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Quantitative Analysis of amino acid oxidation markers by tandem mass spectrometry

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Abstract

Oxidative stress plays a central role in the pathogenesis of diverse chronic inflammatory disorders including diabetic complications, cardiovascular disease, aging, neurodegenerative disease, autoimmune disorders and pulmonary fibrosis. Protein misfolding can lead to chronic endoplasmic reticulum (ER) stress which can exacerbate oxidative stress. This can trigger apoptotic cascades resulting in chronic inflammatory disorders. Despite intense interest in origins and magnitude of oxidative stress, ability to quantify oxidants has been limited because they are short-lived. We have developed quantitative mass spectrometry (MS) based analytical strategies to analyze stable end-products of protein oxidation. These molecules provide quantitative and mechanistic assessment of degree of oxidative stress in cell cultures, tissues and biofluids of animal models of disease and human samples. Our studies support the hypothesis that unique reactive intermediates generated in localized microenvironments of vulnerable tissues promote end-organ damage. The ability to quantify these changes and assess response to therapies will be pivotal in understanding disease mechanisms and monitoring efficacy of therapy.

Keywords

Oxidative stress; oxidized amino acids; gas chromatography mass spectrometry; endoplasmic reticulum stress; electrospray ionization tandem mass spectrometry

1. Introduction

Oxygen forms the basis of aerobic life but is only sparingly reactive by itself. However, it is well-recognized that it can be activated by cellular metabolism to form reactive compounds termed as “reactive oxygen species” (ROS) which in turn can form a variety of intermediates including “reactive nitrogen species” (RNS). While physiological levels of such oxidants have a beneficial role in cellular signaling and host defense, excess oxidants can lead to pathological consequences.

Oxidative stress is due to the dysregulation in the synchronized balance between the oxidant generating systems and the antioxidant defense in quenching the excessive free radicals. Proteins, lipids and deoxyribonucleic acid (DNA) are the major targets of oxidative damage, eventually leading to the dysfunction of cellular physiology, as well as stimulating apoptosis. Alternatively, detrimental effects of ROS may be mediated by aberrant “redox signaling” in specific pathophysiological contexts. This latter effect may be attributed to disruption of the normal physiological roles of ROS in cell signaling (Thannickal, 2010).

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During the past two decades, considerable evidence has implicated oxidative stress in several distinct human disorders, including aging (Beckman and Ames, 1998; Muller *et al.*, 2007), atherosclerosis (Baynes and Thorpe, 2000; Pennathur and Heinecke, 2007), neurodegenerative diseases (Butterfield, 2006; Lin and Beal, 2006), diabetes (Baynes and Thorpe, 1999; Brownlee, 2001; Houstis *et al.*, 2006; Jay *et al.*, 2006; Vivekanandan-Giri *et al.*, 2008), pulmonary fibrosis (Hecker *et al.*, 2009) and end-stage renal disease (Cottone *et al.*, 2008; Percy *et al.*, 2005). A major focus of research has dwelt on the importance of ER stress and its link with oxidative stress. The unfolded protein response (UPR) is a signaling pathway that is triggered in response to protein misfolding in the ER. It has been noted that UPR is activated in response to oxidative stress as an adaptive mechanism (Malhotra and Kaufman, 2007) and antioxidants reduce ER stress (Back *et al.*, 2009; Malhotra *et al.*, 2008; Song *et al.*, 2008). Persistent ER stress and oxidative stress in the susceptible individual can lead to end-organ damage (Fig. 1). Oxidative stress has also been implicated in many chronic fibrotic diseases as well (Thannickal, 2010). Indeed, fibrosis is a major cause of organ failure and death accounting for roughly 45% of mortality in the United States population (Wynn, 2004). Therefore, understanding pathways of oxidative damage and designing specific and sensitive methods of detecting oxidized biomolecules is of critical importance. In this article, we will discuss our work in developing tandem mass spectrometric (MS/MS) methods to quantify specific amino acid oxidation markers that provide mechanistic information on oxidative pathways operative *in vivo*. We will outline sample preparation from cell cultures, tissue and other biospecimens, preparation of internal standards, liquid chromatography and MS conditions and parameters for quantitative analysis of amino acid oxidation markers.

