

## Note d'application

# A Generic 2D-UPLC-MS Assay for the Identification and Quantification of Host Cell Proteins in Biopharmaceuticals

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## Abstract

In this application note, a generic UPLC-MS assay that offers comprehensive HCP identification and quantification for biotherapeutic protein samples is described.

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## Benefits

- An efficient and generic UPLC-MS<sup>E</sup> assay is developed for identification and quantification of host cell proteins (HCPs) in biotherapeutics that provides over four to five orders of magnitude in concentration.
- This targeted, high-throughput quantitative assay for selected HCP proteins enables researchers to monitor their abundance changes.

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## Introduction

Most biotherapeutics today are produced by recombinant DNA technology using a well-selected host cell system. Host cells express a large number of their own proteins that can easily contaminate the recombinant protein drug. Even after sophisticated purifications steps, low levels (1 to 100 ppm) of host cell proteins (HCPs) may still remain in the final purified biopharmaceutical product. Because HCPs can sometimes trigger an unpredictable immunogenic response, regulatory guidelines stipulate that they need to be identified and quantified to protect patient safety.

The presence or absence of HCPs in protein drugs can determine whether or not a biopharmaceutical is accepted by the regulatory agencies. For example, in 2008, the European Medicines Agency (EMA) approved a recombinant form of human somatropin only after the manufacturer added additional purification steps to remove the HCPs responsible for immunogenic response in patients.<sup>1</sup> The same agency rejected an interferon biosimilar in 2006 because of insufficient validation for immunogenicity testing.

All analytical methods employed for measuring HCPs face significant challenges due to the wide dynamic range of the protein concentration (four to five orders of magnitude). Some widely used analytical methods, such as process-specific ELISAs and western blots,<sup>2</sup> require prior knowledge regarding the nature of HCP contaminants. In addition, process specific immunoassays are both time consuming (e.g., six months), and expensive to develop (more than \$100K), and are not readily adapted to fully evaluate biopharmaceutical products from different cell types and purification schemes.

Two-dimensional gel electrophoresis coupled to fluorescent staining,<sup>3,4</sup> another popular method for HCP analysis, is only semi-quantitative, has limited dynamic range (two to three orders of magnitude) and requires additional, confirmatory techniques (e.g., mass spectrometry) for HCP identification. Although commercial ELISA kits are developed for generic application to the monitoring of HCPs, they are less specific than the process-specific immunoassays, and cannot offer a complete coverage for all the existing HCPs in the samples.<sup>5,6</sup>

An organization that can demonstrate that it is capable of accurately identifying and monitoring the HCPs in its biotherapeutics is more likely to overcome regulatory hurdles in the acceptance of its products.

In this application note, a generic UPLC-MS assay that offers comprehensive HCP identification and quantification for biotherapeutic protein samples is described. The assay applies an on-line two-dimensional LC approach for peptide separations and a high-resolution and high-mass-accuracy mass spectrometer for protein

identification and quantification.

In contrast to the traditional 2D-chromatography setup schemes that are based on strong cation exchange (SCX) and low pH reversed-phase<sup>7</sup> separation, the 2D method employed here couples a high pH reversed-phase (RP) separation to a low pH RP separation to achieve maximum chromatographic separation and to cope with the complexity and the wide dynamic range that are encountered in the HCP samples.

In addition, a multiplexed data acquisition method ( $MS^E$ ) is employed in the mass spectrometric analysis so low-abundance HCP peptides can be reproducibly sampled and identified without bias.

Furthermore, a fast quantitative assay has been developed based on multiple reaction monitoring (MRM) principles to provide a high-throughput method for monitoring HCP variation in samples from a variety of manufacturing/purification conditions.

In this application note, we evaluate the performance of the assay using monoclonal antibody samples derived from different purification methods.

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## Experimental

### LC conditions

2D-UPLC system:	nanoACQUITY UPLC System with 2D Technology and on-line dilution
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### First LC dimension (Operating at pH 10.0)

Column:	XBridge BEH300 C <sub>18</sub> , 5 $\mu$ m, 1 x 50 mm, (p/n 186003615)
Flow rate:	10 $\mu$ L/min

