Research Article

Journal of PeptideScience

Received: 15 November 2009 Revised: 9 December 2009 Accepted: 22 December 2009 Published online in Wiley Interscience:

(www.interscience.com) DOI 10.1002/psc.1212

The oxidative products of methionine as site and content biomarkers for peptide oxidation

Wansong Zong, Rutao Liu,* Meijie Wang, Pengjun Zhang, Feng Sun and Yanmin Tian

Biomarkers for peptide/protein oxidation under oxidative stress (OS) hold both incredible application potential as well as significant challenges. In this article, liquid chromatography and mass spectrometry were applied to establish a new method for evaluating the oxidation site and degree of peptide oxidized, with its oxidative product serving as biomarker. In the three model peptides, peptide FMRF (containing a methionine) was prone to undergo oxygen addition under UV/H_2O_2 oxidization, forming a sulfoxide (FM(O)RF) with a stable chromatographic peak separate from the model peptides. The oxidation content of FMRF, expressed as $S_{FM(O)RF}/(S_{FM(O)RF} + S_{FMRF})$, is positively correlated with oxidation time. Based on sequence analysis of FM(O)RF, the oxidation mechanism (site and extent) of FMRF under UV/H_2O_2 oxidization was explicitly clarified. By comparing the specific injury to each model peptide, we found that the oxidative products of Met-containing peptides are good biomarkers for OS. This research not only expands the range of biomarkers for OS, but also provides an efficient and accurate method for evaluating oxidation damage to peptides and even proteins. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: oxidative stress; biomarker; peptides; UV/H_2O_2 oxidation; LC–MSn; oxidative product

Introduction

Under endogenous or exogenous stimulation, excessively produced reactive oxygen species (ROS), such as H_2O_2, HO, HO_2, HClO, and O_3 can destroy the dynamic equilibrium between oxidants and anti-oxidant systems and induce oxidative stress (OS) [1–5]. In the case of OS, ROS can oxidize biomolecules (lipids, nucleic acids, peptides, and proteins) and thus induce apoptosis, cancer, arteriosclerosis, and other diseases [2,4–6]. Thus, clarifying oxidation mechanisms of biomolecules caused by OS not only favors understanding of disease processes, but also is beneficial to disease prevention, early diagnosis and treatment [4,6,7].

Biomarkers can reflect the physiological, biochemical, immunological, and genetic characteristics in the oxidation processes, and therefore they are key indexes for evaluating oxidative damage induced by OS [7–14]. Compared with the widely studied biomarkers for lipid [9,11] or nucleic acid oxidation [13], biomarkers for peptide and protein oxidation has been a limiting factor in research on the oxidation mechanisms [8,9]. Although 2,4-dinitrophenylhydrazine, nitrotyrosine, and dityrosine have been widely used as biomarkers for peptide and protein oxidation, the traditional spectroscopic and immunological techniques for detecting them have low sensitivity and can only determine the total carbonyls, nitrotyrosine, or dityrosine in oxidized peptides [9,11]. In addition, they cannot provide specific information for the oxidation sites, let alone the mechanism and degree of oxidation for each site. As such, novel biomarkers that can indicate the oxidation site and oxidation degree for peptides have unparalleled advantages in clarifying the oxidation mechanisms of peptides.

Tandem mass spectrometry (MS/MS) research has confirmed that peptides can be oxidized, forming carbonyl, hydroxyl, and sulfoxide containing products that are stable and time-dependent [15–18]. However, these products have not been treated as potential biomarkers for OS. In this study, liquid chromatography/mass spectrometry (LC/MS) and MS/MS assays were developed to examine the feasibility of using peptide oxidation products as biomarkers to probe the oxidation sites and oxidation degrees of peptides. The oxidation of target peptides was simulated by exposing peptides to UV/H_2O_2 oxidation, then the peptides and their oxidative products were separated and identified by LC/MS. The sites and degree of oxidation were further obtained by LC/MS and MS/MS analyses.

