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LC-MS

TECHNICAL NOTE

Quantitative targeted nano- and capillary-flow LC-MS peptide analysis using the Vanquish Neo UHPLC System coupled to a triple quadrupole mass spectrometer

Authors

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Keywords

Thermo Scientific Vanquish Neo UHPLC system, Thermo Scientific TSQ Altis, Thermo Scientific EASY-Spray PepMap Neo column, HeLa protein digest, PRTC standard, nano-flow LC-MS, capillary-flow LC-MS, selected reaction monitoring (SRM)

Goal

Demonstrate robust performance of the Thermo Scientific™ Vanquish™ Neo UHPLC System coupled to a Thermo Scientific™ TSQ Altis™ Triple Quadrupole Mass Spectrometer for capillary-flow LC-MS/MS based peptide quantification and compare it to a standard nano-flow LC-MS/MS based method.

Introduction

Nano-flow LC combined with mass spectrometry has been a mainstay for analyzing proteins and peptides for at least 20 years, particularly for proteomics research. The main advantage that nano-flow brings compared to analytical flow rates is increased sensitivity. Tryptic digestion of biological samples and tissues produces final sample mixtures of low volume, high complexity and with target peptide concentrations ranging from low attomole to high femtomole per microliter levels.

In other areas of analytical science, such as clinical research applications, the adoption of nano-flow LC/MS has been limited. In such fields, limited robustness, repeatability, ease-of-use, and throughput are considered the main obstacles to nano-flow LC-MS adoption.[1,2] The Vanquish Neo UHPLC system offers a flow range to support applications from nano- to micro-flow, which increases productivity for high sensitivity LC-MS workflows and produces the highest quality results for both LC-MS experts and novice users alike.

In addition to next-generation Vanquish Neo LC hardware, advances in nano-flow LC capillary and column technology improve separation quality and lab productivity. 1500 bar compatible Thermo Scientific™ nanoViper™ Fitting Systems and plug-and-spray Thermo Scientific™ EASY-Spray™ PepMap Neo thermostatted columns combine to maximize robustness, repeatability, and useability of low-flow LC-MS.[3] One challenge that cannot easily be met with nano-flow LC-MS is the high sample throughput requirement necessary for targeted quantitation assays in the large sample cohort analysis. These demands can be fulfilled, however, by switching to capillary-flow LC (capLC) which affords robust, high throughput analysis coupled with the levels of sensitivity attributed to low-flow LC-MS.[4]

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Low-flow LC enhances sensitivity compared to high-flow applications due to several factors. The sample is diluted in less mobile phase during the separation, so analytes are eluted at a higher concentration. Electrospray ionization (ESI) behaves as a concentration-dependent detector,[6] so there is an increase in signal intensity with column ID decrease. In addition, de-solvation efficiencies are greater at lower flow rates and the emitter can physically be positioned much closer to the inlet of the mass spectrometer, so that a greater proportion of the analyte is ionized and enters the mass spectrometer. While nano-flow exploits these effects most efficiently, capillary-flow LC still benefits from them strongly including the higher sample loading capacity compared to nanoLC-MS. Moreover, the sensitivity loss may be partially offset by the shorter gradients achieved using capillary-flow LC, resulting in sharper chromatographic peaks, higher signal-to-noise, and improved resolution.

The purpose of this technical note is to determine whether quantitative LC-MS performance can be maintained when moving from nano-flow to capillary-flow rates. HeLa protein digest was chosen as a matrix of reasonable complexity for targeted peptide quantification. Commercially available heavy labeled peptides were spiked into a HeLa protein digest. Thermo Scientific™ Pierce™ Retention Time Calibration Mixture (PRTC) contains peptides with a wide range of hydrophobicities to elute across the entire LC gradient and doesn't contain endogenous peptides of the HeLa protein digest matrix.

The Vanquish Neo UHPLC system was used to compare results for nanoLC-MS and capLC-MS as it allows evaluation of both methods on the same hardware platform without hardware modifications. To maintain a sufficient number of scans across each peak for quantification, the mass spectrometer must be capable of acquiring data at a high acquisition rate. The TSQ Altis MS is capable of scanning at 600 SRMs/second while maintaining sensitivity and consistent performance. It is therefore the ideal detector to evaluate the performance of the Vanquish Neo UHPLC system at both nano- and capillary-flow rates. The criteria proposed in the FDA Bioanalytical Method Validation Guidance for Industry[7] were used to estimate if the target analyte is quantifiable. The limits for accuracy should be within 20% of the nominal concentration (n=5) and precision within 20% CV (n=5). Finally, the robustness of the capLC-MS method was evaluated by monitoring the consistency of retention times, peak widths, peak asymmetries, and peak areas for hundreds of injections over several days.

Materials and methods

PRTC peptides were spiked into the HeLa protein digest standard to generate a dilution series of 0.01, 0.1, 1, 10, and 100 fmol/µL PRTC in 166 ng/µL HeLa protein digest matrix. This dilution series was used to evaluate the linearity of nanoLC-MS and capLC-MS methods.

Pre-mixed Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard was reconstituted by adding 50 µL of 0.1% formic acid (FA) in water. The vial was subsequently sonicated for 2 min, followed by aspirating and releasing 10-times with a pipette to fully reconstitute the sample at a final concentration of 200 ng/µL of HeLa protein digest containing 100 fmol/µL PRTC.

