Analysis of Host Cell Protein Using Automatic Offline Fractionation and LC-MS/MS

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Introduction

Most biopharmaceuticals such as monoclonal antibodies today are produced from genetically modified host cell systems. A large number of low level (1-100 ppm) host cell proteins (HCPs) can still remain in the final products even after multiple purification steps. Since HCPs can induce an adverse immunogenic response, identification and quantification of HCPs are required to secure the quality of products. While HCPs are traditionally analyzed by anti-HCP ELISA, LC/MS analysis could provide more in-depth characterization of HCPs throughout the biopharmaceuticals production process. In this study, a workflow coupling automated offline peptide fractionation and LC/MS was demonstrated. Both high pH reverse phase (high-pH RP) fractionation and strong cation exchange (SCX) fractionation were evaluated followed by low pH reverse phase LC/MS peptide mapping analysis. These results was also compared to 1D-LC/MS analysis without offline fractionation.

Experimental

AssayMAP Sample Preparation
A human IgG1 mAb produced from CHO cells in a hollow fiber bioreactor was denatured with urea, reduced with TCEP, alkylated with iodoacetamide, digested with trypsin, and cleaned up by C18 columns in bulk. Digested and de-salted mAb samples were dried down, and resuspended in 0.1% TFA in Water.
The mAb digest was fractionated with RP-S cartridge (reverse phase) or SCX cartridge using the Protein Sample Prep Workbench Fraction application on the AssayMAP Bravo. For high pH reverse-phase fractionation, RP-S cartridges were first primed with 70% ACN, 0.1% TFA and equilibrated with 0.1% TFA. 100 µg of digested mAb was then loaded onto each cartridge, and fractionated into 6 fractions. For SCX fractionation, SCX cartridges were first primed with 500 mM ammonium formate (pH 6.8), equilibrated with 10 mM ammonium formate (pH 3), 100µg of digested mAb was then loaded onto each of the cartridges, and fractionated into 6 fractions.
A total of 15 µg mAb digest were injected for each set of LC/MS experiment with 5 replicate runs.

	High-pH RP Fractionation	SCX Fractionation
Fraction	**% Acetonitrile**	**mM NH_4HCO_2**
0	Flow Through	Flow Through
1	0	10
2	10	55
3	15	100
4	20	160
5	30	220
6	100	500

Data Analysis
Results were searched against the *Cricetulus griseus* NCBI database appended with the mAb sequence using Spectrum Mill with a 1% FDR filter. The protein IDs had to have at least 2 peptides/identification. Each identified peptide had to be confirmed with MS/MS from at least 3 out of 5 replicate runs.

LC Conditions
Agilent 1290 Infinity II UHPLC with an Agilent Eclipse Plus C18 column (1.8 µm, 2.1×100 mm, PN 959758-902)

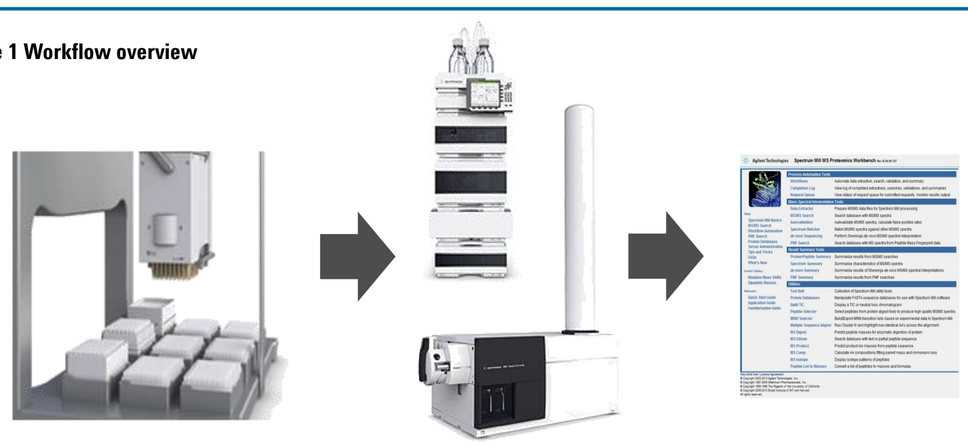
LC Parameters	
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in Acetonitrile
Gradient	0 - 5 min → 2% B 65 min → 25% B 75 min → 40% B 80 min → 90% B
Stop time	85 min
Post time	5 min
Column Temperature	60°C
Flow rate	0.4mL/min

MS Conditions:
Agilent 6550 iFunnel Q-TOF System with Agilent JetStream ion source.

