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Fraction of Soluble Polysaccharides from *Inula crithmoides* by Sequential Extraction

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Abstract: Water-soluble polysaccharides were extracted sequentially from *Inula crithmoides*. Three methods were applied: H_2O, $K_2C_2O_4H_2O$ and NaOH. Each fraction was purified by precipitation in ethanol followed by ultrafiltration of recovered solids dissolved in water through a membrane with a molecular weight cut off of 100000, are purified by precipitation with ethanol followed by ultrafiltration to obtain respectively carbohydrates INPU, IOPU and IBPU. Elemental analysis demonstrated that the extracted products were polysaccharides exempt from proteins. Obtained products were characterised by their structural (sugar composition, degree of methylation (DM) and molar mass of acid equivalent unit) and macromolecular characteristics (molar mass and intrinsic viscosity). The sugar composition indicates that all these polymers are pectins made up mainly of acid galacturonic (between 33.8 and 64.8%). Determination of the percentage of HG and RG-I of the functions extracted shows a mixture of these two types of pectin. The macromolecular features of these compounds have been characterized by size-exclusion chromatography coupled with multi-angle laser light-scattering detection (SEC/MALLS).

Key words: Water-soluble polysaccharides, *Inula crithmoides*, sugar composition, degree of methylation, macromolecular characteristics

INTRODUCTION

For many years pectins have been the subjects of extensive research. Pectins have a number of applications in the pharmaceutical, cosmetic and food industries.

These numerous applications and justify the search for new sources of pectins. Present research is part of a valorisation project on the water-soluble polymers extracted from the Tunisian flora. With this objective we have undertaken a study about the extraction, purification and physico-chemical characterisation of the polysaccharides from this plant, as to our knowledge no paper on this topic has been published yet. *Inula crithmoides* is a plant from the halophyte group and the family of Compositae. Its period of flowering is from August at October. It is widespread in Mediterranean basin, in maritime meadows and salt-water marshes (Pottier Alapettite, 1979) and in Tunisia it is abundant in Tabarka, Carthage, Gabès, Oasis of Djérid (Clintock *et al*., 1976).

The research carried out on *Inula crithmoides*, primarily concerned the ecology of halophytes in the Mediterranean coast of Damietta, Egypt (Serag, 1999), pharmacological evaluation of the dichloromethanol extract from *Inula crithmoides* (Barrachina *et al*., 1995), chlorinated tymol derivatives from Inula (Marco *et al*., 1993) and edaphic characteristics of salt meadow vegetations eastern regions of Spain (Boira, 1995).

However, no data exist concerning the macromolecular properties of polysaccharides extracted from *Inula crithmoides*.

In the present study, extraction, purification of polysaccharides is reported. Our interest has been focused on the physico-chemical characterisation using several techniques such as: viscosity in dilute solutions and steric exclusion chromatography equipped with double detection refractometric and multiangle laser light scattering (SEC/MALLS). The general aim of this was to understand the structure we report about the dependence of the extraction procedure on the chemical and physico-chemical characteristics of pectic polysaccharide isolated from *Inula crithmoides*, including sugar composition, DM and molar mass and intrinsic viscosity.

MATERIALS AND METHODS

Materials: The plant was collected in the area of Soliman (Cap Bon Tunisian) in March, 2005.

One thousand grams of sheets of finely cut plant was washed for 1 h in 4 L of boiling 95% ethanol and the mixture was then allowed to stir mechanically overnight at room temperature. After filtration through a G2 sintered

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glass funnel, the residue washed with ethanol (5×500 cm^3) and acetone (3×300 cm^3)-named AIS - was dried, to be used as stock material for extraction (Forni *et al*., 1994).

Water-soluble polymers were obtained from AIS after extraction for 2 h in hot water (1:30 w:w, 80°C) with mechanical stirring. The mixture was filtered on canvas and then on Celite through a G2 sintered glass funnel. The residue (R1) will be treated later on during the extraction with oxalate while the filtrate was precipitate by adding an excess of ethanol (40:60 v:v). After settling for 12 h at 4°C, the insoluble material was removed by centrifugation (5000 rpm) for 30 min to yield the fraction INP was dialysed by ultrafiltration on a Minitan cell (Millipore) using membranes with a molecular weight cut-off of 100000 in order to eliminate the remaining salts, as well as polymers (proteins and polysaccharides) of low molar mass. Polymer in retentate (named INP) was finally obtained (Joye and Luzio, 2000; Majdoub *et al*., 2001a, b).

