Articles

Electron Caption Dissociation of Proteins Initiated by Photoelectrons Generated from 266 nm UV Laser Radiation on an ICR Cell Wall

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It was demonstrated that ECD MS can be successfully applied to Bovine ubiquitin (8.6 kDa, 76 amino acids) and Pseudomonas aeruginosa azurin (13.9 kDa, 128 amino acids, 1 disulfide bond) cations using photoelectrons emitted by irradiating a 266 nm UV laser light on the edge of an ion cyclotron resonance (ICR) cell trapping plate hole. The photoelectron ECD method cleaved protein backbone bonds as effectively as conventional ECD MS methods that employed a tungsten (W) wire or heated dispenser cathode as an electron source. This method offers the advantage of providing electrons for ECD MS without an apparent electron source.

Key Words : 266 nm UV laser, Electron capture dissociation (ECD), Ubiquitin, Azurin, FTICR MS

Introduction

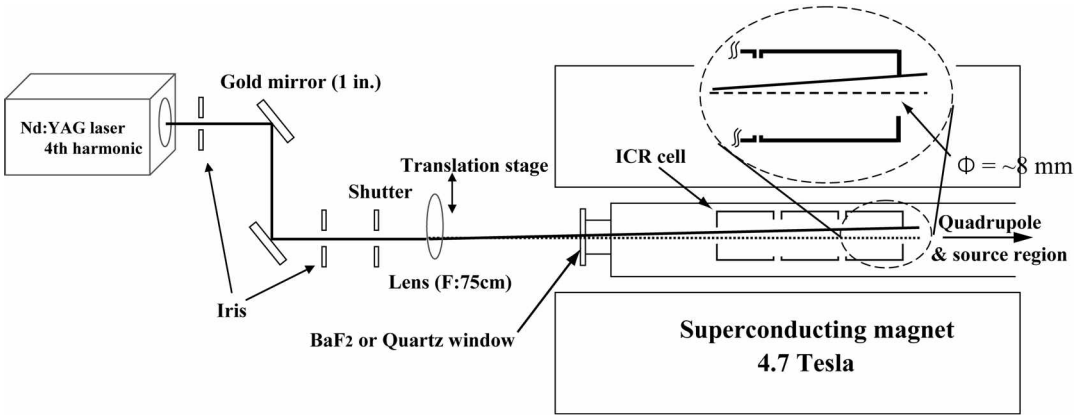
Electron capture dissociation mass spectrometry (ECD MS) is a useful tandem mass spectrometry technique, which allows analysis of large intact protein cations, as well as small peptide ions.[1-5] Compared to traditional energetic heating MS/MS methods, such as collisionally activated dissociation (CAD) and infrared multiphoton dissociation (IRMPD), ECD provides far more extensive backbone fragmentations of protein/peptides. It has been previously demonstrated that ECD MS can be used to characterize as large as 45 kDa proteins.[6] In addition, ECD has the unique characteristics of preserving weak inter/intramolecular non-covalent interactions and posttranslational modifications (PTMs) while cleaving strong covalent backbone bonds of proteins/peptides.[7-11] This unique property is known to be due to its fast dissociation mechanism occurring prior to energy randomization, though this mechanism is still under debate. Thus, ECD and analogous electron transfer dissociation (ETD) methods[12] are now widely used for analysis of protein conformations and phosphorylation/glycosylation PTMs.

Many researchers have made efforts towards improving ECD efficiency. Initially, the McLafferty group used a tungsten (W) or rhodium (Rh) wire/ribbon for production of electrons.[1-3,7,8] Later, Zubarev *et al.* successfully employed an indirectly heated dispenser cathode to increase not only the number of emitted electrons but also the cross-section of electron emission.[13] Invention of a hollow cathode further improved the utility of ECD, as it allowed simultaneous use of IRMPD and ECD.[14] However, ECD users often suffer from the instability or reproducibility of ECD operation. It has been known that such problems are mainly due to electric field distortion caused by the e^--charging effect around the electron source support block.

