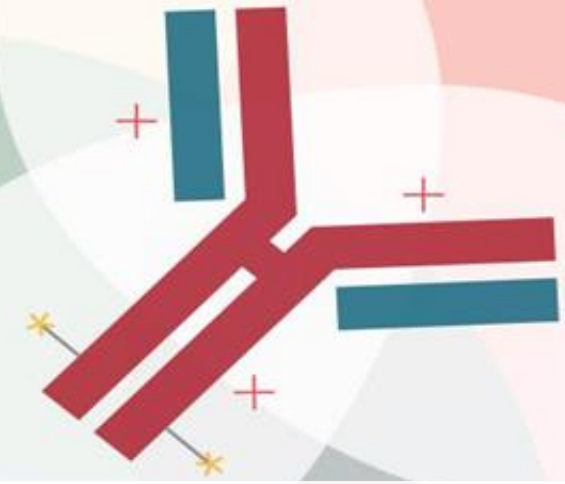

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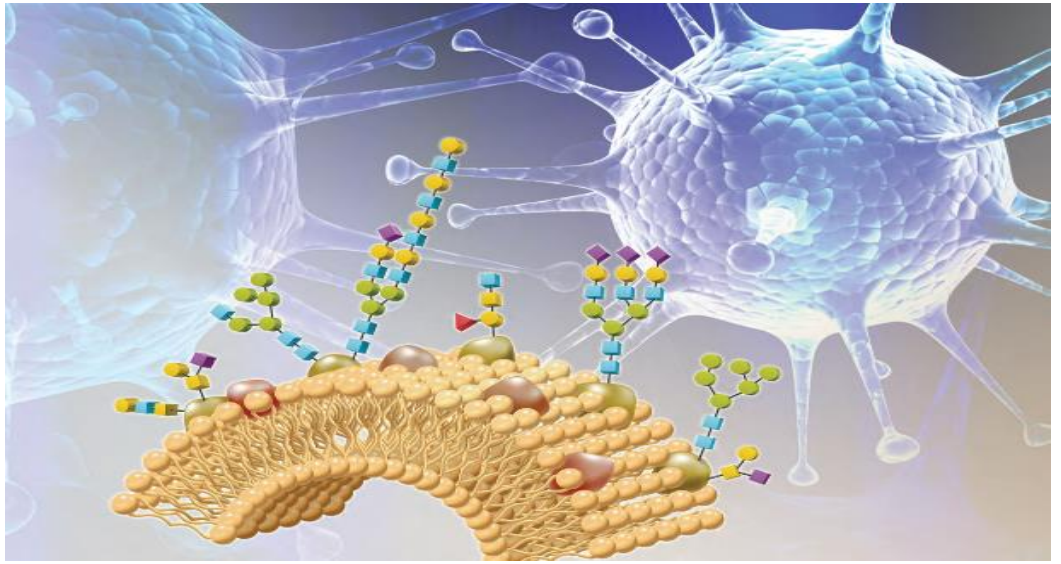
Ion Chromatography based analytical strategies for unravelling glycan complexity in biologics

Global BioPharma Summit

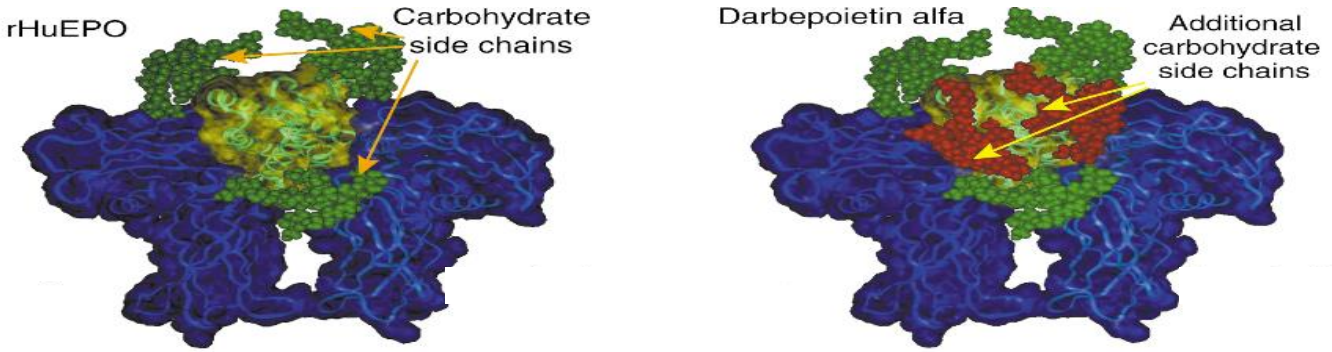
High Complexity, High Risk

- Glycans represent the most complex post-translational modifications to determine
  - They have the largest impact upon efficacy, safety and stability
  - Regulatory guidelines mean glycans have to be fully profiled
- No single analytical technique can provide all the necessary information required



Glyco-engineering to improve biopharmaceuticals

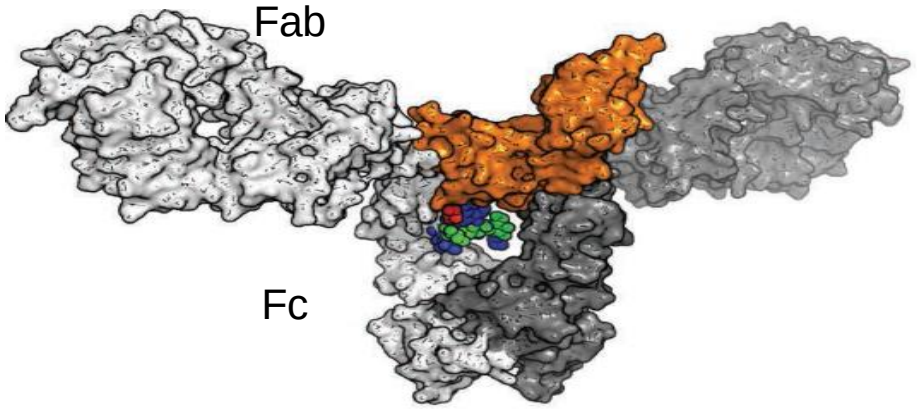
EPO:

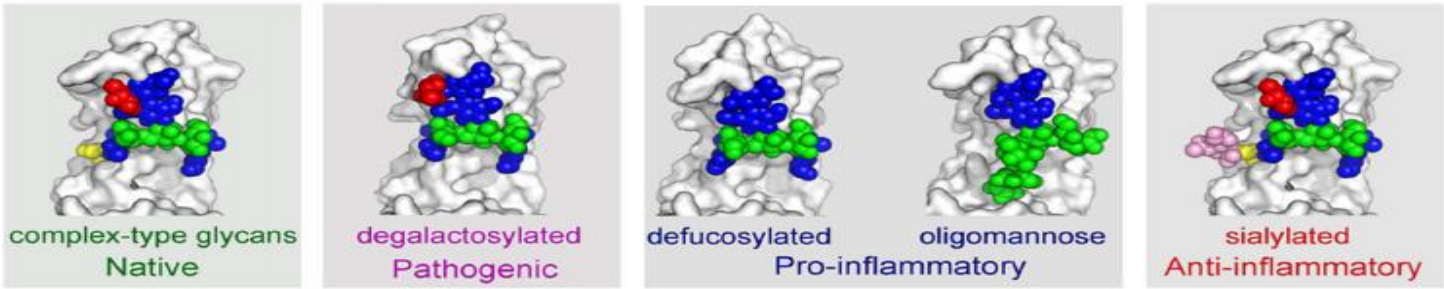


$T_{1/2}$ = 19h

$T_{1/2}$ = 32h

Therapeutic antibodies: Fc glycans determine function



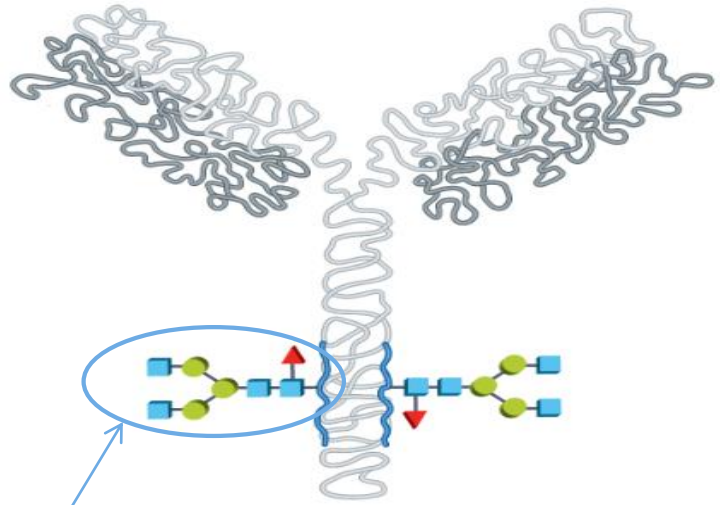


ThermoFisher SCIENTIFIC

Characterization and Confirmation of Biological Products

ICH (Q6B) recommended 6 test approaches for characterization and confirmation of biological products:

- Amino acid sequence
- Amino acid composition
- Terminal amino acid sequence
- Peptide map
- Sulfhydryl group(s) and disulfide bridges
- **Carbohydrate structure**



- *"For glycoproteins, the carbohydrate content and Structure (neutral sugars, amino sugars, and sialic acids) is determined."*

HPAE-PAD for Carbohydrate Analysis

HPAE-PAD refers to a technique where non-derivatized carbohydrates (ranging from simple sugars to complex carbohydrates) are analyzed by High-Performance Anion-Exchange (HPAE) chromatography coupled with Pulsed Amperometric Detection (PAD).

