Effect of the Seed Morphology on the Separation Yield, Chemical Characteristics and Thickening Capacity of Carob (*Ceratonia siliqua L.*) Gums

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ABSTRACT— ***The present work aims at bringing new insights into the galactomannan physicochemical characteristics through an unprecedented approach: relationship between the morphology of carob seeds and corresponding gums chemical and physical properties. Therefore, four commercial carob seeds with different granulometries were studied. Their endosperms (Locust bean gum, LBG) were evaluated in term of yield, chemical composition, sugar composition and macromolecular and rheological properties. Acid dehulling furnished a gum yield in the range of 39.4–42.0%. The greater size of the seeds allowed an easier dehulling and a production of more and purer gum flour. The viscosity of 1% LBG aqueous solutions varied widely from 903 to 2081 mPas and appeared to be influenced by the seed size. The mannose to galactose ratio, of the extracted gums, did not show clear difference that may explain the difference in viscosity. However, the macromolecular size values for intrinsic viscosity [η] and radius of gyration (Rg) of the extracted gums, increased from 11.73 to 13.64 dl.g^{-1} and from 71.7 to 76.6 nm, respectively, with increasing seed morphology. Moreover, the total galactomannan content varied from 74.88 to 83.79 with increasing seed size. The best thickening properties were observed for the gum from the seeds with the highest size, in consistence with their macromolecular features, combining probably with their total galactomannan content. The physicochemical characteristics of locust bean gum flours were found to be dependent on the carob seeds morphology.***

Keywords— Carob seed morphology; galactomannan; sugar composition; intrinsic viscosity; apparent viscosity

1. INTRODUCTION

Locust bean gum (LBG, E410) is a galactomannan extracted by grinding the endosperm portions of the seeds of the legume plant *Ceratonia siliqua* L mainly grown in Mediterranean regions.
Galactomannans are widely used as additives in the food (ice cream, pet food, and others) and non-food (paper, textile, pharmaceutical, cosmetic and others) industries, due to their ability to yield high viscosity at low concentrations [12, 24, 25, 36]. Being non-digestible, they are considered as dietary fibre in foods [9, 14, 15]. In more recent times, LBG is used as a fat substitute in mayonnaise.
Galactomannans are linear polysaccharides based on a β-(l→4)-mannan backbone to which single D-galactopyranosyl residues are attached via α-(l→6) linkages [10, 16]. The galactose side branches are not spaced uniformly [10, 13, 31]. The average mannose to galactose ratio (M/G) in LBG is approximately 3.5. Carob seed galactomannans differ in their mannose to galactose ratio (M/G) and their molecular size, depending on the regional localization, the variety and age of the plant (tree), the growth conditions (climate, soil) [4, 6, 17, 21, 26, 27, 30, 35] and the method used for gum extraction [12, 29] or for gum purification [1, 9, 10, 15, 16, 22, 23].

However, very little is known about the relationship between the morphology of carob seeds and the corresponding chemical and physical properties of gums. The molecular structure (the degree of mannose substitution with galactose) and the molecular size affect water solubility, influence ability to intermolecular association, and also control the rheological properties of LBG [12].

The objective of this study was to compare the composition and physicochemical properties of the carob gums extracted from seeds of different morphologies. The characteristics of the extracted gums were evaluated in terms of yield, chemical composition, mannose to galactose ratio and macromolecular features and solution viscosity.

2. MATERIAL AND METHODS

2.1. Raw material and extraction procedures

Raw material*:* The carob seeds used in this study were commercial grains belonging to the same population of Malaga region. They were provided by Tropical Agriculture S.A. (Malaga, Spain) and gauged beforehand according to their morphology (length x thickness). For example, a carob seed with morphological characteristic of 5.5x3, meaned that its length ranging between 5.5-6 mm and its thickness ranging between 3-3.5 mm relating to the sieves used. According to **Figure 1**, seeds presented different morphologies: from less or more spherical to less or more flat. Over 60% had a length (or diameter) of 7 mm. Class 4.5 x 7 was the most important.
Four types of seeds of different and increasing sizes were selected for the study (Figure 1*).

