High Sensitivity Host Protein Quantitation in an IgG1 Monoclonal Antibody Preparation via Data-Independent Acquisition

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Monoclonal antibodies (mAb) are major target-oriented biotherapeutics that are used to treat an array of human diseases. Current therapeutic monoclonal antibodies are immunoglobulin G (IgG) 1 derivatives, typically produced in mammalian cell culture using Chinese hamster ovary (CHO) or other cells[1]. Process-related impurities and other trace contaminants that co-purify during downstream processing must be characterized and monitored to control product quality, potency, and safety.

The detection and quantitation of host cell protein (HCP) contaminants is an area of particular concern, as these contaminants can elicit an adverse response in patients. EMA (European Union) regulations have been in effect since 1997 and reflect ICH guidelines that the removal of HCPs should be proven:

"6.2 Validation of the purification procedure - …. The ability of the purification process to remove other specific contaminants such as host-cell proteins … should also be demonstrated".

Historically, HCPs have often been monitored using immuno-affinity assays (typically ELISA) based on polyclonal serum generated against the whole proteome of the host cell culture, or against a subset of the proteome obtained by enrichment under conditions similar to the product purification process. This approach is inherently biased toward the detection of highly-expressed HCPs, and HCPs that are highly immunogenic in the animal species used in reagent generation. Results of these tests can be misleading, as low-level HCPs are equally dangerous to the patient, and the human immune system might react differently to that of the animal. Failure to detect an immunogenic contaminant can lead to product intolerance or adverse reactions in patients, which threatens patient health as well as clinical trial outcomes. Biopharmaceutical developers have substantial motivation to ensure that they have unbiased analysis of the HCP complement of a product. Modern mass spectrometry has emerged as an attractive tool to improve the analysis of HCPs in a rapid and efficient manner that also

provides greater certainty. It would also be advantageous to identify the specific proteins individually, as well as quantify them. However, until recently it was unclear whether or not available mass spectrometry solutions could provide the sensitivity and dynamic range necessary to detect and quantify trace HCP contaminants amongst an enormous excess of biotherapeutic protein, and at speeds to complete confident analyses in a reasonable time span. Moreover, conventional data-dependent acquisition methods adopted from shotgun proteomics for the detection of peptides from FASTA database searching techniques are inherently stochastic, leading to poor run-to-run reproducibility, particularly at the MS/MS level. A commercially viable mass spectrometry-based solution for HCP analysis must avoid biases, operate without needing to know the contaminant proteins prior to establishing the assay (e.g. ELISA), and retain the beneficial aspects of speed, sensitivity, and consistency.

Here we leverage the high speed and sensitivity of a TripleTOF® mass spectrometer, combined with powerful data-independent SWATH® acquisition to demonstrate unbiased, highly-reproducible quantitation of model host cell protein contaminants in an IgG1 sample down to the level of ten parts per million (ppm, wt/wt contaminant/product) Method fidelity and throughput were maintained via the use of micro-scale LC (20 ul/min) and short analytical runs (less than 45 minutes.)

Advantages of the TripleTOF® System with SWATH® Acquisition for Host Cell Protein Analysis

- **Comprehensive, unbiased quantitation with simultaneous MS/MS information:** Amounts of any number of host cell proteins can be quantified in a single run.
- **Sensitivity and speed:** Host cell proteins can be quantified at low PPM levels, using LC gradients as short as thirty minutes.
- **Throughput and reliability:** The speed and sensitivity of the TripleTOF system allow the use of microflow LC for this assay, eliminating the problems with reliability and throughput that are typically seen with nano-scale LC.

Experimental Design

Sample Preparation: 1 mg of monoclonal mouse IgG1 isotype (Waters, Milford, MA) was denatured with urea, reduced with TCEP, and alkylated with MMTS. The resulting denatured protein was digested with trypsin at 37˚C for four hours. A constant concentration of this digest was spiked with a commercial six-protein digest mixture (Michrom, Auburn, CA), at a range of concentrations.

