Performance, Carry-Over and Robustness Evaluation of a Newly Available LC-MS/MS Setup for Bottom-Up Proteomics Profiling

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Introduction

Nano-flow liquid chromatography is the workhorse of LC-MS based discovery proteomics, providing higher sensitivity through better electrospray ionisation compared with capillary- or analytical-flow systems. Nano-flow liquid chromatography systems should provide high resolution chromatography while keeping carry-over and overhead times low, hence maximizing data acquisition efficiency. In addition, the system should be able to operate for extended periods of time with minimal maintenance and trouble-shooting required. All these points are achieved not only by careful optimisation of the separation methods but also by the design and construction of the system itself. Here, the newly available *proteoElute* LC coupled with the *PepSep Advanced* nano-flow columns were evaluated for carry-over, robustness and performance for discovery bottom-up proteomics applications.

Materials and Methods

Materials

HEK 293 and C. *elegans* digest were produced in-house using standard operating procedures. All experiments were carried out using a proteoElute (Bruker) LC, C18 PepSep Advanced (25 cm x 75 mm x 1.5 mm) (PepSep), *PepSep Captive Spray 2 Emitter (PepSep)*, and a *timsTOF HT (Bruker)* MS. *dia-PASEF* data was analysed with *Spectronaut 21 (Biognosys)* using *direct-DIA*. Statistical analysis and visualisation were performed using R.

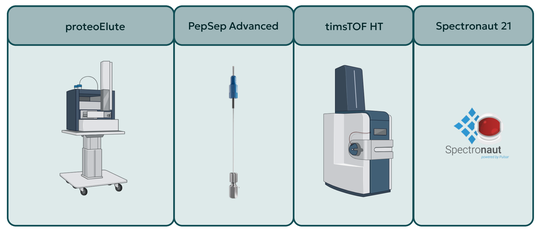


Figure 1: Instrumentation summary.

Methods

Robustness and Performance Evaluation

HEK 293 tryptic digest was fractionated into 10 fractions by HPRP chromatography. Each fraction was measured in triplicate with 8 different gradient lengths ranging from 5 to 144 minutes for a total of 240 runs acquired continuously over ~12 days, with no intervention on the system. For performance evaluation, unfractionated HEK 293 tryptic digest was measured in triplicates with the same methods as above.

Carry-Over Evaluation

The proteoElute has a range of options to mitigate the carry-over. The carry-over mitigation effect of the following options of the proteoElute and their interactions was evaluated: “Extended wash” (on or off), “Flush injection port” (on or off), and “Toggle wash in gradient” (off, 3 steps, 6 steps). The experimental unit consisted of the following sequence of injections: 800 ng HEK 293, 60 ng C. *elegans,* and three blanks. Each condition was acquired in triplicatewith a 21-minutes gradient. The total quantity of HEK- or *C. elegans*-specific peptides carried over was assessed (**Figure 3**).

Results

Robustness and Performance Evaluation

The 240 injections were carried out without interruption or any maintenance task on the system. The retention times proved to be **stable over the 12 days of acquisition** with peptide retention time deviation consistently under 1% (**Figure 2, A**). Median CV of protein quantities across replicates was stable at ~20% for all methods and across the sequence (**Figure 2, B**). The profiling of unfractionated HEK 293 digest led to the average identification of **58’409 precursors and 5’892 protein groups with 5-minutes gradient** with 70% of the protein groups having a CV < 20%. With the **144-minutes gradient, 249’154 precursors and 10’620 protein groups were identified in average**, with 93% of protein groups with a CV < 20% (**Figure 2, C**).

