Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 851 (2007) 124–139
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Review

# Detection of *S*-nitrosothiols in biological fluids: A comparison among the most widely applied methodologies☆

Daniela Giustarini[a], Aldo Milzani[b], Isabella Dalle-Donne[b], Ranieri Rossi[a,*]

[a] *Department of Neuroscience, Pharmacology Section, Via A. Moro 4, University of Siena, 53100 Siena, Italy*
[b] *Department of Biology, Via Celoria 26, University of Milan, 20133 Milan, Italy*

Received 17 June 2006; accepted 20 September 2006
Available online 10 October 2006

**Abstract**

Many different methodologies have been applied for the detection of *S*-nitrosothiols (RSNOs) in human biological fluids. One unsatisfactory outcome of the last 14 years of research focused on this issue is that a general consensus on reference values for physiological RSNO concentration in human blood is still missing. Consequently, both RSNO physiological function and their role in disease have not yet been clarified. Here, a summary of the values measured for RSNOs in erythrocytes, plasma, and other biological fluids is provided, together with a critical review of the most widely used analytical methods. Furthermore, some possible methodological drawbacks, responsible for the highlighted discrepancies, are evidenced.


*Keywords:* Reviews; Nitric oxide; *S*-Nitrosoalbumin; *S*-Nitrosohemoglobin; *S*-Transnitrosation reactions

## Contents



## 1. Introduction

The function of nitric oxide (NO) in the activation of guanylyl cyclase and in mediating vasorelaxation is now well-established,

☆ This paper is part of a special issue entitled "Analysis of the L-arginine/NO pathway", guest edited by D. Tsikas.

* Corresponding author. Tel.: +39 0577 234198; fax: +39 0577 234208.
*E-mail address:* ranieri@unisi.it (R. Rossi).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.031

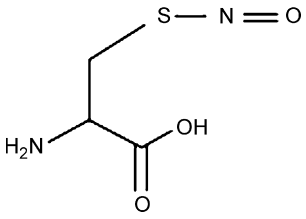


Fig. 1. Chemical formula for *S*-nitrosocysteine, the simplest low-molecular-mass *S*-nitrosothiol.

as also recognized by the award of the Nobel Prize to Furchgott, Ignarro and Murad in 1998 for their discovery about the actions of NO in the cardiovascular system. However, NO and its derivatives may be involved in a much broader range of pathophysiological actions [1]. NO is not detectable per se in human circulation at the basal state because of its short half-life (<0.1 s) in that it rapidly undergoes a series of chemical reactions with various types of biomolecules [2]. However, many storage forms of NO have been postulated to exist in mammalian tissues. Recent studies have focused on the role of intravascular NO-derived molecules that could stabilize NO bioactivity and contribute to blood flow and oxygen delivery [3]. Among these, a pool of derivatives of NO, i.e., *S*-nitrosothiols (RSNOs), where a nitroso moiety is covalently bound to the sulfhydryl group (SH) of proteins or low-molecular-mass (LMM) thiols, has been identified in various tissues (reviewed in Ref. [1]). Chemically, *S*-nitrosothiols are thioesters of nitrite with close analogy with nitrite esters of alcohols (Fig. 1). The evidence that RSNOs possess many of the biological activities of NO itself (e.g., in causing arterial and venous smooth muscle relaxation, in inhibiting platelet aggregation) by activating guanylyl cyclase has stimulated a wealth of research into the biological significance of these molecules. Since RSNOs are relatively stable and release NO under various conditions, they are considered to be a buffering system that controls intra- and extracellular functions of NO increasing its range of actions in terms of time and space [4].

## 2. Formation and catabolism of *S*-nitrosothiols

*S*-Nitrosothiols cannot be produced by the simple reaction of NO with free thiol groups. The formation of RSNOs observed in oxygenated buffers [5] was supposed to be due to the oxidation of NO to dinitrogen trioxide ($N_2O_3$). This molecule can be produced in environments containing both NO and $O_2$, and $N_2O_3$ is considered a good nitrosating agent, since it reacts with thiols at physiological pH to yield RSNOs and nitrite [1,6]. Differently from the gas phase, the formation of $N_2O_3$ from autooxidation of NO in aqueous phase is rather unlikely [6,7]. Thus, the mechanism(s) leading to the RSNO formation in vivo are still largely unknown. Nevertheless, in the presence of reduced thiols at mM-concentrations in incubates with nitric oxide synthase (NOS), formation of nM- to μM-concentrations of RSNOs has been reported [8–10]. Moreover, intravenous infusion of aqueous solution of NO gas was shown to be accompanied by systemic and hemodynamic effects [11], which are mediated by the transport of NO stored as RSNOs, thus further suggesting an in vivo mechanism that can convert a percentage of NO into RSNOs. However, it is unclear what could be the chemical mechanism(s) involved. Among the various possible routes, catalysis due to metal ions in traces, which can serve as electron acceptors, has been hypothesized (Fig. 2) [1]. Alternatively, serum albumin has been shown to catalyze the production of *S*-nitrosothiols by oxidizing, within the protein hydrophobic core, NO to $NO^+$, which is then transferred either to the sulfhydryl group of Cys34 of albumin itself or to other thiols (LMM or protein thiols) [4,12].

A controversial theory, in which hemoglobin (Hb) was suggested to be capable of preserving, transporting, and exporting NO activity through the formation of *S*-nitrosohemoglobin (Hb-SNO) as an intermediate, has been proposed for RSNO production in vivo. This theory suggests that NO binds rapidly to the heme group of deoxygenated Hb to form nitrosylhemoglobin (Fe-NO Hb). Once Fe-NO Hb is oxygenated (and undergoes a T to R transition), some of the NO on the heme group is transferred to the β-93 cysteine to form Hb-SNO [13,14]. Upon deoxygenation, some of the NO (actually $NO^+$) on the cysteine is transferred to erythrocytic glutathione or thiol groups on anionic exchanger (AE1) protein and, finally, it is exported from the red blood cell (RBC) to effect vasodilation, thus actively participating in the regulation of blood flow [15]. Nevertheless, the proposed mechanism seems to have numerous shortcomings, and many researchers in the field were unable to obtain the same results as originally proposed [16–20]. Finally, the lack of an allosterically

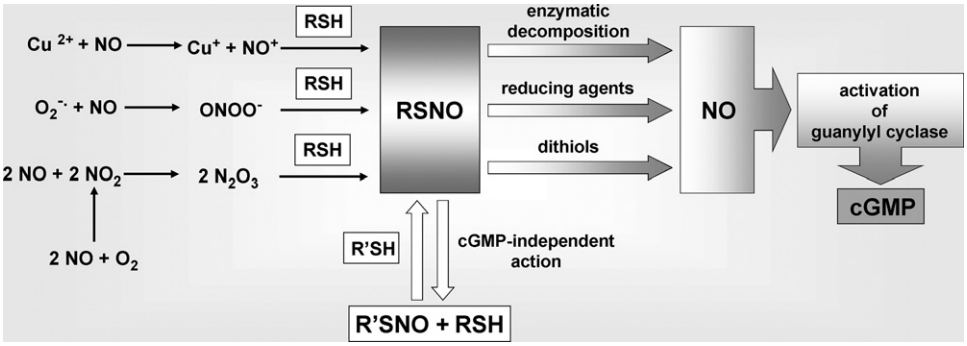


Fig. 2. Summary of proposed main pathways for the formation, decomposition and biological action of *S*-nitrosothiols (RSNOs). $N_2O_3$, dinitrogen trioxide; $NO_2$, nitrogen dioxide; $NO^+$, nitrosonium ion; $ONOO^-$, peroxynitrite; RSH and R′SH, thiols; ($O_2^{\bullet-}$) superoxide anion.

controlled intramolecular transfer of NO from the heme group to cysteine has recently been further demonstrated [20].

Different reducing agents such as transition metal ions, ascorbate, superoxide ($O_2^{\bullet -}$), thiols and direct light can mediate NO release from RSNOs. Some enzymes have also been studied for their ability to decompose RSNOs (e.g., formaldehyde dehydrogenase, protein disulfide isomerase, γ-glutamyl transferase) and the occurrence of an *S*-nitrosoglutathione (GSNO) reductase has been proven from bacteria to mammals [21]. Additionally, the ability of dithiols has been evidenced (specifically thioredoxin and lipoic acid) to decompose some RSNOs with concomitant generation of nitroxyl ($NO^-$), the one-electron reduction product of NO [22]. However, how NO is released from RSNOs in vivo is not clear. Free metal ion-dependent NO release could be physiologically unlikely, since concentrations of free metal ions are kept low. On the other hand, some copper-containing cell-surface proteins, such as ceruloplasmin, would be able to decompose RSNOs, enhancing their NO-mimetic activity (Fig. 2) [23,24]. Thiols can also have an important role in RSNO metabolism since they are ubiquitous and reach millimolar concentrations in the intracellular environment [17]. They can react with RSNOs by transnitrosation, a reaction in which the nitroso group is exchanged between *S*-nitrosothiol and another thiol (Fig. 2). Since the susceptibility to decomposition of LMM RSNOs is much greater than that of protein *S*-nitrosothiols (PSNOs) and, additionally, some LMM RSNOs (e.g., *S*-nitrosocysteine, Cy-SNO) can also cross cellular membranes [25], these *S*-transnitrosation reactions appear to have a pivotal role in RSNO metabolism and to influence their biological activity.

## 3. Mechanism(s) of action of *S*-nitrosothiols

It is still to be elucidated whether RSNOs can be simply considered a by-product of the metabolism of NO itself or can have an active role in the regulation of biological pathways. Beyond their supposed feature to function as NO stores, RSNO are able to deliver NO and to act via guanylyl cyclase activation, there is a large wealth of research as well about RSNO capability to act via a guanylyl cyclase-independent pathway. The guess is that NO, through the formation of RSNOs, can induce post-translational modifications on sulfhydryl groups of cysteine residues in proteins, thus contributing to the regulation of their function (Fig. 2). Dozens of examples of proteins undergoing *S*-nitrosation have been reported in the literature [1,26–28]. However, it should be underlined that most examples of protein cysteine *S*-nitrosation have been described only in purified proteins or in cells treated with supraphysiological concentrations of NO donors or LMM RSNOs (e.g., Cy-SNO), thus simply demonstrating that the protein has a reactive SH group that can be nitrosated [1]. Some evidence of *S*-nitrosated proteins in vivo in various tissues has also been reported [29–32], but these results should be taken with caution. In fact, the intracellular environment contains millimolar concentrations of glutathione, therefore, as transnitrosation reactions are governed by second order reversible kinetics, the $K_{eq}$ of which are close to unit, it is unlikely that protein *S*-nitrosation is stable under these conditions [1]. It has recently been reported that *S*-nitrosation is targeted enzymatically to specific protein sites (thioredoxin may catalyze this reaction), thus diminishing the non-specificity of a simple chemical reaction [33]. However, as the enzymatic catalysis cannot influence the *S*-transnitrosation equilibria, this issue requires further analysis.

## 4. From NO to RSNO: what is the yield?

Even if the complete chemical mechanism has not yet been fully elucidated, it is widely accepted that most RSNOs arise from NO and, ultimately, from NOS activity, given that the enzyme *S*-nitrosothiol synthase, even if postulated, has not yet been identified. In any case, it is not of secondary importance to evaluate how much NO can be converted into RSNOs to have an idea, with acceptable approximation, of the levels of RSNOs occurring in tissues and biological fluids. NO administered intravenously at 30 μmol/min resulted in an increase in RSNOs in peripheral blood of ≈30 nM (corresponding to an increase of about 50% over measured basal values) [11]. Additionally, after treatment of RAW 264.7 cells with lipopolysaccharide [34] or of epithelial cells with NO-donors [35], less than 0.01% of the released NO was transformed into RSNOs. The amount of NO that is delivered from endothelial cells into blood stream is calculated to be $10^3$ to $10^4$ NO molecules/$\mu m^2$ s [19]. Consequently, it seems to be unlikely that RSNO concentration in blood and other biological fluids can be at the μM- rather than at the nM-range. Nevertheless, higher production of NO, typically by the inducible NOS, as occurs for example during sepsis, may increase the basal concentration of RSNOs [36], although the quantitative aspect of this is still to be elucidated.

