Application Note: 451

Absolute Quantitation of Targeted Endogenous Salivary Peptides using Heavy Isotope-labeled Internal Standards and High-Resolution Selective Reaction Monitoring Mass Spectrometry

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Key Words

- TSQ Vantage
- Absolute Quantitation
- AQUA
- H-SRM
- Salivary Peptide

Introduction

Salivary gland secretions have significant potential as a noninvasive source of disease biomarkers and recent compilations of the salivary protein repertoire became valuable resources for parallel studies focusing on oral and systemic diseases.[1] One novel finding shows the presence of a complex suite of peptides and protein fragments in the low molecular weight fraction (< 10kDa) of which a subset have biological activity.[2] However, the expression levels and absolute quantitative amounts of these biologically active peptides among different salivary gland secretions have not been yet investigated. Recently, we have demonstrated the utility of high-resolution selected reaction monitoring (H-SRM) method on a triple quadrupole instrument for highly selective and sensitive quantitation of targeted peptides in complex matrices.[3] In this study, a targeted peptide quantitation assay is created for a triple quadrupole mass spectrometer to determine the natural abundance of six peptides in sixteen samples from the salivary gland secretions of eight donors. In order to get absolute quantitative information from the targeted peptides, isotopically labeled peptides were used as internal standards. To increase the detection capabilities of the assay, an H-SRM approach was employed using the Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer in which the mass resolution on the first quadrupole (Q1) was set to 0.4 FWHM.

Goal

To develop a highly sensitive, selective, accurate and precise method for absolute quantitation of endogenous salivary peptides on a triple quadrupole mass spectrometer by using isotopically labeled peptides as internal standards.

Experimental Conditions

Peptide Selection and SRM Method Design

Six endogenous salivary peptides (DSHAKRHHGY, DSHAKRHHGYK, DSHAKRHHGYKR, HEKHHSHRGYR, GRPQGPPQQGGHQQ & AAPDEKVLDSGFR) that showed potential as oral disease biomarkers were selected as the quantitative targets.[2] For each targeted peptide, an isotopically labeled counterpart was synthesized [(DSHA*KRHHGY, DSHA*KRHHGYK, DSHA*KRHHGYKR, HEKHHSHR*GYR, GRPQGP*PQQGGHQQ, AAP*DEKVLDSGFR (Thermo Fisher Scientific, Ulm, Germany)] according to the AQUA peptide quantification strategy.[4] For optimization of the H-SRM assay, each heavy peptide was infused by syringe pump into the mass spectrometer. Most abundant precursor ions were selected and two to four most abundant fragment ions were chosen for each precursor ion, yielding a total of 46 H-SRM transitions for targeting the six peptide pairs (Table 1).

Sample Preparation

Parotid saliva/secretion (PS) and sublingual/submandibular (SMSL) gland secretions were collected from eight human donors, yielding sixteen saliva samples (eight for PS series and eight for SMSL series). The low-molecular-weight fraction was prepared by ultrafiltration (nominal molecular weight limit 10,000) and used without further clean-up. The six isotopically labeled peptides were mixed together to make a 50 fmol/µL AQUA peptide stock mixture. 2.5 µL of the stock AQUA mixture and 2.5 µL of TFA were spiked into a 20 µL aliquot of prepared low-molecular-weight fraction from each salivary gland secretion of eight donors respectively. The final concentration of spiked AQUA peptides was 5 fmol/µL in each saliva sample and contained 10% TFA. Each saliva sample was diluted from 20 µL to 25 µL.

