ASMS 2015

ThP 528

Analysis of Therapeutic Proteins Using Hydrophobic Interaction / Reversed Phase 2D-LC/MS with Multiple Heart-Cutting

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Introduction

Hydrophobic interaction chromatography (HIC) is a popular approach for both downstream processing and analytical scale analysis of mAbs. In the latter case, HIC has the ability to elucidate the presence of various species, for example aggregates or oxidized variants. HIC separations are also gaining popularity for determining the drug-to-antibody ratio (DAR) of antibody-drug conjugate (ADC) preparations. Such investigations often benefit from mass measurement using MS, but the high salt conditions used for HIC separations are completely incompatible with ESI. In this work, we demonstrate the coupling of HIC to MS using a 2D-LC system which affords multiple heart-cutting (MHC) and subsequent desalting/separation using reversed-phase chromatography on-line with TOF MS.

2D HIC-RP/MS Methods

Samples were analyzed using a 1290 Infinity 2D-LC platform as depicted below. A 4.6 x 100 mm HIC column was used in the first dimension (^{1}D) and a 2.1 x 50 mm RP-mAb C4 reversed-phase column was used in the second dimension (^{2}D).

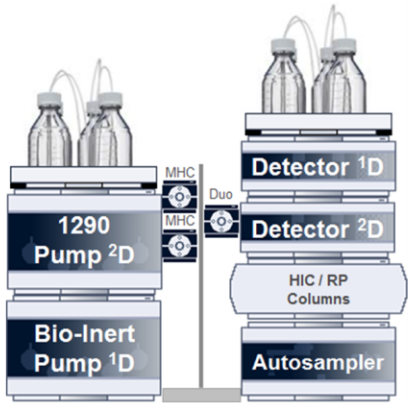


Figure 1: 1290 Infinity 2D-LC Platform

The system is plumbed with three external valves. The Duo valve interfaces the first and second dimension via two MHC valves. The MHC valves contain 6 sample loops each which permit the storage of up to 10 fractions of interest from the ^{1}D. Using this approach, the investigator is able to perform ^{2}D separations of any desired run time. A schematic of the MHC valve and loop configuration is shown in Figure 2.

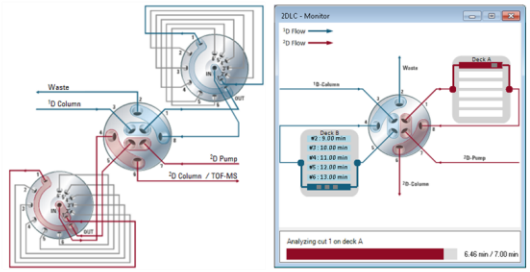
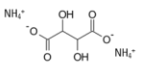


Figure 2. (Left) Schematic of MHC setup for 1290 Infinity 2D-LC system. (Right) The MHC monitor displays peaks stored for analysis in the ^{2}D.

HIC separations were performed using *ammonium tartrate* buffer as opposed to *ammonium sulfate*, which resulted in intense +98 Da adducts in the MS dimension (data not shown, see Xiu *et. al* [1]) .



Ammonium tartrate HIC buffer

The ^{2}D reversed-phase column provided both desalting and additional separation prior to introduction of the sample into the MS system. RP separations were performed using A: 0.01% TFA, 0.1% FA and B: 0.01% TFA, 0.1% FA in ACN at 80 degrees C. The RP column was interfaced directly with a 6224 TOF MS. Programming of the MHC, ^{1}D and ^{2}D separations was controlled through the specialized 2D-LC software shown in Figure 3.

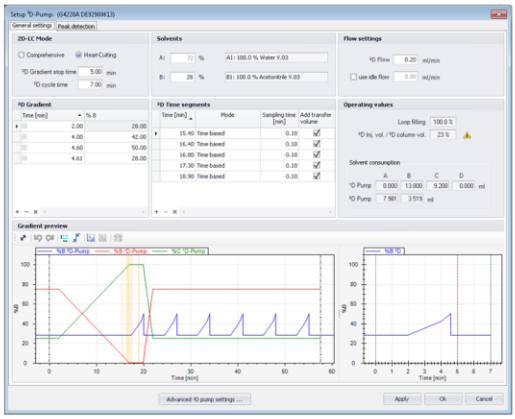


Figure 3. Screenshot of the 2D-LC software used for method programming and visualization.