MS parameters	
Ion mode	Positive ion mode (Centroid)
Drying gas temperature	250 °C
Drying gas flow	14 L/min (nitrogen)
Nebulizer	35 psi
Sheath gas temperature	250 °C
Sheath gas flow	11L/min
Capillary voltage	3500 V
Nozzle voltage	0 V
Isolation Width	Narrow (~1.3 m/z)
Acquisition parameters MS mode	Data were acquired at 2GHz, extended dynamic range mode, mass range 300-1700 (MS) and 100-1700 (MSMS). Exclusion list for mAb peptide precursors were applied.

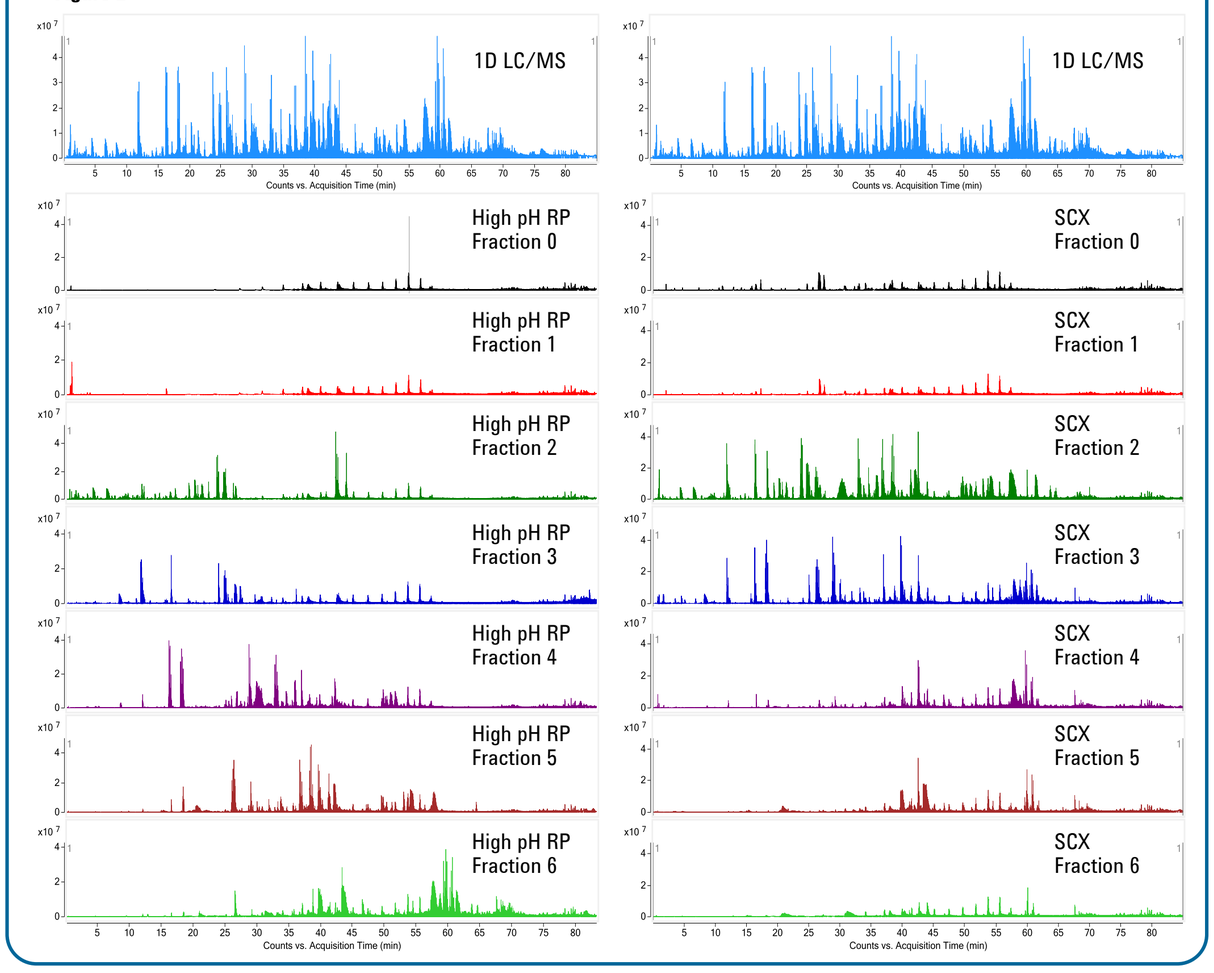
Results and Discussion

Figure 1 Workflow overview



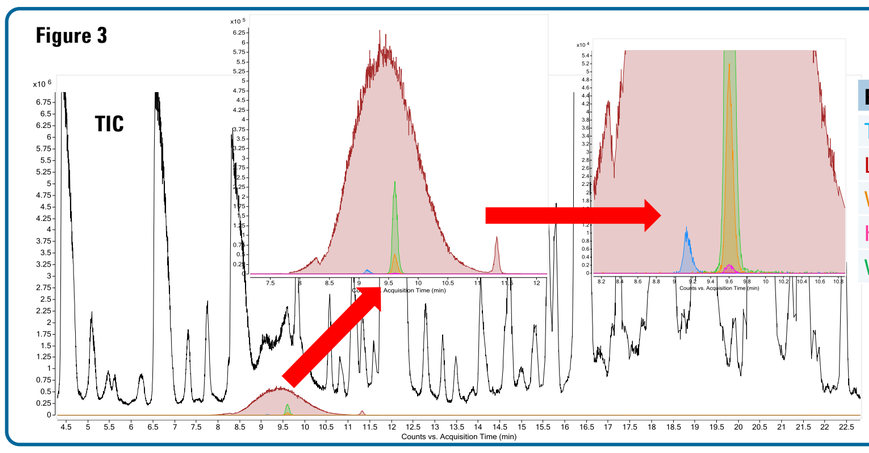
Results and Discussion

Figure 2



Results and Discussion

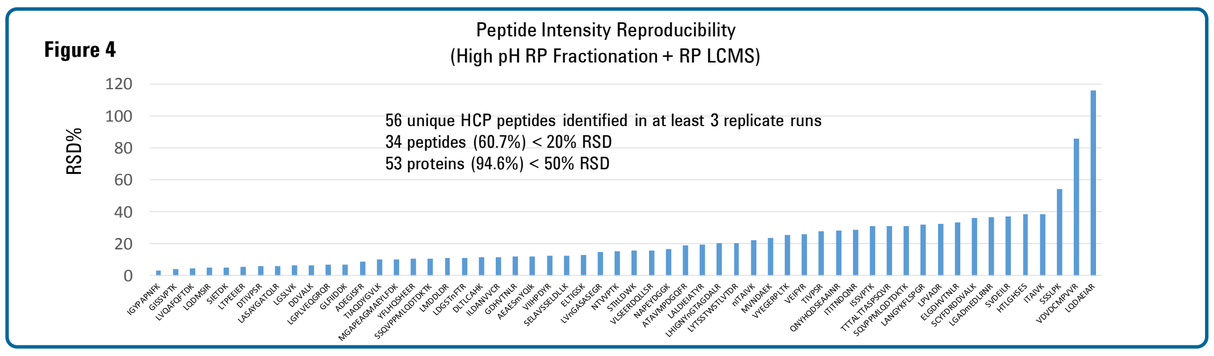
Figure 3



Peptide	Precursor	Intensity
TPVSEKVTK	494.7869	5.33^5
LTPSPR	335.6981	2.49^8
WYPGSVKR	517.7762	1.39^6
KTAEWLTK	528.7610	9.46^3
WYQQKPGK	345.5188	7.36^6

Figure 3 shows extracted ion chromatograms of the precursors for five co-eluting peptides (in colors) and the total ion chromatograms (in black). Each of the peptides was identified with at least one MSMS spectrum. Broad dynamic range (>4 orders) is demonstrated for co-eluting peptides, which is critical for host cell protein analysis when the HCP peptides usually co-elute under extremely intense mAb peptide peaks.

Figure 4



	High pH Fractionation + low pH RP-LCMS	SCX Fractionation + Low pH RP-LCMS	1D-LCMS
Unique Peptide Identified	**56**	**43**	**17**
Total HCP Identified	**17**	**11**	**4**

Conclusions

- A Host cell protein analysis workflow is demonstrated using AssayMAP for automated offline peptide fractionation followed by reverse phase LC/MS.
- With the same injection amount, high pH fractionation with RP LC/MS provided the most host cell proteins identified, comparing to SCX fractionation with RP LC/MS and 1D LC/MS analysis.
- Agilent 6550 iFunnel Q-TOF system provides broad in-spectrum dynamic range (>4 orders) allowing detection and identification of low amount HCP peptides co-eluting with extremely intense mAb peptides.

Reference:
Wang, X. et al., "Host cell proteins in biologics development: Identification, quantitation and risk assessment", *Biotechnol Bioeng.*, 2009, Jun 15; 103(3):446-458