HPAE-PAD for Carbohydrate Analysis

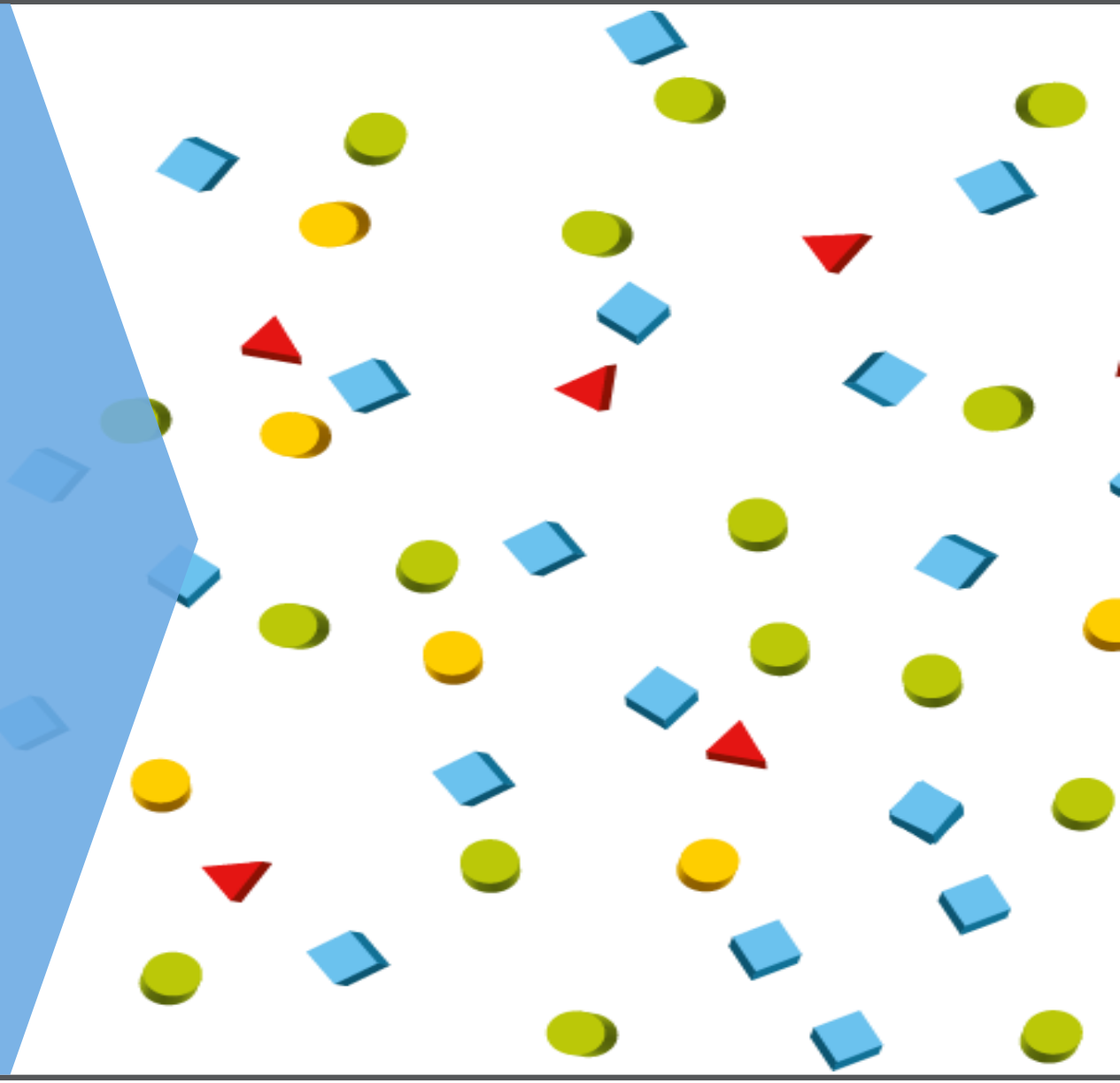
Basics of HPAE

- Carbohydrates are separated as oxyanions at high pH (>12).
- These separations are achieved using sodium hydroxide or sodium acetate gradients in sodium hydroxide.
- Separations of carbohydrate ranging from monosaccharides to oligosaccharides are achieved using our diverse column portfolio
- For more information : www.thermofisher.com

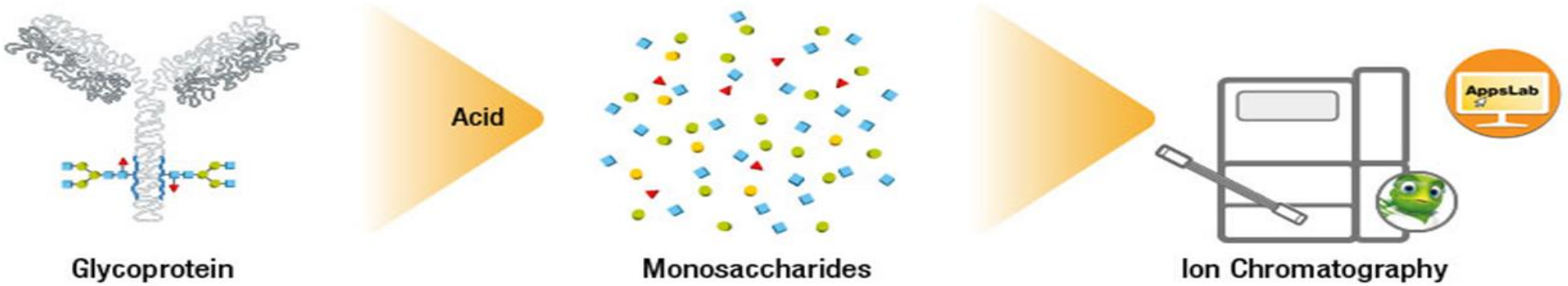
Basics of PAD

- Non-derivatized carbohydrates are detected on a Au working electrode (WE) at high pH by pulsed amperometric detection (PAD)
- PAD applies a series of potentials (a waveform) to a WE
- Pulsed amperometry detects analytes containing functional groups which get oxidized at the applied detection voltage
- For more information : www.thermofisher.com

Monosaccharides & Sialic Acids



Glycoprotein Monosaccharide Analysis by HPAE-PAD



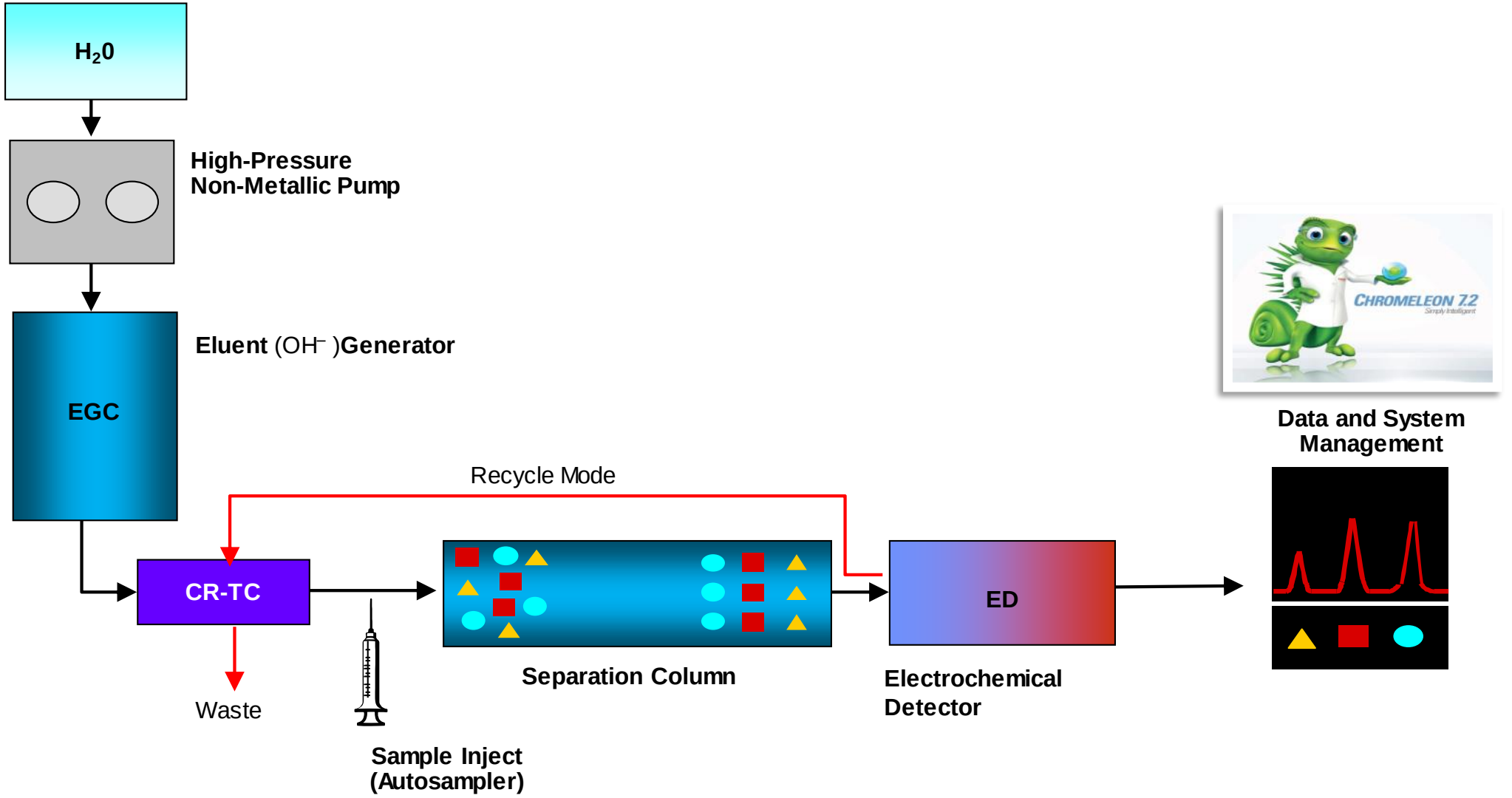
Monosaccharide Analysis Workflow

1. Hydrolyze the sample
2. Remove acid and dry the sample
3. Reconstitute the sample in water
4. Directly inject it for analysis

