MRM QUANTIFICATION OF HOST CELL PROTEINS IN PROTEIN BIOPHARMACEUTICALS

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Catalin E. Doneanu[1], Weibin Chen[1], Alex Xenopoulos[2], Jim Murphy[1], Keith Fadgen[1], StJohn Skilton[1] and Jeff Mazzeo[1]
[1] Waters Corporation, Milford, MA, USA; [2] Millipore Corporation, Bedford, MA, USA

OVERVIEW

A high-throughput LC-MRM assay for quantification of low-abundance HCPs in recombinant biopharmaceuticals in the range of 10 - 1,000 ppm was developed

INTRODUCTION

- Residual host cell proteins (HCPs) are commonly present in minute quantities in the final biopharmaceutical protein (typically expressed as ng/mg of recombinant protein or ppm concentrations)
- HCPs can potentially elicit an unpredictable immune response in patients
- 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.

WORKFLOW OVERVIEW

- Spike a mixture of 5 proteins in the concentration range of 10—1,000 ppm (ng/mg) in the protein biopharmaceutical
- Enzymatic digestion of the protein sample to peptides
- 2D-LC/MS^E with IDENTITY^E to discover contaminant proteins (HCPs)
- Label-free quantification using the best responding top 3 peptides from each protein for comparison against known concentrations of the spiked proteins
- VERIFY^E for mining the MS^E dataset for extracting appropriate MRMs for each HCP
- LC-MRM assay for targeted, high-throughput absolute quantification, after spiking the $^{13}C^{15}N$-isotopically labeled peptides in the protein biopharmaceutical

METHODS

Sample Preparation

A chimeric anti-phosphotyrosine IgG1 monoclonal antibody (PTG1 mAb) was expressed in two different CHO cell lines and purified by Protein A chromatography using two different protocols following manufacturer recommendations (**Protocol I** and **Protocol II**). Four samples labeled 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. 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 PTG1 preparations along with four $^{13}C^{15}N$ isotopically labeled peptides and the resulting protein mixture was denatured with RapiGest (Waters), reduced/alkylated with DTT/IAM and enzymatically digested with trypsin (Promega). The amounts of spiked proteins loaded on-column were: 4,000 fmoles LA (bovine alpha-lactalbumin), 800 fmoles PHO (rabbit phosphorylase b), 320 fmoles ADH (yeast alcohol dehydrogenase), 80 fmoles BSA (bovine serum albumin) and 16 fmoles ENL (yeast enolase).

LC Conditions

A nanoACQUITY™ UPLC® system (Waters) with 2D technology was used for peptide separations. A reversed-phase/reversed-phase (RP/RP) method was developed that uses 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 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 before analyte trapping onto the trap column.
- **Trap column**: A 5-µm Symmetry C_{18} trap (2.7 µl volume) 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 C_{18} 1.7 µm, kept at 65 °C and operated at 12 µL/min.

Fractions were eluted in ten steps (1: 10.8% Eluent B, 2: 12.4%, 3: 14.0%, 4: 15.4%, 5: 16.7%, 6: 18.6%, 7: 20.4%, 8: 25.0%, 9: 30.0%, 10: 50.0% B. Each step was mixed in a 1:10 ratio with 0.1% TFA in water (pH=2.1) before trapping. Low pH separations in the second chromatographic dimension used a 30 min gradient from 7 to 35% acetonitrile (0.1% FA-formic acid). See Figure 1 for the diagram of the 2D-LC system.

MS conditions

A) Discovery and Label-free Quantification: Data independent, alternate scanning LC/MS^E experiments were performed with a SYNAPT G1 HDMS™ mass spectrometer (Waters).

- Acquisition time was 0.5 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: MRM analyses were performed on two platforms: 1) Xevo™ TQ MS instrument coupled with the ACQUITY UPLC system, 2) Xevo™ TQ-S mass spectrometer coupled to a TRIZAIC nanoTile™ and a nanoACQUITY LC system.

- 2.1 x 150 mm BEH130 C_{18} column (1.7 µm particles) operated at 35 °C with a flow rate of 300 µL/min; 3-40% B over 15 min (B: acetonitrile with 0.1% FA; A: water with 0.1% FA).
- nanoTile™ packed with BEH 1.7 µm C18 particles, operated at 45 °C using a flow rate of 3 µL/min; 3-40% B over 7 min, same mobile phases as above.
- Other parameters: ESI potential 3.5 kV, CV 37 V, source 90 °C, MS1/MS2 isolation window 0.75 Da (FWHM), adjustable collision energy depending of precursors/fragment (15-30 ev range) and 10 ms dwell time.

