Advancements in subzero temperature chromatography for HDX-MS

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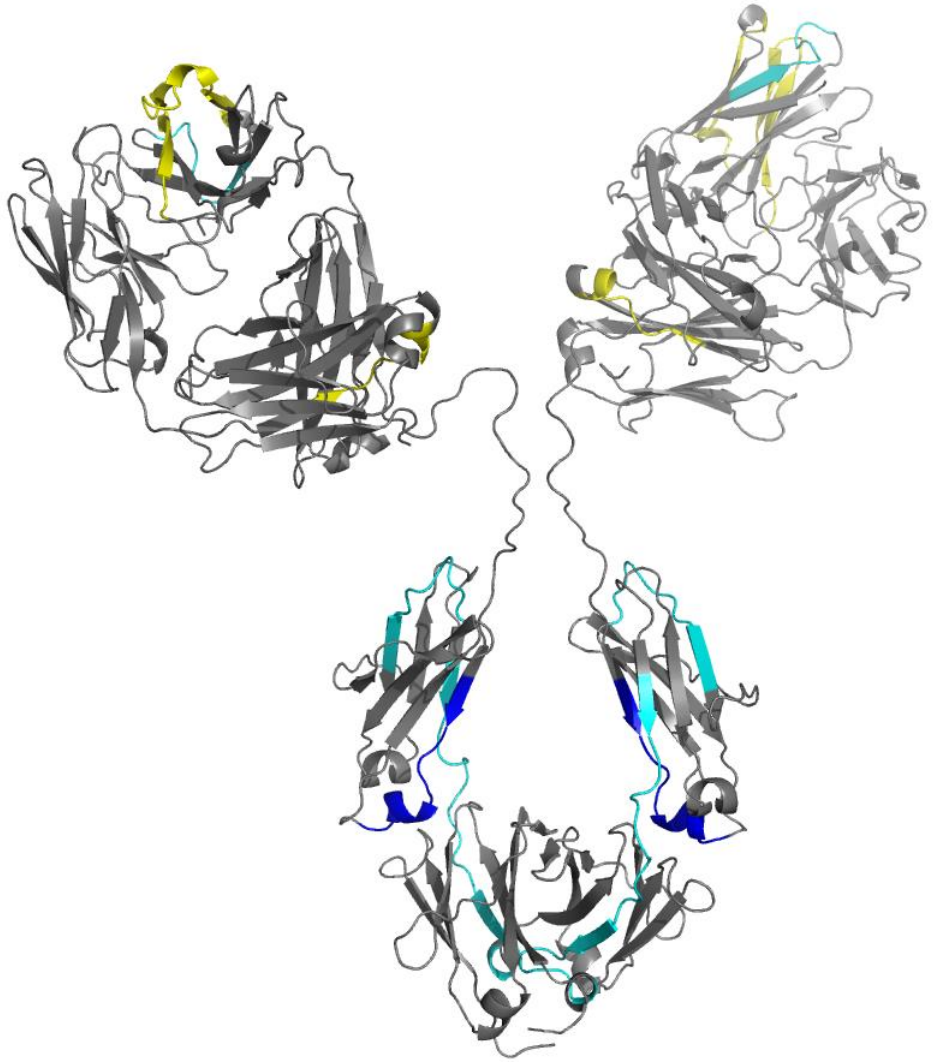
HDX for measuring protein structural dynamics



- conformations of proteins and complexes
- binding interactions
- epitope mapping
- allosteric effects
- folding dynamics
- stability
- biosimilarity