2. Experimental Procedures

2.1 Isotope dilution MS is a highly sensitive and specific method for detecting oxidized biomolecules *in vivo*

ROS and RNS intermediates are difficult to detect *in vivo* because they are extremely short-lived due to their high reactivity with endogenous substrates; however, these oxidized substrates may serve as biomarkers for the activation of relevant oxidative stress pathways. Immunohistochemistry and dihydroethidium fluorescence have been extensively used to study oxidation-specific epitopes and oxidant production. These techniques are highly sensitive, and their ability to provide epitope-specific structural data can localize oxidative events to cell types or to subcellular locations. However, they are nonspecific as antibodies can bind to structurally similar compounds and, at best, only semiquantitative. The major drawback of high performance liquid chromatography (HPLC) based methods, is the appearance of co-eluting structurally similar compounds (Shigenaga *et al.*, 1997). Spectrophotometric or fluorometric detection of 3-nitrotyrosine can be confounded by contribution from tryptophan oxidation or other protein modifications (Guptasarma *et al.*, 1992). In contrast, MS offers a highly sensitive and specific approach to quantify oxidative biomarkers. When combined with isotope dilution, in which a labeled internal standard which is identical to the target analyte except for the heavy isotope is added to the mixture, accurate quantitation can be achieved to low femtomole to attomole levels. Addition of the internal standard also allows normalization for loss of analytes during sample preparation.

2.2 Oxidized amino acid content correlates with degree of oxidative stress *in vivo*

To understand the molecular mechanisms that promote oxidative stress *in vivo*, we first identified the patterns of oxidation products that are formed by well-characterized oxidant-generating model systems. We then characterized patterns of products in tissue and plasma derived from animal models of disease and human samples. Since lipid peroxidation products readily undergo subsequent chain-propagating reactions and lose their initial

oxidant imprint, we chose aromatic amino acids in proteins to study stable end-products of oxidation. Using a combination of free radical generating systems *in vitro* and studying biospecimens from animal models of disease and humans, we and others defined patterns of these oxidative markers that accurately indicate pathways of oxidation that are activated (Back *et al.*, 2009; Bergt *et al.*, 2004; Brennan and Hazen, 2003; Hazen *et al.*, 1997; Leeuwenburgh *et al.*, 1997a; Leeuwenburgh *et al.*, 1997b; Malhotra *et al.*, 2008; Okamura *et al.*, 2009; Pennathur *et al.*, 2005; Pennathur *et al.*, 1999; Pennathur *et al.*, 2001; Pop-Busui *et al.*, 2009; Shishehbor *et al.*, 2003; Shu *et al.*, 2009; Song *et al.*, 2008; Vincent *et al.*, 2009; Wiggin *et al.*, 2008; Zhang *et al.*, 2008; Zheng *et al.*, 2004). We have utilized this molecular fingerprinting strategy to identify tissue-specific pathways of oxidation *in vivo*. The phenylalanine residues in proteins when subjected to glycooxidation or hydroxyl radical damage forms *ortho*-tyrosine and *meta*-tyrosine. Oxidation reactions of tyrosine residues include dimerization (to form *o,o'*-dityrosine; mediated by tyrosine radicals, ROS and RNS), chlorination (to form 3-chlorotyrosine; catalyzed by myeloperoxidase) and nitration (to form 3-nitrotyrosine; mediated by RNS). Quantifying these unnatural amino acids characterizes the underlying oxidant pathway operative in target tissue. The overall scheme for analysis for oxidative amino acids is outlined in Fig. 2. The analytical strategy can be divided into four parts: 1) sample preparation, 2) separation of analytes 3) detection of analytes and data processing, 4) quantification of analytes.

