The application of the thermal energy analyser to the analysis of nitrosamines and organic nitrates

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'The Application of the Thermal Energy Analyser to the analysis of Nitrosamines and Organic Nitrates.'

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ABSTRACT

The thermal energy analyser (TEA) has been used as a detector to analyse five different types of samples of nitrosamines and organic nitrates. Results are presented for the analysis of nitrosodimethylamine in aqueous samples using capillary gas chromatography, with a detection limit of less than 20 ppb. Total nitrosamine content of personal hygiene products was determined using chemical denitrosation coupled to the TEA, with a similar lower limit. Nitroglycerin and pentaerythritoltetranitrate were analysed qualitatively in explosives residues. Attempts were made to quantify nitrosodiethanolamine in a dye, and nitroglycerin in blood, and the initial findings are presented.

Acknowledgements

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CONTENTS

CHAPTER 1 : INTRODUCTION

1.1 Thermal Energy Analyser

The Thermal Energy Analyser[1] (TEA) takes the substances eluted from the GC column and allows the qualitative and quantitative identification of nitrogen containing compounds by pyrolysing them at a strictly controlled temperature in a flash furnace to produce nitric oxide (NO). This radical then passes into a cooled reaction chamber where it reacts with ozone produced in situ to form electronically excited nitrogen dioxide (NO_2*). This can decay back to its ground state by chemiluminescence, emitting radiation of a characteristic wavelength which passes through a filter and is detected by a photomultiplier tube specifically designed to look at these wavelengths. The intensity of the emissions is proportional to the NO concentration, and therefore to the amount of nitroso-compound. The output is sent back to the GC software to be integrated and displayed as a peak area.

Pyrolysis

The TEA has two modes of operation depending upon the type of nitrogen compound to be detected.

Nitrogen mode (Catalytic pyrolysis).

This mode is used when an NH bond rather than an NO bond is present and requires the presence of oxygen in the pyrolyser. The oxygen reacts with the NH to produce the NO radical in the presence of a catalyst at high temperature (850^0C). The catalyst is present in the pyrolyser in both modes of operation but has no action when oxygen is not present.

Nitroso mode (Non-catalytic pyrolysis).

In this mode no oxygen is needed as the NO radical is produced by pyrolysis alone (provided the temperature is appropriate for the stability of the NO bond).

The remainder of the parent nitrogen compound, and any other organic molecules or fragments will not produce NO, and pass through the rest of the system undetected.

For the purposes of this work Nitrogen mode was not used and subsequent discussions will be concerned only with operation in Nitroso mode.

Optimisation of Pyrolysis temperature.

For a particular compound, enough heat energy must be supplied to break the substrate to NO bond. The pyrolysis temperature varies beween different groups of

compounds, and also within each group. Nitrosamines all pyrolyse below 500^0C, whereas the nitrate esters Gtn and Petn produce NO at about 250^0C and 750^0C respectively, and nitroaromatics generally require temperatures of 800^0C. For all these compounds there is an optimum temperature for pyrolysis at which the molar response is highest, but pyrolysis will still occur to a lesser extent at slightly lower temperatures. The optimum temperature can easily be found by injecting a standard solution of the compound to be studied and varying the pyrolyser temperature until the greatest response is obtained. This is then quantitative as long as the conditions are kept constant.

Optimisation of TEA Internal Pressure and Ozone Flow

To obtain the highest degree of sensitivity from the TEA the pressure must be carefully maintained. If the pressure is too high the tiny fraction of NO_2* that actually produce light quanta will be further reduced by deactivation in two and three body collisions (see outline). The former can be made negligible and the latter eliminated by running at pressures of only a few millimetres of mercury, but if the pressure is too low the NO_2* will pass through the system too quickly and is likely to decay after leaving the chamber, in which case it will not be detected. A compromise is made between the two factors.

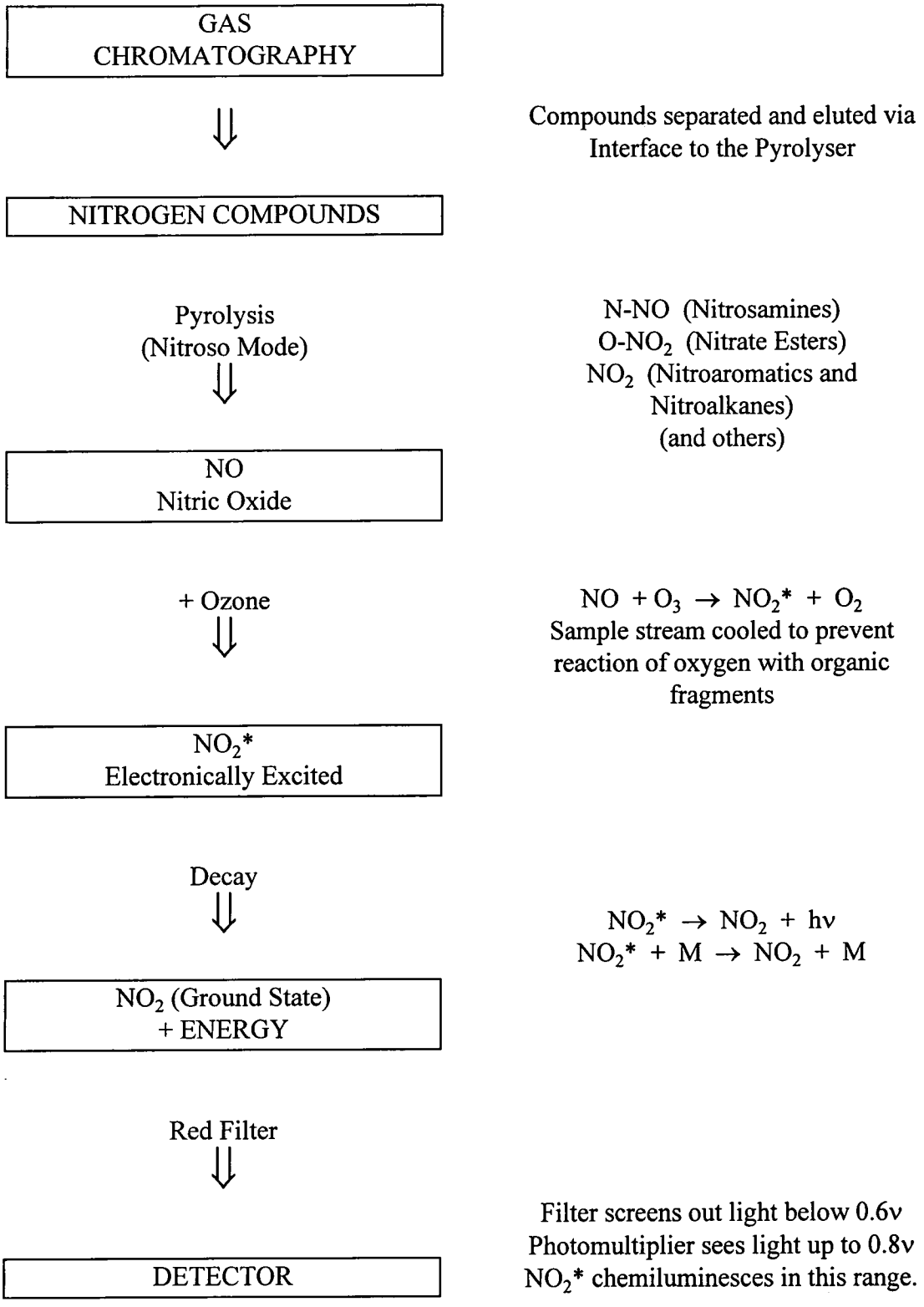
For our system it was found to be desirable to run at a total pressure of 1 mmHg. This required an ozone flow equal to a pressure of 0.6 mmHg, the rest of the pressure

is made up by the flow of carrier gas from the GC, i.e. 1 ml/min for a 30 metre capillary column.

Applications of the TEA

The TEA can be used as a detector for gas or liquid chromatography, or in some cases the output gases from a reaction can be fed directly into the detector. Its major advantage is its selectivity for nitrogen compounds, this means that resolution is greatly increased, with the required compound often appearing as the only peak instead of amongst a forest of others. This in turn allows smaller amounts to be detected. Overall this means that less sample is needed, with less clean-up, it is easier to identify the peak of interest, and the problem of huge solvent peaks at the same retention time is avoided.

Figure 1. How the Thermal Energy Analyser Works[1]



1.2. Nitroglycerin

Nitroglycerin (Glyceryl Trinitrate, Gtn) has two very different uses, it is both a powerful explosive and an effective vasodilatory drug. The first property was utilised by Alfred Nobel in his bid to find the 'ultimate deterrant to war', when he used Gtn to make dynamite. He failed in this respect, Gtn is still a popular explosive today (when used as dynamite) as it is relatively simple to make, but his work did lead to the discovery of Gtns medicinal properties. Workers in his explosives factory, including Nobel himself, suffered terrible headaches which were found to be due to the dilation of blood vessels in the brain caused by excessive GTN exposure.

