Structural characterisation and enzymatic fingerprinting of oligo and polysaccharides

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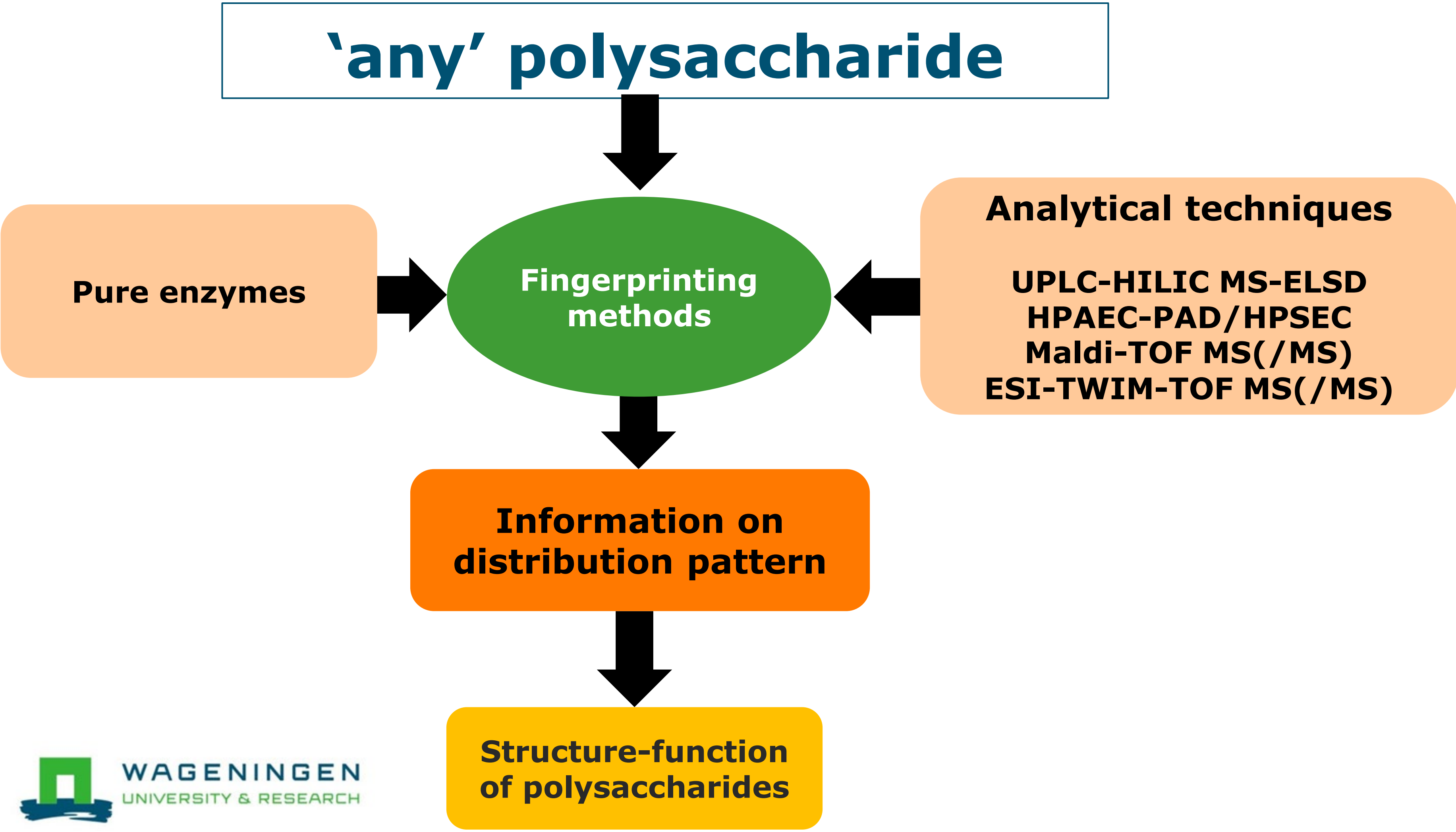
Functionality of oligo and polysaccharides: the chemical fine structure matters!

Fingerprinting polysaccharides to reveal the distribution of substituents → Our Approach



‘any’ polysaccharide

Pure enzymes

Fingerprinting methods

Analytical techniques

UPLC-HILIC MS-ELSD
HPAEC-PAD/HPSEC
Maldi-TOF MS(/MS)
ESI-TWIM-TOF MS(/MS)

Information on distribution pattern

Structure-function of polysaccharides

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Presentation outline

- Enzymatic fingerprinting of **pectin**
    - Enzymatic degradation
    - Identification and quantification of diagnostic oligosaccharides
    - Construction of parental polysaccharide molecules
- Characterisation of prebiotic oligosaccharides
    - Galactooligosaccharides---- PGC-MS identification

Pectin plays an important role as:

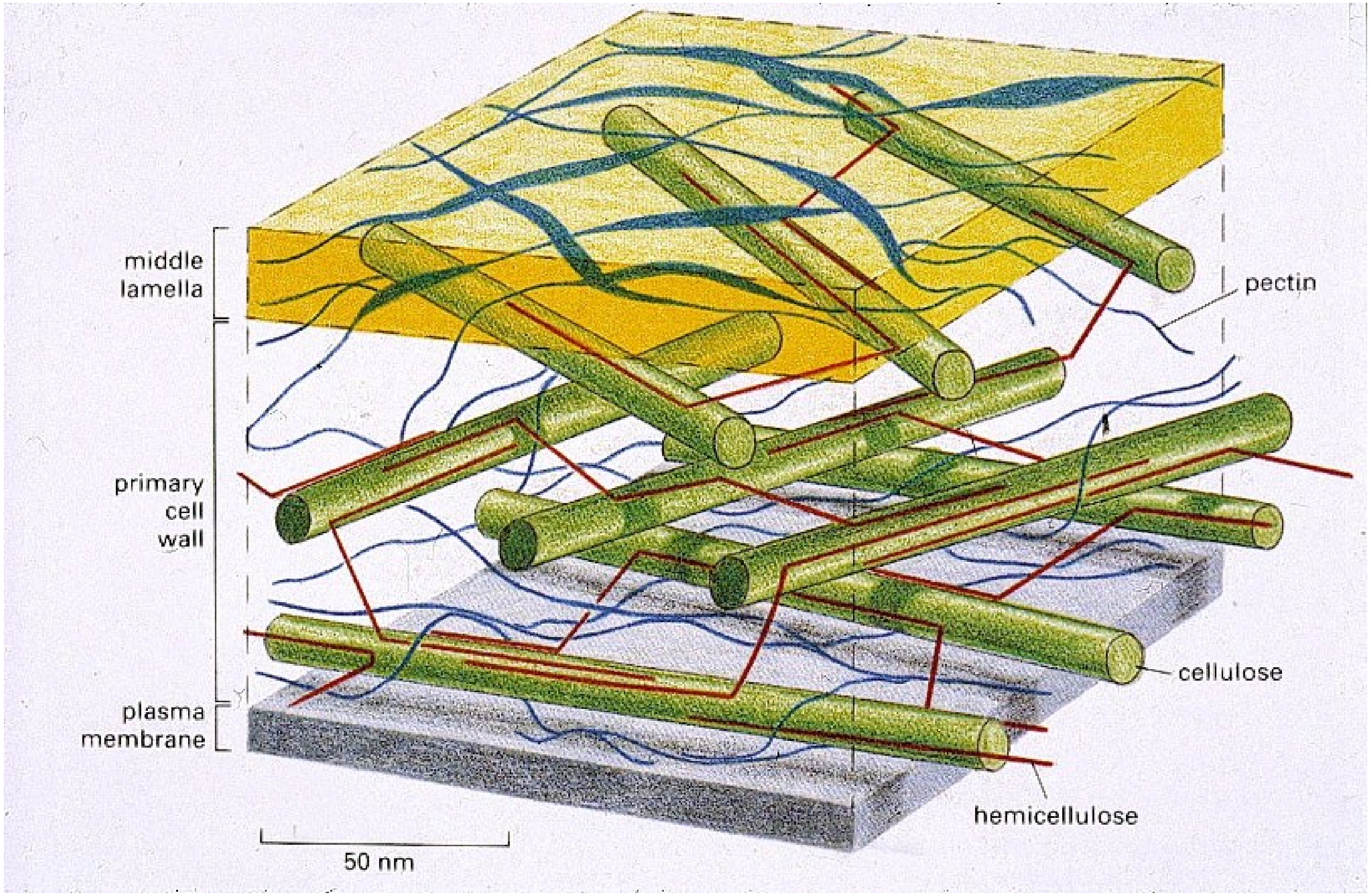
- Cell Wall component
    - Determine (partly) texture of tissues
    - Change during ripening of e.g., fruits (endogenous enzymes)
    - Change during processing due to depolymerization, de-esterification, solubilization, etc.
    - Present in by-products from agro-industry
- Texturizer in many fruit- & vegetable-based food products
- Ingredient for food industry
    - Modification during extraction and down-stream processing
    - Thickener and gelling agent
    - Stabilizer in fruit and milk beverages
- Health promoting component
    - Stimulation immune system
    - Fermentation by gut bacteria

Pectin is part of the plant cell wall

- Pectin – charged, branched
- Hemicellulose – moderately branched, strong interaction with cellulose
- Cellulose
- Lignin – highly complex polymer of phenolic compounds
- Especially ‘pectin’ and ‘hemicellulose’ represent classes of polysaccharides with a broad range of chemical structures and a wide variation in size, charge, branching, etc

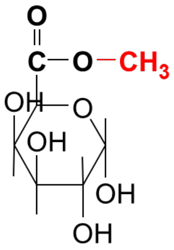
model for the primary plant cell wall

McCann and Roberts (1991)

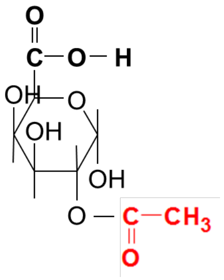


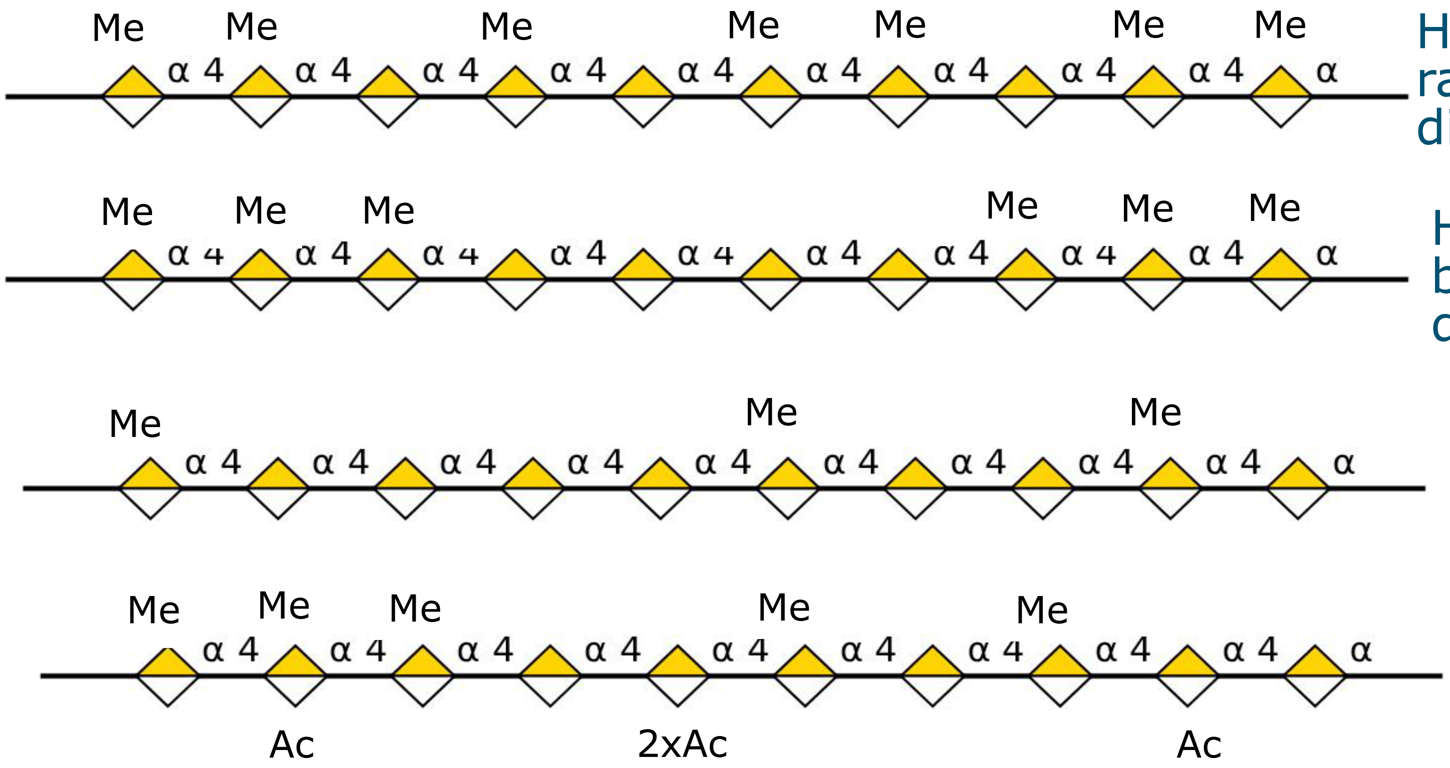
Schematic structure of pectic polysaccharides

Homogalacturonan (HG)



Galacturonic acid





HM Pectin, random distribution

HM Pectin, blockwise distribution

LM Pectin

Acetylated pectin

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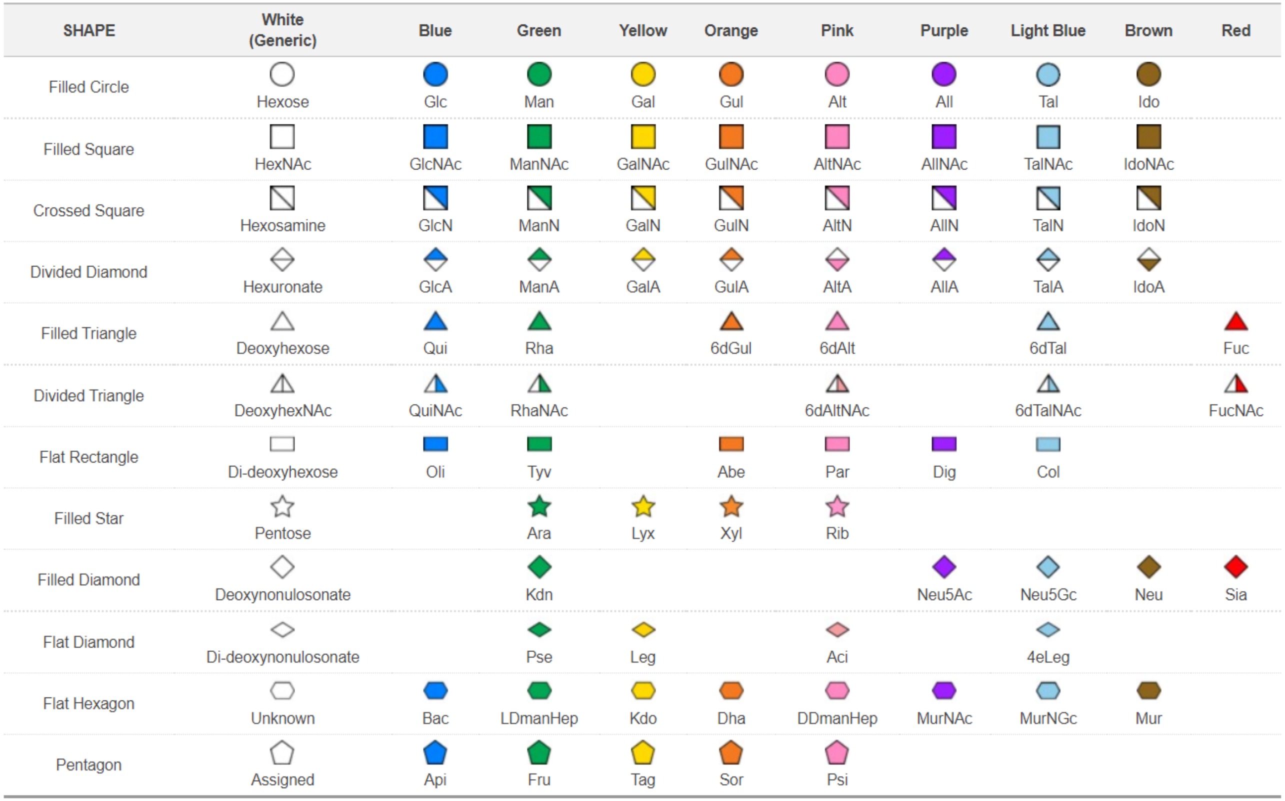
HM: High degree of methyl esterification

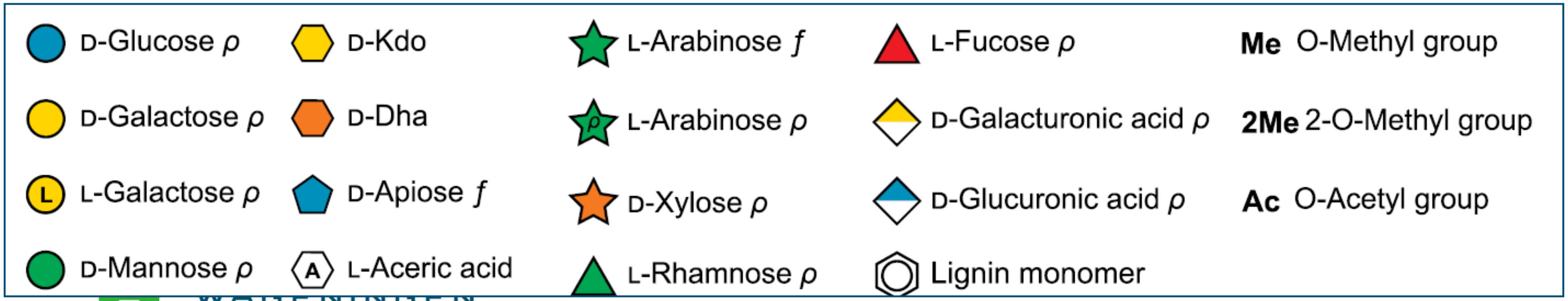
LM: Low degree of methyl esterification

Carbohydrate symbols:
https://www.ncbi.nlm.nih.gov/glycans/snfg.html

Monosaccharide symbol nomenclature



SHAPE	White (Generic)	Blue	Green	Yellow	Orange	Pink	Purple	Light Blue	Brown	Red
Filled Circle	Hexose	Glc	Man	Gal	Gul	Alt	All	Tal	Ido	
Filled Square	HexNAc	GlcNAc	ManNAc	GalNAc	GulNAc	AltNAc	AllNAc	TalNAc	IdoNAc	
Crossed Square	Hexosamine	GlcN	ManN	GalN	GulN	AltN	AllN	TalN	IdoN	
Divided Diamond	Hexuronate	GlcA	ManA	GalA	GulA	AltA	AllA	TalA	IdoA	
Filled Triangle	Deoxyhexose	Qui	Rha		6dGul	6dAlt		6dTal		Fuc
Divided Triangle	DeoxyhexNAc	QuiNAc	RhaNAc			6dAltNAc		6dTalNAc		FucNAc
Flat Rectangle	Di-deoxyhexose	Oli	Tyv		Abe	Par	Dig	Col		
Filled Star	Pentose		Ara	Lyx	Xyl	Rib				
Filled Diamond	Deoxynonulosonate		Kdn				Neu5Ac	Neu5Gc	Neu	Sia
Flat Diamond	Di-deoxynonulosonate		Pse	Leg		Aci		4eLeg		
Flat Hexagon	Unknown	Bac	LDmanHep	Kdo	Dha	DDmanHep	MurNAc	MurNGc	Mur	
Pentagon	Assigned	Api	Fru	Tag	Sor	Psi				



- D-Glucose *p*
- D-Galactose *p*
- L-Galactose *p*
- D-Mannose *p*
- D-Kdo
- D-Dha
- D-Apiose *f*
- L-Aceric acid
- L-Arabinose *f*
- L-Arabinose *p*
- D-Xylose *p*
- L-Rhamnose *p*
- L-Fucose *p*
- D-Galacturonic acid *p*
- D-Glucuronic acid *p*
- Lignin monomer

