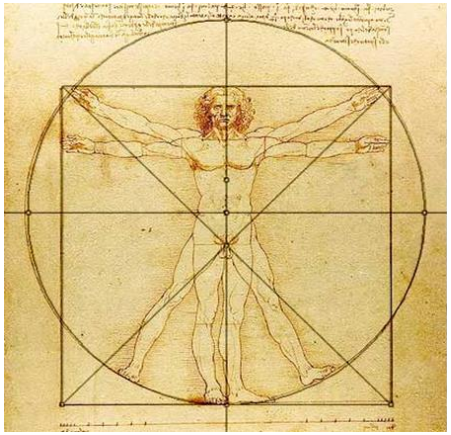
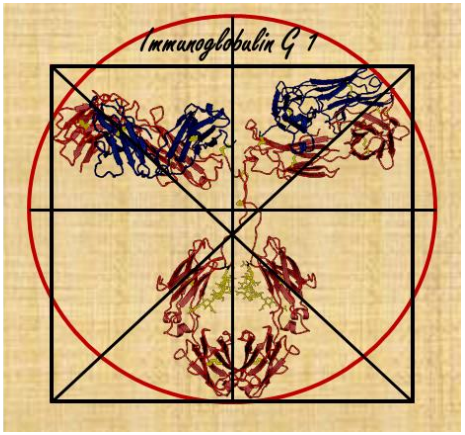
Rapid magnetic bead based sample preparation for automated and high throughput N-glycan analysis of therapeutic antibodies





Csaba Váradi[1], Clarence Lew[2] and András Guttman[1,2]

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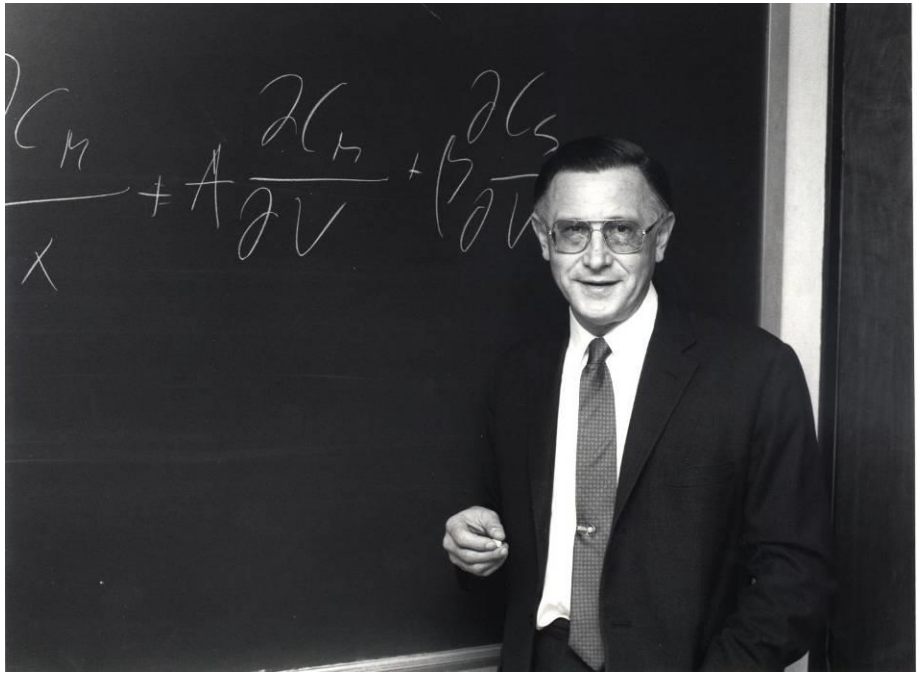
[1]*Sciex Separations, Brea, CA*

HLBS Horváth Laboratory of Bioseparation Sciences

SCIEX

In Memoriam Professor Csaba Horváth



1930 - 2004

1952	M.S., Technical University Budapest (BME)
1952-56	Faculty member at BME
1957-61	Farbwerke Hoest
1961-63	Ph.D. at J.W.Goethe University, Frankfurt
1963-64	Harvard Medical School
1964-70	Yale University
1970-79	Associate Professor, Dept. Eng. Yale
1979-2004	Professor of Chemical Engineering, Yale

Llewelyn West Jones Jr. Professor of Chem. Eng. 1993-1998
Roberto C. Goizueta Professor of Chem. Eng. 1998-2004

International Recognition

1978 Dal Nogare Award
1978 Commemorative Tswett Medal (USSR)
1980 M.S. Tswett Award in Chromatography
1982 Humboldt Award for Senior US Scientists
1983 American Chemical Society National Chromatography Award
1986 Chromatography Award of the Eastern Analytical Symposium
1990 Member of the Hungarian Academy of Science
1994 A.J.P. Martin Gold Medal
1994 Fellow of the AIChE
1997 Halász Medal Award
2000 Michael Widmer Award of the New Swiss Chemical Society
2001 American Chemical Society National Award
2002 Cross of Honor for Arts and Sciences of the Austrian Republic
2003 Torbern Bergman Medal of the Swedish Chemical Society
2003 Heureka Price of the Hungarian Chemical Society
2004 Member of the US National Academy of Engineering

Top 10 Pharmaceutical Products Dominance of Proteins

Top 10 products by sales in 2008

Rank	Product	Company	Therapeutic Subcategory	Technology	WW sales ($m)
1	Lipitor	Pfizer + Astellas + Almirall	Anti-hyperlipidaemics	Chiral chemistry	13,507
2	Plavix	BMS + Sanofi-Aventis	Platelet aggregation inhibitors	Small molecule chemistry	9,447
3	Advair	GlaxoSmithKline	Other bronchodilators	Small molecule chemistry	7,828
4	Enbrel	Wyeth + Amgen + Takeda	Other anti-rheumatics	Recombinant product	6,455
5	Diovan	Novartis + Ipsen	Angiotensin II antagonists	Small molecule chemistry	5,825
6	Rituxan	Roche	Anti-neoplastic MAbs	Monoclonal antibody	5,481
7	Remicade	SGP + J&J + Mitsubishi Tanabe	Other anti-rheumatics	Monoclonal antibody	5,293
8	Nexium	AstraZeneca	Antacids & anti-ulcerants	Chiral chemistry	5,200
9	Epogen/Procrit	J&J + Amgen + Kirin	Anti-anaemics	Recombinant product	5,162
10	Avastin	Roche	Anti-neoplastic MAbs	Monoclonal antibody	4,818

2008
1 of top 5
5 of to 10
28% of top 100

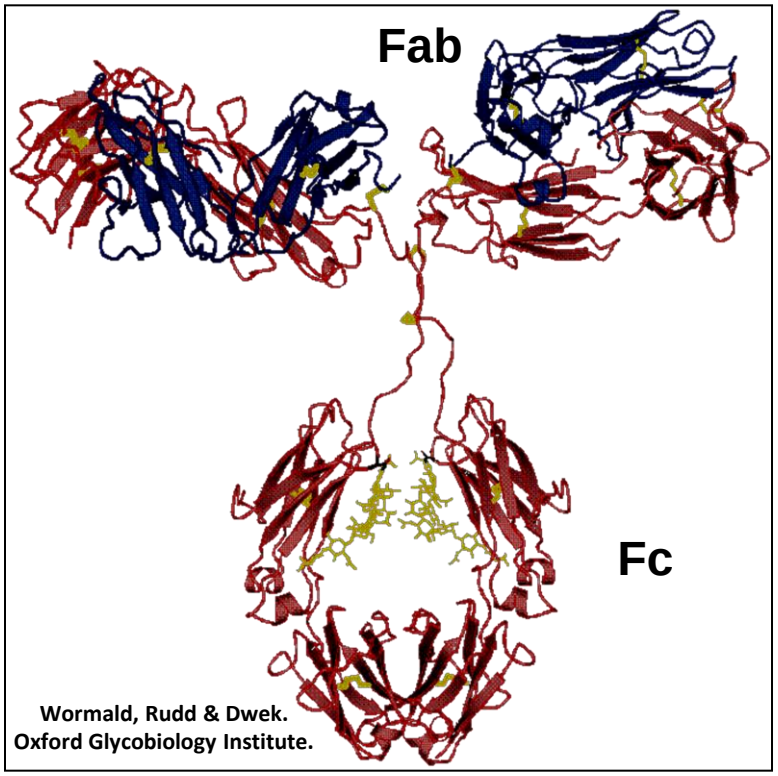
Top 10 products by sales in 2014

Rank	Product	Company	Therapeutic Subcategory	Technology	WW sales ($m)
1	Avastin	Roche	Anti-neoplastic MAbs	Monoclonal antibody	9,232
2	Humira	Abbott + Eisai	Other anti-rheumatics	Monoclonal antibody	9,134
3	Rituxan	Roche	Anti-neoplastic MAbs	Monoclonal antibody	7,815
4	Enbrel	Wyeth + Amgen + Takeda	Other anti-rheumatics	Recombinant product	6,583
5	Lantus	Sanofi-Aventis	Anti-diabetics	Recombinant product	6,386
6	Herceptin	Roche	Anti-neoplastic MAbs	Monoclonal antibody	5,796
7	Crestor	AstraZeneca	Anti-hyperlipidaemics	Small molecule chemistry	5,739
8	Spiriva	Boehringer Ingelheim	Anti-cholinergics	Small molecule chemistry	5,552
9	Remicade	SGP + J&J + Mitsubishi Tanabe	Other anti-rheumatics	Monoclonal antibody	5,220
10	Gleevec/Glivec	Novartis	Other cytostatics	Small molecule chemistry	5,136

