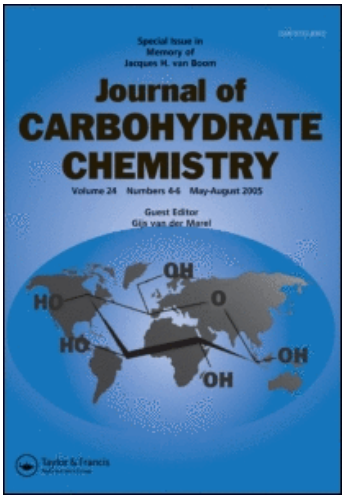

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^{13}C CP/MAS NMR SPECTROSCOPY IN THE ANALYSIS OF PECTINS

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ABSTRACT

^{13}C CP/MAS NMR spectra of powder commercial pectins were recorded and interpreted. NMR spectral results were applied for the calculation of galacturonic acid content (GalA), degrees of methylation (DM) and acetylation (DA). In most cases NMR values from GalA and DM agreed with values obtained by conventional methods (photometry and HPLC). The determination of DA using NMR data was successful only in the case of sugar beet pectin with high acetyl content. The C-6 carbon region was studied in detail and the chemical shift and the shape of peaks in this region were strongly influenced by the ratio of uronic carboxyl forms. Correlation between C-6 carbon chemical shift and DM values for pectins in their protonated form was evaluated.

INTRODUCTION

Pectin is a structural polysaccharide of plant cell walls. It plays an important role in softening and loss of tissue cohesion during ripening of vegetables and fruits. Pectins are widely used in the food industry as gelling and stabilising agents.[1,2] Basically pectins are polymers of (1→4) linked α-D-galacturonic acid that are partially methyl esterified.

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). The galacturonic acid backbone is sometimes interrupted by L-rhamnose residues while some pectins are also *O*-acetylated or contain neutral sugar side chains. Structural analysis of pectins from different sources is very important for use in food technology and characterisation of industrial scale raw materials.[3,4]

Preparation of pectin solutions for conventional NMR analysis is difficult. Although pectins are soluble in water, their solutions are very viscous and this complicates applying NMR solution techniques for their analysis.[5] Solid state ^{13}C NMR has proven to be a valuable technique in structural and conformational analysis of polysaccharides[6,7] and has been applied in the study of chain conformation of pectin macromolecules in solid and gel states.[8] Solid state NMR methods were also used in investigation of plant cell wall materials containing pectic compounds. Van Gorsel[9] used solid state NMR spectroscopy in determination of the loss of cell wall integrity due to chilling. These changes are associated with changes of pectin structure in cell walls. Irvin *et al.*[10] studied apple cell wall samples using ^{13}C CP/MAS NMR techniques and showed that polyuronide content and degree of methylation remained constant during ripening of apple tissues. On the basis of solid state ^{13}C NMR spectroscopy Hoagland and Pfeffer[11] estimated pectin content in carrot fibres and methylation degree of carrot pectin.

The presented contribution summarises results of ^{13}C CP/MAS NMR spectroscopy of commercial pectins with different structural properties.

RESULTS AND DISCUSSION

The polygalacturonic acid and pectins studied and their carbon chemical shifts are presented in Tables 1 and 2, respectively. Pectins with high or low content of methoxyl groups were assigned as HM or LM. The ^{13}C CP/MAS NMR spectra of polygalacturonic acid (**1**), citrus pectin **2** and sugar beet pectin **5** are presented in Figure 1. The spectrum of **1** was used as the basis for the interpretation of related pectin spectra (Table 2, Figure 1). The intense signal at 171.6 ppm was assigned to C-6 carbon of COOH group and the resonance at 101.1 ppm to the anomeric C-1 carbon. The peaks in the range of 60 to 90 ppm are from carbons of pyranoid ring.[4,12] The C-4 carbon signal at 79.0 ppm is close to the resonances between 67-71 ppm from other ring carbons.

