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應用手冊

Improving the Detection of Thyroglobulin in Human Plasma for Clinical Research by Combining SISCAPA Enrichment and Microflow LC-MS

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Abstract

This application note investigates if the ionKey/MS System operating in a dual-pump trapping configuration can provide reductions in LLOQ levels for Thyroglobulin (Tg).

Benefits

- ionKey/MS configured for dual-pump trapping is well suited to analyze SISCAPA eluents
- Sub 1 ng/mL quantitation level of thyroglobulin is achieved using 10x less plasma than the comparable standard flow method
- Accuracy is highly correlated with the values obtained from the standard flow method but offers higher levels of precision LC over 4 replicates
- Dual-pump trapping significantly reduces cycle times to under 7 minutes allowing a similar number of samples to be run in the same time frame as the best in literature standard flow method
- Microflow is a viable and attractive solution for clinical research

Introduction

Current research immunoassays for Thyroglobulin (Tg) may be subject to high false negative rates in a significant portion of the sample population due to the presence of endogenous anti-Tg autoantibodies (Tg AAbs) that block the binding epitope resulting in the reporting of a negative result in the immunoassay. The prevalence of these negative results has lead researchers to look for alternative analytical approaches that can improve the quality of the result.

Stable isotope standards and capture by anti-peptide antibodies (SISCAPA) enrichment for Tg combined with standard flow LC-MS has been implemented as an alternative approach in clinical research labs. The high analytical selectivity and specificity of the capture step using anti-peptide antibodies specific for a proteolytic peptide unique to Tg greatly enhances the detection and quantitiation of Tg down to levels of approximately 1 ng/mL or 1.52 amol/µL. However, standard flow LC-MS requires 200–400 µL of plasma to reach these relevant LOQ levels, a very large volume of sample.

Microflow LC-MS, exemplified by the ionKey/MS System, operating at 10's of µL/min offers substantial analytical

sensitivity benefits over standard flow using less starting plasma in sample-limited applications.[1] Accordingly, we investigate here if the ionKey/MS System operating in a dual-pump trapping configuration can provide reductions in LLOQ levels for Tg, using less plasma while maintaining the requisite accuracy, precision, and throughput exemplified by published standard flow LC-MS assays. The dual-pump trapping configuration was explicitly chosen due to the ability of the set-up to handle relatively large injection volumes compared to iKey Separation Device volume, reduce carryover coming from the sample loop and trap column, and decrease cycle time by affording load ahead capability on the trap column and independent washing and equilibration of the trap column and the iKey Separation Device.

Experimental

LC conditions

MS system:	Xevo TQ-S operating in MRM Mode with Unit Mass Resolution
Ionization mode:	ESI Positive
Capillary voltage:	Optimized through infusion of analyte of interest
Source temp.:	100 °C
Cone gas flow:	50 L/Hr
Nano gas flow:	Off
Collision energy:	Optimized through infusion of analyte of interest, see Table 1
Cone voltage:	Optimized through infusion of analyte of interest, see Table 1

Chromatography software:	MassLynx 4.1
Quantification software:	TargetLynx

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Sample preparation

The generic SISCAPA enrichment workflow coupled with ionKey/MS is detailed in Figure 1. The sample preparation detailed in this application note was performed by SISCAPA Assay Technologies following their

recommended procedures.

1. Plasma sample is digested using trypsin. Any potential auto Tg antibodies are digested along with the target, Tg, to their corresponding peptides.

2. A highly selective and specific antibody against a proteotypic peptide unique to Tg with the amino acid sequence FSPDDSAGASALLR (FSP) is conjugated to a magnetic bead support.

3. A stable isotope standard (SIS) of the FSP peptide and the bead-conjugated antibody is added to the plasma digest.

4. The FSP peptide and SIS are selectively enriched by the anti-peptide antibody bead complex in an automated fashion in the 96-well plate format.

5. The beads are then washed to remove unbound matrix material and the bound peptides are released using acid elution.

6. The resulting eluent is subjected to microflow LC-MS using the conditions described below:

Peptide	Precursor	Product	CE	Cone voltage
FSP.light	708.8	768.5	27	30
FSP.heavy	703.8	758.5	27	30
FSP.light	708.8	697.4	27	30
FSP.heavy	703.8	687.4	27	30
FSP.light	**708.8**	**591.8**	**21**	**30**
FSP.heavy	**703.8**	**586.8**	**21**	**30**

Table 1. Optimized MRM transition parameters for the heavy and light versions of FSP. The qualifier MRMs are shown in bold. These parameters were optimized thru infusion using the onboard fluidics of the Xevo TQ-S and an infusion iKey Separation Device.

