

Analysis of the L-Arginine/Nitric Oxide Pathway: The Unique Role of Mass Spectrometry

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Abstract: Nitric oxide (NO) is a gaseous radical molecule. In human organism NO is produced in various cells from L-arginine by the catalytical action of NO synthases (NOS). The L-arginine/NO pathway powerfully contributes to maintain multiple physiological functions, including vascular tone, platelet function and neurotransmission. The metabolic fate of NO is very complex due to the participation of numerous compounds resulting from the ability of NO to react practically with any biomolecule to produce biologically active metabolites (e.g. S-nitrosothiols) and biologically inactive metabolites (e.g. nitrate). The concentration in biological fluids and tissues of members of the L-arginine/NO family is of particular interest, as it may characterize the status of this pathway in health and disease as well as to monitor the progress of pharmacological interventions. Thus, measurement of the NO metabolites nitrate and nitrite is suitable to assess NO synthesis *in vivo*. On the other hand, measurement of the circulating NOS inhibitor asymmetric dimethylarginine (ADMA) was found to reliably identify pathological conditions associated with NO-related endothelial dysfunction. Among the various analytical methods currently available for the analysis of the L-arginine/NO family, mass spectrometry (MS)-based approaches such as gas chromatography-mass spectrometry (e.g. GC-MS/MS) and liquid chromatography-mass spectrometry (e.g. LC-MS/MS) emerged indispensable analytical tools for the reliable quantitative analysis of the whole NO family. The present article discusses the currently available analytical methods especially emphasizing the importance of the MS technology to the NO field of research.

Keywords: L-arginine, nitric oxide (NO), nitrate, asymmetric dimethylarginine, 3-nitrotyrosine, mass spectrometry.

INTRODUCTION

L-Arginine (L-Arg) is a semi-essential amino acid physiologically involved in the urea cycle. In addition, L-Arg undergoes multiple metabolic fate, e.g. it is utilized in the synthesis of peptides and proteins. Methylation of the guanidino group of L-Arg in proteins leads to mono- and

oxidation of one N-atom of the guanidino group of L-Arg to the small free-diffusable, radical, bioactive gas NO, with L-citrulline (L-Citr) being the second reaction product (Fig. (1)). The mechanism of this reaction is not yet completely understood. By means of mass spectrometry (MS) it was found that one oxygen atom from one molecule of molecular

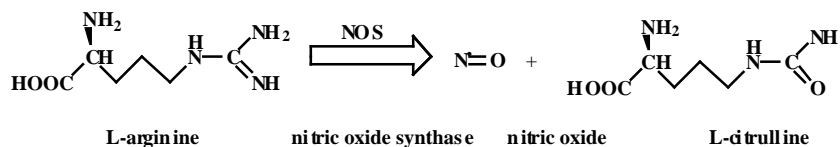


Fig. (1). Biosynthesis of nitric oxide (NO) from L-arginine. The enzyme NO synthase (NOS) catalyzes the oxidation of L-arginine to NO and L-citrulline by using molecular oxygen. For simplicity, other participants in this reaction including cofactors are not shown. More details are given in the text.

dimethylated L-Arg analogs. Protein metabolism recovers unchanged L-Arg and the methylated L-Arg analogs.

In 1987, the L-Arg/nitric oxide (NO) pathway has been discovered [1]. NO is generally accepted to be identical with the endothelium-derived relaxing factor (EDRF), which had been discovered already in 1980 [2]. NO synthase (NOS, EC 1.14.13.39), which is expressed in various cells including endothelial cells [3], was found to catalyze the unique

oxidation of one N-atom of the guanidino group of L-Arg to the small free-diffusable, radical, bioactive gas NO, with L-citrulline [4,5]. Oxidation of L-Arg to NO seems to proceed *via* intermediate formation of *N*^G-hydroxy-L-arginine. This compound was isolated from NOS incubates [6], and has been shown to be a substrate for NOS [7].

Interestingly, only a very small part of endogenous L-Arg (of the order of 0.2%) is oxidized by NOS to NO *in vivo* in humans [8]. However, endogenous NO production is obviously sufficient and highly regulated to maintain permanently various important physiological functions, such as modulation of vascular tone, platelet function, and neurotransmission, to name a few [9]. These functions are mainly mediated by cGMP [10].

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Table 1. Summary of the members of the L-arginine/nitric oxide pathway^a.

Compound	Abbreviation	Oxidation number of N	
<i>L-Arginine and derivatives</i>			
L-Arginine	L-Arg	-3	
N ^G -Hydroxy-L-arginine	NOHA	-1	
L-Citrulline	L-Citr		
Agmatine	AGMA		
N ^G -Monomethyl-L-arginine	NMMA	-3	
N ^G ,N ^G -Dimethyl-L-arginine	ADMA	-3	
N ^G ,N ^{G'} -Dimethyl-L-arginine	SDMA		-3
Monomethylamine	MMA	-3	
Dimethylamine	DMA	-3	
<i>NO and derivatives</i>			
Nitric oxide	NO	+2	
Nitrogen dioxide	NO ₂	+4	
Superoxide anion	O ₂ ⁻		-1/2 (O)
Peroxyntirite anion	ONOO ⁻	+3	-1 (OO)
Nitrite	NO ₂ ⁻	+3	
Nitrate	NO ₃ ⁻	+5	
Nitroxyl anion	NO ⁻	+1	
Dinitrogen oxide	N ₂ O	+1	
<i>Nitro and nitroso compounds</i>			
S-Nitrosohemoglobin	SNOHb	+3	
S-Nitroglutathione	GSNO ₂	+5	
S-Nitrosoglutathione	GSNO	+3	
S-Nitroalbumin	SNO ₂ ALB	+5	
S-Nitrosoalbumin	SNALB	+3	
S-Nitrocysteine	SNO ₂ C	+5	
S-Nitrosocysteine	SNC	+3	
3-Nitro-L-tyrosine	3-NT or NO ₂ Tyr	+5	
3-Nitro-4-hydroxyphenylacetic acid	NHPA	+5	
3-Nitrotyrosinoalbumin	NTALB	+5	

^aThis list does not include all known members of the L-Arg/NO pathway reported in the literature

Chemistry, biology and pharmacology [11] of the L-Arg/NO pathway are very complex. NO undergoes a series of chemical reactions with biomolecules belonging to various classes of compounds. A key step in the metabolic fate of NO is its reaction with the superoxide anion (O₂⁻), which may also be produced by NOS [12]. This extremely

rapid reaction leads to the formation of peroxyntirite (ONOO⁻) which may initiate additional reactions. The most preferred targets for NO and ONOO⁻ are thiols and aromates, in particular the amino acids L-cysteine (L-Cys) and L-tyrosine (L-Tyr), as well as the heme moiety of enzymes. Nitrogen-containing products formed in these reactions include S-nitrosothiols (RSNO) and S-nitrothiols (RSNO₂), 3-nitro-L-tyrosine (3-NT) and nitros(yl)ated hemoglobin, the majority of which may exert NO-like biological activities.

Many endogenous compounds, such as the N^G-methylated analogs of L-Arg, namely N^G-monomethyl-L-arginine (NMMA) and N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA), may inhibit NOS-catalyzed production of NO in a concentration dependent manner [13]. The metabolic fate of these compounds is, therefore, of particular interest. Thus, enzymic hydrolysis of ADMA to dimethylamine (DMA) and L-Citr by the dimethylarginine dimethylaminohydrolase (DDAH) is assumed to be the major inactivating pathway of ADMA [14]. DMA and a significant part of unmetabolized ADMA are eliminated by the kidney [14].

The complexity of the L-Arg/NO pathway (Table 1), the involvement of chemically labile substances including NO itself (t_{1/2} 0.1 s in human circulation [15]) and the occurrence of some of these compounds at very low concentrations in biological fluids represent a great challenge for analytical chemistry. Reliable quantitative determination of the L-Arg/NO pathway in different *in vitro* and *in vivo* conditions requires the use of highly specific, sensitive and accurate analytical approaches. At present, all of these requirements are best fulfilled by the MS technology which is characterized by inherent accuracy. MS in combination with modern chromatographic techniques, notably capillary gas chromatography (i.e. GC-MS and GC-MS/MS) and more recently with liquid chromatography (i.e. LC-MS and LC-MS/MS), turned out to be the most useful analytical approach in this topic. Members of the L-Arg/NO family (Table 1) include gaseous substances (e.g. NO, N₂O, DMA) as well as nonvolatile, highly polar and electrically charged organic and inorganic compounds (e.g. NO₂⁻, NO₃⁻, 3-nitro-L-tyrosine), many of them such as the S-nitrosothiols (e.g. S-nitrosocysteine) being thermally labile. Despite this heterogeneity, MS-based approaches are in principle applicable to any member of the L-Arg/NO family. Thus far, MS-based analytical methods have been reported for the majority of the members of the L-Arg/NO pathway.

The present article discusses recent developments in the analytical chemistry, biochemistry and pharmacology of the L-Arg/NO pathway, especially focussing on and emphasizing the contribution of the MS methodology to this topic. On the basis of current achievements, the importance and role of the MS technology to the research of the L-Arg/NO pathway in the near future is outlined.

PIONEER ROLE OF MASS SPECTROMETRY IN DISCOVERING L-ARGININE-DERIVED NO AND ELUCIDATING THE MECHANISM OF NO FORMATION

Hibbs and colleagues showed that L-Arg is the precursor for nitrite/nitrate in macrophages [16]. The same year, i.e.

