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Equilibrium studies of galactomannan of *Cassia fastuosa*[1] and *Leucaena leucocephala* and Cu^{2+} using potentiometry and EPR spectroscopy

A.L.R. Mercê[a,*], S.C. Lombardi[a], A.S. Mangrich[a], F. Reicher[b], B. Szpoganicz[c], M.R. Sierakowski[a]

[a]*Departamento de Química, Centro Politécnico, Universidade Federal do Paraná, CP: 19081, CEP: 81531-990, Curitiba, Pr, Brazil*
[b]*Departamento de Bioquímica, UFPR, Curitiba, Pr, Brazil*
[c]*Departamento de Química, Universidade Federal de Santa Catarina, Brazil*

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The binding constants of Cu^{2+} and galactomannans composed of sugar units of mannose and galactose from the seeds of *Cassia fastuosa* and *Leucaena leucocephala* were determined by potentiometric titrations and inspected by EPR spectroscopy. Two complexed species were found in both systems, one moiety being the metal ion (M) and the other moiety a sugar monomer of the polysaccharide (L), non-protonated, ML, and protonated, MHL. The values for the logarithms of the binding constants are in the range of 15 units for ML for both employed galactomannans, and 8.0 for MHL for Man:Gal medium average ratio 4:1 galactomannan (*C. fastuosa*) and 6.8 for Man:Gal medium average ratio 2.6:1 one (*L. leucocephala*).

The EPR spectra have shown one octahedral complex species linked through the oxygen atoms of the hydroxyl groups of the monomer with tetragonal deformation. The A and g obtained values were $A_{\parallel} = 178G$, $g_{\parallel} = 2.260$, and $g_{\perp} = 2.050$ and $A_{\perp} = 30G$ for CU^{2+} with the galactomannans. This was assigned to be ML_2 complex which was only detected in the solid state due to the formation of insoluble products in solution, above pH value of 7.0.

The extension of formation of complexed species of galactomannans with Cu^{2+} is probably much more affected by the pH variation than by the degree of substitution of monomers of the chain of the biopolymers studied so far.

INTRODUCTION

Polysaccharides from the seeds of various plants species have long been used in industries as a way to replace more expensive and less effective products. Some examples are the food industries, where some polysaccharides are being used as food additives due to their different rheological behaviour according to changes in the physical chemical situations (Pilnik and Rombouts, 1985).

Galactomannans are a polysaccharide of (1 → 4) β-D-mannan total or partially substituted at 0–6 by α-D-galactopyranosil units. The ratio between the two sugar units and the physico-chemical properties can vary depending upon the plant. These biopolymers have different physiological functions in the seeds, one is to maintain a minimum humidity inside the reproductive cell, and the other is to provide a stock food to the germinal seeds (Dea *et al.*, 1986; Dey, 1978; Dea and Morrison, 1974).

The interaction of metal ions and polysaccharides has applications in analysis (electrophoresis, chromatography and NMR shift reagents), industry (sequestration of metal ions), medicine, coordination chemistry and biochemistry (Yano *et al.*, 1985).

*Corresponding author. Fax: 55 41 267 4236; e-mail: anamerce@quimica.ufpr.br
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Galactomannans are an important agent for metal transport, as are all biodegradable biopolymers complexed to metal ions (Gonzalez-Davila *et al.*, 1990). Whenever these complexes break down, metal ions are released. Many other industrial applications of polysaccharides are discussed in the literature (Sandford and Baird, 1983), including some applications in agricultural chemicals, as flowable pesticides and suspension fertilizers. In the latter use the complexed polysaccharide in the soil can also act as a slow release fertilizer both for organic and inorganic micronutrients for plants. Some previous studies (Mercê *et al.*, 1996a, b) have presented results for a trial slow-release organic fertilizer derived from substances with complexing ability with metal ions.

Reports in the literature (Menjivar, 1986; Conway *et al.*, 1983) have shown guar gum and its hydroxypropyl derivatives to be the most extensively used polysaccharides in commercial fracturing fluids. Hydraulic fracturing is a widely used method for stimulating the production of oil and gas formations. It is also reported that metal crosslinked polysaccharide gels are superior to non-crosslinked polysaccharide solutions.

