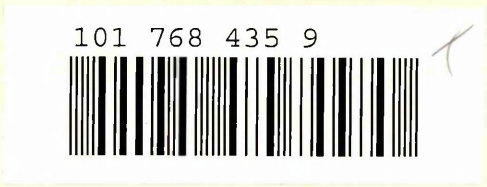

REFERENCE

Development and Application of Isotopically-Selective Assays for Nitric Oxide Metabolites

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Abstract

The importance of nitric oxide (NO) in neurotransmission[1], immunological defense[2], and as a vasodilator in the regulation of blood pressure[3] is well known, so there is a high interest in methodology for NOS turnover measurements, or methods that help to discover new metabolites of nitric oxide. Use of L-arginine with a ^{15}N stable label allows determination of the NO pathway throughput, but requires sensitive detection methods which are capable of isotopic discrimination. The Griess assay for nitrite has been combined with surface enhanced resonance Raman spectroscopy (SERRS) to allow more sensitive determination of nitrite, and also quantitative discrimination between ^{14}N and ^{15}N forms. The method was optimised for use with silver sol prepared from silver nitrate and citrate. The method was applied to the analysis of urine, serum and culture medium with recoveries of 96%, 85% and 95% respectively. Good reproducibility was achieved following pretreatment of samples by solid phase extraction. The limit of detection for nitrite was 5 nmol/l and the response was linear up to at least 10 µmol/l. In terms of isotopic discrimination between the ^{14}N and ^{15}N isotopes, ^{15}N nitrite was detectable at isotopic ratios of greater than 1:20 ^{15}N : ^{14}N. The time of analysis, excluding derivatisation by the Griess assay, was approximately 5 minutes. The method will be useful in metabolic tracer studies using stable labels. An alternative assay using ion chromatography – mass spectrometry allowed the detection of nitrite and nitrate without prior derivatisation, and showed good discrimination between nitrogen isotopes. Nitrite and nitrate were separated from each other and from matrix components by suppressed ion chromatography. Post

column oxidation and a chloride trap column were employed to improve senstivity and selectivity. The limit of detection for ^{14}N-nitrite and ^{14}N-nitrate was 200 nmol/l and the detection limit for ^{15}N-nitrite and ^{15}N-nitrate 50 nmol/l. Nitrate recovery was 93% from urine and 94% from serum. Recovery of nitrite was 96% from urine and 107% from serum.

The IC-MS assay was used in a pilot study of primary pulmonary hypertension, in which NOS turnover was found to be significantly lower in the patient group than in the controls. The assay was also used in a study of NO donors where the donor compounds as well as nitrite and nitrate could be detected.

CONTENTS

Abbreviations

Alb - serum albumin

CE – capillary electrophoresis

cGMP – cyclic GMP

DETAnonoate - 2,2´-(hydroxynitrosohydrazono)bis-ethanamine

EDRF - endothelial-derived relaxing factor

GC – gas chromatography

GIRMS – Gas isotope ratio mass spectrometry

GSH – glutathione

Hb – haemoglobin

HbO_2 – oxy-haemoglobin

HPLC – high performance liquid chromatography

IC – ion chromatography

IC-MS ion chromatograpy – mass spectrometry

L-NMMA – NG-methyl-L-arginine

n.d. - not detectable

NO – nitric oxide

NOS - nitric oxide synthase

MbO_2 – oxy-myoglobin

MetHb - met-haemoglobin

MS – mass spectrometry

PAPAnonoate - 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine

PPH – primary pulmonary hypertension

SERS – surface enhanced Raman spectroscopy

SERRS – surface and resonance enhanced Raman spectroscopy

sGC – soluble guanylate cyclase

UV – ultra-violet

VIS – visible

1. Introduction

1.1 Nitric oxide and metabolism

1.1.1. Overview

Nitric oxide (NO) is an important molecule with numerous metabolic functions, such as neurotransmission[1] immunological defense[2] and as the endothelial-derived relaxing factor (EDRF)[3,4] NO is produced from oxidation of L-arginine in the NO-pathway catalysed by three isoforms of nitric oxide synthase (NOS). In vivo, NO is excreted mainly as nitrite and nitrate in the urine. However, nitrolysation of proteins to nitrosothiols has been an area of great interest over the past years. Research has shown an impairment of NO production in several diseases such as disorders of the systemic and pulmonary circulations[5], diabetes[6], septic shock[7] and cancer[8].

1.1.2. Properties of NO

Nitric oxide (NO) is a colourless, toxic, non-flammable gas. Its boiling point is -152°C and it is solid below -164°C. NO is in equilibrium with its dimer (N_2O_2). Because of its odd number of electrons it has paramagnetic properties and is a so-called stable radical. NO is not very soluble in water (73.4 ml/l), but it is relatively soluble in lipids. As a small, uncharged molecule NO can diffuse quickly in biological systems. The diffusion constant of NO in aqueous solution is 3300$\mu m^2 s^{-1}$ at 37°C. These properties allow NO to penetrate cell membranes and pass through tissue. As a free radical, NO reacts quickly with compounds containing unpaired electrons such as molecular oxygen, superoxide or transition metals. In principle NO may react by electron gain to form the nitroxyl anion NO^-, and by electron loss to form NO^+, the nitrosonium ion[9].

1.1.3. The nitric oxide pathway and nitric oxide synthases

NO is produced in cells by the nitric oxide pathway through the catalysed oxidation of L-arginine to L-citrulline[10] (Figure 1.1).

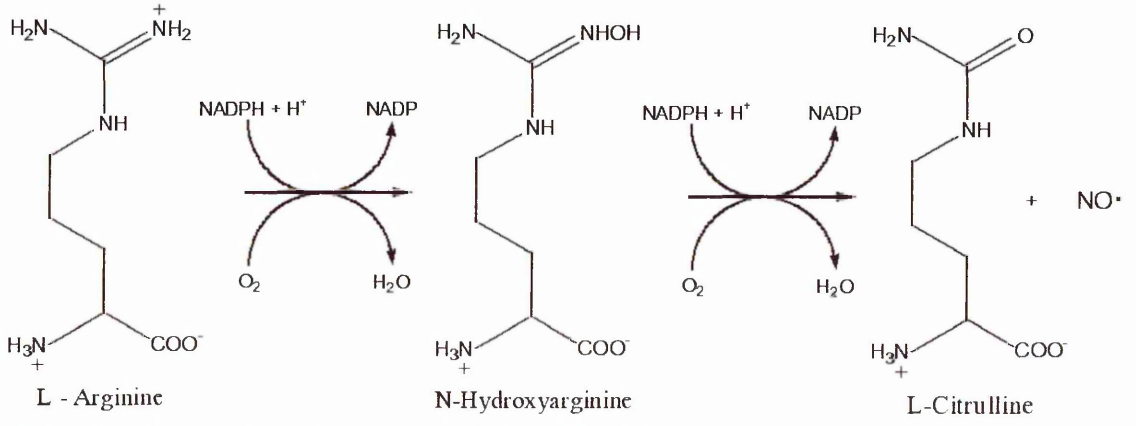

Figure 1.1: Formation of nitric oxide by the NO pathway

The catalysed 5 electron oxidation of the guanidino nitrogen by molecular oxygen leads to the production of equimolar amounts of NO and L-citrulline. The formation of NO is thought to be a two-step biochemical reaction. One of the guanidino-nitrogens of L-arginine is oxidised to N-hydroxyarginine followed by further oxidation to L-citrulline and nitric oxide.

This reaction is catalysed by a family of enzymes called Nitric Oxide Synthases (NOS):

a) neuronal (nNOS)	Type I
b) inducible (iNOS)	Type II
c) endothelial (eNOS)	Type III

There are different nomenclatures for the NOS isoforms[11]. They are categorised in two groups: as calcium/calmodulin-dependant NOS (cNOS), that is constitutively expressed and dependant on calcium/calmodulin, versus iNOS where the letter 'i' indicates that its expression is inducible and calcium/calmodulin-independent[12]. The calcium/calmodulin-dependent isoforms are named neuronal NOS (nNOS) and endothelial NOS (eNOS) refering to the type of cells where they were first discovered. The abundance of an enzyme is not specific to an organ or type of tissue[13]. Another nomenclature enumerates the enzymes in the order in which they were first cloned. The first isoform purified and cloned was nNOS (Type I), followed by iNOS (Type II) and finally eNOS (Type III)[12]. Three genes encode NOS in human, cow, rat and mouse, where each NOS gene

is located on a different chromosome[14]. Among these, 39% of the 1144 residues in the shortest isoform are universally conserved. Across species, the homology between equivalent isoforms averages 90% ± 6%, while within species the homology between isoforms averages 53% ± 2%. While nNOS and eNOS are expressed constitutively to form NO in small amounts, the expression of iNOS is induced upon exposure to inflammatory stimuli and in larger amounts than the other two constitutive isoforms[15].

The nitric oxide synthases show a high degree of similarity with the cytochrome P_{450} enzymes, but require additional cofactors namely flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and tetrahydrobiopterin (BH_4) to pass electrons from reduced nicotinamide adenosine dinucleotide phosphate (NADPH) substrate to the heme[12]. eNOS has an extra site for myristoylation since it is usually membrane bound[12], while the two other isoforms can be found in the cytosol[16]. All three enzymes possess the same binding sites for the cofactors including calcium and calmodulin[17]. iNOS does require calcium and calmodulin to produce NO, but their affinity for NOS binding site is high and they are almost permanently bound to the enzyme[12].

In healthy cells, when iNOS is not induced it produces approximately the same amount of NO as the two other isoforms (1 [µmol/l])/min/mg protein NO at 37°C). However, upon exposure to inflammatory cytokines, NO production is greatly enhanced[12]. The rate of NO production from endothelial cells in situ or in culture was measured at around 0.8 pmol min^{-1} mg-1 [18]. With an average mass of 1.5 kg of endothelial tissue in the whole body, the daily production of NO by eNOS would amount to 1728 µmoles. The administration of ^{15}N-labelled L-arginine to

healthy volunteers showed that 0.34% of all dietary arginine was used for NO synthesis within the splanchnic region[19].

1.1.4. Non-enzymatic reactions that form NO

Under acidic conditions NO can be formed from nitrite by disproportionation, which has lead to the idea that nitrite could act as a significant source of NO[20]:

$$NO_2^- + H^+ \longrightarrow HNO_2$$

$$3HNO_2 \longrightarrow 2NO + NO_3^- + H^+ + H_2O$$

Equation 1.1: Disproportionation of nitrite under acidic conditions

Nitrite is the conjugated base of nitric acid, a weak acid with a pKa of approximately 3.8. Under pysiological conditions, small quantities of nitric acid will be present. The disproportionation of nitric acid generates NO and nitrate.

In the stomach, high NO concentrations have been detected attributed to reduction of nitrite from saliva or dietary sources in contact with acidic gastric juice[21].

1.1.5. Reaction of NO with oxygen

The autoxidation of NO in aqueous environments forms mainly nitrite and only very little nitrate[22]. The overall reaction is as follows:

$$4NO^{\cdot} + O_2 + 2H_2O \longrightarrow 4H^+ + 4NO_2^-$$

Eqution 1.2: Autoxidation of NO to nitrite

NO can react with oxygen to $ONOO^{\cdot}$, which can further react with NO to form $NO_2^{\cdot}$ radicals:

$$2NO^{\cdot} + O_2 \longrightarrow ONOO^{\cdot}$$

$$ONOO^{\cdot} + NO^{\cdot} \longrightarrow ONOONO$$

$$ONOONO + \longrightarrow 2NO_2^{\cdot}$$

Equation 1.3a: Formation of $NO_2^{\cdot}$ via $ONOO^{\cdot}$

Alternatively, a dimerisation of NO and reaction of the dimer with oxygen to N_2O_4 can occur, which can break down to $NO_2^{\cdot}$:

$$2\ NO^{\cdot} \longrightarrow N_2O_2$$

$$N_2O_2 + O_2 \longrightarrow N_2O_4$$

$$N_2O_4 \longrightarrow 2NO_2^{\cdot}$$

Equation 1.3b: Formation of $NO_2^{\cdot}$ via dimerisation

A third reaction does exist to form $NO_2^{\cdot}$ by the oxidation of NO with molecluar oxygen:

$$2NO + O_2 \longrightarrow 2NO_2^{\cdot}$$

Equation 1.3c: Formation of $NO_2^{\cdot}$ via oxidation

$NO_2^{\cdot}$ resulting from either one of the reactions above (1.3a, 1.3b, 1.3c) reacts further to form nitrite:

$2NO_2^{\cdot} + NO^{\cdot} \longrightarrow N_2O_3$

$N_2O_3 + H_2O \longrightarrow 2NO_2^{-} + 2H^{+}$

Equation 1.4 Formtion of nitrite from $NO_2^{\cdot}$

An alternative reaction for NO decomposition in physiological fluids has been suggested by Gaston et al.[23], where NO oxidation forms both nitrite and nitrate. NO reacts with molecular oxygen (O_2) first to form nitrogen dioxide (NO_2). Dimerisation of NO_2 forms dinitrogentetroxide (N_2O_4), which may act as an intermediate to generate nitrite and nitrate:

$2NO + O_2 \longrightarrow 2NO_2$

$2NO_2 \longrightarrow N_2O_4$

$N_2O_4 + H_20 \longrightarrow HNO_2 + HNO_3$

Equation 1.5: Oxidation of NO to nitrite and nitrate

1.1.6 Reaction of NO with superoxide

In aqueous solution, NO can react with superoxide anions ($O_2^{-\cdot}$) to form peroxynitrite ($ONOO^-$)[22] at near-diffusion-limited rate of $6.7x10^9$ l mol^{-1} s^{-1}.

$NO^{\cdot} + O_2^{-\cdot} \longrightarrow ONOO-$

Equation 1.6: Formation of peroxynitrite

The reaction of NO with superoxide is a simple combination of two radicals. It can theoretically act as a sink for NO, but in aerobic environments the formation

of peroxide is outcompeted by the autooxidation of NO to form nitrite[22].

Peroxynitrite can oxidise free thiol groups by nitrosylation[24], induce membrane lipid oxidation[25] or oxidise DNA[26]. Peroxynitrite (pK_a = 6.8) decomposes rapidly under acidic conditions to form nitrogen dioxide and hydroxyl radicals (OH)[27]:

$$ONOO^- + H^+ \longrightarrow ONOOH$$

$$ONOOH \longrightarrow NO_2 + OH$$

Equation 1.7: Decomposition of peroxynitrite

The reaction progresses via an intermediate with radical-like activity. The reaction products are strong oxidants and may be responsible for lipid peroxidation[28]. Lymar et al report the formation of nitrosoperoxycarbonate ($ONO_2CO_2^-$) from reaction of peroxynitrite with carbon dioxide (CO_2)[29].

$$ONOO^- + CO_2 \longrightarrow ONO_2CO_2^-$$

Equation 1.8: Formation of nitrosoperoxycarbonate

The rate constant for this reaction was determined to be $3x10^4$ l mol^{-1} s^{-1}. This is sufficiently large that this reaction can progress in physiological fluids where the total carbonate concentration is typically 25mM or greater. Nitrosoperoxycarbonate can act as a nitrating agent and NO_2^+ resulting from its decay may be responsible for the nitration of tyrosine under pathological conditions[29].

1.1.7 Reaction of NO with proteins

An important physiological target of NO is haemoglobin (Hb), since NO binds avidly to iron II present in the porphyrin ring to form nitrosyl-haemoglobin under anaerobic conditions[30]. Of all possible ligands of Hb, NO has the highest affinity, being 1500 times higher than that of CO[31]. The half-time for dissociation of the first NO molecule from $Hb_4(NO)_4$ at 19°C is about 8h at pH 9 and 3h at pH 6[32]. The rate constant for the combination of haemoglobin with the first molecule of NO is 3.0×10^7 l mol^{-1} s^{-1} [33]. NOHb concentrations of 0.5 µmol/l in arterial blood and 0.9 µmol/l in venous blood were reported[34]. NOHb is converted rapidly in the presence of oxygen and methaemolgobin reductase to HbO_2 via MetHb within the red blood cells[35].

When Hb is added to an aqueous solution the half life of NO decreases significantly[36]. The reaction of NO with oxy-haemoglobin (HbO_2) has virtually no back-reaction and the reaction-products are Hb and nitrate[37]. This differs from the degradation of NO as found in aqueous solution, where mainly nitrite is formed and is the explanation for the short half life of NO in vascular systems[38]. In biological systems, oxy-myoglobin (MbO_2) can take the place of oxy-haemoglobin[39]. The combination of NO and free oxy-haemoglobin is almost instantaneous[40]. The rate of this second order reaction has been determined to be 3.4×10^7 M^{-1} s^{-1} [41]. The rate constant for NO uptake by human red blood cells was reported to be 0.167 M^{-1} s^{-1}, but is influenced by the shape, the orientation of the membrane molecules and the intracellular haemoglobin concentration[42]. Therefore the reaction of NO with oxy-haemoglobin in the vasculature is limited by its diffusion into the red blood cells and is significantly slower than the reaction of NO with free oxy-haemoglobin.

One of the main targets for the physiological effects of NO is soluble guanylate cyclase(sGC), a protein containing a haem group with a porphyrin ring as found in Hb. NO triggers a conformational change in the enzyme, stimulating the production of cyclic-GMP (cGMP), which leads to smooth muscle dilatation[23]. sGC can act as a NO acceptor, because the structural environment of the His-ligated haeme-group allows the formation of a pentacoordinate ferrous nitrosyl complex even under aerobic conditions[43].

Free thiol groups on biological molecules such as proteins, polypeptides or amino acids are an important target for NO. Cysteine is the sole thiol source in humans either in proteins such as albumin or haemoglobin, peptides such as glutathione or as free cysteine. NO may react with free thiol groups to form nitrosothiols[44]:

$$4NO + O_2 + 2RSH \longrightarrow 2RSNO + 2NO_2^- + 2H^+$$

Equation 1.9: Formation of S-nitrosothiols

Under anaerobic conditions NO reacts with thiols to form disulphide, dinitrogen monoxide and nitrogen[45]:

$$RSH + B^- \longrightarrow RS^- + BH$$

$$RS^- + NO \longrightarrow RSNO^-$$

$$RSNO^- + H^+ \longrightarrow RSNOH$$

$$2RSNOH \longrightarrow RSN(OH)N(OH)SR$$

$$RSN(OH)N(OH)SR \longrightarrow RSSR + N_2 + H_2O$$

Equation 1.10: Reaction of NO with thiols under anaerobic conditions

The reaction can occur in two ways. Either the loss of the radical electron forms NO^+ causing a strong hydrophilicity and reactivity towards most biological R-SH species[46]. The uptake of an electron forms NO^- and allows reaction with electropositive R-SH species found in the presence of ferrous ion or other transition metals[47]. In blood, this reaction has to be put in context with the oxidation of NO to nitrate by HbO_2 or the formation of nitrosyl-Hb, which are two competing reactions to the formation of s-nitrosothiols. The reaction of NO with the cysteine-ß93 of the ß-subunit forming S-nitrosohaemoglobin (SNO-Hb) is oxygen dependent and facilitated in the oxygenated status, whereas deoxygenation is accompanied by an allosteric transition in SNO-Hb that releases the NO group[48]. Zhang et al reported that the formation of S-nitrosothiols in the presence of oxyHb or oxyMb is only a mixing artifact and that most of the NO was oxidised to nitrate, which seems to limit the formation of S-nitrosothiols under aerobic conditions[49]. Reichenbach et al pointed out the possibility of S-nitrosothiol formation in the presence of met-Mb, but have not demonstrated it under physiological conditions[50].

Simon et al. determined the total amount of S-nitrosothiols in human plasma and found about 7 µmol/l S-nitrosothiols of which 96% were S-nitrosoproteins and 82% of the S-nitrosoproteins were S-nitroso-albumin[51]. The total level of glutathione (GSH+GSSG) in plasma is about 20µmol/l, with 85% being GSH[52]. In plasma S-nitrosoglutathione was reported to be present at a concentration of between 0.02-0.2 µmol/l and have a half-life of 8 minutes[53],[54]. S-nitrosocysteine was found at levels between 0.2-0.3 µmol/l and the half-life was determined to

be below 1 minute[54,55]. S-nitrosohaemoglobin concentration was determined at a concentration of 0.3 µmol/l in arterial blood or 0.003µmol/l in venous blood with a half-life of less that 1 minute[48,54].

S-Nitrosothiol	*Concentration*
S-nitrosoalbumin	0.74[51]
S-nitrosoglutathione	0.02-0.2[53,54]
S-nitrosocysteine	0.2-0.3[54,55]
S-nitrosohaemoglobin	0.3(arterial), 0.003(venous)[48,54]

Table 1.1: Reported concentrations of S-nitrosothiols in human serum

Feelisch et al reported the presence of N-nitrosoproteins in human plasma of healthy volunteers at a concentration approximately 5-fold greater than the S-nitrosothiols[56]. It appeared that both the N- and S-nitroso moieties were associated with the albumins. This finding not only raises questions about the origin and physiological function of this class of compounds, but also whether the presence of these potentially mutagenic compounds increases the risk of cancer[57]. Previously, N-nitroso compounds have been only found to be generated endogenously under infectious or inflammatory conditions either via NO-mediated nitrosation, intermediate formation of peroxynitrite, or bacterial action[58].

Other protein targets are metallo proteins that bind NO and oxidise it to nitrate[59]. For example, the inactivation of aconitase by NO binding to Fe-S clusters regulates iron metabolism[60].

1.1.8 Metabolic Fate of nitric oxide

Yoshida et al studied the metabolic fate of both inhaled ^{15}NO and ^{15}NO injected into the blood of rats[61]. The ^{15}N label was found in high levels in the blood serum, red blood cells and urine of the rats and at low levels in the trachea, lung, muscle and kidneys. It was demonstrated that inhaled and injected NO rapidly enters circulating red blood cells to become oxidised to nitrate and excreted in the urine and that neither NO nor its metabolites are stored in larger amounts in any tissues for longer periods.

It has been demonstrated by Liu et al. that the reaction of NO with oxygen within the hydrophobic regions of membranes is approximately 300 times more rapid than in the surrounding aqueous medium[62]. This suggests that hydrophobic sites such as biological membranes play an important role for the metabolic fate of NO.

Even though NO is able to diffuse across distances of more than 100μm in tissue, the haemoglobin present in the circulating red blood cells is a major sink for NO[63]. This creates a diffusion gradient towards the vasculature. Even after NO has diffused into the smooth muscle, it can rapidly diffuse back along the diffusion gradient to the red blood cells[27]. The half life of NO in blood has been determined to be not longer than 1.8 ms[64]. The half life of nitrate in blood is about 8 hours[65]. The normal nitrate level in serum is approximately 30 μmol/L, but may go up to 1000μmol/L under pathological conditions[66]. In urine, the total nitrate concentration ranges from 250-1000μmol/L[67]. In balance studies, where rats were given injections of ^{15}N-labelled NO, nitrite or nitrate, or inhaled NO, approximately 90% of the administered ^{15}N was recovered as nitrate in the urine within 48 hours[68]. Yoshida et

al. reported that a certain amount of the nitrate formed in the blood is discharged into the oral cavity through the salivary glands and transformed to nitrite[69]. Part of this nitrite is converted to NO gas in the stomach. Nitrate in the intestine is partly reduced to ammonia through nitrite, readsorbed into the body, and converted to urea.

Unlike in whole blood, where both NO and nitrite are oxidised to nitrate, in plasma free of Hb NO is oxidised almost completely to nitrite, where it remains stable for several hours[70]. This raises the question whether the oxidation of NO by oxyhaemoglobin progresses stepwise via nitrite or leads directly to nitrate. Intratracheal administration of ^{13}N-nitrite to rats or mice revealed that 70% of the ^{13}N was converted to ^{13}N-nitrate, but 27% remained as nitrite and that the conversion occurred within the red blood cells[71]. In a study using ^{15}N-labelled L-arginine, it has been demonstrated that in fasted young volunteers approximately 90% of the circulating nitrite is a result of the L-arginine pathway[72]. This may include NO, peroxynitrite or S-nitrosothiols as possible sources for nitrite. Inhibition of eNOS by NG-methyl-L-arginine (L-NMMA) diminished nitrite concentration in human forearm vasculature of healthy volunteers under basal conditions[73]. Demoncheaux et al. have shown that nitrite is in equilibrium with nitric oxide under physiological conditions and is therefore both a sink and source of NO[74]. The half-life of nitrite in human blood is 110 s and is pH and temperature dependant, but independent of the free haemoglobin concentration[75]. The normal level of nitrite in serum is about 4.4 µmol/L[76]. Urinary nitrite is usually not detectable except upon urinary infection[77]. The oxygen uptake of haemoglobin by nitrite under aerobic conditions occurs via a slow single-electron transfer from nitrite to the bound

molecular oxygen of haemoglobin, followed by autocatalysis in which either superoxide, peroxide, nitrogen dioxide, peroxynitrite or metHb radical may be involved[35, 78, 79, 80, 81].

In a metabolic study where ^{15}N-labelled nitrite and nitrate was given to rats, small amounts of ^{15}N-urea were found as a NO metabolite in the urine[68]. The ^{15}N-urea content was lower after the ^{15}N-nitrate injection than after the ^{15}N-nitrite injection. This suggests that nitrite entered the urea cycle and that conversion from nitrate to nitrite occurred. NH_3 and the urea subsequently produced from it are quantitatively significant nitrogen precursors for nitrate[82]. It has been shown in rats that continuous infusion of ammonium acetate leads to the formation of nitrate from NH_3[82].

The effect of nitrosated proteins on the fate of NO is still a matter of great debate. Several enzymes have been described to break down S-nitrosothiols in vitro. Xanthine oxidase can break down S-nitrosothiols to peroxynitrite in the presence of xanthine[83]. Glutathione peroxidase and thioredoxin reductase may generate NO from S-nitrosothiols[84,85]. CuZn-superoxide-dismutase has been shown to represent a physiological catalyst for the release of NO from low-molecular weight S-nitrosothiols, such as S-nitrosoglutathione, but not S-nitrosoproteins such as S-nitrosoalbumin[86]. Formaldehyde dehydrogenase can use S-nitrosothiols as substrate and produces hydroxylamine in the process[87]. Gamma-glutamyl transpeptidase converts s-nitrosoglutathione to S-nitrosocysteinyl-glycine, that has a greater membrane permeability[88]. Some groups suggest the occurence of transnitrosation. The infusion of NO inhibitors was reported to result in a rapid decay of S-nitrosoproteins with the formation of

low-molecular S-nitrosothiols[89]. It has been shown that the incubation of S-nitrosocysteine or S-nitrosoglutathione with human plasma resulted in a rapid decomposition of these low-molecular compounds and virtually all NO was recovered as S-nitrosoalbumin[90]. The transfer of NO from albumin to L-cysteine was directly determined in rabbit plasma, indicating that in vivo an intensive NO exchange between high and low molecular weight thiols may occur[55].

S-nitrosothiols can be metabolised in inorganic reactions. Iron in combination with free thiol groups may contribute to both breakdown of and formation of S-nitrosothiols[91]. Reaction with copper can release nitric oxide from S-nitrosothiols[92]. The presence of Cu^{2+} and more importantly Cu^{+} in the circulatory system stimulates the breakdown of S-nitrosothiols to NO and disulfide[93]. Reducing agents such as glutathione and ascorbate can facilitate this process by chemical reduction of the transition metal ions. Superoxide has been shown to cause breakdown of the S-nitrosothiols CysNO and GSNO, but under normal physiological conditions the concentrations at which superoxide ions are present may be too low to provide a significant sink for NO[94].

Besides molecular oxygen, other reactive oxygen species such as superoxide were suggested to be involved in the breakdown of NO[95]. Since superoxide is produced by endothelial cells and macrophages, both of which are capable of generating NO as well, the reaction of NO with superoxide to peroxynitrite may be a metabolic sink for NO despite the short half-life of superoxide and its low concentration in biological systems. It has been shown that purified constitutive, endothelial NOS forms simultaneously NO and superoxide, the ratio depending

on the concentration of the substrate L-arginine and the availability of cofactors[96]. Peroxynitrite will only be formed when NO and superoxide are formed at equal rates[97].

1.1.9 Nitric oxide and the nervous system

NO is produced in the central nervous system (CNS) and in many parts of the brain by nNOS located in the neurons[1], where it can both link local blood flow to neuronal activity and modulate neurotransmitter release.

nNOS binds with postsynaptic density proteins in the brain[98], where it creates the link between postsynaptic glutamate binding and NO synthesis[99]. The binding of glutamate to N-methyl-D-aspartate-(NMDA)-type receptors causes an increase in intracellular Ca^{2+} levels, which activates nNOS to generate NO. NO completes the positive feedback loop by diffusing back to the presynaptic terminals, where it causes the release of glutamate[100]. This process of long-term-potentiation (LTP) seems to depend on cGMP, which regulates Ca^{2+} levels by regulating the cyclic nucleotide-gated ion channels and therefore the release of glutamate from the presynaptic terminals[101].

NO is an important neurotransmitter in the peripheral nervous system (PNS). nNOS may contribute to sensory transmission and is found in some peripheral nerves where it may contribute to sensory transmission[102]. It may play a role in nitrergic transmission within non-adrenergic, non-cholinergic (NANC) neuro-effector nerves[103]. NO appears to cause dilatation of the stomach as a response to an increased intragastric pressure. NO is responsible for the relaxation of the

corpus cavernosum and is a key factor in the development of penile erection in humans[104]. It has been suggested that not only NO but also S-nitrosothiols mediate signal transduction[105].

1.1.10 Nitric oxide in the immune system

NO is produced as part of the immunological defense mechanism against pathogens, such as tumour cells, bacteria, fungi and helminths[106]. NO is mainly, but not exclusively, produced when macrophages are acivated by cytokines or endotoxin and gene transcription of iNOS is induced. This results in a sustained production of NO and diffusion to the target cells[107]. The cytotoxic effect is achieved by the combination of nitric oxide with metal-containing active centres in key enzymes in target cells[108]. Not only NO itself, but also S-nitrosothiol as a NO donor may have a role in immunological defense, for instance an antimicrobial effect in the airway lining fluid in human airways[109].

Hierholzer et al. reported that endotoxin-induced nitric oxide initiates an inflammatory response in the liver[110]. In severe cases lethal concentrations of nitric oxide are produced by the tissue of the vascular wall which can lead to hypotension, septic shock and organ failure.

Since the mechanism of cytotoxicity of NO is not specific to a target, NO producing cells have to protect themselves against it. Pre-stimulated macrophages tolerated high NO levels through increased breakdown of NO by superoxide[111].

Escherichia coli resists high NO levels by activation of the redox-sensitive

transcriptional regulator SoxR, that leads to a defense response that oxidises NO and thus protects the bacteria against macrophages[112].

1.1.11 Nitric oxide in the vascular system

In the cardiovascular system two functions can be attributed to NO. It acts as a vasodilator responsible for regulating blood pressure[3] and control of platelet aggregation[113]. The cardiovascular NO, which regulates blood pressure, originates mainly but not only from eNOS in the endothelial cells[114]. The eNOS actvity under normal physiological conditions is regulated by chemical as well as mechanical factors[115].

Increase in intracellular Ca^{2+} increases eNOS activity, while Ca^{2+} antagonists such as acetylcholine or bradykinin decrease it. NO may diffuse through the endothelial cell membranes into the adjacent smooth muscle cells to activate sGC by binding to the heme. The cGMP produced upon sGC activation leads to increased levels of 3', 5' -cyclic monophosphate (cGMP) and thus to smooth muscle relaxation and a lower vascular tone[116]. The effect is localised and any NO coming in contact with the blood stream may either be quickly oxidised by HbO_2 to nitrate and Hb[37] form HbNO[31] or nitrosylate thiol groups on proteins[46]. S-nitroso-Hb has been suggested by recent studies to have a role in blood pressure regulation itself or serve as a storage for NO that buffers its effect[117]. Hb may be S-nitrosylated in the lung and NO released from erythrocytes in the tissues. This way S-nitrosohaemoglobin may regulate blood flow in the lung in response to oxygen tension by releasing NO in the arterioles[118]. GSNO has been proven to be a selective and potent inhibitor of platelet activation in humans[119]. The significantly slower reaction rates of

superoxide with nitrosothiols in comparison to the reaction rate of superoxide with NO are consistent with the hypothesis that the formation of nitrosothiols stabilises NO and thus these compunds can serve as a carrier or buffer of NO[94]. The concept of S-nitrosothiols as buffers or carriers of NO has not yet completely been established. The thiol N-actelyl-cysteine did not modulate NO-mediated responses in the human forearm circulation[120]. The in vitro production of NO from S-nitrosothiols does not always correlate with the extent of its vasodilatory effects[54].

Cells of the endothelium can respond to mechanical stress caused by pressure or shear force with an immediate release of NO as a result of increased Ca^{2}+ influx[121]. NO production is therefore positively fedback by blood pressure.

Inhibition of eNOS may lead to hypertension but also to glomerular damage, because NO inhibits platelet aggregation by raising the concentration of cyclic AMP in the platelets in a mechanism involving cGMP and prostacyclin[122].

1.1.12 Nitric oxide related diseases

NO may play an important role in cancer, as it is involved in the process of carcinogenesis. In the early phase of the development of a tumour NO may mediate DNA damage[123]. It supports tumour progression through induction of angiogenesis[124] and may suppress the immune response in the late phase of cancer development[8]. It is a very controversial subject how NOS activity is implicated in tumour progression and NOS activity and while some groups report increased NOS activity[125], while others report decreased NOS activity[126].

A hyperdynamic circulation is often seen in patients with liver disease and can be the reason for the death of these patients[127]. This is a result of high cardiac

output and low systemic as well as pulmonary vascular resistance[128]. Nitrite and nitrate levels in blood and urine of these patients are increased, which has been linked to NO overproduction[129]. It is assumed that endotoxins and cytokines induce NOS and cause widespread vasodilation[130]. However, the mechanism of the systemic vasodilation is still unclear, since endotoxin increases the expression of NOS, but cirrhosis does not[131].

In patients with essential hypertension, whole-body nitric oxide production is significantly lower than in healthy people under basal conditions[132]. Interestingly, L-arginine therapy seems to prevent hypertension and reduces systolic and diastolic pressures in animals and humans suffering from essential hypertension[133].

Patients suffering from hypoxic lung disease and pulmonary hypertension show reduced nitric oxide production in the pulmonary endothelium. It is thought that nitric oxide released by the pulmonary endothelium may contribute to the characteristically low pulmonary vascular resistance[5]. Indeed, inhalation of nitric oxide gas at a concentration of 40 ppm has a powerful selective vasodilating effect[134].