Benefits of HPAE-PAD

1. Monosaccharide composition can screen for changes in glycosylation
2. Sensitive detection with no derivatization
3. Allows measurement of total sugars and amounts of specific monosaccharides
4. Same system can analyze variety of carbohydrates

Simple Workflow using Ion Chromatography Systems



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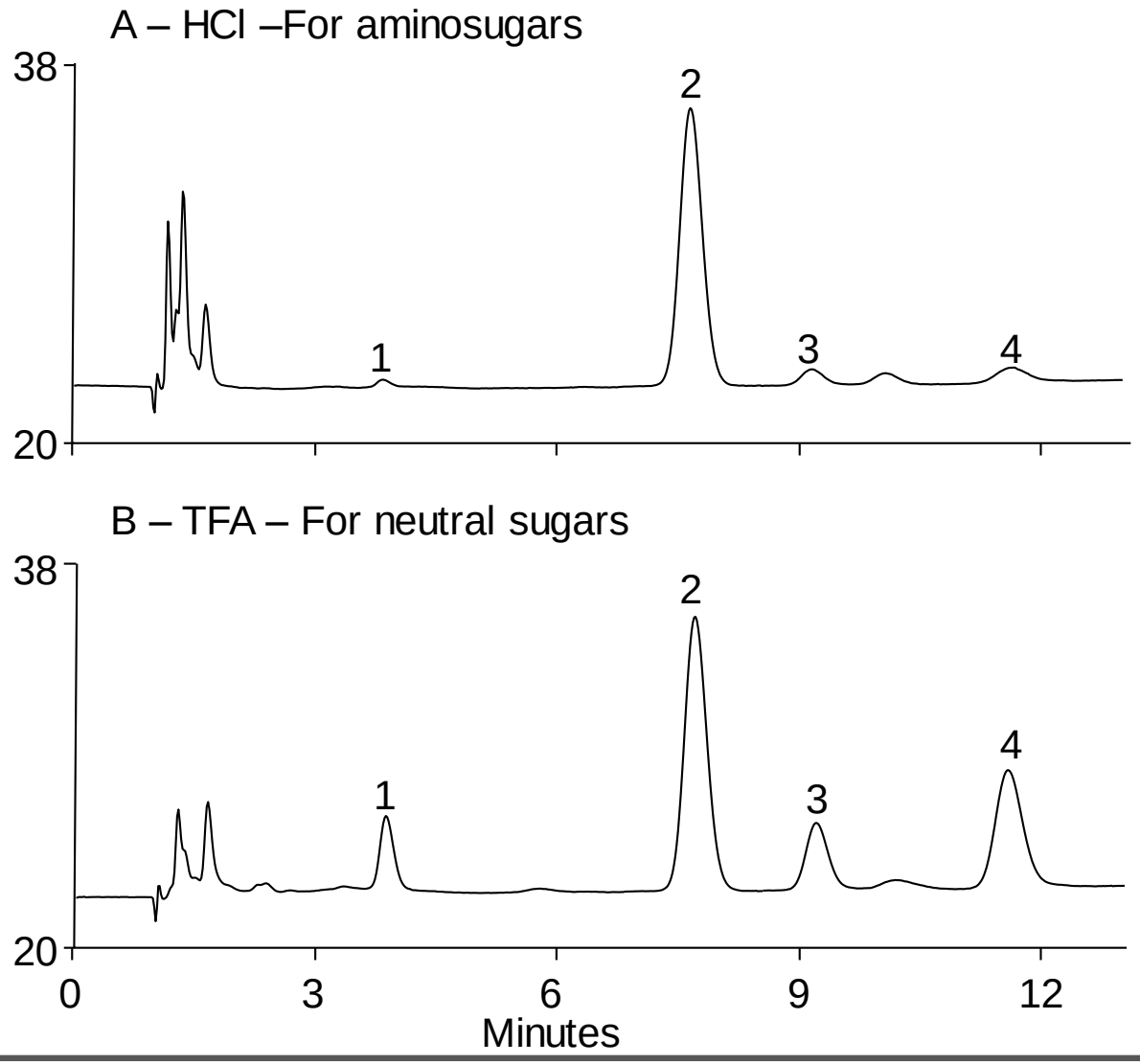
Monosaccharide Compositional Analysis of hIgG



Column:	Dionex CarboPac PA20 + Thermo Scientific™ Dionex™	AminoTrap™ column
Eluent:	10 mM KOH	
Flow Source:	EG50 + CR-ATC	
Flow Rate:	0.5 mL/min	
Inj. Volume:	10 µL (**2 µg**)	
Detection:	PAD (Au) Disposable Waveform A (TN21)	
Temperature:	30 °C	
Sample:	A) 6N HCl hydrolyzate B) 4N TFA hydrolyzate	
Peaks:	1. Fucose 2. Glucosamine 3. Galactose 4. Mannose	

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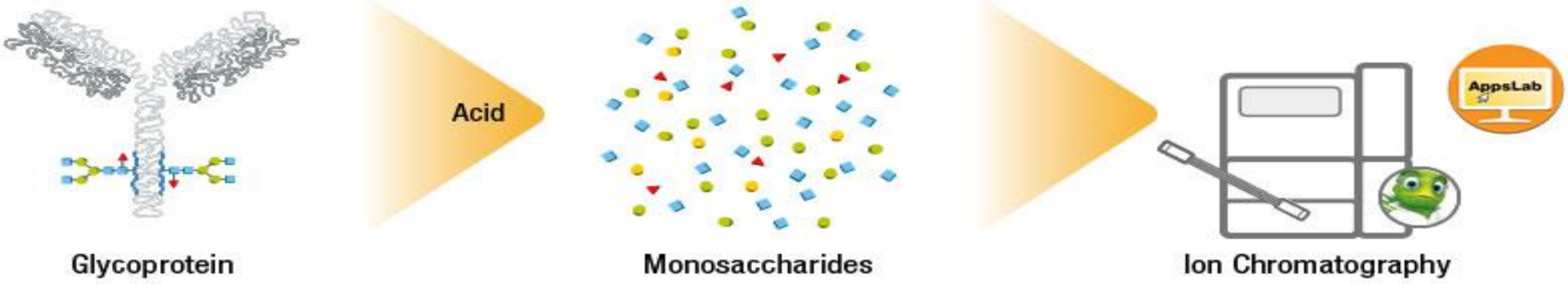
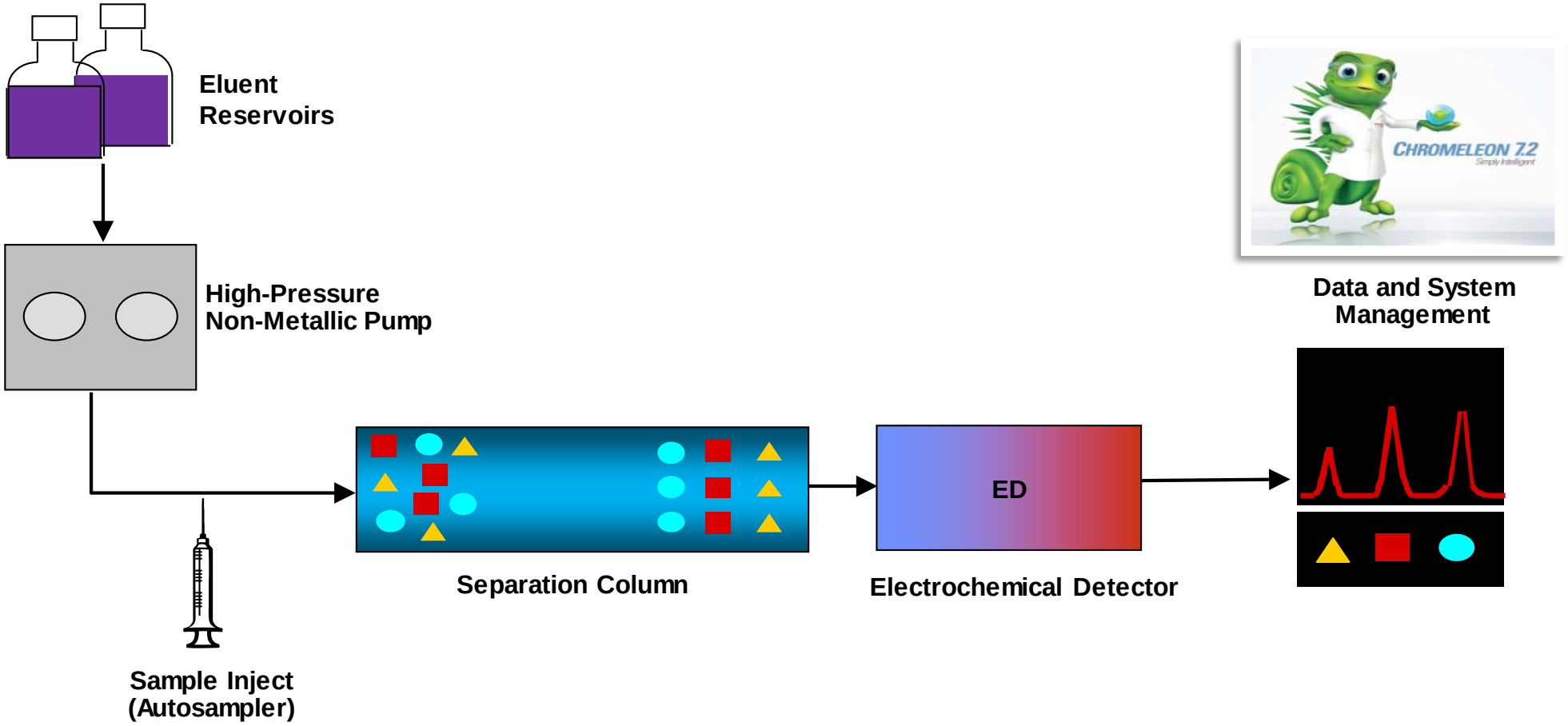
Basic HPAE-PAD Glycoprotein Sialic Acid Analysis