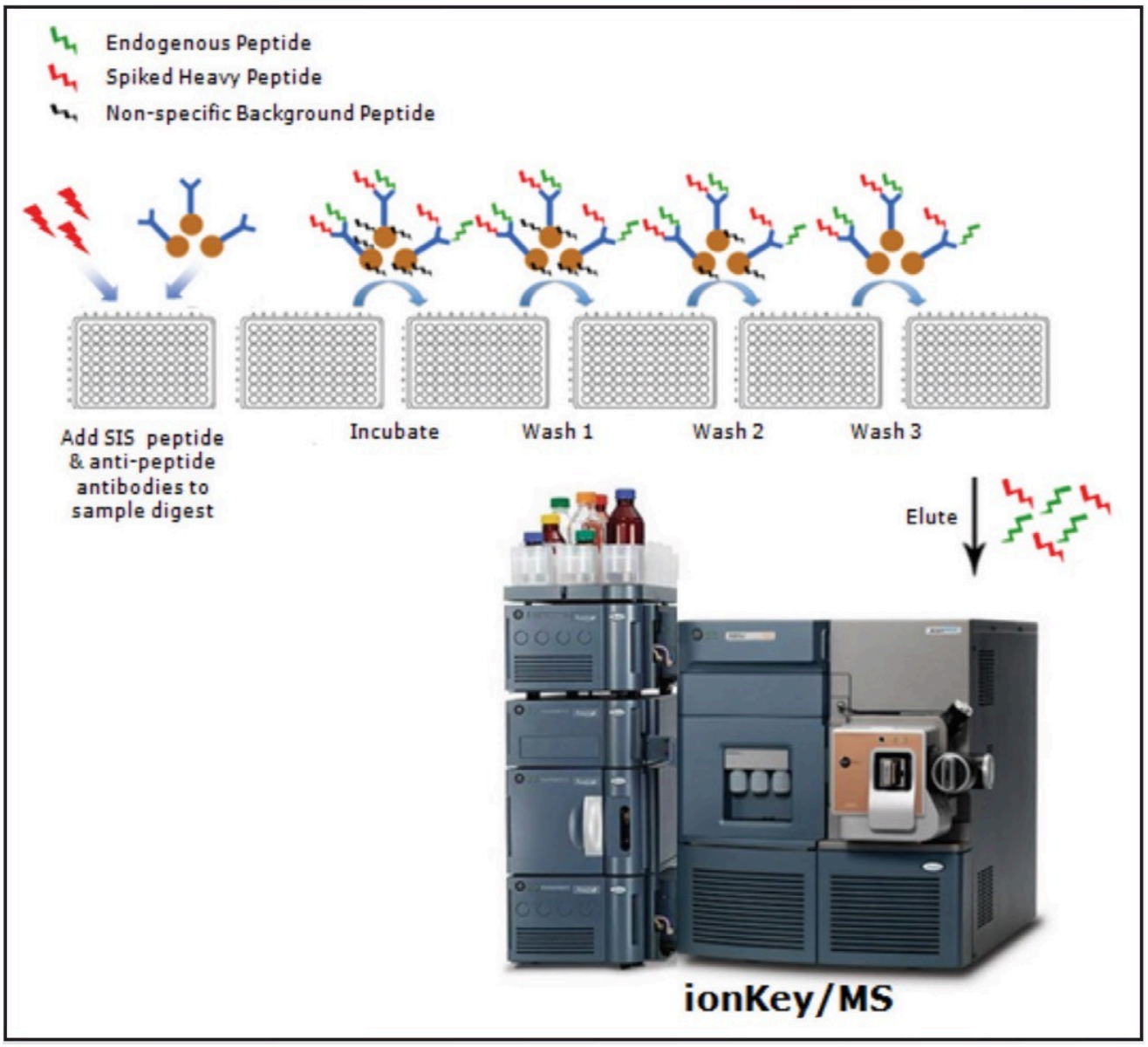


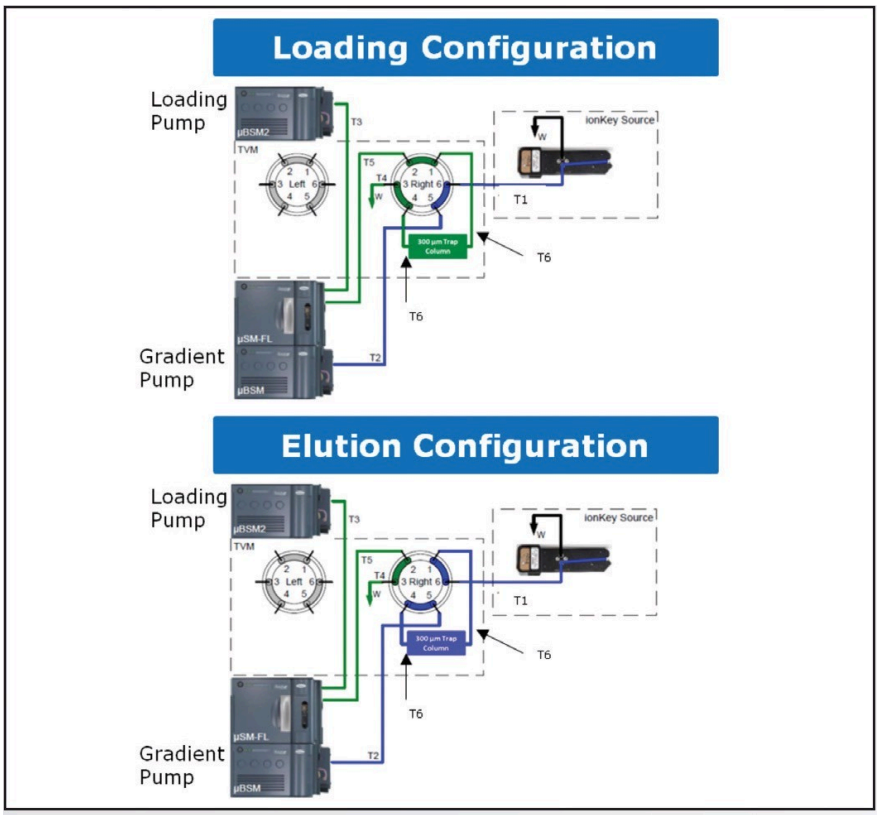
Figure 1. Analytical workflow employed combining SISCAPA enrichment and microflow LC-MS using the ionKey/MS System for the sensitive detection of Tg.