Mobile phase A:	20 mM ammonium formate in water (pH 10)
Mobile phase B:	Acetonitrile
Step-elution gradient:	A 10-step elution gradient to fractionate the peptides in the first dimension at pH 10 is undertaken. The percentage of mobile phase B in each step is: 10.8, 12.4, 14.0, 15.0, 16.7, 18.6, 20.4, 25.0, 30.0, and 50.0%, respectively.
Diluting solution:	0.1% TFA in Milli-Q water, 100 $\mu$ L/min flow rate
Trap column:	Symmetry C <sub>18</sub> , 0.5 x 20 mm, 5 $\mu$ m (2.7 $\mu$ L internal volume)

## Second LC dimension (Operating at pH 2.4)

Column:	ACQUITY UPLC BEH C <sub>18</sub> , 0.3 x 150 mm, 1.7 $\mu$ m (p/n 186002605)
Column temp.:	65 °C
Flow rate:	12 $\mu$ L/min
Mobile phase A:	0.1% FA in Milli-Q water (pH 2.4)
Mobile phase B:	0.1% FA in ACN
Gradient elution:	7 to 35% of mobile phase B in 30 min

## MS conditions

## HCP discovery platform

MS system:	SYNAPT HDMS
Acquisition time:	0.5 s
m/z range:	50 to 1990
ESI spray voltage:	2.6 kV
Cone voltage:	37 V
Source temp.:	120 °C
Low-energy fragmentation:	5eV (fixed)
High-energy fragmentation:	Collision Energy ramp between 15 and 35 eV

## High-throughput MRM assay platform

LC system:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH300 C <sub>18</sub> , 2.1 x 150 mm 1.7 µm packed (p/n 186003687)
Column temp.:	35 °C
MS system:	Xevo TQ MS
ESI spray voltage:	3.5 kV
Cone voltage:	37 V

Source temp.:	90 °C
MS1/MS2 isolation window:	0.75 Da (FWHM)
Dwell time:	20 to 30 ms

## Informatics

ProteinLynx Global SERVER (PLGS) 2.4

## Sample preparation

### MIX-4 protein digest standard

The MIX-4 protein digest standard was prepared by diluting stock solutions of the individual MassPREP protein digests of ADH (p/n 186002328), PHO (p/n 186002326), BSA (p/n 186002329), and ENL (p/n 186002325) in 20 mM ammonium formate, pH 10, to achieve a final concentration of 20 nM ADH, 4 nM PHO, 1 nM BSA, and 0.2 nM ENL.

### HCP sample preparation

A chimeric anti-phosphotyrosine IgG1 monoclonal antibody (PTG1 mAb) was expressed in two different Chinese hamster ovary (CHO) cell lines and purified by Protein A chromatography using two different protocols following manufacturer recommendations.

Among the six samples analyzed, four samples labeled as A1, B1, A2, and B2 were expressed in DG-44 CHO cells, while two samples labeled C and D were expressed in CHO-S cells. Two different purification protocols were followed. Samples A1/A2, and B1/B2 were biological replicates, grown under identical conditions.

Five protein standards (LA, PHO, ADH, BSA, and ENL) were spiked in 250 µL of PTG1 (5 to 10 mg/mL) and the resulting protein mixture was denatured with 0.1% *Rapi*Gest for 15 min at 60 °C, reduced with 10 mM DTT for 30 min at 60 °C, alkylated with 20 mM IAM for 30 min (at RT) and digested overnight (37 °C) with porcine trypsin (Promega) using a 1:20 (w/w, enzyme:protein) ratio. After digestion, the *Rapi*Gest surfactant was decomposed by adding 5 µL of pure TFA and the samples were incubated for 30 min at 37 °C and centrifuged (10 min at 10,000 rpm) to separate the insoluble component of *Rapi*Gest by precipitation. After adjusting the pH of the supernatant

solution to pH 10 using a solution of 2 M ammonium formate (pH 11), the digest volume was brought to 1 mL using 20 mM ammonium formate (pH 10). The amounts of spiked protein digests loaded on-column using a 100  $\mu$ L sample loop were: 4000 fmoles of LA (bovine alpha-lactoglobulin), 800 fmoles of PHO, 320 fmoles of ADH, 80 fmoles of BSA, and 16 fmoles of ENL.

## Internal standards for MRM analysis

For MRM quantification, three  $^{13}\text{C}^{15}\text{N}$ -isotopically labeled peptides (Sigma Aldrich) were spiked at a concentration of 20 nM into 250  $\mu$ L of PTG1 digest that was prepared following the digestion protocol described above.

