Sensitive signature peptide quantification in a complex matrix using accurate mass spectrometry

Featuring the ZenoTOF 7600 system and SCIEX OS software

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This technical note describes the achievement of low amol/µL level of quantification for signature peptides in rat plasma, with improvements in MS/MS sampling efficiency on an accurate mass spectrometer. Additionally, with the availability of TOF MS/MS data, further enhancement in sensitivity was achieved by the summation of several highly abundant fragment ions for quantification. As a result, a 2-fold improvement in LLOQ and an overall linear dynamic range (LDR) of greater than 4.3 orders of magnitude (Table 1) was achieved.

Traditional workflows for quantitative bioanalysis of peptides and proteins, such as immunological assays, have been displaced by LC-MS/MS analysis using triple quadrupole mass spectrometers. While the triple quadrupole platform has been the gold standard for most bioanalytical workflows, offering great sensitivity and quantitative performance, accurate mass spectrometry has been increasingly suggested for quantitative bioanalysis.[1,2] However, accurate mass spectrometry platforms, such as traditional time-of-flight (TOF) systems, often lack sensitivity due to limited duty cycle in between TOF pulses.

With the introduction of the ZenoTOF 7600 system, the improvement in MS/MS sampling efficiency offers a robust and sensitive platform to support routine peptide and protein quantification. Here, the Zeno trap controls the ion beam from the collision cell which facilitates greater ion transmission to the TOF accelerator, improving the duty cycle to ≥90% (classical TOF is below 30%). As a result, this enhances the overall MS/MS sampling efficiency enabling the ZenoTOF 7600 system to be highly advantageous for quantitative bioanalysis workflows that can benefit from the accessibility of the full product ion profile and the improvement in sensitivity using the Zeno trap.[3,4]

In this technical note, 3 peptides were selected as model analytes to evaluate the quantitative performance of signature peptides on the ZenoTOF 7600 system. Ultra-low LLOQs, ranging from 0.025 fmol/µL to 0.05 fmol/µL were achieved with an LDR greater than 4.3 orders of magnitude. The measured upper limit of quantification (ULOQ) ranged from 500 fmol/µL to 2000 fmol/µL. Overall, the assay demonstrated outstanding accuracy, precision and linearity, highlighting the robustness and performance of the developed method for the quantification of signature peptides.

Key features of the signature peptide quantification workflow using the ZenoTOF 7600 system and SCIEX OS software

- Achieve low-amol/µL level quantification of signature peptides in complex matrices using the Zeno MRM^{HR} workflow
- Reach enhanced sensitivity by summation of multiple highly abundant fragment ions with the availability of TOF MS/MS data and improvements in MS/MS sampling efficiency using the Zeno trap
- Confirm GLP-level accuracy and precision for quantitative workflows with greater than 4.3 orders of magnitude in LDR using the ZenoTOF 7600 system
- Easily acquire, process and manage data on a single platform using the SCIEX OS software

Table 1. A summary of the quantitative performance of the signature peptide assay on the ZenoTOF 7600 system.

Peptide Sequence	Charge state	Fragment ions used for quantification	LLOQ (fmol/µL)	ULOQ (fmol/ µL)	LDR (orders)
FNWYVDGVEVHNAK	*3+*	*b2, y6 and y4*	*0.05*	*2000*	*4.6*
*AGLIVAEGVTK**	*2+*	*y6*	*0.025*	*500*	*4.3*
*LGLDFDSFR**	*2+*	*y5, y6 and y8*	*0.025*	*500*	*4.3*

Methods

Sample preparation: Plasma proteins were precipitated with cold methanol. After centrifugation, the supernatant was discarded while the pellet was solubilized in 200 mM ammonium bicarbonate in 10:90 (v/v) methanol/water. Digestion was performed using trypsin. After 1 hour at 60°C, the solution was acidified by adding formic acid.[5] The digested plasma was diluted by 200x using 5:1:94 (v/v/v) acetonitrile/formic acid/water. Synthesized peptides (Table 2) were spiked into the digested plasma solution and followed by serial dilution in the matrix. The final injection volume was 10 µL.

