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REVIEW ARTICLE

Analytical methods for 3-nitrotyrosine quantification in biological samples: the unique role of tandem mass spectrometry

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Abstract Reactive-nitrogen species, such as peroxynitrite ($ONOO^-$) and nitryl chloride (NO_2Cl), react with the aromatic ring of tyrosine in soluble amino acids and in proteins to form 3-nitrotyrosine. The extent of nitration can be quantified by measuring 3-nitrotyrosine in biological matrices, such as blood, urine, and tissue. This article reviews and discusses current analytical methodologies for the quantitative determination of 3-nitrotyrosine in their soluble and protein-associated forms, with the special focus being on free 3-nitrotyrosine. Special emphasis is given to analytical approaches based on the tandem mass spectrometry methodology. Pitfalls and solutions to overcome current methodological problems are emphasized and requirements for quantitative analytical approaches are recommended. The reliability of current analytical methods and the suitability of 3-nitrotyrosine as a biomarker of nitrative stress are thoroughly examined.

Keywords Misidentification · Nitration · Peroxynitrite · Quantification · Tandem mass spectrometry · Tyrosine · Validation

Introduction

Formation of 3-nitrotyrosine

The encounter of the radical gas nitric oxide (·NO) with oxygen in its various forms in aqueous media is fateful for NO's life and bioactivity. With molecular oxygen (O_2), physically dissolved in biological systems, NO undergoes autooxidation to form nitrite (NO_2^-) (see reaction 1), a chemically still reactive species. In erythrocytes, NO reacts with molecular oxygen (O_2) bound to hemoglobin, i.e., with oxyhemoglobin ($Hb[Fe^{2+}]O_2$), to form the chemically quite inert anion nitrate (NO_3^-) (see reaction 2). Upon meeting superoxide ($O_2^{-\cdot}$), which is ubiquitous in biological systems and chemically highly reactive, peroxynitrite ($ONOO^-$) is formed (Huie and Padmaja 1993) (see reaction 3). Peroxynitrite is the conjugate base of the extremely labile and highly reactive peroxynitrous acid (ONOOH; $pK_a \approx 6.8$). The $ONOO^-$/ONOOH system is a very strong oxidant and a potent nitrating agent, and as such harmful to cells (Ischiropoulos 1998; Radi et al. 2001). $ONOO^-$/ONOOH-induced modifications of biomolecules may lead to alterations in signaling pathways, impaired function, toxicity, and to lipid peroxidation (Niki 2009). A well-known example for loss of enzyme activity by nitration of tyrosine residues being involved in the catalytic process is represented by cyclooxygenase (COX). We (Tsikas 2008) and others (Zou et al. 1997) found that in vitro nitration of Tyr385 of COX by using a very high molar excess of synthetic peroxynitrite over COX is associated with little loss of activity. Although mechanistically very interesting, the physiological and pharmacological significance of this kind of inhibition of enzyme activity remains to be demonstrated.

In aqueous phase and within a large pH range, $ONOO^-$/ONOOH decomposes via highly reactive, probably radical intermediates including the gaseous radical nitrogen dioxide ($\cdot NO_2$) to finally produce nitrate, nitrite, and O_2 (Pfeiffer et al. 1997; Kissner and Koppenol 2002) (see reaction 4). In biological systems including blood, nitrite can be reduced to NO and oxidized to nitrate, for instance in erythrocytes by oxyhemoglobin, and to nitrylchloride

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(NO_2Cl) possibly via the catalytical action of myeloperoxidase (MPO) (see reaction 5).

$$4\,{}^{\cdot}NO + O_2 + 2\,H_2O \rightarrow 4\,NO_2^- + 4\,H^+ \quad (1)$$

$${}^{\cdot}NO + Hb\left[Fe^{2+}O_2\right] \rightarrow NO_3^- + Hb\left[Fe^{3+}\right] \quad (2)$$

$${}^{\cdot}NO + O_2^{\cdot-} \rightarrow ONOO^- \quad (3)$$

$$4\,ONOO^- \rightarrow 2\,NO_3^- + 2\,NO_2^- + O_2 \quad (4)$$

$$NO_2^- + HOCl \rightarrow \left[NO_2^+Cl^-\right] + HO^- \quad (5)$$

$$ONOO^- + GSH \rightarrow GSNO + HOO^- \quad (6)$$

$$3\,ONOO^- + 4\,GSH \rightarrow 2\,NO_2^- + NO_3^- + 2\,GSSG + 2\,H_2O \quad (7)$$

Nitrating agents, such as $ONOO^-$/ONOOH and other reactive-nitrogen species (RNS) including nitrylchloride (Halliwell 1997), preferentially react with the aromatic ring of free (soluble amino acid) and protein-incorporated tyrosine to form soluble 3-nitrotyrosine (NO_2Tyr) and protein-incorporated 3-nitrotyrosine (NO_2TyrProt), respectively (Fig. 1). Both NO_2Tyr and NO_2TyrProt are chemically fairly stable. The underlying mechanisms leading to 3-nitrotyrosine formation by $ONOO^-$/ONOOH are not fully understood. They may include involvement of both radicals, such as $\cdot NO_2$, and cationic intermediates, such as $^+NO_2$. Nevertheless, tyrosine-nitration seems to yield a single reaction product, i.e., 3-nitro-L-tyrosine (Fig. 1). NO_2Tyr and NO_2TyrProt in biological fluids and tissues are thought

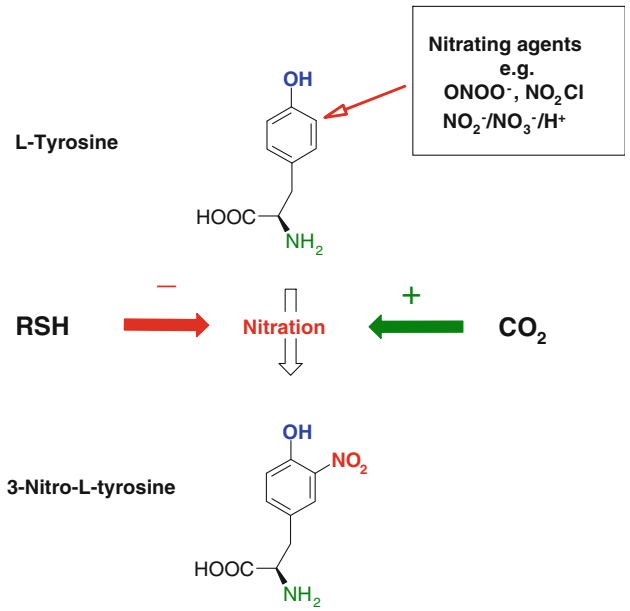

Fig. 1 Nitration of L-tyrosine to 3-nitro-L-tyrosine by the nitrating agents peroxynitrite ($ONOO^-$), nitryl chloride (NO_2Cl), nitrous acid (HONO), and nitric acid ($HONO_2$). Peroxynitrite-induced formation of 3-nitro-L-tyrosine is inhibited (−) by thiols (RSH) and elevated (+) by CO_2 (usually supplied as bicarbonate)

to be footprints left by RNS, i.e., to be biomarkers of nitrosative/nitrative stress. It has been reported that both MPO and peroxynitrite can abundantly nitrate a particular tyrosine residue, for instance Tyr192 in apolipoprotein A-I (apoA-I), with Tyr192 being equally chlorinated (Shao et al. 2005). Interestingly, in that study Tyr192-chlorination, but not Tyr192-nitration was found to be associated with loss of cholesterol efflux activity of apoA-I. However, another group found no correlation between tyrosine-nitration and tyrosine-chlorination of high-density lipoprotein (Pennathur et al. 2004). Regarding nitrative stress in apoA-I deficient mice, see the study by Parastatidis et al. 2007.

Nitrate, nitrite, and nitrogen oxygen gases (NO_x) are ubiquitous in nature. Thus, formation of NO_2Tyr and NO_2TyrProt is not exclusively related to NO produced endogenously from L-arginine, and the concentration of these species in biological systems may not specifically indicate altered activity of particular enzymes including NO synthase (NOS). It is worth mentioning that nitration of tyrosine can also occur at low pH values under physiological conditions or ex vivo/in vitro upon sample acidification during sample treatment (Fig. 1). The latter, i.e., the artifactual formation of NO_2Tyr and NO_2TyrProt is a widely recognized major methodological problem and is addressed below.