The NO released by iNOS accounts for wide-spread vasodilation in septic shock, as well as for the hypotensive state induced by cytokine therapy in patients with cancer[135]. iNOS is induced in endothelial and smooth-muscle vessel cells by certain cytokines and low doses of nitric oxide synthase inhibitors may acutely reverse the hypertension seen in these patients[7].

Diabetes mellitus is often accompanied by vascular diseases such as ischaemic

heart disease, cerebrovascular disease and peripheral vascular disease[6]. It is controverial how endothelial dysfunction and NOS activity is related to diabetes. Some groups report that high glucose levels increased activity[136]. Others report that hypercholesterolemia, which is associated with diabetes may cause endothelial dysfunction through increases in the production of superoxide and other free radical species that inactivate NO[137]. Some groups report no difference[138].

Pathogenesis of multiple sclerosis may be connected to free radicals including NO[139]. The induction of NOS may be increased by elevated levels of various cytokines in the cerebrospinal fluid as well as in the blood[140]. Yamashita et al found significantly elevated levels of the NO metabolites nitrite and nitrate in cerebrospinal fluid, especially in the late phase of multiple sclerosis exacerbation[141].

1.2. Analytical methods in nitric oxide research

1.2.1. Overview

Numerous methods and techniques have been developed for the analysis of NO itself, its metabolites or the determination of NOS turnover. The most popular types, despite their problems, are colorimetric assays for nitrite and nitrate derived from the Griess assay. However, nitrite and nitrate in body fluids may also arise from dietary intake and other sources. As a consequence, isotope labelling experiments with L-$[^{15}N]_2$-arginine are preferred to measure whole body nitric oxide turnover. Previous methods of nitrite and nitrate analysis with isotopic discrimination have included GC-MS after derivatisation to nitroaromatic compounds or continuous-flow gas isotope ratio mass spectrometry (GIRMS) after reduction to ammonia then conversion to nitrogen. There have been many more methods developed for the determination of NO metabolites but this chapter will focus on the most important ones.

1.2.2. The Griess assay and derived methods

The most widely used method for nitrite determination is still the Griess assay, a colorimetric procedure first described by P. Griess in 1879, based on the formation of an azodye and quantification by measuring the dye's absorbance at 540 nm[142]. Figure 1.2 shows the formation of the Griess product. First, nitrite is diazotised with sulfanilamide under acidic conditions. This diazonium salt is coupled to 1-naphthylethylenediamine by forming an azobond. The resulting diazo-dye (Griess product) has an absorbance maximum at 540 nm in its protonated form under acidic conditions and at 496 nm in its non-ionic form under neutral or basic conditions. The problem with this method is that it is very sensitive to matrix effects, since it does not include proper separation of the matrix from the analytes.

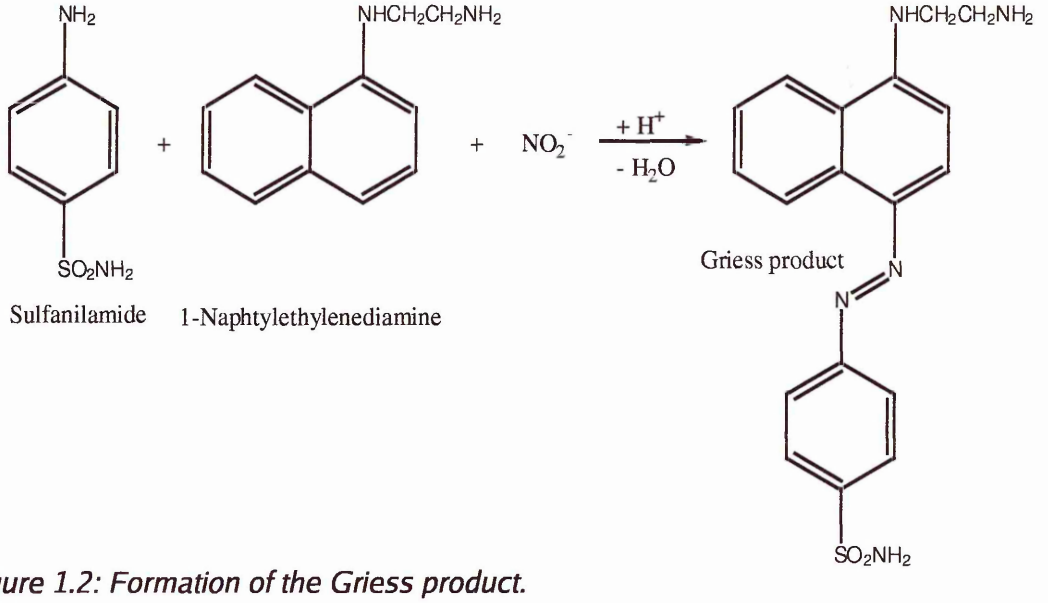

Figure 1.2: Formation of the Griess product.

Oxidants or reductants, such as ascorbic acid[143] or formate[144], present in the

sample can oxidise or reduce the azo-dye and thus reduce the color intensity[145]. The decomposition of the diazo-dye is catalysed by copper or zinc, which also leads to an under-determination if these species are present in the sample matrix[146]. The low pH needed in the reaction to form the diazo salt may lead to the formation of nitrosothiols in matrices being rich in protein or amino acids, such as serum or urine[147]. Finally, it is not possible to discriminate between the ^{14}N and ^{15}N forms with this assay.

To aid the detection of nitrate from biological fluids and improve automation the Griess assay was modified by Green at al[148]. In this flow-injection system, nitrate can be analysed by passing the sample through a column packed with copperised cadmium to reduce nitrate to nitrite. The conversion of nitrate to nitrite was reported to be almost 100%. The reported lower detection limit for nitrite and nitrate was 1µmol/l. Validation data for this method were only presented for nitrate after 40-fold dilution, and so the method may therefore suffer from the same problems as the classic Griess assay when nitrite is analysed. Nitrite levels in serum are usually around a few µmol/l[76] and the sample can not be diluted at such a high ratio, otherwise nitrite concentration is diluted to levels far below the limit of detection of this method. Other groups have observed reduction of nitrate beyond nitrite or incomplete conversion of nitrate to nitrite, due to loss of reducing capability of the cadmium column over time leading to poor reproducibilities and limits of detection[149].

Instead of reducing nitrate chemically to nitrite, the conversion can be done through enzymatic reduction with nitrate reductase[150]. In this process, even

though the enzyme can be immobilised on a column the conversion ranged between ~30% with nitrate reductase extracted from Escherichia coli and ~64% with nitrate reductase from Aspergillus. This problem was partly solved for urine, but not for serum, by using C_{18} solid phase extraction leading to a recovery of ~96%[151].

1.2.3. Gas Isotope Ratio Mass Spectrometry

The current "gold-standard" for the isotope dicriminative measurement of nitrate is a gas isotope ratio mass spectrometry (GIRMS) based assay[152]. First, total nitrate is measured as described by Green et al. by reducing nitrate to nitrite on a reduction column of copperised cadmium and a subsequent Griess assay[148]. In a second step the ratio $^{14}N/^{15}N$ is determined by GIRMS. Nitrate is preconcentrated on an ion exchange resin, converted to ammonia by reaction with Devarda's alloy for 6 days and then converted by combustion to nitrogen at 1000°C and analysed by GIRMS. The precision of the $^{14}N/^{15}N$ ratio measurement was ±0.0004% with the mass spectrometer used and the calibration curve was linear between 20µmol/l to 1000µmol/l of total nitrate. The between-day coefficients of variation ranged from 0.41% to 0.72%. Other validation data was not presented by the authors and the limit of detection and recoveries are not available. Since this assay relies on the Griess assay for total NO_x^- measurements, it suffers form the same problems as described before. Additionally, the simutaneous measurement of ^{15}N nitrite, ^{15}N nitrate, ^{14}N nitrite and ^{14}N nitrate is not possible. Further, this technique depends on expensive

instrumentation which is not routinely available (the isotope ratio mass spectrometer), is complicated and takes several days to complete.

1.2.4. Gas Chromatography – Mass Spectrometry

The first assay for the determination of nitrite and nitrate in saliva and blood by gas chromatography was developed by Tesch et al[153]. Nitrate and nitrite are converted to volatile aromatic compounds for instance by reaction with benzene. The derivatives are separated by gas chromatography and detected by an electron capture detector, which allowed a limit of detection of 0.9 µmol/l. But this method is not interference free. Others have found that it is prone to interferances from non nitrate sources giving rise to nitrobenzene[154]. Also isotopic discrimination between the ^{14}N and ^{15}N forms is not possible. Mass spectrometric detection after gas chromatographic separation for nitrite and nitrate determination from biological fluids first was introduced by Tsikas et al.[147]. In this method reduction of nitrate to nitrite was required, but later the same group developed a method that allowed simultaneous determination of nitrite and nitrate in biological fluids by gas chromatography – mass spectrometry in a single derivatisation procedure[155]. Nitrite and Nitrate are derivatised by pentafluorobenzyl bromide to the nitro and nitric acid ester pentafluorobenzyl derivatives, respectively in aqueous acetone prior to GC/MS analysis. Even though Tsikas et al. advise that this method should be a reference method for nitrite and nitrate measurements, the validation data mentioned were incomplete. It was only reported that accurate, interference-

free and sensitive (50 fmol of [^{15}N]-nitrite and [^{15}N]nitrate were detected at signal-to-noise ratios of 870:1 and 95:1).

It can also be used to analyse two other possible NO metabolites: S-nitro- and S-nitroso-glutathione. ^{15}N-nitrite and ^{15}N-nitrate are needed as internal standards and therefore this technique can not be used for stable label studies with ^{15}N-L-arginine, where these species or the ^{14}N/^{15}N ratio is of primary interest.

1.2.5 Ion chromatography

Various ion chromatographic methods have been developed for nitrite and nitrate determination, not only for biological fluids. A common problem for nitrite analysis by ion chromatograpy in biological matrices is the naturally high chloride concentration. The similar size/charge ratio of nitrite and chloride leads to coelution of a massive chloride peak, making nitrite undetectable. Manufacturers of solid phase extraction systems offer cartridges packed with a silver-based resin to precipitate chloride prior to analysis, but warn that nitrite recovery is usually low due to conversion of nitrite to nitrate.

This led to the development of an ion chromatographic method for the determination of nitrite and nitrate in body fluids by Monaghan et al[156] in which nitrite and nitrate are separated using a chloride gradient elution on a Carbopac PA-100 column and detected directly by UV absorption at 214nm. With this setup, no interfering peak resulting from chloride is seen as it would be the case with other eluent/detector systems. One major advantage here is that sample

treatment is minimal. No derivatisation or chemical processing is needed that could alter the sample. Centrifugal ultrafiltration is all that is required as sample pretreatment. It is not only useful for nitrite and nitrate but also for other analytes such as lactate or actetate. Calibration curves were linear in the applied working range up to 30µmol/l and the limit of detection was of 250 nmol/l for both nitrite and nitrate.

In human saliva chloride is present at a lower concentration, which allows nitrite and nitrate analysis by ion chromatigraphy using conductivity detection and a carbonate eluent (Helaleh et al.[157]). At these lower chloride levels the nitrite peak can still be seen on the tail of the chloride peak. The recoveries of nitrite and nitrate ranged between 95% and 101%. The calibration was linear over the applied working range and the limit of detection was 325 nmol/l and 540 nmol/l , for nitrite and nitrate, respectively.

1.2.6 Chemiluminescence

One of the few assays to measure NO concentrations directly is chemiluminescence. To obtain chemiluminescence, gaseous NO is reacted with ozone to give nitrogen dioxide in an excited state(NO_2*) and the emitted light from chemiluminescence can be measured with a photomultiplier. The detector response is usually linear to the NO concentration over a very wide range.

This is of special interest for the measurement of exhaled nitric oxide[158]. Breath analysers for NO are commercially available that can measure concentrations

as low as 1ppb. Exhaled air can be collected directly from a single full exhalation or during tidal breathing.

Nitrite and nitrate can also be measured by chemiluminescence after reduction to NO with hot acidified vanadium trichloride[159]. However, this assay is not without problems, since it requires derivatisation of nitrite and nitrate under acidic conditions. Nitroso-compounds might lead to both over- or underestimation and bad recoveries. The reduction of Nitroso-compounds to NO leads to overestimation[160] and the sequestration of the NO produced from nitrite or nitrate reduction by thiols to underestimation[161]. These effects are particularily strong when protein is not removed prior to analysis. Therefore Sen et al. have extended this method by coupling a reversed-phase liquid chromatography system with a chemiluminescence system as the detector[162]. Now, that nitrite is separated from the matrix it can be used for selective, interference-free nitrite determinations from biological fluids with a limit of detection of 0.02 µmol/l.

1.2.7 Capillary electrophoresis

A method for the detection of nitrate from urine by capillary electrophoresis was first developed by Wildman[163]. However, this method was too insensitive for the measurement of basal nitrite. For this purpose, the method had to be modified for the simultaneous determination of basal nitrite and nitrate by Leone et al.[164]. An endoosmotic-flow modifier was applied for the separation of nitrite and nitrate from the matrix and direct UV detection at a wavelength of 214nm was

used. At this wavelength chloride is not detected and no interfering peak was observed, even though chloride coelutes with nitrite due to its similar charge/size ratio at the pH of the buffer system. A major advantage of this technique is that only minimal sample preparation by centrifugal ultrafiltration through a 5kD filter is required. Calibration curves were linear in the investigated concentration range of up to 50µmol/l for nitrite and up to 400µmol/l for nitrate. However, the validation data was incomplete in that proper recovery data from spiked samples was not presented and the limit of detection was not reported.

Zunic et al. also developed a method for the simultaneous detection of nitrite and nitrate in human serum and cerebrospinal fluids by capillary electrophoresis with UV detection at 214nm using a simple borate buffer system at a pH of 10[165]. With this method a limit of detection of 0.57 and 0.43 µmol/l was obtained for nitrite and nitrate, respectively, with a standard capillary instead of one with a detection window with an extended light path designed for UV detection. Calibration curves were linear in the investigated concentration range up to 500 µmol/l. The mean recovery of nitrite and nitrate from human serum was 86.6% for nitrite and 97.4% for nitrate. Recoveries from cerebrospinal fluid were similar with 92.6% and 104.5% for nitrite and nitrate, respectively.

1.3 Raman Spectroscopy

1.3.1 Overview

Although the Raman effect was already discovered by C. V. Raman in 1928[166] it was not widely used as an analytical tool until high energy light sources, namely lasers, became available. Still, because of the lack of sensitivity due to the low abundance of the Raman effect it was used as a qualitative technique to obtain structural information rather than for quantification. Since then techniques have been discovered to enhance the Raman effect, improving sensitivity and making it a valuable tool for trace analysis. Surface enhancement, where the analyte has to be brought in close proximity to a metal surface, was first observed by M. Fleischmann in 1974[167]. Resonance enhancement relies on the use of a light source that emits in the absorption range of the analyte and with both effects combined a very high sensitivity and even single molecule detection can be achieved[168].

1.3.2 The Raman Effect

Raman spectroscopy is often compared to infrared spectroscopy, because the resulting spectra obtained from both techniques look very similar. Even though both measure molecular vibrations, the principles underlying the two methods are fundamentally different. In infra-red spectroscopy infra-red radiation is shone on a sample. A functional group of a molecule only absorbs infra red radiation, if the energy of the incident radiation equals the energy difference between two vibrational levels(resonance).

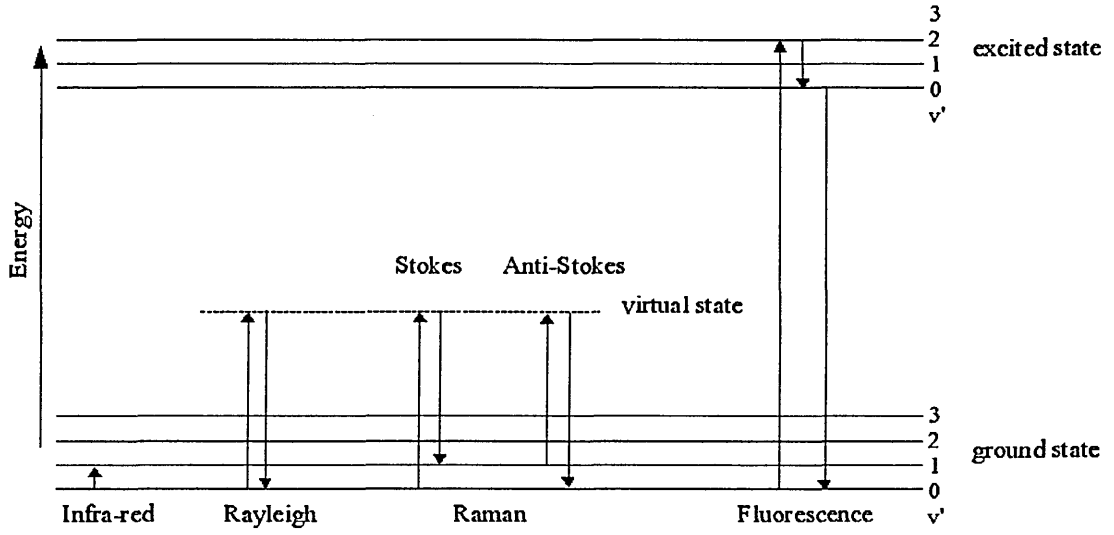

Figure 1.3: electronic transition in raman scattering

Raman Spectroscopy, however, is a two photon process[169]. The incident photon is absorbed by the molecule initially to emit a new photon immediately afterwards. Unlike infra-red spectroscopy, this is an off-resonance process. Absorption does not depend on the wavelength of the incident radiation and does not result in an electron transition to a full excited state such as $\pi \rightarrow \pi^*$, but to an unstable, virtual meta-state. Only very few photons undergo this Raman-process, which is the

reason for the low sensitivity of plain Raman spectroscopy. As a net-result a wavelength shift, the so-called "Raman-shift", of the incident radiation can be observed that corresponds to the energy difference between to vibrational states. Another difference to infra-red spectroscopy is that energy can either be absorbed by the molecule (Stokes) or taken from it (Anti-Stokes), resulting in an electron transition from an excited vibrational state to an unexcited one.

The Raman effect depends on the interaction of the electric field of the incident photon with a polarisable group, it leads to a polarisation of the electrons in the molecule and an induced dipole is created. Thus, in Raman spectroscopy the signal intensity is proportional to the polarisability of the functional group. Functional groups like the diazo group, where the atoms involved have the same electronegativity, are easy to polarise and scatter well. Functional groups which already have a strong dipole such as carbonyl groups are bad scatterers, because more energy is needed to displace the electrons from the atom with higher electronegativity, and to polarise this group.

1.3.3 Quantitative Raman spectroscopy

In principle, the nature of an emission technique makes Raman spectroscopy suitable to trace analysis[170]. In absorbance spectroscopy, a greater technical effort has to be made to discriminate between these two large signals in order to measure low concentrations (double beam instruments etc.). The lower limit in Raman spectroscopy is reached when the signal becomes so weak that it can not be distinguished from the noise. With curently available multichannel detectors it is less than 10 counts[171]. Calibration curves in Raman spectroscopy,

being an emission technique, can be obtained by simply plotting the intensity on the Raman signal against concentration. Internal standards can be used to correct for signal variation and other disturbances.

In comparison to IR spectroscopy, the occurance of water does not pose a problem, such that the analysis of aqueous solutions is possible. Since a monochromatic light source emitting at a wavlength in the visible spectrum is used, cuvettes, optical fibers or other parts of the optics can be made of rather inexpensive materials that are also better adapted to the working environment, and both single and multichannel detectors are available.

The major weakness that Raman spectroscopy shares with other emission techniques is its development as a single beam technique, where correction for variations from source, sample and optics are not made. This can lead to a lower robustness of the method.

Additionally, Raman scattering is rather weak compared to processes like absorption, but resonance and surface enhancement which appear in certain situations can make up for the weak Raman scattering. When they are achieved they not only increase sensitivity, they are also very specific for the analyte.

1.3.4 Instrumentation

A typical Raman spectrometer is comprised of four major parts, the laser light source, the collection optics, the dispersive element and the detection unit[171]. The collimated laser beam is passed through a line filter to remove any unwanted laser lines or sidebands.

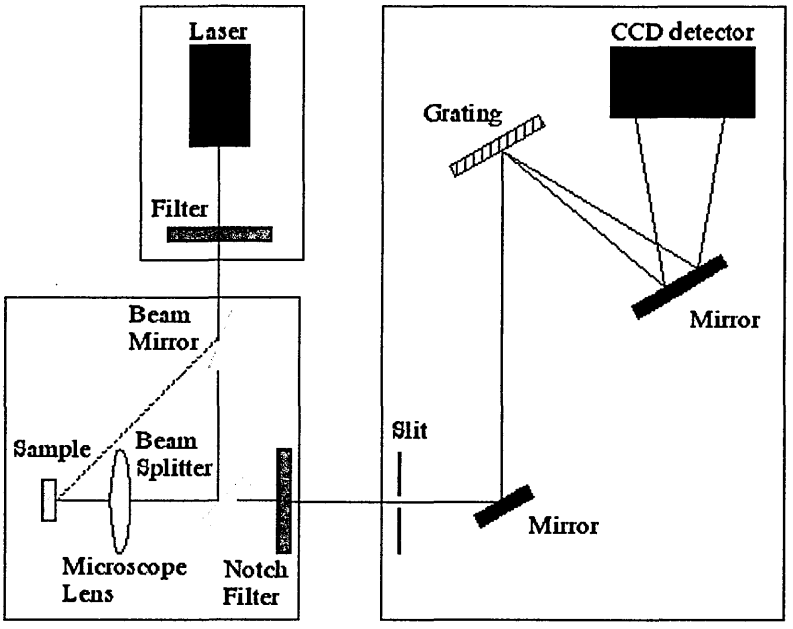

Figure 1.4: Diagram of a singly-dispersive Raman spectrometer

A beam splitter is used to redirect the laser beam towards the sample to achieve a 180° setup. A microscope is used to focus the laser light onto the sample. The scattered light from the sample is passed through the microscope again, through the beam splitter and notch filter. This is a narrow rejection band filter that main purpose is to prevent Rayleigh scattered light and reflected laser light from reaching the detection unit. The detection unit can either be a dispersive one with a grating as shown here or a FT detection system.

Laser sources can be continuous or pulsed lasers. Pulsed lasers are mostly used in the UV range, while continuous lasers are predominant for excitation in the visible range. Most applications in Raman spectroscopy involve continuous gas ion lasers such as argon, krypton or helium-neon lasers, but also solid state lasers. Currently available gas ion lasers can provide Raman excitation in the

range from 450nm to 800nm, where lasers at 514nm, 632nm and 740nm are standard wavelengths. Lasers in the UV range are not used very often, because of the higher cost. They have uses for instance for resonance Raman measurements of compounds that absorb only in the UV region. Near infra-red lasers are also available. The advantage of these lasers is that they practically avoid fluorescence. However the less energetic excitation wavelength mean a lower Raman efficiency and less sensitivity.

The laser is usually focused onto the sample with a Raman microscope, which is usually a normal laboratory microscope. A small laser spot size on the sample is desirable, in order to simplify the collection process and to maximise the power density on the sample. In a confocal arrangement a variable aperture is placed behind the microscopes objective, so that the collected light has to pass through it and out-of-focus light is removed. This also tends to minimise fluorescence as only light from a very small volume of the excited sample is collected and the rest is ignored.

The detection system can be a photomuliplier in the simplest case (Single-Channel Systems). However, better sensitivity can be obtained with array detectors (Multi-Channel Systems). The array commonly consists of 1024 elements and has an intensifier coupled to it. All spectral elements are observed at the same time, which effectively means a longer time of analysis and therefore a greater sensitivity. A trade off has to be made between resolution and bandwidth, which is the major drawback of array detectors. Modern Raman spectrometers have charge coupled devices (CCD) and charge injection

devices(CID) which further improves sensitivity. They are usually cooled to reduce background noise.

1.3.5 Surface enhancement

Surface enhancement was first seen by Fleischmann in 1974 from pyridine on an electrochemically roughened silver electride, and recognized as an extreme enhancement of Raman[167]. The analyte has to be brought into close proximity to the metal surface, either by force or preferably by adsorption onto the metal surface. Two mechanisms have been proposed to account for the SERS effect and an enhanced signal intensity electromagnetic enhancement and chemical enhancement. Electromagnetic enhancement is thought to be due to an increase in the electrical field at the adsorbate because of excitation of the surface plasmons in the metal surface by the incident radiation[172]. Chemical enhancement is thought to be a charge transfer between metal and the adsorbate[173]. Electromagnetic enhancement is a major contributor to the observed enhancement however chemical enhancement also plays a significant role. Since both effects occur combined, it is not yet known to what extent each of them actually contribute to the enhanced signal produced.

The electromagnetic enhancement mechanism is thought to involve the creation of a surface plasmon on the substrate surface, such as a metal colloid, which transfers energy through an electric field to the target molecules[172].

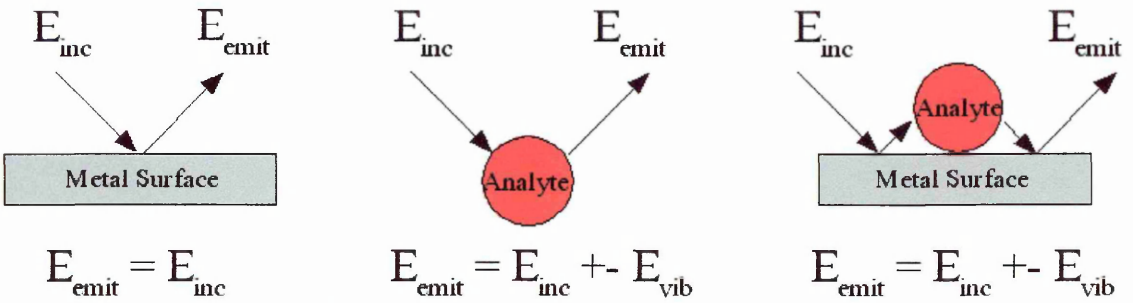

Figure 1.5: Mechanism of electromagnetic enhancement

Electromagnetic radiation striking the colloidal surface, without analyte being present, results in Rayleigh scattering. Rayleigh scattering is a 2-photon process, like Raman scattering, which involves the creation of surface plasmon modes. Unlike Raman scattering, Rayleigh scattering is an elastic process where a photon of identical frequency to that of the the incident photon is expelled following the creation of a surface plasmon on the metal surface. When considering the effect of the incident radiation on the target analyte only, an inelastic Raman Scattering process is observed. In the case of conventional Raman scattering no metal particles are present and the target analyte interacts directly with the incident electromagnetic field. In this case the process is inelastic i.e. the frequency of the incident and resultant photon are not identical due to variations within the vibrational energy levels of the target analyte. Considering the combined effects of the incident laser on the adsorbed analyte and the metal surface, the process is again an inelastic process called surface enhanced Raman scattering (SERS). Incident radiation interacts with both the metal to create a surface plasmon and the target analyte where the variations in the vibrational levels of the molecule result in a photon of a different frequency

being returned to the metal and inelastically scattered[172]. The combination of incident radiation being absorbed by the large area of the colloid surface, and the vibrational energy of the molecule, results in significantly increased scattering power observed as SERS. Electromagnetic surface enhancement is only obtained with metals that have a similar plasmon resonance frequency as the Raman excitation. Various substrates have been investigated, but the most popular ones are silver and gold either in form of an electrode[174] or in form of a colloid[175] especially with excitation in the visible spectrum. The extent of enhancement depends greatly on the surface of the substrate, for instance the degree of roughness of an electrode, or the size, shape and aggregation state of colloidal particles. Often effects resulting from preferred orientation of the analyte to the substrate can be observed.

As well as the electromagnetic enhancement mechanism which is active during the SERS process, there is a chemical enhancement mechanisim which contributes considerably to the observed SERS signal[173]. The chemical enhancement mechanism involves the incident radiation striking the roughened metallic surface resulting in a photon being excited within the metal to a higher energy level. From this excited state, a charge transfer process to an electronic-vibrational level of the same energy within the target analyte takes place. Variations in vibrational energy states occur resulting in the transfer of a photon of different frequency being passed back to the metallic energy levels, and returned to the ground state of the metal.

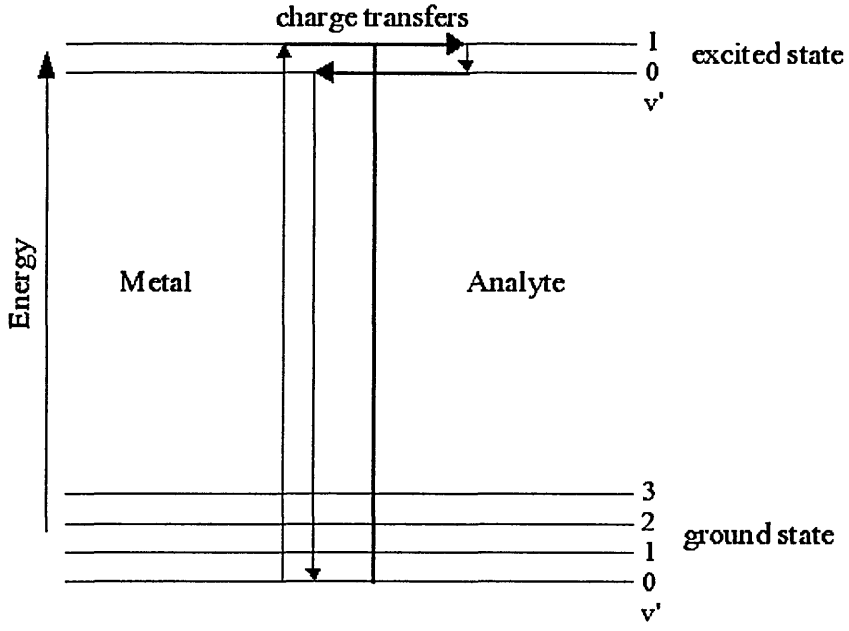

Figure 1.6: electron transitions in chemical surface enhancement

In terms of the chemical enhancement mechanism, the definition is still under debate however, it has been widely proposed that the target molecule must in some way adsorb onto the SERS substrate surface for the enhancement mechanisms to be applied. Once adsorbed, it is not yet resolved whether or not a parallel, but different mechanism is occuring in order to increase the vibrational energies of the molecules for SERS.

1.3.6 Resonance enhancement

In non-resonant Raman scattering, an electron transition to a non-allowed virtual state occurs. The likelihood for this phenomenon to happen is rather low, which explains why non-resonant Raman spectroscopy is not very sensitive. When the energy of the incident light approaches the energy required for an

allowed electronic transition, however, the observed spectrum changes, showing an increase in intensity for certain vibrational bands. This phenomenon is known as resonance Raman scattering or the resonance Raman effect. Its existence was predicted by Placzek in 1934[176] and it was observed for the first time by Shorygin et al. in 1947[177]. The enhanced Raman signal originates from the chromophore, which undergoes an electronic transition upon excitation as shown in Figure 1.7. There are at least two mechanisms responsible for resonance enhancement[177]. The first one, Franck-Condon enhancement, involves a single electronic state. A component of the normal coordinate of the vibration is in direction in which the molecule expands during an electronic excitation.

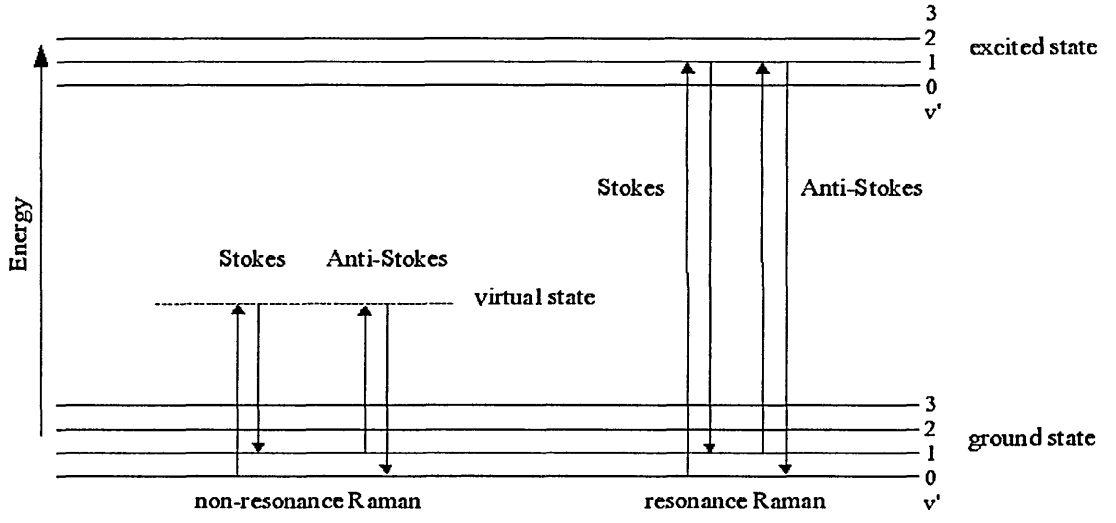

Figure 1.7: electron transitions in resonance raman scattering

The more the molecule is able to expand along the axis when it absorbs light, the greater the enhancement. The second type involves two electronic states

and is called vibronic enhancement.

There is not a distinct frequency where resonance enhancement starts for a certain vibration, but at lower wavelengths an effect called pre-resonance Raman scattering can be observed. It can start at wavelengths of 50-100 nm below the resonance frequency.

In conjunction with surface enhancement, energy transfer can occur from the excited states of the molecule to the metal surface, causing fluorescence quenching enabling the use of a wide range of chromophores[178]. The increase in sensitivity often permits the use of low-power lasers, which reduce the likelihood of analyte photo-decomposition.

1.4 Ion Chromatography – mass spectrometry

1.4.1 Overview

The development of interfaces for the combination of liquid chromatography and mass spectrometry did not lead automatically to methods utilising mass spectrometry as a mode of detection for ion chromatography. The high content of involatile compounds in the eluents traditionally used for ion chromatography were the reason for this incompatibility. Today, two ways are known by which ion chromatography may be made compatible with mass spectrometry. Either a volatile buffer system is used as an eluent for ion chromatography[189], or a suppressor module is employed post column to remove the ionic modifier from the eluent before entering the interface to the mass spectrometer[194].

