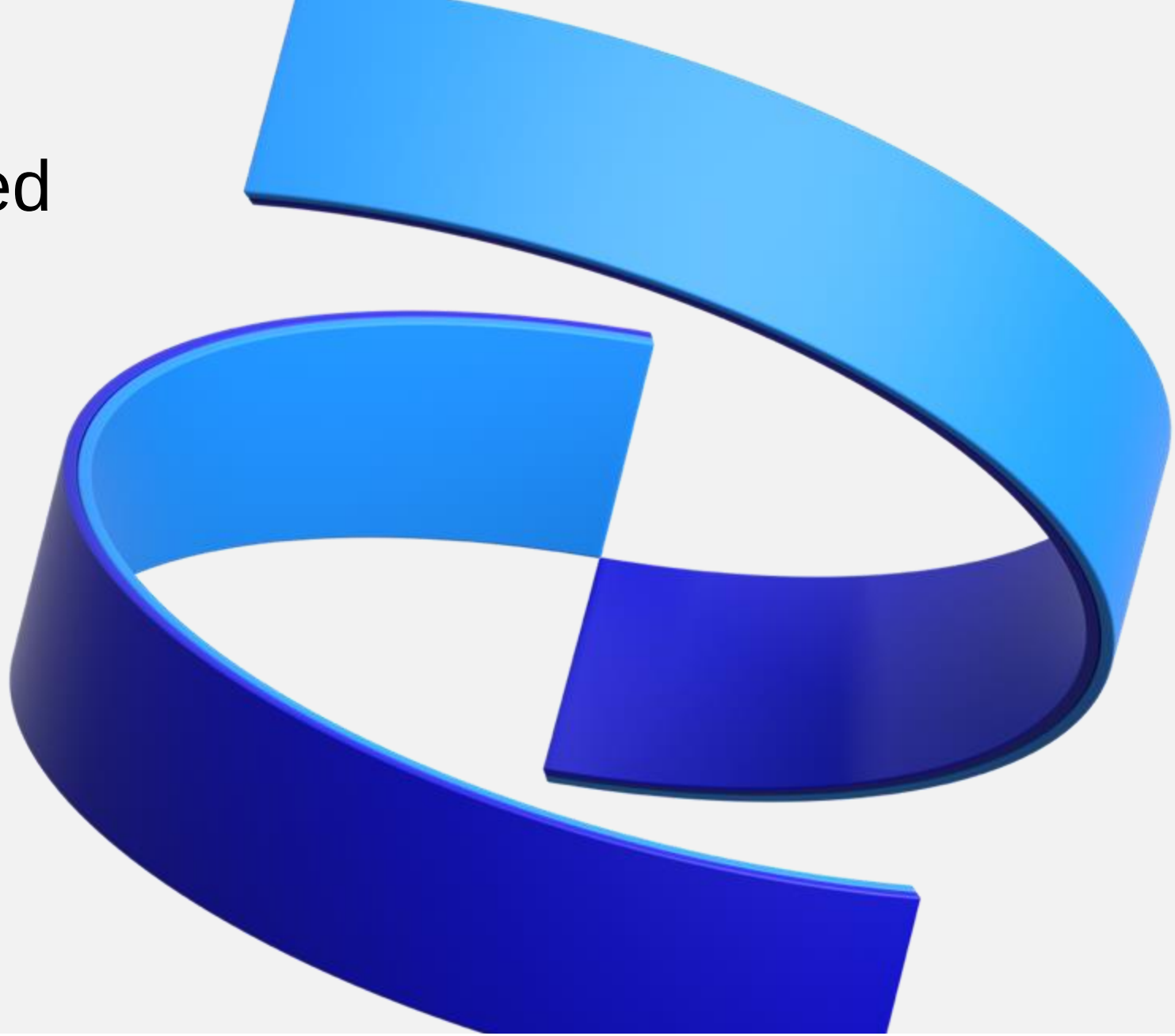
# Identification of N-Linked Glycans from Bovine α-Thrombin Using a Combination of LC-MS Techniques



Dennis Gessmann (presenter)
with Andrew Saati & Naomi Biok

14 October 2022

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer’s Hospital Business Unit (HBU) and Thrombin-JMI®

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer’s Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

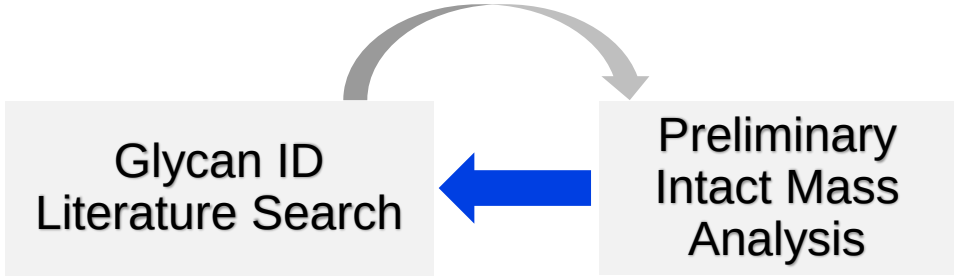
***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***

Preliminary Intact Mass Analysis

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

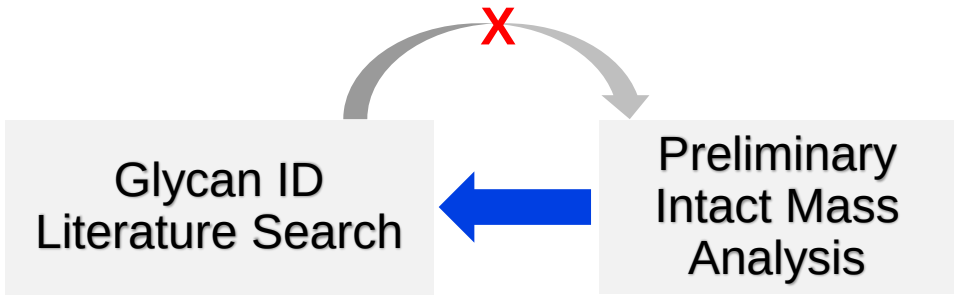
***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***



# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

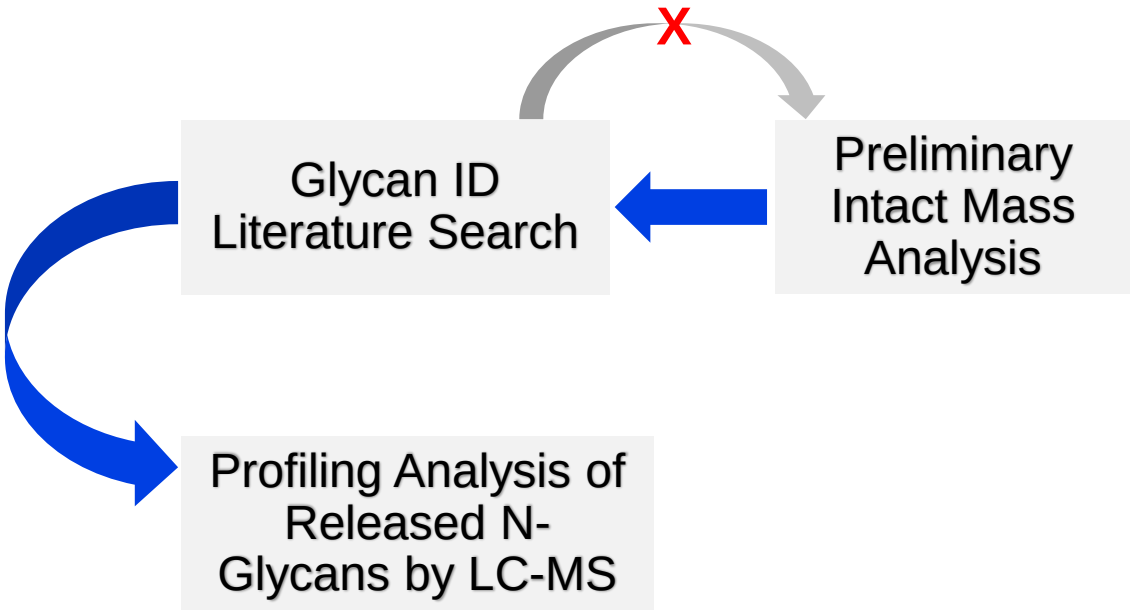
***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***



# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

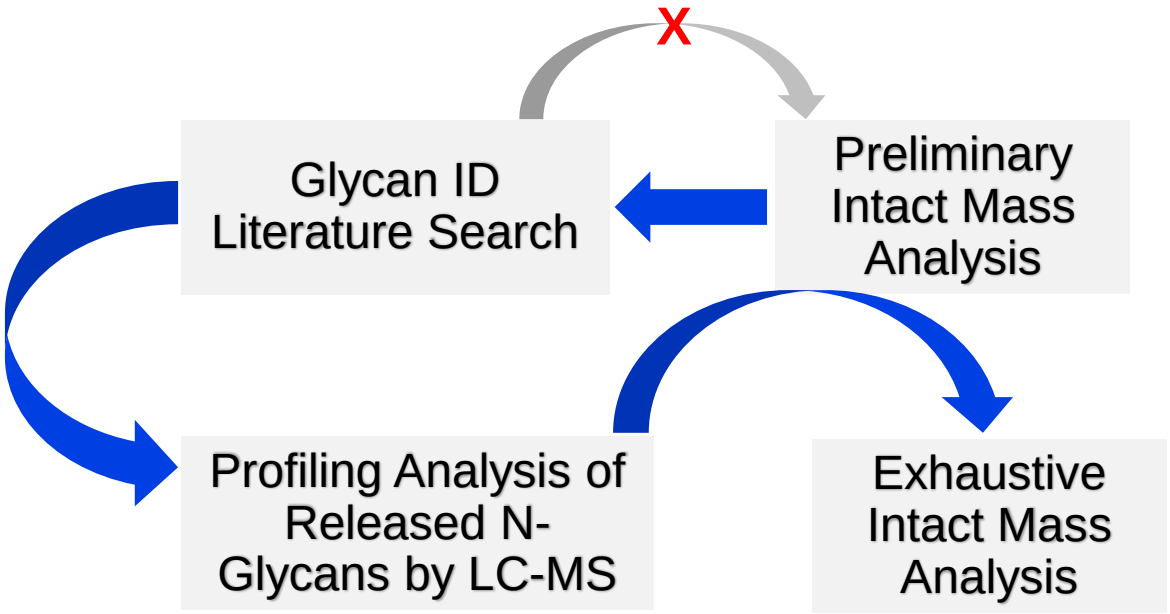
***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***



# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

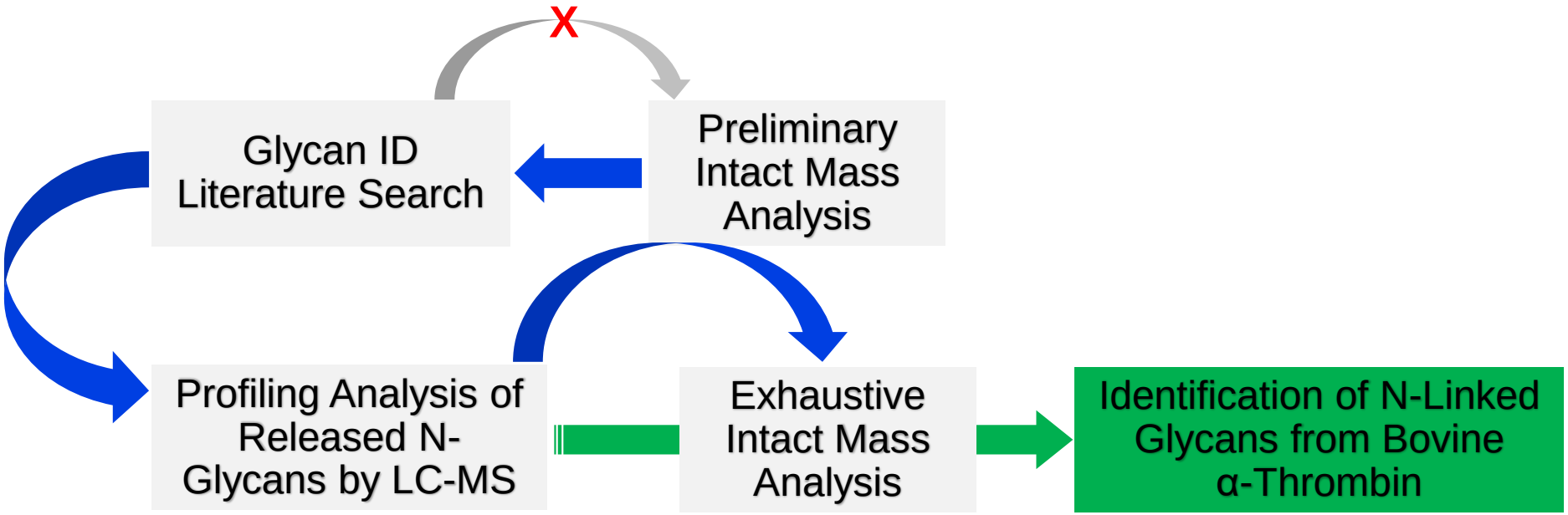
***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***



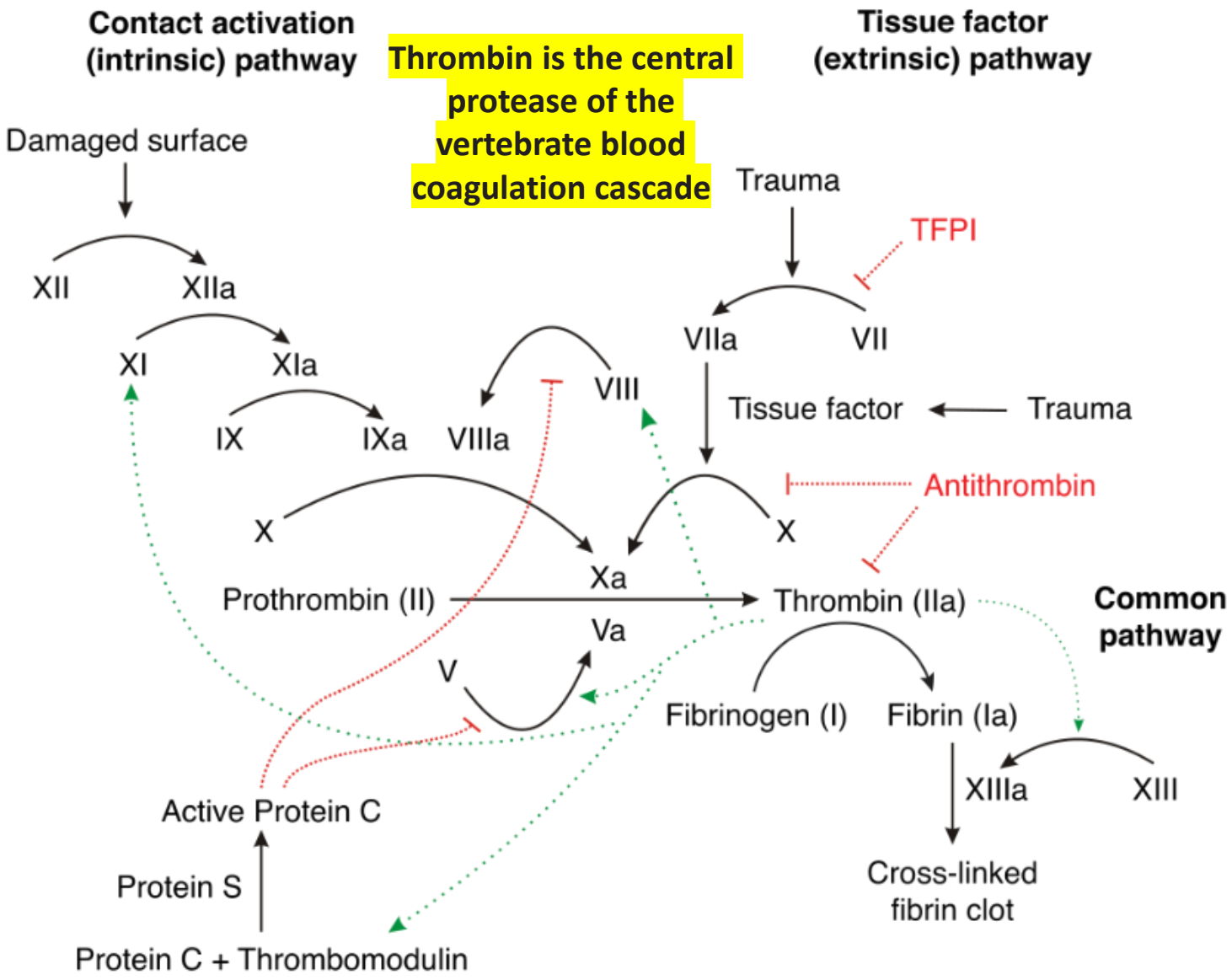
# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***



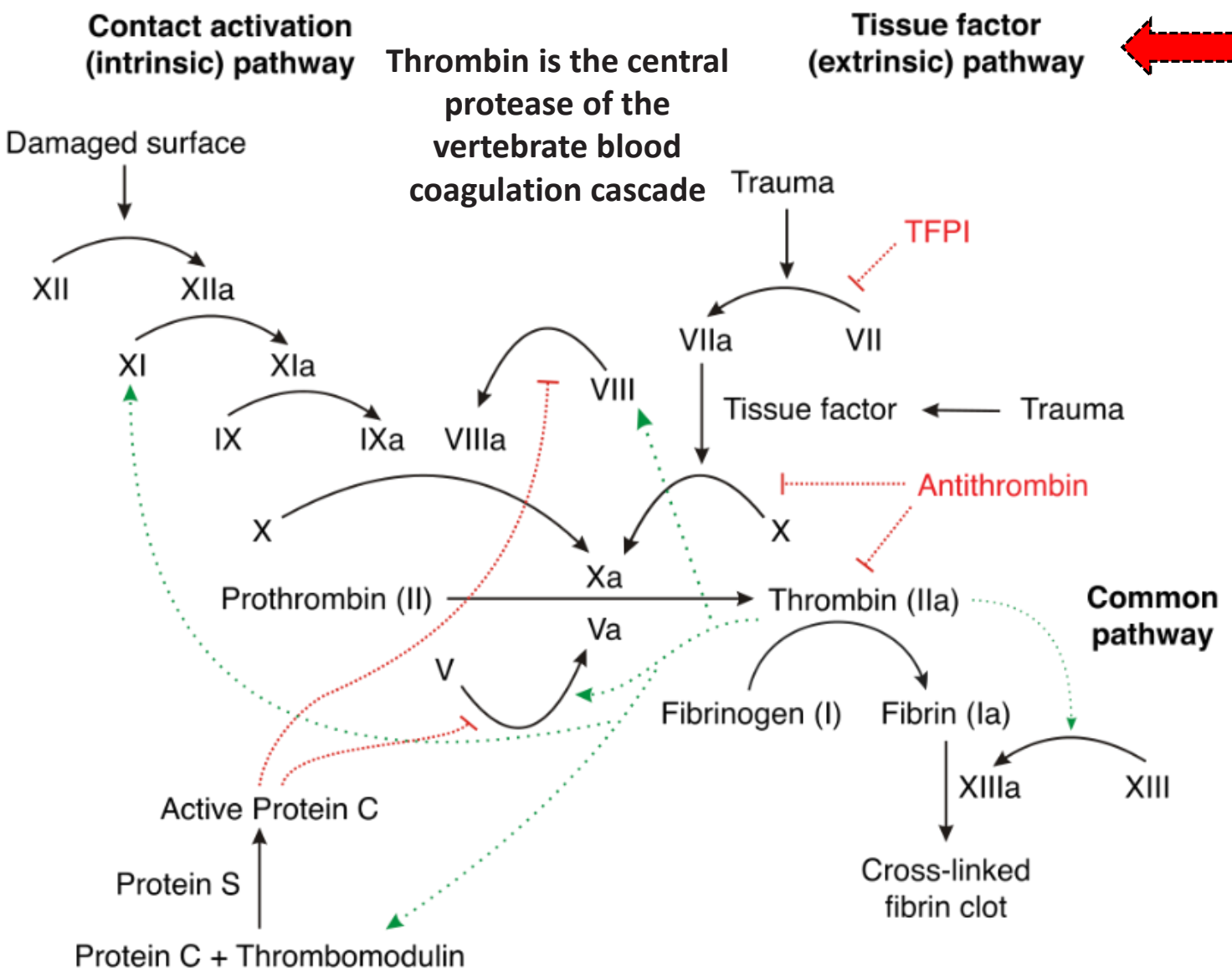
# Biological Activity of Thrombin






Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

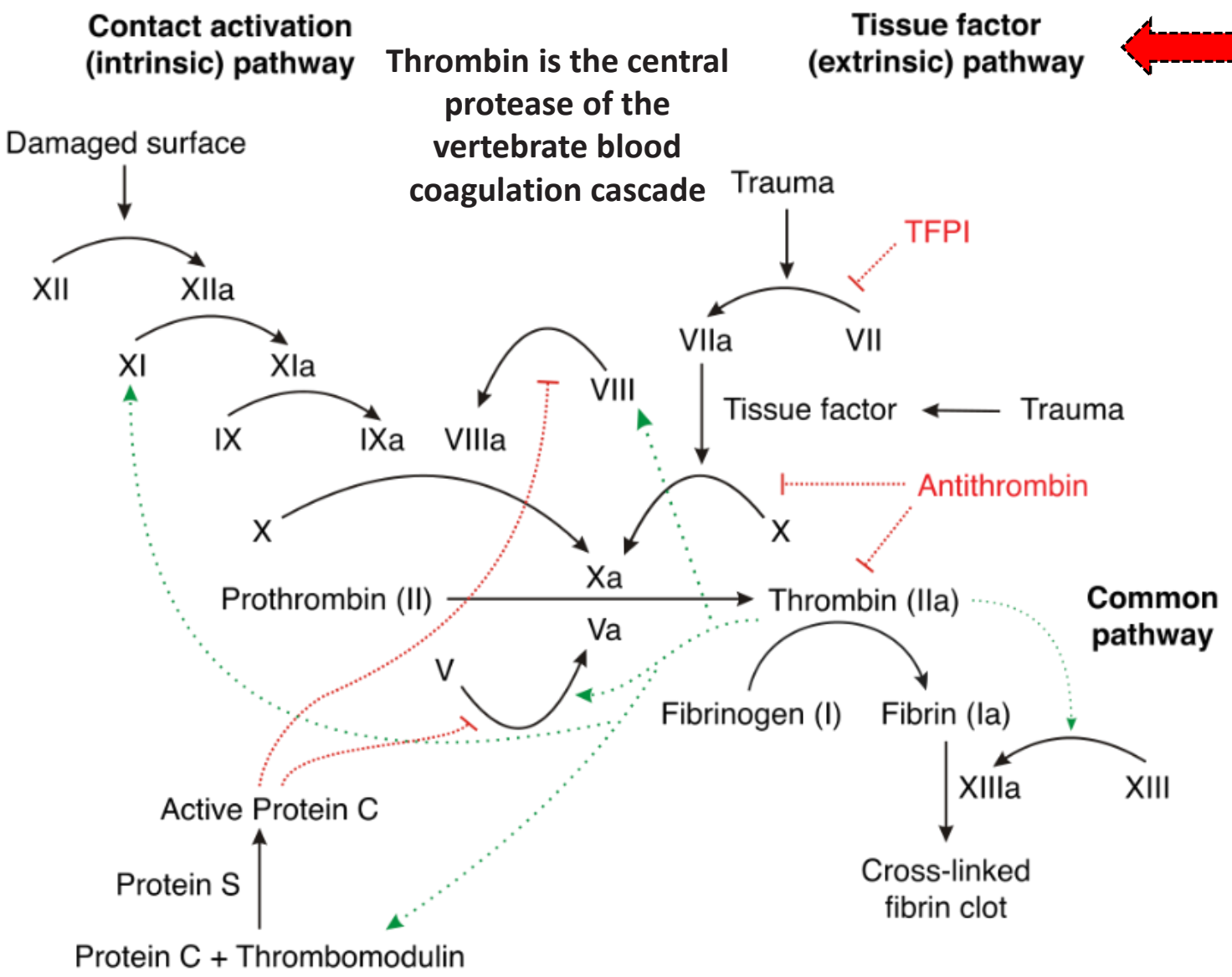
# Biological Activity of Thrombin






Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

# Biological Activity of Thrombin




Activated by External Trauma

e.g. exposure of TF to circulating blood and binding to factor VII



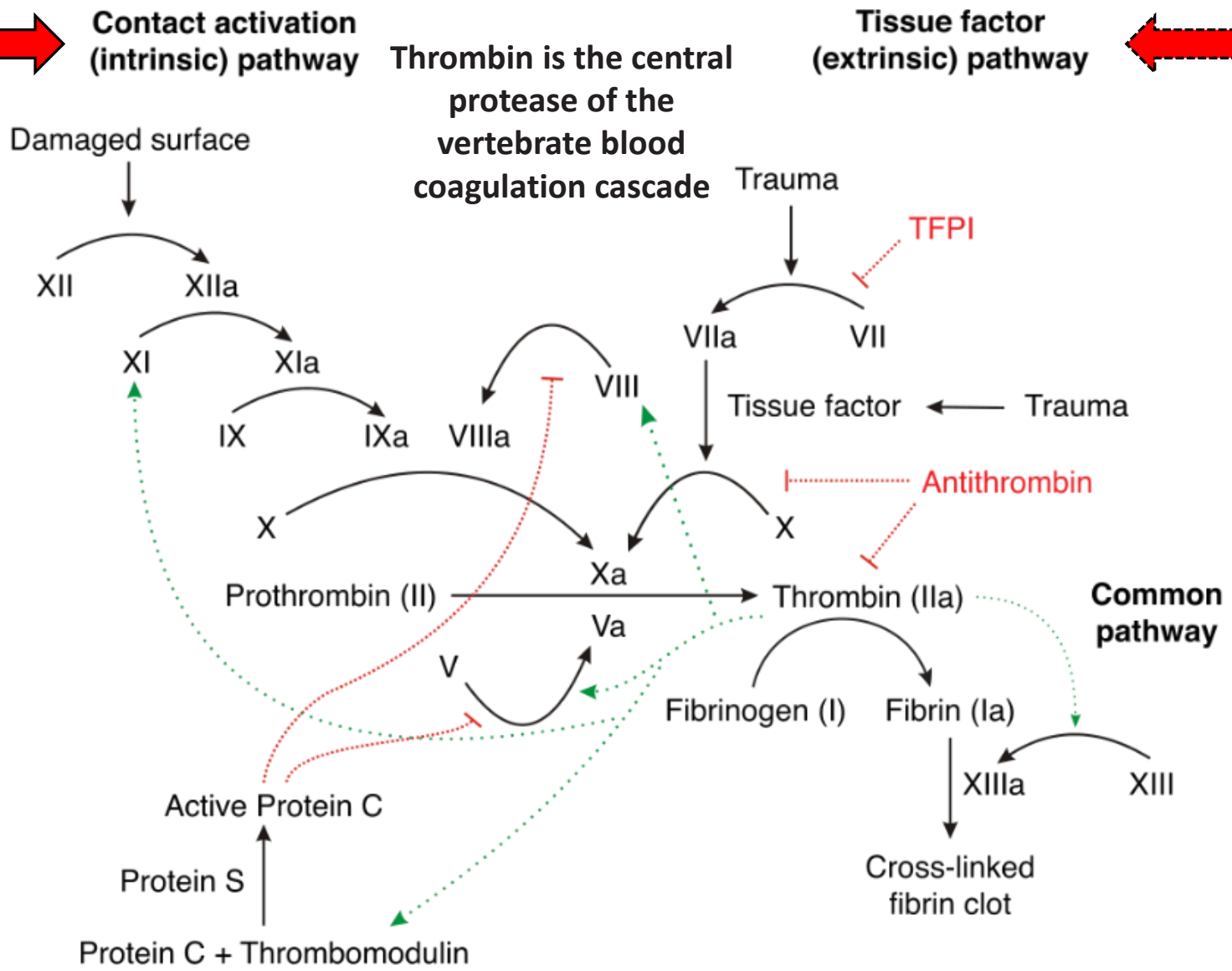
Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

# Biological Activity of Thrombin

Activated by Internal Trauma

Activated by External Trauma

e.g. exposure of TF to circulating blood and binding to factor VII






Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

# Biological Activity of Thrombin

Activated by Internal Trauma

e.g., binding (contact) of collagen to factor XII

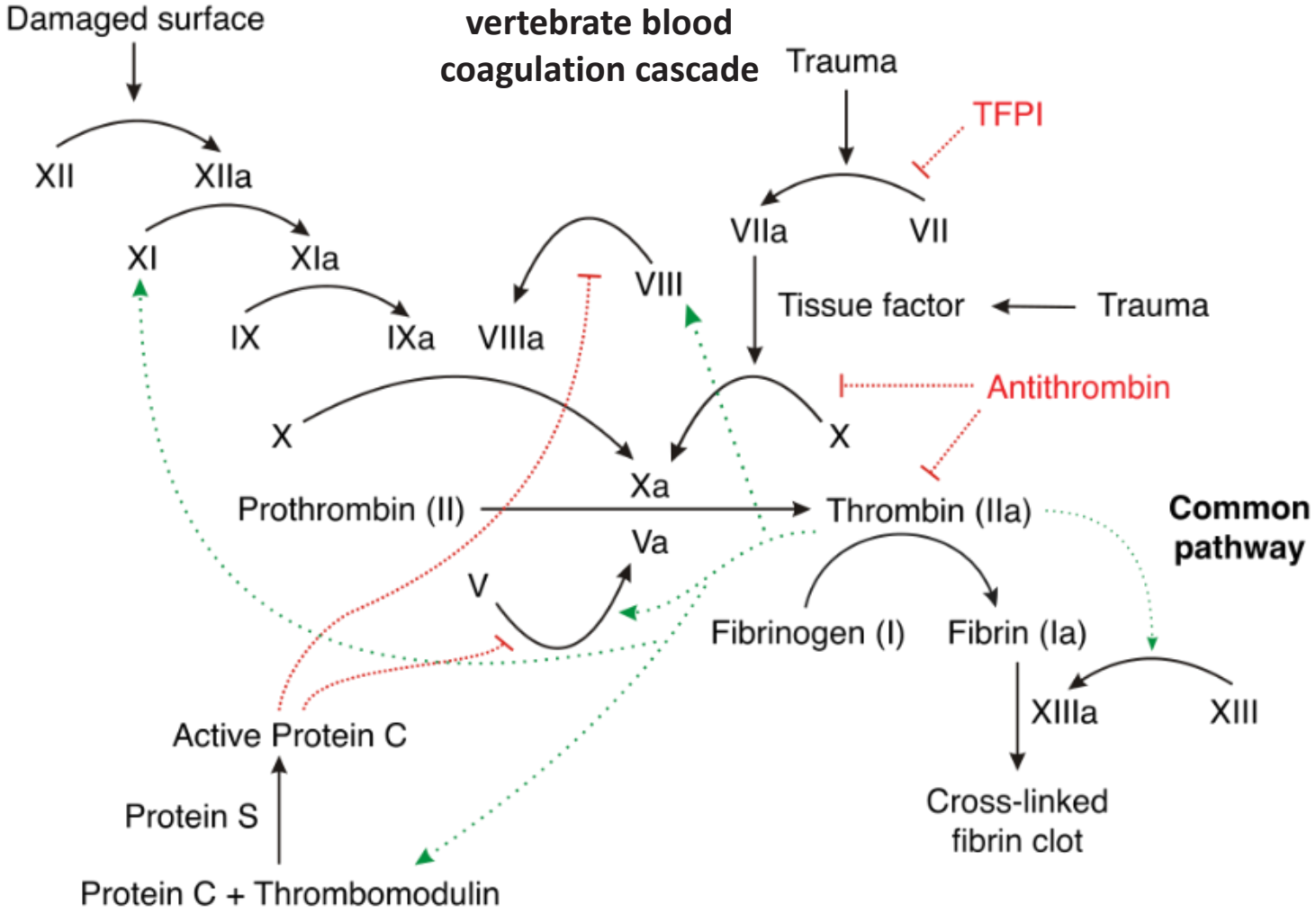
**Contact activation (intrinsic) pathway**

**Thrombin is the central protease of the vertebrate blood coagulation cascade**

**Tissue factor (extrinsic) pathway**

Activated by External Trauma

e.g. exposure of TF to circulating blood and binding to factor VII






Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

# Biological Activity of Thrombin

Activated by Internal Trauma

e.g., binding (contact) of collagen to factor XII

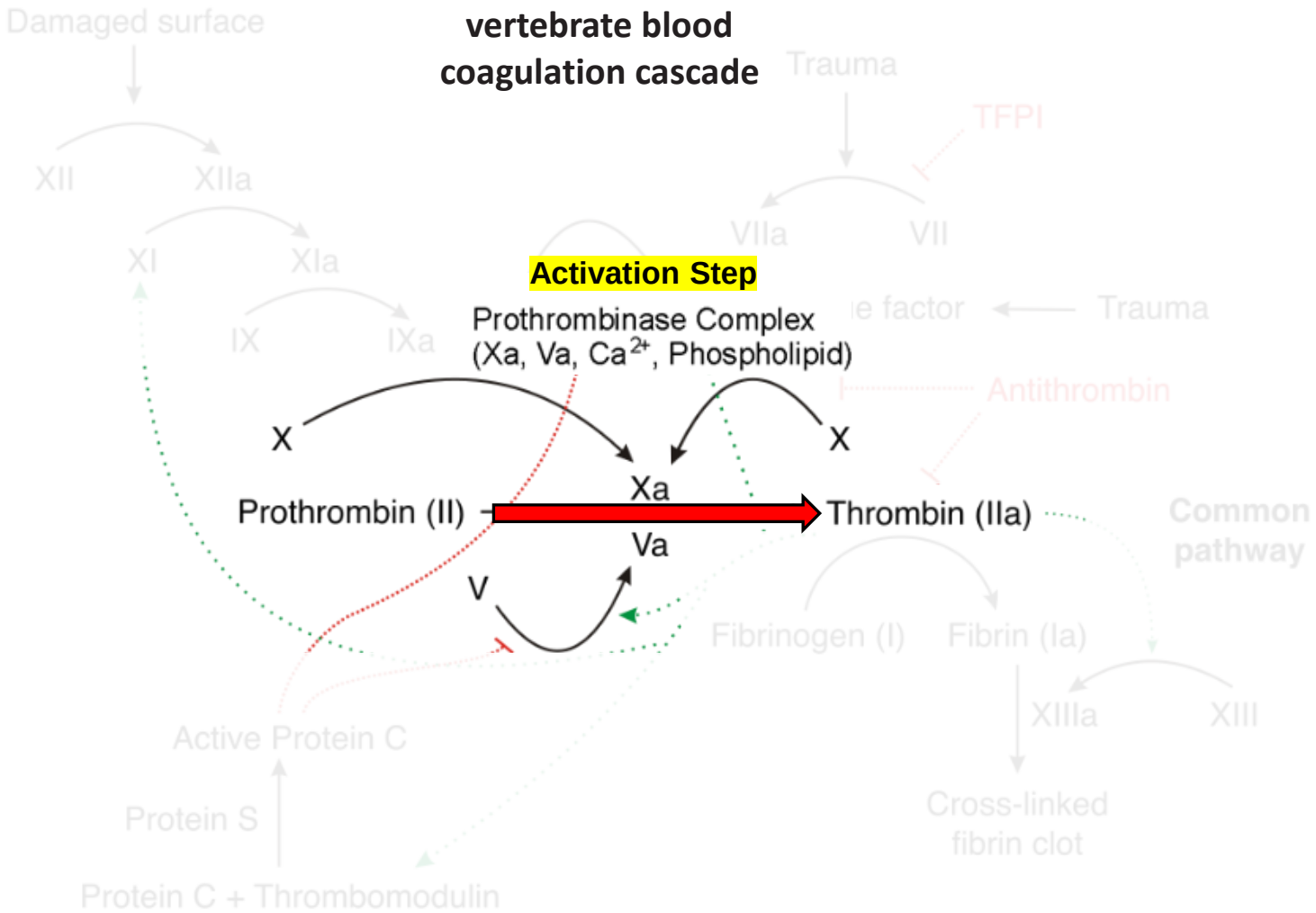
**Contact activation (intrinsic) pathway**

**Thrombin is the central protease of the vertebrate blood coagulation cascade**

**Tissue factor (extrinsic) pathway**

Activated by External Trauma

e.g. exposure of TF to circulating blood and binding to factor VII






Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

# Biological Activity of Thrombin

Activated by Internal Trauma

e.g., binding (contact) of collagen to factor XII

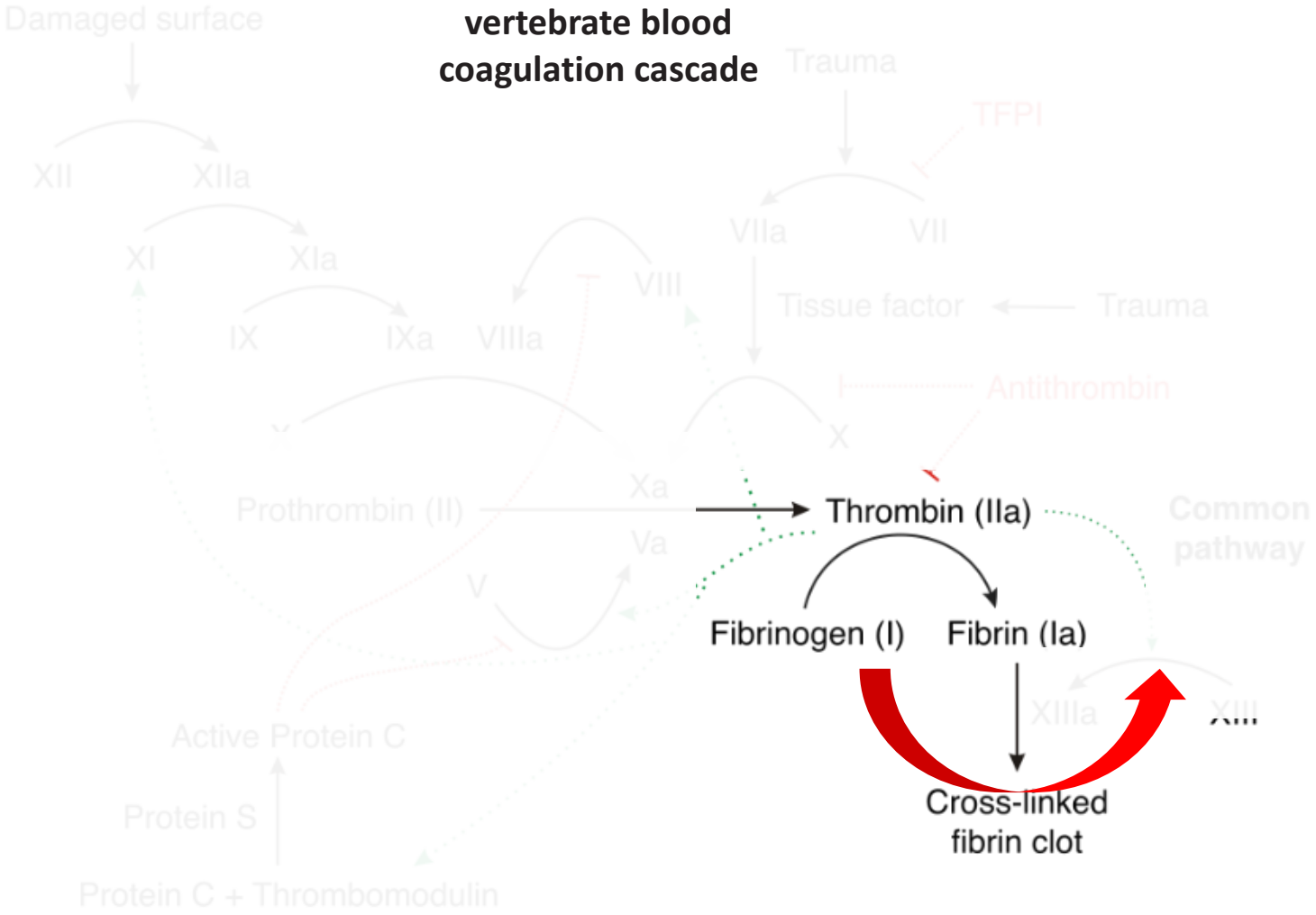
**Contact activation (intrinsic) pathway**