1. Hydrolyze under mildly acidic conditions or treat with a neuraminidase
2. Remove acid and dry the sample
3. Dissolve the dried the sample in DI water and inject or just inject for the enyzme digest
4. Separate on either a CarboPac PA20 column set or a Fast Sialic Acid CarboPac PA20 column and detect by PAD

Simple Workflow using Ion Chromatography Systems



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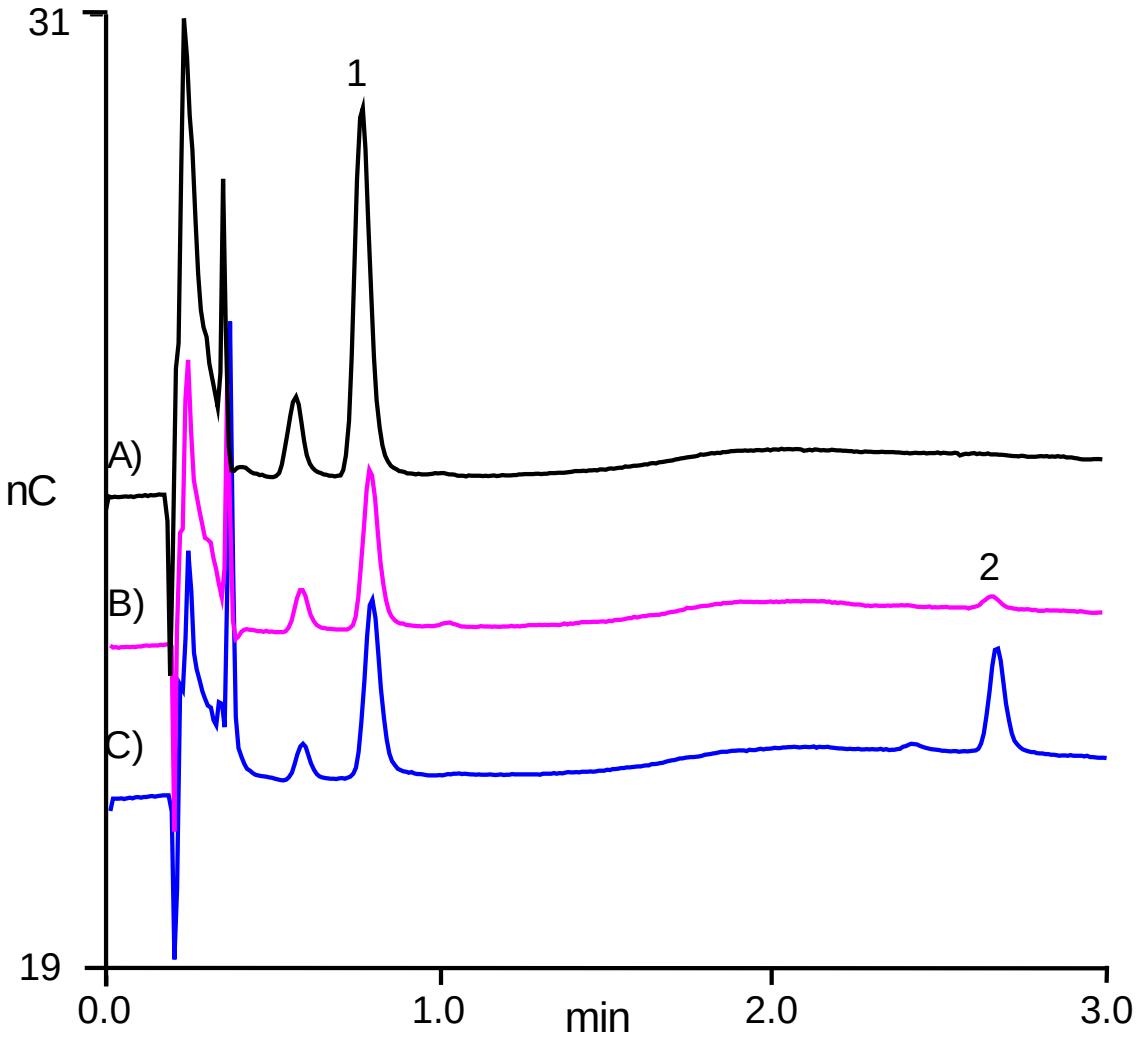
Separation of Sialic Acids



Column: Dionex CarboPac PA20 Fast Sialic Acid, 3 x 30 mm
Eluent: 70-300 mM acetate in 100 mM NaOH
Temperature: 30 °C
Flow Rate: 0.5 mL/min
Inj. Volume: 4.5 µL (full loop)
Detection: PAD

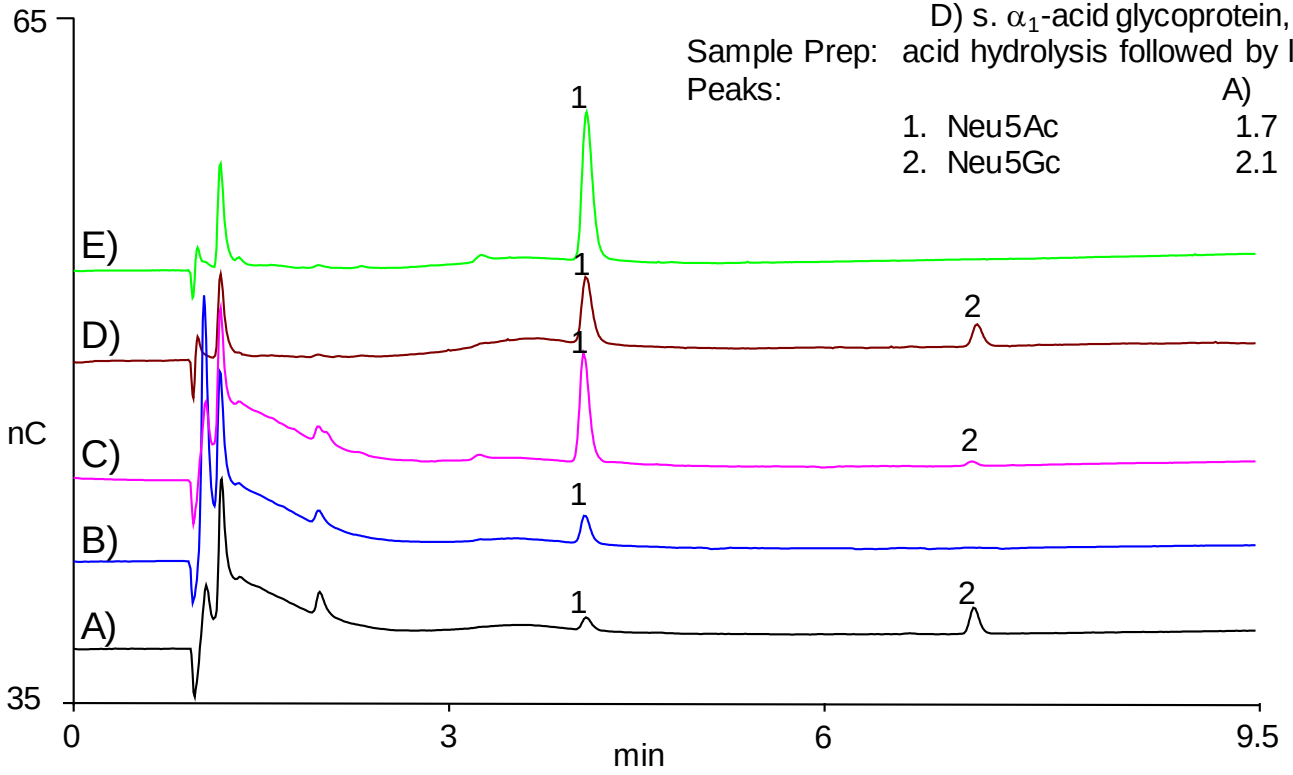
Samples: A) human α_1-acid glycoprotein, 23 ng protein
B) fetuin, 18 ng protein
C) s. α_1-acid glycoprotein, 7.9 ng protein

Peaks:		A)	B)	C)
1.	Neu5Ac	13	5.6	6.1 pmol
2.	Neu5Gc	----	0.20	1.2

Separation of Glycoprotein Acid Hydrolyzates

Column: Dionex CarboPac PA20 guard, 3 x 30 mm
Dionex CarboPac PA20, 3 x 150 mm
Eluent: 70-300 mM acetate in 100 mM NaOH from 0-7.5 min, 300 mM acetate in 100 mM NaOH from 7.5-9.0 min, 300-70 mM acetate from 9.0-9.5 min. 7 min of equilibration at 70 mM acetate in 100 mM NaOH
Temperature: 30 °C
Flow Rate: 0.5 mL/min
Inj. Volume: 10 µL
Detection: PAD, Au (Disposable)
Samples: A) b. apo-transferrin, B) h. transferrin, C) fetuin,
D) s. α_1-acid glycoprotein, E) h. α_1-acid glycoprotein
Sample Prep: acid hydrolysis followed by lyophilization and dissolution
Peaks:

	A)	B)	C)	D)	E)
1. Neu5Ac	1.7	4.4	18	15	37 pmol
2. Neu5Gc	2.1	ND	0.39	2.6	ND



A 10% signal offset has been applied.
ND = Not Detected

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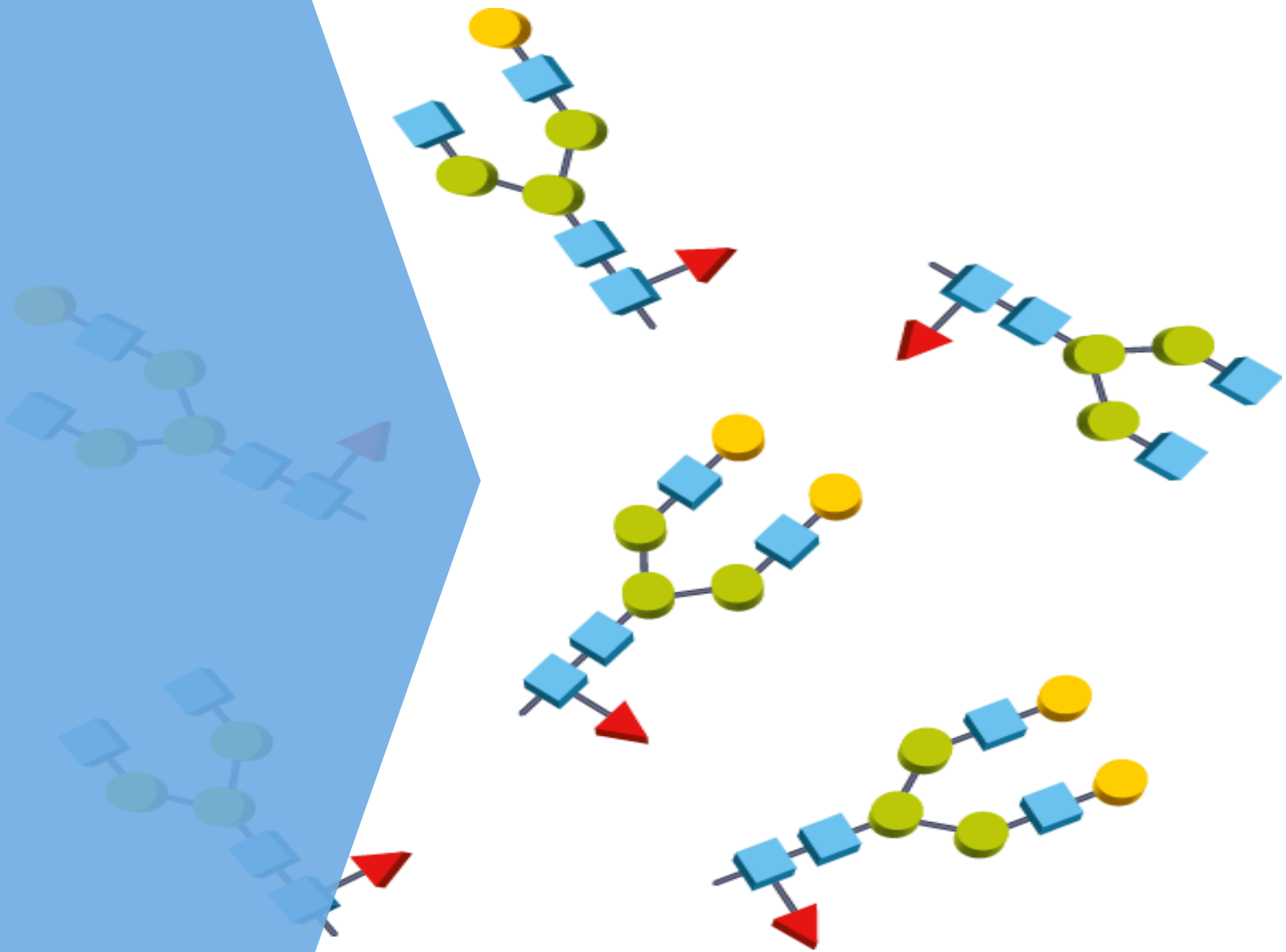
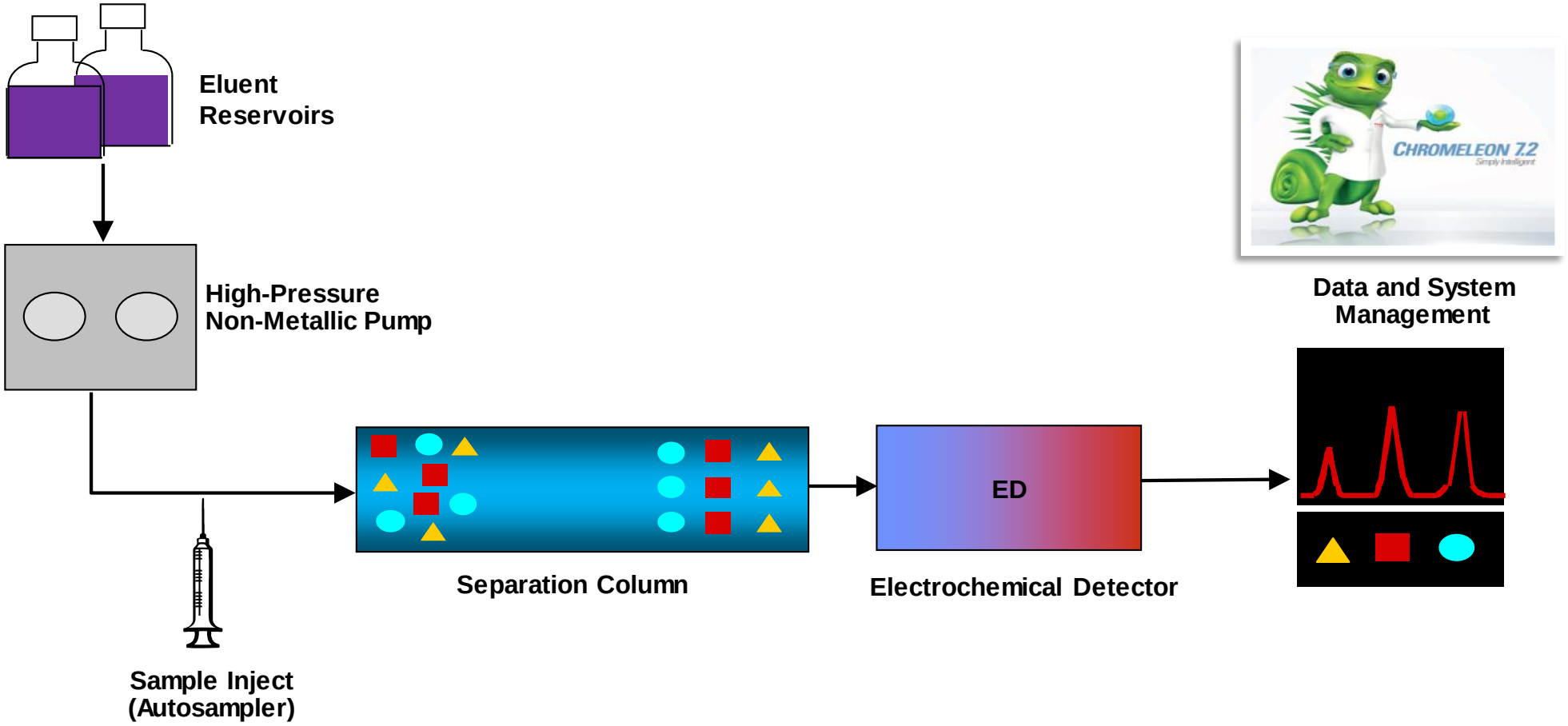
Unlabeled glycans

- O-linked & N-linked



Simple Workflow using Ion Chromatography Systems



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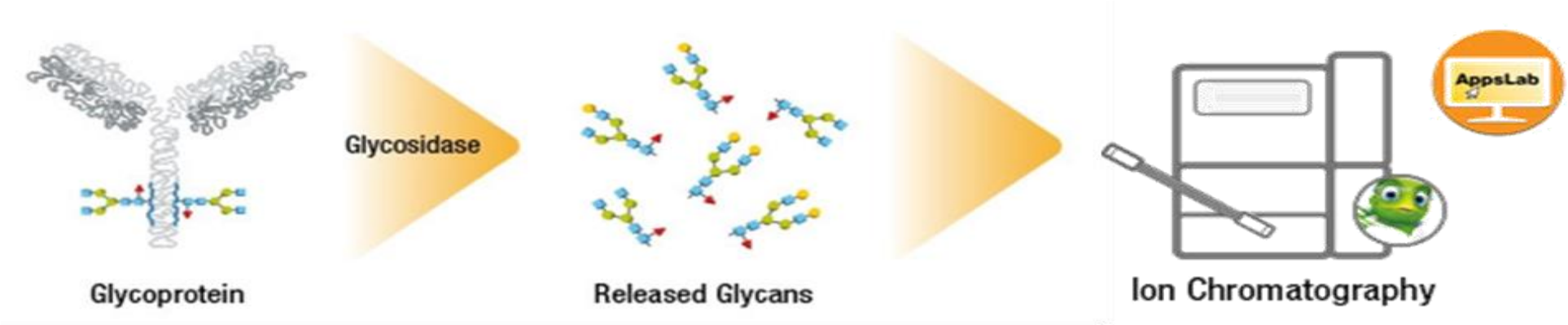
Unlabeled N-Glycan Analysis using HPAE-PAD



Benefits of Ion Chromatography

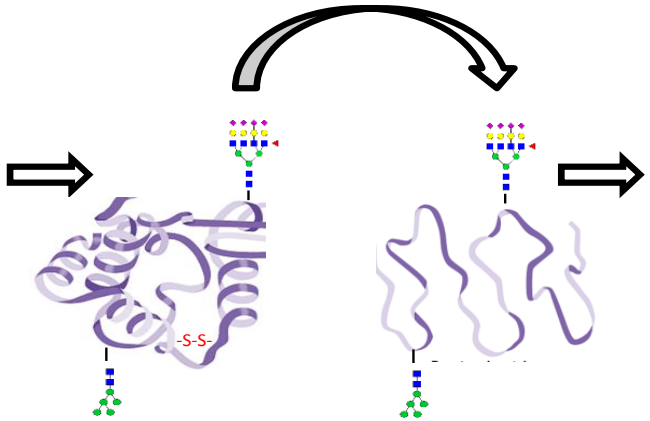
- Direct detection for profiling released glycans
- New, rapid workflow to release glycans from 200µg of glycoproteins with excellent reproducibility
- Sensitive separations based on charge, linkage, position, and fucosylation