Me O-Methyl group

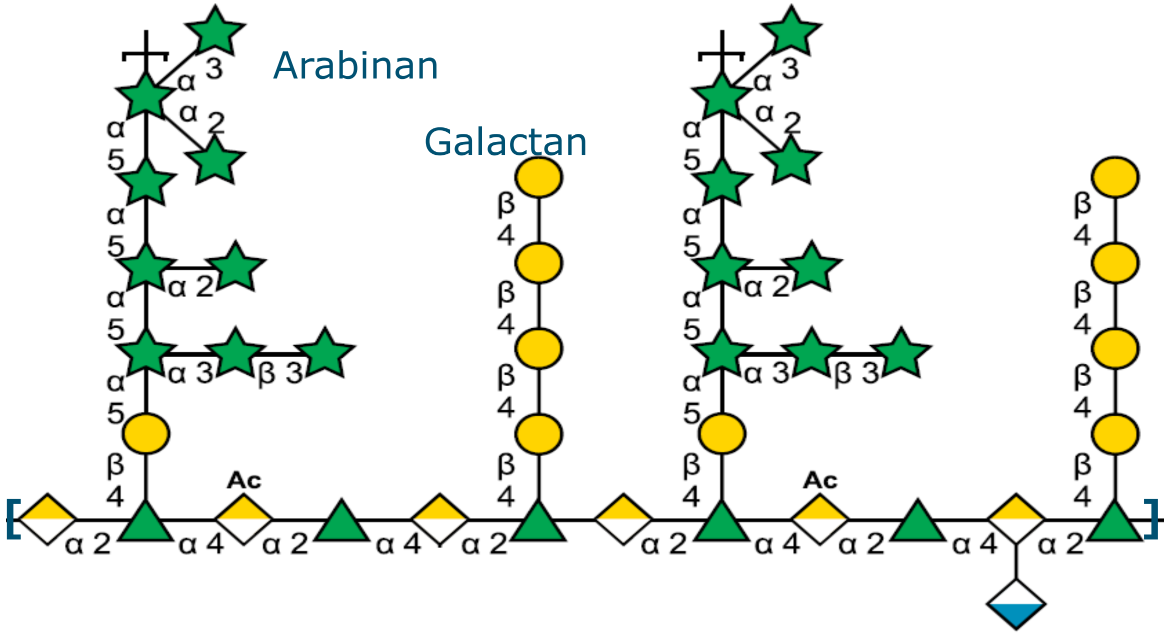
2Me 2-O-Methyl group

Ac O-Acetyl group

Carbohydrate symbols:
https://www.ncbi.nlm.nih.gov/glycans/snfg.html

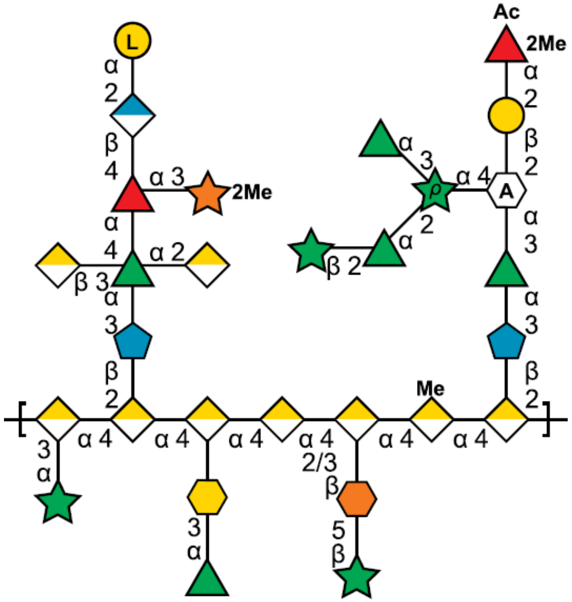
Schematic structure of pectic polysaccharides

Rhamnogalacturonan I (RG I)

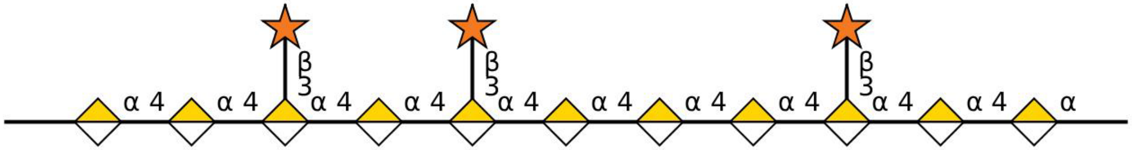

Arabinan

Galactan

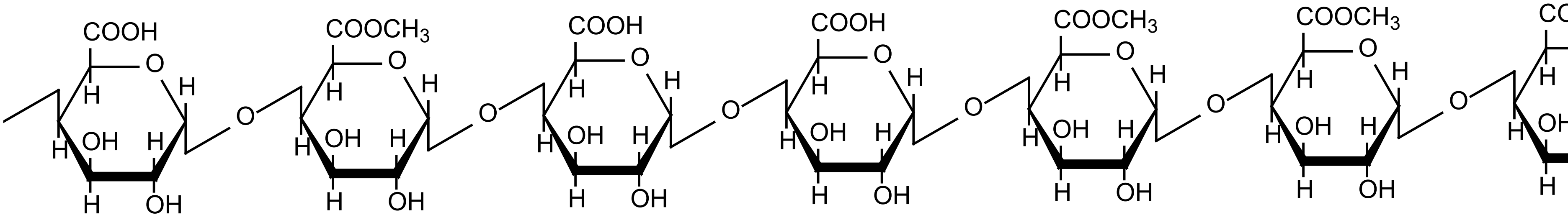
Rhamnogalacturonan II (RG II)



Xylogalacturonan



Changing pectin characteristics in plant material



- Biosynthesis of fully methyl esterified pectin
- Tailoring of molecular weight, methyl esterification and interactions during growing/ripening/storage/processing
  - In planta : enzymes
  - During processing
    - @ home : chemically = cooking
    - @ industry : both chemically and enzymatically (processing aid!)

Pectin structure

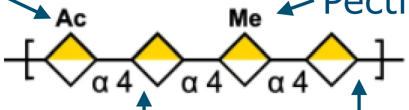
- Commercial pectin is extracted from citrus peel and apple pomace
- Commercial pectin is mainly homogalacturonan with methyl-esters
- Structure depends on:
    - origin raw material (orange, lemon, grapefruit, apple, sugar beet)
    - possible endogenous enzyme activity in raw material (pPME),
    - method of extraction,
    - sugar composition, DM, Mw, DB etc.

- Techno- and bio functionality strongly depends

  on level and distribution of methyl-esters

Pectinases – a variety of enzymes

Homogalacturonan



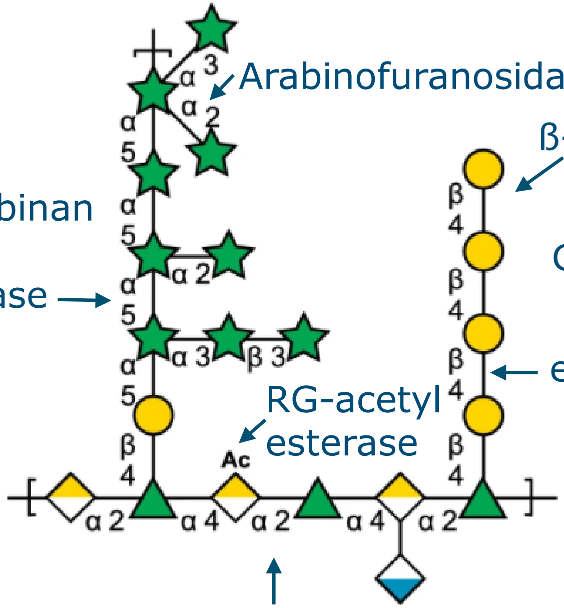
Pectin acetyl esterase

Pectin methyl esterase

polygalacturonases

Pectin & pectate lyases

Rhamnogalacturonan I



Arabinofuranosidase

ß-galactosidase

Arabinan

Galactan

endo-arabinanase

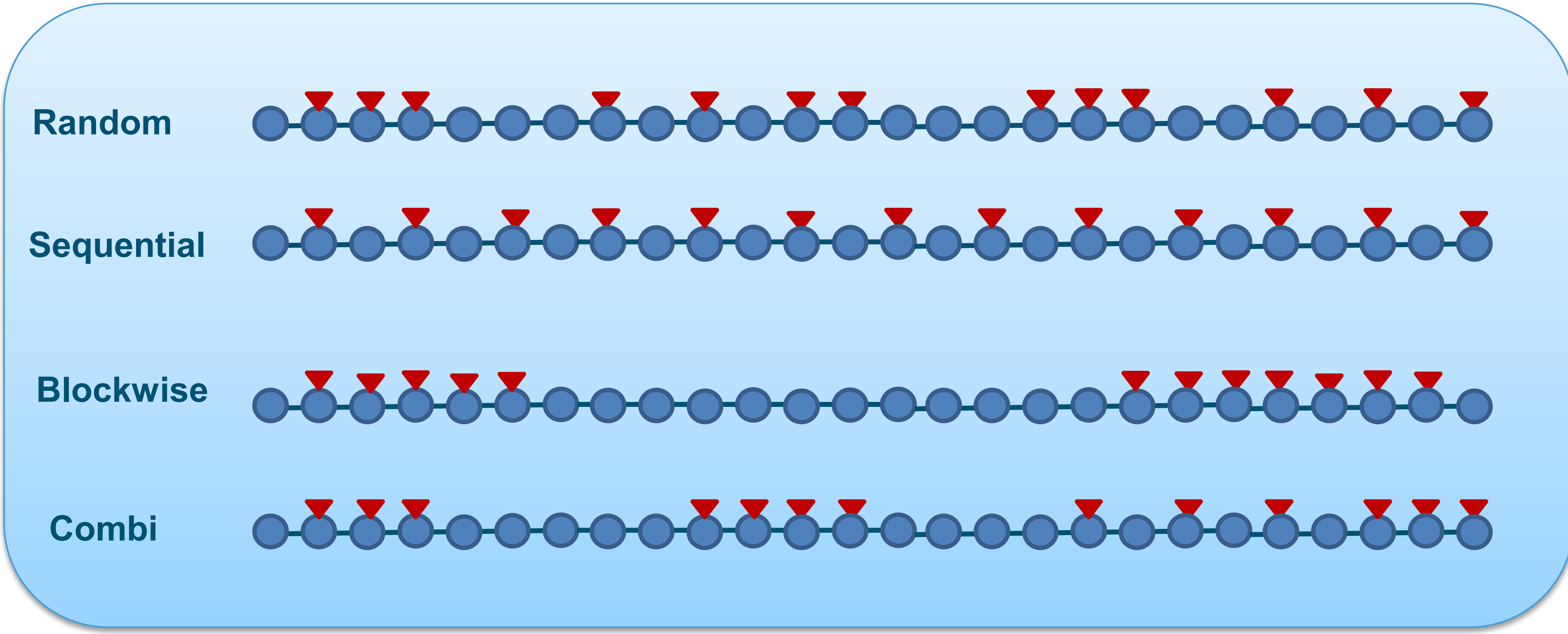
endo-galactanase

RG-acetyl esterase

RG-hydrolase
RG-lyase

Characterisation of pectins

Distribution of methyl esters: Homogalacturonan 50% DM

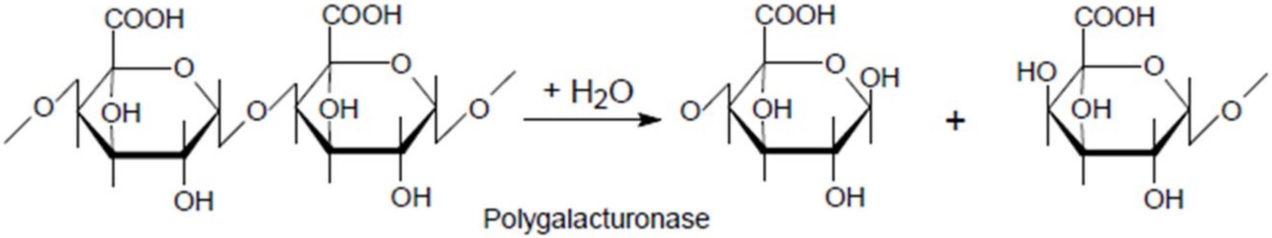


● = **Galacturonic acid (GalA)**

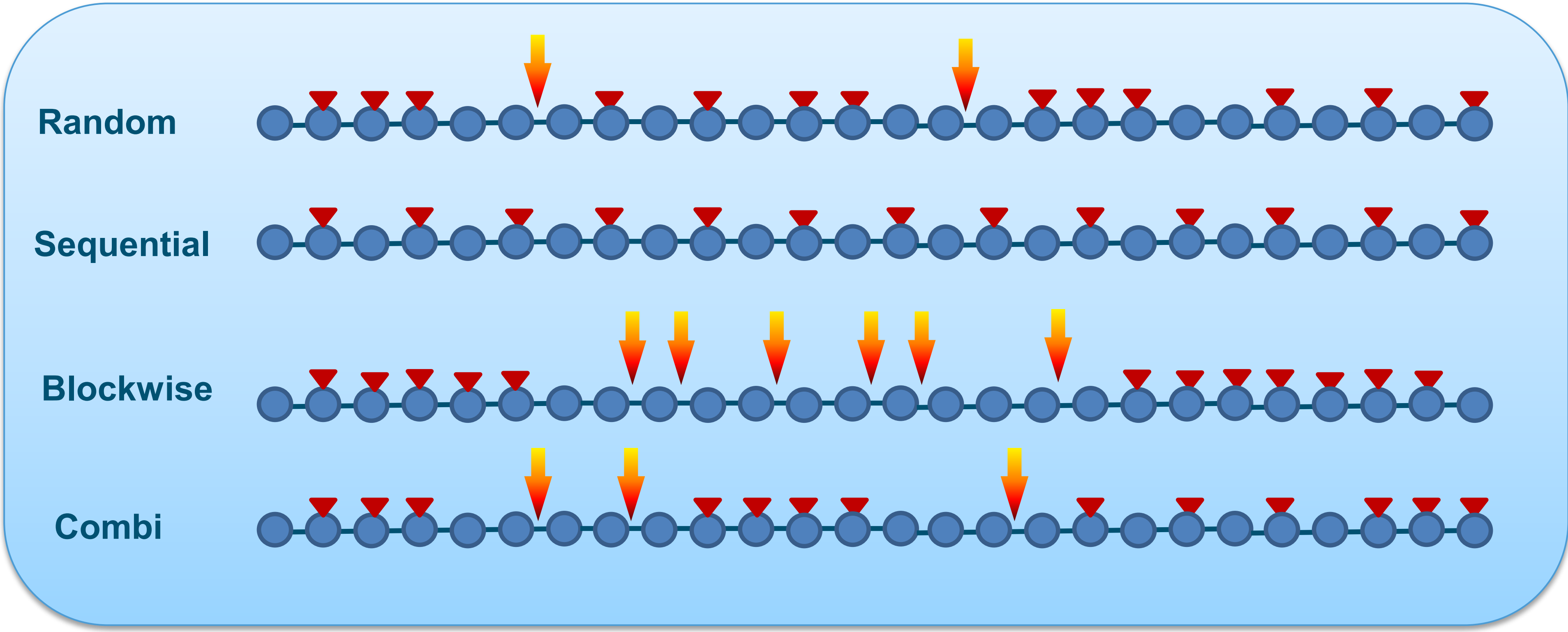
▼● = **methyl-GalA**

Homogalacturonan:

Finger printing of methyl ester groups using PG

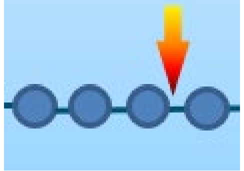


Release of saturated galacturonic acid oligomers



● = Galacturonic acid (GalA)

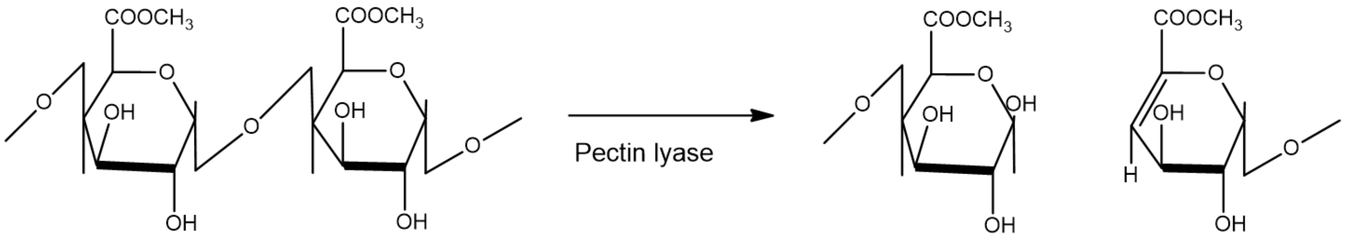
▼● = methyl-GalA



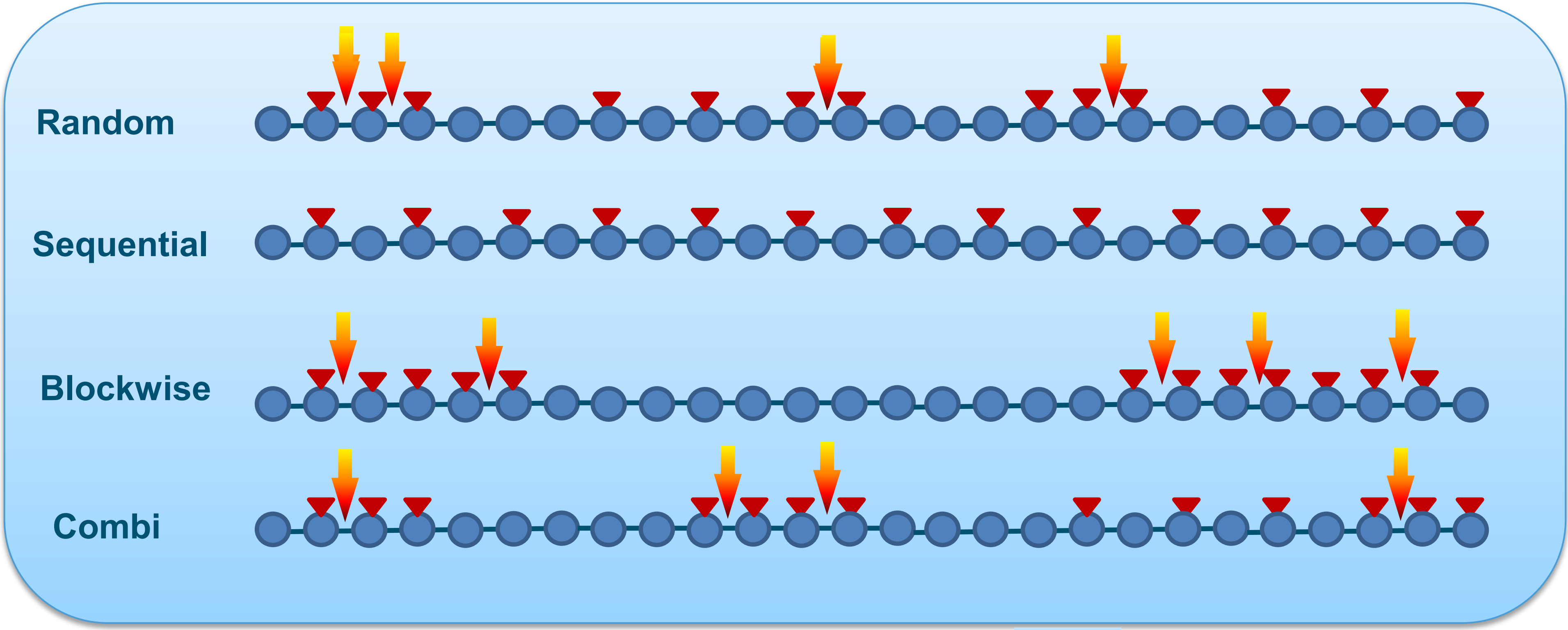
= site of attack endo-PG
(Kluyveromyces fragilis)

Homogalacturonan:

Finger printing of methyl ester groups using PL

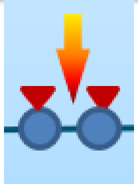


Release of **unsaturated** galacturonic acid oligomers



● = Galacturonic acid (GalA)

