A flexible microflow set-up for the identification and quantitation of proteins in complex samples using Zeno SWATH data-independent acquisition (DIA)

Microflow proteomics using the SCIEX M5 MicroLC system and the ZenoTOF 7600 system

Remco van Soest[1] and Patrick Pribil[2]
[1]SCIEX, USA; [2]SCIEX, Canada

This technical note demonstrates a trap-and-elute microflow LC approach to quantitative proteomics using the SCIEX M5 MicroLC system and the ZenoTOF 7600 system. Protein identification and quantitation were demonstrated using Zeno SWATH DIA with a K562 human cell lysate digest across a range of sample loads, gradient lengths and flow rates, resulting in equivalent data to what has been published previously using other LC systems in combination with the ZenoTOF 7600 system. The M5 MicroLC system offers a high degree of injection volume and method flexibility, significant robustness and quantitative reproducibility, making it an ideal HPLC solution for high-throughput proteomics research when combined with the ZenoTOF 7600 system.

High-throughput in-depth analysis of proteins is essential in proteomics research. DIA has emerged as the preferred analysis method for many researchers. With the introduction of Zeno SWATH DIA on the ZenoTOF 7600 system, researchers now have access to unparalleled speed, sensitivity and accuracy for protein identification and quantitative precision, particularly when quantitation is performed on MS/MS fragments. It has been previously shown that high numbers of proteins can be identified and quantified from cell lysate digests with Zeno SWATH DIA using a combination of the Evosep One system and the ZenoTOF 7600 system[1]. The system described here achieves similar numbers of proteins identified and quantified, see Figure 1, but offers more method flexibility.

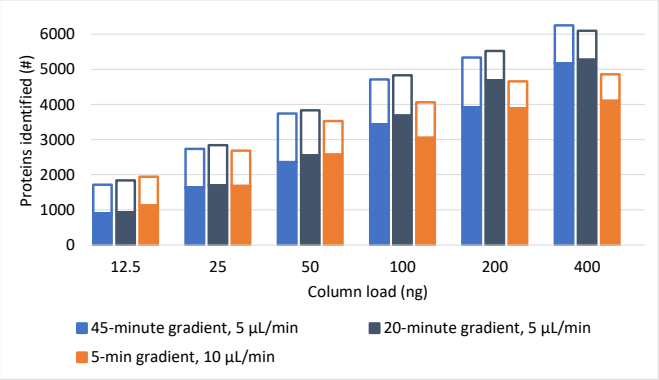

Figure 1. Protein groups identified and quantified for different gradients and sample loads of the K562 digest standard. The solid portion of each bar indicates how many protein groups were quantified with a CV <20%.

Figure 2. The SCIEX M5 MicroLC system.

Key features of using the M5 MicroLC system with the ZenoTOF 7600 system for proteomics

- Full control of the M5 MicroLC system with SCIEX OS software, including records of LC methods, pressures and flow rates
- Using a 50 µL loop and a trap-and-elute set-up, injections from 1 to 40 µL can be made with online desalting
- Zeno SWATH DIA on the ZenoTOF 7600 system combines the speed, sensitivity and quantitative reproducibility needed for robust detection and quantitation of proteins with high throughput
- Almost 80% of the protein groups identified and quantified in 400 ng of a K562 digest with a 45-minute gradient could be identified and quantified using a 5-minute microflow gradient method with a 7-minute cycle time (200 samples per day)
- Excellent retention time (CV <0.5%) and peak area (CV <13%) reproducibility were observed for peptides tracked across 45 replicate injections with a 20-minute gradient method

Methods

Sample preparation: Human K562 cell lysate tryptic digest was acquired from Promega and a 10 ng/µL dilution was prepared in water with 0.1% formic acid. A mixture of 20 isotopically labeled synthetic peptides (PepCalMix, MS Synthetic Peptide Calibration kit, SCIEX) was added to a final concentration of 0.5 fmol/µL for each.

Chromatography: A trap-and-elute microflow LC workflow was set up on a SCIEX M5 MicroLC TE ultralow flow system. A Phenomenex trap column was used (MicroTrap C18, 10 x 0.5 mm, P/N: 05N-4252-AF). With the 20- and 45-minute gradients, a Phenomenex Kinetex XB-C18 (150 x 0.3 mm, P/N: 00F-4496-AC) analytical column was used at 5 µL/min, while with the 5-minute gradient, a Kinetex XB-C18 (50 x 0.3 mm, P/N: 00A-4496-AC) column was used at 10 µL/min. Mobile phases A and B were water and acetonitrile, respectively, each with 0.1% formic acid. Trapping and desalting were performed for 1 minute at 50 µL/min with mobile phase A.

