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# Linear mannan in the endosperm of *Schizolobium amazonicum*

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**Abstract**

The seed endosperm from the Leguminosae *Schizolobium amazonicum* was shown to consist of three different parts: (i) the embryo side or interior section; (ii) the seed coat side or exterior section; and (iii) an intermediate zone rich in galactomannan with a 3:1 Man/Gal ratio located between the two sides of the seeds. The exterior section was submitted to a series of hot water and alkaline extractions and the remaining product investigated by monosaccharide analysis, $^{13}C$ NMR, X-ray and electron diffraction, together with electron microscopy. After an extraction with 16% NaOH, a linear β(1 → 4) microfibrillar mannan could be isolated in this portion of the seeds. This is the first time that a pure mannan fraction has been found in the seed of a Leguminosae. © 2001 Elsevier Science Ltd. All rights reserved.

*Keywords*: Mannan; Galactomannan; *Schizolobium amazonicum*; Endosperm seed; Leguminosae

## 1. Introduction

A number of angiosperms contain other glycans, such as xyloglucans and mannan-based polysaccharides, instead of starch, as the main reserve of their seeds. In these components, the family of mannans is the most widespread and consists of 4 subfamilies, namely glucomannans, galactomannans, galactoglucomannans and linear mannans containing less than 5% of galactose (Hegnauer & Grayer-Barkmeijer, 1993; Stephen, 1983). In glucomannans and galactoglucomannans, the polymer backbone consists of a linear chain of D-mannosyl and D-glucosyl residues linked β(1 → 4), in contrast with mannans and galactomannans, where the main chain contains only D-mannosyl residues linked β(1 → 4) (Avigad & Dey, 1997). As opposed to linear mannans, galactomannans main chains can be substituted very heavily with galactose, containing from ~20% to nearly complete substitution (Reid & Edwards, 1995).

In higher plants, pure mannans have been described in non-leguminous seeds (Stephen, 1983). They are typically found in the endosperms of Palmae such as *Phytelephas macrocarpa* and dates, as well as in coffee beans and seeds of some *Umbelliferae* species (Hopf & Kandler, 1977; Wolfrom, Laver & Patin, 1961). Seeds with deposits of linear mannan in their endosperm are hard and resistant to mechanical damage and retain their resistance even after being expose to water, due to its high insolubility in water (Reid & Edwards, 1995; Stephen, 1983). Mannan resembles cellulose in the conformation of its individual molecular chains and also displays some crystalline polymorphism (Chanzy, Grosrenaud, Vuong & Mackie, 1984; Nieduszynski & Marchessault, 1972), which distinguishes mannan I and mannan II which are frequently encountered together. Mannan I—sometimes called mannan A—is highly crystalline and has a low molecular weight (degree of polymerization ~15) (Aspinall, 1959; Chanzy, Pérez, Miller, Paradossi & Winter, 1987; Meier, 1958). It is soluble in aqueous 6% NaOH (w/w) and adopts a granular morphology (Chanzy et al., 1984, 1987). In addition, mannan I is anhydrous and possesses a density of 1.63 that makes it one of the most dense polysaccharides (Atkins, Farnell, Burden, Mackie & Sheldrick, 1988; Chanzy et al., 1987; Nieduszynski & Marchessault, 1972). Mannan II—sometimes called mannan B—is of high molecular weight (degree of polymerization ~80) (Aspinall, 1959; Mackie & Sellen, 1969; Meier, 1958), is less crystalline and displays a microfibrillar morphology. It is insoluble in aqueous NaOH even at a high concentration and is normally revealed after mannan I has been extracted (Chanzy et al., 1984; Meier, 1958). The crystals of mannan II are less dense than those of mannan I and in addition, they contain some water molecules in their lattice (Millane & Hendrikson, 1994; Yui, Ogawa & Sarko, 1992).