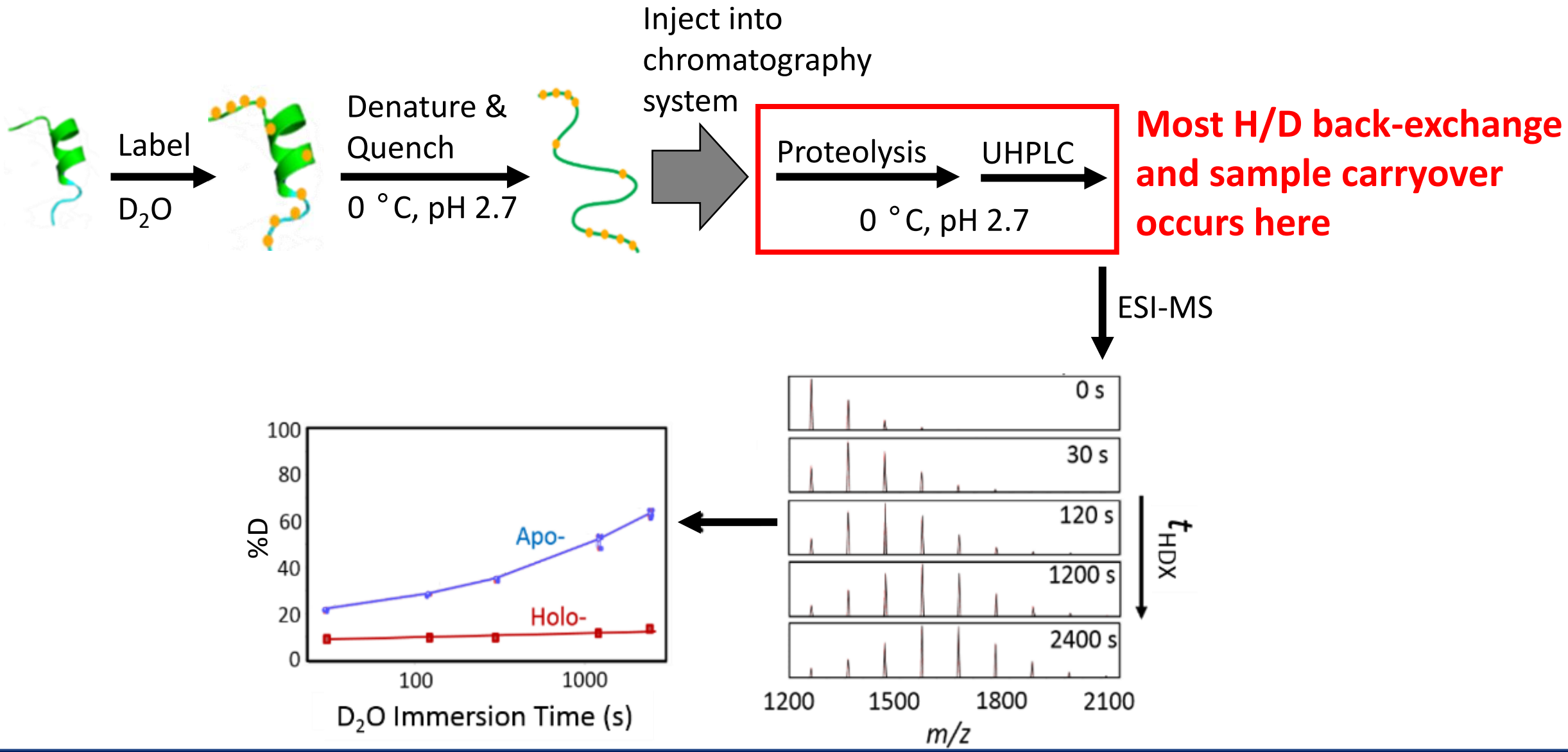
Problem: Back-exchange

- Loss of deuterium label impairs dynamic range of HDX
- Back-exchange can cause poor reproducibility between labs
- Poor temperature regulation causes unsuitable repeatability
- Conventional HDX-MS systems operating at pH 2.7 and 0 °C can lose 30-40% D in 15 min

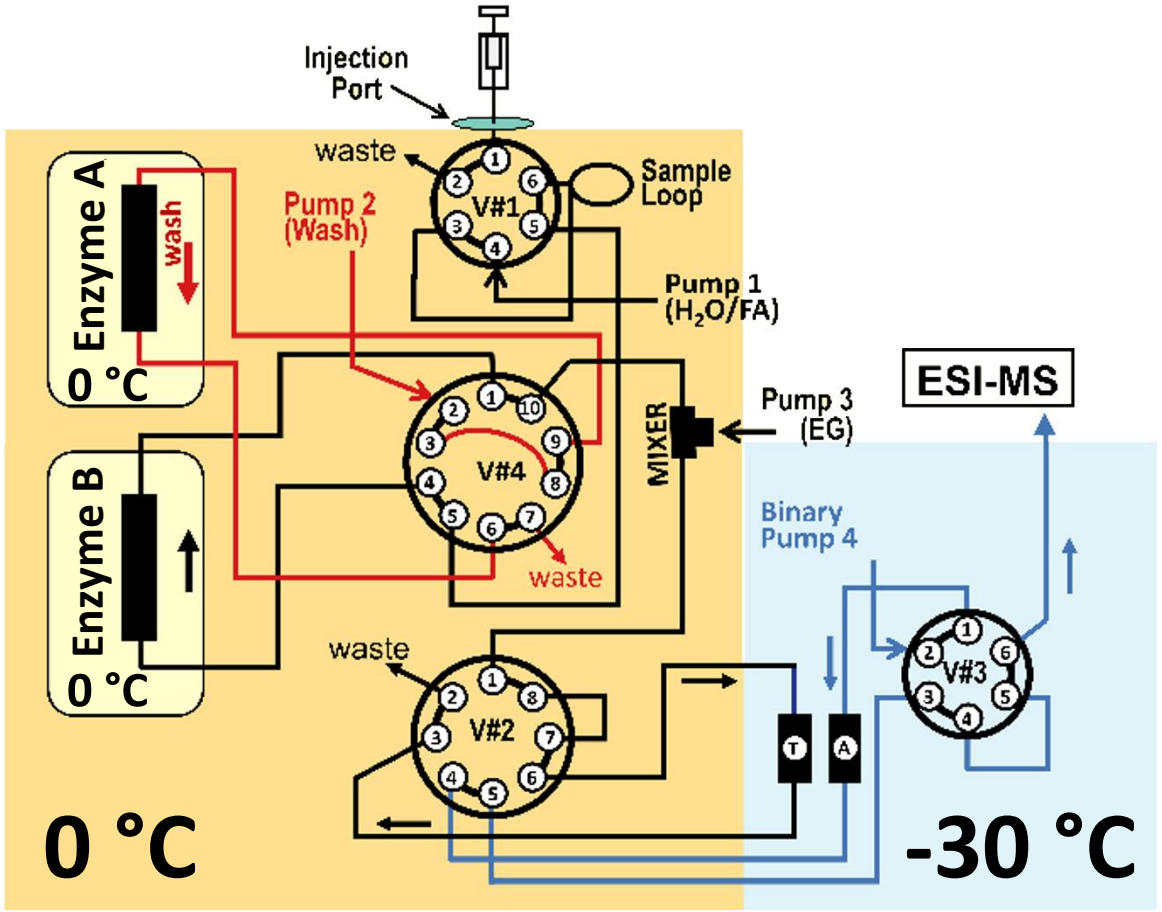
Challenges

- Subzero temperatures can reduce back-exchange, but buffer modifiers are needed to prevent freezing
- Solvents like methanol can impair peptide retention on reversed phase trap columns (>40% MeOH by volume needed for -30 °C)

HDX-MS Workflow



Dual-enzyme, subzero temperature HDX-MS system



Zone	T ± SD, ˚C
Preparation	0.00 ± 0.06
Analytical	-30.00 ± 0.02
Enzyme A	0.000 ± 0.002
Enzyme B	0.000 ± 0.002