1.4.2 Types of chromatography

Ion chromatography itself can be divided into three groups:

- ion exchange chromatography
- ion-pair chromatography
- ion-exclusion chromatography

Ion exchange chromatography requires an ionic stationary phase of the opposite charge to the analyte. The mobile phase usually consists of a salt gradient and the ions from it compete with the analyte for the active sites. The separation process depends on electrostatic interaction between the ionic analyte and the counter-charged ionic stationary phase[179]. As the ionic analyte moves through the stationary phase, it can replace the counter-ions at the stationary phase and, after some time, is replaced by an ion from the mobile phase and enters the mobile phase again. For instance, when a hydroxide eluent is used on an anion exchange column, initially all the immobilised quarternary ammonium groups have a hydroxide anion attached. When the analyte(X^-) is injected, it exchanges with the hydroxide ions at the active sites.

$$R\text{-}N^+R_3OH^- + X^- \longrightarrow R\text{-}N^+R_3X^- + OH^-$$

Equation 1.11: The ion exchange process

The ion-exchange process is an equilibrium that depends on the affinity of the analyte toward the stationary phase. The constant describing the equilibrium process is the selectivity coefficient (K):

$$K = \frac{[X^-]_s\,[OH^-]_m}{[OH^-]_s\,[X^-]_m}$$

K	– selectivity coefficient
$[X^-]_s$, $[X^-]_m$	– analyte concentration in stationary/mobile phase
$[OH^-]_s$, $[OH^-]_m$	– hydroxide concentration in the stationary/mobile phase

Equation 1.12: Equilibrium constant

The different selectivity coefficients of the analytes lead to different retentions and thus to separation. Apart from ion-exchange processes, non-ionic interactions can be observed between the analytes and the stationary phase, most importantly adsorption. Both effects are usually superimposed and influence selectivity.

Ion exclusion chromatography, unlike ion exchange chromatography, is based on non-ionic interactions between the analyte and the stationary phase[180]. De-ionised water is mostly used as the mobile phase and a cation exchanger as stationary phase. The separation mechanism is based on three effects: Donnan exclusion, steric exclusion and adsorption. An hydration shell is formed around the ionic groups of the stationary phase called the Donnan layer. The Donnan

membrane has a partial negative charge and is only permeable for uncharged compounds, for instance water molecules. Fully dissociated anions are excluded from the stationary phase, because of their negative charge. If the analyte is a weak acid it will be protonated and not be affected by the Donnon exclusion. So this technique is particularily useful for the separation of weak inorganic and organic acids, but can even be applied to polar organic compounds such as alcohols, amino acids or carbohydrates.

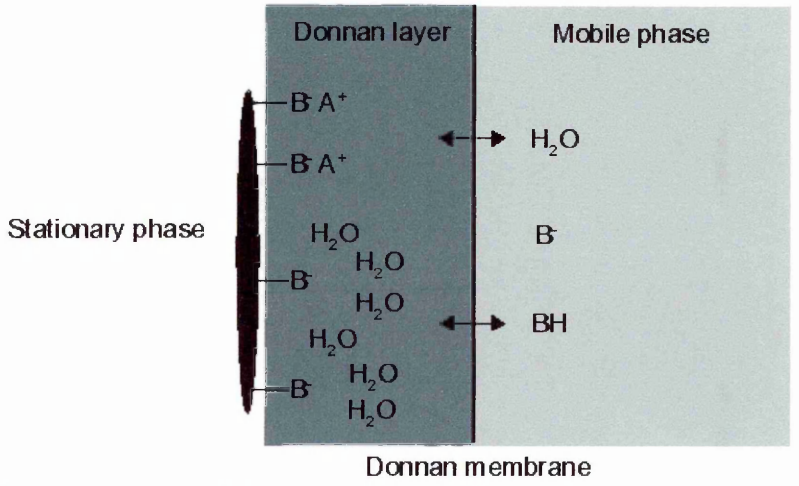

Figure 1.8: The mechanism of ion-exclusion chromatography

Ion-pair chromatography is usually done with a common reversed-phase column. The mobile phase contains a ion-pair reagent that usually consists of a hydrophobic and a ionic moeity. This process has not yet been fully explained. It has been suggested that the ion-pair reagent will arrange to a dynamic layer on the reversed phase material presenting the ionic part towards the mobile phase[181]. This gives the stationary phase ion-exchange properties. It has also suggested that the ionic analyte forms a complex with the hydrophobic ion-pair

reagent[182]. These ion-pairs are retained on the non-polar stationary phase. In any case, separation can be modified through a change in the concentration of the ion-pair reagent or the organic modifier in the mobile phase.

1.4.3 Stationary Phases for anion exchange chromatography

Ion exchange materials for ion chromatography are usually described by their support material, the pore size, the exchange capacity and degree of hydrophobicity[185].

The capacity of a packing material is commonly expressed in milli equivalent per gram resin (mequiv/g) rather than mmol/g and means number of ion-exchange charges present per gram of resin. A higher capacity usually means a longer retention time, but high capacity columns are also preferred when samples of high salt content are to be analysed.

Packing resins can either be microporous or macroporous. Microporous or gel-type packings have a pore size below 20 A and macroporous packings have a pore size between 20 – 400A. Macroporous materials are usually preferred, because they are mechanically more stable due to their higher degree of crosslinking and are not subject to swelling when organic solvents are used in the eluent. The pore size affects the selectivity, through a size exclusion mechanism.

The support material should be stable through a wide pH range to allow flexibillity in the pH of the eluent. Very alkaline or acidic conditions often used to suppress or enhance the dissociation of the analytes. Silica based packing

materials, as used in normal or reversed phase chromatography, can only be used in a pH range from 2-8, otherwise hydrolysis will occur and cause damage, so most packings for ion chromatography are based on organic polymers that can be used in a wide pH range. Styrene/divinylbenzene copolymers are the most widely used substrate materials, since they are stable in a pH range from 0-14. The substrate particles can be functionalised directly on their surface. The most common type of functionalisation is surface amination to produce an anion exchanger[183]. A higher efficiency than with directly surface-modified packings can be obtained with "pellicular" substrates[179]. These stationary phases are also called latex-based ion exchangers and are currently state of the art. Figure 1.9 shows schematic of a latex-based anion exchanger.

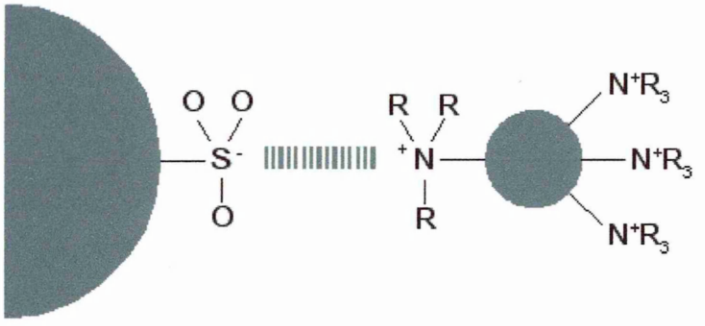

Figure 1.9: Pellicular latex-agglomerated anion exchanger

These materials consist of a surface sulphonated poly-styrene/divinylbenzene substrate particle with diameters between 5µm and 25µm. Fully aminated porous polymer beads of high capacity, the latex particles, of a size of 0.1µm are agglomerated on the surface of the substrate by both electrostatic and van-

der-Waals interactions. The latex-particles carry the actual anion exchange groups. The ion-exchange capacity of such a material is determined by size of the substrate particle, the size of the latex beads and the degree of coverage on the substrate surface.

The surface sulphonation of the substrate prevents the diffusion of inorganic species into the inner part of the stationary phase through Donnan exclusion, so the diffusion process is only controlled by the functional groups of the latex partices[184]. Different functional groups can be bonded to the latex particles and alter the selectivity. Depending on the functional groups bound to the quarternary amino group non-ionic interactions between the analyte and the funcional group can be reduced or increased. For instance a more hydrophobic functional group leads to longer retention for polarizable ions such as nitrate, bromide or iodide[185]. For iodide, which is easily polarisable due to its large radius a hydrophilic functional group is required to avoid long retention times and tailing. For ions that are not very polarisable, such as fluoride or chloride, the type of functional group does not affect selectivity.

1.4.4 The ion chromatographic system

An ion chromatography system is not much different from a classic liquid chromatography system[185]. The materials used have to be resistant to the corrosive eluents that are often used in ion chromatography and so instead of stainless steel polymers such as Teflon or PEEK are used. The detectors that are commonly used for ion chromatography include conductivity as the most

popular one, but also UV-VIS absorbance detection, electrochemical detection and fluorescence detection.

For conductivity detection, either an eluent with a low background conductivity is used (non-suppressed ion chromatography), or a suppressor module is used to reduce the background conductivity of the eluent (suppressed ion chromatography). A popular type of suppressor is an in-line membrane ion exchanger. For instance, for anion exchange chromatography a sodium hydroxide eluent can be used together with an post-column cation exchanger and conductivity detection.

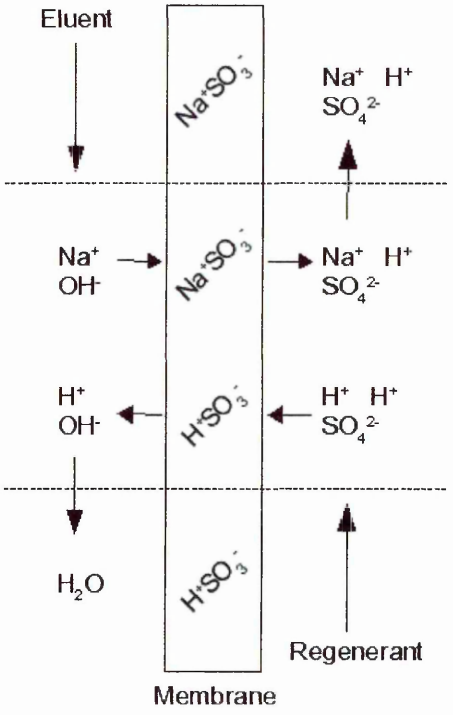

Figure 1.10: Membrane suppressor for anion exchange chromatography

The suppressor will reduce the background conductivity of the eluent by exchanging sodium for hydronium ions, that recombine with the hydroxide ions

to form water. Modern suppressors are designed as membrane suppressors with a membrane that consists of a sulphonated resin for anion exchange chromatography. The eluent is passed along a semipermeable membrane, where the actual ion exchange occurs. The driving force for the diffusion of the sodium ions is provided by the continuous removal of the diffused protons by reaction with the hydroxide from the eluent. To maintain the charge balance the sodium ions have to diffuse into the regenerant. This yields an eluate which is low in total ion concentration, suitable for introduction to a mass spectrometer.

1.4.8 Mass spectrometry as a detector for chomatography

i) Ion sources

The purpose of the ion source is to ionise the analyte prior to analysis in the mass spectrometer[186]. This can be achieved by ionising a neutral molecule through electron ejection, electron capture, protonation, deprotonation, adduct formation or the transfer of a charged species from a condensed-phase to a gas-phase. The two most important points for consideration are the internal energy transferred during the ionisation process and the physicochemical properties of the analyte that can be ionised. Ionisation techniques that are extremely energetic may cause extensive fragmentation, whilst other "softer" techniques only produce molecular species. In a liquid-phase ion source the analyte, which is in solution, is introduced by nebulisation as droplets into the mass spectrometer through various vacuum-pumping stages. Examples of this type of ion source are electrospray, sonic spray, particle beam, thermospray,

and atmospheric pressure chemical ionisation.

A popular type of ion source when coupling liquid chromatography to mass spectrometry is electrospray ionisation (ESI)[187]. ESI is said to be a 'soft' technique since little to no fragmentation of the analyte takes place under low voltages (i.e. the molecular ion tends to remains intact). Large amounts of solvent have to be stripped from the analyte molecules in a desolvatisation process. Desolvation is achieved gradually by thermal energy at relatively low temperatures. The eluent is sprayed into the ion source of the mass spectrometer and high electric fields are applied during nebulisation and desolvatisation. The microdroplets are charged by the voltage applied to the tip of the gas probe. Additional gas probes can assist to desolvate the analytes. During the desolvatisation as the droplets shrink the charge goes over to the analyte ionising the same. The orifice of the mass spectrometer is also protected by a nitrogen stream, the so-called gas curtain.

The interface to the mass spectrometer has been the major problem in the past, when ion chromatography and mass spectrometry are coupled. ESI is effective at desolvating eluents of relatively low volatility (e.g. water). A further problem with ion chromatography is the presence of a high content of involatile salts in the eluent, which can easily block the skimmer inlet to the vacuum phase of the mass spectrometer. For some heavier elements, the inductively-coupled plasma mass spectrometer (ICP-MS), is possible. Because of the high temperature of the plasma no special interface is necessary. Yamanaka et al. used an ICP-MS as a detector for ion chromatography and developed a a method for the

determination of bromate, iodate and other halogen anions in drinking water[188]. The ICP-MS instrument provides a very hard method of ionisation in the plasma torch, fragmenting the analyte into its atoms and so can be used as an element-selective detector. Other sample introduction systems have become available that allow other types of mass spectrometry to be used as a detector for ion chromatography. For non-suppressed ion chromatography volatile organic acids such as formic acid may be used as the ionic component in the eluent, and do not block the mass spectrometer interface. Chaimault et al[189] reported the separation and detection of 20 underivatised amino acids by ion-pair chromatography with detection by pneumatically assisted electrospray mass spectrometry. The eluent was made compatible by using volatile perfluoro heptane- and octane acid as ion-pair reagents. Huber et al[190] also used ion-pair chromatography with pneumatically assisted electrospray mass spectrometry for the analysis of nucleic acids with triethylammonium acetate as the volatile ion-pair reagent. Charles et al[191] developed a method using electrospray tandem mass spectrometry with ion chromatography for simultaneous analysis of oxyhalide ions in water. A volatile ammonium nitrate solution served as eluent and allowed the on-line coupling with negative ion electrospray mass spectrometric detection. Eluents with non volatile compounds can be used with IC-MS ion suppressor modules are employed to remove the problem ion. Ion suppressor modules were initially developed to be used for ion chromatography in conjunction with conductivity detection, but also provide an elegant solution for mass spectrometric detection. Hsu et al[192] demonstrated the successful

coupling of ion-exchange chromatography and mass spectrometry with a particle beam mass-spectrometry through the use of a membrane suppressor for continuous desalting. A mixed-phase column, having both reversed-phase and ion-exchange capabilities was employed. The particle beam interface was found to exhibit an analyte carryover especially for anionic compounds, that was independent of the choice of column or the use or absence of a membrane suppressor. Corr et al[193] accomplished the separation and detection of 13 inorganic anions by IC using an anion exchange column with a carbonate-bicarbonate mobile phase, on-line suppressed conductivity detection, and mass spectrometric detection using an ion spray atmospheric pressure ionization source. Roehl et al[194] used a sodium hydroxide eluent for suppressed ion chromatography and electrospray ionisation mass spectrometry for the anaysis of anionic environmental pollutants in water. Nowadays, suppressed ion chromatography systems are widely available and will provide lower detection limits when coupled with mass spectrometry than unsuppressed ion chromatorgraphy systems, due to the complete removal of the ionic component.

ii) Mass analyser

After ions are generated they are separated according to their masses in the mass analyser. Quadrupoles are among the most commonly used mass analysers for LC-MS systems and are more compact, fast, less expensive, and more robust than most other types of mass spectrometers[195]. One major advantage of using a quadrupole is its ability to perform high scan rates so that

a mass spectrum can be obtained in a short time, which is important for recording chromatograms. The quadrupole consists of four parallel cylindrical or hyperbolic rods, equally spaced around a central axis, that serve as electrodes. Opposing sets of rods are connected to a DC source, so that one pair is negatively charged and the other pair positively. An AC voltage component with a 180° phase shift is superimposed on the DC voltage between both pairs. As ions transit the quadrupole, both AC and DC voltages on the rods are changed simultaneously while maintaining their ratio constant. Hence through the application of controlled AC and DC voltages to opposing sets of poles, a mass filter is generated. Only those ions of a limited range of m/z (i.e. of a certain m/z) will pass through the mass filter, while all other ions collide with the rods and are converted to neutral molecules. Quadropoles are low resolution instruments allowing mass resolutions of approximately 1000, meaning that a mass of 100.1 can still be distinguished from a mass of 100.

1.5 Aims and objectives of the present work

Existing methods for measuring NOS turnover are either not capable of the isotopic discrimination required for metabolic studies using isotope labels, or have issues with loss or artifical generation of the analyte due to sample derivatisation, or are simply very expensive and time consuming. The aims of this work were to develop better techniques for stable isotope labelling experiments, in particular experiments employing ^{15}N-L-arginine, and to apply them to clinical studies. Two techniques were selected as possible solutions, surface enhanced resonance Raman spectroscopy (SERRS) and ion chromatography – mass spectrometry (IC-MS).

SERRS was chosen as an extension to the Griess assay, with Raman spectroscopy for detection instead of absorbance spectrometry. The Griess dye is an appropriate molecule for Raman spectroscopy for four reasons:

- The Raman effect relies on a change in polarisability of a bond with vibration, and so the π -cloud in an azo group is a good scatterer.
- Irradiation in the absorbance band of the Griess dye should lead to resonance enhancement of the scattering.
- The amino groups present on the Griess dye allow adsorption to silver and so surface enhancement can be used to multiply resonance enhancement by several orders of magnitude.
- Extending the Griess assay by SERRS not only makes it more sensitive and selective, but also allows isotopic discrimination between ^{15}N nitrite and ^{14}N

nitrite.

Suppressed IC-MS was selected for isotopic discriminative measurements of $^{14}N/^{15}N$-nitrite and $^{14}N/^{15}N$-nitrate based on previous work with IC and absorbance detection[196]. The anticipated advantages of IC-MS were:

- This method does not rely on pre-column derivatisation and so should be less susceptible to interference.
- Mass spectrometry should provide sensitive detection.
- Mass spectrometry should provide good discimination between ^{14}N and ^{15}N forms.
- The method might allow detection of other analytes of interest.

After method development, application to laboratory clinical samples was intended, e.g. in clinical studies of primary pulmonary hypertension, where the suppressed NOS turnover is thought to be the reason for hypertension.

2. Development of a Raman Spectroscopic Method

2.1 Experimental

2.1.1 Materials

Sulphanilamide (99%+), paraformaldehyde (95%+), 1-naphthylethylenediamine (98%+) and ^{15}N-sodium nitrite (99%) were obtained from Aldrich (Poole, UK). ^{14}N-sodium nitrite (99%) and hydrochloric acid were obtained from BDH (Poole, UK). Silver nitrate (99%+) was obtained from Lancaster (Eastgate, UK) and trisodium citrate (99-100%) from Prime Chemicals (Rotherham, UK). Analar-grade trisodium phosphate was obtained from BDH (Poole, UK). Distilled and deionised water was produced with a Waters Nanopure MilliQ system (Milford, USA) connected to a still. 100mg sized reversed phase C_{18} solid phase extraction cartridges were from International Sorbent Technologies (Glamorgan, UK) and centrifugal ultrafiltration cartridges with a molecular weight cut off of 5000 from Vivascience (Lincoln, UK). Arginine-free culture medium (RPMI1640, GIBCO, UK) was used for the experiments with the macrophages.

2.1.2 Equipment

Raman spectra were acquired using a Renishaw Raman System 2000 spectrometer, which comprised an integral Raman microscope (Olympus BH2 system), a stigmatic single spectrograph, and a Peltier-cooled CCD detector (400x600 pixels). The holographic notch filters allowed a lower spectral limit of approximately 100 cm^{-1}. The excitation wavelengths used were 514nm and

632nm, from 25 mW gas lasers.

2.1.3 Procedures

2.1.3.1 Preparation of silver colloid

Citrate based silver colloid was prepared based on the method described by Lee and Meisel[197]. 90 mg of silver nitrate were dissolved in 500 ml distilled water and heated to boiling under stirring. A 35 mmol/l citrate solution (10mL) was added quickly and the solution was kept boiling for 60 minutes with continous stirring. Colloid suspension was kept in the dark and used within 3 weeks.

Borohydride based silver colloid was prepared by adding 100ml of aqueous silver nitrate solution (2.5mmol/l) dropwise to 300ml of an ice water cooled aqueous sodium borohydride solution(2mmol/l). The solution was boiled for 60 minutes to remove excess borohydride and made up to 500ml with distilled water.

2.1.3.2 Sample cleanup

Urine samples were pretreated by C_{18} solid phase extraction. The cartridge was conditioned with 1ml methanol followed by 1 ml distilled water. 1 ml of urine was loaded on a cartridge and the fluid collected. Serum samples were additionally deproteinised by ultra-filtration with a 5kD cut-off filter at a speed of 5000 rpm at a temperature 3°C.

2.1.3.3 Griess Reaction

Nitrite standards were prepared with degassed MilliQ-water and kept sealed from air. Griess reactions were performed in sealed vials. For the recovery studies samples were spiked to 5µmol/l nitrite from a degassed nitrite stock solution. For the Griess reaction, 1ml of a 60 mmol/l aqueous sulfanilamide solution in 100 mmol/l HCl was added to 1ml sample followed by 1mL of a 5 mmol/l aqueous napthylethylenediamine solution. The samples were allowed to react in the dark for 15 minutes at room temperature, then neutralised with 500µl of a 200 mmol/l trisodium phosphate solution to stop the reaction. Samples were then passed through a C_{18} solid phase extraction cartridge to separate the dye from salts which would interfere with colloid aggregation in SERS. The cartridge was conditioned with 1 ml methanol followed by 1 ml degassed, distilled water. 1 ml sample was loaded and rinsed with 1 ml degassed, distilled water to leave the neutral dye on the cartridge. The dye was then eluted with 1 ml methanol, the methanol was evaporated under nitrogen and 1 ml 150 mmol/l sodium nitrate solution added as aggregant. Prior to analysis, 6 volumes of silver colloid were added to 1 volume of sample.

2.1.3.4 Raman Measurements

The laser was focussed through a 20 times objective on the sample solution and spectra were recorded for 100 seconds. When sampling from a cuvette, the aggregated sample was placed quickly in the cuvette so that no air was left and it was focussed onto the solution through the wall. When sampled from a

wellplate, the aggregated sample was imediately filled into one of the chambers on the wellplate and the laser was focussed in the center of the solution. When a dried sample was measured from a microscope slide a drop of the aggregated sample was placed on the microscope slide and it was dried at 40°C.

2.1.3.5 Activated Macrophages

Macrophages were prepared and activated by Dr. T.M. Stevanin (University of Sheffield). Supernatants from 12 day old human monocyte derived macrophages maintained in RPMI 1640 arginine-free culture medium were infected with 250 µL of a bacterial suspension containing approximately $3x10^7$ bacteria (MC58 wild type). After an incubation time of 20h, at 37 °C and 5% CO_2, the supernatants were then treated with 2% paraformaldehyde for 15 minutes at 37 °C to fix the bacteria.

Sample cleanup and Raman measurements were performed by the author. The samples were spun down and a dual C_{18} SPE cleanup was perfomed on them prior to Raman analysis as described above.

2.2 Results and Discussion

2.2.1 Sampling

Three different ways of acquiring Raman spectra have been studied. Firstly one can keep the aggregated sample in a cuvette, secondly a wellplate can be used or thirdly, the sample can be dried down onto a microscope slide after mixing with the aggregant.

Raman spectra can be taken from a liquid sample in a filled cuvette with a confocal setup of the microscope. Here, the focus of the laser beam has to be set just behind the glass wall. When the laser penetrates the sample solution too deeply, intensity is lost because the transmission through the colloid is low. Keeping the sample in a cuvette during the Raman measurement has the advantage that the sample does not evaporate. This is especially advantageous when samples contain organic solvents, methanol for instance.

Raman spectra can be taken from a liquid sample filled into a well plate by simply focussing on top of the solution. Care must be taken when sampling from a wellplate to focus in the center of the wellpate, because the surface of the liquid will not be even, but will form a meniscus. Sample evaporation can occur in this setup, but is low for purely aqueous solutions. However, samples containing organic solvents, for instance methanol, showed a significant reduction in volume by evaporation.

Laser irradiation in Raman spectrometry can lead to dye bleaching, but no problems with photobleaching were observed with liquid-suspension samples at the laser intensities used. Diffusion in and out of the beam and energy transfer

to solvent were possible with the aqueous suspension used, which probably accounts for this stability.

The third way of aquiring Raman spectra is to filter or dry the colloid down onto a microscope slide or another solid surface and then focus on the dry silver particles that have dye adsorbed on them. This will greatly increase the dye and colloid concentration, and may lead to enhanced sensitivity and simpler sample handling. However, attempts to improve sensitivity by drying down the colloid suspension were unsuccessful because photobleaching of the dye was observed with solid dried samples. Very intense spectra could be obtained for short measurement times, but it was not possible to sample for longer than a few seconds without destroying the dye completely. Another disadvantage is that the colloid aggregation or more precisely the size of the colloid particles could not be controlled. Due to the longer time needed to dry the sample under a nitrogen stream, the precipitated colloid particles were much bigger than the ones present in the liquid samples.

2.2.2 Aggregation control

i) Control of interferences

It is well known that the ionic strength of the sample solution influences colloid aggregation and hence signal intensity in SERRS[198]. Controlling aggregant type and concentration is therefore of critical importance and optimisation is required. Initial experiments indicated that, when biological samples were analysed, the recoveries were very low and variable. The problems of poor

reproducibility with serum and urine samples were found to be due to varying ionic strengths between samples which differentially affected aggregation.

A sample preparation step was devised to both extract (and if necessary concentrate) the Griess dye, and remove salts and acids which would cause irreproducible aggregation. By removing the salt-laden matrix ,then susequently adding a uniform type and concentration of aggregant, aggregation could be controlled to give good reproducibility. A dual C_{18} SPE cleanup was performed on the urine samples as described in Figure 2.1.

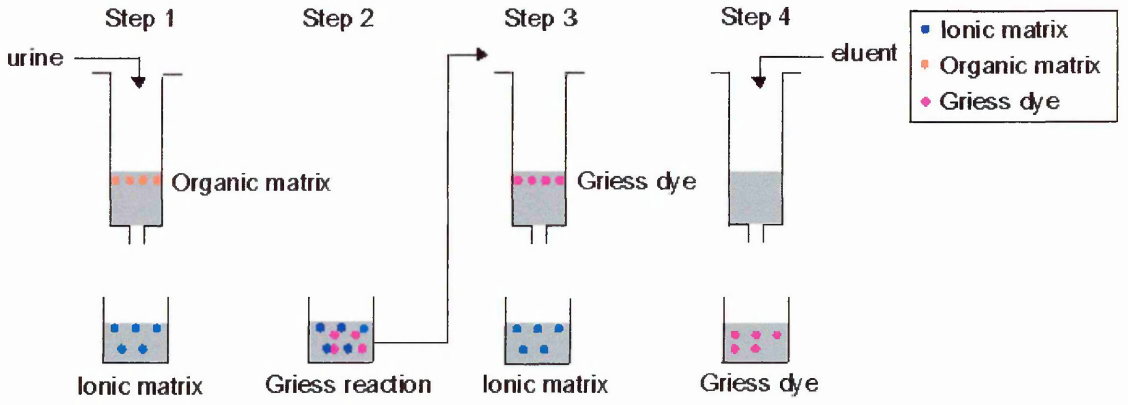

Figure 2.1: SPE sample cleanup for SERRS samples

After conditioning, urine was loaded on the first C_{18} SPE cartridge to retain the organic portion of the matrix. The ionic filtrate was collected and the Griess reaction was performed on it. The analyte was so converted from an ionic species to an organic one. Since the organic portion of the matrix was already removed, a second SPE step could selectively separate the analyte from the remaining portion of the matrix. For serum samples that are usually high in protein an ultra-filtration step has to be employed before the dual C_{18} SPE cleanup. Proteins can not be completely removed with a C_{18} cartridge.

When a SPE separation was performed on the samples after the Griess reaction the dye was retained on the cartridge and thus separated from the acidic aqueous solvent. So after elution with metanol the dye was present in the preferred neutral form, with a more useful SERRS spectrum than the acidic form. After the sample pretreatment the aggregant (i.e. sodium nitrate) was then used for aggregation as described in the experimental section to achieve uniform aggregation between all samples and calibration standards.

ii) Selection of aggregant

Chloride has been reported as an aggregant ion[198], but in this work no significant enhancement was seen with chloride, bromide or iodide as aggregants in a concentration range from 0 to 160mmol/l. Organic acids such as acetate or citrate are not useful as aggregants here because they show bands at 1400 cm^{-1} from the interaction of carboxylate groups with the silver surface, which interfere with the diazo band at 1420 cm^{-1}. Smith et al[199] observed a band at 1400 cm^{-1} for the COO vibration and the appearance of shoulders at 1412 and 1370 cm^{-1}, which could also be seen in a spectrum of acetate (figure 2.2).

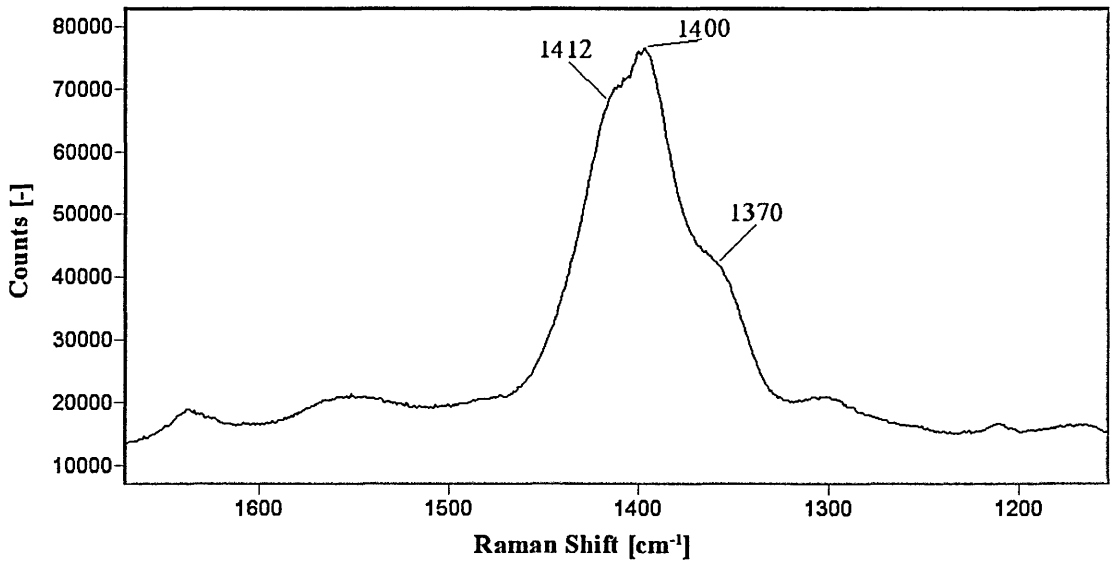

Figure 2.2: SERRS spectrum of 50mmol/l actetate.

Sodium nitrate was selected as aggregant here, because it is in the silver colloid already (from the production process), and it does not show significant Raman bands in the frequency range examined. The amount of nitrate added to the sample as aggregant is an important parameter and was optimised as shown in figure 2.3.

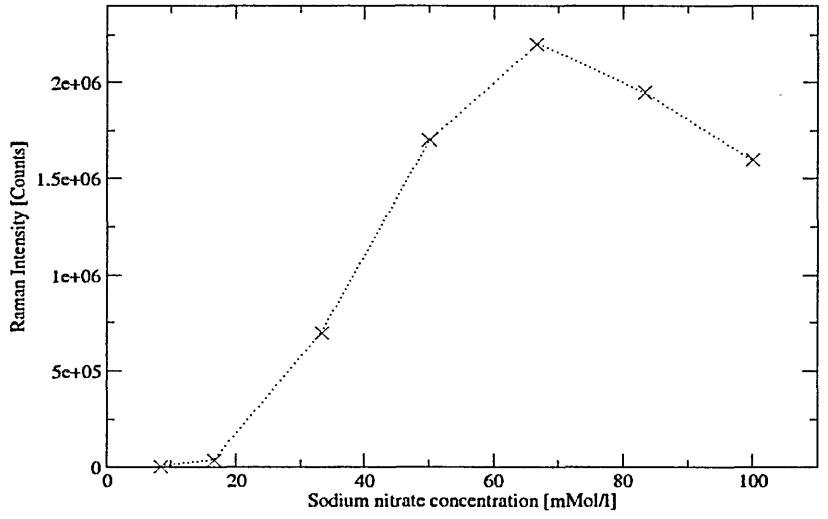

Figure 2.3: Raman intensities for a 100nM standard at different sodium nitrate concentrations.

Usually, there is an optimum aggregant concentration, which gives the strongest signal. Variation of aggregant concentration showed an increase in signal intensity up to 65 mmol/l sodium nitrate, with a decrease thereafter as the silver colloid became unstable and started to precipitate. In the presence of methanol, less aggregant was needed to obtain optimum aggregation (25mmol/l).

An additional problem arises from the citrate used to generate the silver colloid. Citrate itself adsorbs to the silver colloid and generates an interfering band at 1400cm^{-1} with shoulders at 1412 and 1370 cm^{-1}, as shown in Figure 2.4.

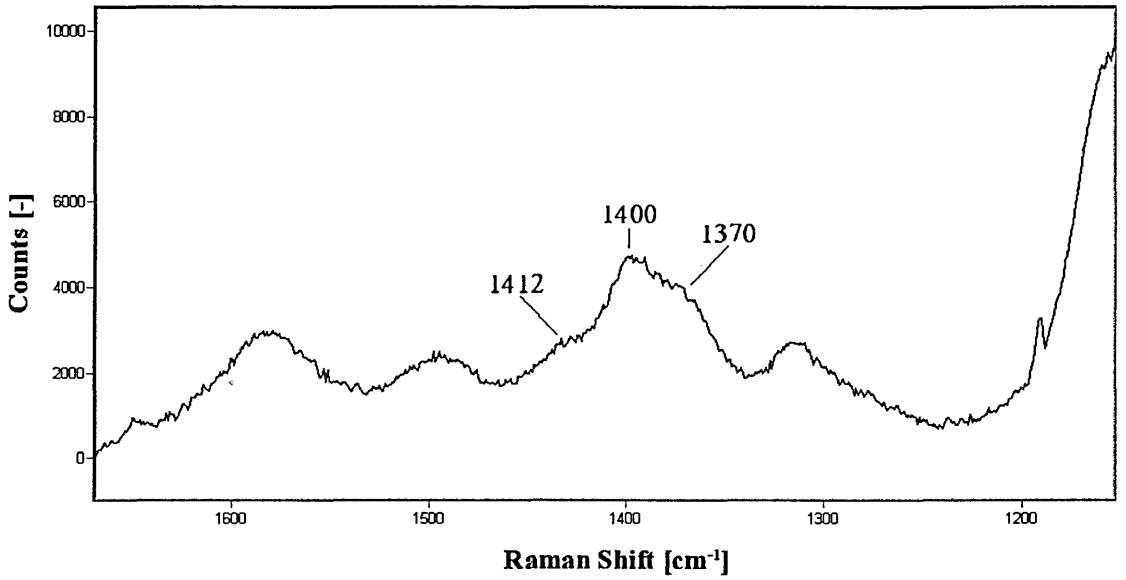

Figure 2.4: Bands obtained from silver colloid aggregated with 20mmol/l sodium nitrate.

