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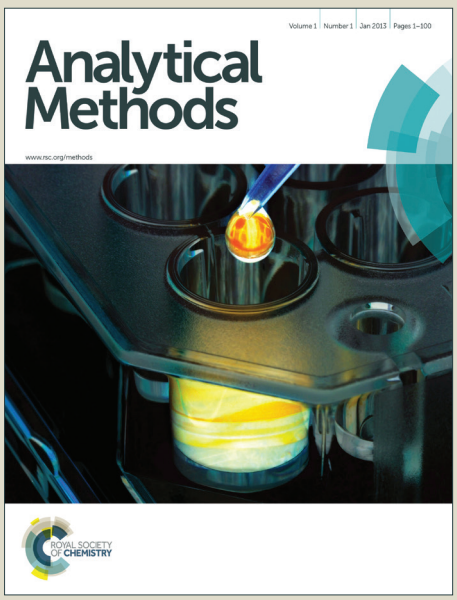

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PAPER

Quantitative and Structure Analysis of Pectin in Tobacco by ^{13}C CP / MAS NMR Spectroscopy

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A new method utilizing ^{13}C CP/MAS NMR spectra was developed for the simultaneous quantitative and structure analysis of pectin in tobacco. The conditions of acid extraction of tobacco sample and parameters of spectra instrument were optimized. The C-6 carbon region of NMR spectra was studied in detail and the chemical shift and the area of peaks were applied for the calculation of polygalacturonic acid content (PGalA), degrees of methylation (DM) and acetylation (DAc). The quantitative results showed that the average recovery was 92.5% with the RSD of 4.4%～4.9%. Therefore, this NMR method could provide important information about both amount and structure of pectins.

1. 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, 3]. In the cigarette industry, the content of pectin is a useful index of quantity for characterizing tobacco blends. For a high content of pectin tobacco sample, it becomes soften and sensitive to mildew at a high humidity of air whereas rigid and brittle at a low humidity of air [4]. The main structural element of pectins is galacturonan a linear polymer of α (1→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). Both the degree of methylesterification (DM) and degree of acetylation (DAc) have a profound impact on functional properties and pectins obtained from different sources reveal significant differences in their structural and technological properties [5].

For the sucking quality of tobacco, pectin is a kind of unfavourable substance. 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 [6]. Therefore, the quantitative and structure analysis of pectin are very important for the tobacco quality and processing.

The quantitative determination of pectin in tobacco often includes several steps: extraction, hydrolyzation and chromatography or photometry analysis [7-9]. At present, the acid extraction and enzymatic hydrolysis of pectin followed by IC analysis is the tobacco industry standard method in China [10]. But it is very time-consuming, troublesome, and moreover, there has no structure information of pectin during these processes.

NMR spectroscopy is an informative method for characterizing the composition and sequence of the polysaccharide units [11]. It has the potential to enable quantitative determination of functional groups in a complex material because all equivalent nuclei potentially give rise to signals of equal intensity regardless of their chemical environment. Up to now, this NMR technique has been applied in the study of the conformation of pectin macromolecule chains in solid and gel states [12-14]. The preparation of pectin solutions for NMR analysis is difficult. Although pectin is well soluble in water, its solution is very viscous which complicates the application of NMR solution techniques for its analysis. Solid-state ^{13}C NMR has proven to be a valuable technique in structural and conformational analysis of polysaccharides. Many methods were also used in investigation of plant cell wall materials containing pectin compounds [4, 15].

In this work we present a new method for the simultaneous quantitative determination and structure analysis of pectin in tobacco by ^{13}C CP/MAS NMR spectra. On the basis of solid state ^{13}C NMR spectroscopy, pectin content in extracted tobacco, its composition, degree of esterification and acetylation were estimated by peak fitting analysis of C-6 region of the spectra.

2. Experimental

2.1 Chemicals and materials

The standard samples of polygalacturonic acid (PGA) and potassium salts 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

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distilled water was used throughout the work. Flue-cured tobacco samples were from Zimbabwe and Guizhou, China. Oriental and Burkey tobacco samples were from Xinjiang and Hubei, China, respectively.

