

# Ultra-sensitive host cell protein detection using CESI-MS with SWATH<sup>®</sup> acquisition

*Sub-ppm HCP sensitivity through an orthogonal CE separation and reduced ion suppression*

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## KEY BENEFITS

- Detection of host cell proteins from limited sample quantities in the early development phase
- Orthogonal separation mechanism for additional method development or detection validation
- Improvement in sensitivity through reduced ion suppression and higher ionization efficiency at ultra-low flow rates

## Introduction

Host cell proteins (HCPs) are undesired impurities in biologic preparation processes and can negatively affect biotherapeutic quality, potency, and safety. Identification and quantification of HCPs within therapeutic monoclonal antibody (mAb) preparations remain a challenge with a need for improved sensitivity and specificity. Mass spectrometry, particularly data-independent acquisition such as provided by SWATH acquisition, provides a robust and sensitive means to quantify HCPs in the parts-per-million (ppm) range. The integration of capillary electrophoresis (CE) and electrospray ionization (ESI) into one process (CESI) presents the possibility to improve the sensitivity of HCP quantitation through reduced ion suppression and improved ionization efficiency at ultralow nanoliter per minute flow rates.

We demonstrate the use of CESI-MS with SWATH acquisition for the ultrasensitive detection of host cell proteins (HCPs) in a representative mAb preparation. To simulate HCPs, we spiked a mAb digest with digested protein standards over a concentration range of sub-ppm to parts per thousand (ppth) concentrations. HCP proteins at ppth concentrations were identified with a CESI-MS information-dependent acquisition (IDA) run to generate an ion library for proteins/peptides. Identical CESI separations were performed with the HCP dilution series using SWATH acquisition. The ion library and SWATH data were used to screen for peptides and fragment ions which best represent the HCP concentrations. The concentration-indicative peptides and ions were then used to generate calibration curves for the HCPs. HCPs were quantified down to the low ppm range and in some cases even into the sub-ppm range, representing detection of HCPs over three orders of magnitude.



CESI 8000 Plus High Performance Separation Module coupled to a TripleTOF<sup>®</sup> 5660 System via a NanoSpray<sup>®</sup> III source, CESI adapter, and OptiMS Silica Surface Cartridge

## Experimental

- **Sample Preparation:** A representative mAb and 17 protein standard mixture were digested separately to peptides at 1 mg/mL using a 4-hour digestion protocol with RapiGest, DTT, iodoacetamide, and trypsin. The digested protein standard mixture was spiked into the digested mAb throughout the ppth ppm, and ppb range to represent contaminant host cell proteins. The peptide preparations were then diluted to 250 µg/mL in 125 mM ammonium acetate, pH 4.
- **CESI Conditions:** CESI experiments were carried out with a SCIEX CESI 8000 Plus system (P/N A98089) equipped with a temperature controlled auto sampler and a power supply with the ability to deliver up to 30 kV. An OptiMS Silica Surface Cartridge (P/N B07367) with a porous tip was used for infusion and peptide mapping experiments. Solutions of 10% acetic acid were employed as the background electrolyte (BGE) and conductive liquid. After pressure injection of ~25

ng peptides, sample stacking was performed using transient isotachopheresis (t-ITP). CESI separations were performed at 20 kV. The same CESI separation conditions are used for IDA identification and SWATH acquisition quantitation runs.

- MS/MS Conditions:** A SCIEX TripleTOF® 5600+ system with a NanoSpray® III source and CESI adapter (P/N B07366) controlled by Analyst® TF 1.7 Software were used. HCP identification was performed with information dependent acquisition (IDA) with 15 MS/MS cycles (100 and 50 ms accumulation times for MS and MS/MS, respectively). HCP detection and quantification were performed using data-independent SWATH acquisition with 30 constant window width scans (30 *m/z* with 1 *m/z* overlaps) from 300 – 1200 *m/z* using 150 and 50 ms accumulation times for MS and SWATH scans, respectively.
- Data Analysis:** High resolution MS and MS/MS spectra were analyzed using SCIEX ProteinPilot™, PeakView®, and MultiQuant™ software.

