditions on pectin from tobacco has an important significance on accessing the overall tobacco quality and processing.

NMR spectroscopy is an informative method for characterizing the composition and sequence of the polysaccharide units[16]. Up to now, this NMR technique has been applied in

the study of the conformation of many macromolecule chains in solid and gel states[17,18]. In this paper, the influence of extraction conditions on the degree of methylation and degrees of acetylation, yield and purity of pectin in tobacco was investigated by ^{13}C CP/MAS NMR spectra. The optimum conditions for extracting pectin from tobacco such as, pH value, heating time and temperature were studied. From this research, not only the changes of the structure of pectin in this procedure could be obtained, but also the key factors in the extraction could be control to get the highest yield and purity pectin from tobacco sample.

EXPERIMENTAL

The standard sample of polygalacturonic acid (PGalA) and potassium salt of citrus pectins (PGAS) were used as product without purification from J&K Chem-Tech Ltd (Shanghai, China). All reagents employed were of analytical grade quality and distilled water was used throughout the work. Flue-cured tobacco sample was from Zimbabwe and Guizhou, China. Oriental and Burkey tobacco samples were from Xinjiang and Hubei, China, respectively.

Pectin extracted from tobacco sample[19]: 10 g of weighed, oven-dried tobacco sample was extracted by ultrasound with 100 mL deionized water for 0.5 h, rinsed with 40 °C deionized water two times and filtered with a funnel. Then the residue was adjusted with 1 M HCl to pH 2 and kept for 1.5 h at 85 °C. After the hydrolysis, the extract was filtrated and adjusted with 1 M NaOH to pH 3.5, followed by washing and sedimentation with ethanol solution (1:1, v/v), acetone and ethanol in turn. The residue was dried at 40 °C and weighed to calculate the yield of pectin. The product was ground to power about 150 μm and stored under phosphorus pentaoxide for the NMR analysis.

^{13}C CP/MAS NMR spectroscopy: High resolution ^{13}C CP/MAS NMR spectra of samples were measured using Bruker AVANCE AV 400 spectrometer operating at 400 MHz employing a double-tuned solid-state probe equipped with 4 mm (o.d.) spinners. The ^{13}C CP/MAS spectra were recorded using ^{13}C rf-field strengths of 62.5 kHz and a spin rate of 15 kHz. The spectra were obtained applying the following parameters: 2 ms contact time, recycle delay of 2s, acquisition time of 25.4 ms and sweep width of 30 kHz. The typical number of scans was 1024 for the CP/MAS spectra. All spectra were referenced to the carbonyl peak of glycine at 176.03 ppm. The spectra were apodized by Lorentzian line broadenings of 10 Hz. Fitting of line widths using a Lorentzian line shape was performed by the built-in procedure in the MestReNova 6.1.1 software.

Chemical shift calculation and peak fitting: The ^{13}C carbon chemical shifts were calculated by MestReNova 6.1.1 software for model structures. Decomposition of ^{13}C CP/MAS NMR spectra in the regions of 169-178 ppm was pursued by peak fitting module of MestReNova 6.1.1 software. The results of peak separation were applied to obtain the degrees of methylation, degrees of acetylation values and the ratio of the pectin ester on the basis of the relative areas of separated peaks.

RESULTS AND DISCUSSION

^{13}C CP/MAS NMR spectra of pectin: The ^{13}C CP/MAS NMR spectra of pectin samples (N1, N2) and the standard samples of polygalacturonic acid (STD) were presented in Fig. 1. The chemical shifts of pectins carbon resonances were summed up in Table-1. The resonances at 176-168 ppm were assigned to C-6 carbons of galacturonic units and the peak had stable location, enough intensity and little interference. Therefore, the purity or content of pectin could be calculated from the integral of these region carbon resonances between 168 and 180 ppm *versus* the sample weights. On the other hand, the resonances at 101 and 79 ppm arised from glycosidic bond carbons C-1 and C-4, respectively. The peaks at 67-72 ppm came from the other carbons of pyranoid ring[20]. An intense resonance at 53 ppm (Table-1) represented methyl carbons of the methyl ester $COOCH_3$ in the spectra of pectinates. This resonance band increased with subsequent methyl esterification and had been used for the estimation of degrees of methylation values.