This prompted the first investigations into Gtn, and it was realised that it could be a useful medicinal tool when its effects were targeted correctly. Gtns therapeutic properties were first utilised in 1874, and its success has ensured that it is still used today to treat heart conditions such as angina pectoris and congestive heart failure. A number of other nitrate drugs (such as Isosorbide Dintrate, Isdn and Pentaerythrytol-tetranitrate, Petn) have also been found to be effective, and all are available in a variety of dosage forms and may be used in conjunction with one another. GTN is available in sublingual tablets and sprays to give an almost instant but short-lived rise in blood levels, suitable for immediate treatment, whilst slow release transdermal patches and ointments, oral capsules, or controlled intravenous delivery can be used for a more prolonged prophylactic effect

The effects and actions of Gtn are not yet fully understood, and there are many areas which require further elucidation. The fate of Gtn in the body - where and how quickly it is metabolised, and the amounts and activites of the metabolites produced are important questions which have not been answered conclusively. The disposition of active species in the blood is also important when considering how effective a calculated dose of Gtn will be. Studies have shown that Gtn is rapidly degraded in the body to its di- and mono-nitrate metabolites.[2] The liver is mainly responsible for the high clearance rate of the drug by first pass metabolism,[3,4] but Gtn is also lost in blood to a lesser extent. This happens in two distinct stages; firstly, a significant amount of Gtn is very rapidly bound by blood proteins, secondly, denitration occurs and metabolites are formed, which can themselves be distributed into the erythrocytes.[2] Blood clearance is not of great overall importance in the body, but becomes more significant when samples are taken for analysis, and will be discussed later. On reading the literature, it is hard to draw any solid conclusions as to how haemodynamically active Gtn and its metabolites really are. Some of the conclusions put forward suggest that Gtn is the principal active molecule,[5-7] whilst some suggest that it plays a fairly small role and the metabolites are more significant in causing clinical effects.[8-10]

The relationship between dosage form and pharmacological effects has been widely studied, conclusions can be drawn from observation of patients physical responses to

the drug as well as from analytical studies on blood samples. Early work in this area put forward the argument that oral Gtn is completely destroyed by first pass metabolism and therefore had no clinical utility.[3] Subsequent work has proved that clinical effects are produced, although it is now thought these may be mostly due to the activity of Gtn metabolites.[10] The effects and duration of each of the dosage forms are now quite well documented,[8,10-12] and the efficacy of new formulations can be tested against these results.

It is known that Gtn exerts its beneficial effects by causing relaxation of the vascular smooth muscle, but the detailed mechanisms of how this occurs are not entirely known. The mode of action is thought to be via nitric oxide, which is the final active metabolite produced from Gtn, and has been shown to produce clinical effects when administered *in vivo*.[6] Investigations into this may also shed some light on the problem of tolerance, a phenomenon which occurs in patients undergoing continuous nitrate therapy, where clinical effects are seen to decline in response to a constant dosage. It is thought this may occur by metabolite inhibition of Gtn biotransformation, possibly due to the depletion of free thiol groups which may be necessary for this to take place, and investigations are ongoing.

There is still much to be discovered about Gtn before it can be said that patients are really getting the maximum benefits from Gtn therapy, and as one pair of writers

commented in their review of the Gtn literature in 1984[5] "nitroglycerin assay is one of the great challenges of modern drug analysis". This is still true today.

1.3. Pentaerythritoltetranitrate

Pentaerythritoltetranitrate (Petn) has very similar chemical properties to Nitroglycerin, it too is a potent vasodilator and is often used in conjunction with other nitrate drugs to ease certain heart conditions. It is also a powerful explosive, most commonly used to detonate other explosives. It is chemically stable and in its pure form is a granular white powder which can be set off by friction. It is prepared simply by introducing pentaerythritol into concentrated nitric acid, cooling, precipitating with weaker nitric acid, and then recrystalising from acetone.

Petn can be mixed with another explosive called RDX (cyclo-1,3,5-trimethylene-2,4,6-trinitramine) to form Semtex. Particles of the two are bound with an adhesive to form a durable, pliable explosive. Semtex is insoluble in water and hence weathers well, it is odourless and does not show up on x-rays or in metal detectors and is therefore especially popular with terrorists. as well as with commercial explosives experts. It is also inexpensive - licensed blasters can buy it for £13/lb, and experts working on the Paris TWA jet explosion last year speculated that as little as 2lbs of strategically placed Semtex could have caused the disaster.

Because of its particulate nature, and the fact that it contains adhesive, even though Semtex appears to be a homogeneous material it tends to leave behind

residues on contact. These can easily be transferred from one surface to another, and may remain for many months due to their insolubility in water. Experiments under controlled conditions have shown that after contact with Semtex, traces may still be detectable after more than fifty successive hand prints.[46] Traces like these may be swabbed off with acetone and analysed, usually by GC with either TEA or MS, and detection limits as low as 100 picograms have been reported,[22] although modifications were made to the TEA to sharpen the peaks in this case.

1.4. N-Nitrosamines

N-Nitrosamines are a group of compounds which have come to public attention because of their carcinogenic properties. They were first discovered in 1863[14] but as they are of use only in a limited number of reactions, were not considered to be of any great interest. Since the discovery of their toxicity in the 1950s,[27] however, they have been widely studied and are often used as models for studying the mechanisms of carcinogenesis. It has been found that of the 130 or so nitrosamines known, at least 80% of them are potent carcinogens, inducing tumours in a wide range of target cells and organs in nearly all animal species, including primates.[14,16,17] This has led to concern about human Nitrosamine exposure and the possible effects on humans, and has prompted a great deal of research into their occurrence in the environment and the workplace, and in commercial products. These studies have shown that there are many ways in which we come into contact with nitrosamines in our everyday lives.

They are found in items such as foods, beverages, tobacco, drugs, cosmetics and toiletries, detergents, industrial and agricultural chemicals and rubber products , and both the people that use these products, and those that are involved in their manufacture are at risk of exposure.[18-21]

Nitrosamines are formed from a wide variety of precursors and nitrosating agents, making them fairly ubiquitous. Primary, secondary and tertiary amines, amine oxides, alkanolamides,and other amine type compounds can react with nitrite salts, nitrous acid, oxides of nitrogen or other nitrosamines, and depending on the precursor and catalysts present, nitrosation can occur in acidic, neutral or alkaline conditions.[14,22]

$$R'R''NH + HNO_2 = R'R''NNO + H_2O$$

Humans are exposed to preformed nitrosamines in a number of different ways, including inhalation, dermal absorption and ingestion. Tobacco contains many volatile amines such as nicotine, which can be nitrosated either during the long curing process or on combustion, leading to high concentrations of nitrosamines.[23] Many cosmetic formulations such as creams. lotions, make-up and shampoos use alkanolamines like di- and triethanolamine to help form stable emulsions, and as nitrosating agents are often also present in these products, many contain nitrosamines such as Nitrosodiethanolamine (Ndela), some at levels of up to 48 ppm,[24,25] and it has been shown that Ndela can be readily absorbed through the skin.[26] Certain rubber products can cause contamination of items which come into contact with them - foods

packaged in elastic rubber netting may contain nitrosamines which migrate from the netting into the food during storage and on cooking.[25] This also occurs in baby bottle nipples, where nitrosamines migrate into the milk during sterilisation.[27] Foods such as meat and fish which are cured using nitrates can contain various nitrosamines, depending on which amines are present, due to nitrosation by nitrite produced by bacterial reduction of nitrate.[14,18]

A number of industrial working environments have shown nitrosamine levels which give cause for concern. They may use products already contaminated with nitrosamines, or have a high nitrosating potential due to the presence of precursors and nitrosating agents in chemicals and the atmosphere. The highest levels are found in leather tanneries, where workers can be exposed to a daily dose of up to 440 μg/person/day.[19] Metal workers using synthetic cutting fluids, and those involved in their manufacture can be exposed to high levels of Ndela by absorption through the skin. In the rubber industry nitrosamines are produced during the vulcanization process, and again contaminate both the working environment and the final product.

Nitrosamines can also be synthesised *in* vivo, but little is known about this. In the acid environment of the stomach amines present in many foods can react with nitrite from the food or present in swallowed saliva to form carcinogenic nitrosamines, and it has been suggested these may contribute to the pathogenesis of gastric cancer.[28]

International standards for restriction of nitrosamine levels have been introduced, and as a result there has been an increasing demand for robust methods for routine quantitative analysis of a great variety of products and environments to ensure compliance. It is also essential to identify which nitrosamines are present were contamination exists, so changes in working practices or use of chemicals can be introduced to minimise or eliminate the risk of exposure.