Instrumental set-up

In attempts to decrease cycle time and allow more samples to be run on the ionKey/MS System, the configuration chosen was a dual-pump trapping configuration as shown in Figure 2. In dual-pump trapping a dedicated binary solvent manager plumbed with larger I.D. transfer lines handles the loading of the trap column. A second binary solvent manager is dedicated for gradient elution of the analyte of interest off of the trap column to the iKey Separation Device. Due to the fact that the loading pump is plumbed with larger I.D. transfer lines, this loading step can occur at a faster flow rate without reaching the pressure limit of the system. The optimized

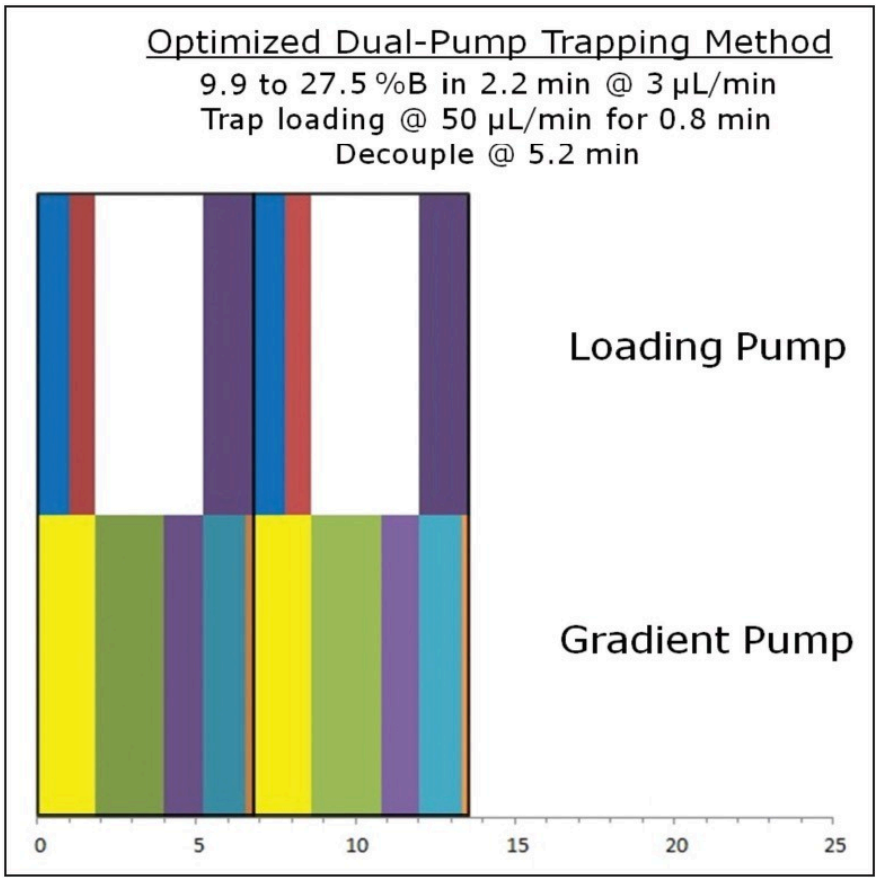
loading flow rate in this method was found to be 50 µL/min, however, flow rates of up to 70 µL/min are possible. Furthermore as we employ two dedicated pumps, the loading of the trap column by the loading pump can be overlapped with the equilibration of the iKey Separation Device by the gradient pump, effectively cancelling out the sample loading time from the total cycle time, resulting in considerable analytical time savings. After the set loading time, the valve is switched to the elution configuration as seen in Figure 2 and 3, and the gradient pump forms a gradient that back flushes the analyte off the trap column to the iKey Separation Device. During this elution step the loading pump is in line with the sample loop and can be used to flush the loop at a high flow rate with any mixture of mobile phase which should help manage carryover.

The dual-pump trapping configuration also allows heart cutting type experiments in which the trap is decoupled from the iKey Separation Device just after the last analyte of interest elutes by switching the trapping valve back into the loading configuration. Decoupling is beneficial from an analytical column and MS optics cleanliness standpoint as any later eluting matrix components such as proteins and phospholipids will be directed to waste.



#	Length	I.D.	Fittings	Part #	Order #
T1	40"	40 µm	V-F	430004188	700010399
T2	30"	25 µm	M-V	430003619	700009872
T3	30"	40 µm	M-V	430003620	700009873
T4	31"	.01"	PEEK	430004090	700009892
T5	26"	40 µm	V-V	430003658	700009881
T6	6"	40 µm	V-V	430003649	700009878

Figure 2. The dual-pump trapping set-up contains a dedicated binary solvent manager for fast sample loading onto the loop and trap column and a dedicated gradient elution pump. In this set-up line T3 has a 40 µm I.D. which affords a loading flow rate of 50 µL/min. The gradient transfer line, T2, remains a 25 µm I.D..