Chromatography: Samples were analyzed using the Eksigent ekspert™ 425 System and a ChromXP column (0.5 mm x 10 cm C18-CL, 3µm 120Å.) The HPLC gradient is shown in Table 1. A flow rate of 20 µL/min was used. Solvent A consisted of 2% acetonitrile and 0.1% formic acid, solvent B consisted of 98% acetonitrile with 0.1% formic acid. 10ug of antibody were loaded per run. The column was maintained at 45 ºC.

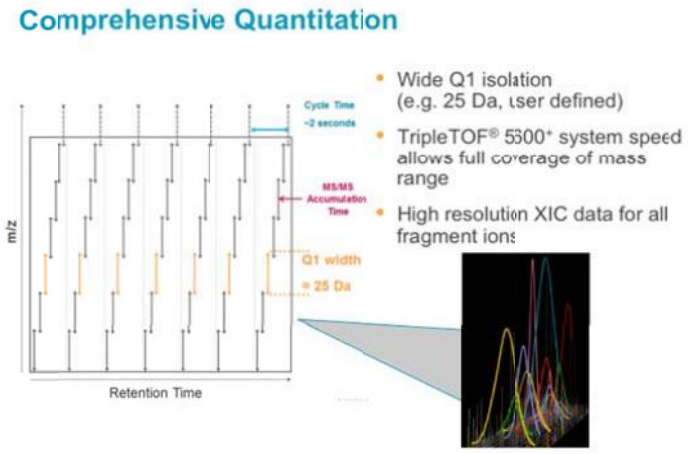

Figure 1. SWATH® Acquisition Obtains MSMS data on all ions. By rapidly stepping the MS/MS isolation window across the mass range, fragment ion chromatograms are collected for all observed ions within chromatographic peaks.

Time	Solvent A	Solvent B
0	95	5
1	95	5
25	65	35
30	10	90
35	10	90
36	95	5
41	95	5

Table 1: LCMS Gradient Profile

Mass Spectrometry: Unmodified and spiked mAb digests were analyzed using a TripleTOF 5600 system. An information dependent acquisition (IDA) LC-MS/MS method was used for initial peptide identification and to generate a peptide library for SWATH acquisition data interrogation. This IDA method consisted of a high resolution TOF MS survey scan followed by 20 MS/MS per second with a minimum accumulation time of 50 msec. SWATH data-independent acquisitions were subsequently performed in triplicate on each sample, using a 20 Da Q1 window width, to obtain quantitative MS/MS chromatograms for every precursor ion between 400 and 1200 m/z.

Data Processing: IDA data files were searched using ProteinPilot™ Software against a database containing the sequence of the antibody and the sequences of the model 'host' cell proteome. Quantitative analysis was performed using the search results as a peptide library to inform SWATH peptide fragment ion chromatogram extraction using the SWATH Acquisition tool inside of PeakView® Software.

Peptide Mapping and Library Generation

The levels of each model host cell protein in our various spiked samples are shown in Table 2, expressed as parts per million (ppm.) Our qualitative characterization runs using IDA analysis were performed on the 'Level 1' sample, where model HCPs ranged from approximately 100-500 ppm. These initial runs were used to catalog IgG1 and model HCP-derived peptides, and to generate a peptide library to inform subsequent SWATH analysis. Antibody sequence coverage was 100% for Light Chain and 99.5% for Heavy Chain (Figure 2.) An advantage with this approach is that the peptide libraries produced in this step can be used indefinitely in any subsequent SWATH analysis. In general, the IDA runs should rarely need to be repeated, except for example, when making substantial changes to the protein expression or purification processes that are used. In a functional sense, this step can be considered analogous to the common practice of generating a polyclonal ELISA reagent against partially enriched HCPs, but the IDA method avoids the detection biases introduced with such a reagent.

Level	Serum Albumin	Lacto-peroxidase	Carbonic Anhydrase	Glutamate Dehydrogenase	Alpha Casein	Lacto-globulin
1	415	485	182	350	148	114
2	207	242	91	175	74	57
3	104	121	46	88	37	29
4	52	61	23	44	18	14
5	26	30	11	22	9	7

Table 2: Model HCP levels in samples, expressed as parts per million.