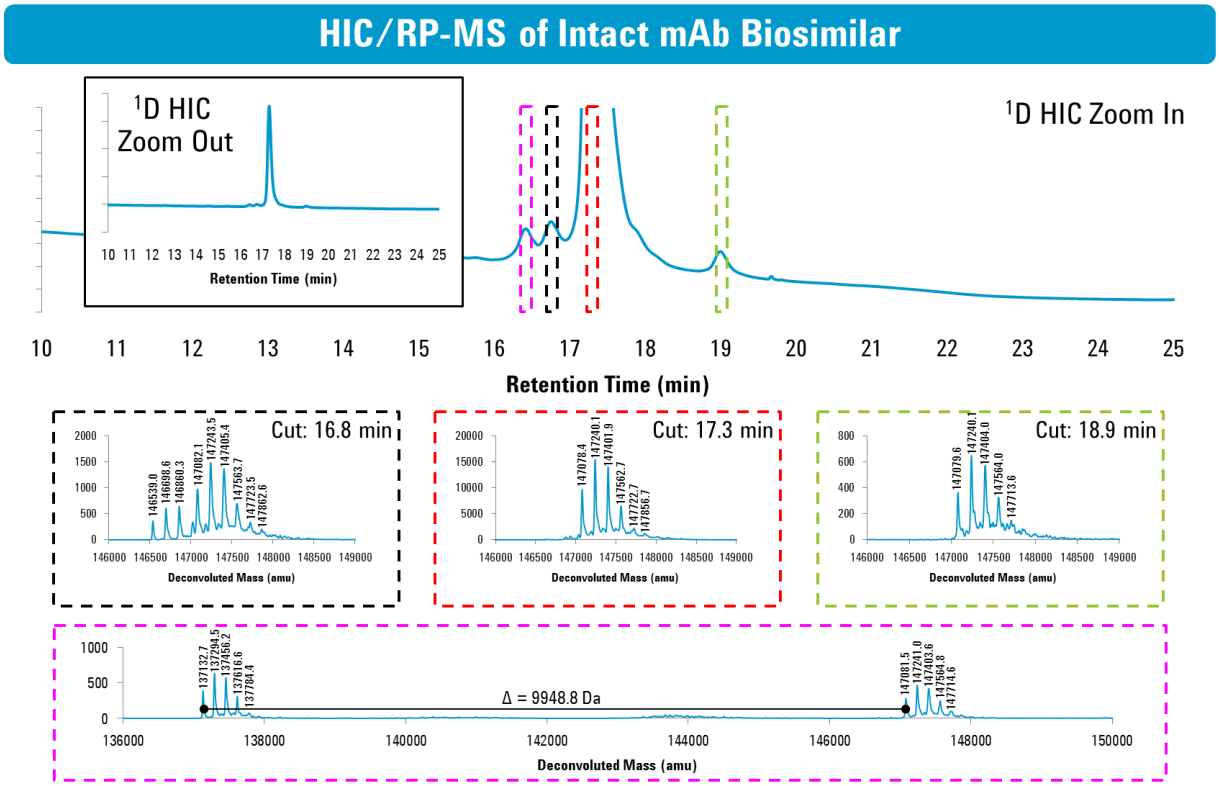


Figure 4. Analysis of a mAb biosimilar using HIC revealed multiple satellite peaks (see zoom in) as well as a major peak (see zoom out). Heart-cuts from the first dimension at 16.4, 16.8, 17.3, and 18.9 min were stored and then separated on the second dimension followed by MS detection. Various impurities were detected that ranged in mass difference from the main peak.

