Pectins from Apple Pomace – Characterization by ^{13}C and ^{1}H NMR Spectroscopy

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Abstract: *Pectins were extracted from apple pomace flour with 5% (w/v) aqueous citric acid solutions under different time and temperature according to an experimental design (factorial 2^2 with triplicate of central point). Monosaccharide composition of fractions was determined by colorimetric analysis and gas chromatography. The structure of pectins was studied by NMR spectroscopy. The degree of esterification (DE=30.5-55.9), determined by FT-IR spectroscopy, was indirectly correlated with increasing temperature and time of extraction, showing that drastic conditions for extraction promote hydrolysis of esterified units. High content of galacturonic acid is consistent with the smooth region of the polysaccharide. ^{13}C and ^{1}H NMR spectroscopy confirmed the presence of uronic acids in the free and methyl ester forms. NMR data also showed the presence of arabinan and galactan as side chains.*

Resumo: *Pectinas foram extraídas de farinha de bagaço de maçã com solução aquosa de ácido cítrico a 5% (p/v) em diferentes tempos e temperaturas, definidos por um planejamento fatorial 2^2 com triplicata no ensaio central. A composição monossacarídica das frações obtidas foi determinada por métodos colorimétricos e cromatografia gasosa, A estrutura das pectinas foi estudada por espectroscopia de ressonância magnética nuclear (RMN). O grau de esterificação (DE=30.5-55.9), determinado por FT-IR, variou inversamente ao aumento de temperatura e tempo de extração, demonstrando que as condições mais drásticas podem promover hidrólise do grupo éster. Elevados conteúdos de ácido galacturônico indicam o predomínio de região lisa no polissacarídeo. Os dados da espectroscopia de RMN de ^{13}C e ^{1}H são consistentes com a presença de ácidos urônicos na forma livre e metoxilada. Os dados de RMN também indicam a presença de arabinana e galactana como cadeias laterais.*

Introduction

Pectins are a complex and structurally diverse group of heterogeneous polysaccharides found in the primary cell wall and middle lamellae of most plants. These polysaccharides provide them with mechanical strength and flexibility due to their interaction with other cell wall components.[1] Pectins are widely used as functional food ingredient and are listed among the ingredients of many food products. Worldwide annual consumption is estimated to be around 45 million kilograms, with a global market value of at least 400 million Euros.[2] In the food industry, pectins are known primarily as a gelling agent and are widely used in the production of jams and jellies, fruit juice, confectionary products and bakery fillings. The other major use of pectins is for the stabilization of UHT-treated drinkable yogurts and for blends of milk and fruit juice due its stability at pH values below 4.3.[3,4] In all application areas the fine structure of the pectins deeply affects their functionality[5], and their composition varies with the source, as

well as with the conditions used during extraction.[6,7] Currently, citrus peel and apple pomace are the major sources of extracted pectins, whilst other potentially valuable sources remain largely unused because of certain undesirable structural properties.

The fine structures of pectins can be extremely heterogeneous between plants, between tissues, and even within a single cell. The fundamental constituents of pectins are homogalacturonan (HGA) and rhamnogalacturonan (RGI). The chain lengths of the various domains can vary considerably, and the sugar composition of RG I can also be highly heterogeneous.[8] Their dominant structural feature is a main chain of poly-α(1→4)-D-galacturonic acid occasionally interrupted by α(1→2)-linked α-L-rhamnopyranose residues. A considerable proportion of galacturonic acid residues of the backbone is methylesterified. The homogalacturonan parts of the polymer are referred to as "smooth" regions while the rhamnose–rich zones are called "hairy" regions, as the latter sugars carry neutral oligosaccharide side chains frequently composed by D-Gal*p* and L-Ara*f* sugars.[9-12]

Pectins are classified as low methoxyl (LM) or high methoxyl (HM) according to their degree of esterification. The former contains between 25–50% methoxylated carboxyl groups, the latter, between 50–80%. The molecular structure determines the properties of pectin fractions: high methoxyl pectins require high soluble solids present as co-solute and acid media in order to form gel, while low methoxyl pectins produce gel in the presence of cations such as calcium.[6,8]