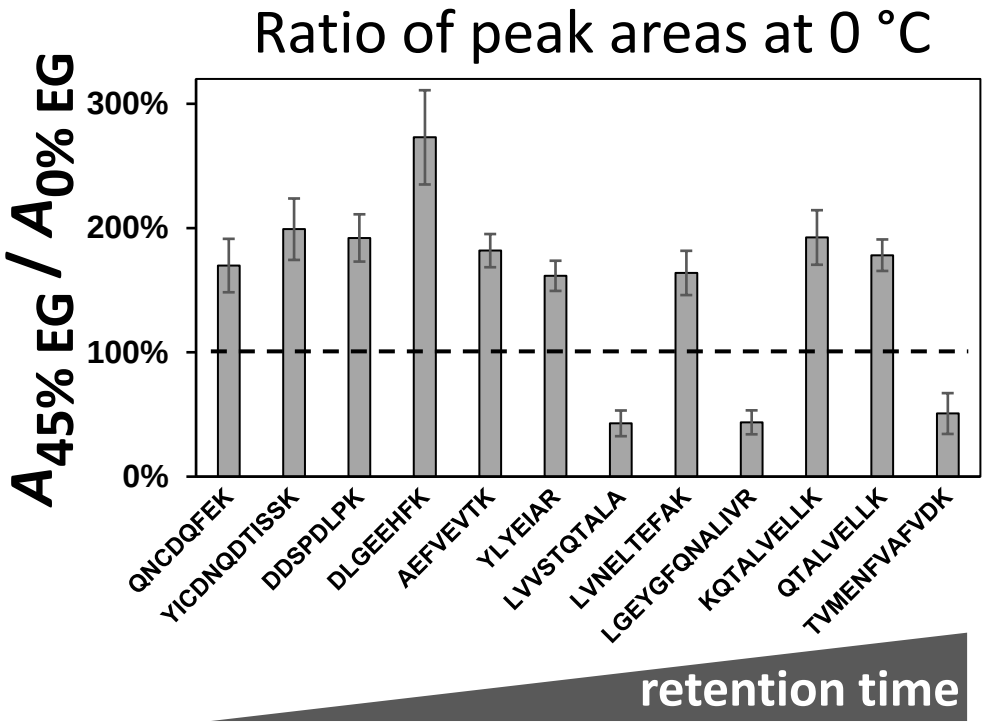
Precise temp. control

- Trap and analytical columns at subzero temp.
- Backflushing of all columns is supported
- Concurrent cleaning with analytical runs
- Enzyme column cleaning with binary pump
- Allows combinations of proteases, glycosidases, and enzyme columns at independent temps. needed for activity

Does EG affect ESI-MS signal?

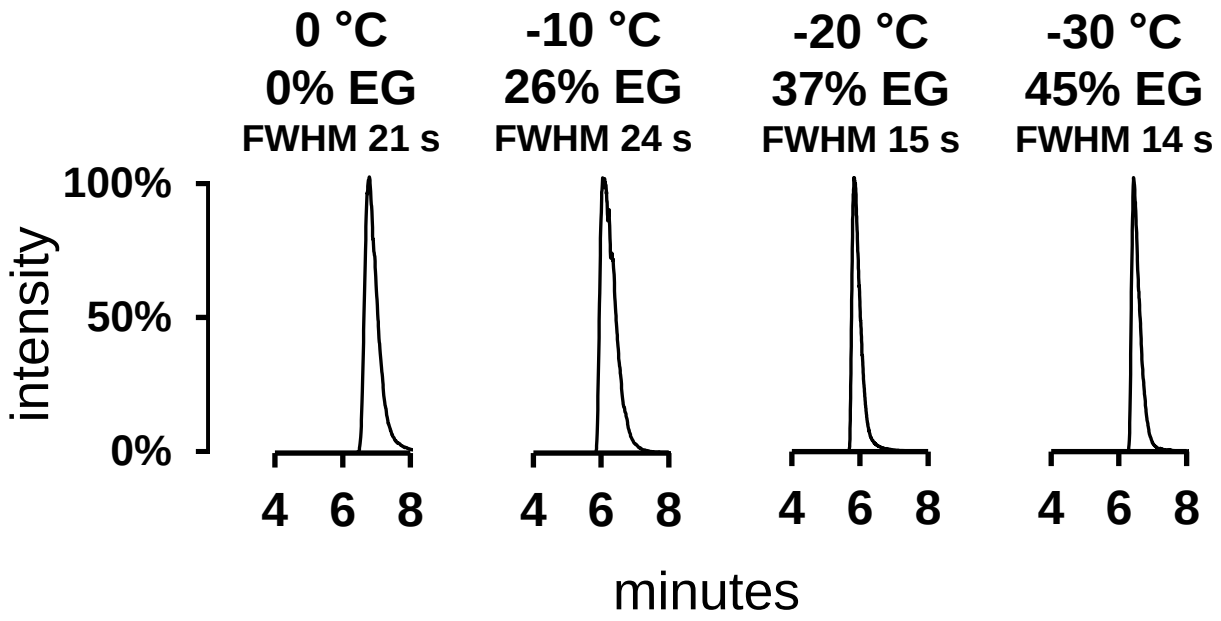
BSA tryptic peptides measured at 0 °C with 0% EG and 45% EG in aqueous mobile phase