We now deal with some physiochemical features of seeds of the tree *Schizolobium amazonicum*, which belongs to the

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Leguminosae family, subfamily Caesalpinoideae. This tree is quite abundant in the north of Brazil where it is known as "pinho cuiabano" (Corrêa, 1984). The seeds resemble other albuminous leguminous seeds, in the sense that they are rich in a galactomannan. Its 3:1 (Man:Gal) ratio, which represents ~50% of total dry endosperm, has been previously characterized (Ganter, Heyraud, Petkowicz, Rinaudo & Reicher, 1995). In addition to this polysaccharide, a linear (1→5) linked α-L-arabinofuranan has also been isolated (Petkowicz, Sierakowski, Ganter & Reicher, 1998). The latter, which is an unusual polymer, was found also in *Schizolobium parahybae* seeds (Zawadzki-Baggio, Sierakowski, Corrêa & Reicher, 1992) and it has been suggested that *S. amazonicum* and *S. parahybae* are very closely related (Petkowicz et al., 1998; Rizzini, 1986).

The extraction and disencrustation of some mannan-rich solid fragments from the seeds of *S. amazonicum* are described. These fractions are analyzed by monosaccharide analysis, $^{13}C$ NMR, X-ray and electron diffraction, together with electron microscopy.

## 2. Materials and methods

### 2.1. Polysaccharide source

Seeds of *S. amazonicum* were collected in the National Forest of Tapajós, Pará, Brazil, and supplied by EMBRAPA (Empresa Brasileira de Agropecuária)—CPATU—Belém, where a specimen is deposited in the Herbarium (IAN), under number 158478.

### 2.2. Polysaccharide isolation

The seeds of *S. amazonicum* were treated with water at 100°C, for 30 min and then kept at 4°C until swelling took place. Thereafter, the seed coats were removed and each side of the endosperm separated from the embryo. When placed in hot water (65°C) for 6 h, each side of the endosperm became delaminated. Then, it was isolated, an interior section adjacent to the embryo and an exterior one next to the seed coat. A water-soluble mucilaginous substance acted as a glue between the two sections.

Both sections of the endosperm were isolated and submitted to exhaustive hot aqueous (65°C) extractions. The insoluble residue from the aqueous extractions for the exterior section was successively extracted with aqueous 4, 6, 8 and 16% NaOH (w/w), at 100°C, each step taking 2 h. These fractions were named M-1, M-2, M-3 and M-4, respectively. For the interior section, only aqueous extractions (65°C) were carried out, but the tissues became more fragile and were not studied further.

At each step of disencrustation, samples of the exterior fraction were collected and thoroughly washed or dialyzed against distilled water until neutral, and were stored as an aqueous suspension using drops of toluene as preservative. When necessary, the samples were freeze-dried.

### 2.3. Sugar composition analyses

Each sample was solubilized and partially hydrolyzed with 72% (w/w) aqueous $H_2SO_4$ (1 h, 0–4°C) and water then added to reach 0.25 M. The samples were then fully hydrolyzed at 100°C (Saeman, Moore, Mitchell & Millet, 1954). The hydrolyzates were neutralized with $BaCO_3$ and analyzed by high-performance liquid chromatography (HPLC) on a CHO-682 column (Interchim) eluted with pure water at 85°C. HPLC was performed in an equipment that included a Model 6000 A pump, an U6K injector and a R-401 refractometric detector from Waters. Peak areas were calculated using a Chromatopac C-RIB integrator (Shimadzu).

### 2.4. Smith degradation

A sample of fraction M-4 (25 mg) was dissolved in 1.0 M NaOH (5 ml), acetic acid (1.0 M) then added to pH 7, followed by 5 ml of $NaIO_4$. After 7 days oxidation at room temperature, the product was dialyzed, reduced with $NaBH_4$ and dialyzed. Na ions were removed, and the insoluble faction was immediately treated again with $NaIO_4$ and later reduced again with $NaBH_4$. Samples subjected to one and two cycles of oxidation/reduction were hydrolyzed, reduced, and the products analyzed by the GLC of derived alditol acetates.

### 2.5. Methylation analysis

Fraction M-4 (30 mg) was methylated twice by the method of Haworth (Lindberg, 1972), and the per-*O*-methylated product was hydrolyzed (Bouveng & Lindberg, 1960) and analyzed by GLC-MS as partly derived *O*-methylated alditol acetates. The GLC-MS analysis was performed using a Varian chomatograph model 3300 equipped with an OV-225 capillary column (0.25 mm i.d. × 30 m) linked to a Finnigan-MAT mass spectrometer.