The properties of pectins result from variations in the structures at the molecular level, which may often be identified by spectroscopic methods.[6] Although determining the complete structure of a carbohydrate usually requires the application of chemical and spectrometric methods, nuclear magnetic resonance (NMR) spectroscopy is the most powerful technique for this kind of study. NMR has been extensively used to characterize the hydration properties of proteins and carbohydrates.[13] It is a non-destructive technique and requires far less material than other methods.[6,14]

Experimental

Ripe apples (variety Gala) obtained in the local market, were milled and pressed for extraction of the cloudy juice. The residual pomace was dried, ground and sieved (80 mesh) to be used as raw material.

Pectins were isolated from apple pomace (20g) by extraction with (200 mL) aqueous citric acid (5% w/v), in different times (30 or 80 min) and temperatures (50 or 100ºC) according to an experimental design [15] with triplicate of central point (55 min; 75 ºC). The pectins were precipitated with ethanol (400 mL) and after ~12 hours, filtrated and dried at 35-40 ºC [16]. Both standard and apple pomace pectins were dried (under vacuum over P_2O_5 at 40º) prior to Fourier transform-infrared (FT-IR) analysis. FT-IR spectra were recorded using Shimadzu 8400 FTIR equipment, with 4 cm^{-1} of resolution, by using solid and powdered

samples. The degree of esterification (DE) is defined as follows: (number of esterified carboxylic groups/number of total carboxylic groups) x 100. DE is inferred from the ratio of the area of the band at 1740 cm^{-1} (corresponding to the number of esterified carboxylic groups) over the sum of the areas of the bands at 1740 and 1630 cm^{-1} (corresponding to the number of total carboxylic groups). The band areas were determined using Shimadzu software.[17,18]

To determine monosaccharide composition, pectin samples were hydrolyzed with trifluoroacetic acid, and the residues were reduced and acetylated. The resulting alditol acetates was analyzed by gas chromatography (GC) using an HP-5890 series II Gas Chromatograph at 220 ºC (FID and injector temperature, 250 ºC) with a DB-210 capillary column (0.25 mm i.d. x 30m), film thickness 0.25 µm; the carrier gas was nitrogen. Uronic acid was estimated by the *m*-hydroxybiphenyl method, as described by Blumenkrantz and Asboe-Hansen.[19]

NMR spectroscopy was performed with a Varian Mercury-300 spectrometer operating in the Fourier transform mode, using D_2O as solvent for the sample contained in a tube of 5 mm i.d. at 60ºC. Chemical shifts were expressed in δ (ppm) relative to DDS (sodium-4,4-dimethyl-4-silopentane-1-sulphonate) reference. ^{13}C NMR experiments were performed using frequency at 75.45 MHz; pulse width 18867.9 Hz; pulse power 45º; relaxation delay 1.132 µsec; number of scans 25600; acquisition delay 0.868 sec; FT size 32768; total time 15h24min; pulse sequence s2pul; FID resolution 0.847 Hz; pulse interval 24.8 µsec; decoupled ^{1}H at 300.058 MHz in the continuous mode. The conditions used for ^{1}H NMR experiments were frequency at 300.057 MHz; pulse width 4498.4 Hz; pulse power 45º; relaxation delay 10 sec; number of scans 80; acquisition delay 3.641 sec; FT size 65536; total time 18min and 24sec; pulse sequence s2pul; FID resolution 0.126 Hz; pulse interval 60.6 µsec; decoupled ^{1}H at 300,058 MHz (off during acquisition delay and on during delay).

Results and Discussion

The yield of pectins obtained from apple pomace ranged from 5.7 to 16.8%. The increase in yield was directly correlated with the increase in temperature and time of extraction (Table 1), being the temperature the main factor.

Table1. Experimental design with factors level, gravimetric yield and degree of extracted pectins.

