

# Could It Be Enzymes?

## Fons Voragen

5<sup>th</sup> European Symposium on Enzymes in Grain Processing (esEGP5)

Norwich UK

31.03.08 – 02.04.08



**WAGENINGEN UNIVERSITY**

**AGROTECHNOLOGY AND FOOD SCIENCES**

**LABORATORY OF FOOD CHEMISTRY**

# Start Cereal Xylan Research Food Chemistry Wageningen

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- Mid '80ties
- Effective Bread Improver introduced by Puratos Belgium
- Active Ingredient? Chemical? No!
- **Could It Be An Enzyme?**
- Screening protocol for assaying enzymes produced by *Rhizopus Oligosporus* during Tempeh fermentation and by *Aspergillus awamori* for xylan conversion ( EU programm: Energy from Biomass) available at Lab. of Food Chemistry.
- Strong xylanase activity in Improver!!!
- Race for more knowledge of xylans and xylanases started.
- This was also the start of research in this area in our laboratory.



# Main Research area's

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- **Structural Characterization of (Glucurono-)Arabinoxylans in Water Unextractable Solids (WUS) from:**
  - Wheat: flour and bran (**Baking, Feed, Valorization**)
  - Barley and malt (**Brewing**)
  - Sorghum and Maize (**Brewing, Feed**)
- **Mode of action of endo-xylanases and accessory enzymes from *Aspergillus awamori* CMI 142717 and from *Bifidobacterium adolescentis* DSM 20083**
- **Peroxidase catalyzed conjugation of peptides, proteins and polysaccharides via endogenous and exogenous phenols.**
- **Progress Analysis Techniques**





# Role of arabinoxylans in Grain processing and Grain products

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- **Baking (Wheat/rye)**
  - Milling behaviour
  - Dough characteristics
  - Loaf volume
  - Crumb and crust characteristics (staling)
- **Starch-gluten separation**
- **Filtration wheat starch hydrolysates**
- **Gluten quality**
- **Ingredient (gelling/ thickening/ NDO)**
- **Brewing**
  - Starch conversion (accessibility)
  - Filtration wort/beer
  - Haze formation
- **Food/ feed**
  - Dietary fibre
  - Anti-nutritional effects (limited access nutrients)



# Xylanases in cereal processing

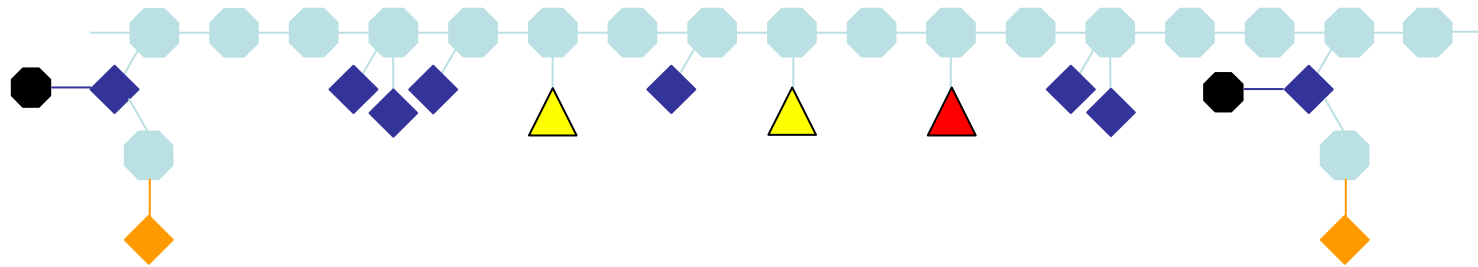
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- **Baking: Dough handling and bread quality.**
- **Feed digestibility.**
- **Starch-Gluten separation (Wheat).**
- **Filtration wheat starch hydrolysates.**
- **Gluten quality.**
- **Brewing: Starch accessibility, wort filtration and haze prevention (adjuncts).**
- **By product utilization.**
- **Ingredient modification .**





# General structure of (glucurono)arabinoxylans from Grains

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  $\beta$ -Xylp

  $\alpha$ -Araf

  $\alpha$ -GlcA

 4-OMeGlcA

 Feruloyl or  
coumaryl group

     
Galp-(1 $\rightarrow$ 4)-Xylp-(1 $\rightarrow$ 2)-  
[5-*O*-(*trans*-feruloyl)-Araf  
(Saulnier *et al.*, 1995)



# WUS yield (% ds flour) and WUS composition (% ds WUS) of some Grains

	Wheat		Barley		Rye		Sorghum	Maize
	flour	bran	whole grain	spent grain	Whole grain	bran	Whole grain	whole grain
<b>Yield</b>	<b>1.2</b>	<b>58</b>	<b>7.6</b>	<b>58</b>	<b>14.8</b>	<b>54</b>	<b>5.3</b>	<b>8.7</b>
<b>Protein</b>	<b>2</b>	<b>5</b>	<b>32</b>	<b>5</b>	<b>10</b>	<b>3</b>	<b>7</b>	<b>7</b>
<b>Glucose</b>	<b>15</b>	<b>24</b>	<b>36</b>	<b>21</b>	<b>28</b>	<b>29</b>	<b>34</b>	<b>33</b>
<b>starch</b>	<b>3</b>	<b>1</b>	<b>4</b>	<b>2</b>	<b>13</b>	<b>3</b>	<b>3</b>	<b>8</b>
<b>cellulose</b>	<b>6</b>	<b>16</b>	<b>4</b>				<b>12</b>	<b>22</b>
<b>(1→3,1→4)-β-glucan</b>	<b>6</b>	<b>3</b>	<b>21</b>	<b>}18</b>	<b>}14</b>	<b>}24</b>	<b>3</b>	<b>nd</b>
<b>arabinose + xylose</b>	<b>74</b>	<b>50</b>	<b>29</b>	<b>38</b>	<b>31</b>	<b>48</b>	<b>38</b>	<b>53</b>
<b>ara/xyl</b>	<b>0.6</b>	<b>0.7</b>	<b>0.7</b>	<b>0.6</b>	<b>0.6</b>	<b>0.4</b>	<b>0.9</b>	<b>0.8</b>
<b>phenolic acid</b>	<b>0.3</b>	<b>0.8</b>	<b>0.4</b>	<b>0.8</b>	<b>nd</b>	<b>nd</b>	<b>0.8</b>	<b>1.7</b>
<b>uronic acids</b>	<b>+</b>	<b>3.2</b>	<b>+</b>	<b>3.3</b>	<b>1.0</b>	<b>3.0</b>	<b>6.5</b>	<b>8.5</b>
<b>acetyl groups</b>	<b>+</b>	<b>1.2</b>	<b>0.5</b>	<b>0.7</b>	<b>nd</b>	<b>0.8</b>	<b>2.4</b>	<b>4.9</b>

nd = not determined



# Phenolic ester groups

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- Ferulic acid and, to a lower content, coumaric acid.
- Esterified to the *O*-5 position of arabinose residues.
- 1 Ferulic ester group per 300 to 600 arabinose residues.
- Prevailing in outer layers (aleurone, pericarp).
- Polysaccharide -Polysaccharide cross-linking through oxidative dimerization of esterified ferulic acid residues.
- Cross-linking to lignin, protein?
- Extractable, quite selectively, for ca 15% from WUS with 0.05 M Barium hydroxide at 20 °C with 50% loss of ferulic acid.



# Characterization of arabinoxylans

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- **Arabinose/xylose ratio (?)**
- **Relative amounts of un-/mono-/disubstituted xylopyranosyl residues.**
- **Presence of (4-*O*-Me)-glucuronic acid residues (bran, corn, sorghum, rice).**
- **Presence of coumaric, ferulic acid or acetyl (?) residues.**
- **Homogeneity of arabinoxylan fraction.**
- **Distribution of substituents along AX backbone.**
- **Molecular size distribution.**



# Structure elucidation of AX: General Strategy

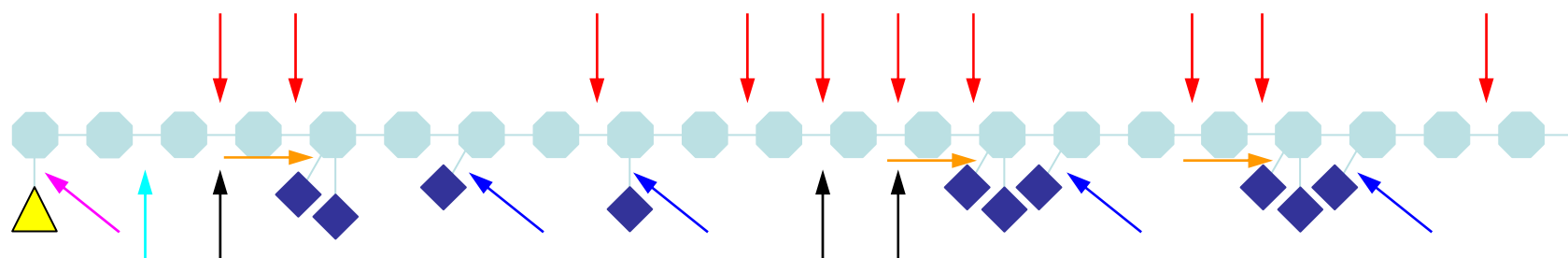
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- **Sugar (D/L) composition.**
  - **Glycosidic linkage composition:**
    - Methylation analysis.
    - In sequence periodate oxidation/reduction/hydrolysis.
  - **NMR analysis on polymeric level.**
  - **Enzymatic fragmentations:**
    - Isolate (all) fragments (HPLC methods).
    - Elucidate absolute structure of fragments.
  - **Maldi-TOF MS, MS/MS and NMR Techniques, Accessory Enzymes.**
- ⇒ **Reconstruct Parental Polysaccharide!**







# **Xylanases and Accessory Enzymes**



# Sites of attack of enzymes active on (glucurono)arabinoxylans



Site of attack of:

- |   |                                     |   |                                  |
|---|-------------------------------------|---|----------------------------------|
|  | Endo-(1,4)- $\beta$ -D-xylanase I   |  | (1,2)- $\alpha$ -D-Glucuronidase |
|  | Endo-(1,4)- $\beta$ -D-xylanase III |  | AXH-m                            |
|  | Glucuronoxylanhydrolase             |  | AXH-d3                           |
|   |                                     |   | Acetyl and Feruloyl esterases!   |



# Xylanases and Accessory Enzymes

<b>Enzyme</b>	<b>EC-Number</b>	<b>GH-family</b>	<b>R/I</b>	<b>Mr kD</b>	<b>pI</b>	<b>pHopt.</b>	<b>Topt. oC</b>	<b>Substrate Specificity</b>
<b>Endo-Xylanase I</b> <i>A.awamori</i>	3.2.1.8	10	R	39	5.7 – 6.7	5.5 – 6.0	55	Hetero-xylans
<b>Endo-Xylanase III</b> <i>A.awamori</i>	3.2.1.8	11	R	26	3.3 – 3.5	4.0	45 - 50	Hetero-xylans
<b>B-Xylosidase</b> <i>A.awamori</i>	3.2.1.37	3	R	110	4.2	6.5	70	Xylan & X-Oligo's
<b>AXHm3</b> <i>A.awam.</i> <i>Bi.adolesc.</i>	3.2.1.55	51	I	32 160	-	5.0 6.0	50 37	Arabino-xylans
<b>AXHd3</b> <i>Bi.</i> <i>adolescentis</i>	3.2.1.55	43	I	100	-	6.0	30	Arabino-xylans
<b>ArafA</b> <i>A. niger</i>	3.2.1.55	51	R	128	6 – 6.5	4.1	-	A,AG & AX oligo's
<b>ArafB</b> <i>A.niger</i>	3.2.1.55	54	R	60	5.5 - 6	3.7	-	A, AG & AX Poly- / oligo
<b>XAE</b> <i>A.niger</i>	3.1.1.72	1	-	30.5	3.0 - 3.2	5.5 – 6.0	50	Birchwood Xylan
<b>Endo-GX/ GAXhydrolase</b>	3.2.1.8	5	R?	45	-	6.5 - 7	-	Glucurono-xylan
<b>α-Glucuronidase</b>	3.2.1.139	67	I	91 -150	4.6-5.3	3.5 – 6.5	-	Oligomeric GAX

