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RESEARCH ARTICLE

Study on ^{13}C MultiCP/MAS ssNMR Analysis of Tobacco Pectin

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Abstract: ***Background*:** As one of the most important economic crops, tobacco products have a long history and dominate the development of the world economy. Pectin, as a complex colloidal substance widely present in plant cell walls, its content is an important factor affecting the safety of tobacco smoking.

***Objective*:** This study aimed to analyze the content and structure of pectin in tobacco samples.

***Methods*:** In this study, tobacco pectin was extracted by ultrasonic-assisted ionic liquid extraction, and the ^{13}C MultiCP/MAS NMR spectral analysis of pectin was conducted.

***Results*:** The type of extractant, duration of ultrasonication, extraction temperature, and solid-liquid ratio were optimized. Under the conditions of using 1-Butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4) as the extractant, the solid-liquid ratio of 1:20 g/mL, and the ultrasonic power of 600 w for 30 min at 30°C, the yield of 23.7% of tobacco stem pectin and the purity of 54.2% could be obtained. The optimized MultiCP sequence parameters, with 10 CP cycles of 1.0 ms and the repolarization time of 50 ms could obtain high-resolution spectra within a time of 1.0 h. The C-6 peaks of the pectin in spectra were fitted using the spectral deconvolution technique and calculated the methyl-esterification (DM) of the tobacco pectin, which was generally less than 50% and belonged to the low methyl esterification pectin. The pectin content of the tobacco sample was calculated using the standard curve method with the addition of dimethyl sulfone (DMS) as an internal reference. The results of this method were consistent with the colorimetric method.

***Conclusion*:** The ^{13}C MultiCP/MAS NMR method has the advantages of being green, fast, and accurate and provides a new technical tool for quantitative and qualitative studies of cell wall substances in tobacco samples.

Keywords: Solid-state NMR, ^{13}C MultiCP/MAS, pectin, tobacco, ionic liquid extraction, quantitative and structural analysis.

1. INTRODUCTION

Pectin is widely distributed in the middle layer of the plant cell wall and can be used as a gelling agent, thickener, and stabilizer [1-3]. It is also suitable for producing packaging films and coatings [4]. Pectin macromolecule is a linear chain consisting of (1,4)-α-D-galacturonic acid, partially methyl-esterified at the carboxyl group of C-6 [5], and the degree of methyl-esterification (DM) refers to the number of methyl-esters on the backbone [6]. As a crucial structural parameter, DM plays an important role in the efficacy of pectin, especially in its gelation and stability [7]. The main chemical components in tobacco include alkaloids, sugars, organic acids, proteins, *etc* [8]. Among them, pectin polysaccharide is a hydrocolloid substance that plays an important role in the stability of tobacco tissue structure. However, tobacco pectin produces harmful gases such as formaldehyde and formic acid during burning and smoking [9]. These gases are hazardous to consumers' health and harm the safety of tobacco smoking [10]. Therefore, it is important to establish a rapid and accurate analytical method to quantify the pectin content in tobacco.

The methods of determining pectin content are divided into chemical and instrumental analytical methods. Chemical methods include titration and gravimetric methods. The titration method is suitable for the determination of pure pectin and has limitations in use [11]; the gravimetric method is safe and reliable, but the operation is time-consuming and laborious [12]. The common instrumental methods include UV spectrophotometry [13], high-performance liquid chromatography (HPLC) [14, 15], and near-infrared spectroscopy

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(NIR) [16]. These methods are fast and accurate. However, the disadvantage is that the sample preparation is cumbersome; most of them need acidolysis or enzymolysis, and the structural information of plant macromolecules cannot be obtained [17]. Nuclear Magnetic Resonance (NMR) technology offers certain advantages in analyzing material structure [18]. In particular, liquid NMR is a powerful tool for the structural study of organic compounds, and rich molecular structure information can be obtained by analyzing the spectra [19]. However, it's difficult to find a suitable solvent to dissolve the sample. In contrast, the solid-state NMR (ssNMR) technique is a valuable tool for analyzing the structure and conformation of polysaccharides. It can also be employed to study insoluble biomass samples [20]. This technique has been used in food science [21, 22], agricultural science [23], pharmaceutical chemistry [24, 25], material science [26], and other fields [27]. However, due to the low sensitivity and low spectral resolution, it is challenging to meet the requirements of quantitative analysis in a relatively short period [28]. To achieve these objectives, the magic-angle-spinning (MAS) technique is used [29]. This technique homogenizes the anisotropic spin interactions between different nuclei and reduces the broadening of resonance lines.

At present, the most commonly used quantitative method is direct polarization (DP), which often obtains reliable quantitative information by setting the cycle delay greater than 5 times the maximum longitudinal relaxation time(T_1) in the sample [30]. However, for biomolecules, it usually takes tens of hours to obtain quantitative ^{13}C DP/MAS ssNMR spectra with good signal-to-noise ratios (SNR), which makes this method difficult to generalize for bulk measurements. Therefore, most NMR studies of biomolecules do not rely on DP but use cross-polarization (CP) [31]. Although CP reduces the detection time, the accuracy of quantification is poor for different types of nuclei. This is because, under the same experimental conditions, the cross-polarization efficiency of abundant (I) and dilute (S) nuclei are different. They have different CP kinetic behaviors [32]. Based on the above problems, researchers have proposed the method of multiple-cross polarization (MultiCP) to achieve S nuclei detection in a short time [33]. The concept of MultiCP was initially introduced by Gerstein in 1985 [34]. A quantitative detection technique based on MultiCP was proposed by Wu *et al*. in 1989 [35]. In 2014, Schmidt-Rhor *et al*. named this method MutiCP and further optimized it in 2017 to reduce signal loss due to 90° pulse resonance deviation and excitation pulse width accuracy using the 180° compensated pulse technique [33, 36]. Through the MultiCP process, the CP efficiencies of different S-nuclei in the sample system gradually increase and tend to the same level, thereby achieving quantitative information. The method is based on heteronuclear dipole interactions, so it is sensitive to nuclear spacing and molecular mobility and can obtain high-resolution NMR carbon spectra of solid materials [37]. Currently, the MultiCP method has been used to detect the crystallinity of cellulose [38], to quantify the component content of valine/methionine mixtures [39], and to determine the proportion of carbon-containing functional groups in kerogen [40]. However, the experimental parameters can affect the accuracy of measuring results for complex natural samples, thus need to be optimized.

