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Review

Measurement of the NO metabolites, nitrite and nitrate, in human biological fluids by GC–MS☆

Steve M. Helmke[a], Mark W. Duncan[a,b,*]

[a] *Department of Pediatrics, University of Colorado at Denver and the Health Sciences Center Aurora, CO 80045, USA*
[b] *Division of Endocrinology, Diabetes & Metabolism, University of Colorado at Denver and the Health Sciences Center Aurora, CO 80045, USA*

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Abstract

In this article we critically review the development and application of gas chromatography–mass spectrometry (GC–MS) techniques to the measurement of the nitric oxide (NO) metabolites, nitrite and nitrate, in human biological fluids. Our focus is on the issue of the fitness of any analytical strategy to its intended purpose and the validity of the analytical results generated. The accuracy, precision, recovery, selectivity and sensitivity of the various methods are evaluated and the potential pitfalls, both specific to the methods, and general to the area, are considered. Several examples of the applications of these techniques to clinical investigations of NO physiology are also critically evaluated.

Keywords: Reviews; Nitration; Alkylation; Stable isotopes; Clinical investigations; Mass spectrometry

Contents

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* Corresponding author at: Department of Pediatrics and Division of Endocrinology, Diabetes & Metabolism, University of Colorado at Denver and the Health Sciences Center Aurora, CO 80045, USA. Tel.: +1 303 724 3343; fax: +1 303 724 3420.
E-mail address: mark.duncan@uchsc.edu (M.W. Duncan).

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1. Introduction

Nitric oxide (NO) has emerged as a key regulatory molecule in the vasculature, the brain and the immune system. However, because NO is a reactive gas with an *in vivo* half-life of less than 0.1 s [1], its direct measurement presents a considerable analytical challenge. In the presence of oxygenated hemoglobin, oxygen and superoxide, circulating NO is rapidly converted to both nitrite (NO_2^-) and nitrate (NO_3^-). The NO_2^- anion is further oxidized to NO_3^-, and this is excreted as the major NO metabolite in urine. Therefore, in practice, the production of NO is most readily assessed by quantifying one or both of the anions derived from it.

Plasma and serum NO_2^- have been shown to be useful measures of endothelial NO production while urinary NO_3^- is believed to be a useful non-invasive measure of systemic NO production (reviewed in [2,3]). Despite their deceptively simple structures, both NO_2^- and NO_3^- themselves present a substantial analytical challenge. Numerous strategies (reviewed elsewhere in this volume) have been reported for the measurement of NO_2^- and/or NO_3^- including colorimetric assays (e.g. the Griess reaction), chemiluminescent assays, HPLC with UV or electrochemical detection and capillary electrophoresis. This review focuses on strategies based on gas chromatography–mass spectrometry (GC–MS), evaluates this analytical pairing and critically assesses some of the methods based on it.

2. Gas chromatography–mass spectrometry—an overview

GC–MS is commonly employed for the quantitative analysis of trace components because the instruments are increasingly affordable, widely available and highly reliable. When engaged optimally, GC–MS is highly selective, sensitive, especially in the negative ion electron capture (NIEC) mode, and delivers both accurate and precise data over a wide dynamic range. Although direct analysis is limited to compounds that are volatile and thermally stable at readily accessible temperatures, GC–MS is also applicable to species such as NO_2^- and NO_3^- following their chemical modification. This principle is discussed in greater detail in the sections that follow. The availability of stable (or non-radioactive) isotopes markedly enhances the potential of GC–MS because these can be incorporated into labeled internal standards that are both chemically and physically equivalent to the native forms. Further, stable-isotope labeled compounds can be administered to humans in order to trace the pathways for NO generation and metabolism [4].

Apart from the drawbacks associated with volatility, there are few other limitations intrinsic to GC–MS. The most obvious additional concerns relate to the cost and complexity of this approach. Some stable-isotope labeled compounds and derivatization reagents can be expensive, but rarely are the costs prohibitive. More significant is the fact that skilled individuals are essential to realizing the full potential of GC–MS and the importance of this factor cannot be overstated. While many researchers highlight the unique benefits of GC–MS-based analytical strategies, it is also important to acknowledge that these assays are not always accurate, precise and sensitive. The application of GC–MS does not, by itself, assure valid data and without rigorous attention to detail and careful validation, even the most powerful of analytical instruments can be inappropriately applied to generate meaningless data. There are many examples of this across analytical chemistry, and unfortunately, some from areas related to the scope of this review.

3. General considerations when applying GC–MS to NO_2^- and NO_3^- determinations

In this review article we evaluate existing applications of GC–MS to the study of NO and its metabolic pathway. We focus on the issue of the fitness of any analytical strategy to its intended purpose and the validity of the analytical results that are generated. Unfortunately, and all too frequently, analytical methods are not thoroughly validated before they are applied directly to complex biological problems and consequently, erroneous data are generated that redirect research and confound our understanding of the biology. The importance of the validation process, and of objective and quantifiable measures of assay performance such as accuracy, precision, recovery, selectivity and the lower limit of quantification, cannot be overstated. Also, given the unique aspects of NO_2^- and/or NO_3^- analysis, we also address the potential pitfalls of indirect measures of analysis that require chemical modification of the target species to a measurable entity, e.g. the reaction of nitrate and nitrite anions with toluene or pentafluorobenzyl bromide.

4. GC–MS strategies for the analysis of the anions NO_2^- and NO_3^-

As already stated, NO is not measured directly by GC–MS, but the anions NO_2^- and NO_3^- are employed as indirect measures of NO levels. Further, at least by GC–MS, neither NO_2^- nor NO_3^- is analyzed directly, but they must first be converted to higher molecular weight neutral molecules amenable to GC analysis. To date, published reports have focused on quantifying NO_2^-, NO_3^-, or both, either measured separately, or as their sum. This approach assumes that other (endogenous or contaminating) sources of NO_2^- and NO_3^- are insignificant relative to that formed from NO, or at the very least, that these contributions can be defined and corrected for to provide meaningful values for NO production. However, as we will discuss later, other direct and indirect sources of NO_2^- and NO_3^- are potential sources of error with all these determinations and these have compromised some studies. Further, artifactual inter-conversion by inappropriate sample handling or processing prior to analysis is an additional potential pitfall that must be avoided.

