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# Top-Down Proteomics

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The reversed-phase columns used for bottom-up proteomics of peptides don't work with many proteins. Some don't elute at all while others elute in peaks 15 minutes wide. Progress in top-down proteomics requires alternative modes of chromatography. Examples:

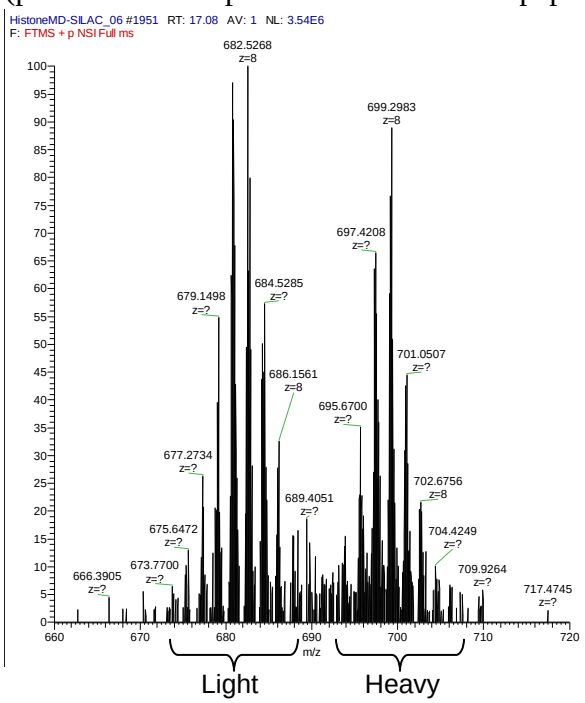
## WCX-HILIC of Histone isoforms: Histone H3(2-51)

Over 700 variants of H3 have been identified with various combinations of 17 types of **post-translational modifications** (PTM's). Most of the PTM's are in residues 2-51. H3 is typically analyzed via that fragment from Glu-C digestion ("middle-down" proteomics). Success in this identification requires good separation of the variants by chromatography prior to MS. This is accomplished with capillaries of our WCX material, **PolyCAT A**. Inclusion of 60-70% ACN superimposes hydrophilic interaction and hence sensitivity to variations in polarity (*e.g.*, methylation) as well as charge. WCX materials lose their (-) charge below pH 4. WCX-HILIC can then be performed with a totally volatile mobile phase with a decreasing gradient of ACN (tuning down the hydrophilic interaction) and pH (uncharging the PolyCAT A). The proteins can then be eluted directly to MS.

Full MS spectra (= intact mass) of histone H3 fragments: The two peak clusters represent light and heavy (= isotopically heavy Arg & Lys residues) SILAC histone H3 tails differing by 1 methyl-group (= 14 Da). The 2-µm PolyCAT A material [*RIGHT*] affords sharp peaks with half as many unresolved variants complicating the mass spectrum as with the 3-µm material's results [*LEFT*].

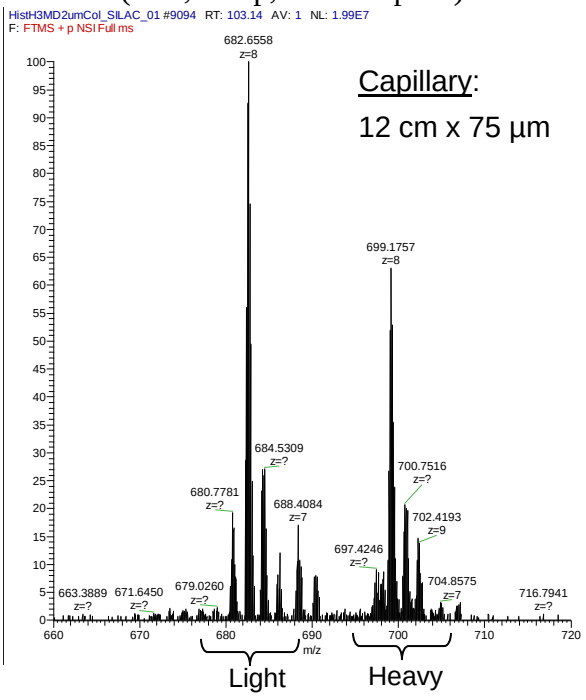
**3 µm, 1500-Å PolyCAT A**

(peak not well-separated from other H3 peptides)