2.2 Pectin extracted from tobacco sample

10g of weighed, oven-dried tobacco sample was extracted by ultrasound with 100 mL deionized water for 0.5h, rinsed with 40℃ deionized water two times, and then filtered with a funnel. Then the residue was adjusted with 1M HCl to pH 2.0 and kept for 1.5h at 85℃. After the hydrolysis, the extract was filtrated and adjusted with 1M 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℃ and weighed to calculate the yield of pectin. The product was ground to power about 150μm and stored under phosphorus pentaoxide for the NMR analysis.

2.3 ^{13}C NMR spectroscopy

High resolution ^{13}C NMR spectra of samples were measured using Bruker AVANCE AV 400 spectrometer operating at 400MHz employing a double-tuned solid-state probe equipped with 4 mm (o.d.) spinners. The ^{13}C CP/MAS and DP/MAS spectra were recorded using ^{13}C and ^{1}H 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, acquisition times of 25.4ms, number of scans of 1024 and sweep width of 30 kHz. Recycle delays of 2s and 128s were employed for the CP/MAS and DP/MAS experiments, respectively. All spectra were referenced to the carbonyl peak of glycine at 176.03 ppm. The spectra were apodized by Lorentzian line broadenings of 10Hz. Fitting of line widths using a Lorentzian line shape was performed by the built-in procedure in the MestReNova 6.1.1 software.

2.4 Sample preparation and Analysis

A dried portion of the pectin standard or tobacco residue from acid extraction was loaded into the NMR rotor cell and weighed to within ±0.1 mg on an analytical balance. Typically, the signal-to-noise ratio for the C-6 resonance at 170 ppm in our tobacco spectra was in the range of (50-100):1. Approximately 80 mg of the pectin material was encapsulated in a cylindrical cell machined from aluminum nitride ceramic rod to precisely fit inside the 4 mm zirconia NMR rotor. The same rotor end caps were used for all measurements, and the zirconia rotor itself was always oriented in the same direction inside the NMR probe stator.

2.5 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 DM, DAc values and the ratio of the pectin ester on the basis of the relative areas of separated peaks.

3. Results and discussion

3.1 ^{13}C NMR spectra of pectin

Both the CP/MAS and DP/MAS NMR techniques were performed to analyse the pectin from tobacco samples. These spectra of pectin samples were displayed in Fig. 1. Obviously, a very good spectral resolution was obtained that facilitated detailed assignment of different functional groups present in pectin. When comparing the spectra of CP/MAS and DP/MAS, it was noted that both of them displayed similar features, except for the intensity of part carbons, whereas the resonance at 135 ppm (spinning side bands) was not present in the CP/MAS spectrum. In general, solid state NMR spectra are not always quantitative. The NMR signal of some ^{13}C nuclei may be diminished or be rendered completely unobservable, especially when the CP technique is used. However, if the loss of NMR signal intensity is equal across all functional groups, their relative ratios will not be changed, even though total signal is decreased. Smernik and Oades [16] have investigated the quantitative reliability of solid state ^{13}C NMR spectra to different materials and classified the materials into three categories based on their chemical structures: one of them is those materials that give quantitative signals with both CP and Bloch decay (BD) NMR techniques. These materials include cellulose, pectin, chitosan, lignin, and palmitic acid. That is to say pectin can be quantitatively measured by CP technique. Of course, DP is more accurate to perform the quantitative analysis. In our experiments, as recycle delays of 128s was employed for the DP/MAS, it took about two days (over 36 h) to complete a test whereas 34min for the CP/MAS. The advantage of CP over the DP technique is improved sensitivity, mainly brought about by a shorter delay required between consecutive scans recycle delay. For our tobacco samples, the improved sensitivity offered by the CP technique is required to obtain a solid state ^{13}C NMR spectrum in a realistic timeframe.