0 °C	0 % EG
-10 °C	26 % EG
-20 °C	37 % EG
-30 °C	45 % EG



Added EG has no negative impact

Effects of EG & temperature on chromatography



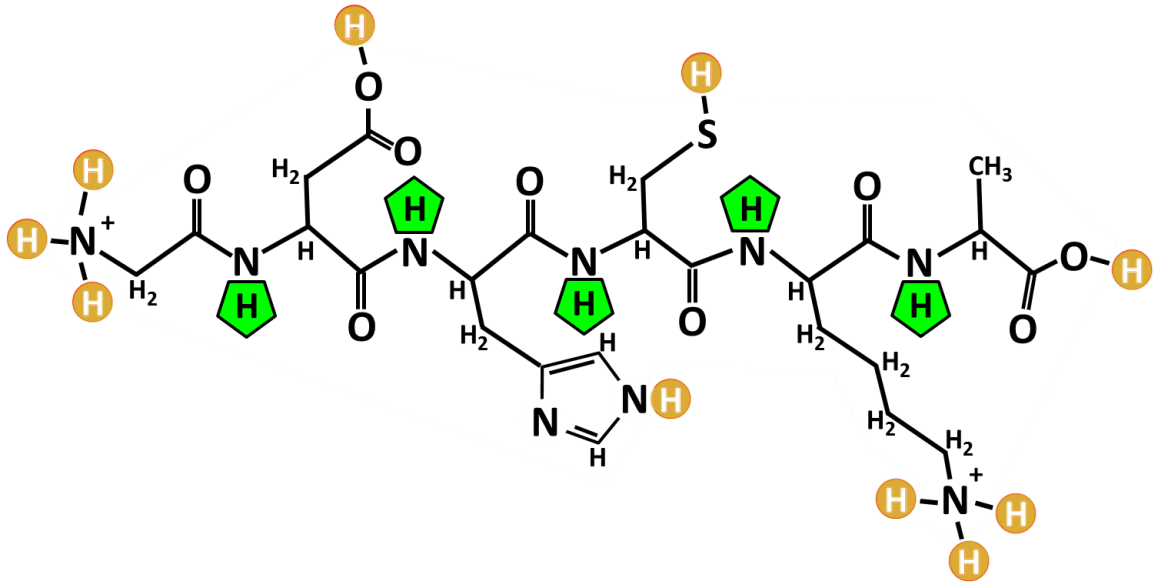
pressure	**240 bar**	**400 bar**	**550 bar**	**950 bar**

- Decrease in temperature increases retention time
- Peptide separation not degraded
- Pressure increases with EG and lower temperature
- Thermo Vanquish pump and stainless steel tubing used to meet pressure requirements
- LC Pump capacity dictates trap and analytical column specifications

Trap column: 2.1 mm x 20 mm, 2.6 µm

Analytical column: 2.1 mm x 30 mm, 2.6 µm

HDX sites

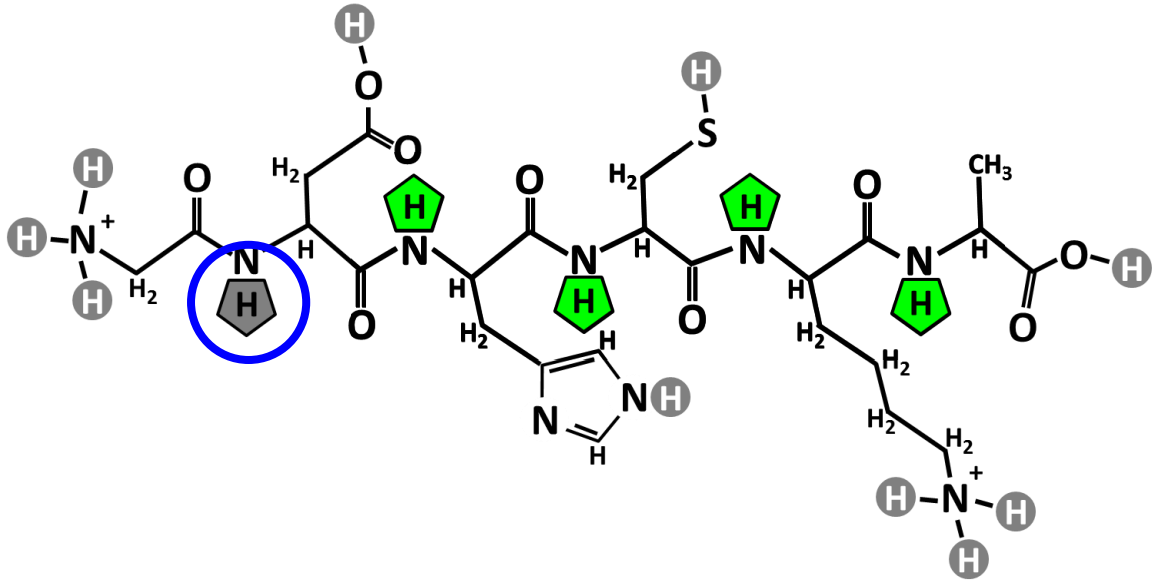


Gly – Asp – His – Cys – Lys – Ala

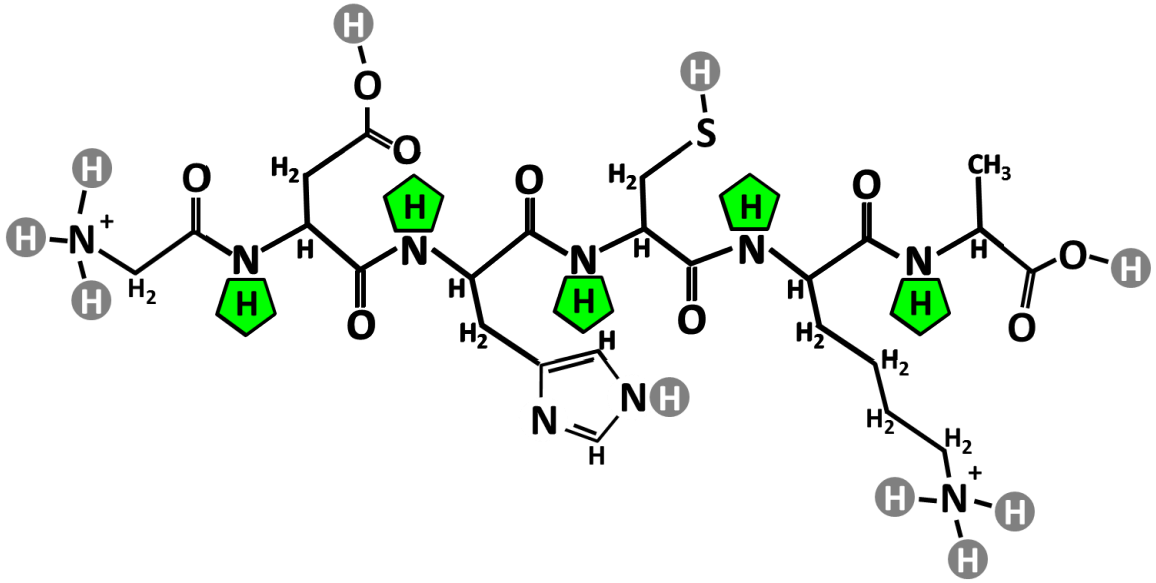
HDX rate of side chains is too fast (t_{ex} < 1 ms)

