

A UNIQUE LC-MS ASSAY FOR HOST CELL PROTEINS IN BIOLOGICS

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INTRODUCTION

- Residual host cell proteins (HCPs) from recombinant production are a major component of biopharmaceutical process-related impurities. HCPs can elicit an immune response in patients.
- The composition of HCPs is extremely heterogeneous and changes with production and purification procedures.
- European Union guidelines for HCPs require producers to identify and quantify such process-related impurities (<http://www.emea.europa.eu>).
- Current analytical methods for measuring HCPs, (typically ELISA, gels, blots), are expensive, subjective, time-consuming to develop, and require prior knowledge about the identities of the contaminant proteins.
- An efficient UPLC/MS^E assay is presented that identifies and quantifies unknown HCPs over 4-orders of magnitude in concentration. This assay is a generic procedure that can easily be re-applied to different cases.**

Workflow Overview

- Enzymatic digestion of sample to peptides
- 2D-LC/MS^E with IDENTITY^E to discover contaminant proteins
 - 2D-LC allows more sample loading
 - Develop specific host cell protein databases
 - Top 3 peptides for absolute label-free quantitation,
 - Data mined for MRMs using VERIFY^E
- Sample digests are analyzed by Tandem quadrupole MS for targeted, high-throughput quantitation (e.g. using isotopically labeled peptides)

METHODS

Sample Preparation

Five proteins were spiked into a biosimilar monoclonal antibody (*Trastuzumab*): **ENL** (yeast enolase), **LA** (bovine beta-lactoglobulin), **ADH** (yeast alcohol dehydrogenase), **PHO** (rabbit glycogen phosphorylase b), **BSA** (bovine serum albumin). The resulting protein mixture was denatured with RapiGest (15 min at 80 °C), reduced with 10 mM DTT (30 min at 60°C), alkylated with 20 mM IAM (30 min at RT) and enzymatically digested with Promega trypsin (1:20 w/w ratio) at 37°C overnight.

LC Conditions

A nano ACQUITYTM UPLC system (Waters) with 2D technology was used for peptide separations. A reversed-phase/reversed-phase (RP/RP) method was developed that used the pH of the mobile phases to change the selectivity of a peptide separation in two separate dimensions [1,2].

- First Dimension** (1D) pH=10. 1.0 mm x 50 mm XBridge C₁₈ column (5 μm particles); flow 10 μL/min. Mobile phase 20 mM ammonium formate in water (Solvent A) and ACN (Solvent B).
- Online dilution** of the eluent from 1D before trapping onto 2D column.
- Trap column:** A 5-μm Symmetry C₁₈ trap (0.5 x 20 mm) was used to trap peptides between the two LC dimensions.
- Second Dimension** (2D) pH=2.4. 0.3 mm x 150 mm analytical column BEH C₁₈ 1.7 μm, Flow at 4 μL/min.

Fractions were eluted in five steps (1 = 10.8% Eluent B; 2 = 14.0% B; 3 = 16.7% B; 4 = 20.4% B; 5 = 50.0% B). Each step was mixed in a 1:10 ratio with 0.1% TFA in water before trapping. Low pH separations in the second chromatographic dimension used a 90 min gradient from 3 to 40% acetonitrile (0.1% FA-formic acid). See Figure 1 for diagram.

MS conditions

A) Discovery and Label-free Quantitation: Data independent, alternate scanning LC/MS^E experiments were performed with a SYNAPT HDMS mass spectrometer (Waters).

- Acquisition time was 0.7 sec MS and MS^E.
- M/z range: 50-1990.
- Fixed CE at 5 eV for MS; CE ramp 15 - 35 eV for high-energy MS^E.
- ProteinLynx Global Server (PLGS) 2.4 with Identity^E Informatics [3].

B) Targeted Quantitation: MRMs analyses were performed on a Waters Xevo TQ tandem quadrupole mass spectrometer coupled with the ACQUITY UPLC system.

- 2.1 x 150 mm BEH130 C₁₈ column (1.7 μm particles) at 35 °C
- Flow rate of 300 μL/min; 3-40% B over 10 min (B: acetonitrile with 0.1% FA; A: water with 0.1% FA)
- Xevo TQ parameters: Potential 3.5 kV, CV 37 V, source 90°C, MS1/MS2 isolation window 0.75 Da (FWHM), adjustable collision energy (3-40% ACN, 0.1% FA).

SYNAPT HDMS mass spectrometer coupled to the 2D nanoACQUITY UPLC system for HCP Identification



RESULTS

Chromatographic Performance of the 2D-LC System

Figure 1: Fluidic configuration for 2-dimensional chromatography and on-line dilution.

