Evaluating stability of human monoclonal antibody in rat cell cultures using a surrogate peptide LC-MS/MS approach

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INTRODUCTION

In vitro stability evaluations of a biopharmaceutical, such as a monoclonal antibody (mAb), can be useful in the early stages of drug development. Analytically, however, the direct quantitative measurement of a mAb-based drug is more difficult than small molecule quantitation, due to greater structural complexity of protein biopharmaceuticals in comparison to small molecule drugs. Our approach to mAb LC-MS/MS quantification in a traditional test system for predicting drug stability in vitro involved incubations of rat liver cells (suspended or cultured primary hepatocytes) with human SiLu™Lite mAb, followed by trypsin digestion using Waters ProteinWorks™ eXpress direct digest kit and quantitation of five tryptic mAb surrogate peptides.

METHODS

Intact human monoclonal antibody (mAb) standards

Intact SiLu™Lite mAb and stable isotope-labeled SiLu™ mAb standards were purchased from Sigma-Aldrich. ProteinWorks™ eXpress direct digest kit was purchased from Waters Corporation. Figure 1 and Table 1 detail relevant information about the mAb standards used.

Figure 1. Human SiLu™Lite mAb sequence

>light chain

QSALTQPRSVSGSPGQSVTISCTGTSSDIGGYNFVSWYQQHPGKAPKLMIYDATK**RPSGVPDR**FSGSKSGNTASLTISGLQAEDEADYYCCSYAGDYTPGVVFGGGTK**LTVLGQPK**AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNK**YAASSYLSLTPEQWK**SHRSYSCQVTHEGSTVEKTVAPTECS

>heavy chain

EVQLVESGGGLVQPGGSLRLSCVASGFTLNNYDMHWVRQGIGKGLEWVSKIGTAGDRYYAGSVKGRFTISRENAKDSLYLQMNSLRVGDAAVYYCARGAGRWAPLGAFDIWGQGTMVTVSSASTK**GPSVFPLAPSSK**STSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNK**ALPAPIEK**TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Variable regions: in red
Conserved regions: in blue

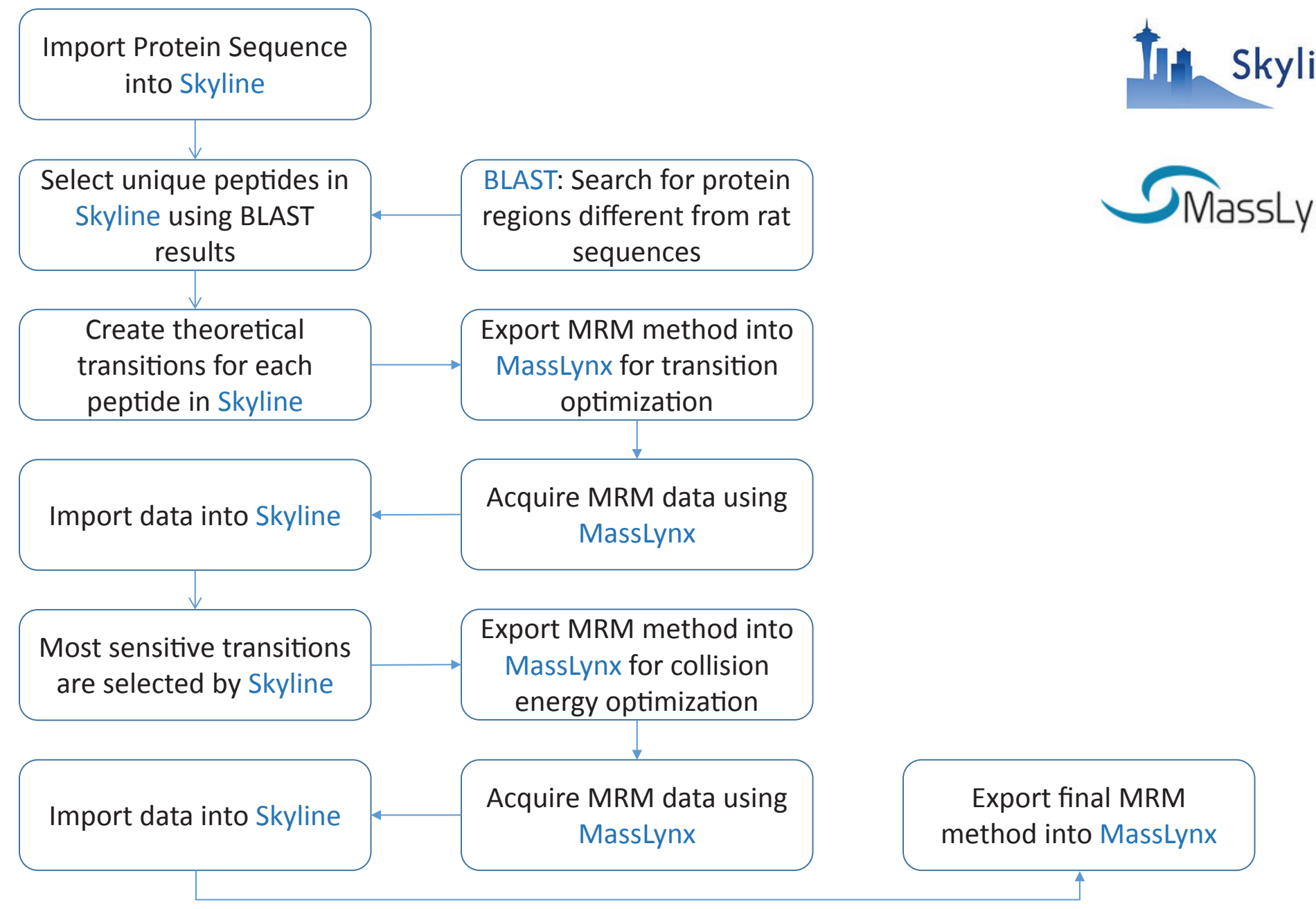
Table 1: Human SiLuLite™ mAb (analyte) and stable isotope-labeled SiLu™ mAb (internal standard, IS) composition

	Human SiLuLite™ mAb	Human SiLu™ mAb (IS)
Chemical formula (intact protein, non-reduced):	$C_{6374}H_{9864}N_{1708}O_{1992}S_{46}$	$C_{6002}H_{9864}N_{1548}O_{2008}S_{52}[^{13}C_{372}][^{15}N_{160}]$
Molecular weight (average):	143,767.8 g/mol	144,744.4 g/mol

Surrogate peptide selection – Method development

Both light and heavy chain sequences of SiLu™Lite mAb were compared to the database of rat protein sequences using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/). Regions of SiLu™Lite mAb established as different from proteins endogenous to the test system were used to generate a list of tryptic peptides of convenient length. Peptides specific to the variable or conserved regions of SiLu™Lite mAb light and heavy chains (underlined in Figure 1) were selected for method development and LC-MS/MS quantitation using Skyline and MassLynx software (Table 2). Figure 2 depicts the workflow used to generate the method for quantitation of the surrogate peptides selected.