Data-Independent Quantitation of Model Host Cell Proteins via SWATH® Acquisition

Following our IDA runs, we re-analyzed our samples using data-independent SWATH Acquisition. Quantitative analysis was performed via peptide fragment ion chromatogram extraction using the SWATH Acquisition tool in PeakView® Software, using the peptide library data to inform f agment ion selection. Figure 3 shows an example of SWATH data representing a peptide from Serum Albumin. Aligned extracted ion chromatogram (XIC) traces for nine peptide fragment ions are shown, for each of the HCP spike levels. The peptide is easily detectable at the 10 PPM level, and visibly absent in the control sample lacking model HCPs (F).

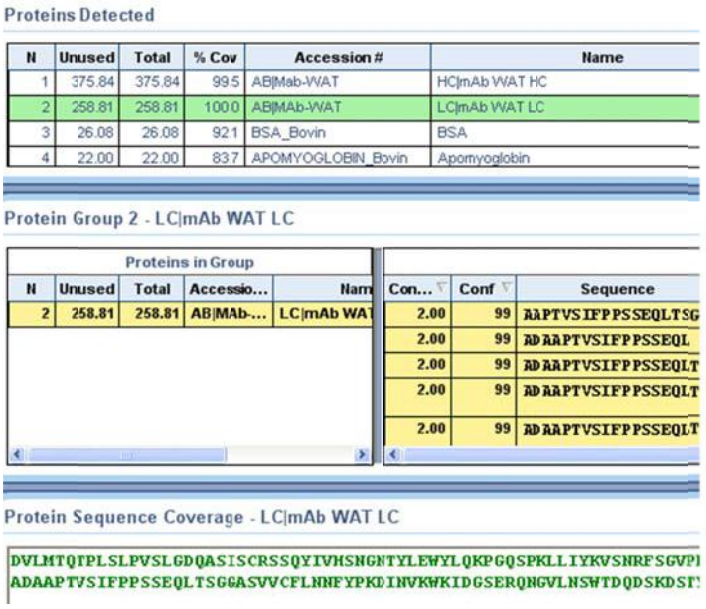

Figure 2. Antibody coverage in peptide mapping runs. Extremely high coverage of the heavy and light chains are obtained using Protein Pilot.

For each of the model HCPs, three to four peptides were used for SWATH® quantitation. It is important to note that the MS/MS information is used here for quantitation, rather than just confirmation of peptide identity, providing superior acuity compared to MS1-based quantitation. In all cases, protein-level quantitation reflects summation of the individual peptide signals. We also selected four peptides from the antibody itself for quantitation. Despite up to a 100,000 fold difference in abundance between the product and the contaminants, the quantitation of both HCPs and antibody was highly reproducible. Further, quantitation of the antibody served as an internal loading control, allowing for normalization of the HCP signals, thereby eliminating minor differences in HCP signal response arising from variations in sample loading, etc. This made it possible to compare HCP levels in sets of runs performed many days apart with high quantitative accuracy, without undue concern for maintaining identical states with respect to the LC and MS systems. Such capability would also allow experiments to be run when needed rather than having to dedicate a system to HCP analysis alone for a number of days. Variations in antibody response across samples run on different days were generally less than ten percent.

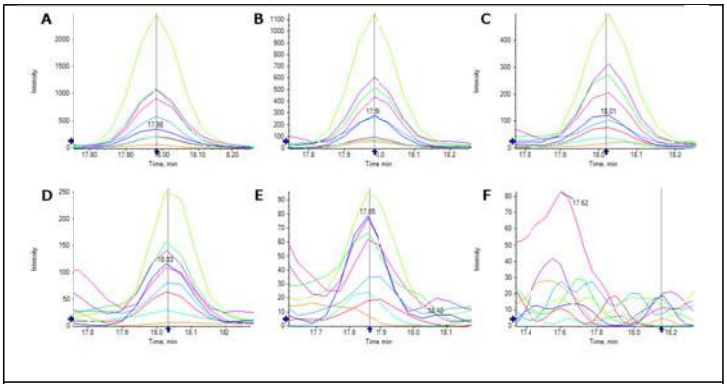

Figure 3. SWATH XIC traces for a peptide from serum albumin. Fragment ion XICs for the peptide SLHTLFGDELCK are shown. Traces correspond to protein levels of A: 415 ppm, B: 207 ppm, C: 104 ppm, D: 52 ppm, E: 26 ppm, and F: a negative control sample containing no model HCP peptides.