NO_2^- and NO_3^- are chemically inter-convertible by redox reactions. For analytical purposes, the controlled oxidation of NO_2^- to NO_3^- is usually accomplished by treatment with hydrogen peroxide (H_2O_2), and the controlled reduction of NO_3^- to NO_2^- is usually performed using cadmium. In early methods only one species was analyzed directly. For example, NO_2^- was measured directly, then redetermined following conversion of NO_3^- to NO_2^-. The difference between these two

determinations was used to calculate NO_3^- levels. As a specific example, Tsikas et al. [5] first analyzed NO_2^- directly, then reanalyzed after conversion of NO_3^- to NO_2^- by reduction with cadmium. From the direct NO_2^- determination, and the sum of NO_2^- and NO_3^-, both the NO_2^- and NO_3^- content of the original sample could be determined. Conversely, Gutzki et al. [6] only determined NO_3^- directly. Samples were pretreated with H_2O_2 to convert any NO_2^- to NO_3^- and the analysis gave the sum of NO_2^- and NO_3^-.

The addition of a chemical conversion step, however, adds a potential source of error. Because the second species is calculated by difference, the errors associated with both separate determinations are incorporated in this value. This is especially significant when determining NO_2^- levels by difference because the amount of NO_2^- is small relative to NO_3^-. Subtracting a large value for NO_3^- from the large total for $NO_2^- + NO_3^-$ yields a small value for NO_2^- with a substantial error associated with it. Consequently, methods designed to measure both NO_2^- and NO_3^- directly, independently and simultaneously, are preferable because they are more expedient and offer increased accuracy and precision. Two general strategies have been developed to convert NO_2^- and NO_3^- to heat-stable, volatile derivatives, i.e., nitration and alkylation and these are discussed in some detail below.

4.1. Nitration

In this approach an acid is used to catalyze the nitration of an aromatic compound by electrophilic substitution. Benzene [7], trimethoxybenzene (TMB) [6] and toluene [8,9] have all been used as substrates in this reaction and a representative reaction with toluene is shown in Fig. 1. Because NO_2^- does not react under these conditions, it must be converted to NO_3^- before it can be assayed by this method [6]. The first application of GC–MS as a tool to analyze NO_3^- was reported by Green et al. [7] and here sulfuric acid was used to catalyze the reaction of $^{14}NO_3^-$ and $^{15}NO_3^-$ with benzene [10]. The yield of nitrobenzene was only about 50%, but this was sufficient to determine the $^{14}NO_3^-/^{15}NO_3^-$ ratio. Nitrobenzene was resolved by GC and no interfering substances were found to co-chromatograph. Selected ion monitoring (SIM) in NIEC mode was performed for ^{14}N-nitrobenzene (*m/z* 123) and ^{15}N-nitrobenzene (*m/z* 124) and the ratio of the peak heights was used to calculate the $^{14}NO_3^-/^{15}NO_3^-$ ratio in the original sample. Several correction factors had to be incorporated into the calculations to account for the naturally occurring stable isotopes (e.g. ^{13}C, ^{15}N, ^{17}O, ^{2}H) that appear at higher *m/z* values, but these are straightforward and are based on natural isotope abundances. However, the tendency for nitroaromatics to lose hydrogen from the aromatic ring is problematic because it causes peaks to appear at lower *m/z* values and the extent of these losses varies depending on ion source conditions (e.g. GC column flow rates). Consequently, the loss was determined daily by running a standard sample of ^{14}N-nitrobenzene and a correction factor was then incorporated into the calculations for the $^{14}NO_3^-/^{15}NO_3^-$ ratio. A calibration curve was generated by adding 0–80 atom percent excess $^{15}NO_3^-$ to a known amount of $^{14}NO_3^-$ in aqueous solution and the measured atom percent excess matched that predicted. A similar curve was generated by adding $^{15}NO_3^-$ to human urine, but the authors did not report on the obvious next step: the measurement of NO_3^- in urine samples.

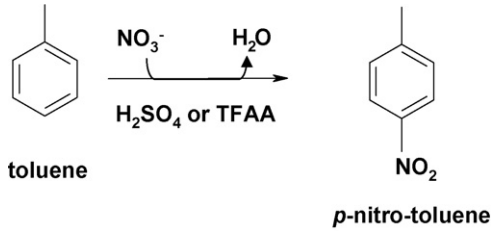

Fig. 1. Nitration of toluene. In the presence of H_2SO_4 or TFAA catalyst, NO_3^- reacts with aromatic rings to form the corresponding nitro-aromatic compound. In this example the substrate is toluene and for clarity only the *p*-isomeric product is shown.

Rhodes et al. attempted to employ the sulfuric acid-catalyzed nitration of benzene to measure the $^{14}NO_3^-/^{15}NO_3^-$ ratio in human plasma but they reported severe interferences [11]. They identified one potential interfering substance as *N*-nitrosothioproline and suggested that the presence of other nitrosamines was also problematic. Presumably these compounds could decompose to yield free NO_3^- during the sulfuric acid step and thereby contribute to an artifactual increase in the apparent abundance of NO_3^-.

In a variation of this approach, trifluoromethanesulphonic acid was used as the catalyst for the nitration of benzene and the method was applied to the measurement of plasma and urinary NO_3^- [12]. Ringqvist et al. found no differences between healthy volunteers and women with primary Raynaud's phenomenon [12]. However, they did report an unexplained seasonal variation in plasma NO_3^- (i.e., higher in winter) but with no associated variation in urinary NO_3^-. They did not report any assessment of their errors, their analytical range, or provide other data to validate their method. Further, the investigators seemed to be unaware of the interferences reported by Rhodes et al. [11] and perhaps related problems could account for the unexplained variations that were observed.