CHAPTER 2 : ANALYSIS OF ORGANIC NITRATES

2.1. Nitroglycerin analysis in blood

2.1.1. Previous work on GTN analysis in blood

The history of GTN analysis has been frought with problems. Early attempts to analyse GTN used colorimetric methods, and were unable to distinguish between organic and inorganic nitrates. Quantitative analysis did not begin in earnest until the late 'sixties, when gas chromatography with electron capture detection was first being investigated. Packed columns were used initially, but generally produced less than perfect results as the sensitivity was quite low and analysis times very long.[29] Chromatograms were often badly affected by interference peaks, and this problem was partly overcome by using non-polar solvents like hexane and pentane to try and exclude most of the relatively polar contaminants found in blood, but this meant many repetetive extractions due to the low partition coefficient of GTN.[29,30] A general detection limit of 0.5 ng/ml can be reached with this method when using 2 ml samples with careful clean-up procedures and ideal operating conditions.[31]

Substituting capillary columns in place of packed columns gives extra sensitivity[10,32,24] but the amount of sample injected is smaller, hence better extraction techniques are needed to obtain a sufficient degree of concentration, and the problem

of contamination is still not solved. Use of different solvents means that the concentrations of GTN and its metabolites in plasma can be determined seperately or simultaneously which can provide much information about GTN metabolism.[10, 33,34] A disadvantage of this technique is that GTN often partially decomposes in the hot injector port or on the heated column.[35-37] This problem can be solved in part by cryofocussing, ie. having a relatively cool injector port and a cool column and allowing the sample to slowly move onto the column to form a band before starting the temperature program. A compromise is made between the amount of decomposition and the amount of sample getting onto the column. This also allows the injection of slightly larger samples, up to a limit of about 5μl when the peak becomes too broad.

Gas chromatography (packed or capillary) can be combined with mass spectrometry, and gives a better chromatogram with good linearity and a typical sensitivity of 0.05 ng/ml. Unfortunately setting up a GC-MS system is expensive in terms of both time and money, and often requires synthesis of specific internal standards, which means this method is not always suitable for routine clinical analysis.[38,39]

A real step forward in GTN analysis is the application of the thermal energy analyser (TEA), (see Chapter 1). This detector is specific and highly sensitive for nitrogen, which is a major breakthrough in reducing the problems of sample clean-up and chromatographic interference. The result is high quality chromatograms, with a

detection limit of 0.05ng/ml.[40-42] A minor disadvantage in using the TEA is that the peaks obtained are slightly broader than those produced by other detectors. Modifications to the TEA have been proposed and documented which result in sharper peaks,[13] but in general this is only worth considering when working with peaks very close to the signal to noise ratio. The TEA can also be used as a detector for liquid chromatography, which has the advantage of eliminating partial decomposition of GTN found with GC.

Even with the best analytical instruments available, GTN analysis can still be complicated by other factors, which must be taken into account when interpreting results. Sample handling is absolutely crucial, and the final result can vary widely depending on the procedures used. The key point is that GTN is rapidly degraded in whole blood at body temperature with a half life of 3 min at therapeutic concentrations[2]. The rate of degradation is slower at lower temperature, the half life being 27 min at 2^0C, it is therefore essential to cool the sample and separate out the plasma containing GTN as soon as possible. A short delay may result in significant underestimation of GTN concentration. It is usual to take the sample directly into a chilled container, (which must contain heparin to prevent coagulation), centrifuge immediately (preferably in a refrigerated centrifuge and for as short a time as possible), remove the plasma and freeze in dry ice, the sample can then be kept for at least a month before analysis.[5,24,32] When the sample is analysed it is recommended

that extraction of the sample should be started whilst the sample is still cold after thawing, and that heat should be avoided at all times during the procedure.

Loss of GTN from blood can be reduced by adding chemical inhibitors or stabilizers. Previous methods used silver nitrate[30] but whilst this stabilizes GTN, it also causes gelling whole blood samples making them difficult to work with, and there are worries that silver nitrate may nitrate glycerol found in the sample. A more satisfactory method of stopping GTN degradation is addition of iodoacetamide immediately after sampling, and when chilled as well the rate of degradation falls to zero due to inhibition of enzymes involved in GTN metabolism.[2]

A problem which was highlighted in the early days of GTN analysis is the adsorption of GTN by plastics.[5] This was initially discovered when patients receiving intravenous GTN from plastic infusion sets were found to need much higher doses than expected to produce clinical results.[44] Subsequent work showed that up to 85% of GTN was lost when delivered in this way, and similar losses occurred when stored in plastic containers, therefore GTN samples should be kept in glass at all times. This also means that results of some early GTN analyses should be regarded with caution when making comparisons.

2.1.2. Analysis of GTN in blood

2.1.2.1. Experimental

The aim of this piece of work was to develop a method that could be used for routine analysis of GTN levels in clinical blood samples. It was intended that the method would be developed in four stages :

- optimising the chromatography to obtain the best possible detection limit and reproducible calibrations
- using water spiked with GTN to develop a sample extraction that was reproducible and efficient
- using spiked whole blood to make sure the method worked on 'real' samples
- obtaining clinical samples to test for GTN content

Due to lack of time only the first stage was completed, although one clinical blood sample was obtained and analysed, to give an indication as to whether the method was working yet, but unfortunately this did not prove very successful due to a number of complications. The preliminary findings are presented, along with suggestions for further investigations.

Reagents

GTN was kindly supplied by South Cleveland Hospital in two forms - Tridil® solution for intravenous infusion, 500 μg/ml in an aqueous solution containing 10 % alcohol, and a metered sublingual spray formulated in alcohol, 400μg/dose). Petroleum ether (boiling range 30 - 40^{0}C) and toluene were from Sigma. Water was laboratory distilled

1). Chromatography and calibration

GC-TEA calibration standards were prepared by directing the GTN spray into toluene and diluting as necessary. Multiple solutions were made and compared to determine the reproducibility of the spray dosage. The reproducibility of the injected standards was ± 10 %, which takes into account the reproducibility of both the injection and the dosage of the spray.

GC - TEA Conditions

A 30 m × 0.32 mm I. D. DB 5.625 (5 % phenyl) methylpolysioxane capillary column with a stationary phase film thickness of 0.25 μm (J & W Scientific, CA. USA) was used. The temperature was held at 45^0 C for 3 min and then temperature programmed at a rate of 50^0 C /min to 200^0 C and held for 5 min. The injector was operated in splitless mode for 3 min then in split mode with a split ratio of 20 : 1 for the rest of the run. An injection port temperature of 155^0 C was used. The carrier gas was

nitrogen at a flow rate of 1 ml/min. The TEA pyrolyser was set at 500^0 C and the interface at 225^0 C. The total internal pressure was 1 mmHg.

2). Extraction procedure

Petroleum ether was used for the extraction. N-pentane or n-hexane are often favoured by many researchers, but cost considerations led to this choice of solvent, and although there may be a small difference in extraction due to the branched isomers present in the petroleum fraction, this did not present a problem here. Tridil solution was diluted as needed at various levels down to 20 ppb and 5 ml aliquots used as test samples. 10 ml of pet. ether was added and the mixture was vortexed for 1 min, then placed in a -30^0C freezer for 30 mins to freeze the aqueous layer. The ether layer was removed and evaporated under nitrogen to about 1 ml, then transferred quantitatively to a graduated vial, 100 µl of toluene was added and the solution concentrated to 100 µl. 1 µl was injected onto the GC - TEA.

Blood sample

The sample was taken from a patient receiving an intravenous dosage of 0.36 mg / hour of GTN (using Tridil solution), which is intended to give a blood concentration of 80 ppb at equilibrium.

For the analysis, a 5 ml sample was taken into a chilled tube, centrifuged immediately and a gel plug used to separate the plasma and cells. The tube was kept on ice for the 30 min journey between the hospital and the laboratory, and analysed

immediately. Unfortunately no heparin had been added therefore the plasma had coagulated, and required further centrifugation for 15 min at 4000 rpm. 2.5 g of supernatent were recovered, to which 10 ml of pet. ether followed by 5 ml of saturated brine were added. On addition of the brine the sample coagulated again and had to be centrifuged for a further 20 min. The aqueous layer was then frozen, and the sample was concentrated in the same way as described above.