## 5. Quantitative analysis of *S*-nitrosothiols

### 5.1. General concerns

There are a large number of challenges that investigators have to face when approaching the quantification of RSNOs in biological systems. Most of them are connected to their very low in vivo levels. As a consequence of this, the presence of nitrite, iron-nitrosyl and other compounds, occurring in tissues and also as contaminants in buffers and chemicals, makes the detection of RSNOs artifact-prone, as some procedures may lack selectivity [37]. Moreover, the chemical lability of RSNOs in the presence of thiols, ascorbic acid or metals further complicates the problem. The analytical difficulties are mirrored by the incontrovertible observation that reported values by different research groups cover some orders of magnitude also when analyzing the same tissue or biological fluid. It is wondering that one research group found 20–30 nmol/mg PSNO (i.e., 1.5–2 mM) in endothelial cells [38] or rat kidney [39], a value that corresponds to the levels of cellular glutathione (GSH) itself. Conversely, Bryan et al. [40] found only 10–100 nM of RSNOs in almost all the analyzed tissues. Therefore, an apparent gap of four-to-five orders of magnitude exists. One can guess that such four-order difference is limited to tissues or cells where RSNO measurement is more difficult, due to the preparative phase for sample

Table 1
Reported *S*-nitrosothiol levels in plasma or serum in health and disease measured by different methodologies

| Sample | *S*-Nitrosothiol | Concentration (μM) | Methodology | Cleavage | Publication year | Reference |
|---|---|---|---|---|---|---|
| Human | RSNO | 7.19 ± 5.73 | Chemiluminescence | Photolysis | 1992 | [41] |
| Human | PSNO | 6.92 ± 5.45 | Chemiluminescence | Photolysis | 1992 | [41] |
| Rabbit | Cy-SNO | 0.221 ± 0.259 | EC/HPLC | No | 1994 | [70] |
| Rat | PSNO | 1.20 | Spectrophotometer (Griess) | $HgCl_2$ | 1996 | [120] |
| Human[a] | RSNO | 2.00 ± 0.800 | Spectrofluorometric | Unspecified | 1997 | [52] |
| Human RA[a] | RSNO | 3.17 ± 2.30 | Spectrofluorometric | Unspecified | 1997 | [52] |
| Human OA[a] | RSNO | 2.35 ± 1.27 | Spectrofluorometric | Unspecified | 1997 | [52] |
| Human[a] | RSNO | 0.450 ± 0.400 | Fluorimeter (DAN) | $HgCl_2$ | 1997 | [71] |
| Human sepsis[a] | RSNO | 1.30 ± 0.800 | Fluorimeter (DAN) | $HgCl_2$ | 1997 | [71] |
| Mice | LMM RSNO | 5.00 | Spectrophotometer (Griess) | $HgCl_2$ | 1997 | [121] |
| Human | RSNO | 0.321 ± 0.139 | Chemiluminescence | $HgCl_2/Vd^{3+}$ | 1998 | [91] |
| Rat[a] | PSNO | 0.930 ± 0.360 | Chemiluminescence | $Cu^+$/Cys | 1998 | [88] |
| Human | RSNO | 0.220 ± 0.190 | HPLC (Griess) | $HgCl_2$ | 1998 | [105] |
| Human | Alb-SNO | 0.181 ± 0.150 | GC–MS | $HgCl_2$ | 1999 | [94] |
| Human hepatic diseases | Alb-SNO | 0.161 ± 0.274 | GC–MS | $HgCl_2$ | 1999 | [94] |
| Human CRF | Alb-SNO | 0.147 ± 0.055 | GC–MS | $HgCl_2$ | 1999 | [94] |
| Human | GSNO | <0.100 (LOD) | HPLC (OPA) | β-Me | 1999 | [67] |
| Rat | GSNO | <0.100 (LOD) | HPLC (OPA) | β-Me | 1999 | [67] |
| Human[a] | LMM RSNO | <0.025 (LOD) | Chemiluminescence | $Cu^+$/Cys | 2000 | [122] |
| Human[a] (arterial blood) | PSNO | 0.045 ± 0.014 | Chemiluminescence | $I_2/I^-$ | 2000 | [122] |
| Human[a] | PSNO | 0.063 ± 0.013 | Chemiluminescence | $I_2/I^-$ | 2000 | [122] |
| Rat | Alb-SNO | 0.120 ± 0.052 | HPLC (Griess) | $HgCl_2$ | 2000 | [36] |
| Rat | LMM RSNO | <0.020 (LOD) | HPLC (Griess) | $HgCl_2$ | 2000 | [36] |
| Human | Alb-SNO | 0.062 ± 0.024 | HPLC (Griess) | $HgCl_2$ | 2000 | [104] |
| Human | LMM RSNO | <0.020 (LOD) | HPLC (Griess) | $HgCl_2$ | 2000 | [104] |
| Human | RSNO | 0.028 ± 0.007 | Chemiluminescence | $Cu^+/I_2/I^-$ | 2000 | [72] |
| Human[a] (arterial blood) | PSNO | 0.024 ± 0.009 | Chemiluminescence | $I_2/I^-$ | 2001 | [123] |
| Human[a] | PSNO | 0.035 ± 0.013 | Chemiluminescence | $I_2/I^-$ | 2001 | [123] |
| Human | RSNO | 0.250 ± 0.200 | Chemiluminescence | $HgCl_2$/KI | 2001 | [51] |
| Human hypercholesterolemia | RSNO | 0.550 ± 0.260 | Chemiluminescence | $HgCl_2$/KI | 2001 | [51] |
| Rat | RSNO | 0.051 ± 0.006 | Chemiluminescence | $Cu^{2+}/I_2/I^-$ | 2001 | [124] |
| Rat biliary cirrhosis | RSNO | 0.206 ± 0.059 | Chemiluminescence | $Cu^{2+}/I_2/I^-$ | 2001 | [124] |
| Human | PSNO | 0.020–0.050 | HPLC (DAN) | $HgCl_2$ | 2001 | [56] |
| Human | Alb-SNO | 4.20 ± 1.00 | Fluorimeter (DAF-2) | Photolysis | 2001 | [45] |
| Human pregnancy | Alb-SNO | 5.10 ± 0.700 | Fluorimeter (DAF-2) | Photolysis | 2001 | [45] |
| Human pregnancy | RSNO | 9.40 ± 1.50 | Fluorimeter (DAF-2) | Photolysis | 2001 | [45] |
| Human pre-eclampsia | Alb-SNO | 6.30 ± 1.40 | Fluorimeter (DAF-2) | Photolysis | 2001 | [45] |
| Human pre-eclampsia | RSNO | 11.1 ± 2.90 | Fluorimeter (DAF-2) | Photolysis | 2001 | [45] |
| Rat | RSNO | 0.002 | Chemiluminescence | $I_2/I^-$ | 2002 | [82] |
| Guinea pig | RSNO | 0.025 | Chemiluminescence | $I_2/I^-$ | 2002 | [82] |
| Monkey | RSNO | 0.001 | Chemiluminescence | $I_2/I^-$ | 2002 | [82] |
| Human | RSNO | 5.93 ± 0.750 | Spectrophotometer (Griess) | $HgCl_2$ | 2002 | [47] |
| Human diabetes | RSNO | 7.64 ± 0.790 | Spectrophotometer (Griess) | $HgCl_2$ | 2002 | [47] |
| Rat | RSNO | 1.78 ± 0.760 | Chemiluminescence | $Cu^+$/Cys | 2002 | [125] |
| Rat | RSNO | 0.050–1.00 | NO electrode | $Cu^{2+}$ | 2002 | [4] |
| Human | RSNO | 0.040 ± 0.007 | Chemiluminescence | $Cu^+/I_2/I^-$ | 2002 | [126] |
| Human | RSNO | 0.015 ± 0.006 | Chemiluminescence | $Cu^+/I_2/I^-$ | 2002 | [11] |
| Human | RSNO | 0.0072 ± 0.0011 | Chemiluminescence | $I_2/I^-$ | 2002 | [3] |
| Human SCD | PSNO | 0.0023 ± 0.0005 | Chemiluminescence | $I_2/I^-$ | 2002 | [53] |
| Human | RSNO | 0.450 ± 0.450 | Fluorimeter (DAN) | $HgCl_2$ | 2003 | [50] |
| Human HD | RSNO | 2.25 ± 1.17 | Fluorimeter (DAN) | $HgCl_2$ | 2003 | [50] |
| Rat | Alb-SNO | 0.0012 ± 0.0003 | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Human | Alb-SNO | 0.0072 ± 0.0011 | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Guinea pigs | Alb-SNO | 0.0252 ± 0.0051 | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Human | RSNO | 9.00 | Fluorimeter (DAN) | $HgCl_2$ | 2003 | [49] |
| Human CRF | RSNO | 12.0 | Fluorimeter (DAN) | $HgCl_2$ | 2003 | [49] |
| Human CRF before HD | RSNO | 8.10 | Fluorimeter (DAN) | $HgCl_2$ | 2003 | [49] |
| Human CRF after HD | RSNO | 8.60 | Fuorimeter (DAN) | $HgCl_2$ | 2003 | [49] |
| Rat | RSNO | 0.00135 ± 0.00046 | Chemiluminescence | $I_2/I^-$ | 2004 | [40] |
| Human (left ventricle) | RSNO | 10.8 ± 3.10 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human (pulmonary artery) | RSNO | 9.20 ± 3.70 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human CHF (left ventricle) | RSNO | 14.7 ± 4.20 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human CHF (pulmonary artery) | RSNO | 12.9 ± 3.10 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human HD | RSNO | 1.77 ± 0.320 | Fluorimeter (DAN) | $HgCl_2$ | 2004 | [128] |

Table 1 (*Continued*)

| Sample | *S*-Nitrosothiol | Concentration (μM) | Methodology | Cleavage | Publication year | Reference |
|---|---|---|---|---|---|---|
| Cat | Alb-SNO | <0.050 (LOD) | Fluorimeter (DAN) | $HgCl_2$ | 2004 | [81] |
| Cat | GSNO | <0.100 (LOD) | HPLC (UV) | No | 2004 | [81] |
| Rat | LMM RSNO | $0.010 \pm 0.006$ | NO electrode | $Cu^{2+}$ | 2004 | [112] |
| Rat | RSNO | 0.200 | NO electrode | $Cu^{2+}$ | 2004 | [112] |
| Human | Alb-SNO | $0.00059 \pm 0.00031$ | Chemiluminescence | $I_2/I^-$ | 2004 | [43] |
| Human pregnancy | Alb-SNO | 2.90 | Biotin-switch | Ascorbic acid | 2005 | [46] |
| Human pre-eclampsia | Alb-SNO | 7.20 | Biotin-switch | Ascorbic acid | 2005 | [46] |
| Human | RSNO | 0.090 | EPR spectrometry | MGD | 2005 | [114] |
| Human RA | RSNO | 0.109 | EPR spectrometry | MGD | 2005 | [114] |

[a] Serum. CHF: congestive heart failure; CRF: chronic renal failure; DAN: 2,3-diaminonaphthalene; EPR: electron paramagnetic resonance; HD: hemodialysis; β-Me: 2-mercaptoethanol; MGD: *N*-methyl-D-glucamine dithiocarbamate; OPA: *o*-phthalaldehyde; RA: rheumatoid arthritis; SCD: sickle cell disease.

analysis; however, a similar discrepancy among different laboratories is present also when biological fluids are analyzed (Tables 1–3).

Stamler et al. first reported the presence of 7 μM RSNOs in plasma of healthy humans, suggesting that *S*-nitrosoalbumin is the most abundant circulating *S*-nitrosothiol [41]. During the past 14 years, dozens of other studies measured the concentrations of RSNOs both in healthy people and subjects suffering from various diseases: from an analytical standpoint, results mirror the difficulties of researchers in the field. Apart from the initial difficulties one faces with new methodologies and validation procedures, paradoxically, still now most of the papers reporting measurement of RSNOs in human blood or other tissues are frequently preceded by an "in situ" validation of the applied analytical procedure. Furthermore, in these papers [42–44] we can frequently find data supporting the thesis that early published results are affected by artifacts because of the use of inappropriate methods.

