Enhancing Host-Cell Protein Detection in Biotherapeutics Using HILIC Enrichment and Proteomic Analysis

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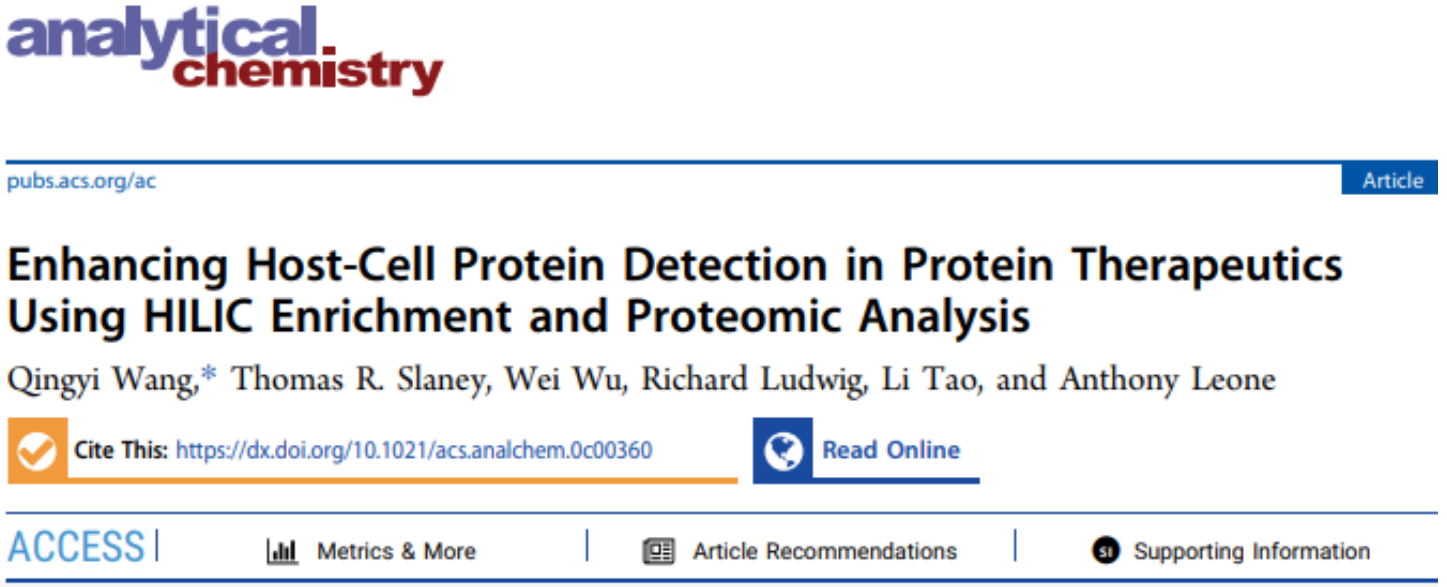
Bristol Myers Squibb™

Acknowledgements:
Bristol Myers Squibb Co-op Students

1. Dr. Qingyi Wang, Ph.D.



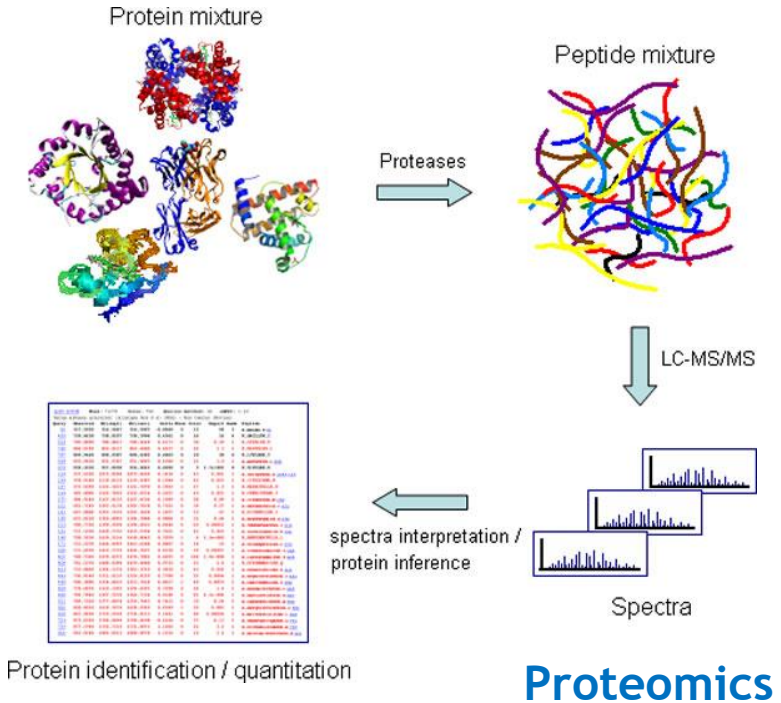
2. Dr. John Crellin, Ph.D.
   — Manuscript In-Process

Outline

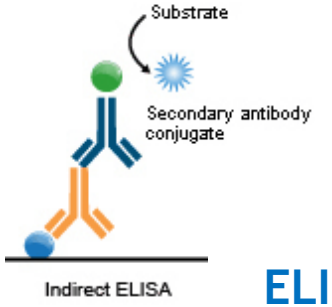
- Host-Cell Protein (HCP) Characterization by LC-MS
- HCP Enrichment Using HILIC
- Method Demonstration with mAbs and Fusion Proteins
- Automation of Analysis and Quantitation
- Future Directions