Figure 2. Method development workflow with BLAST, Skyline and MassLynx



- Import Protein Sequence into Skyline
- Select unique peptides in Skyline using BLAST results
- BLAST: Search for protein regions different from rat sequences
- Create theoretical transitions for each peptide in Skyline
- Export MRM method into MassLynx for transition optimization
- Acquire MRM data using MassLynx
- Import data into Skyline
- Most sensitive transitions are selected by Skyline
- Export MRM method into MassLynx for collision energy optimization
- Acquire MRM data using MassLynx
- Import data into Skyline
- Export final MRM method into MassLynx

Table 2: SiLu™Lite mAb surrogate peptide data

Peptide #	Surrogate peptide sequence	SiLu™Lite mAb (analyte)		SiLu™ mAb (IS)	
		Elemental composition	MW	Elemental composition	MW
1	RPSGVPDR	$C_{36}H_{62}N_{14}O_{12}$	882.4672	$C_{24}H_{62}N_6O_{12}[^{13}C_{12}][^{15}N_8]$	902.4837
2	LTVLGQPK	$C_{39}H_{70}N_{10}O_{11}$	854.5226	$C_{33}H_{70}N_8O_{11}[^{13}C_6][^{15}N_2]$	862.5368
3	YAASSYLSLTPEQWK	$C_{81}H_{118}N_{18}O_{25}$	1742.8516	$C_{75}H_{118}N_{16}O_{25}[^{13}C_6][^{15}N_2]$	1750.8658
4	GPSVFPLAPSSK	$C_{55}H_{87}N_{13}O_{16}$	1185.6394	$C_{49}H_{87}N_{11}O_{16}[^{13}C_6][^{15}N_2]$	1193.6536
5	ALPAPIEK	$C_{39}H_{67}N_9O_{11}$	837.4960	$C_{33}H_{67}N_7O_{11}[^{13}C_6][^{15}N_2]$	845.5102

Analytical method

All samples were analyzed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) with a Waters Xevo TQ-S tandem quadrupole mass spectrometer, for acquisition of multiple reaction monitoring (MRM) data, in positive mode with electrospray ionization. Tryptic peptides were separated using a Waters Acquity UPLC®Peptide BEH C18, 300 Å (2.1 x 150 mm, 1.7 µm) column and mobile phases A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A chromatographic gradient ramped from 10 to 50% B over 6 minutes at 0.3 mL/min. The capillary voltage applied was 3.0 kV, and the cone voltage was 35 V. Table 3 shows the LC-MS/MS parameters for the five surrogate peptides selected.

Table 3: Optimized LC-MS/MS parameters for the SiLu™Lite mAb surrogate peptides

Peptide #	Surrogate peptide sequence	Charge	MRM transition		Collision energy (eV)	Retention time (min)
			SiLu™Lite mAb (analyte)	SiLu™ mAb (IS)		
1	RPSGVPDR	2+	442.2>709.4	452.3>719.4	15	5.52
2	LTVLGQPK	1+	855.5>542.3	863.5>550.3	31	3.51
3	YAASSYLSLTPEQWK	2+	872.4>687.3	876.4>695.4	15	4.79
4	GPSVFPLAPSSK	2+	593.8>418.2	597.8>426.2	21	4.46
5	ALPAPIEK	1+	838.5>654.4	846.5>662.4	30	3.41

Suspended rat hepatocyte incubations

Human SiLu™Lite mAb (25 µg/mL) was incubated with pooled suspended rat hepatocytes (1 million cells/mL) in Williams' E medium supplemented with GlutaMAX-1 (2 mM) and HEPES (0.1 mM). Reactions were initiated by combining medium containing SiLu™Lite mAb and medium containing hepatocytes. The incubation temperature was 37°C, and the incubation atmosphere was a 95:5 mixture of air and CO_2, 95% relative humidity. After 0, 15, 30, 60 and 120 min of incubation, reactions were stopped by transferring aliquots to ProteinWorks™ tubes (Waters Corp.) containing digestion buffer and internal standard. The samples were heated immediately to 80°C for 10 min.

Sample and calibration standard preparation for suspended rat hepatocyte incubations

Calibration standards (1 – 30 µg/mL) were prepared by serial dilution in Williams' E medium. Samples were prepared for LC-MS analysis using Waters ProteinWorks™ eXpress digest kit with the 3-step protocol. The internal standard concentration was approximately 10 µg/mL. Briefly, 35 µL hepatocyte incubation samples were transferred into ProteinWorks™ tubes containing 135 µL digestion buffer with internal standard. The tubes were heated to 80°C for 10 minutes, digested with trypsin (45°C for 2 hours), then centrifuged at 920 RPM for 1 min. The samples were treated for another 15 min with an inactivation reagent at 45°C.

Cultured rat hepatocyte incubations

Cell culture medium (MCM+) containing human SiLu™Lite mAb (0.007, 0.07, 0.7 and 1.4 µM) was applied to cultured rat hepatocytes on the first day of treatment and the media was replaced after 24 hours. The culture multiwell plates were incubated at 37°C, and the incubation atmosphere was a 95:5 mixture of air and CO_2, 95% relative humidity. Following the second treatment, equal volumes of spent medium were collected from triplicate 0, 0.25, 3, and 24 hour samples and flash frozen with no other components in the analysis plate until further sample preparation.

Sample and calibration standard preparation for cultured rat hepatocyte incubations

Calibration standards (0.1 – 100 µg/mL; 0.0007-0.7 µM) were prepared by serial dilution in MCM+ media. Samples and SiLu™Lite mAb calibration standards were prepared for LC-MS analysis using Waters ProteinWorks™ eXpress digest kit with the 3-step protocol as described for suspended hepatocyte samples above. Where applicable, samples were diluted using appropriate dilution factors for the method range prior to analysis. SiLu™Lite mAb was quantified by back calculation of concentrations against duplicate calibration curves using the simplest appropriate weighting and regression algorithm based on analyte/internal standard peak area ratios.

