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Review

Analysis of nitrite and nitrate in biological fluids by assays based on the Griess reaction: Appraisal of the Griess reaction in the L-arginine/nitric oxide area of research☆

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Abstract

In the Griess reaction, first reported by Johann Peter Griess in 1879 as a method of analysis of nitrite ($\mathbf{N}O_2^-$), nitrite reacts under acidic conditions with sulfanilic acid ($HO_3SC_6H_4NH_2$) to form a diazonium cation ($HO_3SC_6H_4–N{\equiv}\mathbf{N}^+$) which subsequently couples to the aromatic amine 1-naphthylamine ($C_{10}H_7NH_2$) to produce a red–violet coloured ($\lambda_{max} \approx 540$ nm), water-soluble azo dye ($HO_3SC_6H_4–N{=}\mathbf{N}–C_{10}H_6NH_2$). The identification of nitrite in saliva has been the first analytical application of this diazotization reaction in 1879. For a century, the Griess reaction has been exclusively used to identify analytically bacterial infection in the urogenital tract, i.e. to identify nitrite produced by bacterial reduction of nitrate (NO_3^-), the major nitrogen oxide anion in human urine. Since the discovery of the L-arginine/nitric oxide (L-Arg/NO) pathway in 1987, however, the Griess reaction is the most frequently used analytical approach to quantitate the major metabolites of NO, i.e. nitrite and nitrate, in a variety of biological fluids, notably blood and urine. The Griess reaction is specific for nitrite. Analysis of nitrate by this reaction requires chemical or enzymatic reduction of nitrate to nitrite prior to the diazotization reaction. The simplicity of the Griess reaction and its easy and inexpensive analytical feasibility has attracted the attention of scientists from wide a spectrum of disciplines dedicated to the complex and challenging L-Arg/NO pathway. Today, we know dozens of assays based on the Griess reaction. In principle, every laboratory in this area uses its own Griess assay. The simplest Griess assay is performed in batch commonly as originally reported by Griess. Because of the recognition of numerous interferences in the analysis of nitrite and nitrate in biological fluids and of the desire to analyze these anions simultaneously, the Griess reaction has been repeatedly modified and automated. In recent years, the Griess reaction has been coupled to HPLC, i.e. is used for post-column derivatization of chromatographically separated nitrite and nitrate. Such a HPLC-Griess system is even commercially available. The present article gives an overview of the currently available assays of nitrite and nitrate in biological fluids based on the Griess reaction. Special emphasis is given to human plasma and urine, to quantitative aspects, as well as to particular analytical and pre-analytical factors and problems that may be associated with and affect the quantitative analysis of nitrite and nitrate in these matrices by assays based on the Griess reaction. The significance of the Griess reaction in the L-Arg/NO pathway is appraised.

Keywords: Reviews; Diazotization; Fluorescence; HPLC; Interferences; Quantitation; Reduction; Spectrophotometry

Contents

Abbreviations: L-Arg, L-arginine; DAF-2, 4,5-diaminofluorescein-2; DAF-2 DA, 4,5-diaminofluorescein-2 diacetate; DAF-2T, DAF-2-triazol; DAN, 2,3-diaminonaphthalene; ECD, electron capture detection; EDRF, endothelium-derived relaxing factor; FAD, flavin adenine dinucleotide; FIA, flow injection analysis; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LOD, limit of detection; NAT, 2,3-naphthotriazole; NED or NEDA, *N*-(1-naphthyl)ethylenediamine; NEM, *N*-ethylmaleimide; NO, nitric oxide; QC, quality control; SAN, sulfanilamide; TCA, trichloroacetic acid

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1. The L-arginine/nitric oxide pathway – nitric oxide, nitrite, nitrate

The endothelium-derived relaxing factor (EDRF), discovered in 1980 [1] and identified as nitric oxide (NO) or a labile nitroso species (*R*-NO, *R* unknown moiety) in 1987 [2,3], has been shown to be produced from the oxidation of one of the terminal guanidino N atoms of L-arginine (L-Arg) in various cells [4–9]. NO has been shown to be an intermediate in the oxidation of L-Arg to nitrite and nitrate in macrophages [9]. By means of GC–MS and orally administered L-[guanidino-$^{15}N_2$]-arginine to humans it was demonstrated that ^{15}N-labelled nitrate was excreted in the urine, thus demonstrating that L-arginine is a precursor for nitrate biosynthesis in humans [10–14]. NO synthase (NOS, EC 1.14.13.39) is the enzyme responsible for the conversion of L-Arg to NO and L-citrulline (L-Citr), and is expressed in various cells [15–17]. Investigations on the underlying mechanism of NOS-catalyzed conversion of L-Arg to L-Citr and NO by GC–MS revealed that the O atoms in the ureido group of L-Citr and in NO originate from dioxygen (O_2), not from water [18–20].

Chemistry and biochemistry of NO and its redox-activated forms have been reviewed in the past [21,22]. In aqueous phase, free of biological material, NO exclusively autoxidizes to nitrite [23]. In this reaction incorporation of O from H_2O into NO seems to take place during hydrolysis of an intermediate, such as N_2O_4 [23,24]. The half-life of NO in aqueous phosphate-buffered solution of pH 7.4 was estimated to be 130 s [25].

One of the most important reactions of NO is that with haemoglobin. The ratio of rates of NO binding and release for $Fe^{(II)}$-haemoglobin is 5–6 orders of magnitude greater than that of O_2. NO reacts with oxyhaemoglobin ($HbFe^{(II)}O_2$) to produce methaemoglobin (MetHb, $HbFe^{(III)}$) and nitrate. In blood of healthy humans, NO metabolism has been shown to depend upon the oxygenation of red cell haemoglobin [26]. The uptake of NO into the red blood cells with subsequent conversion to nitrate is the major metabolic pathway for endogenously formed NO [27]. Because nitrate itself is biologically completely inactive, in contrast to nitrite, oxidation of NO in erythrocytes to nitrate by oxyhaemoglobin is the most effective and definite inactivating metabolic fate of NO. ^{15}NO has been shown to oxidize in human blood both to [^{15}N]nitrate and [^{15}N]nitrite, with [^{15}N]nitrate being by far the major oxidative metabolite of ^{15}NO [24]. Erythrocytes seem to possess a high oxidative capacity for NO and nitrite. The half-life ($t_{1/2}$) of [^{15}N]nitrite (range 0–109 μM) in whole blood was determined by GC–MS to be 11–13 min [24]. An assay based on the Griess reaction has provided a similar value for the half-life of externally added nitrite (at 2.5 μM) in whole blood [28]. The L-Arg/NO pathway has been shown by GC–MS to be the major source of plasma nitrite in humans [29] and of urine nitrite in mice [30].

Assessment of NO synthesis is of particular interest, as it may characterize the status of the L-Arg/NO pathway in health and disease as well as may allow monitor the progress of pharmacological interventions. NO can be directly measured in the human circulation upon stimulation [31], however, endogenous NO at the basal state has not been reliably quantified in the blood, thus far. The half-life of NO in vivo in the circulation is most likely shorter than 0.1 s [32]. By contrast, nitrite and nitrate are stable metabolites of NO, present both in blood and urine, and accessible to analysis. Quantitative determination of the concentration of nitrite and nitrate in biological fluids, notably plasma, serum and urine, is the most suitable method to assess NO synthesis in vivo. Studies in vivo in humans and mammals indicate that circulating nitrite rather than nitrate reflects endothelial-dependent NO synthesis in humans and mammals [33–35]. It is clearly

established that the major urinary metabolite of NO is nitrate. In general, the excretion rate of nitrate in the urine is a reliable non-invasive method to assess gradually long-lasting changes in whole body NO synthesis under basal conditions as well as upon pharmacological or physical treatment [36–38].

2. The Griess reaction

2.1. The original Griess reaction – a historical view

The most famous and most frequently used method of analysis of nitrite and nitrate is based on the Griess reaction [39]. Johann Peter Griess (1829–1888) was a German Chemist. In the original work reporting on the synthesis of novel pigments, Griess described in detail the synthesis of the azo dye "Diazobenzolamidonaphtol" starting from diazobenzene and naphthylamine [40]. In order to increase the water-solubility of the azo dye, diazobenzene was replaced by diazobenzene sulfonic acid which was prepared from sulfanilic acid [39]. In 1879, Griess introduced this reaction for the identification of "traces" of nitrous acid [39]. In the original Griess reaction, nitrite reacts with sulfanilic acid under acidic conditions, i.e. with nitrous acid, to form a diazonium ion which couples to α-naphthylamine to form a readily water-soluble, red–violet coloured azo dye; the molecular formula of the reaction product had been reported by Griess to be $C_{16}H_{13}N_3SO_3$ (Fig. 1). Griess applied this reaction to 5–10-fold diluted saliva which turned red after addition of the reagents [39]. Because of the easy feasibility of the test, Griess suggested its use as experimental demonstration in lectures. Three decades later, a direct relationship was found by means of the Griess reaction between the presence of nitrite in the urine and the occurrence of urinary tract infection [41]. Since that time and until the discovery of the L-Arg/NO pathway in 1987, detection of bacteriuria in urology has been the main application field of the Griess nitrite assay [42].

2.2. Variants of the original Griess reaction

The original Griess reaction is a diazotization reaction. Instead of the originally used reagents, i.e. sulfanilic acid and α-naphthylamine, the so called Griess reagents, derivatives of these nitrosable and coupling components, notably sulfanilamide and *N*-(1-naphthyl)ethylenediamine (NED or NEDA), can be used (Fig. 2). The use of NED as a coupling component for sulfanilamide in the Griess reaction has been first suggested by Bratton and Marshall in 1939 [43], although originally intended for the analysis of sulfanilamide in blood and urine rather than of nitrite. The molar absorptivity for the purified azo dye formed in the diazotization reaction of aqueous nitrite, sulfanilamide and NED has been determined to be $\varepsilon = 47{,}500\ M^{-1}\ cm^{-1}$ at 545 nm [43]. The molar absorptivity for the azo dye formed in the diazotization reaction of aqueous nitrite, sulfanilamide and NED has been reported to be $\varepsilon = 41{,}000\ M^{-1}\ cm^{-1}$ at 540 nm [28]. The azo group, i.e. –N=N–, is the most important chromophore group of the azo dye molecule. NED was found to offer several advantages over other coupling components, such as *N*,*N*-dimethyl-1-naphthylamine in terms of reproducibility, greater rapidity of coupling, increased sensitivity, increased acid-solubility of the azo dye, and pH-independence of the colour in the pH range of 1–2 [43]. Introduction of an aqueous solution of NO in an aerobic solution of sulfanilamide (25 mM) and NED (2.5 mM) in 100 mM phosphate buffer of pH 7.4 was found to yield an absorbance band at 496 nm which increased with increasing NO concentration. Acidification of the buffered solution resulted in an absorbance band at 546 nm indicative of the corresponding azo dye [43]. The molar absorptivity of the diazonium cation

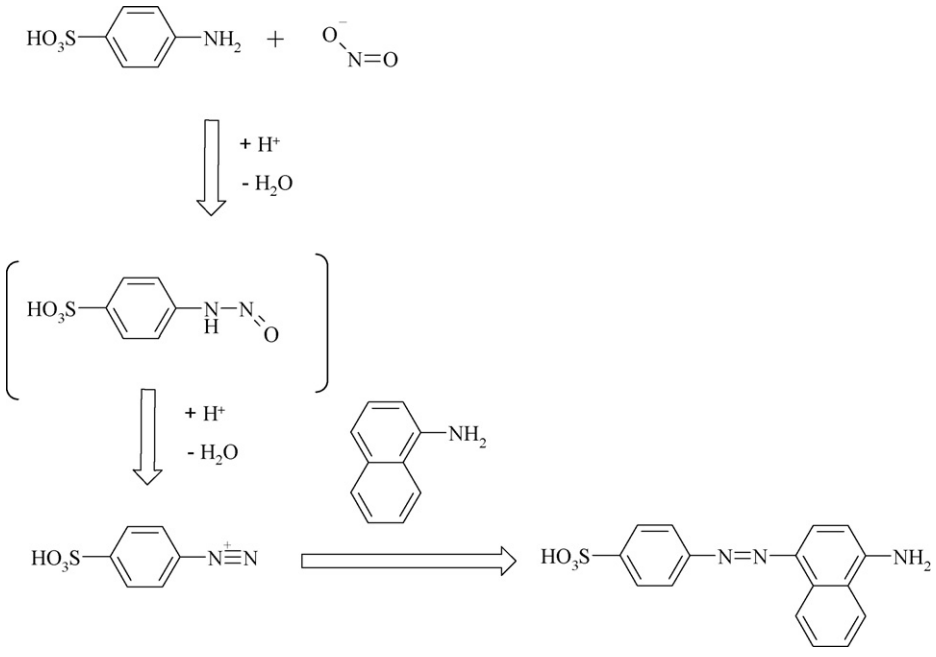

Fig. 1. The Griess reaction originally reported by Griess in 1879 [39]. Under acidic conditions nitrite reacts with the amino group of sulfanilic acid to form the diazonium cation, which couples to α-naphthylamine in *para*-position to form the azo dye.

