" THE ISOLATION AND ESTIMATION OF LOW MOLECULAR WEIGHT N-NITROSAMINES IN BIOLOGICAL MATERIALS "

by

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A Thesis

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CONTENTS

SUMMARY

Low molecular weight N-nitrosamines were detected by ultraviolet, infrared, nuclear magnetic resonance, mass spectral, thin-layer chromatographic and gas liquid chromatographic means. A method for the estimation of dimethylnitrosamine, diethylnitrosamine, ethyl-n-propylnitrosamine and di-n-propylnitrosamine has been developed. The method involves the isolation of the nitrosamines in an aqueous distillate by freeze-drying. After extraction of the nitrosamines from the aqueous distillate by means of dichloromethane, their separation and quantitative estimation are achieved by gas liquid chromatography of the extract. The procedure can be extended down to the ppb range. Dimethylnitrosamine was isolated from an extract of Solanum incanum and identified by gas liquid chromatography on four different columns, infrared and nuclear magnetic resonance spectroscopy.

1. INTRODUCTION

In 1956 Magee and Barnes [1] demonstrated that dimethylnitrosamine was highly carcinogenic in a wide range of animal species. Up to that time there had been little interest in the chemistry of the alkyl nitrosamines, and virtually none in their analysis. The potential danger represented by the presence of N-nitrosamines in foods has been widely recognised. The International Agency for Research on Cancer at Lyons in France has already convened several international meetings on the subject of this review, the latest of which was held in Heidelberg, Germany in October, 1971.

Nitrosamines are relatively easily formed from nitrite or nitrous gases and secondary amines under the conditions normally pertaining in most biological tissues. Under certain conditions tertiary amines also produce nitrosamines. The ubiquitous presence of amines and nitrite in foods, and even in the human body (e.g. stomach), has led to a greatly increased interest in the separation, isolation and identification of nitrosamines in the environment.

Despite the wide range of techniques available to the analytical chemist, this problem, viz the separation and analysis of nitrosamines, has proved to be far more difficult than was first thought would be the case. The low molecular weight nitrosamines, despite having relatively high boiling points, are extremely volatile. This, together with their occurrence in very low concentrations (mostly a few ppb), renders their accurate analysis very difficult. The most difficult part of the development of a viable analytical technique has been concerned with the "clean-up" procedure prior to actual separation of the nitrosamines, their identification and estimation. The wide diversity of methods of

clean-up and final analysis, which have been applied by a large number of workers in a short space of time, makes it desirable at this stage to review and assess the whole field. Carcinogenic nitroso-compounds have previously been reviewed with emphasis on their biological activity by Magee [2, 3], Magee and Schoental [4], Druckrey et al [5] and Magee and Barnes [6]. In 1969 Fishbeim and Falk [7] briefly reviewed the chromatography of nitrosamines; while in 1971 Walters [8] reviewed some limited aspects of the analytical progress up to that time and Wassermann [9] surveyed the analytical procedures for nitrosamines.

1.1 METHODS OF ISOLATION

1.11 Methods depending essentially on solvent extraction

The efficient isolation of nitrosamines from the materials in which they occur is an essential prerequisite to their identification and estimation. Many of the methods reported in the literature are laborious and inefficient and often lead to the formation of artefacts. In addition, the lack of quantitative data in many of the publications renders impossible a true evaluation of the relative merits of the various procedures.

Isolation of nitrosamines by extraction with an organic solvent has been frequently used. It has the prime disadvantage that usually many other substances, many of which could interfere with subsequent analysis of nitrosamines, are extracted at the same time from most biological materials. Neurath et al [10] established that dichloromethane is a most effective nitrosamine solvent, the efficiency of extraction from aqueous solutions being increased when the latter is saturated with sodium chloride. Sander et al [11, 12] and Sander and Seif [13] used

dichloromethane for the extraction of nitrosamines from bacterial cultures and the stomach contents of rats, and humans. After evaporation of the dried dichloromethane extract, the residue was dissolved in a small volume of acetone. Lijinsky et al [14] used dichloromethane for the extraction of the reaction mixture resulting from the admixture of a nitrite with drugs. The extract, after acid-washing, was concentrated in a stream of nitrogen. This procedure can lead to significant losses of nitrosamines [15]. In the analysis of cigarette smoke, Serfontein and Hurter [16] used a dichloromethane extract of the smoke condensate. The extract was washed with acid and alkali and concentrated after drying. Johnson et al [17] and Rhoades and Johnson [18] used a similar procedure. Percolation of finely ground samples of beans and maize, to which had been added nitrosamines, with dichloromethane gave low recoveries [19].

Neurath et al [20] trapped cigarette smoke in successive traps containing cotton wool, water and pentane. Soxhlet extraction of the cotton wool with pentane yielded a solution which, after washing with acid and alkali, was concentrated. The contents of the water and pentane traps were extracted with dichloromethane and ether respectively. The authors subsequently concluded [10] that artefacts were produced during the condensation of the smoke.

Soxhlet extraction has been used by several workers for the examination of wheat flour for nitrosamines. (Marquardt and Hedler [21], Thewlis [22], Hedler and Marquardt [23] and Eisenbrand et al [24]). Du Plessis and Nunn [15] extracted food samples with dichloromethane in a Soxhlet apparatus; subsequently, passage of the extract through a heated U-tube packed with glass beads removed the high boiling components.

Using solutions containing 30 ppm of diethylnitrosamine, a recovery of 90% of the latter was recorded after passage through the heated U-tube.

1.12 Methods depending essentially on distillation

Several investigators have taken advantage of the volatility of many nitrosamines when attempting isolation of these compounds from biological materials. Steam distillation from acid or alkaline media has often been used. Heath and Jarvis [25] effected quantitative separation of dimethylnitrosamine from spiked animal tissues by steam distillation of the alkaline homogenate. Neuenhofer et al [26] used a similar procedure when separating nitrosamine from acidified liver and kidney homogenates, while Preussmann [27] separated nitrosamines from the ferrous complex of EDTA and ascorbic acid by steam distillation after the addition of alkali to the mixture.

Foreman et al [28, 29] used steam distillation as a preliminary clean-up procedure to separate nitrosamines from canned meat which had been digested with sodium hydroxide. The recoveries of dimethylnitrosamine and diethylnitrosamine, in the first 50 ml of distillate from spiked samples, were over 90% and about 100% respectively. The distillate was made alkaline and redistilled. After acidification, the latter distillate was passed through an ion-exchange column (Amberlite GC 120). The eluate, after being made alkaline, was successively redistilled to smaller volumes to achieve concentration of the nitrosamines. Although the resin column removed basic contaminants, a number of interfering substances remained. Foreman et al [29] extracted African spirits with dichloromethane and, after evaporation of most of the organic phase, the remaining alcohol was removed by oxidation. The mixture was made

alkaline and distilled to half its original volume, so as to increase the concentration of any nitrosamine present. This procedure successfully removed the alcohol, but other interfering compounds remained. The recovery of nitrosamine from spiked alcoholic solutions was very variable (20 - 80%), demonstrating the inefficiency of the method for the examination of alcoholic samples. Möhler and Mayrhofer [30] found that steam distillation of acidified food samples was an inefficient method for the isolation of nitrosamines from the food matrix, while Du Plessis and Nunn [19] found that a combination of steam distillation followed by extraction of the distillate with dichloromethane gave inconsistent results.

Eisenbrand et al [31] found that steam distillation at atmospheric and reduced pressures of aqueous solutions of volatile nitrosamines gave a good recovery from alkaline, neutral and acid media. Steam distillates of alkaline food samples contained more interfering compounds than the distillates from neutral or acid samples [32]. These investigators preferred steam distillation of food samples under neutral or acid conditions, despite the enhanced possibility of artefact formation. No artefacts were, however, detected in the distillates. These distillates were extracted with dichloromethane, the latter being subsequently concentrated in a similar manner to that of Osborne [33]. Bogovski et al [34], when examining the retardation of nitrosamine formation by tannins, also employed distillation from an acid medium.

Keybets et al [35] digested spinach with sodium hydroxide and isolated nitrosamines by steam distillation and dichloromethane extraction of the steam distillate. They recorded the procedure as being very inefficient, since the analysis of spinach samples spiked with dimethyl-

nitrosamine and diethylnitrosamine resulted in respective losses of 70 - 80% and 30 - 40%. Further, the number of interfering compounds was found to increase [32] when an excess of alkali was added to the sample before distillation. Hedler et al [36], using a similar method for the analysis of soya beans, omitted the evaporation of the dichloromethane solution. Sen et al [37] steam distilled spinach, and extracted the distillate at an alkaline pH with dichloromethane and subsequently concentrated the latter to a small volume under a stream of nitrogen.

Kröller [38] partially purified the steam distillate of an acetone concentrate by precipitation with lead acetate and strontium hydroxide. The filtrate was extracted with dichloromethane. The extract, after washing with acid and alkali, was further purified by extraction with ether and n-hexane. Food-stuffs were extracted in a Soxhlet apparatus with dichloromethane and the steam distillate of the concentrated extract was extracted with n-heptane. The organic phase, after washing with acid and alkali, was concentrated to a small volume. Undoubtedly these procedures resulted in high losses of nitrosamines [29] and are of little use for the analysis of trace amounts of nitrosamines.

Ender and Ceh [39, 40, 41] extracted fish preparations with hot distilled water and, after removal of fats on cooling, proteins and basic products were precipitated with phosphotungstic acid. The filtrate was made alkaline and was redistilled several times to successively smaller volumes. The distillate was acidified prior to the final distillation. Schuller [42] extracted wheat meal with dichloromethane, followed by concentration of the extract in a rotary evaporator. Alternatively, the extraction was carried out in a Soxhlet apparatus. Recoveries of dimethylnitrosamine were 75% and 57% respectively. Nitrosamines were

finally separated by preparative TLC, the most favourable average overall recovery of diethylnitrosamine, although not stated, appears to have been 46%.

Möhler and Mayrhofer [30] found the direct extraction of a food sample with either ether or dichloromethane to be rather ineffective. These authors steam distilled acidified meat and cheese. The dichloromethane extracts of the distillates were washed with acid and alkali and the neutral extracts concentrated under a stream of nitrogen. Greatest losses of nitrosamine occurred during washing and concentration of the dichloromethane extract. Overall recoveries of nitrosamine were only 20 - 40%. A more efficient procedure was used by Sen et al [37] but all the interfering compounds were not removed. Wheat flour was extracted with ether in a Soxhlet apparatus and the dried ethereal extract concentrated in a stream of nitrogen. The recoveries of diethylnitrosamine, at concentrations of 10 - 4000 ppm, ranged from 65 - 90%.

Eisenbrand [43] extracted wheat flour in a Soxhlet apparatus with dichloromethane. The concentrated organic extract was made alkaline and the acidified steam distillate, after saturation with sodium chloride, was redistilled twice. The distillate was extracted with dichloromethane, and the latter concentrated to a small volume using a Kuderna-Danish evaporator in a stream of nitrogen. This clean-up procedure did not remove all of the interfering compounds. The nitrosamines were finally separated by preparative TLC and quantitatively determined by derivative formation [44]. The overall recoveries of 22ppb dimethylnitrosamine, 22 ppb diethylnitrosamine and 34 ppb di-n-amylnitrosamine added to wheat flour, were 69, 82 and 62% respectively.

A few attempts, incorporating ion-exchange chromatography as a

partial clean-up method, were only of limited effect since a number of neutral contaminants remained in the extract. Sen et al [45] used this method partially to clean-up the filtrate of acidified gastric juice. The effluent from Amberlite CG-120 was made alkaline and was passed through Amberlite IRA-400. After the effluent had been made alkaline it was extracted with peroxide-free ether or dichloromethane. The dried organic extract was concentrated to a small volume. The concentrate was subsequently analyzed according to the method of Sen et al [37].

Sen et al [37] extracted cheese, meat and fish homogenates with dichloromethane. The solvent was evaporated, the residues steam distilled, and the acidified distillates passed through an ion-exchange resin (Amberlite CG-120). The eluates, after being made alkaline, were again extracted with dichloromethane, dried and the solvent evaporated to small volumes in a stream of nitrogen. The recoveries of nitrosamines, added to the foods at concentrations of 5 - 25 ppm, varied from 30 - 80%. Sen et al [46], when analyzing uncooked fish samples, modified the method of Sen et al [37]. A polyamide column was used in conjunction with the ion-exchange resin and an extract of the eluate was subsequently fractionated on an alumina column. This method removed more contaminating compounds than the previous procedure. The steam distillate obtained from an aqueous extract of cooked fish samples was analyzed in a similar manner. Towards the end of this investigation, the latter method was abandoned after it was found that steam distillation of food samples in the presence of nitrite and large amounts of secondary amines produced nitrosamine artefacts. The artefacts were formed even under strongly alkaline conditions. In an attempt to overcome this difficulty

cooked fish samples were solidified with excess anhydrous sodium carbonate and sodium sulphate, and the extraction then proceeded as for uncooked samples.

Sen [47] surveyed a number of Canadian foods (wheat, cheese, fish, smoked fish, spinach, mushrooms, sauerkraut, meat products and alcoholic drinks) for their nitrosamine content. The products were extracted according to the method of Sen et al [37]. After the alkaline extract had been heated until all the solvent had evaporated, the residue was distilled. The acidified distillate was purified by ion-exchange chromatography using a Rexyn 101-polyamide column. The effluent was extracted with dichloromethane and the extract concentrated. The latter, in n-hexane, was passed through an alumina column, after which the adsorbed nitrosamines were eluted with dichloromethane.

Commercial samples of cheese, anchovy paste, cured meat (Kaselar) and Westphalian rye-bread were examined by Freimuth and Gläser [48]. The nitrosamines were isolated in a manner similar to that of Sen et al [37], but the ion-exchange procedure was not used. The recovery of added nitrosamine, estimated by the method of Daiber and Preussmann [49], was 60 - 70%.

Ender et al [50] and Ender [51] used cellulose column chromatography to clean up ethanolic extracts obtained from cod skin and herring meal. The water-soluble extracts were obtained either by fractional ethanolic extraction or by dialysis through cellophane membranes.

Du Plessis et al [52] used the dry column chromatography method of Loev and Goodman [53] partially to clean up a methanolic extract of the fruit of Solanum incanum. The positions of standard nitrosamines in the column were determined by the extinction of a fluorescent compound added

to the alumina. The corresponding nitrosamine zone, in a column to which the fruit extract was added, was extracted with ether and the ether concentrated. Dimethylnitrosamine was isolated from this extract by preparative TLC and GLC. Crosby et al [32] found that only a moderate clean-up was achieved by dry column chromatography.

Eisenbrand et al [54] separated nitrosamines and lipids in acetonitrile-saturated n-heptane by liquid-liquid extraction. Three extractions of the n-heptane phase with acetonitrile were sufficient to concentrate more than 95% of all but one of eleven nitrosamines into the polar phase. A moderate loss of nitrosamine occurred when the acetonitrile was concentrated to a small end-volume. Dimethylnitrosamine, the most volatile dialkylnitrosamine, was recovered in 85% yield when 200 ml of acetonitrile containing 0.38 ppm was concentrated to 3 ml. The acetonitrile extract was evaporated in admixture with alumina and the nitrosamines were desorbed from the alumina with methanol. This clean-up method was applied to a wheat flour extract [54]. It did not effectively purify the extract, and evaporation of acetonitrile in the presence of alumina did not prevent loss of nitrosamine.

Food samples, suspected of containing nitrosamines, were distilled by Walters [8] prior to isolating the nitrosamines from the distillate. Recoveries of nitrosamines (low molecular weight), on distillation of a 20% saline solution, varied from 70% for diethylnitrosamine to 100% for di-n-butylnitrosamine. The recovery of some cyclic and high molecular weight nitrosamines was very poor. A variety of foods, incubated under conditions approximating to those occurring in the human stomach, were analyzed for nitrosamines either by distillation under reduced pressure or by repeated extraction with dichloromethane. Sodium chloride

was added as an aid to nitrosamine distillation. In the light of recent experiments by Du Plessis and Nunn [19] this step appears to be unnecessary. The simultaneous distillation and extraction of aqueous food samples was effected by Issenberg and Tannenbaum [55] who used the apparratus of Likens and Nickerson [56]. The dichloromethane extract was concentrated to a small volume under a stream of nitrogen, or in a Kuderna-Danish evaporator. The recoveries of dimethylnitrosamine and diethylnitrosamine added to foods at a level of 10 ppb were 60% and 70% respectively.

Howard et al [57] employed a detection method relying on a specific response to nitrogen compounds. The application of such a procedure should require fewer clean-up steps and thus the recovery of nitrosamines should be increased. Dimethylnitrosamine in smoked fish was recovered by distillation of the filtrate obtained from samples which had been digested in methanolic potassium hydroxide. The distillate was extracted with dichloromethane and the dried organic phase concentrated in a Kuderna-Danish evaporator. The extract was further concentrated under a stream of nitrogen and the concentrate, in pentane, was passed through a Celite 545 column as the final step of nitrosamine isolation. The recovery of dimethylnitrosamine, added at a level of 10 ppb, was 70 - 80%. This procedure was extended [58] to include six to nine volatile N-nitrosamines. After the sample had been digested as before, the filtrate was extracted with dichloromethane in a liquid-liquid extractor. The extract was made alkaline, the solvent evaporated, and the residue distilled. The distillate, after washing with acid and alkali, was dried and concentrated in a Kuderna-Danish evaporator, the final concentration being effected in a stream of nitrogen. The extract was further purified

by chromatography on a silica gel column using an ether-dichloromethane mixture as eluant. The ether-dichloromethane eluate was concentrated as above. The recoveries of nine nitrosamines, added at levels of 10 ppb to salmon roe, were 70 - 114%.

The procedure of Howard et al [57] for the estimation of nitrosamines in fish was modified by Fazio et al [59] to improve the clean-up and recovery of dimethylnitrosamine. The modified procedure included acidification of the distillate before the extraction with dichloromethane, and an alkali washing of the dichloromethane extract before it was dried. The average recoveries of dimethylnitrosamine, added at levels of 10 ppb to 400 g of salmon, shad and sable, were 75%, 78% and 77% respectively. A more rigorous clean-up procedure was used for the GLC/MS confirmation of the presence of dimethylnitrosamine in fish. Three dichloromethane extracts of the first portion of distillate obtained [57] were dried and the combined extracts concentrated in a Kuderna-Danish evaporator. The concentrate, together with the second portion of distillate, was made alkaline and distilled. This distillate was extracted three times with dichloromethane, dried, concentrated and further purified by column chromatography as before [57].

Fiddler et al [60] employed the digestion and distillation procedures of Howard et al [57] to estimate dimethylnitrosamine in ham. The distillate was twice washed with hexane and after being acidified with trifluoroacetic acid, it was extracted three times with dichloromethane. The combined extracts were washed with dilute alkali. The dichloromethane was dried and concentrated in a Kuderna-Danish evaporator. The remaining alkaline layer was extracted with dichloromethane. The dried extract was added to the Kuderna-Danish evaporator and concen-

trated to a small volume.

Ender and Ceh [39] found that dimethylnitrosamine, even at a concentration of 1 μg in 1000 ml of water, was quantitatively adsorbed by activated charcoal when the particle size was at an optimum. The adsorbed dimethylnitrosamine could easily be quantitatively removed from the charcoal by steam distillation, or extraction with dichloromethane. The authors did not state what the optimum particle size for the charcoal was, and the absence of any quantitative data for the adsorption and desorption of dimethylnitrosamine from charcoal has discouraged its use in clean-up procedures.

Walters et al [61] performed a quantitative polarographic estimation of the adsorption by activated carbon of volatile and non-volatile nitrosamines in aqueous biological homogenates. Aqueous solutions of seventeen nitrosamines were examined and most nitrosamines were almost quantitatively removed from an aqueous 10 ppm solution which had been shaken for 2 h with activated carbon. However, the nitrosamines studied were less easily desorbed from the carbon. Boiling methanol removed from 0 - 100% of the adsorbed nitrosamines. Apart from unsaturated aldehydes, substances which affected the polarographic estimation of nitrosamines were not desorbed by boiling methanol. The method was not applied to biological extracts.

Casselden et al [62] used fractional distillation to increase the concentration of aqueous solutions of nitrosamines after methanol and salt had been added. The nitrosamine-rich fraction was collected between the alcohol and aqueous phases. The concentrations of low molecular weight nitrosamines were increased 30-fold while those of the higher homologues were increased 15 to 20-fold. Nitrosamines added at

low levels to muscle mince were recovered in increased concentration. This simple method, although suitable for qualititative analysis by a procedure specific for nitrosamines, would be difficult to quantize owing to the numerous factors which could influence the appearance of the nitrosamine in the small fraction distilling between the alcohol and the water. Walters et al [63] confirmed that dimethylnitrosamine fractionally distilled from an aqueous solution containing 5% methanol, was concentrated almost 40-fold. However, only 50% of the diethlynitrosamine originally present was recovered. Crosby et al [32] used fractional distillation when investigating East African spirits for the presence of nitrosamines.

Williams et al [64] combined vacuum distillation, solvent extraction and fractional distillation to examine a potable spirit. Two standard ethanol-water mixtures (35 : 65), the first containing 1 ppm of each of dimethylnitrosamine and diethylnitrosamine, and the second 2 ppm of each nitrosamine, 420 ppm of palmitic acid and 50 ppm of acetic acid, were vacuum distilled almost to dryness. Each distillate was collected in an ice-cooled trap connected in a series with two solid carbon dioxide-acetone traps. The combined contents of the traps were neutralized, salts added and, after being diluted with water, were extracted three times with dichloromethane. The combined dried extracts were fractionally distilled to remove ethanol and concentrated to 500 µl. The final concentrate was stored under nitrogen in sealed ampoules. During all stages of the analysis light was excluded as much as possible. The recoveries of the two nitrosamines were 20% and 30% respectively. The organic acids did not affect the recovery and in the light of quantitative vacuum distillation analyses by Du Plessis and Nunn [19], the greatest loss of

nitrosamine would have occurred during fractional distillation [28]. The distillate from the potable spirit was collected in two fractions, the first distilling up to 31° and the second at 31 - 32°. Each distillate, after extraction with pentane to remove a number of interfering compounds, was then treated as above. Dimethylnitrosamine and diethylnitrosamine were found to be almost insoluble in pentane.

Vacuum distillation, first applied to nitrosamine analysis by Lyderson and Nagy[65] has recently been quantitatively investigated by Du Plessis and Nunn [19] and successfully combined [33] with a more specific method of detecting nitrosamines. Lyderson and Nagy [65] found that the recoveries of dimethylnitrosamine and diethylnitrosamine distilled from aqueous alkaline samples were 95% and 100% respectively. When this method was applied to a spiked fish sample 20 - 60% of the dimethylnitrosamine was lost, which was attributed to a reaction of the nitrosamine with some components of the fish. Devik [66], Heyns and Koch [67, 68], Heyns et al [69], Williams et al [64] and Scanlan and Libbey [70] reported the use of vacuum distillation for the examination of biological materials suspected of containing nitrosamines.

Low molecular weight dialkylnitrosamines in aqueous food samples were isolated by freeze-drying [19], the distillate being collected in a trap cooled in liquid air. The nitrosamines in the distillate were extracted with dichloromethane, and separated and estimated gas chromatographically. When this method was applied to cellulose powder, beans, marrow and fish, spiked with nitrosamines at levels ranging from 125.6 to 0.24 ppm, recoveries ranged from 79.6% to 101%. In the same investigation the extraction of nitrosamines from aqueous solutions with dichloromethane was reinvestigated. The recoveries of dimethylnitrosamine,

diethylnitrosamine, ethyl-n-propylnitrosamine and di-n-propylnitrosamine, added at levels of 7.4 to 16.3 ppm to 50 ml water, and extracted with five 5 ml aliquots of dichloromethane, were 77.5%, 94.4%, 91.4% and 92.0% respectively. Eisenbrand et al [71] concentrated rigorously dried dichloromethane solutions, spiked with nitrosamines, in various ways. When the solutions were concentrated in a Kuderna-Danish evaporator, the most efficient method of concentration used, the recoveries of all four nitrosamines tested exceeded 92%.

Osborne [33] used vacuum distillation to separate nitrosamines from meat. Of 16 nitrosamines added to meat at levels of 1 - 40 ppb, the amounts of low molecular weight nitrosamines (dimethylnitrosamine to di-n-butylnitrosamine) recovered in the distillate, varied from 58% to 102% while the recoveries of higher molecular weight nitrosamines (up to methyloctylnitrosamine) varied from 33% to 73%. The distillate from a meat sample was extracted with dichloromethane and the extract was concentrated in a Kuderna-Danish evaporator. The concentrate was reconcentrated after the addition of n-hexane. The above procedure has also been applied by Telling et al [72], Hill [73] and Epstein [74].

1.2 METHODS OF DETECTION AND ESTIMATION

1.21 Thin-layer chromatography

Owing to its speed, simplicity and moderate sensitivity, thin-layer chromatography (TLC) has been extensively employed for the detection of nitrosamines. Preussmann et al [75, 76] detected nitrosamines by means of a simple procedure which was subsequently adopted by a number of workers who lacked more sensitive equipment. The TLC characteristics of about 60 nitrosamines were determined on silica gel (250 μ)

which had been activated for 1 h at 100°. Dialkylnitrosamines were separated using hexane : ether : dichloromethane :: 10 : 3 : 2, as the mobile phase, while cyclic nitrosamines were separated with a similar mobile phase in the proportions 5 : 7 : 10 respectively. Alkylaryl-nitrosamines and diarylnitrosamines were separated by hexane : ether : dichloromethane :: 10 : 3 : 2. The chromatographed nitrosamines were sprayed with a mixture of 5 parts of 1.5% diphenylamine in ethanol and one part of 0.1% palladium chloride in 0.2% sodium chloride solution. When the sprayed plate was exposed to ultraviolet (UV) irradiation (240 nm) for a few minutes, nitrosamines formed blue-violet spots against a colourless background. An active support, such as silica gel, diatomaceous earth or aluminium oxide, was necessary when this technique was used. Sensitivities of about 1 - 2 μg for volatile nitrosamines and 0.5 μg for the less volatile nitrosamines were claimed. This spray did not, however, detect methylallylnitrosamine or diallylnitrosamine. Amines, amino-acids, secondary carbonyl compounds and phenols did not interfere with the determination. Quinonoid and nitro-compounds produced brown colours, while acids and dicarboxylic acids produced brown colours. Terpenes, unsaturated hydrocarbons, α - β unsaturated carbonyl compounds, alkyl- and phenylbenzoyl derivitives also hindered the detection of nitrosamines.

Chromatographed nitrosamines were also detected by exposing the plate to UV irradiation and subsequently spraying with Griess reagent [77, 78]. The reagent comprised 1 part of 0.1% sulphanilic acid in 30% acetic acid and 1 part of 0.1% α-naphthylamine in 30% acetic acid. Nitrosamines produced red spots a few minutes after spraying, with sensitivities of 0.5 μg for the low molecular weight ones. N-nitroso-

N, O-dimethylhydroxylamine could not be detected when using this method, while any other compound containing a nitroso-group was detected.

Since the interfering compounds were not detected by both of the above methods, the authors concluded that a positive result from both was specific for N-nitroso-compounds. Other authors [27, 37] have found that the above method is not specific for nitrosamines and should only be used as a preliminary test for the presence of nitrosamines. The R_f-values of a number of nitrosamines, separated on silica gel, are given in Table I.

Neurath et al [79], using lithium aluminium hydride, reduced N-nitrosamines to the corresponding hydrazines. Condensation of the hydrazide with 5-nitro-2-hydroxybenzaldehyde produced a yellow hydrazide. The hydrazides were separated by TLC on silica gel G (250μ) plates, which had been activated for 2 h at 100°, with carbon tetrachloride : ethylacetate :: 95 : 5, as mobile phase. Chloroform : methanol :: 95 : 5, was used to separate the hydrazides derivatives of 4-amino-1-methyl-piperazine and 4-amino-morpholine. An alkali spray increased the intensity of the spots so that 0.5 μg of the resulting yellow-brown hydrazide could be detected. The sensitivity was further increased by spraying with potassium hexacyanoferrate, so that 0.05 μg of the blue product could be detected.

