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Carob (*Ceratonia siliqua* L) Seed Tegument Separation and Analysis: Comparison with Locust Bean Gum Hot-Water-Insoluble Residue Monosaccharide's Composition

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Abstract—.**The proximate composition of the carob seed hull were analyzed in order to evaluate the effect of carob seed tegument fragments (as contaminants) on locust bean gum (LBG, a galactomannan usually used as food additive) composition. It has been observed that the hot-water-insoluble residue, recovered after LBG flour dissolution and centrifugation, contains about 71-100% of total arabinose, xylose and rhamnose present in LBG crude flour. These results suggest the presence of non-galactomannan polysaccharides in LBG flour. The separation of the seed components by boiling water pre-treatment furnished ~30% of brown coat. The seed coat sugar composition, determined by GC analysis after partial acid hydrolysis, shown that arabinose (~20%) and xylose (6%) are the major monosaccharides in the hull polysaccharides (theorically from hemicelluloses fraction (≥20%)).**

It can suggest that the minors sugars as arabinose, xylose and rhamnose, usually determined in carob gum analysis, came from the small pieces of seed husk remaining in gum flour during its primary extraction process.

Index Terms— **carob seed hull; gum contaminants, hot-water-insoluble residue, sugars composition,**

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I. INTRODUCTION

The carob seed is obtained by crushing the fruit pod of the carob tree (Ceratonia siliqua L.) found in Mediterranean regions. The carob trees are a valuable resource for reforestation of degraded zones where fire, erosion and desertification are a threat. In fact, Carob tree grows well even on poor, sandy and calcareous soil. The economic importance of this species resulting from industrial utilisation of the Locust Bean Gum (LBG, E410) obtained by crushing the endosperm of the seeds. Locust bean gum (LBG) was the first galactomannan used both industrially and in food products (ice cream, baby foods and pet foods) [1-3]. Both its economical and ecological value makes carob one of the most important Mediterranean trees [4].The seed is composed of the husk (30-33%), the endosperm (42-46%) and the germ (23-25%) [5]. The first stage of the endosperm (LBG source) extraction involves removal of the tight-fitting brown coat; either by thermo-mechanical, by chemical treatment [6-10]) or by process combining seeds milling and gum dissolution/purification [11-12]. In all procedures, the process must drastically reduce or avoid, as possible, the presence of impurities from hull and germ pieces, in LBG flour. This seems to be the most technical and economical difficulties which have to be met in carob gum extraction [13].

da Silva and goncalvez [11] had studied chemical composition of crude, purified and the 80°C insoluble residue of LBG samples. The analysis of these three samples shown the following sugar composition (without galactose and mannose contents): crude LBG [rhamnose (0.15%), arabinose (1.38%), xylose (0.32%), and glucose (2.48%)], purified LBG [Rhm (0%), Ara (0.39%), Xyl (0.09%), and Glc (1.01%)], and the 80°C insoluble residue [Rhm (1.55%), Ara (15.61%), Xyl (2.32%), and Glc (7.72%)]. According to these authors, if the sugar composition of the pellet is taken into account, it is plausible to consider the presence of hemicelluloses (such as xyloglucans) and pectic substances (such as arabynans, galactans and arabynogalactans) in the crude gum, which are concentrated in the sediment.
Unfortunately, there is no literature data on the origin of non-galactmannan monosaccharides in LBG flour: natural presence or contamination? There is not either data on carob seed hull composition which could permit to compare seed gum flour and seed hull sugars composition.

The purpose of this study was to analyze the proximate

composition of carob seed husk. Then, the monosaccharide composition in the hull polysaccharide, determined by means of GLC method after acid hydrolysis, was analyzed and compared with the hot water-insoluble components from LBG flour. At the other hand, particular attention was given to the dietary fibers content of carob seed hull, which was determined by non-enzymatic-gravimetric methods, in order to evaluate the insoluble hemicelluloses fraction.

