Analysis of NO and its metabolites by mass spectrometry. Comment on 'Detection of nitric oxide in tissue samples by ESI-MS' by Z. Shen, A. Webster, K. J. Welham, C. E. Dyer, J. Greenman and S. J. Haswell†

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Recently, Shen *et al.* (*Analyst*, 2010, **135**, 302) reported on a flow injection analysis (FIA) ESI-MS/MS approach for the determination of the short-lived gaseous nitric oxide (NO) in biological samples. This method is based on the reaction of NO, and presumably of other NO-derived oxides such as N_2O_3, with the vicinal amino groups of methylpiperazinobenzendiamine to form a benzotriazole derivative. Under MS/MS conditions, the protonated derivative loses molecular nitrogen (N_2) from the triazole ring and the product ion formed is utilized for quantitative analyses. This seems to be the first ESI-MS/MS method for authentic NO detection and quantification. However, the ESI-MS/MS method reported by Shen *et al.* deserves some critical discussion.

Nitric oxide (NO) is endogenously produced from L-arginine by the action of NO synthases (NOS), as well as from the reduction of nitrite by not yet fully understood enzymatic and chemical reactions.[1] Until present, not only NO but also its metabolites nitrite, nitrate and *S*-nitrosothiols (thionitrites) have been measured by GC-MS-based methods.[2] By contrast, LC-MS-based methods are almost entirely absent for NO, nitrite and nitrate so far, unlike for other members of this pathway.[1,2] To our knowledge, Shen *et al.*[3] reported on the first FIA-ESI-MS/MS approach for authentic NO after spontaneous reaction of NO with the vicinal amino groups of the probe methylpiperazinobenzendiamine, an aromatic *o*-diamine reagent, to form a benzotriazole derivative. Interestingly, under MS/MS conditions, this derivative loses most likely molecular nitrogen (N_2) from the triazole ring, with one N atom of N_2 stemming from NO. Previously, similar intramolecular diazotization reactions have been used for the analysis of NO and nitrite by various techniques including GC-MS and HPLC with fluorescence (HPLC-FL) detection.[1,4] Given the potentially high specificity of the MS/MS process, it is reasonable to assume that this particular MS/MS method should allow for highly specific detection of NO and would be superior to other non-MS-based methods that utilize similar derivatization reactions.[4]

However, from an analytical point of view the article by Shen *et al.*[3] suffers from some noteworthy shortcomings and misunderstandings. First, title and abstract of the article suggest that ESI-MS, *i.e.*, simple MS, allows for quantitative analysis, although in fact only ESI-MS/MS, *i.e.*, tandem MS, has been shown to suit for NO detection. Second, renunciation of the LC step in MS/MS-based methods, as has been done by Shen *et al.*[3] for NO detection and by Weaving *et al.*[5] for L-arginine and its methylated metabolites in biological fluids, to give another example for this pathway, is very tempting, but is potentially fraught with danger due to unknown interferences. Therefore, it is indispensable that the ability of leaving out the LC step in MS/MS techniques be demonstrated experimentally and be reported.[6] Such an evidence has not been presented by Shen *et al.*[3] The utility of simple MS has to be demonstrated by presenting close results agreement from parallel MS and tandem MS/MS analyses before application to biological samples.

Although assays based on inter- and intra-diazotization reactions including the Griess reaction are very frequently used in the analysis of NO and its metabolites nitrite and nitrate (after reduction to nitrite) and *S*-nitrosothiols,[7] the underlying mechanisms are incompletely investigated and poorly understood. Obviously, diazotization and related reactions and the subsequent detection of the final derivatives are associated with competing reactions of nucleophiles, including water and reductants such as thiols and ascorbic acid, with potential intermediates such as diazonium cations.[8] In addition, aromatic *o*-diamine reagents such as the newly used methylpiperazinobenzendiamine[3] and the frequently used 2,3-diaminonaphthalene (DAN), are not specific for NO and NO derivatives, but may react with various substances as has been demonstrated for instance for selenium compounds.[9,10] It cannot be excluded that in FIA-ESI-MS/MS such derivatives and other matrix constituents may interfere with NO analysis due to rearrangement and fragmentation during ESI. Such derivatives may then artefactually contribute to NO. Consequently, serious interference and lacking analytical accuracy due to unknown recovery of the diazo or triazo derivatives may result.[8] We found that the FIA-ESI-MS spectrum of the diazo derivative of the very simple reaction mixture of nitrite, sulfanilamide and *N*-(1-naphthyl)ethylendiamine shows that diazotization reaction mixtures may contain much more substances than the reagents and the desired reaction product (see ESI, Fig. S1†).