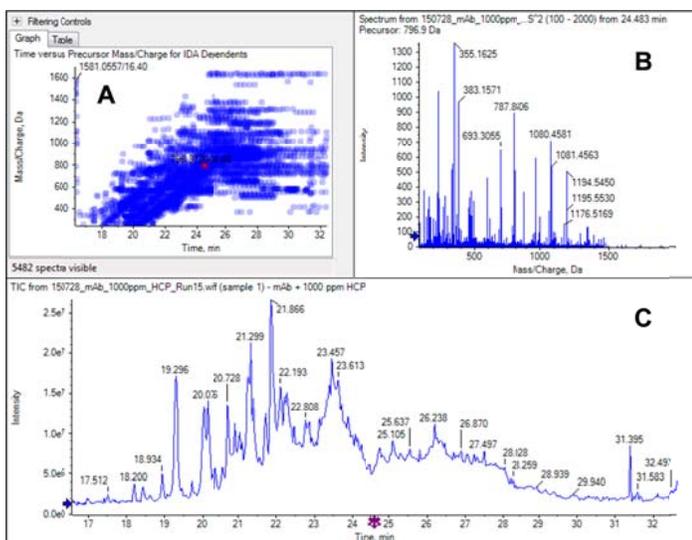


Figure 1. Raw data from a CESI-MS IDA run on the 1 parts per thousand protein standard and mAb sample preparation. The raw data is represented as (A) a heat map of the peptides selected for MS/MS, (B) a representative peptide MS/MS spectra, and (C) a total ion electropherogram (TIE) of the CESI peptide separation.

## Results

The first step for a host cell protein experiment is identifying proteins with an IDA experiment while present at higher concentrations. In practice, this experiment would be performed on a partially purified mAb preparation. Here we simulated the partially purified preparation with protein standards spiked in at 1 parts per thousand by mass relative to the mAb. Representative CESI-MS IDA data displayed by PeakView in IDA Explorer is shown in Figure 1. CESI-MS IDA runs allow for identification of peptides within representative host cell proteins which yield good

MS/MS spectra at lower concentrations. ProteinPilot search results from the IDA experiment is shown in Figure 2. The peptide fragment ions from protein and peptides database matches can later be evaluated as good quantitative representatives of a HCPs concentration from the SWATH acquisition data.

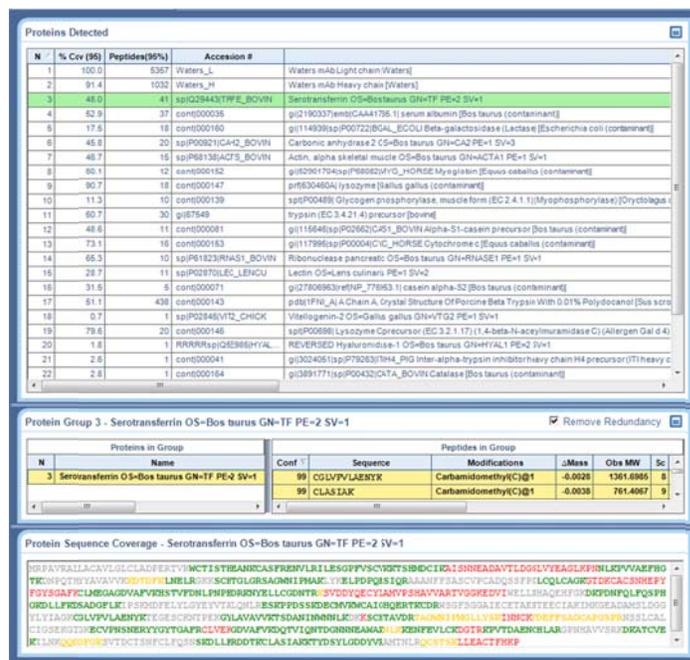


Figure 2. Identification of representative mAb and protein standards from CESI-MS IDA runs using ProteinPilot™. Peptides shown in green within the protein sequence represent the best candidates for quantitation using SWATH™ acquisition since they have the best MS/MS signals. These peptides are imported into the PeakView® SWATH microapp for evaluation.

