

Characterization of Phospho- and Glycopeptides by CESI-MS: A Powerful Technique for Increasing Sensitivity

- *Increased sensitivity across 3 logs of dynamic range with a low LOD*
- *Reduction in ion suppression bias*

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Sensitivity in characterization of complex or biologically significant samples is an essential parameter for an overall understanding of their heterogeneity, function, and molecular identity. Techniques including capillary electrophoresis (CE) and liquid chromatography (LC) either as standalone techniques or hyphenated with mass spectrometry (MS) have been applied in biopharmaceutical and proteomics research. CE has been shown to provide extremely efficient separations but lacked the ability to easily confirm identity without the use of known standards against which to compare. In this regard, CESI-MS, the integration of CE with electrospray ionization (ESI) into a single dynamic process within the same capillary, has been used to reach further into the realm of sensitivity (Busnel *et al.*, 2010). Whereas conventional CE-MS technology introduces samples into the MS using flow rates in the hundreds of nanoliters (nl) per minute range, CESI-MS can perform this task at ultra-low flow rates of <30 nl/min. This ultra-low flow rate enables an overall increase in sensitivity due to the improved presentation of poorly ionizing molecular species like glycoproteins and phosphopeptides, effectively increasing ionization efficiency and minimizing ionization bias (Moini, 2007; Schmidt *et al.*, 2003)). In order to further investigate this technique, the potential benefits of CESI-MS for the analysis of biologically significant glycopeptides and also phosphoproteomics have been studied.

Many biologically relevant molecules like immunoglobulins (IgGs) can be classified as glycoproteins because they consist of a protein with covalently attached oligosaccharides. Glycosylation is a very important characteristic in IgGs as it helps define biological activity, clearance, and physicochemical properties. Because glycosylation occurs fairly heterogeneously, the dynamic range of glycans making up a population of glycoforms associated with a specific IgG can be quite broad, requiring highly sensitive analytical techniques for proper characterization. This requirement, for example, is necessary in cases where an IgG exists at low titer, such as when dealing with antigen-specific species.

Using a transient isotachopheresis (t-ITP) method to enhance assay sensitivity, digested IgG samples were separated and analyzed by CESI-MS. The t-ITP-CZE separation provides a stacking effect prior to separation enabling greater sensitivity in the analysis due to concentration of material. Interestingly, the t-ITP-CZE system provided a separation on the basis of the attached glycan (Fig. 1). In contrast, glycopeptides from the different IgG subclasses were found to co-migrate.