Gutzki et al. [6] quantified both anions by GC–MS/MS in a study aimed at measuring NO_2^- and NO_3^- derived from NO produced by endothelial cells. The approach was based on NO_3^- reacting with TMB to form 1-nitro-2,4,6-trimethoxybenzene (NTMB). Trimethoxybenzene was selected in preference to benzene as the nitration substrate because fewer side products were generated and the acid-to-sample ratio could be reduced to fivefold [10]. $^{15}NO_2^-$ was chosen as the internal standard and the conversion of NO_2^- to NO_3^- was accomplished by treating the samples with H_2O_2. Because samples may have already contained some NO_3^-, the assay measured the sum of NO_2^- and NO_3^-. The recovery of $^{15}NO_2^-$ as ^{15}NTMB was determined to be $71 \pm 4\%$. NTMB was resolved by GC and subjected to NIEC ionization. MS or MS/MS were performed on a triple-stage quadrupole mass spectrometer. The most abundant ions in the spectrum of NTMB were the molecular anion at *m/z* 213 and its product ions $[NTMB\text{-}NO_2]^-$ at *m/z* 167 and NO_2^- at *m/z* 46. Under MS/MS conditions the spectrum of NTMB showed

almost complete fragmentation of the precursor ion to the product ions. Therefore, in the selected reaction monitoring (SRM) mode the transition 213 → 46 was monitored for ^{14}NTMB and the transition 214 → 47 for ^{15}NTMB, each with a dwell time of 60 ms. The peak height ratio for the two ion chromatograms was then used to calculate the $^{14}NO_2^-/^{15}NO_2^-$ ratio and a correction factor was applied to account for the small amount of naturally occurring ^{15}N and ^{17}O found in NO_2^-. To construct a calibration curve, 25–2500 ng/ml NO_2^- was added to phosphate buffered saline (PBS) containing 250 ng/ml $^{15}NO_2^-$. The assay was applied to human endothelial cell culture perfusates and NO_2^- and NO_3^- were found to increase three-fold after bradykinin stimulation. However, the amounts found in unstimulated cell perfusates did not differ significantly from those found in PBS alone. This indicated that the PBS buffer already contained measurable NO_2^- and NO_3^-, a serious pitfall discussed below, and therefore this made it difficult to know the actual fold increase.

Wennmalm et al. described methods to measure both NO_2^- and NO_3^- in separate assays [8]. Plasma was subjected to ultrafiltration and chromatography; urine was purified using C_{18} cartridges prior to assay. For NO_3^-, sulfuric acid-catalyzed nitration was used with toluene as the substrate and $^{15}NO_3^-$ was used as the internal standard. Following GC, SIM analysis was based on *m/z* 136 (nitrotoluene, NT) and *m/z* 137 (^{15}NT). The NO_2^- assay employed $^{15}NO_2^-$ as the internal standard and the hydrochloric acid-catalyzed reaction of NO_2^- with hydralazine to form tetrazolo[1,5-α]phthalazine. Following GC, SIM was performed at *m/z* 171 and *m/z* 172 for tetrazolo[1,5-α]phthalazine and the corresponding ^{15}N-labeled compound, respectively. While this approach looked encouraging, details of the assay characteristics and its validation were limited.

A significant problem with sulfuric acid-catalyzed nitration assays was revealed when it was observed that there was considerable interference caused by nitroarginine analogs [13]. These analogs, notably N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA), are commonly used as NO synthase (NOS) inhibitors. It was found that sulfuric acid caused these compounds to decompose to NO_3^-, artifactually increasing the measured NO_3^- concentration. Consequently, these interfering compounds must be completely removed from urine or plasma samples by solid-phase extraction (SPE) on cation exchange cartridges prior to performing this assay.

The nitration approach was further refined by Smythe et al. [9]. They employed trifluoroacetic anhydride (TFAA) as the catalyst in place of sulfuric acid and used toluene as the nitration substrate. Although a mechanistic study was not undertaken, the authors proposed two alternative pathways for the reaction, both beginning with the formation of trifluoroacetyl nitrate from TFAA and NO_3^-. First they proposed that trifluoroacetyl nitrate could dissociate to form a trifluoroacetic acid (TFA) anion and a nitronium cation (NO_2^+), the later undertaking electrophilic aromatic nitration of toluene. Alternatively, they suggested that the trifluoroacetyl nitrate could form a cyclic six-membered transition state with toluene which would then dissociate to yield TFA and nitrotoluene. Regardless of the specifics of the mechanism, however, the strength of this approach is that under these milder reaction conditions decomposition of the nitroarginine analogs to NO_3^- was minimal. Therefore, sample clean up with SPE was not required before performing this GC–MS assay. In the presence of TFAA, NO_3^- was quantitatively converted to nitrotoluene (NT) with the formation of the *ortho-* (*o-*), *meta-* (*m-*) and *para-* (*p-*) isomers in the proportion 57:3:40, respectively. All three isomers were resolved by GC. $^{15}NO_3^-$ was used as the internal standard for GC–MS. While there was insufficient *m*-NT formed for precise quantification, the assay could be performed utilizing either (or both) the *o*-NT and/or *p*-NT isomers. In this first report by Smythe et al. electron ionization (EI) was employed and under these conditions both *p*-NT and *m*-NT generated molecular ions at *m/z* 137, while the corresponding *p*-^{15}NT and *m*-^{15}NT isotopomers generated molecular ions at *m/z* 138. The *o*-NT exhibited the well-known "*ortho*-effect" leading to the loss of OH to give the base peak at *m/z* 120. The *o*-^{15}NT produced the corresponding ion at *m/z* 121. For quantification, peak areas were measured from the SIM chromatograms and were then corrected for the naturally occurring stable isotope contribution present in the native compounds and for the amount of $^{14}NO_3^-$ found in the $^{15}NO_3^-$ internal standard. For the assay utilizing the *para* isomer the peak area ratio *m/z* 137/138 was measured; for the assay utilizing the *ortho* isomer the peak area ratio *m/z* 120/121 was measured. Both ion pairs gave linear calibration curves over the range 0–1000 pmol. The limit of quantification (LOQ) was 100 fmoles. In a refinement of the method, NIEC was employed [14] and under these conditions the molecular anion of *o*-NT could be monitored at *m/z* 137. The corresponding *o*-^{15}NT molecular ion could be monitored at *m/z* 138. Using these peak areas, a calibration curve was produced of equivalent quality to that generated in EI mode.

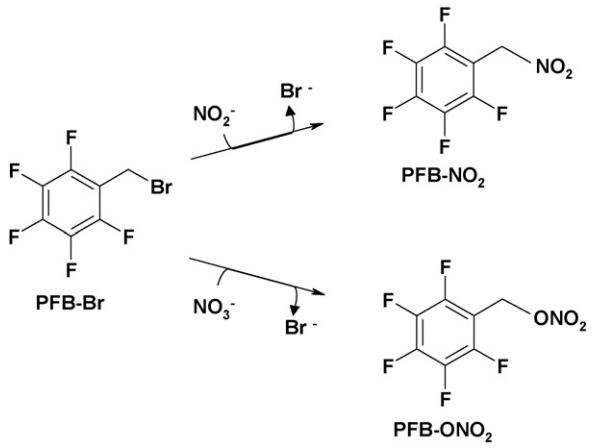

Fig. 2. Alkylation by PFB-Br of NO_2^- and NO_3^-. The strong alkylating agent PFB-Br can react with either NO_2^- or NO_3^- to form the corresponding products PFB-NO_2 or PFB-ONO_2.

