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Application News

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Protein Quantitation / LCMS-9030

Direct Quantitation of Intact Proteins By Multiple Ion Chromatogram Method on Q-TOF Mass Spectrometer

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❑ Introduction

With the growth of global protein therapeutic market, it is particularly important to accurately and precisely monitor the protein levels throughout the process of production, characterization, and clinical uses. Over the last 20 years, mass spectrometry has become a powerful analytical technique for protein study. In quantifying intact proteins, selected ion monitoring (SIM) [1] and multiple reaction monitoring (MRM) [2] have been reported by using the triple-quadrupole (QQQ). Quadrupole time-of-flight (Q-TOF) is more sensitive and specific than QQQ, and it is commonly used in profiling analysis. However, due to a lower dynamic range, there is lack of quantitative studies for intact proteins by using Q-TOF. In this study, we aimed to investigate and evaluate the performance of multiple ion chromatogram (MIC) for quantification of intact proteins using Shimadzu LCMS-9030 Q-TOF mass spectrometer.

❑ Experimental

A. Protein standards

Protein standard MSRT2 set including ribonuclease B (~15.0 kDa), insulin (5.8 kDa), lysozyme (14.3 kDa), and β-lactoglobulin A (18.4 kDa) was purchased from Sigma-Aldrich. Ribonuclease B is a mix of five sialylated glycans (man5, 6, 7, 8, and 9).

B. Sample preparation and analytical conditions

Protein standards were dissolved in Milli-Q water with 0.1% trifluoroacetic acid (TFA) (stock solution, 200 µg/mL) and diluted to different working solutions for calibration curves, ranging from 1 to 200 µg/mL. The samples were directly injected and analyzed by a Shimadzu LCMS-9030 Q-TOF mass spectrometer. The analytical conditions are shown in Table 1.

C. MIC method development

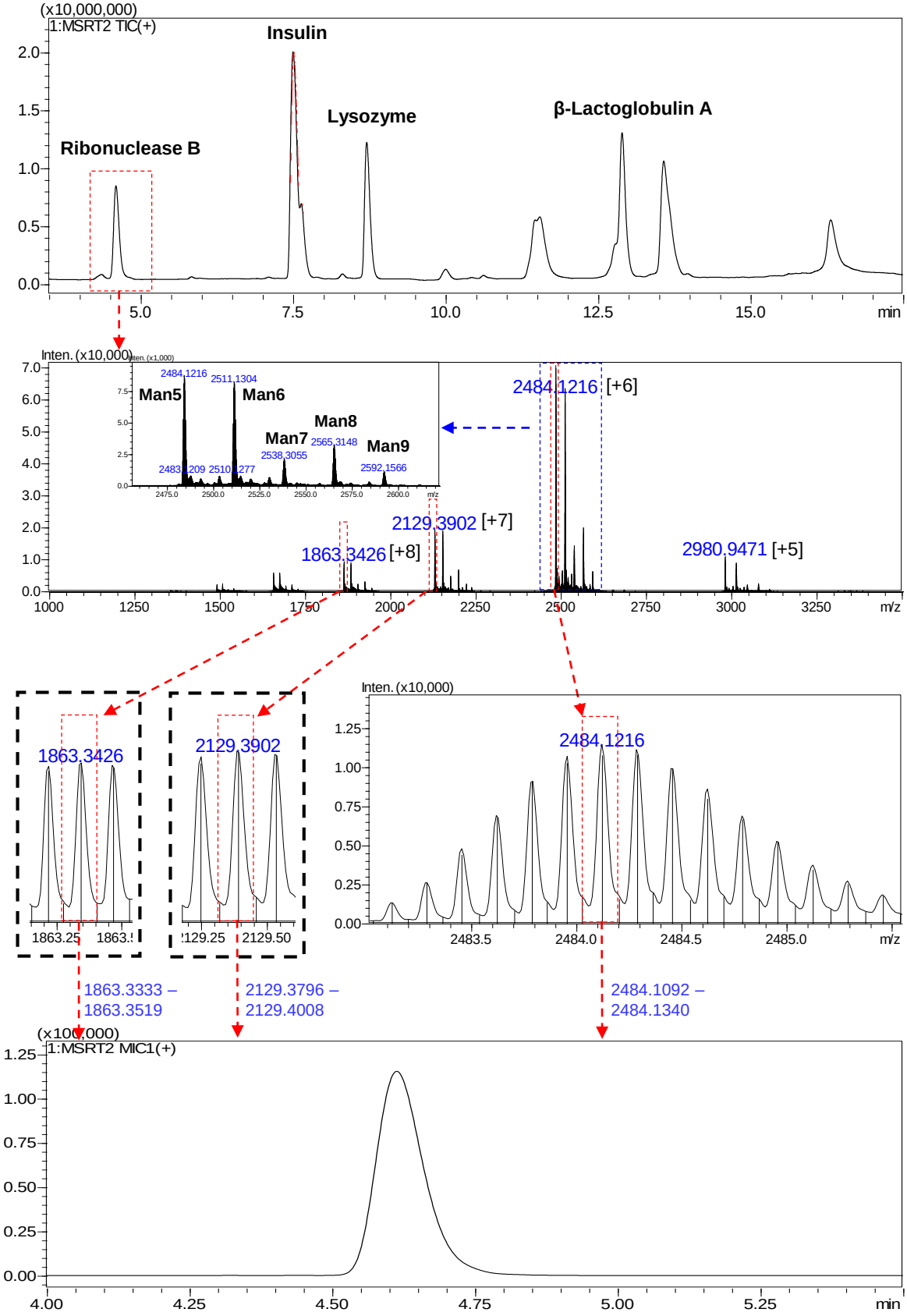
In MIC method development, three highest intensity ion signals were first selected from multiple-charged ion envelope of each protein, and further the most abundant monoisotopic mass from each respective multiple-charged ion was chosen and superposed as MIC, with 5 ppm tolerance of *m/z*. The procedure is shown in Figure 1, taking ribonuclease B (man5) as an example. The MIC settings are summarized in Table 2.