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## Results and Discussion

### Chromatographic performance of the 2D-UPLC system

A schematic diagram illustrating the operation of the 2D-UPLC system during sample loading, sample elution from the first dimension, and sample separation from the second dimension is shown in Figure 1A–C. Peptide samples are loaded under basic conditions (pH 10) on the first-dimension column (Figure 1A) and fractionated by RP chromatography using the 10-step elution with increasing acetonitrile concentrations (Figure 1B). Each peptide fraction is diluted (on-line) with a solution containing 0.1% TFA (pH 2.1) at a 1:10 ratio so the peptides eluted from the first dimension can be temporarily retained on a trapping column. The peptides are then separated on the second-dimension analytical column using a 30-min gradient under acidic conditions (Figure 1C).

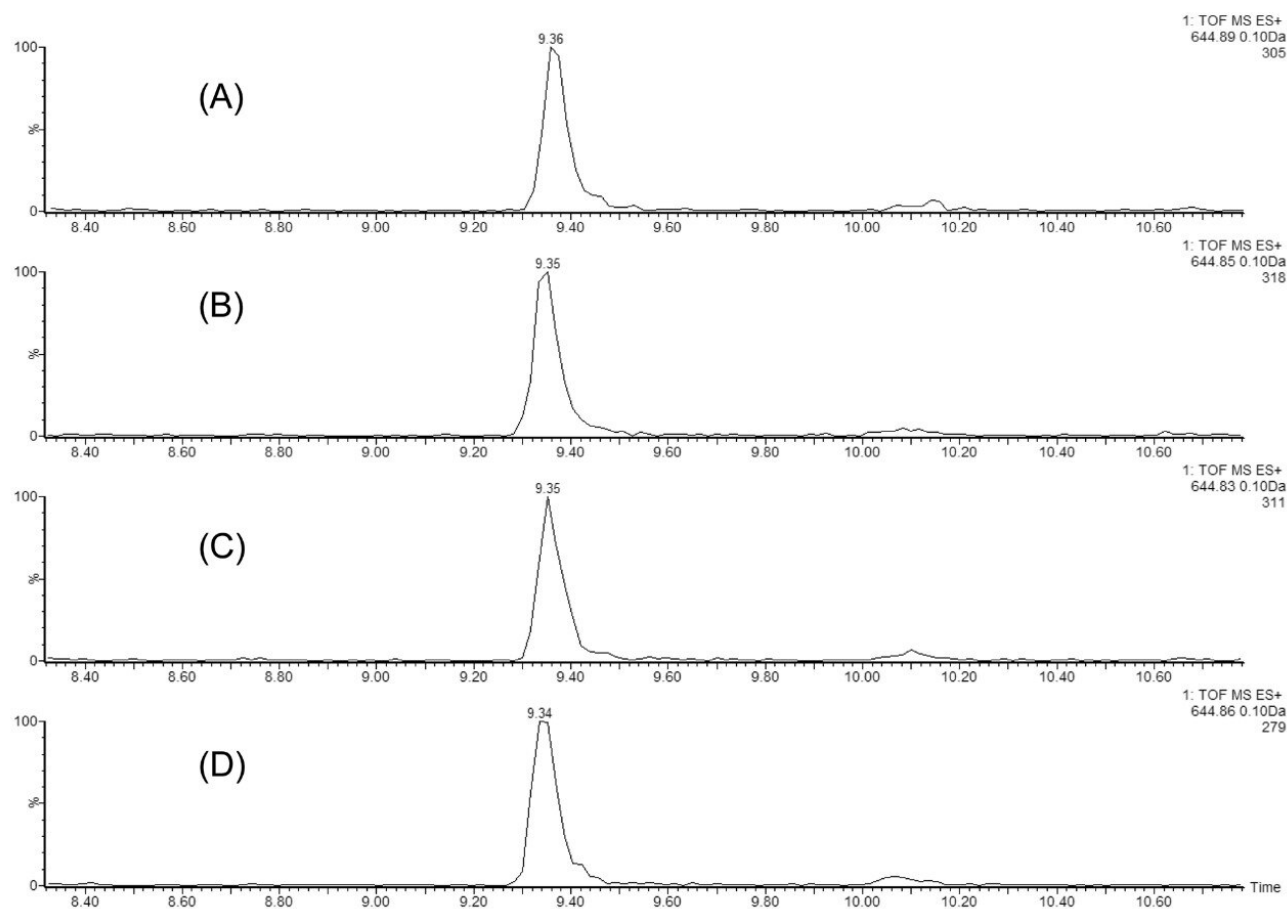




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*peptide fractionation using the first chromatographic dimension (high pH reversed-phase), and peptide trapping; (C) peptide separation in the second dimension (low pH reversed-phase).*