RESULTS

Suspended rat hepatocytes

Table 4 shows the calibration curve parameters for the five surrogate peptides for the experiments with suspended rat hepatocytes. The calibration standards for all peptides had accuracy ranges within ± 25 % of the theoretical concentrations with quadratic 1/x regression and weighting ($r^2 \geq 0.99$). Calibration curve plots for all five surrogate peptides are shown in Figure 3.

Table 4: Calibration curve parameters for surrogate peptides (1 – 30 µg/mL) from SiLu™Lite mAb prepared in Williams' E medium

Peptide #	Surrogate peptide sequence	Weighting	Quadratic Fit (r^2)	% Accuracy (calculated)
1	RPSGVPDR	1/x	0.990	87.8 to 120
2	LTVLGQPK	1/x	0.994	87.2 to 117
3	YAASSYLSLTPEQWK	1/x	0.991	88.1 to 122
4	GPSVFPLAPSSK	1/x	0.990	91.4 to 114
5	ALPAPIEK	1/x	0.994	92.0 to 118

Figure 4 shows representative extracted MRM chromatograms for the five surrogate peptides in a 30 µg/mL SiLu™Lite mAb reference standard prepared using the Waters ProteinWorks™ eXpress digest kit and protocol. Figure 5 shows representative extracted MRM chromatograms for the peptide 5 (**ALPAPIEK**) in the reference standards within 1-30 µg/mL concentration range. Peak shape and retention times were appropriate and reproducible.

Metabolic stability results for the incubations of SiLu™Lite mAb in suspended rat hepatocytes over 120 min are graphically represented in Figure 6. Overall, there was negligible substrate loss observed over the 120 min incubation time course. Some variability was observed in the early time point samples for peptides 1 (**RPSGVPDR**) and peptide 2 (**LTVLGQPK**). Reasons are currently unclear.

Figure 3: Calibration curves for SiLu™Lite mAb surrogate peptides in Williams' E medium

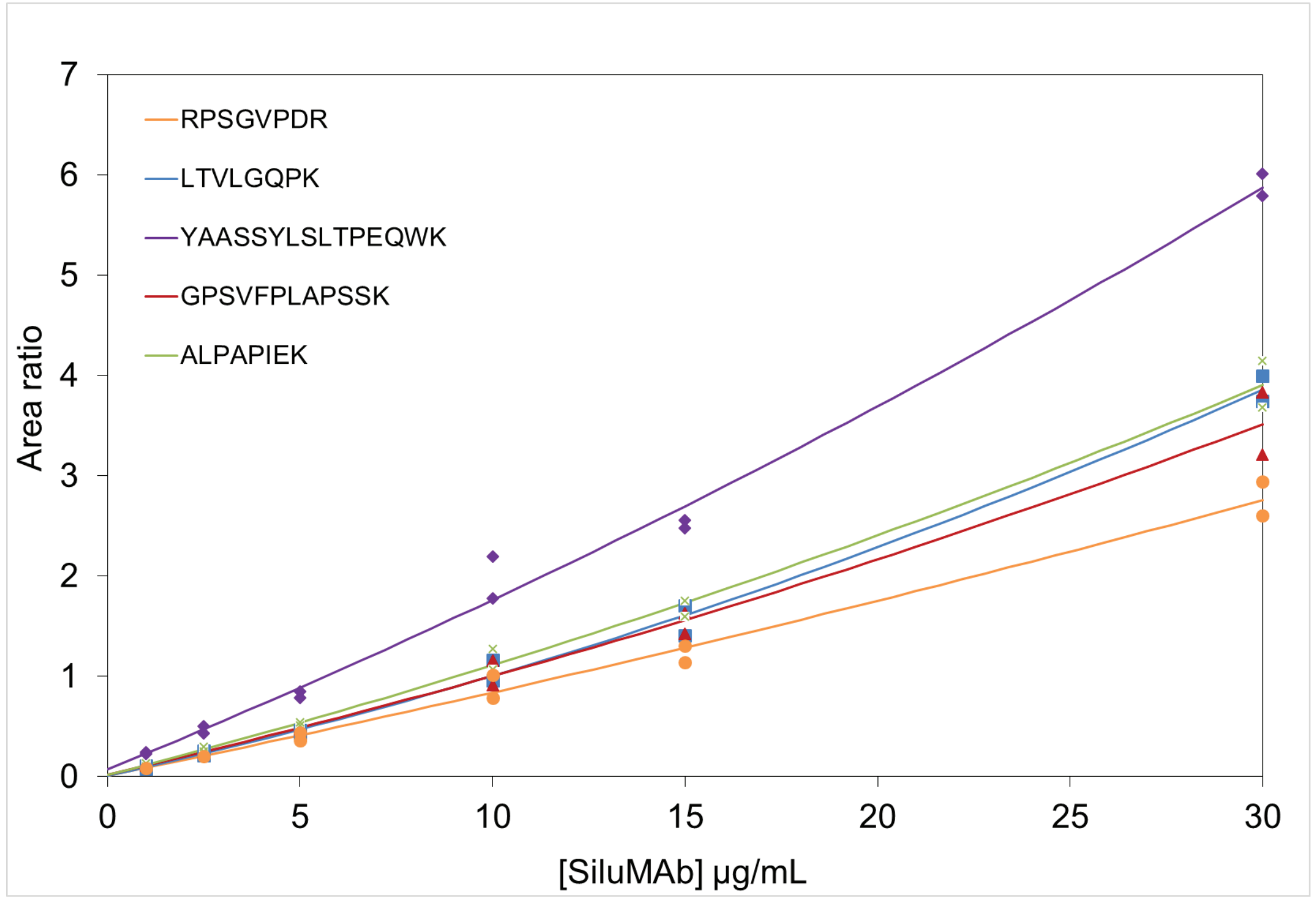
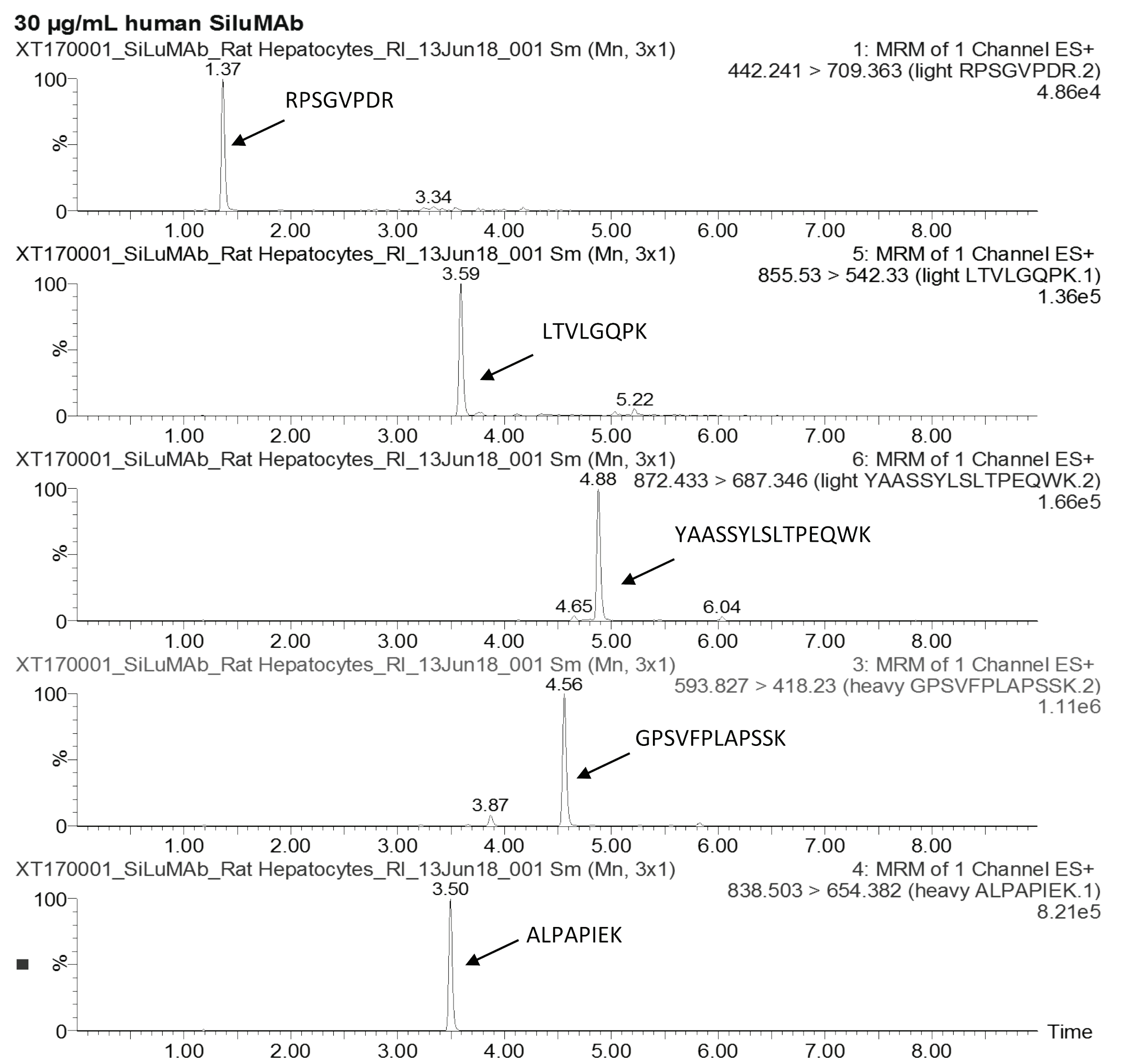


