New structural features of *Acacia tortuosa* gum exudate

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ABSTRACT

Acacia tortuosa produces a clear gum, very soluble in water. Previous reports showed that it was constituted by four fractions, one of them an arabinogalactan–protein complex. The elucidation of the *A. tortuosa* gum structure by the combination of classical chemical methods, size exclusion chromatography and NMR spectroscopy, was the objective of this investigation. The data obtained show that the heteropolysaccharide is an arabinogalactan type II, highly ramified, with lateral chains at C-2 as well as at C-6 of the galactose 3-O residues; mono-O-substituted galactoses were not detected. There are residues of mannose, the arabinose, pyranose predominantly, is terminal and 2-O-linked. The abundance of the 4-O-methyl-α-D-glucuronic acid was not previously reported. The proteic fraction is probably represented by an arabinogalactan–protein complex that binds poorly with β-glucosyl Yariv reagent, and two glycoproteins. The NMR spectra suggest that the carbohydrate links to hydroxyproline through the galactose (galactosylation).

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1. Introduction

Polysaccharides possess interesting applications as hydrocolloids, mainly due to their ability to (a) retain water, (b) increase the viscosity of aqueous dispersions, (c) form viscoelastic materials and (d) provide a suitable texture for specific commercial products. For this reason, they are widely used as moisture-control products, thickeners, gelling agents, stabilizers of different kinds of dispersion, crystallization inhibitors and even as emulsifiers (Braun & Rosen, 2010; Imeson, 2010; Phillips & Williams, 2009).

Only a few plant species are cultivated, at present, to obtain gums used in the food industry as additives: *Acacia senegal*, source of arabic gum; *Astragalus* spp., source of tragacanth; *Cyamopsis tetragonolobus*, source guar gum; *Ceratonia siliqua*, source of locust bean gum.

Acacia tortuosa (Leguminosae), a tropical American specie, located in Venezuela, produces a clear gum, very soluble in water (León de Pinto, Martínez, De Bolaño, Rivas, & Ocando, 1998; León de Pinto, Martínez, Ortega, Villavicencio, & Rojas, 1993). Previous studies demonstrated that the gum contains mainly a polysaccharide and a proteinaceous material (Beltrán, León de Pinto, Martínez, & Rincón, 2005). The polysaccharide contains galactose (69%), arabinose (13%), xylose (<1%) and uronic acid (18%). The nitrogen content is relatively high (6%), in comparison with those reported for *A. senegal* (0.29%) gum and for other *Acacia gummiferae* gums, such as, *Acacia seyal* (0.14%), *Acacia gerrardii* (1.8%), *Acacia robusta* (2.8%) and *Acacia tortilis* (1.9%) (Beltrán, León de Pinto, Martínez, & Rincón, 2005; Islam, Phillips, Sliivo, Snowden, & Williams, 1997; Sidigg, Osman, Al-Assaf, Phillips, & Williams, 2005). The amino acid composition showed high proportions of hydroxyproline, serine, lysine and glycine; threonine glutamine, proline, alanine, valine, isoleucine, tyrosine, phenylalanine and histidine are also found as minor constituents, similar to those reported for *A. seyal*, *A. gerrardii*, *A. robusta* and *A. tortilis* (Beltrán, León de Pinto, Martínez, & Rincón, 2005). Size exclusion chromatography, with multiangle laser light scattering data demonstrated that *A. tortuosa* gum is a highly polydisperse system (Beltrán, León de Pinto, Martínez, Picton, et al., 2005). The weight average molar mass (Mw = 410,000 g mol^{-1}) and the number average molar mass (Mn = 170,000 g mol^{-1}) are within the typical ranges reported for many *Acacias* (Al-Assaf, Phillips, & Williams, 2005).

