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Impact of purification and fractionation process on the chemical structure and physical properties of locust bean gum

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ABSTRACT

Crude locust bean gum (CLBG) was purified and fractionated into two parts: the first was obtained by solubilization in water at 25 °C (GM25) and the second consisted in a further extraction at 80 °C on the residual impoverished fraction (GM80). The complete structural characterization has shown that GM80 possessed relatively longer chain lengths than GM25, a slightly lower degree of galactose substitution and a somewhat sharper galactosyl distribution in substituted and unsubstituted regions. A physical behavior analysis was carried out on solubilization kinetics, viscosity, viscoelasticity and formation of associated gels with xanthan or carrageenan. The average structure of GM80 generated larger intra-chain, inter-chain and inter-molecular interactions, resulting in the appearance of a stronger network. Small structural differences therefore generated very different physical behaviors. This study thus allowed to establish, in a precise and complete manner, fractionation–purification–structure–function relationships of galactomannans extracted from carob.

1. Introduction

Locust bean gum (LBG) is an additive (E410) used mainly in the food industry for its rheological, texturing and gelling properties. The combination of LBG with other polysaccharides – such as xanthan and carrageenan unable to jellify alone – has also been used in industry for a long time. Specific force gels may be formed by this economically interesting technique to give unique properties for specific applications (Gillet, Blecker, Paquot, & Richel, 2014). Locust bean gum is made from the endosperm of the seeds of the carob tree (*Ceratonia sliliqua* L.). The endosperm is composed of reserve polysaccharides (hemicelluloses) called galactomannans.

Galactomannans consist of a β-(1 → 4) D-mannopyranosyl backbone substituted to varying degrees in α-(1 → 6) with single D-galactopyranosyl residues (McCleary, 1980). This basic structure is the same for all galactomannans. However, three elements allow distinction between these polysaccharides: the degree of substitution of galactose, the molecular sizes, and the distribution pattern of galactosyl substituents along the main chain of mannans. Different kinds of distributions, existing in nature, have been proposed in the literature: (a) the regular arrangement, (b) the random and (c) uniform layout of blocks (Fig. 1). However, intermediate structures between these three "types" may also exist (Dea & Morrison, 1975). The few methods to evaluate distribution patterns of lateral galactosyls on galactomannans are quite conventional techniques: determination of degree of blockiness, [^{13}C]-NMR (nuclear magnetic resonance) analysis or computer modeling after enzymatic hydrolysis (Baker & Whistler, 1975; Daas, Grolle, van Vliet, Schols, & De Jong, 2002; Grasdalen & Painter, 1980; Izydorczyk & Biliaderis, 1996; Lazaridou, Biliaderis, & Izydorczyk, 2000; Manzi, Ceredo, & Shoolery, 1986; McCleary, 1979; Painter, Gonzalez, & Hemmer, 1979). These studies show that the galactomannans block structure is not quite accurate (Gillet, Blecker, et al., 2014). The fine structure of the galactomannans locust bean is most likely composed of "smooth" zones (low substituted) and "hairy" zones (much denser in side galactosyls, without being systematically adjacent).

The production process of locust bean gum consists in obtaining a crude gum by grinding endosperms. Then purification treatments can take place in order to increase the proportion of galactomannans and eliminate odors, impurities and endogenous

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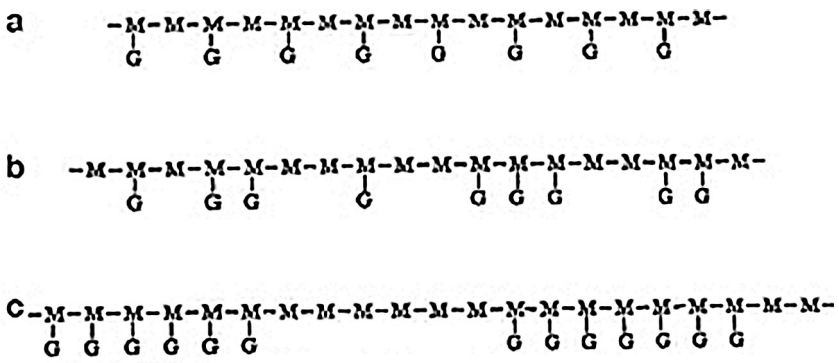

Fig. 1. Different theoretical kinds of galactosyl distributions proposed in the literature: (a) the regular arrangement, (b) the random arrangement, and (c) uniform layout of blocks.
According to Dea and Morrison, 1975, with the permission from Elsevier.

enzymes (Lopes da Silva & Gonçalves, 1990). Clarifying is then the most common process. It consists of a dissolution of the gum in water, soda or acetic acid, followed by a filtration (Kawamura, 2008) or a centrifugation step (Dea & Morrison, 1975) to remove insoluble materials. Galactomannans are then precipitated using solvents such as ethanol (McCleary & Matheson, 1974), isopropanol (Andrade, Azero, Luciano, & Goncalves, 1999; Azero & Andrade, 2002; Dea & Morrison, 1975) or methanol (Rafique & Smith, 1950). Precipitation of galactomannans can also be obtained by the formation of cupric complexes (Andrews, Hough, & Jones, 1952; McCleary & Matheson, 1974), barium complexes (Kapoor, 1972) or by precipitation in borate buffer (Barker, Stacey, & Zweifel, 1957).

The purification step has a major influence on the composition and properties of locust bean gums. During clarification, the dissolution temperature is a crucial parameter which influences the average chemical structure of the resulting gum that is also correlated to the physical properties observed in aqueous solution or dispersion. Thus, a locust bean gum purified at "cold" temperature (about 20 °C) exhibits properties significantly different from those of "hot" extracts (above 40 °C) (McCleary, 1988). The solubility properties are sometimes used for successively re-extracting the same crude LBG to obtain fractions of structural narrower distributions. This subtractive fractionation provides specific properties of gums (Gillet, Blecker, et al., 2014). Temperature is not the only factor impacting on the properties of the clarified/fractionated locust bean gum. The type and amount of solvent used during precipitation also play an important role (Wielinga, 1990).