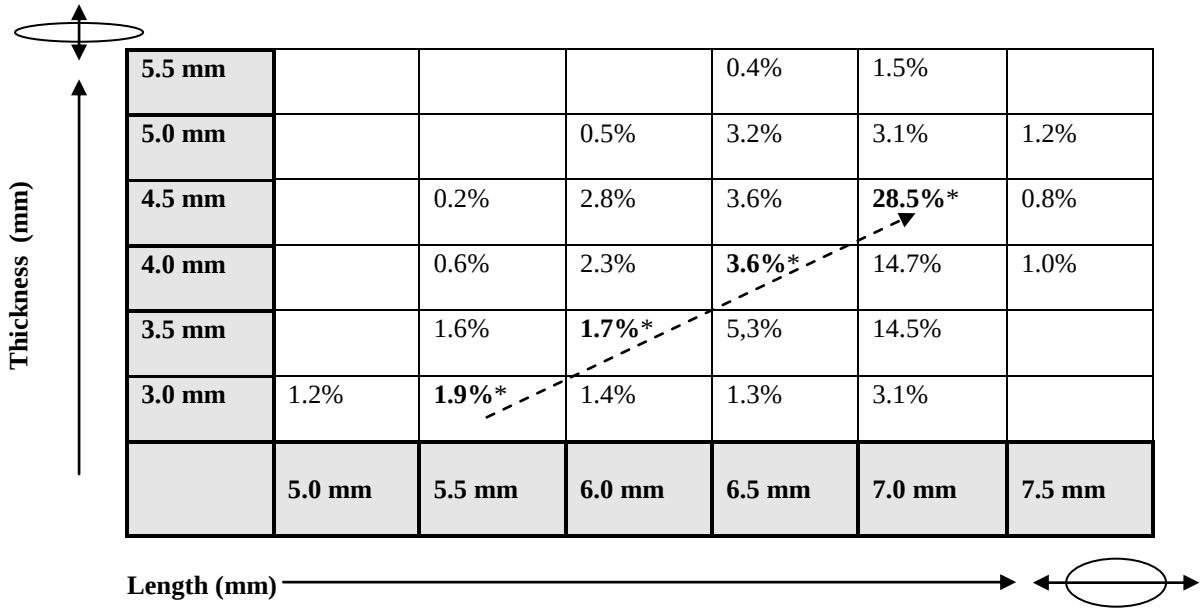

Figure 1: Distribution of the commercial carob seeds (%weight) according to their morphology.
(*) Samples selected for the study

Gum extraction procedure*:* Locust seeds gums (LBG) were prepared under the following conditions: Whole seeds (100g) were macerated in 60 ml of H_2SO_4/H_2O 60/40 v/v solution at 60 °C for 1 h. This treatment with acid at an elevated temperature carbonized the hull which is removed by an extensive washing and rubbing operation in water and through a metallic sieve of 2 mm. The dehusked seeds were dried at 100 °C for 30 min and then briefly crushed (10 seeds dehusked/ 3 s) with a laboratory mill (model MF 10, IKA, Staufen, Germany) to separate the two endosperms thereby releasing the friable germ. The germ fractions were then sifted off from the unbroken endosperm halves with a 2 mm sieve. The pieces of endosperm were ground to pass through a 0.125 mm sieve. This final product was referred to as locust seeds gums from acid dehulling pre-treatment (LBGa). Acid dehulling procedure was found to give better results in terms of gum viscosity and molecular properties than boiling water pretreatment as previously described [11, 12, 29].