1987, Palmer *et al.* suggested that EDRF and NO are identical species [17]. Also, on the basis of similarity of biological and chemical properties (e.g. reaction with hemoglobin to produce nitrosylhemoglobin) of EDRF and NO, Ignarro *et al.* presented evidence in 1987 that the EDRF produced and released from artery and vein is NO or a labile nitroso species [18]. Definite evidence that nitrite/nitrate are derived exclusively from the terminal guanidino N-atoms of L-Arg in activated macrophages was presented by Iyengar *et al.* by means of GC-MS in 1987 [19]. In their experiments the authors incubated macrophages with ^{15}N -labelled L-arginine, i.e. L-[guanidino- $^{15}\text{N}_2$]-arginine, and measured ^{15}N enrichment in cell culture supernatants of nitrite and nitrate after their conversion to nitrobenzene [20,21], and of *N*-nitrosomorpholine which was formed from the reaction of macrophage-derived nitrosyl ($^{\cdot}\text{NO}$) and morpholine externally added to capture nitrosyl groups. In 1988, Palmer *et al.* demonstrated by MS studies using L-[guanidino- $^{15}\text{N}_2$]-arginine that vascular endothelial cells synthesize NO from L-Arg [22]. Interestingly, formation of NO in that study was demonstrated directly, i.e. by measuring ^{15}NO itself (i.e. *m/z* 31) without any derivatization step. Almost at the same time and by means of the same methodology, i.e. MS technology in combination with the use of L-[guanidino- $^{15}\text{N}_2$]-arginine, it was definitely demonstrated that L-Arg is a physiological precursor of endothelium-derived nitric oxide (EDNO) [23, 24], and that NO is an intermediate in the oxidation of L-Arg to nitrite and nitrate in macrophages [25].

The same methodology has also been applied to humans, rats and ferrets [26,27]. The group of Tannenbaum demonstrated that humans, to whom L-[guanidino- $^{15}\text{N}_2$]-arginine was given as an oral dose, excreted ^{15}N -labelled nitrate in the urine, thus definitely demonstrating that L-arginine is a precursor for nitrate biosynthesis in humans [26,27]. In addition, this group found that nitrate synthesized by mammalian cells *in vivo* undergoes losses similar to those for exogenous nitrate. In that study nitrate was measured by the GC-MS method of Green *et al.* [21], i.e. after conversion of nitrate to its nitrobenzene derivative.

By means of GC-MS, chemiluminescence and diazotization, Moncada's group demonstrated that endogenous L-Arg-derived NO is present in the exhaled air of humans [28]. To demonstrate this, the authors trapped NO as nitrosothiopropine using an aqueous solution of thiopropine and analysed them in the positive chemical ionization mode. These experiments suggested that NO may play both vascular regulatory and host defense roles in pulmonary physiology and pathophysiology.

After having demonstrated that L-Arg is the precursor of NO and L-Citr, it was of major interest to delineate the underlying mechanism of this reaction. Two mechanisms of NO and L-Citr formation from L-Arg had been proposed [16,25]. Both mechanisms predicted incorporation of an O atom from water to form L-Citr. The only possibility to test the proposed mechanisms was the use of MS in combination with the use of ^{18}O -labelled water, i.e. H_2^{18}O , and ^{18}O -labelled molecular oxygen, i.e. $^{18}\text{O}_2$. Two independent researcher groups have addressed this issue almost at the same time [4,5,29]. Incorporation of ^{18}O into NO and L-Citr would form species being by 2 atomic mass units heavier

than in case of ^{16}O incorporation. These distinctly weighing species can be easily separated and quantified by MS. GC-MS experiments by Kwon *et al.* [4] clearly demonstrated that the O atom in the ureido group of the L-Citr product of macrophage NOS originates from dioxygen (O_2), not from water (H_2O). Leone *et al.* [5] and Stuehr *et al.* [29] demonstrated unequivocally that both constitutive and inducible NOS incorporate molecular oxygen into both NO (measured as nitrosomorpholine) and L-Citr, excluding reaction mechanisms that hinge on water to provide ureido oxygen. In addition, the experiments by Stuehr *et al.* provided solid evidence that *N*^G-hydroxy-L-arginine is an intermediate in the biosynthesis of NO from L-Arg [29]. Interestingly, the redox cofactors NADPH and tetrahydrobiopterin have been found to be required for the synthesis of NO from either L-Arg or *N*^G-hydroxy-L-arginine [29]. With L-Arg, the synthesis of 1 mol of NO was coupled to the oxidation of 1.5 mol NADPH; whereas with *N*^G-hydroxy-L-arginine, only 0.5 mol of NADPH was oxidized per 1 mol of NO formed [29]. Investigations by Pufahl *et al.* [6] using ^{15}N -labelled *N*^G-hydroxy-L-arginine and by Klatt *et al.* [7] clearly established that *N*-hydroxylation is the first step in the conversion of L-Arg to NO.

MS was of fundamental importance to elucidate the NOS-catalyzed conversion of L-Arg to NO and L-Citr. Nevertheless, the mechanism of the second step of this reaction, i.e. the oxidation of the guanidino C atom of *N*^G-hydroxy-L-arginine to form L-Citr and NO, still remains almost completely unresolved. Presumably, the oxidation of the guanidino C atom of *N*^G-hydroxy-L-arginine produces a highly unstable intermediate, such as the *N*^G-hydroxy-*C*^G-hydroxy-L-arginine, that has been not accessible to GC-MS analysis because of the required derivatization which is usually performed at high temperatures. Perhaps is LC-MS better appropriate to investigate this issue.

ASSESSMENT OF NITRIC OXIDE SYNTHESIS FROM L-ARGININE *IN VIVO* IN HUMANS

GC-MS experiments using L-[guanidino- $^{15}\text{N}_2$]-arginine in animals by Tannenbaum's group showed that only a very small part of orally or intravenously administered L-[guanidino- $^{15}\text{N}_2$]-arginine is converted to ^{15}N -labelled nitrate [27]. In ferrets, only approximately 0.1 % of the administered L-[guanidino- $^{15}\text{N}_2$]-arginine dose were found to be incorporated in urinary nitrate under basal conditions [27].

The order of magnitude of L-Arg involvement in the L-Arg/NO pathway in humans, in particular in comparison to the urea cycle, was investigated by Hibbs and colleagues in patients receiving interleukin-2 (IL-2) therapy by means of GC-MS by using L-[guanidino- $^{15}\text{N}_2$]-arginine [8]. For this purpose very large amounts of L-[guanidino- $^{15}\text{N}_2$]-arginine (i.e. 3.9 g, 18.4 mmol) were administered intravenously. Nitrate and urea were analysed by GC-MS in the electron ionization mode as described by Green *et al.* [21] (for nitrate) and by Tserng and Kalhan [30] (for urea). Before IL-2 mediated induction of NO synthesis, only 0.1 % of the infused L-[guanidino- $^{15}\text{N}_2$]-arginine molecules were excreted into the urine as ^{15}N -labelled nitrate during the

subsequent 24-h period. In comparison, 17 % of the infused L-[guanidino-¹⁵N₂]-arginine molecules were excreted into the urine as ¹⁵N-labelled urea during the same period. After completion of the induction course of IL-2, 0.7 % of the infused L-[guanidino-¹⁵N₂]-arginine dose was excreted into the urine as ¹⁵N-labelled nitrate. This represents a seven-fold increase in incorporation of ¹⁵N-labelled terminal guanidino nitrogen atoms of L-[guanidino-¹⁵N₂]-arginine into urinary ¹⁵N-labelled nitrate. On the other hand, urinary excretion of ¹⁵N-labelled urea did not increase (16 %) upon IL-2 induction in that study. The same methodology has also been used by Katz *et al.* to investigate the L-Arg/NO pathway in patients with congestive heart failure and in healthy subjects serving a control [31]. After infusion of L-[guanidino-¹⁵N₂]-arginine (40 μmol/kg), 24-h urinary excretion of ¹⁵N-labelled nitrate was found to be decreased in the patients both at rest (2.2 versus 8.0 μmol/24 h) and during submaximal exercise (2.4 versus 11.4 μmol/24 h) compared with control subjects [31]. In accordance with Hibbs *et al.* [8], Katz *et al.* [31] found that 24-h urinary excretion of ¹⁵N-labelled urea at rest in the patients did not differ from that in the healthy subjects (1.1 versus 1.2 mmol/24 h).

¹⁵N-Labeling experiments with intravenously administered L-[guanidino-¹⁵N₂]-arginine in patients suffering from essential hypertension and MS analysis of ¹⁵N-labelled nitrate were also carried out by Forte *et al.* [32]. In that study L-[guanidino-¹⁵N₂]-arginine (200 mg, 1.14 mmol) was administered intravenously over 10 min, urinary nitrate was extracted and converted to ammonia which was further converted to nitrogen gas by combustion. Unlabelled and ¹⁵N-labelled nitrogen, i.e. ¹⁴N₂ and ¹⁵N₂, respectively, were measured by MS. Forte *et al.* [32] found that 36-h urinary excretion of ¹⁵N-labelled nitrate was significantly lower in the hypertensives than in the normotensives (1313 versus 2133 nmol), suggesting that basal NO synthesis is diminished in patients with essential hypertension. Forte *et al.* [32] confirmed the finding by Hibbs *et al.* [8] that much less than 1 % of administered L-[guanidino-¹⁵N₂]-arginine is excreted into the urine as ¹⁵N-labelled nitrate.