RSNO concentration has been reported to be altered in patients suffering from many different pathologies such as pre-eclampsia [45,46], diabetes [47,48], end-stage renal disease [49,50], hypercholesterolemia [51], rheumatoid arthritis [52], sickle cell disease [15,53], chronic obstructive pulmonary disease [54], and cystic fibrosis [55]. These findings were from assessing RSNOs in a variety of biological fluids: blood, plasma, sputum, saliva, bronchoalveolar lavage fluid (BALF), synovial liquid, or cerebrospinal fluid (see Tables 1–3). Considering these data as a whole, it is impossible to demonstrate that the variation in the levels of RSNOs may serve as an indicator of the evolution of the disease, with the consequent attribution of diagnostic/prognostic importance. The three-to-four orders of magnitude divergence in basal levels (Tables 1–3), severe artifacts occurring during preparative pre-analytical phases, analytical shortcomings, and, more generally, the insufficient attention by some authors, reviewers and editors of scientific journals to the analytical chemistry of NO (and other NO-related molecules) pose reasonable concerns on most of these findings of clinical research and their ensuing conclusions on the involvement of RSNOs in physiology and pathology [16,56–58]. Thus, it is possible that measurement of RSNOs in different biological fluids may have a clinical relevance, but this can be understood with certainty only once some evident methodological drawbacks are solved.

### 5.2. Sample manipulation and pre-analytical phase

Not less important than the use of the appropriate analytical technique is the actual sample processing. The importance of adding SH-alkylating agents such as *N*-ethylmaleimide (NEM) to prevent *S*-transnitrosations and chelators to avoid metal-mediated degradation in the samples before analyses has been highlighted [59]. In procedures requiring acidification for protein precipitation and/or sample derivatization, nitrite is transformed into nitrous acid ($pK_a = 3.4$), which is a strong nitrosating agent, able to artificially lead to an increase in RSNOs [60]. Thus, the use of NEM or other thiol blockers in large excess over matrix concentrations of thiols is particularly recommended. Analogously, some additional critical points should be considered during sample manipulations. The collection of samples in chilled tubes to be immediately kept and maintained at 2–4 °C, sample handling in the dark to prevent degradation, as well as avoiding freeze/thawing process that may elicit artifactual production of RSNOs are highly recommended [61,62].

In addition, a careful consideration should be paid to the choice of commercially available chemicals and solutions that may contain considerable amounts of nitrite [63]. Moreover, because some anticoagulants, beyond being contaminated with nitrite, may also influence the quantification of RSNOs [64,65], this possible interference with the analytical phase must be verified. Finally, ultrafiltration procedures have been reported to deliver some NO metabolites ($NO_x$) such as nitrite and nitrate from cellulose filters, thus leading to an overestimation of the real RSNO value [66].

### 5.3. The most common analytical procedures

*S*-Nitrosothiols in biological fluids have been quantified by a wide spectrum of different techniques, e.g., chemiluminescence, spectrophotometry, HPLC, gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), fluorimetry, electron paramagnetic resonance (EPR) spectroscopy, and immunological methods. Some methods are directed to measurement of total *S*-nitrosothiols, whereas others may discriminate between PSNOs and LMM RSNOs. Most of the developed techniques involve the conversion of the *S*-nitroso group to nitrite (e.g., by $HgCl_2$ action) or its reduction to NO and finally the detection of NO itself

Table 2
Reported *S*-nitrosothiol levels in red blood cells in health and disease measured by different methodologies[a]

| Sample | *S*-Nitrosothiol | Concentration (μM) | Methodology | Cleavage | Publication year | Reference |
|---|---|---|---|---|---|---|
| Rat (arterial) | RSNO | 3.40 ± 1.55 | Spectrophotometer (Griess) | $HgCl_2$/Cd | 1997 | [76] |
| Rat (arterial) | Hb-SNO | 0.144 ± 0.012 | Chemiluminescence | $I_2/I^-$ | 2002 | [82] |
| Rat | Hb-SNO | 0.037 ± 0.011 | Chemiluminescence | $I_2/I^-$ | 2002 | [82] |
| Human (arterial) | Hb-SNO | 0.046 ± 0.017 | Chemiluminescence | $I_2/I^-$ | 2002 | [87] |
| Human | Hb-SNO | 0.069 ± 0.011 | Chemiluminescence | $I/I_2^-$ | 2002 | [87] |
| Rat | RSNO | 5.1 ± 2.87 | Spectrophotometer (Griess) | $HgCl_2$/Cd | 1997 | [76] |
| Human (arterial) | Hb-SNO | 0.161 ± 0.042 | Chemiluminescence | $I_2/I^-$ | 2000 | [122] |
| Human | Hb-SNO | 0.142 ± 0.029 | Chemiluminescence | $I_2/I^-$ | 2000 | [122] |
| Human (arterial) | Hb-SNO | 0.099 ± 0.036 | Chemiluminescence | $I_2/I^-$ | 2000 | [129] |
| Human | Hb-SNO | 0.117 ± 0.054 | Chemiluminescence | $I_2/I^-$ | 2000 | [129] |
| Rat | Hb-SNO | 0.025 ± 0.021 | Fluorimeter (DAN) | $HgCl_2$ | 2000 | [36] |
| Rat (LPS-treated) | Hb-SNO | 0.605 ± 0.162 | Fluorimeter (DAN) | $HgCl_2$ | 2000 | [36] |
| Human (arterial) | Hb-SNO | 2.50 | Chemiluminescence | Photolysis | 2002 | [14] |
| Human | Hb-SNO | 0.30 | Chemiluminescence | Photolysis | 2002 | [14] |
| Human | Hb-SNO | 1.930 ± 0.281 | Fluorescence (DAF-2) | $HgCl_2$ | 2002 | [14] |
| Human | Hb-SNO | <0.050 (LOD) | Chemiluminescence | $I_2/I^-$ | 2003 | [130] |
| Rat | Hb-SNO | 0.072 ± 0.033 | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Rat (arterial) | Hb-SNO | 0.288 ± 0.025 | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Guinea pig (arterial) | Hb-SNO | 0.431 ± 0.099 | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Human | Hb-SNO | <0.001 (LOD) | Chemiluminescence | $I_2/I^-$ | 2003 | [127] |
| Rat | Hb-SNO | 0.123 ± 0.016 | Chemiluminescence | $I_2/I^-$ | 2004 | [40] |
| Human (left ventricle) | Hb-SNO | 6.15 ± 1.81 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human (pulmonary artery) | Hb-SNO | 6.70 ± 1.76 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human CHF (left ventricle) | Hb-SNO | 13.7 ± 3.21 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human CHF (pulmonary artery) | Hb-SNO | 6.90 ± 2.08 | Chemiluminescence | $I_2/I^-$ | 2004 | [92] |
| Human | Hb-SNO | 22 | Chemiluminescence | $I_2/I^-$ | 2004 | [48] |
| Human type 1 diabetes | Hb-SNO | 13.2 | Chemiluminescence | $I_2/I^-$ | 2004 | [48] |
| Human | RSNO | 0.177 ± 0.107 | Chemiluminescence | $Cu^+$/Cys | 2005 | [90] |
| Human SIRS and ARDS | RSNO | 3.70 ± 1.97 | Chemiluminescence | $Cu^+$/Cys | 2005 | [90] |
| Human | Hb-SNO | 3.52 | Chemiluminescence | Photolysis | 2005 | [44] |
| Human hypoxiemia | Hb-SNO | 0.235 | Chemiluminescence | Photolysis | 2005 | [44] |
| Human | RSNO | 0.700 | Chemiluminescence | Photolysis | 2005 | [15] |
| Human mild SCD | RSNO | 0.280 | Chemiluminescence | Photolysis | 2005 | [15] |
| Human severe SCD | RSNO | 0.187 | Chemiluminescence | Photolysis | 2005 | [15] |
| Human | Membrane PSNO | 5.10 | Chemiluminescence | Photolysis | 2005 | [15] |
| Human mild SCD | Membrane PSNO | 1.50 | Chemiluminescence | Photolysis | 2005 | [15] |
| Human severe SCD | Membrane PSNO | 0.300 | Chemiluminescence | Photolysis | 2005 | [15] |
| Sheep[b] | RSNO | 0.025 | Amperometry | $Cu^{2+}/Cu^+$ | 2005 | [115] |
| Human | Hb-SNO | 0.054 | Chemiluminescence | $I_2/I^-$ | 2006 | [74] |
| Human (arterial) | Hb-SNO | 0.050 | Chemiluminescence | $I_2/I^-$ | 2006 | [74] |

ARDS: acute respiratory distress syndrome; CHF: congestive heart failure; DAN: 2,3-diaminonaphthalene; RA: rheumatoid arthritis; SCD: sickle cell disease; SIRS: systemic inflammatory response syndrome.

[a] All values are referred to the whole blood volume. For the original data referring to red blood cells (RBC) values, the blood values have been calculated by considering a hematocrit value of 45%. For the original data expressed as mol NO/mol Hb, values have been calculated by considering 300 g/l of hemoglobin (if not differently specified) in RBCs. Additionally, for the calculations the Hb was considered as a tetramer (if not differently specified). For membrane RSNO a protein concentration of 3 mg/ml blood was considered. Some of these values (reported without SD) were approximately deducted from graph data.

[b] Value measured in whole blood.

or nitrite formed from NO. Alternatively, a procedure for *S*-nitrosoglutathione determination by detection of the glutathionyl moiety after its cleavage with 2-mercaptoethanol has been proposed [67]. It requires a pre-column derivatization with *o*-phthalaldehyde (OPA) followed by HPLC separation and fluorescence or UV detection. Differently, the biotin-switch method involves the cleavage of the S–NO bond by ascorbic acid, the biotinylation of the nascent thiol and the immunological detection of the biotin tag [68]. To the authors' knowledge, only a few methodologies have been applied for the direct measurement of RSNOs. Specifically, GSNO has been detected in biological fluids by LC–MS in human plasma [69]; however, the authors reported only a comparison between the tracings of plasma from arterial and venous blood and any reference to the concentrations found was lacking. Additionally, *S*-nitrosocysteine was directly measured in rabbit plasma, before and after the in vivo intravenous administration of *S*-nitrosoalbumin, by HPLC coupled to an electrochemical detector (ECD) with a dual Au/Hg electrode [70]. Blood samples were immediately centrifuged, then plasma was microfiltered and injected into the HPLC/ECD instrument.

In the present review, we have tried to critically analyze the techniques applied for *S*-nitrosothiol detection in biological fluids. We hope this analysis will set the stage for further

Table 3
Reported *S*-nitrosothiol levels in different biological fluids in health and disease measured by different methodologies

| Sample | *S*-Nitrosothiol | Concentration (μM) | Methodology | Cleavage | Publication year | Reference |
|---|---|---|---|---|---|---|
| Human BALF | RSNO | 0.240 ± 0.075 | Chemiluminescence | Photolysis | 1993 | [131] |
| Human tracheal aspirate | RSNO | 0.530 ± 0.110 | Chemiluminescence | Photolysis | 1993 | [131] |
| Human BALF lung transplant | RSNO | 0.830 ± 0.063 | Chemiluminescence | Photolysis | 1993 | [131] |
| Human BALF pneumonia | RSNO | 4.43 ± 1.40 | Chemiluminescence | Photolysis | 1993 | [131] |
| Human tracheal aspirate ECMO | RSNO | 0.056 ± 0.027 | Chemiluminescence | Photolysis | 1993 | [131] |
| Human synovial fluid RA | RSNO | 10.1 ± 2.90 | Spectrofluorometric | Unspecified | 1997 | [52] |
| Human synovial fluid OA | RSNO | 8.20 ± 3.52 | Spectrofluorometric | Unspecified | 1997 | [52] |
| Human urine | RSNO | <0.150 (LOD) | Fluorescence (DAN) | $HgCl_2$ | 1997 | [71] |
| Human urine sepsis | RSNO | 8.90 ± 3.90 | Fluorescence (DAN) | $HgCl_2$ | 1997 | [71] |
| Human BALF | RSNO | 0.080 | Chemiluminescence | $Cu^+$/Cys | 1999 | [132] |
| Human BALF CF | RSNO | Not detectable (LOD not reported) | Chemiluminescence | $Cu^+$/Cys | 1999 | [132] |
| Exhaled breath | RSNO | 0.110 ± 0.020 | Spectrophotometer (Griess) | $HgCl_2$ | 2001 | [55] |
| Exhaled breath severe asthma | RSNO | 0.810 ± 0.060 | Spectrophotometer (Griess) | $HgCl_2$ | 2001 | [55] |
| Exhaled breath mild asthma | RSNO | 0.080 ± 0.010 | Spectrophotometer (Griess) | $HgCl_2$ | 2001 | [55] |
| Exhaled breath CF | RSNO | 0.350 ± 0.070 | Spectrophotometer (Griess) | $HgCl_2$ | 2001 | [55] |
| Exhaled breath COPD | RSNO | 0.240 ± 0.040 | Spectrophotometer (Griess) | $HgCl_2$ | 2001 | [55] |
| Human sputum | RSNO | 38 | Spectrophotometer (Griess) | $HgCl_2$ | 2004 | [54] |
| Human sputum, COPD | RSNO | 60.4 | Spectrophotometer (Griess) | $HgCl_2$ | 2004 | [54] |
| Human gastric juice | RSNO | 0.204 | Chemiluminescence | $Cu^+$/GSH | 2005 | [114] |
| Human synovial fluid | RSNO | 0.308 | EPR spectrometry | MGD | 2005 | [114] |

BALF: bronchoalveolar lavage fluid; CF: cystic fibrosis; COPD: chronic obstructive pulmonary disease; DAN: 2,3-diaminonaphthalene; ECMO: extracorporeal membrane oxygenation; EPR: electron paramagnetic resonance; MGD: *N*-methyl-D-glucamine dithiocarbamate; OA: osteoarthritis; RA: rheumatoid arthritis.

validation and consensus on methodologies, and will lead to a clearer understanding of the physiological relevance of RSNOs in vivo.