Rapid workflow for native glycan preparation for analysis

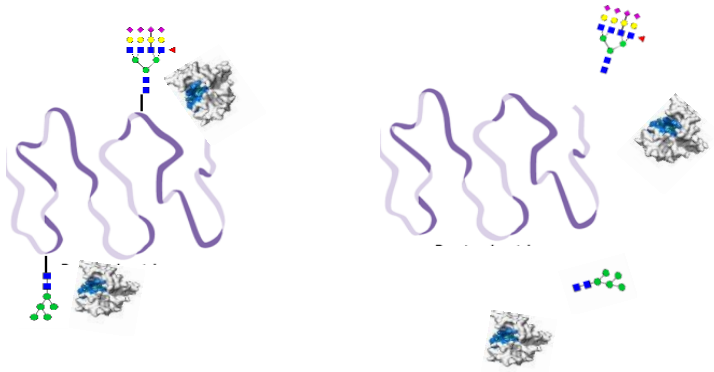
Digestion

- 200 µg glycoprotein
- HEPES buffer at pH 7.9
- TCEP
- Proprietary detergent

Denaturation, reduction at 90 °C for 5 min

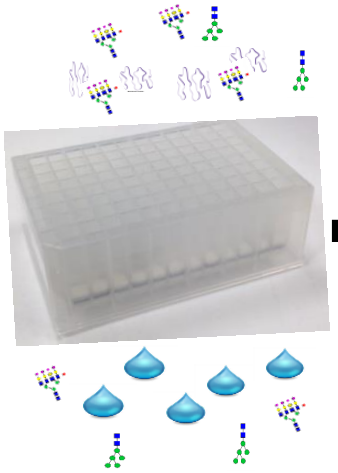


Digestion with PNGase F at 50 °C for 15 min



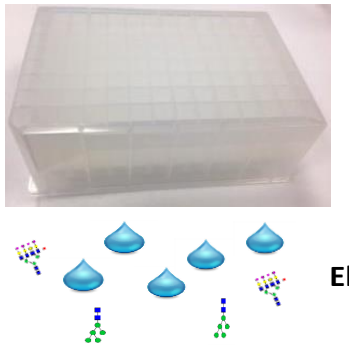
Clean Up

Protein removal on Hypersep Hypercarb Plate



Elution in 50 % MeCN and 0.1 TFA

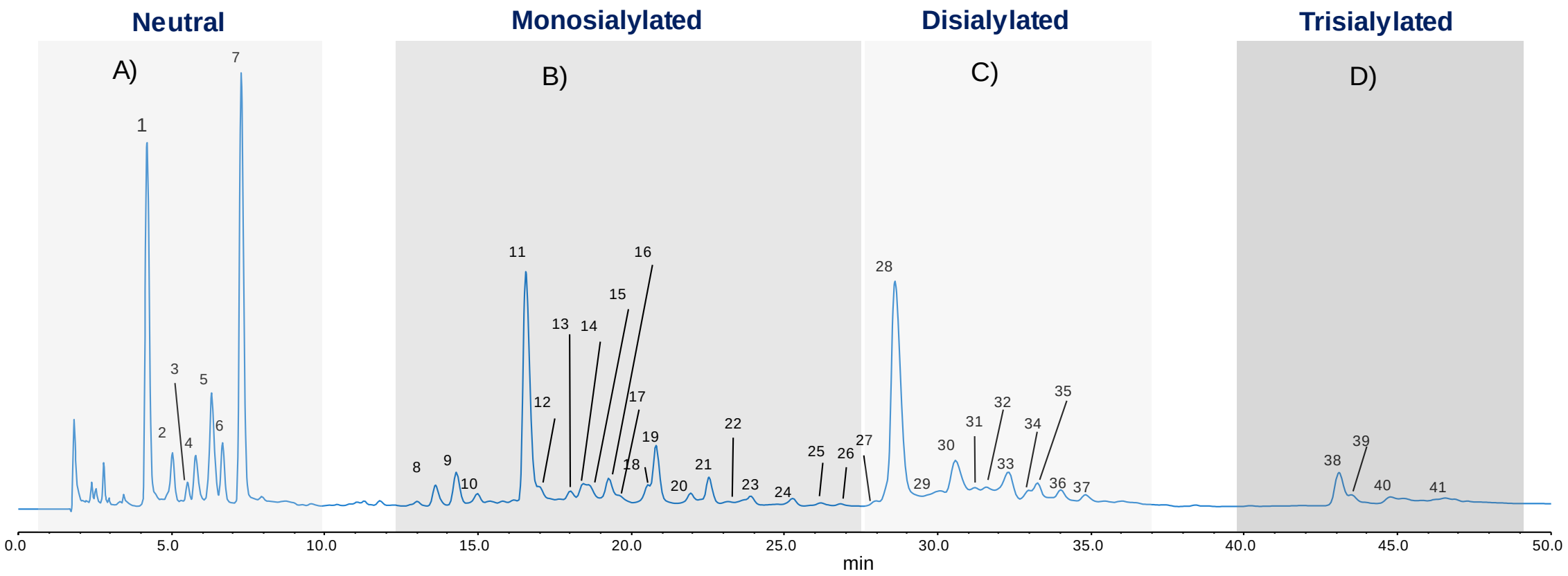
Elute MeCN concentration is adjusted to 90 % and load onto Hypersep Diol Plate



Elution of glycans in water

Analysis of native glycans using IC S-5000+ coupled to Q Exactive Mass Spectrometer

Sensitive HPAE-PAD Analysis Based on Charge



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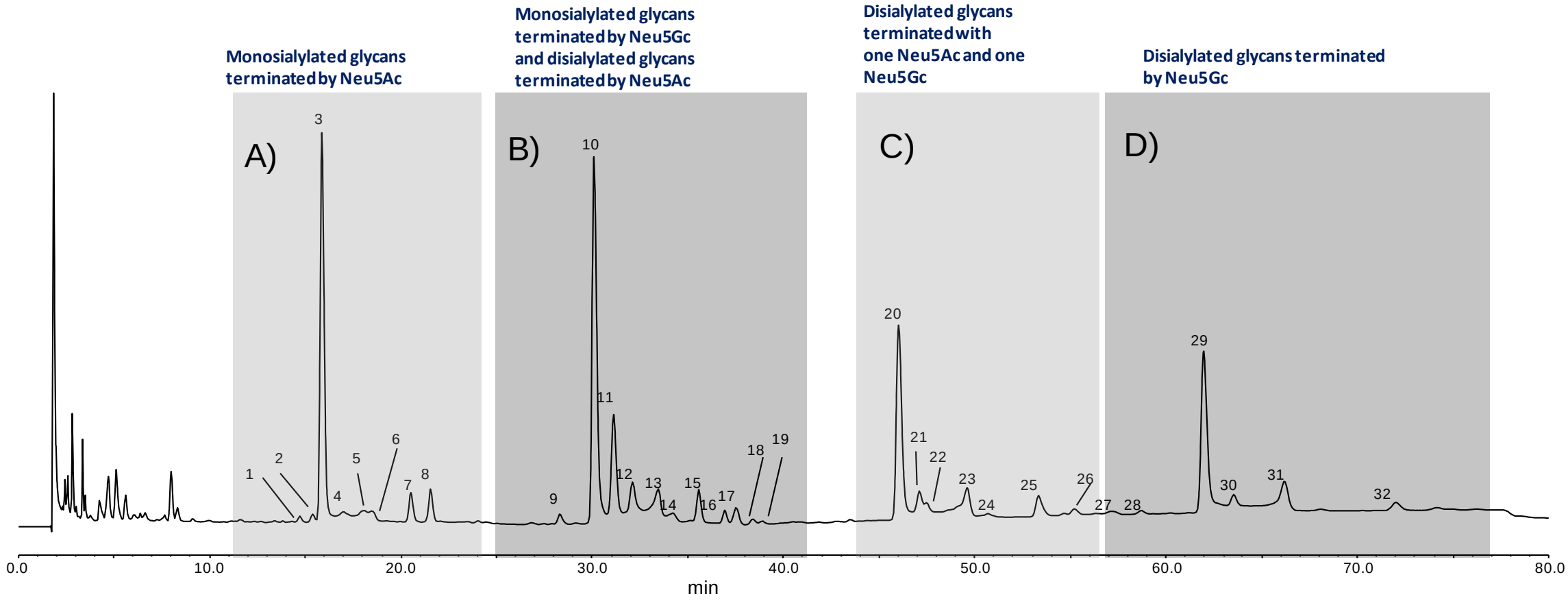
Sensitive HPAE-PAD Analysis Based on Charge and Linkage