II. Material and Methods

A. Raw materials

In this study, the carob seeds used (length: 5.5-6 mm x thickness 3.5-4 mm) were obtained from T.A.S.A., Parque Arboretum de Algarrobos (Malaga, Spain). The carob seed hull was obtained after an aqueous thermal pre-treatment of carob seeds under the following conditions: 100 g (~780 seeds) of whole seeds immersed in 800 ml of boiling water during 60 min. The swelled seeds, without tegument disruption, were then easily separated manually to obtain the husk, endosperm and germ components. A pure flour of seed coat (hull, testa) was then obtained by drying (100°C/30 min) and milling (IKA A10) the hull fraction.

At the other hand, a commercial sample of locust bean gum (GRINSTED LBG 047, extra high analytical grade, lot number 3121659 from DANISCO Denmark) was used to obtain hot water-insoluble residue (containing non-galactomannan components).

B. Carob gum hot water-insoluble residue isolation

In order to obtain and analyse the LBG hot water-insoluble residue (containing non-galactomannan components), a dissolution/centrifugation method was applied as follow: A 10 g sample of powdered commercial gum was gradually added to strongly stirred distilled water (500 ml), at room temperature. The dispersion was then heated at 80°C ± 2°C for 30 min in a water bath, with stirring. The resulting solution was centrifuged at 2800 ×g at 20°C for 30 min, to separate the supernatant and the insoluble residue fraction (pellet). The sediment (LBG S80°C, insoluble matter containing non-galactomannan components) was recovered, dried and ground to a fine powder for further analysis. The solubilized galactomannan fraction (LBG F80°C) was precipitated by pouring into two volume excess of ethanol 96%, dried at 40°C in an air circulated owen and ground to a fine white powder.

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C. Proximate composition

Moisture content of samples was determined by oven-drying, using an air-circulated oven at 106 °C, for 24 h. All values were calculated on a dry-weight basis. The *ash* content of the carob gum was determined gravimetrically after dry mineralization at 600°C for 12 h. *Protein* content was determined by the Kjeldahl method [14], after mineralization with a Digestion System 20 (Tecator AB, Höganäs, Sweden) and distillation by a Kjeltec Auto 1030 Analyser (Tecator AB, Höganäs, Sweden).

D. The constituent Neutral Sugar analysis by Gas Chromatography (GC)

For the determination of monosaccharide composition, the carob seed hull flour was partially hydrolysed using trifluoroacetic acid (2 M TFA, 2 h at 110 °C). Note that it is a partial acid hydrolysis of the seed tegument flour but a hydrolysis of hemicellulosic and pectin polysaccharides fraction. In fact that cellulose component hydrolysis or carbohydrate complete hydrolysis necessities 72%H_2SO_4 [15]. The neutral sugars, rhamnose (Rhm), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc), excluding the glucose derived from the cellulose (CEL), released by acid hydrolysis of the polysaccharides, were separated and quantitatively determined by gas–liquid chromatography (GLC) after reduction with $NaBH_4$ and acetylation [16-17], [8]. The alditol-acetate derivatives were dissolved in dichloromethane and 1µL samples injected into Hewlett–Packard Agilent 6890 series gas chromatograph equipped a high performance capillary column, BP1-methylsiloxane (30 m x 0.32 mm, 0.25 µm film thickness, Scientific Glass Engineering, S.G.E. Pty. Ltd., Melbourne, Australia) with CP-Sil 5 CP as stationary phase. Helium was used as the carrier gas at a flow rate of 1.6 ml/min. The injection temperature was 290 °C and the column 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 ionization detector (FID) (Hewlett Packard, USA), at 320 °C. 2-desoxy-D-glucose (purity > 99.5%, Sigma Chemical Co., St Louis, MO, USA) was used as an internal standard.

E. Dietary fibre composition by Non-Enzymatic-Gravimetric Method (AOAC 920.86)

Insoluble hemicellulose, cellulose and lignin were determined by the non-enzymatic-gravimetric method, to AOAC 920.86/AACC 32.10 method, described by Van Soest [18].