Carbon dioxide (CO_2) and thiols, such as glutathione (GSH) are physiologically present at mM-concentrations in all types of cells and have diametrically opposite effects on tyrosine-nitration by $ONOO^-$/ONOOH. Thus, CO_2 increases the extent of tyrosine-nitration, whereas thiols act as inhibitors of nitrotyrosination (Figs. 1, 2) (see reactions 6, 7). The positive effect of CO_2 is most likely due to the intermediate formation of nitrosoperoxycarbonate ($ONOOCO_2^-$). $ONOO^-$/ONOOH is a very strong oxidant toward thiols, and thiol-oxidation to disulfides by peroxynitrite is by far more abundant than tyrosine-nitration and thiol-nitros(yl)ation (Fig. 2). The second product of the reaction of $ONOO^-$/ONOOH with thiols is nitrite (see reaction 7). One-electron oxidations by peroxynitrite are attributed to reactive intermediates, such as the ${}^{\cdot}CO_3^-$ and ${}^{\cdot}NO_2$ radicals (Lymar and Hurst 1998; Goldstein and Czapski 1999; see also Ducrocq et al. 1999; Gow et al. 1996; Lemercier et al. 1997; Uppu et al. 1996) formed by homolytically decomposed $ONOOCO_2^-$. On the other hand, CO_2 is an apparently potent inhibitor of *S*-nitros(yl)ation and thiol-oxidation (Fig. 2b). Under physiological conditions, CO_2 and thiols would compete for $ONOO^-$/ONOOH and their relative concentration could be decisive whether tyrosine-nitration or thiol-nitros(yl)ation would prevail. Given the abundant occurrence of thiols in intra- and extra-cellular compartments from the quantitative point of view thiol-oxidation would be the most important reaction of peroxynitrite

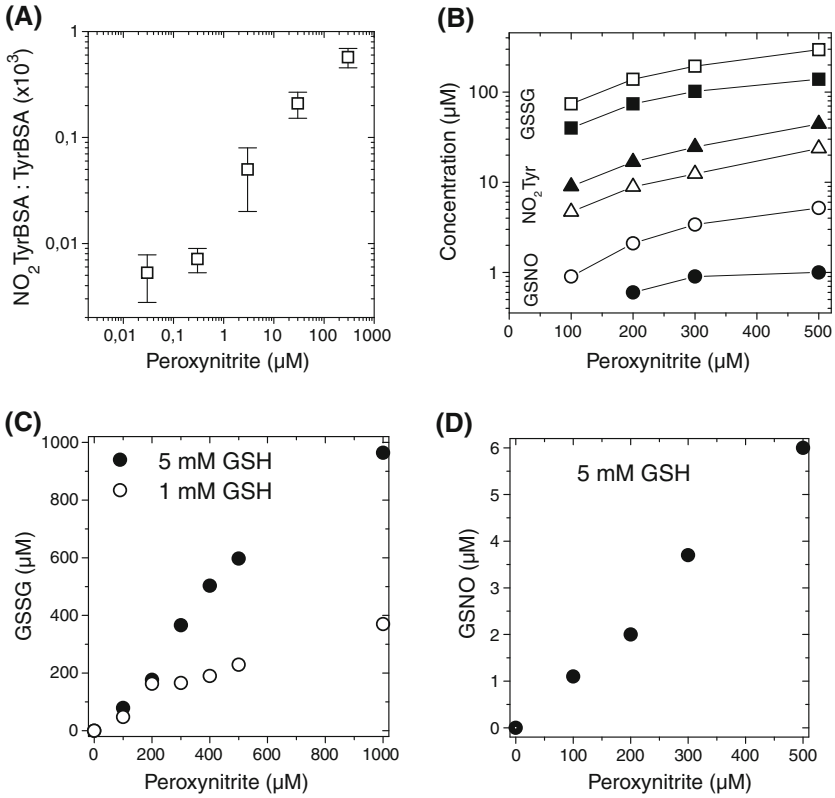

Fig. 2 **a** Formation of 3-nitrotyrosine bovine serum albumin (NO_2TyrBSA), **b** soluble 3-nitro-L-tyrosine, **b**, **d** *S*-nitrosoglutathione (GSNO), and **b**, **c** glutathione disulfide (GSSG) depending on peroxynitrite concentration in the absence and in the presence of glutathione (GSH) at 1 or 5 mM. Note the logarithmic scale on the *y* axis in **a** and **b**. Part of the graphs shown here were reconstructed from previously reported work (Tsikas et al. 2003; Tsikas 2006)

(Fig. 2) (see Denicola et al. 1996; Jourd'heuil et al. 1999; Lymar and Hurst 1998; van der Vliet et al. 1998; Zhang et al. 1997). This may be a plausible explanation for the very low concentrations of 3-nitrotyrosine and *S*-nitrosothiols in various biological fluids, as well as for the formidable analytical challenge caused by these nitr(os)ated compounds (Tsikas et al. 2002; Tsikas 2006; Ryberg and Caidahl 2007; Giustarini et al. 2007).

Sites of tyrosine-nitration in peptides and proteins

L-Tyrosine is a proteinogenic amino acid and may be present in a large number in proteins. For example, human serum albumin (HSA) contains 18 tyrosine residues (Peters 1985), whereas hemoglobin comprises 7 tyrosine moieties, to name two abundant circulating proteins; the angiotensins I and II, representatives of oligopeptides, bear each 1 tyrosine molecule. Given the high reactivity and the most likely low specificity of endogenous nitrating agents, the likelihood of multi-nitration of tyrosine in proteins is expected to be rather high. Indeed, mass spectrometry investigations on tyrosine-nitration in bovine serum albumin (BSA) by the exogenous nitrating agent tetranitromethane indicated that 4 out of 21 tyrosine residues are nitrated to a much higher degree than a fifth tyrosine residue located at position 163 (Petersson et al. 2001; see also Sarver et al. 2001). Also, similar investigations revealed that peroxynitrite nitrates various tyrosine residues in HSA, of which two were found to be more abundantly nitrated, i.e., Tyr138 and Tyr411 (Jiao et al. 2001). Tyr138 and Tyr411 of HSA were also found to be nitrated by tetranitromethane (Wayenberg et al. 2009). However, in that work the identification of 3-nitrotyrosine by MALDI-TOF has not been reported in satisfactory detail, notably from the quantitative point of view.

It is reasonable to expect that tyrosine-nitration in proteins may vary considerably with respect to the number and position of tyrosine in macromolecules in vivo, and all the more in vitro, depending upon the physicochemical properties of the nitrating agent used; e.g. various peroxynitrite salts, such as the tetramethylammonium salt, tetranitromethane, or nitronium tetrafluoroborate ($[NO_2^+BF_4^-]$). It is obvious that synthetic nitrotyrosinated proteins would be rather analytically poorly defined reference standards and different from those produced endogenously (see below). Indeed, reliable quantification of NO_2TyrProt in biological systems represents a significant analytical challenge, notably when using immunological assays that utilize synthetically nitrated proteins as calibrators. Although much less reactive, the other proteinogenic aromatic amino acids, i.e. phenyl alanine and tryptophan (see for instance Ikeda et al. 2007), are also attacked by nitrating agents to produce the corresponding nitro-derivatives.

The site-specificity of tyrosine-nitration in macromolecules has been the subject of investigation in recent years. These studies provided important information, for instance they showed that there may be some preference for tyrosine-nitration. But the impact of these studies is rather limited because most of them were proteomic studies without real reference to quantitative issues (reviewed by Bigelow and Qian 2008; Abello et al. 2009; Yan 2009). Moreover, it has been recently recognized that multiple factors can contribute to the misidentification of tyrosine-nitration by shotgun proteomics (Stevens et al. 2008; Prokai 2009). Also, it is worth mentioning that many in vitro studies on protein nitration utilized very high concentrations of nitrating agents, whereby ignoring the significant protecting effect of endogenous antioxidants, notably of thiols (Palamalai et al. 2006). The results of a selection of those studies are summarized in Table 1 and discussed briefly below.

The fact that two (Tyr24 and Tyr42) out of three tyrosine residues (Tyr24, Tyr42, and Tyr140) of the α-chain of human hemoglobin are nitrated by nitrite/hydrogen peroxide, with cysteine and methionine residues also being oxidized (Chen et al. 2008), argues against a high site-specificity of tyrosine-nitration and other oxidative modifications in proteins. It is interesting to note that identification and characterization of sites of nitration in proteins may lead to very different results, even by using highly sophisticated analytical approaches and techniques, such as tandem mass spectrometry (MS/MS). For instance, nitration of BSA (that contains 19 tyrosine residues) with tetranitromethane resulted in 3 sites of tyrosine-nitration when tryptic peptides were measured directly, but it resulted in 6 nitrated tyrosine molecules when analyzed after reduction of the nitro-group to the amino group (Ghesquière et al. 2006). Sharov et al. (2006) observed by LC–MS/MS that in vitro nitration of rat skeletal muscle glycogen phosphorylase b (Ph-b) by peroxynitrite resulted in nitration of 12 tyrosine residues (i.e., at positions 51, 52, 113, 155, 185, 203, 262, 280, 404, 473, 731, and 732), while in vivo in the rat only one (at position 113 in young rats) or 3 tyrosine (at positions 113, 161, and 573 in old rats) residues were presumed to be endogenously nitrated. Similar discrepancies between endogenous and in vitro tyrosine-nitration in proteins have been reported by another group (Kanski and Schöneich 2005; Kanski et al. 2005a, b). The claim that not only the number, but also the position of nitrated tyrosine residues differ in vitro and in vivo would indicate that tyrosine-nitration may occur distinctly different in living organisms than in vitro in cells and in cell-free media. In addition to the studies mentioned above, the author refers to some studies addressing analysis of nitrated proteins (Amoresano et al. 2007; Chatterjee et al. 2009; Fontana et al. 2006; Ghosh et al. 2006; Hong et al. 2007; Kim et al. 2006; Nuriel et al. 2008; Petre et al. 2005; Rabbani and Thornalley 2008). It is worth mentioning the two recent studies which identified factors that contribute to the misidentification of protein tyrosine-nitration by shotgun proteomics and proposed manual validation criteria (Stevens et al. 2008). These authors also pointed out to the particular importance of the synthesis and unequivocal structural MS/MS characterization of nitrotyrosinated peptides in proteomics studies. In this context, the author of the present review refers to recent work providing evidence that all nitropeptides reported in a previous article (Hong et al. 2007) were false positives (Prokai 2009).