Table 1: Solvents and additives

Reagent	Grade	Supplier	Part Number
Thermo Scientific™ Pierce™ HeLa Protein Digest Standard, 20 µg	N/A	Thermo Fisher Scientific	88329
Thermo Scientific™ Pierce™ Retention Time Calibration Mixture (PRTC), 0.5 pmol/µL	N/A	Thermo Fisher Scientific	88320
Thermo Scientific™ Pierce™ HeLa Digest/PRTC Standard, 10 µg and 5 pmol	N/A	Thermo Fisher Scientific	A47997
Acetonitrile with 0.1% formic acid	Optima™ LC-MS	Fisher Chemical	LS120-212
Isopropanol	Optima™ LC-MS	Fisher Chemical	A461-212
Formic acid	Optima™ LC-MS	Fisher Chemical	A117-50
Water with 0.1% formic acid	Optima™ LC-MS	Fisher Chemical	LS118-212
Water	Ultra-Pure, 18.2 MΩ at 25°C	Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	

LC-MS analyses were performed on a Vanquish Neo UHPLC system (Table 2) connected to a TSQ Altis mass spectrometer. A Thermo Scientific™ EASY-Spray™ NG Source (PN ES081) was used to couple LC and MS.

Table 2: Vanquish Neo UHPLC system configuration

Module	Part Number
Vanquish Neo UHPLC system- Binary pump N, Split sampler NT, solvent rack, Vanquish system controller, ship kit and system base with drawer	VN-S10-A-01
Vanquish display	6036.1180

The Vanquish Neo UHPLC system was set up in nano/capillary-flow direct injection mode with 20 µm ID nanoViper capillaries using the solvents listed in Table 3. A nano EASY-Spray PepMap Neo column (75 µm ID × 15 cm, C18, 2 µm, 100 Å, PN ES75150PN) was used for the nano-flow experiments and a capillary EASY-Spray PepMap Neo column (150 µm ID × 15 cm, C18, 2 µm, 100 Å, PN ES150150PN) was used for the capillary-flow experiments. The LC method details are described in Tables 4, 5, and 6. MS method details are described in Table 7. Selected reaction monitoring (SRM) transitions for HeLa and PRTC peptides were used from the default method templates available via the TSQ Altis instrument method editor.

The LC-MS system was operated with Thermo Scientific™ Chromeleon™ 7.2.10 MUd. Alternatively, Thermo Scientific™ SII for Xcalibur™ 1.5.1 can be used.

Table 3: Solvents used for nano- and capillary-flow LC-MS experiments

Solvent	Composition
Eluent A	100% water, 0.1% formic acid
Eluent B	80% acetonitrile, 20% water (v/v), 0.1% formic acid
Weak wash liquid of metering device	100% water, 0.1% formic acid
Strong wash liquid of metering device	80% acetonitrile, 20% water (v/v), 0.1% formic acid
Weak (outer) needle wash liquid	100% water, 0.1% formic acid
Strong (outer) needle wash liquid	80% acetonitrile, 20% water (v/v), 0.1% formic acid
Rear seal wash liquid	75% isopropanol, 25% water (v/v), 0.1% formic acid

Table 4: LC method parameters for nano- and capillary-flow LC-MS methods

Category	Parameter	Value Nano-flow	Value Capillary-flow
Sample Loading	Fast loading	Enabled	Enabled
	Mode	CombinedControl	FlowControl
	Flow	0.5 µL/min	3.0 µL/min
	Pressure	800 bar	-
	Loading volume	Automatic	Automatic
	Injection volume	1 µL	2 µL
Sample pick-up	Outer needle wash mode	After Draw	After Draw
	Outer needle wash time (strong)	3.0 s	3.0 s
	Outer needle wash speed (strong)	80.0 µL/s	80.0 µL/s
	Outer needle wash time (weak)	5.0 s	5.0 s
	Outer needle wash speed (weak)	80.0 µL/s	80.0 µL/s
	Draw speed	0.2 µL/s	0.2 µL/s
	Draw delay	2.0 s	2.0 s
	Dispense speed	3.0 µL/s	3.0 µL/s
	Vial bottom detection	Enabled	Enabled
Column Equilibration	Fast equilibration	Enabled	Enabled
	Mode	CombinedControl	FlowControl
	Flow	0.5 µL/min	3.0 µL/min
	Pressure	800 bar	-
	Equilibration factor	5	5
Temperature	EASY-Spray column temperature	40 °C	40 °C
	Autosampler temperature	5 °C	5 °C

Table 5: The parameters of nano-flow 27-min gradient method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	0.3	3
27.0	27.0	0.3	40
Column wash phase			
27.1	0.1	0.3	100
32.1	5.0	0.3	100

Table 6: The parameters of capillary-flow 12.4-min gradient method

Time (min)	Duration (min)	Flow rate (µL/min)	%B
Gradient separation phase			
0.0	0.0	3.0	4
8.0	8.0	3.0	20
12.0	4.0	3.0	35
12.4	0.4	3.0	50
Column wash phase			
12.6	0.2	3.0	99.0
13.6	1.0	3.0	99.0

Table 7: MS method parameters for nano- and capillary-flow LC-MS method

Parameter	Value Nano-flow	Value Capillary-flow
Source parameters		
Ion source type	NSI	NSI
Spray voltage (static, positive)	1900 V	1900 V
Sweep gas	0 psig	0 psig
Ion transfer tube temperature	275 °C	275 °C
Scan parameters		
Acquisition mode	SRM (selected reaction monitoring)	SRM (selected reaction monitoring)
Polarity	Positive	Positive
Cycle time	0.8 s	0.5 s
Use calibrated RF lens	Enabled	Enabled
Q1 resolution (FWHM)	0.7 amu	0.7 amu
Q3 resolution (FWHM)	1.2 amu	1.2 amu
CID gas	1.5 mTorr	1.5 mTorr
Source fragmentation	0 V	0 V
Chromatographic peak width	8 s	6 s
Use chrome filter	Enabled	Enabled

Results and discussion

The primary reason for moving from nanoLC to capLC is to improve throughput. The increase of linear velocity for capLC methods also reduces the contribution of gradient delay volume, column re-equilibration time, and sample loading times. We observed a significant reduction of the analysis time achieved by moving from nanoLC to capLC methods. The time for the first peak to elute is decreased by more than 4 minutes for capLC-MS methods. The time for the last eluting peak was reduced by around 10 minutes. Column re-equilibration was reduced approximately by 4 minutes and the total time for the analysis of 10 injections is 203 minutes (capLC) compared to 504 minutes (nanoLC). Thus, a 60% reduction in total analysis time (includes sample pick-up, sample loading, column equilibration, gradient elution and column wash steps) is achieved using capLC (Figure 1). Some peptide pairs had lower chromatographic resolution in capLC in comparison with nanoLC (e.g. PRTC_02, HeLa_01, and PRTC_07) but this did not reduce the linearity and limit of quantitation (LoQ) of the capLC method.