In practice, one of the most important steps in structural analysis is to extract the "target" analyte from the sample [41]. Among the extraction methods for plant pectin, the most common industrial extraction technique is acid extraction [42]. This method is carried out under acidic, high-temperature conditions. Although the extraction efficiency is high, it pollutes the environment and is highly corrosive to the equipment [43]. Enzymatic extraction is a specialized extraction method with high extraction efficiency. However, it is costly [44]. In recent years, ionic liquids (ILs) have gained considerable attention as a designable "green" solvent in various fields [45], particularly the chemical industry, due to their good solubility and thermal stability [46]. They have been widely used to extract and separate biologically active substances. Ultrasonic-assisted extraction (UAE), a green separation technology, has rapidly developed in recent years [47]. It uses ultrasonic waves to enhance the speed of media molecules' movement, penetration, and interaction, thus promoting the solubilization of substances in plant tissues. The combination of ILs and UAE can fully utilize both advantages. Compared to the traditional acid extraction method, UAE-IL extraction offers a shorter extraction cycle and lower energy and solvent consumption. It provides a new method for the extraction of pectin in tobacco [48].

In this study, tobacco pectin was extracted using UAE-ILs extraction. The content and structure of tobacco pectin were analyzed using the ^{13}C MultiCP/MAS ssNMR technique. The experimental parameters of the MultiCP pulse sequence were optimized, and a suitable internal reference was selected to achieve rapid and accurate quantitative analysis of tobacco pectin. The methyl esterification information in the pectin samples was obtained using the deconvolution technique.

2. MATERIALS AND METHODS

2.1. Instruments, Materials and Reagents

Bruker Advance Ⅲ HD 400WB solid-state nuclear magnetic resonance spectrometer (Bruker, Germany), with Bruker 4 mm ^{1}H-^{13}C double resonance MAS probe. TU-1901 UV spectrometer (Beijing PuXi General Instrument Co., Ltd), DF-101S thermostatic bath (Gongyi Yuhua Instrument Co., Ltd), KJD-30L ultrasonic instrument (Changzhou Kejingda Washing Machine Co., Ltd), JA2003 electronic balance (Shanghai Shunyu Hengping Instrument Co., Ltd.), TDL-5-A centrifuge (Shanghai Anting Scientific Instrument Factory), DZF-ZB vacuum drying box (Beijing Ever Bright Medical Treatment Instrument Co., Ltd), XW-80A vortex oscillator (Jiangsu Xinkang Medical Instrument Co., Ltd), DHG-9140A drying oven (Shanghai Jinghong Experimental Equipment Co., Ltd), RE-52AA rotary evaporator (Shanghai Yarong Biochemical Instrument Factory).

Tobacco stems, flue-cured tobacco, and reconstituted tobacco are from Jiangxi Province of China. Burley tobacco samples are from the Hubei Province of China. Oriental tobacco samples are from Yunnan Province of China. All the above samples are provided by Jiangxi China Tobacco Industry Co. The tobacco samples were dried at 40°C for 2 h, crushed, sieved by a 40-mesh sieve, and sealed.

1-Butyl-3-methylimidazole tetrafluoroborate ([Bmim]BF_4, 97%); 1-butyl-3-methylimidazole bromide ([Bmim]Br, 97%); 1-butyl-3-methylimidazole chloride ([Bmim]Cl, 97%); cellulase (enzyme mixture, ≥1000 unit/g); polygalacturonic acid; dimethyl sulfone (DMS, 99%) was purchased from Aladdin (Shanghai, China). Sodium-3-(trimethylsilyl)propionate (TMSP, 98% atom) was purchased from Rhawn (Shanghai, China). Other chemical reagents used in this work were of analytical grade and the secondary distilled water was used throughout the work.

2.2. Pectin Extraction Method

Tobacco samples (2.5 g) were treated with 40 mL of [Bmim]BF_4 solution (1.0 M) and sonicated at 30°C for 30 min. Subsequently, 200 mL of distilled water was added to dissolve the extracted pectin and filtered to remove the precipitate. Following this, 30 mg of cellulase was added to the filtrate, which was then placed in a drying oven at 45°C for 12 h. After the hydrolyzed cellulose was removed by filtration, the resulting solution was the pectin solution. To further refine the pectin, it was heated to 80-90°C for decompression distillation, with approximately 150 mL of water being removed. Then, 200 mL of anhydrous ethanol was added to the remaining pectin solution and precipitated for 3-4 h. The mixture was centrifuged at 4000r/min for 5 min, with the precipitate being washed with 100 mL of anhydrous ethanol 2-3 times. The washed precipitate was put into a vacuum oven, drying at 55°C for 12 h. After that, the pectin sample was obtained [49]. Overall, pectin extraction procedures are schematized in Fig. (**1**).

