BIOCHEMICAL DIAGNOSIS OF DISEASES CAUSING OLIGOSACCHARIDURIA BY MASS SPECTROMETRY

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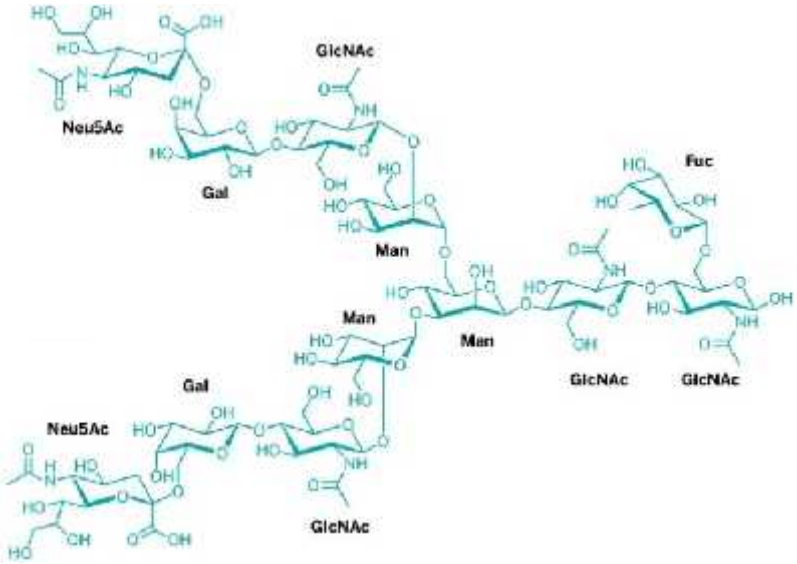
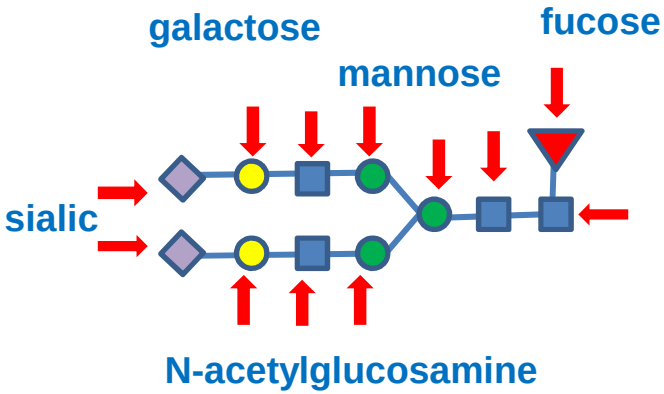
The oligosaccharides are polymers of a small number of monosaccharides

For example:
Biantennary N-glycan, composed of:





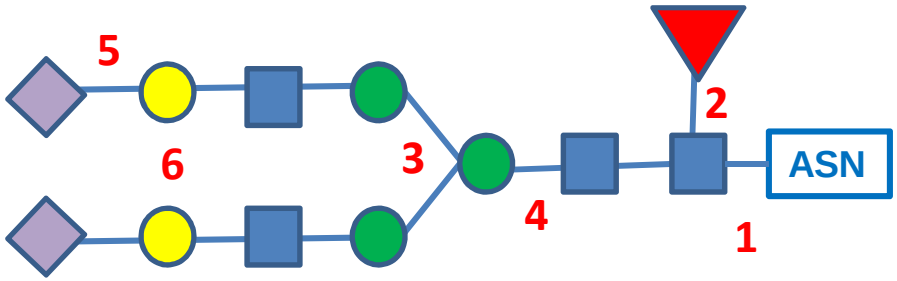
The oligosaccharidurias are a group of lysosomal storage dieases that excrete oligosaccharides above normality.

Depending on the affected lysosomal enzyme, different oligosaccharides accumulate in tissues, organs… and are excreted in urine.

These enzymes are involved in the catabolism of:

- **N-linked glycoproteins (Oligosaccharidoses)**
- **O-linked glycoproteins (Oligosaccharidoses)**
- **Glycosphingolipids**
- **Others**