Load pump events	Time (min)	Gradient pump events	Time (min)
Trap equilibration/sample loading onto loop	0.95	iKey equilibration	1.75
Trap loading	0.8		
Wash loop	3.4	Gradient	2.2
Wash trap	1.6	Wash iKey Separation Device	1.2
		Delay volume	1.35
		Housekeeping	0.25
Cycle time	6.75	Cycle time	6.75

Figure 3. Schedule of events in the optimized dual-pump trapping workflow.

Results and Discussion

The analytical sensitivity of the ionKey/MS System in the dual-pump trapping configuration, using the parameters defined previously, was first evaluated using synthetic standards of the light and heavy FSP peptides.

A 6 point calibration curve was created comprising a concentration range of 2,000 amol/µL down to 0.64 amol/µL utilizing a 1 in 5 dilution with 3% acetonitrile in 0.1% formic acid. Each calibration point was run in triplicate and we observed an excellent linear response and reproducibility over the calibration range as detailed in Figure 4 with the 13 amol level having a coefficient of variation (CV) of approximately 16%. This data reinforces the ability of the platform and method described to analyze synthetic standards of FSP.

amol on column	Avg light area	CV	Avg heavy area	CV
40000	1343852.3	4.9%	1181870.0	4.4%
8000	267515.0	1.1%	234463.0	0.8%
1600	43533.7	4.8%	39211.7	1.5%
320	8312.3	2.0%	7147.7	2.6%
64	1681.0	4.9%	1614.0	1.3%
12.8	246.0	16.3%	254.0	16.4%

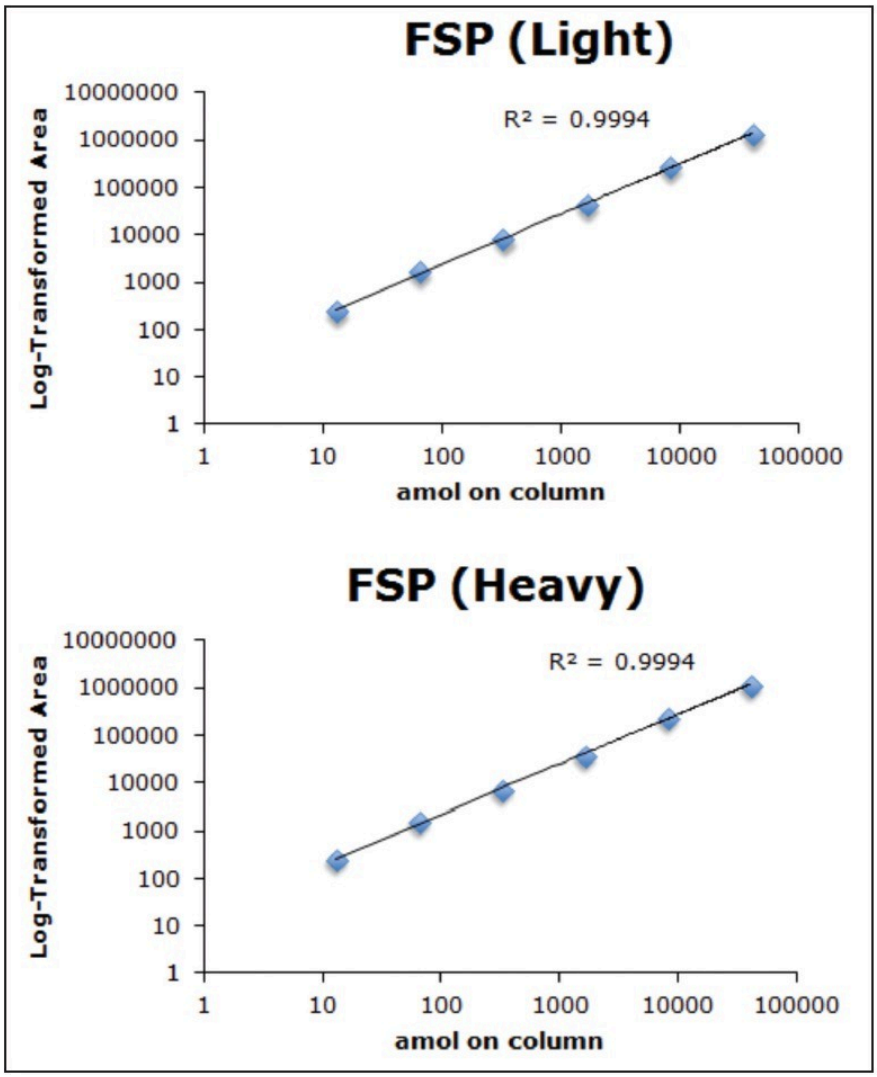


Figure 4. Calibration curve expressed in amol on column for the analysis of the synthetic standards of both the heavy and light versions of FSP. We observed an excellent linear response and reproducibility when working with these standard variants of the peptide.