Common Analytical Approaches

- Methods often combine:
  - Immunoassays
  - Electrophoresis
  - LC-MS

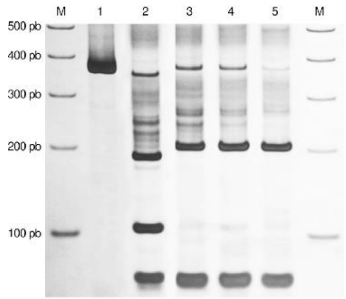


Proteomics

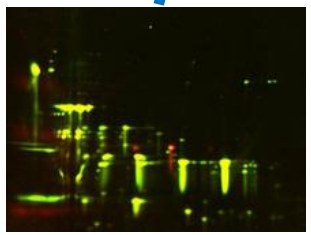


ELISA

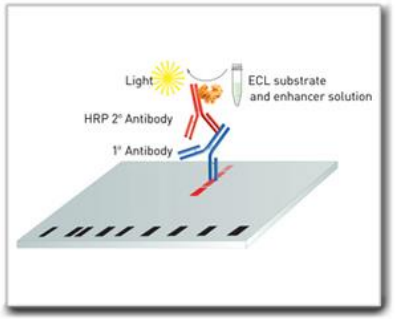
SDS PAGE



HCPs



2D-differential in-gel electrophoresis

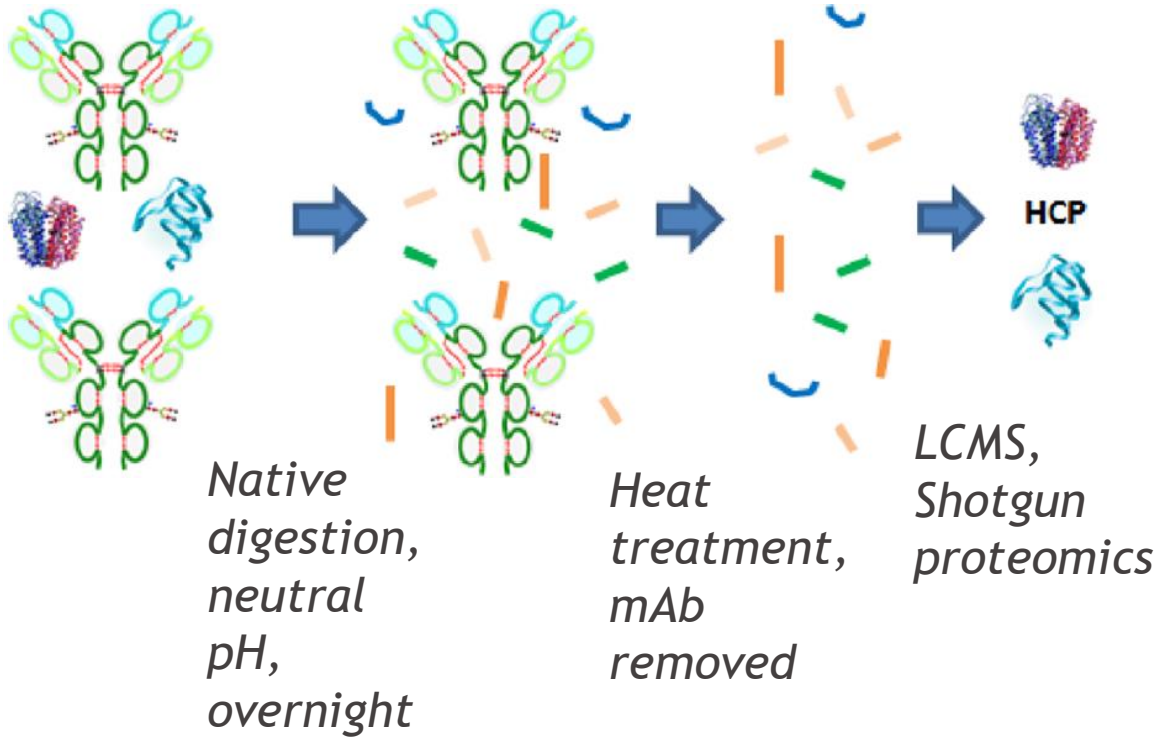


Western Blot

Problem: Dynamic range of HCPs vs. Therapeutic Protein

- After trypsin digestion ("Bottom-up"), 10^5-10^6 fold difference in HCP vs. Therapeutic peptide concentration
- **Strategies:**
  - Better MS instruments
    - Ion mobility, higher resolution, better dynamic range, faster duty cycle
  - Increasing LC resolution
    - Longer gradient, multiple columns, 2D-LC
  - Enriching residual HCPs before digestion
    - mAb removal by affinity purification
    - Precipitating mAb after native digestion ("Lilly method")
    - **Depleting the mAb by other sample preparation strategies**