Rheological studies are seen in the literature as well as those concerning the determination of thermodynamic values, such as enthalpy changes, entropy of ionization of some materials derived from polysaccharides. Fewer studies are seen regarding the determination of equilibrium constants measured in aqueous solutions using potentiometry (Wang *et al.*, 1994; Bos *et al.*, 1994; Fernandes *et al.*, 1992; Baba *et al.*, 1974).

Measurements of the zeta potential (Sakuma *et al.*, 1990), calorimetry (Dobetti and Delben, 1992; Delben *et al.*, 1989), UV absorption spectrophotometry (Dobetti and Delben, 1992), circular dicroism measurements (Sakuma *et al.*, 1990), X-ray fluorescence measurements (Masri *et al.*, 1974) and potentiometry (Baba *et al.*, 1974) have been reported in the literature in order to find binding constants for some biopolymers and some model compounds for biological cells and some metal ions.

Other binding studies using electronic paramagnetic ressonance (EPR) of Mn(II) on gellan, kappa and iota carragennan gels have been reported in the literature. The results help understand how strongly the metal ion is bound to the ligands and which one coordinates to the metal ion (Tsutsumi *et al.*, 1993).

This study is an attempt to understand the chelating ability of galactomannans towards Cu^{2+} with varying pH. The binding constants were determined using potentiometry and EPR spectra were obtained from the insoluble products of species formed in those systems.

The galactomannans employed were of mannose:galactose (Man:Gal), average ratio of 4:1 and 2.6:1, respectively from the seeds of *Cassia fastuosa* (Tavares, 1994) and *Leucaena leucocephala*.

EXPERIMENTAL

Material

All chemicals used were of analytical-reagent grade and were used without further purification. All solutions were made with bi-distilled, deionized and CO_2-free water. The galactomannans employed, Man:Gal ratios of 4:1 and 2.6:1, were extracted from seeds of *Cassia fastuosa* and *Leucaena leucocephala*, respectively, as described by Tavares (1994). The monosaccharide composition was obtained by analysis of their alditol acetates composition (Wolfrom and Thompson, 1963; Blakeney *et al.*, 1983). The resulting alditol acetates were analysed by GLC-MS with a model 3300 Varian equipped with an OV-225 capilary column (0.25 mm i.d. × 30 m) linked to a Finnigan Trap model 419 mass spectrometer unit at 70 eV. Injections were carried out at 50 °C and the column then programmed (4.0 °C/min) to 220 °C.

A portion of extracted and dried galactomannan was dissolved with the aid of a vortex in a proper quantity of warm water to obtain a final concentration of 5 g/l of the biopolymer. At this concentration the viscosity of the solution was low enough to provide rapid diffusion of the species in the system. The solution was then transferred to the reaction vessel and was titrated in the absence and in the presence of the metal ions solutions, with KOH solution. The remaining stock solution was kept under refrigeration for up to 5 days. Whenever necessary a freshly prepared aqueous solution was employed. The number of moles of this solution was calculated using the molecular weight of either one galactopyranose or manopyranose portion. The monomeric sugar portions of the biopolymers are referred as the ligand (L) throughout this work.

The monomers mannose and galactose employed were obtained from Merck, Germany, and were used without prior purification.

Metal solutions were made from nitrate salt (Carlo Erba, Brasil) and their concentrations were determined following the literature (Schwarzenbach and Flaschka, 1969). The aqueous KOH (Merck, Brasil) 0.1 mol/l, carbonate-free solution was standardized against potassium hydrogen phthalate (Carlo Erba, Brasil). KNO_3 (Baker and Adamson, USA) was the supporting electrolyte to maintain the ionic strength (μ) at 0.100 mol/l.

