

# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900

Open access books available

185,000

International authors and editors

200M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index  
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?  
Contact [book.department@intechopen.com](mailto:book.department@intechopen.com)

Numbers displayed above are based on latest data collected.  
For more information visit [www.intechopen.com](http://www.intechopen.com)



# Mass Spectrometry Strategies for Structural Analysis of Carbohydrates and Glycoconjugates

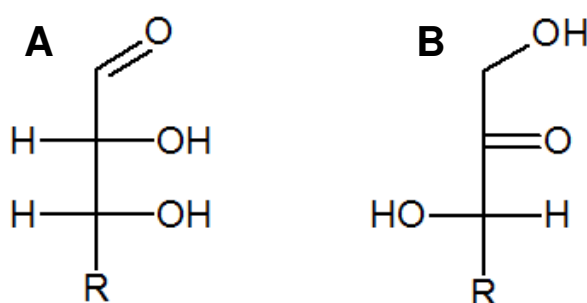
Guilherme L. Sassaki and Lauro Mera de Souza

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/55221>

## 1. Introduction

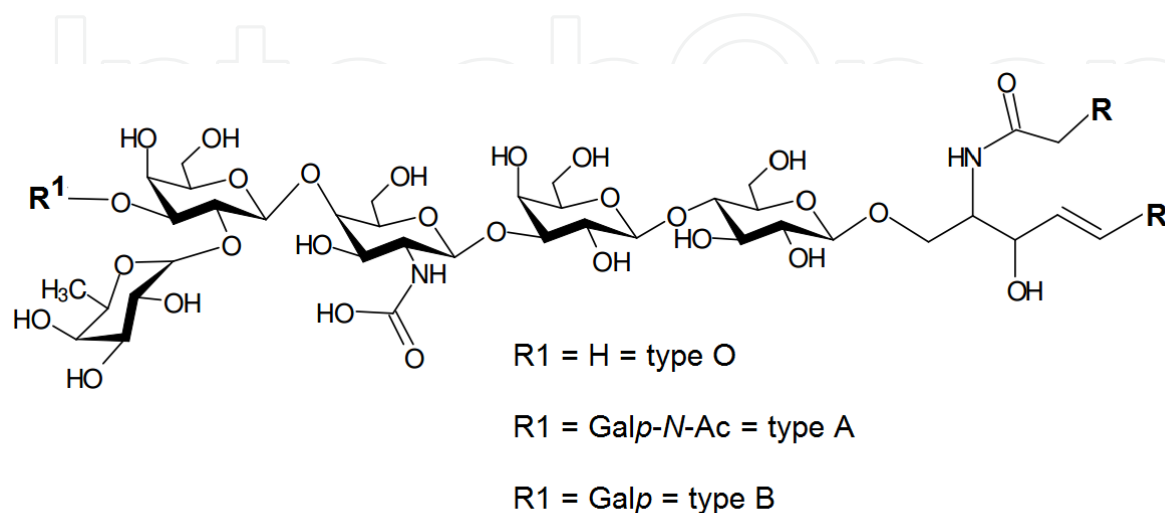
Carbohydrates are compounds rich in hydroxyl groups, being a monosaccharide a building block for complex carbohydrates. Beside the large amounts of hydroxyl groups, two chemical functions define the organic class, and even the simplest carbohydrate must contain either an aldehyde (polyhydroxyaldehyde) or ketone (polyhydroxyketone) functions (Fig. 1).



**Figure 1.** Functional groups defining carbohydrates (A) aldose (B) ketose

Carbohydrates can be naturally found as free monomers named monosaccharides (e.g. glucose, fructose), linked itself forming oligosaccharides such as the disaccharides sucrose and lactose, or larger structures containing hundreds of monosaccharides, the polysaccharides. Carbohydrates are the most abundant biomolecules worldwide, since they are found as structural matrix of plants (cellulose) and invertebrates (chitin). Other biological roles of

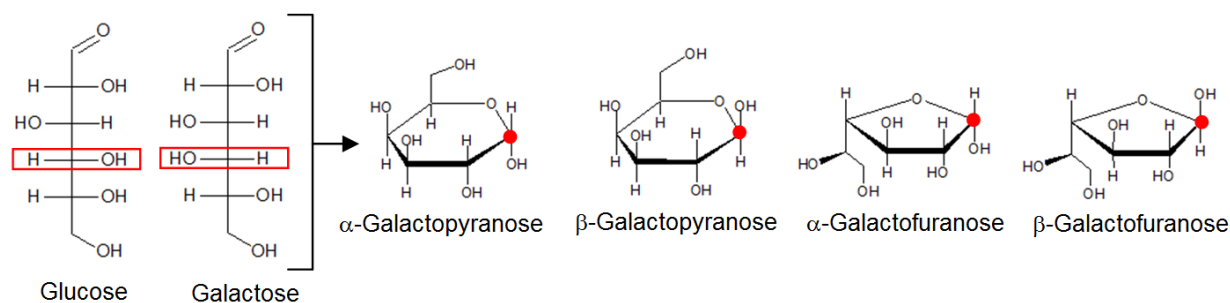
carbohydrates are storage and transport of energy (e.g. starch, glycogen and sucrose). Despite many roles of carbohydrates are not well understood, it is well accepted that carbohydrates contain the codes for cell-cell recognition. To understand the importance of carbohydrates as code molecules it is important to emphasize that the blood typing results from different types of oligosaccharides on the surface of blood cells (Fig. 2).



**Figure 2.** Oligosaccharides involved in the blood typing

Because of their constitution and architecture, each monosaccharide offers many linkage sites, for example a simple hexose such as glucose, in its cyclic form of glucopyranose, maintains four free hydroxyl groups (i.e. *O*-2, *O*-3, *O*-4 and *O*-6) and every one are available to be attached for other monosaccharides. Thus, different from other linear macromolecules, namely proteins and nucleic acids, the chemistry of carbohydrates is quite complex, considering the presence of several epimers, enantiomers, anomeric configurations and branches, with variable linkage possibilities. Also, their biosynthesis is considered extremely complex, since it lack any template. Nevertheless, carbohydrates can be found associated with virtually any other molecule being found linked to proteins, lipids, nucleobases, and several compounds from the secondary metabolism.

The diversity of the carbohydrates is firstly defined by the massive possibilities of monosaccharide hydroxyl configurations, producing many stereocenters, allowing different monosaccharides with same backbone, just alternating the hydroxyl configuration. In solution, the monosaccharides with carbon chains higher than tetroses tend to cyclize. For example, a simple monosaccharide (galactose, a hexose or arabinose, a pentose) is frequently found in two different ring configurations: galactopyranose (or arabinopyranose) and galactofuranose (or arabinofuranose), an analogy to pyran and furan rings. Nevertheless, when in the ring configuration, an additional stereocenter is formed on the C1, named the anomeric carbon (C1 for the aldoses or C2 for ketoses). In this case, two other configurations named as anomers  $\alpha$  and  $\beta$  are possible and, according to ring configuration, the monosaccharide could be presented as  $\alpha$ -galactopyranose,  $\beta$ -galactopyranose,  $\alpha$ -galactofuranose and  $\beta$ -galactofuranose (Fig. 3).



**Figure 3.** Monosaccharide epimers (glucose and galactose, epimers in C-4) and the diversity of configuration that can be assumed by a monosaccharide. Red circles indicate the anomeric carbons

### 1.1. Mass spectrometry in carbohydrate analysis

In the early of mass spectrometry development, the limitation of ionization modes avoided the carbohydrate analysis. Electron ionization (EI), formerly called electron impact, is one of the oldest ionization modes, developed by Dempster in 1918, this ionization is well used organic compounds [1-2]. Nevertheless, it requires volatile and thermally stable compounds hindering the carbohydrate analysis, because they are non volatile compounds. In 1963, Wolfrom and Thompson introduced a method to make monosaccharides suitable for the “in gas phase analysis”, developed for gas chromatography (GC) [3-4]. It consisted to introduce acetyl radicals to any free hydroxyl making a non volatile monosaccharide in a volatile derivative at the GC conditions. A common problem in monosaccharide derivatization is the random formation of anomers ( $\alpha$  and  $\beta$ ) and ring configuration (furanose and pyranose). Since this hampers the GC analysis of monosaccharides, an additional step was included, which consist into reduce the anomeric carbon with  $\text{NaBH}_4$ , yielding an aditol, which could be further acetylated [3-4]. These methods allowed monosaccharides to be analyzed by GC, but also met the requirements of the electron ionization for volatile compounds, making the hyphenated GC-MS a powerful tool for monosaccharide composition. Latter, these methods were combined with a previous alkylation of carbohydrates, becoming the main means for interglycosidic linkage analysis.

Considering that electron ionization promotes an extensive fragmentation of analytes, EI alone is not suitable for mixture analysis. Chemical ionization (CI) is able to make mixture analysis simpler than EI, however this is suitable only for mixtures containing compounds with different molecular mass. Therefore, for the monosaccharide analysis the coupling of gas chromatography with mass spectrometry was, actually, a perfect “wedding”, considering that mass spectrometry (EI or CI) alone would not be able to describe their isomers but, on the other hand, the separation of gas chromatography can ensure the analysis.

After the 80s, when Baber and coworkers introduced a new ionization source [5], called “fast atom bombardment” (FAB), the oligosaccharides could be analyzed in their intact form. The method consists in to dissolve samples in a non volatile matrix, to which a beam of accelerated atoms is triggered. This produces lower fragmentation than EI, allowing to observe the ion of

entire oligosaccharides, which can be post selected and fragmented individually. Currently, many types of ionization sources can be employed to carbohydrate analysis, but the main ones remains to be “matrix-assisted laser desorption ionization” (MALDI) and “electrospray ionization” (ESI). The later having a special advantage because it is easy for coupling with liquid chromatography systems, improving greatly the analytical potential of both.

### 1.2. Obtaining enriched saccharide extracts

Carbohydrates are essentially hydrophilic compounds, thus being extracted from their matrix in water. However, inter- and intramolecular interactions of some polysaccharides avoid their solubility in water. On the other hand, many glycans are found attached to different molecules, such as lipids, being insoluble in water, but well in organic solvents such as methanol-chloroform. For the most mass spectrometry analysis of carbohydrates, the entire polysaccharide is not suitable or not interesting to be analyzed, because unlike proteins which have a defined molecular mass, the polysaccharides are polydisperse. Thus, carbohydrates must be analyzed as oligosaccharides, which can be found as naturally occurring compounds, or then produced by controlled mild hydrolysis or enzymatic degradation. Most of glycoconjugates can be analyzed directly in its native composition, but oligosaccharides from glycoproteins should be released prior MS analysis.

### 1.3. Chemical releasing oligosaccharides

Chemical reactions for releasing oligosaccharides can be achieved by partial hydrolysis of polysaccharides, a nonselective process that lead to different oligosaccharides. There are many ways to get partial hydrolysis, but it is important to adapt the method for the polysaccharide of interest, considering that different polysaccharides will have different behaviors [6]. Acids such as trifluoroacetic acid or chloridric are always a good choice since they are easy to eliminate after hydrolysis. Another alternative to be considered is the controlled Smith degradation. This is of particular importance to degrade specific carbohydrates, those containing free vicinal hydroxyl. Thus depending on the overall polysaccharide backbone, it is possible to breakdown the main chain releasing the side chain oligosaccharides [7-8].

Oligosaccharides from glycoprotein can also be obtained by chemical releasing. This can be done by  $\beta$ -elimination, which consists in to submit the glycoproteins to alkali degradation under reductive condition, for example by adding  $\text{NaBH}_4$ . This process can release *O*-linked oligosaccharides only, converting the unit that was linked to the protein in its alditol [9]. Anhydrous hydrazine is a suitable reagent for releasing unreduced *O*- and *N*-linked glycans. The method introduced in 1993 by Patel and coworkers allows releasing selectively *O*- and *N*-linked glycans in dependence of temperature, 60 °C (5 h) for *O*-linked and 95 °C (4 h) for *N*-linked glycans [10-11].

### 1.4. Enzymatic releasing oligosaccharides

Unlike many chemical releasing methods, the enzymatic catalysis can be well controlled, are selective and reproductively. It consists in the use of enzymes to cleave specific glycosidic

linkages. The range of enzymes for polysaccharides cleavage is varied as well as the polysaccharides. Thus, some information about the structure is needed, such as the monosaccharide composition and glycosidic linkage. Glycans from glycoproteins can also be released by enzymatic catalysis. This can be done by using endoglycosidases such as peptide *N*-glycosidase F (PNGase F) and peptide *N*-glycosidase A (PNGase A). Glycan from glycolipids can also be released with endoglycoceramidase, however this is not necessary, considering that most of glycolipids can be analyzed as entire structures [12-13].

## 2. Analysis of carbohydrates

### 2.1. Monosaccharide composition

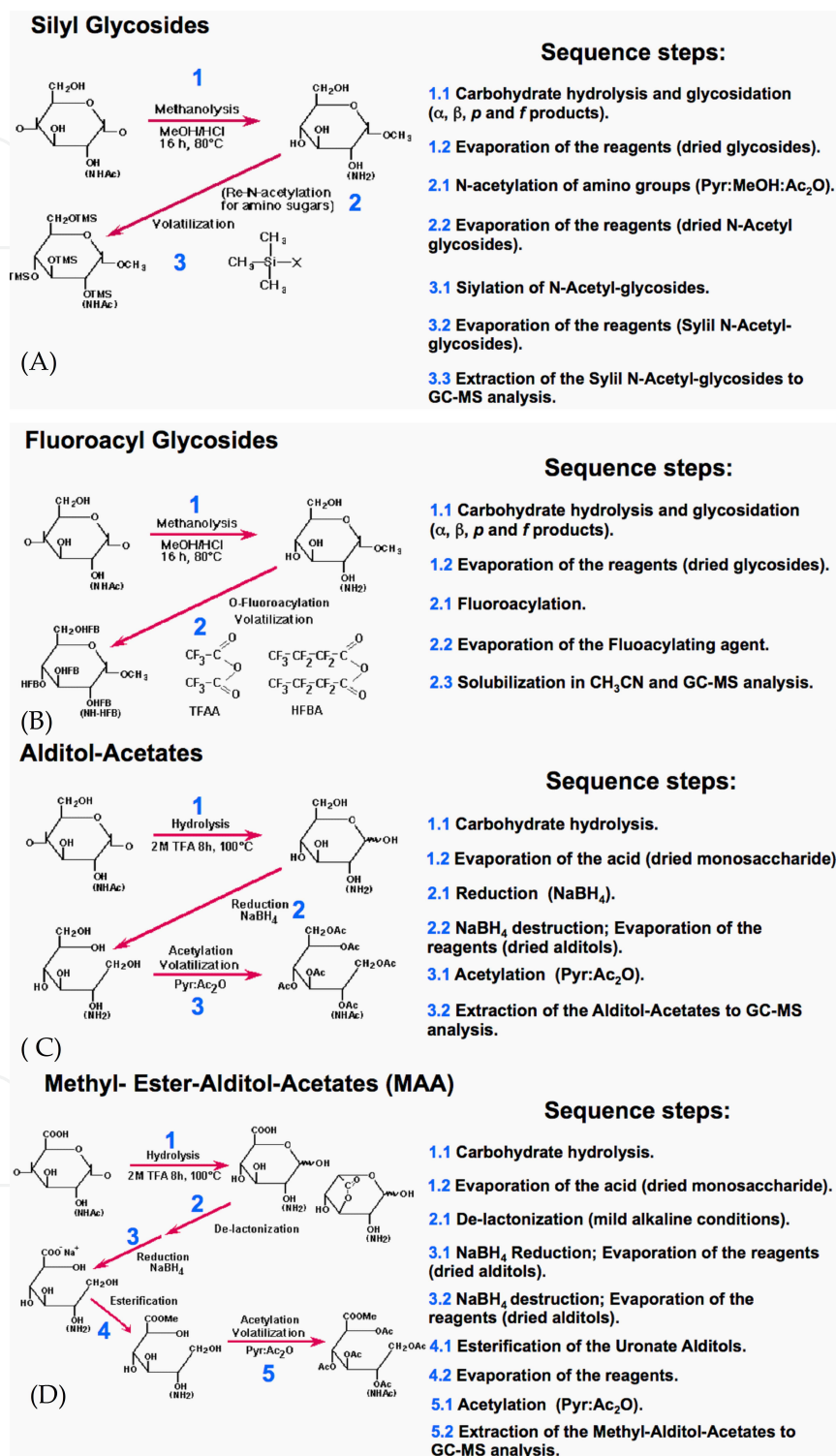
The first step in the carbohydrate analysis is to know the type and amounts of monosaccharides that compose a particular structure of interest. The methods for it including complete hydrolysis that can be performed with chloridric acid, trifluoroacetic acid, sulfuric acid and so on, preferably with an easily removable acid. Mass spectrometry of carbohydrates gives new perspectives for their structural analysis and for the use of GC-MS, the chemical derivatization gave “wings” to non-volatile compounds, making possible to determine the chemical structure of native glycans, which can be constituted by oligosaccharide chains, fatty acid and amino acids. GC-MS can provide highly resolved chromatograms, providing the identification and quantification of compounds in fmol quantities. Carbohydrates are usually converted to derivatives by silylation, acylation and alkylation [14-16].

