The Use of Ion Mobility Enabled QTof HRMS for the Quantification of Trace Protein Impurities in Biotheraputics

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INTRODUCTION

- Analysis of low-levels (1-100 ppm) protein impurities (e.g. host cell proteins – HCPs) in protein biotherapeutics is challenging.
- Ligand binding assays (LBAs) typically provide the total HCP concentration, and they cannot identify and measure individual HCP contaminants.
- Quantification based on low resolution tandem mass spectrometers is also challenging due to potential interference from other co-eluting peptides when they share the "same" precursor > fragment transition.
- In this poster, ion mobility enabled QTof HRMS platform was used. A high selectivity (HS) MRM assay, which combines ion mobility separation of peptide precursors with high-resolution (Rs~40,000) MS-detection of peptide fragments, was explored.
- The capabilities of this approach was investigated for the quantification of low abundance peptide standards spiked in a monoclonal antibody (mAb) digest from 0.1 nM to 100 nM. Data suggest it has the sensitivity and dynamic range (>log 3) typically achieved in HCP analysis. All six peptide standards were detected at concentrations as low as 0.1 nM in the presence of a high-abundance peptide background. When considering the MW of spiked-in peptides, the LOQ of this assay is at the 1-2 ppm level.

METHOD

Sample Preparation. A licensed mAb biosimilar (Inflectra, 10 mg/mL) was denatured with 0.04% RapiGest surfactant (60°C, 15 min), reduced with 20 mM DTT (60°C, 1h), alkylated with 10 mM IAM (RT, 30 min) and digested with a mixture of Lys-C and porcine trypsin (Promega) overnight. A MassPREP digest standard containing six rabbit phosphorylase (PHO) peptides (Waters P/N 186006011) was spiked post-digestion in the mAb digest at the following concentrations: 0.1, 1. 10, and 100 nM. At 10 µL injections, the amounts of each peptide loaded on-column were **1**, 10, 100 and 1,000 **fmoles**, while the amount of matrix (Inflectra digest) stayed constant at 2 µg. This corresponds to a targeted **LLOQ of 1-2 ppm**

LC Conditions. An ACQUITY™ UPLC® H-class system equipped with a CSH (charged-surface hybrid) C18 column (2.1 x 150 mm, 1.7 µm particles) was used to separate the mAb digest at a flow rate of 200 µL/min and a column temperature of 60 °C. Gradient elution was performed from 1% to 40% mobile phase B in 30 min and the total LC runtime was 50 min. The mobile phase composition was: Solvent A: 0.1% FA (formic acid) in DI water and Solvent B: 0.1% FA in ACN.

MS Conditions. Data-independent acquisition following precursor level ion mobility separations (HDMS^E) were performed on a VION IMS QTof mass spectrometer, over the m/z range of 100-2000 with an acquisition time of 0.5 sec. One HS-MRM acquisition method designed to monitor all six PHO peptides was created following the Tof-MRM optimization experiments .

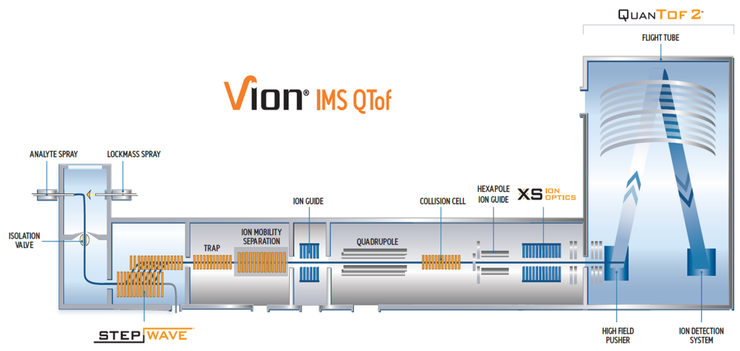


Figure 1. Diagram of the VION IMS QTof mass spectrometer. In the high selectivity MRM acquisition mode (HS-MRM) the precursors of the peptide that is being quantified are separated from other co-eluting (interfering) peptide precursors in the ion mobility cell, isolated by the quadrupole and fragmented with a fixed collision energy in the collision cell. The signal produced by the peptide fragment ions is enhanced by adjusting the pusher frequency and peptide quantification is performed using the high MS resolution (>40,000) signals produced by the most intense fragment ion of each peptide.

