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Coupled Mass Spectrometric Strategies for the Determination of Carbohydrates at Very Low Concentrations: The Case of Polysaccharides Involved in the Molecular Dialogue Between Plants and Rhizobia

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Additional information is available at the end of the chapter

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1. Introduction

Polysaccharides play important biological roles in all living beings. They act as membrane and cell wall constituents, afford physical and chemical protection from the environment, ensure nutrition storage or compose the antigens that increase or repress defenses during infection processes, protein folding, molecular recognition, cell adhesion, etc. [1,2]. With regard to their major biological roles it appears a priority to understand their structure/activity relationship. Unfortunately, even though their relevance has been clearly demonstrated, the structural analysis of such compounds remains difficult. Actually, even though they are often highly soluble in water, their polarity due to the presence for instance of hydroxyls, carboxyls and sulfates, makes them quite difficult to handle. These polar groups are involved in intra and inter-molecular hydrogen bonding, ionic interactions and for lipopolysaccharides (LPS) hydrophobic interactions. Such molecular interactions are sources of solubility problems and can lead to the formation of aggregates, making their analysis more difficult (enormous apparent size, broadening of NMR signals, ionization difficulties, and so on).

In the past, analysis of polysaccharides was mostly achieved through Gas Chromatography coupled to Mass Spectrometry (GC-MS) of the PS hydrolysates. This technique remains widely used, due to its simplicity, but the development of high field Nuclear Magnetic

Resonance (NMR) and cryoprobes have opened new avenues. Unfortunately, NMR presents major drawbacks such as sample purity, sample complexity and concentration requirements. However, in the last decade, the development of highly sensitive, high-throughput Mass Spectrometers (MS) and software able to make global approaches (“omics”) on very complex mixtures, have induced a new interest for the MS analyses (especially for the study of protein glycosylation). This time, the instruments mostly use soft ionization techniques associated to powerful MS² or ⁿ capacity analyzers experiments on electrospray ionization coupled to tandem mass spectrometers (ESI-MS/MS) or matrix assisted laser desorption and ionization coupled to a time of flight analyser (MALDI-ToF) [3]. Finally, the recent appearance of ToF and FTICR (Fourier transform ion cyclotron resonance) analyzers on the market allowed access to high resolution measurements enabling the determination of the exact molecular masses (with ToF error of less than 5 ppm and FTICR less than 0.5 ppm). Exact masses give access to the elementary composition of the molecule that is extremely precious when the analyte is present in a complex biological matrix at low concentrations precluding study by MS/MS.

Beside the evolution of structural analysis, separations techniques have also been enhanced especially by using higher resolution techniques (narrow bore columns, high pressure tolerant or acido-basic tolerant chromatographic phases). This progress has made it possible to couple chromatographic and MS systems on-line.

In this chapter, we will describe the classical MS couplings like GC-MS or HPLC-MS, but also focus on unusual but useful polysaccharide analysis systems like capillary electrophoresis coupled with mass spectrometry (CE-MS) or high performance anion exchange chromatography coupled with mass spectrometry (HPAEC-MS) that we have developed. Finally, we will present the practical uses of these techniques in a fundamental application: the analysis of the exo-, lipo- and capsular polysaccharides of *Sinorhizobium meliloti*.

2. Structural analyses using mass spectrometry

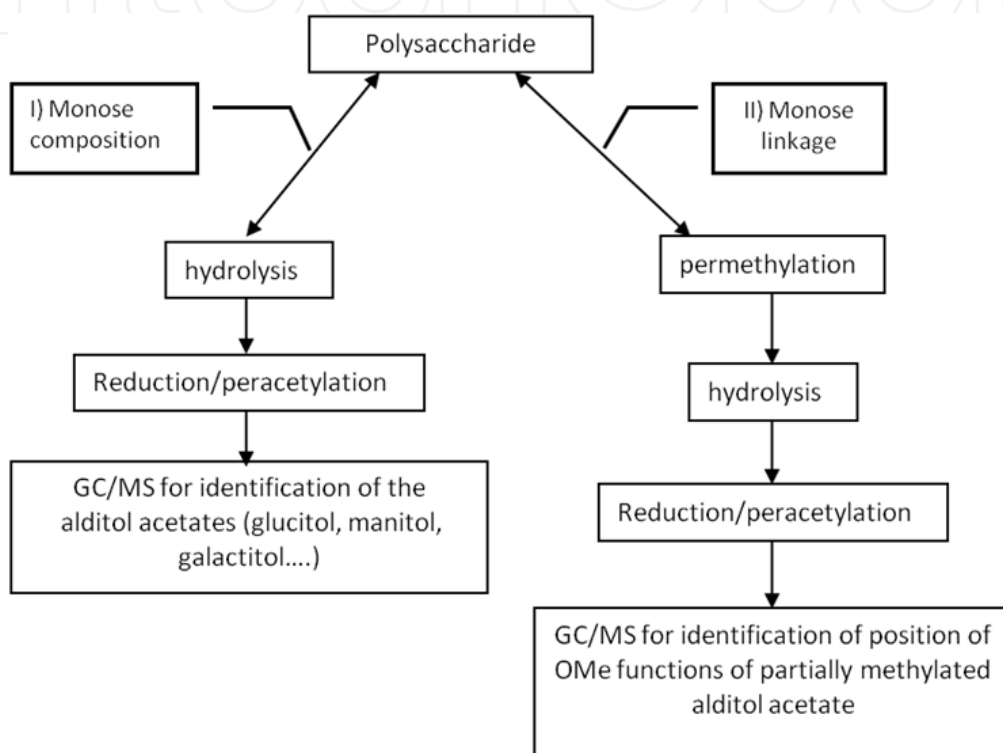
2.1. Gas chromatography coupled to mass spectrometry

The analysis of polysaccharides is relatively easy to run using ESI-MS and ESI-MS/MS to identify the concatenation of polysaccharide in hexose, deoxy-hexose, pentose, acidic sugars (polyhydroxy carboxylic acids), but it does not provide the polysaccharide composition (glucose, mannose, fructose...) mainly because many monosaccharides have isobaric masses, and often lack an ion (X and A) to identify how the monosaccharides are branched. GC-MS using electron ionization (EI) and chemical ionization (CI) is well suited for such a purpose. Some recent reviews have been published which gather and summarize the different numerous strategies developed in the literature for sugar analysis [4, 5, 6].

Because polysaccharides are mainly composed of aldoses, ketoses, deoxymonosaccharide, amino-monosaccharide and acidic monosaccharide, GC-MS analysis is based on the hydrolysis of the polysaccharides followed by derivatization and GC/MS analysis to identify

the different sugar units through their migration times and/or mass spectra. Among the hydrolyzed monosaccharides some cannot be observed like uronic acids, ulosonic acids (*e.g.* Kdo), 4-amino-monosaccharide, and other charged species. Acids cannot be observed because their sodium salts are not volatile. Their treatment must be adapted for study by GC-MS. We describe below the main treatments for the study of polysaccharides: hydrolysis, derivatization, and we evoke the case of the acidic sugars.

Scheme 1 presents the general strategy which is used in GC-MS to analyze polysaccharides by determining the nature of the sugars, the relative quantities and the mode of attachment.



Scheme 1. General strategy used in GC-MS to analyze polysaccharides

2.1.1. Quality and relative quantities of monosaccharides: alditol acetate

To identify the nature of the monosaccharide, hydrolysis using H_2SO_4 , HCl or trifluoroacetic acid (weaker than the others but easy to evaporate) is performed. After a reduction step using sodium borohydride [7], acetylation with acetic anhydride [8] results in alditol acetates which can be identified using GC retention time. GC-MS with chemical ionization using ammonia confirms the mass of the pseudo molecular ions $(\text{M}+\text{H})^+$ and $(\text{M}+\text{NH}_4)^+$ of the alditol acetates. The chromatographic peaks help to identify the relative number of monomers composing the polysaccharide. Reduction with NaBD_4 allows easy identification of the anomeric carbon.

The difference in the hydrolysis rate between the sugars results in certain difficulties *i.e.* uronic acids being partially hydrolyzed, the monosaccharide linked to it is underrepresented. The same difficulties also exist for 2-acetamidohexoses which are

partially *N*-deacetylated under excessive acid concentrations. The 2-aminohexoses obtained are not hydrolyzed and the carbohydrates linked to them are underrepresented [9].

For the acidic sugars, a modified strategy can be used, it consists first in methyl esterification of the uronic acids using DMSO and methanol or diazomethane. The methoxy group (or any other substitution) is a good leaving group for reduction of esters with sodium borohydride to yield aldehydes and, subsequently, their respective neutral sugars. This way, the reduction of the esters by NaBD₄ prior to hydrolysis yields a neutral polysaccharide allowing total hydrolysis and, after acetylation, identification by GC-EI-MS of the CD₂OCOCH₃ moiety bearing the monosaccharides [10,11,12] characteristic of the initial acidic sugars. Alternatively, methanolysis is also used to release monosaccharides as methylglycosides with esterified carboxyl groups. Conditions of methanolysis are presented in table 1.

Table 1 presents different hydrolysis conditions, reduction and acetylation on different kinds of polysaccharides.

Hydrolysis conditions	Reduction and acetylation conditions	Polysaccharide	ref
10 M HCl (80 °C, 30 min)	Excess of NaBH ₄ (2 h, 20 °C), acetylated with a 1:1 Ac ₂ O–pyridine mixture (100 °C, 1 h)	LPS of <i>Providencia alcalifaciens</i> O12	[13]
100 µL sample, 50 µL of 0.7 M 4-methylmorpholine borane (MMB) and 100 µL of 6 M TFA, 80 °C, 30min. Then, 50 µL of 0.7M MMB. evaporation and addition of 200 µL 2 M TFA and 500 µL of acetonitrile. Dried again.	Dried sample +100 µL acetic anhydride and 100 µL TFA, reaction at 50 °C for 10 min. Then 1mL toluene to each dried sample . After dissolution in 1.5 mL CH ₂ Cl ₂ and wash with 0.5M Na ₂ CO ₃ and water. Sample are then dissolved in CH ₂ Cl ₂ spiked with internal standards	Agar containing 3,6-anhydrogalactose	[14]
Sample was suspended in 2 N H ₂ SO ₄ heated under vacuum at 100 °C for 3 h. 50 µL of water containing 500 ng monosaccharide internal standard and 1 mL H ₂ O was added. Then, 2 mL 50% <i>N,N</i> -dioctylmethylamine are added to neutralize the remaining acid. After centrifugation, the top layer was purified on a C18 column.	Add 50 µL of 100 mg/mL NaBD ₄ and store at 4 °C overnight. Evaporate with methanol/acetic acid under vacuum. Then add 300 µL of acetic anhydride, react at 100 °C for 13–16 h. Quench with 750 µL H ₂ O for 1 h at room temperature. Extract with 1 mL of chloroform (CHCl ₃) Remove aqueous phase, add 0.8mL of cold 80% NaOH, mix, elute with CHCl ₃ on a Chem-Elut column.	Agar containing 3,6-anhydrogalactose	[14]

Hydrolysis conditions	Reduction and acetylation conditions	Polysaccharide	ref
2 mg sample treated with 0.5 mL TFA 2 M for 1 h at 121 °C	TFA removed by evaporation, then NaBH ₄ reduction and then acetylation with Ac ₂ O–pyridine (1:1, v/v; 2 mL) at room temperature for 12 h	Exopolysaccharide containing glucuronic acid	[15]
Methylesterification: 10mg sample dissolved in 5mL DMSO add diazomethane. Freeze- dry. For reduction, dissolve 2mL imidazol/HCl 1M pH 7 at 0°C, 2 drops of octanol add 200mg KBH ₄ 2h. Add acetic acid until pH 6, dialyze and freeze dry. Add 0.5mL TFA 4N at 100°C, 4h.	Evaporate sample, add NH ₄ OH 0.1N and 1mg KBH ₄ 2h 20°C. Then add acetic acid until pH 6, co-distillate with methanol. Acetylate dried residue with pyridine/acetic anhydride (Ac ₂ O) (50-200µL) 12h, 20°C. Dry the sample in presence of toluene.	Polysaccharide from <i>Bacillus circulans</i>	[12]
1 mg sample hydrolyzed with 1 M TFA at 100°C for 8 h, evaporate to dryness.	Reduce the dried sample with excess of NaBH ₄ and acetylate with Ac ₂ O–pyridine (1:1, v/v; 2 mL) at room temperature for 12 h.	Exopolysaccharide from surface-liquid culture of <i>Clonostachys rosea</i>	[16]
1mg polysaccharide fraction in 2 M TFA at 100 °C for 8 h, followed by evaporation to dryness.	Dry residue reduced NaBH ₄ or NaB ₂ H ₄ and acetylated with Ac ₂ O-pyridine (1:1, v/v) at room temperature for 12 h.	Polysaccharide containing 3-O-methylated mannogalactan	[17]
1 mg mycelium 6 h at 100 °C with 3 mL 6 M HCl. Dry at 40°C	Add to dry sample, 20 µg inositol in 0.8 mL water, and 50 µl NaBH ₄ (10 mg/ mL in 2 M ammonia), at 37 °C, 90 min. Add 2 mL acetic acid in methanol (1:200 v/v). After drying, add 300 µl Ac ₂ O, 100 °C for 15 h. In ice-bath add 750 µl of water. Extract with CHCl ₃ .	Chitin polysaccharide from mycelium.	[18]
Methanolysis	Derivatization		
4.5mg of freeze-dried fibers and pulps suspended in 2mL of 2M solution of HCl in anhydrous CH ₃ OH, 100 °C 4 h. Add 80µL pyridine. Add 1mL sorbitol 0.1mg/mL.	Dissolve dried sample in 70µL pyridine. Add 150µL hexamethyldisiloxane and 80µL trimethylchlorosilane for 12 h at room temperature.	O-acetyl-(4-O-methylglucurono)xy lan from <i>Agave sisalana</i>	[19]

Hydrolysis conditions	Reduction and acetylation conditions	Polysaccharide	ref
Add MeOH 0.5M HCl (acetyl chloride 140mL + 4mL MeOH) at 80 °C for 16 h. dry under a stream of nitrogen gas.	300 µl of TriSilR reagent are added to dried material, at 80 °C for 20min and the reagents removed with a nitrogen stream. 1mL hexane extraction of the sample, dry and add hexane.	Major grape polysaccharides	[20]
2mL of 0.01, 0.125, 0.25 or 0.5M HCl in MeOH added to 10mg sample, heated at 85°C. Neutralize with Ag ₂ CO ₃ , dry the supernatant		Gelling carrageenans and agarose	[21]
Add 1.5mg sample to 0.5mL of MeOH/HCL (15mL MeOH and 0.4mL acetyl chloride). Heat at 80°C for 24h and dry.	Add to the dry sample 0.5mL of pyridine, hexamethyldisilazane, trimethylchlorosilane (9:3:1, v/v/v), 80°C 2h. Dry and add 0.5mL hexane.	Plant gums	[22]
Add 2mL 0.5M HCl/MeOH to 5mg sample at 80°C. Neutralize to pH 6.	30µL/30µL pyridine and BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) added to dry sample, 2h 20°C.	Polysaccharides containing uronic acids.	[12]

Table 1. asd

Scheme 2 presents different ions obtained for alditol acetate in EI. Generally, no molecular ion and only very low abundance of $(M-CH_3COO)^+$ are observed in the spectrum. Stereoisomers give very near spectra and the base peak is the acetylium ion (CH_3CO^+) at m/z 43. The primary fragments are formed by cleavage of the carbon chain and then these odd mass number ions may lose an acetic acid (loss of 60 mass units) or a ketene (loss of 42 mass units) or an acetic anhydride (loss of 102 mass units) [23].