OLIGOSACCHARIDOSES (1): N-Linked Glycans Catabolism Defects



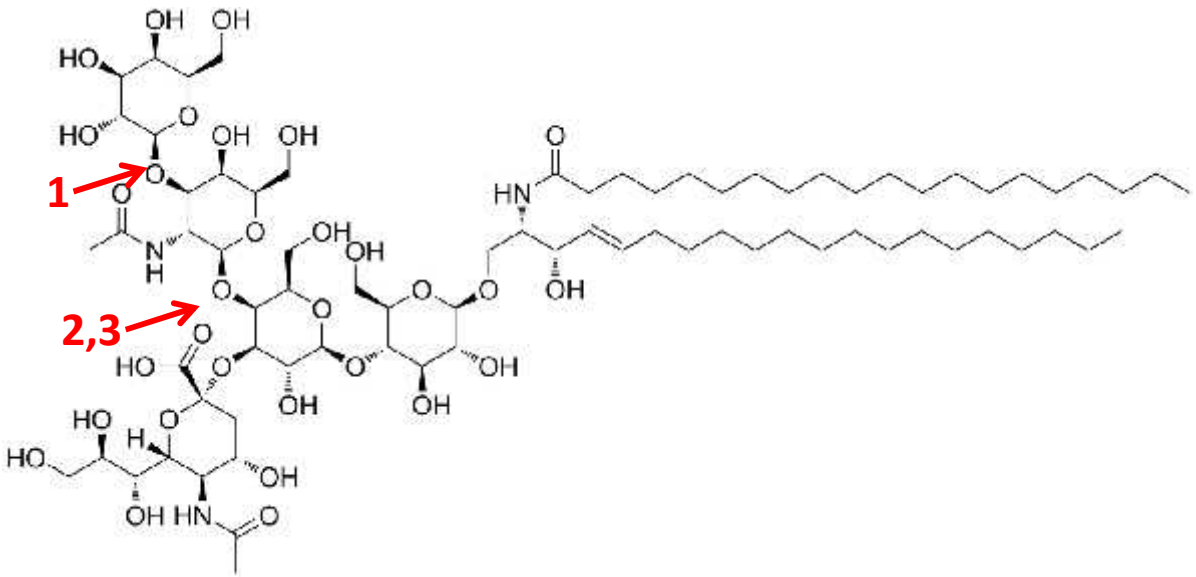
Deficiency	Disease
1. Aspartylglucosaminidase	aspartylglucosaminuria
2. Alpha-L-fucosidase	fucosidosis
3. Alpha-mannosidase B	alfa-mannosidosis
4. Beta-mannosidase	beta-mannosidosis
5. Alfa-Neuraminidase	sialidosis (mucolipidosis I)
6. Cathepsin	galactosialidosis
7. Sialin	sialuria (Salla disease, SASD)

OLIGOSACCHARIDOSES (2): O-Linked Glycans Catabolism Defects

Deficiency	Disease
Alfa-N-acetylgalactosaminidase	Schindler disease Kanzaki disease

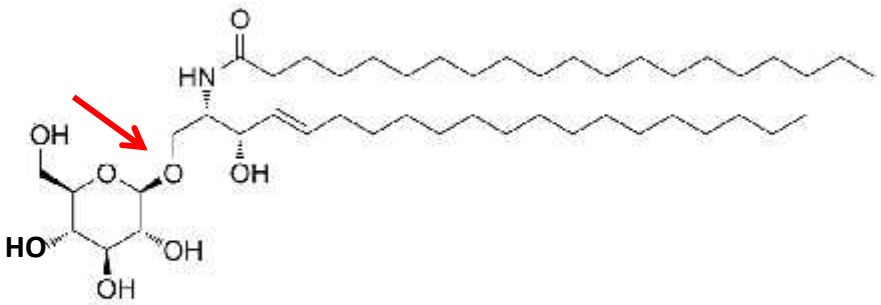
Other Oligosaccharidurias: Glycosphingolipids catabolism defects
Concerning the catabolism of Gangliosides

Deficiency	**Disease**
	Gangliosidosis
1. Beta-galactosidase	**GM1**
2. Beta-Hexosaminidase A isoenzyme	**GM2 (TaySachs disease)**
3. Beta-Hexosaminidase A+B isoenzymes	**GM2 (Sandhoff disease)**



Other Oligosaccharidurias: Glycosphingolipids catabolism
Catabolism of Cerebrosides

Deficiency	**Disease**
	Cerebrosidosis
Glucocerebrosidase	**Gaucher disease**



Other Oligosaccharidurias

Deficiency	Disease
	Multiple lysosomal deficiency
N-Ac-glucosamine-1P-transferase	Mucolipidosis type II, III (I-cell)
	Glycogen storage diseases (glycogenosis)
Alfa-(1,4)-glucosidase	Pompe disease (GSD 2)
Glucose-6-phosphatase	GSD 1a
Debrancher enzyme	GSD 3
Brancher enzyme	GSD 4
Hepatic phosphorylase kinase (alpha-sub.)	GSD 9a

Oligosaccharide profile in urine samples of controls

To keep in mind that:

There is a wide variation in the excretion of oligosaccharides even in healthy controls or non-lysosomal storage disease patients.

This variation could be the result of diet, parenteral nutrition, pregnancy, and drug use as well as blood group types.

Urine from breast-milk-fed children, contains a wide range of oligosaccharides from milk.
This also happens in urine samples of the mothers during breastfeeding.

The excretion of oligosaccharides decreases as age increases.

MASS SPECTROMETRY TECHNIQUES (MS)

- Mass spectrometry (MS) is an analytical technique that measures the *m/z* (mass-to-charge) ratio of the molecules present within a sample.

- **These molecules must be ionized**

- ***m/z* and molecular ion are coincident when charge is +/-1**

- ***m/z* does not change between isomers, so:**
  - **Glucose, galactose, mannose: are Hexoses (Hex)**
  - **N-Acetylglucosamine, N-acetylgalactosamine: are N-AcetylHexosamines (HexNAc)**
  - **It seems there is no isomers of fucose, sialic, glycosylneuraminic, glucuronic acids…**

MS/MS TECHNIQUES (Tandem mass spectrometry)

- MS/MS is an analytical technique that measures the *m/z* of a molecular ion and its fragments.