In the present study, as an alternative method, we employed a photoelectron effect to generate electrons inside an ion cyclotron resonance (ICR) cell with a 266 nm pulsed UV laser radiation. This method is convenient because electrons can be supplied without an electron source support block which can potentially inhibit the ECD operation through accumulation of electrons. UV laser light can produce photoelectrons when it is incident on the wall of the ICR cell if the photon energy is enough to overcome the so-called work function (Φ) of the ICR cell metal. Thus, similarly to thermal electrons generated on a heated surface of W or Rh wire/ribbon, photoelectrons are also expected to play the role of initiating ECD MS, whereby ECD using photoelectrons will be referred to as "*photoelectron ECD*" in the later description. From the historical standpoint, the discovery of ECD MS was fortuitously made in 1998 by the McLafferty group while performing a 193 nm UV photodissociation experiment on large protein cations.[15] Since then, however, no particular effort has been made to utilize a UV light for electron emission.

Experimental

Experiments were conducted on a 4.7 T electrospray ionization-Fourier transform ion cyclotron resonance mass spectrometer (ESI-FTICR MS: Varian, Lake Forest, CA, USA). A detailed experimental setup can be found else-



Scheme 1. Experimental setup. Note that the off-centered lens position presumably led to the laser's hitting the outer-rim of the opening in the ICR cell trap plate.

where.[16-20] In brief, protein samples were dissolved to a concentration of 20 μM in a water: methanol: acetic acid solution. ESI was performed using a direct infusion method at a rate of 0.5-1 μL/min through a 100 μL Hamilton syringe driven by a syringe pump (Harvard Apparatus 22, Holliston, MA, USA). A potential of 2.0-2.4 kV was applied between an emitter and capillary ion entrance. Ions were then transferred into an ICR cell through an RF-only quadrupole ion guide. Desired protonated pseudo-molecular ions were isolated using a single stored waveform inverse Fourier-transform (SWIFT).

A simplified laser setup diagram is shown in Scheme 1. For the pre-selected and stored ions, a pulsed 266 nm UV light was irradiated at a repetition of 10 Hz for 10-20 s. The pulsed 266 nm UV light was made using a home-built fourth harmonic generation device (1064 nm → 532 nm → 266 nm) from a pulsed fundamental Nd:YAG laser (Quantel, Brilliant b, France) beam. The pulse width of the fundamental laser was ~7 ns. The average single pulse energy of the UV beam was adjusted to be ~5 mJ. A mechanical shutter was placed in the laser beam path to control the laser irradiation time. Using a CaF_2 lens (f = 75 cm) positioned in front of the magnet bore, the 266 nm beam was focused into the ICR cell through a BaF_2 window and carefully aligned using a counter-propagating He-Ne laser so that the center of the focused beam, ~1 mm in diameter, coincided with the symmetrical axis of the ICR cell. Under this perfect aligned condition, no photoelectron was generated because the diameter of the UV laser beam was less than that (~8 mm) of the opening of the ICR cell trapping plate. Thus, in order to generate photoelectrons, we slightly tilted the laser beam path by sliding the center of the CaF_2 lens in a direction normal to the symmetrical axis of the ICR cell. It was experimentally found that the maximum production of fragmentations occurred when the center position of lens was approximately 3 mm off from the symmetric axis.

ECD experiments were performed for *Bovine* ubiquitin (76 amino-acid residue, monoisotopic mass = 8,564.5 Da) and *Pseudomonas aeruginosa* azurin (128 amino-acid residues, monoisotopic mass = 13,934.8 Da). *Bovine* ubiquitin (Sigma, Seoul, Korea) and *Pseudomonas aeruginosa* azurin samples (Fluka, Seoul, Korea) were used without further purification step.

Results

To facilitate the comparison of backbone cleavage efficiencies between the conventional and photoelectron ECD methods, ECD MS experiments were carried out for *ubiquitin* cations that were previously well characterized by the conventional ECD methods. Furthermore, *Pseudomonas aeruginosa* azurin (128 amino-acids) cations were also subjected to ECD MS, for the first time, to demonstrate its backbone cleavage capability for larger proteins.