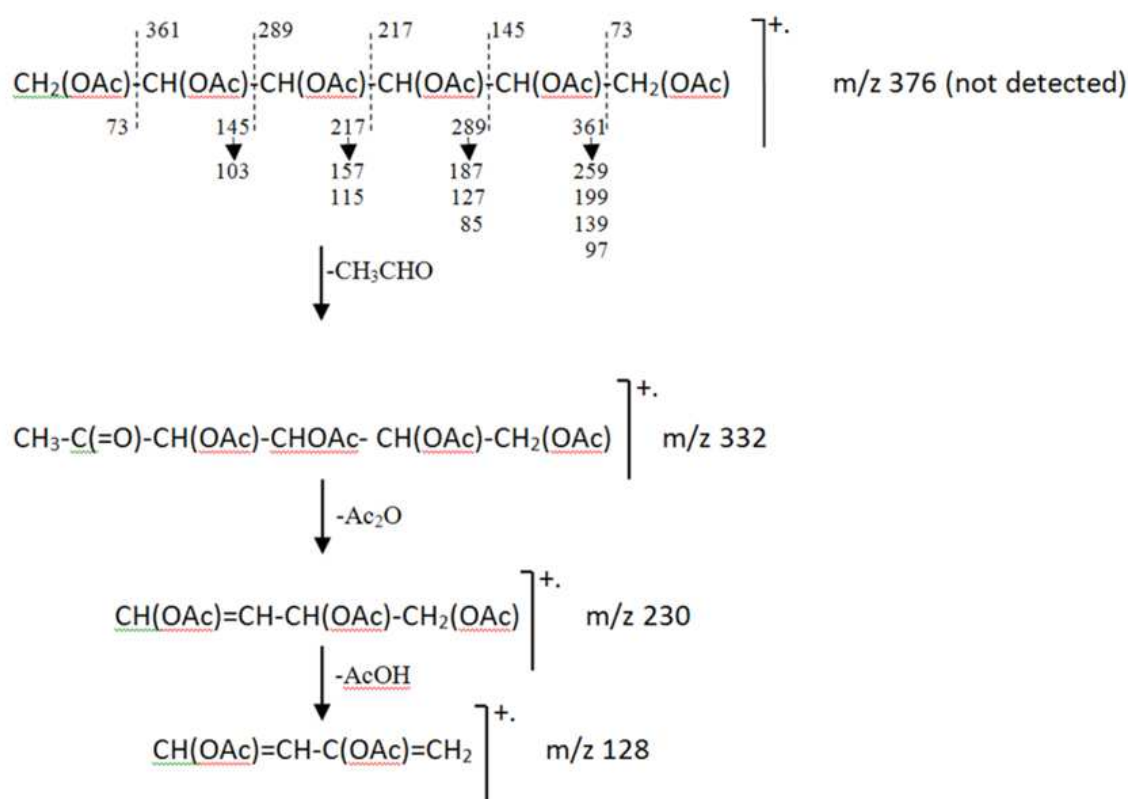
If deoxycarbons are present, alfa cleavage resulting in methyl radical loss is insignificant [24]. Some even mass number ions come from rearrangements resulting in the loss of acetaldehyde (loss of 44 mass units), acetic acid, acetic anhydride and ketene from the same mass number molecular ions [25].

When an acetamido group is present, the main ions are due to alfa cleavages induced by the N atom [26].

Consequently comparison of the mass spectrum (EI or CI) and the migration time to standards allows the monosaccharides to be identified.

Trimethylsilyl derivatives can also be used for alditol derivatives [27, 28]. No molecular peaks can be detected. As for most TMS derivatives in EI-MS, small $M-15$ ions are identified and m/z 103 $((CH_3)_3SiOCH_2^+)$ and m/z $(n \times 102) + 1$ (n being the number of carbon atoms in the

chain of the alditol fragment) are recorded with their corresponding ion coming from a loss of 90 amu ((CH₃)₃SiOH).



Scheme 2. Main fragmentation of hexitol peracetate.

Monosaccharides can also be analyzed prior to reduction by NaBH₄ or NaBD₄, under their cyclic forms, but the chromatograms are more complicated due to the hemiacetal group in sugars which leads to multiple structures [29].

2.1.2. Quality and relative quantities of monosaccharides: derivatives of cyclic forms of monosaccharides

Most of time the polysaccharide is submitted to a methanolysis and after the sample is silylated (see Table 1). With ammonia, CI can be used to identify the (M+H)⁺ and (M+NH₄)⁺ ions and can be identified by comparison of the migration times of standard the sugar. It is known that the relative intensity of the (M+NH₄-CH₃OH)⁺ ion allows the assignment of pyranoside and furanoside structure [30]. Other fragmentation ions exist resulting from the loss by (M+NH₄)⁺ or (M+H)⁺ of CH₃OH or a single or two (CH₃)₃SiOH molecules.

EI is frequently used and the resulting fragmentation has been extensively reported [30, 31]. If only very rare molecular ions are present in the spectrum, pentose and hexose are characterized by (M-CH₃)⁺ ions and losses of CH₃OH and (CH₃)₃SiOH from this last ion. Deoxyhexoses give M⁺ and (M-CH₃O)⁺. Glucuronic and galacturonic acids also exhibit (M-CH₃)⁺ ions in the spectrum and losses of CH₃OH and (CH₃)₃SiOH but also an m/z 234 ion is

characteristic of acidic derivatives resulting from a complex McLafferty-type rearrangement of trimethylsilyl group to the carboxyl function. Finally, an m/z 204 ion is closely related to ring size, being favored by a six-atom cyclic structure [32].

For pyranose forms the relative intensity of this ion (compared to base peak) varies between 30 and 100% while for furanose it is lower (5%), while m/z 217 is very intense for furanose forms.

These fragmentations and migration times of the TMS derivatives will help to determine the species that are difficult to characterize owing to their migration times.

2.1.3. The attachment mode

2.1.3.1. Synthesis of partially methylated alditol acetate

The use of methylated alditol acetate is a convenient protocol to identify the mode of attachment, it has been well reviewed by Hellerqvist and Sweetman [33]. The first step of this study consists in a permethylation of the polysaccharide, using finely powdered sodium hydroxide and methyl-iodide. A recent article shows that presolubilization of 100-300 μ L of a solution of 1mg/mL polysaccharide with 5 μ L of anhydrous glycerol dried prior to derivatization, offers better methylation yields [34]. Recently, solid-phase spin column permethylation and solid phase capillary permethylation were described [35, 36] and are presented in Table 2. These permethylation processes are derived from the classical method described by Costello [37, 38]. After the permethylation, the polysaccharide is hydrolyzed. For example: the polysaccharide is dried, then 1.8mL glacial acetic acid is added and the sample is briefly sonicated; 0.20mL of 2M sulfuric acid are added and heated at 100°C for 9h. Then acetic acid can be removed in a rotary evaporator several times by adding water and keeping the temperature below 40°C. An equimolar amount of BaCO₃ is added to remove the sulfuric acid, and the precipitate is filtered and washed in a minimum volume of water. Then washings and filtrates are combined and about 5mg of NaBH₄ (or NaBD₄) are added, the reaction is driven for at least 1h. Finally, per-acetylation is driven as reported in table 1.

GC/MS using ammonia CI and then EI are performed, to determine the molecular mass and the structure of the partially methylated acetate alditol respectively. Following this protocol, the positions of the O-methyl functions reflect the initial position of the free OH functions of the polysaccharides.

2.1.3.2. Fragmentation rules of partially methylated alditol acetate in EI

In EI, no molecular ions can be observed. The rules include amino sugars and predict the formation of ions formed by cleavage of the C-C chain. i) The major ion corresponds to the cleavage between acetyl-N-methylaminated carbon and O-methylated carbon, with charge retention predominantly on the aminylated carbon, ii) an intense ion comes from two O-methylated carbons with charge retention on either carbon (the intensities of both ions are inversely proportional to the size of the fragment), iii) weaker ions are due to the cleavage between two C atoms one acetylated the other methoxylated which keeps the charge. These

ions may lose ketene, acetic acid or methanol molecules. Table 3 gives the different masses of the different possible ions. With the knowledge of the $(M+H)^+$ or/and $(M+NH_4)^+$ ion, obtained in GC:CI/MS and the fragment ions from the EI spectrum, the position of the methyl and the attachment of the different sugars can be identified. Combining these data with ESI-MS/MS gives the correct sequence of the polysaccharide.

Permethylation Conditions	Polysaccharides	ref
Dissolve in 200 μ L DMSO, powdered NaOH (5 mg) and 200 μ L CH ₃ I. Stir vigorously for 30 min and left overnight at room temperature. extract with CHCl ₃ .	matesaponin	[39]
Microspin column are placed in microcentrifuge vial, filled with acetonitrile. Pack with NaOH beads in acetonitrile. Centrifuge the spin column with acetonitrile then DMSO. Suspend dried polysaccharide in DMSO, CH ₃ I, H ₂ O (70.8; 26.4; 2.8%) spin the column at 800rpm for 30s. Recycle the 8x. Washed it with 100- μ L aliquot of acetonitrile, at 10 000 rpm for 0.5 min. Extract column with CHCl ₃ .	Nglycans from serum proteins	[40,41]
Add 10 mg polysaccharide in 1mL DMSO to 1mL CH ₃ I and 20mg powdered NaOH for 30min. Stir vigorously and maintain at 25°C overnight. Stop with H ₂ O, neutralize with acetic acid, dialyse (8 kDaMr cut off) against distilled H ₂ O and freeze-dry. Extract with CHCl ₃ .	3-O-methylated mannogalactan from <i>Pleurotus pulmonarius</i>	[42]
Suspend 100-300 μ g of dry polysaccharide in 100 μ L DMSO. Mix 400 μ L CH ₃ OH and 100 μ L 50% NaOH, and 2mL DMSO and vortex. Centrifuge at 3500 rpm for 1 min to get the NaOH pellets. Wash 5x with 2 mL DMSO. Mix the polysaccharide and the 500 μ L NaOH slury solution with 100 μ L CH ₃ I. Evaporate, and clean with 2mL H ₂ O and 2mL CH ₂ Cl ₂ .	Pn6B, Pn14, Dextran polysaccharides	[43]

Table 2. Permethylation of polysaccharides.

CH ₂ =O ⁺ CH ₃ m/z 45	CH ₂ OCH ₃ CHO ⁺ CH ₃ m/z89	CH ₂ OCOCH ₃ CHO ⁺ CH ₃ m/z 117	CH ₂ OCOCH ₃ CH=NH ⁺ (COCH ₃) m/z 144	CH ₂ OCOCH ₃ CH=NH ⁺ (COCH ₃)CH ₃ m/z 158
CH ₂ OCH ₃ CHOCH ₃ CH=O ⁺ CH ₃ m/z 133	CH ₂ OCOCH ₃ CHOCH ₃ CH=O ⁺ CH ₃ m/z 161	CH ₂ OCH ₃ CHOCOCH ₃ CH=O ⁺ CH ₃ m/z 161	CH ₂ OCOCH ₃ CHOCOCH ₃ CH=O ⁺ CH ₃ m/z 189	CH ₂ CH ₃ CHOCOCH ₃ CH=O ⁺ CH ₃ m/z 131
CH ₂ OCH ₃ CHOCH ₃ CHOCH ₃ CH=O ⁺ CH ₃ m/z 177	CH ₂ OCOCH ₃ CHOCH ₃ CHOCH ₃ CH=O ⁺ CH ₃ m/z 205	CH ₂ OCH ₃ CHOCOCH ₃ CHOCH ₃ CH=O ⁺ CH ₃ m/z 205	CH ₂ OCH ₃ CHOCOCH ₃ CHOCOCH ₃ CH=O ⁺ CH ₃ m/z 233	CH ₂ OCOCH ₃ CHOCOCH ₃ CHOCOCH ₃ CH=O ⁺ CH ₃ m/z 261

Table 3. Principal fragmentations in EI/MS of the C-C chain of permethylated alditol acetate
We do not present the ions which can lose CH₃OH, CH₂CO, CH₃COOH.

2.2. ElectroSpray ionisation mass spectrometry (ESI-MS) analyses

Using electrospray technology, the analyte is introduced into the mass spectrometer as a solution delivered by a syringe pump (direct input) or as a fraction eluted from a liquid chromatography (HPLC/UPLC). The analyte solution passes through a needle on which a high potential difference is applied (classically 3 kV). This produces a spray of droplets with a surface charge of the same polarity as the needle. The charged droplets shrink as the solvent evaporates. Charge promiscuity then produces continual explosions of the droplets into smaller ones until they reach the gas phase. During this process, the molecules present in the solution are concentrated and this often produces a suppression effect. This effect consists in the masking of one compound by another present in a mixture. The suppression effect is minimal when all the compounds together are chemically equivalent in terms of hydrophobicity or acido-basicity. As hydrophilic compounds stay longer in the dissolved form during the ESI process, the more hydrophobic compounds will generally suppress the more hydrophilic compounds. In the positive ion mode, basic compounds will easily suppress more acidic compounds. As carbohydrates are very hydrophilic and often acidic, only two possibilities exist to analyze them without suppression: a separation of the sugars based on their acidity (ion exchange chromatography) or derivatization of the hydroxyls to generate more hydrophobic compounds. The latter techniques include derivatization such as permethylation [44;45] or metal adduction [46;47] which has been extensively studied. Structural analysis of underivatized saccharides using negative mode ion electrospray ionization has also been investigated [48].

A systematic nomenclature for labeling fragment ions observed in MS/MS has been introduced by Domon and Costello [49] (Figure 1).

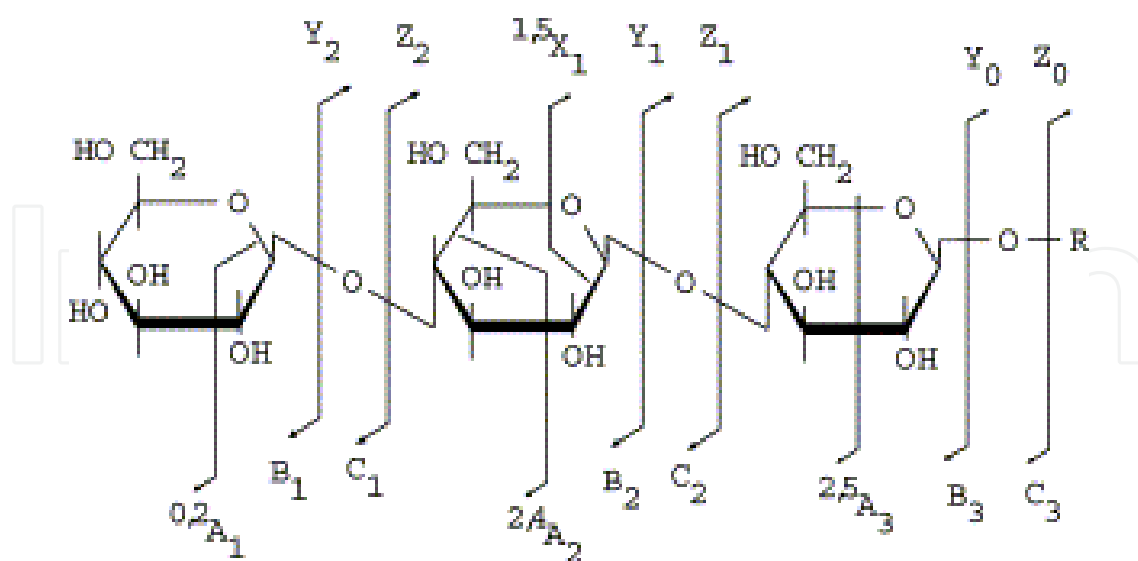


Figure 1. Systematic nomenclature for labeling fragment ions observed in MS/MS following Domon and Costello.