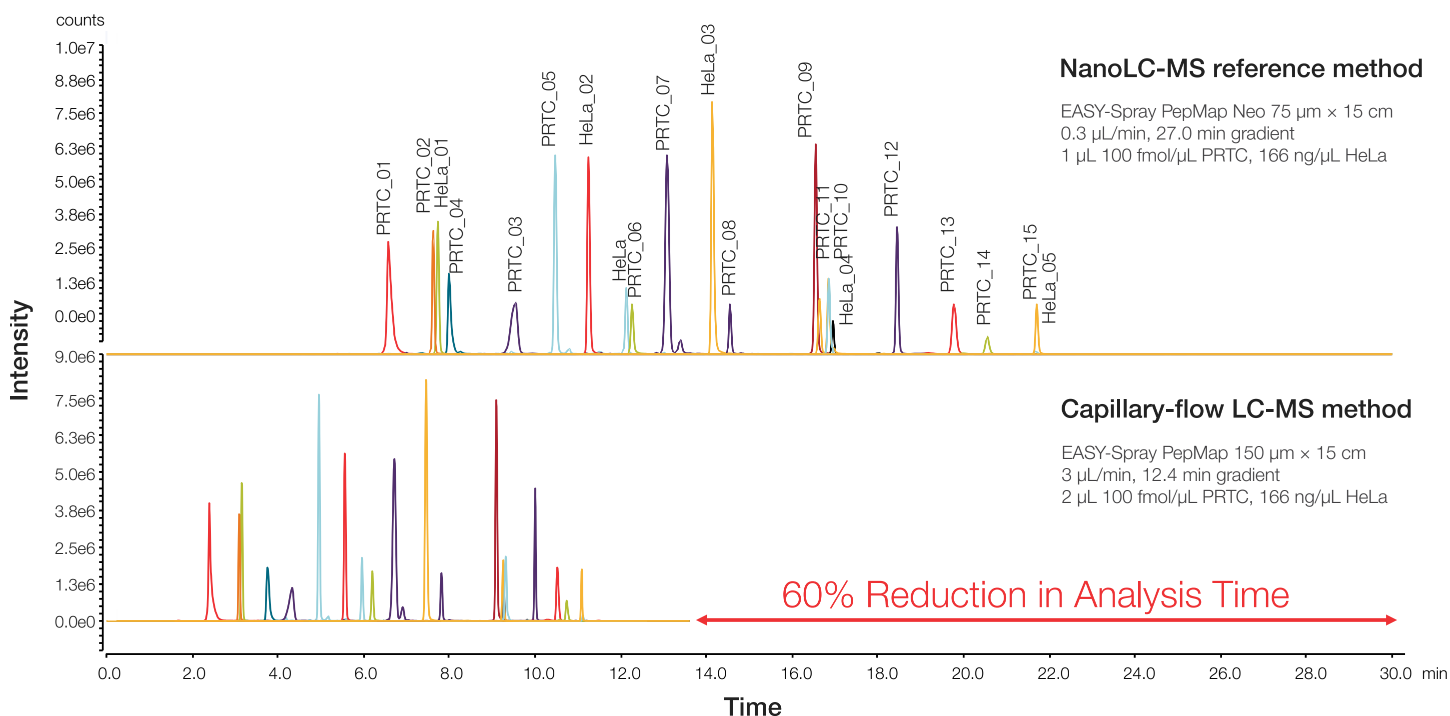

Figure 1: The 60% reduction in total analysis time allows increasing the sample throughput moving from the nano- to the capillary-flow LC-MS method.

To evaluate linearity, calibration curves were constructed by serial dilution of PRTC spiked in HeLa digest, from 0.01–100 fmol/µL. After that 1 µL (nanoLC) or 2 µL (capLC) of each standard was loaded onto the respective column (n = 5). Figure 2 shows examples for two peptides within the linear range for both nanoLC and capLC methods. On the right-hand side of Figure 2 the lower end of the calibration curve is zoomed in.