Ubiquitin. ESI mass spectrum showed a multitude of ubiquitin pseudo molecular ions ranging from 6+ to 12+ charge states (spectrum not shown here). The distribution of charge states was bimodal, where one mode covers the charge states from 9+ to 12+ peaking at the 10+ charge state, and the other from 6+ to 8+. The former represents a charge state distribution for unfolded conformers of ubiquitin, whereas the latter for folded conformation. Higher proton densities are known to lead to opening the conformations of protein cations through charge-to-charge repulsions.[8] In general, an ESI solution with high water content, such as water: methanol (95:5 v/v), is known to shift the charge state distribution to lower charge states, *i.e.*, native-like folded conformations, and the solution with high methanol content (49:49:2 water:mathanol: acetic acid v/v/v) favorably produces the higher charge state ions with unfolded conformations.[21] Experimental observations were consistent with this previously mentioned report.

Figure 1 represents the ECD MS mass spectra obtained using photoelectrons generated by irradiating a 266 nm UV laser pulse into the ICR cell, wherein (a), (b), and (c) are the ECD product mass spectra for ubiquitin (a) 7+, (b) 9+, and (c) 11+ ions, respectively. As shown in Figure 1, photoelectron ECD for ubiquitin cations induced the dissociations of protein backbone bonds mainly between an amide nitrogen and alpha carbon (NC_α), thereby producing ***c*** and ***z*** ions. However, there were some minor ***a***, ***b***, ***x***, and ***y*** ions. As in conventional ECD, each ECD mass spectrum shows singly

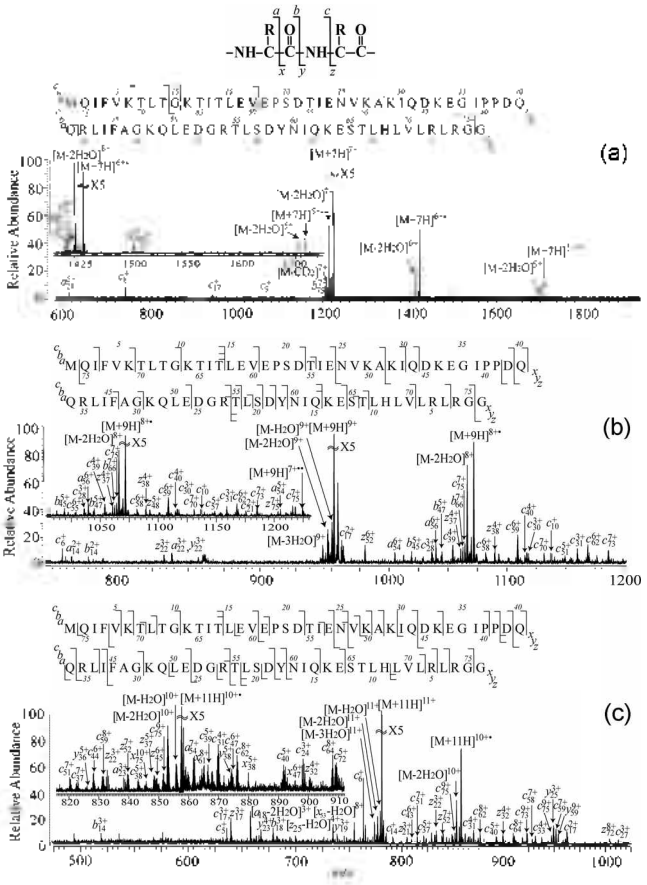


Figure 1. Photoelectron ECD MS spectra for (a) 7+, (b) 9+, and (c) 11+ *Bovine* ubiquitin cations. For detailed cleavage information, enlarged partial mass spectra are denoted in the insets. The overall backbone cleavages are displayed in the linear sequence.

and doubly reduced ions, $[M+nH]^{(n-1)\cdot\cdot}$ and $[M+nH]^{(n-2)\cdot\cdot\cdot}$, respectively.[2,4,8] Retrospectively, the observation of reduced ions offers evidence that the electron capture process occurred upon irradiation of the 266 nm laser light. The backbone fragmentation efficiency varied depending on the charge states of the ubiquitin cations. For ubiquitin 7+ ions, only a few fragments were observed, whereas more fragments were produced for the higher charge states (9+ and 11+ ions). The enhanced ECD efficiencies for the higher charge state cations can be related to the increased electron capture efficiencies of the protein cations with higher proton densities, which are know to be triply proportional to the charge of protein cations under study. Our experimental results are consistent with those previously reported by the McLafferty group in which a W wire/ribbon electron source was employed.[4] The observed numbers of ECD fragments for the three charge states largely corroborated the McLafferty group's results: 7+ ions, 8 (photoelectron ECD) versus 13 (conventional ECD); 9+ ions, 31 versus 42; 11+ ions, 45 versus 42 inter-residue cleavages.[8]