### 2.6. Nuclear magnetic resonance spectroscopy

The $^{13}C$ NMR spectrum was recorded at 50°C with a Bruker AC-300 spectrometer at 75 MHz, after dissolving fraction M-4 in a 50% (w/w) urea-$D_2O$. Chemical shifts are expressed as ppm relative to the resonance of DSS (sodium 4,4-dimethyl-4-silopentane-1-sulfonate), as internal standard.

### 2.7. Electron microscopy

Dispersed fragments of fraction M-4 were treated for 1 h in dilute TFA at 60°C. They were then washed extensively and suspended in distilled water. After sonication, drops of this suspension were deposited on carbon coated electron microscopy grids and allowed to dry. Some of these grids were shadowed with W/Ta at an angle of 30°. The grids were observed with a transmission electron microscope (TEM) Philips CM 200 CRYO operated at 80 kV for

imaging purpose and 200 kV and low dose conditions for electron diffraction determination. The calibration of the electron diffraction diagrams was carried out with a gold standard.

### 2.8. X-ray diffraction

Films consisting of stacks of dried residues, after different extraction steps, were X-rayed with a Wharus flat film vacuum camera mounted on a Philips 1720 X-ray generator operated at 30 kV and 20 mA with Ni-filtered CuKα radiation. For recording of hydrated X-ray patterns, some specimens were pre-hydrated at 95% relative humidity for 48 h. These specimens were then sealed inside thin glass capillaries before X-raying. Calibration was carried out with calcite.

## 3. Results and discussion

When the exterior section of the endosperm of *S. amazonicum* was treated with an aqueous solution of NaOH of increasing concentration, the mannose composition of the residual polysaccharide gradually increases. The figures, shown in Table 1, indicate a steady drop in galactose and glucose contents when extraction mediums ranging from 4 to 16% NaOH were used. After 16% concentration, the mannose content of the corresponding M-4 fraction, which represents ~20% of the total dry endosperm, is nearly 98% with only a trace of glucose and a lesser amount of galactose. After methylation analysis of M-4, GC-MS showed acetates of 2,3,6-$Me_3$-Man (94.9%), 2,3,4,6-$Me_4$-Man (1.2%), $Me_2$-Man (2.2%) and 2,3,4,6-$Me_2$Gal (1.7%). Periodate oxidation followed by reduction and acid hydrolys afforded, after the second cycle, erythritol and traces of glycerol. These figures together with the data presented in Table 1 indicate that the fraction M-4, the residue resulting from the extraction with 16% NaOH is essentially a linear mannan according to the definition (Stephen, 1983).

Fraction M-4 was further examined by $^{13}$C NMR spectroscopy. Taking advantage of its solubility in concentrated aqueous urea (Grosrenaud, 1980), we were able to solubilize enough of the M-4 fraction to record its $^{13}$C NMR spectrum in 50% (w/w) of urea in $D_2O$. The spectrum (Fig. 1) consists of six major signals corresponding to the six carbons of a