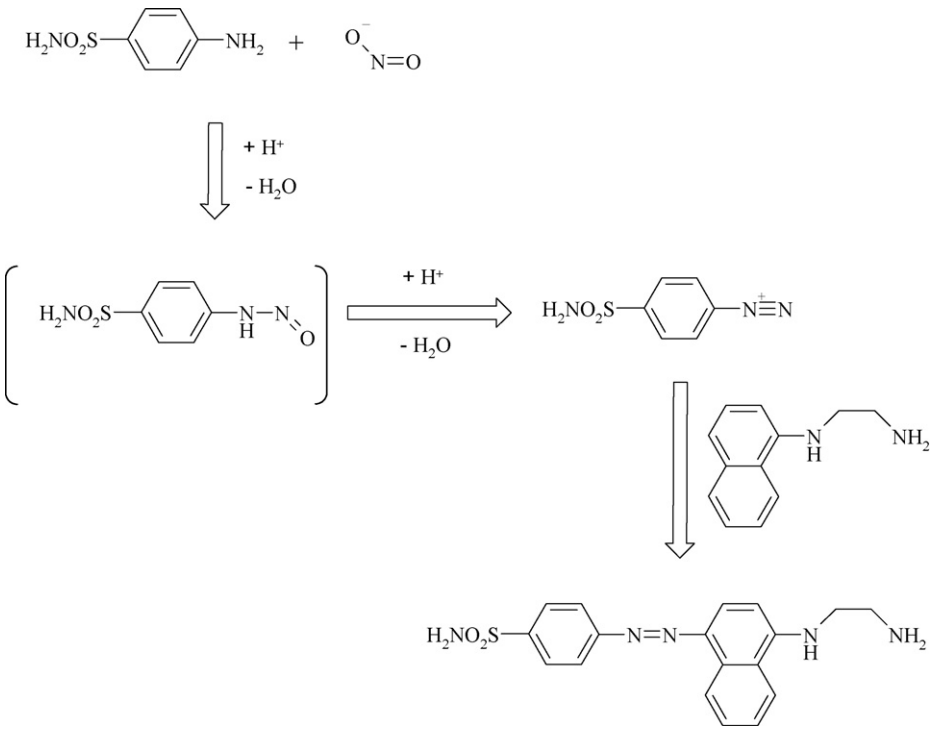

Fig. 2. The modified, currently most commonly used Griess reaction. Under acidic conditions nitrite reacts with the amino group of sulfanilamide to form the diazonium cation, which couples to *N*-(1-naphthyl)ethylenediamine in *para*-position to form the corresponding azo dye.

formed was estimated to be $\varepsilon = 950\,M^{-1}\,cm^{-1}$ [44], i.e. about 50-fold smaller than of the corresponding azo dye [43].

The modified Griess reaction using sulfanilamide and NED is currently the most frequently used Griess assay in quantitative analyses. This method has been established as a European standard for determining nitrite in water (European standard, water quality–determination of nitrite–molecular absorption spectrometric method. 1993. EN 26777). Sulfanilic acid or sulfanilamide can be replaced by sulfathiazole (a sulfanilamide derivate) [45] or by 4,4′-diamino-diphenylsulfone (i.e. dapsone) [46]. Interestingly, endogenous substances present in human feces, presumably nitrosamides and *N*-nitroso compounds, can form upon incubation with nitrite at physiological pH ether-soluble Griess reagent-positive material [47].

The Griess reaction, carried out in acidic solution with sulfanilic acid and NED, played an important role, in addition to GC–MS, chemiluminescence and spectrophotometry using haemoglobin, in the identification of NO released from endothelium and formed in macrophages [3,5,8]. It was reported that under the experimental conditions used, the Griess assay was 100-fold more sensitive for NO and labile nitroso compounds than for nitrite [3]. However, another group found that the Griess assay consisting of an HCl acid solution of sulfanilic acid and NED was not adequately sensitive to measure NO at submicromolar concentrations, and that EDRF is best considered a measure of nitrite and mixed nitroxides [48]. For discussion of this and related issues concerning NO measurement in biological models, see the review article by Archer [49]. In principle, the Griess assay can also be used to determine NO trapped by haemoglobin after separation of nitrosylhaemoglobin by gel filtration chromatography [50].

The diazotization reaction can also occur intra-molecularly, i.e. by using a single compound that contains two amino groups in *ortho*-position, such as 2,3-diaminonaphthalene (DAN) [51,52], or one amino group and a nitrosable nitrogen atom, such as hydralazine [53]. Remarkably, Griess reported the first intra-molecular diazotization reaction of nitrite with 1,2-diaminobenzene (phenylendiamine) leading to benzotriazol [39]. Interestingly, the diazotization of 2,3-diaminonaphthalene yields the stable and highly fluorescent compound 2,3-naphthotriazole (NAT) (Fig. 3), while diazotization reactions involving sulfanilic acid and naphthylamine or their derivatives produce azo dyes absorbing light in the visible range, i.e. around 540 nm.

The diazotization reaction is specific for nitrite and strictly depending on the presence of acid. Mechanistically, the diazoti-

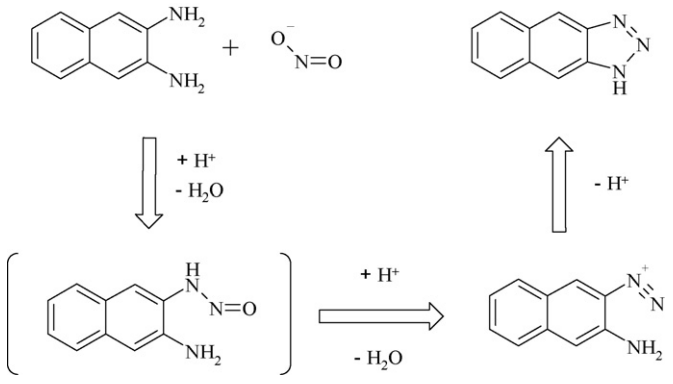

Fig. 3. The intra-molecular diazotization reaction of nitrite in acidic solution carried out with 2,3-diaminonaphthalene.

zation reaction represents an electrophilic aromatic substitution reaction, with the diazonium ion (e.g. produced from sulfanilamide) being the electrophilic agent and the second aromatic amine (e.g. NED) being the nucleophilic base. The electrophilic attack of the diazonium ion takes place at *para*-position with respect to the amino group of the aromatic base, i.e. the coupling component, (Figs. 1 and 2). Diazotization reactions are also feasible with aliphatic amines. However, the corresponding diazonium ions are unstable due to the lack of mesomerization – unlike the aromatic diazonium ions – and release readily molecular nitrogen (N_2).

Diazonium ions are targets for many nucleophiles including water and inorganic anions, such as chloride. In some circumstances, such as in the presence of high concentrations of $CuCl_2$ (e.g. 100 mM) and long incubation times (e.g. 1 h) [54], the diazonium ion formed from HCl-acidified, nitrite-containing matrices and an externally added aromatic amine, such as *p*-bromoaniline can be converted into *p*-bromochlorobenzene which can be analyzed by GC and electron capture detection (ECD) with high sensitivity. An interesting case for an alternative fate of a diazonium ion is represented by 5-aminofluorescein (5AF) which is highly fluorescent. 5AF has a primary amino group which can be diazotized by nitrite in acidic solution. After completion of the diazotization – preferably performed in 1–2 M HCl solution, the reaction mixture is alkalinized with NaOH (1 M final concentration), with the diazonium cation being converted into the hydroxylamine which stays in equilibrium with its conjugate base anion [55]. The LOD of this method has been reported to be about 5 nM of nitrite [49]. A similar reaction leading to a highly fluorescent (LOD, 0.65 nM) and alkaline-stable diazotate anion has been reported more recently [56].

Further interesting variation of the Griess reaction is that reported by Saltzman [57]. In this method, a mixture of sulfanilic acid, NED and acetic acid is used to trap nitrogen dioxide (NO_2) from the atmosphere by drawing the air directly through the Griess reagent mixture and measuring the colour formed (550 nm). Nitric acid was found not to react with the Griess reagents, and it is likely that NO does not produce the azo dye in the absence of air under the experimental conditions used [57]. The reaction of NO_2 with Griess type reagents has also been investigated by Huygen [58]. Atmospheric NO_2 was also trapped using an alkaline solution and determined spectrophotometrically using a modified version of the Saltzman procedure as well as spectrofluorometrically using 5AF in HCl acid solution [59], with both methods having produced comparable concentrations for atmospheric NO_2 of the order of 1 ppbv [59].

Besides vicinal aryl diamines, such as 1,2-diaminobenzene and DAN, the Griess reaction has also been performed using a single "classical" Griess reagent. Thus, performance of the Griess reaction with 1-naphthylamine has been reported to produce an azo dye that can be extracted with chloroform and detected at 560 nm [60], suggesting that 1-naphthylamine serves both as the diazotizing and the coupling component. Recently, it was reported that performance of this reaction with NED instead of 1-naphthylamine produced a new compound that possesses weak fluorescence [61]. By this method as little as 1.5 nM of nitrite could be detected in water.

2.3. Spectrophotometric and spectrofluorometric methods of nitrite and nitrate

Until 1963 over 50 colorimetric methods had been reported for the determination of nitrite and nitrate in a variety of matrices; many based on the Griess reaction [62]. At that time, more than 20 of the newer methods had been reported to give higher molar absorptivity, e.g. $\varepsilon = 620{,}000\ M^{-1}\ cm^{-1}$, with nitrite than any of the Griess type of reagents [62]. To discuss the currently available spectrophotometric and spectrofluorometric methods for the analysis of nitrite and nitrate is beyond the aim of the present article. Here, it is referred to some selected research papers [62–69], as well as to selected review articles [22,49,62,70–73]. Many of these methods are based on the nitrosation/nitration of compounds producing strongly UV–vis absorbing or highly fluorescent species. Some more recently reported spectrofluorometric methods are based on the diazotization reaction.

Of particular interest are methods that involve an intramolecular diazotization reaction in fluorogenic species, such as 4,5-diaminofluorescein-2 (DAF-2) or its cell-permeable diacetate (DA) derivate (DAF-2 DA) that produce the highly fluorescent DAF-2-triazol (DAF-2T) [73–77], analogous to the diazotization reaction occurring in DAN (Fig. 3). In contrast to the methods aiming at producing specifically fluorescence or increasing native fluorescence, there have also been reported less selective methods, the fluorescence quenching methods, in which diazotization and other reactions, such as oxidation of the fluorogenic species by NO suppress considerably fluorescence, allowing thus indirect analysis of nitrite or NO [60,78–83].

2.4. Analysis of nitrate by the Griess reaction

Analysis of nitrate by assays based on the Griess reaction requires reduction of nitrate to nitrite prior to diazotization. As a result of the preceding reduction step, the Griess assay provides the sum of nitrite and nitrate both in batch and in automated Griess assays not separating nitrite from nitrate, and nitrate quantity has to be calculated by difference. In Griess assays using various reagents including the DAN and in some related nitrite-specific assays, reduction of nitrate to nitrite in various matrices including biological fluids is accomplished by means of chemicals [35,46,84–108] and the enzyme nitrate reductase (EC 1.6.6.2.) from different sources, such as *Aspergillus* species [46,52,109–133] (Table 1).

