

# The Quantitative Power of SCIEX Triple Quad and the QTRAP® 6500+ LC-MS/MS System for the Bioanalysis of Biotherapeutics

**Featuring the SCIEX Triple Quad™ and QTRAP 6500+ Systems with IonDrive™ Turbo V Ion Source and SCIEX OS Software 1.6.1**

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With a rising number of biopharmaceuticals on the horizon, there is also an increasing demand for the bioanalysis of such analytes. Supporting the discovery and development for a range of compounds, bioanalysis via liquid chromatography coupled to mass spectrometry (LC-MS) shows advantages over traditional ligand binding assays such as multiplexing possibilities, wider dynamic range, higher specificity as well as higher selectivity to name a few.

While different approaches of LC-MS based quantitation are being investigated by scientists, quantitation using peptides as surrogates (bottom-up approach) via MRMs is still the widest applied approach being used. This approach not only offers a very high sensitivity with low limits of quantitation, but also a great linear dynamic range combined with low CVs to ensure confidence in reliable results.

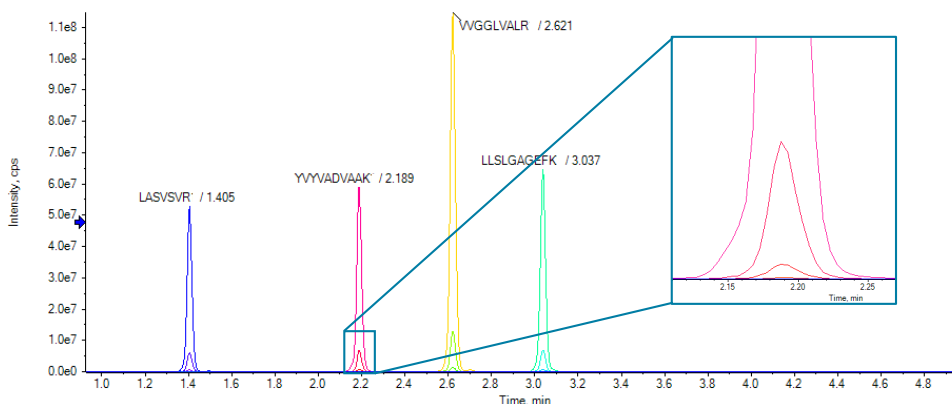
Here, the suitability of the SCIEX Triple Quad/QTRAP 6500+ System for the analysis of peptides in positive as well as negative ionization mode was investigated. Furthermore, the intra-spectra dynamic range of isotopic peptides eluting at the same retention time was determined (Figure 1).



**ExionLC™ UHPLC system coupled to the SCIEX QTRAP 6500+ LC-MS/MS System and SCIEX OS Software 1.6.1.**

## Key Features of the Bioanalysis of BioTherapeutics

- The SCIEX Triple Quad and QTRAP 6500+ Systems offer superior quant power for Bioanalysis reaching low LLOQs, wide LDR and tight CVs
- IonDrive Turbo V Ion Source ensure efficient heat transfer for maximal ionization for both positive and negative ionization mode
- Comprehensive quantitation with SCIEX OS-MQ combining ease-of-use with confidence in the results



**Figure 1. Extracted ion chromatograms (XICs) for all peptides and their isotopologues for the 6x5 LC-MS/MS peptide reference mix. Right hand side: Exemplary zoom-in to lower abundant isotopologues for the peptide YVYADVAAK.**

## Methods

Three different experiments were performed in order to demonstrate the quantitative power of the SCIEX Triple Quad and QTRAP 6500+ Systems for the bioanalysis of biopharmaceuticals: MRM methods were optimized for the 6×5 LC-MS/MS Peptide Reference mix from Promega. This reference mixture was used to show instrument's intra-spectra linear dynamic range and sensitivity. Furthermore methods for signature peptides in plasma matrix were setup and a dilution curve was analyzed showing the instruments power for quantitation in positive, as well as negative ionization mode within a real life sample matrix. All measurements were performed in triplicates.