2014
5 of top 5
7 of to 10
50% of top 100

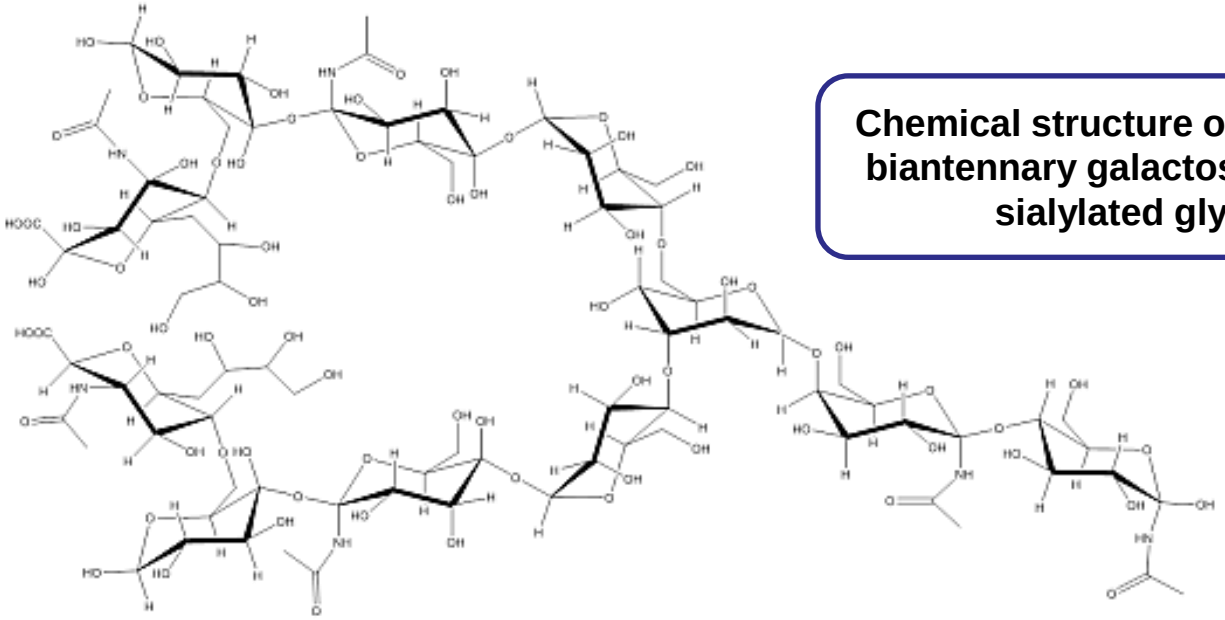
EvaluatePharma

IgG1

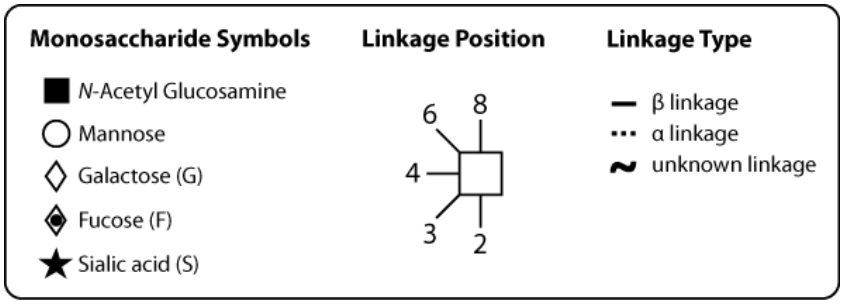


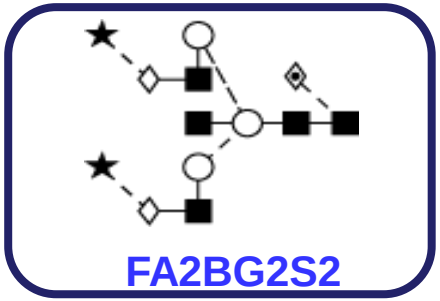
- One N-linked glycosylation site at Asn 297.
- Variability depending on cell line and expression conditions.
- Structural diversity: Fuc, Gal, Neu5AC, GlcNAc
- Glycosylation determines:
  - biological activity,
  - physicochemical properties,
  - ADCC and CDC functions.
- Importance of IgG glycosylation analysis in:
  - clone selection,
  - product characterization,
  - lot release

Symbolic representation of glycans



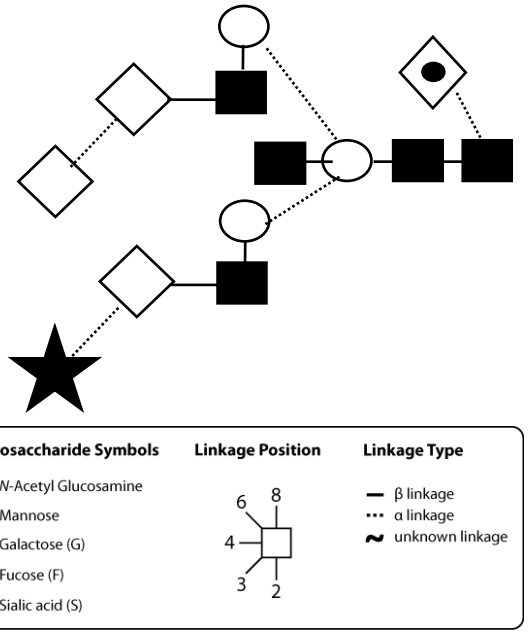
Chemical structure of a complex biantennary galactosylated and sialylated glycan

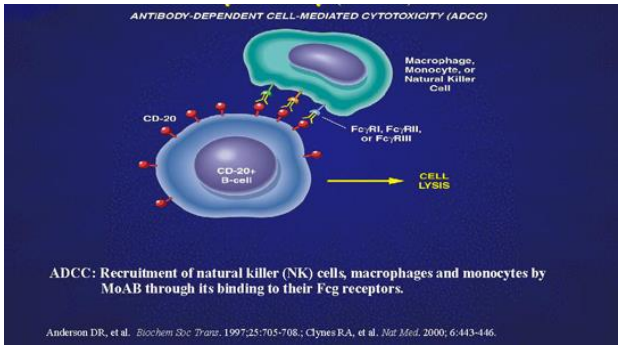




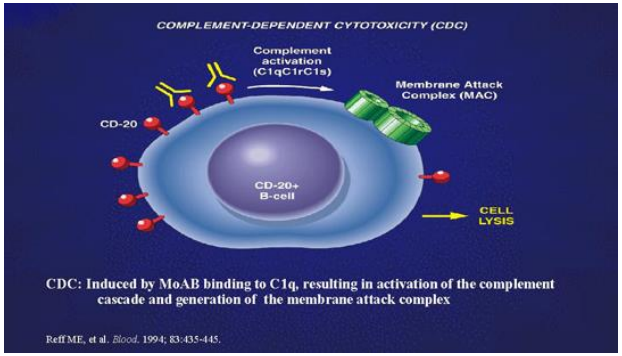
Functionality of terminal sugar residues of N-glycans on IgG

- Core fucose: Enhanced ADCC activity
- Bisecting GlcNAc: Enhanced ADCC activity
- Sialic acid: Suppression of ADCC and anti-inflammatory
- Galactose: Placental transport; enhanced CDC
- GlcNAc/mannose: Ligand for Mannose Binding Protein
- α1,3-gal, NGNA: Antigenic