**Thrombin is the central protease of the vertebrate blood coagulation cascade**

**Tissue factor (extrinsic) pathway**

Activated by External Trauma

e.g. exposure of TF to circulating blood and binding to factor VII






Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

# Biological Activity of Thrombin

Activated by Internal Trauma

e.g., binding (contact) of collagen to factor XII

**Contact activation (intrinsic) pathway**

**Thrombin is the central protease of the vertebrate blood coagulation cascade**

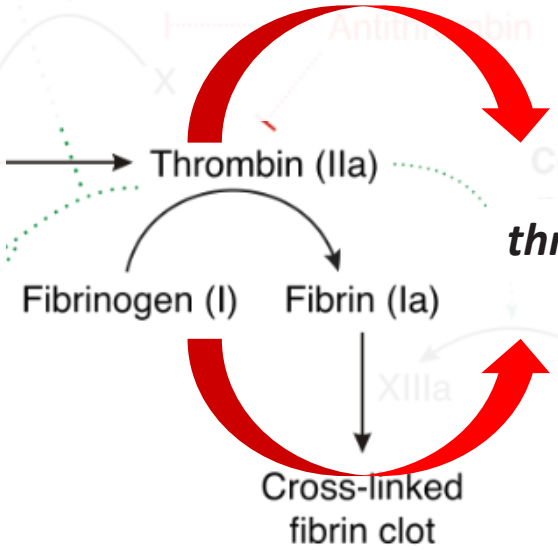
**Hemostasis, stop of bleeding, is achieved**

**Tissue factor (extrinsic) pathway**

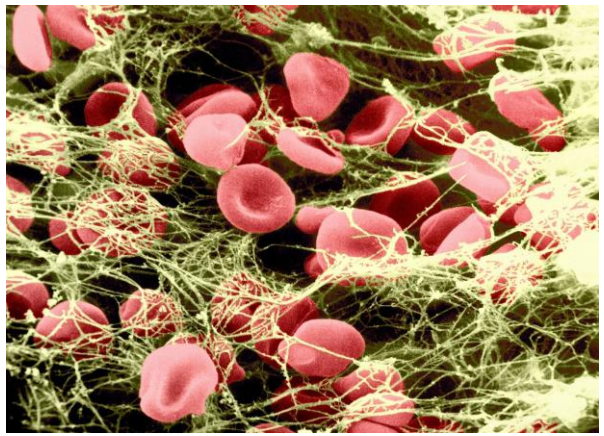
Activated by External Trauma

e.g. exposure of TF to circulating blood and binding to factor VII

**Platelet Activation**




***thrombos*, greek for clot**

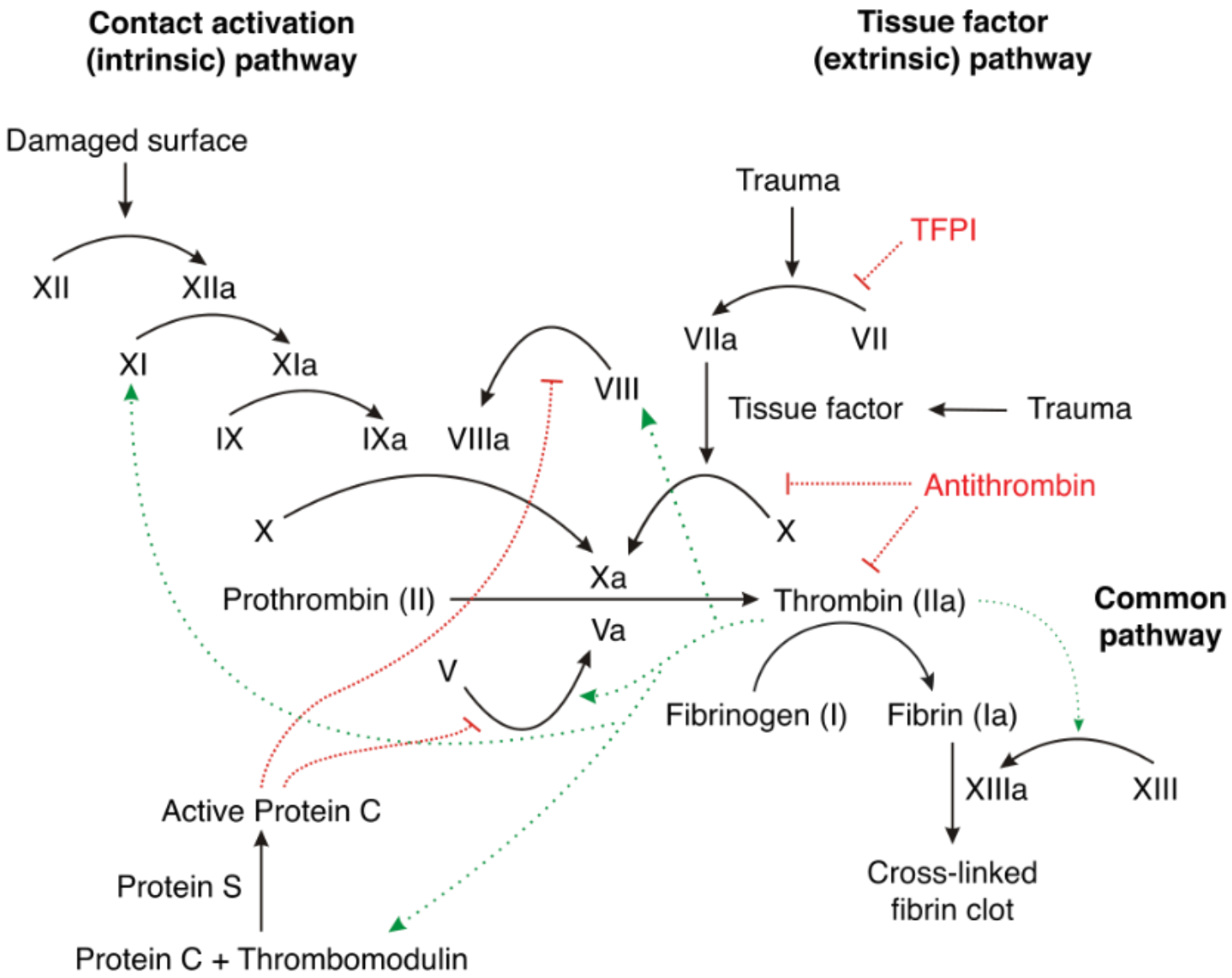


https://www.britannica.com/science/fibrin

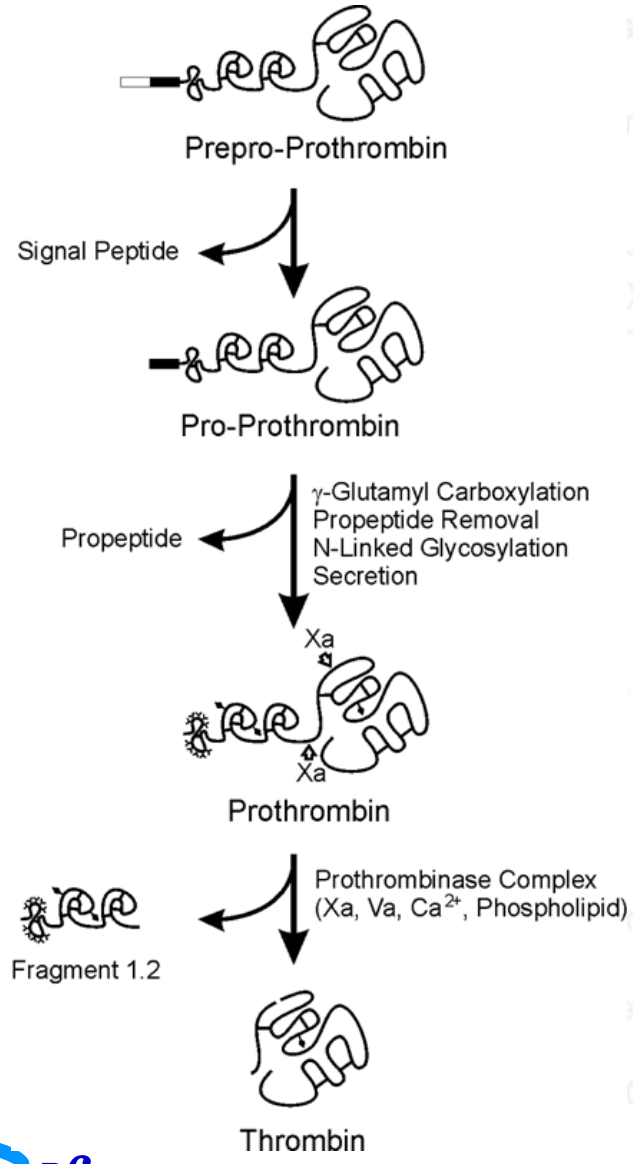


Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599.

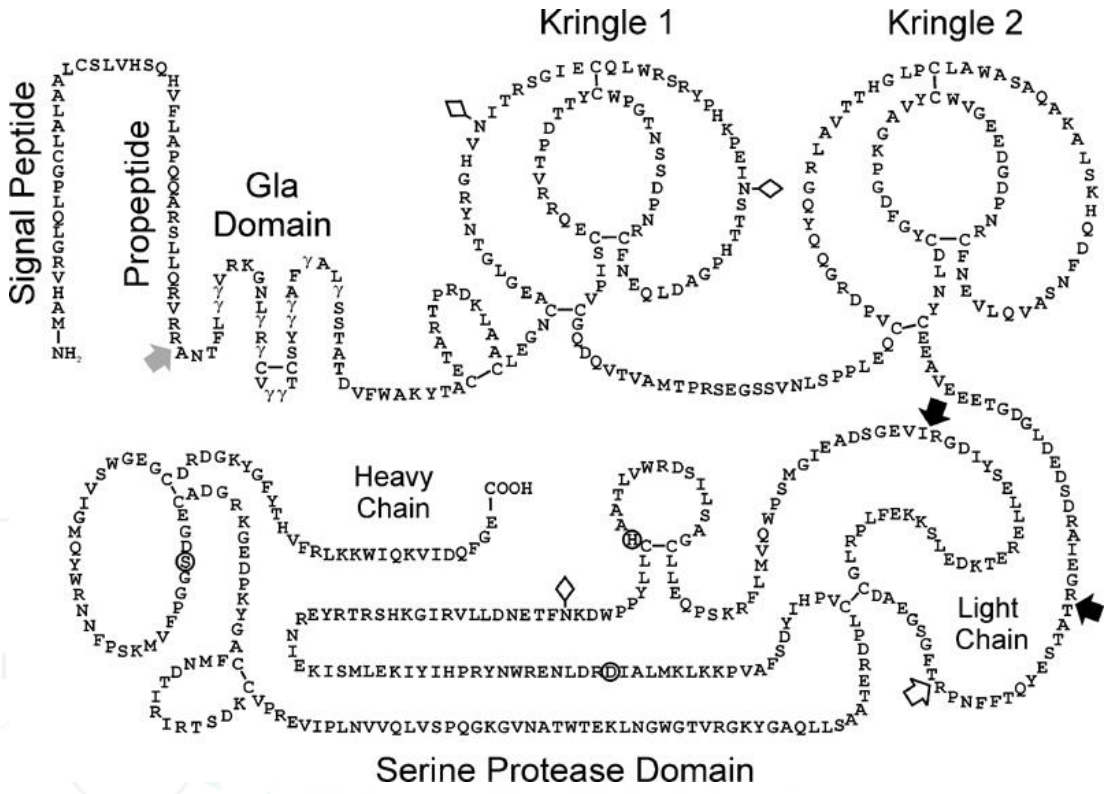
# Biosynthesis of α-Thrombin



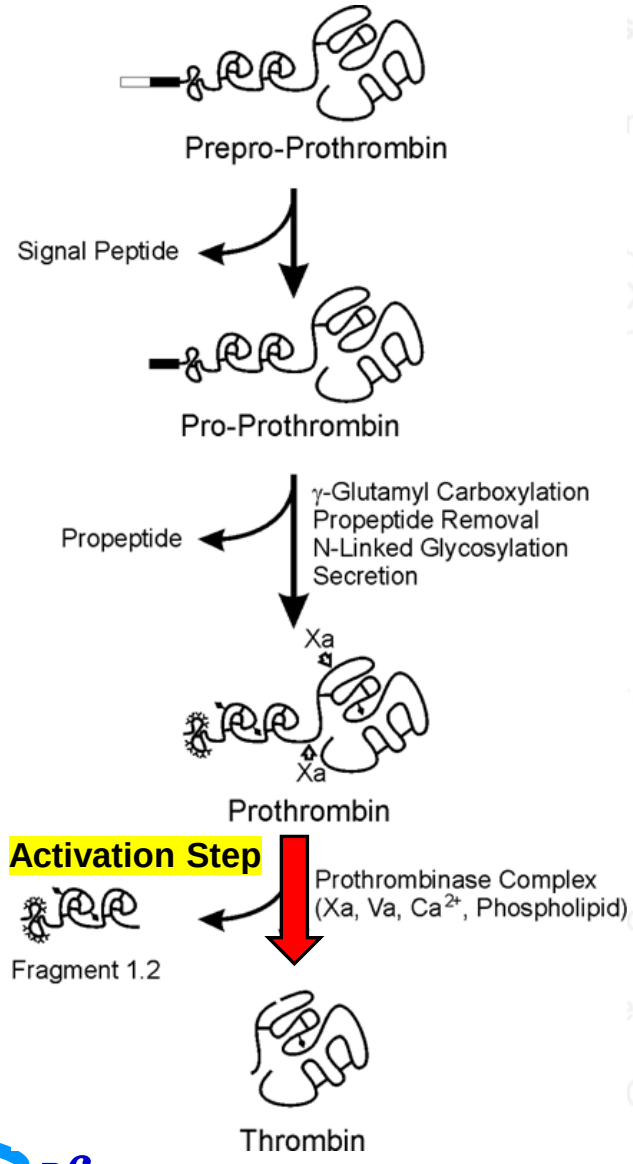
# Biosynthesis of α-Thrombin



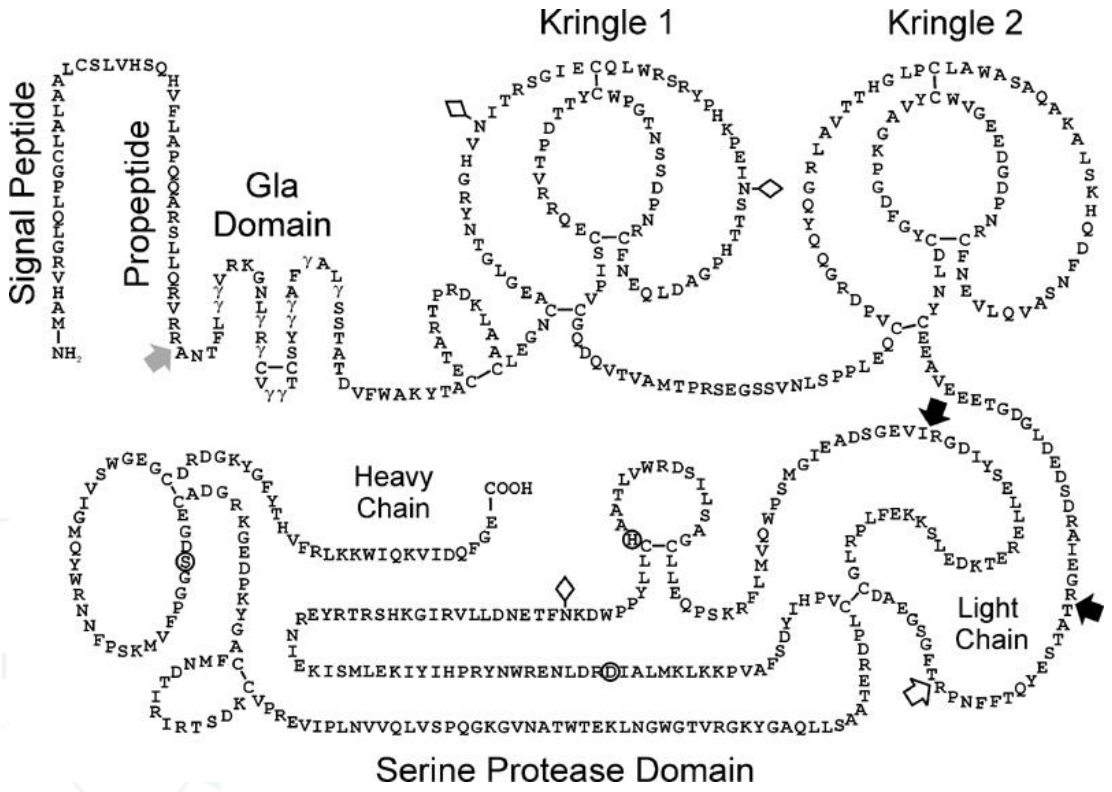

*Amino acid sequence of human Prepro-Prothrombin*



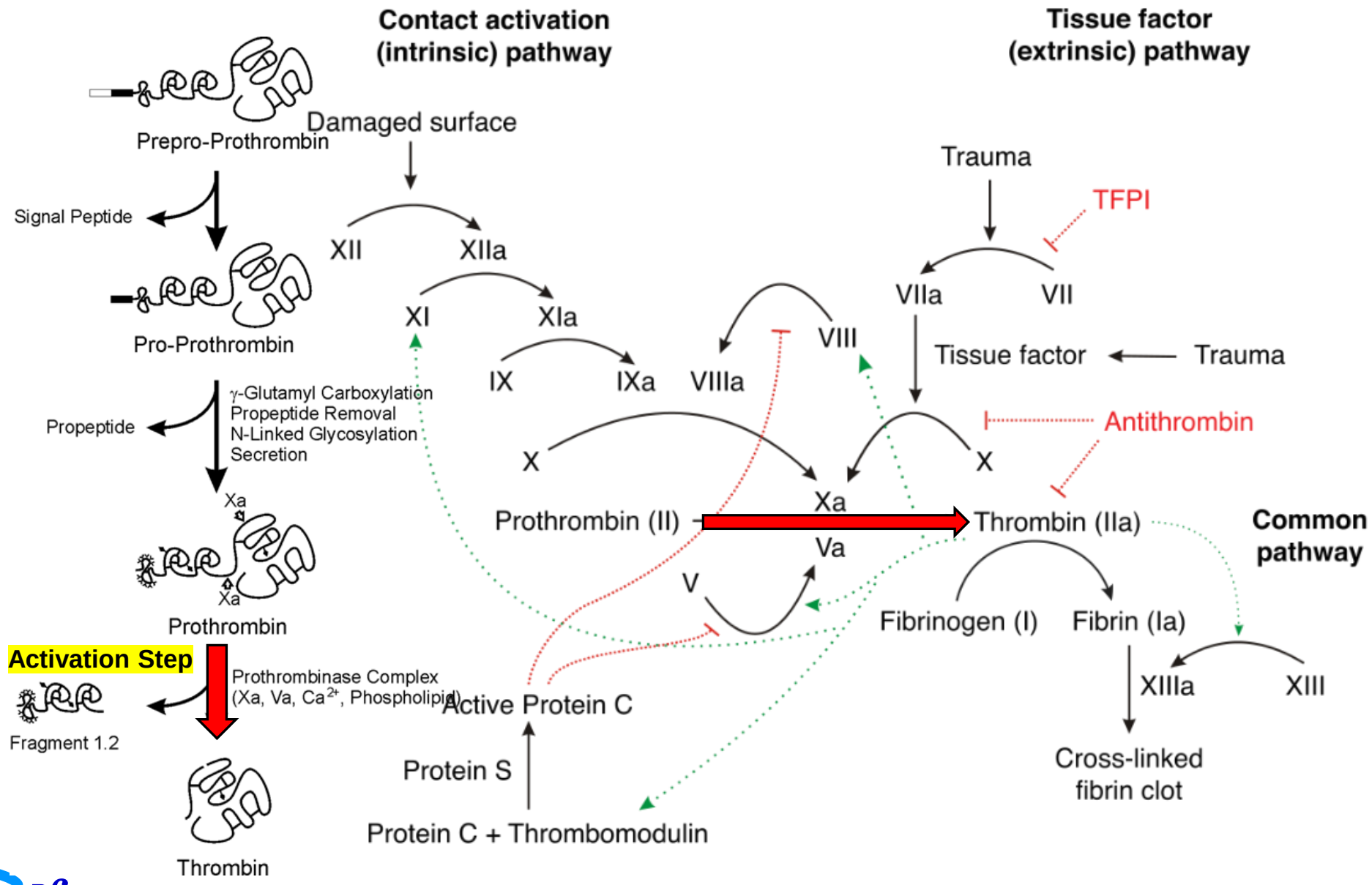
# Biosynthesis of α-Thrombin



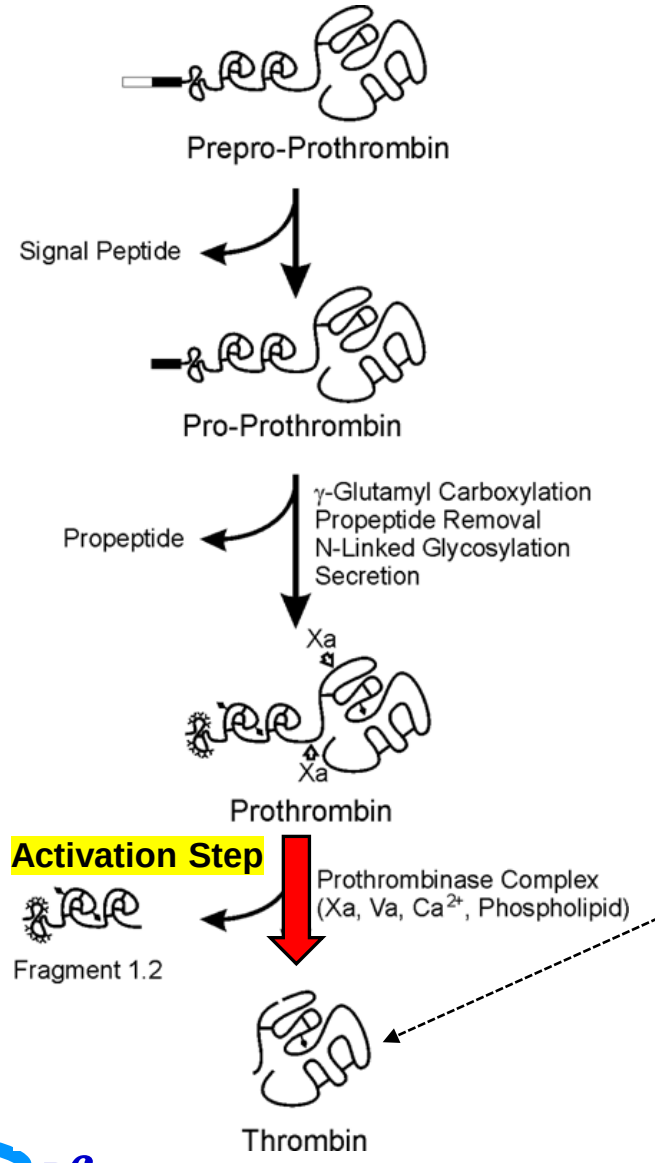

*Amino acid sequence of human Prepro-Prothrombin*



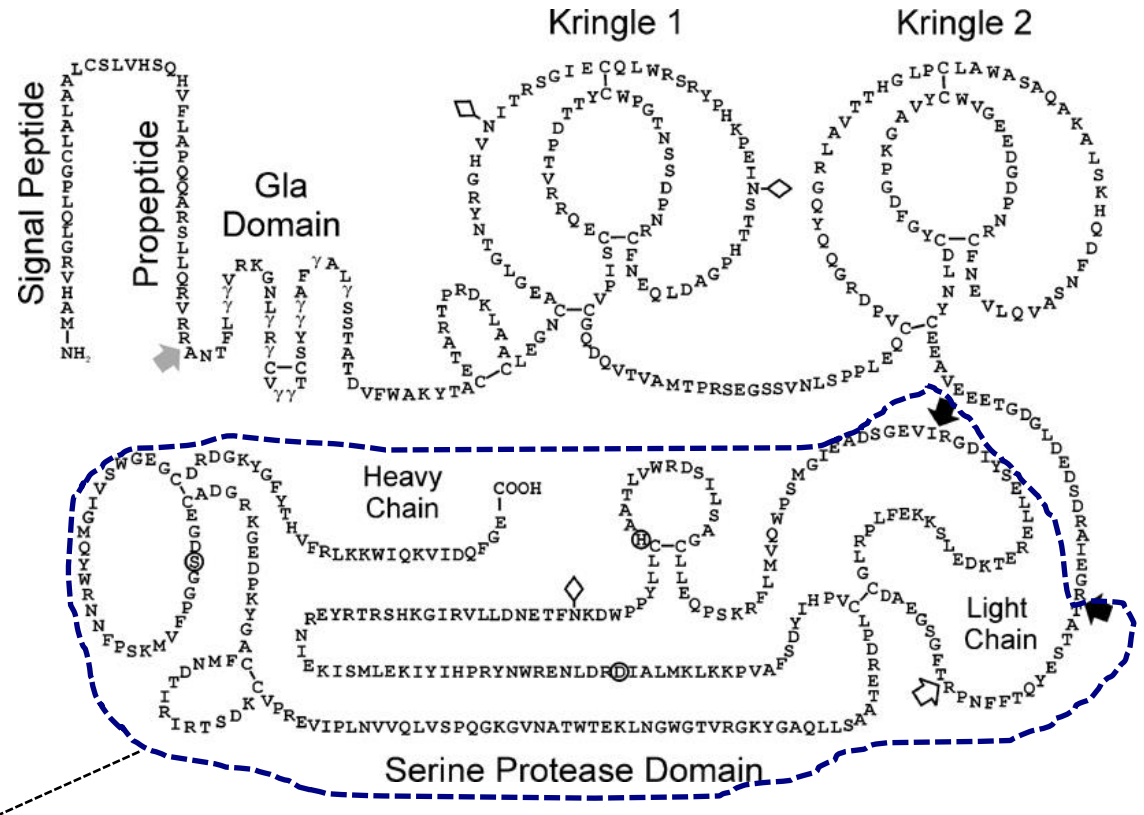
# Biosynthesis of α-Thrombin



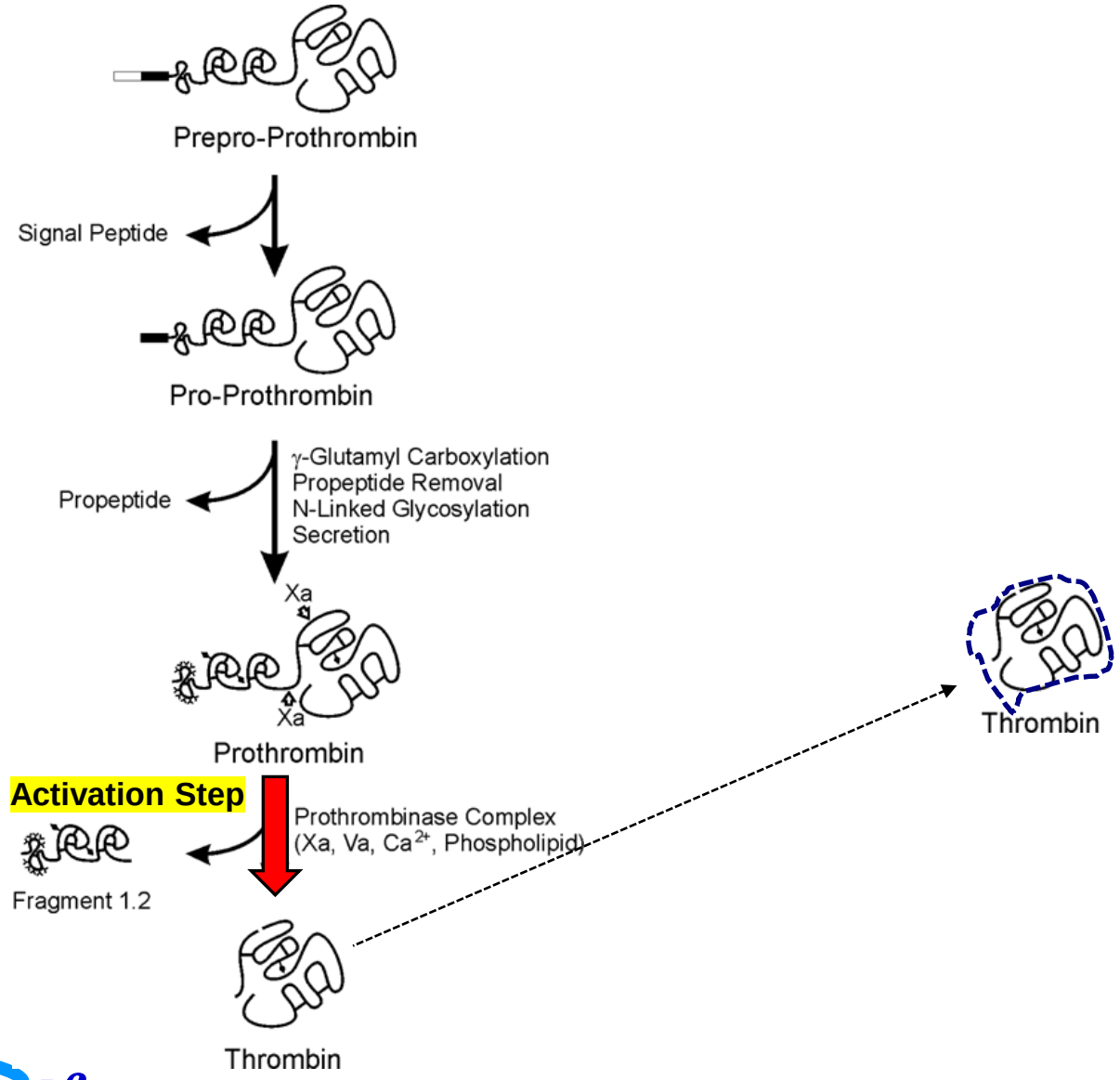
# Biosynthesis of α-Thrombin



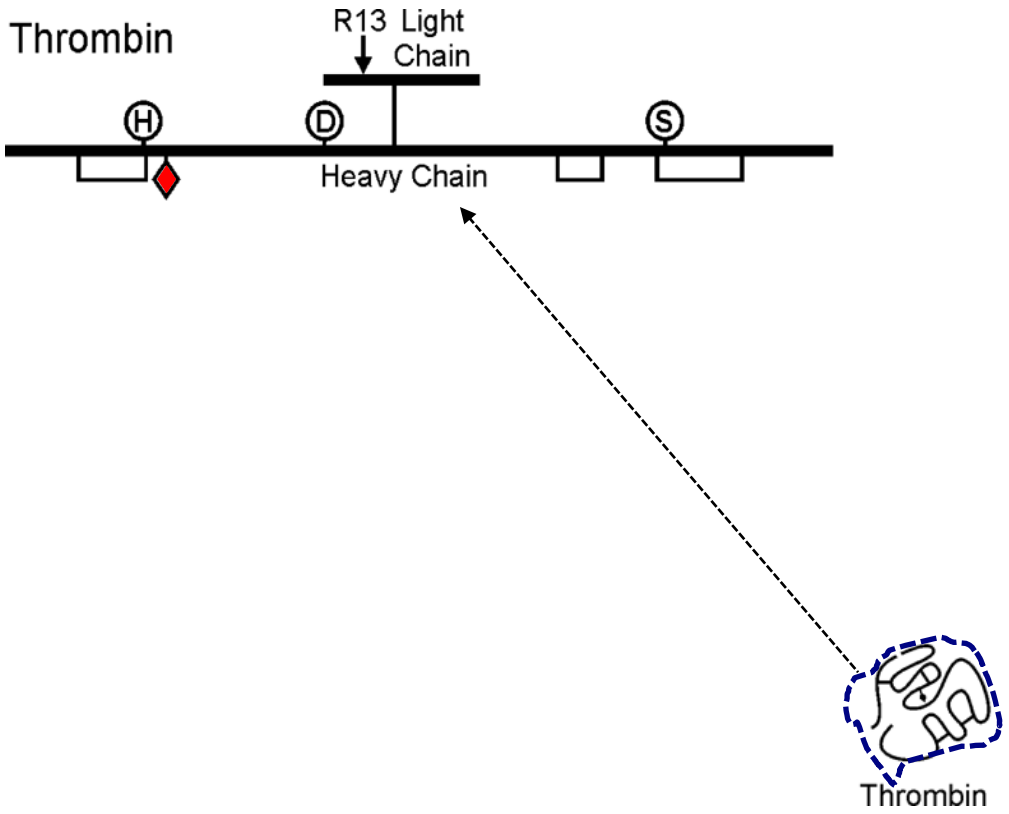

*Amino acid sequence of human Prepro-Prothrombin*



# Biosynthesis of α-Thrombin



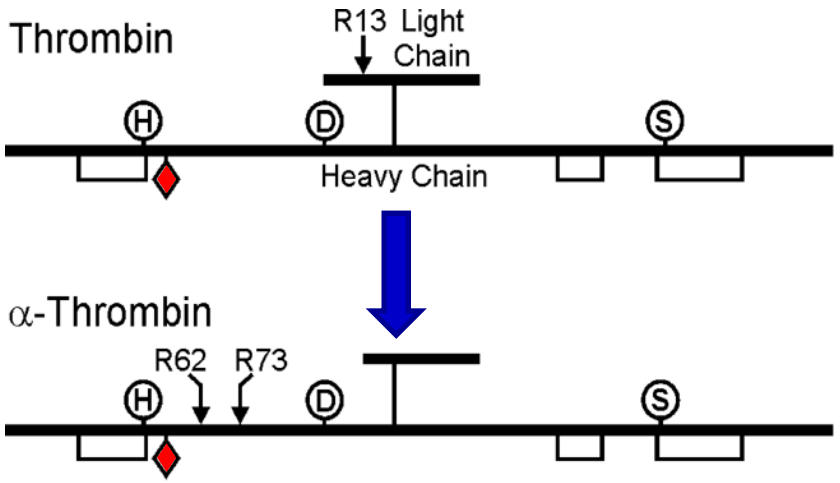
# Biosynthesis of α-Thrombin






Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

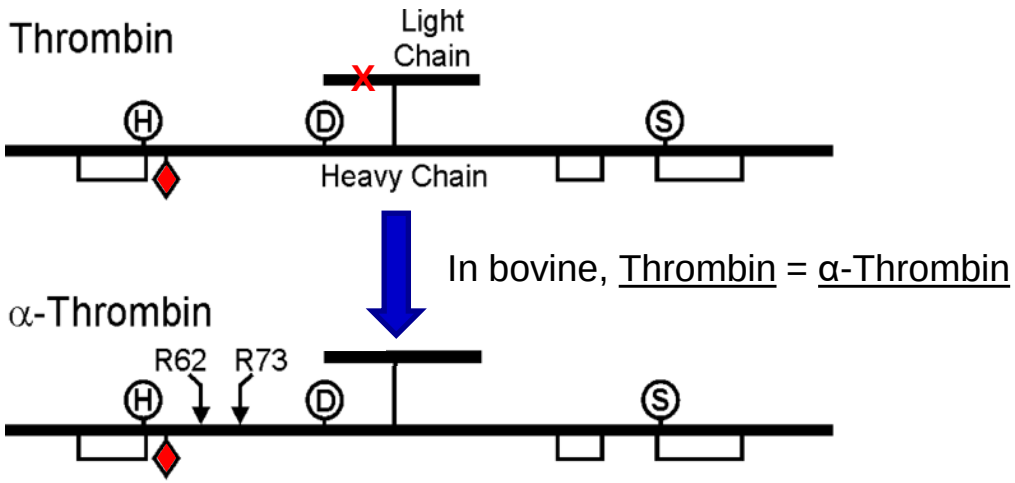
# Biosynthesis of α-Thrombin






Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

# Biosynthesis of α-Thrombin

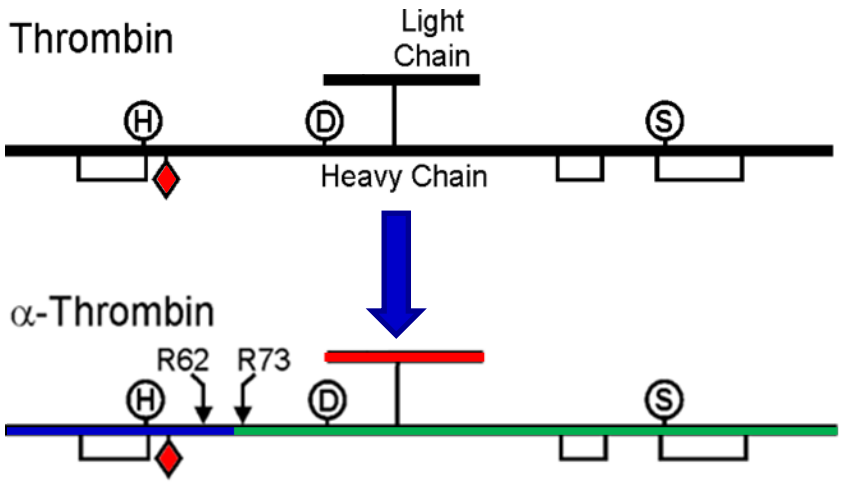



In bovine, Thrombin = α-Thrombin

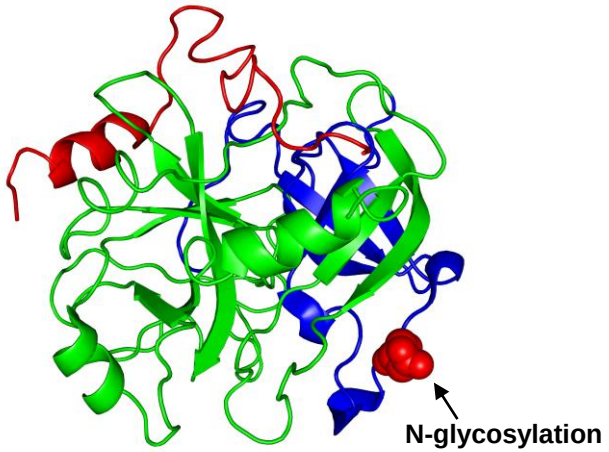


Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

# Biosynthesis of α-Thrombin: Protein Structure and Function

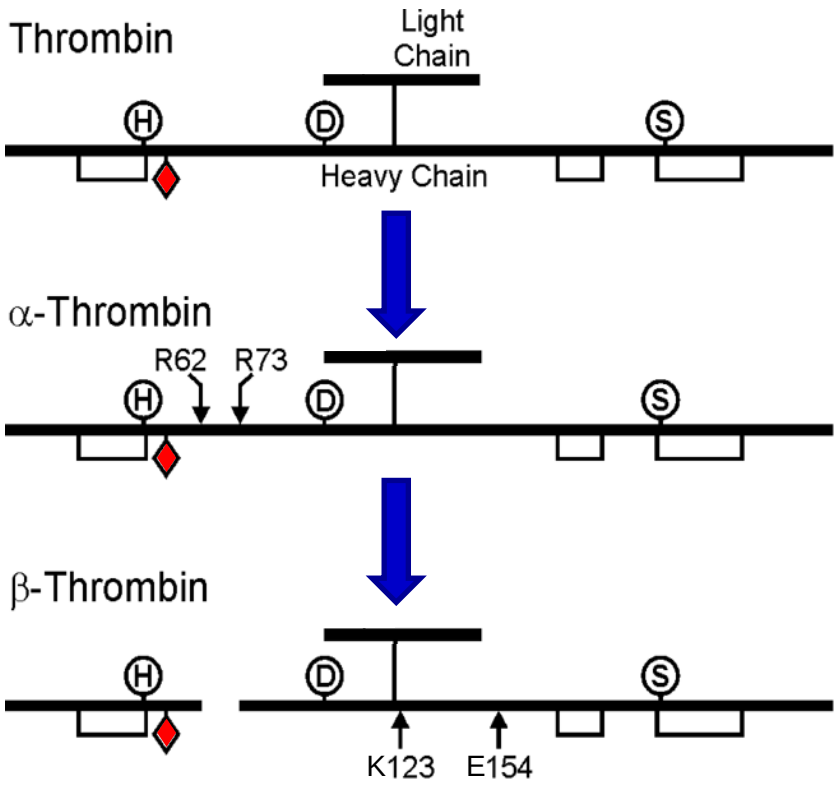



**A Serine Protease (H-D-S) with a Heavy and Light Chain and one N-Glycosylation Site**




PDB DOI: 10.2210/pdb1HRT/pdb

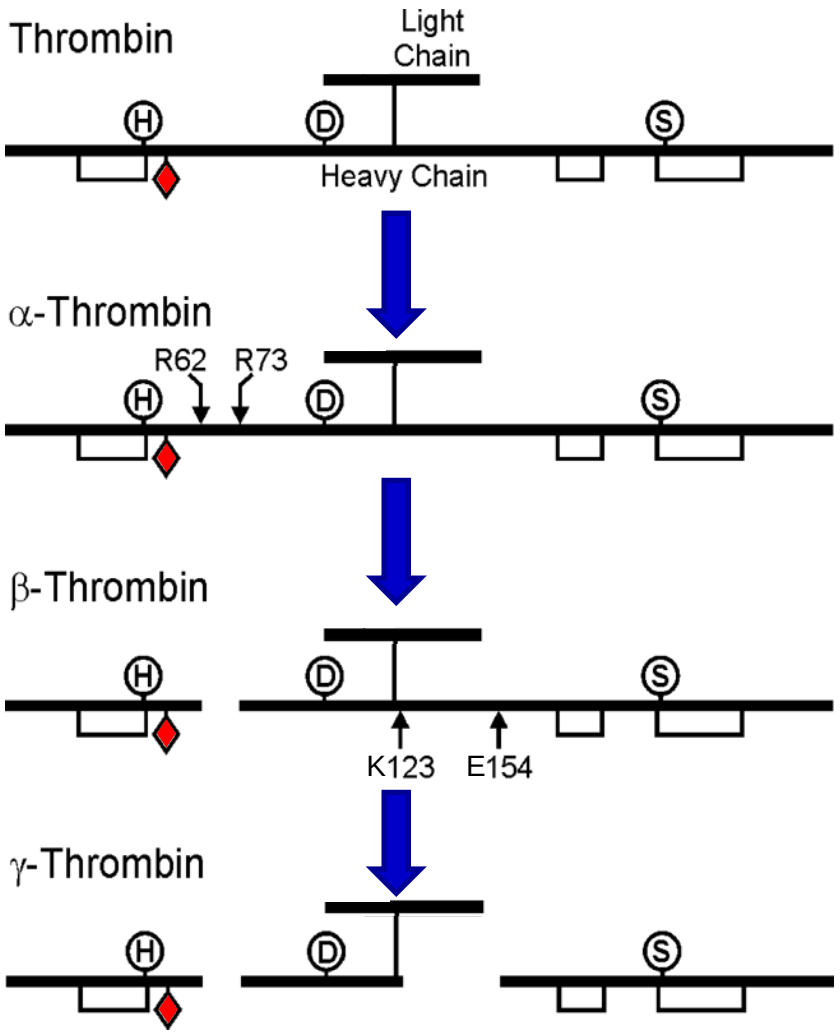
# Biosynthesis of α-Thrombin: Autolytic Products






Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

# Biosynthesis of α-Thrombin: Autolytic Products






Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

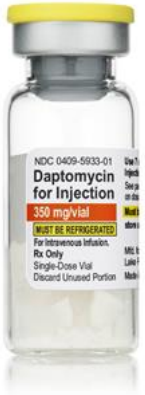
- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products





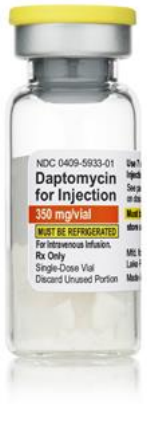

THROMBIN-JMI® (thrombin, topical, bovine origin)

# Pfizer’s Hospital Business Unit (HBU)

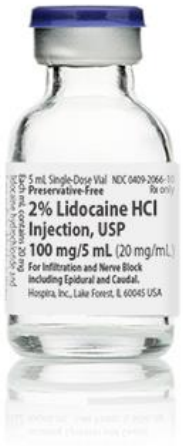
*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products
  - Anesthesia





# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products
  - Anesthesia
  - Uniquely differentiated Hospital products

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products
  - Anesthesia
  - Uniquely differentiated Hospital products

# Pfizer’s Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer’s HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer’s only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products
  - Anesthesia
  - Uniquely differentiated Hospital products

## THROMBIN-JMI®

# Pfizer's Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer's HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer's only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products
  - Anesthesia
  - Uniquely differentiated Hospital products

## THROMBIN-JMI®


→ a topical product of bovine origin

# Pfizer's Hospital Business Unit (HBU)

*Delivers Vital Medicines Where They Are Needed Most*

## Pfizer's HBU (est. 2019)

- A global business with one of the broadest portfolios of medicine in the industry
- Pfizer's only customer- and channel-centric business
- Broad Portfolio
  - Anti-infectives
  - Sterile injectables
  - Surgical products
  - Anesthesia
  - Uniquely differentiated Hospital products

## THROMBIN-JMI®

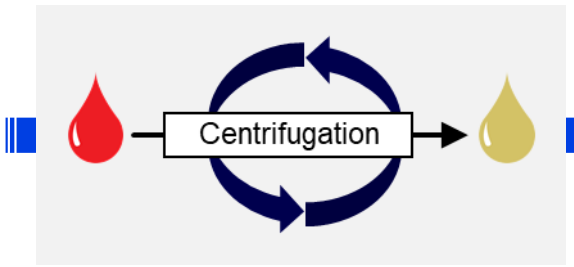

Surgical products → a topical product of bovine origin

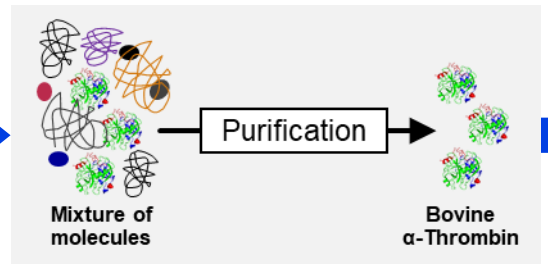
## Indication and Usage

Indicated to aid hemostasis whenever oozing blood and minor bleeding from capillaries and small venules is accessible and control of bleeding by standard surgical techniques (such as suture, ligature, or cautery) is ineffective or impractical.

# THROMBIN-JMI®: Manufacturing

Stef Bennett - The Hungry Aperture/Getty Images/iStockphoto





## Bovine

- Bovine plasma used to manufacture Thrombin-JMI® is sourced solely from animals of U.S. origin
- USDA inspections ensure cattle are disease free and fit for human consumption

## Bovine Plasma

- Thrombin-JMI® does not contain nor is derived from specified risk materials defined in Commission Decision 97/534/EC

## Purification

- Isolate Prothrombin
- Convert Prothrombin to Thrombin
- Viral Inactivation
- Chromatography Purification
- Chromatography Polishing
- Viral Filtration
- Bulk Thrombin

## Thrombin-JMI®

- THROMBIN-JMI is available
  - 5,000 and 20,000 IU vial
  - Pump Spray Kit
  - Syringe Spray Kit
  - Epistaxis Kit
  - Gelfoam-Thrombin Kit
  - Gel-flow-Thrombin Kit

# Presentation Overview

- Biological Activity & Biosynthesis of α-Thrombin
- Pfizer's Hospital Business Unit (HBU) and Thrombin-JMI®
- Manufacturing of Thrombin-JMI® from bovine

***Why was there a need to identify N-linked glycans from bovine α-Thrombin?***

Preliminary Intact Mass Analysis

# FDA - Guidance for Industry For the Submission of CMC and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma or Serum-Derived Products

https://www.fda.gov/media/124397/download

**Characterization**

→ identity, stability and consistency

# FDA - Guidance for Industry For the Submission of CMC and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma or Serum-Derived Products

https://www.fda.gov/media/124397/download

## Characterization

→ identity, stability and consistency

*In general, characterization may include, but need not be limited to the following:*

- Potency Assays
- Chromatographic Assays
- Electrophoresis, e.g., SDS-PAGE
- Immunoblot Analysis
- Fluorescence Activated Cell Sorter (FACS) Analysis
- Enzyme-Linked Immunosorbent Assay (ELISA)

FDA - Guidance for Industry For the Submission of CMC and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma or Serum-Derived Products

https://www.fda.gov/media/124397/download

**Characterization**

→ identity, stability and consistency

*In general, characterization may include, but need not be limited to the following:*

- Potency Assays
- Chromatographic Assays
- Electrophoresis, e.g., SDS-PAGE
- Immunoblot Analysis
- Fluorescence Activated Cell Sorter (FACS) Analysis
- Enzyme-Linked Immunosorbent Assay (ELISA)

FDA - Guidance for Industry For the Submission of CMC for a Therapeutic Recombinant DNA-Derived Product or a Monoclonal Antibody Product for in vivo use

https://www.fda.gov/media/77528/download

**Characterization/Proof of Structure**

→ identity, […], stability and consistency

FDA - Guidance for Industry For the Submission of CMC and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma or Serum-Derived Products

https://www.fda.gov/media/124397/download

**Characterization**

→ identity, stability and consistency

*In general, characterization may include, but need not be limited to the following:*

- Potency Assays
- Chromatographic Assays
- Electrophoresis, e.g., SDS-PAGE
- Immunoblot Analysis
- Fluorescence Activated Cell Sorter (FACS) Analysis
- Enzyme-Linked Immunosorbent Assay (ELISA)

FDA - Guidance for Industry For the Submission of CMC for a Therapeutic Recombinant DNA-Derived Product or a Monoclonal Antibody Product for in vivo use

https://www.fda.gov/media/77528/download

**Characterization/Proof of Structure**

→ identity, […], stability and consistency

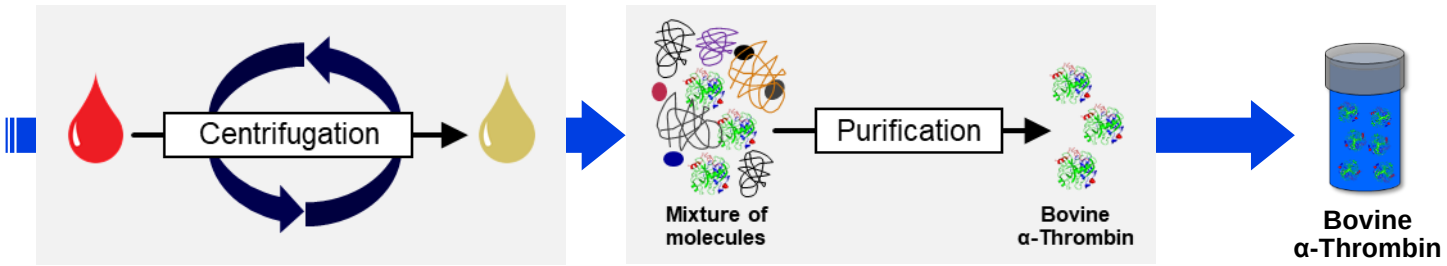
*[…] are not necessarily limited to the following:*

- Amino Acid Analysis
- Amino Acid Sequencing, entire sequence or amino- and carboxyterminal sequences
- Peptide Mapping
- Determination of Disulfide Linkage
- SDS-PAGE (Reduced and Non-Reduced)
- Isoelectric Focusing
- Conventional and HPLC e.g., Reverse-Phase, Size Exclusion, Ion-Exchange, etc
- Mass Spectroscopy
- Assays to detect product-related proteins including deamidated, oxidized, cleaved, and aggregated forms and other variants e.g., amino acid substitutions, adducts/derivatives
- Assays to detect residual host proteins, DNA, reagents
- Immunochemical Analyses
- Assays to quantitate bioburden, endotoxin

# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

## Preliminary Intact Mass Analysis

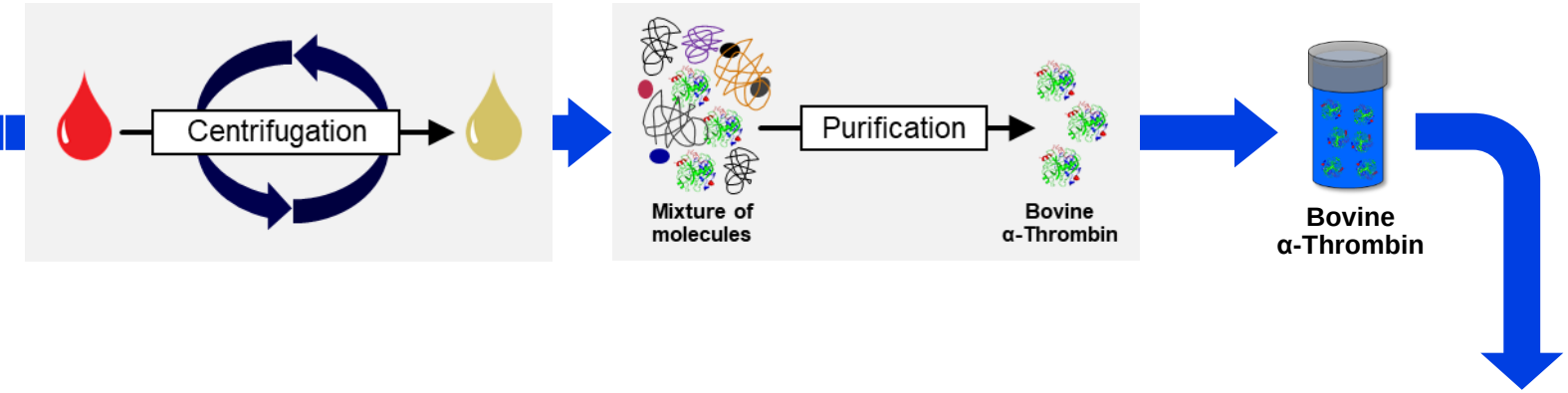
Stef Bennett - The Hungry Aperture/Getty Images/iStockphoto

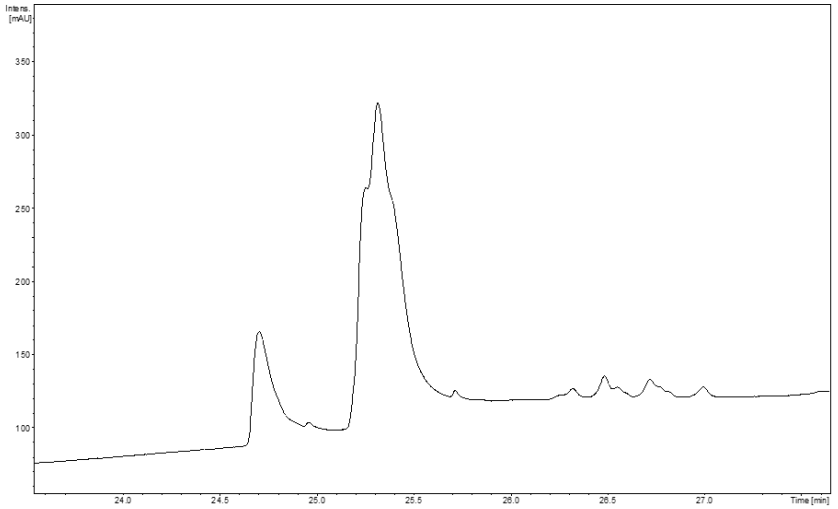


# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

## Preliminary Intact Mass Analysis

Stef Bennett - The Hungry Aperture/Getty Images/iStockphoto




UV Chromatogram

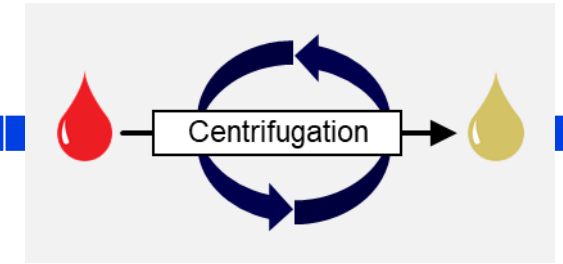
**LC Separation**

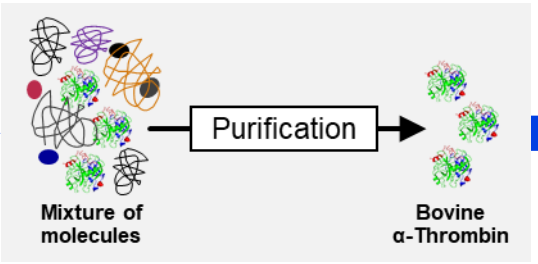
Waters H-Class UPLC

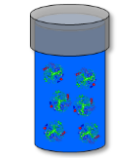
# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

## Preliminary Intact Mass Analysis

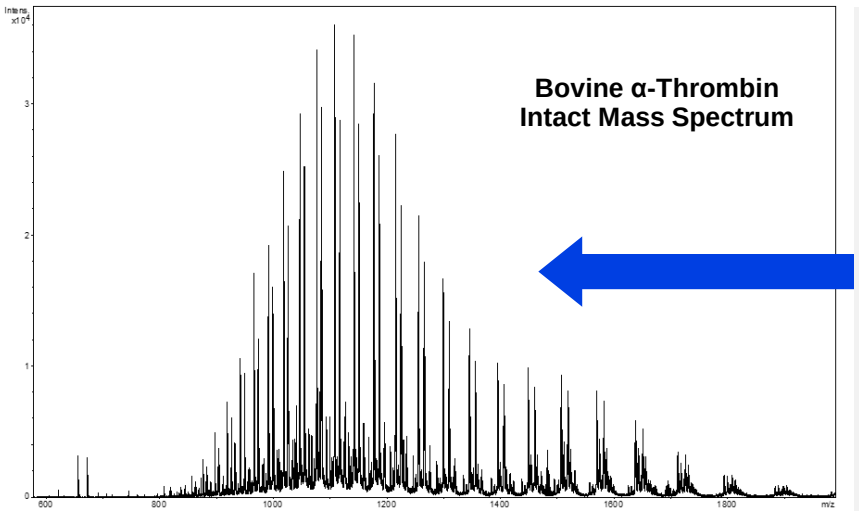
Stef Bennett - The Hungry Aperture/Getty Images/iStockphoto



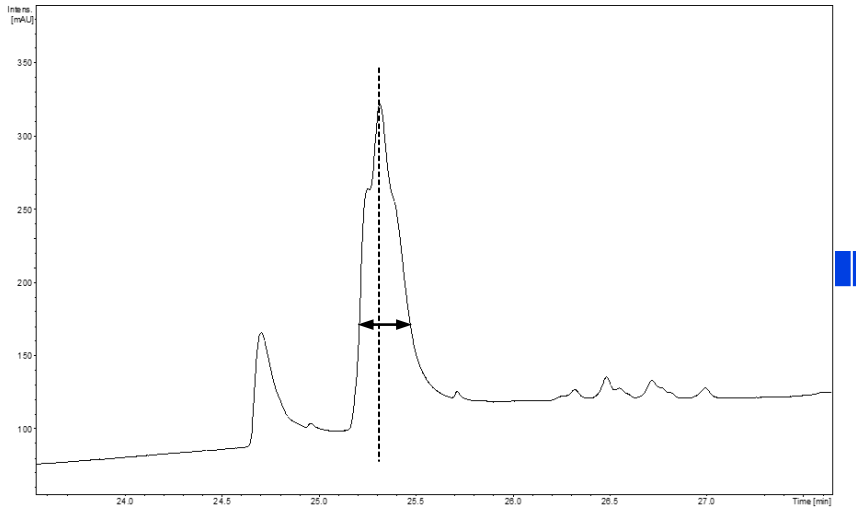
Bovine α-Thrombin




Mass Spectrum

MS Analysis


Bruker maXis II MS



UV Chromatogram

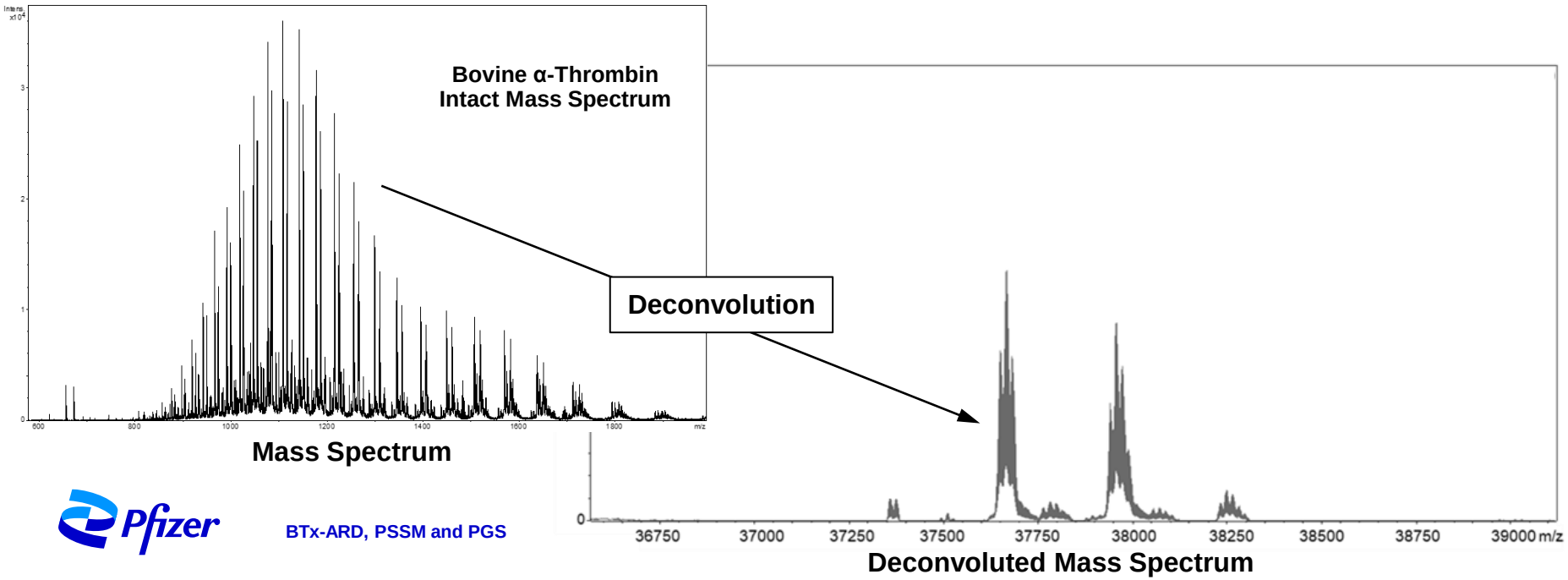
LC Separation

Waters H-Class UPLC

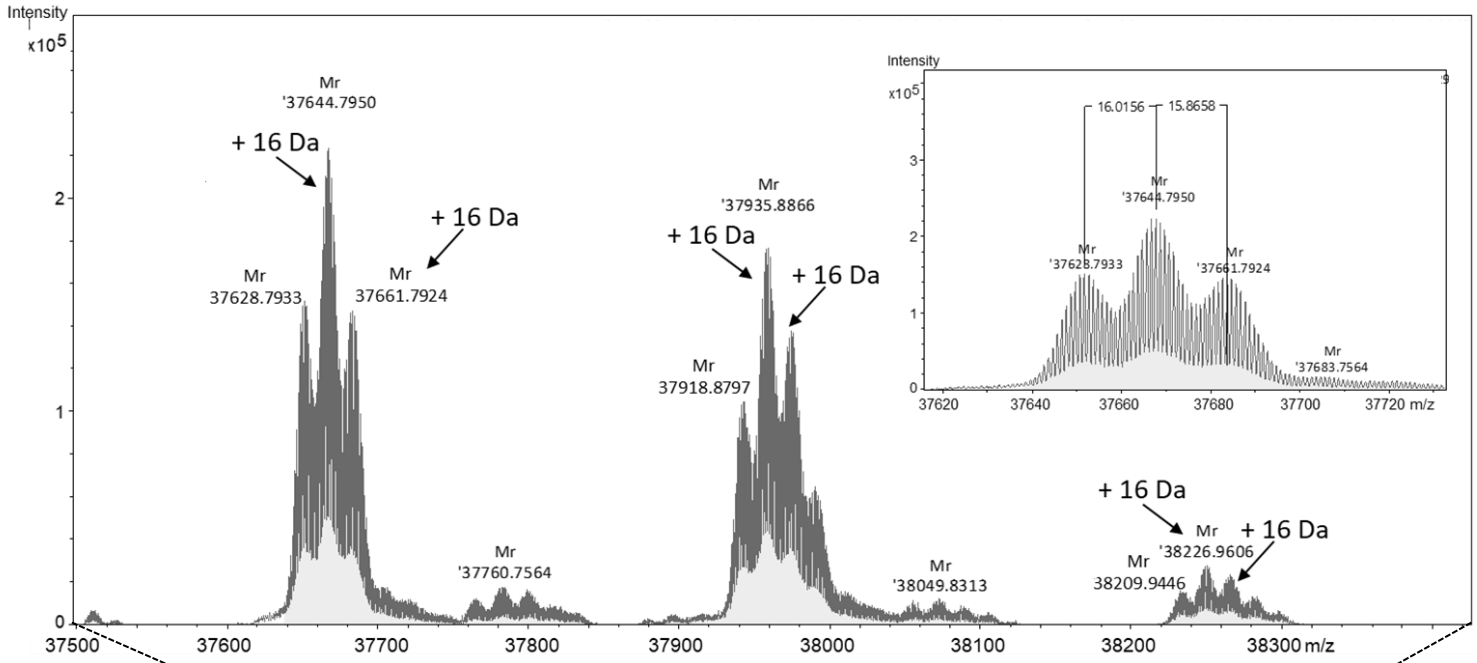
# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

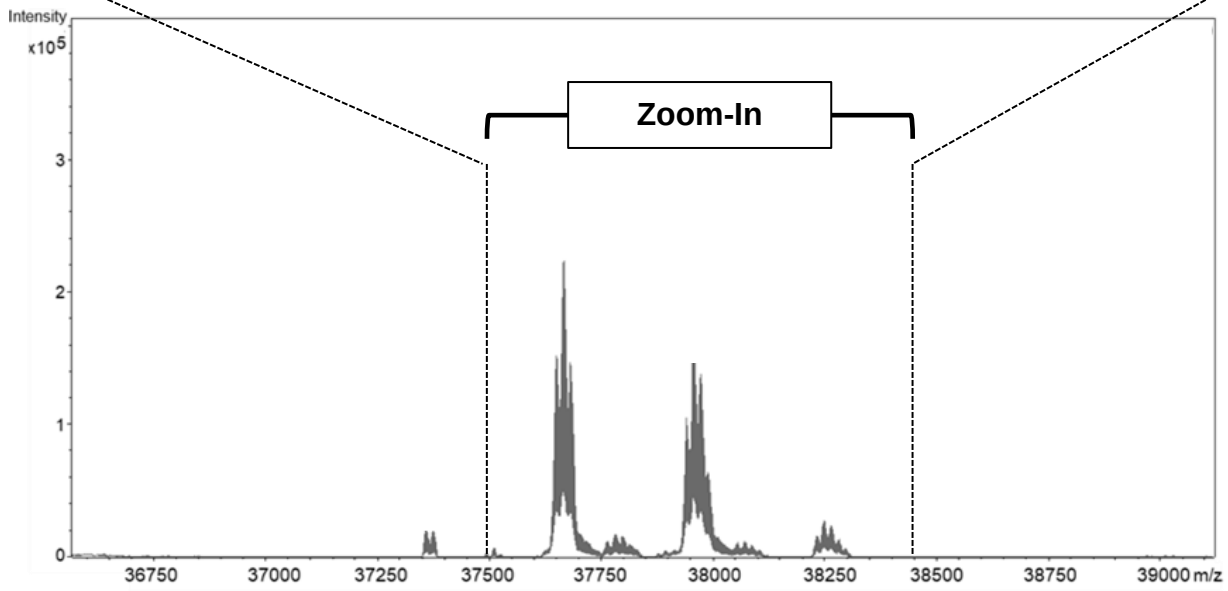
## Preliminary Intact Mass Analysis

Stef Bennett - The Hungry Aperture/Getty Images/iStockphoto



# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

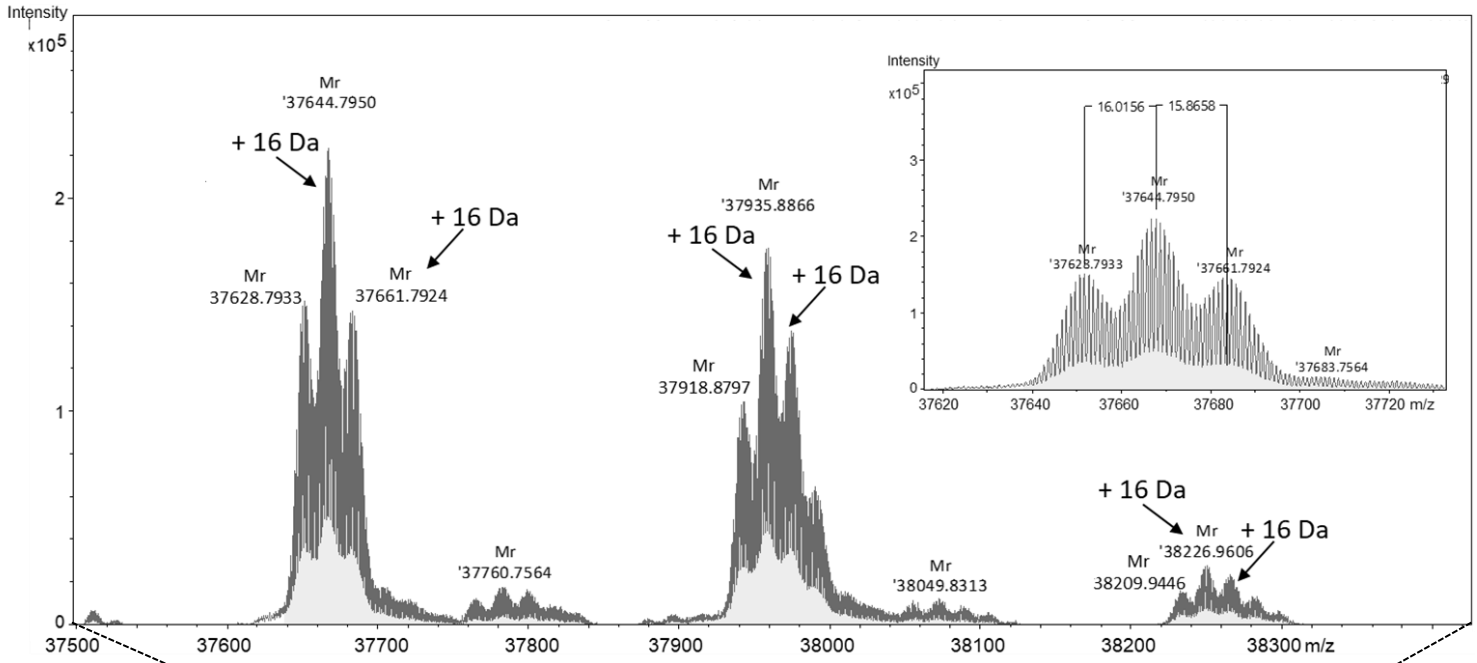


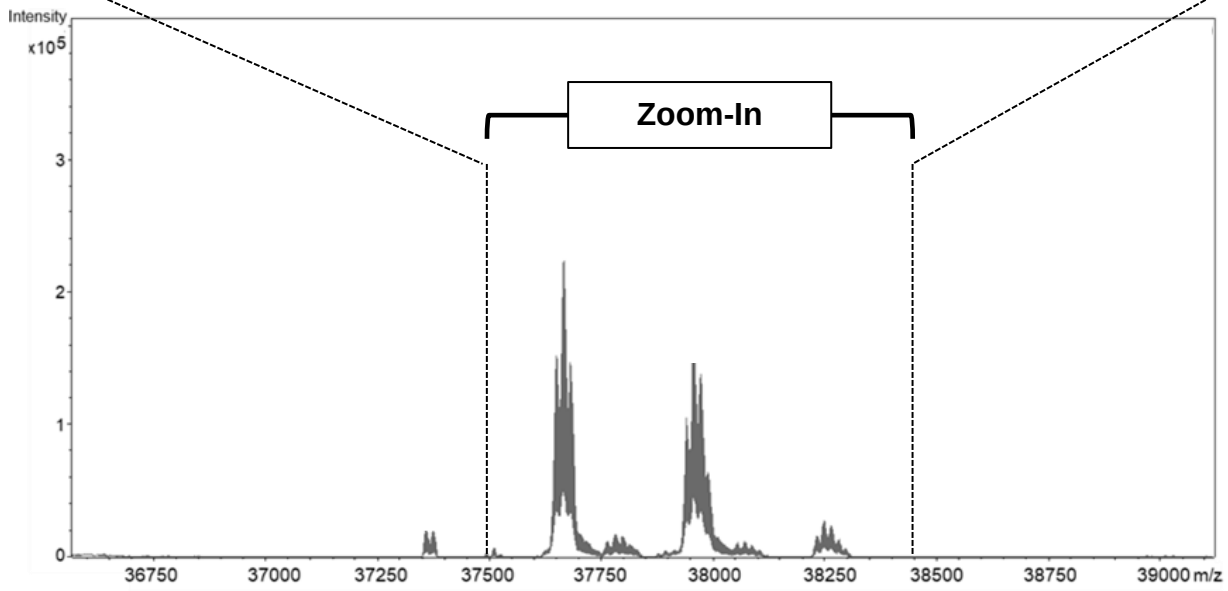




Deconvoluted Mass Spectrum

# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

Protein Modification?

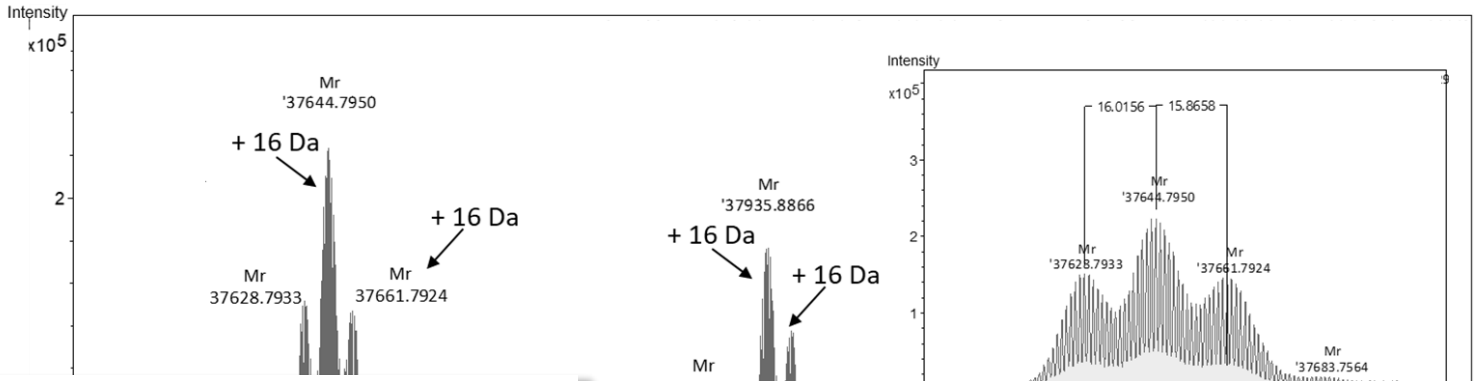



Deconvoluted Mass Spectrum

# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

Protein Modification?