**2 µm, 1000-Å PolyCAT A**

(nice, sharp, isolated peak)

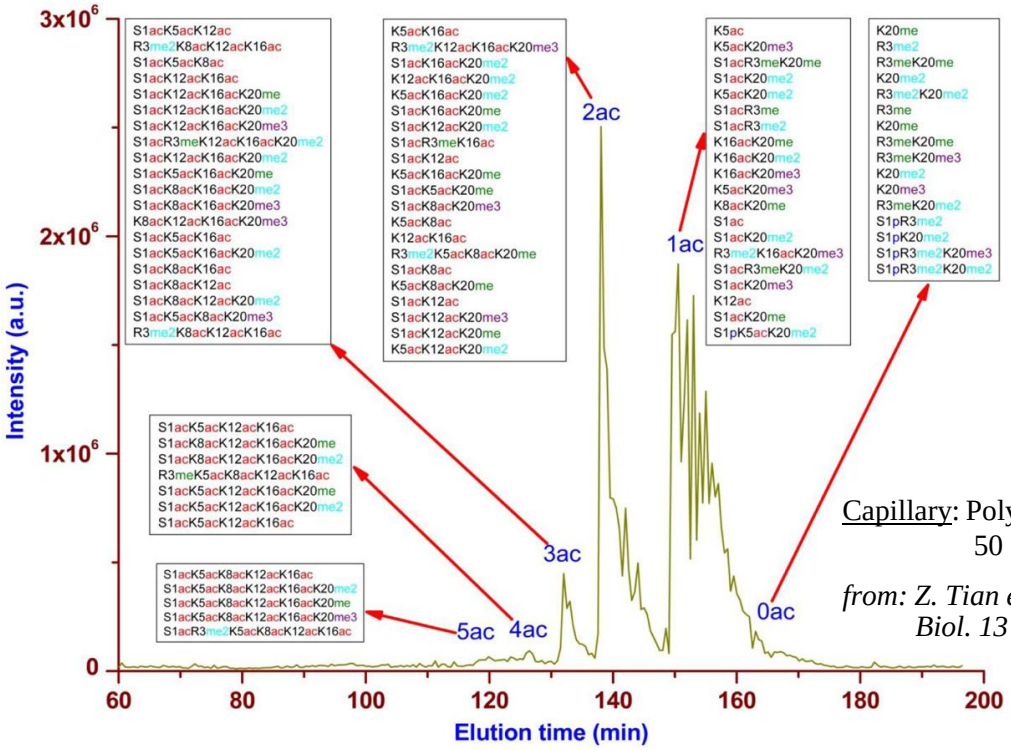



Capillary:

12 cm x 75 µm

(*data courtesy of S. Sidoli and B.A. Garcia, Univ. of Penn.*)

# WCX-HILIC of Histone isoforms: Histone H4




Capillary: PolyCAT A, 5-µm, 1000-Å; 50 cm x 100 µm

*from: Z. Tian et al., Genome Biol. 13 (2012): R86*

Histone H4 features acetylation on Lys. The more highly acetylated isoforms have less (+) charge and elute earlier in WCX-HILIC. The example above shows the elution positions of histone H4 isoforms with numerous PTM's in addition to acetylation on Lys.