Quantitative analytical methods have greatly contributed to evaluate some physiological, pathophysiological, and potential therapeutic roles of NO. The basis for the development of quantitative methods is the proper knowledge of the metabolism and elimination of NO. Experimental and clinical studies by Wennmalm *et al.* showed that the major metabolic pathway for endogenously formed NO includes uptake into the red blood cells and oxidation to nitrate which is eliminated from the plasma via the kidneys [33]. By means of GC-MS-validated chromatographic methods, Wennmalm *et al.* showed that inhalation of NO by healthy volunteers caused increase in plasma nitrate from 26 to 38 μM after 60 min [33]. By contrast, plasma nitrite did not increase during NO inhalation in that study; after 30 and 60 min of inhalation, the plasma levels of nitrite were 1.1 and 1.2 μM, respectively, with the basal plasma level of nitrite being 1.3 μM [33].

Definite evidence that the L-Arg/NO pathway is the major source of plasma nitrite in humans was presented by Rhodes *et al.* by means of ¹⁵N-labelling GC-MS experiments [34]. The GC-MS methods previously used in ¹⁵N-labelling

GC-MS experiments were based on the conversion of nitrate to nitrobenzene or other nitroaromatics. This method, however, does not allow direct detection of nitrite, because nitrite has to be oxidized to nitrate prior to the nitration reaction. Direct GC-MS analysis of nitrite was possible after the discovery of an alternative derivatization reaction using pentafluorobenzyl (PFB) bromide (PFB-Br) as the derivatization agent [35]. Several years later it has been discovered that this derivatization agent can also be applied to derivatize nitrate [36]. By these reactions nitrite and nitrate are converted to their volatile and strongly electron-capturing PFB derivatives [35,36]. The derivatization reactions for the GC-MS analysis of nitrite and nitrate are described below in detail. Infusion of L-[guanidino-¹⁵N₂]-arginine into fasted humans and GC-MS analysis of plasma nitrite as PFB derivative unequivocally demonstrated that as much as 90 % of circulating nitrite is derived directly from the L-Arg/NO pathway in fasted humans [34]. Rhodes *et al.* showed that the GC-MS method of Tesch *et al.* for nitrate [20], which involves conversion of nitrate to nitrobenzene, is subject to interference from non-nitrate sources in plasma. Nevertheless, the results of this group endorse the use of nitrite and nitrate as quantitative indices of NO production in human plasma [34]. Indeed, ¹⁵N-labelling experiments in animals with intravenously or orally given L-[guanidino-¹⁵N₂]-arginine and GC-MS analysis of nitrite and nitrate as PFB derivatives revealed that nitrite and nitrate are circulating and excretory metabolites of L-Arg-derived NO [37-39]. Circulating nitrite rather than nitrate turned out to reflect endothelial-dependent NO synthesis in humans and mammals [40-42].

Recent methods of analysis based on the use of stable-isotope labelled L-Arg and L-Citr to measure metabolism of L-Arg-derived NO have been reviewed by Luiking and Deutz [43]. The authors concluded that this technique is the most accurate method to study quantitative changes in the NO production rate in both healthy and diseased humans [43].

QUANTITATIVE ANALYSIS

General Considerations

GC analysis of nitrite, nitrate and other members of the L-Arg/NO family (Table 1) requires their conversion into volatile and thermally stable derivatives. The choice of chemical derivatizing agents is mainly directed to the functional groups of the molecules and to the method of detection. Negative-ion chemical ionization (NICI) in GC-MS allows for highly sensitive analysis. Maximum sensitivity can be achieved by using derivatizing agents possessing electron-capturing elements such as fluorine. Fluorine-containing derivatizing agents such as pentafluorobenzyl bromide (for nitrite, nitrate), pentafluoro-propionic anhydride (for 3-nitro-L-tyrosine, ADMA and other amino acids), and pentafluorobenzoyl chloride (for DMA) have found wide application to the NO field of research (see below).

The S-nitroso group of S-nitrosothiols is thermally highly unstable. In general, chemical derivatives of these NO metabolites, which still contain the S-nitroso groups, are not

accessible to GC-MS analysis. Therefore, the *S*-nitroso group of *S*-nitrosothiols must be converted into more suitable species such as nitrite. However, this proceeding requires elimination of blank nitrite or accurate determination of its concentration and subtraction from the total value (i.e. blank nitrite + *S*-nitrosothiol-derived nitrite). Because nitrite levels are usually far above *S*-nitrosothiol levels, such analytical methods may become complex, time-consuming and more importantly inaccurate by interfering nitrite.

Severe analytical problems may originate from the easy and abundant artifactual formation of *S*-nitrosothiols and 3-nitro-L-tyrosine, in particular under acidic conditions. Therefore, many precautions have to be taken, including use of further chromatographic procedures. Finally, another analytical hurdle is the biological matrix and the laboratory materials used. The procedures used to draw blood, generate and storage plasma or serum must be standardized and consider all potential experimental conditions that may influence the reliability of the analytical method. Impressive indicators of existing analytical difficulties in the field of NO research are the wide reported ranges for basal levels of many members of this family. Thus, circulating *S*-nitrosothiols have been detected in plasma of healthy humans at concentrations between 1 nM and 10 μ M, which covers a range of four orders of magnitude. Also, 3-nitro-L-tyrosine basal levels in human plasma have been reported to range between 1 nM and at least 60 nM. Even for circulating nitrite basal levels have been reported to range between 100 nM and 10 μ M. The specific problems with these and other members of the L-Arg/NO family are discussed below separately. The issue concerning the wide variability of published basal levels for many members of the L-Arg/NO family, in particular for *S*-nitrosothiols and 3-nitro-L-tyrosine, is currently intensively discussed in the literature [44-51]. Unlike 3-nitro-L-tyrosine, circulating *S*-nitrosothiols have evaded the definition of reference intervals to date. Therefore, at least the physiological and pathological roles of these biologically potent NO derivatives are at present uncertain and questionable.

The most promising technology to achieve accurate quantitative determination of several members of the L-Arg/NO pathway in various biological fluids and tissue is the use of MS. The MS approach is the single technology in which quantification is carried out by using stable-isotope labelled analogs of the endogenous compounds. These so-called internal standards, e.g. ^{15}N -labelled nitrite (i.e. $^{15}\text{NO}_2^-$) for the analysis of endogenous nitrite (i.e. $^{14}\text{NO}_2^-$), are added to the respective biological matrix and undergo all chemical and physical changes during the whole analytical process, such as extraction, derivatization and chromatographic separation. Thus, endogenous compounds and internal standards behave almost identically until their separation in the mass spectrometer according to their distinctly different mass-to-charge (m/z) ratios, e.g. m/z 46 for $^{14}\text{NO}_2^-$ and m/z 47 for $^{15}\text{NO}_2^-$. In all the other non-MS approaches, quantification must be performed by using calibration curves which have to be generated in matrices distinctly different from the biological matrices.

The following sections concisely describe the principles and applications of MS-based analytical methods for a series

of members of the L-Arg/NO family and discuss the importance of these methods in defining reference values and evaluating non-MS-based analytical methods.

NITRITE AND NITRATE

Prior to identification of nitrite and nitrate as the major metabolites of endogenous NO, these anions have attracted attention because of their cancerogenic potential. Analytical methods for the quantitative determination of nitrite and nitrate in biological fluids available in this area of research have been adapted to the L-Arg/NO pathway. In addition novel methods based on different principles, including spectrophotometry, fluorometry, high-performance liquid chromatography (HPLC), capillary isotachopheresis and capillary electrophoresis (CE), and GC-MS have been developed and are currently used in the NO field in experimental and clinical studies. Methods of analyses of dietary and NO-derived nitrite and nitrate from the clinical biochemistry point of view have been reviewed by Ellis *et al.* [52].

The most famous and most frequently used method to measure nitrite and nitrate is based on the Griess reaction which is a diazotization reaction and is known since 1879 [53]. Analysis of nitrate by the Griess reaction requires preceding reduction to nitrite. Nevertheless, assays based on the Griess reaction, in particular the so-called batch Griess assays, are subject to numerous interferences [54]. In this assay, interferences may occur even when nitrate is analyzed in freshwater, in particular after reduction with cadmium to nitrite [55]. Interferences many also include anticoagulants and other preanalytical factors despite use of nitrate reductase for the reduction of nitrate to nitrite [56].

Among the analytical methods reported so far, only very few are suitable for the quantitative measurement of nitrite and nitrate in biological fluids, in particular in plasma, serum and urine of humans. Basal levels for circulating and excretory nitrite and nitrate generated by various analytical methods including GC-MS methods, which are published in chemical analysis-oriented journals or are sufficiently described in non-analytical journals, are summarized in Table 2 and Table 3, respectively. Obviously, these methods have generated diverging values for nitrite and nitrate in human plasma and serum. The applicability to human urine of the majority of the analytical methods listed in (Table 2) has not been demonstrated so far. In case of collecting 24-h urine, urinary nitrate levels are expressed as $\mu\text{mol}/24\text{ h}$; when urine is collected by spontaneous micturition, urinary excretion of nitrate should be corrected for urinary creatinine (Table 3). Only very few non-GC-MS assays reported have been thoroughly validated by GC-MS [20,33,57] or other methods [42].

In principle, two derivatization reactions were found to be useful for the analysis of nitrite and nitrate by GC-based techniques such as GC-MS (Fig. (2)). The first method utilizes an aromatic compound such as benzene or trimethoxybenzene which serves as electrophile, requires concentrated sulfuric acid as the catalyst, and yields volatile, thermally stable, and electron-capturing nitroaromates (Fig. (2A)) [20,21,58,59]. The second reaction uses PFB-Br as the

Table 2. Nitrite and nitrate concentrations (in μM) in blood plasma/serum of healthy humans at the basal state as measured by mass spectrometry-based and non-mass spectrometry-based assay methods reported in the literature.