# Mode of action of AXH<sub>m</sub> and Araf A & B

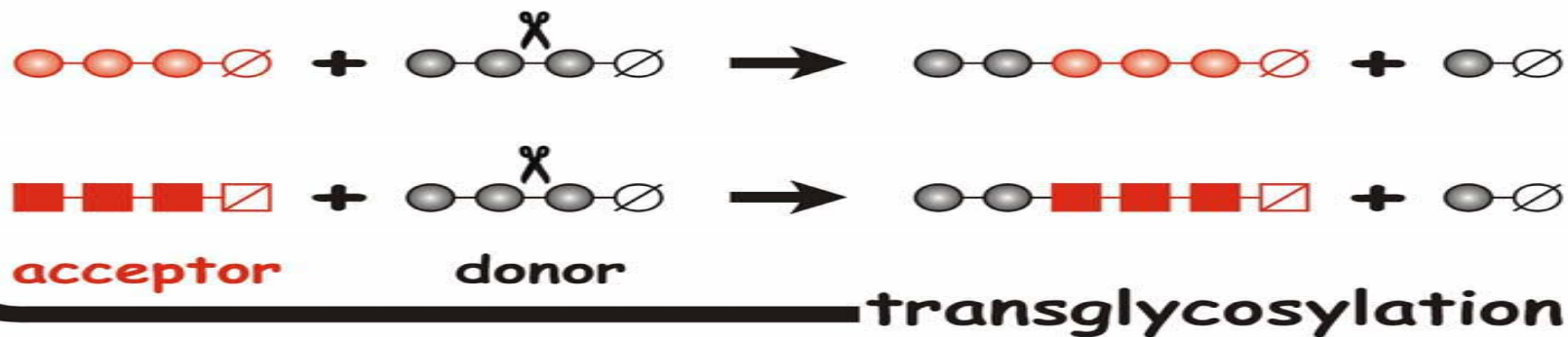
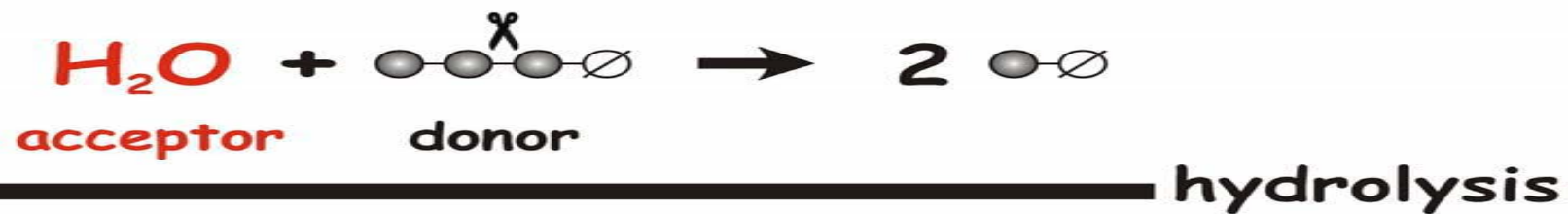
Enzyme: Substrate	AXH	ArafA	ArafB
Arabinan	-	-	+
Arabinan oligomers	-	+	+
Arabinoxylan	+	-	+
Arabinoxylan oligomers: Only $\alpha$ -(1,2 <u>or</u> 1,3) on terminal and non-terminal single substituted Xylopyranosyl units	+	+	
Only terminal single substituted Xylp			+





## Retaining glycosidases can be used for oligosaccharide synthesis

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# **Structural Characterization**



## Other Structural data on WU-AX

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- **Enzyme degradation studies using endoxylanases and arabinoxylanhydrolase with known modes of action.**
- **Absolute structures oligosaccharides established (RU Utrecht). Simulation of arabinoxylan chain and their degradation.**
- **From mode of action xylanases, from the occurrence of 1 or 2 unsubstituted xylose residues at non-reducing end and 2 at reducing end and the release of xylobiose, -triose and-tetraose it can be estimated which proportions of the unsubstituted xylose residues are present as clusters of 3, 4, 5, 6 and 7 contiguous residues.**
- **From analysis of periodate oxidation/reduction/acid hydrolysis studies:**
  - **ca 50% of substituted xylose residues present as isolated units**
  - **ca 40% of substituted xylose residues present as blocks of 2 contiguous xylose residues and 9% as blocks of 3.**



# Isolation and fractionation of AX

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## Flour/Bran

↓ Aqueous extraction

- Water-extractable AX

- ▶ Protein removal (mild!)

- ▶ Starch removal

- ▶ AGP removal

- ▶ AEC/SEC

- ▶ Graded  $(\text{NH}_4)_2\text{SO}_4$  or EtOH precipitation

↓ Remove Fat, Protein, Starch ↓

- Water-Unextractable CWM

- ▶ Specific  $\text{Ba}(\text{OH})_2$  extraction incl.  $\text{NaBH}_4$

- ▶ Graded alkaline extraction

- ▶ AEC/SEC



# AX in Ba(OH)<sub>2</sub> extracts as % of WUS (16 h, RT, 260 mM NaBH<sub>4</sub>)

AX-Fractions Grain (fraction)	WUS	BE % AX	BEn	K1	K4	R	Remarks.
Wheat Flour	1.2 (75)	68	11	9.6	7.8	3.8	Various populations: a/x, UA. Oxidative Cross-linking
Wheat bran	58 (50)	40 (0.77)					
→ FAX: Ca/Ba(OH) <sub>2</sub> 2h RT ▶ 0.4 % Fer.	58 (50)	4/7					
Barley (Dehusked)	7.6 (49)	63 (0.72)	15	13	4	6	
Malt	1.2 (78)	61 (0.68)	11	11	6	6	
Sorghum: polished	1.1 (46)						
Whole grain	5.3 (58)	53 (1.1)	5.3	18.8	13.6	9.1	Ca. 10% UA
Maize meal	8.7 (62)	21 (1.1)	18	-	-	46.3	Ca. 8.5 % UA

↑                      ↑



# WU-AX from Wheat Flour and Bran

Whole Wheat flour contains  $\text{ca } 10\%$  ds AX:

## 1.3% in flour (75% WUS)

Extract	yield (%)	mole %		A/X	Un	M	Di
		A	X				
BE	82	34	66	.51	64	20	14
Et-20	20	26	74	.36	70	22	7
Et-30	33	31	69	.46	67	20	12
Et-40	18	35	65	.55	65	18	17
Et-50	16	40	59	.68	59	15	25
Et-60	10	44	55	.80	52	14	33



## & 8.7% in bran (90%WUS)

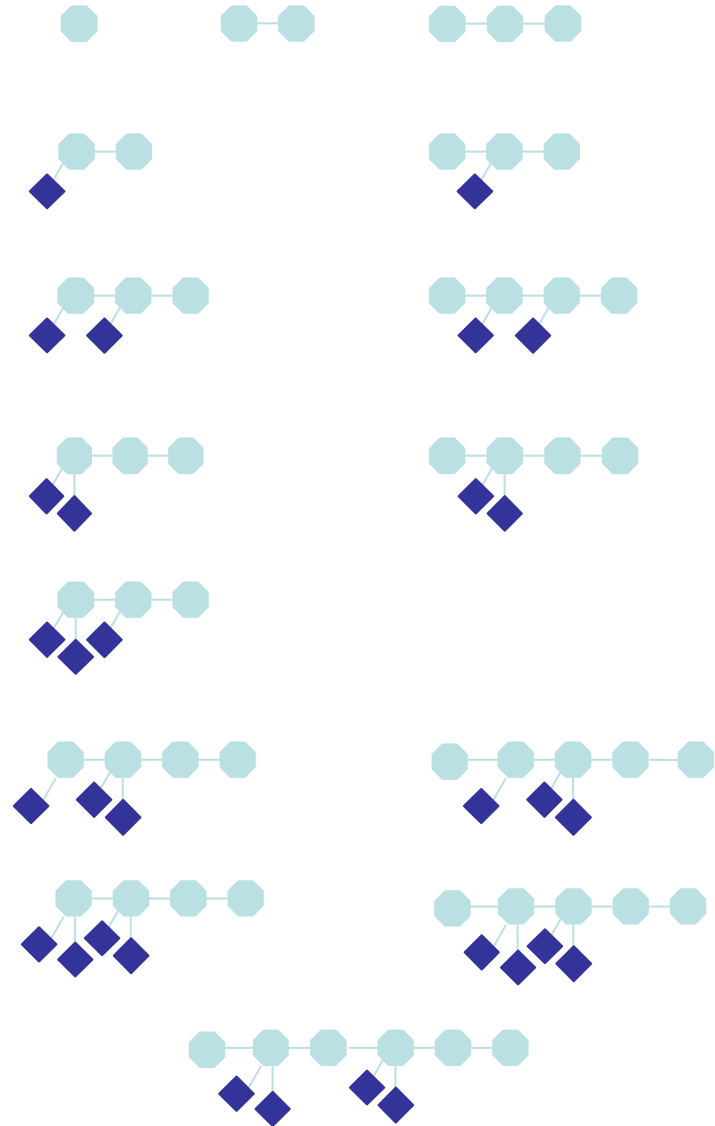
Extract	Yield (%)	mole %		A/X	Un	M	Di
		A	X				
BE	29	42	58	.72	53	20	16
Et-10-20	30	16	82	.20	83	15	2
Et-60-70	44	49	45	1.08	23	25	52

BE → 2.6% GlcUA  
Et-10-20 → 1.2% GlcUA  
Et-60-70 → 4 % GlcUA

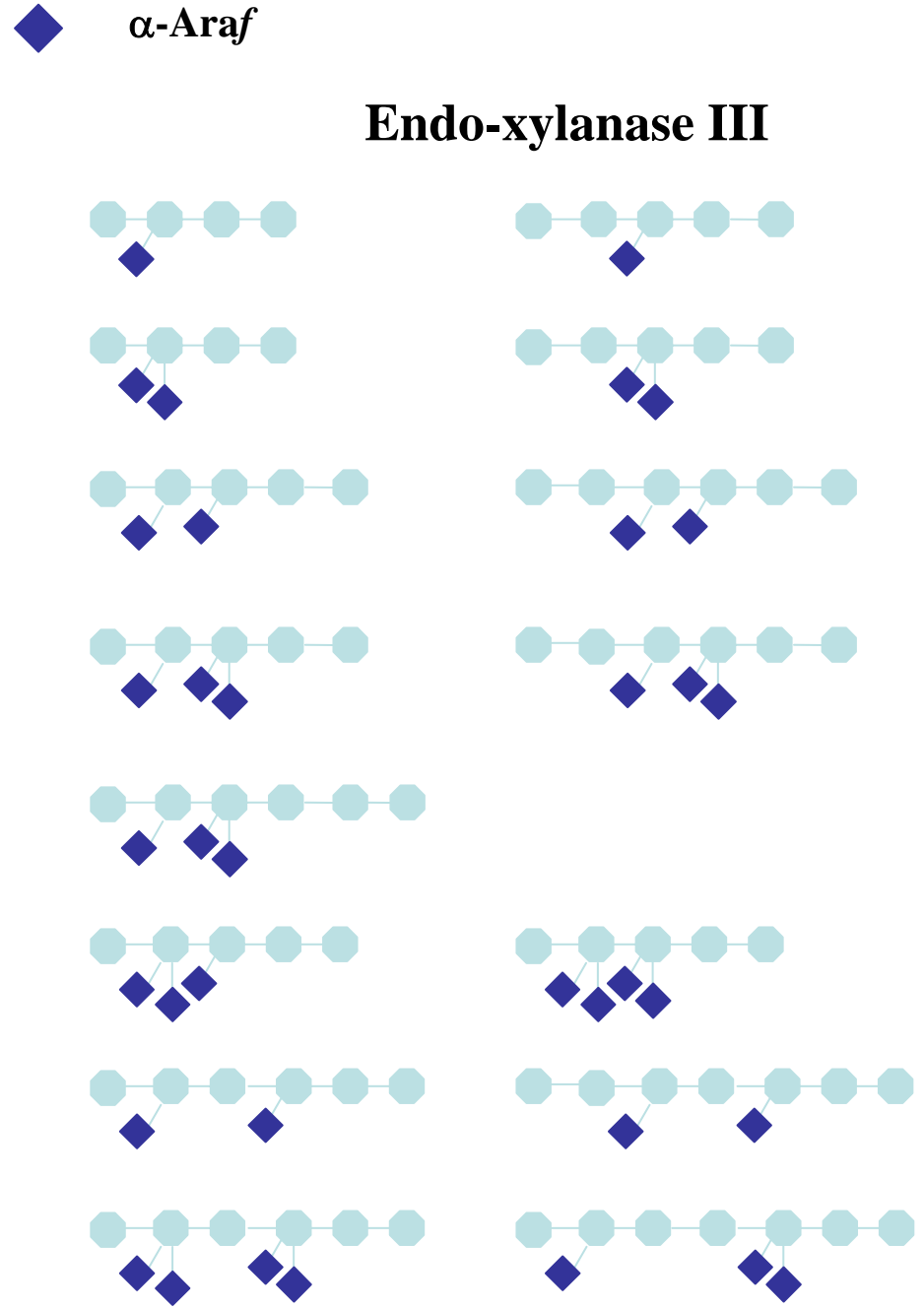




## Endo-xylanase I



## Endo-xylanase III

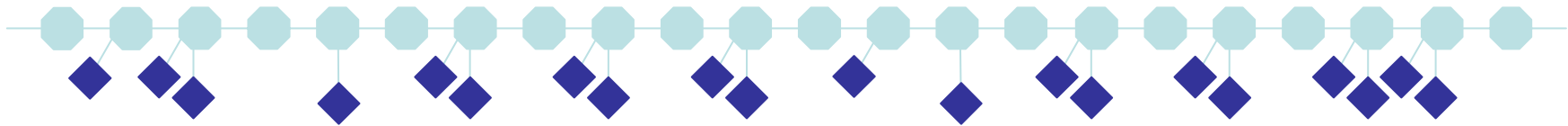




# Wheat AX: reconstructed parental polysaccharides:

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**region A:**



**region B:**





# Variations in AX from different wheat cultivars and within cultivars

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- Variation in content of arabinoxylans.
- Variation in A/X ratio (minor).
- Within and between each variety AX's differ in the amounts of un-, 2-O and 3-O mono-, and double substituted xylosyl residues.
- Differences in molecular weight between varieties and populations occur.
- Variations in ferulic acid content.