2.1.2.2. Results

See fig. 2

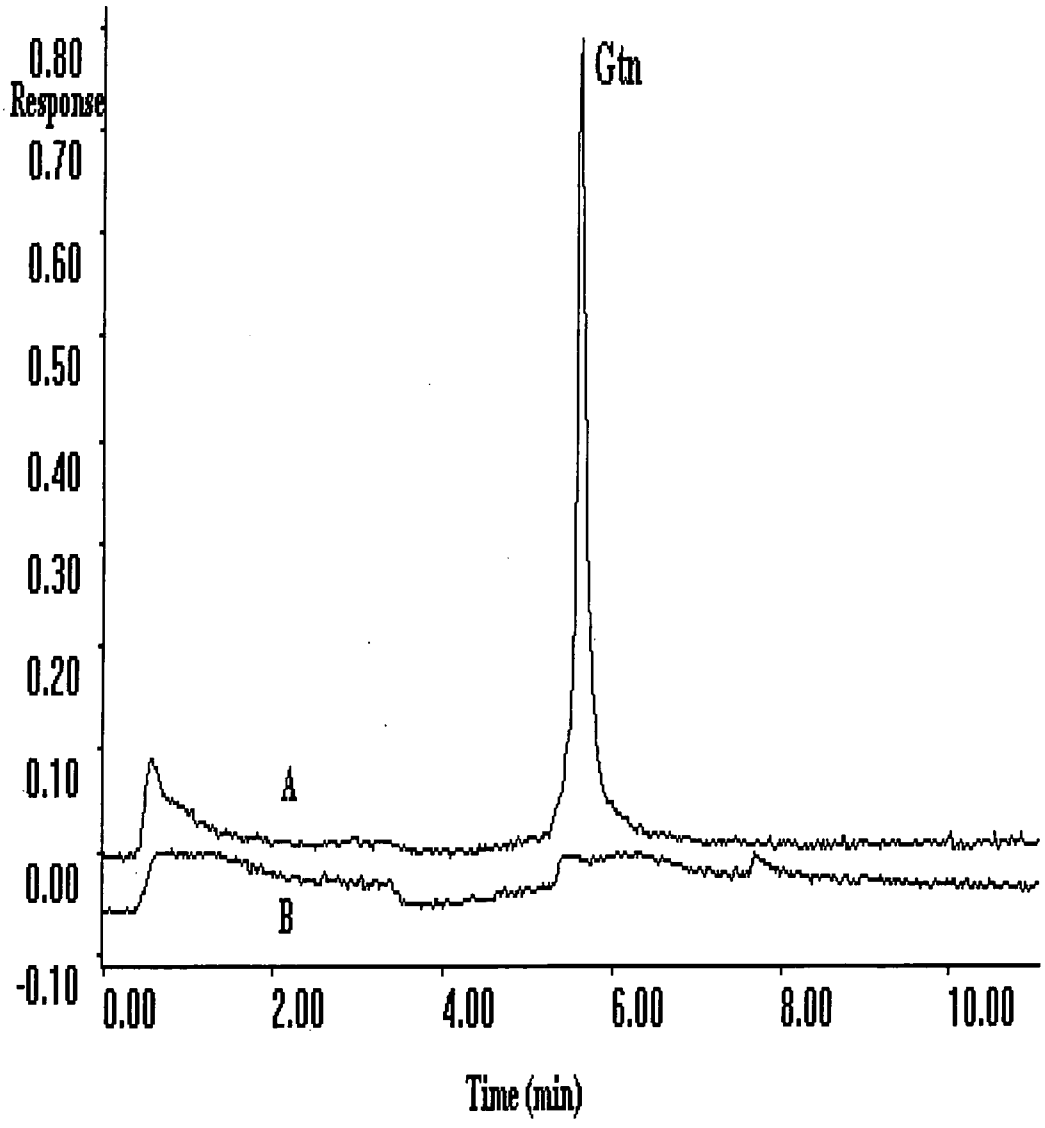
Trace A shows the result of an extraction on water spiked with GTN at 80 ppb, and gives a recovery of 50 %. Trace B shows the result of extracting the blood sample. The chromatogram shows a possible trace of GTN, but there is no peak which can be integrated. The chromatogram is very clean which is a positive sign, indicating that potentially the detection limit could be quite low.

2.1.2.3. Discussion

It was never expected that a perfect result would be obtained from this analysis as it would be unlikely that the method for extraction of spiked water samples would transfer to blood samples without modifications, so this analysis was simply meant to show what progress had been made. There are obvious reasons why no GTN was detected. The fact that the plasma coagulated twice and had to be re-spun means that a large amount of GTN may have been bound up with the coagulated proteins and

spun down with them and therefore not been recovered in the supernatent. GTN is metabolised in plasma to a small extent[6], so the extra time spent at room temperature during centrifugation will have reduced the content further. Other investigators have used sodium chloride to increase the partition coefficient of GTN into the solvent[12], and not encountered any problems, but it is possible that the coagulation occurred because the sample had to be re-centrifuged before extraction and had warmed up considerably before the addition of the NaCl solution. Further work on spiked blood samples is needed to solve these problems, possibly using iodoacetamide to inhibit GTN degradation. The analysis should be straightforward if there are no complications due to coagulation, so heparin must not be forgotten, and sodium chloride probably should be forgotten, as it is only added to increase the extraction, and is not absolutely necessary.

Fig. 2.


0.80
Response
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
Gtn
A
B
0.00
2.00
4.00
6.00
8.00
10.00
Time (min)

2.2 Analysis of GTN and PETN in their explosive forms

The analysis of GTN and PETN as explosives is much simpler than when dealing with samples in blood. In many cases traces of suspected explosives can simply be swabbed, brushed or vacuum filtered off a surface, dissolved in a suitable solvent and analysed directly,[43] they may be mixed with other components, such as mixers like woodmeal, and these can often be identified under a microscope.[47]

2.2.1. Experimental

Four samples were provided for qualitative GTN/PETN analysis.

Reagents

Pure PETN crystals were supplied with the samples. GTN was kindly supplied by South Cleveland Hospital as a metered sublingual spray formulated in alcohol.

Acetone supplied by BDH

GC - TEA Conditions

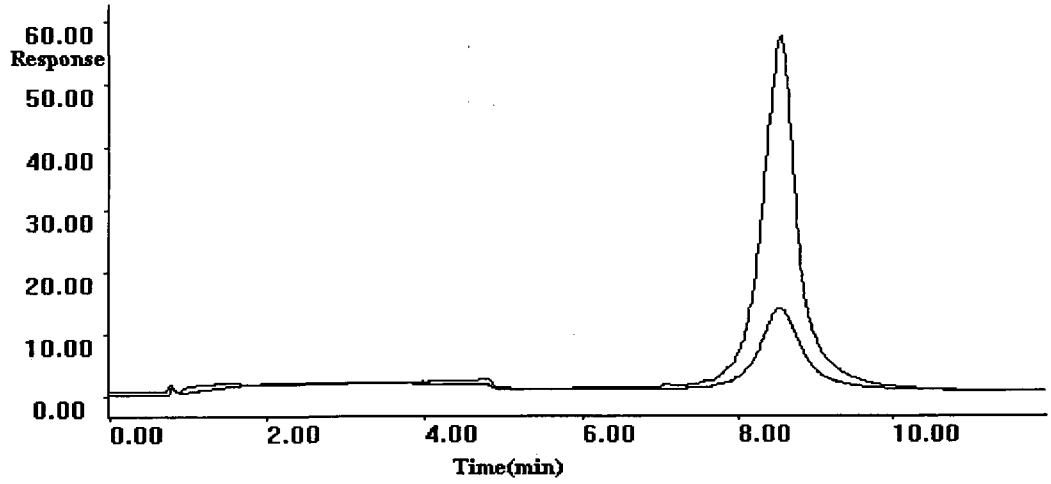
A 30 m × 0.32 mm I.D. DB 5.625 (5 % phenyl)methylpolysiloxane capillary column with a stationary phase film thickness of 0.25 μm (J & W Scientific, CA USA) was used. The temperature was held at 45°C for 4 min and then temperature programmed at a rate of 40°C/ min to 200°C and held for a further 4 min. The injector was

operated in splitless mode for 4 min , then split for the rest of the run with a split ratio of 20:1. An injection port temperature of 170°C was used. The carrier gas was nitrogen at a flow rate of 1 ml/min. The TEA pyrolyser was set at 750°C and the interface at 275°C. The total pressure was 1 mmHg.

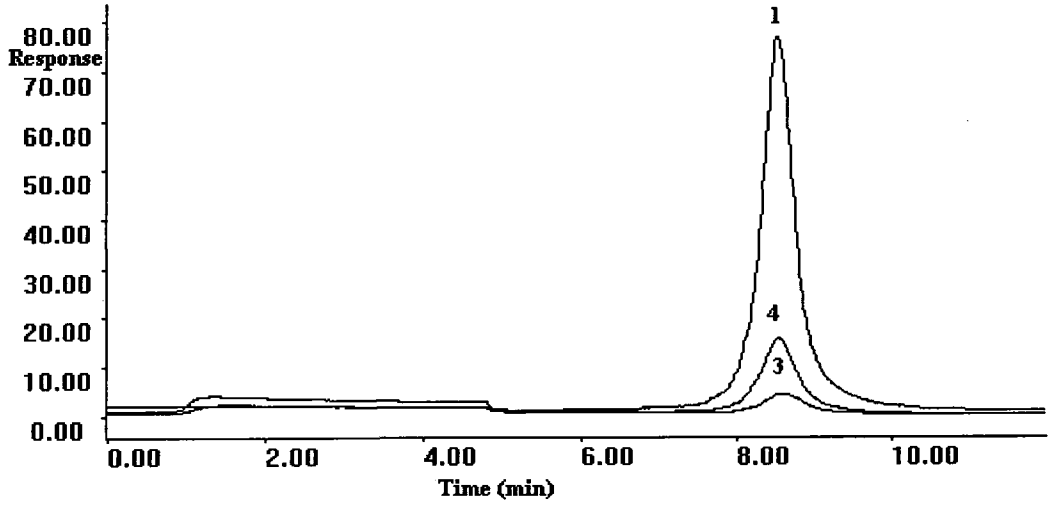
2.2.2. Results

An examination of the following chromatographs shows that samples 1, 3, and 4 contained PETN, and sample 2 contained GTN