UNIMOD protein modifications for mass spectrometry

Help

Unimod

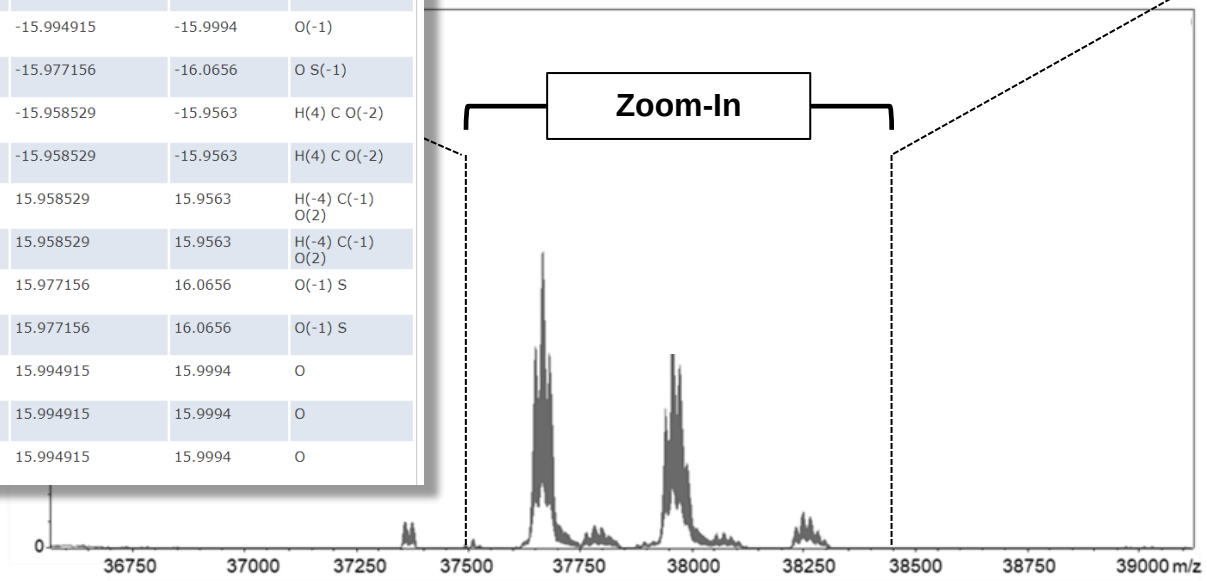
Logged as **Guest** Log out Change password Advanced search

Search for: Any field | Contains | 15.9 | Search | Show all

Details found: 16 Page 1 of 1

Records Per Page:: 20

| | Accession # | PSI-MS Name | Interim name | Description | Monoisotopic mass | Average mass | Composition |
|---|---|---|---|---|---|---|---|
| View | 1096 | | Phe->Met | Phe->Met substitution | -16.027929 | -15.9778 | C(-4) S |
| View | 447 | Deoxy | semialdehyde | reduction | -15.994915 | -15.9994 | O(-1) |
| View | 648 | | Ser->Ala | Ser->Ala substitution | -15.994915 | -15.9994 | O(-1) |
| View | 678 | | Tyr->Phe | Tyr->Phe substitution | -15.994915 | -15.9994 | O(-1) |
| View | 548 | | Cys->Ser | Cys->Ser substitution | -15.977156 | -16.0656 | O S(-1) |
| View | 559 | | Asp->Val | Asp->Val substitution | -15.958529 | -15.9563 | H(4) C O(-2) |
| View | 1081 | | Glu->Xle | Glu->Leu/Ile substitution | -15.958529 | -15.9563 | H(4) C O(-2) |
| View | 670 | | Val->Asp | Val->Asp substitution | 15.958529 | 15.9563 | H(-4) C(-1) O(2) |
| View | 1128 | | Xle->Glu | Leu/Ile->Glu substitution | 15.958529 | 15.9563 | H(-4) C(-1) O(2) |
| View | 420 | Carboxy->Thiocarboxy | thiocarboxy | thiocarboxylic acid | 15.977156 | 16.0656 | O(-1) S |
| View | 654 | | Ser->Cys | Ser->Cys substitution | 15.977156 | 16.0656 | O(-1) S |
| View | 35 | Oxidation | Hydroxylation | Oxidation or Hydroxylation | 15.994915 | 15.9994 | O |
| View | 540 | | Ala->Ser | Ala->Ser substitution | 15.994915 | 15.9994 | O |
| View | 569 | | Phe->Tyr | Phe->Tyr substitution | 15.994915 | 15.9994 | O |



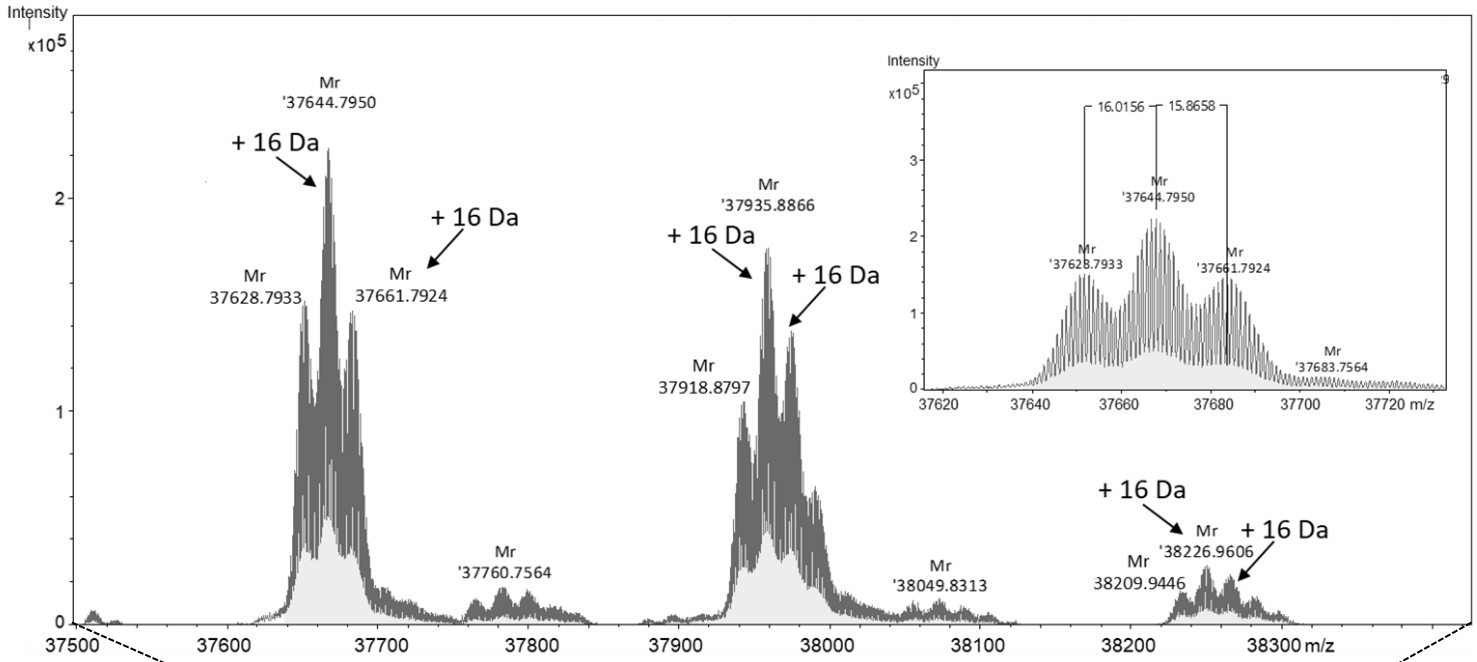

Deconvoluted Mass Spectrum

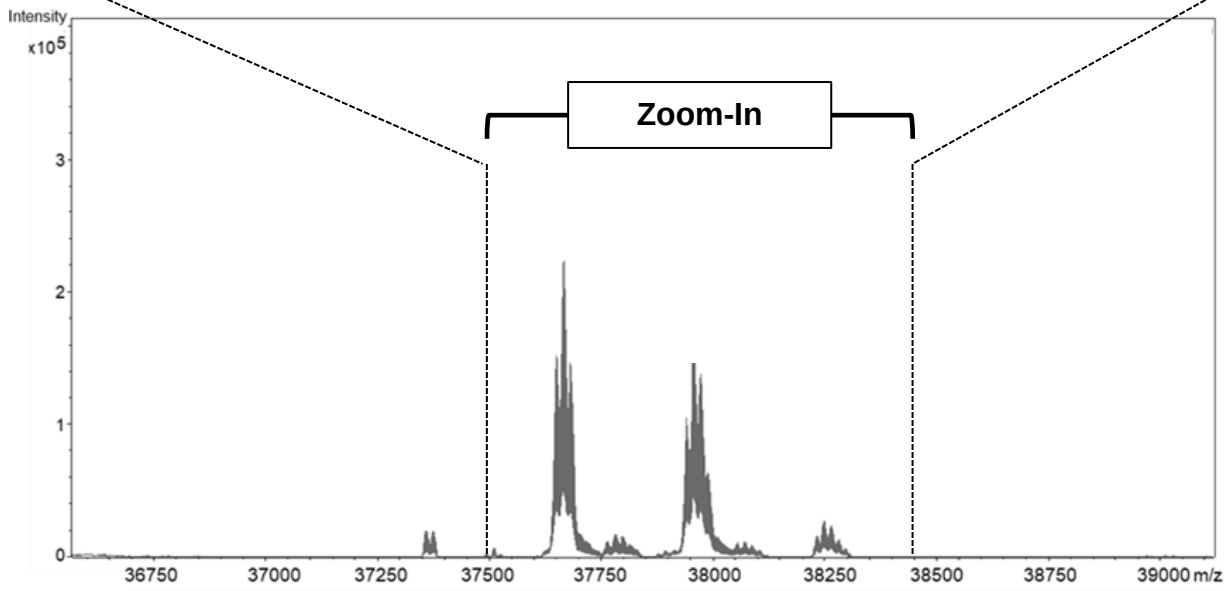
# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

Protein Modification?

Presence of NeuGc in non-human mammals

Chou et al PNAS 1998
95(20): 11751-11756



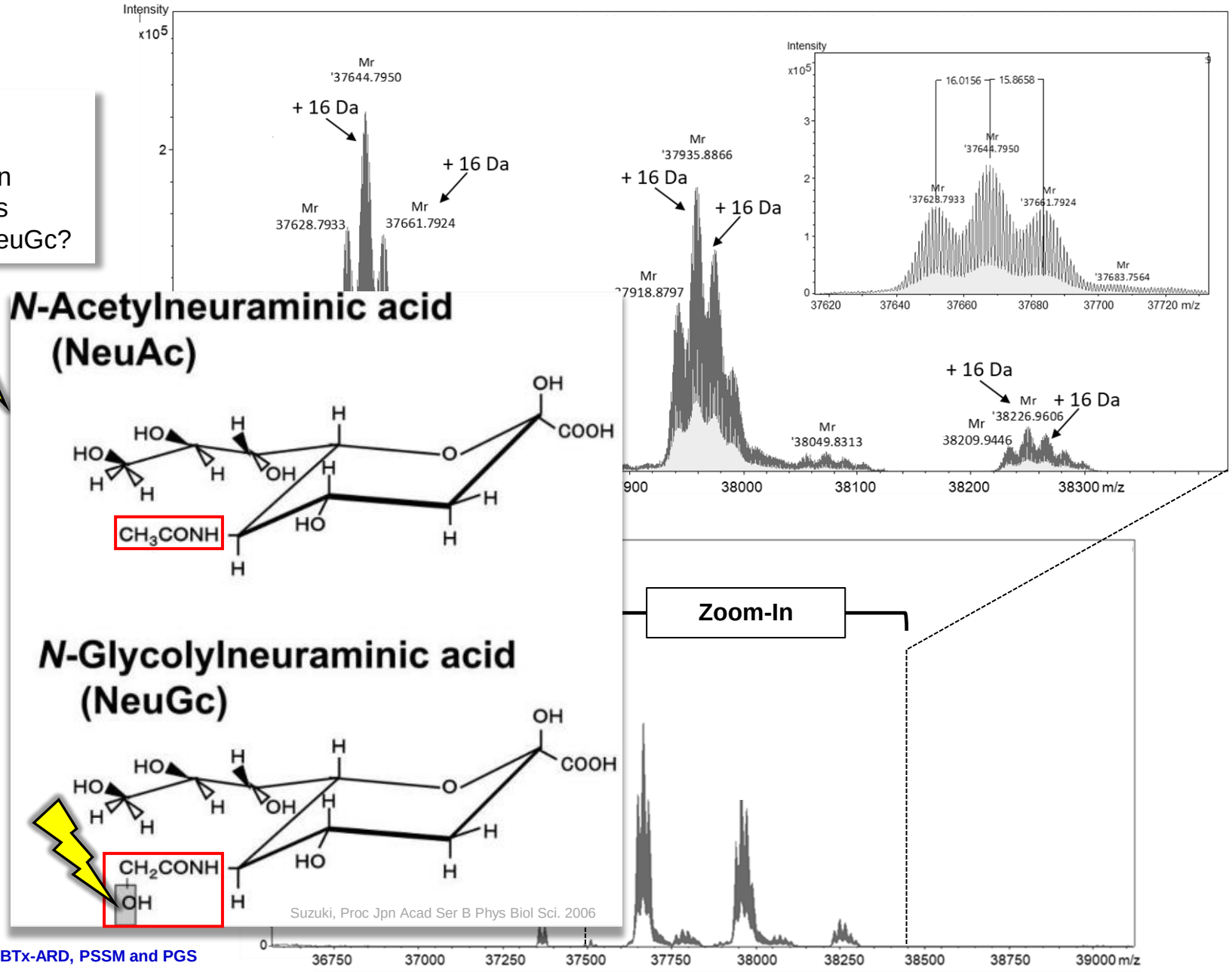

Deconvoluted Mass Spectrum

# Why was there a need to identify N-Linked glycans in bovine α-Thrombin?

Protein Modification?

Presence of NeuGc in non-human mammals
→ mix of NeuAc & NeuGc?

Chou et al PNAS 1998
95(20): 11751-11756




**N-Acetylneuraminic acid (NeuAc)**




**N-Glycolylneuraminic acid (NeuGc)**




Suzuki, Proc Jpn Acad Ser B Phys Biol Sci. 2006

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Davie et al *An Overview of the Structure and Function of Thrombin*, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006**

Both bovine and human prothrombin contain three N-linked carbohydrate chains, two of which are located in kringle domain 1 and one of which is present within the serine protease domain. The latter of these has been the focus of structural analyses that have identified slight differences between human and bovine carbohydrate as well as minor microheterogeneity within the single carbohydrate chain attached to human thrombin.[53–55]

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Davie et al *An Overview of the Structure and Function of Thrombin*, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006**

Both bovine and human prothrombin contain three N-linked carbohydrate chains, two of which are located in kringle domain 1 and one of which is present within the serine protease domain. The latter of these has been the focus of structural analyses that have identified slight differences between human and bovine carbohydrate as well as minor microheterogeneity within the single carbohydrate chain attached to human thrombin.[53–55] This oligosaccharide chain is linked to Asn53 in human thrombin (thrombin heavy-chain numbering) and has been characterized as a sialated biantennary glycan of the *N*-acetyl-lactosamine prototype, with fucose present in 50% of the carbohydrate chains.[55]


54. Mizuochi T, Fujii J, Kisiel W, Kobata A. Studies on the structures of the carbohydrate moiety of human prothrombin. J Biochem (Tokyo) 1981;90:1023–1031

55. Nilsson B, Horne MK III, Gralnick HR. The carbohydrate of human thrombin: structural analysis of glycoprotein oligosaccharides by mass spectrometry. Arch Biochem Biophys 1983;224:127–133

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Davie et al *An Overview of the Structure and Function of Thrombin*, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006**

Both bovine and human prothrombin contain three N-linked carbohydrate chains, two of which are located in kringle domain 1 and one of which is present within the serine protease domain. The latter of these has been the focus of structural analyses that have identified slight differences between human and bovine carbohydrate as well as minor microheterogeneity within the single carbohydrate chain attached to human thrombin.[53–55] This oligosaccharide chain is linked to Asn53 in human thrombin (thrombin heavy-chain numbering) and has been characterized as a sialated biantennary glycan of the *N*-acetyl-lactosamine prototype, with fucose present in 50% of the carbohydrate chains.[55]

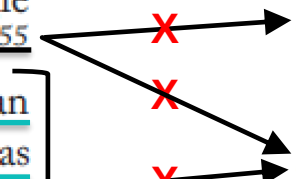

54. Mizuochi T, Fujii J, Kisiel W, Kobata A. Studies on the structures of the carbohydrate moiety of human prothrombin. J Biochem (Tokyo) 1981;90:1023–1031

55. Nilsson B, Horne MK III, Gralnick HR. The carbohydrate of human thrombin: structural analysis of glycoprotein oligosaccharides by mass spectrometry. Arch Biochem Biophys 1983;224:127–133

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Davie et al *An Overview of the Structure and Function of Thrombin*, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006**

Both bovine and human prothrombin contain three N-linked carbohydrate chains, two of which are located in kringle domain 1 and one of which is present within the serine protease domain. The latter of these has been the focus of structural analyses that have identified slight differences between human and bovine carbohydrate as well as minor microheterogeneity within the single carbohydrate chain attached to human thrombin.[53–55] This oligosaccharide chain is linked to Asn53 in human thrombin (thrombin heavy-chain numbering) and has been characterized as a sialated biantennary glycan of the *N*-acetyl-lactosamine prototype, with fucose present in 50% of the carbohydrate chains.[55]



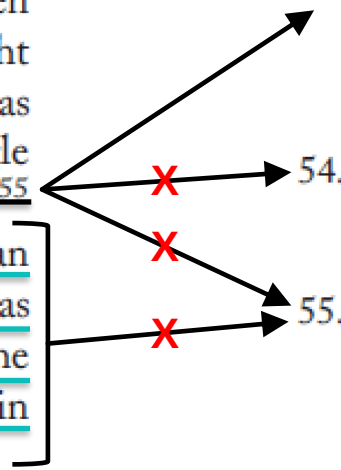

54. Mizuochi T, Fujii J, Kisiel W, Kobata A. Studies on the structures of the carbohydrate moiety of human prothrombin. J Biochem (Tokyo) 1981;90:1023–1031

55. Nilsson B, Horne MK III, Gralnick HR. The carbohydrate of human thrombin: structural analysis of glycoprotein oligosaccharides by mass spectrometry. Arch Biochem Biophys 1983;224:127–133

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Davie et al *An Overview of the Structure and Function of Thrombin*, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006**

Both bovine and human prothrombin contain three N-linked carbohydrate chains, two of which are located in kringle domain 1 and one of which is present within the serine protease domain. The latter of these has been the focus of structural analyses that have identified slight differences between human and bovine carbohydrate as well as minor microheterogeneity within the single carbohydrate chain attached to human thrombin.[53–55] This oligosaccharide chain is linked to Asn53 in human thrombin (thrombin heavy-chain numbering) and has been characterized as a sialated biantennary glycan of the *N*-acetyl-lactosamine prototype, with fucose present in 50% of the carbohydrate chains.[55]




53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

54. Mizuochi T, Fujii J, Kisiel W, Kobata A. Studies on the structures of the carbohydrate moiety of human prothrombin. J Biochem (Tokyo) 1981;90:1023–1031

55. Nilsson B, Horne MK III, Gralnick HR. The carbohydrate of human thrombin: structural analysis of glycoprotein oligosaccharides by mass spectrometry. Arch Biochem Biophys 1983;224:127–133

# Bovine α-Thrombin N-Glycan ID: Literature Search

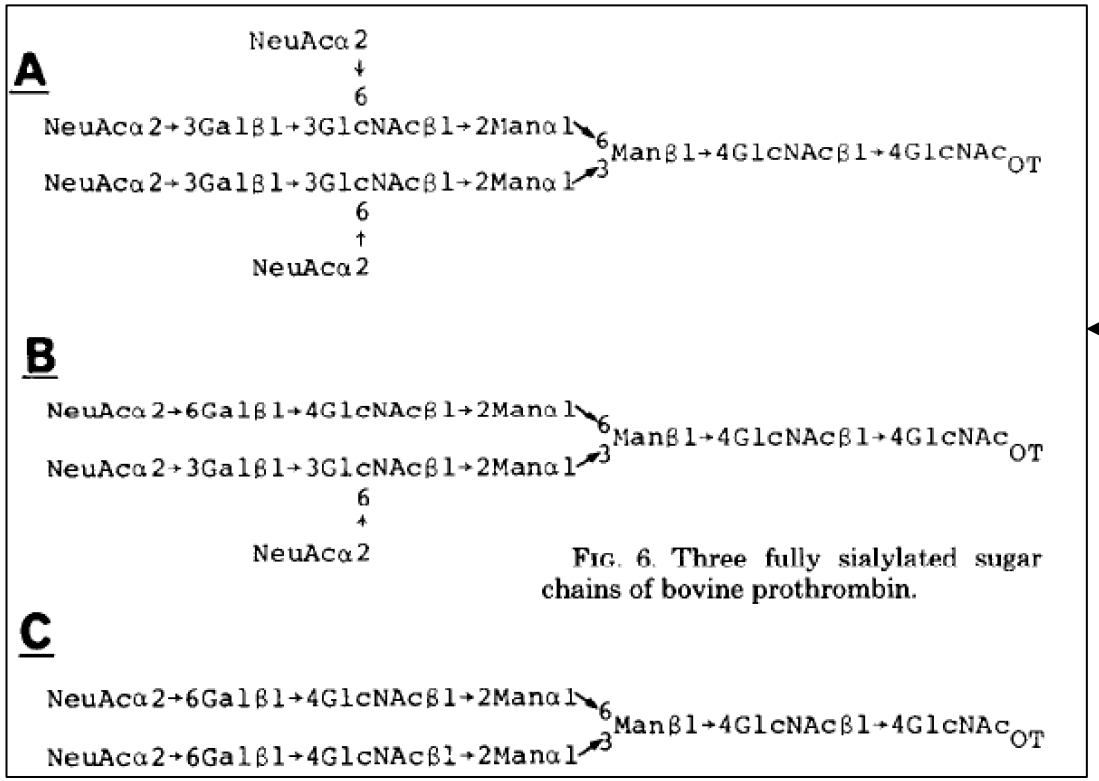



FIG. 6. Three fully sialylated sugar chains of bovine prothrombin.

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

# Bovine α-Thrombin N-Glycan ID: Literature Search

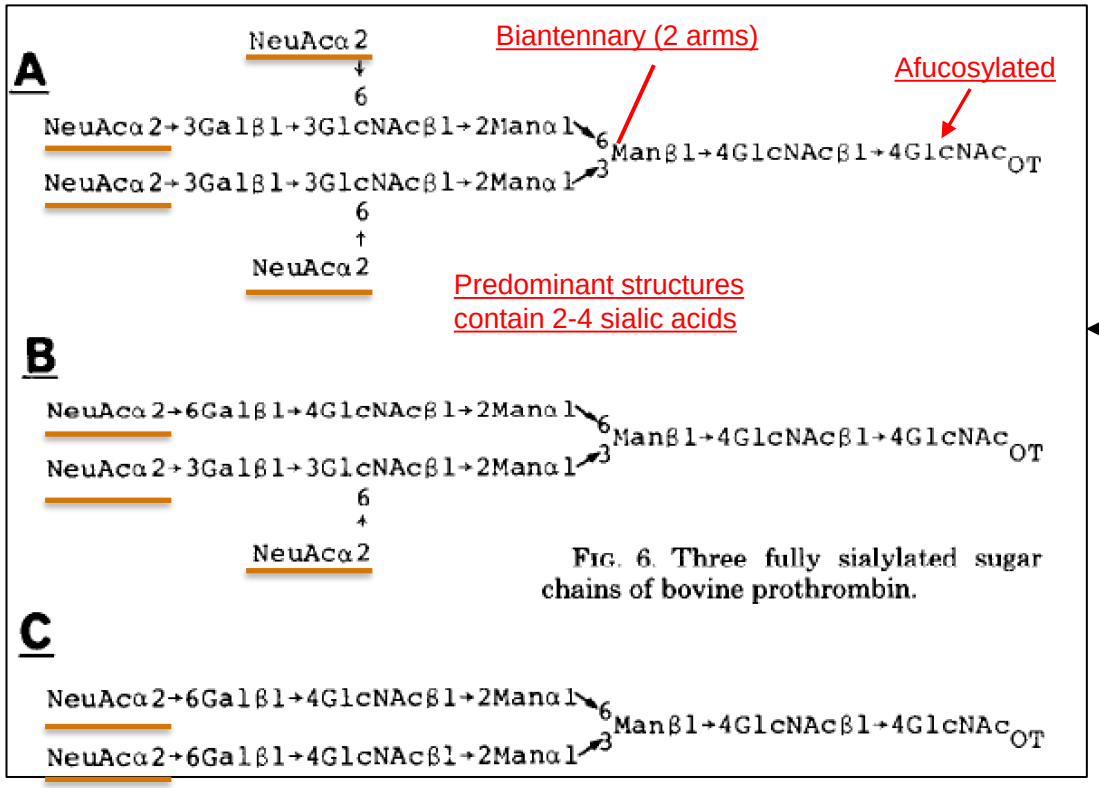



FIG. 6. Three fully sialylated sugar chains of bovine prothrombin.

Biantennary (2 arms)

Afucosylated

Predominant structures contain 2-4 sialic acids

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

**G2 + NeuAc**

# Bovine α-Thrombin N-Glycan ID: Literature Search

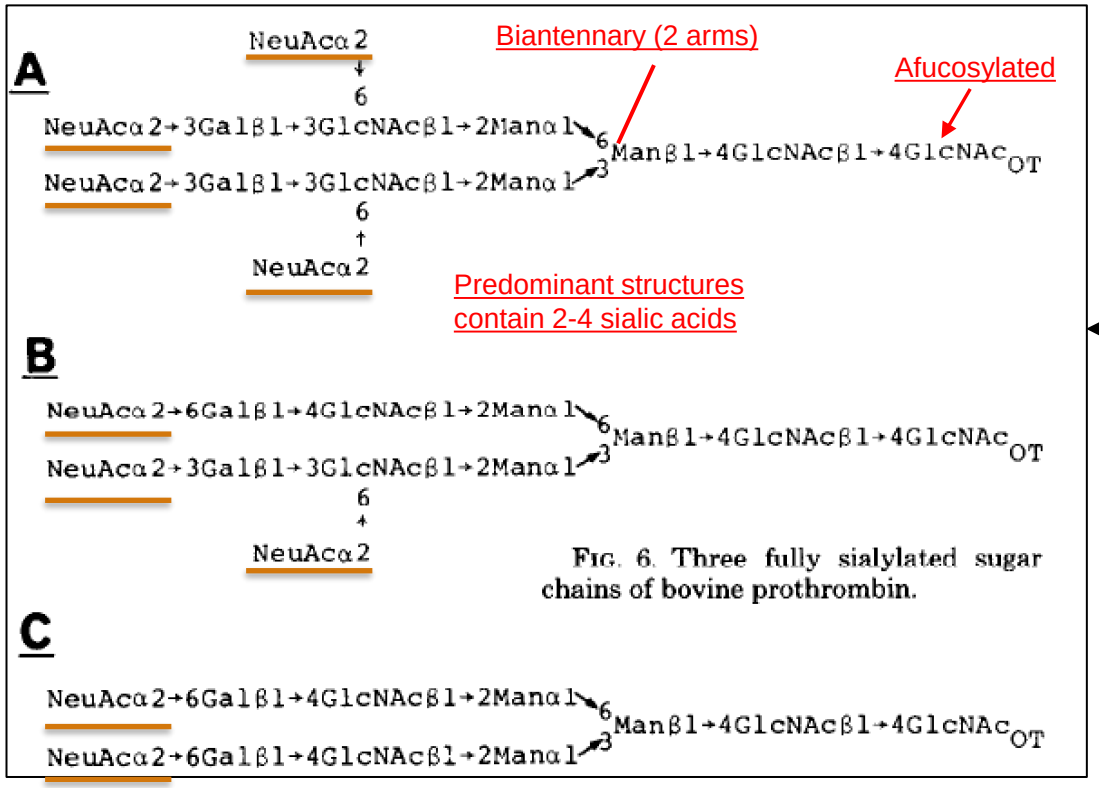



FIG. 6. Three fully sialylated sugar chains of bovine prothrombin.

Biantennary (2 arms)

Afucosylated

Predominant structures contain 2-4 sialic acids

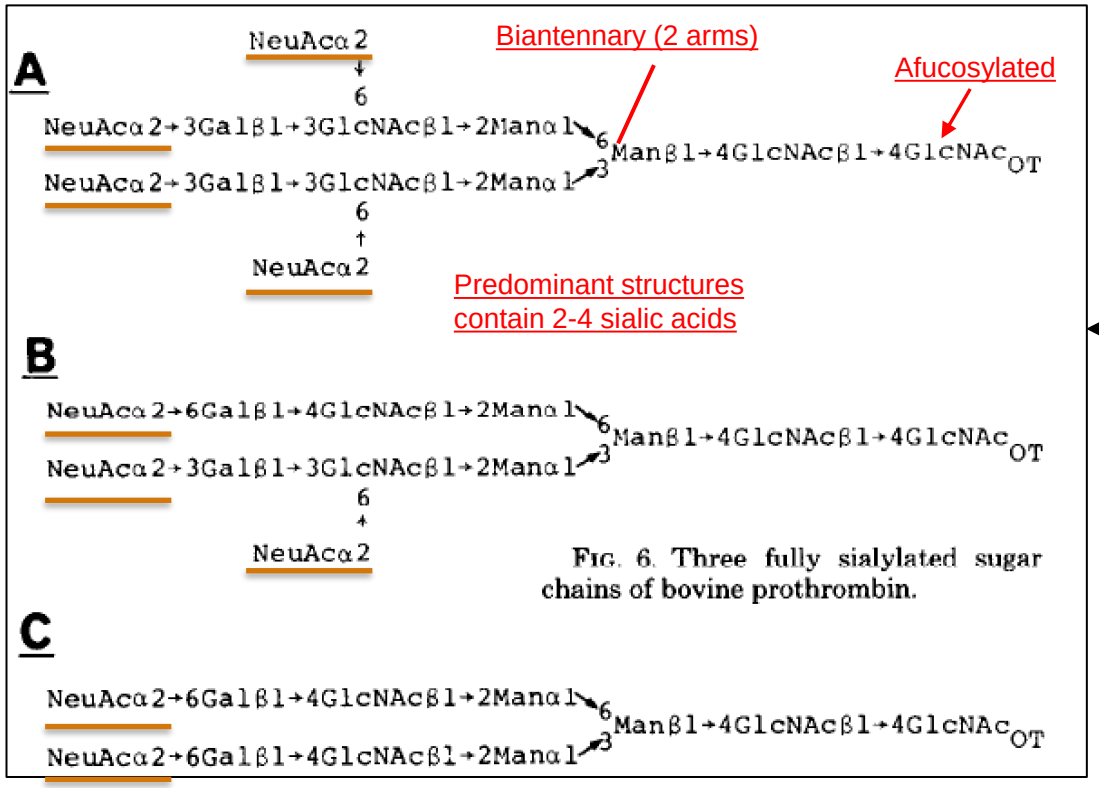
53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

**G2 + NeuAc**

31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

# Bovine α-Thrombin N-Glycan ID: Literature Search




Biantennary (2 arms)

Afucosylated

Predominant structures contain 2-4 sialic acids

FIG. 6. Three fully sialylated sugar chains of bovine prothrombin.

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

**G2 + NeuAc**

**G2 + NeuGc and/or NeuAc**

31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

# Bovine α-Thrombin N-Glycan ID: Literature Search

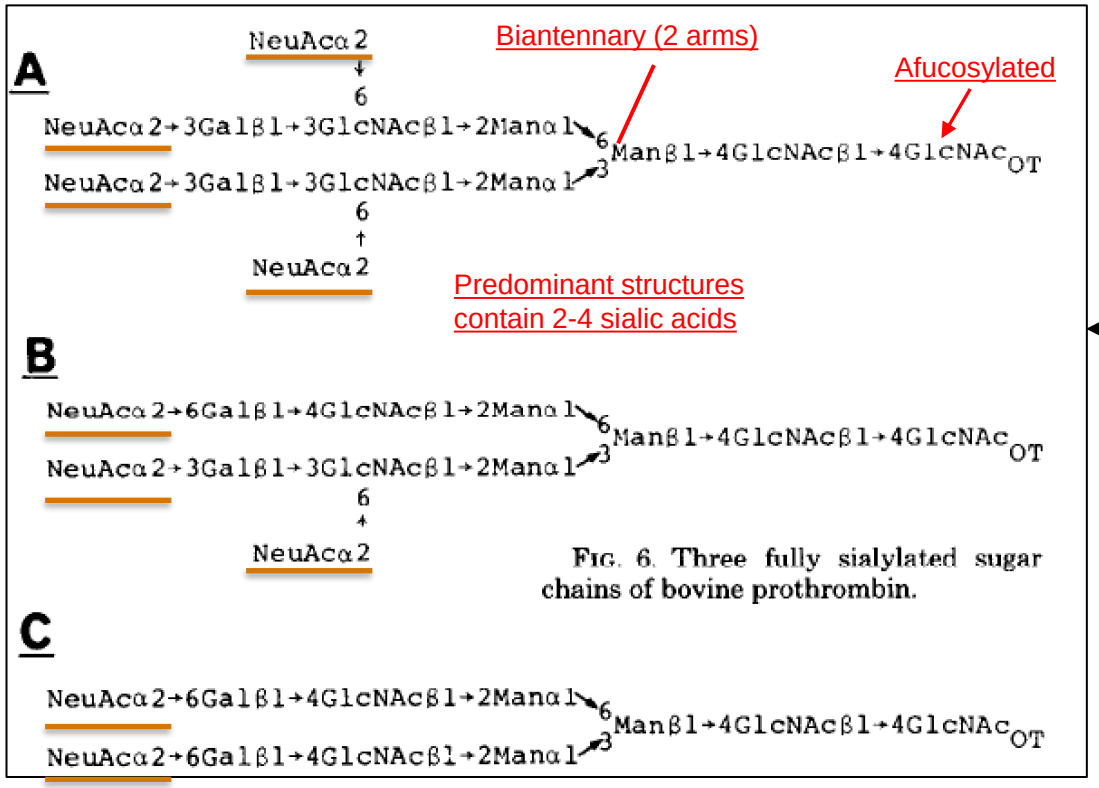



FIG. 6. Three fully sialylated sugar chains of bovine prothrombin.

Biantennary (2 arms)

Afucosylated

Predominant structures contain 2-4 sialic acids

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

**G2 + NeuAc**

X

**G2 + NeuGc and/or NeuAc**

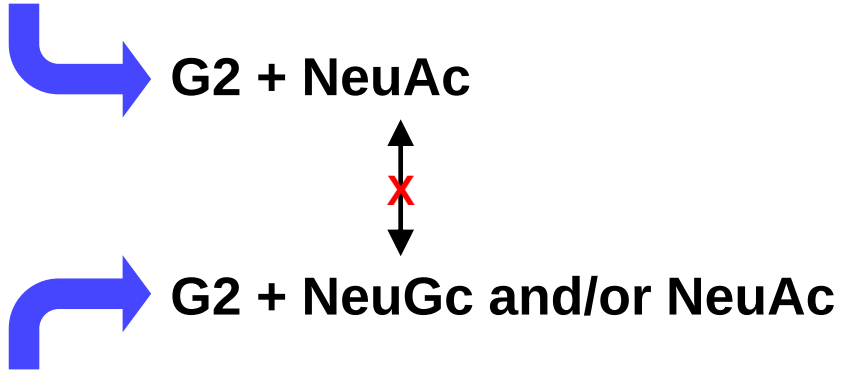
31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Thrombin from Bovine Plasma: Sigma PN T4648**

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425




31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

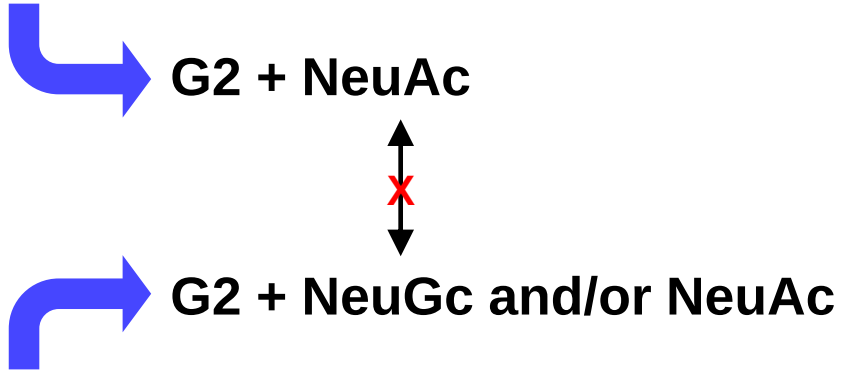
# Bovine α-Thrombin N-Glycan ID: Literature Search

**Thrombin from Bovine Plasma: Sigma PN T4648**

→for bovine glycan

**Magnusson - Thrombin and Prothrombin, The Enzymes Vol. 2 1971**

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425




31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

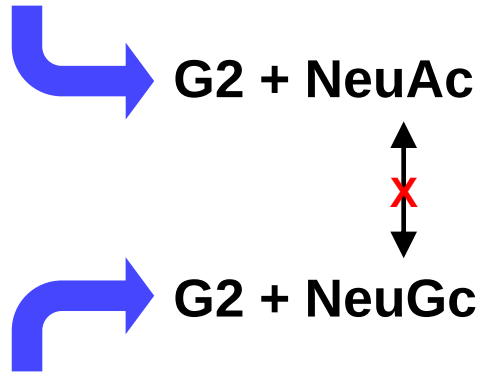
# Bovine α-Thrombin N-Glycan ID: Literature Search

**Thrombin from Bovine Plasma: Sigma PN T4648**

→for bovine glycan

**Magnusson - Thrombin and Prothrombin, The Enzymes Vol. 2 1971**

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425




31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

# Bovine α-Thrombin N-Glycan ID: Literature Search

**Thrombin from Bovine Plasma: Sigma PN T4648**

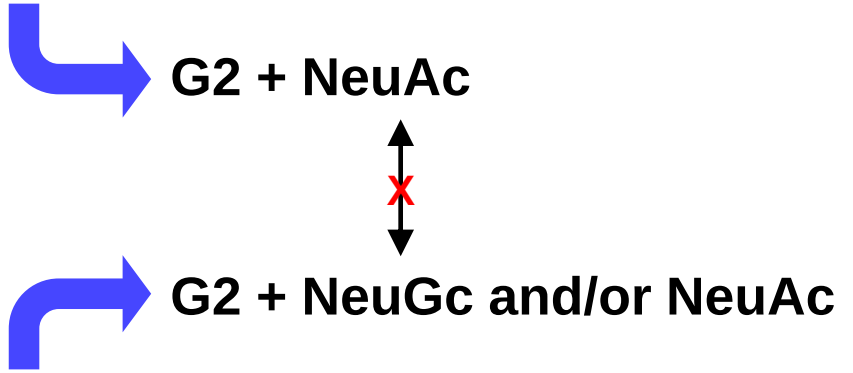
→for bovine glycan

**Magnusson - Thrombin and Prothrombin, The Enzymes Vol. 2 1971**

→for MW via SDS PAGE

**Malkowski et al, Protein Science, 1997**

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425




31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247,** 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

# Bovine α-Thrombin N-Glycan ID: Literature Search

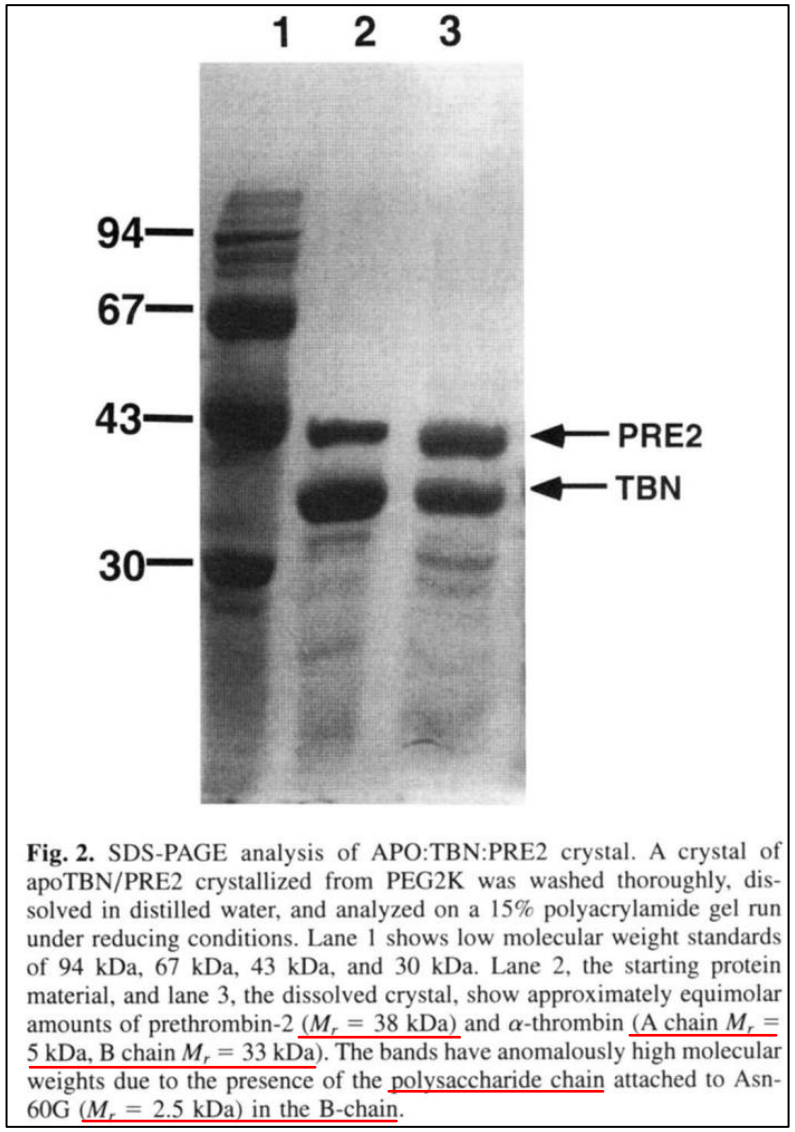



**Fig. 2.** SDS-PAGE analysis of APO:TBN:PRE2 crystal. A crystal of apoTBN/PRE2 crystallized from PEG2K was washed thoroughly, dissolved in distilled water, and analyzed on a 15% polyacrylamide gel run under reducing conditions. Lane 1 shows low molecular weight standards of 94 kDa, 67 kDa, 43 kDa, and 30 kDa. Lane 2, the starting protein material, and lane 3, the dissolved crystal, show approximately equimolar amounts of prethrombin-2 ($M_r$ = 38 kDa) and α-thrombin (A chain $M_r$ = 5 kDa, B chain $M_r$ = 33 kDa). The bands have anomalously high molecular weights due to the presence of the polysaccharide chain attached to Asn-60G ($M_r$ = 2.5 kDa) in the B-chain.