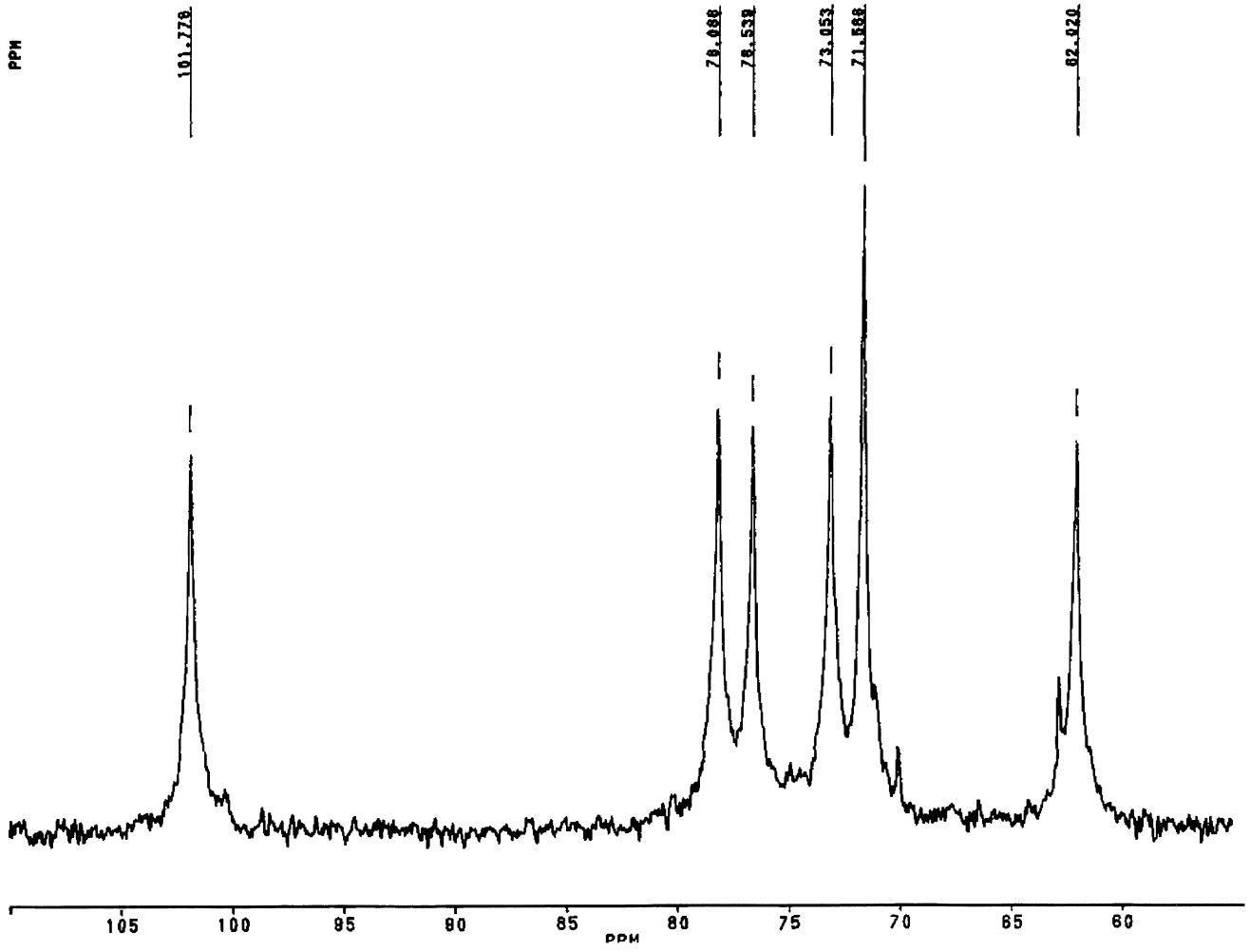


Fig. 1. $^{13}$C NMR spectrum of *Schizolobium amazonicum* mannan in 50% urea ($D_2O$).

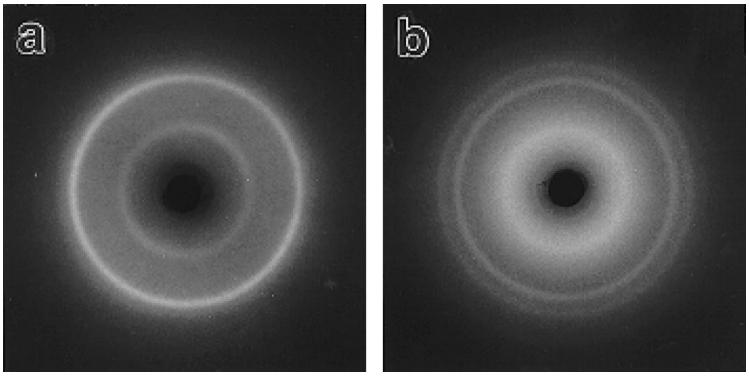


Fig. 2. The X-ray diffraction pattern of fraction M-4: (a) anhydrous conditions; and (b) hydrated conditions.