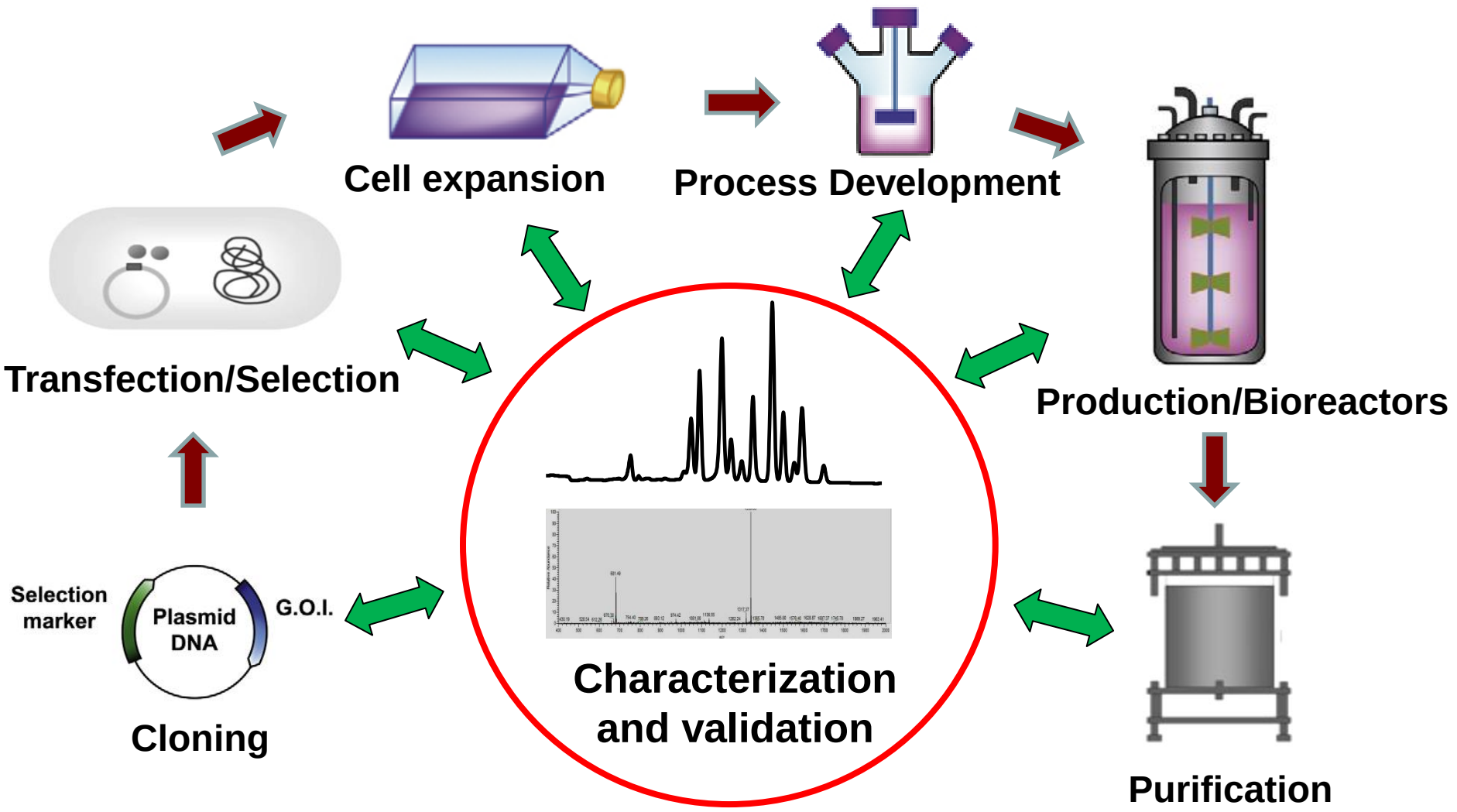


ADCC



CDC

The manufacturing process of recombinant therapeutic proteins



Alterations in glycosylation processing

- Many factors contribute to alterations in glycan processing on recombinant glycoproteins
  - Expression levels of the processing enzymes in the host cell line
  - Monosaccharide nucleotide donor levels
  - Cell signaling pathways: cytokines/hormones, drugs, media components
  - Loss of cellular organelle organization due to e.g., pH changes
  - Mutations in genes, gene silencing, overexpression
  - Bioprocessing environment such as temperature, oxygen level, etc.
- Sugars are involved in key aspects of bioprocessing
  - Importance of Process Analytical Technology
- Quality by design:
  - Importance of sugar function relationship
  - Selection of cell lines with appropriate glycosylation
- Proper analytical toolset to ensure that the product has correct glycosylation for optimal activity

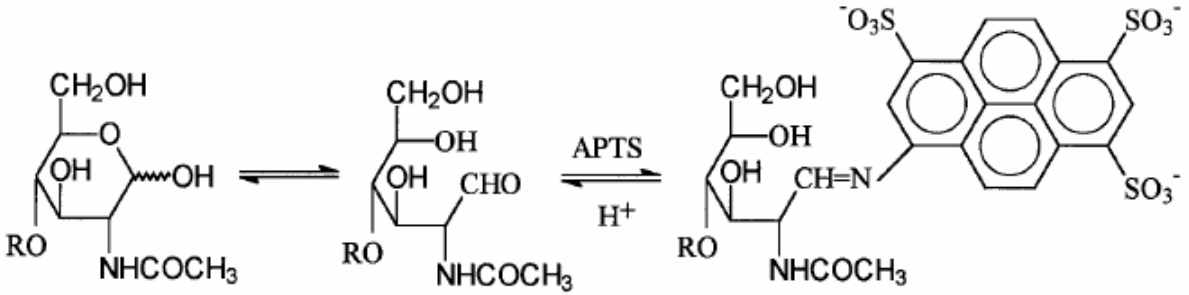
Glycan analysis options

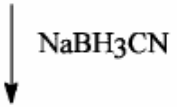
***CHALLENGE*: complex, diversified structures; no chromophore / fluorophore groups; mostly not charged**

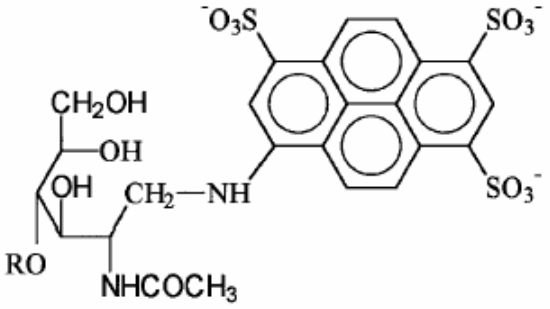
Analytical methods in glycan analysis:

- **Structural characterization options:** MS and NMR
- **HPLC**: - HPAE/PAD
  - Normal phase and HILIC (HPLC and UPLC)
  - Graphitized carbon (HPLC and chipLC)
- **Capillary Electrophoresis**
- **Hyphenated Methods: LC-MS, CE-MS, CESI-MS**

Glycan Labeling by APTS







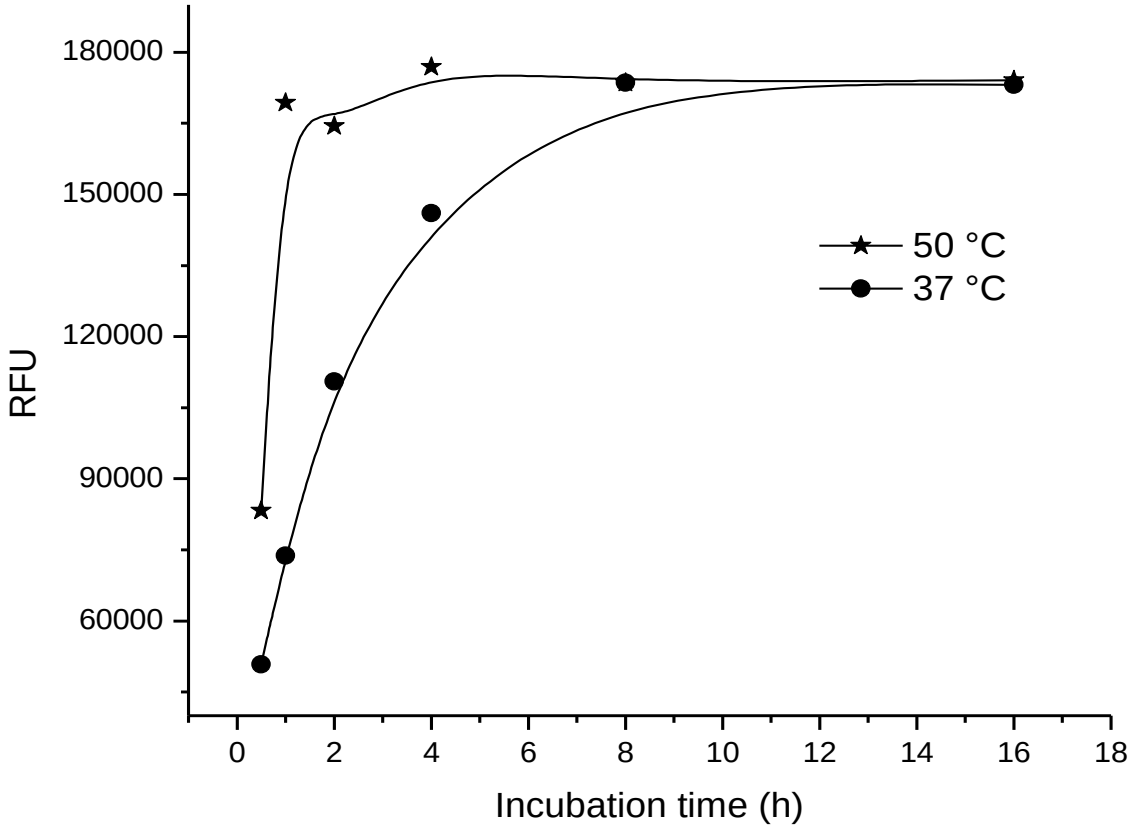
Purpose:

Introduction of label and charge

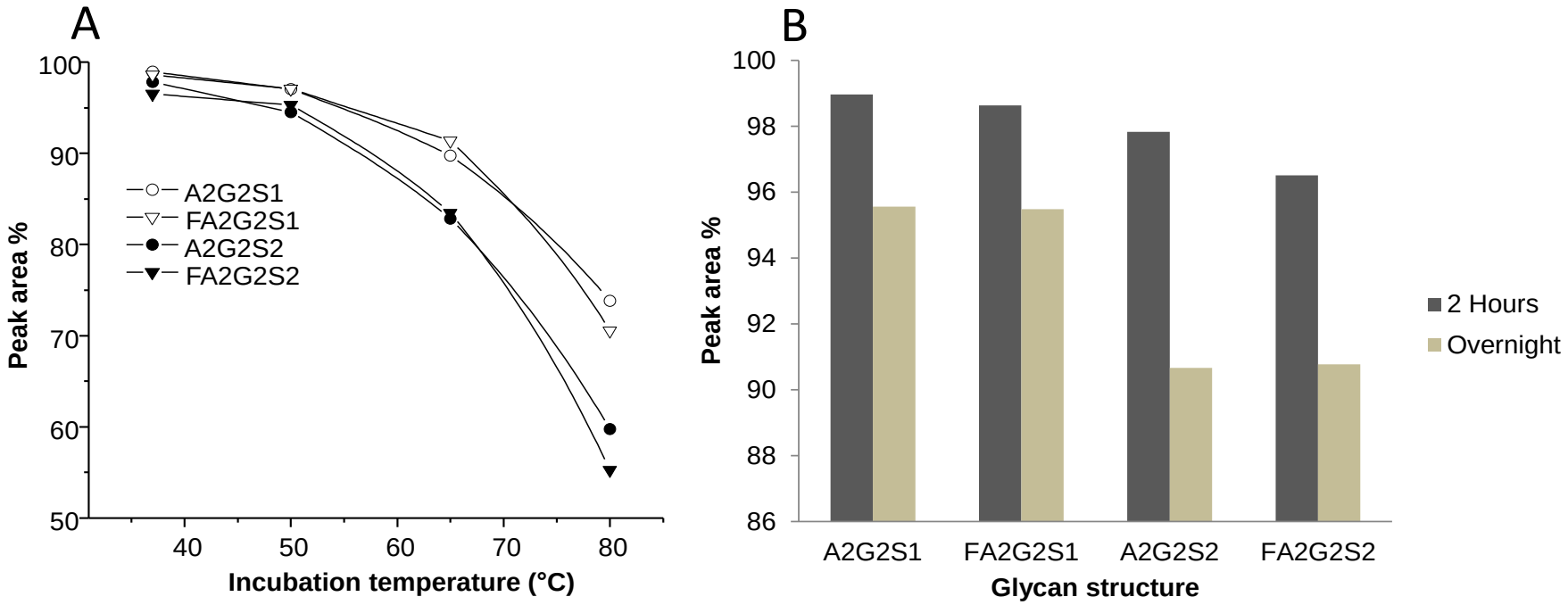
- **Reductive amination**
- **Sugar reducing ends only**
- **ex 488 / em 520 nm LIF/LedIF, excellent sensitivity**
- **Simple, one step reaction**
- **Great efficiency (over 90%) under optimized conditions**

  (reagent concentration, time, temperature, pH, solvent)
- **Non-selective: uniform labeling for most structures**
- **Easy quantification: one fluorophore per sugar molecule**

Average fluorescent intensity as a function of PNGase F digestion time at 37ºC and 50ºC.