A. tortuosa gum aqueous dispersions (15–40% w/v) displayed shear-thinning non-Newtonian flow properties at 20 °C, fitting the Sisko model, since a tendency towards a high-shear limiting Newtonian viscosity was observed. The aqueous dispersion gum of *A. tortuosa* (40% w/v) exhibited clear viscoelastic properties from

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5 to 25 °C (Muñoz et al., 2007). The surface tension of *A. tortuosa* gum (42.6 mN/m), at a given concentration (0.5% w/v) (Muñoz et al., 2007), is below the value reported for gum arabic (46.9 mN/m) at the same concentration (Huang, Kakuda, & Cui, 2001); the emulsifying properties of this last gum (Ibanoglu, 2002; Tan, 2004) have been related to a an arabinogalactan–protein complex (Picton, Bataille, & Muller, 2000). The fractionation and characterization of *A. tortuosa* gum suggested the presence of an arabinogalactan (AG) and an arabinogalactan–protein complex (AGP) (Beltrán, León de Pinto, Martínez, Picton, et al., 2005).

Structural studies, by sugar composition, methylation analysis and 1D-NMR spectroscopy, suggested that the backbone of the polysaccharide structure is mainly a β-D(1 → 3) galactan (León de Pinto et al., 1998). This nucleus also contains arabinose and uronic acid residues; the presence of these residues, vulnerable to periodate oxidation, requires a further study (León de Pinto et al., 1998).

This work reports new structural features of *A. tortuosa* gum exudate by the combination of classical chemical methods, size exclusion chromatography and 1D- and 2D-NMR spectroscopy.

2. Materials and methods

2.1. Origin and purification of gum samples

Gum from *A. tortuosa* (L) Wild (uveda), was collected in Zulia State (East of Maracaibo lake), Venezuela, South America, by the authors in January–March, 2012. The identification of voucher specimens was confirmed by Dr. Lourdes Cárdenas de Guevara, a botanical taxonomist of the Universidad Central de Venezuela. The gum exudate was purified as described previously (León de Pinto et al., 1998).

2.2. General methods

Neutral sugar compositions were determined by HPLC, with an IR detector (Waters 410, 35 °C) and a carbohydrate column (Waters, WAT 044355, oven 35 °C), an Acetonitrile:water (80:20) mixture was used as eluent (flow 1 ml/min). The amino acid content was determined with a post-column derivatizer (Pickering laboratories) adapted to a Perkin Elmer 785 UV–visible detector (570 nm), a sodium cation exchange column (Pickering Laboratories) and buffer solutions (pH: 3.8 and 7.14) as eluent (0.5 ml/min) were used. The uronic acids values were determined by the Blumenkrantz–Asboe-Hansen method (Blumenkrantz & Asboe-Hansen, 1973). The total protein and carbohydrate contents were evaluated, using the Lowry and phenol–sulfuric methods respectively (Dubois, Geilles, Hamilton, Reberts, & Smith, 1956; Lowry, Rosenbough, Farr, & Randall, 1951).

2.3. Classical methods

2.3.1. Preparation of the degraded gums A and B

Purified gum (7.2 g) was hydrolyzed with sulfuric acid (5 mM, 290 ml, 96 h, 100 °C). After cooling, neutralization and filtration, the solution was dialyzed against running tap water (48 h), using a Spectra/Por molecular membrane tubing (MWCO 12–14,000). The degraded gum A was obtained by freeze-drying. This hydrolysis gives information about the structure of the lateral chains.

Degraded gum B was prepared by a drastic Smith-degradation of degraded gum A. Degraded gum A (1.5 g) was subjected to oxidation (50 ml, 0.25 M, $NaIO_4$), reduction (0.5 g, $NaBH_4$) and then acid hydrolysis (0.5 M, H_2SO_4) at room temperature for 2 days. The product was obtained by freeze-drying, after dialysis against tap water (48 h). This drastic degradation is used to obtain information about the nucleus of the studied polymer.

2.3.2. Methylation analysis of the original gum

The original gum was permethylated by two successive methylation processes (Haworth, 1915; Purdie & Irvine, 1903). The corresponding alditol acetates were prepared from the permethylated polysaccharide, as described previously (León de Pinto et al., 2000). The alditol acetates were analyzed by GC–MS in a Hewlett Packard HP6890 GC coupled with a Hewlett Packard 5973A Mass-selective detector. The gas chromatograph was equipped with a capillary HP-5MS column (30 m × 0.25 mm i.d 0.25 mm). The carrier gas was Helium (1 ml/min). The oven temperature was raised from 70 to 260 °C at a rate of 6 °C/min and then to 280 °C at a rate of 25 °C and held for 2 min.