2.3 Sample Preparation

Oxidized amino acids can be measured from proteins derived from a variety of different samples including tissue, biofluids (plasma, urine, cerebrospinal fluid), and cell culture. In general, plasma and urine measurements provide systemic levels of oxidant stress and tissue determinations provide local levels of oxidant stress. During procurement, it is important to ensure that samples are acquired in a sterile and uniform fashion. It is extremely important to prevent *ex-vivo* oxidation as it will confound interpretation. Sample preparation typically entails isolation of proteins, hydrolysis to liberate individual amino acids, sample clean-up with solid phase extraction and processing prior to further analysis. Processing the samples is a double-edged sword because each step will result in some degree of analyte loss. It is imperative to add internal standards prior to processing to account for these losses.

2.3.1 Buffers and solutions required for sample preparation—Antioxidant buffer (Buffer A): consists of 100 μ M diethylenetriaminepentaacetic acid (DTPA), 50 μ M butylated hydroxyl toluene (BHT) in 1% (v/v) ethanol, and 10 mM 3-amino-1,2,4-triazole in 50 mM sodium phosphate buffer, pH 7.4. DTPA is a metal-chelator and prevents metal-catalyzed oxidation reactions; BHT is a lipid soluble antioxidant; aminotriazole is a peroxidase inhibitor. Fresh antioxidant buffer is prepared prior at the start of the experiment.

Delipidation buffer (Buffer B) consists of water, methanol and water saturated ether in 1:3:7 (v/v/v) ratio.

2.3.2 Tissue collection and protein isolation—The animals are perfused with antioxidant Buffer A prior to harvesting the tissue samples. This step ensures the removal of red blood cells and prevents *ex-vivo* oxidation. The tissue samples are immediately stored in Buffer A in -80°C until analysis. Prior to the start analytical procedure, the tissue samples are thawed, minced and washed several times in aliquots of freshly prepared Buffer A. The samples are then homogenized with a hand tissue homogenizer. It is important to make sure that no residual tissue pieces are present. The tissue lysates, are once again sonicated using stainless steel probe sonicator (Omni ruptor-250, Omni International, Marietta, GA). The tissue lysate is centrifuged at 1000g for 10 minutes at 4°C . The supernatant containing soluble proteins is aliquoted and stored for further processing.

2.3.3. Protein isolation from cell cultures—Prior to harvesting attached cells, the plates are washed several times with Buffer A. Following treatment with trypsin, the cells are diluted with Buffer A and spun briefly for 5 minutes at 1000g. The supernatant is discarded and the cell pellet is overlaid with fresh Buffer A and then resuspended and the process is repeated two more times. Subsequently, the cell suspension is subjected to sonication using stainless steel probe sonicator. The cell lysate is centrifuged at 1000g for 10 minutes at 4°C. The supernatant containing soluble proteins is aliquoted and stored for further processing.

2.3.4. Protein precipitation, delipidation and hydrolysis of lysates derived from biological samples—All subsequent procedures are carried out at 4°C. Tissue or cell lysates, are taken in pyrolyzed hydrolysis vials and the volume is adjusted to 1ml with 50mM phosphate buffer, pH 7.4. For plasma, 5µl is diluted with 50mM phosphate buffer (pH 7.4) to a final volume of 1 mL in pyrolyzed hydrolysis vials. Subsequently the protein is precipitated by addition of trichloroacetic acid (10% final concentration). After centrifugation at 3000g for 10 minutes at 4°C, the protein precipitate is delipidated with Buffer B. This step ensures removal of lipids which can interfere with sample analysis. Following centrifugation, the lipid fraction is discarded. The protein pellet is dried in the fume hood under nitrogen to remove traces of the solvent. 4N Methanesulfonic acid pretreated with benzoic acid (10mg/ml) is added to the delipidated protein sample. Known concentration of isotope labeled internal standards of *ortho*-tyrosine and *meta*-tyrosine, *o,o'*-dityrosine, 3-chlorotyrosine, 3-nitrotyrosine, $^{13}\text{C}_6$ phenylalanine and $^{13}\text{C}_6$ tyrosine are spiked into the samples for absolute quantification of the modified amino acids and their precursors found in the biological samples. An alternate labeled precursor amino acid, [$^{13}\text{C}_9$, $^{15}\text{N}_1$]tyrosine, is added to monitor potential artifact formation which usually is a problem mainly for tyrosine halogenation, dimerization and nitration products. The samples are hydrolyzed at 100°C in sand bath for 24 hours.