Next, to demonstrate that the platform is compatible with SISCAPA eluates and can actually detect endogenous Tg in human plasma, we performed a plasma titration experiment where varying amounts of pooled human plasma were digested followed by SISCAPA enrichment of the FSP peptide. Accordingly, it is expected that the

PAR value, or the ratio of the endogenous light FSP to the heavy SIS FSP added after digestion at a consistent concentration, should increase linearly when plotted against pooled human plasma amount. The results shown in Figure 5 show the expected linear response was achieved for human plasma amounts down to 40 µL, with no observed backpressure fluctuations in the iKey Separation Device. Accordingly, it can be concluded that the platform is compatible, robust, and analytically sensitive for endogenous FSP in human plasma enriched using the SISCAPA workflow. Furthermore, as a positive control, the experiment was replicated on an Agilent 1290/6490 QqQ instrument operating in the standard flow regime and utilizing the recommended method parameters for the instrument. A high linear correlation of $R^2 = 0.998$ as seen in Figure 6 was achieved between the two platforms confirming the accuracy of the PAR values as measured on the ionKey/MS System. Additional evidence of the agreement between the PAR values obtained on the standard flow and ionKey/MS can be visualized in the Bland-Altman plot seen in Figure 7. Agreement between all measurements is within the 95% confidence interval. Furthermore, the ionKey/MS System showed better precision across 4 replicates than the standard flow system. This suggests microflow offers tangible improvements in the precision of measurement of FSP while maintaining the accuracy expected of the conventional standard flow approach.

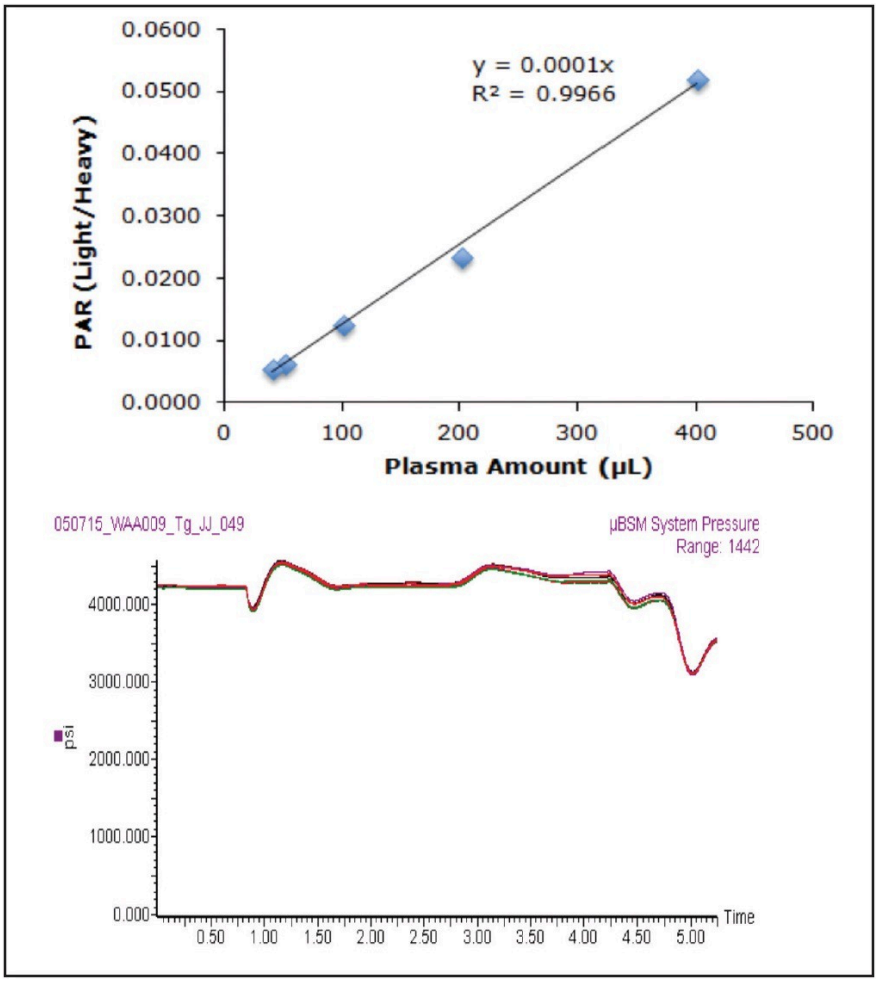
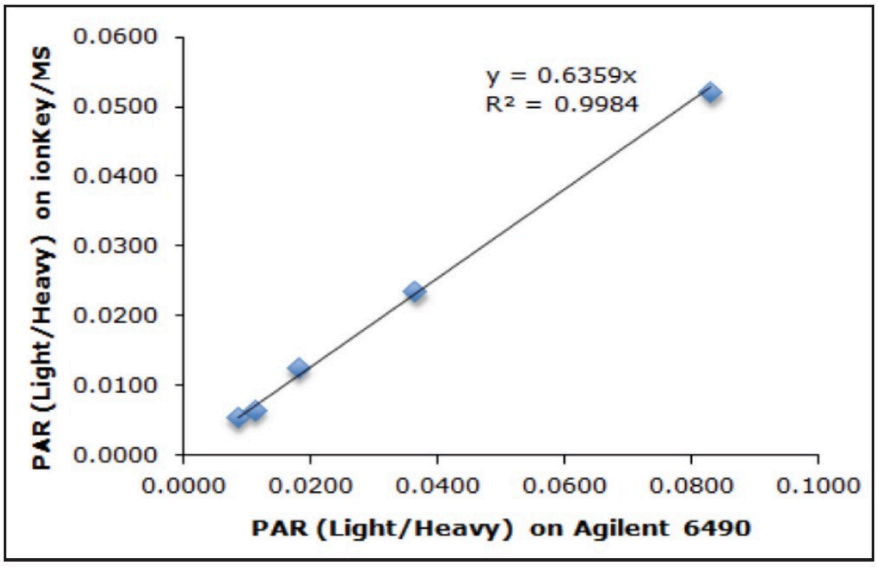


Figure 5. A linear response was achieved for pooled human plasma amounts down to 40 µL with no observed backpressure fluctuations demonstrating the ionKey/MS System as described is compatible, robust, and sensitive for SISCAPA eluates.