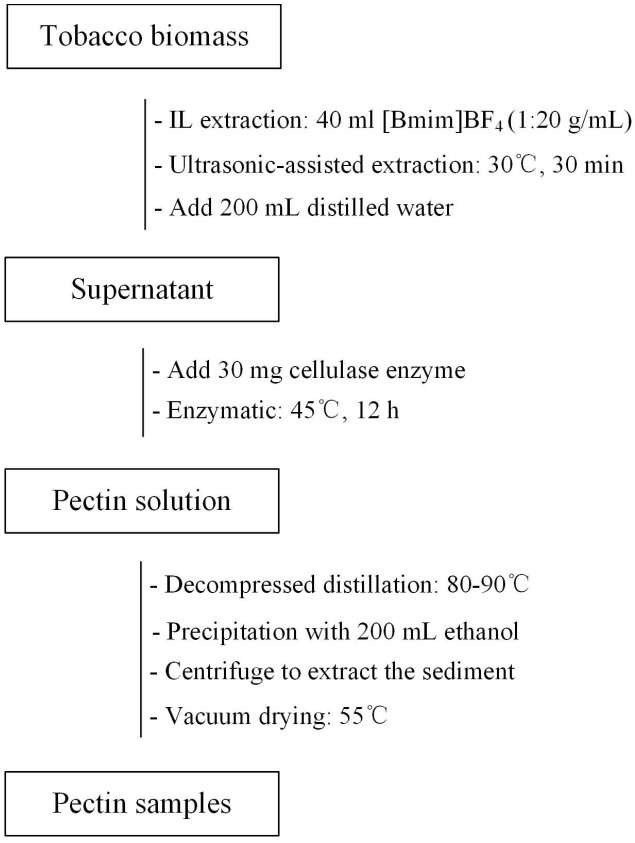

Fig. (1). Flowchart of pectin extraction from tobacco samples.

The yield and purity of tobacco pectin extracted by ultrasonic-assisted ionic liquid were calculated according to the following equations (**1**, **2**) [50, 51] respectively:

$$Pectin\ yield(\%) = \frac{W_1}{W_0} \times 100 \quad \text{(Eq. 1)}$$

$$Pectin\ purity(\%) = \frac{W_2}{W_1} \times 100 \quad \text{(Eq. 2)}$$

where W_0 represents the weight of the dried tobacco sample, W_1 represents the weight of extracted pectin, and W_2 represents the mass of galacturonic acid in pectin extract.

2.3. Solid-state Nuclear Magnetic Resonance Analysis of pectin

All samples in the study were analyzed on a Bruker 400 MHz AVANCE AV III spectrometer with a probe size of 4 mm. All spectra were measured at a spinning frequency of 14 kHz. For the acquisition of ^{13}C DP/MAS ssNMR spectra, 1024 scans were performed over a time of 29 h with a recycle delay of 100 s ($5T_1$). The optimal experimental parameters for the MultiCP sequence were set as follows: 10 periods of 1.0 ms CP, the duration of repolarization period of 50 ms, a setting of the number of scans to 2048, and a relaxation delay of 1.0 s. The ramp for CP was implemented in 11 steps and a 1% amplitude increment. The measuring time of MultiCP requires 1.0 h.

For each NMR quantitative test, 100 mg of tobacco pectin and 5 mg of DMS were accurately weighed, mixed, and carefully ground into a homogeneous powder. Then, the samples were tightly packed in the 4 mm rotor for measuring the spectra. The obtained NMR spectra were optimized using MestReNova14.3.3 software, including adjustment of the window function, phase correction, baseline correction, and curve smoothing [52].

2.4. Spectral Deconvolution Analysis

The decomposition of ^{13}C MultiCP/MAS NMR spectra in the regions of 180-165 ppm was pursued by MestReNova14.3.3 software. Three Lorentzian/Gaussian peaks were introduced. After several iterations, the residuals gradually decreased until the fit converged. The results of peak separation were applied to obtain the DM (Eq. 3) [53].

$$DM(\%) = \frac{A_{COOCH3}}{Ac-6} \times 100 \quad \text{(Eq. 3)}$$

Where A_{COOCH3} represents the integral area of the methyl-substituted galacturonic acid, $A_{C\text{-}6}$ represents the integral area of the C-6 peak.

2.5. Standard Curve of ^{13}C MultiCP/MAS ssNMR

To construct a mass-based calibration curve for pectin, a series of mass gradient mixtures of polygalacturonic acid (10, 20, 30, 40, and 50 mg) and 5 mg of DMS were prepared. To maintain the same filling volume, part of the sample was homogenously mixed with NaCl and then loaded into a 4 mm rotor. The spectrum of each mixture was acquired using the ^{13}C MultiCP/MAS ssNMR. The standard curve was plotted using the mass of polygalacturonic acid as the x-axis and the ratio of the integrated area of the C-6 (180-165 ppm) peak to the DMS (~42.5 ppm) peak in the spectrum as the y-axis [54].

2.6. Colorimetric Method Analysis of Pectin

To verify the experimental results of NMR, the GalA content was determined using the m-hydroxy diphenyl colorimetric method reported by Blumenkrantz [55]. The analytical procedure is detailed in the Supplementary Material, the colorimetric calibration curve is provided in Fig. (**S1**), and Table **S1** shows the moisture content of different types of tobacco samples. In addition, the accuracy of the results was verified by the sample spiking test, and the results are listed in Table **S2**.

3. RESULTS AND DISCUSSION

3.1. Ultrasonic-assisted Ionic Liquid Extraction of Pectin

The extraction process has the most significant effect on the yield and purity of pectin [56]. In this study, conditions such as the type of ILs, the duration of ultrasonication, the extraction temperature, and the solid-liquid ratio were optimized to achieve the best extraction results.

An ionic liquid is a non-molecular solvent composed of ions. It usually consists of organic cations containing heteroatoms and inorganic or organic anions [57]. The insoluble pectin constituents can be directly contacted and hydrolyzed into soluble pectin under acidic conditions and thus released from plant tissues [58]. Therefore, three representative acidic hydrophilic ILs were selected for the extraction of tobacco stem samples in this experiment: [Bmim]Cl, [Bmim]Br, and [Bmim]BF_4, and the pH values of these selected ILs are shown in Table **S3**. According to the reference [59], the concentration of ILs was chosen to be 1.0 mol/L. The extraction results of three ILs were compared under the conditions of ultrasonication at 30°C for 30 min, and the solid-liquid ratio of 1:20 g/mL, as shown in Fig. (**2**). The highest yield and purity of pectin was obtained using [Bmim]BF_4 extractant. This is because the cations such as Ca^{2+} and Mg^{2+} in the tobacco stem samples bind to pectin through ionic bonding and have a confining effect on pectin. The [Bmim]BF_4 extractant has strong binding ability with Ca^{2+}, $Mg^{2+,}$ and other ions, which can increase the solubility of pectin and thus improve the yield [59]. Therefore, [Bmim]BF_4 was chosen as the extractant in this study.