Methods

The potentiometric titrations were carried out under an inert atmosphere of water–KOH saturated nitrogen (White-Martins, Brasil) in an water-jacketed vessel maintained at 25.0 ± 0.1 °C, Microquimica, MQBTC 99-20 (Florianópolis, Brasil) in the absence and in the presence of 0.02 and 0.04 mmol of Cu^{2+}. The pK_w used was 13.78 at 25.00 ± 0.1 °C. A Metrohm manual piston buret was used to deliver

the titrant, standard KOH-free CO_2, and the pH values were directly measured with a Micronal (SP, Brasil) model B-375 pH meter fitted with an Analyser (SP, Brasil) glass and calomel reference electrodes calibrated with standard HCl and KOH solutions to read log $[H^+]$ directly (Martell and Motekaitis, 1992).

Both biopolymers were put in the presence of a known excess of strong acid (HCl, aqueous solution, 0.1 mol/l, Merck, Brasil) to make sure that all potential basic sites were protonated whenever a titration was carried out. This same procedure was followed during the titration of the monomers.

At constant increments of volume of titrant, 0.10 ± 0.02 ml, added to the system adjusted to 0.100 mol/l constant ionic strength (μ), the correspondent pH (Martell and Motekaitis, 1992) was read and plotted to calculate the values of the binding constants of the system.

The peeled and crushed seeds of *C. fastuosa* and *L. leucocephala* were treated with water at 100 °C for 15 min and submitted to exhaustive aqueous extration at 25 °C for 24 h. The solution was treated with 0.1 mol/l sodium chloride, precipitated with ethanol, washed with ethanol and acetone, and dried at 25 °C. The total protein was measured according to Hartree (1975). The infrared (IR) spectrum of the galactomannan from *L. leucocephala* was recorded by an FTIR Bomem double-beam spectrometer with KBr pellets.

The EPR spectra of solid galactomannans and their extracted products completed to Cu^{2+} were recorded at room temperature of 25 °C using a Brucker EPR X-band spectrometer, model ESP 300-E, 9.7 GHz, 100 kHz field modulation, Germany.

The complexes of Cu^{2+} with Man:Gal ratios 4:1 and 2.6:1 were obtained as follows. To a magnetic stirred and warm aqueous solution of biopolymer, either Man:Gal ratio 4:1 or ratio 2.6:1, a matching number of millimoles of solid copper nitrate was added. A small amount of KOH solution was then added until pH was higher than 7.0. The solution was left to cool, and after 2 h the precipitate obtained was centrifuged and dried at 60 °C in an oven. The solids were taken to the EPR.

Data treatment

The first protonation constants of the acidic sites of galactomannan was taken from literature (Martell and Smith, 1994) for their monomers alone, mannose and galactose, and the second protonation constant was optimized by the calculations further employed using the microcomputer program Best7 (Martell and Motekaitis, 1992).

The basic algorithm in Best7 is stated in Eq. (1):

$$T_i = \sum_{j=1}^{NS} e_{ij}\beta_j \prod_{K=1}^{j} [C_k]^{e_{ij}} \tag{1}$$

which is a statement of the mass balance of the *i*th component in terms of the *j*th species summed over all species present, *NS*. Each species concentration consists of a product of the overall stability constant and individual component concentrations $[C_k]$ raised to the power of the stoichiometric coefficient e_{ij}. The set of simultaneous equation obtained is solved for each component $[C_k]$. The value of $[C_k]$ is particular when it represents the calculated concentration of H^+, which is then compared with the measured hydrogen ion concentration. The standard deviation in pH units is obtained by Eq. (2) (Martell and Motekaitis, 1992):

$$\delta_{\text{fit}} = (U/N)^{1/2} \tag{2}$$

where $N = \Sigma w$ as in Eqs. (3) and (4) (Martell and Motekaitis, 1992):

$$U = \sum w(\text{pH}_{\text{obs}} - \text{pH}_{\text{calcd}})^2 \tag{3}$$

$$w = 1/(\text{pH}_{i+1} - \text{pH}_{i-1})^2 \tag{4}$$

The millimole was used to express the quantities of reagents and the hydrolysis constants employed for Cu^{2+} were obtained from literature (Baes and Mesmer, 1976) and were fully employed in calculations with Best7.