Plasma (µL)	ionKey/MS CV	Agilent 6490 CV
400	6.8%	17.2%
200	6.5%	9.7%
100	9.6%	12.5%
50	13.9%	17.7%
40	6.0%	36.2%

Figure 6. The experiment above was replicated on a standard flow Agilent System as a positive control and an excellent correlation was obtained. The high correlation and better precision across 4 replicates proves that microflow offers tangible benefits in the analysis of Tg over the conventional standard flow approach.

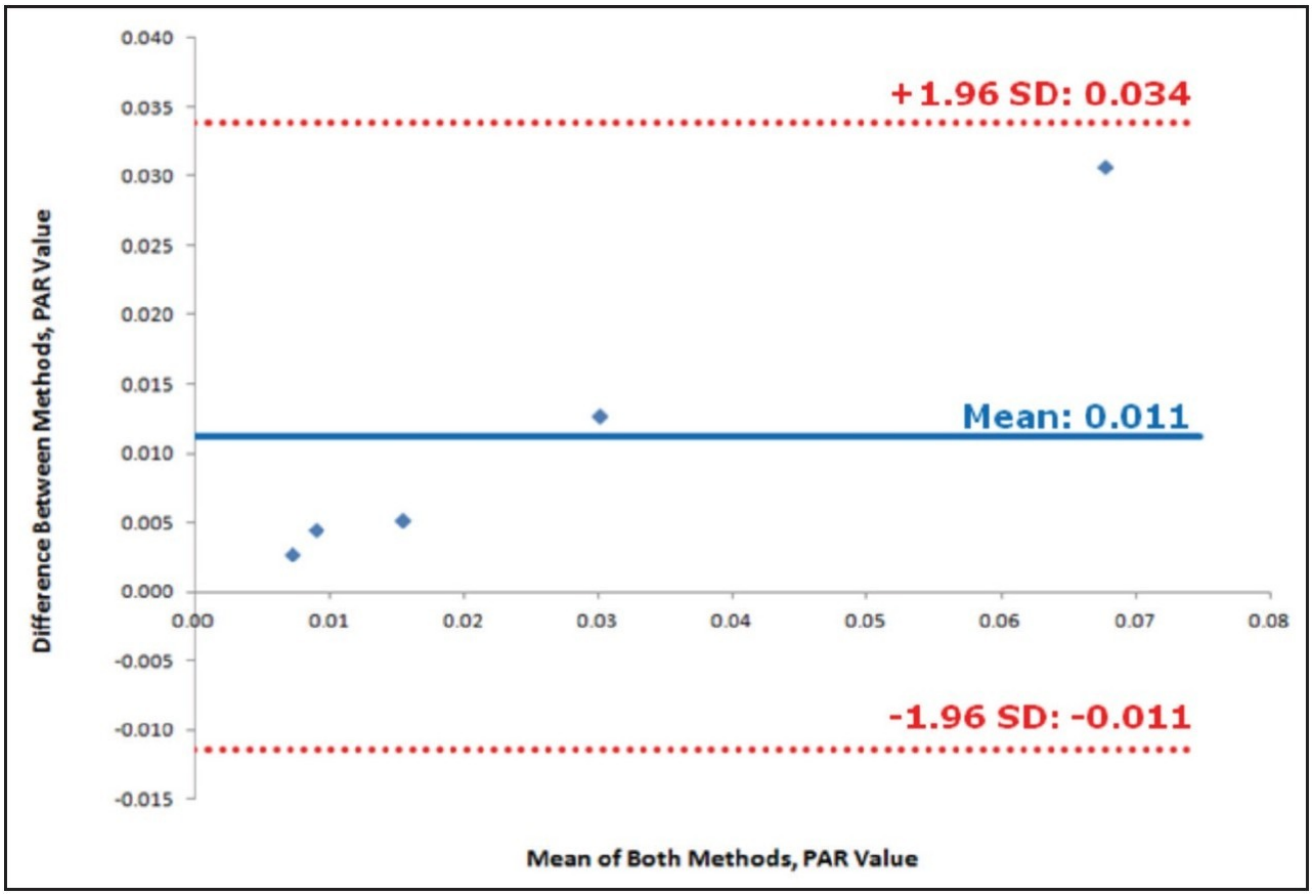


Figure 7. Bland-Altman Plot showing all differences between the standard flow Agilent method and the microflow method measurements of the FSP PAR value lie within the upper and lower confidence intervals. Agreement is therefore expected for 95% of the samples.