Most frequently used chemical reductors include cadmium (Cd) in various forms, such as powder, sponge, wire and activated columns, and vanadium chloride (VCl_3 or VCl_3/VCl_4) (Table 1). Reduction with Cd can be performed under acidic (HCl acid or acetic acid) and alkaline (ammonium chloride buffer, e.g. pH 8.8) conditions, in batch and automated Griess assays. Reduction with vanadium chloride is performed under HCl-acidic conditions (Table 1). Nitrate reductase-catalyzed reduction of nitrate to nitrite is most frequently performed in the presence of the cofactor NADPH, the concentration of which may vary from 1 up to 1000 μM (Table 1). Oxidized NADPH, i.e. $NADP^+$, has been shown to interfere in the subsequent diazotization of nitrate-derived nitrite [114]. Application

Table 1
Summary of selected chemical and enzyme-based methods of reduction of nitrate to nitrite used in assays based on the Griess reaction

(A) Chemical reduction

Reductor	pH	Batch/automated	Species/matrix	Efficiency (%)	"Lifetime"	Reference
Cd column	Alkaline	Batch	A/P	≈100	Indefinite	[84]
Cd wire	Alkaline	Automated	Water	≈100	1000 samples	[85]
Cd filings/sponge	N.R.	Batch	Water	≈100	N.R.	[86]
Cd column	Alkaline	Automated	A, H/U, P, Se, Sa	≈100	800–1000 samples	[87]
Cd column	Alkaline	Automated; FIA	A/Se	≥ 95	N.R.	[88]
Cu-plated Cd column	Alkaline	Automated; HPLC	A/U, Se	≈100	N.R.	[90]
Cd powder	Alkaline or acidic	Batch	H/U, P	30–80	N.A.	[95]
Cu-plated Cd filings	N.R.	Automated; HPLC	H/P	≈100 (as recovery)	N.R.	[98]
VCl_3	Acidic (5% HCl)	96-well plate	H/S	N.R.	N.A.	[100]
Cu–Cd alloy	Alkaline	96-well plate	A, H/S	≈100	N.A.	[102]
VCl_3/VCl_4 (0.05 M in 1 M HCl)		Batch	Water	N.R.	N.A.	[108]

(B) Enzymatic reduction

Enzyme/organism	Cofactor/recycling system	Batch/automated	Species/matrix	Efficiency (%)	Reference
K. pneumoniae	No/no	Batch	H/Se	≈100	[109]
NR from *Aspergillus*	NADPH (40 μM)	96-well plate	H/P	≈90	[52]
NR from *Aspergillus*	NADPH (≈130 μM)	Batch	H/Se	N.R.	[111]
NR from *Aspergillus*	NADPH (50 μM); FAD (5 μM)	Batch	H/P	85–100	[113]
NR from *Aspergillus*	NADPH (1 μM)/G6PDH	96-well plate	A, H/U	N.R.	[114]
NR from *Aspergillus*	NADPH (500 μM)/no	Batch	A, H/B	≈100	[116]
NR from *Aspergillus*	NADPH (950 μM)/G6PDH	Batch	EC culture	≈100	[118]
NR from *Aspergillus*	NADPH (80 μM)/no	Batch	H/U, S, CSF	N.R.	[119]
NR (commercial kit, Cayman)		96-well plate	A/P	N.R.	[120]
NR from *Aspergillus*	NADPH (3 μM)/no	96-well plate	A/brain tissue	≈100	[121]
NR from *P. stutzeri*	Dissimilatory enzyme	96-well plate	H/P	≈100 (as recovery)	[125]
NR from *Aspergillus*	NADPH (1 μM)/G6PDH	Batch; HPLC	A/brain tissue	N.R.	[126]
NR from *E. coli* MC1061	No/no	Batch	A/Se	60–70	[132]

A, animal; H, human; U, urine; P, plasma; Se, serum; Sa, saliva; B, blood; NR, nitrate reductase; N.A., not applicable; FIA, flow injection analysis; G6PDH, glucose-6-phosphate dehydrogenase; EC, endothelial cells; CSF, cerebrospinal fluid.

of NADPH at very low concentrations (e.g. 1 μM) in nitrate reductase incubates minimizes interferences originating from the NADPH/$NADP^+$ system. NADPH-recycling enzymes, such as glucose-6-phosphate dehydrogenase (G6PDH) are frequently used to continuously provide sufficient reduction equivalents [73,114,121,126] (Table 1). For the sake of completeness, it should be mentioned that in few assays using nitrate reductase concentration of nitrate is estimated indirectly, i.e. on the basis of oxidation of NADPH (added at 100 or 240 μM) in the presence of FAD (added at 2.5 or 5–10 μM) by measuring the decrease in absorbance at 340 nm, but not by measuring nitrate-derived nitrite by the Griess reaction [110,112].

2.5. Analysis of thionitrites – S-nitrosothiols – and alkyl nitrites by the Griess reaction

Special analytical attention has been paid to endogenous low- and high-molecular-mass *S*-nitrosothiols or thionitrites (*R*-*S*-N=O), such as *S*-nitrosocysteine, *S*-nitrosoalbumin and *S*-nitrosohaemoglobin, because of their potent NO-related biological actions including vasodilation and inhibition of platelet aggregation [134–139]. It is noteworthy that besides NO, labile, NO–releasing nitrosothiols, notably *S*-nitrosocysteine [140], have been considered to be identical with EDRF [2,3]. The Griess reaction has been applied by several groups to analyze various *S*-nitrosothiols in biological fluids and tissues. Prerequisite for the application of the Griess reaction is the chemical conversion of the *S*-nitroso group into nitrite, either directly or indirectly via NO formation. The most frequently used method is based on the use of an aqueous solution of $HgCl_2$ as proposed by Saville [141]. The most crucial point in such assays is the elimination of ubiquitous *S*-nitrosothiol-unrelated nitrite [135]. One of the most frequently used reagents to remove endogenous and blank nitrite, the concentration of which may exceed by far that of *S*-nitrosothiols, is ammonium sulfamate which strictly requires acidic conditions [43,135]. However, acidic conditions favour artifactual formation of *S*-nitrosothiols from nitrite and ubiquitous thiols, so that preceding addition of thiol-alkylating species, such as *N*-ethylmaleimide (NEM) becomes mandatory [135]. As a result of this and because of the very low content of biological material on *S*-nitrosothiols and the instability of the *S*-nitroso group, specific and sensitive measurement of this class of NO metabolites/derivates by Griess assays gets very difficult. Both batch and automated Griess assays using spectrophotometric and fluorometric detection have been applied to measure various *S*-nitrosothiols [46,116,142–145]. It is noteworthy that for *S*-nitrosoglutathione (GSNO) the diazotization reaction, carried out in phosphate-buffered saline both with sulfanilamide/NED and DAN, in the presence of $HgCl_2$ or $CuCl_2$, has been reported to proceed at neutral pH [145].

Eventually, it should be mentioned that alkyl nitrites, the homologous of thionitrites, may interfere with the analysis of nitrite or *S*-nitrosothiols. Thus, treatment of solutions of decyl nitrite in acetone, acetonitrile, dioxane or tetrahydrofuran with aqueous sulfanilamide (290 mM) for 1 min followed by aqueous NED (13.5 mM) and spectrophotometric detection at 550 nm has been shown to produce the azo dye [146]. Organic nitrate drugs, such as pentaerythrityl tetranitrate (PETN) have been shown to contribute artefactually to endogenous nitrite and nitrate when added to serum but not to urine [147] as measured by GC–MS [148]. So far, alkyl nitrites and nitrates from endogenous sources have not been reported. Potential artefactual contribution of alkyl nitrites to nitrite and nitrate during sample treatment and analysis by the Griess assay, GC–MS and other techniques should be considered when organic nitrites and nitrates are used or administered.

2.6. The significance of the Griess reaction in the L-arginine/nitric oxide pathway of the human organism – a literature search

In addition to the assays based on the Griess reaction and the other spectrophotometric and spectrofluorometric assays mentioned above, reported methods of analysis of nitrite, nitrate and *S*-nitrosothiols include chemiluminescence, HPLC with various detection techniques, CE, CZE, GC, and GC–MS (reviewed in refs. [22,24,28,49,62,70–73,93,103,115,139,144,145,149–155]). However, no other methodology like that utilizing the Griess reaction has been so frequently and widely used, impressively underlining the popularity of this analytical approach among scientists from various disciplines. A literature search in the database PubMed in the period under review between 1950 and 2006 showed that about 90% of the listed articles obtained by using the search term "Griess" were related to the L-Arg/NO pathway, with about 8% of which being found to be closely related to analytical chemistry (Table 2). In this search, articles related to the diagnosis of urinary infection were considered unrelated to the L-Arg/NO pathway. A search using the term "Greiss"–in the Anglo-Saxon countries sometimes "Greiss" is used instead of the correct name "Griess" in relation to the diazotization reaction discovered by Johann Peter Griess–resulted in 245 articles of which approximately 50% were related to the L-Arg/NO pathway. Combination of the term "Griess" or "Greiss" with other frequently used expressions in this context, such as "Assay", "Reaction" or "Reagent", yielded articles in high percentage portions being related to the L-Arg/NO pathway, of which even considerably higher rates of up to 63% for the combination "Griess AND Serum" dealt with analytical issues. Considering all single "Griess" or "Greiss" and all combined search terms, containing or non-containing these terms, in average 77% of the articles obtained were found to be connected to the L-Arg/NO pathway, with 23% of which being published in chemical analysis-oriented journals or having dealt significantly with analytical issues (Table 2).

Interestingly, a search in PubMed using combinations of the term "Griess" with a variable search term representing a human disease or condition revealed that assays based on the Griess reaction have been and are currently frequently applied to measure nitrite and nitrate in human biological fluids in the period under review (1950–2006). Thus, the search term "Griess AND [name of disease]" resulted in the following entries (n): $n = 91$ for "cardiovascular disease", $n = 85$ for "infection", $n = 60$ for "can-

Table 2
Summary of the results from a literature search in PubMed[a] in relation to the reaction of Griess[b] and other related diazotization reactions, to nitrite and nitrate with special emphasis to the L-arginine/nitric oxide pathway[c] and analytical chemistry

Search term(s)	Citations *In toto*	Related to L-Arg/NO Pathway	Related to analytical chemistry
I. Griess			
Griess	1009	893 (89%)[d]	71 (8%)[e]
Griess and assay	608	588 (97%)	67 (11%)
Griess and blood	333	322 (97%)	32 (10%)
Griess and HPLC	50	50 (100%)	11 (22%)
Griess and nitric oxide	833	832 (100%)	41 (5%)
Griess and nitrate	270	252 (93%)	49 (19%)
Griess and nitrite	583	535 (92%)	58 (11%)
Griess and plasma	136	134 (99%)	14 (10%)
Griess and reaction	565	533 (94%)	41 (8%)
Griess and reagent	164	144 (88%)	31 (22%)
Griess and serum	134	112 (84%)	18 (16%)
Griess and urine	57	40 (70%)	25 (63%)
		(92 ± 9%)	(17 ± 16%)
II. Greiss			
Greiss	245	121 (49%)	4 (3%)
Greiss and assay	95	75 (79%)	5 (7%)
Greiss and reaction	61	60 (99%)	3 (5%)
		(76 ± 25%)	(5 ± 2%)
III. Nitrite			
Nitrite and DAF[f]	10	10 (100%)	1 (10%)
Nitrite and DAN[g]	15	15 (100%)	9 (60%)
Nitrite and diaminofluorescein	16	16 (100%)	2 (13%)
Nitrite and fluorescence	317	121 (38%)	39 (32%)
Nitrite and fluorometry	179	119 (66%)	23 (19%)
Nitrite and spectrophotometry	694	177 (26%)	61 (34%)
		(86 ± 34%)	(28 ± 18%)
IV. Nitrate			
Nitrate and DAF	5	2 (40%)	1 (50%)
Nitrate and DAN	11	7 (64%)	4 (57%)
Nitrate and diaminofluorescein	5	5 (100%)	1 (20%)
Nitrate and fluorescence	392	47 (12%)	18 (38%)
Nitrate and fluorometry	171	33 (19%)	9 (27%)
Nitrate and spectrophotometry	694	97 (14%)	34 (35%)
		(42 ± 35%)	(38 ± 14%)
		(77 ± 31%)	(23 ± 18%)

[a] PubMed, Public Medline (http://www.ncbi.nlm.nih.gov/entrez/query). Period under review: 1950–2006.
[b] Greiss: in the Anglo-Saxon countries, the name *Griess* sometimes is written as *Greiss*.
[c] Articles related to diagnosis of urinary infection were considered unrelated to the L-Arg/NO pathway.
[d] Percentage of total.
[e] Percentage of "L-Arg/NO Pathway".
[f] DAF, Diaminofluorescein.
[g] DAN, Diaminonaphthalene.

cer", $n = 32$ for "rheumatism", $n = 25$ for "eye disease", $n = 24$ for "kidney diseases", $n = 15$ for "bacteriuria", $n = 7$ for "helicobacter", $n = 4$ for "schizophrenia", $n = 3$ for "Alzheimer disease", and each $n = 1$ for "Parkinson disease" and "erectile dysfunction", to name a few. The wide spectrum of diseases connected with the use of Griess assays and the high search rate obtained for cardiovascular and kidney diseases in particular emphasize which important role researchers attributed to the L-Arg/NO pathway in diseases in general and in NO-related endothelial dysfunctions in particular.