The spectra of all pectins, with the exception of LM citrus pectin **8**, had an intense resonance at 53 ppm representing methyl carbons of the methyl ester $COO\underline{C}H_3$ (Table 2). Sugar beet pectin **5** also had signals at 20.9 and 24.5 ppm from methyl carbons of acetyl

Table 1. The specification of pectin samples

No	Specification of samples
1	polygalacturonic acid, Koch-Light Lab., England
1a	potassium salt of **1**
2	citrus pectin, Koch-Light Lab., England
2a	potassium salt of **2**
3	apple pectin, Smiřice, Danisco Ingr., Czech Republic
4	sugar beet pectin T, Sweden
5	sugar beet GENU pectin type BETA[a]
6	LM citrus GENU pectin type VIS-L[a]
7	HM citrus GENU pectin type USP-H[a]
8	LM citrus GENU pectin type LM-1912CS[a]
9	LM amidated citrus GENU pectin type LM-101AS[a]
10	LM amidated citrus GENU pectin type LM-102AS[a]
11	LM amidated citrus GENU pectin type LM-104AS[a]
12	HM citrus GENU pectin type A medium rapid set[a]
13	HM citrus GENU pectin type B rapid set[a]
14	HM citrus GENU pectin type BA-KING[a]
15	HM citrus GENU pectin type D slow set[a]

a. Copenhagen Pectin Factory, Denmark

groups OCO$\underline{C}H_3$, typical for this pectin. Signals at 17.6 and 17.4 ppm from sugar beet pectins (samples **4** and **5**, respectively) probably belonged to methyl carbons of rhamnose residues (Table 2). Assignment of pectins' spectra signals discussed here were in agreement with literature assignment.[12,13]

NMR spectral results were applied to determine some important pectin characteristics in comparison with those obtained by conventional methods. The content of galacturonic acid units (GalA), the degree of methylation (DM) and acetylation (DA) 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 to the integral intensity of signals in the range of 60 to 110 ppm. This region contains signals of all pyranoid ring carbons.

Table 2. ^{13}C CP/MAS NMR chemical shifts (in ppm) for polygalacturonic acid and pectins.

Sample	Chemical shift (ppm)						
	C-1	C-2,3,5	C-4	C-6	$COO\underline{C}H_3$	$O\text{-}CO\underline{C}H_3$	CH_3-Rha
1	101.12	69.10	79.83	171.61	-	-	-
1a	100.84	71.81	79.27	175.60	-	-	-
		68.91					
2	100.97	66.93	79.00	171.00	53.33	-	-
2a	100.29	69.28	79.69	174.92	53.41	-	-
				171.15			
3	101.61	71.03	sh[a]	171.55	53.55	-	-
4	101.07	66.95	sh	175.59	53.68	-	17.57
5	100.93	70.08	sh	171.19	53.23	20.95	17.38
6	101.03	69.05	sh	171.26	53.25	-	-
7	101.08	69.01	sh	171.08	53.29	-	-
8	101.21	69.79	79.16	171.75	-	-	-
9	101.21	69.25	76.98	171.30	53.36	-	-
10	101.43	69.10	79.43	171.39	53.56	-	-
11	101.11	69.04	78.76	171.16	53.43	-	-
12	100.89	66.95	79.24	170.61	53.25	-	-
13	100.98	69.17	sh	170.57	52.85	-	-
14	100.73	69.11	sh	170.66	53.10	-	-
15	100.62	66.87	sh	171.08	53.48	-	-

a. shoulder of C-2,3,5 peak

Combined acid and ester C-6 carbon signals can also be used as a standard for the DM estimation.[5] Pfeffer *et al.*[14] applied solution ^{13}C NMR spectroscopy in the determination of the relative proportions of free and methylated carboxyl groups in pectin gels as the ratio of peak areas at 172.8 ppm (COOH) relative to the areas either at 171.3 ppm ($\underline{C}OOCH_3$) or 53.7 ppm ($COO\underline{C}H_3$). In the case of the CP/MAS technique, the signals of C-6 carbons in acid and ester forms could not be resolved and for determination of DM we could only use the resonance of the methyl ester carbon. The ratio of the