The intensity of these bands increased with increasing concentration of the sodium nitrate aggregant. For measurements of nitrite concentrations below 20 nmol/l it was best to work at aggregant concentrations of 20mmol/, to reduce the size of these interfering bands.

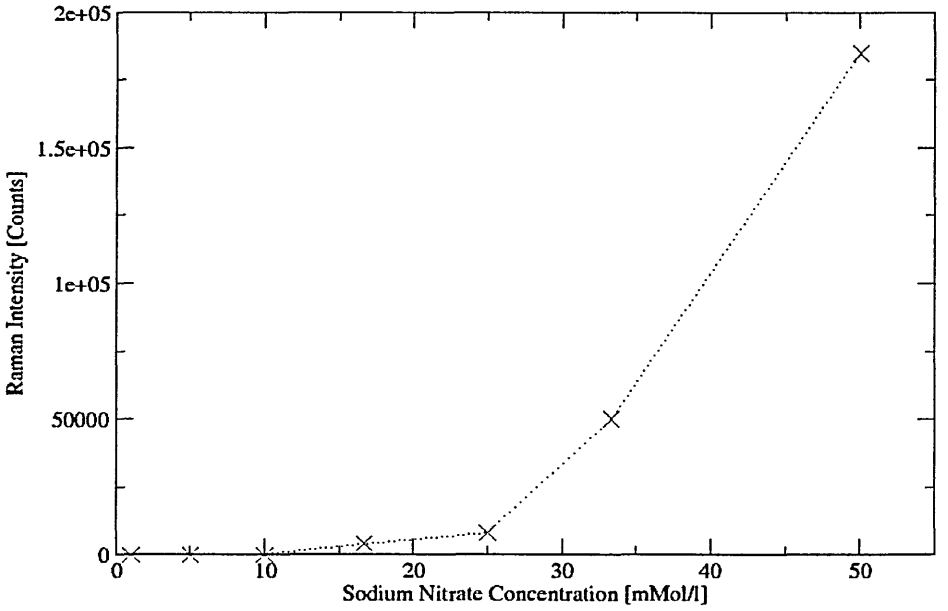

Figure 2.5: Intensity of the citrate bands at different sodium nitrate concentrations

The citrate peaks disappeared when colloid prepared by the borhydride method was used. The use of citrate for colloid preparation can not be omitted currently, because citrate is not only used to generate the silver colloid, but also to stabilise it once it is produced. The silver colloid produced by the borohydride method was found to be inferior in both stability and sensitivity of the SERRS measurements it was used for. The colloid produced by the borohydride reaction was not as dense as the one produced by the citrate method, settled down too quickly after aggregation and was not stable for longer than a few hours.

2.2.3 Acidic versus neutral

The Griess assay must be performed under acidic conditions otherwise the

diazonium salt is not formed and the reaction will not take place. As shown in figure 2.6, the N=N stretch band is much weaker in the protonated form than in the neutral form.

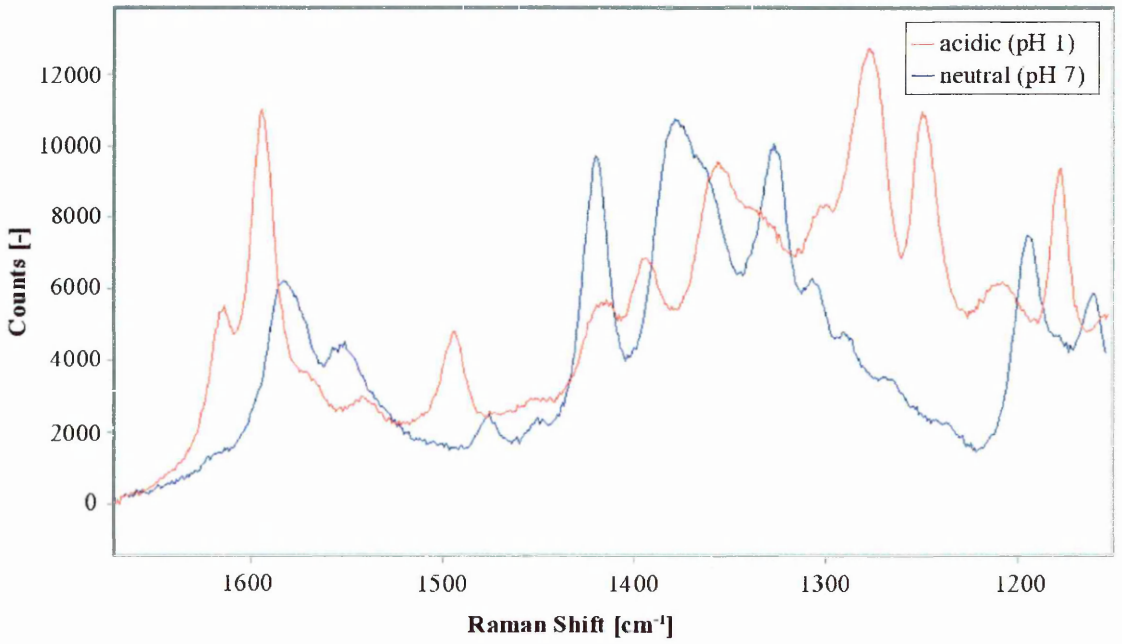

Figure 2.6: Raman spectra taken under acidic and neutral conditions

Although the C-N band (1279 cm^{-1}) is stronger in the spectrum of the protonated form it is not useful for isotopic discrimination because it overlaps strongly with another band. Acid can be used for aggregation of the silver colloid, it is undesirable both because the silver colloid will be dissolved slowly and because the spectrum obtained for the Griess dye in its protonated form is less useful than the spectrum obtained for the Griess dye in neutral form.

2.2.4 Choice of laser source

At neutral pH, the visible absorbance maximum of the Griess dye is at 496nm,

while in acid solution the protonated form of the dye absorbs maximally at 550nm, so a laser source emitting at a wavelength of 514nm would be expected to give a greater resonance enhancement than a laser source emitting at 632nm.

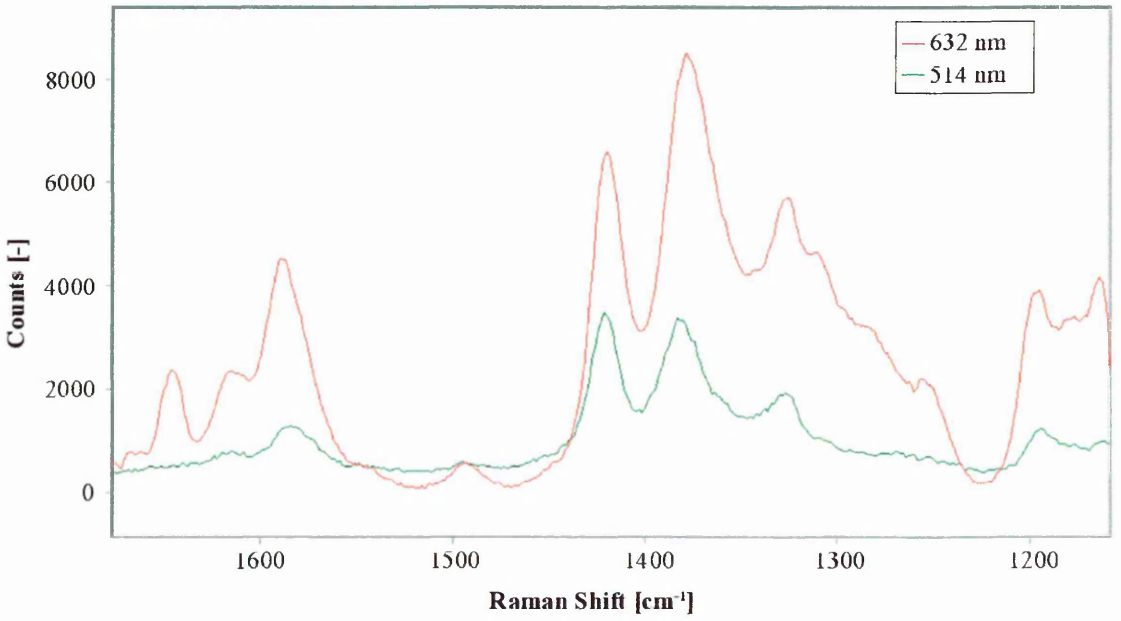

Figure 2.7: Raman spectra taken with different laser sources. Green: 514nm, red: 632nm.

However, when choosing a laser source for this Raman method it is important to use the dye in the presence of silver colloid, since the optimum for the dye adsorbed to silver may differ from the optimum for the free dye. This is because the colloid surface plasmon absorption is also important. For the Griess dye adsorbed to silver colloid, strong resonance and surface enhancement can be seen for the chromophore and the band corresponding to the N=N stretch (1420 cm^{-1}) is strong. Overall sensitivity was worse at 514nm than at 632nm, so a red laser was preferred. This may indicate more efficient excitation of the dye via

the silver surface plasmon absorption, and for stronger surface enhancement may have been obtained with the laser source emitting at 632nm. Resonance between the laser light and the surface plasmons created on the surface of the silver colloid is required to achieve surface enhancement.

2.2.6 Quantitative analysis

For quantitative measurements, the band seen for the N=N stretch at 1420 cm^{-1} is more useful than the band corresponding to the C-N vibration seen at 1279cm^{-1}, which is weaker at neutral pH. The band area was used to determine nitrite concentration. A limit of detection of 5 nmol/l nitrite was achieved under these conditions, which is easily adequate for most nitrite analyses. It represents a considerable enhancement over the limit of detection achieved by absorbance measurement of the Griess dye, even though much lower detection limits (down to 8 x 10^{-16} mol/l) have been reported using SERRS with other dyes[168]. Calibration curves with freshly prepared colloid were linear up to 10 µmol/l with good correlation (r = 0.995 and higher). This linearity and reproducibility shows what can be achieved with SERRS if colloid quality and aggregation conditions can be controlled, and the results did not need correction procedures[200] or flow injection systems[201] to achive good quantification. To achieve this reproducibility it was necessary not only to introduce the SPE dye extraction step to control colloid aggregation and sample pH, but also only to use silver colloid for a few weeks after production. Old colloid (months of storage) started to show non linear calibration curves even

though the same limit of detection could be obtained with it. Recovery data from spiked urine, serum and cell culture media measured by absorbance spectroscopy and SERRS are shown in Table 2.1. Recoveries (n=5) from spiked urine averaged 96% (range from 91.6% to 98.7%), from serum 85% (range from 79.8% to 90.3%) and from culture medium 95% (range from 88.7% to 101.2%).

	Urine recovery [%] Absorbance	***Urine recovery [%] SERRS***	***Serum recovery [%] SERRS***	***Culture media recovery [%] SERRS***
Sample 1	95.8	91.6	81.3	101.2
Sample 2	92.5	94.4	79.8	97.5
Sample 3	96.3	97.7	89.2	88.7
Sample 4	91.9	97.3	90.3	93.9
Sample 5	98.5	98.7	84.5	92.7
mean	**95**	**96**	**85**	**95**

Table 2.1: Recoveries from biological fluids by the Griess assay with detection by absorbance spectroscopy and SERRS.

The average nitrite concentration of serum samples was 1.8 µmol/l nitrite, while the average nitrite concentration of urine samples was 750 nmol/l. These recoveries from complex biological matrices such as urine and serum were good, allowing the method to be used for such samples. Similar recoveries were found using the colorimetric Griess assay, indicating that the more elaborate sample preparation for SERRS did not adversely affect recovery.

2.2.7 Isotopic discrimination

Isotopic discrimination can be achieved in vibrational spectroscopy because the resonance frequency of a ^{15}N labelled diazo group ($^{15}N=^{14}N$) will be lower than the resonance frequency for an unlabelled diazo group ($^{14}N=^{14}N$). Figure 2.8 compares the SERRS spectrum from dye prepared with 20 nmol/l ^{15}N-nitrite with the SERRS spectrum from dye prepared with 20 nmol/l ^{14}N-nitrite.

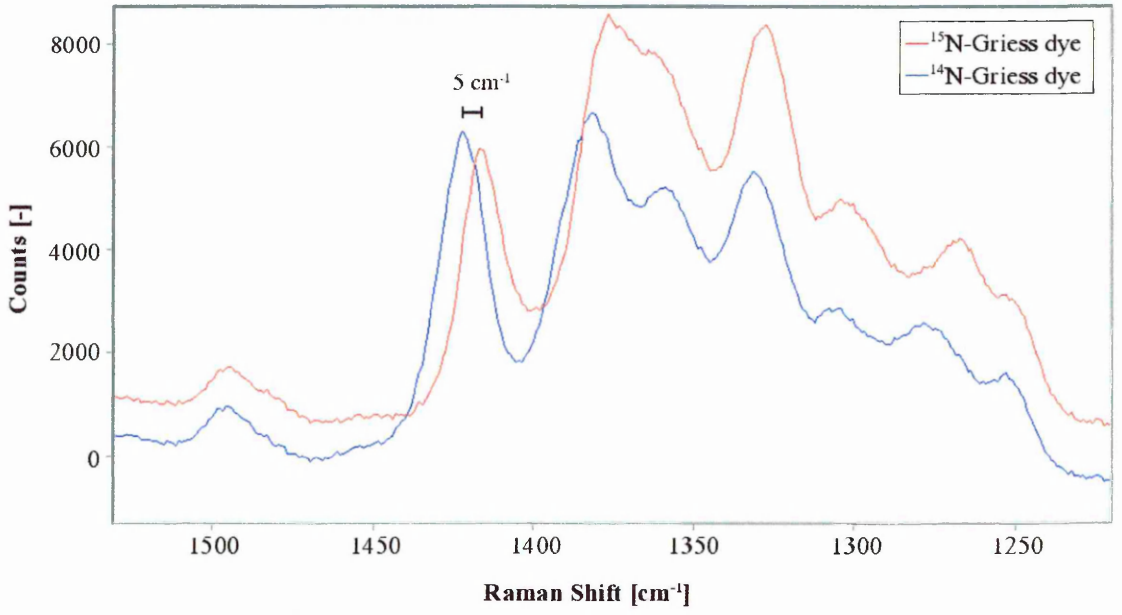

Figure 2.8: Spectra of the Griess dye prepared from 20nmol/l ^{15}N-nitrite (red) and Griess dye prepared from 20nmol/l ^{14}N-nitrite (blue).

The bandshift resulting from one nitrogen substitution was 5 cm^{-1} for the N=N stretch and 10 cm^{-1} for the C-N stretch, which overlaps with another band. No band shifts were observed for any other bands in the spectrum. Biswas et al[202] reported band shifts of 18-34 cm^{-1} for the azo stretch in different azo dyes upon substitution of one of the nitrogens by ^{15}N. Presumably the structural environment of the azo group in the Griess dye results in a less dramatic shift

upon ^{15}N substitution than in the dyes examined by Biswas et al.

When a mixture of labelled and unlabelled Griess product was analysed, the two bands could not be resolved completely but always appeared as one band, shifting to lower peak wavenumber as the proportion of ^{15}N labelled dye increased. Fourier self deconvolution, 2nd derivative analysis or band fitting did not enhance the $^{15}N/^{14}N$ resolution obtained. So the band maximum frequency was used to obtain the $^{15}N/^{14}N$ ratio as shown in Figure 2.9.

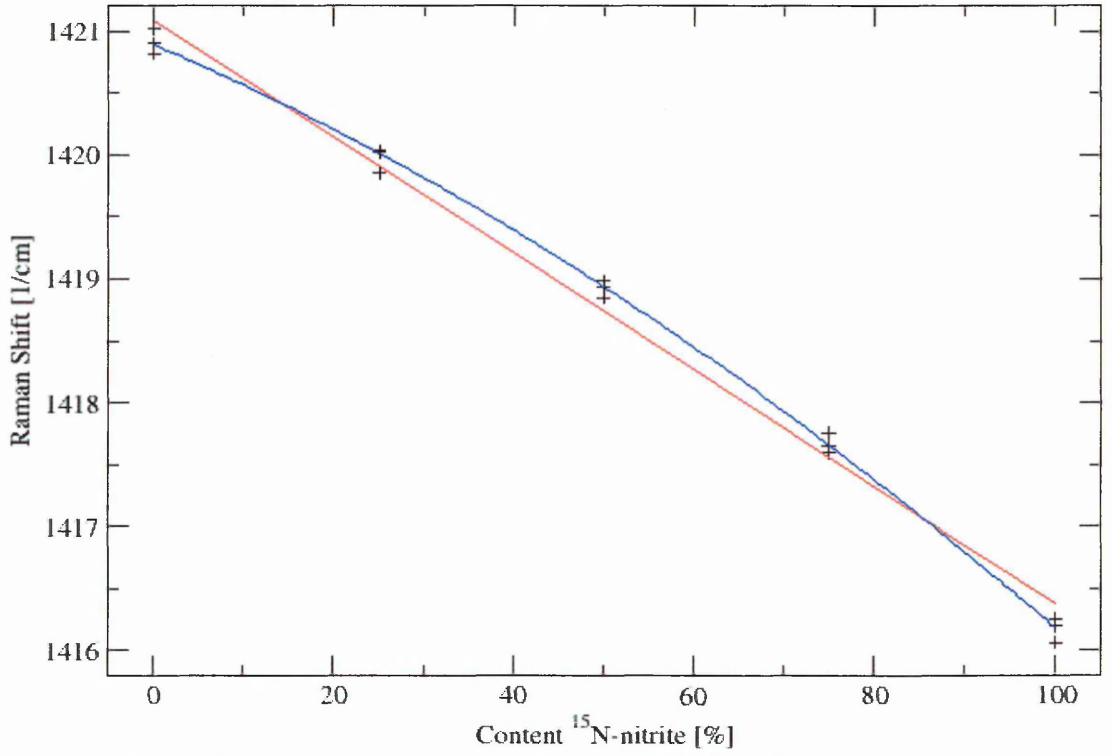

Figure 2.9: Calibration curve to determine the ^{14}N-nitrite/^{15}N-nitrite ratio. Triplicates were measured for each standard concentration. Red: linear regression, Blue: non-linear regression (quadratic)

A near linear relationship was seen between the band maximum frequency and the ^{15}N content, with a correlation coefficient greater than 0.996. This allowed

detection of ^{15}N nitrite content to a minimum of 5%. Improvements in band resolution, either by hindering of rotation or by computer aided sharpening may help, though noise may be increased.

2.2.8 Cell Culture Media

12 day old human monocyte derived macrophages were incubated with ^{15}N-labelled L-arginine. On activation of macrophages, iNOS is expressed and can produce NO in large quantities[203]. Subsequently, nitrate as a major NO metabolite should be determinable easily with the developed SERRS method. However, total nitrite levels in these samples were low ranging at about 500 nmol/l, being identical to the nitrite background levels of the cuture medium alone and no ^{15}N-nitrite was detectable. When the samples were spiked to 500 nmol/l ^{15}N-nitrite the usual band shift could be observed, which means that in fact no significant amounts of nitrite were present in the samples.

One possible explanation is that the macrophages were not activated properly and thus did not express iNOS, even though a prolonged in vitro cultivation of 10-12 days was reported to trigger NO production in macrophages[204]. Without such an *in vitro* cultivation procedure, or if the procedure is insufficient, macrophages are not activated. As a result no nitrite would have been produced and only background nitrite would have been detectable.

Another possibility is that the macrophages were activated, but the produced nitrite was converted to other compunds, for instance by oxidation to nitrate during the incubation process. In this case, since nitrite is an intermediate in the

oxidation process, the ^{14}N-nitrite present at the beginning of the experiment would have been oxidised to nitrate as well and being replaced by ^{15}N-nitrite. Total nitrite was determinable in the samples being only ^{14}N-nitrite. This indicates that activation probably failed in the samples and no iNOS was expressed. Further indication that the activation of the macrophages may have failed comes from western blots perfomed from the macrophages. These did not clearly show the presence of iNOS after incubation.

2.3 Conclusions

It has been shown that highly sensitive measurements of nitrite are possible using an extension of the Griess assay with surface and resonance enhanced Raman spectrometry. In summary:

- Resonance and surface enhancement were achieved by adsorbing the Griess dye in its neutral form onto colloidal silver and aggregating the colloid prior to excitation with a 632 nm laser. No improvement was seen by employing a green laser with an excitation wavelength close to the absorbance maximum of the Griess dye.

- Sampling was most convenient with a wellplate. High reproducibility between samples and standards was achieved by separating the Griess product from the ionic matrix by solid phase extraction prior to aggregation.

- Calibration curves were linear with correlation coefficients higher than 0.995. In spite of interfering bands from citrate present in the silver colloid, a limit of detection of 5 nmol/l for nitrite was obtained. Isotopic discrimination between the ^{15}N and ^{14}N isotopes in the Griess dye was possible at $^{15}N/^{14}N$ isotopic ratios of 1:20 or greater.

- ^{15}N-nitrite could be detected in urine, serum and cell culture medium with recoveries of 96% (range from 91.6% to 98.7%), 85% (range from 79.8% to

90.3%) and 95% (range from 88.7% to 101.2%) respectively.

- It was not possible to detect ^{15}N-nitrite derived from ^{15}N-L-arginine by activated macrophages. However, the problem was identified as failure to induce iNOS and not the analytical method itself.

- This simple and straightforward method is a valuable tool for high-throughput analysis of NO turnover in biological studies, where higher concentrations of ^{15}N-L-arginine can be applied. It is, however, not useful for *in vivo* human studies where ^{15}N-nitrite is usually present at levels below the ratio of isotopic discrimination that is possible with this method.

3. Development of an IC-MS method

3.1 Experimental

3.1.1 Materials

Nitrite and nitrate standards were prepared by dissolving an appropriate amount of Analar-grade ^{14}N-sodium salt (BDH) or the ^{15}N-sodium salt (99 atom% Aldrich). HPLC-grade methanol (Fisher) and chloride-free sodium hydroxide (puriss. p.a, Fluka) was used to prepare the eluents. Milli-Q water with a resistance of greater than 18MΩ was used for all solutions. 30% hydrogen peroxide solution (BDH, UK) was used to prepare the solution for post-column oxidation. For sample preparation, 200 mg C_{18} solid phase extraction cartridges (International Sorbent Technologies LTD, Glamorgan, UK) and ultrafiltration cartridges with a molecular weight cut off of 5000 (Vivascience, Lincoln, UK) were used.

3.1.2. Equipment

3.1.2.1. Ion chromatograph

A Dionex suppressed ion chromatography system equipped with a Dionex ASRS-II Ultra anion-suppressor and a Dionex AS-11HC column with a diameter of 4mm and a length of 25 cm together with a guard column was used. PEEK capillary (Suppelco, UK) was used to connect the components. An ESA 5020 guard cell (ESA Inc, USA) was used for electrochemical oxidation.

3.1.2.2. Mass Spectrometer

The mass spectrometers used were a Micromass Platform instrument and a Micromass Quattro instrument both equipped with an electrospray interface. A

Harvard syringe pump (Harvard Apparatus, USA) was used for the direct infusions.

3.1.3 Procedures

3.1.3.1 Direct infusion experiments

A syringe pump was directly connected to the electrospray interface of the mass spectrometer via HPLC PEEK tubing. A solution of the analyte of a concentration of 50 µmol/l was prepared in degassed water containing 50% methanol. The solution was infused at a flowrate of 10µmol/l. 500 µl were pumped through the suppressor module at a higher flowrate first, before mass spectra were recorded to displace the void volumn in the suppressor module. For direct infusion through the suppressor module, the suppressor was fitted between the syringe pump and the electrospray interface.

3.1.3.2 Mass spectrometer setup

The mass spectrometer was tuned for the detection of nitrite, nitrate and the NO donor compounds. A capillary voltage of 2 kV, a cone voltage of 30 V was used. 0.25 kV were used for the HV Lens, 2.5 V for the ion energy and 1 V for the ion energy ramp. The source temperature was 85°C and a mass resolution of 15 was used. A dwell time of 0.5s was used for the m/z=62 mass trace and a dwell time of 2.0s was used for the m/z=63 mass trace.

3.1.3.3 Sample preparation

Urine samples were pretreated by C_{18} solid phase extraction using 200 mg C_{18} solid phase extraction cartridges (International Sorbent Technologies LTD, Glamorgan, UK). A cartridge was conditioned with 1ml methanol followed by 1 ml distilled water. 1 ml of urine was loaded on a cartridge and the fluid collected for injection. Serum samples were deproteinised by ultra-filtration using ultrafiltration cartridges with a molecular weight cut off of 5000 (Vivascience, Lincoln, UK) at 5°C and a speed of 6000 rpm. The filtrate was used for analysis.

3.1.3.4 Suppressed IC-MS with MS detection

The eluent was prepared by adding methanol to Milli-Q water and degassing for 15 minutes. The concentrated hydroxide solution was then added quickly to minimise contact with air. The eluents were kept under helium to prevent any carbon dioxide dissolving leading to the formation of carbonate. Standards were made up from degassed eluent (mathanol/water 30:70) without sodium hydroxide. A flowrate of 0.5 ml/min was used for chromatography and a split of 1:15 was applied by using a T-piece to reduce the flowrate for the mass spectrometer.

For urine samples a two step gradient was used in the analysis beginning with a sodium hydroxide concentration of 20mmol/l for 20 minutes, then 40mmol/l for 30 minutes and finally a 10 minutes equilibration step at a concentration of 20mmol/l. The methanol concentration in the eluent was 30%.

For serum samples a linear gradient from 15mmol/l to 20mmol/l sodium

hydroxide within the first 20 minutes of the run was used and then followed the same procedure as for urine samples.

3.1.3.5 IC-MS with post-column oxidation

Post-column oxidation was achieved through an auxiliary HPLC pump that fed in hydrogen peroxide solution at a low flowrate via a T-piece after the column and before the suppressor module. For urine and serum samples 0.5% hydrogen peroxide solution was added at a flowrate of 0.1 ml/min.

3.1.5.6 IC-MS with oxidation and chloride removal

Chloride trap columns were prepared by packing a 4mm guard column with the packing material from a Dionex OnGuard II Ag SPE cartridge. 10 ml of degassed MilliQ-water were pushed through the chloride trap column to remove any mobile silver ions from the resin, prior to use. The chloride trap column was placed between the suppressor and the eluent split.

3.1.3.7 IC-MS with electrochemical oxidation

The ion chromatograph was equipped with two Dionex AS-11 columns with the electrochemical cell in between them. The suppressor was fitted after the second column and before the split and mass spectrometer. A potential of 1V was applied to the electrochemical cell. An isocratic method was applied using an eluent consisting of degassed MilliQ-water containing 30% methanol at a sodium hydroxide concentration of 20 mmol/l.

3.1.5.8 Recovery Study

Urine and serum samples from independent batches were spiked to 10µmol/l ^{15}N-nitrite and ^{15}N-nitrate in separate sets (n=5) prior to sample cleanup. Pairs of spiked and unspiked samples were analysed by ion chromatography with post-column oxidation and a chloride trap. Recovery was calculated by substracting the concentration of analyte found in the unspiked sample or from the amount of analyte found in the spiked sample and dividing by the spike concentration.

3.2 Results and Discussion

3.2.1 IC-MS with oxdiation and chloride removal

The initial direct infusion experiments showed strong mass bands for nitrite and nitrate for both the ^{14}N and ^{15}N species as shown in figure 3.1.

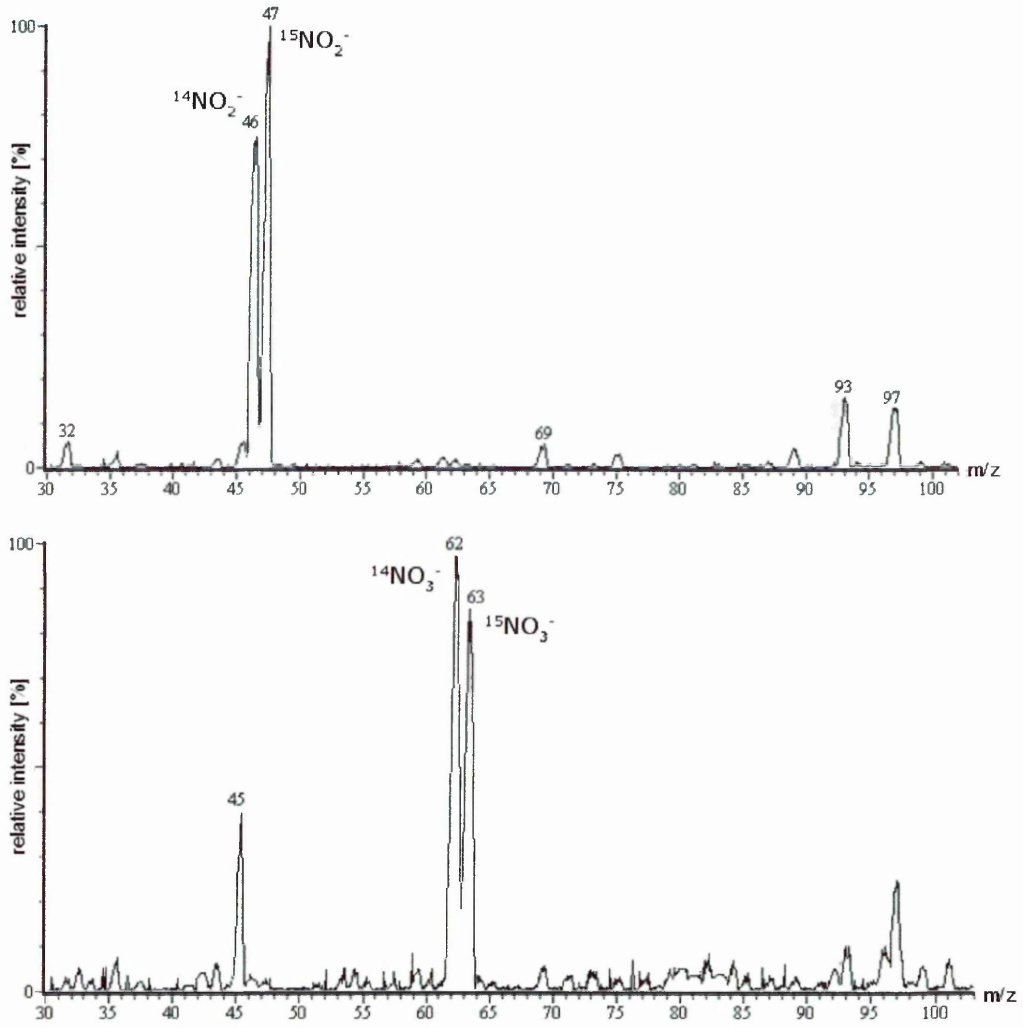

Figure 3.1: Mass spectra obtained from direct infusion of a solution containing ^{14}N-nitrite and ^{15}N-nitrite (50µmol/l each) (top) and ^{14}N-nitrate and ^{15}N-nitrate (100µmol/l each) (bottom).

When it was attempted to measure nitrite by IC-MS, the sensitivity for nitrate was very low on the m/z = 46 and m/z = 47 mass trace and even though nitrite

was present in at a high concentration no peak was seen in the chromatogram. It was suspected that nitrite itself does not ionise very well in the ion source of the mass spectrometer, when the suppressor module was used. So the direct infusion experiment was repeated, and the experimental stup changed so that direct infusion occurred through a suppressor module, as it was used in the IC-MS measurement.

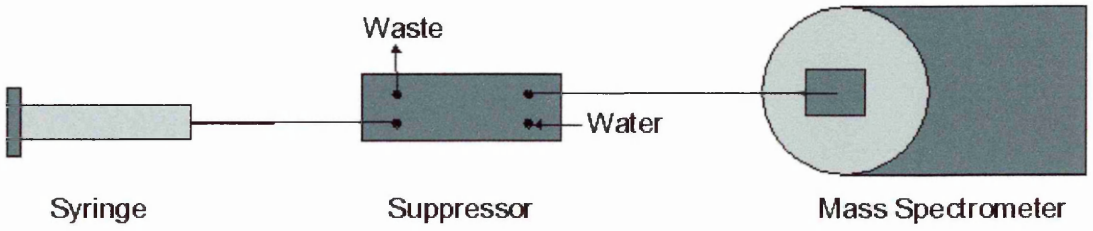

Figure 3.2: Experimental set up for direct infusion with a suppressor module

Figure 3.3 shows the mass spectrum obtained from direct infusion experiments with a solution containing 50µmol/l ^{14}N-nitrite and ^{15}N-nitrite. The mass bands for ^{14}N-nitrite and ^{15}N-nitrite ions are approximately 100 times smaller when a suppressor module was used for direct infusion. Two effects could be observed in this experiment that are responsible for the low sensitivity for nitrite, the oxidation of nitrite to nitrate and the formation of the water adduct. Since a mixture of ^{14}N-nitrite and ^{15}N-nitrite was infused the occurance of two adjacent mass bands of similar size indicates a conversion product of nitrite. The occurance of the mass bands at m/z = 62 and m/z = 63 indicates some oxidation to nitrate. The mass bands at m/z = 80 and m/z = 81 result from a nitrate/water adduct. An adduct of water and nitrite molecules was not observed.