Table 1: Analytical conditions on LCMS-9030

LC conditions		
Column	:	ProteCol-G C8 (100 mm x 2.1 mm; 3 µm)
Column temperature	:	50 °C
Mobile phase A	:	0.1% TFA in water
Mobile phase B	:	0.1% TFA in acetonitrile
Flow rate	:	0.3 mL/min
Gradient program	:	Time (min) %B 0.0 20 20.0 60 20.5 80 23.5 80 24 20
Injection volume	:	5 µL
MS conditions		
Interface	:	Heated EST
Interface voltage	:	4 kV
Interface temperature	:	300 °C
Nebulizing gas	:	Nitrogen, 3.0 L/min
Heating gas flow	:	Zero Air, 10.0 L/min
DL temperature	:	250 °C
Drying gas flow	:	Nitrogen, 15.0 L/min
Heat block temperature	:	400 °C
MS mode	:	Positive
Mass range	:	1000 – 3500 m/z

Table 2: MIC settings for intact proteins with 5 ppm tolerance

Protein	MIC No.	Peaks	Start m/z	End m/z
Ribonuclease B (man5)	MIC1	1	1863.3333	1863.3519
		2	2129.3796	2129.4008
		3	2484.1092	2484.1340
Ribonuclease B (man6)	MIC2	1	1883.5913	1883.6101
		2	2152.6760	2152.6976
		3	2511.1178	2511.1430
Ribonuclease B (man7)	MIC3	1	1903.9708	1903.9898
		2	2175.8233	2175.8451
		3	2538.2928	2538.3182
Ribonuclease B (man8)	MIC4	1	1924.2276	1924.2468
		2	2198.9763	2198.9983
		3	2565.3020	2565.3276
Ribonuclease B (man9)	MIC5	1	1944.4848	1944.5042
		2	2221.9890	2222.0112
		3	2592.1436	2592.1696
Insulin	MIC6	1	1162.5307	1162.5423
		2	1452.9133	1452.9279
		3	1936.8812	1936.9006
Lysozyme	MIC7	1	2044.5438	2044.5642
		2	2385.1347	2385.1585
		3	2861.9615	2861.9901
β-Lactoglobulin A	MIC8	1	1670.4030	1670.4198
		2	1837.3422	1837.3606
		3	2041.3810	2041.4014



Total ion chromatogram (TIC) of intact proteins

Mass spectrum of ribonuclease B with 5 post-translational modifications (PTMs). Three highest intensity multiple-charged ion signals [+6, +7, +8] were selected for MIC development

The most abundant monoisotopic mass with 5 ppm tolerance of ribonuclease B (man5) was superposed as MIC

Generated MIC of ribonuclease B (man5) for quantitative analysis

Fig. 1 The procedure for multiple ion chromatogram (MIC) development, taking ribonuclease B (man5) as an example.

