

A New Methodology for Targeted Peptide Quantitation in Complex Mixtures Using a High Resolution Triple Quadrupole Mass Spectrometer

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Introduction

A common endpoint for a biomarker discovery experiment is a list of putative marker proteins. A reasonable next step is to then perform targeted quantitative measurements of these proteins in an expanded patient population to assess their validity as markers. Analytical accuracy and precision are required for the unambiguous quantitative analysis of targeted proteins from very complex mixtures. Wide dynamic range, high sensitivity, and robustness are critical for high throughput detection of low abundance proteins. A selected reaction monitoring (SRM) workflow, based on tandem mass spectrometry, allows highly sensitive and selective quantitation of unique peptides from proteins of interest in complex biological matrices and is indispensable for biomarker validation.¹⁻⁴ The major advantage of this approach is a low detection limit, i.e. the ability to measure low levels of peptides in complex mixtures with high chemical background, while maintaining excellent quantitative accuracy and precision.

As there are no available standards for most proteins of interest, it is necessary to confirm the identity of observed peaks in SRM experiments for protein quantitation. Traditionally, an SRM-triggered full scan MS/MS was used to confirm the identity of a targeted peptide⁵ via database search. An additional benefit of acquiring a full scan MS/MS spectrum is to enable method refinement for the selection of precursor-product transitions to ensure maximum sensitivity. However, the quality of the MS/MS scan is compromised when peptide peaks co-elute with isobaric chemical interferences from background ions.² Such an approach also suffers from worse detection limits compared to those observed for an SRM experiment. In addition, the long cycle time limits the utility of the approach for simultaneous peptide identity confirmation and quantitation in a single LC run.⁵

An alternative approach to confirm the identity of a targeted peptide is to increase the number of monitored *b*- or *y*-type ions from each peptide.⁶ This approach produces the best results with regard to sensitivity, duty cycle, and selectivity by using a triple quadrupole mass spectrometer in SRM mode while providing high confidence confirmation of the identity of targeted peptides by enabling tag searching.

The overall success of either method is dictated by the instrument's ability to select the precursor ion. Both methods require precursor ions to pass through Q1 and dissociate via ion-neutral collision. Thus, reduction of chemical interference passing through Q1 increases the

quality of the detected signal. Most commercial instruments use unit resolution (0.7 FWHM) for precursor ion selection. The Thermo Scientific TSQ Quantum Ultra is the only triple quadrupole mass spectrometer that can employ high resolution (0.2 FWHM) for precursor ion selection, allowing for highly selective reaction monitoring (H-SRM). This dramatically reduces the chemical interferences from the background while maintaining high transmission efficiency.⁷ In addition, this instrument can monitor over 300 H-SRM transitions per segment of LC run and trigger Quantitation-Enhanced Data-DependentTM MS/MS (QED-MS/MS) for peptide sequence confirmation.⁴ These unique benefits make the TSQ Quantum Ultra an ideal instrument for targeted quantitative proteomics.

In this study, we demonstrate the instrumental advantages afforded by the TSQ Quantum Ultra for highly selective and highly sensitive confident targeted peptide quantitation in whole human serum digests. A new approach for simultaneous targeted peptide identification and quantitation, based on high resolution precursor ion isolation and time alignment of multiple fragment ions from targeted peptides, is described. This approach is also compared to the traditional SRM-triggered MS/MS approach for peptide quantitation, QED-MS/MS on a TSQ Quantum Ultra, and MRM-triggered Enhanced Product Ion Scan (EPI MS/MS) from a 4000 Q TRAP[®] (Applied Biosystems, Foster City, CA). The analytical precision of the H-SRM assay was evaluated vs. that of the SRM assay on the TSQ Quantum Ultra and on the 4000 Q TRAP using the same transitions and dwell times. Lastly, the quantitative accuracy of the H-SRM assay was evaluated.

Goal

Develop a fast, robust H-SRM based workflow for accurate, quantitative analysis of many targeted proteins for the purpose of biomarker verification and validation in complex mixtures.

Experimental Conditions

Sample Preparation

An aliquot of 25 μ L of human serum (Sigma Corp., St. Louis, MO) was used. The serum sample was diluted 40-fold with 975 μ L of 100 mM ammonium bicarbonate buffer, ultra-filtered (10,000 MW cut off) and enzymatically digested. The digested mixtures were vacuum-dried and reconstituted in 250 μ L water containing 0.1% formic acid (10 \times dilution).

Key Words

- TSQ Quantum UltraTM
- H-SRM workflow
- QED-MS/MS
- Targeted Quantitative Proteomics

LC

Nano-HPLC for the TSQ Quantum Ultra

Pump: Thermo Scientific Surveyor™ MS pump with MicroAS autosampler

Column: 75 µm × 100 mm C18

Post-split flow rate: 300 nL/min

Buffer A: 0.1% FA in 2% Acetonitrile

Buffer B: 0.1% FA in Acetonitrile

Gradient: 2% B to 50% B in 90 min

Sample loading: 2 µL directly loaded on column

Nano-HPLC for the 4000 Q TRAP

Pump: Eksigent™ nanoHPLC with integrated autosampler

Column: 100 µm × 100 mm C18

Flow rate: 300 nL/min

Buffer A: 0.1% FA in 2% Acetonitrile

Buffer B: 0.1% FA in Acetonitrile

Gradient: 2% B to 50% B in 90 min

Sample loading: 2 µL directly loaded on column

MS

TSQ Quantum Ultra

Thermo Scientific Ion Max™ source equipped with a column adapter for nanoflow (New Objective).

H-SRM: Q1, 0.2 FWHM; Q3, 0.7 FWHM

SRM: Q1, 0.7 FWHM; Q3, 0.7 FWHM

Q2, 1.5 mTorr; Dwell times, 20 ms, 10 ms,

and 5 ms; For 318 transitions H-SRM assay

5 ms dwell time was used resulting in the cycle time of 2.2 s

QED-MS/MS:

Scan event 1 (SRM): Q1 and Q3, 0.7 FWHM;

Q2, 1.5 mTorr; Dwell time, 10 ms

Scan event 2 (QED-MS/MS): DD precursor mass

from scan event 1; Signal threshold 5,000 counts;

Q1 and Q3, 0.7 FWHM; Q2, 1.5 mTorr;

CE, 0.034 × precursor mass m/z + 3.134;

Dynamic exclusion: repeat, 2; duration, 60 s;

Exclusion time, 60 s; exclusion list size, 50;

Cycle time, 3.84 s.

4000 Q TRAP*

NanoSpray™ source for nanoflow.

MRM: Q1, 0.7 FWHM; Q3, 0.7 FWHM;

Q2, high pressure

Dwell times, 20 ms, 10 ms, and 5 ms

EPI MS/MS:

Scan event 1 (MRM): 0.7 FWHM for Q1 and Q3;

Q2, high pressure; Dwell time, 10 ms;

Scan event 2 (EPI MS/MS): DD precursor mass from scan

event 1; Signal threshold, 3000 cps Q1;

Q1 and Q3, low resolution (1.5 FWHM); Q2, high

pressure; CE, 30 with spread of 6; Dynamic fill, on;

repeat, 1; Exclusion time, 60 s; Cycle time, 6.5 s.

Database Searching

To facilitate comparison of results, all data were processed with Mascot™ software (Matrix Sciences) using SwissProt database (taxonomy: Human) and the following search parameters:

Mass values: Monoisotopic

Protein Mass: Unrestricted

Peptide Mass Tolerance: ± 1.2 Da

Fragment Mass Tolerance: ± 0.6 Da

No Missed Cleavages

Instrument type: ESI-Quad

Report top hits: auto.

*The 4000 Q TRAP mass spectrometer was operated according to Whetton and co-workers⁵ and the 4000 Q TRAP operating manual. The experiments were performed in an independent third-party laboratory by a certified, SCIEX®-trained operator.

The screenshot displays the SRM workflow software interface. On the left, a vertical toolbar contains icons for: 'Add proteins', 'Digest Proteins', 'Add/Edit Modifications for a peptide', 'Add/Edit Transitions for a peptide', 'Generate Quantum Sequence and Method', and 'Process RAW file'. The main workspace is divided into several panels:

- Add proteins to the data set:** A dialog box with a 'Protein Description' field, a 'Paste protein sequence' text area containing a long amino acid sequence, and an 'Add' button.
- Digestion Parameters:** A dialog box for configuring digestion settings. It includes a 'Digestion Enzyme' dropdown set to 'Trypsin (KR)', a 'Number of flanking amino acids to be ignored' set to 15, 'Peptide Length Constants' from 7 to 25, and 'Peptide M+ Constants' from 400.2 to 1800.2. There are checkboxes for 'Cys', 'Met', 'NXT/NGS', and 'RP/KP'. Below these are options for 'Which mass to use?' (Monoisotopic or Average) and 'Number of missed cleavage sites allowed'. At the bottom, there are fields for 'Use Database to filter redundant sequences' and 'Eliminate peptides occurring more than 2 times in the database', along with a 'Digest' button.
- Transition Editor:** A table for defining SRM transitions. It has columns for 'Peptide sequence', 'Precursor mass', and 'Product mass'. The table contains several rows of data, such as:

Peptide sequence	Precursor mass	Product mass
LSLQLGLLFGAPFLNR	1152.0605	676.0960
GVAAAAATGAMR	1159.0705	656.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049
YVDFVNR	1162.0705	671.3049

Figure 1: Peptide selection and SRM transition design using SRM workflow software.

Peptide Selection and SRM Transition Design

There are two basic approaches for peptide selection and SRM transition design.³ If the targeted protein has been detected in a previous LC-MS/MS experiment, peptides identified from these experiments that are unique to that protein are subsequently selected for SRM assay design. Alternatively, if no LC-MS/MS data are available for the targeted protein, the peptide selection and SRM assay design are performed *in silico* using the known protein sequences (hypothesis-based approach). We have developed an SRM workflow software for targeted protein quantitation⁸ that can be used for predicting candidate peptides and choosing multiple fragment ions for SRM assay design, for building an instrument method and a sequence file, and also for automatic peptide identity confirmation and quantitative data processing (Figure 1). This software was used to design all the SRM assays described in this work.

Our first goal was to evaluate the sensitivity advantages of an H-SRM assay. High-confidence detection of targeted peptides was achieved using time alignment of specific multiple fragment ions from each peptide. This approach was compared with a traditional SRM-based assay in which the SRM signal triggers a full scan MS/MS event (QED-MS/MS on the TSQ Quantum Ultra and EPI MS/MS on 4000 Q TRAP) for peptide confirmation. For this, 24 serum proteins with plasma concentrations ranging from 3.40E5 pg/mL (vitronectin) to 1.25E9 pg/mL (haptoglobin)⁹

were selected. One to four proteotypic peptides^{1,3,4} were used to monitor each targeted protein. The selected peptide sequences contained no Cys, Met or other commonly modified residues and had a mass range of 800–2400 Da (a total of 50 peptides, Table 1). Five to seven y -ions from each peptide were monitored yielding a total of 318 H-SRM transitions. Because no full scan MS/MS data are acquired in this H-SRM approach, it was necessary to make sure that the selected y -ion series would uniquely identify each targeted peptide. To do this, we searched selected y -ions and a precursor ion from each peptide against the human SwissProt database using Mascot Sequence Query and confirmed that the targeted peptide was unique and had a high peptide score. When using QED-MS/MS or EPI MS/MS to confirm the identity of targeted peptides, two to four y -ions from each peptide were monitored in the SRM survey scan (a total of 152 SRM transitions) used to trigger the MS/MS acquisition.

Our other goal was to evaluate the quantitative reproducibility and accuracy of the H-SRM assay by comparing the results of an H-SRM assay on the TSQ Quantum Ultra with those from an SRM assay on the TSQ Quantum Ultra and an MRM assay on the 4000 Q TRAP using variable dwell times. Thirteen serum proteins with varying concentrations were targeted for these experiments. A total of 61 SRM transitions, designed from 20 unique peptides representing these proteins, were used.

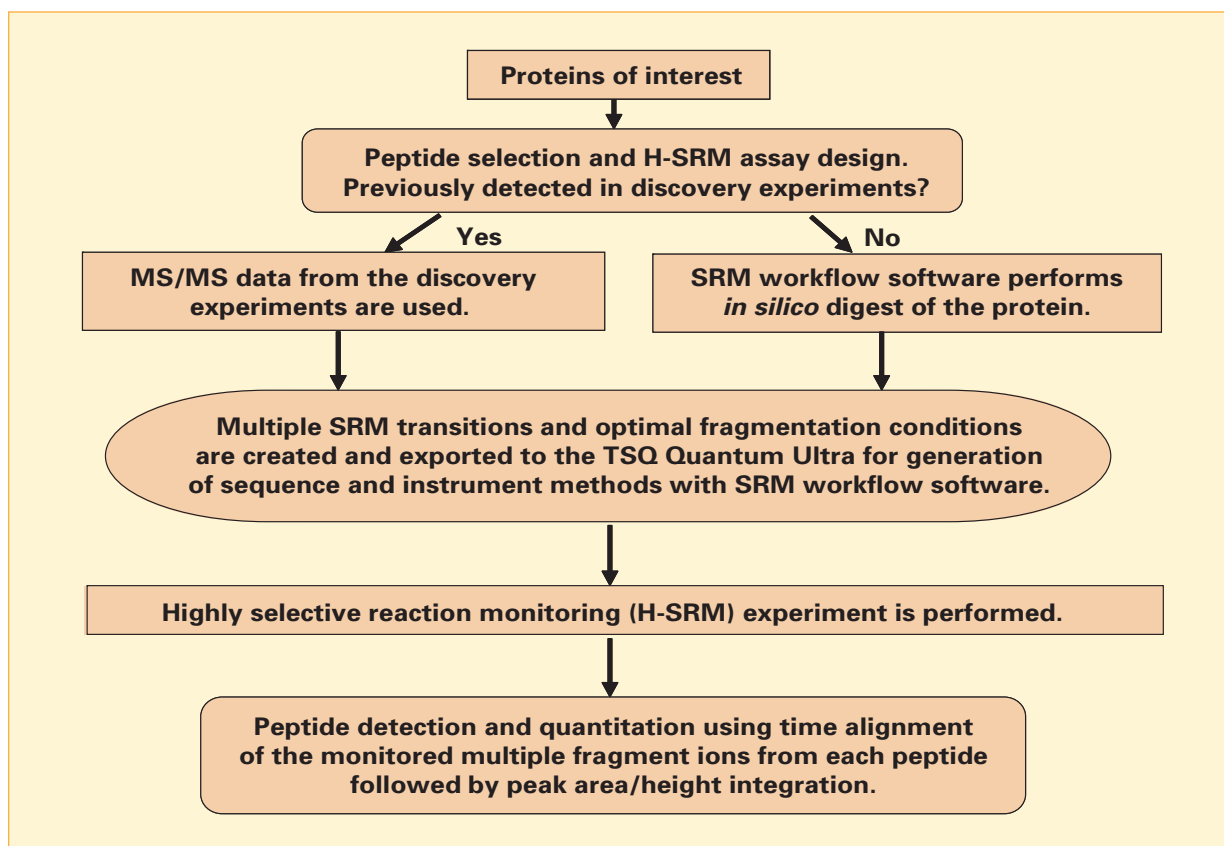


Figure 2: H-SRM workflow for simultaneous peptide detection and quantitation.

Results and Discussion

Evaluation of the H-SRM Based Workflow for Targeted Peptide Quantitation

The proposed H-SRM workflow is shown in Figure 2. The main advantage of high resolution isolation (0.2 FWHM) is its ability to selectively detect low abundance peptide peaks within a complex background.³ The identity of the peptide is confirmed by monitoring multiple selected fragment ions with the same retention time. There is no

limit on the number of fragment ions that can be selected for each peptide with this approach. Typically, four to seven y-type fragment ions from each targeted peptide are sufficient to maintain high assay specificity. The enhanced selectivity of the high resolution precursor ion isolation obviates the need for a time-consuming full scan MS/MS event. The shorter duty cycle with H-SRM (2.2 s for a 318 H-SRM assay using a 5 ms dwell time setting) allows for simultaneous peptide confirmation and quantitation

#	Protein	Peptide	RT	Multiple H-SRM TSQ Quantum Ultra 50 peptides identified	QED-MS/MS TSQ Quantum Ultra* 36 peptides identified	EPI MS/MS 4000 Q TRAP* 32 peptides identified
1	Alpha-1-acid-glycoprotein2	WFYIASAFR	55.24	✓	✓	✓
		TEDTIFLR	36.68	✓	✓	✓
2	Alpha-2-antiplasmin	LGNQEPGGQTALK	25.65	✓	✓	✓
		FDPSLTQR	29.80	✓	X	✓
3	Alpha-2-HS-glycoprotein	HTLNQIDEVK	26.27	✓	✓	✓
		FSVVYAK	31.03	✓	✓	✓
4	AMBP	ETLLQDFR	42.73	✓	✓	✓
5	Apolipoprotein M	AFLTPR	36.58	✓	✓	X
		WYHLTEGSTDLR	42.22	✓	X	✓
6	Apolipoprotein H	ATVVYQGER	18.96	✓	✓	✓
		EHSSLAFWK	36.27	✓	✓	X
7	Apolipoprotein L1	NEADELR	17.09	✓	X	✓
		VAQELEEK	17.78	✓	✓	✓
8	Human coagulation factor XII	TEQAAVAR	32.03	✓	X	X
		EQPPSLTR	24.38	✓	✓	✓
9	Complement component 4A preproprotein	VDFTLSSER	37.17	✓	✓	✓
		DSSTWLTAFLK	67.72	✓	✓	X
		DFALLSLQVPLK	68.19	✓	✓	✓
		VGDTLNLNLR	41.22	✓	✓	X
10	Complement component 1 inhibitor precursor	LLDSLPSDTR	50.54	✓	X	X
		TNLESILSYPK	49.50	✓	X	X
11	Complement component1, s sub component	TINVPLR	33.60	✓	X	X
		EDTPNSVWEPK	35.87	✓	✓	✓
12	Complement factor B	YGLVTYATYPK	41.55	✓	✓	✓
		STGSWSTLK	27.77	✓	✓	✓
		EELLPAQDIK	38.65	✓	✓	✓
13	C-type lectin domain family 3, member B	NWETEITAQPDGGK	40.42	✓	X	X
		LDTLAQEVALLK	54.10	✓	✓	✓
14	Gelsolin isoform b	HVVPNEVVQR	29.32	✓	✓	✓
		TGAQELLR	26.98	✓	✓	✓
		PALPAGTEDTAK	27.29	✓	X	X
15	Haptoglobin	TEGDGVYTLNDK	30.86	✓	✓	✓
		VGYSVSWGR	33.55	✓	✓	✓
16	Hemoglobin alpha chain	VGAHAGEYGAELER	30.58	✓	X	X
17	Hemopexin	NFPSPVDAEFR	45.50	✓	✓	✓
18	Histidine-rich glycoprotein	DGYLFQLLR	60.97	✓	✓	✓
		QIGSVYR	19.16	✓	✓	✓
19	Inter-alpha-trypsin inhibitor heavy	AAISGENAGLVR	30.37	✓	✓	X
		EVAFDLEIPK	52.24	✓	✓	✓
		LDAQASFLPK	39.53	✓	✓	X
20	Kininogen 1	TVGSDTFYSFK	40.83	✓	✓	X
		QVVAGLNFR	37.73	✓	✓	✓
		DFVQPPTK	29.97	✓	X	X
21	Plasma kallikrein	VSEGNHDIALIK	30.86	✓	X	X
22	Serum amyloid P-componenet	VGEYSLYIGR	40.41	✓	✓	✓
		IVLGOEQDSYGGK	31.70	✓	✓	✓
23	Vitronectin	VDTVDPYPR	33.10	✓	X	X
		FEDGVLDPDYPR	42.36	✓	✓	✓
24	Zinc alpha-2-glycoprotein 1	AGEVQPELR	28.19	✓	✓	✓
		EIPAWVFPDPAQITK	61.47	✓	X	X

* Peptides were identified by Mascot 2.2 search against SwissProt database

Table 1: Amino acid sequence and retention time of 50 peptides representing 24 targeted proteins. The comparison of identified peptides with three different methods is also shown.

within a single LC run while maintaining the maximum sensitivity for detection. In order to evaluate the advantages of the H-SRM based methodology, we compared the results from three different experiments for detecting the same 50 peptides representing 24 proteins in human serum (Table 1). The first two experiments were based on the traditional SRM-triggered full scan MS/MS approach using two high performance tandem mass spectrometers: TSQ Quantum Ultra and 4000 Q TRAP. One hundred and fifty-two transitions (2-3 transitions per peptide, 1-4 peptides per protein) for each SRM survey scan were used. The third experiment utilized 318 SRM transitions (5-7

transitions per peptide, 1-4 peptides per protein) without Data Dependent full scan MS/MS. Both high resolution (0.2 FWHM) and unit resolution (0.7 FWHM) were used for the 318 SRM assay in order to evaluate the benefits of H-SRM for eliminating interference from serum background ions.

Figure 3 shows the total ion chromatogram of the QED-MS/MS experiment on the TSQ Quantum Ultra (top) and the EPI MS/MS experiment on the 4000 Q TRAP (bottom). The MS/MS data from the TSQ Quantum Ultra were searched against the SwissProt database using SEQUEST® and Mascot. Both search engines produced

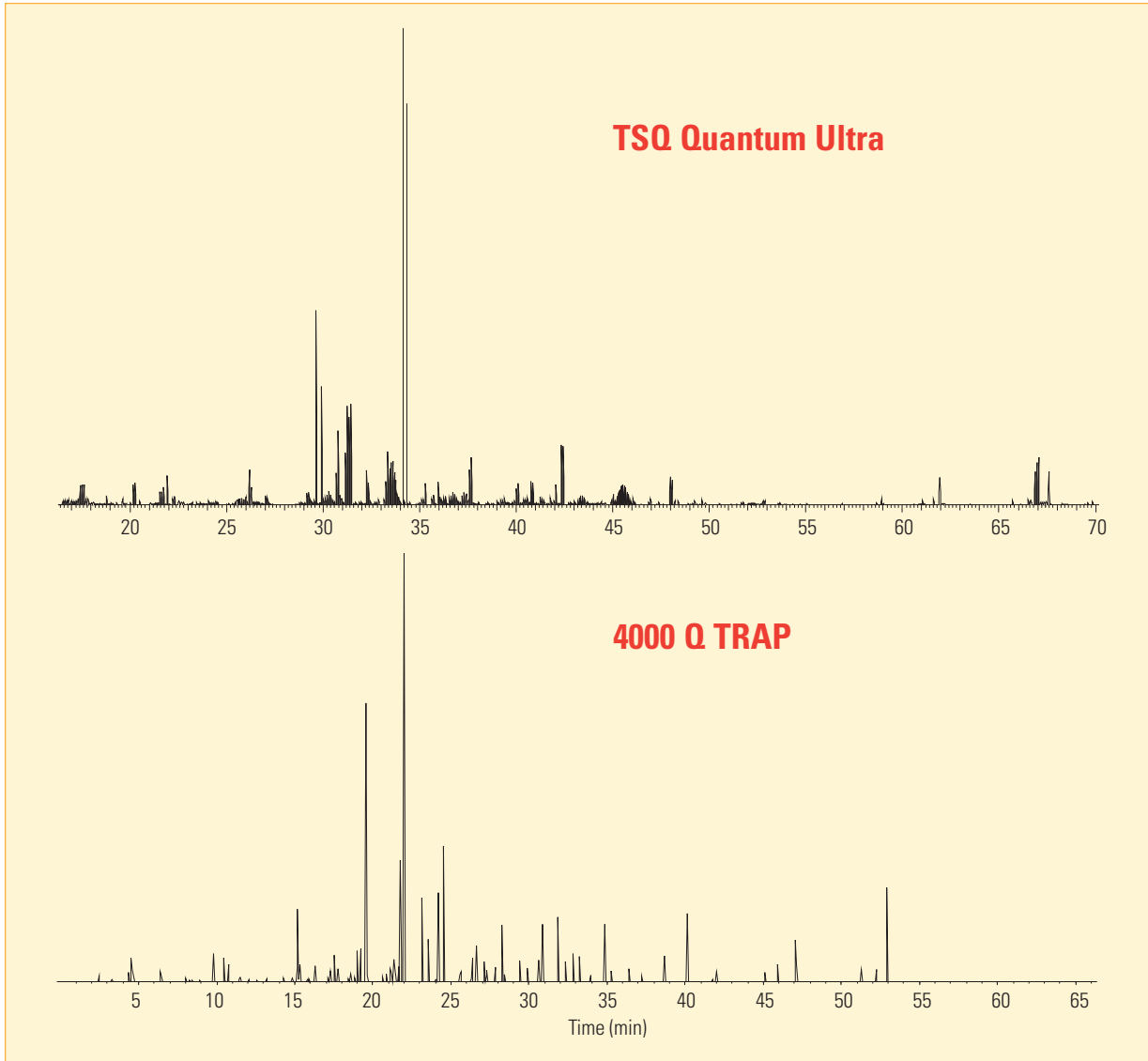


Figure 3: Total ion chromatogram for identifying 50 peptides using QED-MS/MS on TSQ Quantum Ultra (top) and EPI MS/MS on 4000 Q TRAP (bottom)

similar results. The MS/MS data from the 4000 Q TRAP were searched with ProteinPilot™ and Mascot against the same SwissProt database. ProteinPilot gave poor results due to lack of charge state confirmation, but better search results were obtained by Mascot. Tables 2 and 3 summarize the Mascot search results for QED-MS/MS data on the TSQ Quantum Ultra and the EPI MS/MS data on the 4000 Q TRAP, respectively. Both experiments identified

21 of the 24 targeted proteins (complement component 1 inhibitor, plasma kallikrein and hemoglobin alpha chain were not identified with $P < 0.05$). However, using the TSQ Quantum Ultra, we identified four more peptides (36 total) than the 4000 Q TRAP (32 total).

Unlike the 4000 Q TRAP, which must use low resolution isolation (1.5 FWHM) for both Q1 and Q3 to obtain sensitive full scan MS/MS spectra,^{2,5} the TSQ

Protein Hits	Protein Description	Protein Score	Pep Rank	Pep Exp <i>m/z</i>	Pep Exp <i>Mr</i>	Pep Calc <i>Mr</i>	Peptide Score	Sequence
1	C04A_HUMAN Complement C4-A precursor	167	1	527.26	1052.51	1052.51	30	VDFTLSSER
			1	557.82	1113.63	1113.61	50	VGDTLNLNLR
			1	672.40	1342.79	1342.79	48	DFALLSLQVPLK
			1	684.36	1366.71	1366.71	34	DSSTWLTAFLVK
2	HPT_HUMAN Haptoglobin precursor	115	1	490.75	979.49	979.49	36	VGYVSGWGR
			1	656.31	1310.61	1310.60	80	TEGDGVYTLNDK
3	ITIH1_HUMAN Inter-alpha-trypsin inhibitor heavy chain H1 precursor	104	1	545.30	1088.59	1088.59	32	LDAQASFLPK
			1	580.81	1159.61	1159.61	24	EVAFDLEIPK
			1	579.32	1156.63	1156.62	48	AAISGENAGLVR
4	KNG1_HUMAN Kininogen-1 precursor	102	2	502.29	1002.57	1002.56	11	VWAGLNFR
			1	626.30	1250.59	1250.58	92	TVGSDTFYSFK
5	GELS_HUMAN Gelsolin precursor	83	1	444.25	886.49	886.49	49	TGAQELLR
			1	638.34	1274.67	1274.71	34	HVVPEVNVQOR
6	FETUA_HUMAN Alpha-2-HS-glycoprotein precursor	81	1	407.23	812.45	812.44	24	FSVYAK
			1	598.82	1195.63	1195.62	57	HTLNQIDEVK
7	SAMP_HUMAN Serum amyloid P-component precursor	59	1	578.80	1155.59	1155.59	10	VGEYSLYIGR
			1	697.35	1392.69	1392.69	49	IVLGQEQQDSYGGK
8	A1AG1_HUMAN Alpha-1-acid glycoprotein 1 precursor	55	1	497.76	993.51	993.51	43	TEDTIFLR
			1	580.81	1159.61	1159.58	11	WFYIASAFR
9	CFAB_HUMAN Complement factor B precursor	54	1	483.75	965.49	965.48	10	STGGSWSTLK
			1	578.32	1154.63	1154.62	22	EELLPAQDIK
			1	638.34	1274.67	1274.65	23	YGLVYATYPK
10	APOL1_HUMAN Apolipoprotein-L1 precursor	37	1	473.25	944.49	944.48	37	VAQELEEK
11	HRG_HUMAN Histidine-rich glycoprotein precursor	40	1	411.73	821.45	821.44	14	QIGSVYR
			2	562.81	1123.61	1123.60	26	DGYLFQLLR
12	APOH_HUMAN Apolipoprotein H	40	1	511.77	1021.53	1021.52	26	ATVVYQGER
			1	552.78	1103.55	1103.54	14	EHSSLAFWK
13	HEMO_HUMAN Hemopexin precursor	34	1	610.81	1219.61	1219.60	34	NFSPVDAAFR
14	AMBP_HUMAN AMBP protein precursor	32	1	511.27	1020.53	1020.52	32	ETLLQDFR
15	C1S_HUMAN Complement C1s subcomponent precursor	25	1	686.82	1371.63	1371.63	25	EDTPNSVWEPK
16	APOM_HUMAN Apolipoprotein M	24	1	409.25	816.49	816.49	24	AFLTTPR
17	ZA2G_HUMAN Zinc-alpha-2-glycoprotein precursor	19	1	564.29	1126.57	1126.56	19	AGEVQEPFLR
18	VTNC_HUMAN Vitronectin precursor	18	1	711.83	1421.65	1421.65	18	FEDGVLDPDYPR
19	TETN_HUMAN Tetranectin precursor (TN)	17	1	657.39	1312.77	1312.76	17	LDTLAQEVALLK
20	A2AP_HUMAN Alpha-2-antiplasmin precursor	15	1	656.85	1311.69	1311.68	15	LGNQEPGGQTALK
21	FA12_HUMAN Coagulation factor XII precursor	15	1	464.25	926.49	926.48	15	EQPPSLTR

Table 2: Search results of QED-MS/MS data on the TSQ Quantum Ultra using Mascot against the SwissProt database. Twenty-one out of 24 proteins were identified.

Quantum Ultra can acquire sensitive QED-MS/MS spectra by using unit resolution (0.7 FWHM) or high resolution (0.2 FWHM) for precursor ion isolation. Figure 4 shows two examples of SRM-triggered QED-MS/MS spectra and the search results with SEQUEST (a: high abundance protein of haptoglobin and b: low abundance protein of vitronectin). Both targeted peptides (TEGDVGYTLNDK and FEDGVLDPDYPR) were confidently identified. When

using higher resolution (0.7 FWHM) for precursor ion isolation, the full scan MS/MS spectrum is less contaminated by fragments from isobaric co-eluters, which significantly improves its quality. This is clearly shown for the QED-MS/MS spectrum of m/z 684.4 (isolation width 0.7 FWHM), Figure 5 (a) and the EPI MS/MS spectrum of m/z 684.4 (isolation width 1.5 FWHM), Figure 5 (b). The QED-MS/MS spectrum produced a good match with

Protein Hits	Protein Description	Protein Score	Pep Rank	Pep Exp m/z	Pep Exp M_r	Pep Calc M_r	Peptide Score	Sequence
1	CFAB_HUMAN Complement factor B precursor	137	1	483.80	965.59	965.48	43	STGSWSTLK
			1	578.30	1154.59	1154.62	26	EELLPAQDIK
			1	638.30	1274.59	1274.65	68	YGLVYATYPK
2	SAMP_HUMAN Serum amyloid P-component precursor	115	1	578.80	1155.59	1155.59	53	VGEYSLYIGR
			1	697.40	1392.79	1392.69	62	IVLGQEQDSYGGK
3	HPT_HUMAN Haptoglobin precursor	109	1	490.80	979.59	979.49	26	VGYSVSWGR
			1	656.30	1310.59	1310.60	83	TEGDVYTLNDK
4	GELS_HUMAN Gelsolin precursor	85	1	444.30	886.59	886.49	39	TGAQELLR
			1	638.40	1274.79	1274.71	46	HVVPNEVVQR
5	FETUA_HUMAN Alpha-2-HS-glycoprotein precursor	85	1	407.20	812.39	812.44	32	FSVVYAK
			1	598.80	1195.59	1195.62	53	HTLNQIDEVK
6	A2AP_HUMAN Alpha-2-antiplasmin precursor	78	1	407.20	812.39	812.44	31	FDPSTLQR
			1	598.80	1195.59	1195.62	48	LGNQEPGGQTALK
7	A1AG1_HUMAN Alpha-1-acid glycoprotein 1 precursor	78	1	497.80	993.59	993.51	42	TEDTIFLR
			1	580.80	1159.59	1159.58	36	WFYIASAFR
8	APOL1_HUMAN Apolipoprotein-L1 precursor	77	2	423.70	845.39	845.39	39	NEADELR
			1	473.30	944.59	944.48	38	VAQELEEK
9	CO4A_HUMAN Complement C4-A precursor	76	1	527.30	1052.59	1052.51	37	VDFTLSSER
			1	672.40	1342.79	1342.79	30	DFALLSLQVPLK
10	AMBP_HUMAN AMBP protein precursor	59	1	511.30	1020.59	1020.52	59	ETLLQDFR
11	ITIH1_HUMAN Inter-alpha-trypsin inhibitor heavy chain H	56	1	580.80	1159.59	1159.61	56	EVAFDLEIPK
12	TETN_HUMAN Tetranectin precursor (TN)	55	1	657.40	1312.79	1312.76	55	LDTLAQEVALLK
13	C1S_HUMAN Complement C1s subcomponent precursor	51	1	686.80	1371.59	1371.63	51	EDTPNSVWEPAK
14	VTNC_HUMAN Vitronectin precursor	44	1	711.80	1421.59	1421.65	44	FEDGVLDPDYPR
15	ZA2G_HUMAN Zinc-alpha-2-glycoprotein precursor	39	1	564.30	1126.59	1126.56	39	AGEVQPELRL
16	HRG_HUMAN Histidine-rich glycoprotein precursor	37	1	562.80	1123.59	1123.60	37	DGYLFQLLR
17	HEMO_HUMAN Hemopexin precursor	34	1	610.80	1219.59	1219.60	34	NFPPSPVDAAFR
18	APOH_HUMAN Apolipoprotein H	31	1	511.80	1021.59	1021.52	31	ATVYQGER
19	APOM_HUMAN Apolipoprotein M	29	1	531.10	1590.28	1589.78	29	WIYHLTEGSTDLR
20	FA12_HUMAN Coagulation factor XII precursor	24	1	464.30	926.59	926.48	24	EQPPSLTR
21	KNG1_HUMAN Kininogen-1 precursor	18	1	502.30	1002.59	1002.56	18	QVVAGLNFR

Table 3: Search results of EPI MS/MS data on 4000 Q TRAP using Mascot against SwissProt database. Twenty-one out of 24 proteins were identified.

Mascot, confirming the identity of the targeted peptide DSSTWLTAFLVK (Mascot P-score of 34). On the other hand, the EPI MS/MS spectrum from the 4000 Q TRAP was contaminated with fragments from isobaric precursors

and the quality of the MS/MS spectrum was too poor (Mascot P-score of 5) to confidently identify the same peptide. A much longer cycle time for EPI MS/MS (6.5 s vs. 3.8 s for QED-MS/MS) is an additional disadvantage.

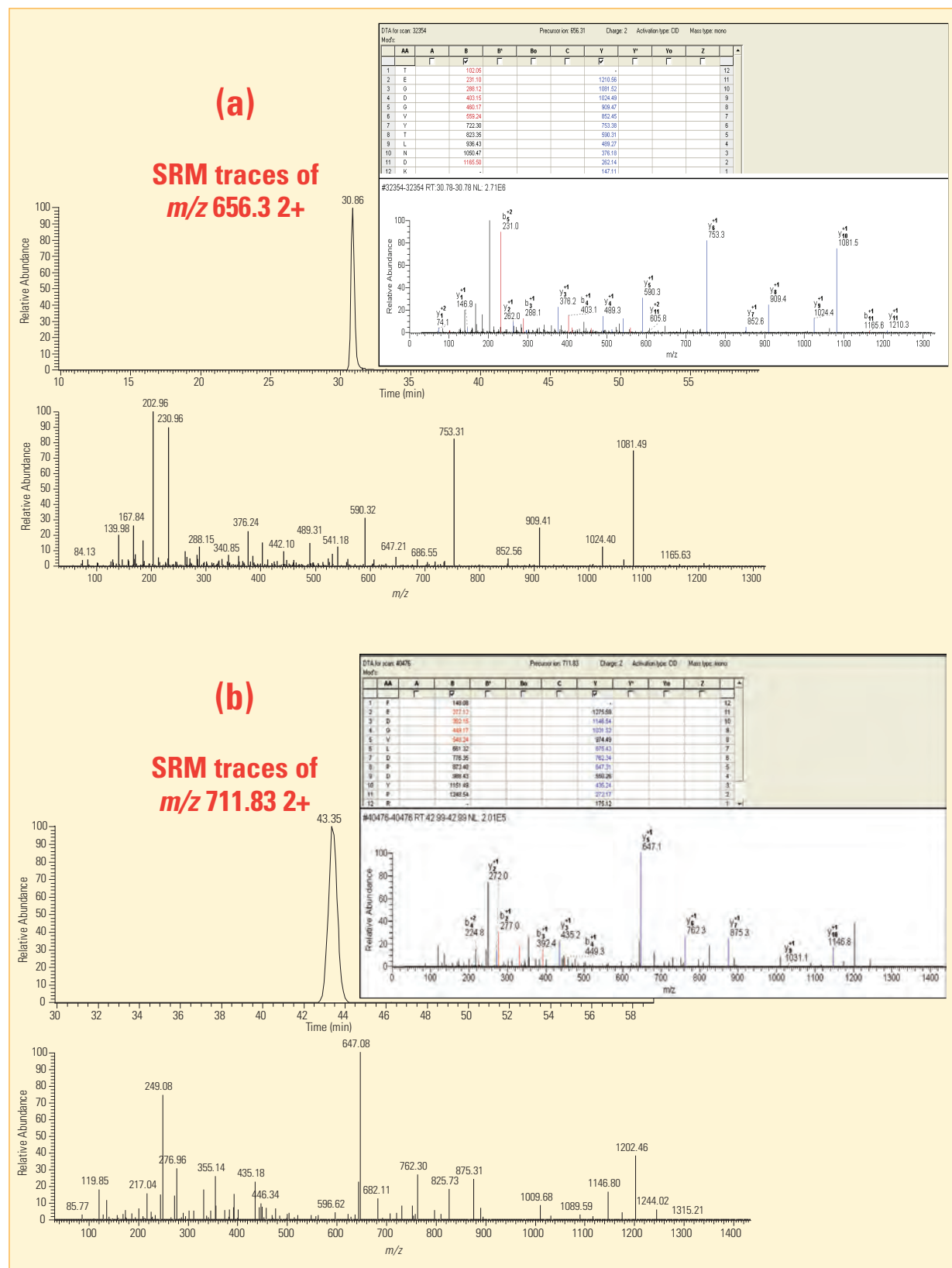


Figure 4: Two examples of identifying targeted peptides by QED-MS/MS. The insets show the SEQUEST search results. (a) SRM transition for peptide TEGDGVYTLNDK representing haptoglobin (1.25 mg/mL in plasma⁹) triggered the QED-MS/MS acquisition. (b) SRM transition for peptide FEDGLVDPYPR representing vitronectin (340 ng/mL in plasma⁹) triggered the QED-MS/MS acquisition.

This explains why the TSQ Quantum Ultra with QED-MS/MS identified more peptides (36 out of 50) than did the 4000 Q TRAP with EPI MS/MS (32 out of 50) (Table 2, 3).

Although the TSQ Quantum Ultra was able to identify several more targeted peptides by using QED-MS/MS compared with the 4000 Q TRAP, it was still challenging to identify all 50 targeted peptides from the whole serum

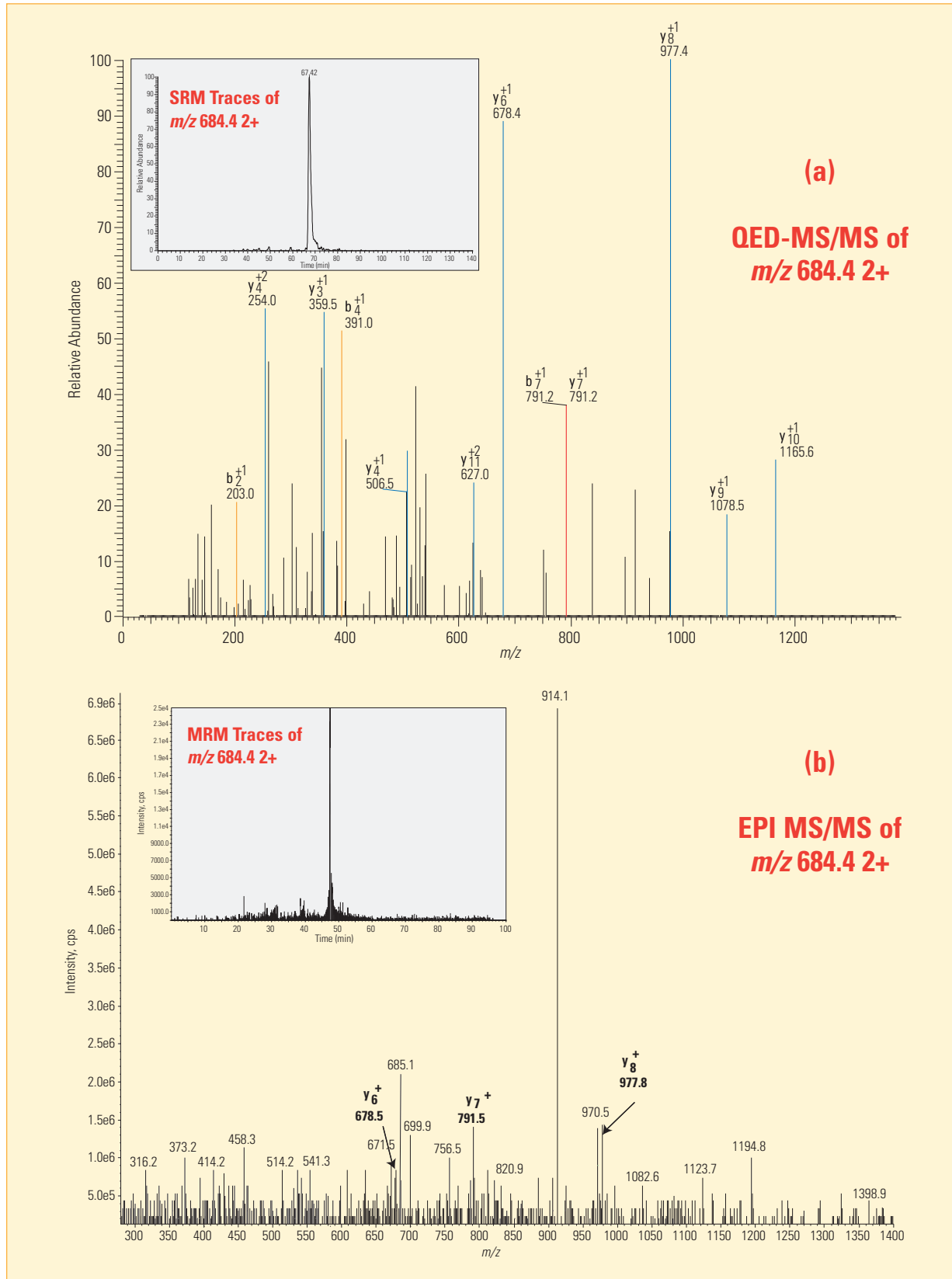


Figure 5: Comparison of a QED-MS/MS spectrum from the TSQ Quantum Ultra (a) with an EPI MS/MS spectrum from the 4000 Q TRAP (b) for the precursor ion of m/z 684.4 (+2). The targeted peptide DSSTWLTFVLK from complement component 4A preproprotein was confidently confirmed by high quality QED-MS/MS with a Mascot P-score of 34 (a). But, it was not confirmed by EPI MS/MS because contaminating fragment ions in the spectrum resulted in a low P-score of 5 (b).

digests. This is due to the limitations of the traditional approach to detection of low abundance peptides in complex backgrounds, specifically the challenge of confidently identifying peptides that co-elute with isobaric interferences.² The difficult task of identifying all 50 targeted peptides from 24 proteins in complex whole serum digests was accomplished only by using our proposed new approach, which relies on high resolution isolation for detection of low abundance peptides, and time alignment of multiple fragment ions for confirmation of the peptides' identities. Figure 6 shows one example of

a low abundance peptide (LLDSLPSDTR representing complement component 1 inhibitor), which was not identified by the traditional approach, but was successfully detected using H-SRM. The H-SRM TIC (top) showed single peaks with approximately *12 times higher* signal-to-noise ratio as compared with the SRM TIC (bottom). The precursor identity was confidently confirmed by time-aligning five γ -ions; these were sufficient to uniquely identify this peptide using a Mascot Sequence Query against SwissProt database (Figure 7). By rendering full scan MS/MS unnecessary, and together with high resolution

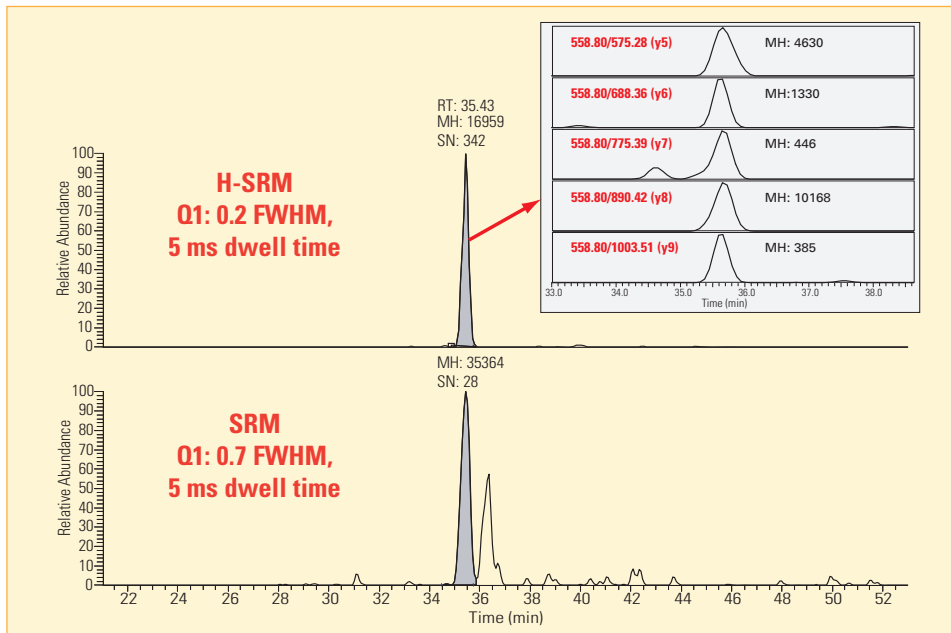


Figure 6: The peptide LLDSLPSDTR representing complement component 1 inhibitor was confirmed by using time alignment of its multiple fragment ions (inset). Compared with SRM, the H-SRM assay improved S/N significantly and reduced non-specific interference from the serum background dramatically which allowed for unambiguous detection of the targeted peptide.

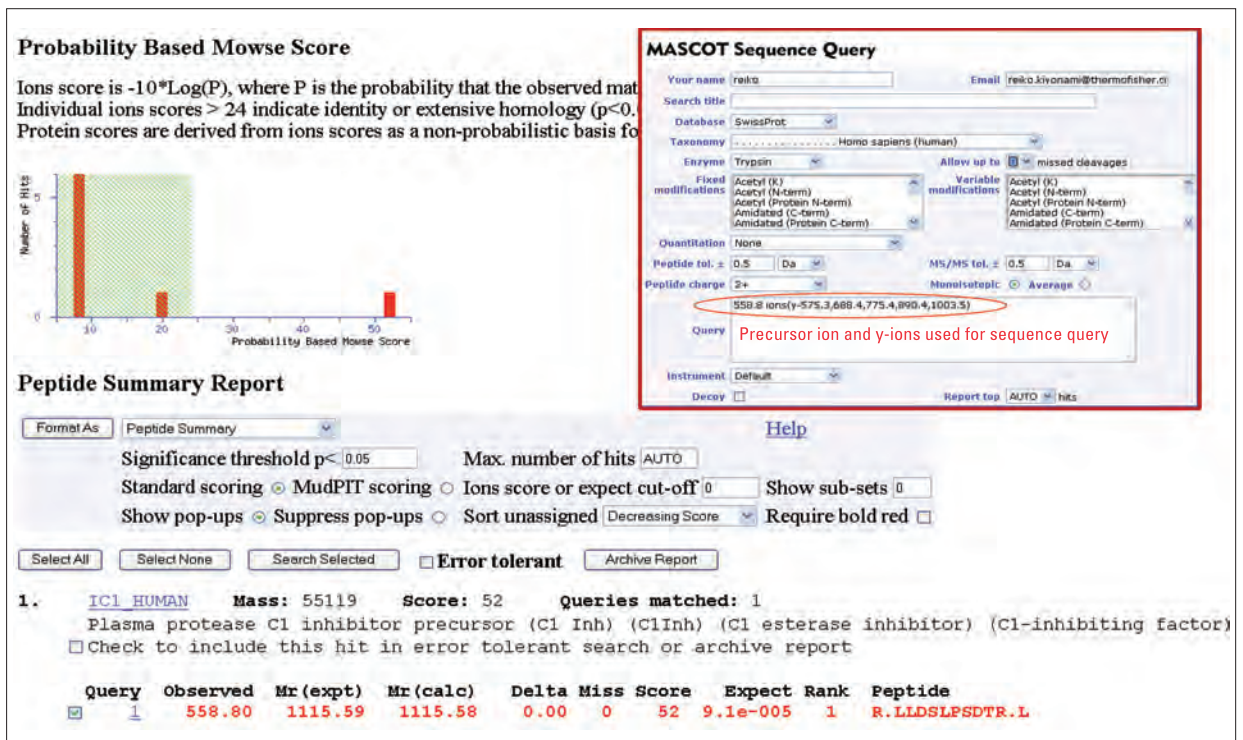


Figure 7: The Mascot sequence query result using five selected γ -type fragment ions from targeted peptide LLDSLPSDTR.

isolation, this approach provides a significant advantage for fast and unambiguous identification of targeted peptides with much lower detection limits. Table 1 presents a summary of the targeted peptides that were confirmed by the three different methods. All 50 peptides were detected with confirmed identities using time alignment of multiple fragment ions from the H-SRM assay, compared to 36 peptides detected using QED-MS/MS on the TSQ Quantum Ultra and only 32 peptides using EPI MS/MS on the 4000 Q TRAP (Figure 8).

The shorter H-SRM cycle time (2.2 s vs. 3.9 s for QED and 6.5 s for EPI in this study) allows for simultaneous peptide identification and quantitation within a single

LC-MS/MS run. For example, in the analysis run using 318 H-SRM transitions, fourteen scans were acquired across the eluted peak of the peptide LLDSLPSDTR, which is more than sufficient to provide reproducible quantitative results (Figure 9). In addition, monitoring multiple product ion transitions enables rapid and reliable method refinement as opposed to relying on the necessity of having a clean and abundant full scan product ion spectrum in the conventional approach. From the collection of SRM transitions, determination of the most selective and sensitive transitions are straightforward, ion ratios can be calculated for validation, and the retention times are provided.

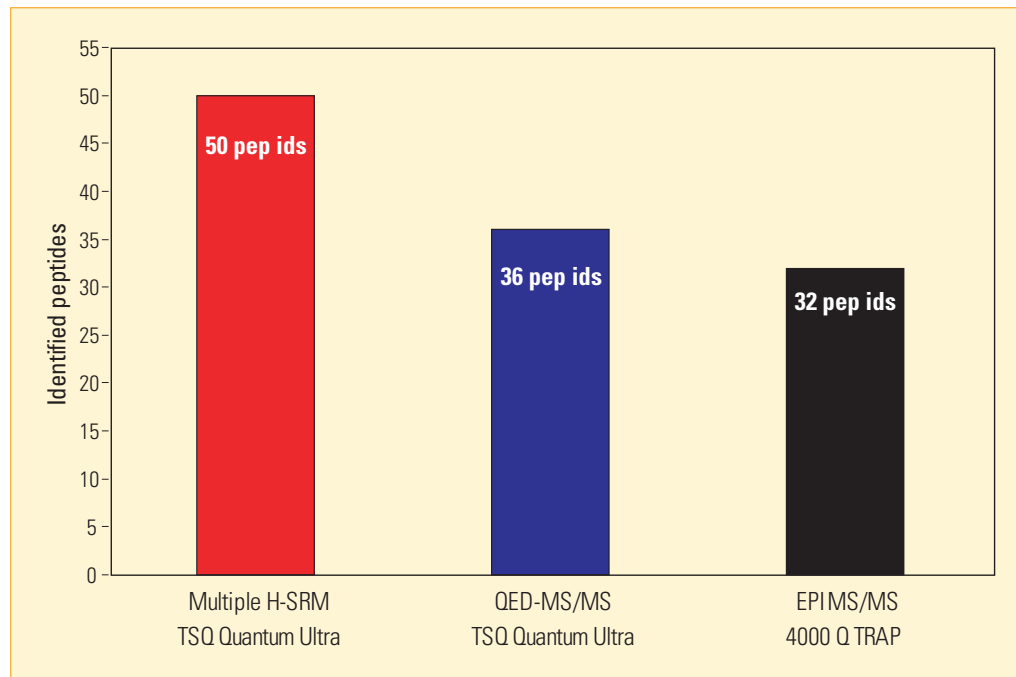


Figure 8: A comparison of the number of peptides identified using three different methods.

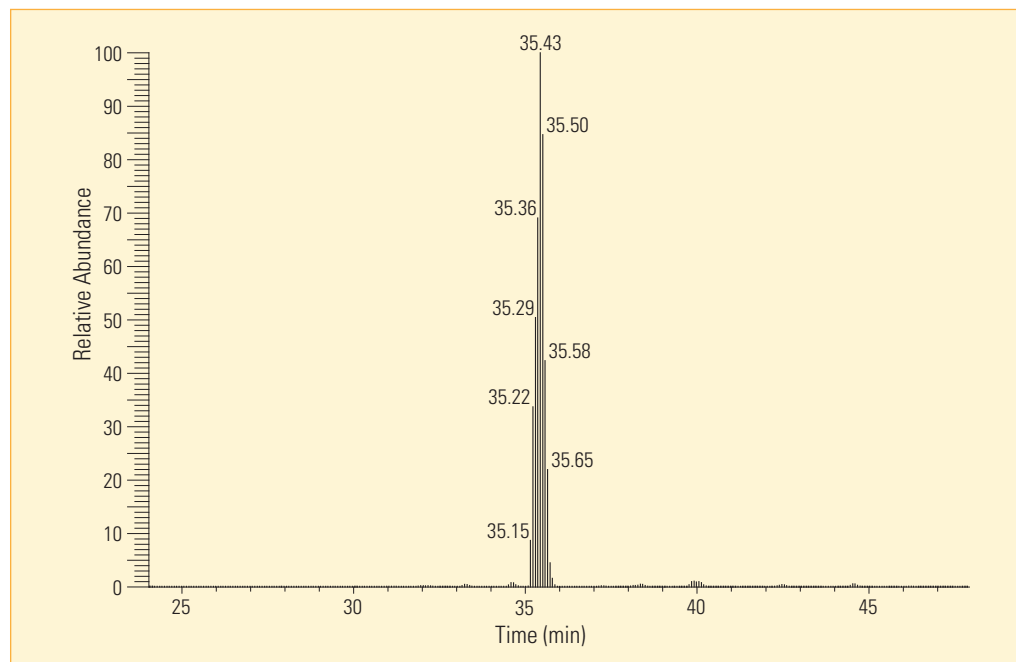


Figure 9: Selected SRM traces for the peptide LLDSLPSDTR (m/z 558.8) representing complement component 1 inhibitor. Fourteen scans were acquired in this 318 H-SRM assay using 5 ms dwell time.

H-SRM Assay Precision at Different Dwell Times

A total of 61 SRM transitions, designed for 20 unique peptides representing 13 proteins, were used to test the precision of the H-SRM assay (Table 4). The H-SRM assays were performed in triplicate at each dwell time (5 ms, 10 ms, 20 ms) using the TSQ Quantum Ultra. The same SRM assays with unit resolution (0.7 FWHM) were also conducted on both the TSQ Quantum Ultra and the 4000 Q TRAP in triplicate at each dwell time. No significant signal loss was observed using shorter dwell times on the TSQ Quantum Ultra for both H-SRM and

SRM assays. For the H-SRM assay, the average signal loss was 12% when dwell time was reduced from 20 to 5 ms. The peptide VGEYSLYIGR showed the largest variation, losing 28% of signal intensity at 5 ms dwell time compared with 20 ms dwell time (Figure 10). However, a significant signal loss was observed on the 4000 Q TRAP using shorter dwell times. On average, 45% of signal intensity was lost at 5 ms dwell time compared with 20 ms dwell time (Figure 11). In the worst case, an 80% signal loss was observed for the peptide DFVQPPTK using the 4000 Q TRAP.

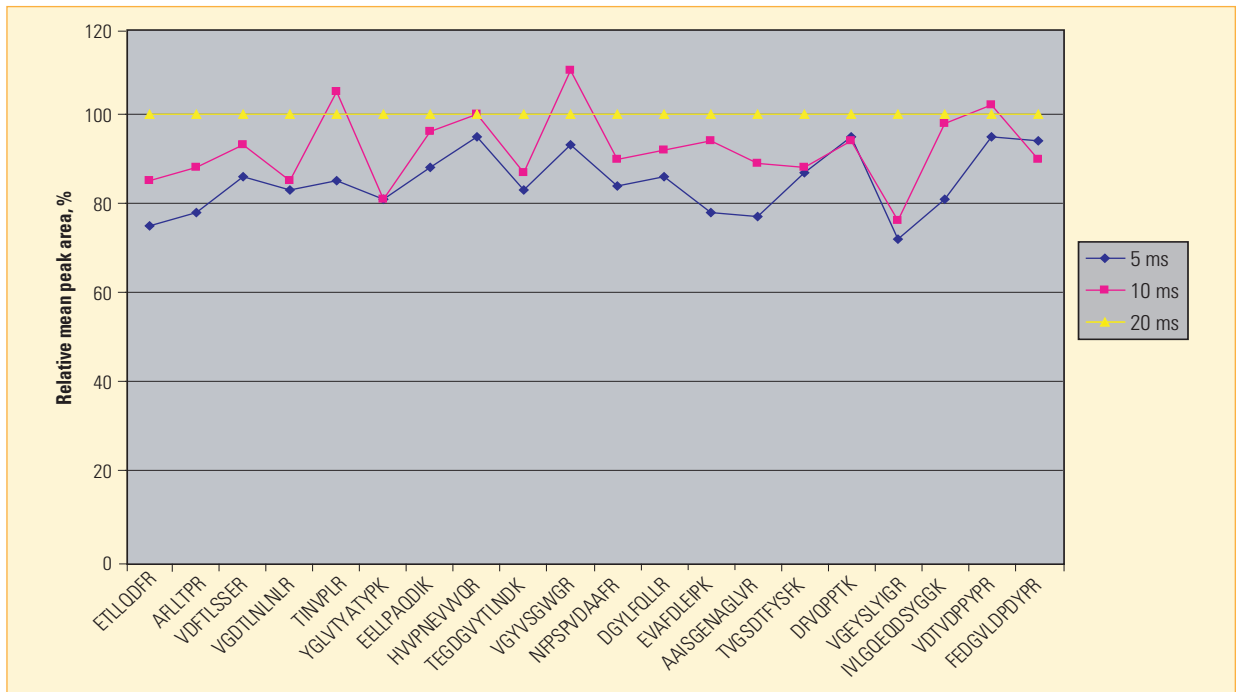


Figure 10: Relative change of averaged raw peak areas (n=3) for 20 serum peptides across different dwell times using H-SRM (Q1, 0.2 FWHM) on the TSQ Quantum Ultra.

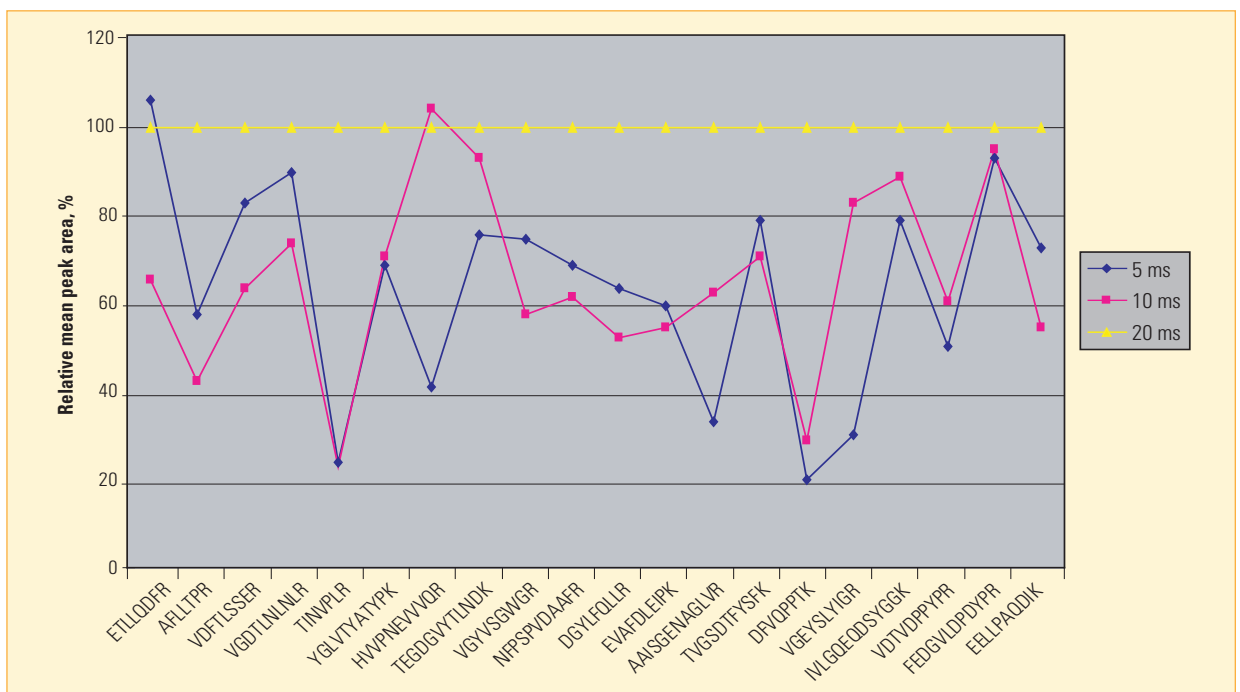


Figure 11: Relative change of averaged raw peak areas (n=3) for 20 serum peptides across different dwell times using MRM (Q1, 0.7 FWHM) on the 4000 Q TRAP.

The mean raw peak area and % CV variation of the H-SRM and SRM assays at different dwell times acquired on the TSQ Quantum Ultra and that of the MRM assay on the 4000 Q TRAP are summarized in Tables 4 and 5, respectively. Figure 12a-c shows comparisons of % CV results based on these summaries. The H-SRM assay on

the TSQ Quantum Ultra achieved higher assay precision for all dwell times by dramatically reducing non-specific interferences from the serum background. The reproducibility of the H-SRM assay was excellent and approximately 95% of peptides had CV values of <15% for all three dwell times.

Peptide	H-SRM						SRM					
	Mean Area		% CV		Mean Area		% CV		Mean Area		% CV	
	5 ms	10 ms	5 ms	10 ms	5 ms	10 ms	5 ms	10 ms	5 ms	10 ms	5 ms	10 ms
ETLLQDFR	5306979	5987942	14.4	2.9	7087222	14.2	17870264	4.8	16144537	6.5	21012034	9.4
AFLTPR	2343721	2667912	12.2	19.4	3021895	2.9	8517811	23.0	8855961	23.8	14242461	17.7
VDFTLSSER	4086351	4453567	6.4	4.2	4770800	12.8	12937371	15.2	14081989	10.4	19662053	17.0
VGDTLNLNLR	4538032	4656495	1.3	7.3	5500169	10.6	14469971	7.0	12926076	10.6	15606329	15.4
TINVPLR	6376783	7902119	7.0	11.7	7490320	9.4	20953244	26.4	26101149	19.6	34728733	26.3
YGLVYATYPK	5167312	5154807	10.6	9.1	6359996	17.3	16683221	9.3	15339340	8.7	19709122	10.2
EELLPAQDIK	14983067	16315794	4.6	11.1	17005929	4.7	42928800	17.6	43722426	19.1	61123790	12.7
HVVPNEVVQR	3546385	3770675	14.0	15.2	3734683	3.3	15122237	11.1	16106096	2.9	2297115	4.8
TEGDGVYTLNDK	15685232	18022858	3.0	7.5	14891978	0.7	37338334	17.2	44867791	27.5	53152981	3.5
VGYVSGWGR	10903887	129683137	15.5	11.4	117215733	6.2	283555682	36.8	306682051	26.0	209563509	26.7
NFPSPVDAAFR	60599383	64702391	2.6	6.3	71769403	9.4	133984579	12.2	180677905	17.6	187419331	11.4
DGYLFQLLR	3727491	4003966	7.1	3.0	4353635	10.0	10095454	10.4	10115674	13.2	9540360	4.9
EVAFDLEIPK	7159915	8615872	8.0	5.9	9203943	1.4	14376170	6.4	20521361	9.1	20219695	6.1
AAISGENAGLVR	3453932	4457886	10.9	13.3	4001541	8.8	13310551	32.8	12433933	11.4	16585939	8.9
TVGSDTFYSFK	5916403	5984698	9.2	1.9	6819773	16.4	21682693	8.5	22430868	8.3	32129349	12.5
DFVQPPTK	11800371	11611580	2.3	12.7	12340433	6.5	40375911	27.4	47175236	17.7	56171827	11.2
VGEYSLYIGR	214563	228545	13.3	5.7	300006	9.5	799390	8.9	843707	14.8	849741	14.8
IVLGQEQDSYGGK	1398887	1987759	7.3	13.6	1732344	9.6	5262561	33.2	7277734	16.1	9109180	7.5
VDTVDPYPR	27542274	31921012	17.5	7.6	29024927	7.8	61910771	34.2	93163311	23.8	117774430	13.5
FEDGVLDPDYPR	26678354	25506256	8.7	8.9	28376037	11.8	73859095	6.8	63441464	13.2	78488053	16.9

Table 4: Mean peak area values and CVs for three replicate analyses across three dwell times on the TSQ Quantum Ultra with H-SRM assay and SRM assay.

Peptide	MRM					
	Mean Area		% CV		Mean Area	
	5 ms	10 ms	5 ms	10 ms	5 ms	10 ms
ETLLQDFR	66833	41643	5.7	30.8	62820	14.2
AFLTPR	7500	5488	8.4	6.9	12860	13.3
VDFTLSSER	48433	37490	5.7	7.0	58267	20.7
VGDTLNLNLR	66233	54697	3.8	20.0	73433	7.2
TINVPLR	6735	6434	41.7	30.1	26880	8.8
YGLVYATYPK	42135	43367	11.0	7.7	60857	13.8
EELLPAQDIK	238860	174350	12.4	4.5	318610	5.1
HVVPNEVVQR	1542	3854	42.2	13.8	3715	11.1
TEGDGVYTLNDK	174050	211440	11.0	8.1	228033	12.2
VGYVSGWGR	871667	673367	10.4	8.0	117033	22.5
NFPSPVDAAFR	352800	313600	25.8	10.8	508300	25.0
DGYLFQLLR	24300	20290	11.1	13.5	38123	9.4
EVAFDLEIPK	59600	54497	16.3	15.5	99900	14.5
AAISGENAGLVR	15367	28600	28.4	18.5	45387	15.1
TVGSDTFYSFK	21320	26910	6.1	3.3	37660	6.1
DFVQPPTK	6343	9099	34.9	32.3	29920	2.2
VGEYSLYIGR	809	2167	26.1	56.7	2603	6.1
IVLGQEQDSYGGK	10881	12219	5.2	21.3	13753	15.7
VDTVDPYPR	8830	10445	56.3	7.5	17200	9.9
FEDGVLDPDYPR	219984	224810	10.7	12.5	235647	6.3

Table 5: Mean peak area values and CVs for three replicate analyses across three dwell times on the 4000 Q TRAP.

The SRM assays both on the TSQ Quantum Ultra and the 4000 Q TRAP also gave acceptable quantitative precision at 20 ms dwell time yielding 80% of peptides with CV values <20%. Although the variation of % CVs for shorter dwell times (5 ms and 10 ms) of the SRM/MRM assay on both platforms were higher compared to the H-SRM assay, the data on the TSQ Quantum Ultra showed less variation compared with that from the 4000 Q TRAP.

All the peptides quantitated had CVs of <30% using the TSQ Quantum Ultra at 10 ms. Conversely, only 85% of the peptides had CVs <30% on the 4000 Q TRAP at 10 ms. At 5 ms dwell time, 80% of peptides had CVs <30% on both platforms, but the 4000 Q TRAP showed much wider variation for the remaining 20% of peptides (Figure 12a-c).

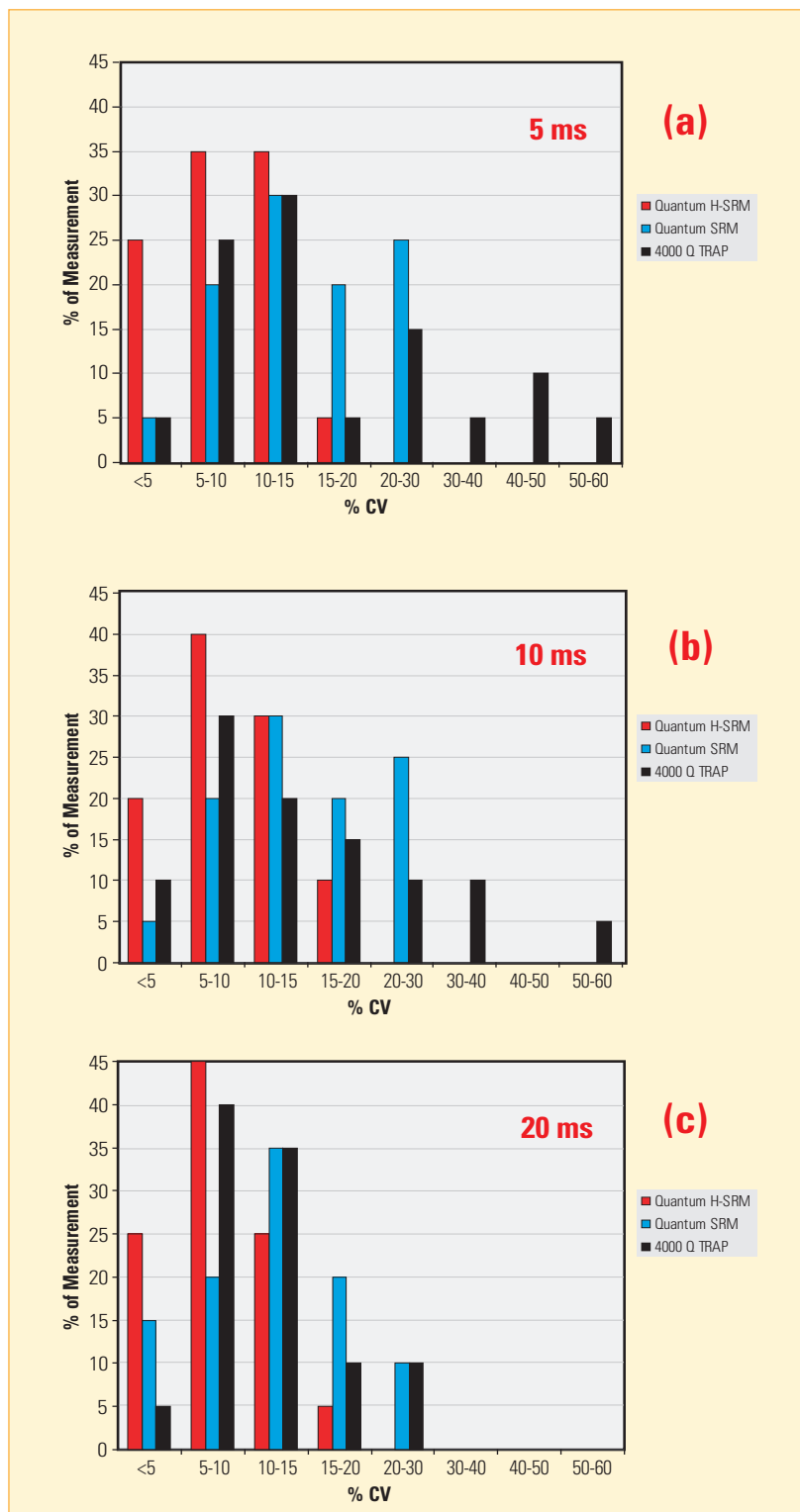


Figure 12: Assay precision (% CV) comparisons between TSQ Quantum Ultra and 4000 Q TRAP across different dwell times.

Quantitative Accuracy

The excellent analytical precision of the H-SRM assay coincided with high quantitative accuracy. Table 6 shows the relative quantitative results for 13 targeted proteins (A, 1 μ L injection and B, 2 μ L injection) with the H-SRM assay. On average, an error of $\pm 4\%$ (n=3) was observed.

	Protein	Sample A (1 μ L) Mean Area (n=3)	Sample B (2 μ L) Mean Area (n=3)	B/A Ratio Observed	B/A Ratio Expected	% Error of Ratio*
1	AMBP			1.94	2.00	-3.0
	ETLLQDFR	7640072	14663553	1.94		
2	Apolipoprotein M			2.09	2.00	4.5
	AFLTPR	3549729	7445575	2.09		
3	Complement C4-A			2.14	2.00	7.0
	VDFTLSSER	7356641	15764496	2.14		
4	Complement C4-B			2.12	2.00	6.0
	VGDTLNLNLR	6522740	13834291	2.12		
5	Complement component1 q			1.82	2.00	-9.0
	TINVPLR	11992826	21609996	1.82		
6	Complement factor B			2.07	2.00	3.5
	YGLVTYATYPK	5585992	11391670	2.04		
	EELLPAQDIK	21306581	44757519	2.10		
7	Gelsolin isoform b			1.93	2.00	-3.5
	HVVPNEVVQR	2310372	4464778	1.93		
8	Haptoglobin			1.99	2.00	-0.5
	TEGDGVYTLNDK	22164154	45309950	2.04		
	VGYSVSGWGR	185320005	371465118	2.00		
	NFSPVDAAFR	66257716	128839733	1.94		
9	Histidine-rich glycoprotein			1.92	2.00	-4.0
	DGYLFQLLR	2768997	5323262	1.92		
10	Inter-alpha-trypsin inhibitor heavy			2.12	2.00	6.0
	EVAFDLEIPK	6577817	14680692	2.23		
	AAISGENAGLVR	4909825	9834816	2.00		
11	Kininogen 1			1.95	2.00	-2.5
	TVGSDFYSFK	6089979	12656487	2.07		
	DFVQPPTK	18825551	34420368	1.83		
12	Serum amyloid P-component			2.00	2.00	0.0
	VGEYSLYIGR	453911	934968	2.06		
	IVLGQEQDSYGGK	2239569	4376917	1.95		
13	Vitronectin			1.96	2.00	-2.0
	VDTVDPYPR	36737187	71271250	1.94		
	FEDGVLDPDYPR	27485185	54140444	1.97		

* % of Error of Ratio = (Observed B/A ratio-Expected B/A ratio)/Expected B/A ratio x 100

Table 6: Relative quantitative results of targeted serum proteins with H-SRM assay for sample A (1 μ L of serum) and sample B (2 μ L of serum).

Conclusions

A new approach using the H-SRM assay on a TSQ Quantum Ultra triple quadrupole mass spectrometer for simultaneous qualitative and quantitative targeted protein analysis was evaluated. This approach gave excellent sensitivity, analytical assay precision and quantitative accuracy for targeted protein quantitation in whole human serum by dramatically reducing non-specific interference from serum background ions. The H-SRM assay greatly improves assay specificity and detection limits thereby offering significant advantages for biomarker verification and validation studies.

1. The proposed H-SRM assay for peptide detection and confirmation with time alignment of multiple fragment ions from each peptide had lower detection limits than the traditional approach for peptide detection and identification using SRM-triggered MS/MS data acquisition and provided wider quantitative dynamic range. All 50 targeted peptides were identified using the H-SRM methodology. Traditional approaches identified only 36 peptides (QED-MS/MS on TSQ Quantum Ultra) and 32 peptides (EPI MS/MS on 4000 Q TRAP). The new approach also allowed for efficient simultaneous targeted peptide peak identification and quantitation in a single LC run.

2. The H-SRM assay provided significant improvement of S/N ratios resulting in unambiguous detection of targeted peptides. Excellent analytical precision was obtained for all selected dwell times (5 ms, 10 ms, and 20 ms) on the TSQ Quantum Ultra. Approximately 95% of the peptides gave CVs <15% for all three dwell times. No significant signal loss was observed when using shorter dwell times.

3. The H-SRM assay provided the widest dynamic range for targeted peptide quantitation. The dynamic range for detecting proteins in this study was over four orders of magnitude.

4. The quantitative accuracy of the H-SRM assay was excellent. The average relative quantitation error was $\pm 4\%$.

5. Aided by higher resolution precursor ion isolation and faster cycle time, the TSQ Quantum Ultra provided high quality SRM-triggered QED-MS/MS data and identified more targeted peptides by a Mascot search compared with the 4000 Q TRAP.

6. Monitoring multiple γ -type product ions (four or more) provided the most selective and sensitive means of further method refinement to determine proteotypic peptides and corresponding transitions including fragment ion ratios, which could subsequently be used for validated methods.

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