2.4. NMR spectral studies of the degraded gums A and B

Experiments were recorded in a Bruker Avance DRX-400 NMR spectrometer operated at 500.13 and 125.77 MHz, for ^1H and ^{13}C NMR, respectively. Studies included correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC). The degraded products (50–100 mg) were dissolved in deuterium oxide (0.5–1.0 ml); the analyses were performed at probe temperature (27 °C). NMR data were reported in ppm (δ) down field from Me_4Si; 1,4-dioxane was used as internal standard.

2.5. Size exclusion chromatography of the original gum

Size exclusion chromatography (SEC) was used in order to obtain the different molecular weight fractions of the original gum, this technique was performed in an open column, packed with Sephacryl S-500 HR (Sigma), and sodium chloride solution (0.5 M) was used as a carrier (0.5 ml/min). The protein, at 280 nm, and carbohydrate (by sulfuric acid method, at 490 nm) contents were monitored with an UV–visible spectrometer. The elution profile was obtained plotting absorbance vs elution volume (León de Pinto, Sanabria, Martínez, Beltran, & Igartuburu, 2002).

2.6. Yariv reaction of the original gum

Aliquots (4 and 40 μg) of the original gum, were subjected to rocket electrophoresis in agarose gels (1% w/w) containing β-glucosyl Yariv reagent, which selectively precipitates and stains AGPs as they move through the gel. Gum arabic was used as standard (Komalavilas, Zhu, & Nothnagel, 1991).

Table 1
Sugar composition of *Acacia tortuosa* original gum and its fractions.

Sample Sugar	Proportion (% m/m)			
	OG[a]	F1[b]	F2[c]	F3[d]
Galactose	53 ± 1.5	32 ± 0.6	48 ± 0.3	52 ± 0.7
Arabinose	28 ± 1.5	38 ± 0.2	39 ± 0.6	32 ± 0.8
Xylose	2 ± 0.26	6 ± 0.2	2 ± 0.2	3 ± 0.5
Mannose	7 ± 0.5	13 ± 0.2	5 ± 0.3	5 ± 0.3
Uronic acid	10 ± 0.26	11 ± 0.3	6 ± 0.2	8 ± 0.4
Total	100	100	100	100

[a] OG: Original gum.
[b] F1: Fraction one.
[c] F2: Fraction two.
[d] F3: Fraction three.

3. Results and discussion

3.1. Sugar composition of the original gum

Sugar composition of *A. tortuosa* gum showed galactose (53%), arabinose (28%), xylose (2%), mannose (7%), and uronic acid (10%) (Table 1). This composition of sugars differs from that reported before (León de Pinto et al., 1998) because it was difficult to separate galactose and mannose under the conditions employed using paper chromatography. The use of HPLC improved the resolution.

3.2. Methylation analysis of the original gum

Methylation analysis of *A. tortuosa* gum exudate (Table 2) showed the presence of 3-O, 2,3-di-O and 3,6-di-O- substituted galactose and terminal and 2-O-arabinose, as furanose and pyranose residues. These results suggest that the structure is a branched one with points of ramification on C-2 and/or C-6 of the 3-O-β-D-galactose residues. Nevertheless, the methylation data differ from those published previously (León de Pinto et al., 1998), which suggested the presence of a less ramified structure with ramification points at C-6 of the 3-O-galactose residues. In addition, they did not detect 2-O-linked arabinose. The complexity of the chromatographic profile of the methylglycosides used in the previous work made their identification difficult, in contrast to the greater simplicity and precision obtained for the alditol acetates (Churms, 1990).