Optimization of fluorophore labeling conditions

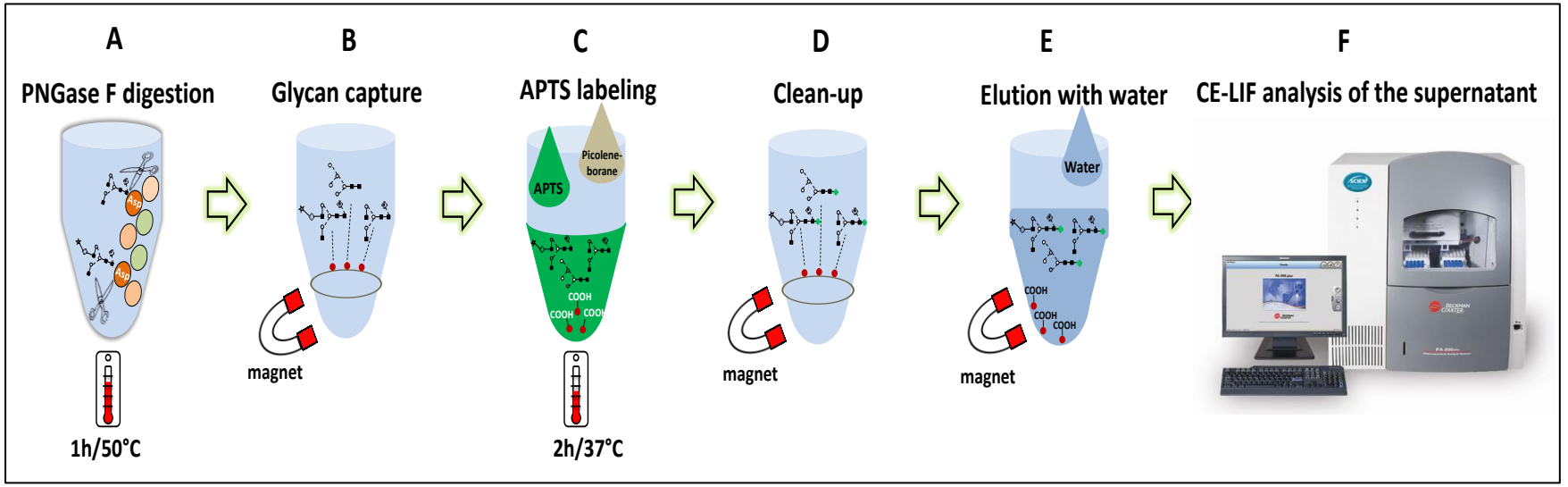


APTS labeling efficiency study.

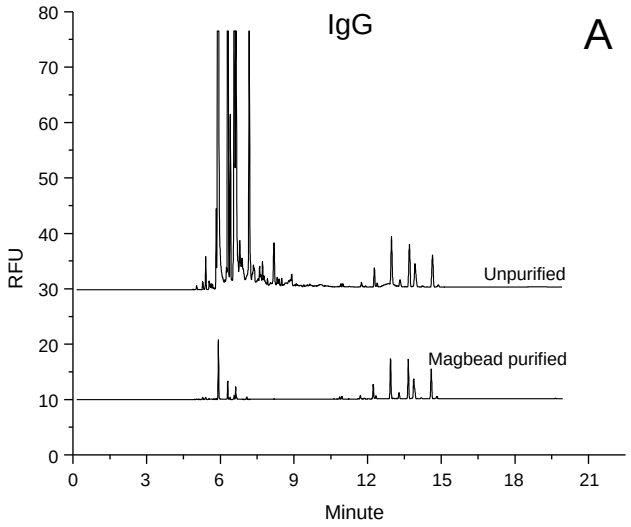
Section A			
AcOH conc	15%	20%	25%
A2G2S1	1.9	10.0	10.9
A2G2S2	1.1	5.3	5.9
FA2G2S1	6.6	14.9	15.5
FA2G2S2	3.2	13.0	13.5
Section B			
APTS conc	20 mM	40 mM	80 mM
3 Ladders avg	14.6	39.3	46.0
Section C			
Labeling strategy (37 °C)	2h, 15% AcOH, 20 mM APTS	2h, 20% AcOH, 40 mM APTS	O/N, 15% AcOH,20 mM APTS
IgG	9.5	24.2	52.9
Fetuin	18.9	28.2	66.3
RNase B	13.2	23.5	63.3

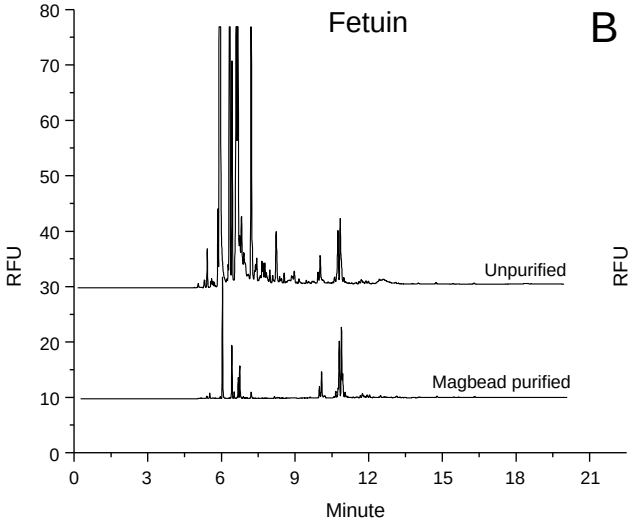
[a]The numbers in the table represent the average signal intensity of 3 labeling reactions.

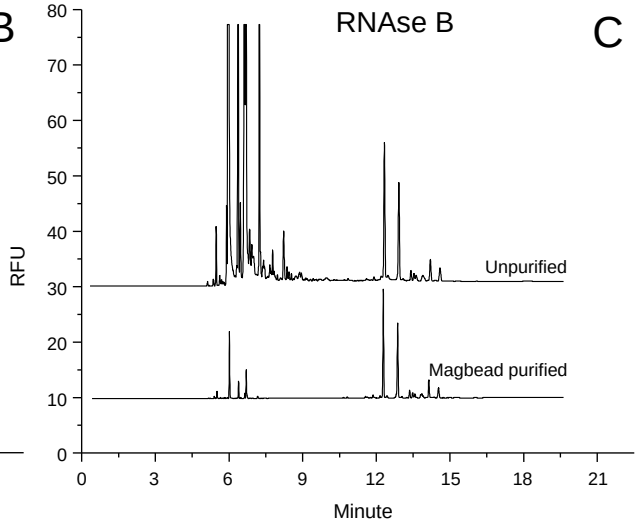
Magnetic bead based sample preparation for N-glycosylation analysis by CE-LIF



Magnetic bead based APTS clean-up for glycoproteins with complex, sialylated and high mannose structures







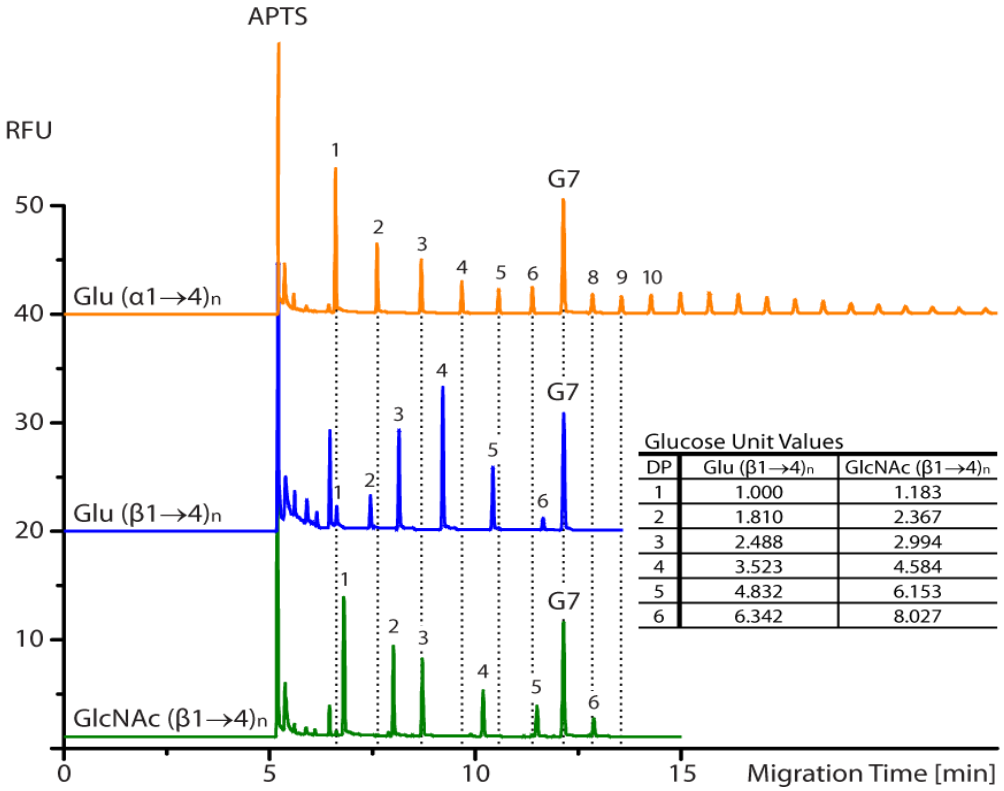
Comparison between the magnetic bead and the traditional overnight methods in the peak area%

