

Efficient separation and accurate spotting of NBS Labeled Peptides Using the Micro HPLC AccuSpot System

This application uses the Micro HPLC AccuSpot system to help facilitate relative quantitation of expressed proteins with NBS reagents. Chicken egg-white lysozyme was used as a model protein and 100µg each of the protein was labeled using ¹³CNBS and ¹²CNBS reagents respectively. After labeling, they were mixed, reduced, alkylated and trypsin digested, then gel-filtered to remove excess reagents. Next, the Micro HPLC was used with reverse chromatography for separation, after which AccuSpot was used to automatically spot samples onto MALDI plates. Finally, samples were analyzed using AXIMA-CFR.

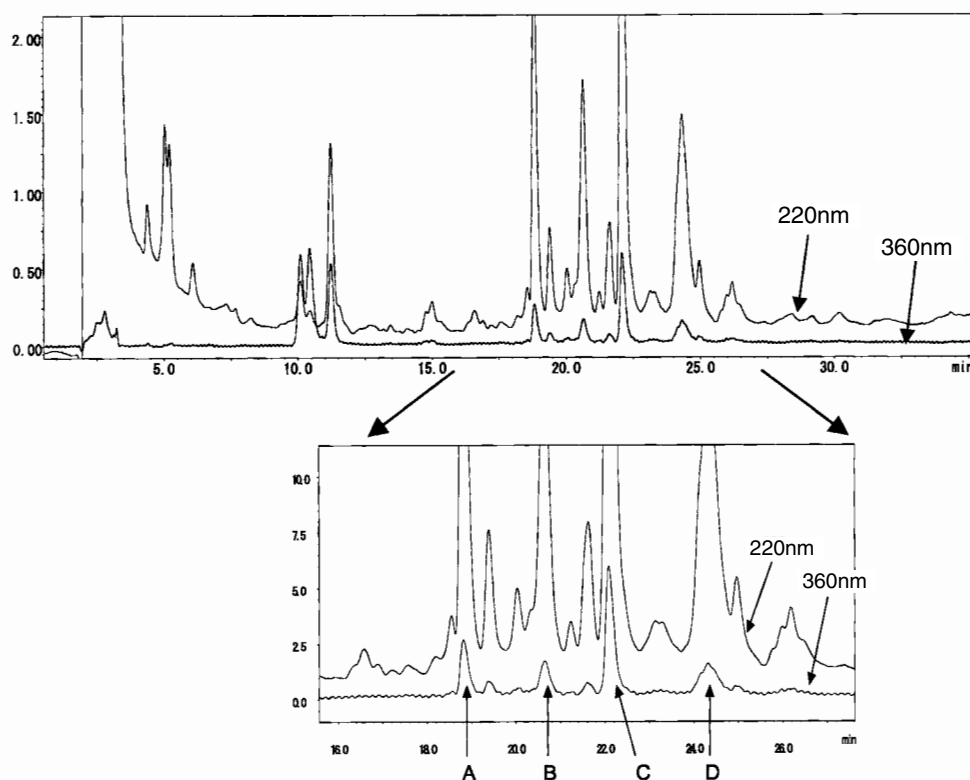


Figure 1 HPLC chromatogram of the processed egg-white lysozyme

HPLC Conditions

System	: Shimadzu Micro HPLC AccuSpot	Column	: Develosil ODS-HG-5 (0.5 x 100mm)
Eluent	: A: 0.1 % aqueous TFA, B: 0.1% TFA acetonitrile solution	Flow rate	: 10 µL/min
Gradient	: A/B; 80/20-20/80 (30min)	Detection	: 220nm, 360nm
Column Temp	: Ambient		
Injection volume	: 1/50 the peptide mixture volume obtained after gel-filtration according to standard protocol for ¹³ CNBS reagent (equivalent to 4µg of sample)		