2.3.5. Solid phase extraction of oxidized amino acids from biological samples—Chrom-P columns (ENVI-ChromP, 3 mL, Supelco., Bellefonte, PA) are activated with 100% methanol. Methanol is allowed to flow through gravity in the columns, to remove any trapped air bubbles. The column is preconditioned four times with 2ml of 0.1% trifluoroacetic acid (TFA) in water at pH 4. The acid hydrolysate is subjected to solid phase extraction and washed with 2ml of 0.1% TFA of pH 4. The amino acids bound to the column are eluted with 50% methanol. The eluent is collected in clean glass vial and dried at 60°C under vacuum. The dried samples are resolubilized in 50µl of the 0.1% TFA.

2.4. Preparation of authentic and isotope labeled standards

Isotope labeled L- $^{13}\text{C}_6$ phenylalanine and L- $^{13}\text{C}_6$ tyrosine are obtained from Cambridge Isotope Laboratories (Andover, MA) and are used to synthesize all isotopically labeled standards. The overall synthetic strategy is outlined in Fig. 3. Isotopically labeled *ortho*-tyrosine and *meta*-tyrosine were synthesized using [$^{13}\text{C}_6$] phenylalanine, copper, and hydrogen peroxide (H_2O_2) as described previously (Huggins *et al.*, 1992). *o,o'*- $^{13}\text{C}_{12}$ dityrosine was prepared with - [$^{13}\text{C}_6$] tyrosine, horseradish peroxidase (HRP), and H_2O_2 as described previously (Leeuwenburgh *et al.*, 1997b; Pennathur *et al.*, 2005; Pennathur *et al.*, 1999). L-3-Chloro $^{13}\text{C}_6$ tyrosine was synthesized by reacting L- $^{13}\text{C}_6$ tyrosine with an equimolar concentration of hypochlorous acid (HOCl) in the presence of 100 mM Cl^- in 20 mM phosphate buffer (pH 4.0). Reactions were carried out for 60 min at 37°C. They were initiated by adding HOCl and terminated with 0.1 mM methionine (Gaut *et al.*, 2002). Excess of tetranitromethane (TNM) was used to synthesize [$^{13}\text{C}_6$] 3-nitrotyrosine from 2mM L- $^{13}\text{C}_6$ tyrosine in 50mM Tris buffer (pH 8.0). The mixture was vortexed for 1 hour at room temperature (Gaut *et al.*, 2002). The resulting amino acids were isolated using solid-

phase extraction with a 3-mL C18 column (Supelco Inc, Bellefonte, PA). Concentrations of amino acids were determined by comparison with authentic standards using HPLC and monitoring of A_{276} . Further confirmation is obtained by verifying characteristic MS spectra by electrospray ionization tandem mass spectrometry (ESI/MS/MS)

2.5 Separation and detection of analytes

The two main separation platforms used are either gas chromatography (GC) or liquid chromatography (LC) coupled with MS.

2.5.1. Gas Chromatography/Mass Spectrometry (GC/MS)—GC/MS is a commonly used platform for measuring oxidized amino acids. As separation in GC occurs in an oven at high temperatures, analytes need to be volatile and thermally stable and it is therefore necessary to derivatize samples prior to analysis. The amino acids are converted to either heptafluorobutyl derivatives or trimethylsilyl derivatives prior to analysis. The analytes entering the source in GC/MS are ionized by electron impact (EI) or chemical ionization (CI) and the mass analyzer is usually a single quadrupole. While necessary, it is important to keep in mind that derivatization is additional sample processing that can result in analyte loss and artifact formation and this is one of the major drawbacks of GC/MS. Additionally, GC/MS has limited mass range and therefore not suitable for larger molecular weight compounds such as *o,o'*-dityrosine. For these reasons, we no longer employ GC/MS for routine analysis of oxidized amino acids.