Strategy: Native digestion with precipitation depletion of mAb from HCP peptides

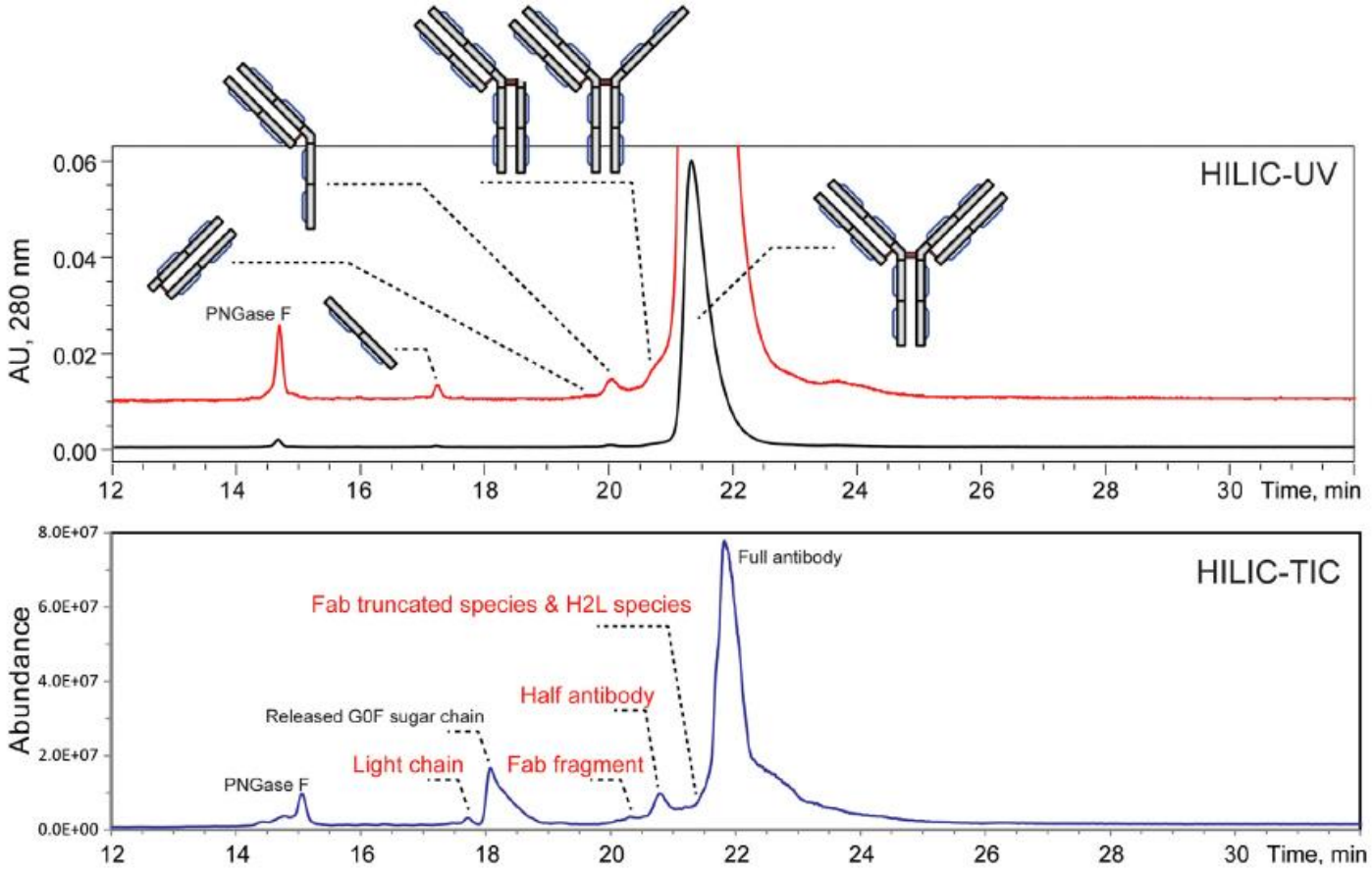


- Published by L. Huang *et al.* at Eli Lilly
- Benefits:
  - Simple, improves sensitivity
  - Universal for mAb therapeutics
- Limitations:
  - Heat labile HCPs lost
  - HCPs co-precipitating with mAb
  - Digest-resistant DS only (i.e. mAbs)
  - Incomplete removal of mAb

Anal. Chem. **2017**, *89*, 5436-5444.

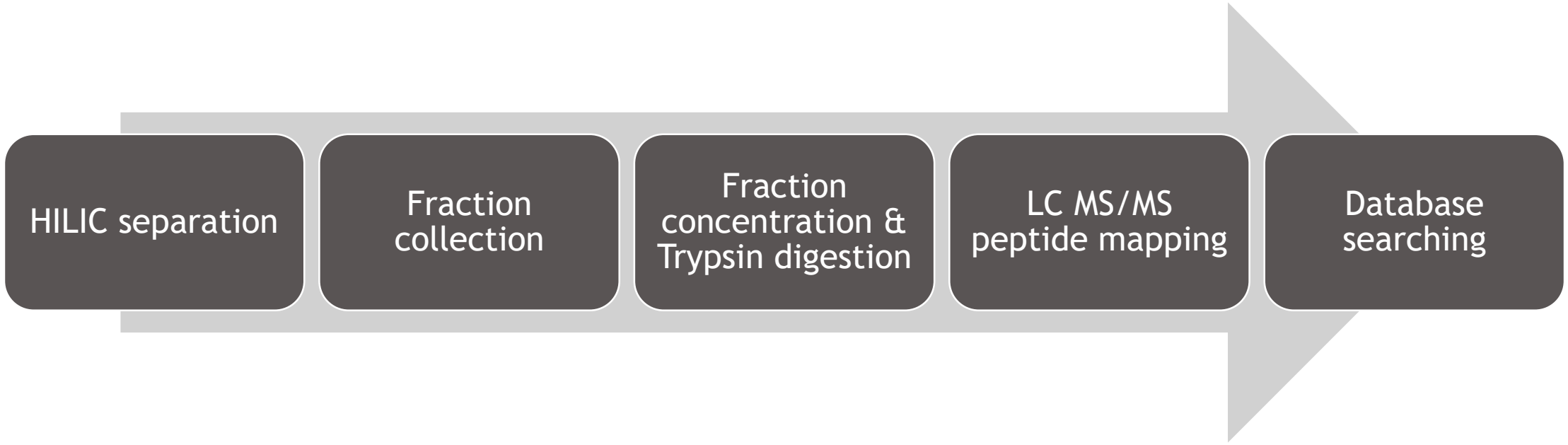
New method for therapeutic protein separation using HILIC (Hydrophilic Interaction Chromatography)

- Characterization of LMW impurities in therapeutic mAb with HILIC
  - Mostly determined by protein size
  - Denaturing conditions (high organic)
- **Can we apply the same method to separate HCPs from the mAb?**