3.3. NMR spectroscopy of the degraded gums A and B

Application of 1D- and 2D-NMR spectroscopy to the degraded products, obtained from the original gum, led to complete previous reports (León de Pinto et al., 1998). Signal assignments were based on chemical evidence and comparisons with model compounds (Agrawal, 1992; Bock & Pedersen., 1983; Brewer, Hunter, & Lajoie, 1998; Martínez et al., 2003; Scott, Faulker, Rubins, & Buschman, 2000).

Degraded gum A, was constituted by galactose (70%), arabinose (9%) and uronic acids (21%). The COSY spectrum showed the spin systems H-1/H-2: 4.50/3.60; 4.50/3.45; 4.50/3.35; 4.50/3.20 (Fig. 1a). The anomeric proton (4.5 ppm) is directly linked to a carbon that resonates at 102.41 ppm (Fig 1b). These NMR data suggested the existence of non-reducing 3-O-β-D-galactose residues, which are probably in four different chemical environments.

There were correlation signals 5.28/3.6, in the COSY spectrum (Fig. 1a) related to the presence of the resonances at 5.28/92.41 and 5.28/68 in HMQC and HMBC spectrum, respectively (Fig. 1b

Table 2
Methylation analyses of the original gum from *Acacia tortuosa*.

O-Methyl sugars	Tr[a]	Type of linkage	Characteristic fragments (*m/z*)
2,3,5-Me$_3$-Ara[b]	1.00	Araf[d](1→	45, 71, 87, 101, 117, 129, 161
2,3,4-Me$_3$-Ara[b]	1.04	Arap[e](1→	87, 101, 117, 161
3,5-Me$_2$-Ara[b]	1.12	→2)Araf[d](1→	45, 71, 87, 101, 129, 161, 189
3,4-Me$_2$-Ara[b]	1.15	→2)Arap[e](1→	87, 101, 117, 129, 189
2,3,4,6-Me$_4$-Gal[c]	1.31	Galp[f](1→	71, 87, 101, 117, 129, 145, 161, 205
2,4,6-Me$_3$-Gal[c]	1.32	→3) Galp[f](1→	45, 101, 117, 129, 161, 233
4,6-Me$_2$-Gal[c]	1.38	→2,3)Galp[f](1→	45, 85, 101, 115, 129, 161, 261
2,4-Me$_2$-Gal[c]	1.46	→3,6)Galp[f](1→	87, 117, 129, 189, 233

[a] Retention times relative to 1,4-di-O-acetyl-2,3,5-tri-O-methyl arabinitol (15.95 min).
[b] Ara: Arabinose.
[c] Gal: Galactose.
[d] Araf: Arabinofuranose.
[e] Arap: Arabinopyranose.
[f] Galp: Galactopyranose.