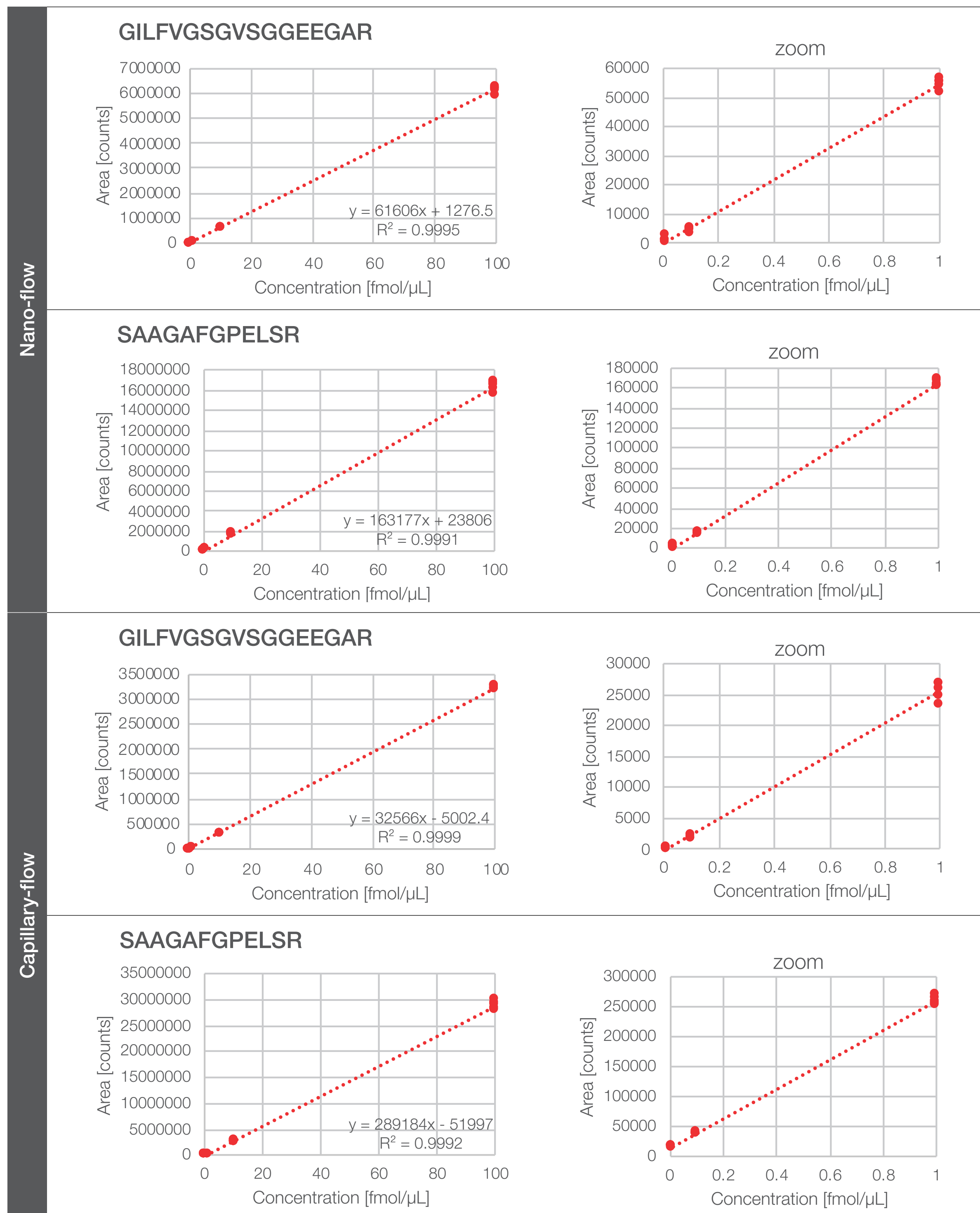

Figure 2: Representative calibration curves for two PRTC isotope labeled peptides spiked in HeLa protein digest for capillary-flow and nano-flow LC-MS in the range from 0.01 to 100 fmol/µL, with zoomed view on the right side focusing on the calibration levels from 0.01 to 1 fmol/µL (5 replicates)

The column used for nano- and capillary-flow methods had the same 15 cm column length and 2 µm particle size and an inner diameter of 75 µm ID for nanoLC-MS and 150 µm ID for capLC-MS. The larger inner diameter increases the loading capacity of the column, allowing increased injection amounts of the spiked HeLa protein digest. Both nano- and capillary-flow methods provide a linear response across the same concentration range, even though the absolute response is different between the two flow rates. The high correlation coefficients for 15 PRTC peptides were obtained for nano- and capillary-flow LC-MS method (Figure 3).

One advantage of moving to capillary-flow rates is that the chromatographic peaks become sharper with a reduction of gradient length (Figure 4) and an increase of flow rate. Three peptides covering the elution range for typical tryptically digested peptides have been selected to compare PWHM for nanoLC (top) and capLC (bottom). The time (in seconds) corresponds to the peak width at half-height. In all cases, the peak width at half-height is 75–80% narrower with capLC compared to the corresponding nanoLC experiments, which corresponds to a 0.6–1.2s lower peak width. Sharper peaks improve the signal-to-noise ratio as well as improve the chromatographic peak resolution from matrix interferences.