In summary, proteomic studies have delivered recently a plethora of published modifications of proteins by RNS. There have been proposed different approaches to identify sites of tyrosine-nitration in macromolecules of endogenous sources in vivo as well as in vitro. However, the majority of these studies were designed to provide qualitative rather than quantitative data, often prone to misidentifications even in this regard (Stevens et al. 2008; Prokai 2009). Despite some indication of potential sites of preferential tyrosine-nitration in proteins, actually we lack convincing evidence of noteworthy site-specific protein tyrosine-nitration. To achieve this goal, we need sophisticated, elegant, proper and validated quantitative approaches, well-conceived studies, and performance of MS/MS analyses by highly skilled investigators. In particular, we should have in mind that precise and accurate quantification of modified proteins are of critical importance to many areas of proteomics (Duncan 2007; Stevens et al. 2008; Duncan et al. 2009; Prokai 2009).

Occurrence, metabolism, and elimination of 3-nitrotyrosine

So far, 3-nitrotyrosine has been detected in numerous biological tissues and fluids including plasma, urine, cerebrospinal fluid (CSF), and exhaled air condensate (see for example the review article by Ryberg and Caidahl 2007). Figure 3 shows schematically the currently known formation and oxidative metabolism pathways of 3-nitrotyrosine. First, formation, metabolism, and elimination of 3-nitrotyrosine have been investigated in rats. Intraperitoneal injection of tetranitromethane led to formation of 3-nitrotyrosine in plasma proteins and hemoglobin (Ohshima et al. 1990). 3-Nitrotyrosine (NO_2Tyr), orally given to rats, was found to be metabolized to 3-nitro-4-hydroxyphenyllactic acid (NHPL) and 3-nitro-4-hydroxyphenylacetic acid (NHPA) which were excreted in the urine, with NHPA being identified as the major urinary metabolite of 3-nitrotyrosine in the rat (Ohshima et al. 1990). This group also reported that NHPA is excreted in the urine of smokers and non-smokers at mean excretion

Table 1 Reported sites of tyrosine-nitration in various proteins in vitro and in vivo

Reference and year	Protein	Nitrating agent	Approach	Tyrosine position	Remark
Jiao et al. (2001)	Albumin (HSA)	Peroxynitrite	HPLC, MS/MS	138, 411 (and other non-identified)	In vitro
Petersson et al. (2001)	AT-II; BSA	Tetranitromethane	MALDI, MS/MS	Qualitative analyses	
Sarver et al. (2001)	BSA	Tetranitromethane	MALDI	Qualitative analyses	Laser-induced decomposition
Pietraforte et al. (2004)	Human HbO_2	Nitrite	MS/MS	2% Tyr-nitration in HbO_2	Relative yield
Kanski et al. (2005a)	Creatine kinase	Peroxynitrite	MS/MS	82 In vitro; 14 and 20 in vivo	Concern[a]
Park et al. (2005)	p65 of NF-κB	Nitroprusside	MS	66, 152	
Shao et al. (2005)	apoA-I	MPO, peroxynitrite	MS/MS	192 (susceptible to chlorination)	In vitro
Tedeschi et al. (2005)	Alpha-tubulin	None	MALDI-TOF	161, 357 (in vivo)	PC12 cells
Fujigaki et al. (2006)	IDO	Peroxynitrite	MS/MS	15, 345, 343	Cells
Ghesquière et al. (2006)	Bovine serum albumin (BSA)	Tetranitromethane	MS/MS	355, 364, 424 286, 364, 376, 393, 424, 475	3 or 6 Tyr residues
Sharov et al. (2006, 2009)	Phosphorylase b	Peroxynitrite	MS/MS	51, 52, 113, 155, 185, 203, 262, 280, 404, 473, 731, 732 (in vitro); 113, 161, 573 (endogenous)	
Webster et al. (2006a, b)	Recomb. p38 MAPK	Peroxynitrite	MS	132, 245, 258	45-Da increase
Hsiai et al. (2007)	LDL apo-B-100	Peroxynitrite	MS/MS	144, 2,524, 3,295, 4,116, 4,211	None
Lin et al. (2007)	CYP2B6, CYP2E1	Peroxynitrite	MS/MS	244, 268, 354, 380	Heme modification
Nakagawa et al. (2007)	Cytochrome C	Peroxynitrite	MALD-TOF	74 (targeted)	High concentrations
Tedeschi et al. (2007)	Peripherin	None	MALDI-TOF	17, 376	Endogenous
Hong et al. (2007)	N-RAP	None	MS/MS	5970	Concern[b]
	Myosin heavy chain polypeptide 6	None	MS/MS	114	Concern[b]
	Tropomyosin	None	MS/MS	491	Concern[b]
	Neurofibromin	None	MS/MS	221	Concern[b]
Chen CL et al. (2008)	Complex II	Peroxynitrite	MS/MS	56, 142	Post-ischemia
Chen HJ et al. (2008)	Human Hb	Nitrite/H_2O_2	MS/MS	α24, α42, β130	3 of 7 Tyr residues
Ulrich et al. (2008)	Eosinophil	None, NO_x/H_2O_2	HR-MS	349, 33	Eosinophils
Danielson et al. (2009)	α-Synuclein	None	MS/MS	39 (125, 133, 136)	Cellular model
Liu et al. (2009)	Complex I	Peroxynitrite	MS/MS	247, 47, 53	In vitro
Matalon et al. (2009)	Surfactant D	Peroxynitrite	MS	314 (cross-linking)	In vitro, mice
Wayenberg et al. (2009)	Albumin (HSA)	Tetranitromethane	MALDI-TOF	138, 411	In vitro, pure HSA

[a] Concern with quality of spectra and, hence, possibly misidentified

[b] Concern: endogenous, misidentified

Note with regard to both concerns see Stevens et al. 2008 and Prokai 2009

rates of 2.7 μg/day (17.8 nmol/day) and 2.9 μg/day (19.1 nmol/day), respectively. From this data, a mean urinary NHPA concentration of 15 nM in humans is calculated. This order of magnitude (i.e., 2.3 nmol of NHPA per mmol of creatinine) was confirmed by GC–MS/MS in young healthy volunteers (Keimer et al. 2003). More recently, healthy subjects were found to excrete unchanged NO_2Tyr into the urine at mean excretion rates of 0.5 nmol/mmol creatinine (Tsikas et al. 2005) or 1.4 nmol/mmol creatinine (Kato et al. 2009). These findings support the idea that NHPA could indeed be the major urinary metabolite of 3-nitrotyrosine. However, thus far there is no study to investigate whether in urine NO_2Tyr or NHPA is better suitable as a biomarker of nitrative stress. Interestingly, it has been recently reported that NO_2TyrProt as well as protein-incorporated 3-bromo-tyrosine, i.e., BrTyrProt, are excreted in the urine of humans (Chen and Chiu 2008). Quantitative aspects of 3-nitrotyrosine occurrence in biological systems are discussed in detail below.

For the sake of completeness it should be mentioned that oxidation of tyrosine may also lead to formation of dityrosine in addition to 3-nitro-, 3-chloro-, and 3-bromotyrosine. Dityrosine has been reported (Orhan et al. 2004, 2005) to be excreted in human urine at mean rates of about 10 and 6 nmol/mmol creatinine as measured by LC-APCI-MS/MS using ion trap and the quadrupole technology,

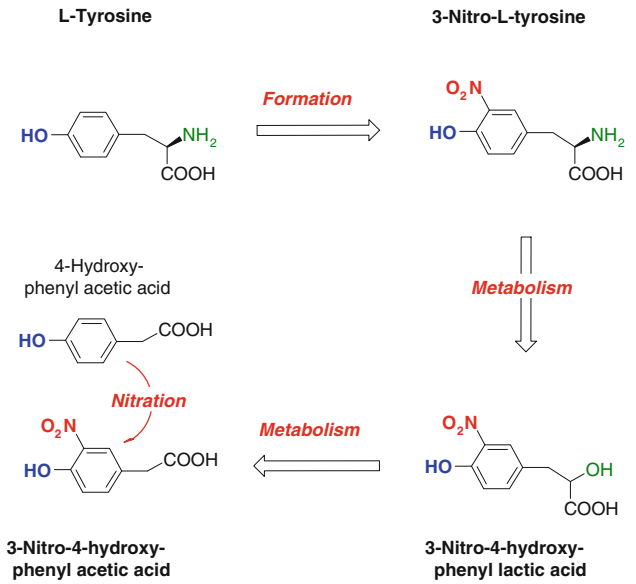

Fig. 3 Formation of 3-nitro-L-tyrosine from L-tyrosine and metabolism of 3-nitro-L-tyrosine to 3-nitro-4-hydroxyphenyllactic acid and 3-nitro-4-hydroxyphenylacetic acid. 3-Nitro-4-hydroxyphenylacetic acid can also be formed from 4-hydroxyphenylacetic acid

respectively. This excretion rate is comparable to that of NHPA and about 10 times higher than that of NO_2Tyr (see above). Data on dityrosine are very scarce; this modified tyrosine is not further considered in the present review.

