



brief technical report

retention time reproducibility using a splitless nanoLC coupled to a FT-ICR mass spectrometer

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introduction

Biomarker discovery requires the comparison of control and disease samples to elucidate differences and ultimately identify candidate biomarkers. Our laboratory has focused on the low molecular weight fraction of serum as a potential repository for these biomarkers. This type of analysis is not amenable to the incorporation of internal standards for each molecular species. Instead, the absolute abundance of each molecular species derived from control and disease populations are compared; each species is defined as a mass and retention time pair. Recently we demonstrated this approach using a 10 kDa cutoff filter with the high mass precision and accuracy of FT-ICR.¹ However, uncorrected retention time did not prove useful as a result of poor reproducibility and the number of components detected. We have also demonstrated that a 50 kDa filter contributes a significant enhancement in the number of species detected which will place higher demands on retention time as a parameter to define unique species.²

Previous work utilizing nanoLC gradients required a flow splitter prior to the chromatographic column to achieve nanoLC flow rates. This approach is not always able to provide a constant flow to the nanoLC column due to backpressure differences between the nanoLC column and the splitter. This results in a systematic drift in retention time; 5 replicate analyses of ~500 molecular species in pooled ovarian cancer sera showed 95% of these species to have retention time variability of ± 54 seconds (expressed as a 95% confidence interval of the mean of retention time calculated separately for each species).¹ However, the confidence interval of the mean would have continued to increase due to further increases in column backpressure as additional analyses were carried out.

Modern HPLC instrumentation has the capability of providing excellent retention time reproducibility at higher flow rates but the capability to reproducibly provide a splitless nanoLC organic gradient has only recently become available. This advance is due to pumps that can provide nanoLC flow rates without splitting (in one example these nanoliter per minute flow rates are achieved by continuous monitoring of the pressure differential across a flow restrictor). This approach will obviate the need to post-process data using chromatographic alignment algorithms.

application:	Biomarker discovery
objective:	Increase retention time reproducibility of nano HPLC separations of peptides to enable the use of absolute retention time for the identification of biomarkers
technology:	A splitless NanoLC-1D™ system, with active flow control and real-time column backpressure monitoring, coupled to an FT-ICR mass spectrometer
results:	Use of the direct pumping NanoLC-1D system increased retention time reproducibility by an order of magnitude compared to data obtained with a splitter-based nano HPLC system

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In conventional (1 mL/min) splitless HPLC systems, column backpressure is utilized as a diagnostic to monitor column health. However, the operator is blind in nanoLC systems utilizing a solvent splitter to the dynamic conditions of the nanoLC column and unable to confidently assess flow rate at sub microliter/min flow rates. The new splitless nanoLC pumps provide a true column backpressure read out which has allowed us to easily diagnose and monitor the condition of the nanoLC column.

In this report, we have examined the capability of one of a new generation of nanoLC pumps that provides splitless nanoLC gradients at nanoLC flow levels. An Eksigent NanoLC-1D pump with autosampler (Eksigent Technologies, LLC, Livermore, CA) was coupled to an IonSpec 9.4T FT-ICR mass spectrometer (IonSpec, Lake Forest, CA) equipped with a modified dual Z-spray ESI source (Waters, Milford MA).² Data analysis procedures detailed elsewhere¹ were applied to seven replicate nanoLC-FT-ICR-MS chromatograms of tryptic BSA digest. **Figure 1** shows the retention time reproducibility (95% confidence interval of the mean) and the total ion abundance (right-hand y-axis) versus retention time, showing no dependence between either of them. The diagnostic capabilities of column backpressure monitoring have allowed us to obtain excellent retention time reproducibility: 27 of 29 BSA fragments detected have a 95% CIM of ± 4.5 seconds. This is an order of magnitude improvement over the previous result of ± 54 seconds.¹ The retention time reproducibility afforded by this instrumentation allows the use of absolute retention time as an additional constraint for the elucidation of putative biomarkers. With the analysis of increasingly complex mixtures imminent, this additional specificity will prove to be an important orthogonal constraint to the high mass measurement accuracy and precision provided by FT-ICR-MS.

References

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Figure 1: Retention time reproducibility (95% confidence interval of the mean) versus retention time for 29 BSA tryptic fragments identified in seven replicate nano-LC-FT-ICR chromatograms (left axis, squares). A representative total ion chromatogram is also plotted (right axis, line).