Figure 4: Extracted MRM mass chromatograms for a 30 µg/mL SiLu™Lite mAb reference standard showing five surrogate peptides in Williams' E medium



Cultured rat hepatocytes

Table 5 shows the calibration curve parameters for the five surrogate peptides monitored for experiments with cultured rat hepatocytes. The calibration standards for all five peptides had accuracy ranges within ± 25 % of the theoretical concentrations with linear or quadratic 1/x regression ($r^2 \geq 0.99$). The calibration curve plots for the surrogate peptides are shown in Figure 7.

Table 5: Calibration curve (0.1-100 µg/mL; 0.0007-0.7 µM) parameters for surrogate peptides from SiLu™Lite mAb in cultured rat hepatocytes

Peptide #	Surrogate peptide sequence	Regression and weighting algorithm	Regression Fit (R^2)	% Accuracy (calculated)
1	RPSGVPDR	Linear 1/x	0.995	76.4 to 121
2	LTVLGQPK	Quadratic 1/x	0.991	86.4 to 112
3	YAASSYLSLTPEQWK	Linear 1/x	0.991	78.6 to 118
4	GPSVFPLAPSSK	Linear 1/x	0.995	87.5 to 115
5	ALPAPIEK	Linear 1/x	0.990	85.9 to 116

Figure 5: Extracted MRM mass chromatograms for ALPAPIEK for SiLu™Lite mAb calibration curve in Williams' E medium