**Sample Preparation for the 6×5 Peptides Reference Mix:** The 6×5 LC-MS/MS peptide reference mix was obtained from Promega and diluted in 5% acetonitrile/1% formic acid (v/v) by a factor of five. Final injection volume was 5 µL which is equal to 1 pmol on column for the highest abundant isotopologue.

**Sample Preparation for Peptides in Matrix:** Rat plasma was digested according to Ouyang, Z et al.<sup>1</sup> Briefly, plasma proteins were precipitated with cold methanol. Upon centrifugation, supernatant was discarded. The pellet was resolved in 200 mM ammonium bicarbonate in 10 % (v/v) methanol:water. Digestion was performed using trypsin. After one hour at 60°C, the solution was acidified by adding formic acid. The digested plasma was diluted by 200× using 5 % acetonitrile / 1 % formic acid (v/v). Synthesized peptides (Table 9) were spiked into the digested plasma solution and followed by serial dilution in matrix. Final injection volume was 10 µL.

**Chromatography:** Details for the chromatography are listed in Table 1-3.

Table 1. Chromatographic Conditions for all Analyses.

Parameter	Value
Column	Phenomenex bioZen Peptide XB-C18 50×2.1 mm; 2.6 µm
Mobile Phase A	Water with 0.1 % formic acid
Mobile Phase B	Acetonitrile with 0.1 % formic acid
Flow Rate	500 µL/min
Column Temperature	40 °C
Injection Volume	5 µL / 10 µL

Table 2. Gradient for the 6×5 LC-MS/MS Peptide Reference Mix.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
0.0	95	5
3.5	75	25
3.6	10	90
4.1	10	90
4.2	95	5
5.0	95	5

Table 3. Gradient for the Peptides in Matrix.

Time [min]	Mobile Phase A [%]	Mobile Phase B [%]
0.0	95	5
0.5	95	5
5.5	60	40
5.6	10	90
6.0	10	90
6.1	95	5
7.0	95	5

**Mass Spectrometry:** The source conditions were optimized for peptide analysis in positive and negative ionization mode for the respective flow rate (see Table 4). Suitable transitions were chosen for each peptide and each isotopologue and the declustering potential (DP), collision energy (CE) and the cell exit potential (CEP) were tuned for best intensity (see Tab. 8 and 9 in the appendix).

**Data Processing:** Data were processed with SCIEX OS 1.6.1. Peak integration was performed using the MQ4 algorithm.

Table 4. MS Parameters.

Parameter	Positive Mode	Negative Mode
GS1	65 psi	65 psi
GS2	65 psi	65 psi
CUR	35	35
TEM	500°C	500°C
IS	5500 V	-4500 V
CAD	high	high

## Intra-Spectrum Dynamic Range Evaluation with 6×5 LC-MS/MS Peptide Reference Mix

The peptide mix consists of several peptides with different hydrophobicities spanning gradients commonly used for peptide analysis. For each peptide there are five isotopologues of the same peptide sequence present which differ in the number of stable heavy-labeled amino acids (<sup>13</sup>C, <sup>15</sup>N). Furthermore, these five isotopologues differ in the molar ratios covering four orders of magnitude (Figure 1) and therefore being well suited to assess LC-MS performance for the analysis of peptides.

The whole range of isotopologues could be quantified with the SCIEX Triple Quad 6500+ System instrument while keeping CVs tight and achieving a great linearity (Figure 2, Table 5). From a signal-to-noise perspective, the 6500+ shows the potential to detect and quantify lower amounts and span a range of over five orders of magnitude (Figure 3).