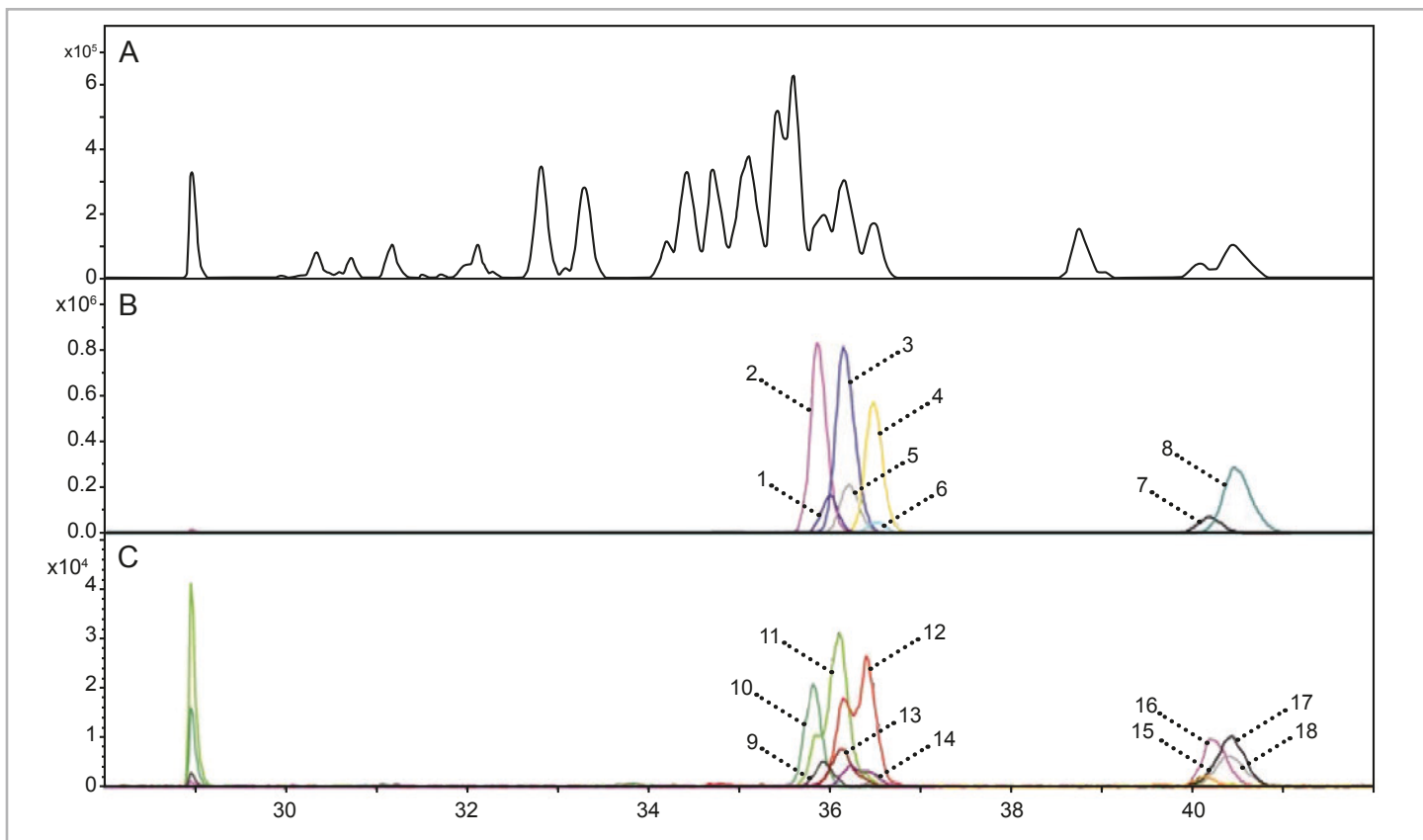
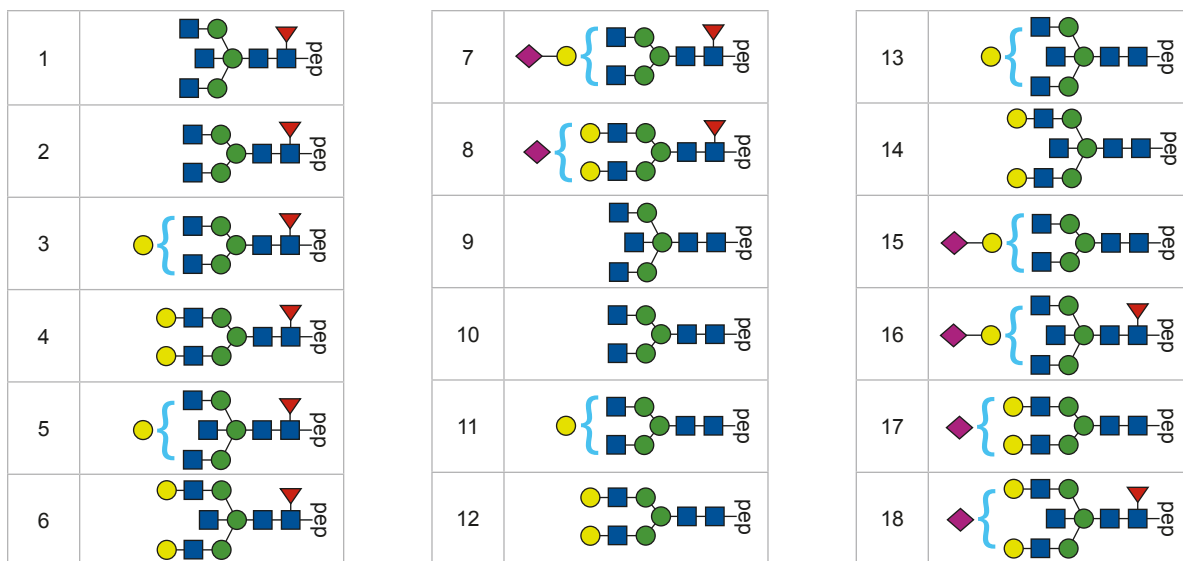


Figure 1. (A) Base peak electropherogram of a tryptic digest of total IgG purified from human serum; (B) extracted ion traces of the high concentration IgG1 glycopeptide species including assignment; (C) extracted ion traces of the low concentration IgG1 glycopeptide species including assignment. Blue square, N-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, N-acetylneuraminic acid.