## Complex GAX: specific problems

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- Limited degradation or no degradation at all with pure enzymes and mixtures of pure enzymes.
- Some fragments isolated and identified.
- Complex side chains.
- Fragmentation with pure and partially purified enzyme fractions (*Humicola insolens*):
  - Digests complex mixtures of oligosaccharides.
  - Structural information with MALDI-TOF MS.
  - Due to use of not well defined enzyme mix loss of information.



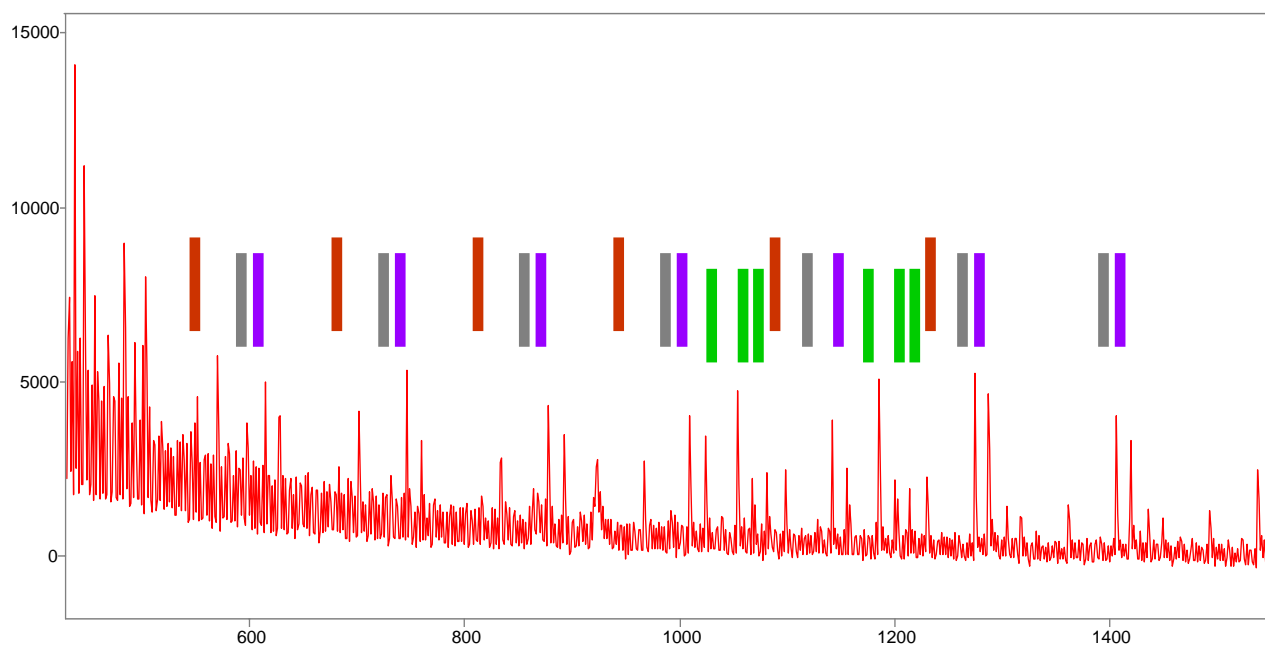
# Maldi ToF MS spectrum of an arabinoxylan digest from the BE fraction of maize kernels

**Pent<sub>n</sub>**

**Pent<sub>n</sub>GlcA**

**Pent<sub>n</sub>GlcA OMe**

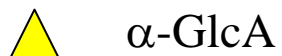
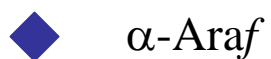
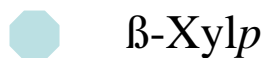
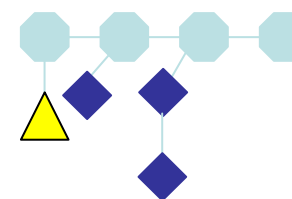
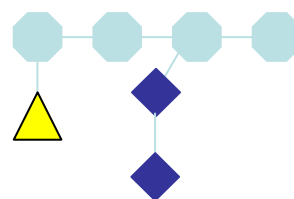
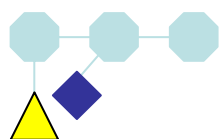
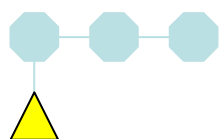
**Pent<sub>n</sub>GlcA<sub>2</sub> OMe<sub>m</sub>**





# Identified sorghum glucurono-arabinoxylan oligosaccharides

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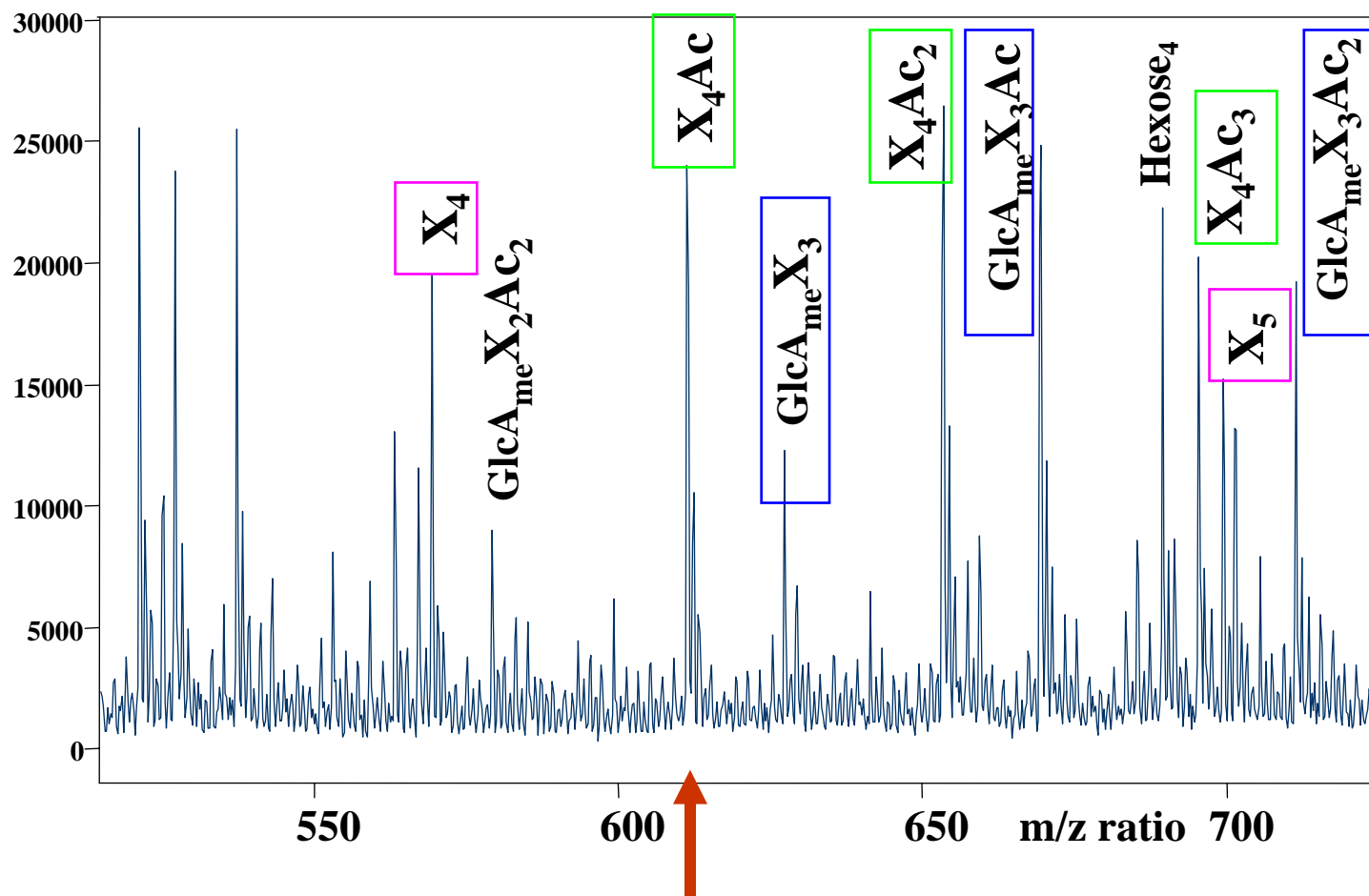




# Part of the Maldi-TOF-mass spectrum of an *Eucalyptus* wood hydrolysate

(175 °C; 18 minutes.)