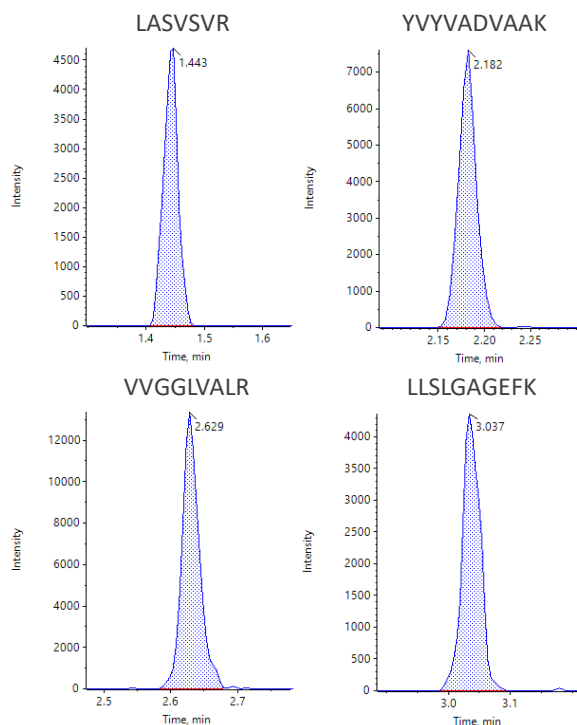


Figure 3. XICs for the lowest isotopologue of 0.0001× of the 6×5 LC-MS/MS peptide reference mix for each of the peptides being monitored.

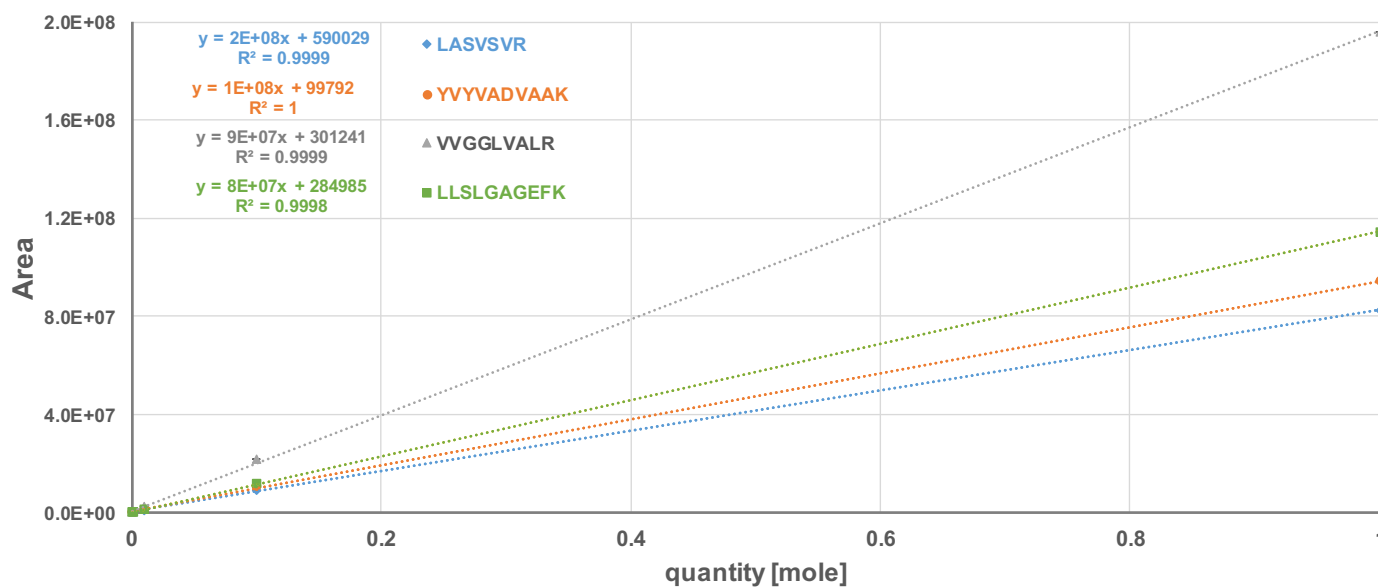
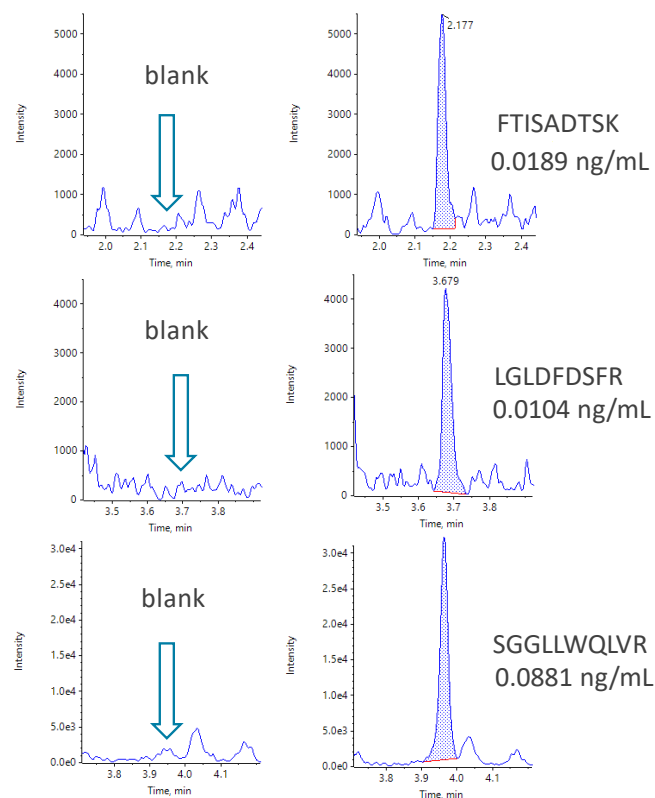