HDX rate of amide backbone is more easily measured (t_{ex} = 10 s to hours)

%D calculation: Should we rethink D_{max}?



Gly – Asp – His – Cys – Lys – Ala



Gly – Asp – His – Cys – Lys – Ala

$$\%D = \frac{m_{t,HDX} - m_{0\%}}{m_{100\%} - m_{0\%}} \times 100\%$$

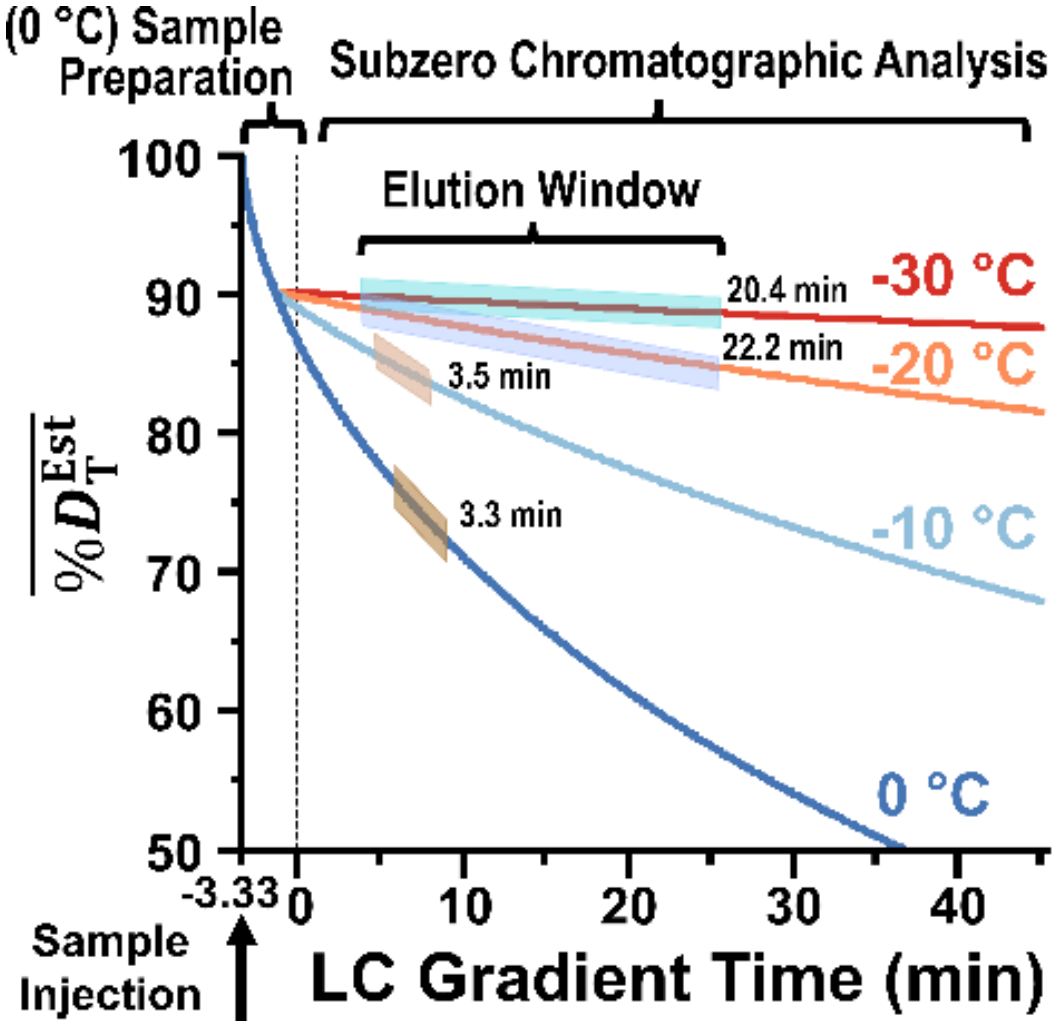
90% D using n-1 exchangeable backbone amides

72% D using all exchangeable backbone amides

HDX-MS characteristics at $T \leq 0$ °C

LC gradient duration, min	8	8	8	8	40	40
$T_{\text{Zone 2}}$, °C	0	-10	-20	-30	-20	-30
Elution window, min	3.30 ± 0.02	3.5 ± 0.1	4.40 ± 0.02	5.30 ± 0.07	22.2 ± 0.2	20.4 ± 0.2
Average %D for 12 peptide set	72 ± 11	83 ± 9	90 ± 10	90 ± 10	87 ± 9	89 ± 10
%D improvement		11 ± 14	18 ± 15	18 ± 15	15 ± 14	17 ± 15
%D improvement for high 3 peptides		18 ± 3	27 ± 1	24 ± 3	26.7 ± 0.7	25.3 ± 0.3