Acidic moieties strongly influence the fragmentation energetics and patterns of tandem mass spectrometric daughter ion formation. Non-sulfated compounds dissociate in the

positive mode into abundant ions through B and Y type fragmentation resulting from the cationisation of the glycosidic oxygen [50], these fragmentations being facilitated on 4- and 6-linked reducing terminal residues [51]. In the negative mode deprotonated neutral carbohydrates produce C- and Z-type ion fragments [52], when the acidic sugars undergo B- and Y-type fragmentation [53]. Therefore, interpretation of the CID spectra implies knowledge of the fragmentation pathway, and the use of mass tables of the common monosaccharide building blocks is often essential to build the sequence of unknown structures. Of course, understanding the biosynthetic pathway of oligosaccharides helps to avoid making mistakes in the sequence [54].

2.2.1. Direct Input (DI/MS)

As explained just before, analyzing carbohydrate mixtures by direct introduction is a challenge due to the easy suppression of these compounds by numerous other natural compounds like proteins, aminoacids, lipids or other differently charged or decorated saccharides.

In comparison to MALDI, ESI produces less in-source fragmentation of acidic glucans and other fragile ions and is easily coupled on one side to tandem MS allowing structural investigations and on the other side on-line to liquid chromatography. As this ionization technique is very soft, true MS (without fragmentation) can be obtained. However, MALDI produces less complex charge state patterns and less multiple cation adducts and suppression effects. Actually, the analyte is not in solution with the salts and the deposit is quite heterogenic indicating places where suppression effects could be less important [55]. The polymeric complexity of carbohydrates often produces an overlap of the ESI charge state patterns making them extremely difficult to analyze [56] even with software such as MaxEnt (1 and 3). As a result, except for acidic glucans, ESI analysis of glycans requires chromatographic pre-separation of the sample.

2.2.2. High-resolution liquid chromatography coupled to Mass Spectrometry (HPLC-MS)

Normal phase HPLC on naked inorganic oxides was the technique first developed but it exhibited numerous drawbacks (peak tailing, retention time shifts, etc.), therefore, reversed phase HPLC was developed and proved to be suitable for most all bioanalytical solutes. However, a problem remained: how to create retention in RP-HPLC for polar compounds without dewetting the C₁₈ phase [57]. Lack of retention of hydrophilic compounds is almost always due to solvophilicity. Actually, the polar functions enter more favorable dipolar interactions with the polar RP-HPLC eluent, than with the stationary phase [58]. When the lack of retention is due to charge, retention can easily be achieved on ion-exchange chromatography (see next paragraph) or by using ion pairing that does not require specific apparatus. Using this technique, glycosaminoglycans polymers up to a polymerization degree of 40 can be observed on reversed phase ion pairing HPLC [59]. Unfortunately, ion pairing has been shown to reduce ESI-MS sensitivity [60].

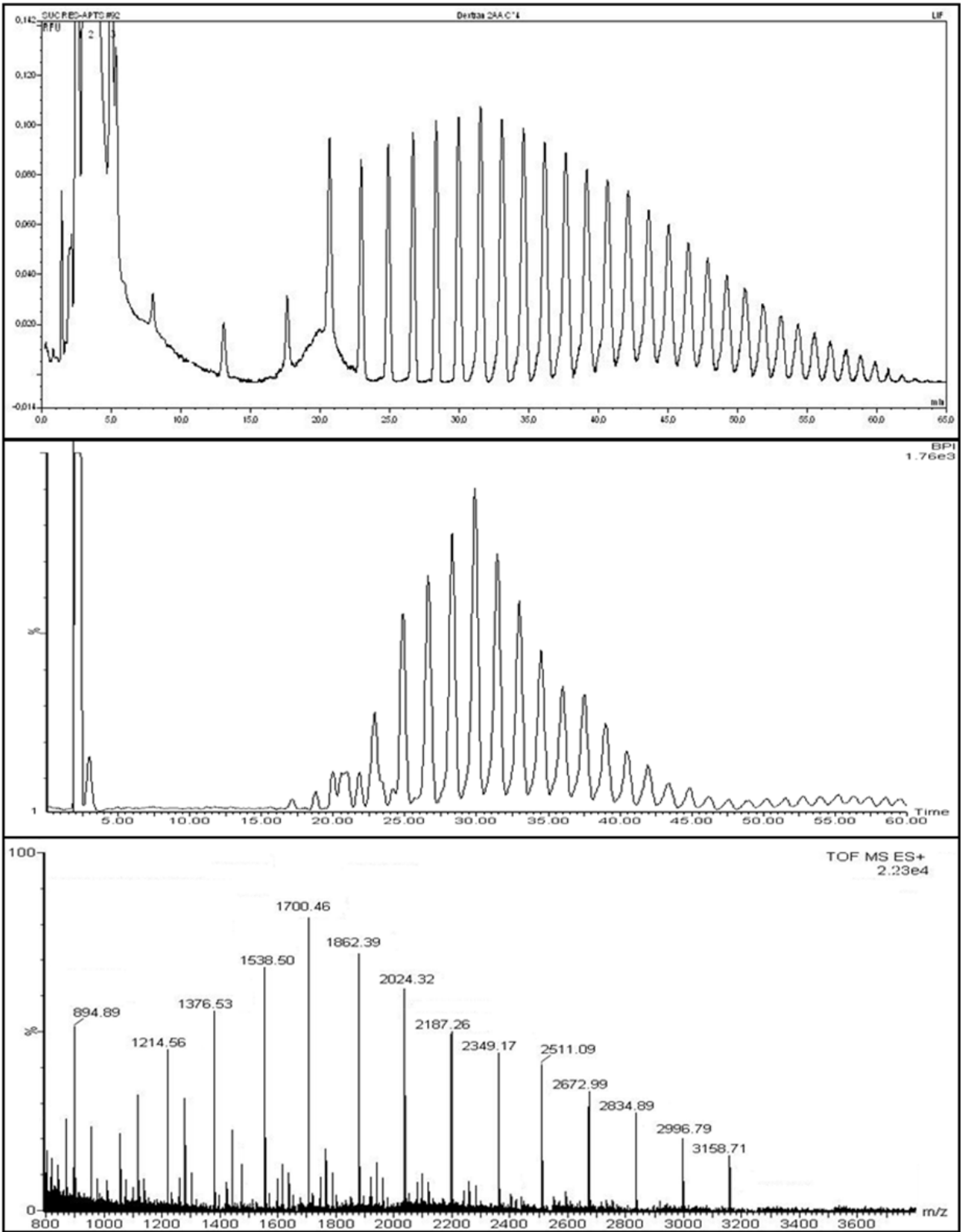


Figure 2. HILIC/LIF/ESI-MS analysis of 2-AB labeled glucose ladder. At the top: improved LIF detection of the neutral saccharides, in the middle: positive mode ESI-MS total ion current, at the bottom: spectrum obtained by summing all the spectra of the compounds eluting between 15 and 50 minutes.

The greatest problem concerns the highly hydrophilic but still neutral analytes. The classical way to analyze them is to convert them into more hydrophobic compounds by a chemical reaction [61]. In addition to the retention of the compounds, such organic derivatization improves their detection (UV absorption or fluorescence emission) (figure 2). The main drawbacks of this approach are that the separation of all the compounds is only based on the same hydrophobic tail, derivatization is time-consuming, and quantitative labeling cannot be systematically achieved. Therefore, a new strategy has been developed and is based on a new stationary phase. In practice, the stationary phase is polar and attracts the more polar part of the eluent that will act as the retentive phase. The complete eluent is relatively hydrophobic but is a sufficiently good solvent to allow distribution between the stationary and mobile phases. This technology is named hydrophilic interaction chromatography (HILIC). The quite neutral hydrophobic/hydrophilic balance of the eluent allows an easy interfacing with ESI-MS unlike the normal phase HPLC (NP-HPLC) working with totally organic solvents which are not compatible with the nebulization process [62].

2.2.3. High Performance Anion Exchange Chromatography coupled to Mass Spectrometry (HPAEC-MS)

High performance anion exchange chromatography (HPAEC) using sodium hydroxide or acetate based eluents is a well-established technique for determining underivatized carbohydrates [63;64] and high-performance separation of alditols, mono- and oligosaccharides ranging from 2 to 60 mers have been described using pulsed amperometric detection (PAD) [65]. The identification of individual carbohydrates is usually performed by comparison of their retention times with those of reference samples. In biological samples complex overlapping occurs: acidic sugars are eluted in the "oligosaccharide domain" making this approach very uncertain. Moreover, the nature of sugar moieties and the variability of glycosidic linkages makes the retention times of the oligosaccharides unpredictable. For these reasons, coupling of HPAEC-PAD with ESI-Q-ToF (ESI coupled to a hybrid tandem mass spectrometer constituted of a quadrupole and a ToF) MS was required to collect structural information on the PS sequences. Two detections are necessary. PAD allows, from retention time, the nature of the saccharide (neutral, acidic, oligosaccharidic) to be determined while ESI-Q-ToF MS gives access to the composition. Coupling HPAEC with MS presents a technological challenge, due to the non-volatility and high conductance of the sodium hydroxide or acetate dissolved in water, used as eluent. To avoid this limitation, a commercial desalting device using a selective cation exchange membrane and a regenerant can be installed on-line between the column and the MS [71,66]. The use of this on-line desalter with oligosaccharide separations with MS has only been rarely described [67, 68, 69, 70, 71].

We have developed HPAEC and ESI-Q-ToF MS conditions to perform efficient on-line coupling of the two techniques [72]. PA1 ion exchange columns have been used for oligosaccharide analysis even though they are designed for monosaccharides, because the PA 200 column is not available in the narrowbore size. Two different gradients have been used at a flow rate of 0.3 mL/min. For neutral carbohydrates: during the first 15min NaOH concentration increased from 10 to 50mM, and during the following 5 min up to 80mM,

finally, during the last 15 min the concentration of NaOAc increased from 0 to 90 mM while NaOH remains constant, return to the initial conditions was done over 10 min. A wait of 35 min between two injections is necessary to equilibrate the column. To separate acidic sugars, the gradient started with a NaOAc concentration ramp increasing from 0 to 90 mM in 15 min while the concentration of NaOH was kept constant at 80 mM, the concentration of NaOAc (90 mM) and NaOH (80 mM) were then held constant for 5 min then 5 min to return to initial conditions. A wait of 15 min between two injections was necessary to equilibrate the column.

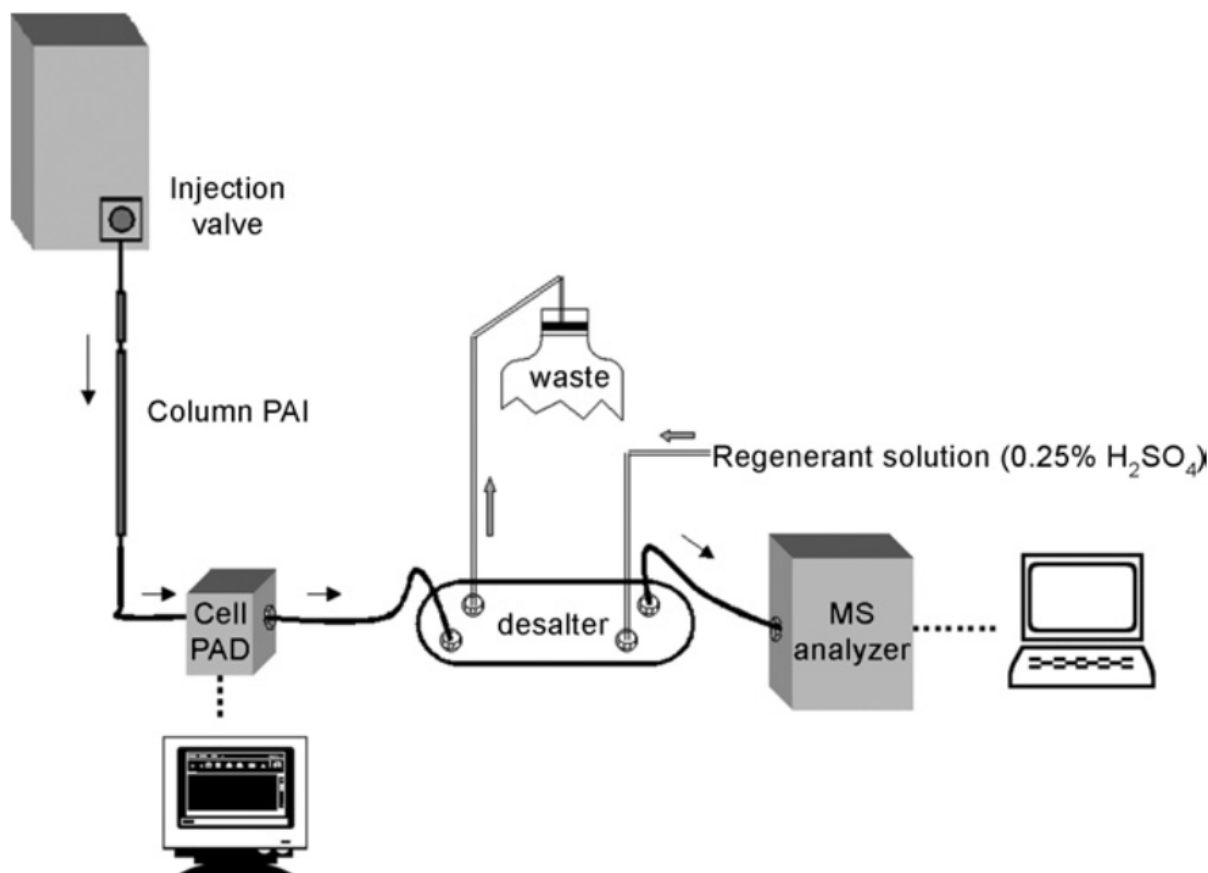
Due to the low amounts of polysaccharide that can be dissolved, no post-column split could be used to couple ESI-Q-ToF with an analytical sized column. This methodology was never used for the analysis of heteropolysaccharides.

Performing HPAEC for carbohydrate analyses implies the use of a NaOH and NaOAc concentration gradient. At high pH (>13.5), carbohydrates are in the anionic form allowing their retention on the phase. Electrocatalytic oxidation mediated by NaOH at the surface of the gold electrode occurs by application of a positive potential. The current generated is then proportional to the carbohydrate concentration. Therefore obtaining a PAD signal, implies placing the suppressor after the PAD but before the MS (Scheme 3). Since a high ionic content is not compatible with electrospray ionization mode, the salt level was reduced using an on-line 4 mm ASRS (anion self-regenerating suppressor) desalter. The desalting efficiency and the pH of the mobile phase after desalting were checked. The peak broadening due to the additional void volume of the ASRS and the reference electrode cavity was then investigated. Desalting efficiency was followed through a conductometer as a second detector. The pH was followed each minute (using the combination pH reference electrode in the electrochemical cell of the PAD unit) to be sure that the residual conductivity was not due to acetic acid. When the regenerant used in the ASRS is pure water at a flow rate of 2 mL/min, it is possible to suppress NaOH up to 100 mM (at a flow rate 0.3 mL/min), but it does not provide the suppression of 100 mM NaOAc (the pH increased up to 13 at 25 min). In contrast, the use of 0.25% H₂SO₄ in water at a flow rate of 2 mL/min can maintain the conductivity below 300 μ S for the entire analysis. Desalting of the NaOH eluent resulted in the measurement of a stable pH of 7; during the NaOAc gradient, the pH decreased regularly to pH 3. During conductimetric detection, a current is continuously applied to the membrane (50 mA). No effect of regeneration current on the membrane regeneration efficiency was observed.