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Protein	Peptide	Precursor *m/z* (Q1)	Charge	Product *m/z* (Q3)	Ion Type	Charge
Peptide 1	DSHAKRHHGY	403.20	3	335.80	y_8	3
		403.20	3	364.90	y_9	3
		403.20	3	399.20	y_6	2
		403.20	3	434.70	y_7	2
Heavy Peptide 1	DSHA*KRHHGY	404.53	3	337.27	y_8	3
		404.53	3	366.17	y_9	3
		404.53	3	399.20	y_6	2
		404.53	3	436.67	y_7	2
Peptide 2	DSHAKRHHGYK	334.70	4	252.63	M-18	4
		334.70	4	330.36	y_9	3
		334.70	4	378.54	y_4	2
Heavy Peptide 2	DSHA*KRHHGYK	335.70	4	252.60	M-18	4
		335.70	4	331.36	y_9	3
		335.70	4	379.90	y_4	2
Peptide 3	DSHAKRHHGYKR	373.75	4	344.94	y_{11}	4
		373.75	4	369.19	M-18	4
		373.75	4	430.57	y_{10}	3
Heavy Peptide 3	DSHA*KRHHGYKR	374.75	4	345.90	y_{11}	4
		374.75	4	370.18	M-18	4
		374.75	4	431.90	y_{10}	3
Peptide 4	HEKHHSHRGYR	361.68	4	304.82	y_7	3
		361.68	4	357.18	M-18	4
		361.68	4	388.20	y_6	2
		361.68	4	456.73	y_7	2
Heavy Peptide 4	HEKHHSHR*GYR	364.30	4	308.17	y_7	3
		364.30	4	359.68	M-18	4
		364.30	4	393.20	y_6	2
		364.30	4	461.75	y_7	2
Peptide 5	GRPQGPPQQGGHQQ	491.65	3	409.72	b_8	2
		491.65	3	496.26	b_5	1
		491.65	3	526.23	y_5	1
		491.65	3	879.41	y_8	1
Heavy Peptide 5	GRPQGP*PQQGGHQQ	493.65	3	412.70	b_8	2
		493.65	3	496.26	b_5	1
		493.65	3	526.23	y_5	1
		493.65	3	879.41	y_8	1
Peptide 6	AAPDEKVLDSGFR	468.95	3	421.60	y_{11}	3
		468.95	3	631.80	y_{11}	2
		702.86	2	466.24	y_4	1
		702.86	2	631.82	y_{11}	2
		702.86	2	793.42	y_7	1
Heavy Peptide 6	AAP*DEKVLDSGFR	470.95	3	423.60	y_{11}	3
		470.95	3	634.80	y_{11}	2
		705.86	2	466.24	y_4	1
		705.86	2	634.80	y_{11}	2
		705.86	2	793.42	y_7	1

Table 1: SRM transition list used for targeting the six native peptide/isotopically labeled peptide pairs

LC/MS

Nano-HPLC

Pump:	Thermo Scientific Surveyor MS pump equipped with MicroAS autosampler
Column:	PicoFrit™ C18 column from New Objective (75 µm x 100 mm)
Post-split Flow Rate:	300 nl/min
Buffer A:	0.1% Formic acid/H_2O
Buffer B:	0.1% Formic acid/100% ACN
Gradient:	0% B to 95% B in 30 min
Injection Volume:	2 µL

MS

Mass Spectrometer:	TSQ Vantage with a standard Ion Max source equipped with a column adapter for nanoflow (New Objective)
H-SRM Set Up:	Q1, 0.4 FWHM; Q3, 0.7 FWHM; Q2, 1.5 mTorr (Ar), Dwell time, 20 ms
CE:	Optimized by using each AQUA peptide

Note: Each sample was run in triplicate

Results

Optimal Q1 Resolution

The main advantage of high resolution isolation is its ability to selectively detect low abundance peptide peaks within a complex background. Nearly all commercial instruments are used with unit resolution (0.7 FWHM) for precursor ion selection and would otherwise suffer significant signal loses when using higher resolution (0.4 FWHM or 0.2 FWHM) isolation. The Thermo Scientific TSQ Series are well known for higher resolution for precursor ion selection while maintaining high transmission efficiency.[5] To determine if the H-SRM improves quantitative precision for targeted peptides, pooled saliva sample was spiked with all six internal standards, analyzed at three different Q1 resolutions (0.7 FWHM, 0.4 FWHM & 0.2 FWHM). Figure 1 shows an example of SRM trace for heavy peptide GRPQGP*PQQGGHQQ (10 fmol on column) at three different Q1 resolutions. The interference peak observed while using unit resolution (0.7 FWHM) was eliminated when using higher resolution (0.4 FWHM and 0.2 FWHM). The signal-to-noise ratio was greatly improved while using higher selectivity for Q1 precursor ion. After evaluating all the target peaks, 0.4 FWHM resolution at Q1 was found to be selective enough to reduce chemical interferences from the saliva background.