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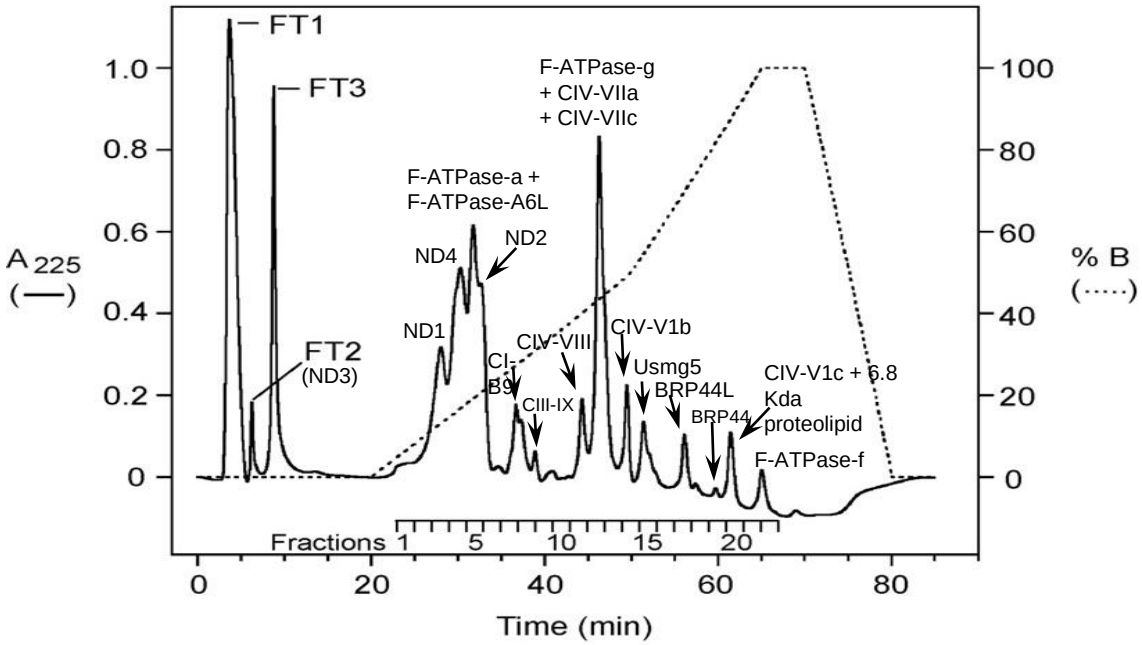
# HILIC-MS of Mitochondrial Membrane Proteins




*from: J. Carroll et al., PNAS 103 (2006) 16170*

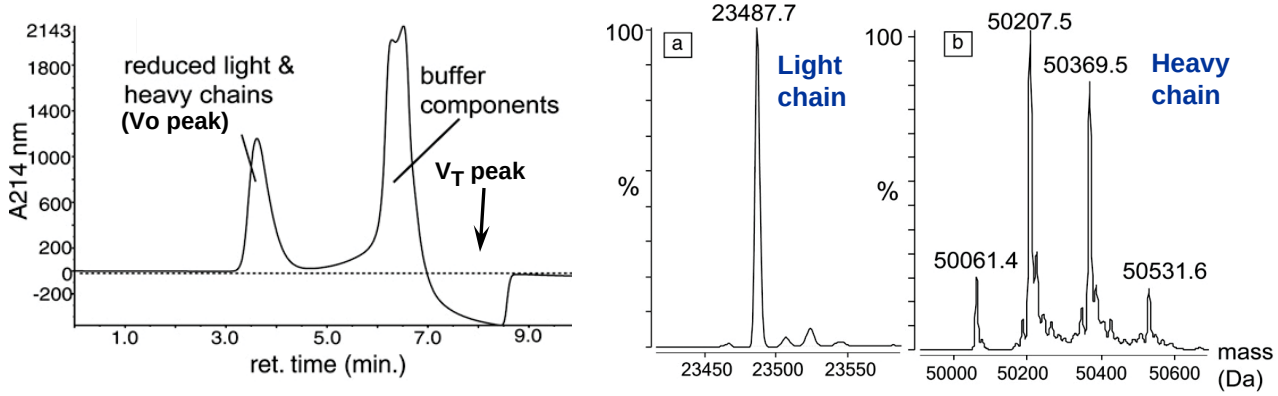
HILIC tends to works well for proteins that don't normally occur in aqueous media, such as membrane proteins, apolipoproteins, etc. In this case, a blend of 63% 2-propanol + 22.5% ACN was used, along with 0.5% hexafluoro-2-propanol and 20 mM NH4-formate (pH 3.7). A decreasing organic gradient was run.

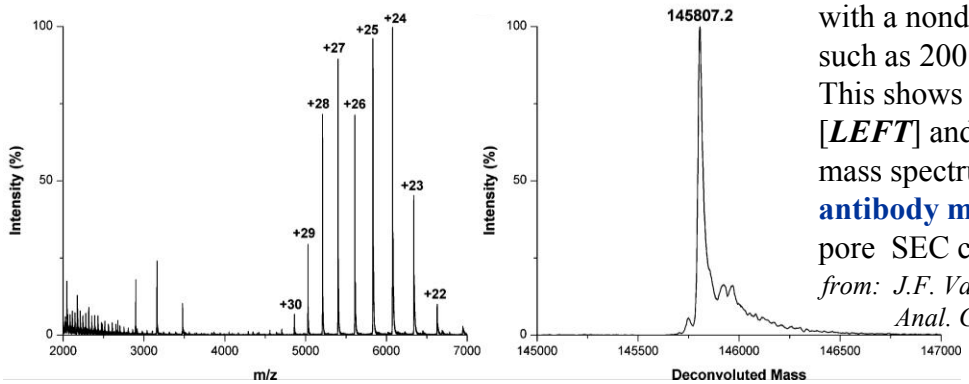
Column: **PolyHYDROXYETHYL A**, 5 µm, 300 Å; 100x1.0 mm