2.2. Proximate composition

The *moisture* content of the carob gum was determined gravimetrically after drying the material (500 mg) in an oven at 105 °C for 24 h. The *ash* content of the carob gum (3 g) was determined gravimetrically after dry mineralization at 600 °C for 12 h. The *lipid* content of the carob gum (3 g) was determined by extraction with chloroform/methanol (2/1 v/v) as described by Folch et al. [19]. The solvent was removed by rotatory evaporation at 35-40°C under reduced

pressure. The extracted lipids were dried in a desiccator to constant weight and determined gravimetrically. The *protein* content of the carob gum (150 mg) was determined by the Kjeldahl procedure, after mineralization (with a 1000 KJELTABS MQ tablet and a Digestion System 20, 1015 Digester, Tecator AB, Höganäs, Sweden) and distillation (by a Kjeltec Auto 1030 Analyser, Tecator AB, Höganäs, Sweden) with a conversion factor of 5.87 according to Anderson [2]. The *nitrogen free* extract (as carbohydrate content) was estimated by difference.

2.3. Sugar composition by Gas-liquid Chromatography

The monosaccharides in carob gum were determined as their alditol-acetate derivatives by gas-liquid chromatography (GLC) analysis after hydrolysis of polysaccharides with 1 M H_2SO_4 sulphuric acid (100 °C / 2h) according to the slightly modified method previously described [1, 5, 12]. Optimum hydrolysis time is dependent on a balance between the rate of release of hydrolysable polysaccharides and the degradation of monosaccharides that occurs during prolonged treatment under experimental conditions. The sugars in the hydrolysate (0.4 ml) were reduced to their corresponding alditols by adding 2 ml of DMSO containing 2% $NaBH_4$. Reduction was performed for 90 min at 40 °C. The excess of sodium borohydride was then destroyed by adding 0.6 ml glacial acetic acid. Acetylation was then performed with acetic anhydride (4 ml, 10 min at room temperature) in the presence of 1-methylimidazole (0.4 ml) as a catalyst. Acetylation was stopped with 10 ml deionized water and the acetylated alditols were partitioned between dichloromethane (4.0 ml) and water. After the phase separation, the lower one was removed with a pasteur pipette and putted (1 ml) in a septum-cap vial.

2-deoxy-D-glucose was employed as internal standard and standards of different carbohydrates (L(+)-rhamnose, D(–)-arabinose, D(+)-xylose, D(+)-mannose, D(+)-glucose and D(+)-galactose from Fluka Chemie (Buchs, Switzerland)) were used. The analyses were accomplished using a Hewlett–Packard Agilent 6890 series gas chromatograph equipped with a HP1 column (30 m×0.32 mm, film thickness 0.25 µm). Derivatized extracts (1.0 µl) in dichloromethane were injected on-column. Helium was used as the carrier gas with a flow of 1.6 ml/min. The injection temperature was 290 °C and the temperature program was: 1 min at 120 °C, linear increase in 4 min to 220 °C and finally in 35 min to 290 °C and this temperature was then maintained for 4 min. Compounds were detected using a flame ionisation detector at 320 °C.

2.4. Macromolecular characteristics by Size exclusion chromatography (SEC)

The gums were solubilized (0.1% w/v on a dry weight basis) in deionized water at 80°C for 30 min under mechanical stirring. Insoluble material was removed by centrifugation at 9400 × g (14000 rpm in a Centrifuge BECKMAN J-21C, rotor JA14) for 30 min at 20°C and filtration through a 0.45 μm membrane filter prior to the injection onto the column and final polymer concentration determination. The weight-average molecular weight (M_w) and intrinsic viscosity [η] of carob gums were determined using high performance size exclusion chromatography (HPLC Waters 2690 ALLIANCE) equipped with a $TSKGMPW_{XL}$ column (TosoHaas Co. Ltd., Tokyo, Japan) and coupled with refractive index (RI, Model 2410, Waters Corporation, Milford, USA), viscosity and right angle laser light-scattering (Dual Detector, Model 270, Viscotek, Houston, USA) detectors. The distribution of the molecular weights (MW) was calibrated using solutions of known MW standard dextran. The columns was thermostated at 30 °C, the flow rate was of 0.7 ml/min, the mobile phase was 0.05 M $NaNO_3$ with 0.05% NaN_3 as conservator and the injection volume was of 100 µ1.

