Native ion exchange chromatography directly coupled to Orbitrap mass spectrometry allows surface charge discrimination and online detection of intact proteins

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ABSTRACT

Purpose: To demonstrate an integrated platform for direct-coupling of native ion exchange separations with Orbitrap mass spectrometry.

Methods: Weak cation exchange performed in native conditions, coupled directly to the mass spectrometer, for charge variant analysis of the commercially-available therapeutic monoclonal antibody, Trastuzumab.

Results: Unmodified Trastuzumab was found to be separated from a singly-deamidated form by several minutes. Separation of near-isobaric variants improves observed mass accuracies. WCX-MS method compatible with ReSpect deconvolution and Sliding Window analysis allowed automatic detection of unmodified and deamidated versions of the top 3 glycoforms of Trastuzumab.

INTRODUCTION

Therapeutic proteins are microheterogeneous mixtures in which several unique, yet near-isobaric molecular compositions may be present simultaneously. Conventional denaturing intact protein MS utilizes aqueous/organic mixtures at low pH for sensitive ESI; however, complex protein spectra may be obscured by m/z interferences of overlapping successive charge states arising from denatured protein masses. “Native” MS, utilizing aqueous buffers within physiological pH ranges, dramatically improves complex intact protein ESI spectra by reducing charge state values to simplify spectra. Chromatographic separation is largely incompatible with native MS due to common denaturants and/or buffer salts which suppress protein ionization. In this report we describe a powerful analytical platform for characterizing intact protein mixtures using native ion exchange LC coupled directly to Orbitrap MS. Separation of near-isobaric variants allows improved mass accuracy of proteins.

MATERIALS AND METHODS

Sample Preparation

Trastuzumab sample was not subjected to pre-treatment for intact mass analysis. For peptide mapping, Trastuzumab sample was reduced and alkylated, and then digested using trypsin.

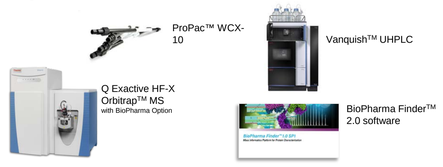
LC-MS Methods

Liquid chromatography for intact protein analysis was performed using a Thermo Scientific™ Vanquish™ Horizon UHPLC system coupled to a Thermo Scientific™ ProPac™ WCX-10 (weak cation exchange) column (4 x 250 mm) (**Figure 1**). Buffer ‘A’ was comprised of 50 mM ammonium acetate, approximately pH = ~ 7; Buffer ‘B’ was comprised of 50 mM ammonium acetate, adjusted to approximately pH = ~10. For Trastuzumab charge variant MS analysis, 1 uL of stock concentration sample (21 mg/mL) was injected directly onto the column and protein desalting was performed on-line. The column eluent was connected directly to the ionization source of the mass spectrometer. Mass spectrometry analysis was performed using a Thermo Scientific™ Q Exactive™ HF-X Orbitrap™ mass spectrometer operated in High Mass Range mode for intact protein analysis. A Thermo Scientific™ Orbitrap Elite™ mass spectrometer was used for peptide mapping analysis. Data dependent acquisition (DDA) employing the top 15 method was used for CID MS/MS.

Data Analysis

Intact protein data were analyzed using a combination of Sliding Window and ReSpect™ algorithms in Thermo Scientific™ BioPharma Finder™ 2.0 software. Peptide mapping data were analyzed using BioPharma Finder software. Peptide LC-MS spectra were data searched against the Trastuzumab amino acid sequence, allowing for deamidation (N, D) as a variable modification.

Figure 1. Platform for on-line native WCX-MS method. Essential instrumentation for our platform includes the ProPac WCX-10 weak cation exchange column, the Vanquish Horizon UHPLC, the Q Exactive HF-X Orbitrap mass spectrometer, and BioPharma Finder data analysis software.



RESULTS

Ion Exchange Offers Unique and Powerful Separations Mode for Proteins

In the vast majority of cases, LC-MS intact protein analysis is performed using reversed phase liquid chromatography (RP-LC) as the means of chromatographic separation. RP-LC allows proteins to be concentrated on-column, de-salted on-line, and then separated on the basis of hydrophobicity. Although RP-LC is suitable for separating many protein mixtures, this method often struggles to separate protein variants, which may have essentially equivalent hydrophobic properties with subtle differences in chemical compositions, which can include protein charge and size.