1. Principle

Reflux degradation/extraction was conducted on the samples in neutral detergent for 90 min to remove starch, proteins, lipids, and soluble fibres (as pectin and soluble hemicelluloses). For Neutral Detergent Fiber (NDF) determination, all chemical digestate solution is filtered through crucible using vacuum, and NDF residue (containing insoluble Hemicullose, cellulose and lignin) is weighed. To obtain Acid-Detergent Fiber (ADF) (containing cellulose and lignin), samples were treated with Acid-Detergent to remove starch, protein, lipid, and pectic and hemicelluloses substances, and then filtered, dried, and weighed. Then, to obtain Acid-Isoluble Fiber (AIF) (containing lignin), the residue (ADF) is treated with sulphuric acid 72% for 3h to remove celluloses substances and then filtered, dried, and weighed. NDF, ADF, and AIF residue values are corrected for ash.

2. Preparation of Neutral Detergent Fiber (NDF)

0.5-1 g sample, ground to pass 1 mm screen, were weighed

into a suitable container (250 mL fritted flask) for refluxing. 100 ml acid-detergent solution (~ 100 ml ND/1g sample) and ~0.5 g sodium sulfite with a rubber spatula were added. The solution was then heated to boiling; and samples were refluxed for 90 min. Boiling was adjusted to an even level, to reduce foaming. Sample was filtered on a previously dried (furnace-dried 500°C/2h in, let cooled and then oven-dried at 105°C/12h) and tared crucible, using light suction. Residue was washed twice with hot water (90-100° C) and twice with EtOH 95%; and then dried at 100°C for 8 h (or overnight), cooled in a desiccator, and weighed. Yield of recovered neutral detergent fiber was reported as cell-wall constituents or as crude dietary fiber. Noncell-wall material was estimated by subtracting this value from 100. Ash was determined by incinerated crucible 2 hr at 500°C. Ash content was reported of neutral-detergent fiber.

3. *Preparation of Acid-Detergent Fiber (ADF)*

5-1 g sample, ground to pass 1 mm screen, was weighed into a 250 mL fritted flask for refluxing apparatus. 100 ml acid-detergent solution was added for 1g of sample weighed. Sample was heated to boiling and heat was reduced to limit foaming. Samples was refluxed for 90 min and filtered on a previously tared crucible, using light suction (vacuum admited until/after crucible has been filled). The filtration residue was washed twice with hot water (90-100° C) and twice with EtOH 95%; and then dried at 100°C 8 h or overnight, cooled in a desiccator, and weighed. Yield of recovered acid detergent fiber (ADF) was reported as cellulose and lignin content

4. *Isolation of Acid-Insoluble Lignin (AIL)*

Crucible was placed in a 50 ml beaker for support. An amount of about halfway (3 cm high) with 72% H_2SO_4 was added to the crucible containing the acid-detergent fiber (ADF) and stirred with a glass rod after 10 min. Crucible with glass rod remain in crucible was let at ambient temperature for 1h. Thus, at hourly intervals (as acid drains away), the crucible was refilled with 72% H_2SO_4, and stirred. After 3 hI, the crucible containing the acid-detergent fiber (ADF) was filtered with vacuum, and washed with hot water until free from acid. Stirring rod was also rinsed and removed. Crucible was dried at 103°C and weighed after cooling in a desiccator. Then, crucible was ignited in a muffle furnace at 500°C for 2 h. Crucible was placed when still hot into desiccator, cooled and weighed. Yield of recovered acid insoluble lignin was reported as lignin fraction

The content of cellulose and hemicellulose can be calculated from the contents of NDF, ADF and lignin, as follow: Hemicellulose is weight of NDF residue less weight of ADF residue, while Cellulose is weight of ADF residue less weight of lignin.

All values were determined at least in duplicate and standard deviation (SD) was calculated.

III. Results and Discussion

A. *Carob seed gum hot-water-insoluble material chemical composition*

The chemical feature of the insoluble residue recovered after LBG flour dissolution/centrifugation (as described in section 2.2) is shown in table 1. Total yield of the sediment (LBG S80°c) accounted for 15.51% while the soluble and refined galactomannan (LBG F80°c), accounted for 75.04%. The lost part (difference to 100% of initial material was about 10%) eliminated with purification process may constituted of a part of small molecules of galactomannan, of lipids, proteins and mineral components. The appearance of the insoluble residue indicates the presence of high amount of small pieces of carob seed brown hull (previously present in the crude gum as contaminant). According to the protein content, it could be calculated that this insoluble residue contains ~97% of the total protein content (6% in crude LBG), showing that protein was drastically eliminated from the refined fraction. This protein may reflect a possible contamination by germ fractions, in addition of the natural presence of structural proteins and enzymes from the endosperm (da Silva & Gonçalves, 1990[11]; McCleary & Matheson 1974[19]; 1975[19]). It is well established that carob seed germ (embryo) contains a high (>50%) protein content [21-22], [13].

