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Exploring the benefits of differential mobility separation and SWATH data independent acquisition for complex proteomic sample analysis



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INTRODUCTION

The past decade has seen several introductions of commercial systems offering a mobility-based separation technique coupled to mass spectrometry. These techniques have introduced the concept of gaining some level of structural information related to the compound, with the ability to measure collision cross section (CCS), thus providing information beyond elemental composition via accurate mass. The advent of these systems has also presented the user with the ability to separate isobaric compounds and therefore supplement the chromatographic separation in several application areas. In proteomic analysis, combined mobility and ToF detection methods such as PASEF, are routinely used to improve sensitivity and selectivity by increasing the effective peak capacity of the LC separation in data independent analysis (DIA) workflows (1-3). These techniques offer the ability to discriminate between ions based on their charge state, prior to mass selection for MS/MS analysis. Another approach commonly used to achieve a similar effect relies on differential mobility separation techniques such as FAIMS and DMS (4-6). In these techniques, discrimination of singly charged species is easily achievable, and depending on the instrument geometry, additional charge state separation can be achieved. In the current work, we investigate the combination of planar differential mobility with SWATH DIA.

MATERIALS AND METHODS

Sample Preparation:
K562 digested samples were obtained from SCIEX and reconstituted according to supplied protocol.

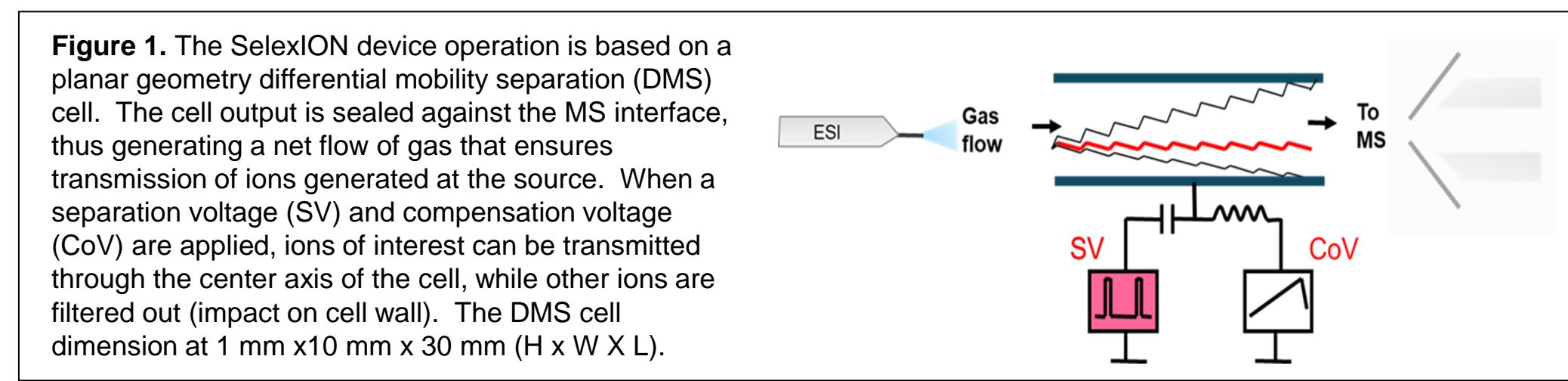
HPLC Conditions:
All separation were performed on an Evosep One system using the 100 and 200 sampled per day (SPD) workflows. Samples were loaded on Evtip using the protocol supplied by the vendor.

MS Conditions:
All analysis were performed a TripleTOF 6600 system equipped with a SelexION device. The OptiFlow Pro ion source was operated with the microflow probe (1-50 µL/min) with ISV and temperature set at 4500V and 200°C, respectively. The acquisition software was Analyst TF 1.8.1.

The differential mobility separation (DMS) is based on a planar geometry (Fig. 1) where a separation voltage (SV) is applied along with a compensation voltage (CoV) to ensure transmission of ions along the center axis. The majority of the data was either collected in transmission mode (SV=CoV=0V) or in separation mode with SV set to 3800V. The resolving gas (DMR) was set to low (10 psi) to provide enhanced separation and an average FWHH of 1.5V to minimize overlap between CoV steps.

SWATH DIA was performed with a fixed window of 25amu with an accumulation time of 20ms, and the precursor range was from 400 to 950. Replicated injections (n=3) were performed at different CoV values ranging from 10.5 to 36V, using increments of 1.5V.