The species distribution curves were drawn with the microcomputer program SPE (Martell and Motekaitis, 1992). In general three titrations have been made, one with the ligand alone, and two others with ligand and metal in different ratios. These ratios were also optimized by trial and error, until no further minimization of the standard deviation in the calculations of program Best7 were obtained. All reported results are the average of triplicate potentiometric titration experiments.

RESULTS AND DISCUSSION

The model that best fit the potentiometric titrations results for the two different sources of galactomannans and the metal ion Cu^{2+} was the one in which the metal ion took two H^+ ions from the hydroxyl groups of either mannose or galactose random moiety of the biopolymer structure. The protonation constants of the monomers are high enough to prevent the determination by potentiometry, so the values were taken from literature in which UV–vis spectroscopy was used (Martell and Smith, 1994). Also the potentiometric attempts on the monomers and Cu^{2+} showed no complexation at all in the experimental conditions employed, so they left no result for further comparison with those obtained with the biopolymers.

The average binding constants calculated using the main algorithm in Eq. (1), in this work, followed the general equation:

$$\text{M} + n\text{L} \rightleftharpoons \text{ML}_n \qquad K_n = [\text{ML}_n]/[\text{M}]\cdot[\text{L}]^n \tag{5}$$

where M is the metal ion and L, the monomeric sugar portion of the biopolymer, and n is the average number of this portion that is able to bind to a metal ion. So ML and ML_2 represent 1 mol of Cu^{2+} bound to one and two

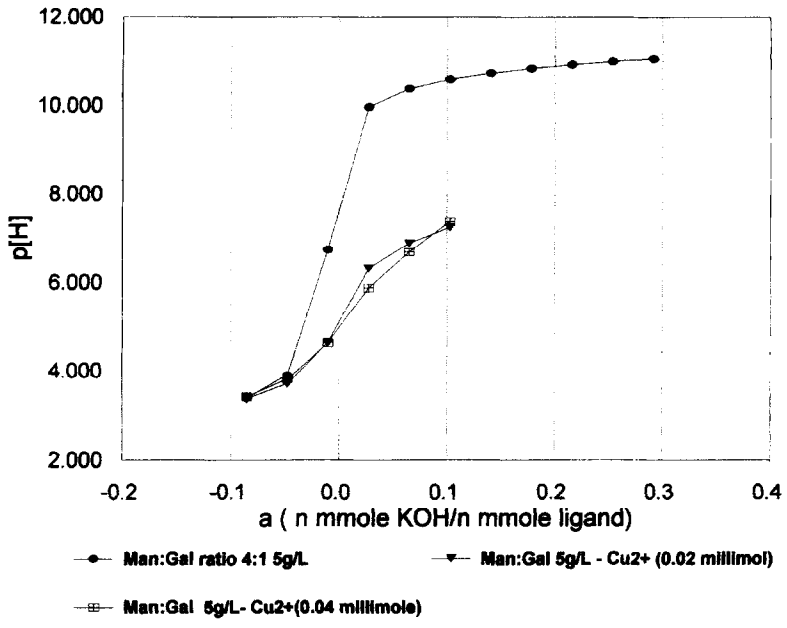

Fig. 1. Potentiometric pH profile of a solution of 5 g/l Man:Gal ratio 4:1 galactomannan with 0.01 and 0.02 mmol of Cu^{2+}. $T = 25°C$, $\mu =$ 0.100 mol/l (KNO_3).

monomeric sugar portions that have been depleted of two H^+ ions from hydroxyl groups, respectively. MHL represents the same as ML but only one hydroxyl group has lost the H^+ ion which is then coordinated to Cu^{2+}.