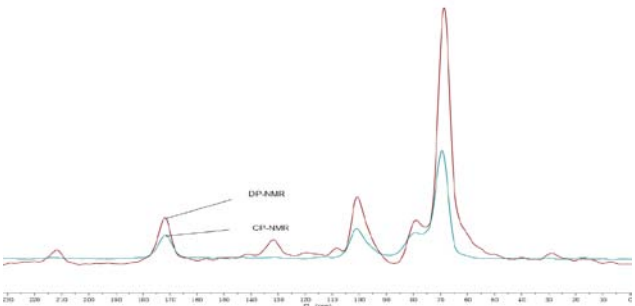

Fig. 1 ^{13}C CP/MAS and DP/MAS NMR spectra of samples of PGA.

The ^{13}C CP/MAS NMR spectra of pectin samples (N1, N2) were presented in Fig. 2. 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, while O<u>C</u>OCH$_3$ carbons of acetyl groups in acetylated samples were also situated in this region. The resonances at ~101 ppm 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 [17]. The NMR spectra of potassium pectate and potassium pectinates (STD2) were also shown in Fig. 2. There were double resonances observed at ~175 ppm, which presented ionized form and methyl ester pectinate. An intense

resonance at ~53 ppm (Table 1) represents methyl carbons of the methyl ester $COO\underline{C}H_3$ in the spectra of pectinates. This resonance band increases with subsequent methyl esterification and has been used for the estimation of DM values.

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	$COO\underline{C}H_3$	$O\text{-}CO\underline{C}H_3$
STD1 (PGA)	101.09	69.25	79.06	171.19		
STD2 (PGAS)	99.15	68.51 71.05	77.58	174.94 176.72		
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.

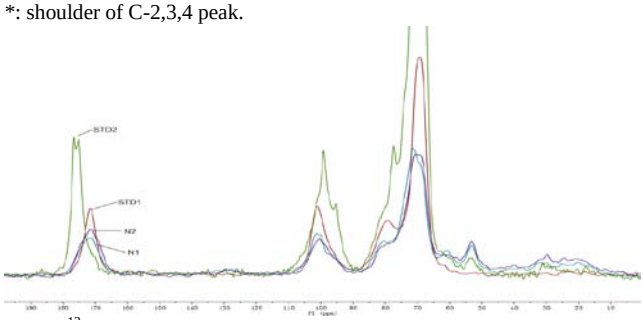

Fig. 2 ^{13}C CP/MAS NMR spectra of PGA, PGASS and pectin samples extracted from tobacco.

3.2 Optimization of extraction conditions of the tobacco pectin

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 [18]. 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 [19]. Extraction with chelating agents has the disadvantage that it is difficult to remove residual chelators. Alkaline extraction could decrease the DM, DAc, and the length of the galactronic acid main chain by ß–elimination. Most investigators have selected conditions for extraction that yield the highest quantity of the pectin with desired properties [20-21]. The highest amount of pectin is generally obtained by hot acid extraction and it is also the most convenient approach for industrial extraction. In our study, hot acid extraction was selected for the study of extraction efficiencies from tobacco sample. To optimize the conditions of extraction, a three-level orthogonal array design (OAD) was employed [22]. The pH value, the heating time and temperature were the optimized variables with the constant sample amount (10 g). The results of average pectin yields and purity were presented in Table 2.