Data Analysis:
All data analysis was performed using the desktop version of DIA-NN 1.8.1-b11 (<https://github.com/vdemichev/DiaNN>) using the same library from previously published work (7). Protein/peptide output list from DIA-NN (*.tsv files) were compared in a pairwise fashion using in-house developed Jupiter notebook generating a venn-diagram representation of the unique identifications made



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RESULTS

Figure 2 is a 2D representation of the peptide ion signal detected from the LC-DMS-MS analysis of a K562 cell digest (average over 10 min) performed at SV=3800. The y-axis represents the CoV range covered (10-38) and the x-axis represents the precursor m/z range. Similar to other mobility-based separation techniques, the singly charged ion signal can easily be separated from ion signal that is originating from multiply charged species. From this figure, we can observe that species of lower m/z value (400-600) are observed over a wider range of CoV values when compared to those of higher m/z value (700-1000). This suggests that one could determine an optimum combination of CoV SWATH windows that would provide selective coverage of the peptide domain and maximize duty cycle to support fast LC analysis, as depicted in Figure 2 (right side).

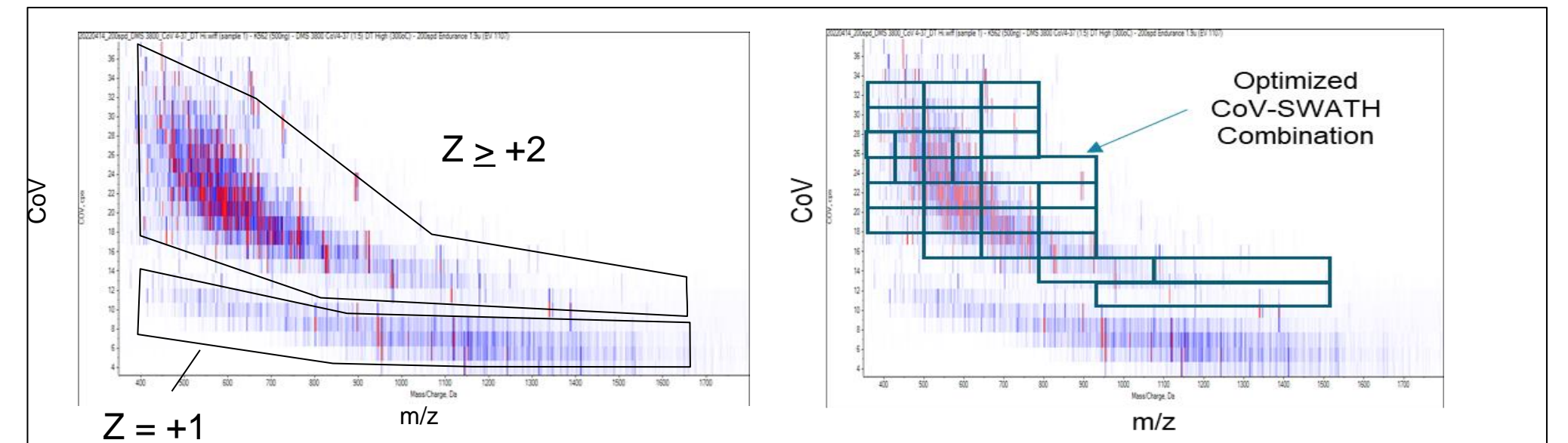


Figure 2. The LC-DMS-MS of K562 digest with SV 3800 and CoV ranging from 2 to 40V. This is a 2D representation by averaging 10 min of data to demonstrate the distribution of precursors versus CoV. Region for various charge states are highlighted (left panel) and potential CoV SWATH windows are shown (right panel)

In order to determine CoV-Precursor window combinations for optimum DMS SWATH DIA, we first performed replicate (n=3) injections at fixed CoV value with precursor windows of 25 amu across the entire mass range. Chromatographic separation of K562 digest was performed with the Evosep system using the 100 SPD workflow. The analysis was performed from CoV 10.5 to 36V, using a step of 1.5V (Figure 3). Including the analysis with DMS deactivated (transmission mode), a total of 57 injections was made to provide us with a complete coverage of the CoV space over the entire precursor range. DIA-NN was used to generate lists of proteins/peptides detected at each CoV setting and these lists were subjected to a pair-wise comparison to find similarity between them. Table 1 shows that at the peptide levels, common peptides are observed only at adjacent CoV values (within FWHH). When CoV values are far apart, very few common peptides are observed (single digit number). Table 2 shows a similar comparison at the protein level. In this case, a significant number of common proteins (10-20% range) can be found even when CoV values are far apart. This suggests that covering a wide range of CoV values could still offer some benefit in terms of protein identification.