A critical chromatographic parameter in multidimensional chromatographic separations is the reproducibility of peptide fractionation during an extended period of operation. This is illustrated in Figure 2 using a MIX-4 protein digest, which contains 20 nM of ADH, 4 nM of PHO, 1 nM of BSA, and 0.2 nM of ENL in 20 mM ammonium formate (pH 10). Figure 2 shows the extracted mass chromatograms of the T43 peptide from ENL protein (VNQIGTLSESEIK, monoisotopic peak  $[M+2H]^{2+}$  of 644.86), from the second dimension (low pH) separations in four consecutive injections (experiments). In each experiment, a five-step fractionation using 10.8, 12.4, 15.4, 18.6, and 50% Eluent B (100% ACN) was performed in the first dimension. T43 ENL peptide is eluted only in Fraction 4, demonstrating great reproducibility of the first dimension fractionation. In addition, the retention time reproducibility (0.05% RSD) for the same peptide over 48 hours of separation demonstrates the stability of the 2D-UPLC system.



*Figure 2. Reproducibility of 2D-UPLC for four consecutive experiments: extracted mass chromatograms of T43 peptide from ENL (VNQIGTLSESEIK,  $m/z$  for  $[M+2H]^{2+}$ : 644.86) eluted only in Fraction 4 out of 5 (using 18.6% ACN). Second dimension chromatography runs were performed at 12  $\mu\text{L}/\text{min}$  using a 30-min gradient (7–35% ACN, 0.1% FA). Twenty femtomoles of ENL digest was loaded on column.*

In the second experiment, we investigated the ability of the 2D-UPLC system to maintain good chromatographic performance, independent of the number of fractionation steps in the first dimension. Figure 3 displays the extracted mass chromatograms of T43 peptide generated from the second dimension separations under four fractionation schemes: “simulated 1D” (single step elution from 10.8 to 50% of Eluent B), 3-step, 5-step, and 10-step fractionations, respectively. The system demonstrates the highly reproducible retention time over the different operation schemes (0.15% RSD). The 10-step fractionation experiment revealed an important aspect regarding the high pH/low pH 2D separation of peptides: peptides can elute entirely within a single fraction (5/10) even with a relatively narrow elution step-gradient (1.9% B). As shown in Figure 4, no T43 peptide could be

detected in the previous or the following fractions.