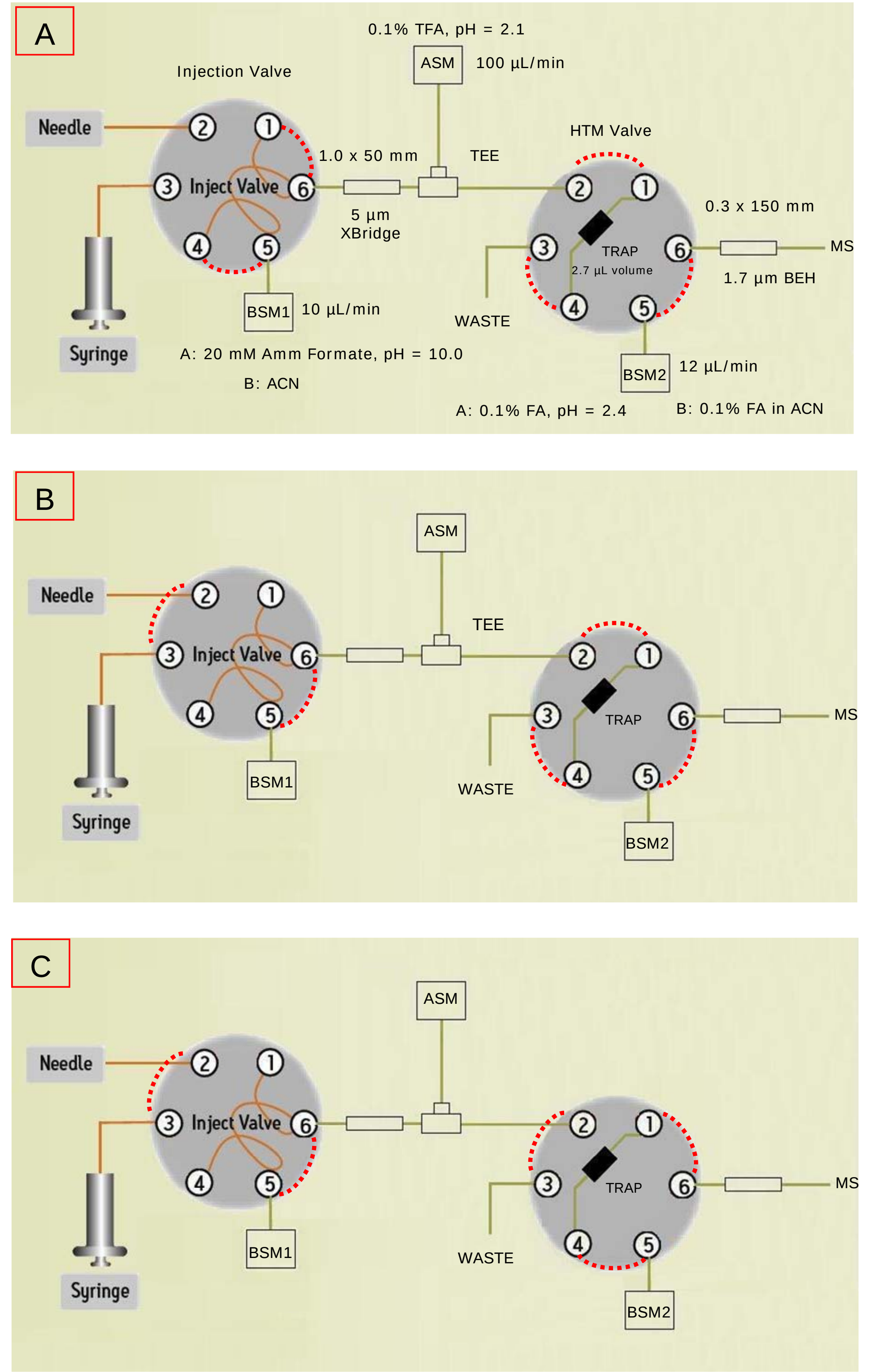

Figure 1: Fluidic configuration for 2-dimensional chromatography with on-line dilution: (A) Sample loading; (B) Peptide elution from 1D (in 10 steps) followed by trapping; (C) Peptide separation in the second dimension.