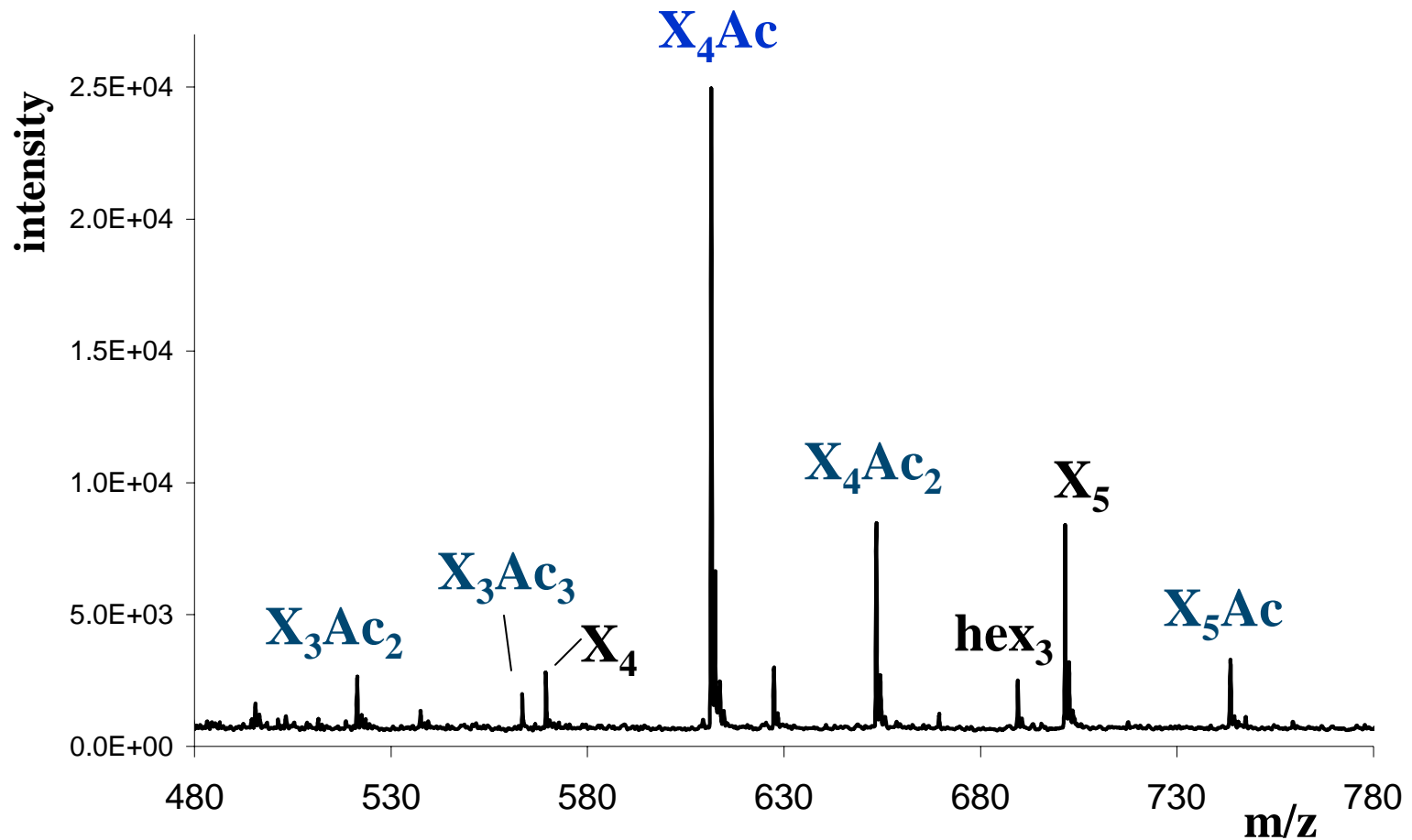
500 - 750 Dalton range





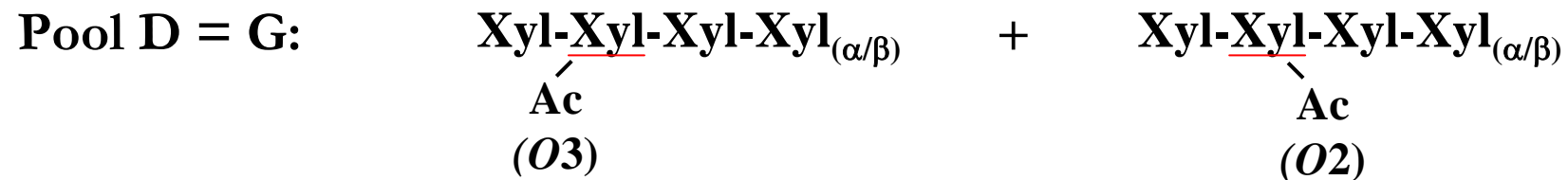
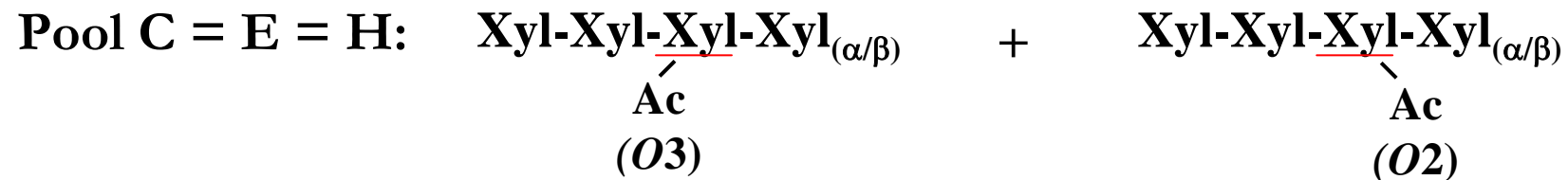
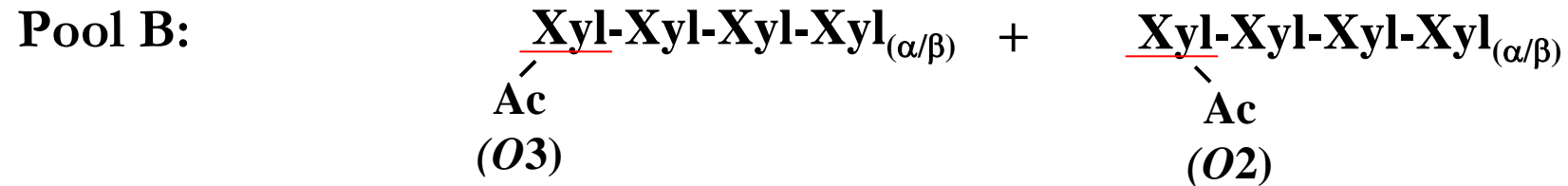
# Size-exclusion fractionation of neutral O-acetylated XOS

MALDI-TOF mass spectrum pool X<sub>4</sub>Ac





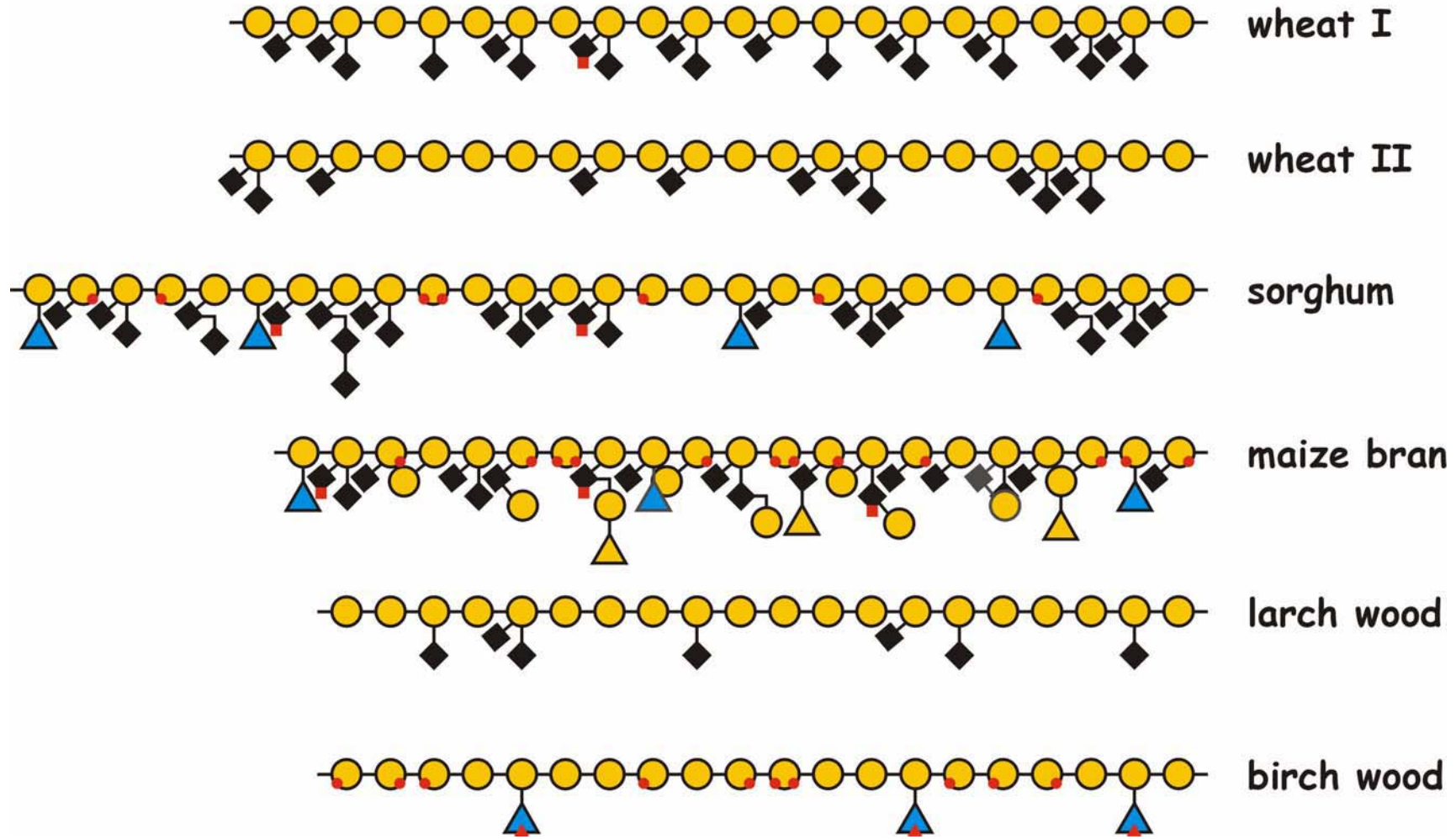
# Summary results NMR (1D + 2D)



- \* no separation between *O3*- and *O2*- acetylated X<sub>4</sub> by RP?
- \* *O*-acetyl-migration after separation by RP?



# Variations in xylan structure

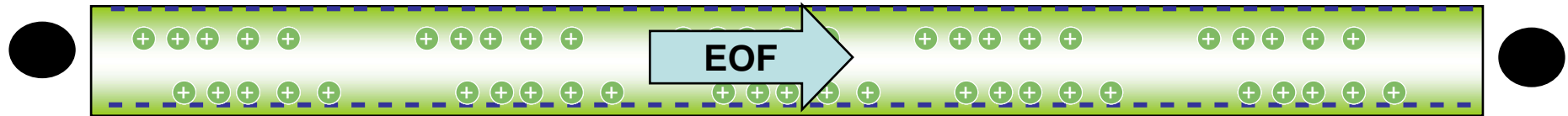




# **Analytical Developments**



# Capillary Electrophoresis



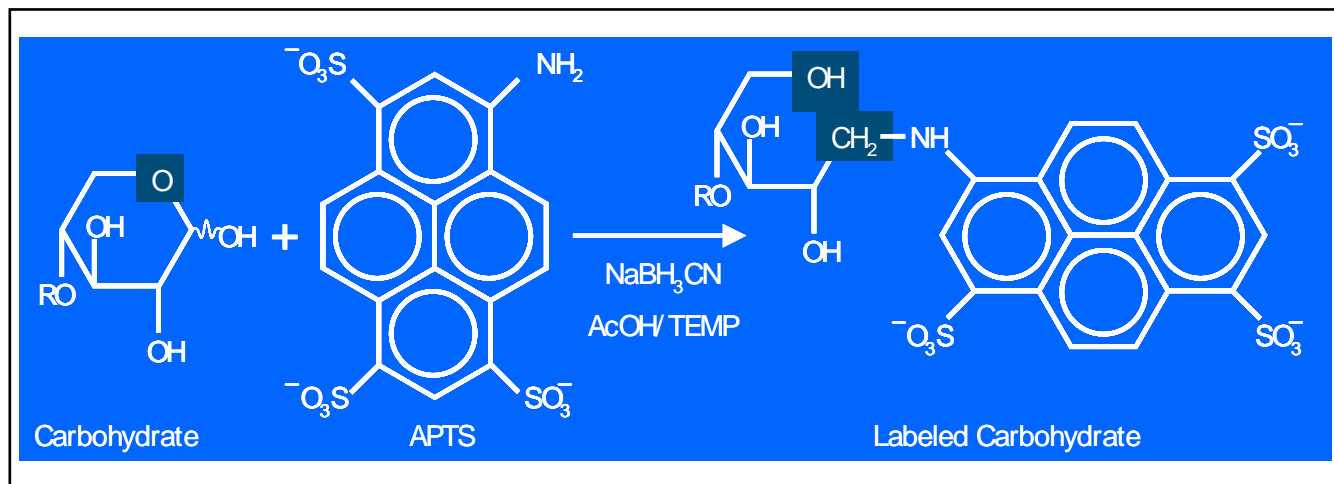
- Separation based on charge
  - Most positive structures elute first
  - Neutral structures elute with Electro Osmotic Flow (EOF)
  - Negative structures can elute, depending on the EOF
- Polyvinyl alcohol-coated capillary
  - Coating is reducing the charge of the wall





# Labelling with APTS

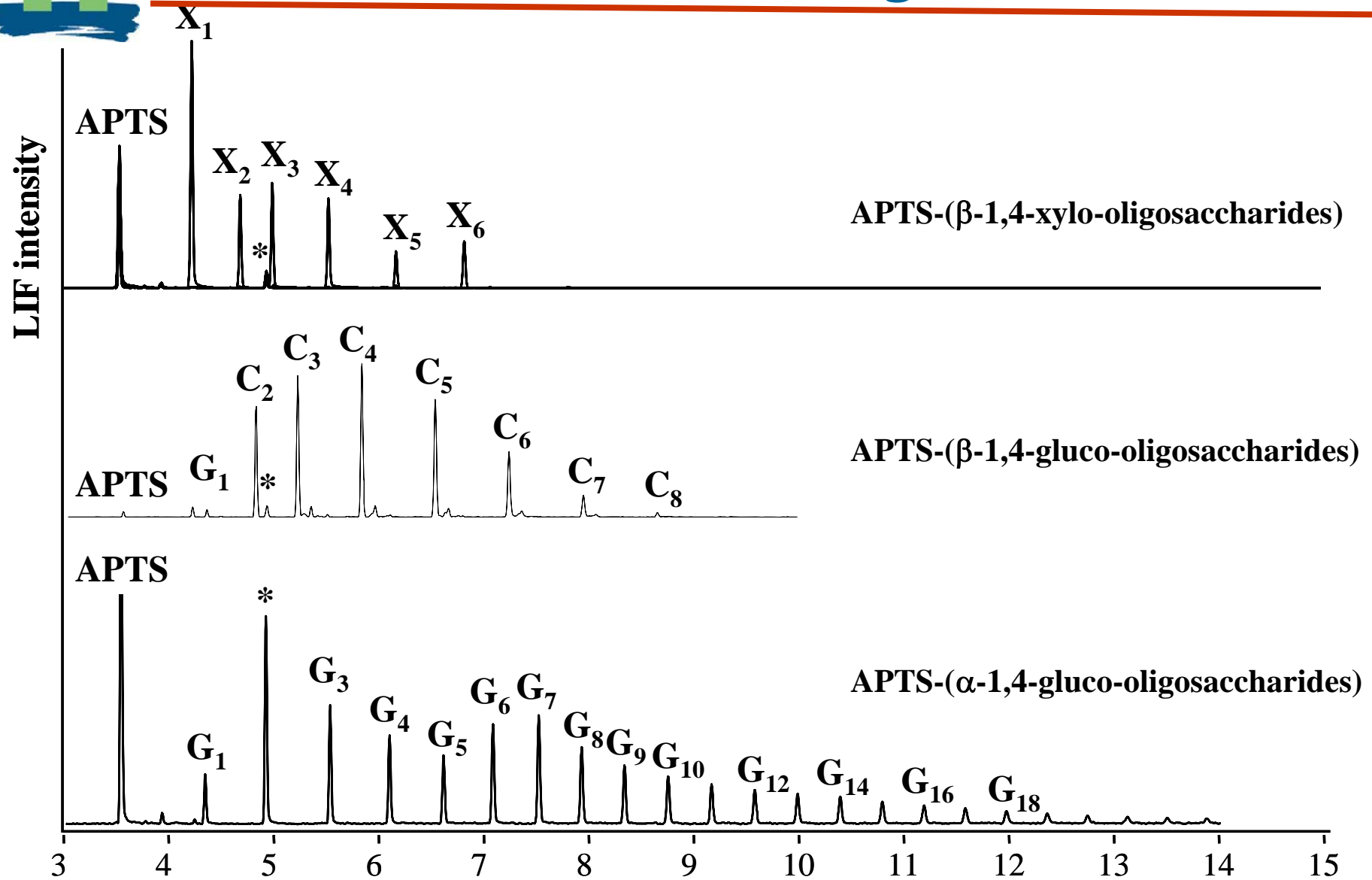
- Not every sugar molecule is charged
  - Labeling with APTS
    - To give charge to a sugar molecule
    - To increase signal (fluorescent)
    - To allow quantification on mole basis