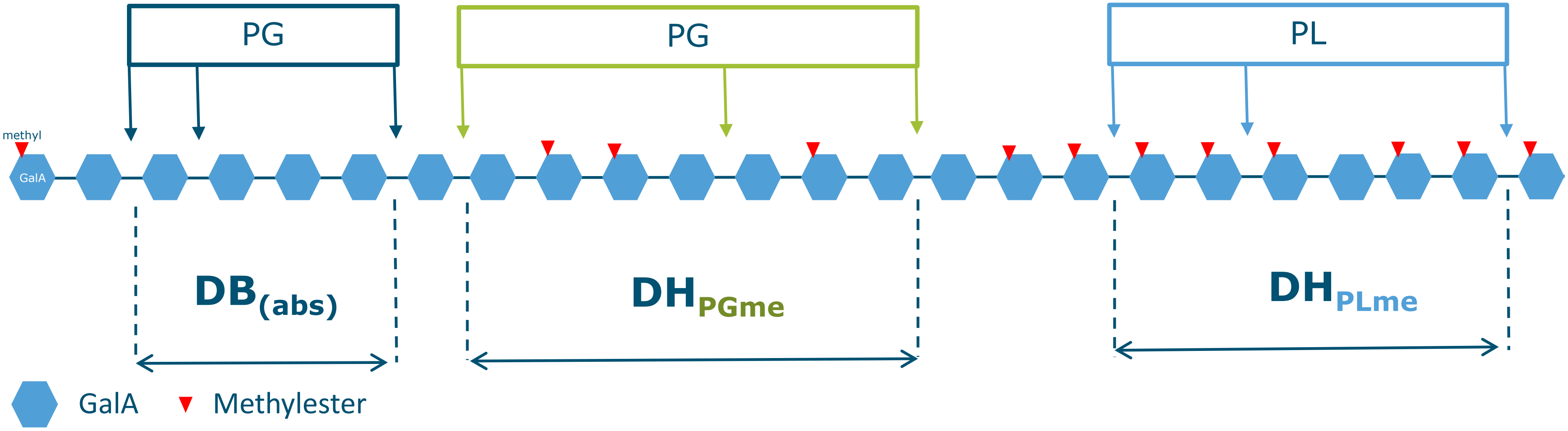
▼● = methyl-GalA



=site of attack pectin lyase

Descriptive parameters

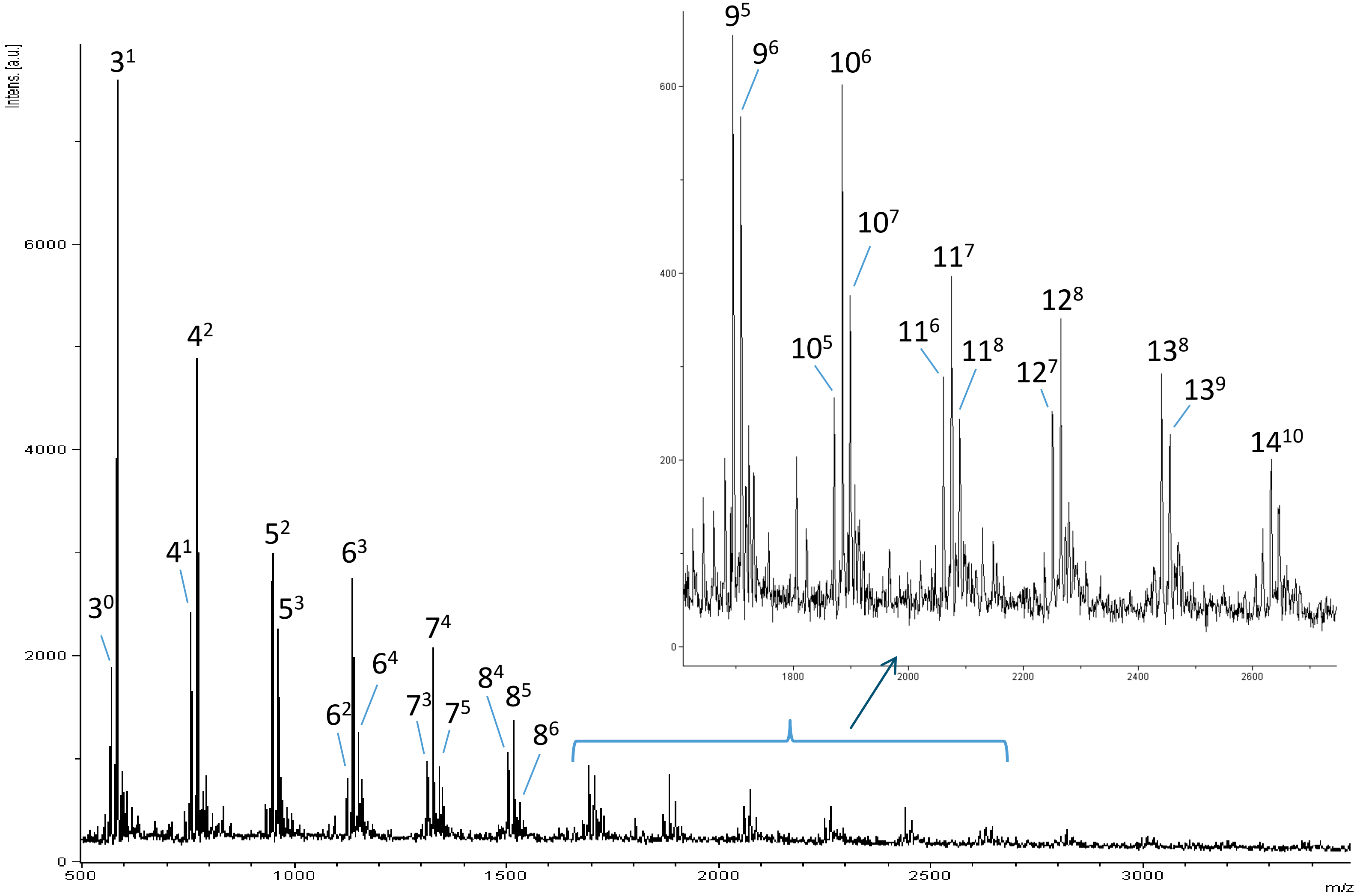
Degree of Blockiness and Degree of Hydrolysis



- **DB** quantifies unsubstituted mono-, di- and tri GalA oligomers as released by PG
  → Information on non-esterified regions, relative to non-esterified GalA
- **DB absolute → Information on non-esterified regions within pectin, relative to total GalA**
- **$\mathrm{DH}_{\mathrm{PGme}}$** represents PG released methylesterified segments
  → Information on specific (partly) methyl esterified regions
- **$\mathrm{DH}_{\mathrm{PLme}}$** represents PL released highly methylesterified oligomers from the pectin
  → Information on highly methyl esterified regions

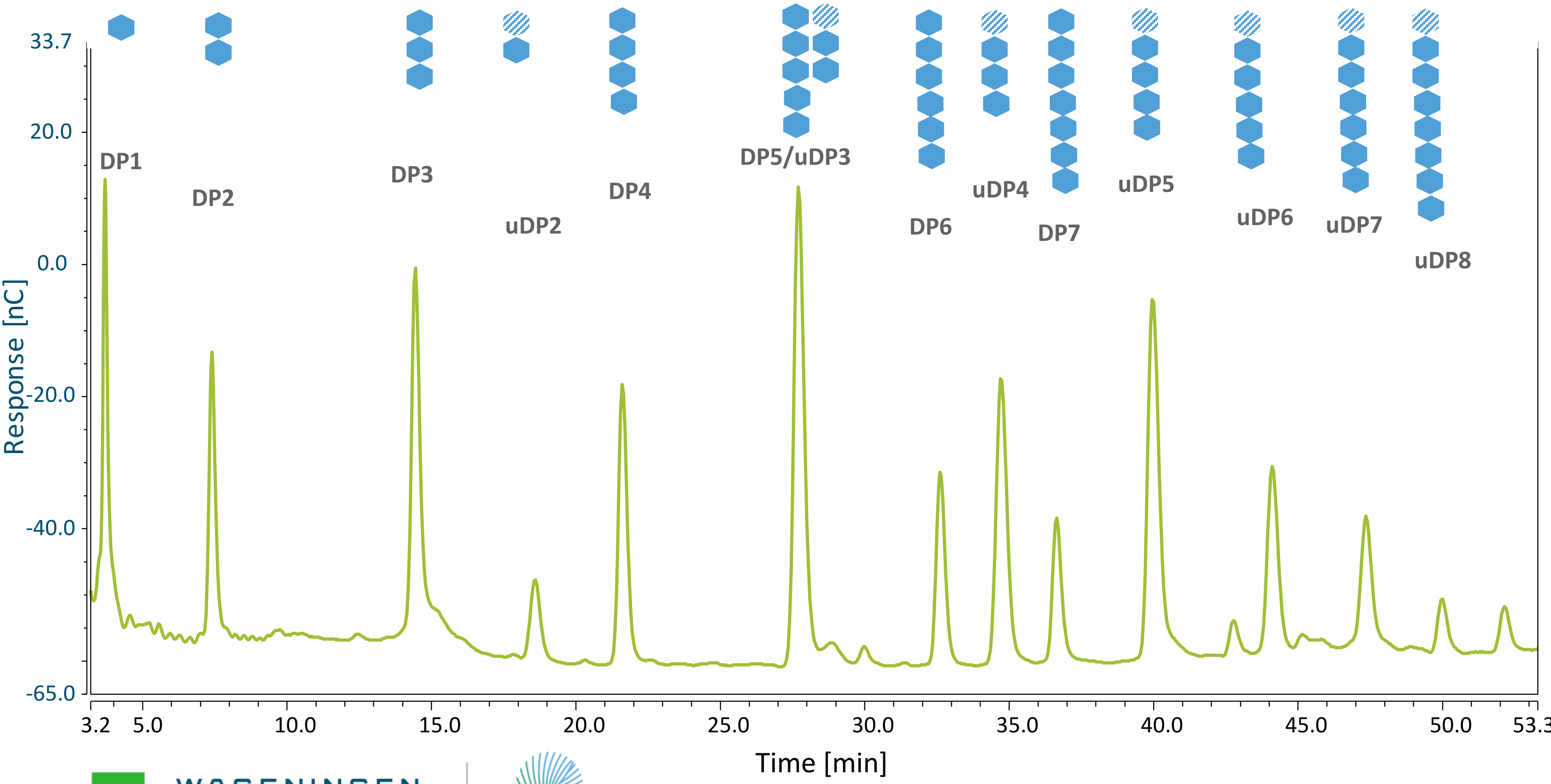
Daas et al, 2000, 2001
Jermendi, Beukema et al. 2021

MALDI TOF MS of random esterified DM70 pectin digested by endo-PG



4^2= GalA$_4$ 2 methyl

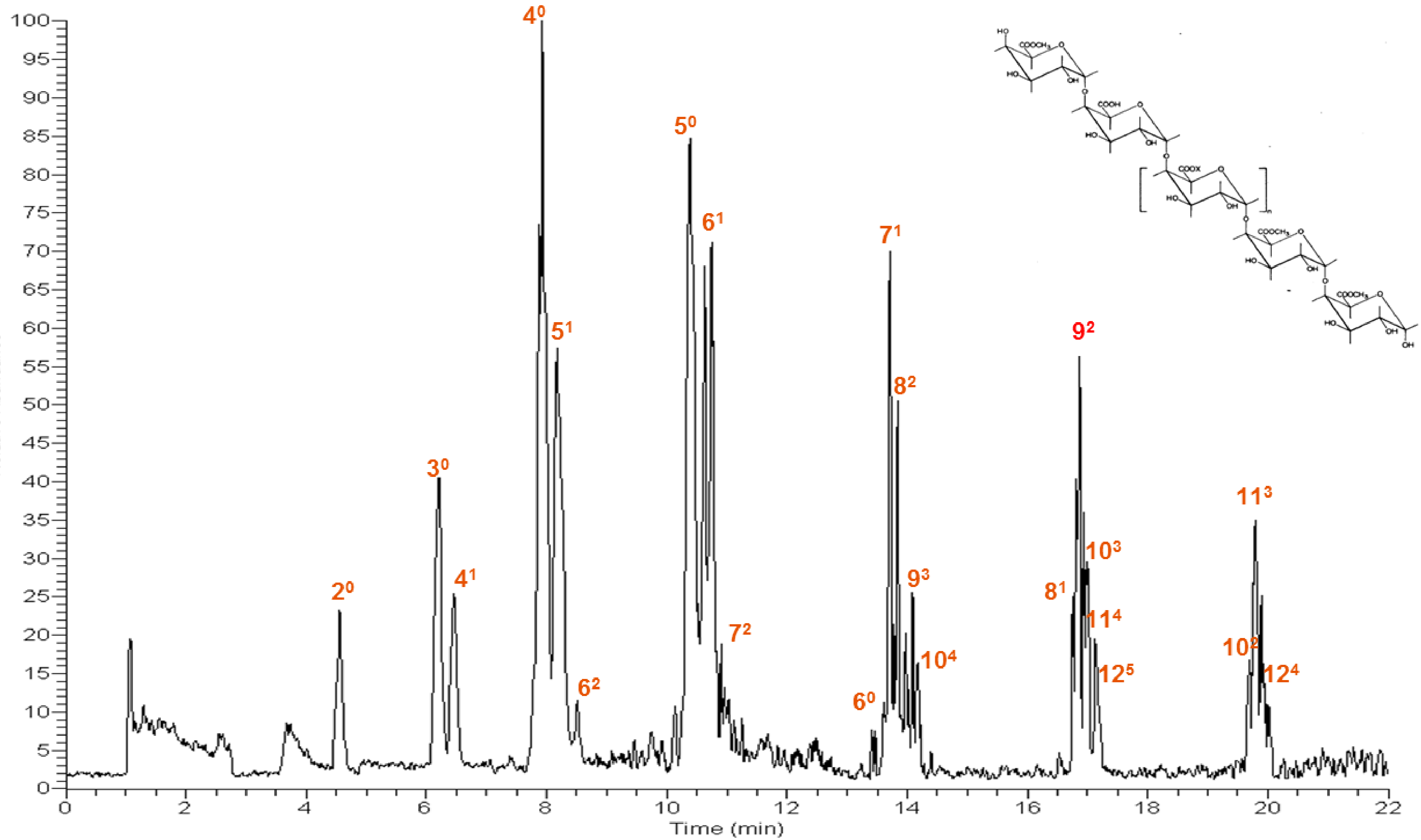
HPAEC-PAD elution profile of the DM~60 pectins after enzymatic degradation



⬢ Saturated GalA

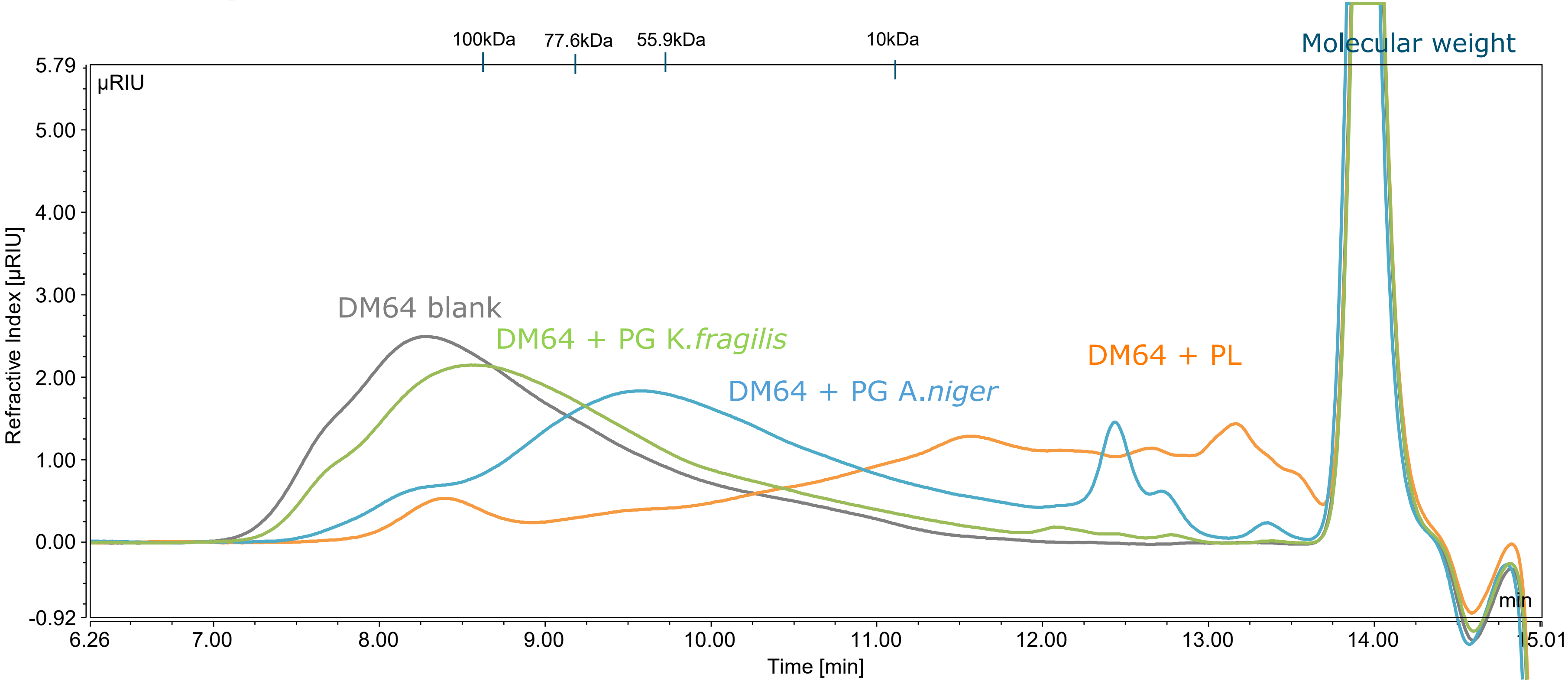
▨ Unsaturated GalA

HILIC HPLC-Iontrap-MS^n method: methyl-esterified pectin oligomers



5^1= GalA$_5$ 1 methyl

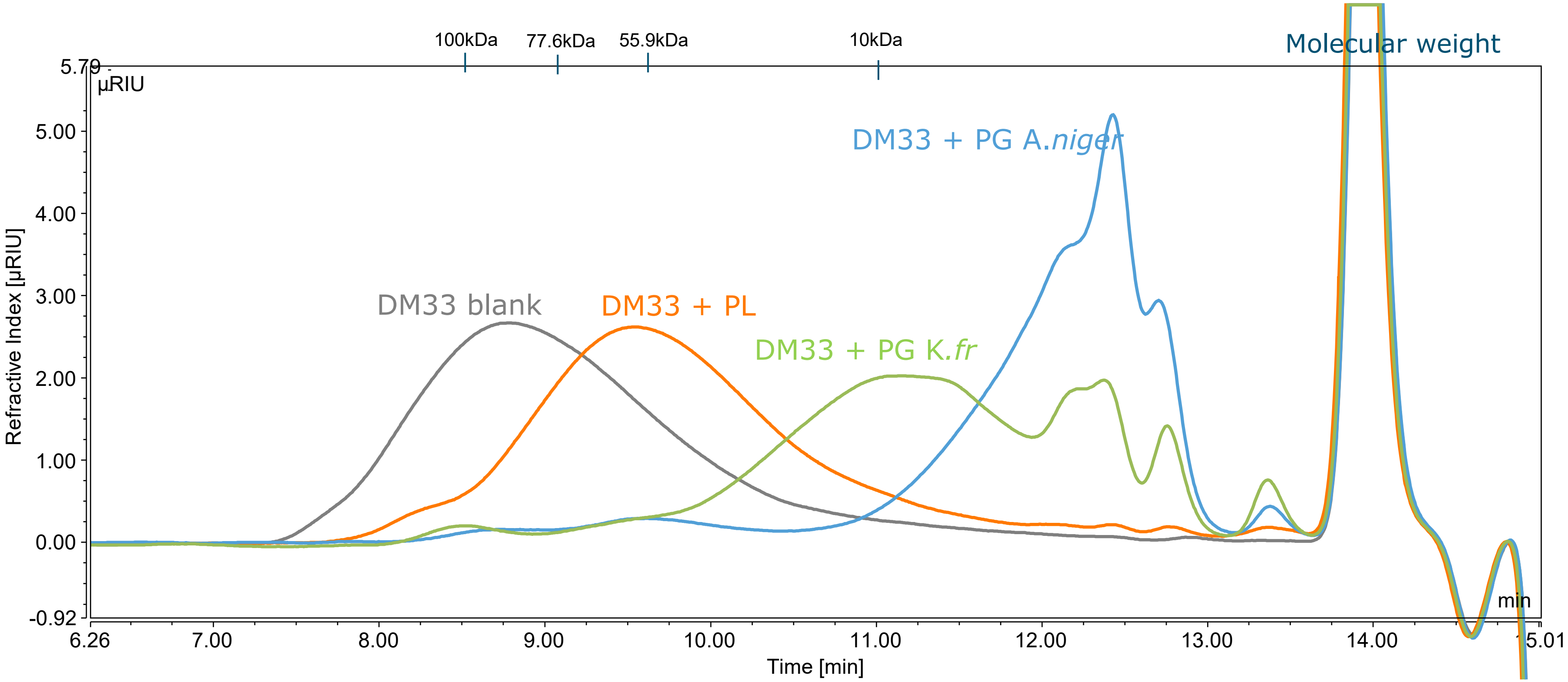
HPSEC elution profiles of high esterified DM64 pectin incubated with PGs and a PL



PL degrades substantially HM pectin

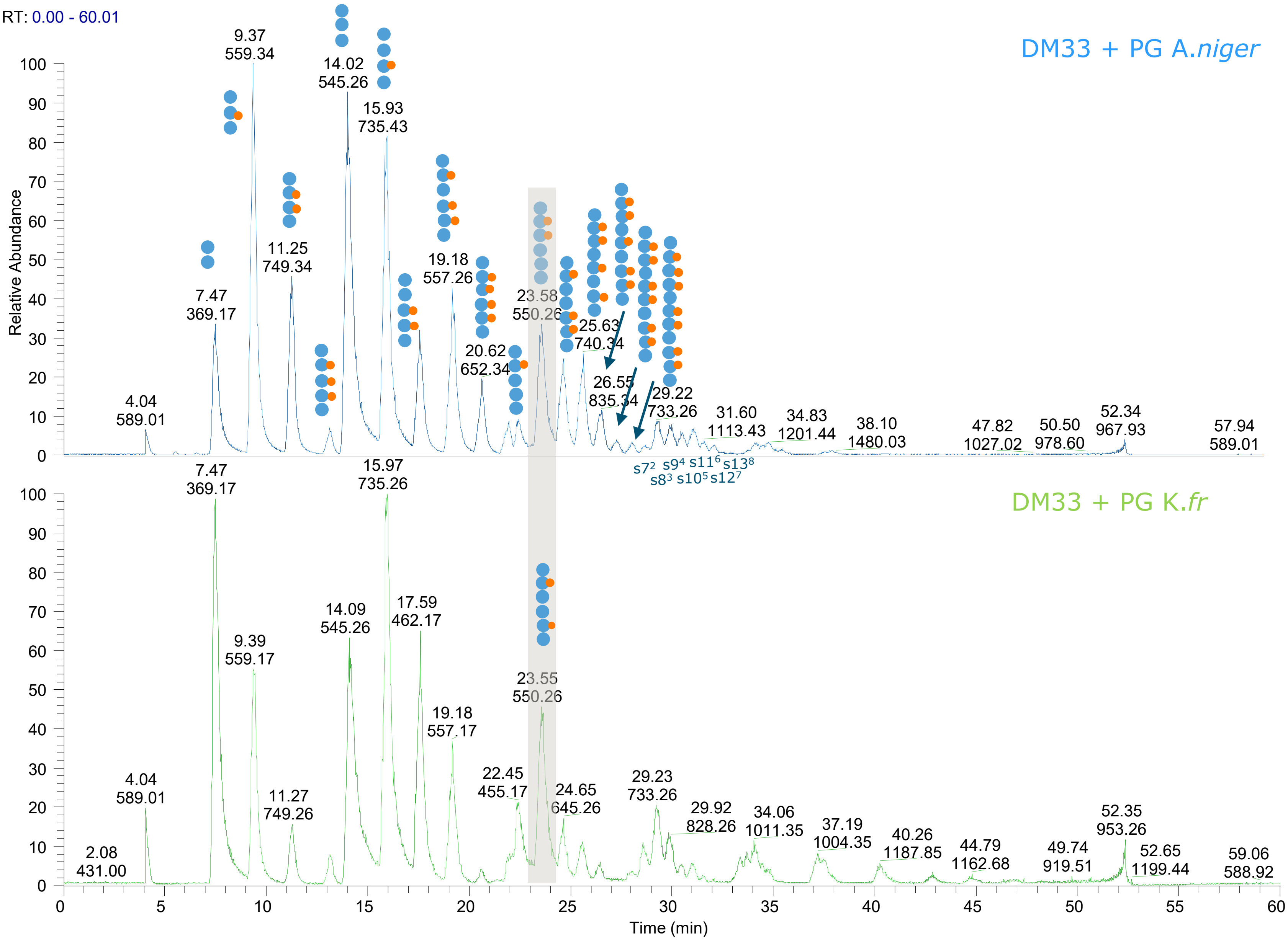
PG degrades HM pectin only slightly and degradation depends on origin of PG