Materials and Methods

Materials

Peptides Phe-Met-Arg-Phe (FMRF, 598.76 Da), Asp-Arg-Val-Tyr-Val-His-Pro-Phe (DRVYVHPF, 1032.18 Da), and Arg-Pro-Pro-Gly-Phe-Ser-Pro-Tyr-Arg (RPPGFSPYR, 1076.23 Da) were purchased from GL Biochem Inc. (Shanghai, China) and had a purity at least of 95%. Thiourea, 30% H_2O_2, methionine (Met), and trifluoroacetic acid (TFA) were ordered from Sinopharm Chemical Reagent Inc. (Shanghai, China). HPLC methanol and HPLC acetonitrile were purchased from Merck (Germany). All reagents were prepared with Millipore ultrapure water and no buffer was used.

* *Correspondence to: Rutao Liu, School of Environmental Science and Engineering, Shandong University, Jinan 250100, People's Republic of China. E-mail: rutaoliu@sdu.edu.cn*

School of Environmental Science and Engineering, Shandong University, 27 Shanda South Road, Jinan 250100, People's Republic of China

Journal of PeptideScience

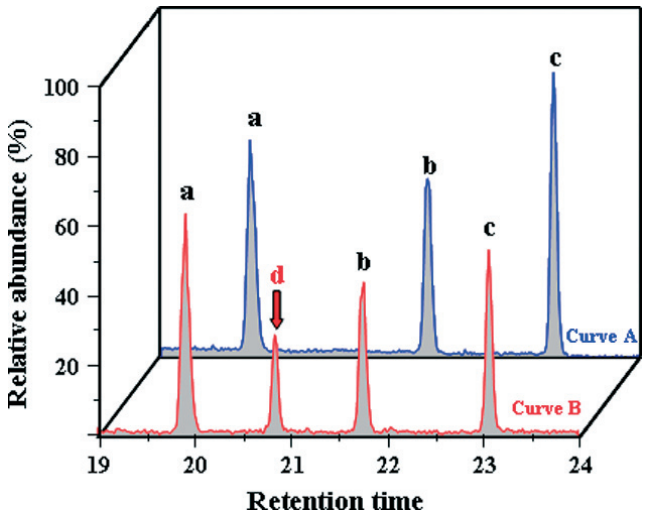


Figure 1. LC/MS chromatograms of (A) the control sample and (B) the oxidized sample exposed to UV/H_2O_2 oxidation for 15 min. Peak a, RPPGFSPYR; b, DRVYVHPF; c, FMRF; and d, FMRF + 16 Da.

UV/H_2O_2 Oxidation of Model Peptides

Immediately after mixing 200 µL (60 pmol/µL) DRVYVHPF, RPPGFSPYR and FMRF with 20 µL 1.5% H_2O_2, the samples were exposed to a 100 W UV lamp. After exposure to radiation for a certain period of time, the oxidation reaction was stopped by adding 340 µl stop solution (thiourea 100 mmol/l and Met 2 mmol/l). Stop solution was also added in to the control sample to prevent the post-oxidation in the processes of sample preparation and chromatographic separation. All samples were stored at 4 °C before LC/MS or MS/MS analysis.

LC/MS Analysis of Native and Oxidized Peptides

Chromatographic separations were performed on a HPLC (Waters 2695) and the mobile phases used were: (i) 90% acetonitrile and (ii) 10% acetonitrile. Both mobile phases contain 0.1% TFA. Samples (10 µl) were loaded onto a Great Eur-Asia C_{18} column (4.6 mm × 250 mm, 5 µm particle size, 120 Å) then eluted at a flow rate of 0.5 ml/min. Peptides were eluted using 100% B for 6 min, then mobile phase B was gradually reduced to 50% over 15 min. After a 5-min isocratic elution, mobile phases were rapidly switched to the initial conditions to equilibrate the column for 10 min. The column temperature was set at 30 °C.