Based on  
Chen, et al (1998)



# CE-LIF of different oligosaccharides



– Migration behavior not as straightforward as expected

Time (min)



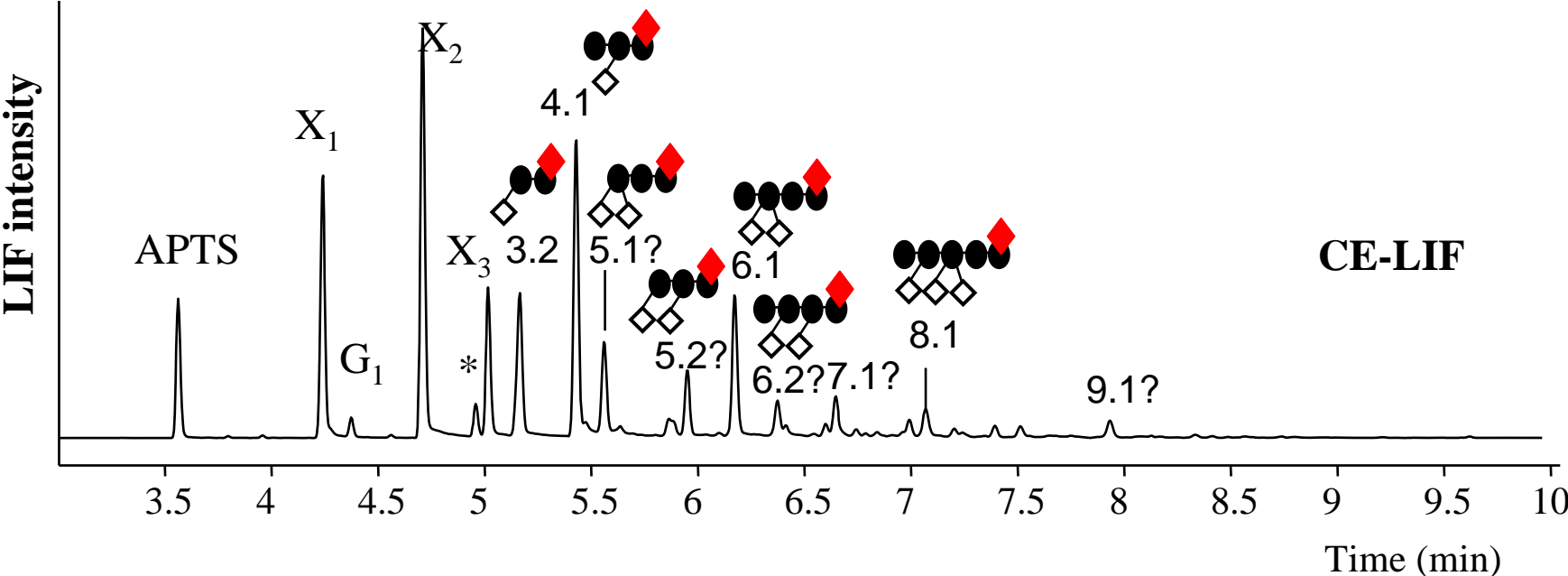
# CE-MS coupling: Consequences

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- Longer capillary (to connect to MS; partially without cooling)
- No LIF detection (UV detection possible instead)
- Adapt capillary (fused silica instead of coated silica)
- Adapt buffer (volatile, lower pH)
- Find good interface

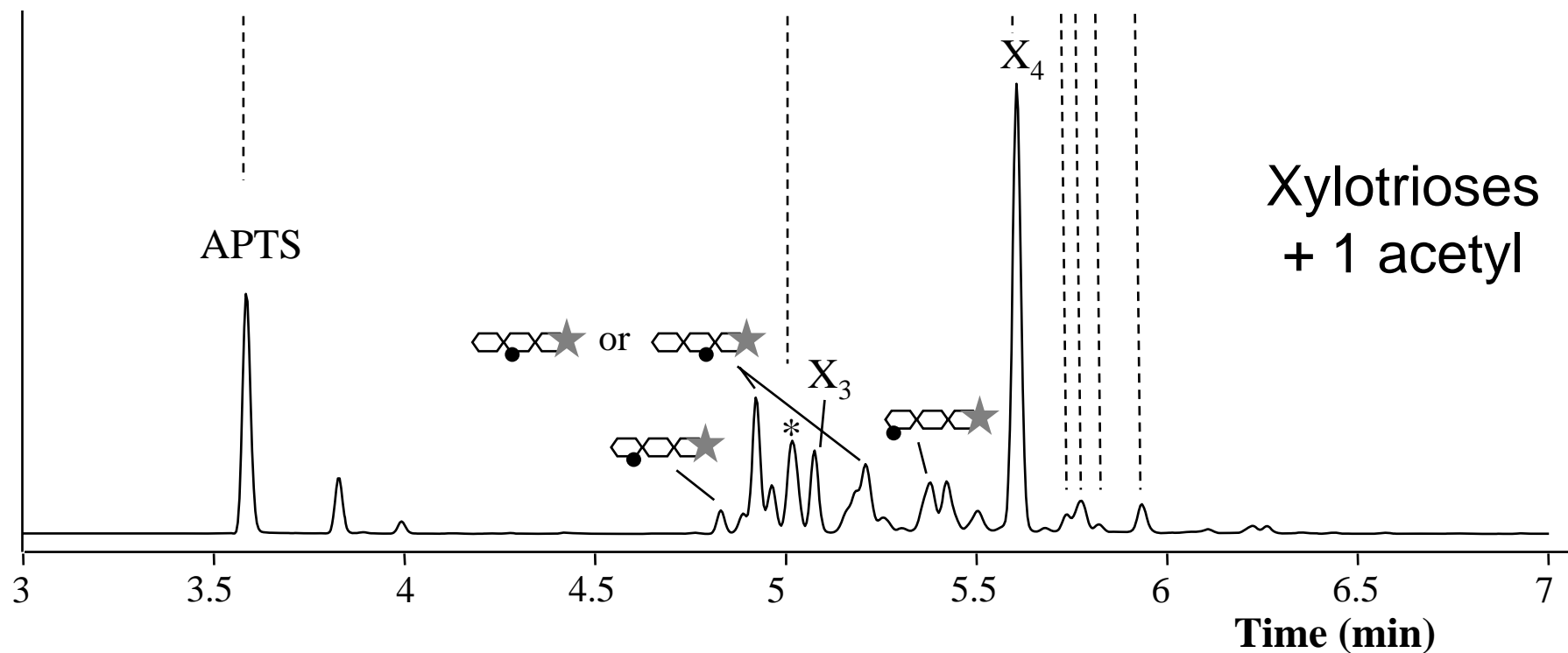


# CE-LIF(MS) of arabinoxylan-oligosaccharides



? = needs to be confirmed

# CE-LIF of acetylated xylo-oligosaccharides





# Fermentation of different xylo-oligosaccharides (XOS)

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**Have various substituents of XOS an influence on ability and rate of fermentation and on the production of SCFA (plus lactate)?**

## **Material & Methods:**

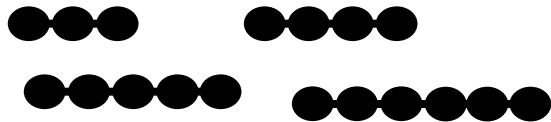
- \* faecal inocula of four human volunteers**
- \* four mixtures of differently substituted XOS and a blank (without sugar added)**
  
- each XOS-mixture and blank was incubated with each inocula (20 incubation-mixtures)**
  
- 94 hours of incubation: at 10 times samples were taken**
  
- samples analysed:**
  - sugars: HPAEC and MALDI-TOF mass spectrometry**
  - short chain fatty acids (SCFA) and lactate: HPLC**



# XOS-mixtures:

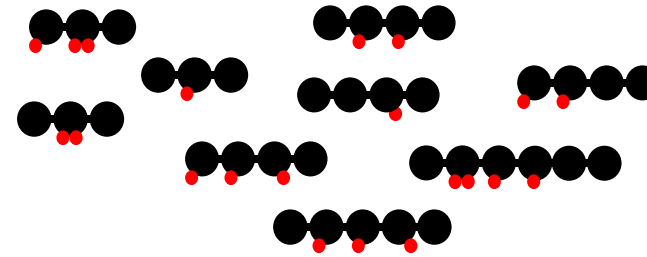
non-substituted XOS: **nXOS**

DP 3 - 15



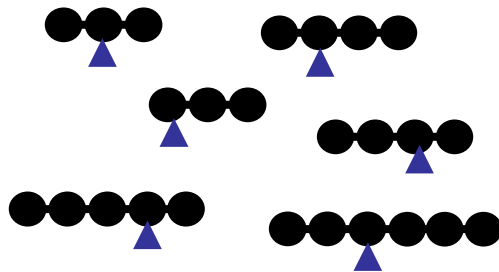
acetylated XOS: **AcXOS**

DP 3 - 15



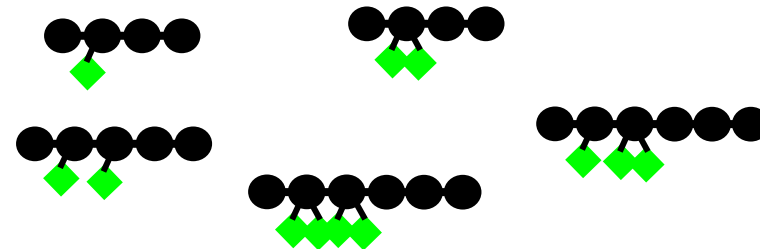
4-*O*-methylglucurono-XOS: **GlcA<sub>me</sub>XOS**