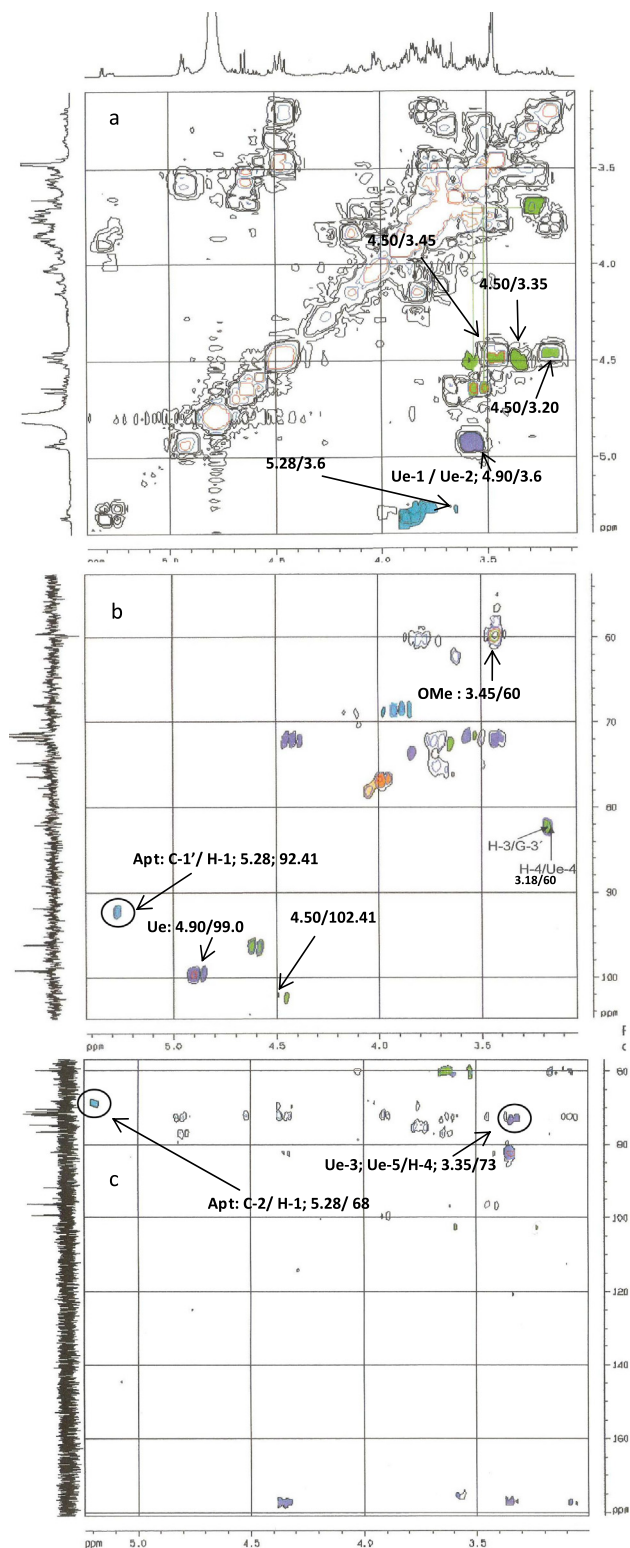**Fig. 1.** NMR data for *Acacia tortuosa* degraded gum A. (a) COSY, (b) HMQC, (c) HMBC. Apt = Terminal arabinopyranose. Ue = 4-O-Methyl-α-ether. OMe = Methoxyl group.

and c). The signals at 5.28, 92.41 and 68 ppm were assigned to H-1, C-1 and C-2 of terminal β-L-arabinopyranose residues, respectively.

On the other hand, the COSY spectrum (Fig. 1a) showed that a resonance at 4.90/3.6 correlated with that which appeared at
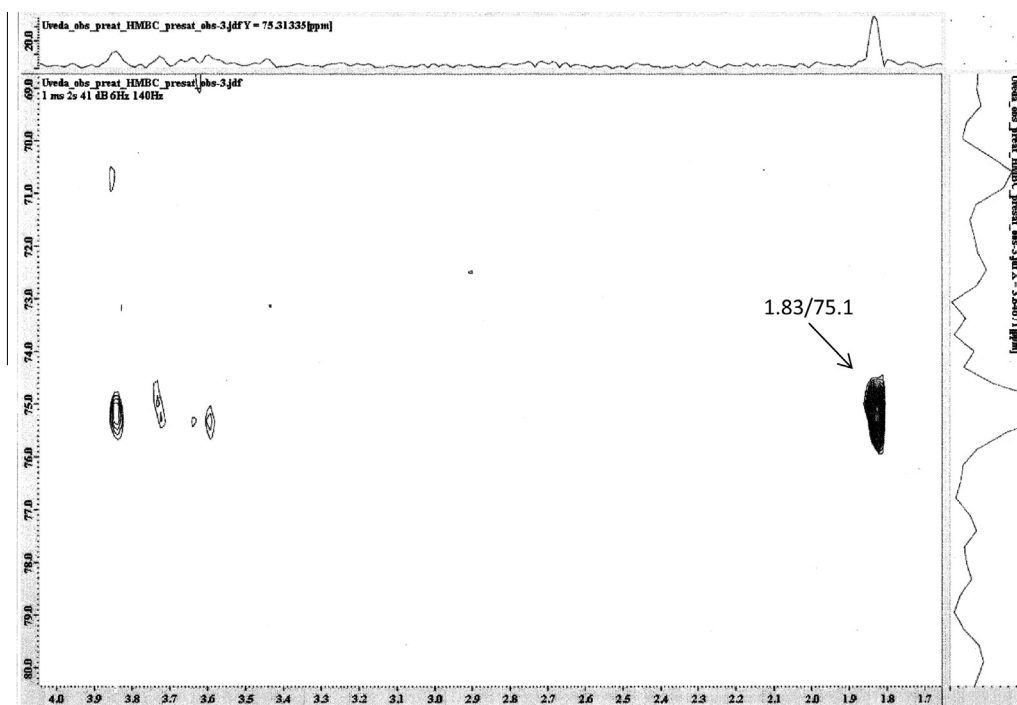
4.90/99.0 ppm in HMQC spectrum (Fig. 1b). The signals at 4.90 and 99.0 ppm were assigned to H-1 and C-1 of 4-O-methyl-α-D-glucuronic acid. In addition, other resonances were observed assignable to this uronic acid, i.e., 3.45/60; 3.35/73 (Fig. 1b and c).