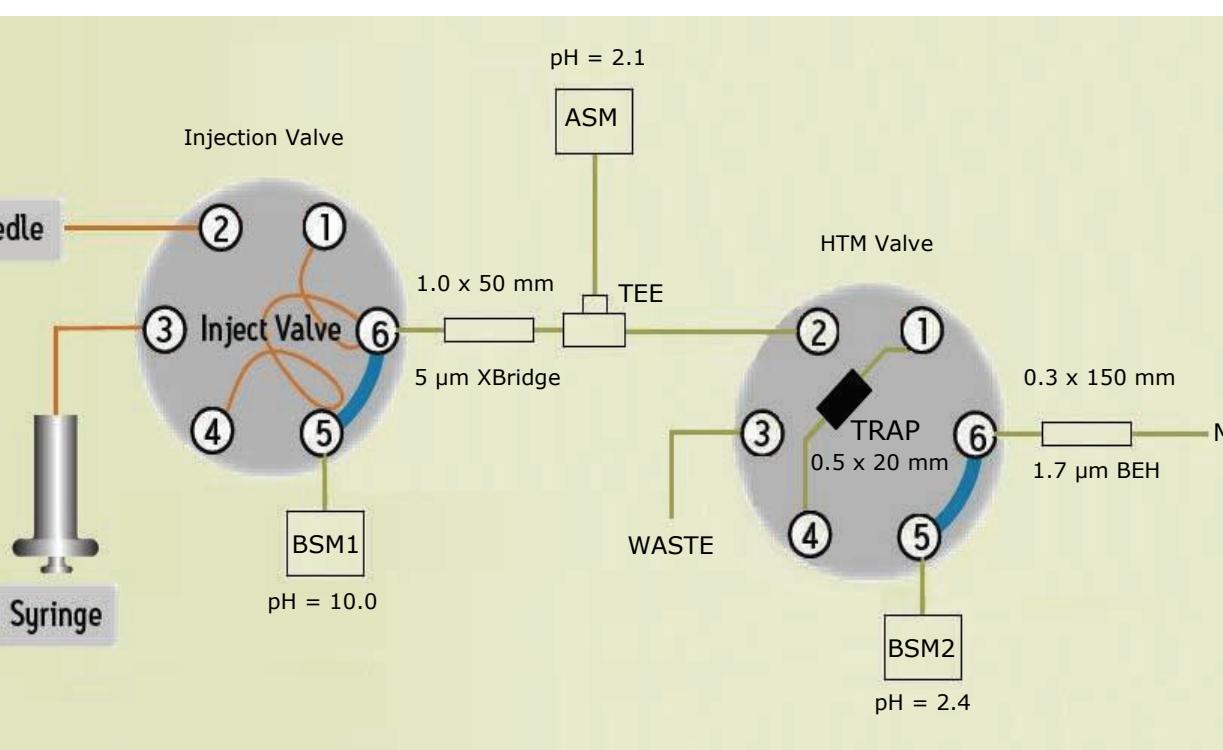


Figure 2. Comparability of Chromatographic performance

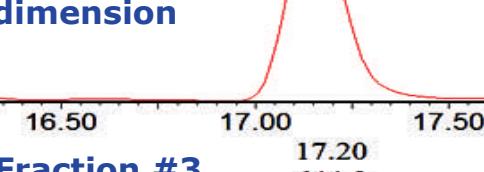
Extracted mass chromatograms of the T43 ENL peptide (VNQIGTLSESIK, M_H²⁺ = 644.86) from triplicate 2D runs. Only the 2nd dimension chromatograms are shown. This peptide was only eluted in Fraction #3 in the 1st dimension chromatography. Totally 60 fmoles of ENL digest were loaded on column in each 2D experiment.

- (A) direct injection on a 0.3x 150 mm BEH column;
- (B) "simulated" 1D run using a single elution step (50% Eluent B) for the high-pH elution;
- (C) Fraction 3 of the 2D-LC run. All separations used a 30 min gradient (3-40% ACN, 0.1% FA).

(A) 1D Separation with direct inj.



(B) 2D run with a single-step elution in the 1st dimension



(C) Fraction #3 from a 2D-LC run

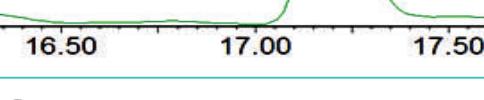
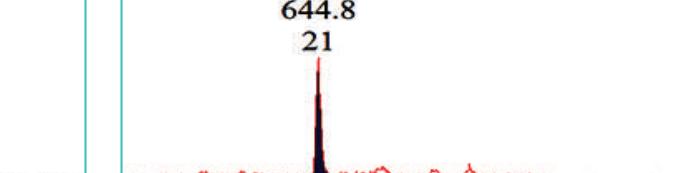


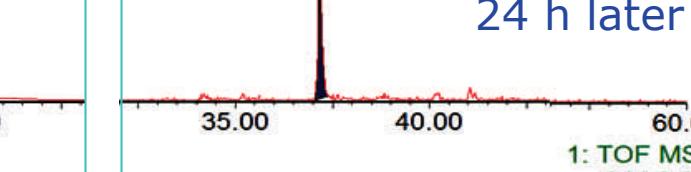
Figure 3. Reproducibility of 2D chromatography

Extracted mass chromatograms of the T43 ENL peptide (VNQIGTLSESIK, M_H²⁺ = 644.86) from triplicate 2D runs. Only the 2nd dimension chromatograms are shown. This peptide was only eluted in Fraction #3 in the 1st dimension chromatography. Totally 60 fmoles of ENL digest were loaded on column in each 2D experiment.