The coupling of both PAD and MS detectors offers two advantages. First, the safety of the mass spectrometer, intrusion of salt in the source and in the mass analyzer can dramatically affect the performance of the mass spectrometer, because only a small quantity of salt produces signal suppression. Therefore, when signal is present on PAD but disappears on MS, it is an indication that desalting is no longer sufficient. The second advantage, as discussed in the next paragraphs, is that the MS and PAD sensitivity are complementary.

As we performed on line PAD and MS analyses, the first before and the second after desalting, we measured peak broadening, and separation efficiency. A 120 μ L void volume

was measured between the PAD and the ESI source with 30% peak broadening but without loss of efficiency. Since the flow rate of 0.3 mL/min is compatible with the ESI source, no split was necessary.



Scheme 3. Coupling scheme. The PAD is placed on-line with the MS, but before the desalting device to ensure the electrocatalytic process.

Analysis of acidic sugars opens up the possibility of using the positive or negative mode for MS detection. Theoretically, detection in the negative mode decreases the noise due to the increased specificity, and therefore increases the signal-to-noise ratio (S/N) and the sensitivity. The sugars we are looking for contain a carboxylic acid function and are easily ionisable in the negative mode. We studied the response factor of a commercial acidic oligosaccharide: 6'-sialyl-lactose; surprisingly, the TIC intensities were almost identical for ESI+ and ESI-. Only a sensitivity factor difference of two was measured at 5 µg/mL, and the positive ionization mode did not exhibit significantly more noise. In the positive mode, the presence of at most one sodium adduct was observed (as counter-ion of the acid). As both MS modes gave similar results, the behavior using MS/MS was investigated in both modes. However, collision energy is lower in the positive mode, which could be an advantage to obtain a better sensitivity, since high energy ions are difficult to refocus.

The detection sensitivity using HPAEC-PAD or HPAEC-ESI-Q-ToF MS is dependent on the nature of the sugar. The first fast oxidation step occurring on the gold electrode (PAD) involves the aldehyde of the carbohydrate, resulting in the formation of a carboxylic acid

and the production of two electrons. The second fast oxidation reaction is the cleavage of the C1-C2 bond, followed by conversion of C2 and C6 to the corresponding carboxylates, resulting in the production of 6 electrons (most efficient response) [72]. Therefore the predictive response factors are: Hex>6-desoxyHex>HexA>2-desoxyHex>ulosonic acids (Figure 3). For ESI-Q-ToF MS, the ionization occurs on the glycosidic linkage and is often facilitated through the presence of acid functions close to the ionization site, implying the most sensitive response for the ulosonic acids.

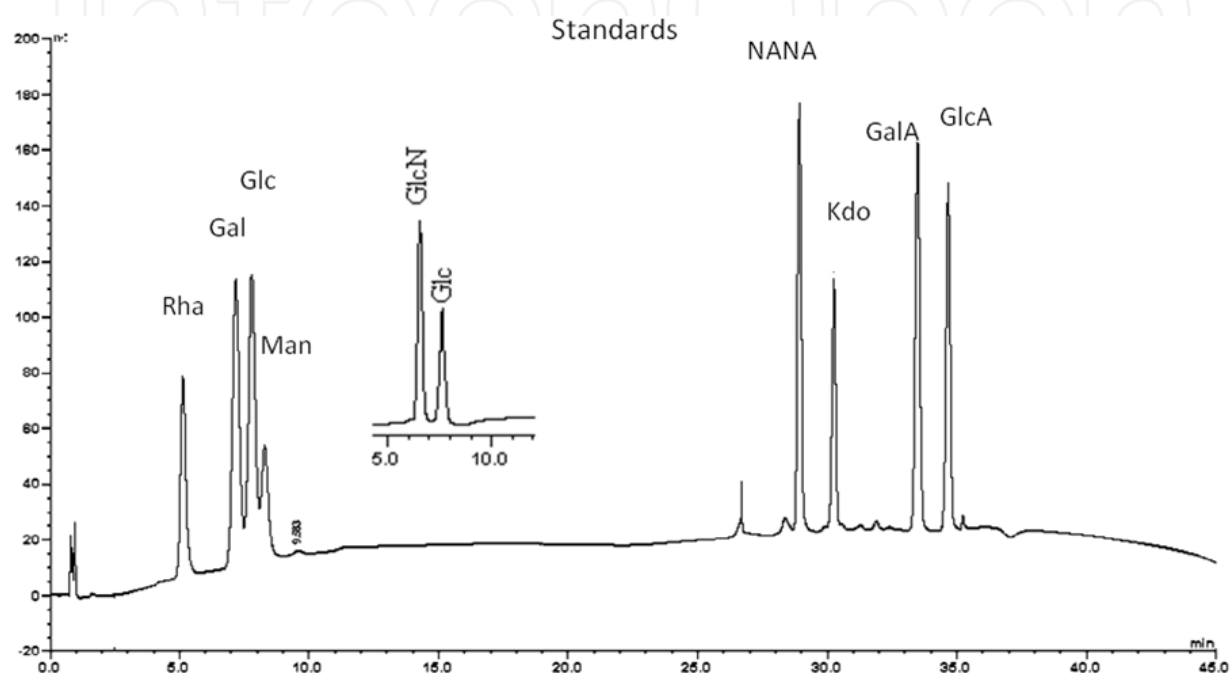


Figure 3. Chromatogram obtained by PAD detection of common sugars : Rhamnose (Rha), Galactose (Gal), Glucose (Glc), Mannose (Man), N-Acetyl Neuraminic acid (NANA), 2-Keto,3-deoxyoctulosonic acid (Kdo), galacturonic acid (GalA) and glucuronic acid (GlcA).

The concentration response of ESI-MS is often not linear and is very variable from one sugar to the other. For this reason, a quantification curve and the limit of detection (LOD) for each type of saccharide standard were measured for the five standards in solutions ranging from 200 to 2 µg/mL with an injection volume of 5 µl.

The HPAEC-ESI-Q-ToF MS response measurements surprisingly indicate that uronic acids respond weaker than expected, even less than hexoses. The LOD of GalA was not satisfying, unlike those obtained for all other saccharides.

2.3. FAB MS and FAB MS/MS of carbohydrates

The fast atom bombardment (FAB) ionization is less and less used due to the fact that the MS suppliers no more produce this type of sources. Nevertheless, this ionization process is of interest, because it is significantly more energetic than MALDI and ESI. In consequence, fragmentation can be observed in the ion source for natively charged glycans and be applied well to positive or ions. In the positive mode sodium cationized species are easily analyzed,

while in negative mode it is the deprotonated ions that are observed with greatest abundances for those containing acid residues. Permethyated glycans are very easily ionized but do not result in “*in-source*” fragmentations, collision induced dissociation spectrum in MS/MS can be used to obtain such fragmentation, the nomenclature of the different fragmentations was proposed by Domon and Costello [49]. FAB ionization requires quantities of native glycans above 10nmol, and 10 times less for permethylated species [73].

Using FAB-MS and -MS/MS linkage position can be determined like with ESI, as well as the position of sulfate or other substitutions on the polysaccharide [74]. The internal fragmentation of the monosaccharide cycles (A and X) are useful for such topic.

In the eighties, periodate oxidation was reintroduced to address sequence problem. Oligosaccharides can be sequentially oxidized with periodate and reduced with sodium borodeuteride and permethylated or peracetylated [75]. The bindings of monosaccharide residues can be determined on the basis of the sequences of the primary and secondary ions.

An original attempt of determination of the anomeric configuration was done using methyl glycopyranosides and $[M+Ag]^+$ ions. It was observed that the unimolecular decompositions of the ions showed dramatic changes related to the relative positions of both the hydroxyl group on C2 and the anomeric methoxyl group. When these groups were in the *cis* position, methanol was exclusively expelled. When they were in the *trans* position, silver hydride was preferentially lost [76].

2.4. CE-MS

2.4.1. Capillary electrophoresis and laser induced fluorescence.

Capillary electrophoresis, due to its high resolving power and sensitivity, has been useful in the analysis of intact and derived oligosaccharides and polysaccharides affording concentration and structural characterization data essential for understanding their biological functions. As most glycans do not have any strong chromophores on their structures and have low extinction coefficient they are difficult to detect using UV absorbance. Indirect UV has been used, it is based on using a chromophore in the electrolyte resulting in negative peaks but because the chromophore must have a mobility very near that of the sugars it is often difficult to ensure an adequate detection limit [77, 78]. Labelling with a chromophore is very useful, because Laser induced fluorescence (LIF) [79,80] can be used on monosaccharides as well as on polysaccharides. CE experiments are comparable to GC experiments to identify monosaccharides. Acetylation or silylation of alditol is replaced by an amino reduction step using an amino fluorescent dye and $NaBH_3CN$. Most of the time 1-aminopyrene-3,6,8-trisulfonate (APTS) is used for monosaccharide, their migration time allow the identification of the different derivatized alditols, it is very convenient for small quantities of natural polysaccharides and specially polysaccharides extracted from DOC PAGE. An acidic compound is more easily prepared than with classical alditol strategies for GC analysis [81].

For polysaccharides the same labeling reaction can be used and the different compounds can be easily separated. Recently, optimization of the boric acid concentration (which increases the charge of the polysaccharide by complexation) and linear polyacrylamide concentration in the buffer yielded a separation of 12 polysaccharides of very similar structures, most with baseline separation in less than six minutes [85]. Using this technique, high throughput glycosylation can be performed [82]. Thirty-two IgG N-glycans were analyzed using a combination of weak anion exchange enrichment and exoglycosidase digestion. Aminobenzamide and Aminopyrenyltrisulfonate labeling of polysaccharide followed by a UPLC on 1.7 μm HILIC or CE-LIF respectively have been compared. Combination of these data demonstrated that complete structural annotation is possible within a total analysis time of 20 min due to the advantageous orthogonality of the separation mechanisms [83]. This work confirms the use of glycosidase in CE-LIF studies for the determination of the structure of polysaccharides. Significant labeling improvements were made by replacing the conventionally used acetic acid catalyst for NaBH_3CN reduction with citric acid. Using a 1:10 glycan to fluorophore molar ratio resulted in a 95% derivatization yield at 55°C with only 50 min reaction time and negligible terminal sialic acid loss [84].

2.4.2. Capillary electrophoresis and mass spectrometry.

A large number of CE-MS and CE-MS/MS experiments were driven for polysaccharide structural determination and have been extensively reviewed recently by Y. Mechref [85]. Different interfaces between the CE-LIF instrument and MS are commercially available. Most works use APTS labeling and ESI ionization, although MALDI is also used but with lab-made instruments [86]. A very interesting study concerning polysaccharides from IgG was realized. Figure 4 presents a schema of the CE-MS system with on-line LIF detection adjusted 20 cm from the ESI tip. A sheath liquid consisting of a 50:50 isopropanol:water (0.2% ammonia) was added coaxially to the separation capillary at flow-rate 4 $\mu\text{L}/\text{min}$. CE was performed with PVA coated capillaries (60 cm total length \times 50 cm effective length \times 50 μm ID) and a running buffer of 40 mM *alpha*-aminocaproic acid, pH 4.5 (adjusted with acetic acid) + 0.02% hydroxypropylmethylcellulose (HPMC) with an applied voltage of -30 kV were used [87]. The inherent mass accuracy of the MS permitted the identification of major and minor glycans using their $(\text{M}-\text{H})^-$ ions. This study demonstrated for the first time the ability to attain CE-MS separation efficiency somewhat comparable to that commonly observed in CE-LIF analysis. As seen in figure 4 the four early migrating species are clearly visible in both the online LIF and MS electropherograms. The MS electropherograms suffer from a shift in migration time and some loss in separation efficiency, which is due to dead volumes originating from the addition of the MS detector.

Neutral markers to label acidic polysaccharide can be used. Their main advantage will be to allow the separation of the acidic polysaccharide following the number of acid residues [88], 2-aminoacridone was proposed in place of APTS. Nakano *et al* [89] used 9-fluorenylmethyl chloroformate (Fmoc-Cl), during the digestion of a glycoprotein by PNGase which can label the released 1-amino function of the first GlcNac residue. It prevents reductive amination.

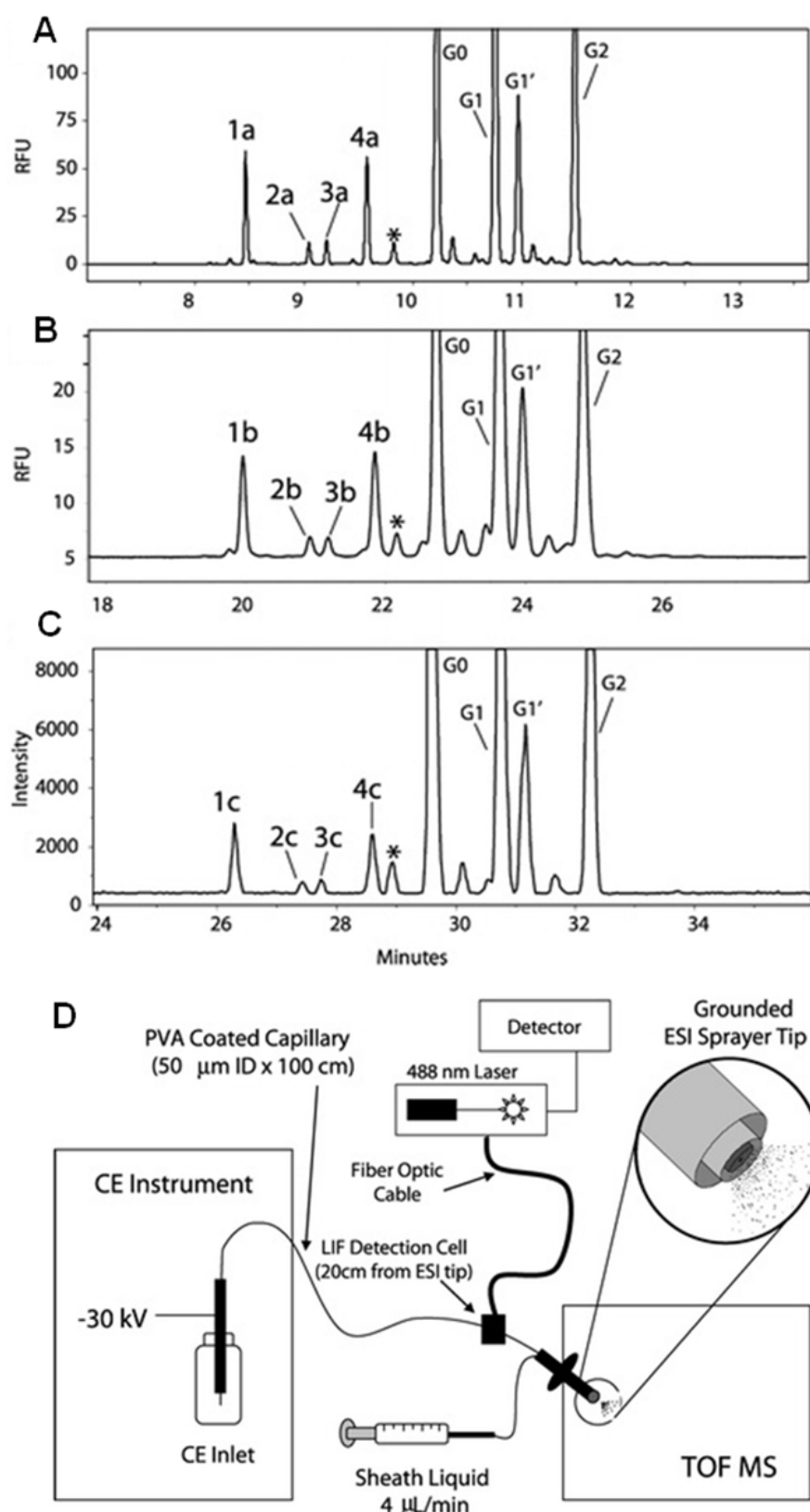


Figure 4. Expanded-scale electropherograms of rMab 1: A) standard CE-LIF electropherogram using a 60 cm capillary, B) CE-LIF trace obtained on-line with MS detection and C) CE-MS base peak electropherogram. D) Scheme of the CE-MS system with on-line LIF detection.