The methoxyl signal (59.33; 60.00 ppm) did not invert in the DEPT 135 spectrum (data not shown). The NMR spectroscopy data demonstrated the abundance of 4-O-methyl-α-D-glucuronic acid in the structure of the A. tortuosa gum which was not described in the previous work (León de Pinto et al., 1998).

Degraded gum B was constituted by galactose (60%), arabinose (10%) and uronic acids (30%) and represents the backbone of the structure of the gum from A. tortuosa. The COSY spectrum (Fig. 2a) shows the spin correlations 4.46/3.47 and 4.40/3.47 ppm, due probably to H-1/H-2 of 3-O-β-D-galactose residues in different environments. According to the HMQC spectrum (Fig. 2b) the H-2 proton

(3.47 ppm) is directly linked to C-2 (72.25 ppm) as was expected. Spin correlations G-4/H-4, G-5/H-5 and G-6/H-6 were also observed, as has been reported for other polysaccharide gums (León de Pinto et al., 2002; Martínez et al., 2003) (Fig. 2b).

There were observed correlations at 4.03/3.88/3.65 and 4.03/68; 3.88/68, (Fig. 2a and b) respectively, probably due to terminal β-L-arabinopyranose, which was not detected in the previous work (León de Pinto et al., 1998). These residues may be removed by periodate oxidation, but steric hindrance and the formation of hydrogen linkage between some sugar residues may preclude its elimination, as has been described for other polysaccharides gums (Martínez, León de Pinto, Rivas, & Ocando, 1996).

The COSY spectrum of degraded gum B exhibited the spin system protons 4.60/3.80 (Fig. 2a) due to β-D-glucuronic acid. The protons (4.60 and 3.78 ppm) are linked directly to C-1 (104.5 ppm) and C-2 (75.00 ppm), respectively, as has been shown by the HMQC spectrum (Fig. 2b).

The DEPT-135 (data not shown) showed an inverted signal (69.97 ppm), assignable to C-6 β-D-galactose, which may be linked to the uronic acids and/or the terminal β-L-arabinopyranose residues in the backbone of the structure, as a confirmation of the persistence of these residues in the nucleus.

3.4. Isolation and characterization of the fractions from the original gum

Fractionation by size-exclusion chromatography (open column) of the original gum, showed three main fractions that contain carbohydrate and protein (Table 3). Fraction 1 contains the highest carbohydrate content (86% m/m), but lower protein proportion (12% m/m) with a predominance of serine and hydroxyproline (Table 3), which suggests the presence of an AGP complex in A. tortuosa gum, as was described previously (Beltrán, León de Pinto, Martínez, Picton, et al., 2005). The carbohydrate composition of this fraction, showed a galactose:arabinose ratio of 32:38, which is still in the range reported for an AG type II (Table 1) (Fincher, Stone, & Clarke, 1983; Gaspar, Johson, Mckenna, Bacic, & Schultz, 2001).