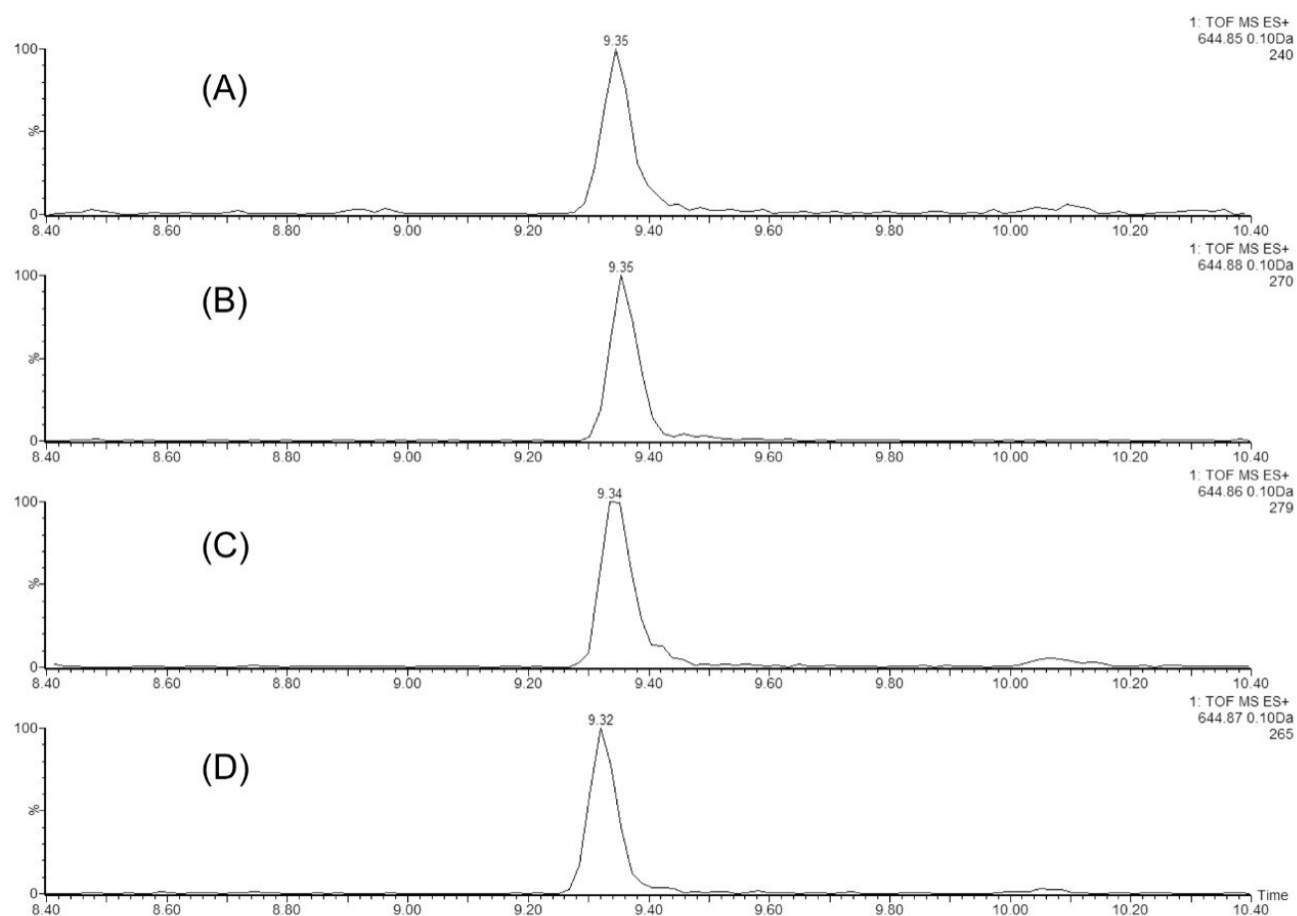
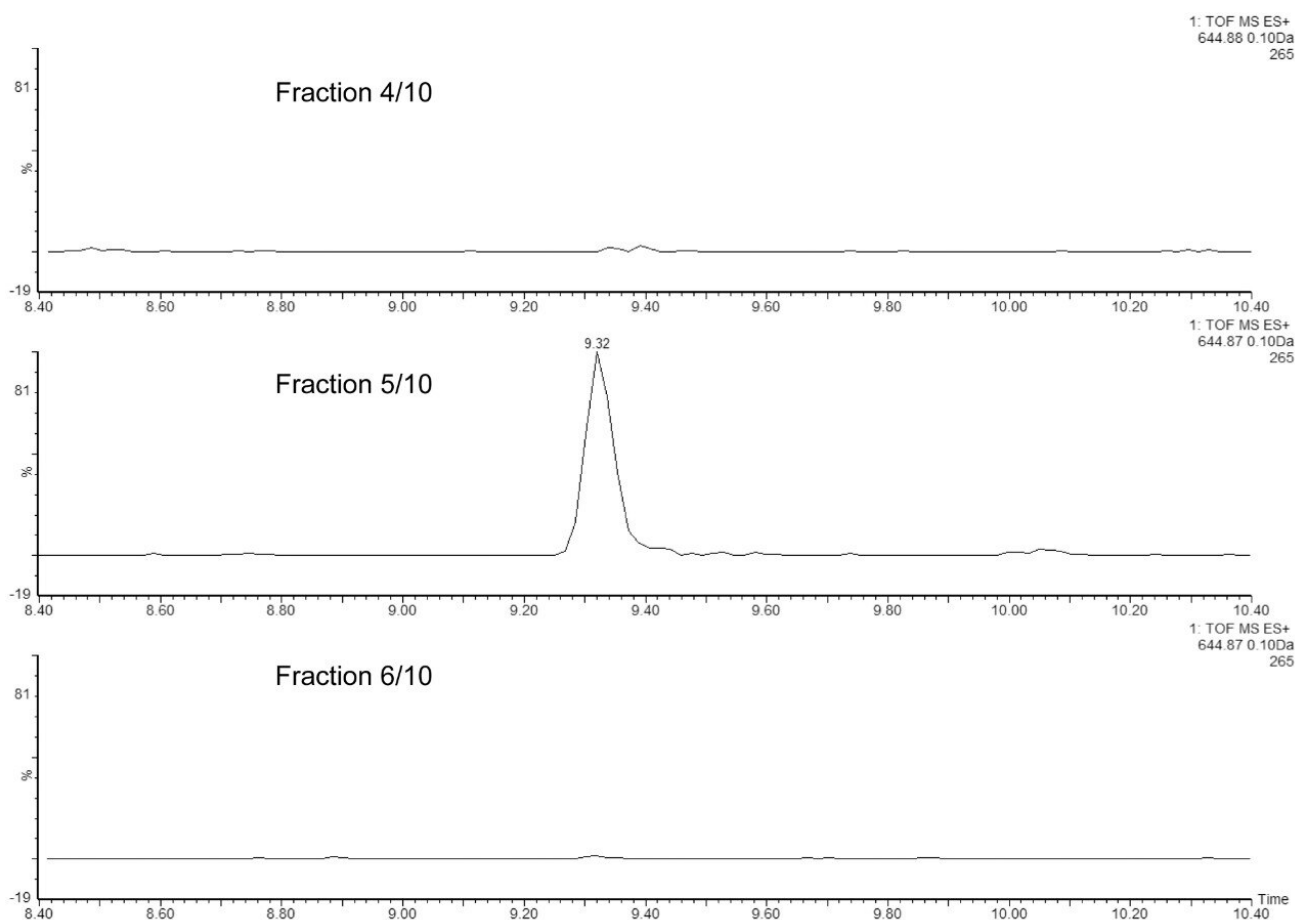