For the 20- and 45-minute gradients, the gradient was from 3% B to 35% B, followed by a 1-minute ramp to 80% B, a 2-minute wash at 80% B, a ramp down to 3% B over 1 minute and a 6-minute re-equilibration. Figure 3 shows the gradient table and graph for the 45-minute gradient method programmed in SCIEX OS software.

The 5-minute gradient was from 3% B to 33% B. After 5 minutes, the trap column was switched back online with the gradient loading pump. The trap was washed with 80% B for 30 seconds at 50 µL/min and then was re-equilibrated with mobile phase A. The analytical column was not cleaned and was re-equilibrated during the trap washing. Analytical column washing was unnecessary, as the sample was loaded on the separate trap column and switched offline from the column for washing before any hydrophobic contaminants were eluted to the analytical column. The analytical column was held at 40°C. A 50 µL loop was used for 1.25-40 µL injections. Triplicate injections were run for each sample.

Mass spectrometry: The ZenoTOF 7600 system was operated in positive mode using the OptiFlow Turbo V ion source with a microflow probe and 1-10 µL/min electrode. The source conditions were Gas 1 60 psi, Gas 2 20 psi, curtain gas 35 psi and temperature 225°C. The electrospray voltage was 4,500 V at 5 µL/min and 5,500 V at 10 µL/min. The Zeno SWATH DIA parameters used with the different gradients are listed in Table 1. The declustering potential was 80 V, CAD gas was 7 and time bins to sum was set to 8 for all gradients. The collision energy for TOF MS was 10 V. For MS/MS, the dynamic collision energy for charge state 2 was used.

Data processing: Zeno SWATH DIA data were processed using DIA-NN software, version 1.8.1[2]. Searches were performed using a combined K562/HeLa spectral library generated previously using high-pH fractionation followed by data-dependent acquisition (DDA) analysis on the Zeno TOF 7600 system[3]. A 1% false discovery rate (FDR) was used for all searches. The resulting DIA-NN software output files were further processed with Excel to calculate the number of identified proteins in at least 2 of the 3 replicates and the number of proteins quantified with CVs >20%. The Analytics module of SCIEX OS software was used to analyze 15 of the spiked-in PepCalMix peptides. For each PepCalMix peptide, the most intense fragment of the +2 charge state was used to extract ion chromatograms to determine retention time and peak area reproducibility.