Several theories correlate purification process with chemical structure (Azero & Andrade, 2002; Gaisford, Harding, Mitchell, & Bradley, 1986; Kök, 2007; Lopes da Silva & Gonçalves, 1990; Pollard & Fischer, 2006) or with physical behavior of galactomannans (Azero & Andrade, 2002; Gaisford et al., 1986; Izydorczyk & Biliaderis, 1996; Lopes da Silva & Gonçalves, 1990; Tako, Asato, & Nakamura, 1984; Wielinga, 1990), but the process–structure–properties relationship has not been thoroughly investigated. Some authors have established the relationship between chemical structures and properties in solution/dispersion (Azero & Andrade, 2002; Bresolin, Milas, Rinaudo, & Ganter, 1998; Dakia, Wathelet, & Paquot, 2010; Dea, Clarck, & McCleary, 1986; Gaisford et al., 1986; Izydorczyk & Biliaderis, 1996; Lazaridou et al., 2000; Lopes da Silva & Gonçalves, 1990; Monteiro, Rebelo, de Cruz e Silva, & Lopes da Silva, 2013; Richardson, Willmer, & Foster, 1998; Rinaudo, 2001). Unfortunately, the structural characteristics mostly determined are molecular weight and degree of substitution of galactose. The distribution pattern of lateral substituents is ignored in most cases, although it seems likely to impact profoundly the physical characteristics of a galactomannan solution and dispersion (solubility, viscosity, gel formation, etc.). On the other hand, most studies are based on a commonly accepted chemical structure for locust bean gum (blocks distribution or "smooth" and "hairy" regions) to interpret physical measurements carried out, without checking that the studied samples really have the structure assumed (Cheetham & Mashimba, 1988; Dakia, Blecker, Robert, Wathelet, & Paquot, 2008; Dea & Morrison, 1975; Dea, McKinnon, & Rees, 1972; Dea et al., 1977; Doublier & Launay, 1981).

This study aims therefore to completely establish links between the purification/fractionation process of carob galactomannans and their "structure–property" relationship. Elucidations of the fine chemical structure (including distribution pattern of lateral substituents) of different samples of galactomannans, obtained by temperature subtractive fractionation, are correlated with the characterization of their physical behavior, through the study of solubilization kinetics or network formation in viscous dispersions and associated gels.

2. Materials and methods

2.1. Materials

Locust bean gum (LBG) was obtained from PFW Ltd. (Greenford, UK) as the product sold under the trademark HERCOGUM N1. α-Galactosidase enzyme (EC 3.2.1.22) has been provided by Megazyme International (Bray, Irlande). All others chemicals were purchased from commercial suppliers and used as received.

2.2. Purification and temperature fractionation

A 1% massic dispersion of CLBG was prepared by mixing 10 g of gum in 1 L of distilled water with an Ultra-Turrax homogenizer (IKA, Staufen, Germany) at 25 °C. The dispersion was then mixed by a 400 rpm magnetic stirrer for 3 h at 25 °C before being centrifuged (8000 × *g*, 25 °C, 30 min) to remove insoluble materials. The supernatant was precipitated by pouring into a two-volume-excess of absolute ethanol. The precipitated material was then filtered, washed twice with ethanol and acetone, resolubilized in 20 °C water by mixing, freeze-dried and milled until a fine grinding was obtained (<1 mm) (Fig. 2). This fraction is called GM25. The pellet obtained after centrifugation (about 6 g of dry materials) was resuspended in 1 L of distilled water at 80 °C and underwent the same procedure of impoverishment at 80 °C to give the GM80 fraction. This subtractive fractionation was realized in triplicate.

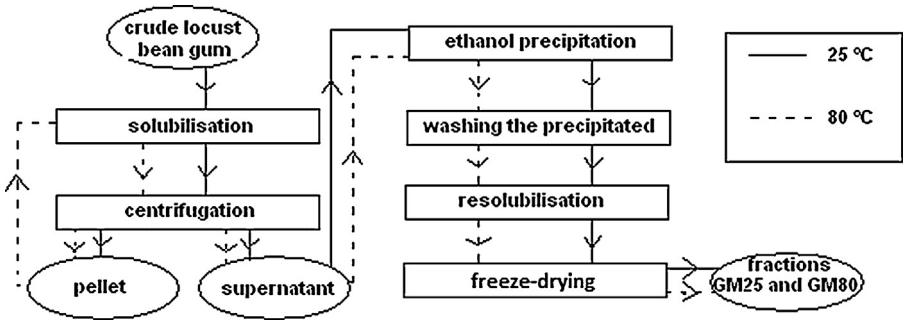

Fig. 2. Illustration of the purification and temperature fractionation process.

2.3. Analyze of composition

2.3.1. Moisture content

1 g of all the fractions was held in an oven at 105 °C and weighted regularly until readings stabilized. All values were then expressed on a dry matter basis.

2.3.2. Protein analysis

Protein contents in the CLBG and fractions GM25 and GM80 were estimated by the Kjeldahl procedure, after mineralization (with a 1000 KJELTABS MQ tablet and a Digestion System 20, 1015 Digester, Tecator AB) and distillation (by a Kjeltec Auto 1030 Analyser, Tecator AB) with a conversion factor of 5.87 according to Anderson (1986).

2.3.3. Fat and ash analyses

A mixture of chloroform and methanol (respectively, in proportion (v/v) 2:1) at 65 °C was used according to the Soxhlet procedure to extract the fat in approximately 5 g of sample, for 5 h. For ash analysis, 3 g of each sample were burned to ash for 12 h in a muffle furnace at 600 °C.

2.3.4. Crude fiber

Crude fiber (cellulose and lignin) was determined by the acid-detergent fiber method (ADF) of Van Soest (1963) in a Fibertec system (Brussels, Belgium) using cetyltrimethylammonium bromide in 1 M sulphuric acid previously standardized as the acid detergent solution. 1 g of each sample was analyzed twice.

2.3.5. Total hemicellulose analysis

The amount of total hemicellulose was estimated as the remainders of the samples after the other substances (protein, fat, ash, crude fiber and moisture) were accounted for. This fraction was considered as totally consisting of galactomannans (Lopes da Silva & Gonçalves, 1990).

2.4. Structural characterization of GM25 and GM80

2.4.1. Measurement of mannose/galactose ratio

Mannose/galactose (M/G) ratios were calculated using an adaptation of the method described by Blakeney, Harris, Henry, and Stone (1983). 50 mg of each sample were hydrolyzed by 3 mL of 1 M sulphuric acid, in a sealed Sovirel tube at 105 °C for 3 h. The resulting aldoses were reduced in alditol acetates. 2-Deoxyglucose was used as internal standard. Gas chromatography analyses were performed on a HP Agilent 6890 series chromatograph (Agilent Technologies, Diegem, Belgium) with a flame ionization detector equipped with HP-1 capillary column (30 m × 32 mm, 0.25 film thickness,) coated with methylsiloxane. 2 μL-samples were injected in splitless mode. Initial temperature of 120 °C in the system was increased to 220 °C at a rate 4 °C/min. Helium was used as the carrier gas with a flow rate of 1.6 mL/min. The temperature of the injector and the detector were 290 °C and 320 °C, respectively.

