IDENTIFICATION AND QUANTIFICATION OF HOST CELL PROTEIN IMPURITIES IN HIGH PURITY MONOCLONAL ANTIBODIES DOWN TO 1 PPM: AN INTER-LABORATORY STUDY

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INTRODUCTION

- Host cell protein impurities (HCPs) in biopharmaceuticals raise immunogenicity, stability, and drug efficacy concerns, even when present at ppm levels.
- Regulatory guidances mandate that downstream processes are optimized to clear HCPs to the lowest levels that can be practically achieved, and that sensitive assays are in place to demonstrate clearance.
- The ELISA and immunoassay based HCP detection methods widely adopted today typically report total HCP burden, and often lack broad specificity required for comprehensive HCP monitoring.
- LC-MS based approaches for identification and quantification of HCPs in biopharmaceuticals have now been realized [Refs. 1-5, 7] to enable comprehensive HCP discovery and monitoring of individual HCP protein impurities.
- In this study, a novel analytical approach (2DLC-HDMS^E), that combines high pH/low pH reversed phase (RP/RP) separations with orthogonal ion mobility (IM) gas phase separations, is presented as a means to maximize analytical peak capacity and achieve robust low ppm HCP analysis.
- Our results indicate that LC-MS assays are now able to attain comparable sensitivity to traditional immunological based HCP assays, while offering discrete HCP identification and monitoring.

METHODS

Sample preparation

Three murine derived mAbs: a NIST reference mAb standard; Remicade, a commercial Infliximab (Innovator); and Inflectra (its approved Biosimilar) biotherapeutics were analyzed. Samples (2.5 mg mAb) were denatured with RapiGest™ SF (60 °C, 15 min), DTT reduced (60 °C, 1h), alkylated with IAM (RT, 30 min) and digested with a mixture of Lys-C and porcine trypsin (Promega) overnight. Four protein digest standards were spiked post-digestion such that the amounts loaded on-column using a 250 uL injection were: 5,000 fmol ADH (yeast alcohol dehydrogenase), 1,000 fmol PHO (rabbit phosphorylase b), 250 fmol BSA (bovine serum albumin) and 50 fmol ENL (yeast enolase).

2D-LC configuration

ACQUITY™ UPLC® M-Class system with 2D technology was used for reversed-phase/reversed-phase (RP/RP) 2D-LC separations of the antibody digest.

First Dimension (1D) pH 10: 1.0 x 50 mm XBridge C_{18} column (5 µm particles), 10 µL/min flow. Mobile phase: 20 mM ammonium formate in water (Solvent A) and ACN (Solvent B).

Online dilution (1:10) of the eluent from 1D prior to the trap column.

Trap column: 0.3 x 50 mm packed with 5-µm Symmetry C_{18} particles.

Second Dimension (2D) pH 2.4: 0.3 x 150 mm analytical column CSH C_{18} 1.7 µm, kept at 60 °C and operated at 10 µL/min.

Fractions were eluted in ten steps of increasing organic strength, with each step diluted online to 1:10 ratio with 0.1% TFA in water (pH=2.1) before trapping. Low pH separations in the second chromatographic dimension used a 40 min gradient from 3 to 40% acetonitrile (0.1% formic acid).

MS and data processing

Data-independent acquisition (MS^E) were performed on a SYNAPT G2-S mass spectrometer:

- Acquisition cycle time was 0.5 sec, m/z range: 100-1990.
- Fixed CE at 5 V for MS scans; CE ramp 20-45 eV for MS^E scans.
- For IMS (HDMS^E) enabled acquisition, a fixed wave velocity (650 m/s) and fixed wave height (40 V) applied.
- Ion mobility specific CE were applied during HDMS^E acquisition [6]
- Data Processing: ProteinLynx Global Server (PLGS) 3.0.2

