Accurate Quantitation of Deamidated Peptides to Accelerate Formulation Process Development in Therapeutic Proteins

Michael Peddicord and Difei Qiu, Bristol-Myers Squibb, New Brunswick, NJ
Ming Gu and Yongdong Wang, Cerno Bioscience, Norwalk, CT

Overview

- LC-MS analyses (80 min and 12 min total run times) were performed on a tryptic digest of a monoclonal antibody (mAb).
- The digestion was performed with elevated temperature and pH in order to induce increased levels of deamidation.
- Through peak shape calibration and spectral accuracy calculation, relative quantitation of 9 deamidated peptides was performed.
- The accuracy of quantitation by spectral accuracy was compared with that obtained from classic peak area integration using accurate mass extracted ion chromatograms (XIC's).

Introduction

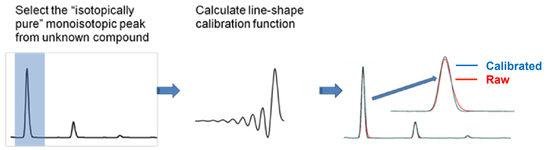
During the development of proteins and monoclonal antibodies as therapeutics, it is critical to have an understanding of the degree of protein degradation during formulation and storage of drug product. One key degradation pathway commonly monitored in evaluating protein stability is the deamidation of asparagine residues. LC-MS tryptic peptide mapping has been routinely used to monitor the formation of deamidated degradation products, and typically employs extended chromatographic methods (1.5 hours or longer) to attempt to achieve baseline separation of all the native peptides from their deamidated analogues. Even with shallow gradients the baseline separation of all deamidated peptides and their native analogs is difficult. To ensure an accurate relative quantitation using XIC's, a baseline separation is required due to the overlap of the native peptide's ^{13}C isotope with the monoisotopic peak of the deamidated product. Partially separated deamidated peptides cannot be quantitated accurately and those which co-elute with the native may not be detected. Alternative rapid quantitation of the deamidated peptides based on a spectral accuracy calculation of overlapped mass spectral signals from both deamidated and native peptides was demonstrated previously (ASMS poster 2013), but its quantitative results had not yet been systematically studied or reported. In this presentation, quantitation results by classic LC peak integration and spectral accuracy calculations will be compared. In addition, true peak purity of base-line resolved peaks by both LC separation and high mass accuracy XIC will be carefully examined.

Methods

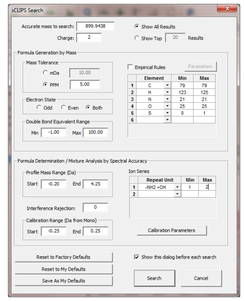
- **Samples:** A mAb sample was denatured (8M Guanidine), reduced (DTT), alkylated (Iodoacetate), and digested with trypsin (Promega). The samples were incubated at 60°C and pH 8.5 for 4 hours to induce elevated levels of deamidation.
- **HPLC:** The sample was chromatographed using a Waters Acquity and a Waters BEH C18 column (2.1 mm x 100 mm; 1.7 μm particles). The column was held at 45 °C. Mobile phase A was water with 0.1% TFA and mobile phase B was acetonitrile with 0.085% TFA. Flow rate was 0.4 mL/min. Methods of 80 minutes and 12 minutes were used.
- **MS Data Acquisition:** LC-MS data were acquired in profile mode with a mass range from *m/z* 200 to 2000 and resolving power of 35,000 (FWHM) on a Thermo Q-Exactive Orbitrap mass spectrometer.
- **MassWorks data processing:** MS spectra were exported from Xcalibur to MassWorks to perform peak shape calibration through sCLIPS (self Calibrated Lineshape Isotope Profile Search) based on the monoisotope peak. Through the peak shape calibration, spectral accuracy can be calculated and utilized to perform exact mixture analysis between calibrated spectra and theoretically calculated spectra for the purpose of quantitation of deamidated peptides.

Results and Discussion

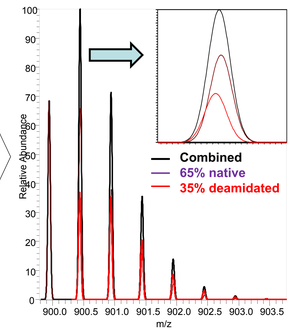
I. Illustration of the Approach



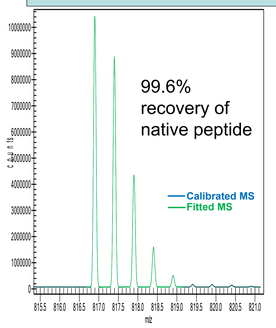
- Monoisotopic peak of the unknown itself is used as "the standard" for peak shape calibration.
- "The standard" is as close in *m/z* and time of measurement to the unknown as possible.
- Peak-shape-only calibration without mass adjustment.