Applying this technique to a scenario in which high sensitivity is necessary to analyze discrete differences in glycosylation, pathogenic IgG1 alloantibodies produced by pregnant women against platelet antigens of the fetus were analyzed. The IgGs in these samples demonstrate low core fucosylation, a characteristic feature of potential pathogenic importance as it promotes platelet destruction and is known to enhance ADCC (Shields et al, 2002). Trypsin digested samples were

analyzed by CESI-MS and showed variability in core fucosylation and galactosylation (Fig 2). These findings were consistent with previously performed nano-RPLC-MS studies which found heterogeneity in expression of core fucosylation and galactosylation (Wuhrer et al, 2008). Remarkably, CESI-MS was able to perform these analyses across 3 logs of dynamic range with a lower LOD even following a 20-fold sample dilution.

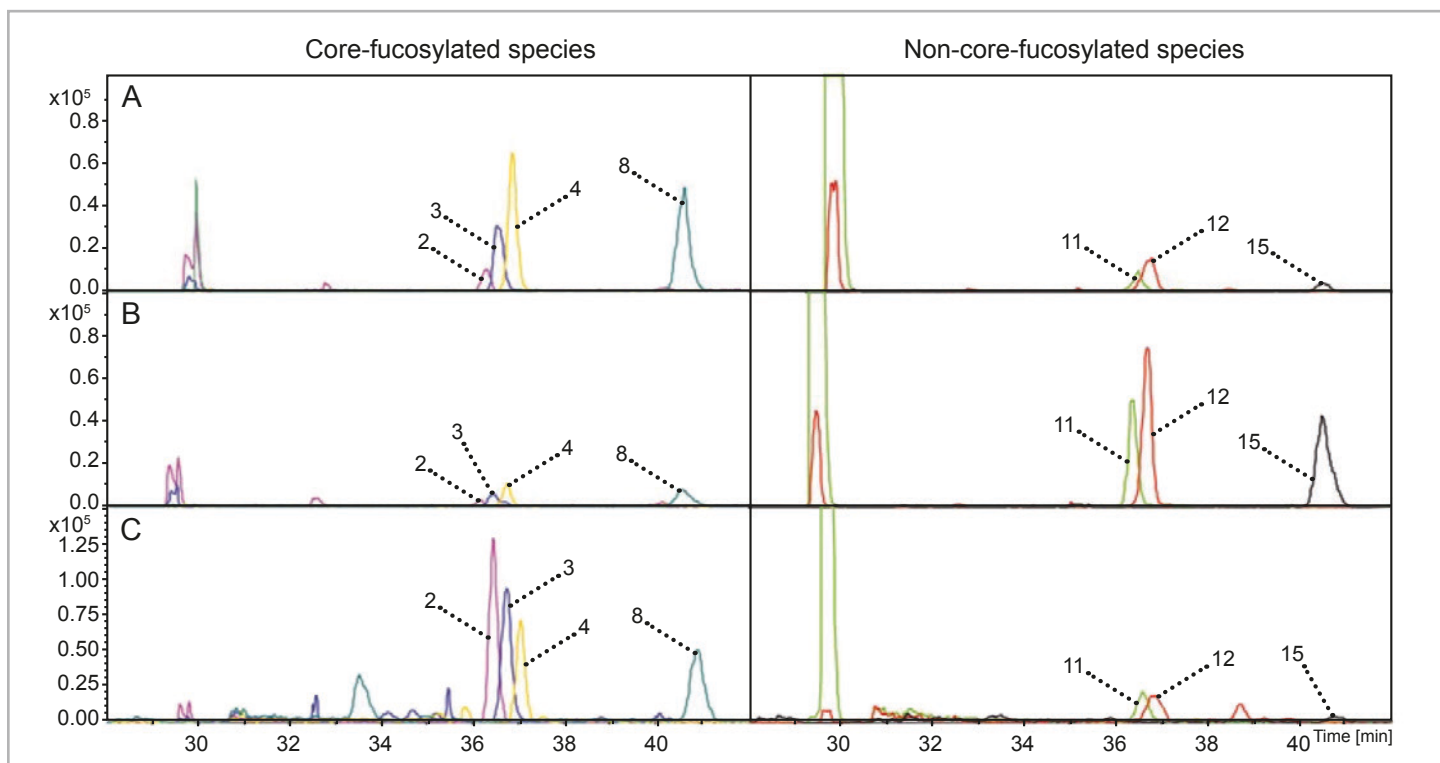


Figure 2. IgG Fc glycosylation profiles of HPA1a alloantibodies purified from sera of three pregnant women. Extracted ion traces are given of the major core-fucosylated (left) and non-core-fucosylated (right) glycopeptide species. (A) The first HPA1a glycosylation profile is high in fucosylation, galactosylation, and sialylation, (B) the second shows low fucosylation with high galactosylation and sialylation, and (C) the third shows low galactosylation and sialylation with high fucosylation. Blue square, N-acetylglucosamine; red triangle, fucose; green circle, mannose; yellow circle, galactose; purple diamond, N-acetylneuraminic acid.

Protein phosphorylation has long been associated with cellular function and cell signaling networks. Because phosphorylation is generally transient in nature, phosphorylated proteins are rare relative to non-phosphorylated proteins, making analysis difficult when interrogating heterogeneous protein populations. Study of phosphorylated protein variants by MS can be difficult due to co-elution or co-migration through a