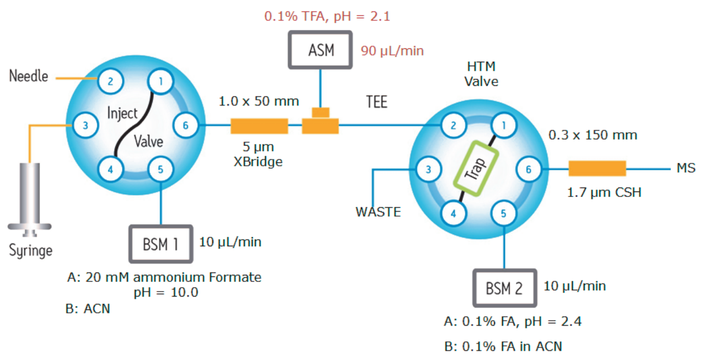
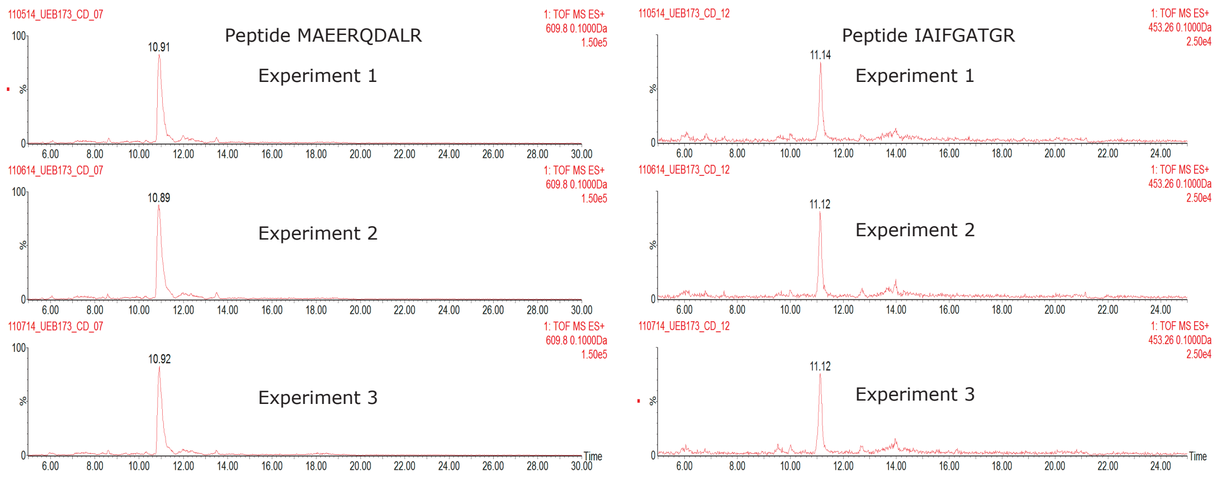


Figure 1. Fluidic configuration for two-dimensional high pH/ low pH RP/RP chromatography, employing pre-trap on-line dilution.

SYNAPT G2-S HDMS system coupled to an ACQUITY UPLC M-Class with 2D technology.

ANALYTICAL DEVELOPMENT

Reproducibility of the 2D LCMS^E HCP Discovery Methodology



Peptide MAEERQDALR (m/z = 609.80, +2) from NSFL1 cofactor p47 (conc. *7 ppm*), eluting in 1D fraction 3 of 10

Peptide IAIFGATGR (m/z = 453.26, +2) from flavin reductase (NADPH) (conc. *2 ppm*), eluting in 1D fraction 8 of 10.

Figure 2. LC separation reproducibility of the 2DLC chromatographic system. Extracted mass chromatograms were generated for the monoisotopic peaks of two low-abundance HCP peptides. The peptides reproducibly elute in the same fraction at consistent retention times over the course of the triplicate HCP discovery experiment.

Ion Mobility (HDMS^E) acquisition simplifies MS^E spectral complexity of HCP peptide fragments of an HCP Peptide Presursor Ion (m/z 877.92, 2+ Charge)