### 5.3.1. Spectrophotometric detection

The simplest procedure to determine RSNOs is based on a modification of the reaction described by Griess more than one century ago. Under acidic conditions (pH < 3), sulfanilamide reacts with nitrite forming a diazonium cation which, in turn, reacts with *N*-(1-naphthyl)ethylenediamine yielding a diazo molecule characterized by a strong absorbance around 540 nm [65]. Some authors have also suggested that variations, e.g., the replacement of sulfanilamide with dapsone, increase the performance of the method [71]. Since this procedure is essentially based upon the detection of nitrite, the concentration of which in biological fluids is largely higher than that of RSNOs, it is absolutely necessary to remove the pre-existing nitrite before RSNO analysis [72]. This can be achieved by different procedures, e.g., ultrafiltration or gel-filtration of samples to remove low-molecular-weights compounds [36,37]. Alternatively, sulfamate can be added to samples at 5–10 mM final concentrations, thus reducing nitrite to $N_2$ (Fig. 3) [73]. All these procedures may have significant drawbacks: the materials applied for the ultrafiltration may release RSNO metabolites such as nitrite [66], which may interfere with the measurement. Additionally, it is not so easy to completely eliminate the nitrite background because some proteins such as hemoglobin have specific binding sites for nitrite [37,74]. Sulfamate is able to eliminate nitrite, but sample acidification is required (Fig. 3). Under acidic conditions, nitrite is protonated into nitrous acid that nitrosates SH groups producing RSNOs; thus, NEM or other molecules able to alkylate free thiols must be used before sample acidification [72].

The nitroso moiety of RSNOs is generally transformed into nitrite by the action of $HgCl_2$. It is obvious that, when sulfamate is used, its excess may remove also nitrite produced de novo from the cleavage of RSNOs, leading to a possible underestimation of *S*-nitrosothiols. Even if the reaction of nitrite with sulfamate is slower than that with sulfanilamide, mainly because of the high concentrations of sulfanilamide [75] used in the assay procedure (i.e., a final concentration of 0.5 wt.% sulfanilamide), this possible interference should not be neglected. In some cases, the use of $HgCl_2$ has been coupled with the addition of cadmium probably to facilitate the cleavage process [76]. However, it should be considered that cadmium is able to reduce nitrate to nitrite in a pH-dependent manner, allowing its detection by reaction with

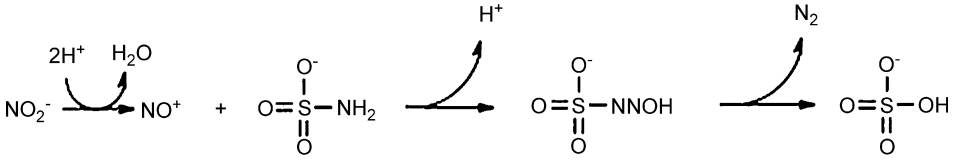


Fig. 3. Schematic representation of the reaction between sulfamate and nitrite. Nitrite is converted to $NO^+$ at acidic pH and then $NO^+$ reacts with ammonium sulfamate. Nitrogen and sulfuric acid are the products of the reaction.

the Griess reagents. Consequently, over-estimated values can be obtained.

By the use of the spectrophotometric detection after derivatization with the Griess solution, RSNOs have been measured in plasma, BALF, urine, blood (Tables 1–3). Some ready-to-use colorimetric detection kits are also available (e.g., Oxonon, Alexis Biochemicals) [54,55]. However, the detection limit of the method close to 0.2 μM of nitrite [56,77] represents the main limit that hampers application of the Griess assay on most biological samples. In our experience, the Griess method is not sensitive enough to allow RSNO measurement in normal human plasma or whole blood, suggesting that levels of RSNOs in human blood are lower than 0.2 μM [56]. To confirm this, the addition of standard solutions of *S*-nitrosoalbumin (Alb-SNO) or *S*-nitrosohemoglobin (0.5–10 μM) to the same samples readily allowed their detection with the appearance of the typical red-purple color [56,77]. We think that this easy experiment should be a starting point to grossly indicate the RSNO levels in samples to be analyzed and, in particular, to confirm data obtained by other techniques when the measured concentrations are above the detection limit of the spectrophotometric Griess method.

#### 5.3.2. Fluorimetric detection

Nitrite derived from decomposed RSNOs can be detected by means of the diazotization reaction of 2,3-diaminonaphthalene (DAN), a reaction that takes place intramolecularly, because the DAN molecule contains two amino groups in *ortho*-position. This reaction yields 2,3-naphthotriazole (NAT), a stable fluorescent compound, whose fluorescence is measured using an excitation of 365 nm and emission of 405 nm [71]. Fluorescent procedures based on this diazotization reaction have been used to measure nitrite, nitrate and *S*-nitrosothiols ([65,71] and Tables 1 and 2). Basically, the same possible interferences described for spectrophotometric RSNO detection can also affect fluorimetric methods, with the major problems being the lack of selectivity and the scarce reproducibility. This is probably due to the fact that some of the applied procedures for nitrite removal by treatment with sulfamate were carried out without sample acidification [49,50,71]. This generated a significant pitfall, since an absolute requirement for sulfamate to trap nitrite is an acidic pH [78]. Additionally, some doubts have also been posed about the employment of sulfamate to remove the nitrite background. Some data, in fact, seem to indicate that this action should be preferentially achieved by using sulfanilamide [72].

An alternative fluorimetric assay has been applied to determine RSNOs in plasma samples from normal pregnant and pre-eclamptic women [45]. By this procedure, RSNOs are determined after the delivery of NO by UV radiation and its reaction with 4,5-diaminofluoresceine (DAF-2), which was declared by the authors themselves to specifically react with NO but not with other $NO_x$ under the applied experimental conditions [45]. However, the original method [79] was not validated in plasma but only used for evidencing NO production within cells. Nevertheless, it has also been reported that photolysis in the presence of free thiols enhances NO and RSNO formation from nitrate, suggesting that most of the RSNOs measured by this methodology are likely due to such artifact [80].

Declared detection limit of the fluorimetric methods is as low as 20 nM [71]. Great discrepancies in reference values can be observed for RSNOs measured in body fluids by these fluorimetric methods by analyzing the data in Table 1. Marzinzig et al. [71] and Wlodek et al. [49] found mean values of 450 and 8800 nM in plasma of healthy subjects, respectively, and more generally plasmatic levels of RSNOs assessed with the above described procedures ranged from undetectable [81] to 9.2 μM [45].

#### 5.3.3. Chemiluminescence

Gas-phase chemiluminescence is undoubtedly the most widely applied method for RSNO detection, mainly because of its suitability in terms of low detection limit [82]. Briefly, it consists of an indirect analysis of RSNOs by measuring the NO derived from the cleavage of the S–NO bond. The NO gas is drawn by a vacuum into a reaction chamber, where it is oxidized with ozone to form excited $NO_2^*$, which rapidly decays back to its ground state, emitting near-infrared radiation and can be quantified by a photomultiplier [83]. This methodology can be applied to measurements in biological samples by taking advantage of the low solubility of NO in aqueous solutions (close to 2.0 mM at 20 °C, [84]): in fact, NO has a partition coefficient between gaseous and aqueous phases of about 20. Thus, NO dissolved in the liquid phase can be displaced by bubbling an inert gas through the specimen [85]. Different procedures have been proposed to release NO from RSNOs, the most frequently used of which are photolysis and chemical methods involving use of $Cu^+$/cysteine or iodine/triiodide [59]. The preferential choice of the reductive agent is a quite delicate aspect since these procedures greatly differ in terms of reducing properties, which in turn influence both the efficiency and the specificity of the reaction. For example, it has been observed that photolysis is not specific for the cleavage of the S–NO bond, but it can also cause the release of nitroso groups from nitrosamine, dinitrosyl–iron complexes, leading to a possible overestimation of the results [86]. Moreover, it has also been reported that nitrate can be reduced to NO by photolysis in the presence of thiols [80]. These observations might explain the high concentration of RSNO measured by photolysis-chemiluminscence methods in biological samples [15,41,44]. The substitution of photolysis with chemical reduction seems to be able (at least in part) to overcome these methodological problems. In particular, recent data seem to indicate the cleavage of the S–NO bond with $I_2/I^-$ (triiodide method), coupled with some additional steps during sample preparation, as the most appropriate procedure for RSNO analysis by chemiluminescence [59]. Specifically, since HI derived by the use of $I^-$ in acidic environment is able to reduce nitrite, generating indefinite amounts of NO, the pre-treatment of samples with acidified sulfanilamide is carried out. In fact, acidified sulfanilamide, by reacting with nitrite, forms a diazonium ion that is not reduced to NO by HI, largely increasing the selectivity of the procedure [72]. *S*-Nitrosothiols can be further distinguished from nitrosyl complexes by reaction of the sample, prior to the addition of acidified sulfanilamide, with and without mercuric chloride, which converts S–NO to nitrite. Finally, RSNOs are measured as the difference of the chemiluminescence signal

between samples pre-treated or not treated with $HgCl_2$ [37]. By this procedure, RSNOs have been measured both in plasma and in RBCs. Because of the instability of Hb-SNO in reducing environments when whole blood or RBCs have to be analyzed [87], sample pre-treatment with a Hb-SNO stabilizing solution containing $K_3Fe(CN)_6$, NEM, and Nonidet-P-40 is required [37].

RSNO concentration both in plasma and RBCs measured with this methodology was found to be several orders of magnitude lower than that obtained by photolysis/chemiluminescence (see data in Tables 1 and 2).

The use of $Cu^+$/cysteine as cleaving agents can have some advantages, since the neutral pH necessary to perform the reductive step ensures that nitrite, nitrate and probably other $NO_x$ are not detected, possibly rendering this procedure highly specific for RSNO detection [88]. Cysteine has two functions. First, by *S*-transnitrosation, it transforms all RSNOs into Cy-SNO; the $NO^+$ equivalent is then reduced by $Cu^+$, forming NO (and $Cu^{2+}$) with the transnitrosation equilibrium that favors *S*-nitrosocysteine because of the excess of Cys. Second, Cys reduces $Cu^{2+}$, regenerating $Cu^+$ [89]. The method, as stated by the authors, appears not to be affected by NO release from Fe-NO Hb, rendering it particularly suitable for RSNO detection in blood [42,88,90]. However, an accurate validation of this procedure has not yet fully carried out, in particular, its selectivity needs to be proven.

The use of $HgCl_2$ for RSNO cleavage and the successive reduction of nitrite with vanadate or $I^-$ has also been adopted [51,91]. However, the authors did not remove the blank nitrite present in their samples, thus de facto they measured nitrite plus *S*-nitrosothiols. The levels of RSNOs were thus assessed after subtraction of the signal obtained from samples untreated with $HgCl_2$. However, this proceeding affects the accuracy of the method and this procedure has been abandoned in more recent years.