4.2. Alkylation

In an alternative strategy, NO_2^- and NO_3^- can be alkylated by reaction with pentafluorobenzyl bromide (PFB-Br) [5,15] as shown in Fig. 2. This reaction can be performed with either acetone [5] or acetonitrile [16], serving as the solvent. The reac-

Table 1
Plasma and urinary NO_2^- and NO_3^- concentration ranges, means, and SD measured by GC–MS in 12 healthy volunteers on an unrestricted diet

		Plasma (μM)	Urine (μmol/mmol creatinine)
NO_2^-	Range	2–5	0.2–1
	Mean ± SD	3.65 ± 0.84	0.49 ± 0.25
NO_3^-	Range	50–120	30–300
	Mean ± SD	68.0 ± 17.4	109.5 ± 61.7

Data were taken from [5].

tion of PFB-Br with NO_2^- is the basis of the method as originally described [5] and the mechanism involves nucleophilic attack of pentafluorobenzyl bromide by NO_2^-. This results in the loss of Br^- and formation of α-nitro-pentafluorotoluene (PFB-NO_2). In order to measure the NO_3^- anion by this reaction it has to be reduced to NO_2^-, for example, with cadmium. The PFB-NO_2 is extracted into toluene and the sample run by either GC–MS or GC–MS/MS. Negative ion electron capture (NIEC, sometime incorrectly called negative ion chemical ionization) works well for the strongly electron capturing PFB-NO_2 and this yields a spectrum dominated by the NO_2^- anion (*m/z* 46) and the PFB anion (*m/z* 181). For the analysis of plasma and urine, samples are spiked with the internal standards $^{15}NO_2^-$ and $^{15}NO_3^-$ and these yield PFB-$^{15}NO_2$ with the subsequent formation of the PFB ion at *m/z* 181 and the $^{15}NO_2^-$ ion at *m/z* 47. For quantification the instrument is operated in the SIM mode and the peak area ratio *m/z* 46/47 is measured. In this original study the investigators reported a linear calibration curve over the range 0–200 ng NO_2^- and 0–10 μg NO_3^- with RSD values of less than 4%. Both NO_2^- and NO_3^- could be quantified in human plasma and urine by this method (see Table 1).

In later studies it was shown that the PFB-Br-based assay was free from several interferences that commonly plague other approaches [17]. For example, reduced thiols (e.g. cysteine, *N*-acetyl-cysteine) and reduced glutathione, which may interfere with the Griess assay, did not compromise this approach. Further, there was no interference from nitroaromatics (e.g. 1,2-dimethoxy-4-nitrobenzene or 3-nitro-L-tyrosine) or from nitroso compounds (e.g. *N*-nitrosomorpholine) that interfere with nitrobenzene-based methods [11]. However, there was interference from the nitroarginine analogs L-NAME and L-NNA and these compounds had to be removed from samples by SPE before reduction of NO_3^- to NO_2^- [13]. Interferences were found to be due to the generation of free NO_2^- from these compounds during the cadmium reduction step [15].

The PFB-Br-based assay was also adapted for GC–MS/MS operation on a triple-stage quadrupole mass spectrometer [18]. The first quadrupole was used to select for the PFB-NO_2 precursor ion (*m/z* 226) and an intense product ion at *m/z* 66 was selected in the third quadrupole for detection. Similarly, PFB-$^{15}NO_2$ (*m/z* 227) produced a strong product ion at *m/z* 67. A SRM assay was implemented based on the transitions 226 → 66 for $^{14}NO_2^-$ and 227 → 67 for $^{15}NO_2^-$. Although GC–MS/MS provided enhanced selectivity over previous GC–MS methods, problems associated with the initial identification of the product ions, and especially the low overall sensitivity, limited the usefulness of this approach.

5. Simultaneous analysis of NO_2^- and NO_3^- by GC–MS

A very significant advance in the measurement of NO_2^- to NO_3^- was made by Tsikas in 2000 [15] when they described the first method for the simultaneous quantification of both NO_2^- and NO_3^-. Their approach is an important improvement because it avoids a chemical conversion required by previous assays, i.e., the oxidation of NO_2^- to NO_3^- prior to aromatic nitration, or the reduction of NO_3^- to NO_2^- prior to alkylation. As already discussed, these conversions complicate the assay and serve as a source of error. Tsikas and colleagues adopted a thoughtful alternative strategy based on the fact that under optimal conditions, both NO_2^- and NO_3^- can react with PFB-Br as shown in Fig. 2. The bromine of PFB-Br is a strong leaving group and nucleophilic attack can be initiated in several ways: i.e., the N of NO_2^- can react readily with PFB-Br to form α-nitro-pentafluorotoluene (PFB-NO_2) – the basis of the original method – but in addition, the O of NO_3^- can also initiate nucleophilic attack on PFB-Br, albeit much more slowly, to form the corresponding nitric acid ester of pentafluorotoluene (PFB-ONO_2). Therefore, at elevated temperatures and higher PFB-Br concentrations, both NO_2^- and NO_3^- will react directly with PFB-Br. The structures of these putative products, PFB-NO_2 and PFB-ONO_2, were confirmed by EI GC–MS after HPLC separation. However, the higher temperatures required to produce PFB-ONO_2 also caused PFB-NO_2 to begin to breakdown in a time-dependent manner. Therefore, a reaction time of 1 h was chosen as a compromise: i.e., long enough for PFB-ONO_2 to accumulate but short enough to avoid excessive loss of PFB-NO_2. The PFB-NO_2 and PFB-ONO_2 were well resolved by GC and with NIEC the major fragment ion from PFB-NO_2 was NO_2^- (*m/z* 46); the major fragment from PFB-ONO_2 was the NO_3^- anion (*m/z* 62) with little NO_2^- (*m/z* 46) evident. The NIEC spectrum of PFB-O$^{15}NO_2$ is shown in Fig. 3. Prior to analysis, samples were spiked with $^{15}NO_2^-$ and $^{15}NO_3^-$ to serve as internal standards and these produced the corresponding PFB derivatives at 1 Da higher mass, i.e., *m/z* 47 and 63. This assay is performed in the SIM mode while monitoring *m/z* 46, 47, 62 and 63, each with a dwell time of 50 ms. The peak area ratio *m/z* 46/47 was used to generate the NO_2^- calibration curve; the peak area ratio *m/z* 62/63 to generate the NO_3^- calibration curve. Tsikas and colleagues employed this assay to measure NO_2^- and NO_3^- in human plasma and urine with excellent performance parameters: recovery (accuracy) was near 100% and precision (RSD) less than 4% for NO_3^- and 3% for NO_2^-. A representative GC–MS chromatogram for the simultaneous quantification of NO_3^- and NO_2^- in human plasma is shown in Fig. 4. The investigators found no evidence that excess NO_2^- or NO_3^- interfered with the determination of the other species. Nitroarginine analogs (e.g. L-NNA) do not interfere with this assay because there is no conversion step involving reduction or oxidation that might lead to artifactual generation of NO_2^- or NO_3^-. The omission of a reduction step also avoids the