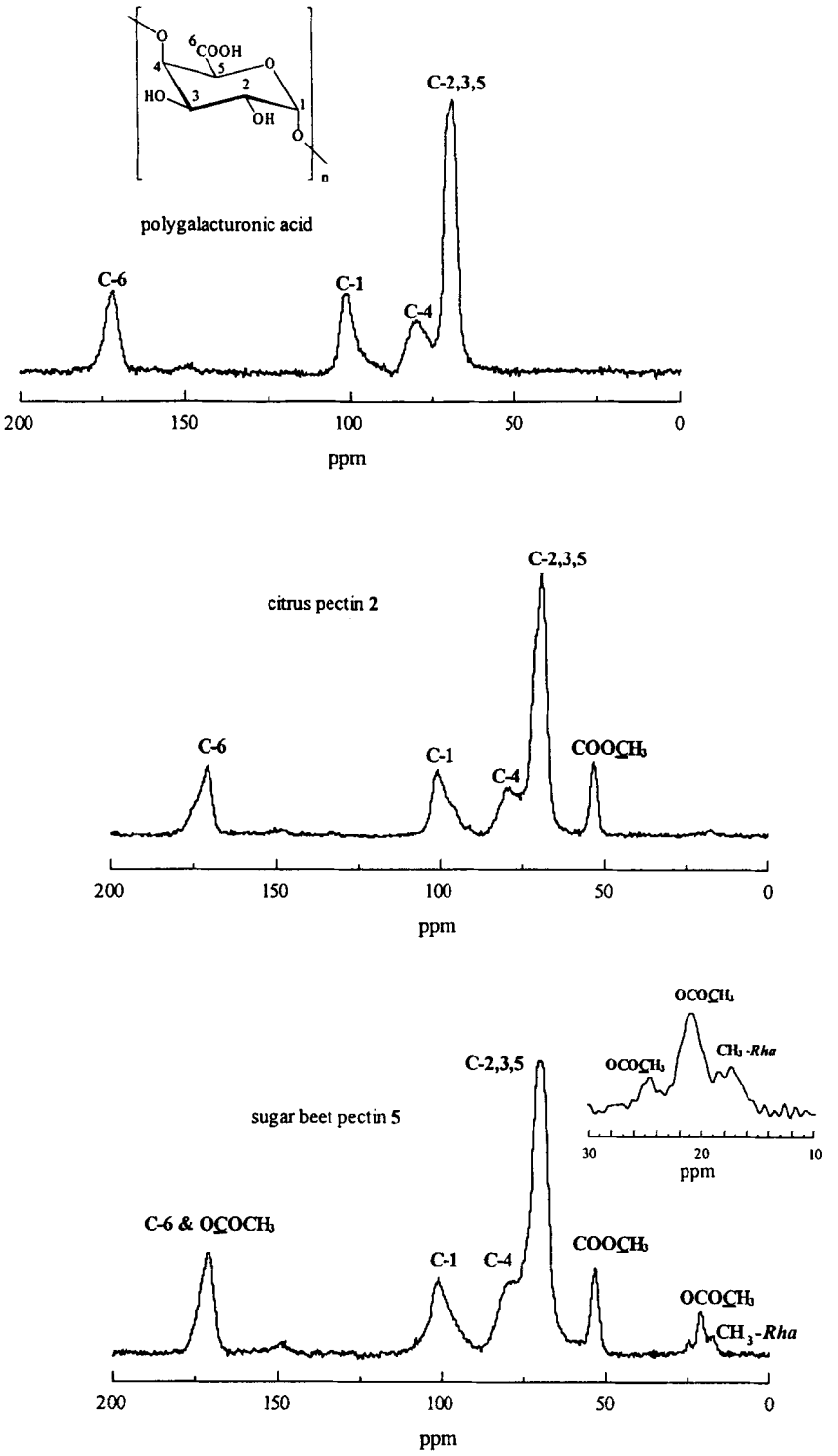


Figure 1. ^{13}C CP/MAS NMR spectra of polygalacturonic acid (**1**) and pectins **2** ar with resonance assignments.

Table 3. Galacturonic acid content (% w/w) of pectin samples determined by ^{13}C CP/MAS NMR spectroscopy and photometry with 3-hydroxybiphenyl (520 nm).