- **Calibrating for high spectral accuracy**
- **Exact mixture deconvolution**
- **Quantitative analysis**

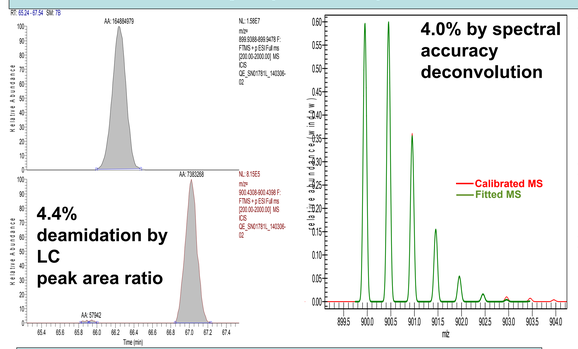


II. Evaluation with A Pure Peptide



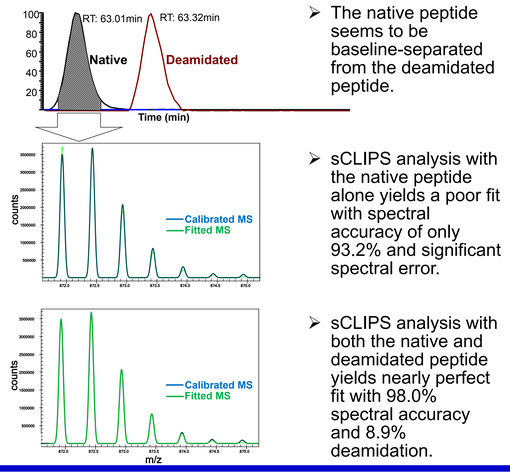
- A spectral accuracy calculation is performed on non-deamidated peptides. An example is shown on the left which has an amino acid sequence of FSGSGSGTDFTLTISR.
- High spectral accuracy of 99.4% achieved.
- Several non-deamidated peptides were recovered with no false positive detection of the deamidated peptides.

III. Quantitation of Fully Separated Peptides with Deamidation



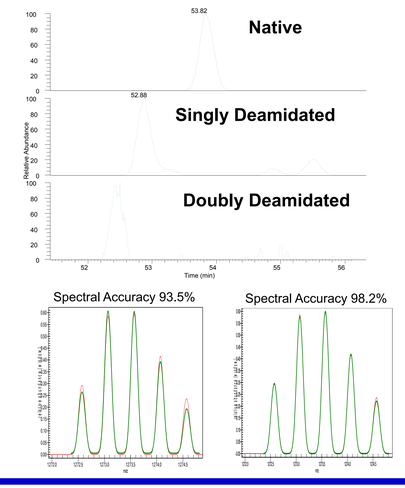
- A spectral accuracy calculation is performed on averaged spectra including both native and deamidated peptides.
- High spectral accuracy of 98.4% achieved.
- Excellent quantitation agreement between XIC peak area ratio and sCLIPS spectral accuracy analysis.

IV. Detection of Co-elution with LC Separation



- The native peptide seems to be baseline-separated from the deamidated peptide.
- sCLIPS analysis with the native peptide alone yields a poor fit with spectral accuracy of only 93.2% and significant spectral error.
- sCLIPS analysis with both the native and deamidated peptide yields nearly perfect fit with 98.0% spectral accuracy and 8.9% deamidation.

V. Identification of Multiply Deamidated Peptides



- Three possible deamidated sites in this peptide: GFYPSDIAVEWESNGQPENNYK
- Original quantitation by both XIC peak area integration/ratio and spectral accuracy calculation considered only the singly deamidated peptide.
- Including only the singly deamidated peptide, calculated a spectral accuracy that was found to be surprisingly low at 93.5%, with significant lack of fit observed. However, high spectral accuracy of 98.2% was achieved when both singly and doubly deamidated peptides are included in the sCLIPS analysis.
- The doubly deamidated peptide was indeed confirmed by high mass accuracy XIC and quantified to be about 10% by spectral accuracy calculation.

VI. Summary of Quantitation Results

Native Peptide Sequences	Deamidated (%)		
	XIC Peak Integration	Spectral Accuracy Quantitation	
	80 min run	80 min run	12 min run
GLEWVTFISYDGNNK	22.2	20.2	21.2
NTLYLQMNSLR	3.2	2.3	2.4
FNWYVDGVEVHNAK	6.8	5.5	7
VVSVLTVLHQDWLNGK	66.3	67.9	66.9
NQVSLTCLVK	8.5	10	8.6
GFYPSDIAVEWESNGQPENNYK	67.4	69.9	72.1
WQQGNVFSCSVMHEALHNHYTQK	12.2	13	N/A*
SGTASVVCLLNNFYPR	3.9	4	4.9
VDNALQSGNSQESVTEQDSK	11.2	11	13

- Some quantitation variations may be caused by different background ions involved in the peak area integration and spectral averaging for the spectral accuracy calculation.
- * No quantitation results due to interference ions.

Conclusions

- The spectral accuracy approach for the quantitation of deamidated peptides provides accurate results compared with those obtained by classic peak area integration using accurate mass XIC's.
- This approach performs a spectral deconvolution of overlapped spectra and enables accurate quantitation of deamidated peptides with minimum LC separation (12-min run) or possibly through infusion without separation at all.
- Base-line resolved peaks even when using accurate mass XIC's (5ppm) does not guarantee that there aren't co-eluted deamidated or native peptides under each seemingly separated peak. The spectral accuracy calculation will serve as a powerful tool to detect and quantify deamidation for co-eluting peptides.

References

(1) Piliang Hao , Jingru Qian , Bamaprasad Dutta , Esther Sok Hwee Cheow , Kae Hwan Sim , Wei Meng , Sunil S. Adav , Andrew Alpert , and Siu Kwan Sze ; Enhanced Separation and Characterization of Deamidated Peptides with RP-ERLIC-Based Multidimensional Chromatography Coupled with Tandem Mass ; *J. Proteome Res.*, 2012, 11 (3), pp 1804–1811.

(2) Ming Gu, Accurate Quantitation of Deamidated Peptides by Mass Spectral Accuracy. Proceedings of the 61st ASMS Conference on Mass Spectrometry and Allied Topics, Minneapolis, MN, 2013.

(3) Yongdong Wang and Ming Gu, The Concept of Spectral Accuracy for MS. *Anal. Chem.* 2010, *82,* 7055–7062