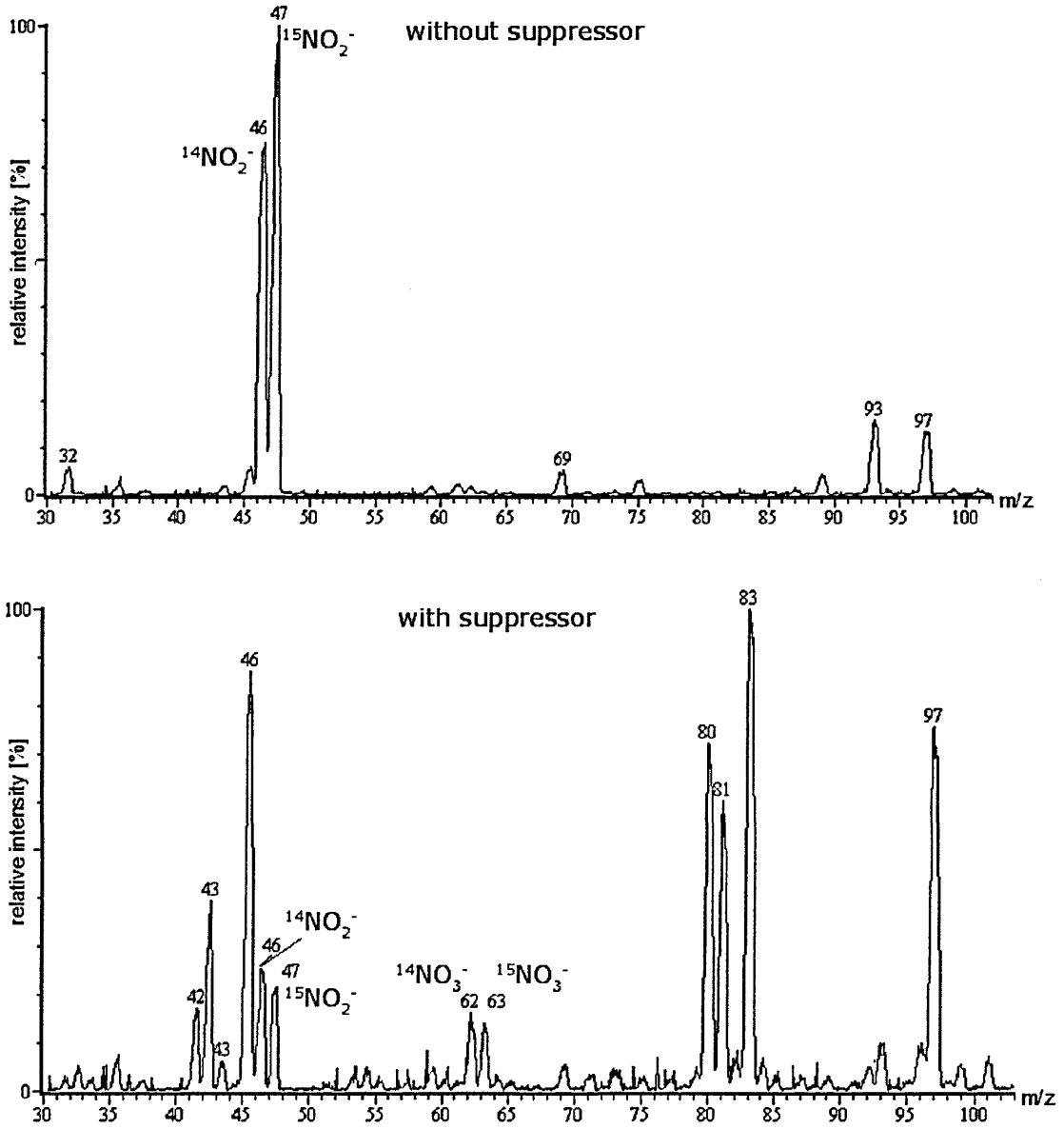

Figure 3.3: Mass spectra obtained from direct infusion of a solution containing ^{14}N-nitrite and ^{15}N-nitrite (50µmol/l each) directly (top) and through the suppressor module (bottom).

Both effects are relatively weak and the bands originating from the conversion of nitrite to nitrate are even smaller than the bands for nitite ions. Even though a major converion product of nitrite could not be identified, the experiment

showed that the suppressor module introduces changes to the eluent that increase the reactivity of nitrite. The suppressor module is essentially a cation exchanger and lowers the pH of the eluent by 1 to 2 pH units, since it is exchanging omnipresent cations in the eluent to hydronium ions. The slightly acidic conditions introduced by the suppressor module may allow the formation of nitrous acid and its disproportionation to nitric oxide and nitrate in the ion source and simple catalysis of the oxidation to nitrate, which was observed to some extent. Other reactions are also possible in the environment of the ion source of a mass spectrometer that can yield different charged and uncharged molecules or fragments as conversion products of nitrite, thus diminish the sensitivity.

The post-column oxidation of nitrite to nitrate presents an elegant solution to this problem.

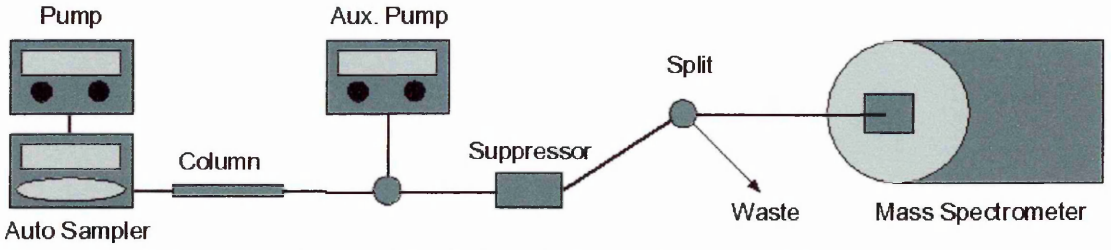

Figure 3.4: Instrumental setup for chemical post-column oxidation

The setup differs from classic IC instruments that hydrogen peroxide solution is mixed into the eluent stream after the column. Nitrate did not show a loss in sensitivity when the suppressor module was employed or post-column oxidation was used. Furthermore, both species could be measured in one mass trace,

which yields general enhancement in sensitivity for quadrupole mass spectrometers because the dwell time does not have to be split between two mass channels. The amount of hydrogen peroxide added post-column to the eluent stream was optimised to maximise and if possible achieve complete conversion. A solution of a nitrite and nitrate concentration of 25µmol/l was injected and for each run the concentration of the added hydrogenperoxide was increased. Figure 3.5 shows the plot.

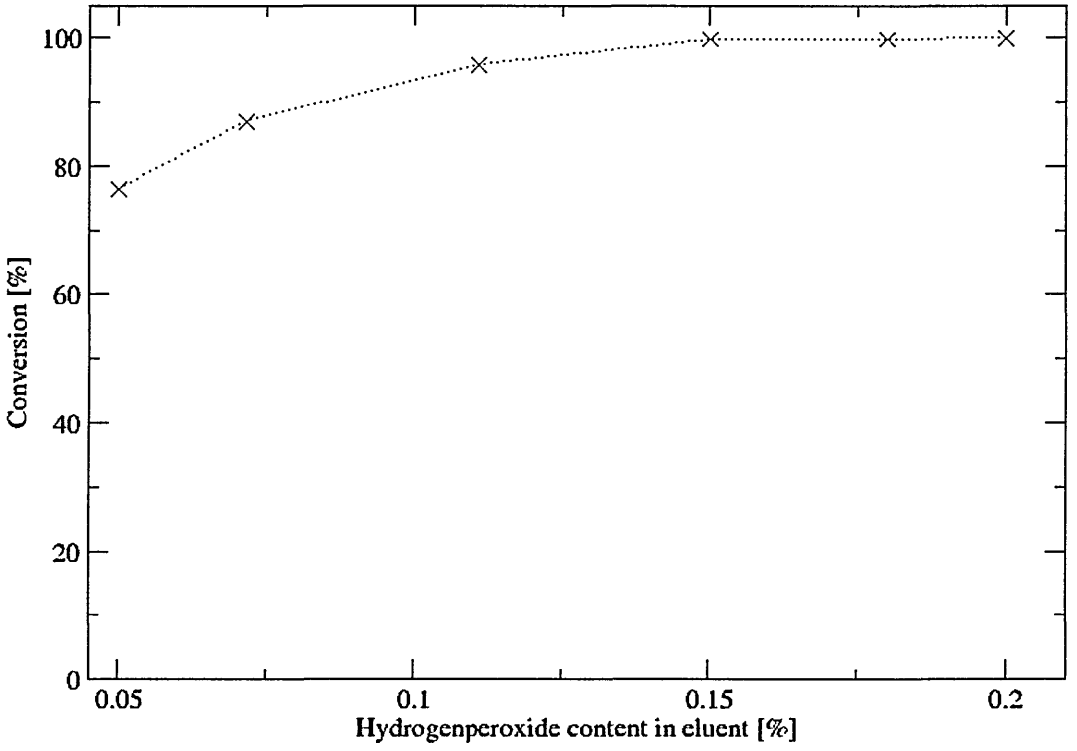

Figure 3.5: Optimisation of post-column nitrite conversion

Complete conversion from nitrite to nitrate by post-column oxidation was observed at a hydrogen peroxide concentration higher than 0.2%, even at high nitrite concentrations of 1000 µmol/l. This also simplified the method greatly,

because only one calibration curve was needed to measure both anions. However, the use of post-column oxidation did not solve the problem completely, as recoveries for nitrite from serum and urine were still below far below 50% as shown in Figure 3.6.

Further investigation revealed that chloride, which has a similar solvated charge to size ratio to nitrite and is present in very high concentrations of approximately 125 mmol/l in serum, coelutes with nitrite and could not be separated by applying a longer gradient.

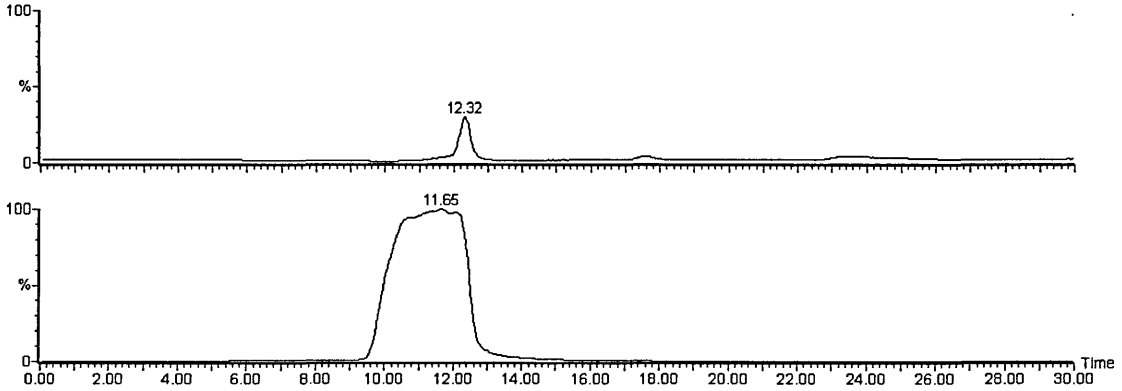

Figure 3.6: Coelution of chloride with nitrite

Normally, chloride and nitrite can be separated on anion exchange columns with hydrophobic head groups since nitrite is polarisable, while chloride is not. Due to the organic solvent in the eluent, the interaction between nitrite molecules and the head groups is much less than compared to when eluents without organic solvents are used, which leads to a lower resolution between nitrite and chloride. The organic solvent is needed to aid desolvation in the interface of the mass spectrometer and can not be left out. The resolution

between nitrite and chloride in biological fluids is also a problem because the high amounts of chloride lead to strong peak broadening. The coelution of chloride not only results in suppression of the ionisation of nitrite in the ion source of the mass spectrometer, but also increases the reactivity of nitrite in the ion source. Since all cations are exchanged to hydronium ions, the chloride ions enter the ion source as hydrochloric acid together with the nitrite ions causing very acidic conditions.

It was tried to remove chloride as a sample pre-treatment step using a combination of a Dionex OnGuard Ag and OnGuard SCX SPE cartidges. The SCX cartrigde is needed to trap any silver ions released from the OnGuard Ag cartridge, which would damage the analytical column. But it was found that a major part of the nitrite is oxidised to nitrate, which resulted in a very poor recovery of 10-50% for nitrite. Oxidation of nitrite to nitrate even happened with nitrite standards made up in degassed water and might have to do with trace metals or silver ions catalysing the oxidation that are present in the silver resin or trapped on the strong cation exchange material of the OnGuard SCX cartridge. Pre-column sample cleanup with combined Onguard Ag/SCX cartridges is therefore not suitable for nitrite analysis.

Since oxidation is desirable after separation on the column and interfering chloride could not be removed by a pre-column sample treatment step, this gave rise to the idea of a chloride trap column that could be used to remove all chloride post-column combined with post-column oxidation to measure nitrite. The instrumental setup is shown in Figure 3.7.

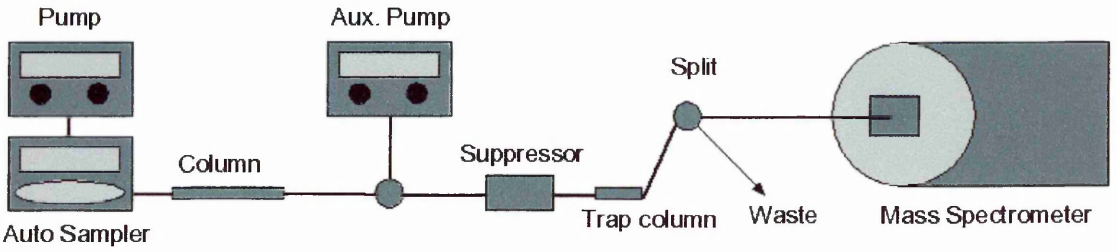

Figure 3.7: Intrumental setup for IC-MS with post-column oxidation and a chloride trap column

When employing both a chloride trap column and post column oxidation, all four analytes could be measured in one run, as shown in figure 3.8.

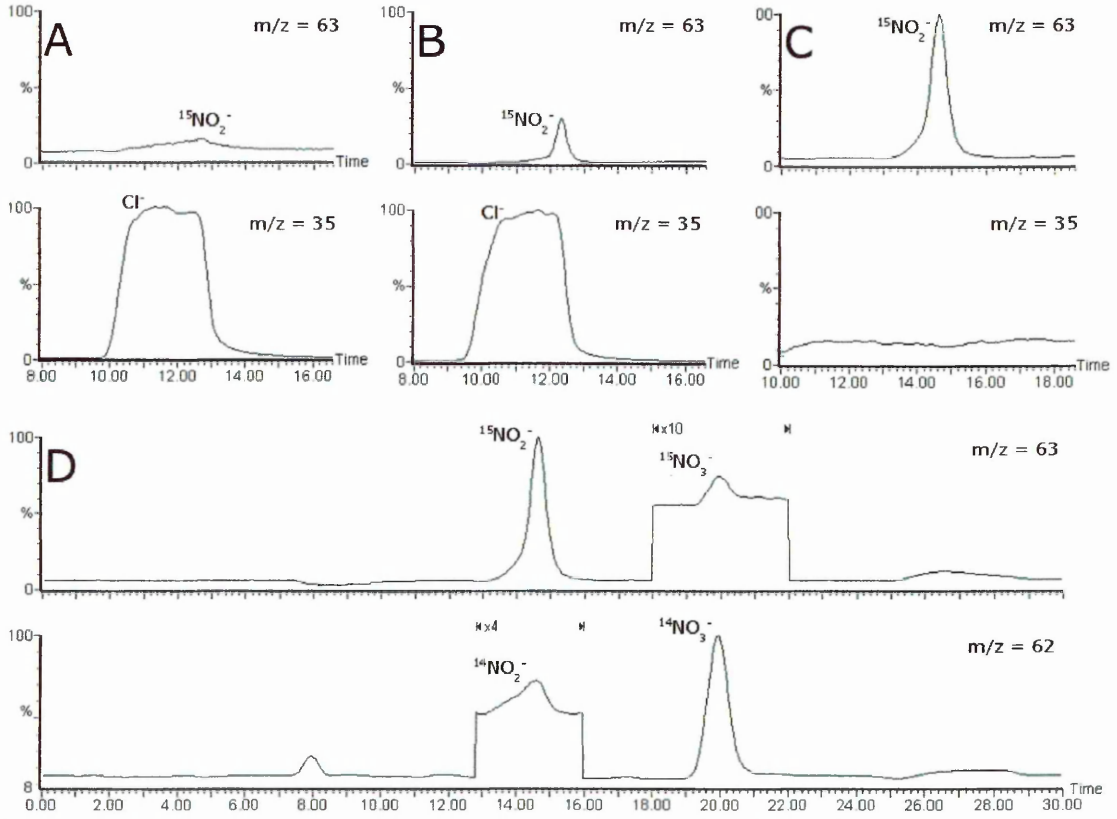

Figure 3.8: Chromatograms of a serum sample spiked to 10µM ^{15}N-nitrite analysed by IC-MS (A), by IC-MS with post-column oxidation (B) and by IC-MS with a chloride trap and post-column oxidation (C,D).

Nitrite eluted after 14.5 minutes, nitrate after 20 minutes and the overall time for each analysis was 60 minutes including a high concentration gradient step to elute high affinity matrix compounds and including an equilibration step at the end of the gradient. The nitrite peak showed some fronting, since chloride was removed post column and is therefore migrating with nitrite along the column during the separation process causing peak deformation. The retention time for nitrite and nitrate was 1.5 minutes longer when a chloride trap column and post-column oxidation were used, because of the extra tubing and void volume. This particular serum sample (figure 3.8) contained 1 µmol/l ^{14}N-nitrite and 50 µmol/l ^{14}N-nitrate. Endogenous ^{15}N-nitrate could also be seen since the natural abundance of the ^{15}N isotope is 0.360%. No endogenous ^{15}N-nitrite could be detected, because the nitrite level in serum samples is much lower than nitrate levels and subsequently the ^{15}N-nitrite concentration was below the detection limit. The limit of detection for ^{14}N-nitrite and ^{14}N-nitrate was 200 nmol/l and the detection limit for ^{15}N-nitrite and ^{15}N-nitrate 50 nmol/l. Lower background noise levels on the m/z=63 mass trace were resonsible for the better limit of detection for the ^{15}N species as well as the longer dwell time. Calibration curves were linear to concentrations up to 1000 µmol/l with correlation coefficients of 0.9995 and better. An example is shown in figure 3.9.

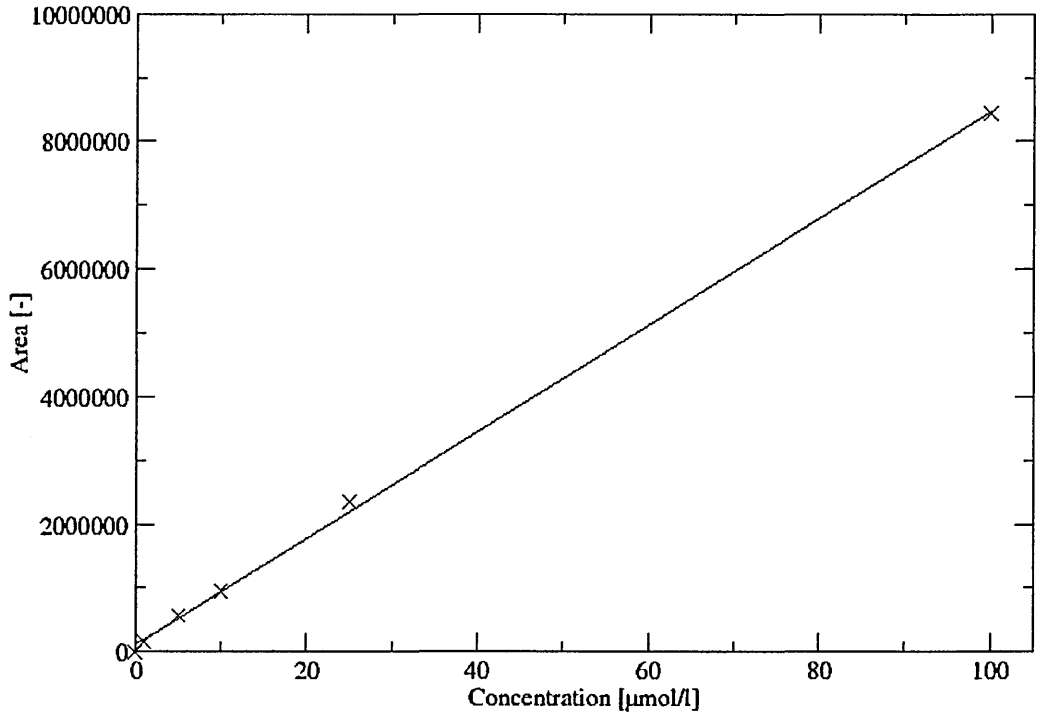

Figure 3.9: Typical calibration curve for ^{14}N-nitrate (r^2 = 0.9996)

Occasionally, large signals were seen at m/z = 45 resulting from the formation of formate from the oxidation of methanol, but were not found to interfere with the measurements.

To determine whether any interferences on the m/z = 63 mass trace occur at high ^{14}N-nitrate levels due to resultant broadening of the mass band at m/z= 62 a series of standards was injected where the ^{15}N-nitrate concentration was kept fixed at 10µmol/l in each and the ^{14}N-nitrate concentration was increased in each up to 1000µmol/l (Figure 3.10).

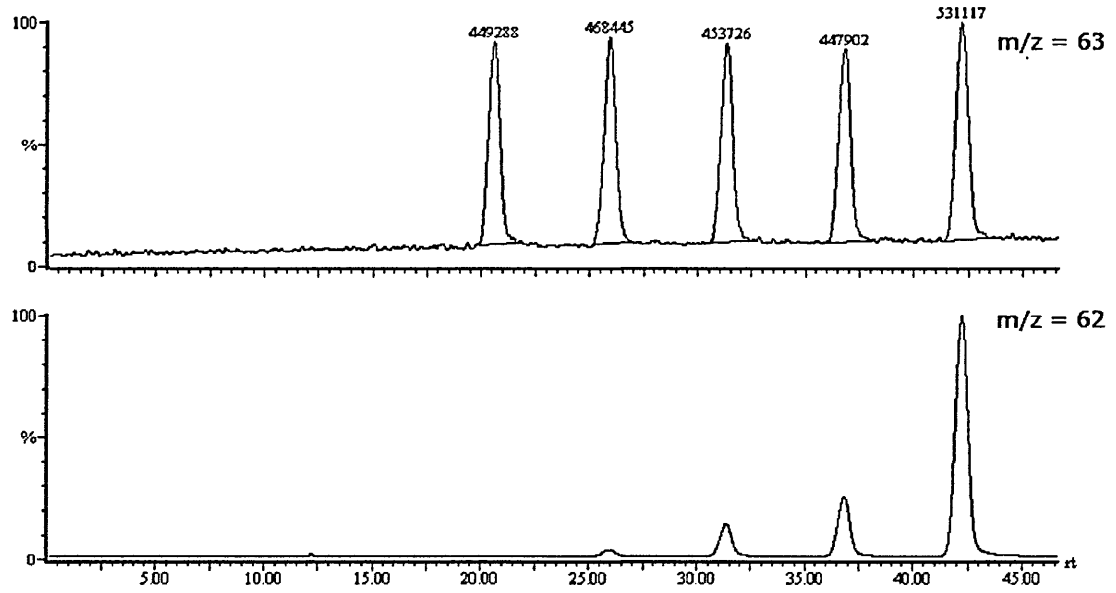

Figure 3.10: Chromatogram of standards with fixed ^{15}N-nitrate concentration (10µmol/l) and increasing total nitrate concentration(0,10,50,100,500 µmol/l). Values indicate peak area.

The peak area for the ^{15}N-nitrate peaks stayed constant except for the last injection (500µmol/l nitrate) which introduced measurable amounts of endogenous ^{15}N-nitrate (1.8µmol/l endogenous ^{15}N-nitrate). The concentration for the last ^{15}N-nitrate peak was 11.7µmol/l, which matched the theoretical 11.8 µmol/l of 10µmol/l spiked and 1.8µmol/l endogenous ^{15}N-nitrate. These data show that there was no significant overlap of the m/z=62 mass band into the m/z=63 band at the mass resolution used. The recoveries (Table 3.1) were determined from urine (n=5) and serum samples (n=5) spiked with ^{15}N-nitrite and ^{15}N-nitrate (10µmol/l). The mean recovery of nitrate was 92.8% (range from 89.3 to 96.2) from urine and 94.1% (range from 88.6 to 99.9) from serum. The mean recovery of nitrite was 95.5% (range from 90.7 to 98.5) from urine and 106.7% (range from 102.9 to 110.3) from serum.

	urine		*serum*	
	Recovery [%] nitrite	Recovery [%] nitrate	Recovery [%] nitrite	Recovery [%] nitrate
Spike 1	90.7	89.3	110	91.6
Spike 2	94.8	96.2	103.7	99.9
Spike 3	98.3	90.1	110.3	93.3
Spike 4	98.5	95.4	102.9	88.6
Spike 5	95.1	92.8	106.5	96.9
mean	**95.5**	**92.8**	**106.7**	**94.1**

Table 3.1: Recoveries of nitrite and nitrate from urine and serum

All biological samples had a high ^{14}N-nitrate content and so endogenous ^{15}N-nitrate (0.360%) was always visible. To measure the excess ^{15}N-nitrate resulting from ^{15}N-L-arginine infusion on top of endogenous ^{15}N-nitrate the endogenous ^{15}N-nitrate concentration must be calculated (0.36% of the ^{14}N-nitrate concentration) and substracted from the measured ^{15}N-nitrate concentration. This calculation requires that the instrumental precision is adequate. To test this the ratio between the peak area of the endogenous ^{15}N-nitrate peak and the peak area of the ^{14}N-nitrate peak of a nitrate standard were measured at different concentrations (Table 3.2).

Nitrate concentration [µmol/l]	***n***	***Peak area ratio [%]*** $^{15}NO_3^-/^{14}NO_3^-$	***SD***
100	9	0.59	0.047
250	7	0.61	0.019
500	7	0.6	0.021
summed data	23	0.6	0.029

Table 3.2: Ratios of the ^{15}N-nitrate peak area over the ^{14}N-nitrate peak area of nitrate standards

When the limit of detection is defined as three times the standard deviation, the ratio of ^{15}N-nitrate to ^{14}N-nitrate in a sample has to exceed the mean value plus three times SD, i.e. 0.69% to determine non-endogenous ^{15}N-nitrate. In other words, if a sample contains 100µmol/l nitrate then ^{15}N-nitrate levels have to exceed 414.6 nmol/l with 54.6µmol/l non-endogenous ^{15}N-nitrate on top of the 360 nmol/l endogenous ^{15}N-nitrate.

The measured ratio of the ^{15}N-nitrate and ^{14}N-nitrate peak areas did not match the natural isotope ratio of 0.360%. When the instrument was changed to an equal dwell time on both channels, the ^{15}N/^{14}N peak area was 0.28%. To check whether this was due to a difference in the quantitative instrument response between m/z = 63 and m/z = 62 at the different dwell times used for these two readings, samples of known total nitrate concentration was analysed using pure ^{15}N-nitrate as calibrant for the m/z = 63 trace.

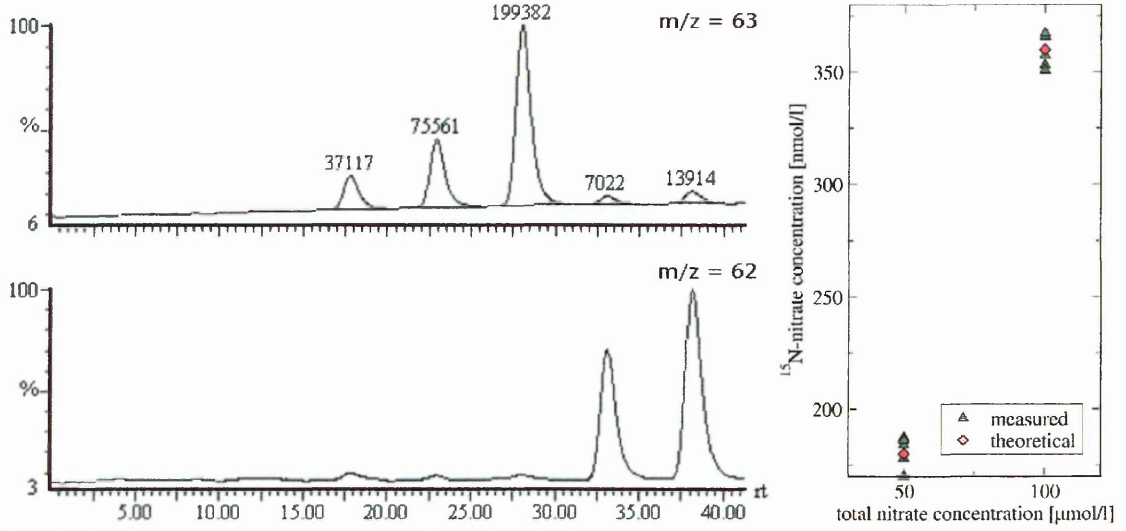

Figure 3.11: Determination of endogenous ^{15}N-nitrate in samples of known total nitrate concentration. Left: Peak 1-3 are 1, 2, 5 µmol/l ^{15}N-nitrate standards. Peak 4,5 are 50, 100 µmol/l normal nitrate standards. Values indicate peak area. Right: Red diamonds: theoretical endogenous ^{15}N-nitrate concentration(assuming 0.36% ^{15}/^{14}N relative abundance), Green circles: ^{15}N-nitrate concentration found in 50 and 100 µmol/l normal nitrate standards (n=8).

The results (Figure 3.11) show that the measured ^{15}N-nitrate content of the sample was indeed 0.360%, so it seems that the instrumental response is different at m/z = 63 and m/z = 62 when different dwell times were used.

3.2.2 IC-MS with electrochemical oxidation

An alternative method has been developed using electrochemical oxidation rather than chemical oxidation by hydrogen peroxide solution. The electrochemical cell was placed after the column and before the suppressor, where sodium hydroxide from the eluent is present. If the electrochemical cell was placed after the suppressor, no conversion of nitrite to nitrate was observed. The instrumental setup in detail is shown in figure 3.12.

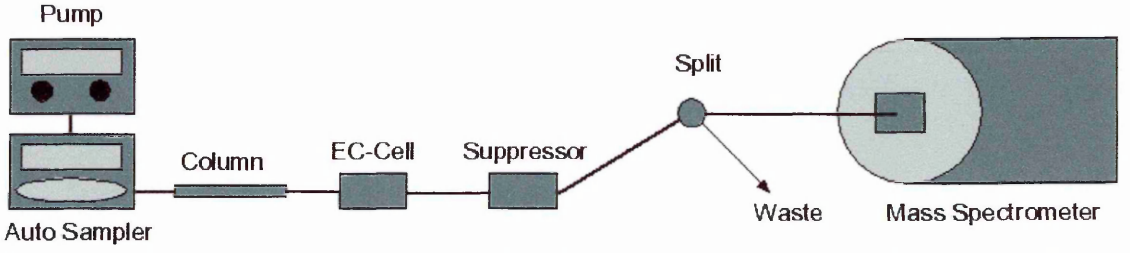

Figure 3.12: Intrumental setup for IC-MS with electrochemical post-column oxidation.

Different voltages can be applied to the electrochemical cell and the conversion of nitrite to nitrate is a function of the applied voltage. So, initially optimisations had to be done to achieve maximum, and if possible, complete conversion from nitrite to nitrate. Figure 3.13 shows a plot of the applied voltage versus the conversion.

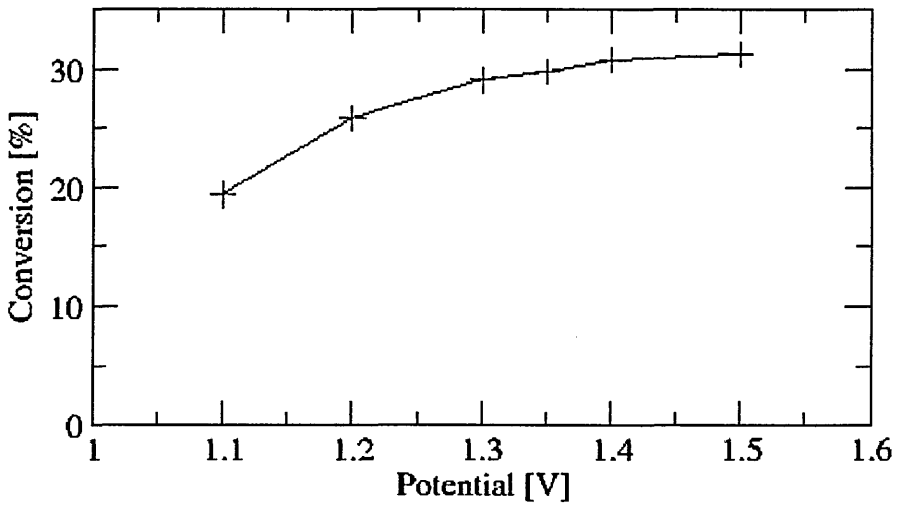

Figure 3.13: Conversion of nitrite to nitrate in the electrochemical cell at different potentials

The conversion efficiency increases with the potential applied to the electrode of the electrochemical cell and seems to eventually reach a maximum of approximately 30%. A potential of 1.3V was chosen for future measurements, because higher voltages will not gain significantly higher conversion and the high currents that occur in the cell at higher voltages can damage the cell.

Even though the conversion rate was much lower than for chemical oxidation this method was still interesting, because a different setup was possible, that did not require a chloride trap column. The electrochemical cell was placed between two columns, because the oxidation of nitrite to nitrate is base catalysed. No significant conversion of nitrite to nitrate was found if the cell was placed after the suppressor module. The instrumental setup is shown in detail in figure 3.14.

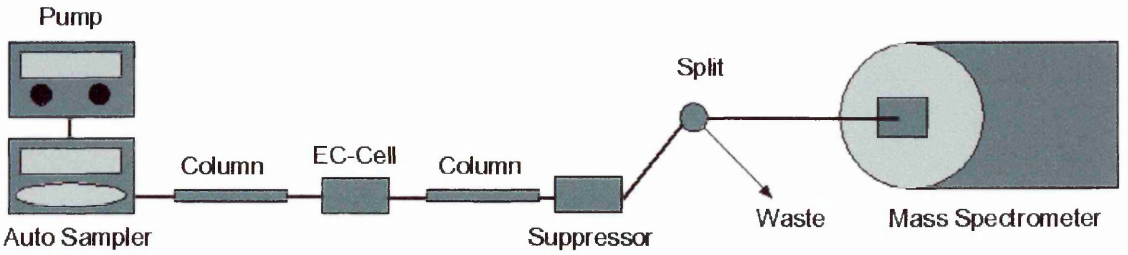

Figure 3.14: Intrumental setup for IC-MS with electrochemical post-column oxidation using two columns

Nitrate was separated from chloride and nitrite on the first column. Nitrite and chloride coelute from the first column and enter the electrochemical cell simultaneously, where nitrite will be converted to nitrate. On the second column the nitrate derived from nitrite oxidation can be separated from chloride. Figure 3.15 shows the separation of nitrite and nitrate from chloride in a serum sample spiked to 20µmol/l nitrite.