It is worth mentioning that origin and metabolism of NO_2Tyr, NO_2TyrProt, and other oxidatively modified soluble and protein-incorporated tyrosine residues are poorly investigated and little understood. In particular, it is actually unknown whether and to which extent NO_2Tyr derives from proteolyzed NO_2TyrProt or from nitrated soluble tyrosine.

Reported analytical methods and concentrations of 3-nitrotyrosine in biological samples

A wide spectrum of physicochemical and immunological methods is currently available for NO_2Tyr and NO_2TyrProt in plasma, urine, and tissue (for reviews see Duncan 2003; Tsikas and Caidahl 2005; Ryberg and Caidahl 2007; Tsikas 2008). These methods and the 3-nitrotyrosine concentrations resulted from their application to plasma, urine, CSF, and exhaled breath condensate (EBC) are summarized in Table 2 for NO_2Tyr and in Table 3 for NO_2TyrProt. Figure 4 illustrates a selection of the reported NO_2Tyr concentrations in human plasma since the first report by Kamisaki et al. in 1996 until the end of 2009 as measured by different analytical approaches. These data reveal several orders of magnitude range for reported concentrations of NO_2Tyr and NO_2TyrProt in almost all analyzed biological matrices of humans and rats in baseline conditions.

Reportedly, the lowest plasma NO_2Tyr concentration in humans was measured by LC–MS/MS to be 0.6 nM (Svatikova et al. 2004), whereas one of the highest human plasma NO_2Tyr concentration reported so far was measured by HPLC–UV to be 5,300 nM (Fatouros et al. 2004). In human CSF, NO_2Tyr concentration was measured to be 0.35 nM by GC–MS/MS (Ryberg et al. 2004) and 1.4 nM by HPLC-ECD (Tohgi et al. 1999). Diverging NO_2Tyr concentrations have also been reported for EBC; e.g., each about 0.04 nM by GC–MS/MS (Lärstad et al. 2005) and LC–MS/MS (Göen et al. 2005), but 35 nM by means of a fluorescence method (Balint et al. 2001). In healthy humans, urinary excretion rate of NO_2Tyr has been reported to be 0.4–1.4 nmol/mmol creatinine as measured by GC–MS/MS (Tsikas et al. 2005) and LC–MS/MS (Kato et al. 2009).

Considerable discrepancy has also been observed for NO_2TyrProt (Table 3). For instance, the molar ratio of NO_2TyrProt to TyrProt has been determined to be $35{:}1 \times 10^6$ by GC–MS (Frost et al. 2000), but only $1.2{:}1 \times 10^6$ by GC–MS/MS (Keimer et al. 2003; Tsikas et al. 2003). So far, the lowest concentration in human plasma for tyrosine-nitrated albumin has been reported to be only about 0.2 nM as measured by ELISA (Wayenberg et al. 2009), which is almost 100 times lower than the estimated concentration of tyrosine-nitrated albumin of about 20 nM as measured by affinity chromatography coupled to GC–MS/MS (Keimer et al. 2003). Interestingly, NO_2TyrProt has been reported to be excreted in the urine at a concentration of about 800 pg/ml (Radabaugh et al. 2008) or with a NO_2TyrProt to TyrProt molar ratio of $97{:}1 \times 10^6$ (Chen and Chiu 2008) as measured by LC–MS/MS in both studies.

In consideration of the unique specificity of the MS/MS methodology and of the sensitivity of modern GC–MS/MS and LC–MS/MS instruments, a NO_2Tyr concentration of the order of 1 nM (Fig. 4) and a molar ratio of $1{:}10^6$ for NO_2TyrProt/TyrProt in plasma appear useful guide numbers for soluble and protein-associated 3-nitrotyrosine, respectively (Tsikas and Caidahl 2005). These numbers may be a proper measure to prove both the quality and the reliability of analytical methods for 3-nitrotyrosine and the results they deliver in basic and in clinical studies. Below, major demands on analytical methods for reliable quantitative analysis of NO_2Tyr and NO_2TyrProt in biological samples are discussed in detail.

Demands on analytical methods for reliable 3-nitrotyrosine measurement

The source and nature of the analytical problems, shortcomings, and pitfalls associated with 3-nitrotyrosine

Table 2 Reported concentrations for soluble 3-nitrotyrosine (NO_2Tyr) and its metabolite 3-nitro-phenylacetic acid (NHPA) in biological fluids of humans and other species

Species	Matrix	Concentration (nM)	Method	LOD/LOQ	1st Author and year
Human	Plasma	31	HPLC-FL	N.R./6 nM	Kamisaki et al. (1996)
Human	Plasma	<5	HPLC-ECD	0.1 pmol/5 nM	Ohshima et al. (1999)
Human	Plasma	2.8	GC–MS/MS	4 amol/0.125 nM	Schwedhelm et al. (1999)
Human	CSF	1.4	HPLC-ECD	?	Tohgi et al. (1999)
Human	Plasma	64	GC–MS	N.R.	Frost et al. (2000)
Human	Plasma	4.4	LC–MS/MS	1 fmol/4.4 nM	Yi et al. (2000)
Human	Plasma	14	HPLC–UV	N.R./N.R.	Strand et al. (2000)
Human	EBC	35	Fluorescence	?	Balint et al. (2001)
Human	Plasma	11	GC–MS	70 amol/N.R.	Gaut et al. (2002)
Human	Plasma	N.D.	LC–MS/MS	3.2 fmol/1.6 nM	Gaut et al. (2002)
Rat	Plasma	<1.4	LC–MS/MS	73 fmol/1.4 nM	Delatour (2002a)
Human	Plasma	<100	Amino acid analyzer	N.R./100 nM	Ohya et al. (2002)
Human	Plasma	135	EIA	2 nM/N.R.	Inoue et al. (2002)
Human	Plasma	0.74	GC–MS/MS	30 amol/0.3 nM	Söderling et al. (2003)
Human	Plasma	0.73	GC–MS/MS	4 amol/0.125 nM	Tsikas et al. (2003)
Human	Plasma	0.64	GC–MS/MS	4 amol / 0.125 nM	Keimer et al. (2003)
Human	Urine, NHPA	2.3 nmol/mmol	GC–MS/MS	4 amol/0.125 nM	Keimer et al. (2003)
Human	Plasma	9.4	LC–MS/MS	22 fmol/N.R.	Ahmed et al. (2003)
Human	Plasma	6.5	LC–MS/MS	22 fmol/N.R.	Thornalley et al. (2003)
Human	Plasma	5.4	GC–MS	N.R.	Pannala et al. (2003)
Human	Plasma	5,300	HPLC–UV	200 nM/N.R.	Fatouros et al. (2004)
Human	Plasma	0.6	LC–MS/MS	N.R./N.R.	Svatikova et al. (2004)
Human	Urine	<3.3 nmol/mmol	LC–MS/MS	25 nM/N.R.	Orhan et al. (2004)
Rat	Urine	440 nM ?	Stacking CE	90 nM/440 nM	Maeso et al. (2004)
Human	CSF	0.35	GC–MS/MS	30 amol/0.3 nM	Ryberg et al. (2004)
Human	Plasma	<0.4	LC–MS/MS	22 fmol/0.4 nM	Ahmed et al. (2005)
Human	Plasma	1	LC–MS/MS	100 fmol/N.R.	Nicholls et al. (2005)
Human	Urine	0.46 nmol/mmol	GC–MS/MS	4 amol/0.125 nM	Tsikas et al. (2005)
Human	EBC	40–1,000 pM	LC–MS/MS	/40 pM	Göen et al. (2005)
Human	EBC	4–40 pM	GC–MS/MS	3 amol/0.6 pM	Lärstad et al. (2005)
Human	Plasma	0.75	GC–MS/MS	4 amol/0.125 nM	Tsikas (2006)
Human	EBC	0.5–7 nM	GC–MS and HPLC-ECD	220 and 200 pM/N.R.	Celio et al. (2006)
Human	Urine, NHPA	1 μmol/day	GC–MS	?	Pannala et al. (2006)
Human	Liver (MD)	<LOD/LOQ	HPLC-ECD	0.5 nM	Richards et al. (2006)
Human (disease)	Urine	13 nmol/mmol	LC–MS/MS	N.R.	Nicholls et al. (2007)
Human	EBC	0.03–7.5	LC–MS/MS	25 pM/N.R.	Conventz et al. (2007)
Human	Plasma	300–14,000 nM	ELISA	?	Rossner et al. (2007)
Human	Urine	0.3–3 nM	LC–MS/MS	30 pM/N.R.	Radabaugh et al. (2008)
Human	Urine	0.7 nM	ELISA	?	Shimizu et al. (2008)
Human	Urine	0.4 nmol/mmol	GC–MS/MS	4 amol/0.125 nM	Pham et al. (2009)
Human	Urine	1.4 nmol/mmol	LC–MS/MS	?	Kato et al. (2009)
Rat	Plasma	5.4	GC–MS/MS	4 amol/0.125 nM	Magné et al. (2009)
Human	Plasma	2.5–9.1	GC-ECD	5 fmol/2.5 nM	Pavlovic et al. (2009)