column or gradient which can lead to ion suppression in ESI-MS analysis. Additionally, the degree of phosphorylation also impacts overall ionization efficiency which can be adversely affected by the increasing negative charge due to the addition of multiple phosphate groups. Therefore it is assumed that proteins/peptides that are more heavily phosphorylated are poorly ionizing relative to their non-phosphorylated counterparts.

In order to address questions around differential ionization of phosphopeptides, Heemskerk *et al.* (2013) applied ultra low-flow CESI-MS to minimize bias. The objective of the work was to generate a high resolution separation to better discriminate phosphorylated from non-phosphorylated species in an ultra-low flow environment. Initial research demonstrated that CESI-

MS infusion of a mixture of five synthetic peptides containing an increasing number of phosphate groups – 0, 1, 2, 3, or 4 resulted in more equimolar detection at lower flow rates. In order to define the role of flow rate in MS ionization bias of these phosphopeptides, the rate of hydrodynamic infusion was varied from 4.5 to 500nL/min. This range was chosen keeping mind that flow rates below 20nL/min have been shown to decrease ionization bias in relative peak intensities for some peptides resulting in a trend toward an equimolar response, a phenomenon that would be valuable in phosphoproteome analysis (Schmidt *et al.*). The synthetic phosphopeptides used in this study contained 0 to 4 tyrosine residues to accommodate phosphorylation and were designed with C-terminal arginine residues to emulate tryptic fragments commonly analyzed in peptide mapping experiments. CESI-MS achieved significantly improved sensitivity for detection of the synthetic phosphopeptide mixture in conditions where equal sample load and concentration were used. Importantly, a significant reduction in ionization bias between the 5 synthetic peptides was seen when decreasing flow rate from >100nl/min to ~10nl/min.