HPSEC elution profiles of low esterified DM33 pectin after incubation with PGs and PL

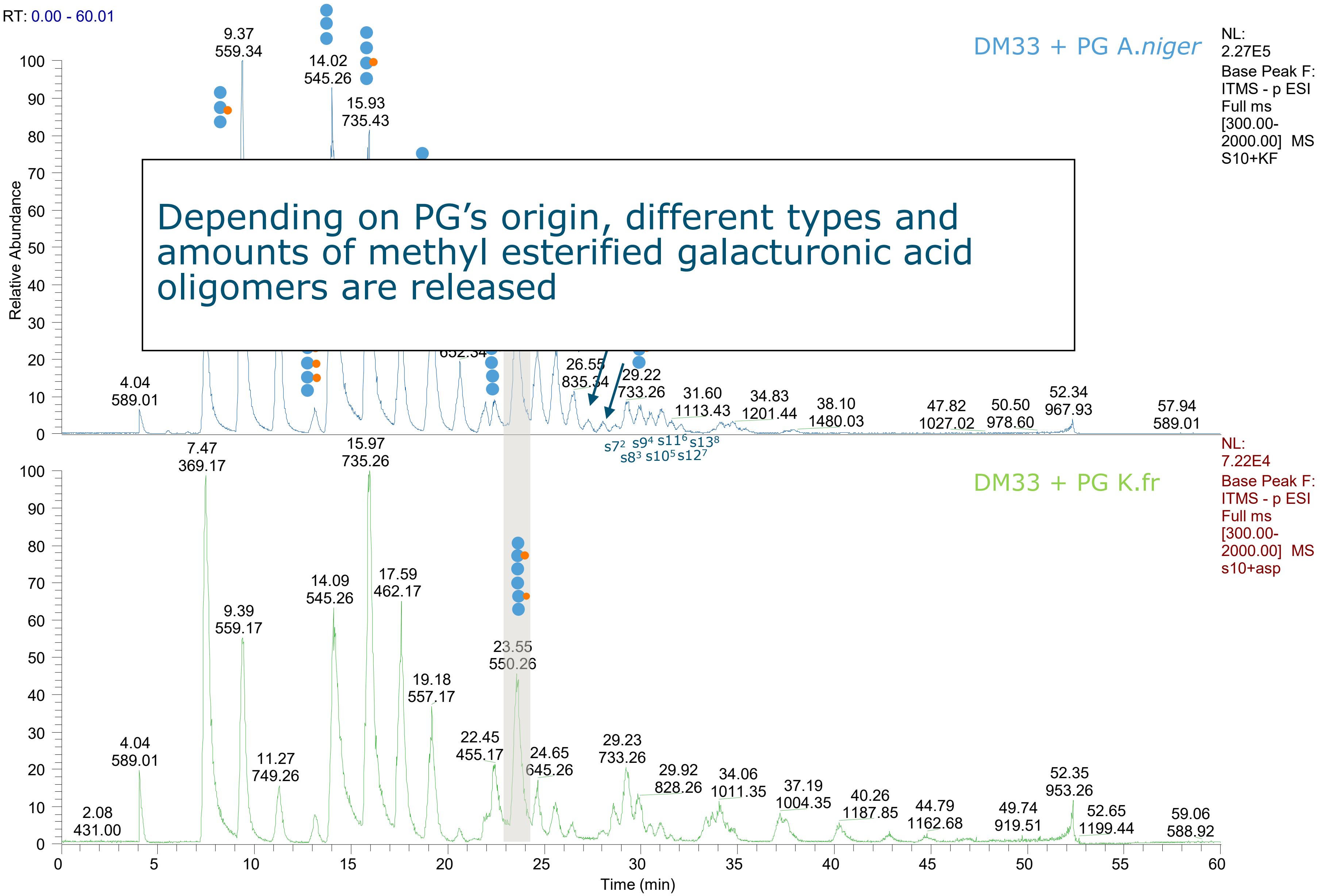


Depending on PG's origin, different Mw profiles are obtained.
PG degrades LM pectin much better than PL

HILIC LC-MS profiles of DM33 pectin incubated with PGs



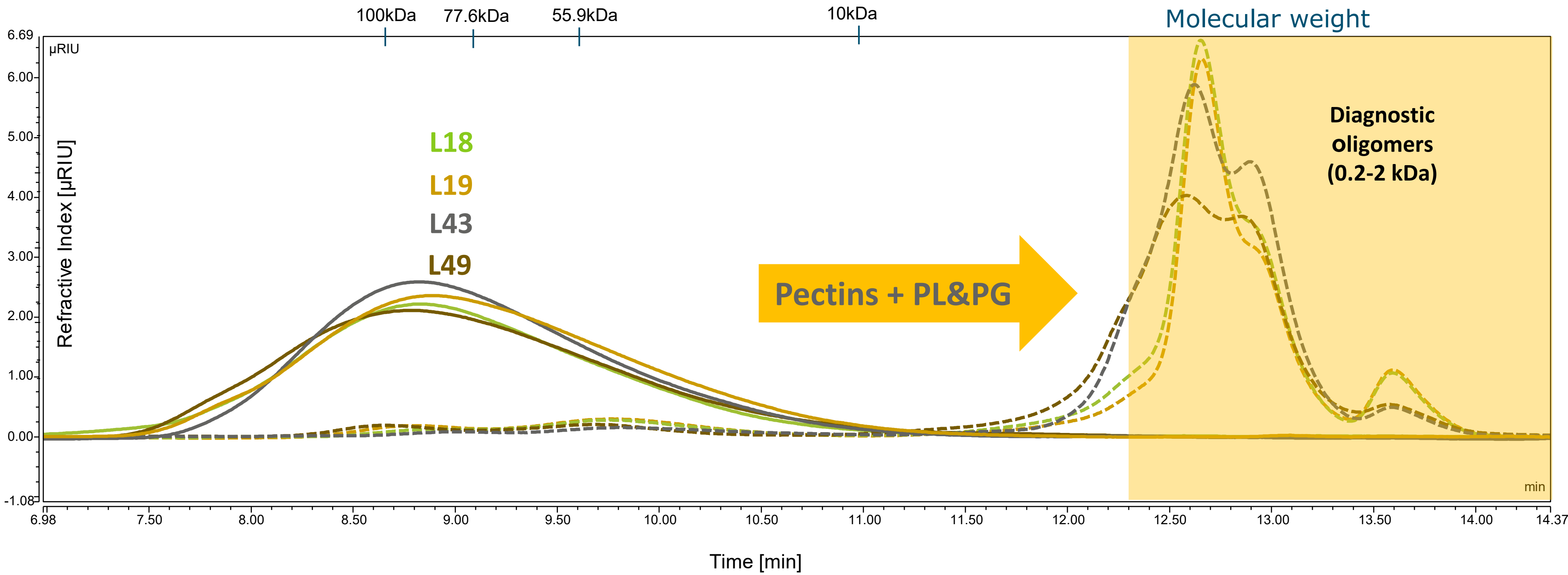
UPLC-MS profiles of DM33 incubated with PG from different origin



Depending on PG's origin, different types and amounts of methyl esterified galacturonic acid oligomers are released

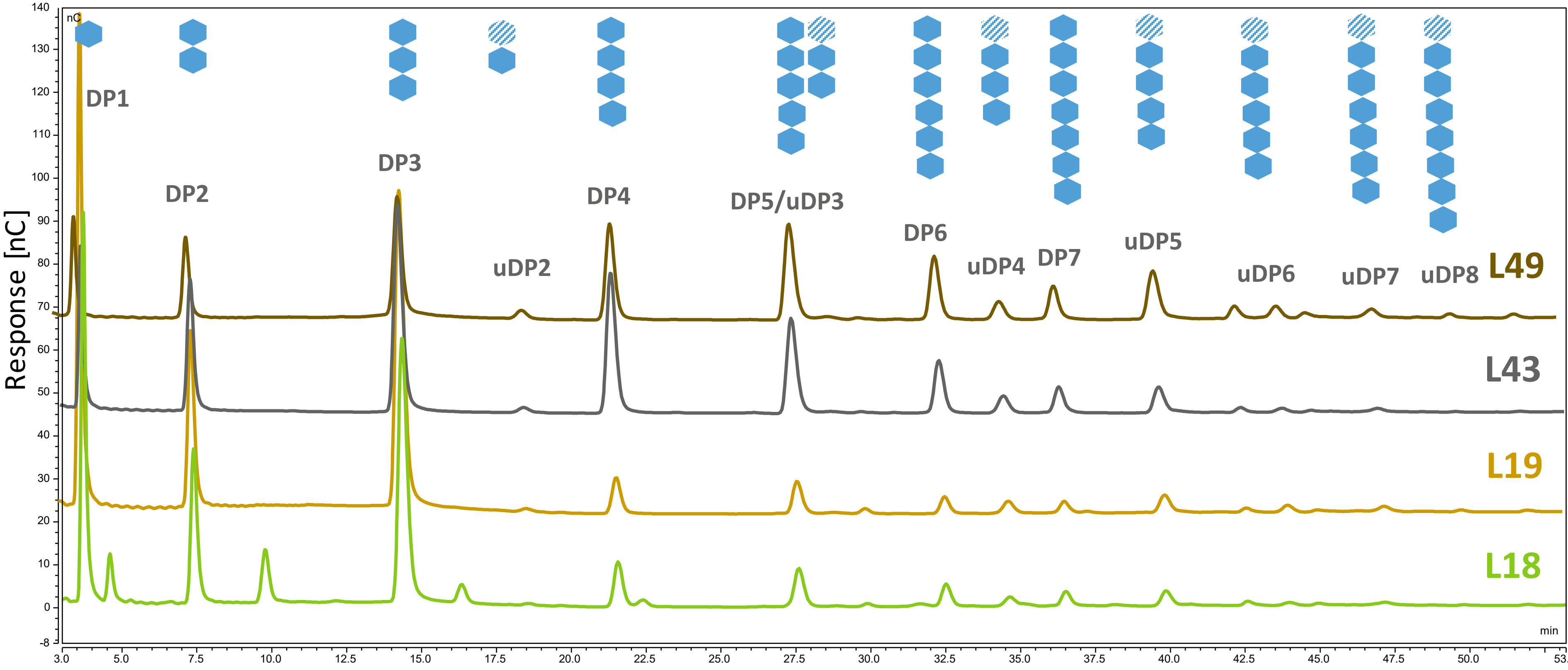
HPSEC-RI elution profiles of Lemon pectins before and after enzymatic degradation by PG + PL

Two times 2 different commercial Lemon pectins:
2 x LM pectins
2 x MM pectins



PG and PL digestion together completely degrade various pectins to mono-, and oligosaccharides

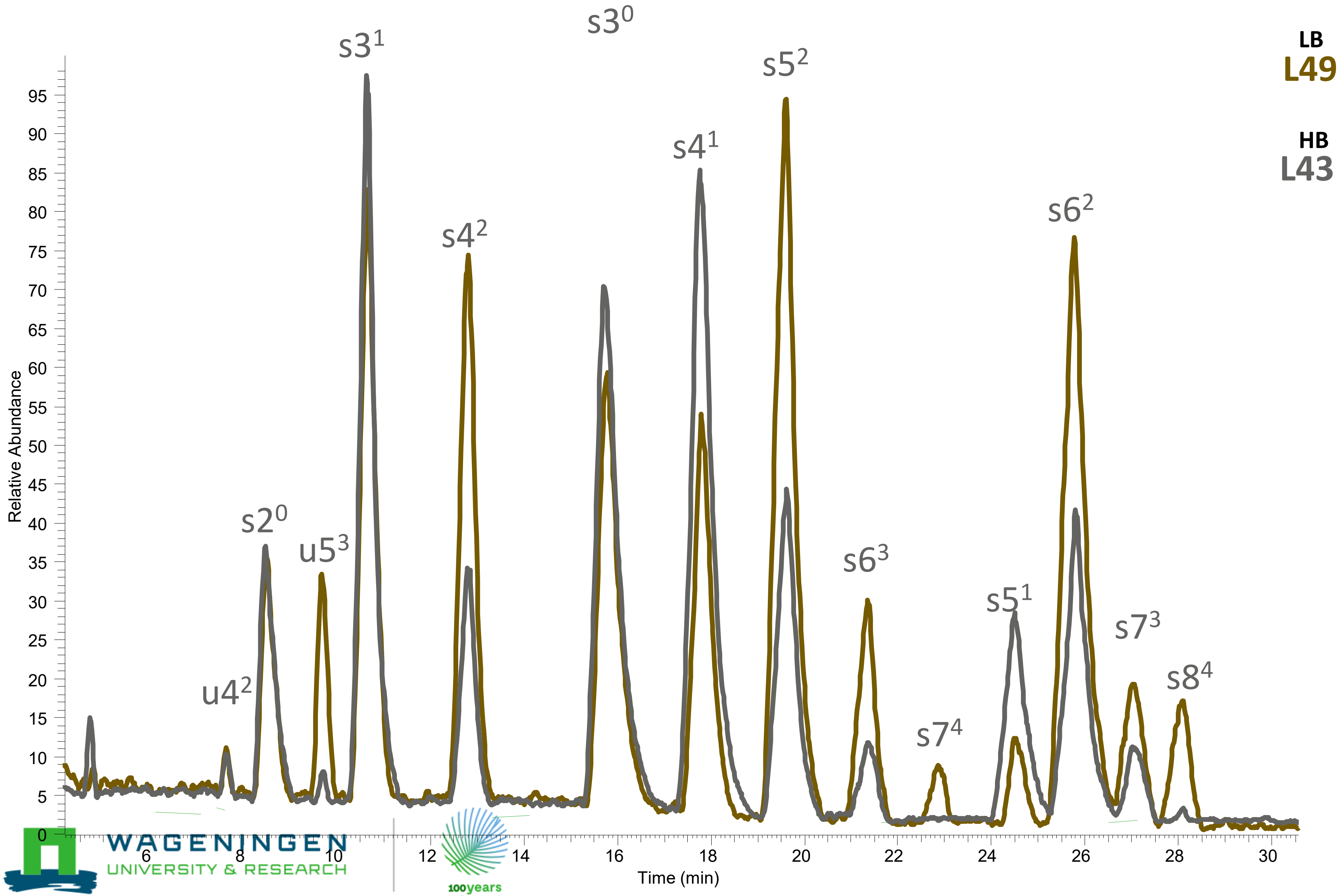
HPAEC-PAD elution profile of the pectins after enzymatic degradation



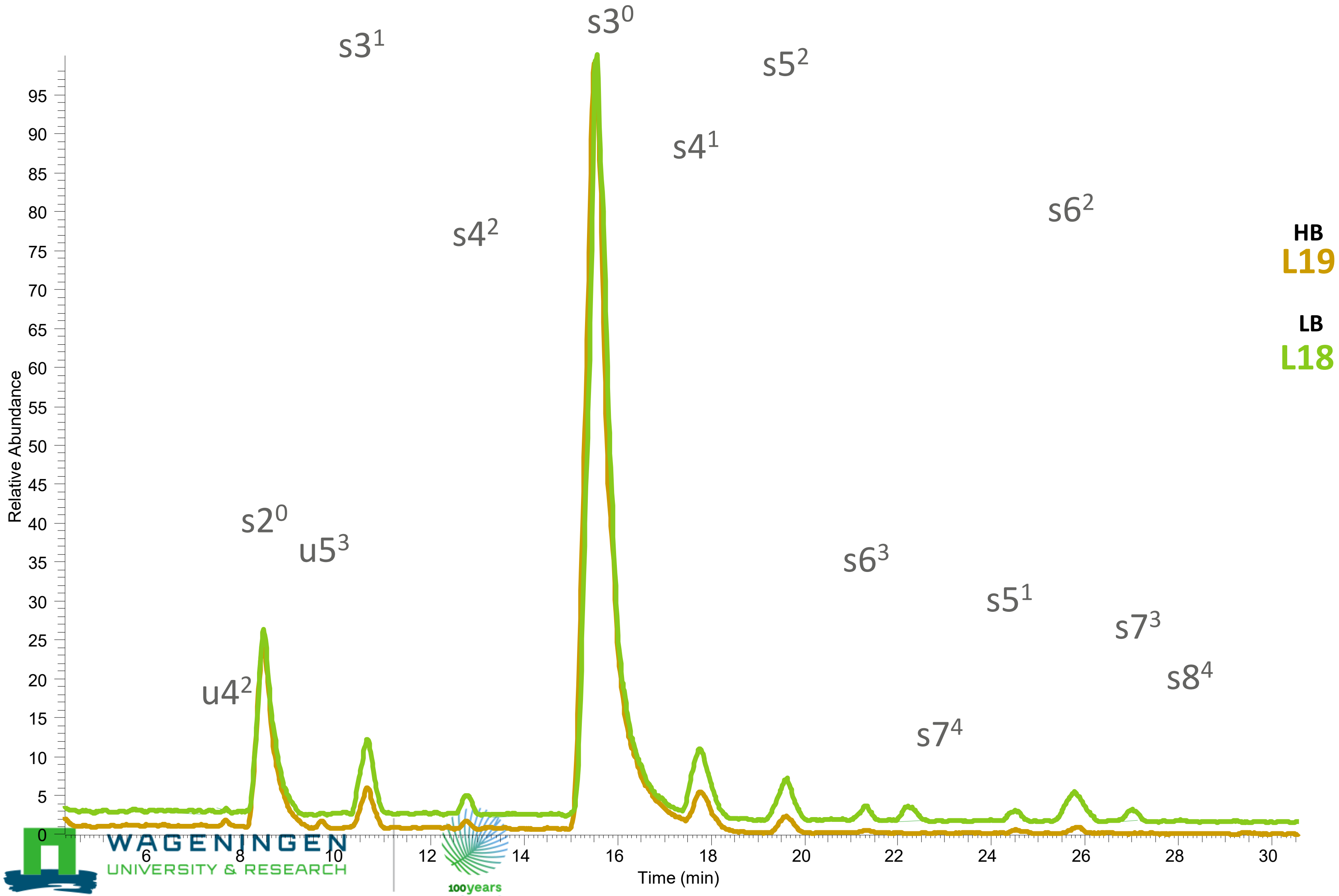
DP = Degree of Polymerisation;
DP1-n = saturated GalA oligomers
uDP1-n= unsaturated GalA oligomers

Nice distinction between PG- (saturated) and PL (unsaturated) oligomers
No information on methyl esters
Availability of standards is limited

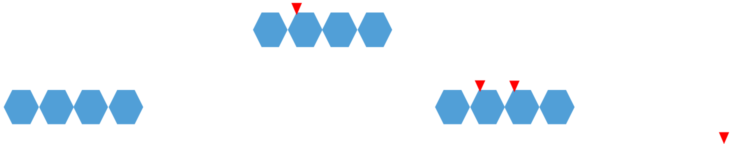
UPLC-HILIC-MS elution profile of pectins after enzymatic degradation



UPLC-HILIC-MS elution profile of pectins after enzymatic degradation

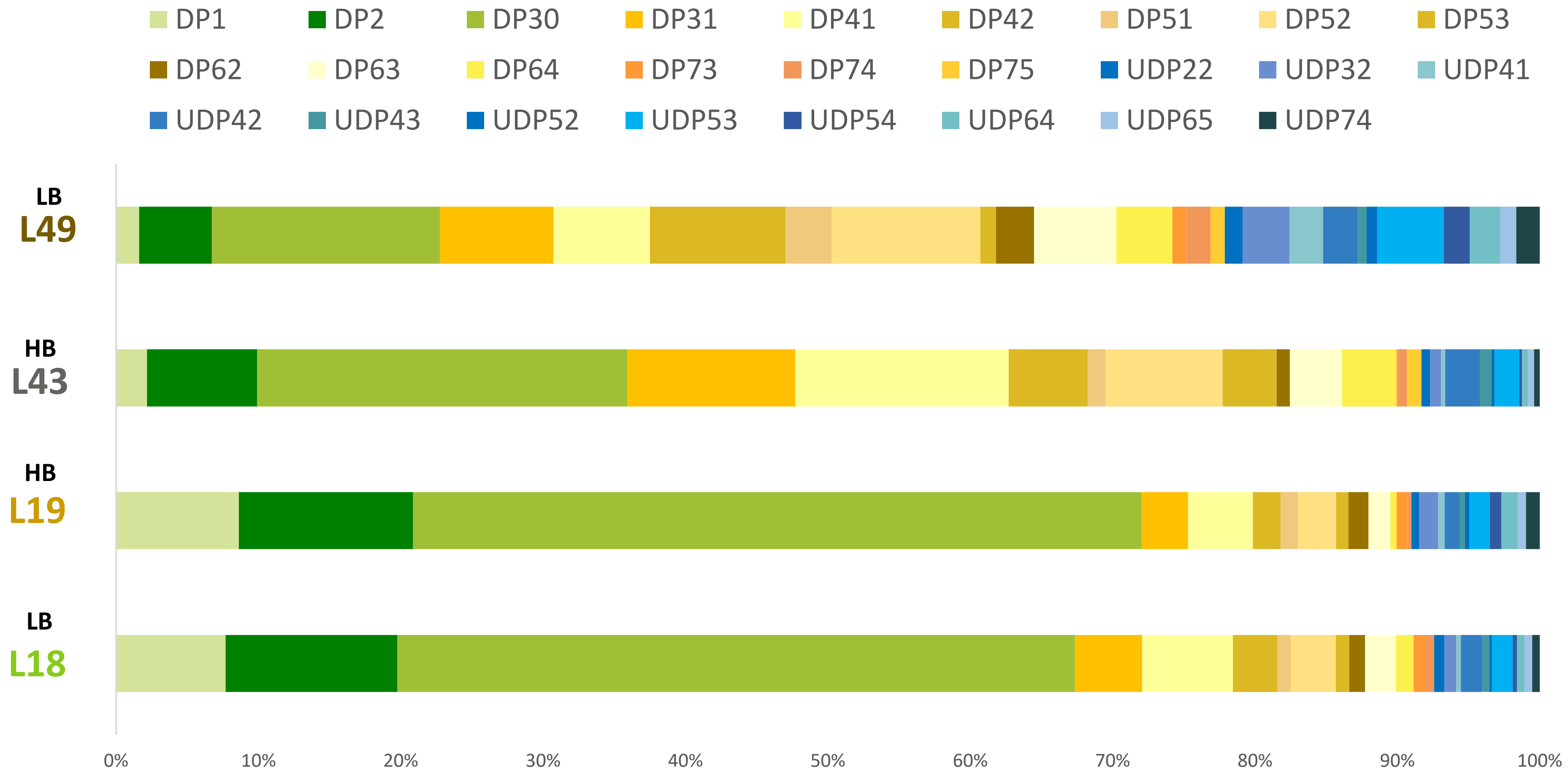


Pectin descriptive parameters



	Code/DM	Origin	Mw	DB	DB$_{abs}$	DH$_{PGme}$	DH$_{PLme}$
LM Pectin	L19	Lemon	75	93	75	21	10
LM Pectin	L18	Lemon	78	80	66	27	8
MM Pectin	L43	Lemon	74	56	32	73	11
MM Pectin	L49	Lemon	(114)	34	18	65	26

Relative abundance of oligosaccharides released from the pectins



Oligomers differ in level, size and methyl-ester patterns, highlighting minor and major differences in methyl-ester distribution of pectins having the same overall degree of methyl-esterification.