2.4.2. Measurement of α-D-galactopyranosyl distribution along the D-mannan backbone

Two different and complementary techniques were used in order to estimate galactose distribution along the mannan main chain. The first one, a method proposed by Baker and Whistler (1975), was an alkaline degradation that gives information about the degree of blockiness of each sample. In this method, gas chromatography analyses were performed on a HP Agilent 6890 series chromatograph coupled to a HP 5973 mass spectrometer selective detector, equipped with HP-1 capillary column (30 m × 32 mm, 0.25 film thickness) coated with methylsiloxane (all devices from Agilent Technologies, Diegem, Belgium). 1 μL-samples were injected in splitless mode. Initial temperature of 90 °C in the system was increased to 220 °C at a rate 4 °C/min. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. The temperature of the injector was 290 °C. Mass detector was conducted with electronic impact (EI) mode at 70 eV, source and quadrupole temperatures were set at 230 °C and 150 °C, respectively. Identification of 1,5-di-o-acetyl-2,3,4,6-tetra-o-methyl-D-mannitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-mannitol, with levels of certainty of 91% and 83% respectively, was performed using a NBS mass spectral database.

The second method used was based on the [^{13}C]-NMR spectroscopy works of Grasdalen and Painter (1980) together with Manzi, Ceredo, & Shoolery (1986) and Izydorczyk and Biliaderis (1996). The [^{13}C]-NMR spectroscopy was performed on a Varian Unity 600 MHz instrument spectrometer (Oxford, UK) operated at 90 °C to diminish viscosity and, thereby, line-width. The fractions (1.5%, w/v) were dissolved in H_2O containing 10% D_2O. 50 000 pulses were collected; pulse acquisition time was 0.865 s and r.f. pulse angle was 90 °C. Chemical shifts were expressed relatively to external DSS.

The crude locust bean gum was hydrolyzed (HCLBG) by α-galactosidase to obtain a comparative control in the analysis. HCLBG was obtained from a 5 g/L CLBG dispersion in acetate buffer. 500 mL of enzyme (65 U/mg) were added to 0.5 L of dispersion and stirred for 20 h at 40 °C and a pH of 4.68. HCLBG was recovered by precipitation in two volumes of ethanol, dried at room temperature and milled until a fine grinding was obtained (<1 mm).

2.4.3. Measurement of molecular weight

The scattering light from a polydisperse macromolecular solution can be used to measure the molecular weight (Mw) distribution. This is usually obtained by the Zimm method, i.e. the scattering intensity is measured at different angles and concentrations, followed by an extrapolation to zero angle and zero concentration. This procedure gives the absolute value of the weight average Mw (Viebke, 1995). Measurements were

determined in triplicate using a high performance size exclusion chromatography (HPSEC) HP 1200 series apparatus (Agilent Technologies, Palo Alto, CA, USA) equipped with a TSK-gel GMPWxl column (30 cm × 7.5 mm; particle size 13 μm) (Tosoh Corporation, Tokyo, Japan), coupled to a BI-DNDC refractometer and a BI-MwA multi-angle laser light scattering (MALLS) (both from Brookhaven Instruments Co., Holtsville, NY, USA).

The GM25 and GM80 fractions samples were solubilized (0.5 mg/mL) in deionized water and heated at 90 °C overnight under mechanical stirring. Similarly the samples were also solubilized in phosphate buffer (50 mM KH_2PO_4:NaOH 1:3, pH 8), in order to estimate molecular aggregation (Richardson et al., 1998). Deionized water and phosphate buffer were, respectively, used as eluent, with a flow rate of 1 mL/min at 40 °C. The samples were filtered through the 0.45 μm internal filter of the device. A refractive index increment (dn/dc) of 0.147 mL/g was determined experimentally and used for SEC-MALLS/RI determinations.

2.5. Physical properties in solution/dispersion

Three main physical properties of galactomannans in solution/dispersion, related to their industrial potential applications, were investigated.

2.5.1. Solubilization kinetics

Samples were prepared at a concentration of 0.5% (w/w) on a dry weight basis at 25 °C and 80 °C, respectively for 5 min, 10 min, 30 min, 20 min, 120 min, 180 min and 15 min, 30 min, 60 min, 120 min, 180 min, under magnetic stirring. Then, the corresponding dispersion was centrifuged at 5000 × *g* during 15 min to remove the insoluble material. The supernatant recovered and the final polymer concentrations were determined as total solids dried at 105 °C for 24 h in an air-circulating oven. Percentage of solubilization was determined by the ratio between supernatant massic concentration and initial massic concentration.

2.5.2. Viscosity and viscoelasticity

Rheological properties of carob gum were characterized at 25 °C on carob gum dispersions prepared as follows: 1% on a dry weigh basis in distilled water at 90 °C, under mechanical stirring for 3 h and cooled to room temperature before measurements. Rheological measurements were performed using a rheometer (Anton Paar, Graz, Austria) equipped with a temperature control system and with a cone and plate geometry (4° cone angle, 50 mm plate diameter, 102 μm gap). Each sample (approximately 3 mL) was placed in the sensor system for measurement at 25 °C. Curves of shear stress (τ) as a function of the shear rate (D), were obtained with the following program: 300 s from 0.3 to 300 s^{-1}. The parameters of flow index (n) and consistency (K) are obtained by solving the equation of Herschel–Bulkley: $\tau = \tau_0 + K(\gamma)^n$ (made by *Gabriel Data Analysis – GDA* program); τ, τ_0 and γ are, respectively, the shear stress, the yield stress and the shear rate.

Oscillatory tests were performed at 25 °C using the same equipment. Variations in G' (dynamic elastic modulus), G'' (dynamic viscous modulus) and η^* (complex viscosity) were recorded as a function of frequency, thus obtaining the characteristic mechanical spectra. Frequency sweeps from 0.03 to 30 Hz were performed at constant strain within the linear viscoelastic range (γ = 1 Pa) as realized by Dakia et al. (2008). All the measurements were performed at least in duplicate.