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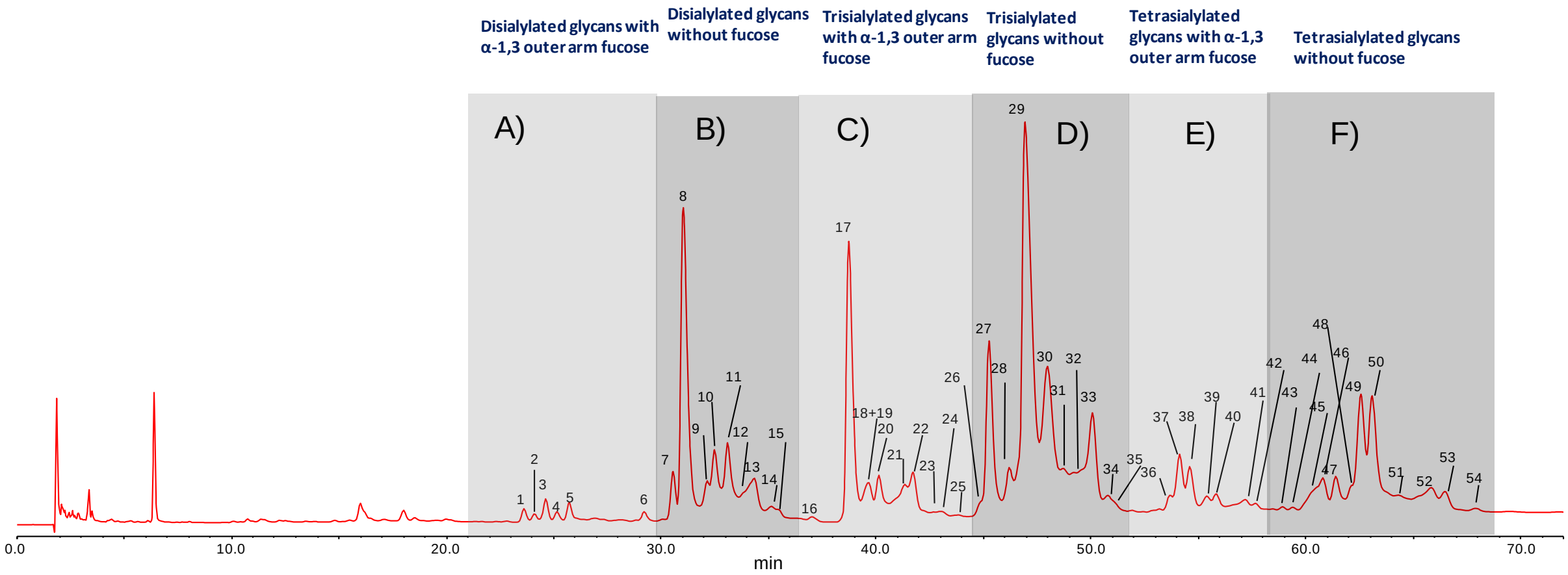
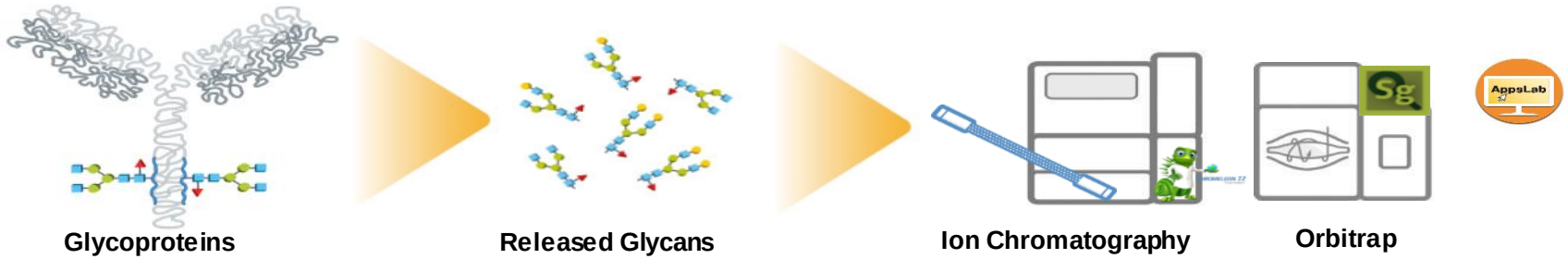
Sensitive separations based on charge, position, and fucosylation



ThermoFisher SCIENTIFIC

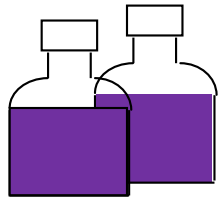
Unlabeled N-Glycan Analysis using HPAE-PAD/MS



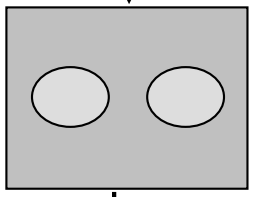
Benefits of Ion Chromatography

- HPAE-PAD/MS is an excellent tool to profile and annotate complex mixture of native glycans released from different glycoproteins
- Easy interface with MS provides in-depth characterization of all released glycans including low abundant N-linked glycans
- Excellent reproducibility of peak area distribution of glycans released from denatured mAb.

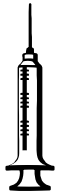
Unlabeled N-Glycan Analysis using HPAE-PAD/MS



Eluent
Reservoirs



High-Pressure
Non-Metallic Pump

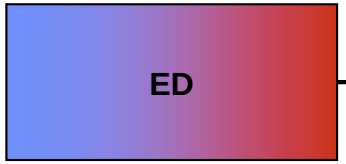


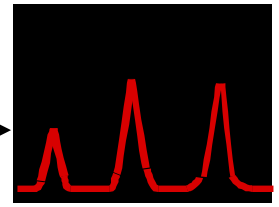
Sample Inject
(Autosampler)

Separation Column

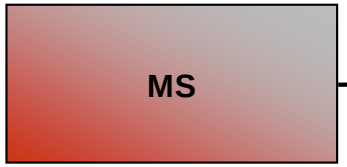
Data and System
Management

Electrochemical Detector

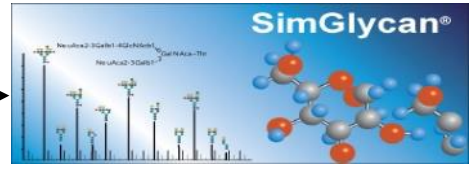


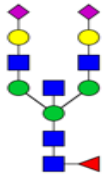


Structural information by
comparing with known glycan standards



QE Mass Spectrometer





Structural Identification

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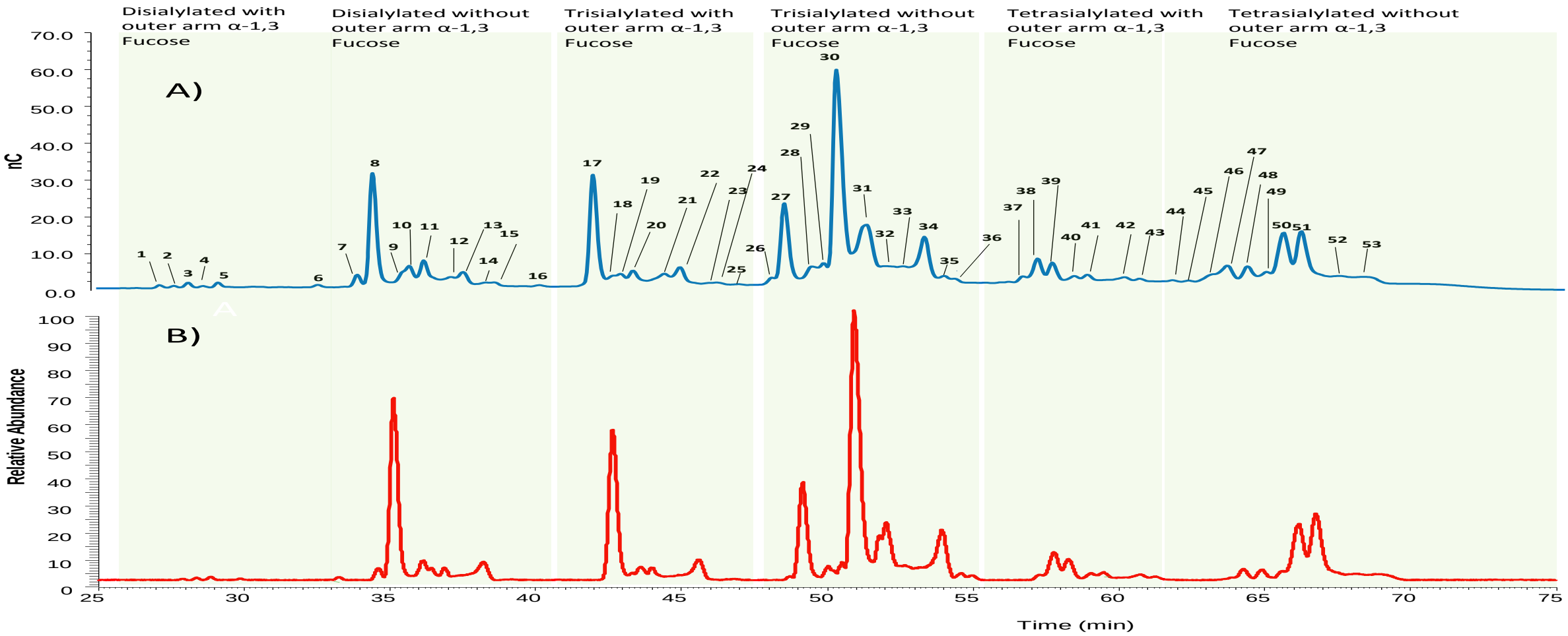
Label-free Analysis of N-linked Glycans by HPAE-PAD-MS

PAD (A) and MS base peak (B) chromatograms of hAGP glycans



HPAE-PAD is able to separate highly complex glycan mixtures based on charge, linkage, & fucose