After hydrolysis, many derivatization methods can be applied, such as the silylation (Fig. 4A), which was firstly used for carbohydrates by Sweley and coworkers [17], becoming very popular because it is simpler, rapid and applicable for all carbohydrate, including monosaccharides, alditols, uronic acids, deoxy-monosaccharides, amino sugars and can be extended to oligosaccharides [15-18]. The problem of silylation and fluoroacylation is the formation of many isomers from each monosaccharide, due to the mutarotation and the glycosidation reaction (Fig. 4B). In order to resolve this problem, carbohydrates have been converted to alditols, dithiocetals, aldonoitrile and other derivatives, these reactions are well described by Knapp (1979) [18] and Biermann & McGinnis (1989) [15].

A classical method for monosaccharide analysis by gas chromatography, introduced by Wolfrom and Thompson (1963) [3-4], includes a reduction step, which will avoid the mutarotation, consequently the  $\alpha$ ,  $\beta$ , pyranose and furanose isomers to each monosaccharide. This is followed by an acetylation (Fig. 4C) of hydroxyl groups that makes monosaccharides (as alditol acetate derivatives) suitable for gas chromatography and mass spectrometry with electron ionization. So the alditol-acetates are commonly used for identification and quantification [15,19]. Sasaki and coworkers (2008) provide modification in this method (Fig. 4D), creating the MAA (methyl-esters-alditol-acetates), which extended to lipid, amino acids and uronic acids [20]. Since some steps are needed to obtain the derivatized analytes, the follow schemes show the simplified pathway of some carbohydrates derivatives. The fragmentation profile from alditol acetates allows to easily distinguishing among monosaccharide class, i.e. a pentose



from a hexose or deoxyhexose. However within the monosaccharide class, it is very difficult distinguish among them, being essential the chromatography separation.



**Figure 4.** Schematic representation of monosaccharide derivatization methods (A) silylation, (B) fluoroacylation, (C) acetylation and (D) acetylation modified method.

The choice for a derivatizing procedure will depend of the nature of glycoconjugate. Generally, how much more complex is the structure, silylation and fluoroacylation are the desired procedures. However these procedures give rise to complex chromatograms and EI-MS profile, which normally led to misunderstand results. So, why to choose these procedures? Since both methods use glycosidation in mild conditions, avoiding carbohydrate degradation, mainly of ketosugars and also provide esterification of carboxylic groups on acidic sugars. By these properties, the high volatile the fluoracyl (HFBA, PFPA and TFA) and silyl (BSTFA and MBTFA) they are commonly used for structural analysis of complex sialyl glycans, although they are unstable and some cares should be done prior to analysis. So where is the field of application for the alditol-acetates? They are used for carbohydrate quantification and identification for heteropolysaccharides and non-degradable sugars. In the case of acidic ones they normally are carboxi-reduced by carbodiimide and  $\text{NaBH}_4$  [21], which provide the correspondent alditol prior acetylation and GC-MS analysis, moreover it has been observed in our research laboratory that the alditol-acetates are cheap, easy and stable for ~30 years to date, which became an exceptional tool as unique standard and for quantification assays where stability and repetitions are need. Also the EI-MS profile aids to fit the monosaccharide classes, giving primary and secondary fragments that are key ions for identification and with lesser artefacts formation than the silyl and fluoracyl derivatives (Fig. 5-7 and Table 1) [15,19-20,22-23].

Since many derivatives could be prepared, each method has advantages and problems due the physical and chemical properties of the carbohydrates. In order to analyze the carbohydrates without formation of many isomers, the MAA had demonstrated robustness for qualitative and quantitative analysis of carbohydrates. The silylated or fluoroacylated derivatives are a great option to obtain high volatile sugars, including oligosaccharides, however, they are not stable as the acetylated ones. Moreover, MAA use simple and less expensive reagents in comparison to the others.

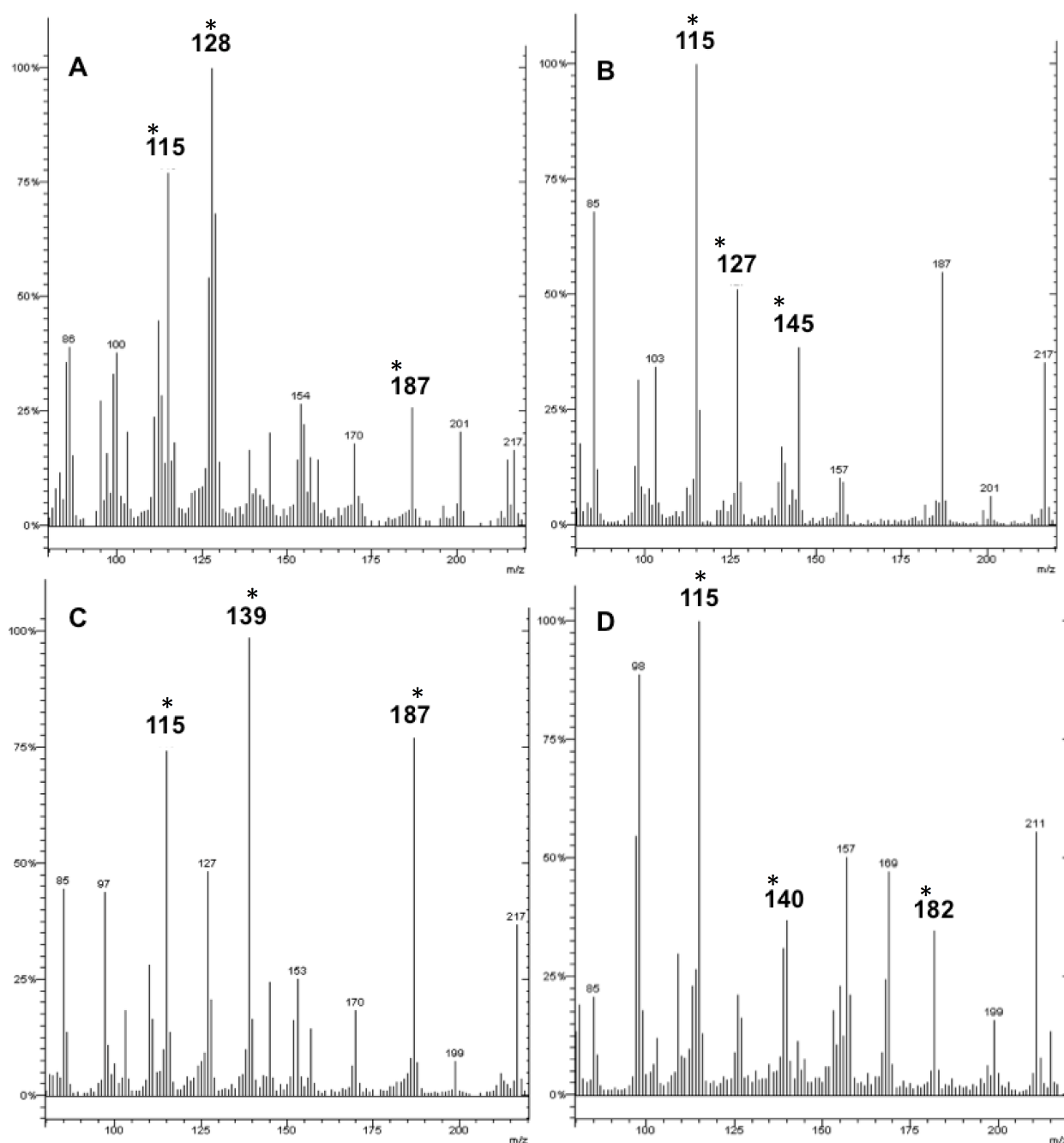
## 2.2. Interglycosidic linkage analysis: partially methylated alditol acetates

Combined with NMR spectroscopy, methylation analysis is the most utilized method for determination of structure of complex carbohydrates, providing the linkage analysis and structure of monosaccharide units in oligo-, complex glycans. Per-O-methylation of carbohydrates has been carried out by several methods such as those of Haworth, Kuhn et al., Hakomori, and nowadays by Ciucanu and Kerek [24-27].

Per-O-methylated products can be converted to partially O-methylated alditol acetates (PMAAs) via successive hydrolysis, reduction/acetylation and identified by GC-MS using their characteristic GC retention times and EI-MS fingerprints [28]. The method is extremely sensitive, requiring low amounts as 50  $\mu\text{g}$  of glycan. PMAAs were refined by Carpita and Shea by use of sodium borodeuteride in the reduction step [19], avoiding the problem of mass symmetry from different partially O-methylated aldoses (Fig. 8).

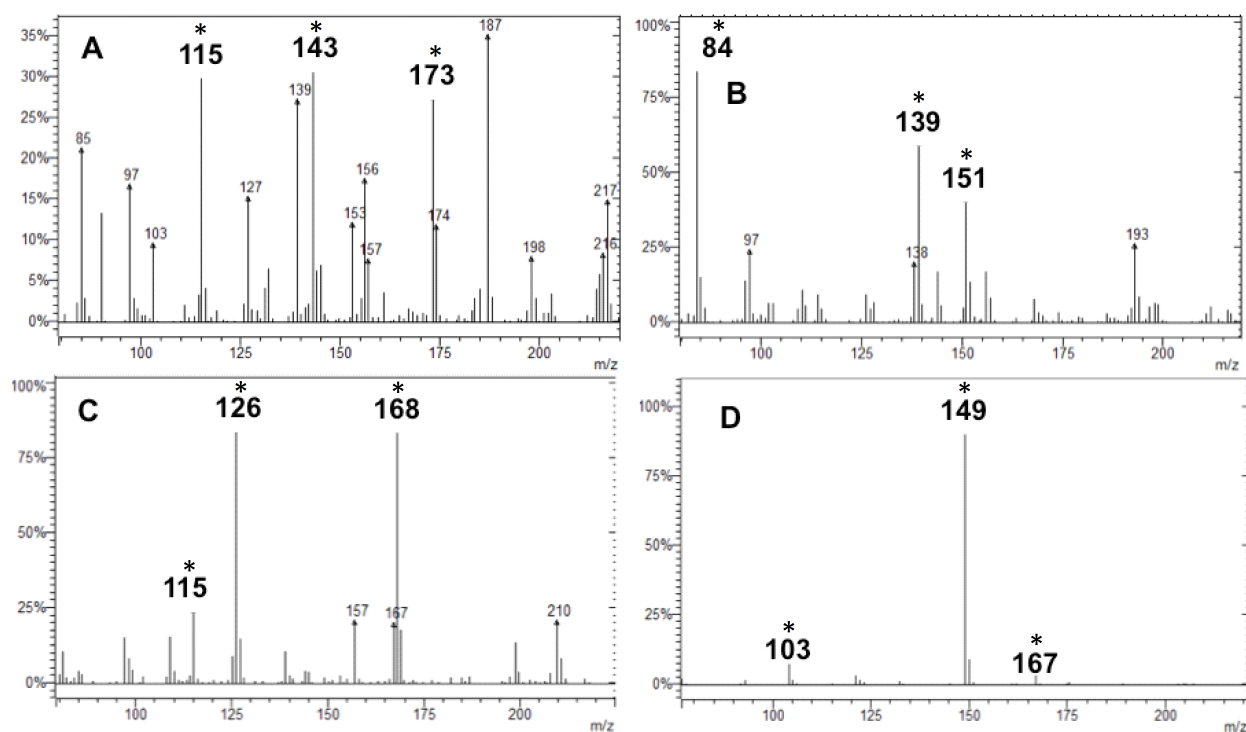
Usually, in glycan analysis more than one type of monosaccharide is present, which makes necessary to compare the retention times of the PMAAs, with standards. The common



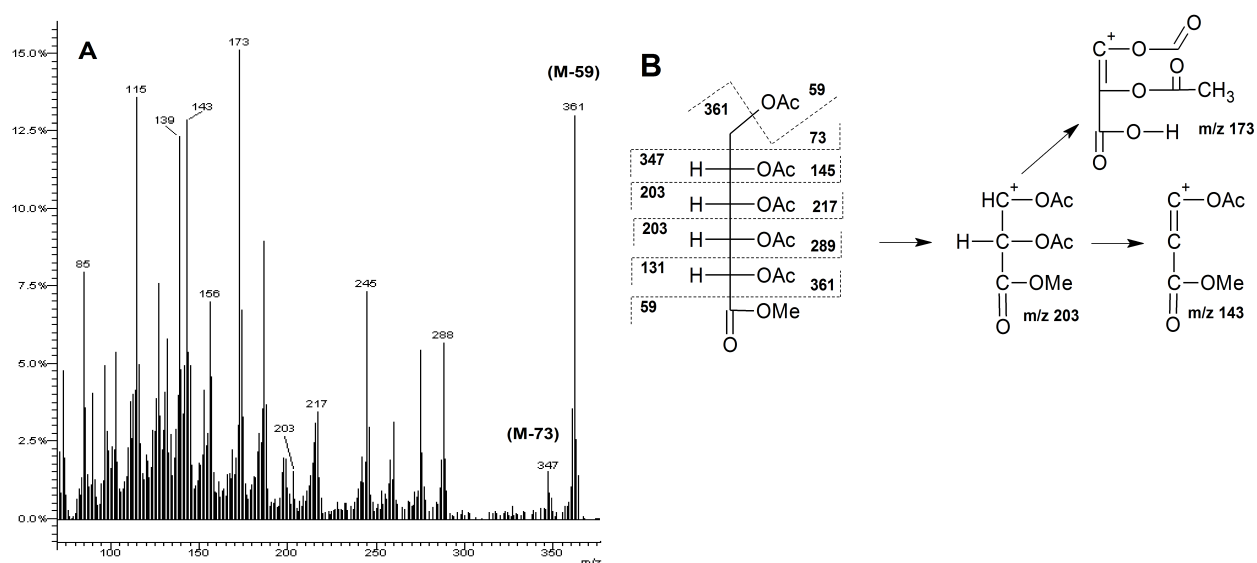


**Figure 5.** EI-MS profile of some alditol-acetates (80-220  $m/z$ ), key ions for identification are identified with (\*) at the MS spectra. (A) 6-deoxy-hexitol; (B) pentitol; (C) hexitol; (D) non-reduced hexitol.

procedure is synthesize or acquire authentic standard for each monosaccharide. However this can be time consuming and expensive if it done individually. An alternative strategy was simultaneously provides partial *O*-methylation of each individual monosaccharide and convert it to a mixture of PMAAs. Recently, Sasaki et al. [29-30] produced series of PMAAs of Glc, Man, Gal, Ara, Xyl, Fuc, and Rha, and have identified them in the C-1 deuterated form and the EI-MS and retention time profile of the pyranosidic and furanosidic ring conforma-



**Figure 6.** EI-MS profile of some alditol-acetates (80-220  $m/z$ ), key ions for identification are identified with (\*) at the MS spectra. (A) Me-ester-hexitol; (B) Hexaminitol; (C) inositol; (D) fialate.