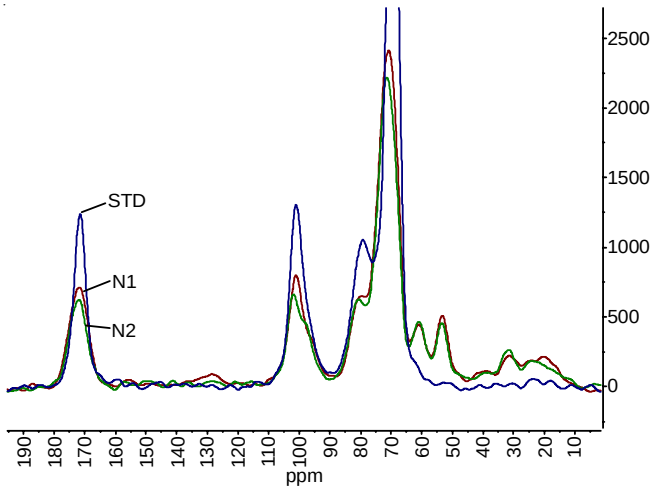

Fig. 1. ^{13}C CP/MAS NMR spectra of polygalacturonic acid, polygalacturonic acid-standard sample and pectin samples extracted from tobacco

TABLE-1
CP/MAS ^{13}C NMR CHEMICAL SHIFTS (IN ppm) FOR POLYGALACTURONIC ACID AND PECTINS

Sample	Chemical shift (ppm)					
	C-1	C-2, 3, 5	C-4	C-6	$COO\underline{C}H_3$	$O\text{-}CO\underline{C}H_3$
STD (PGA)	101.09	69.25	79.06	171.19		
N1	101.55	71.07	80.59	171.10 174.13	53.36	20.48
N2	101.91	71.10	Sh*	169.19 171.41 174.44	53.57	21.26

*: Shoulder of C-2,3,4 peak

Effect of pH value on the yield and purity of pectin in tobacco acid hydrolysis: As the extraction process gives the most important effect on the yield, purity, structure and composition of pectin, therefore, optimization of extraction conditions is the key factor in analysis of tobacco pectin[15]. In present study, hot acid extraction was selected for the study of extraction efficiencies from tobacco sample. The pH value, the heating time and temperature were the optimized variables with the constant sample amount (10 g). Fig. 2 showed the yield and purity of pectin obtained from tobacco acid extract under different pH values. According to this figure, the yield of pectin in tobacco showed an apparent difference under different pH values. Evidently, the yield of pectin increased with the acid strength. The average of yield of pectin varied from 4.61 to 10.38 % with the pH value at 1.5-2.5. Pectin might not be hydrolyzed completely with weaker acid strength (pH > 2.5). The yield of pectin increased with the acid strength and the highest yield (10.38 %) was obtained at pH 1.5 for 1.5 h at 85 °C, whereas the lowest yield (4.08 %) resulted from 2 h extraction at pH 2.5 and 75 °C. But other nonpectic compounds (such as cellulose and hemicelluloses) may also be solubilized from the cell wall at high acid strength, which may lead to the high yield of pectin[21]. In addition, great changes had been observed in the reaction of acid hydrolysis at pH 1. The obtained product was white powder at the yield of 84.8 %. But the later determination showed that the purity of PGalA in the extraction was very low (7.44 %). Constenla *et al.*[22] showed that the optimal pH values used to get higher yield of pectin were different from different plants. A similar effect was obtained by Pagan *et al.*[23] in the extraction of peach pomace pectins, contrary to soy hull pectin where the yields decreased with increasing acid strength.