2.5.2 Liquid Chromatography/Mass Spectrometry (LC/MS)—Biomedical mass spectrometry has recently been revolutionized by the advent of ionization methods that permit introduction of large, complex biomolecules into the mass spectrometer in liquid solutions. ESI is perhaps the most powerful of the new ionization methods for coupling LC with MS. In ESI/MS, large, complex, ionic molecules are introduced into the ion source as liquid solutions as a fine spray of charged droplets. This permits direct MS analysis of intact, simple and complex analytes. Since high temperatures and need for volatility are not involved, sample derivatization is not needed.

The triple Quadrupole (QQQ) is a versatile mass analyzer. The basic subunit consists of 4 parallel metal rods – hence the term quadrupole. A radiofrequency voltage is applied to the rods and only ions within a particular mass to charge ratio (m/z) range will be able to traverse through the quadrupole to the detector. Ions outside the defined m/z range spiral out of control and are destroyed when they hit a rod. A QQQ combines 3 quadrupoles in series and allows MS/MS to be performed. In MS/MS, the first quadrupole is used to scan across a preset m/z range and select a user-defined parent ion of interest which is then fragmented in the second quadrupole by colliding with an inert gas such as nitrogen through the process of collision-induced dissociation (CID). The fragment daughter ions are sorted in the third quadrupole for analysis. Several functional groups (e.g. H_2O , $COOH$, etc) characteristically fragment during CID, and thus analysis of the daughter ions can yield structural information about the parent ion. In addition, as compounds have unique fragmentation patterns, QQQ with multiple reaction monitoring (MRM) can be used to experimentally confirm potential analytes. QQQ is very sensitive and provides the most precise quantitation in the MRM mode and therefore ideally suited for analysis of oxidized amino acids.

3. Results

3.1 LC/MS/MS detection of oxidized amino acids using authentic standards

Oxidized amino acids are quantified by LC/ESI/MS/MS in the MRM mode. MS experiments were performed using an Agilent 6410 Triple Quadrupole mass spectrometer

coupled with an Agilent 1200 HPLC system (Agilent Technologies, New Castle, DE), equipped with a multimode source. C-18 column from Agilent with 1.8 μ m particle size and 4.6 $\times$ 50mm dimension is used for the HPLC separation. The amino acid hydrolysate is subjected to reverse phase separation utilizing the following parameters. The mobile phase is 0.1% formic acid (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The column was first equilibrated with 100% Solvent A. The gradient for the HPLC run is as follows: isocratic 2% acetonitrile for 0.5 to 3 minutes, and then a linear gradient of 10% to 95% acetonitrile, for 3 to 7 minutes with a flow rate of 0.6ml/min. Under these chromatography conditions, authentic compounds and isotopically labeled standards exhibited retention times identical to those of analytes derived from tissue samples. The limit of detection (signal/noise >10) was < 100 fmol for all of the amino acids.

Positive LC/ESI/tandem MS was performed using following parameters: capillary spray voltage 4000 V, drying gas flow 11 L/min, drying gas temperature 350°C and nebulizer pressure 45 psi. Flow injection analysis was used to optimize the fragmentor voltage. Optimal fragmentor voltage for each amino acid in MS2 scan mode was obtained. Mass range between m/z 100 and m/z 400 was scanned to obtain full scan mass spectra.

Figure 4 depicts the MS/MS spectrum from *ortho*-tyrosine (Panel A), *o,o'*-dityrosine (Panel B), 3-chlorotyrosine (Panel C) and 3-nitrotyrosine (Panel D). *meta*-tyrosine has an identical MS/MS spectrum as *ortho*-tyrosine but can be distinguished due to differences in retention time (Table 1). The molecular ion $[M + H]^+$ of *ortho*-tyrosine, *o,o'*-dityrosine, 3-chlorotyrosine and 3-nitrotyrosine are m/z 182, 361, 216 and 227 respectively. The neutral loss of m/z 46 is the most important product ion for all the oxidized amino acids. This yields an intense ion at m/z 136, 315, 170 and 181 respectively. These transitions form ideal candidates for MRM analysis.