DP 3 - 15



arabino-XOS: **AXOS**

DP 3 - 11



● xylose

▲ 4-*O*-methylglucuronic acid

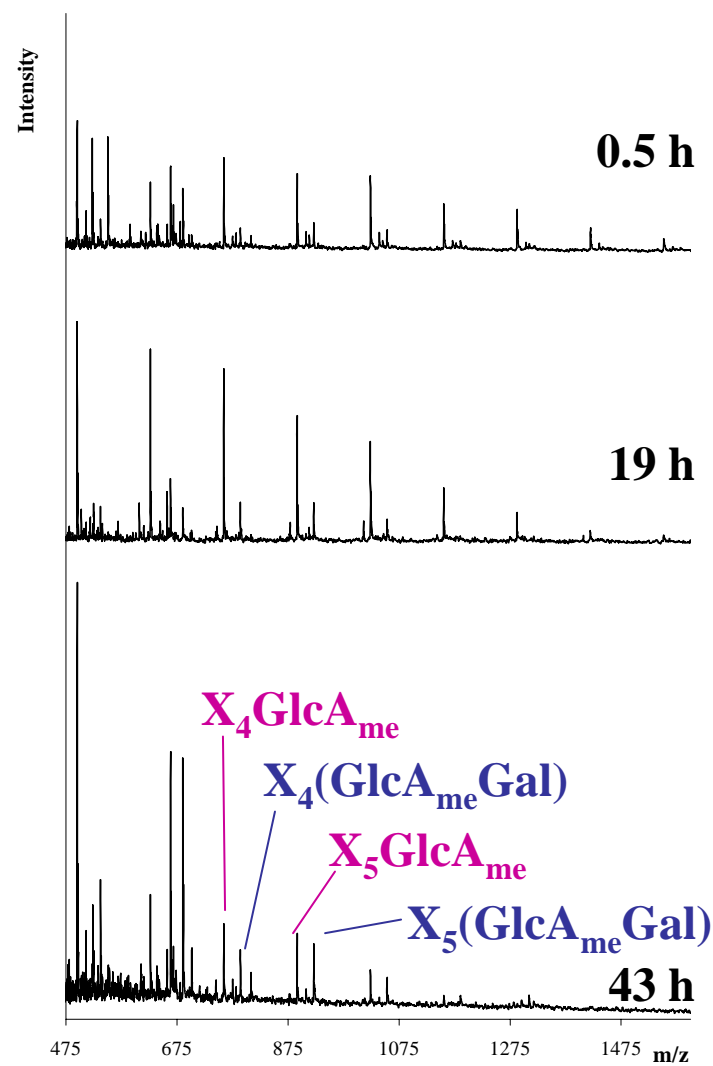
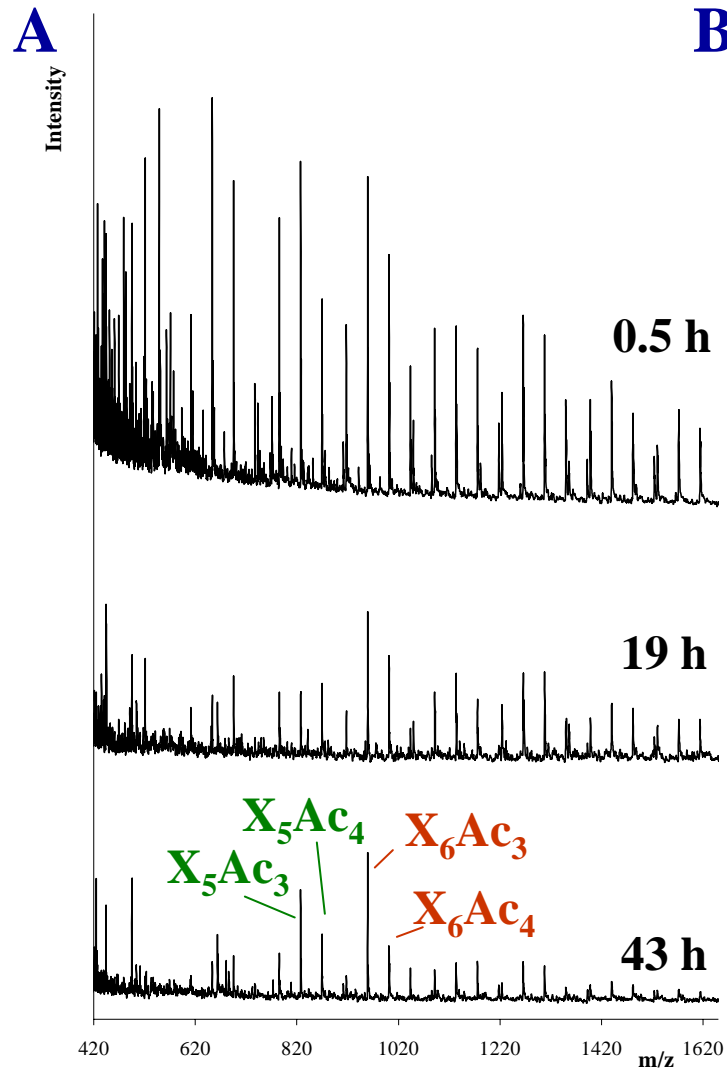
• acetyl group

◆ arabinose



# MALDI-TOF MS: AcXOS (A) and GlcA<sub>me</sub>XOS (B)

at time 0.5 h, 19 h, and 43 h of fermentation by FI 1



**X =**  
xylose

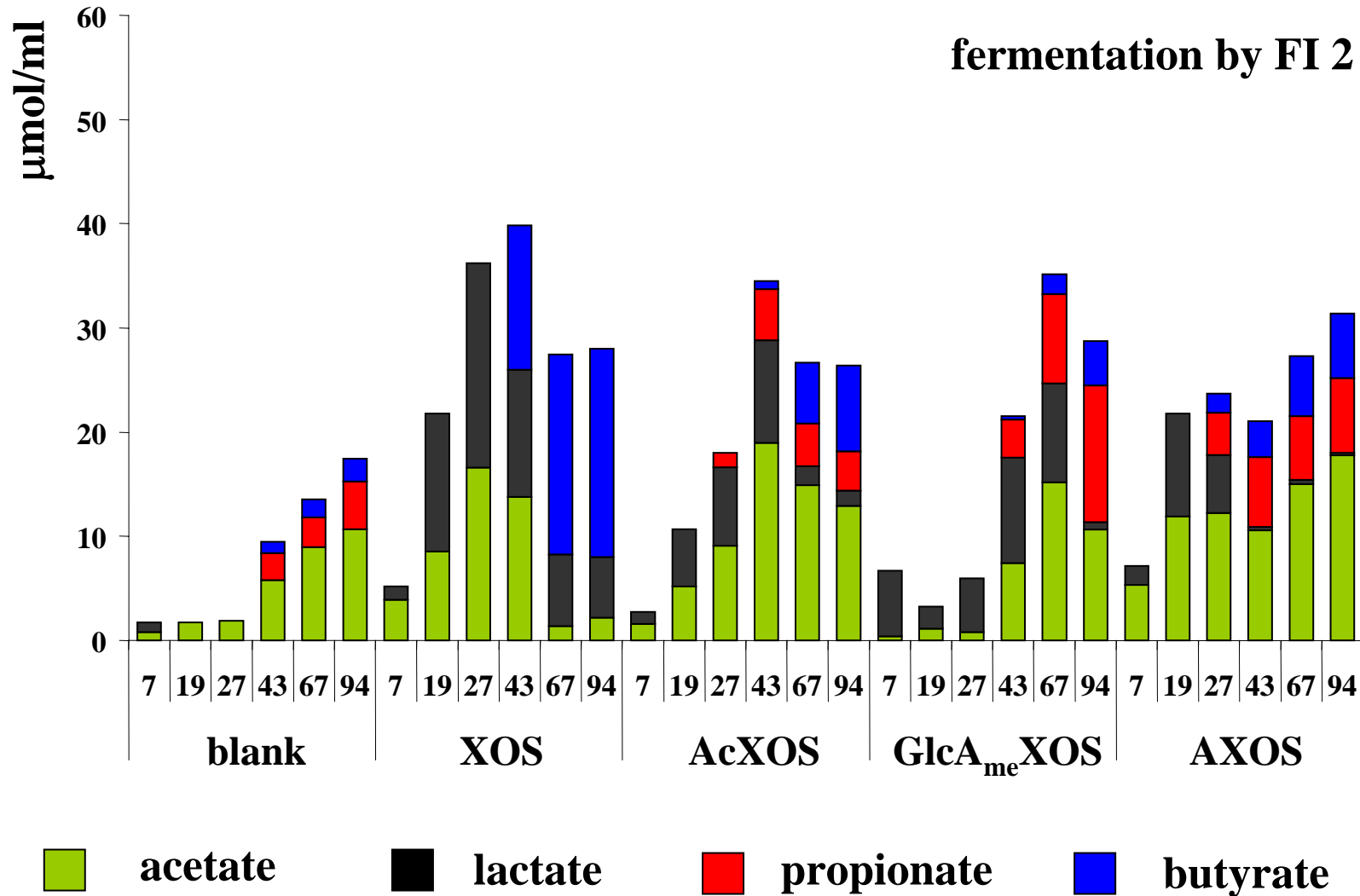
**Ac =**  
acetyl group

**GlcA<sub>me</sub> =**  
4-*O*-methyl-  
glucuronic  
acid

**Gal =**  
galactose



# SCFA and lactate production during fermentation of XOS (2)





- **Peroxidase-catalysed cross-linking of feruloylated Xylans and tyrosine containing peptides**



# *Relevance of Peroxidase*

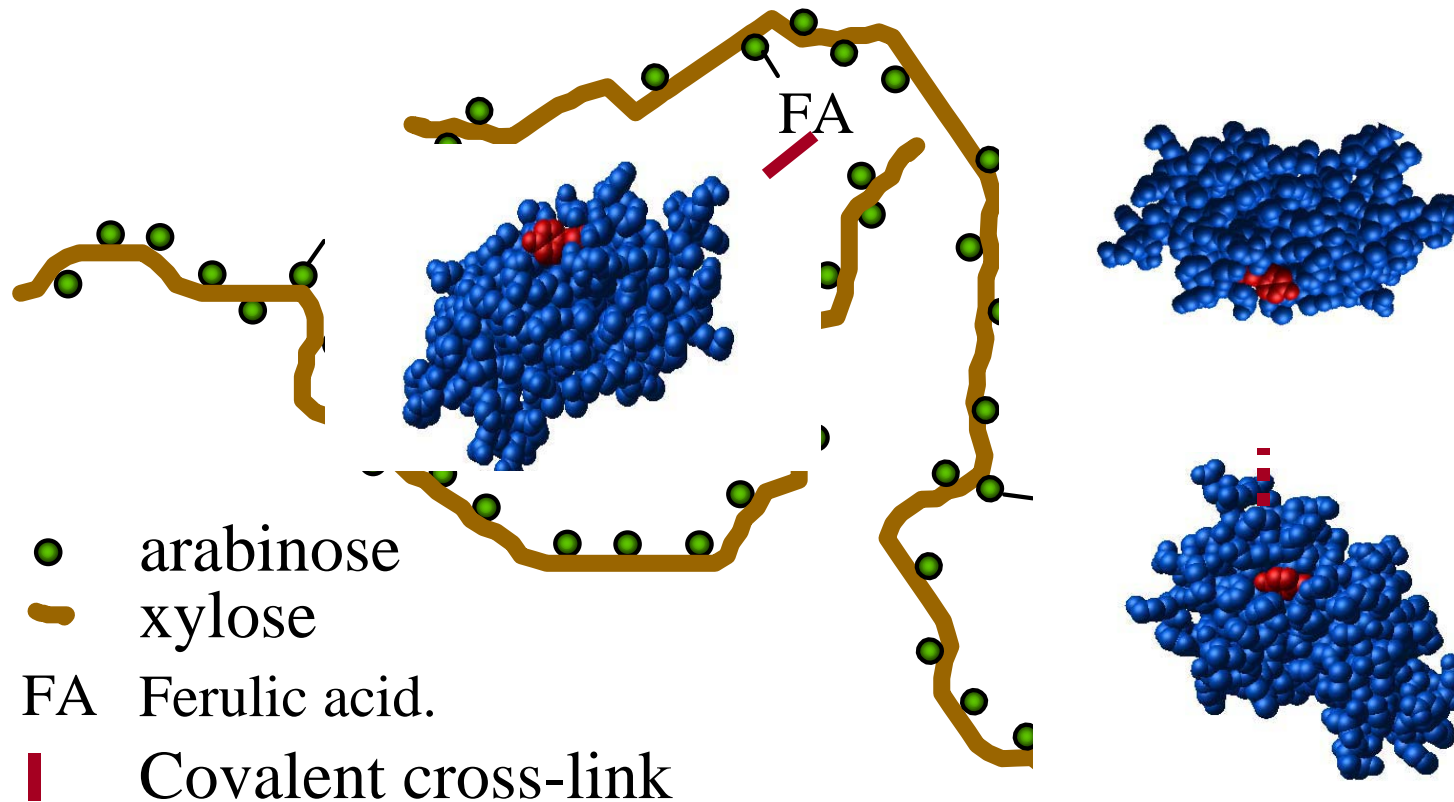
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- Peroxidase can oxidize tyrosine in BLA
  - Peroxidase can oxidize esterified FA in arabinoxylans
  - Radical combination would theoretically lead to coupling of the protein and the arabinoxylan
- ➔ Model studies with tripeptide GYG (protein 'model') and ferulic acid (FA)