LC/MS was performed on a micromass ZQ mass spectrometer (Waters) operated with the electrospray ionization (ESI) source in positive ion mode. The ESI source conditions were set as follows: capillary voltage 3.5 kV, sample cone voltage 55 V, extraction cone voltage 0.5 V, source temperature 110 °C, and cone gas (N_2) 30 l/h. The data were processed using MassLynx software, version 4.1.

MS/MS Analysis of Target Peptides

MS/MS were also obtained in positive ion mode by direct injection of samples into an LCQ Fleet mass spectrometer (ThermoFisher, USA) using a syringe pump at 5 µl/min. During the MS/MS scan, the collision energy was set at 40 eV and the MS/MS scan range was automatically adjusted according to the molecular weight of the parent ion.

Results and Discussion

LC/MS Analysis of the Native and Oxidized Peptides – Discovery of the Oxidative Products from Model Peptides

The chromatographic separation of model peptides and their oxidative products, coupled with the full scan mass spectrum of corresponding chromatography peaks, led to the identification of oxidative products of the model peptides.

It can be seen from Figure 1 (curve A) that the intact model peptides have three, well-separated characteristic peaks between 19 and 25 min, with out evident oxidation. The full scan mass spectra of these successively eluted peaks (Figure 2) verify that peaks a, b, and c correspond to peptides DRVYVHPF, RPPGFSPYR, and FMRF, respectively. For samples exposed to UV/H_2O_2 oxidation, a new peak appeared at 20.84 min, a decreased intensity peak was observed for FMRF, while DRVYVHPF and RPPGFSPYR remained unchanged (curve B, for 5 min oxidation). The new peak must be associated with the oxidation of FMRF. Comparing their MS results, peak d (*m/z* of 616 Da) has an increase of 16 Da over FMRF (*m/z* of 600 Da), indicating a difference in oxygen atom compared with that of FMRF.

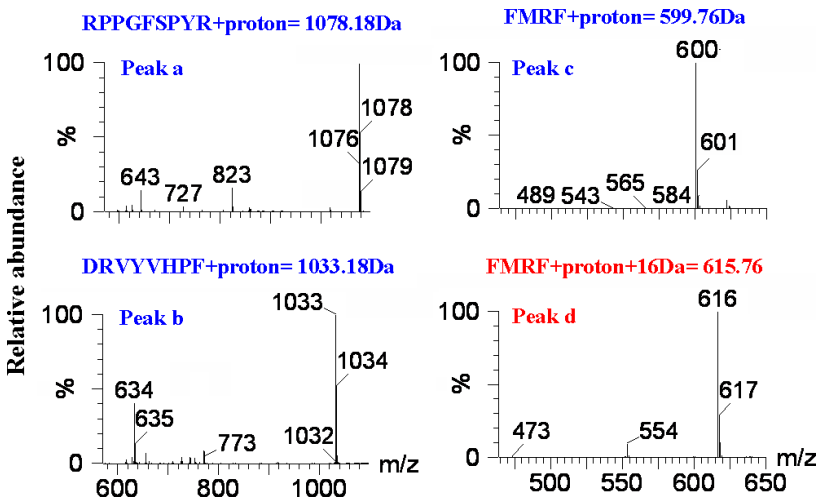


Figure 2. Full scan mass spectra of the corresponding chromatographic peaks in Figure 1 (see Figure 1 for assignments). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

We also found that the peptides DRVYVHPF and RPPGFSPYR could be oxidized by UV/H_2O_2, forming oxidation products with an added O atom. However, these oxidation products have lower abundance and are not separated from total ion chromatogram (TIC) peaks of the model peptides. The Met residue is more reactive to UV/H_2O_2 oxidation than the other amino acid residues in the model peptides [3,5]. A valid biomarker should appear in a sufficient amount to be easily detected and to have a highly efficient separation. Therefore, the oxidation product (with an added O atom) of FMRF was selected as a prospective biomarker for OS.