Fraction	Temperature (°C)	Time (min)	Yield[1] (%)	DE[2]
1	50	30	5.7	55.9
2	100	30	14.0	32.5
3	100	80	16.8	30.5
C*	75	55	7.2	41.8

[1]Based of dry apple flour [2]Determined by FT-IR
*Central point of experimental design (values are average of three experiments)

The DE of apple pomace pectin obtained by FT-IR spectroscopy (Table 1) shows that an increase in temperature promotes a decrease in DE. This behaviour is in agreement with

results obtained by Smout et al. [20] for pectin obtained from carrots.

The monosaccharide composition of pectins as determined by GC (gas chromatography) and galacturonic acid by Blumenkrantz and Asboe-Hansen method is shown in Table 2. The fractions have the same monosaccharide units in different amounts and galacturonic acid content varying from 36.7 to 42.5%. The qualitative profile of the pectic fractions was similar to that described by Schols et al.[10]

Table 2. Monosaccharide composition[1,2] of apple pectin fractions extracted with citric acid 5 % (w/v)

Fraction	Uronic acid[2]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
	(mol %)							
1	38.6	5.5	-	28.7	7.4	-	11.0	8.8
2	36.7	5.6	-	8.5	14.0	-	18.0	17.2
3	42.3	5.6	-	5.7	14.4	0.9	16.6	14.5
C*	42.5	5.4	1.2	22.5	7.9	-	10.6	9.9

[1]Neutral Sugar determined by GC.
[2]Determined by Blumenkrantz and Asboe-Hansen method.
*Central point of experimental design.

^{13}C and ^{1}H NMR spectroscopies were applied to investigate the fine structure of pectin. NMR chemical shift data and their assignments based on the literature data[21-25] are shown in Table 3. Anomeric carbon (C-1), carbon C-6 in the galacturonic acid, esterified and non-esterified, was further unequivocally established by ^{13}C NMR spectrum analysis for all fractions (Figure 1), which showed characteristic signals of polysaccharides like pectins.

Characterization of Fraction 1

Fraction 1 accounted for 5.7% (of dry matter) and contained 38.6% of galacturonic acid (Table 2). Neutral sugars were also present, mainly arabinose (28.7%) and galactose (11.0%).

In the ^{13}C NMR spectrum, the signals in the anomeric region, at δ 102.4, 102.7 and 110.0 ppm correspond to C-1 of β-xylopiranosil, galacturonic acid, and α-arabinofuranosyl residues, respectively (Table 3). The signals at δ 80.2 (C-2), 83.4 (C-4) and 63.7 (C-5) were attributed to α-arabinofuranosyl. In the low field region, typical signals are observed for the C-6 carboxyl group of galacturonic acid units at 176.0 ppm (esterified) and 173.0 ppm (not esterified).