LOD limit of detection, *LOQ* limit of quantification, *N.M.* not measured, *N.R.* not reported, *SPE* solid-phase extraction, *N.D.* not detectable, *MD* microdialysis sample, *?* unknown

Table 3 Reported circulating protein-associated 3-nitrotyrosine (NO_2TyrProt as 3-nitrotyrosine/tyrosine molar ratio, i.e. $\times 1{:}10^6$, in pmol/mg or in nM) levels in health and disease measured by various methodologies in methodological and clinical studies

Species	Matrix	Content	Method	Hydrolysis	1st Author and year
Human, HC	Plasma	28–193 pmol/mg	HPLC-FL	HCl solution	Kamisaki et al. (1996)
Human, HC	Plasma	5.1 pmol/mg	HPLC/GC-TEA		Petruzzelli et al. (1997)
Human, H Smokers	Plasma	571 pmol/mg	HPLC/GC-TEA		Petruzzelli et al. (1997)
Human, HC	Plasma	N.D.	ELISA		ter Steege et al. (1998)
Human, celiac disease	Plasma	1,270 nM	ELISA		ter Steege et al. (1998)
Human, HC	Plasma	120 nM	ELISA		Khan et al. (1998)
Human, systemic sclerosis	Plasma	293 nM	ELISA		Khan et al. (1998)
Human, HC	Plasma	$35 \times 1{:}10^6$	GC–MS		Frost et al. (2000)
Human, diabetes type II	Plasma	251 nM	ELISA		Ceriello et al. (2001)
Human	Plasma	170 pmol/mg	Immunology/WB		Pignatelli et al. (2001)
Rat, HC	Plasma	$4–18 \times 1{:}10^6$	LC–MS/MS		Delatour et al. (2002b)
Human, HC	Plasma	0.2–0.8 pmol/mg	LC/MS/MS	Enzymes	Lorch et al. (2003)
Human, HC	Plasma	0.6 pmol/mg	GC–MS/MS	Enzymes	Söderling et al. (2003)
Human, HC	Plasma	$1.55 \times 1{:}10^6$	GC–MS/MS	Enzymes	Tsikas et al. (2003)
Human, HC	Plasma	$1.2 \times 1{:}10^6$	GC–MS/MS	Enzymes	Keimer et al. (2003)
Human, HC	Plasma	5.8 pmol/mg	GC–MS	NaOH	Pannala et al. (2003)
Human, HC	Plasma	$8 \times 1{:}10^6$	GC–MS	Sulfonic acid	Pennathur et al. (2004)
Human, HC	Plasma	$0.6 \times 1{:}10^6$	LC–MS/MS	Enzymes	Ahmed et al. (2005)
Human, HC	Plasma	$1.40 \times 1{:}10^6$	GC–MS/MS	Enzymes	Tsikas et al. (2005)
Human, renal disease	Plasma	$1.78 \times 1{:}10^6$	GC–MS/MS	Enzymes	Tsikas et al. (2005)
Human, HC	Plasma	1.8 ng/mg protein	GC–MS	NaOH	Kingdon et al. (2006)
Human, HC	Urine	$97 \times 1{:}10^6$	LC–MS/MS	HCl	Chen and Chiu (2008)
Human, HC	Plasma	200–1,000 pg/ml	LC–MS/MS	Enzymes	Radabaugh et al. (2008)
Human, HC	Urine	60–800 pg/ml	LC–MS/MS	Enzymes	Radabaugh et al. (2008)
Human, asphyxia	Plasma	150 pM	ELISA		Wayenberg (2009)

HC healthy control, *N.M.* not measured, *LOQ* limit of quantitation, *N.D.* not detectable

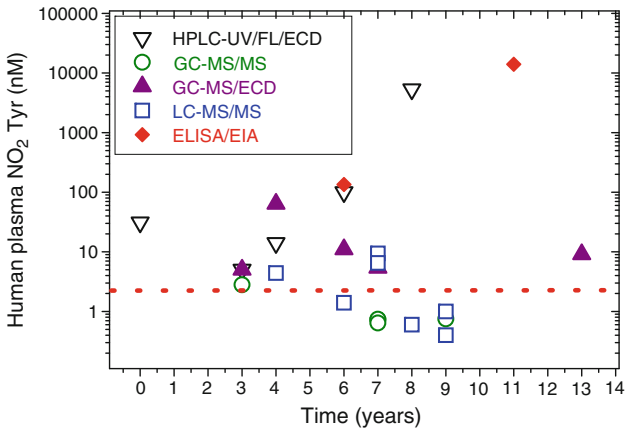

Fig. 4 Reported concentrations for 3-nitro-L-tyrosine (NO_2Tyr) in plasma of healthy humans as measured by means of various analytical techniques. The period between 1996 (year *zero*) and 2009 (year *13*) was considered. The *horizontal dotted line* at about 2 nM indicates the approximate upper level for plasma NO_2Tyr concentration in healthy humans at the basal state as suggested by the tandem mass spectrometry (MS/MS) methodology. Note the logarithmic scale on the *y* axis

analysis include (Fig. 5): (1) abundant artifactual formation of 3-nitrotyrosine during sample treatment; (2) lack of sensitivity; (3) lack of specificity; (4) inadequate or even completely absent method validation, notably of immunological assays; (5) inadequate review of the analytical component of the work during the publication process; (6) misplaced confidence in and uncritical use of commercially available assays that have not been adequately validated; (7) failure to consider and rationalize contradictory results originating from the use of reliable methods, i.e., GC–MS/MS and LC–MS/MS; and (8) an uncritical adoption and propagation of experimental findings derived from the use of unreliable methods (Tsikas 2010a). These issues are discussed in detail below.

Artifactual tyrosine-nitration

Due to the high reactivity of the phenolic ring of the precursors of NO_2Tyr, NO_2TyrProt, and NHPA and the ubiquity of nitrite, nitrate, and tyrosine, 3-nitrotyrosine

species are readily and abundantly formed from their precursors under certain experimental conditions, such as acid-catalyzed proteolysis or esterification/etherification in aqueous media. Artifactual 3-nitrotyrosine formation can be minimized by separating tyrosine from nitrite and nitrate, for instance by HPLC (Schwedhelm et al. 1999), or by converting 3-nitrotyrosine to 3-aminotyrosine prior to derivatization (Söderling et al. 2003) (Fig. 6); these different methods have led to very similar results for NO_2Tyr in human plasma, i.e., to basal concentrations of about 1 nM (Fig. 4) underlining the high efficiency of these measures toward artifactual 3-nitrotyrosine formation.

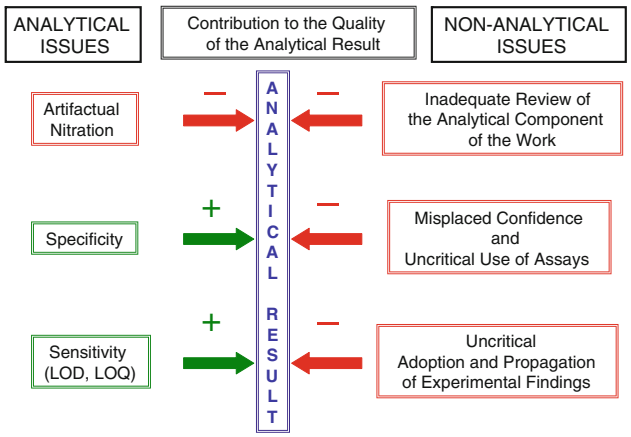

Fig. 5 Schematic of the main analytical factors (*left panel*) and non-analytical factors (*right panel*) that may contribute positively (+) or negatively (−) to the quality of the analytical results for 3-nitro-L-tyrosine

As NO_2Tyr and $NO_2TyrProt$ values are commonly normalized to Tyr and TyrProt values, respectively, simultaneous analysis of tyrosine and 3-nitrotyrosine is very tempting. However, it should be considered that samples that contain NO_2Tyr and $NO_2TyrProt$ also contain Tyr and TyrProt at a very high excess over 3-nitrotyrosine, e.g., 40,000-fold in plasma and up to about 8,000,000-fold in proteolysates, respectively. Analysis of tyrosine-containing NO_2Tyr and $NO_2TyrProt$ samples is fraught with a certain danger for artifactual formation of 3-nitrotyrosine. For instance, in GC–MS-based methods in which samples are derivatized and extracts are injected mostly in the heat, abundant nitration of aromatic and non-aromatic compounds is possible (Tsikas et al. 2009a). Therefore, separation of 3-nitrotyrosine from tyrosine at an early stage of the analytical process is highly advisable (see also below).