Serfontein and Hurter [80] reduced nitrosamines to hydrazines with lithium aluminium hydride. The hydrazines were converted to the 4-nitroazobenzene-4'-carboxylic acid hydrazides according to the method of Neurath and Doerk [81]. The hydrazide derivatives were separated by multiple TLC on silica gel with petroleum ether (b.p. 60 - 80°) : chloroform :: 5 : 9, as the mobile phase. When this method was applied to a neutral extract of cigarette smoke condensate, three hydrazide deriva-

tives of nitrosamines were detected after 5 to 7 consecutive runs. The hydrazides were derived from dimethylnitrosamine, diethylnitrosamine and N-nitrosopiperidine. These tentative results were supported by gas liquid chromatography (GLC) using a single liquid phase.

Tobacco smoke condensate was also examined by Neurath et al [20] using the method of Neurath et al [79] and Neurath and Doerk [81]. Nitrosamines were not detected in normal blend cigarette smoke condensate. but hydrazides corresponding to three nitrosamines were detected in the condensate of tobacco containing a large amount of nitrite and volatile bases. One hydrazide had an R_f-value identical with the hydrazide derivative of methyl-n-butylnitrosamine. The UV and infrared (IR) spectra of a larger sample of the above hydrazide derivative, purified and concentrated by preparative TLC, supported this conclusion. The other hydrazides were not identified. No hydrazides were found in the condensate, but a portion of the nitrosamines present may have been artefacts [10]. It was suggested [10] that oxidation of NO to NO_2 in the trapping apparatus led to the formation of these artefacts. Neurath et al [10] re-examined tobacco smoke condensate using the above method and the method of Neurath and Doerk [81], and established that an equimolar mixture of NO and NO_2 and a secondary amine produced two moles of the corresponding nitrosamine. This reaction was temperature sensitive, dependent on time and required the exclusion of oxygen. Dimethylnitrosamine, N-nitroso-pyrrolidine and methyl-n-butylnitrosamine, which were identified in cigarette smoke condensate, were probably artefacts produced in the trapping system.

Kröller [38] chromatographed extracts of tobacco smoke condensate, cheese and flour on silica gel G with hexane : ether : dichloromethane ::

10 : 4 : 1, as the mobile phase. Nitrosamines were detected by the method of Preussmann et al [75, 76]. Another method of detection required the spraying of the chromatographed plate with 10% hydrochloric acid. After exposure to UV irradiation, the plate was sprayed with 0.2% ethanolic ninhydrin reagent. Coloured spots appeared after the plate had been dried for 1 h at 100°. The detection limit, 0.5 μg of nitrosamine, was the same as for the method of Preussmann et al [75,76]. As ninhydrin reacts with amines, the presence of the latter in an extract will lead to an erroneous result. A combination of the above two methods is not specific for nitrosamines and the above cited identification of N-nitroso-piperidine and di-n-butylnitrosamine in tobacco smoke condensate can only be tentatively accepted.

Schoental and Gibbard [82], employing the method of Preussmann et al [75, 76], failed to detect nitrosamines in Chinese incense smoke.

The coupling of gas chromatography and TLC using the method of Preussmann et al [75, 76] to detect nitrosamines eluted onto the TLC plate, was proposed by Mayrhofer and Möhler [83]. This method has not been adopted by other workers owing to the lack of sensitivity of the TLC detection of nitrosamines.

Preussmann [27] used TLC [75, 76] to investigate the oxidation of twelve nitrosamines. Under the conditions used, Fenton's reagent and alkaline peroxidisulphate did not oxidise nitrosamines. Apart from the arylalkylnitrosamines, the oxidative conditions of Undenfriend et al [84] had an effect on all the nitrosamines investigated. Each nitrosamine formed several unidentified oxidation products with lower R_f-values than the initial materials.

Kadar and Devik [86] showed that Devik [66], when investigating

the formation of N-nitrosamines by the Maillard reaction, had erroneously claimed to have identified nitrosamines after using the diphenylamine-palladium chloride spray technique of Preussmann et al [75, 76]. Heyns and Koch [67, 68] and Heyns et al [69] applied the method of Preussmann et al [75, 76] when duplicating the work of Devik [66]. Four nitrosamines (dimethylnitrosamine, diethylnitrosamine, di-n-propylnitrosamine and di-n-butylnitrosamine), a mixture of pyrazines, acetylfuran, furfuryl alcohol, acetylpyrrole and the products from the Maillard reaction were examined on a silica gel PF using the developing solvent of Devik [66]. Apart from the nitrosamines, no other substances gave a positive (violet) colour.

The unsatisfactory nature of many of the procedures used for the isolation and detection on nitrosamines is exemplified by the results of several workers who examined extracts of flour. Marquardt and Hedler [21] used the method of Preussmann et al [75,76] to detect nitrosamines in an extract of flour. After preparative TLC of the extract, the identity of a spot with an R_f-value corresponding to diethylnitrosamine was corroborated by TLC of the 5-nitro-2-hydroxybenzaldehyde derivative of the spot [79]. The mobile phase was carbon tetrachloride : ethyl acetate :: 95 : 5. A brief report [85] corroborated the presence of diethylnitrosamine in a flour extract [21]. Thewlis [22], using procedures of isolation and detection published by Marquardt and Hedler [21], did not detect any nitrosamines in flour and bread, even after the samples had been exposed to nitrogen oxides. Diethylnitrosamine was tentatively identified in a flour sample which had been spiked with diethylamine and exposed to nitrogen oxides. The sample was heated for 1 h at 70° prior to analysis. The procedure could detect 1 μg

of flour. Hedler and Marquardt [23] used the method of Preussmann et al [75, 76] to examine extracts of wheaten plants, grain and flour as well as milk and cheese. Diethylnitrosamine was not detected in unheated German flour, but an extract of heated flour gave a coloured spot with an R_f-value corresponding to that of diethylnitrosamine. The diethylnitrosamine contained an impurity which was also rendered visible when the above method was used. Each of the flour extracts examined also exhibited a faintly coloured spot with an R_f-value similar to that for the impurity in diethylnitrosamine. The authors implied that the correspondence of TLC spots in the flour extracts with an impurity in the diethylnitrosamine standard was additional evidence for the existence of diethylnitrosamine. Other workers used pure nitrosamines or purified the nitrosamine standards until no interfering spots appeared when TLC was employed. The method of Neurath et al [79] was also used to corroborate the presence of diethylnitrosamine in flour and cheese extracts. When this method was applied, very small amounts of hydrazide were detected by TLC [79]. A spot was obtained which had the same R_f-value as the hydrazide derivative obtained from diethylnitrosamine. Owing to the complexity of the extracts and the possibility of artefact formation during extraction, it is unjustifiable to conclude that the hydrazide was derived from diethylnitrosamine. Flour samples from Thewlis [22] were examined and, in contrast to the reults of Thewlis, Hedler and Marquardt [23] found evidence for diethylnitrosamine in all cases. This difference in results cannot easily be explained in terms of a difference in analytical technique. Thewlis [22] employed a similar method and obtained only slightly less extract from unheated flour samples, so that the concentrations of nitrosamines should have been similar in the flour extracts of

both workers. Thewlis [22] did, however, obtain much more extract from heated flours so that any nitrosamine, which had been extracted, would have been in reduced concentration. Hedler and Marquardt[23] also presented tentative evidence for the occurrence of diethylnitrosamine in wheaten plants, grain, milk and cheese. Interfering spots were produced by fatty acids, present in ripened cheese, when the diphenylamine - palladium chloride spray of Preussmann et al [75, 76] was used. Möhler and Mayrhofer [30] examined extracts of flour, meat and cheese but failed to detect any nitrosamines when the method of Preussmann et al [75, 76] was used. Sen et al [37] initially used the method of Preussmann et al [75, 76] when investigating extracts of wheat, flour cheese, fish and spinach. The nitrosamine standards (1 - 5 μg) and the food extracts (100 - 200 μg) were spotted in duplicate on each plate. The plates were chromatographed in n-hexane : ether : dichloromethane, the proportions of the constituents in the mobile phase being varied according to the nitrosamine standard used [75, 76]. Nitrosamines were detected by developing one half of the chromatographic plate with Griess reagent [77, 78] and the other half with ninhydrin reagent. The R_f-values of several nitrosamines separated on silica gel, are given in Table I. The diphenylamine-palladium chloride spray [75, 76] gave many false positive results for nitrosamines, while Griess reagent [77, 78] and ninhydrin reagent did not detect these interfering compounds. In contrast to N-nitrosamines, organic nitrites, nitroso-compounds and free amines gave positive results with Griess and ninhydrin reagents before the chromatographic plate had been exposed to UV irradiation. The authors were thus usually able to distinguish between N-nitrosamines and interfering compounds when using the latter two spray reagents. They considered this combination to be

more specific for the detection of N-nitroso-compounds. The concentrations of nitrosamines were estimated by comparison of the intensity of the unknown spot with the intensity of a standard spot. The sensitivity of the method was about the same as other TLC methods [75, 76, 79]. Any TLC spot detected by both methods was concentrated and purified by preparative TLC. The detection limit of a particular nitrosamine was dependent on its volatility. While dimethylnitrosamine, when added to different foods, could only be detected at or above 0.15 ppm, diethylnitrosamine and di-n-propylnitrosamine were each detected at levels of 50 ppb when added to different foods. No nitrosamines were detected in spinach, unheated flour, or flour samples which had been heated for 6 h at 150 - 160 °. A few extracts of cheese and herring gave positive results for nitrosamines with Griess reagent [77, 78] but owing to the small amount of sample, no confirmatory tests were possible.

Freimuth and Gläser [48] used the method of Preussmann et al [75,76] as a qualitative identification of nitrosamines in cheese, anchovy paste, cured meat (Kaselar) and Westphalian rye-bread. The samples were separated on silica gel G (Merck) with hexane : ether : dichloromethane :: 10 : 3 : 2 as mobile phase. Preparative TLC was combined with the method of Daiber and Preussmann [49] as a quantitative procedure for estimating nitrosamines. The sensitivity of the TLC method was 40 to 50 µg/ kg.

Sen and Dalpe [87] detected nitrosamines with ninhydrin and N-(1-naphthyl)-ethylenediamine hydrochloride - sulphanilic acid.

Sander [88] was the first to suggest that nitrosamines could be formed in the human stomach if both nitrite and secondary amines were present. Sen et al [37] used their method [45] for the detection of

nitrosamines in the stomach contents of humans, rabbits and rats. Diethylamine and sodium nitrite were incubated with gastric juice and the diethylnitrosamine produced was estimated by previously published methods [37]. The identity of the diethylnitrosamine was confirmed by GLC. The highly acid human and rabbit gastric juices produced more diethylnitrosamine than did the less acid rat gastric juice.

Sander [89] demonstrated that nitrite-reducing bacteria (Proteus vulgaris, Escherichia coli and Serratia marcescens) were capable of synthesizing nitrosamines. Nitrosamines produced from diphenylamine, N-methylaniline and di-n-propylamine were detected with Griess reagent [77, 78], isolated by the method of Preussmann et al [75, 76] and quantitatively determined according to the method of Sander [90]. Nitrosation of secondary amines was greatest at low pH levels, while dialkylamines produced less nitrosamine than arylamines.

Sakshaug et al [91] reported that the hepatoxic effect of a herring meal was due to the high concentration of dimethylnitrosamine in the meal. Dimethylnitrosamine was isolated by Ender et al [50] from herring preserved with sodium nitrite. The nitrosamine was identified by GLC, UV and IR studies. The method of Preussmann et al [75, 76] and the hydrazine method of Ender and Ceh [39] were employed to obtain [50] further evidence for the existence of dimethylnitrosamine in hepatoxic herring meal.

Twenty-three samples of cooked and uncooked fish were examined by Sen et al [46] for nitrosamines, which were detected by the method of Sen et al [37, 45]. Samples of smoked and canned fish, with no added nitrite, did not appear to contain any nitrosamines. A variety of nitrosamines (dimethylnitrosamine, diethylnitrosamine and di-n-propylnitros-

amine) were detected in fish samples which had been spiked with 200 ppm of sodium nitrite. All three nitrosamines were never present in one sample. These results, although supported by GLC, were not entirely satisfactory, since towards the end of the investigation it was found that the formation of artefacts was promoted by the extraction procedure.

Smoked fish and meat, cheese and mushrooms were examined by Ender and Ceh [39]. The two qualitative methods of Neurath et al [20, 79] and two quantitative methods, the hydrazine method [39] and the method of Preussmann et al [75, 76] were used. The authors claimed to have detected nitrosamines in samples of smoked fish, smoked meat and mushrooms. No nitrosamines were identified in cheese. It was stated that the nitrosamines isolated were chemically related to dimethylnitrosamine, but it is uncertain whether the qualitative and quantitative methods were all applied to each sample, and further, the authors stress that the extractive procedure may have led to the formation of artefacts.

Stored spinach may develop a high concentration of nitrite [92]. Keybets et al [35], using the method of Preussmann et al [75, 76], examined spinach for the presence of nitrosamines which would be formed by the reaction of secondary amines and nitrite. The R_{ST} values (where $R_{ST} = X$), which were more reliable than R_f-values, were used for characterisation of nitrosamines.

$$X = \frac{\text{distance between unknown spot and start}}{\text{distance between standard spot and start}}$$

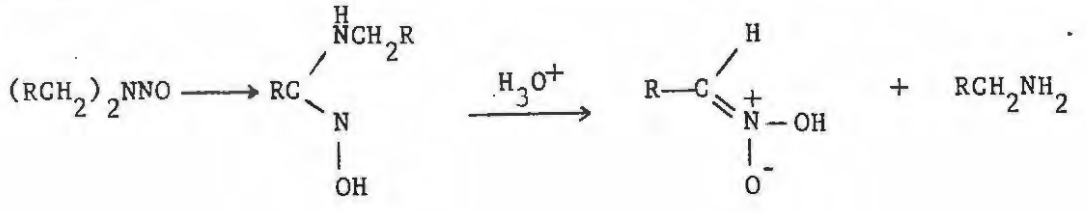
Although the technique of isolation resulted in a 70 - 80% loss of dimethylnitrosamine and a 30 - 40% loss of diethylnitrosamine, the method was claimed to be sensitive to 0.5 ppm dimethylnitrosamine and 0.1 ppm diethylnitrosamine which had been added to spinach. Nitrosamine were

not detected in stored spinach samples. Diethylnitrosamine was detected in nitrite-bearing spinach (pH 3.1) which had been stored for a week after diethylamine had been added. Other spinach samples spiked with nitrite and diethylamine also contained diethylnitrosamine. It appeared that nitrosamine was only produced under severe conditions since a one hundred-fold excess of diethylamine produced less than one percent of the theoretically predicted amount of diethylnitrosamine.

African alcoholic spirits, often produced under unusual conditions, was stated to contain nitrosamines [93], identification being effected by TLC. The fruit of Solanum incanum, used to curdle milk by herdboys in the Transkei, was shown to contain dimethylnitrosamine [19]. The method of Preussmann et al [75, 76] was used to detect the nitrosamine zone on TLC plates. This zone was concentrated by preparative TLC, dry column chromatography [53] and preparative GLC. Final identification was effected by GLC, nuclear magnetic resonance (NMR) and IR spectroscopy.

The transplacental passage of diethylnitrosamine after intracardial injection (10 - 30 mg) was studied by Mohr et al [94] who used the method of Preussmann et al [75, 76].

Neuenhofer et al [26] determined the hydroxamic acids derived from the fermentation of four nitrosamines by liver or kidney homogenates. Dimethylnitrosamine, diethylnitrosamine, di-n-butylnitrosamine and N-nitrosohexamethyleneimine were estimated by the method of Preussmann et al [75, 76]. The nitrosamines were first rearranged to the corresponding amidoxime which was rapidly hydrolyzed to an amine and a hydroxamic acid.



After reaction, the samples were chromatographed on cellulose with ether and butanol : acetic acid : water :: 5 : 1 : 4, as mobile phases, the hydroxamic acid zone being revealed by spraying with ferric chloride solution, and identified by comparison with the R_f- value of an authentic sample.

A quantitative investigation of the thin-layer chromatography of dimethylnitrosamine, diethylnitrosamine and diamylnitrosamine was carried out by Eisenbrand et al [71]. Estimations were by polarography or UV spectrophotometry. When solutions of nitrosamines were hand-spotted losses were comparable with those obtained by automatic streaking on TLC plates. Estimations were effected after steam-distillation of the adsorbant (silica gel GF_{254} and PF_{254}), as nitrosamines were almost quantitatively removed from silica gel by steam distillation (minimum recovery was 96.5%). The results were more reproducible at 4°, when 76% of the dimethylnitrosamine and 89.5% of the diethylnitrosamine were recovered. Dry dichloromethane was found to be the best solvent. When the thickness of the adsorbant was increased to 0.6 mm and the plates were chromatographed at 4° in the dark, the results were more reproducible and the maximum loss of nitrosamines was 10%. Eisenbrand [43] applied these conditions to the separation of nitrosamine zones from wheat flour by preparative TLC.

Hedler et al [36] examined the components of a soyabean oil extract by TLC, on silica gel PF, with hexane : ethyl acetate : dichloromethane :: 5 : 2 : 2 as mobile phase. R_f-values of nitrosamine standards chromatographed under these conditions are given in Table 11. A GLC analysis revealed that the components were not successfully separated when preparative TLC was used. Serious losses of nitrosamines occurred

when spiked extracts were chromatographed by TLC. When 5 μg of each of dimethylnitrosamine, di-n-propylnitrosamine and di-n-butylnitrosamine were chromatographed the recoveries were 84%, 64% and 59% respectively.

1.22 Liquid chromatography

Parris [94] separated (during one minute) a cyclohexane solution of dibutylnitrosamine, nitrosopyrrolidine and nitrosopiperidine on a column packed with Ether Permaphase with heptane (flow: 1.5 ml/min.) as the mobile phase. The nitrosamines were detected by UV but the response of cyclohexane interfered with the detection of dibutylnitrosamine. When the column was packed with uncoated Zipax all three nitrosamines were separated, by weak adsorption, from the solvent front. The separation required about 10 minutes. Twenty nanograms of each compound could be detected.

1.23 Gel filtration chromatography

Eisenbrand et al [24] applied gel filtration chromatography (Sephadex LH-20, column eluted with methanol : water :: 1 : 1 4.3 ml/ h) to the separation of nine dialkylnitrosamines. Under the conditions used, six symmetrical dialkylnitrosamines were separated over a span of 240 min and three methylalkylnitrosamines were separated during 120 min. Detection was by UV absorption at 230 - 235 nm. When the method was applied to an extract of wheat flour, UV-absorbing contaminants masked the elution range in which nitrosamines were expected.

1.24 Gas liquid chromatography - Mass spectrometry

Although GLC is a very powerful technique for the separation of mixtures, great care should be exercised when attempting to use

it as a method for the identification of the individual components of such a mixture. Comparison of the retention times of an unknown with those of a standard on three liquid phases, preferably differing in polarities, is often accepted as proof of the identity of the unknown with the standard. Even if this routine is followed, the "identification" of the unknown can only be regarded as tentative until an absolute method has been applied to the problem. There are several reports of the "identification" of nitrosamines using the retention time on a single liquid phase compared with a standard. Until more rigorous methods are applied such results must remain purely tentative. The most satisfactory method for the identification of nitrosamines in the concentrations in which they appear to occur (usually ppb), is provided by gas chromatograph - mass spectrometer linked systems (GLC-MS). These conclusions are evident from the results reviewed in this section.

Serfontein and Hurter [16] separated synthetic mixtures of dimethylnitrosamine, diethylnitrosamine and di-n-propylnitrosamine and investigated dichloromethane extracts of cigarette smoke condensate. The authors found a number of peaks, one of which had a retention time corresponding to that of N-nitrosopiperidine. The column (150 in X $^{5}/_{16}$ in copper spiral tubing) was packed with 10% polyethylene glycol 4000 on Celite (acid washed) 35 - 65 mesh and was maintained at 100°. The injection block temperature was 110° and the detector temperature was 105°. The nitrogen carrier gas flow was 20 ml/ min at an inlet pressure of 1.5 atm. The gas chromatograph was fitted with a flame ionization detector (FID).

The fallibility of single-column gas chromatographic identifications of nitrosamines was demonstrated by Kadar and Devik [86], Heyns

and Koch [67, 68], Heyns et al [69], Williams et al [64] and Scanlan and Libbey [70]. Devik [66] had claimed to have identified diethylnitrosamine in roasted potato starch spiked with D-glucose and one of three amino-acids. It was subsequently found [86, 64, 67 - 70] that pyrazines, which may occur in heated food [96], could be confused with nitrosamines. Kadar and Devik [86] recorded the retention times (relative to dimethylnitrosamine) of a number of nitrosamines and pyrazines on a polyethylene glycol adipate column (25% on diatomite, 60 - 70 mesh) maintained at 150°. Several pyrazines had retention times almost identical with those of nitrosamines.

Williams et al [64] also demonstrated the inadequacy of simple polarographic and GLC methods when applied to the detection of nitrosamines in alcoholic beverages. Gas chromatography was carried out on an F & M 810 gas chromatograph fitted with an FID and a 12 ft X 1/8 in copper column packed with 10% Carbowax 20 M on 60 - 80 mesh Chromosorb W and programmed at 8°/ min from 65 - 210° with a carrier flow (nitrogen) of 40 ml/ min. Preparative GLC was accomplished on an instrument constructed in the laboratory [97] and fitted with specially designed traps [98]. The column (20 ft X 3/4 in) was packed with 5% Carbowax 20 M and programmed at 4°/ min from 100 - 210° with a nitrogen carrier flow of 500 ml/ min. Dimethylnitrosamine and diethylnitrosamine were separated satisfactorily and the detection limits were 0.5 ppm. A trace component with a retention time close to that of dimethylnitrosamine was detected (GLC) in potable spirit before it had been concentrated. This corroborated a preliminary polarographic examination. Under the GLC conditions used, the retention time of hexanol was very close to that of dimethylnitrosamine. A further trace component was found to have a

retention time close to that of diethylnitrosamine.

GLC-MS was used to examine the gas chromatographic peaks attributed to dimethylnitrosamine and diethylnitrosamine. An LKB 9000 mass spectrometer (equipped for GLC-MS) was fitted with a column which was identical with that used for analytical GLC. The chromatographic parameters varied for different analyses. A typical example used temperature-programming (6°/ min) from 65 - 210° with a helium carrier gas flow of 30 ml/ min. The mass spectra of dimethylnitrosamine and diethylnitrosamine agreed with the published spectra of Collin [99]. Dimethylnitrosamine and diethylnitrosamine were not detected in the distillate or any of the extracts by GLC-MS and no higher aliphatic nitrosamines were detected. The components detected in the unconcentrated spirit, with retention times close to those of dimethylnitrosamine and diethylnitrosamine, were confirmed to be hexanol and ethyl octanoate respectively.

When the GLC effluent was continuously monitored for ions 74 and 102 (the parent ions of dimethylnitrosamine and diethylnitrosamine respectively) small peaks occurred at 74 and 102 when hexanol and ethyl octanoate respectively were eluted. An extract which apparently contained nitrosamines (from polarographic analysis) was split into eleven fractions by preparative GLC. When these fractions were re-examined polarographically only the sixth fraction ostensibly contained nitrosamine. A GLC-MS analysis of this fraction showed that the polarographic activity was due to furfural. This conclusion was confirmed by UV spectroscopy.

Heyns and Röper [100, 101] and Heyns et al [69] developed a specific procedure for nitrosamine analysis using GLC-MS. The gas chromatograph (Perkin-Elmer F6), fitted to a mass spectrometer (Atlas CH4), was

equipped with two capillary columns, a 25 m PDEGS-column and a 25 m "R-column", containing Ucon LB 550X, treated with potassium hydroxide. The efficiencies of the two columns were tested before the GLC was linked to the mass spectrometer. Two mixtures of 18 dialkylnitrosamines and 7 cyclic nitrosamines and arylalkylnitrosamines and higher molecular weight dialkylnitrosamines were separated ("R-" and PDEGS-columns respectively) under isothermal and programmed conditions. Temperature-programming was used to achieve a more satisfactory separation. Under isothermal conditions, the operating parameters of the two columns, apart from temperatures (160 and 180° respectively), were identical, with nitrogen inlet pressures of 0.6 kp/ cm^2, flows of 1.7 ml/ min and split ratios of 1 : 50. The same procedure was used for temperature-programmed separation ("R-column", 80 - 160° at 5°/ min, isothermal for 104 min; PDEGS-column, 150 - 180° at 5°/ min, isothermal for 24 min). Nitrogen was at an inlet pressure of 0.4 kp/ cm^2 with a flow of 1 ml/ min and a split ratio of 1 : 50. The retention times of the eighteen nitrosamines varied from 7 - 95 min when the GLC ("R-column") was coupled to the mass spectrometer. The column was temperature-programmed from 65 - 140° in 15 min and the run was continued while the column was cooled to 100° over 33 min, after which it was heated to 160° in 12 min and the final 60 min of the run was carried out under isothermal conditions. The helium inlet pressure was 0.43 kp/ cm^2, the flow was 1 ml/ min and the split ratio was 1 : 50. The seven nitrosamines were separated during 40 min on the PDEGS-column attached to the mass spectrometer, with the column temperature being programmed from 80 - 180° at 5°/ min with a final 20 min run under isothermal conditions. The mass spectrometer was operated with an electron energy of 70 eV. These conditions gave satisfactory separations of nitrosamine

mixtures with a gas chromatographic detection limit of 0.2 to 0.5 μg / nitrosamine, while the detection limit for mass spectrometry was 0.01 μg / nitrosamine.

Heyns and Koch [67, 68] and Heyns et al [69] used the above procedure to investigate the Maillard reaction between glucose and three amino-acids, previously studied by Devik [66]. Dichloromethane extracts of the volatile Maillard products, when examined by GLC-MS, did not contain any nitrosamines, although the GLC detection limit for dimethylnitrosamine was 0.07 μg. Pyrazines, pyrroles and furanes were identified.

Scanlan and Libbey [70] also investigated products from the Maillard reaction. They employed a similar procedure to that used by Heyns and Koch [67, 68]. GLC analyses were performed on a stainless steel column (12 ft X 0.085 in ID) packed with 2.5% butanediol succinate on Chromosorb G (acid-washed, silanised) 100 - 120 mesh with a flow rate of 25 ml/ min of helium (at 100°). The column temperature was initially held at 90° for 10 min and was then programmed at 4°/ min to 175°. The injector temperature was 200° and the FID temperature was 210°. The GLC was linked to a mass spectrometer. Numerous mass spectra recorded (70eV) at the known retention times of dimethylnitrosamine, diethylnitrosamine and di-n-butylnitrosamine did not reveal the presence of these compounds. Many pyrazines, found [96] to occur in heated foods, were recorded.

Saxby [102] successfully separated pyrazines from nitrosamines by means of a precolumn reactor. The teflon precolumn (10 cm X 3 mm OD) contained 10% cuprous thiocyanate which formed a stable pyrazine complex. Four pyrazines (pyrazine, methylpyrazine, 2,5-dimethylpyrazine and 2,6-dimethylpyrazine) and two nitrosamines (methylethylnitrosamine and di-n-propylnitrosamine) were separated on a teflon column (2 m X 3 mm OD)

packed with 10% diethylene glycol adipate on Chromosorb W (acid-washed, silanized) 80 - 100 mesh with nitrogen as carrier gas (flow : 35 ml/min). The column was temperature-programmed from 50 to 180° at 5°/ min. Under these conditions the retention times of methylethylnitrosamine and 2,5-dimethylpyrazine were identical. The precolumn selectively removed the four pyrazines.