Figure 2. Quantitation Results for the 6×5 LC-MS/MS Peptide Reference Mix. Mean areas of the XICs of each transition versus the molar quantity of each isotopologue for the four different peptides being monitored. A linear fit was applied to each isotopologic dilution with R<sup>2</sup> value being indicated in the graph (n = 3).

**Table 5. Quantitation Results for the 6×5 LC-MS/MS Peptide Reference Mix (n = 3).**

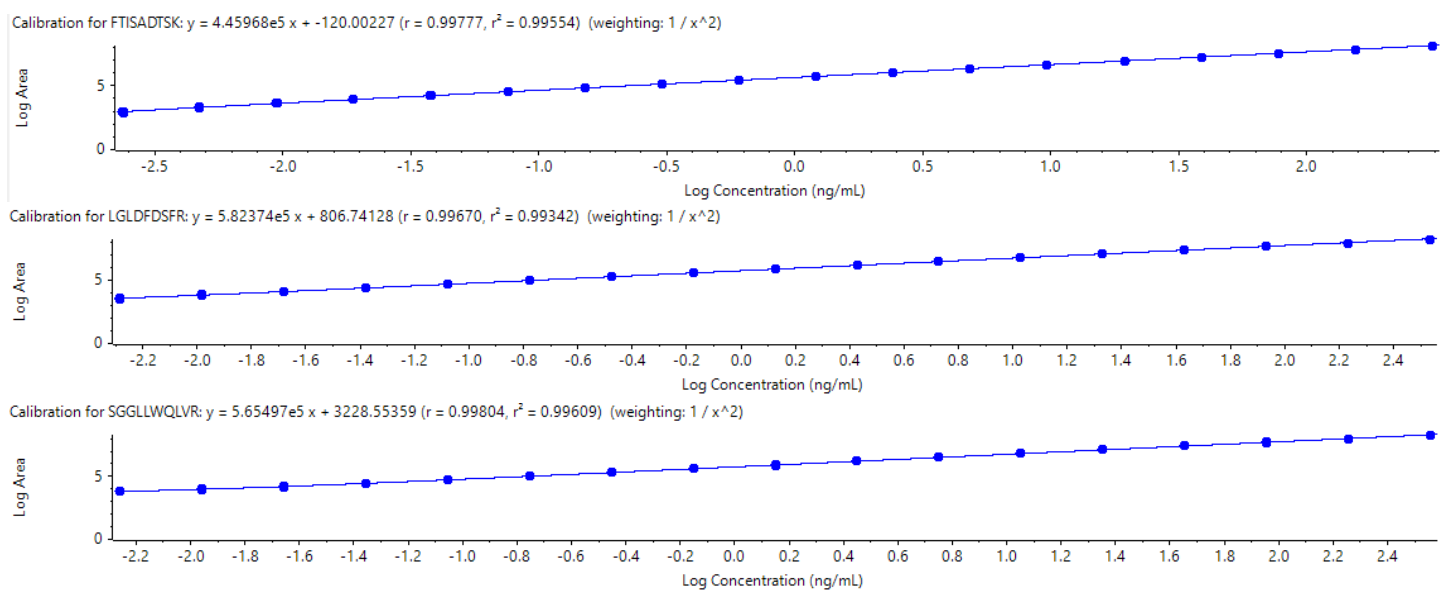
peptide	molar ratios	Area <sub>mean</sub>	%CV
LASVSVR	1	8.3E+07	2.9
	0.1	9.3E+06	3.8
	0.01	9.8E+05	2.0
	0.001	8.8E+04	6.3
	0.0001	9.0E+03	6.5
YVYVADVAAK	1	9.4E+07	3.0
	0.1	1.0E+07	1.4
	0.01	1.1E+06	2.1
	0.001	1.1E+05	8.4
	0.0001	1.0E+04	18.2
YVYVADVAAK	1	2.0E+08	2.7
	0.1	2.2E+07	2.3
	0.01	2.3E+06	1.4
	0.001	2.3E+05	5.2
	0.0001	2.3E+04	1.9
LLSLGAGEFK	1	1.1E+08	1.0
	0.1	1.2E+07	1.7
	0.01	1.2E+06	2.7
	0.001	1.2E+05	5.2
	0.0001	1.1E+04	13.5