The application of the Griess reaction to the quantitative determination of nitrite and nitrate in human body fluids, notably blood and urine, in the frame of the L-Arg/NO pathway is the focus of attention of the next sections of this article. For a review of analytical methods beyond the Griess reaction-based assays for the measurement of nitrite and nitrate in body fluids and their clinical applications from the clinical biochemistry point of view, see the article by Ellis et al. [72].

3. Nitrite and nitrate concentrations in human biological fluids measured by assays based on the Griess reaction

3.1. General aspects

In 1996, Viinikka described in his review the state of art of the assessment of NO production on the basis of the measurement of nitrite and nitrate in human blood and urine [93]. Viinikka noticed that "The scientific literature on NO is extensive, but our knowledge of its (patho)physiological role in man is still fragmentary". On the basis of the observation that the mean concentrations of nitrite in the blood of healthy adults reported until 1996 varied from non-detected to 4.2 μM, and those of nitrate from 19.7 to 44 μM measured by various methodologies including those based on the Griess reaction, Viinika stated that "Problems concerning the measurement of NO production in vivo are one reason for this lack of knowledge". In his review article, Viinikka ascertained that "the technical measurement of nitrite/nitrate is obviously reliable, but there are problematic pre-analytical factors", with the most significant and not totally avoidable being the confounding effect of diet-derived nitrate. Viinikka concluded that "better methods for measuring the production of NO in vivo would be welcome" [93].

On the basis of the great variability of nitrite and nitrate concentrations in human plasma, serum and urine reported during the last 10 years [155], we must admit that we are confronted today largely with the same problem. Moreover, we must assess that not only the measurement of nitrite and nitrate is an analytical challenge, but further members of the L-Arg/NO pathway, notably the *S*-nitrosothiols and 3-nitrotyrosine [135,139,156], cause a lot of worry. In the area of *S*-nitrosothiol research, reported basal concentrations in healthy humans range from non-detectable (i.e. a few nM) to 10 μM, i.e. vary by almost four orders of magnitude [137,139]. Unlike nitrite and nitrate, the extraordinary great variability of the *S*-nitrosothiol concentrations cannot be attributed to dietary intake, but it is rather due to analytical and pre-analytical issues, notably readily artefactual formation of *S*-nitrosothiols – even under storage conditions in the cold [157,158] – and their chemical lability, as well as due to lack of specificity, selectivity, and sensitivity of the analytical detection methods.

The following discussion will, therefore, focus on analytical and pre-analytical issues as a potential source for the reported diverging nitrite and nitrate concentrations observed from the use of the Griess reaction – mostly performed with the combination of sulfanilamide (SAN) with *N*-(1-naphthyl)-ethylenediamine (NED) or with 2,3-diaminonaphthalene alone – in the analysis of human plasma, serum and urine rather than on the effect of diet. It is assumed that the problem of dietary nitrate and nitrite is well-recognized and appropriately considered and documented in clinical and methodological studies. In this discussion, basal concentrations obtained from the use of other methodologies including GC–MS as a reference approach will also be considered.

3.1.1. Factors influencing formation, stability and reaction fate of diazonium ions, intermediates and azo dyes in the Griess reaction

Kinetics and mechanism of the Griess reaction have been intensely studied and reported in 1979 by Fox [159], who investigated the formation of azo dyes from nitrite and various nitrosable substances and coupling reagents including SAN and NED in different conditions performed in 1.5% acetic acid. In these investigations, SAN and NED (and other Griess reagents) had been applied at concentrations regularly used at that time for the analysis of nitrite in aqueous phase, e.g. 1 and 0.2 mM, respectively [160], with nitrite concentration being fixed at 10 μM. The results of the study of Fox [159] showed that the recovery of the azo dye formed from the reaction of nitrite with SAN and NED is dependent upon a number of factors, namely: (1) relative concentration of SAN and NED (Fig. 4); (2) reaction of nitrite

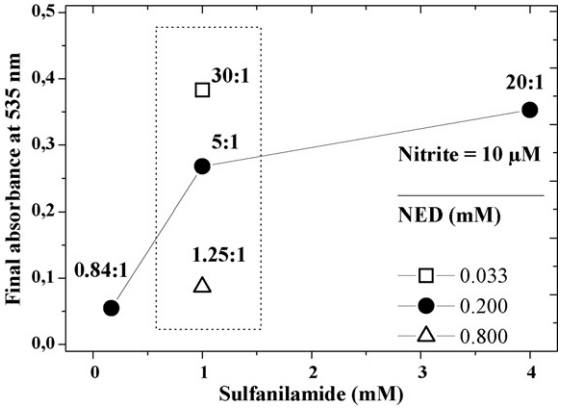

Fig. 4. Final absorbance at λ_{max} 535 nm of the azo dye formed from the reaction of nitrite (10 μM) in 1.5% acetic acid with sulfanilamide (SAN) and *N*-(1-naphthyl)ethylenediamine (NED) at varying concentrations and molar ratios. The Figure was constructed by using the data of Table 5 concerning the combination of SAN and NED reported by Fox [159]. The number above the symbols refers to the molar ratio of SAN to NED. The concentration of the reagents SAN (1 mM) and NED (0.033, 0.200 and 0.800 mM; within the dotted rectangle) had been normally used for the analysis of nitrite at that time [160].

Table 3
Composition of the Griess reagents sulfanilamide (SAN) and *N*-(1-naphthyl)ethylenediamine (NED) or its dihydrochloride salt (NEDD) used in selected literature reports in batch and automated Griess assays for the analysis of nitrite and/or nitrate in biological fluids

Griess reagents	SAN/NED	Assay	Reference
58 mM SAN/0.5 M H_3PO_4 + 3.9 mM NEDD/water	14.9	Automated	Green et al. [87]
100 mM SAN/1.5 M H_3PO_4 + 8 mM NED	12.5	Batch	Oudkerk Pool et al. [111]
5.8 mM SAN/250 mM H_3PO_4 + 0.5 mM NED	11.6	Batch	Moshage et al. [113]
58 mM SAN/0.5 M H_3PO_4 + 3.9 mM NEDD/0.5 M H_3PO_4	14.9	Automated/HPLC	Muscará and de Nucci [90]
29 mM SAN/1.34 mM NED/0.34 M HCl	21.6	Automated/HPLC	Yamada and Nabeshima [94]
232 mM SAN/10.8 mM NED/0.27 M HCl	21.5	Automated/FIA	Schulz et al. [162]
29 mM SAN/1.34 mM NED/0.34 M HCl	21.6	Automated/HPLC	Kurioka et al. [163]
29 mM SAN/1.34 mM NED/0.34 M HCl	21.6	Automated/HPLC	Ishibashi et al. [98]
116 mM SAN/1.35 M HCl + 3.9 mM NEDD/water	29.7	Batch	Miranda et al. [100]
58 mM SAN/3 M HCl + 5.4 mM NED/water	10.7	Batch	Sastry et al. [102]
348 mM SAN[a] + 10.8 mM NED	>32	Batch	Giustarini et al. [28]

[a] A saturated solution of SAN in ethanol and 0.6 M H_3PO_4 were used.

with the sulfonamide group of SAN, and reaction of nitrite with NED; (3) formation of more than one pigment; (4) oxidation of the diazonium ion intermediate; (5) oxidation of the azo dye; (6) reduction of the diazonium ion by cysteine (at 200 μM), NADH (at 10 μM) and ascorbate (at 25 μM); (7) formation of semistable nitroso-reductant intermediates; (8) pre-reaction of SAN with nitrite, if SAN and NED are not added simultaneously; and (9) pH and temperature, with optimum values being 2.5–3.5 and room temperature, respectively.

With the exception of acetic acid and consequently of the pH value at which the Griess reaction took place in the investigations of Fox [159], the other experimental conditions are comparable to those used in various batch and automated Griess assays for the quantitative determination of nitrite and nitrate in biological fluids of humans and animals (Table 3). We may, therefore, reasonably assume that most of the above mentioned factors will also influence nitrite analysis in biological fluids. The observations of Fox [159] may not only explain the previous findings of this group that Griess assays yielded different amounts of nitrite [161], but they may also explain diverging values for nitrite reported in the literature (see below). The kinetic and mechanistic studies of Fox suggest that measured nitrite is a function of both sample preparation and development of the colour, with interactions occurring between the two [159]. Even in "pure" aqueous phase, i.e. in a non-biological matrix, three physiological substances, i.e. cysteine, ascorbate and NADH, with the latter also being frequently used in nitrate determination by Griess assays, have been found to interfere with nitrite analysis [159]. The foregoing factors are not the entirety of exogenous and endogenous factors or reactions – nitrite itself is highly reactive in acidic solutions and nitrous acid may be converted into NO and nitrate – that may affect nitrite measurement.

Fox's investigations were restricted to aqueous solutions of nitrite. Thus, multiple interfering factors arising from the absolutely essential chemical or enzymatic reduction of nitrate as well as from the diversity and individuality of the biological matrices (see below), had not been considered at all in that study [159]. Nevertheless, the conclusions drawn by Fox in 1979 are still valid: "Only under identical conditions can inhomogenous multiple reactions give identical yields of a given product. In view of the many possible reactions involved in the Griess reaction, the importance of control of the reaction conditions is readily apparent." [159]. Thus far, the kinetics and mechanism of the Griess reaction have been investigated almost exclusively by spectrophotometry. The recent advances made in the LC–MS technology should allow investigate the Griess reaction and related diazotization reactions using different nitrosable and coupling components in aqueous phase.

Most of the conditions of the Griess reaction can be defined, controlled and optimized by the analyst both in batch and automated assays (Table 3). But conditions and factors dictated by the individuality of the biological matrix are hard to control. It is likely that the control of the conditions of the Griess reaction and the elimination of potentially interfering species from endogenous and exogenous sources is best accomplished by those automated methods, in which additional chromatographic separation steps are included, such as a column packed with a Dowex cation-exchange resign [87], or HPLC columns packed either with an anion-exchanger [90] or with a polystyrene polymer [94]. In the automated flow injection analysis (FIA)-Griess method, the nitrite-containing sample is subjected to the Griess reaction without any preceding chromatographic step. In this method, the Griess reagent mixture (e.g. 232 mM SAN + 10.8 mM NED in 0.27 M HCl [162]) is continuously pumped through the system by means of an HPLC pump at a constant flow rate. Considering a volume of about 44 μl for the reaction loop (90 cm × 0.25 mm i.d.) [162], it is calculated that the Griess reaction proceeds for only 2.6 s in such a FIA system. It has been reported that the Griess reaction is incomplete under such conditions [162]. Nevertheless, standardized conditions and a 500-fold higher sensitivity compared to the batch Griess assay have been reported to allow reliable determination of nitrite in biological fluids including blood [162].