The residue (R1) is treated with the oxalate $K_2C_2O_4H_2O$ (0.5%) at 25°C, after filtration, precipitation and freeze-dried, we obtained IOPU. The residue (R2) is treated in basic medium with (NaOH; pH = 12, t = 2 h, 4°C) to give product IBPU (Fig. 1).

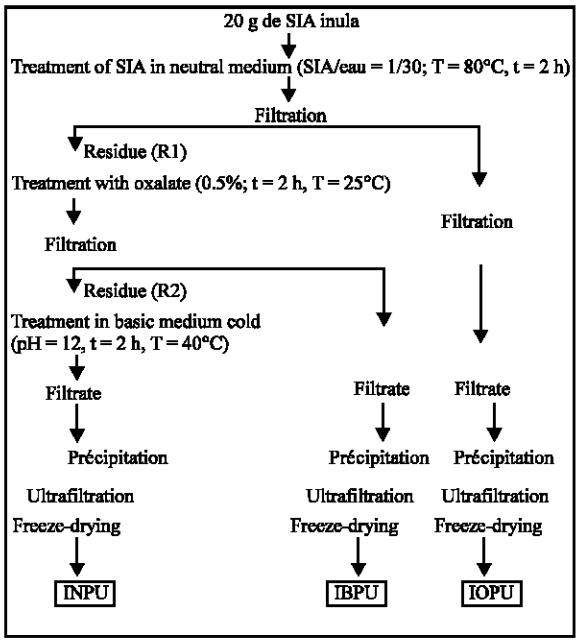

Fig. 1: Procedure diagram used for extraction and purification of water-soluble polysaccharides from *Inula crithmoides*

Sugar analysis: Neutral sugars were determined as anhydroglucose by the phenol-sulfuric acid assay (Dubois *et al*., 1956). Correction was made for interference from GalA. Total uronic acids were assayed as anhydrogalacturonic acid using m-phenylphenol colour reagent. All fractions (0.5 mg) were methanolysed in 2 M anhydride acid in methanol (24 h, 80°C) for measurement of individual sugars, using myo-inositol as internal standard. They were silylated (4°C overnight in 1% trimethylchlorosilane in N, O-bis trimethylsilyl-fluoroaceaamide) and analysed by gas liquid chromatography on capillary columns DB 225 (J.W. Instruments) with nitrogen as vector gas and air-hydrogen mixture as fuel (Goubet *et al*., 1995).

Degree of methylation: DM was defined as the number of moles of acetic acid or methanol per 100 mol of GalA.

The obtained DM values were confirmed using a FT-IR method described previously (Monsoor *et al*., 2001; Kravtchenko *et al*., 1992).

AEW determination: The anionic groups of polysaccharide samples (0.5 g in 100 cm^3 of distilled water) were first converted into their acidic form by percolating onto a strongly acidic ion exchange resin (Dowex 50X8)and then titrated under nitrogen by NaOH, in the presence of 0.15 M NaCl to minimise the polyelectrolyte effect by screening the charges on the chain. The molar mass of the Acid Equivalent Unit (AEW) was determined for each sample from the potentiometric titration according to:

$$AEW = \frac{m}{C_{NaOH} \cdot V_e}$$

where, m is the mass of the polymer sample, V_e the NaOH volume of the endpoint.

Viscosimetric measurements: To determine the intrinsic viscosity [η] of dilute polysaccharide solutions (C inferior to the overlap concentration C*), the flow time of the polymer solutions at different concentrations was measured in 1 M NaCl at 25°C using an viscometer (TI1, Sematech, France). Preliminary experiments have shown that the flow time of a given polyelectrolyte did not vary when NaCl concentration was as large as 1 mol L^{-1}. The reduced viscosity (η_{sp}/C) was then plotted against the polymer concentration (Fig. 2). The intrinsic viscosity was derived from the intercept (C = 0) and the Huggins constant (k') was inferred from the slope. It was subsequently checked that the starting concentration C_0 was inferior to the critical concentration C* by calculating its value using the equation (Morris *et al*., 1980): $C^* = 1.5/[\eta]$.