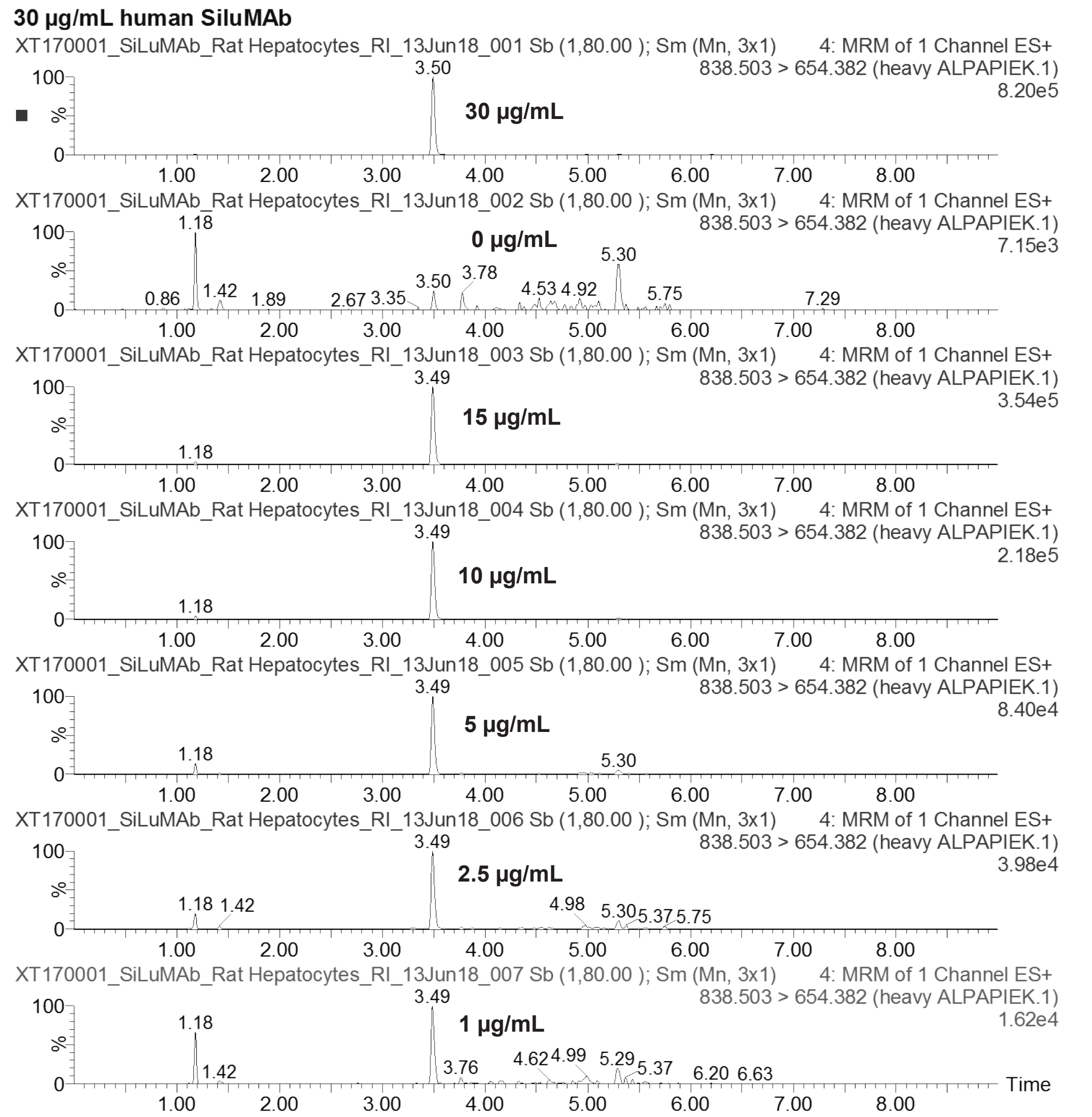


Figure 6: Metabolic stability of SiLu™Lite mAb (25 µg/mL) in suspended rat hepatocytes (1 million cells/mL) incubated for up to 120 min based on surrogate peptide quantitation