Quantitative Linearity and Detection Limits

Figure 4 shows the signal response for each model HCP across the sets of triplicate runs. Figure 5 highlights the CVs observed for each protein and concentration level. At the higher load levels, CVs were all between 3 – 7%. Even at the lowest level, where the HCP proteins were present at 7-30 ppm, the average CV was only 7%, and the most variable protein had a CV of 12%. These results indicate extremely high quantitative reproducibility, independent of protein identity, suggesting that this approach should be broadly applicable for the unbiased quantitation of HCPs down to low ppm levels.

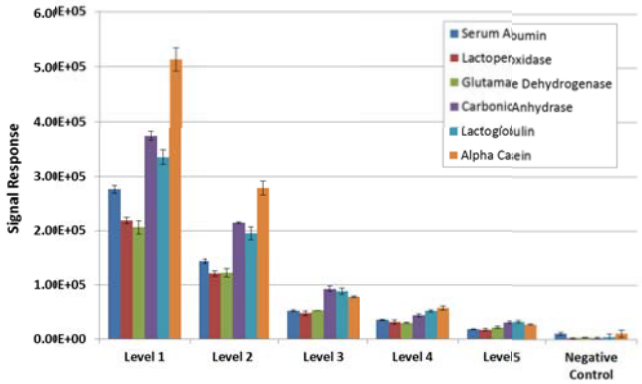

Figure 4: Protein level signal responses. Peptide level signals for each protein were summed, providing a direct measure of concentration. Values shown are the mean and standard deviations (error bars) of triplicate runs.

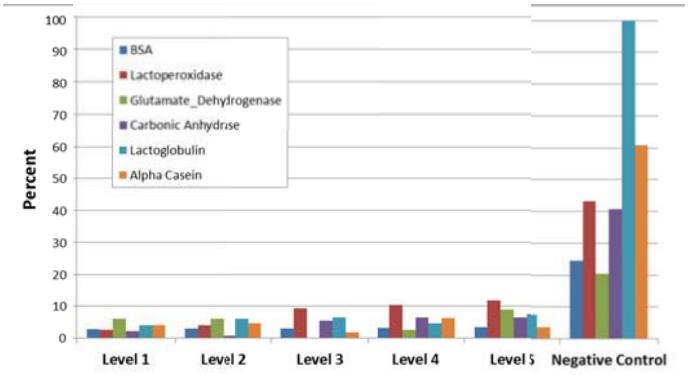

Figure 5: Protein level coefficients of variation across replicates. Consistently low CVs across the model HCP contamination levels indicate excellent quantitative accuracy down to the lowest tested level (protein present at 7-30 ppm). The very high CV values observed in the negative control confirm that the software could not find a reliable signal as expected.

Conclusions

Current analytical strategies for monitoring the levels of host cell protein contaminants in biologics present an unpalatable choice. One can have sensitivity and speed at the cost of detection blind spots and biases (ELISA), or unbiased detection at the individual protein level, at the cost of extremely low throughput and poor reproducibility (typical MS approaches derived from proteomics.) The approach we present here, relying on the power of MS/MS^{ALL} via SWATH® Acquisition, combines the key strengths of both of these approaches, while eliminating the liabilities. Because a single SWATH Acquisition method can target the entire precursor peptide mass range, any number of target proteins can be quantified in a single run. Further, this comprehensive quantitative profiling eliminates problems with run-to-run variability that are inevitable with conventional data-dependent acquisition strategies derived from shotgun proteomics. Critically, these advantages do not need to come at the cost of throughput, reliability, or sensitivity. As demonstrated here, MS/MS^{ALL} via SWATH acquisition on the TripleTOF 5600+ system can deliver consistent, low ppm-level quantitation of host cell proteins in runs that are well under an hour, at flow rates in the tens of microliters per minute, thereby eliminating the complications and reliability issues associated with nano-scale LC.

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