- **Identification, specificity, sensitivity are improved**

- **Ionization is achieved by:**
  - **-MALDI (Matrix-Assisted Laser Desorption/Ionization)**
  - **-ESI (electrospray ionization)**

- **Mass analysis (mass analysers) by:**
  - **TOF/TOF (time of flight)**
  - **QQQ (triple quadrupole)**
  - **MS^n (*MS raised to n*, by an ion trap)**

- **MS/MS can be coupled to:**
  - **-liquid chromatography**
  - **-capillary electrophoresis**

→Useful to resolve isobaric structures

MS/MS Techniques: MALDI/TOF

Samples are dry and fixed on a metal plate

This fact makes difficult to couple with online separation techniques

Offline purification methods are needed (SPE)

Ionization and analysis in positive, negative, protonated or deprotonated but also adducts with Na+ or K+

Different matrix and reagents to derivatize (or without derivatization) could be used

Sialic acid could fragment depending on the energy imparted

TOF is accurate mass analyser, with higher resolution than quadrupoles, and a wider mass range (>2000 m/z)

TOF/TOF could identify fragments (sugars), and then the sequence, in the oligosaccharide

MS/MS Techniques: TRIPLE QUADRUPOLE

There are different modes to analyse the samples

- MRM mode: multiple reaction monitoring (the analysis of 1 molecular ion and 1 specific fragment)

- Neutral loss mode: Analysis of a family of compounds with a common loss after fragmentation)

- Ion precursor mode:

Analysis of a family of compounds with a common fragment

- **The oligosaccharides can be derivatised.**

This oligosaccharide profile is strongly informative,

The abnormal compounds can be identified and also be quantified

So, it is a good alternative to traditional tests like TLC

Continuing with Oligosaccharidurias and MS/MS Techniques…

Sensitivity of ESI decreases as the mass of oligosaccharides increases, displaying a poor ionization

But: Derivatization can improve the efficiency (sensitivity, shape of chromatographic peaks…) of the analysis

There are several kinds of derivatization, e.g.:

-permethylation:

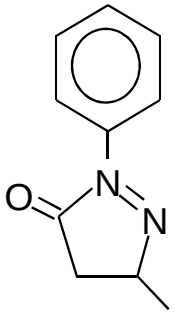
-that enables uniform ionization
- and improves sensitivity of neutral and acidic oligosaccharides

-PMP (1-phenyl-3-methyl-5-pyrazolone)

-to analyse sialylated and neutral oligosaccharides
-to produce a common fragment with *m/z* 175 in all product ion scans

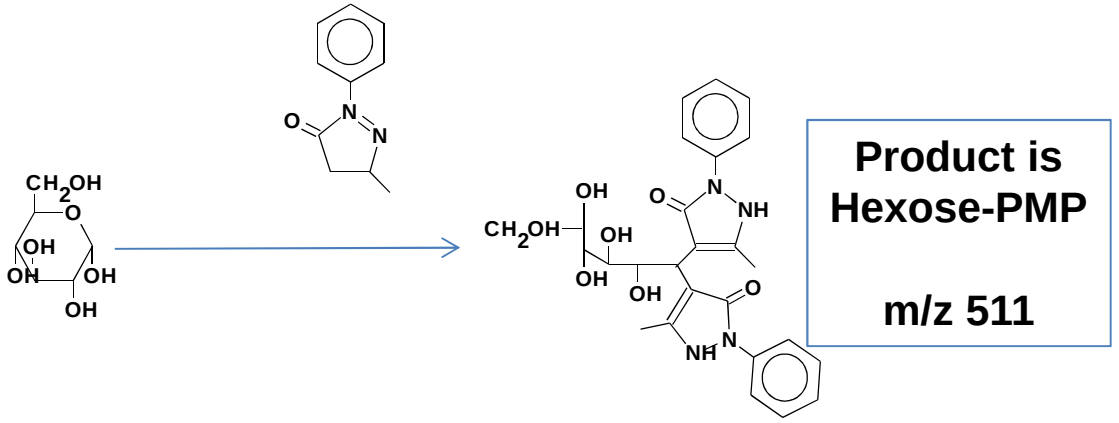
→ So, selective analysis can be done by Precursor Ion mode

Derivatisation with PMP (1-phenyl-3-methyl-5-pyrazolone)



PMP

PMP reacts with a reducing sugar:

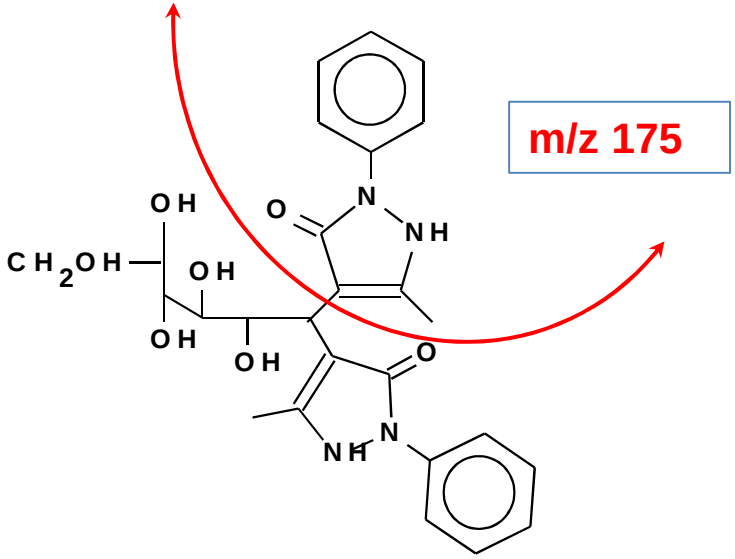


Product is Hexose-PMP

m/z 511

PMP (1-phenyl-3-methyl-5-pyrazolone)