On the other hand, the monosaccharide's analysis of sediment (insoluble material), the refined gum and the crude gum revealed (Table 1) the following composition (without galactose and mannose contents): the hot water-insoluble residue (sediment) [arabinose (3.54%), xylose (0.17%), rahmnose (0.02%) and glucose (0.17%)], the 80°C soluble (purified) LBG [arabinose (0.12%), xylose (0.07%), rahmnose (0.00%) and glucose (0.65%)], and the crude LBG [arabinose (0.75%), xylose (0.23%), rahmnose (0.12%) and glucose (2.12%)].

From these results, it could be calculated that the sediment contains about 97% of total Ara, 71% of total Xyl and 100% of total Rhm of LBG crude flour. Otherwise, it can be observed evident difference in the total galactomannan (Man + Gal) content (~50% in LBG insoluble matter compared to ~ 90% in the LBG soluble fractions), suggesting the presence of high level of impurities (contaminants from germ and hull fractions) in the insoluble. In view of these results, it can be assumed that the "minor" sugars content reflects the evident presence of non-galactomannan polysaccharides.

Our observations are comparable to those of da Silva and Gonçalves [11] whom reported also a higher level of arabinose (15.61%) in LBG insoluble fraction. Difference with our arabinose value (3.54%) may be attributed to the origin and purity of the gum flo-"&ur. The sample used in this paper is an extra high analytical grade of locust bean gum in which contaminants (germ and hull fractions) are probably drastically reduced, compared to a commercial food grade [8].

Table 1: Yields, proximate composition and sugars composition of LBG (crude, purified and sediment) samples (g/100g on a dry matter (DM) excepted moisture)

	Crude LBG	Soluble fraction (purified) LBG F80°C	Insoluble (sediment) LBG S80°C
Yield (g/100g DM)	100	75.04 ± 3.11	15.51 ± 1.47
Moisture	9.85 ± 0.41	9.91 ± 0.12	11.27 ± 0.01
Ashes	1.02 ± 0.15	0.13 ± 0.03	0.61 ± 0.17
Proteins	6.03 ± 0.02	0.83 ± 0.00	30.55 ± 2.67
Rhamnose	0.12 ± 0.03	0.00 ± 0.00	0.02 ± 0.00
Arabinose	0.75 ± 0.02	0.12 ± 0.02	3.54 ± 0.02
Xylose	0.23 ± 0.05	0.07 ± 0.01	0.17 ± 0.02
Mannose (M)	66.11 ±2.01	73.27 ± 3.35	36.61 ± 4.04
Glucose	2.12 ± 0.02	0.65 ± 0.02	0.17 ± 0.01
Galactose (G)	17.16 ± 0.53	17.81 ± 1.04	12.43 ± 1.71
Galactomannan (M+G)	83.27	90.08	49.04

All values were determined in triplicate (means ±SD). All measurements on a dry weight basis

B. Carob seeds hull chemical composition

Results obtained from carob seed hull investigation (Table 2) revealed that the seed constituents' separation, after boiling water pre-treatment, furnished ~30% of brown coat, ~50% of off-white endosperm and ~20% of yellow germ, on dry weight basis. The carob seed hull presented a low content of moisture (0.12%), ash (2.34%), lipids (0.54%) and protein (3.71%). Therefore, the nitrogen free extract determined as total carbohydrate, shows that hull contain a higher content of carbohydrate (93.29%).

Monosaccharide's analysis of carob seeds tegument revealed the presence of arabinose (20.29%) and xylose (6.00%) as major sugars and minor quantities of rhamnose (1.11%), mannose (0.67%), glucose (1.23%) and galactose (1.58%).