**Figure 4. XICs for blanks and respective LLOQ (S/N ~ 10) side by side for the peptides FTISADTSK, LGLDFDSFR and SGLLWQLVR in plasma matrix monitored in positive ionization mode.**

## Peptide Quantitation in Plasma Matrix (Positive Ionization Mode)

Detecting and quantifying analytes in biological matrices such as plasma can be much more challenging than in neat solvent. To evaluate the instrument quantitation performance when analyzing matrix samples, synthetically produced peptides from the SCIEX PepCal Mix (LGLDFDSFR\* and SGLLWQLVR\*; P/N 5045759) as well as one synthetic signature peptide derived from an antibody sequence (FTISADTSK) were spiked into digested rat plasma.

Even in the challenging matrix, a linear dynamic range of up to 5 orders of magnitude with great accuracy and %CV was achieved (Figure 5 and Table 6). The peptide concentrations down to 0.0104 ng/mL have been detected reproducibly making the instrument perfectly suitable for overcoming the challenges during the bioanalysis of biopharmaceuticals. The XICs for the respective LLOQs and matrix blanks are shown in Figure 4.



**Figure 5. Quantitation Results for the Peptide FTISADTSK, LGLDFDSFR and SGLLWQLVR in Plasma Matrix in Positive Mode.** Log-log plot of XIC areas versus the concentration in ng/mL. A linear fit was applied to all calibration curves using a  $1/x^2$  weighting. Regression details and  $R^2$  value being indicated in the graph for each peptide ( $n = 3$ ).

**Table 6. Quantitation Results for the Peptides in Plasma Matrix in Positive Ionization Mode.** Accuracy, %CV and linear dynamic range (LDR) for each concentration per peptide ( $n = 3$ ).