Because of the very short and greatly varying half-life of NO in biological fluids, autoxidation of NO to nitrite and additional

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† Electronic supplementary information (ESI) available: positive-ion FIA-ESI-MS spectrum of the reaction mixture from the diazotization of nitrite with sulfanilamide and *N*-(1-naphthyl)ethylendiamine. See DOI: 10.1039/c0an00411a

reactions of NO with various biomolecules may be important determinants of inaccuracy in quantitative analyses of NO due to varying and substantial loss of the target analyte (Fig. 1). In MS-based methods such difficulties may be overcome by using stable-isotope labelled analogs, for instance ^{15}N-labelled NO, nitrite or *S*-nitrosothiols (Fig. 2). Unfortunately, Shen *et al.*[3] did not use any stable-isotope labelled NO or NO donor, such as ^{15}NO or an *S*-[^{15}N]nitrosothiol (see below), or any other unrelated compound for use as internal standard as has been done by others in LC-MS/MS.[11,12] We should keep in mind that the inherent accuracy of the MS/MS technique cannot compensate for omission of the internal standard.

The recovery rate of the triazole derivative from methyl-piperazinobenzendiamine and synthetic NO or from NO produced in biological samples has not been reported by Shen *et al.*[3] The recovery rate is best determined by using synthetic triazole derivatives, for instance from the reaction of methyl-piperazinobenzendiamine with nitrite or [^{15}N]nitrite in acidic medium. Given the poor NO donating properties of *S*-nitrosothiols such as *S*-nitroso-*N*-acetyl-penicillamine (SNAP)[13] (Fig. 3) and the potential for direct reaction of the *S*-nitroso group of *S*-nitrosothiols[14,15] with the probe, the utility of such NO donors as calibrators is rather questionable. In the particular case of SNAP, it should be emphasized that regarding diazotization, SNAP has been reported to be about 500 times less effective compared to NO.[13]

Analytical methods intended for quantitative measurement in biological systems require thorough validation before use in experimental and/or clinical studies.[16] The validation process should include determination of linearity, accuracy, precision, lower limit of quantification (LLOQ), stability of analytes, and study of potential interferences. The quality of analytical methods and the value of the quantitative results generated by using insufficiently and improperly validated analytical methods are very difficult to assess. Generation of potentially expectable results in *in vitro* or *in vivo* studies, *e.g.*, differences in NO production rates between hypoxia and normoxia, and/or aorta and intestine,[3] is not a dependable criterion for analytical reliability.[17] Differences found in various experimental conditions, such as in normoxia and hypoxia, may be due to the dependency of the diazotization reaction rather than due to different NO production rates. Thus, detection of NO instead of nitrite (and/or nitrate) may lead to misinterpretation despite use of sophisticated MS/MS techniques.