Authors	Nitrite	Nitrate	Method	Ref.
Tesch <i>et al.</i> 1976	N.R. ^a	N.R.	GC-ECD	[20]
Green <i>et al.</i> 1982	N.R.	15 – 60	Griess (automated)	[21]
Farell <i>et al.</i> 1992	0.14	N.R.	CL	[63]
Kanno <i>et al.</i> 1992	N.R.	22	Griess (automated)	[64]
Hibbs <i>et al.</i> 1992	32 (nitrite+nitrate)		Griess (batch)	[8]
Wennmalm <i>et al.</i> 1993	1.3	26	HPLC-UV	[33]
Misko <i>et al.</i> 1993	N.R.	N.R.	Fluorometry	[65]
Leone <i>et al.</i> 1994	0.45	41	CE	[66]
Tsikak <i>et al.</i> 1994	1.8	38	GC-MS	[67]
Rhodes <i>et al.</i> 1995	0.6	37	GC-MS (?)	[34]
Ueda <i>et al.</i> 1995	3.3	52	CZE	[68]
Moshage <i>et al.</i> 1995	1.3 – 13	4 – 45	Griess (batch)	[69]
Preik-Steinhoff, Kelm 1996	0.58	25	HPLC-ECD	[70]
El Menyawi <i>et al.</i> 1998	1.1 (nitrite+nitrate)		HPLC-UV	[71]
Smythe <i>et al.</i> 1999	N.R.	8 - 81	GC-MS	[61]
Tsikak <i>et al.</i> 1999	0.55	27	HPLC-UV	[72]
Tsikak <i>et al.</i> 1999	N.R.	27	GC-MS/MS	[62]
Lauer <i>et al.</i> 2001	0.3	25	FIA-Griess	[41]
Kleinbongard <i>et al.</i> 2003	0.3	24	FIA; CL; HPLC	[42]

^aN.R., not reported.**Table 3. Nitrite/nitrate concentrations (μM , $\mu\text{mol}/\text{mmol}$ creatinine or $\mu\text{mol}/24$ h) in urine of healthy humans at the basal state as measured by mass spectrometry-based and non-mass spectrometry-based assay methods reported in the literature.**

Authors	Nitrite/Nitrate	Method	Ref.
Radomski <i>et al.</i> 1978	768 μM	Colorimetry	[73]
Green <i>et al.</i> 1982	250 – 2000 μM	Griess (automated)	[21]
Kanno <i>et al.</i> 1992	124 $\mu\text{mol}/\text{mmol}$	Griess (automated)	[64]
Hibbs <i>et al.</i> 1992	690 $\mu\text{mol}/24$ h	Griess (batch)	[8]
Wennmalm <i>et al.</i> 1993	20 – 170 μM	HPLC-UV	[33]
Bode-Böger <i>et al.</i> 1994	104 $\mu\text{mol}/\text{mmol}$	GC-ECD	[74]
Tsikak <i>et al.</i> 1994	49 – 109 $\mu\text{mol}/\text{mmol}$	GC-MS	[67]
Moshage <i>et al.</i> 1995	990 μM	Griess (batch)	[69]
Smythe <i>et al.</i> 1999	23 – 1865 μM	GC-MS	[61]
Tsikak <i>et al.</i> 1999	178 – 2889 μM	GC-MS/MS	[62]
Tsikak 2004	1100 μM	HPLC-UV	[57]

derivatization agent. Nucleophilic substitution of bromide in PFB-Br by nitrite and nitrate leads to the formation of the nitro PFB derivative (PFB-NO₂) and nitric acid ester PFB derivative (PFB-ONO₂), respectively (Fig. (2B)) [35,36].

Under the strong acidic conditions of the nitration reaction, nitrite can also be converted to the same nitroaromatic derivative, but maximum recovery requires preceding oxidation of nitrite to nitrate, for instance by means of H₂O₂ [59]. This derivatization method has found

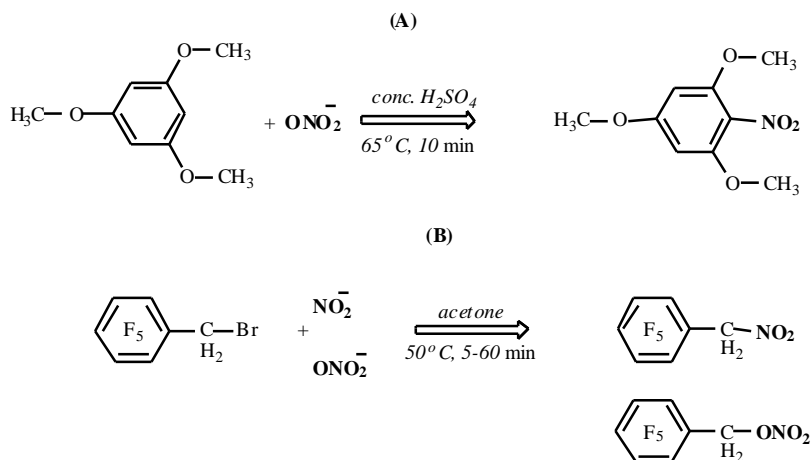


Fig. (2). Derivatization reactions of nitrite and nitrate for gas chromatographic analysis. (A) Nitration of trimethoxybenzene by means of concentrated sulfuric acid. (B) Nucleophilic substitution of Br from pentafluorobenzyl bromide by nitrite and nitrate to produce the nitro derivative and the nitric acid ester, respectively. For more details see the text.

application to the quantitative measurement of nitrite and nitrate in various biological fluids, but has severe drawbacks.

A major shortcoming of this derivatization reaction is that nitrite can not be accurately determined in the presence of high excess (e.g. 40-fold or higher) of nitrate over nitrite, which is the situation in human plasma/serum and in particular in urine. Moreover, in contrast to the method involving use of PFB-Br, the nitration reaction can not be used for the simultaneous analysis of nitrite and nitrate. On the other hand, conversion of nitrite, nitrate, and their ^{15}N -labelled analogs into the corresponding PFB derivatives proceeds equally effective in every biological matrix and does not require any sample pretreatment [36]. During derivatization of matrices rich in proteins, such as plasma and tissue homogenates, protein precipitation readily occurs, but this does not affect derivatization. Simultaneous determination of nitrite and nitrate in human plasma by GC-MS after extractive alkylation with PFB-Br using tetradecyldimethylbenzylammonium chloride as the phase-transfer catalyst with 1,3,5-tribromobenzene derivatives as the internal standard has also been reported [60]. However, extractive alkylation of nitrite and nitrate by PFB-Br is not necessary and seems not to represent an improvement of the conventional „one-phase“ derivatization procedure [35,36].

The second drawback of the nitration reaction is that unknown endogenous compounds may interfere with the nitrate measurement, because they decompose to form nitroaromatics during derivatization [34]. Nitration of aromatics has been improved by using trifluoroacetic anhydride instead of concentrated sulfuric acid as the catalyst and toluene as the electrophile [61]. This nitration reaction produces three nitrotoluene isomers. Smythe and colleagues reported on the usefulness of the derivatization reaction to analyze nitrate in human plasma and urine [61]. It is unknown whether this alternative nitration reaction can be applied to specifically measure nitrite.

Further major advantage of analyzing by GC-MS of nitrite and nitrate as PFB derivatives over nitroaromatics, such as nitrobenzene and nitrotoluene, originates from the unique

ionization and fragmentation of PFB- NO_2 and PFB- ONO_2 under NICI conditions. Unlike the nitroaromatics, PFB- NO_2 and PFB- ONO_2 ionize to produce nitrite (NO_2^- , m/z 46) and nitrate (NO_3^- , m/z 62), respectively. MS detection of these ions is superior over detection of other ions such as the nitrobenzene cation ($\text{C}_6\text{H}_5\text{NO}_2^+$, m/z 123) [21] that contain carbon atoms. Analysis of nitrite and nitrate as PFB derivatives by using their ^{15}N -labelled analogs yields linear calibrations over a large concentration range covering at least two orders of magnitude, and allows simple calculation of endogenous NO_2^- and NO_3^- concentrations [62]. By contrast, analysis of nitrate as nitrobenzene requires correction because of considerable contribution of the ^{13}C -isotope (1.1% natural abundance) to the ^{15}N -isotope [21]. This is of particular importance in ^{15}N -labelling experiments, in which the $^{15}\text{N}/^{14}\text{N}$ ratio in nitrite and nitrate is required.

Extensive studies on interferences, including use of the highly specific GC-MS/MS technology, have shown that the GC-MS quantification of urinary and circulating nitrite and nitrate as PFB derivatives is free of any interference [36,62]. Thus, no other compounds from urine and plasma found to coelute with the PFB derivatives of nitrite and nitrate and to produce the ions with m/z 46 and m/z 62. Also, nitrite and nitrate found not to interfere with each other either during derivatization or GC-MS analysis [36].