Foreman et al [28] employed a GLC analysis of simple dialkylnitrosamines which does not seem to have yet been adopted by other workers in the field. The steam-distillate of corned beef, spiked with 1 ppm of each of dimethylnitrosamine and diethylnitrosamine, was directly analyzed on a column packed with water-stable microporous polymer beads (Chromsorb 101). The recoveries of dimethylnitrosamine and diethylnitrosamine from the corned beef were 95 and 97% respectively. Six nitrosamines (dimethylnitrosamine, methylethylnitrosamine, diethylnitrosamine, di-n-propylnitrosamine, methylhydroxyethylnitrosamine and di-n-butylnitrosamine) were separated on the above column. A lower limit of detection of 10 µg per 10 µl injection was obtained. A Varian Aerograph 1200 gas chromatograph, equipped with an FID, was fitted with a 6 ft X 1/8 in OD stainless steel column packed with Chromosorb 101 (oven temperature, 200°; carrier gas flow rate, 25 ml/ min).

The presence of dimethylnitrosamine and diethylnitrosamine in meat, cheese and flour was investigated by Möhler and Mayrhofer [30] using a Beckman GC 2A gas chromatograph equipped with a thermal conductivity detector (TCD). The column, 1.8 m long, contained a DEGS-impregnated packing maintained at 160°.

Kröller [38] examined cheese, flour and cigarette smoke extracts using a Perkin-Elmer 116 chromatograph equipped with a TCD. The columns

(2 m x 3 mm ID), connected in tandem, were packed with 5% Silicone Rubber SE 32 and 15% Silicone DCO, both on Celite 545 60 - 100 mesh. The column temperature was 200°. The helium carrier gas (inlet pressure 2.5 atm) had a flow of 36 ml/ min. Retention times were recorded for 1 µl injections of a 0.5% n-hexane solution of diethylnitrosamine, di-n-propylnitrosamine, di-isopropylnitrosamine, di-n-butylnitrosamine, di-isobutylnitrosamine and di-n-pentylnitrosamine. The retention times were excessively long, varying from 315 sec for dimethylnitrosamine to 1013 sec for di-n-pentylnitrosamine. The above nitrosamines were also examined on a Perkin-Elmer F20 gas chromatograph equipped with an FID. The nitrosamines were separated on a glass column (2 m X 2.7 mm ID) packed with 1% Silicone Gum SE 30 on acid-washed DMCS-treated Chromosorb G 80 - 100 mesh with nitrogen as carrier gas. The oven and injector temperatures were 165 and 260° respectively. The inlet pressures for nitrogen, hydrogen and oxygen were 1.5, 2.0 and 1.5 atm respectively. The retention times of the nitrosamine standards were very short, varying from 45 sec for dimethylnitrosamine to di-n-pentylnitrosamine. The author stated that both gas chromatographic procedures confirmed the presence of diethylnitrosamine in flour. Di-n-propylnitrosamine and di-isopropylnitrosamine were identified in commercial Dutch cheeses but no nitrosamines were detected in cheeses prepared from potassium nitrate and boiled milk.

Sen et al [46] examined the formation of nitrosamines in nitrite-treated fish. The various fish extracts (smoked haddock, cod, hake, mackeral, canned mackeral and salmon) were examined on two 5 ft X 1/8 in stainless steel GLC columns. The first column, packed with 6% Reoplex 400 on Chromosorb W 60 - 80 mesh, was operated at 105° with a nitrogen

carrier gas flow of 27 ml/ min. The second column, packed with 10% Carbowax 20 M on Chromosorb W (HMDS-treated) 60 - 80 mesh, was operated at 103 - 105° with a nitrogen carrier gas flow of 26 ml/ min. No nitrosamines were detected in control samples not treated with nitrite, but nitrosamines were apparently detected in samples which were treated with nitrite (50 - 200 ppm). Dimethylnitrosamine, diethylnitrosamine and di-n-propylnitrosamine were apparently detected. These authors also examined a dichloromethane extract of steam-distilled fish samples and subsequently found that nitrosamines could be formed from nitrite and excess secondary amines even under strongly alkaline conditions[57]. Steam-distillation was abandoned and three fish samples were extracted with dichloromethane. These extracts still apparently contained dimethylnitrosamine although two of them at reduced concentration.

When Petrowitz [85] analyzed an ethereal extract of flour [21] he identified diethylnitrosamine as the product obtained from preparative TLC of the extract. The nitrosamine was identified on three different columns at two to three different temperatures (60 to 150°). The columns varied from 2 ft to 6 ft in length and were packed with Silicone Gum Rubber, Carbowax 20 M and Apiezon L. Diethylnitrosamine tailed on Silicone Gum Rubber and Apiezon L. The limit of detection was about 50 μg of diethylnitrosamine on a 6 ft Carbowax 20 M column at 150°. There was a linear relationship between peak area and the amount of diethylnitrosamine from 50 - 500 μg . The F and M gas chromatograph, model 500, fitted with a TCD maintained at 100°, had a helium carrier gas flow of 100 ml/ min.

Neurath et al [10] published important quantitative gas chromatographic determinations of nitrosamines, and they investigated methods of

trapping and extracting nitrosamines from cigarette smoke condensate. They estimated the losses of nitrosamines on evaporation of solutions of 10 mg dimethylnitrosamine and diethylnitrosamine in 1 1 to 3 1 n-pentane dichloromethane and mixtures of these two solvents. The solutions were concentrated at atmospheric pressure to 30 ml, and then made up to a volume of 50 ml with dichloromethane. When n-pentane was used as solvent, the losses of dimethylnitrosamine and diethylnitrosamine were high (90 - 95%). These high losses were strikingly reduced when some dichloromethane was present in the n-pentane. The lowest losses (less than 5%) occurred when dichloromethane was used as solvent. Distribution constants were determined by gas chromatography for a 1 : 1 mixture (10 ml of each phase) of an organic phase and an aqueous solution of 25 µg of each of six nitrosamines (dimethylnitrosamine, methylethylnitrosamine, diethylnitrosamine, di-n-propylnitrosamine, methylbutylnitrosamine and di-n-butylnitrosamine). The amount of nitrosamine in the organic phase (n-pentane and dichloromethane) increased on ascending the homologous series (distribution constant, K_D, of dimethylnitrosamine for pentane - water is =.33, K_D for diethylnitrosamine is 0.5 and K_D for di-n-butylnitrosamine is 99.0). K_D also increased when water was saturated with sodium chloride. The highest K_D-value for nitrosamines in an organic phase was achieved for dichloromethane - water saturated with sodium chloride, when K_D for dimethylnitrosamine was 10.0, K_D for diethylnitrosamine was 39.0 and K_D for di-n-propylnitrosamine was 99.0. Other distribution constants were determined for one or more nitrosamines in cyclohexane - 90% methanol, cyclohexane - methylnitrite, dichloromethane - 1% HCl, ether - water and ether - water saturated with sodium chloride. The reaction between dimethylamine and nitrogen oxides

was found to produce dimethylnitrosamine when equimolar amounts of NO and NO_2 were present. No methylnitrite was detected. When methanol was also present some methylnitrite was formed. Methyl-n-butylnitrosamine was detected in solvent traps (n-pentane or dichloromethane) containing 200 mg/ litre of methylbutylamine exposed to nitrogen oxides. The amount of methyl-n-butylnitrosamine formed was dependent on the temperature. Methylnitrite was only formed when very high concentrations of the amine and NO_2 were used. A Perkin-Elmer F6 Fractometer, fitted with an FID, was used in these analyses. The 1 m column, with helium as carrier gas, was packed with Marlophene on Teflon. Dimethylnitrosamine and methyl-n-butylnitrosamine were estimated under isothermal conditions (160°) while dimethylnitrosamine, formed in the equilibrium reaction between dimethylamine and nitrogen oxides, was estimated by temperature-programmed GLC using an initial temperature of 40° for 2 min and programming at 30°/ min to 160°.

Sen [103] and Althorpe et al [104] developed procedures for the GLC determination of nitramines produced from the corresponding nitrosamines. Sen [103] used a Varian Aerograph Model 1200 gas chromatograph equipped with a tritium electron capture detector (ECD). The 6 ft X $^1/8$ in stainless steel column, packed with 10% Carbowax 20 M on Chromosorb W (HMDS-treated) 60 - 80 mesh, was kept at 152°, with the injector at 225° and the detector at 210°. The nitrogen carrier flow was 24 ml/ min. The nitrosamine was converted to the corresponding nitramine by a modified procedure of Emmons and Ferris [105]. The conversion of dimethylnitrosamine to dimethylnitramine was dependent on the conditions used, but was reproducible when the conditions were controlled. When the reaction was allowed to proceed for 24 h about 60% of the theoretical

yield was obtained, while the yield was about 50% for a reaction proceeding overnight at room temperaure. The relative amount of nitramine obtained did not vary when the sample size was diminished from 5 μg to 1 μg nitrosamine. About 8 pg of dimethylnitramine (corresponding to 16 pg dimethylnitrosamine) could be detected and there was a linear relationship between the peak height of dimethylnitramine and the amount present. The linear relationship did not hold for amounts of dimethylnitramine greater than about 100 pg. This method was used to confirm the identity of dimethylnitrosamine isolated from smoked hake [46].

Althorpe et al [104] used a procedure similar to the above [103] to prepare nitramine derivatives of nitrosamines. A reaction time of 3.5 h was used. A 5 ft PEG 20 M column, operated at 140°, was used for separating nitrosamines and nitramine derivatives. The efficiency of the conversion reaction was 72 - 85% for all the nitrosamines tested except N-nitrosopiperidine, where only 25% of the nitramine was obtained. The retention time of the nitramine was generally 2 to 2.5 times that of the corresponding nitrosamine (e.g. dimethylnitrosamine and dimethylnitramine retention times were 4.0 and 9.8 min respectively; di-n-propylnitrosamine and di-n-propylamine 9.5 and 20.5 min). The response (using peak height and peak area) was determined for nitrosamines by flame ionization detection and the response of the corresponding nitramine was measured by ECD. Increases in sensitivity varied from 65 times (peak height) for the nitramine of di-isopropylnitrosamine to 545 times for the nitramine of dimethylnitrosamine.

Castegnaro et al [106] modified the procedure of Sen [103] by using methyl ethyl ketone peroxide to oxidize nitrosamines to nitramines. The nitramine was estimated on a Pye 104 gas chromatograph fitted with an

ECD detector and a 20 ft X 1/8 in stainless steel column, packed with 10% FFAP on Chromosorb W (DMCS-treated). The column was operated isothermally for 45 min, and was then temperature-programmed at 10°/ min to 200° so that dimethylphthalate, a high boiling contaminant originating from the procedure of formation of the nitramine, was eluted.

Preparative GLC was used by Du Plessis et al (52) who identified dimethylnitrosamine in an extract of Solanum incanum. A Beckman GC-4 gas chromatograph, fitted with dual FID's and a stream splitter, was equipped with dual glass columns 1.19 m X 12.5 mm ID, packed with 15% Carbowax 1000 on Gas Chrom P 80 - 100 mesh. The oven temperature was 100° and the nitrogen carrier gas flow rate was 115 ml/ min. Under these conditions dimethylnitrosamine had a retention time of 4.9 min. A second separation of the solanaceous extract on polyethylene glycol succinate and gas chromatography of the neutralized extract supported the previous conclusion. The extract was then chromatographed on the Carbowax columns and the fraction with retention time of 4.9 min was collected in a porous plug (107) of deuterochloroform. The identity of the trapped nitrosamine was confirmed by NMR and IR.

An unusual preparative method was proposed by Mayrhofer and Möhler (83), who observed that nitrosamines emerging from the effluent pipe of a gas chromatograph could be adsorbed on activated silica gel plates, which should then be developed according to the method of Preussmann et al (75, 76). The detection limit of dimethylnitrosamine and diethylnitrosamine by GLC was 5 µg nitrosamine in 0.05 ml dichloromethane. The amount of nitrosamine adsorbed on the silica gel plates was about 60 - 70%. The authors used the above method when analyzing 10 µg of a nitrosamine mixture in 1 ml of solvent. A Beckman GC 2A

gas chromatograph was equipped with TCD and fitted with columns 1.8m long, packed with DEGS, at an oven temperature of 160° and a hydrogen pressure of 15 psi.

Johnson et al [17] used a selective nitrogen detector to investigate the presence of nitrosamines in cigarette-smoke condensate. Even with a selective nitrogen detector, the complex extract produced numerous interfering peaks. They mentioned having developed a detector selective for amine compounds. This amine detector, based on the Coulson Liquid Conductivity nitrogen detector (Tracor Inc.), was described by Rhoades and Johnson [18]. In the normal mode of operation the detector reduces organic nitrogen to ammonia which is absorbed in a small continuous flow of deionized water. The variation in conductivity of the deionized water is detected. The detector was modified so that amine-type compounds were selectively pyrolyzed to ammonia. Using an inert argon carrier gas, the operating parameters of the standard were altered so that other types of organic nitrogen compounds produced little or no ammonia. When cigarette tar, dissolved in dichloromethane, was spiked with dimethylnitrosamine, diethylnitrosamine and methyl-n-butylnitrosamine, the nitrosamines appeared as sharp peaks against a fluctuating background. With the detector operating in the normal mode, a great number of interfering peaks appeared, which masked the nitrosamines. The sensitivity of the detector permitted the analysis of a few nanograms of nitrosamine. The chromatographic column, 4 ft long, was packed with 10% Carbowax 1540 on Gas Chrom Q 80 - 100 mesh and temperature-programmed from 70 - 150° at 5°/ min. The argon carrier gas was saturated with water at room temperature to prevent tailing of the dimethylnitrosamine.

Sen [47] detected nitrosamines with the Coulson Liquid Conductivity nitrogen detector and noting that other nitrogen compounds also responded to the detector, was of the opinion that other methods should be used to confirm the presence of nitrosamines. The sensitivity was fair, as 0.02 ppm of each of diethylnitrosamine. di-n-propylnitrosamine and N-nitrosopyrrolidine could be detected in a 2 µl sample. A meat extract was analyzed with the above detector which was maintained at 500°. The analysis took place on a Micro Tek MT 2000 gas chromatograph fitted with 4 ft X 1/4 in U-shaped glass columns packed with 3% Carbowax 20 M on Gas Chrom Q, 80 - 100 mesh. The temperature of the column was maintained at 50° for 3 min and was then temperature-programmed at 15°/ min to 155°. The helium carrier gas had a flow of 60 ml/ min and the injector was maintained at 200°. A Varian Aerograph (model 2740) gas chromatograph fitted with an FID and a 6 ft X 1/8 in stainless steel column packed with 10% Carbowax 20 M on Chromosorb W, 60 - 80 mesh (HMDS-treated) was also used to detect nitrosamines in a meat extract. The column temperature was programmed from 150 - 170° at 15°/ min and the flow rate of the nitrogen carrier gas was 25 ml/ min. The detector and injector were both at 215°. Dimethylnitrosamine, isolated from uncooked salami, was detected on this column with the temperature set at 105°. Dimethylnitrosamine, isolated from a smoked dried sausage, was converted to the corresponding nitramine [103] and was detected by a tritium ECD on the same column with the temperature set at 155°. Nitramines were also determined (ECD) on a 5 ft X 1/8 in stainless steel column packed with 6% Reoplex 400 on Chromosorb W, 60 - 80 mesh. Other GLC parameters were identical to those above [47].

Howard et al [57] developed a procedure with a sensitivity of 10 ppb for estimating nitrosamines. A 2.74 m X 4 mm ID coiled glass column, packed with 10% Carbowax 1540 + 3% KOH on Gas Chrom P 100 - 120 mesh, was fitted to a Barber-Colman model 5000 gas chromatograph. The injector was kept at 185° and the detector at 220°. The argon carrier gas, hydrogen and air were maintained at 43 ml/ min, 40 ml/ min and 300 ml/ min respectively. The column was temperature-programmed from 80° (initial time of 3 min) to 120° at 10°/ min. A modified thermionic detector was also fitted. This consisted of a platinum-iridium coil, coated with KCl, fitted over the jet tip of the detector. With this modification 4 ng of dimethylnitrosamine could be detected. A recovery (corrected for apparent dimethylnitrosamine present) of 70 - 80% was obtained when smoked nitrite-treated chub samples were spiked with 10 ppb dimethylnitrosamine. The dimethylnitrosamine was not bound to protein in uncooked chub since it could be successfully recovered from a spiked sample which had stood overnight. Analysis of smoked nitrite-treated chub showed an apparent dimethylnitrosamine content of 1 - 2 ppb. The same amount of dimethylnitrosamine was detected in chub without the nitrite treatment. Under alkaline conditions neither dimethylamine hydrochloride nor trimethylamine hydrochloride reacted with nitrite. These results conflict with those of Sen et al [46]. Three fish samples were spiked with trimethylamine hydrochloride (300, 700 and 1500 ppm) and 2500 ppm sodium nitrite. Having been smoked, the samples were extracted in the usual way and the apparent dimethylnitrosamine values were 3 - 5 ppb. These authors record that they could obtain a mass spectrum of dimethylnitrosamine by analysis of a solution containing 10 ppb or higher in a

gas chromatograph linked to a mass spectrometer. Since all the amounts of apparent dimethylnitrosamine were below 10 ppb its identity was not confirmed.

Fazio et al [59] modified the procedure of Howard et al [57] to study raw, smoked and nitrite - and/ or nitrate-treated smoked fish. A Barber-Colman 5000 Series Selectra gas chromatograph, modified with a KCl thermionic detector [57] was fitted with a 2.74 m X 4 mm ID coiled glass column packed with 10% Carbowax 1540 on Gas Chrom Q 100 - 120 mesh. Using the same operating parameters previously described [57], the retention time of dimethylnitrosamine was 14.5 min. A tandem GLC-MS arrangement, adapted from Damico and Barron [108], was equipped with a three-way valve so that the solvent could be vented before it entered the mass spectrometer. The gas chromatograph fitted to the mass spectrometer was equipped with a 9 ft X $^{1}/_{8}$ in glass coiled column, having packing and operating parameters the same as those described above. An identifiable mass spectrum could be obtained from 50 ng of dimethylnitrosamine. In the fish samples examined, the identity of the gas chromatographic peak corresponding to dimethylnitrosamine was confirmed by mass spectrometry.

Fiddler et al [60] estimated dimethylnitrosamine in ham using a procedure with a sensitivity of 25 ppb. A Varian Aerograph model 1740-1 gas chromatograph was equipped with a standard FID and an alkali flame ionization detector (AFID). Hydrogen flows were 40 and 63 ml/ min respectively and air flows were 400 and 240 ml/ min respectively. Dual stainless steel columns (9 ft X $^{1}/_{8}$ in), packed with 15% Carbowax 20 M-TPA on Gas Chrom P 80 - 100 mesh, were installed. The oven temperature was 115° and the helium carrier flow rate was 47 ml/ min. Injector port and detector temperatures were 190 and 205° respectively. The reproduci-

bility characteristics of the AFID were erratic and operating conditions, (especially hydrogen flow) critically affected sensitivity. During the course of the day sensitivity of the detector declined by 20%. The sensitivity could be improved by allowing the detector to cool overnight and manipulating hydrogen and air flows. The internal standard method was used to estimate dimethylnitrosamine. 2,3,5,6-Tetramethylpyrazine was selected as an internal standard since it had a favourable retention time and responded to variations in detector sensitivity in an analogous manner to dimethylnitrosamine. Although halogenated hydrocarbons tail when used with an AFID, dichloromethane was used in the assay since its use facilitated the clean-up and concentration of the sample. An internal-external standard ratio procedure was used to overcome the variations in sensitivity of the AFID. An aliquot of internal standard was added to the ham extract and the standard solution. The peak height of dimethylnitrosamine in the ham sample was normalized by dividing it by the result obtained from multiplying the dimethylnitrosamine to 2,3,5,6-tetramethylpyrazine ratio in the standard solution by the peak height of 2,3,5,6-tetramethylpyrazine in the ham sample. The AFID response was linear over a range of 4 - 80 ng dimethylnitrosamine. Ten hams were studied and the chromatograms of all the sample extracts contained either no peaks or very small peaks with retention times in the general area of dimethylnitrosamine. These peaks were not identified but if they corresponded to dimethylnitrosamine the levels in the samples were less than 1 ppb. This procedure could not be directly applied to foodstuffs that were likely to contain pyrazines since these compounds could distort the peak heights of the internal standard.

Pensabene et al [109] reported the GLC retention times and mass

spectral data for 25 nitrosamines. A Perkin-Elmer 800 gas chromatograph was equipped with dual stainless steel columns 10 ft X $^{1}/8$ in OD and flame ionization detectors. The first column, packed with 5% Carbowax 20 M-TPA on Sopelcoport 100-120 mesh, was used with a helium flow rate of 60 ml/ min. Hydrogen and air flows were 24 and 430 ml/ min respectively. The column was isothermally operated for 5 min at 100° and was then temperature-programmed to 185° at 5°/ min. The second column, packed with 5% OV-1 on Anakrom ABS 35 - 50 mesh, was used with a helium flow rate of 118 ml/ min. The hydrogen and air flow rates were 40 and 430 ml/ min respectively. The column was temperature-programmed from 70° to 185° at 5°/ min. Methyl esters of straight chain acids (C_6 to C_{16}), with even numbers of carbon atoms, were also chromatographed to indicate the retention times of nitrosamines under different conditions. Mass spectra were recorded on a Du Pont 21-492 mass spectrometer at an ionizing voltage of 70 eV and an ion source temperature of 210°. The mass spectra generally agreed with the published spectra of Collin [99] and Schroll et al [110]. The characteristic fragmentation patterns of several cyclic nitrosamines have been recorded by ApSimon and Cooney [111].

Hedler et al [36] examined an extract of soyabean oil on a Hewlet Packard nitrogen detector (detector temperature of 400°). The separation was carried out on a column packed with Carbowax 20 M on Chromosorb, using helium as carrier gas, and temperature-programmed from 70 - 160° at 10°/ min. Under these conditions dimethylnitrosamine, diethylnitrosamine, di-n-propylnitrosamine and di-n-butylnitrosamine were separated within six minutes, with retention times of 1.90, 2.53, 3.89 and 6.01 min respectively. The limit of detection in 1 µl of methanol was 5 ng of each nitrosamine. When 2 µl of soyabean extract were chromatographed,

two substances with retention times corresponding closely to two of the four standard nitrosamines were detected. The peak heights of these two substances were enhanced when the extract was spiked with dimethylnitrosamine and di-n-butylnitrosamine. The amounts of the two substances in the extract were estimated by comparing their peak areas with the areas given by measured weights of the pure nitrosamines. The identity of the dimethylnitrosamine was confirmed by mass spectrometry.

Lijinsky et al [14] estimated N-nitroso compounds on three 6 ft X 1/8 in stainless steel columns. The gas chromatograph, fitted with an FID, used helium carrier gas with an inlet temperature at or below 200°. Low molecular weight nitrosamines were analyzed on 28% Pennwalt 223 + 4% KOH on Chromosorb P, temperature-programmed from 140 - 160°. Low and medium molecular weight nitrosamines were analyzed on 15% DEGS on Chromosorb W temperature-programmed from 100 - 170°. All nitrosamines, particularly high molecular weight nitrosamines, were analyzed on 8.4% DEGS on Chromosorb W, temperature-programmed from 80 - 180°.

Hill [73] analyzed the urine of Wistar rats with bladder infections. Each rat was fed 0.5 mg piperidine on two successive days and given drinking water containing 5 mg/ ml nitrate. A similar diet for control rats did not contain any piperidine. The method of Telling et al [72] was used to transfer any nitrosamine that may have formed from the urine to hexane. The N-nitrosopiperidine, estimated by GLC-MS, was present in half of the test rats, but was absent in the control rats. The proportion of piperidine nitrosated (0.5%) by the in vivo system was comparable with that in an in vitro system, using the same amounts of reactants and the same strain of bacterium.

Osborne [33] reported the results of Telling et al [72]. Samples

of luncheon meat were examined for their volatile nitrosamine content. The nitrosamines were analyzed on a Pye 104 gas chromatograph fitted with an FID and a 5 ft column packed with 10% Carbowax 20 M on acid-washed celite. The column was operated isothermally at 170° and the nitrogen carrier gas had a flow rate of 36 ml/ min at an inlet pressure of 28 psi. When the gas chromatograph was connected to a mass spectrometer, several peaks, which chromatographically could be ascribed to nitrosamines, were shown not to be nitrosamines. Hydroxyacetone could be confused with dimethylnitrosamine or diethylnitrosamine and benzaldehyde with methyl-isobutylnitrosamine. Extracts from the vacuum distillation of various foods were examined on the same gas chromatograph fitted with a 5.5 m X 4 mm ID glass column packed with 10% Carbowax 20 M on silanized celite (100 - 120 mesh). The column was temperature-programmed from 100 - 200° at 2°/ min and the helium carrier gas had a flow rate of 40 ml/ min. The column effluent was split 10 : 1, the larger amount of effluent passed to an AEI MS902 mass spectrometer fitted with a Watson and Biemann separator [112, 113] and the smaller amount of effluent passed to an FID. Nitrosamines were identified by monitoring the occurrence of NO^+ peaks in the high resolution mass spectrum. Sixteen nitrosamines were examined by this procedure. The sensitivity for nitrosamines, when monitoring the NO^+ peak was 0.05 to 0.1 µg. In many cases, where the molecular ion had a greater abundance, 0.01 to 0.025 µg of nitrosamine could be detected when the molecular ion was monitored. For a 10 µl injection, this was equivalent to 0.5 to 1 ppb of individual nitrosamine in the sample.

The presence of dimethylnitrosamine in samples of sable, salmon and shad, previously identified by GLC [57], was confirmed [58] by GLC-MS. A Barber-Colman 5000 gas chromatograph, fitted with a modified

KCl thermionic detector [57], and with other GLC parameters as previously described [57], had the column temperature-programmed from 80 - 180°. The apparent dimethylnitrosamine content of meat samples was found to be 1 - 5 ppb, with only one meat sample containing 5 ppb dimethylnitrosamine, the identity of which was confirmed by mass spectrometry. The authors warned against the unreliability of retention times and R_f-values for the identification of nitrosamines. In many extracts of ham and frankfurters a GLC peak corresponding to diethylnitrosamine was shown not to be a nitrosamine by GLC-MS.

Sen [47] analyzed nitramines, obtained from the oxidation of nitrosamines, by mass spectrometry.

Eisenbrand [44] and Preussmann [114] used the method of Eisenbrand and Preussmann [115] to hydrolyze nitrosamines to the corresponding amine and nitrosylbromide. The amine was extracted and coupled with heptafluorobutyrylchloride. Eleven derivatives were separated in less than 15 min on a Varian 1520 B gas chromatograph equipped with an FID and tritium ECD. The 10 ft X $^1/_8$ in column, packed with 5% SE-30 on Chromosorb W (acid-washed) 80 - 100 mesh, was initially operated at 80° and then temperature-programmed at 10°/ min to 160°. Nitrogen , the carrier gas, had a flow of 18 - 20 ml/ min. The injector temperature was 180°. These authors also used these conditions for the quantitative estimation of nitrosamines. The lower limit of detection on the ECD was 100 - 200 pg and the standard curves were linear over the range 1 - 100 ng.

1.25 Polarography

Polarography is one of the more sensitive methods for the detection and estimation of nitrosamines. Nitrosamines may be qualitatively detected in acid solution at a concentration of about 0.05

ppm [8]. The well-formed half-waves disappear at a pH above about 2-3 [116, 117]. The method is most suitable for the determination of single N-nitrosamines since their half-wave potentials fall within a narrow range of about 0.3 volts, which precludes their individual estimation when two or more are present in a mixture (see Table II). Walters [8] obtained concordant results when diethylnitrosamine was estimated by polarography and GLC.