3.1.2. Methods of quantification

In general, the method of quantification is one of the most crucial procedures in the whole analytical method. With the single exception of the mass spectrometry methodology that utilizes stable-isotope labelled analogs of the analytes to be quantitated, calculation of concentration of analytes by other analytical

approaches is based on the use of calibration curves. Because of the physiological occurrence of nitrite and nitrate in biological matrices, calibration curves for nitrite and nitrate have to be prepared in matrices different from the biological matrices, regularly in aqueous solution, mostly in "nitrite-free" distilled water or buffers used for dilution. This method of quantification is based on the assumption that endogenous and exogenous compounds and other factors present in the biological matrix do not affect at all the Griess reaction. Commonly, quantification of nitrate in Griess assays is also based on the use of calibration curves which are generated by using aqueous solutions of nitrite but not of nitrate. Therefore, absolute requirement for accurate calculation of nitrate concentrations at the basal state is knowledge of the exact reduction recovery rate.

Particularly in batch Griess assays, in which the interference by numerous known and unknown substances is presumably maximum, the reduction of nitrate to nitrite is mostly incomplete, with the reduction recovery rate being unknown, variable and rather low. This has been shown by various groups and assays, e.g. by HPLC with on-line post-column Cd reduction and Griess reaction (recovery, 26%) [90], and by GC–MS using ^{15}N-labelled nitrate as internal standard (recovery, 30–80%) [95]. In batch Griess assays, recovery rate of nitrate reduction by Cd has been shown to vary greatly even in diluted urine samples, with dilution factors of up to 1:100 (v/v) not completely eliminating interferences [95]. In theory, dilution factors higher than 100-fold could further suppress interference, but the final concentration of urinary nitrate could be of the same order of nitrate present in water or buffers used for dilution and could therefore lead to inaccurate concentrations [95].

Interestingly, automated Griess assays involving either a "clean-up" column [87] or a HPLC column for the separation of nitrite and nitrate [90,98,163], on-line Cd reduction column and post-column derivatization utilizing the Griess reaction have been reported uniformly to provide almost quantitative recovery of excretory and circulating nitrate (Table 1). This could be due to the use of authentic nitrate for calibration, especially in the HPLC-Griess method, so that the quantitative reduction recovery may become insignificant if the reduction step is reproducible.

3.1.3. External validation

Use of nitrite calibration curves to quantify nitrate in biological fluids by Griess assays may add considerably to the variability of the basal nitrate concentrations in biological fluids reported in the literature so far (see next sections). Incomplete reduction of nitrate to nitrite or inaccurate determination of the reduction recovery rate in the respective biological matrix will inevitably produce inaccurate results. Batch and automated Griess assays and other methods of analysis of nitrite and nitrate should be validated in addition by proven and fully validated methods independent of the Griess reaction. One of the most appropriate methodologies for external evaluation is the GC–MS technology, because of its inherent accuracy and unique characteristic of providing "absolute" values in biological fluids, notably of endogenous substances including nitrite and nitrate [87,95,164,165].

We compared a frequently used batch Griess assay utilizing SAN (37.5 mM), NED (12.5 mM) and 4 M HCl as the Griess reagents with a GC–MS method [166] by quantitating nitrate in 33 human urine samples after 1:10- and 1:100-dilution (v/v) with NH_4Cl buffer and reduction of nitrate by Cd powder under alkaline conditions (5 wt.% NH_4Cl buffer, pH 8.8) [95]. Nitrate concentrations in all urine samples subjected to this comparison were in a relevant concentration range (128–2298 μM as measured by GC–MS [166]). The results from the statistical reanalysis of the original data [95] by regression analysis and by the more reliable method of Bland and Altman [167] are shown in Fig. 5. Linear regression analysis between urinary nitrate concentrations measured by the Griess assays and by GC–MS showed moderate correlation, considerable variability and mean recovery rates of the order of 40% (slopes) in the Griess assay (Fig. 5A and B). Plotting of the data by the method of Bland and Altman revealed inacceptable great deviation and a systematic error in the Griess assay, which was pronounced in the 1:100-diluted urine samples (Fig. 5C and D). Similar results were also observed [24] by comparing a batch Griess assay according to Guevara et al. [119] with a GC–MS method [166] for plasma nitrate in the frame of a clinical study as measured in portions of the same plasma samples by two independent investigator groups [168].

3.2. Nitrite and nitrate in urine

Nitrite and nitrate concentrations have been determined by various analytical methods both in urine from spontaneous micturition and in urine collected over a certain period of time, such as over 24 h. In methodological papers nitrite and nitrate concentrations in urine are usually expressed in units of concentration (μM or mM). In clinical studies nitrite and nitrate concentrations in urine are corrected for creatinine excretion (e.g. μmol of nitrite or nitrate per mmol of creatinine; μmol/mmol), notably in spontaneous urine, or are expressed as an excretion rate (e.g. μmol of nitrite or nitrate per h or 24 h). Except for urine collected over 24 h, comparison of quantitative methods of analysis of nitrite and nitrate on the basis of urinary concentrations of these anions is only useful and meaningful when comparing data corrected for creatinine excretion and/or time of collection. Thus, nitrate concentration in urine of healthy adults typically ranges between 100 and 3000 μM, i.e. 30-fold, whereas creatinine- and collection time-corrected values usualy range between 50 and 150 μmol/mmol, and between 500 and 1200 μmol/24 h, i.e. about 3-fold, as reported by various methods including those based on the Griess reaction and GC–MS [27,155]. It should be emphasized that dietary intake of nitrate and nitrite may considerably contribute to circulating and excretory nitrate and nitrite. Thus, healthy young volunteers with uncontrolled nitrate diet excreted in average 110 μmol/mmol creatinine, whereas the same group under standardized low-nitrate diet excreted in average 49 μmol/mmol creatinine as measured by GC–MS [166]. Data obtained from the use of Griess assays usually represent the sum of nitrite and nitrate. In non-infected urine nitrate contributes by approximately 99% to the sum of nitrite and nitrate, so that the anion measured is largely nitrate.

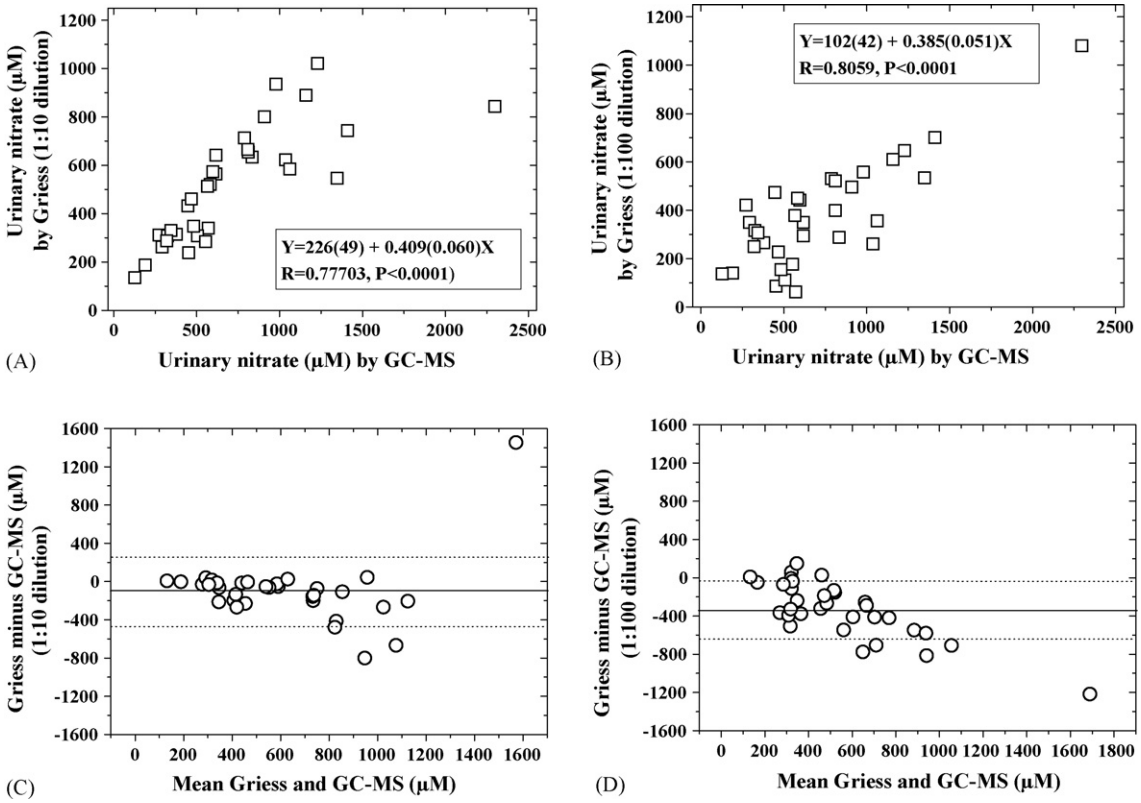

Fig. 5. Comparison of a common batch Griess assay (SAN, 37.5 mM; NED, 12.5 mM; 4 M HCl) with a GC–MS method [166] for nitrate in urine samples ($n = 33$) from healthy and ill humans. Urine samples were diluted (1:10 and 1:100, v/v) with 5 wt.% NH_4Cl buffer, pH 8.8, nitrate was reduced to nitrite by Cd powder [95], and nitrite was quantified in these samples by measuring absorbance at 540 nm and by GC–MS. (A) and (B), linear regression analysis between the nitrate concentration observed by the Griess assay and by GC–MS in urine samples diluted 10-fold (A) and 100-fold (B). (C) and (D), plotting according to the method of Bland and Altman [167] between the difference in the nitrate concentrations measured by the Griess assay and by GC–MS vs. the mean nitrate concentration measured by the methods in urine samples diluted 10-fold (C) and 100-fold (D). Horizontal lines show the mean difference (solid) and the ±1 S.D. range (dot). Data were taken from previous study [95].

3.2.1. Nitrite concentrations in urine

Except for bacteriuria [41] and in contrast to nitrate, quantitative data on urinary nitrite in physiological conditions from the application of Griess assays and other analytical methods are exceptionally rare [155]. The urinary nitrite concentrations of the order of 0.5 μmol/mmol creatinine or 6 μmol/24 h, which were provided by a GC–MS method [166] that allows for direct determination of nitrite, i.e. independently of nitrate, may serve as a reference point in the following discussion on urinary excretion of nitrite by healthy humans with uncontrolled nitrate diet. These data correspond to an approximate nitrite concentration of 2 μM in urine collected for 24 h. Moshage et al. reported nitrite concentration of 4 μM in urine of healthy humans, however, the batch Griess assay used (LOD, 1.5 μM in distilled water) had not been validated for urine samples [113]. In our hands, nitrite concentration in urine samples from healthy young volunteers of low-nitrate diet ranges between 200 and 1600 nM, with the corresponding nitrate concentrations ranging between 200 and 2200 μM as measured by GC–MS [24]. Thus, this range of concentration for urinary nitrite is below the range of LOD values of the majority of batch Griess assays. This and the obvious need to dilute urine samples in batch Griess assays may explain the rarity of urinary nitrite concentrations in physiological urine from humans. On the other hand, automated Griess assays, such as the HPLC-Griess assay have been reported to have considerably lower LOD values and to allow quantification of nitrite in human plasma at the basal state of the order of 100 nM [98]. In theory, the HPLC-Griess method should allow quantitative determination of nitrite in urine of healthy humans. Kurioka et al. have used the commercially available HPLC-Griess method to measure nitrite and nitrate in urine of healthy humans [163]. Injection of an aliquot (not explicitly stated by the authors) of urine samples diluted 1:19 in purified water and treated with an equal volume of ethanol into the HPLC-Griess system yielded creatinine-corrected nitrite concentrations ranging between 1.9 and 8.2 μmol/mmol for the subjects investigated [163]. These nitrite excretion rates correspond to nitrite concentrations of 14 ± 5 μM and are approximately 10–20 times higher than those measured by GC–MS [147,155,166]. The nitrite concentrations repoprted by Kurioka et al. [163] are not supported by the findings of Li et al. [169] who found by HPLC analysis and fluorescence detection using the DAN reagent that nitrite is present in urine of untreated rats at 682 ± 66 nM, nor by the findings of Sohn and Fiala [123] who could not measure nitrite in urine of untreated rats above 2000 nM, i.e. the LOD of the method. Interestingly, urinary nitrite excretion may increase up to 2-fold after oral administration for 5 days of organic nitrates, such as pentaerythrityl tetranitrate (PETN, 80 mg/day) or isosor-

bid dinitrate (ISDN, 30 mg/day) to healthy humans as measured by GC–MS [147].

