ACQUISITION MODE CHARACTERIZATION FOR THE QUANTITATIVE AND QUALITATIVE ANALYSIS OF CROSS-LINKED PEPTIDES BY TARGETED AND UNTARGETED LC-IM-MS

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INTRODUCTION

Cross-linking mass spectrometry (XLMS) is an emerging structural biology technique. It can be used to gain structural insights into proteins and complexes that cannot easily be studied by high resolution structural techniques. Most studies to date have focused on the identification of the cross-linked peptides using DDA based LC-MS techniques. The primary challenges are low abundance and suppression by the more intense linear, non-crosslinked peptide ion background during analysis. Here, the quantitative aspects of XLMS are investigated and contrasted for various LC-MS acquisition modes. In addition, it will be demonstrated how structural information in terms of collisional cross section and ETD sequence annotation could be obtained and utilized to aid identification.

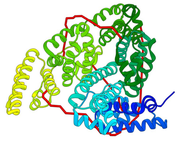

Figure 1. Secondary 3D structure cross-linked bovine serum albumin. Yellow-blue gradient = BSA; red = chemical cross-linker(s).

METHODS

Standards and samples

Cannabinoid metabolite standards were obtained from Sigma-Adrich and spiked in human urine from 5-5000 ppb (on-column amounts). A four-protein tryptic digest mixture (ENO1 (yeast), ADH (yeast), BSA (bovine) and PYGM (rabbit) spiked into *E.coli* digest matrix from 4.5 amol to 4.5 pmol.

Cross-linking

Bovine serum albumin (BSA) was cross-linked and reduced, alkylated, and digested as previously described [1]. Serial dilutions were conducted in a four-protein digest mixture (see above) that served as a surrogate matrix. Pre-crosslinking and digestion BSA spike amount estimates were from 4.5 amol to 450 fmol.

MS conditions

MS:	Synapt G2-S*i* enabled with ion mobility (IM) separation, SONAR scanning quadrupole DIA and ETD functionality
polarity:	ESI (+ve)
method(s):	MS, HDMS, DDA, HDDDA, TofMRM, TofMRM$_{EDC}$, HDMRM, MSE, HDMSE, SONAR, graphically summarized in Figure 2.
ETD reagent:	4-nitrotoluene [2].

LC conditions

Metabolite separations were conducted with an AQUITY I-Class system and a HSS T3 1.8 µm 2.1 x 100 mm column operated at 0.4 mL/min using a 10 min reversed phase gradient. An M-Class system was used for all peptide separations (ordinary protein digest and XL applications) using a BEH C18 1.7 µm 0.3 x 100 mm column with a 30 min reversed phase gradient at 5 µL/min.

Informatics workflows

All quantitative LC-MS data were peak-detected with Skyline-daily, illustrated in Figure 3, and processed with custom python scripts. Qualitative analyses and interpretation of the data were conducted with MassLynx operating software

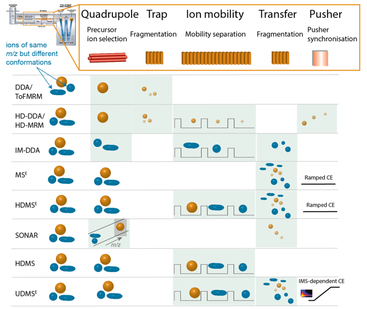

Figure 2. Acquisition methods principles. The shaded areas highlight analyzer regions where additional separation (selectivity/sensitivity) or isolation (selectivity) was achieved. CID can be conducted in both the trap and transfer regions (acquisition mode dependent).

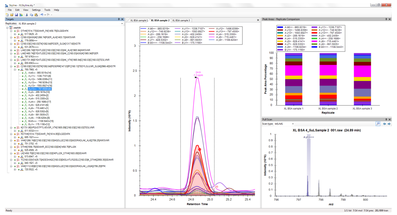

Figure 3. Data driven peak detection and integration (MSE acquisition tryptic digested XL-BSA standard).