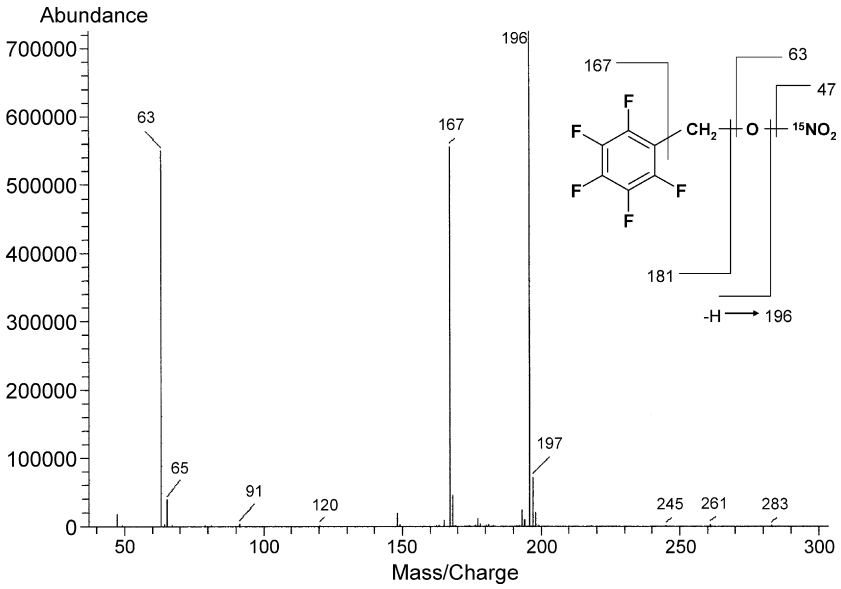

Fig. 3. NIEC spectrum of the reaction product of $^{15}NO_3^-$ and PFB-Br. The fragment pattern confirms the product as PFB-O$^{15}NO_2$. The fragment peak at *m/z* 63 for $^{15}NO_3^-$ was used as the internal standard in SIM for quantification of NO_3^-.

artifactual generation of NO_2^- from nitroso compounds [19]. By avoiding acidification and any redox reactions this assay is able to simultaneously determine both NO_2^- and NO_3^- without any of the previously described interferences and the use of stable-isotope internal standards enhanced precision and accuracy. Currently, this approach is the best available method for quantifying NO_2^- and NO_3^- in biological samples.

A potentially important modification of the PFB-Br alkylation approach has been reported by Kage et al. in which the above reaction was carried out in the presence of a phase-transfer catalyst, i.e., tetradecyldimethylbenzylammonium chloride (TDMBA) [16]. In their original description only NO_2^- was measured directly; to measure NO_3^- it was first reduced to NO_2^- by hydrazine sulfate in the presence of Cu^{2+} and Zn^{2+}. Subsequently, Kage et al. demonstrated that by increasing the amount of PFB-Br present, both NO_2^- and NO_3^- could be simultaneously analyzed as their PFB-NO_2 and PFB-ONO_2 derivatives, respectively [20]. The inclusion of TDMBA as the phase-transfer catalyst greatly increased the rate at which NO_3^- reacted with PFB-Br and therefore increased the yield of PFB-ONO_2. Just as importantly, the yield of the PFB-NO_2 derivative formed from NO_2^- was also increased because it no longer underwent the previously observed decomposition.

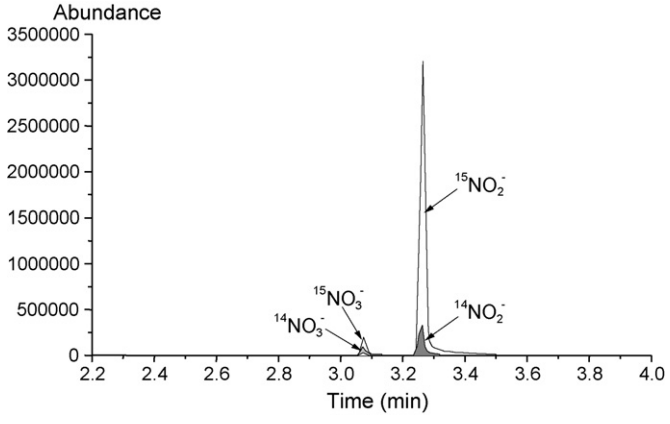

Fig. 4. GC–MS chromatogram of the simultaneous quantification of NO_2^- and NO_3^-. A representative partial chromatogram from the quantification of NO_2^- and NO_3^- in human plasma is shown. The internal standards were added to the plasma at final concentrations of 80 μM $^{15}NO_3^-$ and 10 μM $^{15}NO_2^-$.

Despite these very positive attributes, there are compromises associated with the assay as described by Kage et al. that limit its utility. No stable-isotope labeled standard was included and the investigators relied on 1,3,5-tribromobenzene (TBB) as their internal standard. Because PFB-ONO_2, PFB-NO_2, and TBB are all well resolved under the GC conditions employed, the internal standard cannot account for ion source events. Further, the ions monitored are derived by different pathways: *m/z* 62 for NO_3^- (from PFB-ONO_2), *m/z* 46 for NO_2^- (from PFB-NO_2) and *m/z* 79 (for the Br^- ion derived from TBB). Quantification was performed using peak area ratios *m/z* 62/79 for NO_3^- and *m/z* 46/79 for NO_2^-. While NO_3^- could be measured, the assay was not sensitive enough to measure basal NO_2^- levels in human plasma. As a consequence of these factors, assay precision was around 10%.

6. GC–MS, cross-validation and analytical errors

Historically, the Griess assay has been employed to measure NO_2^- and NO_3^- (after its reduction to NO_2^-). Under acidic conditions NO_2^- undergoes a diazotization reaction to form a visible chromophore that can be quantified [21]. However, approaches based on this reaction are subject to interferences and errors. By contrast, quantitative analyses based on chromatography followed by mass spectrometry and utilizing stable isotope internal standards offer the highest levels of precision and accuracy available. Specificity arises from chromatographic resolution coupled with the selectivity afforded by selected mass (or reaction) detection. For example, under optimal condition a

selected reaction ion chromatogram offers a flat baseline except for well-defined peaks corresponding to the species of interest. For these reasons GC–MS methods, and most especially GC–MS/MS assays operating in SRM mode, are often considered the "gold standard" against which all other methods can be compared. For example, GC–MS methods have been used to cross-validate other approaches for determining NO_2^- and NO_3^-, including the Griess assay and various HPLC based methods.

An extensive study has compared NO_2^- determinations in human plasma and urine obtained by the Griess assay with those generated by GC–MS [17]. It was established that, under the acidic conditions of the Griess assay, reduced glutathione and several thiols in plasma (e.g. cysteine and *N*-acetyl-cysteine) reacted with NO_2^- to form *S*-nitroso compounds that artifactually lowered the apparent NO_2^- level. In addition, NO_2^- was also shown to react with serum albumin to form *S*-nitrosoalbumin further reducing the measured NO_2^- level. In fact, after only 1 h at the acidified conditions required for the Griess reaction, no detectable NO_2^- remained in samples as measured by GC–MS.

In order to measure urinary NO_3^- by the Griess assay it has to first be reduced to NO_2^- and this is a potential source of error. For example, when a calibration curve of unlabeled NO_3^- was constructed in aqueous buffer, the yield of NO_2^- was assumed to be the same as when the reaction was done on NO_3^- in urine [17]. However, such assumptions do not have to be made for GC–MS assays when a stable-isotope labeled standard is included. For example, when $^{15}NO_3^-$ is added to urine prior to reduction, the yield of $^{15}NO_2^-$ is exactly the same as the yield of NO_2^- from the urinary NO_3^-. Therefore, when comparisons were made, the values obtained by the Griess assay were only 30–80% of those obtained from the same samples when analyzed by GC–MS [17]. Substances present in urine presumably interfered with the reduction reaction and lowered the yield of NO_3^- compared to that obtained in aqueous buffer. However, because the stable-isotope labeled internal standard, $^{15}NO_3^-$, is subject to the same interference as endogenous NO_3^-, this "corrects" for the lower yield.

GC–MS has also been used to cross-validate various HPLC-based approaches. For example, NO_2^- was reacted with *N*-acetylcysteine (NAC) in the presence of hydrochloric acid to form *S*-nitroso-*N*-acetylcysteine (SNAC) and this product was analyzed by HPLC with UV detection at 333 nm [22]. NO_3^- was assayed after reduction to NO_2^- and conversion to SNAC. There was excellent agreement between values obtained by this method and by a GC–MS method [17]. Urinary NO_3^- has also been measured by isocratic, anion-pairing, reversed-phase HPLC without any derivatization and results generated by this method were shown to be in good agreement with those obtained by GC–MS [23].

7. Pitfalls in the analysis of NO_2^- and NO_3^- by GC–MS

There are numerous potential pitfalls in the analysis of NO production by the measurement of NO_2^- and NO_3^-. Some of these arise because of the unique requirements of a GC–MS method, others are more fundamental and are common to all strategies.

First, and foremost, as with any trace analysis, care must be taken to avoid contamination of equipment or reagents with the target analytes. While it may seem obvious, it must be emphasized that special care should be exercised in collecting and processing samples for analysis because equipment and reagents can be contaminated with considerable amounts of NO_2^- and NO_3^-. Some citrate and EDTA monovettes used for drawing human blood samples have been reported to contain significant amounts of NO_2^- and NO_3^- (i.e., up to 9 nmol NO_2^- and 18 nmol NO_3^-) [17,24]. Glass tissue culture vials as received from the manufacturer also reportedly contain variable amounts of NO_3^- ranging up to 6 nmol/vial [14]. In this instance the contamination could be removed by three washes with deionized water "free" of NO_2^- and NO_3^-. A cell culture medium used to grow a macrophage cell line was found to contain mM-concentrations of NO_3^- [14] and this completely obscured the increase in NO_3^- levels arising from the interferon-stimulated production of NO. Even double-distilled water was reported to contain 1.2 μM NO_2^- and 2.8 μM NO_3^- [5]. As an example of the importance of these factors, a potentially interesting observation of NO production by blood during dialysis was clouded because it was not possible to exclude contamination of the dialysis fluid [25].

Second, it must be kept in mind that the NO_2^- and NO_3^- present in the human biological fluids can arise from sources other than NO production and these include diet and environmental factors. Many foods, especially processed meats and beverages, including tea, beer and wine, contain significant amounts of NO_3^- [26]. A typical diet has been estimated to result in the ingestion of about 2000 μmol NO_3^- per day while the production of NO_3^- from NO is *ca.* 1000 μmol per day; however, on a NO_3^- restricted diet the intake of NO_3^- can be reduced to less than 200 μmol/day [27]. Plasma NO_3^- concentrations of individuals on restricted diets were reportedly around 30 μM, but levels increased within 1 h to about 100 μM [28], or even 200 μM [29] after ingestion of NO_3^--rich food. Upon resumption of a NO_3^--restricted diet concentrations returned to the restricted diet values after 48 h [29]. Blood levels of NO_2^- and NO_3^- can be increased significantly following ingestion of NO_3^--contaminated water [30] or the inhalation of air polluted with automotive exhaust [16]. In defense of his HPLC method, El Menyawi suggested that the low concentrations of serum NO_3^- in his control Thai subjects relative to European subjects could be the result of a much lower exposure to NO_3^- from foods and/or pollutants [31]. However, under most circumstances pollution is probably a minor contributor in comparison to dietary NO_3^- intake and endogenous production of NO. Consequently, it has been suggested that if plasma NO_3^- is to be used as a measure of NO production, then subjects should be placed on a restricted diet for at least two days prior to the study.