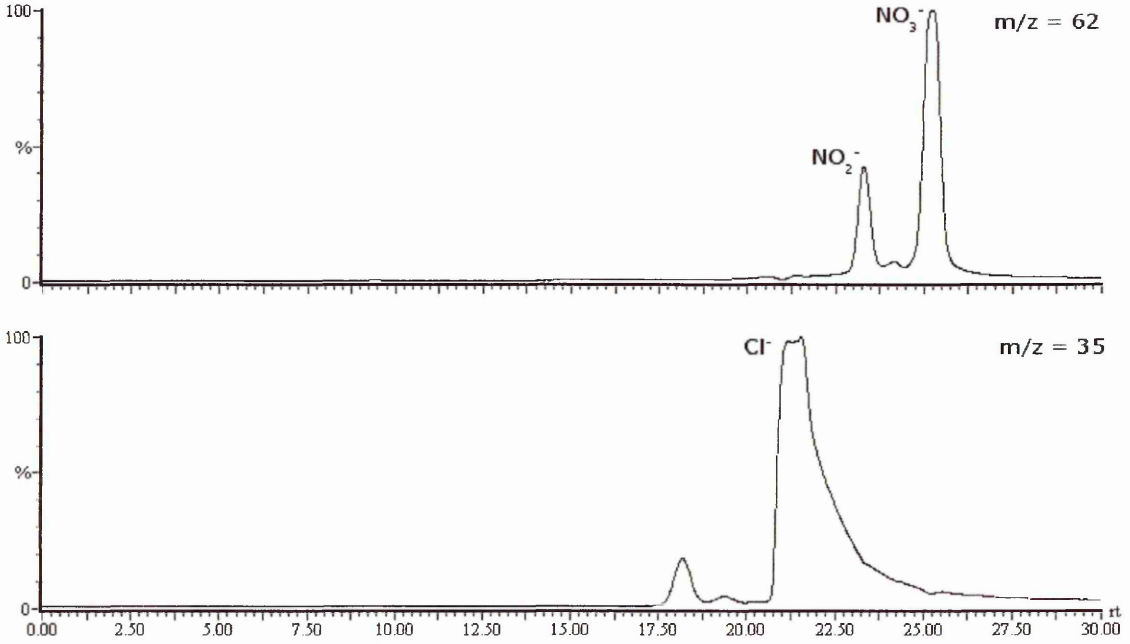

Figure 3.15: Chromatogram of a serum sample spiked to 20 µmol/l nitrate analysed by IC-MS using two columns and electrochemical on-line oxidation

Chloride elutes after 21.5 minutes, nitrite elutes after 23.3 minutes and nitrate after 25.2 minutes. Nitrite and nitrate are detected on the same mass trace of m/z = 62. Chloride is detected on the m/z = 35 mass trace. The advantage of this setup is that no chloride trap is required and the oxidation is easier to handle than in case of chemical oxidation. A similar setup would not have been possible with chemical oxidation because the hydrogen peroxide solution would have damaged the column quickly. Another advantage of this setup is that electrochemical oxidation is "cleaner" than chemical oxidation and no reactants need to be added to the eluent that could affect ionisation in the ion source of the mass spectrometer. The disadvantage is that only a conversion of 30% could be achieved with the electrochemical cell, however the conversion was constant over the investigated concentration range which did not result in non-linear calibration curves. Further disadvantages are the high backpressure and longer retention times due to the two columns. Since the first method using chemical oxidation and a chloride trap gave better overall performance, it was the preferred method.

3.3 Conclusions

It has been shown that ion chromatography coupled to mass spectrometry is a valuable tool for the determination of nitrite, nitrate from biological matrices such as blood serum or urine. A trap column has been designed to remove chloride, the major interferant, and post-column oxidation has been used to enhance the detection of nitrite. The developed methodology has several advantages over previous methods (see 3.2):

- No sample derivatisation is required and sample pretreatment is minimal. The existing techniques needed derivatisation of nitrite/nitrate to measure total nitrite/nitrate concentrations as well as to determine the $^{14}N/^{15}N$ ratio of the species. This does not only consume more time and complicate the method, but also introduces risk of false assessments due to incomplete derivatisation, losses through oxidation or losses through side reactions. The method presented here does not require more than a simple sample cleanup and therefore reduces the risk of nitrite/nitrate losses, thus yielding a faster and more reproducible assay.

- Nitrite and nitrate can be determined simultaneously and independently. If nitrite and nitrate levels are analysed by the Griess assay, every sample has to be measured twice and nitrite concentration subtracted from the total NO_X concentration after nitrate to nitrite reduction. This is not needed for the IC-MS method. Nitrite and nitrate and their ^{15}N species can be analysed in one

run.

- Only standard analytical tools are needed. Unlike the method presented by Forte et al.[152], which requires rather expensive equipment, this method works with a normal ion chromatograph and a simple quadrupole mass spectrometer that is available in most analytical laboratories.

- The method has good validation data for biological matrices. Detection limit, linear range and recoveries were excellent for serum and urine, which shows that this method is ideal for complex matrices, not only because the chromatographic separation makes detection of the analytes easier, but also because the chloride trap column removes the major interferent quantitatively.

- The method can easily be automated. Only standard ion chromatography and mass spectrometry equipment is needed for this method, which makes it compatible with autosampling and automated sample pretreatment technologies.

4. Application of IC–MS

4.1 Introduction

4.1.1 Clinical studies

There is evidence that primary pulmonary hypertension (PPH) is caused by a deficiency in nitric oxide release by endothelial nitric oxide synthase (NOS) in the lung tissue[205]. It is therefore important to compare whole body NOS turnover in PPH patients with that in healthy controls. Forte at al.[132] have successfully used ^{15}N-L-arginine infusion to study NOS turnover in patients with essential hypertension, with detection of the ^{15}N label in nitrite and nitrate via gas isotope ratio mass spectrometry (GIRMS). This chapter reports the use of Forte's infusion technique with the developed IC-MS methodology described in the previous chapter in a preliminary study of patients suffering from primary pulmonary hypertension compared to healthy controls as proof of principle of the assays usefulness in clinical studies.

4.1.2 Laboratory studies

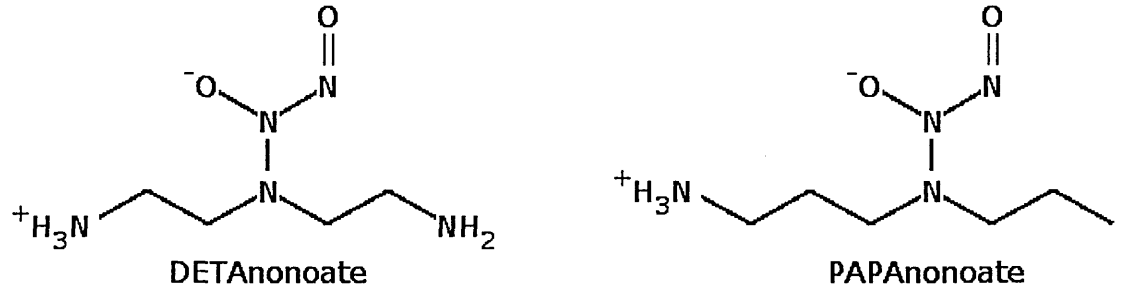

Figure 4.1: The NO donor drugs DETAnonoate and PAPAnonoate

Various compounds have been proposed as possible nitric oxide donors or buffer stores. The kinetics of donor breakdown and buffer NO adduction/release

are of interest, and the IC-MS system was used to study three such compounds. Detection of the NO donors DETAnonoate, PAPAnonoate and S-nitroso-cysteine in simple solution was achieved and the breakdown kinetics of the NO donor drugs DETAnonoate and PAPAnonoate was investigated via subsequent nitrite and nitrate formation.

4.1 Experimental

4.1.1 Materials

As described in chapter 3.

4.1.2 Equipment

As described in chapter 3.

4.1.3 Procedures

4.1.3.1 Volunteers and Patients

Four female patients with primary pulmonary hypertension (median mean pulmonary artery pressure 54 mm Hg, average age 42.75 years, SD = 5.1) were recruited. All were on warfarin, diuretics and calcium channel blockers. Four age-matched female, healthy and non-smoking volunteers (average age 36 years, SD = 9.1) were investigated. A diet low in nitrite and nitrate was adopted by all, vegetables and conserved meat being avoided. ^{15}N-L-arginine was given via an indwelling venous cannula as a primed (10µmol/kg) and then a constant (10µmol/kg/h) intravenous infusion whilst supine. Urine was collected over 48 hours, from 12 hours before until 36 hours after an intra-venous infusion of L-[^{15}N]$_2$-arginine (300 mg, 99 atom% enrichment, Mass Trace, MA, USA). Written informed consent was obtained, the study having approval of the South Sheffield Ethics Committee (see 8. Ethical approval). Patients were recruited and procedures carried out by Drs. S. Wharton, R.Vancoe, J.M. Wong and D.Kiely from the Pulmonary Vascular Research Centre, Royal Hallamshire

Hospital.

4.1.3.2. Sample preparation

Urine samples were pretreated as described in in chapter 3.

4.1.3.3 IC-MS measurements

The cleaned up urine samples were processed as described in chapter 3. The ^{14}N-nitrate calibration curve was established form a series of nitrate standards. The ^{15}N-nitrate present at a ratio of 0.36% in the nitrate standards was used to establish the ^{15}N-nitrate calibration curve. This was possible because the total ^{15}N-nitrate levels were only up to twice as high as the endogenous ^{15}N-nitrate levels, since the clinical study was designed to only provide a small pool of labelled arginine for safety reasons. Direct calibration with ^{15}N-nitrate standards showed indeed an endogenous ^{15}N-nitrate of 0.36% in nitrate, as described in chapter 3. Samples were diluted if concentrations were not in the linear working range. To calculate ^{15}N-nitrate from NOS turnover, endogenous ^{15}N-nitrate was determined from the ^{14}N-nitrate concentration in the sample and subtracted from the total measured ^{15}N-nitrate. All results were normalised by urine volume.

4.1.3.4 NO-donors

Stock solutions of 10mmol/l DETAnonoate and PAPAnonoate in 0.1 mmol/l aqueous sodium hydroxide were prepared. Cysteine was dissolved in 0.1mmol/l phosphate buffer solution (pH 7.4) to prepare a 1mmol/l stock solution.

Appropriate amounts of the stock solutions were added to 5ml of the phosphate buffer solution to obtain a NO donor concentration of 100µmol/l. The sample was incubated in a shaking water bath at 37°C. At the desired time points 1ml of the solution was removed and stored at -30°C.

4.1.3.4 Statistical analysis and calculation procedure

Because of the small number of replicate samples, range errors are used to indicate error ranges. Statistical comparisons were done with Students t-test preceeded by an f-test for equal variance. Linear regression was used for the calibration curves.

4.2 Results and Discussion

4.2.1 IC-MS measurements

Figure 4.2 shows a chromatogram of a patient urine sample from the 12-24 hours collection after ^{15}N-L-arginine infusion.

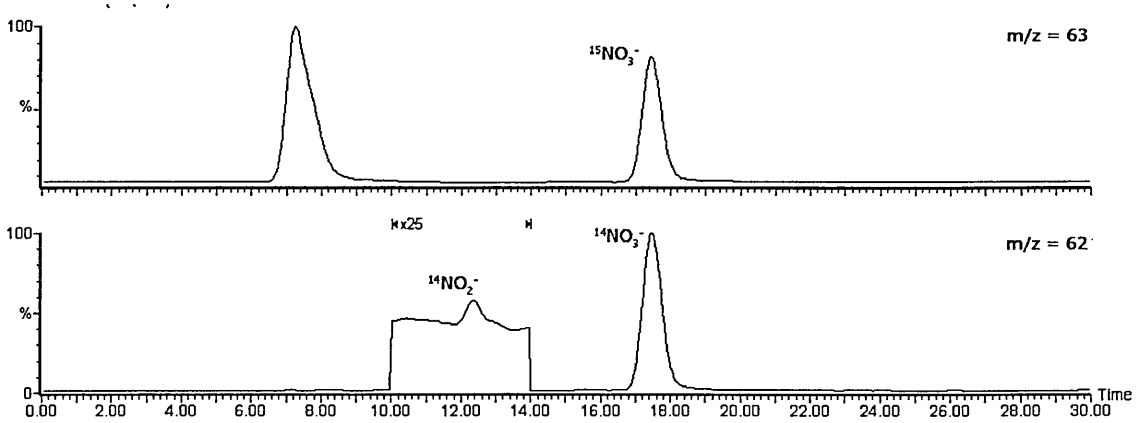

Figure 4.2: Chromatogram of a patient urine sample 12-24 h urine collection after ^{15}N-L-arginine infusion analysed by IC-MS using a chloride trap and post-column oxidation.

Nitrite eluted after 12.5 minutes and nitrate after 17.5 minutes. In this sample the ^{14}N-nitrite concentration was 2.04 µmol/l, ^{14}N-nitrate concentration was 1070 µmol/l, ^{15}N-nitrite was not detectable and ^{15}N-nitrate concentration was 6.7 µmol/l. ^{15}N-nitrite may be mostly oxidised to nitrate in the urine samples and so the ^{15}N-nitrite levels were below the detection limit. Occasionally, high nitrite levels were found, but the level of ^{15}N-nitrite found was about 0.36% of the 14N-nitrite, suggesting that the 15N-nitrite seen was not from the labelled L-arginine infusion (values shown in figure 4.3). This shows that the nitrite in these samples was not a result of endothelial NOS turnover, but must have come from another yet unidentified source such as possibly bacterial nitrite production resulting from urinary infection.

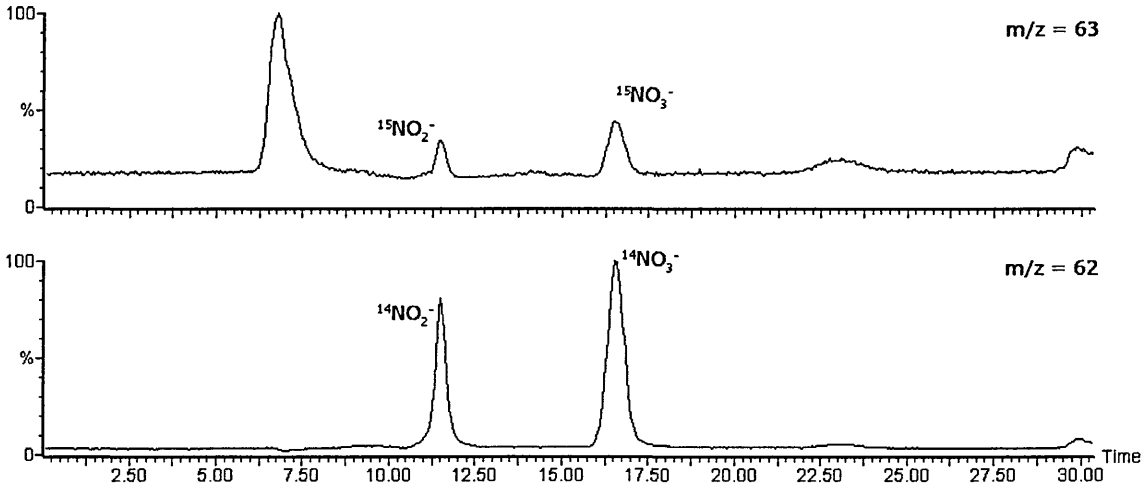

Figure 4.3: Chromatogram of a urine sample that showed high nitrite concentration. ^{14}N-nitrite concentration was 74 µmol/l, ^{15}N-nitrite 259 nmol/l.

For this study however the occasional high nitrite samples had no effect on the overall result, because the technique allowed to discriminate between ^{15}N-L-arginine derived nitrite and nitrite from other sources. None of the previously available techniques are immune to this source of error, and even the ones using mass spectrometry to ^{14}N/^{15}N discrimination require determination of total NO_X (Chapter 1.2) by a technique which is not isotopically selective.

4.2.2 NOS turnover in healthy subjects

In the healthy subjects 10.65 µmol ^{15}N-nitrate resulting from ^{15}N-L-arginine was excreted within 36 hours after the infusion, with a range from 3950 µmol/l to 17772 µmol/l between the individuals. Assuming that one ^{15}N-nitrate molecule is produced from one ^{15}N-L-arginine molecule, about 0.6% of the administered ^{15}N-L-arginine was excreted as ^{15}N-nitrate. ^{15}N-nitrate originating from ^{15}N-L-arginine was detectable in all samples and all samples contained a detectable amount of endogenous ^{15}N-nitrate. Table 4.1 shows the ^{15}N-nitrate excretion at the investigated time intervals.

$^{15}NO_3^-$ excretion [nmol]				
subject	**0-12h**	**12-24h**	**24-36h**	**total**
1	1262	1464	1224	3950
2	1191	3948	2112	7251
3	1293	4260	12219	17772
4	7924	788	4894	13606

Table 4.1: $^{15}NO_3^-$ excretion in healthy subjects

Compared to other studies of NOS turnover employing ^{15}N-L-arginine, the amount of excreted ^{15}N-nitrate by healthy subjects in this study was several times higher. Table 4.2 compares the values to previously published ones.

	$^{15}NO_3^-$ excretion[nmol]	**^{15}N-L-arginine conversion [%]**	**n**
Forte[152]:	1642 ±83	0.138 ±0.005	11
Demoncheaux[206]:	4216 ±2189	0.246 ±0.128	6
Dürner:	10650 ±5044	0.622 ±0.295	4

Table 4.2: $^{15}NO_3^-$ excretion in healthy subjects reported by Forte et al, Demoncheaux at and this study. Values are shown in mean standard deviation although the the lower two rows have only a small sample number.

Forte at al. used the Griess reaction to assess total urinary nitrate levels and in a second step used gas isotope ratio mass spectrometry to determine the ^{15}N-nitrate fraction as described by Green at al.[207]. Green et al. showed recovery data for urine that was diluted 1:40 using the flow injection system which reduces nitrate to nitrite through a cadmium column and then derivatises nitrite with the Griess reaction.

However, Forte at al. did not follow the method described by Green et al. and diluted urine 1:10 prior to injection, but crucially did not present any recovery data. Matrix effects deminishing the formation of the Griess dye may have lead to significantly lower results. The individual-to-individual variation reported by Forte at al is also surprisingly small, compared to other studies (Table 4.2). Demoncheaux et al. used a similar approach, by using the colorimetric Griess assay to determine total nitrate levels and then measuring the ^{14}N-nitrate/^{15}N-nitrate ratio by GC-MS after derivatisation as described by Tsikas et al.[147] The results are closer to those been found in this study, but still are dependant on the Griess assay which is not ideal for biological matrices.

4.2.3 NOS turnover in PPH patients

In the group of 4 PPH patients an average of only 3.2 µmol ^{15}N-nitrate was excreted within 36 hours after the infusion, with a range from 70µmol/l to 6338µmol/l resulting from 1720µmol/l ^{15}N-L-arginine infused (table 4.3). Assuming one ^{15}N-nitrate molecule is produced from one ^{15}N-L-arginine molecule, about 0.2% of the administered ^{15}N-L-arginine was excreted as ^{15}N-nitrate. ^{15}N-nitrate resulting from ^{15}N-L-arginine was not detectable in four patient samples. These samples also had lower ^{14}N-nitrate levels resulting in the endogenous ^{15}N-nitrate levels being below the detection limit (Table 4.3).

$^{15}NO_3^-$ excretion [nmol]				
subject	0-12h	12-24h	24-36h	total
1	2500	2056	1782	6338
2	50	n.d.	n.d.	50
3	1208	n.d.	n.d.	1208
4	1346	846	2938	5130

Table 4.3: $^{15}NO_3^-$ excretion in the investigated PPH patients (n.d. = not detectable)

Undetectable ^{15}N-nitrate levels have not been observed during the recovery study and in the samples obtained from the healthy subjects that were analysed at the same time. Overall the amount of excreted ^{15}N-nitrate was lower in the PPH patients group, than in the healthy volunteers (section 4.2.2). Figure 4.3 shows the ^{15}N-nitrate excretion of the healthy controls compared to the PPH patients. A f-test indicated a similar variance between the sample sets, and the -value for a one-tailed t-test was 0.04. This indicates a significant difference between the two groups, although the sample number was very low.

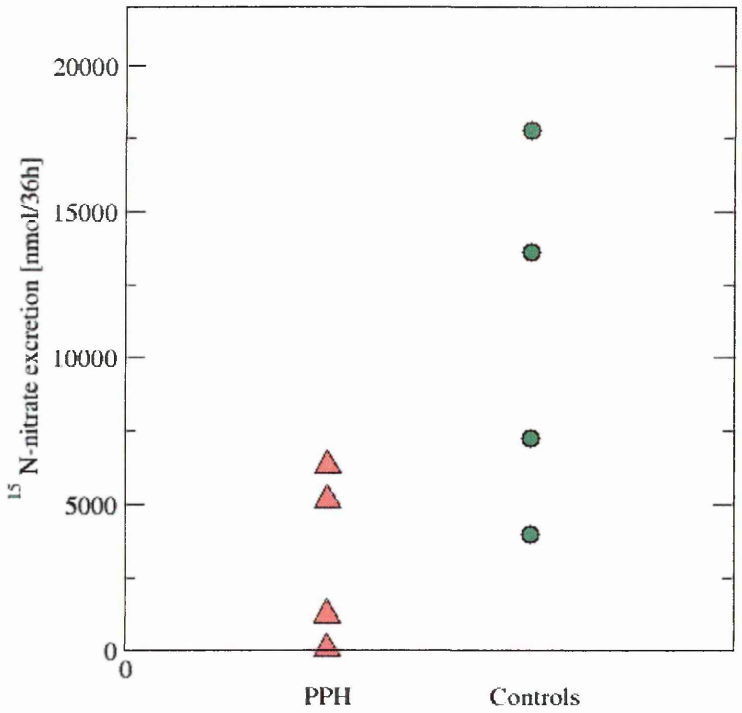

Figure 4.4: ^{15}N-nitrate excretion in PPH patients (red triangles) and healthy controls(green circles).

This finding confirms earlier results using gas chromatography - mass spectrometry obtained by Demoncheaux et al[208]. Median 12 hours recovery of urinary ^{15}N-nitrate over 36 hours was reported to be 16 times lower in the PPH patients (n=6) than in the healthy controls(n=4).

Forte at al. compared ^{15}N-nitrate excretion in healthy subjects (n=11) to patients with essential hypertension (n=11) and found a significantly lower excretion by the patients[132], where 200mg ^{15}N-L-arginine were administered and samples taken in the same intervals as in this study. A mean ^{15}N-nitrate excretion of 2.1µmol was determined in the healthy subjects and 1.3µmol in the patients respectively. Again in this study, even though the general trend is the same, much higher amounts of ^{15}N-nitrate could be detected. This may indicate less

sample loss due to the simpler sample preparation required by the ion-chromatography based method.

Kaneko et al.[209] measured biochemical reaction products of NO in the lung in PPH patients (n=8) and healthy controls (n=8) by chemiluminescence after conversion to NO without a stable label. A reduction in the levels of NO reaction products was found in the PPH patents (0.69µmol/l SE = 0.21) compared to the healthy controls (3.3 µmol/l SE = 1.05). This finding reflects the same general trend with the results in this study.

Neither the healthy controls nor the patients showed complete clearance of ^{15}N-after 36 hours of observation (Figure 4.5).

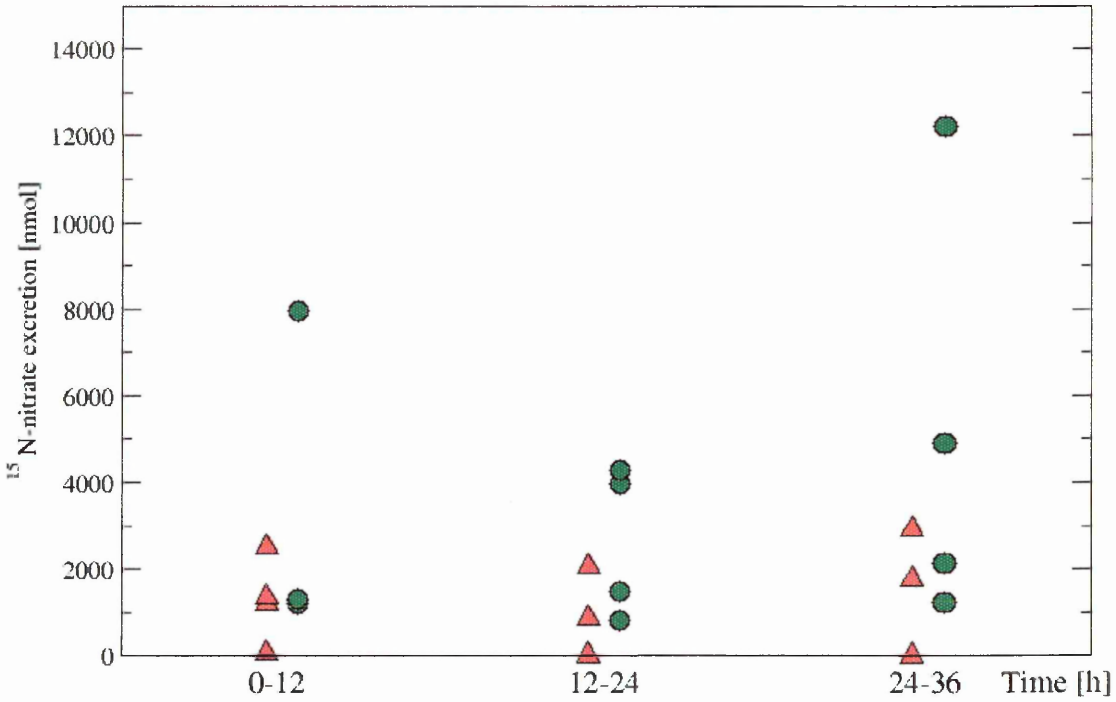

Figure 4.5: ^{15}N-nitrate excretion in urine in healthy subjects(green circles) and patients(red triangles) over time.

In the PPH patients the highest amount of ^{15}N-nitrate was excreted towards the

end of the experiment (24-36h). Similar experiments by Forte et al[152,132] showed almost total clearance of ^{15}N after 36 hours in healthy subjects as well as hypertensive patients, while the experiments by Demoncheaux et al[208] agree with the results obtained here in that ^{15}N-nitrate from ^{15}N-L-arginine was still detectable in the 24h-36h interval.

4.2.3 Detection of nitric oxide donors

This technique was not only found to be useful for the determination of nitrite and nitrate, but it was also possible to detect nitric oxide generating compounds such as pharmaceutical nitric oxide donors and what were thought to be nitric oxide carriers, primarily nitrosylated peptides or amino acids. The oxidative stress through the post-column oxidation step probably generates nitrate from the nitric oxide donating moiety in the nitric oxide donor molecule, which can be detected with the developed detection system. It was not possible to detect these compounds directly with the mass spectrometer, because the suppressor module removed the analytes from the eluent stream in their cationic form while they migrated through it.

Figure 4.6 shows a chromatogram obtained from a phosphate-buffered solution of PAPAnonoate after 30 minutes of incubation.

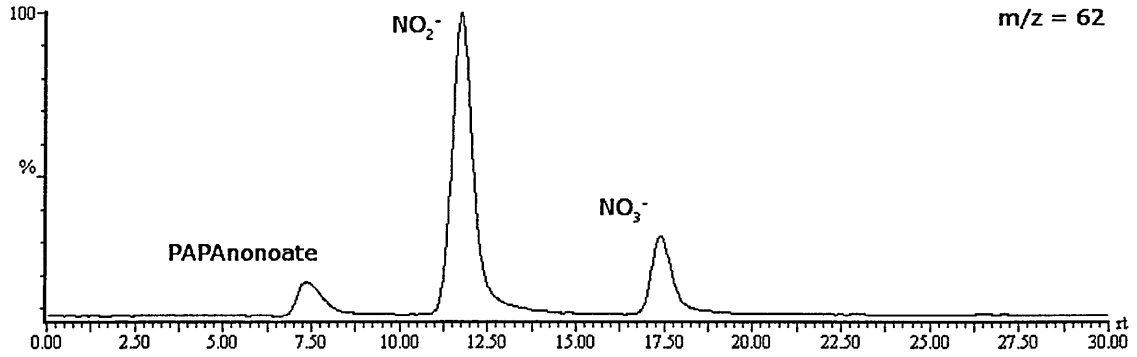

Figure 4.6: Chromatogram of a buffered PAPAnonoate solution after incubation for 30 mins analysed by IC-MS using a chloride trap and post-column oxidation

The NO donor compound PAPAnonoate eluted after 7.5 minutes, nitrite after 12 minutes and nitrate after 17.5 minutes. PAPAnonoate eluted first, since it is a

weak anion, and might not have been separated from other possibly interfering compounds when analysed from more complex sample matrices. Figure 4.7 shows a chromatogram obtained from a phosphate-buffered solution of DETAnonoate after 30 minutes of incubation.

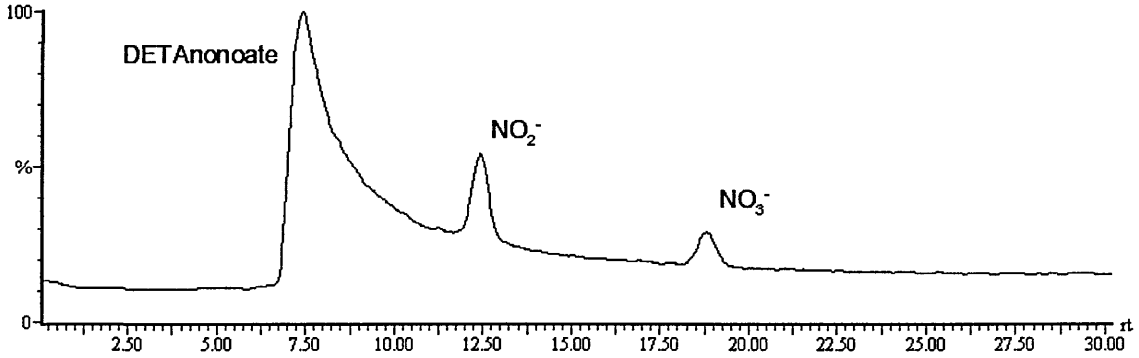

Figure 4.7: Chromatogram of a buffered DETAnonoate solution after incubation for 30 mins analysed by IC-MS using a chloride trap and post-column oxidation

The retention times are similar to the ones from the PAPAnonoate sample, but DETAnonoate, which elutes first after approximately 7.5 minutes shows strong peak tailing, so a Dionex AS11 HC column might not be ideal for this analyte.

The nitric oxide carrier S-nitroso-cysteine gave a sharp peak as shown in Figure 4.8.

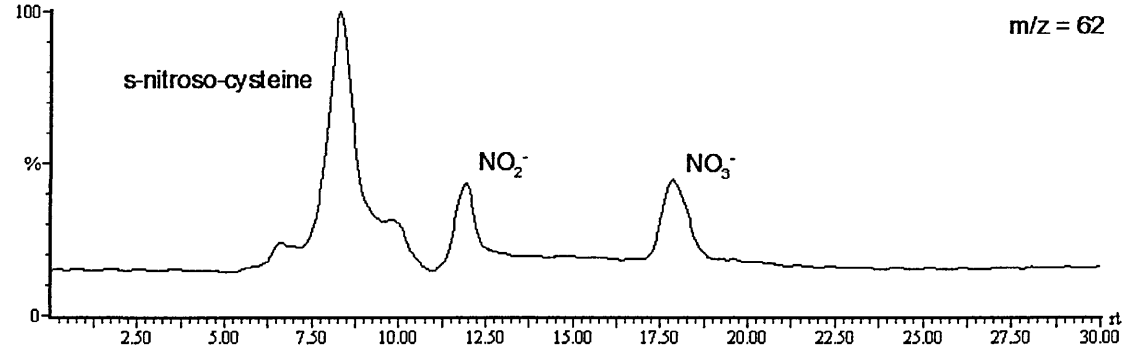

Figure 4.8: Chromatogram of a buffered S-nitroso-cysteine solution analysed by IC-MS using a chloride trap and post-column oxidation

The analytes were detected on one mass trace which increased sensitivity of the quadrupole mass spectrometer, since it allowed a higher dwell time. The rate of nitrate generation from the NO donors in the post-column oxidation process was only 32% for DETAnonoate and 11.5% for PAPAnonoate compared to the complete conversion of nitrite to nitrate. The rather high sodium hydroxide concentration of 20 mmol/l used in this method allowed short retention times and so a short analysis time for nitrite and nitrate analysis. A low concentration gradient step will be needed to separate the NO donor compounds from each other and from possibly interfering matrix components if the NO donor compounds itself were to be analysed in mixture or from more complex matrices.

The degradation of PAPAnonoate and DETAnonoate and subsequent nitrite and nitrate generation was studied in phosphate-buffered solution. Figure 4.9 shows the chromatograms of the DETAnonoate samples taken at different incubation times. The peak for DETAnonoate is decreasing with time and the peaks for nitrite and nitrate are increasing with time. The DETAnonoate peak is difficult to integrate, because of the strong peak tailing. Although the nitrite peak was found on its tail, integration of the nitrite peak was not complicated. DETAnonoate is a rather slow NO donor with a half-life of approximately 20 hours[210]. So as expected only very low nitrite and nitrate levels could be measured over the duration of the experiment as shown in Figure 4.10.