Also the complexing capacity of the biopolymer was inferred to be those of the hydroxyl groups heading toward the same direction in a plane of each monomeric sugar portion. As these monomeric portions are randomly distributed, the determined values are averages for these repeating portions. The repeating sugar portion mannose, the C-2 and C-3 hydroxyl groups in axial position could support well a complexation with a metal ion, and in galactose, C-3 and C-4 hydroxyl groups could be responsible for holding one metal ion, as long as they are deprived from their H^+ ions with increasing pH.

The literature has shown a Man:Gal ratio for *Leucaena leucocephala* of 2:1 (McCleary, 1979), although the one extracted in this work has shown a ratio of 2.6:1. This is related to the physyological aspects of the vegetable, the place where the vegetable grows and also the time of harvest. Total protein of 8.4% m/m following Bitter and Muir (1962) and 6.0% m/m of uronic acid (IR bands in 1640 and 1360 cm^{-1}) in the Man:Gal ratio 2.6:1 galactomannan were also found. For the Man:Gal ratio 4:1 galactomannan the total protein was 4.9%.

The potentiometric pH profiles of the Man:Gal ratios 4:1 and 2.6:1, in the absence and in the presence of Cu^{2+}, are depicted in Figs. 1 and 2, respectively. These profiles have given way to the binding constants for each case, depicted in Table 1 for Man:Gal ratio 4:1, and in Table 2 for Man:Gal ratio 2.6:1 galactomannan. Those curves show a buffer region after pH values around 7 due to the formation of insoluble products in the aqueous media. Thus species bearing more than 1 mol of L per 1 mol of metal ion could not be detected (for instance, ML_2) as the mathematical relations take into account the influence of pH on the complex formation in those systems.

Taking a look at the calculated binding constants in 1 and 2, it is seen that Cu^{2+} ions interact with both galactomannans the same intensity, no matter the average ratio of mannose to galactose of the biopolymer. This can lead to an assumption that the complexation in the range of one cyclohexane ring of the monomer of the biopolymer, per metal ion, is not influenced by which one of the monomers is binding Cu^{2+}. The independence of the composition of *N*-carboxymethyl chitosans in the binding towards Cu^{2+} was previously reported (Dobetti and Delben, 1992).

Fig. 3 depicts the distribution diagram of a saturated solution of Man:Gal ratio 4:1 and Cu^{2+} in percent of species present from pH values of 2.0–10.0, total metal

Table 1. Logarithms for the binding constants of the complexes between Man:Gal ratio 4:1 galactomannan with Cu^{2+}. $T = 25$ °C and $\mu = 0.100$ mol/l (KNO_3)

	log*K* Man:Gal ratio 2.6:1 with Cu^{2+}
ML/M × L	15.0 ± 0.2
MHL/ML × H	8.0 ± 0.2
ML2/ML × L	Non-detected

Table 2. Logarithms for the binding constants of the complexes between Man:Gal ratio 2.6:1 galactomannan with Cu^{2+}. $T = 25$ °C and $\mu = 0.100$ mol/l (KNO_3)

	log*K* Man:Gal ratio 2.6:1 with Cu^{2+}
ML/M × L	15.6 ± 0.1
MHL/ML × H	6.8 ± 0.1
ML2/ML × L	Non-detected