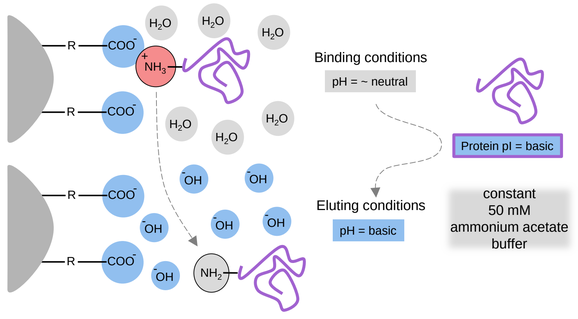
Reversed phase chromatography is an inherently denaturing form of separation due to the presence of organic solvent, which is often adjusted to very low pH (< 3). Denaturing conditions produce mass spectra which are observed as broad distributions of highly charged species at relatively lower m/z ranges. Denaturing mass spectra allow sensitive protein identification; however, the close spacing of successive charge states in denaturing conditions can pose issues when attempting to resolve highly complex mixtures or near-isobaric species, such as antibody drug conjugate samples **(Reference 1**).

Recently, size exclusion chromatography (SEC) in native conditions has been directly coupled to mass spectrometry. This method produces native mass spectra similar to static nanospray or infusion methods, where relatively lower charge states are observed as more narrow distributions at relatively higher m/z. Although native SEC-MS offers on-line desalting and an automatable route to producing native mass spectra, SEC generally offers low resolution separation and does not allow on-column sample concentration.

Ion exchange is a well understood and powerful mode of protein separation. This form of chromatography is routinely used in the BioPharma industry to monitor for variant forms of therapeutic monoclonal antibodies, and in this context is referred to as ‘charge variant analysis’. Charge variant analysis with traditional UV detection allows an intact biotherapeutic protein sample to be characterized in terms of charge variant content, in which the presence of ‘acidic’ or ‘basic’ variants can be determined relative to a ‘main’ peak.

Traditional ion exchange buffers include sodium, potassium, and phosphate salts, which cause severe ion suppression for electrospray mass spectrometry, and thus are considered to be LC-MS incompatible. Ion exchange resins can be either strong or weak, and elution can proceed by a gradient of salt, pH, or a hybrid of both salt and pH.

Figure 2. Weak cation exchange (WCX) performed in native mass spectrometry friendly conditions. In neutral conditions cationic (basic) proteins have a net positive charge while terminal carboxylic groups have a net negative charge, which allows efficient binding as proteins are loaded on to the column. As pH is increased, cationic protein variants become net neutral according to their isoelectric point and elute from the column.



Elution by pH gradient in native WCX-MS method

We introduce a native ion exchange chromatography mass spectrometry method, which utilizes pH elution (**Figure 2**). Native conditions in the mobile phases was achieved by buffering with volatile salt (50 mM ammonium acetate) and maintaining a limited range of pH. These criteria are consistent with other ion exchange methods with optical detection which allow proteins to retain non-covalent structure in order to achieve chromatographic discrimination based on protein surface charge. In this method volatile salt can efficiently substitute for traditional buffer salts to allow cationic proteins to efficiently bind to a solid phase support and then become eluted in conditions of increasing pH.

In initial testing we found that our WCX LC-MS method is able to perform charge variant separation of multiple intact monoclonal antibodies with a range of isoelectric points (**Figure 9**).We focused on the commercially-available therapeutic monoclonal antibody sample, Trastuzumab, and found that a shallow linear gradient was sufficient to separate numerous charge variant species (**Figure 3**).

Figure 3. LC conditions for pH-elution in native WCX-MS method. (A) Trastuzumab sample without pretreatment was injected onto a ProPac WCX-10 column for LC-MS analysis. As the protein sample is loaded buffer salts are not retained on the column and are detected early in the LC-MS run. Thus, protein samples are automatically desalted. A 20 min gradient of 1-8% Buffer B produced several chromatographic peaks corresponding to multiple charge variants of Trastuzumab.

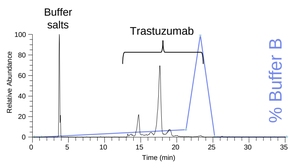
Buffer A
50 mM AmAce, pH 7

Buffer B
50 mM AmAce, pH 10

Column
ProPac WCX-10
4 x 250 mm

Flow rate
0.3 mL/min

Time (min)	% Buffer B
0	1
1	1
21	8
23	100
25	1
35	1

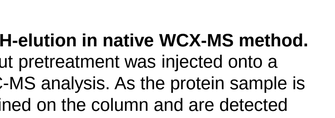


Comparison of native pH-elution WCX-MS method to published salt-elution WCX-UV

The resulting chromatogram from our pH-elution native WCX-MS method was compared to a previously published study which also involved analysis of Trastuzumab using salt-elution on a WCX column with UV detection (**Figure 4**) (**Reference 2**). We found the two datasets to be highly similar. Harris et al. used fractionation and peptide mapping to find that Trastuzumab consists of an expected “main” peak which is altogether unmodified, and an ‘acidic’ early-eluting peak was attributed to a single deamidation on one single light chain, specifically at residue D30 (**Figure 4B**). We sought to reproduce these results by intact mass analysis using our pH-elution native WCX-MS method.