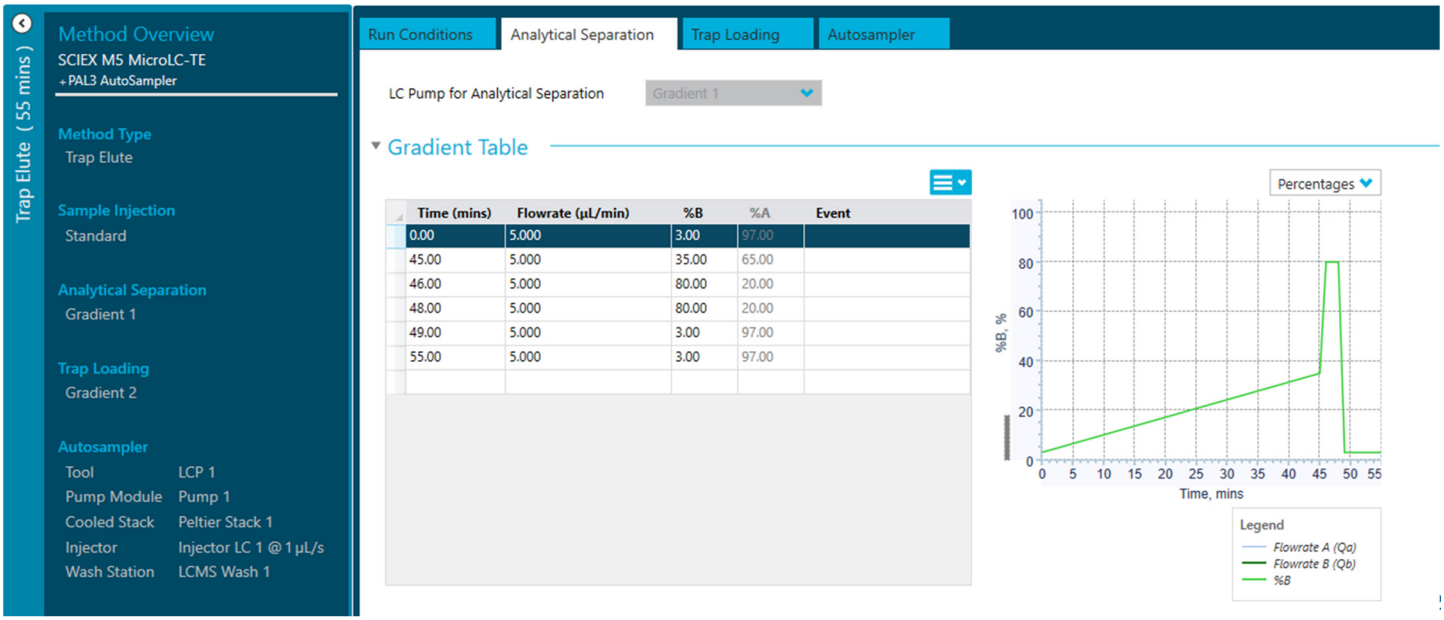

Figure 3. Method table in SCIEX OS software for the 45-minute gradient.

Table 1. MS parameters for the different gradient methods.

Parameter	45-min	20-min	5-min
TOF MS range (m/z)	*400 - 1,500*	*400 - 1,500*	*400-1,500*
TOF accumulation (s)	*0.1*	*0.1*	*0.05*
MS/MS range (m/z)	*140 -1,800*	*140-1,750*	*140-1,750*
*# variable Zeno SWATH DIA windows**	*85*	*65*	*56*
MS/MS accumulation (s)	*0.013*	*0.013*	*0.012*
Total scan time (s)	*1.63*	*1.28*	*1.02*

** The windows used are available on request.*

Identification and quantitation of proteins using different length gradients

Figure 4 shows the total ion chromatograms (TICs) for the different injected amounts of K562 digest using the 5-minute gradient. Figure 1 summarizes the number of protein groups identified and quantified with a CV <20% in at least 2 of 3 replicates for the different gradient lengths and sample loads. For the 400 ng sample load, the 45-minute gradient resulted in the most identifications (6,253 protein groups), although the number of identified protein groups was similar to the 20-minute gradient (6,099). With the 5-minute gradient, 4,858 protein groups were identified. This is 78% of the number measured for the 45-minute gradient but the throughput was more than 8 times higher, as the total cycle time for the 5-minute gradient was 7 minutes. At the lowest (12.5 ng) load, the most protein groups were identified with the 5-minute gradient. This is likely due to the narrow chromatographic peaks and short column that result in spectra with better S/N ratios for the at this low injected amount. Approximately 83-86% of the identified protein groups at the 400 ng load could be quantified with a CV <20% using the different methods. In comparison, ~50-58% of protein groups were quantifiable with a CV <20% at the lowest 12.5 ng load.

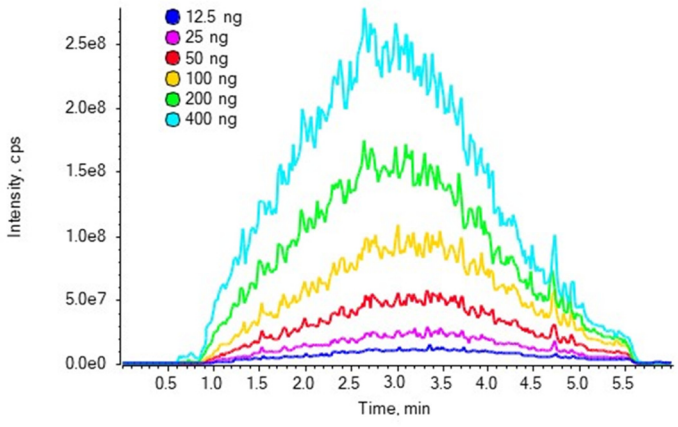

Figure 4. TICs of K562 digest injections at different loads analyzed with the 5-minute gradient method.

Reproducibility of retention times and MS/MS peak areas from 20- and 5-minute gradients

45 replicate injections of 50 ng K562 digest (5 µL injection volume) were made with the 20- and 5-minute methods to determine the retention time and MS/MS peak area reproducibility of the LC-MS system. Figure 5 shows an example of the chromatography for the 20-minute gradient, and figure 6 plots the retention times for all 45 runs. Table 2 summarizes all reproducibility data. High retention time reproducibility was achieved with CVs <0.47% for the 20-minute gradient and <1.6% for the 5-minute gradient. In addition, high peak area reproducibility was achieved, indicated by CVs <13% and <17% for the 20-minute and 5-minute gradients, respectively.