HDX-MS characteristics at $T \leq 0$ °C

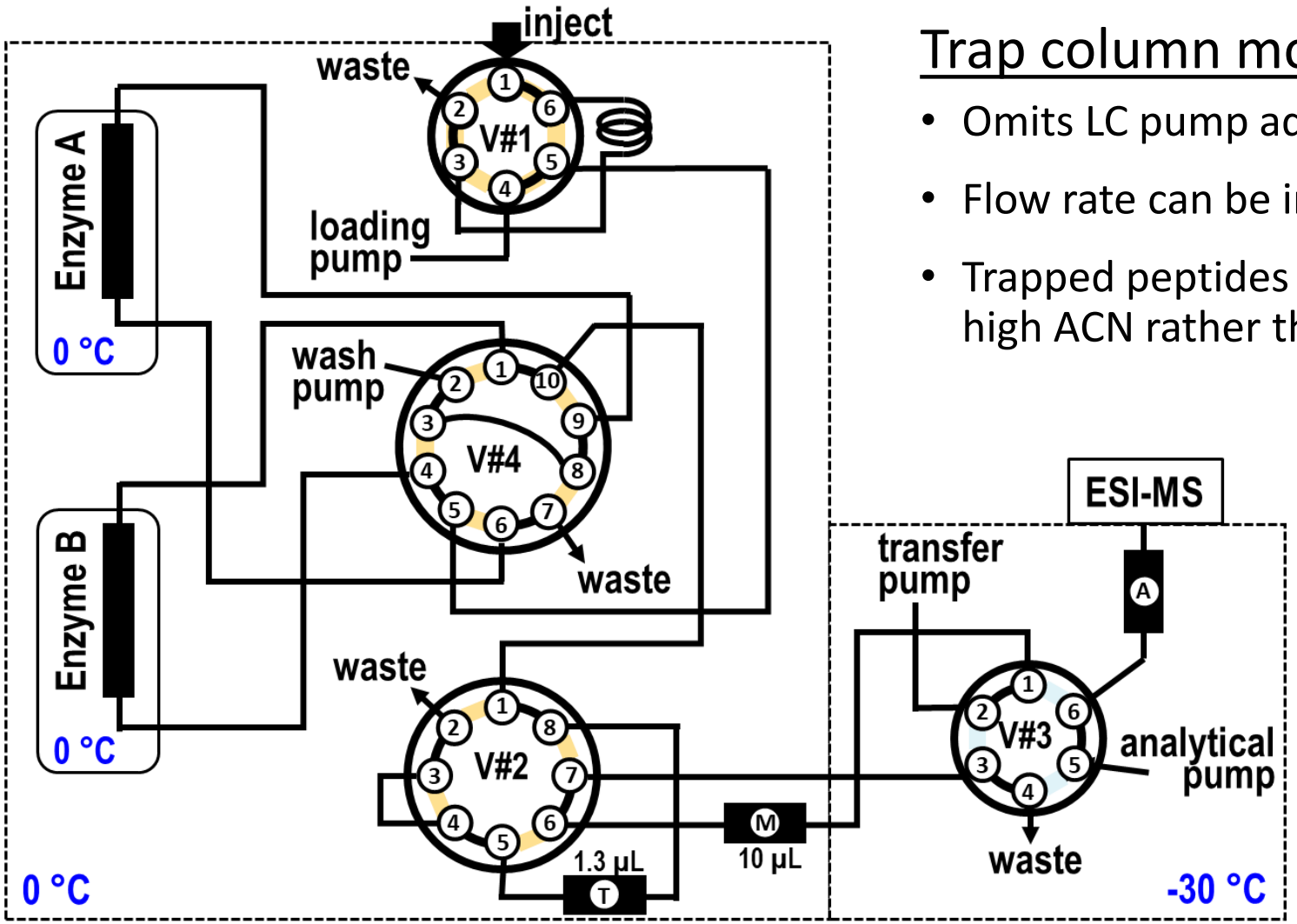


- Most of back-exchange for -30 °C measurements comes from sample prep zone
- Gradients at -30 °C could be extended with minimal back-exchange

HILIC analytical separations to reduce pressures

- Backpressures at -30 °C were suitable for latest UHPLC pumps, but methods to reduce pressures could more easily facilitate adoption of subzero chromatography for HDX-MS and improve its robustness.
- Reversed phase liquid chromatography (RPLC) gradients start with ≈97% water.
- Hydrophilic interaction liquid chromatography (HILIC) gradients elute with ≤50% water or polar solvent.
- Greater fraction of water requires more ethylene glycol to prevent freezing at subzero temperatures. Both water and ethylene glycol increase backpressures.
- HILIC can use methanol in place of water for elution.