The possibility of measuring nitrite independently of nitrate, even at nitrite-to-nitrate molar ratios of 1:1000, enabled both to demonstrate the physiological presence of nitrite in urine of healthy volunteers and to quantitate its excretion rate. Thus, healthy young volunteers with uncontrolled nitrate diet were found to excrete 5.7 μmol of nitrite per day, equivalent to a creatinine-corrected excretion rate of 0.49 $\mu\text{mol}/\text{mmol}$ creatinine [67]. The corresponding values for urinary nitrate were 1226 $\mu\text{mol}/24$ h and 109 $\mu\text{mol}/\text{mmol}$ creatinine, whereas the plasma levels were 3.7 μM for nitrite and 68 μM for nitrate. In urine and plasma samples of volunteers on standardized low-nitrate diet significantly lower nitrite and nitrate levels were measured

compared with those determined in the volunteers with uncontrolled nitrate diet (i.e. approximately half of those) [67]. That the L-Arg/NO pathway is the major source of urinary nitrite was demonstrated *in vivo* in the mouse by giving orally L-[guanidino-¹⁵N₂]-arginine (10 mg/100 ml drinking water) and measuring the ratio of *m/z* 47 to *m/z* 46 by GC-MS [38]. GC-MS quantitative determination of nitrite and nitrate as PFB derivatives in serum and urine of healthy volunteers who received organic nitrates (i.e. isosorbide dinitrate or pentaerythryl tetranitrate) presented evidence that organic nitrates are metabolized in part to nitrite which is then eliminated by the kidney [75]. Further applications of this GC-MS approach to experimental and clinical studies in the frame of the L-Arg/NO research have been discussed elsewhere [76].

S-NITROSOTHIOLS

In 1992, Stamler *et al.* reported for the first time that NO circulates in plasma of healthy human primarily as *S*-nitrosoalbumin (SNALB) at a concentration of about 7 μ M [77]. On the basis of this finding, Stamler *et al.* suggested that SNALB may be a physiological reservoir of NO, by which NO-related biological actions such as vasodilation are regulated in humans [77]. This highly interesting finding has initiated much scientific work in this area. However, until today there is no solid confirmation of Stamler's group originally reported values and hypothesis. Further endogenously occurring *S*-nitrosothiols are *S*-nitrosohemoglobin (SNOHb) and *Fe*-nitrosohemoglobin (HbFeNO) the concentration of which in blood has been reported to be of the order of 32 - 894 nM [78]. One of the most characteristic chemical reaction of *S*-nitrosothiols is the transfer of its nitrosyl group (⁺NO) to other thiols [79]. Therefore, formation of a single *S*-nitrosothiol, e.g. SNALB or SNOHb, would necessarily lead to formation of *S*-nitrosothiols of all endogenous thiols, including *S*-nitrosocysteine (SNC), *S*-nitrosoglutathione (GSNO) and *S*-nitrosoproteins. In addition to the potential physiological roles of *S*-nitrosothiols [50,80-83], this class of compounds has also attracted attention because of its therapeutic potential as NO donating drugs [84,85]. Interestingly, *S*-nitrosothiols such as the synthetic *S*-nitroso-*N*-acetylpenicillamine (SNAP) have been shown *in vivo* in the rabbit not to induce tolerance, unlike the organic nitrate glycerol trinitrate [86].

Numerous analytical methods have been developed for the quantitative analysis of *S*-nitrosothiols in relevant biological matrices. The majority of the analytical methods available to nitrite and NO has also been extended to *S*-nitrosothiols. These methods involve conversion of the *S*-nitroso group to nitrite, e.g. by means of the Saville reaction originally developed to determine thiols [87], or reduction of the *S*-nitroso group to NO. These analytical methods have been used to quantitate in particular circulating *S*-nitrosothiols in health and disease, and in experimental and clinical studies. Divergence in basal levels of the extent of four orders of magnitude, severe analytical shortcomings and numerous pitfalls, and not unimportantly insufficient payment of attention by reviewers and editors of non-analytical journals to the analytical chemistry in the NO

clinical research, reasonably question scientific findings concerning physiology and pathology of *S*-nitrosothiols [44,45,47-49,51].

Analogous to nitrite and nitrate, only very few groups worldwide considered the MS technology in their research, and only relatively rarely have been applied MS-based analytical methods to *S*-nitrosothiols until today. Due to the thermal lability of the *S*-nitroso group, *S*-nitroso compounds lost this functional group during GC-MS. In addition, the majority of these compounds are non-volatile. These properties hinder the GC-MS analysis of endogenous low-molecular-mass *S*-nitrosothiols. From this point of view, LC-MS should be considered more appropriate for the MS analysis of native and derivatized low-molecular-mass *S*-nitrosothiols including *S*-nitrosoglutathione and *S*-nitrosocysteine [88,89,90]. Kluge *et al.* showed by LC-MS that *S*-nitrosoglutathione is physiologically present in rat cerebellum and quantified it by using *S*-[¹⁵N]nitrosoglutathione as internal standard [88]. This group also clearly demonstrated by LC-MS artifactual abundant formation of *S*-nitrosoglutathione during sample treatment [88]. Tsikas *et al.* have shown by LC-MS/MS formation of *S*-[¹⁵N]nitrosoglutathione in the cytosol of intact human blood cells upon incubation with *S*-[¹⁵N]nitrosocysteine, indicating existence of a selective transport system for *S*-nitrosocysteine in human erythrocytes [89]. *S*-Nitrosothiols (RSNO) and *S*-nitrothiols (RSNO₂) have almost identical chromatographic and spectrophotometric properties [90,91]. Thus, in principle we do not certainly know whether the substances expected to be *S*-nitrosothiols are indeed *S*-nitrothiols or *S*-nitrosothiols. These compounds can be formed by the reaction of thiols with peroxyxynitrite (ONOO[•]). By means of LC-MS/MS, Balazy *et al.* showed that *S*-nitrosoglutathione (GSNO₂), but not *S*-nitrosoglutathione (GSNO) is formed from the reaction of glutathione (GSH) with peroxyxynitrite [90]. Nevertheless, the reaction of thiols with peroxyxynitrite, the reaction products formed and the underlying mechanisms are insufficiently investigated and incompletely understood. Eventually, it should be pointed out that LC-MS is the only methodology that allows qualitative and quantitative analysis of high-molecular mass *S*-nitrosothiols such as *S*-nitrosohemoglobin [92]. By means of the electrospray ionization (ESI) LC-MS methodology, Ferranti *et al.* demonstrated *in vitro* in hemolysates that -Cys93 of hemoglobin is the preferred site of *S*-nitrosylation by externally added *S*-nitrosocysteine [92].

Scharfstein *et al.* reported that *S*-nitrosocysteine is physiologically present in rabbit plasma at a mean basal concentration of about 200 nM [93]. This observation and the physiological urinary excretion of cysteine as *N*-acetylcysteine prompted investigations on the occurrence of *S*-nitroso-*N*-acetylcysteine (SNAC), i.e. the mercapturate of *S*-nitrosocysteine. SNAC was measured in plasma and urine of healthy humans by means of GC-MS and by using double-labelled *S*-nitroso-*N*-acetylcysteine, i.e. *S*-[¹⁵N]nitroso-*N*-[²H₃]acetyl-cysteine, as the internal standard [94]. Despite the high sensitivity of this GC-MS method, SNAC was not present in human plasma and urine at concentrations above 1 nM, suggesting that the mercapturic acid pathway of *S*-nitrosocysteine does not exist in humans

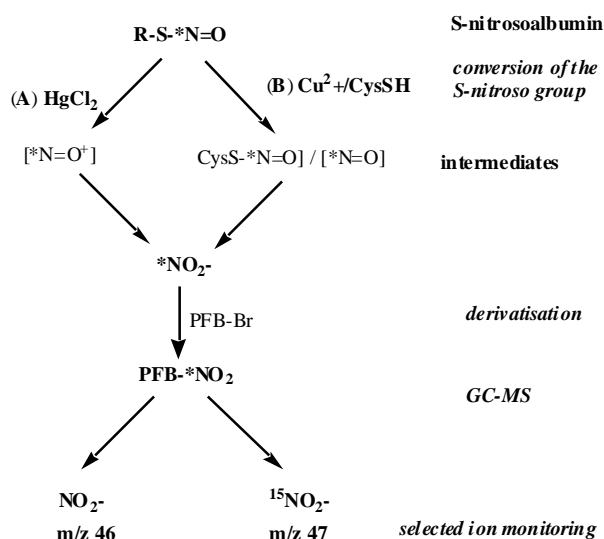


Fig. (3). Schematic of the principles of the GC-MS methods for the analysis of S-nitrosoalbumin (RSNO, R=albumin-Cys34) in human plasma using ¹⁵N-labelled S-nitrosoalbumin (RS¹⁵NO, R=albumin-Cys34) as internal standard. After affinity-column chromatography extraction, endogenous S-nitrosoalbumin and internal standard are converted either by HgCl₂ (A) or cysteine/Cu²⁺ (B) to nitrite (NO₂⁻) and ¹⁵N-labelled nitrite (¹⁵NO₂⁻), respectively, via different intermediates. Subsequently, NO₂⁻ and ¹⁵NO₂⁻ are converted to their pentafluorobenzyl (PFB) derivatives by means of PFB-Br and analyzed by GC-MS by selected ion monitoring of *m/z* 46 and *m/z* 47, respectively. Asterisks indicate the ¹⁴N and ¹⁵N isotopes of the N atoms of the S-nitroso group and nitrite. CysSH, cysteine.

[94]. On the other hand, S-[¹⁵N]nitroso-N-[²H₃]acetyl-cysteine intravenously administered to a rat could be detected in rat plasma by this GC-MS method [94].