Panel A	Magbead protocol			Overnight protocol		
IgG	Avg Area %	STDEV	RSD%	Avg Area %	STDEV	RSD%
FA2G2S2	1.19	0.04	3.7	1.21	0.09	7.0
FA2BG2S2	1.23	0.03	2.2	1.20	0.03	2.6
FA2(3)G1S1	1.71	0.06	3.8	1.73	0.10	5.8
FA2G2S1	7.45	0.25	3.3	7.48	0.31	4.1
FA2BG2S1	1.74	0.16	9.0	1.65	0.17	10.4
FA2	22.12	0.47	2.1	22.23	0.15	0.7
FA2B	3.97	0.11	2.8	3.97	0.04	1.1
FA2(6)G1	22.93	0.40	1.8	23.01	0.60	2.6
FA2(3)G1	11.59	0.06	0.5	11.57	0.17	1.5
FA2B(6)G1	4.86	0.41	8.5	4.69	0.14	3.1
FA2B(3)G1	1.02	0.09	9.1	1.07	0.06	5.4
FA2G2	18.13	0.47	2.6	18.30	0.12	0.6
FA2BG2	1.46	0.04	3.0	1.40	0.04	2.6
Panel B	Magbead protocol			Overnight protocol		
RNase B	Avg Area %	STDEV	RSD%	Avg Area %	STDEV	RSD%
Man5	43.45	0.62	1.4	43.90	0.37	0.8
Man6	33.39	0.36	1.1	33.50	0.25	0.7
Man7*	3.56	0.13	3.6	3.51	0.04	1.0
Man7**	2.58	0.08	3.2	2.52	0.07	2.7
Man7***	2.18	0.03	1.2	2.22	0.11	5.1
Man8	8.60	0.30	3.5	8.22	0.13	1.6
Man9	6.00	0.35	5.8	6.04	0.23	3.8
Panel C	Magbead protocol			Overnight protocol		
Fetuin	Avg Area %	STDEV	RSD%	Avg Area %	STDEV	RSD%
Peak 1	6.95	0.28	4.0	5.08	0.25	4.9
Peak 2	15.46	0.58	3.7	11.43	0.47	4.1
Peak 3	3.19	0.07	2.1	3.24	0.10	3.1
Peak 4	3.97	0.11	2.8	4.11	0.18	4.3
Peak 5	25.93	0.59	2.3	27.90	0.32	1.2
Peak 6	32.57	0.30	0.9	32.67	0.21	0.7
Peak 7	8.45	0.14	1.6	12.73	0.67	5.3
Peak 8	3.32	0.12	3.5	3.17	0.14	4.5

Magnetic bead method timeline

- PNGase F Digestion	60 min
- Glycan capture	10 min
- APTS labeling	120 min
- Cleanup	10 min
- Elution	10 min
- CE-LIF	20 min
Total analysis time	**230 min**

Standardization



Glucose Unit Values

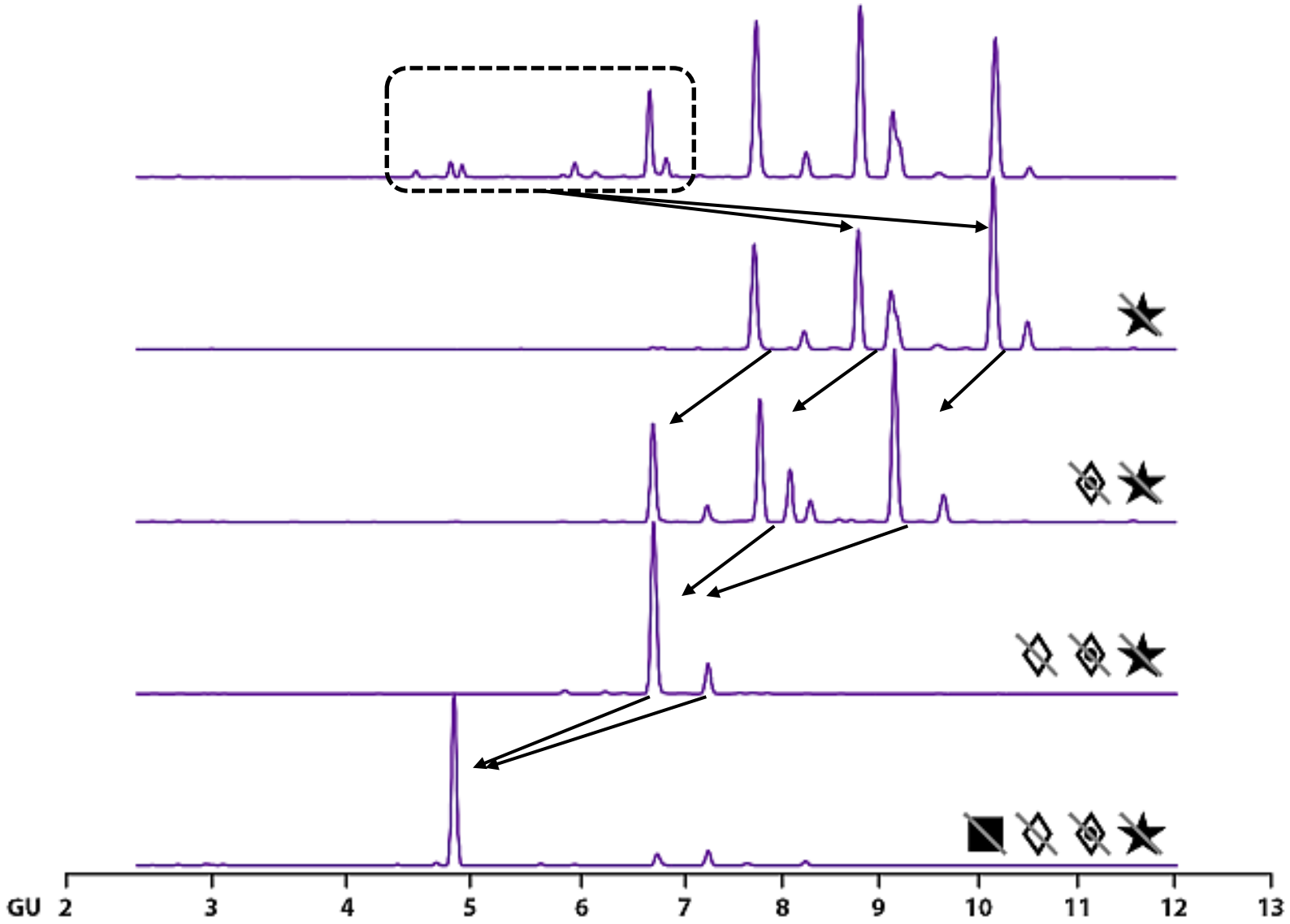
DP	Glu (β1→4)n	GlcNAc (β1→4)n
1	1.000	1.183
2	1.810	2.367
3	2.488	2.994
4	3.523	4.584
5	4.832	6.153
6	6.342	8.027

- Glucose Unit

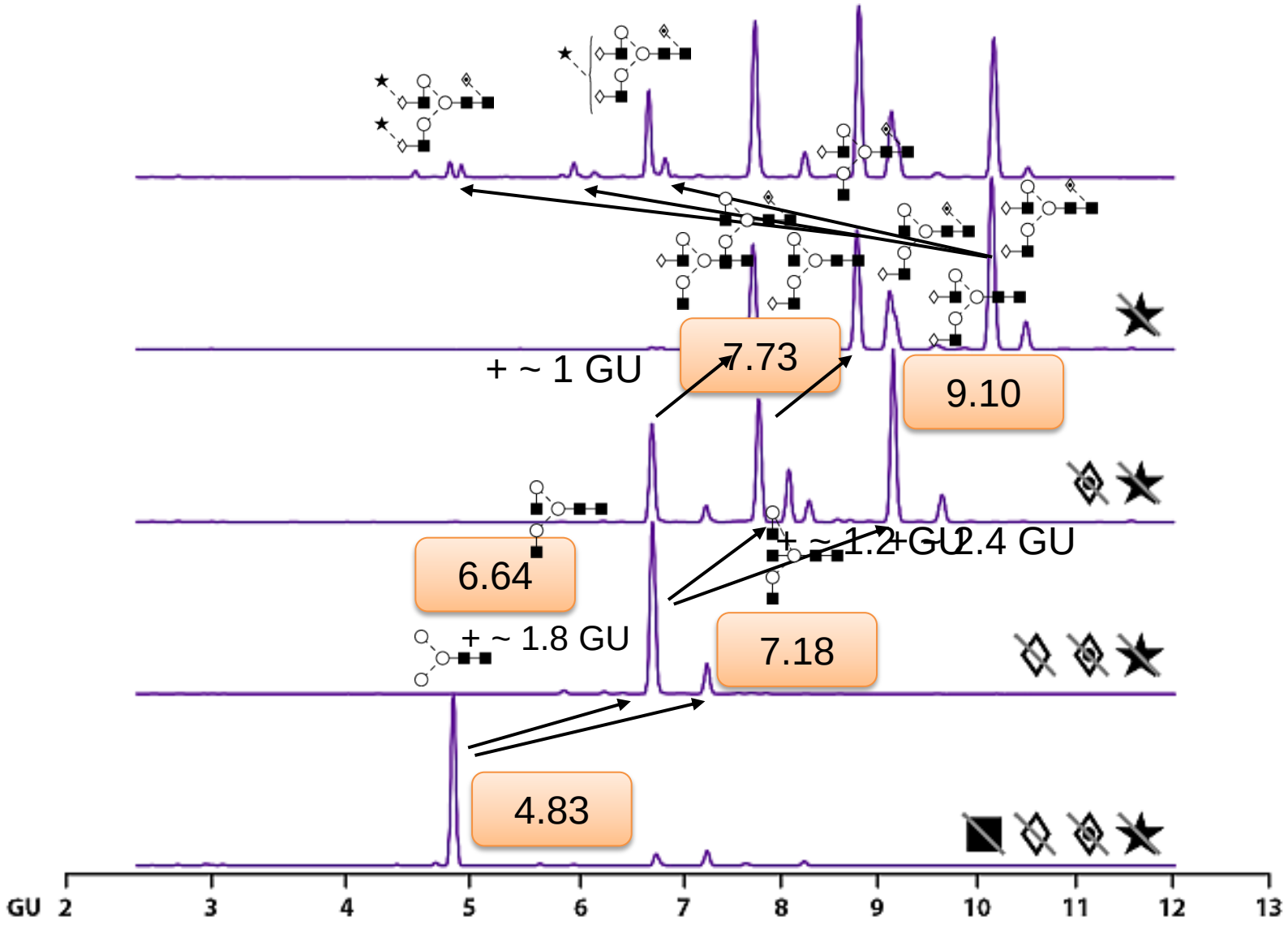
$$GU_x = G_n + \frac{t_x - t_n}{t_{n+1} - t_n}$$