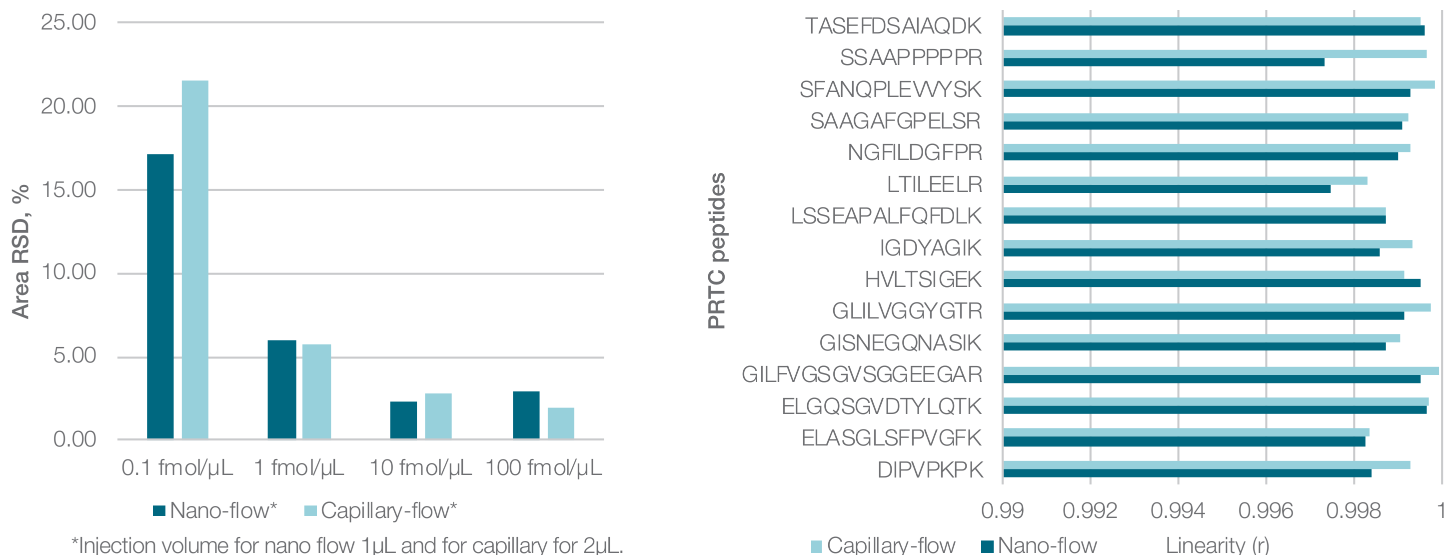

Figure 3: The left graph shows the area precision for 15 PRTC isotope labeled peptides at various concentration levels and 5 replicate injections for each concentration. The right graph shows the linearity for 15 PRTC peptides for the nano- and capillary-flow calibration curves with 5 concentration levels from 0.01 to 100 fmol/µL and 5 replicate injections for each concentration level. Considering 15 PRTC peptides (n = 5), the average linearity (r) was 0.9988 for nano-flow and 0.9993 for capillary-flow LC-MS.

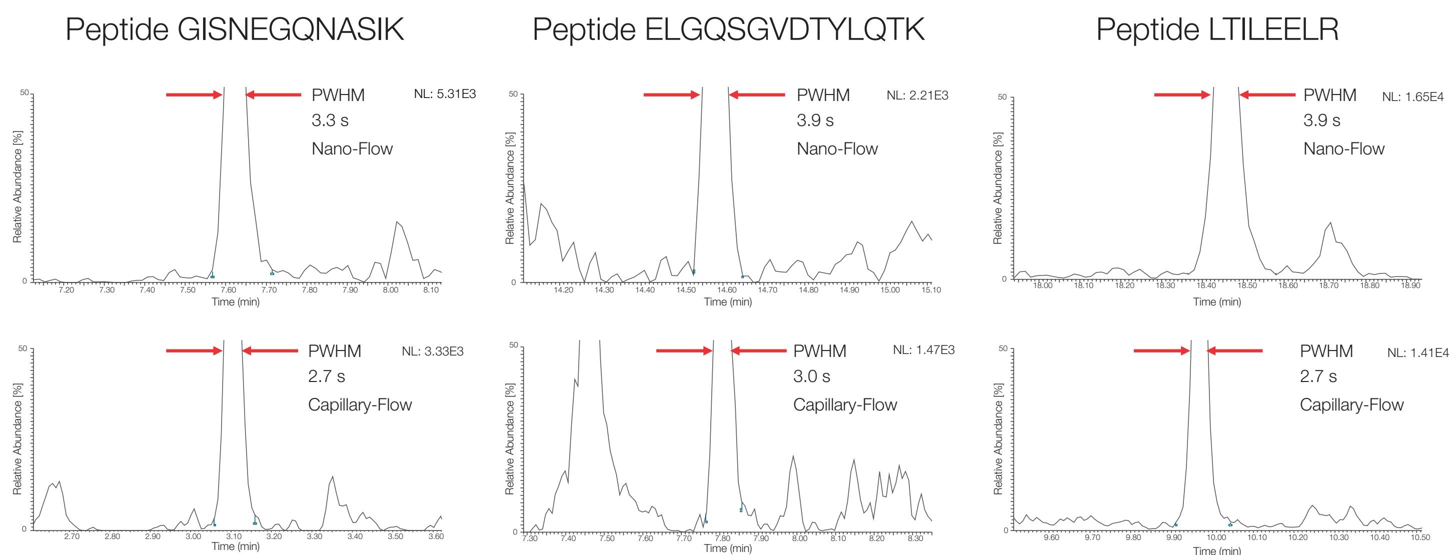

Figure 4: Peak properties for 3 representative peptides of nano- (top) compared to capillary-flow LC-MS method (bottom). The vertical scale (signal intensity) has been zoomed to 50% of the peak height.

Having sharper peaks requires sufficient MS acquisition speed for precise peak integration. The TSQ Altis MS can scan at 600 SRMs per second and, therefore, can quantify multiple analytes simultaneously. Figure 5 shows the same three peptides from Figure 4 with the number of scans across the peak. In all cases, there are at least 12 scans across each peak, giving an excellent quantitative performance.

Both the FDA Guidelines for Bioanalysis and the EMA Guideline on Bioanalytical Method Validation define limits of quantitation as having precision (CV) and accuracy (bias) <20%. Based on these criteria, the lowest quantifiable concentration level was determined for both nanoLC-MS and capLC-MS methods (Table 8).