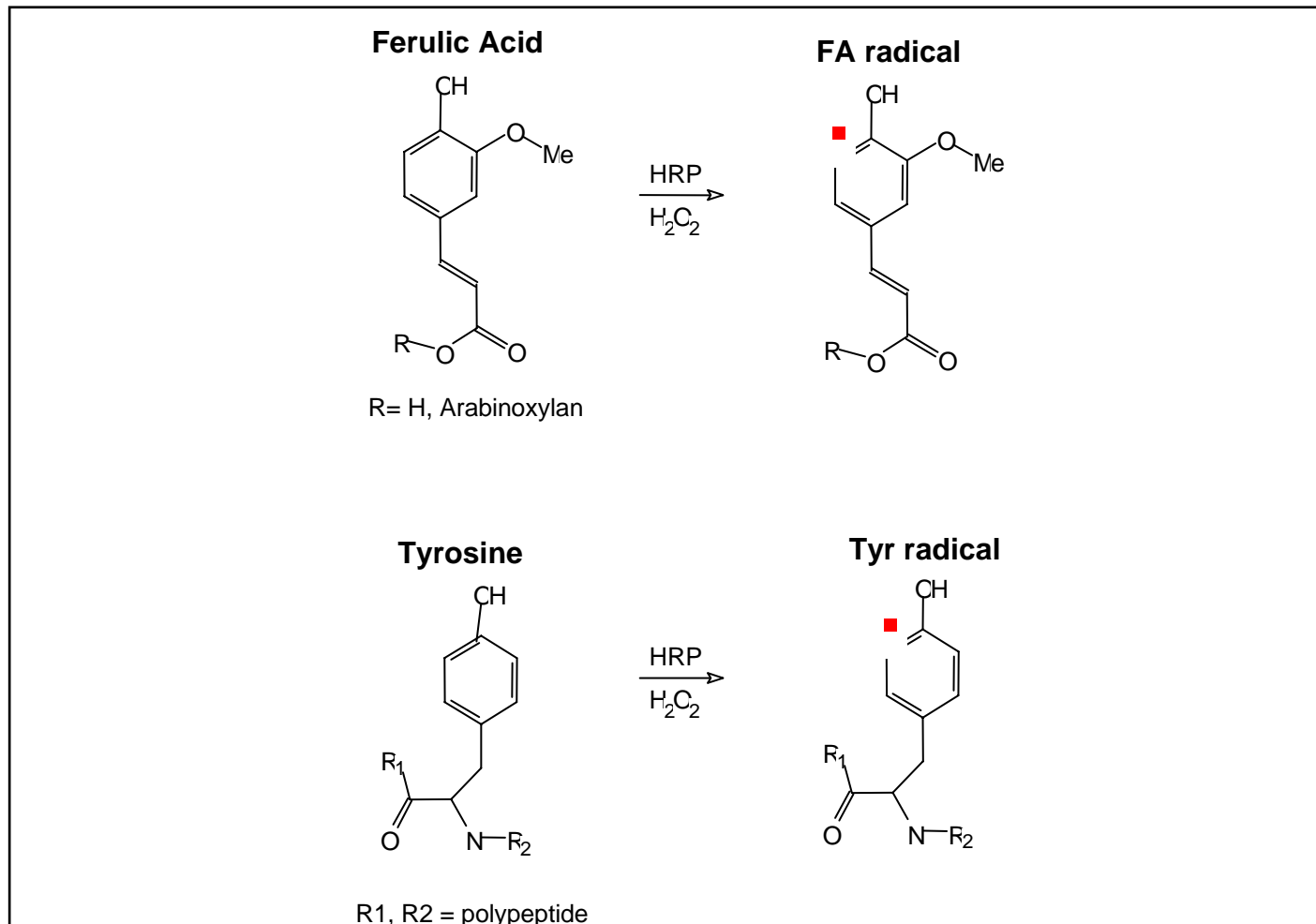
# Covalently linked Protein-Carbohydrate network ?

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# Enzymatic oxidation of ferulic acid and tyrosine

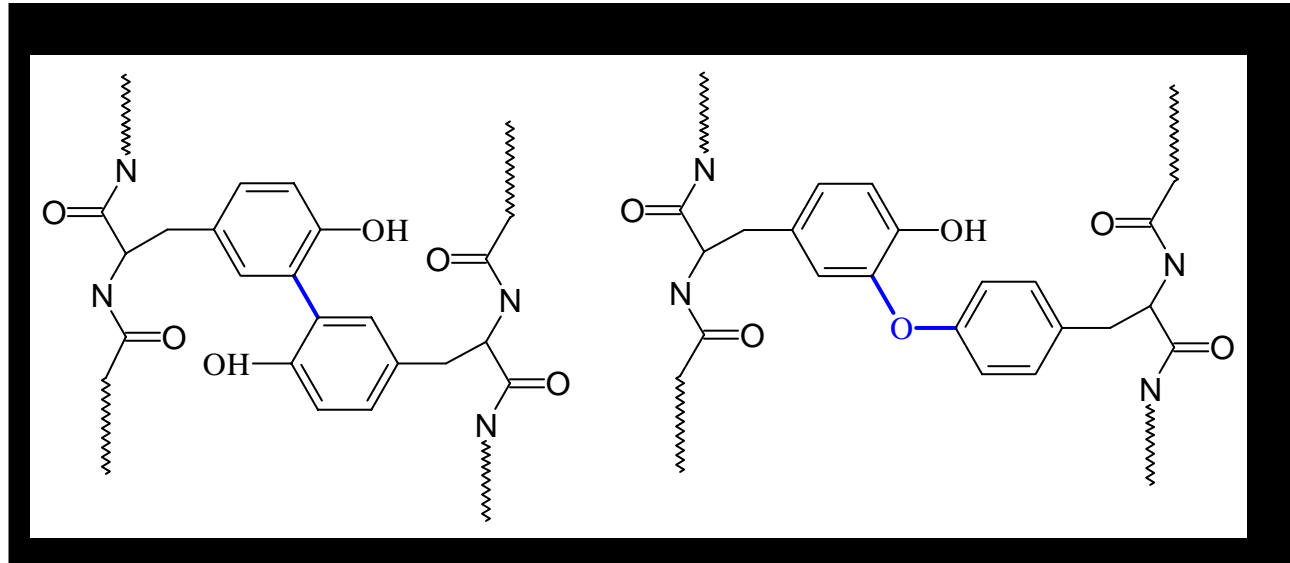
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# Tyrosine free radical combinations

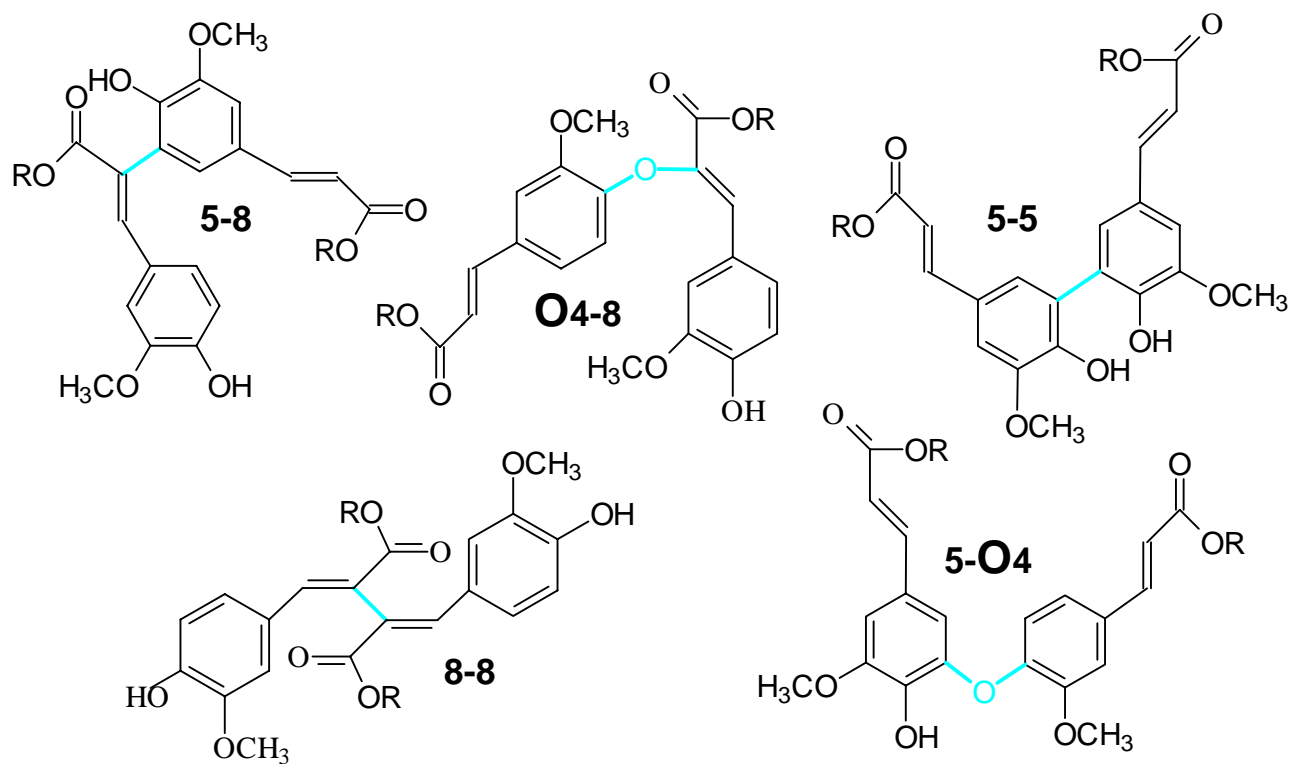
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Catalyzed by peroxidase both *in vivo* as *in vitro* for tyrosine containing :

- amino acids
- peptides
- proteins

# Ferulic acid free radical combination

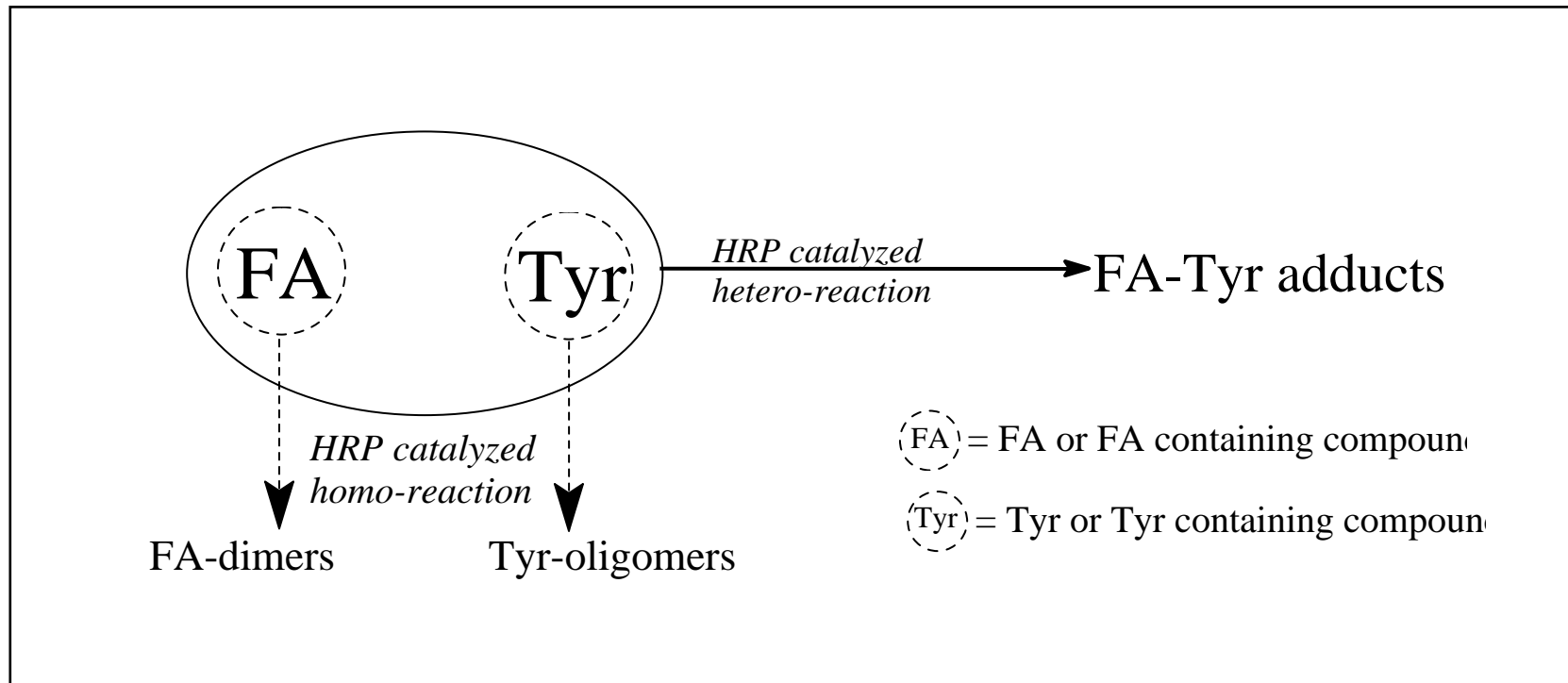


Catalyzed by peroxidase both *in vivo* and *in vitro*



# Need for kinetic control of the incubation

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# Kinetically controlled incubation

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- Oxidation FA > 3000 fold faster oxidation GYG
- Full conversion of FA > 100 fold faster than that of GYG
- Continuous addition of small FA aliquots to a GYG-containing incubation-mixture is the only way to prevent FA-oligomerization solely
- equal populations of GYG and FA free radicals, favouring hetero-reaction GYG-FA



# Conclusion model studies GYG and FA

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- 2FA and nGYG can be cross-linked at low [FA] (Oudgenoeg et al., J. Agric. Food Chem., 49, 2001)
- xFA and 1 GYG can be cross-linked at high [FA] (Oudgenoeg et al., J Biol Chem, 24, 2002)



# Modification of the protein BLA with ferulic acid

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- Can the methods applied with FA and GYG be extrapolated to modification of an actual protein ?
- Primarily dependent on accessibility of the tyrosines in the protein to be modified