To further study the analytical sensitivity of the platform in terms of LLOD and LLOQ, a reverse curve was generated by titrating the heavy FSP peptide from 5,000 amol/µL down to 0.75 amol/µL and spiking synthetic light peptide at a constant level in human plasma. The LLOD, defined in this work as the point below which the CV is consistently above 30% for FSP, was determined to be 15 amol on column. The LLOQ, defined in this work as the point below which the CV is consistently greater than 20%, was 45 amol column. Representative chromatograms for the LLOD and LLOQ levels along with the reverse curve can be seen in Figure 8. The LLOQ of 45 amol is slightly higher than that estimated in the synthetic standard FSP work as one would expect due to the influence of the matrix.

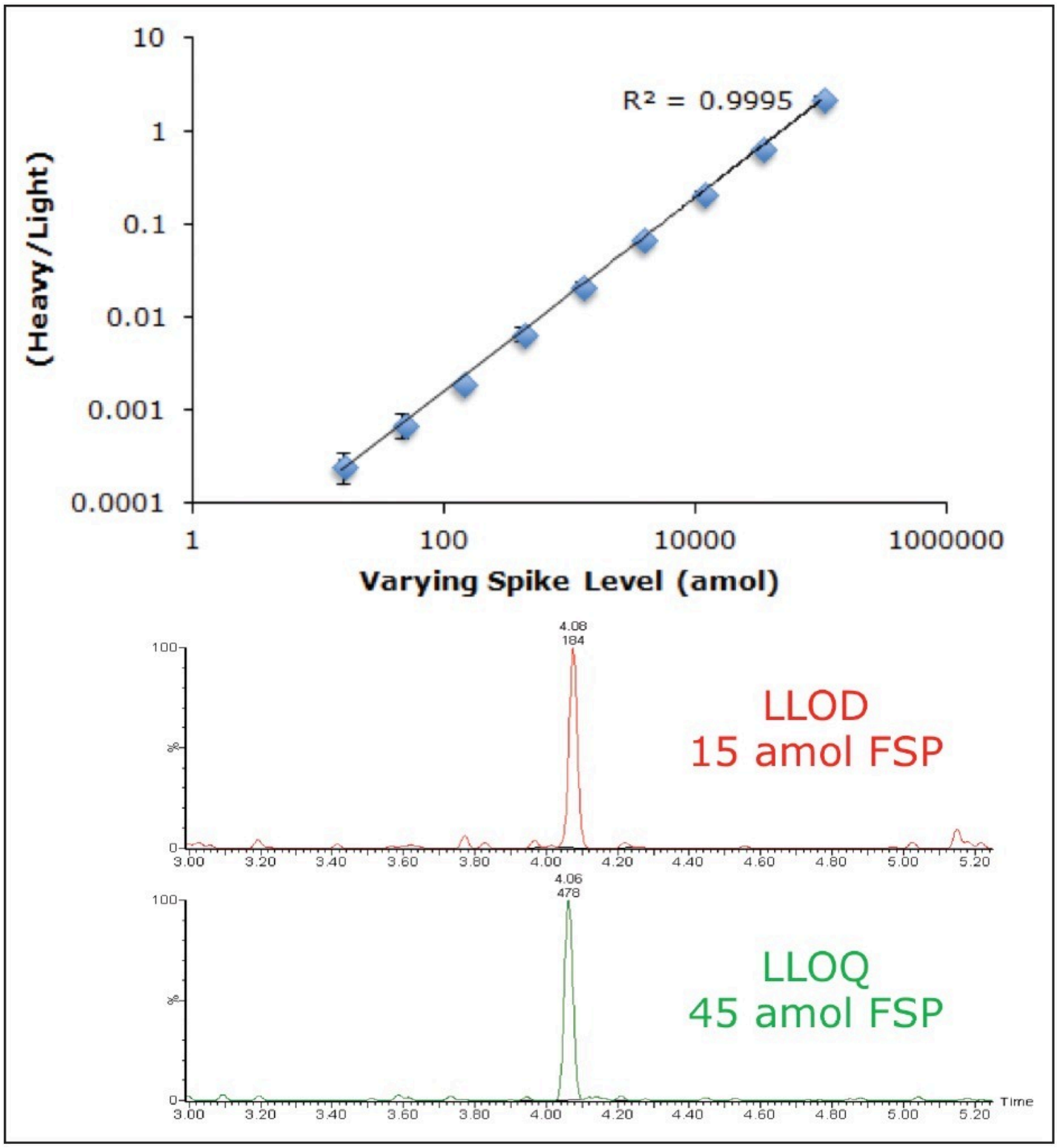


Figure 8. A reverse curve was generated to determine the approximate LLOD and LLOQ of the peptide measurement. The LLOD is 15 amol FSP on column. The LLOQ is 45 amol FSP on column.

A final curve was generated by titrating purified Tg protein in bovine plasma known to be deficient in Tg from 152 to 0.152 amol/µL followed by SISCAPA enrichment in attempts to get an estimated LLOQ value for the entire assay including the digestion step. The LLOQ of the method using only 50 µL of plasma as shown in Figure 9 is

estimated to be approximately 1.18 amol/µL (0.78 ng/mL). Accordingly, the method achieves quantitation levels of Tg in bovine serum of 1.52 amol/µL (1 ng/mL) with ease.