2.5.3. Gel formation in association with other polysaccharides

To study the formation of gels by galactomannans in association with another polysaccharide, xanthan was used. As described by Bresolin et al. (1998), mixtures of xanthan (4 g/L) and galactomannans (2 g/L) are able to form a gel. Such mixtures were performed with GM25 and GM80 fractions, during 3 h under mechanical stirring. Two temperatures of dissolution were tested: 25 and 80 °C. After cooling to 20 °C, samples were analyzed with a TA-XT2 texture analyzer (Stable Micro-Systems, Haslemere, UK). Penetration experiments were performed by means of a 15 mm diameter flat circular probe, penetrating twice for 5 s into the gel at a speed of 60 mm/min (penetration depth = 5 mm). The total work of penetration, indicated as the gel strength and taken as the surface obtained under the force deformation curve (Van Camp & Huyghebaert, 1995), was averaged using two repeated determinations. Hardness is defined as the peak force during the first compression cycle (Blecker, Paquot, & Deroanne, 2000).

3. Results and discussion

3.1. Chemical characterization

The purification process and the temperature fractionation developed here were efficient to remove impurities and to ensure high-quality galactomannan contents (confirmed by gas chromatography analysis) (Fig. 2). Indeed, GM25 and GM80 fractions were very pure and consisted exclusively in galactomannans (about 99.9%). After purification, proteins were found in the residual pellet (RP) after the second extraction and ashes were located in the filtrate. GM25 fraction, GM80 fraction, the pellet and the filtrate represented, respectively, 32.6%, 33.3%, 18% and 11% of the dry starting material. All results were presented in Table 1 wherein the data set to zero corresponds to values below the limit of detection of the devices.

3.2. Structural characterization

To study the structure of GM25 and GM80 fractions, M/G ratio was determined and related to the distribution of galactosyl residues along the mannose main chain ("fine structure of galactomannan"). To allow a comparison of the obtained results (Table 2), measurements were also performed on two other contrasted galactomannans:

- A guar gum, much more substituted, with a more regular distribution of galactosyls residues (fewer "hairy" zones).
- An α-galactosidase-hydrolyzed crude locust bean gum (HCLBG). This enzyme eliminates easily accessible galactosyls residues (located between one or more free mannosyls residues). Thus, enzymatic attack avoids highly substituted areas because few binding sites are available.

M/G ratios were obtained by the method of Blakeney et al. (1983). They indicated that guar gum is much more substituted (at least twice) than carob gums. GM25 and GM80 fractions had M/G ratios of 2.85 and 3.84, respectively. The ratio for GM80 was 1 unit higher than that of GM25. However, these two fractions presented lower ratios than that determined for CLBG (4.07). The RP of the purification process contained very low substituted galactomannans (M/G ratio = 4.50 – more than GM25 and GM80 fractions), which contribute significantly to the ratio value for crude gum (M/G ratio = 4.07). The action of α-galactosidase on crude locust bean gum seems thus quite effective since it increased the value of the M/G ratio from 4.07 to 5.98. This HCBLG fraction was very slightly substituted.

Analysis of [^{13}C]-NMR spectra of these fractions can provide interesting information about fine structure of galactomannans. M/G ratios can also be determined by considering the intensities of C-1 mannose and galactose signals in [^{13}C]-NMR spectra (Manzi, Ceredo, & Shoolery, 1986). This method provides results relatively

Table 1
Production yields of GM25 and GM80 fractions (as % of initial dry matter) and their chemical characterization.

		CLBG	GM25	GM80
Yields		100	32.57	33.25
Chemical composition				
	Protein	6.00 ± 0.03	0.03 ± 0.02	0.03 ± 0.01
	Fat	2.10 ± 0.11	0	0
	Ash	0.31 ± 0.05	0.03 ± 0.01	0.03 ± 0.01
	Fiber	2.20 ± 0.20	0	0
	Galactomannans[a]	89.39 ± 0.24	99.94 ± 0.02	99.94 ± 0.01

[a] Obtained by difference.

close to those obtained by Blakeney method (Blakeney, Harris, Henry, & Stone, 1983) (Table 2).

Spectra also showed that resonance from C4 of D-mannose residues were split, in evident dependence upon the nearest neighbor probabilities ("diad frequencies") of D-galactosyl groups along the mannan chains (Fig. 3). Diad frequencies were obtained by integrating C_4(Man) peak areas. F11, F21/F12 and F22 gave, respectively, the di-, mono- or non-substituted mannose pair proportions (Table 2). The percentages of total lateral substituents obtained by C_4(Man) peak analysis [F11 + (F21 or F12)/2] were fairly well correlated with M/G ratios. Thus, guar gum has a high proportion of di-substituted (F11) or mono-substituted (F12/F21) di-mannosyls and very few pairs without substituents. This makes sense since guar gum consists of a large proportion of side galactosyls residues (M/G = 1.5). Therefore consecutive "smooth" mannosyl residues remain limited. This gum has a fairly homogeneous profile. GM25 and GM80 fractions present a similar profile, different from guar gum. It indicates a much larger proportion of non-substituted di-mannosyls, explained by high M/G ratios. However, GM80 fraction has a higher proportion of F11 and F22 than GM25 while its M/G ratio is more important. This clearly indicates that the distribution of galactosyls on the mannose main chain does not respond to a simple statistical law. Hydrolyzed locust bean gum (HCLBG) also has a [^{13}C]-NMR spectrum having the same morphology as GM25 and GM80 fractions. However, the proportion of non-substituted pairs is much more important. Splitting of the C-6 substituted D-mannose resonance provides, therefore the basis for determining the next-nearest-neighbor probabilities (triad frequencies) (Fig. 3). However, the spectrum is not sufficiently resolved to accurately quantify and interpret the results. At this stage, it appears that particular distribution patterns may occur, since they cannot be explained by the M/G ratio alone. However, it is nevertheless not possible to be sure of the localization of these non-, mono- or di-substituted mannosyl pairs on the main chain. It is therefore necessary to use the determination of the degree of blockiness for more detailed informations on chemical structures.

The study of the degree of blockiness is focused on α-D-galactopyranosyl side-chain groups distribution. It was determined by measurement of o-acetyl-o-methyl-D-mannitol derivatives by GC–MS, which gives a characteristic of the distribution pattern in the parent galactomannan:

- D-mannopyranosyl units substituted at o-6 with α-D-galactopyranosyl groups and at O-4 by a D-mannopyranosyl group without substitution at O-6 will produce 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-mannitol **(1)**,
- whereas, if the D-mannopyranosyl attached at O-4 bears a D-galactopyranosyl group at o-6, this D-mannopyranosyl residue will give rise to 1,4,5-tri-o-acetyl-2,3,6-tri-o-methyl-D-mannitol **(2)**.