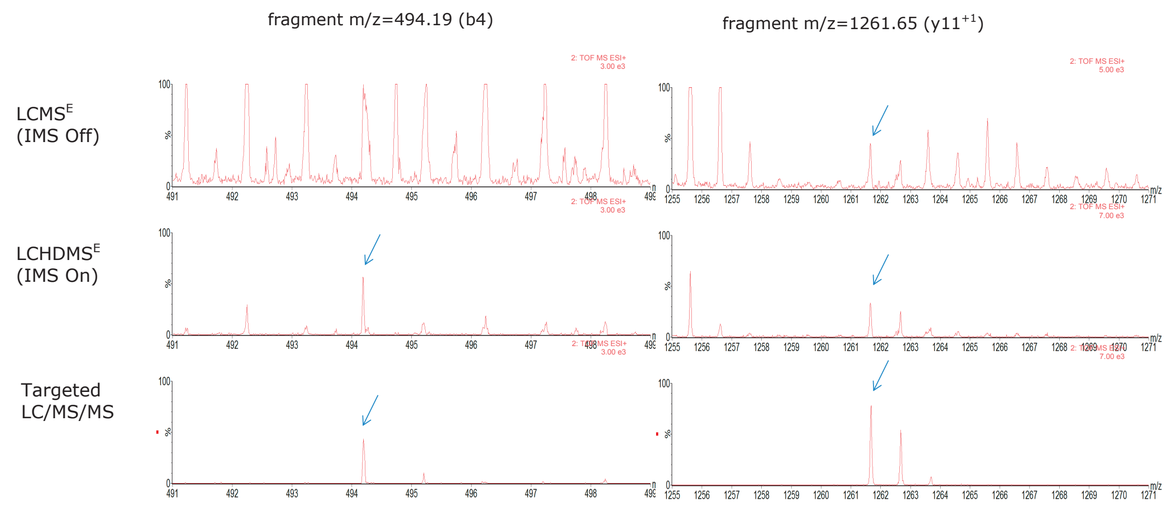


Figure 3. Enabling Ion Mobility during 2DLC-MS^E acquisition (i.e. 2DLC-HDMS^E) separates peptide MS ions in the gas phase, and simplifies the resulting MS^E fragmentation spectra. The complex MS^E fragmentation spectra (TOP) seen without IMS separation, are greatly simplified by enabling IMS (MIDDLE), enabling clear ion detections. Confirmatory targeted MS/MS (2 m/z isolation window) independently validates fragment ion assignments. Example: NSFL1 Cofactor p47 peptide SYQDPSNAQFLESIR from NIST mAb.

HCP ANALYSIS OF NIST MAB REFERENCE STANDARD

Detecting low level (sub 20 PPM) HCPs in the NIST mAb Reference Standard from multiple peptides

No crt	Average HCP conc (ppm)	HCP Identification	Sequence Coverage (%)	MW (kDa)	Peptide Sequence	Monoisotopic peak (charge)	RT (min)	Drift time (ms)	2D Fraction no
1	16	Clathrin interactor 1	7.3		ELVEFAQDDDRLR	535.93 (+2)	11.7	3.3	1
					SLENYHFVDEHGKDDQGINIR	475.03 (+5)	10.2	1.9	3
					YVGVSSDSVGGFR	665.33 (+2)	11.8	3.5	4
2	14	Low affinity IgG gamma Fc region receptor	32.7	36.7	SQVQASYTFK	579.79 (+2)	7.2	3.5	3
					TLHQSKPVTITVQGPK	578.67 (+3)	6.2	3.5	5
					EMGETLPEEVGEYR	819.87 (+2)	17.8	5.1	5
					ISFFHNEK	511.26 (+2)	6.5	3.2	6
					HPEALDEETEHDYQNHI	692.96 (+3)	8.1	3.7	6
					ATVNDSGEYRCQMEQTR	682.30 (+3)	15.6	4.1	6
					EDTVTLTCEGTHNPGNSSTQWFHNGR	737.09 (+4)	9.2	4.1	6
3	13	Stress induced phosphoprotein 1	9.0	62.6	LAYINPDLALEEK	744.90 (+2)	17.2	4.5	5
					ALSAGNIDDALQCYSEAIK	680.33 (+3)	17.9	3.7	5
					ALDLDSSCKEAADGYQR	633.62 (+3)	24.8	3.6	9
4	12	Glucose-6-phosphate isomerase	10.6	62.7	ELFEADPER	553.26 (+2)	16.7	3.5	4
					HFVALSTNTAK	594.82 (+2)	5.6	3.6	5
					AVLHVALR	439.78(+2)	7.3	2.7	7
					VWFVSNIDGTHIAK	529.62 (+3)	15.7	3.4	9
					TLASLSPETSLFIIASK	889.50 (+2)	23.5	5.2	9
5	7	NSFL1 cofactor p47	28.2	40.7	EFVAVTGTEEDR	676.84 (+3)	16.1	4.5	1
					LGAAPEEESAYVAGER	824.89 (+2)	10.2	4.8	1
					MAEERQDALR	609.80 (+2)	10.9	3.7	3
					LAHGGQVNLDMEDHRDEDFVKPK	530.86 (+5)	9.1	3.0	3
					SYQDPSNAQFLESIR	877.92 (+2)	15.5	5.1	3
					SPNELVDDLFK	638.82 (+2)	19.1	3.5	4
					DLIHDQDEEEEEEEGQR	700.63 (+3)	17.6	3.9	4
6	7	Beta-2-microglobulin	22.7	13.8	TVYWDRDM	543.24 (+2)	10.9	3.2	1
					VEMSDMSFSK	580.75 (+2)	10.3	3.5	3
					TPQIQVYSR	546.29 (+2)	7.9	3.3	3
7	5	Serine/arginine-rich splicing factor 7	15.7	30.8	VYVGNLGTGAGK	568.31 (+2)	7.5	3.5	4
					NPPGFAFVEFEDPRDAEDAVR	793.37 (+3)	20.2	4.4	4
8	5	Transketolase	8.3	67.6	TSRPENAIIYSNNEDFQVGQAK	827.74 (+3)	11.3	4.1	3
					NMAEQIIQEIYSQVQSK	670.34 (+3)	26.6	3.8	8
					VLDPFTIKPLDRK	514.64 (+3)	12.9	3.3	9
9	4	Adenylate kinase 2	11.3	26.5	LEAYHTQTTPLVEYYR	661.99 (+3)	12.9	3.5	5
					AVLLGPPGAGK	490.34 (+2)	9.5	3.0	6
10	3	Prostaglandin reductase 1	8.2	35.6	MEGFIVNR	483.25 (+2)	9.7	2.9	3
					TGPCPQGPAPEVVIYQQLR	1055.54(+2)	17.1	5.4	6
11	2	Flavin reductase (NADPH)	12.6	22.2	LPSEGPQPAHVVVGDVR	586.32 (+3)	10.2	3.6	3
					IAIFGATGR	453.26(+2)	11.1	3.4	8
12	1	Peroxiredoxin 5	25.7	21.8	VGDAIPSVEVFEGEPGK	865.44 (+2)	17.8	5.1	3
					THLPGFVEQAGALK	734.40 (+2)	12.2	4.5	6
					ALNVEPDGTGLTCSLAPNILSQL	795.08 (+3)	29.1	4.0	8