# Size-Exclusion Chromatography: Examples with antibodies

SEC can be performed using volatile solvents. Here, antibody light and heavy chains all elute in the total exclusion volume peak (Vo) from a **PolyHYDROXYETHYL A** column with a pore diameter of 200 Å and a mobile phase of 50 mm formic acid [***LEFT***]. The Vo peak is sent to a mass spectrometer and deconvoluted mass spectra are obtained for the light and heavy chains [***RIGHT***]

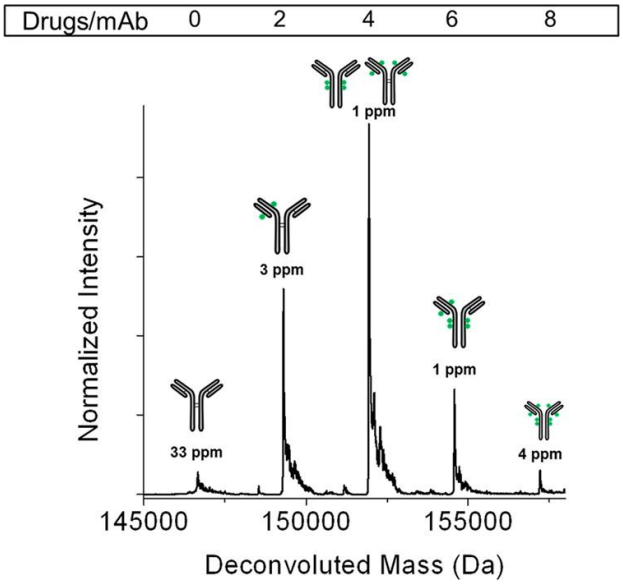
*from: L.J. Brady et al., J. Am Soc. Mass Spectrom. 19 (2008) 502*




It is also possible to perform SEC with a nondenaturing mobile phase such as 200 mM ammonium acetate. This shows the raw unprocessed [***LEFT***] and deconvoluted [***RIGHT***] mass spectrum of an **intact antibody molecule**. Here, a 300-Å pore SEC column was used.