On the other hand, fractions 2 and 3 present higher protein content, with low amounts of hydroxyproline, which suggests that they could be glycoproteins (Table 3).

Yariv reaction gave a diffuse halo for the original A. tortuosa gum (data not shown), distinct from the well defined halo produced by gum Arabic, which demonstrates that the studied gum binds poorly to β-glucosyl Yariv reagent; this behavior has been reported for A. seyal gum and cashew-nut tree exudate gum (Islam et al., 1997; Pereira et al., 2007). This result suggests that A. tortuosa gum could possess an AGP weakly stained by the β-glucosyl Yariv reagent.

3.5. NMR spectroscopy of the proteic fractions from degraded gum B

COSY and HMQC spectra from degraded gum B (Fig. 2a and b) gave interesting information about the proteic fraction present in

Fig. 2. NMR spectral data for *Acacia tortuosa* degraded gum B. (a) COSY, (b) HMQC. Apt = Terminal arabinopyranose residues. Ue = 4-O-Methyl-α-D-glucuronic acid, G = galactose residues, Hα/Hβ = protons α and β amino acids, Pro = proline, Leu = leucine, Val = valine, Cγ/Hγ pro = proline carbon and proton γ.

Table 3
Carbohydrate and protein contents of *Acacia tortuosa* original gum and its fractions.

Sample	Yield (% m/m)	Carbohydrate (% m/m)	Protein (% m/m)
[a]OG		51 ± 0.5	42 ± 0.5
[b]F1	13	86 ± 0.5	12 ± 0.3
[c]F2	46	42 ± 0.7	53 ± 0.5
[d]F3	41	34 ± 0.4	63 ± 0.3

[a] OG: Original gum.
[b] F1: Fraction one.
[c] F2: Fraction two.
[d] F3: Fraction three.

Fig. 3. HMBC spectrum from *Acacia tortuosa* degraded gum B.

the backbone structure of the *A. tortuosa* gum. The COSY spectrum (Fig. 2a) showed correlations, probably due to amino acids present in the nucleus. There were observed signals (4.1–4.9/1.1–1.9, Fig. 2a) which could be assigned to protons from α/β of amino acids (Brewer et al., 1998). The correlation 2.54/2.04; 2.36/2.00 ppm (Fig. 2a) may also correspond to Hβ/Hσ of proline (Scott et al., 2000). In addition, the HMQC spectrum of degraded gum B (Fig. 2b) revealed that the protons at 2.54 and 1.27 ppm are linked to the carbons (39.41 and 19.00 ppm) assignable to Cβ and the – CH_3 group of aliphatic amino acids, such as leucine and valine, respectively, while protons at 1.84 ppm are probably linked to a C-γ of proline (Scott et al., 2000).

The HMBC spectrum of degraded gum B (Fig. 3) showed a main correlation signal at high field, 1.83/ 75.10 ppm which may suggest the presence of a covalent linkage between galactose and hydroxyproline residues. The signal at 75.1, assignable to C-γ of hydroxyproline, was shifted to low field due the galactosylation, and was scalar-correlated, through three linkages, with H-α (Tan et al., 2010). The presence of this linkage between the protein and carbohydrate, in *A. tortuosa* gum, was not reported previously (León de Pinto et al., 1998).

4. Conclusions

The heteropolysaccharide of the *A. tortuosa* gum is an AG type II, highly ramified, with lateral chains at C-2 as well as at C-6 of the galactose 3-O residues; mono-O-substituted galactoses were not detected. There are residues of mannose; the arabinose (pyranose predominantly) is terminal and 2-O-linked.

The abundance of the 4-O-methyl-α-D-glucuronic acid in its structure was not previously reported.

The proteic fraction is probably represented by an AGP complex, that binds poorly with β-glucosyl Yariv reagent, and two glycoproteins, isolated and characterized according to their composition. There were some amino acid signals, well distinguished from the carbohydrate in the NMR spectra. In the same way, the NMR data suggested that the carbohydrate links to the hydroxyproline through the galactose (galactosylation).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015. 02.124.

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