For 7+ ions, the fragmentation yield was relatively poor, because ECD was not effective for these folded conformations.[3,8] In general, ECD is known to preserve intramolecular noncovalent interactions and thus causes minimal

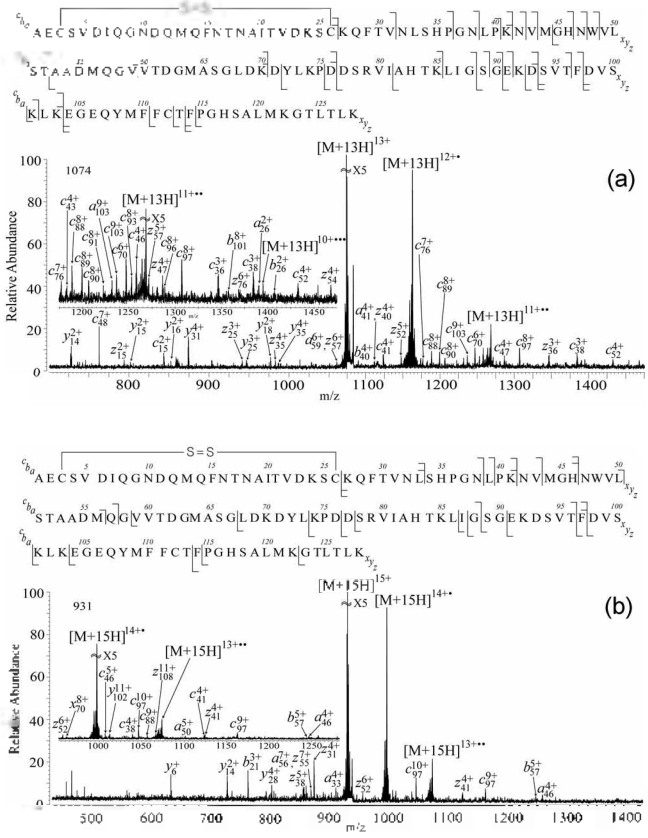


Figure 2. Photoelectron ECD MS spectra for (a) 13+ and (b) 15+ *Pseudomonas aeruginosa* azurin cations. For detailed cleavage information, enlarged partial mass spectra are denoted in the insets. The overall backbone cleavages are denoted in the linear sequence.

backbone dissociation for folded conformers, as manifested by singly and doubly reduced peaks in Figure 1. It is also worthwhile to mention that the spectrum shows some difference to data obtained previously using a heated dispenser cathode electron source.[19] The reason for this difference lies on the fact that a heated cathode in our previous ECD source was situated just behind the ICR cell, so that blackbody radiations corresponding to high temperatures activated ubiquitin 7+ ions into unfolded conformers.

Azurin. Photoelectron ECD was also performed for *Pseudomonas aeruginosa* azurin consisting of 128 amino-acid residues with one disulfide bond between ^{3}cysteine and ^{26}cysteine. ESI mass spectrum showed a broad charge state distribution from 8+ to 18+. Due to the broad charge state distribution, the abundance of each charge state was relatively low compared to ubiquitin. Of the charge states, abundant 13+ and 15+ charges were subject to the photoelectron ECD. Figure 2 (a) and (b) show the ECD mass spectra for the 13+ and 15+ charge states, respectively. Each charge state gave rise to 52 and 35 fragments upon photoelectron ECD, most of which were *c* and *z* ions. For the 15+ charge state, the occurrence of *y* ions was relatively frequent. Each ECD spectrum of the 13+ and 15+ charge states revealed 35 and 27 inter-residue cleavages, respectively. The lower number of cleaved inter-residue bonds for the 15+ charge states was presumably due to the relatively low abundance compared to

the 13+ charge state.