3.2. Quantitation of oxidized amino acids by MRM analysis

The amino acids are quantified using isotope dilution LC/MS/MS in the MRM mode. The retention times and the MRM transitions utilized for quantification of the authentic compounds, internal standards and artifact monitoring analytes are outlined in Table 1. Extracted ion chromatograms are derived from the MRM transitions are shown in Fig. 5. The ion at m/z 136 and its respective isotopically labeled internal standard ion at m/z 142 were used to quantify *ortho*-tyrosine and *meta*-tyrosine. *o,o'*-dityrosine is analyzed using the m/z 315 ion and its isotopically labeled internal standard ion at m/z 327. 3-Chlorotyrosine is quantified using ion at 170 m/z and its respective isotopically labeled internal standard ion at m/z 176. 3-Nitrotyrosine is quantified using the ion at m/z 181 and its respective isotopically labeled internal standard ion at m/z 187. To quantify phenylalanine and tyrosine (the precursor amino acids), the sample was diluted 1 in 1000 and analyzed in a separate injection as these precursor amino acids are typically present 1000 to 10,000 fold excess to their oxidized counterparts. Phenylalanine, the precursor amino acid for *ortho*-tyrosine and *meta*-tyrosine, was quantified using the ion at m/z 126 and the isotopically labeled internal standard ion at m/z 132. The ion at m/z 136 and the isotopically labeled internal standard ion at m/z 142 were used to quantify tyrosine, the precursor amino acid for *o,o'*-dityrosine, 3-chlorotyrosine and 3-nitrotyrosine. We constructed an external calibration curve that used each amino acid as a standard and the corresponding isotopically labeled amino acid as an internal standard. The ratio of ion currents for each amino acid divided by that of the internal standard was a linear function of unlabeled amino acid for all ranges over which the amino acids were measured. The limit of detection (signal/noise >10) was <100 fmol for all the amino acids. Potential artifact during sample work-up is usually an issue with *o,o'*-dityrosine, 3-chlorotyrosine and 3-nitrotyrosine determination. Therefore, we routinely add L-[$^{13}\text{C}_9$, ^{15}N]tyrosine, a isotopically distinct tyrosine. We monitor artifact formation by analyzing appearance of m/z 334 (dimerization: *o,o'*-dityrosine artifact), m/z 179

(chlorination artifact), and m/z 190 (nitration artifact). The amount of artifact is then subtracted from the measured oxidized amino acid to correct for this issue. We do not routinely monitor for artifact formation for *ortho*-tyrosine and *meta*-tyrosine. If this is an issue, an isotopically distinct phenylalanine can be added to monitor this possibility. Finally, we normalize the oxidation markers to the precursor amino acids so that changes in tissue volume, amount of protein or extraction efficiency are accounted for.

4. Conclusions

Many lines of evidence implicate oxidative stress in chronic diseases. However, accurate measurement of oxidant generation *in vivo* is challenging. The power of tandem mass spectrometry offers a unique opportunity to quantify low levels of oxidized biomolecules from complex bio specimens. We have utilized this approach to precisely assess oxidative stress in biological samples ranging from cell cultures, biofluids and tissue from animal models of disease and humans. This analytical approach thus has a broad applicability to study a variety of disorders in which oxidative stress plays a causal role. More studies are needed to evaluate the utility of these markers to predict disease progression and to determine efficacy of potential interventions.

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Abbreviations

BHT	butylated hydroxytoulene
CI	chemical ionization
CID	collision-induced dissociation
DNA	deoxyribonucleic acid
DTPA	diethylenetriaminepentaacetic acid
EI	electron impact ionization, ESI, electrospray ionization
ER	endoplasmic reticulum
GC/MS	gas chromatography/mass spectrometry
HPLC	high performance liquid chromatography
LC/MS	liquid chromatography/mass spectrometry
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
m/z	mass to charge ratio
RNS	reactive nitrogen species
ROS	reactive oxygen species
QQQ	triple quadrupole
HRP	horse radish peroxidase

TNM	tetranitromethane
TFA	trifluoroacetic acid
UPR	unfolded protein response

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Figure 1. Roles of oxidative stress and endoplasmic reticulum (ER) stress in chronic disease
Interaction between environmental and genetic factors, promote ER and oxidative stress. Eventual damage to proteins, lipids and DNA can lead to end-organ damage and chronic disease.