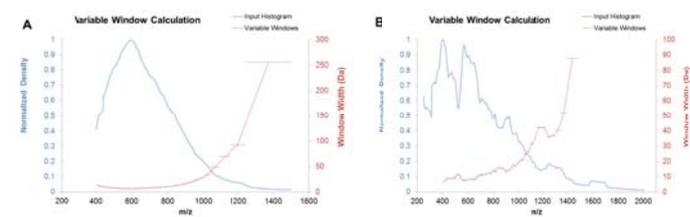
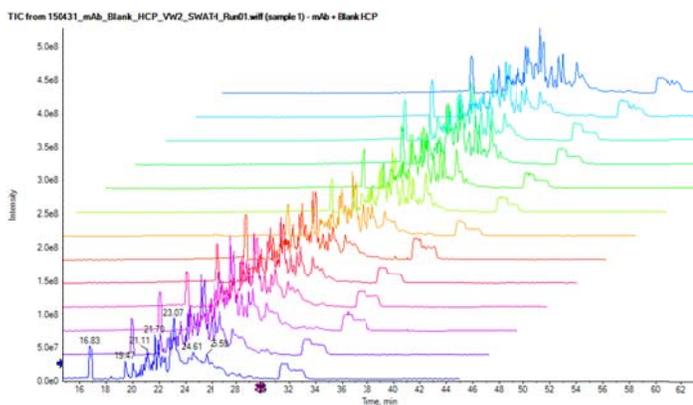
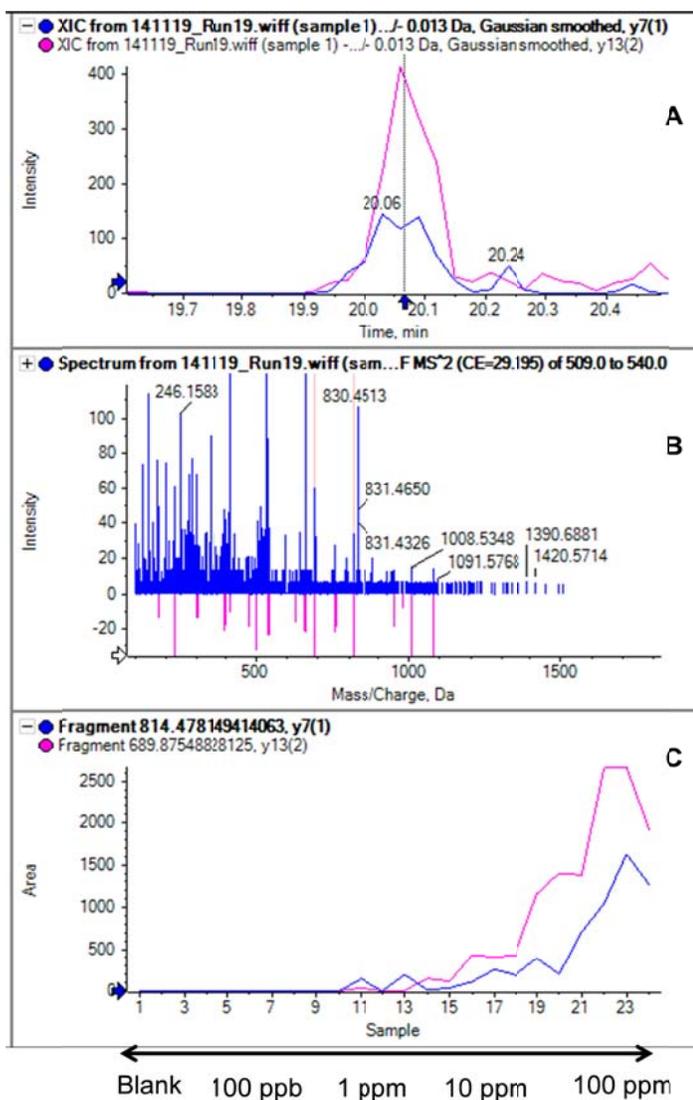


Figure 3. Calculation of variable SWATH™ acquisition window widths based on IDA runs of a (A) yeast lysate and (B) 1 parts per thousand HCP/mAb preparation.