❑ Results and Discussion

Six-point calibration curves for the proteins (except insulin, 4 points) were generated in duplicates (Figure 2). Linearities, linear range, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and matrix effect were determined (Table 3). Respective linearities (R^2 >0.997) were achieved for ribonuclease B (man5, 6, 7, and 8), lysozyme, and β-lactoglobulin A in the range of 5 – 200 μg/mL, ribonuclease B (man9) in the range of 20 – 200 μg/mL, and insulin in the range of 2 – 50 μg/mL. Calibration linear ranges were different for

all proteins because of their different response factors. LOD and LOQ for the proteins ranged from 0.32 to 5.40 µg/mL and 1.13 to 17.99 µg/mL, respectively (Figure 3). The accuracy and precision tests were performed at low (n = 4) and medium (n = 4) concentrations within their respective calibration ranges. The errors in accuracy are under 20%, and the prevision is demonstrated with less than 15% RSD.

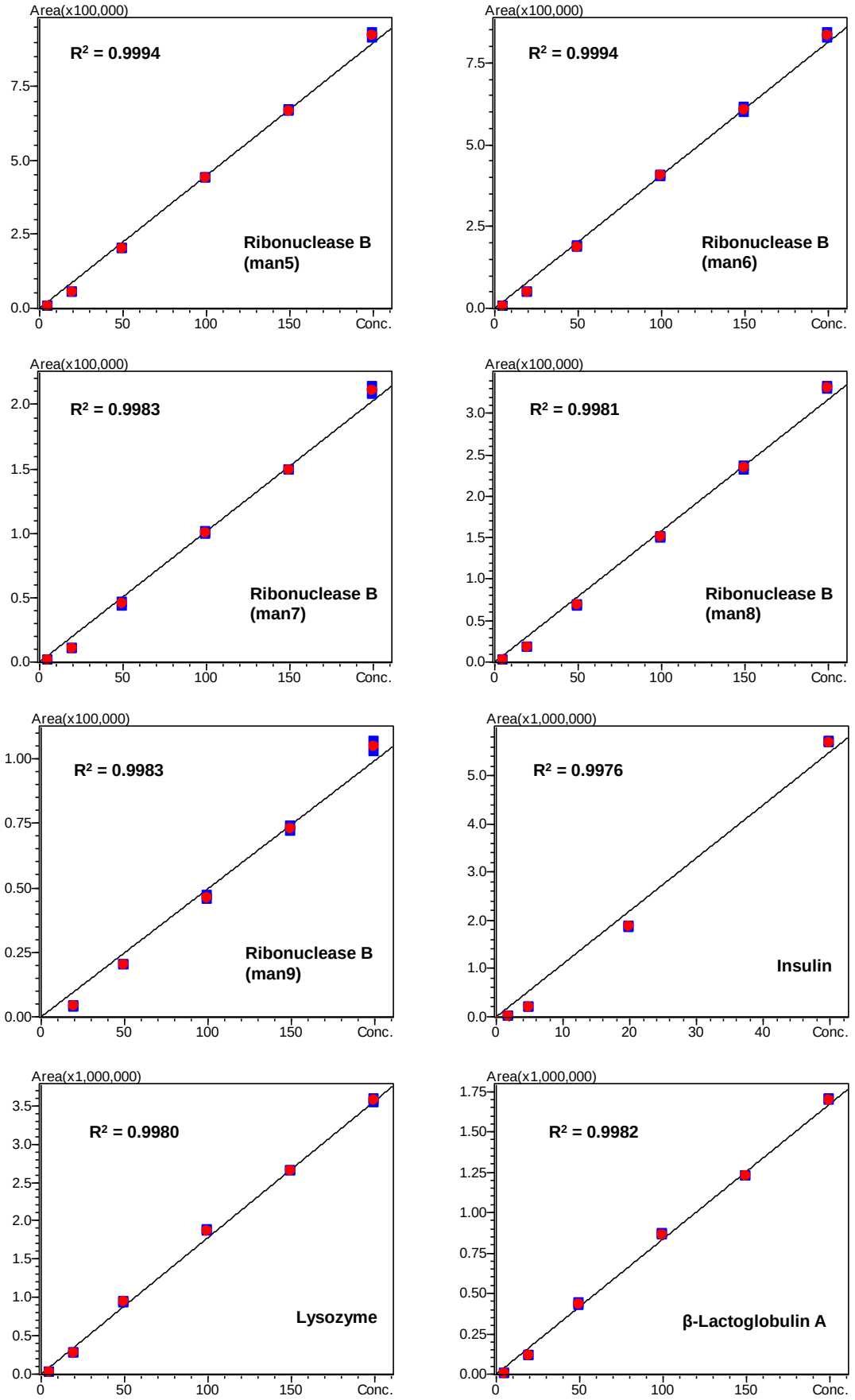
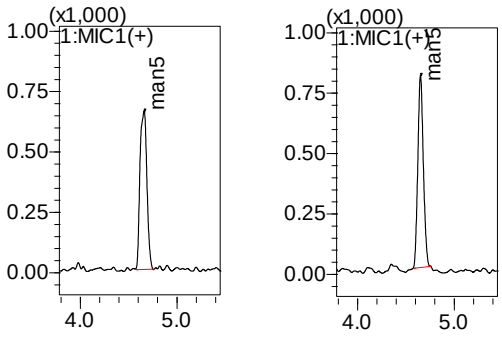
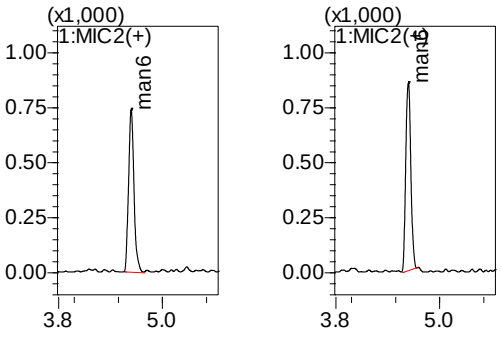