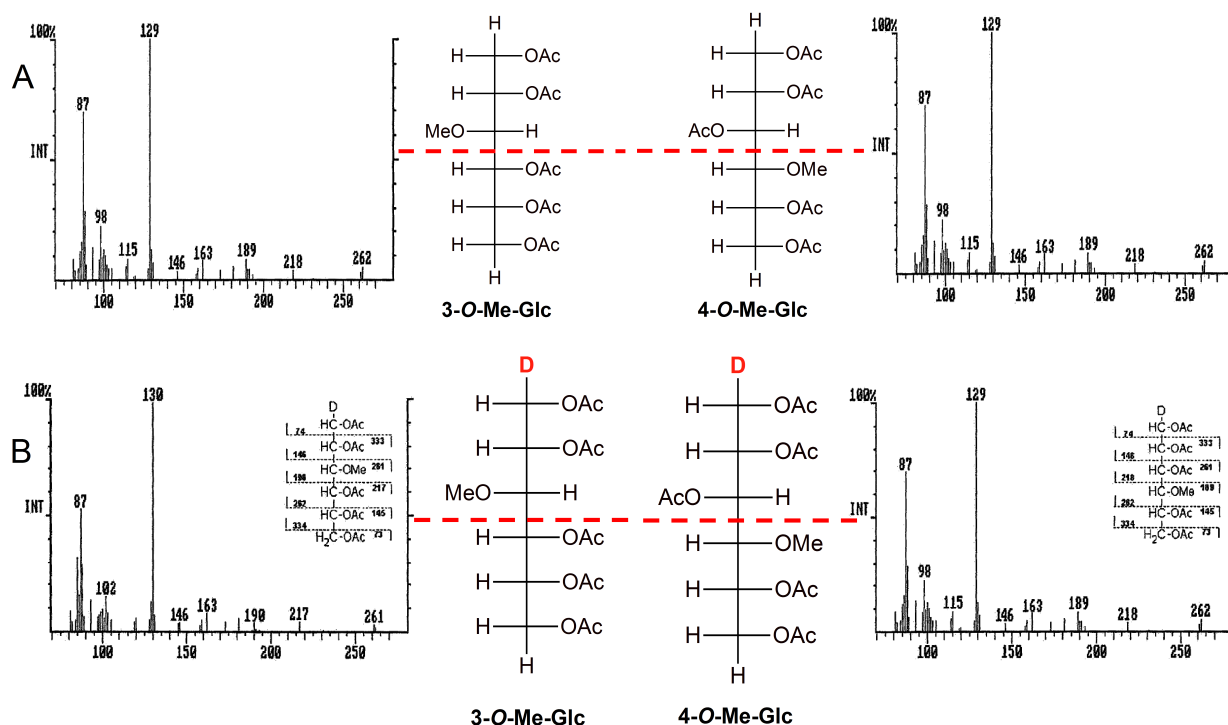
**Figure 7.** (A) EI-MS spectrum of 1-Me-ester-hexitol acetate (B). Primary and secondary fragmentations of the key ions on EI-MS spectrum.

tions, using the Purdie methylation ( $\text{Ag}_2\text{O}-\text{MeI}$ ) [31]. So, what is the big advantage of the proposed work? The answer is a simple rationalization and rapid identification of the PMAA. Supposing that a glycan has the following monosaccharide composition: Xyl, Ara, Glc and Gal. Performing the methylation by Ciucanu and Kerek, you will have from EI-MS the following

Methyl-Esters- Alditol-Acetates	Rt	Reporter ions (m/z)
2-Deoxy-Rib	11.52	245-232-159-145-142-129-117-100-82-57
Rha	13.92	317-215-201-187-170-155-145-129-128-115-112-103-99-95
Fuc	14.17	317-215-201-187-170-155-145-129-128-115-112-103-99-95
Ara	14.27	303-217-187-145-140-127-115-103-98-95
Xyl	14.59	303-217-187-145-140-127-115-103-98-95
2-Deoxy-Glc	15.72	317-303-217-201-159-141-129-115-112-103-95
GalA	16.88	361-287-245-217-187-173-156-143-139-127-115-103-97
4-O-Me-GlcA	16.02	333-319-261-217-175-143-139-133-127-115-101-85
ManA	17.07	361-287-245-217-187-173-156-143-139-127-115-103-97
GulA	18.2	361-287-245-217-187-173-156-143-139-127-115-103-97
GlcA	17.13	361-287-245-217-187-173-156-143-139-127-115-103-97
IdoA	18.57	361-287-245-217-187-173-156-143-139-127-115-103-97
Man	17.91	375-289-273-259-217-187-170-153-145-139-127-115-103-97-85
Gal	18.15	375-289-273-259-217-187-170-153-145-139-127-115-103-97-85
Glc	17.98	375-289-273-259-217-187-170-153-145-139-127-115-103-97-85
m-Ins	17.62	375-211-199-168-157-139-126-115-109-97
Glc-HepA	20.23	433-420-359-317-275-259-217-197-187-173-169-143-139-127-115-103
Man-Hep	19.78	448-332-290-218-187-182-170-139-127-115-109-103-97-85
Glc-Hep	20.12	448-332-290-218-187-182-170-139-127-115-109-103-97-85
Perseitol	20.54	448-332-290-218-187-182-170-139-127-115-109-103-97-85
GalNAc	19.88	374-318-259-193-168-151-139-126-114-102-97-84
GlcNAc	19.74	374-318-259-193-168-151-139-126-114-102-97-84
ManNAc	19.93	374-318-259-193-168-151-139-126-114-102-97-84
NAc-Neu*	23.48	518-504-476-444-330-318-259-186-172-154-139-97
NAc-Neu*	24.05	518-504-476-444-330-318-259-186-172-154-139-97

GC-MS analysis of MAA on CP-Sil-8CB (DB-1) capillary column. \*Retention time (*R<sub>t</sub>*) in min, at 100 °C to 180 °C (10 °C min<sup>-1</sup>, then held for 5min.) and to 320 °C (10 °C min<sup>-1</sup>, then held for 5 min). NAc-Neu\* derivatives formed during reduction of the ketone group. EI-MS were obtained using an Ion-trap at 220 °C, transfer line 320 °C and internal ionization.

**Table 1.** Retention times and EI-MS reporter fragments obtained by mass spectrometry from the MAA derivatives adapted from Sasaki et al. (2008)



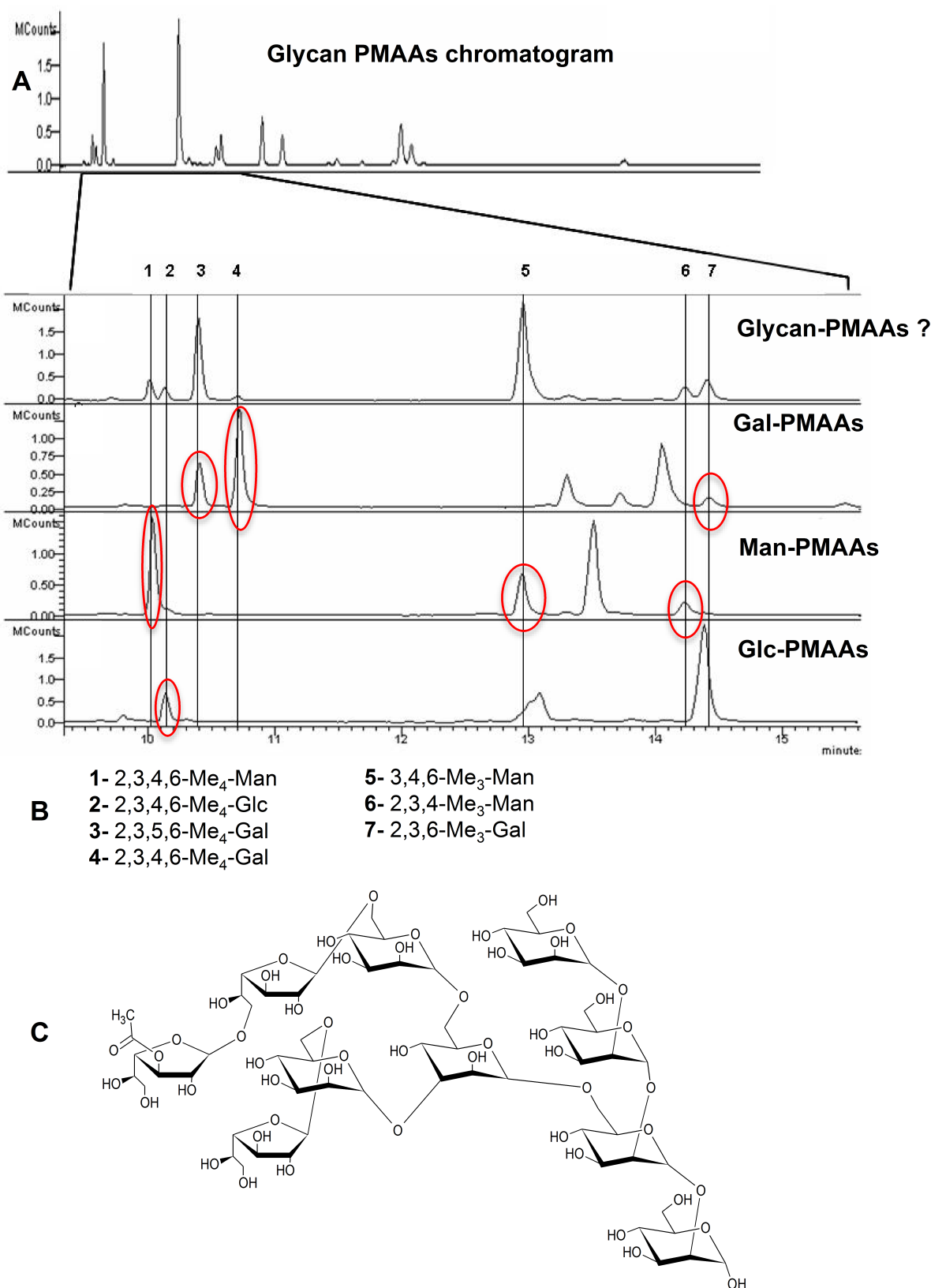
**Figure 8.** (A) Non deuterated PMAAs both derivatives have the same EI-MS profile. (B) C-1 deuterated PMAA, the differentiation between the mass symmetrical derivatives is possible, the EI-MS profile are different.

answer: PMAAs-Hexitol and PMAAs-Pentitol, and their methylation distribution for linkage analysis. However, information of the type of monosaccharide is difficult to assign and normally misunderstood. Although, if you inject, individually, the mixtures of PMAAs from Xyl, Ara, Glc and Gal, the analysis will be easier, since now you have the *R<sub>t</sub>* and the EI-MS profile of PMAA from each monosaccharide, an example for this application is demonstrate in Figure 9. From these PMAAs mixtures of individual monosaccharide is possible to create an EI-MS library for interglycosidic linkage (Fig. 10).

### 2.3. Oligosaccharide analysis

With the introduction of FAB-MS in early 80's [5], the analysis of carbohydrates swerved and oligosaccharides could be analyzed as entire structures, considering that FAB source, unlike EI, is able to transfer the entire oligosaccharides to the gaseous phase, ionizing then. Nowadays, the most used instrumentation are based on MALDI or ESI-MS ionization, considered soft ionization techniques that leads to formation of less unintended fragmentation during the ionization process, which are also called as in-source fragmentation.

Most of carbohydrates lacks acidic or basic functional groups, being lesser ionizable than peptides, for example. However, during the evolution of mass spectrometry of carbohydrates, many chemical reactions were developed to enhance the ionization and consequently the MS signal. Common derivatives processes included per-*O*-methylation or per-*O*-acetylation of entire oligosaccharides [33], these enhance their transfer to gaseous phase

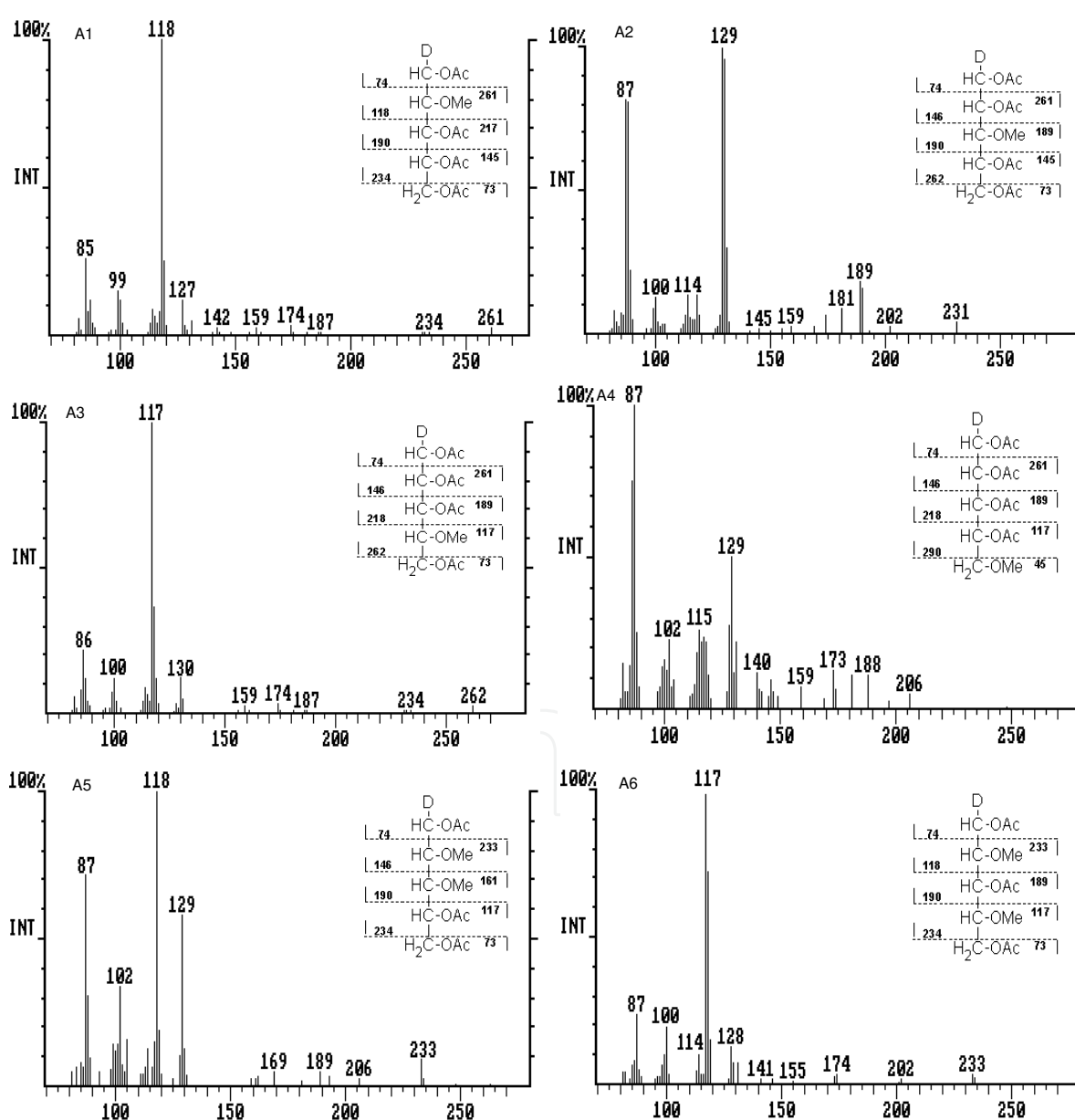


**Figure 9.** (A) GC-MS chromatogram of an exopolysaccharide obtained from *Exophiala jeanselmei* [32] (B) Identified PMAA from standard mixtures of Gal, Man and Glc, based on their Rt(s) and EI-MS profile (C). Hypothetical structure of the glycan based on methylation and NMR data.

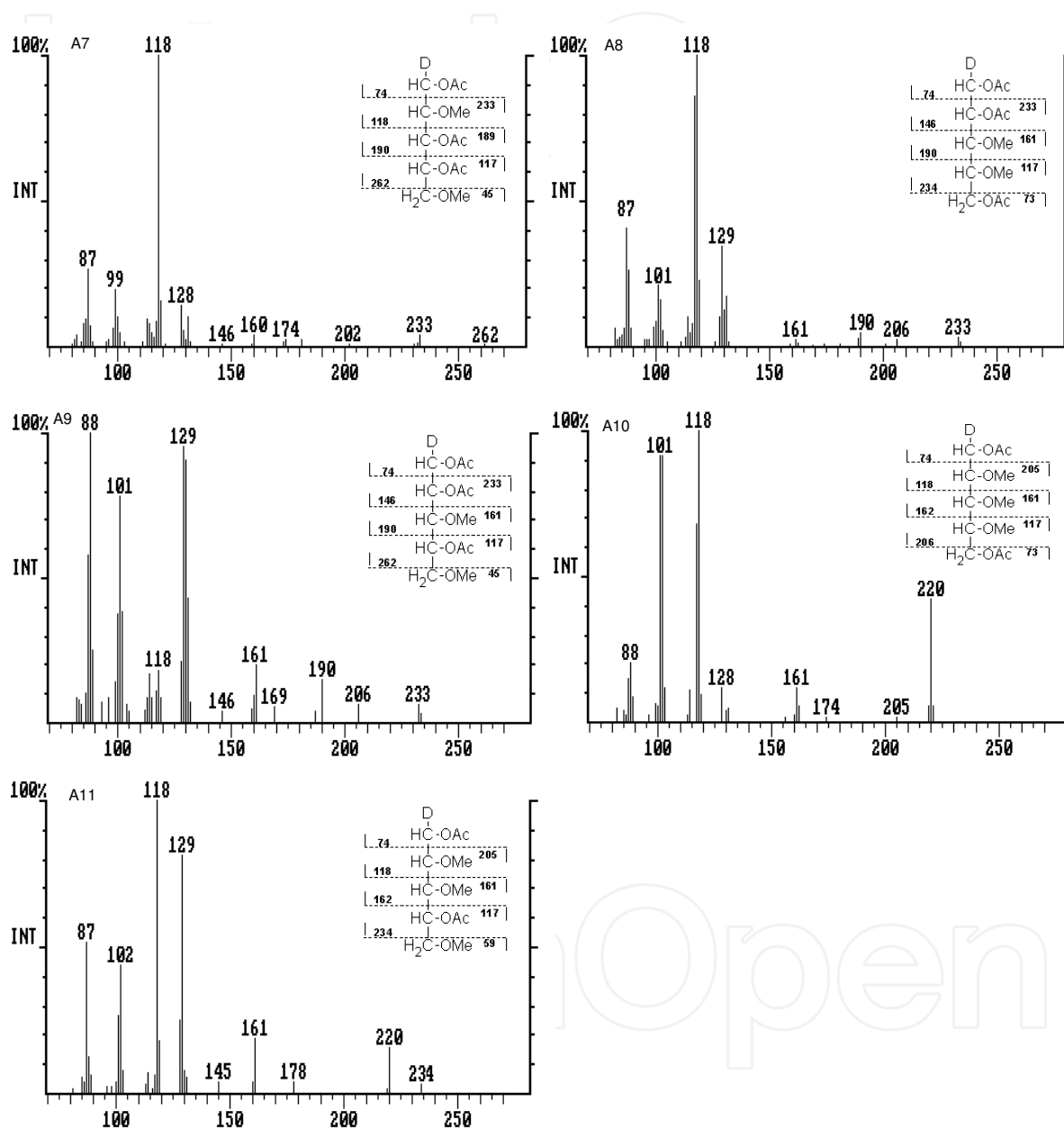


during ionization and, also, per-*O*-methylation allows to easier detection of the branch sites via fragmentation.