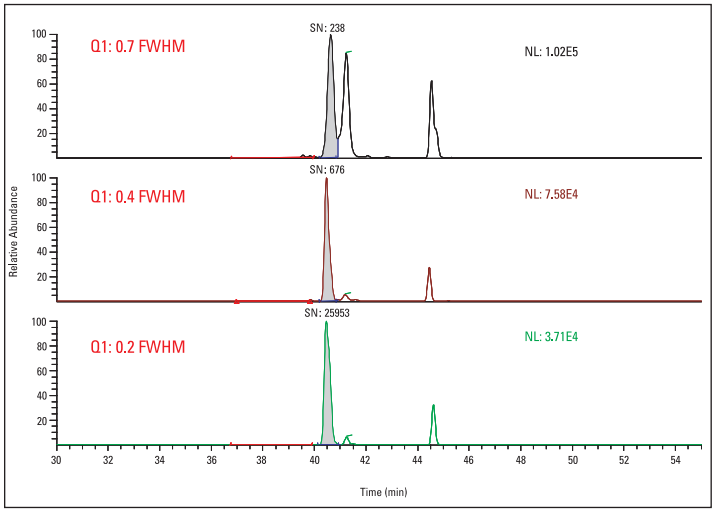

Figure 1: Comparison of the chromatographic traces from the analysis of the heavy peptide GRPQGP*PQQGGHQQ at three different Q1 resolutions

Detection Limit and Linear Dynamic Range for Targeted Endogenous Peptides

With the AQUA strategy, the absolute quantitation of the native peptide is determined by comparing the abundance of the known AQUA internal standard peptide to the abundance of the native peptide.[4] To determine the detection limit and quantitative linearity of targeted native saliva peptides, the six heavy AQUA peptides were mixed and spiked into the pooled saliva sample over a concentration range of four orders of magnitude (0.05 fmol, 0.5 fmol, 5 fmol, 50 fmol and 100 fmol per µL of pooled saliva sample). By using the new RF-only ion ring technology (S-lens) for maximum ion transmission, the TSQ Vantage can deliver highly sensitive and reproducible quantitative results for very low-level analytes.[6] In our experiment, both excellent sensitivity and wide linear dynamic range were achieved. Figure 2a shows the summed SRM chromatographic traces of HEKHHSHR*GYR/ HEKHHSHRGYR peptide pair with 50 attomoles of HEKHHSHR*GYR loaded on column. Figure 2b shows the heavy peptide peak clearly detected by the retention time (RT) alignment of monitored multiple fragment ions from the heavy labeled peptide. Figure 3 shows the calibration curve of AQUA-labeled peptide HEKHHSHR*GYR across the concentration range of 0.05 fmol to 100 fmol on column by using the native peptide in the pooled saliva sample as the internal standard. Excellent linearity was obtained over four orders of magnitude (R^2=0.9962).