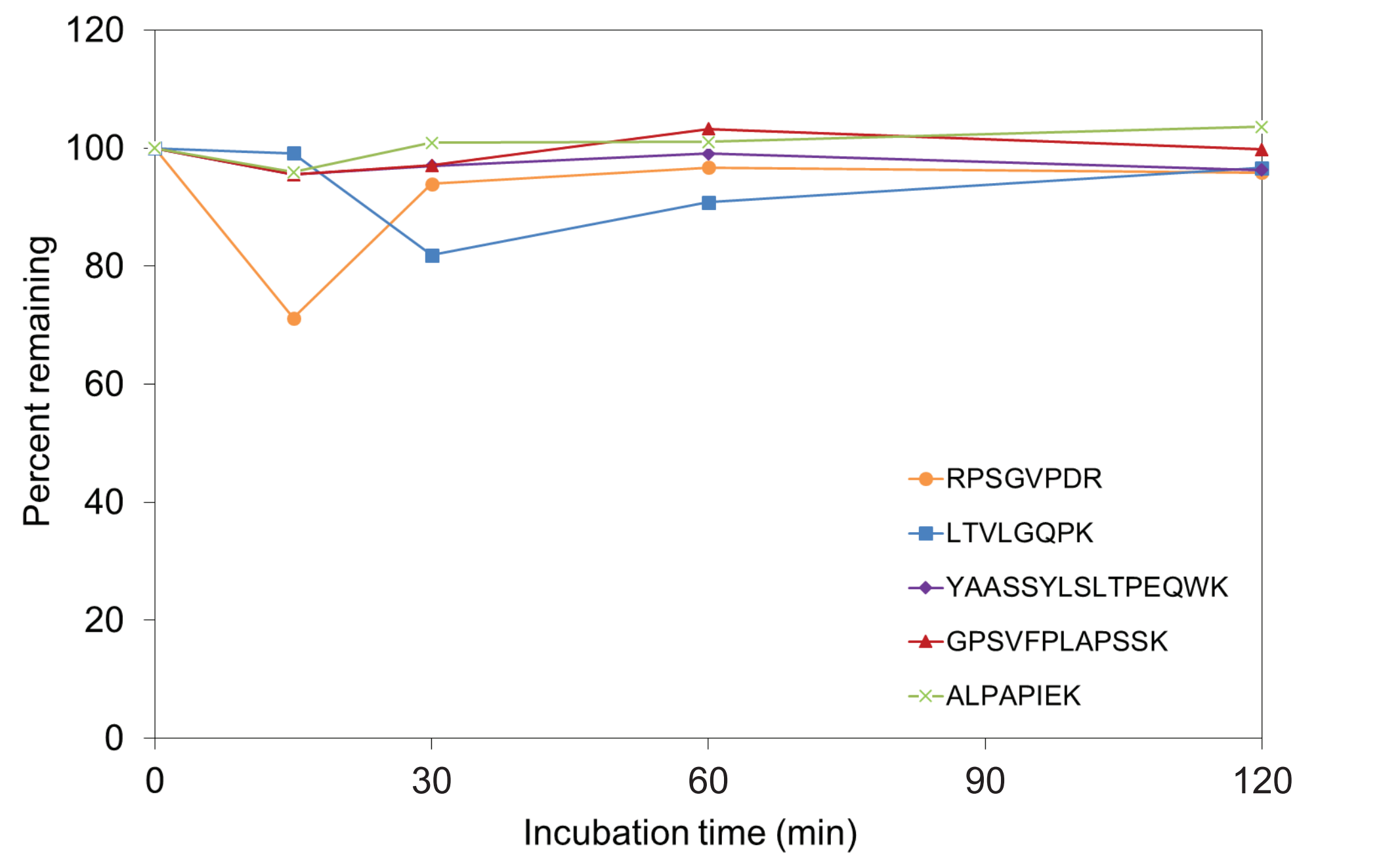


Figure 7: Calibration curves for SiLu™Lite mAb surrogate peptides in MCM+ medium

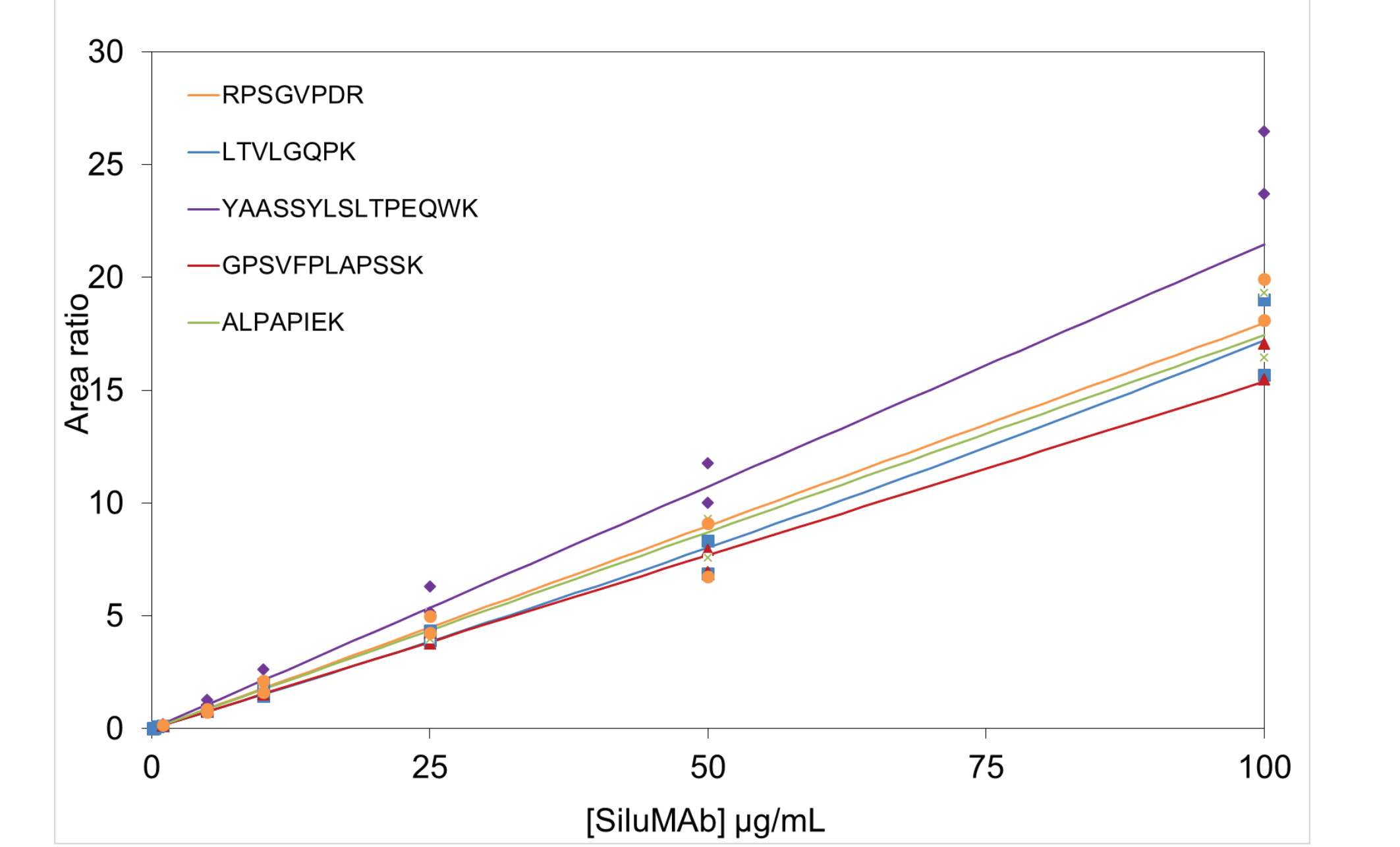


Figure 8 shows representative extracted MRM chromatograms for peptide 3 (YAASSYLSLTPEQWK) from the 0 h samples of cultured rat hepatocytes treated with SiLu™Lite mAb at 0.007, 0.07, 0.7 and 1.4 µM concentrations. Representative extracted MRM chromatograms for peptide 3 (YAASSYLSLTPEQWK) following treatment with 0.7 µM SiLu™Lite mAb and collected at 0, 0.15, 3 and 24 h post treatment are shown in Figure 9. Similar to suspended hepatocytes, experiments with cultured hepatocytes resulted in appropriate and reproducible peptide peak shape and retention times. The quantitation data obtained agreed between the triplicates analyzed for each peptide at designated concentrations and treatment timepoint, showing robustness of the digestion procedure as well as the LC-MS/MS method.

Stability results from sample analysis of SiLu™Lite mAb in cultured rat hepatocytes over the course of 24 h are shown in Table 6 for all the surrogate peptides. Stability results for SiLu™Lite mAb (0.07 µM) incubated with cultured rat hepatocytes and monitored by five surrogate peptides are graphically represented in Figure 10. Overall, SiLu™Lite mAb was stable in cultured rat hepatocytes within the 0.007 to 1.4 µM concentration range over the incubation time course and no detectable substrate loss was observed.

Figure 8: Extracted MRM mass chromatograms for peptide 3 (YAASSYLSLTPEQWK) in 0 min cultured hepatocyte samples treated with SiLuLite™mAb at varying concentrations