Figure 3. Chromatographic performance (e.g., RT reproducibility and peak width) is maintained during first-dimension fractionation: mass chromatograms of ENL T43 peptide obtained under four fractionation conditions: (A) "simulated" 1D run using a single elution step (from 10.8 to 50% ACN); (B) fraction 2 out of 3 (from 10.8 to 18.6% ACN); (C) fraction 4 out of 5 (from 15.4 to 18.6% ACN); (D) fraction 5 out of 10 (from 15.4 to 16.7% ACN). All separations used a 30 minute-gradient (7–35%ACN, 0.1% FA).



*Figure 4. Performance of peptide fractionation at high pH conditions: mass chromatograms of ENL T43 peptide from three consecutive experiments using a 10-step fractionation in the first dimension: Fraction #4 corresponds to a step elution from 14.0 to 15.4% ACN; Fraction #5 was from 15.4 to 16.7% ACN; Fraction #6 was from 16.7 to 18.6% ACN. All separations were performed using a 30 min gradient (7–35% ACN, 0.1% FA). The amount loaded on-column was 20 fmoles of ENL digest.*

The chromatographic performance of the 2D-UPLC setup can be maintained, regardless of the number of fractionation steps. The reproducibility of the 2D-UPLC setup with respect to the retention time, peak width/shape, and the intensity of the ESI-MS signal within four consecutive experiments demonstrates the absolute trust a laboratory studying HCPs can place in the system. Overall, the data demonstrates the ability of the high pH XBridge Column to retain peptides over the duration of the fractionation experiment (six to 10 hours) without measurable sample losses.

## Identification of HCPs in mAb preparations

To test the analytical capability of the 2D-UPLC/MS<sup>E</sup> technology for identification of low-abundance HCPs in biopharmaceuticals, we analyzed six mAb (PTG1) samples that were expressed by DG-44 (samples labeled A1, A2, B1, and B2) and CHO-S hamster cell lines (samples C and D).

Each of the samples was purified by two different protein-A chromatography columns. This single-step purification protocol was not designed to achieve fully optimized purification for the highest purity of mAb, rather it provided good test samples to examine the capability of the LC-MS assay to identify HCPs from relatively complex biological samples.

A relatively large number of CHO proteins (about 40) were co-purified (see Table 1) with the mAb target. Five proteins standards (originating from other species than the host hamster cells) were spiked in the PTG1 preparations before tryptic digestion. These protein standards serve as an internal control to probe the dynamic range of the assay and to provide internal references for quantification of HCPs using the summed signal of the three best responding peptides of each protein identified in the analysis.<sup>8</sup>

As shown in Table 1, a total of 37 HCPs across six PTG1 preparations were identified. Because the CHO protein database is not available in the public domain, these HCP proteins were identified using the mouse/hamster homology search. The measured HCP concentrations varied widely, from 10 to 3000 ppm. Four out of five spiked protein standards (except the lowest abundance ENL) were identified in all samples (two out of three replicates). Most of the HCPs identified in this study were high-abundance CHO proteins.