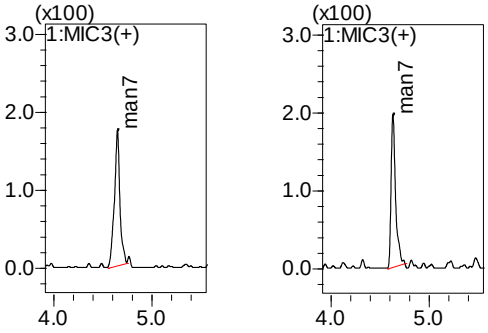
Fig. 2 Calibration curves of intact proteins



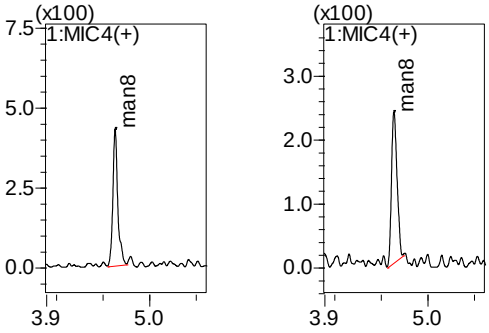
Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
5	3,125	4.670	18.78	2.66	2.38
5	3,114	4.660	23.83	2.10	



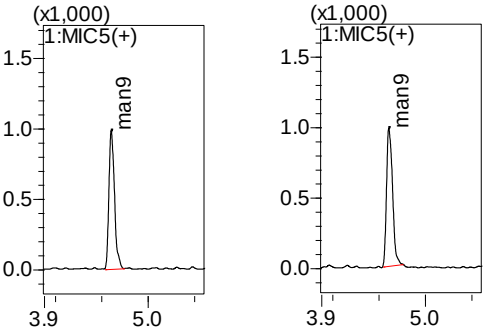
Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
5	3,125	4.655	41.51	1.20	1.11
5	3,413	4.655	49.39	1.01	



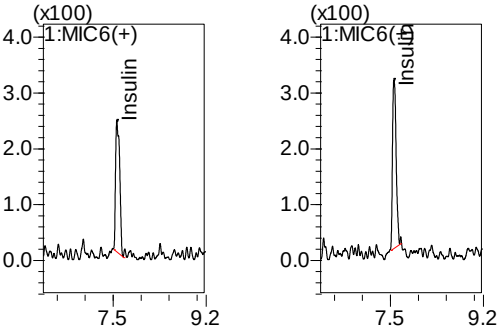
Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
5	674	4.658	15.74	3.18	3.11
5	621	4.638	16.43	3.04	



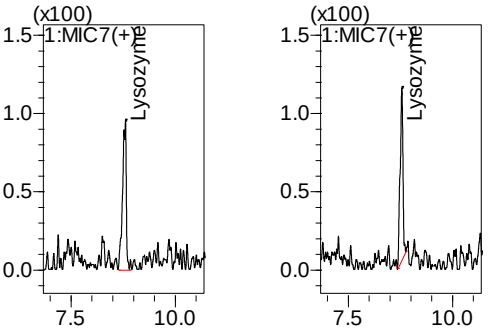
Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
5	1,564	4.638	19.23	2.60	3.55
5	978	4.643	12.12	4.50	