β-(1 → 4) linked D-mannan. In addition, there are some minor signals that can be attributed to the 2% of remaining galactosyl residues. In Table 2, our assignments are compared with those already in literature for solutions of linear mannan or its oligomers (Gorin, 1973; Rochas, Taravel & Turquois, 1990; Usui, Mizuno, Kato, Tomoda & Miyajima, 1979). They are also compared with the assignment of the solid state spectra $^{13}C$ NMR CP/MAS of crystalline mannan I (Jarvis, 1990; Marchessault, Taylor & Winter, 1990). These comparisons confirm the close match existing between the chemical shifts in the spectra of our sample and those of the literature data for linear mannan.

Fraction M-4 was examined by X-ray diffraction under dry and hydrated conditions. The two patterns, shown in Fig. 2 are slightly different. The dry pattern contains two fairly sharp and strong rings, calibrated at 0.771 and 0.445 nm. The hydrated pattern presents three rings calibrated at 0.800 (diffuse) 0.467 (strong) and 0.404 nm (strong). The values obtained for the hydrated pattern correspond closely to the strongest diffraction spots obtained on the mannan II from *Acetabularia crenulata* (Frei & Preston, 1968) or those of konjac glucomannan (Chanzy, Grosrenaud, Joseleau, Dubé & Marchessault, 1982; Yui et al., 1992), that diffract as mannan II. The strong ring at 0.445 nm and its absence at 0.40 nm in the dry pattern is also consistent with the dry pattern of mannan II (Chanzy et al., 1984).

The ultrastructure of the fragments of the M-4 sample can be revealed by transmission electron microscopy. Fig. 3a and b are examples of this structure. In Fig. 3a, which corresponds to the border of a cell, the microfibrillar nature of the sample is clearly observed. The microfibrils are of a small size and are intertwined in the cell wall. The insert in Fig. 3a corresponds to an electron diffraction diagram recorded on one square micron of the specimen without maintaining the sample under hydrated conditions. This diagram displays a strong ring calibrated at 0.445 nm. Such a diffraction ring is typical of the electron diffractograms of mannan II samples recorded under similar conditions (Chanzy et al., 1984). In Fig. 3b, obtained at higher magnification but after shadowing, the microfibrillar nature of the sample is further revealed. The microfibrillar morphology is consistent with similar observations made on mannan II from *A. crenulata*, from *Codium fragile* (Chanzy et al., 1984) or on mannan II from *P. macrocarpa* (Chanzy et al., 1984; Meier, 1958).

The results clearly demonstrate the existence of a microfibrillar crystalline mannan II fraction in the endosperm of *S. amazonicum*. This is the first time that a pure linear mannan is observed in a Leguminosae. In the seeds of this tree, the linear mannan occurs on the exterior side of the endosperm, a part of the tissue that resists to swelling and extraction with alkali. Interestingly, no cellulose was found in this

Table 1
Monosaccharide composition from the residue of the exterior section of the endosperm of *S. amazonicum* after successive treatments with alkali (tr, trace)

| Fraction | Treatment (% NaOH) | Monosaccharide composition[a] (%) | | |
|---|---|---|---|---|
| | | Man | Gal | Glc |
| M-1 | 4 | 88.9 | 7.2 | 3.9 |
| M-2 | 6 | 92.3 | 4.6 | 3.0 |
| M-3 | 8 | 94.1 | 3.6 | 2.3 |
| M-4 | 16 | 97.9 | 2.1 | tr |

[a] Determined by HPLC.

Table 2
Comparison of the $^{13}C$ chemical shifts (ppm) for fraction M-4 in urea solution with those from ivory nut mannan (NaOD solution) and *Phoenix dactylifera* (solid state)

| | δ (ppm) | | | | | |
|---|---|---|---|---|---|---|
| Mannan | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
| *S. amazonicum* (M-4) | 101.8 | 71.6 | 73.0 | 78.1 | 76.5 | 62.0 |
| Ivory nut[a] | 101.7 | 72.2 | 73.5 | 78.8 | 78.8 | 62.1 |
| *Phoenix dactylifera*[b] | 101.7 | 69.9 | 72.1 | 81.0 | 75.9 | 61.9 |

[a] NaOD solution (Gorin, 1973).
[b] In vivo determination (Jarvis, 1990).