*from: J.F. Valliere-Douglass et al., Anal. Chem. 84 (2012) 2843*

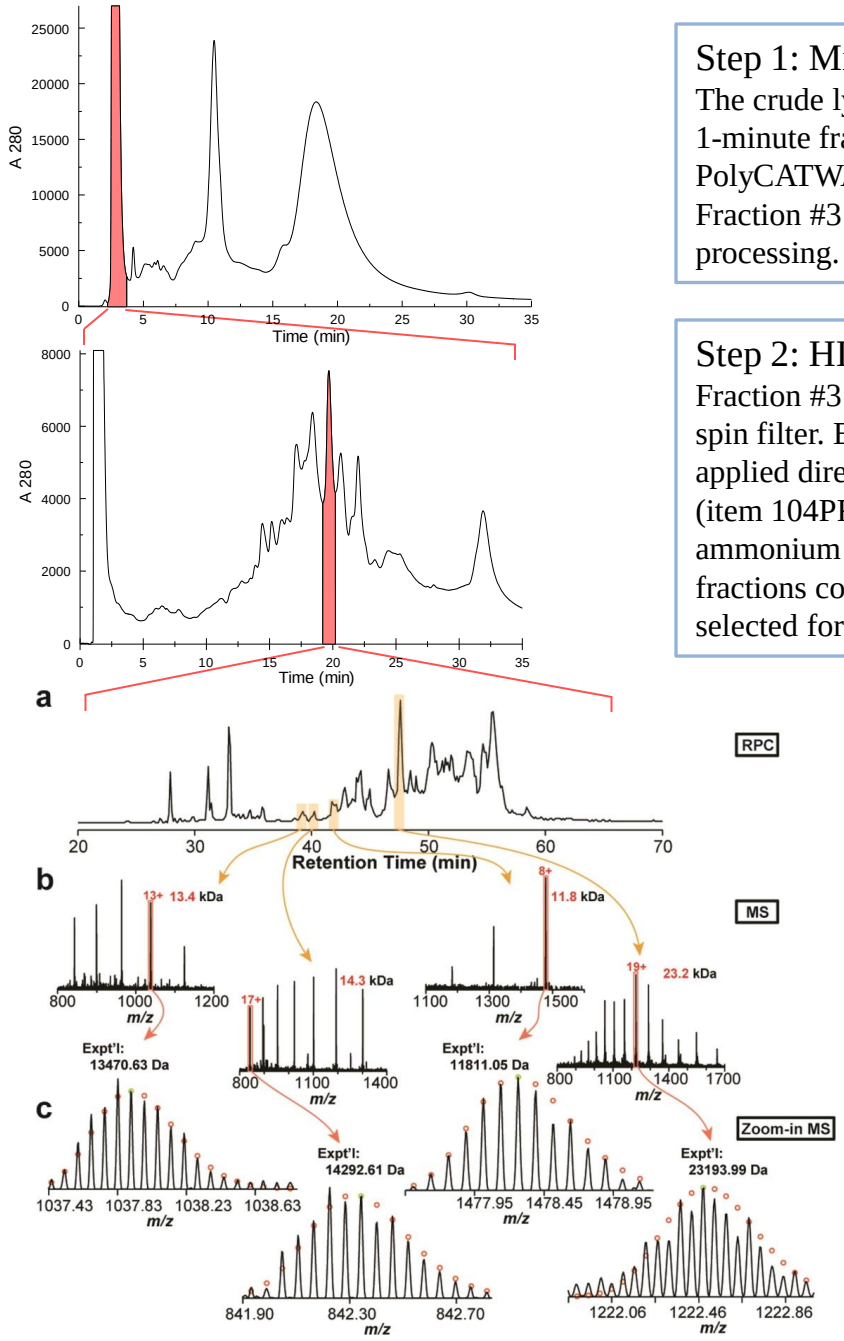



Here, nondenaturing SEC-MS is used to ascertain the distribution of **antibody-drug conjugates (ADC's)** with varying numbers of drug molecules conjugated to the antibody. This data was obtained with a 150x0.3-mm capillary of PolyHYDROXYETHYL A (5-µm, 300-Å) for microscale analysis of 2 µg of sample.

*from: S.M. Hengel et al., Anal. Chem. 86 (2014) 3420*

# 3-D Protein Fractionation: An IEX-HIC-RPC Sequence

Sample: HEK 293 cell lysate




## Step 1: Mixed-bed IEX

The crude lysate was separated into 35 1-minute fractions with a salt gradient on a PolyCATWAX column (item 204CTWX0510). Fraction #3 [*colored*] was selected for further processing.

## Step 2: HIC

Fraction #3 was concentrated with a 10 kDa spin filter. Extra salt was added and it was applied directly to a PolyPROPYL A column (item 104PR0315). A decreasing gradient of ammonium tartrate was used, again with 35 fractions collected. Fraction #20 [*colored*] was selected for further processing.

## Step 3: RPC

HIC fraction #20 was desalted with a 10 kDa filter and applied to a PLRP-S capillary (100 mm x 100 µm). Proteins were eluted with an ACN gradient in 0.25% formic acid into a Q Exactive Orbitrap mass spectrometer. Representative mass spectra are shown for the indicated peaks, along with zoom-in spectra with unit mass isotopic resolution.

## RESULT:

Starting with all 35 HIC fractions from IEX Fraction #3, 640 intact proteins were identified.
201 of these id's were nonredundant (some proteins were identified in more than one HIC fraction).

A 3D analysis of all 35 IEX fractions would have required 35x35 = 1225 RPC runs. Many more proteins would have been identified, at the cost of more time and effort. There is a tradeoff involved. It is still worthwhile; omitting the HIC step (with an IEX-RPC sequence only) resulted in just 47 nonredundant id's.

(*data adapted from S.G. Valeja, L. Xiu, Z.R. Gregorich, H. Guner, S. Jin, and Y. Ge, Anal. Chem. online 4/13/2015 [doi: 10.1021/acs.analchem.5b00657]*)