**Thrombin from Bovine Plasma: Sigma PN T4648**

→for bovine glycan

**Magnusson - Thrombin and Prothrombin, The Enzymes Vol. 2 1971**

→for MW via SDS PAGE

**Malkowski et al, Protein Science, 1997**

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

**G2 + NeuAc**

X

**G2 + NeuGc and/or NeuAc**

31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

# Bovine α-Thrombin N-Glycan ID: Literature Search

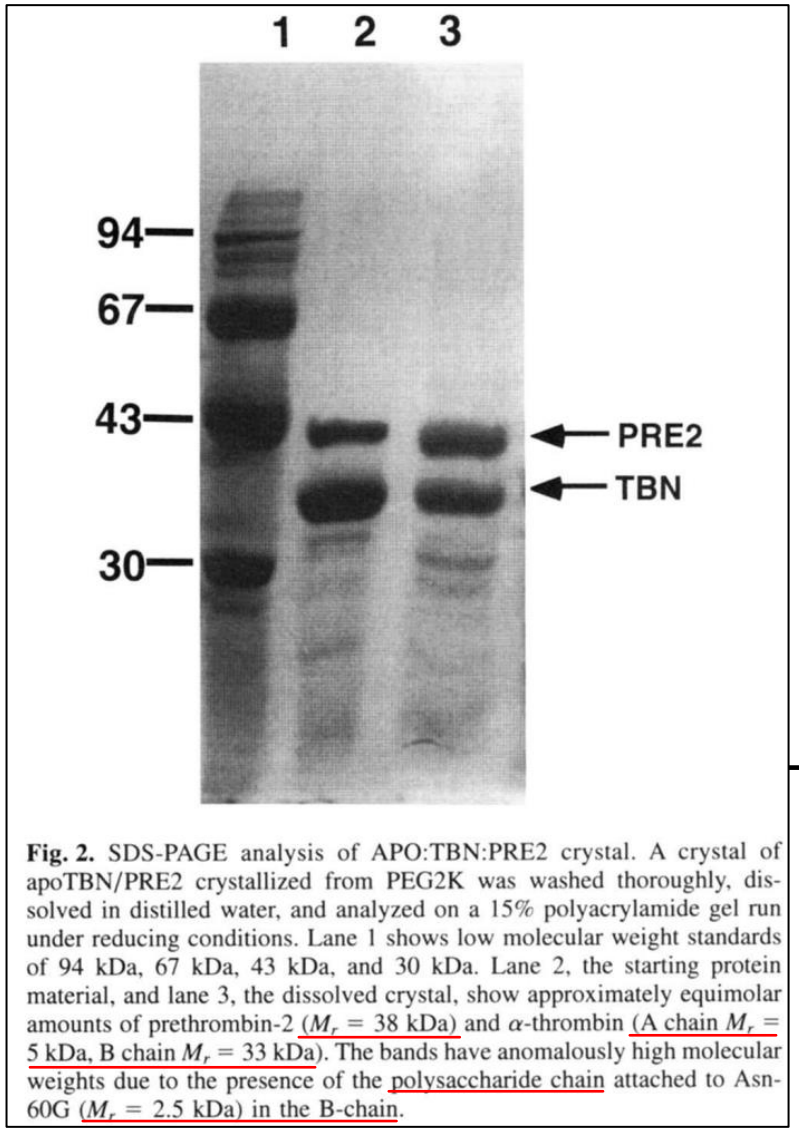



**Fig. 2.** SDS-PAGE analysis of APO:TBN:PRE2 crystal. A crystal of apoTBN/PRE2 crystallized from PEG2K was washed thoroughly, dissolved in distilled water, and analyzed on a 15% polyacrylamide gel run under reducing conditions. Lane 1 shows low molecular weight standards of 94 kDa, 67 kDa, 43 kDa, and 30 kDa. Lane 2, the starting protein material, and lane 3, the dissolved crystal, show approximately equimolar amounts of prethrombin-2 ($M_r$ = 38 kDa) and α-thrombin (A chain $M_r$ = 5 kDa, B chain $M_r$ = 33 kDa). The bands have anomalously high molecular weights due to the presence of the polysaccharide chain attached to Asn-60G ($M_r$ = 2.5 kDa) in the B-chain.

**Thrombin from Bovine Plasma: Sigma PN T4648**

→for bovine glycan

**Magnusson - Thrombin and Prothrombin, The Enzymes Vol. 2 1971**

→for MW via SDS PAGE

**Malkowski et al, Protein Science, 1997**

→ **MW of N-Glycan = 2.5 kDa**

53. Mizuochi T, Yamashita K, Fujikawa K, Kisiel W, Kobata A. The carbohydrate of bovine prothrombin. Occurrence of Gal beta 1 leads to 3GlcNAc grouping in asparagine-linked sugar chains. J Biol Chem 1979;254:6419–6425

**G2 + NeuAc**

X

**G2 + NeuGc and/or NeuAc**

31. Nelsestuen, G. L., and Suttie, J. W. (1972) *J. Biol. Chem.* **247**, 6096–6102

Nelsestuen and Suttie (31) reported that bovine prothrombin contains both *N*-glycolyl and *N*-acetylneuraminic acid.

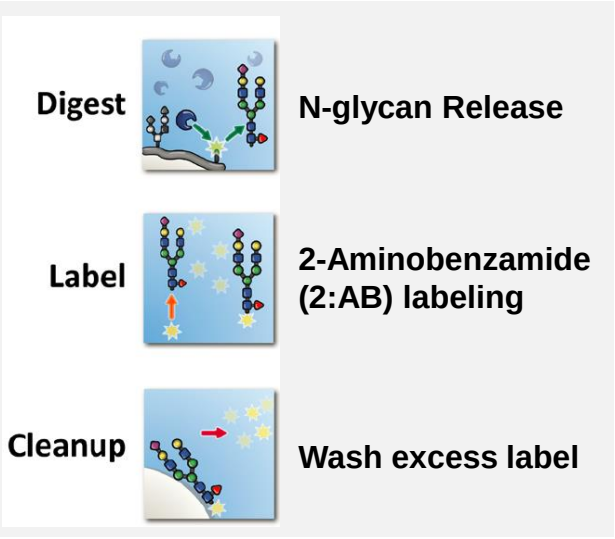
# Objectives

## Profiling Analysis of Released N-Glycans by LC-MS

- Characterize N-glycan profile from bovine plasma thrombin
- Determine if 16 Da mass differences from the intact thrombin analysis is related to N-glycan heterogeneity

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

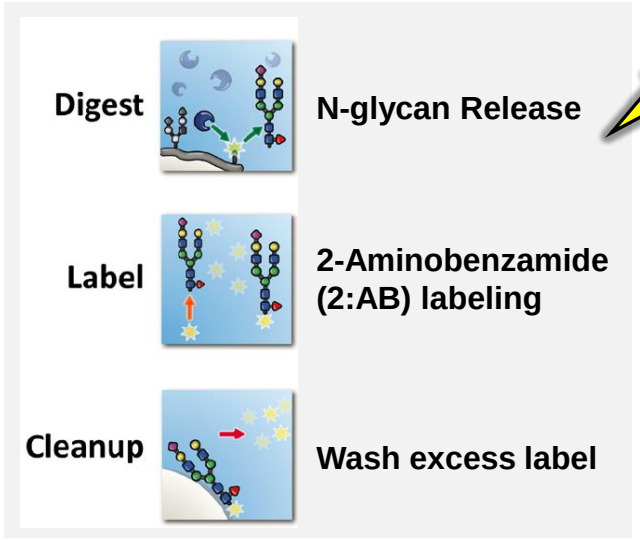
## 2-AB Labeled N-Glycan Analysis



# Profiling Analysis of Released N-Glycans by LC-MS: 2:AB

## 2-AB Labeled N-Glycan Analysis

**Native enzymatic release via PNGase F at 37°C overnight**



# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

## 2-AB Labeled N-Glycan Analysis by NP-AEX-MS

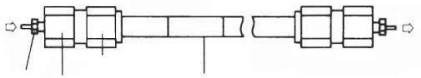
**Digest** — **N-glycan Release**

**Label** — **2-Aminobenzamide (2:AB) labeling**

**Cleanup** — **Wash excess label**

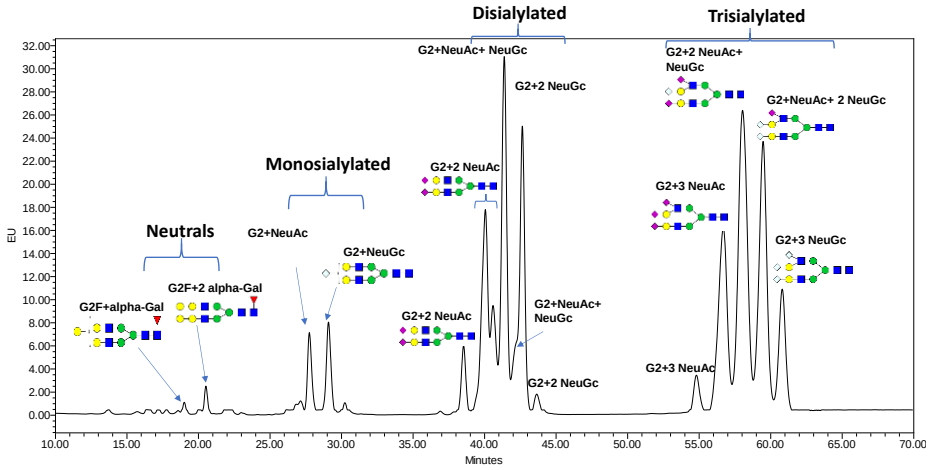
**Waters H-Class Bio UPLC**

**NP-AEX Separation**



**Chromatography geared toward highly sialylated (charged) N-glycans**

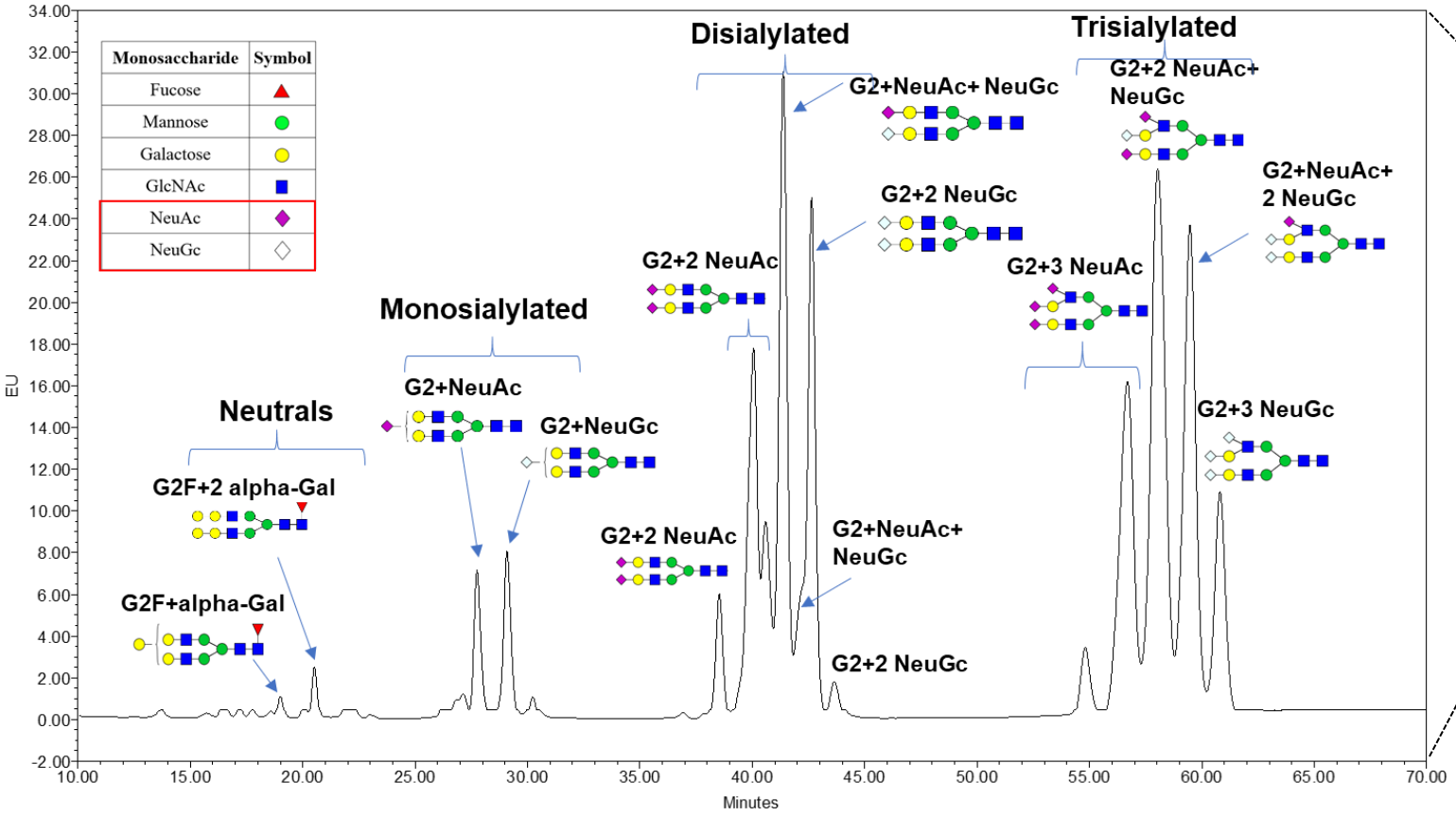
**Thermo Exactive Plus EMR**

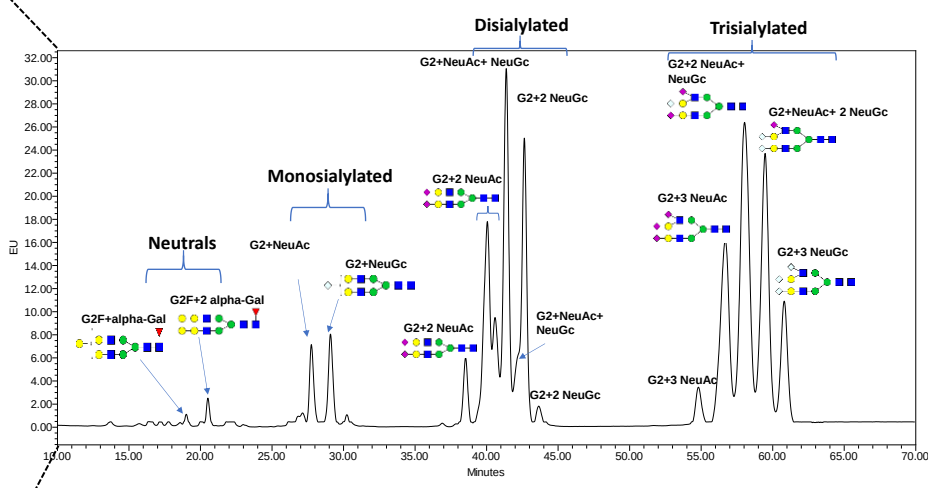


# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**



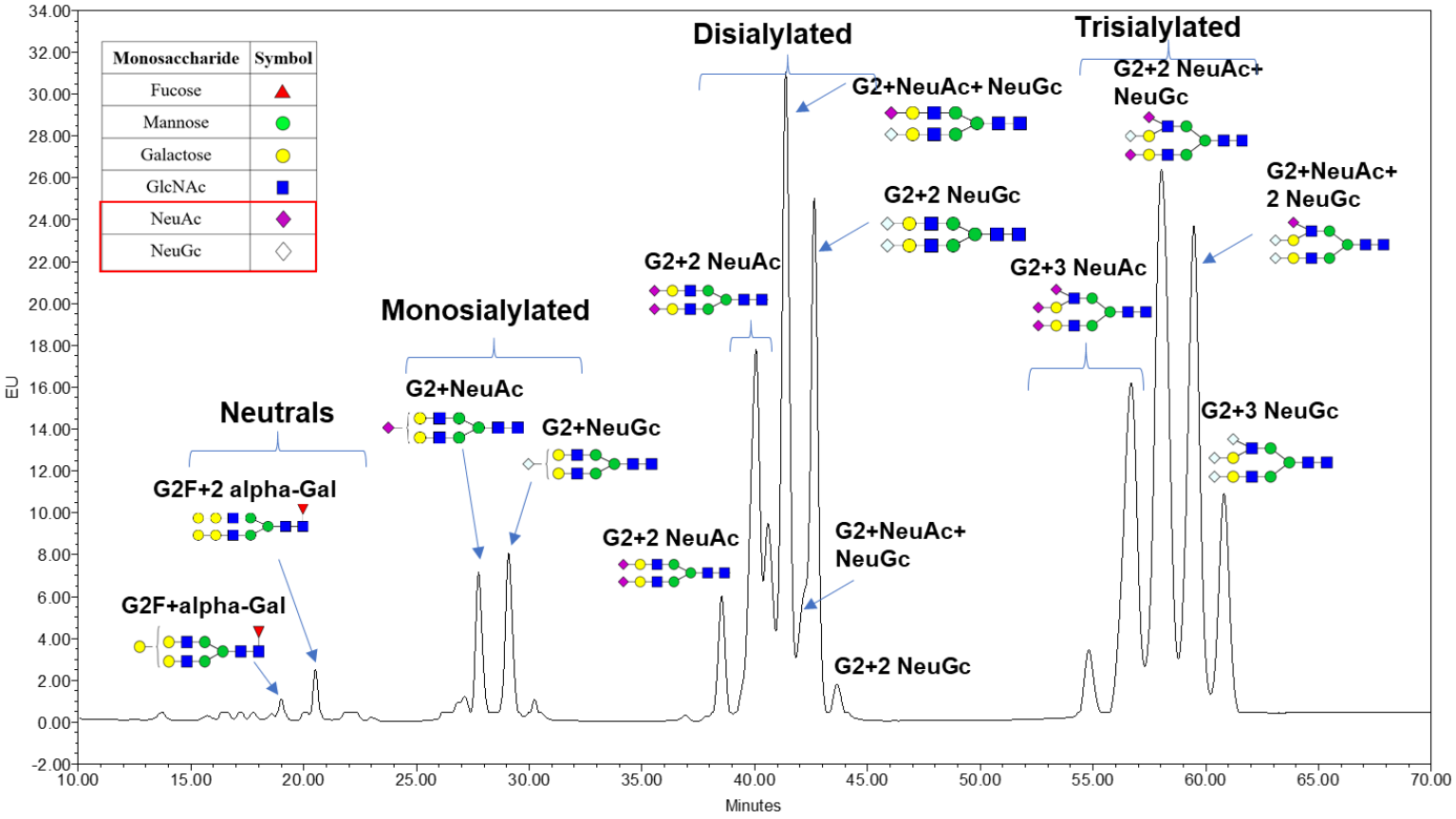
# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**

- **Sialic acids are mixture of NeuAc and NeuGc**

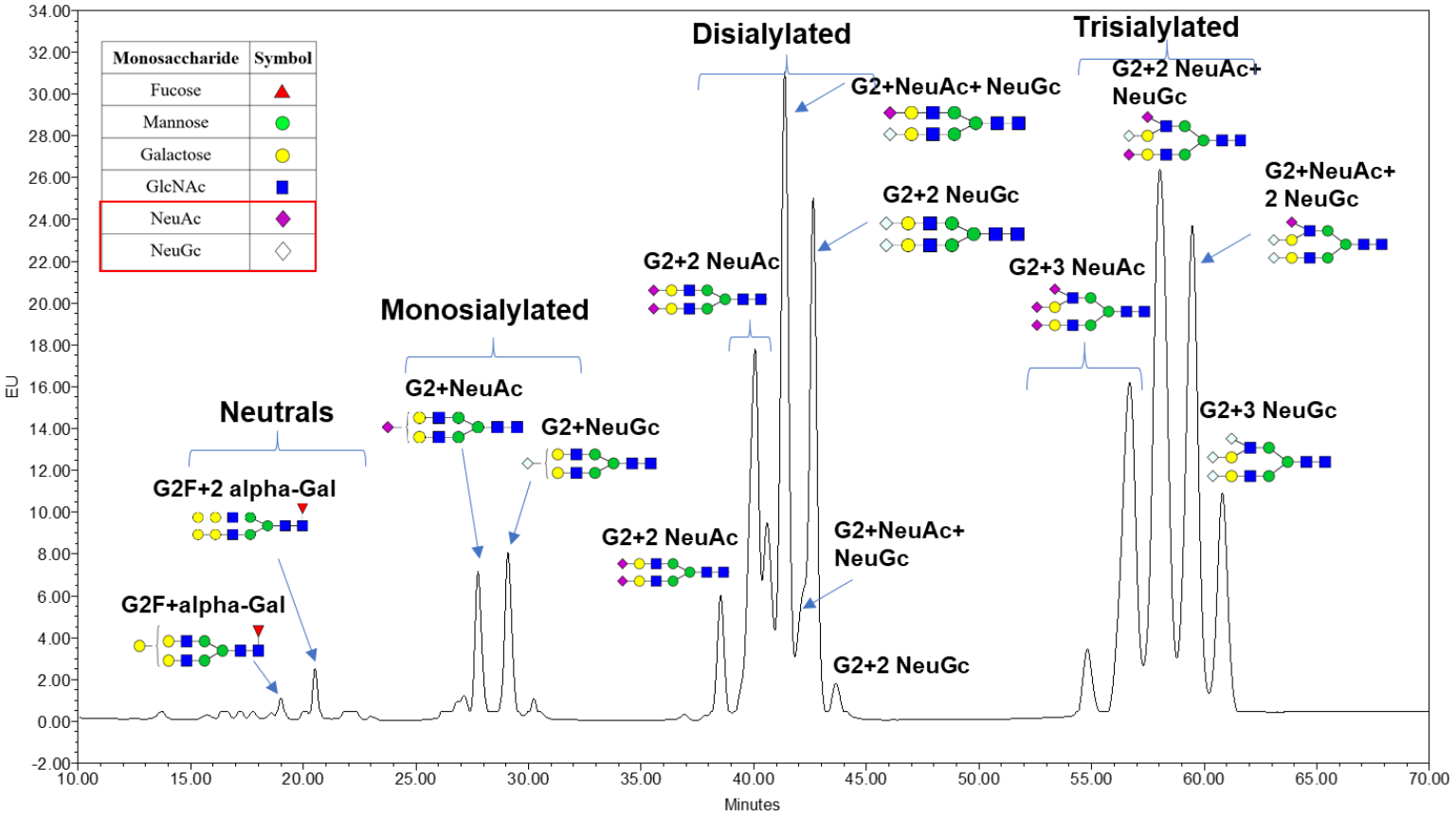
►16 Da mass difference supports intact data



# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**




- **Sialic acids are mixture of NeuAc and NeuGc**

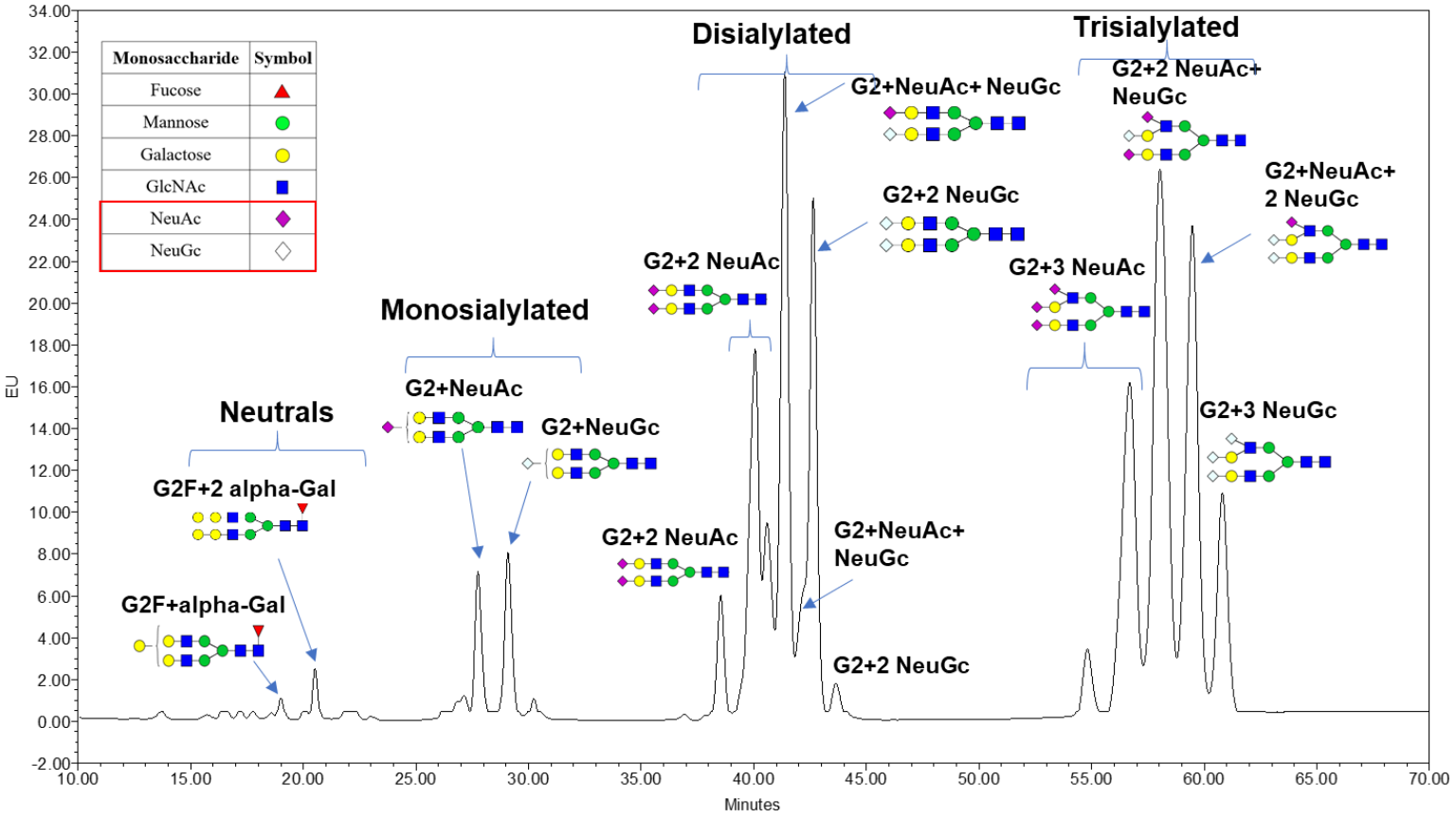
►16 Da mass difference supports intact data

- **Predominant structures contained two and three sialic acids**

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**




- **Sialic acids are mixture of NeuAc and NeuGc**

►16 Da mass difference supports intact data

- **Predominant structures contained two and three sialic acids**

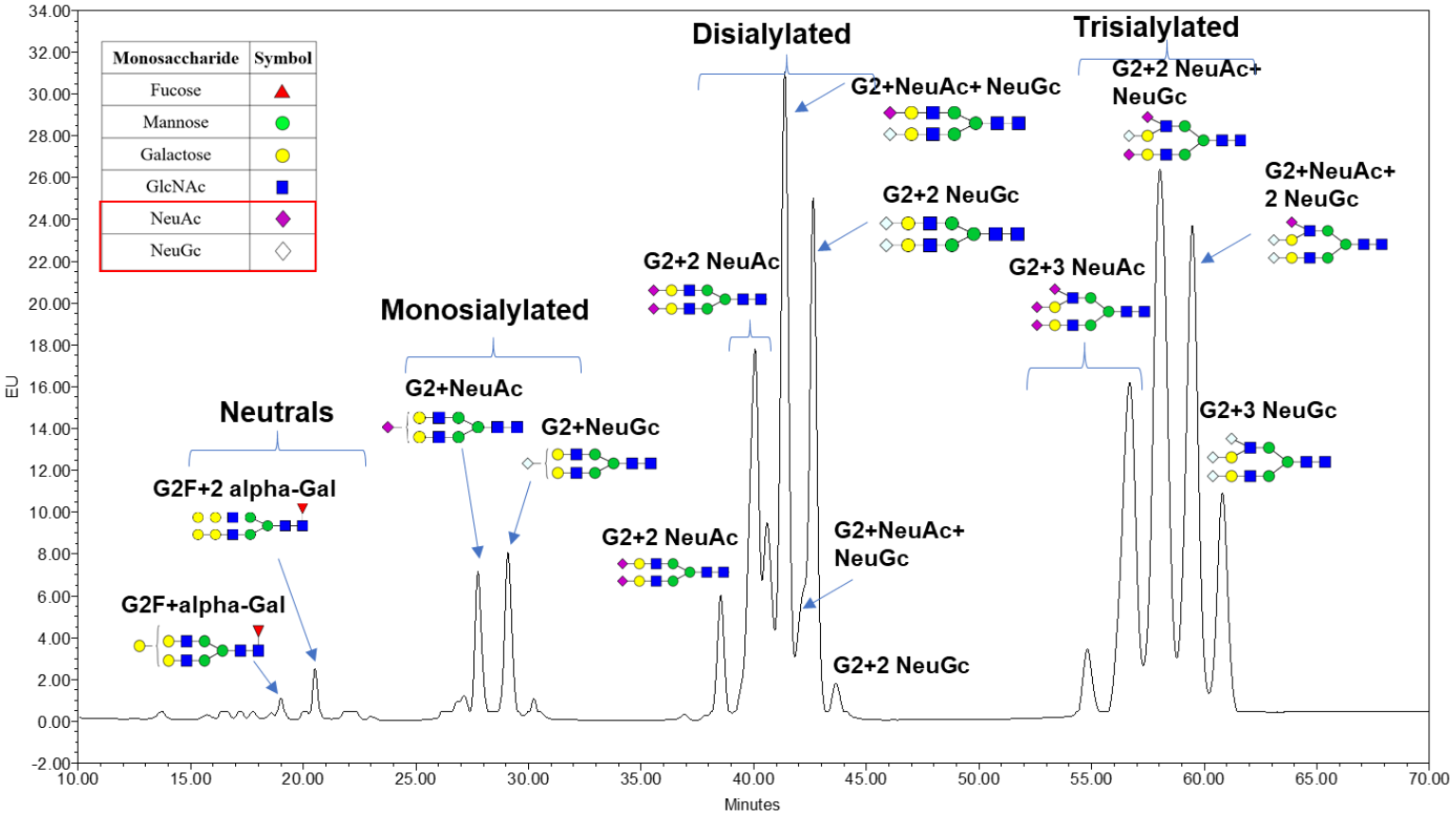
- **Multiple peaks observed per monosaccharide composition**

►Trisialylated structures are much wider

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**




- **Sialic acids are mixture of NeuAc and NeuGc**

►16 Da mass difference supports intact data

- **Predominant structures contained two and three sialic acids**

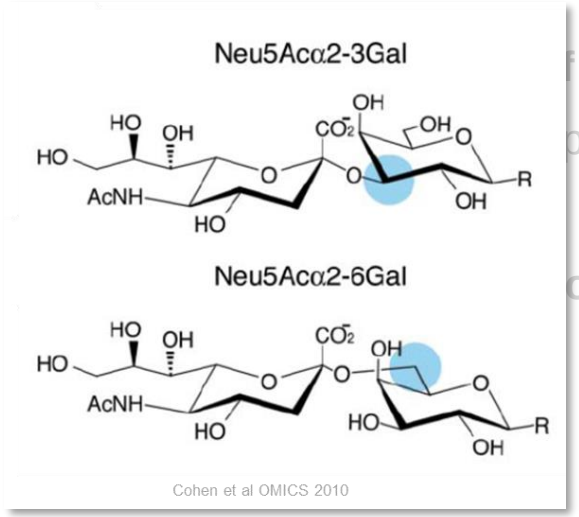
- **Multiple peaks observed per monosaccharide composition**

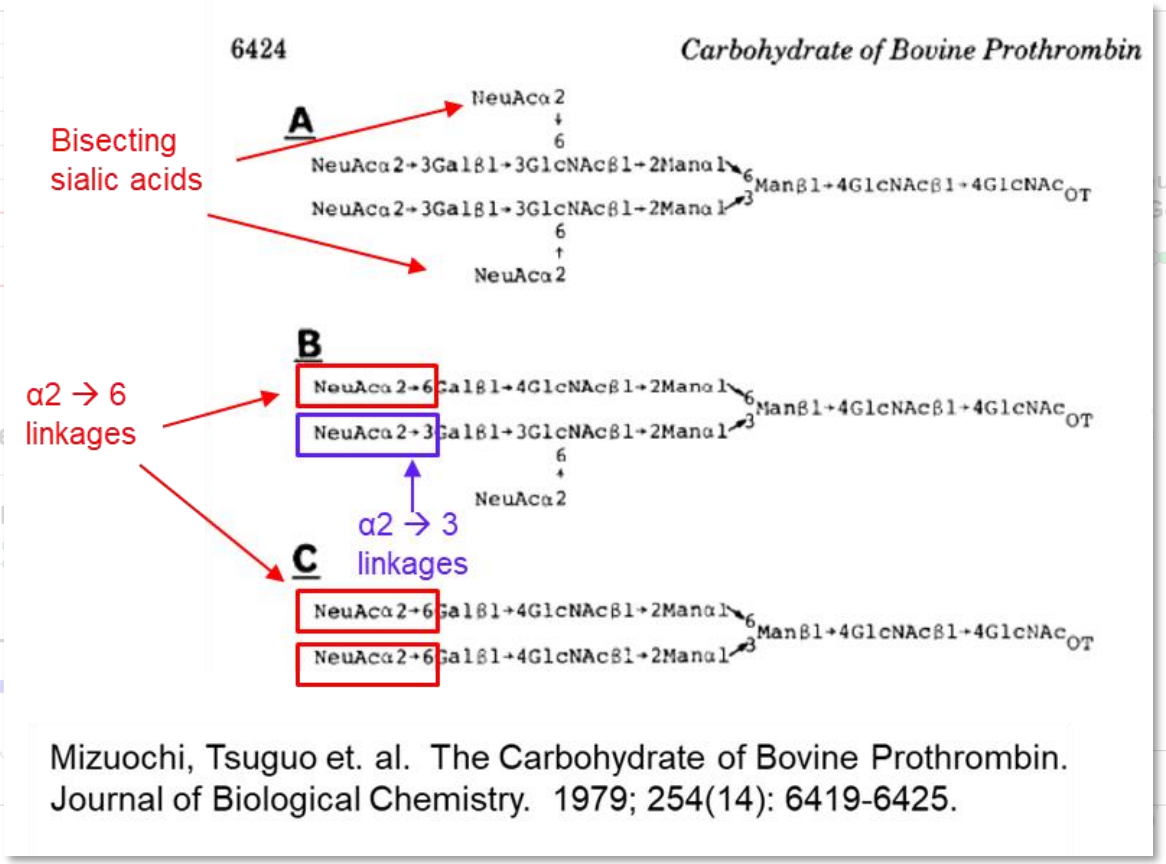
►Trisialylated structures are much wider

Why?

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans**




- **Multiple peaks observed per monosaccharide composition**

►Trisialylated structures are much wider

Why?