3.2.2. Nitrate concentrations in urine

Since early in the 20th century, the origin of nitrate in the urine has been intensely investigated. Thus, in the study of Mitchell et al. [170], subjects received for a period of 220 days a diet consisting largely of foods containing low amounts of nitrate equivalent to a daily intake of about 250 μmol of NO_3^-. Volumetric analysis of urine nitrate after its conversion to NO resulted in an average daily excretion of about 800 μmol of NO_3^-, with marked individual differences (range 300–1400 μmol of NO_3^-) [170]. This order of excretion rate of nitrate in healthy humans has been confirmed almost 80 years later by methods based on HPLC [27] and GC–MS [166]. The investigations of Mitchell et al. showed that humans consistently excreted more nitrate than they ingested, in many cases from 3 to 6 times the amount taken by food [170]. By means of the automated Griess assay [87] as well as by GC–MS, Green et al. found that under conditions of constant low ingestion of nitrate (<180 μmol/day) the amount of nitrate excreted in urine (range 690–980 μmol/day) was an average of 4-fold greater than the amount ingested [171]. Mitchell et al. [170] and Green et al. [171] concluded that nitrate in human urine is not derived entirely from nitrate in the food, but is endogenously synthesized by a relatively constant rate and independently of nitrate ingestion, with the variability in urinary excretion being due to differences in the intensity of nitrate production [170]. Interestingly, Mitchell et al. reported that in their study no nitrite had been detected in fresh urine [170].

In consideration of the relatively great variation in the nitrate concentrations in urine, especially when measured in spontaneous urine and expressed in absolute concentrations (i.e. μM or mM), a reliable appraisal of methods of analysis of nitrate in urine and a comparison among each other on the basis of measured and reported concentration values is rather meaningless, except external evaluation is performed by alternative methods (Fig. 5). Nevertheless, reported urinary nitrate concentrations obtained from the use of satisfactorily validated Griess assays and some other assays are summarized in Table 4.

3.3. Nitrite and nitrate in plasma, serum and whole blood

3.3.1. Nitrite and nitrate concentrations in plasma, serum and whole blood in humans at the basal state

Tables 5 and 6 summarize reported concentrations of nitrite and nitrate in the circulation of healthy humans and untreated animals as measured by batch and automated Griess assays. Nitrite and nitrate have been commonly measured in plasma or serum and rarely in whole blood. The most frequently used sample treatment procedure was deproteination by means of chemicals, such as $ZnSO_4$/NaOH, acids or ultrafiltration. The reported LOD values range between 2 and 10 nM in automated FIA methods [152,162] and HPLC using DAN [169], and 50 nM [28] to 1.5 μM [113] mostly in batch Griess assays. Nitrite concentration in the circulation ranges from non-detected or 0.1 to 13 μM (Table 5), whereas concentration of circulating nitrate ranges from non-detected or 4 to 108 μM (Table 6). Great differences are found for circulating nitrite even when apparently the same methodology was used, e.g. between 0.22 μM [28] and 9 μM in batch assays, or between 0.1 μM and up to 20 μM in automated Griess assays, with the majority of them having yielded clearly lower concentrations for plasma of serum nitrite than batch assays. Measurement of circulating nitrite and nitrate by analytical approaches other than Griess assays also yielded similar ranges and variability even when the same methodology has been applied [72,93,155]. Typically, the GC–MS methodology provides values of about 0.5 μM for nitrite and 42 μM for nitrate in serum [147], and 1.8 μM (range 1.1–3.3) for nitrite and 38 μM (20–68) for nitrate in plasma of healthy young volunteers on standardized low-nitrate diet [166]. Thus, independently of the

Table 4
Summary of reported urinary basal concentrations of nitrate in healthy humans and untreated animals measured by assays based on the Griess reaction and by other selected methodologies

Reference	Species	Sample treatment	Assay	Nitrate
Mitchell et al. [170]	Human	$FeCl_3$/HCl	Volumetry	300–1400 μmol/day
Radomski et al. [172]	Human	Nitration	Colorimetry	768 ± 279 μM
Green et al. [171]	Human	HCl/Dilution (1:20)	Griess (automated; Cd column)	690–980 μmol/day
Green et al. [87]	Human	Dilution (1:20)	Griess (automated; Cd column)	250–2000 μM
Phizackerley and Al-Dabbagh [109]	Human	$ZnSO_4$/NaOH	Griess (batch; enzymatic)	188–1098 μM
Hibbs et al. [12]	Human	Dilution (1:10)	Griess (batch; enzymatic)	690 μmol/day
Kanno et al. [36]	Human	Dilution	Griess (automated; Cd column)	124 μmol/mmol creatinine
Gilliam et al. [110]	Human	Dilution (1:40)	NADPH oxidation	1111 ± 223 μM
Wennmalm et al. [27]	Human	SPE; Dilution	HPLC-UV	470–1130 μM
Tsikas et al. [166]	Human	Cd reduction	GC–MS	49–109 μmol/mmol creatinine
Bories and Bories [112]	Human	Dilution (?)	NADPH oxidation	480–3000 μM
Moshage et al. [113]	Human	Dilution (?)	Griess (batch; enzymatic)	990 μM
Guevara et al. [119]	Human	Dilution (1:50)	Griess (batch; enzymatic)	370–2520 μM
Kurioka et al. [163]	Human	Dilution (1:19)	Griess (HPLC; Cd column)	122–335 μmol/mmol creatinine
Li et al. [169]	Rat	Reductase	HPLC (DAN reagent)	605 ± 69 μM
Connoly et al. [173]	Human	Dilution (1:20)	HPLC-UV	2270–4180 μM
Golikov and Nikolaeva [107]	Human	NaOH/$ZnSO_4$	Griess (batch; Cd granules)	51 ± 5 μmol/mmol creatinine
Tsikas [165]	Human	Dilution (1:4)	HPLC-UV	300–1800 μM

SPE, solid-phase extraction.

Table 5
Summary of reported circulating basal concentrations of nitrite in humans and other species by assays based on the Griess reaction and by some selected methodologies

Reference	Species	Matrix	Sample treatment	Assay	Nitrite (μM)	LOD
Rath and Krantz [174]	Human	Blood	$ZnSO_4$/NaOH	Batch	2.1 ± 1.4	N.R.
Green et al. [87]	Human	Plasma	SSA	Automated	N.D.	1 μM
Phizackerley and Al-Dabbagh [109]	Human	Serum	N.R.	Batch	3.3 ± 2.1	N.R.
Moshage et al. [113]	Human	Plasma	Dilution; $ZnSO_4$	Batch	1.3–13	1.5 μM
Muscará and de Nucci [90]	Rat	Serum	Ultrafiltration	Automated	N.R.	75 nM
Dembny et al. [120]	Human	Plasma	Ultrafiltration	Batch	≈9	N.R.
Schulz et al. [162]	Human	Blood	NaOH; Ultrafiltration	Automated (FIA)	0.1–0.4	2 nM
Ishibashi et al. [98]	Human	Plasma	Methanol (1:1)	Automated (HPLC); Cd	0.2 ± 0.1	0.1 μM
Li et al. [169]	Rat	Plasma	Ultrafiltration	Automated (HPLC)	0.7 ± 0.1	10 nM
Miranda et al. [100]	Human	Serum	Ultrafiltration	Batch; VCl_3	4.9 ± 1.2	0.5 μM
Sastry et al. [102]	Human	Serum	NaOH/$ZnSO_4$	Batch; Cu-Cd alloy	3.7 ± 0.6	1.0 μM
Kleinbongard et al. [152]	Human	Plasma	Dilution/ultrafiltr.	Automated (FIA)	0.2 ± 0.02	10 nM
Pinto et al. [129]	Human	Serum	Ultrafiltration	Automated; enzym.	5–20	0.7 μM
Giustarini et al. [28]	Human	Plasma	NEM; TCA	Batch	0.22 ± 0.07	50 nM

N.D., not determined; N.R., not reported; SSA, sulfosalicylic acid.

methodology used, concentration of circulating and excretory nitrite (see Section 3.2.1) are almost of the same order, whereas circulating nitrate concentrations are several times lower than urinary concentrations (Table 4), regularly 1–2 orders of magnitude smaller.

3.3.2. *Identification of pre-analytical factors influencing quantitative analysis*

Unlike in urine, the Griess reaction cannot at all be performed in whole or diluted blood. Quantitative determination of nitrite and nitrate in whole blood, plasma or serum requires a series of analytical procedures to eliminate interfering blood constituents, notably haemoglobin and plasma proteins. Pioneering work on the blood-nitrite content has been done in the thirties and forties of the 20th century. Because of the instability of nitrite in acidic media, Rath and Krantz [174] prepared a clear, protein-free blood filtrate by centrifuging whole blood (10 ml) after treatment first with 4.5 wt.% $ZnSO_4$ and then by 1 M NaOH in a proportion of 1:2.5:1.5 (v/v), which was subjected to the Griess reaction performed with sulfanilic acid and α-naphthylamine in acetic acid solution. A study of a series of 170 human subjects (9–91 years of age, mean 51 years) hospitalized for traumatic injuries and operative treatment revealed physiological occurrence of nitrite in venous blood at 2.05 ± 1.36 μM (mean ± S.D.) within the range of 0.11 to 6.3 μM. Similar mean blood nitrite concentrations were also measured in dogs (2.07 μM, $n = 69$), steers (2.04 μM, $n = 5$), and monkeys (2.39 μM, $n = 14$) [174]. Rath and Krantz found that 32 aged subjects (average age, 73.5 years) had clearly lower blood nitrite concentrations, i.e. 1.27 ± 0.43 μM, with no correlation existing between arterial pressure and nitrite concentration in blood. The blood nitrite concentrations measured by Rath and Krantz using the batch Griess reaction are almost 10 times higher than those reported recently by Dejam et al. [175] for blood nitrite in young healthy volunteers measured by reductive chemiluminescence (see below).

In their article, Rath and Krantz pointed out that "During all analytical procedures scrupulous caution must be exercised to avoid contamination of the sample with extraneous nitrite from air, water and glassware." [174]. Rath and Krantz recommended not to use any anticoagulant if immediately after blood is withdrawn it is placed in the flask containing the $ZnSO_4$ solution, and not to use higher dilution of the blood, "since the nitrite ion is present in extremely minute amounts in normal blood" [174]. Thus, already several decades ago the most important pre-analytical factors had been recognized as potential source of interference in the quantitative determination of nitrite in blood

Table 6
Summary of reported circulating basal concentrations of nitrate in humans and other species by assays based on the Griess reaction and by some selected methodologies

Reference	Species	Matrix	Sample treatment	Assay	Nitrate (μM)[a]	LOD
Green et al. [87]	Human	Plasma	SSA	Automated; Cd column	15–60	1 μM
Phizackerley and Al-Dabbagh [109]	Human	Serum	N.R.	Batch; enzymatic	34 ± 20	N.R.
Oudjerk Pool et al. [111]	Human	Serum	Dilution (1:6)	Batch; ezymatic	13–108	0.4 μM
Pratt et al. [88]	Dog	Serum	SSA	Automated (FIA); Cd	6–14	25 nM
Bories and Bories [112]	Human	Serum	Dilution (1:5)	NADPH oxidation	0–42	5 μM
Moshage et al. [113]	Human	Plasma	Dilution; $ZnSO_4$	Batch; enzymatic	4–45	1.5 μM
Guevara et al. [119]	Human	Serum	Methanol/ether	Batch; enzymatic	35 ± 10	N.R.
Dembny et al. [120]	Human	Plasma	Ultrafiltration	Batch; enzymatic	≈49	N.R.
Ishibashi et al. [98]	Human	Plasma	Methanol (1:1)	Automated (HPLC); Cd	27 ± 5	0.1 μM
Miranda et al. [100]	Human	Serum	Ultrafiltration	Batch; VCl_3	38 ± 8	0.5 μM
Sastry et al. [102]	Human	Serum	NaOH/$ZnSO_4$	Batch; Cu–Cd alloy	44 ± 4	1.0 μM
Pinto et al. [129]	Human	Serum	Ultrafiltration	Automated; enzymatic	14–52	2.3 μM

[a] Usually, the sum of nitrite + nitrate is reported. SSA, sulfosalicylic acid; dilution, v/v.

by the Griess assay: (1) blood volume; (2) proteins; (3) contamination; and (4) anticoagulation. We must ascertain whether the majority of the currently available analytical methods of nitrite and nitrate are susceptible to these basic pre-analytical factors. Thus, the importance of control of the sample treatment conditions and the associated phenomena is readily apparent. Minimizing of interferences and sample contamination are decisive in the accurate quantitative analysis of nitrite and nitrate in blood, plasma and serum.