Modifications to subzero LC system for HILIC



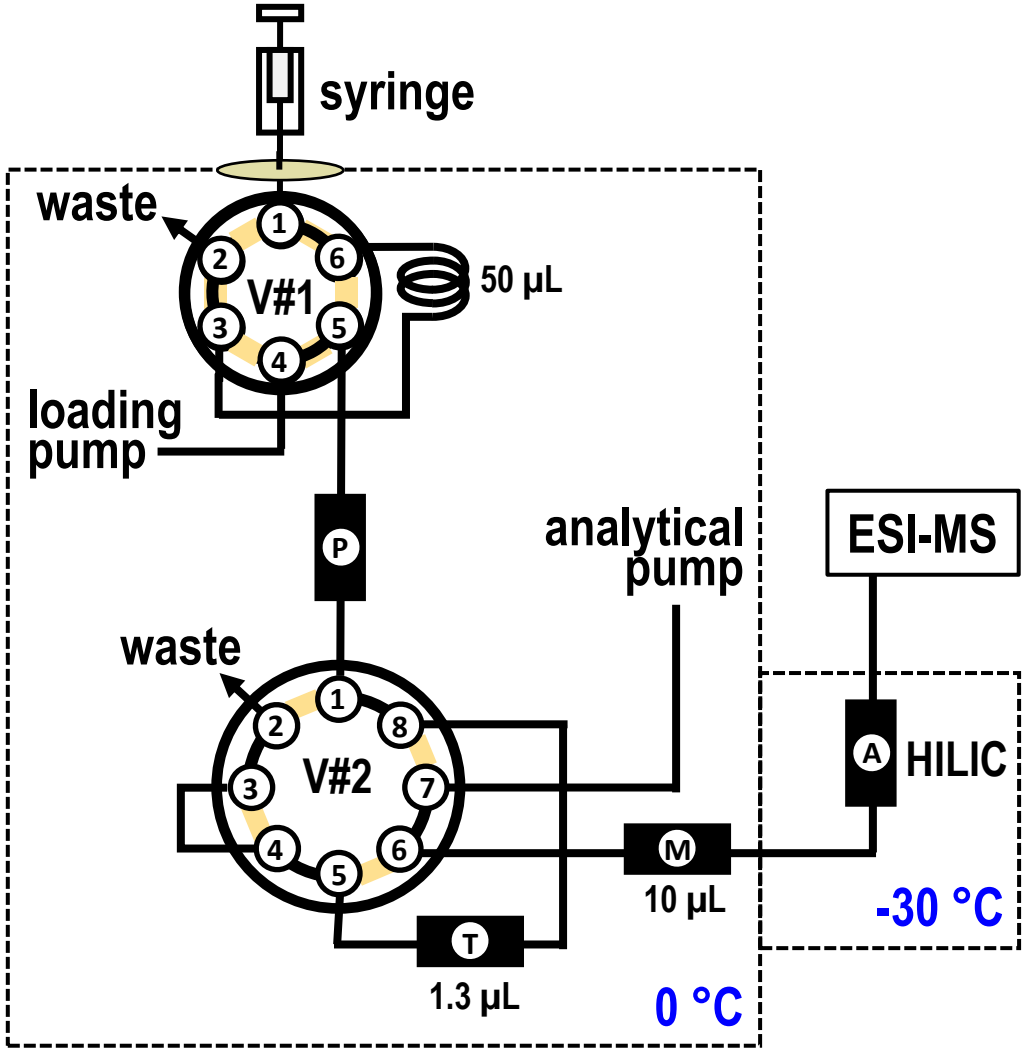
Trap column moved to prep chamber

- Omits LC pump adding EG at mixing T before trap
- Flow rate can be increased from 50 to 200 µL/min
- Trapped peptides immediately elute to HILIC column with high ACN rather than slowly eluting like in RPLC

	RPLC	HILIC
Proteolysis time, temp.	120 s, 0 °C	60 s, 0 °C
Desalting time, temp.	80 s, -30 °C	
Transfer time, temp.		10 s, 0 °C

- Microtrap and 10 µL mixer used to reduce concentration of water during sample loading

Simplest 2 valve, 2 pump system for HILIC



HILIC can reduce backpressures

$T_{\text{Zone 2}}$, °C	Mode	Aqueous/polar solvent volume fractions,	Loading pump pressure, bar	Analytical pump pressure, bar
0	RPLC	100% H_2O	70	240
-20	RPLC	63% H_2O, 37% EG	200	550
-20	HILIC	63% H_2O, 27% EG, 10% MeOH	50	130
-30	RPLC	55% H_2O, 45% EG	240	950
-30	HILIC	63% H_2O, 27% EG, 10% MeOH	50	320
-30	HILIC	100% MeOH	50	60

Mixtures contain a volume fraction of 0.1% FA.