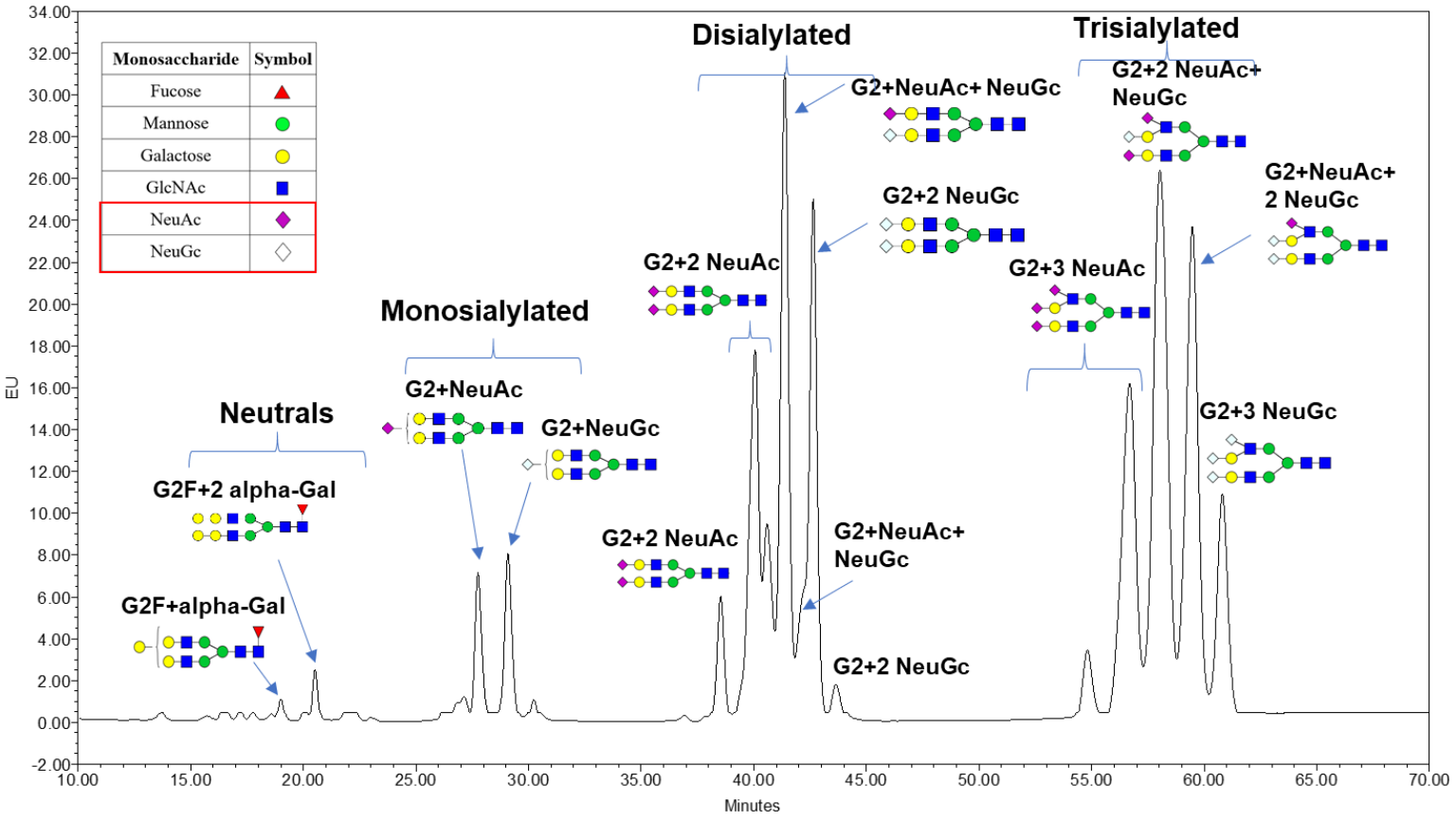
►Sialic Acids are located on the terminal Gal or antennal GlcNAc residues

►Sialic Acids contain α2-3 and α2-6 linkages

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**




- **Sialic acids are mixture of NeuAc and NeuGc**

►16 Da mass difference supports intact data

- **Predominant structures contained two and three sialic acids**

- **Multiple peaks observed per monosaccharide composition**

►Trisialylated structures are much wider

Why?

►Sialic Acids are located on the terminal Gal or antennal GlcNAc residues

►Sialic Acids contain α2-3 and α2-6 linkages

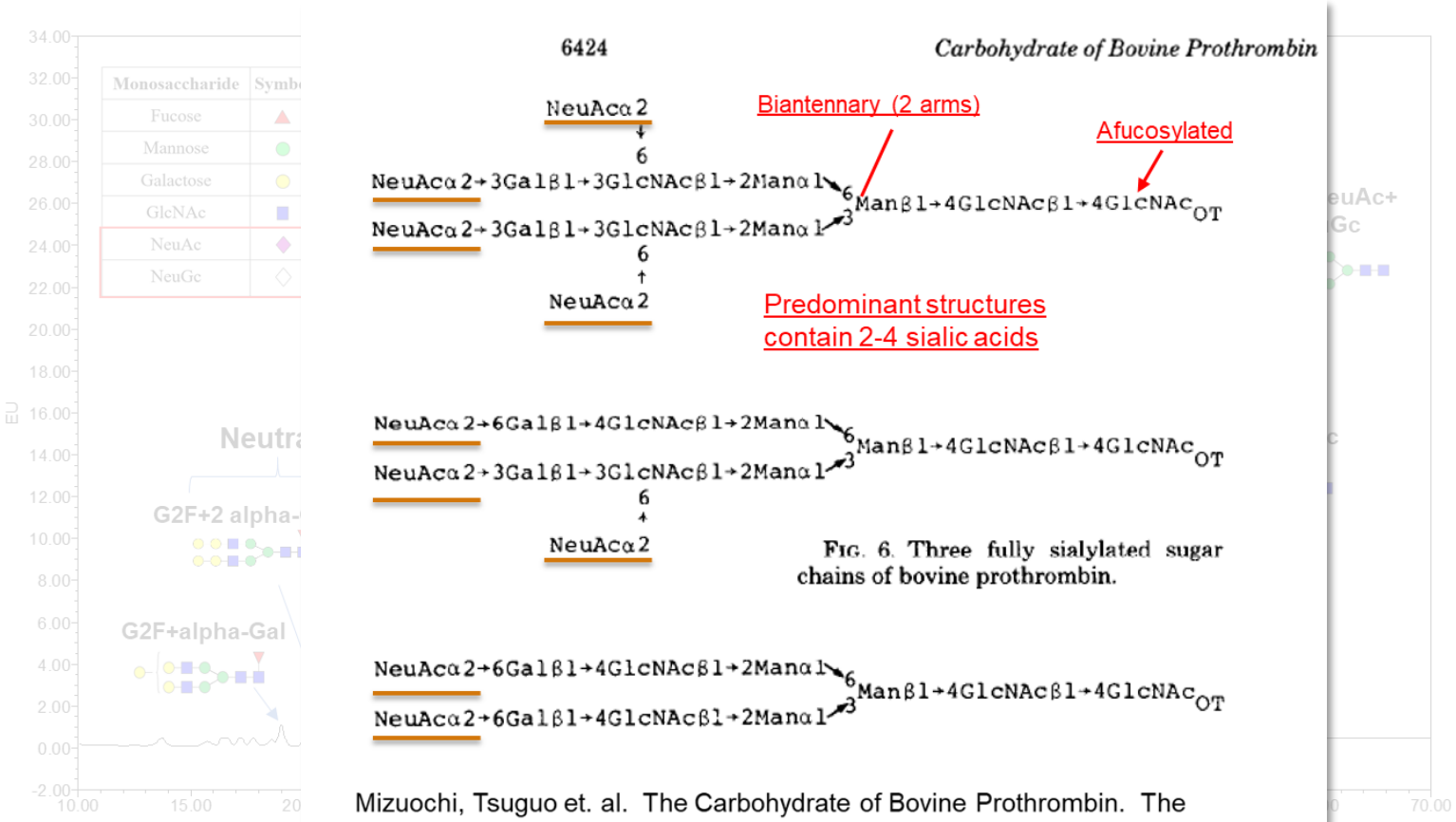
►These differences result in multiple elution positions for the same N-glycan composition

►Wider peaks due to bisecting GlcNAc

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**




6424 *Carbohydrate of Bovine Prothrombin*

Biantennary (2 arms)

Afucosylated

NeuAcα2→3Galβ1→3GlcNAcβ1→2Manα1→6(3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$

NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$

Predominant structures contain 2-4 sialic acids

NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$

FIG. 6. Three fully sialylated sugar chains of bovine prothrombin.

Mizuochi, Tsuguo et. al. The Carbohydrate of Bovine Prothrombin. The Journal of Biological Chemistry. 1979; 254(14): 6419-6425.

- **Sialic acids are mixture of NeuAc and NeuGc**

►16 Da mass difference supports intact data

- **Predominant structures contained two and three sialic acids**

- **Multiple peaks observed per monosaccharide composition**

►Trisialylated structures are much wider

Why?

►Sialic Acids are located on the terminal Gal or antennal GlcNAc residues

►Sialic Acids contain α2-3 and α2-6 linkages

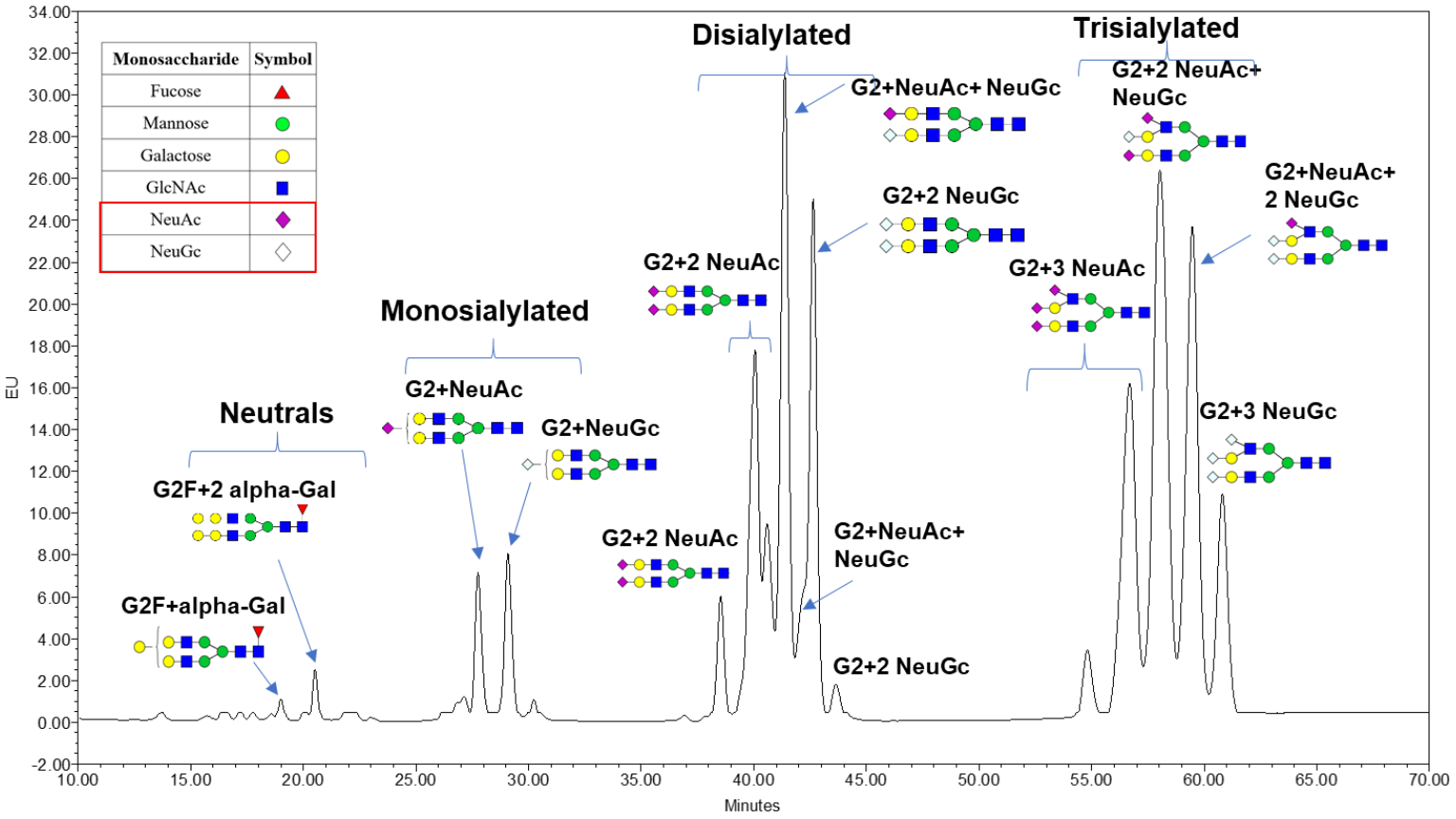
►These differences result in multiple elution positions for the same N-glycan composition

►Wider peaks due to bisecting GlcNAc

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS**

**Exhaustive Elucidation of N-Glycans**




- **Sialic acids are mixture of NeuAc and NeuGc**

►16 Da mass difference supports intact data

- **Predominant structures contained two and three sialic acids**

►Tetrasialylated structures not detected

- **Multiple peaks observed per monosaccharide composition**

►Trisialylated structures are much wider

Why?

►Sialic Acids are located on the terminal Gal or antennal GlcNAc residues

►Sialic Acids contain α2-3 and α2-6 linkages

►These differences result in multiple elution positions for the same N-glycan composition

►Wider peaks due to bisecting GlcNAc

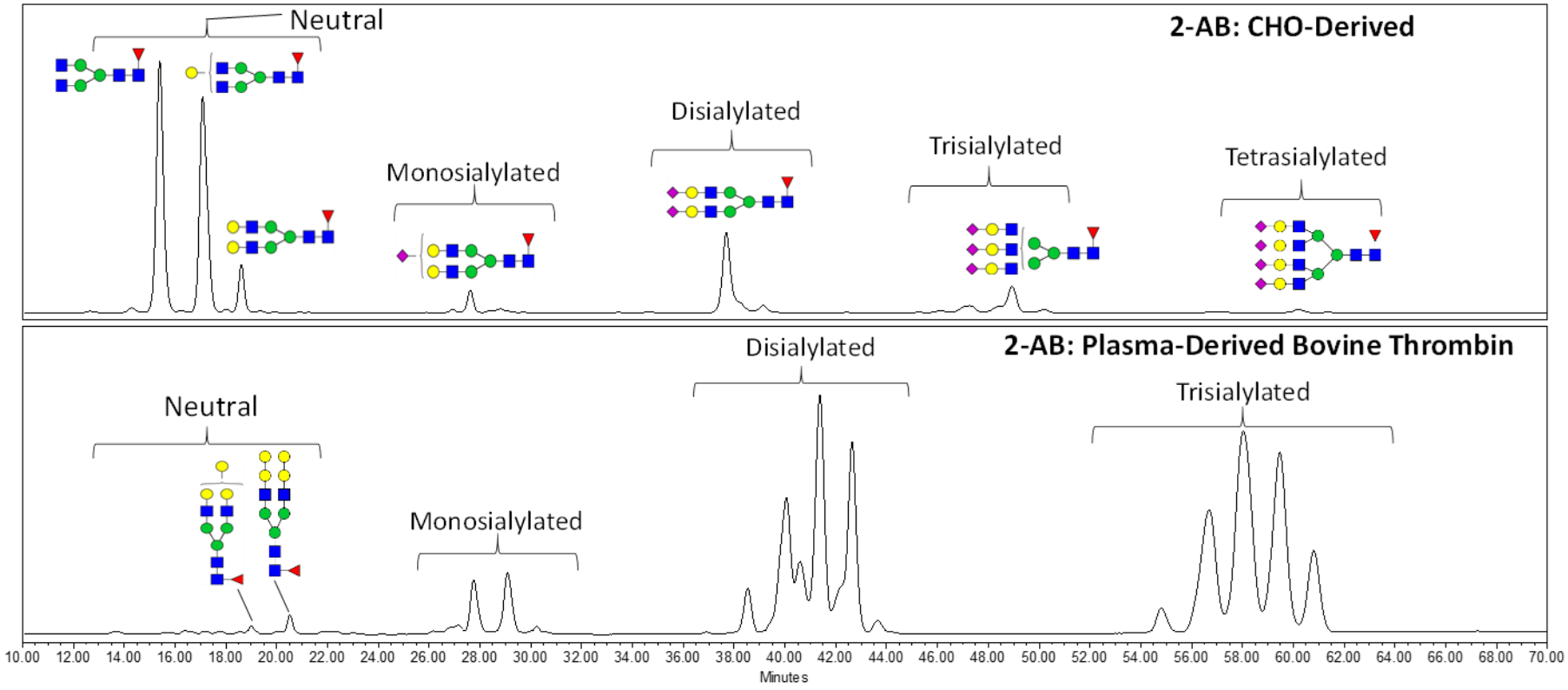
# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein**

**Are tetrasialylated structures recovered from a CHO-derived protein?**

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein**



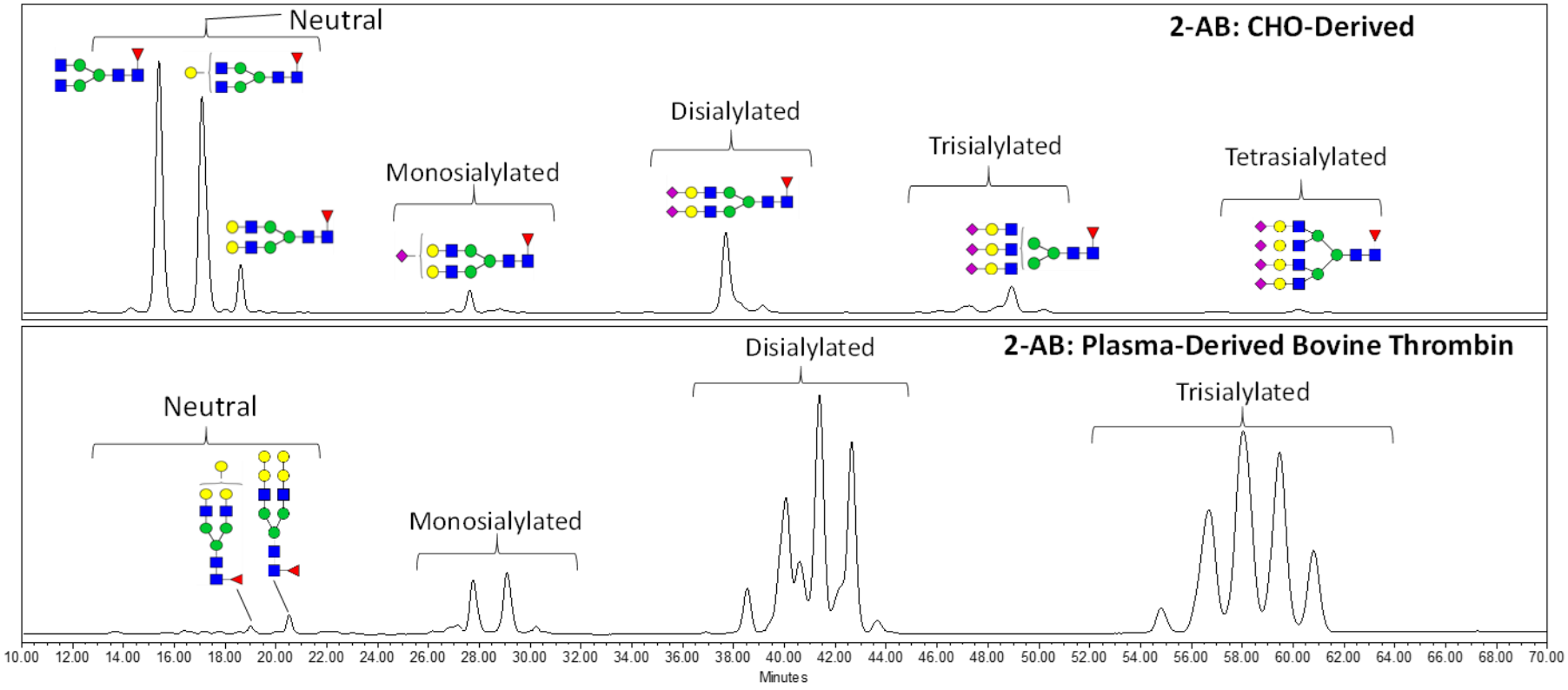

**Are tetrasialylated structures recovered from a CHO-derived protein?**

**►Yes.**

Conformational differences of N-glycans between bovine and CHO likely account for differences in retention times of charged glycans.

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein**




**Are tetrasialylated structures recovered from a CHO-derived protein?**

►**Yes.**

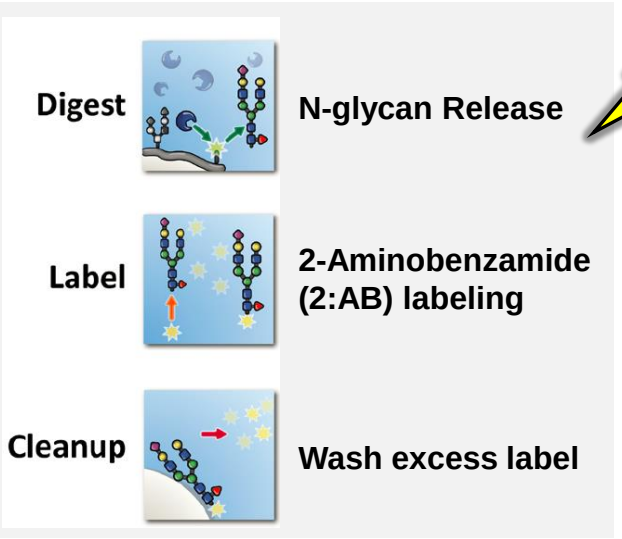
Conformational differences of N-glycans between bovine and CHO likely account for differences in retention times of charged glycans.

►Why are tetrasialylated structures not detected?

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein**

**Native enzymatic release via PNGase F at 37°C overnight**




**Are tetrasialylated structures recovered from a CHO-derived protein?**

►**Yes.**

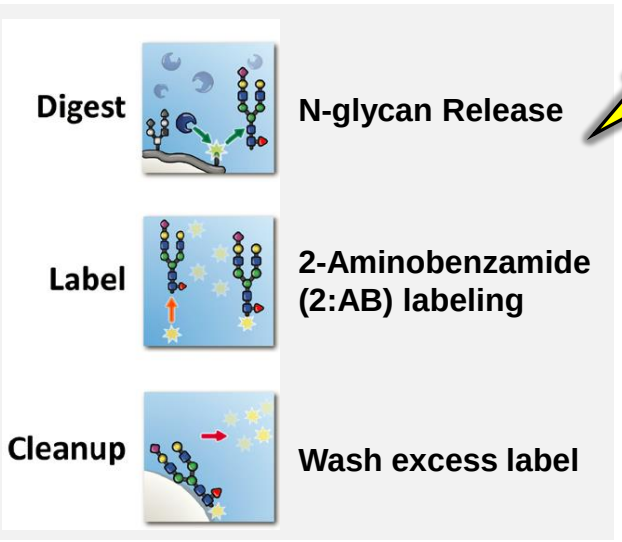
Conformational differences of N-glycans between bovine and CHO likely account for differences in retention times of charged glycans.

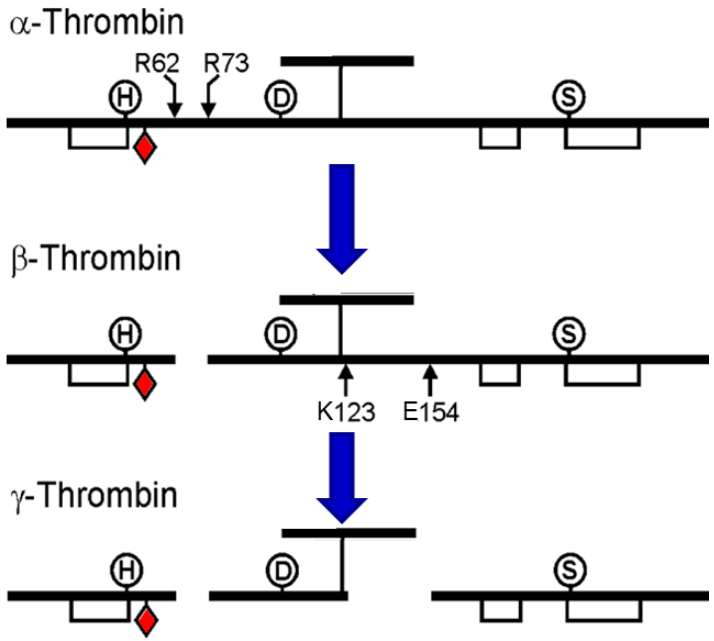
►Why are tetrasialylated structures not detected?

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein**

**Native enzymatic release via PNGase F at 37°C overnight**




**Are tetrasialylated structures recovered from a CHO-derived protein?**

►**Yes.**

Conformational differences of N-glycans between bovine and CHO likely account for differences in retention times of charged glycans.

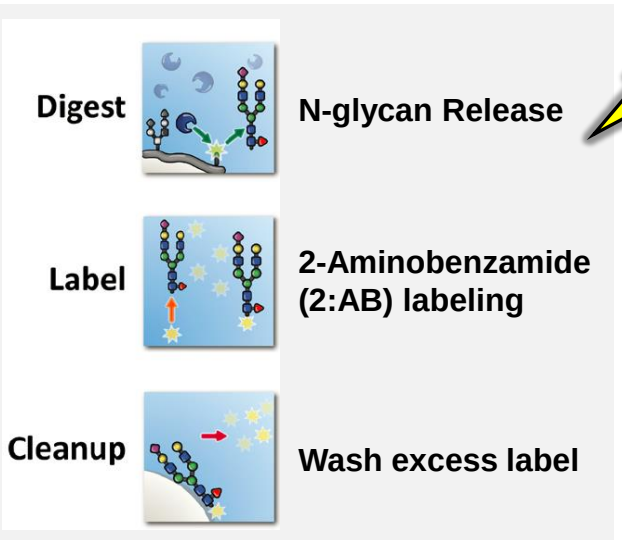
►Why are tetrasialylated structures not detected?



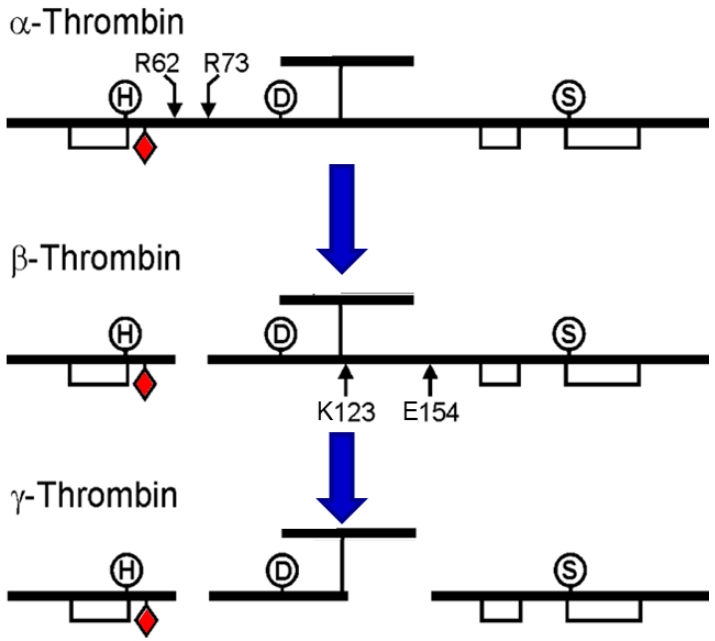
Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

# Profiling Analysis of Released N-Glycans by LC-MS: 2-AB

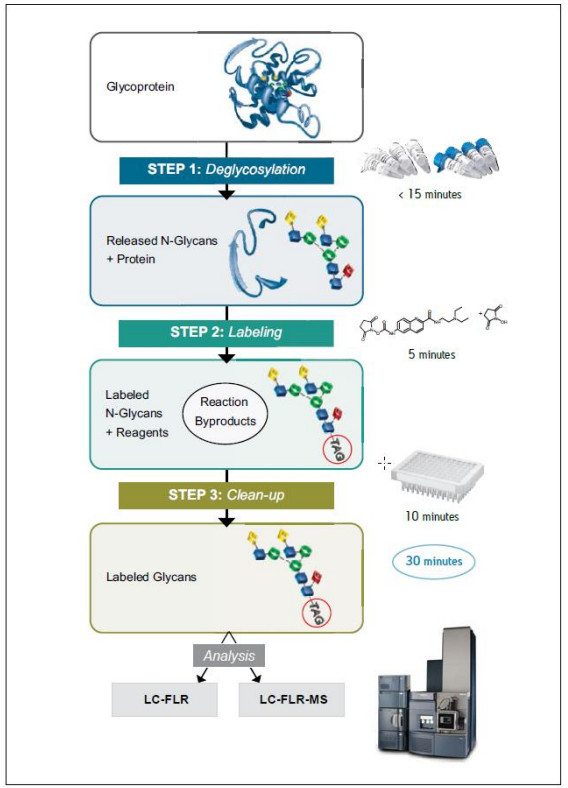
**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein** → **Use of a More Efficient Release & Labeling Process**




**Native enzymatic release via PNGase F at 37°C overnight**




## Waters RapiFluor Kit



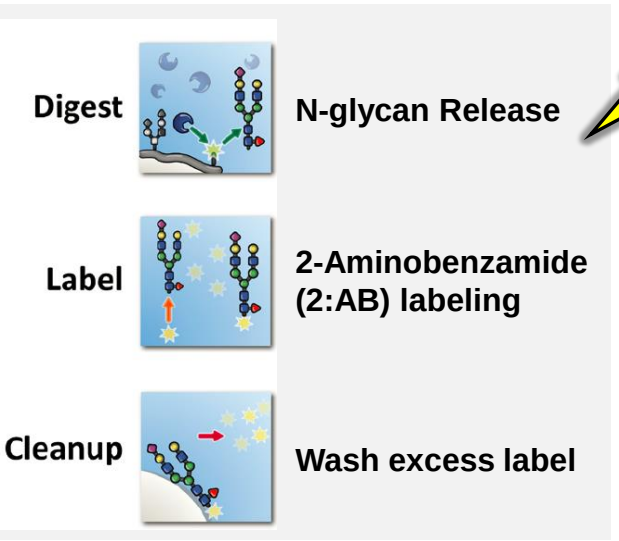

www.waters.com



Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

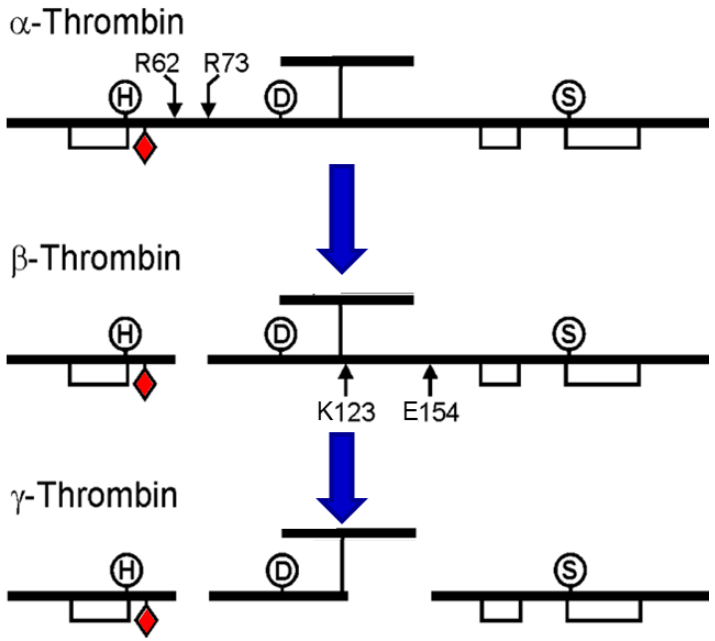
# Profiling Analysis of Released N-Glycans by LC-MS: RapiFluor

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein** → **Use of a More Efficient Release & Labeling Process**

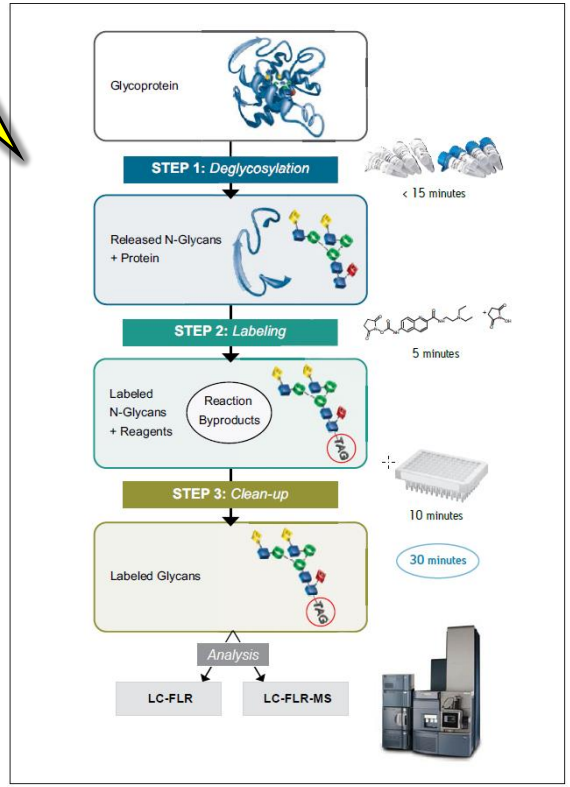



**Native enzymatic release via PNGase F at 37°C overnight**

**Protein Denaturation followed by enzymatic release at 50°C for <15 min**




## Waters RapiFluor Kit



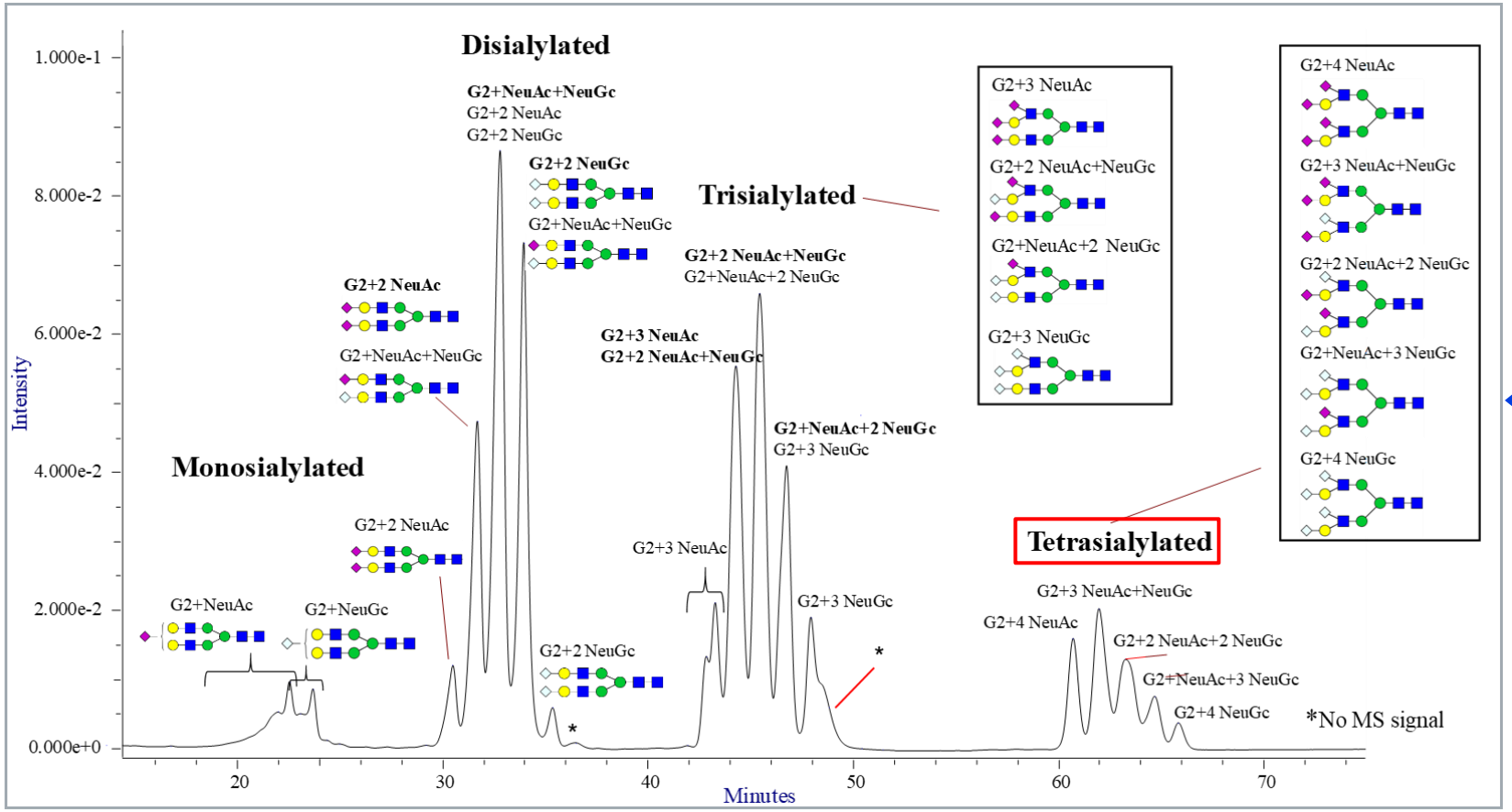

www.waters.com



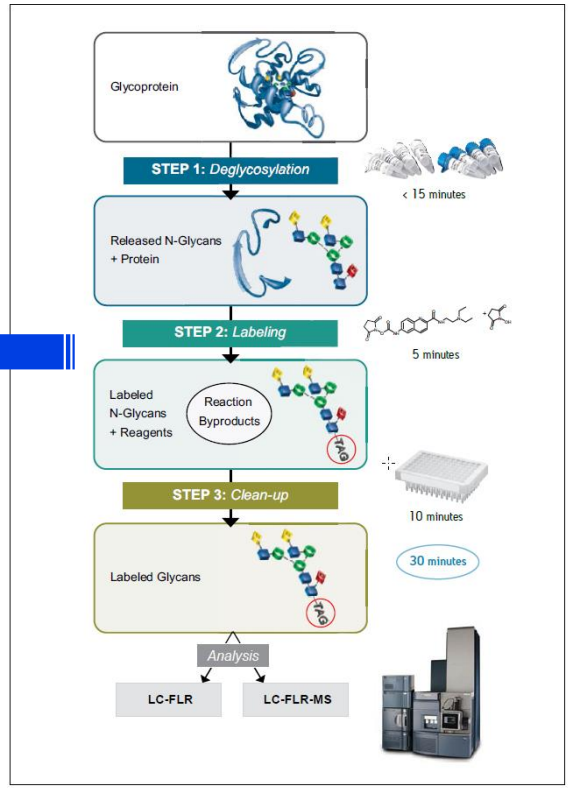
Davie et al, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006

# Profiling Analysis of Released N-Glycans by LC-MS: RapiFluor

**2-AB Labeled N-Glycan Analysis by NP-AEX-MS** → **Exhaustive Elucidation of N-Glycans** → **Comparison to CHO-Derived Protein** → **Use of a More Efficient Release & Labeling Process**