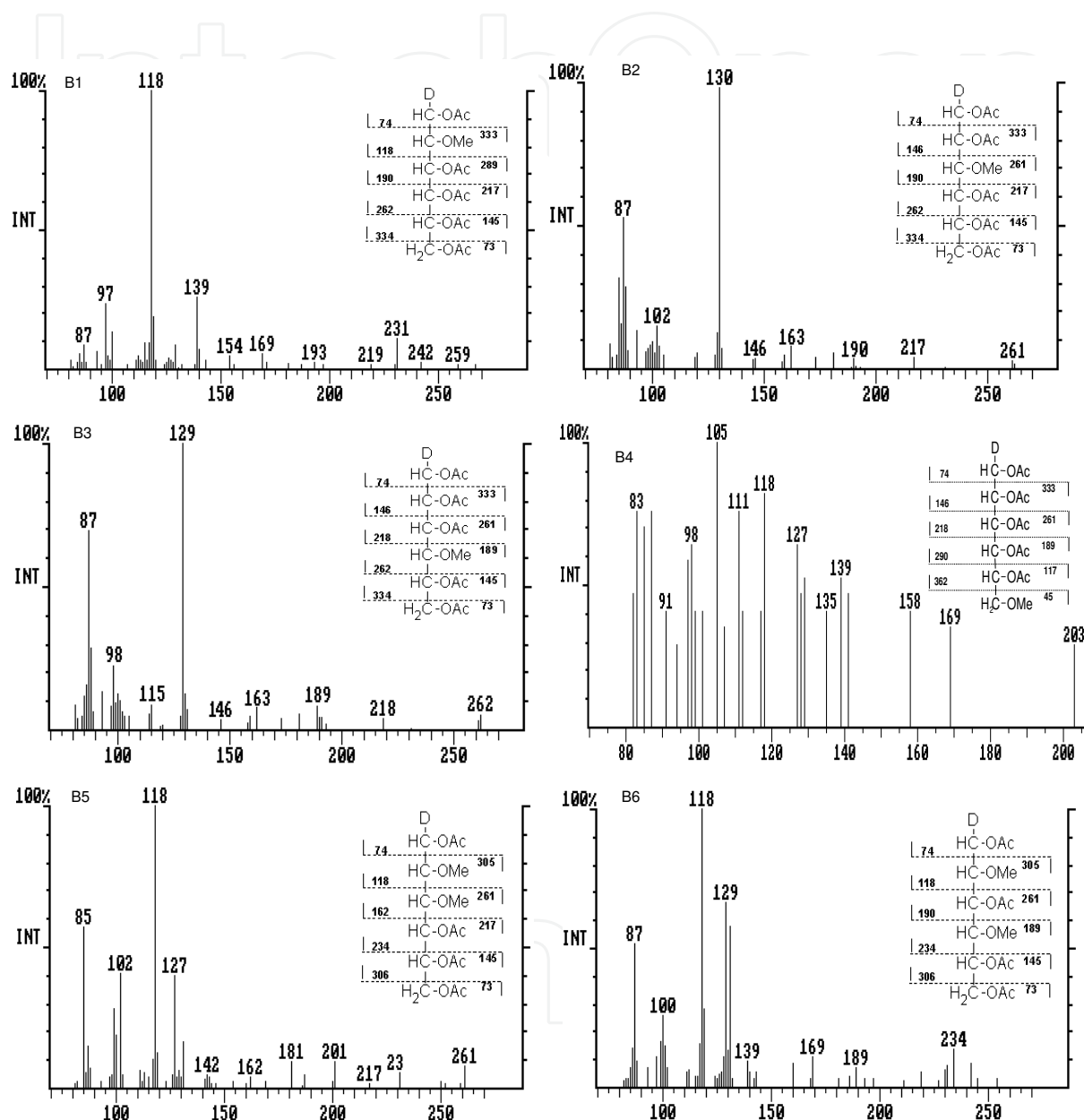
IntechOpen



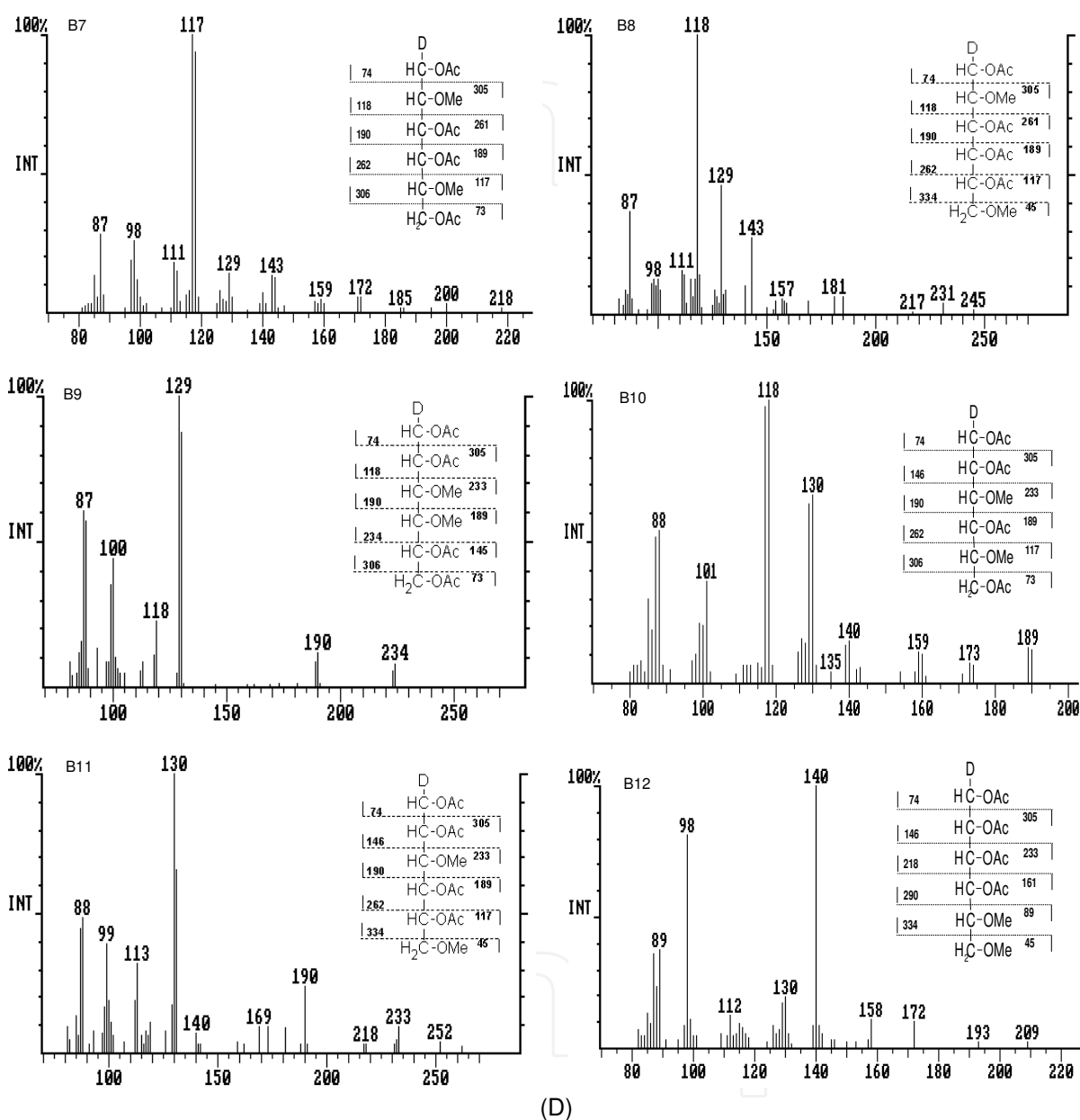
(A)



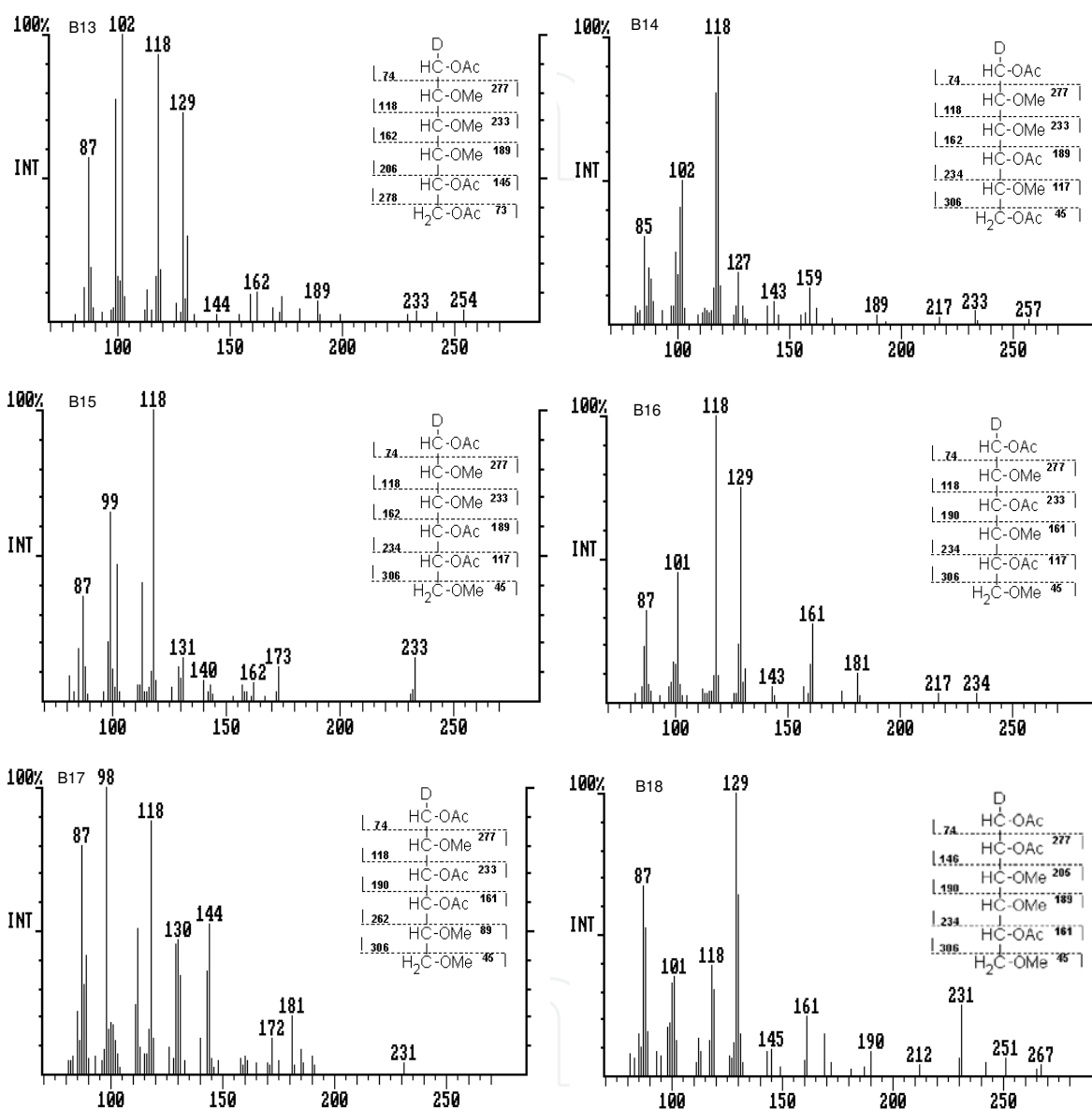
(B)



(C)

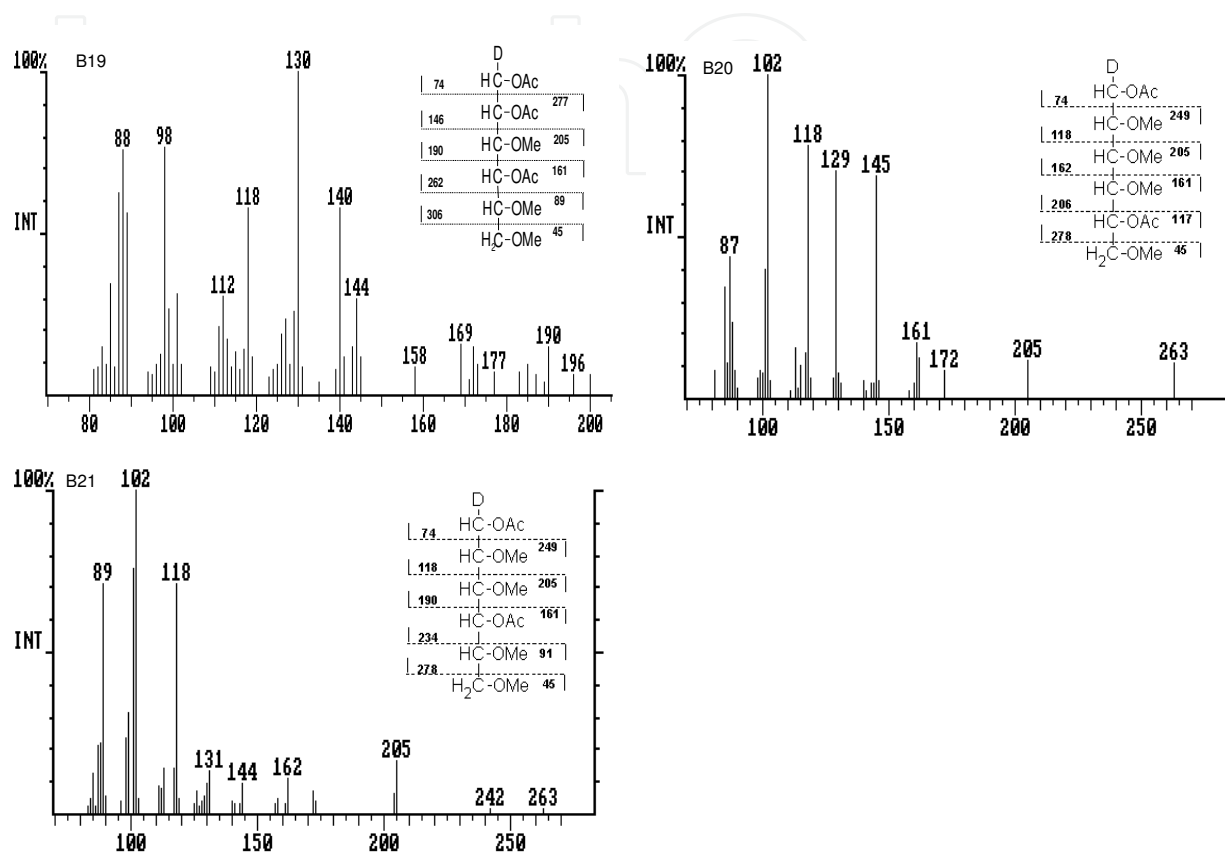


(D)