Table 2. List of peptide targets.

Peptide Sequence	Description
FNWYVDGVEVHNAK	*Conserved sequence in human immunoglobulin G (IgG)*
*AGLIVAEGVTK**	*Synthetic peptide with C terminal K heavy isotope labeled ($C^{13}N^{15}$)*
*LGLDFDSFR**	*Synthetic peptide with C terminal R heavy isotope labeled ($C^{13}N^{15}$)*

Chromatography: The separation was performed at a flow rate of 0.4 mL/min using an ExionLC system. A Phenomenex bioZen Peptide XB-C18 column (2.1 x 50 mm, 2.6 µm, 100 Å) was used for separation. The column oven temperature was set to 40°C. The mobile phase A consisted of 0.1% formic acid in water, while the mobile phase B was composed of 0.1% formic acid in acetonitrile. Gradient conditions are summarized in Table 3. A volume of 10 µL was injected for analysis. All samples were analyzed in triplicate.

Table 3. LC gradient conditions.

Time (min)	Mobile phase B (%)	Mobile phase A (%)
0	*97*	*3*
1	*97*	*3*
4	*60*	*40*
4.1	*10*	*90*
5.1	*10*	*90*
6.1	*97*	*3*
8	*97*	*3*

Mass spectrometry: Data were acquired in positive mode using Zeno MRM^{HR} on a ZenoTOF 7600 system. Collision energy (CE), source and MS parameters were optimized for all the signature peptides. A summary of the source and MS parameters and the Zeno trap settings is displayed in Table 4. Additionally, the MRM^{HR} parameters and fragments used for quantification for each of the signature peptides are summarized in Table 5. Unit Q1 resolution was used for the analysis.

Table 4. Source and MS conditions.

Parameter	Value	Parameter	Value
Curtain gas	*30 psi*	*Source temp.*	*600˚C*
Ion source gas1	*50 psi*	*Ion source gas 2*	*60 psi*
CAD gas	*7*	*Ion spray voltage*	*5500 V*
MS accumulation time	*80 ms*	*MS/MS accumulation time*	*10 ms*
TOF MS start mass (m/z)	*400*	*TOF MS stop mass (m/z)*	*800*
TOF MS/MS start mass (m/z)	*100*	*TOF MS/MS stop mass (m/z)*	*1200*
Zeno threshold	*20,000 cps*		

Table 5. MRM^{HR} parameters and fragments used for quantification.

Peptide	Q1 mass (*m/z*)	Fragment mass (*m/z*)	DP (V)	CE (V)
FNWYVDGVEVHNAK (b2)	*560.27*	*262.118*	*80*	*30*
FNWYVDGVEVHNAK (y4)	*560.27*	*469.252*	*80*	*30*
FNWYVDGVEVHNAK (y6)	*560.27*	*697.363*	*80*	*30*
AGLIVAEGVTK (y6)*	*533.32*	*612.344*	*80*	*32*
LGLDFDSFR (y5)*	*540.27*	*681.322*	*80*	*34*
LGLDFDSFR (y6)*	*540.27*	*796.351*	*80*	*34*
LGLDFDSFR (y8)*	*540.27*	*966.457*	*80*	*34*

Data processing: Data were processed using the Analytics module in SCIEX OS software with the MQ4 integration algorithm. A $1/x^2$ weighting was used for quantification.

Zeno trap provides greater sensitivity

In traditional TOF MS/MS acquisition, ions are lost between TOF MS spectra acquisitions, resulting in a much lower MS/MS sampling efficiency and sensitivity than MRM on a triple quadrupole mass spectrometer. The Zeno trap increases ion transmission by providing control of the ion beam from the collision cell into the TOF accelerator (Figure 1).