## Waters RapiFluor Kit




www.waters.com

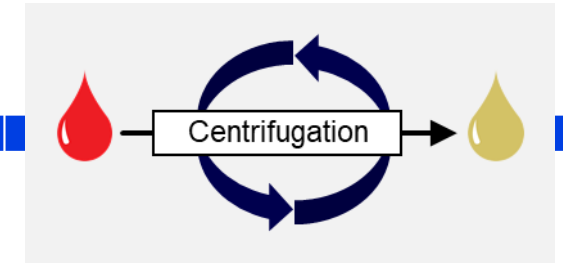
► **Tetrasialylated structures were effectively recovered**

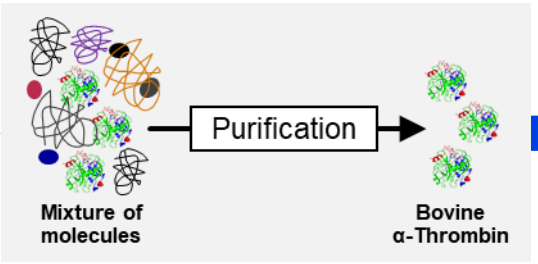
# Profiling Analysis of Released N-Glycans by LC-MS

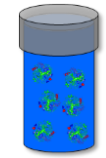
- Sialic acid residues observed were a combination of NeuAc and NeuGc which differ by 16 Da
- N-glycans from bovine plasma α-Thrombin are comprised of the complex-type, afucosylated, biantennary structure (G2) with one to four sialic acid residues
- Multiple elution positions for the same N-glycan compositions are due to sialic acids located on the terminal Gal or bisecting GlcNAc residues with α2→3 and/or α2→6 linkages

# Preliminary Intact Mass Analysis of Bovine α-Thrombin by LC-MS

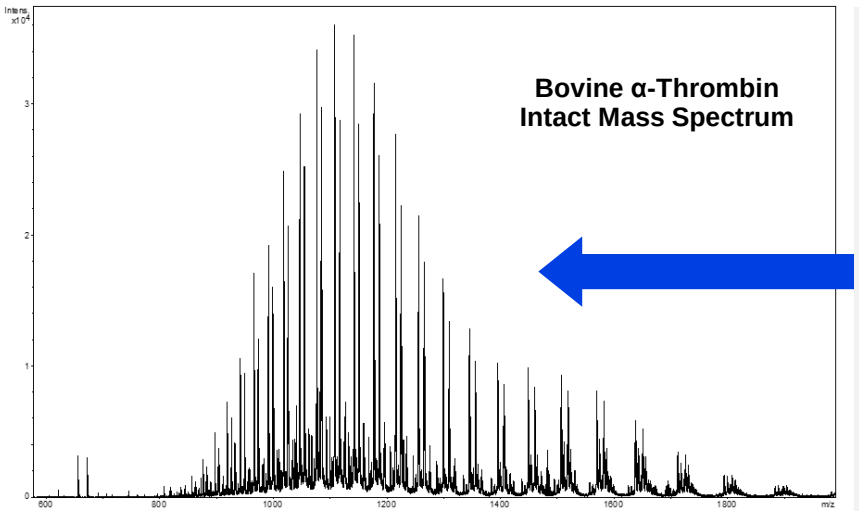
Stef Bennett - The Hungry Aperture/Getty Images/iStockphoto



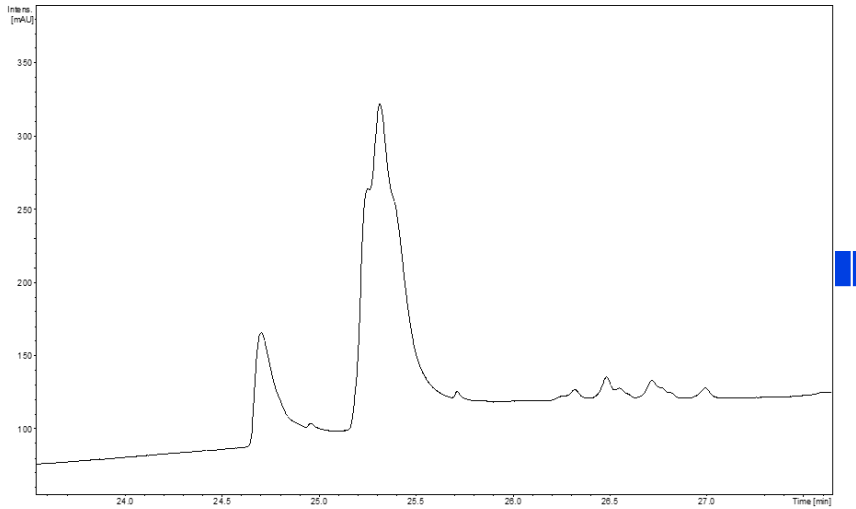
Bovine α-Thrombin




Mass Spectrum

MS Analysis


Bruker maXis II MS

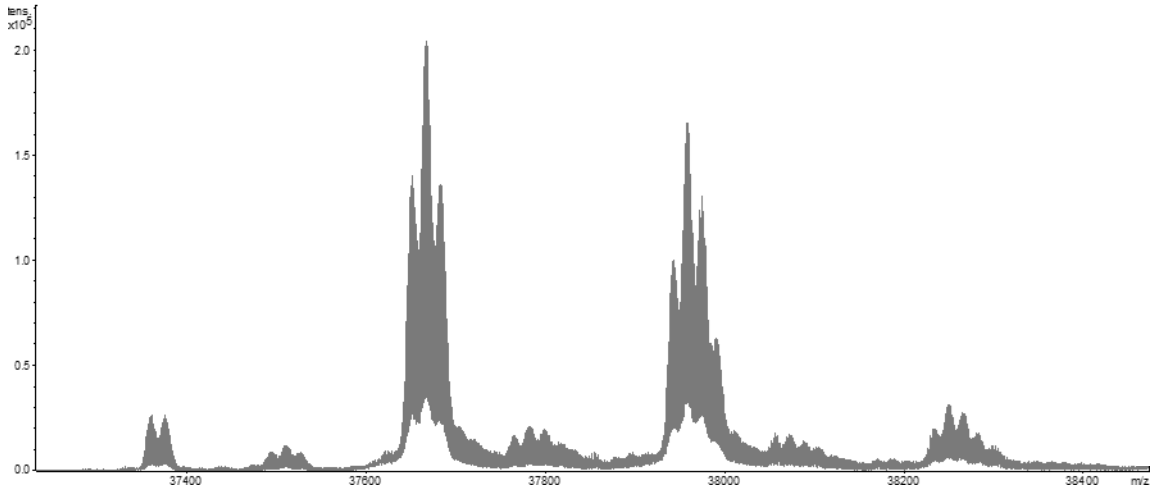


UV Chromatogram

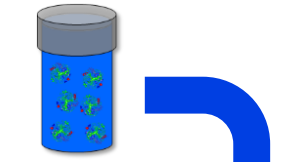
LC Separation

Waters H-Class UPLC

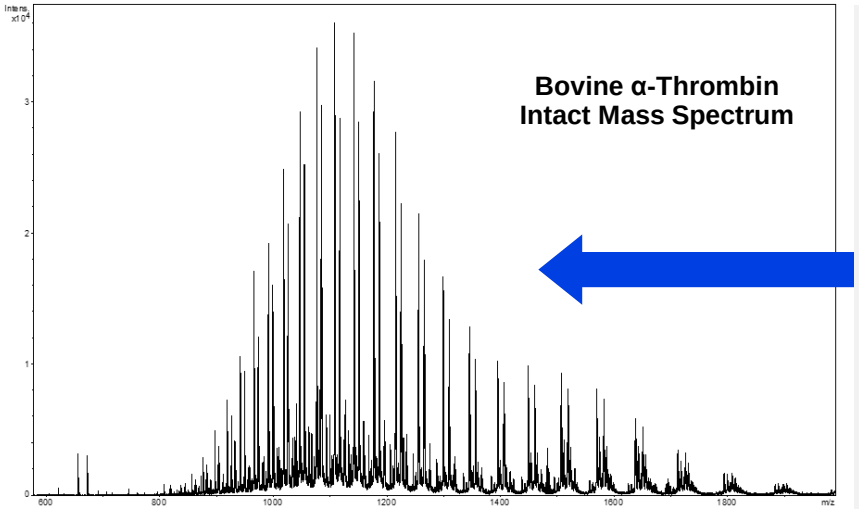
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS




Deconvoluted Mass Spectrum



Bovine
α-Thrombin

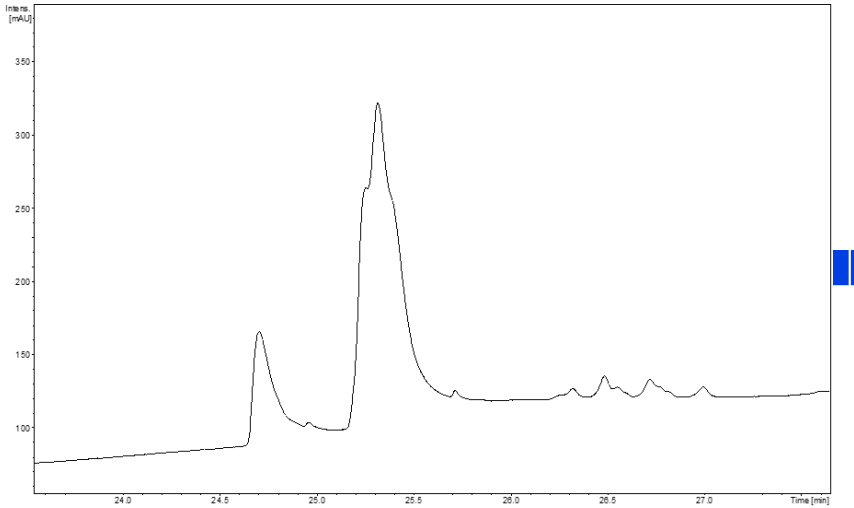



Bovine α-Thrombin
Intact Mass Spectrum

Mass Spectrum

MS Analysis


Bruker maXis II MS

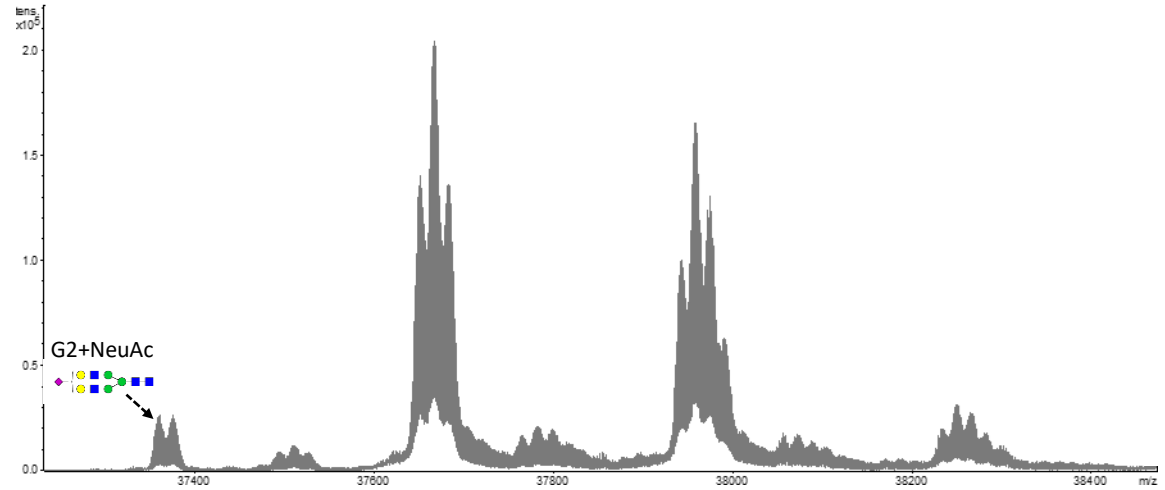



UV Chromatogram

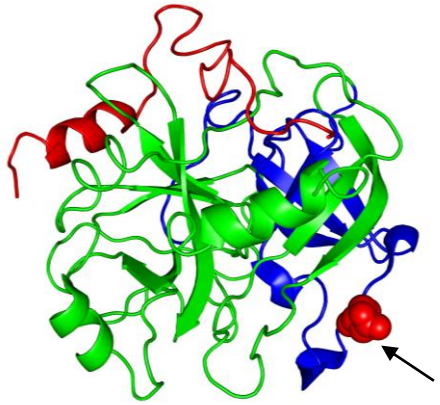
LC Separation

Waters H-Class UPLC

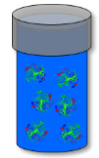
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS



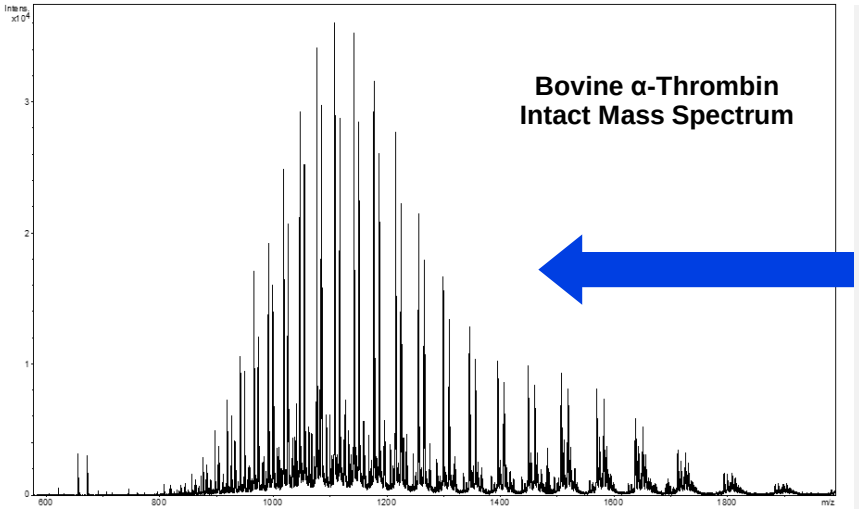

α-Thrombin



N-glycosylation site

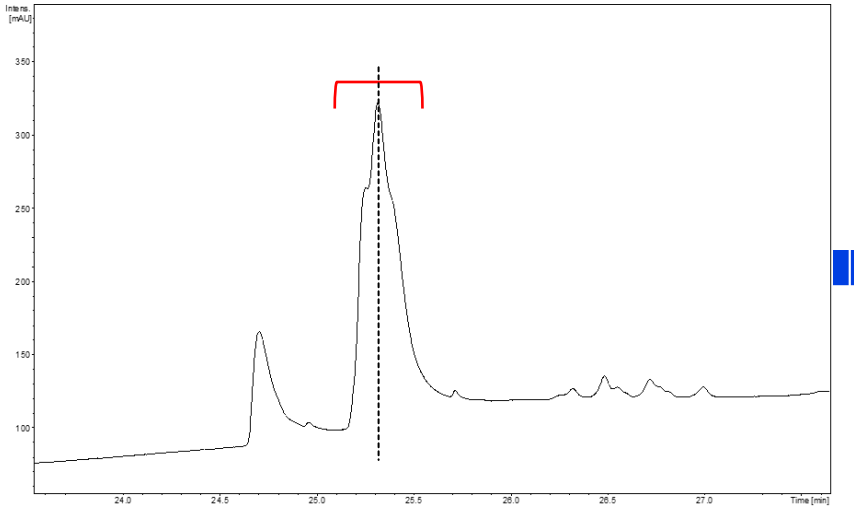


Bovine α-Thrombin




MS Analysis

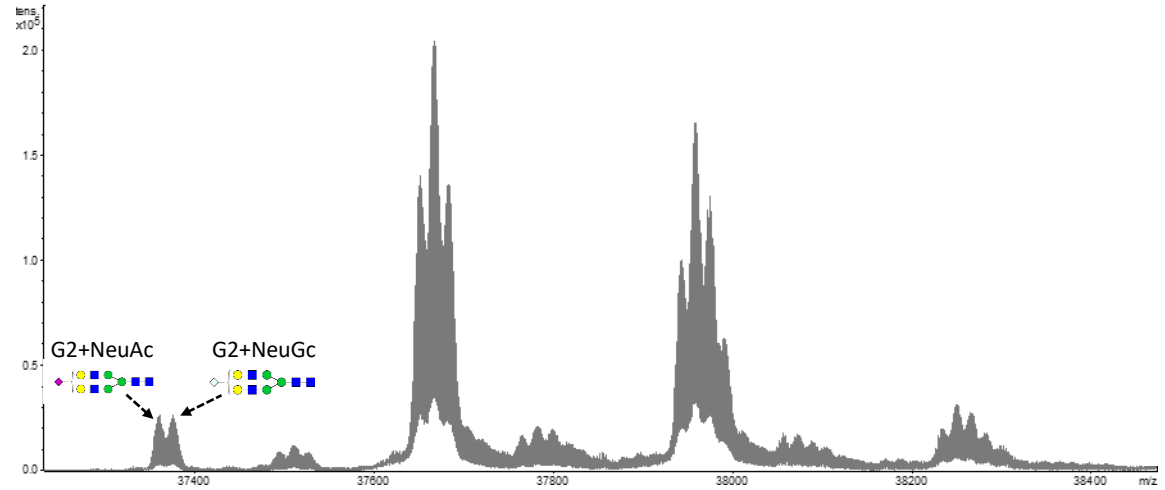

Bruker maXis II MS



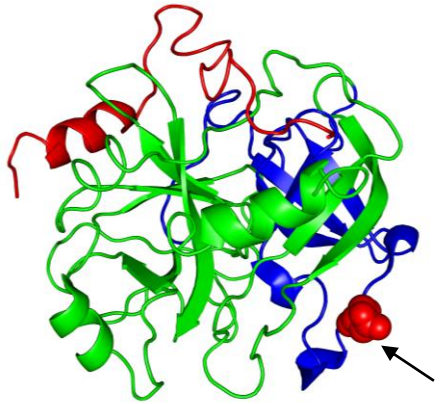

LC Separation

Waters H-Class UPLC

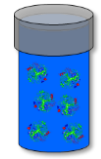
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS



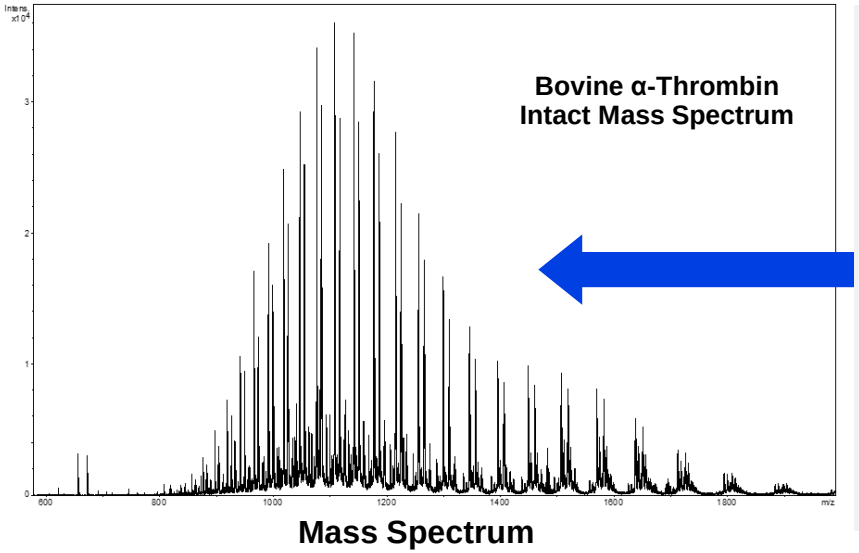

α-Thrombin



N-glycosylation site

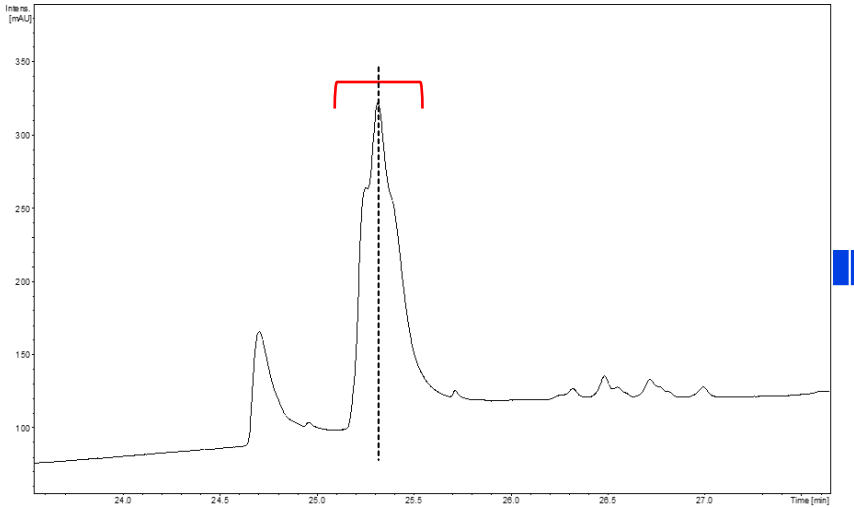


Bovine α-Thrombin




MS Analysis

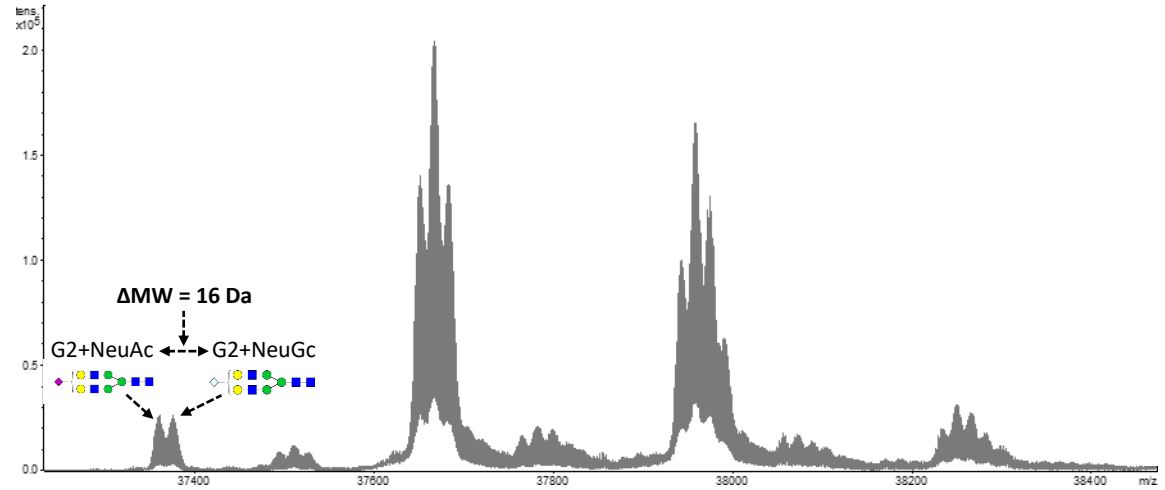
Bruker maXis II MS




LC Separation

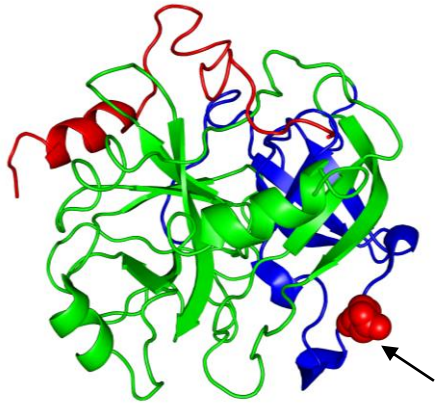
Waters H-Class UPLC

# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS

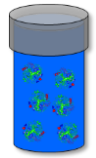



Deconvoluted Mass Spectrum

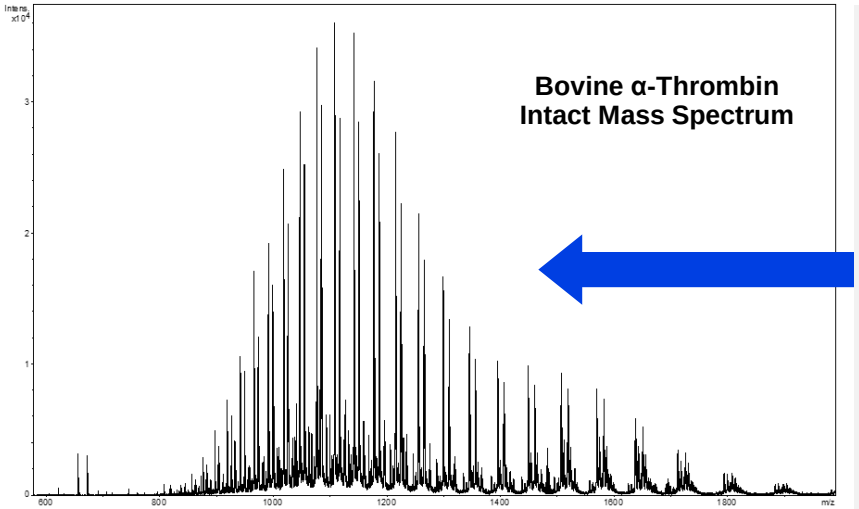
α-Thrombin



N-glycosylation site



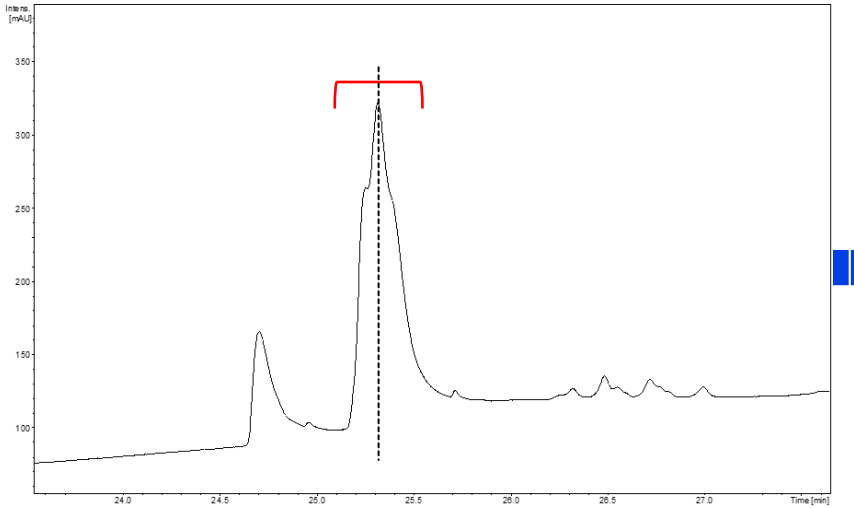
Bovine α-Thrombin




Mass Spectrum

MS Analysis


Bruker maXis II MS

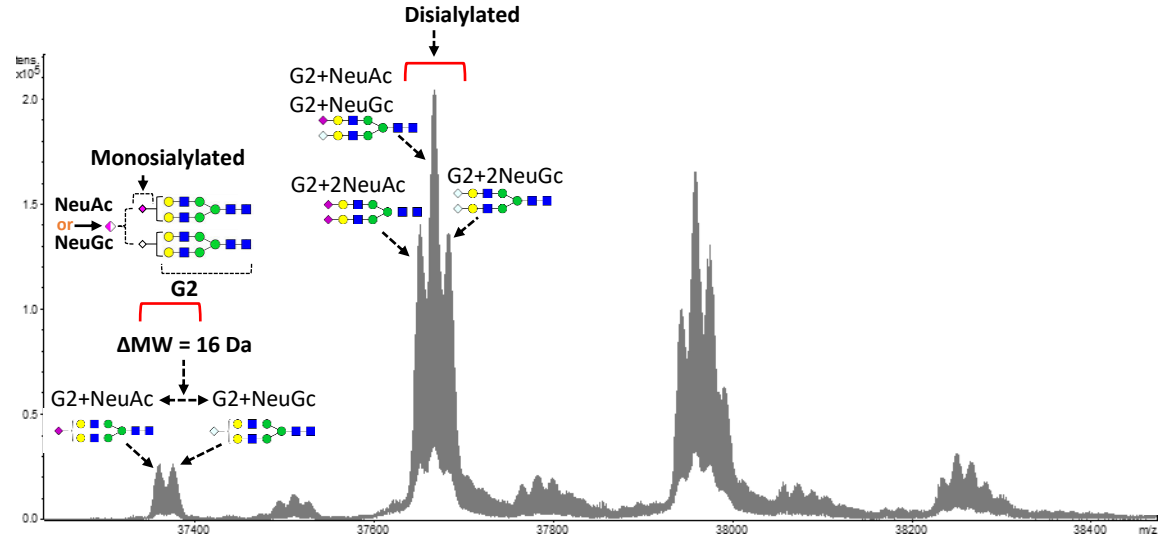



UV Chromatogram

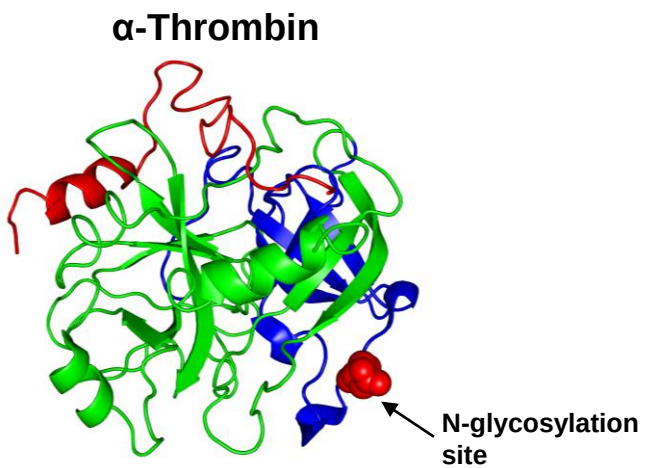
LC Separation

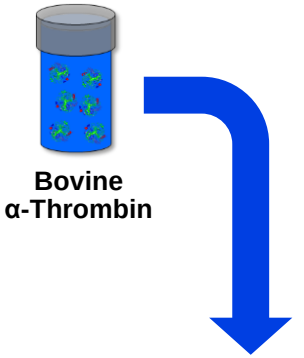
Waters H-Class UPLC

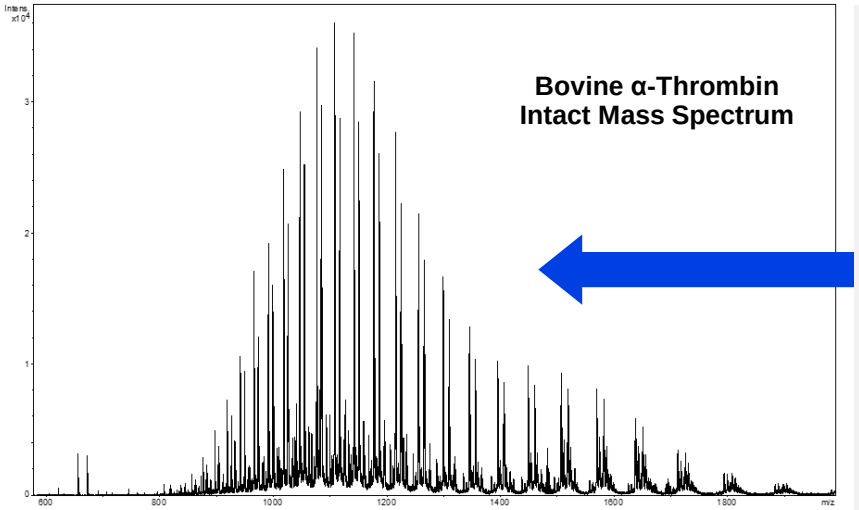
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS




Deconvoluted Mass Spectrum



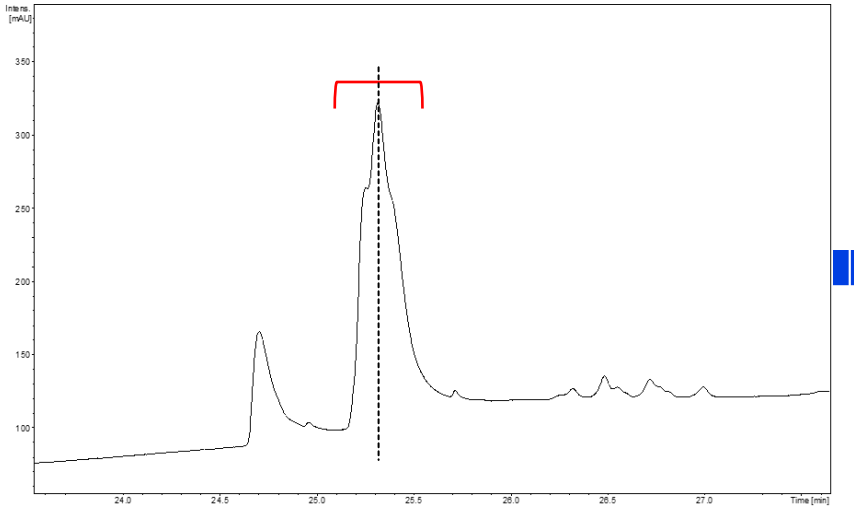




Mass Spectrum

MS Analysis


Bruker maXis II MS

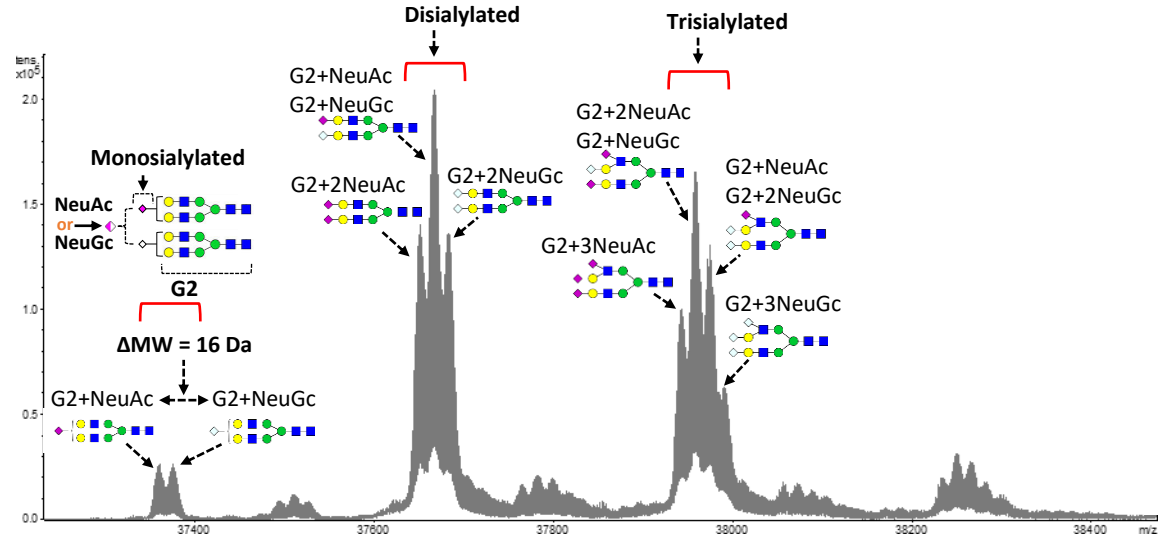



UV Chromatogram

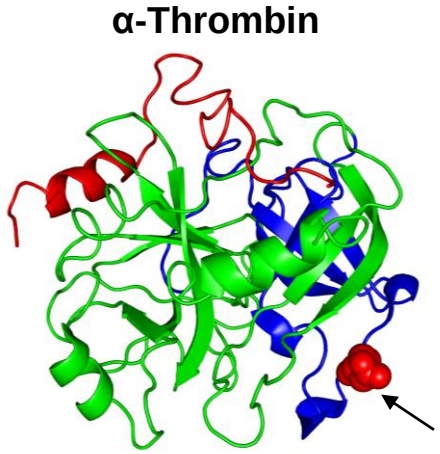
LC Separation

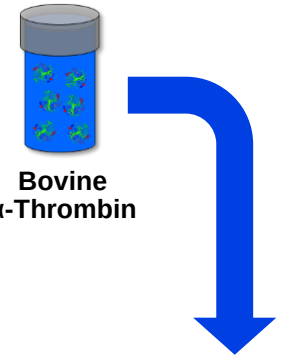
Waters H-Class UPLC

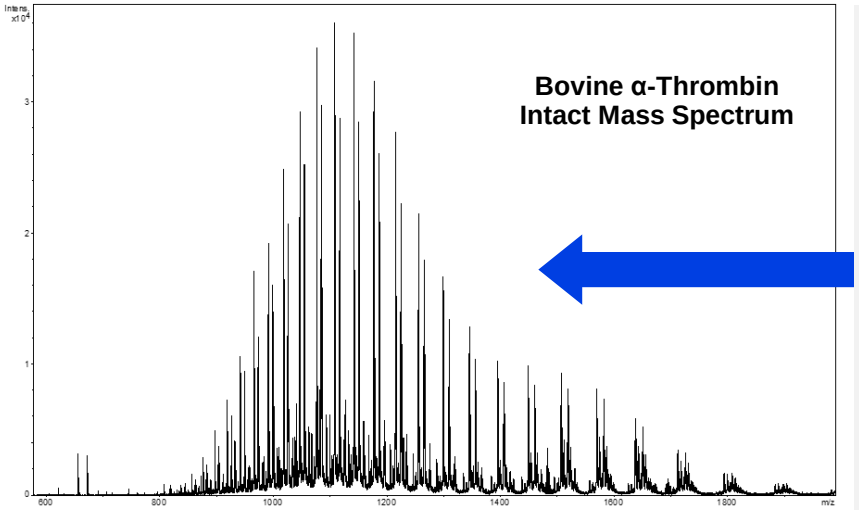
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS




Deconvoluted Mass Spectrum



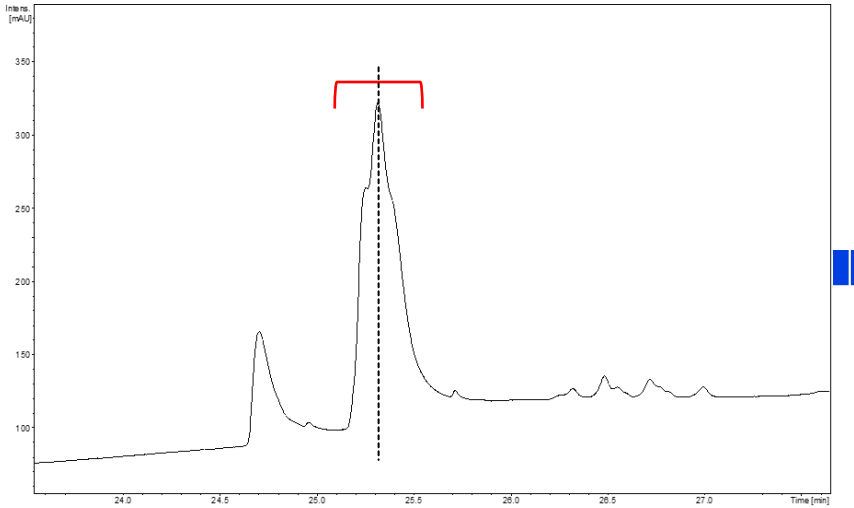




Mass Spectrum

MS Analysis


Bruker maXis II MS

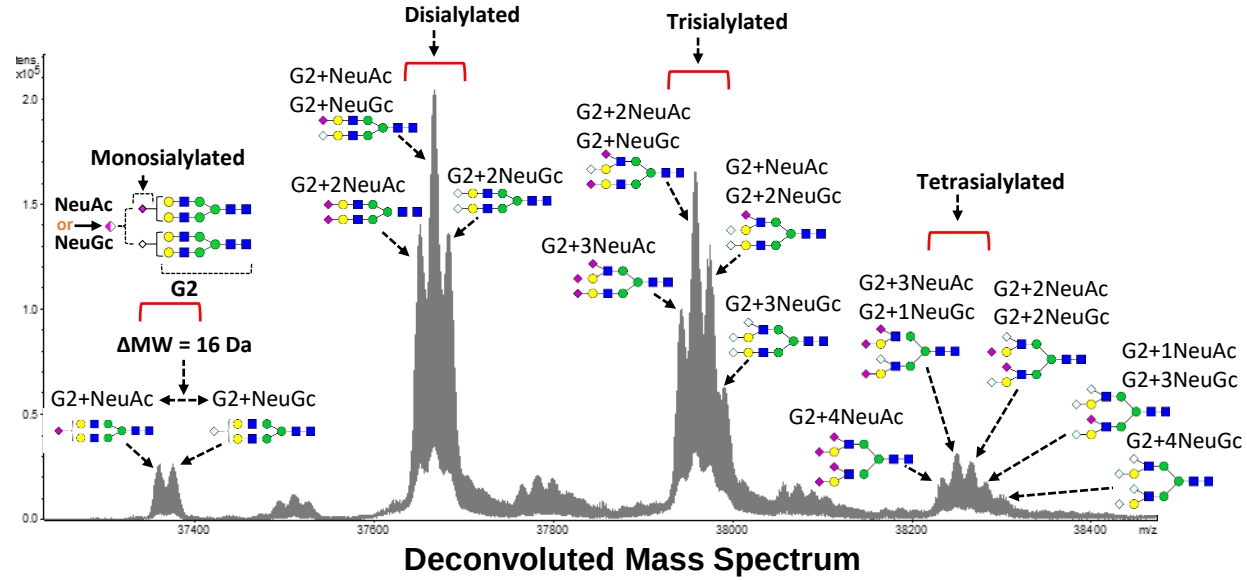


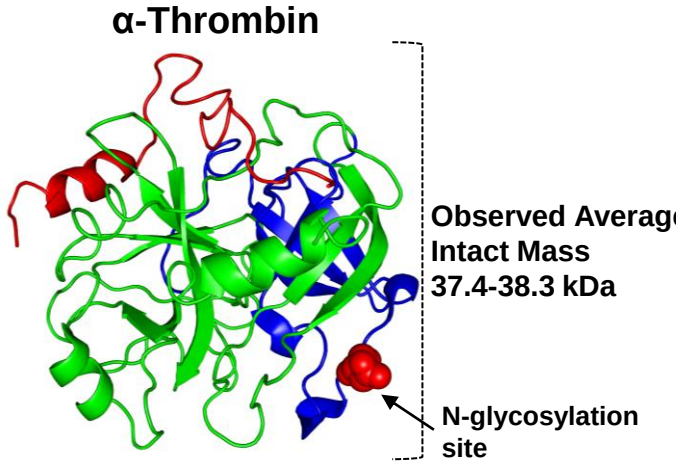

UV Chromatogram

LC Separation

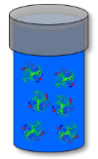
Waters H-Class UPLC

# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS

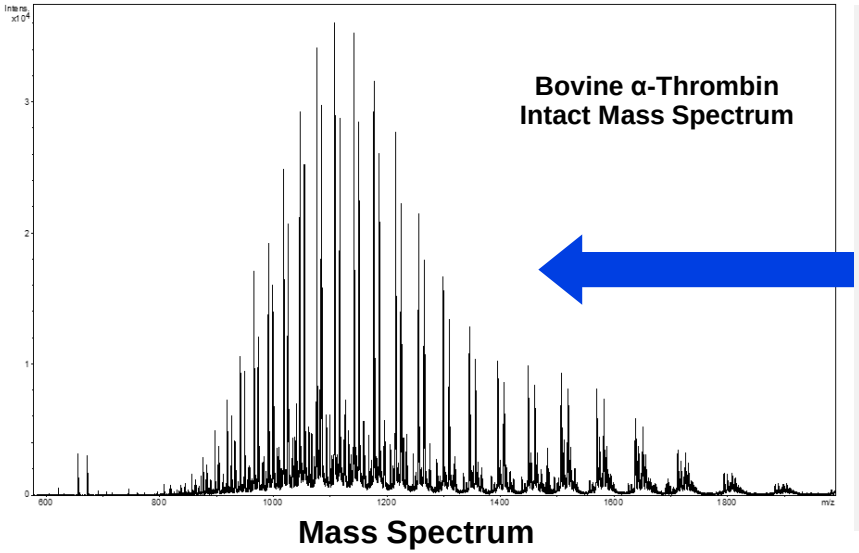



Observed Average Intact Mass 37.4-38.3 kDa

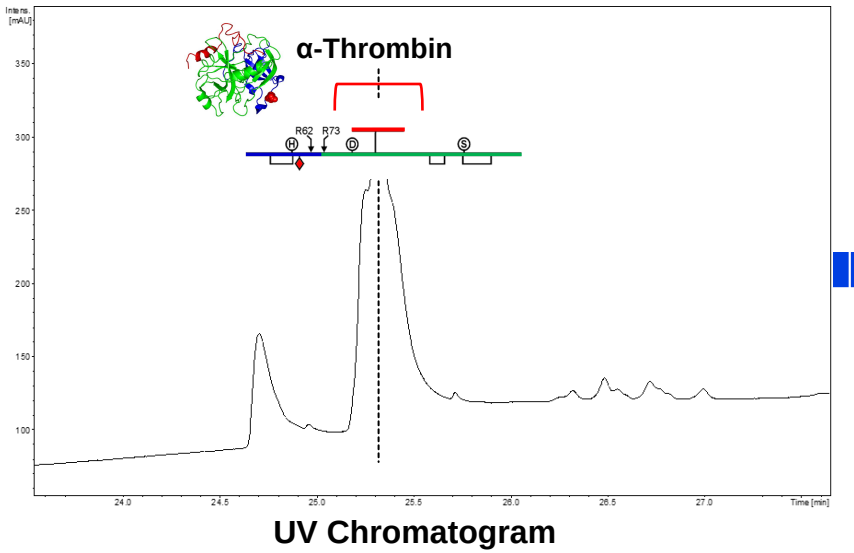


Bovine α-Thrombin




MS Analysis

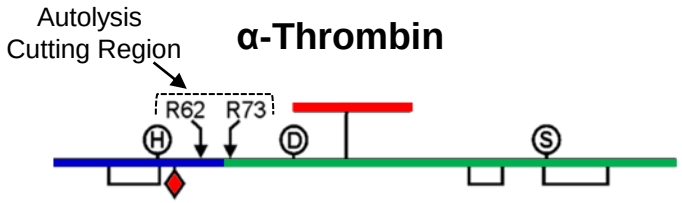
Bruker maXis II MS

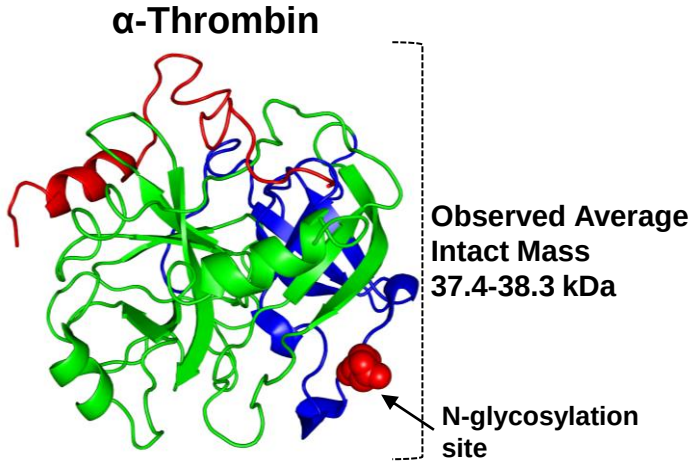


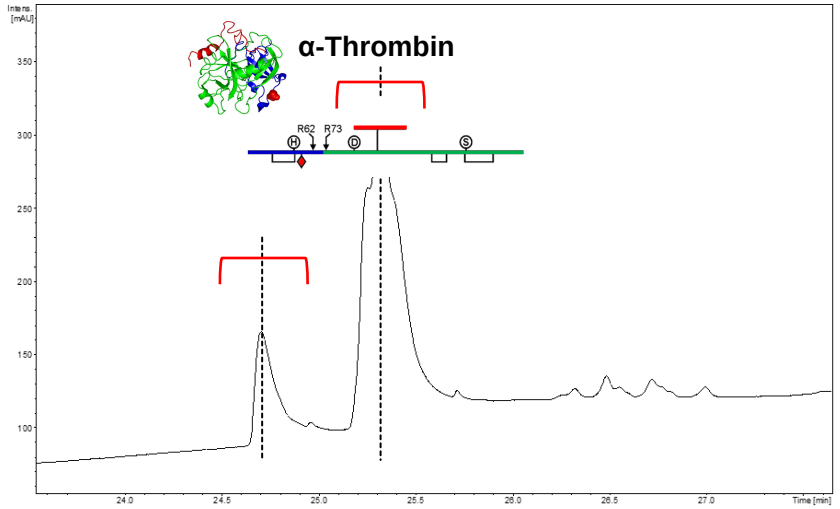

LC Separation

Waters H-Class UPLC

# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS

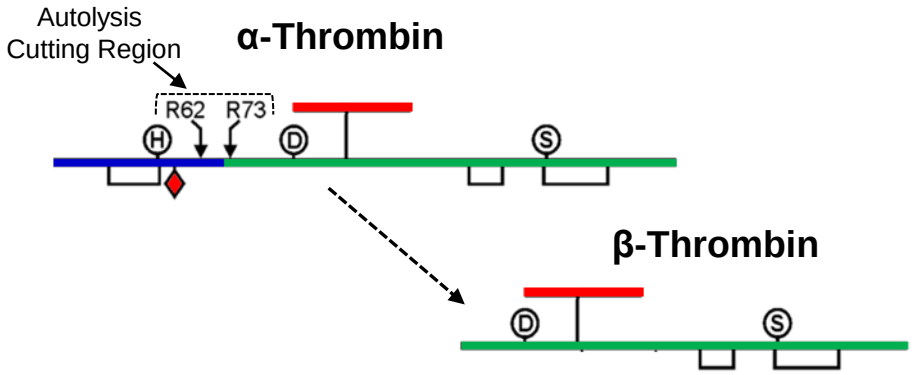


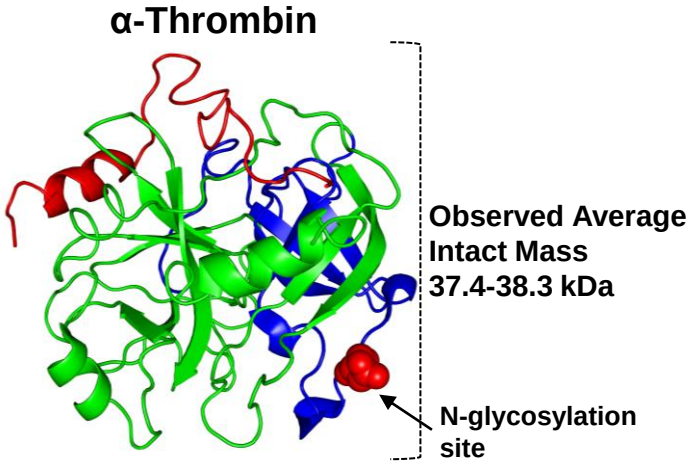



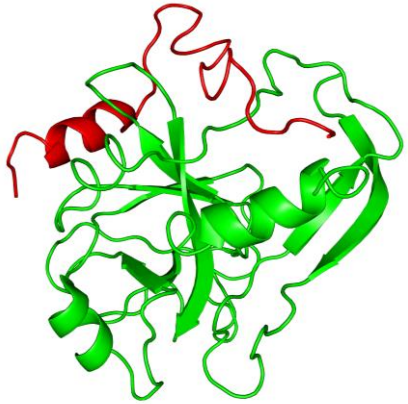




UV Chromatogram

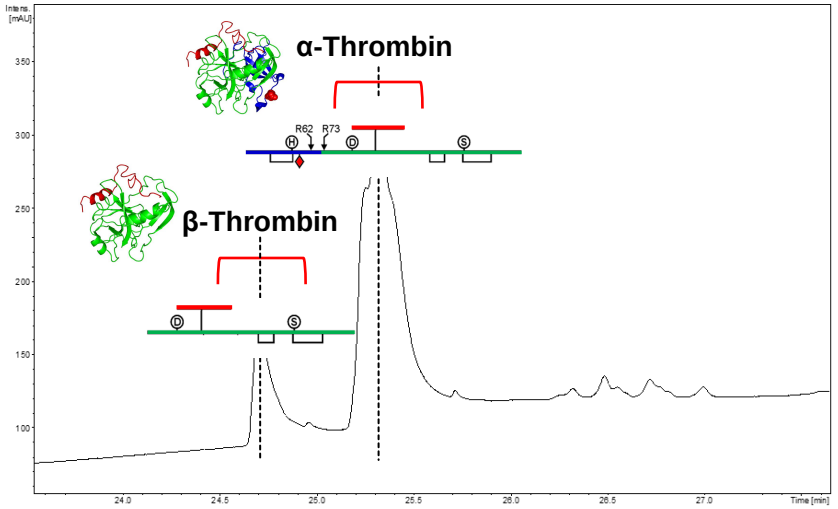
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS



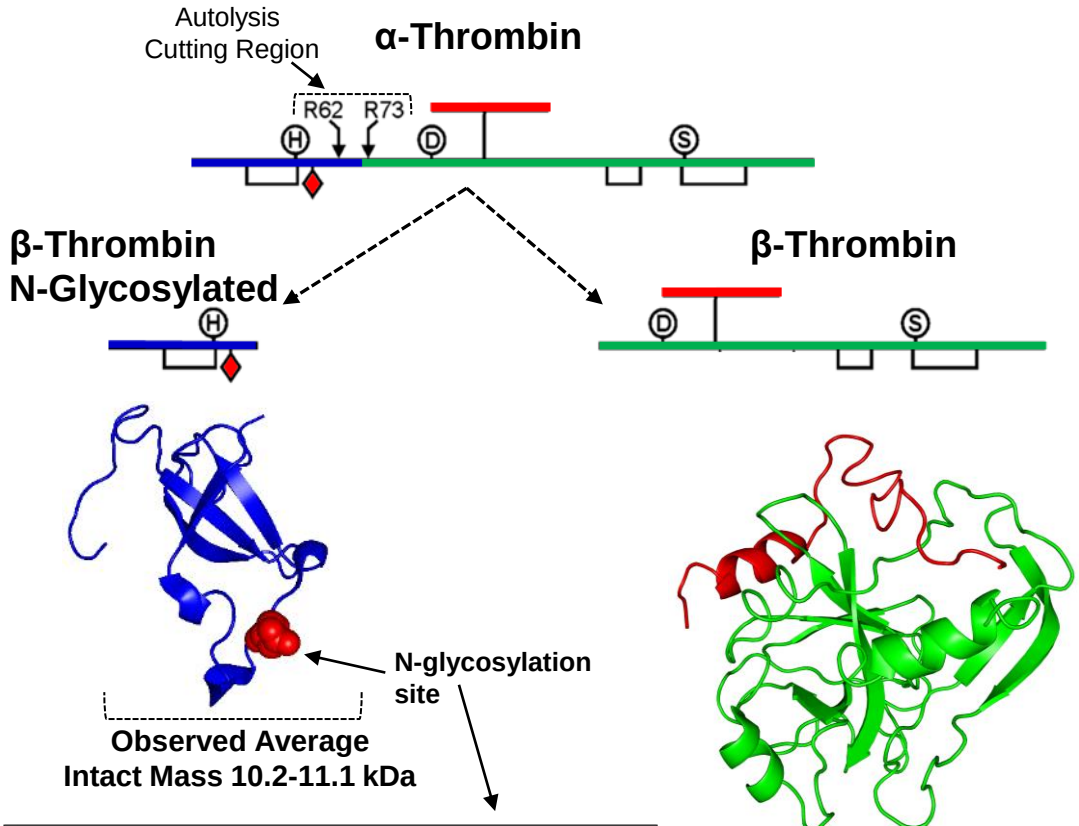



Observed Average
Intact Mass 27 kDa



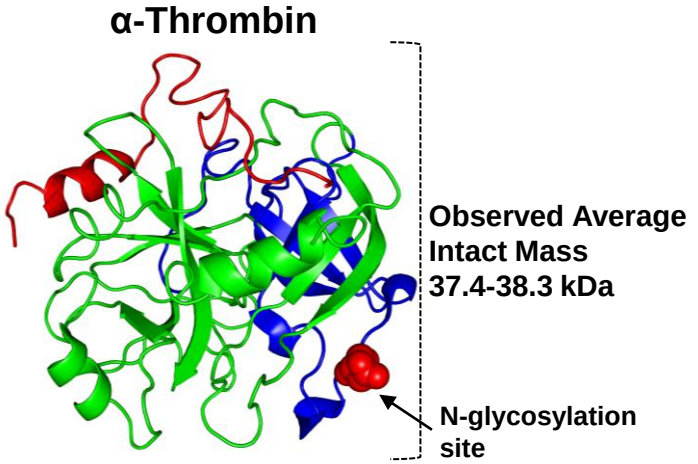

UV Chromatogram

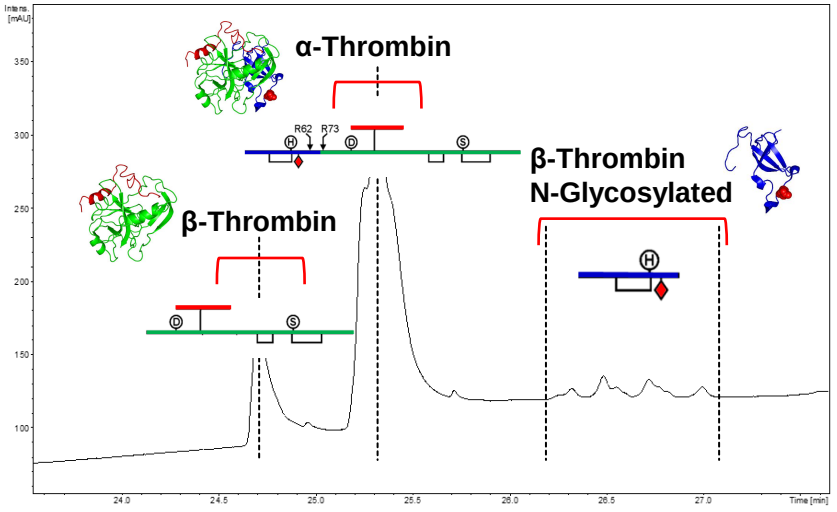
# Exhaustive Intact Mass Analysis of Bovine α-Thrombin by LC-MS



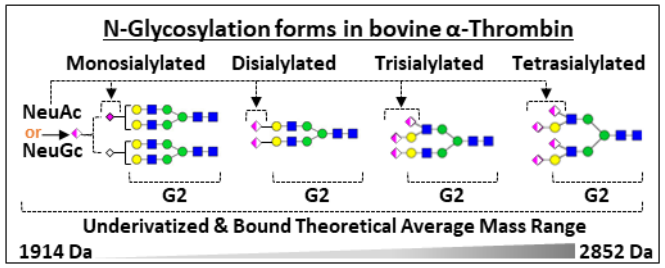



Observed Average
Intact Mass 27 kDa



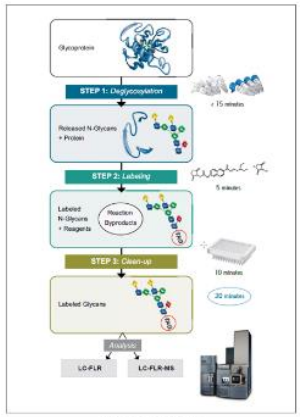

UV Chromatogram

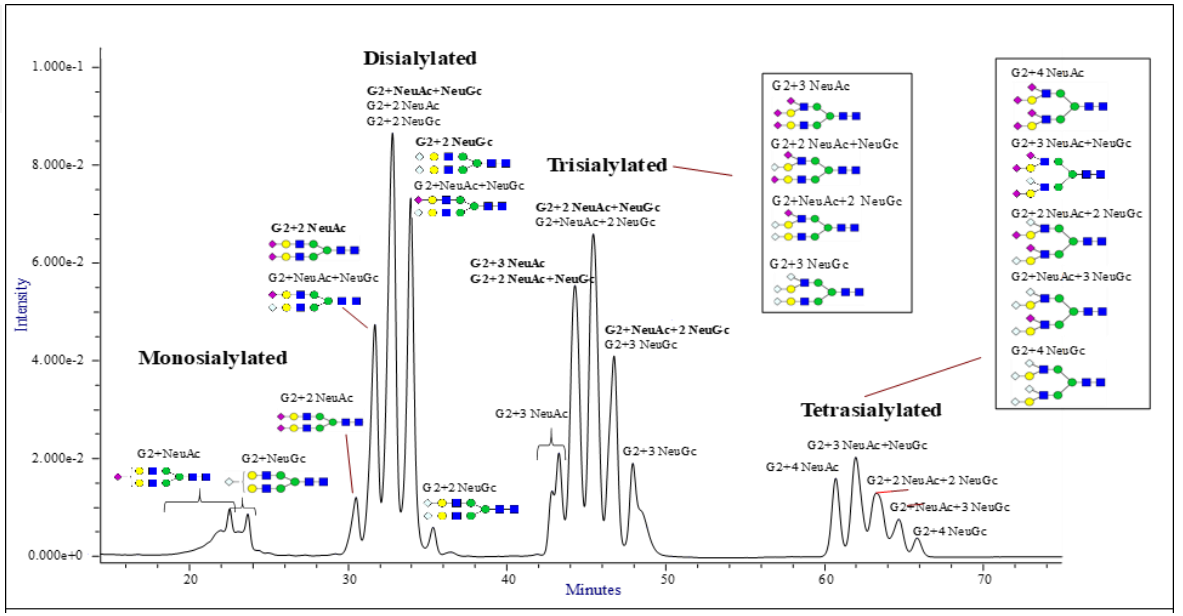


# Identification of N-Linked Glycans from Bovine α-Thrombin Using a Combination of LC-MS Techniques

## Profiling Analysis of Released N-Glycans by LC-MS

**Waters RapiFluor Kit**

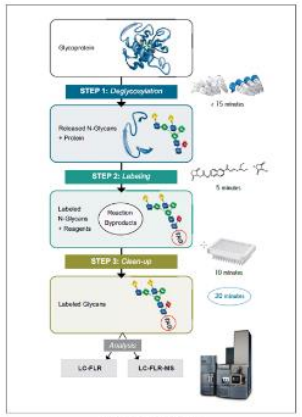


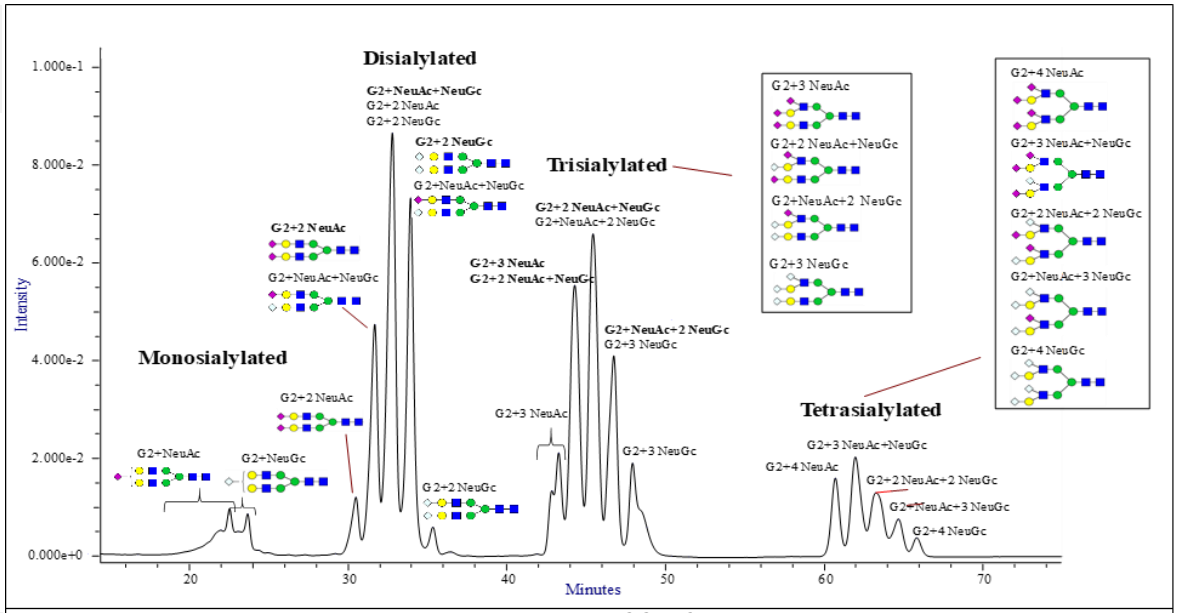
# Identification of N-Linked Glycans from Bovine α-Thrombin Using a Combination of LC-MS Techniques

## Profiling Analysis of Released N-Glycans by LC-MS

**Waters RapiFluor Kit**




www.waters.com

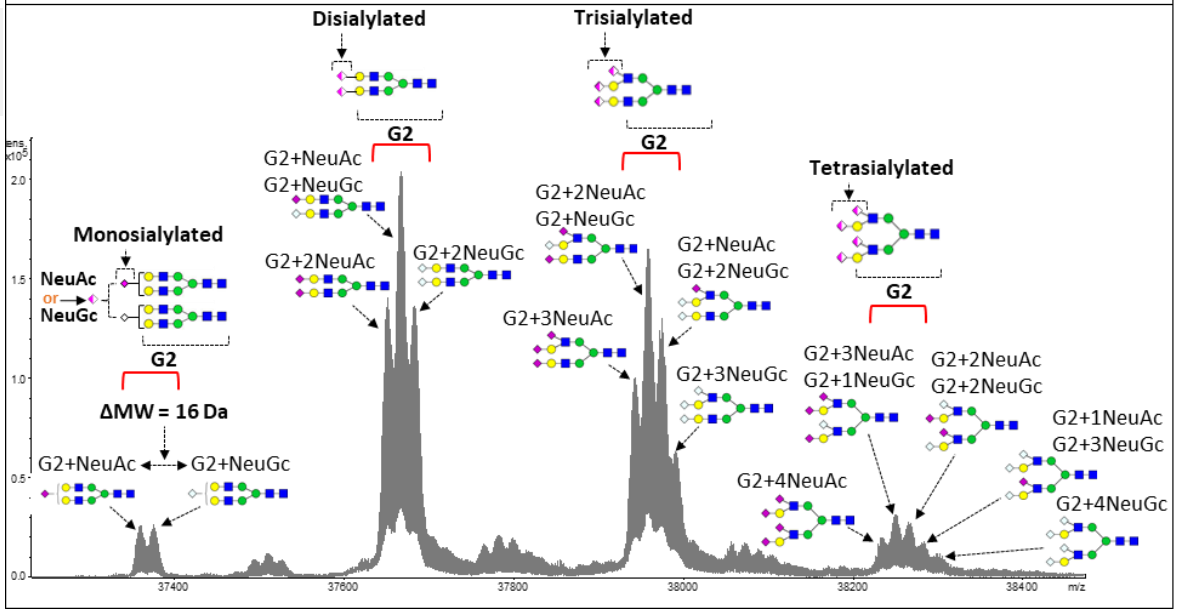



## Exhaustive Intact Mass Analysis

**LC Separation** **MS Analysis**

Waters H-Class UPLC

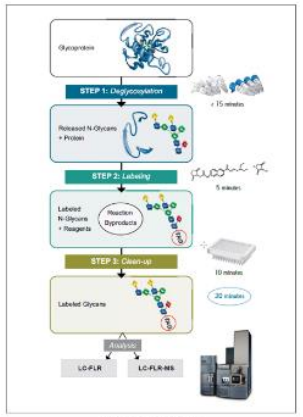

Bruker maXis II MS

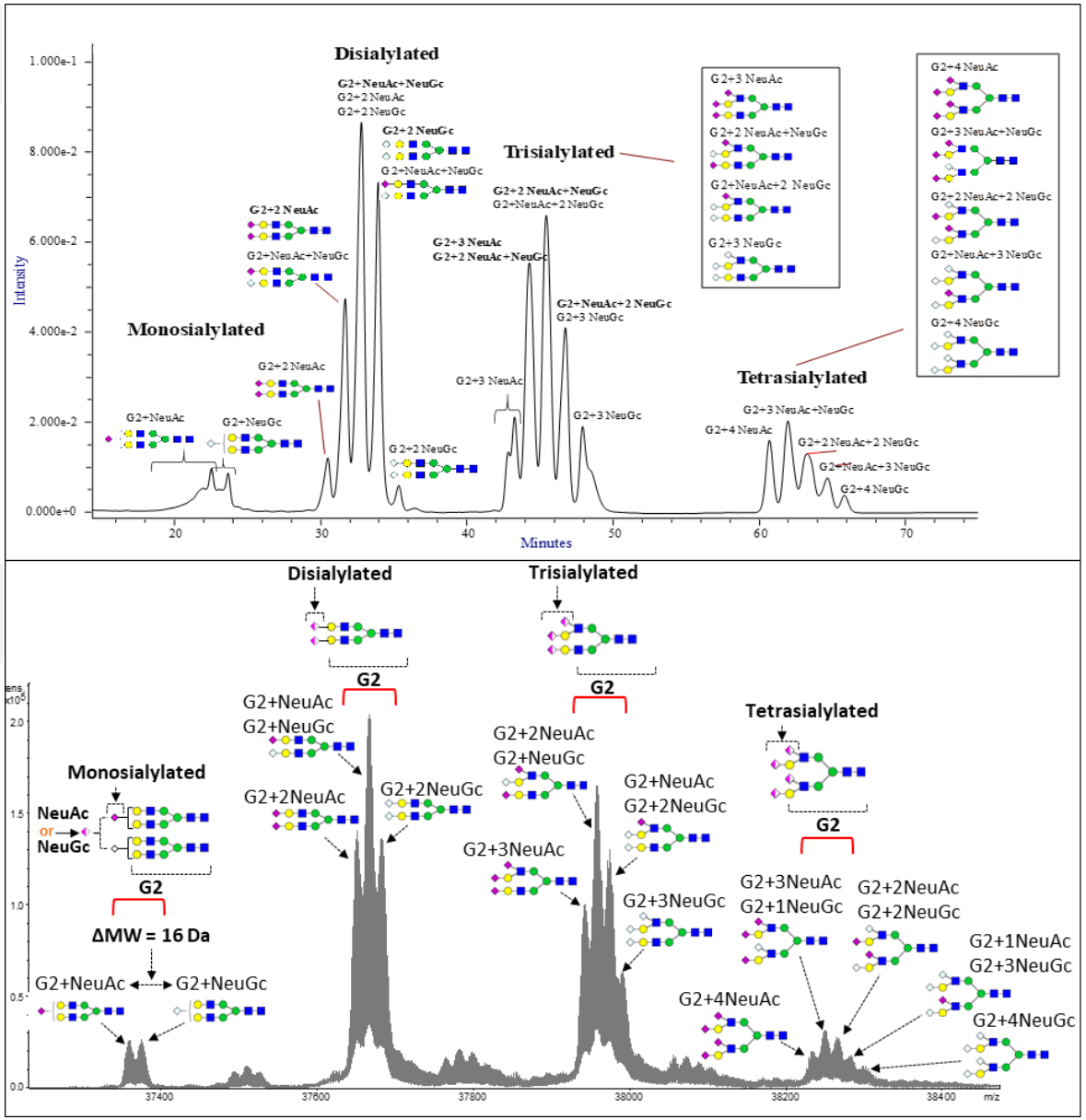


# Identification of N-Linked Glycans from Bovine α-Thrombin Using a Combination of LC-MS Techniques

## Profiling Analysis of Released N-Glycans by LC-MS

**Waters RapiFluor Kit**



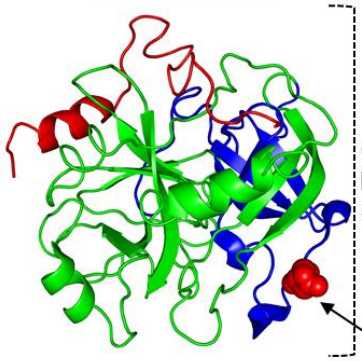




## Exhaustive Intact Mass Analysis

**LC Separation** | **MS Analysis**


**Waters H-Class UPLC** | **Bruker maXis II MS**

**α-Thrombin**

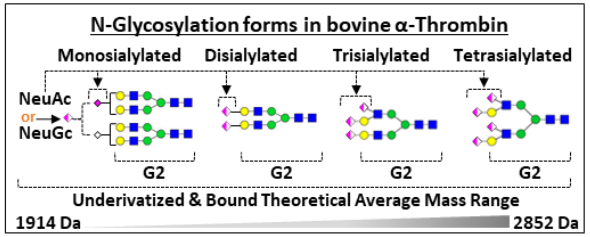


**Observed Average Intact Mass 37.4-38.3 kDa**

**N-glycosylation site**

**N-Glycans**

- complex-type, afucosylated, biantennary structure, G2, with 1 to 4 sialic acid residues
- Sialic acid residues: NeuAc and NeuGc
- Sialic acid residues: located on the terminal Gal or via bisecting GlcNAc residues with α2→3 and/or α2→6 linkages



# Acknowledgements

Jason Rouse
Matthew Thompson

Nick Ellis

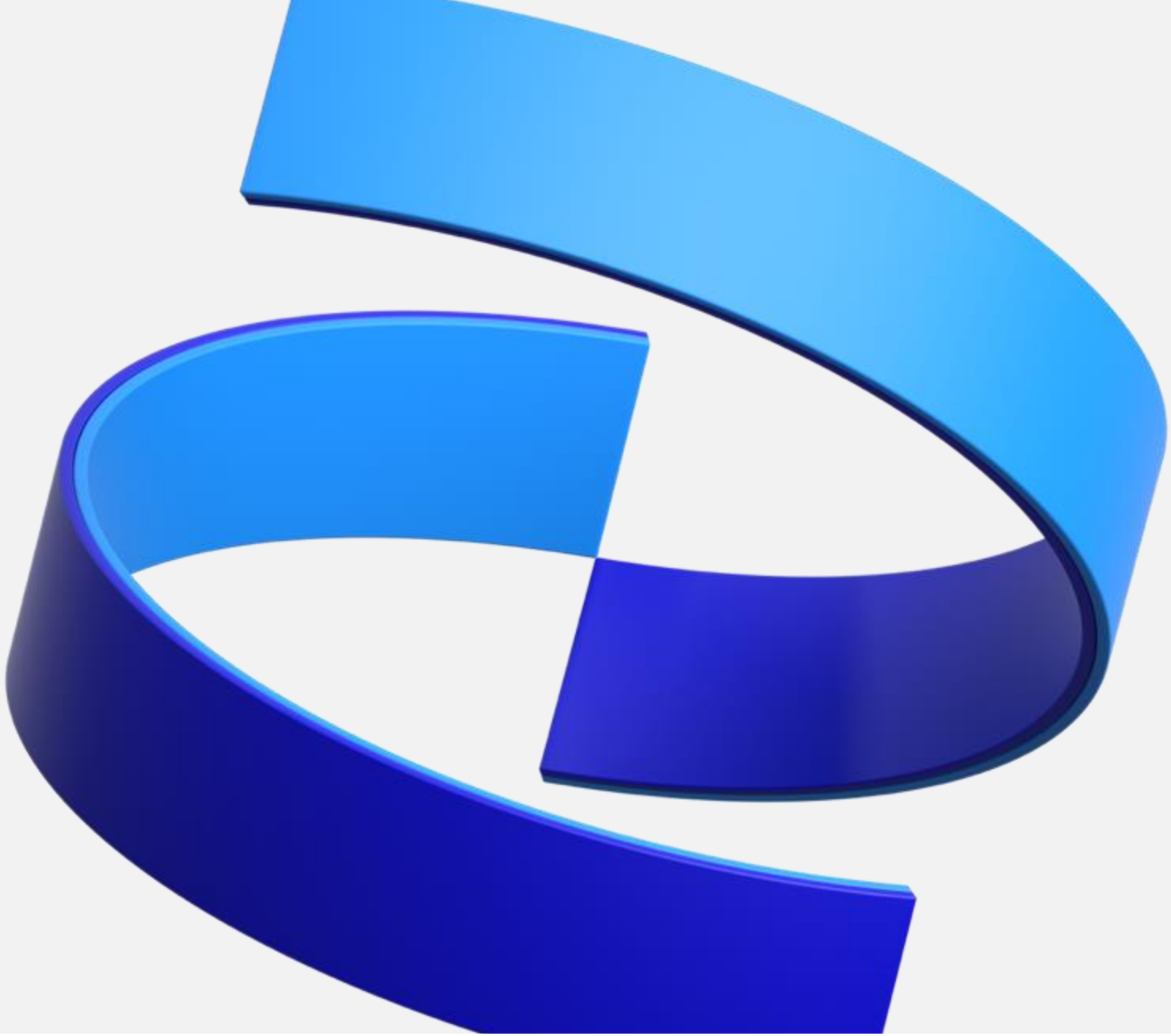
Kaffa Cote
Aimee Nicole
Himakshi Patel

## References

1. Clinical use of topical thrombin as a surgical hemostat. Biologics. 2008, 4, 593-599
2. Davie et al An Overview of the Structure and Function of Thrombin, Seminars in Thrombosis and Hemostasis/Volume 32, Supplement 1 2006
3. Terrab and Pawlak, The Journal of Applied Research Vol. 7, No.3, 2007
4. Thrombin-JMI® U.S. Physician Prescribing Information, Revised: 4/2019
5. Mizuochi et al The carbohydrate of bovine prothrombin. J Biol Chem 1979
6. Nelsestuen et al - The Carbohydrates of Prothrombin 1972 J Bio Chem
7. Magnusson - Thrombin and Prothrombin, The Enzymes Vol 2 1971
8. Malkowski et al, Protein Science, 1997
9. Chou et al PNAS 1998 95(20): 11751-11756
10. Cohen et al OMICS 2010
11. Suzuki, Proc Jpn Acad Ser B Phys Biol Sci. 2006
12. Bovine α-Thrombin Structure PDB DOI: 10.2210/pdb1HRT/pdb

# Thank You

# Questions?



# Disclosure

Authors have no conflict of interest to declare.