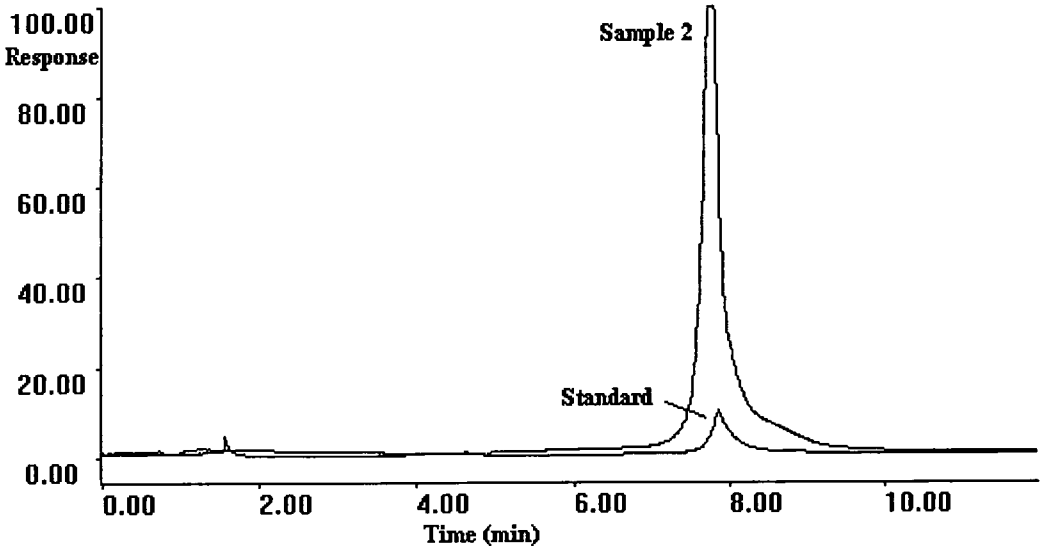
PETN Standard : 2 different sized injections of the standard



Samples 1, 3, and 4, all containing PETN



GTN standard, and Sample 2, containing GTN



CHAPTER 3 : ANALYSIS OF N-NITROSAMINES

3.1. Previous nitrosamine analysis

There are so many N-nitrosamines occurring in such a wide variety of products and environments that the main challenge involved in their analysis is finding a suitable way to extract and clean-up the sample prior to analysis. Matrices such as soap, lipstick, rubber, air, bacon, gastric juice, and pesticides are just a few examples of those which come under scrutiny. The problem is usually finding a suitable solvent system for the extraction, but in some cases extra separation using column chromatography or ion exhange chromatography is necessary as well.[5] Analysis by GC-TEA has been established as a firm favourite for looking at volatile nitrosamines, and there are many well documented methods spanning a period of nearly twenty years.[27,48,49] Non-volatile nitrosamines, which can't be analysed by GC or HPLC, can be analysed by reacting them chemically in a sealed vessel to produce nitric oxide, and passing this straight into the chemiluminescence detector,[28,45] and this method is also useful for screening products for nitrosamines which may be present, although it does not distinguish between them. Some nitrosamines contain groups such as -OH which need derivatising before they can be run by GC, to prevent unwanted interactions with the column, and this requires an extra step in the work up of a sample.[50]

Nitrosamines are still being found in new places , so there will be a need for new methods in the future. There is also a constant need for monitoring of products

and places that have been found to be contaminated, so established methods which are easy to use and commercially viable are always in demand.

3.2. Analysis of N-Nitrosodimethylamine in aqueous samples

Four samples were provided for analysis of Ndma content. The samples were water based but contained small amounts of other dissolved organic compounds which were not named. A level of less than 50 ppb was expected in all the samples and the results were to be quoted as less than a maximum.

3.2.1. Experimental

GC-TEA Conditions

A 30 m × 0.32 mm I. D. DBWAX (polyethylene glycol) capillary column with a stationary phase film thickness of 0.5 μm (J & W Scientific CA. USA) was used. The temperature was held at 75^0 C for 1 min and then temperature programmed at a rate of 25^0 C/min to 200^0 C and held for 3 min. The carrier gas was nitrogen at a flow rate of 1 ml/min. The injector was operated in splitless mode for 0.65 mins and then in split mode with a split ratio of 20:1 for the rest of the run. An injector port temperature of 200^0 C was used.

The TEA was operated in Nitroso mode, the pyrolyser was kept at 500^0 C and the interface at 225^0 C. The total pressure in the detector was 1mmHg.

Reagents

All water was laboratory distilled. Ether and toluene (HPLC Grade) and Ndma (99.7% purity) were supplied by Sigma. Nitrogen (Medical Grade) was from B.O.C.

Calibration of GC - TEA

A standard stock solution of 1000 ppm Ndma in toluene was made by adding 100 mgs to 100 mls of solvent and shaking thoroughly. Aliquots were then diluted successively with toluene to 1 ppm, 100 ppb and 10 ppb. These standards were kept sealed in glass at 0-5 C in the dark. Injections of the standards were performed daily to ensure stability of the solutions and reproducibility of analysis. The day to day repeatability was within ± 10 %

Extraction Procedure

An aliquot of sample (30-50 mls) was shaken for two minutes with an equal amount of diethyl ether and allowed to settle, the ether layer was then removed and the procedure repeated twice. The ether layers were combined and evaporated down to approximately 10 mls on a rotary evaporator at 38 C, no vacuum was used as the equipment available did not allow sufficient control to a desirable level of vacuum which would not increase the vapour pressure of the Ndma to an unacceptable level. 1 ml of toluene was added then the sample was transferred quantitatively to a graduated vial and evaporated under a stream of nitrogen to 1 ml. The addition of toluene meant that there was very little ether in the final

sample, and so the problems associated with injecting such a volatile solvent were avoided. 1 µl was injected onto the GC -TEA.

Determination of Extraction Efficiency

The amount of sample available was limited, therefore most of the preparative work to determine the extraction efficiency was done using aliquots of water spiked with Ndma at various levels. A volatile solvent was required for the extraction to reduce Ndma losses during concentration. Ether was used in preference to Dichloromethane, which formed an emulsion when shaken with the samples and would not settle back into two separable phases. It was found that during the concentration stage, when the sample was taken down to less than 1 ml a large proportion of sample was lost, so the evaporation was curtailed at the 1 ml stage. As low levels of Ndma were expected in the samples, fairly large volumes (30 - 50 mls) had to be used in order to obtain a sufficient degree of concentration. Aliquots of water were spiked with Ndma from 200 ppb down to 20 ppb and the extraction procedure above was followed (Table 1). Each of the samples were then spiked in the same manner to determine whether the traces of organic compounds present would affect the extraction efficiency. The spike was done at a higher concentration than was expected in the sample (200 ppb), to make sure the Ndma result would be easily quantifiable (Table 2). The samples were then analysed for Ndma content and the results were compared.

3.2.2. Results

Table 1 : Results from water spiked with Ndma :

Volume H_2O	Ndma Added	Concentration Recovered	Concentration Factor	Extraction Efficiency
50 mls	200 ppb	3.1 ppm	50	31 %
30 mls	50 ppb	220 ppb	15*	30 %
50 mls	20 ppb	280 ppb	50	28 %

(* Sample only taken to 2 mls)

Table 2 : Results from samples spiked with Ndma (50 fold concentration) :

Sample	Ndma Added	Concentration Recovered	Extraction Efficiency
1	200 ppb	5.1 ppm	51 %
2	200 ppb	3.8 ppm	38 %
3	200 ppb	4.2 ppm	42 %
4	200 ppb	4.9 ppm	49 %

Table 3 : Results of sample analysis:

Sample	Amount Recovered	Efficiency Assumed	Ndma in Sample
1	37 ppb	51 %	2 ppb
2	-	38 %	-
3	98 ppb	42 %	8 ppb
4	91 ppb	49 %	4 ppb

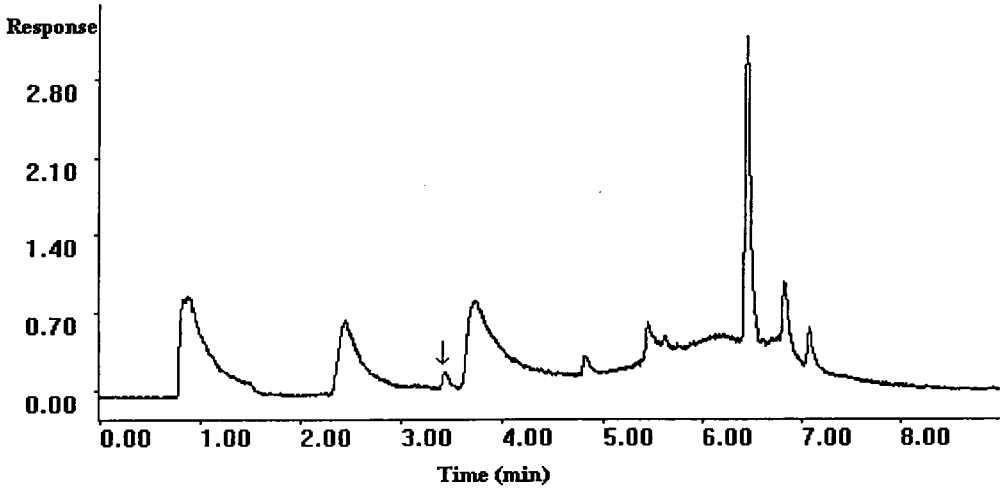
The chromatograms which follow show the results of the extraction of each of the four samples, in each case an arrow points to the retention time of Ndma. The spiked extraction for sample 1 is also shown to give a comparison.