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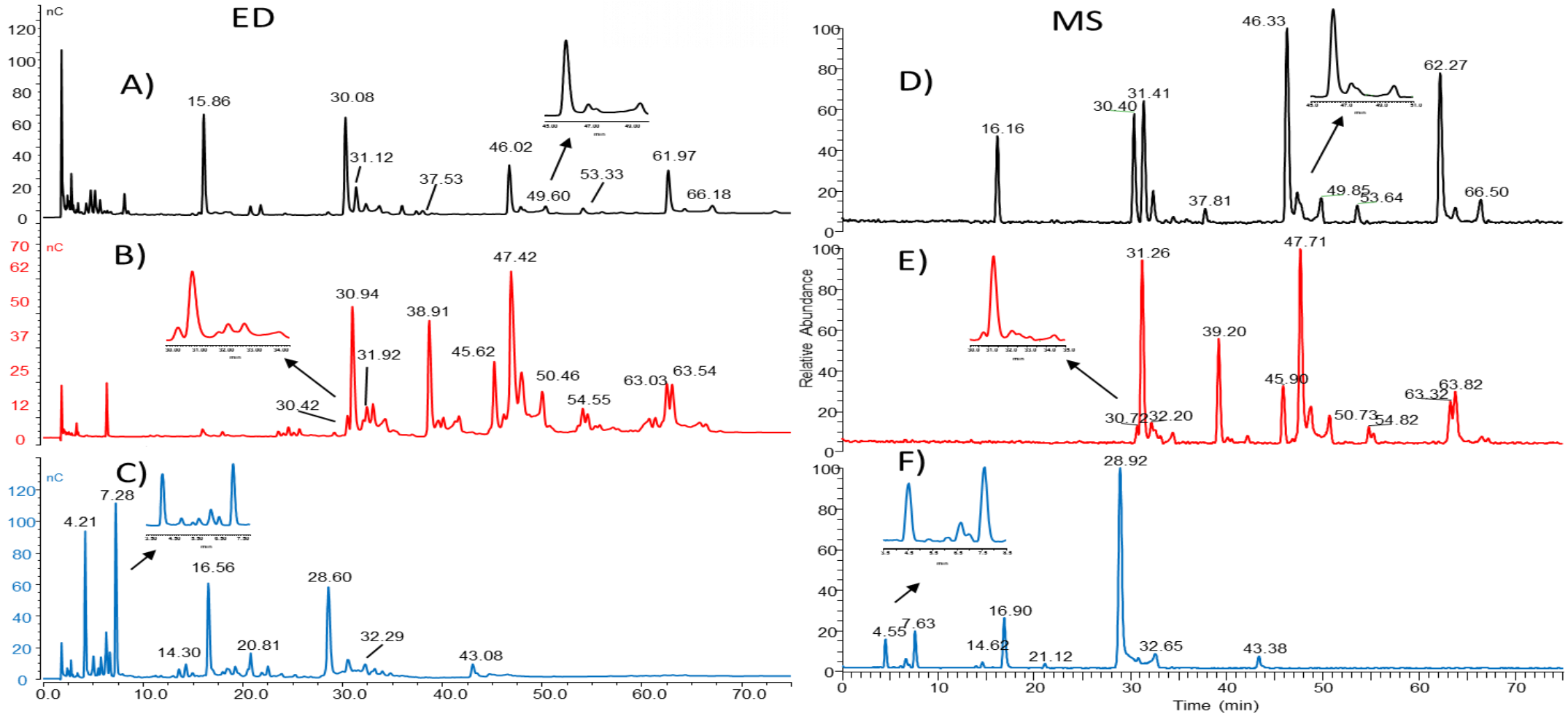
Label-free Analysis of N-linked Glycans by HPAE-PAD-MS



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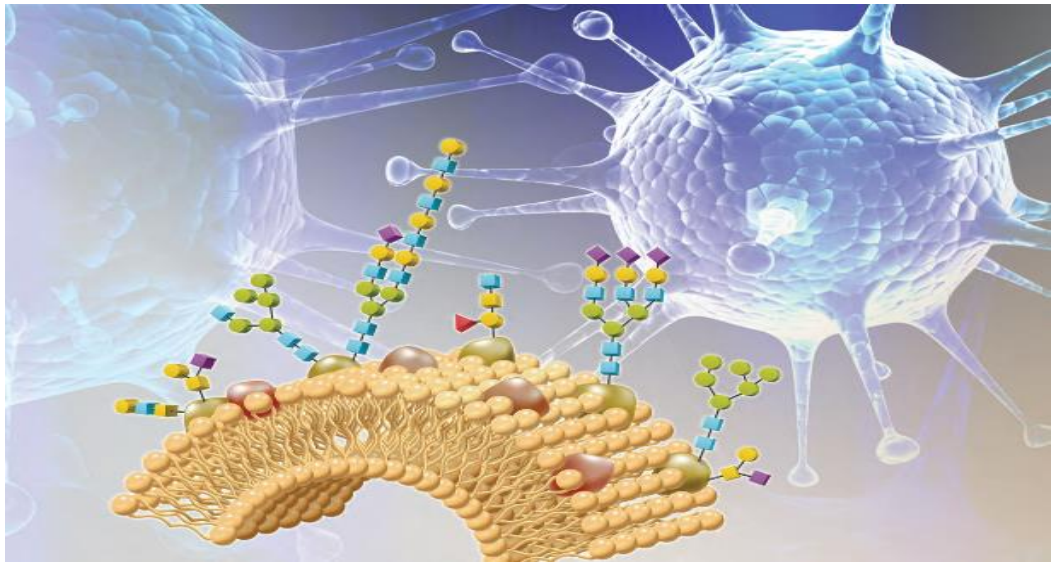
IC for BioPharma Applications



Sensitive carbohydrate analysis of recombinant glycoproteins, biosimilars, antibiotics, and glycoconjugate vaccines

Analytes ranging –

- Carbohydrate (mono-, di-, polysaccharide) analysis
- Sialic acid analysis
- Asn (N-)-linked oligosaccharides
- Ser/Thr (O-)-linked oligosaccharide
- Sugar phosphates (mannose-6-phosphate)
- Sugar alcohols

Ion Chromatography for Pharmaceutical Analysis

Applications

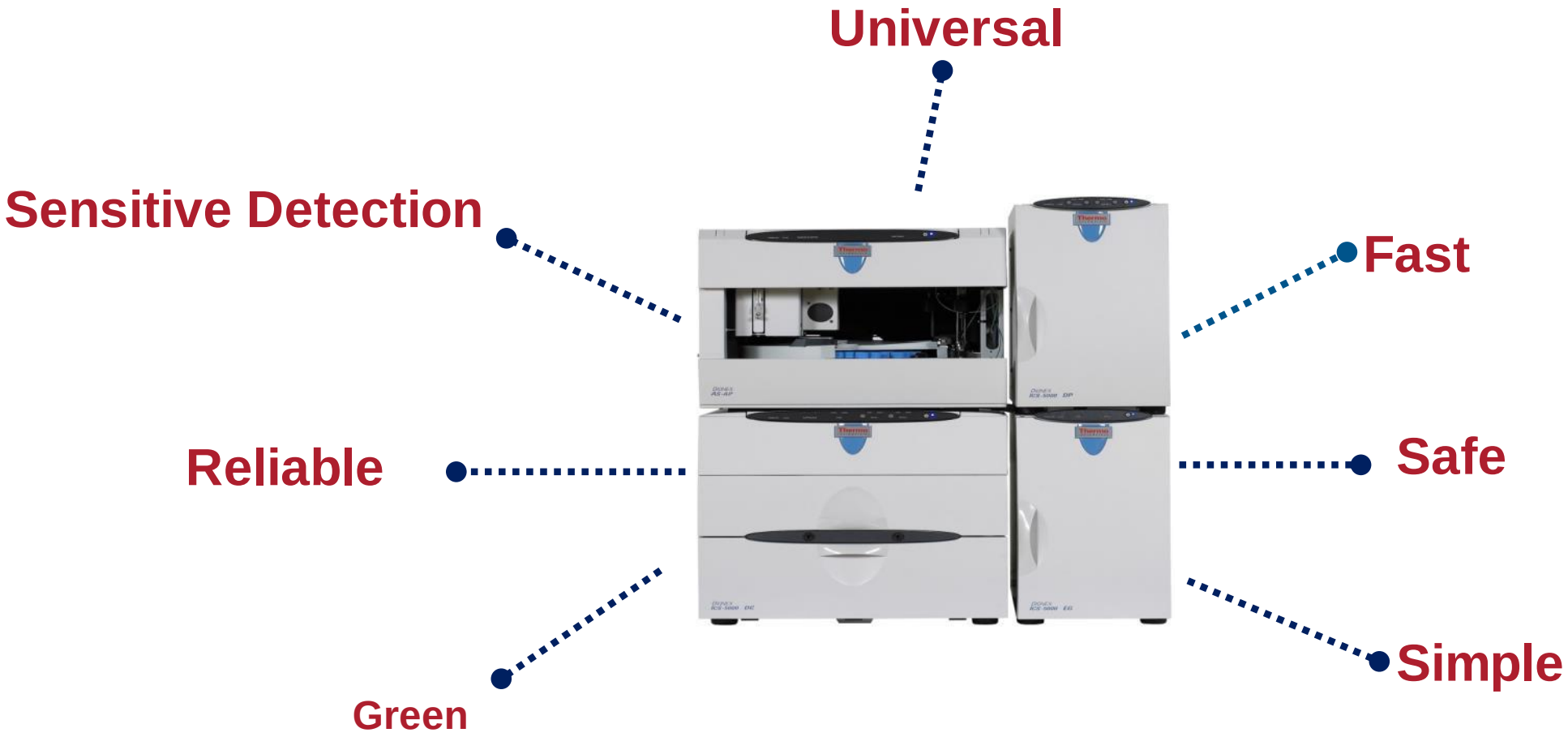
- Counterion determinations
- Impurity analysis
- Drug substance assay
- Measuring excipients in drug products

Benefits of IC

- Speed
- Reproducibility
- Accuracy
- Safety
- Cost effectiveness

One solution for all drug development stages

Ion Chromatography – Making It Easier To Get Results



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Conclusions

- HPAE-PAD is a fast carbohydrate analysis method
  - Directly quantify non-derivatized carbohydrates with high sensitivity and selectivity
- HPAE-PAD is the one system for monosaccharides, sialic and other sugar acids, sugar phosphates, sugar alcohols, sulfate sugars, aminoglycoside antibiotics, oligosaccharides (charged and neutral), and small polysaccharides
- With innovations, such as HPIC and CarboPac columns, carbohydrates are analyzed in as little as 5 minutes

Questions?

- **THANK YOU**