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100years
1918 — 2018

Pectin’s functionality:
Methyl ester level and distribution matters:

- Immune modulation - Pectins in Toll-like receptor activation and inhibition
    - Pectins with blockwise distributed GalA residues strongly induce TLR2-1 signaling in a pectin structure dependent way

- Fermentation characteristics – shaping the human gut microbiome
    - Differently methyl esterified pectins stimulate different gut bacteria and fermentation differs in speed (= location), type and level of metabolites formed.

Sahasrabudhe et al, 2018
Beukema et al, 2020
Beukema et al, 2022

Enzymatic fingerprinting of polysaccharides

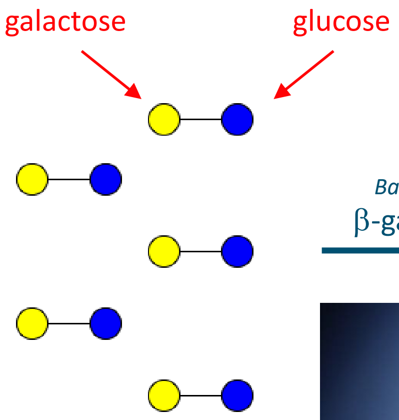
- Approach successfully applied on:
- Pectins
- Arabinoxylans
- Derivatised starches (Crosslinked, HP-, Ac-, RS3)
- Galactomannans
- Alginates
- Xanthan

Oligosaccharide research

UPLC-HILIC-MS characterisation of oligosaccharides

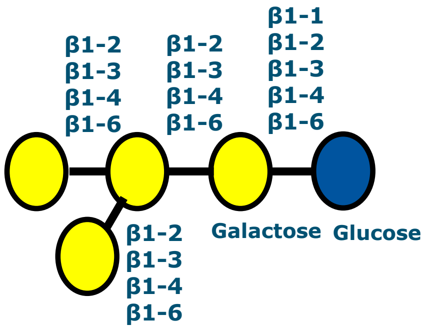
Prebiotische OS: Galacto-oligosachariden GOS

Enzymatically produced from lactose



lactose

Bacillus circulans
β-galactosidase



Complex mixture of
oligomers DP 2-8;
Many different isomers

Commercial syrup Vivinal-GOS contains 57% GOS, 21% lactose, and 22% galactose + glucose.

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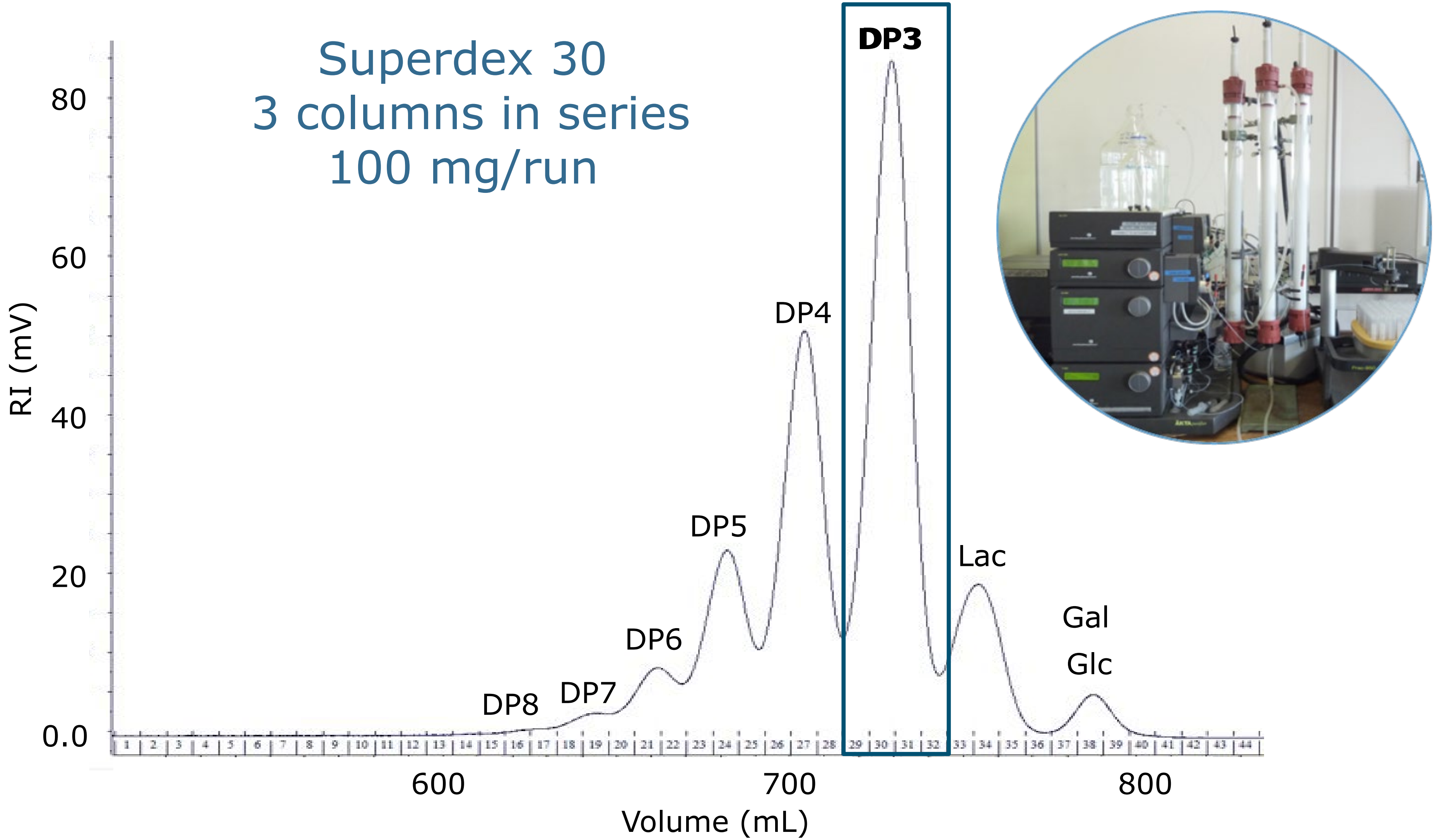
Preparative SEC of GOS

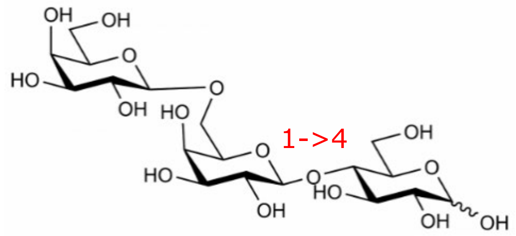


Superdex 30
3 columns in series
100 mg/run

HPLC-MS analysis of oligomers

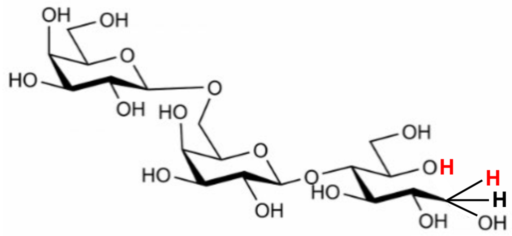
- Complex elution pattern and often broad peaks due to (partly) separation of alfa/beta anomers.
- Labelling / reduction of reducing end of oligosaccharide
- Label-enhanced annotation of MS-fragments

GOS Pre-treatment before UHPLC-PGC-MS Analysis

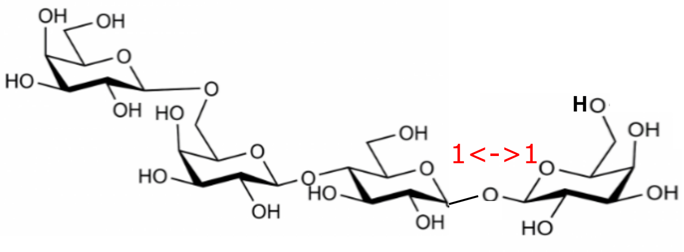


Galactosyl-6-lactose (6'GL)
m/z 504

1. $NaBH_4$ reduction
2. SPE

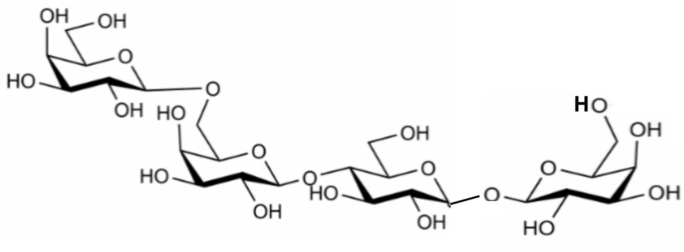


Reduced 6'GL
m/z 504 + 2



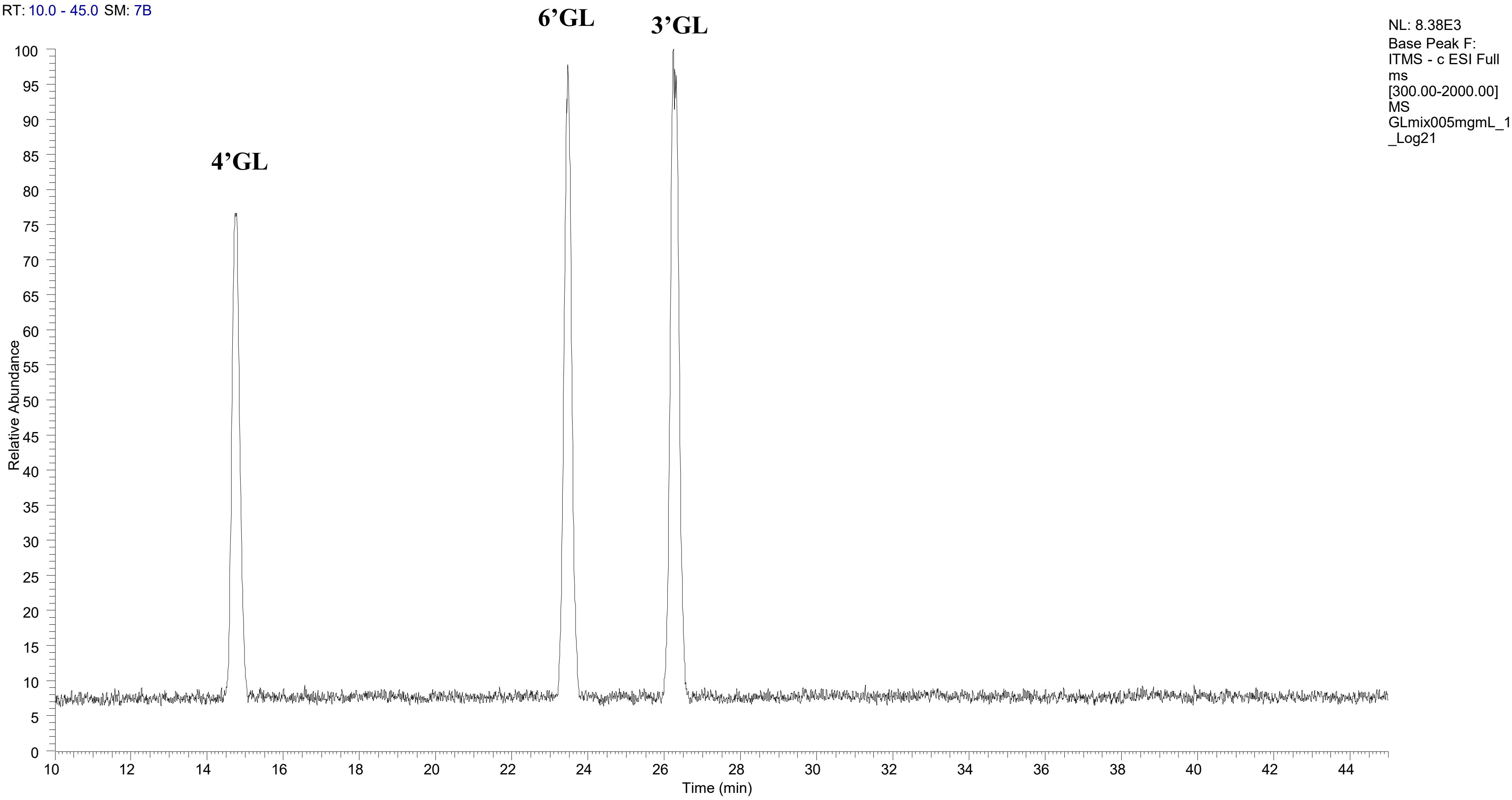
DP4 GOS
m/z 666

1. $NaBH_4$ reduction
2. SPE

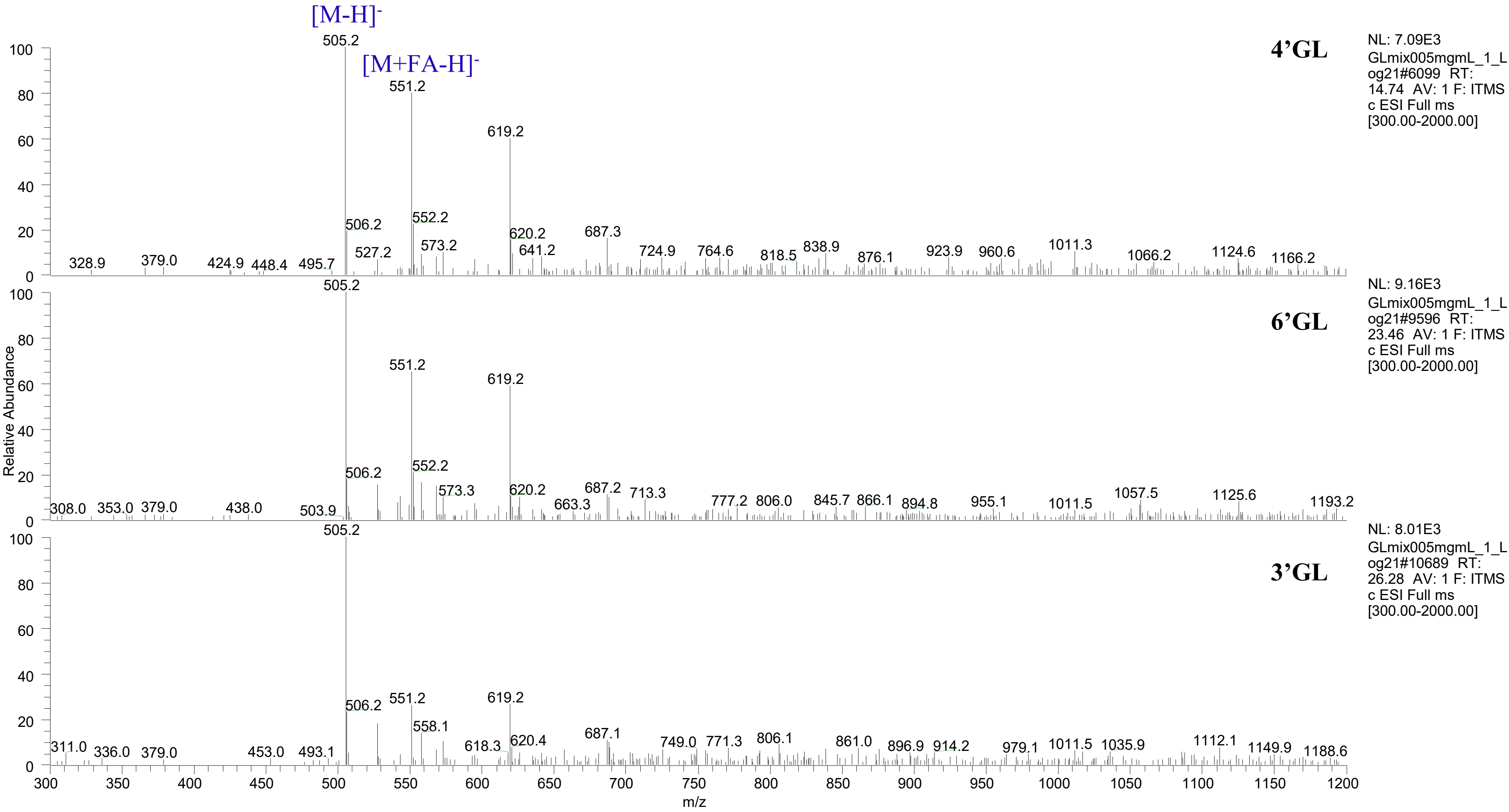


Non-reduced DP4 GOS
m/z 666

PGC-MS elution patterns of reduced 3′, 4′, 6′ Galactosyl Lactose standards

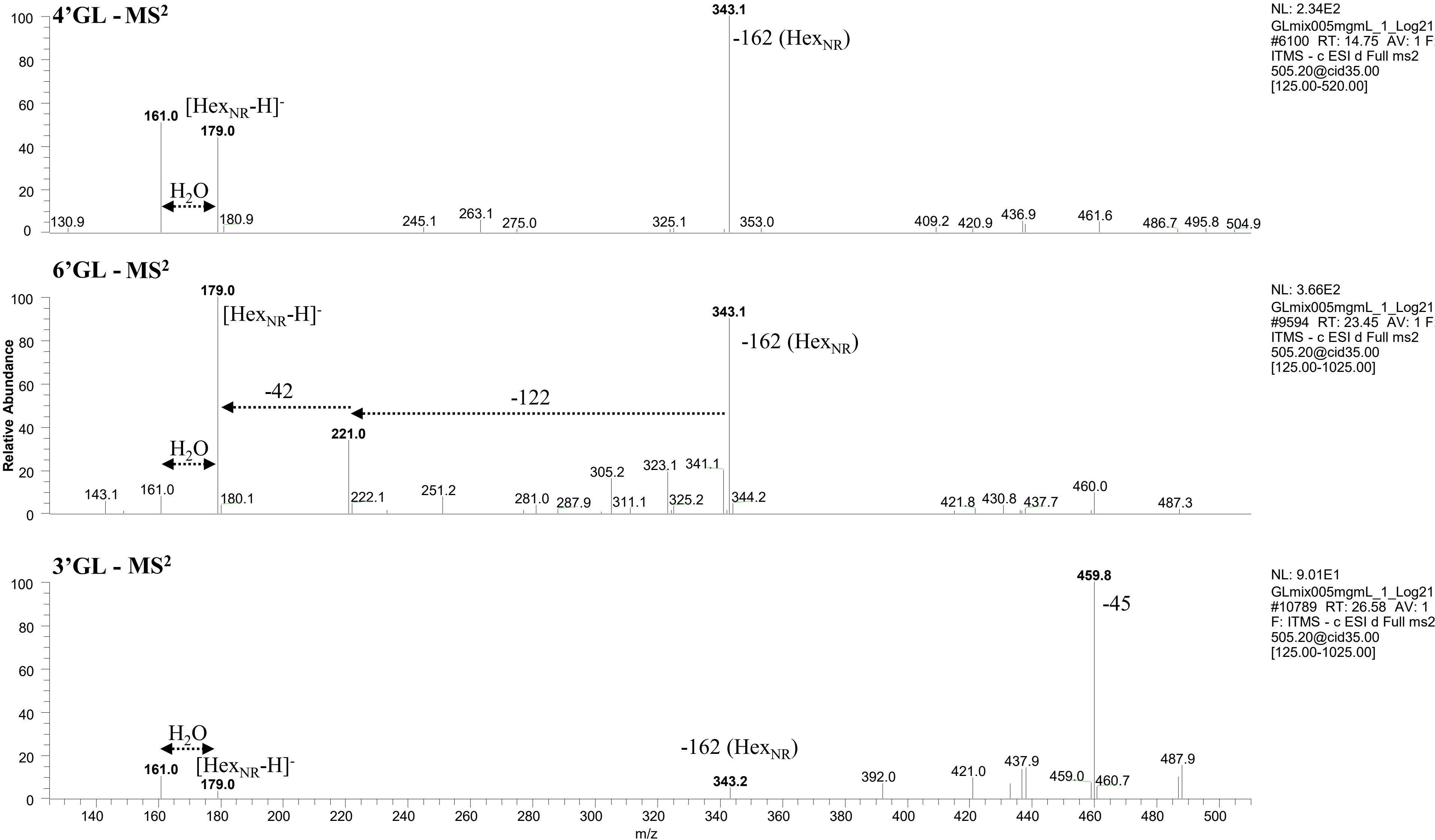


Mass spectra of reduced 3’, 4’, 6’ Galactosyl Lactose standards (negative mode)