Polysaccharide molar mass determination by size exclusion chromatography (SEC): Solutions were

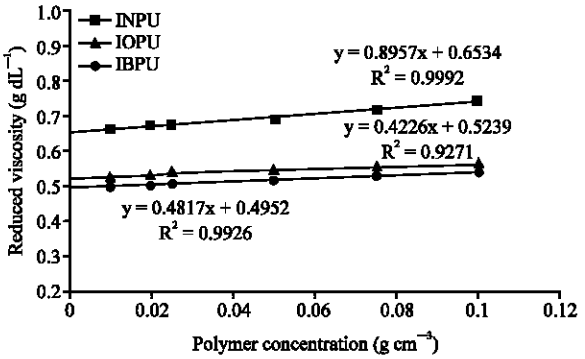

Fig. 2: Reduced viscosity (ηsp/C) measurements were conducted in 1 M NaCl at 25°C

prepared by dissolving the polysaccharide samples overnight in 0.1 M $NaNO_3$. The pH was adjusted to the desired value by adding phosphate buffer (I = 0.025 mol L^{-1}; pH 6.3).

The polymer concentration of solutions (0.1 wt. %) was chosen such that the overlap concentration of polymer C* was not exceeded (see viscometric measurements), but large enough to give a measurable light scattering response.

Analyses of polysaccharides were performed on a chromatographic setup including a pump (Waters, 515), a TSK-GEL GMPWXL column (Tosohaas) and a multi-angle laser light scattering (MALLS) detector (Dawn™ DSP, Wyatt Technology C_o), coupled with an interferometric refractive index detector (Optilab, Wyatt Technology C_o). The samples injected through a 0.1 cm^3 loop were eluted at 40°C with a flow rate of 0.5 cm^3 min^{-1}.

The light scattering data were analysed using ASTRA software (Wyatt Technology C_o).

RESULTS AND DISCUSSION

Extraction: Extraction of polysaccharides from *Inula crithmoides* was carried out using three different techniques (Fig. 1). The first method was mainly based on the boiling-water treatment of AIS while the second one consisted in the treatment with oxalate and the third in basic medium cold. In all cases, the final purification was carried out by elimination of the remaining low molar mass compounds using the retention of the polysaccharide fraction on ultrafiltration membranes with a high molecular weight cut off (100,000).

Table 1 shows the yields of the recovered polymer obtained via the three techniques of extraction. The yields were calculated in comparison with the corresponding mass of dry plants. Elemental analysis of the purified INPU, IOPU and IBPU samples showed that these

Table 1: Comparison of yields of the procedures used to extract polysaccharides from *Inula crithmoides*

Extraction	Sample	Yield (wt.-%)	Sample	Yield (wt.-%)
H_2O	IN	20.90	INPU	2.84
$K_2C_2O_4$	IO	8.15	IOPU	1.90
NaOH	IB	7.82	IBPU	1.69

Table 2: Sugar compositions of polysaccharides extracted from *Inula crithmoides*

Sample	INPU (%)	IOPU (%)	IBPU (%)
Ara	9.6	4.9	7.7
Rha	12.9	7.1	8.8
Fuc	8.3	2.1	0.5
Xyl	8.4	4.0	2.4
GalUA	33.8	64.8	43.8
GlcUA	0.2	0.2	0.3
Man	0.2	0.1	0.0
Gal	24.9	15.7	35.1
Glc	1.6	1.0	1.5
SNP	18.7	7.5	4.7

SNP: Sucre Non Pectique

polymers consist of polysaccharides exempt from proteins as no nitrogen could be detected in their elemental analysis. This result demonstrated the efficiency of the purification by ultrafiltration.

The first step was the separation of small organic compounds from polymers by extraction with hot ethanol. The residue AIS had about the same mass as the starting plants in the dry state.

About 10% of AIS was water soluble, the remaining part being mainly fibres. The polysaccharides were then roughly isolated from proteins and salts by precipitation, giving the INP, IOP and IBP fractions. As can be seen in Table 1, the polymer yield in these cases was very similar. On the other hand, the yield of the purified fraction after ultrafiltration was low especially for (IBPU). The highest yield of pectic substances was obtained with H_2O.