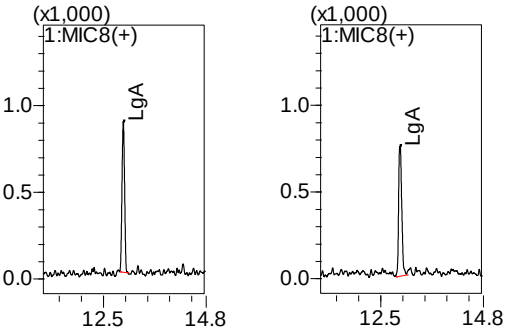
Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
5	4,070	4.609	45.58	4.39	4.38
5	4,343	4.613	45.73	4.37	



Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
2	1,199	7.582	19.23	2.26	1.98
2	1,345	7.578	12.12	1.71	



Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
2	583	8.826	5.11	3.91	3.75
2	503	8.803	5.57	3.59	



Conc. (μg/mL)	Area	RT	S/N	LOQ	Ave
5	3,516	12.969	20.08	2.49	2.60
5	3,664	12.958	18.48	2.71	

Figure 3 Determination of LOQ of intact proteins by MIC method on LCMS-9030

Table 3: Evaluation of method performance for MIC-based intact protein quantification on LCMS-9030

Protein	Linearity (R^2)	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)	Prepared conc. (µg/mL)	Measured conc. (µg/mL)	Accuracy (%)	Prevision (% RSD)	Matrix effect Serum (%)	Matrix effect Urine (%)
Ribonuclease B (man5)	0.9994	5 - 200	0.63	2.10	20	18.73 ± 0.63 (n=4)	93.67	5.41	153	140
					100	102.28 ± 0.71 (n=4)	102.28	0.75		
Ribonuclease B (man6)	0.9994	5 – 200	0.63	2.10	20	18.84 ± 0.29 (n=4)	94.22	2.38	149	135
					100	103.06 ± 0.82 (n=4)	103.06	0.85		
Ribonuclease B (man7)	0.9983	5 – 200	0.91	3.04	20	17.64 ± 0.77 (n=4)	88.21	8.05	163	141
					100	102.44 ± 1.78 (n=4)	102.44	1.89		
Ribonuclease B (man8)	0.9981	5 – 200	1.35	4.50	20	18.91 ± 0.45 (n=4)	94.55	4.41	155	143
					100	102.07 ± 1.65 (n=4)	102.07	1.77		
Ribonuclease B (man9)	0.9983	20 - 200	5.40	17.99	20	22.88 ± 1.02 (n=4)	114.38	12.22	165	141
					100	102.19 ± 3.36 (n=4)	102.19	3.84		
Insulin	0.9976	2 - 50	0.51	1.71	6	7.17 ± 0.03 (n=4)	119.46	0.82		
					20	17.02 ± 0.97 (n=3)	85.12	6.97	182	158
Lysozyme	0.9980	5 - 200	1.08	3.59	20	17.26 ± 0.58 (n=4)	86.30	3.77	172	137
					100	106.98 ± 0.61 (n=4)	106.98	0.58		
β-Lactoglobulin A	0.9982	5 - 200	0.34	1.13	20	16.25 ± 0.30 (n=4)	81.27	2.41	123	132
					100	98.39 ± 0.67 (n=4)	98.27	0.71		

The matrix effect is an important consideration when quantifying analytes from biological samples using ESI mass spectrometry. In this study, human serum and urine were used as sample matrices to evaluate the specificity and the feasibility of the MIC method. Matrix effect was evaluated at a concentration of 20 µg/mL. A general increase in response for all the eight proteins was observed both in serum and urine matrices. In such cases of direct quantification of intact proteins using the introduced MIC method, it is important to assess the prevalence of matrix effects. However, this is no different than standard best practices used for MRM methods.

❑ Conclusions

We propose a straightforward method based on the MIC of Q-TOF mass spectrometer to directly and quickly quantify intact proteins without the need for enzymatic digestion and purification. By combining several multiple-charged ion signals of an intact protein with high resolution TOF mass spectrometry, the methodology demonstrated the feasibility for use in quantifying intact proteins ranging from few thousands to 18.4 kDa, and possibly extended to higher mass. The results of method performance evaluation indicates good accuracy and precision, and high sensitivity – down to 2 µg/mL.

❑ Reference

1. Mao Y, Moore RJ, Wagnon KB, Pierce JT, Debban KH, Smith CS, Dill JA, Fuciarelli AF, "*Analysis of α2u-globulin in rat urine and kidneys by liquid chromatography-electrospray ionization mass spectrometry*", Chem. Res. Toxicol. 11 (1998): 953-961.
2. Wang EH, Combe PC, Schug KA, "*Multiple Reaction Monitoring for Direct Quantitation of Intact Proteins Using a Triple Quadrupole Mass Spectrometer*", J. Am. Soc. Mass Spectrom. 27 (2016): 886-896.

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