Low-molecular-mass and high-molecular-mass S-nitrosothiols are accessible to GC-MS analysis after conversion of the S-nitroso group into nitrite by means of HgCl₂ or Cu¹⁺ ions (e.g. produced *in situ* from Cu²⁺ ions by cysteine); the corresponding S-[¹⁵N]nitroso analogs are used as internal standards [95-98] (Fig. (3)). Specific analysis of a

certain S-nitrosothiol by this method requires its chromatographic separation prior to the treatment with HgCl₂ or Cu²⁺/cysteine. The main interfering compound in methods measuring S-nitrosothiols is ubiquitous nitrite which is abundantly present as contamination in laboratory chemicals and materials including distilled water. Thus, „blank“ nitrite may exceed by far the concentration of endogenous S-nitrosothiols such as S-nitrosoalbumin [96]. Removal of nitrite is possible, e.g. by means of ammonium sulfamate in acidic solution. However, in the presence of thiols this reaction competes with rapidly preceding S-nitrosylation [91]. Artifactual formation of S-nitrosothiols during this procedure seems to be not completely avoidable [88,91].

Special attention has been paid to S-nitrosoalbumin due to its potential function as a carrier and storage of NO [77]. GC-MS methods have been developed, validated and applied to quantitate S-nitrosoalbumin levels in plasma of healthy and diseased humans [95,96,98]. These methods use S-[¹⁵N]nitroso-labelled albumin which is added to plasma or even blood and serves as internal standard. Endogenous S-nitrosoalbumin and the internal standard are selectively isolated from plasma by affinity extraction, e.g. on HiTrapBlue Sepharose affinity columns [95,96,98]. By this step native albumin and other modified albumin molecules, such as 3-nitrotyrosinoalbumin (see below), are also extracted, but they do not interfere with the measurement of S-nitrosoalbumin. The S-nitroso groups of endogenous S-nitrosoalbumin and the internal standard present in the eluate of affinity column extraction are converted to nitrite and ¹⁵N-labelled nitrite, respectively, are derivatized with PFB-Br and analyzed by GC-MS [96,98]. In addition, blank nitrite is also accurately quantified and its value subtracted. By means of this GC-MS method the concentration of S-nitrosoalbumin in plasma of healthy humans was determined to be within the range 156 - 205 nM [96,98]. In patients suffering from liver and chronic renal disease, endogenous S-nitrosoalbumin levels were found to be of the same order of magnitude [96]. This GC-MS was the first to question the S-nitrosoalbumin levels in plasma of healthy humans of the order of 7000 nM previously reported by Stamler *et al.* [77]. More recent studies question even the level of 150 nM and suggest that

Table 4. Summary of basal levels of S-nitrosothiols (RSNO) [(i.e. S-nitrosoalbumin (SNALB) or total S-nitrosothiols (PSNOs)] in plasma of healthy humans reported in the literature.

Authors	RSNO (nM)	Method	Ref.
Stamler <i>et al.</i> 1992	7000 ± 5000 (SNALB)	CL (photolysis)	[77]
Marzinzig <i>et al.</i> 1997	450 ± 400 (PSNOs)	Fluorometry	[99]
Tsikas <i>et al.</i> 1999	181 ± 150 (SNALB)	GC-MS	[96]
Marley <i>et al.</i> 2000	28 ± 7 (PSNOs)	CL (Copper)	[100]
Tyurin <i>et al.</i> 2001	4200 ± 1000 (SNALB)	Fluorophotometry	[101]
Cannon <i>et al.</i> 2001	24 - 35 (PSNOs)	CL (I ₃ ⁻)	[102]
Moriel <i>et al.</i> 2001	250 ± 200 (PSNOs)	CL (KI)	[103]
Rossi <i>et al.</i> 2001	20 - 50 (PSNOs)	Fluorometric HPLC	[44]
Massy <i>et al.</i> 2003	450 ± 440 (PSNOs)	Fluorometry	[104]
Wlodek <i>et al.</i> 2003	8800 (PSNOs)	Fluorometry	[105]

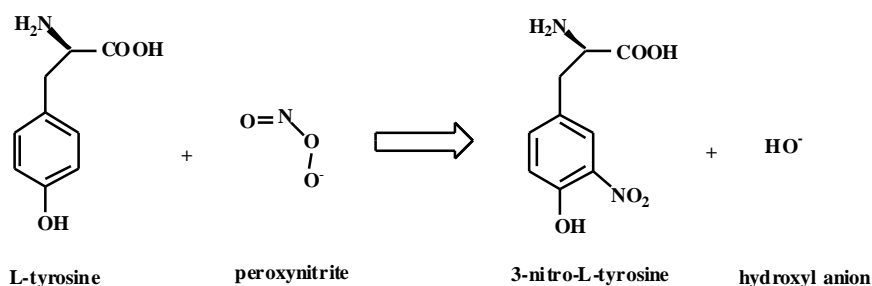


Fig. (4). Formation 3-nitro-L-tyrosine from the reaction between L-tyrosine and peroxynitrite (ONOO⁻).

the basal levels of *S*-nitrosothiols in human plasma are far below 150 nM, i.e. of the order of even only 1 nM which is very close to the detection limits of the methods used. (Table 4) summarizes reported values for *S*-nitrosoalbumin and total *S*-nitrosothiols measured in human plasma or serum at the basal state.

The main reports about the biochemical, physiological, pathological and therapeutic properties of *S*-nitrosothiols in the cardiovascular system in humans and animals have been recently critically analyzed and discussed in detail by Giustarini *et al.* [106]. Several analytical shortcomings may have contributed to the greatly diverging plasma levels of *S*-nitrosothiols listed in (Table 4). Massy *et al.* [104] and Wlodek *et al.* [105] applied in their studies the procedure originally described by Marzinzig *et al.* [99], which uses ammonium sulfamate at neutral pH to eliminate nitrite. However, elimination of nitrite by ammonium sulfamate stringently requires acidic conditions [48]. Thus, in those studies unremoved nitrite present in the samples at different concentrations may be the major contributing fraction to *S*-nitrosothiols. Interestingly, Dejam *et al.* recently found that thiols may enhance NO and *S*-nitrosothiol formation from nitrate during photolysis [107], suggesting considerable contribution of nitrate to *S*-nitrosothiols when photolysis-based chemiluminescence is used for their determination. Photolysis-induced nitrate-dependent contribution to *S*-nitrosothiols has been shown to be avoidable by means of *N*-ethylmaleimide (NEM) and HgCl₂ which remove thiols [107].

The above mentioned GC-MS method [96] has been used to investigate *S*-nitrosylation of endogenous albumin *in vivo* in the rat [37]. Intravenous infusion of *S*-[¹⁵N]nitrosoglutathione in the rat led to formation of *S*-[¹⁵N]nitrosoalbumin in rat plasma *in vivo*, with [¹⁵N]nitrate and [¹⁵N]nitrite being also formed. Stopping of the infusion caused immediate fall in the concentrations of circulating *S*-[¹⁵N]nitrosoalbumin, [¹⁵N]nitrate and [¹⁵N]nitrite [37]. From that experiment the half-life of *S*-[¹⁵N]nitrosoalbumin *in vivo* in the rat was estimated to be 10 – 20 min. *In vitro* in human blood, the half-life of *S*-[¹⁵N]nitrosoalbumin (added at 25 μM) was determined to be 5.5 h [95].

The origin of endogenous *S*-nitrosothiols is still uncertain. In the presence of glutathione at mM-concentrations in NOS incubates, two researcher groups reported formation of *S*-nitrosoglutathione at μM-concentrations [108,109]. In NO synthase incubates containing glutathione and L-[guanidino-¹⁵N₂]-arginine as the substrate,

formation of *S*-[¹⁵N]nitrosoglutathione at only nM-concentrations could be demonstrated by GC-MS [110]. In accordance with an alternative mechanism, the first *S*-nitrosothiol is *S*-nitrosohemoglobin which is formed from the intramolecular transfer of the nitrosyl group of *Fe*-NO-hemoglobin to the sulfhydryl group of -Cys93 [81]. Further studies involving application of reliable analytical methods such as LC-MS are required to solve this challenging analytical problem.

3-NITRO-L-TYROSINE

Soluble L-tyrosine (Tyr) and L-tyrosine residues in proteins (TyrProt) are attacked by various reactive nitrogen and oxygen species including peroxynitrite (ONOO⁻) and nitryl chloride (NO₂Cl) to form 3-nitro-L-tyrosine (NO₂Tyr) and protein-associated 3-nitro-L-tyrosine (NO₂TyrProt), respectively (Fig. 4) [111,112]. Circulating NO₂Tyr and NO₂TyrProt are widely used as biomarkers of oxidative stress in humans. Different MS-based and non-MS-based analytical methods are currently available for the quantitative determination of NO₂Tyr and NO₂TyrProt in human plasma and serum (Table 5). In non-immunological methods, NO₂TyrProt is hydrolyzed to its amino acids by means of acids, bases or enzymes. Because the number of nitrated L-Tyr molecules in proteins is not known, NO₂TyrProt values are usually expressed as the ratio of 3-nitro-L-tyrosine to L-tyrosine molecules of the respective protein, i.e. NO₂Tyr / TyrProt, or as amount of NO₂TyrProt per amount of protein, e.g. as pmol/mg (Table 5). Application of these methodologies to healthy normal humans revealed basal levels for circulating NO₂Tyr ranging between 0.4 and 64 nM, i.e. by two orders of magnitude (Table 5). Great discrepancies were also found for NO₂TyrProt, thus NO₂TyrProt was either “not detectable” or the molar ratio of NO₂TyrProt / TyrProt amounted up to 1:35 × 10⁶. Similar to the *S*-nitrosothiols (see above), the wide range of basal plasma levels of NO₂Tyr and NO₂TyrProt in humans clearly demonstrates the existence of severe analytical problems, even in MS-based methods.