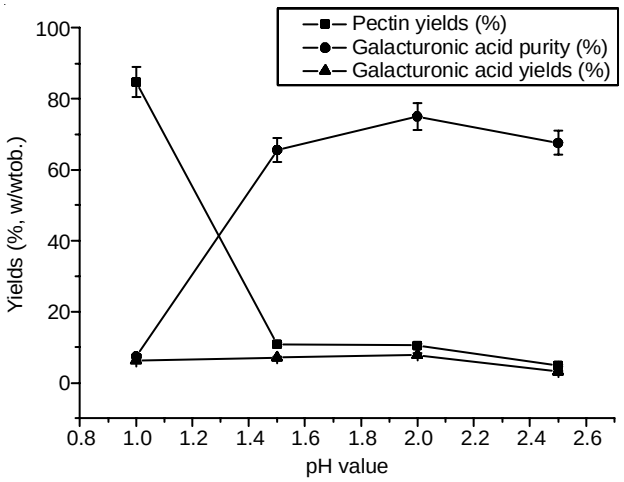

Fig. 2. Effect of pH values on the yield and purity of pectin in tobacco acid hydrolysis

Galacturonic acid was the main component of the pectin. Its quantification allowed the estimation of the purity of pectin from tobacco. As shown in Fig. 2, the pH value also had a significant effect on the pectin purity. Though the yield of pectin from tobacco was higher at pH 1.5 than at pH 2, the purity of pectin from tobacco wasn't the same. At pH 2, the contents of polygalacturonic acid were always superior to that found at pH 1.5. Three hypotheses can be proposed[21]. First, few pectins had been extracted at pH 1.5. Second, other nonpectic compounds has been solubilized from the cell wall at pH 1.5 and precipitated with alcohol. Finally, at the lowest pH value, the extracted pectins had been degraded to small molecular weight compounds that had not been precipitated with the ethanol. In this study, the second reason may be possible. Hwang *et al.*[24] also reported that the pectins from acid hydrolysis were most likely to contain notable amounts of free neutral polysaccharides with high molecular weights that were distinguished from polysaccharides covalently incorporated to the side chains of pectin.

Effect of temperature on the yield and purity of pectin in tobacco acid hydrolysis: The effect of temperature on the yield and purity of pectin from tobacco was investigated with the temperature at 65-105 °C. As shown in Fig. 3, the yield of pectin increased with the temperature and the yield (10.56 %) obtained at 95 °C was always higher than that obtained at 75 °C (9.87 %) or 85 °C (10.54 %). However, some researchers observed that pectin yields of sugar beet were lower at 90 °C than those at 80 °C and were related to some degradation of pectin at 90 °C[22,25]. Here, a similar effect was obtained at temperature above 95 °C. With respect to the purity of pectin, the extraction temperature influenced the content of polygalacturonic acid of the extract evidently as well. Indeed, the purity of polygalacturonic acid obtained at 95 °C (78.4 %) was the highest with the temperature at 65-105 °C for 1.5 h at pH 2. The lower purity of pectin may contain nonpectic substances or degraded fractions from the pectins. Marcon *et al*[26] observed that the arabinan, usually present in side-chains of the hairy regions of pectin, was partially removed in the conditions of extraction. Similar results were reported by Habibi *et al.*[27]. They detected that small amount of arabinose extraction at 80 °C for 60 min with HCl.

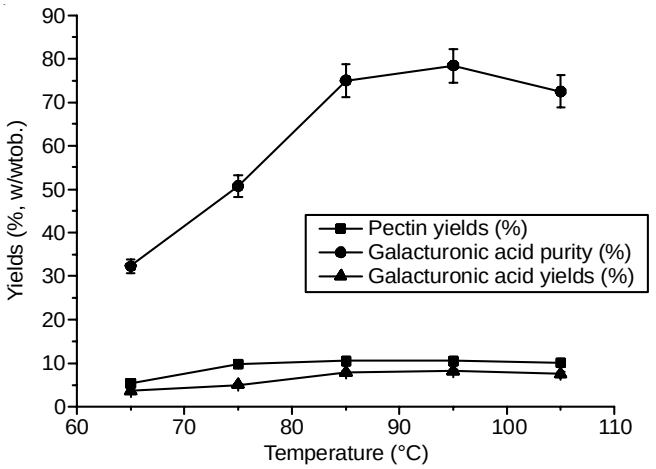

Fig. 3. Effect of temperature on the yield and purity of pectin in tobacco acid hydrolysis