It was also notable that there was observed no mass spectral peak either in Figure 2(a) or (b) that indicated the cleavage of a disulfide bond between the ^{3}cysteine and ^{26}cysteine. Other groups have previously demonstrated that ECD can successfully cleave a disulfide bond between two cysteine amino-acid residues.[2,4,22] However, in our experiments for azurin cations, any fragment ion that can support the evidence of the cleavage of the disulfide bond was not observed. Presumably, relatively compact conformations of 13+ and 15+ charge states azurin limited the access of an electron to this disulfide bond.

Discussion

The photoelectron ECD MS method employing a 266 nm UV laser for photoelectron generation was shown to be as effective as the conventional ECD MS methods that utilized a W wire/ribbon or dispenser cathode for an electron source in terms of the overall backbone cleavage patterns and the number of cleaved backbone bonds, at least, for ubiquitin and azurin cations. The photon energy of the 266 nm wavelength laser beam is just enough for producing photoelectrons because the work-function of the ICR cell wall made of gold-plated copper (Φ_{copper} = 4.7 eV) was similar to the single photon energy of a 266 nm laser light, *c.a.* 4.66 eV.[23] The power dependence of fragment abundance was also measured, but a reliable dependence could not be determined because fragments were observed only in a relatively narrow power range, *e.g.*, 3-6 mJ/pulse. Furthermore, the laser power could not be increased to greater than 6 mJ/pulse, due to the low damage threshold of the employed BaF_2 or quartz laser window.

Here we should mention that any production of fragments shown in Figure 1 and 2 was, indeed, not observed under the condition of perfect laser alignment in our photoelectron ECD experiments for the large protein cations. The optimal production of ECD fragments was achieved when the lens position was 3 mm off from the center-line. At this lens position, it appears that the edge of the UV Gaussian beam irradiated the outer-rim of the ICR cell opening. We also empirically found that when the position of the lens was slid by more than 3 mm, a bunch of electrons were generated to damage a preamplifier electric circuit because the central part of the UV Gaussian beam hit the ICR cell wall. Even though it required a very careful adjustment to achieve the optimal lens position, the once established lens position led to a stable ECD MS operation over a long period of time without the need to readjust the lens position. The day-to-day ECD efficiency was also very stable.

The UV laser radiation on the ICR cell wall could also generate a plume of metal ions as in laser ablation experiments. However, in the ECD MS mass spectra, no metal ion adduct peak was observed. The Coulombic repulsion between two positively charged protein and metal ions presumably prohibited them from combining to form metal ion adducts. Any interference that might be caused by the possibly generated metal ions was not observed either.

The same experiment was also peformed for small peptides such as *Bradykinin* (RPPGGFSPFR), *Substance P* (RRKPQQFFGLM), and *Angiotensin* II (DRVYIHPF). In contrast to the large protein cations, UV laser radiation gave rise to abundant ***b*** and ***y*** ions, as well as a few ***c*** and ***z*** ions (spectra not shown here). For ***b*** and ***y*** ions, direct UV absorption at the UV chromophoric amino acid residues, *e.g.*, phenylalanine (F) and tyrosine (Y), seemed to be responsible for the backbone fragmentations.[24] For the large proteins discussed above, direct absorption of UV light was less effective in resulting in production of ***b*** and ***y*** ions than for small peptides since the proteins have a much larger number of vibrational modes into which degraded thermal energy could flow. In fact, the photoelectron ECD cleavage pattern for ubiquitin 7+ cations suggested that the effective internal temperature of ubiquitin 7+ cations under the radiation of 266 nm laser was close to a room temperature.[19]

In summary, it was demonstrated that our photoelectron ECD offers an alternative ECD MS method that can be used for characterization of protein cations as large as 8.6 to 13.9 kDa without an apparent electron source such as a W/Rh wire or a dispenser cathode.