2.5. Viscosity measurement

Gum solutions were prepared at 1% concentration on a dry weight basis in distilled water, at 80°C under mechanical stirring for 30 min and cooled at ambient temperature before measurements. Note that heat treatment is required for maximum solubilisation and to achieve the best water binding capacity [20, 28, 32].

Viscosity measurement were performed in a Rotovisco Haake RV20 (Germany) rotational viscometer fitted with a thermostatic bath for temperature control. A Haake CV20 controller was used to program the tests and the sensor System ME30 utilizing a cone/cylinder configuration was used for measurement. Each sample (3 ml) was placed in the sensor system for measurement at 25 °C at the shear rate of 10 s^{-1}.

Descriptive statistics were done and results were expressed as means ± SD. All the measurements were performed at least in duplicate.

3. RESULTS AND DISCUSSION

3.1. Extraction yield

Seeds were dehulled in acid medium and then divided into coats, endosperm and embryo. **Table 1** reports the size (morphology or granulometry) and the number of the seeds in 100 g of dry seeds; and also the yield of endosperm (gum), germ (embryo) and husk (tegument). The number of seeds, for the same weight (100g), varied from 925 for grain

1 to 444 for Grain 4: Grain 1 (925 seeds /100g) < Grain 2 (680 seeds /100g) < Grain 3 (562 seeds /100g) < Grain 4 (444 seeds /100g). The yield in endosperm (gum source) varies slightly from 39.4% for Grain 1 to 42% for Grain 4. The yield in germ showed also a variation from 16.8% for Grain 1 to 20.8% for Grain 4. Variation in endosperm and germ yield is reflected in the yield of the husk (tegument), which was higher (43.8%) in Grain 1 and lesser in Grain 4 (37.2%). From these extraction results, it can be assumed that the biggest seeds were the best for carob gum yield. In addition, in practice the big size of the seeds made the dehulling process easier and permitted to produce more and purer gum flour (with a minor amount of teguments fractions as contaminants).

Table 1. Carob seeds of different granulometries: characteristics and separation yields.

	Grain 1	Grain 2	Grain 3	Grain 4
Morphology (mm)				
(Length x Thickness)	5.5x3	6x3.5	6.5x4	7x4.5
Seeds number in 100 g	925	680	562	444
Endosperm (%)	39.4 ± 1	40.1 ± 3	40.5 ± 3	42.0 ± 5
Germ (%)	16.8 ± 4	18.8 ± 1	20.5 ± 4	20.8 ± 0.5
Husk* (%)	43.8	41.7	39.0	37.2

**Obtained by difference*
Grains 1 to Grain 4 are carob seeds which size increase from Grain 1 to Grain 4.

3.2. Chemical composition

In order to evaluate the seeds (Grain 1, Grain 2, Grain 3 and Grain 4) composition regarding to seeds morphological characteristics, their corresponding endosperms flours (LBG1, LBG2, LBG3, and LBG 4) were evaluated, and the results were reported in **Table 2**.

According to these results, it can be observed that the extracted gums flours do not show evident difference, excepted a relatively high protein content (7.0%) for LBG 1 from the smaller seeds (Grain 1).

Table 2. Carob seeds endosperms (LBG) flours composition (%)

	LBG 1	LBG 2	LBG 3	LBG 4
Moisture	9.18 ± 0.22	9.38 ± 0.30	9.83 ± 0.120	10.17 ± 0.09
Ashes	0.92 ± 0.05	0.78 ± 0.07	0.95 ± 0.03	0.97 ± 0.02
Proteins (N × 5.87)	7.00 ± 0.24	4.90 ± 0.15	5.95 ± 0.84	5.46 ± 0.22
Lipids	1.10 ± 0.02	1.05 ± 0.02	0.90 ± 0.07	0.93 ± 0.03
Nitrogen free extract (by difference)	90.98 ± 0.10	93.23 ± 0.13	92.20 ± 0.31	92.64 ± 0.09

All values were determined in triplicate. All measurements are on a dry weight basis ± SD (except for moisture). LBG 1 to LBG 4 are gums from carob seeds which size increase from Grain 1 to Grain 4.