Secondly, the extraction process conditions were optimized. The yield and purity of pectin from tobacco stem under different conditions were obtained by choosing different levels of duration of ultrasonication of 10, 20, 30 min; extraction temperature of 20, 30, 50°C and solid-liquid ratio of 1:10, 1:20, 1:30 g/mL. The sample size was maintained constant, and the results are shown in Fig. (**3**). It can be seen that the yield of pectin progressively increased with the extension of the duration of ultrasonication at 10, 30, and 50 min, while the purity of pectin slightly decreased at 50 min. This is because a short duration of ultrasonication leads to insufficient hydrolysis, whereas a long time leads to excessive hydrolysis, thereby increasing the fragmentation of pectin molecules and lowering the purity of pectin [60, 61]. Consequently, the duration of ultrasonication was chosen to be 30 min. The extraction temperature also plays a crucial role. At temperatures of 20, 30, and 50°C, the yield and purity of pectin show a trend of initial increase followed by decrease. As the temperature rises, the molecular thermal movement intensifies, enhancing the solvent and solute diffusion ability and greatly accelerating the extraction process. However, higher temperatures will cause degradation of pectin molecules, deepening of color, and browning. It affects its property and structure [62]. Therefore, the extraction temperature was chosen to be 30°C. The solid-liquid ratio determines the contact area of liquid with solid and influences the pectin yield. A low solid-liquid ratio will lead to incomplete immersion of raw materials in the solvent. A high solid-liquid ratio requires large solvent consumption and causes high acidity, which is unbeneficial for the extraction of pectin [63]. Consequently, the optimal process conditions for pectin extraction were: duration of ultrasonication of 30 min, extraction temperature of 30°C, and solid-liquid ratio of 1:20 g/mL, under which the yield was 23.3% and the purity was 54.2%.

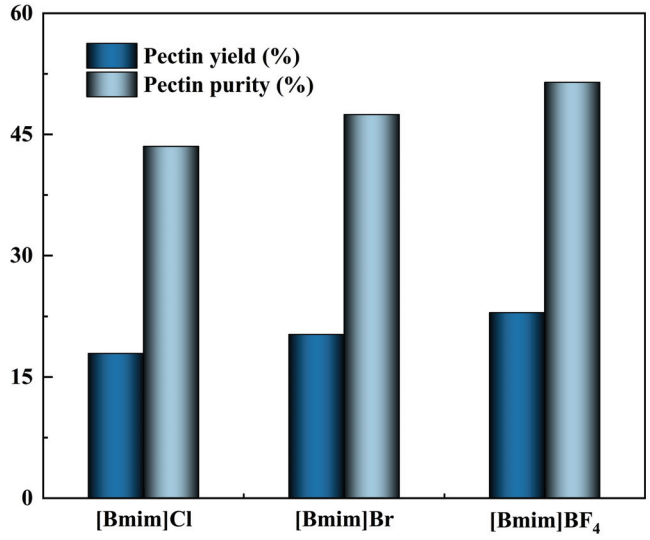

Fig. (2). Effect of different types of ionic liquids on the yield and purity of pectin from tobacco stems. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

To verify the robustness of the extraction method, three parallel experiments were carried out under optimal conditions, and the pectin content of the tobacco stem samples was determined by the colorimetric method. The results were 11.2%, 11.8%, and 11.8%, respectively, with a relative standard deviation of 2.98% (n=3), indicating that the extraction formula is stable.

3.2. Optimization of Parameters of MultiCP/MAS Pulse Sequence

The ^{13}C DP/MAS ssNMR spectrum was chosen to evaluate the ^{13}C MultiCP/MAS ssNMR spectrum due to its high spectrum intensity and reliable quantitative information [64]. During the experimental process of ^{13}C DP/MAS ssNMR, the settings of the relevant experimental parameters directly affect the accuracy of the DP spectrum [65]. Among them, the recycle delay is an important parameter affecting the accuracy of the signal peak area, and its setting is directly related to ^{13}C longitudinal relaxation time ($T_{1,C}$) [66]. Typi-

cally, a delay 3-5 times that of the longitudinal relaxation time is inserted in between every scan of the NMR experiment, which allows full restoration of the nuclear spins to equilibrium along the direction of the polarizing magnetic field. It is guaranteed that the intensity of the signal being integrated is fully proportional to the number of nuclei [67, 68]. In this experiment, the pectin of the tobacco stem was chosen to determine the $T_{1,C}$. The obtained relaxation curve was fitted with the function $I(t)=I(0)\exp(-x/T_{1,C})$ [69] as shown in Fig. (**4**). The $T_{1,C}$ of the C-6 peak (180-165 ppm) was 20.9 s. Therefore, the ^{13}C DP/MAS recycle delay was set to 100 s (~$5T_{1,C}$), and the accurate quantitative spectrum was obtained in 29.0 h. The result is illustrated in Fig. (**5**).

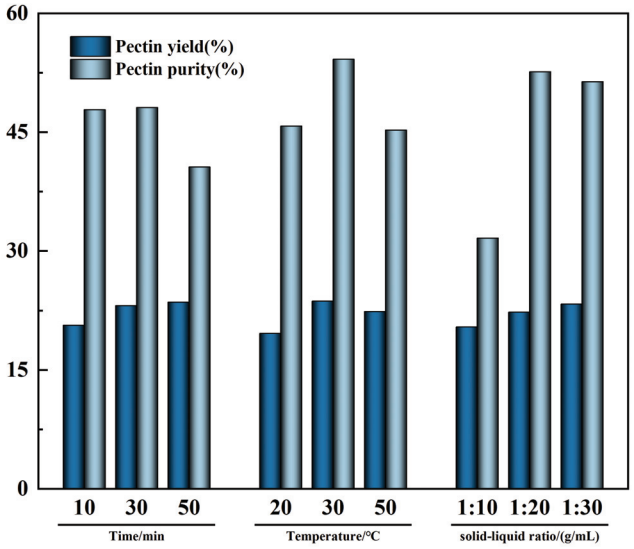

Fig. (3). Effect of time, temperature, and solid-liquid ratio on the yield and purity of pectin from tobacco stems. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