AccuSpot Conditions

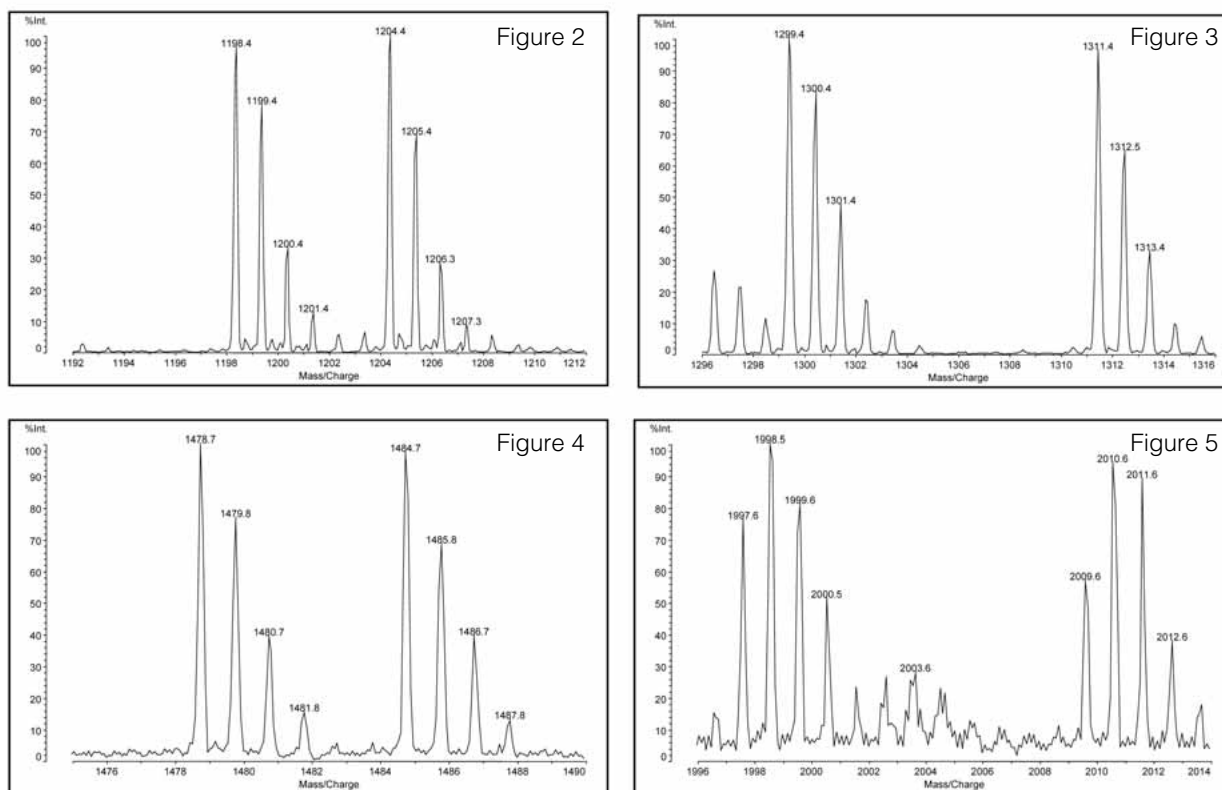
Matrix	: CHCA (7.5mg/mL, 50% CH3 CN-0.05% TFA)	Spotting interval	: 3 seconds
Matrix flow rate	: 10µL/min		
Spot volume	: 1 µL		

The chromatogram is shown in Figure 1. The elution time for the labeled peptides could be determined from detection at 360 nm, which is the characteristic absorption wavelength for NBS labeled peptides.

Among the theoretical fragments after trypsin digestion, those fragments with mass numbers up to 2000 that incorporate tryptophan are the four types listed below.

GTDVQAWIR	(m/z of labeled fragment : ^{12}C 1198.5, ^{13}C 1204.5)
WWCNDGR	(m/z of labeled fragment : ^{12}C 1299.4, ^{13}C 1311.4)
GYSLGNWVCAAK	(m/z of labeled fragment : ^{12}C 1478.6, ^{13}C 1484.6)
IVSDGNGMNAWVAWR	(m/z of labeled fragment : ^{12}C 1981.8, ^{13}C 1993.8)

In this analysis, judging from the detected peaks on HPLC chromatogram (220nm and 360nm), eluted fraction (10-26min) was spotted onto MALDI-plate using AccuSpot and those spots were analyzed with AXIMA-CFR. As a result, the target labeled peptides were detected from the spots corresponding to peaks A through D in Figure 1 (see Figures 2 to 5). The peak pair intensity ratios for these peaks showed good agreement with the set ratio (1:1), as shown in Table 1.



Analytical results from AXIMA-CFR

Table 1 Relative quantitation results

Elution position for HPLC (Fig. 1)	MALDI TOFMS spectrum	Trypsin digest fragment (*: NBS marker)	Labeled fragment mass number (^{12}C NBS, ^{13}C NBS)	Observed ratio (H/L)	Set ratio (H/L)
A	Figure 2	GTDVQAW*IR	1198.5, 1204.5	1.01	1
C	Figure 3	W*W*CNDGR	1299.4, 1311.4	0.95	1
B	Figure 4	GYSLGNW*VCAAK	1478.6, 1484.6	1.06	1
D	Figure 5	IVSDGNGM**NAW*VAW*R (**: Met is oxidized)	1997.8, 2009.8	0.84	1