Numerous workers have attempted to improve the specificity of the polarographic method, since many other compounds may be reduced at the dropping mercury electrode. In this connexion, Heath and Jarvis [25] estimated dimethylnitrosamine in animal tissues in 0.01 M sulphosalicylic acid without encountering any interfering contaminants. A solution of dimethylnitrosamine at a concentration of 1 µg/ ml gave a well-formed half-wave while the calibration curve of diffusion current vs concentration was slightly concave upwards. Polarograms of five nitrosamines were recorded on a Tinsley single unit type V722/14 polarograph, fitted with an ink recorder and a "dilution" type polarographic cell which incorporated an internal saturated-calomel electrode. The cathode dropping time, from a head of 45 cm of mercury, was 4 sec. When determining dimethylnitrosamine in fish products, Lydersen and Nagy [65] removed polarographic contaminants by passing nitrogen through the sample at 50° for 30 min. They used an ammonium sulphate - sulphuric acid buffer and found that the additon of potassium bromide prevented the formation of electrolyte bridges. The mercury pool at the bottom of the cell was used as one of the electrodes.

Devik [66] used the method of Lydersen and Nagy [65] to examine the distillate from roasted potato starch spiked with D-glucose and one

of three amino-acids. The polarogram showed several half-waves, two of which were attributed to a number of nitrosamines. The Maillard Reaction was examined more thoroughly by Kadar and Devik [86] who used the method of Lydersen and Nagy [65]. The mercury pool was used as one of the electrodes and polarograms were recorded at 25°. Under these conditions, the potential of the Hg/ HgBr electrode was 0.10 v against the saturated-calomel electrode. The polarograms of seven pyrazines and seven N-nitroso-compounds were recorded (see Table II). The half-wave potentials, at pH 1.3, of pyrazines and nitrosamines were too similar to permit of their polarographic differentiation. Heyns and Koch[67,68] and Heyns et al [69] corroborated these findings. They recorded the half-wave potentials of four pyrazines and four nitrosamines (see Table II) . The polarograms were recorded on a Radiometer PO4 instrument. The procedure of Lydersen and Nagy [65] was modified as follows. Aliquots (about 10 ml) of a control and an unknown solution were titrated with 0.01 M sulphuric acid. A drop of mixed indicator (100 ml 0.03% methyl red in 70% ethanol and 15 ml 1% methylene blue in water) was used. The titrated solutions were buffered with 5 ml of buffer prepared according to the method of Lydersen and Nagy [65]. Nitrogen was bubbled through the solutions for 5 h to remove oxygen.

Nikulin and Klochkova [117] studied the reduction of diethylnitrosamine at pH 1 - 12. Two-step waves were produced in slightly acidic and neutral media. The second wave could be clearly discerned at pH 6.15, when both protonated and non-protonated molecules were reduced. At a very low pH, only protonated molecules were reduced so that a single wave was produced.

Differential polarography [61] has been advocated as a method for

improving the sensitivity of the polarographic determination of nitrosamines. A Southern Analytical differential cathode-ray polarograph, type A 1660, was used. The voltage sweep was applied simultaneously to two identical cells and the output current of the two cells was opposed. The wave heights corresponding to a range of concentrations of various nitrosamines were observed on the cathode ray graticule and calibration curves were plotted in conjunction with a predetermined amplification factor. The solutions were de-oxygenated with a stream of oxygen-free nitrogen. Two aliquots of acidified sample were compared polarographically. Prior to analysis, one of the aliquots was exposed to UV irradiation for 1.5 to 3 h. The photosensitive nitrosamines were degraded to products with half-waves at more negative potentials while photostable polarographic contaminants were not affected, so that the differential polarogram comprised half-waves due only to the degraded nitrosamines. This method is not specific for nitrosamines since any photosensitive polarographic contaminant may still be detected. Thus unsaturated aldehydes and ketones, some of which gave peak potentials close to nitrosamines, were still detected by differential polarography since they are photosensitive. The authors recorded the polarograms of numerous nitrosamines and compounds related to nitrosamines (see Table II). The authors claimed that the method of Daiber and Preussmann [49] could be used to improve the specificity of differential polarography. Daiber and Preussmann [49] estimated nitrosamines by determining the nitrite released after their photochemical cleavage, but Saxby [102] noted that pyrazines also released some nitrite on photolysis.

Casselden _et al_ [62] used polarography to detect nitrosamines in

a fraction of distilled African spirits. A Southern Analytical differential polarograph, type A 1660, was used. Determinations were made from 4.5 ml of distillate to which 0.5 ml 2 M hydrochloric acid had been added. Methanol (20% v/v) did not significantly alter nitrosamine peak heights, but the potentials of nitrosamines in the presence of methanolic hydrochloric acid were 50 - 100 mv more negative than those observed in aqueous electrolyte. Since a number of workers have shown the inadequacy of the application of polarography to the analysis of nitrosamines, the above recorded detection of nitrosamine in African spirits by polarographic means, is of dubious value.

Williams et al [64] used polarography as a preliminary method to analyse potable spirits. Distilled and undistilled spirits as well as pentane and dichloromethane extracts of the latter were polarographically examined. An Evershed pen-recording polarograph was used to record the half-wave potentials of 5 ml samples in the presence of 0.2 N hydrochloric acid. Under the conditons used, the half-wave potentials of furfural could not be distinguished from those of several nitrosamines.

Mixtures of nitrosamines, especially when in the form of crude extracts, cannot successfully be analyzed by polarography. However, polarography can be used for the estimation of a single nitrosamine, once it has been isolated in a state of purity.

1.26 Ultraviolet Spectroscopy

(i) N-nitroso-compounds

The UV spectra of nitrosamines are characterised by a low-intensity absorption band at about 340 nm and a high-intensity one at about 230 nm. Solvents affect the spectra to a considerable

extent [118]. Many workers have used the intensity of the UV absorption for quantitative estimation of nitrosamines. Preussmann [27] estimated dimethylnitrosamine, methylethylnitrosamine, diethylnitrosamine, di-n-propylnitrosamine, di-isopropylnitrosamine, ethyl-n-butylnitrosamine, ethyl-tert-butylnitrosamine and methylallynitrosamine in aqueous solution. Eisenbrand et al [54], using the absorption at 230 nm, estimated nitrosamines in n-heptane which had been saturated with acetonitrile. The Beer-Lambert law was obeyed over a wide range of concentrations. Du Plessis and Nunn [15] confirmed the observations of Eisenbrand et al [54]. Diethylnitrosamine was estimated in water which had been saturated with carbon disulphide. The absorption at 235 nm was used to estimate from 2 - 30 µg/ ml of diethylnitrosamine in a one cm quartz cell while the absorption at 341 nm was used to estimate from 200 - 2500 µg/ ml of diethylnitrosamine. Dimethylnitrosamine and diethylnitrosamine were estimated in water, pentane and n-hexane by Möhler and Mayrhofer [30]. At the 230 nm absorption maximum, with water as solvent, less than 1 µg /ml of these two nitrosamines could be detected in a one cm quartz cell. Dichloromethane, which absorbs UV radiation in this region, was not a suitable solvent. Nevertheless, Eisenbrand et al [24] estimated nitrosamines in 50% aqueous methanol and in dichloromethane or dichloromethane diluted with n-pentane [31]. The absorption of dimethylnitrosamine at 225 nm and 330 nm in water and at 360 nm in chloroform was used as confirmatory evidence for its presence in an extract derived from herring meal [50]. Mirvish [119] used the UV absorption of several nitrosamines and nitrosamino-acids to estimate the rate of nitrosation of the corresponding amines. The results confirmed the findings of Sander et al [12] who showed that the less basic an amine, the more readily it was nitro-

sated. Williams et al [64] used UV spectroscopy to confirm the presence of furfural in, and the absence of nitrosamines from, a fraction isolated by preparative GLC from an extract of potable spirits.

(ii) Nitroso-derivatives

Coloured nitrosamine derivatives, used to render small amounts of nitrosamines visible, have also been analyzed by UV spectrophotometry. In this way, Neurath et al [20] confirmed the presence of methyl-n-butylnitrosamine in an extract of cigarette smoke condensate. The nitrosamine was reduced to the hydrazine with lithium aluminium hydride. The hydrazine was condensed with 5-nitro-2-hydroxybenzaldehyde, and the derivative examined in a UV spectrophotometer. Möhler and Mayrhofer [30] were unable to reproduce the 20% reduction yield claimed by Neurath et al [20], and reported a 10% yield, which, in their work, precluded the possibility of UV analysis of the 5-nitro-2-hydroxybenzaldehyde derivative of the hydrazine, because of the interference of an excessive amount of 5-nitro-2-hydroxybenzaldehyde.

Ender and Ceh [39, 41], after reduction of the nitrosamine with zinc and hydrochloric acid, condensed the nitrosamine with p-dimethylaminoazobenzene. Although the reduction proceeded for an inconveniently long time (15 - 20 h), yields of 90 - 100% were claimed for dimethylnitrosamine and diethylnitrosamine, with 60% yields of hydrazines for other alkylnitrosamines. The condensation product was estimated at 458 nm. As a control, the method of Daiber and Preussmann [49] was also used to estimate the concentrations of nitrosamines. The levels measured by the hydrazine method were generally 10% lower than the values determined by the Daiber and Preussmann method [49].

A quantitative procedure, involving the formation of nitrite from nitrosamines in aqueous or methanolic solution by exposure to UV irradiation, was developed by Daiber and Preussmann [49]. The nitrite, after reaction with sodium carbonate, was measured with Griess reagent [77, 78] and the absorbance of the reaction product measured at 525 nm. The method, applied to seven nitrosamines, obeyed the Beer-Lambert law. The amount of nitrite formed was 98% of that theoretically predicted. Nitrosamines with an aryl group attached to the nitroso-group yielded only 70 - 80% of the theoretically predicted nitrite. It was possible that some of the nitrite was oxidized to nitrate, resulting in decreased readings when the sample was exposed for a long time. The sensitivity of the procedure was 0.085 μg/ ml of dimethylnitrosamine. When dimethylnitrosamine was cleaved in methanol, the sensitivity was lower, since only about 40% of the predicted amount was formed. When applied to nitrosamides the sensitivity was 2 - 4 μg/ ml. Neuenhofer et al [26], Ender and Ceh [39], Ender [51], Walters [8] and Bogovski et al [34] all used the method of Daiber and Preussmann [49] for nitrosamine estimation. An automated variation of this method was developed by Fan and Tannenbaum [120] and then used by Issenberg and Tannenbaum [55].

Sander [90] irradiated alkaline solutions of nitrosamines in acetone with long-wave UV irradiation, detecting the resulting nitrite with Griess reagent [77, 78]. The yield of nitrite was 30% of the theoretically predicted amount. Inorganic nitrite and esters of nitrous acid interfered with the procedure. Less than 1 μg of nitrosamine could be detected.

Eisenbrand and Preussmann [115] hydrolysed nitrosamines to

amines and nitrosylbromide using hydrogen bromide in glacial acetic acid. After reaction of the nitrosylbromide with sulphanilic acid, the resulting diazonium ion was coupled with N-(naphthyl-(1))-ethylenediamine and the absorbance measured at 550 nm. In tests covering seventeen nitrosamines, the Beer-Lambert law was obeyed and the sensitivity of 1 μg/ ml decreased when the procedure was applied to nitrosamides. Water adversely affected the conversion of nitrosamine to nitrosylbromide. The resulting amine could be detected by the methods of Seiler and Wiechmann [121, 122], Neurath and Doerk [81] and Walle and Ehrsson [123].

1.27 Infrared Spectroscopy

The fundamental vibrations of nitrosamines have been assigned [124]. Rademacher and Lütke [125] recorded the vibrational spectra of seven isotopic dimethylnitrosamines in different states of aggregation and in different solvents. Characteristic frequencies of dimethylnitrosamine occur at 1446 cm^{-1} (N = O stretch), 1291 cm^{-1} (C – N antisym stretch), 1052 cm^{-1} (N – ^{N} stretch), 845 cm^{-1} (C – N sym. stretch) and 684 cm^{-1} (= = N – N bend.). The presence of dimethylnitrosamine in an extract of herring meal was confirmed by IR spectroscopy [50]. Du Plessis *et al* [52] used IR data as part of the confirmatory evidence for the presence of dimethylnitrosamine in an extract of *Solanum incanum*.

1.28 Nuclear Magnetic Resonance Spectroscopy

The polar resonance form of the nitrosamine group makes a large contribution to the resonance hybrid. In nitrosamines the restricted rotation about the N – N partial double bond [126, 127, 128], in conjunction with their planar configuration [129, 130, 131], causes

the magnetic non-equivalence of protons in the two alkyl substituents.

The NMR spectrum of dimethylnitrosamine was recorded in deuterochloroform [52]. The NMR spectrum of a deuterochloroform solution of a GLC fraction obtained from an extract of Solanum incanum contained several peaks, including two at 6.34 and 7.05 ppm, characteristic of dimethylnitrosamine. NMR spectra were taken on a Perkin-Elmer R12 instrument, with the scale set to 10 ppm. Owing to the small amount of dimethylnitrosamine present in the fraction, the sensitivity of the instrument was set to a high level when the spectrum was taken [132].

2. DISCUSSION

2.1 Introduction

The carcinogenic activity of dimethylnitrosamine was first noted by Magee and Barnes [1]. Following the isolation of this compound from fish meal [50] and reports of the occurrence of N-nitroso-compounds in mushrooms [133] and micro-organisms [134, 135], there has been increasing concern about the possible presence of carcinogenic nitrosamines in the environment. The possibility of the formation of these compounds in foodstuffs, and the paucity of procedures for the analysis of these compounds, has stimulated research into methods for their detection and estimation at trace levels in human foods.

Authentic specimens of N-nitrosamines were synthesized (see 3.2) or supplied by Eastman Kodak, Rochester, New York, U.S.A. (DBNA). The various spectra (ultraviolet, infrared, nuclear magnetic resonance and mass) of members of a series of nitrosamines were recorded to permit their final identification in biological material. Not all of these methods were of sufficient sensitivity or specificity to justify their use.

2.11 Ultraviolet spectra of N-nitrosamines

The UV spectra of N-nitrosamines exhibit a high intensity absorption (ε ca. 7000 - 8000) around 235 nm and a low intensity absorption (ε ca. 90) around 360 nm. The low intensity absorption exhibits fine structure in both non-polar and weakly polar solvents [136]. When a more polar solvent is used, there is a marked shift to the blue [137]. The UV spectra of DMNA, DENA, MPNA, EPNA and DPNA were recorded (see 3.17

and 3.31) and found to be very uniform (See Table III). The intensities of the absorptions did not vary to any appreciable extent. Calibration curves, constructed for aqueous solutions of DMNA and DENA (see Figs. 1 - 4), were found to obey the Beer - Lambert law. About 2 ppm of each nitrosamine could be estimated. Although the sensitivity was satisfactory, the similarity of the spectra (a typical spectrum is shown in Fig. 5) excluded estimations of individual nitrosamines in a mixture.

2.12 Infrared spectra of N-nitrosamines

In N-nitrosamines the group frequency of particular interest, for the purpose of identification, is due to the N = O stretching vibration. This vibration, covering a wide range of frequencies [138] (1040 - 1940 cm^{-1}) in inorganic derivatives, occurs in the region 1400 - 1690 cm^{-1} in organic compounds. Although many nitrocompounds occur as dimers and partial vibrational assignments of N-nitrosamines have been based on a dimeric model [118, 126, 139, 140, 141], recent accounts [124, 142, 143] have concluded that dimethylnitrosamine is unassociated in solution. These conclusions may extend to other dialkylnitrosamines as well. N-nitrosamines exhibit an intense band at 1425 - 1460 cm^{-1} which has been assigned [118, 126, 139, 140, 141] to the N = O vibration. This band shifts to a lower frequency as the solvent polarity increases. This solvent effect may be explained by the stabilising influence of polar solvents on the ionic resonance form $R_2\overset{+}{N} = O - \bar{O}$.

IR spectra of DMNA, DENA, MPNA, EPNA and DPNA were recorded (see 3.16 and 3.32) from liquid films and from solutions. In all cases the N = O vibration was prominant and occurred at about 1445 cm^{-1}. In carbon

tetrachloride the N = O vibration appeared at about 1460 cm^{-1}. About 15 µg of DMNA could be detected in 10 µl of deuterochloroform. The moderate sensitivity of this procedure merited its use in confirming the presence of DMNA in an extract of Solanum incanum (see 2.19i).

2.13 Nuclear magnetic resonance spectra of N-nitrosamines

The ionic resonance form of the nitroso-group makes a large contribution to the resonance hybrid so that the N — N partial double bond presents a barrier to rotation of about 23 kcal/ mole for DMNA [126, 127, 128]. The rotational barrier in nitrosamines in conjunction with their planar configuration [129, 130, 131] causes the magnetic non-equivalence of protons in the two alkyl substituents. The nitroso-group exhibits anisotropic diamagnetic shielding [130, 131] such that protons in the plane of the double bond are shifted downfield while those located above the double bond are shifted upfield. On the basis of this observation, alkyl protons at higher fields have been assigned [130, 131] to the cis position.

Although NMR is an important technique of identification, the weakness of its absorption requires the use, even in micro-tubes, of relatively large (ca. 3 mg) samples if a reasonably intense spectrum is to be produced from a single scan with a 60 MHz instrument. Computer averaging [144] of repetetive scans can considerably improve the signal/ noise ratios while Fourier transform procedures [145] drastically shorten the scanning time. The NMR spectrum of DMNA was recorded (see 3.33) in deuterochloroform with tetramethylsilane as an external standard. An acceptable spectrum (see Fig. 30A) was obtained from 4 mg of DMNA dissolved in 18 µl of deuterochloroform.

The comparative insensitivity of the method renders it unsuitable for trace analysis of N-nitrosamines. In an isolated case (see 3.31 and 2.19i), where about 1 mg of DMNA was isolated from Solanum incanum, the identiity of the suspected nitrosamine was confirmed by NMR.

2.14 Mass spectra of N-nitrosamines

The mass spectra of several dialkyl N-nitroso-compounds have been reported [99, 110, 146]. In additon, di-benzyl-N-nitrosamines [146, 147] and cyclic N-nitrosamines [110, 111] have been examined. In the lower dialkylnitrosamines (DMNA to DPNA) the molecular ion is of notable abundance while in the higher molecular weight dialkylnitrosamines, owing to the increased likelihood of alkyl cleavage, the molecular ion is of insignificant relative abundance [148]. Alkyl peaks and additional alkyl fragments are encountered. The NO peak is usually of medium abundance. An M - 17 peak occurred for all nitrosamines examined [111]. Accurate mass measurements [111] of M - 17 fragments from cyclic N-nitrosamines have confirmed that these fragments are due to loss of OH. A six-membered Mc Lafferty type rearrangement [149] has been proposed [111] (see Fig. 6).

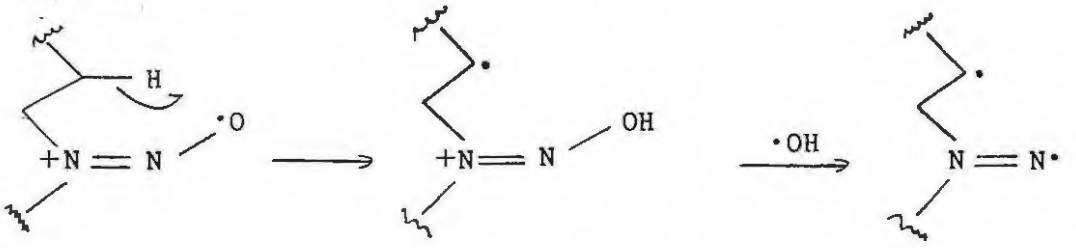


Fig. 6.

Loss of olefin fragments from nitrosamines has been accounted for [111] by Fig. 7.

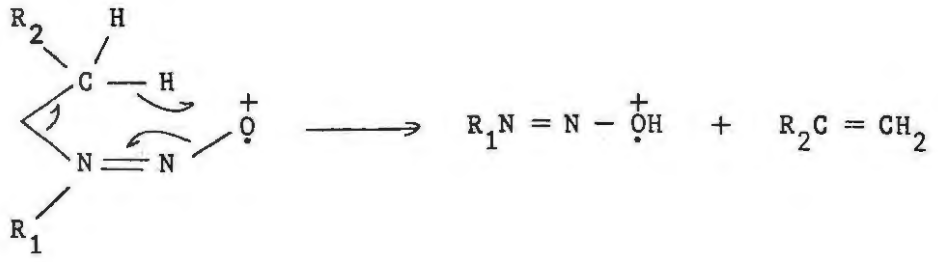


Fig. 7.

A frequently encountered peak at m/e 27 may correspond to ionized HCN.

Low resolution (ca. 100) mass spectra of DMNA, DENA, MPNA, EPNA, DPNA, DBNA and NPIP were recorded at 70 eV (see 3.35). Except for DBNA mass spectra of all the nitrosamines were recorded on both an AEI MS30 and a Hitachi RMS4 mass spectrometer. The mass spectrum of DBNA was recorded on the AEI instrument. The pure samples of nitrosamine were introduced via gas chromatographs coupled to the mass spectrometers. A computer program, written in Fortran IV, was used to display the normalized mass spectra in the form of bar graphs (see Figs. 8 to 20).

(a) Dimethylnitrosamine (Figs. 8 and 9).

The most intense peak was due to the molecular ion at m/e 74. Other prominent peaks occurred at m/e 44, 43, 42, 30 and 28. By analogy with accurate mass measurements [110] of DBNA and N-nitrosomorpholine, the other prominent peaks may be assigned the compositions C_2H_6N, C_2H_5N, C_2H_4N, NO and CH_2N respectively.

(b) Diethylnitrosamine (Figs. 10 and 11).

The base peak, at m/e 102, was produced by the molecular ion. Distinctive peaks appeared at m/e 57, 56, 44, 42 and 30. By consulting accurate mass measurements of cyclic nitrosamines [111] and

and DENA [110], the prominent peaks (except m/e 44) may be assigned the compositions C_3H_7N, C_3H_6N, C_2H_4N and NO respectively. The fragemnt with m/e 44 may have the compositions C_2H_6N or NNO. The latter composition is very unlikely since it would involve the simultaneous cleavage of two bonds and except for MPNA does not occur to any appreciable extent in the mass spectra of other nitrosamines. Accurate mass measurements and deuterium labelling are required to clarify the fragmentation process leading to the formation of this ion.

(c) Methyl-n-propylnitrosamine (Figs. 12 and 13).

The most intense peak occurred at m/e 42. Other conspicuous peaks appeared at m/e 102, 85, 73, 44 and 43. Comparison of accurate mass measurements of DPNA [110] supports a combined composition $C_3H_6 + C_2H_4N$ for the base peak. In a similar way, the other peaks, except for m/e 44 (see 2.14ib), may be assigned compositions $C_4H_{10}N_2O$ (molecular ion), $C_4H_9N_2$ (M - 17), $C_2H_5N_2O$ and $C_2H_5N + C_3H_7$ respectively.

(d) Ethyl-n-propylnitrosamine (Figs. 14 and 15).

The base peak, at m/e 42, may be assigned the complex composition $C_3H_6 + C_2H_4N$ (see 2.14ic). Other prominent peaks occurred at m/e 57, 56, 43, 29 and 27. By comparing accurate mass measurements of DPNA [110], the other peaks except m/e 29 and 27, may be assigned compositions C_3H_7N, C_3H_6N and $C_2H_5N + C_3H_7$ respectively. Deuterium labelling and accurate mass measurements would confirm the compositions of fragments 27 and 29 which may be due to HCN and C_2H_5 respectively.

(e) Di-n-propylnitrosamine (Figs. 16 and 17).

The base peak, at m/e 43, may be assigned the complex

composition $C_2H_5N + C_3H_7$ (see 2.14id). Other distinctive peaks appeared at m/e 130, 70, 42, 41 and 29. Using the same method as above (see 2.14id) the other peaks, except m/e 29 (see 2.14id), may be assigned compositions $C_6H_{14}N_2O$ (molecular ion), C_4H_8N, $C_2H_4N + C_3H_6$ and C_3H_5 respectively.

(f) Di-n-butylnitrosamine (Fig. 18).

The most intense peak occurred at m/e 84. Other prominent peaks appeared at M3e 57, 43, 42, 41 and 29. By consulting accurate mass measurements of DBNA [110], the other prominent fragments, except m/e 29 (see 2.14id), may be assigned compositions $C_5H_{10}N$, $C_4H_9 + C_3H_7N$, $C_3H_5 + C_2H_5N$ and $C_3H_6 + C_2H_4N$ respectively.

(g) N-nitrosopiperidine (Figs. 19 and 20).

The most intense peak occurred at m/e 84. Other prominent peaks appeared at m/e 57, 43, 42, 41 and 29. Comparison of accurate mass measurements of DBNA [110] and N-nitrosomorpholine [110] suggests compositions, except for m/e 55, of $C_4H_8 + C_3H_6N$, $C_3H_6 + C_2H_4N$, $C_3H_5 + C_2H_5N$ and $CH_2N + C_2H_4$ respectively. More information is required before the composition of m/e 55 may be assigned. It may be formed by a mechanism related to Fig. 3, in which case the likely composition is C_4H_7.

(ii) Coupling of a gas chromatograph and mass spectrometer

The most effective methods of qualitative analysis (UV, IR, NMR and MS) are inappropriate for simultaneous analysis of mixtures, while methods which separate multi-component mixtures into single components, e.g. chromatography, are inadequate for unambiguous structural

elucidation. At the present time, chromatographic procedures are most effective for separation while either chemical or spectrometric methods are particularly suitable for identification. The most sensitive of spectrometric methods are IR and MS. A great advantage of chromatography (gas chromatography) is that small quantities are required and thus complete analysis of a mixture could be achieved by passing the separate zones of pure components from a gas chromatograph into the spectrometric apparatus which would produce the spectrum of each component in turn.

Chromatographic separation requires an eluent which dilutes the components of a mixture. It is usually necessary to separate the pure components from the eluent. This separation is achieved in the interface which connects the exit of the gas chromatographic column to the spectrometric apparatus. Gas chromatography - infrared spectroscopy (GC-IR) is the less sensitive of the two spectrometric methods proposed. An acceptable spectrum may be produced from about 20 µg of material [150]. GC - MS is more sensitive : recognisable spectra have been reported from 28 ng of material injected into the gas chromatograph [151]. The interface between the gas chromatograph and the mass spectrometer has to achieve a pressure reduction from about atmospheric pressure at the column exit to about 10^{-5} mm Hg at the ion source. The devices currently employed [152] are inefficient. The membrane separator [153], used in the AEI MS30 instrument, consists of a membrane in which the sample can dissolve while the carrier gas cannot. The efficiency of transfer depends on both the temperature and type of sample. The Watson and Biemann separator [113], used in the Hitachi RMS4 instrument, consists of a porous tube through which the light carrier gas may escape.

(a) Sensitivity of GC - MS

For N-nitrosamines the membrane separator operated most efficiently at 60°. A disadvantage was the threshold effect - repeated injections of dilute N-nitrosamines (typically 1 - 2 μg in 5 μl of dichloromethane) were required to optimize the sensitivity. After four or five injections, 400 ng of DMNA, injected into the gas chromatograph, yielded a molecular ion peak (at maximum attenuation) which was 90% of the base peak occurring at m/e 28 (due to background). The peak width of DMNA (emerging from the gas chromatograph) was 45 sec. This was the most sensitive reading obtained. After the membrane interface had been sensitized to N-nitrosamines, injections of dichloromethane (30 μl) failed to dislodge any traces of material which might have been present in the membrane. When excessive amounts (e.g. 100 - 300 μg) of nitrosamines were used to sensitize the membrane, additional injections of dichloromethane produced "ghost peaks". Although this phenomenon was not observed for traces of N-nitrosamines, one or two blank determinations were performed before an unknown solution was analyzed.

The molecular separator, in the Hitachi RMS4, was operated at 150°. Few unknown samples were analyzed on this machine since the sensitivity was only 1.5 μg of DMNA .

(b) Identification of N-nitrosamines by computer matching of low resolution mass spectra

It is known [154, 155, 156] that the different operating characteristics of mass spectrometers affect the fragmentation pattern of a compound. Further distortions occur when gas chromatographic peaks are scanned, since the eluted component changes concentration during the

scan. This distortion may be minimized by recording the spectrum at or near the peak maximum and by keeping the time of scanning very much less than the elution time of the peak.