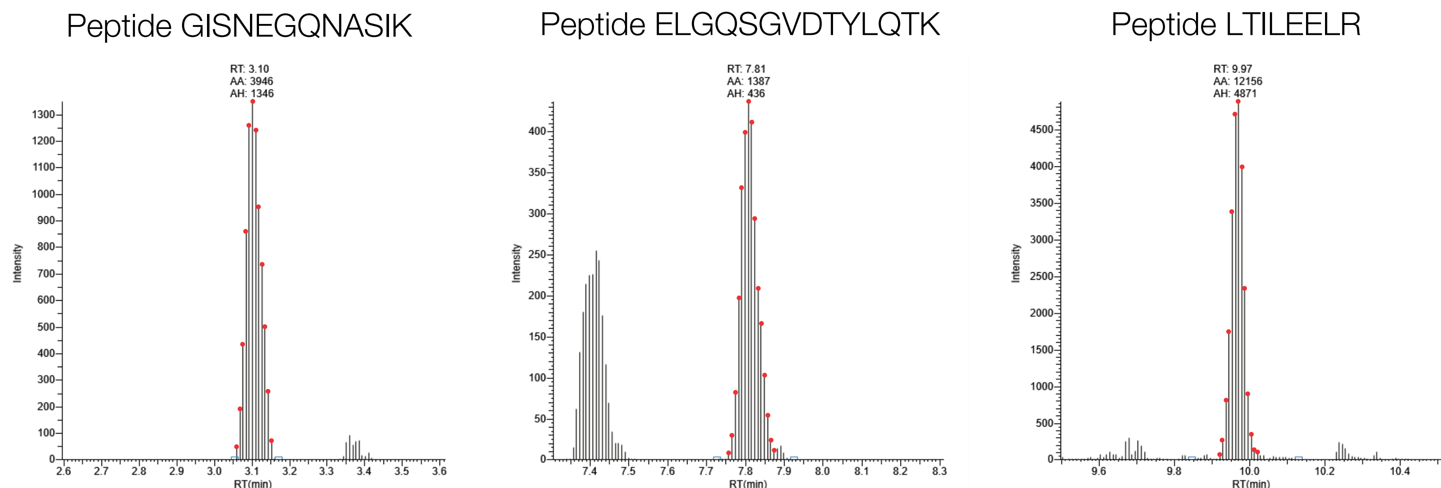

Figure 5: MS acquisition rate determined via the number of MS scans across 3 representative peptide peaks for the fast capillary-flow LC-MS method.

Table 8: Coefficient of variation (CV, %) and bias, % for corresponding PRTC peptide and LoQ values obtained with nano- and capillary-flow LC-MS methods

	NanoLC-MS			CapLC-MS		
	%CV	% Bias	Quantifiable level (amol/µL)	%CV	% Bias	Quantifiable level (amol/µL)
DIPVPKPK	16.3	10.2	100	2.07	7.12	100
ELASGLSFPVGFK	11.2	18.1	1000	17.0	19.1	1000
ELGQSGVDTYLQTK	16.1	18.4	100	14.6	15.2	100
GILFVGSGVSGGEEGAR	17.1	6.02	100	12.3	18.0	100
GISNEGQNASIK	13.6	3.50	100	12.0	12.6	100
GLILVGGYGTR	7.18	4.70	100	5.43	19.5	100
HVLTSIGEK	4.66	14.3	100	4.94	0.89	100
IGDYAGIK	16.3	0.73	100	6.84	15.7	100
LSSEAPALFQFDLK	5.35	17.2	10000	6.55	14.2	10000
LTILEELR	8.90	1.10	1000	7.09	7.80	1000
NGFILDGFPR	4.87	12.9	100	4.41	14.4	1000
SAAGAFGPELSR	7.37	7.30	100	2.53	11.3	100
SFANQPLEVVYSK	7.80	8.97	10	4.92	19.3	100
SSAAPPPPPR	4.79	13.2	100	7.16	0.90	100
TASEFDSAIAQDK	13.0	8.66	100	18.6	0.60	100

The behavior of ESI MS as concentration sensitive detector implies that the narrower column inner diameter results in higher sensitivity. However, as illustrated in Table 8 due to better separation performance the capLC-MS method shows comparable quantifiable performance for all 15 PRTC peptides. The quantifiable concentration was higher using capLC-MS than nanoLC-MS for only three peptides. The higher column capacity and sharper peaks obtained with capLC-MS almost certainly mitigate some of the sensitivity loss when moving from nano- to capillary-flow LC-MS analysis. Additionally, a significant gain in throughput is observed for capLC-MS analysis.

Figure 6 shows examples of limits of detection (LoD) when capLC-MS allows detecting peptide targets even at concentrations 10 times lower than the LoQ. in HeLa protein digest matrix.

Having a rugged and reproducible capLC system is critical for applied scientific laboratories, especially when used for clinical research applications. Figure 7 illustrates the reliability of the Vanquish Neo UHPLC system for over 75 hours of continuous operation. The average retention time RSD, % for all peptides across 200 runs was below 0.28%. There is one peak (PRTC_03) where the retention time shift was observed that is potentially related to the secondary interaction of the peptide with the column material. The average peak area RSD, % for all peptides across 200 runs was 5.3%.

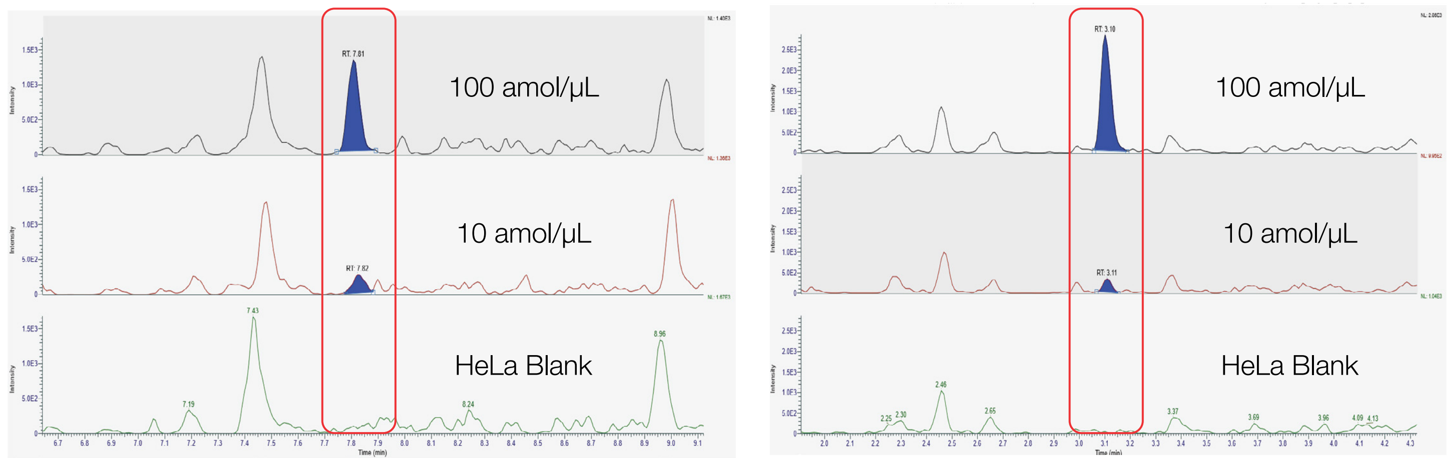

Figure 6: Limit of detection of capillary-flow LC-MS method for ELGQSGVDTYLQTK (left) and GISNEGQNASIK (right) peptides