MS/MS Analysis of the Native and Oxidized Peptides – Identification of the Oxidation Site

Tandem mass spectrometry (MS/MS) is widely used to analyze peptide sequences by comparing the molecular weights of a set of interdependent fragment ions. This superior strategy can also be used to identify the oxidation sites of peptides. Comparing the MS/MS spectra of FMRF and its oxidation product (Figure 3), we find: a shift of +16 Da is detected for the b_2, b_3, and y_3 fragment ions, suggesting the oxidation site is located at the Met residue. The S atom in the Met residue is the main target site of OS (much more active than other atoms), and is always oxidized into a sulfoxide ($-CH_2SCH_3$ is oxidized into $-CH_2SOCH_3$) [3,19]. So FMRF is likely to be oxidized into FM(O)RF.

Under UV/H_2O_2 oxidation, H_2O_2, UV, and HO/HO_2·(decomposed from H_2O_2) can serve as the main oxidizing agents for the oxidation of FMRF [20,21]. Therefore, evaluating their contributions to FM(O)RF will clarify the oxidation mechanism of FMRF. When a mixture of the three model peptides is oxidized by H_2O_2 or UV alone, the corresponding TICs (Figure 4) also have a new peak near 20.84 min for FM(O)RF and a decreased peak for FMRF. Thus, both H_2O_2 and UV can oxidize FMRF. By comparing the oxidative content of FMRF produced by UV, H_2O_2, and UV/H_2O_2, the contribution of HO/HO_2·(decomposed from H_2O_2) can be identified. In the samples oxidized by UV, H_2O_2, and UV/H_2O_2 for 20 min, the degree of oxidation of FMRF by UV or H_2O_2 alone (3.7 and 16.4%) is much less than the 37.5% oxidation induced by the combination of UV/H_2O_2 (shown in Figure 5). The total amount oxidized by H_2O_2 and UV individually (20.1%) is also less than 37.5%. The difference (17.4%) should be attributed to the HO/HO_2·formed by irradiating H_2O_2 with UV light. Thus the oxidation of FMRF under UV/H_2O_2 oxidation can be achieved in a variety of ways (described in Scheme 1).

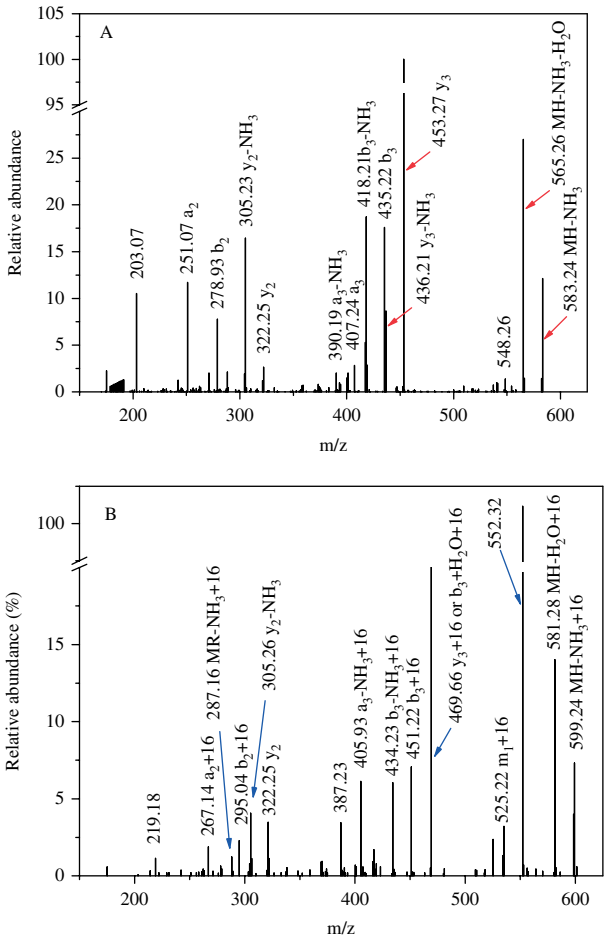


Figure 3. (A) MS/MS spectrum of the protonated FMRF at *m/z* 600.29 ± 0.5 Da. (B) MS/MS spectrum of the protonated FMRF + ^{16}O at *m/z* 616.29 ± 0.5 Da. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Quantitation of the Oxidized Peptide – Validation of the Proposed Biomarker Candidate