Five mass spectra of dimethylnitrosamine, compiled from the above spectra and from reported data [99, 109, 110], were compared by a computer program, written in Fortran IV. Four matching procedures [157] were tested (see 3.37). The simplest of these procedures compared the n largest intensities irrespective of order. The second method compared the n strongest peaks in order of decreasing intensity and made an allowance for the relative positions of peaks with equal m/e values. The third and fourth methods subdivided the spectra into R equal ranges, each containing m mass units. The n most intense peaks in each range were selected. In method 3, the number of agreements irrespective of order was counted in each range and averaged. In method 4 the n strongest peaks in each range were treated in a manner analogous to method 2. Table V summarizes the results obtained when each DMNA spectrum was in turn used as a standard and compared with the remainder . In agreement with published results [157], methods 3 and 4 appeared most satisfactory for comparison of spectra (see Table IV).

The quality of spectra obtained from traces of materials is adversely affected by the high sensitivities required. The high sensitivities employed increase the contributions to the mass spectrum, of background and bleeding of liquid phase from the column. Under these circumstances, the mass spectrum of the compound in question may be swamped by remnants of solvent, especially when the separation from the solvent is slight. In this connexion, a computer program was used to detect variations in successive mass spectra recorded over a gas chromato-

graphic peak (see 3.37ii). An average "background" mass spectrum, computed from spectra scanned on either side of a gas chromatographic peak, was subtracted from the mass spectrum recorded at the top of the peak. With this method, three successive spectra were obtained from 1.0 μg of DMNA in 10 μl of dichloromethane. The respective normalized mass spectra are illustrated in Figs. 21, 22 and 23. Although the molecular ion (m/e 74) of DMNA is clearly evident in the spectrum recorded at the top of the gas chromatographic peak, the remainder of this spectrum does not visually correspond to spectra (see Figs. 8 and 9) of pure DMNA. The degrees of matching between this spectrum and that of pure DMNA (recorded on the AEI instrument) were 0.70, 0.58, 0.50 and 0.45 for the respective methods of comparison. In contrast to these results, the normalized mass spectrum of 1.0 μg of DMNA, obtained after subtraction of an average "background" (see Fig. 24), closely resembled the library of DMNA spectra (see Table IV). When this mass spectrum of DMNA was included in the library and compared with the other spectra, the degrees of matching obtained were similar to those for the conventional spectra (see Table IV). The procedure outlined above may be modified by enhancing the contribution of one of the spectra to the average "background". This would be particularly suited to non-gaussian peak shapes. Although sophisticated computer appliances are available to control [158] mass spectrometers and manipulate data [157], the above procedure is useful for laboratories without access to sophisticated computer programs.

(c) Identification of N-nitrosamines using high resolution mass spectrometry

In low resolution mass spectrometry the m/e ratio of a fragment is determined to the nearest mass unit. On the basis of

$^{12}C = 12.000000$, the isotopic atomic weights are not integral mass numbers. Fragments with the same nominal mass but different elemental compositions will have slightly varying masses if sufficiently careful measurements are performed. Thus the molecular ion of any compound, provided that it is of reasonable intensity, may be used to determine the empirical formula. Simple fragments, once their compositions have been determined, may be used to identify certain classes of compounds[72]. For N-nitrosamines it has been established [72] that a fragment of m/e 30, present in the mass spectrum of all compounds examined, has the compositions NO and CH_4N. The ion CH_4N+ also occurs in the mass spectra of numerous amino-compounds [159] but the fragment NO occurs exclusively in the mass spectra of organic compounds containing NO and NO_2. This fragment may thus be used as further evidence for the contribution of a nitrosamine to the particular mass spectrum in question. Other possible elemental compositions [72] for mass 30 are $C^{18}O$, $^{15}N_2$, ^{13}CHO, H_2N_2, $CH_3{}^{15}N$, $^{13}CH_3N$, CH_4N, $^{13}CCH_5$ and C_2H_6. The resolution of a mass spectrometer is a measure of its ability to separate ions of similar masses. The resolution $\underline{R}$, required to separate two ions $\underline{m}_1$ and $\underline{m}_2$ with a mass difference of Δm, is defined by

$$R = \frac{m_1}{m} \quad \text{or} \quad \frac{m_2}{m}$$

A resolution of 25,000 would be required to separate the ions nearest in mass, NO and $C^{18}O$ (masses of 29.9800 and 29.9992 respectively). Assuming the ion at a nominal mass of 30 has a moderate intensity, the most likely ion, closest in mass to NO, is CH_2O (exact mass : 30.0106) and a resolution of only 2400 would be required to separate them. A second fragment

of notable abundance in the mass spectrum of N-nitrosamines, has m/e 42 and accurate mass measurements [110] have shown its composition to be $C_2H_4N + C_3H_6$. The latter ion is a common fragment from alkyl groups of three or more carbon atoms while the former may arise from numerous nitrogenous compounds [159]. This ion (C_2H_4N), in conjunction with other mass spectral evidence, may still aid in the identification of an N-nitrosamine. The most likely ion, closest in mass to C_2H_4N , is C_2H_2O and a resolution of only 1800 would be required to separate them.

The high resolution (ca. 3400) mass spectrum of DMNA was recorded on the AEI MS30 instrument. The NO fragment at m/e 30 was too low in intensity to warrant an accurate mass measurement. The molecular ion (m/e 74) and the ion at m/e 42 (C_2H_4N) were accurately measured (see 3.38). The deviations of the results from the expected masses were unexpectedly high - they were between 210 and 59 ppm. These unacceptable errors were found to be due to a fault in the focussing system of the reference sample. This fault resulted in peaks which were flat-topped so that very accurate measurements were impossible. This technique will be pursued further when the above fault has been repaired.

2.15 Thin-layer chromatography of N-nitrosamines

The simplicity and relative sensitivity of TLC have contributed to its popularity as a qualitative and quantitative technique. Because of the extreme variability (up to 50%) of R_f-values [160] comparisons of these values for the purpose of qualitative identification must be based on the chromatographic behaviour of substances under identical conditions i.e. parallel and mixed runs on the same TLC plate. Published R_f-values are merely indications of the degree of separation which

may be achieved under defined conditions. Quantitative procedures depending on visual comparison of spots have a variability of 10 - 15% [161].

The mobile phases of Preussmann et al [75, 76] did not adequately separate all the nitrosamines investigated and a mobile phase consisting of carbon tetrachloride : dichloromethane :: 6 : 4, was employed (see 3.34i). This phase confined the dialkylnitrosamines examined to a band with an R_f of 0.2 to 0.6. The nitrosamines were rendered visible with the reagent of Preussmann et al [75, 76]. This procedure, with a sensitivity of 1 µg for DMNA, was used as a preliminary test for the presence of nitrosamines in plant extracts and a preparative adaptation was used to obtain partially cleaned-up plant extracts.

Dilute nitrosamine solutions were unsuccessfully concentrated in KBr wedges (see 3.32ii) . The procedure might be adapted for the analysis of the less volatile high molecular weight nitrosamines.

The method of Neurath et al [79], used to detect 2 to 4 mg of DMNA and DENA (see 3.34ii), was not adopted for trace analysis.

2.16 Gas chromatography of N-nitrosamines

Virtually all chemists engaged in the analysis of organic compounds are familiar with gas chromatography [162] which is a procedure for separating a mixture into its components. The small samples required and the ease of interpretation of results have contributed to its extensive use. The time required for each component to elute (retention time) is a simple means of identification. In certain instances two or more components may have the same retention times. The observation of retention data on three columns of differing polarity reduces the possibility of confusing two compounds. Nevertheless, the most convincing proof that overlapping of eluates has not occurred is provided by additional techniques

(e.g. GC-IR and GC-MS). The many factors which affect retention behaviour (e.g. interactions of solute with solid support, temperature and flow fluctuations and column bleed), necessitate the separation of unknowns and standards under nearly identical conditions. The standard and unknown may be chromatographed in quick succession and the retention times compared or a mixture of the standard and unknown may be chromatographed. If the substance added to the sample was present in the mixture, one of the peaks would be relatively larger than that of the original sample.

The area of a chromatographic peak is proportional to the amount of substance present and in order to use gas chromatography for quantitative analysis, the area of the peak and the proportionality factor have to be determined. In most cases it can be assumed [163] that the proportionality factors for a number of components are identical especially if a relatively small range of closely related compounds is analyzed. In some instances, however, unacceptable differences have been found [164]. In many cases, the measurement of peak areas, even with the aid of integrators, is tedious. Peak heights are frequently substituted for peak areas, even though the former are more dependent on slight changes of operating conditions. These changes are minimized in the internal standard method [165], where the samples of unknown concentration are analyzed together with the standard of known concentration.

(i) Quantitative estimation of N-nitrosamines

In most cases in this investigation the internal standard method [165] was used to estimate N-nitrosamines. Preliminary estimations were occasionally determined from the relationship between concentration and peak area or peak height. In these instances, errors

were minimized by using the mean value obtained from at least five readings. Calibration curves were established by the method of least squares [166]. The degree of linear correlation was gauged from the correlation coefficient [167], r, since when $r = |1|$, all the points lie on a straight line. The following procedures were used to estimate nitrosamines.

(a) DMNA was estimated (see 3.35iia) on column g (see 3.19iiig) without an internal standard. Calibration curves, relating the areas of the peaks due to DMNA to their concentrations, were established at attenuations of 5000, 2000, 200 and 50. Concentrations of DMNA were chosen (see Calibration Table 1) to give three or four well-spaced points on each calibration curve. Each point on the curves represented the mean peak area obtained from three one µl injections. The correlation coefficients were all 0.999, indicating a high degree of linearity.

(b) DMNA was also estimated (see 3.35iib) on column h (see 3.19iiih) with p-xylene as an internal standard. The calibration curve was established at an attenuation of 2. Five well-spaced points were plotted on the calibration curve. Each point represented the mean peak area obtained from four or five one µl injections (see Calibration Table 2). The correlation coefficient was 0.999.

(c) DENA was estimated (see 3.3.5iic) on column i (see 3.19iiii) with cyclohexanone as internal standard. Calibration curves were established at attenuations of 200, 20 and 2. Concentrations of DENA were chosen to give five or six well-spaced points on each calibration curve. Each point represented the mean peak area of four or five determinations (see Calibration Table 3). The correlation coefficients were all 0.999.

(d) Calibration curves for the estimation of DENA, at an attenuation of 20 on column i (see 3.19iiii), were constructed (see 3.35iid) by four methods:

1) from the peak area of DENA
2) from a ratio of peak area of DENA to peak area of internal standard (cyclohexanone)
3) from the peak height of DENA
4) from a ratio of peak height of DENA to peak height of internal standard (cyclohexanone)

Concentrations of DENA were chosen (see Calibration Table 4) to give six well-spaced points on the calibration curves. Each point represented the mean reading of four 1 µl injections. The respective correlation coefficients were all 0.999.

A series of five different concentrations of DENA were estimated from the four calibration curves and the deviations from the known values were compared (see Table V). The mean reading of three 1 µl injections was used for each determination. Estimation of DENA by method 4 appeared slightly more accurate than methods 1 and 2 while method 3 was the least accurate. It is known that slight fluctuations in gas chromatographic parameters affect peak heights to a greater extent than peak areas. These variations will affect the peak heights of DENA and cyclohexanone in a similar way so that a ratio of the two would be largely unaffected. The results obtained agree with these expectations. The precision of the four methods of estimation, as indicated by the scatter of individual determinations about the mean, was noticibly improved for methods 2 and 4 which used internal standards. The greater precision of these two methods must be related to the use of the internal standard

and probably involves the cancelling out of errors due to measurement.

Since the accuracy of method 4 was marginally better than that of method 2 and since the precision of both of these methods was comparable, method 4 was adopted owing to its greater simplicity.

(e) DMNA, DENA, EPNA and DPNA were estimated (see 3.35iie) on column j (see 3.19iiij) at attenuations of 200 and 20 with chlorobenzene as internal standard. All the nitrosamines generated peaks of the same order of magnitude on the gas chromatogram (see Fig. 25 for a typical example). The concentrations of standard solutions were chosen to give five well-spaced points on the calibration curves of DMNA, DENA and EPNA. The calibration curve for DPNA was established from four points since the DPNA peak was offscale for the most concentrated solution. Each point on the calibration curves represented the mean of at least five 1 µl injections (see Calibration Tables 5 and 6 respectively). The correlation coefficients of the least squares lines (see Figs. 26 and 27 respectively) were all greater than 0.999, indicating a high degree of linearity. Thus the standard errors (standard errors of estimates[167]) about the fitted least squares lines were less than 1% in all four cases. With this degree of accuracy, confidence limits [168] about the least squares lines were unnecessary.

The analysis time of less than 8 min, combined with the high sensitivities employed, resulted in marked tailing of solvent through the internal standard, DMNA and DENA peaks (see Fig. 25). These peaks were measured from the interpolated base-line of the tail.

Although the reproducibility of sample volumes is unimportant when the internal standard method is employed, a constant volume of one µl was used. In this way, the precision of calibration data calculated

with and without internal standards could be compared. The coefficient of variation, I, defined by the relationship $I = s/\bar{X}$ where s is the standard deviation and $\bar{X}$ designates the mean value, was used as a measure of precision. Of the sets of coefficients of variation those associated with the internal standard method were the smaller. This confirms the conclusions reached in section 2.16id above. Data from EPNA and DPNA peaks, measured at the higher sensitivity without an internal standard, were more precise than those obtained from DMNA and DENA peaks where tailing by solvent would be expected to affect the reproducibility of base-line interpolations. When the internal standard method was used, at the higher sensitivity, the relationship between degree of tailing and precision was less clear-cut. At the lower sensitivity, there was no correlation between degree of tailing and precision. These results suggest that the accuracy of base-line interpolations is not affected by moderate tailing. There was no evidence, within the range of peak heights studied, of a relationship between peak heights and precision. Thus small peaks (the smallest were ca. 11 mm) could be measured with the same accuracy as large ones. At the lower sensitivity, the precision of measurement of peak heights alone was inferior to the corresponding data obtained at the higher sensitivity. The cause of the variations in precision was traced to the use of different syringes for measuring calibration data. Three different Hamilton type 7101 1.0 µl syringes (Hamilton Co., Whittier, California, U.S.A.) were subsequently tested. For each syringe, peak heights of nitrosamines in a standard solution were measured from ten replicate readings at an attenuation of 200. For the three syringes there were no significant differences between the precisions with which peak height ratios were measured

(see Table IX). For one of the syringes, the precision with which peak heights alone were measured was anomalous. When the precision of this syringe was statistically compared with that of the other two, there was a significant difference at the 5% level (Fisher's distribution). The use of this particular syringe (with a different plunger) to establish calibration curves at an attenuation of 200 would account for the observed variations in precision. These variations may be related to an imperfection in the barrel of the syringe.

(f) Small concentrations of DMNA, DENA, EPNA and DPNA were occasionally estimated from the above calibration curves (see 2.16ie) without the addition of an internal standard. This method was only resorted to when the addition of an aliquot of internal standard would have rendered the nitrosamine peaks too small to measure. In order to use the above calibration curves (see 2.16ie), the height of the internal standard was required. This was measured from five replicate readings obtained from a solution containing the same amount of internal standard used for constructing the calibration curves. Errors in the estimation of nitrosamine peak heights were minimized by taking the mean value of at least five readings. The ratios of peak heights of nitrosamines to peak heights of internal standard were calculated and the amounts of nitrosamines estimated in the usual way. When a solution of the above four nitrosamines (see 2.16ie) of known concentration was estimated by the above procedure, the estimations did not differ by more than 5% of the known values. The nitrosamines could have been estimated from a graph relating peak heights of nitrosamines to concentration, but this procedure would have required the construction of a further series of calibration curves. Although this procedure negated the advantages of the internal

standard method, it was convenient to use and possible muddling of calibration curves was avoided.

(g) DENA and NPYROL were estimated (see 3.35iig) on column k (see 3.19iiik) with naphthalene as internal standard. The concentration of each nitrosamine was chosen such that each of them generated peaks of the same order of magnitude on the gas chromatogram. Calibration curves for the nitrosamines were established, from five well-spaced points, at respective attenuations of 160 and 40 (see Calibration Table 7). Each point represented the mean value of seven 1 µl injections. The correlation coefficients of the least squares lines through the data points were 0.999 and 0.996 respectively. The standard errors about the means were less than 1% in both cases.

2.17 Isolation of N-nitrosamines

(i) Extraction of N-nitrosamines from aqueous solutions

Under ideal conditons, the partition coefficient, K_D, is independent of the amount of solute used. In this situation, provided that an equilibrium is reached between the extrahend and the extractant, the number of extractions required to attain a desired efficiency is governed by K_D and the relative volumes of the two phases.

Three readily available solvents - dichloromethane, ether and carbon disulphide - were tested (see 3.41i) for efficiency in the extraction of DENA from an aqueous solution. The concentration of DENA was estimated (see 2.16ic and 3.35iic) from the mean value of three determinations. In accordance with previous results [10], it was found that dichloromethane was the best solvent. The respective recoveries (mean values from two separate experiments) were 86.6, 50.4 and 37.8%

respectively. When 20 ml of an aqueous solution of DENA (11.7 ppm) was extracted (see 3.41ii) with different volumes of dichloromethane under similar conditions, the recoveries of DENA ranged from 50.4 to 95.3% (see Fig. 28). An almost quantitative (94.9%) recovery of DENA was obtained when the above aqueous solution was extracted (see 3.41ii) with three successive portions (5 ml) of dichloromethane. These results indicated that the value of the partition coefficient (estimates ranged from 9.2 to 20.3) was such that DENA was almost quantitatively transferred into dichloromethane after three successive extractions.

The efficiencies of extraction of DMNA, DENA, EPNA and DPNA from two different volumes (50 ml and 5 ml) of aqueous solution were determined under controlled conditions (see 3.42). Initially, nitrosamine concentrations were measured in each successive extract and subsequently, in separate experiments, the concentrations were measured in the combined extracts.

The aqueous solutions were extracted with five successive portions (5.0 and 2.0 ml respectively) of dichloromethane. Two separate periods (12 min and 4 min) of shaking were tried, and as the difference between them was negligible (compare Tables VI and VII), the shorter time was used in all subsequent work. For the larger volume (50 ml) of aqueous solution, over the range of concentrations studied (7.0 to 16.3 ppm), only DMNA was not recovered in greater than 90% yield after three extractions. For the smaller volume (5 ml) of aqueous solution, over the same range of concentrations, the recoveries of all four nitrosamines exceeded 71% after only two extractions. The concentrations were too low to be measured in subsequent extractions. The results, obtained from a minimum of four experiments, are tabulated in Tables VI to X. Fig. 29 illustrates the results obtained in Table VIII.

Further examination of the results obtained from the successive extraction of 50 ml of aqueous solution (see Table VIII and Fig. 29) revealed that DMNA was more extractable as the concentration diminished while the reverse situation was encountered for the remaining nitrosamines. The anomalous behaviour of the nitrosamines studied may be explained by differences in the degree of association or dissociation in the two phases. DMNA, over the range of concentrations studied, may exist as a dimer in aqueous solution since a linear relationship was found between the square of the amount of DMNA extracted and the concentration. The results obtained in section 2.11, together with those of other UV studies [24, 27, 54] in which the Beer-Lambert law was obeyed, appear to contradict these tentative conclusions. Further experiments are required before any definite conclusions may be reached.

With the above technique, the lower limit of quantitative estimation was about 3 ppm. The need for a sensitive method for the estimation of nitrosamines at the ppb level stimulated two approaches (a and b below) for improving the sensitivity :

(a) The response of the GLC detector could be enhanced. This may be achieved either by altering the chemical structure of nitrosamines, thereby rendering them sensitive to ECD detection, or by modifying the detector.

Since little was known about the chemistry of nitrosamines, and because of the need for rapid development of an analytical procedure, the former approach was not investigated. Subsequently, Sen[103] and Althorpe et al [104] converted nitrosamines to nitramines, a few pg of which were detected by ECD. The method was not, however, placed on a quantitative basis.

The latter approach proved unsuccessful in this laboratory. The use (see 3.19iii1) of a Perkin-Elmer halogen phosphorus detector did not lead to any improvement in sensitivity when a dichloromethane solution of nitrosamines was chromatographed and detected. This result conflicted with previous reports [57, 59]. After this procedure had been abandoned, it was learnt [169] that dichloromethane was an unsatisfactory solvent for use with this type of detector.

(b) The concentration of nitrosamines, after isolation from foods, could be increased. Concentration of rigorously dried dichloromethane solutions through fractionating columns (see 3.52ii) or a Kuderna-Danish evaporator (3.52iii) was successfully employed (see Table XI). The use of a vacuum-jacketed vigreaux column led to results comparable with those obtained from the Kuderna-Danish evaporator. Hence, in certain cases where nitrosamines are known to be present at the ppb level, the amount can be estimated by the above method, providing that interfering substances have first been removed.

The findings of Eisenbrand et al [44] record that evaporation of nitrosamines solutions, in the ordinary way or in a vacuum, leads to excessive losses. These findings were confirmed (see Tables XII, XIII and XIV).

(ii) Estimation of N-nitrosamines in biological materials

The selective extraction of nitrosamines from biological materials, and the subsequent clean-up of these solutions prior to analysis, has been a serious obstacle in many methods so far devised. Five methods of extraction were investigated :

(a) percolation of a finely ground sample with dichloromethane ;

(b) extraction of the sample with dichloromethane in Soxhlet apparatus;

(c) steam distillation followed by dichloromethane extraction of the distillate;

(d) preparative dry-column chromatography;

(e) freeze-drying of the sample followed by dichloromethane extraction of the distillate .

(a) In the percolation procedure (see 3.62i) the recovery of DENA, added to finely ground maize, was unacceptably low (see Table XV). When dichloromethane, ether or carbon disulphide were used as extractants. The recovery was not significantly improved when rates of percolation were varied.

(b) Soxhlet extraction of cellulose powder or finely ground maize, spiked with DENA (see 3.62ii), was effected in the normal way with dichloromethane as solvent. The recoveries, although satisfactory (greater than 95%) for cellulose powder, proved inconsistent for the finely ground maize (see Table XVI). Some extracts, obtained from maize, were freed from high boiling components by flash distillation (see 2.18 and 3.63ii). In experiments with dichloromethane extracts containing 30 ppm of DENA recoveries of 90% were recorded. This procedure was abandoned in favour of the simpler, and more efficient , freeze-drying technique .

(c) Steam distillation of comminuted Solanum incanum fruit, spiked (see 3.62ii) with DENA, was hindered by frothing of the homogenized mass. When distillation proceeded at a pace slow enough to reduce frothing to an acceptable level, the recovery of DENA in the distillate was low (58%).

The remote possibility of artefact formation or chemical interference ruled out the inclusion of an anti-foaming agent. Shortly after this, Sen [47] discovered that steam distillation of food samples in the presence of excess nitrite and large amounts of secondary amines produced large amounts of nitrosamines. The procedure was then abandoned.

(d) Dry column chromatography [53] is a procedure for the rapid preparative separation of a mixture. This technique was used (see 3.13 and 3.62iv) to clean-up methanolic plant extracts. Preliminary experiments (see 3.13) established that the R_f-values of DMNA, DENA, EPNA and DPNA (chromatographed on deactivated alumina packed in flexible nylon tubes) all occurred in a section (nitrosamine fraction) with R_f-values of 0.55 to 0.75. The effectiveness of this technique was tested with a methanolic extract of Solanum incanum containing a known amount of DMNA and with a solution of DMNA of known concentration. The nitrosamine fractions obtained were divided into two equal portions and extracted with three successive aliquots of dichloromethane or ether. The amounts of DMNA recovered at this stage were in the region of 50% (see Table XVII). After the solutions had been concentrated under vacuum at 0° to final volumes of about 4 ml, the overall recoveries had declined to about 10% (see Table XVII). It was not very effective [32] in removing contaminants and the freeze-drying procedure for isolating nitrosamines was substituted.

(e) Although Lydersen and Nagy [65] successfully used vacuum distillation at 40° to recover DMNA added to fish, the procedure was not in general use. The advantages to be gained by the separation of volatile material from a biological sample prompted the use of the freeze-drying

procedure. In this technique (see 3.14), the sample, after fine comminution in a Waring blender, was introduced into flasks and immediately frozen by rotating the flasks in liquid air. It was then freeze-dried in the normal way, the distillate being collected in a trap cooled in liquid air. After thawing, the distillate was extracted with dichloromethane and the nitrosamines estimated by GLC (see 2.16ie and 3.35iie). Preliminary experiments (see 3.62va) showed that the recoveries of DENA, added to tomatoes, marrow and fish (haddock and stockfish) exceeded 90%. The effectiveness of the technique was then tested (see 3.62vb) by freeze-drying aqueous solutions of nitrosamines (DMNA, DENA, EPNA and DPNA), and a mixture of cellulose powder and water containing these four nitrosamines. The latter mixture was intended to simulate a biological sample. Recoveries were measured from experiments performed in triplicate and the GLC estimations were also performed in triplicate. Relative deviations between both individual experiments and estimations were seldom more than 4.5% and never more than 7%. The recoveries of nitrosamines (see Table XVIIIA) ranged from 80% for the most volatile (DMNA) to 101% for the least volatile (DPNA) of the nitrosamines tested. Acceptable recoveries of DMNA, DENA, EPNA and DPNA were recorded from spiked samples (see 3.62vb) of beans, marrow and haddock (see Table XVIIIA,B). The recoveries were partly dependent on the concentration of the nitrosamines, slightly lower recoveries being obtained at lower concentrations.

In the above-mentioned materials, few substances giving rise to interfering GLC peaks were encountered. Clear qualitative detection of the above four nitrosamines was possible down to levels of 500 ppb. The lower limit for quantitative work was 3 ppm but this range can be extended (see 2.17ib) by careful concentration of the rigorously dried solution. The effectiveness of this technique depends upon the complete

distillation of all the nitrosamines and cannot be used for the less volatile nitrosamines. Recently preliminary studies [170] have shown that higher molecular weight nitrosamines can be recovered satisfactorily by a process similar to that of Lydersen and Nagy [65].

2.18 Clean-up of extracts

Many of the substances which interfere with the GLC determination of nitrosamines may be removed, either by washing with acid and alkali (see 2.19i and 3.63i), or by flash distillation (see 3.63ii).

Reactive pre-columns [171, 172] may be used to remove certain groups of components before gas chromatography. The feasibility of removing substances containing three types of functional group, was investigated. Carbonyl compounds (cyclohexanone, acetone, acetaldehyde and n-butyraldehyde) were removed (see 3.63iiia) by semicarbazide hydrochloride. Alkanes (n-pentane, n-heptane, n-octane and n-nonane) were removed by Molecular Sieve type 5A (see 3.63iiib). Boric acid and berylium acetylacetonate did not remove ethanol or n-butanol.

Further experiments, in which pre-columns are attached to a GLC-MS system, are in progress.

2.19 Survey of Transkeian plants for the possible presence of N-nitrosamines

The possible relationship of cancer in the Transkei to the presence of nitrosamines in foodstuffs eaten by the Bantu and the initiation of field experiments, has been discussed [173]. The plant material collected for analysis is listed (see Table XIX) according to the local Bantu names since much of it is not yet botanically classified.

Field experiments and suitable laboratory facilities were separated

by a distance too great to warrant the transportation of samples as they were collected. A simple extraction procedure, comminution with methanol (see 3.71i), was adopted. Two extraction procedures, percolation and freeze-drying (see 3.71ii and 3.71iii respectively), were generally used in this laboratory.

