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Detecting Carbamylation of Intact Proteins and Peptides by LC/MS (/MS)

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Introduction

Characterization of pharmaceutical proteins is an important analytical challenge that must be met in pharmaceutical and biotechnology companies today. Small chemical variations in proteins can be the difference between an active and an inactive therapeutic. Reversed-phase chromatography coupled with mass spectrometry has rapidly become the technique of choice for the determination of post-translational modifications. We have developed a simple and rapid LC/MS method for identifying the presence and degree of carbamylation of intact proteins or peptides in a mixed sample using either single quadrupole or Q-TOF type instrumentation.

Experimental Protein Conditions

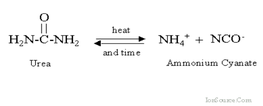
Waters 996 PDA Detector

Waters ZQ™ Mass Detector
–Source = ESI (+), Capillary (kV) = 3.3, Cone (V) = 25
–Temperature (°C), Source = 150, Desolvation = 350
–Gas flow (L/Hr), Cone = 50, Desolvation = 500
–Scan Mode
or Micromass Q-TOF II

Cytochrome C, Carbonic Anhydrase, and Horse Heart Myoglobin
–Intact and Carbamylated 1 hr. 98 °C in 2M Urea

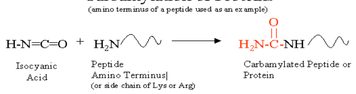
Separation Method:
–Symmetry 300® C_{18} 5.0 µm 4.6 X 50 mm
–A: 0.1% Formic acid in water
–B: 0.1% Formic acid in ACN
–Gradient from 15% B to 80% B in 15 min.
–0.5 ml/min, Split Flow to ~0.2ml/min diverted to ZQ

Decomposition of Urea

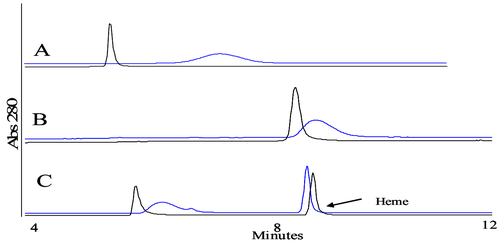


The general reaction mechanism by which proteins become carbamylated.

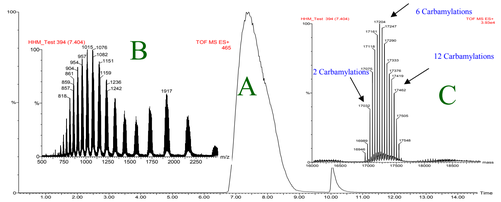
Carbamylation of Proteins
(amino terminus of a peptide used as an example)



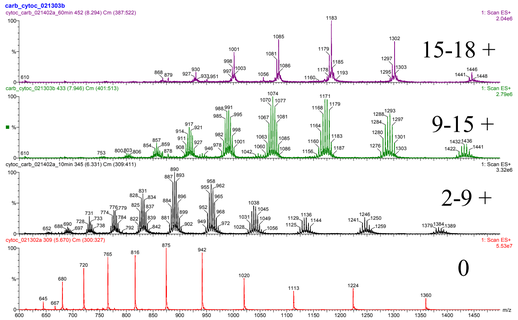
The overlay chromatograms of proteins separated on Delta-Pak™ C_{18}, 5 µm, 4.6 x 50 mm column. The black trace represent the protein unmodified while the blue trace represents the carbamylated sample.
(A) Bovine Cytochrome C (B) Carbonic Anhydrase (C) Horse Heart Myoglobin



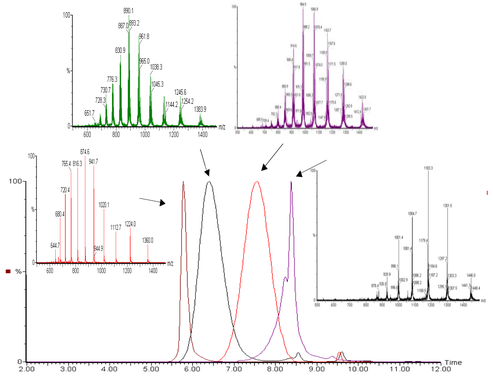
A) The LC/MS total ion chromatograph of a mixed carbamylated sample of Horse Heart Myoglobin separated by Waters Symmetry 300® C_{18} column and analyzed on a Micromass Q-Tof II. B) The mass spectral envelope created by a mixed sample of carbamylated Horse Heart Myoglobin. C) The MaxEnt1 deconvoluted spectrum of the Horse Heart Myoglobin protein envelope showing unmodified horse heart myoglobin at 16946 Da and including protein species with 1-12 carbamylations.



The protein mass spectral ion envelopes created by increasing amounts of carbamylation of Bovine Cytochrome C. The number on the left indicates the number of carbamylations per protein in the sample mixture.