This high protein content in LBG 1 reflected a high level of germ fractions, remaining in some dehusked seeds during the gum extraction. In fact, it was difficult or imposible to separate germ from endosperms for some very small seeds. Note that germ is a good protein (>50%) source [11]. This observation confirm that smalls seeds constituents (coat, endosperm and germ) separation is more difficult that the big ones.

3.3. Viscosity determination

To perform a homogeneous comparison of viscosity measurements, all LBG samples were prepared at 80°C/30 min, at 10 $g.l^{-1}$ concentration, and measured at 25°C at the shear rate of 10 s^{-1} with a rotational rheometer. As it can be

seen at **Figure 2**, the apparent viscosity values expressed in mPas (cps) of these extracted gums (LBG1 to LBG4), for similar concentration (1%), increase (LBG 1 (903 mPas) < LBG 2 (1417 mPas) < LBG 3 (1828 mPas) < LBG 4 (2081 mPas)) with increasing seeds size (Grain 1 (5.5x3 m) < Grain 2 (6x3.5) < Grain 3 (6.5x4) < Grain 4 (7x4.5)).

The fact that the gum solution viscosity increases with increasing seed size suggested that there is a relation between these two parameters. Also, these results shown that there may be a possible relevant difference between these galactomannan samples, perhaps regarding to their M/G ratio and their macromolecular size. Because, according to some authors [30], higher mannose to galactose ratio (M/G) and a higher macromolecular size, lead to higher thickening capacity.

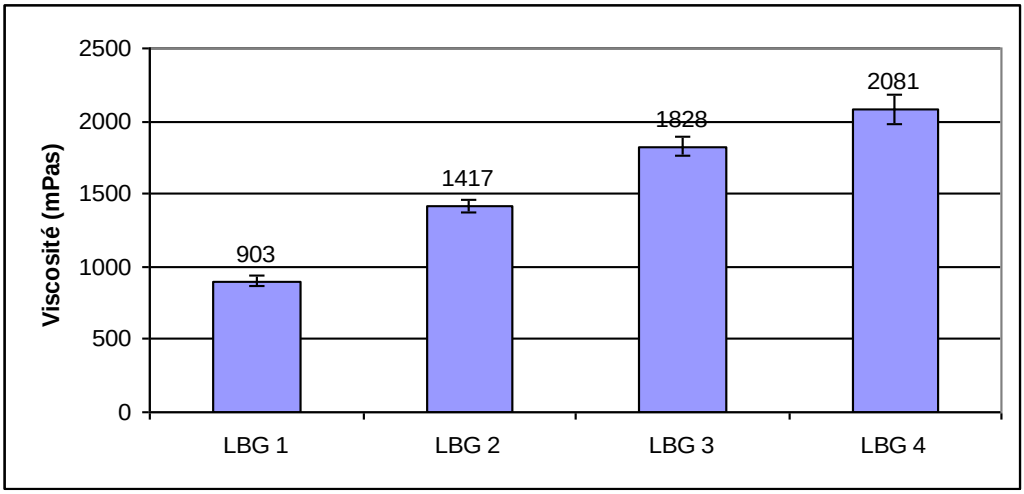

Figure 2. Viscosity measurement at 25°C at 10 s^{-1} shear rate for extracted LBG samples prepared at 1% concentration at 80°C/30 min. *LBG 1 to LBG 4 are gums from carob seeds which size increase from Grain 1 to Grain 4*

3.4. Sugars composition

To correlate the observed viscosity with the M/G ratio parameter, monosaccharide residues in gums flours were identified and quantified (**Table 3**).

The results showed that LBG 1 contains a high content of minor sugars (arabinose, xylose, rhamnose, and glucose) when it's compared to LBG 4. If it is reasonable to argue that these minor sugars were the products of acid hydrolysis of non-galactomannan polysaccharides, it is possible that these minor sugars come from the hydrolysis of seeds coat carbohydrates. Seed coat fine fractions were gum contaminants [11, 12], and the minor sugars level may indicate the degree of contamination. These analytical results showed again that the big seeds made the dehusking process easier and permited to produce purer gum flour.