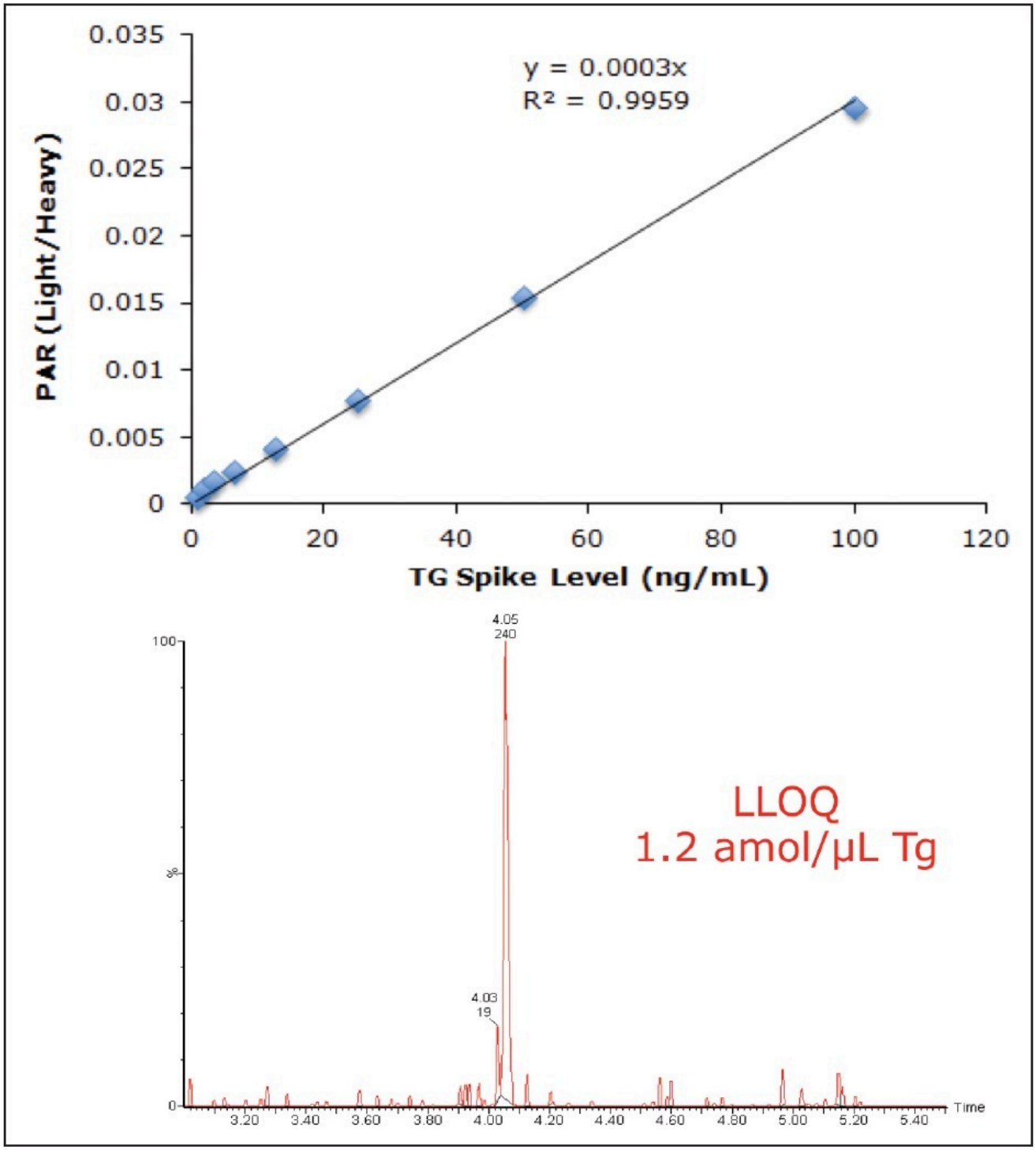


Figure 9. A curve was generated by titrating purified Tg in bovine plasma from 152 to 0.152 amol/µL. The LLOQ of the assay including the digestion step using 50 µL of sample is estimated to be 1.2 amol/µL (0.78 ng/mL).

Conclusion

Use of an optimized SISCAPA enrichment that is highly specific for a signature peptide of Tg combined with LC-MS using a vetted dual-pump trapping ionKey/MS System provides a sub 1.52 amol/µL (1 ng/mL) quantification limit of Tg protein with a cycle time of 6.75 min. This quantification limit is comparable with the best in literature for standard flow. However, the ionKey/MS System methodology outlined above also offers a few tangible benefits to the standard flow method including; a simplified enrichment procedure, the use of ten times less starting plasma prior to enrichment, an injection volume that is two times smaller, and significantly less solvent consumption. Additionally, evidence is provided in a head-to-head comparison with standard flow in which the microflow approach offers highly correlated PAR measurements while being significantly more precise across 4 replicate measures. Furthermore, the cycle time on the microflow system is only 0.25 min longer than the standard flow method outlined in the literature allowing a similar number of samples to be run in the same time frame but with higher analytical sensitivity and lower sample volumes.

We therefore conclude that the ionKey/MS System operating in the dual-pump trapping configuration does provide acceptable LLOQ levels for Tg using significantly less plasma while maintaining the requisite accuracy, better precision, and throughput levels exemplified by standard flow LC-MS methods. Accordingly, microflow is a viable and attractive solution for clinical research.

References

1. Lame, M.E., Chambers, E.E., Improving a High Sensitivity Assay for the Quantification of Teriparatide in Human Plasma Using the ionKey/MS System, Library Number Waters Application Note, 720004948EN, 2015.

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