MS^2 mass spectra of reduced 3’, 4’, 6’ GL

MS/MS spectra of *m/z* 505 [M-H]$^-$



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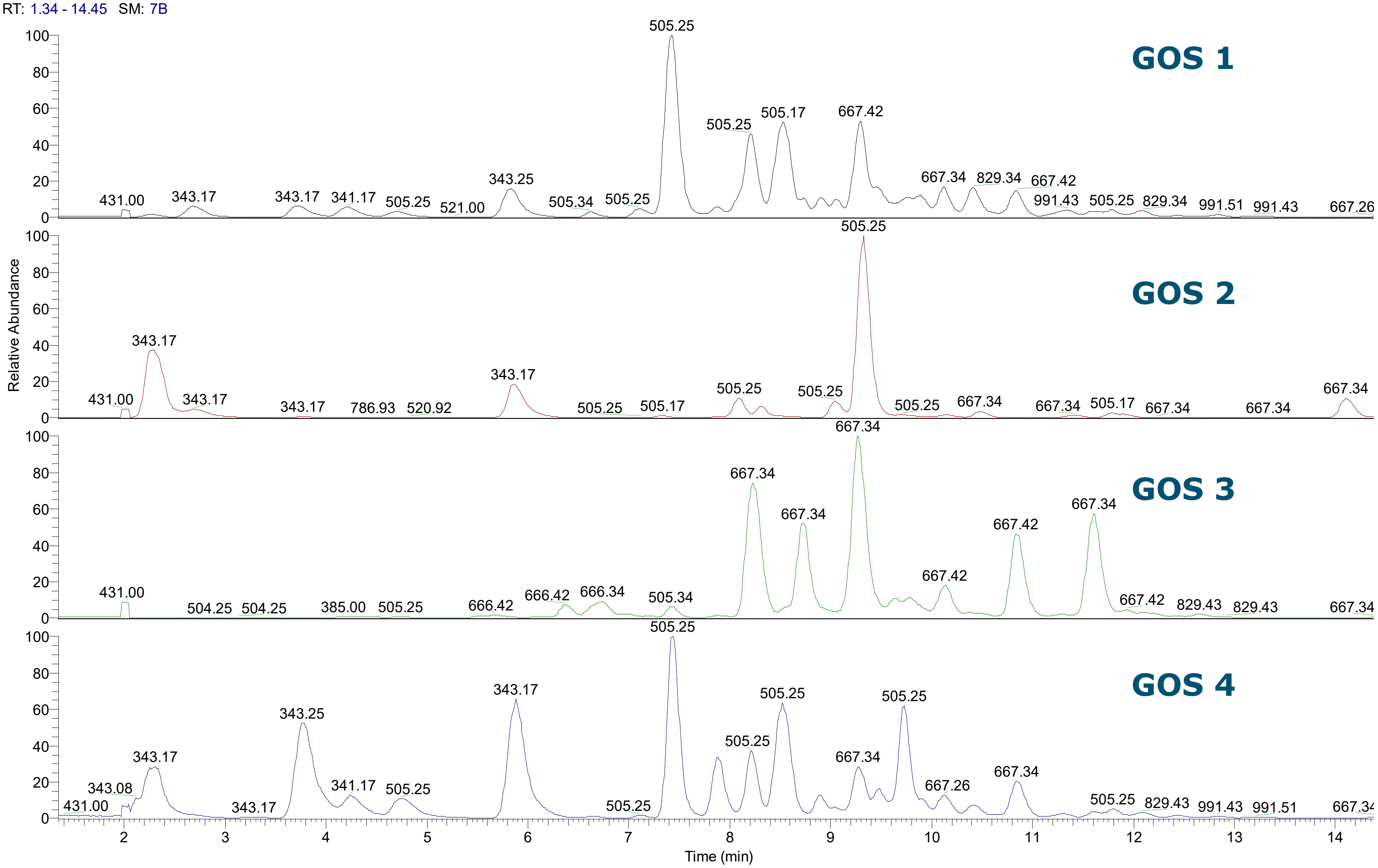
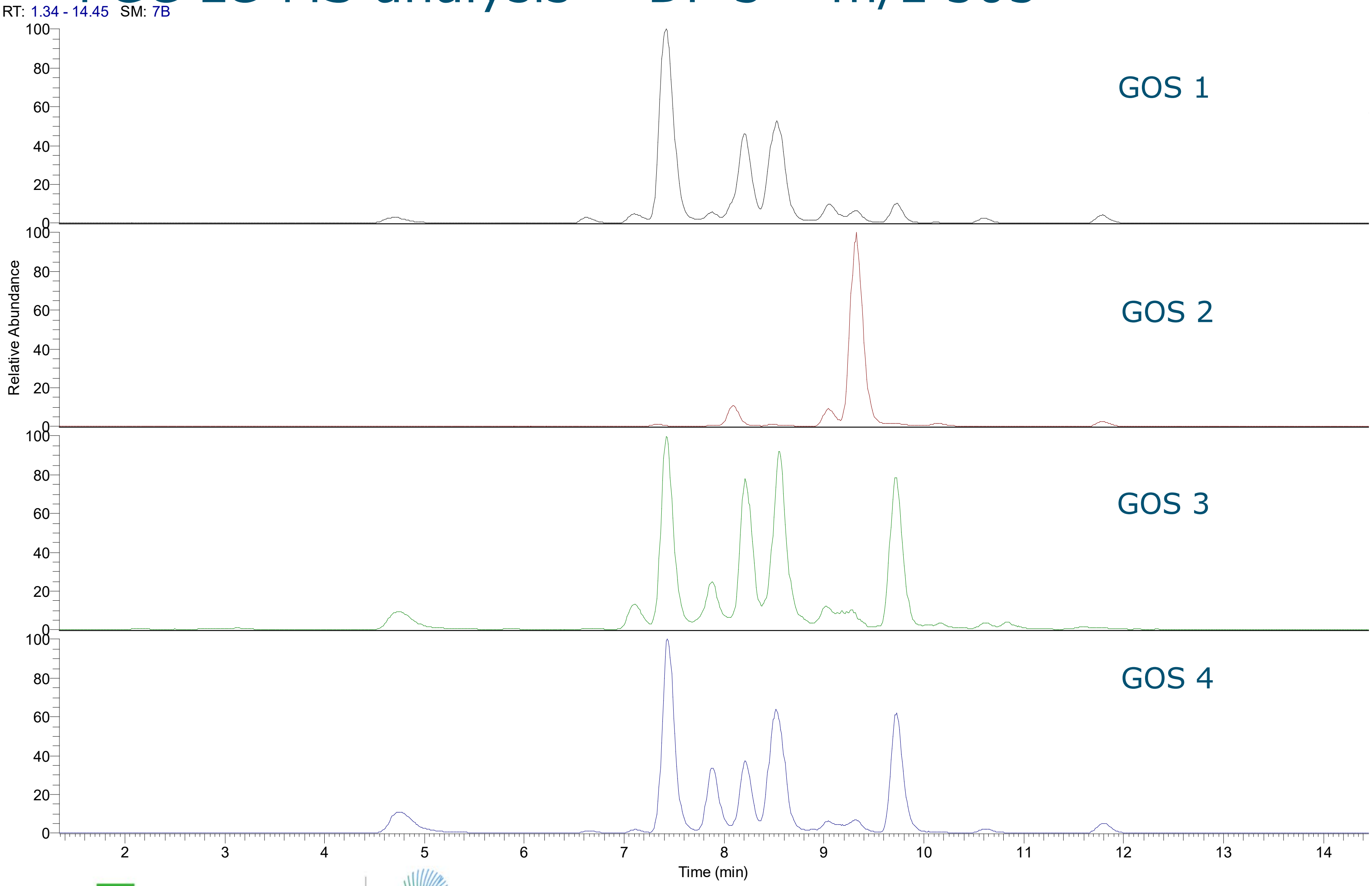
1918 — 2018

Compositional differences of different GOS samples PGC LC-MS analysis - all oligomers



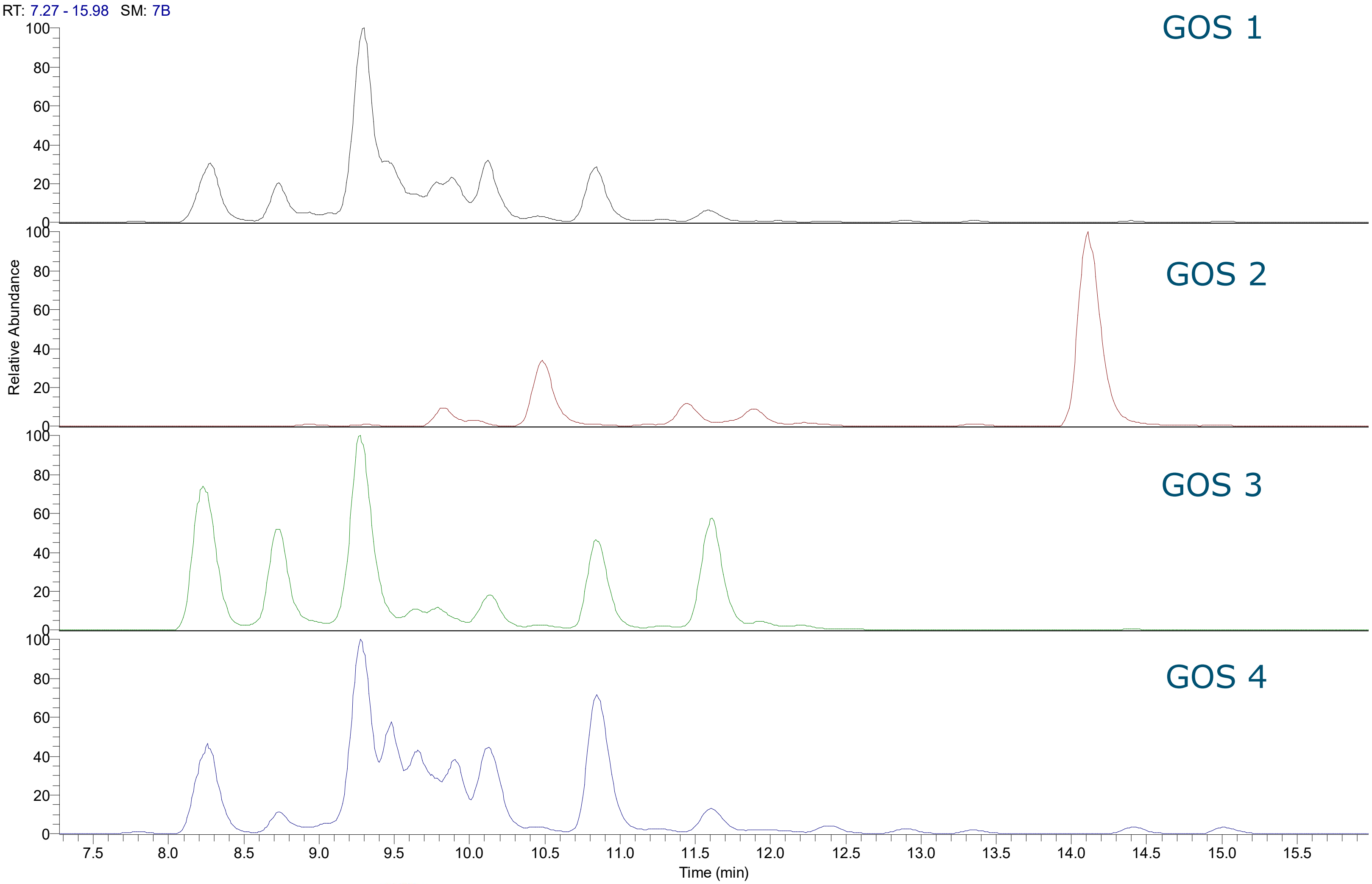
Compositional differences of GOS

PGC LC-MS analysis - DP 3 – m/z 505

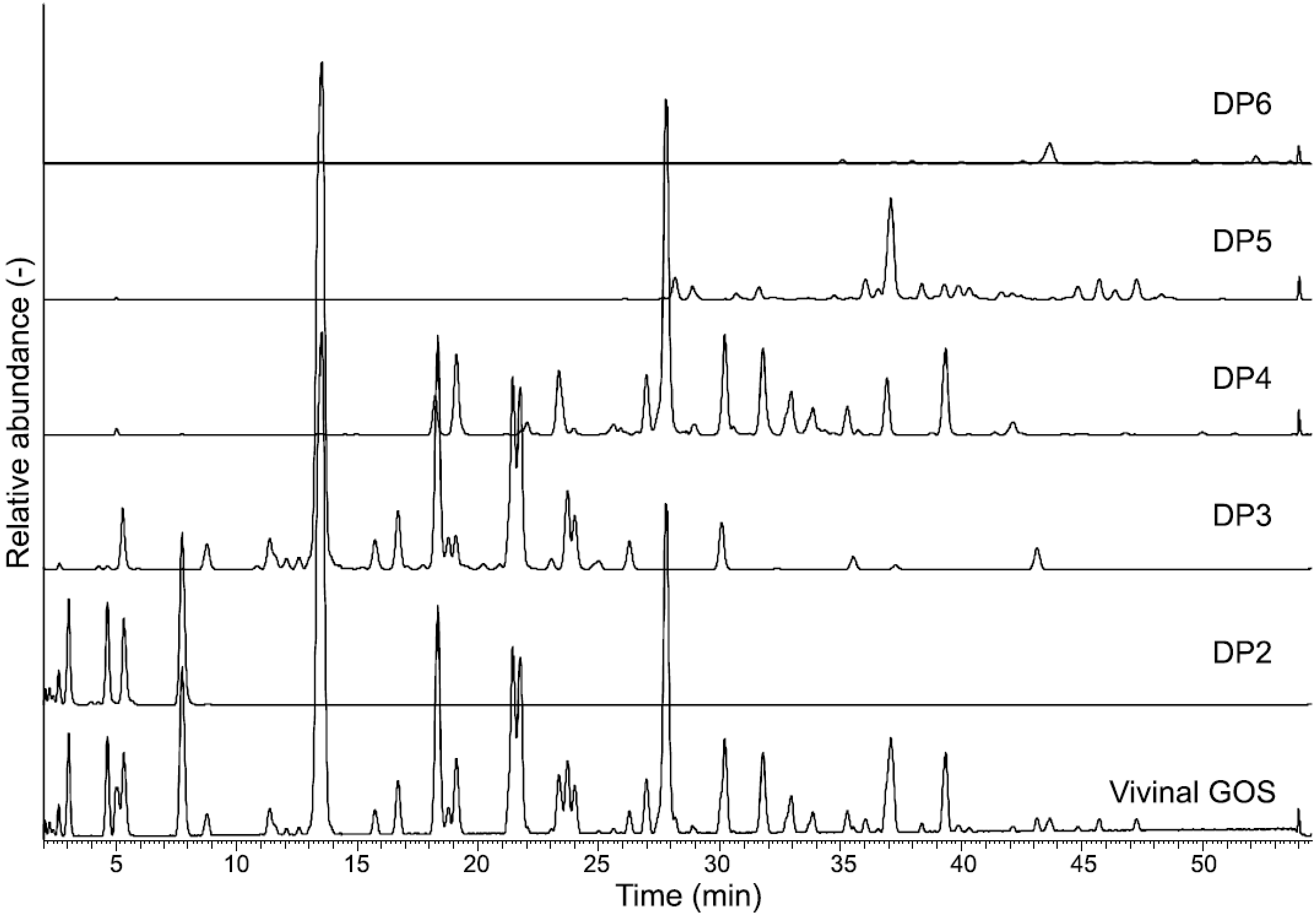


Compositional differences of GOS

PGC LC-MS analysis - DP 4 – m/z 667

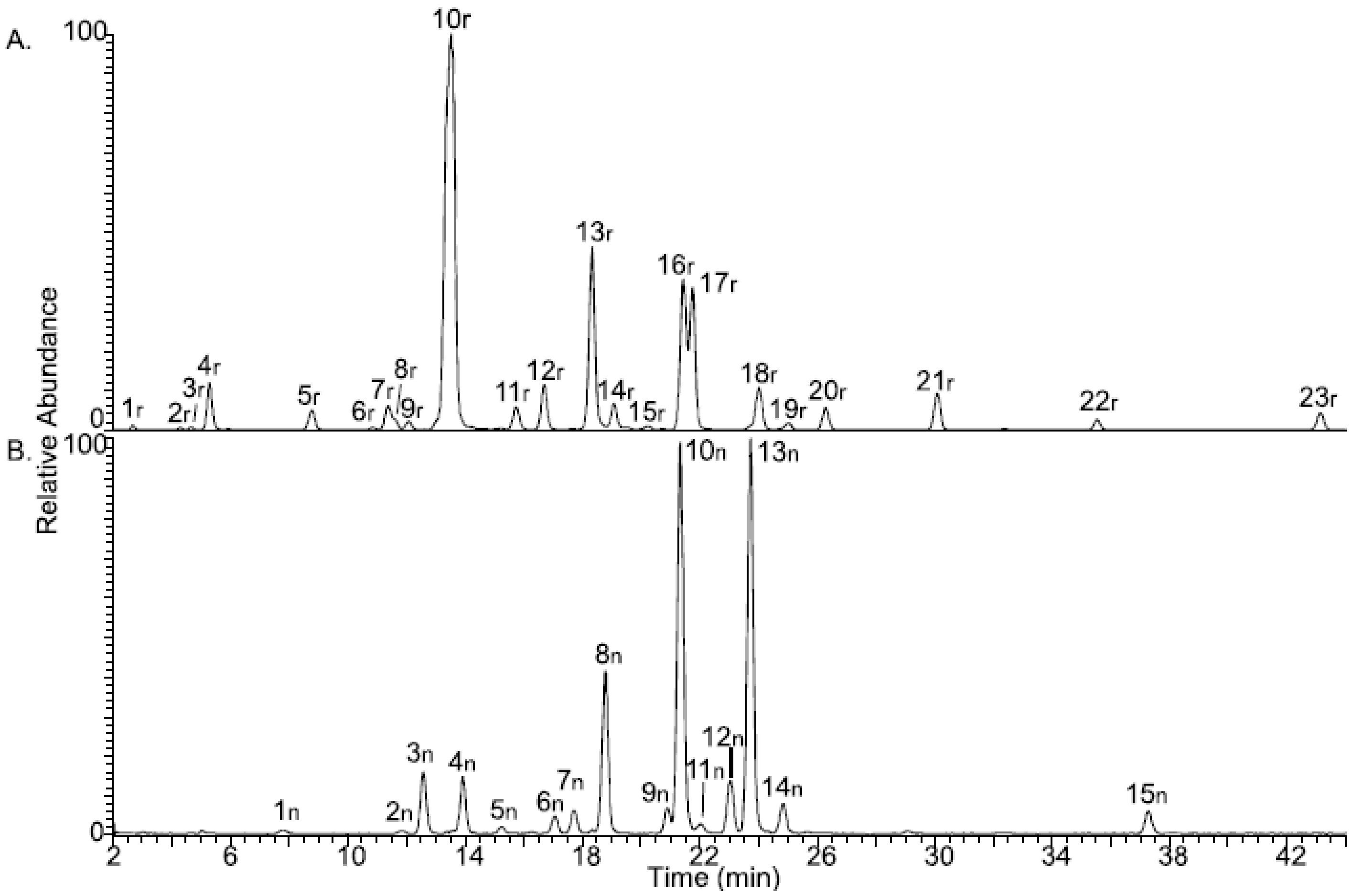


UHPLC–PGC–MS profile of reducing and non-reducing Vivinal GOS



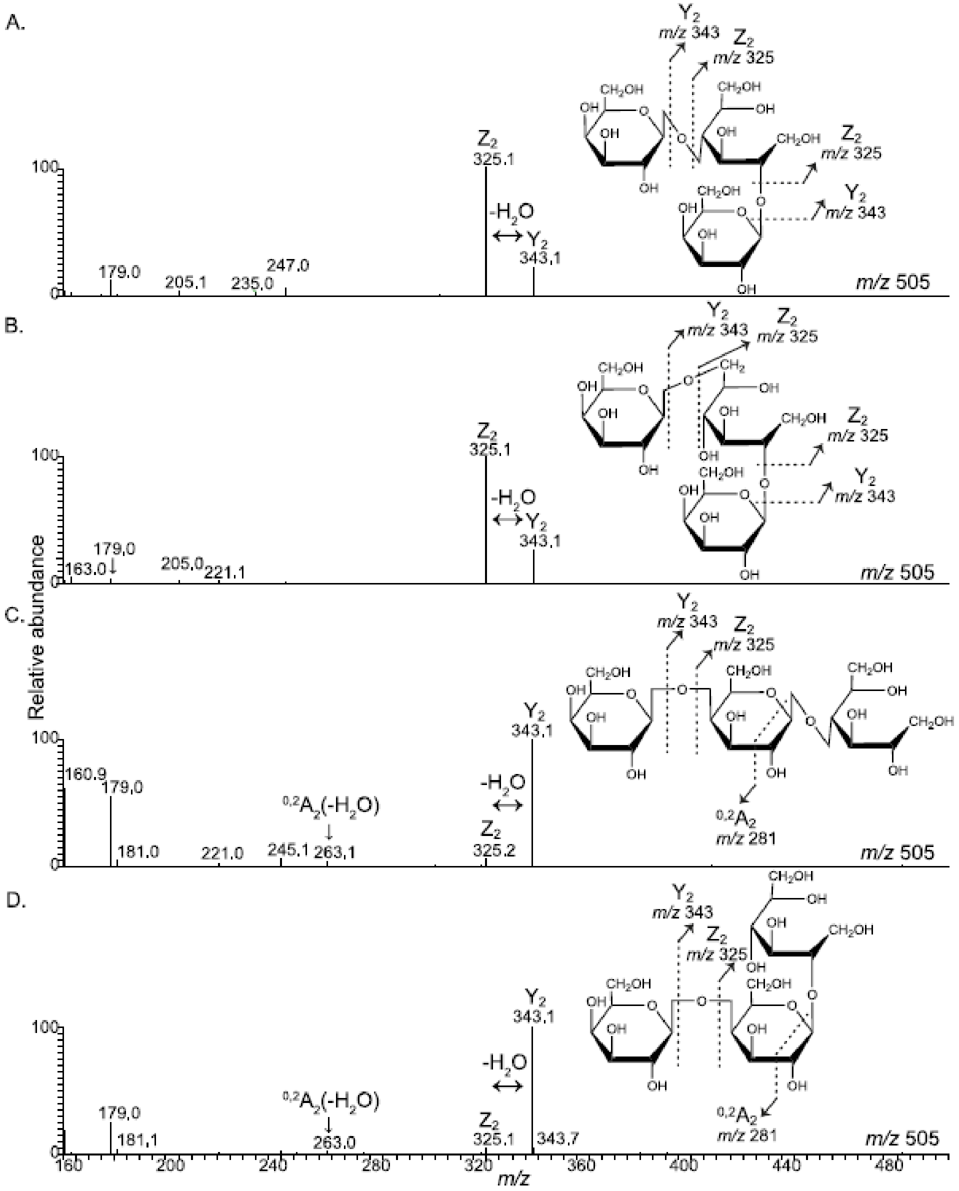
Logtenberg et al, 2020

UHPLC–PGC–MS profile of Vivinal GOS DP3 with the selection of (A) reducing [m/z 505 and 551 (M + FA)] and (B) nonreducing [m/z 503 and 549 (M + FA)] isomers