Using a very simple 20 mM phosphate buffer (pH 8.5), the authors separated the different polysaccharides following the number of sialic acids, then MS and MS/MS spectra identified the composition of each polysaccharide thanks to $(M+H)^{2+}$ or $(M+H)^{3+}$ ions. An example of the mass electropherogram and the different mass spectra are presented in figure 4 which concerns the glycans from bovine fetuin. In this study the authors showed that this method can be used to identify polysaccharides from glycoprotein extracted from an SDS PAGE separation.

3. Polysaccharides of *Sinorhizobium meliloti*

Polysaccharides are commonly found at the surface of Gram negative bacteria (Figure 5). The aim of our work is to elucidate the structure of the polysaccharides from the surface of bacteria. Rhizobia are Gram negative bacteria living in soil and able to establish a symbiotic interaction with leguminous plants, known as nitrogen fixing symbiosis [90]. During this mutual interaction, bacteria bring combined nitrogen, in the form of ammonia directly transformed from atmospheric N_2 , to the plant. In turn the plant provides hydrocarbons and develops new organs on its roots which host the bacteria: the nodules [91,92]. During the early stages of establishing symbiosis, a molecular dialogue takes place. First, the partners in the soil are recognized. The plant exudes flavonoid compounds and the neighboring rhizobia respond by secreting Nod Factors (lipochitooligomers) [93]. Nod factors play a major role during the physical contact between the bacteria and the root hairs, and also trigger the organogenesis of the nodule [94]. Then the bacterial threads can invade the root to colonize the nodule. At this stage, the rhizobial surface polysaccharides are essential [95,96,97,98]. To enlarge our knowledge about their role, it is necessary to determine their

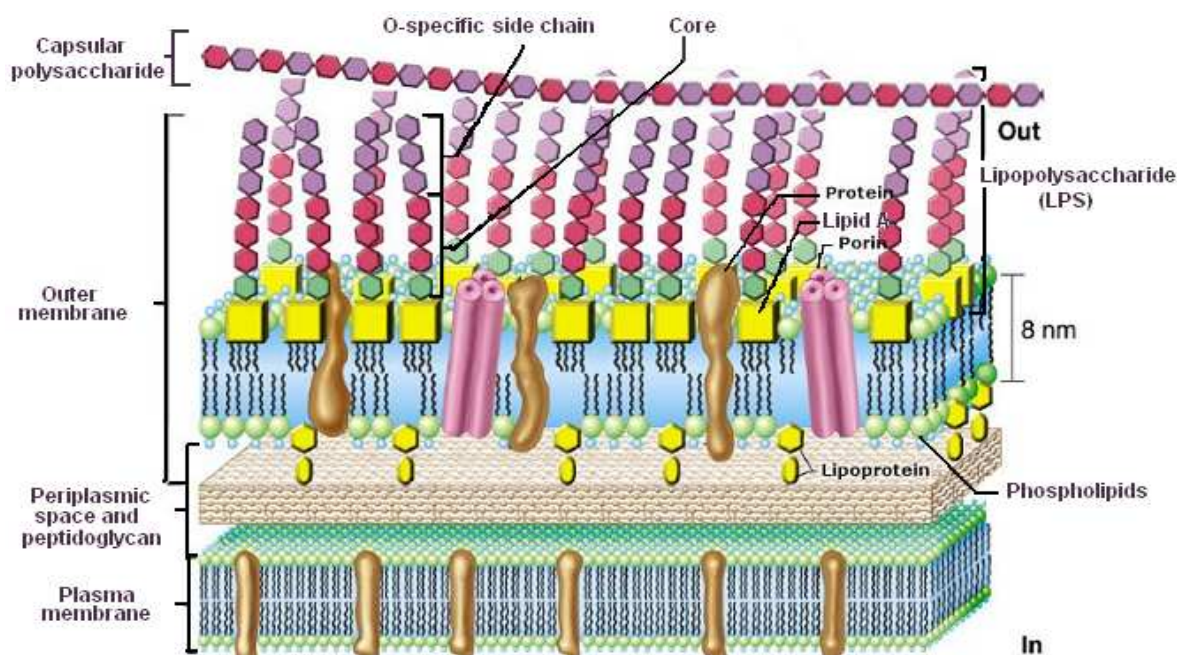


Figure 5. Scheme of the surface of Gram negative bacteria. The EPSs are not represented here; they are over the capsular polysaccharides.

structure. Their structural characterizations will be described below. They generally consist in analysis of composition by Gas Chromatography coupled with Mass Spectrometry (GC-MS), and sequence analysis made principally by Mass Spectrometry (MS). The choice of the MS coupling, mode or instrument depends on the nature of the polysaccharide (size and composition) as well as on the type of information we want to accede.

Sinorhizobium meliloti –the European model of rhizobia - has an external surface where 3 types of polysaccharides can be observed: exopolysaccharide (EPS), capsular polysaccharide (CPS) and lipopolysaccharide (LPS). Each class of PS have to be investigated alone, because their physico-chemical properties do not allow their simultaneous detection.

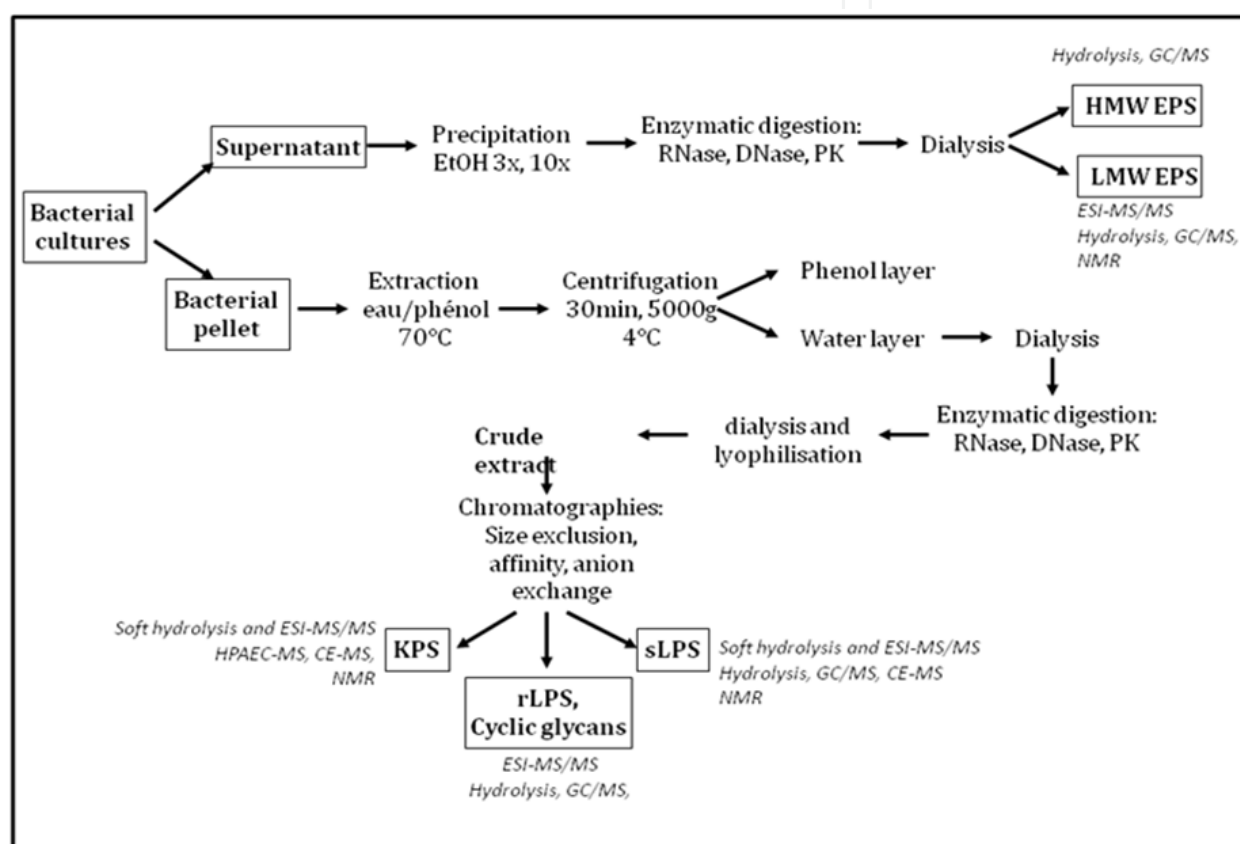


Figure 6. Isolation protocol for the different polysaccharides. In italic : characterization methods.

3.1. Exopolysaccharides of *Sinorhizobium meliloti*

3.1.1. Isolation of the EPS

The EPS are generally composed of many repetitions of 8 to 12-mers of hexose-subunits in a linear and/or branched form. This can be more or less substituted by O-acetyl, succinyl and pyruvyl groups. Their composition and structure is species specific and depends on the growth conditions [99]. The EPS are produced by the bacteria during the stationary phase of growth [99].

When the EPS of *S. meliloti* were studied, the cultures were stopped 5 hours after the stationary phase, by centrifugation. The supernatant, containing the EPS, was isolated and

lyophilized. When concentrated enough (increased viscosity but well dissolved), the EPS were precipitated, first with 3 volumes of ethanol. The pellet so formed was centrifuged and kept, the supernatant was concentrated. The pellet constitutes the high molecular weight (HMW) EPS fraction. The EPS left in the treated supernatant were then precipitated with 10 volumes of ethanol, the pellet was centrifuged and kept. This is the low molecular weight (LMW) EPS fraction. Each fraction was resuspended in water and subjected to proteinase K digestion (3.5 g/L at final concentration) for 4h at 36°C. The mixtures were then dialyzed against water. The pellets thus obtained were lyophilized, and once dried, used for structural analysis determination.

3.1.2. Characterization of the EPS

To learn about the monosaccharide composition, the polysaccharides must be first hydrolyzed. This chemical reaction is carried out in acidic conditions. A solution of EPS in water was acidified by 98% TFA to a final concentration of 10%, and the mixture was kept at 100°C for 2h. At the end of the treatment, the leaving acid has to be evaporated. To help this elimination in a N₂ stream, repetitive additions of isopropanol are necessary.

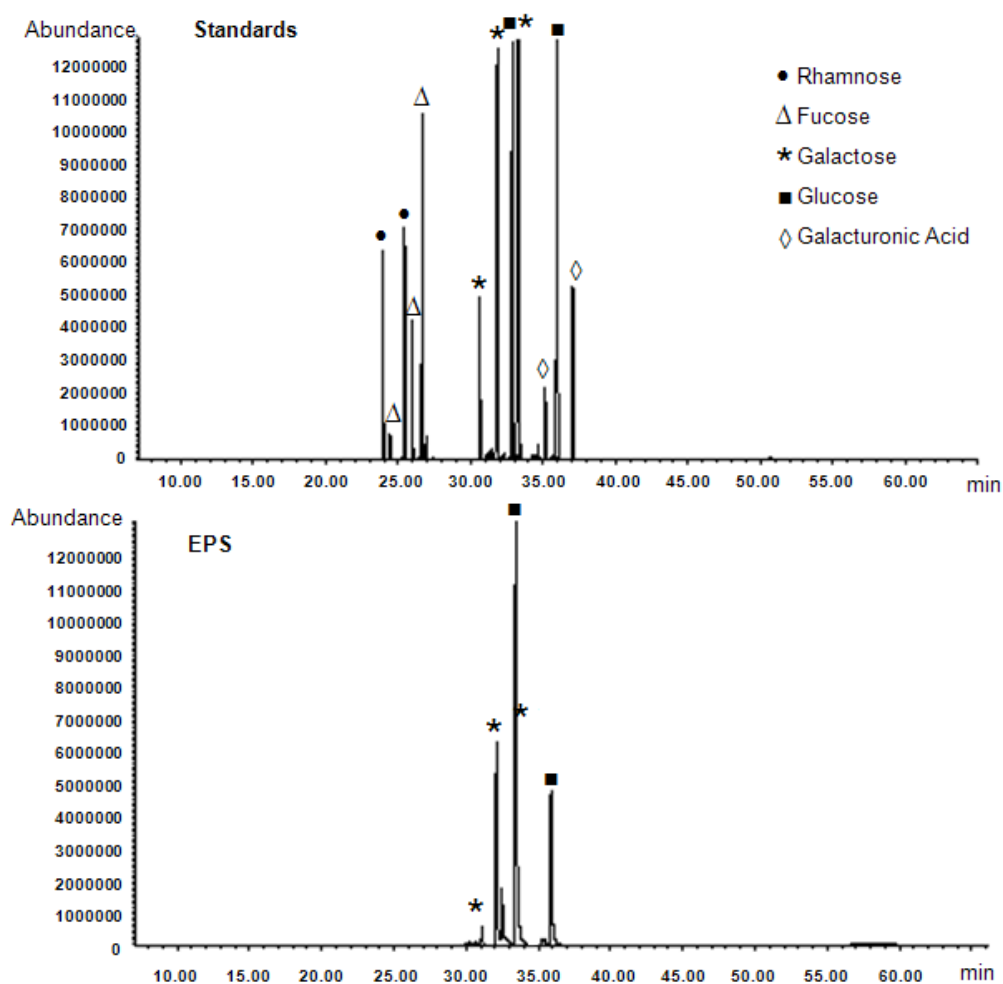


Figure 7. Chromatograms obtained by GC-MS for silylated standard saccharides and silylated hydrolyzed EPS LMW of *Sinorhizobium meliloti* 1021. EPS is made of Glucose and Galactose.