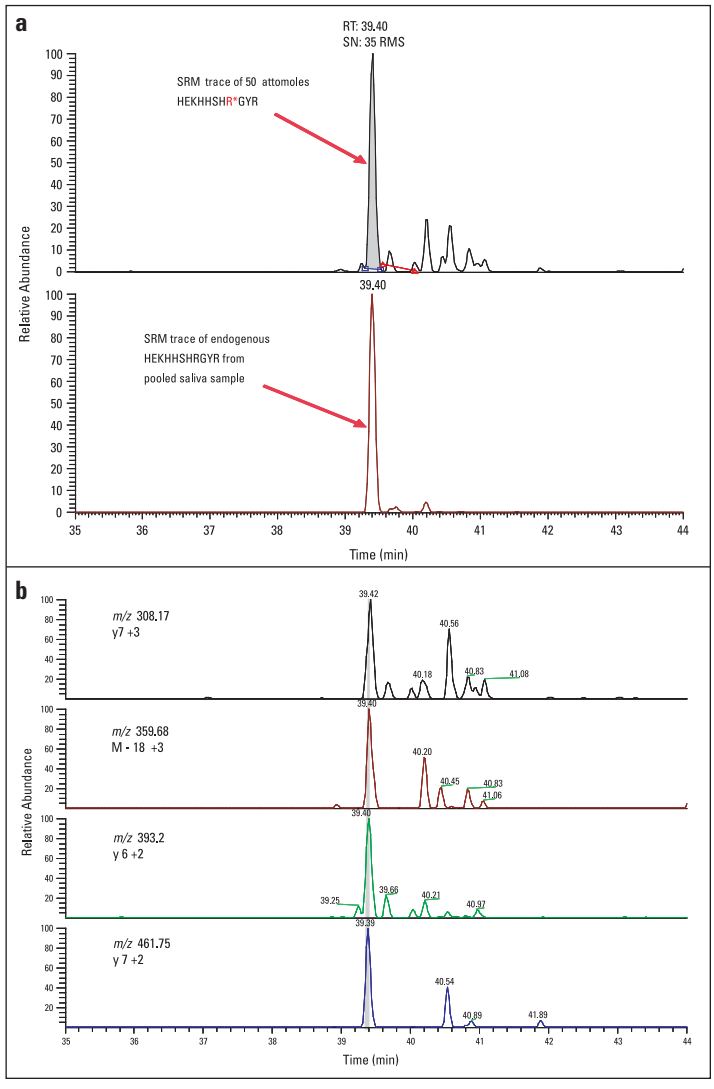

Figure 2: a) SRM chromatograms of the HEKHHSHR*GYR/ HEKHHSHRGYR peptide pair (50 attomoles of HEKHHSHR*GYR on column). b) Individually monitored fragment ion chromatograms from the heavy peptide.

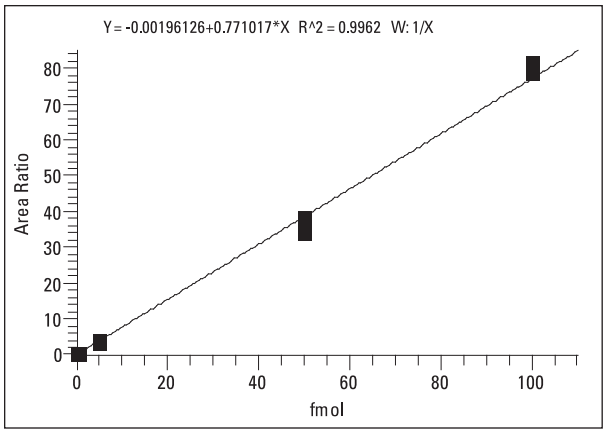

Figure 3: Linear calibration curve for the heavy peptide HEKHHSHR*GYR (50 amol to 100 fmol on column), n=3

Targeted Native Peptide Confirmation and Absolute Quantitation by AQUA

The targeted peptide peak from each sample was confirmed by using both the retention time and multiple fragment ion ratios from the co-eluting isotopically labeled internal standard (Figure 4). Five targeted peptides were detected in all sixteen saliva samples. The H-SRM assay was performed in triplicate for each saliva sample. The sixth targeted peptide (AAPDEKVLDSGFR) was detected only in Y PS and Y SMSL saliva samples. Extracted ion chromatograms were generated and the analyte peaks integrated. The quantities of endogenous peptides were calculated using the mean-peak-area ratios (n=3) of endogenous peptide/isotopically labeled peptide pairs multiplied by the absolute amount of internal standard (5 fmol/µL) and 1.25X dilution factor. Figure 5 shows an example of how to calculate the absolute amount of peptide AAPDEKVLDSGFR using this strategy. Although this native peptide existed at a very low concentration level (calculated 60 attomoles on column), the peak was clearly detected with a signal-to-noise ratio of 20. Table 2 shows an example of the absolute quantitation results of six targeted peptides from the saliva sample of Y SMSL. The analytical precision across the three replicate runs was good. The relative standard deviation (RSD) of five peptides was less than 12% (average 8.5%). The RSD of low level peptide AAPDEKVLDSGFR was also within acceptable range (25%).

