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Multiple ion chromatogram (MIC) for direct quantification of intact proteins using Q-TOF mass spectrometry

TP 658

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1. Overview

- A MIC method was developed for direct quantitative analysis of intact proteins using LCMS-9030 Q-TOF mass spectrometry
- This MIC assay can be used to accurately and precisely monitor intact protein levels throughout the process of production, characterization, and clinical uses

2. Introduction

Mass spectrometry (MS) has emerged as a powerful analytical technique for direct quantification of intact proteins. In quantifying intact proteins, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) methods have been reported by using triple-quadrupole (QQQ) mass spectrometry. Because of a low dynamic range, there is lack of studies of intact protein quantification using quadrupole time-of-flight (Q-TOF) mass spectrometry. However, Q-TOF has the advantages of high-resolution accurate mass. In this study, we aimed to identify, investigate, and demonstrate the performance of multiple ion chromatogram (MIC) for direct quantification of intact proteins using a Shimadzu LCMS-9030 Q-TOF mass spectrometer.

3. Methods

3.1 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).

3.2 Sample preparation and analytical conditions

Protein standards were dissolved in the 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.

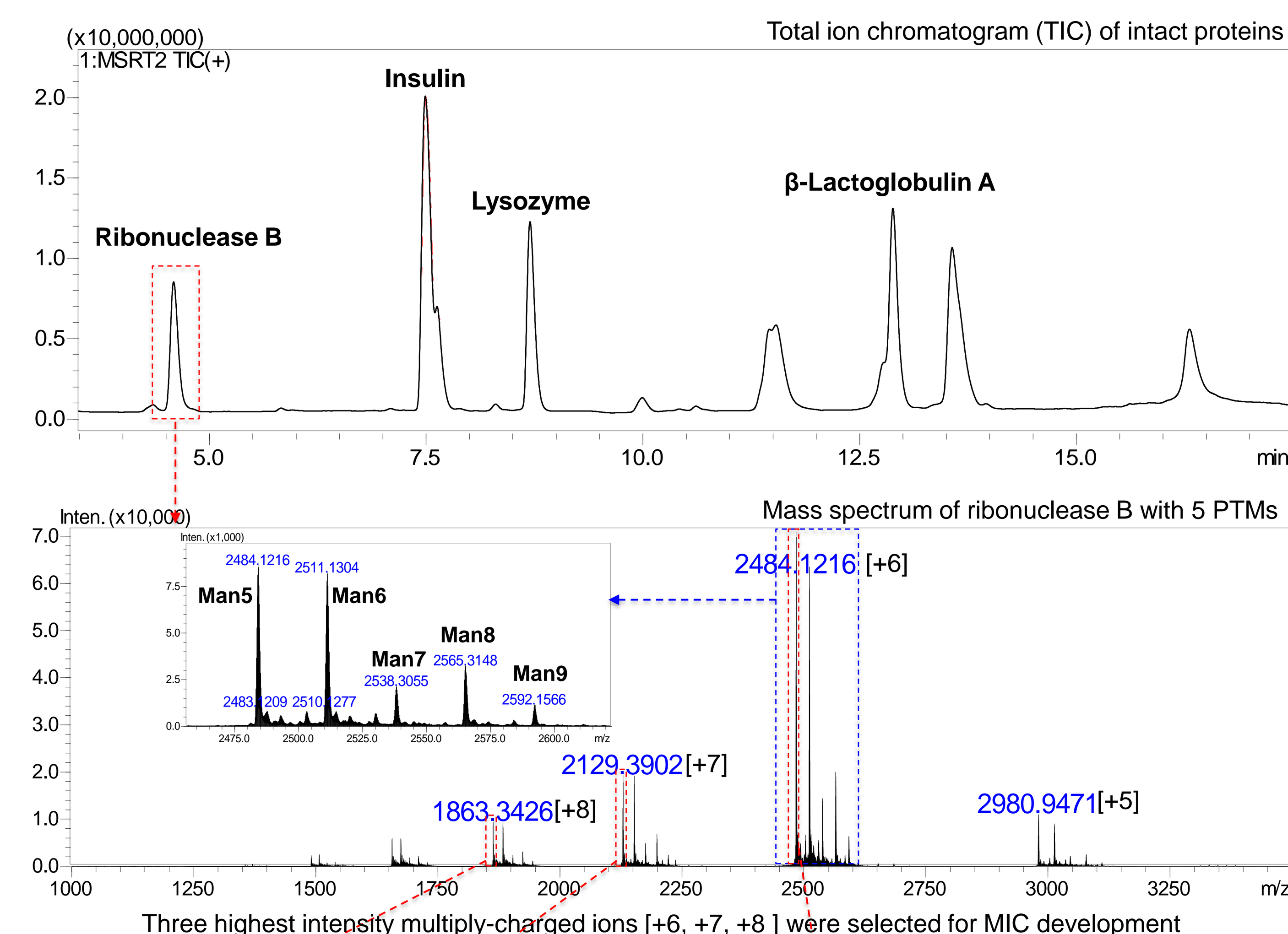
Table 1. Analytical conditions on LCMS-9030