Figure 4. Previously published salt-elution WCX-UV data shows similar chromatography as pH-elution WCX-MS method. (A) Trastuzumab sample was analyzed by WCX-UV and fractionated for peptide mapping, which identified a main peak and an acidic peak corresponding to a single deamidation at residue D30. We have found similar main (*) and acidic (^) peaks in our WCX-MS analysis. (B) The amide side chain of asparagine can become converted to aspartic acid via a succinimide intermediate, resulting in a very small change in mass (0.98 Da) and a modest decrease in protein isoelectric point, becoming more acidic.

A



Harris et al., 2001
WCX-UV
0 - 200 mM NaCl gradient
in 20 mM $NaPO_4$

Q Exactive HF-X BioPharma
WCX-MS
pH 7-10 gradient (1-8%)
in 50 mM $NH_4CH_3CO_2$

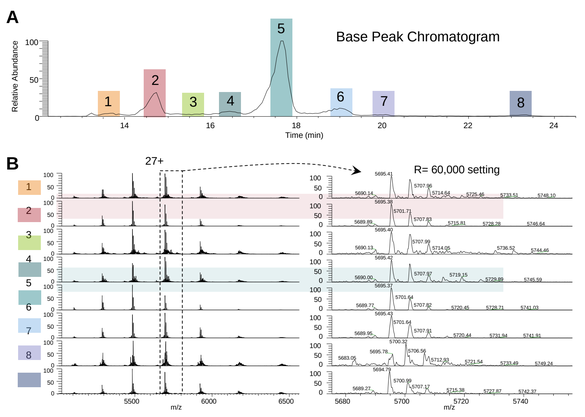
B

Buffered volatile salt conditions allow ‘native’ mass spectra

By using native conditions we aim to preserve tertiary structure and thus separate proteins based on surface charge. Preservation of tertiary structure also allows proteins to yield native mass spectra, which simplifies and improves spectral quality. Native ion exchange WCX-MS produces protein spectra at higher m/z ranges which increases inter-charge state separation and significantly minimizes m/z interferences compared to reverse phase LC-MS. By coupling native pH-elution WCX separation directly to a Q Exactive HF-X Orbitrap mass spectrometer we were able to combine high resolution native mass spectra with high resolution ion exchange chromatography. Intact masses can easily be ascribed to each of the chromatographically-separated charge variants (**Figure 5A**).

A major benefit of combining these two technologies is ability to separate near isobaric variants which would normally co-elute by reversed phase or size exclusion. By removing “impurities” we were able to generate mass spectra of purified variants, which allowed us to achieve very high mass accuracies for intact mass analysis. An example of this is the nearly identical masses observed for the deamidated (Peak 2) and unmodified (Peak 5) Trastuzumab species (**Figure 5B**).

Figure 5. Native mass spectra are produced using WCX-MS method. (A) Base Peak Chromatogram for WCX-MS analysis of Trastuzumab. We have identified 8 chromatographic peaks which correspond to unique charge variants. **(B)** Averaged spectra corresponding to unique charge variants of Trastuzumab. Spectra are highlighted which correspond to the two most abundant chromatographic peaks, Peak 2 and Peak 5.



Sliding Window Deconvolution can distinguish deamidated vs. unmodified Trastuzumab

We analyzed our WCX-MS intact protein spectra using ReSpect deconvolution combined with Sliding Window analysis in BioPharma Finder 2.0. To allow the software to automatically recognize deamidated from unmodified forms we used very low mass tolerances (4 ppm) for ReSpect, Sliding Window, and Sequence Matching. We automatically identified many species with less than 3 ppm mass accuracy, including the 3 most abundant glycoforms in both unmodified and deamidated forms. This mass accuracy is a benefit of ion exchange separation, Without chromatographic separation these near-isobaric species **(Δ mass = 0.98 Da, ~6.5 ppm)** would yield tremendous overlap in mass spectra, and this mass interference would result in an increased error in observed mass.

Figure 6. BioPharma Finder 2.0 settings for ReSpect deconvolution and Sliding Window analysis.

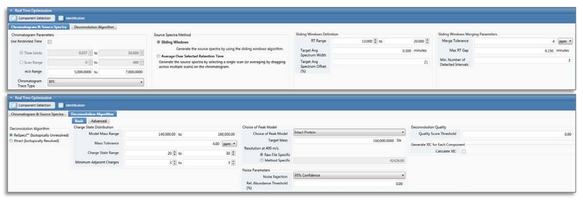


Figure 7. Sliding Window ReSpect deconvolution results of WCX-MS analysis of Trastuzumab. (A) Deconvolved spectrum of species identified in WCX-MS analysis of Trastuzumab. **(B)** Sliding Window extracted chromatograms of deconvolved components, showing top 3 glycoforms in both deamidated and unmodified forms.