Thus, if the α-D-galactopyranosyl groups are isolated from each other along the D-mannan backbone, compound **(1)** would result from the D-mannopyranosyl residues to which they are attached. On the other side, application of the reaction sequence to a "block type" of pattern of substitution would yield mainly compound **(2)**, even if compound **(1)** would be present too (at the beginning of each block). The ratio between compounds **(1)** and **(2)** will give therefore the degree of blockiness of each sample, which represents the percentage of side galactosyls having at least one substituted mannosyl as neighbor. An important ratio therefore reflects a structure necessarily consisting of many successively substituted mannosyls ("blocks") grouped in "hairy" regions.

Guar gum is highly substituted (M/G = 1.51) and it has therefore many di- or mono-substituted di-mannosyls. Determination of the degree of blockiness (Table 2) indicated yet that a small proportion of these pairs are contiguous. Indeed, only 40% of lateral substituents possess neighbors successively substituted ("blocks"). In contrast, carob samples, yet much less substituted, have a substantially higher proportion of side galactosyls arranged in "blocks" (70% for GM25), whereas such distribution reached 83% for GM80 and 96% for HCLBG. This indicates that the enzyme hydrolyzed all "isolated" galactosyl substituents in the "smooth" or "densely substituted" areas, without being able to attack successively substituted residues. Proportion of non-substituted di-mannosyls therefore increased significantly. Again, the M/G ratio does not seem to be a sufficiently accurate parameter for structural characterization.

The chain length is also an important aspect of the structure. During polysaccharides analysis, the distribution of molecular weight measured is highly dependent on the sample preparation and measurement technique conditions (Gillet, Blecker, et al., 2014). A unique and absolute weight average molecular weight

Table 2
Fine structural characterization of different galactomannans: GM25, GM80, HCLBG and guar gum (Guar).

Structural characterization	Guar	GM25	GM80	HCLBG	CLBG	RP
M/G ratio (GC-FID)[a]	1.51 ± 0.01	2.85 ± 0.01	3.84 ± 0.01	5.98 ± 0.01	4.07 ± 0.03	4.50 ± 0.03
M/G ratio ([^{13}C]-NMR)[b]	1.70 ± 0.02	2.98 ± 0.04	3.50 ± 0.01	6.55 ± 0.04	/	/
Degree of blockiness (%)[c]	40.00 ± 1.23	70.00 ± 2.04	83.00 ± 2.47	96.00 ± 3.15	/	/
F11 diad frequencies (%)[b]	41.28 ± 1.25	12.35 ± 1.17	15.65 ± 0.28	6.13 ± 0.18	/	/
F12 or F21 diad frequencies (%)[b]	46.53 ± 0.54	35.52 ± 0.95	30.35 ± 0.59	21.01 ± 1.49	/	/
F22 diad frequencies (%)[b]	12.19 ± 0.71	52.10 ± 0.20	54.00 ± 0.31	72.85 ± 1.66	/	/

[a] Blakeney et al. (1983).
[b] Manzi et al. (1986).
[c] Baker and Whistler (1975).

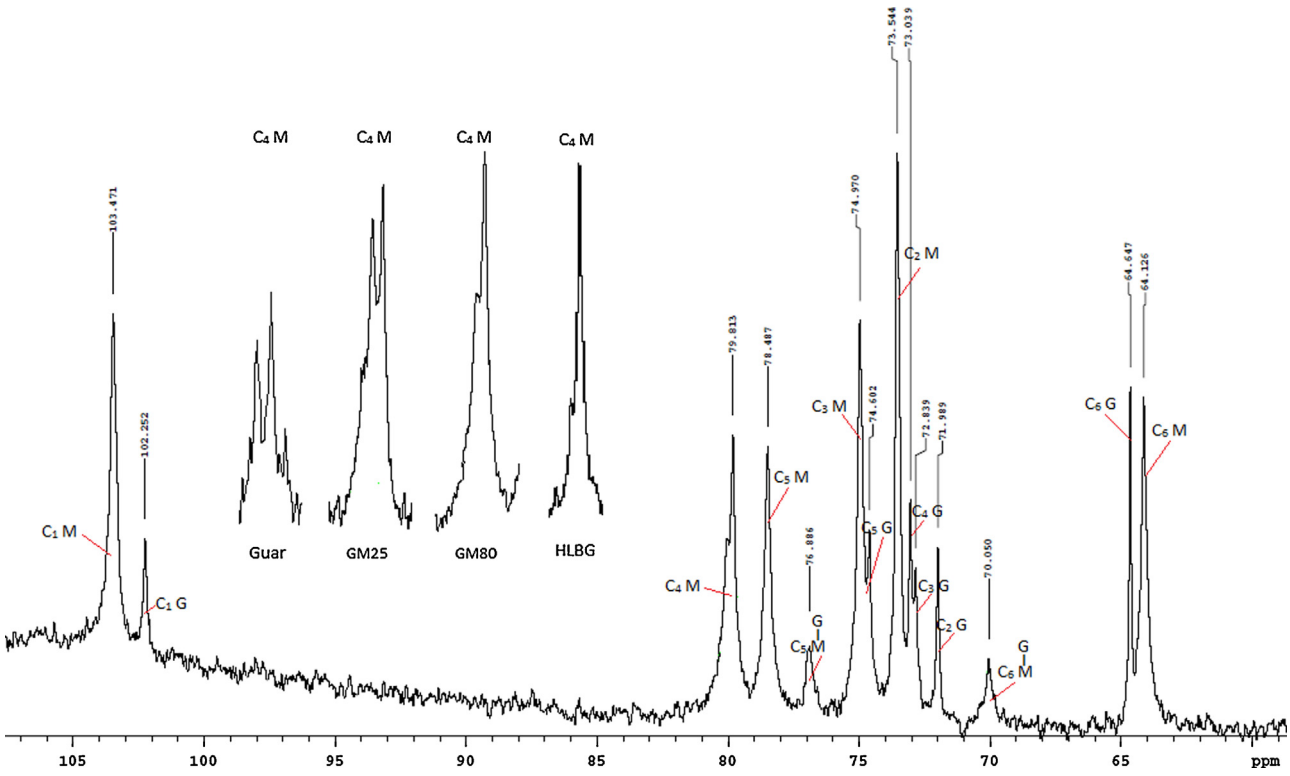

Fig. 3. [^{13}C]-NMR spectrum of different galactomannans: GM25, GM80, HCLBG and guar gum. Focus on mannosyl fourth carbon (C_4).