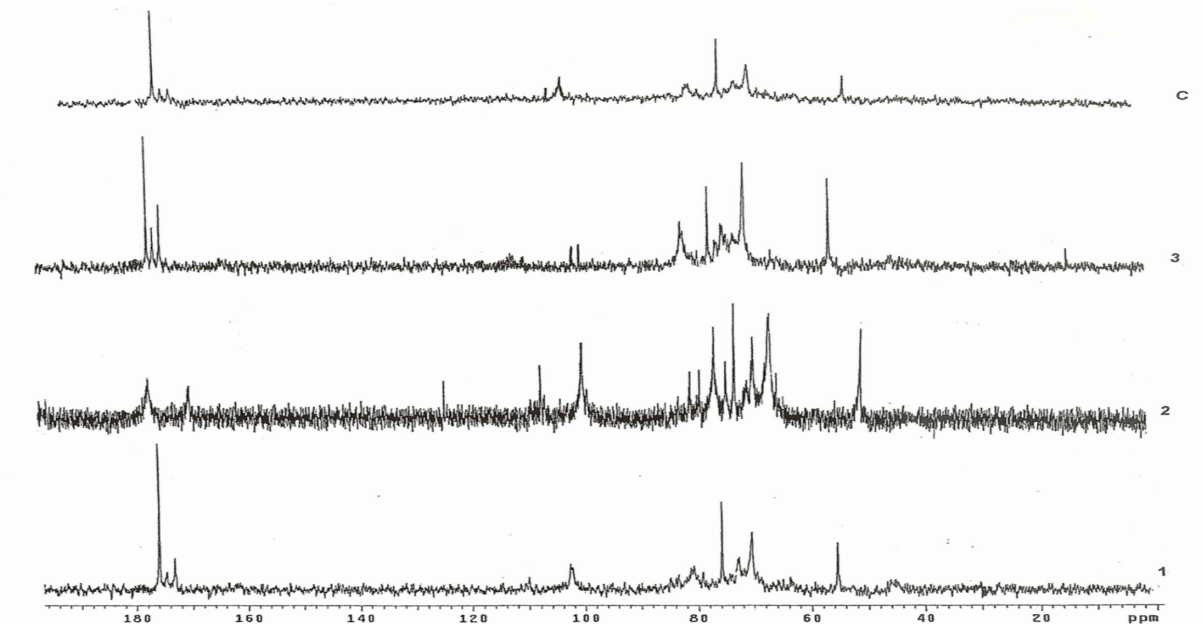

Figure 1. ^{13}C NMR spectra of fractions 1, 2, 3 and C from apple pomace.

Table 3. ^{13}C-NMR and ^{1}H-NMR data for pectins from apple pomace

Fraction	Glycosil residue	Chemical shift C/H (ppm)						
		1	**2**	**3**	**4**	**5**	**6**	***O*-Me**
1	Gal*p*A	102.7/4.81	70.4/3.65	69.7/3.82	79.2/4.30	72.8/4.43	173.0	
							176.0	55.4/3.36
	Ara*f*	110.0/5.23	80.7/ND	ND/4.11	83.4/ND	63.7/ND		
	Gal*p*		ND/3.31		ND/3.36	75.78/ND		
	Xyl*p*	102.4/ND						
	Rha*p*				ND/3.20		ND/1.18	
2	Gal*p*A	100.1/ND	70.5/ND	71.5/ND	78.7/ND	75.6/ND	170.6	
							178.5	52.9/ND
	Ara*f*	107.6/ND						
3	Gal*p*A	100.6/4.99	70.2/3.75	72.8/4.14	79.9/4.32	74.2/4.49	172,9	
		4.81					175.6	55.1/3.67
	Ara*f*		80.6/ND	77.4/ND	81.0/4.25	64.4/3.85		
	Gal*p*	106.6/ND	ND/3.39	ND/3.68				
	Xyl*p*	102.1/ND						
	Rha*p*				ND/3.34		18.7/1.22	
C*	Gal*p*A	101.6/4.80	70.2/4.00	ND/4.19	79.2/4.32	73.0/4.41	175.7	
		4.77					172.9	55.1/3.67
	Ara*f*		80.3/ND	75.8/4.13	81.5/ND	ND/3.38		
	Gal*p*	ND/4.51	ND/3.32	ND/3.65		75.54/ND		
	Xyl*p*	102.15/ND						
	Rha*p*				ND/3.33		ND/1.59	

*Central point of experimental design.

The signal at δ 55.4 ppm corresponds to *O*-methyl ester groups (*O*-Me) at C-6.[18,22] The assignment of the hydrogens of galacturonic acid unit appears at δ 4.81 (H-1), 3.65 (H-2), 4.30 (H-4), 4.43 (H-5). The signal at δ 3.36 was attributed to *O*-Me groups. The H-4 signal of rhamnose (3.2 ppm) suggests the presence of unsubstituted residues such as side chains in this fraction.

Characterization of Fraction 2

Fraction 2, sugar composition (Table 2), is composed essentially of galacturonic acid, galactose, and rhamnose, in the ratio 37:18:5, suggesting the presence of a rhamnogalacturonan substituted with galactan side-chains. The arabinose was detected in a smaller amount than that in Fraction 1, indicating that the arabinan, usually present in side-chains of hairy regions of pectin, was partially removed in the conditions of extraction. These results were similar to those reported by Habibi et al.[25], who detected small amounts of arabinose extraction at 80 ºC for 60 min with HCl.