FTISADTSK				LGLDFDSFR				SGLLWQLVR			
conc. [ng/mL]	Accuracy	%CV	LDR	conc. [ng/mL]	Accuracy	%CV	LDR	conc. [ng/mL]	Accuracy	%CV	LDR
310	87.8	2.5		342	82.1	0.7		361	88.7	0.9	
155	94.1	1.3		171	91.9	1.1		180	97.5	1.3	
77.5	96.7	1.3		85.5	97.1	1.8		90.2	100.5	1.5	
38.7	98.5	1.0		42.7	100.1	1.2		45.1	102.8	1.1	
19.4	99.7	2.0		21.4	102.3	2.0		22.6	103.2	1.0	
9.68	100.1	2.5		10.69	103.0	1.4		11.28	103.9	0.5	
4.84	100.7	1.1		5.34	103.3	0.4		5.64	103.5	2.0	
2.42	100.4	2.0		2.67	104.7	0.8		2.82	103.6	0.4	
1.21	99.7	1.2	5.1	1.34	104.0	1.0	4.8	1.41	100.3	2.8	4.8
0.605	100.5	2.1		0.668	102.6	2.2		0.705	102.0	1.5	
0.303	100.8	1.0		0.334	103.9	0.8		0.352	103.5	2.1	
0.151	101.5	1.8		0.167	104.7	3.7		0.176	98.8	4.9	
0.0757	104.3	1.3		0.0835	102.9	3.8		0.0881	95.7	2.3	
0.0378	106.9	6.0		0.0417	104.6	4.0		0.044	96.4	1.6	
0.0189	104.1	4.4		0.0209	94.4	6.9		0.022	92.2	10.8	
0.00946	102.6	3.9		0.0104	95.9	13.6		0.011	109.5	7.1	
0.00473	106.7	13.2		0.00521	102.2	15.1		0.0055	97.8	2.9	
0.00236	95.7	12.9									

## Peptide Quantitation in Plasma Matrix (Negative Ionization Mode)

Some analytes might not ionize well in positive ion ionization mode or the S/N can be negatively impacted because of matrix interference. In such cases reaching LODs and LLOQs necessary for bioanalytical studies can be a burden to scientists. The versatility of the instrumentation in combination with very strong quantitative power in both modes become even more important as negative ionization mode can be a solution.

Here, the performance for three peptides from the SCIEX PepCal Mix (AVYFYAPQIPLYANK\*, LGLDFDSFR\* and SAEGLDASASLR\*; P/N 5045759) was evaluated in negative ionization mode. Although the linear range is dependent on the analytes, it could be demonstrated that an LDR of up to 4.5 orders of magnitude was achievable (Table 7). Low LLOQs and %CVs could be achieved as well (Table 7). The XICs for the respective LOQs and matrix blanks are shown in Figure 4.

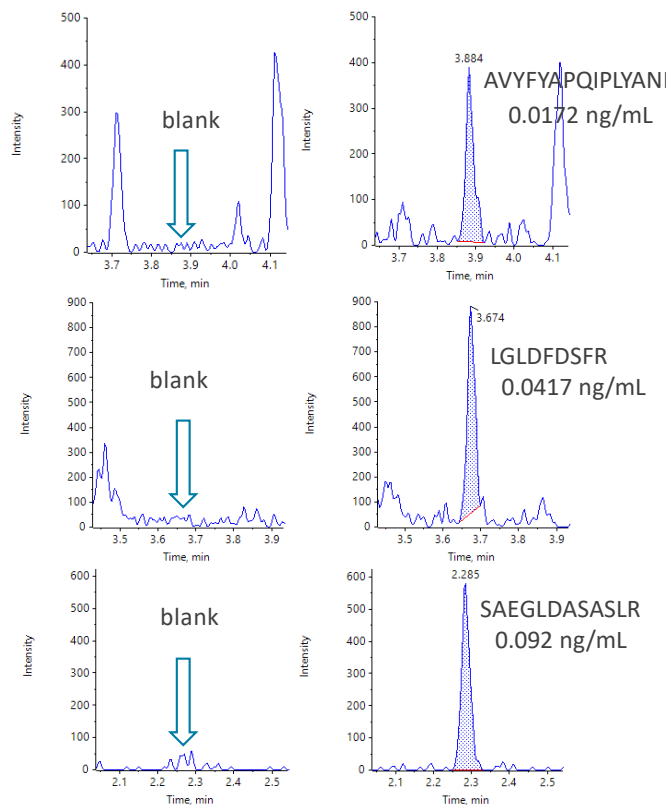


Figure 6. XICs for blanks and respective LLOQ (S/N ~10) side by side for the peptides AVYFYAPQIPLYANK, LGLDFDSFR and SAEGLDASASLR in plasma matrix monitored in positive ionization mode.