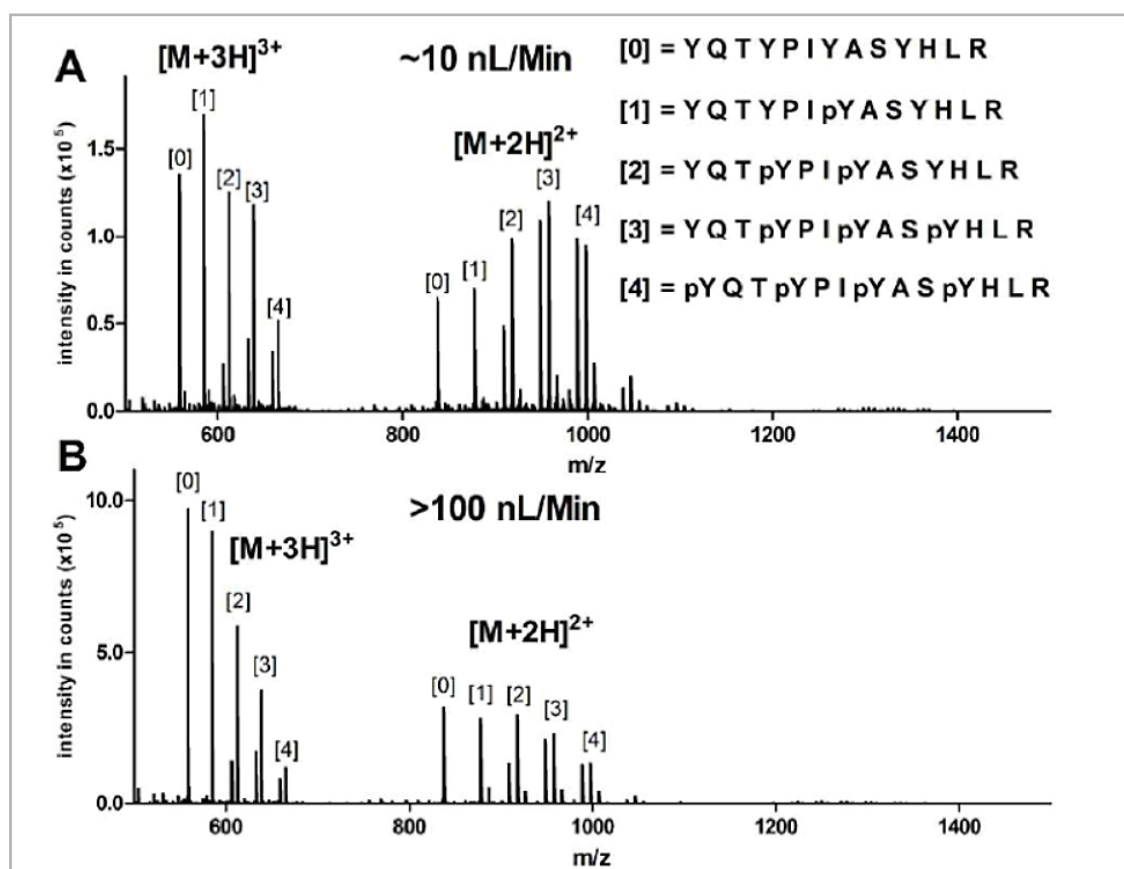


Figure 1. Typical mass spectra of the model phosphopeptide mix infusion at flows below 10 nL/min and above 100 nL/min.

Further experimentation was performed to examine this phenomenon using a more complex sample. To accommodate this, a bovine milk digest was analyzed using transient isotachopheresis (t-ITP). Bovine milk is a complex biological sample with a large proportion of the protein content corresponding to α S1, α S2, β and κ caseins, on which there exist abundant phosphorylations. t-ITP-ESI-MS allowed for 250 nl of sample (~37% of the capillary) to be injected, resulting in protein stacking and therefore higher concentration for introduction into the MS. As higher concentration protein samples have a tendency to precipitate or interact with the capillary surface, CESI-MS was performed using a separation capillary with a chemically neutral surface. While the neutral surface helps minimize protein-wall interactions, it also serves to virtually eliminate electroosmotic flow (EOF), helping extend the separation window and therefore maximize the resolving capability. Loading 5ng of the bovine milk digest, CESI-MS was capable of identifying 13 phosphopeptides, including 2 multi-phosphorylated peptides, in comparison to only 5 phosphorylated peptides identified using nano-LC-MS (Heemskerk et al, 2012). In fact, even a 10x increase in sample load in LC-MS could not equal the sensitivity of CESI-MS.

These data serve to illustrate the powerful capability of CESI-MS technology to separate complex phosphopeptide species from one another.

Taken together, the data presented in this Technical Note using the CESI 8000 High Sensitivity ESI Module describes the capability of an ultra-low flow environment to attain significant increases in sensitivity while decreasing ionization bias among analytes. As specifically highlighted, both phosphopeptides and glycoproteins, which generally are challenging to analyze using mass spectrometry, can be efficiently ionized and detected with minimal bias in CESI-MS. It should also be noted that no sample pre-concentration was performed to achieve this sensitivity increase and ionization improvement, thus simplifying the overall workflow from sample to data.

Analysis characteristics	Number of phosphopeptides	1P	2P	Unique phosphopeptides
5 ng milk digest tITP-MS	12	10	2	5
5 ng milk digest nano-LC-MS	5	5	0	1
50 ng milk digest nano-LC-MS	9	9	0	3

Table 1. A comparison of the detected phosphopeptides from a bovine milk digest.
(Table adapted from Heemskerk et al, 2012)

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