In LC/MS analysis, the relative amounts of analytes (peptides), usually expressed by their TIC peak areas, depend on their protonation efficiency [22,23]. The arginine residue in FMRF or FM(O)RF is the main site where the proton is located, but is not the main oxidative site. So UV/H_2O_2 oxidation has no significant

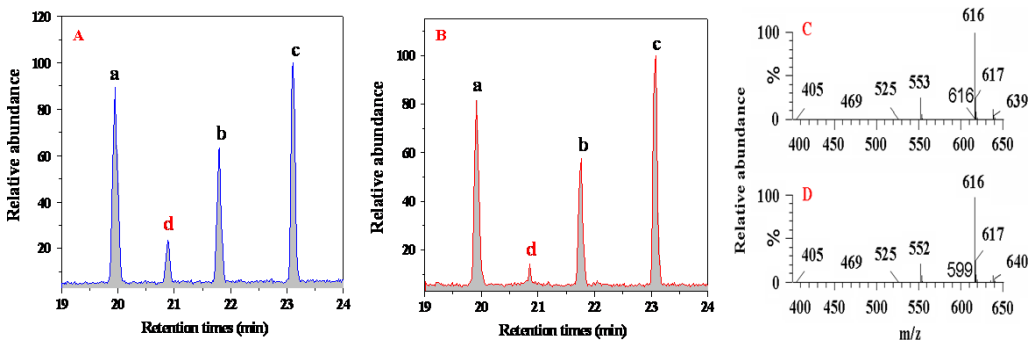


Figure 4. (A) LC/MS chromatogram of the oxidized sample exposed to H_2O_2 for 20 min. (B) LC/MS chromatogram of the oxidized sample exposed to UV light for 20 min. (C) Averaged full scan mass spectrum corresponding to peak 'd' in (A). (D) Averaged full scan mass spectrum corresponding to peak 'd' in Figure 1(B). This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Journal of PeptideScience

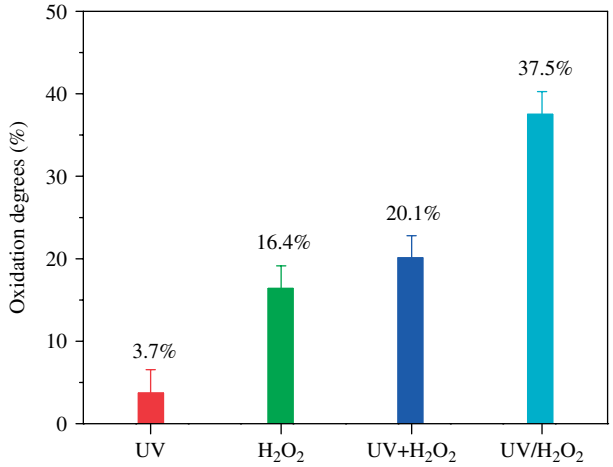
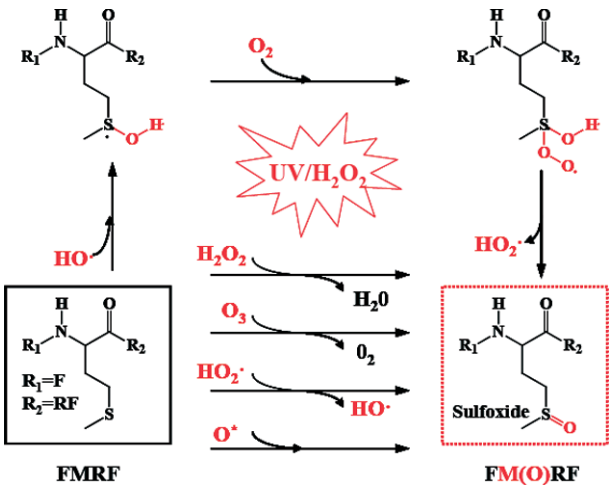


Figure 5. Oxidation degrees of FMRF induced by H_2O_2, UV and UV/H_2O_2 for 15 min.