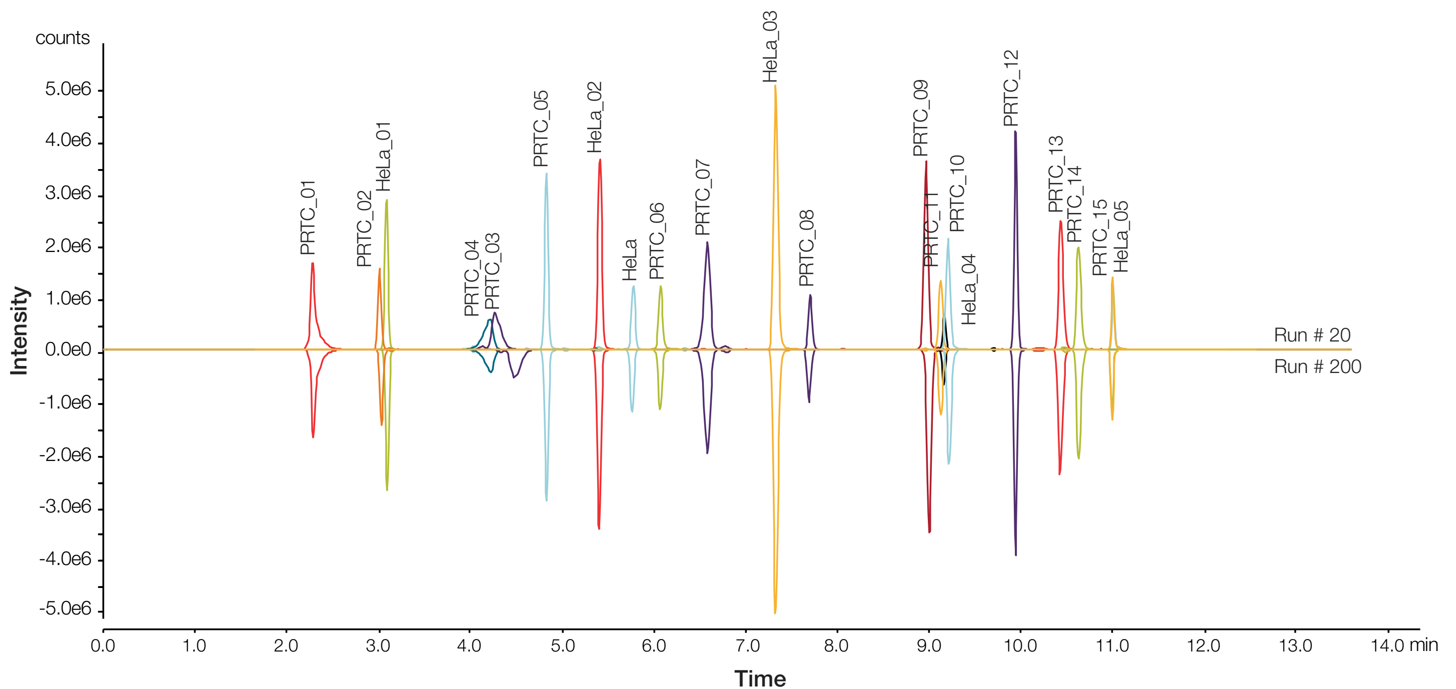

Figure 7: Capillary-flow LC-MS method long term robustness test over 75 hours and 200 runs of HeLa/PRTC sample.

Figure 8 further confirms the consistency of retention time, peak area, peak width, and peak asymmetry across the 200 runs with average peak width at half maximum of 3.3 s and average peak asymmetry of 1.1 for all PRTC peptides, highlighting the robustness of the LC-MS setup.

The overall sample carry-over performance for an LC-MS setup, especially when targeting low concentration analytes, is crucial for robust and reliable analysis. Two main contributors to the overall sample carry-over are the carry-over introduced from the LC system and the column carry-over. The sample carry-over was determined from a blank injection after a high concentration injection of 400 fmol PRTC. Figure 9 shows the contribution of LC carry-over and column carry-over. The total carry-over for most of the peptides was below 0.1% after high concentration PRTC injection. To isolate the carry-over sources blank runs with and without injection procedures were performed. The LC system carry-over contribution was below 0.07% for all peptides.