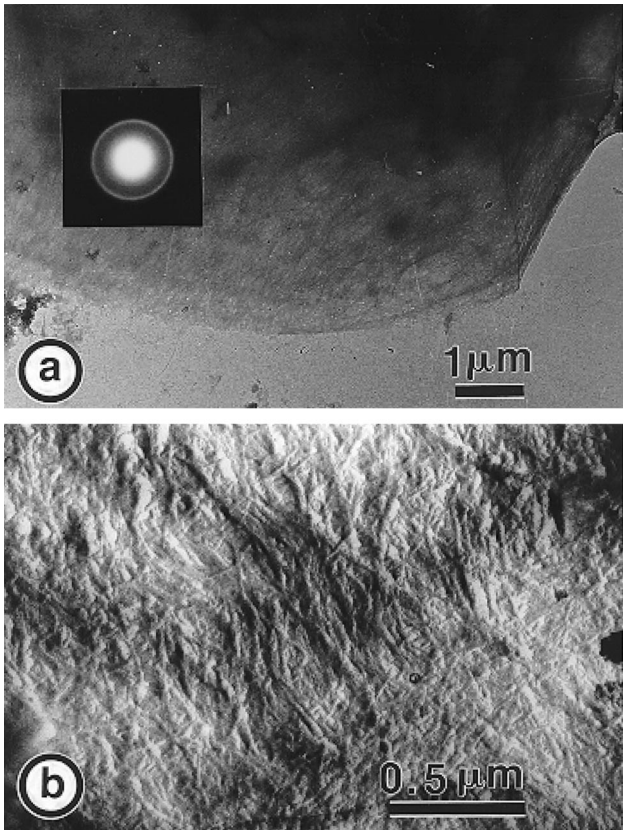


Fig. 3. Fragments of fraction M-4 observed by electron microscopy. (a) Low dose image; insert—typical electron diffraction pattern recorded on one square micron of the specimen. (b) As in (a), but after shadowing with W/Ta.

tissue. Models for cell-wall architecture include cellulosic walls, in which the skeletal component, cellulose, is cemented in a matrix of hemicellulosic polysaccharides (Reid, 1997). The absence of cellulose in the *S. amazonicum* endosperm is unusual. It remains to be seen if the mannan II fraction from the seeds of *S. amazonicum* has the role of reserve for the development of the embryo or that of skeletal material for the endosperm cell wall and if other Leguminosae seeds present the same type of morphology and structure.

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## References

Aspinall, G. O. (1959). Structural chemistry of the hemicelluloses. *Advances in Carbohydrate Chemistry*, *14*, 429–468.

Atkins, E. D. T., Farnell, S. A., Burden, C., Mackie, W., & Sheldrick, B. (1988). *Biopolymers*, *27*, 1097–1105.

Avigad, G., & Dey, P. M. (1997). In P. M. Dey & J. B. Harborne, *Plant biochemistry* London: Academic Press (pp. 143–204).

Bouveng, H., & Lindberg, B. (1960). Hydrolysis of methylated polysaccharides. *Methods in Carbohydrate Chemistry*, *5*, 287–298.

Chanzy, H. D., Grosrenaud, A., Joseleau, J. P., Dubé, M., & Marchessault, R. H. (1982). Crystallization behavior of glucomannan. *Biopolymers*, *21*, 301–319.

Chanzy, H., Grosrenaud, A., Vuong, R., & Mackie, W. (1984). The crystalline polymorphism of mannan in plant cell walls and after recrystallisation. *Planta*, *161*, 320–329.

Chanzy, H., Pérez, S., Miller, D. P., Paradossi, G., & Winter, W. T. (1987). An electron diffraction study of mannan I. Crystal and molecular structure. *Macromolecules*, *20*, 2407–2413.

Corrêa, M. P. (1984). *Dicionário de Plantas Úteis do Brasil*, vol. 3. Rio de Janeiro: Imprensa Nacional (pp. 36).

Frei, E., & Preston, R. D. (1968). *Proceedings of the Royal Society of London*, *169*, 127–145.

Ganter, J. L. M. S., Heyraud, A., Petkowicz, C. L. O., Rinaudo, M., & Reicher, F. (1995). Galactomannans from Brazilian seeds: characterization of the oligosaccharides produced by mild acid hydrolysis. *International Journal of Biological Macromolecules*, *17*, 13–19.