About the ozone-chemiluminescence technique, it is necessary to verify its selectivity and accuracy. Some doubts have been cast on the pre-treatment of samples with acidified sulfanilamide, since it has been observed that hydrochloric acid per se is able to reduce the chemiluminescence signal [42]. Nevertheless, this statement has been recently challenged [74], demonstrating that RSNOs are stable at acidic pH values and that acidic sulfanilamide does not interfere with RSNO titration. Additionally, it has underlined the scarce NO yield from Alb-SNO: 78% by $I_2/I^-$ chemiluminescence, and 34% by $Cu^+$/Cys chemiluminescence [42,82,88]. Recent data also suggest that use of $Cu^+$/cysteine and $I_2/I^-$ reagents underestimate the concentration of Hb-bound NO in blood, because of the auto-capture of NO by deoxygenated Hb/cell-free heme in the reaction chamber [42]. To avoid this problem, the same authors suggest to add $K_3Fe(CN)_6$ to the original triiodide reagent, so as to oxidize heme and generate methemoglobin, which binds NO with lower affinity and, consequently, is a less potent scavenger of NO. Therefore, the released NO can escape auto-capture in the reaction chamber. Alternatively, in order to avoid NO auto-capture, carbon monoxide has been added to the inert gas flow through the reflux chamber [90]. Nevertheless, the problem to avoid the NO auto-capture by heme by the addition of $K_3Fe(CN)_6$ was also considered for the triiodide assay, as clearly reviewed by Yang et al. [37]. It is not clear why Rogers et al. [42] and Doctor et al. [90] did not consider these aspects in their recent experiments. Moreover, by means of this procedure, total NO (i.e., Hb-SNO, Fe-NO Hb, and any nitrite bound to Hb) has been measured in human blood and levels ranging from 445 to 600 nM have been found [42], which are largely lower than that reported by the same authors for RSNOs in previous works [47,48,92]. The paper by Rogers et al. [42] as well has recently been challenged and the conclusions put into question [74], suggesting that most of the differences reported were simply due to the nitrite contained in RBCs that was co-titrated with RSNO.

Finally, it has been reported that some of the problems of incomplete NO yield from RSNOs, when $Cu^+$/Cys are exploited as cleaving agents, could be due to the low solubility of CuCl at neutral pH and the ease by which $Cu^+$ can be oxidized to $Cu^{2+}$. As a consequence, the authors advised to substitute the cysteine with the ascorbate [89], an efficient reducing agent of $Cu^{2+}$ but not of nitrite.

From data of Tables 1–3, great discrepancies among basal values measured with the different analytical procedures based on chemiluminescence are evident. These discrepancies are still present when comparing levels detected by using techniques that involve similar procedures for the S–NO cleavage. This indicates that sample manipulation and pre-analytical phase are probably critical steps. This is particularly evident in samples rich in hemoglobin (whole blood or RBCs).

#### *5.3.4. Mass spectrometry*

Among the analytical methods available for RSNO analysis, mass spectrometry (MS)-based approaches, such as gas chromatography–mass spectrometry, and liquid chromatography–mass spectrometry, are emerging as fundamental tools, in particular as a consequence of their inherent selectivity and accuracy. These features of MS-based methods are due to the use of stable-isotope labeled analogues of the endogenous compounds as internal standards. Due to the thermal lability of the *S*-nitroso group, the necessity of derivatization to render these compounds volatile, and the fact that most of RSNOs in biological fluids are located in protein cysteine moieties, GC–MS is difficult to be applied. From this point of view, LC–MS should be considered more appropriate for the MS-analysis of RSNOs, but also this approach is limited because of the technical difficulties linked to the necessity of detecting *S*-nitrosated proteins in a sample containing a complex mixture of proteins. On the other hand, a direct analysis of LMM RSNOs, even if easier, is hampered by their very low levels in vivo.

GC–MS methods have been developed, validated and applied to quantify *S*-nitrosoalbumin levels in plasma of healthy and diseased humans (for a review see Ref. [93]). The RSNO cleavage was carried out by $HgCl_2$ or $CuSO_4$/cysteine; nitrite produced was derivatized with pentafluorobenzyl bromide (PFB bromide) by means of a substitution reaction that leads to the formation of the nitro-PFB derivative, which can be then analyzed by GC–MS. These methods use *S*-[$^{15}$N]nitrosoalbumin as an internal standard. The standard is added to samples at the appropriate final concentration and undergoes all physical changes during the whole analytical process. Endogenous molecules and their

respective stable-isotope labeled analogues behave almost identically until their separation in the mass spectrometer according to their mass-to-charge ratios. By means of this method, the concentration of *S*-nitrosoalbumin in plasma of healthy humans was determined to be within the range 150–205 nM [94,95]. Since endogenous nitrite will also contribute to RSNOs, *S*-nitrosoproteins (and thus proteins) are separated by extraction on HiTrapBlue Sepharose affinity columns. As stated above, these GS–MS-based analytical procedures offer high selectivity and accuracy. However, being indirect measurements, titration of nitrite released from RSNOs may have some drawbacks essentially due to the presence of nitrite in all the used buffers, co-eluting nitrite, and other $NO_x$ that can form nitrite during sample manipulation. Therefore, blank nitrite is to be accurately assessed and its value subtracted.

LC–MS-based methods are able to detect *S*-nitrosothiols without derivatization; however, such methods are limited to detection of LMM RSNOs (e.g., GSNO [100,101]) or Hb-SNO produced in vitro [96]. Consequently, these procedures have actually found little application in RSNO detection. This reflects the intrinsic feature of the LC–MS-based technology coupled with the fact that most of RSNOs in biological fluids are probably bound to proteins. In other words, LC–MS can be useful to detect LMM RSNOs, but their concentrations are presumably too low to be reliably measured in most biological samples. Notwithstanding, some papers, in which an effort at improving the sensitivity and selectivity of proteomic approaches based on mass spectrometric techniques has been done, have been recently published [97–99].

### 5.3.5. *High-performance liquid chromatography*

Chromatographic separation before RSNO detection may represent a useful tool to increase the specificity of the methodology. Additionally HPLC would allow direct simultaneous measurements of different RSNOs (for example, by discriminating among various LMM RSNOs), without any need of derivatization, by simply detecting spectrophotometrically UV or visible absorbance at 335 or 545 nm, respectively. However, this methodology is actually limited by the low extinction coefficients of RSNOs (e.g., 920 $M^{-1}\,cm^{-1}$ at 335 nm [102] and 17.2 $M^{-1}\,cm^{-1}$ at 545 nm for GSNO [103]). Moreover, most RSNOs in biological samples are likely represented by *S*-nitrosoproteins, which elute with different retention times and the non-nitrosated proteins have absorbance spectra that overlap those of PSNOs, rendering impracticable any direct analysis [60]. Therefore, HPLC separation has been coupled with the indirect spectrophotometric detection of RSNOs by reaction with the Griess reagents [36,104,105]. $HgCl_2$ post-column is used to decompose RSNOs and successively the diazo compound is detected after HPLC separation. This is an improvement compared to the classical spectrophotometric analysis with the Griess reagents, as HPLC can ensure a lower detection limit due to minor interference by background spectra (due to the chromatographical fractionation of the molecules contained in the sample). However, it presents some of the possible drawbacks (e.g., nitrite interference, artificial formation of RSNOs) previously described for the spectrophotometric Griess method. The problem of nitrite contamination was solved by subtracting the peak area obtained with or without pre-treatment with $HgCl_2$ [105], with consistent troubles of reproducibility and quantification, mainly because nitrite is much more abundant than RSNOs in analyzed samples. Other authors pre-treated samples by ultra-filtration thus eliminating the pre-existing nitrite [36,104] but, as above mentioned, the ultra-filtration step is not always artifact-free. Interestingly, a post-column derivatization procedure for LMM RSNO analysis has been proposed [36,104]: LMM RSNOs were separated by ultra-filtration and loaded onto HPLC equipped with a RPC18 column; the eluate was mixed before detector with the Griess solution containing $HgCl_2$ and the diazo compound developed was detected at 540 nm. A similar procedure was adopted also for PSNO analyses only changing the chromatographic column. In both cases, the detection limit was 20 nM [36,104]. Although the above described methods yielded divergent values for RSNOs in human plasma (Table 1), they suggest, as general information that RSNO physiological concentrations are rather at the nanomolar level. In particular, the method proposed by Jourd'heuil et al. was applied for the detection of LMM RSNOs and gave the fundamental information that their levels both in human and rat blood are under the declared detection limit of the method (i.e., 20 nM) [36,104].

HPLC for detection of RSNOs can be further improved in terms of sensitivity and detection limit by coupling HPLC with the fluorimetric analysis. We have developed a procedure suitable for the measurement of PSNOs by reversed phase-HPLC coupled with fluorescent detection following reaction of nitrite with DAN under acidic conditions [56]. As previously described, the relatively weak fluorescent DAN agent reacts rapidly with nitrite at acidic pH to yield the highly fluorescent derivative NAT. In our procedure, plasmatic proteins are separated by acetone precipitation (thus eliminating the basal nitrite levels of the sample), nitrite is delivered from PSNOs by $HgCl_2$ treatment and, successively, incubation with DAN under acidic conditions is carried out after blocking free thiols with an excess of NEM. 2,3-Naphthotriazole is then separated by HPLC on a C18 column and detected by fluorescence. Our data seem to indicate such a methodology as a possible alternative to the chemiluminescence technique, at least for PSNO detection in plasma samples. The sensitivity and the relative linearity of this method are confirmed by analyses obtained from human plasma spiked with 20–200 nM of authentic human *S*-nitrosoalbumin (Fig. 4). Human Alb-SNO was obtained by reaction between GSNO and human albumin as previously described [106]. Briefly, human albumin, dissolved in Na/K phosphate buffer (0.1 M, pH 7.4) at a final concentration of 30 mg/ml, was incubated with GSNO (1 mM final concentration). After a 90 min incubation, excess of GSNO was removed by passing reaction mixture through Sephadex PD-10 columns (Pharmacia Biotech, Sweden). Alb-SNO titer was carried on by colorimetric determination of nitrite after decomposition of the S–NO bond with $HgCl_2$.

Human blood was obtained from healthy donors and immediately centrifuged (20 s at 10,000 × *g*) for plasma analyses. Plasma was then spiked with different amount of Alb–SNO and rapidly treated with NEM (10 mM final concentration). After 30 s from NEM addition, proteins were precipitated by

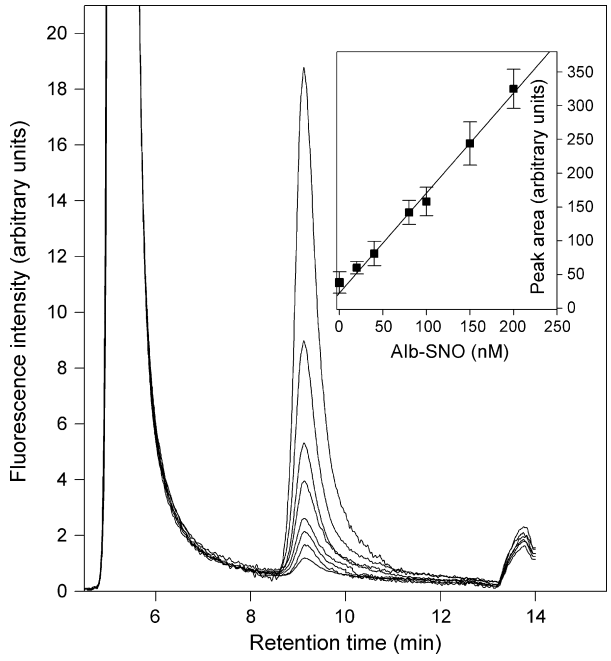


Fig. 4. HPLC elution profile of 2,3-naphthotriazole obtained from nitrite released by $HgCl_2$ cleavage of plasmatic PSNO reaction with DAN. Human blood was obtained from healthy donors and immediately centrifuged for plasma analyses. The peaks around 9 min retention time represent 2,3-naphthotriazole, the tracings are representative of a typical experiment in which the following samples (from the lower to the upper) were analyzed: blank (human plasma pretreated with $HgCl_2$ before acetone precipitation), unspiked human plasma, human plasma spiked with 20, 40, 80, 100, 150, 200 nM (final concentrations) *S*-nitrosoalbumin (Alb-SNO). In the inset, the correlation between mean ± S.D. of measured peak areas and the final concentration of added Alb-SNO is shown.

the addition of 4 volumes of acetone. After centrifugation and discarding of supernatant, pellets were washed twice with acetone, resuspended in the presence of 2 mM $HgCl_2$ (final concentration) and, after 2 min, again precipitated with acetone. Supernatants were then acidified with 20 mM HCl (final concentration) and incubated with 5 μg/ml DAN (final concentration) for 5 min. The solution was neutralized with a mixture of 1.0 M HEPES, pH 7.0/1.25 M NaOH (6:1, v/v), loaded onto HPLC (Sephasil C18 column), eluted isocratically (55 vol.% methanol and 45 vol.% 50 mM Hepes, pH 7.0) and detected by fluorescence (excitation at 363 nm and emission at 430 nm wavelength).