Ions are gated then released based on potential energy. Generally, higher *m/z* ions are released first then followed by lower *m/z* ions. A wide range of ions now arrive in the accelerator to be pushed during the same pulse resulting in a 10-fold increase in MS/MS sampling efficiency.[6]

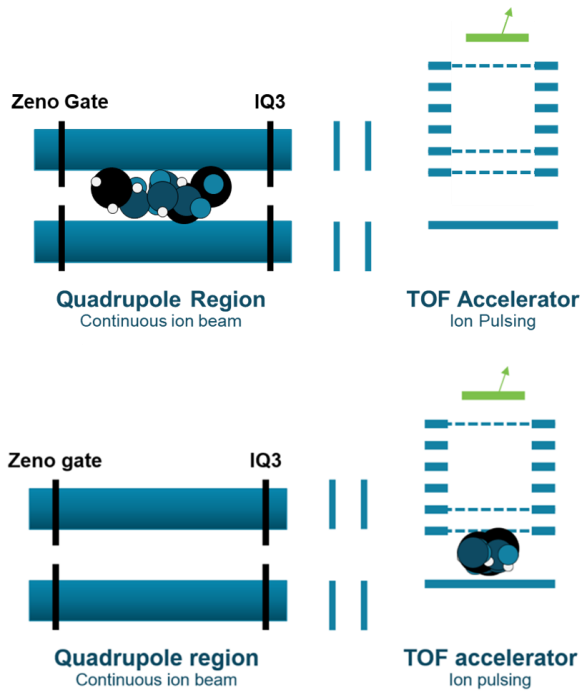

Figure 1. Zeno trap enables ion beam control from the collision cell prior to entrance into the TOF accelerator. Gains in ion transmission improve overall MS/MS sampling efficiency.

Overview of the signature peptide workflow

FNWYVDGVEVHNAK, AGLIVAEGVTK* and LGLDFDSFR* were used as model peptides to evaluate the quantification of signature peptides on the ZenoTOF 7600 system. The peptides were spiked into processed rat plasma at concentrations ranging from 0.025 fmol/µL to 2000 fmol/µL.

Quantification was performed using the following strategies: 1) using the most sensitive fragment ion and 2) using the sum of multiple highly abundant fragment ions. For the former method, fragment ions y6, y8 and y6 were used for the quantification of peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. For the latter strategy, the XIC responses from fragment ions y5, y6 and y8 were added for the quantification of peptide LGLDFDSFR*. For the quantification of peptide FNWYVDGVEVHNAK, the XIC responses from fragment ions b2, y4 and y6 were added.

All calibration points were measured in triplicate. For the determination of the LLOQ, a %CV value of less than 20% and accuracy between 80% and 120% was required. For all other concentrations, a %CV value of less than 15% and accuracy between 85% and 115% of the nominal concentration was required. This criteria was applied to both quantification strategies.