Table 2. Retention time and peak area reproducibility for PepCalMix peptides.

	20-min		5-min	
Peptide	Retention time CV (%)	Area CV (%)	Retention time CV (%)	Area CV (%)
AETSELHTSLK	*0.306*	*7.17*	*1.08*	*12.1*
AGLIVAEGVTK	*0.314*	*5.87*	*0.643*	*8.03*
AVGANPEQLTR	*0.263*	*6.46*	*0.890*	*9.78*
DGTFAVDGPGVIAK	*0.202*	*7.32*	*0.556*	*10.6*
GAYVEVTAK	*0.283*	*9.39*	*1.00*	*10.3*
GFTAYYIPR	*0.182*	*7.07*	*0.579*	*10.6*
IGNEQGVSR	*0.473*	*12.7*	*1.62*	*17.3*
LDSTSIPVAK	*0.309*	*7.10*	*0.779*	*11.4*
LGLDFDSFR	*0.138*	*5.82*	*0.422*	*9.03*
LVGTPAEER	*0.331*	*6.90*	*1.16*	*9.42*
SAEGLDASASLR	*0.317*	*9.21*	*0.799*	*10.3*
SGGLLWQLVR	*0.124*	*5.61*	*0.379*	*8.88*
VFTPLEVDVAK	*0.158*	*5.47*	*0.497*	*8.92*
YDSINNTEVSGIR	*0.370*	*13.2*	*0.717*	*17.5*
YIELAPGVDNSK	*0.306*	*5.87*	*0.619*	*8.82*

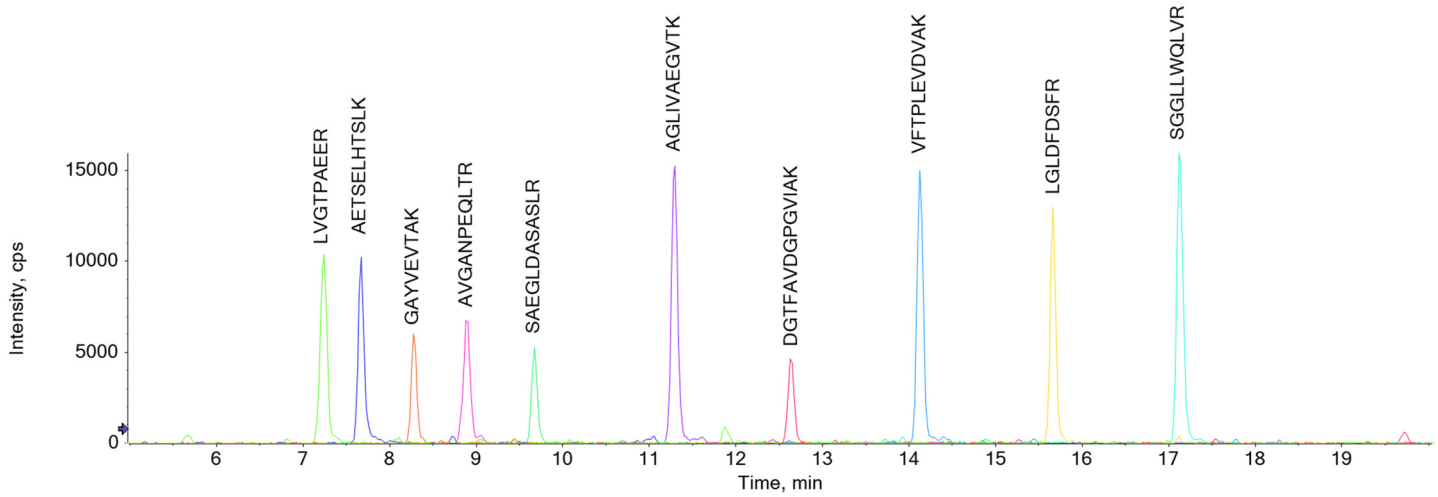

Figure 5. XICs of the MS/MS fragments for 10 of the PepCalMix peptides. These data are from a 50 ng K562 sample spiked with 2.5 fmol of each of the PepCalMix peptides.