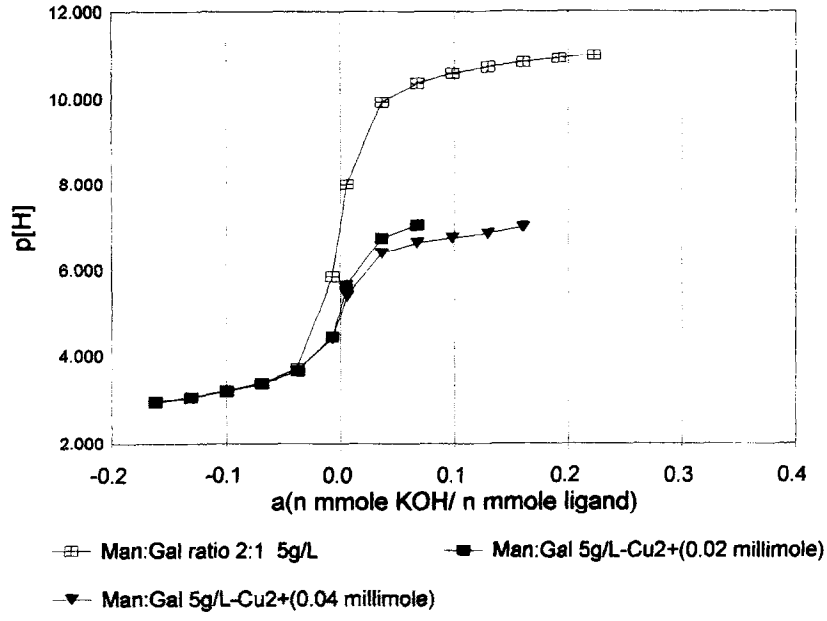

Fig. 2. Potentiometric pH profile of a solution of 5 g/l Man:Gal ratio 2.6:1 galactomannan with 0.01 and 0.02 mmol of Cu^{2+}. $T = 25°C$, $\mu =$ 0.100 mol/l (KNO_3).

concentration set at 100%. The species in the distribution diagram for Man:Gal ratio 2.6:1 galactomannan matches the values of pH in Fig. 3, except for the percentages. In this Fig. 3, one can see the maximum formation of the protonated complex MHL between the monomeric sugar portions (L) and Cu^{2+} (M) at pH value of 6.9; the other species detected, ML, at pH = 10.0. All species are present (>20%) in both acidic and basic regions for both galactomannans employed.

In Figs. 4 and 5 one can see the EPR spectra of Man:Gal ratio 4:1 galactomannan alone and complexed to Cu^{2+}, respectively. The EPR spectra of Man:Gal ratio 2.6:1 and Cu^{2+} showed the same features as those in Fig. 5. The spectrum for Man:Gal ratio 4:1 showed some octahedral iron impurity (g = 2.054), which also showed up in the copper complex spectrum (Fig. 5). These impurities have probably arisen from residues of proteic material of the vegetable from which the galactomannan was extracted. In Fig. 5 the EPR parameters indicate an octohedral structure with tetragonal deformation (z-axis elongated, A and g

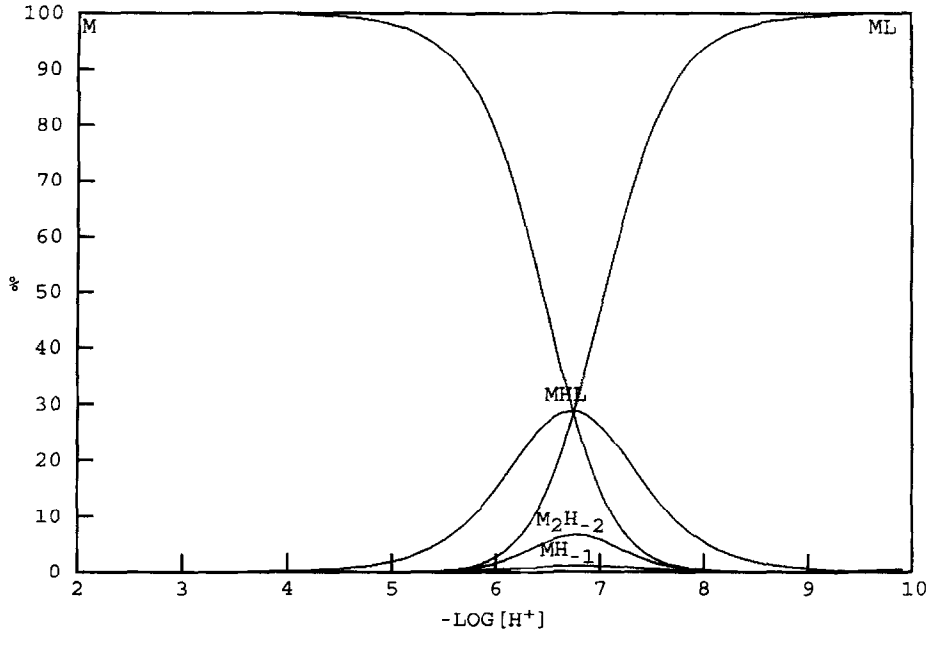

Fig. 3. Species distributions of saturated solution of Man:Gal ratio 4:1 (L) with Cu^{2+} (M) of pH values from 2.0 to 10.0. %, percentage of a species present, with the metal concentration set at 100%. H_{-x} represents $(OH)_x^-$. M represents the uncomplexed Cu^{2+}.