After the OAD procedure had been conducted, a graph with the average yield and purity of pectin from acid extraction tobacco sample under the same pH value, heating time or temperature level was drawn (Fig. 3) to examine the key variable. According to Fig. 3, the yield of pectin in tobacco showed an evident difference under different pH values. The average of yield of pectin varied from 4.61% to 10.38% with the pH value at 1.5~2.5. The highest yield (10.98%) was obtained at pH 1.5 for 1.5 h at 85℃, whereas the lowest yield (4.08%) resulted from 2.0 h extraction at pH 2.5 and 75℃. Evidently, the yield of pectin increased with the acid strength. But other nonpectic compounds, such as cellulose and hemicelluloses, may also be solubilized from the cell wall at high acid strength and form the impurity substance in the extraction process, which may lead to the high yield of pectin [19]. On the other hand, the results showed that the pH value had a significant effect on the pectin purity (calculated as PGalA, %). At pH 2.0, the average of content of PGalA was superior to that found at pH 1.5 and 2.5. The extraction temperature also influenced the yield and purity of pectin in tobacco. Indeed, the PGalA content was higher at 95℃ than that at 85℃ and 75℃. The highest content of PGalA was found at 85℃ for 2.0 h at pH 2.0. However, it was reported that pectin yields of sugar beet were lower at 90℃ than those at 80℃, and were related to some degradation of pectin at 90℃. [23, 24] With respect to the influence of the extraction time, the yield increased up to 1 h and became constant thereafter. The similar results

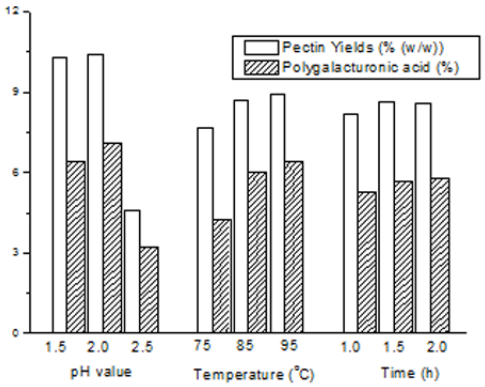

Fig. 3 The effects of pH value, temperature and heating time on the yield and purity of pectin in tobacco acid hydrolysis.

Table 2 Effect of pH value, temperature and heating time on the average pectin yields and purity from acid extraction tobacco sample (n = 3)

No.	pH Value	Temp (℃)	Time (h)	Pectin Yields* (% (w/w))	PGalA (%)
1	1.5	75	1.0	9.32	4.92
2	1.5	85	1.5	10.87	7.13
3	1.5	95	2.0	10.98	7.19
4	2.0	75	1.5	9.87	5.62
5	2.0	85	2.0	10.59	7.85
6	2.0	95	1.0	10.68	7.76
7	2.5	75	2.0	4.08	2.24
8	2.5	85	1.0	4.67	3.11
9	2.5	95	1.5	5.09	4.29

* The yield and PGalA were calculated by weight ratio of pectin crude product and PGA units to tobacco, respectively. These values were the average of three times under these conditions.

could be observed at pH 1.5 as well as pH 2.0. According to Fig.3, there was no evident difference between the 2.0 h and 1.5 h extraction. Therefore, the optimal conditions to extract pectin from tobacco were pH 2.0 for 1.5 h at 85℃. Under these conditions, the content of PGalA was 7.91%.

3.3 The evaluation of quantitative analyses of pectin in tobacco

Quantitative analyses of pectin extracted from tobacco sample were performed by ^{13}C CP/MAS NMR utilizing the above described procedures. Measurements were made on each of at least three separately weighed samples for each sample. A calibration curve for the pectin analysis was established from the ^{13}C CP/MAS NMR spectra of a set of 6 PGA standard samples with weights ranging from 10-80 mg. The integral of the region carbon resonance between 168 and 180 ppm was plotted versus the sample weights. Linear regression analysis was performed with the intercept constrained to pass through zero, yielding a straight line A=645.39×m - 339.28 with a correlation coefficient r^2 = 0.9996. Limits of detection (LOD) and limits of quantifi- cation (LOQ) were calculated at 0.42mg/g and 1.27 mg/g with signal to-noise ratios of 3 and 10, respectively.

To evaluate the accuracy of NMR spectra analyses of pectin, two pectin extraction samples (1# and 2#) added with PGA standard sample were performed by ^{13}C CP/MAS NMR spectra. Each sample was analyzed three times separately and the results were showed in Table 3. According to the Table, the average recovery of PGA was 97.3% with the RSD of 3.0%～4.2%, which indicated that the ^{13}C CP/MAS NMR was an accurate method for determination of GalA amount.