VION IMS QTof MS system

RESULTS

Workflows

1. Acquiring HDMSe data using Pepmap workflow
   - RT | m/z | Fragments | ccs | dt
2. CE optimization using TOF MRM workflow
   - Best fragment | Best CE
3. TOF MRM Data Acquisition and Quantification
   - precursor | Fragment

Step 1 . Collect HDMSe data to determine RT, MZ, CCS, and 3 major fragments m/z to be used for Tof MRM method development.

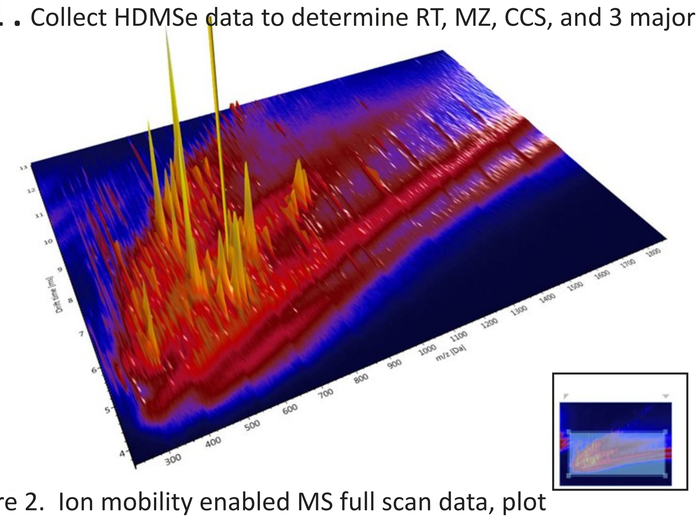


Figure 2. Ion mobility enabled MS full scan data, plot of m/z vs drift time vs response. The plot shows high level matrix peaks having the same m/z or retention time.

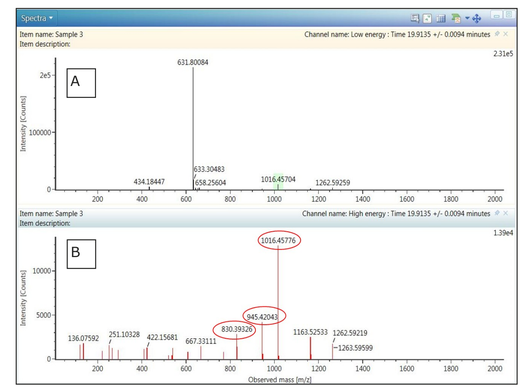


Figure 3. Example of HDMS^E data: (A) Low-energy spectrum showing the Pep 6 precursor ion; (B) high-energy fragmentation spectrum of the same peptide, displaying the top 3 most abundant fragments ions (circled) selected for Tof-MRM collision energy (CE) optimization.

Step 2. Tof MRM CE optimization to determine the best precursor > fragment pair for each peptide.

Peptide Sequence	Retention time	Precursor/ Charge	Fragment/ Charge	Optimum CE (V)
2. TCAYTNHTVLPEALER	16.3	625.6404 (+3)	714.3781(+1)	24
4. LLSYVDDEAFIR	21.2	720.8721(+2)	964.4734(+1)	22
5. LITAIGDVVNHDPVVGDR	19.8	630.6742(+3)	832.4236(+2)	20
6. VFASYEEYVK	17.8	631.8006(+2)	1016.4571(+1)	24

Table 1. Results of the Tof-MRM CE optimization experiment: the most abundant fragments of each PHO peptide with the corresponding optimized collision energy.

Step 3 . Tof MRM Quantification Results.