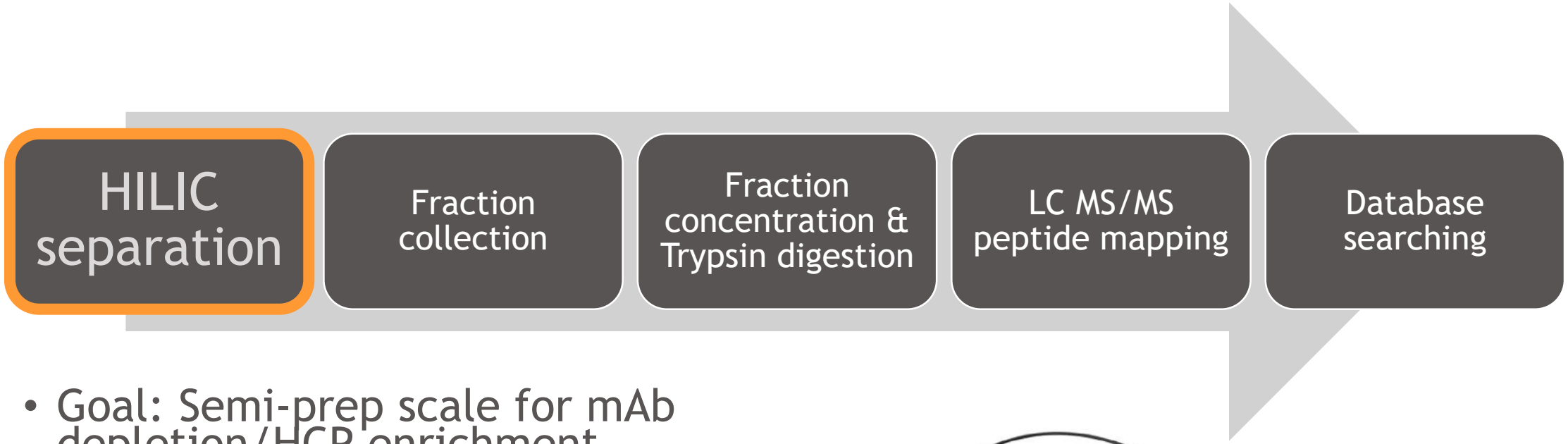


J. Pharm. Biomed. Anal. **2018**, *154*, 468-475.

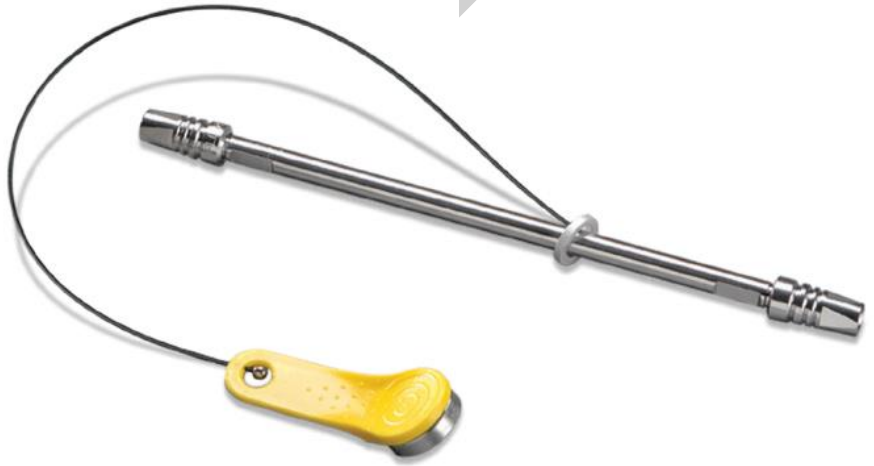
Proposed protocol



HILIC separation



- Goal: Semi-prep scale for mAb depletion/HCP enrichment
- Column:
  - Waters Acquity UPLC BEH Glycoprotein Amide column, 300 Å, 1.7 µm, 2.1 x 150 mm
- Mobile phase:
  - A/B: water/acetonitrile + 0.1% TFA