The ^{13}C NMR data for Fraction 2 are shown in Table 3. The spectrum shows signals characteristic of C-6 galacturonic acid residues at δ 178.5 and 170.6 ppm corresponding to the esterified and non-esterified forms, respectively. The presence of esterified carboxyl groups in the galacturonic acid residue is confirmed by the signal at 52.9 ppm due to methoxyl groups. In the range of the anomeric carbon region (C-1, 90-110 ppm), the signals at δ 100.1 and 107.6 ppm were assigned to C-1 of galacturonic acid and α-arabinofuranosyl, respectively. The signal at δ 125.4 ppm was attributed to phenolic compounds, frequently found in apple.

Characterization of Fraction 3

Fraction 3, pectin extracted under more drastic conditions (100 ºC for 80 min), yielded 42.8% of galacturonic acid. The neutral sugar composition yielded rhamnose (5.6%), galactose (16.6%), mannose (0.9%), glucose (14.5%), xylose (14.4%) and arabinose (5.7%). The lack of arabinose found can be explained by the hydrolysis of the arabinofuranosyl linkages during the acidic conditions of extraction.[25]

The ^{13}C NMR spectrum of Fraction 3 present a relative simplicity (Table 3). It shows two major peaks in the anomeric region. The signals at δ 100.6 and 102.1 ppm are characteristic of galacturonic acid and β-xylopiranosil C-1 carbon, respectively. The signals at δ 175.6 ppm and 172.9 ppm were attributed to esterified and non-esterified carboxyl groups, the signals at δ 18.7 ppm to the C-6 carbon of Rha*p,* and at 55.1 ppm to the *O*-methyl group in the carboxylic methyl esters. The ^{1}H NMR assignments of galacturonic acid unit appear at δ 4.81 and 4.99 (H-1), 3.75 (H-2), 4.14 (H-4), 4.32 (H-4), 4.49 (H-5). The signals at δ 3.68 and 3.67 were attributed to methyl ester, suggesting a different electronic environment for the galacturonic residues of esterified units.

Characterization of Fraction C

Fraction C is composed of galacturonic acid, arabinose, galactose and small amount of rhamnose (Table 2) in the ratios 42:22:11:5. The ^{13}C NMR spectrum of this fraction (Table 3) shows two major signals at δ 175.7 and 172.9 ppm corresponding to C-6 carbon of esterified and non-esterified carboxyl groups of galacturonic acid. The three main signals in the anomeric region, at δ 107.1; 102.1 and 101.6 ppm, were assigned to C-1 carbon of arabinofuranosyl, xylopiranosil and α-D-galacturonic acid, respectively. Median region signals at δ 80.3, 75.8 and 81.5 ppm were assigned to C-2, C-3 and C-4 of α-arabinofuranosyl, respectively. These data are compatible with ^{13}C-NMR data reported by otherauthors.[24-26]

^{1}H NMR show signals at δ 4.80 (H-1), 4.00 (H-2), 4.19 (H-4), 4.32 (H-4) and 4.41 (H-5), attributed to galacturonic acid. The signals at δ 4.51 and 1.59 ppm correspond to H-1 and H-6 of galactopiranosyl and rhamnopiranosyl, respectively. The rhamnose H-4 signal at δ 3.3 ppm suggest the presence of unsubstituted rhamnose residues like those shown by Renard, Crépeau and Thibault.[27]

Conclusion

The association of chemical analysis, chromatography and spectrometric methods was effective to identify some features of the structure of pectins from apple pomace. The ^{13}C NMR and GC data confirm the presence of high contents of D-galacturonic acid as seen in the linear polymeric region. The amounts of galactose and arabinose determined by GC and the occurrence of α-arabinofuranosyl in the ^{13}C NMR suggest the presence of galactans and arabinans as side chains. The presence of glucose and xylose may be associated with the extraction of hemicellulose from the cell wall.

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