3.3.3. Discussion of the effects of pre-analytical, analytical and other factors on the quantitative analysis of circulating nitrite and nitrate

Quantitative determination of nitrite in whole blood necessitates immediate processing after blood is withdrawn from a blood vessel in order to prevent oxidation of nitrite to nitrate. The need for immediate sample treatment could be one reason for the very rare measurement of nitrite and nitrate in whole blood.

Oxidation of nitrite in blood can be prevented by alkali alone [162] or in combination with $ZnSO_4$ for protein precipitation [174], or by other means, such as by ferricyanide [103,175]. An automated Griess assay (FIA), that involved treatment of blood (1.5 ml) with 0.1 M NaOH and subsequent neutralization with 1 M phosphoric acid, and protein separation by ultrafiltration [162], as well as a reductive chemiluminescence-based method that involved treatment of human whole blood with ferricyanide [175], resulted in blood nitrite concentrations ranging between 100 and 400 nM [162] and 176 ± 17 nM [175], which are about 10 times smaller than those measured by Rath and Krantz [174]. Furthermore, the chemiluminescence method revealed higher nitrite concentrations in erythrocytes (288 ± 47 nM) as compared to plasma (121 ± 9 nM) suggesting that erythrocytes are the major intravascular storage sites of nitrite in human blood [175]. This apparent accumulation of nitrite in human erythrocytes seems to collaborate with observations of Recchia et al. [176], who found that in dogs the ratio of the sum of nitrite + nitrate in the erythrocytes to that in plasma ranges between about 4:1 and 15:1, with decreasing plasma CO_2 pressure and bicarbonate concentration decreasing this ratio, i.e. theoretically shifting it to a ratio of 1:1 for bicarbonate-free plasma. However, the findings of Dejam et al. [175] and Recchia et al. [176] are contradictory to those of Parks et al. [177], who demonstrated that radioactive ^{13}N-labelled nitrite and nitrate are distributed uniformly on a per-volume basis between plasma and blood cells in vivo in mice and rabbits.

Uneven distribution of nitrite and nitrate between blood plasma and red blood cells, bicarbonate-dependent distribution of nitrite and nitrate in blood compartments, and possibly further, yet unknown factors that may potentially influence nitrite and nitrate transport across the red blood cell membrane [79,178] could have two consequencies: (1) they could explain, at least in part, the greatly diverging nitrite and nitrate concentrations in plasma and serum reported so far (Tables 5 and 6); and (2) they could be strong arguments in favour of measuring nitrite and nitrate in whole blood rather than in plasma or serum. This insufficiently investigated and contested issue should be further addressed in forthcoming investigations.

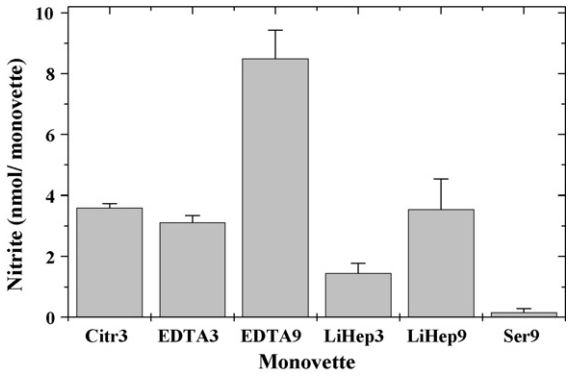

Fig. 6. Nitrite content of various commercially available monovettes (Sarstedt, Germany) as measured by GC–MS. Values are taken from ref. [30] and are presented as mean ± S.E., $n = 5$ each. Abbreviations: Citr, citrate; LiHep, lithium heparin; Ser, serum; 3 and 9, monovette volume of 3 and 9 ml.

3.3.3.1. Blood, serum or plasma? The role of anticoagulants. The effects of commercially available monovettes commonly used to generate plasma or serum from blood on nitrite and nitrate concentrations have been investigated by several groups. Most frequently used anticoagulanting agents include EDTA, citrate and heparin which are placed within monovettes. The blood volume that can be withdrawn with such monovettes regularly amounts to 3 or 9 ml. Commercially available monovettes have been shown to contain considerable and varying amounts of nitrite and nitrate [30,95,155]. Although all monovettes were found to contain considerably higher amounts of nitrate (2–17 nmol) than nitrite (0.17–8.5 nmol, Fig. 6), the relative contribution of the monovettes to blood nitrite is several times higher than to nitrate. The lowest nitrite content was measured in serum mononovettes followed by monovettes containing heparin, EDTA and citrate. It can be calculated that when such monovettes would be filled with blood the nitrite present as a contamination in the monovettes would contribute to endogenous nitrite by 0.019 μM in serum monovettes, 0.3–0.4 μM in heparin monovettes, 0.94–1.04 μM in EDTA monovettes, and even 1.2 μM in citrate monovettes. Except for serum monovettes, the contribution of the other monovettes to endogenous nitrite may reach and even exceed the endogenous nitrite concentration in dependence upon the blood volume drawn. Indeed, the nitrite concentration in serum and plasma from blood of a healthy volunteer has been determined to be 0.796 ± 0.086 μM in serum (9-ml monovettes), 1.167 ± 0.086 μM in EDTA plasma (3-ml monovettes) and 1.66 ± 0.044 μM in citrated plasma (3-ml monovettes) [155]. On the assumption that exogenous nitrite is evenly distributed between plasma and erythrocytes consideration of the monovette-derived nitrite leads to corrected mean nitrite values of 0.78, 0.82 and 1.26 μM, respectively. Whether commonly used anticoagulants may influence distribution of nitrite and nitrate in blood, is at present unknown. Measurement of nitrite in serum may be associated with the lowest artefactual contribution. However, precautions should be taken to prevent oxidation

of nitrite to nitrate during serum generation. It should be pointed out that reported serum nitrite concentrations are not generally lower than plasma concentrations, independently of the assay used (see Table 5 and ref. [155]), suggesting that aditional experimental parameters may influence serum nitrite concentrations. Thus, in healthy humans at the basal state mean nitrite concentrations have been reported to be 4.9 μM [100] and 3.7 μM [102] in serum, but only 0.22 μM in plasma [28] as measured by batch Griess assays. Frequently, the procedures used to draw blood and generate plasma and serum samples are inadequately reported, and the influence of this important pre-analytical step on the reported nitrite and nitrate concentrations cannot be reliably evaluated.

Besides contribution to endogenous nitrite, anticoagulants have been reported to interfere with the analysis of nitrate in plasma by alternative mechanisms. Dembny et al. reported that heparin (at 14.3 U/ml) but not EDTA (at 3.8 mM) interfered with the determination of nitrate but not of nitrite in rat and human plasma, with the inteference being based on the inhibition of nitrate reductase-catalyzed reduction of nitrate to nitrite as observed by using a batch Griess assay [120]. However, this method has not been sufficiently characterized and the plasma nitrite concentrations of about 9 μM are among the highest measured in rat and humans so far (Table 5). Ricart-Jané et al. investigated the effects of anticoagulants and other pre-analytical factors in the quantification of nitrate in plasma by a commercially available batch Griess assay involving nitrate-reductase catalyzed reduction of nitrate [128]. Although this work neither reported on a final, interference-free and validated method nor reported on basal concentrations of nitrite and nitrate in rat or human plasma, it showed that numerous parameters may interfere and affect quantitative analysis of nitrite and nitrate by the batch Griess assay by largely unresolved mechanisms [127]. In accordance with Ricart-Jané and colleagues these parameters may include: (i) kind of anticoagulating agent; (ii) ultrafiltration of plasma; (iii) stability and amount of nitrate-reductase to be added to plasma; (iv) calibration by nitrate instead of nitrite; (v) and subtraction of the "background" absorbance at 540 nm in case of using plasma without ultrafiltration [127].

3.3.3.2. Sample deproteination by protein precipitation, ultrafiltration or microdialysis. In the absence of any other materials formation of azo dyes via the Griess reaction proceeds in aqueous solutions of nitrite rapidly and is almost quantitative. However, the presence of even a single additional compound, such as ascorbate, cysteine or NADPH, may significantly affect quantification of nitrite [159]. The more complex the nitrite-containing matrix is the higher is the probability for interferences for nitrite in the Griess reaction by mechanisms related and unrelated to the diazotization reaction itself. Thus, the requirement for an additional step in the analysis of nitrate, e.g. batch nitrate reduction by Cd, may enormously enhance the susceptibility for interferences [86]. Potential, mechanism-based interferences may become significant in any of the various steps of the diazotization reaction as discussed by Fox [159]. In protein-containing matrices, such as plasma, serum or cultured cells, proteins may considerably interfere with the quantitative determination of nitrite and nitrate when measured by the Griess reaction. Interfering proteins may "positively" contribute to nitrite due to intrinsic absorbance around 540 nm (e.g. by haemoglobin, myoglobin, NOS [50,179]). Protein-dependent interference may be "negative" due to reaction of nitrite with reactive moieties of proteins, notably tyrosine and cysteine, with nitrite nitrosating and nitrating proteins under the acidic conditions required for the Griess reaction [95,156]. A major interference seems to be haemoglobin, which at only 10 μM has been shown to completely abolish the diazotization of nitrite with DAN [52]. Eventually, the incompatibility of proteins with assays involving HPLC is readily apparent.

Simple dilution of the protein-rich matrix has also been performed, but it is unlikely that it eliminated protein-related interferences. Moreover, in particular batch Griess assays may become inapplicable to nitrite in diluted plasma or serum samples, if sensitivity is insufficient. Therefore, proteins are most frequently eliminated either by precipitation using organic or inorganic chemicals, or by ultrafiltration using commercially available cartridges of various cut-off values, such as 10 or 20 kDa [46,52,100,120,121,125,127,152,162,169]. It is worth mentioning that measurement of NO formation in vivo is frequently performed by combining the microdialysis technique with the Griess reaction [94,126,180–183]. Ultrafiltration devices with cut-off values of 3, 5, 10, 12, and 30 kDa are commercially available from many suppliers. The majority of such ultrafiltration cartridges have been shown to be contaminated with nitrite and nitrate. Thus, analysis by ion-exchange HPLC with UV absorbance detection at 214 nm on the third wash of the tested devices with water (0.5–3.5 ml) revealed presence of nitrite and nitrate from "not detectable" (LOD, each 3 pmol) to 0.7 μM nitrite and 13.6 μM nitrate [184]. This HPLC method yielded mean plasma nitrite and nitrate concentrations in healthy humans of 3.5 and 47 μM, respectively [184]. Contamination of ultrafiltration devices with nitrite and nitrate may well explain "unexpected" findings showing up to 205% higher nitrite and nitrate concentrations in ultrafiltered as compared to non-ultrafiltered plasma samples [128]. Inorganic chemicals used for deproteination include $ZnSO_4$/NaOH [102,174] and $HgCl_2$/Na_2CO_3 [84]. Organic chemicals used for protein precipitation include acids, such as trichloroacetic acid (TCA) [28,84] and sulfosalicylic acid [87,88], and organic solvents, such as methanol alone [98] or in combination with diethylether [119]. It should be noted that not only inorganic acids, such as HCl [95], but also strong organic acids, such as TCA may cause significant loss (of about 85%) of nitrite in plasma under conditions used for protein precipitation apparently through nitration of proteins [84]. While use of thiol-specific agents, such as *N*-ethylmaleimide may prevent loss of nitrite through *S*-nitrosylation of thiols, C- and N-nitrosation/nitration of proteins in acidified samples is difficult to avoid.