Detection sensitivity: limits of detection and quantitation

Given the pM-to-nM concentration of 3-nitrotyrosine in biological samples, highest detection sensitivity is an indispensable prerequisite for accurate quantification of NO_2Tyr in many biological matrices, notably plasma, EBC, and CSF (Table 2). Highest sensitivity for 3-nitrotyrosine, in terms of the limit of detection (LOD) and in particular of the limit of quantitation (LOQ), is provided by GC–MS/MS and LC–MS/MS. The reported 3-nitrotyrosine methods based on GC–MS/MS and LC–MS/MS in recent years allow detection of 3-nitrotyrosine in plasma as little as 0.04 nM. This LOQ value is about 7 to 10 times lower than the mean concentration of NO_2Tyr in plasma samples

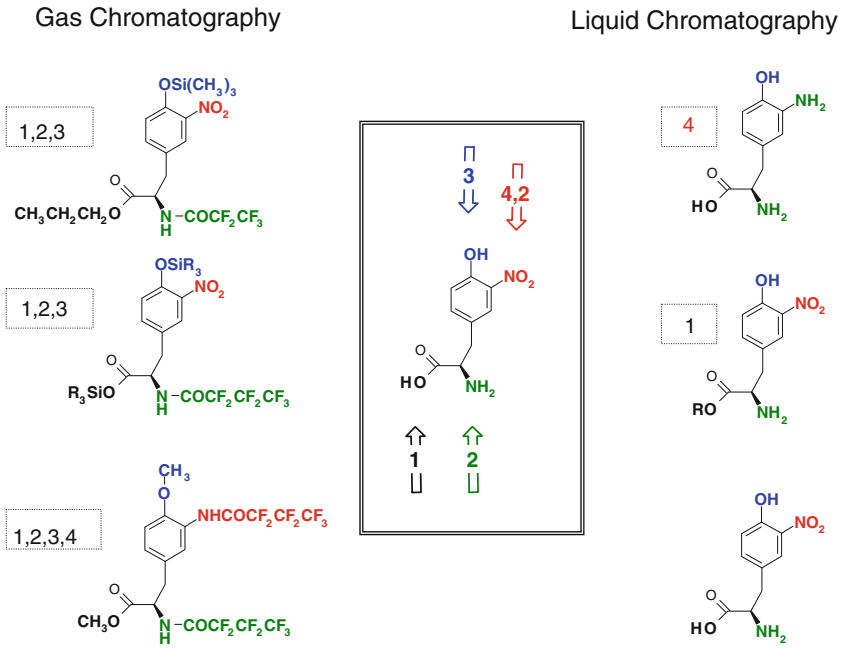

Fig. 6 Frequently used derivatization procedures for 3-nitro-L-tyrosine and commonly measured 3-nitro-L-tyrosine derivatives in analytical techniques based on gas chromatography (*left panel*) and liquid chromatography (*right panel*). *1* esterification, *2* acylation, *3* etherification, *4* reduction of nitro-group to amino group

of healthy humans (Table 2). First, improvement of sensitivity of LC–MS/MS for many analytes including 3-nitrotyrosine has been achieved by butyl esterification (Delatour et al. 2002a). About 10 years ago, LC–MS/MS instruments did not allow quantification in plasma less than about 4 (Yi et al. 2000) and 2 nM (Gaut et al. 2002; Delatour et al. 2002a) of 3-nitrotyrosine (Table 2). However, recent advances in this technology lowered the LOD and LOQ values for 3-nitrotyrosine by two and one order of magnitude, respectively. This appreciable sensitivity enhancement may make esterification of 3-nitrotyrosine from biological samples superfluous, if analysis is performed on modern LC–MS/MS instruments. From the quantitative perspective, LOQ rather than LOD is useful as a criterion for methods comparison and evaluation (Tsikas 2009a).

Besides lacking specificity (see below), due to co-eluting/migrating interfering contaminants, it is noteworthy that analytical methods, such as HPLC, GC, and CE coupled with detection systems different from tandem mass spectrometers, for instance with fluorescence, electrochemical detectors, or even mass spectrometers, are frequently used for NO_2Tyr quantification in human plasma and other biological samples, although their LOD and LOQ values are considerably higher than required. Consequently, such approaches are not able to provide accurate NO_2Tyr concentrations in basal conditions and their appropriateness in intervention and clinical studies is very limited (Tsikas 2009b). The reported NO_2Tyr concentrations measured in biological samples by such approaches, including GC–MS (see next section), should be treated with caution, at least those being measured in basal conditions.

As mentioned above, the level of the LOQ value of a NO_2Tyr method is of pivotal importance for the reliable quantification of NO_2Tyr in most biological samples including plasma. One possibility to lower the LOQ value is to perform additional chromatographic steps. For instance, in our GC–MS/MS method for NO_2Tyr we incorporated a HPLC step prior to final GC–MS/MS analysis. Admittedly, the combination of HPLC with GC–MS/MS renders the whole analytical method very time-consuming. However, it is worth mentioning that HPLC, in addition to minimizing artifactual formation of 3-nitrotyrosine by separating NO_2Tyr from tyrosine, nitrite, and nitrate, considerably lowers the method's LOQ by eliminating numerous endogenous substances (Tsikas and Caidahl 2005). In consideration of the analytical challenge of NO_2Tyr quantification in biological samples, additional steps that ensure reliable NO_2Tyr results are necessary and tolerable despite considerable complication of the method and increase of the analysis time. It cannot be emphasized strongly enough that reliability rather than rapidity should have first priority in 3-nitrotyrosine analysis (Duncan 2003; Tsikas 2008).

Specificity

In GC–MS/MS- and LC–MS/MS-based methods, high specificity is mainly provided and ensured by MS/MS, where collision-induced dissociation (CID) yields fragments, the product ions, from a given precursor ion. All or some of the product ions are characteristic for the analyte. MS/MS minimizes or eliminates the likelihood that other structurally related or unrelated substances that coelute with the analyte of interest, e.g. NO_2Tyr, because of insufficient chromatographic separation by GC or LC, contribute to the analyte.

GC–MS-based methods are compromised by potentially interfering compounds (Tsikas 2009a, b). Such interference has been demonstrated for NO_2Tyr in a GC–MS method in which other sources of interferences including artifactual NO_2Tyr formation are excluded. A proper means to identify potential interferences in GC–MS is analysis of the same samples by GC–MS/MS (Figs. 7, 8) and comparison of the results offered by the methods (Fig. 9). Lack of reports that LC–MS-based methods are applicable to NO_2Tyr in plasma and other biological matrices is supportive of the inappropriateness of LC–MS for NO_2Tyr quantification in most biological systems.

Eventually, 3-nitrotyrosine is considerably less abundant than other tyrosine derivatives including 3-bromotyrosine, and 3-chlorotyrosine (Gaut et al. 2002). These halogenated tyrosines may be potential interferences in the 3-nitrotyrosine analysis by low-specificity approaches.

These few examples suggest that the number of substances in biological samples with the potential to interfere with 3-nitrotyrosine analysis is likely to be much higher than presently known, and that method's specificity needs to be enhanced by proper measures, even in those approaches that are generally considered highly specific. Such a measure could be the specific extraction of NO_2Tyr by means of immunoaffinity chromatography for which, however, no material is commercially available so far (Radabaugh et al. 2008; Tsikas 2010b).