RESULTS

Acquisition

The results shown in Figure 4 summarize the spectral quality of the obtained data. MSE, HDMSE and SONAR are DIA variants, whereas TofMRM and HDMRM would classify as PRM techniques. Enhanced Duty Cycle (EDC) promotes sensitivity for a given ion *m/z* by increasing the MS duty cycle. Note that 2D/3D peak detection was not applied, which would provide improved S/N and a reduced number of contaminant product ions for the DIA centric methods.

One of the benefits of the addition of IMS to a schema is shown in Figure 5, illustrating reduced noise at MS1 and MSn levels, as well as more exclusive product ion assignment when fragmentation is applied post IMS separation. In addition, inclusion of IMS and fragmentation pre IMS can increase instrument duty cycle and hence sensitivity. For example, when applied in HDDDA mode, the pusher is synchronized with the velocity of all singly charged product ions and can increase sensitivity up to a factor of 10 [3].

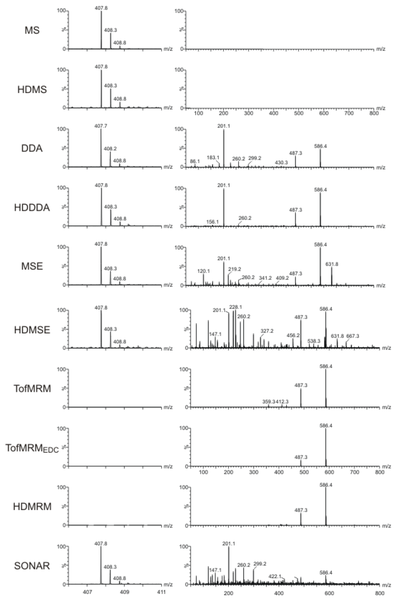

Figure 4. 1D extracted MS1 and MSn spectra for doubly charged DIVGAVLK (ADH) spiked into E.coli matrix, eluting at 16.6 min. MSE data, and HDMSE and SONAR data can be additionally 2D and 3D peak detected/extracted, respectively.

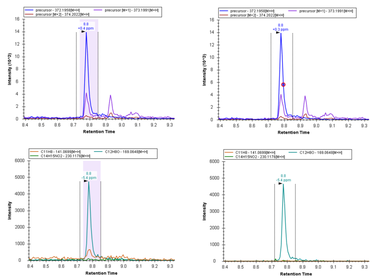

Figure 5. 1D (left) and 2D (right) extracted HDMSE XIC chromatograms of five ppb 3-(4-Methylnaphthalene-1-carbonyl)-1-pentylindole spiked in urine.

Isotopic correction, LLOD and dynamic range

Dynamic range was extended by applying an isotopic correction method for MS1 based acquisition techniques, as shown in Figures 6 and 7. In short, unsaturated isotope detection was used to correct for skews in isotopic distributions, increasing the dynamic range by 35% on average for HDMS acquisitions. In general, the correction effect was most noticeable for the IM enabled methods.

An MS2 based correction based variant [4] was used to correct MS1 signals for methods were both MS1 and MS2 data were available at equal integration times (MSE, HDMSE and SONAR). In the example shown in Figure 7, the correction was found to be peptide dependent and ranged from ~ 5 - 10, with an average variation of 10.2% and increasing dynamic range by at least one order.

A semi-quantitative summary in terms of averaged dynamic range and LLOD using both correction methods for the metabolite and peptides samples is provided in Figure 8.

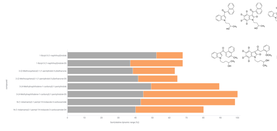

Figure 6. Uncorrected (grey) and blank/background subtracted and isotopic corrected (grey + orange) MS1 based quantitation relative dynamic range for metabolite standards spiked in urine.