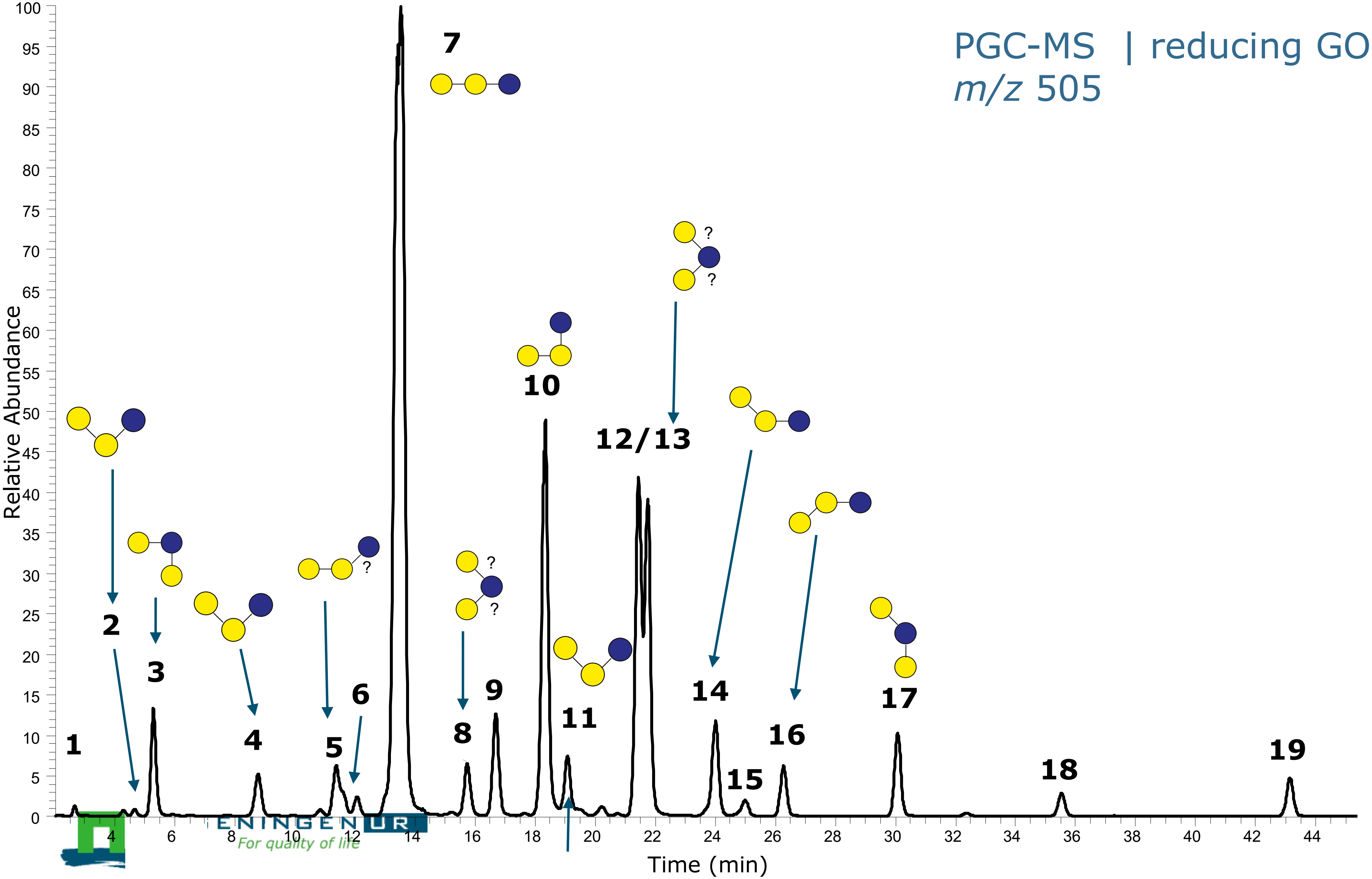


Logtenberg et al, 2020

Fragmentation spectra (ESI MS^2) in negative mode of GOS DP3 isomers (A) 4r, (B) 21r, (C) 10r, and (D) 13r



Characterized GOS DP3 isomers



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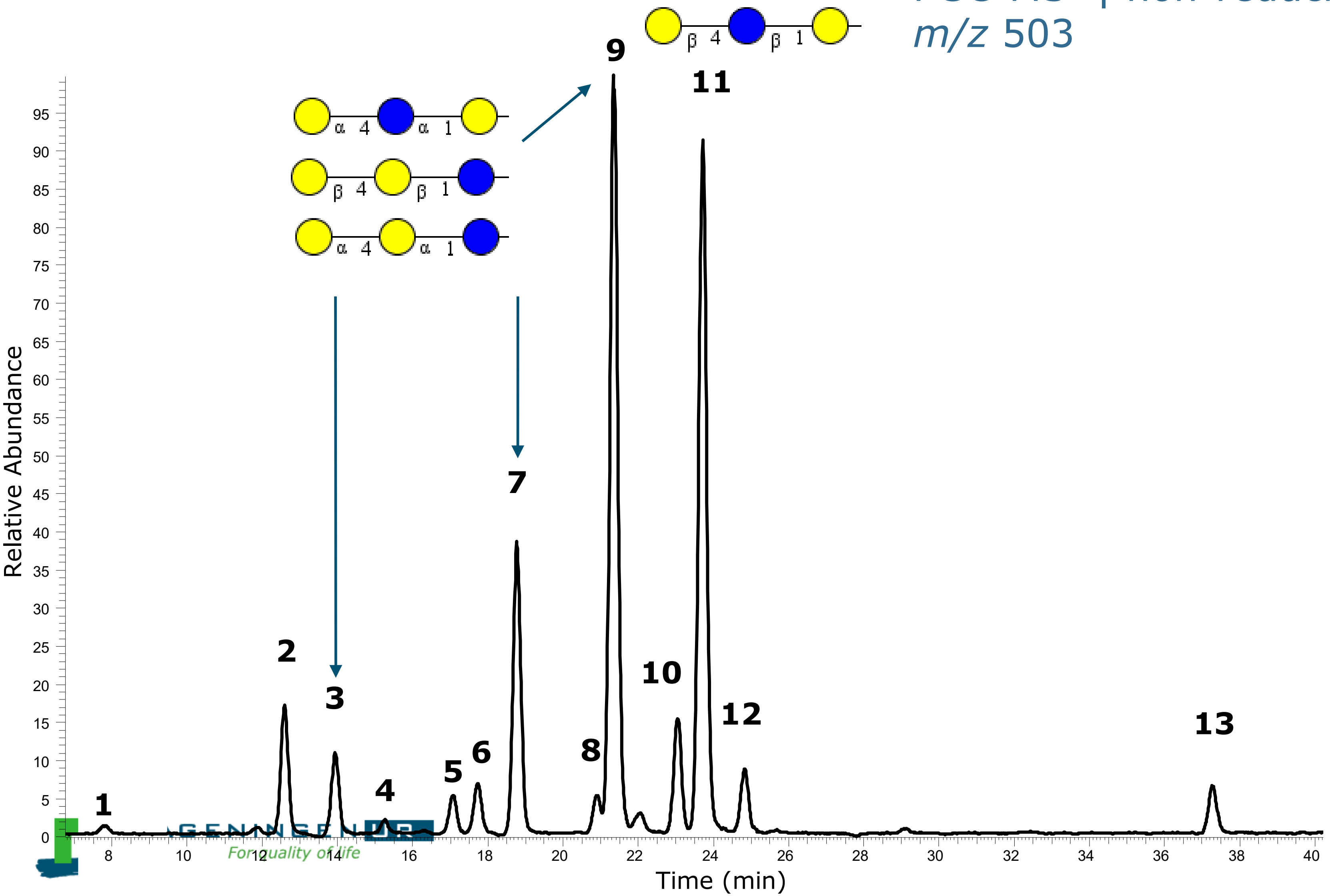
For quality of life

GOS DP3 isomers with (1-1)-linkage

PGC-MS | non-reducing GOS
m/z 503

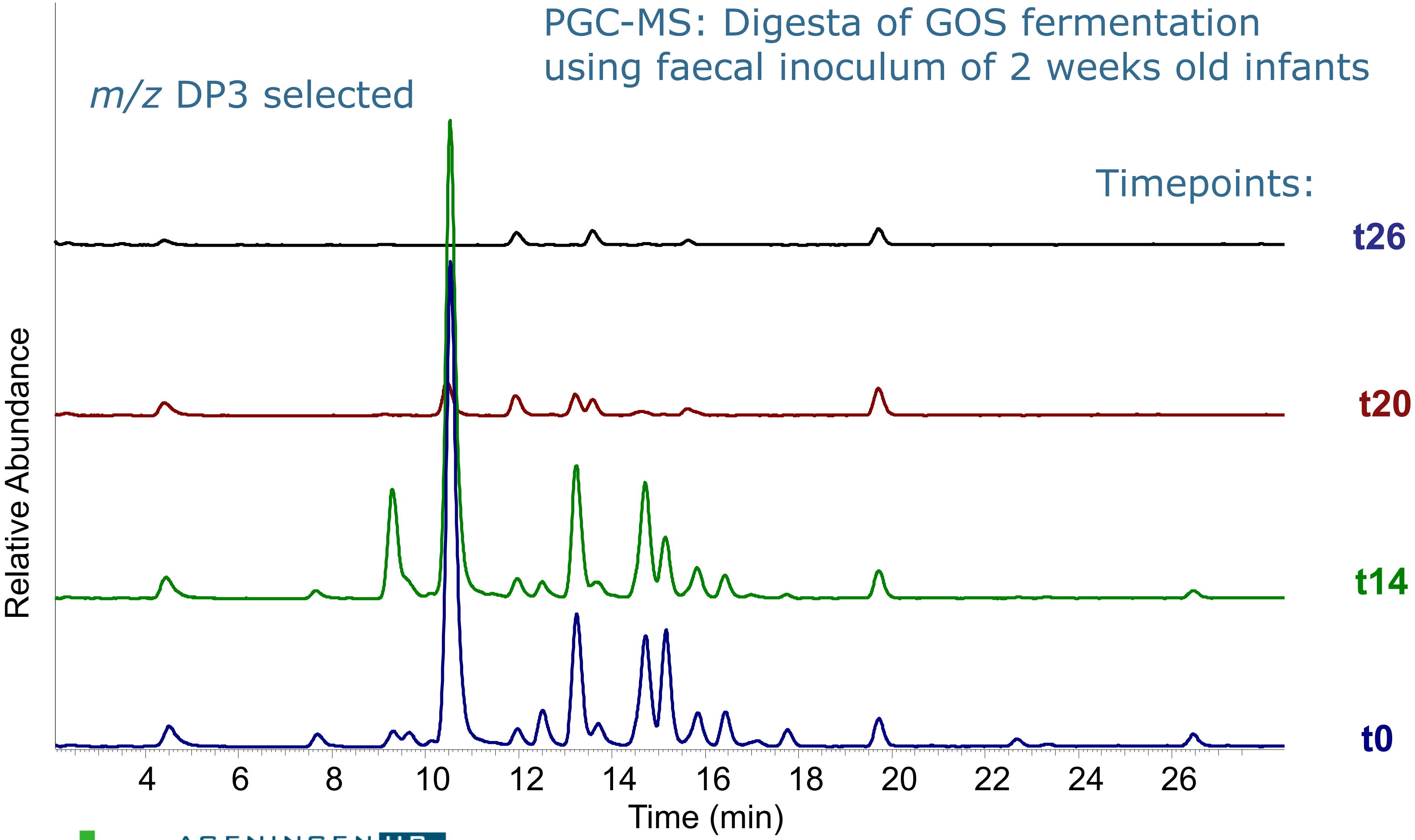


GOS's functionality: Size and linkages matters

- Fermentation characteristics – shaping the human gut microbiome
  - Differently FOS structures stimulate different gut bacteria and fermentation differs in speed (= location) and in type and level of metabolites formed.
- Immune modulation / strengthening barrier function

Sahasrabudhe et al, 2018
Beukema et al, 2020

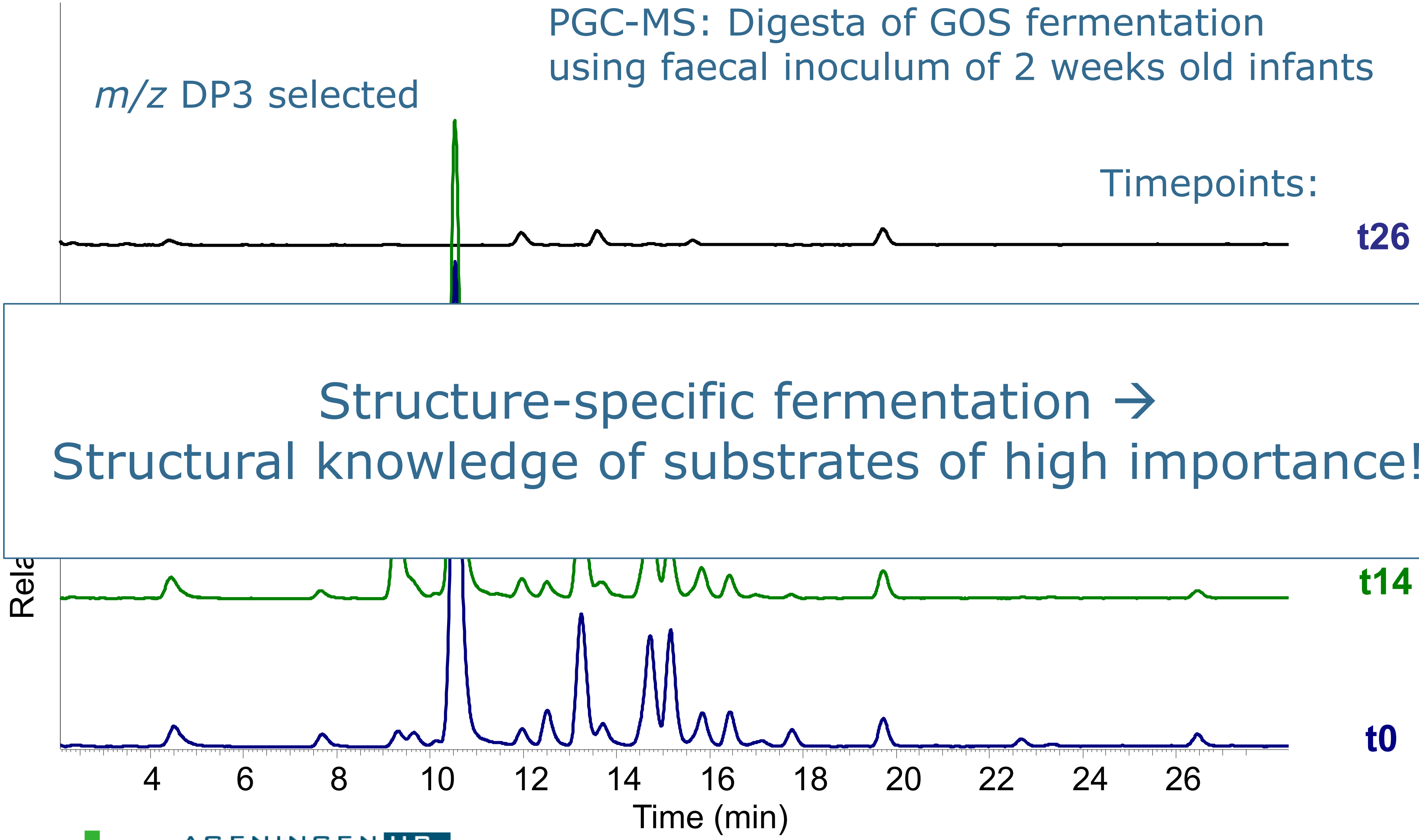
In vitro fermentation using human inoculu shows isomer-specific degradation



PGC-MS: Digesta of GOS fermentation using faecal inoculum of 2 weeks old infants

m/z DP3 selected

In vitro fermentation using human inoculum shows isomer-specific degradation



PGC-MS: Digesta of GOS fermentation using faecal inoculum of 2 weeks old infants

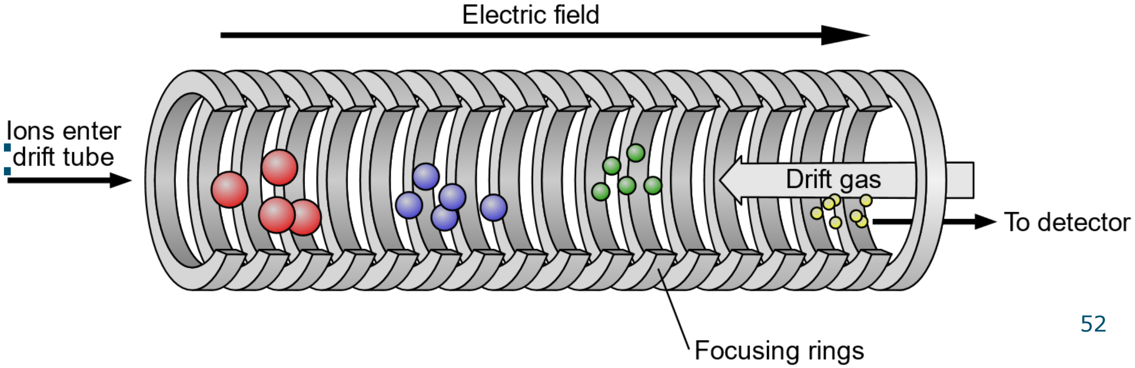
m/z DP3 selected

Structure-specific fermentation →
Structural knowledge of substrates of high importance!