Scheme 1. Oxidation mechanisms of FMRF induced by UV/H_2O_2.

influence on FMRF and FM(O)RF protonation and the oxidative content of FMRF can be expressed as $S_{FM(O)RF}/(S_{FM(O)RF} + S_{FMRF})$. The relationship between degree of oxidation and oxidation time is shown in Figure 6.

In general, the content of oxidized FMRF varies with the oxidation time. In the first 15 min, there is an excellent linear relation ($R = 0.9927$) between the degree of oxidation and oxidation time. However, due to the consumption of H_2O_2 by the oxidation reaction and UV photolysis, its concentration reduces and the oxidation rate of FMRF gradually slows down (corresponding to a gentle curve). The above results indicate that it is practicable to evaluate the oxidation content of FMRF by the ratio $S_{FM(O)RF}/(S_{FM(O)RF} + S_{FMRF})$.

Conclusions

Based on LC/MS and MS/MS, the oxidation mechanisms (site and extent) of FMRF, DRVYVHPF, and RPPGFSPYR under UV/H_2O_2 oxidization were explicitly clarified. Compared with the slightly oxidized DRVYVHPF and RPPGFSPYR, FMRF is susceptible to UV/H_2O_2 oxidization and undergoes oxygen addition forming

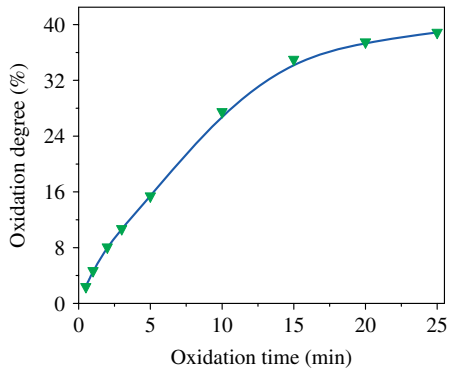


Figure 6. Dose–response curve for the oxidation degree of FMRF exposed to UV/H_2O_2 oxidation with the extended time.

a sulfoxide containing peptide FM(O)RF. The oxidized content of FMRF, expressed as $S_{FM(O)RF}/(S_{FM(O)RF} + S_{FMRF})$, is linearly correlated with oxidation time for the first 15 min. The rapid separation and accurate identification of FM(O)RF indicate that oxidized peptides can be treated as novel biomarkers to evaluate the sites and degrees of oxidation of peptides. By comparing the specific state of each model peptide, we also found that oxidative products of Met-containing peptides could be a suitable biomarker for OS. Coupling with enzymatic digestion, this technique can be further used to screen biomarkers for evaluating the oxidative mechanisms of proteins.

Acknowledgements

This work is supported by NSFC (20607011, 20875055), the Fok Ying Tong Education Foundation (111082), NCET-06-0582 and Foundation for Middle Young Scientists, and Key Science-Technology Project in Shandong Province (2007BS08005, 2008GG10006012) is also acknowledged. The authors thank Dr Pamela Holt for editing the manuscript for English.