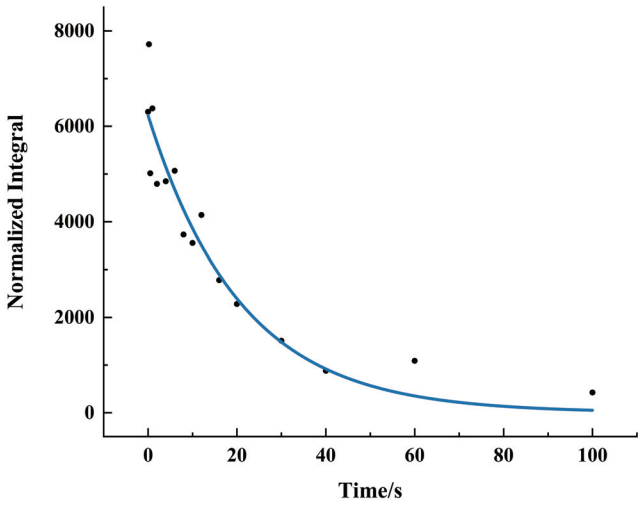

Fig. (4). Mono-exponential $T_{1,c}$ fitting of pectin. R^2=0.9043, $T_{1,C}$=20.90 s. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

In the MultiCP sequence, important experimental parameters include the contact time of CP blocks (t_p), the duration of the repolarization period between each CP block (t_z), and the number of CP blocks (n). Due to the complex kinetics of polarization transfer in MultiCP, the difference in t_p of CP blocks often produces large differences, so the optimization of t_p is the most critical [33]. In our test, the t_p of 0.010 ms, 0.10 ms, 0.30 ms, 0.50 ms, 0.80 ms, 1.0 ms, 1.3 ms, 1.5 ms, and 2.0 ms were designed for the pectin of tobacco stem sample. Combined with ^{13}C DP/MAS ssNMR spectra, the differences between MultiCP and DP spectra at the C-6 peak of pectin were compared (three representative spectra were selected with t_p of 0.10 ms, 1.0 ms, and 1.3 ms, respectively), as depicted in Fig. (**5**). At 180-165 ppm, the peak intensity under t_p at 0.10 ms was weak, and there was a large difference compared with the DP spectra. As t_p increased, the peak intensity subsequently increased, and the difference with the DP spectrum lessened. When t_p increased to 1.0 ms, the spectrum agreed well with the DP spectrum. However, when t_p increased to 1.3 ms, the signal intensity did not significantly enhance. As t_p lengthens, the polarization transfer efficiency due to the spin-lattice relaxation of the ^1H nucleus in the rotational coordinate system decreases, which leads to the weakening of the ^{13}C signal intensity. Considering all factors, t_p of 1.0 ms is appropriate for pectin of different types of tobacco samples, including tobacco stem.

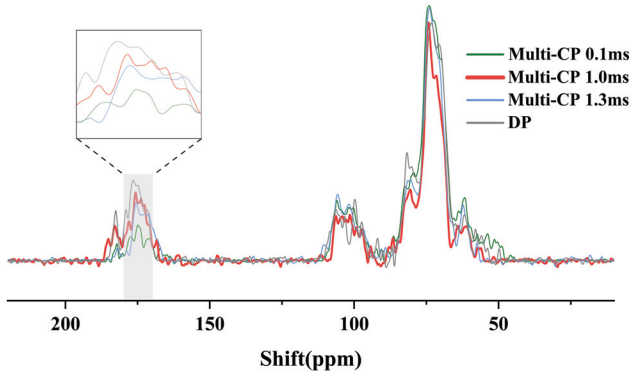

Fig. (5). ^{13}C MultiCP/MAS ssNMR spectra and ^{13}C DP/MAS ssNMR spectrum of tobacco stem samples. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The crucial innovation of the MultiCP approach is repeated blocks of CP separated by periods of duration t_z, during which the ^1H magnetization can recover to a near-equilibrium value [33]. Therefore, the setting of t_z is needed to ensure that most of the ^1H magnetization is restored to the thermodynamic equilibrium state while minimizing the decay of the ^{13}C magnetization. In addition, the setting of t_z directly affects the experiment time of MultiCP, so the setting of t_z should not be too long. Since, typically $t_z \approx 2T_{1,H}$, at which time about 63%~86% of ^1H magnetization relaxation is recovered [70]. The $T_{1,H}$ of tobacco pectin was measured, and the curve was fitted using the $I(t)=I(0)+P\exp(-x/T_{1,H})$ function, and the result is shown in Fig. (**6**). $T_{1,H}$=21 ms was measured, so t_z was set to 50 ms for this experiment.

The final optimized parameters determined in this work are t_p of 1.0 ms and t_z of 50 ms. 2048 scans and 10 CP blocks were chosen to obtain the spectra within 1.0 h. Under the

optimal parameters, the measurement time is greatly shortened compared to DP/MAS with high SNR.

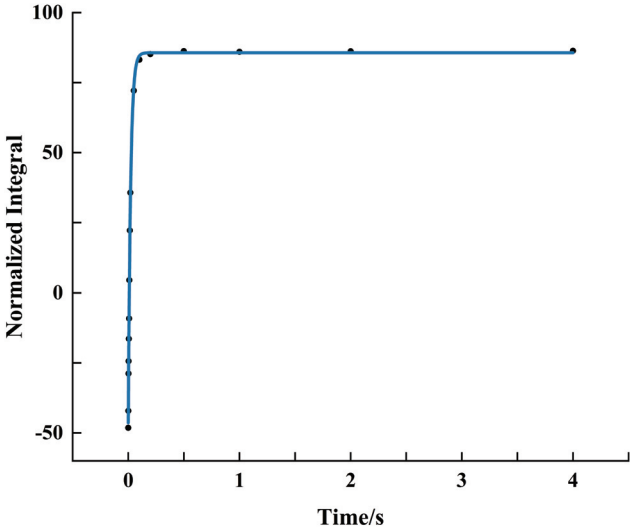

Fig. (6). Triple exponential $T_{1,H}$ fitting of pectin. R^2=0.9979, $T_{1,H}$=21 ms. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.3. Attribution of Pectin ^{13}C MultiCP/MAS ssNMR Spectra