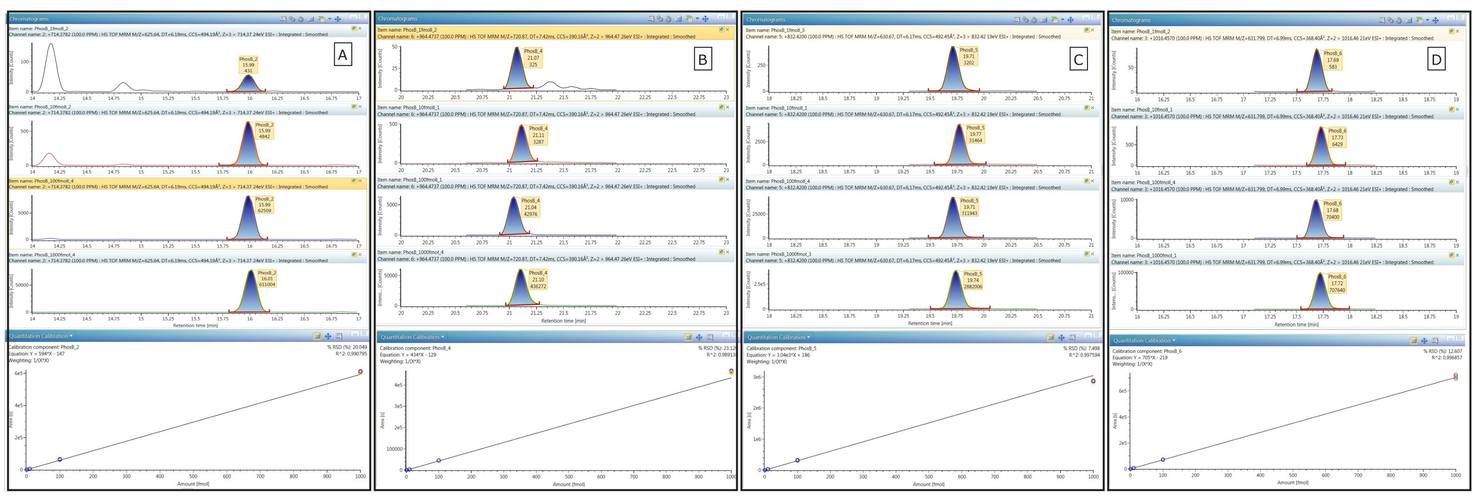


Figure 4. HSMRM chromatograms and calibration curves for the 4 PHO peptides spanning 3 orders of magnitude (0.1, 1, 10 and 100 nM). (A) Pep 2; (B) Pep 4; (C) Pep 5; (D) Pep 6.