Figure 2. SYNAPT HDMS™ mass spectrometer coupled to the 2D nanoACQUITY UPLC® system.

RESULTS

HCP Identification Method

- **LC-MS^E Data**: a combined MS^E dataset collected from 10-step 2D RP/RP separations performed in triplicate.
- **Protein Identification Informatics**: PLGS 2.4 featuring Identity^E Bioinformatics.
- **Database**: protein database was compiled from 12,943 mouse Swiss Prot entries, 654 Golden hamster proteins, the sequences of LA, ADH, PHO, BSA, ENL, porcine trypsin, protein A (*S aureus*) and the heavy and light chain sequence of PTG1. The final randomized database contained 27,212 entries.

Prot no	Protein Description	Protein concentration (ppm): DG-44 cells A1	A2	B1	B2	CHO-S cells 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	Laminin subunits Mus musculus			31	71		
6	Actin cytoplasmic isoforms Mesocricetus auratus Golden hamster	907	956	813	877	287	45
7	**Clusterin Mus musculus**	1010	1068	658	855	537	185
8	**Nidogen-1 Mus musculus**	38	263	42	300		
9	Glycogen phosphorylase b rabbit - PHO	621	423	536	659	801	621
10	Plasminogen activator inhibitor 1 RNA binding protein Mus musculus			400	464		
11	Lipoprotein lipase Mesocricetus auratus Golden hamster	1168	481	669	464		
12	78 kDa glucose regulated protein Golden hamster	201	341	168	463		
13	**Glyceraldehyde 3-P dehydrogenase G hamster**	448	771	573	442	104	31
14	Nascent polypeptide associated complex subunits Mus musculus			494	440		40
15	T complex protein 1 subunits Mus musculus	100	271	95	296		
16	Serine protease HTRA1 Mus musculus	565	265	471	286		
17	High mobility group protein isoforms Mus musculus			113	278		59
18	40S ribosomal protein S3 Mus musculus	354	323	268	278		
19	Alpha-lactalbumin bovine - LA	120	260	155	270	200	104
20	Lysosomal alpha glucosidase Mus musculus	40	385	152	266		
21	Nuclease sensitive element binding protein 1 Mus musculus		17	296	208		
22	Pyruvate kinase isozyme M2 Mus musculus	242	332	143	196		
23	Activated RNA polymerase II transcriptional coactivator p15			132	161		
24	Heat shock protein HSP 90 beta Mus musculus	76	114	121	154		
25	Nucleophosmin Mus musculus			96	145		
26	Insulin like growth factor binding protein 4 Mus musculus			150	129		51
27	Complement C1q tumor necrosis factor related protein 4	53	51	164	104		
28	Eukaryotic translation initiation factor isoforms Mus musculus	26	16	239	98		17
29	Alcohol dehydrogenase yeast - ADH	72	59	81	83	101	77
30	Interleukin enhancer binding factor 2 Mus musculus			71	81		
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	5945	7190	12897	15302	1151	1020
	mAb purity	**94.05**	**92.81**	**87.10**	**84.30**	**98.85**	**98.98**

Table I. HCP concentrations (expressed in ppm or ng HCP/mg protein) measured across six PTG1 mAB preparations using the three best responding peptides in ESI-MS. Samples labeled A1, A2 and C were purified using **Protocol I**, while B1, B2 and D were processed using **Protocol II**. 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.

Sample no	Sample ID	CHO Cell Line	Protein conc (mg/mL)	No of HCPs Identified	Common HCPs	HCP conc (ppm)	PTG1 purity (%)
1	A1	DG44	5.00	21	4	5945	94.05
2	A2	DG44	7.37	23	4	7190	92.81
3	B1	DG44	5.79	30	4	12897	87.10
4	B2	DG44	4.47	30	4	15302	84.30
5	C	CHO-S	3.88	5	4	1151	98.85
6	D	CHO-S	5.00	9	4	1020	98.98

Table II. Summary of HCP identifications provided by the MS^E analysis.