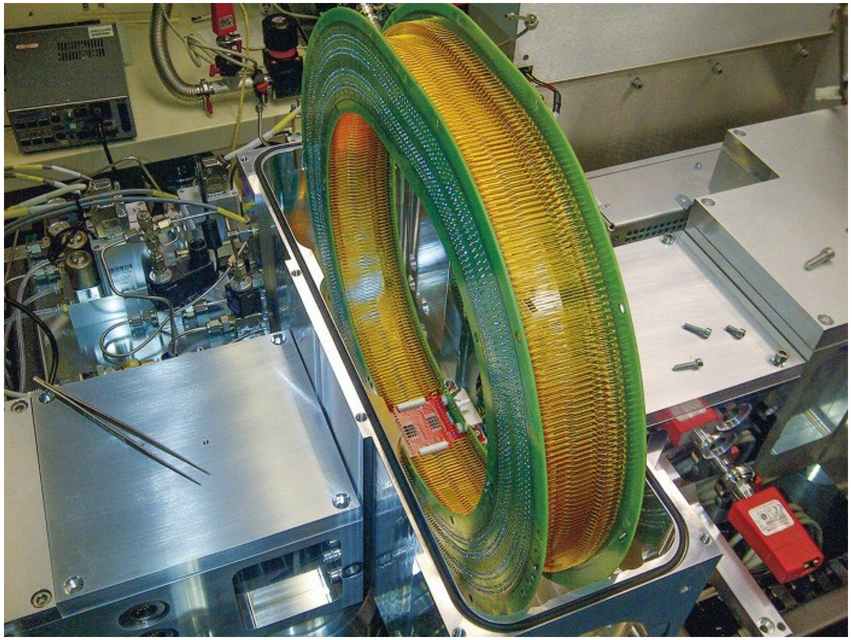
Ion mobility mass spectrometry

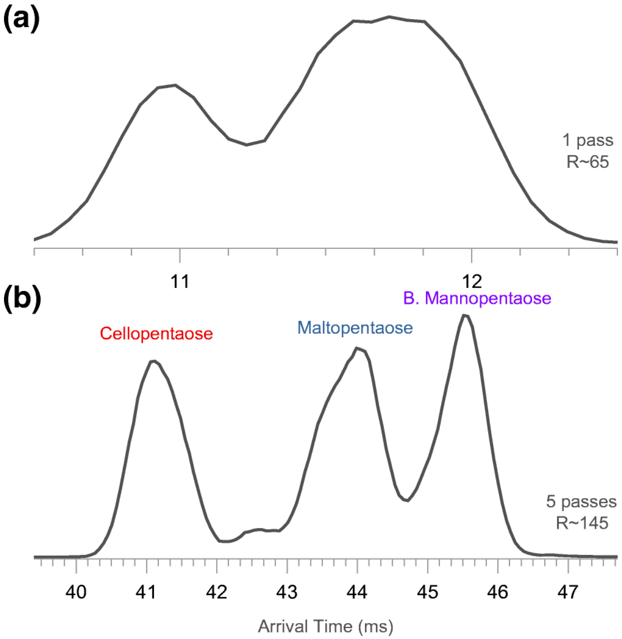
- Ions pushed through tube by electric field
- Drift gas at low pressure/counter flow
- Friction with drift gas slows ions down

- Friction depends on:
  - Size
  - Shape



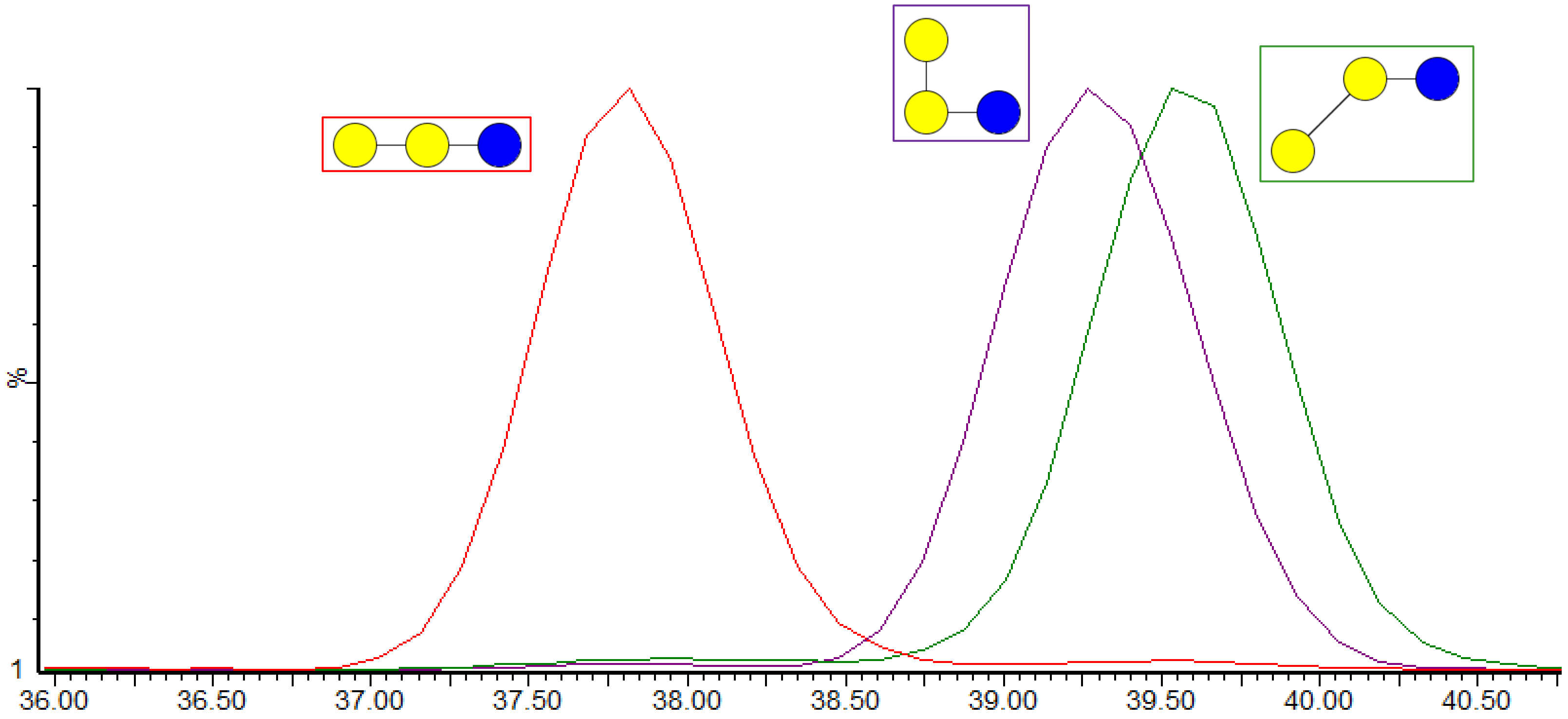
Cyclic IMS: multipass separations



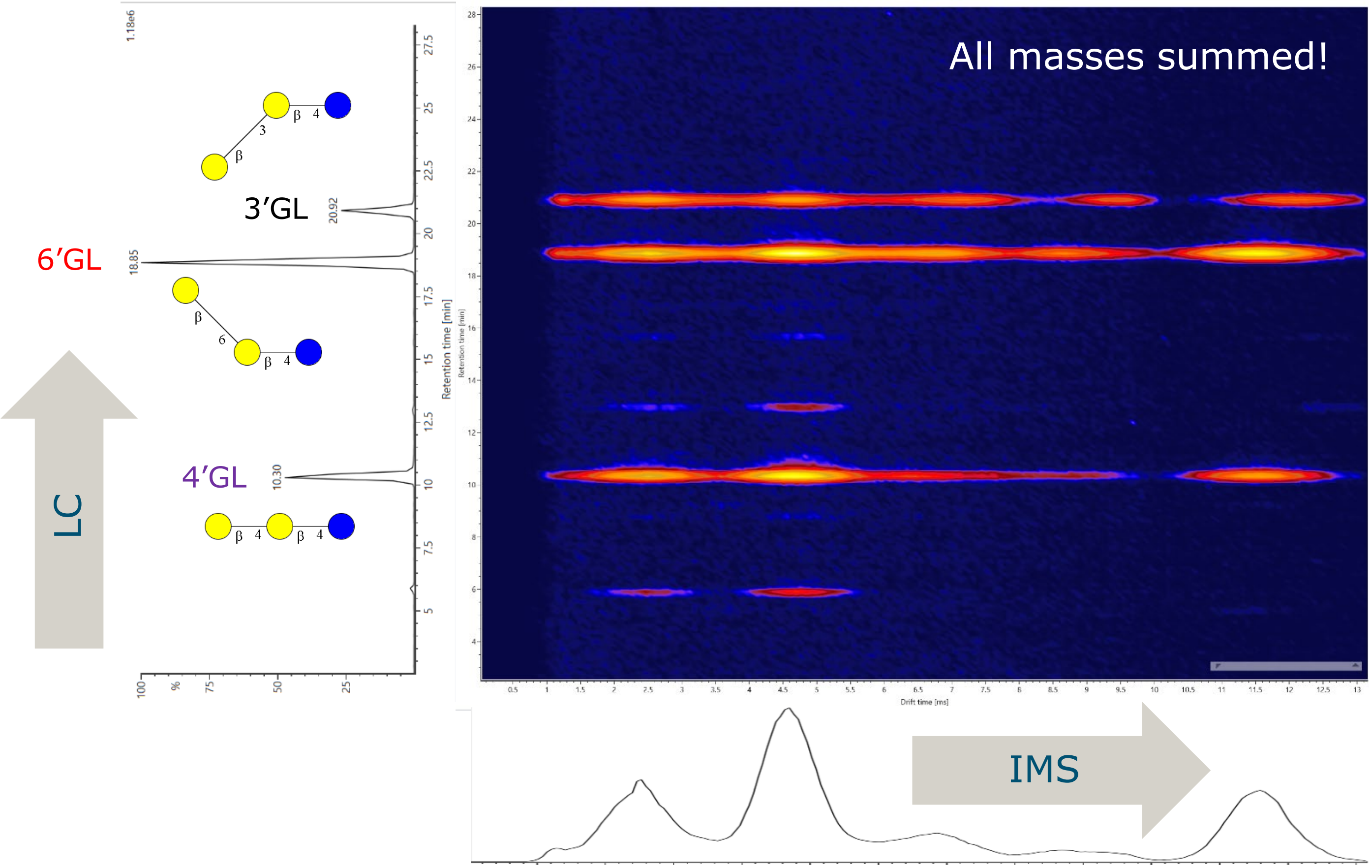


- Increased resolution

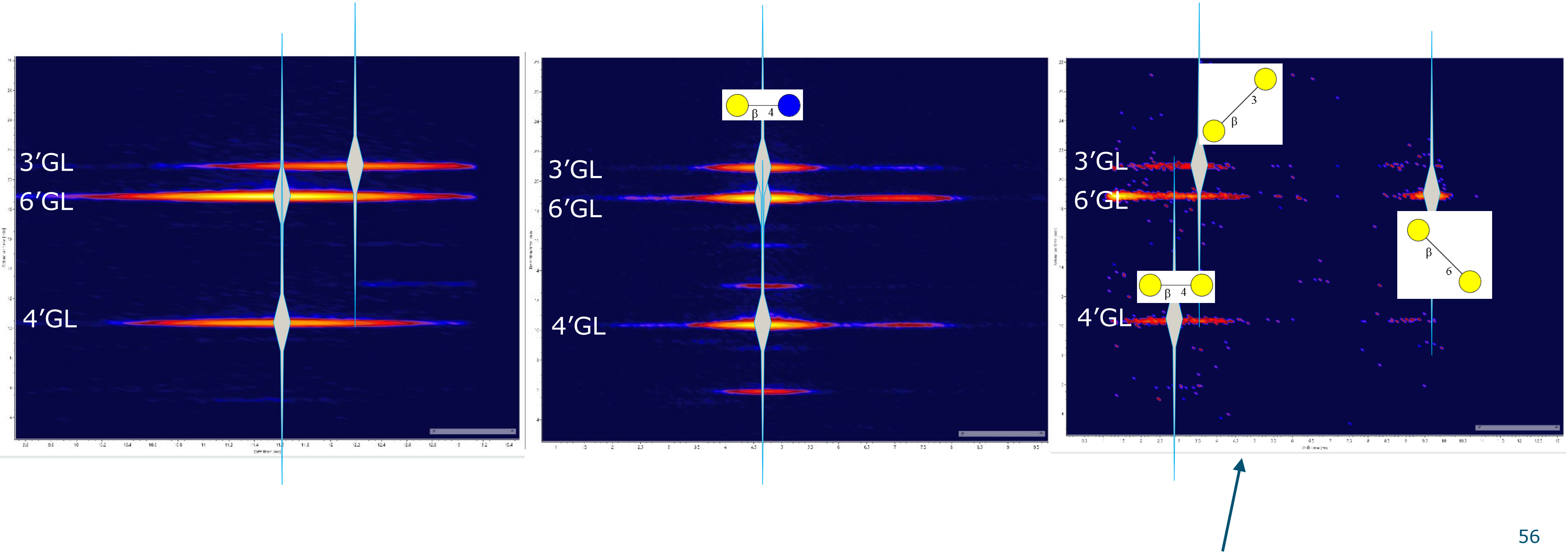
Cyclic IM MS of Galactosyl Lactose standards
Influence of linkage type on drift time



PGC-IM-MS of GL: data structure



LC-IM-MS: Marker ions



Structural marker ions for linkage type

Conclusions

- Enzymatic fingerprinting and subsequent analysis of diagnostic oligomers is quite powerful in characterising a wide range of polysaccharides.
- UHPLC-MS^n using a HILIC BEH amide and PGC can be employed to separate a wide variety of oligomeric structures.
- Depending the research question, a whole array of mass spectrometric techniques are available and useful
    - Maldi TOF (TOF) MS
    - Ion trap MS-MS-MS- ..... MS^n
    - Ion mobility TWIM MS

Thanks for your attention!

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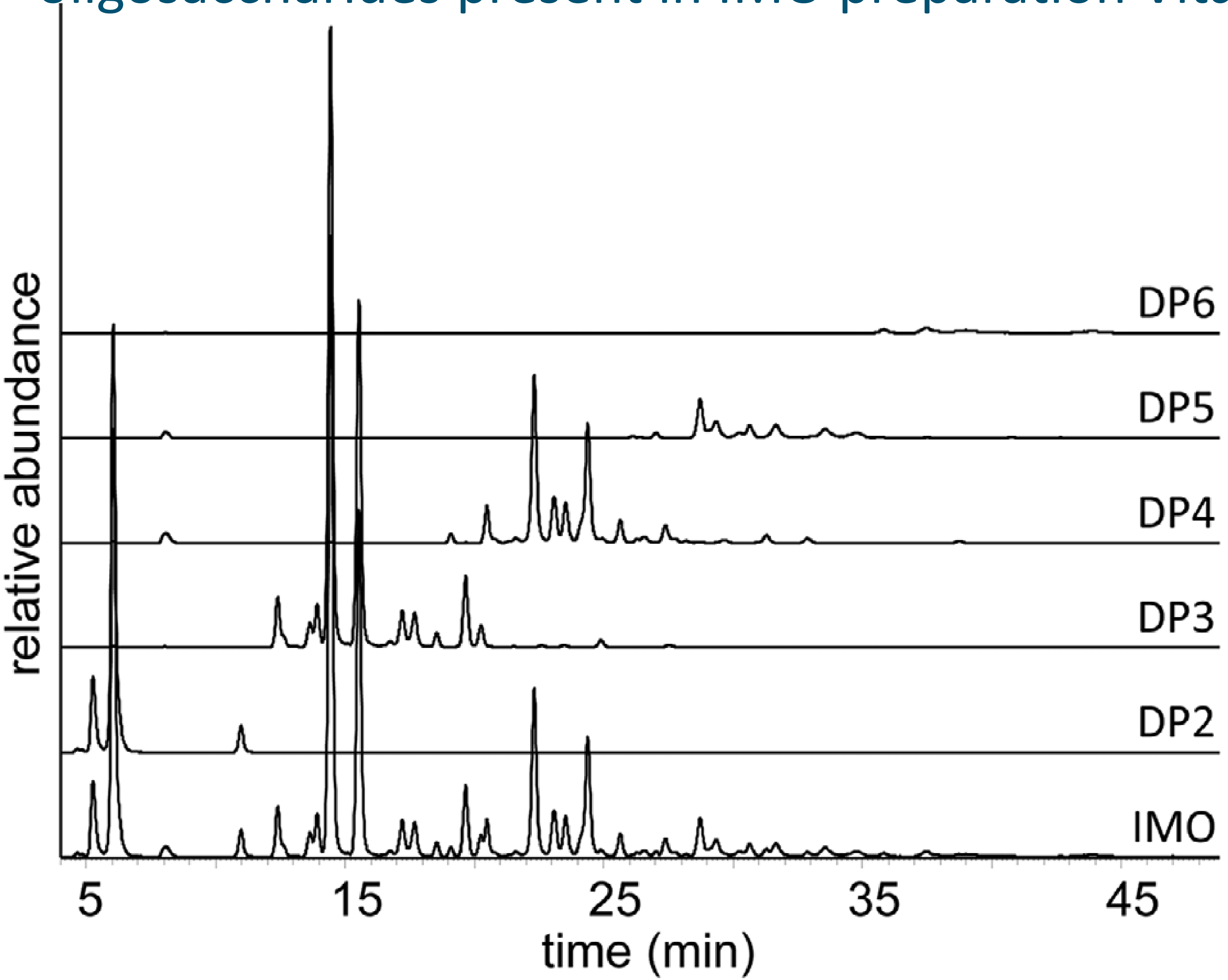
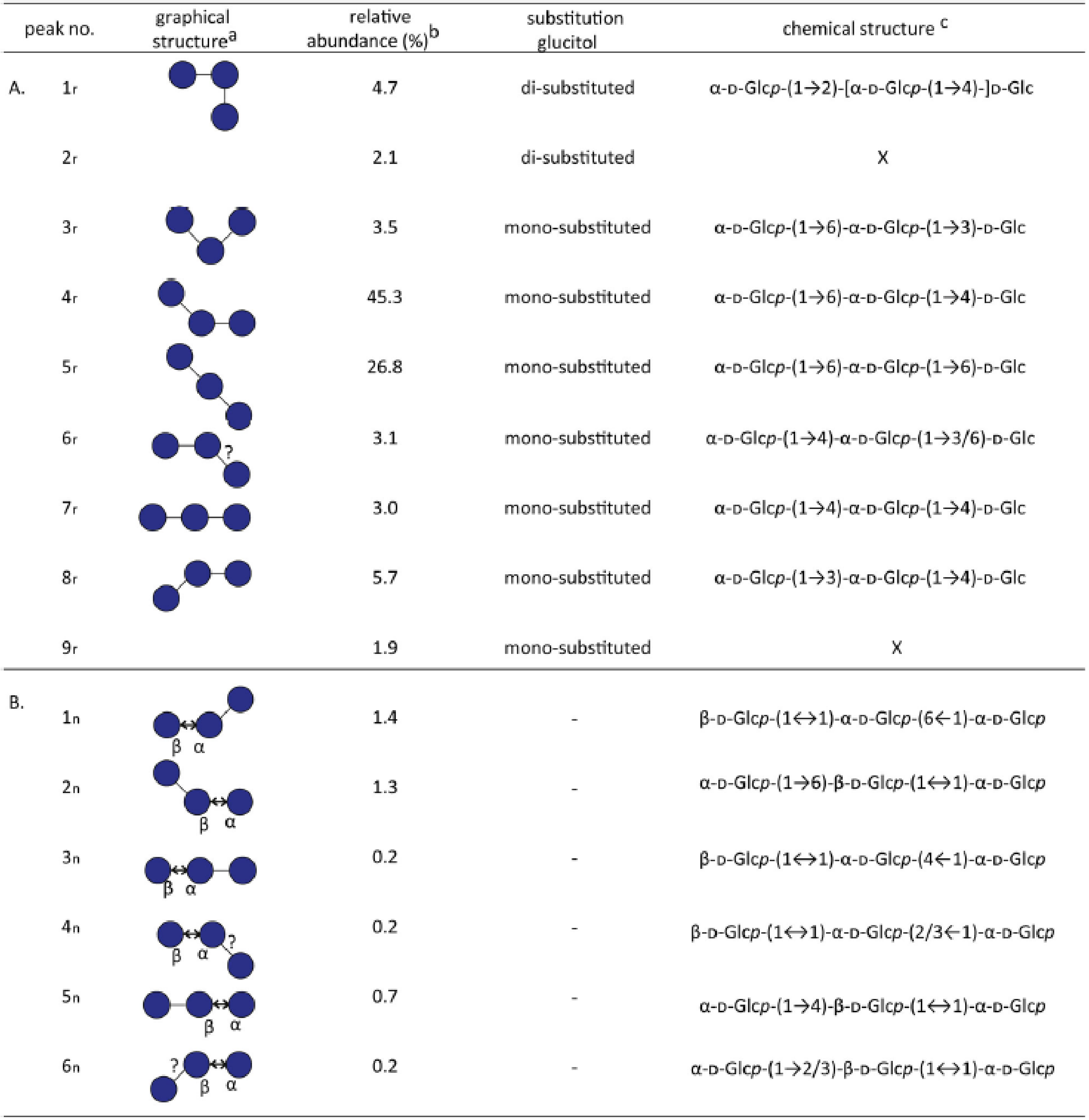
UHPLC-PGC-MS profile of reducing and non-reducing oligosaccharides present in IMO preparation Vitafiber



Overview of reducing (A) and non-reducing (B) DP3 isomers present in the IMO preparation Vitafiber.



	peak no.	graphical structure[a]	relative abundance (%)[b]	substitution glucitol	chemical structure [c]
A.	1_r		4.7	di-substituted	α-D-Glc*p*-(1→2)-[α-D-Glc*p*-(1→4)-]D-Glc
	2_r		2.1	di-substituted	X
	3_r		3.5	mono-substituted	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-D-Glc
	4_r		45.3	mono-substituted	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc
	5_r		26.8	mono-substituted	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→6)-D-Glc
	6_r	?	3.1	mono-substituted	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3/6)-D-Glc
	7_r		3.0	mono-substituted	α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc
	8_r		5.7	mono-substituted	α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-D-Glc
	9_r		1.9	mono-substituted	X
B.	1_n	β α	1.4	-	β-D-Glc*p*-(1↔1)-α-D-Glc*p*-(6←1)-α-D-Glc*p*
	2_n	β α	1.3	-	α-D-Glc*p*-(1→6)-β-D-Glc*p*-(1↔1)-α-D-Glc*p*
	3_n	β α	0.2	-	β-D-Glc*p*-(1↔1)-α-D-Glc*p*-(4←1)-α-D-Glc*p*
	4_n	β α ?	0.2	-	β-D-Glc*p*-(1↔1)-α-D-Glc*p*-(2/3←1)-α-D-Glc*p*
	5_n	β α	0.7	-	α-D-Glc*p*-(1→4)-β-D-Glc*p*-(1↔1)-α-D-Glc*p*
	6_n	? β α	0.2	-	α-D-Glc*p*-(1→2/3)-β-D-Glc*p*-(1↔1)-α-D-Glc*p*

PGC-MS elution profiles of three IsoMalto OS preparations,