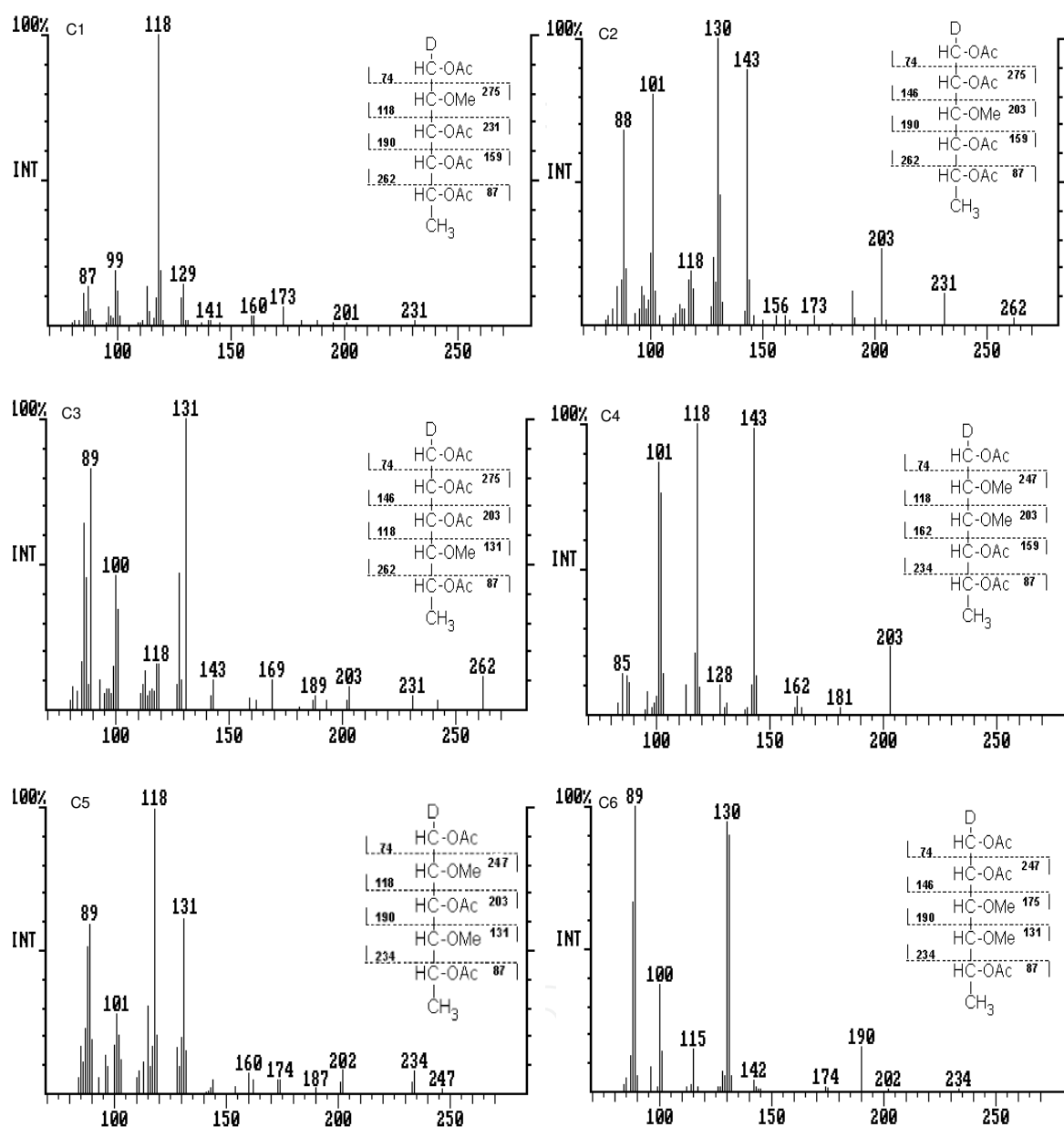
(E)



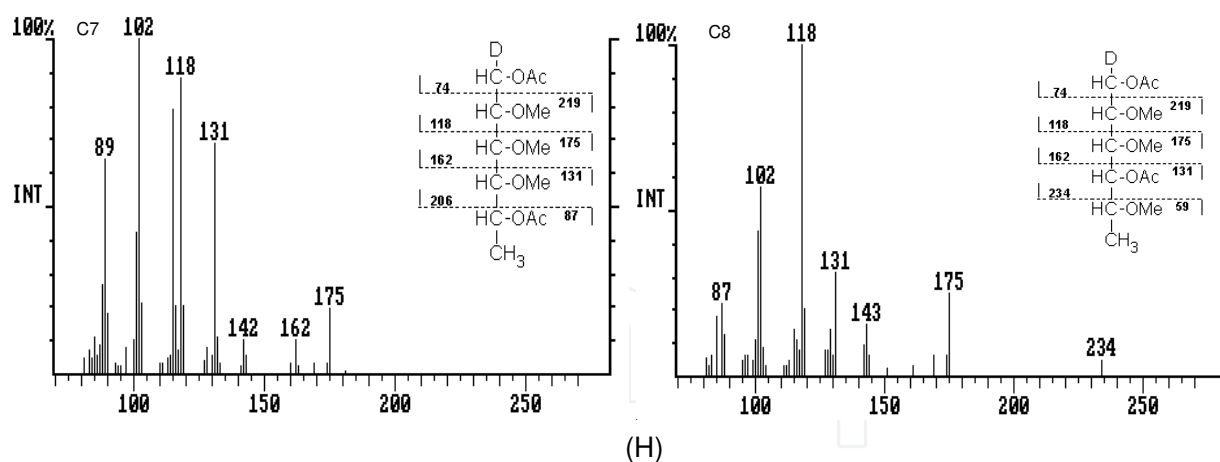


(F)

IntechOpen



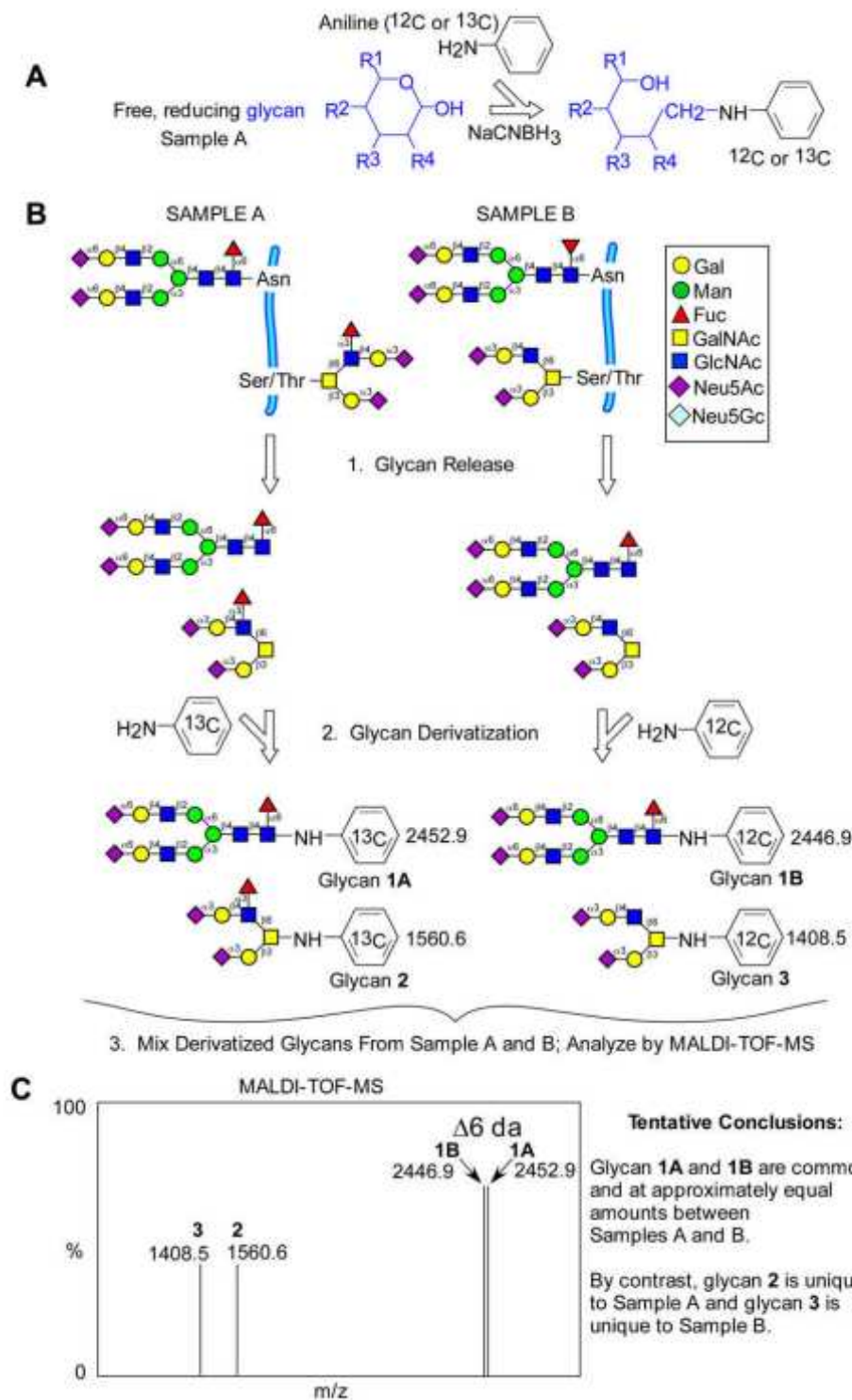
(G)



**Figure 10.** (A) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2-MePentitol (A1), 3-MePentitol (A2), 4-MePentitol (A3), 5-MePentitol (A4), 2,3-Me<sub>2</sub>Pentitol (A5), 2,4-Me<sub>2</sub>Pent (A6), 2,5-Me<sub>2</sub>Pentitol; (B) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2-MePentitol (A7), 3,4-/2,3-Me<sub>2</sub>Xyl (A8), 3,5-Me<sub>2</sub>Pentitol (A9), 2,3,4-Me<sub>3</sub>Pentitol (A10), 2,3,5-Me<sub>3</sub>Pentitol (A11); (C) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2-MeHexitol (B1), 3-MeHexitol (B2), 4-MeHexitol (B3), 6-MeHexitol (B4), 2,3-Me<sub>2</sub>Hexitol (B5), 2,4-Me<sub>2</sub>Hexitol (B6), 2,5-Me<sub>2</sub>Hexitol; (D) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2,5-Me<sub>2</sub>Hexitol (B7) 2,6-Me<sub>2</sub>Hexitol (B8), 3,4-Me<sub>2</sub>Hexitol (B9), 3,5-Me<sub>2</sub>Hexitol (B10) 3,6-Me<sub>2</sub>Hexitol (B11), 5,6-Me<sub>2</sub>Hexitol (B12); (E) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2,3,4-Me<sub>3</sub>Hexitol (B13), 2,3,5-Me<sub>3</sub>Hexitol (B14), 2,3,6-Me<sub>3</sub>Hexitol (B15), 2,4,6-Me<sub>3</sub>Hexitol (B16), 2,5,6-Me<sub>3</sub>Hexitol (B17), 3,4,6-Me<sub>3</sub>Hexitol (B18); (F) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 3,5,6-Me<sub>3</sub>Hexitol (B19), 2,3,4,6-Me<sub>4</sub>Hexitol (B20), 2,3,5,6-Me<sub>4</sub>Hexitol (B21); (G) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2-Me6-Deoxy-Hexitol (C1), 3-Me6-Deoxy-Hexitol (C2), 4-Me6-Deoxy-Hexitol (C3), 2,3-Me<sub>2</sub>6-Deoxy-Hexitol (C4), 2,4-Me<sub>2</sub>6-Deoxy-Hexitol (C5), 3,4-Me<sub>2</sub>6-Deoxy-Hexitol (C6); (H) – EI-MS patterns at  $m/z$  80 to 270 of PMAA standards, deuterated at C-1: 2,3,4-Me<sub>3</sub>6-Deoxy-Hexitol (C7), 2,3,5-Me<sub>3</sub>6-Deoxy-Hexitol (C8).

A good option to insert an ionizable group on the oligosaccharides chains is the reductive amination. In this reaction, a reductive medium is enriched with a compound containing a primary amine. Virtually any amino-compound can be used, but especially those containing aromatic rings such as 2-aminoacridone, 2-aminobenzamide (2-AB), 2-aminopyridine, 2-aminoquinoline, 4-aminobenzoic among other are of peculiarly interesting, since the insertion of a chromophore also allows detection by ultra violet detectors, common in liquid chromatography. This reaction is considered quantitative, and in an elegant experiment, Xia and coworkers employed a glycan reductive isotope labeling (GRIL) methodology using conventional <sup>12</sup>C-anilin (called light) and <sup>13</sup>C<sub>6</sub>-anilin (called heavy) [34]. After the oligosaccharides being obtained from different sources, they were labeled via reductive amination using the heavy and light reagents. Then, the oligosaccharides from both sources could be combined and analyzed together by high performance liquid chromatography with mass spectrometry detection (HPLC-MS) and MALDI with time of flight (TOF) detector. Since the oligosaccharides were isotopically labeled, the difference of 6 mass units (m.u.) observed on MS, indicated the heavy- and light labeled oligosaccharides, allowing monitor any change in the amount of specific oligosaccharides (Fig. 11).

Reductive amination using a chiral reagent, such as 2-aminopropanol, has been employed to resolve enantiomeric monosaccharides. After being aminated with one of enantiomers (R or S) the amino-alditol formed is acetylated being suitable for GC-MS analysis, even without

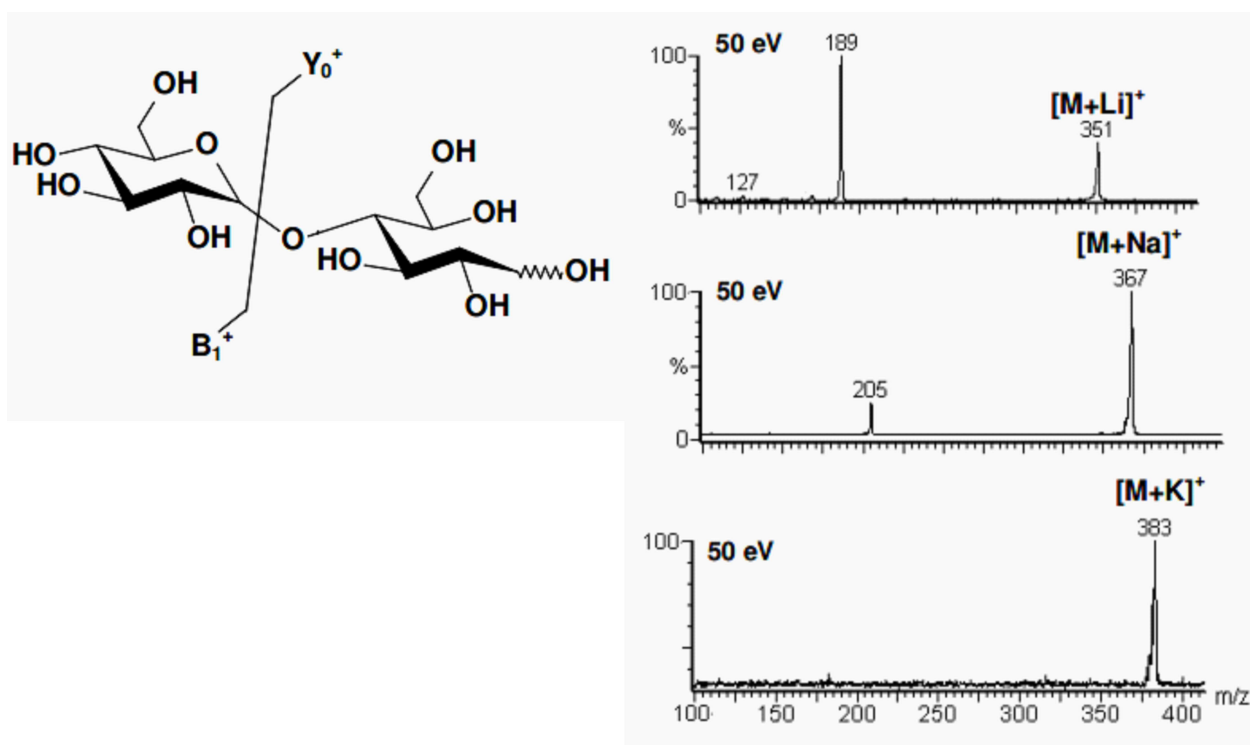


**Figure 11.** Schematic representation of the glycan reductive isotope labeling (GRIL). (A) Aniline labeling through reductive amination, (B) Glycan release and derivatization with  $^{13}\text{C}$  and  $^{12}\text{C}$  aniline in different samples followed of sample mixture, (C) analysis of the glycan mixture. (Reproduced from Xia et al, Analytical Biochemistry, 387, 162-170, 2009)

using chiral columns. This is especially employed to determine the D/L-galactose ratio from algae agarans.

With the increase of sensitive of mass spectrometers, oligosaccharides do not need to be derivatized to become analyzable by MS. In several applications, neutral oligosaccharides from different sources are analyzed in their native structure. To aid the ionization procedures, the neutral oligosaccharides are cationized mainly by using adducts of alkaline metal, such as  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Li}^+$ , other applications also use  $\text{NH}_4^+$ . The inconvenient of this method is to locate the reducing end of free oligosaccharides, since sometimes it is similar to non-reducing end(s). When possible a simple reduction will provide an increment of 2 m.u. and the terminus reduced site can be easily differentiated.

Another disadvantage of the method concerns the fragmentation process, considering that the cationization with the referred metals produces complex oligosaccharide-cation with lower stability than a protonation of an amino group, for example. This is well observed during the fragmentation of oligosaccharides complexed with  $\text{K}^+$ , since the complex is broken faster than glycosidic linkages, and the oligosaccharide fragments are lost as neutral loss (NL). However,  $\text{Na}^+$  and  $\text{Li}^+$  adducts provide good fragmentation, being  $\text{Li}^+$  better than  $\text{Na}^+$  for the most application (Fig. 12).