Table 1 indicates that the HCP composition/concentration significantly depends on the cell lines used for PTG1 expression. Total HCP concentrations for samples C and D produced by the CHO-S cell line are significantly lower than the total HCP concentrations measured for DG-44 (samples A1, A2, B1, and B2). In addition, the protocols used for Protein A purification of PTG1 also influences the HCP composition/concentration as suggested by Table 1.

Prot no	Protein Description	Protein concentration (ppm)					
		DG-44 cells				CHO-S cells	
		A1	A2	B1	B2	C	D
1	Nucleolin Mesocricetus auratus Golden hamster		191	1615	3034		
2	Heterogeneous nuclear ribonucleoprotein isoforms Mus musculus			1459	2413		
3	Elongation factor isoforms Mus musculus	304	745	1705	1354	142	532
4	Procollagen C endopeptidase enhancer 1 Mus musculus			1655	1265		
5	Actin cytoplasmic isoforms Mesocricetus auratus Golden hamster	907	956	813	877	287	45
6	Clusterin Mus musculus	1010	1068	658	855	537	185
7	Glycogen phosphorylase b rabbit - PHO	621	423	536	659	801	621
8	Plasminogen activator inhibitor 1 RNA binding protein Mus musculus			400	464		
9	Lipoprotein lipase Mesocricetus auratus Golden hamster	1168	481	669	464		
10	78 kDa glucose regulated protein Mesocricetus auratus Golden hamster	201	341	168	463		
11	Glyceraldehyde 3 phosphate dehydrogenase Golden hamster	448	771	573	442	104	31
12	Nascent polypeptide associated complex subunits Mus musculus			494	440		40
13	Nidogen 1 Mus musculus	38	263	42	300		
14	T complex protein 1 subunits Mus musculus	100	271	95	296		
15	Serine protease HTRA1 Mus musculus	565	265	471	286		
16	High mobility group protein isoforms Mus musculus			113	278		59
17	40S ribosomal protein S3 Mus musculus	354	323	268	278		
18	Alpha-lactalbumin bovine - LA	120	260	155	270	200	104
19	Lysosomal alpha glucosidase Mus musculus	40	385	152	266		
20	Nuclease sensitive element binding protein 1 Mus musculus		17	296	208		
21	Pyruvate kinase isozyme M2 Mus musculus	242	332	143	196		
22	Activated RNA polymerase II transcriptional coactivator p15			132	161		
23	Heat shock protein HSP 90 beta Mus musculus	76	114	121	154		
24	Nucleophosmin Mus musculus			96	145		
25	Insulin like growth factor binding protein 4 Mus musculus			150	129		51
26	Complement C1q tumor necrosis factor related protein 4	53	51	164	104		
27	Eukaryotic translation initiation factor isoforms Mus musculus	26	16	239	98		17
28	Alcohol dehydrogenase yeast - ADH	72	59	81	83	101	77
29	Interleukin enhancer binding factor 2 Mus musculus			71	81		
30	Laminin subunits Mus musculus			31	71		
31	Guanine nucleotide binding protein subunits Mus musculus	52	86	44	78		
32	Serum albumin precursor bovine - BSA	46	25	47	57	61	46
33	Cofilin Mus musculus	15	39	21	53		
34	Peroxiredoxin 1 Mus musculus	31	33	39	49		
35	Heat shock cognate 71 kDa protein Mus musculus	122	212				
36	Heat shock protein HSP 90 alpha Mus musculus	71	83				
37	Tubulin isoforms Mus musculus	122	147			81	60
	TOTAL ppm HCPs (without considering the spiked proteins)	5945	7190	12897	15302	1151	1020
	PTG1 purity	94.05	92.81	87.10	84.30	98.85	98.98

Table 1. HCP concentrations (expressed in ppm or ng HCP/mg protein) measured across six PTG1 mAb

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*preparations using the three best responding peptides in ESI-MS. Samples labeled A1, A2 and C were purified on a ProSep-vA column, while B1, B2, and D were processed on a MabSelect Sure column. Samples A1/A2 and B1/B2 were biological replicates produced by DG-44 hamster cells, while samples C and D were expressed in CHO-S hamster cells grown under identical conditions. High abundance proteins are highlighted in red (> 1,000 ppm), medium abundance are either in yellow (500–1000 ppm) or green (100–500 ppm) and low-abundance HCPs are highlighted in grey (< 100 ppm). The mAb purity for each preparation is displayed at the bottom of each sample column.*

## High-throughput monitoring of HCPs by UPLC/tandem quadrupole MS

Once a purification process is established, organizations need to monitor the known HCPs to prove that their process is well-controlled. They may also need to demonstrate to the regulator that the claims made for the product are consistent throughout a number of batches.