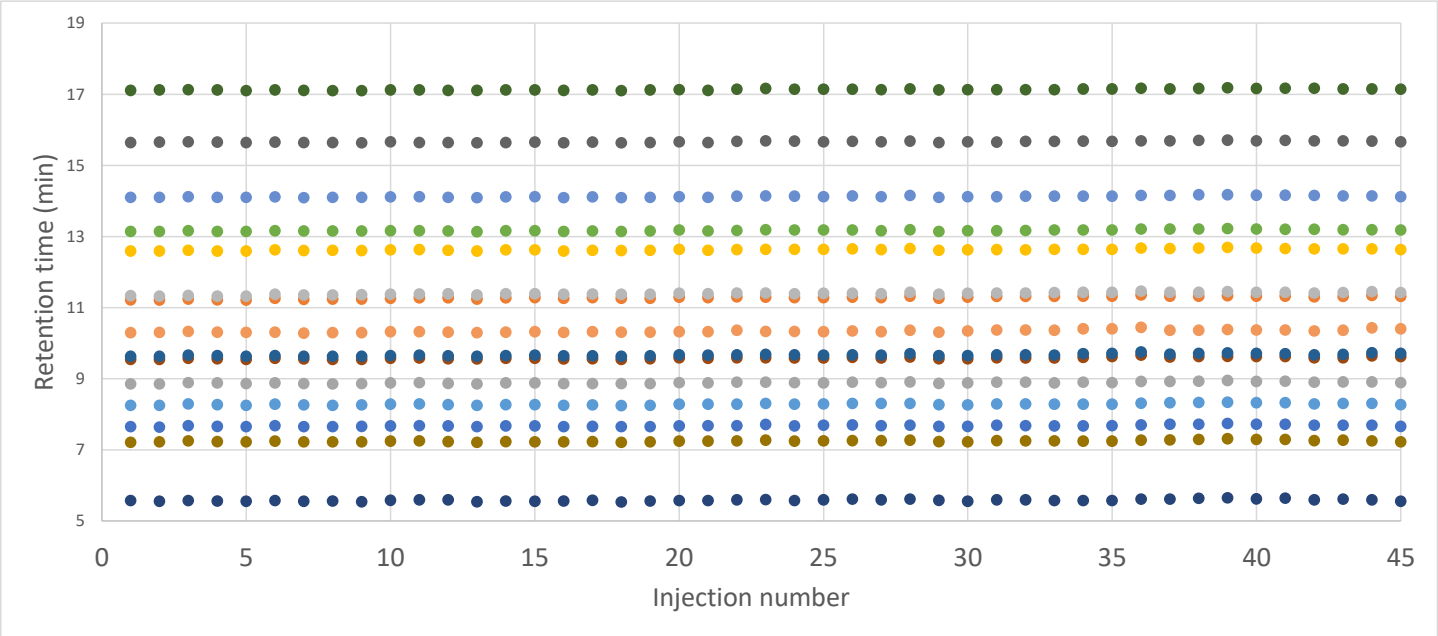

Figure 6. Retention time reproducibility for the 20-minute gradient. The retention times for 15 PepCal Mix peptides were plotted as a function of injection number. The CV observed was between 0.12% and 0.47%.

Conclusions

- The M5 MicroLC system is a flexible microflow solution for proteomics experiments, allowing trap-and-elute experiments with a wide range of injection volumes and gradient lengths
- With a 45-minute gradient, 6,253 protein groups were identified in 400 ng of a K562 human cell lysate digest and 83% of the protein groups (5,164) were quantified with a CV <20%
- With a 5-minute microflow gradient (7-minute cycle time), 4,858 protein groups were identified in 400 ng of a K562 human cell lysate and 4,102 protein groups were quantified (CV <20%)
- The 5-minute method provided 8 times higher throughput than the 45-minute method, with ~80% of the protein groups identified and quantified at a sample load of 400 ng
- A minimal reduction in the number of protein groups identified and quantified (<20% CV) was seen with a 20-minute gradient method, compared to the 45-minute gradient method
- At low sample loads, the 5-minute gradient method enabled the identification and quantitation (CV <20%) of more protein groups, compared to the 20-minute or 45-minute gradients
- The M5 MicroLC system coupled with the ZenoTOF 7600 system provides excellent retention time and peak area reproducibility

References

1. High-throughput quantitative proteomics using Zeno SWATH data-independent analysis (DIA) and the Evosep One system. SCIEX technical note, RUO-MKT-02-14799-A.
2. Demichev V *et al.* (2019) DIA-NN: neural networks and interference correction enable deep proteome coverage in high throughput. *Nature Methods*, **17**, 41-44.
3. Large-scale protein identification using microflow chromatography on the ZenoTOF 7600 system. SCIEX technical note, RUO-MKT-02-14415-A.

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices