

Strategies for comprehensive analysis of amino acid biomarkers of oxidative stress

Review Article

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Summary. Despite the wide interest in using modified amino acids as putative biomarkers of oxidative stress, many issues remain as to their overall reliability for early detection and diagnosis of diseases. In contrast to conventional single biomarker studies, comprehensive analysis of biomarkers offers an unbiased strategy for global assessment of modified amino acid metabolism due to reactive oxygen and nitrogen species. This review examines recent analytical techniques amenable for analysis of modified amino acids in biological samples reported during 2003–2007. Particular attention is devoted to the need for validated methods applicable to high-throughput analysis of multiple amino acid biomarkers, as well as consideration of sample pretreatment protocols on artifact formation for improved clinical relevance.

Keywords: Oxidative stress – Modified amino acids – Analytical techniques – Comprehensive analysis – Multiple biomarkers

Introduction

Oxidative stress has long been hypothesized as an underlying mechanism associated in normal ageing processes, as well as implicated in the etiology of many diseases (Mak and Newton, 2001; Markesbery, 1997). Since reactive oxygen and nitrogen species (e.g., hydroxyl radical, peroxynitrite) are intrinsic by-products of aerobic metabolism and immune defense, living organisms have developed natural mechanisms for neutralizing reactive intermediates and repairing oxidative damage while maintaining cellular redox balance. Oxidative damage is attributed to deficiencies in enzymatic or non-enzymatic antioxidant cellular defenses coupled with increased exposure to reactive oxygen/nitrogen species (i.e., ROS/RNS) derived from endogenous and exogenous sources (Valko et al.,

2007). Chronic oxidative stress can induce irreversible changes in normal cellular metabolism, which has been implicated in the pathogenesis of cardiovascular (Brennan and Hazen, 2003; Ceriello and Motz, 2004) and neurodegenerative disorders (Markesbery and Lovell, 2006; Sultana et al., 2006).

The identification, quantification and validation of putative biomarkers indicative of oxidative stress remain an on-going challenge in analytical chemistry. An ideal biomarker represents a stable product of oxidative/nitrosative stress, which is present at low but quantifiable concentrations in cells or biofluids from healthy patients (Dalle-Donne et al., 2006). Moreover, a biomarker that is indicative of a specific disorder and useful for early detection, progression or intervention of disease is highly desirable in a clinical setting. Numerous biomarkers of lipid, fatty acid, DNA and protein oxidative/nitrosative damage have been examined, including F₂-isoprostanes (Basu, 2004), modified nucleosides (Orhan et al., 2004), aldehyde protein adducts (Carini et al., 2004) and carbonylated proteins (Dalle-Donne et al., 2003), respectively. In general, biomarkers derived from protein modifications have been most extensively reported as they represent the major biopolymer constituent and functional component of cells. In this case, analytical methods are required to quantify modified amino acids present in intact protein (Shishehbor et al., 2003a). This is important for understanding of the specificity of amino acid oxidation and its impact on protein conformational stability, activity

or function. Alternatively, analysis is performed by comparison of the relative amounts of free and total modified amino acids present before and after protein hydrolysis, respectively (Mita et al., 2004). This option is often used as a general strategy to assess oxidative stress status for global changes in amino acid metabolism. However, the clinical significance of such an analysis will ultimately depend on the type of sample (e.g., cell, plasma, urine), requirement of sample pretreatment (e.g., proteolysis, chemical derivatization), performance of analytical technique (e.g., selectivity, sensitivity) and method to normalize measured amino acid concentration levels among different biological samples (e.g., total protein mass, unmodified amino acid). These factors are relevant to ensure reliable quantification of low levels of modified amino acids in complex and variable samples.

Oxidation/nitrosation processes occur primarily with reactive amino acid side chains within protein or free amino acid pools. The mechanism and relative reactivity of specific amino acids with different ROS/RNS have been discussed in detail in recent reviews (Alvarez and Radi, 2003; Davies, 2005; Marnett et al., 2003; Stadtman and Levine, 2003). In general, most ROS/RNS are electrophilic species and thus undergo preferential reactions with electron-rich amino acids, including Tyr, Phe, Trp, His, Cys and Met. Indeed, these reactive amino acid classes (e.g., thiols, indoles, etc.) also serve as functional antioxidants present at physiologically relevant concentrations in biofluids or cells with activity comparable to classic antioxidants, such as ascorbic acid (Herraiz and Galisteo, 2004; Meucci and Mele, 1994). There is increased recognition that oxidative damage to proteins may contribute to the development and/or progression of various diseases as a result of protein misfolding, aggregation and inactivation (Dalle-Donne et al., 2006; Valko et al., 2007). However, a direct causal relationship between a specific diseased state and ROS/RNS has yet to be conclusively demonstrated in vivo. This is primarily due to the lack of a suitable biomarker whose concentration change can be directly correlated to disease within a heterogeneous population. The difficulty in establishing such a link is only compounded by the complexity of human diseases and the analytical challenges facing current biomarker analysis. To date, the majority of studies have focused on the analysis of a *single* modified amino acid as a biomarker for oxidative stress. New strategies are urgently needed for simultaneous analysis of multiple amino acid biomarkers as a way to enhance specificity, accuracy and reliability when assessing dysregulated metabolism in a clinical setting.

Extensive reviews of redox proteomics related to amino acid modifications have described the current challenges in assessing biomarkers of oxidative stress (Dalle-Donne et al., 2005). The aim of this review is to provide an overview of analytical techniques suitable for modified amino acid analysis reported during 2003–early 2007. Particular attention is devoted to strategies that permit comprehensive and high-throughput analysis of amino acid biomarkers associated with oxidative/nitrosative stress with minimal sample pretreatment. Similar to emerging metabolomic initiatives (Ryan and Robards, 2006), unbiased analysis of global amino acid metabolism in biological samples involving known and unidentified biomarkers of oxidative stress offers a promising framework for future studies.

Amino acid side chain oxidation/nitrosation

Tyr is one the most reactive targets involved in oxidative stress generating several clinically relevant modified amino acid biomarkers. Nitration of tyrosine to produce 3-nitrotyrosine (3-NO₂-Tyr) represents the most widely quantified amino acid modification. Indeed, 3-NO₂-Tyr has been utilized as a putative biomarker for a diverse spectrum of chronic disorders, ranging from asthma, cardiovascular disease, cystic fibrosis, diabetes to Parkinson's disease (Table 1). 3-NO₂-Tyr may be generated by different reactive pathways and oxidants, including peroxynitrite (ONOO⁻), myeloperoxidase (MPO) and other metalloproteins (Dalle-Donne et al., 2005; Duncan, 2003; Mohiuddin et al., 2006; Pietraforte et al., 2003). Once formed in vivo, 3-NO₂-Tyr is metabolized to 3-nitro-4-hydrophenylacetate (NHPA) prior to urinary excretion. However, NHPA is not a useful indicator of 3-NO₂-Tyr formation as it is also produced by in vivo nitration of excess *p*-hydroxyphenylacetic acid (Halliwell and Whiteman, 2004) or via increased dietary nitrate intake (Vliet, 2006). Although 3-NO₂-Tyr formation has been linked to protein modification, it has also been detected in apparently healthy individuals. This provides evidence that low level tyrosine nitration may not only be involved in pathological processes due to RNS, but may also play an endogenous role in cell signal transduction pathways (Dalle-Donne et al., 2005).

3,3'-Dityrosine (Di-Tyr) is a Tyr oxidation product produced by coupling of two Tyr radicals and represents a selective biomarker of oxidatively-modified proteins in vivo. Hydroxyl radicals (OH[•]), ONOO⁻, UV/γ-irradiation, MPO/H₂O₂-Tyr systems and other oxidant stressors may all result in Di-Tyr production (Brennan and Hazen,

Table 1. Summary of quantitative analyses for oxidative/nitrosative stress reported using a *single* modified amino acid biomarker

Analyte	Disease state	Free or protein bound	Sample type	Sample pretreatment	Analytical technique	Reference
3-Cl-Tyr	Atherosclerosis, coronary artery disease	Protein bound	Human aortic tissue	Acid hydrolysis, SPE, TBDMS derivatization	GC-NICI-MS/MS	Bergt et al. (2004)
3-Cl-Tyr	Low birth weight, lung/bacterial infection	Protein bound	Human endotracheal aspirate	Acid hydrolysis, SPE, Boron trifluoride/ <i>N</i> -propanol + TFA/ethyl acetate derivatization	GC-NICI-MS	Buss et al. (2003)
Di-Tyr	–	Free	Human urine	None	LC-APCI-MS/MS	Orhan et al. (2004)
Di-Tyr	Hyperlipidemia	Free	Human plasma	None	FL λ_{ex} (nm): 325 λ_{em} (nm): 400	Chien et al. (2004)
Di-Tyr	Obstructive sleep apnea	Free	Human urine	SPE	LC-FL λ_{ex} (nm): 288 λ_{em} (nm): 430	Jordan et al. (2006)
L-DOPA	–	Free	–	None	Voltammetry with Au nanoparticle	Raj et al. (2003)
L-DOPA	–	Free	–	None	Cyclic voltammetry	Fang et al. (2005)
L-DOPA	–	Free	–	None	Cyclic voltammetry	Milczarek and Ciszewski (2004)
L-DOPA	–	Free	–	None	Cyclic voltammetry	Zhang et al. (2005)
L-DOPA	–	Free	–	None	CE-ECD with diamond micro-electrode	Shin et al. (2003)
L-DOPA	–	Free	–	None	ECD with Au/albumin/polyphenol oxidase doped electrodes	Miscoria et al. (2005)
L-DOPA	–	Free	–	None	ECD with LiMn ₂ O ₄	Leu and Lin (2006)
L-DOPA	Chronic hemodialysis	Protein bound	Human plasma	Delipidate	FL λ_{ex} (nm): 430 λ_{em} (nm): 512	Sutherland et al. (2003)
L-DOPA	Chronic hemodialysis	Protein bound	Human plasma	Acid hydrolysis	LC-FL λ_{ex} (nm): 280 λ_{em} (nm): 320	Sutherland et al. (2003)
MetSOx	Hyperglycemia	Free	Rat plasma	SPE, TBDMS derivatization	GC-MS	Mashima et al. (2003)
NFK	Alzheimer's disease	Free	Human cerebral spinal fluid	None	LC-FL λ_{ex} (nm): 330 λ_{em} (nm): 437	Ahmed et al. (2006)
NFK	Diabetes	Free	Human plasma, urine	None	LC-FL λ_{ex} (nm): 330 λ_{em} (nm): 437	Ahmed et al. (2005)
NHPA	–	Free	Rat urine	Organic extraction, TLC, PFB derivatization	GC-NICI-MS	Mani et al. (2003)
NHPA	–	Free	Human urine	SPE, Heptafluorobutyl/TBDMS derivatization	GC-NICI-MS	Pannala et al. (2006)
3-NO ₂ -Tyr	–	Free	Human liver microdialysate	None	LC-ECD	Richards et al. (2006)
3-NO ₂ -Tyr	–	Free	Human exhaled breath condensate	SPE	LC-ESI-MS/MS	Goen et al. (2006)
3-NO ₂ -Tyr	–	Free	Human plasma	Heptafluorobutyric amide/TBDMS derivatization	GC-NICI-MS	Pannala et al. (2003)

(continued)

Table 1 (continued)

Analyte	Disease state	Free or protein bound	Sample type	Sample pretreatment	Analytical technique	Reference
3-NO ₂ -Tyr	–	Free	Human plasma	SPE, Heptafluorobutyryl/TBDMs derivatization	GC–NICI–MS	Pannala et al. (2006)
3-NO ₂ -Tyr	–	Protein bound	Human plasma	Base hydrolysis, SPE, Heptafluorobutyric amide/TBDMs derivatization	GC–NICI–MS	Pannala et al. (2003)
3-NO ₂ -Tyr	–	Free	Human plasma	Heptafluorobutyric acid/trimethyl diazomethane derivatization	GC–NICI–MS/MS	Soderling et al. (2003)
3-NO ₂ -Tyr	Asthma	Free	Human exhaled breath condensate	SPE, Heptafluorobutyric anhydride/TMS derivatization	GC–NICI–MS/MS	Larstad et al. (2005)
3-NO ₂ -Tyr	Asthma, cystic fibrosis	Free	Human exhaled breath condensate	Heptafluorobutyric anhydride/trimethyl diazomethane derivatization	GC–NICI–MS/MS	Celio et al. (2006)
3-NO ₂ -Tyr	Asthma, cystic fibrosis	Free	Human exhaled breath condensate	None	LC–ECD	Celio et al. (2006)
3-NO ₂ -Tyr	Asthma	Free	Human exhaled breath condensate	SPE, Butanol/acetyl chloride derivatization	LC–MS/MS	Baraldi et al. (2006)
3-NO ₂ -Tyr	Coronary artery disease	Protein bound	Human plasma	Desalt and delipidate, Acid hydrolysis, SPE	LC–ESI–MS/MS	Shishohor et al. (2003b)
3-NO ₂ -Tyr	Diabetes	Free	Human plasma	None	LC–UV λ_{abs} (nm): 280, 335	Wang et al. (2004)
3-NO ₂ -Tyr	Diabetes	Free	Rat urine	None	CE–UV λ_{abs} (nm): 214	Maeso et al. (2004)
3-NO ₂ -Tyr	Diabetes	Protein bound	Human plasma	None	ELISA λ_{abs} (nm): 450	Bo et al. (2005)
3-NO ₂ -Tyr	Eales' disease	Protein bound	Human blood monocytes	Acid hydrolysis	LC–UV λ_{abs} (nm): 365	Rajesh et al. (2003)
3-NO ₂ -Tyr	Hypercholesterolemia	Free, protein bound	Human plasma	None	ELISA Chemiluminescence	Pereira et al. (2004)
3-NO ₂ -Tyr	Neonatal chronic lung disease	Free, protein bound	Plasma lung tissue	Desalt and delipidate, Acid hydrolysis, SPE	LC–ESI–MS/MS	Munson et al. (2005)
3-NO ₂ -Tyr	Obstructive sleep apnea	Free	Human plasma	SPE	LC–MS/MS	Svatikova et al. (2004)
3-NO ₂ -Tyr	Parkinson's disease	Free	Human cerebral spinal fluid	None	LC–ECD	Isobe et al. (2006)
3-NO ₂ -Tyr	Peripheral vascular disease	Protein bound	Human plasma	None	ELISA	DaRos et al. (2003)
3-NO ₂ -Tyr	Systemic lupus erythematosus	Free	Rabbit sera	None	LC–UV	Khan and Ali (2006)
5-OH-Trp	–	Free	Rat serum	None	LC–ESI–MS	Koppiseti et al. (2005)
5-OH-Trp	–	Free	Human plasma	SPE	CE–ESI–MS	Peterson et al. (2004)
5-OH-oxindole	–	Free	Mammalian sera, rat tissues	SPE	LC–ECD	Papy-Garcia et al. (2003)
5-OH-indole-acetic acid	Chronic brain injury, Huntington's disease	Free	Human plasma	Organic extraction	LC–FL λ_{ex} (nm): 280	Christofides et al. (2006)
ortho-Tyr	Chronic renal failure, Diabetes	Free	Human plasma, urine	None	λ_{em} (nm): 345 LC–FL λ_{ex} (nm): 275 λ_{em} (nm): 305	Molnar et al. (2005b)

2003; Giulivi et al., 2003). Indeed, Di-Tyr formation within oxidatively-modified intracellular protein is considered an endogenous marker for selective proteolysis (Giulivi and Davies, 1993). The reaction mechanism and formation rates of Di-Tyr have been recently reviewed (Malencik and Anderson, 2003). Since the 3-3' chemical bond of Di-Tyr is resistant to hydrolysis once liberated from degraded protein, it is excreted in nearly quantitative yields in urine (Dalle-Donne et al., 2005). Halogenated Tyr metabolites, such as 3-chlorotyrosine (3-Cl-Tyr) and 3-bromotyrosine (3-Br-Tyr), have also been proposed as putative oxidative stress biomarkers of human disease (Mohiuddin et al., 2006). 3-Cl-Tyr and 3-Br-Tyr are produced from the reaction with hypochlorous acid (HOCl) and hypobromous acid (HOBr), respectively, although a defined mechanism remains unclear (Hawkins et al., 2003). 3-Cl-Tyr is considered a specific biomarker of MPO-catalyzed halogenation (Brennan and Hazen, 2003). The in vivo metabolism of these halogenated biomarkers is currently unknown although detoxification by dehalogenase enzymes and glutathione-S-transferase has been proposed (Dalle-Donne et al., 2006).

Another important oxidized Tyr biomarker is 3,4-dihydroxytyrosine (*L*-DOPA), which may be produced in vivo by radical oxidation and/or via enzymatic transformation by tyrosine hydroxylase during catecholamine biosynthesis (Molnar et al., 2005a). A variety of other oxidized Tyr derivatives including dopamine, dopamine quinone, 5,6-hydroxyindol, 5,6-hydroxy-3-oxoindol have also been examined (Giulivi et al., 2003). The quantification of these metabolites is particularly relevant in Parkinson's disease patients receiving Levodopa (*L*-DOPA) therapy. Long-term treatments with *L*-DOPA have been demonstrated to induce neurotoxicity in patients receiving this therapy since it can undergo autooxidation to generate ROSs, as well as be incorporated as a Tyr substitute during protein biosynthesis (Valko et al., 2007). Thus, dietary and pharmaceutical intervention can play important roles in modulating oxidative stress associated with amino acid metabolism and protein biosynthesis.

The electron-rich aromatic amino acids, Phe and Trp are also important targets for ROS/RNS. Phe can undergo oxidation in vivo to form two major positional isomers of Tyr, namely *ortho*- and *meta*-Tyr. These stable Tyr isomers have been used as an indirect measure of the extent of OH[•] radical formation (Molnar et al., 2005a) which have been associated with ageing, as well as different chronic diseases (Gurer-Orhan et al., 2006). Several in vivo and in vitro oxidized/nitrosated Trp metabolites have also been quantified as described in recent reviews

(Dalle-Donne et al., 2005; Yamakura and Ikeda, 2006). In vitro nitrosation of Trp residues in proteins by ONOO⁻ and peroxidase/H₂O₂/nitrate resulted in the production of 1-, 4-, 5-, 6- and 7-NO₂-Trp isomers (Yamakura and Ikeda, 2006). The highest yielding isomer, 6-NO₂-Trp, is stable to acid hydrolysis and represents a potential biomarker for in vivo studies, although its biological role remains unclear (Yamakura and Ikeda, 2006). In addition, oxidants including HOCl and UV radiation may oxidize Trp to kynurenine (KYN) and *N*-formyl-*L*-kynurenine (NFK) (Hawkins et al., 2003). The KYN metabolic pathway has important implications in neurological disorders, including Parkinson's and Alzheimer's disease (Mackay et al., 2006; Stoy et al., 2005). Quantification of hydroxylated Trp metabolites 5-hydroxy-tryptophan (5-OH-Trp) and 5-hydroxy-indoleacetic acid can provide insight into the efficacy of Trp loading diets as treatments for neurological disorders, such as Huntington's disease (Christofides et al., 2006).

Met protein residues are also readily modified to produce two major oxidized species, methionine sulfoxide (MetSO_x) and methionine sulfone (MetSO_n). The mechanism, rates of formation and diseases associated with elevated levels of these products has been reviewed elsewhere (Davies, 2005; Schoneich, 2005). Met oxidation to MetSO_x is reversible since methionine sulfoxide reductase A and B (MsrA/MsrB) selectively reduces free MSO_x. (Galeva et al., 2005). The reversible nature of this oxidation has led researchers to postulate that Met oxidation acts as a redox switch involved in protein function and repair (Davies, 2005; Marnett et al., 2003). Ageing processes have recently been reviewed in this context (Friguet, 2006). Much attention is currently focused on identifying the specific sites of Met oxidation in proteins using MS based proteomic assays (Choi et al., 2006). In contrast, few publications have focused on quantifying the relative levels of free and protein bound Met oxidation products. The conflicting opinions on the usefulness of measuring modified Met biomarkers for oxidative stress (Davies, 2005; Halliwell and Whiteman, 2004) may be partially responsible for this trend. Table 1 summarizes major modified amino acids that have reported as putative biomarkers of oxidative stress in association with different disease from 2003 to early 2007, which also highlights the selection of analytical technique, sample type and sample pretreatment. Interestingly, only about 30% of these studies have utilized more than one single modified amino acid as a biomarker for clinical investigation with far fewer using more than three biomarkers, which are summarized separately in Table 2.

Table 2. Summary of quantitative analyses for oxidative/nitrosative stress using *multiple* modified amino acid biomarkers

Analyte	Disease state	Free or protein bound	Sample type	Sample pretreatment	Analytical technique	Reference
3-Br-Tyr	Asthma	Free	Human urine	SPE, Heptafluorobutyl derivatization	GC-NICI-MS	Mita et al. (2004)
3-Cl-Tyr	Churg-Strauss syndrome	Free	Human urine	SPE, Heptafluoro-TBDMs derivatization	GC-NICI-MS/MS	Higashi et al. (2004)
3-Br-Tyr	Asthma	Protein bound	Human airway epithelial cells	MnSOD isolation, Desalt and Delipidate, Acid hydrolysis, SPE	LC-ESI-MS/MS	Comhair et al. (2005)
3-Cl-Tyr						
Di-Tyr						
<i>meta</i> -Tyr						
3-NO ₂ -Tyr						
<i>ortho</i> -Tyr						
3-Cl-Tyr	Atherosclerosis	Protein bound	Human plasma	Lipid extraction, Acid hydrolysis	LC-ECD	Yamaguchi et al. (2005)
3-NO ₂ -Tyr						
3-Cl-Tyr	Atherosclerosis	Protein bound	Rat serum	Lipid extraction, Acid hydrolysis	LC-ECD	Yamaguchi et al. (2006)
3-NO ₂ -Tyr						
3-Cl-Tyr	Cardiovascular disease	Protein bound	Aortic atherosclerotic lesion, human serum	Acid hydrolysis, SPE	LC-ESI-MS/MS	Zheng et al. (2004)
3-NO ₂ -Tyr						
3-Cl-Tyr	Alzheimer's disease	Protein bound	Human brain tissue	Acid hydrolysis, SPE, <i>N</i> -propyl heptafluorobutyl derivatization	GC-NIEC-MS	Green et al. (2004)
Di-Tyr						
<i>ortho</i> -Tyr						
Di-Tyr	–	Free	Cat urine	SPE, <i>N</i> -butyl derivatization	LC-ESI-MS/MS	Marvin et al. (2003)
3-NO ₂ -Tyr						
Di-Tyr	Atherosclerosis	Protein bound	Human carotid artery plaques	Acid hydrolysis, SPE, Ozonolone derivatization	GC-NICI-MS/MS	Morton et al. (2003)
3-NO ₂ -Tyr						
Di-Tyr	Atherosclerosis	Protein bound	Human plasma	Acid hydrolysis, SPE, <i>N</i> -propyl perheptafluorobutyl derivatization	GC-NICI-MS	Shishchikov et al. (2003a)
<i>ortho</i> -Tyr						
3-NO ₂ -Tyr	Alzheimer's disease	Free	Human cerebrospinal fluid	None	LC-ESI-MS/MS	Ahmed et al. (2006)
Di-Tyr						
MetSOx						
3-NO ₂ -Tyr	Diabetes	Free	Human plasma, urine	None	LC-ESI-MS/MS	Ahmed et al. (2005)
Di-Tyr						
3-NO ₂ -Tyr	Renal failure	Free	Human plasma	None	LC-ESI-MS/MS	Thornalley et al. (2003)
MetSOx						
Di-Tyr						
3-NO ₂ -Tyr	Diabetes	Protein bound	Rat frontal cortex retina tissue	Acid hydrolysis, SPE, <i>N</i> -propyl heptafluorobutyl derivatization	GC-NICI-MS/MS	Pennathur et al. (2005)
Di-Tyr						
<i>meta</i> -Tyr						
<i>ortho</i> -Tyr						
3-NO ₂ -Tyr						
L-DOPA	Parkinson's disease	Protein bound	Human plasma	Acid hydrolysis	LC-UV/FL/ECD	Rodgers et al. (2006)
<i>meta</i> -Tyr					λ_{ex} (nm): 280 λ_{em} (nm): 320	

L-DOPA	–	Free	Human plasma, urine	None	LC-ESI-MS/MS	Piraud et al. (2005)
5-OH-KYN						
L-DOPA	Diabetes	Protein bound	Human cataractous lenses	Acid hydrolysis	LC-FL λ_{ex} (nm): 275 λ_{em} (nm): 305	Molnar et al. (2005a)
meta-Tyr						
ortho-Tyr	–	Free	Human saliva	None	LC-FL λ_{ex} (nm): 275 λ_{em} (nm): 305	Chen et al. (2006)
meta-Tyr						
ortho-Tyr	Global ischemia	Free	Rat cardiac effluent	None	LC-ECD	Biondi et al. (2006)
meta-Tyr						
ortho-Tyr	Hypoxic ischemia encephalopathy	Free	Human cerebrospinal fluid	N-propyl heptafluoryl derivatization	GC-NICI-MS	Ogihara et al. (2003)

In vivo protein biosynthesis with oxidized amino acids

Free circulating levels of modified amino acids are likely the result of protein oxidation and degradation with subsequent release of stable oxidized metabolites. However, there is growing evidence that free oxidized amino acids may also be incorporated into newly synthesized proteins in place of their unmodified precursors. Acid hydrolysis and LC-MS/MS analysis of Chinese hamster ovary cells incubated with *meta*-[^{14}C]-Tyr in the presence of physiological concentrations of Phe and Tyr demonstrated modified amino acid uptake and incorporation into cellular protein (Gurer-Orhan et al., 2006). Ozawa et al. showed that *E. coli* bacterial growth on a DOPA-rich, Tyr-free medium had over a 90% uptake of DOPA at each Tyr site based on MS and ^{15}N -HSQ-NMR characterization (Ozawa et al., 2005). In a rigorous study, Rodgers et al. (2006) demonstrated *L*-DOPA derived protein biosynthesis in Parkinson's disease patients. Blood was separated into erythrocyte, lymphocyte and protein fractions and analyzed for *L*-DOPA content. The lymphocyte fraction showed elevated levels of *L*-DOPA being incorporated into protein. In contrast, erythrocytes which cannot synthesize new protein showed no appreciable increase in *L*-DOPA content. The concentration of *meta*-Tyr, a biomarker used to assess oxidative damage, remained constant between diseased and healthy patients in all fractions. This work presented the first direct evidence of *L*-DOPA protein biosynthesis in Parkinson's disease patients receiving this therapy (Rodgers et al., 2006). Together, these studies offer an alternative explanation for generation of protein-bound modified amino acids as opposed to direct modification of reactive amino acids within susceptible long-lived protein exposed to ROS/RNS. Further studies are needed to better distinguish the source and fate of modified amino acid metabolites in cells.

Quantitative analysis of oxidized/nitrosated amino acids

The challenges in performing reliable analysis of different classes of oxidized/nitrosated amino acids are immense. It has been suggested that determination of oxidative protein modifications are an order of magnitude more difficult than analyzing DNA damage (Halliwell and Whiteman, 2004). In general, analytical techniques require excellent sensitivity and selectivity for quantifying nanomolar levels of modified amino acids in complex biological samples. This often necessitates off-line sample pretreatment steps prior to analysis to enhance concen-

tration sensitivity while minimizing interferences, such as solid-phase extraction (SPE) and chemical derivatization. Several studies have demonstrated that bias is a major concern since 3-NO₂-Tyr, 3-Cl-Tyr and 3-Br-Tyr may be generated as artifacts during protein acid hydrolysis and/or chemical derivatization steps (Dalle-Donne et al., 2006; Halliwell and Whiteman, 2004). Removing minor amounts of oxidizing or nitrating agents (e.g., NO₂⁻) by desalting prior to hydrolysis (Baraldi et al., 2006) or employing alkaline conditions to hydrolyze protein can minimize this effect (Pannala et al., 2003). This latter precaution apparently has not been adopted in many studies as summarized in Table 1. Also, protein digests performed under anaerobic conditions in the gas phase using mercaptoacetic acid and hydrochloric acid (Rodgers et al., 2006) has also been reported to prevent undesired modifications. Moreover, the types of equipment used to perform acid hydrolysis can also impact the extent of nitration with Eppendorf[®] more suitable than borosilicate or polypropylene tubes in this regard (Morton et al., 2003). Recently, it has been reported that repeated freezing and thawing samples containing phosphate or nitrate can also contribute to Tyr nitration (Halliwell and Whiteman, 2004). Thus, all sources of artifacts must be considered when validating an analytical method to reliably quantify amino acid biomarkers of oxidative stress.

Rigorous assessment of artifact generation during sample handling can readily be assessed by using deuterated amino acid analogues with MS characterization. Baraldi et al. used both 3-NO₂-[¹³C₆]-Tyr and d₃-Tyr as internal standards in their LC-MS analysis of 3-NO₂Tyr in asthmatic and healthy children (Baraldi et al., 2006). The use of an isotopomer as internal standard allowed for 3-NO₂-Tyr artifacts to be assessed by monitoring the d₃-3-NO₂-Tyr signal, while 3-NO₂-[¹³C₆]-Tyr was used to improve method precision. Alternatively, conversion of 3-NO₂-Tyr to *N*-acetyl-3-amino-Tyr prior to acid hydrolysis allows endogenous 3-NO₂-Tyr to be selectively extracted from complex matrices (Duncan, 2003). In a GC-negative-ion chemical ionization (NICI)-MS analysis of 3-NO₂-Tyr in human plasma, Soderling et al. (2003) employed a complex sample pretreatment protocol where 3-NO₂-Tyr was initially reduced with dithionite to 3-amino-Tyr and then derivatized with heptafluorobutyric acid and trimethylsilyldiazomethane to produce the corresponding di-*O*-methyl-di-*N*-fluorobutyryl Tyr derivative. In vitro generation of 3-NO₂-Tyr during sample handling/derivatization resulted in the formation of a di-*O*-methylmono-*N*-fluorobutyryl Tyr derivative. GC-NICI-MS analysis of the two different heptafluorobutyric acid/trimethylsilyldiazomethane adducts permitted selective identification of both in vivo and in vitro

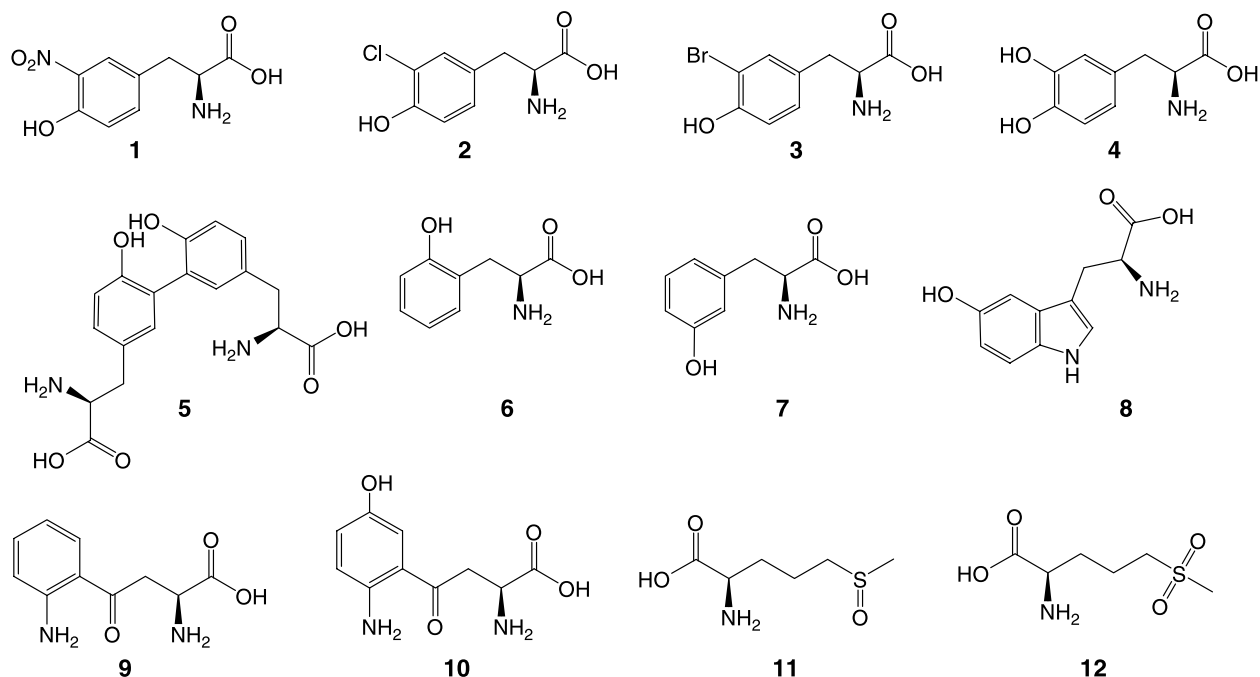


Fig. 1. Chemical structures of some major modified amino acids used as biomarkers of oxidative stress: **1**, 3-NO₂-Tyr; **2**, 3-Cl-Tyr; **3**, 3-Br-Tyr; **4**, *L*-DOPA; **5**, Di-Tyr; **6**, *ortho*-Tyr; **7**, *meta*-Tyr; **8**, 5-OH-Trp; **9**, KYN; **10**, 5-OH-KYN; **11**, MetSO_x and **12**, MetSO_a

production of 3-NO₂-Tyr, while also improving overall detector response.

Analysis of multiple amino acid biomarkers of oxidative stress

To date, there have been numerous reports and reviews of methods used for targeted analysis of single biomarkers based on modified amino acids, (Brennan and Hazen, 2003; Giulivi et al., 2003; Hawkins et al., 2003; Malencik and Anderson, 2003; Pietraforte et al., 2003; Stadtman and Levine, 2003) as summarized in Table 1. However, with the emergence of metabolomics (Ryan and Robards, 2006) as a new paradigm in disease prognosis and drug development, analytical techniques amenable to comprehensive analysis of modified amino acid metabolism are needed. In general, hyphenated separation techniques coupled to MS offer unsurpassed selectivity for multiple modified amino acid analysis relative to detection methods based on electrochemical, UV absorbance and fluorescence (Giulivi et al., 2003; Malencik and Anderson, 2003). Immunoassay techniques based on ELISA (Troxler et al., 2004) offer excellent sensitivity and selectivity for trace analysis, but are difficult to independently validate due to changes in the heterogeneity and cross-reactivity of antibodies derived from different sources. A review of different analytical techniques suitable for the analysis of *multiple* amino acid biomarkers of oxidative stress is discussed below. Methods that offer rapid and high-throughput analysis of small amounts of sample with minimal off-line sample pretreatment are highly desirable in clinical applications.

GC-MS

GC-MS is one of the most widely reported techniques for modified amino acid analysis due to its high separation efficiency, extensive MS database and long history of reliable and validated use. However, the polar nature and low volatility of amino acids requires their chemical derivatization prior to separation. Perfluorinated and silylated derivatization agents are routinely employed to overcome these limitations, but often require high temperatures and long reaction times that are not compatible with thermally-labile species. Moreover, chemical derivatization can also result in incomplete or multiple labeling of amino acids, which complicates MS interpretation. The problem of artifact generation of oxidized amino acids during sample pretreatment is also a significant concern. The quantification of free and protein-bound 3-Cl-Tyr

and 3-Br-Tyr in acid hydrolysates of urine and plasma of asthmatic patients using GC-NICI-MS (Mita et al., 2004) highlights these issues. The metabolites were initially converted to their heptafluorobutyryl derivatives, extracted in ethyl acetate and further derivatized to their corresponding *tert*-butyldimethylsilyl derivatives prior to NICI with methane gas. After extensive sample pretreatment steps, the technique possessed a 30-fold linear dynamic range (1.2–36 ng · mL⁻¹) with no apparent artifact formation of 3-Cl-Tyr or 3-Br-Tyr. The same technique was successfully applied to quantify urinary free 3-Cl-Tyr and 3-Br-Tyr in patients afflicted with Churg-Strauss syndrome (Higashi et al., 2004). The *n*-propyl heptafluorobutyryl derivatives of free *meta*- and *ortho*-Tyr in the cerebrospinal fluid of newborn infants with hypoxic ischemic encephalopathy was also reported by isotope dilution GC-NICI-MS using a similar sample preparation procedure (Ogihara et al., 2003). Selected ion monitoring (SIM) at 417.1 and 595.1 Da allowed both singly and doubly labeled *n*-propyl heptafluorobutyryl derivatives of each Tyr isomer to be quantified (Ogihara et al., 2003). These studies demonstrate that GC-NICI-MS in the SIM mode can be used to quantify specific isomeric biomarkers with low detection limits.

Shishehbor et al. (2003b) quantified protein-bound levels of 3-Cl-Tyr, Di-Tyr and *ortho*-Tyr by GC-NICI-MS after conversion of metabolites to their *n*-propyl, perheptafluorobutyryl derivatives. Gas-phase methane sulfonic acid hydrolysis of the precipitated protein and isotopic [¹³C₆]- and [¹³C₁₂]-internal standards was used to generate and validate free oxidized amino acids. Potential interfering species were removed from acid hydrolysate by solid-phase extraction prior to analyte derivatization. Pennathur et al. employed a similar sample preparation for analysis *ortho*-Tyr, *meta*-Tyr, 3-NO₂-Tyr and Di-Tyr in proteins derived from the retina tissue of hyperglycemic rats (Pennathur et al., 2005). The formation of 3-Cl-[¹²C₉, ¹⁵N]-Tyr, *ortho*-[¹²C₉, ¹⁵N]-Tyr and Di-[¹²C₁₈, ¹⁵N₂]-Tyr was found to be negligible during sample preparation and analyte labeling (Shishehbor et al., 2003b). The inclusion of multiple internal standard isotopomers is particularly necessary for GC-MS analyses of hydrolyzed proteins as discussed previously. The application of GC-MS with negative-ion electron capture (NIEC) detection with perfluorinated derivatization agents can increase method sensitivity as illustrated in the simultaneous analysis of 3-Cl-Tyr, Di-Tyr and *ortho*-Tyr derived from the hippocampus of Alzheimer's patients brains (Green et al., 2004). After tissue preparation and acid hydrolysis, amino acids

were extracted using anion-exchange chromatography and converted to their *n*-propyl, perheptafluorobutyl derivatives. A LOD of 1 fmol for 3-Cl-Tyr was reported for the optimized GC–NIEC–MS method (Green et al., 2004).

LC based methods

Unlike GC, reverse-phase (Ahmed et al., 2005) and anion-exchange (Yamaguchi et al., 2005) LC allows tuning of the selectivity of the separation by varying the composition of the mobile phase. Moreover, chemical derivatization of amino acids is not required, which allows their rapid analysis using different detector formats, including native fluorescence (Chen et al., 2006), ECD (Yamaguchi et al., 2006) and ESI-MS (Marvin et al., 2003).

LC–FL

Tyrosine and tryptophan metabolites possess significant intrinsic fluorescence properties when using UV excitation. However, high efficiency separation of minor modified amino acids in complex samples is critical to reduce interference of co-eluting species when using native FL detection. The analysis of free urinary *meta*- and *ortho*-Tyr metabolites derived from diabetes mellitus and renal failure patients highlights this problem (Molnar et al., 2005a). For instance, *meta*-Tyr was not quantified in samples due to co-elution with an unknown metabolite and/or presence at concentration levels below the limit of detection (LOD) of the method. In a similar study,

meta-, *ortho*-Tyr and *L*-DOPA were analyzed by LC–FL from acid hydrolysates of protein derived from cataract lenses of diabetic mellitus (DM) and non-DM patients. The isocratic LC separation employed did not permit baseline resolution of all sample components. After correcting for total protein content, the reported *meta*-Tyr concentration was approximately 14-times lower than *ortho*-Tyr in DM patients (Molnar et al., 2005a). Chen et al. used LC–FL to determine free *meta*- and *ortho*-Tyr in the saliva of subjects who chewed two major types of *Areca quid* (Chen et al., 2006). Adequate resolution achieved in this study allowed for nanomolar concentration levels to be determined in saliva. It is important to note that in vitro and in vivo oxidative processes can influence the relative amounts of each Tyr isomer derived from Phe oxidation. Nearly equimolar concentrations of *meta*- and *ortho*-Tyr are produced in vitro while more than a 10-fold difference has been reported after in vivo oxidation (Davies, 2005; Halliwell and Whiteman, 2004; Molnar et al., 2005a). These studies illustrate the need for careful experimental design with particular attention to chromatographic conditions used to separate and quantify multiple amino acid biomarkers by LC–FL.

LC–ECD

LC coupled to electrochemical detection (ECD) offers good selectivity and sensitivity for the determination of oxidized/nitrosated amino acid biomarkers. However, the

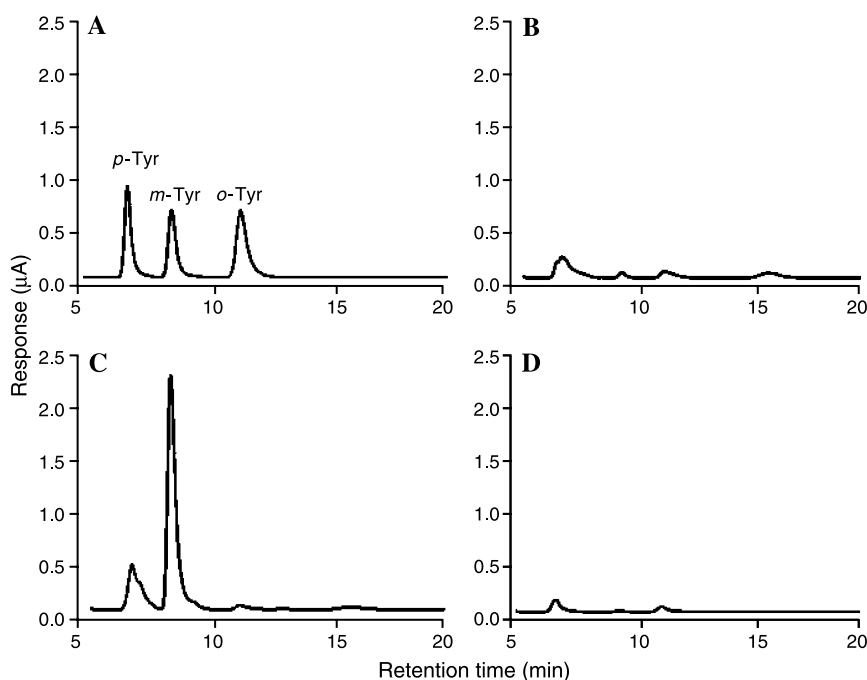


Fig. 2. Representative LC–ECD chromatograms of *meta*-, *ortho*- and *para*-Tyr as **A** 1 μM standard solutions, **B** rat cardiac effluent prior to the induction of 30 min total global ischemia; **C** 40 s after ischemic reperfusion and **D** 5 min after ischemic reperfusion. Reprinted from Biondi et al. (2006), Hydroxylation of D-phenylalanine as a novel approach to detect hydroxyl radicals: application to cardiac pathophysiology. Cardiovasc Res 71: 322–330; with permission from the European Society of Cardiology

use of ECD requires careful control of detection conditions. 3-NO₂-Tyr and 3-Cl-Tyr have been determined in low-density lipoproteins (LDL) from plasma after acid hydrolysis using anion-exchange LC with ECD detection (Yamaguchi et al., 2005, 2006). Accurate quantification of elevated levels of these biomarkers, along with other non-protein oxidative biomarkers, allowed the researchers to conclude that oxidative and nitrosative stress was associated with disease progression. The selective hydroxylation of Phe was investigated in the cardiac effluent of rats before and after total global ischemia by LC-ECD (Biondi et al., 2006). The reported detection limits under 10 fmol for *meta*- and *ortho*-Tyr allowed researchers to demonstrate that Phe hydroxylation can be utilized to assess oxidative stress triggered by the onset of cardiac ischemia, as depicted in Fig. 2. However, care must be taken to ensure the assay is free from interference of co-eluting metabolites. Dysregulated Trp metabolism via the kynurenine pathway in patients with chronic brain injury and Huntington's disease has also been examined by analysis of 3-hydroxytryptophan, xanthurenic acid and 3-hydroxyanthranilic acid by LC-ECD (Forrest et al., 2004; Mackay et al., 2006; Stoy et al., 2005). These studies demonstrated the relevance of the oxidative kynurenine metabolism and the role of its toxic metabolites in progression of various neurological disorders.

LC-MS

LC-MS offers distinct advantages compared to photometric and electrochemical detection formats in terms of selectivity required for oxidative/nitrosative amino acid biomarker analyses in complex biological samples. LC coupled with ion-trap (IT) (Munson et al., 2005), triple-quadrupole (TQ) (Baraldi et al., 2006) and time-of-flight (TOF) (Marvin et al., 2003) mass analyzers have been reported primarily when using ESI interfaces. Each mass analyzer offers MS/MS capabilities with low detection limits when operated in selective ion and/or multiple reaction monitoring (SIM/MRM) modes. Baseline resolution is only required for analytes possessing very similar MS or MS/MS properties, such as isobaric and isomeric species. The inclusion of isotopically labeled amino acids allows in vitro production of oxidized/nitrosated amino acids during sample handling to be accurately quantified using isotope-dilution. The advantages of LC-MS/MS for targeted analysis of oxidative stress biomarkers, including oxidatively modified free and protein-derived amino acids has been recently reviewed (Watson et al., 2003).

Off-line sample preparation is often reduced using LC-MS/MS as chemical labeling is often not required. However, LC-MS analysis of multiple modified amino acids from complex samples is challenging due to high background noise and ion-suppression. To reduce these deleterious effects, chemical labeling may be employed prior to chromatography. Marvin et al. developed an LC-ESI-MS/MS technique with isotope dilution in MRM mode for the quantitation of 3-NO₂-Tyr and Di-Tyr in cat urine (Marvin et al., 2003). To circumvent the high background noise with the urine sample, 3-NO₂-Tyr was butylated prior to analysis, resulting in a 6-fold increase in 3-NO₂-Tyr MS response. However, Di-Tyr was analyzed in its native state as doubly- and triply-butylated derivatives resulting in a lower MS response. The limit of quantitation (LOQ) for 3-NO₂-Tyr and Di-Tyr derived from protein acid hydrolysates were reported as 14.5 and 140 nM, respectively. The authors compared data derived from both Q-TOF and TQ mass spectrometers and showed the Q-TOF was advantageous in terms of its increased mass accuracy, reduced ion suppression and ability to perform full scan product ion mode analyses. Thornalley et al. (2003) quantified multiple advance glycation endproducts (AGEs), including 3-NO₂-Tyr and MetSO_x, in rats with induced diabetes and healthy humans by LC-ESI-MS/MS using a TQ mass analyzer operated in MRM mode. The free metabolites were analyzed in their native form and as *N*-acetyl conjugates in human plasma and urine, respectively. A gradient elution with two reverse phase columns in series was used to aid in the separation of the more hydrophobic AGE metabolites prior to ESI ionization. The extensive chromatography and inclusion of isotopically labeled standards allowed LODs of 0.022 and 0.15 pmol and recoveries of 88 and 102% to be realized for 3-NO₂-Tyr and MetSO_x, respectively. This same technique was used for the selective determination of free MetSO_x, NO₂-Tyr and Di-Tyr in the plasma of diabetic (Ahmed et al., 2005) and Alzheimer's (Ahmed et al., 2006) patients. Figure 3 illustrates the use of isotopically labeled internal standards in these studies for peak identification and assessment of artifact nitration during sample preparation.

An attractive alternative to off-line chemical labeling for background matrix reduction is the use of solid-phase extraction (SPE) prior to LC-MS/MS analysis. SPE simultaneously preconcentrates the oxidized/nitrosated amino acids of interest while reducing the extent of in vitro modification during labeling. This protocol was used in the quantification of protein-bound 3-NO₂-Tyr, 3-Cl-Tyr, 3-Br-Tyr, Di-Tyr, *ortho*-Tyr and *meta*-Tyr in

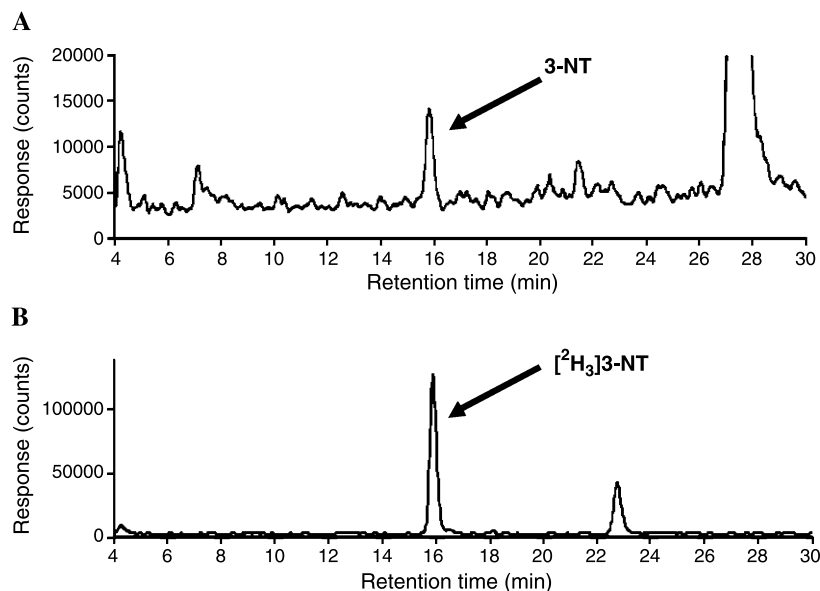


Fig. 3. Representative LC-MS chromatograms demonstrating the use of isotopically labeled internal standards for peak identification, where (A) is enzymatic CSF protein hydrolysate and (B) with 5 pmol 3-NO₂-[²H₃]-Tyr internal standard added. Reprinted from Ahmed et al. (2006), Protein glycation, oxidation and nitration adduct residues and free adducts of cerebrospinal fluid in Alzheimer's disease and link to cognitive impairment. *J Neuro Chem*, 92: 255–263; with permission from Blackwell Publishing Ltd

superoxide dismutase (MnSOD) using stable isotope dilution LC-ESI-MS (Comhair et al., 2005). Isotopically labeled amino acids were added to the protein sample which was then delipidated, desalted and hydrolyzed under argon with methanesulfonic acid. SPE was performed to isolate and pre-concentrate the hydrolyzed oxidative/nitrosative products prior to LC-MS/MS analysis. This sample pretreatment allowed for all six biomarkers to be quantified in MnSOD isolated from epithelial cell brushings of mild asthmatic patients.