Under optimized conditions of instrumental parameters, the standard sample of polygalacturonic acid and the pectin sample extracted from tobacco stems were analyzed using ^{13}C MultiCP/MAS ssNMR. The spectra are obtained as shown in Fig. (**7**). The signal peaks of the spectra were attributed. Among them, 19 ppm is the signal after acetylation of O-2 and O-3, and 54 ppm is the signal of carboxyl methyl esterification of pectin. 65-75 ppm is the C-2,3,5 peaks of pectin, 78 ppm is the signal of the C-4 peak of pectin, 102 ppm is the signal of the C-1 peak, and the C-6 peak region of pectin at the chemical shift of 180-165 ppm [71].

Of these, the carboxyl C-6 carbons of galacturonic units are present as carboxylic acid (COOH), ester ($COOCH_3$), and carboxylate anion (COO^-) [72]. The difference in chemical shifts of these peaks is significantly less than the sum of their half-width, so the resonance signals overlap. To obtain quantitative and qualitative information, a split-peak fit to the C-6 region of pectin is required. This process requires deconvolution of the spectra using Mestrenova14.3.3 software. In this experiment, several hybrid Lorentz-Gaussian functions were mainly used as fitting models for split-peak fitting of the C-6 peaks [73]. The fitting results are shown in Fig. (**8**), which decompose the signal in the region of 180-165 ppm into three spectra with different displacements, namely -$\underline{C}OO^-$ (δ175-173 ppm, FIT1), -$\underline{C}OOCH_3$ (δ~172 ppm, FIT2), -$\underline{C}OOH$ (δ~169 ppm, FIT3). The DM of pectin was calculated by the ratio of the integrated area of $\underline{C}OOCH_3$ to the total integrated area of the C-6 peak. Three sets of deconvolution treatments of extracted pectin from tobacco stem samples were done in parallel in the experiment, and the results are shown in Table **1** (Table **S4** for deconvolution parameters), which shows that the DM of the tobacco stem pectin is less than 50%, and it is a low methyl esterified (LM) pectin.

3.4. Quantitative Analysis of Pectin

The C-6 peak position of pectin is stable and less interfering, and a strong linear correlation exists between the peak area and the galacturonic acid content of the pectin molecule. Quantitative analysis can be effectively achieved

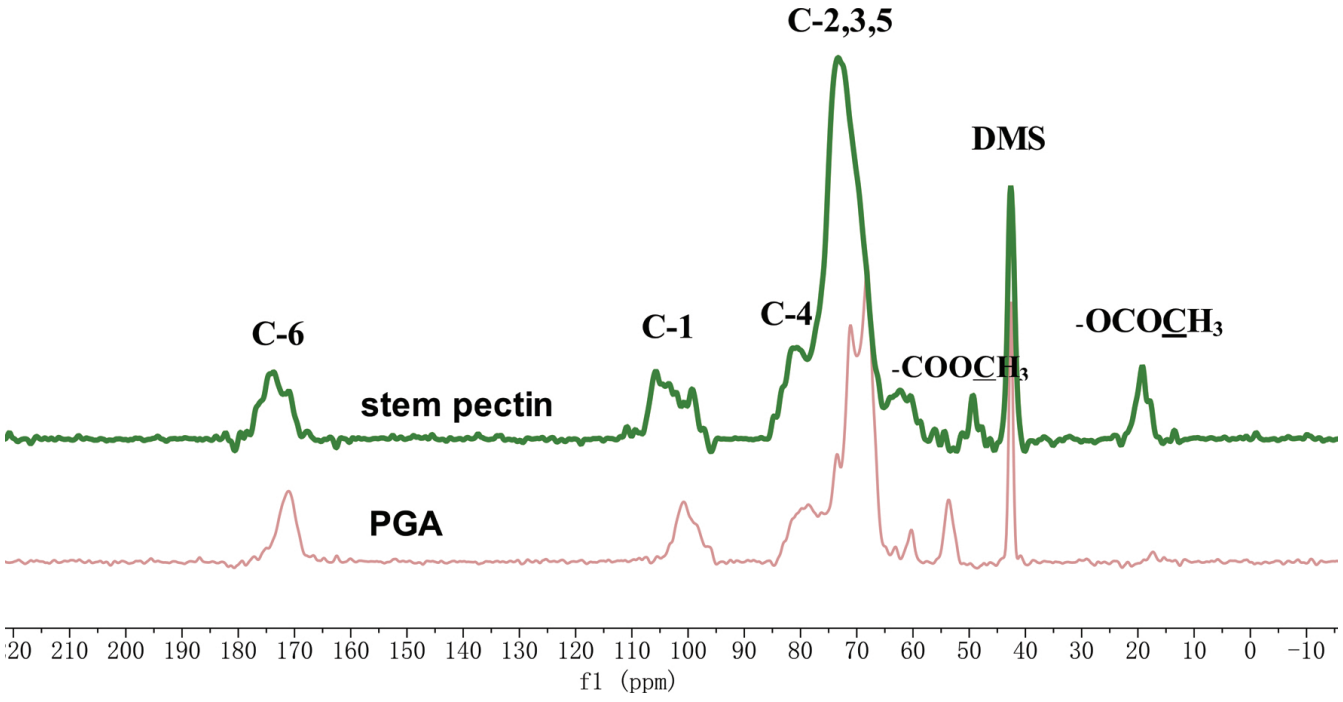

Fig. (7). ^{13} C MultiCP/MAS ssNMR spectra of polygalacturonic acid (PGA) and pectin extracted from tobacco stems. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

by adding an internal reference to the NMR sample. The properties required of suitable internal reference in NMR are as follows: (1) favorable chemical inertness and stability; (2) suitable chemical shift in a vacant region of the spectrum for avoiding peak overlaps; (3) small line width, even a small amount of internal reference can produce a considerable intensity; (4) good relaxation characteristics[74]. Based on the above criteria, some possible internal reference compounds were screened out: TMSP (T_1 =4.0 s), L-alanine (T_1 >3.5 s), adamantane (T_1 =1.9 s), and DMS (T_1 =0.17 s). These compounds have good signal resolution and fulfill the above criteria[75]. In this study, the signals from TMSP and DM on spectra were examined (Fig. (**S2**)). The spectrum for DMS shows a signal sharp peak at 42.5 ppm, whereas TMSP has signal peaks at both 0 ppm and 180 ppm [52, 76]. Since the signal peak of TMSP partially overlaps with the C-6 region, it may affect the quantification of the C-6 peak. Therefore, DMS was chosen as the internal reference for the experiment.