Third, and perhaps most critically, the artifactual production, consumption, or inter-conversion of NO_2^- and NO_3^- during sample handling and processing must be avoided. As we have already discussed, sulfuric acid, used to catalyze the nitration reaction, will artifactually generate NO_3^- as a break-

down product from *N*-nitroso-thioproline and presumably other nitroso-amines [11], and from the NOS inhibitors, L-NAME and L-NNA [9,13]. In the alkylation approach as it was originally described, a reduction step using cadmium was required [5]. However, this causes the artifactual generation of NO_2^- as a breakdown product from these NOS inhibitors [15] and from the nitroso compounds, *S*-nitrosoglutathione and *S*-nitrosocysteine [19]. By contrast, acidification of samples can cause the artifactual diminution of NO_2^- because it then reacts with thiol compounds, including cysteine, *N*-acetyl-cysteine, reduced glutathione, and serum albumin, to form the corresponding *S*-nitroso compounds [17]. Consequently, if deproteinization of samples is performed then it should be done at neutral pH by precipitation with organic solvents, such as acetone or acetonitrile; precipitation with strong acids such as trichloroacetic acid (TCA) should be avoided. The unintended and therefore artifactual reduction of NO_3^- to NO_2^- has not been reported, but artifactual oxidation of NO_2^- to NO_3^- can occur and should be avoided. In whole blood, NO_2^- is rapidly oxidized to NO_3^- [32] and therefore, when preparing plasma or serum, blood should be centrifuged immediately at low temperature [2]. It has also been reported that freezing samples in sodium phosphate buffer can cause conversion of NO_2^- to NO_3^-, at least as measured by the Griess assay [33]. The effect could be minimized by quick freezing with liquid N_2, and reportedly does not occur when potassium phosphate buffers are employed. However, the conditions tested were somewhat artificial and optimized to promote freezing artifacts. No oxidation of NO_2^- to NO_3^- was observed using an alkylation-based GC–MS assay with conditions more typically encountered with human samples, for example, basal concentrations of plasma NO_2^- [34].

8. Contributions of GC–MS assays for NO_2^- and NO_3^- to the biological sciences

Because MS can discriminate between stable isotopes, GC–MS has been able to make some fundamental contributions to our understanding of NO pathways [4,35]. Stable isotopes have been employed as tracers allowing the elucidation of NO pathways in human subjects. Even before the importance of NO was fully recognized, the GC–MS assay of Green et al. [7] was used to establish that L-[*guanidino*-$^{15}N_2$]-arginine was the precursor of $^{15}NO_2^-$ and $^{15}NO_3^-$ produced by activated macrophages in cell culture [36]. Similarly, oral ingestion of L-[*guanidino*-$^{15}N_2$]-arginine was used to demonstrate that labeled arginine is the precursor of urinary $^{15}NO_3^-$ in humans [37,38] and of urinary $^{15}NO_2^-$ and $^{15}NO_3^-$ in mice [24]. GC–MS was also used to demonstrate that the major metabolic fate of inhaled ^{15}NO in humans is first oxidation to $^{15}NO_3^-$ in the plasma, then excretion in urine [39].

Internal standards labeled with stable isotopes have also allowed accurate and precise determinations of the NO_2^- and NO_3^- levels in human plasma and urine [5,9,18]. The concentrations of NO_2^- and NO_3^- in the plasma and urine of 12 healthy volunteers on an unrestricted diet were measured by the alkylation-based GC–MS approach [5] and the range of values along with their means and SD are shown in Table 1. Values obtained from a control group receiving a standardized low-NO_3^- diet were halved. These values were confirmed by GC–MS/MS [18] and are consistent with those obtained in other GC–MS studies [9] and other reliable assays for plasma and urinary NO_2^- and NO_3^- (reviewed in [3]). While cross-validation studies and extensive surveys of diverse populations have not been performed, these concentrations are generally considered to represent the normal ranges for these analytes and significantly different values should be considered suspect.

9. Employing these assays to improve our understanding of the role of NO, NO_2^- and NO_3^-

The measurement of NO_2^- and NO_3^- by GC–MS has been applied to investigations of several clinically relevant conditions, including transplant rejection [40], hemodialysis [25], euglycemic hyperinsulinemia [41], stroke [42], erectile dysfunction [43], peripheral occlusive arterial disease [44], Zellweger syndrome [45], sepsis [28], depression [46], bronchopulmonary dysplasia [47] and hypertension [48]. However, when a complex biological scenario is investigated with these assays, the findings need to be interpreted carefully, with both the study design and the performance characteristic of the assay kept in mind. For example, some studies have employed the nitration-based approach which, without careful attention to detail, may be prone to interferences. Other investigations have employed the alkylation approach incorporating a reduction reaction which itself may lead to interferences. Only a handful of reported studies (e.g. [44,47]) have employed what we might now consider an optimal method, e.g. the simultaneous and independent determination of NO_2^- and NO_3^- by alkylation in the presence of stable isotope standards [15]. At other times study design has been compromised because key factors have not been carefully considered in advance. Critical review of all aspects of the published work is therefore prudent, if not essential, when piecing together the complex biology associated with the NO pathway. In this section several published studies are presented and critically evaluated in this light.

The nitration of trimethoxybenzene [6] was used to determine NO_3^- in the urine of cardiac transplantation patients being monitored for rejection by right heart biopsies [40]. The NO_3^- concentration was normalized to the creatinine concentration and the NO_3^-/creatinine ratio was observed to exhibit a trend toward a positive correlation with the degree of rejection. However the considerable inter- and intra-individual variation precluded the use of this assay for the diagnosis of acute graft rejection. Nitration-based assays are subject to interferences as previously discussed and also the patients were not on NO_3^- restricted diets. These factors may have contributed to the considerable variation in the measurements.

Nitration of toluene [8] was used to determine NO_3^- in plasma and the effluent dialysis fluid of patients undergoing hemodialysis [25]. In this study there was an apparent increase in NO_3^- leaving the dialyzer and the authors speculated that NO may have been generated within the system by blood cells exposed to reduced concentrations of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA). How-

ever, these investigators could not rule out the possibility that the NO_3^- came from the dialysis fluid itself because this reagent was not checked for contamination.