(Mw) therefore has little meaning. However, values obtained from identically prepared samples, analyzed by the same technique in the same conditions, can be compared among themselves. The results of fractions GM25, GM80 and CLBG determined by SEC-MALLS analyses in water or buffer, are presented in Table 3. Degrees of polymerization (DP), polydispersity index (PI) and radii of gyration (*Rg*) were also presented.

Polydispersity index (PI) was determined by Mw/Mn ratio. In water, GM25 fraction had a higher Mw than GM80 fraction. It was also more polydisperse. However, CLBG had the highest Mw value (1018 kDa) and PI (1.56). Fairly similar values were already determined by SEC coupled to triple detection (Pollard et al., 2007). CLBG is composed of a broad distribution of galactomannans, some with a very high Mw. These are excluded of GM25 and GM80 fractions and are found in the pellet (Fig. 2).

The same measurements were performed in phosphate buffer. The role of the buffer is to avoid any galactomannans self-association (Morris, 1990) or polymer aggregation (Richardson et al., 1998). GM25 fraction had a Mw value close to that obtained in water. The Mw value of CLBG determined in the buffer was considerably lower. This may evidence inter-chain hydrophobic interactions in water that artificially increased the value obtained. Richardson et al. (1998) already demonstrated such behavior, even at low concentrations. In contrast, the GM80 fraction exhibited higher Mw in the buffer than in water. This phenomenon could be explained by the presence of intramolecular cooperative bonds in water (and not inter-chain), generated by a rearrangement of hydrophobic regions in the galactomannans. These bonds would disappear in buffer, thereby increasing hydrodynamic volume and Mw. The buffered medium could also promote dissolution and thus help to keep a part of galactomannans – probably the longest – solubilized, than they would not be in water. The results obtained in the buffer seem to be the most robust and representative of reality, since under these conditions, Mw value of CLBG (770 kDa) was between the low values of GM25 fraction (760 kDa) and higher values of GM80 fraction (910 kDa). The CLBG is highly polydisperse (PI = 1.23) while the two others fractions had a more narrow distribution. The degree of polymerization (DP) of GM80 is also higher than GM25 fraction. DP can however be adjusted for the M/G ratio. It is thus possible to estimate the average length of the mannose main chain of GM25, GM80 and CLBG fractions. They were respectively equivalent to 3480, 4465 and 3825 mannosyls residues. The radii

Table 3
Structural data of GM25 and GM80 solutions (0.5 mg/mL) determined at 40 °C by SEC-MALLS: weight average molecular weight (Mw), number average molecular weight (Mn), polydispersity index (PI), radius of gyration (Rg).

	Water			Phosphate buffer		
	GM25	GM80	CLBG	GM25	GM80	CLBG
M_w (kDa)	736.7 ± 15.1	699.6 ± 49.5	1017.9 ± 162.8	761.1 ± 9.3	911.3 ± 54.2	772.2 ± 9.8
M_n (kDa)	590.3 ± 15.1	636.5 ± 52.6	696.7 ± 86.2	655.9 ± 13.9	897.7 ± 67.2	631.7 ± 12.9
DP_w[a]	4548 ± 93	4319 ± 306	6283 ± 1005	4698 ± 57	5625 ± 335	4767 ± 60
DP_n[a]	3644 ± 155	3929 ± 325	4307 ± 532	4049 ± 86	5541 ± 415	3899 ± 80
PI	1.25 ± 0.03	1.10 ± 0.04	1.46 ± 0.07	1.16 ± 0.03	1.02 ± 0.02	1.23 ± 0.04
$\langle Rg \rangle_w$	91.8 ± 1.5	94.2 ± 3.4	99.2 ± 2.5	91.7 ± 1.7	106.9 ± 4.2	89.3 ± 2.8
$\langle Rg \rangle_n$	100.1 ± 1.5	100.6 ± 1.5	107.6 ± 2.3	98.2 ± 2.2	109.5 ± 3.5	96.4 ± 4.0

[a] Equivalent hexose units.

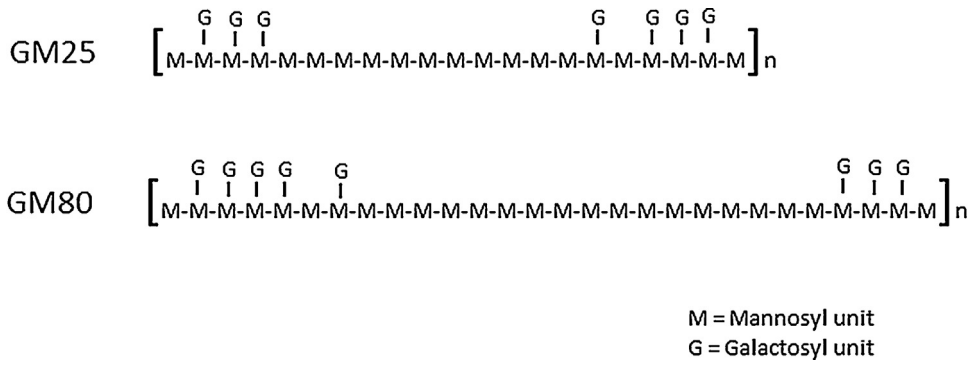

Fig. 4. Plausible schematic representation of GM25 and GM80 fractions structures. M and G are, respectively, mannosyl and galactosyl units.

of gyration (*Rg*) were also determined. It is the average distance between one end of the chain and the center of mass of the structure. The GM80 fraction had the highest value of *Rg* (107 nm). This is in accordance with a study through computer prediction showing that richest galactomannans in long and "smooth" regions have a more extended random coil conformation in aqueous solution (Petkowicz, Reicher, & Mazeau, 1998).

The fractionation process thus generated two fractions of different chemical structures, for which a schematized structure is proposed in Fig. 4: the GM25 fraction consisted of galactomannans composed of shorter chains, richer in galactosyl, which were distributed at 70% inside "block" structures (grouped in "hairy" regions); The GM80 fraction consisted of longer galactomannans less substituted in galactosyls, although these were concentrated to 83% as substituted blocks (also grouped in "hairy" regions).