In comparison with LBG insoluble residue composition (3,54%Ara in this paper and 15,61%Ara according to da Silva and Gonçalves [11], it is evident that arabinose and xylose usually determined in LBG analysis came from the tegument fine fractions remaining in the crude gum. It can be also proposed that (the high part of) the minor sugars (Rhm, Ara, Xyl, Glc) determined during LBG flour analysis came from carob seed hull polysaccharides.

Table 2: Yields, proximate composition and sugars composition of carob seed husk (g/100g on DM)

	Carob seed husk
Yield (g/100g DM)	30
Moisture	0.12 ± 0.90
Ashes	2.34 ± 0.21
Total proteins	3.71 ± 0.42
Lipids (neutral et polar)	0.54 ± 0.70
Nitrogen free extract included fibres	93.29 ± 0.55
Rhamnose	1.11 ± 0.05
Arabinose	20.29 ± 0.12
Xylose	6.00 ± 0.09
Mannose	0.67 ± 0.09
Glucose	1.23 ± 0.02
Galactose	1.58 ± 0.03

C. Carob seed husk dietary fibre (NDF) composition

Dietary fibre consists of a variety of non starch polysaccharides, resistant to hydrolysis by human alimentary enzymes, which include all indigestible polysaccharides (cellulose, hemicelluloses, pectins, β-glucans, gums) and lignin [23-25].

Table 3 showed the percentage of each components of the total crude fibre (determined as Neutral Dietary Fiber (NDF) by chemical-gravimetric method) in carob seed hull. It could be observed that insoluble hemicelluloses, cellulose and lignin fractions accounted for 20%, 33% and 9%, respectively.

The results suggested that it is evident that arabinose and xylose usually determined in LBG flour analysis came from (the hydrolysis of) the hemicellulosic polysaccharides (≥20%) in the carob seed tegument fine fractions. In fact that cellulose component hydrolysis or carbohydrate complete hydrolysis necessities 72%H_2SO_4 [15].

We could speculate, regarding the high level of arabinose and xylose in hull (as described in section 3.2, Table 2), that the major hemicelluloses in the carob seed hull polysaccharides could, theorycally, be arabinan and xylan components. In addition, the presence in hull, of rhamnose, glucose, galactose

and mannose, but in small proportion, could be attributed to the presence of a more complexe polysaccharides.

da Silva and Gonçalves [11], have previously reported a possible presence of other polysaccharides such as pectic substances (i.e. galactanes and arabinogalactans) which probably influence, in their paper, the total galactose content in LBG.

Table 3: Carob seed husk crude dietary fibre composition (g/100g on DM) by chemical-gravimetric method

	NDF: insoluble Hemicelluloses, Cellulose, Lignin	Composition of NDF		
		Insoluble Hemicellulloses	Cellulose	Lignin
Carob grain husk	62%	20%	33%	9%

NDF = Neutral Dietary Fiber

In general, in view of the LBG insoluble polymer and seed hull composition results (Tables 1, 2, 3), it can be assumed that the minor sugars (Ara, Xyl, Rhm and Glc) in LBG, originates from the pectin and hemicelluloses components in carob seed hull fractions.

We hope our data and interpretation stimulate some further work over structural investigation of the isolated hemicellulosic polysaccharides (using sequential extraction with alkali, as described by Ray *et al.* [25], to confirm our hypothesis and to gain a clearer picture of the entire composition of carob seed hull. Hull structural description could permit to propose a sequential enzymatic attack in carob seed dehulling process

IV. Conclusions

The hot water insoluble residue separated after LBG flour dissolution/centrifugation contains about 97% of total arabinose, 71% of total xylose and 100% of total rhamnose, suggesting the evident presence of non-galactomannan polysaccharides in LBG flour.

The carob seed coat partial hydrolysis revealed the presence of arabinose and xylose as major sugars, with a level of 20.29% and 6.00%; and minor quantities of rhamnose (1.11%), mannose (0.67%), glucose (1.23%) and galactose (1.58%). The dietary fibres (NDF) composition revealed the presence of at least 20% of insoluble hemicelluloses.

This suggests that the minor sugars, particularly arabinose and xylose, usually determined during LBG flour analysis, originate (theorycally from pectin and hemicellulosic polysaccharides) from hull fractions remained in the carob gum flour during its primary extraction process.

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