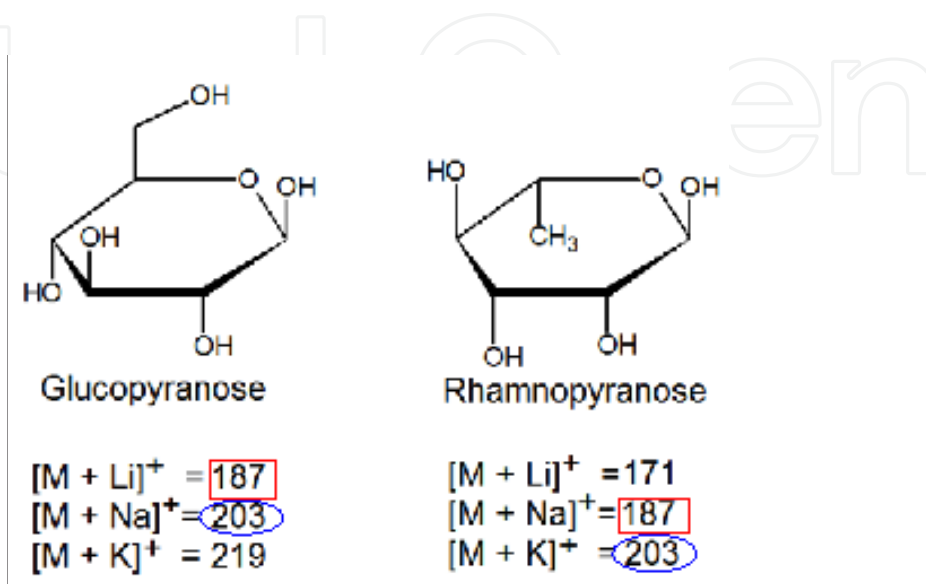


**Figure 12.** CID-MS (50eV) profile of a reduced disaccharide using  $\text{Li}^+$ ,  $\text{Na}^+$  and  $\text{K}^+$  as adducts.

$\text{Na}^+$  is the main adduct naturally occurring in the samples, although vegetables have great amounts of  $\text{K}^+$ . This may cause severe misinterpretation mainly from low resolution MS-spectra, because the difference between both ( $\text{Na}^+$  and  $\text{K}^+$ ) is 16 m.u., similar to that observed from a hexose (e.g. glucose) and a deoxyhexose (e.g. rhamnose). Although not found naturally in the samples,  $\text{Li}^+$  can be added to improve the MS detection, but it can also cause misinter-



pretation, since the mass  $\text{Li}^+$  is 16 m.u. lower than  $\text{Na}^+$  and 32 m.u. lower than  $\text{K}^+$  (Fig 13). To avoid these erroneous interpretations, it is strongly recommend the use of a cation exchange resin to eliminate any residual  $\text{Na}^+$  or  $\text{K}^+$ , and then the cation of interest can be added in lower amounts, avoiding the signal suppression. Using these simple steps, any neutral oligosaccharide or glycoconjugate can be easily analyzed by MS (data from our own experience)



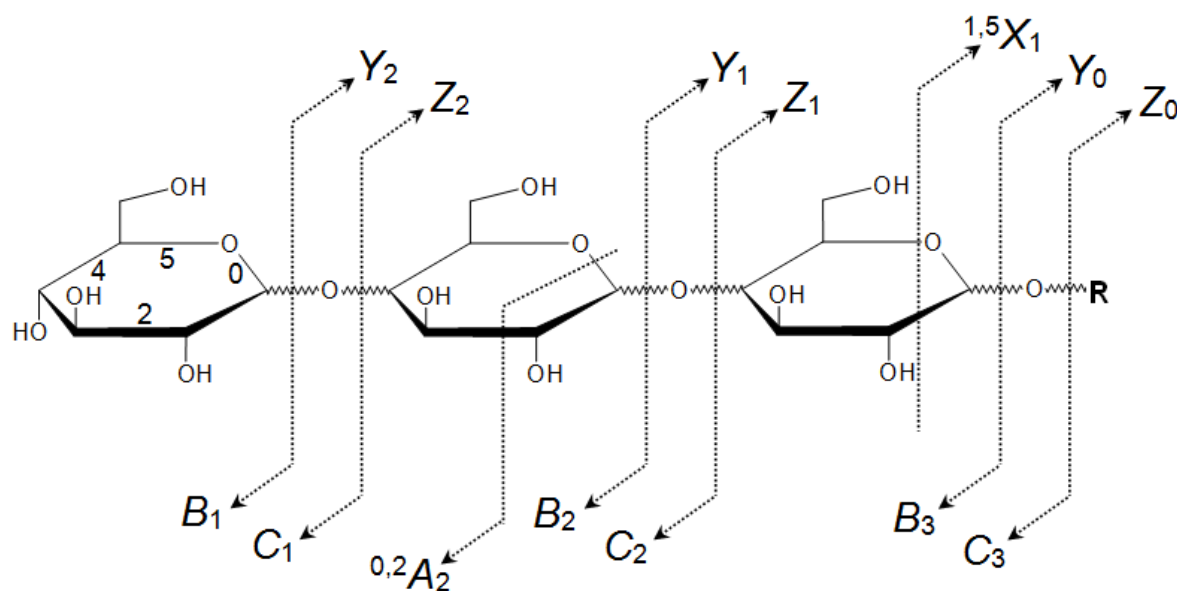
**Figure 13.** Common problems found in carbohydrate analysis with using alkali metals adducts.

Oligosaccharides or glycoconjugates containing acidic monosaccharides such as uronic and neuraminic acids can be directly analyzed by negative ionization mass spectrometry, since they will easily appear as deprotonated ions. Negative ions can also be formed during ionization of neutral carbohydrates, these can be formed by adduct with anions, such as  $\text{Cl}^-$   $[\text{M} + \text{Cl}]^-$ , or then by deprotonation of some hydroxyl group to form  $[\text{M} - \text{H}]^-$  or  $[\text{M} - 2\text{H} + \text{Na}]^-$ .

## 2.4. Tandem-MS of oligosaccharides and glycoconjugates

Carbohydrates can complex with different adduct ions to produce charged molecules. However this complexation is, sometimes, not so stable to overcome the high energy events of fragmentation process, such as in collision induced dissociation (CID). Thus, the fragmentation pathway of carbohydrates is directly affected by ionization methods, but conserves similar characteristics; the fragmentation of carbohydrates occurs mainly on the glycosidic linkages, involving the dissociation of the two adjacent monosaccharides. Considering that the charge is not necessarily retained on a specific monosaccharide residue, this cleavage can releases fragment-ions from both sides, the reducing and non-reducing ends. These fragments can undergo to other fragmentation cycles giving rise the internal fragment-ions. Less abundant, the cross-ring fragmentation occurs with a double cleavage, in which the carbon-carbon or carbon-oxygen linkage is broken within the monosaccharide ring. Although not always observed, this kind of fragments may aid to describe the interglycosidic linkages.

To sort these different types of fragments observed in tandem-MS of glycoconjugates, Domon and Costello (1988) [35] proposed a nomenclature to assign them. Thus, following their nomenclature, when the charge is retained on carbohydrate non-reducing end, and the fragment-ions contain exclusively the glycan moiety, they are named *A*, *B* and *C*. But if the charge is retained on the aglycone (not carbohydrate) moiety, these fragments are termed *X*, *Y* and *Z* (Fig 14). The ions *B*, *C*, *Z* and *Y* are those produced by glycosidic cleavage, and they are accompanied by subscript numbers that refer to the number of monosaccharide residues that the fragment means (e.g. ion  $B_2$  means a fragment containing two monosaccharide residues lacking aglycone, counted from the non-reducing end, whereas  $Y_2$  means a fragment containing two monosaccharide residues linked to aglycone, counted from aglycone). Additional symbols  $\alpha$  and  $\beta$  can be used to designate branched oligosaccharides. The fragment-ions *A* and *X* are those from the cross-ring cleavage, being accompanied by subscript numbers referring to the number of monosaccharide residues, preceding by the superscript numbers indicating the position from which bonds within monosaccharide rings were cleaved (e.g. a fragment assigned as  $^{0,2}A_2$  indicates a cleavage occurring on bond positions 0 and 2, containing two monosaccharides residues, as shown on Fig. 14).

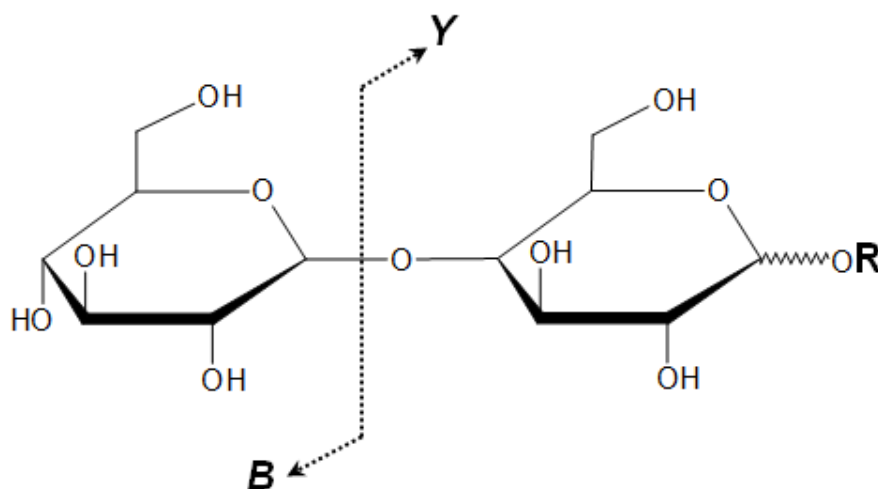


**Figure 14.** Cleavage profile of oligosaccharides and the fragment-ion nomenclature, R should be any compound different of a carbohydrate, named aglycone

For the most oligosaccharides and glycoconjugates  $\text{Li}^+$  seems to be a good choice [36], because the complex [oligosaccharide or glycoconjugate +  $\text{Li}^+$ ] is not missed when undergoes the CID, allowing the use of less energy to produce good fragment-ions. Actually, not only oligosac-

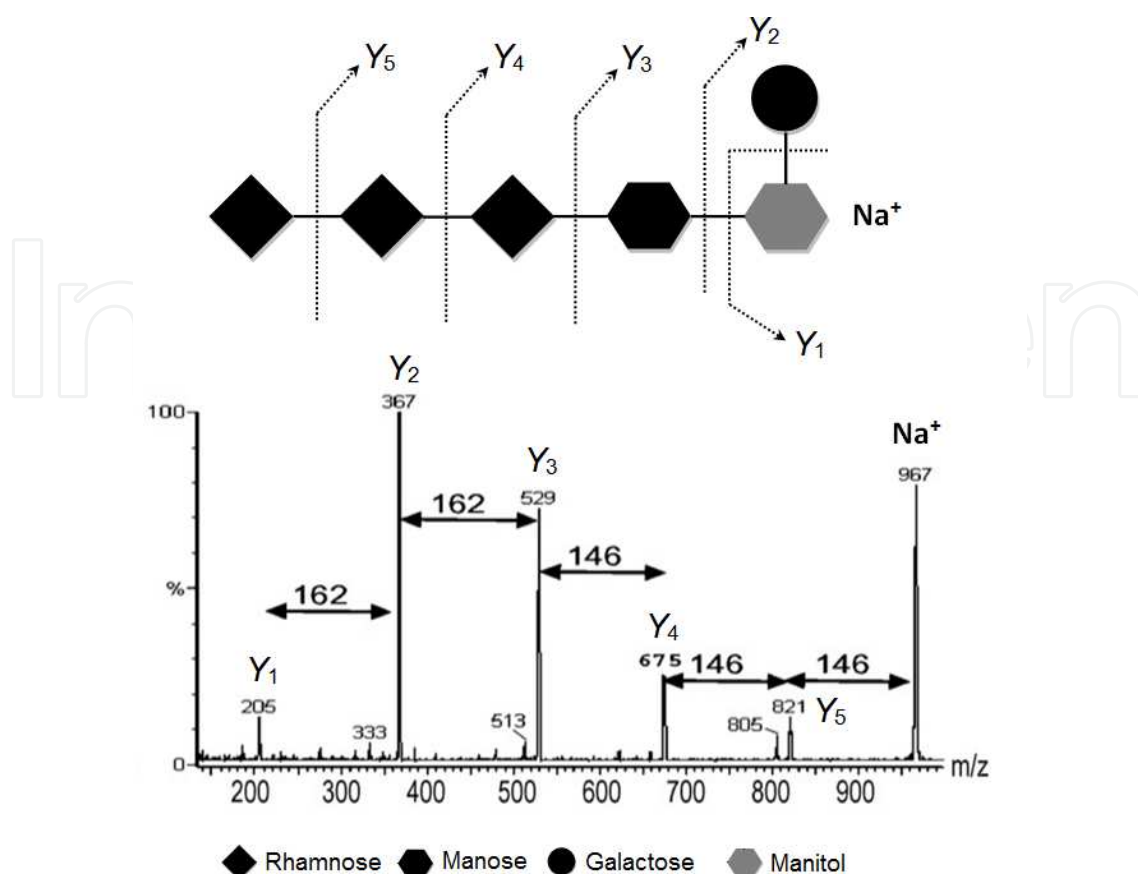
charide or glycoconjugates are better fragmented using  $\text{Li}^+$  as adduct, even some stable ether bond can be broken.

Despite the large amounts of  $\text{K}^+$  in samples from vegetables,  $\text{Na}^+$  adducts are the most common ion in carbohydrate analysis and both can be used to produce  $[\text{M} + \text{Na}]^+$  or  $[\text{M} + \text{K}]^+$ . However,  $\text{K}^+$  adducts are lesser stable than  $\text{Li}^+$  or  $\text{Na}^+$ , and the complexation is lost during CID, thus  $\text{K}^+$  usually produces poor fragments or, they are missed. Although the varied cleavage possibilities, the fragments often observed for the oligosaccharide are those from glycosidic linkage cleavage, where the oxygen is retained on the fragment from reducing end, yielding mainly *B* or *Y* fragments, according to the place where the charge will remain lodged (Fig.15).



**Figure 15.** Typical cleavage site of oligosaccharides

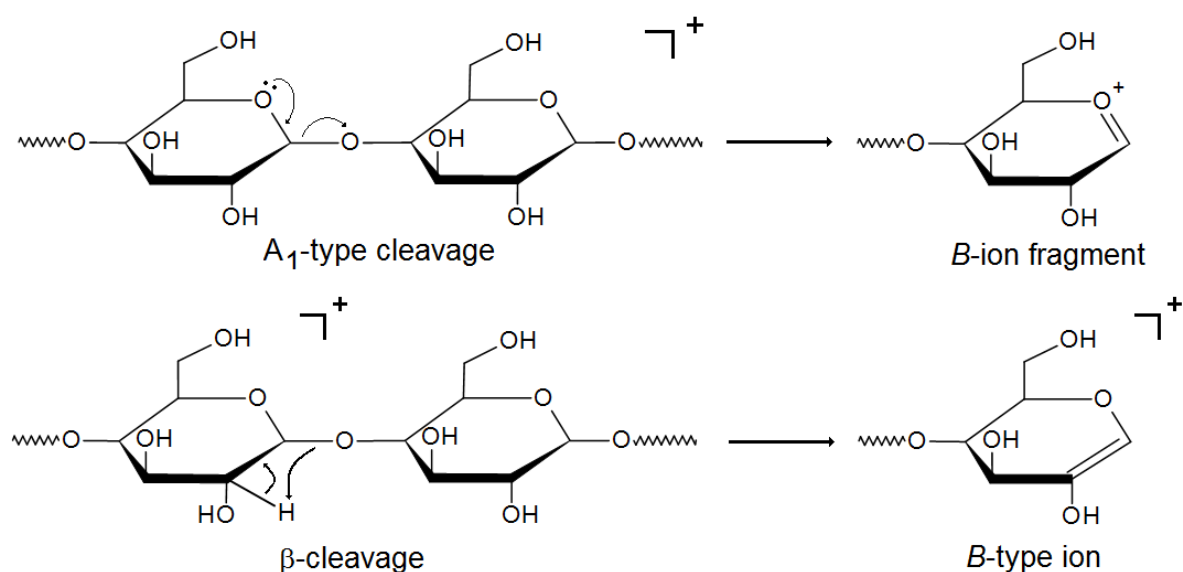
In the oligosaccharides obtained by reductive  $\beta$ -elimination the reduced terminus seems to create a favorable environment to lodge metal adducts [34]. Also, the reduction gives an increment of 2 m.u., useful to differentiate both, non-reducing and the reduced ends. Additionally, with adduct placed on the reduced end all fragment-ions formed will be *Y*-type and no internal fragments will be produced, simplifying the spectrum interpretation (Fig. 16).