Method validation

A major problem fraught with the measurement of NO_2Tyr is the use of inadequately or even entirely non-validated methods for measuring NO_2Tyr and NO_2TyrProt in complex biological samples. The quality of the NO_2Tyr data generated by such methods and the conclusions derived from their application are actually unknown. When discussing method validation issues, it is advisable to distinguish between two categories of analytical techniques;

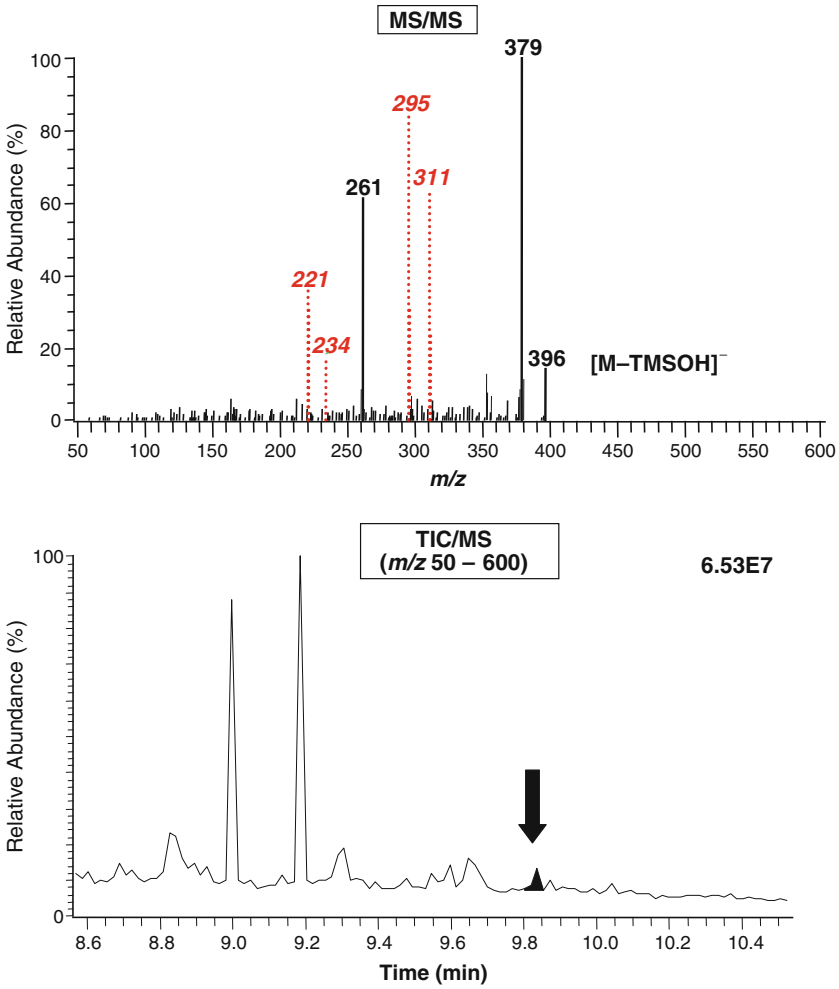

Fig. 7 GC–MS/MS-based identification of interferences in the analysis of 3-nitro-L-tyrosine in human plasma by GC–MS. Product ion mass spectrum (*upper panel*) from the GC peak eluting at 9.84 min and indicated by the *arrow* (*lower panel*). The parent ion at *m/z* 396 ([M-TMSOH]$^-$) of the *n*-propyl-pentafluoropropionyl-trimethylsilyl ether derivative of the compounds eluting at 9.84 min was subjected to collision-induced dissociation. The product ions at *m/z* 379 and 261 indicated by *solid line* are due to 3-nitro-L-tyrosine. The product ions at *m/z* 311, 295, 234, and 221 indicated by *dashed line* and in *italics* are due to unknown interference. This figure was reconstructed from the Fig. 1 of the article by Schwedhelm et al. 1999. *TIC* total ion current, *TMSOH* trimethylsilanol

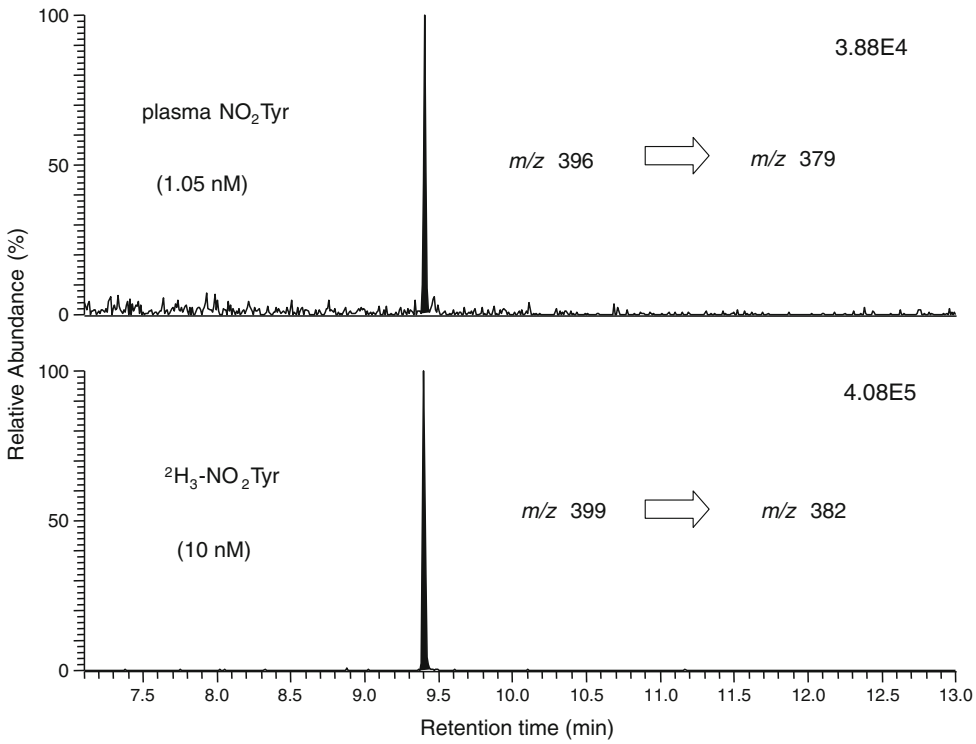

Fig. 8 Partial GC–MS/MS chromatograms from the analysis of endogenous soluble 3-nitro-L-tyrosine (*upper panel*) in plasma of a healthy subject using 3-nitro-L-[$^2H_3$]tyrosine as the internal standard (*lower panel*). Analysis was performed as described in Tsikas et al. 2003

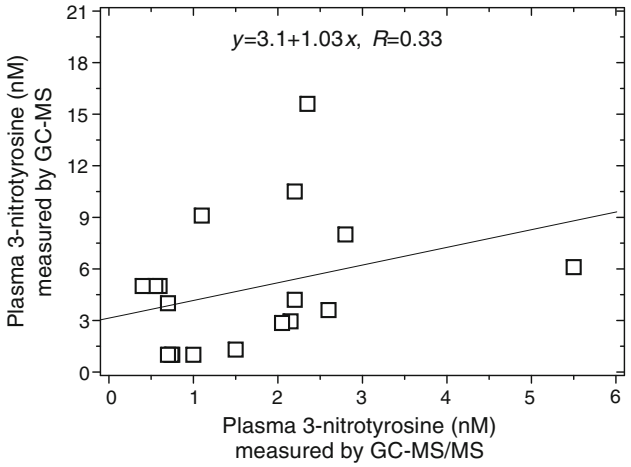

Fig. 9 Linear regression analysis between 3-nitro-L-tyrosine values measured in plasma samples of 18 healthy volunteers at the basal state by GC–MS and those measured by GC–MS/MS in the same samples as described elsewhere (Tsikas et al. 2003). In these plasma samples there was no correlation between GC–MS and GC–MS/MS. The higher 3-nitro-L-tyrosine concentrations measured by GC-MS indicate abundant interference

i.e. physicochemical analysis-based methodologies and immunology analysis-based assays.

The first category includes in-house developed instrumental methods including HPLC, GC, and CE with different detection systems from commercial sources. In most of the reported methods belonging to this class, the analytical performance in terms of linearity, precision, accuracy, sensitivity (LOD and LOQ), specificity, and interference studies including investigations on artifactual formation of 3-nitrotyrosine has been adequately reported. Thus, the reader is able to evaluate the validity of the reported analytical approach. Diametrically opposite to the above-mentioned instrumental techniques, the analytical performance of commercially available assays for 3-nitrotyrosine is scantily reported by the manufacturer and the user (Duncan 2003). Interestingly, commercial availability and adherence to the operating instructions seem to offer many investigators a guarantee for generation of valid analytical data and to lull them into security. Nitrotyrosine determination is not the only one of such procedures, but it is an outstanding example of where commercially available assays can confound rather than inform (Tsikas 2010a). In addition to lacking proper validity, both commercially available and home-made NO_2TyrProt assays suffer from using calibrators of which the chemical identity and quantity is mostly unknown and most likely greatly differing among laboratories. Furthermore, we may reasonably assume that synthetic routes led to tyrosine-nitrated proteins distinctly different from their endogenous congeners. Thus, one may not be surprised that immunological assays for 3-nitrotyrosine provide formidably different results which may range over three orders of magnitude (Safinowski et al. 2009; Wayenberg et al. 2009).

In summary, the very low concentration of NO_2Tyr and NO_2TyrProt, the high potential for abundant artifactual formation and the high risk for interference, and the small changes in NO_2Tyr and NO_2TyrProt concentrations due to disease or intervention make high demands on analytical methods intended for accurate and specific quantification in basic and in clinical research. Because of these special requirements, investigators need to validate thoroughly and carefully both adopted and newly developed physicochemical and immunological analytical methods for NO_2Tyr and NO_2TyrProt (Thompson et al. 2002; Duncan 2003; Tsikas 2006; Araujo 2009; Dewé 2009; Stöckl et al. 2009; Van Eeckhaut et al. 2009). Especially in immunological assays and for a fairly acceptable comparison of results, synthetic NO_2TyrProt used as calibrators need to be fully characterized structurally, notably regarding sites of tyrosine-nitration and other kinds of oxidative modifications that might potentially occur during synthesis.