- Instrument independent
- Tentative structural elucidation

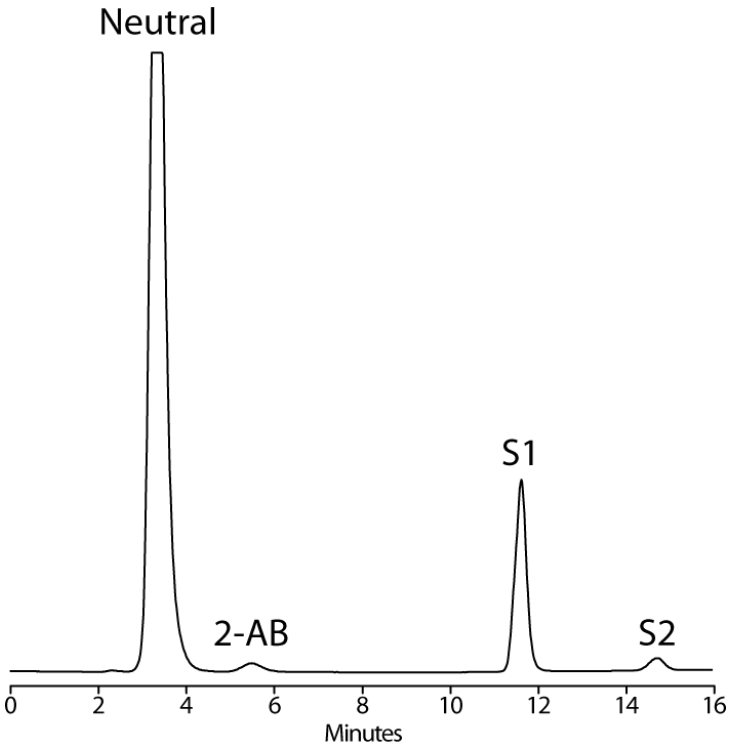
Sequential Digestion

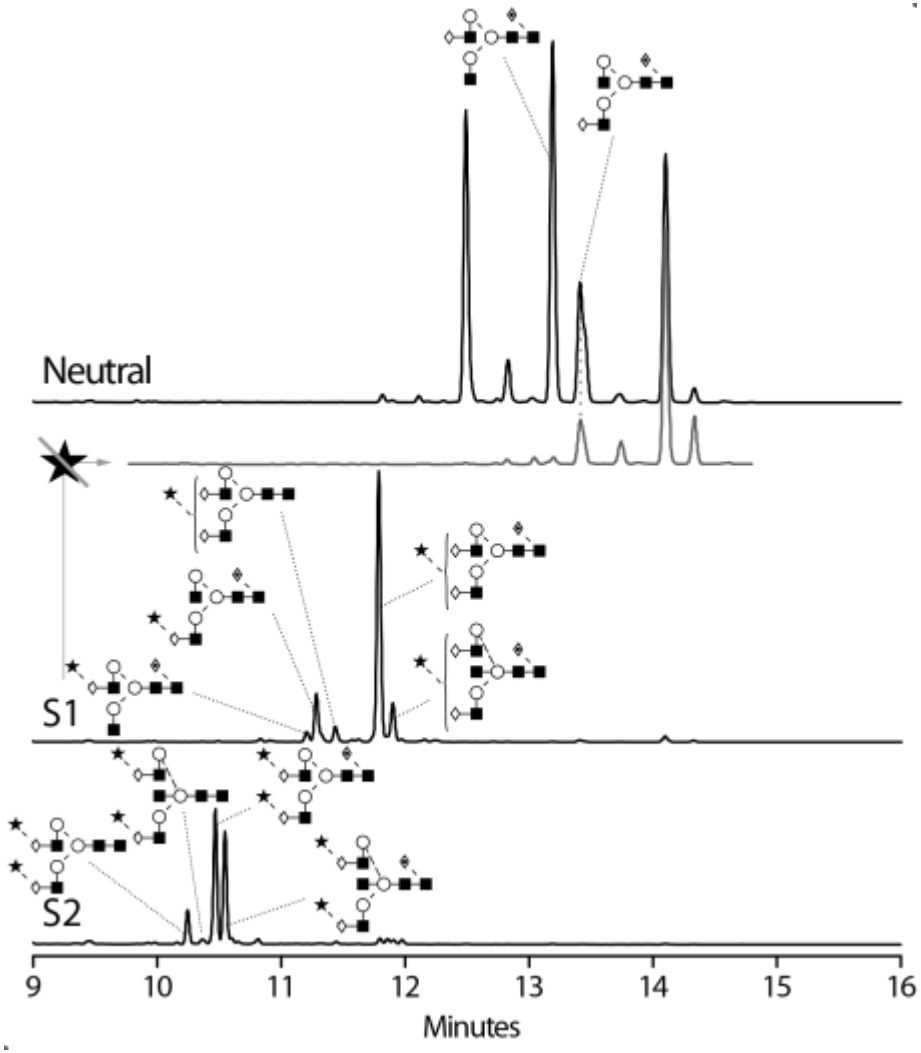


Bottom-up Identification



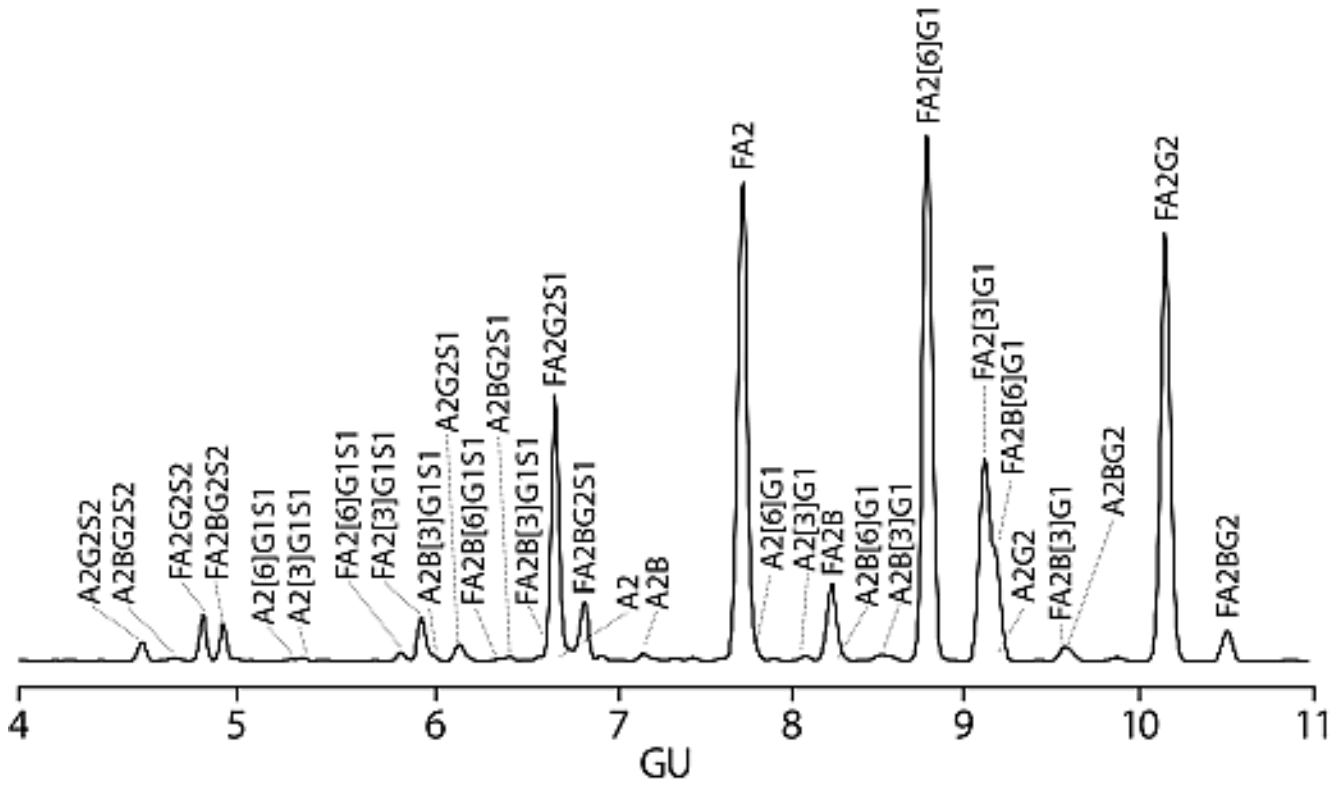
AEC Fractionation



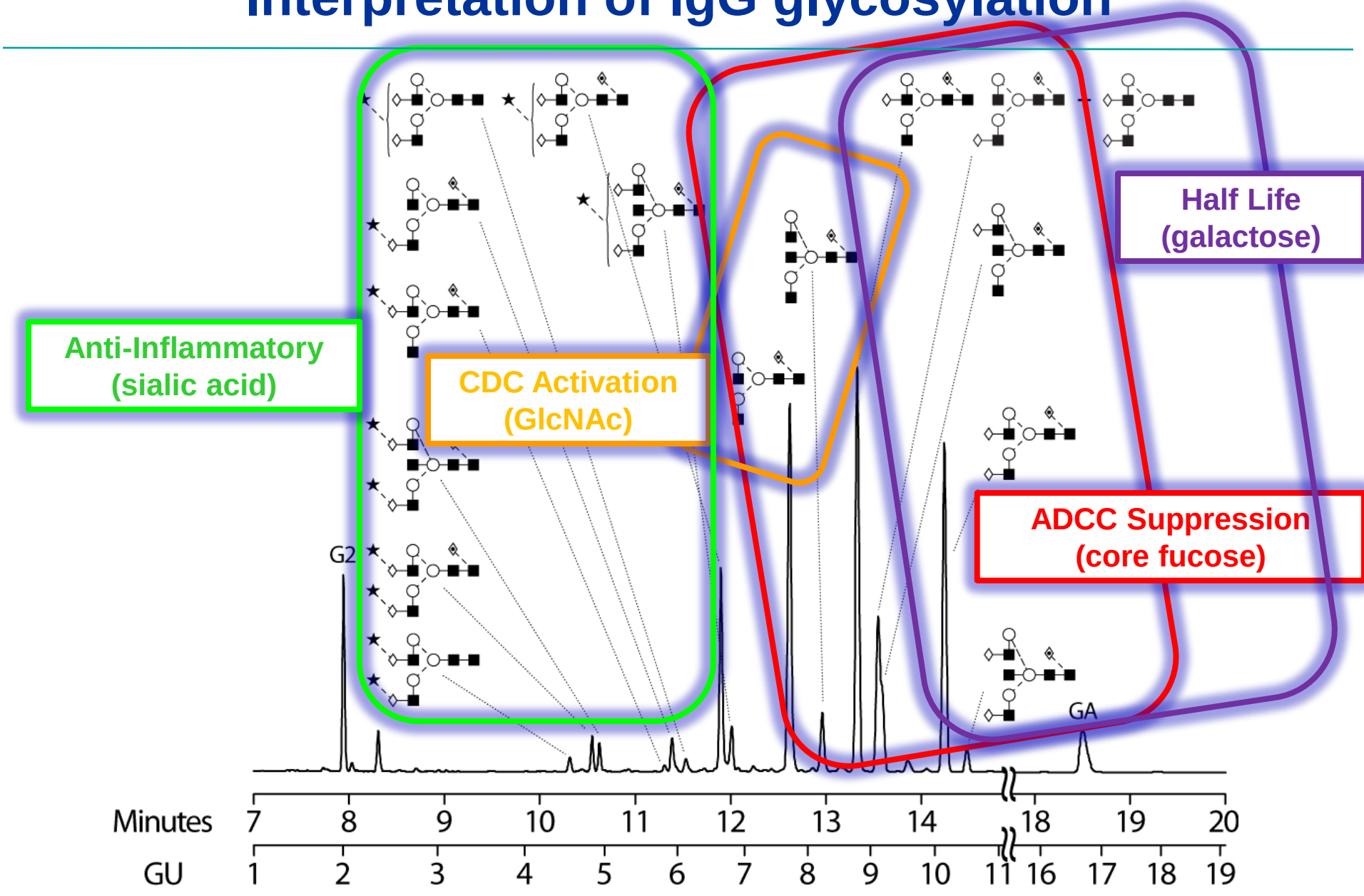


Identified Structures

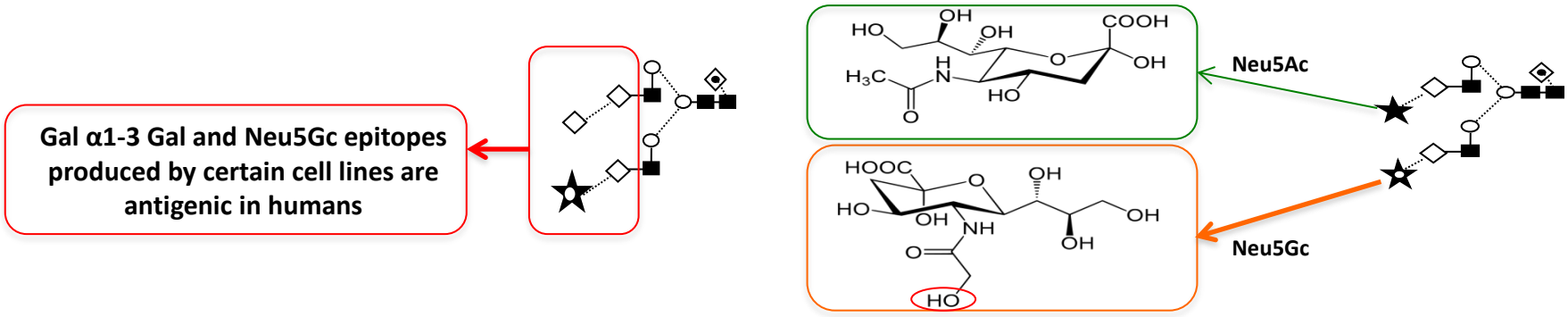
36 possible structures on IgG heavy chain: Combinations of core fucosylation, galactosylation, sialylation and the presence of a bisecting GlcNAc.