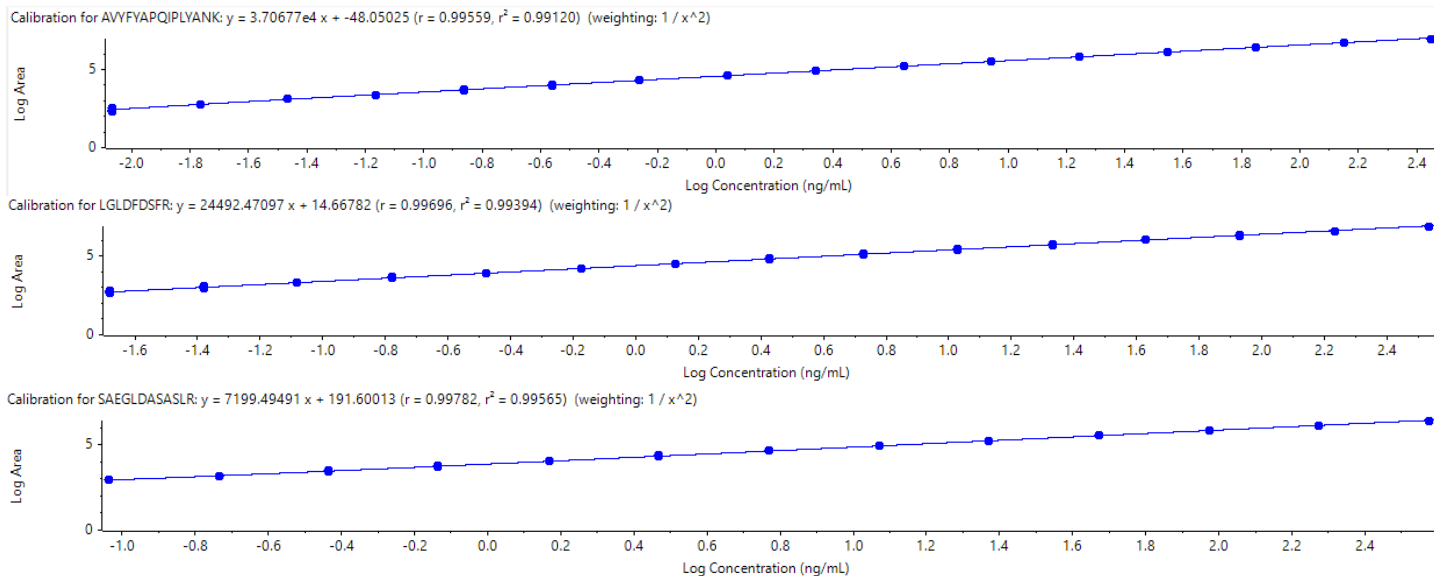


Figure 7. Quantitation Results for the Peptide AVYFYAPQIPLYANK, LGLDFDSFR and SAEGLDASASLR in Plasma Matrix in Negative Mode. Log-log plot of XIC areas versus the concentration in ng/mL. A linear fit was applied to all calibration curves using a  $1/x^2$  weighting. Regression details and  $R^2$  value being indicated in the graph for each peptide ( $n = 3$ ).

**Table 7. Quantitation Results for the Peptides in Plasma Matrix in Negative Ionization Mode.** Accuracy, %CV and linear dynamic range (LDR) for each concentration per peptide ( $n = 3$ ).