The total galactomannan (GM) content, calculated as the sum of mannose and galactose, ranged from 74.88 for LBG 1 to 83.79 for LBG 4. It can be hypothesized that LBG 4 with its relatively high level of GM content gave a greater viscosity, due to the additional material (GM) being solubilised.

The values of M/G ratios changed slightly between LBG 1 (3.15) and LBG 4 (3.24). This range is similar to those reported by others researchers [9, 12].

Nevertheless, the difference in viscosity (described in section 3.3, fig.2) between LBG 1, LBG 2, LBG 3 and LBG 4 samples was not clearly explained by the general monosaccharide's composition.

Table 3. Monosaccharide composition (% on dry matter) of LBG from seeds of different granulometries

	LBG 1	LBG 2	LBG 3	LBG 4
Rhamnose	0.26 ± 0.07	0.00 ± 0.00	0.08 ± 0.08	0.00 ± 0.00
Arabinose	1.61 ± 0.08	1.24 ± 0.01	1.01 ± 0.10	0.98 ± 0.02
Xylose	0.64 ± 0.15	0.40 ± 0.00	0.39 ± 0.10	0.29 ± 0.00
Mannose	56.82 ± 1.34	62.04 ± 0.33	62.84 ± 1.18	64.04 ± 0.29
Glucose	2.81 ± 0.02	2.53 ± 0.18	2.26 ± 0.31	2.46 ± 0.02
Galactose	18.06 ± 0.31	19.70 ± 0.50	19.04 ± 0.74	19.75 ± 0.14
Galactomannan*	74.88	81.96	81.88	83.79
Man/Gal Ratio	3.15	3.15	3.29	3.24

All values were determined in triplicate. All measurements are on a dry weight basis ± SD.
LBG 1 to LBG 4 are gums from carob seeds which size increase from Grain 1 to Grain 4.
**Total galactomannan (GM) content calculated as the sum of Mannose and Galactose.*

3.5. Macromolecular characteristics

To correlate the observed viscosity with the macromolecular size, the macromolecular properties of the carob seeds gums were analyzed and shown in **Table 4**.

Table 4. Measurements of macromolecular characteristics of LBG samples by SEC (viscotek).

	LBG 1	LBG 2	LBG 3	LBG 4
M_w (Da)	0.942×10^6	0.900×10^6	0.929×10^6	0.973×10^6
Pi = M_w / M_n	1.33	1.32	1.20	1.13
[η] (dl/g)	11.73	13.00	13.32	13.64
Rg (nm)	71.7	73.1	74.3	76.6

LBG 1 to LBG 4 are gums from carob seeds which size increase from Grain 1 to Grain 4.

It can be observed that the estimates molecular weights (Mw) values, apart from LBG1, seems increased from LBG 2 to LBG 4 with increasing seeds size: LBG 2 (0.900×10^6 Da) < LBG 3 (0.929×10^6 Da) < LBG 4 (0.973×10^6 Da).

The polydispersity index (Pi), ($Pi=M_w/M_n$, related to unhomogeneity of polysaccharides) values decreased from LBG 1 to LBG 4 showing an increase in macromolecules homogeneity, with increasing seeds size: LBG 1 (1.33) < LBG 2 (1.32) < LBG 3 (1.20) < LBG 4 (1.13).

The intrinsic viscosity [η] and the radius of gyration (Rg) (a type of molecular size measurement, depending on the dimensions and the extension of the polymer chain) values increased from LBG 1 to LBG 4 showing an increase in hydrodynamic volume, with increasing seeds size: LBG 1 (11.73 dl/g and 71.7 nm) < LBG 2 (13.00 dl/g and 73.1 nm) < LBG 3 (13.32 dl/g and 74.3 nm) < LBG 4 (13.64 dl/g and 76.6 nm).

According to these results (M_w, [η], Rg), LBG4 from the bigger seed seems contain a great number of high and expanded molecules than LBG1, LBG2 and LBG3 samples and this could promote interchain associations and increase its rheological properties.