Structural analysis: sugar and functionality composition: The sugar composition of the isolated polysaccharides reported in Table 2 shows that they were mainly composed of Gal A (33.8 to 64.8%). These results confirm the pectic nature of the obtained polysaccharides as the mole fraction of Gal A was higher than 65 mol- % (May, 1990). The ammonium oxalate fraction was found to consist mainly of pectin, heavily branched with mostly arabinose and xylose units (65% of the galacturonosyl residues substituted).

A significantly higher GalA mole fraction featured the ammonium oxalate fractions (IOPU) with respect to other fractions INPU and IBPU. Rha (7.1 to 12.9%) and two other neutral sugars specific of pectins, galactose (15.7 to 35.7%) and arabinose (4.9 to 9.6%), were also found. The presence of Fucose and Xylose suggests that the structure of INPU is formed by a mixture of pectin (81.3%) and hemicellulose (18.7%) of xylofucane type. Moreover,

Table 3: Compositions HG and RG-I of polysaccharides extracted from *Inula crithmoides*

Sample	%HG [a]	%RG-I [b]	Gal/Rha	Ara/Rha
INPU	20.9	60.4	1.9	0.7
IOPU	57.7	34.8	2.2	0.7
IBPU	35.0	60.3	4.0	0.9

[a]HG = GalU-Rha., [b]RG-I = (2 × Rha) + Gal + Ara

Table 4: Functional analysis of polysaccharides extracted from *Inula crithmoides*

Sample	DM	Gal A (%)	AEW	pKa_0
INPU	21.5	33.8	472	1.73
IOPU	19.0	64.8	307	1.98
IBPU	5.5	43.8	270	2.44

DM results are expressed as mol-% of GalA

the significant rhamnose rate indicates the presence of a rhamnogalacturonane I (RG-I) (between 34.8 and 60.8% are five times the percentage of rhamnose) (Table 3).

In pectins, GalA units can be partly methylated on C6 and/or bear O-acetyl groups on C2 or C3 (Rombouts and Thibauet, 1986). Acetylation of pectins is mostly confined to the RG-1-like region while methyl esterification mainly occurred on smooth HG blocks (M'sakni *et al*., 2005). The polymer properties of pectin strongly depend on charge density (electrostatic repulsion between GalA units) and on esterification (hydrophobic association). DM determined for the different samples are reported in Table 3. The obtained DM value was in the range 6 to 22 mol-%. Pectin extracted in neutral water generally has a DM value higher than 50 mol-%. Such a lower DM is close to those observed for pectins extracted from citrus with pH 5 succinate-oxalate buffer and from sugar-beet cell walls with imidazole or carbonate buffer (Mary *et al*., 2000).

DM was either determined by titration of GalA acid functions (AEW). As seen in Table 4, an excellent correlation is observed between DM and AEW the consistency of the different data obtained in the structural analysis of the isolated polysaccharides.

Molar mass characterization and conformation of the isolated polysaccharides: The chromatograms of INPU and IOPU introduce practically only one elute population respectively at the neighbourhoods of 7 and 7.5 mL. This population is of strong mass this is explained by the great intensity of signal of diffusion of the light. These chromatograms show the purity of these two products extracted starting from Inula in spite of the presence from trace from composed of low mass.

As can be seen, the function log (molar mass) = f (Ve) calculated from the experimental values is linear over the major part of the peak with a deviation for higher elution volumes (Fig. 3). This phenomenon is indicative of non-SEC effects under the present experimental conditions due

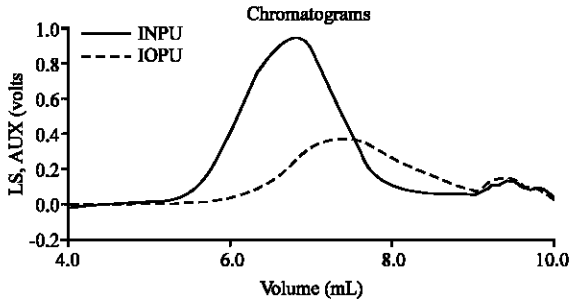

Fig. 3: Molar mass as a function of the elution volume for INPU and IOPU samples

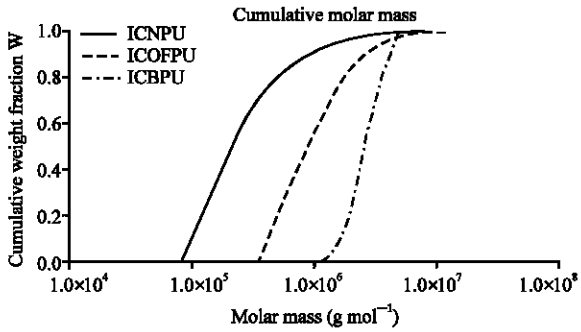

Fig. 4: Cumulative molar mass for polysaccharides extracted from *Inula crithmoides*

to partial polymer adsorption on the chromatographic support likely caused by hydrophobic interaction (Fig. 4).