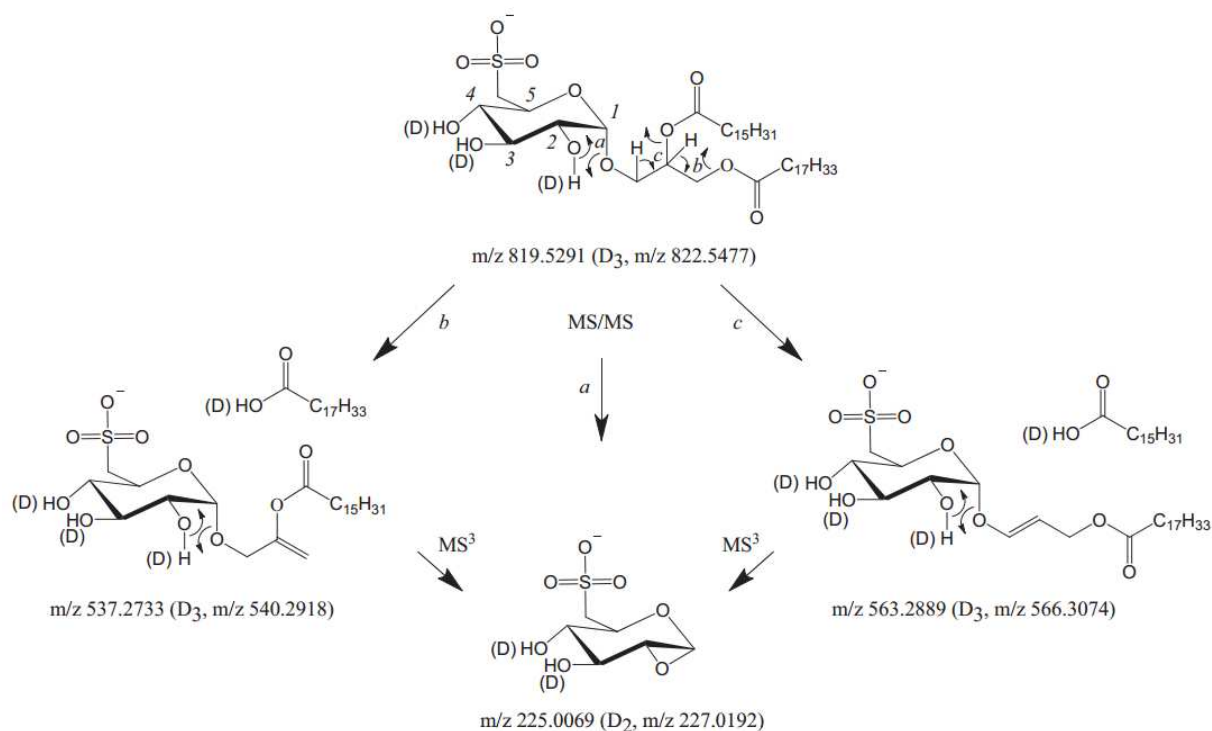
**Figure 16.** CID-MS profile of a reduced oligosaccharide from *Scedosporium prolificans* (Adapted from Barreto-Bergter et al., International Journal of Biological Macromolecules, 42, 93-102, 2008.)

The difference between the fragments indicates the type of monosaccharide, and for common non-derivatized oligosaccharides, a simple rule describes their monosaccharide sequence as follows: differences of 132 m.u. indicate a pentose, 146 m.u. indicates a deoxyhexose and 162 m.u. indicates a hexose (Fig.16). This occurs because the oxygen remains on the Y-type fragments, being recovered as a hydroxyl group via  $H^+$  transfer from the adjacent unit which, on the other hand, appears as a dehydrated residue to give  $150 - 18 = 132$  Da for pentoses,  $164 - 18 = 146$  Da for dexoyhexoses and  $180 - 18 = 162$  Da for hexoses. This can be employed for de most monosaccharides even those complex. Since in the ionization with alkali metals, the complexation occurs randomly, B-type fragments is frequently observed, but not similar to classical formation of B-fragments that involves the oxonium formation via  $A_1$ -type cleavage pathway, instead the mechanism is similar to a  $\beta$ -cleavage with a double bond between C1 and C2 (Fig 17) [33].

For the negatively charged sulfonolipids, an alternative fragmentation pathway involving an epoxide formation between C1 and C2 are also described. To study this fragment, Zhang and coworkers [37] promoted a deuterium exchange on the free hydroxyl with an increment of 3 m.u. in the entire sulfonolipid, thus they were able to observe a lost of a deuterium during CID-MS (Fig 18).



**Figure 17.** Typical cleavage mechanism observed in oligosaccharides leading to formation of *B* fragment-ions.

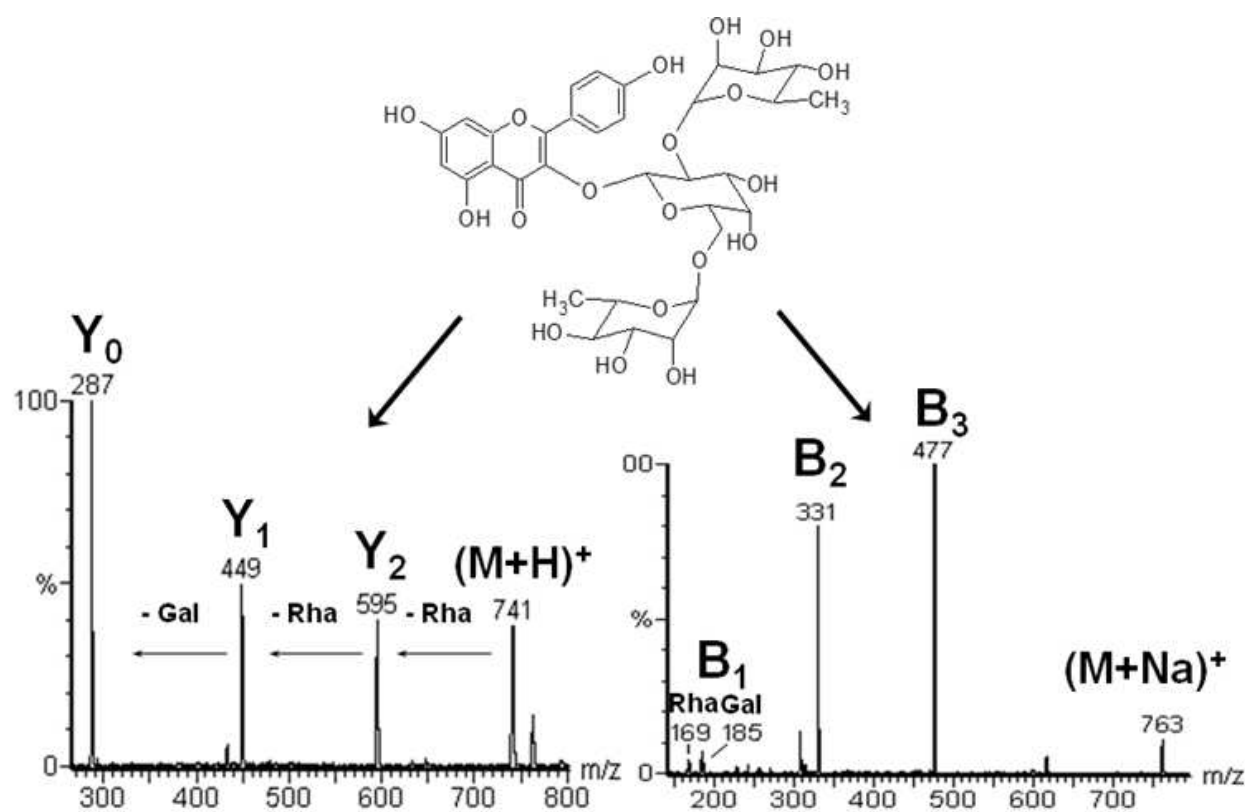


**Figure 18.** Cleavage mechanism observed for sulfoglycolipids that leads to formation of an epoxide between C-1 and C-2 (Reprduced from Zhang et al., International Journal of Mass Spectrometry, 316–318, 100–107, 2012.)

The ionization of glycoconjugates will vary according to the nature of aglycone moiety, but the glycan moiety enables ionization with the adducts cited above. Therefore, the choice for a specific ionization mode will constrain the fragmentation behavior. To exemplify, let's



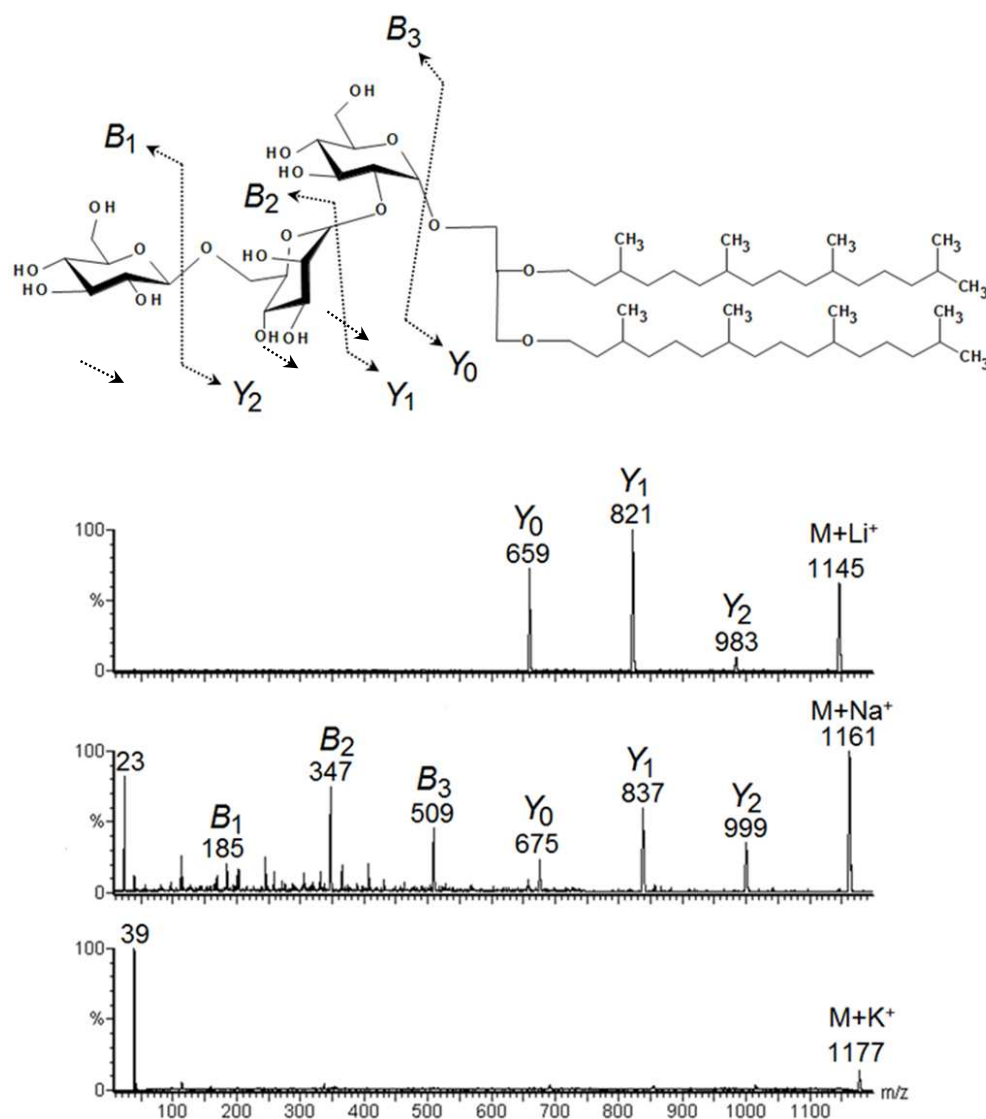
consider a glycoconjugate in which the charge is permanently retained on aglycone moiety. All the observed fragment-ions should be *X*, *Y* or *Z*, missing those of *A*, *B* and *C*. However, the *A*, *B*, *C* fragment-ions may be important to describe the structure. This occurs in glycosides from flavonoids, which can be ionized in the negative mode  $[M-H]^-$  or positive mode  $[M+H]^+$ . In both situations, the charge is retained on the flavonoid (aglycone) and consequently only the fragment-ions *X*, *Y* and *Z* are produced. Considering that glycans can be attached in any hydroxyl group from flavonoid and sometimes in more than a single glycosylation site, the formation of *B* or *C* fragment-ions is critical to confirm the presence of the oligosaccharide structure, as shown in Fig. 19. To overcome this situation, the flavonoid-glycosides can be ionized with  $Na^+$  or  $Li^+$ , since they have great affinity to the hydroxyl group from carbohydrates, they will provide mainly the fragments from the series *A*, *B*, *C*, complementing the information obtained from  $[M+H]^+$  (Fig. 19) [38].



**Figure 19.** Changes in fragmentation behavior by using different adducts. The protonated ion gives rise to *Y*-type fragments whereas sodiated ion produces *B*-type fragments. The  $B_3$  fragment confirms the presence of the trisaccharide moiety, being missed in protonated ion. (Adapted from Souza et al., Journal of Chromatography A, 1207, 101-109, 2008.)

As it was previously considered,  $Li^+$  adducts produces better fragments from glycosides than other common alkali metals; though each situation must be evaluated as unique. It is well established that glycolipids produces good ionization and fragmentation as adduct of  $Li^+$ . However, Souza and Sassaki studying the fragmentation behavior of a glycolipid from *Haloarcula marismortui* [39], an Archaea, noted the absence of the series *A*, *B*, *C* in the fragmen-

tation profile of a triglycosylarchaeol (TGA), when  $\text{Li}^+$  was used, but these ions were well produce with  $\text{Na}^+$  and all fragments were poorly produce with  $\text{K}^+$  (not published data). Unlike acylglyceroglycolipids or sphingoglycolipids from other living cells, lipids from Archaea contain two branched chains in ether linkage with glycerol called archaeol. The ether linkages might create an environment to accommodate the charge  $[\text{M}+\text{Li}]^+$  and thus allowing the formation of only Y-type fragment-ions (Fig. 20)



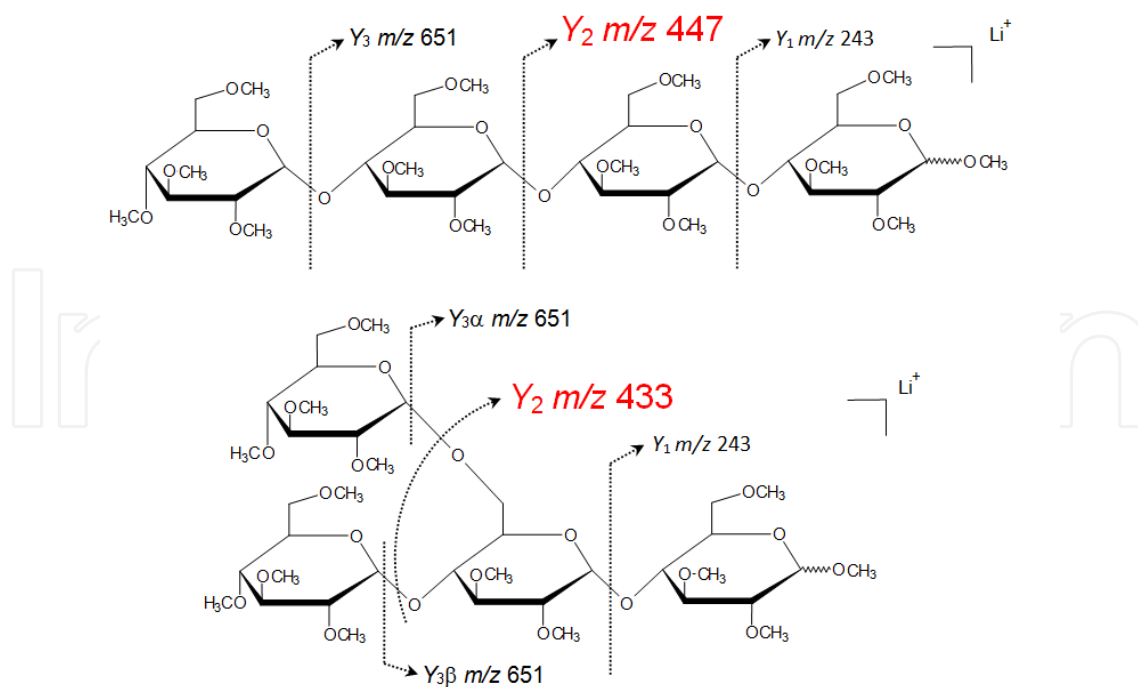
**Figure 20.** Fragmentation behavior of a triglycosylarchaeol from *Haloarcula marismortui* using different alkali metals as adducts. Not published data.