The plot of peak area versus concentration suggests a good linearity; the concentration of PSNOs in human plasma at the basal state obtained from these data is estimated to be 34 ± 15 nM [56], a value near to the detection limit of the method (i.e., 20 nM). This concentration was calculated after subtraction of the area given by "blank reactions" obtained by analyzing the same plasma samples without addition of Alb-SNO, but pretreated with $HgCl_2$ (before acetone precipitation) in order to eliminate all present *S*-nitrosothiols. This blank was greater than that obtained with reagents only. The presence of this "not-eliminable" blank peak is therefore the shortcoming of the method, in particular because its intensity may vary between experiments. We found that it could be reduced by a preparative purification of the DAN reagent through C18 columns and using as much as possible nitrite-poor buffers. Furthermore, the "not-eliminable" blank peak is also influenced by nitroso gases ($NO_x$) present in the atmosphere, since its intensity increases with time after dry powders of DAN and $HgCl_2$ are dissolved. However, we cannot exclude that some of the nitrite present in plasma samples was still present despite the acetone precipitation and washing. Therefore, we are trying to clarify these aspects before proposing our method as valid and reproducible for PSNO detection in plasma. In any case, values for basal plasma PSNOs we have found by our method agree with those reported in some recent papers (see Table 1). This further indicates that the physiological concentration of these molecules in human plasma is at the low nanomolar level rather than at the micromolar level.

### *5.3.6. Other analytical procedures*

To understand how *S*-nitrosation is involved in NO-mediated post-translational modification of proteins, the proteomic analysis of *S*-nitrosated proteins is fundamental. In fact, a key issue to evaluate the occurrence and, in case, the putative role of this protein modification is the availability of a methodology allowing the detection not only of the total amount of *S*-nitrosothiols in a tissue, but also the *S*-nitrosation of each single protein. For this purpose, the biotin-switch assay has been developed [107]. This semi-quantitative methodology has been used to reveal the occurrence of single nitrosated proteins in tissues and cells; furthermore, it has recently been applied to the quantitative detection of Alb-SNO in human plasma as well, yielding values in healthy pregnant women close to 3 μM [46]. This procedure includes three main steps: (i) the alkylation of free thiols by the thiol-specific reagent methyl-methanethiosulfonate (MMTS); (ii) the reduction of *S*-nitrosothiols by ascorbate (after MMTS being removed); (iii) the reaction of the newly formed thiols with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propionamide. Biotinylated proteins are then revealed by Western blot using a peroxidase-conjugated anti-biotin antibody [68]. However, it has recently been evidenced that *S*-nitrosated proteins have different reactivity towards ascorbate and that, under the suggested conditions, ascorbate is able only to cleave a minor percentage of PSNOs, so that the detected species may represent the proteins that are sensitive to ascorbic acid reduction, rather than the *S*-nitrosated protein pool [108]. To overcome this problem, it has been suggested that the assay should be modified by drastically incrementing both the concentration of ascorbate (to 10–50 mM) and the time of incubation (up to 3–5 h). Unfortunately, to date, the selectivity of the procedure to cleave the S–NO bond has been poorly evaluated. Considered that the concentration range of PSNOs is likely to be much lower than that of protein disulfides, it is of fundamental importance to demonstrate that ascorbic acid is selective for RSNOs, i.e., it is unable to reduce various S–S bridges abundantly present in plasma. Jaffrey et al. stated that, in their experiments, ascorbic acid failed to reduce 5,5′-dithiobisnitrobenzoic acid (DTNB) as well as glutathione disulfide [107]. However, in a recent paper, it has been shown that ascorbate can reduce the disulfides of tubulin as well as those of DTNB [109]. In addition, some newly published data show that ascorbate per se can interfere with the biotinylation reaction by giving false-positive results [110]. All these critical aspects concerning the

employment of the biotin-switch assay for PSNO detection have also been recently reviewed and discussed [111]. Application of this assay to detect Alb-SNO in human plasma revealed levels of 2.9 and 7.2 μM in healthy pregnant women and in pre-eclampsia, respectively [46]. These values are significantly higher (about three orders of magnitude) than those considered to be physiological in human plasma [111].

An alternative assay for plasmatic RSNOs has been proposed, which is essentially based on the formation of NO from RSNOs by using $CuCl_2$; the released NO is in turn detected electrochemically with an NO electrode [4,112]. Values measured with this method are in the 50–1000 nM range for RSNOs and 10 nM for LMM RSNOs in rat plasma. The discrimination between PSNOs and LMM RSNOs was provided by using different concentrations of $Cu^{2+}$ since PSNOs are less prone to be cleaved by $Cu^{2+}$. Therefore, RSNOs (using high concentrations of $Cu^{2+}$) and LMM RSNOs (at low concentrations of $Cu^{2+}$) were measured by this method. However, also in this case, the authors did not adequately describe and validate the applied procedure nor watchfully weighed up all the possible interferences. Moreover, it has been reported that $Cu^{2+}$ releases NO from RSNO with low efficiency [113], thus the percentage of NO released from RSNOs by this procedure is unknown. These shortcomings and the fact that quantification is based on the use of a single, usually LMM RSNO, question the utility of this method for quantitative analysis of RSNOs in complex biological samples such as plasma.

Recently, an EPR-based analytical procedure has been reported to provide a sensitive assay for RSNOs. *S*-Nitrosothiols are homolytically cleaved by *N*-methyl-D-glucamine dithiocarbamate (MGD) at alkaline pH. The released NO is spin trapped by MGD complexed with $Fe^{2+}$, the resulting adduct is then measured by EPR spectrometry [114]. The method, with a detection limit of 50 nM, was shown not to be influenced by external added nitrite and nitrate or 3-nitrotyrosine. However, even if the procedure could be relevant, an adequate validation is still to be performed, actually in analogy to many other methods for RSNO detection. Calibration curves were constructed with GSNO and not with PSNOs, which are likely to be the main constituent of the RSNO pool. Additionally, the calibration curve was built in the range of 300–1000 nM and the assay precision was determined by using 1 μM GSNO, which is an irrelevantly high concentration. Considering that the authors measured mean values of 90 nM in human plasma by their method [114], it is not clear why they did not produce the calibration curve with concentrations added within a relevant range, i.e., the measured plasma values.

Finally, a direct amperometric procedure for RSNO analysis in biological fluids has been recently described [115]. This method looks promising and combines the use of polymer films containing the redox couple $Cu^{2+}/Cu^{+}$ with the employment of an amperometric NO sensor. RSNOs are decomposed to NO by $Cu^{+}$ with successive NO revealing by the amperometric detector. No interference with nitrite in the range 0.1–100 μM has been observed. Hematic RSNO level of 25 nM in sheep blood has been measured by this procedure. The available data about this new methodology are insufficient to really appreciate if it could be a real improvement for quantitative RSNO detection. Calibration curves have been performed only in buffers by adding 0.2–40 μM standard RSNOs, a concentration range largely higher than that measured in human (and sheep) blood. In addition, the various RSNOs tested were found to produce different detection signals at a molar basis, so that the authors rightly stated that their method is of semi-quantitative value [115].

## 6. Conclusions and future prospects

The application of the above discussed methods for RSNO detection in biological fluids undoubtedly has provided greatly differing values at the basal state. RSNOs have been the subject of intense study following initial reports that plasma contains 7 μM *S*-nitrosoalbumin [41]. Over the last 14 years, the reported measured levels both in plasma and whole blood randomly bounced from low nanomolar to values even greater than those originally proposed by Stamler and co-workers. It is evident that one of the main reasons why a general agreement about the physiological range of RSNOs in human blood (and in other tissues as well) is still lacking, is the availability of numerous methodologies being inappropriately validated prior to use in animal and human studies that could render them universally applied. Since the performance of the methodology applied to RSNO investigation has the potential to alter our perception of some biological processes, a probationary period during which the investigators test the suitability of the method itself and ascertain its limitations is necessary. Many analytical and pre-analytical aspects need to be addressed and defined in order to develop a suitable methodology for RSNOs. Specifically, there are pre-analytical steps to standardize: (i) avoidance of nitrite contamination and artifactual RSNO formation or degradation during sample manipulation; (ii) definite clarification of the need and kind of sample storage before analysis. Additionally, some analytical factors should also be taken into consideration: e.g., the eventual release/formation of NO and nitrite from molecules other than RSNOs, the heterogeneity of RSNOs themselves including the RSNO serving as the calibrator, the selectivity of the method, which is also challenged by the presence of mixtures of hardly distinguishable iron-nitrosyl and *S*-nitrosothiols in plasma and blood. Disregard and/or inadequate consideration of these aspects as a whole have probably generated the disappointing state of the art on RSNO detection presented in Tables 1–3.

To pick the best methodology is a difficult task for the reader. In our opinion, the perfect analytical procedure does not exist at the moment, even if some methods could be better than others. Gas-phase chemiluminescence-based procedures are suitable in terms of detection limit over other techniques, but some aspects should be fully clarified: e.g., the yield of NO from RSNOs, the best cleaving agent(s), the possible interference by other potential NO-releasing molecules and, in particular, why it gives some divergent results when applied by different laboratories. However, if micromolar levels of RSNOs are supposed to be present in samples, the derivatization with the Griess reagents, which has been recently updated for nitrite (and RSNO) detection in the presence of large amounts of proteins, with the advantage of

being time-saving and having a detection limit of 100–200 nM [77], could represent the first choice.

One could probably wonder how it may be possible that, using poorly validated and artifact-prone analytical procedures, many research groups found differences in the measured levels between control subjects and those affected by a particular disease (Tables 1–3). One reason could be the different procedures applied for the collection and storage of samples for control group compared to the pathological one. Another important issue that may explain generation of greatly differing results from the application of apparently the same analytical method could be inadequate adoption and validation of the respective analytical method in the own laboratory. But also many other factors should be considered such as differences in $NO_x$ concentrations, thiol status, diet, and circadian rhythm. Accurate quantitative determination of RSNOs in biological fluids is a great analytical challenge and requires highly qualified skillful personnel and meticulous experimental work.

The frequent use of inappropriate, non-validated and/or artifact-prone analytical techniques in research articles indicates that poor research has survived the peer-review process. Because of the significant importance we would like to discuss this issue in more detail. An aspect of this is the use of inappropriate or incorrect methods. Once incorrect, artifact-enriched analytical procedures become common, it is hard to stop them from spreading through the literature like a genetic mutation. Many editors have wrestled with the problem of authors objecting to a reviewer's criticism on the grounds that the same methods have appeared in previous articles, possibly in the same journal. In addition, many readers seem to assume that articles published in peer-reviewed journals are scientifically sound and methodologically correct, despite much evidence of the contrary. Therefore, it is important that misleading work is identified after publication. Undoubtedly, the ultimate value of an article rests with the reader. Editors should give special attention to the papers (in particular letters) making criticism to previously published methodologies. A problem that frequently hampers an adequate space to letters discussing methodologies is that demonstrating (or suggesting) a paper that contains one or more major analytical flaws may challenge its findings. Thus, as one can imagine, such letters/commentaries are not generally welcome by the editors, since they may be a clear admission that the peer-review process has failed.

Bioanalytical procedures are key determinants in generating reproducible and reliable data. It is essential to apply well-characterized and satisfactorily validated analytical methods, which, constantly undergoing changes and improvements, represent the cutting edge of the technology. Bioanalytical methods must be validated to demonstrate their reliability and reproducibility. In addition to the linearity, detection and quantification limits, analysts should investigate and include in the validation process of their analytical methods particular analytical measures: (i) selectivity; (ii) accuracy; (iii) precision; (iv) recovery; and (v) stability [17,116].