Column	ProteCol-G C8 (100 mm x 2.1 mm; 3 µm)
Flow rate	0.3 mL/min
Mobile phase	A: 0.1% TFA in water B:0.1% TFA in acetonitrile
Elution mode	Gradient elution, 20%B (0 min) → 60%B (20 min) → 80%B (20.5 min) → 80%B (23.5 min) → 20%B (24 min) → 20%B (30 min)
Oven temp.	50°C
Injection vol.	5.0 µL

Interface & temp.	Heated ESI, 300°C
Mass range	m/z 1000-3500 (+)
Interface voltage	4 KV
Heat Block temp.	400°C
DL temp.	250°C
Nebulizing gas flow	N_2, 3.0 L/min
Drying gas flow	N_2, 15.0 L/min
Heating gas flow	Zero air, 10.0 L/min

3.3 MIC method development

In MIC method development, three highest intensity ion signals were first selected from multiply-charged ion envelope of each protein, and further the most abundant monoisotopic mass from each respective multiply-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. MIC settings are summarized in Table 2.



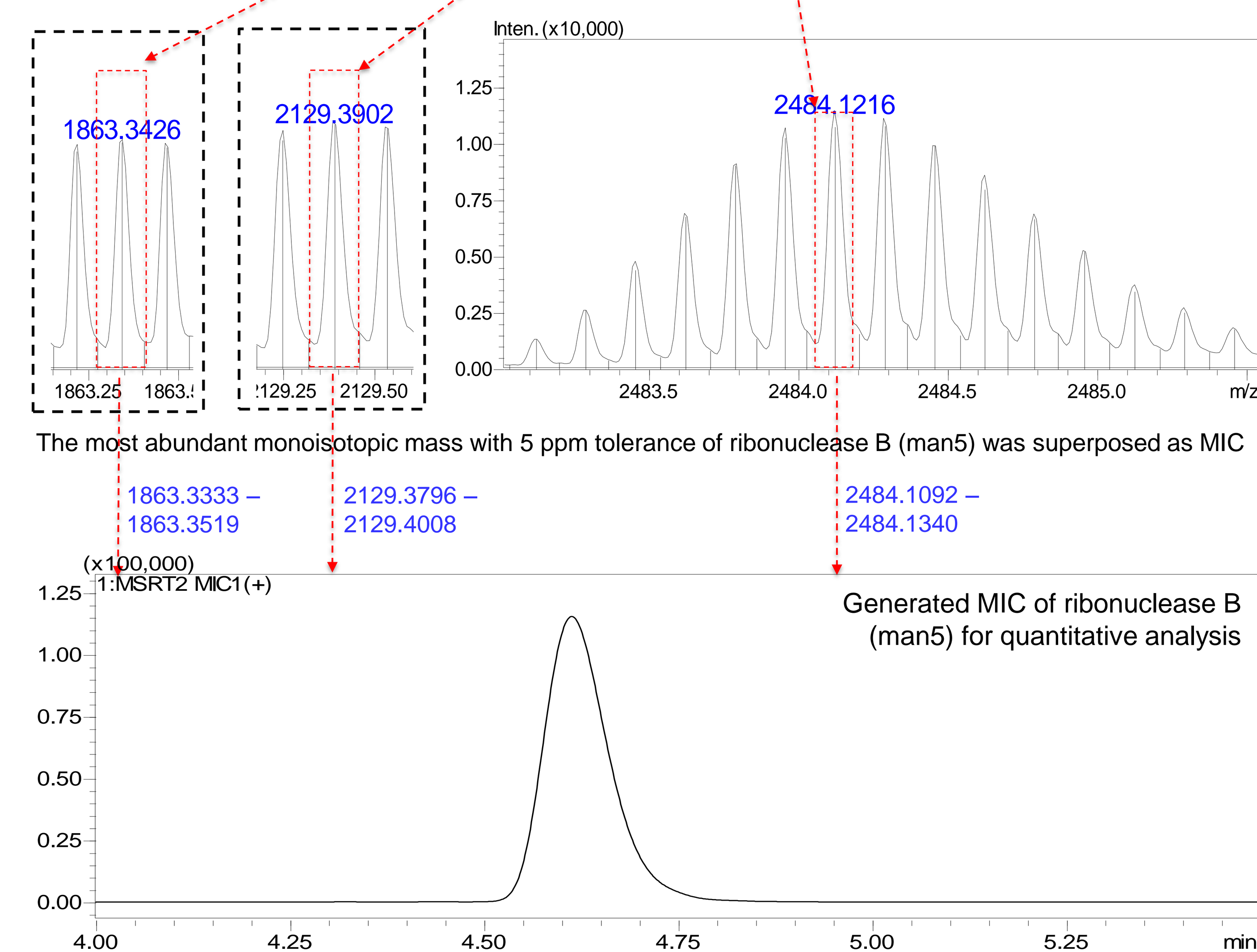


Figure 1. The procedure for multiply ion chromatogram (MIC) development, taking ribonuclease B (man5) as an example.

Table 2. MIC settings of intact proteins

Protein	MIC No.	MIC settings		
		Peak No.	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

4. Results