Table 3. Recovery and repetition data of the analytical method for pectin with GalA or tobacco standard

Sample	Pectin Content (%)	Spiking level (PGalA/mg)	Recovery (%)	RSD (%)	Mean Recovery (%)
1#	7.91	20	95.5	4.2	97.3
		40	96.7	3.9	
2#	7.10	20	103.2	3.4	
		40	93.6	3.0	
3#	7.91	169.2	93.4	4.4	92.5
4#	7.91	507.6	91.5	4.9	

To verify the accuracy of whole method, a tobacco sample added with 2g and 6g standard tobacco sample (marked by IC method [10]) was performed with acid hydrolysis, purification and NMR spectra analysis. Measurements were also made on each of three separately weighed samples for each sample and the results were showed as Table 3 (3# and 4#). The results revealed that the average recovery was 92.5% with the RSD of 4.4%～4.9%. These data demonstrated that the proposal method can be used for the tobacco pectin with a suitable degree of precision.

3.4 Structure analysis of pectin in tobacco sample

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 belongs to carboxyl C-6 carbons of galacturonic unit that are present as carboxylic acid COOH, carboxylate anion COO^-, or ester $\underline{C}OOCH_3$ [25]. The content of polygalacturonic acid units (PGal A), DM and DAc of pectin samples were evaluated as the ratio of integral intensity of the C-6, $COO\underline{C}H_3$ and O-$CO\underline{C}H_3$ carbons, respectively. Jarvis and Apperley measured ^{13}C CP/ MAS NMR spectra of solid pectin acid, sodium pectate and the acid form of pectin and reported that the carboxyl resonance was at 171.6 ppm for the protonated and esterified forms and 176 ppm for the ionised form [26]. Our measurements showed that PGA standard had a single signal at 171.2 ppm that represented COOH carbons, whereas the potassium salt was observed at 176.7 ppm (as showed as Fig.4), consisted with the above reported pectin spectral results. C-6 carbon signals of pectin from tobacco extraction often had a rather complicated shape due to the variable content of COOH, $COOCH_3$ and COO^- groups. All these resonances overlapped one another because the shift difference between them was significantly less than the sum of their half widths. Therefore, decomposition of the C-6 region of this spectrum was necessary. We used the multiple mixed Lorentzian-Gaussian functions fitting model. These spectra had two signals in the carboxyl region (Fig. 4(b) (c)). The peak at 171 ppm indicated carboxylate anion carbons and the peak at 174 ppm belonged to carbonyl carbons of methyl ester groups. On the other hand, tobacco pectin, as a kind of HM pectin, had a resonance signal at 171 ppm with a lowfield shoulder due to high content of methyl ester groups together with some free carboxyls in acid and salt forms. Therefore, the ratio of pectin acid in the protonated and esterified forms, the constituent of tobacco pectin could be got from the decomposition of C-6 spectra by the multiple mixed Lorentzian-Gaussian fitting model.