The analytical methods listed in (Table 5) differ not only in their basic principle, but their also differ from one other with respect to sensitivity, selectivity, specificity and accessibility to interferences. The main sources of interference are artifactual acid-catalyzed formation of NO₂Tyr and NO₂TyrProt during sample treatment and co-elution of unknown substances with NO₂Tyr in the chromatographic systems used. Considerable interference may occur in GC-MS methods due to co-elution of unknown

Table 5. Summary of basal levels of free and protein-associated 3-nitro-L-tyrosine in plasma of healthy humans reported in the literature in methodological articles.

Authors	NO ₂ Tyr (nM)	NO ₂ TyrProt/TyrProt (x 1:10 ⁶)	Method	Ref.
Kamisaki <i>et al.</i> 1996	31	N.R. ^a	HPLC-FL	[113]
Fukuyama <i>et al.</i> 1997	N.R.	N.R.	HPLC-UV	[114]
ter Steege <i>et al.</i> 1998	N.R.	N.R.	ELISA	[115]
Schwedhelm <i>et al.</i> 1999	2.8	N.R.	GC-MS/MS	[116]
Frost <i>et al.</i> 2000	64	35	GC-MS	[117]
Yi <i>et al.</i> 2000	< 4.4	N.R.	LC-MS/MS	[118]
Gaut <i>et al.</i> 2002	11	N.R.	GC-MS	[119]
Tsikakos <i>et al.</i> 2003	0.73	1.55	GC-MS/MS	[120]
Söderling <i>et al.</i> 2003	0.74	0.6 (pmol/mg)	GC-MS/MS	[121]

^a N.R., not reported.

compounds which produce ions with the same *m/z* value like the 3-nitro-L-tyrosine derivative [120]. These interferences can be effectively eliminated by tandem mass spectrometry such as GC-MS/MS [120].

Application of the GC-MS/MS methodology by two research groups revealed almost identical mean levels for NO₂Tyr in human plasma at the basal state of the order of 1 nM (Table 5) [120,121]. The lower limits of quantitation (LOQ) of these methods were clearly below 1 nM, i.e. 0.125 nM [116] and 0.3 nM [121]. That NO₂Tyr circulates in human plasma at a concentration of about 1 nM is supported by two LC-MS/MS methods [118,122,123]. Yi *et al.* quantitated NO₂Tyr in human plasma by LC-MS/MS without any derivatization step. However, the authors could not detect NO₂Tyr at concentrations above the LOQ of the method which was 4.4 nM [118]. Delatour *et al.* reported that the sensitivity of the LC-MS/MS method could be increased by derivatizing 3-nitro-L-tyrosine to its butyl ester [122,123]. This group has not determined NO₂Tyr in human normal plasma but in rat plasma, in which NO₂Tyr was determined by LC-MS/MS at a concentration of 1.5 nM in 4 of 8 animals; the value of 1.5 nM is virtually the LOQ value of the method [122]. More recently Svatikova *et al.* have found by LC-MS/MS that NO₂Tyr occurs in plasma of healthy humans at 0.7 nM [124].

On the basis of the data provided by GC-MS/MS [116,120,121] and LC-MS/MS [118,122,123,124], the use of a range of 0.5 to 3 nM for NO₂Tyr and the order of magnitude of 0.6 pmol/mg plasma protein or a molar ratio of 0.4 to 1.6x1:10⁶ for NO₂TyrProt in plasma of healthy humans as reference values reasonably appear justified. From this point of view, at present circulating NO₂Tyr seems to be best quantifiable in human plasma at the basal state by the GC-MS/MS technology because of its considerably higher sensitivity as compared with the LC-MS/MS technology and because of its higher specificity as compared with the simple GC-MS.

By providing accurate levels for NO₂Tyr and NO₂TyrProt in plasma of humans and animals at the basal state, and by pointing out analytical shortcomings and

pitfalls, the MS technology, notably the tandem mass spectrometry (i.e., MS/MS) technique, greatly contributed to a solid knowledge of 3-nitro-L-tyrosine's physiology thus far. MS-based and non-MS-based analytical approaches to the analysis of 3-nitrotyrosine have been recently critically discussed [125]. MS-based analytical methods, in particular GC-MS/MS in combination with HPLC [116,120], are much more complex, time-consuming and have a relatively low throughput as compared with antibody-based approaches. This may limit the type of studies that can be performed by means of MS-based approaches. However, as Dr Duncan rightly concluded [125]: "Nevertheless, it is far preferable to have limited good quality data, than it is to be flooded with complex and confounding results".

METHYLATED L-ARGININE ANALOGS

Endogenous methylated L-arginine analogs include monomethylarginine (*N*^G-monomethyl-L-arginine; NMMA), asymmetric dimethylarginine (*N*^G,*N*^G-dimethyl-L-arginine; ADMA) and symmetric dimethylarginine (*N*^G,*N*^G-dimethyl-L-arginine; SDMA) (Fig. (5)). All methylated L-arginine analogs are inhibitors of various NOS isoforms including neuronal NOS [126]. Due to the potential regulatory role of methylated L-arginine analogs on NOS activity, special attention has been paid to analytical approaches to the quantitative determination of NMMA, ADMA and SDMA in biological fluids, notably human plasma. The most frequently used methods to quantitate L-arginine and its methylated analogs include HPLC with fluorescence detection, in particular with pre-column derivatization with *o*-phthaldehyde (OPA) (Table 6). Application of these HPLC approaches to clinical studies revealed elevated circulating levels of ADMA in various diseases. Furthermore, circulating ADMA levels were found to correlate with the degree of endothelial-dependent NO-related dysfunction. On the basis of the data from application of HPLC approaches there is accumulating evidence that ADMA is an emerging cardiovascular risk factor [136].

Problems associated with the lack of appropriate stable-isotope labelled analogs of methylated L-Arg have hampered

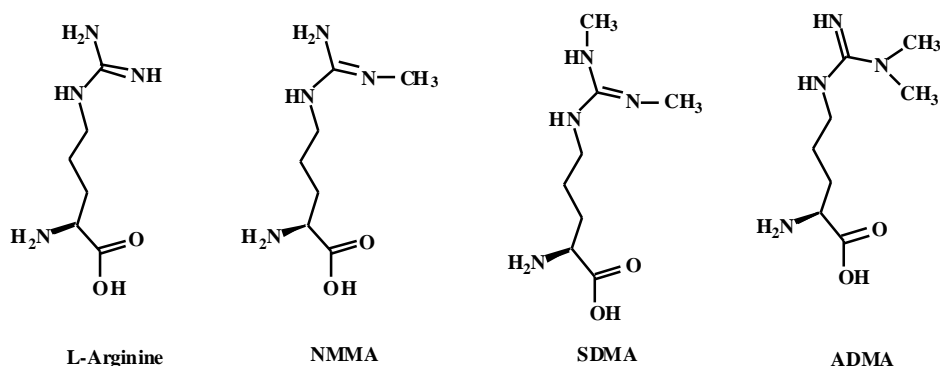


Fig. (5). Chemical structures of L-arginine and its N^G -methylated analogs. NMMA, monomethylarginine (N^G -monomethyl-L-arginine); SDMA, symmetric dimethylarginine (N^G, N^G -dimethyl-L-arginine); ADMA, asymmetric dimethylarginine (N^G, N^G -dimethyl-L-arginine).

Table 6. Summary of basal levels of asymmetric dimethylarginine (ADMA) in plasma of healthy humans reported in the literature in methodological articles.

Authors	ADMA (nM)	Method	Ref.
Pettersson <i>et al.</i> 1997	580 ± 20	HPLC-FL (OPA)	[127]
Tsikas <i>et al.</i> 1998	520 ± 210	HPLC-FL (OPA)	[128]
Vishwanathan <i>et al.</i> 2000	124 ± 47	LC-MS/MS (ESI)	[129]
Teerlink <i>et al.</i> 2002	420 ± 60	HPLC-FL (OPA)	[130]
Tsikas <i>et al.</i> 2003	390 ± 60	GC-MS/MS	[131]
Martens-Lobenhoffer & Bode-Böger 2003	453 ± 128	LC-MS (ESI)	[132]
Fleck <i>et al.</i> 2003	660 ± 40	HPLC-FL (OPA)	[133]
Heresztyn <i>et al.</i> 2004	440 ± 80	HPLC-FL (AccQ Fluor)	[134]
Albsmeier <i>et al.</i> 2004	600 ± 76	GC-MS	[135]

the development and use of MS-based approaches to their quantitative analysis. However, in recent years these problems have been successfully overcome. Today, various MS-based approaches such as GC-MS, LC-MS and their variants, i.e. GC-MS/MS and LC-MS/MS, are available (Table 6). Application of these approaches to humans revealed basal plasma levels ranging between approx. 100 and 600 nM, with the majority of them being within the range of 400 to 600 nM. The first LC-MS/MS method for ADMA has been reported by Vishwanathan *et al.* [129]. In this method, ADMA, SDMA, NMMA and L-Arg are analyzed without any derivatization. In the LC-MS method by Martens-Lobenhoffer and Bode-Böger, ADMA, SDMA, NMMA and L-Arg are analyzed as OPA derivatives [132]. In both methods, $^{13}\text{C}_6$ -Arg is used as internal standard for all compounds analyzed [129,132]. The first GC-MS/MS method for ADMA has been reported by Tsikas *et al.* using *de novo* synthesized $^2\text{H}_3$ -methyl ester ADMA as the internal standard [131]. The first application of a real internal standard for ADMA, i.e. $^2\text{H}_6$ -ADMA, for the quantitative determination of ADMA in human plasma by GC-MS has been reported by Albsmeier *et al.* [135]. Unlike other

members of the L-Arg/NO family, such as 3-nitrotyrosine and S-nitrosothiols (see above), application of MS and HPLC approaches provided very similar ADMA levels in plasma and serum of healthy humans (Table 6).