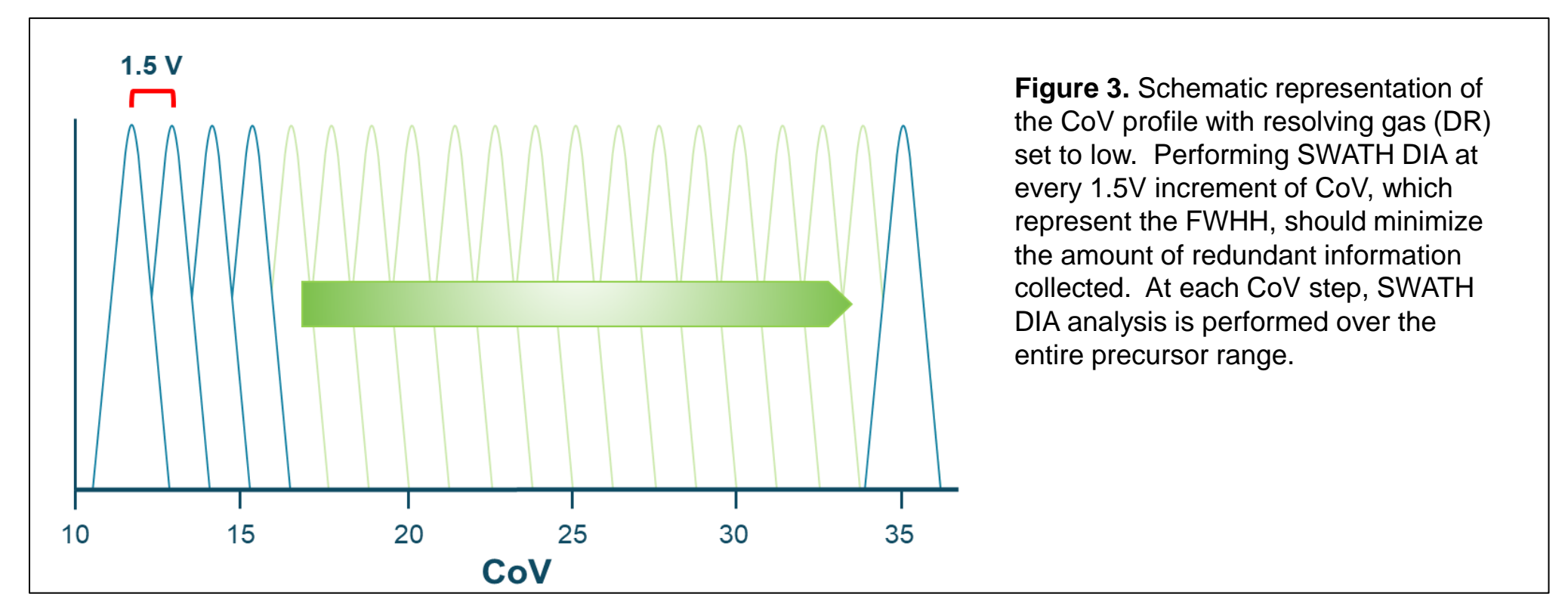


Figure 3. Schematic representation of the CoV profile with resolving gas (DR) set to low. Performing SWATH DIA at every 1.5V increment of CoV, which represent the FWHH, should minimize the amount of redundant information collected. At each CoV step, SWATH DIA analysis is performed over the entire precursor range.

Peptide Level	# of Common IDs versus DMS conditions										
CoV	Total Pept	DMS OFF	CoV 12	CoV 15	CoV 18	CoV 21	CoV 24	CoV 27	CoV 30	CoV 33	CoV 36
10	332	332	0	0	0	0	0	0	0	0	0
15	180	85	95	0	0	0	0	0	0	0	0
20	526	188	338	148	0	0	0	0	0	0	0
25	110	55	55	55	0	0	0	0	0	0	0
30	237	127	110	127	0	0	0	0	0	0	0
35	147	47	47	47	0	0	0	0	0	0	0
38	237	237	0	0	0	0	0	0	0	0	0

Table 1. Number of common peptides at different CoV values with the DMS operated at SV=3800 or set to transmission mode (DMS OFF). K562 digest acquired on Evosep at 100 spd.