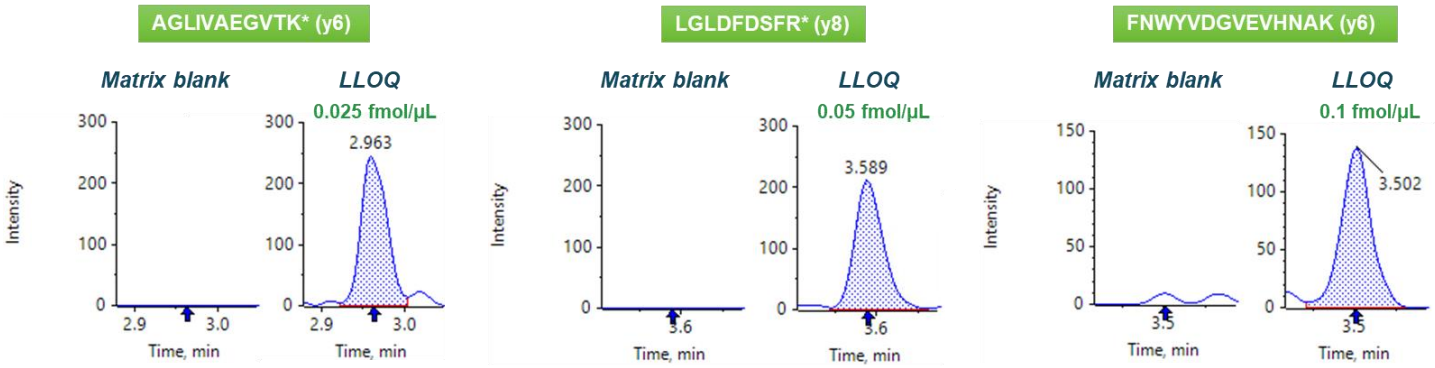

Figure 2. Extracted ion chromatograms (XICs) of the matrix blank and LLOQ using Zeno MRM^{HR}. Using the single fragment ion approach, an LLOQ of 0.025 fmol/µL, 0.05 fmol/µL and 0.1 fmol/µL was achieved for peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No matrix interferences were observed in the blank.

Quantification using a single fragment ion

In this workflow, quantification of signature peptides was performed using the Zeno MRM^{HR} workflow. For the following discussion, the most abundant fragment ion was used for the quantification of the signature peptides.

LLOQs of 0.025 fmol/µL, 0.05 fmol/µL and 0.1 fmol/µL were achieved for peptides AGLIVAEGVTK*, LGLDFDSFR* and FNWYVDGVEVHNAK, respectively (Figure 2). No interferences were observed in the matrix blank.

The linear range for the single fragment ion approach covered concentrations from 0.025 fmol/µL to 2000 fmol/µL (Figure 3). An LDR greater than 4 orders of magnitude was observed for each of the calibration curves.

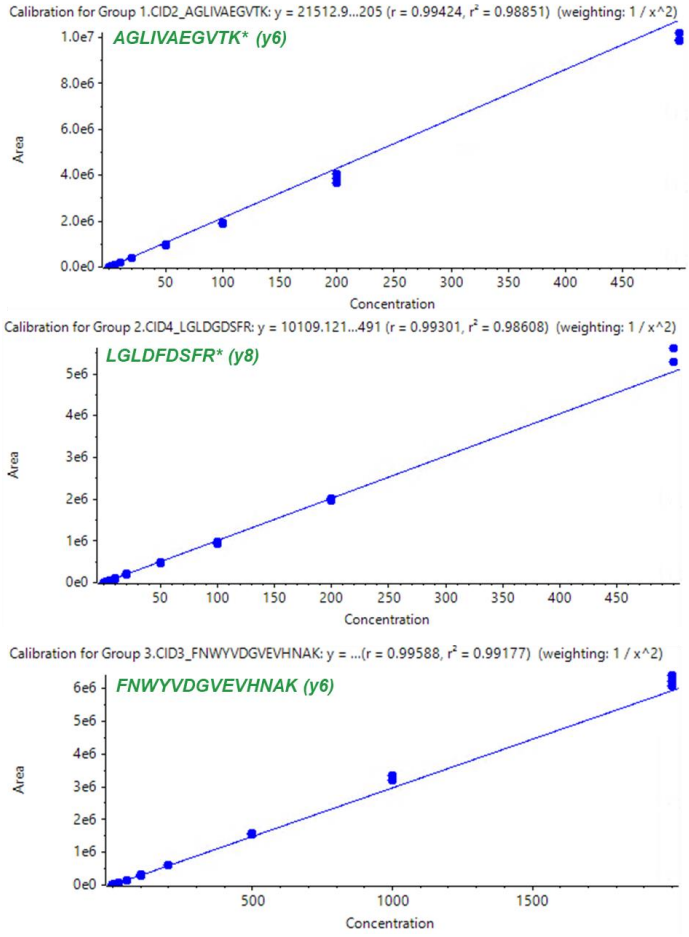

Figure 3. Calibration curves for the signature peptides using single fragment ion quantification. The linear range covered 0.025 fmol/µL to 2000 fmol/µL with an overall LDR of greater than 4 orders of magnitude.

Calculated concentrations for each calibration point were within ±15% of the nominal value (Table 6). The overall accuracy of the LLOQ was within 8% of the nominal concentration, indicating a highly accurate quantification platform for peptides at low concentrations. As shown in Table 6, the %CV value was less than 15%, demonstrating high reproducibility.