The summary for all the calculated results of each native peptide in the eight PS series samples and the eight Y SMSL series samples are shown in Tables 3 and 4, respectively. Abundances of peptides varied significantly between donors and glandular sources. The peptide AAPDEKVLDSGFR was unique to the gland secretions of one donor. Another peptide HEKHHSHRGYR was consistently more abundant in sublingual/submandibular than in parotid saliva. The clinical significance for these abundance variations are under further investigation.

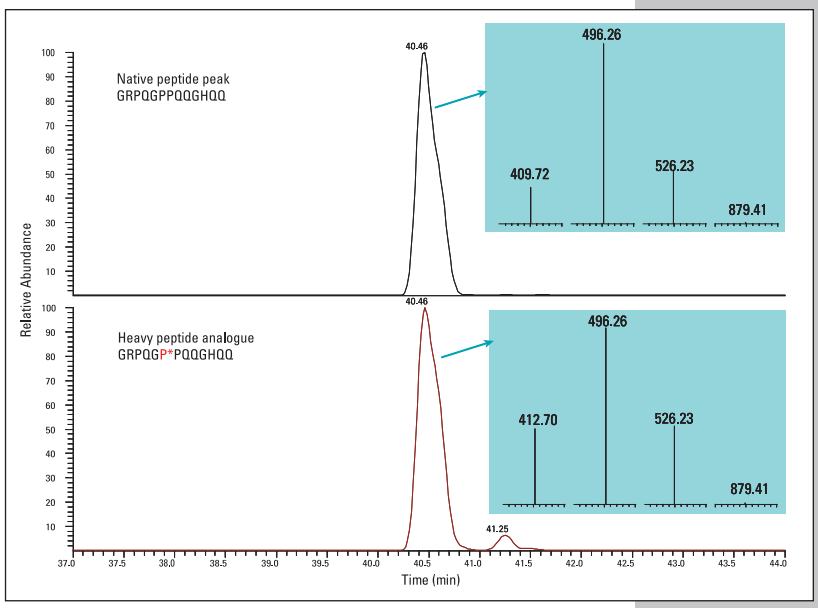

Figure 4: Confirmation of targeted native peptide peaks using both RT and multiple fragment ion ratios of the co-eluting heavy isotopic-labeled peptide analogue

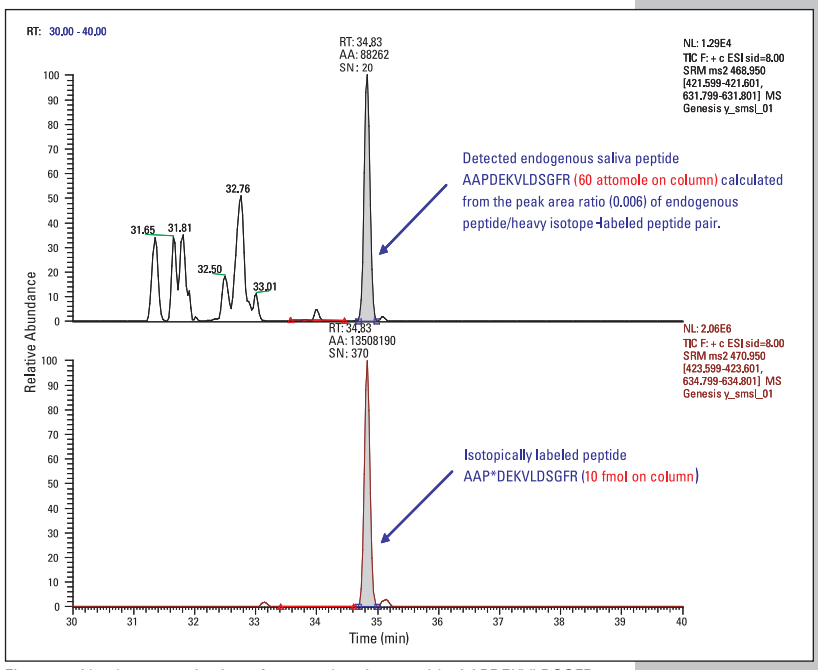

Figure 5: Absolute quantitation of targeted native peptide AAPDEKVLDSGFR by using the isotopically labeled peptide as an internal standard

Peptide	Sequence	Ratio (Native/Labeled) n=1	Ratio (Native/Labeled) n=2	Ratio (Native/Labeled) n=3	Mean Ratio (Native/Labeled) Mean	Calculated Native Amount* fmol/µL	RSD %
Peptide 1	DSHAKRHHGY	10.866	11.591	11.308	11.255	70.3	3.25
Peptide 2	DSHAKRHHGYK	11.094	12.754	13.050	12.299	76.9	8.57
Peptide 3	DSHAKRHHGYKR	4.269	3.408	3.681	3.786	23.7	11.62
Peptide 4	HEKHHSHRGYR	0.063	0.079	0.079	0.074	0.5	12.48
Peptide 5	GRPQGPPQQGGHQQ	3.423	3.284	2.998	3.235	20.2	6.70
Peptide 6	AAPDEKVLDSGFR	0.006	0.008	0.005	0.006	0.04	25.46

** Calculated native peptide amount = mean ratio of (Native/Labeled) x 5 fmol/µL x 1.25*

Table 2: Absolute quantitative results of six targeted peptides from the saliva sample of Y SMSL using the AQUA strategy

Peptide	Sequence	Y PS fmol/µL	E PS fmol/µL	EL PS fmol/µL	KR PS fmol/µL	DO PS fmol/µL	KS PS fmol/µL	LN PS fmol/µL	S PS fmol/µL
Peptide 1	DSHAKRHHGY	13.2	1.4	33.2	20.6	104.1	6.1	50.4	11.9
Peptide 2	DSHAKRHHGYK	44.9	13.1	56.3	47.4	71.5	24.5	66.7	35.7
Peptide 3	DSHAKRHHGYKR	25.9	3.0	66.7	22.8	98.5	6.9	51.2	24.6
Peptide 4	HEKHHSHRGYR	2.5	0.2	5.7	3.7	7.6	0.7	3.1	2.1
Peptide 5	GRPQGPPQQGGHQQ	62.9	36.2	124.5	48.1	30.8	37.7	48.8	46.1
Peptide 6	AAPDEKVLDSGFR	0.0006							

Table 3: Quantitative summary for the parotid saliva samples of eight human donors

Peptide	Sequence	Y SMSL fmol/µL	E SMSL fmol/µL	EL SMSL fmol/µL	KR SMSL fmol/µL	DO SMSL fmol/µL	KS SMSL fmol/µL	LN SMSL fmol/µL	S SMSL fmol/µL
Peptide 1	DSHAKRHHGY	70.3	0.6	9.1	52.6	75.7	33.6	2.2	35.3
Peptide 2	DSHAKRHHGYK	73.1	3.5	42.4	59.2	112.2	58.5	10.3	59.0
Peptide 3	DSHAKRHHGYKR	23.7	0.6	17.7	4.2	58.8	38.1	2.3	41.5
Peptide 4	HEKHHSHRGYR	0.500	0.003	0.200	0.034	0.700	0.400	0.090	1.430
Peptide 5	GRPQGPPQQGGHQQ	20.2	4	74.0	183.8	34.0	353.5	16.8	85.9
Peptide 6	AAPDEKVLDSGFR	0.04							

Table 4: Quantitative summary for the sublingual/submandibular saliva samples of eight human donors

Conclusions

1. A 46-transitions H-SRM assay on a new TSQ Vantage triple quadrupole mass spectrometer was used to successfully determine the absolute amounts of six targeted endogenous peptides from parotid and sublingual/submandibular gland secretions of eight human donors using the AQUA strategy without any prior peptide enrichment or sample clean up.
2. The H-SRM assay was highly sensitive and enabled to detect the native salivary peptides at less than 10 attomole/µL level.
3. The analytical precision of the H-SRM assay was good. The RSDs of five peptides were less than 12% (average 8.5%). The RSD of the low-level peptide AAPDEKVLDSGFR was also within acceptable range (25%).
4. Significant abundance variations for each targeted endogenous peptide were observed between donors and between glandular sources. The biological meaning for these variations is a subject of further investigation.

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