(i) Solanum incanum

TLC (see 3.12) of the methanolic extract (see 3.71i) did not reveal any spots with R_f-values corresponding to those of N-nitrosamines. Preparative TLC (see 3.12) of the methanolic extract yielded an almost colourless oil which gave a positive Lassaigne's test for nitrogen. When a portion of this oil was repeatedly spotted onto a TLC plate and chromatographed as described below (3.12), a spot corresponding to DMNA appeared 7 min after spraying with the spray reagent of Preussmann et al [75, 76]. Other very faint spots suggested the presence of DENA and DPNA. The spot corresponding to DMNA increased in intensity when authentic material was added to a portion of the oil. The IR spectrum of this oil (8% in carbon tetrachloride) revealed several peaks (3012 wsh, 1287 m, 1040 w, cm^{-1}; s, strong; m, medium; w, weak; sh, shoulder) co-incident with the IR spectrum of authentic DMNA. The minute amount of material recovered, after many weeks of preparative TLC, led to this procedure being replaced by dry-column chromatography (see 3.13 and 3.62iv) which is more suited to preparative applications. A "nitrosamine-rich fraction" (145 mg of a colourless oil) was produced from 360 g of methanolic plant extract.

The oil (3% in carbon tetrachloride) was separated on column a (see 3.17ia) and found to contain at least eleven components, with retention times which varied from 0.7 to 4.2 min. A peak occurring at 1.4

min corresponded with the retention time of DMNA. The peak tentatively assigned to DMNA increased in intensity when authentic material was added to the sample. The examination was repeated on column b (see 3.17ib) and the results were the same - the peak tentatively assigned to DMNA corresponded with the retention time of authentic material and increased in intensity when authentic material was added. Gas chromatography was repeated on column c (see 3.17iic). The same chromatographic results were obtained, a peak at 3.9 min increasing in intensity when authentic DMNA was added. A further repetition on column d (see 3.17iiid) gave the same results - a peak at 4.9 min increased in intensity when authentic material was added. This peak was still prominent (see Fig. 29) after 200 µl of the oil (3% in carbon tetrachloride) had been neutralized with dilute sulphuric acid and dilute sodium hydroxide. The fraction with a retention time of 4.9 min (on column d) was collected (see 3.19iiid) in a porous plug [174] of 25 µl of deuterochloroform (spectroscopic grade) in a tube immersed in liquid air. After successive injections, 1 mg of this fraction had accumulated in the porous plug. The deuterochloroform solution was subsequently warmed to room temperature and sealed in a micro-ampoule (10 X 1.7 mm ID) which was placed in a standard NMR tube having a 1.7 cm nylon plug [174] at the bottom. A solution of tetramethylsilane (2% in deuterochloroform), added to the NMR tube to cover the ampoule, served as an external standard. The NMR spectrum (see Fig. 30B) several peaks, including two at 6.34 and 7.05 ppm which were characteristic of the spectrum of authentic DMNA (see Fig. 30A) taken under the same conditions. The deuterochloroform solution was transferred to a micro-infrared cell and the IR spectrum (see Fig. 31) contained fifteen

peaks co-incident with those of authentic DMNA (see Fig. 32). These results showed that DMNA was present in the sample of Solanum incanum examined. Owing to the mild conditions of extraction and concentration used, there appears to be little likelihood of the DMNA being an artefact.

(ii) Maize

The extract (see 3.71ii) was freed from high boiling components (see 3.63ii) and examined by GLC (column i; see 3.17iiii). A peak occurred at 4.63 min which corresponded with the retention time of DENA under the same conditions. This sample was not examined further and there is insufficient evidence to conclude that DENA was present in the extract.

(iii) Beans

The extract was prepared and chromatographed as above (2.19ii) and DENA was tentatively identified in the extract.

(iv) Other plants

There was no evidence of low molecular weight nitrosamines in the remaining plant extracts.

2.20 Conclusion

An extensive survey, using GLC-MS, for the presence of nitrosamines in the above plants (see Table XIX) and other biological material, is in progess.

3 EXPERIMENTAL

3.1 GENERAL

3.11 Glassware was cleaned in hot chromic acid.

3.12 Thin layer chromatography was performed on glass plates (7 X 14 and 20 X 20 cm) coated with Silica Gel G (Merck) containing calcium sulphate as binder. A Desaga TLC applicator was used to apply 1 g and 6 g of coating to the respective plates which were developed in a preserving jar and a Shandon T.L.C. Chromatank respectively. For preparative work, the Silica Gel was sucked up from the developed plates with a glass funnel (4 cm diameter) joined by PVC tubing to a Buchner flask connected to a waterpump.

3.13 Dry-column chromatography [53] improved the isolation of nitrosamine-rich fractions from plant extracts. In this technique, the plant extract was chromatographed (eluent : dichloromethane) on a column comprising a flexible nylon tube (supplied by Walter Coles and Co., Walworth, London, England) packed with deactivated neutral alumina. The neutral alumina (Merck) was heated (500 to 600^{o} for 3 to 4 h) after which it was allowed to absorb 10% by weight of water. The positions of DMNA, DENA and DPNA were judged by carrying out a parallel experiment on a quartz tube (18 X 0.5 in ID) packed with 1% of a fluorescent indicator (Woelm fluorescent green indicator). Under UV irradiation (256 nm) the above nitrosamines were rendered visible as a dark, broad band against a fluorescent background. The results obtained with Silica Gel as adsorbent were not reproducible and alumina was used in subsequent experiments. The above nitrosamines were chromatographed on alumina supported in a flexible nylon tube (48 X 1 in ID) which was subsequently

cut up into sections (R_f 0.55, 0.55 to 0.75, 0.75) and extracted with dichloromethane. The first section was extracted with 500 ml while the second and third sections were each extracted with 250 ml of dichloromethane. The filtered extracts were examined by UV spectrophotometry. The nitrosamines were found exclusively in the section with R_f 0.55 to 0.75 (nitrosamine fraction). Identical results were obtained with a 48 X 2 in ID flexible nylon tube packed with alumina. Plant extracts (see section 3.71i for method of preparation) were chromatographed on the latter columns. The nitrosamine fraction was cut out and eluted with ether. The filtered extract was evaporated under vacuum (water-pump) at less than 0°.

3.14 Freeze-drying of samples. In this technique the material, after fine comminution in a Waring Blender, was introduced into flasks and immediately frozen in liquid air. The flasks, enclosed within gauze jackets in case of an implosion while under vacuum, were then freeze-dried in the normal way, the distillate being collected in a trap cooled in liquid air.

3.15 Description and method of use of freeze-drying apparatus.

Plates number 1, 2, 3 and 5 show the whole or portions of a 1 m rule.

Plate number 1 shows a high vacuum pump (Edwards "Speedivac" High Vacuum Pump Model 2SC30), mounted on a trolley, fitted with a liquid air trap for protecting the pump from moisture. The liquid air container (Dewar Flask) is shown in the lowered position.

Plate number 2 depicts the top of the trolley pump. The hole at X allows liquid air to be poured, through a funnel, directly into the

Dewar.

Plate number 3 depicts the apparatus for freeze-drying large samples (up to 400 g of distillate can be collected in the condenser). The sample to be freeze-dried is apportioned, as a slurry, into two 2-litre round-bottomed flasks. Each flask is then rapidly rotated in liquid air in order to distribute the sample in an even layer around the bottom half of the flask.

Plate number 4 shows the freeze-drying apparatus (for large samples) connected to a waterpump. Initially the condenser is lowered about halfway into a 7-litre Dewar flask containing liquid air. To complete the freeze-drying, the condenser is lowered further when a thick ring of condensate (about 200 g) has accumulated. This prevents the condenser from becoming clogged by a ring of condensate before the freeze-drying is completed. The condenser is shown fully lowered in the plate. (Aqueous samples freeze-dry satisfactorily at pressures of < 0.3 mm Hg).

Plate number 5 shows the apparatus for freeze-drying small samples (about 50 g). The inlet to the condenser is attached by means of a Quickfit joint.

Plate number 6 depicts three small condensors fitted into one 7-litre Dewar Flask.

3.16 IR spectra were recorded on a Beckman IR-8 instrument equipped with a reference beam attenuator (RIIC no. AT-02). The sample chamber was purged with dry nitrogen before the spectra were measured from thin films between KBr discs (25 mm diameter), micro KBr discs (5 mm diameter) or micro liquid cells (RIIC no. MC-03). The micro cells were mounted in a beam condenser (RIIC no. C-21).

3.17 UV spectra were recorded (slow scan speed) on a Unicam SP.800 B instrument. Samples and reference materials were contained in matched UV Grade Silica cells (1 cm) equipped with teflon stoppers.

3.18 NMR spectra were recorded on a Perkin-Elmer R12 instrument with the scale set to 10 ppm.

3.19 Gas chromatography, unless otherwise stated, was performed with liquid phases supplied by Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A. The following instruments were used:

(i) A Beckman GC-2A gas chromatograph equipped with an FID and a Texas Instruments Servo/Writer II recorder fitted with a Disc Model 200 integrator. With nitrogen as carrier gas, the following conditions were used:

(a) Stainless steel column (1.83 m X 4.8 mm ID) packed with 3% ECNSS-M on Gas Chrom Q 80 - 100 mesh. Respective oven and injector temperatures were 100 and 160°. Nitrogen, hydrogen and air flow rates were 164, 57 and 400 ml/min respectively.

(b) Stainless steel column (1.83 m X 4.8 mm ID) packed with 14% HI-EFF-4BP on Gas Chrom CLA 80 - 100 mesh. Respective oven and injector temperatures were 100 and 200°. Nitrogen, hydrogen and air flow rates were 164, 40 and 351 ml/min respectively.

(ii) A Beckman GC-4 gas chromatograph equipped with dual FID's and a Beckman 10 in recorder. With nitrogen as carrier gas, the following conditions were used:

(c) Dual glass columns (1.07 m X 4.8 mm ID) packed with 14.5% HI-EFF-1BP on Gas Chrom P 80 - 100 mesh. Respective oven, inlet, inlet line, detector and detector line temperatures were 100, 173, 114, 140 and 133°. Nitrogen, hydrogen and air flow rates were 91, 67 and 172 ml/min

respectively.

(d) Dual glass columns (1.19 m X 12.5 mm ID) packed with 15% Carbowax 1000 on Gas Chrom P 80 - 100 mesh. Respective oven, inlet, inlet line, detector and detector line temperatures were 100, 200, 196, 250 and 248°. Nitrogen, hydrogen and air flow rates were 115, 60 and 233 ml/min respectively.

(e) Dual glass columns (1.19 m X 12.5 mm ID) packed with 14.5% HI-EFF-1BP on Gas Chrom P 80 - 100 mesh. Other GLC parameters were identical with (d).

(f) Dual glass columns (1.17 m X 4.1 mm ID) packed with 15% Carbowax 1000 on Gas Chrom P 80 - 100 mesh. Respective oven, inlet, inlet line, detector and detector line temperatures were 120, 210, 202, 245 and 240°. Nitrogen, hydrogen and air flow rates were 50, 80 and 262 ml/min respectively.

(iii) A Perkin-Elmer 900 gas chromatograph equipped with dual FID's and a Hitachi QPD_{54} recorder. An effluent stream splitter (20 : 1) was fitted to the outlet of one of the columns. With nitrogen as carrier gas, the following conditions were used:

(g) Dual glass columns (1.18 m X 4.0 mm ID) packed with 5% HI-EFF-4BP on Gas Chrom P 80 - 100 mesh. Respective oven, injector and manifold temperatures were 120, 173 and 283°. Nitrogen, hydrogen and air flow rates were 11, 24 and 224 ml/min respectively.

(h) The operating conditions were the same as (g) except that the nitrogen, hydrogen and air flow rates were 9, 27 and 300 ml/min respectively.

(i) Dual glass columns (1.18 m X 4.0 mm ID) packed with 15% HI-EFF-4BP on Gas Chrom P 80 - 100 mesh. Respective oven, injector and manifold

temperatures were 150, 224 and 251°. Nitrogen, hydrogen and air flow rates were 13, 24 and 351 ml/min respectively.

(j) Operating conditions were the same as (i) except that nitrogen, hydrogen and air flow rates were 14, 27 and 350 ml/min respectively.

(k) The columns were identical with (i). Respective oven, injector and manifold temperatures were 180, 250 and 320°. Nitrogen, hydrogen and air flow rates were 14, 37 and 250 ml/min respectively.

(l) Operating conditions were the same as (i) except that a Perkin-Elmer Halogen-Phosphorus Detector Accessory (supplied by Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.) was operated in conjunction with one of the FID's.

(iv) A Perkin-Elmer 900A gas chromatograph equipped with dual FID's and a Hitachi QPD_{54} recorder. Helium was used as carrier gas since the instrument was linked by a fritted glass separator [113] to a mass spectrometer (Hitachi - Perkin-Elmer RMS4). An effluent stream splitter (20 : 1) was fitted to the outlet of the column leading to the mass spectrometer. In this way, 95% of the column effluent passed towards the mass spectrometer and the remaining 5% was detected in the FID. The operating conditions were the same as (i) and the temperature of the separator was 200°.

(v) A Pye Series 104 gas chromatograph was linked by membrane separator (157) to an AEI MS30 mass spectrometer. Helium was used as carrier gas and all the column effluent passed towards the mass spectrometer. The following conditions were used :

(m) Glass column (1.52 m X 4.8 mm ID) packed with 3% SE-30 on Gas Chrom Q 100 - 120 mesh (supplied by Pye Unicam Ltd., Cambridge, England). The respective oven, inlet and separator temperatures were 140, 170 and 40°. The helium flow rate was 41 ml/min.

(n) Glass column (1.52 m X 4.8 mm ID) packed with 5% F.F.A.P. on

Chromosorb G 80 - 100 mesh (supplied by Perkin-Elmer Corp.). The respective oven, inlet and separator temperatures were 140, 170 and 40°. The helium flow rate was 46 ml/min.

(o) Glass column (1.52 m X 4.8 mm ID) packed with 3% 2-CEMS on Chromosorb G 80 - 100 mesh (supplied by Perkin-Elmer Corp.). The respective oven, inlet and separator temperatures were 130, 170 and 40°. The helium flow rate was 48 ml/min.

(p) Glass column (2.72 m X 4.8 mm ID) packed with 1.5% OV-17 on Gas Chrom Q 80 - 100 mesh. The respective oven, inlet and separator temperatures were 140, 170 and 40°. The helium flow rate was 35 ml/min.

(q) The operating conditions were the same as (p) except that the column was temperature programmed from 130 to 260° at 20°/min.

(r) Glass column (2.72 m X 4.8 mm ID) packed with 1% HI-EFF-4BP on Gas Chrom CLA 80 - 100 mesh. The respective oven, inlet and separator temperatures were 145, 170 and 40°. The helium flow rate was 56 ml/min.

3.20 Mass spectrometry was performed with two instruments:

(i) A Hitachi - Perkin-Elmer RMS4 instrument, equipped with a solid probe inlet, was fitted to a Perkin-Elmer 900A gas chromatograph by means of a fritted glass separator [113]. Mass spectra were recorded (Honeywell Model 2106 Visicorder) at an ionising voltage of 70 eV.

(ii) An AEI MS30 instrument, equipped with a solid probe inlet and an all-glass heated inlet system, was fitted to a Pye Series 104 gas chromatograph by means of a membrane separator [153]. Mass spectra were recorded (AEI U.V. Series 10-430S recorder) at an ionising voltage of 70 eV.

3.2 PREPARATION OF N-NITROSAMINES

N-nitrosamines were prepared according to standard procedures and

and fractionally distilled until pure according to gas chromatography. The boiling points are tabulated in Table III.

3.3 DETECTION AND SEPARATION OF N-NITROSAMINES

3.31 UV spectrophotometry

Solutions were prepared in volumetric flasks by diluting weighed portions of each nitrosamines (DMNA, DENA, MPNA, EPNA and DPNA) with solvent. The solvents were : DMNA, MPNA, EPNA and DPNA - water ; DENA - water and water saturated with carbon disulphide. The UV spectra of all the nitrosamines exhibited a low intensity absorption at ca. 333 nm and a high intensity absorption at ca. 233 nm (see Table III). Calibration curves were established, by the method of least squares [167], for DMNA and DENA from both maxima using respective nitrosamine concentrations of 115 to 2166 and 2.4 to 30.3 μg/ ml (see Figs. 1 to 4).

3.32 IR spectroscopy

(i) Liquid phase spectra of all the above N-nitrosamines (see 3.31) were recorded from pure liquids and from solutions of nitrosamines in carbon tetrachloride. The spectrum of DMNA was also recorded in deuterochloroform. Fig. 25 illustrates the IR spectrum of DMNA recorded from pure liquid.

(ii) Concentration of dilute N-nitrosamine solutions in KBr wedges. A KBr wedge (Harshaw Chemical Co., Cleveland, Ohio, U.S.A.) was placed, base down, in a vial containing a solution (0.5 ml) of DMNA (10 mg). The migration of the solution to the tip of the wedge and the evaporation of the solvent (at room temperature) served to concentrate the solute. The solute was further concentrated at the tip of the wedge by

the addition of solvent (carbon tetrachloride, dichloromethane, or methyl bromide) when the initial solution was exhausted. The tip of the wedge was compressed into a micro-disc (5 mm diameter) which was examined by IR spectrophotometry.

3.33 NMR spectrophotometry

NMR spectra were obtained from milligram quantities of materials contained in micro-ampoules (100 X 1.7 mm ID). A solution of material was introduced (micro-syringe) into the ampoule which was sealed after it had been cooled in liquid air The sealed ampoule was placed in a standard NMR tube with a 1.7 cm nylon plug at the bottom. A solution of tetramethylsilane (2% in deuterochloroform), added to the tube to cover the ampoule, served as an external standard.

3.34 Thin-layer chromatography

(i) Separation of N-nitrosamines. DMNA, DENA, DPNA, NPIP and DPHNA were chromatographed with hexane : ether : dichloromethane :: 4 : 3 : 2, carbon tetrachloride : hexane : chloroform ether :: 10 : 3 : 2 : 1 and carbon tetrachloride : dichloromethane :: 6 : 4 as solvents. The N-nitrosamines were rendered visible by spraying with Preussmann's reagent [75, 76].

(ii) Separation of N-nitrosamine derivatives. The N-nitrosamines were reduced to hydrazines which were condensed with 5-nitro-2-hhdroxy-benzaldehyde and the yellow hydrazones were separated by TLC with carbon tetrachloride : ethyl acetate :: 95 : 5 as solvent [79].

(a) Preparation of 5-nitro-2-hydroxybenzaldehyde. Dimethoxymethane was coupled with p-nitrophenol to yield 5-nitro-2-hydroxybenzylchloride which was oxidized to the aldehyde.

(b) Preparation of dimethoxymethane. The method of Houben [175], described in Organic Syntheses [176], was used to prepare a 56% yield of dimethoxymethane (b.p. 42 - 46°).

(c) Preparation of 5-nitro-2-hydroxybenzyl chloride. The method described in Organic Syntheses [177] was used to prepare a 74% yield of 5-nitro-2-hydroxybenzyl chloride (m.p. 128°).

(d) Sommelet oxidation of 5-nitro-2-hydroxybenzyl chloride. The procedure of Angyal et al [178] was employed to prepare a 53% yield of 5-nitro-2-hydroxybenzaldehyde (m.p. 127°).

3.35 Gas chromatography

(i) Detection of N-nitrosamines. N-nitrosamines were detected on a variety of columns (see section 3.19).

(ii) Quantitative estimation of N-nitrosamines. The method of least squares [159] was used to establish the best straight line through the data points.

On column (g) without an internal standard

(a) DMNA (retention time : 2.58 min) was estimated from calibration curves established at attenuations of 5000, 2000, 200 and 50. Standard solutions of DMNA in ether were prepared in volumetric flasks by diluting weighed portions of nitrosamines (see Calibration Table 1). Concentrations of DMNA were chosen to give three or four well-spaced points on the individual calibration curves. Each point represented the mean peak area obtained from three one µl injections.

On column (h) with an internal standard

(b) DMNA (retention time : 3.58 min) was estimated from a calibration

curve established (at an attenuation of 2) by means of the internal standard method [160]. Standard solutions of DMNA in carbon disulphide were prepared as above (3.35iia). An aliquot of the internal standard (p-xylene ; retention time : 1.69 min), sufficient to generate a peak of 100 mm (at an attenuation of 200) on the gas chromatogram, was added to each of the solutions of DMNA. Concentrations of DMNA were chosen to give five well-spaced points on the calibration curve (see Calibration Table 2). Each point represents the mean peak area obtained from four or five one µl injections.

On column (i) with an internal standard

(c) DENA (retention time : 3.26 min) was estimated by the above method (3.35iib) at attenuations of 200, 20 and 2. Ether was used as solvent and cyclohexanone (retention time : 4.79 min) was the internal standard. Concentrations of DENA were chosen to give five or six well-spaced points on each calibration curve. Each point represents the mean peak area obtained from four or five one µl injections. Calibration Table 3 contains the data used to construct the respective calibration curves.

(d) DENA was also estimated (3.35iic) with dichloromethane as solvent, at an attenuation of 20. Calibration curves for the estimation of DENA were constructed by four methods : 1) from the peak area of DENA 2) from a ratio of peak area of DENA to peak area of internal standard 3) from the peak height of DENA 4) from a ratio of peak height of DENA to peak height of internal standard. Concentrations of DENA were chosen to give six well-spaced points on the calibration curves. Each point represents the mean reading of four one µl injections. Calibration Table 4 contains the data used to construct the respective calibration curves.

On column (j) with an internal standard

(e) DMNA, DENA, EPNA and DPNA (retention times : 3.34, 4.56, 5.74 and 7.23 min respectively) were estimated in dichloromethane solutions. Standard solutions of the nitrosamines, containing the internal standard (chlorobenzene ; retention time : 1.98 min), were prepared. The concentration of each nitrosamine was chosen such that they all generated peaks of the same order of magnitude on the gas chromatogram (Fig. 25). Two sets of calibration curves were constructed at attenuations of 200 and 20. Concentrations of nitrosamines were chosen to give five well-spaced points on each calibration curve. Each point represents the mean peak height obtained from a minimum of five one μl injections. The rapid analysis time, combined with the high sensitivity employed, resulted in the tailing of the solvent through the DMNA and DENA peaks. These peak heights were measured from the interpolated base-line of the tail. Since the injected solutions contained the four nitrosamines under examination, simultaneous plotting on all four calibration curves was possible. Calibration Tables 5 and 6 contain the data used to construct the respective calibration curves. A table for interpolation, using a least squares fit [167] to a straight line relating the concentration of nitrosamine to h (10 X peak height of nitrosamine / peak height of internal standard), was calculated from a computer programme written in Extended Mercury Autocode. The calibration curves were redetermined when the column was repacked.

On column (i) with an external standard

(f) DMNA, DENA, EPNA and DPNA. Solutions of these nitrosamines, without any internal standard, were estimated on the above (3.35iie)

calibration curves. The mean reading obtained from five one μl injections of chlorobenzene, diluted to the same concentration as that used above (see 3.35iie), was used to obtain the ratio h.

On column (k) with an internal standard

(g) DENA and NPYROL (retention times : 2.32 and 7.41 min respectively) were estimated in dichloromethane solutions at respective attenuations of 160 and 40. Standard solutions of the nitrosamines, containing the internal standard (naphthalene; retention time 5.65 min), were prepared as above (see 3.35iib). The concentration of each nitrosamine was chosen (see Calibration Table 7) such that both of them generated peaks of the same order of magnitude on the gas chromatogram. Calibration curves were constructed from five well-spaced points, each point representing the mean value of seven determinations.

3.36 Mass spectrometry

(i) Hitachi - Perkin-Elmer RMS4 mass spectrometer linked to a Perkin-Elmer 900A gas chromatograph. Mass spectra were recorded after pure nitrosamines (DMNA, DENA, MPNA, EPNA, DPNA and NPIP) had been introduced into the gas chromatograph. A computer programme, written in Fortran IV, was used to display the normalised mass spectra in the form of bar graphs (Figs. 8, 10, 12, 14, 16 and 19 respectively). The detection limit for DMNA in dichloromethane was 1.5 μg (introduced into the GLC).

(ii) AEI MS30 mass spectrometer linked to a Pye Series 104 gas chromatograph. An identical procedure (see 3.36i) was used to record and normalise mass spectra of the above nitrosamines (see 3.36i). The normalised mass spectra are displayed in Figs. 9, 11, 13, 15, 17 and 20. Fig. 18 illustrates the mass spectrum of DBNA. The detection limit for DMNA

in dichloromethane was 400 ng (introduced into the GLC).

3.37 Identification of N-nitrosamines by computer matching of low resolution mass spectra

(i) Pure N-nitrosamines. A computer program written in Fortran IV, was used to identify an unknown N-nitrosamine by matching its mass spectrum against a library file of standard N-nitrosamine spectra. The standard spectra were compiled from the above mass spectra (3.36i and 3.36ii) and from data reported by Collin [99], Schroll et al [110], Pensabene et al [109] and ApSimon et al [111]. N-nitrosamines of the same molecular weight were matched according to four matching procedures (160) :

Method 1 : the m/e values of the n largest intensities were compared irrespective of order. The number of agreements, A, was noted. The degree of matching, P_1, was determined from :

$$P_1 = \frac{A}{n}$$

Method 2 : made allowance for the relative positions of peaks with equal m/e values. The degree of matching, P_2, was determined from :

$$P_2 = \frac{1}{n^2} \sum_{k=1}^{A} \left(n - \left|i - j\right|_k\right)$$

where A was the number of agreements irrespective of order, and i and j were the positions in the respective sets of the kth pair of equal m/e values.

Method 3 : the mass spectrum was divided into R equal ranges. Each

range contained m mass units. The n most intense peaks were selected in order of increasing intensity. The number of agreements A_r in m/e values, irrespective of order, were counted in each range r. The degree of match for each range was calculated as in method 1 and then averaged over the R ranges to give the degree of matching P_3.

$$P_3 = \frac{1}{R} \sum_{r=1}^{R} \frac{A_r}{n}$$

Method 4 : treated the n strongest peaks within each range in a similar manner to method 2 and the degree of matching, P_4, was determined from :

$$P_4 = \frac{1}{R} \sum_{r=1}^{R} \frac{1}{n^2} \sum_{k=1}^{A_r} (n - |i - j|_k)$$

Each DMNA mass spectrum was in turn compared (methods 1 to 4) with the remainder of the library. The results are set out in Table IV.

(ii) Impure N-nitrosamines. A computer program written in Fortran IV, was used to detect variations in successive mass spectra recorded over a gas chromatographic peak. An average "background" mass spectrum, computed from spectra scanned on either side of a gas chromatographic peak, was subtracted from the spectrum recorded at the top of the peak. This method was used to obtain three successive mass spectra from 1.0 μg of DMNA in 10 μl of dichloromethane. Figs. 21, 22 and 23 illustrate the normalised mass spectra and Fig. 24 shows the mass spectrum of DMNA obtained after subtraction of the average "background". The latter mass spectrum was compared with the library of spectra (see Table IV).

3.38 Identification of N-nitrosamines using high resolution mass spectrometry

High resolution (ca. 3400) mass measurements, with perfluorokerosene and DMNA as standards, were used to confirm the elemental composition of ions 44 and 74 in the mass spectrum of DMNA.

3.4 EXTRACTION OF N-NITROSAMINES FROM AQUEOUS SOLUTIONS

3.41 DENA

(i) Suitable solvents. Aqueous solutions (20 ml) of DENA (11.7 ppm) were extracted with aliquots (20 ml) of carbon disulphide, ether or dichloromethane in a separating funnel (50 ml) by shaking for 8 min. After settling (10 min) the solvent was run off, dried (15 min over anhydrous sodium sulphate) and the DENA estimated by GLC (see 3.35iic). The amount of DENA in the aqueous phase, after extraction with carbon disulphide, was estimated by UV spectrophotometry (see 3.31).

(ii) Extraction with dichloromethane. The above (3.41i) solution (20 ml) was used to determine the efficiency of extraction of 2, 5, 10, 15, 20 and 25 ml aliquots of dichloromethane. The mean results from two separate experiments are shown graphically in Fig. 28. A similar method was used to extract 20 ml of aqueous solution (3.41i) with three successive portions (5 ml) of dichloromethane. The amount of DENA was measured in the combined extracts.