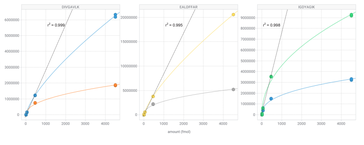

Figure 7. Uncorrected and corrected MS1 IM assisted DIA (HDMSE) quantitation examples for tryptic ADH1 peptides spiked into E.coli digest matrix. The linear calibration curves are from 450 amol - 450 fmol on-column, representing ≥ 3 orders linear dynamic range.

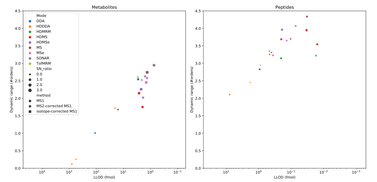

Figure 8. LLOD vs. dynamic range results (average values for all analytes and replicates) summary for metabolite (left) and peptide (right) samples spiked in urine and E.coli matrix, respectively.

Quantitative and qualitative XL-BSA analysis

The acquisition methods with the best dynamic range and LLOD were selected for quantitative analysis of tryptic digested XL-BSA and their MSn performance characteristics summarized in Figure 9.

Additional qualitative XL sequence information was obtained by operating the MS instrument in ETD mode of acquisition and one of the obtained spectra shown in Figure 10, illustrating near complete coverage or both arms by predominant detection of y and z ions .

Figure 9. MSE (grey), HDMSE (blue) and TofMRM (orange) quantitative MSn data analysis results summary XL-BSA peptide analysis. The dashed lines represent the estimated LLOD (average plus three sigma blank (four) samples).

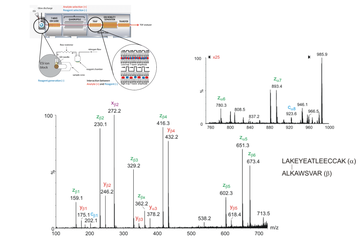

Figure 10. ETD setup, shown inset top left [2], and partial annotation LC-ETD-MS spectrum of [M+5H]$^{5+}$ of LAKEYEATLEEC-CAK_ALKAWSVAR (red = cross-linked residues; blue = CAM).

Stoichiometry/Hi(n) XL-BSA analysis

The amount of the XL-BSA peptides was estimated using a modified Hi(n) approach. Using the three most abundant serial BSA peptides, an average molar amount response factor was estimated [5]. XL and disulfide linked peptides ionize differently compared to linear peptides and was accounted for [6] .

As proof a concept, shown in Figure 11, the molar amount of two XL-BSA peptides was estimated, suggesting about equal stoichiometry and a molar abundance that is at least ten-fold lower that those of serial peptides. Conformation of these findings will be confirmed using standards.

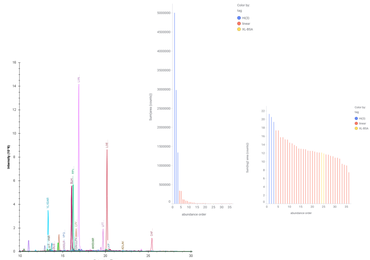

Figure 11. DIA (MSE) MS1 XICs and single charge state abundance distribution BSA peptides (45 fmol/µl spiked into a four-protein digest mixture. Hi(n) calibration peptides are highlighted in blue, linear peptides in red and XL peptides (LAKEYEATLEECCAK_ALKAWSVAR and CCTKPESERMPCTEDYLSLILNR_SLGKVGTR (orange = M(Ox)). Concentration values are pre crosslinking reaction and digestion.

CONCLUSION

- **The quantitative performance of the acquisition methods of an IM enabled quadruple oa-ToF instrument have been characterized for metabolite and peptide samples**
- **PRM and DIA (with and without IM) style methods demonstrated the best overall quantitative performance and were applied for the quantitative analysis of XL peptides to estimate abundance**
- **Additional confirmation of XL peptides was obtained through ETD fragmentation and sequence annotation**
- **A custom label-free Hi(n) quantitation method afforded XL stoichiometry analysis and amount estimation**

References

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