Sample 1 is much dirtier than the others, and may contain quite high levels of other nitrosamines which were not tested for in this case, the peak at ca 6.5 min is probably the C12 alkyl-nitrosamine, and is present in a significant amount.

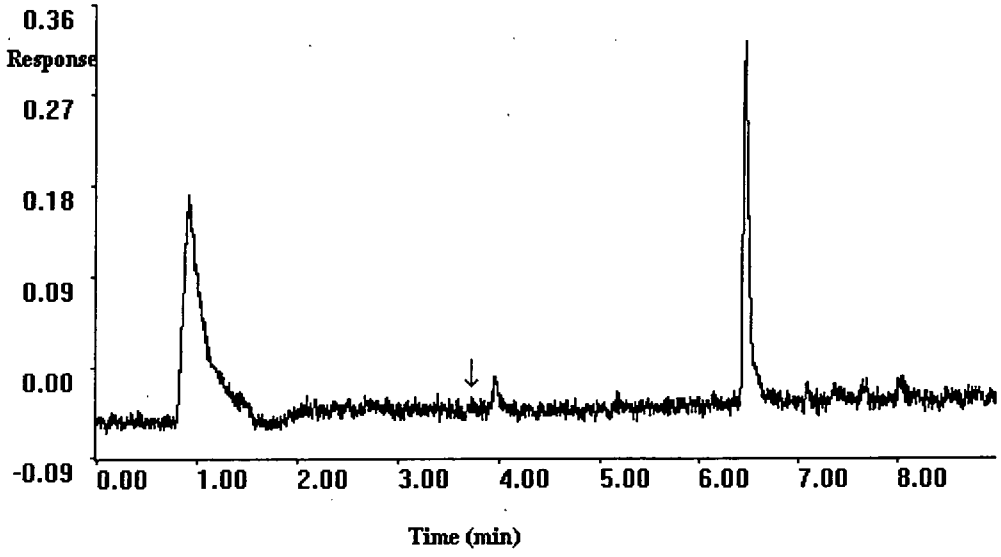
Sample 2 also shows the peak at ca 6.5 min, but at a much lower level. There is no Ndma, the tiny peak at nearly 4 min is at a slightly later retention time and corresponds to the contaminant peak which appears after Ndma in the other samples.

Samples 3 and 4 both show low levels of Ndma, and another contaminant, which appears to be decomposing on the column and forming a tail.

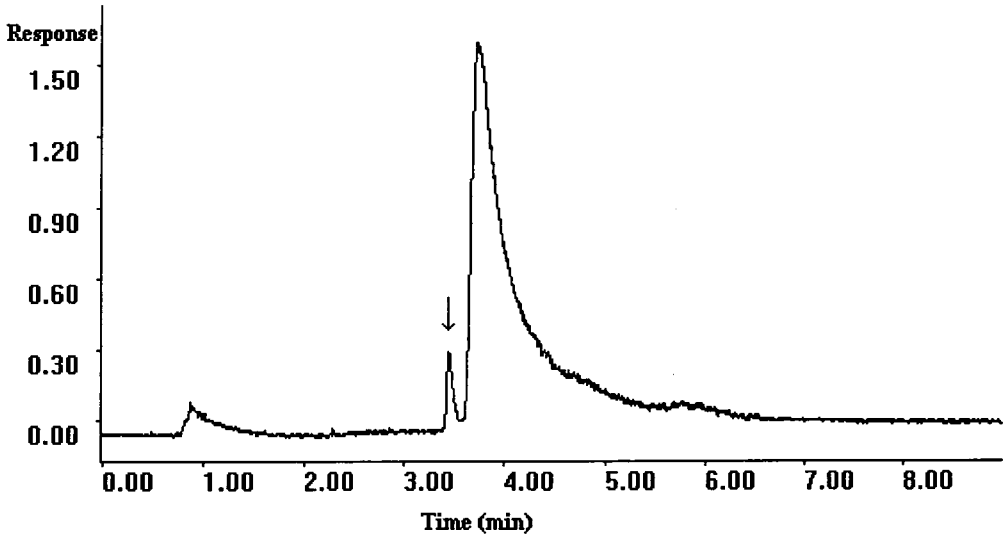
Sample 1



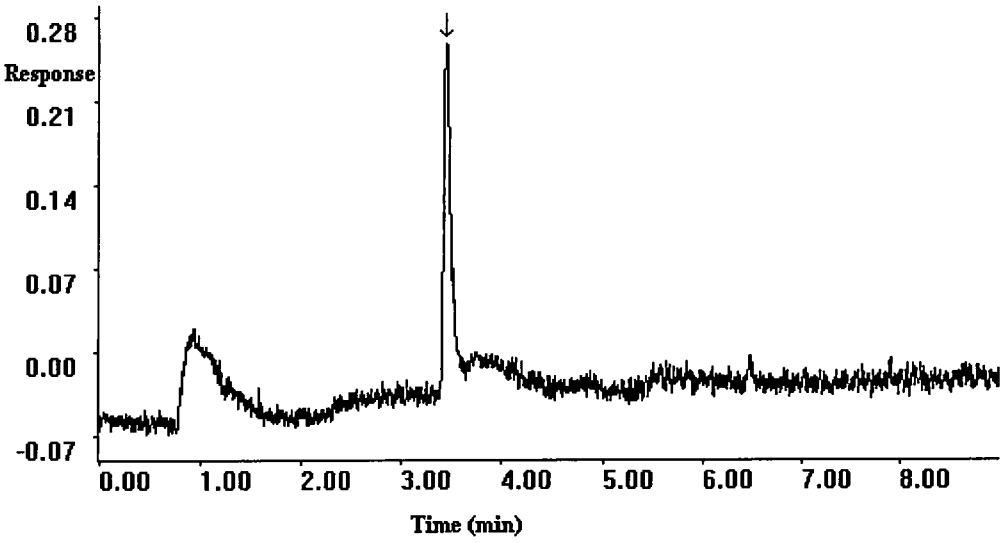
Sample 2



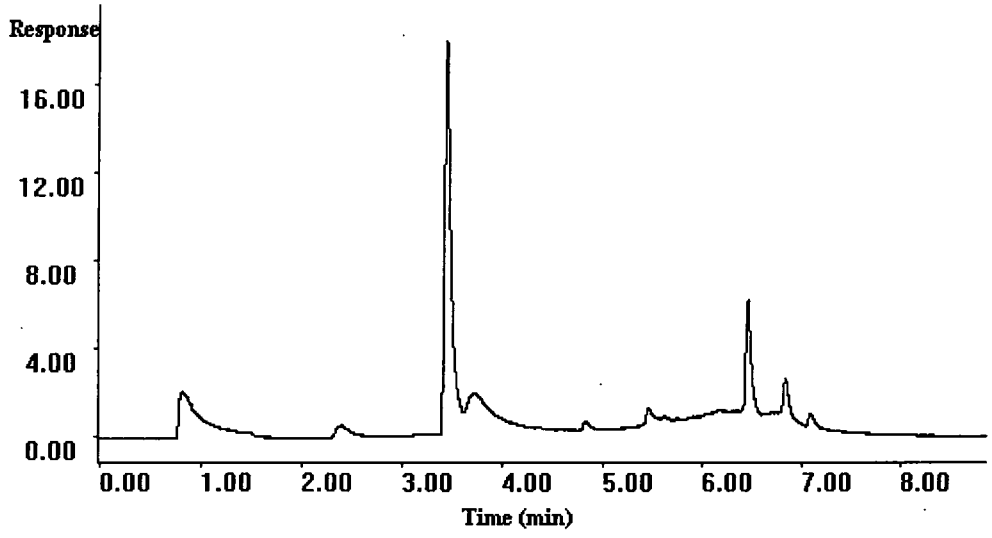
Sample 3



Sample 4



Sample 1 spiked at 200 ppb



The fact that the extraction efficiency is much less than 100 % is not a major problem, but it is the limiting factor determining the sensitivity of the method and means that the overall detection limit of the method is not as low as it could be. Further work on a more effective solvent which gives a better partition coefficient for Ndma could improve the method and allow even smaller amounts to be detected.