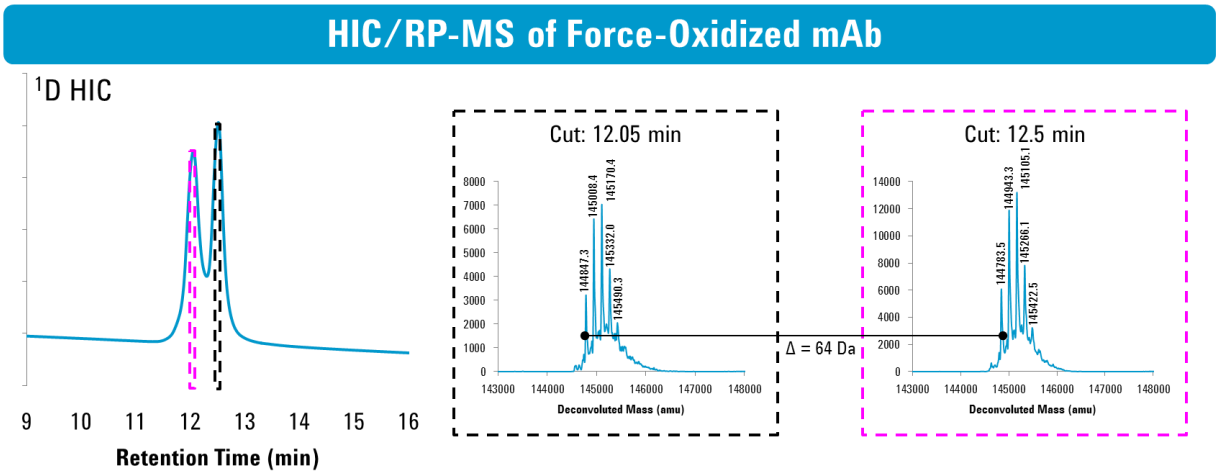


Figure 5. A mAb was treated with 1% TBHP for 72 hours at 37 °C and analyzed using the HIC/RP 2D-LC/MS system. TBHP treatment resulted in a new peak at RT 12.05 min. Mass measurements of a heart-cut of this peak and the main peak at RT 12.5 min are shown in the magenta and black boxes, respectively. The mass of the oxidized mAb is 64 Da higher than the untreated mAb, indicating four potential sites of oxidation under these conditions.