3.42 DMNA, DENA, EPNA and DPNA

The efficiency of extraction from two different volumes of aqueous solution was determined. In the first instance, nitrosamine concentrations were measured in each successive extract and subsequently, in a separate experiment, the concentrations were measured in the combined

five extracts. The aqueous solutions (50 and 5 ml), containing known amounts of all four nitrosamines, were extracted with five successive portions (5.0 and 2.0 ml respectively) of dichloromethane in glass-stoppered test tubes (10 X 3 and 8 X 3 cm respectively) for an exact period of time. Nitrosamine concentrations were measured after two separate periods of shaking (12 and 4 min) and, as the difference between them was negligible, the shorter time was used in all subsequent work. After settling (4 min), the dichloromethane was run off, dried (5 min over anhydrous magnesium perchlorate) and the nitrosamines separated and estimated by GLC (3.35iie). The results are tabulated in Tables VI to X. The results given in Table VIII are shown graphically in Fig. 29.

3.5 EVAPORATION OF DICHLOROMETHANE SOLUTIONS OF N-NITROSAMINES

3.51 In a vacuum

(i) Solutions evaporated in round-bottomed flasks. Aliquots (25 ml) of a dichloromethane solution, containing known amounts of N-nitrosamines, were pipetted or weighed into tared 50-ml round-bottomed flasks, and evaporated under vacuum (water-pump) so that a final volume of about 5 ml was reached in 7 to 8 min. The volume of the concentrate was determined by weighing and the nitrosamines estimated as above (3.35iie). The results are tabulated in Table XIII.

3.52 At atmospheric pressure

(i) Solutions evaporated in round-bottomed flasks. Aliquots (25 ml) of nitrosamines in dichloromethane in tared 50-ml round-bottomed flasks were concentrated on a waterbath at 50° to a final volume of about 5 ml

and the nitrosamines estimated as above (see 3.35iie). The results are tabulated in Table XIV. This experiment was repeated with dichloromethane which had been dried over anhydrous magnesium perchlorate for 20 h, and repeatedly distilled until gas chromatographically pure. Aliquots (50 ml) of nitrosamines in dichloromethane in tared 100-ml round-bottomed flasks were concentrated on a waterbath at 65° to a final volume of about 5 ml as above. The nitrosamines were estimated (see 3.35iif) and the results are tabulated in Table XII.

(ii) Solutions evaporated in a Kuderna-Danish evaporator. An aliquot (100 ml) of the dried solution (see 3.51i) was evaporated in a Kuderna-Danish evaporator (250 ml) fitted with a 10-ml collection tube, to a final volume of about 5 ml. The nitrosamines were estimated (see 3.35iif) and the results are tabulated in Table XI.

(iii) Solutions evaporated in flasks fitted with Vigreaux columns. The columns (300 X 15 and 120 X 10 mm), the latter enclosed in a vacuum jacket, were equipped with condensers and fitted to a 100-ml round-bottomed flask flask and a 2-ml pear-shaped flask respectively. Aliquots (50 ml and 2 ml respectively) of a dried dichloromethane solution of nitrosamines (see 3.52i) were concentrated in the above apparatus to final volumes of about 0.6 and 0.2 ml respectively. The volumes were determined by weighing and the nitrosamines estimated as above (see 3.35iie).

3.6 EXTRACTION OF N-NITROSAMINES ADDED TO BIOLOGICAL MATERIALS

3.61 Preparation of spiked material

(i) Pulped food samples were spiked with N-nitrosamines introduced by

means of standard solutions of the latter.

(ii) Finely ground samples were initially extracted in a Soxhlet apparatus with dichloromethane for 24 h. After the solvent had been removed from the sample, in a rotary evaporator at 40°, it was weighed and spiked with a dichloromethane solution of DENA. The spiked sample was equilibrated in a rotary evaporator at atmospheric pressure for 1.5 h at 20°. Dichloromethane was subsequently removed under vacuum (same apparatus at 20°), trapped in a liquid air trap and examined for DENA by GLC (3.35iic). Samples of finely ground maize containing 29.8 and 30.2 ppm of DENA were prepared in this way.

3.62 Recovery of added N-nitrosamines

(i) Percolation with solvents. Portions (45 g) of finely ground mealies, spiked (3.61ii) with DENA (29.8 ppm), contained in glass tubes (6 X 1 in) tapered at one end, were percolated for 50 min with two successive aliquots (50 ml) of solvent (dichloromethane, ether or carbon disulphide) and analysed by GLC (3.35iic). The results are tabulated in Table XV. A similar experiment was performed with portions (50 g) of finely ground maize spiked (3.61ii) with DENA (30.2 ppm), packed in glass tubes (6 X 1 and 24 X 0.5 in) tapered at one end. The columns were percolated (1.5 to 2.5 h) with three successive aliquots (50, 30 and 30 ml respectively) of dichloromethane. The separate percolates were dried and analysed by GLC (3.35iic).

(ii) Soxhlet extraction. Portions (5 g) of cellulose powder, spiked (3.61ii) with DENA (72.1 ppm), were weighed into thimbles (25 X 80 mm) and extracted for 6 h in the normal way with dichloromethane (180 ml). The amount of DENA extracted was estimated by GLC (3.35iic). The

results are tabulated in Table XVI. This experiment was repeated with portions (20 g) of finely ground maize spiked (3.61ii) with DENA (18.0 ppm). The extracts were analyzed as above (3.35iic)

(iii) Steam distillation. Ripe Solanum incanum fruit (50 g), comminuted in a Waring Blender and spiked (3.61i) with DENA (20.1 ppm), was steam distilled in the normal way.

(iv) Preparative dry-column chromatography. A portion (200 ml) of a methanolic extract (3.71i) of Solanum incanum, spiked (3.61i) with 61.9 ppm of DMNA, was concentrated to a final volume of about 50 ml in a rotary evaporator at 20°. In addition to the usual condensate traps the rotary evaporator was fitted with a liquid air trap. The trapped distillates were combined and examined for DMNA by GLC (see 3.35iia). A portion (40 g) of the plant concentrate was separated by dry-column chromatography (see 3.13). The combined nitrosamine fractions (3.13) from the columns were divided into two equal portions and extracted with three successive aliquots (100, 50 and 50 ml) of ether or dichloromethane. The amounts of DMNA in the extracts were estimated by GLC (see 3.35iia). The extracts were concentrated under vacuum at 0° and estimated as above (see 3.35iia). A known amount of DMNA was chromatographed, recovered and estimated in an analogous manner. The results are tabulated in Table XVII.

(v) Freeze-drying

(a) Recovery of DENA from spiked samples of tomatoes, marrow, haddock and stockfish. The material was comminuted with or without the addition of water, depending on its moisture content, in a Waring Blender.

The addition of water to marrow, haddock (fish) and stockfish was essential, as without it the comminuted mass would have been too dry for the thorough mixing-in of the nitrosamine solution. Two samples (100 g) of the comminuted material under examination were placed in two 2-litre flasks and freeze-dried in the normal way (3.15). Another two portions were spiked with aliquots of DENA in dichloromethane. The flasks were stoppered and vigorously shaken (10 min) to ensure thorough mixing. The spiked samples were freeze-dried (3.15) and the distillates analyzed by UV spectrophotometry (3.31).

(b) Recovery of nitrosamines from spiked samples of cellulose, water, beans, marrow and haddock. The above method of freeze-drying (see 3.62va) was used to estimate the recovery of DMNA, DENA, EPNA and DPNA added to the material under examination. The distillates (50 ml aliquots) were extracted with dichloromethane (see 3.42i) and analyzed by GLC (see 3.35iie). The results are tabulated in Table XVIIIA,B).

3.63 "Clean-up" of extracts

(i) Washing with acid and alkali. The organic extract (100 µl) was neutralized by washing with dilute sulphuric acid, dilute sodium hydroxide and water. The neutralized extract was dried with a small swab of desiccated cotton wool.

(ii) Flash distillation. The organic extract was introduced drop-wise into one arm of a U-tube (200 X 10 mm ID), packed with glass beads, suspended in a glycerine bath maintained at about 130°. The U-tube, suitably stoppered and fitted with side arms, was swept continuously with pure nitrogen. The solvent and all volatile components were swept through with the stream of nitrogen and condensed in a trap cooled in liquid air.

The recovery of DENA from spiked (about 30 ppm) dichloromethane solutions was estimated by GLC (3.35iic).

(iii) Removal compounds before gas chromatography. Preliminary experiments were conducted with glass subtraction columns (60 X 4.0 mm ID) packed with a subtractive agent which retained chemicals having a specific functional group. A B5 Quickfit joint at one end of the subtraction column fitted into a corresponding joint in a gas chromatographic glass column (1.12 m X 4.0 mm ID). Other GLC parameters were identical with those for column (i). This method was abandoned in favour of a more convenient approach in which the inlet port glass liner was packed with the desired subtractive agent. The carrier gas inlet to the gas chromatograph was blocked off and a fixture which served as an inlet port was attached to the inlet port of the gas chromatograph by means of a syringe needle. The affixed inlet port consisted of a stainless steel tube (170 X 6 mm ID) equipped with a glass liner, nitrogen inlet, septum cap and a heating jacket which kept the apparatus at the desired temperature (200°). The feasibility of removing three functional groups was investigated.

(a) Elimination of carbonyl compounds. The subtractive agent was 10% semicarbazide hydrochloride on Chromosorb W 80 - 100 mesh.

(b) Elimination of alkanes. The subtractive agent was Molecular Sieve type 5A (supplied by Coast Engineering Laboratory, Redondo Beach, California, U.S.A.). The agent was activated at 300° for 6 h.

(c) Elimination of alcohols. Boric acid (10% on Gas Chrom Q 80 - 100 mesh), berylium acetylacetonate (20% on Chromosorb W 80 - 100 mesh) and naphthyl isocyanate (20% on Chromosorb W 80 - 100 mesh) were tested as subtractive agents.

3.7 DETECTION OF N-NITROSAMINES IN BIOLOGICAL MATERIALS

3.71 Extraction of N-nitrosamines

The following methods were used under varying circumstances throughout this investigation.

(i) Comminution with methanol. The material was comminuted in methanol which had previously been cooled to -30°. After the mixture had been filtered, the extract was stored at -30°. Prior to analysis, the extract was concentrated in a rotary evaporator at a temperature of less than 30°. The materials extracted in this way are Tabulated in Table XIX.

(ii) Percolation with dichloromethane. Ground up samples were extracted according to the method described in section 3.62i. Materials extracted in this way are tabulated in Table XIX.

(iii) Freeze-drying. The material was comminuted with or without the addition of water, depending on its moisture content, in a Waring Blender. The pulped material was then freeze-dried in the normal way (see 3.15).

3.72 Detection of N-nitrosamines

(i) Solanum incanum

(a) TLC. The methanolic extract was treated according to the procedure outlined in section 3.12. A fraction, isolated by preparative TLC, was subsequently examined by IR (see 3.16).

(b) Preparative dry-column chromatography. This procedure (see 3.13) was used to prepare a nitrosamine-rich fraction from 360 g methanolic plant extract.

(c) GLC. The nitrosamine-rich fraction (see 3.72ib) was examined on four different GLC columns (a, b, c and d ; see 3.17). The latter column

was used as a preparative column to prepare about 1 mg of a fraction rich in DMNA. This fraction was examined by IR (see 3.16) and NMR (see 3.18).

(ii) Maize

The finely ground samples were extracted by percolation with dichloromethane (see 3.71ii) and the solution was freed from components by flash distillation (see 3.63ii).

(a) GLC. The dichloromethane extract was examined on column i (see 3.17iiii).

(iii) Beans

The finely ground samples were prepared and analyzed as above (3.72ii).

(iv) Other plants

The remaining plants (see Table XX) were extracted by dry-column chromatography (see 3.13) and analyzed by GLC (see 3.17) on a variety of columns.

APPENDIX I

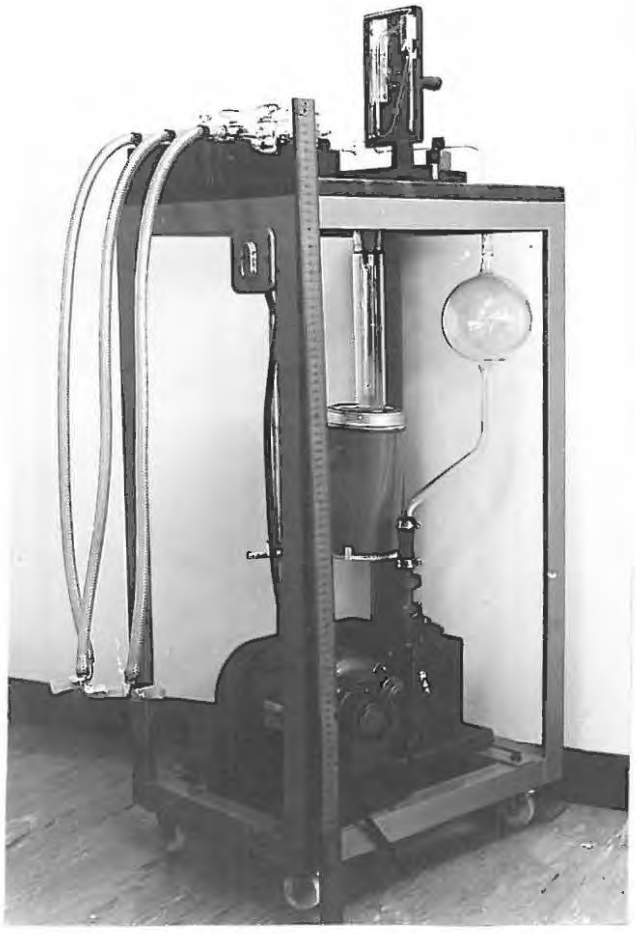


Plate 1

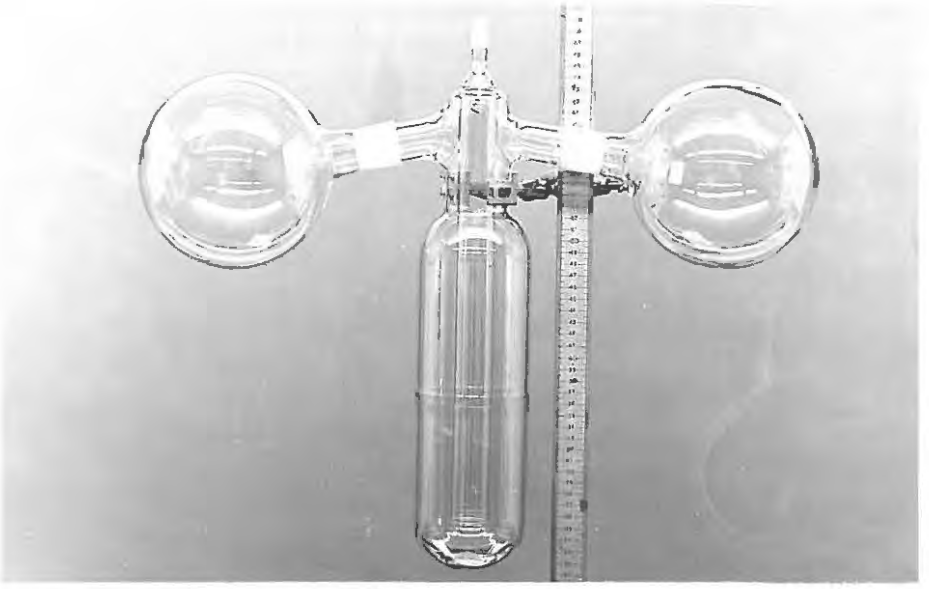


Plate 3

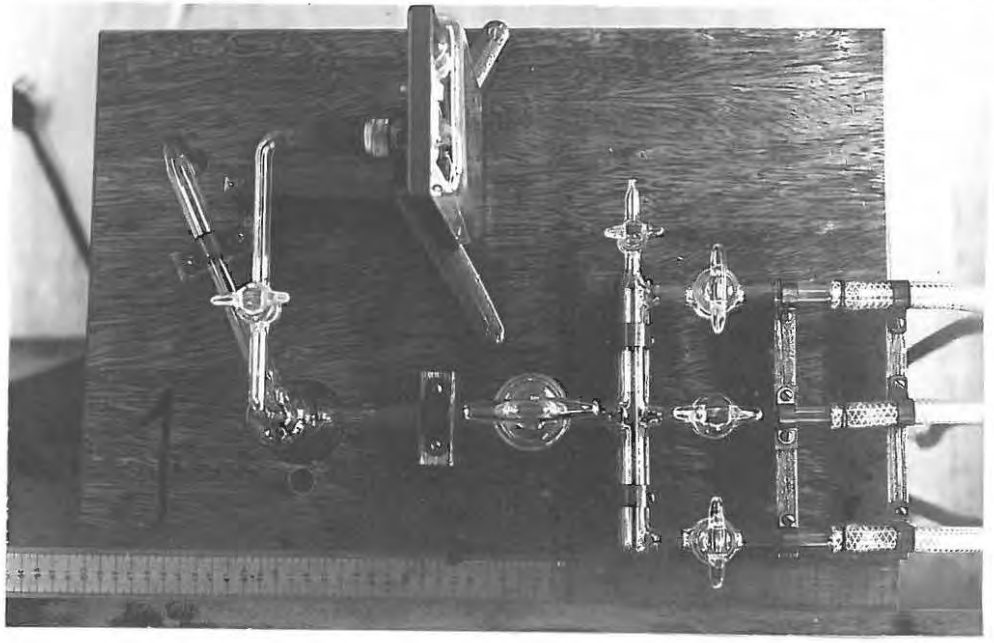


Plate 2

Plate 4

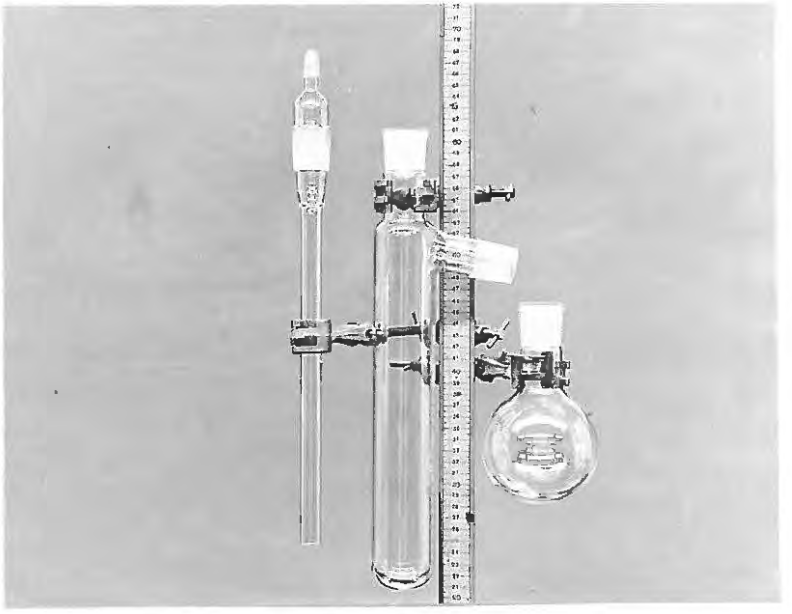


Plate 5

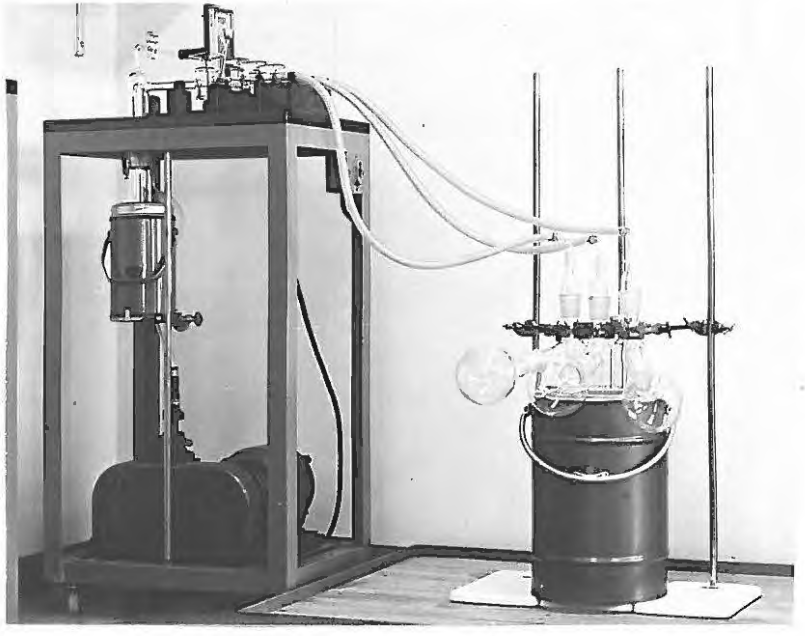


Plate 6

APPENDIX II

Calibration Table 1.

Data used to establish calibration curves for DMNA at attenuations of 5000, 2000, 200 and 50

Attenuation	Concentration of DMNA peak (mg/ ml)	Area of DMNA peak
5000	2.68	169 ± 5
	5.36	351 ± 15
	7.54	460 ± 9
	10.72	658 ± 8
2000	1.34	175 ± 8
	2.68	406 ± 13
	7.54	1076 ± 46
	10.72	1562 ± 53
	(µg/ ml)	
200	74	88 ± 5
	148	202 ± 10
	805	1009 ± 26
	1340	1593 ± 30
50	37	190 ± 21
	74	411 ± 13
	148	880 ± 78

Calibration Table 2.

Data used to establish calibration curves for DMNA

at an attenuation of 2

Concentration of DMNA (μg/ ml)	Area of DMNA peak	Area of p-xylene peak	$\frac{\text{Area of DMNA}}{\text{Area of p-xylene}}$
1.4	284 ± 25	834 ± 17	0.34 ± 0.02
3.36	608 ± 54	742 ± 19	0.82 ± 0.09
7.1	1275 ± 113	775 ± 37	1.65 ± 0.14
8.9	1437 ± 50	712 ± 28	2.02 ± 0.05
10.7	1635 ± 59	717 ± 26	2.28 ± 0.11

Calibration Table 3.

Data used to establish calibration curves for DENA

at attenuations of 200, 20 and 2

Attenuation	Concentration of DENA (μg/ ml)	Area of DENA peak	Area of cyclohexanone peak	$\frac{\text{Area of DENA}}{\text{Area of cyclohexanone}}$
200	47.2	238 ± 11	1513 ± 21	0.16 ± 0.01
	59.0	310 ± 7	1508 ± 11	0.21 ± 0.00
	70.8	365 ± 11	1502 ± 9	0.25 ± 0.01
	144.6	737 ± 16	1523 ± 20	0.48 ± 0.01
	289.2	1517 ± 16	1542 ± 7	0.98 ± 0.01
20	5.9	376 ± 18	1508 ± 25	0.25 ± 0.01
	8.9	492 ± 23	1527 ± 21	0.32 ± 0.01
	11.8	680 ± 28	1485 ± 37	0.46 ± 0.03
	23.6	1313 ± 16	1508 ± 32	0.87 ± 0.01
	35.4	1894 ± 25	1449 ± 13	1.31 ± 0.02
2	0.59	355 ± 47	1420 ± 101	0.25 ± 0.02
	1.18	586 ± 96	1522 ± 77	0.39 ± 0.04
	1.77	825 ± 78	1581 ± 64	0.53 ± 0.03
	2.36	1320 ± 220	1339 ± 228	0.99 ± 0.02
	2.95	1653 ± 168	1495 ± 47	1.12 ± 0.07
	4.13	1885 ± 60	1492 ± 45	1.27 ± 0.02

Calibration Table 4.

Data used to establish calibration curves for DENA

at an attenuation of 20

Concentration of DENA (μg/ ml)	Area of DENA peak	Area of cyclohexanone peak	Area of DENA / Area of cyclohexanone	Height of DENA peak (mm)	Height of cyclohexanone (mm)	Height of DENA / Height of cyclohexanone
5.8	164 ± 29	1358 ± 23	0.12 ± 0.02	21.3 ± 0.02	182.7 ± 1.4	0.12 ± 0.01
11.5	419 ± 26	1395 ± 55	0.30 ± 0.03	46.5 ± 2.4	186.1 ± 4.3	0.25 ± 0.02
17.3	684 ± 41	1405 ± 33	0.49 ± 0.02	71.7 ± 3.4	189.0 ± 3.1	0.38 ± 0.01
23.0	871 ± 75	1367 ± 19	0.64 ± 0.04	96.3 ± 3.8	187.9 ± 3.2	0.51 ± 0.01
28.9	1146 ± 51	1339 ± 31	0.86 ± 0.03	122.8 ± 5.3	187.3 ± 2.9	0.66 ± 0.02
34.6	1381 ± 67	1404 ± 30	0.98 ± 0.03	149.4 ± 4.1	190.0 ± 1.8	0.79 ± 0.03

Calibration Table 5.

Data used to establish calibration curves for DMNA, DENA, EPNA and DPNA at an attenuation of 200

Nitrosamine	Concentration of nitrosamine (μg/ ml)	Peak height of nitrosamine (mm)	Peak height of standard (mm)	Peak height of nitrosamine / Peak height of standard
DMNA	69.5	18.1 ± 0.2	101.5 ± 1.2	1.78 ± 0.01
	116.1	27.9 ± 1.1	92.7 ± 3.3	3.01 ± 0.02
	229.4	64.5 ± 0.8	98.4 ± 0.9	6.55 ± 0.02
	278.0	77.6 ± 2.0	95.1 ± 2.4	8.17 ± 0.03
	573.4	168.9 ± 1.0	98.1 ± 0.7	17.22 ± 0.07
DENA	55.5	23.0 ± 0.3	101.5 ± 1.2	2.26 ± 0.01
	92.7	33.5 ± 1.4	92.7 ± 3.3	3.61 ± 0.03
	183.2	73.3 ± 0.9	98.4 ± 0.9	7.45 ± 0.04
	222.0	86.7 ± 2.2	95.1 ± 2.4	9.11 ± 0.07
	457.9	181.8 ± 1.7	98.1 ± 0.7	18.53 ± 0.12
EPNA	54.2	20.2 ± 0.3	101.5 ± 1.2	1.99 ± 0.01
	90.5	29.0 ± 1.2	92.7 ± 3.3	3.12 ± 0.03
	178.9	63.9 ± 1.0	98.4 ± 0.9	6.49 ± 0.04
	216.8	74.8 ± 2.1	95.1 ± 2.4	7.86 ± 0.08
	447.2	159.3 ± 2.8	98.1 ± 0.7	16.24 ± 0.24
DPNA	100.2	32.2 ± 0.2	101.5 ± 1.2	3.17 ± 0.02
	167.3	47.1 ± 2.0	92.7 ± 3.3	5.07 ± 0.04
	330.7	102.5 ± 1.6	98.4 ± 0.9	10.42 ± 0.08
	400.8	119.5 ± 3.2	95.1 ± 2.4	12.56 ± 0.12
	826.7			

Calibration Table 6.

Data used to establish calibration curves for DMNA, DENA, EPNA and DPNA at an attenuation of 20

Nitrosamine	Concentration of nitrosamine (μg/ ml)	Peak height of nitrosamine (mm)	Peak height of standard (mm)	Peak height of nitrosamine / Peak height of standard
DMNA	7.7	12.1 ± 0.1	98.6 ± 0.4	1.22 ± 0.01
	14.0	28.9 ± 0.3	98.7 ± 0.7	2.93 ± 0.04
	21.0	47.3 ± 0.5	92.9 ± 0.6	4.74 ± 0.04
	28.0	65.8 ± 0.8	96.0 ± 0.9	6.85 ± 0.02
	35.0	82.3 ± 1.1	93.5 ± 1.0	8.80 ± 0.05
DENA	6.2	20.5 ± 0.2	98.6 ± 0.4	2.08 ± 0.02
	11.2	43.2 ± 0.3	98.7 ± 0.7	4.37 ± 0.02
	16.7	65.1 ± 0.3	92.9 ± 0.6	6.53 ± 0.03
	22.2	86.0 ± 1.0	96.0 ± 0.9	8.95 ± 0.03
	27.8	105.2 ± 0.9	93.5 ± 1.0	11.23 ± 0.07
EPNA	5.9	18.9 ± 0.1	98.6 ± 0.4	1.91 ± 0.02
	10.8	39.0 ± 0.2	98.7 ± 0.7	3.95 ± 0.02
	16.3	57.8 ± 0.3	92.9 ± 0.6	5.79 ± 0.02
	21.7	75.6 ± 0.9	96.0 ± 0.9	7.87 ± 0.03
	27.1	92.7 ± 0.6	93.5 ± 1.0	9.91 ± 0.05
DPNA	11.0	30.6 ± 0.2	98.6 ± 0.4	3.10 ± 0.02
	20.0	62.8 ± 0.3	98.7 ± 0.7	6.36 ± 0.03
	30.1	92.9 ± 0.6	92.9 ± 0.6	9.31 ± 0.05
	40.1	120.8 ± 1.5	96.0 ± 0.9	12.57 ± 0.06
	50.1	147.9 ± 0.8	93.5 ± 1.0	15.82 ± 0.08

Calibration Table 7.