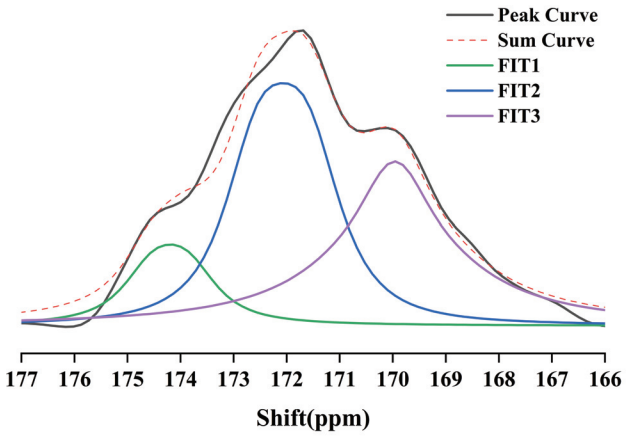

Fig. (8). Deconvolution spectrum of the C-6 spectral region of tobacco stem pectin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Deconvolution analysis and DM of pectin samples from tobacco stems.

Sample	Area [a], 10^3				DM (%)
	$A_{C\text{-}6\ tol}$	A_{COOCH3}	A_{COOH}	A_{COO^-}	
1	35.0	16.7	6.3	12.0	47.7
2	33.3	15.5	6.7	11.1	46.5
3	30.5	14.2	5.6	10.7	46.6

Note: a: Peak area of spectral peak.

In this work, the standard curve method, in addition to an internal reference, was used to obtain more accurate quantitative results. The standard curve is reported in Fig. (**9**), and the curve equation is y= 0.1528x-0.2905 (R^2=0.9944). The limit of detection (LOD) and limit of quantification (LOQ) of pectin were 0.41 mg/g and 0.91 mg/g at 3 times and 10 times SNR, respectively.

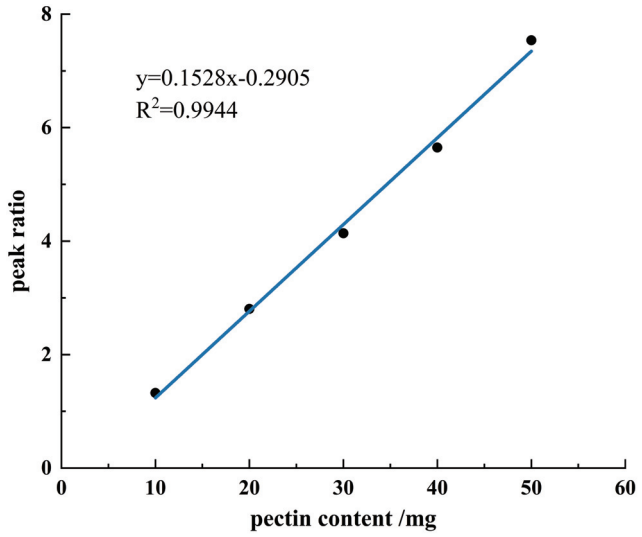

Fig. (9). NMR quantitative standard curve of tobacco pectin. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

To assess the precision of the ^{13}C MultiCP/MAS ssNMR method for the determination of tobacco pectin content, the measurement was repeated 6 times with a pectin sample extracted from tobacco stem, and the results are shown in Table **2**. The mean value of the six measurements was 11.6% with an RSD (n=6) of 2.18%. This indicates that the method has good precision. In addition, to evaluate the accuracy of the method, two levels of polygalacturonic acid standard samples were added to each of the two sets of tobacco stem pectin samples, which were then analyzed by ^{13}C MultiCP/MAS ssNMR. The determination of galacturonic acid content was repeated three times for each sample, and the results are shown in Table **3**. The recoveries of the tobacco pectin ranged from 100.6% to 107.3%, indicating that ^{13}C MultiCP/MAS ssNMR was an accurate method for the determination of tobacco pectin content.

Table 2. Precision of the quantitative method of ^{13}C MultiCP/MAS ssNMR.

No.	Pectin		
	NMR Method (%)	Colorimetric Method (%)	Relative Error (%)
1	11.5	11.7	-1.71
2	11.3		-3.42
3	11.5		-1.71
4	12.0		2.56
5	11.5		-1.71
6	11.8		0.85

3.5. Analysis of Different Types of Tobacco Pectin

A fairly accurate ^{13}C MultiCP/MAS ssNMR method has been applied to analyze the pectin of five varieties of tobacco samples and the obtained spectra are shown in Fig. (**10**). The