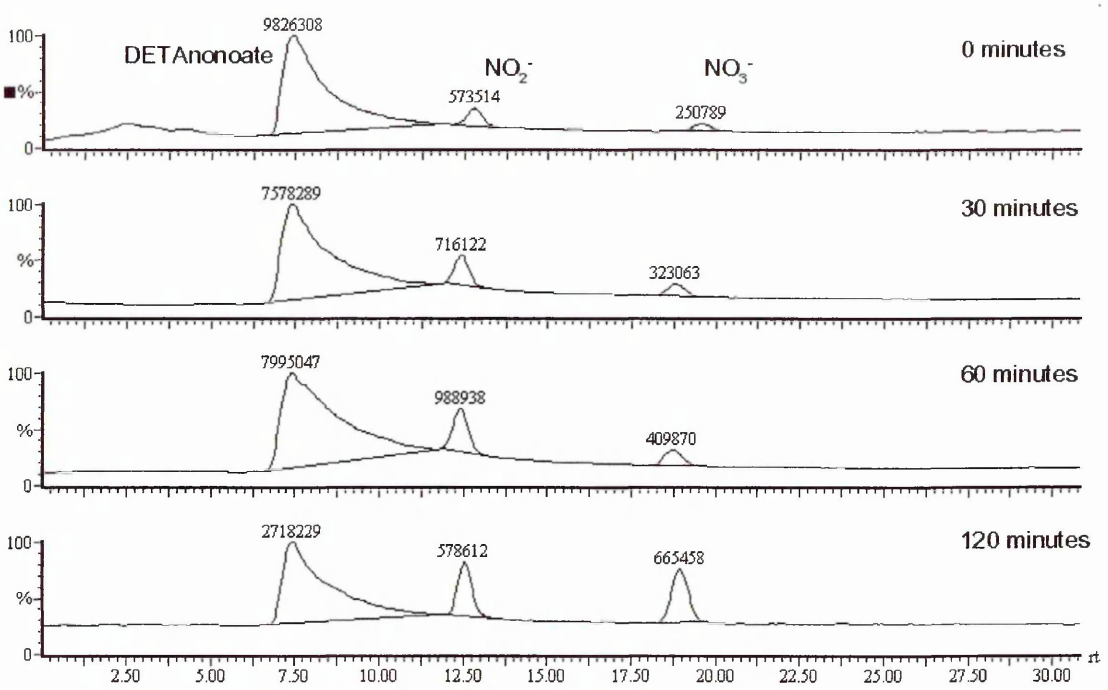

Figure 4.9: Degradation of DETAnonoate after 0, 30, 60 and 120 minutes. Values on peaks indicate peak area.

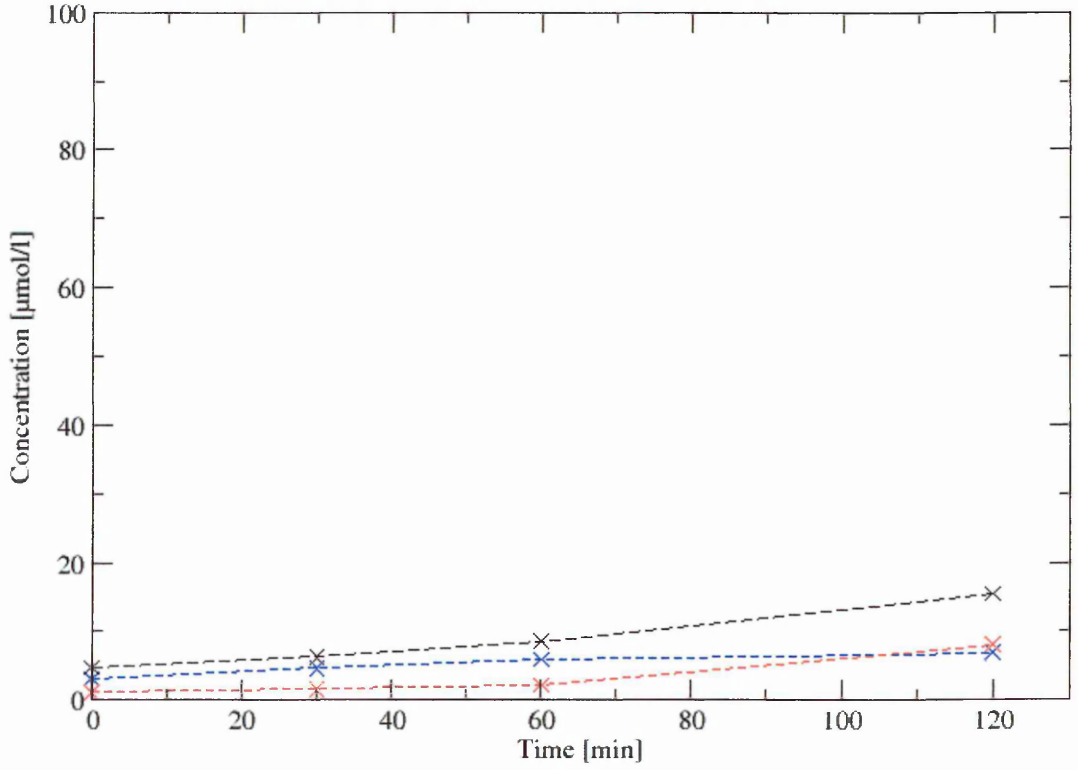

Figure 4.10: Nitrite(blue), nitrate(red) and total NO_X(black) concentration in the buffered DETAnonoate samples. Scaling for comparison to Fig. 4.12.

Initially, the nitrite concentration was about twice as high as the nitrate concentration, which indicates that nitrite is the primary degradation product of NO in buffered aqueous solution. Nitrate appears to be generated by nitrite oxidation and to be a secondary product of NO generated from the NO donor compound. So after approximately 105 minutes the nitrate concentration exceeds the nitrite concentration in the sample solution. Since one molecule of DETAnonoate can generate two molecules of NO, and the starting concentration of DETAnonoate was 100µmol/l, complete conversion should give 200µmol/l. However, due to the long half-life, this concentration was not reached in the experiment.

Figure 4.11 shows the chromatograms of the PAPAnonoate samples taken at different time points.

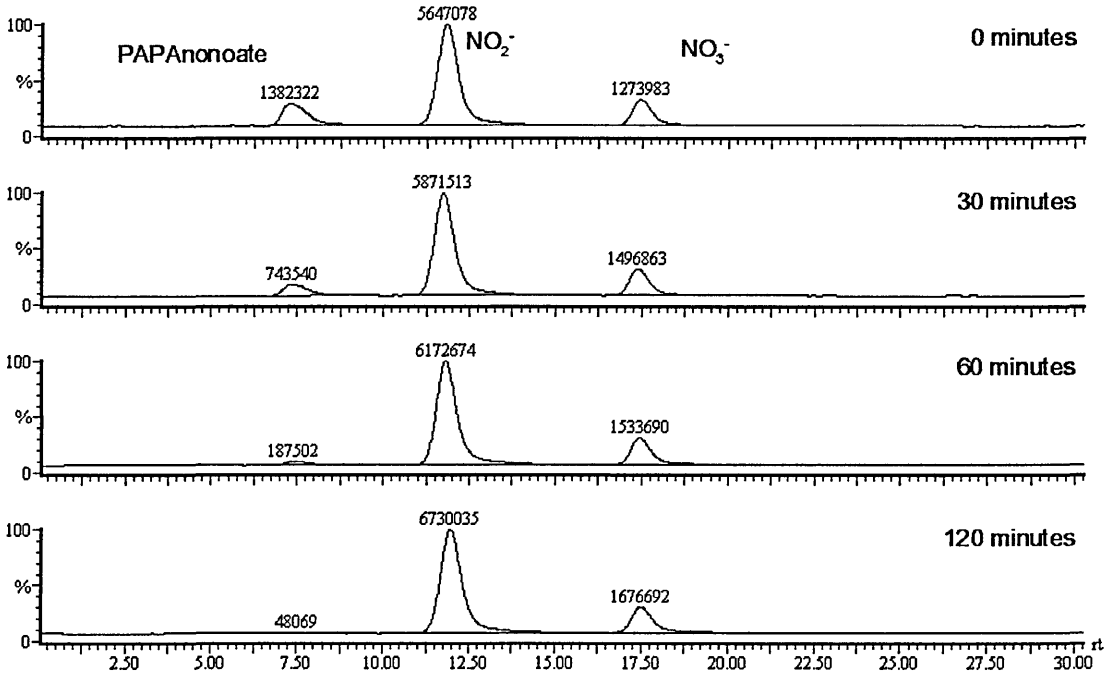

Figure 4.11: Degradation of PAPAnonoate after 0, 30, 60 and 120 minutes. Values on peaks indicate peak area.

The PAPAnonoate peaks at 7.5 minutes disappears almost completely after 120 minutes of incubation. PAPAnonoate is a rather fast NO donor with a half-life of approximately 15 minutes[210]. Due to its instability nitrite and nitrate levels were already very high at the beginning of the experiment and rose only to a fewer extent as shown in Figure 4.12.

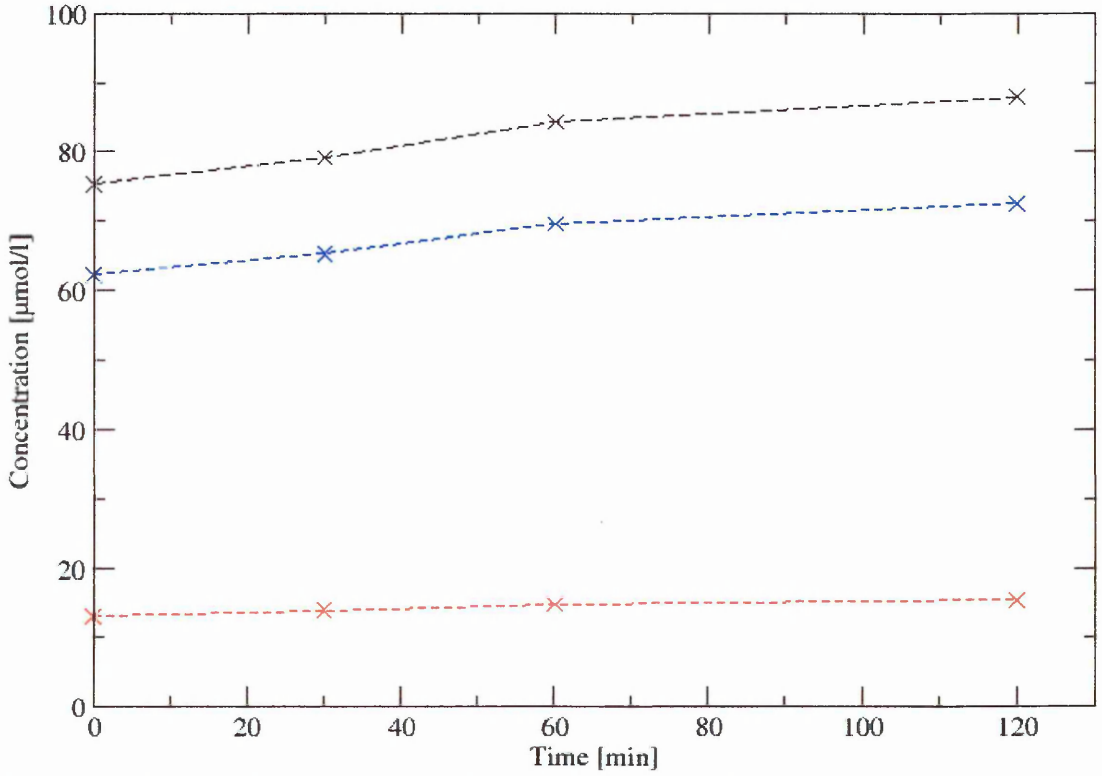

Figure 4.12: Nitrite(blue), nitrate(red) and total NO_X(black) concentration in the buffered PAPAnonoate samples

Due to the short half-life of PAPAnonoate a major fraction was already broken down before the experiment started. Again the primary product of NO degradation appears to be nitite rather than nitrate in aqueous solution. Even though two molecules of NO can be produced from one molecule of

PAPAnonoate and the starting concentration of PAPAnonoate was 100µmol/l, total NO_x levels were only about 84µmol/l after 120 minutes and did not reach the theoretical maximum concentration of 200µmol/l. It is unclear what the reason for this behaviour is. The PAPAnonoate may already have been degraded when it was received from the manufacturer or generally the purity was not as what the label stated it to be.

4.3 Conclusions

The previously developed method involving ion chromatography – mass spectrometry with a chloride trap and post column oxidation was successfully used to study NOS turnover in healthy volunteers and patients suffering from primary pulmonary hypertension. The following conclusions can be drawn:

- at the ^{15}N-L-arginine doses administered ^{15}N-nitrate could be detected in both the healthy volunteers and the patient samples. Endogenous nitrate levels could be easily determined from the ^{14}N-nitrate concentration and simply had to be subtracted. ^{15}N-nitrite was not detected, possibly because the oxidation to nitrate caused ^{15}N-nitrite levels to drop below the limit of detection.

- The use of a label in pulse chase studies means that the method could successfully distinguish between nitrite produced by endogenous sources (e.g. possibly bacterial infection) in some of the samples, where other methods would likely fail.

- The average amount of ^{15}N-nitrate excreted by the healthy subjects was 10.65 µmoles (SD = 5.01 µmol) and 3.186 µmoles (SD = 2.55 µmol) for the PPH patients respectively. In the healthy subjects, 0.622% of the administered ^{15}N-L-arginine was converted to ^{15}N-nitrate in 36h, while the patients group only converted 0.186%.

- Compared to other studies of L-arginine conversion in healthy subjects, significantly higher amounts of ^{15}N-nitrate were detected (10 times more than Forte et al. and 2.5 times more than Demoncheaux et al.). This may be a result of lower losses as this method requires minimal sample preparation in contrast to the other methods that require derivatisation of nitrite and nitrate.

- Other studies assessing NOS turnover in PPH patients and healthy subjects agree with the results shown here in reporting decreased NO production in the patients. Demoncheaux et al[208] found a 16-fold reduction of urinary non-endogenous ^{15}N-nitrate levels, while Kaneko et al[209] found a 4.8-fold reduction of NO metabolites in the lung.

- The excretion patterns do not indicate clearance after 36 h, so it is quite likely that the administered ^{15}N-L-arginine will be present in the body for a much longer time than the experiment. On the other hand ^{15}N-L-arginine could be determined in high levels after 12h after infusion. It would be interesting to observe excretion over a longer time period.

- Other analytes can be measured additionally to nitrite and nitrate. While the other techniques are specific to nitrite and nitrate, the method presented here is capable of measuring other analytes as well. This includes anions such as amino acids, organic acids or inorganic anions. But most importantly it was found that nitric oxide donors and carriers can be detected on the same mass

trace as nitrite and nitrate which makes the technique extremely valuable for drug development and testing or to identify new nitric oxide metabolites.

- The method is specific for NO metabolites and producers. The technique was designed to be specific for nitric oxide metabolites and producers, by using post-column oxidation. All nitric oxide metabolites and producers are detected as nitrate. This not only increases sensitivity for quadrupole mass spectrometers by maximising the dwell time, but can also reduce the number of peaks in the chromatogram.

5. Conclusions

5.1 Overall Conclusions

Two methods have been developed for the determination of NOS turnover, a method based on SERRS and a method based on IC-MS. Both methods offer considerable advantages over previous techniques for the measurement of NOS turnover. The IC-MS method was applied to patient samples.

i) SERRS

The Raman spectroscopic method was found to be a simple and straightforward method for high-throughput analysis of NO turnover in biological studies, where higher concentrations of ^{15}N-L-arginine can be applied (such as in cell cultures) but was of limited use for human studies where ^{15}N-nitrite is usually present at levels below the ratio of isotopic discrimination that is possible with the Raman method.

- Limit of detection of 5 nmol/l for nitrite was obtained and calibration curves were linear up to at least 20µmol/l.
- Isotopic discrimination between the ^{15}N and ^{14}N isotopes in the Griess dye was possible at ^{15}N/^{14}N isotopic ratios of 1:20 or greater.
- The interfering bands resulting from the citrate used to prepare the silver colloid were minimised by optimisation of the aggregant concentration.
- High reproducibility between samples and standards was achieved by separating the Griess product from the ionic matrix by solid phase extraction prior to aggregation. ^{15}N-nitrite could be detected in urine, serum and cell

culture medium with recoveries of 96% (range from 91.6% to 98.7%), 85% (range from 79.8% to 90.3%) and 95% (range from 88.7% to 101.2%) respectively.

- sample turnover time was approximately 5 minutes.
- It was not possible to detect ^{15}N-nitrite derived from ^{15}N-L-arginine by activated macrophages. However, the problem was identified as failure to activate the macrophages and not the analytical method itself.

ii) IC-MS

The method based on ion chromatography – mass spectrometry with a chloride trap column and postcolumn oxidation was suitable for the determination of NO turnover in humans as it was possible to measure ^{15}N-nitrite and ^{15}N-nitrate in the presence of large amounts of ^{14}N-nitrite and ^{14}N-nitrate.

- Limit of detection was 200nmol/l for ^{14}N-nitrite and ^{14}N-nitrate and 50nmol/l for ^{15}N-nitrite and ^{15}N-nitrate and calibration curves were linear up to at least 500µmol/l.
- Isotopic discrimination was only limited by the instrument resolution which is typically 1:1000[195]
- No sample derivatisation was required and sample pretreatment was minimal. The chromatographic separation makes detection of the analytes easier and the chloride trap column removes the major interferent quantitatively.

- Recoveries were excellent for serum and urine,The mean recovery of nitrate was 93% (range from 89% to 96.% from urine and 94% (range from 89% to 100%) from serum. The mean recovery of nitrite was 96% (range from 91% to 99%) from urine and 107% (range from 103%to 110%) from serum.
- sample turnover time was 1 hour.
- other NO related compounds could be detected such as NO carriers and NO donors.

iii) Comparison of techniques

- The SERRS method (LOD 5 nmol/l for nitrite) was more sensitive than the IC-MS method (LOD 50 nmol/l for ^{15}N-nitrite and ^{15}N-nitrate, 200 nmol/l for ^{14}N-nitrite and ^{14}N-nitrate).
- The IC-SM method showed better isotopic discrimination than the SERRS method since the two bands for the $^{15}N=^{14}N$ and $^{14}N=^{14}N$ could not be resolved in the Raman spectrum. Isotopic discrimination in the IC-MS method was only affected by endogenous ^{15}N-nitrate levels and instrument resolution.
- The existing techniques, as well as this SERRS method, need derivatisation of nitrite/nitrate to measure total nitrite/nitrate concentrations as well as to determine the $^{14}N/^{15}N$ ratio of the species. This not only consumes more time, and complicates the method, but also introduces risks of error due to incomplete derivatisation, losses through oxidation or losses through side reactions. The IC-MS method did not require sample derivatisation, but only a simple cleanup procedure.

- the SERRS method is quicker than the IC-MS method. It takes approximately one hour to measure a serum or urine sample by IC-MS including complete elution of the sample matrix from the column and equilibration. It takes only approximately 5 minutes to measure a serum or urine sample by SERRS.

iv) Patient samples

NOS turnover in healthy subjects and patients suffering from primary pulmonary hypertension was investigated using the method based on ion chromatography – mass spectrometry.

- Excretion of ^{15}N-nitrate derived from administered ^{15}N-L-arginine was significantly lower in the patient group than in controls, in agreement with other work[208, 209], suggesting that NOS turnover is indeed lower in the patient group.
- Compared to other studies of L-arginine conversion in healthy subjects, significantly higher amounts of ^{15}N-nitrate were detected (10 times more than Forte et al[132] and 2.5 times more than Demoncheaux et al.[208]). This may be a result of lower losses as this method requires minimal sample preparation in contrast to the other methods that require derivatisation of nitrite and nitrate.

iv) Future Research

The SERRS method could be used for more studies with cell cultures. It is well suited to high throughput work with automated sample handling in multiwell plate assays.

Ion chromatography – mass spectrometry has sufficient selectivity and sensitivity for clinical work, albeit with slower analysis. It may have the potential to replace the existing "gold-standard" of gas isotope ratio mass spectrometry.

The detection of NO metabolites by oxidative postcolumn processing and mass spectrometry could be extended to couple to other modes of chromatography. Ion chromatography is not ideal for organic compounds like NO donors and NO carriers and other types of chromatography will be more suitable for their separation. The oxidative postcolumn processing and detection by mass spectrometry as nitrate is a technique of detection that is specific for all compounds that can be oxidised to nitrate including NO carriers, NO donors and various metabolites. It could, for instance, be used to discover possible unknown metabolites or elucidate the role of S-nitrosothiols in serum.

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7. References

1 Garthwaite J., Boulton C.L.; Nitric Oxide signalling in the central nervous system; Annu. Rev. Physiol.; 1995; 57:683-706

2 Hierholzer C., Harbrecht B., Menezes J.M., Kane J., MacMicking J., Nathan C.F., Peitzman A.B., Billiar T.R., Tweardy D.J.; Essential role of induced nitric oxide in the initiation of the inflammatory response after hemorrhagic shock;J. Exp. Med.; 1998; 187: 917-928

3 Palmer R.M., Ferrige A.G., Moncada S.; Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor; Nature; 1987; 327:524-526

4 Moncada S., Higgs A.; The L-arginine-nitric oxide pathway; New England Journal of medicine; 1993; 329:2002-2012

5 Archer S.L., Tobins J.P., Raij L., Weir E.K.; Hypoxic pulmonary vasoconstriction is enhanced by inhibition of the synthesis of an endothelium derived relaxing factor.; Biochem. Biophys. Res. Comm.; 1989; 164:1198-1205

6 Lakko M., Letho S., Epidemiology of risk factors for cardiovascular disease in diabetes and impaired glucose tolerance; Atherosclerosis; 1998; 137:65-73

7 Petros A., Bennett D., Vallance P.; Effect of nitric oxide synthase inhibitors on

hypotension in patents with septic shock; Lancet; 1991; 338:1557-1558

8 Lejeune P., Lagade C. P., Onier N., Pinard D., Oshima H., Jannin J. F.; Nitric oxide involvement in tumor-induced immunosupprssion; J. Immunol.; 1994; 152: 5077-5083

9 Heslop R.B., Jones K.; A Guide to Advanced Study; Inorganic Chemistry; 1976; 424-432

10 Bredt D.S., Snyder S.H.; Nitric Oxide a physiologic messenger molecule; Ann. Rev. Biochem.; 1994; 63:175-195

11 Nathan C., Xie Q.W.; Nitric Oxide synthases: roles, tolls and controls; Cell; 1994; 78:915-918

12 Stamler J.S., Feelisch M.; Methods in Nitric Oxide Research; Wiley; 1996; 2:19-37

13 Bhgat K., Vallance P.; 1996; J. Royal Soc. Chem.; 89; 667-673

14 Nathan C., Xie Q.W.; Regulation of biosynthesis of nitric oxide; 1994; J. Biol. Chem.; 269:13725-13728

15 Förstermann U., Kleinert H.; Nitric Oxide Synthase: expression and

expressional control of the three isoforms; Naumyn-Schmiedebergs Arch Pharmacol; 1995; 352:351-364

16 Knowles R.; Nitric Oxide Synthases; The Biochemist; 1994; Oct/Nov:3-8

17 Moncada S.; The L-arginine nitric oxide pathway.; Acta Physiol. Scand.; 1992; 145:201-227

18 Kelm M., Feelisch M., Deussen A., Strauer B.E., Schrader J.; Release of endothelium derived nitric oxide in relation to pressure and flow; Cardiovasc. Res.; 1991; 25: 831-836

19 Castillo L., DeRojas T.C., Chapman T.E., Vogt J., Burke J.F., Tannenbaum S.R., Young V.R.; Splanchnic metabolism of dietary arginine in relation to nitric oxide synthesis in normal adult man; Proc. Natl. Acad. Sci. USA; 1993; 90: 193-197

20 Demoncheaux E. A. G., Higenbottam T.W., Foster P.J., Borland C. D. R., Smith A. P. L., Mariott H. M., Bee D., Akamine S., Davies M. B.; Circulating nitrite is a donor for nitric oxide; Clinical Science; 2002; 102:77-83

21 Weizberg E., Lundberg J. O.;Nonenzymatic nitric oxide production in humans; Nitric Oxide; 1998; 2(1):1-7

22 Czapski G., Goldstein S.; The role of the reactions of NO with superoxide and oxygen in biological systems: a kinetic approach; Free Radic. Biol. Med.; 1995; 19(6):785-794

23 Gaston B. Drazen J. M., Loscalzo J., Stamler J. S.; The biology of nitrogen oxides in the airways; Am. J. Respir. Crit. Care. Med; 1994; 149:538-551

24 Radi R., Beckman J. S., Bush K. M., Freeman B. A.; Peroxynitrite oxidation of sulfhydrils: The cytotoxic potential of superoxide and nitric oxide; J. Biol. Chem; 1991; J. Biol. Chem.; 266:4244-4250

25 Radi R., Beckman J. S., Bush K. M., Freeman B. A.; Peroxynitrite-induced membrane lipid peroxidation: The cytotoxic potential of superoxide and nitric oxide; Arch. Biochem. Biophys.; 1991; 288:481-489

26 King P. A., Anderson V. E., Edwards J. O., Gustafson G., Plumb R. C., Suggs J. W.; The stable solid state that generates hydroxyl radical upon dissolution in aqueous solutions: Reactions with proteins and nucleic acid; J. Am. Chem Soc.; 1992; 114:5430-5432

27 Beckman J. S., Koppenol W. H.; Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly; Am. J. Physiol. Cell Pysiol. ;1996; 40:C1424-C1437

28 Radi R., Beckman J. S., Bush K. M., Freeman B. A.; Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitic oxide; Arch. Biochem. Biophys.; 1991; 288(2):481-487

29 Lymar S. V.; Hurst J. K.; Rapid reaction between peroxonitrite ion and carbon dioxide: Implications for biological activity; J. Am. Chem. Soc.; 1995; 117:8867-8868

30 Oda H., Kusumoto S., Nakajima T.; Nitrosyl-hemoglobin formation in the blood of animals exposed to nitric oxide; Arch. Environ. Health; 1975; 30:453-455

31 Olsen J. S.; Stopped-flow, rapid mixing measurements of ligand binding to haemoglobin and red cells; Methods enzymol.; 1981; 76:631-704

32 Gibson Q.H., Roughton F.J.W.; The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin; J.Physiol.; 1957; 136:507-526

33 Gibson Q.H., Roughton F.J.W.; Further studies on the kinetics and equilibria of the reactions of nitric oxide with haemoproteins; Proc. R. Soc. Lond. B. Biol. Sci.;1965; 163:197-205

34 Jia L., Bonaventura C., Bonaventura J, Stamler J.S.; S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control; Nature; 1996; 380:221-226

35 Kosaka H., Uozumi M., Tyuma I.; The interaction between nitrogen oxides and hemoglobin and endothelium-derived relaxing factor; Free Radical Biol. Med.; 1989; 7:653-658

36 Hakim T. S., Sugimori K., Camporesi E.M., Anderson G.; Half-life of nitric oxide in aqueous solutions with and without haemoglobin; Physiol. Meas.; 1996; 17(4): 267-277

37 Wennmalm A., Benthin G., Peterson A-S.; Dependance of the metabolism of nitric oxide (NO) in healthy human whole blood on the oxygenation of its red cell haemoglobin; Br. J. Pharmacol.; 1992; 106:507-508

38 Ignarro L.J., Fukuto J.L., Griscavage J.M., Rogers N.E., Byrns R.E.; Oxidation of nitric oxide: Comparison with enzymatically formed nitric oxide from L-arginine; 1993; Proc. Natl. Acad. Sci.;90:8103-8107

39 Ignarro L.J., Fukuto J.M., Griscavage J.M.; Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine; Proc. Natl. Acad. Sci. USA; 1993; 90:8103-8107

40 Kelm M., Feelisch M., Spahr R., Piper H.M., Noack E., Schrader J.; Quantitative and kinetic characterization of nitric oxide and EDRF release from cultured endothelial cells; Biochem. Biophys. Res. Commun; 1988; 154:236-244

41 Eich R.F., Li T., Lemon D.D., Doherty D.H., Curry S.R., Aitken J.F., Mathews A.J., Johnson K.A., Smith R.D., Philips Jr. G. N., Olson J.S.; Mechanism of NO-induced oxidation of myoglobin and hemoglobin; Biochemistry; 1996; 35:6976-6983

42 Carlsen E., Comroe Jr., The rate of uptake of carbon monoxide and of nitric oxide by normal human erythrocytes and experimantally produced spherocytes, J. Gen. Physiol.; 1958; 42: 83-107

43 Stone J. R., Sands R.H.; Dunham W. R., Marletta M. A.; Electron paramagnetic resonance spectral evidence for the formation of a pentacoordinate nitrosyl-heme complex on soluble guanylate cyclase; Biochem. Biophys. Res. Commun.; 1995; 207: 572-577

44 Goldstein S., Czapski G.; Mechanism of the nitrosation of thiols and amines by oxygenated NO solutions: the nature of the nitrosating intermediates; J. Am. Chem. Soc.; 1996; 118:3419-3425

45 Pryor W. A., Church D. F., Govindan C. K., Crank G.; Oxidation of thiols by nitric oxide and nitrogen dioxide: synthetic utility and toxicological implications; J. Org. Chem; 1982; 47: 156-159

46 Stamler J.S., Singel D.J., Loscalzo J.; Biochemistry of nitric oxide and its redox-

actictivated forms; Science; 1992; 258: 1898-1902

47 Arnelle D., Stamler J.S., NO+ and NO. and NO- donation by S-nitrosothiols: implications for regulation of physiological functions by S-nitrosylation and acceleration of disulfide formation; Arch. Biochem. Biophys; 1995; 318: 279-285

48 Jia L., Bonaventura C., Bonaventura J, Stamler J.S.; S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control; Nature; 1996; 380:221-226

49 Zhang Y., HoggN.; Mixing Artefacts from the bolus addition of nitric oxide to oxymyoglogin: implications for S-nitrosothiol formation; Free Rad. Biol. Med.; 2002; 32:1212-1219

50 Reichenbach G., Sabatini S., Palombari R., Palmerini C. A.; Reaction Mechanism between Nitric Oxide and Glutathione Mediated by Fe(III) Myoglobin; 2001; 5:395-401

51 Simon D.I., Stamler J.S., Jaraki O., Keaney J.F., Osborne J.A., Francis S.A., Singel D.J., Loscalzo J.; Antiplatelet properties of protein S-nitrosothiols derived from nitric oxide and endothelium derived relaxing factor; Arterioscler. Thromb.; 1993; 13:791-799

52 Minamiyama Y., Takemura S., Inoue M.; Effect of thiol status on nitric oxide metabolism in the circulation; Arch. Biochem. Biophys.; 1997; 341:186-192

53 Stamler J.S., Feelisch M.; Methods in Nitric Oxide Research; John Wiley and Sons Chichester; 1996; 19-28

54 Butler A.R., Rhodes P.; Chemistry, analysis and biological roles of S-nitrosothiols; Anal. Biochem.; 1997; 249: 1-9

55 Scharfstein J.S., Keaney Jr. J.F., Slivka A., Welch G.N., Vita J.A., Stamler J.S., Loscalzo J.; In vivo transfer of nitric oxide between a plasma protein-bound reservoir and low molecular weight thiols; J. Clin. Invest; 1994; 94:1432-1439

56 Rassaf T., Bryan N. S., Kelm M., Feelisch M.; Concomitant presence of N-nitroso and S-nitroso proteins in human plasma; Free Rad. Biol. Med.; 2002; 11: 1590-1596

57 Mirvish S. S.; Role of N-nitroso compounds(NOC) and N-nitrosation in etiology of gastric, esophageal, and bladder cancer and contribution to cancer to cancer of known exposures to NOC.; Cancer 1995; 93:17-48

58 Oshima II., Bartsch II.; Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis; Mutat. Res.; 1994; 305:253-264

59 Ignarro L.J., Fukuto J.M., Griscavage J.M., Rogers N.E., Byrns R.E.; Oxidation

of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine; Proc. Natl. Acad. Sci. USA; 1993; 90(17):8103-8107

60 Hentze M. W., Kuhn L. C.; Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress; Proc. Natl. Acad. Sci. USA; 1996; 93:8175-8182

61 Yoshida K., Kasama K., Kitabatake M., Okuda M., Imai M.; Metabolic fate of nitric oxide; Int. Arch. Occup. Environ. Health; 1980; 46:71-77

62 Liu X., Miller M. J. .S., Joshi M.S., Thomas D.D., Lancaster J.R.; Accelerated reaction of nitric oxide with O_2 within the hydrophobic interior of biological membranes; Proc. Natl. Acad. Sci. USA; 1998; 95:2175-2179

63 Lancaster J.R.; A tutorial on the diffusablility and reactivity of free nitric oxide; Nitric Oxide Biol. Chem.; 1997; 1:18-30

64Liu X., Miller M.J.S., Joshi M.S., Sadowska-Krowicka, Clark D.A., Lancaster J.R., Diffusion-limited reaction of free nitric oxide with erythrocytes, J. Biol. Chem.; 1998; 273:18709-18713

65 Wennmalm A., Benthin G., Edlund A.; Metabolism and excretion of nitric oxide in humans An experimental and clinical study; Circ. Res.; 1993; 73: 1121-1127

66 Jungersten L., Edlund A., Petersson A.S., Wennmalm A.; Plasma nitrate as an index of nitric oxide formation in man: analyses of kinetics and confounding factors; Clin. Physiol.; 1996; 16:369-379

67 Weitzberg E., Lundberg J. O. N.; ; Non enzymatic nitric oxide production; Nitric Oxide; 1998; 2:1-7

68 Yoshida K., Kasama K., Kitabatake M., Imai M.; Boitransformation of nitric oxide, nitrite and nitrate; Int. Arch. Occup. Environ. Health; 1983; 52:103-115

69 Yoshida K., Kasama K.; Biotransformation of nitric oxide.; Environ Health Perspect.; 1987; 73:201-205

70 Kelm M., Feelisch M., Grube R., Motz W., Strauer B.E., in: Moncada S., Marletta M.A., Hibbs Jr. J.B., Higgs E.A.; The Biology of Nitric Oxide. Physiological and Clinical Aspects; Portland Press London; 1992; 1:319-322

71 Parks N.J., Krohn K.A., Mathis C.A., Chasko J.H., Geiger K.R., Gregor M.E., Peek N.F.; Nitrogen-13-labelled nitrite and nitrate: distribution and metabolism after intratracheal administration; Science; 1981; 212:58-61

72 Rhodes P.M., Leone A.M., Francis P.L., Struthers A.D., Moncada S.; The L-arginine nitric oxide pathway is the major source of plasma nitrite in fasted

humans; Biochem. Biophys. Res. Commun.; 1995; 209:590-596

73 Kelm M.; Nitric oxide metabolism and breakdown; Biochem. Biophys. Acta; 1999; 1411: 273-289

74 Demoncheaux E. A. G., Higenbottam T. W., Foster P. J., Borland C. D. R., Smith A. P. L., Marriot H. M., Bee D., Akamine S., Davies M.B., Circulating nitrite is a donor for nitric oxide, 2002, Clin. Sci., 102:77-83

75 Grube R., Kelm M., Motz W., Strauer B.E., in: Moncada S., Feelisch M., Busse R., Higgs E.A.; The Biology of Nitric Oxide, Enzymology, Biochemistry and Immunology; Portland Press London; 1994: 4:201-204