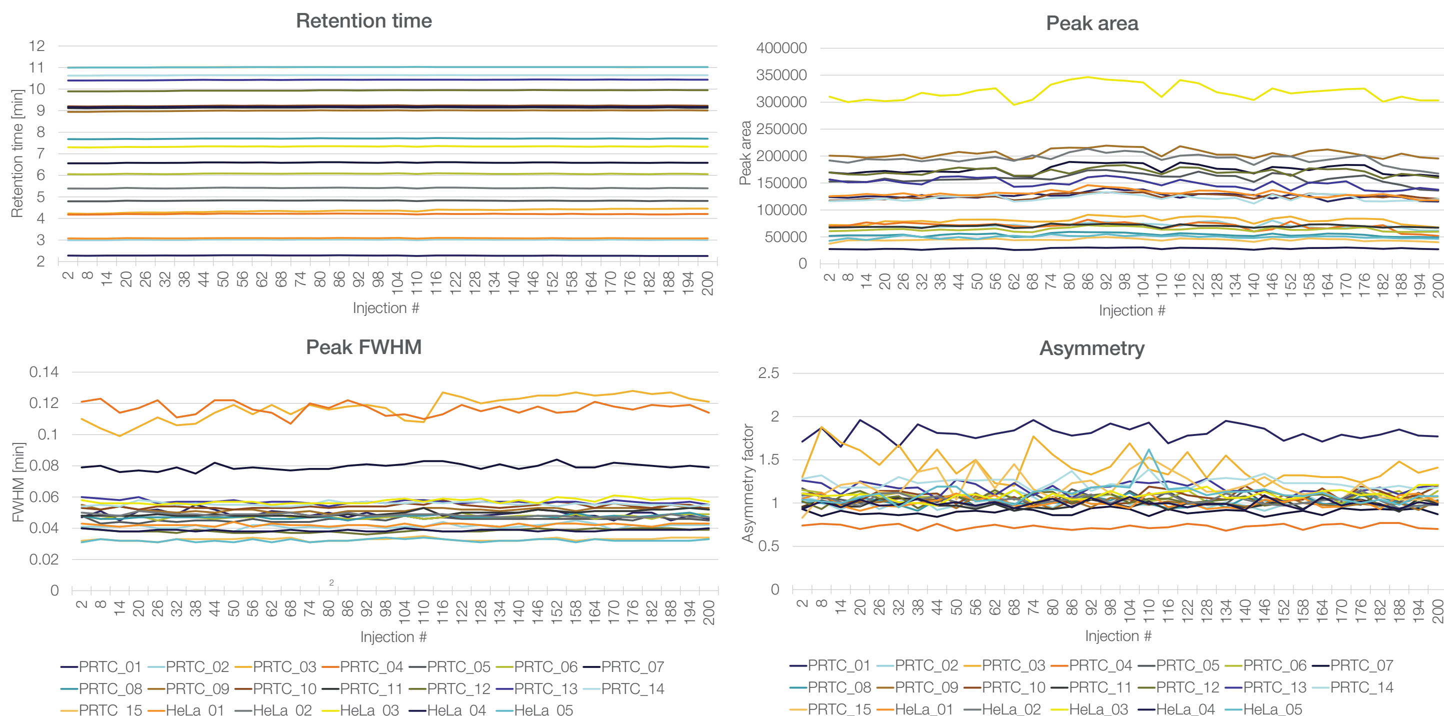

Figure 8: Peak property stability for the capillary-flow LC-MS method across 200 runs and 75 hours of continuous operation

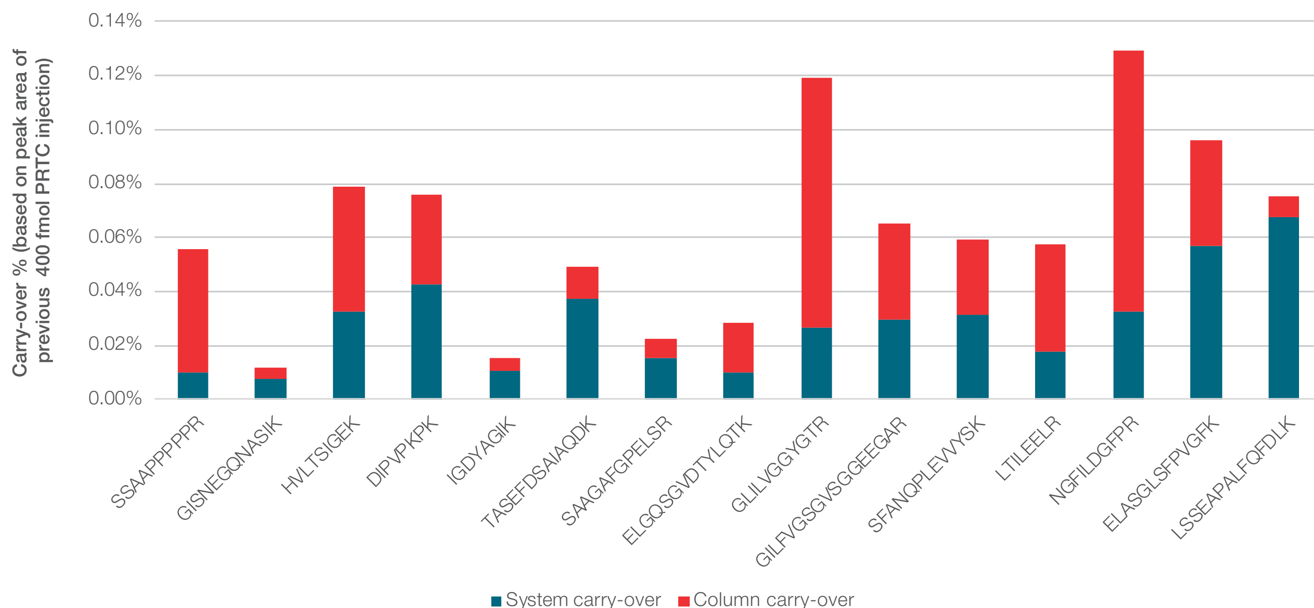

Figure 9: System and column contributions to carry-over observed for PRTC peptides

Conclusions

Based on the precision, accuracy, repeatability, and throughput studies, we have demonstrated that the Vanquish Neo UHPLC system coupled to a triple quadrupole mass spectrometer is an excellent platform for targeted peptide analysis in complex matrices. Furthermore, we have demonstrated that it gives excellent performance at both nano-flow rates and capillary-flow rates, which can be used on the same hardware platform without hardware or fluidics changes. NanoLC-MS applications can be run to achieve the ultimate sensitivity and when additional sample throughput is required, capLC-MS methods can be used to accelerate the sample analysis.

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