### 3. Tandem-MS of derivatized oligosaccharides and glycoconjugates

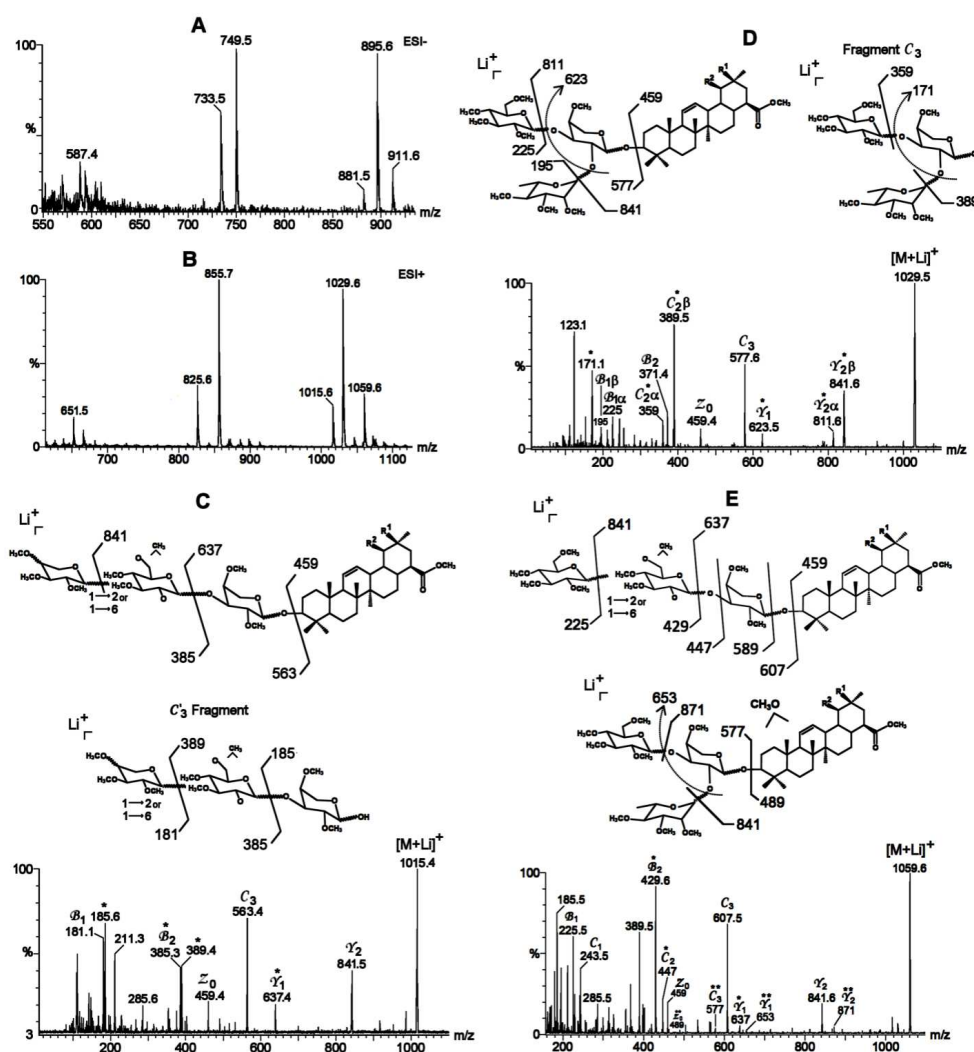
#### 3.1. Per-O-methylation: Sequence and branching information

Per-O-methylation has dual importance in the carbohydrates analysis, the first was previously described, where the partially methylated alditol acetates allow determining the interglycosidic linkages via GC-MS analysis. However, permethylation for entire oligosaccharide analysis allows the using of more volatile solvents, which improves desorption and desolvation of the molecules in techniques such as ESI, enhancing the ion production and sensitive.

The fragmentation profile from per-O-methylated oligosaccharides also contains additional information about linear and branched oligosaccharides, barely observed from the native oligosaccharides. They can be well distinguished from per-O-methylated oligosaccharides, due to the production of different fragment-ions from both chains. This occurs because each methyl radical added to a monosaccharide augment its mass in 14 m.u. thus, the Y-type fragment from the branched unit must have 14 m.u. lesser than other, due to the non-methylated hydroxyl exposing (Figures 21 and 22). This is especially important in glycans from glycoproteins analysis. Another advantage of per-O-methylation concerns about the identification of internal fragments, since the reducing and non-reducing ends can be easily located by the mass increments, but those fragments from internal cleavage will appear with a mass depletion due to the non-methylated hydroxyl exposing.



**Figure 21.** Schematic representation of cleavage sites of branched and non-branched oligosaccharides. It worth noting the fragment-ion Y<sub>2</sub> which are distinctive for both structures

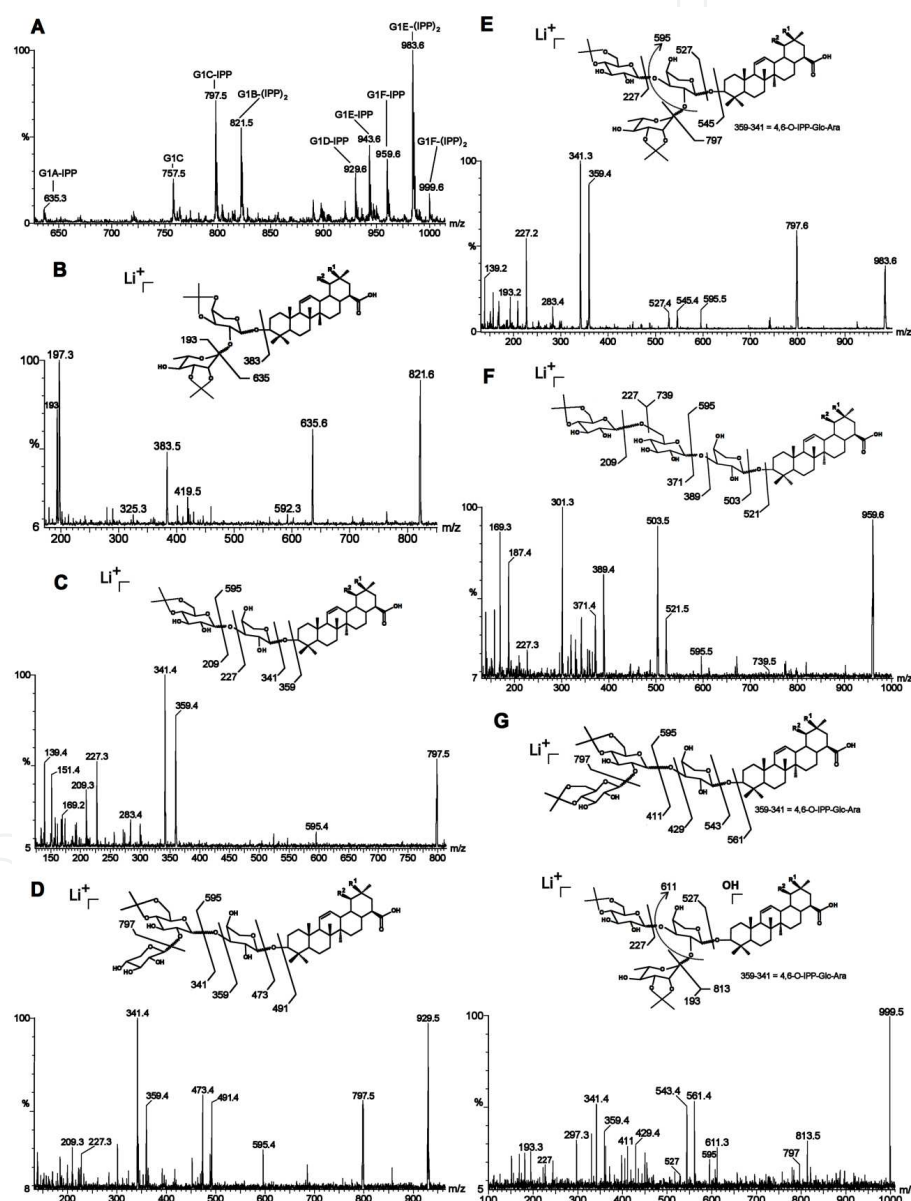


**Figure 22.** Application of per-O-methylation/CID-MS analysis for identification of matesapoinins. (A) Non-derivatized saponins and (B) per-O-methylated. Fragments obtained from derivatized precursor-ions confirm if oligosaccharide chains are linear or branched, as mainly indicated by fragment  $\gamma_1$ , at  $m/z$  637 (C),  $m/z$  623 (D) and  $m/z$  637/653 (E). (Reproduced from Souza et al., Journal of Chromatography A, 1218, 7307-7315, 2011).

### 3.2. Isopropylidene ketals: Interglycosidic linkage and monosaccharide information

The presence of several diastereomers from monosaccharides can cause many misinterpretation due to mass spectrometry cannot distinguished among spatial isomers. This situation is common in plant metabolites that contain a series of galactose and/or glucose glycosides. To overcome this, Souza et al (2012) developed a strategy based on ketal reactions to analyze a mixture of saponins [40]. Since the isopropylidene ketals are formed according to hydroxyl configuration, requiring free vicinal hydroxyl in *cis* configuration, they are selective formed in some monosaccharides. For example, galactopyranose (Galp) has hydroxyl 3 and 4 in *cis* configuration suitable for the ketal formation, whereas glucopyranose (Glc) does not. However, the ketal can be formed on Glc if the hydroxyls 4 and 6 are not linked. Thus, even in mixtures many compounds could be well identified in terms of composition and interglycosidic linkages.

To describe the interglycosidic linkages from a mixture, it is appropriated to have a previous knowledge of the types of linkages that can be finding in the mixture, being easily accessed by PMMA/GC-MS analysis. Thus, with the amount of information obtained by previous analysis, the isopropylidene ketals will indicate exactly those glycosides are attached by galactose or glucose and the type of interglycosidic linkage that unit is involved (Fig 23). These methodologies are under recent development, but good prospects are glimpsed to be extended to other glycosides, including the direct identification of monosaccharides isomers (Fig. 23) employing multiple-stage tandem-MS.



**Figure 23.** Application of isopropylidene ketals in matesapoints analysis (A). Ambiguous interglycosidic linkage observed on PMAA/GC-MS analysis of mixtures can be resolved by ketal formation (B-G). For more details consult Souza et al., *Journal of Chromatography A*, 1218, 7307-7315, 2011 (reproduction).



## Author details

Guilherme L. Sassaki and Lauro Mera de Souza

Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba-PR, Brazil

## References

- [1] Dass, C. Fundamentals of contemporary mass spectrometry. John Wiley & Sons, Hoboken, New Jersey, 2007.
- [2] Hoffmann, E.; Stroobant V. Mass Spectrometry: Principles and Applications. John Wiley & Sons, Chichester, UK. Third Edition 2007.
- [3] Wolfrom, M. L., Thompson, A. *Meth. Carbohydr. Chem.*, v. 2, p. 65-67, 1963.
- [4] Wolfrom, M.L.; Thompson, A. *Meth. Carbohydr. Chem.*, v. 2, p. 211-215, 1963
- [5] Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. *J. Chem. Soc., Chem. Commun.*, p. 325-327, 1981.
- [6] Galanos, C.; Lüderitz, O.; Himmelsbach, K. *Eur. J. Biochem.* v. 8, p. 332-336, 1969.
- [7] Hay, G.W.; Lewis, B.A.; Smith, F. *Methods Carbohydr. Chem.* (1965) 357-361.
- [8] Goldstein, I.J.; Hay, G.W.; Lewis, B.A.; Smith, F. *Methods Carbohydr. Chem.* (1965) 361-370.
- [9] Leitao, E. A.; Bittencourt, V. B.; Haido, R. M. ; Valente, A. P.; Peterkatalinic, J.; Letzel, M.; Souza, L. M.; Barreto-Bergter, E. *Glycobiology*, v. 13, n.10, p. 681-692, 2003.
- [10] Kuraya, N.; Hase, S. *J. Biochem.* v. 112, p.122-126, 1992.
- [11] Patel, T.; Bruce, J.; Merry A.; Bigge, C.; Wormald, M.; Parekh R.; Jaques A. *Biochem.* v. 32, p. 679-693, 1993.
- [12] Tarentino, A.L.; Gomez, C.M.; Plummer, T.H.Jr. *Biochem.* v. 24, p. 4665-4671, 1985
- [13] Altmann, F.; Paschinger, K.; Dalik, T.; Vorauer, K.; *Eur. J. Biochem.* v. 252, p. 118-123, 1998.
- [14] Varki, A.; Freeze, H.H.; Manzi, A.E.; Coligan J.E. (Ed.), *Current Protocols in Protein Science* (1995), John Wiley & Sons, Hoboken, NJ (2001), p. 12.1.1
- [15] Biermann, C.J.; McGinnis, G.D. (Eds.), *Analysis of Carbohydrates by GLC and MS*, CRC Press, Boca Raton, FL (1989)
- [16] Zanetta, J.P.; Timmerman, P.; Leroy, Y. *Glycobiol.* v. 9 p. 255, 1999.

- [17] Sweley, C.C.; Bentley, R.; Makita, M.; Wells, W.W. *J. Am. Chem. Soc.*, v. 85, p. 2497, 1963.
- [18] Knapp, D.R. 1979. Handbook of analytical derivatization reactions. New York: John Wiley & Sons.
- [19] Carpita, N.C.; Shea, E.M.; Biermann, C.J.; McGinnis, G.D. (Eds.), Analysis of Carbohydrates by GLC and MS, CRC Press, Boca Raton, FL (1989), p. 157.
- [20] Sasaki, G.L.; Souza, L.M.; Serrato, R.V.; Cipriani, T.R.; Gorin, P.A.J.; Iacomini, M. *J. Chromatogr. A*, v. 1208, p. 215-222, 2008.
- [21] Taylor, R. L.; Conrad, H. E. *Biochem.* v. 11, p. 1383-1388, 1972.
- [22] Kochetkov, N.K.; Chizhov, O.S. *Adv. Carbohydr. Chem. Biochem.*, v. 21, p. 39, 1966.
- [23] Lönngren, J.; Svensson, S. *Adv. Carbohydr. Chem. Biochem.* v. 29, p. 41, 1974
- [24] Haworth, W.N. *J. Chem. Soc.* v. 107, p. 8-16, 1915.
- [25] Kuhn, R.; Trischmann, H. *Löw Angew. Chem.* v. 67, p. 32, 1955.
- [26] Hakomori, S.I. *J. Biochem.* v. 55, p. 205-207, 1964.
- [27] Ciucanu, I.; Kerek, F. *Carbohydr. Res.* v. 131, p. 209-217, 1984.
- [28] Jansson, P.E.; Kenne, L.; Liedgren, H.; Lindberg, B.; Lönngren, J. *Chem. Commun.* v. 8, p. 1-70, 1970.
- [29] Sasaki, G.L.; Gorin, P.A.J.; Souza, L.M.; Iacomini, M. *Carbohydr. v. 340*, p. 731-739, 2005.
- [30] Sasaki, G.L.; Iacomini, M.; Gorin, P.A.J. *Anais da Academia Brasileira de Ciências*, v. 77, p. 223-234, 2005.
- [31] Purdie, T.; Irvine, J.C. *J. Chem. Soc.* v. 83, p. 1021-1037, 1903.
- [32] Sasaki, G.L.; Czelusniak, P.A.; Vicente, V.A.; Zanata, S.M.; Souza, L.M.; Gorin, P.A.J.; Iacomini, M. *Int. J. Biol. Macromol.*, v. 48, p. 177-182, 2011.
- [33] Dell, A. *Adv. Carbohydr. Chem. Biochem.*, v. 45, p. 19-72, 1987
- [34] Xia, B.; Feasley, C.L.; Sachdev, G.P.; Smith, D.F.; Cummings, R.D. *Anal. Biochem.* v. 387, p. 162-170, 2009.
- [35] Domon B, Costello CE. *Biochemistry*. 1988 Mar;27(5):1534-43 *Res.* v. 340, 731-739, 2005.
- [36] Levery, S.B. *Met. Enzymol.* v. 405, p. 300-369, 2005
- [37] Zhang, X.; Phaner, C.J.; Ferguson-Miller, S.M.; Reid G.E. *Int. J. Mass Spectr.* v 316-318, p. 100-107, 2012.
- [38] Souza, L.M.; Cipriani, T.R.; Serrato, R.V.; Costa, D.E.; Iacomini, M.; Gorin, P.A.J.; Sasaki, G.L. *J. Chromatogr. A*, v.1207, p. 101-109, 2008.
- [39] Souza, L.M.; Muller-Santos, M.; Iacomini, M.; Gorin, P.A.J.; Sasaki, G.L. *J. Lipid Res.*, v. 50, p. 1363-1373, 2009.



- [40] Souza, L.M.; Dartora, N.; Scoparo, C.T.; Cipriani, T.R.; Gorin, P.A.J.; Iacomini, M.; Sasaki, G.L. *J. Chromatogr. A*, v.1218, p. 7307-7315, 2011.

IntechOpen

IntechOpen