76 Michigami Y., Yamamoto Y., Ueda K.; Determination of Nitrite, Sulphate, Bromide and nitrate in human serum by ion chromatography; Analyst; 1989; 114:1201-1205

77 Stamler J.S., Feelisch M.; Methods in Nitric Oxide Research; Wiley; 1996; 5:47-60

78 Doyle M.P., Herman J.G., Dykstra R.L.; Autocatalytic oxidation of hemoglobin induced by nitrite: activation and chemical inhibition; Free Radic. Biol. Med.; 1985; 1:145-153

79 Doyle M.P., Pickering R.A., Dykstra R.L., Nelson C.L., Boyer R.F.; Involvement of peroxide and superoxide in the oxidation of hemoglobin by nitrite; Biochem. Biophys. Res. Commun.; 1982; 105:127-132

80 Kosaka H., Imaizumi K., Imai K., Tyuma I.; Stoichiometry of the reaction of oxyhemoglobin with nitrite; BioChim. Biophys. Acta; 1937; 581:184-188

81 Barnard R.D., The reactions of nitrite with hemoglobin derivatives; J. Biol. Chem.; 1937; 120:177-191

82 Wagner D.A., Moldawer L.L., Pomposelli J.L., Tannenbaum S.R., Young V.R.; Nitrate biosynthesis in the rat; 1985; 232:547-551

83 Trujilli M., Alvarez M., Peluffo B., Feeman B. Radi R.; Xanthine oxidase mediated decomposition of S-nitrosothiols; J. Biol. Chem; 1998; 273: 7828-7834

84 Hou Y., Guo Z., Li J., Wang P.G.; Seleno compounds and glutathion peroxidase catalysed decomposition of S-nitrosothiols; Biochem. Biophs. Res. Commun.; 1996; 228:88-93

85 Nikitovic D., Holmgren A.; S-nitrosoglutathione is cleaved by thioredoxin system with liberation of glutathione and redox regulating nitric oxide; J. Biol. Chem.; 1996; 271:19180-19185

86 Jourd'heuil D., Laroux F. S., Miles A. M., Wink D. A., Grisham M. B.; Effect of Superoxide Dismutase on the Stability of S-nitrosothiols; Arch. Biochem. Biophys.; 1999; 361:323-330

87 Jensen D., Belka G., Du Bois G., S-nitrosoglutathione is a substrate for rat alcohol dehydrogenase class III isoenzyme; J. Biochem.; 1998; 331:659-68

88 Askew S. C., Butler A. R., Flitney F. W., Kemp G. D., Megson I. L.; Chemical mechanisms underlying the vasodilator and platelet anti-aggregation properties of S-nitroso-N-acetyl-DL-penicillamine and S-nitrosoglutathione; Boiorgan. Med. Chem.; 1995; 3:1-9

89 Stamler J. S., Jaraki O., Osbourne J., Simon D.L., Keaney J., Vita J., SingelD., Valeri C.R., Loscalzo J.; Nitric Oxide circultes in mammalian plasma primarily as an S-nitroso adduct of serum albumin; Proc. Natl. Acad. Sci. USA; 1992; 89: 7674-7677

90 Jourd'Heuil D., Hallen K., Feelisch M., Grisham M. B.; Dynamic state of S-nitrosothiols in human plasma and whole blood; Free Rad. Biol. Med.; 2000; 3:409-417

91 Vanin A. F., Malenkowa I. V., Serezhenkow V. A.; Iron catalyses both decomposition and synthesis of S-nitrosothiols: optical and electron paramagnetic reonance studies; Nitric Oxide; 1997; 1:191-203

92 Singh R. J., Hogg N., Joseph J., Kalyanaraman B.; Mechanism of nitric oxide release from S-nitrosothiols; J. Biol. Chem.; 1996; 271:18596-18603

93 Al-Sa'doni H.H., Megson I.L., Bisland S., Butler A.R., Flitney F.W.; Neocuprione, a selective Cu(I) chelator, and the relaxation of rat vascular smooth muscle by S-Nitrosothiols; Br. J. Pharmacol.; 1997; 121:1047-1050

94 Aleryani S., Milo E., Rose Y., Kostka P.; Superoxide-mediated decomposition of biological S-nitrosothiols; J. Biol. Chem.; 1998; 273:6041-6045

95 Sies H.; Oxidative stress: from basic research to clinical application; Am. J. Med.; 1991; 91:31-38

96 Schmidt H. H. H. W., Hofmann H., Schindler U., Shutenko Z.S., Cunningham D.D., Feelisch M.; No NO from NO synthase, Proc. Natl. Acad. Sci. USA; 1996; 14492-14497

97 Miles A.M., Bohle D.S., Glassbrenner P.A., Hansert B., Wink D.A., Grisham M.B.; Modulation of superoxide -dependant oxidation and hydroxylation reactions by nitric oxide, J. Biol. Chem.; 1996; 271:40-47

98 Brenman J.E., Chao D.S., Gee S.H., McGee A.W., Craven S.E., Santillano D.R., Wu Z., Huang F., Xia H., Peters M.F., Froehner S.C., Bredt D.S.;

Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains; Cell; 1996; 84:757-767

99 Bredt, D. S., Christoferson K. S., Brenman J. E.; Jap. J. Pharmacol.; 1997; 75(Suppl. I), 7P(Abst 20)

100 Boulton C., Garthwaite J.; NO signalling and synaptic plasticity; The Biochemist; 1994, 16/5:11-14

101 Boulton C.L., Southam E., Garthwaite J.; Nitric Oxide dependant long term potentiation; Neuroscience; 1995; 69:699-703

102 Duarte I. D. G., Lorenzetti B. B., Ferreira S. H.; Acetylcholine induces peripheral analgesia by the release of nitric oxide. Excerpta Medica; 1989, 1990:165-170

103 Rand M.J.; Nitrergic transmission: nitric oxide as a mediator of non-adrenergic, non-cholinergic neuro-effector transmission; Clin. Exp. Pharmacol. Physiol.; 1992; 19:147-169

104 Ignarro L. J., Bush P. A., Buga G. M., Wood K. S., Fukoto J. M. Rajfer J.; Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle; Biochem. Biophys. Res. Commun.; 1990; 170:843-50

105 Stamler J. S.; Redox Signalling: nitrosylation and related target interactions of nitric oxide; Cell; 1994; 78(6):931-936

106 Wheeler M .A.;, Smith S. D., García-Cardeña G., Nathan C. F., Weiss R. M., Sessa W. C.; Bacterial infection induces nitric oxide synthase in human neutrophils; J. Clin. Invest.; 1997; 99: 110-116

107 Stuehr D.J., Marletta M.A.; Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in responce to Escherichia coli lipopolysaccharide; Proc. Natl. Acad. Sci. USA; 1985; 82:7738-7742

108 Hibbs J.B., Taintor R.R., Vavrin Z.; Synthesis of nitric oxide from a terminal guanidino nitrogen atom of L-arginine: a molecular mechanism regulating cellular proliferation that targets intracellular iron; Excerpta Medica; 1990; 189-223

109 Gaston B., Reilly J., Drazen J. M., Fackler J., Ramden P., Arnelle D., Mullins M. E., Sugarbaker D., Chee C., Singel D., Loscalzo J., Stamler J. S.; Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways; Proc. Natl. Acad. Sci. USA; 1993; 90:10957-10961

110 Hierholzer C., Harbrecht B., Menezes J.M., Kane J., MacMicking J., Nathan C.F., Peitzman A.B., Billiar T.R., Tweardy D.J.; Essential role of induced nitric

oxide in the initiation of the inflammatory response after hemorrhagic shock;J. Exp. Med.; 1998; 187: 917-928

111 Brune B., Gotz C., Messmer U.K., Sandau K., Hirvonen M.R., Lapetina E.G.; Superoxide formation and macrophage resistance to nitric oxide-mediated apoptosis; J. Biol. Chem.; 1997; 272:7253-7258

112 Nunoshiba T., deRojas-Walker T., Wishnok J.S., Tannenbaum S.R., Demple B.; Activation by nitric oxide of an oxidative-stress response that defends Escherichia coli against activated macrophages; Proc. Natl. Acad. Sci. USA; 1993; 90:9993-9997

113 Radomski M.W., Moncada S.; Biological Role of nitric oxide in platelet function; EDICOMPLET; 1991; 45-56

114 Haywood G.A., Tsao P.S., von der Leyen H.E.; Expression of inducible nitric oxide synthase in human heart failure; Circulation; 1996; 93:1087-1094

115 Sase K., Michel T.; Expression and Regulation of Endothelial Nitric Oxide Synthase; Trends Cardiovasc. Med.; 1997; 7:28-37

116 Waldman S. A., Murad F.; Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylase cyclase-cyclic GMP system; J. Cardiovasc. Pharmacol.; 1988; 12: Suppl 5: 115-118

117 Jia L., Bonaventura C., Bonaventura J., Stamler J. S.; S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control; 1996; Nature; 380:221-226

118 Stamler J.S., Jia L., Eu J.P., McMahon T.J., Demchenko I.T., Bonaventura J., Gernert K., Piantadosi C.A.; blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient; Science; 1997; 276:2034-2037

119 Ramsay ., Radomski M., de Belder A., Martin J.F., Lopez-Jaramillo P.; Systemic effects of S-nitroso-glutathione in the human following intravenous infusion; Br. J. Clin. Pharmacol; 1995; 40: 101-102

120 Creager M.A., Roddy M.A., Boles K., Stamler J.S.; N-Acetylcysteine does not influence the activity of endothelium-derived relaxing factor in vivo; Hypertension; 1997; 29:668-672

121 Paltauf-Doburzynska J., Posch K., Paltauf G., Graier W. F.; Stealth ryanodine-sensitive Ca2+ release contributes to activity of capacitative Ca^{2+} entry and nitric oxide synthase in bovine endothelial cells, Journal of Physiology, 1998; 513:369-379

122 Baglis C., Mitruka B., Deny A.; Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage; J. Clin. Invest.; 1992; 90:278-281

123 Nguyen T., Brunson D., Crespi C. L., Penman B. W., Wishnok J. S., Tannenbaum S. R.;DNA damage and mutation in human cells exposed to nitric oxide in vitro; Proc. Natl. Acad. Sci. USA; 1992; 89:3030-3034

124 Montruccio G., Lupia E., de Martino A., Battaglia E., Arese M., Tizziani A. S., Bussolino F., Camussi G.; Nitric oxide mediates angiogenesis induced in vivo by platelet-activating factor and tumor necrosis factor-, Am. J. Pathol.; 1997; 151:557-563

125 Thomsen L. L., Miles D. W., Happerfield L., Bobrow L. G., Knowles R. G., Moncada S.; Nitric oxide synthase activity in human breast cancer; Br. J. Cancer; 1995; 72:41-44

126 Moochhala S. M., Chhatwall V. J. S., Chan S. T. F., Ngoi S. S., Chia Y. W., Rauff A.; Nitic oxide synthase activity and expression in human colorectal cancer; Carcinogenesis; 1996; 17:1171-1174

127 Groszmann R.J.; Hyperdynamic circulation of liver disease 40 years later: pathophysiology and clinical consequences; Hepatology; 1994; 20:1359-1363

128 Herve P., Lebrec D, Brenot F., Simonneau G., Humbert M., Sitbon O., Duroux P., Pulmonary vascular disorders in portal hypertension; Eur. Resp. J.; 1998;

11:1153-1166

129 Guarner G., Soriano G., Tomas A., Bulbena O., Novella M.T., Balanzo J., Vilardell F., Mourelle M., Moncada S.; Increased serum nitrite and nitrate levels in patients with cirrhosis: relationship to endotoxemia; 1993; 18:1139-1143

130 Lumsden A.B., Henderson J.M., Kutner M.H.; 1998; Endotoxin levels measured by chromogenuc assay in portal, hepatic and peripheral venous blood in patients with cirrhosis; Hepatology; 8:232-236

131 Sogni P., Smith A.P.L., Gadano A.Lebrec D., Higambottam T.W.; 1997; Induction of nitric oxide synthase II does not account for excess vascular nitric oxide production in experimental cirrhosis.; Journal of Hepatology; 26:1120-1127

132 Forte P., Copland M., Smith L.M., Milne E., Sutherland J., Benjamin N.; Basal nitric oxide sythesis in essential hypertension; Lancet; 1997; 349:837-842

133 Nakaki T., Hishikawa K., Suzuki H., Saruta T., Kato R.; L-arginine-induced hypotension; Lancet; 1990; 336:696

134 Pepke-Zaba J., Higenbottam T.W., Dinh-Xuan A.T., Stone D., Wallwork J.; Inhaled nitric oxide as a cause of selective pulmonary vasodilation in pulmonary hypertension; Lancet; 1991; 338:1173-1174

135 Moncada S.;The L-arginine nitric oxide pathway.; Acta Physiol. Scand.; 1992; 145:201-227

136 Cosentino F., Hishikawa K., Katusic Z. S., Luscher T. F., High glucose increase nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells; 1997; Cirrculation; 96:25-28

137 Shiode N., Nakayama K., Morishima N., Yamagata T., Matsuura H., Kajijama G.; Nitric Oxide production by coronary conductance and resistance vessels in hypercholesterolaemic patients; Am. Heart J.; 1996; 131:1051-1057

138 Scmetterer L., Findl O., Fasching P., Ferber W., Strenn K., Breiteneder H., Nitric oxide and ocular blood flow in patients with IDDM; 1997; Diabetes; 42:1454-1461

139 Bagasra O., Michaels F. H., Zheng Y. M., Bobroski L. E., Spitsin S. V., Fu Z. F., Tawadros R., Koprowski H.; Activation of the inducible form of nitric oxide synthase in the brains of patients with multiple sclerosis; Proc. Natl. Acad. Sci. USA; 1995; 92:12041-12045

140 Panitch H. S., Hirsch R. L., Haley A. S., Johnson K. P.; Exacerbations of multiple sclerosis in patients with gamma interferon; Lancet; 1987; 1:893-895

141 Yamashita T., Ando Y., Obayashi K., Uchino M., Ando M.; Changes in nitrite

and nitrate (NO_2^-/NO_3^-) levels in cerebrospinal fluid of patients with multiple sclerosis; J. Neuron. Sci; 1997; 153:32-34

142 Griess P.; Bemerkungen zu der Abhandlung der H.H. Weselsky; und Benedikt "Ueber einige azoverbindungen"; Ber. Deutsch. Chem. Ges.; 1879; 12:426-428

143 Leis J. R., Rios A.; Fast formation of NO in reactions of alkyl nitrites with ascorbic acid and analogs; Journal of the Chemical Society, Chemical Communications; 1995;2:169-170

144 Granger D. L., Taintor R. R., Boockvar K. S., Hibbs J. B.; Methods in Enzymology; 1996; 268:142-152

145 Moshage H., Kok B., Huizenga R., Jansen P. L. M.; Nitrite and nitrate determinations in plasma: a critical evaluation; Clin. Chem.; 1995; 41:892-896

146 Sah R.N.; Nitrate-nitrogen determination – a critical review ; Communications in Soil Science and Plant Analysis; 1994; 25:2841-2869

147 Tsikas D. Gutzki F.-M., Rossa S., Bauer H. Neumann C. Dockendorff K. K., Sandmann J. Frolich J. C.; Measurement of nitrite and nitrate in biological fluids by gas chromatography-mass spectrometry and the Griess assay: problems with the Griess assay-solutions with by gas chromatography-mass spectrometry; Analytical Biochemistry; 1997; 244: 208-220

148 Green L. C., Wagner D. A., Glogowski J., Skipper P. L., Wishnok J. S., Tannenbaum S. R.; Analysis of nitrate, nitrite and [15N]Nitrate in biological fluids; Anal. Biochem.; 1982; 126:131-138

149 Marzinzig M., Nussler A. K. Stadler J., Marzinzig E., Barthlen W., Nussler N. C., Beger H. G., Morris S. M., Bruckner U. B.; Improved methods to measure end products of nitric oxide in biological fluids: nitrite, nitrate, and S-nitrosothiols; Nitric Oxide; 1997; 1:177-189

150 Gilliam M. B., Sherman M. P., Griscavage J. M., Ignarro L. J.; A Spectrophotometric Assay for Nitrate Using NADPH Oxidation by Aspergillus Nitrate Reductase; Anal. Biochem.; 1993; 212:359-365

151 Ohkawa S., Ohishi K., Yoneyama T., Hishida A., Kumagai H.; The effect of eliminating interfering substances by solid-phase extraction and sample-pH adjustment in the colorimetric assay of urinary nitrite and nitrate; Anal. Biochem.; 1998; 258:379-381

152 Forte P., Smith L. M., Milne E., Benjamin N.; Measurement of nitric oxide synthesis in humans using L-[$^{15}N_2$]arginine; Methods in Enymology; 1999; 301:92-98;

153 Tesch J. W., Rehg W. R., Sievers R. E.; Microdetermination of nitrates and

nitrites in saliva, blood, water, and suspended particulates in air by gas chromatography; J. Chrom; 1976; 126:743-755

154 Rhodes P. M., Leone A. M., Francis P. L., Struthers A. D., Moncada S.; The L-arginine:nitric oxide pathway is the major source of plasma nitrite in fasted humans; Biophys. Res. Comm.; 1995; 209:590-596

155 Tsikas D.; Simultaneous derivatization and quantification of the nitric oxide metabolites nitrite and nitrate in biological fluids by gas chromatography/mass spectrometry; Anal. Chem.; 2000; 72:4064-4072

156 Moneghan J.M., Cook K., Gara D., Crowther D.; Determination of nitrite and nitrate in human serum; J. Chrom. A; 1997; 770:143-149

157 Helaleh M. I. H., Korenaga T.; Ion chromatographic method for simultaneous determination of nitrate and nitrite in human saliva; J. Chrom. B; 2000; 744.433-437

158 Borland C., Cox Y., Higenbottam T.; Measurement of exhaled nitric oxide in man; Thorax; 1993; 48:1160-1162

159 N.K. Menon, J. Pataricza, T.Binder, R.J. Bing; Reduction of biological effluents in purge and trap micro reaction vessels and detection of endothelium-derived nitric oxide (edno) by chemiluminescence; J. Molec. Cell. Card.; 1991; 23: 389-

393

160 Alpert C., Ramdev N., George D., Loscalzo J.; Detection of S-nitrosothiols and other nitric oxide derivatives by photolysis-chemiluminescence spectrometry; Anal. Biochem.; 1997; 254:1-7

161 Doerr R. C., Fox J. B., Lakritz J. B., Fiddler W.; Determination of nitrite in cured meats bychemiluminescence detection; Anal. Chem.; 1981; 53:381-384

162 Sen N. P., Baddoo P. A., Seaman S. W.; Rapid and sensitive determination of nitrite in foods and biological materials by flow injection or high-performance liquid chromatography with chemiluminescence detection ; J. Chrom. A; 1994; 673:77-84

163 Wildman B. J., Jackson P. E., Jones W. R., Alden P. R.; Analysis of anion constituents of urine by inorganic capillary electrophoresis; J. Chrom.; 1991; 546:459-466

164 Leone A.M., Francis P.L., Rhodes P., Moncada S.; A Rapid and simple method for the measurement of Nitrite and Nitrate in Plasma by High Performance Capillary Electrophoresis; 1994; 200:951-957

165 Zunic G., Spasic S., Jelic-Ivanovic Z.; Simple and rapid method for the measurement of nitrite and mitrate in human plasma and cerebrospinal fluid by

capillary electrophoresis; J. Chrom. B; 1999; 727:73-79

166 Raman C. V., Krishnan K. S.; A New Type of Secondary Radiation, Nature; 1929; 121:501

167 Fleischmann M., Hendra P. J., McQuillian A. J.; Raman spectra of pyridine adsorbed at a silver electrode; Chem. Phys. Lett.; 1974; 26:163-166

168 Kneipp K., Wang Y., Dasari R. R., Feld M. S.; Approach to single molecule detection using surface-enhanced reonance Raman scattering (SERRS): A study using rhodamine 6G on colloidal silver; Appl. Spectrosc.; 1995; 49:780-784

169 J.L. McHale; Molecular spectroscopy; 1999; Prentice hall, New Jersey

170 Vickers T.J., Mann C.K.; Quantitative analysis by Raman spectroscopy; Anal. Raman Spectrosc.; 1991; 114:107-136

171 Chase D.B.; Modern Raman instrumentation and techniques; Anal. Raman Spectrosc.; 1991; 114:21-43

172 Chang R.K., Furtak T.E.; Surface Enhanced Raman Scattering, Plenum Press, New York; 1982

173 Otto A., Mrozek I., Grabhorn H., Akemann W.;Surface enhanced raman scattering; J. Phys. Condens. Matter; 1992; 4:1143-1212

174 Norrod K. L., Sudnik L. M., Rousell D., Rowlen K. L.;Quantitative comparison of five SERS substrates: Sensitivity and limit of detection; Appl Spectrosc.; 1997; 51:994-1001

175 Kellner R., Mizaikoff B., Jakusch M., Wanzenbock H. D., Weissenbacher N.; Surface-enhanced vibrational spectroscopy: A new tool in chemical IR sensing?; Appl. Spectrosc.; 1997; 51:495-503

176 Placzek G.; Handbuch der Radiologie; Akademie-Verlag, Leipzig; 1934; 6:205

177 Shorygin P. P., Krushinskij L. L.; Early Days and Later Development of Resonance Raman Spectroscopy; J. Raman Spec; 1997; 6:383-388

178 Munro C. H., Smith W. E., White P. C.; Qualitative and semiquantitative trace analysis of acidic monoazo dyes by surface-enhanced resonance raman-scattering; Analyst; 1995; 120:993-1003

179 Small H., Stevens T.S., Bauman W.C.; Novel ion exchange chromatographic method using conductimetric detection; Anal. Chem.; 1975; 47:1801

180Wheaton R.M., Baumann W.C.; Ind. Eng. Chem; 1953; 45:228

181 Kraak J. C., Jonker K. M., Huber J. F. K.; Solvent-generated ion-exchange systems with anionic surfactants for rapid separations of amino acids; J. Chrom.; 1977; 142:671

182 Horvath C., Melander W.R., Molnar I., Molnar P.; Enhancement of retention by ion-pair formation in liquid chromatography with nonpolar stationary phases; Anal. Chem.; 1997; 49:2295

183 Gjerde D.T., Fritz J.S., Schmuckler G.; J. Chrom; 1979; 186:509

184 Stevens T.S., Langhorst M.A.; Anal. Chem.; 1982; 54:950

185 Weiss J.; Ion Chromatography, 2nd edition; VCH Publishers, Inc, New York; 1995

186 De Hoffmann E., Stroobant V.; Mass spectrometry, Principles and applications, Second edition; Wiley; 1999

187Fenn J.B., Mann M., Meng C.K.; Science; 1989; 246:64

188 Yamanaka M., Sakai T., Kumagai H., Inoue Y; Specific determination of

bromate and iodate in ozonized water by ion chromatography with postcolumn derivatization and inductively-coupled plasma mass spectrometry; J. Chrom. A; 1997; 789: 259-265

189 P. Chaimbault, K. Petritis, C. Elfakir and M. Dreux; Determination of 20 underivatized proteinic amino acids by ion-pairing chromatography and pneumatically assisted electrospray mass spectrometry; J. Chrom. A; 1999; 855: 191-202

190 Huber C. G., Buchmeiser M. R.; On-Line Cation Exchange for Suppression of Adduct Formation in Negative-Ion Electrospray Mass Spectrometry of Nucleic Acids; Anal. Chem.; 1998.; 70(24): 5288-5295

191 Charles L., Pepin D.; Electrospray ion chromatography tandem mass spectrometry of oxyhalides at sub-ppb levels; Anal. Chem; 1998; 70(2): 353-359

192 Hsu J.; Interfacing ion chromatography with particle beam mass-spectrometry for the determination of organic anionic compounds; Anal. Chem.; 1992; 64:434-443

193 Corr J.J., Anacleto J.F.; Analysis of inorganic species by capillary electrophoresis mass spectrometry and ion exchange chromatography mass spectrometry using an ion spray source; Anal. Chem; 1996;68(13): 2155-2163

194 Roehl R., Slingsby R. Avdalovic N., Jackson P.E.; Applications of ion chromatography with electrospray mass spectrometric detection to the determination of environmental contaminants in water; J. Chrom. A; 2002; 956:245-254

195 Finigan R.E.; Anal. Chem.; 1994; 66:969A

196 Moneghan J.M., Cook K., Gara D., Crowther D.; Determination of nitrite and nitrate in human serum; J. Chrom. A; 1997; 770:143-149

197 P. C. Lee and D. Meisel, J. Phys. Chem., 1982, 86, 3391

198 Ying-Sing Li, Jingcai Cheng, Yu Wang; Surface enhanced Raman spectra of dyes and organic acids in silver solution: chloride ion effect; Spectrochimica Acta Part A; 2000; 56, 2067-2072

199 Munro C.H., Smith W.E., Garner M., Clarkson J., White P.C.; Characterisation of the surface of a citrate-rduces colloid optimized for use as a sustrate for surface-enhanced resonance raman scattering; Langmuir; 1995; 11:3712-3720

200 J. J. Laserna, A. D. Campiglia, J. D. Winefordner; Anal. Chem.; 1989; 61(15); 1697-1701.

201 J.C. Jones, C. McLaughlin, D. Littlejohn, D.A. Sadler, D.Graham, W.E. Smith; Anal. Chem.; 1999; 71; 596-601

202 Nandita Biswas, Siva Umpathy; J. Phys. Chem. A; 2000; 104; 2734-2745

203 Stevanin T.M., Poole R.K., Demoncheaux E. A. G., Read R.C.; 2002; Flavohemoglobin Hmp protects Salmonella enterica serovar typhimurium from nitric oxide-related killing by human macrophages; Infect. and Immun.; 4399-4405

204 Martin J. H. J., Edwards S.W.;Changes in mechanisms of monocyte/macophage-mediatedcytoxicity during culture; J. Immunol.; 1993; 150:3478-3486

205 Giaid A., Saleh D.; Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension; N. Endl. J. Med.; 1995; 333:214-221

206 Demoncheaux E.A.G., Higenbottam T.W., Kiely D.G., Wong J.M., Siddons T., Hall K., Gize A.; Decreased rate of whole body endogenous nitric oxide production in patients with Primary Pulmonary Hypertension; European Respiratory Journal;

207 Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S., Tannenbaum S.R., Analysis of nitrate, nitrite, and [^{15}N]nitrate in bilogical fluids; Anal. Biochem.; 1982; 126:131-138

208 Demoncheaux E.A., Hall K., Garner V., Varcoe R., Wharton S., Spivey A., Higenbottam T.W.; Whole body nitric oxide production is impaired in patients with primary pulmonary hypertension; Thorax; 2000; 55: P96.

209Kaneko F.T., Arroliga A.C., Dweik R.A., Comhair S.A., Laskowski D., Oppedisano R., Thomassen M.J., Erzurum S.C.; Biochemical reaction products of nitric oxide as quantitative markers of primary pulmonary hypertension; Am. J. Respir. Crit. Care. Med.; 1998:158:917-923

210 Fitzhugh A.L., Keefer L.K.; Diazeniumdiolates: pro- and antioxidant applications of the "NONOates"; Free Radic Biol Med.; 2000; 28:1463-1469

SOUTH SHEFFIELD RESEARCH

CJT/LH

Always quote the relevant SSREC Registration Number

03/05/02

Dr. D Bee
Clinical Research Fellow
Respiratory Medicine
E Floor
RHH

Dear Dr. Bee

Ref.: - SS/98/122 - Endogenous vs. exogenous forces of nitric oxide (NO) and higher oxides of nitrogen in liver disease- L-arginine / nitric oxide metabolism in liver disease

Thank you for your letter dated 20/04/02 enclosing an amendment to the above study. This was reviewed by the Ethics Committee on 02/05/02.

Protocol Amendment No.1: Received 20/04/02 ✓

- A dose of up to 85mg/kg ^{15}N-arginine will be infused over 30 minutes. Blood samples will be taken at 0, 10, 20,30,40,50,60,90,120 and 180 minutes for ^{15}N serum enrichment.

Patient Information Sheet; version 2: Received 20/04/02 ✓

Volunteer Information Sheet; version 2: Received 20/04/02 ✓

I can confirm that full Ethics Committee approval still applies to this protocol.

Yours sincerely

Professor C. J. Taylor
Chairman

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Chairman: Professor C J Taylor/ Administrator: Ms K A Khoaz

13/6/00
14/5/00

CJT/JG

23rd May 2000

rofessor T. Higenbottam
onorary Consultant
hest Medicine
HH

ear Professor Higenbottam

e: 97/255 - Anoretic agents and primary pulmonary hypertension

hank you very much for your letter of the 22nd May clarifying the confusion regarding this protocol and 9/128. I can now confirm that you are requesting a protocol amendment to 97/255 rather than 99/128. The ıendment involves altering the analate from plasma to urine for one of the biochemical tests involved in e protocol. This will involve 24 hour urine collections from both subjects and controls. This amendment s previously been considered in error in relation to 99/128 and approved, this approval has now been scinded. I am however happy to provide chairman's approval for this amendment to be extended to /255.

ours sincerely

ofessor C Taylor
airman

Submission of clinical trial protocol to Ethics Committee	**SOUTH SHEFFIELD RESEARCH ETHICS COMMITTEE**
TITLE (succinct & accurate)	**Anoretic Agents and Primary Pulmonary Hypertension**
INVESTIGATORS Name, title & appointment **(first name should be author for communication)**	Professor T W Higenbottam Professor of Respiratory Medicine, Floor F, Medical School, Beech Hill Road SHEFFIELD, S10 2RX Telephone (0114) 271 21 96
AIMS OF STUDY	To identify biochemical and genetic markers for the risk of pulmonary hypertension
POTENTIAL VALUE OF STUDY	Pulmonary hypertension is a progressive and usually fatal disease and only transplantation provides a cure. Early diagnosis may greatly assist the prognosis. Although pulmonary hypertension may be secondary to lung and heart disease, primary pulmonary hypertension is of unknown origin. The aim of this study is to identify potential biochemical markers linked to the development of the disease and identify those individuals at risk. It has been suggested that the epidemiological determined association between the intake of anoretic agents may be due to impaired metabolism of the drug. By genotyping individuals for the alleles of the gene responsible for its metabolism it may be possible to determine whether those individuals who have taken the drug and have impaired enzyme function are at particular risk for developing pulmonary hypertension.
METHODS WITH PARTICULAR EMPHASIS ON: a) Identification, recruitment, number and methods of selection of subjects. b) Procedures c) Measurements d) Storage of Data e) Analysis: statistical methods and power	**a) Subjects** Three groups of 20 subjects will be studied:- 1. Patients characterised for sporadic primary pulmonary hypertension, divided into those who have/have *not* taken anoretics in the previous 3 years. 2. Aget matched positive controls recruited from cardiac myopathy patients awaiting transplantation. 3. Age/sex matched volunteers responding to advertisements. **b) Procedures** Subjects will provide a venous blood sample (40 ml) and a urine sample. For the assessment of the endothelial nitric oxide production the subjects will fast for 12 hours on Milli-Q water and have been on a diet containig no nitrite/nitrate rich food for 2 days before the study. L-arginine or ^{15}N-guanidino-labelled L-arginine will be given via an indwelling venous cannula as a primed (10µmol/Kg) and then a constant (10µmol/Kg/h) intravenous infusion whilst supine. 5 ml blood samples will be taken immediately preceeding infusion and every 20 min thereafter. (Total Volume 45ml) The subjects inhale and exhale through a 2-way non-rebreathing valve with nose-clip in position to collect exhaled breath for nitric oxide estimation. **c) Measurements** Genotyping for fast and slow metabolisers. Plasma levels of 5-HT and its metabolite 5-HIAA using HPLC. Circulating levels of ET-1 by radioimmunoassay system. Urine thromboxane and prostacyclin metabolites will be measured by urine radioimmunoassay. L-arginine is assayed by cation exchange resin column, nitirc oxide nitrite/nitrate ratio by chemiluminescence and mass spectometry. **d) Data storage** Levels from the different metabolites will be recorded in a database system and the files will be stored on hard and floppy disks **e) Analysis** The number of subjects per group was calculated from power equations on the assumption that the incidence of poor metabolisers in primary pulm onary hypertensive patients was raised from 8% in the normal population to at least 50%.

Submission of Clinical trial protocol to Ethics Committee	
Where study to take place	All studies will be conducted at the Royal Hallamshire Hospital, Glossop Road, Sheffield
Duration of study	Each patient's routine consultation (of typically sixty minutes) will be extended by no more than twenty minutes. Endothelial nitric oxide production assessment will be carried out on a further visit lasting 5-6 hours
otential hazards to ubjects	The isotope of L-argine used is non-radiocactive. The amino acid L-argine is one present in a normal diet and no hazards are expected fom its administration.
rocedures of iscomfort and distress or subjects	Insertion of venous cannulae for blood sampling and breathing through a mouthpiece willl involve the usual modest discomfort
ompensation/ ndemnification	Currently no additional compensation has been agreed, and the appropiate 'harm' paragraph has been included on the patient information sheet. The organisations with an interest in the wider use of DNA obtained from the blood samplings have been approached and responses are awaited
inancial arrangements Payment to subjects Payment from company sponsoring the trial	a) to subjects None b) From company None
ccess to Data	The use of a randomised coding system, the key to which will be held only by the investigator, will preclude the need for patient details to be stored with the experimental data. In these circumstances, access need not be restricted
ument Enclosures se enclose examples of any ments given to, or requiring signature of, the subject.	a) Information sheet [copies attached] b) Consent form [1 copy attached] c) CTX/DDX Prof Higenbottam accepts responsibility for the use of unlicensed drugs Are you going to give radioisotopes No d) Protocol 3 copies attached (not supplied to patient) Reciprocal declaration 1 copy attached (not supplied to patient)

nature: T. W. Higenbottam **Date:** 10/10/97

Patent: Improvements to liquid chromatography coupled to mass spectrometry in the investigation of selected analytes; Crowther D., Dürner M.B., Demoncheaux E.; Application No: GB 0319915.5

Publication: Dürner M.B, Demoncheaux E., Higenbottam T., Crowther D.; Quantitative analysis of nitrite and ^{15}N-nitrite in biological fluids by surface enhanced Raman spectroscopy; In submission

Publication: Dürner M.B., Demoncheaux E., Higenbottam T., Crowther D.; Quantitative analysis of nitric oxide metabolites and their ^{15}N labelled isoforms in biological fluids by ion chromatography - mass spectrometry