The results show that at 200 ppb the spiked samples showed a 10 - 20 % greater Ndma extraction efficiency than from water spiked at these levels. Since in water the extraction efficiency was approximately the same over the range of concentrations tested, we may assume that this is the case in the samples, and that the extraction efficiencies for the spiked samples can be applied to the samples

themselves. This leads to the concentrations given in table 3 for the Ndma content of the samples. These figures, are calculated without rounding of intermediate numbers or incorporation of errors (other than the day to day reproducibility of injection of ± 10 %, which is intrinsically included). There is a small error in measurement of the final volume of the sample (± 2%), and also an inherent uncertainty in making assumptions about extraction efficiency, which was estimated to be ± 20%. A final result of less than 20 ppb Ndma was quoted for each of the samples.

3.3. Analysis of apparent total nitrosamine compounds (ATNC) in personal hygiene products

Five samples of personal hygiene products were provided for analysis of total nitrosamine content. The method used was a slightly modified version of that described in a collaborative study organised by the UK Cosmetic Toiletry and Perfume Association (CTPA).[45] This involves chemical denitrosation of N-nitroso compounds in a single reaction with HBr and acetic acid in n-propyl acetate as solvent. The nitric oxide released is passed directly into the thermal energy analyser (without going through the pyrolyser), and the results are fed to the GC as usual for integration. Samples are injected in a mixture of THF and water, depending on the type of matrix involved, but with a minimum of 10 % water to allow the action of sulphamic acid, a powerful nitrite scavenger which removes potential false positive interference from compounds such as nitrite and

nitrite esters. Other N-nitroso species such as C- and S-nitroso compounds can also release NO when treated with Hbr, the risk of a false positive result is unlikely,[45] and can be minimised by examining the formulation of a product to see if the presence of these species is likely. In the case of this study the formulations were not known, and this uncertainty was accepted in the results. In these cases the results are referred to as apparent total nitroso compounds (ATNC). The denitrosation mixture has a finite lifetime determined by the amount of water injected into the system, and the type of sample being analysed, therefore there is a risk of getting a false negative result due to the reaction mixture being spent. This is avoided by injecting a standard after each sample to ensure the system is still working.

3.3.1. Experimental

Reagents

Sulphamic acid, Sodium Nitrite and N-Nitrosodi-isopropylamine (Ndipla, 97 % purity) were from Sigma, n-propyl acetate, HBr in acetic acid (33% w/w), and THF were supplied by Fluka. Water was laboratory distilled.

Denitrosation Apparatus and TEA

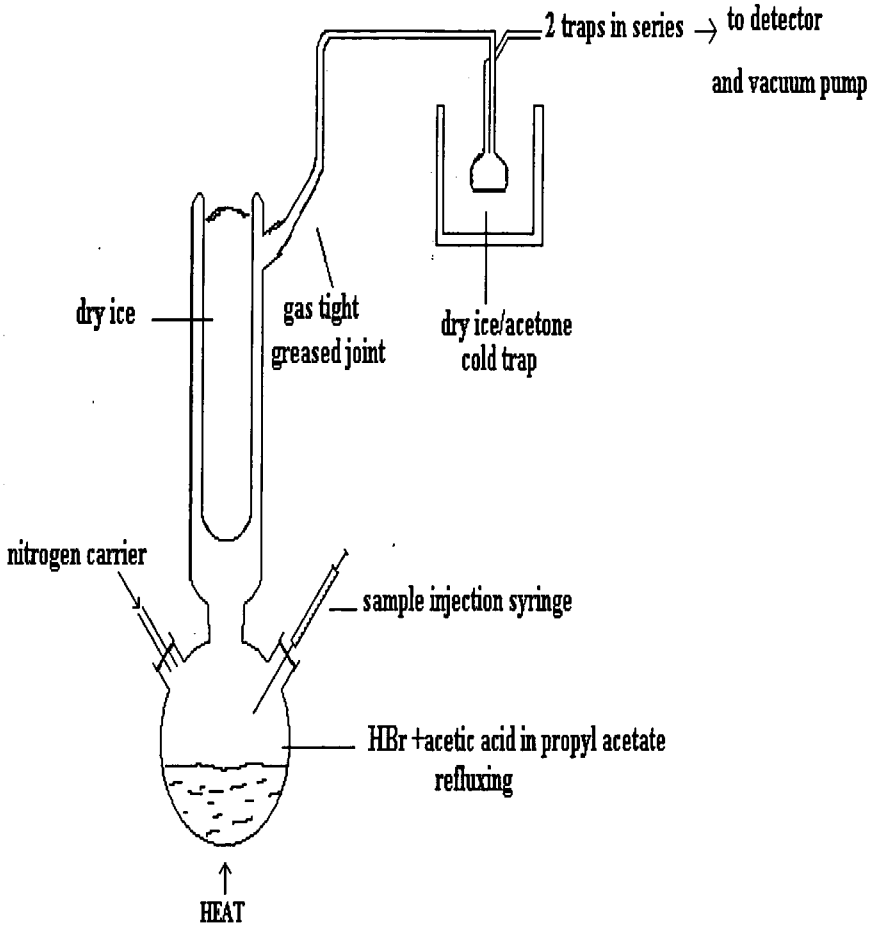
See fig. 3

This apparatus is designed to minimise the dead space between the reaction vessel and the detector so that the NO is sucked through the system as quickly as possible, to try and obtain sharp peaks. Two modifications were made to the

CPTA set-up. A piece of 0.53 mm O.D. megabore fused silica capillary tubing was used to replace the original section of glass tubing which incorporated a length of 0.01 μm glass capillary, which was used to connect the system to the TEA and to control the flow rate. The larger diameter tubing still allowed a good vacuum to be obtained, and the added flexibility meant the apparatus was easier to handle. Initially the apparatus was set up using a spiral water cooled condenser, but this did not to stop the corrosive reactants from passing through the system and attacking the megabore tubing and septa. This was replaced with a cold finger condenser using dry ice/acetone which solved the problem, although the increased volume of the system due to the size of this led to a slight broadening of the NO peaks. This did not cause problems in this work, but may become significant when working quantitatively at the detection limit as integration of peak area would be more difficult.

The flow rate through the megabore tubing allows a total internal pressure in the TEA of 1 mmHg to be sustained.

fig. 3.


2 traps in series → to detector
and vacuum pump
dry ice
gas tight
greased joint
dry ice/acetone
cold trap
nitrogen carrier
sample injection syringe
HBr +acetic acid in propyl acetate
refluxing
HEAT

Sample Preparation

Solutions were made by suspending about 1 - 2 g (accurately weighed) of sample in 5 ml of a mix of 80% THF : 20% water. 500 mg of sulphamic acid was added and the mixture vortexed for 1 minute. The samples were centrifuged at 500 rpm for 5 min and a 250 µl aliquot was immediately extracted into a glass syringe and injected into the reaction vessel. Duplicate analyses were performed on each sample.

Calibration

Samples were calibrated against solutions of Ndipla at 100 and 200 ppb prepared using the same ratio of THF : water as was used for sample preparation, and the same injection volume. Within day reproducibility was within ± 10 %. Preliminary work using successive dilutions of the standards showed that below 20 ppb the peak produced was of poor shape quality and could no longer be integrated accurately, although a rough estimate could be made, therefore 20 ppb was set as the limit of detection, below which any results could be quoted with a lesser degree of confidence.

Verification of Method

To show that the method was discriminating between NO releasing compounds a test was performed. A sample was prepared as normal, and treated with Sulphamic Acid (SA) and injected into the reaction mixture and a peak obtained for ATNC. Sodium nitrite was then added to the sample and it was injected again,

this time the peak produced was off-scale. The sample was then treated again with SA and injected, to produce the peak originally seen. This shows that the method does remove nitrites, and also that treatment with sulphamic acid does not interfere with the end result by affecting the Nitrosamines in any way.

Assay Procedure

The reaction flask was charged with 50 ml n-propyl acetate, 10 ml HBr in acetic, and a few antibumping granules, then purged with nitrogen and heated gently until the solvent was refluxing rapidly. Initially a rush of volatile components results in a very high baseline, which stabilises after about 30 min. The standard is injected twice to ensure the reaction system is working and stable. Samples can then be injected, alternating with the standard, until the standard fails to fall within the limits of reproducibility (either peak area or shape), when the reaction mixture must be renewed. This was generally after injection of about 1 ml of water.