UPLC/MRM-MS methodology can provide this information in a rigorous and objective manner that does not rely on operator interpretation. Furthermore, changes in process methodology may also mean that new ELISAs may take months to develop, whereas UPLC/MRM-MS methods can be changed within minutes to accommodate new proteins.

Therefore, a 20-min UPLC/MRM-MS method was developed on the basis of the discovery results from the 2D-UPLC-MS<sup>E</sup> step. The UPLC/MRM-MS assay is developed to rapidly monitor the concentration changes of the previously identified HCPs prepared from a variety of experimental conditions. Twenty HCPs were selected from the list of identified proteins in the six samples.

In total, 58 transitions for 29 peptides representing the twenty HCPs were monitored in MRM experiments. The assay generated highly reproducible measurements with an average peak area RSD of 13.8% for the entire MRM dataset. The results demonstrate that UPLC/MRM-MS methodology offers an efficient method for high-throughput HCP monitoring during the late stage of biopharmaceutical purification. In addition, an MRM assay provides an easy method for absolute quantification of each individual HCP by using spiked-in isotopically labeled peptides.

It is also important to understand the correlation between the different UPLC-MS techniques used. Three HCPs (out of 20 HCPs monitored in MRM experiment) were quantified using spiked-in isotopically labeled peptides. This method matches the technique used in the industry today to obtain absolute quantification values. The

MRM results correlate well with the  $MS^E$  quantification across all six samples, as shown in Figure 5A–C.

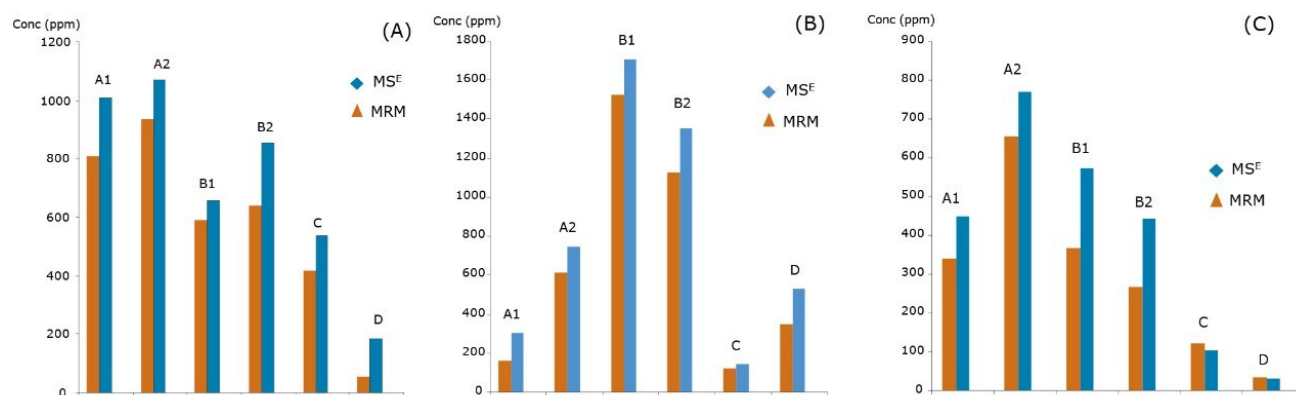


Figure 5. Comparison of HCP quantification between  $MS^E$  and MRM methods: TOF-based quantification ( $MS^E$ ) is based on the precursor signals generated by the average intensity of the three best ionizing peptides against the signals produced by a spiked-in protein standard with a known concentration. MRM quantification is based on using the peak area from the signal of a spiked  $^{13}C^{15}N$ -isotopically labeled peptides with known concentration. Protein concentrations (ppm) measured in six mAb preparations are shown for (A) clusterin, (B) elongation factor 1-alpha, and (C) glyceraldehyde 3-phosphate dehydrogenase.

## Conclusion

- The UPLC- $MS^E$  assay allows the identification and quantification of low-abundance HCP contaminants in biopharmaceuticals over four to five orders of magnitude in concentration.
- Cell lines used for mAb purification can significantly affect the HCPs' identity and concentration.
- Protein A purification of mAbs using different purification protocols produces different HCP patterns.
- A high-throughput UPLC/MRM-MS assay for monitoring targeted HCPs using tandem quadrupole mass spectrometry can be readily implemented.



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