References

1 Andreoli TE. Free radicals and oxidative stress. *Am. J. Med.* 2000; **108**: 650–651.
2 Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 2002; **408**: 239–247.
3 Davies MJ. The oxidative environment and protein damage. *Biochimica et Biophysica Acta* 2005; **17**: 93–109.
4 Seifried HE. Oxidative stress and antioxidants: a link to disease and prevention? *J. Nutr. Biochem.* 2007; **18**: 168–171.
5 Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* 1997; **272**: 20313–20316.
6 Facheris M, Beretta S, Ferrarese C. Peripheral markers of oxidative stress and excitotoxicity in neurodegenerative disorders: tools for diagnosis and therapy? *J. Alzheimers Dis.* 2004; **6**: 177–184.
7 Halling KB, Ellison GW, Armstrong D, Aoyagi K, Detrisac CJ, Graham JP, Newell SP, Martin FG, Van Gilder JM. Evaluation of oxidative stress markers for the early diagnosis of allograft rejection in feline renal allotransplant recipients with normal renal function. *Can. Vet. J.* 2004; **45**: 831–837.
8 Gedik CM, Boyle SP, Wood SG, Vaughan NJ, Collins AR. Oxidative stress in humans: validation of biomarkers of DNA damage. *Carcinogenesis* 2002; **23**: 1441–1446.
9 Donnea ID, Rossib R, Giustarinib D, Milzania A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* 2003; **329**: 23–38.
10 Blumberg J. Use of biomarkers of oxidative stress in research studies. *J. Nutr.* 2004; **134**: 3188–3189.

11 Starke PE, Oliver CN, Stadtman ER. Modification of hepatic proteins in rats exposed to high oxygen concentration. *FASEB J.* 1987; **1**: 36–39.
12 Poli G, Biasi F, Leonarduzzi G. 4-Hydroxynonenal-protein adducts: a reliable biomarker of lipid oxidation in liver diseases. *Mol. Aspects Med.* 2008; **29**: 67–71.
13 Bolin C, Pelaez FC. Assessing biomarkers of oxidative stress: analysis of guanosine and oxidized guanosine nucleotide triphosphates by high performance liquid chromatography with electrochemical detection. *J. Chromatogr. B* 2007; **856**: 121–130.
14 Hung A, Griffin MD, Howlett GJ, Yarovsky I. Effects of oxidation, pH and lipids on amyloidogenic peptide structure: implications for fibril formation? *Eur. Biophys. J.* 2008; **38**: 99–110.
15 Requena JR, Levine RL, Stadtman ER. Recent advances in the analysis of oxidized proteins. *Amino Acids* 2003; **25**: 221–226.
16 Maleknia SD, Brenowitz M, Chance MR. Millisecond radiolytic modification of peptides by synchrotron X-rays identified by mass spectrometry. *Anal. Chem.* 1999; **71**: 3965–3973.
17 Glass RS, Hug GL, Neich CS, Wilson GS, Kuznetsova L, Lee TM, Ammam M, Lorance E, Nauser T, Nichol GS, Yamamoto T. Neighboring amide participation in thioether oxidation: relevance to biological oxidation. *J. Am. Chem. Soc.* 2009; **131**: 13791–13805.
18 Peskin AV, Turner R, Maghzal GJ, Winterbourn CC, Kettle AJ. Oxidation of methionine to dehydromethionine by reactive halogen species generated by neutrophils. *Biochemistry* 2009; **48**: 10175–10182.
19 Xu G, Chance MR. Radiolytic modification of sulfur-containing amino acid residues in model peptides: fundamental studies for protein footprinting. *Anal. Chem.* 2005; **77**: 2437–2449.
20 Sarathy SR, Mohseni M. The impact of UV/H_2O_2 advanced oxidation on molecular size distribution of chromophoric natural organic matter. *Environ. Sci. Technol.* 2007; **41**: 8315–8320.
21 Oppenlander T, Gliese S. Mineralization of organic micropollutants (homologous alcohols and phenols) in water by vacuum-UV-oxidation (H_2O-VUV) with an incoherent xenon-excimer lamp at 172 nm. *Chemosphere* 2000; **40**: 15–21.
22 Mason DE, Liebler DC. Quantitative analysis of modified proteins by LC–MS/MS of peptides labeled with phenyl isocyanate. *J. Proteome Res.* 2003; **2**: 265–272.
23 Guan JQ, Almo SC, Chance MR. Synchrotron radiolysis and mass spectrometry: a new approach to research on the actin cytoskeleton. *Acc. Chem. Res.* 2004; **37**: 221–229.