The rules for validation have been globally harmonized in regulated laboratories of the pharmaceutical industry. However, no precise and binding rules exist in the field of basic research, and method validation procedures are freely applied and interpreted by researchers, reviewers and editors of scientific journals. This is particularly evident in the field of detection of oxidative stress biomarkers but also in the field of NO research. Thus, a careful consideration of the Guideline for Industry Text on Validation of Analytical Procedures would be useful ([116], http://www.fda.gov/cder/guidance/).

The first approach for trying to solve the problem could be the creation of consortia of laboratories that, working together, could define the best methodologies to be used for the accurate measurement of every single NO-derived metabolite. This should be done by evaluating the eventual artifacts of the method and/or of the preparative phases, its precision, accuracy, selectivity and, finally, its suitability to be applied on large-scale clinical studies. Moreover, reference values and ranges for physiological levels should be defined and established for particular RSNOs. This could be reached only through the active collaboration of various laboratories working on the same biological matrix (e.g., blood) and by comparing the different analytical procedures. Not only RSNOs represent a problem child in the area of NO research. For other members of the L-arginine/NO pathway, notably nitrite [65], 3-nitrotyrosine [117,118] and asymmetric dimethylarginine [119], also greatly diverging basal values have been reported so far. In recent years, however, reference values have been suggested for these analytes in human plasma, mainly due to advances in analytical chemistry and collaboration among different groups worldwide. The efforts made in this area may be an incentive to investigators dealing with and interested in RSNOs, which are admittedly a much more problematic class of compounds from the analytical standpoint. Of course, any conclusion drawn from previous and future experiments should be valid only if the analytical methods to determine RSNOs were those recommended by the outcome of this group survey. The same should be done to ascertain whether the levels found in controls are within the indicated ranges. This will greatly help the work of referees and editors of scientific journals as well as other researchers, who aim to repeat the experiments, to confirm, counteract or further ameliorate the reported findings.

## Nomenclature

| | |
|---|---|
| AE1 | anionic exchanger |
| Alb-SNO | *S*-nitrosoalbumin |
| Cy-SNO | *S*-nitrosocysteine |
| DAF-2 | 4,5-diaminofluoresceine |
| DAN | 2,3-diaminonaphthalene |
| DTNB | 5,5′-dithiobisnitrobenzoic acid |
| ECD | electrochemical detector |
| EPR | electron paramagnetic resonance |
| Fe-NO Hb | nitrosylhemoglobin |
| GC–MS | gas chromatography–mass spectrometry |
| GSNO | *S*-nitrosoglutathione |
| GSH | glutathione |
| Hb | hemoglobin |
| Hb-SNO | *S*-nitrosohemoglobin |
| LC–MS | liquid chromatography–mass spectrometry |

| | |
|---|---|
| LMM | low-molecular-mass |
| MGD | *N*-methyl-D-glucamine dithiocarbamate |
| MMTS | methyl-methanethiosulfonate |
| NAT | 2,3-naphthotriazole |
| NEM | *N*-ethylmaleimide |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| OPA | *o*-phthalaldehyde |
| PFB | pentafluorobenzyl |
| PSNOs | protein *S*-nitrosothiols |
| RBC | red blood cell |
| RSNOs | *S*-nitrosothiols |
| SH | sulfhydryl group |

## Acknowledgement

Preparation of this article was supported in part by Fondazione Monte dei Paschi di Siena and by FIRST 2005 (Fondo Interno Ricerca Scientifica e Tecnologica, University of Milan).

## References

[1] N. Hogg, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 585.
[2] D.D. Thomas, X. Liu, S.P. Kantrow, J.R. Lancaster Jr., Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 355.
[3] T. Rassaf, N.S. Bryan, M. Kelm, M. Feelisch, Free Radic. Biol. Med. 33 (2002) 1590.
[4] O. Rafikova, R. Rafikov, E. Nudler, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 5913.
[5] A.R. Butler, F.W. Flitney, D.L.H. Williams, Trends Pharmacol. Sci. 16 (1995) 18.
[6] V.G. Kharitonov, A.R. Sundquist, V.S. Sharma, J. Biol. Chem. 270 (1995) 28158.
[7] P.C. Ford, D.A. Wink, D.M. Stanbury, FEBS Lett. 326 (1993) 1.
[8] H.H.W. Schmidt, H. Hofmann, U. Schindler, Z.S. Shutenko, D.D. Cunningham, M. Feelisch, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 14492.
[9] B. Mayer, S. Pfeiffer, A. Schrammel, D. Koesling, K. Schmidt, F. Brunner, J. Biol. Chem. 273 (1998) 3264.
[10] D. Tsikas, J. Sandmann, K. Denker, J.C. Frölich, FEBS Lett. 483 (2000) 83.
[11] T. Rassaf, P. Kleinbongard, M. Preik, A. Dejam, P. Gharini, T. Lauer, J. Erckenbrecht, A. Duschin, R. Schulz, G. Heusch, M. Feelisch, M. Kelm, Circ. Res. 91 (2002) 470.
[12] A. Nedospasov, R. Rafikov, N. Beda, E. Nudler, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 13543.
[13] A.J. Gow, J.S. Stamler, Nature 391 (1998) 169.
[14] T.J. McMahon, R.E. Moon, B.P. Luschinger, M.S. Carraway, A.E. Stone, B.W. Stolp, A.J. Gow, J.R. Pawloski, P. Watke, D.J. Singel, C.A. Piantadosi, J.S. Stamler, Nat. Med. 8 (2002) 711.
[15] J.R. Pawloski, D.T. Hess, J.S. Stamler, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 2531.
[16] N.S. Bryan, T. Rassaf, J. Rodriguez, M. Feelisch, Nitric Oxide 10 (2004) 221.
[17] D. Giustarini, A. Milzani, R. Colombo, I. Dalle-Donne, R. Rossi, Trends Pharmacol. Sci. 25 (2004) 311.
[18] M.T. Gladwin, A.N. Schechter, Cir. Res. 94 (2004) 851.
[19] J.M. Robinson, J.R. Lancaster, Am. J. Respir. Cell. Mol. Biol. 32 (2005) 257.
[20] K.T. Huang, I. Azarov, S. Basu, J. Huang, D.B. Kim-Shapiro, Blood 107 (2006) 2602.
[21] L. Liu, A. Hausladen, M. Zeng, L. Que, J. Heitman, J.S. Stamler, Nature 410 (2001) 490.
[22] D.A. Stoyanovski, Y.Y. Tyurina, V.A. Tyurin, D. Anand, D.N. Mandavia, D. Gius, J. Ivanova, B. Pitt, T.R. Billiar, V.E. Kagan, J. Am. Chem. Soc. 127 (2005) 15815.
[23] H.H. Al-Sa'doni, I.L. Megson, S. Bisland, A.R. Butler, F.W. Flitney, Br. J. Pharmacol. 121 (1997) 1047.
[24] M.P. Gordge, J.S. Hothersall, A.A. Noronha-Dutra, Br. J. Pharmacol. 124 (1998) 141.
[25] J. Sandmann, K.S. Schwedhelm, D. Tsikas, FEBS Lett. 579 (2005) 4119.
[26] A. Martinez-Ruiz, S. Lamas, Arch. Biochem. Biophys. 423 (2004) 192.
[27] A. Martinez-Ruiz, S. Lamas, Cardiovasc. Res. 62 (2004) 43.
[28] D.J. Singel, J.S. Stamler, Annu. Rev. Physiol. 67 (2005) 99.
[29] D. Yao, Z. Gu, T. Nakamura, Z.Q. Shi, Y. Ma, B. Gaston, L.A. Palmer, E.M. Rockenstein, Z. Zhang, E. Masliah, T. Uehara, S.A. Lipton, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 10810.
[30] H.E. Marshall, K. Merchant, J.S. Stamler, FASEB J. 14 (2000) 1889.
[31] Y. Yang, J. Loscalzo, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 117.
[32] G. Hao, B. Derakhshan, L. Shi, F. Campagne, S.S. Gross, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 1012.
[33] S.R. Tannenbaum, J.E. Kim, Nat. Chem. Biol. 1 (2005) 126.
[34] Y. Zhang, N. Hogg, Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L467.
[35] H.E. Marshall, J.S. Stamler, Biochemistry 40 (2001) 1688.
[36] D. Jourd'heuil, L. Gray, M.B. Grisham, Biochem. Biophys. Res. Commun. 273 (2000) 22.
[37] B.K. Yang, E.X. Vivas, C.D. Reiter, M.T. Gladwin, Free Radic. Res. 37 (2003) 1.
[38] J. Hoffmann, J. Haendeler, A.M. Zeiher, S. Dimmeler, J. Biol. Chem. 276 (2001) 41383.
[39] O. Pechanova, J. Matuskova, D. Capikova, L. Jendekova, L. Paulis, F. Simco, Kidney Int. 70 (2006) 170.
[40] N.S. Bryan, T. Rassaf, R.E. Maloney, C.M. Rodriguez, F. Saijo, J.R. Rodriguez, M. Feelisch, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 4308S.
[41] J.S. Stamler, O. Jaraki, J. Osborne, D.I. Simon, J. Keaney, J. Vita, D. Singel, C.R. Valeri, J. Loscalzo, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 7674.
[42] S.C. Rogers, A. Khalatbari, P.W. Gapper, M.P. Frenneaux, P.E. James, J. Biol. Chem. 280 (2005) 26720.
[43] X. Wang, J.E. Tanus-Santos, C.D. Reiter, A. Dejam, S. Shiva, R.D. Smith, N. Hogg, M.T. Gladwin, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 11477.
[44] T.J. Mc Mahon, G.S. Aheam, M.P. Moya, A.J. Gow, Y.C. Huang, B.P. Luchsinger, R. Nudelman, Y. Yan, A.D. Krichman, T.M. Bashore, R.M. Califf, D.J. Singel, C.A. Piantadosi, V.F. Tapson, J.S. Stamler, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 14801.
[45] V.A. Tyurin, S.X. Liu, Y.Y. Tyurina, N.B. Sussman, C.A. Hubel, J.M. Roberts, R.N. Taylor, V.E. Kagan, Circ. Res. 88 (2001) 1210.
[46] R.E. Gandley, V.A. Tyurin, W. Huang, A. Arroyo, A. Daftary, G. Harger, J. Jiang, B. Pitt, R.N. Taylor, C.A. Hubel, V.E. Kagan, Hypertension 45 (2005) 21.
[47] A.B. Milsom, C.J.H. Jones, J. Goodfellow, M.P. Frenneaux, J.R. Peters, P.E. James, Diabetologia 45 (2002) 1515.
[48] P.E. James, D. Lang, T. Tufnell-Barret, A.B. Milsom, M.P. Frenneaux, Circ. Res. 94 (2004) 976.
[49] P. Wlodek, J. Kucharczyk, M.M. Sokolowska, A. Milkowski, A. Markiewicz, O.B. Smolenski, L.B. Wlodek, Clin. Chim. Acta 327 (2003) 87.
[50] Z.A. Massy, P.T. Borderie, T. Nguyen-Khoa, T.B. Druecke, O.G. Ekindjian, B. Lacour, Nephrol. Dial. Transplant. 18 (2003) 153.
[51] P. Moriel, I.R.O. Pereira, M.C. Bertolami, D.S.P. Abdalla, Free Radic. Biol. Med. 30 (2001) 318.
[52] P. Hilliquin, D. Borderie, A. Hernvann, C.J. Menkes, O.G. Ekindjian, Arthritis Rheum. 40 (1997) 1512.
[53] C.D. Reiter, X. Wang, J.E. Tanus-Santos, N. Hogg, R.O. Cannon III, A.N. Schechter, M.T. Gladwin, Nat. Med. 8 (2002) 1383.
[54] K.M. Beeh, J. Beier, N. Koppenhoefer, R. Buhl, Chest 126 (2004) 1116.
[55] M. Corradi, P. Montuschi, L.E. Donnelly, A. Pesci, S.A. Kharitonov, P.J. Barne, Am. J. Respir. Crit. Care Med. 163 (2001) 854.