Increasing sensitivity using the summation of multiple fragment ions

The accessibility of TOF MS/MS data can be highly advantageous, as fragment ions can be selected based on overall selectivity and sensitivity for quantification. When multiple highly abundant fragment ions are summed from the target peptide, the assay sensitivity can be further enhanced.[3]

The linear range for the multiple fragment ion approach covered concentrations from 0.025 fmol/µL to 2000 fmol/µL (Figure 4). Strong linearity was observed for each of the calibration curves with an LDR of greater than 4.3 orders of magnitude.

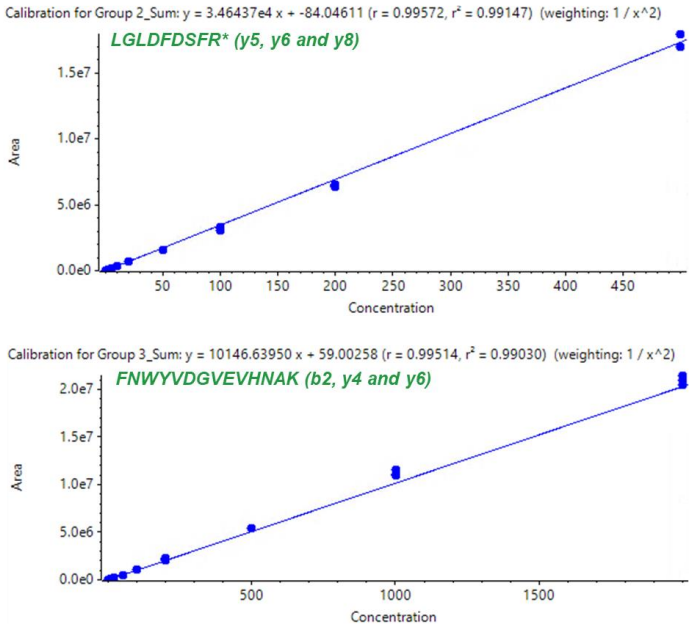

Figure 4. Calibration curves for the signature peptides using multiple fragment ion quantification. The linear range covered 0.025 fmol/µL to 2000 fmol/µL with an overall LDR of greater than 4.3 orders of magnitude.

Table 6. Calculated concentration, precision and accuracy for quantification using a single fragment ion.

	AGLIVAEGVTK*		LGLDGDSFR*		FNWYVDGVEVHNAK	
Concentration (fmol/µL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.025	101.81	13.12	NA	NA	NA	NA
0.05	98.26	14.60	107.89	10.46	NA	NA
0.1	95.82	5.26	114.93	8.42	103.95	13.79
0.2	98.11	7.86	100.17	6.97	NA	NA
0.5	105.30	9.69	100.96	10.42	84.57	12.94
1	99.68	2.91	95.81	1.40	96.83	10.51
2	101.97	3.42	101.73	4.07	91.76	8.17
5	109.58	0.72	99.81	1.44	92.60	4.49
10	104.35	2.45	96.09	7.08	99.28	1.90
20	103.73	4.36	97.55	1.58	102.25	2.49
50	96.32	4.19	92.26	1.74	101.98	1.44
100	94.53	0.81	92.91	4.23	103.62	3.11
200	93.93	4.38	95.87	1.20	103.57	1.77
500	96.61	2.16	104.02	3.42	104.99	0.79
1000	NA	NA	NA	NA	109.50	2.75
2000	NA	NA	NA	NA	105.10	2.56

A 2-fold improvement in LLOQ was achieved when multiple highly abundant fragment ions were summed for quantification (Figure 5). LLOQs of 0.025 fmol/µL and 0.05 fmol/µL were achieved for peptides LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No interferences were observed in the matrix blank.

Calculated concentrations for each calibration point were within ±15% of the nominal value (Table 7). The overall accuracy was within 8% of the nominal concentration at the level of the LLOQ, indicating a highly accurate platform for low-level quantification. As shown in Table 7, the overall %CV value for the acquired data, including the LLOQ, was less than 20%.