Fragmentation of Hexose-PMP by MS/MS



So,
In Precursor Ion mode of m/z 175 analysis,

m/z = 511, that fragmens to m/z 175, will be an Hexose

In the same way…

162 is the molecular mass of one hexose, then,

511+162 = 673 → Hex_2
+162 = 835 → Hex_3
+162 = 997 → Hex_4
…

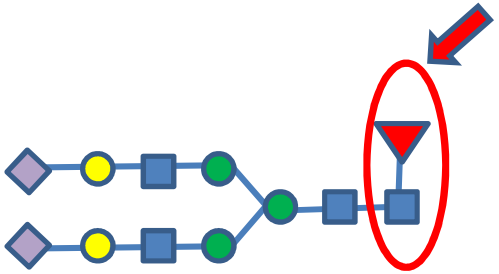
For example: MS/MS = 698/175…Which oligosaccharide could be? We can try to separate the addition into these numbers:

→ 698 = 175 + 377 + 146

Fucose

HexNAc (GlcNAc/GalNAc)

PMP



This oligosaccharide increases it excretion in FUCOSIDOSIS

Of course, previously to the MS/MS analysis, a clean-up is recommended by:

Solid Phase Extraction (SPE)

- **to remove**
  - **salts**
  - **solvents**
  - **reagents**

- **using stationary phases with**
  - **C18**
  - **carbon, carbograph**

Sample preparation and analysis of PMP-oligosaccharides by ESI/MS/MS

(based Ramsay et al. 2005 Analytical Biochem.)

1. **Urine dried under N_2**

2. **+PMP + methyllactose (internal standard) pH 9**

3. **Heated at 70 °C for 90 min**

4. **Sample is washed with chloroform to remove the excess of PMP**

5. **Clean up in SPE C18 cartridge**

6. **Extract is injected by FIA/ESI/MS/MS Sciex4500**
   Precursor ion mode of 175

7. **Quantification by the MRM pair "Molecular ion/175",**
   relating the peak high to that of the internal standard (with known concentration)

   Chemoview software.

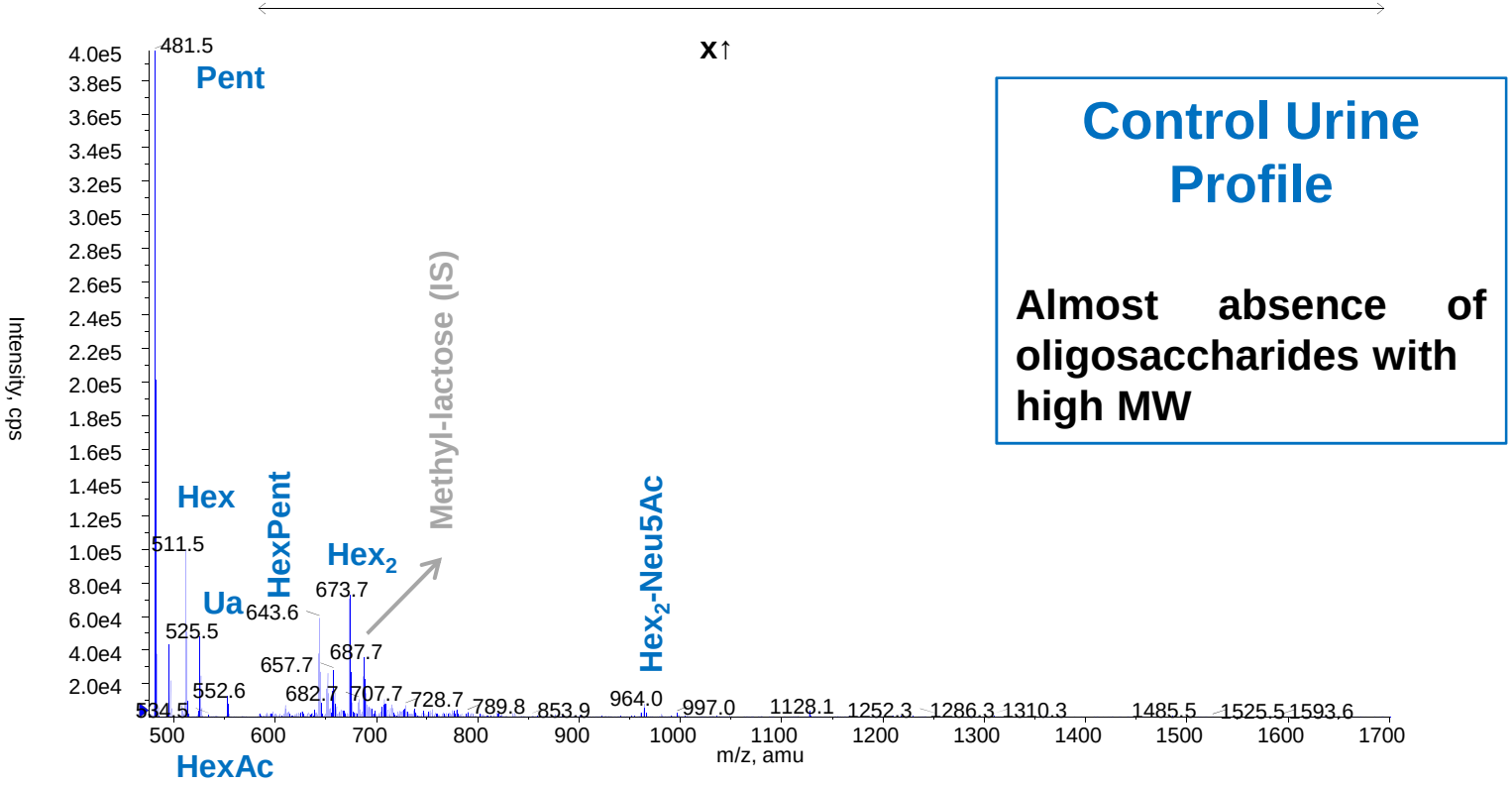
Control Values

Due to the great difference in the excretion of oligosaccharides, several groups of age ranges were established in controls:

< 1 month
2 – 6 months
7 – 12 months
> 1 year

Results of the Analysis of Oligosaccharides in urine by ESI/MS/MS

by Precursor ion scan mode of m/z 175 (PMP derivatized)

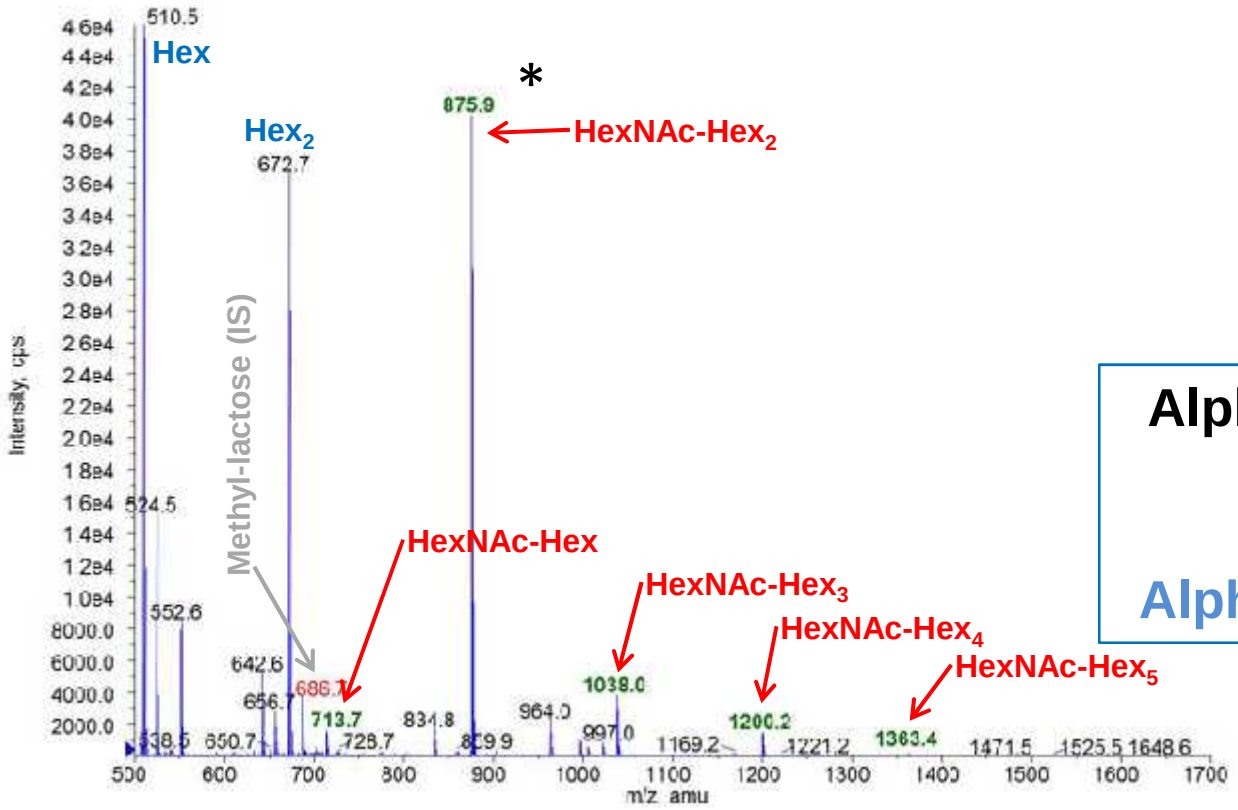


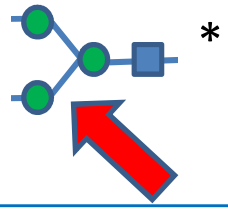
Control Urine Profile

Almost absence of oligosaccharides with high MW

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized





Alpha-mannosidase deficiency

Alpha-mannosidosis

Excretion of OS with 1 HexNAC+ several Hexoses

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ANALISIS DE OLIGOSACARIDOS EN ORINA POR ESPECTROMETRIA DE MASAS EN TANDEM

Ref: *creat:* *10,5* *mmol/L*

ion	analito	*mmol/mol creat*	V.N.
511	Hex1	**24,88**	33,77 ± 33,4
673	Hex2	**19,54**	18,77 ± 18,3
835	Hex3	**0,89**	0,63 ± 0,53
997	Hex4	**0,50**	0,33 ± 0,22
1159	Hex5	**0,02**	0,03 ± 0,04
1321	Hex6	**0,01**	0,02 ± 0,02
1483	Hex7	**0,02**	0,01 ± 0,02
714	HexNAc-Hex	**0,90**	0,57 ± 0,35
876	**HexNAc-Hex2**	**20,39**	**0,15 ± 0,11**
1038	HexNAc-Hex3	**1,86**	0,03 ± 0,03
1200	HexNAc-Hex4	**0,75**	0,02 ± 0,03
1362	HexNAc-Hex5	**0,15**	0,01 ± 0,02
1524	HexNAc-Hex6	**0,02**	0,00 ± 0,01
643	Hex-Pent	**2,59**	2,86 ± 1,68
657	Hex-dHex	**1,58**	3,22 ± 5,44
698	HexNAc-dHex	**0,16**	0,16 ± 0,10
819	Hex2-dHex	**0,10**	0,27 ± 0,27
860	HexNAc-Hex-dHex	**0,19**	0,41 ± 0,66
965	Hex2-dHex2	**0,74**	0,84 ± 0,59
1022	HexNAc-Hex2-dHex	**0,45**	0,12 ± 0,17
1127	Hex3-dHex2	**0,00**	0,02 ± 0,03
1168	HexNAc-Hex2-dHex2	**0,12**	0,14 ± 0,22
1184	HexNAc-Hex3-dHex	**0,05**	0,03 ± 0,04
1330	HexNAc-Hex3-dHex2	**0,01**	0,02 ± 0,03
1346	HexNAc-Hex4-dHex	**0,02**	0,02 ± 0,02
1492	HexNAc-Hex4-dHex2	**0,00**	0,00 ± 0,01
1508	HexNAc-Hex5-dHex	**0,00**	0,00 ± 0,01
964	Hex2-Neu5Ac	**1,17**	0,80 ± 0,47
1005	Hex-HexNAc-Neu5Ac	**0,30**	0,21 ± 0,13
1079	Hex2-HexNAc2	**0,04**	0,03 ± 0,03
1241	Hex3-HexNAc2	**0,05**	0,02 ± 0,02
1282	Hex2-HexNAc3	**0,01**	0,01 ± 0,02
1403	Hex4-HexNAc2	**0,01**	0,01 ± 0,02
1444	Hex3-HexNAc3	**0,00**	0,01 ± 0,02
1532	Hex3-HexNAc2-Neu5Ac	**0,01**	0,01 ± 0,01
1606	Hex4-HexNAc3	**0,00**	0,00 ± 0,01
1647	Hex3-HexNAc4	**0,01**	0,00 ± 0,01
1695	Hex4-HexNAc2-Neu5Ac	**0,01**	0,00 ± 0,01
1345	Neu5Gc-HexNAc-Hex3	**0,04**	0,01 ± 0,02
1695	Hex5-HexNAc3	**0,01**	0,00 ± 0,01
1851	Hex3-HexNAc5	**0,00**	0,00 ± 0,00
525	UA	**8,07**	15,10 ± 14,32
552	HexNAc	**4,40**	5,24 ± 3,87
632	HexNAc-(S)	**0,19**	0,21 ± 0,14

Analysis of Oligosaccharides in urine by ESI/MS/MS

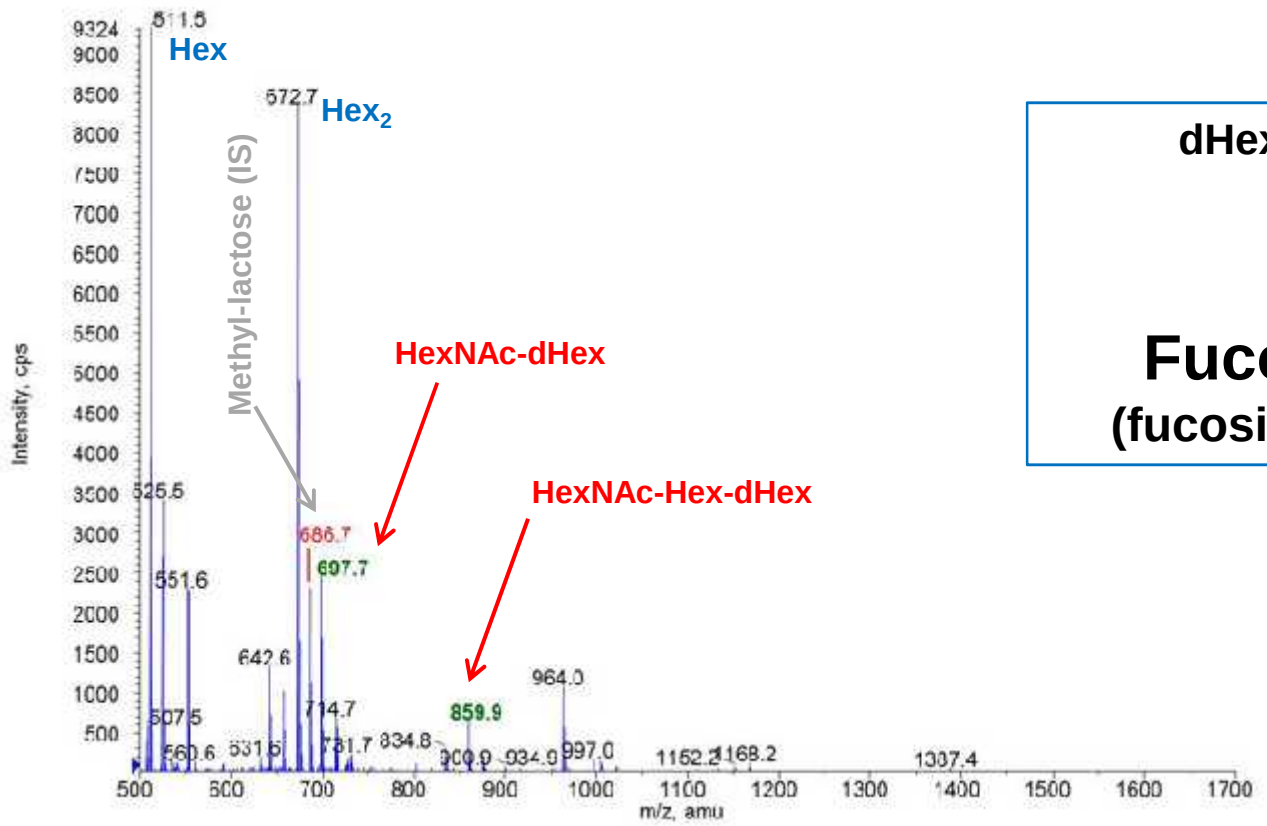
Concentrations (mmol/mol creat.)