ADMA and SDMA have been isolated from and identified in urine of humans by Kakimoto and Akazawa [137]. Carnegie *et al.* reported that healthy humans excreted into the urine 43 μmol of ADMA within 24 h which corresponded to approximately 2.7 $\mu\text{mol}/\text{mmol}$ creatinine as found by LC [138]. Vallance *et al.* identified ADMA and SDMA in human urine by MS and found that the urinary excretion of ADMA is approximately 65 $\mu\text{mol}/24$ h by means of HPLC and CE [13]. Of the MS-based methods reported for the quantitative analysis of ADMA so far, two methods, i.e. GC-MS/MS [131] and LC-MS [132], have been shown to be applicable to human urine. By means of GC-MS/MS, ADMA was detected in urine of healthy humans (n=9) at 25 ± 18 μM which corresponds to 3.4 ± 1 $\mu\text{mol}/\text{mmol}$ creatinine [131]. By means of LC-MS, ADMA concentration in urine of healthy humans (n=15) was measured to be 52 ± 17 μM [132]. These data closely

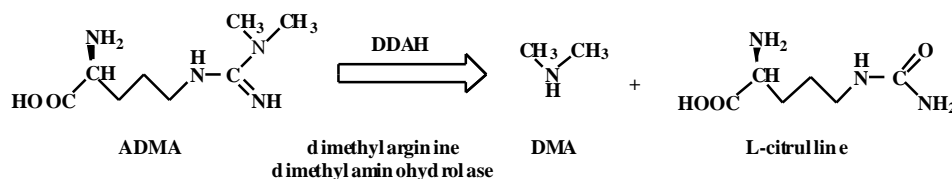


Fig. (6). Biosynthesis of dimethylamine (DMA) from asymmetric dimethylarginine (ADMA). The enzyme dimethylarginine dimethylaminohydrolase (DDAH) catalyzes the hydrolysis of ADMA to DMA and citrulline.

confirm the urinary ADMA levels reported earlier by LC [13,138]. Also, GC-MS/MS confirmed [139] the finding from LC measurements [138] that humans with hepatic diseases excrete more ADMA in the urine than healthy volunteers.

DIMETHYLAMINE

In addition to the elimination of unmetabolized ADMA by the kidneys, there exist a second metabolic pathway of ADMA that involves hydrolysis of the N^G, N^G -dimethyl group of ADMA to form dimethylamine (DMA) and L-citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH; EC 3.5.3.18) (Fig. (6)) [140,141]. DMA is eliminated from the body *via* urinary excretion. Although the origin of urinary DMA is not exclusively the DDAH-catalyzed hydrolysis of ADMA, a recent study in human suggested that the DDAH/ADMA/NO pathway is the major contributor to urinary DMA [14]. Thus, Achan *et al.* estimated that humans generate approximately 300 μmol of ADMA per day, of which approximately 250 μmol is metabolized by DDAH [14]. In that study, urinary DMA was measured spectrophotometrically according to the method of Beal and Bryan [142]. For GC analysis, DMA was converted to its pentafluorobenzamide derivative by means of pentafluorobenzoyl chloride [143]. daCosta *et al.* converted DMA to the tosylamide derivative by means of *p*-toluenesulfonyl chloride and measured DMA in urine and other biological fluids by GC-MS using ^{13}C -labelled DMA as internal standard [144]. Zhang *et al.* utilized the high volatility of DMA and determined it in human urine by head-space GC [145,146]. By this approach, Zhang *et al.* found that the daily urinary excretion of DMA by healthy humans ($n=203$) is 17.4 mg (390 μmol) [146]. Teerlink *et al.* developed a HPLC method with fluorescence detection for the measurement of DMA in serum and urine after precolumn derivatization with fluorenylmethylchloroformate [147]. The concentration of DMA in serum of healthy humans was determined to be 3.3 μM by this method [147]. We modified the GC method of Ripley *et al.* originally described for DMA in foodstuffs [143] and developed a GC-MS method for the quantitative determination of DMA in human urine using $^2\text{H}_6$ -labelled DMA $(\text{CH}_3)_2\text{NH}$ as internal standard (unpublished data). By means of this method we found that DMA is present in urine of healthy volunteers at concentrations ranging between approximately 100 and 900 μM . The creatinine-corrected excretion rate of DMA was determined to be approximately 28 $\mu\text{mol}/\text{mmol}$ creatinine, which corresponds to an approximate daily excretion of 12.6 mg. These data confirm the DMA basal levels in human urine obtained by different analytical methods cited above. Interestingly, we found in healthy humans a tight positive

correlation ($R=0.877$, $P=0.0009$) between the urinary excretion rate of DMA (Y) and that of ADMA (X) with the regression equation $Y=91+14X$, indicating that the mean urinary excretion rate of DMA is approximately 14 times higher than that of ADMA.

MISCELLANEOUS

The analytes and analytical methods discussed in the above sections by far do not cover the complete spectrum of currently known members of the L-Arg/NO family including the enzyme NOS itself and modern approaches such as proteomics, respectively. With the respect to the latter, here it is referred to recent review articles such as the *Nitric Oxide Protocols* [148]. As to short-lived low-molecular-mass species such as NO itself, superoxide and peroxynitrite, these species evaded in principle any quantitative determination *in vivo* in humans by MS-based analytical methods. Non-MS-based methods for NO, superoxide, hydrogen peroxide and peroxynitrite are very popular, but they are largely limited to investigations *in vitro* [149].

CONCLUSIONS AND FUTURE PROSPECTS

Mass spectrometry is not longer restricted to structure elucidation of natural and synthetic compounds and their metabolites. In the field of NO research and other areas of research, the task of mass spectrometry is reaching far beyond the unequivocal delineation of metabolic pathways. Recent advances in mass spectrometry have made this analytical technology the most reliable approach for the accurate, sensitive and even routine quantitative determination of virtually all of the numerous low-molecular-mass and high-molecular-mass members of the L-Arg/NO family in complex biological fluids such as plasma and urine.

Mass spectrometry-based analytical approaches, especially GC-MS/MS and LC-MS/MS, invaluablely helped solving severe analytical problems in this area of research and provided definite reference values for many members of the L-Arg/NO family such as nitrite, nitrate, 3-nitro-L-tyrosine and methylated L-Arg analogs, especially ADMA, the novel cardiovascular risk factor. MS-based analytical approaches infallibly identified minefields and pitfalls in this area of research, and they are, therefore, the most reliable tool to prove the reliability of popular non-MS-based analytical approaches.

The desire for simple and rapid analytical methods for routine analysis in the NO field of research is understandable. However, reliability in terms of accuracy is considered more important than rapidity in particular in clinical research. Therefore, reliability should have priority

over rapidity. It is far preferable to have limited good quality data, than it is to be flooded with complex and confounding results.

At present, the GC-MS and GC-MS/MS technologies are the most appropriate approaches for the analysis of low-molecular-mass members of the L-Arg/NO pathway. In recent years, great advances took place in the LC-MS technology, and it may reasonably be expected that in particular the LC-MS/MS approach will become the most severe competitor for the GC-MS/MS technology with respect to the low-molecular-mass analytes, and will, furthermore, be indispensable for the analysis of high-molecular-mass analytes such as nitrosylated and nitrated proteins.

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ABBREVIATIONS

ADMA	=	asymmetric dimethylarginine
CE	=	capillary electrophoresis
CL	=	chemiluminescence
CZE	=	capillary zone electrophoresis
DDAH	=	dimethylarginine dimethylaminohydrolase
DMA	=	dimethylamine
ECD	=	electrochemical detection
EDNO	=	endothelium derived nitric oxide
EDRF	=	endothelium derived relaxing factor
ELISA	=	enzyme linked immunosorbent assay
ESI	=	electrospray ionization
FIA	=	flow injection analysis
FL	=	fluorescence
GC	=	gas chromatography
GC-MS	=	gas chromatography-mass spectrometry
GC-MS/MS	=	gas chromatography-mass spectrometry-mass spectrometry
GSNO	=	S-nitrosoglutathione
HbFeNO	=	Fe-nitrosohemoglobin
HPLC	=	high-performance liquid chromatography
LC	=	liquid chromatography
LC-MS	=	liquid chromatography-mass spectrometry

LC-MS/MS	=	liquid chromatography-mass spectrometry-mass spectrometry
LOQ	=	(lower) limit of quantitation
MMA	=	monomethylamine
MS	=	mass spectrometry
<i>m/z</i>	=	mass-to-charge ratio
NEM	=	N-ethylmaleimide
NICI	=	negative-ion chemical ionization
NMMA	=	monomethylarginine
NO	=	nitric oxide
NOS	=	nitric oxide synthase
NO ₂ Tyr	=	3-nitro-L-tyrosine
NO ₂ TyrPror	=	protein-associated 3-nitro-L-tyrosine
3-NT	=	3-nitro-L-tyrosine
OPA	=	<i>o</i> -phthaldehyde
PFB	=	pentafluorobenzyl
RSNO	=	S-nitrosothiol
RSNO ₂	=	S-nitrothiol
SNAC	=	S-nitroso-N-acetylcysteine
SNALB	=	S-nitrosoalbumin
SNAP	=	S-nitroso-N-acetylpenicillamine
SNOHb	=	S-nitrosohemoglobin
SNC	=	S-nitrosocysteine
TyrProt	=	protein-associated L-tyrosine

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