Conclusions

It was demonstrated that photoelectron ECD MS can be successfully applied to protein cations, such as ubiquitin and azurin, using electrons generated from radiating a 266 nm UV laser light on an ICR cell wall made of gold-plated copper. As in the conventional ECD MS, the photoelectron ECD mainly induced extensive ***c*** and ***z*** ions for the protein cations. It was experimentally observed that the alignment of the laser beam path was critical in achieving the optimal operation of photoelectron ECD. For photoelectron ECD MS, other UV lasers, such as a 193 excimer laser, are expected to be as effective or better than 266 nm UV light.

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References

1. Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1998**, *120*, 3265.
2. Zubarev, R. A.; Horn, D. M.; Fridriksson, E. K.; Kelleher, N. L.; Kruger, N. A.; Lewis, M. A.; Carpenter, B. K.; McLafferty, F. W. *Anal. Chem.* **2000**, *72*, 563.
3. Oh, H. B.; Breuker, K.; Sze, N. K.; Ge, Y.; Carpenter, B. K.; McLafferty, F. W. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15863.
4. Zubarev, R. A. *Mass Spectrom. Rev.* **2003**, *22*, 57.
5. Cooper, H. J.; Håkansson, K.; Marshall, A. G. *Mass Spectrom. Rev.* **2005**, *24*, 201.
6. Ge, Y.; Lawhorn, B. G.; ElNaggar, M.; Strauss, E.; Park, J.; Begley, T. P.; McLafferty, F. W. *J. Am. Chem. Soc.* **2002**, *124*, 672.
7. Kelleher, N. L.; Zubarev, R. A.; Bush, K.; Furie, B.; Furie, B. C.; McLafferty, F. W.; Walsh, C. T. *Anal. Chem.* **1999**, *71*, 4250.
8. Breuker, K.; Oh, H. B.; Horn, D. M.; Cerda, B. A.; McLafferty, F.

W. *J. Am. Chem. Soc.* **2002**, *124*, 6407.
9. Mirgorodskaya, E.; Roepstorff, P.; Zubarev, R. A. *Anal. Chem.* **1999**, *71*, 4431.
10. Shi, S. D.; Hemling, M. E.; Carr, S. A.; Horn, D. M.; Lindh, I.; McLafferty, F. W. *Anal. Chem.* **2000**, *73*, 19.
11. Häkansson, K.; Cooper, H. J.; Emmett, M. R.; Costello, C. E.; Marshall, A. G. *Anal. Chem.* **2001**, *73*, 4530.
12. Syka, J. E. P.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9528.
13. Tsybin, Y. O.; Häkansson, P.; Budnik, B. A.; Haselmann, K. F.; Kjeldsen, F.; Gorshkov, M.; Zubarev, R. A. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 1849.
14. Tsybin, Y. O.; Witt, M.; Baykut, G.; Kjeldsen, F.; Häkansson, P. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 1759.
15. Guan, Z.; Kelleher, N. L.; O'Connor, P. B.; Aaserud, D. J.; Little, D. P.; McLafferty, F. W. *Int. J. Mass Spectrom. Ion Processes* **1996**, *157/158*, 357.
16. Han, S. Y.; Lee, S. Y.; Oh, H. B. *Bull. Korean Chem. Soc.* **2005**, *26*, 740.
17. Lee, S.; Ahn, S.; Yim, Y.; Kim, B.; So, H.; Oh, H. B. *Bull. Korean Chem. Soc.* **2007**, *28*, 1195.
18. Lee, S.; Han, S. Y.; Lee, T. G.; Chung, G. S.; Lee, D.; Oh, H. B. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 536.
19. Yim, Y.; Kim, B.; Ahn, S.; So, H.-Y.; Lee, S.; Oh, H. B. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 1918.
20. Lee, S. Y.; Chung, G. S.; Kim, J.; Oh, H. B. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3167.
21. Konermann, L.; Douglas, D. J. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1248.
22. Zubarev, R. A.; Kruger, N. A.; Fridriksson, E. K.; Lewis, M. A.; Horn, D. M.; Carpenter, B. K.; McLafferty, F. W. *J. Am. Chem. Soc.* **1999**, *121*, 2857.
23. Weast, R. C. *Handbook of Chemistry and Physics*; CRC Press: Boca Raton, FL, 1988.
24. Oh, J. Y.; Moon, J. H.; Kim, M. S. *J. Mass Spectrom.* **2005**, *40*, 899.