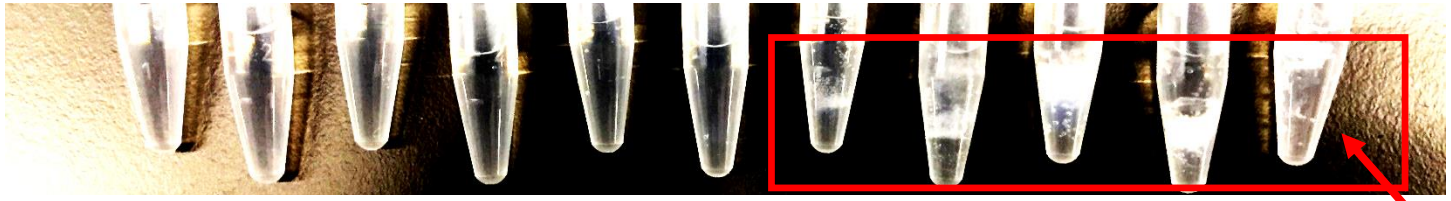


TFA Improves Solubility for Protein in Organic Mobile Phase

MP A	100	90	80	70	60	50	40	30	20	10	0
MP B	0	10	20	30	40	50	60	70	80	90	100

20 mM Ammonium Formate , 0.1% FA

MP A: 100% Aqueous
MP B: 90/10 ACN/Aq

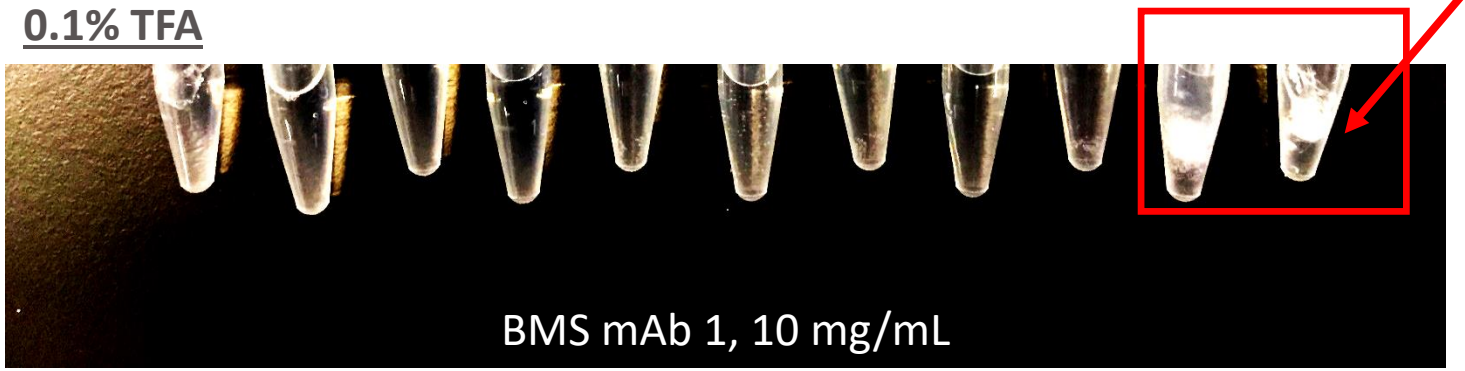


Protein in Ammonium Formate was soluble to only ~50% acetonitrile

Precipitation

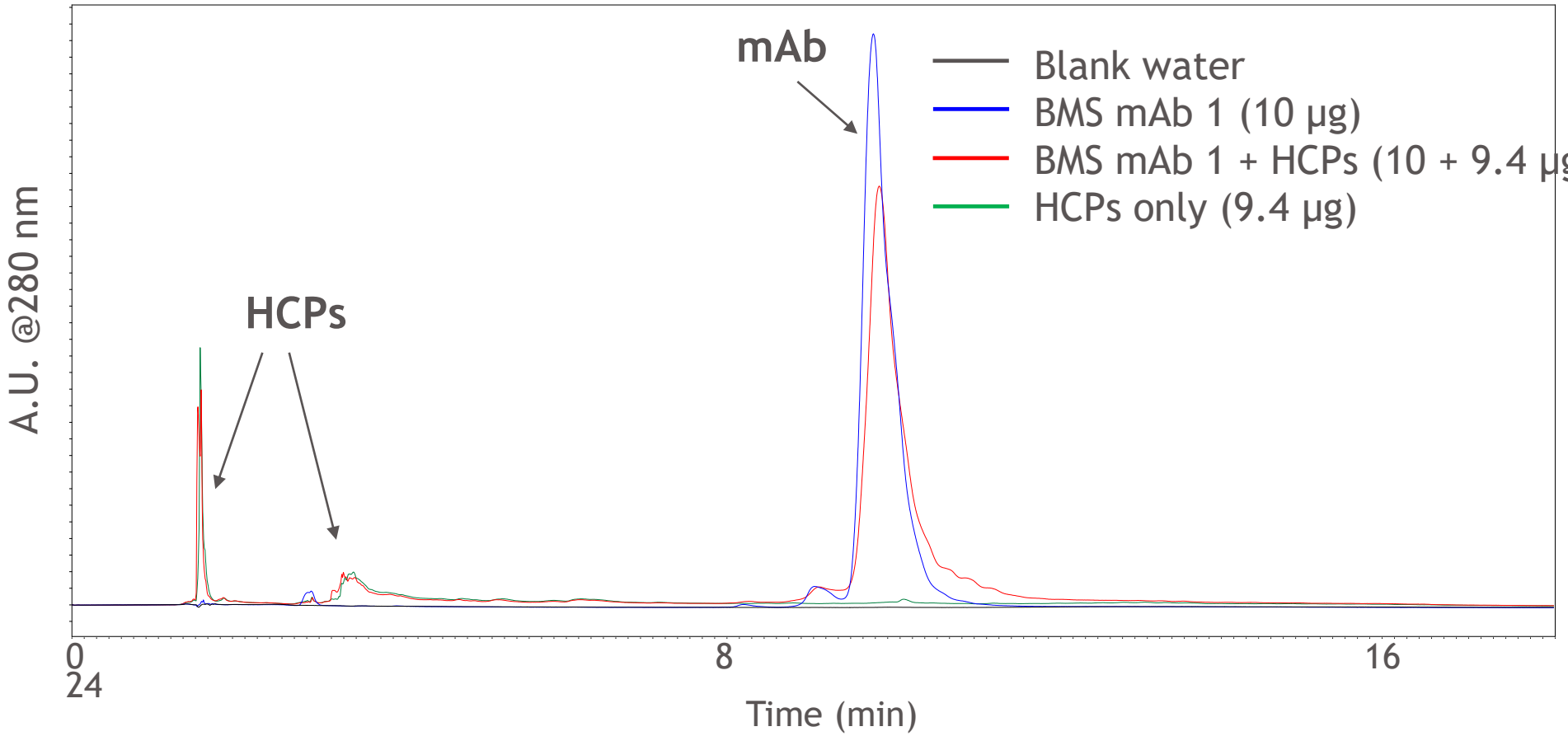
0.1% TFA

MP A: 100% Aqueous
MP B: 100% ACN



Protein in 0.1% TFA was soluble to ~80% acetonitrile

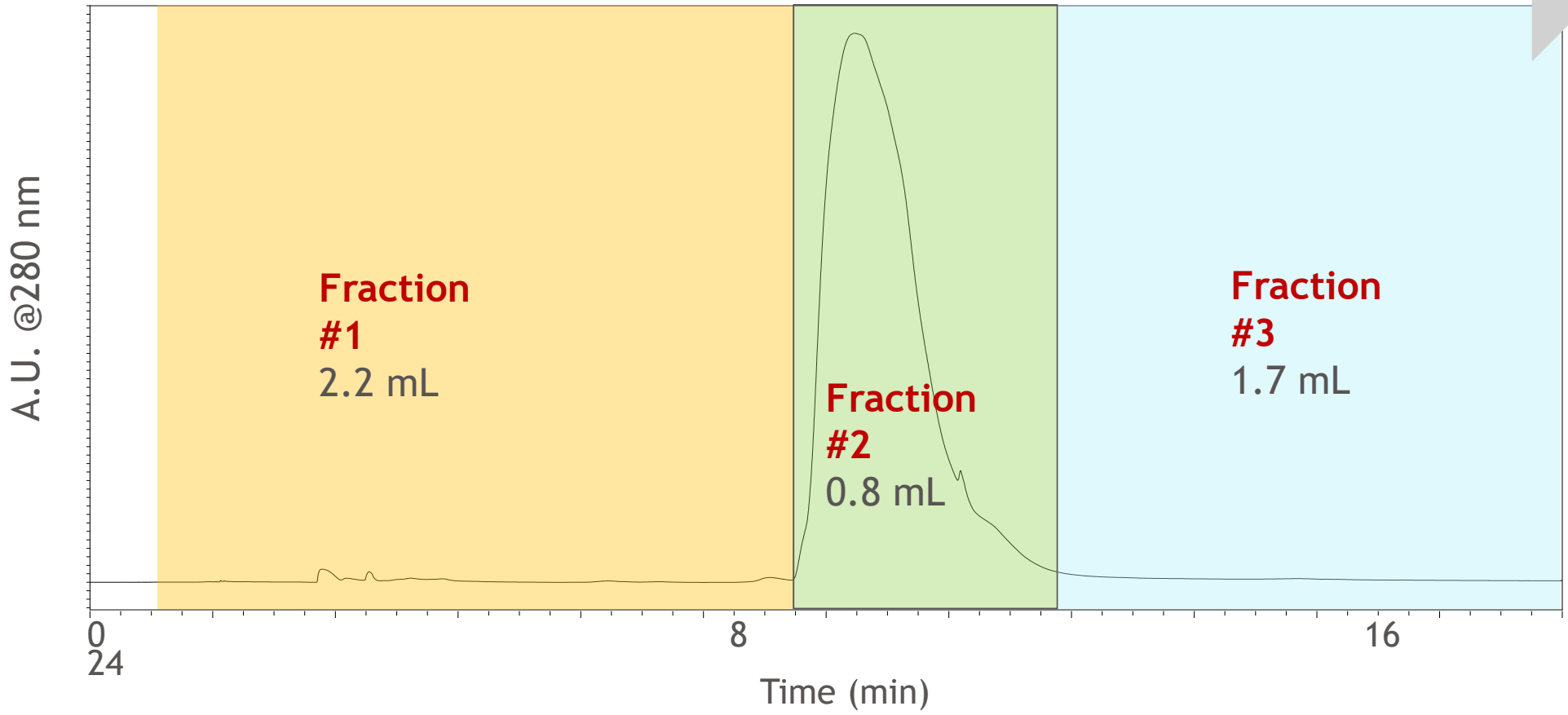
HILIC Separation of HCP standard and mAb



- Most HCPs eluted earlier than mAb
- mAb mostly eluted in a short retention window

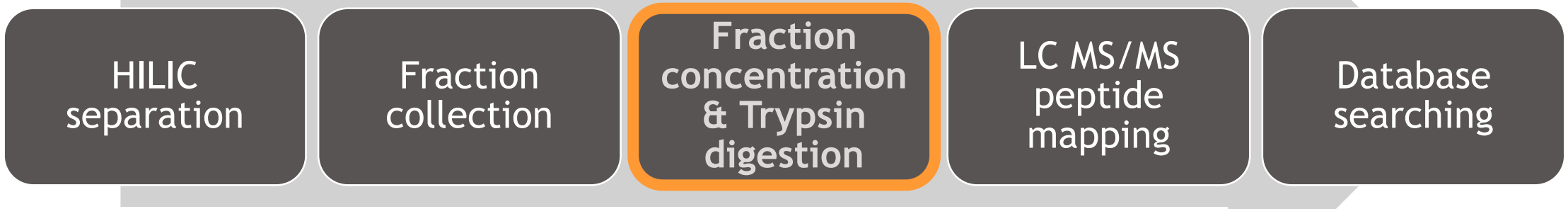
Fraction collection: higher loading; shorter gradient