Effect of time on the yield and purity of pectin in tobacco acid hydrolysis: With respect to the influence of the extraction time, the yield increased up to 1 h and became constant there after with the pH 2 at 85 °C (Fig. 4). Similar results had been observed by Kalpathy and Proctor[25] in the case of extraction of soy hull pectin. The content of PGalA of pectin in tobacco showed slight changes during different

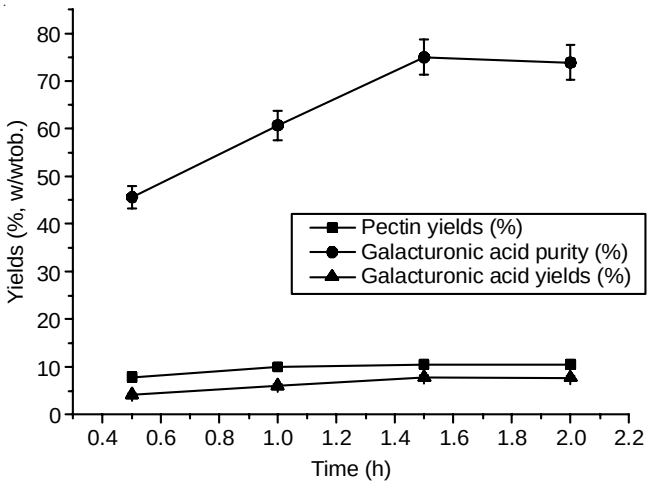

Fig. 4. Effect of time on the yield and purity of pectin in tobacco acid hydrolysis

time. The purity of pectin was increased with time from 0.5 to 1.5 h and decreased slightly with time above 1.5 h. Therefore, 1.5 h was selected as the optimal extract time for the pectin analysis.

Effect of conditions on the structure of pectin in tobacco acid hydrolysis: NMR spectral results were applied to determine some important pectin characteristics in comparison with those obtained by conventional methods. The region of 190-160 ppm of ^{13}C CP/MAS NMR spectrum (Fig. 5) belongs to carboxyl C-6 carbons of galacturonic unit that are present as carboxylic acid COOH, carboxylate anion COO^-, or ester $COOCH_3$[28]. The content of poly-galacturonic acid units (PGal A), the degrees of methylation and degrees of acetylation values of pectin samples were evaluated as the ratio of integral intensity of the C-6, $COOCH_3$ and O-$COCH_3$ carbons, respectively.

$DM = A_{COOCH_3}/A_{C\text{-}6tol} \times 100\ \%$ ($COO\underline{C}H_3$, δ 50-55 ppm)

OR $DM = A_{COOCH_3}/A_{C\text{-}6tol} \times 100\ \%$ ($\underline{C}OOCH_3$, δ 174 ppm)

$DA = A_{OCOCH_3}/A_{C\text{-}6tol}$

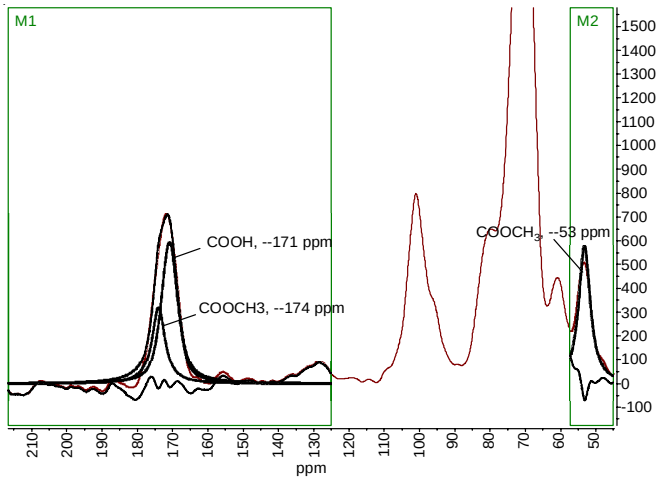

Fig. 5. ^{13}C CP/MAS NMR spectra of pectin in tobacco sample containing methylester groups