[56] R. Rossi, D. Giustarini, A. Milzani, R. Colombo, I. Dalle-Donne, Circ. Res. 89 (2001) e47.
[57] D. Tsikas, J.C. Frölich, Circ. Res. 90 (2002) e39.
[58] D. Tsikas, Circ. Res. 94 (2004) e106.
[59] T. Rassaf, M. Feelisch, M. Kelm, Free Radic. Biol. Med. 36 (2004) 413.
[60] D. Tsikas, F.M. Gutzki, S. Rossa, H. Bauer, C. Neumann, K. Dockendorff, J. Sandmann, J.C. Frölich, Anal. Biochem. 244 (1997) 208.
[61] A. Daiber, M. Bachschmid, C. Kavakli, D. Frein, M. Wendt, V. Ullrich, T. Munzel, Nitric Oxide 9 (2003) 44.
[62] D. Tsikas, J.C. Frölich, Nitric Oxide 11 (2004) 209.
[63] M.M. Pelletier, P. Kleinbongard, L. Ringwood, R. Hito, C.J. Hunter, A.N. Schechter, M.T. Gladwin, A. Dejam, Free Radic. Biol. Med. 41 (2006) 541.
[64] D. Tsikas, Methods Mol. Biol. 279 (2004) 81.
[65] D. Tsikas, Free Radic. Res. 39 (2005) 797.
[66] O. Tashimo, T. Ishibashi, J. Yoshida, H. Tsuchida, M. Nishio, Nitric Oxide 9 (2003) 148.
[67] D. Tsikas, J. Sandmann, D. Holzberg, P. Pantazis, M. Raida, J.C. Frölich, Anal. Biochem. 273 (1999) 32.
[68] R. Jaffrey, S.H. Snyder, Sci. STKE 2001 (2001) PL1.
[69] A.J. Lipton, M.A. Johnson, T. Macdonald, M.W. Lieberman, D. Gozal, B. Gaston, Nature 413 (2001) 171.
[70] J.S. Scharfstein, J.F. Keaney Jr., A. Slivka, G.N. Welch, J.A. Vita, J.S. Stamler, J. Loscalzo, J. Clin. Invest. 94 (1994) 1432.
[71] M. Marzinzig, A.K. Nussler, J. Stadler, E. Marzinzig, W. Barthlen, N.C. Nussler, H.G. Beger, S.M. Morris, U.B. Brückner, Nitric Oxide 1 (1997) 177.
[72] R. Marley, M. Feelisch, S. Holt, K. Moore, Free Radic. Res. 32 (2000) 1.
[73] A. Samouilov, J.L. Zweier, Anal. Biochem. 258 (1998) 322.
[74] X. Wang, N.S. Bryan, P.H. MacArthur, J. Rodriguez, M.T. Gladwin, M. Feelisch, J. Biol. Chem. 281 (2006) 26994.
[75] D. Tsikas, K. Denker, J.C. Frölich, J. Chromatogr. A 915 (2001) 107.
[76] E.F. Funai, A. Davidson, S.P. Seligman, T.H. Finlay, Biochem. Biophys. Res. Commun. 239 (1997) 875.
[77] D. Giustarini, I. Dalle-Donne, R. Colombo, A. Milzani, R. Rossi, Free Radic. Res. 38 (2004) 1235.
[78] D. Tsikas, J.C. Frölich, Nephrol. Dial. Transplant. 18 (2003) 2199.
[79] H. Kojima, N. Nakatsubo, K. Kikuchi, S. Kawahara, Y. Kirino, H. Nagoshi, Y. Hirata, T. Nagano, Anal. Chem. 70 (1998) 2446.
[80] A. Dejam, P. Kleinbongard, T. Rassaf, S. Hamada, P. Gharini, J. Rodriguez, M. Feelisch, M. Kelm, Free Radic. Biol. Med. 35 (2003) 1551.
[81] E.S. Ng, D. Jourd'heuil, J.M. McCord, D. Hernandez, M. Yasui, D. Knight, P. Kubes, Circ. Res. 94 (2004) 559.
[82] M. Feelisch, T. Rassaf, S. Mnaimneh, N. Singh, N.S. Bryan, D. Jourd'heuil, M. Kelm, FASEB J. 16 (2002) 1775.
[83] V. Hampl, C.L. Walters, S.L. Archer, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, John Wiley & Sons, Chichester, UK, 1996, p. 309.
[84] S. Archer, FASEB J. 7 (1993) 349.
[85] A.R. Butler, P. Rhodes, Anal. Biochem. 249 (1997) 1.
[86] C. Alpert, N. Ramdev, D. George, J. Loscalzo, Anal. Biochem. 245 (1997) 1.
[87] M.T. Gladwin, X. Wang, C.D. Reiter, B.K. Yang, E.X. Vivas, C. Bonaventura, A.N. Schechter, J. Biol. Chem. 277 (2002) 27818.
[88] K. Fang, N.V. Ragsdale, R.M. Carey, T. Macdonald, B. Gaston, Biochem. Biophys. Res. Commun. 252 (1998) 535.
[89] E. Nagababu, S. Ramasamy, J.M. Rifkind, Nitric Oxide 15 (2006) 20.
[90] A. Doctor, R. Platt, M.L. Sheram, A. Eischeid, T. McMahon, T. Maxey, J. Doherty, M. Axelrod, J. Kline, M. Gurka, A. Gow, B. Gaston, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 5709.
[91] J.F. Ewing, D.R. Janero, Free Radic. Biol. Med. 25 (1998) 621.
[92] B. Datta, T. Tufnell-Barrett, R.A. Bleasdale, C.J. Jones, I. Beeton, V. Paul, M. Frenneaux, P. James, Circulation 109 (2004) 1339.
[93] D. Tsikas, in: I. Dalle-Donne, A. Scaloni, D.A. Butterfield (Eds.), Redox Proteomics: From Protein Modifications to Cellular Dysfunction and Diseases, John Wiley & Sons Inc., Hoboken, 2006, p. 287.
[94] D. Tsikas, J. Sandmann, F.M. Gutzki, D.O. Stichtenoth, J.C. Frölich, J. Chromatogr. B 726 (1999) 13.
[95] D. Tsikas, J. Sandmann, J.C. Frölich, J. Chromatogr. B 772 (2002) 335.
[96] P. Ferranti, A. Malorni, G. Mamone, N. Sannolo, G. Marino, FEBS Lett. 400 (1997) 19.
[97] X.M. Lu, M. Lu, R.G. Tompkins, A.J. Fischman, J. Mass Spectrom. 40 (2005) 1140.
[98] T.M. Greco, R. Hodara, I. Parastatidis, H.F.G. Heijnen, M.K. Dennehy, D.C. Liebler, H. Ischiropoulos, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 7420.
[99] G. Hao, B. Derakhshan, L. Shi, F. Campagne, S.S. Gross, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 1012.
[100] D. Tsikas, J. Sandmann, S. Rossa, F.-M. Gutzki, J.C. Frölich, Anal. Biochem. 272 (1999) 117.
[101] D. Tsikas, M. Raida, J. Sandmann, S. Rossa, W.G. Forssmann, J.C. Frölich, J. Chromatogr. B 742 (2000) 99.
[102] A. Burg, H. Cohen, D. Meyerstein, J. Biol. Inorg. Chem. 5 (2000) 213.
[103] J.S. Stamler, M. Feelisch, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, John Wiley & Sons, Chichester, UK, 1996, p. 521.
[104] D. Jourd'heuil, K. Hallen, M. Feelisch, M.B. Grisham, Free Radic. Biol. Med. 28 (2000) 409.
[105] R.K. Goldman, A.A. Vlessis, D.D. Trunkey, Anal. Biochem. 259 (1998) 98.
[106] R. Rossi, L. Lusini, F. Giannerini, D. Giustarini, G. Lungarella, P. Di Semplicio, Anal. Biochem. 254 (1997) 215.
[107] S.R. Jaffrey, H. Erdjument-Bromage, C.D. Ferris, P. Tempst, S.H. Snyder, Nat. Cell. Biol. 3 (2001) 193.
[108] Y. Zhang, A. Keszler, K.A. Broniowska, N. Hogg, Free Radic. Biol. Med. 38 (2005) 874.
[109] L.M. Landino, M.T. Koumas, C.E. Mason, J.A. Alston, Biochem. Biophys. Res. Commun. 340 (2006) 347.
[110] B. Huang, C. Chen, Free Radic. Biol. Med. 41 (2006) 562.
[111] M.T. Gladwin, X. Wang, N. Hogg, Free Radic. Biol. Med. 41 (2006) 557.
[112] O. Rafikova, E. Sokolova, R. Rafikov, E. Nudler, Circulation 110 (2004) 3573.
[113] R.J. Singh, N. Hogg, J. Joseph, B. Kalyanaraman, J. Biol. Chem. 271 (1996) 18596.
[114] S.A. Rocks, C.A. Davies, S.L. Hicks, A.J. Webb, R. Klocke, G.S. Timmins, A. Johnston, A.S.M. Jawad, D.R. Blake, N. Benjamin, P.G. Winyard, Free Radic. Biol. Med. 39 (2005) 937.
[115] W. Cha, Y. Lee, B.K. Oh, M.E. Meyerhoff, Anal. Chem. 77 (2005) 3516.
[116] J. Ermer, J. Pharm. Biomed. Anal. 24 (2001) 755.
[117] M.W. Duncan, Amino Acids 25 (2003) 351.
[118] D. Tsikas, K. Caidahl, J. Chromatogr. B 814 (2005) 1.
[119] E. Schwedhelm, Vasc. Med. 10 (2005) 1.
[120] Y. Minamiyama, S. Takemura, M. Inoue, Biochem. Biophys. Res. Commun. 225 (1996) 112.
[121] J.D. MacMicking, R.J. North, R. LaCourse, J.S. Mudgett, S.K. Shah, C.F. Nathan, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 5243.
[122] M.T. Gladwin, J.H. Shelhamer, A.N. Schechter, M.E. Pease-Fye, M.A. Waclawiw, J.A. Panza, F.P. Ognibene, R.O. Cannon III, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 11482.
[123] R.O. Cannon III, A.N. Schechter, J.A. Panza, F.P. Ognibene, M.E. Pease-Fye, M.A. Waclawiw, J.H. Shelhamer, M.T. Gladwin, J. Clin. Invest. 108 (2001) 279.
[124] L.H. Ottesen, D. Harry, M. Frost, S. Davies, K. Khan, B. Halliwell, K. Moore, Free Radic. Biol. Med. 31 (2001) 790.
[125] C. Napoli, G. Aldini, J.L. Wallache, F. de Nigris, R. Maffei, P. Abete, D. Bonaduce, G. Condorelli, F. Rengo, V. Sica, F.P. D'Armiento, C. Mignogna, G. de Rosa, M. Condorelli, L.O. Lerman, L.J. Ignarro, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 1689.
[126] T. Rassaf, M. Preik, P. Kleinbongard, T. Lauer, C. Heiß, B.E. Strauer, M. Feelisch, M. Kelm, J. Clin. Invest. 109 (2002) 1241.
[127] T. Rassaf, N.S. Bryan, R.E. Maloney, V. Specian, M. Kelm, B. Kalyanaraman, J. Rodriguez, M. Feelisch, Nat. Med. 9 (2003) 481.

[128] Z.A. Massy, C. Fumeron, P.T. Borderie, P. Tuppin, T. Nguyen-Khoa, M.O. Benott, C. Jacquot, C. Buisson, T.B. Druecke, O.G. Ekindjian, B. Lacour, M.C. Iliou, J. Am. Soc. Nephrol. 15 (2004) 470.
[129] M.T. Gladwin, F.P. Ognibene, L.K. Pannell, J.S. Nichols, M.E. Pease-Fye, J.H. Shelhamer, A.N. Schechter, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 9943.
[130] K. Cosby, K.S. Partovi, J.H. Krawford, R.P. Patel, C.D. Reiter, S. Martyr, B.K. Yang, M.A. Waclawiw, G. Zalos, X. Xu, K.T. Huang, H. Shields, D.B. Kim-Shapiro, A.N. Schechter, R.O. Cannon III, M.T. Gladwin, Nat. Med. 9 (2003) 1498.
[131] B. Gaston, J. Reilly, J.M. Drazen, J. Fackler, P. Ramdev, D. Arnelle, M.E. Mullins, D.J. Sugarbaker, C. Chee, D.J. Singel, J. Loscalzo, J.S. Stamler, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 10957.
[132] H. Grasemann, B. Gaston, K. Fang, K. Paul, F. Ratjen, J. Pediatr. 135 (1999) 770.