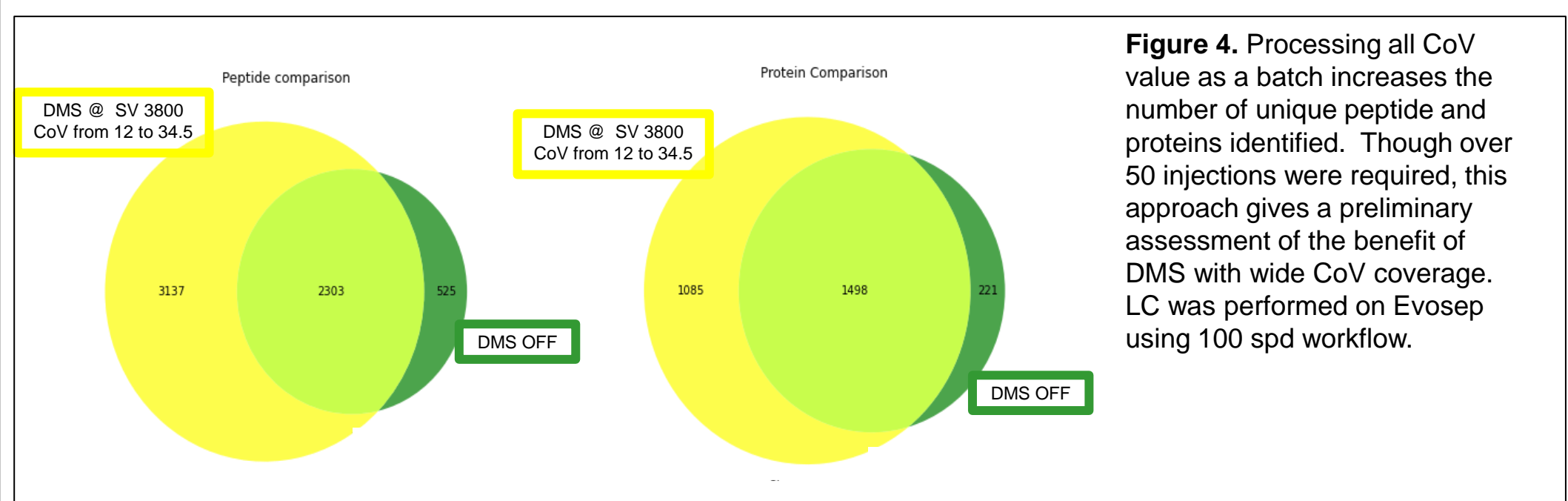


Figure 4. Processing all CoV value as a batch increases the number of unique peptide and proteins identified. Though over 50 injections were required, this approach gives a preliminary assessment of the benefit of DMS with wide CoV coverage. LC was performed on Evosep using 100 spd workflow.

When all DMS active data are processed as a batch (entire CoV range - yellow) and compared to DMS OFF (green), a significantly larger number of unique peptides and proteins are found (Figure 4). The number of unique identification increases by 2x in both cases.

Figure 5 shows the distribution of the peptides with charge state +2 and +3 across the CoV range. Though we did not anticipate a distinct separation between these two charge states, we can see that triply charged peptides will typically be observed at high CoV value, whereas doubly charged ones will typically be observed predominantly at lower CoV values. This can be of benefit when performing SWATH DIA analysis. Based on the identified peptide list, the precursor masses were binned in windows of 50 amu and the number of ID was normalized to the highest number obtained within that window (Figure 6). Applying a threshold of 20%, a total of 70 combinations of CoV and SWATH (70 windows) could be used to capture that majority of the data. With the aim of performing faster LC (200 spd), these 70 windows were split into 3 groups of 23 windows. Using an accumulation time of 20 ms for each CoV SWATH window, this gave us a total cycle time of 0.56 sec (includes MS and settling time). With this cycle time, we ensure that at least 8 to 10 data points were collected across the LC peak (width < 6 sec). Therefore, to cover the entire CoV range and assuming 3 replicate measurements for each group, a total of 9 injections were made, representing approximately 45 min of acquisition time. Figure 7 compares the peptide and protein identification overlap when this data is compared to data acquired with the DMS cell operated in transmission mode. Using the DMS increased the number of unique identification of peptides by 70%, and more than 50% for proteins. We speculate that using a lower threshold (10%), resulting in an additional 23 windows, would have yielded even large gains, while adding 15 min to the total analytical time with current hardware.