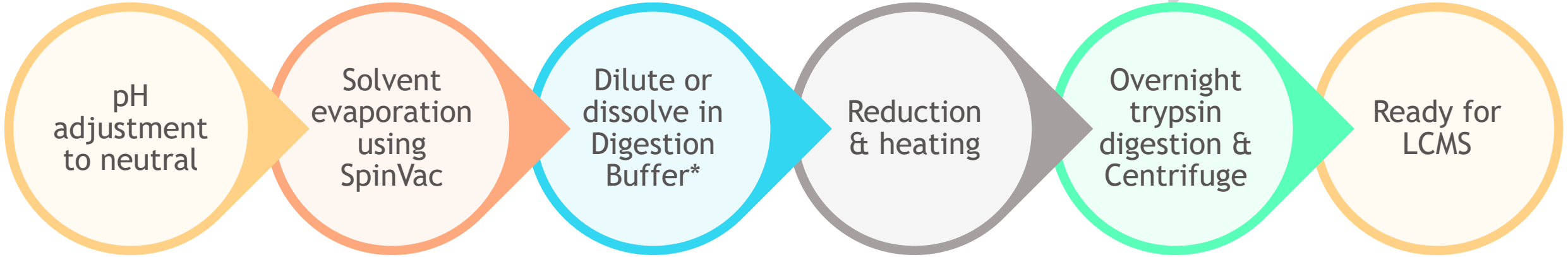
HILIC separation | **Fraction collection** | Trypsin digestion | LC MS/MS peptide mapping | Database searching



- 150 µg BMS mAb 1 per injection
- 24 min gradient
- Collected manually

Fraction concentration & Trypsin digestion





* Digestion Buffer: 2 M urea, 50 mM Tris, pH = 8.0

LC MS/MS peptide mapping

HILIC separation → Fraction collection → Fraction concentration & Trypsin digestion → **LC MS/MS peptide mapping** → Database searching

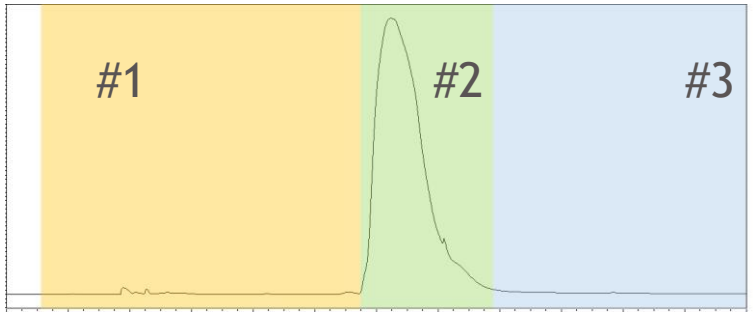
- Gradient and column from Huang *et al.* utilized
  - ~2h at 0.05 mL/min, 0.1% formic acid in ACN/H2O
- Native digestion method (Huang *et al.*) was performed side-by-side on same samples for direct comparison of HCPs detected
  - Same LC-MS/MS method

Orbitrap Fusion Lumos
(Thermo Fisher Scientific)

High resolution
High scan speed

Anal. Chem. **2017**, *89*, 5436-5444.

Results: BMS mAb1 spiked with HCPs



Method	BMS mAb1 + 1*k* ppm HCP	BMS mAb1 + 10*k* ppm HCP
HILIC-enriched HCP method	83 (61)	168 (49)
Fraction #1 only	79 (59)	131 (43)
Native digestion method	41	224
Two methods combined	**102**	**273**

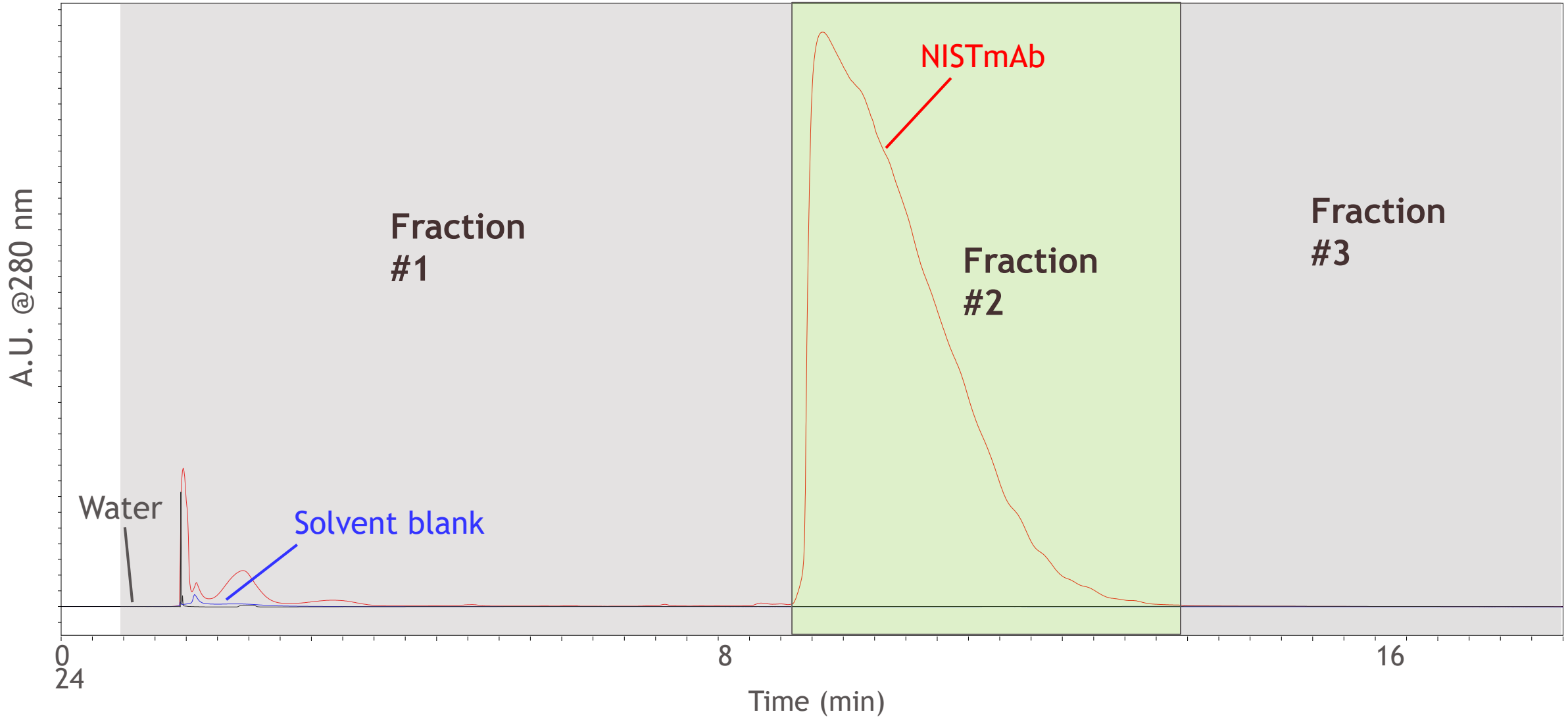
- *Just by # of HCP hits:*
  - *HILIC > Native for 1k ppm HCP sample*
  - *Native > HILIC for 10k ppm HCP sample*
- *HILIC was complementary to Native Digestion method in all samples, expanding number of hits*