Therefore, calculation of the weight-average molar mass reported in Table 5 was done assuming a linear variation of the function (Fig. 5). Mw of polysaccharides extracted in hot water was found to be in the range of 8.3×10^5 g mol^{-1} whereas Mw of the IBPU sample was much higher (2.76×10^6 g mol^{-1}).

In SEC experiments macromolecules are separated according to their dimension (hydrodynamic volume) in a given eluent. For a given elution volume, the higher the molar mass, the more compact the structure of the macromolecule. A comparison of the molar mass variation versus the elution volume for the two samples presented in Fig. 3 clearly shows that IBPU chains display a more compact conformation than the IOPU ones. To get a deeper insight into the conformation of the studied polysaccharides, we plotted the root mean square radius of gyration $\langle rg^2\rangle^{0.5}$ as a function of molar mass (Fig. 6). This variation can be represented as a power law:

$$\langle R_g^2\rangle^{0,5} = (M_w)^x$$

The slope of the double-logarithmic representation of this function gives the value of x, a parameter being

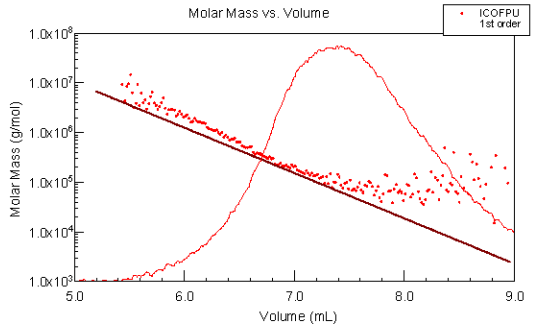

Fig. 5: Relationship between molar mass and volume

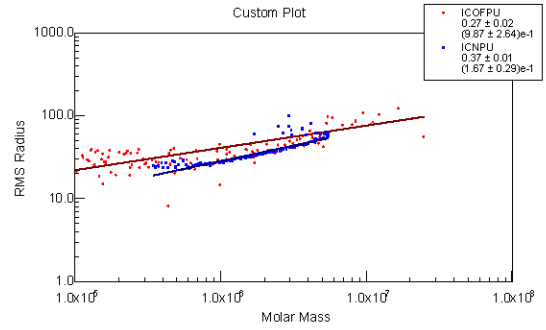

Fig. 6: Relation ship between root mean square radius of gyration $<rg^2>^z$ and molar mass for polysaccharides extracted from *Inula crithmoides*

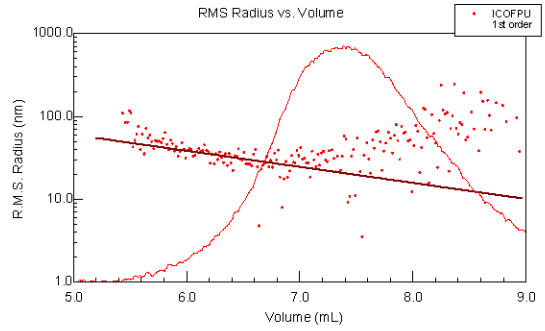

Fig. 7: Relation ship between radius of gyration and volume

characteristic of the polymer conformation (x = 0.3 for a sphere, x = 0.5-0.6 for a random coil and x = 1.0 for a rod). (O'Neill *et al*., 1996; Kohn and Malovikova, 1978). As expected, the polysaccharides extracted from *Inula crithmoides* exhibited a similar conformation with an x value corresponding to a sphere (Fig. 7, Table 5). The presence of the kinked RG-I region linked to linear HG might be responsible for a more flexible complex than pure HG. The low x value found suggests a very intricate compact conformation. To check the validity of this