Evaluation of biological 3-nitrotyrosine concentrations in health and disease from the analytical and review process perspectives

Commonly, and most frequently in studies published in primarily non-analytically oriented journals, the quality of the analytical methodologies used to quantitate nitrative stress is either not considered at all in evaluating the studies outcome or it is considered only tacitly. It is disconcerting that many investigators do not apply solid analytical criteria, but they decide on the quality of the analytical method on the basis of contentious and arbitrary criteria, such as *medical plausibility* and *clinical meaning* (for a discussion see Tsikas 2010a). Strictly speaking, the results published in such studies cannot be really valued. Objective evaluation of studies outcome would be seriously hampered as long as the performance of the analytical methods is not reported with satisfactory details and binding reference values and intervals for NO_2Tyr and NO_2TyrProt evade definition, despite the overwhelming evidence for such values. Thus, the fact of the matter is that the concentration of NO_2Tyr and NO_2TyrProt in plasma of healthy humans is on the threshold of the pM-to-nM range (Tables 2, 3) and their concentration changes only little upon disease, lifestyle including nutrition and physical exercise, and pharmacological treatment, provided reliable analytical methods were used (Table 4). It is advisable and justified to critically evaluate the potential biological significance of the results that originate from studies on 3-nitrotyrosine from the analytical perspective; i.e., on the

basis of the analytical reliability of the methods used. In this context, the NO_2Tyr and $NO_2TyrProt$ concentrations reported by thoroughly validated methods, notably those based on the MS/MS methodology, should serve as the *gold standard*.

Application of improper analytical methods for 3-nitrotyrosine in basic, animal, and clinical studies has led both to highly elevated 3-nitrotyrosine concentrations in many diseases, including asthma, cystic fibrosis, atherosclerosis, Alzheimer's diseases, and smokers to name the most prominent, and to dramatic concentration changes upon pharmacological treatment or physical exercise as compared to health and baseline, respectively. In contrast, most MS/MS-based studies revealed that the changes in 3-nitrotyrosine concentration associated with various disease states and intervention are modest at best (Table 4). From an analytical perspective, the potential role of 3-nitrotyrosine in health and disease and the extent of change of its concentration in animal, interventional, and clinical studies have been discussed elsewhere in detail (Tsikas 2006; Ryberg and Caidahl 2007).

The discussion of analytical methods for 3-nitrotyrosine would be fairly incomplete if issues related to the whole review process mainly in clinical journals and to the increasing use of commercially available *ready-to-use* assays would not be addressed properly. The fact that these particular points are not specific to 3-nitrotyrosine and other nitrated biomolecules (Niki 2009; Tsikas et al. 2009b, c),

Table 4 Some observational, clinical, and experimental studies reporting on changes of 3-nitrotyrosine concentrations

Authors	Approach	Analyte	Species	Matrix	Condition	Change (fold)
Schwemmer et al. (2000)	GC-FID	NO_2Tyr	Human	Urine	Before versus GTN	+3
Troost et al. (2000)	GC–MS/MS	NO_2Tyr	Human	Plasma	Nebivolol versus placebo	±1
Keimer et al. (2003)	GC–MS/MS	NO_2Tyr	Human	Plasma	Before versus after ISDN or PETN	−1.1 to +1.1
Keimer et al. (2003)	GC–MS/MS	NTALB	Human	Plasma	Before versus after ISDN or PETN	±1.0
Keimer et al. (2003)	GC–MS/MS	NHPA	Human	Urine	Before versus after ISDN or PETN	−1.1 to +1.1
Lärstad et al. (2003)	GC–MS/MS	NO_2Tyr	Human	EBC	Health versus asthma	±1.0 to −0.9
Shishehbor et al. (2003a)	LC–MS/MS	$NO_2TyrProt$	Human	Plasma	Patients, CAD versus non-CAD	+1.8
Shishehbor et al. (2003b)	LC–MS/MS	$NO_2TyrProt$	Human	Plasma	Patients, Before after atorvastatin	−1.4
Fahlbusch et al. (2004)	GC–MS/MS	NO_2Tyr/ NTALB	Human	Plasma	Carvedilol versus placebo	±1
Svatikova et al. (2004)	LC–MS/MS	NO_2Tyr	Human	Plasma	Health versus sleep apnea	±1
Göen et al. (2005)	LC–MS/MS	NO_2Tyr	Human	EBC	Smokers versus non-smokers	−0.8
Baraldi et al. (2006)	LC–MS/MS	NO_2Tyr	Human	EBC	Health versus asthma	+5 (NO_2Tyr/ Tyr ratio)
Celio et al. (2006)	GC–MS	NO_2Tyr	Human	EBC	Health versus asthma	−1.7
Tsikas (2006)	GC–MS/MS	NO_2Tyr	Human	Plasma	Health versus renal disease	+2.9
Chen and Chiu (2008)	LC–MS/MS	$NO_2TyrProt$	Human	Urine	Smokers versus non-smokers	±1
Pham et al. (2009)	GC–MS/MS	NO_2Tyr	Human	Urine	Rheumatism versus health	+3.1
Magné et al. (2009)	GC–MS/MS	NO_2Tyr	Rat	Plasma	High-fat meal	−0.5 to +2.8
Safinowski et al. (2009)	ELISA	NO_2Tyr	Human	Plasma	Health, diabetes, meal	±300; +60
Wayenberg et al. (2009)	ELISA	NTALB	Human	Plasma	Normal/mild versus severe NE	+2.0
Nemirovskiy et al. (2009)	LC–MS/MS	NO_2Tyr	Rat	Plasma/Urine	Normal versus CIA	+2.5/+1.4
Pop-Busui et al. (2009)	GC–MS	NO_2Tyr	Human	Plasma	Health versus diabetes	+5 (NO_2Tyr/Tyr ratio)

CAD coronary artery disease, *CIA* collagen-induced arthritis, *GTN* glycerol trinitrate, *ISDN* isosorbide dinitrate, *PETN* pentaerythrityl tetranitrate, *NTALB* 3-nitrotyrosinoalbumin, *NE* neonatal encephalopathy

but obviously apply to every endogenous analyte, does not release us from our responsibility to think about their impact on science. It seems that the importance of the analytical component of the work being considered for publication in primarily non-analytically oriented journals has not been adequately recognized by editors, reviewers, and authors (for a detailed discussion see Tsikas 2006). We all should be aware that the commercial availability of 3-nitrotyrosine assays is no guarantee of analytical reliability (Tsikas 2010a). Thus, there is a pressing need that work considered for publication in clinical journals be reviewed not only from a biological point of view, but with the same emphasis and thoroughness from the analytical standpoints. Adequate description of the analytical methods used in a study provides the reader with the possibility to evaluate the analytical dependability of the assays used and consequently of the results they delivered.

Conclusions and outlook

3-Nitrotyrosine occurs in many biological systems as a soluble amino acid (NO_2Tyr) and as a moiety in proteins and peptides (NO_2TyrProt). Reliable quantification of NO_2Tyr and NO_2TyrProt represents a real analytical challenge to man and machine.

Among the many technologies only very few have really the potential to quantify accurately NO_2Tyr and NO_2TyrProt in biological fluids, such as plasma, urine, CSF, and EBC. At present, the most efficient and dependable approaches are GC–MS/MS (for derivatized NO_2Tyr) and LC–MS/MS (both for derivatized and non-derivatized NO_2Tyr). Thoroughly validated methods based on GC–MS/MS and LC–MS/MS provided valuable information about the quantity of NO_2Tyr and NO_2TyrProt in biological matrices in various conditions (e.g. health, disease, and lifestyle). Unanimously, stable-isotope dilution GC–MS/MS- and LC–MS/MS-based methods revealed that the concentration of NO_2Tyr in human plasma is on the threshold of the pM-to-nM range and changes only very little upon disease or intervention. These important findings are suitable to serve as *the gold standard* and as a measure to test the reliability of alternative techniques, such as GC–MS, HPLC with electrochemical detection, or immunological assays. Immunoassays delivered greatly diverging values for NO_2Tyr and NO_2TyrProt. They need to be cross-validated by evidently reliable GC–MS/MS or LC–MS/MS methods.

Among numerous biomarkers for oxidative stress, 3-nitrotyrosine is generally accepted as a biomarker of nitrative stress (discussed by Dalle-Donne et al. 2006 and Giustarini et al. 2009). However, the utility of 3-nitrotyrosine as a biomarker of nitrative stress is mainly based on observations that resulted from experimental and clinical studies in which no appreciable attention had been paid to the reliability of the analytical methods used. Therefore, a great deal of our present knowledge of the pathophysiology and biochemistry of 3-nitrotyrosine should be put to the test. The mechanisms of formation, metabolism, and elimination of 3-nitrotyrosine, its pathophysiological role, and its appropriateness as a biomarker of nitrative stress need to be re-investigated by using analytical methods that allow for accurate and interference-free quantification of 3-nitrotyrosine, by using relevant concentrations for nitrating agents, and by avoiding the mistakes made in the past, both from an analytical and a review point of view.

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