Table I. List of peptides identified during 2D-LC/HDMS^E analysis of *NIST mAb* identifying HCPs at levels *below 20 ppm*. Detailed information is provided for each peptide regarding the observed monoisotopic m/*z*, charge state, ion mobility drift time, peptide LC retention time from the 2nd-dimension separation, as well as peptide elution fraction from high pH 1st dimension separation.

Consistency of Hi3 Quantification of NIST mAb Reference Standard HCPs across three laboratories

No crt	Accesssion Number	Protein Description	Identified without IM	Average MW (kDa)	Measured HCP Concentration (ppm) Lab I	Lab II	Lab III	Average
1	*P00489*	*Glycogen phosphorylase rabbit (PHO)-1000 fmoles*	*Y*	*97.1*	*155*	*155*	*155*	155
2	*P00330*	*Alcohol dehydrogenase yeast (ADH)-5000 fmoles*	*Y*	*36.7*	*106*	*125*	*164*	132
3	P05064	Fructose biphosphate aldolase A isoform	Y	39.3	113	126	109	116
4	P05063	Fructose biphosphate aldolase C isoform	Y	39.4	106	93	91	97
5	*P02769*	*Bovine serum albumin (BSA)-250 fmoles*	*Y*	*66.3*	*35*	*28*	*20*	28
6	Q99KN9	Clathrin interactor	N	68.5	22	19	7	16
7	P08101	Low affinity IgG gamma Fc region receptor	Y	36.7	10	28	5	14
8	Q60864	Stress-induced phosphoprotein 1	N	62.5	10	16	13	13
9	P06745	Glucose-6-phosphate isomerase	N	62.7	7	15	13	12
10	P01887	Beta-2-microglobulin	N	13.8	5	14	1	7
11	Q9CZ44	NSFL1 cofactor p47	N	40.7	7	10	5	7
12	*P00924*	*Enolase 1 yeast (ENL)-50 fmoles*	*N*	*46.6*	*7*	*7*	*3*	6
13	Q8BL97	Serine/arginine-rich splicing factor 7	N	30.8	6	7	3	5
14	P40142	Transketolase	N	67.6	5	4	6	5
15	Q9WTP6	Adenylate kinase 2	N	26.5	4	6	2	4
16	Q91YR9	Prostaglandin reductase 1	N	35.5	4	3	3	3
17	Q923D2	Flavin reductase (NADPH)	N	22.2	1	3	1	2
18	P99029	Peroxiredoxin 5	N	21.8	1	2	1	1

Table II. Fourteen NIST mAb HCPs were identified in common by all three laboratories performing 2DLC/HDMS^E acquisition. Individual HCP concentrations (range of 1—200 ppm) were calculated by the Hi3 methodology [Ref. 8] from three replicate injections, calibrated by the PHO protein standard, spiked in the NIST mAb post-digestion. Eleven HCPs (highlighted in yellow) required ion mobility based HDMS^E analysis for identification.