Gorin, P. A. J. (1973). Rationalization of carbon-13 magnetic resonance spectra of yeast mannans and structurally related oligosaccharides. *Canadian Journal of Chemistry*, *51*, 2375–2383.

Grosrenaud, A. (1980). Engineering doctoral thesis. University of Grenoble, France.

Hegnauer, R., & Grayer-Barkmeijer, R. J. (1993). Relevance of seed polysaccharides and flavonoids for the classification of the leguminosae: a chemotaxonomic approach. *Phytochemistry*, *34*, 3–16.

Hopf, H., & Kandler, O. (1977). Characterization of the 'reserve cellulose' of the endosperm of *Carum carvi* as a β(1 → 4)-mannan. *Phytochemistry*, *16*, 1715–1717.

Jarvis, M. C. (1990). The $^{13}$C NMR spectrum of (1 → 4)-β-D-mannans in intact endosperm tissue of the data (*Phoenix dactylifera*). *Carbohydrate Research*, *197*, 276–280.

Lindberg, B. (1972). Methylation analysis of polysaccharides. *Methods in Enzymology*, *28*, 178–195.

Mackie, W., & Sellen, D. B. (1969). *Polymer*, *10*, 621–632.

Marchessault, R. H., Taylor, M. G., & Winter, W. T. (1990). $^{13}$C CP/MAS NMR spectra of poly-β-D-(1 → 4) mannose: mannan. *Canadian Journal of Chemistry*, *68*, 1192–1195.

Meier, H. (1958). On the structure of cell walls and cell wall mannans from ivory nuts and from dates. *Biochimica et Biophysica Acta*, *28*, 229–240.

Millane, R. P., & Hendrikson, T. L. (1994). Crystal structures of mannan and glucomannans. *Carbohydrate Polymers*, *25*, 245–251.

Nieduszynski, I., & Marchessault, R. H. (1972). The crystalline structure of poly-β-D-(1 → 4′) mannose: mannan I. *Canadian Journal of Chemistry*, *50*, 2130–2138.

Petkowicz, C. L. O., Sierakowski, M. R., Ganter, J. L. M. S., & Reicher, F. (1998). Galactomannans and arabinans from seeds of Caesalpiniaceae. *Phytochemistry*, *49*, 737–743.

Reid, J. S. (1997). In P. M. Dey & J. B. Harborne, *Plant biochemistry* London: Academic Press (pp. 205–236).

Reid, J. S. G., & Edwards, M. E. (1995). In A. M. Stephen, *Food polysaccharides and their applications* New York: Marcel Dekker (pp. 155–186).

Rizzini, T. (1986). *Árvores e Madeiras Úteis do Brasil*, São Paulo: Edger Blucher (pp. 133).

Rochas, C., Taravel, F. R., & Turquois, T. (1990). NMR studies of synergistic kappa carragennan-carob galactomannan gels. *International Journal of Biological Macromolecules*, *12*, 353–358.

Saeman, J. F., Moore, W. E., Mitchell, R. L., & Millet, M. A. (1954). Techniques for the determination of pulp constituents by quantitative paper chromatography. *Tappi*, *37*, 336–343.

Stephen, A. M. (1983). In G. O. Aspinall, *The polysaccharides* vol. 2. New York: Academic Press (pp. 98–180).

Usui, T., Mizuno, T., Kato, K., Tomoda, M., & Miyajima, G. (1979). *Agricultural Biology and Chemistry*, *43*, 863–865.

Wolfrom, M. L., Laver, M. L., & Patin, D. L. (1961). Carbohydrates of the coffee bean. II. Isolation and characterization of a mannan. *Journal of Organic Chemistry*, *26*, 4533–4535.

Yui, T., Ogawa, K., & Sarko, A. (1992). Molecular and crystal structure of konjac glucomannan in the mannan II polymorphic form. *Carbohydrate Research*, *229*, 41–55.

Zawadzki-Baggio, S. F., Sierakowski, M. R., Corrêa, J. B. C., & Reicher, F. (1992). A linear (1→5)-linked α-L-arabinofuranan from seeds of guapuruvu (*Schizolobium parahybum*). *Carbohydrate Research*, *233*, 265–269.