Sample	GalA, %w/w	
	^{13}C CP/MAS NMR spectroscopy	photometry at 520 nm
1	95	91
2	90	88
3	55	73
4	80	85
5	85	83
6	85	79
7	85	79
8	80	81
9	90	73
10	85	85
11	95	65
12	90	76
13	90	90
14	80	83
15	95	87

integral intensity of the signal at 101 ppm to the integral intensity of the signal at 60-110 ppm was used as a control. The average value of this ratio was 0.215 and the theoretical value was 0.2. The maximum error of the estimation was in an expected range of 10%.

The results of calculations from NMR spectra and the experimental data of conventional methods are reviewed in Tables 3-4. The degree of methylation values calculated from ^{13}C NMR spectra agreed well with those obtained by both conventional methods. The agreement was not so good in the case of galacturonic acid content in samples **3**, **9**, **11** and **12**. It may be connected with the complex nature of the C-6 resonance used in this calculation.

The solid-state NMR technique has been used in determination of the changing degree of *N*-acetylation during deacetylation of chitin and chitosan.[15,16] In both these reports the percentage of deacetylation was calculated by comparing the area of the *N*-$CO\underline{C}H_3$ resonance to the resonances of glucose carbons. As the acetyl content in most

Table 4. The degrees of methylation and acetylation of pectins (%) determined by ^{13}C CP/MAS NMR spectroscopy and conventional methods (photometry and HPLC).

Sample	^{13}C CP/MAS NMR	photometry[a]	HPLC
	A. Degree of methylation, %		
1	0	2	0
2	50	51	46
3	30	34	32
4	15	17	15
5	45	47	41
6	50	46	54
7	60	59	61
8	10	13	-
9	50	46	-
10	35	39	-
11	40	37	-
12	75	68	-
13	80	73	-
14	70	69	-
15	60	64	-
	B. Degree of acetylation, %		
5	30	36	31

a. A-at 570 nm, B-at 540 nm

pectin samples was very low, it was impossible to use CP/MAS technique for the determination of DA in pectins. The degree of acetylation was calculated only for the spectrum of sugar beet pectin **5**. Only in this spectrum was the O-CO<u>C</u>H$_3$ carbon signal higher than the noise level. The DA value of **5** calculated from NMR data was comparable with values reported from conventional methods (Table 4).

DA of natural pectins strongly depend on the plant raw material and the extraction procedure. Matora *et al.* reported[17] that pumpkin and sugar beet pectins extracted with bacterial enzymes had a higher *O*-acetyl content than those extracted with acid, but the enzyme extraction gave significantly higher yields. Highly acetylated pectins have bad gelation properties and must be deacetylated by chemical or enzymatic processing. In this

context we suggest that the solid state NMR can be applied for determination of DA in highly acetylated pectins. Moreover, the values of DA in the process of extraction or acetyl hydrolysis can be monitored by CP/MAS NMR.

Jarvis and Apperley[8] measured ^{13}C CP/MAS NMR spectra of solid pectic acid, sodium pectate and the acid form of pectin and reported that the carboxyl resonance was at 171 ppm for the protonated and esterified forms and 176 ppm for the ionised form. Our measurements showed that polygalacturonic acid (**1**) had a single signal at 171.6 ppm that represented COOH carbons, whereas the potassium salt (**1a**) was observed as a single signal at 175.6 ppm, consisted with the reported pectin spectral results.

C-6 carbon signals of commercial non-purified pectins often had a rather complicated shape due to the variable content of COOH, $COOCH_3$ and COO^- groups. The spectrum of commercial citrus pectin **2** contained a single carboxylic peak at 171 ppm, while the potassium salt of pectin **2a** had two signals in the carboxyl region (Fig. 2). The peak at 174.92 ppm indicated carboxylate anion carbons and the peak at 171.15 ppm belonged to carbonyl carbons of methyl ester groups. LM sugar beet pectin **4** had a strong peak at 175.6 ppm with upfield shoulder near 171.3 ppm (Fig. 3). This suggests that the C-6 carbons of this sample were mostly in the salt form. In contrast, HM citrus pectin **7** had a resonance signal at 171.1 ppm with a lowfield shoulder due to high content of methyl ester groups together with some free carboxyls in acid and salt forms (Fig. 3). The commercial LM citrus pectin **8** was produced by alkaline deesterification of HM pectin. Most of its carboxylic groups were in the salt form (signal at 175.7 ppm). After purification and conversion to the acid form this pectin showed a COOH and $\underline{C}OOCH_3$ carbon resonance at 171.7 ppm (Fig. 4).