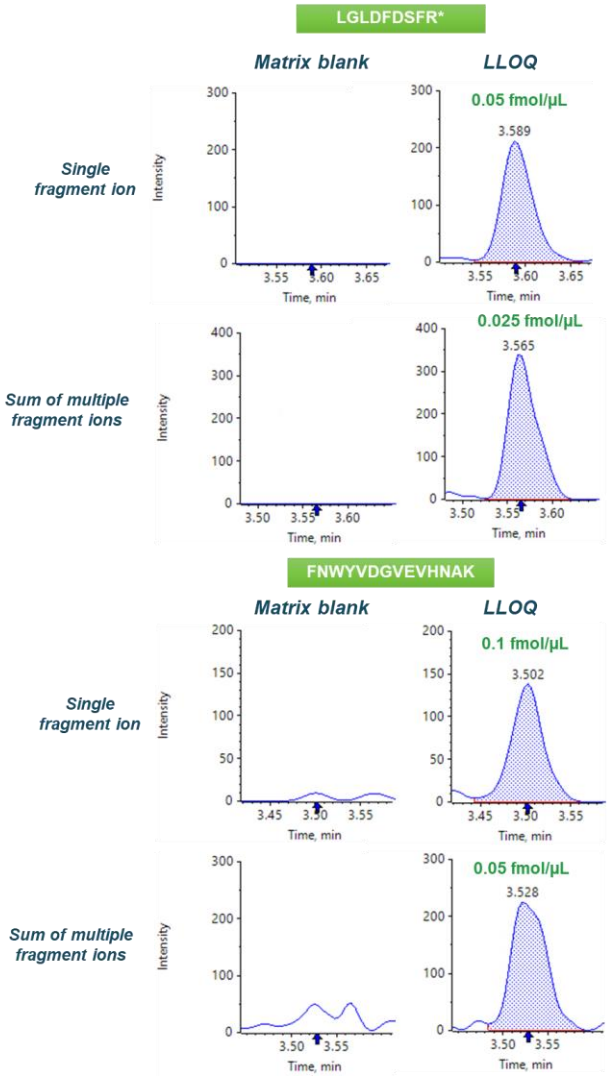

Figure 5. XICs of the matrix blanks and spiked peptides at the LLOQ using summation of multiple fragment ions. A 2-fold improvement in sensitivity was observed when multiple highly abundant fragment ions were summed for quantification. LLOQs of 0.025 fmol/µL and 0.05 fmol/µL were reached for the peptides LGLDFDSFR* and FNWYVDGVEVHNAK, respectively. No matrix interferences were observed in the blank.

Table 7. Calculated concentration, precision and accuracy for quantification using summation of multiple fragment ions.

	LGLDGDSFR*		FNWYVDGVEVHNAK	
Concentration (fmol/µL)	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
0.025	93.84	19.71	NA	NA
0.05	107.80	8.65	107.08	4.45
0.1	106.56	2.66	90.44	9.57
0.2	100.82	5.95	NA	NA
0.5	106.00	8.53	85.74	3.14
1	103.68	1.75	88.83	6.67
2	111.02	4.43	89.43	4.66
5	100.69	2.56	90.38	3.45
10	95.64	5.60	103.43	2.71
20	97.52	1.32	107.77	2.87
50	91.46	1.23	104.44	1.23
100	91.87	4.85	105.68	1.50
200	93.23	1.35	106.39	4.00
500	99.85	3.24	106.89	0.80
1000	NA	NA	110.21	2.56
2000	NA	NA	103.28	2.33

Conclusions

- A highly sensitive signature peptide quantification workflow was developed using the ZenoTOF 7600 system
- Low-amol/µL levels of quantification for signature peptides were reached using a Zeno MRM^{HR} workflow
- Summation of multiple fragment ions enhances the LLOQ up to 2-fold with the availability of TOF MS/MS data and improvements in MS/MS sensitivity using the Zeno trap
- GLP-level accuracy and precision for signature peptide quantification was achieved with greater than 4.3 orders of magnitude in LDR

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