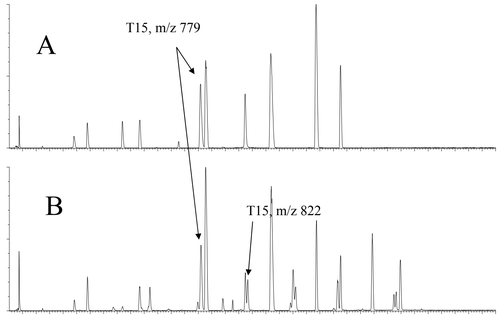
An overlay of the total ion chromatograms showing the separation of Bovine Cytochrome C with varying degrees of carbamylation. As the peaks increase in retention time, there is a corresponding increase in the degree to which the sample is carbamylated. A mass spectral envelope is shown for each peak showing the increased degree of carbamylation as retention increases.



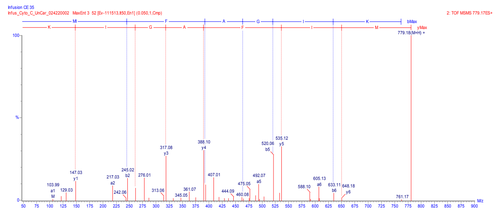
Experimental Peptide Conditions

Waters 996 PDA Detector
Waters ZQ™ Mass Detector
–Source = ESI (+), Capillary (kV) = 3.3, Cone (V) = 30
–Temperature (°C), Source = 150, Desolvation = 350
–Gas flow (L/Hr), Cone = 50, Desolvation = 500
–Scan Mode
Micromass Q-Tof II
–Source = ESI (+), Capillary (kV) = 3.3, Cone (V) = 30
Collision Energy=30
–NanoSpray
–Survey Scan MS 200-1600 m/z, MS/MS 50-1600 m/z
–MaxEnt3 Processed Spectrum
–BioLynx™ Peptide Sequencing
–200nL/min nominal flow
Cytochrome C
–Digested for 4 hours with trypsin then carbamylated for 10 minutes at 98 °C in 2M Urea
Separation Method UV/MS:
–Atlantis™ C_{18} 3 µm 4.6 X 50 mm (186001329)
–A: 0.1% Formic acid in water
–B: 0.1% Formic acid in ACN
–Gradient from 0% B to 40% B in 45 min.

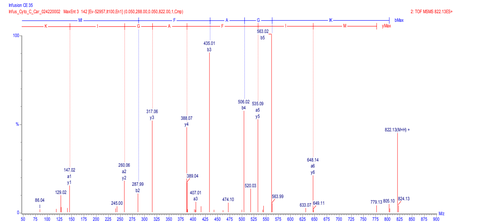
A) The LC/MS total ion chromatogram of Bovine Cytochrome C tryptic peptides separated on a Atlantis™ dC_{18} column. Highlighted is the T15 peptide fragment. B) The LC/MS total ion chromatogram of Bovine Cytochrome C tryptic peptides which have been carbamylated. Highlighted is the T15 peptide which has a 4.6 minute increase in retention due to the carbamylation.

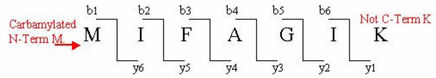


The MS/MS spectrum of the unmodified T15 Bovine Cytochrome C peptide after processing in MaxEnt3 and BioLynx™. The assignment of the a,b and y,z ion series is shown by graphically and in tabular formats.



The MS/MS spectrum of the carbamylated T15 Bovine Cytochrome C peptide after processing in MaxEnt3 and BioLynx™. The assignment of the a,b and y,z ion series is shown by graphically and in tabular formats.





b ion Series				y ion Series			
Ion	m/z un-Carb.	Δ m/z	m/z Carb.	Ion	m/z un-Carb.	Δ m/z	m/z Carb.
b1	N/	-	N/	y1	147	Δ0	147
b2	245	Δ43	287	y2	260	Δ0	260
b3	392	Δ43	435	y3	317	Δ0	317
b4	N/	Δ43	506	y4	388	Δ0	388
b5	520	Δ43	563	y5	535	Δ0	535
b6	633	-	N/	y6	648	Δ0	648
b	N/	-	N/	y	779	Δ43	821

Conclusions

We have shown that this methodology can rapidly:

-Detect carbamylation using intact proteins or peptides with LC/UV and/or LC/MS

-Detect the exact number of modifications per protein in a mixed intact protein sample by LC/MS .

-Detect the exact amino acid(s), that has (have) been modified by LC/MS/MS

-We were unable to detect immonium ions for carbamylated amino acids under the conditions used.

References

1)Golemi, D. et al. (2001). Critical involvement of a carbamylated lysine in catalytic function of class D beta-lactamases. Proc. Natl. Acad. Sci. USA. 98(25):14280-5.

2)Wynckel, A. et al. (2000). Kinetics of carbamylated hemoglobin in acute renal failure. Nephrol. Dial. Transplant. 15(8):1183-8

3)Lippincott, J. et al. (1998). Carbamylation of cysteine: a potential artifact in peptide mapping of hemoglobins in the presence of urea. Anal. Biochem. 267:57-64.

4)Mun, K. et al. (2000). Impaired biological activity of erythopoietin by cyanate carbamylation. Blood Purif. 18:13-17.