Figure 2. Workflow for tandem mass spectrometry based oxidized amino acid analysis

Biological samples are processed and internal standards are added. Subsequently the sample is subjected to either gas (GC) or liquid (LC) chromatography separation. The eluent is ionized by one of several modes of ionization such as EI, CI, or ESI. Following ionization, the resultant mass spectra are derived from the mass analyzers and the analytes of interest are identified. EI, electron impact ionization; CI, chemical ionization; ESI, electrospray ionization; MS/MS, tandem mass spectrometry;

Figure 3. Structures and synthesis of oxidized amino acids

Oxidized amino acids are synthesized from the precursors as described in experimental procedures. H₂O₂, hydrogen peroxide; HOCl, hypochlorous acid; HRP, horse radish peroxidase; TNM, tetranitromethane

Figure 4. Liquid chromatography electrospray ionization tandem mass spectrometry of oxidized amino acids

The samples were separated by reverse phase HPLC and subjected to ESI/MS as described in methods. MS/MS spectra reveal $[M + H]^+$ of *ortho*-tyrosine (Panel A), *o,o'*-dityrosine (Panel B), 3-chlorotyrosine (Panel C) and 3-nitrotyrosine (Panel D) are m/z 182, 361, 216 and 227. The neutral loss of m/z 46 is the most intense product ion for all the oxidized amino acids.

Figure 5. Quantification of oxidized amino acids in the multiple reaction monitoring mode utilizing extracted ion chromatograms

The samples were separated by reverse phase HPLC and subjected to ESI/MS as described in methods. Extracted ion chromatograms were derived from the MRM transitions for *ortho*-tyrosine (Panel A), *o,o'*-dityrosine (Panel B), 3-chlorotyrosine (Panel C) and 3-nitrotyrosine (Panel D) as shown figure. The ratio of ion currents for each amino acid compared with the internal standard (depicted by *) was utilized to quantify the levels of the oxidized amino acids. Note co-elution of authentic oxidized amino acid to the corresponding isotopically labeled internal standard.

Table 1
LC/ESI/MS/MS analysis of oxidized amino acids

The samples were separated by reverse phase HPLC and subjected to ESI/MS as described in Methods. The amino acid analytes, ion transitions utilized for MRM analysis and the retention time are summarized.

Amino acid	Ion Transition	Retention Time
$^{13}\text{C}_6$ <i>ortho</i> -tyrosine	188 → 142	3.55
$^{12}\text{C}_6$ <i>ortho</i> -tyrosine	182 → 136	3.55
$^{13}\text{C}_6$ <i>meta</i> -tyrosine	188 → 142	2.95
$^{12}\text{C}_6$ <i>meta</i> -tyrosine	182 → 136	2.95
$^{13}\text{C}_6$ <i>o,o'</i> -dityrosine	373 → 327	2.25
$^{12}\text{C}_6$ <i>o,o'</i> -dityrosine	361 → 315	2.25
$^{13}\text{C}_6$ chlorotyrosine	222 → 176	4.22
$^{12}\text{C}_6$ chlorotyrosine	216 → 170	4.22
$^{13}\text{C}_6$ nitrotyrosine	233 → 187	4.88
$^{12}\text{C}_6$ nitrotyrosine	227 → 181	4.88
$^{13}\text{C}_9^{15}\text{N}_1$ <i>o,o'</i> -dityrosine (dimerization artifact)	381 → 334	2.25
$^{13}\text{C}_9^{15}\text{N}_1$ chlorotyrosine (chlorination artifact)	226 → 179	4.22
$^{13}\text{C}_9^{15}\text{N}_1$ nitrotyrosine (nitration artifact)	237 → 190	4.88
$^{13}\text{C}_6$ phenylalanine	172 → 126	3.75
$^{12}\text{C}_6$ phenylalanine	166 → 120	3.75
$^{13}\text{C}_6$ tyrosine	188 → 142	1.90
$^{12}\text{C}_6$ tyrosine	182 → 136	1.90
$^{13}\text{C}_9^{15}\text{N}_1$ tyrosine	192 → 145	1.90