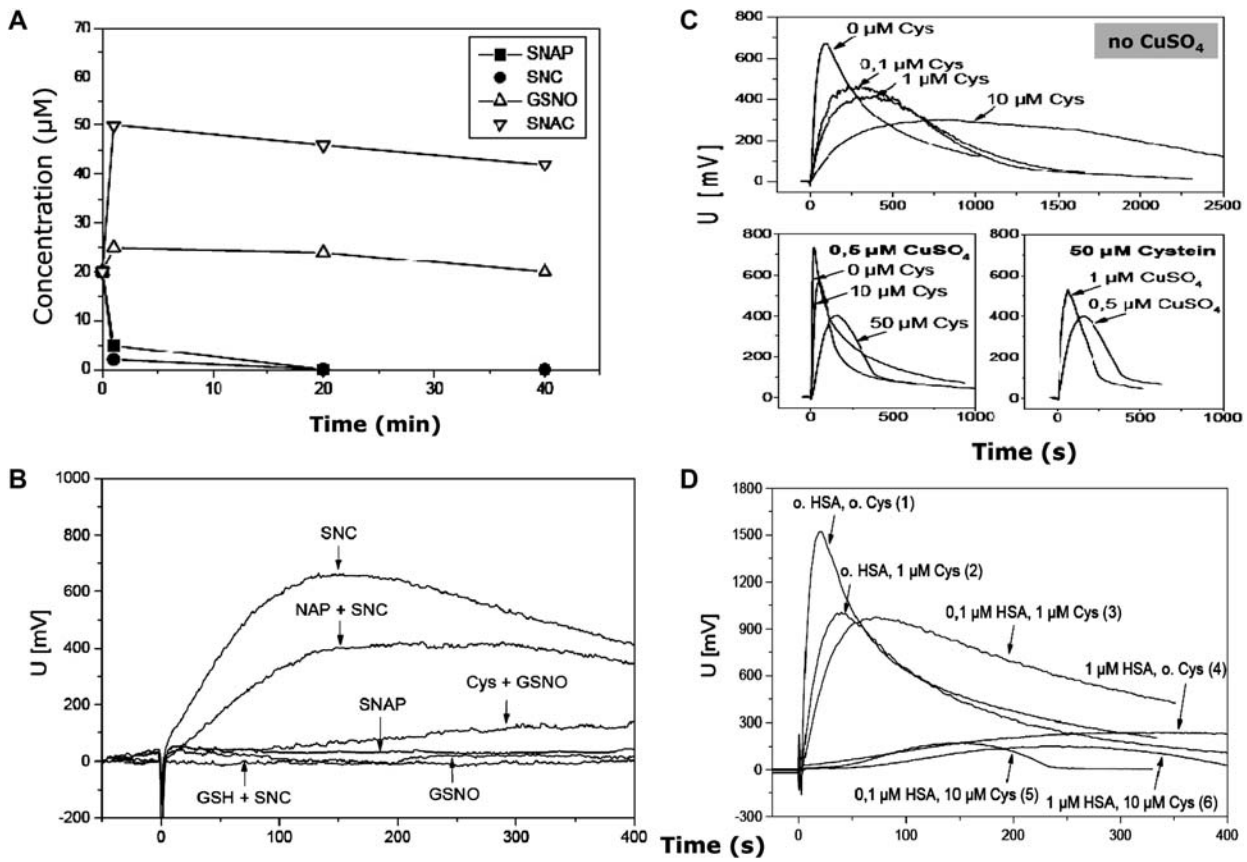

Fig. 1 Stability of various *S*-nitrosothiols, their NO-donating properties and common experimental factors that may greatly affect release and fate of NO in aqueous buffered solutions (0.2 M Tris buffer or 50 mM phosphate buffer, pH 7.4). (A) Concentration time course of SNAP, SNC, GSNO and SNAC upon mixing of 20 μM each with 500 μM each of the corresponding thiols in Tris buffer. (B) Release of NO from SNC, GSNO and SNAP (each 20 μM) and during the reaction of GSH (1 mM) with SNC, cysteine (1 mM) with GSNO, and NAP (1 mM) with SNC in Tris buffer. (C) Effect of cysteine (Cys) and $CuSO_4$ on the NO released from 10 μM SNC in phosphate buffer. (D) Release of NO from SNC in the absence (o) or in the presence of the indicated human serum albumin (HSA) and cysteine concentrations in phosphate buffer. Abbreviations: SNAP, *S*-nitroso-*N*-acetyl-penicillamine; SNC, *S*-nitrosocysteine; GSNO, *S*-nitrosoglutathione; SNAC, *S*-nitroso-*N*-acetyl-cysteine; NAP, *N*-acetyl-penicillamine.