The NMR spectra of pectins with different degrees of methylation in protonated (acid) form showed a difference between resonances for acid and ester C-6 carbons. Protonated pectins had a single peak near 171 ppm, but the C-6 carbon resonances of HM pectins were shifted to higher field in comparison with those of LM pectins. The difference between C-6 carbon chemical shifts of the highest and the lowest esterified pectins (**13**, 170.57 ppm and **8**, 171.75 ppm respectively) was 1.18 ppm (Fig. 5).

The linear correlation between the shift of C-6 carbon and the degree of methylation of pectins was found and is described in Figure 6. The results of correlation for DM values determined by NMR and photometry were similar. For the correlation analysis we used not only spectra of purified pectins in acid form but also those of some non-purified samples that contained most carboxylic groups in protonated and esterified forms according to the shift and the shape of C-6 signals (**2**, **3**, **5**, **6** and **7**). The fact that some citrus pectins (**9**, **10** and **11**) were amidated (15-20 %) and sugar beet pectin **5** was acetylated (about 30 %) did not influence significantly this correlation.

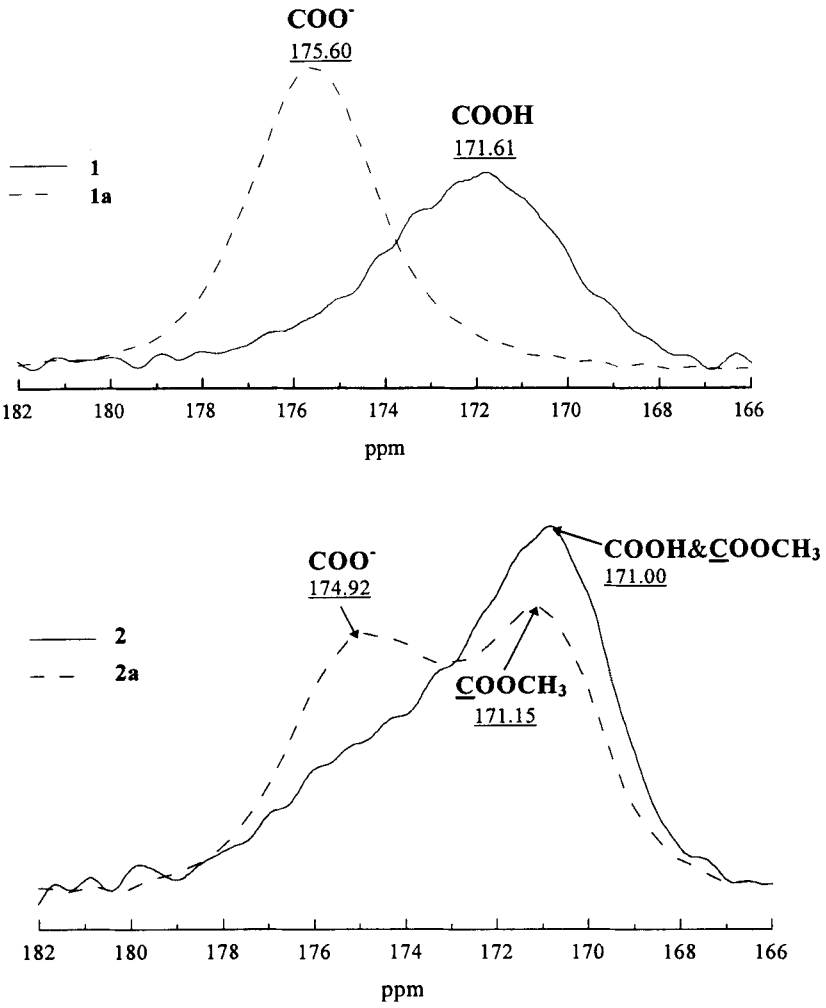


Figure 2. C-6 region of ^{13}C CP/MAS NMR spectra of polygalacturonic acid (**1**), citrus pectin **2** and their potassium salts (**1a** and **2a**, respectively).