- *Parentheses = # HILIC-Unique hits, not in native digestion samples*

Q. Wang *et al*. Anal. Chem. 2020, 92, 10327−10335

Optimized HILIC for large injection volume

NISTmAb Reference Material 8671,
700 µg per injection
24 min gradient



NISTmAb 8671 HCP method performance comparison

Method	NISTmAb
HILIC-enriched HCP method	71 (20)
Fraction #1 only	68 (20)
Native digestion method	91
Two methods combined	111

- Lilly method had higher # of hits in NISTmAb than HILIC
- **HILIC added 20 new hits missed by native digestion alone**
- **Methods again were complementary**
  - **111 detected HCPs by both methods together**

- ***Parentheses = # HILIC-Unique hits, not in native digestion samples***

HILIC improved sensitivity for BMS Fusion Protein 1

Method	BMS fusion protein 1
HILIC-enriched HCP method	21 (7)
Fraction #1 only	17 (6)
Native digestion method	17
Two methods combined	24

- 21 HCPs were found in total from Fraction #1 - #3
- Methods again were complementary, with HILIC returning higher # of hits

Accession #	CHO HCPs not found by Native Digestion method
G3H284	Endoplasmic reticulum resident protein 29
G3HN88	Adenylyl cyclase-associated protein
G3HRT6	Solute carrier family 12 member 1
G3I0Q0	Non-muscle caldesmon
G3ID62	Exostosin-like 2
G3IPK9	Adenylate kinase 2, Mitochondreal

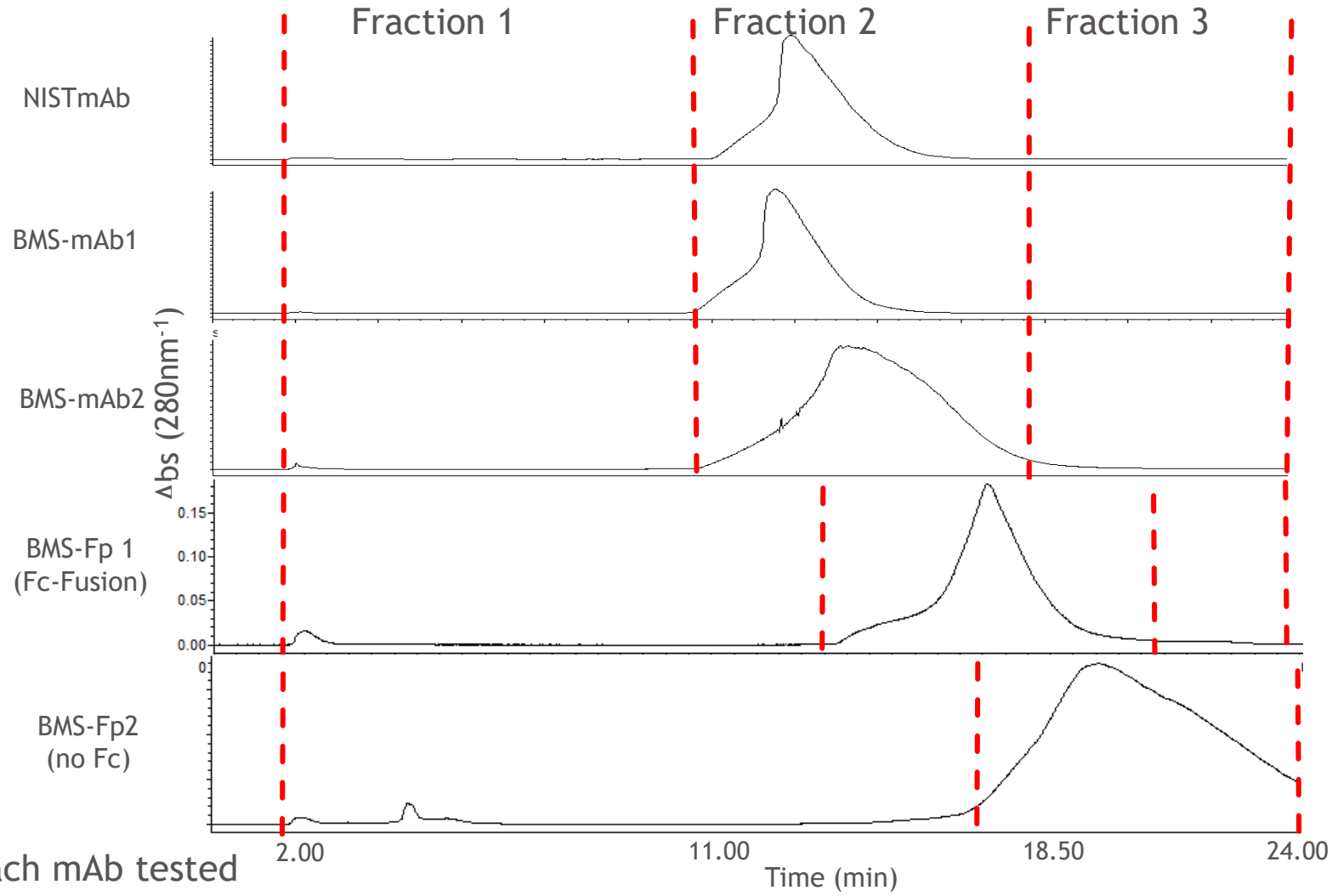
Further optimizations of HILIC-HCP method

- Developing “universal” chromatography conditions
- Improving throughput through automation:
  - Well-plates
  - Fraction collector
  - Plate dryer
- Implementing Quantification Software

A More “Universal” HILIC Diluent and Gradient

- Optimized diluent:
  - 75% ACN, 0.1% TFA

Therapeutic	Concentration (g/L)	Soluble?
NISTmAb 8671	2.5	Yes
BMS-mAb 1	5.0	Yes
BMS-mAb 2	5.0	Yes
BMS-Fp-1	5.0	Yes
BMS-Fp-2	5.0	Yes
BMS-Fp-3	1.0	No