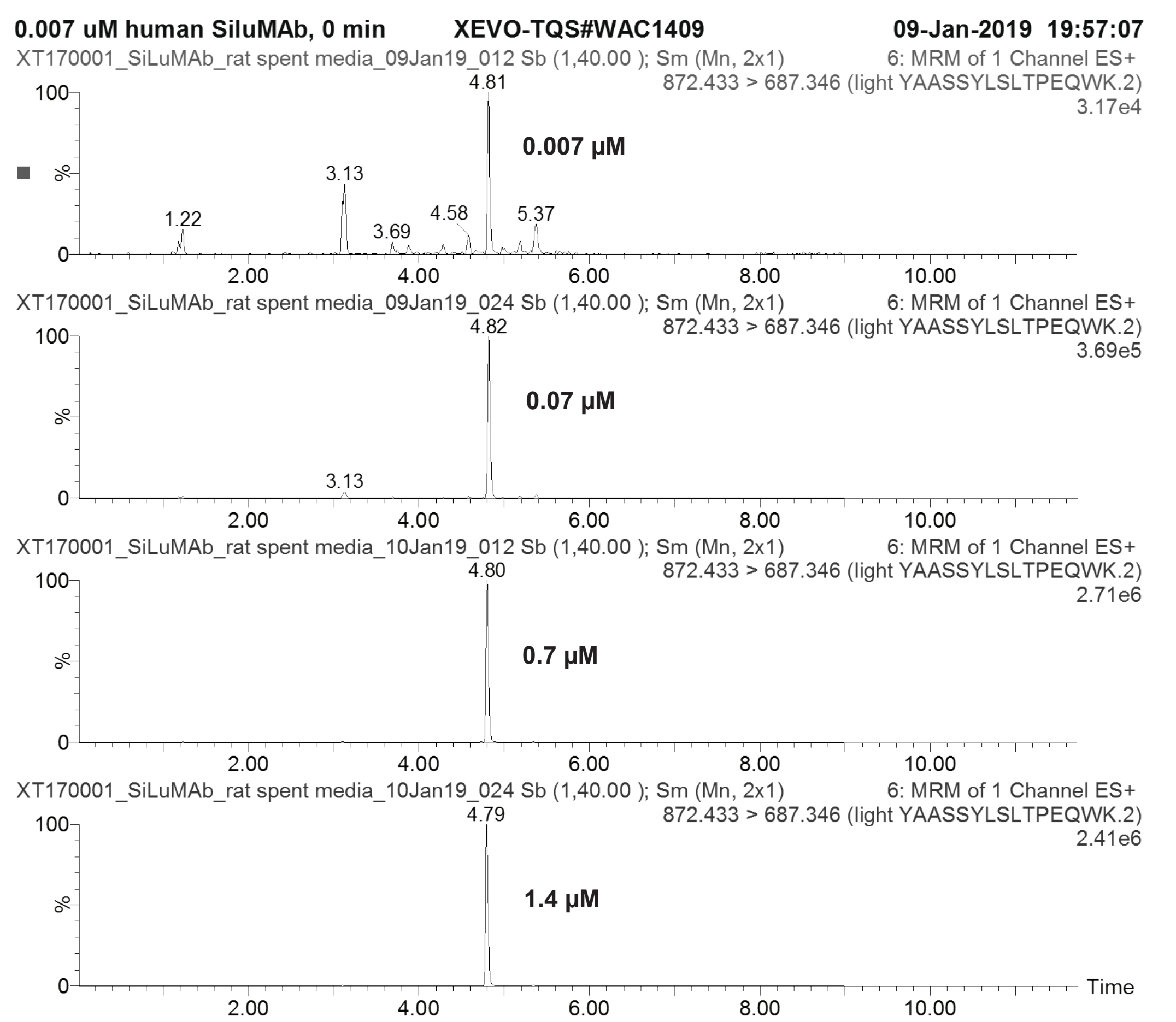


Figure 9: Extracted MRM mass chromatograms for peptide 3 (YAASSYLSLTPEQWK) in cultured hepatocytes treated with 0.07 µM SiLuLite™mAb and collected at 0, 0.25, 3 and 24 h