The correlation could be explained in such a way that the C-6 carbon shift is the average value for acid, amide (if present) and ester forms and depends on their content. On the basis of regression lines (Fig. 6) we tried to approximate the shifts of pure carboxylic (DM = 0 %) and ester (DM = 100 %) C-6 carbon forms. The calculated shifts were 172.05 ppm for COOH and 170.19 ppm for $\underline{C}OOCH_3$ and were lower then those reported for pectin gels (172.8 ppm and 171.3 ppm, respectively).[14] Nevertheless, calculated and experimental differences between resonances of these two carboxyl carbon forms are similar (1.74 ppm and 1.5 ppm, respectively).

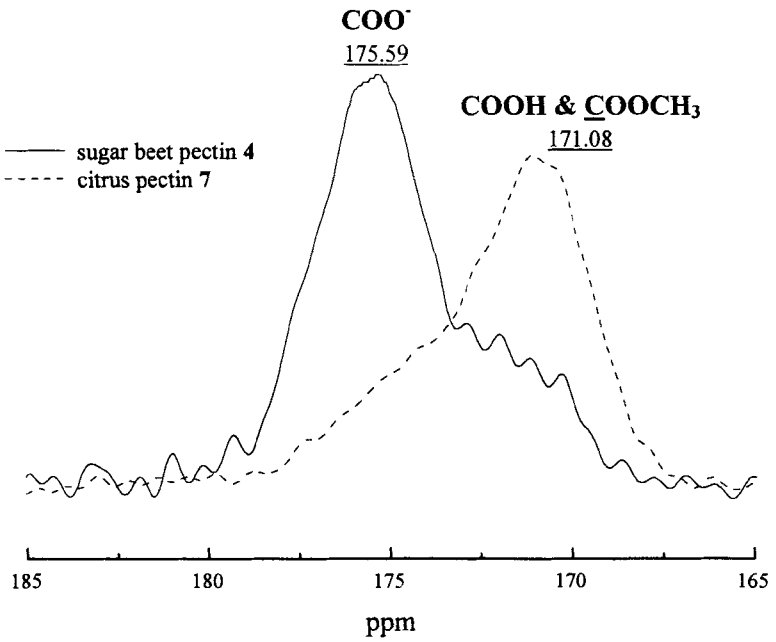


Figure 3. C-6 region of ^{13}C CP/MAS NMR spectra of commercial pectin samples **4** and **7**.

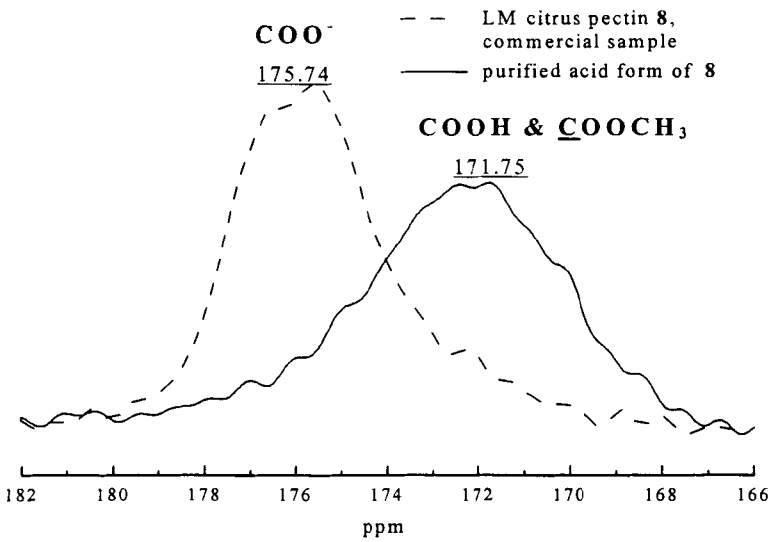


Figure 4. C-6 region of ^{13}C CP/MAS NMR spectra of commercial (salt form) and purified (acid form) LM citrus pectin **8**.

