The Agilent 6495 Triple Quadrupole LC/MS: Peptide Quantitation Performance

Technical Overview

Introduction

Sample complexity and the low concentration of certain biomarkers are the major challenges encountered when performing biomarker verification using multiple reaction monitoring (MRM)-based methods. For this reason, the development of MRM-based analytical methods using stable isotope-labeled standards for the quantitation of peptides in biological fluids has focused on improving method sensitivity and increasing dynamic range by lowering detection limits. Robustness and reproducibility are essential when deploying these methods in high-throughput translational research environments.

This Technical Overview presents the performance of the Agilent 6495 Triple Quadrupole LC/MS for the quantitation of peptides. Sensitivity performance for a synthetic peptide standard was evaluated in both standard flow and nanoflow configurations. The Agilent Jet Stream ionization source was used for standard-flow chromatography, and the Agilent 1260 Infinity HPLC-Chip/MS system was used for nanospray LC/MS. Instrument robustness under challenging conditions was evaluated in the standard flow configuration with direct injection of plasma digest. The instrument was not cleaned or tuned during the three and one half week robustness test.

To assess overall quantitative performance, data from the 6495 Triple Quadrupole LC/MS system were submitted to the MRM Protein Quantitation contest held in conjunction with the Mikromethoden in der Proteinchemie Meeting of July 2014. Contest samples provided by the University of Victoria - Genome BC Proteomics Centre were comprised of lyophilized mouse plasma digests spiked with the light/heavy peptide pairs of six proteins. The results from the participating labs were judged based on quantitative accuracy.

Agilent Technologies

Overview: Agilent 6495 Triple Quadrupole LC/MS Enhancements

Built on Agilent dual-stage iFunnel technology, the 6495 Triple Quadrupole LC/MS delivers attogram to zeptomole limits of detection (LOD) and limits of quantitation (LOQ) over a wide linear dynamic range. The instrument's remarkably low LODs are primarily due to enhanced ion utilization, starting with the iFunnel ion sampling through the high-transmission ion optics, and ending with the high efficiency detector.

The key design features that boost performance for peptide-specific applications include:

- Enhanced Q1 ion optics that improve ion transmission and robustness, and reduce contamination
- Curved, tapered hexapole collision cell that improves MS/MS spectral fidelity
- New detector with high energy conversion dynode (HED) that improves ion detection efficiency and lowers noise at higher HED voltages (±20 kV)
- Extended mass range to 2,250 *m/z* for the analysis of higher molecular weight peptides such as those with modifications such as glycosylation

To learn more about the Agilent 6495 Triple Quadrupole LC/MS system download the brochure at http://www.agilent.com/cs/library/brochures/5991-4541EN_6495_QQQ_Brochure_MedRes.pdf

Quantitation of Peptide Standards in Human Plasma

Sensitivity performance for quantitation of the synthetic peptide standard LVNEVTEFAK from human serum albumin (p/n G2455-85001) was evaluated in both standard flow and nanoflow LC/MS instrument configurations. To minimize loss of the target peptide due to adsorption, the standard curves for 5 amol to 5 pmol on-column were prepared in a simple background matrix of 10 fmol/µL of trypsinized enolase (Sigma, trypsinized using a 2,2,2-trifluroethanol-based protocol developed in-house). All data analysis, including standard curve generation, was performed using Agilent MassHunter WorkStation software.

Standard Flow LC/MS System Evaluation

To evaluate quantitative performance at standard flow rates, an Agilent 1290 Infinity Binary LC system was interfaced to a 6495 Triple Quadrupole LC/MS using the Agilent Jet Stream ionization source. Separations were performed on an Agilent ZORBAX RRHD 300SB-C18 RRHD, 2.1 × 50 mm, 1.8 µm column using a five-minute LC/MS analysis performed in MRM mode. Following a blank injection to establish system cleanliness, replicate (n = 10) injections were made at 10 levels from 5 amol to 5 pmol (Table 1) to evaluate reproducibility. Retention time (RT) reproducibility was determined across all samples (n = 100), and area reproducibility was determined for each level.

Table 1. Precision and accuracy for standard flow LC/MS analysis of the peptide standard LVNEVTEFAK in 10 fmol/µL trypsinized enolase, for all levels tested.

Amount on-column	%RSD (n = 10)	% Accuracy
5 amol	14.0	109.8
7.5 amol	16.0	108.7
15 amol	9.4	105.0
30 amol	9.0	87.1
300 amol	1.6	85.2
3 fmol	1.2	81.4
30 fmol	0.6	86.4
300 fmol	0.7	87.4
3 pmol	2.1	105.6
5 pmol	1.0	97.5

Figure 1 and Table 1 show that the standard flow LC/MS system demonstrated outstanding quantitative performance with:

- Low attomole-level sensitivity with a lower limit of quantitation (LLOQ) of 5 amol on-column (Figure 1A)
- Six orders of linear dynamic range (5 amol–5 pmol on-column) with R^2 = 0.998 for LVNEVTEFAK (Figure 1B)
- Excellent precision and accuracy observed at all levels, including the LLOQ level (Table 1)
- Excellent RT reproducibility (RSD = 0.12 % for n = 100)

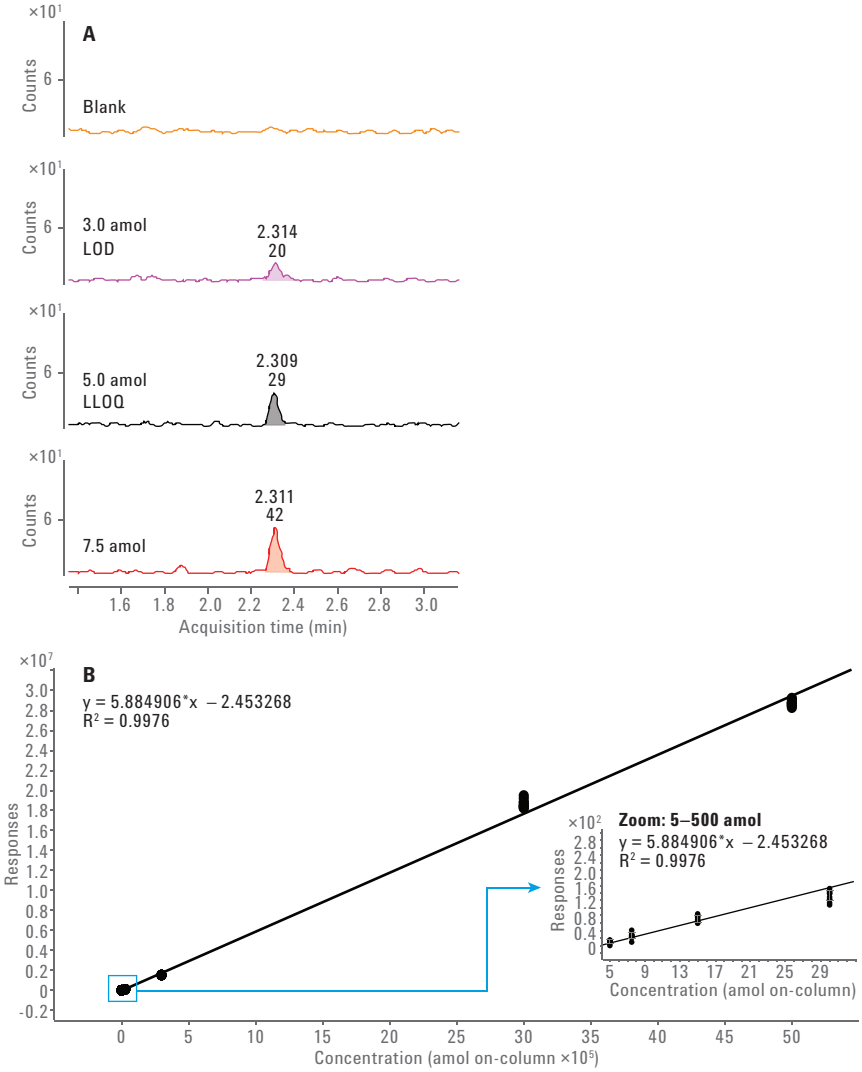

Figure 1. Standard flow LC/MS results for the peptide standard LVNEVTEFAK in 10 fmol/µL trypsinized enolase. A) Stacked extracted ion chromatograms showing the LOD and LLOQ. B) Standard curve with inset showing detail for the range of the curve from 5 to 500 amol.

Nanoflow LC/MS System Evaluation

To evaluate quantitative performance at nanoflow rates, the 6495 Triple Quadrupole LC/MS was interfaced to a 1260 Infinity HPLC-Chip/MS system. Chromatography was performed using a Polaris-HR-Chip-3C18 HPLC-Chip with 20-minute LC/MS runs in MRM mode. Following a blank injection to establish a system background, replicate (n = 5) injections were made at seven levels from 500 zmol to 100 fmol (Table 2) to evaluate %RSDs. Retention time reproducibility was evaluated across all samples, and area reproducibility was calculated for each concentration level.

Figure 2 and Table 2 show that the nanoflow LC/MS configuration provided the ultimate in sensitivity performance including:

- Zeptomole-level sensitivity, with an LLOQ of 500 zmol and an LOD of 250 zmol (Figure 1A)
- Greater than five orders of linear dynamic range (500 zmol–100 fmol on-column) with R^2 = 0.99996 for LVNEVTEFAK (Figure 1B)
- Excellent precision and accuracy observed at all levels, including the LLOQ (Table 2)
- Very good RT reproducibility (RSD = 1.4 % for n = 35)

Table 2. Precision and accuracy for nanoflow LC/MS analysis of the peptide standard LVNEVTEFAK in 10 fmol/µL trypsinized enolase at all levels tested.

Amount on-column	%RSD (n = 5)	% Accuracy
500 zmol	4.5	103.2
1 amol	8.5	80.2
10 amol	5.8	84.6
100 amol	4.7	88.9
1 fmol	1.2	85.6
10 fmol	1.1	97.6
100 fmol	1.7	100.4

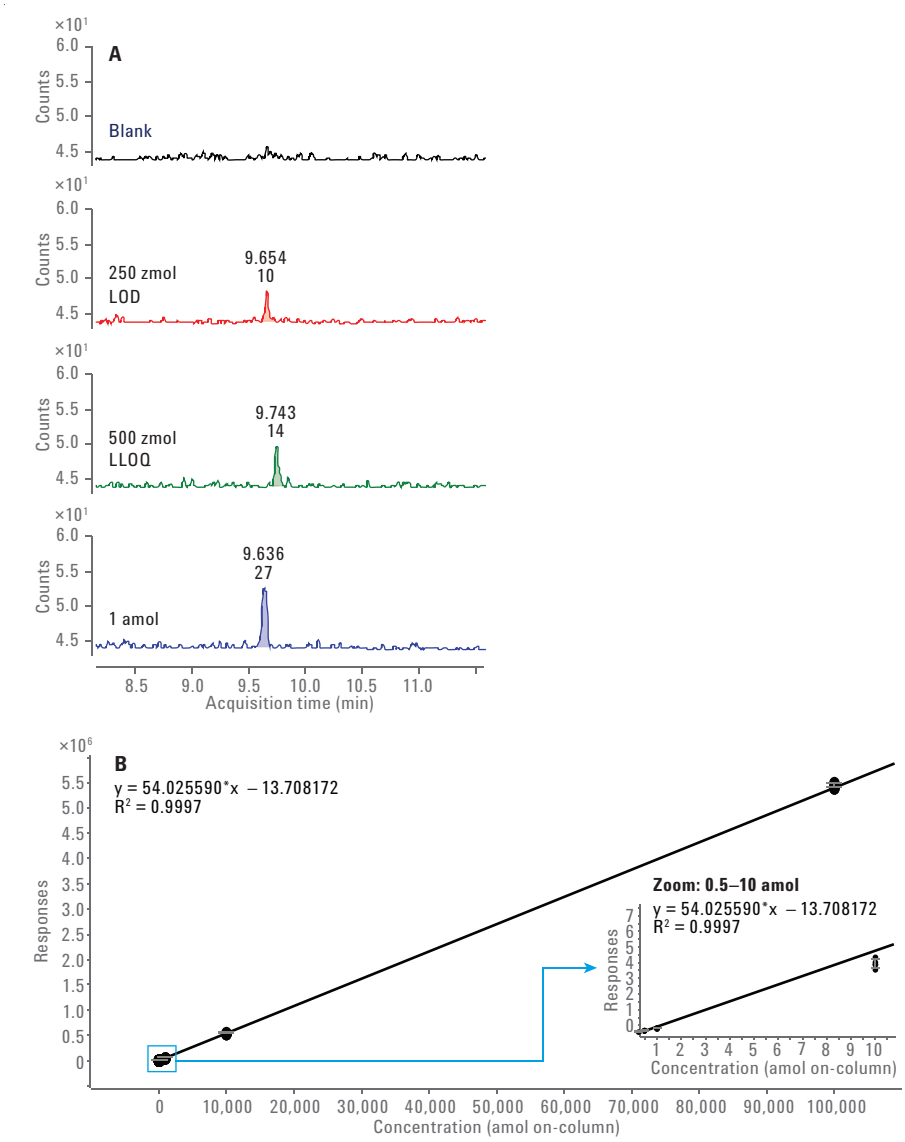

Figure 2. Nanoflow LC/MS results for the peptide standard LVNEVTEFAK in 10 fmol/µL trypsinized enolase. A) Stacked extracted ion chromatograms showing the LOD and LLOQ. B) Standard curve with inset showing detail for the range of the curve from 0.5 to 10 amol.