1: TOF MS ES+ 644.8 0.20Da 250 Area



24 h later



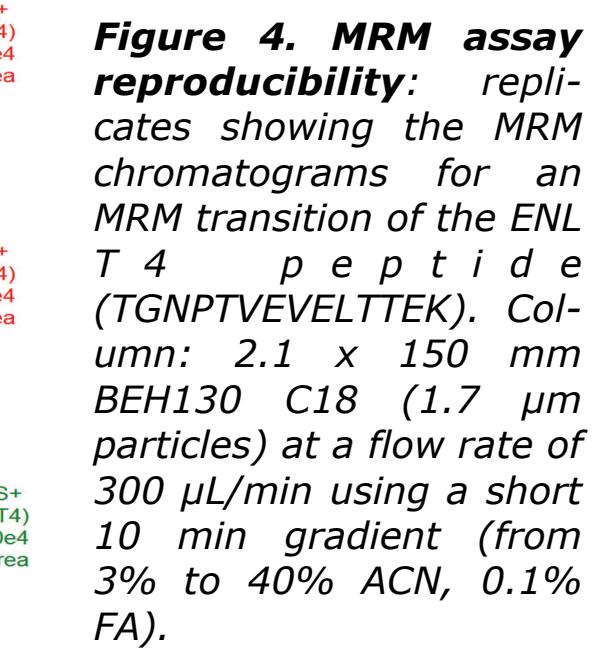
48 h later



Table I. Over Four Orders of Dynamic Range in concentration could be measured using the proteins spiked in the stock solution of a therapeutic monoclonal antibody before digestion

Protein Name	Species	Accession No.	MW (kDa)	Protein Conc. in Sample (fmol/ul)	Dynamic Range (DR) (ppm)	Dynamic Range (Ratio) [log (DR)]
mAb	Humanized	n/a	145.2	32,000	n/a	
LA	Bovine	P00711	16.3	1,000	3,500	285 2.5
ADH	Yeast	P00330	36.8	200	1,600	625 2.8
PHO	Rabbit	P00489	97.3	80	1,675	600 2.8
BSA	Bovine	P02769	69.3	20	300	3,300 3.5
ENL	Yeast	P00924	46.8	4	40	25,000 4.4

The MS^E data was automatically mined using VERIFY^E for the best proteotypic peptides and optimum MRM parameters (e.g. collision energy) for each spiked protein. MRM assays comprising 2 peptides/protein and two transitions per peptide (for a total of 20 MRMs) were then performed on a Xevo TQ mass spectrometer with a 10-min LC separation.



HCP Identification Method

- LC-MS Data:** a combined MS^E data set collected from 5-step 2D RP/RP separation. Samples were analyzed in triplicate.
- Protein Identification Informatics:** PLGS 2.4 featured Identity^E Bio-informatics
- Database:** a decoy database that contains the combination of all the mouse and hamster protein sequences listed in the Swiss-Prot database (<http://www.expasy.ch/sprot/>) with a total 27216 entries (13608 true protein sequence including the sequence of to LA, ADH, PHO, BSA, ENL, porcine trypsin and the heavy and light chain sequence of mAb)
- False Positive Rate of Protein Return: 5%
- The concentration of the HCPs identified was in the range 10 to 50 ppm



Table II. Host Cell Proteins Identified in the Biosimilar Sample

Protein ID	Accession No.	Protein Descriptions	MW (kDa)	# of Pep Matched	Sequ-Coverage (%)
CIRBP_MOUSE	P60824	Cold inducible RNA binding protein	18.6	4	22.5
RAD_MOUSE	O88667	GTP binding protein RAD	33.3	3	22.1
CABP2_MOUSE	Q9JLK4	Calcium binding protein 2	24.2	2	15.2
TWST1_MOUSE	P26687	Twist related protein 1	21.2	2	12.5
NSBP1_MOUSE	Q9JL35	Nucleosome binding protein 1	45.3	2	8.6
NELFE_MOUSE	P19426	Negative elongation factor E	42.5	1	7.5

References

- Gilar M, Olivova P, Daly AE, Gebler JC. *J. Sep. Sci.*, **2005**, 1694.
- Stapels MD, Fadgen K. *Current Trends in Mass Spectrometry, Spectroscopy supplement*, March **2009**.
- Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ. *Proteomics*, **2009**, 1696.