Recently, Piraud et al. (2005a, b) reported the development of a reverse-phase ion-pair LC-ESI-MS/MS technique capable of the simultaneous analysis of 76 amino acids related to inherited amino acid metabolic disorders without off-line preconcentration or chemical derivatization. Prior to injection the free amino acids within healthy human plasma or urine are simply mixed with an aliquot of the mobile phase an injecting onto the column for analysis. An optimized gradient elution consisting of acetonitrile and the ion-pair reagent tridecafluoroheptanoic acid (TDFHA) with an octadecyl-bonded reverse phase column successfully resolved all amino acids from known interfering compounds. The perfluorinated carboxylic acid ion-pair reagent used in this procedure is compatible to ESI-MS due to its high volatility. Under optimal conditions, deuterated amino acids were used for the quantification of both their unlabelled analogues and analytes with a similar structure or retention time (Piraud et al., 2005a). This approach allowed for quantitative analysis of DOPA, KYN and 3-hydroxy-kynurenine using labeled Met, Lys and Lys, respectively. Thus, LC-ESI-MS/MS permits quantitative

analysis of multiple modified amino acid biomarkers in complex human biofluids with minimal sample pretreatment and ionization suppression effects.

CE based methods

CE has not been routinely reported for modified amino acid analysis despite its high separation efficiency, minimal sample consumption and direct compatibility for polar/charged metabolite analysis. Recently, Tilley et al. developed a rapid procedure for the separation of 3-Br-Tyr and Di-Tyr by CE with UV detection (Tilley et al., 2006). The separation was achieved using a 100 mM iminodiacetic acid isoelectric buffer containing the zwitterionic surfactant lauryl sulfate and hydroxypropyl methylcellulose, however the method was limited by poor concentration sensitivity. Large volume sample stacking with CE-UV was reported to enhance concentration sensitivity over two orders of magnitude for trace analysis of minor modified amino acids (Tabi et al., 2005). This method was optimized for high efficiency separations of multiple modified amino acid analysis with nanomolar detection limits using an alkaline borate separation buffer with spermine as an electroosmotic flow modifier. However, application of these methods to real biological samples is constrained by significant interferences due to the limited selectivity of UV absorbance detection.

CE-ESI-MS is a promising platform for amino acid analysis (Saito et al., 2006; Soga et al., 2004) in terms of sensitivity, selectivity and qualitative information provided by MS experiments. Similar to LC-MS, there is

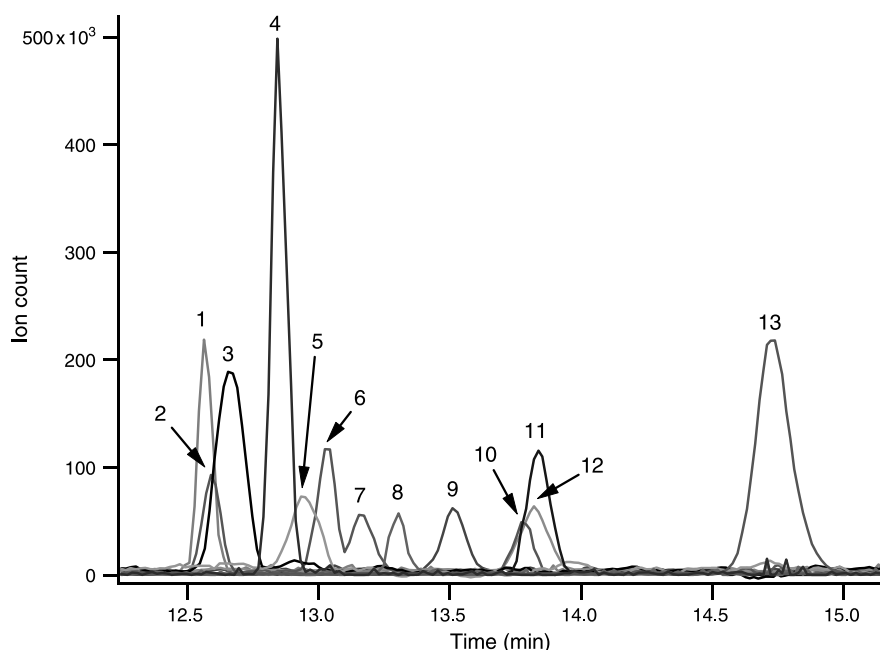


Fig. 4. Simultaneous analysis of multiple amino acid biomarkers of oxidative stress using on-line sample preconcentration with CE-ESI-MS. Overlay extracted ion electropherograms depict 0.1–0.5 μ M of modified amino acids in the presence of their natural analogues (unpublished data). Analyte peak numbering corresponds to: **1**, Met; **2**, *ortho*-Tyr; **3**, Trp; **4**, Phe; **5**, 5-OH-Trp; **6**, Tyr; **7**, *meta*-Tyr; **8**, L-DOPA; **9**, 3-Cl-Tyr; **10**, MetSO_n; **11**, GSSG; **12**, 3-NO₂-Tyr; **13**, GSH. (for a color reproduction of this figure, the reader is referred to the online version of this paper)

typically no requirement for chemical derivatization, thus reducing the potential of in vitro artifact formation. A comparison of the performance of CE-MS relative to GC-MS for amino acid profiling in plant extracts was recently examined by Williams et al. (2007), which demonstrated significant advantages in terms of reduced total analysis times, ease of sample handling and lower cost per sample due to the inexpensive use of fused-silica capillaries and lack of labeling procedures. In addition, off-line sample pretreatment can be further reduced by coupling on-line sample preconcentration and desalting steps directly in-capillary during separation without ionization suppression. Recently, Lee et al. introduced an integrative metabolomic strategy by CE-ESI-MS for unknown low abundance metabolite analysis with nanomolar detection limits (Lee et al., 2007). Computer simulations of amino acid migration behavior were also performed as qualitative tool to support MS for de novo identification of unknown metabolites (Lee et al., 2007). The latter strategy is important for discovery of biologically relevant oxidative stress biomarkers via global analysis of metabolic profiles. Figure 4 depicts an overlay of extracted ion electropherograms using CE-ESI-MS for the analysis of sub-micromolar levels of multiple modified amino acids, including 3-Cl-Tyr, *meta*-Tyr, *ortho*-Tyr, 5-hydroxytryptophan, MetSO_n and L-DOPA in the presence of their unmodified amino acid precursor (unpublished data). This method also highlights the analysis of reduced:oxidized glutathione (GSH:GSSG) ratio as an independent indicator of oxidative stress (Rossi et al., 2006; Shaik and Mehvar, 2006). Note that

CE separations also provide baseline resolution of all three isobaric Tyr positional isomers. Further work is needed to better validate CE-MS as a high-throughput platform for modified amino acid biomarker analysis where sample pretreatment is integrated with chemical analysis in a single-step unlike conventional LC and GC based methods.

Conclusion

The large number of review articles published in 2003–2007 illustrate the growing interest of modified amino acids as putative biomarkers of oxidative stress. However, careful validation of instrumental techniques and sample pretreatment protocols are essential for reliable clinical applications. High-throughput methods amenable for multiple amino acid biomarker analysis are also required for improved specificity in disease diagnosis. Future directions include the development of metabolomic strategies along with multivariate data analyses for unbiased assessment of the impact of oxidative/nitrosative processes on global amino acid metabolism among different biological samples.

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