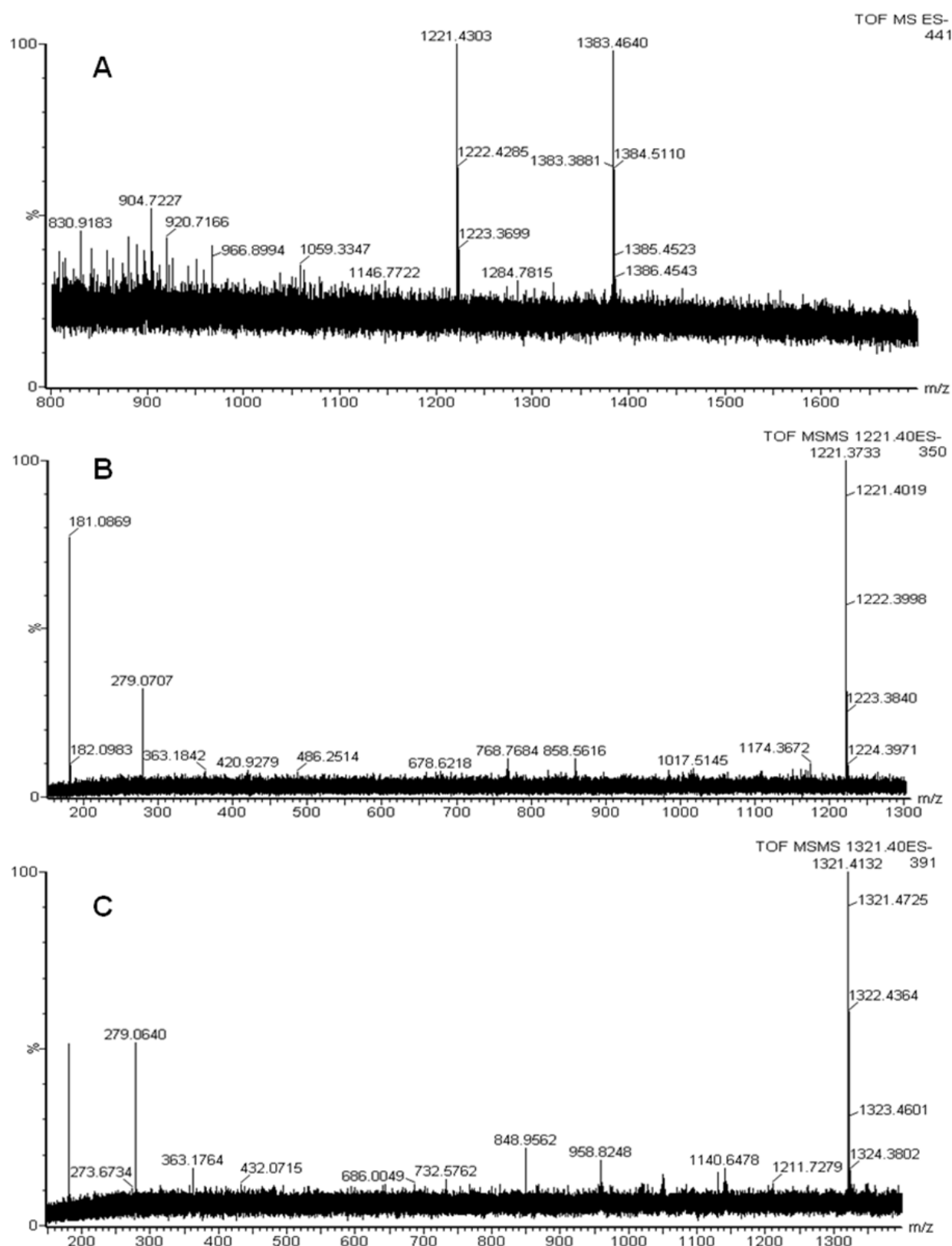


Figure 8. MS analyses of the LMW EPS of *Sinorhizobium meliloti* 1021A) ESI-MS analysis in negative mode B) ESI-MS/MS of ion m/z 1221.4 (the simplest form) amu. C) MS/MS of ion m/z 1321.4 amu.(the succinylated form), m/z 1383 corresponding to the succinylated and acetylated species.

After hydrolysis, the polysaccharides result in a mixture of monosaccharides and can be analyzed by GC-MS after derivatization. In the results presented here, we used the silylation of the sugar hydroxyl groups, which is a facile route. Actually, the protocol is simple, and a large databank is available for MS interpretations. In anhydrous pyridine, a mixture of HMDS and TMCS was added to the hydrolyzed EPS. This reaction was held for 30 min at 70°C [100]. After complete evaporation, the derivatized monosaccharides were injected directly as a hexane solution. The vector gas used was helium and the column was 95% dimethylsiloxane and 5% diphenylsiloxane. The temperature gradient was: 70°C hold for 3 min, increase at 5°C/min to 140°C and at 3°C/min to 240°C, then reach 300°C at 10°C/min, and hold at 300°C for 10 min.

This procedure applied to standard monosaccharides (glucose, galactose, mannose, glucuronic acid and galacturonic acid) allows interpretation of the chromatograms, determination of each sugar pattern (depending on alpha, beta, pyranoside, furanoside or lactone configuration) and the response factor for the different carbohydrates (Figure 7). The monosaccharide composition of the repeated units of LMW and HMW EPSs of *S. meliloti* was thus obtained.

The preparation of a simple solution of intact EPS in acidic methanol allows its ESI-MS analysis. This kind of analysis shows the purity of the EPS and the disparity of the sugars (Figure. 8A). Actually, the polysaccharide diversity is due to a variable degree of polymerization (DP) and to non-carbohydrate substituents. Here, the substituent is probably a succinyl group, adding 100 amu to ion m/z 1221.4 amu to yield the ion m/z 1321.4 amu. The ESI-MS/MS analysis of the ions found in the MS spectrum allows the sequence to be assessed by studying the fragmentation ions (Figure 8 B and C).

3.2. Lipopolysaccharides of *Sinorhizobium meliloti*

LPS have three structural domains: the lipid A, the oligosaccharide core and the O-antigen polysaccharide. The LMW LPS, named rough LPS are composed of lipid A and core polysaccharides, whereas the HMW LPS (smooth LPS) are made of the 3 associated parts [101].

3.2.1. Isolation of the LPS

As LPS are anchored in the outer membrane by lipid A, after growth of *S. meliloti* to the stationary phase, it was necessary to keep the pellet of centrifuged cultures (1L, grown until OD (600nm) 1.5). The pellet resuspended in about 40 mL of water was extracted by phenol at 60°C for 1h [102]. The extraction mixture was centrifuged and the upper phase (aqueous) contained the LPS, and all the other hydrosoluble molecules (DNAs, RNAs, proteins, carbohydrates). Enzymatic digestions were performed to eliminate DNAs, RNAs and proteins, remaining the carbohydrates. Affinity chromatography allowed the isolation of LPS. Then, gel filtration separated the LMW and HMW LPS (respectively rLPS and sLPS).

3.2.2. Characterization of the LPS

In this work, it was the HMW LPS that were studied, so both types of structure had to be determined, the saccharide parts and the lipid A. Soft hydrolysis, in 1% acetic acid at 100°C for 1h, isolated lipid A, separating out of the aqueous solution. A liquid-liquid extraction, with CHCl₃:CH₃OH (3:1), separated the lipidic part from the sLPS.

Structural analysis of lipid A was performed by ESI-MS. The deconvoluted spectrum of doubly-charged ions revealed two series of monocharged ions, with a mass resolution that allowed the identification of the monoisotopic molecular masses (and not only the average molecular masses), i.e.: m/z 2050.41, 2038.39/2036.3, 2022.41, 2010.39 for one series and 1950.41, 1938.39/1936.3, 1922.37, 1910.37 for the second series with sufficient accuracy to allow elemental composition analysis (Figure 9, note that the spectrum presented is not deconvoluted). Accurate mass analysis combined with GC-MS data led to the proposal of a first general structure for lipid A : a di-phosphorylated penta-acylated diglucosamine comprising two 3OH-C14 fatty acids, one 3OH-C18, one 3OH-C19:1 and C27OH-C28 fatty acids for a compound giving a singly charged molecular ion of m/z 1950.4 amu (Figure 10)

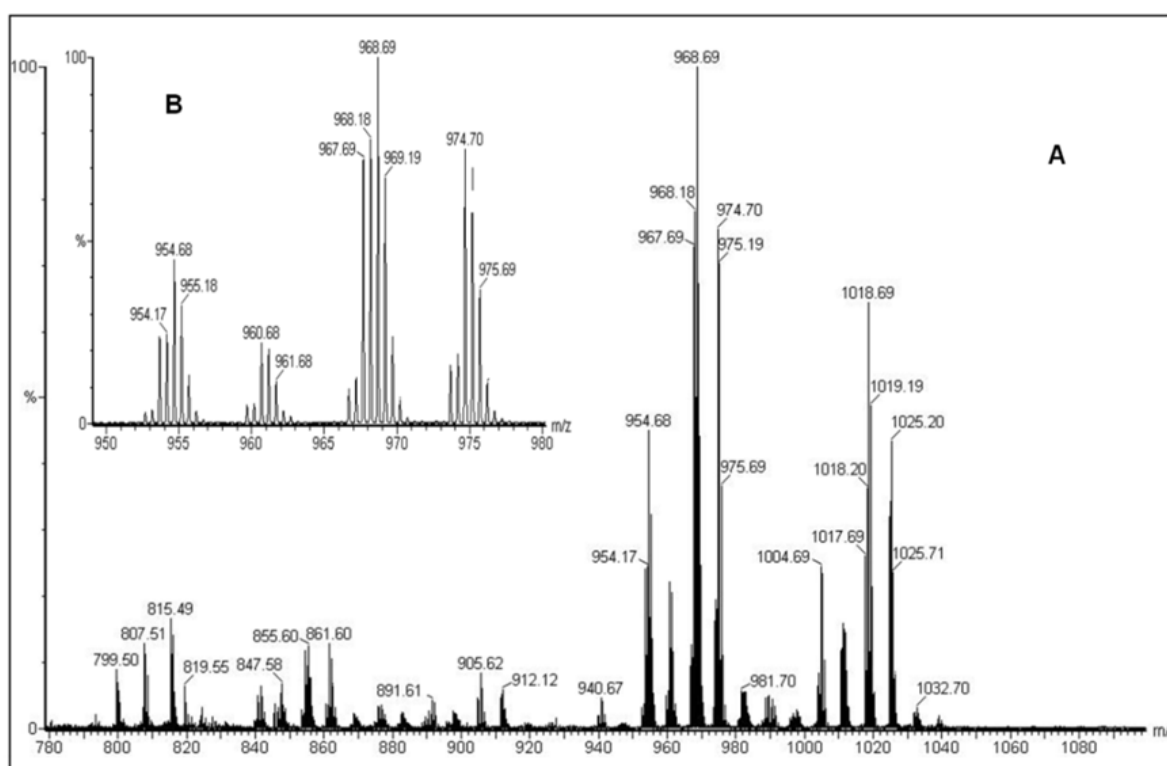


Figure 9. A) Direct ESI-MS spectrum of LPS lipid anchor. Ions ranging from 954 to 976 are assumed to be molecular species and ions observed between m/z 1004 and 1026 are native methoxybutyrate derivatives. Insert B) high-resolution spectrum indicating the double charged state of lipidA.

The difference of 100 amu (50 amu in spectrum shown figure 9) can be attributed to the presence of a 3-methoxybutyrate in the first series. Actually, as the second lipid A distribution was hypothesized to be 3-O-methoxy-butyrate substitution of the 27OH-C28, exact mass measurements were performed. In the mean mass difference found between the

respective members of the two distributions was 100.03 amu, corresponding with high significance to a $C_5O_2H_8$. To confirm this hypothesis, MS/MS analyses of the double charged ion at m/z 1018.2 (Figure 11) have been performed. At low collision energy (15V) m/z 1018.2 generated three major fragments, respectively m/z 2004.4, 1992.4 and 1936.3 amu. M/z 2004 corresponds to the usual neutral loss of methanol (-32 amu), 1992 could be interpreted as a neutral loss of CO_2 (44 amu) after rearrangement. Finally m/z 1936 corresponds to the ketene loss of the 3-methoxy butyrate. Increased collision energy (up to 40V) resulted in the production of the same fragments as observed directly from the parent ion that is not substituted by a 3-O-methoxy-butyrate (m/z 1935.4). The MS analyses of the complete LPS provided, by comparison to lipid A, the mass of the saccharide part.

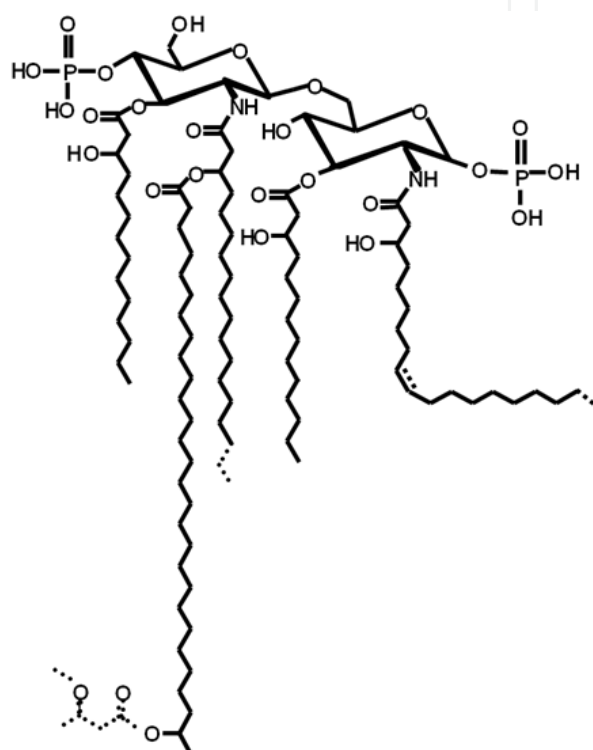


Figure 10. General Lipid A structure of *Sinorhizobium meliloti* 1021's LPS. The dotted lines correspond to the substituent variation found in the sample.

Composition analysis of the saccharide part of the sLPS was performed as for the structural characterization of EPSs. After hydrolysis of the polysaccharide, the monosaccharides obtained were derivatized into alditol acetates, because the O-antigen usually exhibits great sugar diversity.

The process consisted of a reduction by sodium borohydride in ammonia for 1h at room temperature, followed by washing with acetic acid, and an acetic acid in methanol solution [103]. A supplementary wash was made in methanol only, before acetylation. After adding acetic anhydride and pyridine, the reaction stood for 1h at 70°C [104]. Liquid-liquid extraction in water against dichloromethane allowed the isolation of the so-formed alditol acetates in the organic phase. The dried mixture of derivatized monosaccharide was directly analyzed by GC-MS. The temperature gradient started at 110°C and increased at 3°C/min to

reach 300°C. Of course, the same process was performed on standard monosaccharides to establish a short database, helpful for interpreting the chromatogram (Figure 12).