Alpha-mannosidase deficiency

Alpha-mannosidosis

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized



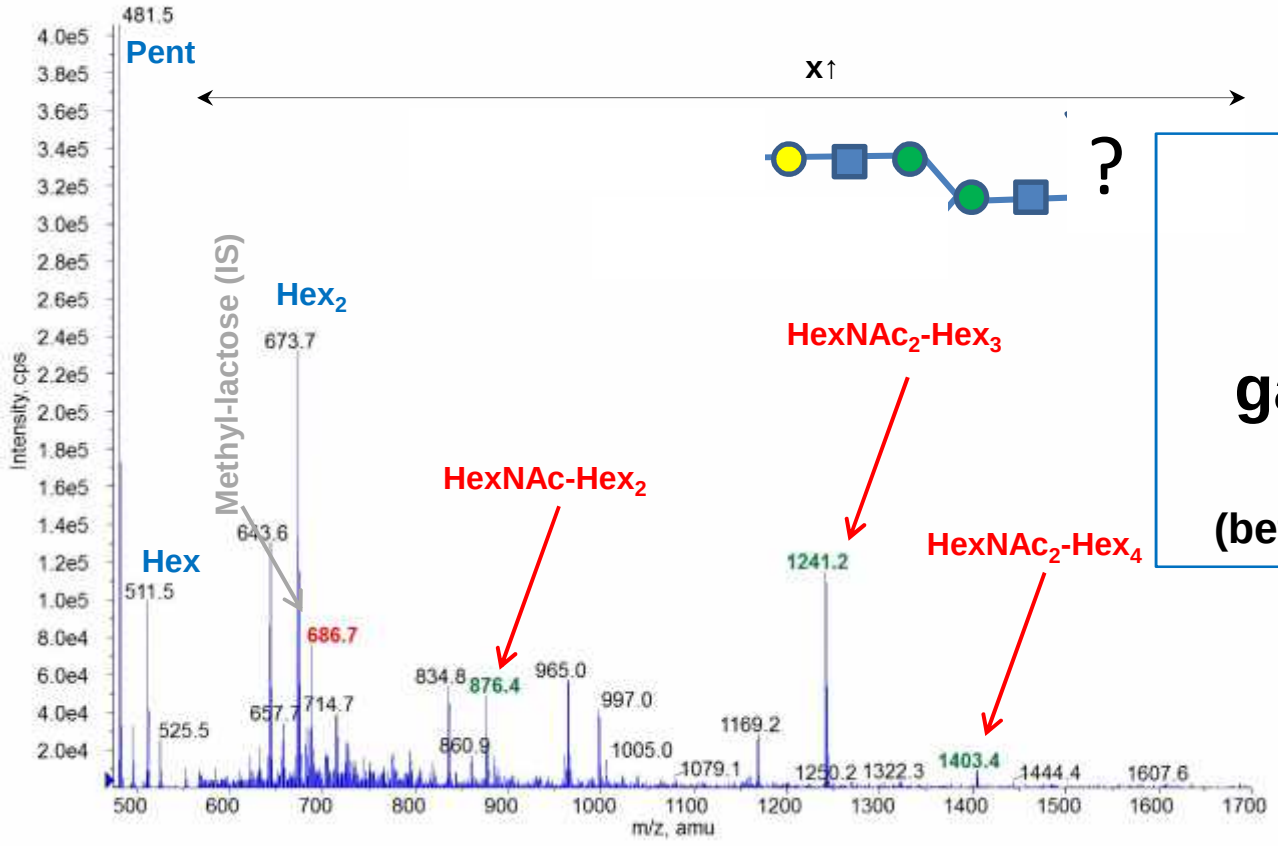
dHex ≡ Fucose

Fucosidosis
(fucosidase defect)

Increased excretion of oligosaccharides with dHex (deoxyhexoses)