HIC/RP-MS of Lys-linked ADC

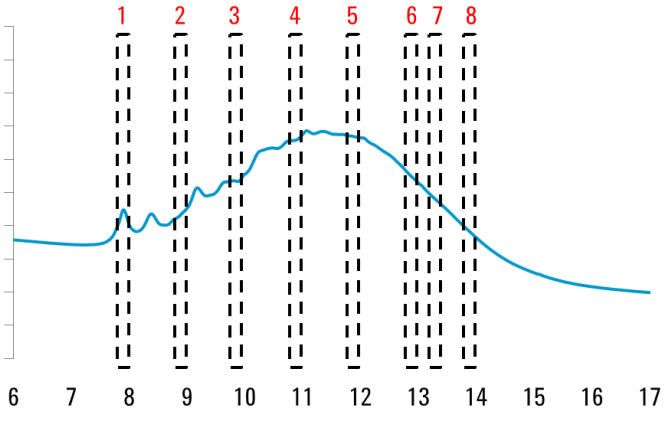
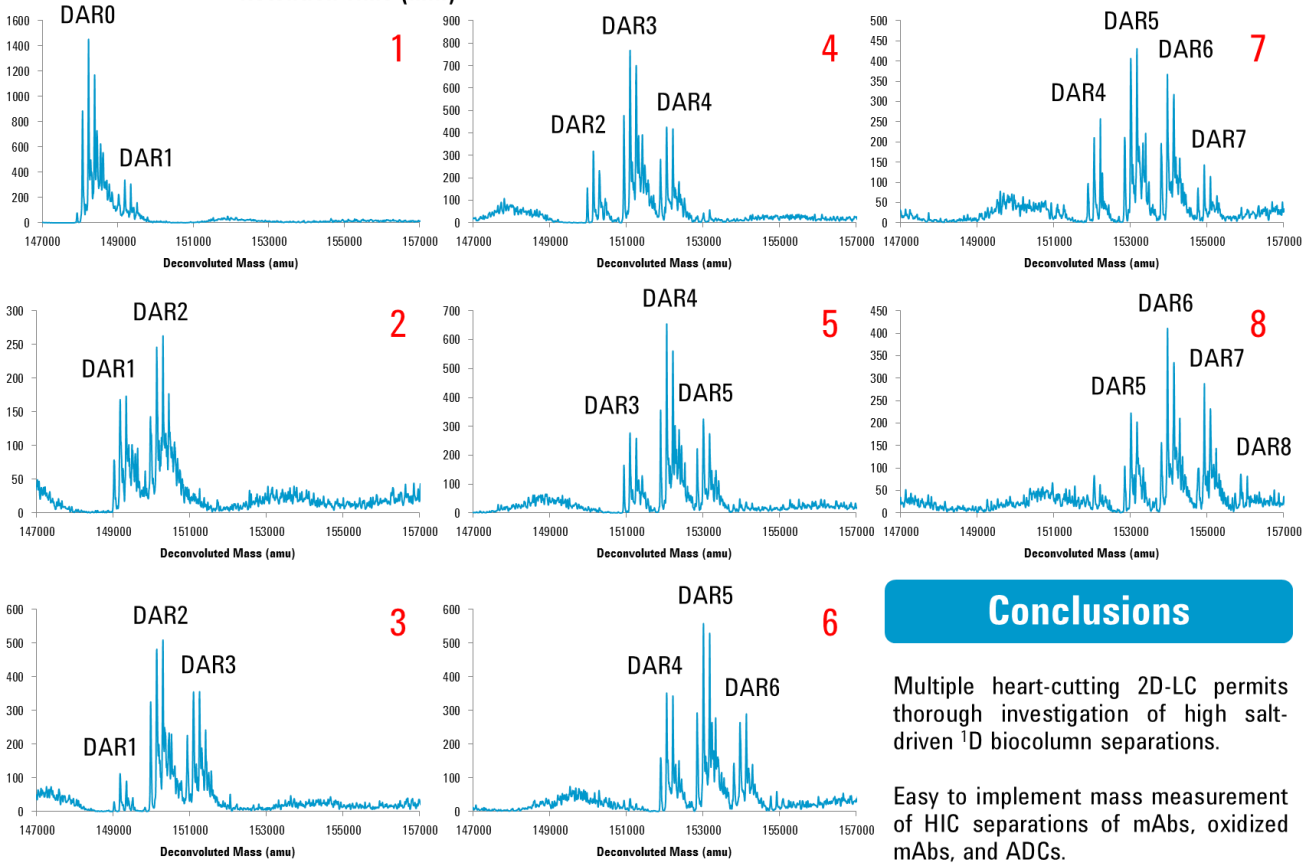


Figure 6. A Lys-linked ADC was analyzed using the HIC/RP 2D-LC/MS system. No deglycosylation was performed prior to analysis. The resulting HIC chromatogram consisted of a very poorly resolved group of peaks eluting over the course of ~7 minutes, reflective of the multiple positional isomers present in the preparation. Eight heart-cuts at RTs 7.9, 8.9, 9.9, 10.9, 11.9, 12.9, 13.4, and 13.9 min were selected and sent to the ^{2}D.

The MS spectra corresponding to the heart-cuts are shown below. mAbs with DAR ranging from 0-8 were observed. The spectra also reflect the glycoform distribution of the mAb in addition to a subpopulation of molecules modified by linker without payload.



Conclusions

Multiple heart-cutting 2D-LC permits thorough investigation of high salt-driven ^{1}D biocolumn separations.

Easy to implement mass measurement of HIC separations of mAbs, oxidized mAbs, and ADCs.

References

1. Xiu, L. *et. al.* Effective Protein Separation by Coupling HIC and RP for Top-down Proteomics. *Anal. Chem.* (2014) 86, 7899-7906.