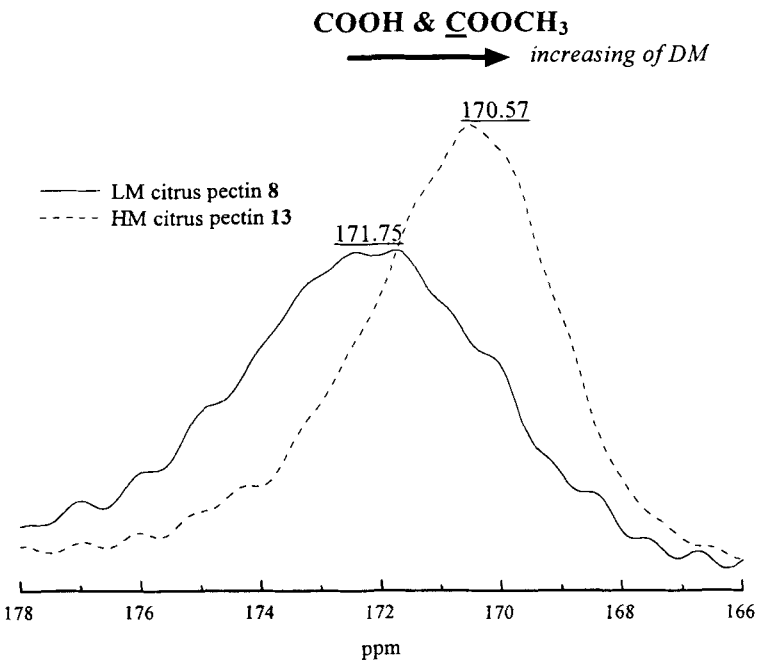


Figure 5. C-6 region of ^{13}C CP/MAS NMR spectra of LM and HM citrus pectins (samples **8** and **13**) in acid form.

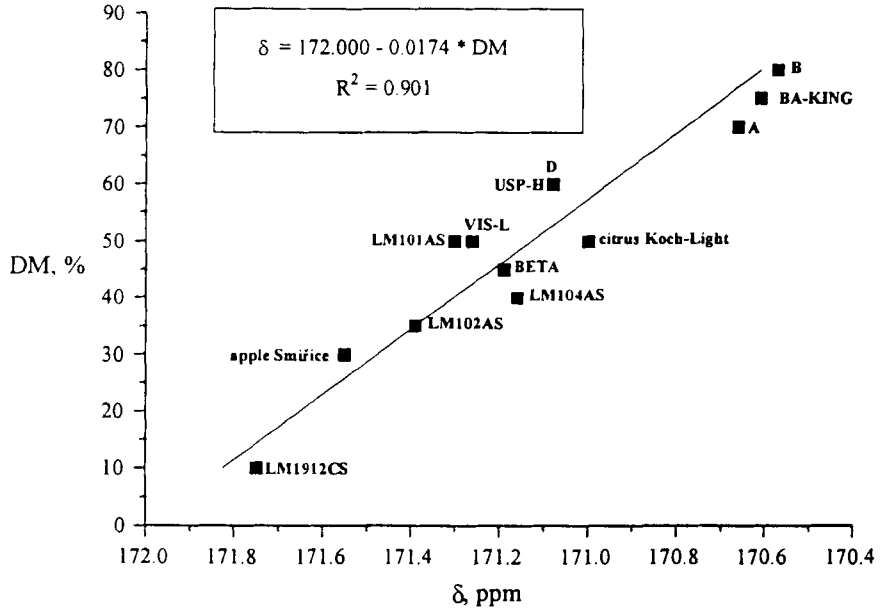
CONCLUSION

In this paper, we have shown the possibilities of ^{13}C CP/MAS NMR spectroscopy in analysis of pectin samples. This method permits to study of some quantitative relations between functional groups in pectin. The best results were obtained in calculation of methylation degrees on the basis of integral intensity of $COO\underline{C}H_3$ carbons near 53 ppm.