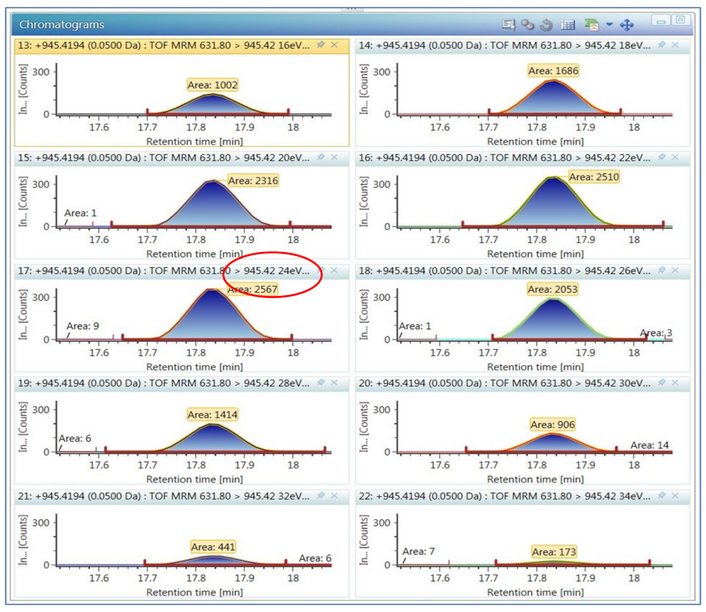
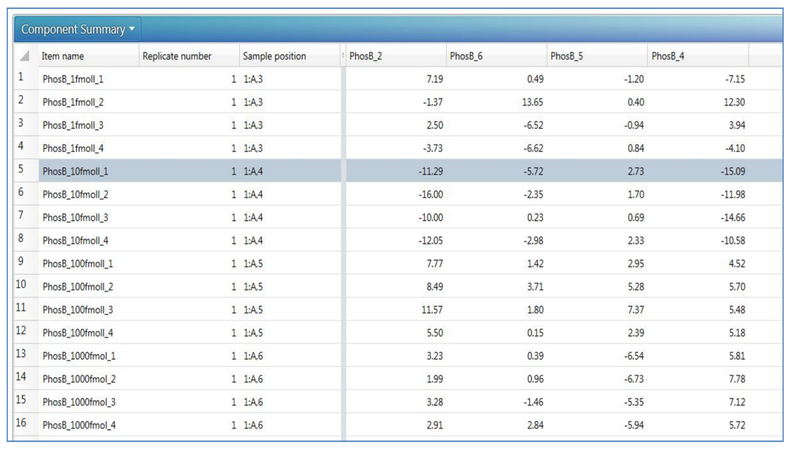


Figure 5. Example CE Optimization results for pep 5. Overlaid chromatograms of 10 collision energies monitored. The CE producing the highest peak area (red circle) was chosen as the CE in the quantification.



	Item name	Replicate number	Sample position	PhosB_2	PhosB_6	PhosB_5	PhosB_4
1	PhosB_1fmoll_1	1	1:A,3	7.19	0.49	-1.20	-7.15
2	PhosB_1fmoll_2	1	1:A,3	-1.37	13.65	0.40	12.30
3	PhosB_1fmoll_3	1	1:A,3	2.50	-6.52	-0.94	3.94
4	PhosB_1fmoll_4	1	1:A,3	-3.73	-6.62	0.84	-4.10
5	PhosB_10fmoll_1	1	1:A,4	-11.29	-5.72	2.73	-15.09
6	PhosB_10fmoll_2	1	1:A,4	-16.00	-2.35	1.70	-11.98
7	PhosB_10fmoll_3	1	1:A,4	-10.00	0.23	0.69	-14.66
8	PhosB_10fmoll_4	1	1:A,4	-12.05	-2.98	2.33	-10.58
9	PhosB_100fmoll_1	1	1:A,5	7.77	1.42	2.95	4.52
10	PhosB_100fmoll_2	1	1:A,5	8.49	3.71	5.28	5.70
11	PhosB_100fmoll_3	1	1:A,5	11.57	1.80	7.37	5.48
12	PhosB_100fmoll_4	1	1:A,5	5.50	0.15	2.39	5.18
13	PhosB_1000fmol_1	1	1:A,6	3.23	0.39	-6.54	5.81
14	PhosB_1000fmol_2	1	1:A,6	1.99	0.96	-6.73	7.78
15	PhosB_1000fmol_3	1	1:A,6	3.28	-1.46	-5.35	7.12
16	PhosB_1000fmol_4	1	1:A,6	2.91	2.84	-5.94	5.72

Table 2. Summary of %deviation from linear curve fitting. All peptides show <15% deviation across the concentration studied, meeting method validation criteria.

CONCLUSIONS

- A simple workflow is presented for the quantification of trace protein impurities in a complex matrix.
- Ion mobility enable MRM quantification produced high sensitive detection.
- All six peptide standards were detected at concentrations as low as 0.1 nM, or 1 femtomole on column, in the presence of 2 ug high-abundance peptide back ground.
- The LOQ of this assay is at the 1-2 ppm level. The linear range is 3-orders from 0.1 nm to 100 nm.
- %Deviations from linear curve fitting were less than 15% across the entire concentration range (0.1-100 nM).

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