3.4. Selected applications of Griess reaction-based assays in clinical and animal studies

In view of the large number of applications of Griess reaction-based assays in experimental and clinical studies, here a few

selected articles will be mentioned and discussed. Two basic criteria for the selection are a close connection to the L-Arg/NO pathway and a satisfactory description and analytical characterization of the applied Griess assays. It is expressly pointed out that the following description is not nearly complete.

Rath and Krantz [174] found by a batch Griess assays that aged subjects (average age, 74 years) had clearly lower blood nitrite concentrations than younger subjects, i.e. 1207 ± 430 nM versus 2050 ± 1360 μM (mean ± S.D.), with no correlation existing between arterial pressure and nitrite concentration in blood. Recently, Kleinbongard et al. [185] found by an automated FIA Griess assay that plasma nitrite concentrations decreased with increasing age, i.e. 284 ± 26 nM in 12 young healthy volunteers (average age, 30 years) versus 148 ± 27 nM (mean ± S.D.) in 12 older patients (average age, 63 years) suffering from endothelial dysfunction, while plasma nitrate concentrations did not differ among the groups, i.e. 26 ± 3 μM versus 22 ± 4 μM (mean ± S.E.). In a step-wise linear regression model mean arterial pressure was identified by Kleinbongard et al. [185] as exerting the greatest influence on plasma nitrite concentration. The basal plasma nitrite concentrations measured by Kleinbongard et al. [185] are about 4 times lower than those measured by Rath and Krantz in blood [174]. Considering an almost uniform distribution of nitrite between plasma and erythrocytes [175,177], the great discrepancy regarding the basal concentrations reported by these groups are presumably due to methodological issues despite the clearly differing populations investigated, unlike the findings concerning arterial pressure and age. In contrast to Kleinbongard et al. [185], Jedličková et al. [186] found by HPLC and ECD that 32 healthy young volunteers (20–40 years of age) and 26 old patients (70–95 years of age) suffering from cardiovascular diseases had very similar plasma (heparin as anticoagulans) nitrite concentrations, i.e. 1317 ± 840 nM versus 1372 ± 914 nM (mean ± S.D.), but clearly different plasma nitrate concentrations, i.e. 19.2 ± 6.4 μM versus 45.4 ± 10.9 μM (mean ± S.D.).

Application of a batch Griess assay involving enzymatic reduction of nitrate to nitrite by *E. coli* reductase in 1:10-diluted urine and in ultrafiltrate from 1:1-diluted serum of healthy volunteers and in non-treated patients with malignant melanoma (MM) or renal cell carcinoma (RCC) revealed similar 24-h urinary output (690, 600, and 521 μmol/day, respectively) and serum nitrite + nitrate concentrations (32, 32, and 36 μM, respectively) [12]. Interleukin-2 administration to the patients caused in that study a striking increase in NO generation as reflected by 24-h urinary nitrite + nitrate excretion (6.5-fold in RCC and 9-fold in MM patients) and by serum nitrite + nitrate concentrations (10-fold in RCC and 8-fold in MM patients). Additional metabolic tracer studies using L-[guanidino-$^{15}N_2$]arginine and GC–MS analysis revealed elevation in [^{15}N]nitrate concentrations both in urine and in serum [12].

Oudkerk Pool et al. [111] investigated NO synthesis in ulcerative colitis and Crohn's disease by measuring nitrite + nitrate concentrations in serum by means of a batch Griess assay after serum dilution (1:6, v/v) with phosphate buffer, enzymatic conversion of nitrate to nitrite with the use of nitrate reductase from *Aspergillus* and oxidation of excess NADPH by phenazine methosulfate. Oudkerk Pool et al. [111] found that median serum nitrite + nitrate concentrations did not differ statistically significantly between ulcerative collitis (34.2 μM, range 15.6–229.4 μM, $n = 75$), Crohn's disease (32.3 μM, range 13.2–143.2 μM, $n = 71$) and healthy controls (28.7 μM, range 13.0–108.4 μM, $n = 33$).

Yamada and Nabeshima [94] applied a HPLC-Griess assay and an in vivo brain microdialysis technique to measure simultaneously nitrite and nitrate concentrations in the cerebellum of conscious rats. The concentrations of both nitrite and nitrate decreased by intraperitoneal injection of the synthetic NOS inhibitor N^G-nitro-L-arginine methyl ester, while administration of L-arginine increased nitrite and nitrate concentrations and diminished the reduction of their concentrations caused by N^G-nitro-L-arginine methyl ester [94].

Rao et al. [121] used a batch DAN-based fluorometric assay to measure nitrite + nitrate in brain tissue of rats before and after traumatic brain injury and cerebral ischemia. Nitrate was reduced to nitrite in ultrafiltered (10 kDa, cut-off) cortical and hippocampal extracts as well as plasma by nitrate reductase in the presence of an NADPH-regenerating system. Both traumatic brain injury and cerebral ischemia were found by the method to elevate nitrite + nitrate concentrations in tissue and in plasma.

An HPLC-Griess assay was used by Ishibashi et al. [98] to measure nitrite and nitrate differences in human coronary circulation with severe atherosclerotic stenosis. Nitrite and nitrate were measured in methanol-deproteinated plasma (1:1, v/v) generated from arterial blood taken at ostium of left coronary artery and from venous blood taken at coronary sinus. The authors found no difference for nitrite but a negative nitrate difference (i.e. nitrate concentration was in venous blood smaller than in arterial blood) in patients with coronary artery disease (CAD) as compared with patients without CAD [98]. Using the same HPLC-Griess method the same group reported later [187] that they found no nitrate difference (34.3 μM versus 34.4 μM) but a positive nitrite difference (140 nM versus 100 nM) between arterial and venous blood taken from aorta and right atrium, respectively, in patients with or without ischemic heart disease.

By measuring plasma nitrite by a FIA Griess assay, the HPLC-Griess method and reductive chemiluminescence Kleinbongard et al. [35,152] investigated in vivo in humans and animals changes of nitrite concentrations upon administration of acetylcholin or NOS inhibitors. Thus, intrarterial infusion of acetylcholin (10 μg/min) was found to increase plasma nitrite from 327 ± 39 to 593 ± 112 nM in humans, while the NOS inhibitor N^G-monomethyl-L-arginine (12 μmol/min) was found to decrease venous plasma nitrite concentration from 315 ± 57 to 136 ± 21 nM [152]. Kleinbongard et al. suggested that plasma nitrite reflects constitutive vascular NOS activity across mammalian species [35].

Interestingly, the Griess assay has also been applied in ophthalmic research. Okuno et al. [188] investigated NO synthesis in the optic nerve head in vivo using microdialysis and the HPLC-Griess assay. A concentric microdialysis probe was inserted into the optic nerve head of a rabbit and was perfused with Ringer's solution. The perfusate dialysate was collected, and nitrite and

nitrate concentrations in the dialysate samples were measured by the HPLC-Griess assay simultaneously. Basal nitrite and nitrate concentrations in the dialysate were determined to be 80 ± 10 and 3959 ± 500 nM, respectively [188]. These concentrations were reduced by intravenously administered N^G-nitro-L-arginine methyl ester and restorted by L-arginine. Intravitreally administered endothelin-1 (ET-1) was found to significantly elevate the concentrations of nitrate, which was inhibited by pretreatment with N^G-nitro-L-arginine methyl ester. Okuno et al. [188] concluded that NO synthesis in the optic nerve head is closely connected with the ET-1 signaling pathway.

Eventually, the use of a commercially available nitrite/nitrate Griess assay kit involving nitrate reductase-catalyzed conversion of nitrate to nitrite in citrated venous plasma should be mentioned and discussed. Elherik et al. [189] used this Griess assay to investigate circadian variation of NO synthesis in young healthy volunteers. This investigator group observed a significant circadian variation in plasma ET-1 concentrations, but only a borderline trend for a circadian variation in nitrite + nitrate plasma concentrations, with higher concentrations at 20.00 h (15.6 ± 8.4 μM), and lower concentrations at 04:00 h (10.9 ± 4.7 μM) [189]. By contrast, quantification of urinary nitrate by GC–MS revealed a statistically significant circadian rhythm of NO synthesis both in healthy and diseased subjects, with the maximum and minimum excretion rates in the urine samples collected from 17:00 to 20:00 h, and 05:00 to 08:00 h, and a 24-h amplitude of approximately 25% [38,155].

4. Concluding remarks and future prospects

The reaction of nitrite with sulfanilic acid and α-naphthylamine under acidic conditions has been discovered by Griess in the 19th century as a method of preparation of pigments, the azo dyes, and has been proposed by him as a method of analysis of nitrite. First urologists, then cancer researchers and more recently investigators from various disciplines are the most frequent users of analytical methods based on the Griess reaction and related diazotization reactions. There is no doubt that assays based on the Griess reaction are the most popular analytical methods for the analysis of nitrite and nitrate in the NO area of research, both in experimental and in clinical studies. The diazotization reaction is usually performed in batch, e.g. in a cuvette and on microplates. In modern assays the Griess reaction is increasingly applied in FIA systems as well as in HPLC systems for post-column derivatization and visible absorbance detection using a mixture of sulfanilamide and NED in HCl solution or for pre-column derivatization with fluorescence detection using the DAN reagent. Incorporation of chemical reductors in the HPLC-Griess and other automated assays based on the diazotization reaction allow for the simultaneous detection of nitrite and nitrate. Today, both batch and automated Griess assays are commercially available and increasingly used in vitro and in vivo studies.

Despite considerable advance in recent years, accurate quantitative determination of nitrite and nitrate in complex biological fluids, such as blood, plasma and urine by assays based on the Griess reaction is still a challenging analytical task. The greatly differing basal concentrations of nitrite in plasma and urine of humans are a firm evidence for the existence of unresolved analytical problems. In all fairness, we must admit that nitrite and nitrate assays based on distinctly different methodologies are also associated with analytical difficulties. Many of the common methodological problems arise from pre-analytical factors including generation and treatment of the biological matrix to be analyzed and from contamination by ubiquitous nitrite and nitrate. The method of quantification remains an inherently crucial analytical shortcoming in assays based on the Griess reaction. Therefore, validation of Griess assays by methodologies based on different principles is essentially important. In addition, one important instrument to monitor the overall reliability of the Griess assays used in clinical studies is the use of a quality control (QC) system. In practice, however, analysis of QC samples in parallel to study samples is rather very rare. Therefore, establishment of QC systems and co-processing of QC samples for nitrite and nitrate in clinical studies is absolutely essential.

There is a general belief that Griess assays are fundamentally reliable for accurate quantitative measurement of nitrite and nitrate in biological samples. In particular, the commercial availability of "ready-to-use" Griess assays is very tempting, and NO researchers are making increasing use thereof. However, commercially availability of Griess assays does not automatically guarantee the accuracy of the results for nitrite and nitrate obtained by these methodologies. Thus, well-recognized and potentially interfering pre-analytical and analytical factors must be adequately considered in the whole analytical process, from sampling to detection. The Griess reaction is very well known in the area of the L-Arg/NO research and is considered in general to run routinely. However, accurate measurement of nitrite and nitrate in biological samples by assays based on the Griess and related reactions requires use of sophisticated analytical procedures by experienced analysts. Eventually, clinically oriented journals have further a responsibility to this area of research to be more attentive to analytical chemistry both from the methodological and the review points of view with regard to authors' own, to adopted and, most important, to commercially available Griess assays.

Over the last years, the NO metabolites *S*-nitrosothiols [136] and nitrite [190] have been proposed to play important, almost identical physiological roles in mammals, including signal transduction and blood flow regulation. Both *S*-nitrosothiols [136] and nitrite [190] have been proposed to represent each the largest intravascular storage form of NO. At present, reported basal concentrations of circulating *S*-nitrosothiols and nitrite range by two to three orders of magnitude, with Griess assays having also contributed to this great divergence. Circulating nitrite and *S*-nitrosothiols have evaded the definition of reference intervals to date. In consideration of the potential roles in blood and tissue, the most urgent and important future prospect in this research area is the establishment of reference values for these closely related NO metabolites. Absolute requirement to reach this goal is the use of reliable, validated, interference-free, specific and sensitive analytical methods in clinical studies. Also, more attention should be paid to the methods of analysis of nitrite

and *S*-nitrosothiols including particularly the Griess assays, both from the analytical and the review point of view, notably in clinical journals.

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