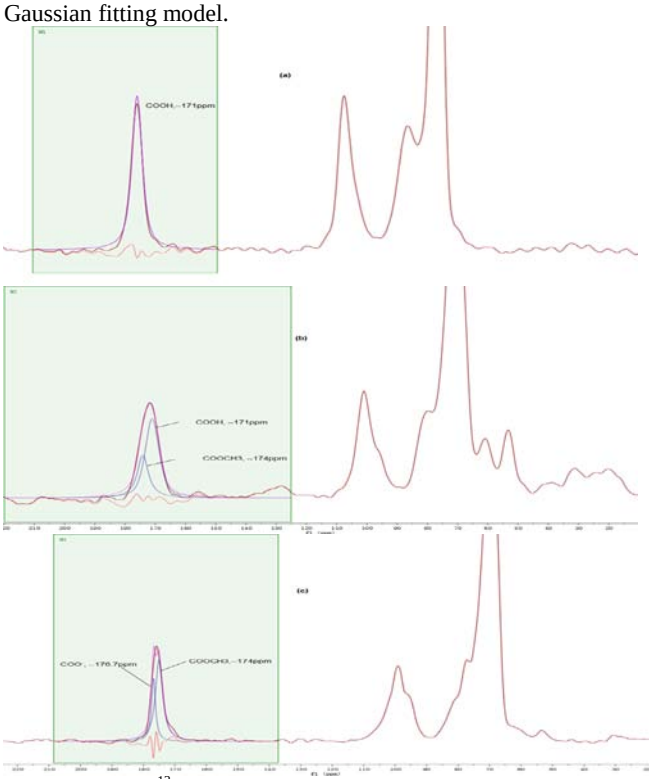

Fig.4 Fitting of ^{13}C CP/MAS NMR C-6 spectral region of pectin in tobacco

Pectin is composed by three main components named rhamnogalacturonan I(RG I), rhamnogalacturonan II(RG II), and

homogalacturnan (HG) [23]. HG is thought to affect cell adhesion and tissue integrity as well as to contribute in signaling. The degree and pattern of methyl esterification of HG affect the cell wall structure and properties with consequences on both the physiological processes of the plants and their resistance to pathogens [27]. In the ^{13}C CP/MAS NMR spectra (Fig. 5), an intense resonance at ~53 ppm represented methyl carbons of the methyl ester COOC$\underline{H}_3$. This resonance band had been used for the estimation of DM values (Table 4) and increased with subsequent methyl esterification. This may reflect methyl ester structure predominating in tobacco pectin samples and was in agreement with the calculations by IR method [23]. On the other hand, decomposition of C-6 region permitted to estimate the relationship between free ionized carboxyl and acetyls in pectate samples, and DM values of tobacco pectin could also be got from the decomposition of 174 ppm regions by the multiple mixed Lorentzian-Gaussian functions fitting model. In fact, the values got by IR method were the total degree of esterification, including degree of methyl and ethyl esterification (DE) [19, 23]. Compared with DM, DE values were often very low in tobacco pectin samples. Although DM could be got from IR method, the NMR method can provide other important information such as DAc values, amount of PGalA and three present form of galacturonic unit. The agreement in the values of DM was not so good between ~53 ppm and ~174 ppm may result in different cross-polarization transfer time of COOC$\underline{H}_3$ and $\underline{C}$OOCH$_3$.

DM=$A_{COOC\underline{H}3}/A_{C\text{-}6tol}$×100 % （COOC$\underline{H}_3$，δ~53 ppm）

OR DM=$A_{COO\underline{C}H3}/A_{C\text{-}6tol}$×100 % （$\underline{C}$OOCH$_3$，δ ~174 ppm）

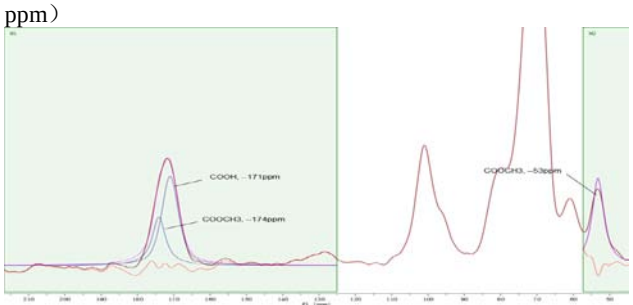

Fig.5 ^{13}C CP/MAS NMR spectra of pectin in tobacco sample containing methylester group

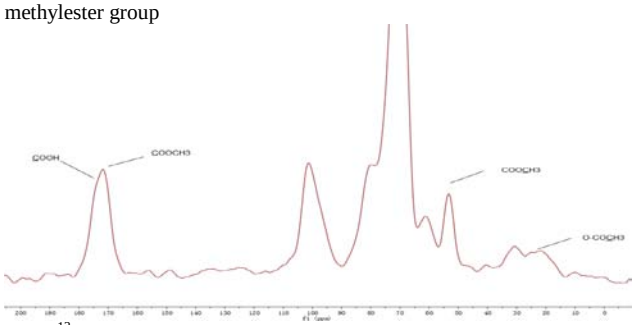

Fig.6 ^{13}C CP/MAS NMR spectra of pectin in tobacco sample containing methylester and acetyl groups