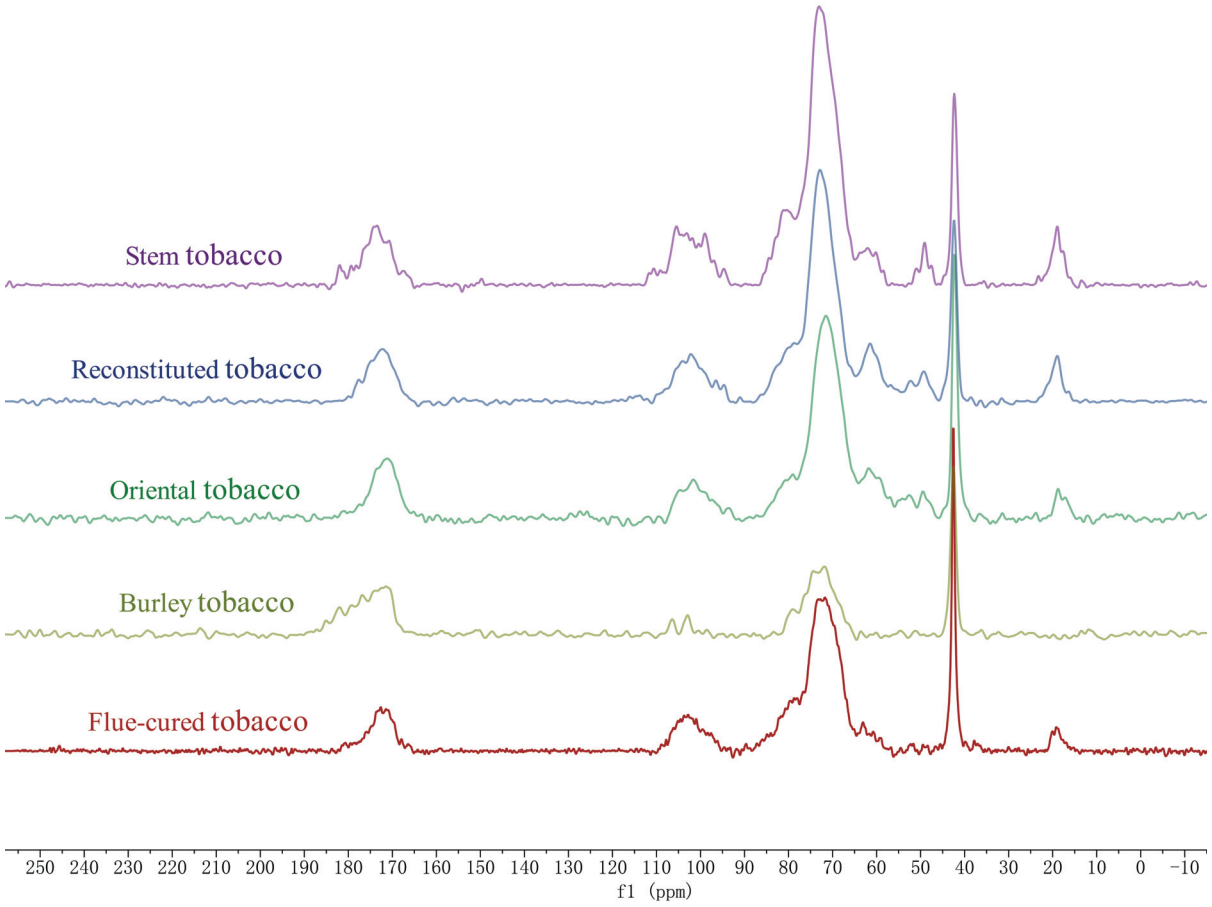

Fig. (10). ^{13}C MultiCP/MAS ssNMR spectra of different types of tobacco samples. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 3. Accuracy of 13CMultiCP/MAS ssNMR quantification methods.

No.	Pectin Content (%)	Spiking Level (mg)	RSD (%)	Recovery (%)	Average Recovery (%)
1#	11.6	7.9	1.17	105.1	104.7
		14.6	1.57	107.3	
2#	11.8	6.4	4.30	106.0	
		12.0	2.66	100.6	

spectra of different types of tobacco samples showed similarity to tobacco stem sample, in which the C-6 peaks of the pectin all had good resolution. The pectin content and DM of the four tobacco samples determined by the ^{13}C MultiCP/MAS ssNMR method (Table **S5** for details of deconvolution parameters) and the pectin content determined by the colorimetric method are listed in Table **4**. It can be seen that the measurements of ssNMR and colorimetric methods are consistent, with relative errors ranging from -3.53% to 2.15%. The DM of the four samples was higher for flue-cured tobacco and reconstituted tobacco and lower for burley and oriental tobacco, indicating a significant difference in the DM of different types of tobacco pectin. Some researchers have used gas chromatography-mass spectrometry to determine the DM of soluble pectin in flue-cured tobacco, burley tobacco, and oriental tobacco, and the results showed that the DM of pectin was higher in flue-cured tobacco and lowest in burley tobacco [77], which was consistent with the results of this work.

Table 4. Comparison of the quantitative results of ^{13}C MultiCP/MAS ssNMR method and colorimetric method for different types of tobacco samples.

Samples	Pectin			
	NMR Method (%)	Colorimetric Method (%)	Relative Error (%)	DM (%)
Reconstituted tobacco	8.2±0.4	8.5	-3.53	57.0
Oriental tobacco	7.8±0.2	7.9	-1.26	40.3
Burley tobacco	9.5±0.4	9.3	2.15	39.0
Flue-cured tobacco	9.7±0.4	10.0	-3.00	53.9

CONCLUSION

In this study, a method of ultrasonic-assisted ionic liquid extraction of pectin is developed, which is more environmentally friendly and efficient than the conventional method. The ^{13}C MultiCP/MAS ssNMR technique is also optimized to analyze the content and structure of tobacco pectin. The detection time of the method is only one-thirtieth of DP. Compared with CP, the method is more sensitive and can obtain quantitative information using DMS as the internal reference, eliminating the effects of signal fluctuation and thus improving the quantitative accuracy. The method has the advantages of being green, fast, and accurate and provides a new tool for determining pectin content and structure in tobacco.

LIST OF ABBREVIATIONS

CP	=	Cross-Polarization
DMS	=	Dimethyl Sulfone
DP	=	Direct Polarization
HPLC	=	High-Performance Liquid Chromatography
ILs	=	Ionic Liquids
MAS	=	Magic-Angle-Spinning
NIR	=	Near-Infrared Spectroscopy
NMR	=	Nuclear Magnetic Resonance
SNR	=	Signal-to-Noise Ratios
UAE	=	Ultrasonic-Assisted Extraction

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of this study are available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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