# Structural features BLA

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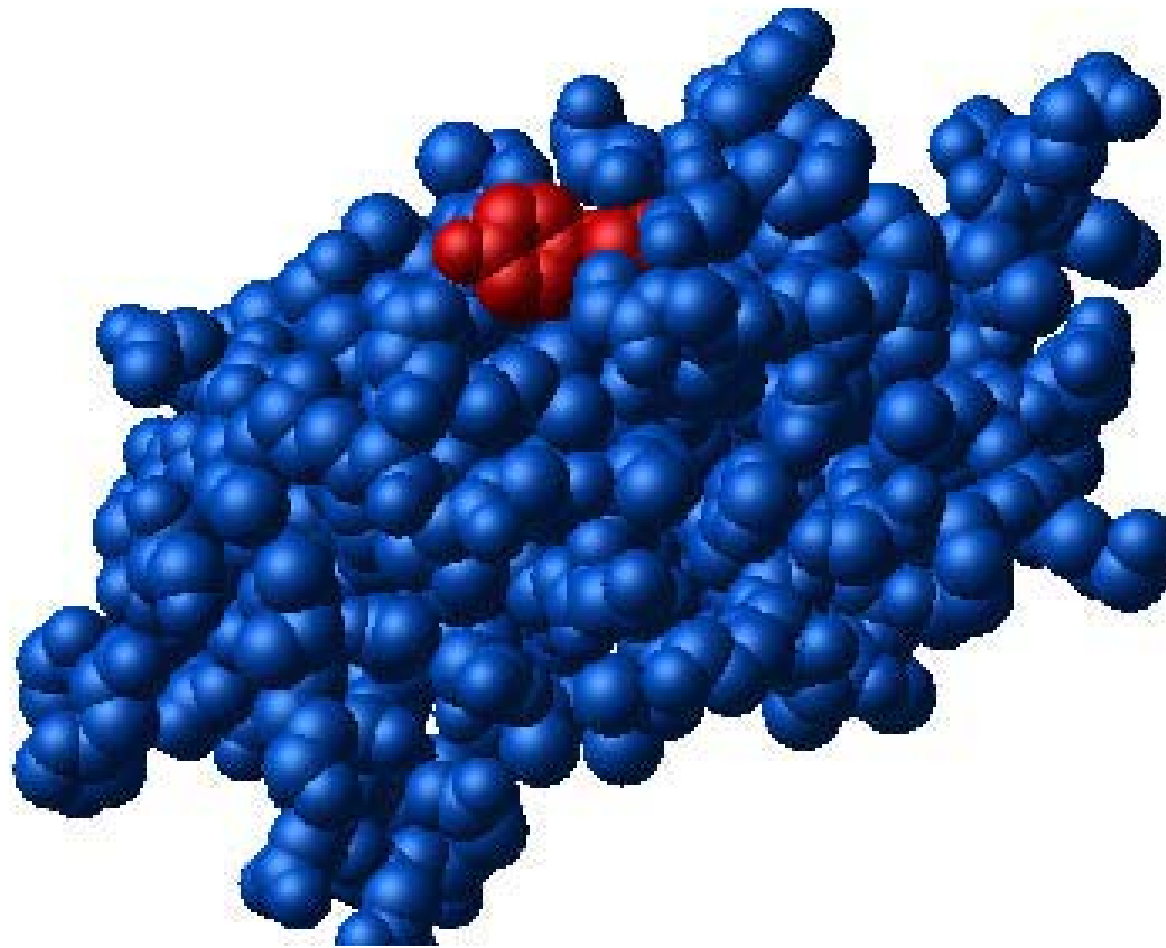
- Holo-BLA ( $\text{Ca}^{2+}$  bound form) is stabilized as compared to apo-BLA ( $\text{Ca}^{2+}$  depleted form)
  - tyrosines in apo-BLA are more exposed
- BLA contains 4 disulfide bridges
  - after breaking the disulfide bonds and carboxymethylation of the cystein sulfides the BLA tyrosines are fully exposed



# BLA: A 123 amino-acid protein

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UV-absorption  
max at 280 nm

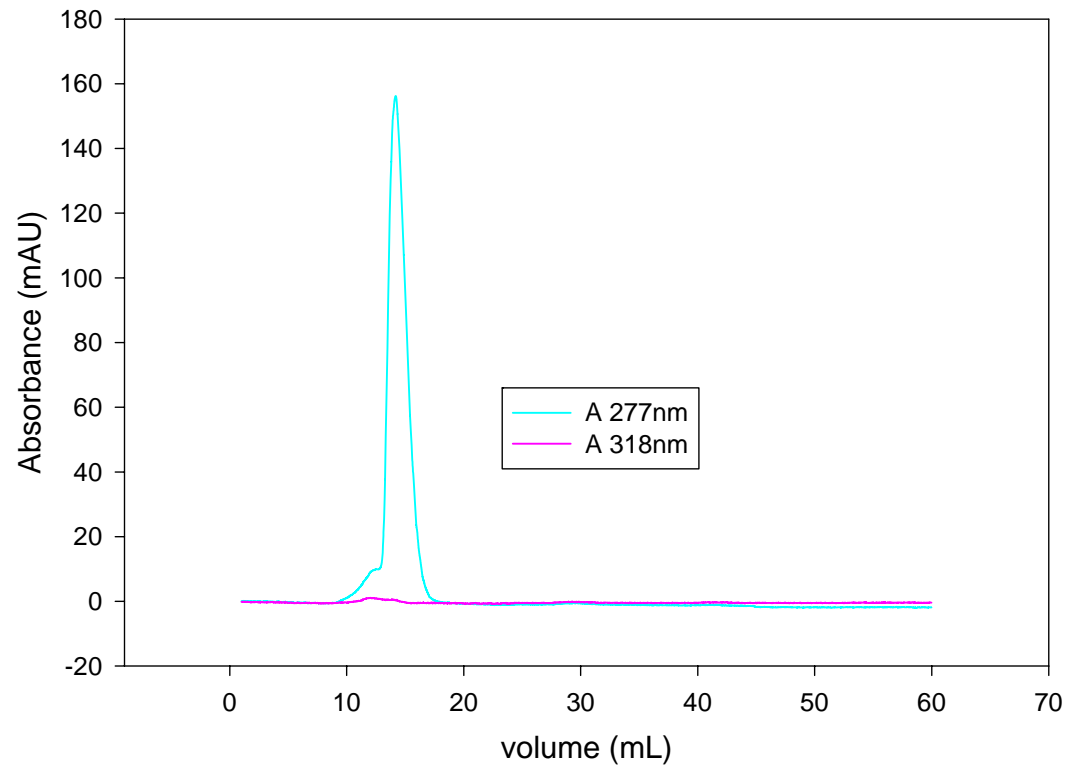


FA spectra  
absorption  
at 280 nm



# Gelfiltration of incubation of holo-BLA with HRP

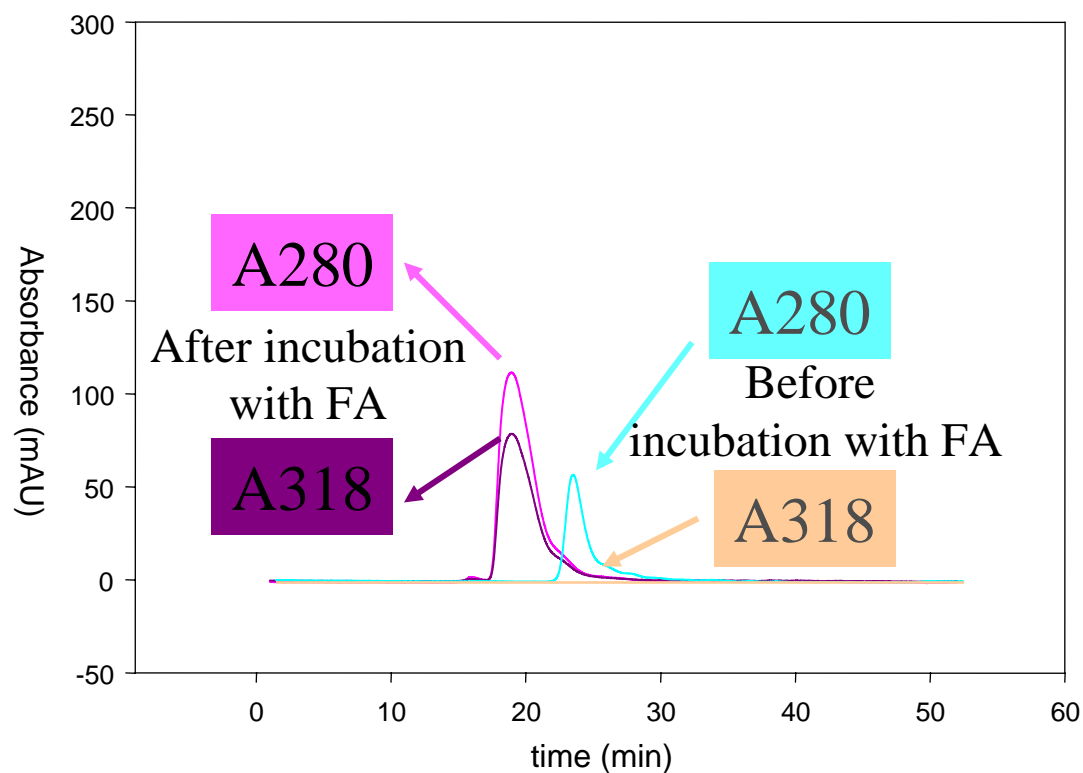
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# Carboxymethylated BLA (proof of principle)

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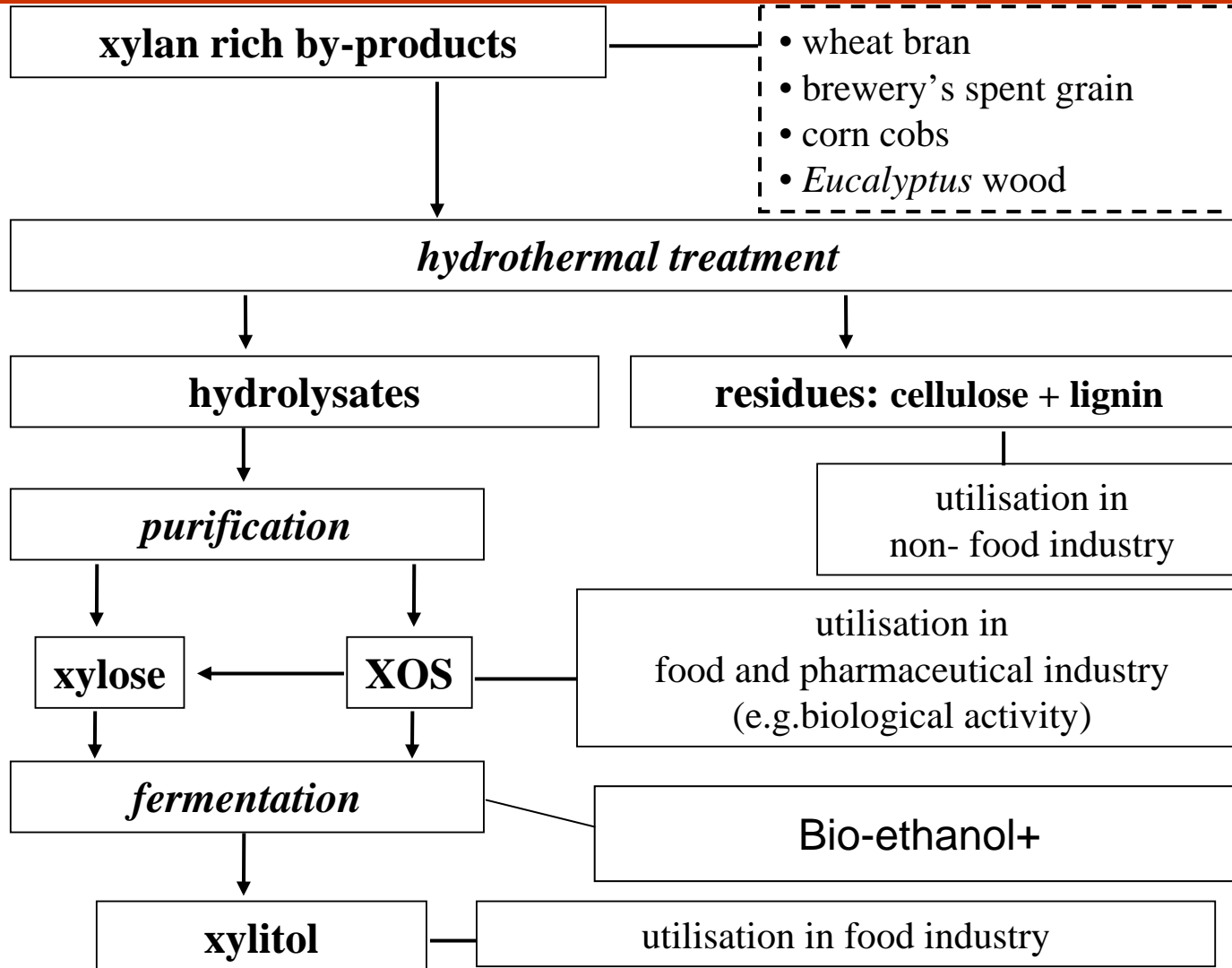
# Summary

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- Tyrosine and Ferulic acid can be covalently linked
- The enzyme Horseradish peroxidase can catalyze this reaction upon kinetic control of the reaction
- Kind of kinetic control governs which of two opposite effects FA exerts on tyrosine templated oligomerization
- Modification of a tyrosine in an actual protein requires distortion of the protein structure and kinetic control



# Xylo-oligosaccharides (XOS) from xylan-rich by-products





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