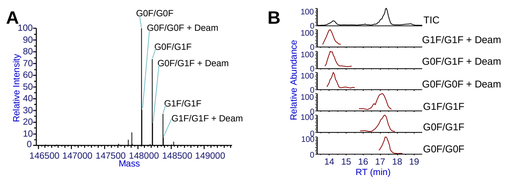


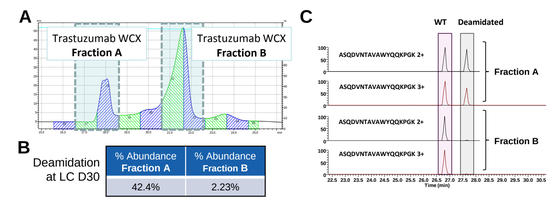
Table 1. Results for ReSpect deconvolution using Sliding Window algorithm

Protein Name	Modification	Average Mass	Theoretical Mass (Da)	Matched Mass Error (ppm)	RT Range
Herceptin intact	2xA2G1F,1xDeamidation	148381.53	148381.80	1.8	13.521 - 14.782
Herceptin intact	1xA2G0F,1xA2G1F,1xDeamidation	148219.47	148219.66	1.3	13.648 - 15.412
Herceptin intact	2xA2G0F,1xDeamidation	148057.69	148057.52	1.2	13.774 - 15.602
Herceptin intact	2xA2G1F	148380.81	148380.82	0.1	15.665 - 17.790
Herceptin intact	1xA2G0F,1xA2G1F	148219.00	148218.68	2.2	15.728 - 17.979
Herceptin intact	2xA2G0F	148056.95	148056.54	2.8	16.800 - 18.421

Fractionation and peptide mapping confirm identity of native WCX-MS intact mass analysis

We repeated our native pH-elution WCX chromatography method to collect fractions of the purported deamidated (acidic) and unmodified (main) peaks for peptide mapping analysis (**Figure 8A**). Despite collecting rather broad fractions, our BioPharma Finder peptide mapping results showed that in Fraction A nearly half of light chain D30 was deamidated, compared to ~2% D30 deamidation in Fraction B (**Figure 8B**). These results were also confirmed manually (**Figure 8C**).

Figure 8. native WCX fractionation and peptide mapping. (A) Deamidated (acidic) and unmodified (main) peaks were collected, trypsin digested, and analyzed by LC-MS/MS. **(B)** Results for BioPharma Finder peptide mapping analysis. **(C)** Manual verification of peptide mapping results.

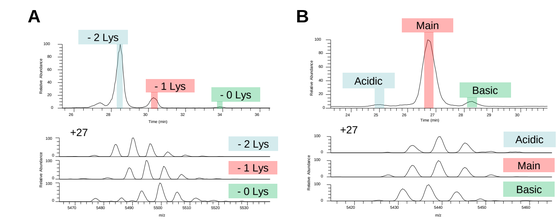


Native WCX-MS analysis useful for other cationic proteins

We tested 2 additional intact IgG1 monoclonal antibody (mAb) samples. We found this method to be sufficient for separating lysine variants as well as basic and acidic forms from these commercially available antibody standards. This result demonstrates that this method is universally applicable for performing charge variant analysis on-line with Orbitrap mass spectrometry (**Figure 9**). It should also be possible to adapt this method to intact mass analysis of other classes of cationic proteins.

This new form of LC-MS has powerful implications for all fields of intact protein mass spectrometry including biopharmaceutical characterization, proteomics, and structural biology.

Figure 9. Native WCX-MS analysis of commercial antibody standards. (A) WCX-MS analysis of NIST monoclonal antibody standard shows separation of known C-terminal lysine variants yields clean mass spectra. (B) WCX-MS analysis of Sigma™ SILu™-Lite mAb shows contributions of both acidic and basic variants which can affect mass accuracy without chromatographic separation.



CONCLUSIONS

We report significant performance advantages of our native ion exchange LC-MS method, which can completely resolve large proteins of virtually the same molecular weight even within a narrow isoelectric range. This includes baseline separation of C-terminal lysine variants as well as acidic and basic variants from the main, or unmodified form. Native WCX-MS has powerful implications for all fields of intact protein mass spectrometry including biopharmaceutical characterization, proteomics, and structural biology.

- Native WCX-MS is a new form of native protein separation (weak ion exchange) coupled to Orbitrap mass spectrometry to allow direct mass measurements of intact proteins
- WCX allows on-line sample concentration and de-salting
- Unmodified Trastuzumab was found to be separated from a singly-deamidated form by several minutes
- Separation of near-isobaric variants can offer tremendous improvements in observed mass accuracies
- ReSpect deconvolution combined with Sliding Window analysis allowed automatic detection of unmodified and deamidated versions of the top 3 glycoforms of Trastuzumab

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TRADEMARKS/LICENSING