Robustness: Complex Sample Analysis

Robustness was evaluated using the standard flow instrument configuration through the direct injection of plasma digest (no SPE cleanup nor immunodepletion) onto an Agilent AdvanceBio Peptide Mapping 2.1 × 100 mm, 2.7 µm column without column eluent diversion at the start or end of analyses.

Protein assay kits (MRM Proteomics from Cambridge Isotope Laboratories) were purchased to provide standard plasma digest and stable-labeled synthetic peptides for 42 proteins at different concentrations in plasma. Both a daily QC kit and a monthly calibration curve kit were used. To assess system robustness, the QC samples were interspersed between sets of hundreds of injections of a high load of plasma digest (40 µg per injection). The recommended kit gradient was used for the QC samples. To accelerate the testing to three and one half weeks, a rapid 15-minute LC/MS method was used to analyze the plasma samples. Over the course of the test, the instrument was not cleaned or tuned, and because of the highly reproducible standard flow chromatography, there was no adjustment of scheduled retention time windows in the dynamic MRM (DMRM) analytical method. The Agilent DMRM algorithm automatically constructs DMRM timetables for multiple analytes throughout the LC/MS analysis based on the retention time window for each analyte, an advantage for high-throughput protein and peptide quantitation.

Figure 3 shows the results for selected peptides in the QC sample, normalized to the response obtained on the first day of the experiment. The system demonstrated excellent robustness with:

- Extremely stable retention times (average of 0.46 %RSD for all peptides across all runs)
- No significant signal degradation observed after 853 injections of 40 µg of plasma digest per injection over three and one half weeks of operation
- Stable response (%RSD = 6–15 for all peptides over all runs)

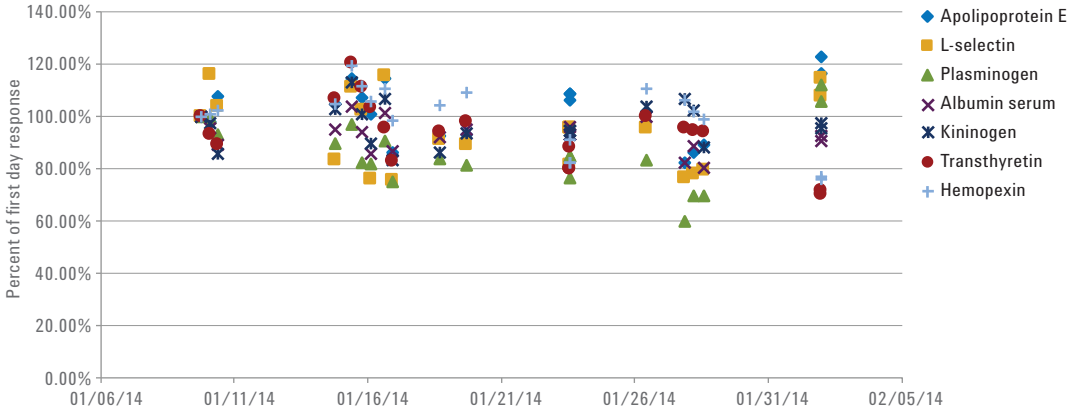

Figure 3. Results for selected peptides in the QC sample, normalized to Day 1 response.

Overall Performance: Analysis of Real-world Samples

To assess the accuracy of quantitative performance for the analysis of a typical biological sample, data from the 6495 Triple Quadrupole LC/MS system were submitted to the MRM Protein Quantitation contest held in association with the Mikromethoden in der Proteinchemie Meeting of July 2014. Contest samples provided by the University of Victoria Genome BC Proteomics Centre were comprised of lyophilized mouse plasma digests (n = 5) spiked with light/heavy peptide pairs of six proteins at different concentrations. The contest rules specified sample rehydration, transitions, and analysis using LC-MRM/MS. The data analysis strategy was chosen at the contestants' discretion, and participants were asked to report both the light natural (NAT) and heavy stable isotopic labeled standard (SIS) peptide peak areas as well as the NAT concentration. The results from the seven sites/systems that participated in the contest were judged based on their quantitative accuracy.

Sample preparation

Rehydrated raw mouse plasma was diluted with 25 mM ammonium bicarbonate. Proteins in the diluted plasma were denatured (deoxycholate), reduced (TCEP), alkylated (iodoacetamide), and tryptically digested. The digests were then spiked with a constant amount of each NAT peptide and variable amounts of SIS peptide for the six proteins (Table 3) for the creation of standard curves. The samples were extracted using solid phase extraction (SPE), concentrated, and desalted.

LC-MRM/MS was performed in the 6495 Triple Quadrupole LC/MS standard flow and nanoflow configurations described above. The NAT and SIS transitions were found and quantified in the same plasma mix. The calculated natural/endogenous concentrations were correlated to the reference peptide to assess quantitative accuracy.

Contest results

Table 4 presents the actual NAT concentration and the Agilent contest results for the target peptides. The 6495 Triple Quadrupole LC/MS systems yielded superior quantitative accuracy for four of the six peptides. With 95.30 % accuracy and 3.3 % CV, the Agilent standard flow system produced the best contest results. The Agilent nanoflow system placed second overall.

Table 3. Standard curve concentration levels for the contest samples.

Protein	UniProt Acc. No.	Peptide	Concentration (fmol/µg of total protein)				
			Std A	Std B	Std C	Std D	Std E
Heparin Cofactor 2	P05546	TLEAQLTPR	20	40	100	200	1,000
Adiponectin	Q15848	GDIGETGVPGAEGPR	0.8	1.6	4	8	40
Apolipoprotein E	P02649	LGPLVEQGR	0.8	1.6	4	8	40
Leptin	P41159	INDISHTQSVSSK	0.032	0.064	0.16	0.32	1.6
Plasminogen	P00747	LFLEPTR	0.032	0.064	0.16	0.32	1.6
Clusterin	P10909	ELDESLQVAER	0.032	0.064	0.16	0.32	1.6

Table 4. Agilent contest results for the target peptides.

Peptide	Actual concentration (fmol/µg)	Standard flow		Nanoflow	
		Concentration determined (fmol/µg)	Quantitative accuracy (%)	Concentration determined (fmol/µg)	Quantitative accuracy (%)
GDIGETGVPGAEGPR	2.49	2.37	95.20	2.30	92.23
LGPLVEQGR	5.49	5.38	97.90	5.42	98.70
ELDESLQVAER	0.45	0.42	92.05	0.37	82.34
TLEAQLTPR	632.79	692.21	91.42	665.93	95.02
INDISHTQSVSSK	0.09	0.09	99.49	0.09	99.49
LFLEPTR	0.03	0.03	95.71	0.03	95.71
	Average		**95.30**		**93.92**

Conclusion

The sensitivity, dynamic range, robustness, and reproducibility of MRM-based LC/MS methods for the quantitation of peptides in biological fluids are important considerations for high-throughput biomarker verification applications. This Technical Overview describes the performance of the Agilent 6495 Triple Quadrupole LC/MS for the quantitation of peptides in plasma for each of these considerations.

Sensitivity performance for the quantitation of a tryptic peptide standard in plasma was evaluated in both standard flow and nanoflow system configurations. The 6495 Triple Quadrupole LC/MS with Agilent Jet Stream Technology provided outstanding sensitivity at standard flow rates with an LLOQ of 5 amol on-column. The highest sensitivity, 500 zmol LLOQ, was achieved at nanoflow rates using the Agilent Infinity HPLC-Chip/MS technology. For the analysis of a complex plasma digest matrix over an extended period of three and one half weeks, the 6495 Triple Quadrupole LC/MS maintained excellent response and RT reproducibility, without cleaning or tuning.

Comparative quantitative performance was assessed through participation in the MRM Protein Quantitation contest held in association with the Mikromethoden in der Proteinchemie Meeting. Results from LC-MRM/MS analyses of contest samples of lyophilized mouse plasma digests spiked with light/heavy peptide pairs of six proteins were judged based on quantitative accuracy. The 6495 Triple Quadrupole LC/MS produced superior results for four of the six peptides, and produced the highest average quantitative accuracy for the six proteins measured.

The unequalled performance of the 6495 Triple Quadrupole LC/MS in peptide quantitation applications is the result of design enhancements including increased precursor ion transmission, improved MS/MS spectral quality, enhanced detection efficiency (HED voltage up to –20 kV), and extended *m/z* range.

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