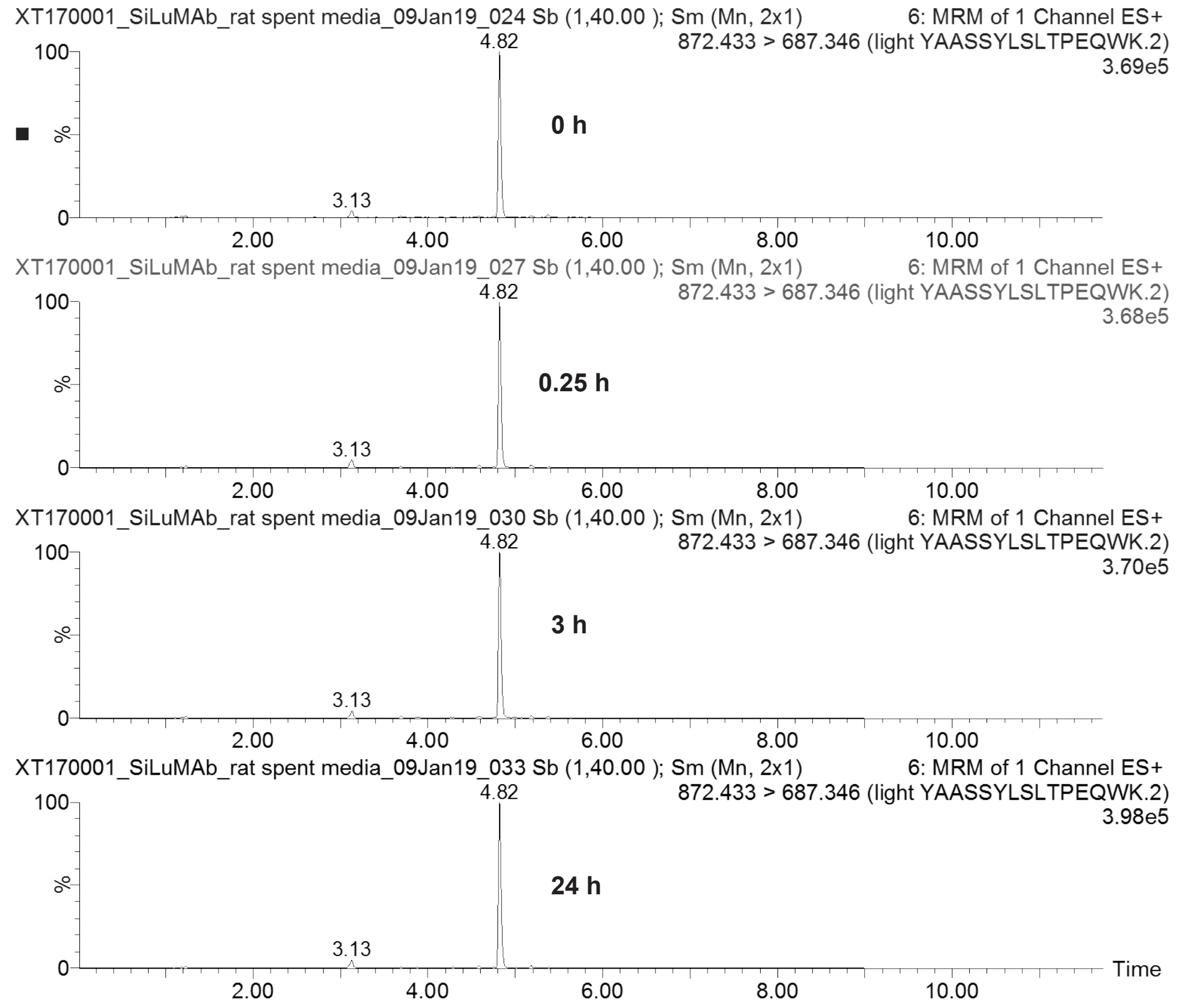
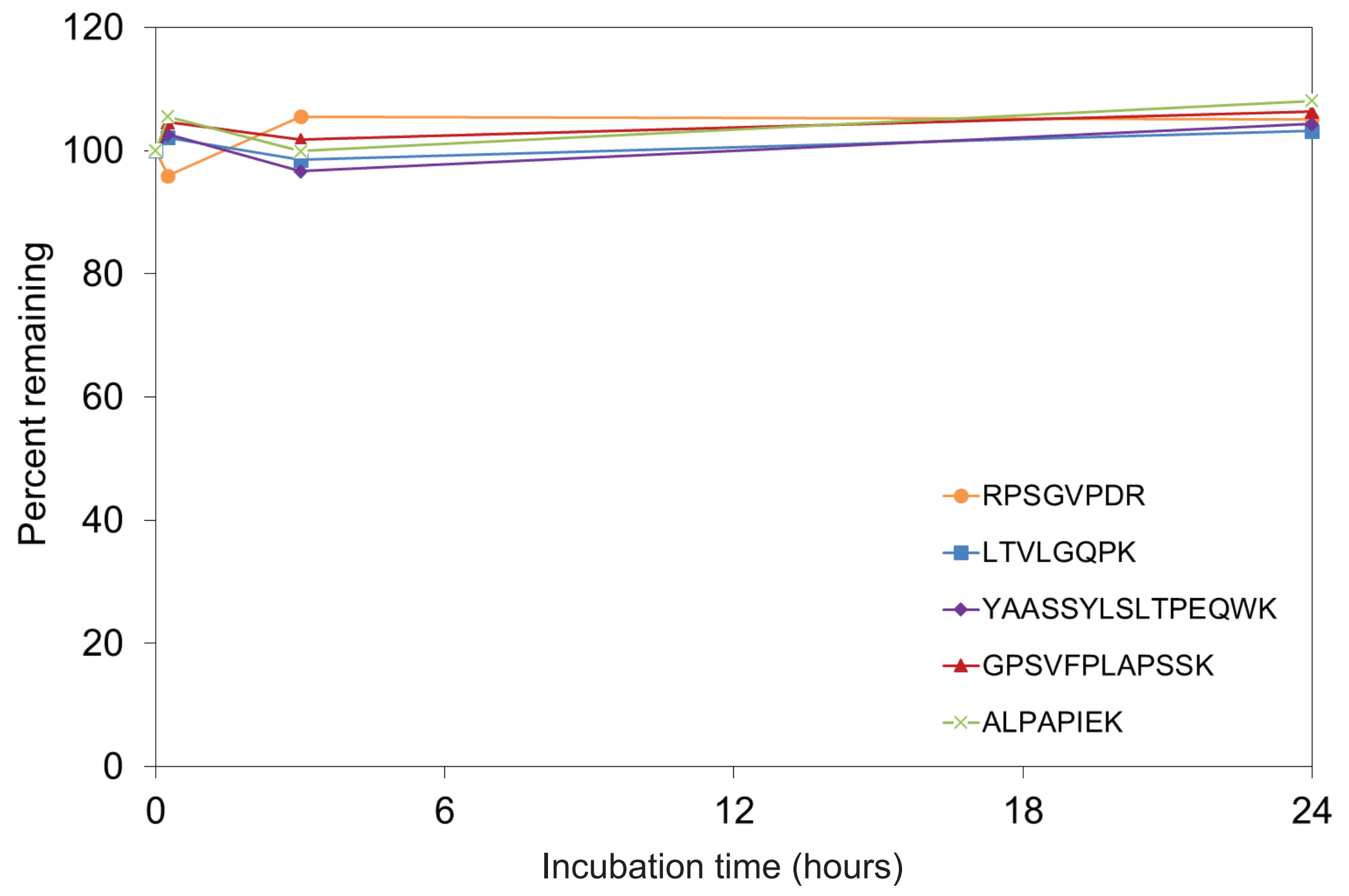


Table 6: Stability of human SiLu™Lite mAb monitored using surrogate peptides in cultured rat hepatocytes incubated for up to 24 h

Silu™mAb (µM)	Time (h)	Substrate (surrogate peptide) remaining (%)				
		RPSGVPDR	LTVLGQPK	YAASSYLSLTPEQWK	GPSVFPLAPSSK	ALPAPIEK
0.007	0	100	100	100	100	100
	0.25	108	94.8	107	96.3	97.8
	3	112	99.6	104	100	107
	24	102	107	110	102	110
0.07	0	100	100	100	100	100
	0.25	95.4	106	103	104	104
	3	107	102	96.7	102	99.9
	24	102	105	104	106	107
0.7	0	100	100	100	100	100
	0.25	93.5	99.2	96.4	99.1	103
	3	99.6	101	99.4	102	103
	24	107	102	103	104	109
1.4	0	100	100	100	100	100
	0.25	101	103	98.7	103	103
	3	102	102	103	104	101
	24	111	99.6	102	103	103

Figure 10: Stability of SiLu™Lite Sigma mAb (0.07 µM) in cultured rat hepatocytes incubated for up to 24 h



CONCLUSIONS

- Stability of a human monoclonal antibody was evaluated in suspended and cultured rat hepatocyte test systems.
- An LC-MS/MS method for the quantitation of mAb in suspended and cultured hepatocytes using five surrogate peptides was successfully developed and applied.
- The calibration curve (**1-30 µg/mL** in Williams' E medium and **0.1-100 µg/mL** in MCM+ medium) accuracy for each peptide was within ± 25 % of the theoretical concentrations with linear or quadratic regression ($r^2 \geq 0.99$).
- The quantitation data obtained for all five of the surrogate peptides yielded consistent results establishing good representation of the intact mAb.
- SiLu™Lite mAb was stable over the relevant incubation time course in suspended and cultured hepatocyte test systems

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