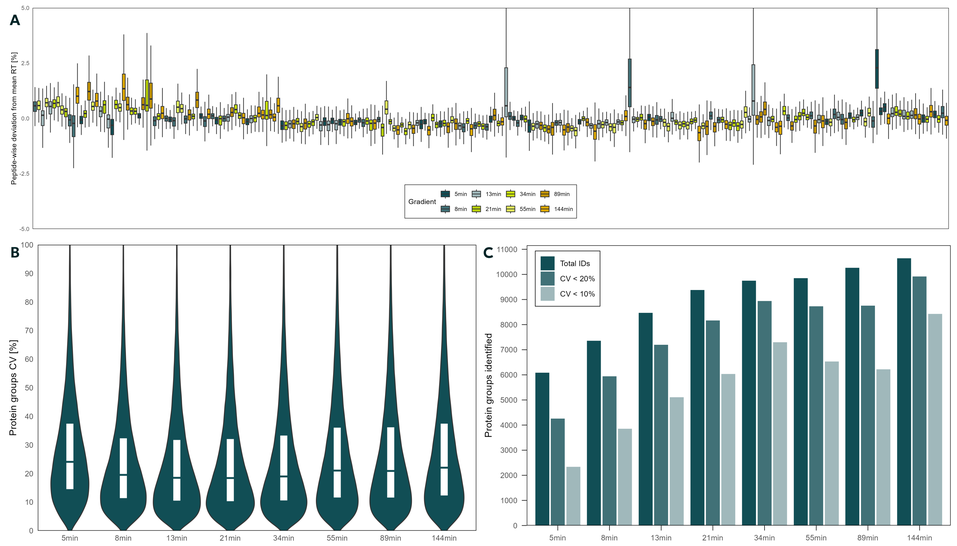


Figure 2: The system showed stable retention time and quantity measurements over a 12 days-long sequence. **(A)** Peptide-wise retention time deviation from mean over acquisitions in the sequence. **(B)** Protein groups CV of each method across the sequence. **(C)** Number of protein groups identified in unfractonated HEK at different CV thresholds.

Sampler Carry-over Evaluation

The carry-over intensity exponentially diminished by 38% for each injection after the HEK injection (i.e. injection distance) independently of the condition. The “extended wash” option was the only factor displaying a significant effect ($p < 0.001$), **reducing overall carry-over by 45%**. The “extended wash” effect had no interaction with the injection distance, meaning it effectively removed all contaminants in the sampler and trap valve (**Figure 4, A**). The extended wash is performed after gradient delivery and takes 5 minutes with the default setting, reducing throughput from 34 samples per day (SPD) to 30 SPD. The residual peptides carried over are mostly on the hydrophobic side of the chromatogram (**Figure 4,** B). **No condition had a significant effect on the number of peptides or proteins identified.** The same conclusions could be drawn from the *C. elegans* “low-load” set with a 65% reduction of carry-over when using the extended wash.

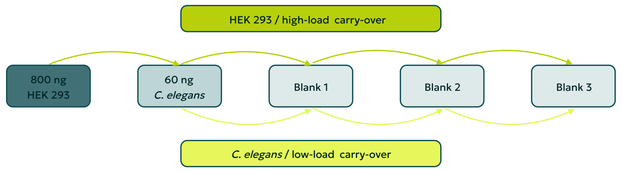


Figure 3: Experimental unit of the carry-over evaluation. The use of samples from two species enables the evaluation of two peptide loads in a single experiment.

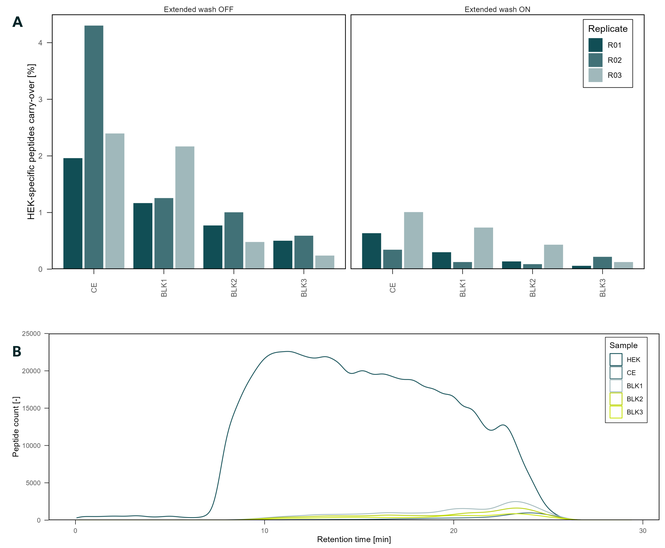


Figure 4: The extended wash option of the LC effectively removes contaminants form the sampler. (A) Human-specific peptides carry-over with and without the extended wash procedure enabled. **(B)** Distribution of HEK peptides over retention time across injections.

Conclusion

- High retention time stability, good quantification precision, and high performance with limited maintenance needed.
- ~8.5k and ~10k protein groups identified at 41 and 19 SPD respectively.
- Total carry-over of less that 1% while loading as much as 800 ng of tryptic digest when using the extended wash option.

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Conflict of Interest Disclosure: Kevin Pacios, Mario Mirabelli, Sebastian Müller, Roland Bruderer are employed by Biognosys or Bruker (see affiliation above).