In general, all values are consistent to those reported by others researchers [7, 12, 18, 27, 33, 34].

Note that the intrinsic viscosity [η] is a macromolecular characteristic directly related to hydrodynamic volume occupied by the macromolecule in a solvent; it represents the volume of solution occupied per unit mass of the macromolecule, which consists of the intrinsic volume occupied by the polymer chain and its excluded free volume. The thickening capacity of the all biopolymer is more important when it mobilizes a large volume of solvent. This is linked to the expansion of the macromolecule in the solvent and depends on its conformation [3, 12, 30, 34]. Therefore, a higher intrinsic viscosity, lead to higher thickening capacity.

Otherwise, LBG 1 compared to LBG 2, LBG 3 and LBG 4 samples indicated a reduction in hydrodynamic volume, since the intrinsic viscosity and the radius of gyration depend on the dimensions and the extension of the polymer chain (Azero & Andrade, 2002). Therefore, the most likely explanation for the small [η] and Rg values (11.73 dl/g and 71.7 nm) and the relatively high M_w value (0.942×10^6 Da) for LBG 1 suggest a presence of great number of small molecules and may also reflect the presence of compacted molecules (self-association through intramolecular

association), and may explain its very less viscosity. It's known that galactomannan macromolecular properties measurements can be complicated by self-association [12, 29, 34].

In general, it can be observed that the mannose to galactose ratios determination did not show evident difference between LBG 1, LBG2, LBG3 and LBG 4 samples, contrary to the analysis of macromolecular structure properties. The hydrodynamic volume [η] and the expansion (Rg) of the extracted gums macromolecules increased with increasing grains morphology (Table 4), and this may explain the difference in viscosity between LBG samples (as described in section 3.3, fig.2). It is noteworthy that there is a relationship between intrinsic viscosity [η] and the apparent viscosity. This relationship was confirmed in this study, where macromolecules with high intrinsic viscosity had high apparent viscosity, and as the intrinsic viscosity increased from one seed morphology to another, apparent viscosity increased as well. The viscosity of a galactomannan used as hydrocolloid thickener is intrinsic viscosity dependent, the relation between [η] and viscosity was confirmed in this study.

At the other hand, perhaps the extraction condition affected the overall result. It can be hypothesized that the endosperm (gum source) from the bigger grains were less influenced (in term of possible partial degradation) by the acid dehulling pre-treatment than the smaller carob seeds.

Considering the effect of the unsubstituted sequences content along the mannan chain backbone on the formation of the intermolecular association that give rise to viscosity [3, 8, 10, 12, 22, 23, 30], it would be interesting in further work to determine the fine structural differences between the galactomannans extracted from seeds with different size (e.g. using 13 CNMR spectroscopy).

4. CONCLUSIONS

This study reports on the chemical characteristics and rheological properties of carob (*Ceratonia siliqua* L) gum (endosperm) from seeds of different morphologies. It mainly examines the effect of carob seed morphology on the structural properties (especially mannose/galactose ratio, macromolecular parameters (intrinsic viscosity, molecular weight,.)) and on the viscosity of gum solutions. To this aim, commercial carob seeds from the same population were used and four classes of seeds of different and increasing sizes were made up for the study.

On the basis of the results obtained, it could be concluded that the seeds of the biggest size appeared to be the best in terms of technological potential: An easier (de)hulling process for gum (endosperm) isolation, the purest gum flour, the best thickening properties (the highest viscosity) at the same solution concentration of 1% (w/v).

Apart from the macromolecular parameters of gum, from seeds of the biggest size, which were clearly higher compared to those of the others, neither the whole chemical compositions nor the mannose/galactose ratios of the different gum flours were likely to account for the significant differences observed in viscosity data. As expected, the bigger the seed size is, the higher the macromolecular ([η], Rg) and rheological (viscosity) properties of derived gum solution are.
This study is relevant and would be useful for the development of a better carob gum production according to the carob seeds size. At the other hand, it can be assumed that the characteristics of carob galactomannan polysaccharide may depend on the seeds morphology, in addition of the origin and the extraction method.

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