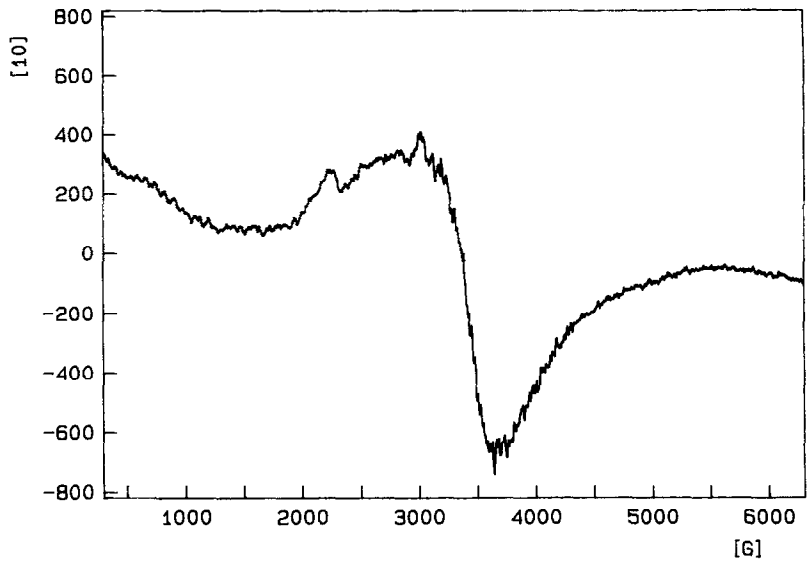

Fig. 4. EPR spectra of Man:Gal ratio 4:1 galactomannan.

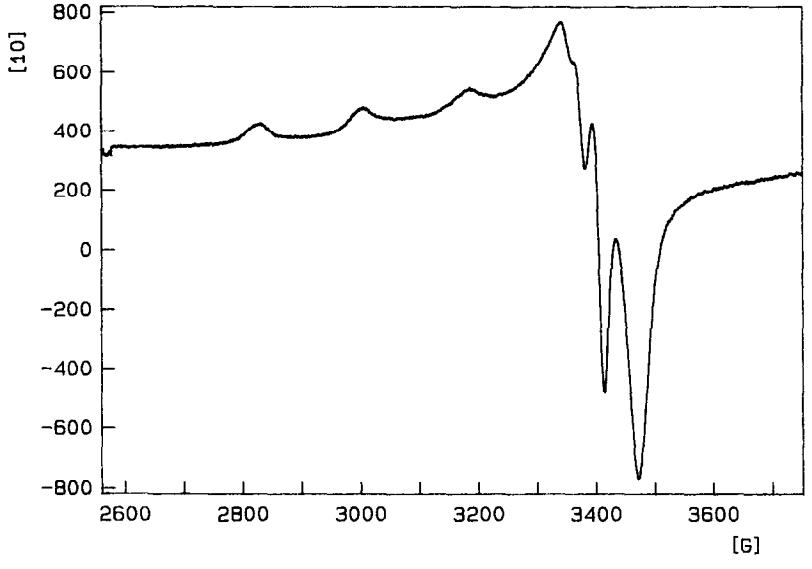

Fig. 5. EPR spectra of the solid products extracted from an aqueous solution having 0.02 mmol of Man:Gal ratio 4:1 galactomannan with 0.02 mmol of Cu^{2+}.

values in Table 3). In both cases, the copper ions are linked through four hydroxyl oxygen atoms in the equatorial plane, having probably two molecules of water in the axial position. This represents the complex ML_2 as it was extracted at a pH value above 7.5, when there was the formation of insoluble products in the aqueous media for the galactomannans employed and Cu^{2+}.