Figure 4 shows a typical chromatogram taken over the lifespan of one charge of the reaction vessel. Peaks 1, 5, 9, 12 and 15 are injections of standard and the rest are samples. It can be seen that the shape of the standard peaks can vary even though the area remains the same, this is also seen in the sample peaks. This is explained by the water present in the reaction vessel, which tends to collect at the bottom, taking with it the HBr reactant, and therefore making the solution non-uniform. This in turn leads to a non-uniform denitrosation reaction where a sudden bumping of the liquid may lead to a faster rate of reaction for a short time, as shown by peak 9. Peak 15 is losing its shape, showing that the mixture is

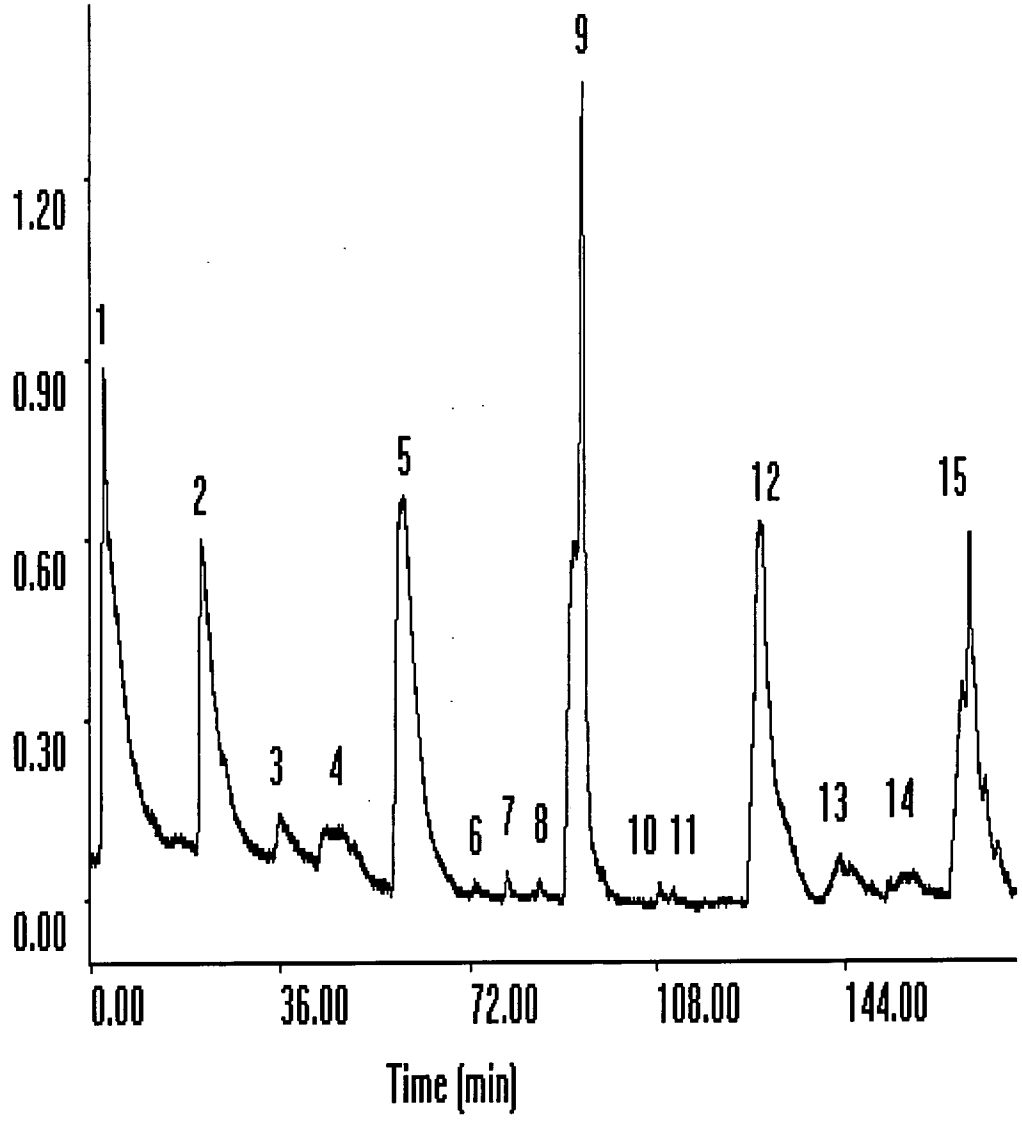
becoming exhausted, but samples 13 and 14 are still acceptable as the area of peak 15 falls within the reproducibility limits of ± 10 %.

3.3.2. Results

Sample	ATNC of Extract (ppb)	ATNC of Sample (ppb)
1	59	180
2	116	365
3	108	260
4	3.2	< 7.5
5	< 3	< 10

As the detection limit for this work is 20 ppb, samples 4 and 5 were quoted as having an apparent total nitrosamine content of less than 20 ppb.

Figure 4.


1.20
0.90
0.60
0.30
0.00
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
0.00
36.00
72.00
108.00
144.00
Time (min)

3.4 Analysis of N-nitrosodiethanolamine (Ndela) in a sample of dye

The dye in question is a preparation of tris(2-hydroxyethyl)-2-nitro-phenylenediamine, which is known to contain traces of nitrosamines as contaminants. This analysis aimed to quantify the amount of Ndela present, and the result was expected to be less than 100 ppb. This proved to be more difficult than first thought, and due to time restrictions this aim was not achieved, but the initial efforts are presented here.

3.4.1 Experimental

To analyse Ndela by GC it is necessary to mask the -OH group by derivatising it, as otherwise it would have a very strong interaction with the column and therefore an excessively long retention time. This is done by adding a silylating agent such as bis-trimethylsilylacetamide (TMSA) and converting the -OH groups to trimethylsilyl groups which are not retained by the column. This analysis is complicated by the fact that the dye contains three -OH groups and two -NH groups, which would cause the dye to be retained on the column if they were not masked, and cause long-term contamination of the column. Any significant amounts of dye present during the silylation process would interfere, as the -OH

and -NH groups would react, possibly in preference to those of Ndela. For these reasons it was important to make sure that the solution to be silylated and injected was as free of dye as possible.

Three different approaches were tried unsuccessfully :

1. Simple solvent extraction

Initially it was thought that it would be possible to extract the Ndela from the dye using a suitable solvent in which Ndela was soluble, but which did not dissolve the dye. It was hoped that the porous, granular nature of the dye would allow circulation of the solvent through the dye, aided by crushing it up, and that when stirred for long enough the extraction would be quantitative. However, a suitable solvent could not be found, as the solubilities of the dye and the nitrosamine were too similar, probably due to the number of -OH groups present in the molecules.

2. Steam distillation and solvent extraction

It was hoped that by choosing a suitable solvent with a boiling point similar to that of Ndela, a distillation could be performed such that the Ndela, which boils at 175-177^{0}C, would be carried over with the solvent, and the dye, which boils at a much higher temperature, would remain behind. 100 ml of petroleum special with a boiling range of 180-220^{0}C was used, to which 10 g of the

dye was added, which had been crushed to enable better removal of the nitrosamine. Approximately 80 ml was distilled over at 130^0C, 100 mmHg, and the distillate was then solvent extracted using 50 ml of methanol, evaporated to dryness, silylated (500 µl of TMSA added and heated to 70^0C for 40 min) and injected onto the GC-TEA.

The first problem with this procedure was that the extraction recovery rate was found to be only about 25 % after performing the distillation using petrol spiked with Ndela at 15 ppm, which means that at the levels expected in the dye, ie. less than 100 ppb, the recovery would probably be significantly lower and we would be very unlikely to be able to detect anything. The dye forms a sludge at the bottom of the vessel when heated, and it is likely that because of the two -OHs on the Ndela and 3 -OHs on the dye, the nitrosamine is mostly retained in the sludge, where it does not freely come into contact with circulating petrol. Another complication was that the petrol was slightly soluble in methanol, and therefore when concentrating down the extractant, it was found that about 3 ml of petrol remained. This limits the concentration factor which can be obtained and subsequently means the detection limit was higher. Water was substituted for methanol, as the petrol is much less soluble and evaporation to a few drops was possible. Unfortunately this meant the evaporation had to be done at a higher temperature which lead to greater losses of Ndela as a proportion of

it reaches its vapour pressure, and this increased as the volume of the solution decreased.

All in all this pocedure was not effective enough to allow a sufficiently low detection limit to perform the analysis.

3. Ion exchange chromatography

A brief attempt was made to separate the Ndela from the dye using a strongly acidic cation exchange resin, in the hope that the amine groups on the dye, when protonated in water, would be retained by the resin and the nitrosamine should pass through. Using water spiked with Ndela it was possible to get a recovery of 50 % after concentration of the eluent and silylation. When the procedure was tried with the dye it was found that very little of the dye was retained on the resin - only ca.0.5 g on 500 g resin, which is a factor of 100 less than the expected amount calculated from the activity of the resin, suggesting that only a small proportion of the dye is being ionised. This again meant that an adequate concentration factor could not be obtained.

3.4.2. Discussion

In the time available an effective method of analysis could not be found due to the similar solubilities of the two compounds. It is possible that column chromatography may work if the correct combination of support and solvent could be obtained, but the

sample would stil need preparing for analysis by GC. High performance liquid chromatography may offer a solution to this analysis, as there would be no problem with the column if the dye was injected, and no need for the tedious derivatisation, making the whole procedure much simpler .

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