3.3. Physical properties

3.3.1. Solubilization kinetics

LBG is an amorphous polymer and its solubility is not dependent on a fusion of crystalline areas. Rather, it is the result of thermodynamic partitioning based on a classical polymer-solvent fractionation, a mechanism that applies only for polysaccharide components with M/G ratio > 2.85 (Gillet, Simon, Paquot, & Richel, 2014; Pollard et al., 2007). Several studies have been focused on the impact of M/G ratio and MW on solubility (Gillet, Simon, et al., 2014; Pollard et al., 2007, 2008; Rinaudo, 2001). These studies indicate that a high galactose substitution and low molecular weights improve the dissolution. Galactomannans with longer chain lengths and poorer in galactose require higher temperatures or longer time to be completely dissolved in water.

The results presented in Fig. 5A are in line with this. GM25 and GM80 fractions dissolve very quickly at 80 °C, as 85–90% yields were obtained in 5 min. Then, solubilization increased more slowly to 95–100% after 180 min. At this solubilization temperature, GM25 fraction was still slightly more soluble than the GM80 fraction, for all the dissolution times. However, extraction yields depended on how GM25 and GM80 fractions were obtained (air drying, grinding, etc.) and, therefore, on their ability to disperse in water (Pollard et al., 2007). At a working temperature of 25 °C, GM25 fraction dissolved much more slowly. However, it reached a yield of 90% after 3 h of dissolution. Under these conditions the GM80 fraction remained insoluble.

The results presented in Fig. 5 are not surprising because GM25 and GM80 fractions were extracted, respectively, at 25 and 80 °C. It seems logical that they are resolubilized to these respective working temperatures. GM80 which is rich in smooth areas, was nevertheless completely insoluble for each dissolution time at 25 °C. The greater presence of galactose or shorter chains may therefore limit intra- and inter-molecular (H-bonds) interactions that lead to aggregation and precipitation of GM80 in these conditions.

3.3.2. Viscosity and viscoelasticity

Apparent viscosity and mechanical spectrum of dispersions can be studied in semi-dilute conditions. Fig. 6A presents steady shear viscosity profiles of GM25 and GM80 fractions. This graph shows the relationship between apparent viscosity η (Pa s) and shear rate (s^{-1}). The viscosity generated by galactomannans dispersions is due to two distinct factors: (i) the interpenetration of the macromolecular chains leading to the creation of "physical" non-specific recovery; (ii) the presence of more specific interactions between macromolecules resulting from intermolecular aggregation phenomena: the hyper-entanglements (Gillet, Blecker, et al., 2014). Apparent viscosity of dispersions in semi-dilute conditions is therefore related to the molecular structure that influences

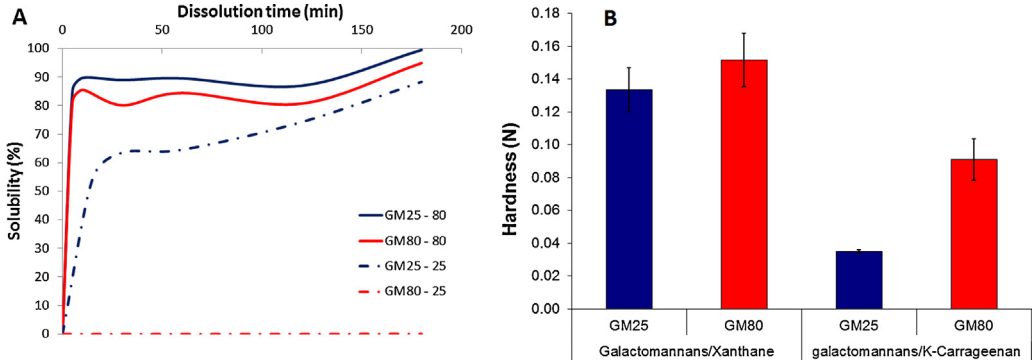

Fig. 5. (A) Evolution of GM25 and GM80 solubility during dissolution time. Dissolution temperatures of 25 °C and 80 °C are shown, respectively, with dotted and continuous lines. (B) Hardness (N) of galactomannan/xanthan (1:2) and galactomannans/carrageenan (1:2) gels generated at 80 °C (6 g/L). Comparison between GM25 and GM80 fractions.

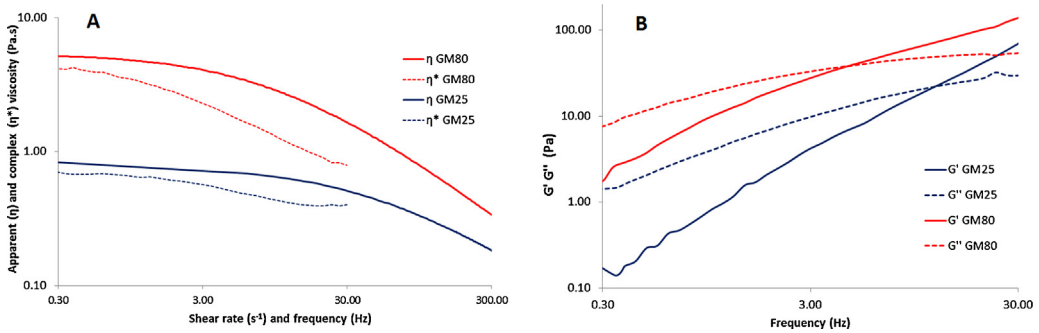

Fig. 6. (A) Steady shear viscosity profile of GM25 and GM80 dispersions (1%). (B) Mechanical spectra of GM25 and GM80 dispersions (1%).

possibilities of interactions (Kapoor, Milas, Taravel, & Rinaudo, 1994; Morris, Cutler, Ross-Murphy, & Rees, 1981; Rinaudo, 2001).

Both fractions studied showed a shear thinning behavior (or pseudoplastic). This means that the apparent viscosity decreased as the shear rate increased. Under imposed shear stress, macromolecules are disentangled more or less quickly – depending on entanglements density and of interactions strength – to end up oriented in the direction of the flow. This macromolecules reorganization results in a decrease of viscosity. Although the shear thinning effect is much stronger for the GM80 fraction ($n_{GM80} = 0.333$ and $n_{GM25} = 0.389$), it possesses a greater apparent viscosity η on the range of shear rates tested ($K_{GM80} = 17.838$ and $K_{GM25} = 7.227$). It is therefore possible to conclude that the molecular structure of the GM80 fraction promotes such intermolecular (aggregation) interactions and therefore hyper-entanglements.