In Fig. 6 it is shown the EPR spectrum of Man:Gal ratio 2.6:1 galactomannan. It suggests the presence of rhombic and octahedral Fe^{3+} ($g = 4.30$ and 2.054, respectively), also present in the spectrum with Cu^{2+}. It also shows another $g = 2.00$, suggesting the presence of free radicals, probably arising from the organic protein matter in this galactomannan.

Table 3. EPR spectra parameters of the complex Man:Gal ratios 4:1 and 2.6:1 galactomannans with Cu^{2+} (refer to Fig. 5)

$A_\parallel$	$g_\parallel$	A	$g_\perp$
178G	2.260	30G	2.050

CONCLUSIONS

It was possible to show in this present work the viability of using potentiometry and the microcomputer program Best7 (Martell and Motekaitis, 1992) in the study of complex properties of a metal ion and biopolymers, in this case, galactomannans. All the results obtained by this technique represent the system studied better using the mathematical model chosen for Best7. This microcomputer program can

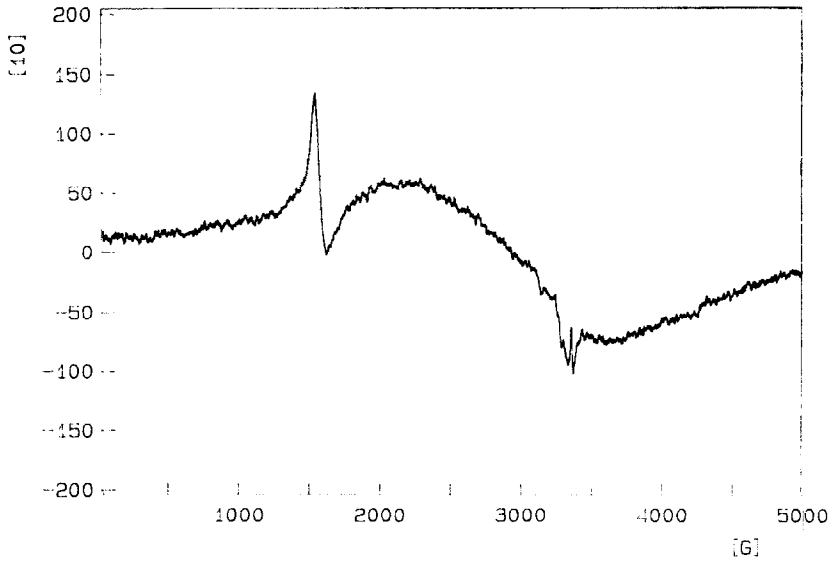

Fig. 6. EPR spectrum of Man:Gal ratio 2.6:1 galactomannan.

also provide an alternative way of getting binding constants either when others fail to give a precise evaluation of a system (Scatchard method; Dobetti and Delben, 1992) or when there is a need for a complicated mathematical model (Langmuir adsorption isotherms; Sakuma *et al.*, 1990; Gonzalez-Davila *et al.*, 1990).

The slight differences between the values for the binding constants for ML (refer to Tables 1 and 2) can be due to the presence of carboxylic acid groups, possibly uronic acid in Man:Gal ratio 2.6:1 galactomannan. These studies are being further developed. One reason for the bigger difference between the binding constants for the species MHL in both galactomannans could be that it is easier to protonate a hydroxyl than a carboxylic group in the monomeric sugar portion of galactomannans. So, the one having some carboxylic acid in its structure would present a lower value for this binding constant.

The number of binding sites of the complexes found in the systems studied was determined by EPR. The ML_2 complex was suggested by the results.

Taking a closer look at the experimental data presented so far, one can assume that the extent of binding of galactomannans of the type employed in this work, and Cu^{2+}, is much more affected by the pH of the solution (the degree of protonation of the chain) than by the degree of substitution of the chain itself.

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