SWATH acquisition can be performed with different *m/z* window configurations to focus on information-rich regions of the *m/z* domain. We investigated the use of constant and variable window widths on the detection of HCPs using CESI-MS. Peptides are generally most abundant in the 400 – 800 *m/z* range, so one of the variable window configurations tested was generated from LC-MS IDA analysis of a common peptide digest from yeast lysate. The other variable window configuration we



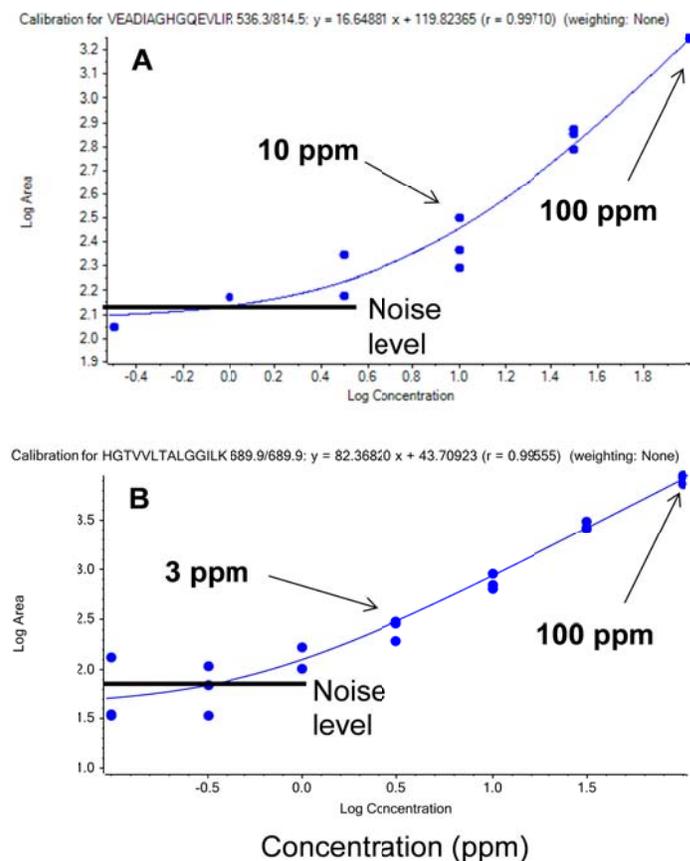
**Figure 4.** Representative total ion electropherograms (TIEs) from CESI-MS SWATH™ acquisition runs including blank and HCP-spiked runs in the ppb to ppt concentration range.



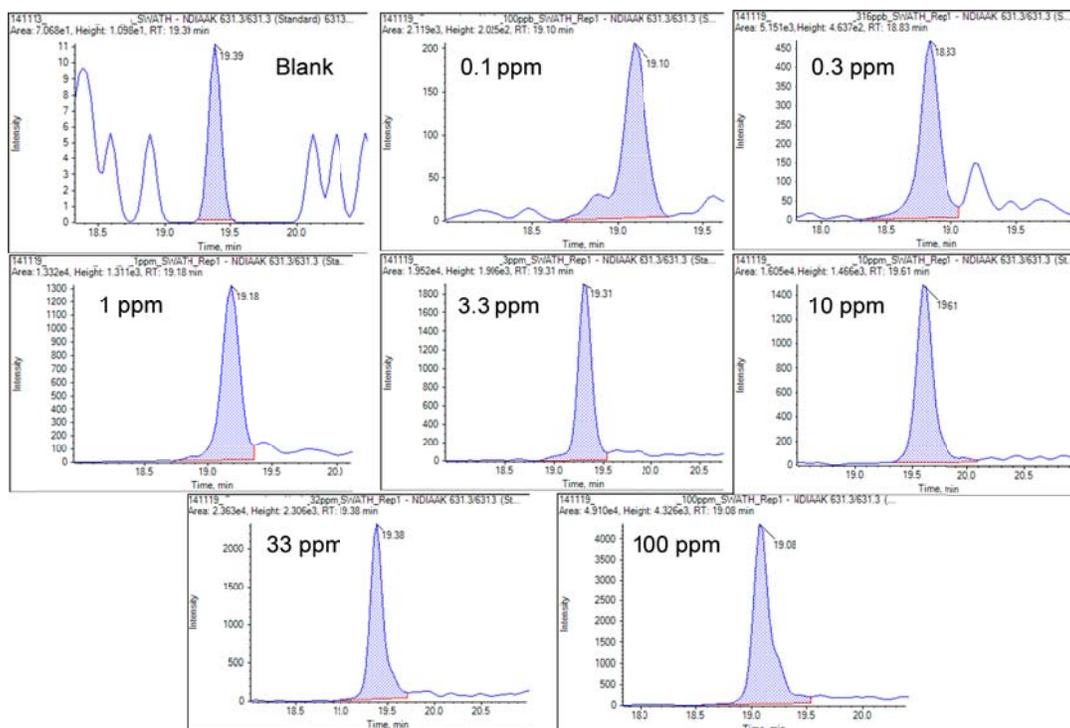
**Figure 5.** (A) Extracted fragment ion electropherograms for myoglobin peptide VEADIAGHGQEVLR y7 and y13 ions. (B) Matching SWATH MS spectra (blue) to previously identified myoglobin peptide fragment ions (pink). (C) Fragment ion peak area plot based on HCP concentration from triplicate runs.