- Gradient and retention windows worked for each mAb tested
- Fusion proteins require specific optimization for solubility and gradient/retention windows

Implementing High-Throughput and Quantitation for HILIC HCP Analysis

HILIC- LC

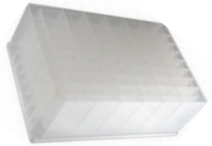
Manual Fractions

SpeedVac

Trypsin Digestion

RP- LC/MS

UPLC w/ Fraction Collector



Well plate Fractions

Heated Plate Dryer

I.S. Protein added + Trypsin Digestion

RP- LC/MS
Quantitation via “Hi-3” method

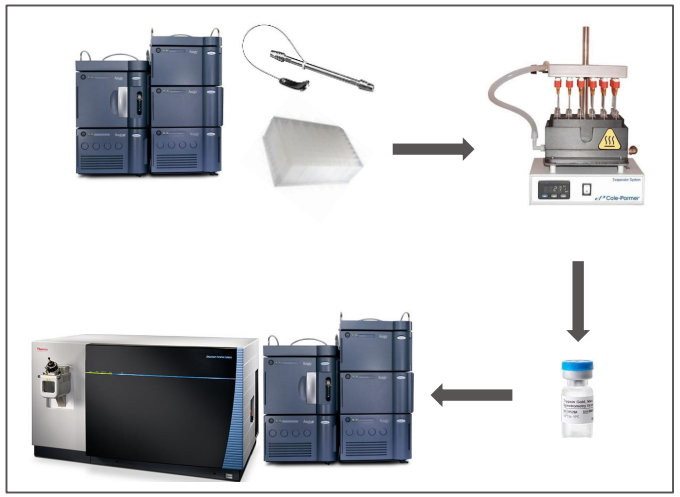
- Throughput improved by automation

Quantitation of Destabilizing HCPs in Protein A-Purified mAb

HCPs of Interest
Carboxypeptidase
Cathepsin D
Cathepsin L1
Lipase
Lipoprotein Lipase
Phospholipase A2
Phospholipase D1
Putative phospholipase B-like 2

Protein	HILIC (ppm)	Native Digest (ppm)
Lipoprotein lipase	104.4 ± 15.7	10.8 ± 15.3
Carboxypeptidase	94.7 ± 20.4	11.9 ± 16.8
Lipase	4.4 ± 5.6	8.4 ± 11.9
Phospholipase B-like	1.8 ± 0.3	6.9 ± 9.8

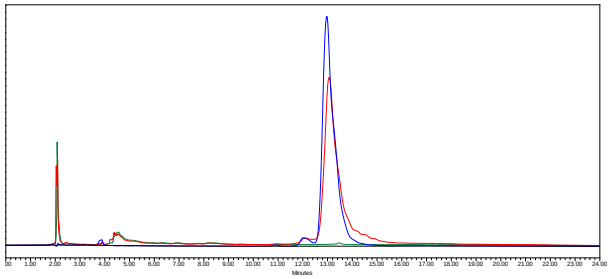
n=3 replicates

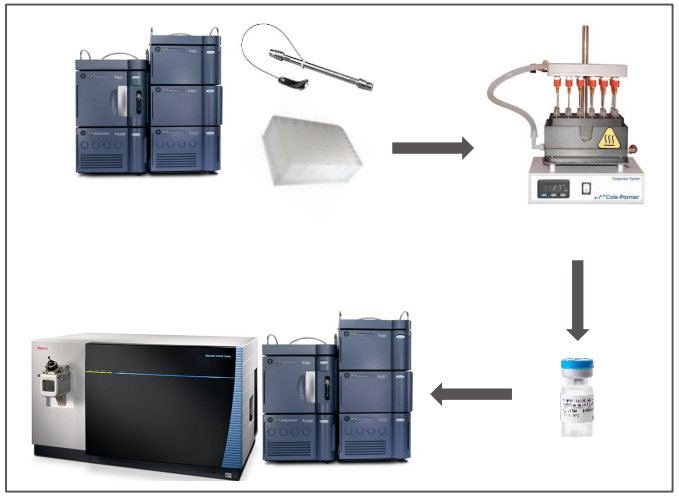


- BMS-mAb2 Protein A eluate searched
- Recovery of different HCPs varied for HILIC vs. Native Digestion
- Agrees with differences in selectivity of methods observed previously

Summary

- A HILIC HCP enrichment method for LC-MS samples was developed for both mAbs and Fusion Proteins
- Our method offers different selectivity for HCPs than the Native Digestion method, improving number of HCPs detected
- Automation and Quantitation improve throughput and capability for screening therapeutic samples
- Future work:
  - Optimizing conditions for fusion proteins
  - Evaluating impact of glycans on HILIC enrichment
  - Demonstrating high throughput analysis for project samples



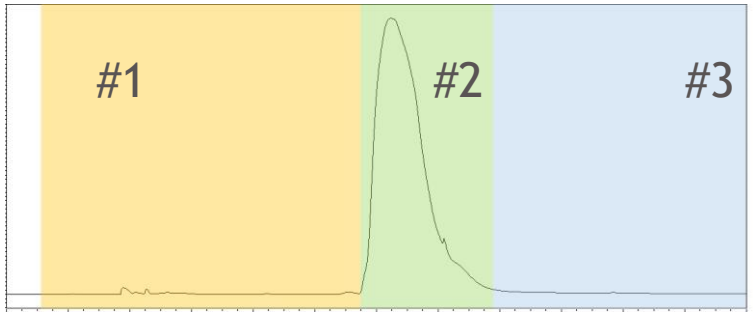


Acknowledgements

- Bristol Myers Squibb Internship and Co-op Program
- Li Tao
- Wei Wu
- Lei Zhang
- Richard Ludwig
- Anthony Leone
- Zhengjiang Li

- Thank you for your time.

Results: BMS mAb1 spiked with HCPs



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HILIC-enriched HCP method	**83 (61)**	**168 (49)**
Fraction #1 only	**79 (59)**	**131 (43)**
Fractions #1 and #2	0 (0)	5 (0)
Fraction #2 only	**2 (1)**	**4 (2)**
Fractions #2 and #3	0 (0)	2 (0)
Fraction #3 only	**2 (1)**	**12 (2)**
Fractions #1 and #3	0 (0)	9 (2)
All three fractions	0 (0)	5 (0)
Native digestion method	**41**	**224**
Two methods combined	**102**	**273**

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  - *HILIC > Native for 1k ppm HCP sample*
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