In the ^{13}C CP/MAS NMR spectra (Fig. 6) of tobacco pectin sample, a weak resonance at ~23 ppm represented methyl carbons of the acetyl ester. This resonance band was used for the estimation of DAc values in tobacco pectin samples. Compared with intense resonance at ~53 ppm, this resonance was weak and unmarked, which indicated a very small amount of acetyl ester groups in tobacco pectin samples and DAc values were low and inaccurate (as shown in Table 4). Only in those spectra of high acetyl content could DAc values be calculated by this method. As a matter of fact, DM and DAc values of natural pectins strongly depend on the plant raw material and the extraction procedure. It has been reported that the pectins extracted with bacterial enzymes had a higher O-acetyl content than those extracted with acid [5, 25]. Highly acetylated pectins have bad gelation properties and must be deacetylated by chemical or enzymatic processing [1]. The solid state NMR may be a good method to monitor the values of DAc in the process of extraction or acetyl hydrolysis.

DAc= $A_{OCO\underline{C}H3}/A_{C\text{-}6tol}$

Table 4. DM data of pectin in tobacco samples by different methods

Sample	DM (%)			DAc (%)
	COOCH$_3$ ~174 ppm	COOC$\underline{H}_3$ ~53 ppm	IR*	O-CO$\underline{C}$H$_3$ ~23 ppm
S1	45.5	50.2	44.6	0.62
S2	43.9	44.5	46.1	1.52
S3	39.8	41.6	42.9	1.05
S4	54.8	58.1	53.5	0.83

*DM (%) = $A_{1740cm}^{-1}/(A_{1740cm}^{-1}+A_{1630cm}^{-1})$ [23]

3.5 Analysis of pectin in tobacco samples

Five tobacco samples from different district and kinds of varieties were extracted and analyzed using conventional ion chromatographic method [10] and proposal ^{13}C CP/MAS NMR spectra method. The results were shown in Table 5. The amounts of PGalA of five samples were in the range of 7.0-9.5% and there was no obvious difference between two methods. These meant the proposal ^{13}C CP/MAS NMR spectra method was also accurate and reliable. More importantly, the structure information such as DM value could be got by the proposal method simultaneously. Of five samples, DM value from Zimbabwe Flue-cured was the highest (53.8%) while Hubei Burley was the lowest (36.6%). According to the Table 5, the yield of pectin extracted from different variety tobacco samples shown obvious differences though they were performed under the same acid extraction condition and procedure. But this didn't effect on the ultimate amounts of PGalA.

Table 5. Analysis of pectin in different tobacco samples by NMR spectra method and ion chromagraphic method

Sample	NMR method				IC method	
	Yields (%)	DM (%)	Pectin (%)	RSD (%)	Pectin (%)	RSD (%)
Guizhou Flue-cured	10.85	52.2	8.3	4.9	8.4	3.9
Zimbabwe Flue-cured	16.25	53.8	8.1	4.1	8.2	3.2
Hunan Oriental	6.10	48.4	7.1	5.1	7.0	2.2
Hubei Burley	16.84	36.6	9.4	3.9	9.5	3.6
Guangzhou Bright	16.48	53.2	9.1	4.6	9.3	4.5

4. Conclusions

In this work, a new ^{13}C CP/MAS NMR spectra method was proposed to analyse the purity and structure of pectin followed

acid extraction from tobacco sample. The method was employed to measure pectin after the extraction of tobacco samples with acid under optimal conditions (pH 2.0 for 1.5 h at 85℃). The C-6 carbon region of NMR spectra was decomposed to study the values of GalA, DM and DAc values and the results were in a good agreement with the conventional chromographic method. Therefore, the NMR method could realize quantitative and structive simultaneous analysis of pectin in tobacco.

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The authors declared no conflict of interest.

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