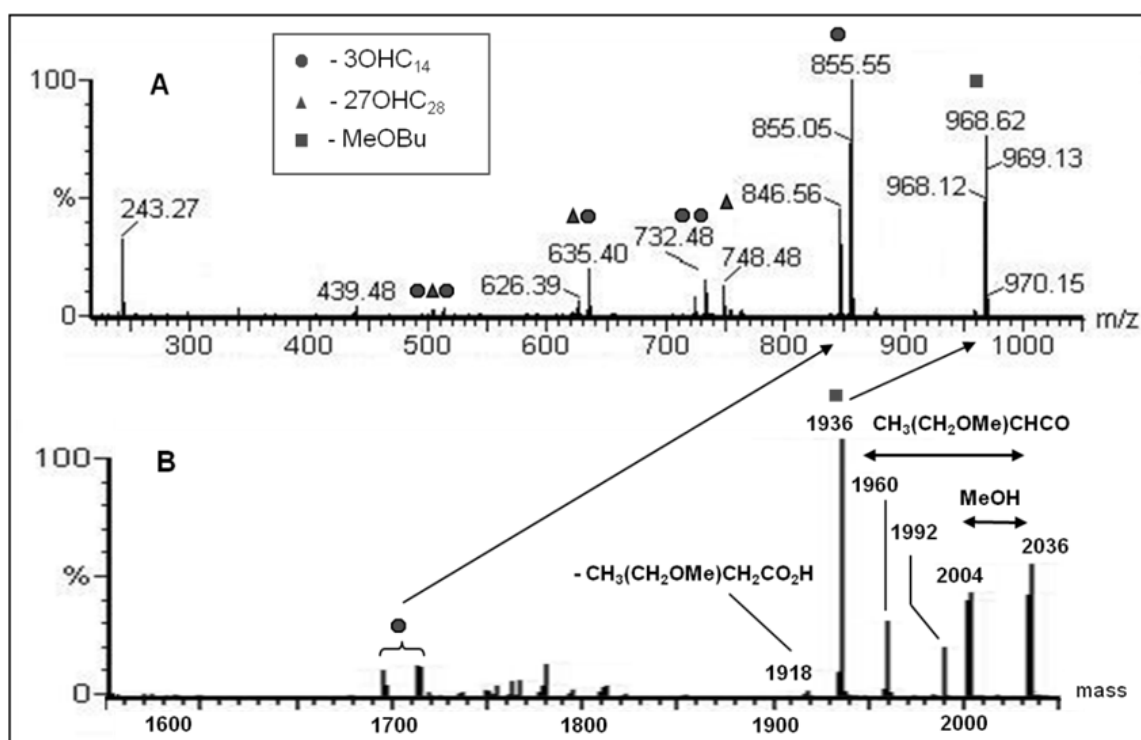


Figure 11. A) CID MS/MS high energy (Ecoll 40V) fragmentation of ion at m/z 1018 results principally in m/z 968 (loss of methoxybutyrate : -MeOBu) double charged species. B) deconvoluted spectrum indicating a characteristic methoxybutyrate fragmentation at lower collision energy (18V).

3.3. Capsular polysaccharides of *Sinorhizobium meliloti*

In rhizobia, capsular polysaccharides are generally composed of a dimer repeating unit, composed of a hexose and a 1-carboxy-2-keto-3-deoxysugar, like Kdo (2-keto-3-deoxy octulosonic acid). Such a structure looks like the K-antigen found in *E. coli*, and was therefore named Kdo-rich capsular polysaccharide (KPS) for *Rhizobia* [105,106].

3.3.1. Isolation of the K type CPS (KPS)

To isolate KPS from bacterial surface molecules, the same protocol as for the isolation of LPS was executed. Surprisingly, the *S. meliloti* 1021 KPSs were also retained by affinity chromatography. Gel filtration allowed enrichment in KPS, but their size is too close to the size of LPS, to be isolated from each other.

3.3.2. Characterization of the EPS

The analysis of carboxy-sugars such as Kdo is extremely difficult with GC-MS, due to their molecular weight and their instability. So, the composition of the KPS of *S. meliloti* was determined by GC-MS and mainly by HPAEC-PAD-MS. The latter technique requires an

HPLC system, an anion exchange column and a desalter, which is required to couple the ion chromatograph with the MS (see part II.2.c). The device is detailed above, in the methodological part. Analysis of sugars with HPAEC needs no derivatization, better for the stability of Kdo. PAD detection is not sufficient to precisely determine the composition of the mixture analyzed, so the system is coupled to a mass spectrometer. The combination of HPAEC-PAD and MS is a challenge, because of the quantity of salt used in HPAEC-PAD, hence the presence of the desalter is essential. The challenge is rewarded by the information provided by this coupling for determining the saccharidic composition, with simple and non-destructive sample preparation.

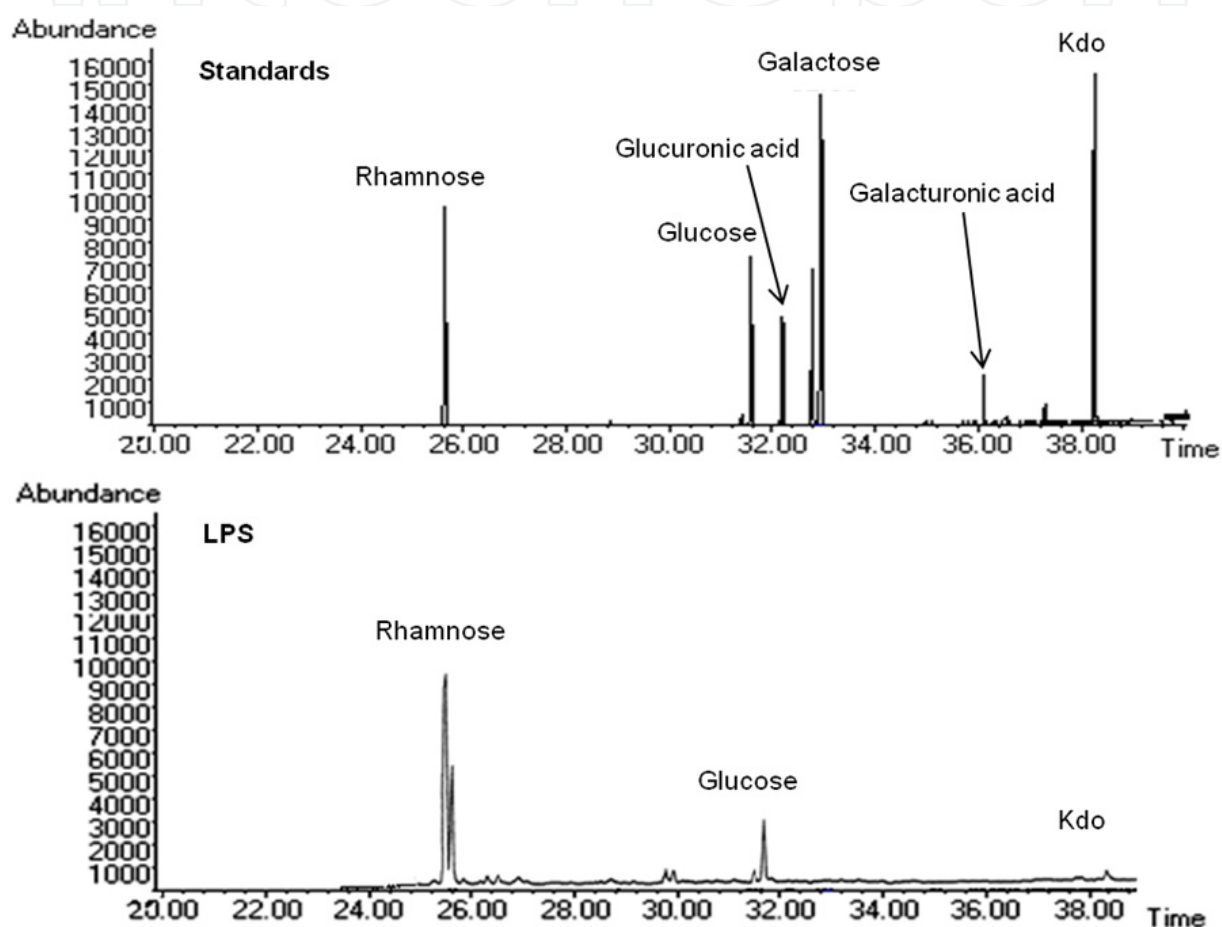


Figure 12. Chromatograms obtained by GC-MS for alditol acetates of standard saccharides and alditol acetates of hydrolyzed LPS of *Sinorhizobium meliloti* 1021. LPS is made of Rhamnose, Glucose and Kdo.

For GC-MS analysis, as the acidic sugar content was high, derivatization with heptafluorobutyric anhydride (HFBA) was necessary [107]. The methanolized polysaccharides were dried and resuspended in anhydrous acetonitrile, a solution of HFBA was added. The reaction was heated to 60°C for 30 min. Evaporation of the leaving HFBA involved several co-evaporations with anhydrous acetonitrile. The final solution was made up in anhydrous acetonitrile and injected directly into the GC-MS. The temperature gradient was 70°C for 3 min, 5°C/min to 100°C, 3°C/min to 240°C and 5°C/min to reach 300°C and hold for 10 min. Figure 13 indicates that the KPS enriched fraction is exclusively composed of Kdo.

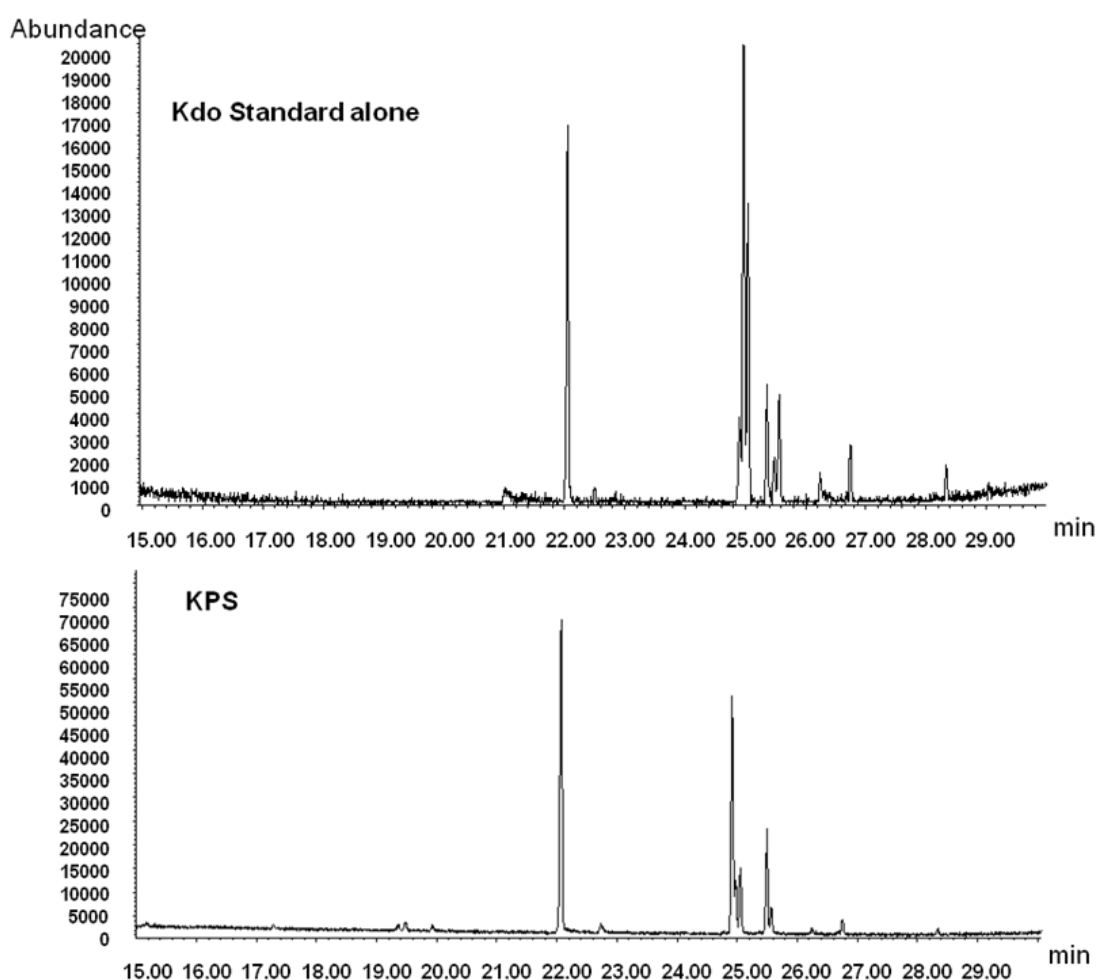


Figure 13. Chromatograms obtained by GC-MS for Kdo standard and methanolyzed KPS of *S. meliloti* 1021, both derivatized by HFBA. KPS is only composed of Kdo.

Unlike GC-MS analyses, in HPAEC-PAD-MS analyses, only soft hydrolysis of the polysaccharide mixture from the size exclusion chromatography (SEC) fractions containing KPS was implemented (1% acetic acid, 100°C, 1h). During the soft hydrolysis, a pellet appeared, indicating the presence of fatty acid on the KPS. MS analysis described below will detail this. The HPAEC-PAD-MS analysis revealed free Kdo and oligo-Kdo, characterized in MS by a mass difference of m/z 220 amu, when ion m/z 221 amu is extracted (Figure 14). The other compounds present in this sample are mostly composed of hexose dimers (m/z 324 amu), revealed when ion m/z 325 amu is extracted. So, the HPAEC-PAD chromatogram allows simultaneous analysis of other polysaccharides in this mixture: glycans, and substituted glycans [72].

A MS analysis confirmed that KPS is a homopolymer of Kdo. Actually, direct ESI-MS in negative mode (Figure 15 A) indicated the presence of many charged compounds. Zooming between m/z 1020 and m/z 1060, multi-charged ions (e.g.: 1035.30 to 1036.05 are quadri-charged ions and 1028.54 to 1029.04 are discharged ions) clearly appear (Figure 15 B). A deconvolution process recovers the native mass of the compounds (Figure 16 A). This reveals a mass difference of 220 amu between the members of the series, typically

corresponding to Kdo. The MS/MS analysis of one of these polymers confirms that it is exclusively composed of Kdo, but also revealed the presence of an anchor explaining the discontinuity between Kdo₁₅ and Kdo₁₇ (Figure 16 B).

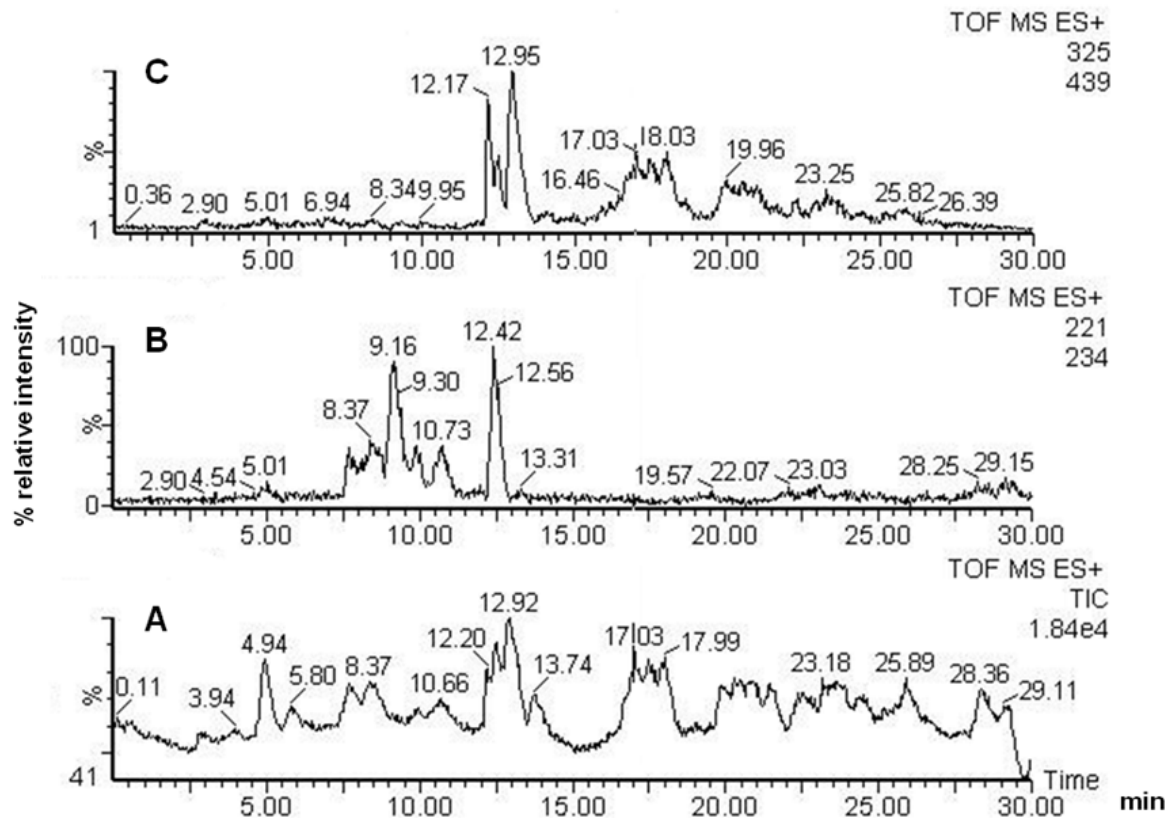


Figure 14. Chromatograms obtained by HPAEC-PAD-MS. A) Total ion current measured by MS. B) Chromatogram obtained when ion m/z 221 is extracted. C) Chromatogram obtained when ion m/z 325 is extracted. These chromatograms revealed that the KPS fraction is composed of polymers of Kdo and of glycans.