HCP ANALYSIS OF REMICADE (INFLIXIMAB) AND ITS BIOSIMILAR, INFLECTRA

No crt	Accesssion Number	Protein Description	Sequence Coverage (%)	Average MW (kDa)	Amount on column fmoles	ng	Concentration ng/mL	ppm	RSD (%)
1	P00489	Glycogen phosphorylase rabbit (PHO) - 1000 fmoles	41.2	97.1	1000	97	1942	92	0.0
2	*P00330*	*Alcohol dehydrogenase yeast (ADH) - 5000 fmoles*	*44.6*	*36.7*	*1981*	*73*	*1454*	*69*	*3.2*
3	*P02769*	*Bovine serum albumin (BSA) - 250 fmoles*	*21.9*	*66.3*	*296*	*20*	*392*	*19*	*10.3*
4	Q6GUQ1	Epidermal growth factor-like protein 8	8.2	33.3	792	26	527	25	14.2
5	Q8CBE3	WD repeat-containing protein 37	4.8	55.1	288	16	317	15	23.1
6	*P00924*	*Enolase 1 yeast (ENL) - 50 fmoles*	*15.7*	*46.6*	*102*	*5*	*95*	*5*	*10.8*
					Total	ng/mL	845		
					Total	ppm		**40**	
					mAb	purity		**99.996%**	

No crt	Accesssion Number	Protein Description	Sequence Coverage (%)	Average MW (kDa)	Amount on column fmoles	ng	Concentration ng/mL	ppm	RSD (%)
1	*P00489*	*Glycogen phosphorylase rabbit (PHO) - 1000 fmoles*	*35.7*	*97.1*	*1000*	*97*	*3884*	*388*	*0.0*
2	*P00330*	*Alcohol dehydrogenase yeast (ADH) - 5000 fmoles*	*40.7*	*36.7*	*2073*	*76*	*3043*	*304*	*4.5*
3	Q6GUQ1	Epidermal growth factor-like protein 8	8.2	33.3	687	23	915	92	18.9
4	P02769	Bovine serum albumin (BSA) - 250 fmoles	19.1	66.3	294	19	780	78	11.1
5	Q8CBE3	WD repeat-containing protein 37	4.8	55.1	142	8	313	31	35.0
6	*P00924*	*Enolase 1 yeast (ENL) - 50 fmoles*	*18.3*	*46.6*	*58*	*3*	*108*	*11*	*26.5*
					Total	ng/mL	1228		
					Total	ppm		**123**	
					mAb	purity		**99.988%**	

Table III. Comparison of HCPs identified and quantified in Remicade and Inflectra. The same 2 HCPs were detected in common for both samples: epidermal growth-factor like protein 8 and WD repeat containing protein 37. Higher levels of the HCPs were detected for both proteins in the biosimilar version, for a total HCP burden roughly three times higher than the innovator.

References

1. Doneanu et. al. *mAbs*, 2012, 4, 24-44.
2. Schenauer, *Anal Biochem*, 2012, 428, 150-157.
3. Protein Downstream Processing, *Methods in Molecular Biology* 1129, 2014, Editor NE Labrou, chapter 25, 341-350.
4. Zhang et. al. *mAbs*, 2014, 6, 659-670.
5. Thompson et. al. *Rapid Commun. Mass Spec*, 2014, 28, 855-860.
6. Distler et. al. *Nat Meth*, 2014, 11, 167-170.
7. Doneanu, et al. Anal Chem, 2015, 87, 10283-10291. (Contains elements of the NIST mAb work in this poster)
8. Silva et. Al. Mol Cell Proteom, 2006, 144-127.

CONCLUSIONS

- An improved generic 2D-LC/HDMS^E assay was developed for quantitative HCP discovery analysis in biopharmaceuticals that incorporates ion mobility separations with 2D-LC/MS^E to extend assay sensitivity by an order of magnitude, allowing for the identification and quantification of HCPs at single digit ppm levels.
- A multi-laboratory trial of this methodology has established the quantitative robustness of the HCP discovery workflow.
- This methodology has been able to establish qualitative similarity of the HCP profile for an innovator biotherapeutic mAb and its approved biosimilar, but demonstrating quantitative differences in HCP levels.

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