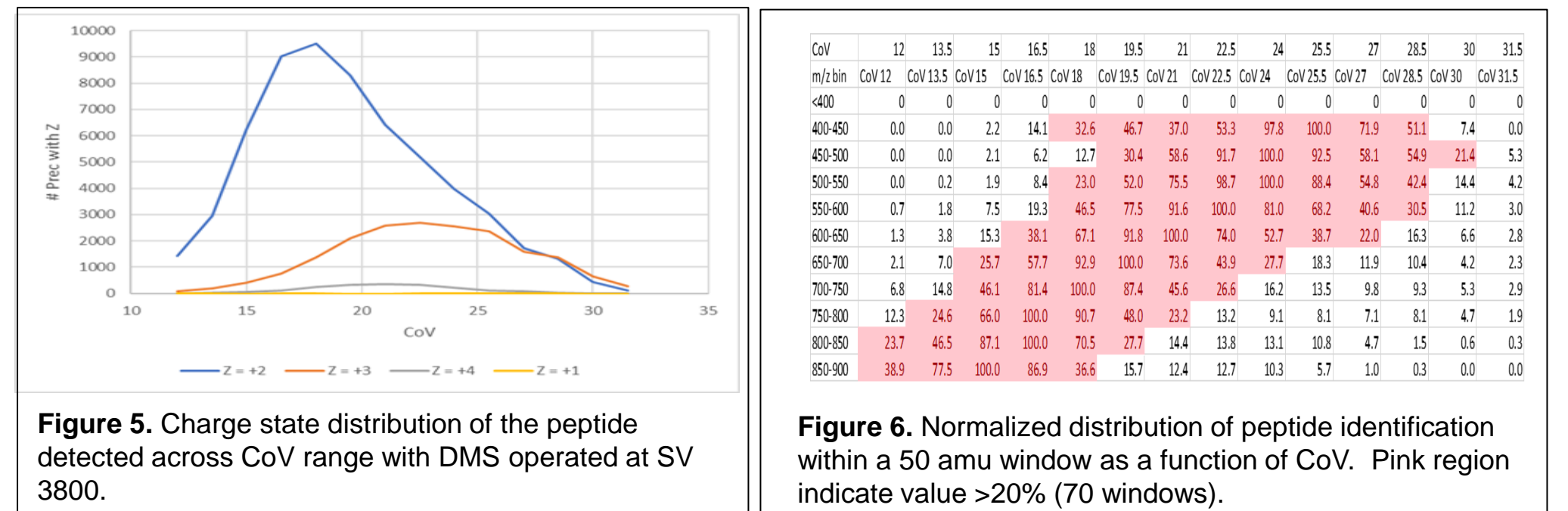


Figure 5. Charge state distribution of the peptide detected across CoV range with DMS operated at SV 3800.

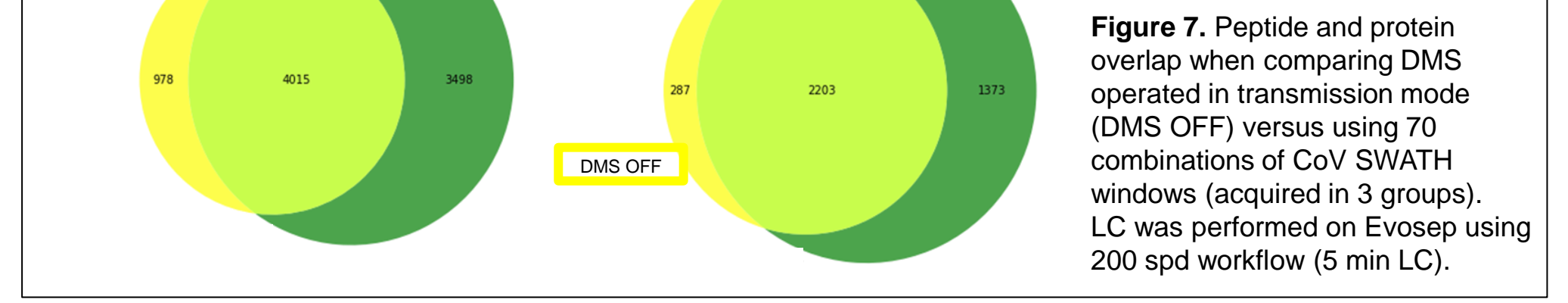


Figure 7. Peptide and protein overlap when comparing DMS operated in transmission mode (DMS OFF) versus using 70 combinations of CoV SWATH windows (acquired in 3 groups). LC was performed on Evosep using 200 spd workflow (5 min LC).

The combination of specific CoV range and wide precursor windows, opens the possibility of operating DMS and SWATH DIA under various LC conditions without sacrificing selectivity. These preliminary data suggest that LC peak capacity increases by factor ranging from 5 to 12 at each CoV value used (8).

Future work on the combination of DMS and SWATH DIA will assess the impact of spectral libraries that are generated with DMS activated on data processing (CoV aware processing). Additional work will also include the incorporation of other instrument features, such as use of Zeno trap, to further improve duty cycle and sensitivity. Finally, the ability to dynamically change the separation capability of the DMS cell by the addition of resolving gas may offer additional flexibility to further supplement the LC peak capacity for many proteomic DIA workflows without sacrificing selectivity.

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