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized



Hex: Man, Gal

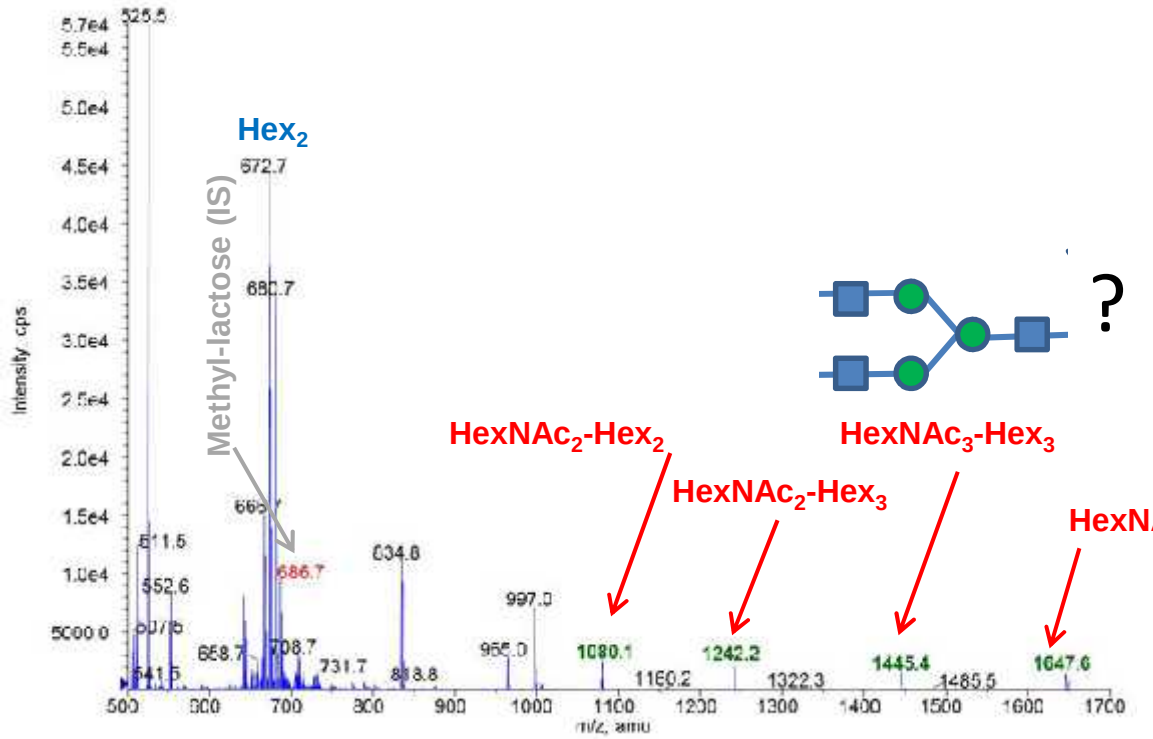
GM1 gangliosidosis

(beta-galactosidase def)

Increased excretion of oligosaccharides with different numbers of Hex and HexNAc

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized



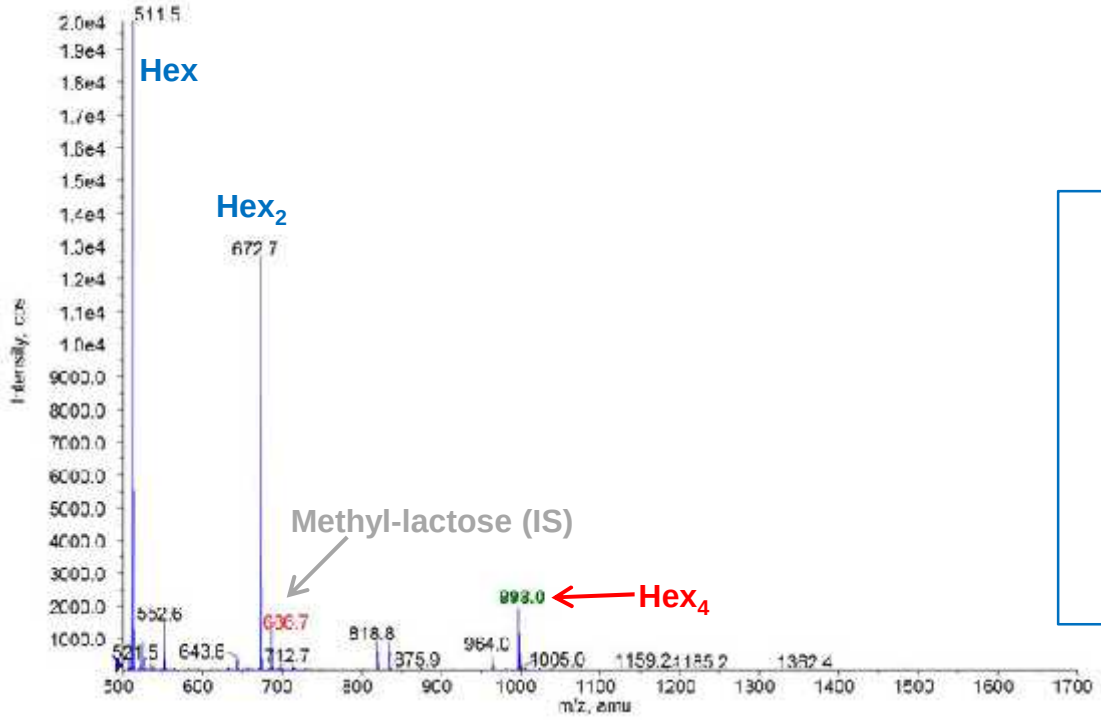
GM2 gangliosidosis Sandhoff

(beta-hexosaminidase def)

Increased excretion of oligosaccharides with different numbers of Hex and HexNAc

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized



Hex_4 ≡ tetraglucose

Pompe Disease (Alpha-glucosidase def GSD 2)

Increased excretion of Hex4

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized

There are some diseases that result in aglycone moieties at the reducing end,

(asparagine, and occasionally threonine and serine), excreted in aspartylglucosaminuria, and Schindler/Kanzaki diseases.

These particular diseases require alternate procedures and internal standards.

Analysis of Oligosaccharides in urine by ESI/MS/MS

Precursor ion scan of m/z 175, PMP derivatized

However, since this method enables the analysis of oligosaccharides with reducing ends,
these following lysosomal storage diseases can be detected:

Mannosidosis
Sialidosis
Galactosialidosis
Fucosidosis
Sialic acid storage disease (SASD)

GM1
GM2 TaySachs
GM2 Sandhoff
Gaucher disease

Mucolipidosis II (I-cell)

Pompe disease