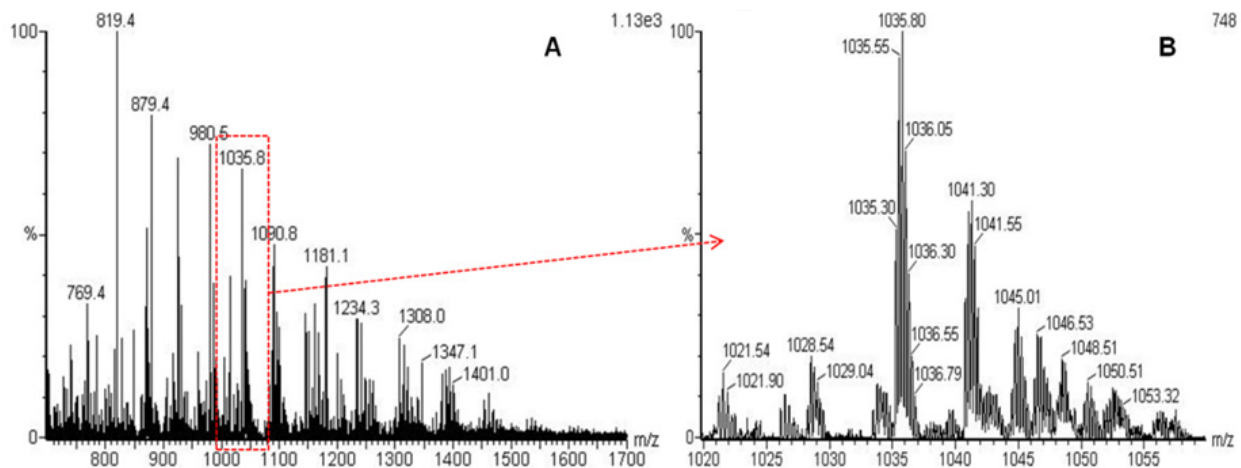


Figure 15. A) ESI-MS spectrum of KPS of *S. meliloti* 1021. B) Zoom on a group of ions revealing the multi-charged ions.

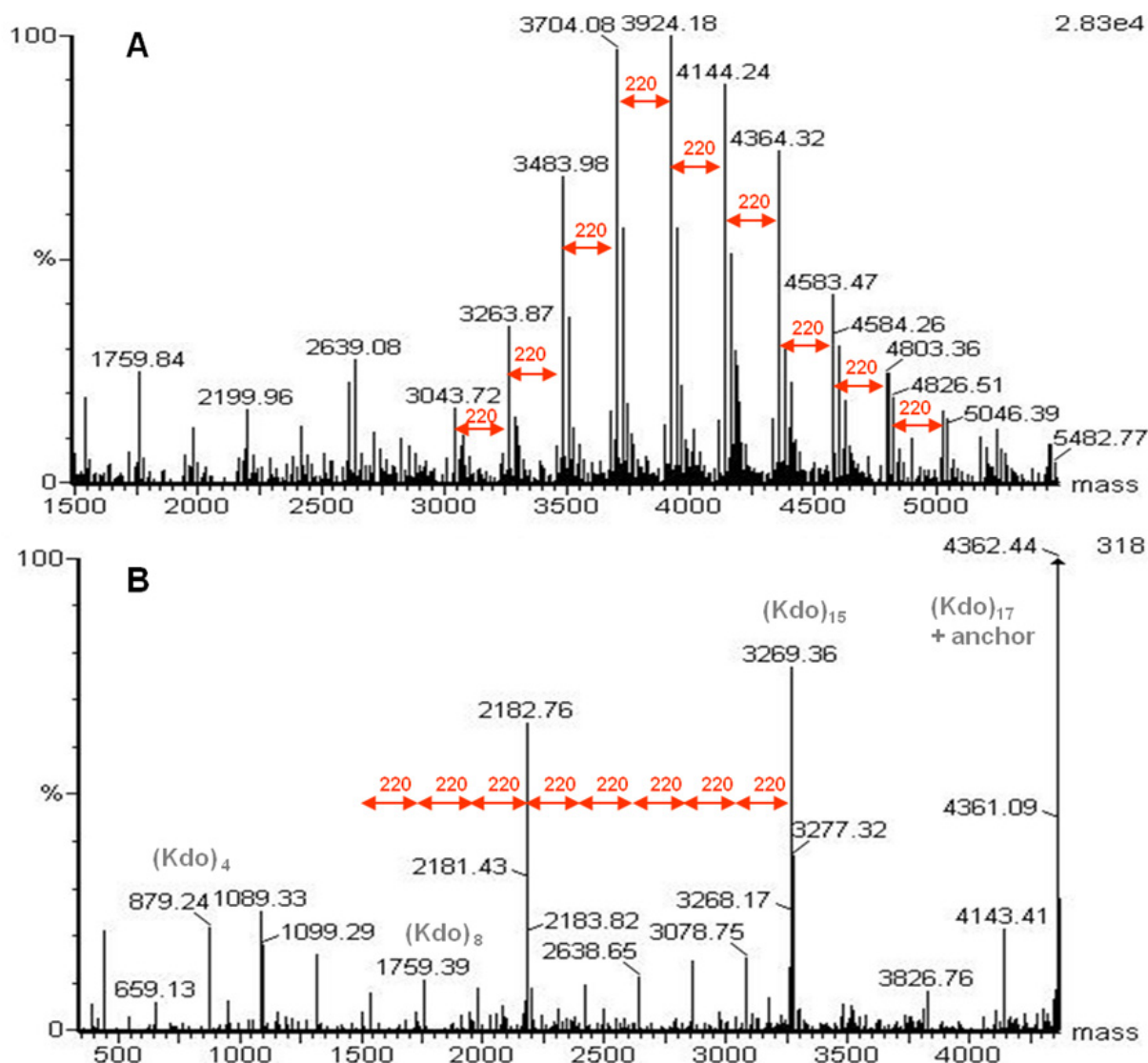


Figure 16. A) deconvoluted spectrum of KPS of *S.mmeiloti* 1021. B) MS/MS analysis of one Kdo-polymer, revealing an anchor on the KPS.

A MS/MS analysis of the smallest Kdo-oligomer revealed the mass of the anchor at m/z 622.4 amu. This ion has been studied separately by MS/MS, after mild hydrolysis on the KPS fraction (Figure 17). Ions m/z 530 corresponds to the loss of the glycerol and 548 to the loss of glycerol minus one water molecule. The interpretation of the spectra, in negative and in positive mode, determined that the anchor was lipidic and composed of a glycerol and phosphoglycerol unit, leading to the structure detailed in figure 18.

For structural characterization of polysaccharides, classical techniques are mostly used. Here, we reported results obtained with chromatography coupled to MS, MS alone and MS/MS. Depending on the characteristics, the type of polysaccharide analyzed, and which data are sought from those samples, it is necessary to adapt the coupled techniques. Advanced techniques, like NMR, are increasingly used for precise characterization. But NMR spectra of polysaccharides are very complex, therefore not easy to interpret. Actually, the heterogeneity in substitution and size is principally the origin of this complexity, especially in 2D NMR.

Moreover, because of the interpretation difficulties, detailed mapping of NMR spectra of polysaccharides in the literature are seldom found. Lastly, as detailed in part I, note that polysaccharide solubility problems can lead to poorly resolved NMR spectra. While NMR can provide useful information about the polysaccharide, MS analysis is not obsolete, because the information provided with MS is unambiguous and confirms NMR spectrum interpretation. In many examples, MS analysis is necessary for an easier interpretation of the NMR spectra. Bacterial polysaccharides are highly complex molecules and many variations occur in one family. The role of polysaccharides from the rhizobia family during nitrogen fixing symbiosis has been demonstrated, as well as the activity of other bacterial polysaccharides during pathogen infection. However, little is known about their structure/activity relationships [108], which implies a long life for polysaccharide structural characterization.

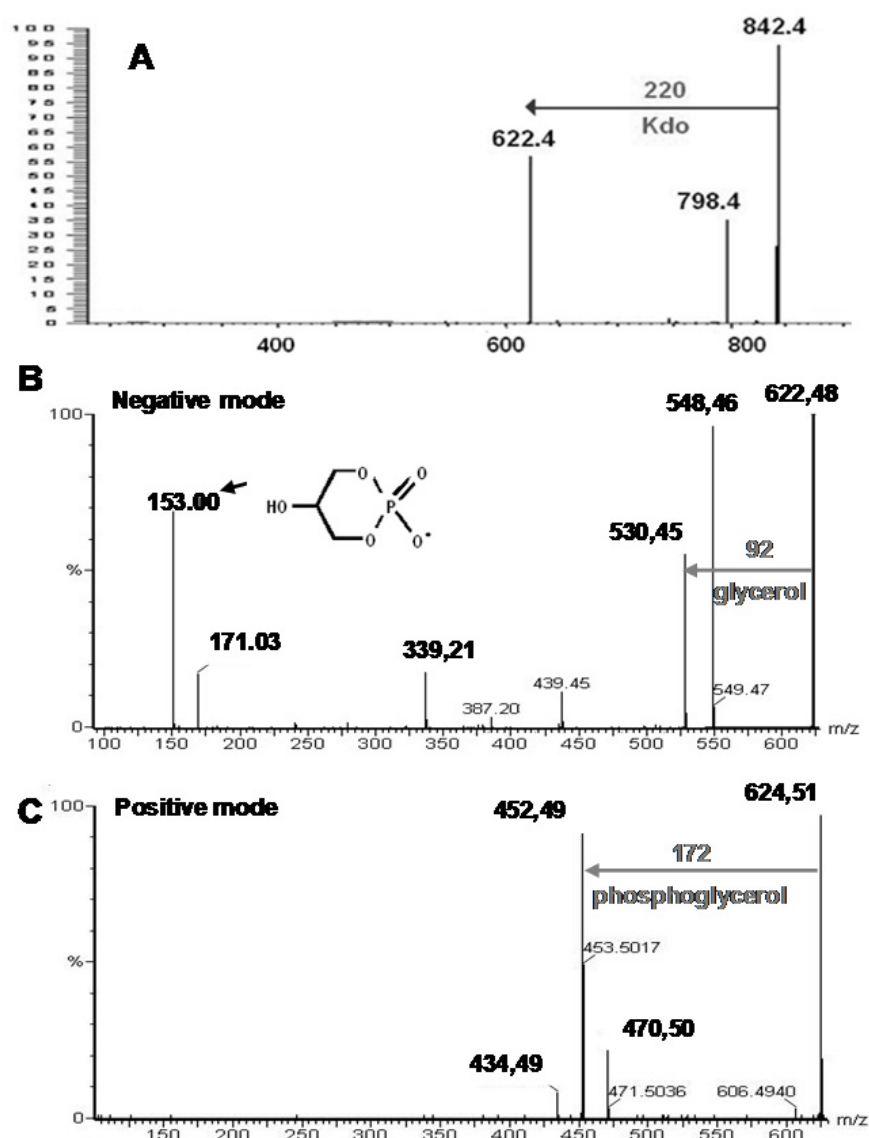


Figure 17. Top, MS/MS analysis of the smallest compound containing Kdo in the KPS, revealing the anchor at m/z 622 amu (A). MS/MS Fragmentation of the lipidic anchor (ion m/z 622 amu) obtained after soft hydrolysis in negative (B) and positive (C) mode. The presence of glycerol and phosphoglycerol in this lipid anchor was thus demonstrated.

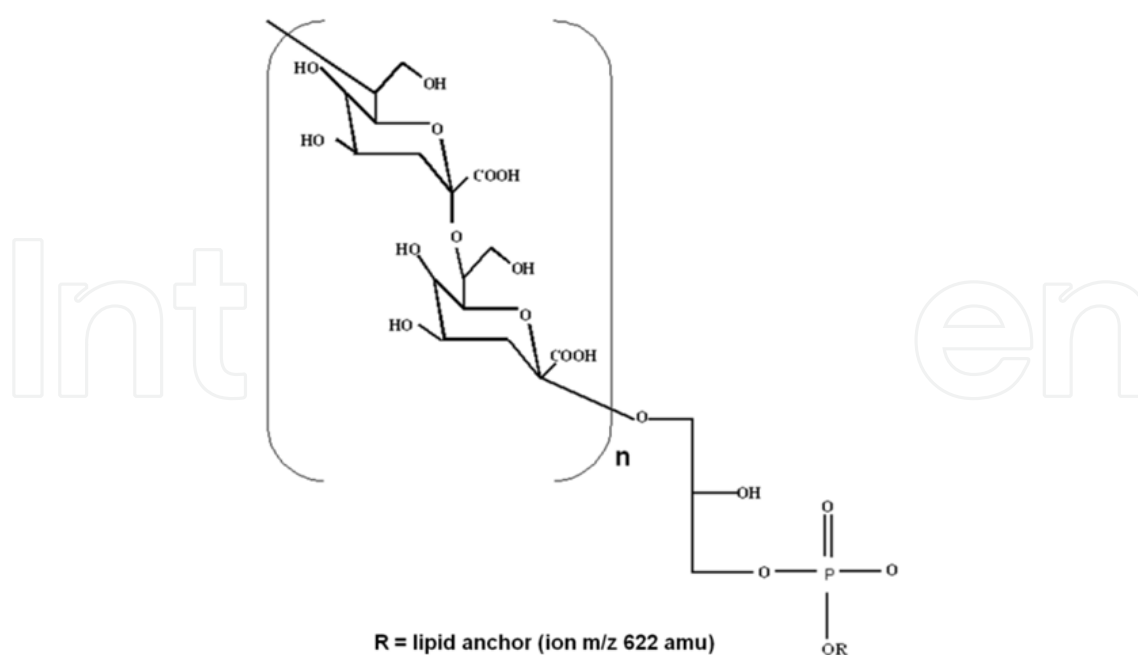


Figure 18. General structure of KPS of *S. meliloti* 1021

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