NO_3^- has also been measured in the cerebral spinal fluid (CSF) of stroke patients [42]. The investigators suggested that early NO production is associated with a smaller infarct volume, indicative of a protective effect, and that late NO production is associated with severe neurological deficits, consistent with a neurotoxic effect. The proposal that NO has a dual effect on ischemic brain damage clearly has important implications, but these findings need to be interpreted with some caution. Of special note, the number of subjects examined was small, the benzene nitration assay [12] employed in this study is prone to interferences, and at this stage, few studies on CSF have been performed. The fate of NO in the brain may be complex, and as the authors acknowledge, some NO formed during ischemia may react with other species rather than being converted to nitrate. Clearly, as the investigators suggest, this work should be confirmed in a larger study [42].

The sum of urinary NO_2^- and NO_3^- (U_{NOx}) has also been employed as a measure of systemic NO production. U_{NOx} was determined by the alkylation approach [5] and by this measure NO production was reduced in patients with uncomplicated essential hypertension [48]. The change in U_{NOx} that normally correlates well with changes in endothelin 1 during euglycemic hyperinsulinemia was not evident in these patients, and the authors suggest that this is consistent with impairment in the ability of insulin to regulate NO production [41].

In a study aimed at determining whether plasma levels of NO_2^- and NO_3^- in the systemic and cavernous blood of male subjects change during different penile conditions, and whether there is a difference in levels between normal males and patients with erectile dysfunction (ED), both the Griess reaction and a GC–MS approach were employed [43]. This work followed up on a previous study by Rossi et al. [49] that reported significantly lower NO_2^-/NO_3^- levels in the penile venous blood of men suffering from psychogenic impotence as assessed by a modification of the Griess assay. In Becker's study, neither NO_2^- nor NO_3^- levels in the peripheral or cavernous blood changed appreciably during developing erection, rigidity and detumescence. Further, there were no significant differences found between NO_2^- and NO_3^- plasma levels in the systemic and cavernous blood samples taken from the normal subjects and patients with ED. By employing two independent analytical methods and incorporating quality control (QC) samples, these investigators provided important information relating to this question. They concluded that NO metabolism in the corpora cavernosa during phases of penile tumescence and rigidity may account for only a minor fraction of local levels of NO_2^- and NO_3^- and therefore the basal levels of NO metabolites in the blood flushing the lacunar spaces of the cavernous body could conceal any local release of NO.

Urinary NO_3^- concentrations in peripheral occlusive arterial disease patients [44] were measured by the simultaneous analysis method [15]. While NO_2^- concentrations were not measured, the method offered the advantage that NO_3^- concentrations could be measured directly without a cadmium reduction step to NO_2^-. The infusion of oxygen was found not to effect urinary NO_3^- concentrations in these patients, unlike prostacyclin, a potent endothelium-derived relaxing factor [44].

U_{NOx} levels were measured by the alkylation method [17] in children born with Zellweger syndrome, a rare peroxisome deficiency disorder [45]. The U_{NOx} was found to be markedly elevated during the first six months of life, then to fall to normal levels. These findings were suggested to be consistent with an initial elevation in NO production, followed by a decrease. The apparent increase in NO was postulated to be due to interactions between the NO pathway and the lipoxygenase and cyclooxygenase pathways, both of which are severely effected in these patients.

Measurements of plasma NO_3^- in sepsis patients [28] have been undertaken by a nitration-based assay [12]. Based on this study, patients could be sorted into two apparent groups: one with very high NO_3^- (i.e., high responders) and another with elevated NO_3^- compared to controls, but not significantly higher than in patients with a localized infection (i.e., low responders). The high responders showed a rapid increase in measured NO_3^- 1 day prior to sepsis, a peak during sepsis, then a decline. There was no clinical difference found between high and low responders and diet did not appear to explain the difference in NO_3^- levels. Interferences associated with substances found in plasma were not discussed, and the reason for an apparent separation of sepsis patients into different groups was unexplained.

The sum of plasma NO_2^- and NO_3^- was measured by the alkylation method [5] in normal healthy volunteers and patients with severe depression [46]. In the patients there was a significant decrease in the sum of plasma NO_2^- and NO_3^- suggestive of a decrease in NO formation. This was correlated with an increase in ADMA, the endogenous NOS inhibitor, which may explain the observed decrease in NO. It was suggested that the various roles of NO in neurotransmitter release and vasoconstriction might account for the previously observed association between depression and coronary heart disease.

Perhaps the best example of a well-designed and executed clinical study is that of Heckman et al. [47]. Urinary NO_2^- and NO_3^- were measured simultaneously by the method of Tsikas [15] in preterm infants with and without bronchopulmonary dysplasia (BPD) [47]. Quality control samples spiked with known amounts of NO_2^- and NO_3^- were run concomitantly. Average recovery (accuracy) was between 89 and 101% and precision (RSD) was below 10% for NO_2^- and below 2% for NO_3^-. The sum of NO_2^- and NO_3^- was not different at 4 weeks, but was significantly higher in BPD infants at 36 weeks. These results do not support the proposal that impaired NO production in preterm infants contributes to BPD. The measured increase in NO metabolites seen later could be an effect of BPD due to pulmonary inflammation or compensatory mechanisms.

10. The future of NO_2^- and NO_3^- analysis by GC–MS

Gaining a better understanding of the biological role of NO is critically important, but to achieve this objective we need accurate and precise methods to quantify NO, or its metabolites.

Despite its simplicity, however, NO has presented an analytical challenge and currently our best approach to assessing it is via the indirect measurement of its primary metabolites, NO_2^- and NO_3^-. The PFB-Br alkylation approach for the simultaneous determination NO_2^- and NO_3^-, when used in conjunction with stable isotope ^{15}N-labeled internal standards [15], is currently the most specific, accurate and sensitive method to measure NO_2^- and NO_3^- by GC–MS. It is free from known interferences and is applicable to most body fluids. Further minor enhancements are likely (e.g. the incorporation of a phase-transfer catalyst such as TDMBA in the derivatization reaction [20], and enhanced mass resolution) that will improve the limit of quantification, selectivity, accuracy and precision of these assays. Hand-in-hand with analytical advances, our understanding of the critical factors in study design (e.g. the impact of drugs, diet and environmental factors) will continue to evolve. Well-designed studies will increasingly employ validated methods and incorporate quality control. Only in this way we will be able to advance our understanding of NO biology.

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