AVYFYAPQIPLYANK				LGLDFDSFR				SAEGLDASASLR			
conc. [ng/mL]	Accuracy	%CV	LDR	conc. [ng/mL]	Accuracy	%CV	LDR	conc. [ng/mL]	Accuracy	%CV	LDR
281	82.1	1.7		342	86.5	0.3		376	88.8	0.7	
141	93.7	1.9		171	92.2	1.0		188	94.3	1.6	
70.3	99.9	0.0		85.5	97.6	1.2		94	98.5	0.5	
35.1	103.9	0.7		42.7	99.8	0.5		47.0	101.3	0.8	
17.6	105.2	0.8		21.4	100.1	0.5		23.5	102.7	2.1	
8.78	106.2	0.4		10.7	101.1	1.1		11.8	102.8	0.7	
4.39	105.7	3.1		5.34	103.3	2.2		5.88	102.8	1.7	
2.20	103.5	0.6	4.5	2.67	102.4	2.5	4.2	2.94	105.7	2.1	3.6
1.10	101.0	2.9		1.34	102.0	1.3		1.47	103.1	5.3	
0.549	103.6	2.8		0.668	100.5	0.4		0.73	103.5	6.2	
0.275	94.3	6.8		0.334	103.7	6.5		0.367	102.1	4.1	
0.137	98.3	8.8		0.167	105.8	4.7		0.184	91.7	4.8	
0.0686	92.6	6.7		0.083	106.2	7.6		0.0918	102.7	7.1	
0.0343	113.1	4.8		0.0417	102.6	12.9					
0.0172	98.1	4.7		0.0209	96.1	14.8					
0.00858	98.9	18.8									

## Conclusions

- The SCIEX QTRAP 6500+ System was demonstrated to have the quantitative power to overcome different challenges in the field of bioanalysis of biopharmaceuticals
- Excellent results in positive and negative ionization mode were achieved proven by lower LLOQs, wider linear dynamic ranges, great accuracies and low %CVs
- The easy-to-use processing software SCIEX OS being compatible with .wiff and .wiff2 data formats speeds up processing enabling getting to answers in a time efficient manner

## References

1. Ouyang Z. *et al.* (2012): Pellet digestion: A simple and efficient sample preparation technique for LC-MS/MS quantification of large therapeutic proteins in plasma. *Bioanalysis* 4(1): 17-28.

## Appendix

**Table 8. MS Parameters for the Transitions of the 6×5 LC-MS/MS Peptide Reference Mix.**

Peptide	Molar Quantity	Q1 m/z	Q3 m/z	DP	CE	CXP
LASVSVR	1	428.3	660.4	50	21	12
	0.1	424.3	656.4	50	21	12
	0.01	420.8	656.4	50	21	12
	0.001	417.8	650.3	50	21	12
	0.0001	414.7	644.4	50	21	12
YVYVADVAAK	1	566.8	864.5	50	22	12
	0.1	562.8	856.6	50	22	12
	0.01	559.8	856.6	50	22	12
	0.001	556.8	850.6	50	22	12
	0.0001	553.8	844.4	50	22	12
VVGGLVALR	1	459.8	708.5	50	24	12
	0.1	456.3	701.4	50	24	12
	0.01	453.3	695.4	50	24	12
	0.001	450.3	695.4	50	24	12
	0.0001	447.3	695.4	50	24	12
LLSLGAGEFK	1	537.3	833.5	50	24	12
	0.1	532.3	823.4	50	24	12
	0.01	528.8	816.4	50	24	12
	0.001	525.3	816.4	50	24	12
	0.0001	521.8	816.4	50	24	12

**Table 9. MS Parameters for the Transitions of the Peptides in Plasma Matrix.**

Ionization mode	Peptide	Q1 m/z	Q3 m/z	DP	CE	CXP
positive	FTISADTSK	485.3	721.4	50	21	12
positive	LGLDFDSFR*	540.3	796.4	50	21	10
positive	SGLLWQLVR*	569.8	711.4	50	21	10
negative	SAEGLDASASLR*	591.8	662.3	-70	-38	-10
negative	LGLDFDSFR*	538.3	529.3	-50	-26	-15
negative	AVYFYAPQIPLYANK*	881.5	755.9	-60	-42	-12

\*heavy labelled peptide

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