Table 5: Macromolecular data for polysaccharides extracted from *Inula crithmoides* obtained by SEC-MALLS (0.025 M $NaNO_3$. pH 6.3. T = 25°C)

Sample	M_n (g mol^{-1})	M_w (g mol^{-1})	$<rg^2>^{0.5}$ (nm)	$d<rg^2>^{0.5}/dM_W$
INPU	2.8 10^5	8.3 10^5	35.5	0.38
IOPU	2.3 10^4	1.9 10^5	34.5	0.28
IBPU	2.46 10^6	2.76 10^6	63.6	0.40

Table 6: Intrinsic viscosity [η] and overlap concentration C* of polysaccharides extracted from *Inula crithmoides*

Echantillons	[η] mL g^{-1}	Rha (%)	K'	C* (g mL^{-1})
INPU	65	12.9	2.08	0.023
IOPU	53	7.1	0.57	0.028
IBPU	50	8.8	1.96	0.030

assumption, polysaccharide intrinsic viscosity [η] and Huggins' constant k' are compared in Table 6. Intrinsic viscosity, a measure of the hydrodynamic volume occupied by a polymer in a given solvent, depends on polymer conformation and molar mass while the Huggins constant is indicative of the chain-chain interaction. Preliminary studies on the influence of the ionic strength indicated that a minimal salt concentration of 1 mol dm^{-3} was necessary for screening the electrostatic repulsion due to the charges located on the GalA residues.

Polysaccharides obtained from *Inula crithmoides* exhibit similar viscometric behaviour with similar intrinsic viscosity (C = 0.7 dL g^{-1}) and values of the Huggins constant (Fig. 2, Table 6). Although IBPU has a higher Mw than the other samples, it displays a significantly lower intrinsic viscosity. This result is an additional proof of its high level of chain compactness in 1 M NaCl. The high Huggins constant found for these polymers is correlated to their associative ability by hydrophobic inter-actions. A stronger effect was observed for INPU. The decrease in intrinsic viscosity and the increase of Huggins' constant both reveal enhanced polymer intra- and inter-molecular associations in comparison with the two other samples IOPU and IBPU.

CONCLUSION

Polysaccharides were extracted from *Inula crithmoides* by selective extraction under mild condition of Ph and temperature and characterized in terms of molecular and macromolecular structures. Three kinds of polysaccharides were extracted Each fraction was purified by precipitation in ethanol followed by ultrafiltration of recovered solids dissolved in water through a membrane with a molecular weight cut off of 100,000, are purified by precipitation with ethanol followed by ultrafiltration to obtain respectively carbohydrates INPU, IOPU and IBPU. It appears from the polymer characteristics and the chemical analysis that the structure of the polysaccharides extracted from *Inula crithmoides* is more

dependent on method of extraction. These exhibited a sugar composition typical of pectins (GalA > 65 mol-%) with a value of degree of methylation DM in the range 6 to 22 mol-%. Mw of polysaccharides extracted in hot water was found to be in the range of 8.3×10^5 g mol^{-1} whereas Mw of the IBPU was much higher (2.76×10^6 g mol^{-1}).

The polysaccharides extracted from *Inula crithmoides* exhibited a similar conformation with an x value corresponding to a sphere. The presence of the kinked RG-I region linked to linear HG might be responsible for a more flexible complex than pure HG.

Further study is now undertaken to obtain more information about their gelling ability, rheological behaviour in semi dilute solutions, study of amphiphilic derivatives of the isolated pectins from *Inula crithmoides* and physico-chemical properties of aqueous dilute solutions, will be published in a forthcoming paper.

ABBREVIATIONS

AIS	:	Alcohol-insoluble substance
IN, IO et IB	:	Polysaccharide extracted, respectively, by H_2O, $K_2C_2O_4$ and NaOH
INP, IOP and IBP	:	The corresponding intermediate precipitated fractions
INPU, IOPU and IBPU	:	Precipitated and ultrafiltred
Ara	:	Arabinose
Gal	:	Galactose
GalA	:	Galact-uronic acid
Glu	:	Glucose
Man	:	Mannose
Rha	:	Rhamnose
Xyl	:	Xylose
HG	:	Homogalacturonan
RG-I	:	Rhamnogalacturonan of type I
DM	:	Degree of methylation

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