Peptide sequence	Protein ID	RT (min)	MRM Transition: Native	13C15N	CV (V)	CE (eV)
SLLNSLEEAK	Clusterin	12.2	552.3 -> 903.5	556.3 -> 911.5	35	21
STTTGHLIYK	Elongation factor	8.3	560.8 -> 831.5	564.8 -> 839.5	35	22
GAAQNIIPASTGAAK	GAD3-PDH	9.6	685.4 -> 702.4	689.4 -> 710.4	35	26
VLFDTGLVNPR	Nidogen-1	12.9	615.8 -> 1018.5	620.8 -> 1028.5	35	24

Table III. Experimental conditions for the MRM assay.

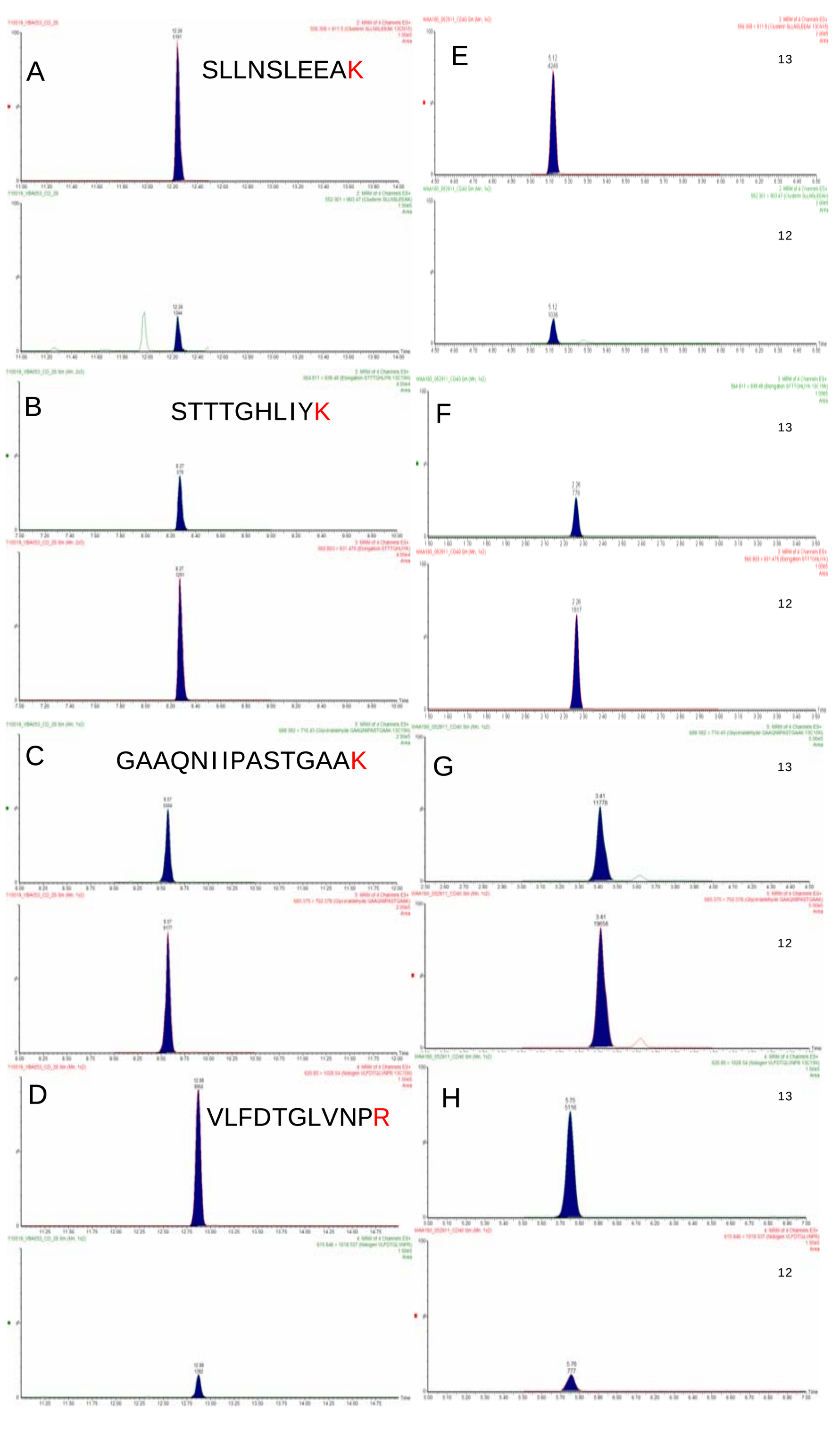

Figure 3. MRM chromatograms recorded for four HCPs measured in sample B1. Each panel displays the best responding transitions for the native and the corresponding $^{13}C^{15}N$-isotopically labeled peptide belonging to (A,E) clusterin, (B,F) elongation factor 1-alpha, (C,G) glyceraldehyde 3-phosphate dehydrogenase and (D,H) nidogen-1. MRMs displayed in panels A-D were recorded using the analytical scale chromatography setup (2.1 mm column ID), while chromatograms presented in panels E-H were obtained on the TRIZAIC nanoTile.

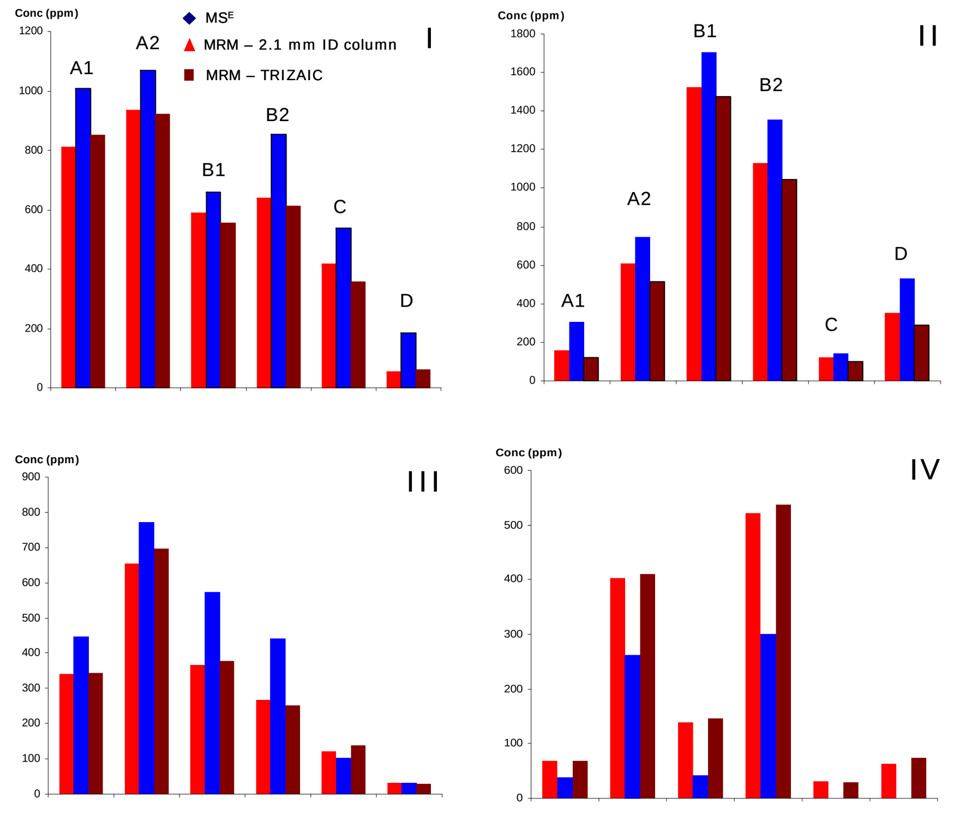

Figure 4. Comparison between MS^E and MRM quantification. TOF-based quantification (MS^E) is comparing the precursor signals in ESI-MS generated by the three best ionizing peptides against the signals produced by a spiked protein with a known concentration. MRM quantification is based on using spiked $^{13}C^{15}N$-isotopically labeled peptides with known concentration. Protein concentrations (ppm) measured in six mAb preparations are shown for (I) clusterin, (II) elongation factor 1-alpha, (III) glyceraldehyde 3-phosphate dehydrogenase and (IV) nidogen-1.

CONCLUSIONS

- The LC/MS^E-based assay allows the identification and quantification of low-abundance HCP contaminants in biopharmaceuticals over four orders of magnitude in concentration.
- TRIZAIC nanoTile in combination with the TQ-S tandem quadrupole shows great promise for high-throughput HCP quantification or monitoring (under 10 min runtime).
- Protein A purification of mAbs using different purification protocols produces different HCP patterns.

References

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3. Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ *Proteomics*, **2009**, 1696.

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