Interpretation of IgG glycosylation

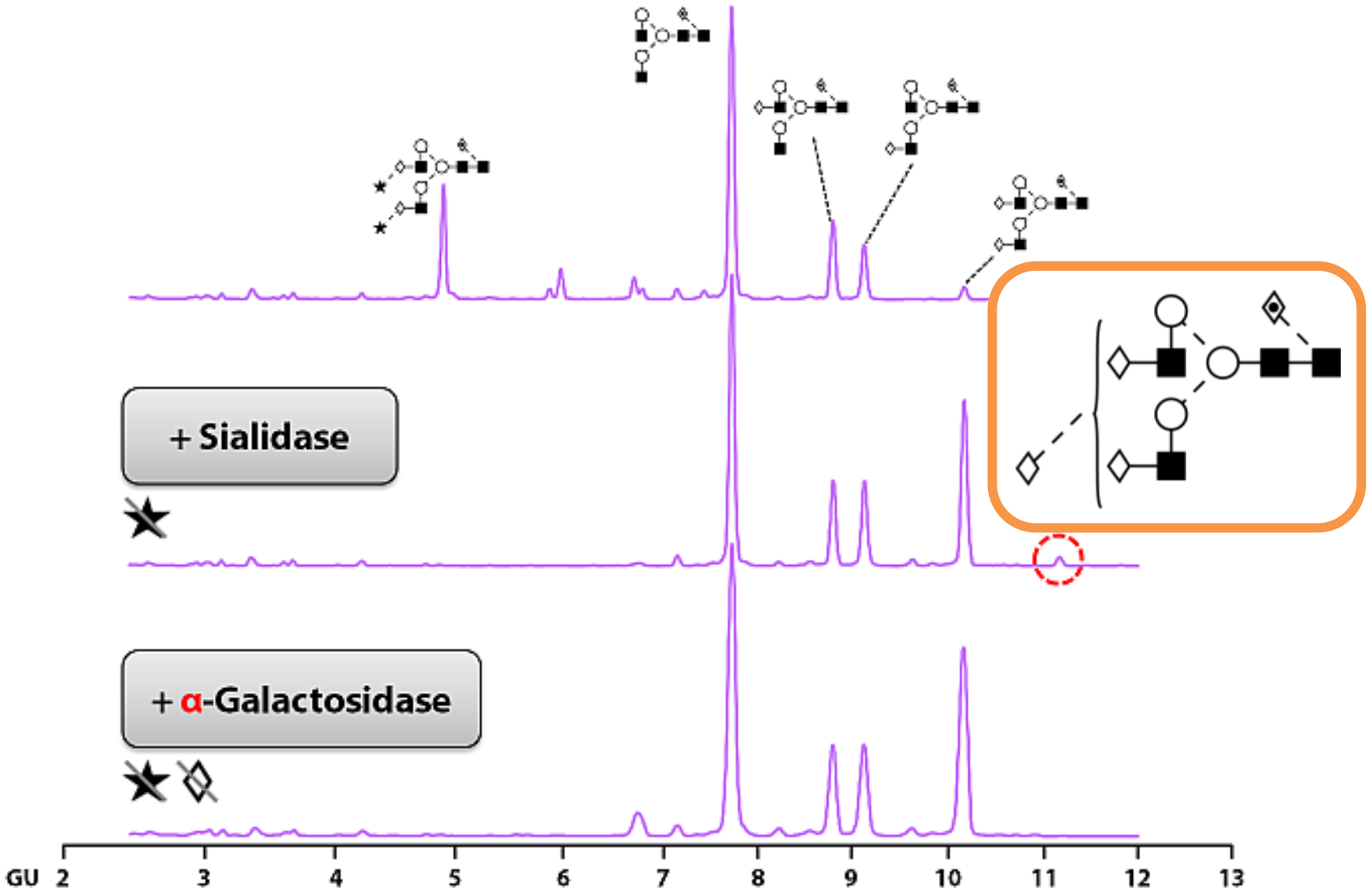


Determination of the presence or absence of potentially immunogenic epitopes

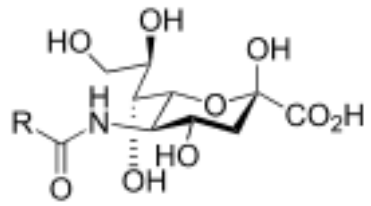


- Certain cell lines are capable of Neu5Gc incorporation or to synthesize galactose-α-1,3-galactose epitopes.
- As glycosylation is subject to cell culture conditions, alterations in the process may result in different levels immunogenic epitopes.