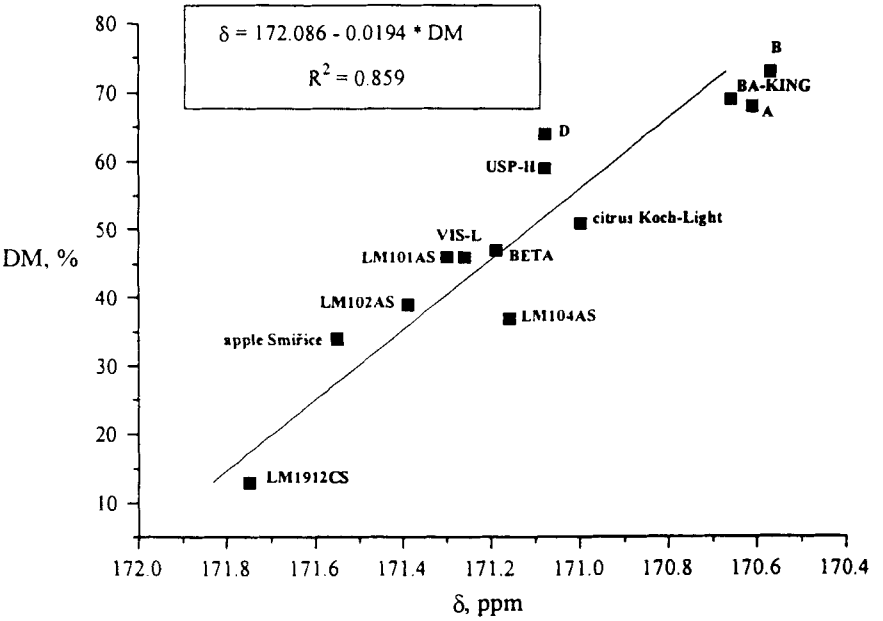
The shift and the shape of C-6 carbon peak can possess additional information about the presence of different functional groups in pectin macromolecules. The resonance signal near 175 ppm indicated a COO^- form of C-6, whereas COOH, $\underline{C}OOCH_3$ and $CONH_2$ groups gave signals in the range of 172-170 ppm. The spectra of pectins in the protonated form (without COO^- groups) appeared as a single C-6 carbon peak whose chemical shift linearly depended on the degree of methylation.

EXPERIMENTAL

Sample preparation. The samples of polygalacturonic acid and pectins are described in Table 1. Samples **1-7** were used for NMR spectroscopy as commercial



a.



b.

Figure 6. Correlation between C-6 shift (δ, ppm) and degree of methylation (DM, %) for pectins in acid form. DM was determined by NMR (**a**) and photometric (**b**) methods.

products without purification. Other samples were purified and converted into protonated (acid) form by washing with 0.1M hydrochloric acid in a mixture of ethanol : water (1:1 v/v). Sample **8** was used for NMR in non-purified and purified (protonated) forms. Potassium salts of samples **1** and **2** were prepared by suspending 0.5 g samples in 150 mL of 0.1M solutions of potassium chloride in 2-propanol : water (1:1 v/v). The suspension was carefully neutralised by addition of small volumes of 1M potassium hydroxide solution with continuous mixing. After the solids settled, the liquid phase was decanted. This procedure was repeated three times until pH did not decrease after resuspending in the base solution. Then the samples were filtered and the residues were washed four times with 40% 2-propanol until the reaction for chloride was negative and then washed with 80% 2-propanol to decrease the water content. All purified samples were air dried at 60 °C and then kept under vacuum over phosphorus pentaoxide.

^{13}C CP/MAS NMR spectroscopy. High resolution ^{13}C CP/MAS NMR spectra of samples were measured using Bruker MSL 200 and DSX 200 spectrometers operating at 50.32 MHz. The spectra were obtained applying the following parameters: 2 ms contact time, recycle delay of 3 s, sweep width of 20 kHz and spinning speed of 4 kHz. All spectra were referenced to the carbonyl peak of glycine at 176. 03 ppm.

Conventional methods. The content of galacturonic acid (GalA) in pectin samples was measured by photometry with *m*-hydroxybiphenyl at 520 nm.[18] This value expressed the total content of uronic carboxylic groups as galacturonic units. The degree of methylation (DM) was determined by photometry with chromotropic acid at 570 nm.[19] The degree of acetylation (DA) of sugar beet pectin **5** was measured by photometry with hydroxylamine (540 nm).[20] Both DM and DA were also determined by an HPLC procedure.[21] The degrees of methylation (DM) and acetylation (DA) of pectins were expressed as the relative content (%) of methoxyl and acetyl groups, respectively.

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