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. LOD and LOQ for the proteins ranged from 0.32 to 5.40 µg/mL and 1.13 to 17.99 µg/mL, respectively. 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.

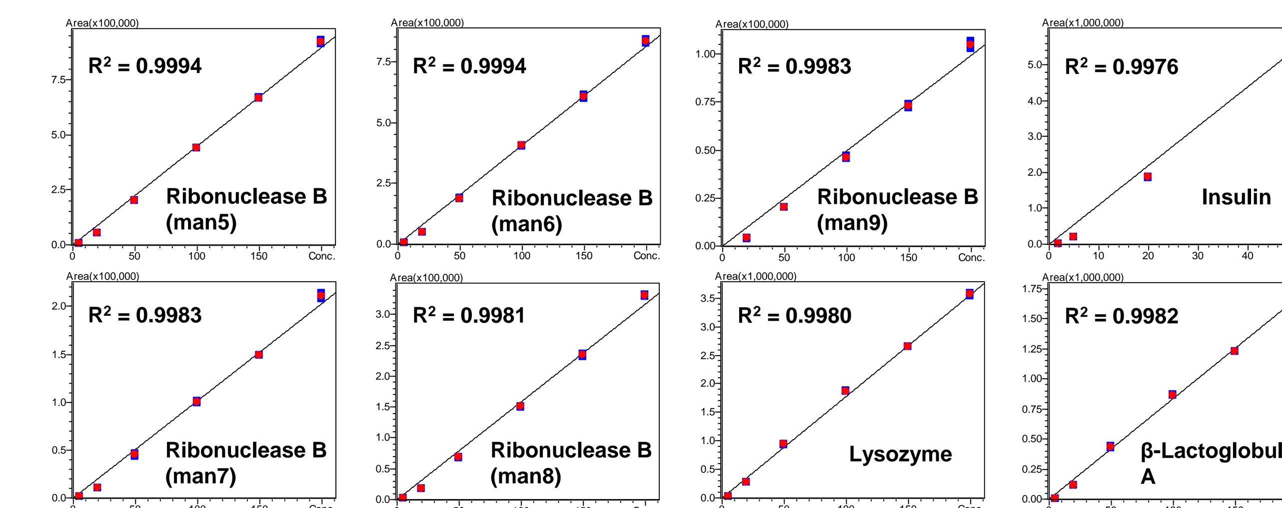


Figure 2. Representative calibration curves of intact protein on LCMS-9030

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 in 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.

Table 3. Method evaluation for MIC-based intact protein quantification

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	-	-

5. Conclusion

We proposed a straightforward method based on the MIC of Q-TOF mass spectrometry to directly and quickly quantify intact proteins without the need for preliminary digestion. By combining several multiply-charged ion signals, the methodology demonstrated a high sensitivity for use in quantifying intact proteins – down to 2 µg/mL. Ion enhancement was observed for target proteins in serum and urine matrices, and therefore matrix effects should be considered in the context of specific applications. However, this is no different than standard best practices used for small molecule quantification.

References

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