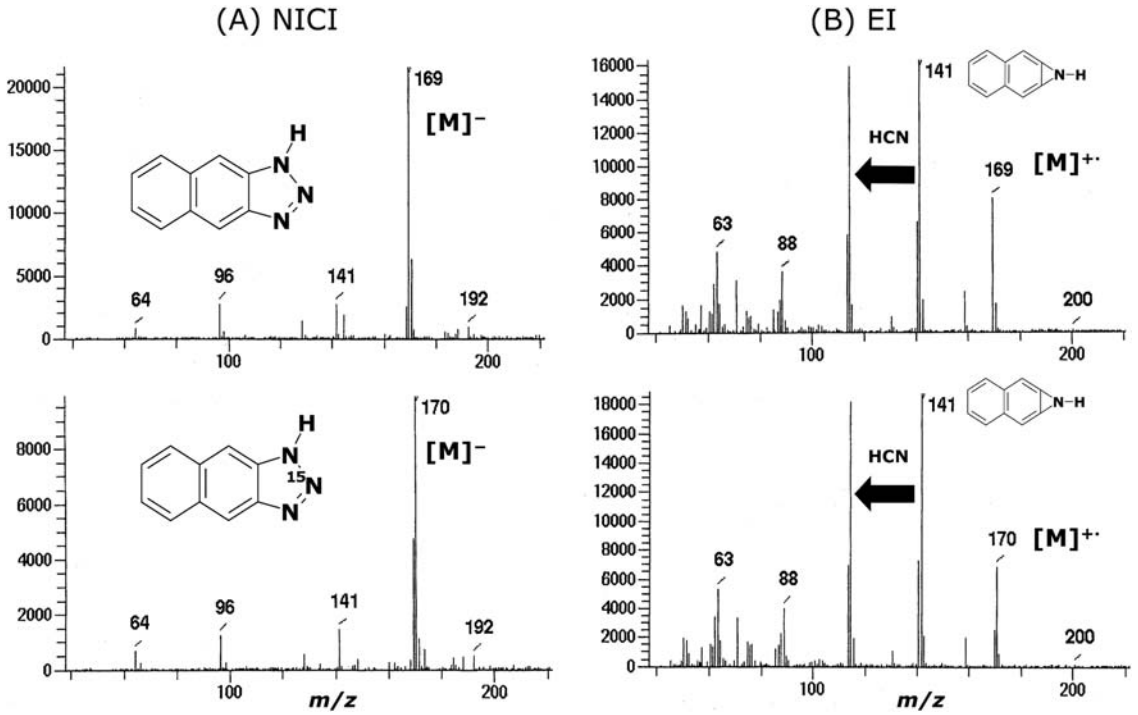

Fig. 2 (A) Negative-ion chemical ionisation (NICI) and (B) electron ionisation (EI) GC-MS spectra of unlabelled (upper panel) and ^{15}N-labelled (lower panel) triazole (DAT) derivatives formed from the reaction of 2,3-diaminonaphthalene (DAN) and unlabelled or ^{15}N-labelled nitrite in acidic aqueous solution. Derivatization was performed as described.[4] After alkalinization, reaction products were extracted with ethyl acetate which was then decanted and dried over anhydrous Na_2SO_4. Aliquots (1 μL) of anhydrous ethyl acetate were injected in the splitless mode and analysed on the Hewlett-Packard MS Engine 5890 (Waldbronn, Germany). The ions of *m/z* 169 for endogenous NO or nitrite and of *m/z* 170 for the internal standard ^{15}NO or ^{15}N-nitrite can be monitored for quantitative measurement in both ionisation modes.

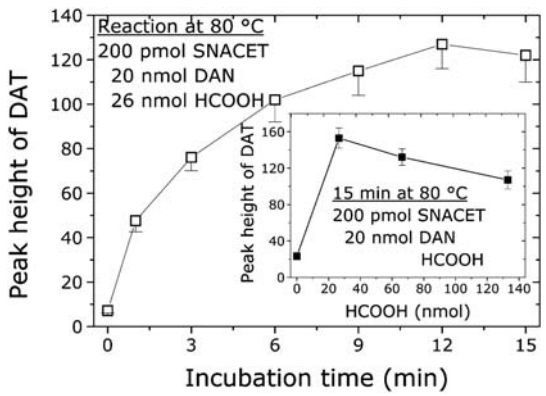

Fig. 3 Formic acid-catalysed formation of the triazole DAT from the reaction of the indicated amounts of 2,3-diaminonaphthalene (DAN) with *S*-nitroso-*N*-acetylcysteine ethyl ester (SNACET) in ethyl acetate at 80 °C. After the respective reaction time or 15 min after addition of the respective amounts of HCOOH (inset), the solvent was evaporated to dryness by means of a nitrogen stream and the residue was reconstituted in 1 mL of the mobile phase. HPLC analysis with fluorescence detection was performed on a Gynkotek HPLC system (Germering, Germany).

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