Analysis of immunogenic epitopes: α1-3 Gal



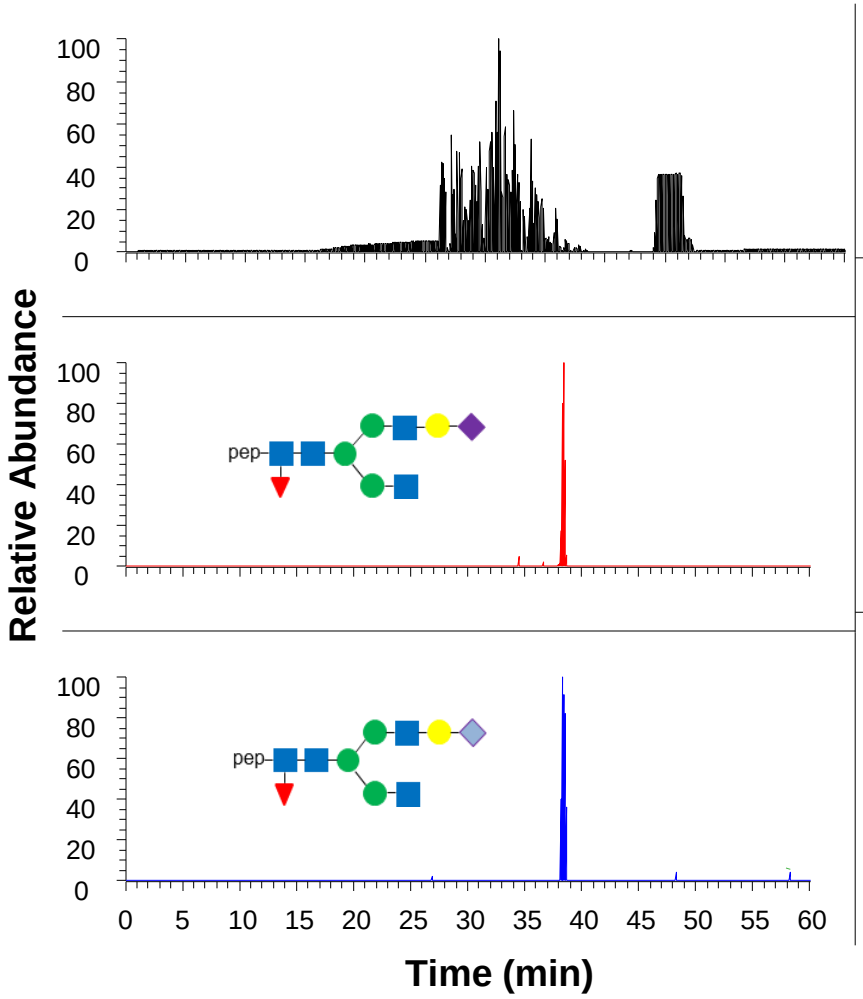
NANA- and NGNA-containing glycoforms detected by CESI-MS

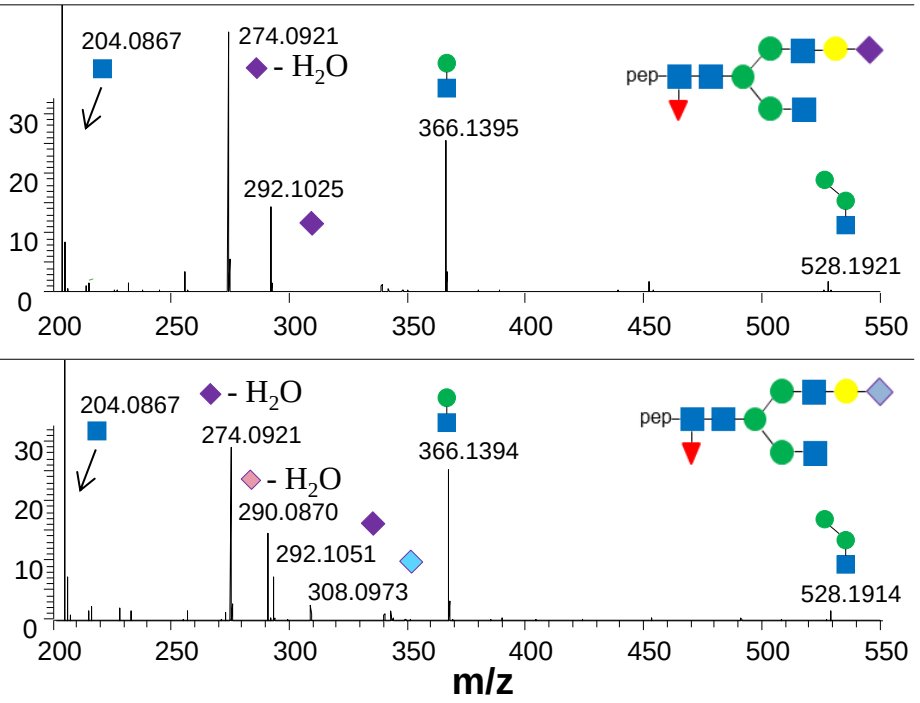


NANA: N-acetylneuraminic acid

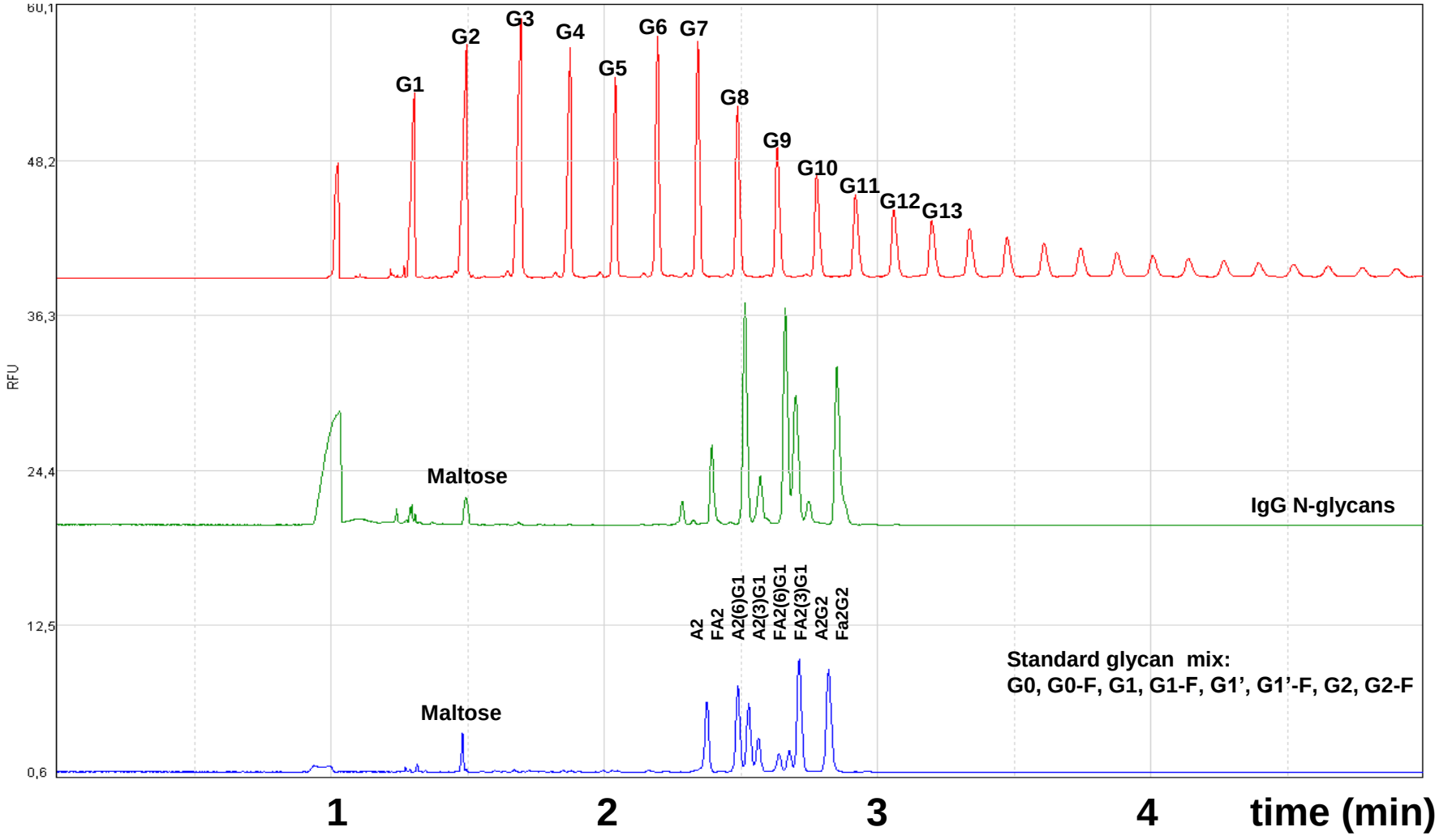
NGNA: N-glycolylneuraminic acid

1 R = -CH_3, Neu5Ac, human
2 R = -CH_2OH, Neu5Gc, mammals





Ultrafast profiling of IgG glycans



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Jo Wegstein
Ted Haxo
Zoltan Szabo