evaluated was generated from the replicate CESI-MS IDA runs from the HCP/mAb preparation as represented in Figure 1. Both variable window configurations are shown in Figure 3. Triplicate SWATH acquisition runs were performed on the dilution series of demonstrative host cell proteins. Figure 4 shows representative total ion electropherograms from the replicate quantitation runs.

Candidate peptides and fragment ions can be selected for quantitation using SWATH Processing in PeakView. Figure 5 shows representative SWATH data selected for quantitation of HCPs. Two extracted fragment ion electropherograms for myoglobin peptide VEADIAGHGQEVLR (Figure 5A) have the same migration profile indicating they are from the same peptide. These two ions were matched to those found in the peptide library from the IDA run (Figure 5B). Additionally, when the fragment ions are considered between triplicate runs of the calibration curve, the appropriate quantitative trend is observed (Figure 5C). Collectively these three pieces of data illustrate the capability of quantifying HCP surrogate peptides based on a few fragment ions.



**Figure 6.** Calibration curves from triplicate CESI-SWATH MS runs of myoglobin peptides (A) VEADIAGHGQEVLR b2 ion and (B) HGTVVLTALGGILK y7 ion.



**Figure 7.** Extracted fragment ion electropherograms for myoglobin peptide NDIAAK in the concentration range from 0.1 ppm to 100 ppm using MultiQuant™.

Due to the very low concentrations of host cell proteins within therapeutic mAb preparations, the sensitivity of mass spectrometric methods are generally pushed to the limit, potentially into the low S/N levels. With the reduced ion suppression of CESI run at ultra-low flow rates (~20 nL/min), it's possible to extend the dynamic range and sensitivity of HCP detection. Figures 6 illustrates the common, expected sensitivities and dynamic ranges for the peptides identified using SWATH acquisition, previously shown with LC-MS methods.<sup>1</sup> That is, from the candidate peptide ion surrogates for HCPs identified using SWATH Processing from CESI-MS runs, two unique myoglobin peptides could be quantified down to low ppm levels (Figure 6). However, there were examples of peptides with even greater sensitivity and dynamic range in our analysis. To specifically show the S/N ratios for one of these quantified peptides we generated extracted fragment ion electropherograms for myoglobin peptide NDIAAK shown in Figure 7. Even at 0.1 ppm peptide NDIAAK has a S/N ratio of ~20, facilitating detection of this representative HCP at the sub-ppm level. Notably, the blank sample run had an intensity of ~10 at the noise level, while the 0.1 ppm sample had an intensity of ~200.

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## Conclusions

An integrated CESI-MS workflow is presented that facilitates the ultra-sensitive detection of representative host cell proteins in a mAb preparation. The combined use of CESI with SWATH acquisition exploits reduced ion suppression and data-independent analysis to create a powerful tool for host cell protein quantitation. This new application adds to the versatility and efficiency of open tube capillary electrophoresis for biologics characterization and complements the detection of HCPs by our high- and micro-flow LC-MS methods.

## References

1. Justin Blethrow and Eric Johansen, High sensitivity host cell protein quantitation in an IgG1 monoclonal antibody preparation via data-independent acquisition with a TripleTOF® System, SCIEX Technical Note, Publication Number 7460213-01.