Methyl-esterification is a common feature of both the homogalacturonan and xylgalacturonan regions in pectins[18]. The effect of pH value, temperature and time on the values of degree of methylation and degree of acetylation of pectin in tobacco acid hydrolysis was investigated and the results were shown in Fig. 6. According to the Fig. 6, the degree of methylation and degrees of acetylation decreased significantly with increasing extraction times. The degree of methylation of the extracted pectins varied from 48.9 to 39.6 %. The highest degree of methylation and degree of acetylation values were obtained for 1h at pH 2.0 and 85 °C. Compared with extraction time, the temperature and pH value had a weaker effect on the degrees of methylation and degree of acetylation. The degree of methylation and degree of acetylation decreased slightly with increasing extract temperature. With the pH value at 1.5-2.5, the values of degree of methylation and degree of acetylation of pectin increased with the pH value and the range of degree of methylation and degree of acetylation of pectin was 42.5-46.9 and 0.96-1.69 %, respectively. As a result, these results confirmed that higher concentrated acid were required for desorption of the less methylated pectins. With an increase in the degree of esterification of pectin chain the charge density rose and desorption of the pectin macromolecules became more difficult. At high acid strength solution (pH < 1.5), methylation and acetylation groups of pectins would be hydrolyzed and eliminated and other nonpectic compounds (such as cellulose and hemicelluloses) may also be solubilized from the cell wall, which may lead to the high yield of pectin, especially for a long time[29]. Therefore, the optimal conditions to extract pectin from tobacco were pH 2 for 1.5 h at 85 °C. Under these conditions, the content of polygalacturonic acid was 7.91 %. The degrees of methylation and degrees of acetylation was 44.6 and 1.52 %, respectively.

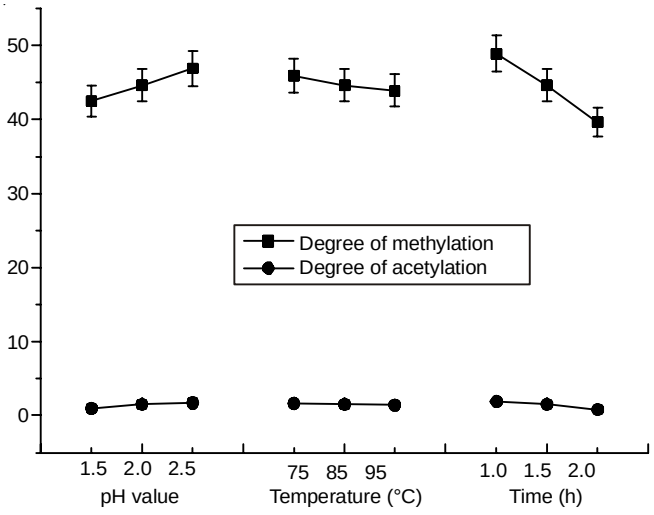

Fig. 6. Effect of pH values, temperature and time on the values of degrees of methylation and degrees of acetylation of pectin in tobacco acid hydrolysis

Conclusion

In this paper, the influence of extraction conditions (pH, temperature and extraction time) on the chemical characteristics of pectin in tobacco was studied by ^{13}C CP/MAS NMR spectroscopy. The results showed that the highest yields (10.87 %) were obtained at pH 1.5 but the purity of pectin got the highest value (78.4 %) at pH 2. The degree of methylation acetylation also confirmed the hydrolysis of pectin under these

extraction conditions. The study showed that the optimal conditions to extract pectin from tobacco were pH 2 for 1.5 h at 85 °C. Under these conditions, the content of polygalacturonic acid was 7.91 %. The degree of methylation and degree of acetylation was 44.6 and 1.52 %, respectively. From the research, the profound understanding the structure of pectin would provide a prerequisite for controlling and manipulating their industrial processing properties.

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