The study of viscoelastic properties is one of the main techniques to highlight entanglements. The viscoelastic behavior of a solution/dispersion is also influenced by the structure of polymers. The mechanical spectra of GM25 and GM80 fraction dispersions are shown in Fig. 6B. The graph is typical of galactomannans in semi-dilute conditions that are viscoelastic fluids generating an entangled network. The loss modulus G'' (Pa) is greater than the storage modulus G' (Pa) at low oscillation frequencies, whereas the reverse is observed at higher frequencies. Beyond the shift point, the oscillation frequency becomes too high and galactomannans chains cannot be dissociated. The GM80 fraction had a much stronger viscoelastic behavior and greater dynamic (complex) viscosity η^* than GM25 fraction (Fig. 6A) whose G' and G'' are lower. The intersection between G' and G'' curves was shifted at lower frequencies for GM80 fraction. This means that it quickly had an elastic dominance behavior generated by entanglements and hyper-entanglements which dissociate less easily.

The relationship between apparent viscosity (η) and complex viscosity (η^*) can also be studied as shown in Fig. 6A. Superimposability of the two viscosities – known as the Cox–Merz rule (Cox & Merz, 1958) – is satisfied for the random coil polysaccharides in which the rheological behavior is controlled by simple physical entanglements (recovery) (Andrade et al., 1999). A greater η^* than η is characteristic of a weak gel (Kulicke & Porter, 1980), while the deviation observed in Fig. 6A is probably due to different types of molecular rearrangement occurring in the two flow patterns over the applied shear rate or frequency range (Richardson & Ross-Murphy, 1987). These rearrangements can be attributed to more specific molecules associations for larger timescale or hyperentanglements than non-specific physical entanglements (Morris et al., 1981). These phenomena are more pronounced for the GM80 fraction for which the η^* curve has a greater deviation than GM25. Both dispersions of galactomannans fractions therefore do not behave as ideal viscoelastic fluids. However, the GM80 fraction structure generated a denser and tougher network, characterized by stronger intermolecular interactions and many more hyperentanglements.

3.3.3. *Formation of associative gels*

When xanthan, galactomannans or carrageenan alone is dissolved at the same concentration as in the mixed gels or at the total concentration of the gels, they do not generate gels. Each fraction alone – regardless of the solubilization temperature – exhibits strength gels near 0.02 N. Only mixed gels are able to generate positive synergies, even when mixed gels are dissolved at 25 °C. In those conditions GM25 and GM80 fractions have gel strengths, respectively, of 0.06 N and 0.03 N. It is therefore possible to generate a gel, even at lower temperatures and under conformational transition temperature of xanthan. The GM80 fraction which is probably not dissolved at 25 °C, presented lower values. Fig. 5B shows the measured force (N) or hardness of mixed gels between GM80 or GM25 fractions and xanthan or carrageenan. Stronger gels were obtained by combining galactomannans in xanthan. The GM80 fraction generated stronger gels (0.152 N) when associated with xanthane at 80 °C. The concentration of solutions, dispersions or gels was invariable. Thus, only the structural characteristics can explain the differences observed in gel formation behavior. Some authors demonstrated a positive correlation between the storage modulus G' of a mixed galactomannan/xanthan gel and the amount of "smooth regions" established in the galactomannan, but they have not been able to determine whether these smooth regions are involved in the junction areas with xanthan (Daas et al., 2002). GM25 and GM80 mixed gels with xanthan seemed to follow the same trend because GM80 fraction – the most organized in "blocks" – generated the strongest gels.

These findings accredit model proposed by Mannion et al. (1992) and Bresolin et al. (1998) wherein interactions between xanthan and galactomannans may be due to two distinct mechanisms. The first occurs at room temperature, giving weak and brittle gels, and is relatively independent of the galactose content of galactomannans. The second mechanism requires higher temperatures to form strong gels highly dependent on galactose composition. Both mechanisms can coexist.

The same findings also arise from the observation of mixed GM25 or GM80/carrageenan gels, although they are weaker. Structure of GM80 fraction appears again to generate stronger interactions with other polysaccharides.

4. Conclusions and perspectives

The choice of the purification/fractionation temperature has a great influence on the average chemical structure of the clarified gum obtained. Both GM25 and GM80 fractions have close structural features but are nevertheless different enough to generate distinct behaviors in solution or dispersion. Typically, galactomannans obtained by extraction at 80 °C presented a greater viscosity, a more elastic behavior and formed stronger gels in aqueous dispersion. It seems thus that a slight increase in chain lengths, M/G ratios and degree of blockiness, favored the appearance of

intra- and inter-chain interactions. These interactions are responsible for precipitation at low temperatures and responsible for the creation of hyper-entanglements and a stronger network when solubilizing temperatures increase progressively. The pattern of substituents distribution seems to be the most important parameter that determines the behavior of a galactomannan in aqueous solution. Its knowledge (in addition to the molecular weight and the degree of galactose substitution) will predict the properties of the gum (or a fraction thereof), and specific applications that could be targeted. A gum fractionated at low temperature may thus enter the composition of mixtures in which the heating is excluded and rapidly bring it viscosity due to its "cold" solubilization. Conversely, a fraction like GM80 would be preferred for the formulation of highly viscous solutions or strong gels, where the temperature increase is permitted.

Based on measurements carried out by solubility determination, rheometer and texture analyzer, an objective determination of which mechanisms and which parts of the structure are involved in interactions are not possible. However, many models suggest that "smooth" regions are responsible of intra-chain, inter-chain and intermolecular interactions, although it was not clearly demonstrated (Cheetham & Mashimba, 1988; Dakia et al., 2008; Dea & Morrison, 1975; Dea et al., 1972, 1977; Doublier & Launay, 1981). To go one step further and to be able to settle this debate, it would be interesting to test our different samples in solid-state NMR. Recent studies have indeed assumed that differences in mobility observed on NMR spectra between hexose polymers would be explained by the presence of molecular interactions located on these hexoses (Vieira & Gil, 2005). It would therefore be possible to establish a correlation between the percentage of smooth areas and the percentage and nature of the least mobile hexoses, and see if the proposed models are verified.

On the other hand, it could be interesting to split the CLBG fraction into other fractions to have a finer observation panel of structure–function relationship of galactomannans obtained by purification. Some studies have thus demonstrated an increase in the M/G ratios and chain lengths as the purification temperature increases gradually (Gaisford et al., 1986; Mannion et al., 1992; McCleary, Clark, Dea, & Rees, 1985; Pollard et al., 2007; Richardson et al., 1998). It would be interesting to complete these results by the determination of other structural characterization parameters such as degree of blockiness and diad (or triad) frequencies. Combinations of GM25 and GM80 in varying proportions may also be studied in order to obtain mixtures of original properties for certain applications.

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