<u>Column dimensions for reversed phase LC</u>

Trap: 2.1 mm x 20 mm, 2.6 µm

RP analytical: 2.1 mm x 30 mm, 2.6 µm

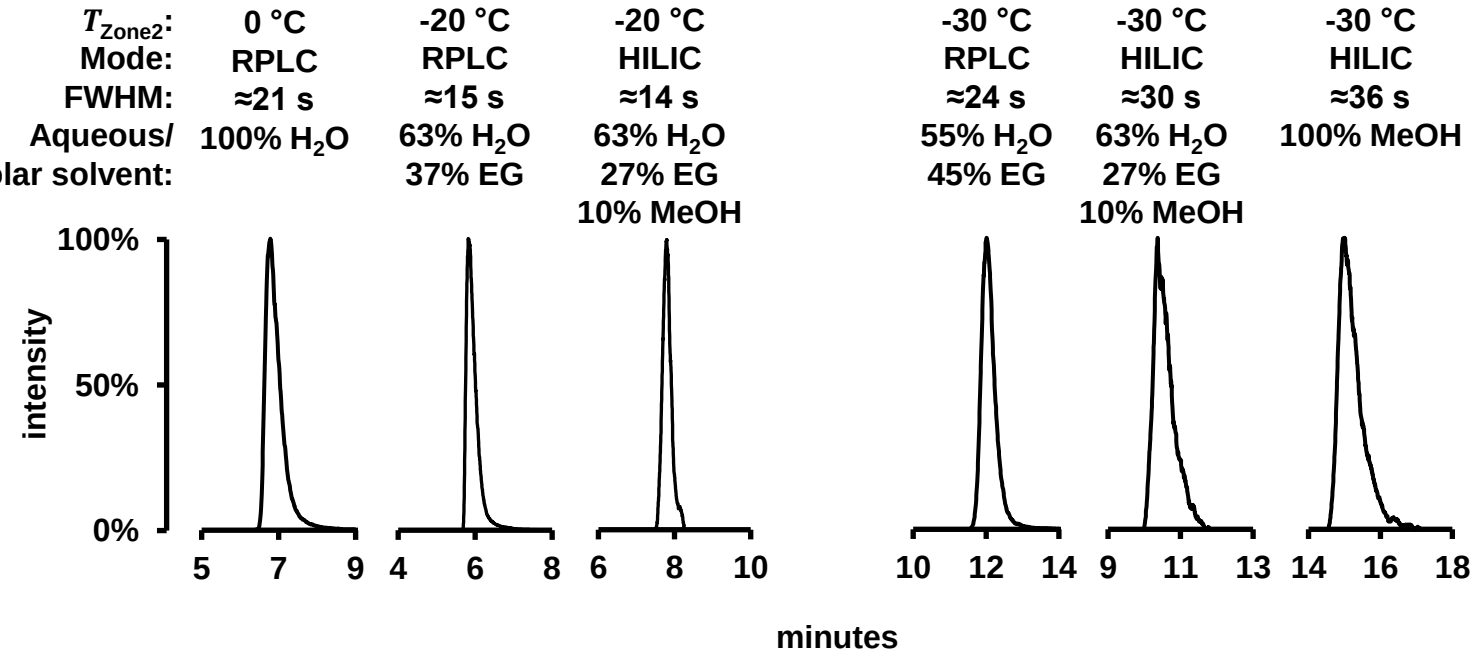
<u>Column dimensions for HILIC</u>

Trap: 0.5 mm x 10 mm, 5 µm

HILIC analytical: 2.1 mm x 100 mm, 1.8 µm

Effects on chromatography

LC peak profiles observed for peptide YLYEIAR^{2+}



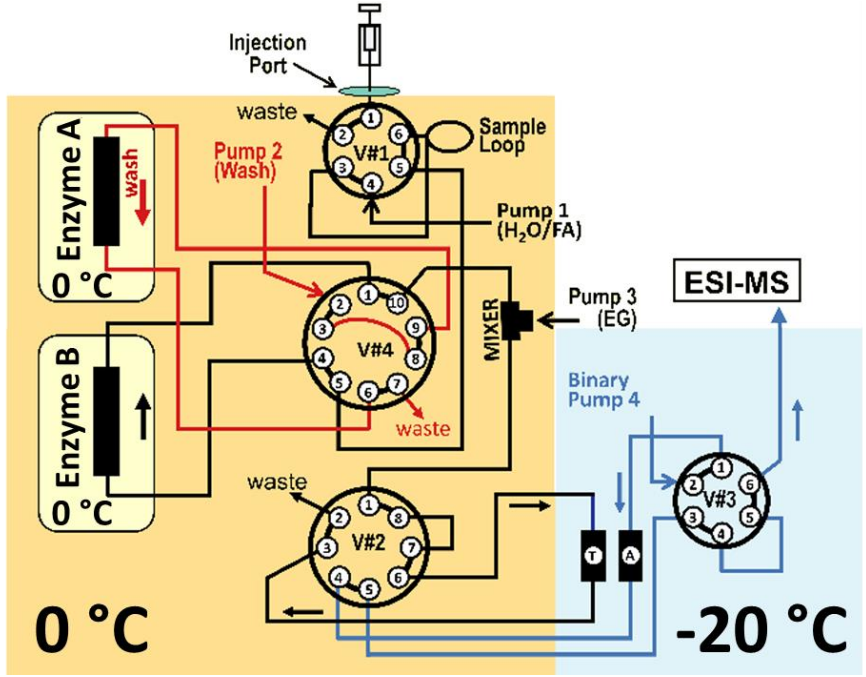
%D recovery in RPLC vs HILIC

LC gradient duration	8 min	8 min	10 min	40 min	30 min	40 min
Method	RPLC	RPLC	HILIC	RPLC	HILIC	HILIC
$T_{\text{Zone 2}}$, °C	0	-20	-20	-30	-30	-30
% EG in polar/aqueous solvent		37	27	45	27	
% MeOH in polar/aqueous solvent			10		10	100
Average %D for 21 peptides	**71 ± 7**	**89 ± 7**	**84 ± 7**	**89 ± 7**	**81 ± 8**	**84 ± 9**
%D improvement		**18 ± 10**	**13 ± 10**	**18 ± 10**	**10 ± 11**	**13 ± 11**
%D improvement for high 3 peptides		**27 ± 2**	**20 ± 2**	**26 ± 1**	**19 ± 1**	**24 ± 2**

Best RPLC condition for gradients <45 min

$T_{\text{Zone 2}}$, °C	Mode	Aqueous/polar solvent volume fractions,	Loading pump pressure, bar	Analytical pump pressure, bar
-20	RPLC	63% H_2O, 37% EG	200	550
-20	RPLC	68% H_2O, 32% MeOH	200	200

Mixtures contain a volume fraction of 0.1% FA.



Solvent after T-mixer: 63% H_2O, 37% EG

<u>Analytical pump</u>

Solvent A: 32% MeOH, 68% H_2O, 0.1% FA

Solvent B: ACN, 0.1% FA

Summary

System allows complex cleaning to remove carryover

2 enzyme column compartments

Rigorous temperature control

Reduced back-exchange

- -30 °C chromatography can enable long gradients to increase peptide detection
- Minimal back-exchange across elution window improves peptide comparability
- Improves reproducibility across labs

HILIC methods to facilitate adoption of -30 °C chromatography

- HILIC analytical separations greatly reduce pressures and allow faster flow rates

More reliable HDX-MS system for biopharma QC programs and protein similarity studies

Publications

1. Anderson, K. W.; Hudgens, J. W. Chromatography at -30 °C for Reduced Back-Exchange, Reduced Carryover, and Improved Dynamic Range for Hydrogen−Deuterium Exchange Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2022.
2. Anderson, K. W.; Hudgens, J. W. Hydrophilic Interaction Liquid Chromatography at Subzero Temperature for Hydrogen-Deuterium Exchange Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2023.
3. Hudgens, J. W. Construction of a Dual Protease Column, Subzero (-30 °C) Chromatography System and Multi-channel Precision Temperature Controller for Hydrogen-Deuterium Exchange Mass Spectrometry. *J. Res. Natl. Inst. Stand. Technol.* 2020.

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