Data used to establish calibration curves for DENA and NPYROL

at respective attenuations of 160 and 40

Nitrosamine	Concentration of nitrosamine (μg/ ml)	Peak height of nitrosamine (mm)	Peak height of standard (mm)	$\frac{\text{Peak height of nitrosamine}}{\text{Peak height of standard}}$
DENA	12.9	9.5 ± 0.9	168.7 ± 5.9	0.06 ± 0.01
	33.8	37.4 ± 2.5	147.6 ± 11.2	0.25 ± 0.01
	67.6	78.5 ± 4.8	156.1 ± 5.9	0.50 ± 0.02
	101.4	113.0 ± 4.9	143.7 ± 4.4	0.79 ± 0.03
	135.2	158.9 ± 14.7	152.1 ± 13.8	1.04 ± 0.02
NPYROL	7.3	7.8 ± 0.4	168.7 ± 5.9	0.05 ± 0.00
	19.2	31.0 ± 4.0	147.6 ± 11.2	0.21 ± 0.01
	38.4	66.2 ± 4.9	156.1 ± 5.9	0.42 ± 0.02
	57.6	90.4 ± 6.4	143.7 ± 4.4	0.63 ± 0.03
	76.8	145.7 ± 15.0	152.1 ± 13.8	0.96 ± 0.03

Table I. Thin-layer chromatography of nitrosamines

Mobile Phase:	n-Hexane/Ether/Dichloromethane		
	4:3:2	5:7:10	10:3:2
Nitrosamine	R_F	R_F	R_F
Dimethylnitrosamine	0.24[a],0.32[b]	0.36[b]	0.10[b]
Methylethylnitrosamine	0.36[a]		
Diethylnitrosamine	0.49[a],0.57[b]	0.55[b]	0.24[b]
Methylpropylnitrosamine	0.46[a],0.48[b]	0.76[b]	0.27[b]
Ethylpropylnitrosamine	0.61[b],0.61[b]	0.87[b]	0.37[b]
Di-n-propylnitrosamine	0.69[a],0.77[b]	0.63[b]	0.38[b]
Di-iso-propylnitrosamine	0.64[a],0.66[b]	0.89[b]	0.41[b]
Methylbutylnitrosamine	0.51[a],0.57[b]	0.83[b]	0.33[b]
Di-n-butylnitrosamine	0.77[A],0.87[b]	0.72[b]	0.46[b]
Methylamylnitrosamine	0.57[a]		
Diamylnitrosamine	0.82[a]		
Methylheptylnitrosamine	0.60[a]		
Methylbenzylnitrosamine	0.53[a],0.58[b]	0.85[b]	0.32[b]
Phenylbenzylnitrosamine	0.93[b]	0.74[b]	0.53[b]
Dibenzylnitrosamine	0.83[b]	0.92[b]	0.63[b]
p-Nitrosomethylaminobenzoic acid			0.00[a]
p-Nitrosomethylaminobenzaldehyde			0.22[a]
N-Nitroso-N-methyl-2-phenylethylamine			0.29[a]
N-Nitrosomethylphenylamine			0.63[a]
N-Nitrosoethylphenylamine			0.72[a]
N-Nitrosodiphenylamine			0.80[a]
N-Nitroso-N-methylpiperazine	0.09[b]	0.04[a],0.28[b]	0.64[b]
Di-N-Nitrosopiperazine	0.09[b]	0.27[a],0.31[b]	0.0[b]
N-Nitroso-N-Carbethoxypiperazine		0.35[a]	
N-Nitrosomorpholine		0.40[a]	
N-Nitrosopyrrolidine		0.41[a]	
N-Nitrosoproline ethyl ester		0.52[a]	
N-Nitrosopiperidine	0.56[b]	0.63[a],0.52[b]	0.23[b]
N-Nitrosoindoline		0.74[a]	

Mobile Phase:	hexane/ethylacetate/dichloromethane
	5:2:2
Nitrosamine	R_F
Dimethylnitrosamine	0.35[c]
Diethylnitrosamine	0.68[c]
Di-n-propylnitrosamine	0.87[c]
Di-n-butylnitrosamine	0.95[c]

a) according to Preussmann et al (75, 76)

b) according to Sen et al (37)

(36)

Table II. Polarographic half-wave potentials (v) of N-nitrosamines

Nitrosamine	a	b	c	d	e
Dimethylnitrosamine	-0.9	-0.94	-0.94	-0.76	-0.68
Methylethylnitrosamine			-0.92	-0.73	
Diethylnitrosamine	-0.88		-0.88	-0.71	-0.62
Di-n-propylnitrosamine			-0.84	-0.65	-0.56
Ethyl-t-butylnitrosamine				-0.75	
Di-n-butylnitrosamine			-0.79		-0.50
Methyl-n-pentylnitrosamine			-0.83		
Di-n-pentylnitrosamine			-0.78		
Dibenzylnitrosamine			-0.75		
Dicyclohexylnitrosamine			-0.86		
4-Picolylmethylnitrosamine			-0.81		
Methyl-2-hydroxyethylnitrosamine			-0.93		
N-nitrosodiethanolamine			-0.90		
N-nitrosopiperidine	-0.79		-0.79		
N-nitrosopyrrolidine	-0.81		-0.84		
N-methyl-N-nitrosoaniline			-0.69		
N-nitrosoproline			-0.79		
1,4-Dinitrosopiperazine			-0.76		
N-nitrosomorpholine	-0.76				
Methylnitrosourethane				-0.49; -0.83	
Ethylnitrosourethane				-0.42; -0.77	

a) Half-wave potential recorded in 0.01 M sulphosalicylic acid (25)

b) Half-wave potential recorded in 50% CH_3COOH (116)

c) Half-wave potential recorded in 0.2 N HCl (61)

d) Half-wave potential recorded in dilute H_2SO_4 under N_2 (86)

e) Half-wave potentials recorded (69) in buffer solution of Lydersen and Nagy (65)

Table III. List of N-nitrosamines

Nitrosamine	Abbreviation	B.P.	UV Data
			λ_{max}, ε in water
Dimethylnitrosamine	DMNA	152°/ 760 mm	332, 99 ; 228, 7000
Diethylnitrosamine	DENA	178°/ 760 mm	333, 87 ; 231, 7800
Methyl-n-propylnitrosamine	MPNA	195°/ 760 mm	332, 89 ; 228, 8100
Ethyl-n-propylnitrosamine	EPNA	199°/ 760 mm	338, 84 ; 232, 7800
Di-n-propylnitrosamine	DPNA	205°/ 760 mm	337, 85 ; 233, 7800
N-nitrosopiperidine	NPIP	215°/ 760 mm	
Phenylmethylnitrosamine	PMNA	122°/ 15 mm	
Phenylethylnitrosamine	PENA	124°/ 15 mm	
Diphenylnitrosamine	DPHNA	66 - 67° m.p.	
N-nitrosopyrrolidine	NPYROL		

Table IV. Comparison of DMNA mass spectra recorded under differing conditions

		A	B	C	D	E	F
A	1		1.00	0.80	0.90	1.00	0.80
	2		0.96	0.80	0.91	0.94	0.80
	3		1.00	0.80	1.00	0.90	0.80
	4		1.00	0.80	1.00	0.90	0.80
B	1	1.00		0.80	0.90	1.00	0.80
	2	0.96		0.78	0.80	0.96	0.73
	3	1.00		0.80	1.00	0.90	0.80
	4	1.00		0.80	1.00	0.90	0.80
C	1	0.80	0.80		0.80	0.80	0.70
	2	0.80	0.78		0.72	0.76	0.63
	3	0.80	0.80		0.80	0.90	0.80
	4	0.80	0.80		0.80	0.90	0.80
D	1	0.90	0.90	0.80		0.90	0.80
	2	0.91	0.80	0.72		0.77	0.72
	3	1.00	1.00	0.80		0.90	0.80
	4	1.00	1.00	0.80		0.90	0.80
E	1	1.00	1.00	0.80	0.90		0.80
	2	0.94	0.96	0.76	0.77		0.74
	3	0.90	0.90	0.90	0.90		0.90
	4	0.90	0.90	0.90	0.90		0.90
F	1	0.80	0.80	0.70	0.80	0.80	
	2	0.80	0.73	0.63	0.71	0.74	
	3	0.80	0.80	0.80	0.80	0.90	
	4	0.80	0.80	0.80	0.80	0.90	

Mass spectrum recorded by :

A : Du Plessis (see Fig. 8)

B : Du Plessis (see Fig. 9)

C : Schroll *et al* (110)

D : Collin (99)

E : Pensabene *et al* (109)

Methods of comparison :

see section 2.14iib for explanation of numbers 1 to 4.

Table V. Estimation of DENA from four different calibration curves

at an attenuation of 20

Concentration of DENA (μg/ ml)					Data used for GLC determination					
Known	Estimated									
	A	B	C	D	A	E	C	F	B	D
7.4	8.0	7.6	8.1	7.7	279 ± 18	1511 ± 21	31.2 ± 1.0	195.5 ± 1.1	0.18 ± 0.01	0.16 ± 0.01
8.9	9.7	9.3	9.8	9.4	348 ± 28	1535 ± 36	38.5 ± 3.0	194.1 ± 2.0	0.23 ± 0.02	0.20 ± 0.02
11.8	11.7	11.2	11.8	11.1	432 ± 7	1575 ± 49	47.5 ± 1.3	197.7 ± 1.0	0.27 ± 0.01	0.24 ± 0.01
15.8	16.4	15.5	16.5	15.9	631 ± 47	1519 ± 44	68.2 ± 4.3	193.5 ± 2.2	0.42 ± 0.02	0.35 ± 0.02
23.6	23.5	21.8	23.0	22.3	926 ± 51	1545 ± 47	97.1 ± 3.7	195.2 ± 2.1	0.60 ± 0.02	0.50 ± 0.01

A : Area of DENA peak ions

B : Area of DENA peak/ area of internal standard

C : Height of DENA peak (mm)

D : Height of DENA peak/ height of internal standard

E : Area of internal standard peak (mm)

F : Height of internal standard peak (mm)

Table VI. Recovery of N-nitrosamines by extraction from 5 ml aqueous solution (bulked extracts).[1]

Nitrosamine	Expt. 1		Expt. 2		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	16.3	11.9	16.3	11.9	71.2
DENA	14.9	12.0	14.9	12.4	81.9
EPNA	7.4	5.8	7.4	6.0	79.7
DPNA	9.9	8.5	9.9	8.2	84.8

[1] Extraction time : 12 min.

Table VII. Recovery of N-nitrosamines by extraction from 5 ml aqueous solution (bulked extracts).[1]

Nitrosamine	Expt. 1		Expt. 2		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	16.3	11.5	16.3	11.6	71.2
DENA	14.9	11.8	14.9	12.2	80.5
EPNA	7.4	5.6	7.4	7.0	85.1
DPNA	9.9	7.7	9.9	8.1	79.8

[1] Extraction time : 4 min.

Table VIII. Recovery of N-nitrosamines by successive extraction from 50 ml aqueous solution.[1]

Nitrosamine	Amount added (ppm)	Extraction 1 found (ppm)	Extraction 2 found (ppm)	Extraction 3 found (ppm)	Extraction 4 found (ppm)	Extraction 5 found (ppm)	Mean recovery %
DMNA	16.3	4.4	7.4	10.1	12.1	13.5	
	16.3	4.2	7.2	9.9	11.8	13.4	
	Mean values						
	16.3	4.3	7.3	10.0	12.0	13.5	82.8
DENA	14.9	11.1	13.6	14.3	14.6	14.6	
	14.9	10.7	13.2	14.0	14.2	14.2	
	Mean values						
	14.9	10.9	13.4	14.2	14.4	14.4	96.6
EPNA	7.4	6.1	6.8	7.0	7.0	7.0	
	7.4	5.9	6.5	6.7	6.7	6.7	
	Mean values						
	7.4	6.0	6.7	6.9	6.9	6.9	93.2
DPNA	9.9	8.5	9.2	9.3	9.3	9.3	
	9.9	8.4	9.0	9.2	9.2	9.2	
	Mean values						
	9.9	8.5	9.1	9.3	9.3	9.3	93.9

[1] Extraction time : 12 min.

Table IX. Recovery of N-nitrosamines by extraction from 50 ml aqueous solution (bulked extracts).[1]

Nitrosamine	Expt. 1		Expt. 2		Expt. 3		Expt. 3		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	16.3	13.0	16.3	13.2	8.9	6.9	8.9	6.6	77.5
DENA	14.9	14.1	14.9	14.1	7.2	6.9	7.2	6.7	94.4
EPNA	7.4	6.6	7.4	6.7	7.0	6.6	7.0	6.4	91.4
DPNA	9.9	9.0	9.9	9.0	12.5	11.5	12.5	11.5	92.0

[1] Extraction time : 12 min.

Table X. Recovery of N-nitrosamines by successive extraction from 5 ml aqueous solution.[1]

Nitrosamine	Amount added (ppm)	Extraction 1 found (ppm)	Extraction 2 found (ppm)	Mean recovery %
DMNA	16.3	6.4	12.1	
	16.3	6.4	12.4	
	16.3	6.5	11.7	
	16.3	7.2	12.7	
		Mean values		
	16.3	6.6	12.2	74.8
DENA	14.9	9.2	12.9	
	14.9	9.0	12.8	
	14.9	9.4	13.0	
	14.9	9.9	13.6	
		Mean values		
	14.9	9.4	13.1	87.9
EPNA	7.4	4.6	6.2	
	7.4	4.5	6.3	
	7.4	4.8	6.4	
	7.4	5.0	6.7	
		Mean values		
	7.4	4.7	6.4	86.5
DPNA	9.9	6.2	8.4	
	9.9	6.1	8.5	
	9.9	6.6	8.7	
	9.9	6.8	9.0	
		Mean values		
	9.9	6.4	8.7	87.9

[1] Extraction Time : 12 min.

Table XI. Recovery of N-nitrosamines from 100 ml dried dichloromethane solution after concentration in a Kuderna-Danish evaporator.[1]

Nitrosamine	Expt. 1		Expt. 2		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	0.38	0.37	0.55	0.52	96.0
DENA	0.25	0.25	0.38	0.34	94.8
EPNA	0.24	0.23	0.36	0.32	92.4
DPNA	0.42	0.40	0.63	0.57	92.9

[1] Mean final volume : 4.5 ml.

Table XII. Recovery of N-nitrosamines from 50 ml dried dichloromethane solution after concentration at 65° on a water-bath.[1]

Nitrosamine	Expt. 1		Expt. 2		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	0.38	0.31	0.38	0.31	81.6
DENA	0.25	0.19	0.25	0.20	78.0
EPNA	0.24	0.18	0.24	0.19	77.1
DPNA	0.42	0.33	0.42	0.35	81.0

[1] Mean final volume : 4.4 ml

Table XIII. Recovery of N-nitrosamines from 25 ml dichloromethane after concentration in a vacuum.[1]

Nitrosamine	Expt. 1		Expt. 2		Expt. 3		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	5.9	4.0	5.9	3.7	5.9	3.5	62.7
DENA	5.3	3.6	5.3	3.3	5.3	3.2	64.2
EPNA	2.5	1.8	2.5	1.6	2.5	1.5	64.0
DPNA	3.6	2.8	3.6	2.3	3.6	2.2	66.7

[1] Mean final volume : 4.2 ml.

Table XIV. Recovery of N-nitrosamines from 25 ml dichloromethane after concentration at 50° on a water-bath.[1]

Nitrosamine	Expt. 1		Expt. 2		Expt. 3		Mean recovery %
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DMNA	5.9	3.8	5.9	3.2	5.9	3.8	61.0
DENA	5.3	3.2	5.3	3.0	5.3	3.3	58.5
EPNA	2.5	1.5	2.5	1.4	2.5	1.5	60.0
DPNA	3.6	2.1	3.6	2.0	3.6	2.2	58.3

[1] Mean final volume : 4.8 ml.

Table XV. Estimation of DENA in percolated extracts of maize

Solvent	Expt. 1		Expt. 2		Mean recovery (%)
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
Dichloromethane	29	19	29	22	72
Ether	28	17	28	15	57
Carbon disulphide	28	11	28	11	39

Table XVI. Estimation of DENA in Soxhlet extracts of cellulose and maize

Cellulose					
Nitrosamine	Expt. 1		Expt. 2		Mean recovery (%)
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DENA	72.1	66.5	72.1	70.7	95.1
Maize					
Nitrosamine	Expt. 1		Expt. 2		Mean recovery (%)
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	
DENA	18.0	14.4	18.0	16.5	85.8

Table XVII. Recovery of DMNA after dry-column chromatography

Extraction with ether

DMNA added to	Unconcentrated extract Added (ppm)	Found (ppm)	Mean recovery (%)	Concentrated extract Added (ppm)	Found (ppm)	Mean recovery (%)
Standard solution	4635	2581		2581	753	
Standard solution	4635	2410		2410	591	
Mean	4635	2496	53.8	2496	672	26.9
Plant extract	4251	2247		2247	567	
Plant extract	4251	2094		2094	608	
Mean	4251	2171	51.1	2171	588	27.1

Overall recovery (%) 13.6

Extraction with dichloromethane

DMNA added to	Unconcentrated extract Added (ppm)	Found (ppm)	Mean recovery (%)	Concentrated extract Added (ppm)	Found (ppm)	Mean recovery (%)
Standard solution	4803	2634		2634	1487	
Standard solution	4803	2208		2208	1136	
Mean	4803	2421	50.4	2421	1312	54.0
Plant extract	4251	2328		2328	1235	
Plant extract	4251	2284		2284	1305	
Mean	4251	2306	54.2	2306	1270	55.1

Overall recovery (%) 27.2

Table XVIIIA Estimation of nitrosamines in freeze-dried water, cellulose and beans

Nitrosamine	Water			Cellulose			Beans						
	Added (ppm)	Found (ppm)	Mean recovery (%)	Added (ppm)	Found (ppm)	Mean recovery (%)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Mean recovery (%)
DMN	44.6	36.8	82.5	44.6	35.5	79.6	44.6	37.2	7.9	7.1	4.5	4.2	88.9
DEN	37.8	34.2	95.5	35.8	33.2	92.7	35.8	33.4	5.3	4.6	3.0	2.6	83.9
EPN	35.1	34.8	99.1	35.1	31.9	99.4	35.1	34.1	5.0	4.8	2.9	2.6	94.3
DPN	62.6	62.2	99.4	62.6	63.2	101.0	62.6	59.7	8.8	8.2	5.0	4.7	94.2

Table XVIIIB Estimation of nitrosamines in freeze-dried marrow and fish (haddock)

Nitrosamine	Marrow							Fish (haddock)						
	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Mean recovery (%)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Added (ppm)	Found (ppm)	Mean recovery (%)
DMN	112.1	103.9	6.7	6.0	5.6	4.8	88.4	53.5	49.8	6.7	6.1	4.5	4.2	92.5
DEN	75.2	65.1	4.5	4.1	3.8	3.3	88.2	43.0	39.2	4.5	4.0	3.0	2.6	84.4
EPN	72.2	65.1	4.3	4.0	3.6	3.3	91.6	42.1	39.2	4.3	4.0	2.9	2.6	92.8
DPN	125.6	115.2	7.5	7.5	6.3	6.2	95.7	75.1	67.6	7.5	6.9	5.0	4.5	90.6

Table XIX. List of Plant Material

Beans

Bikicane

Hlaba or Rwabe

Maize

Nomdlombogi

Nondloloyi

Mhlababangubo

Pumpkins

Umsobosobo

Umsobowenga

Umtuma - Solanum incanum

APPENDIX III

Fig. 1. Relationship between concentration of DMNA and absorbance at 229 nm.

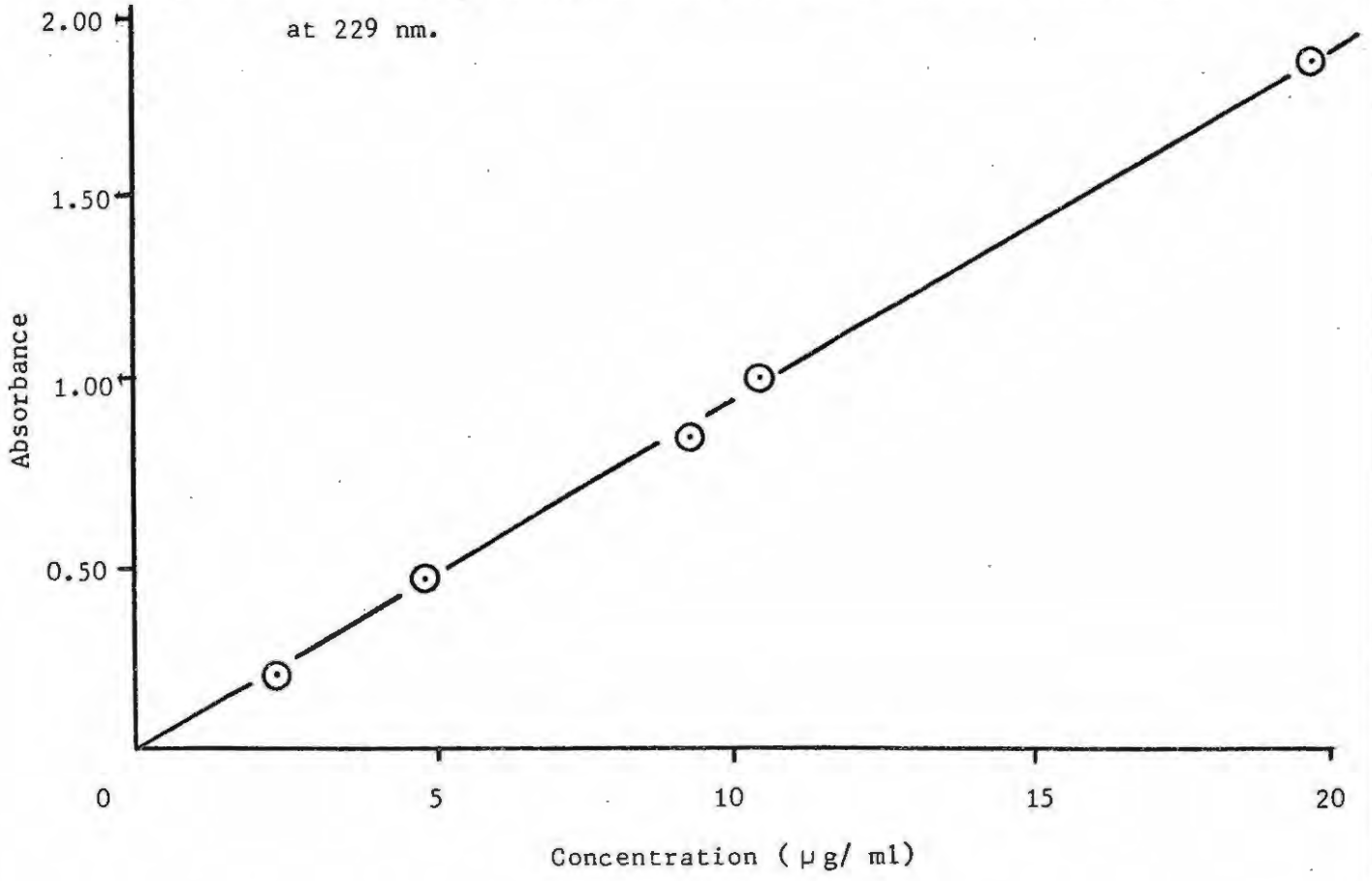


Fig. 2. Relationship between concentration of DMNA and absorbance at 333 nm.

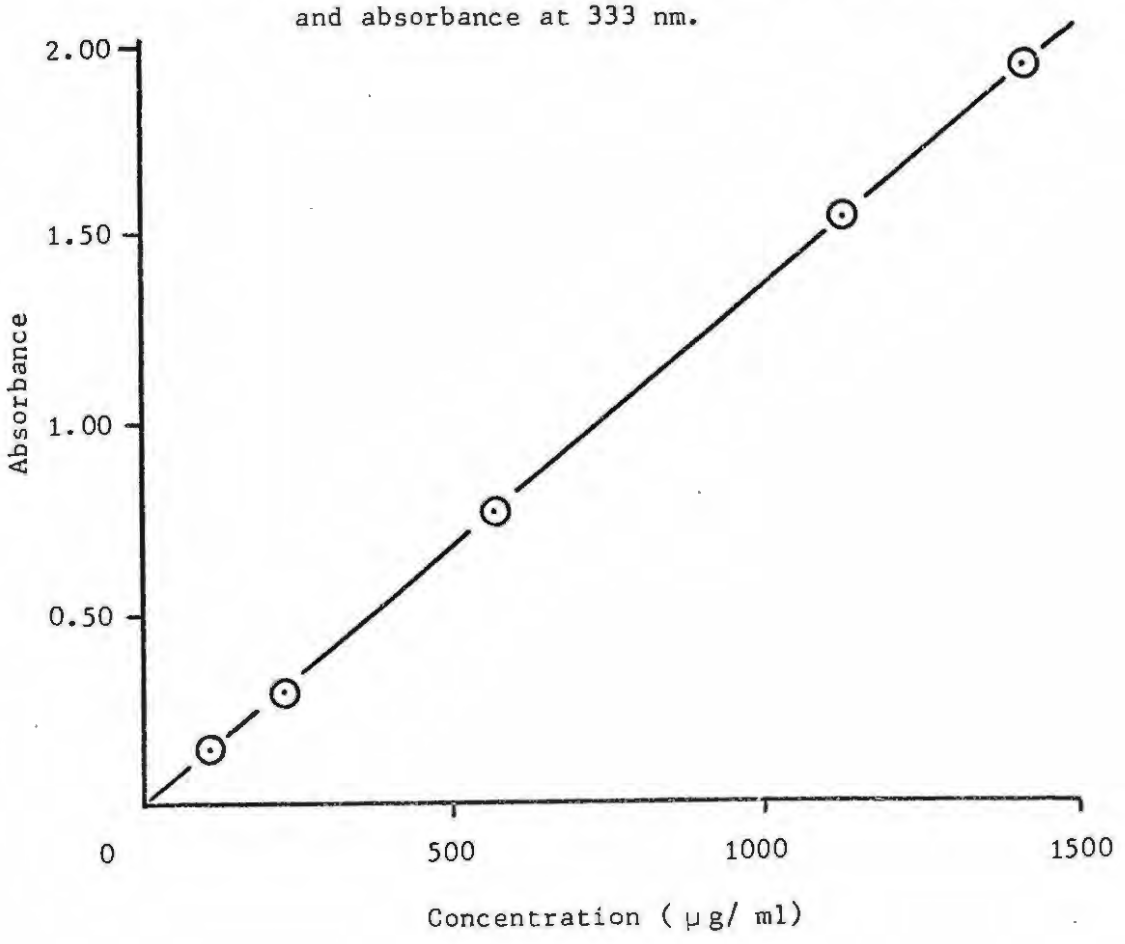


Fig. 3. Relationship between concentration of DENA and absorbance at 235 nm.

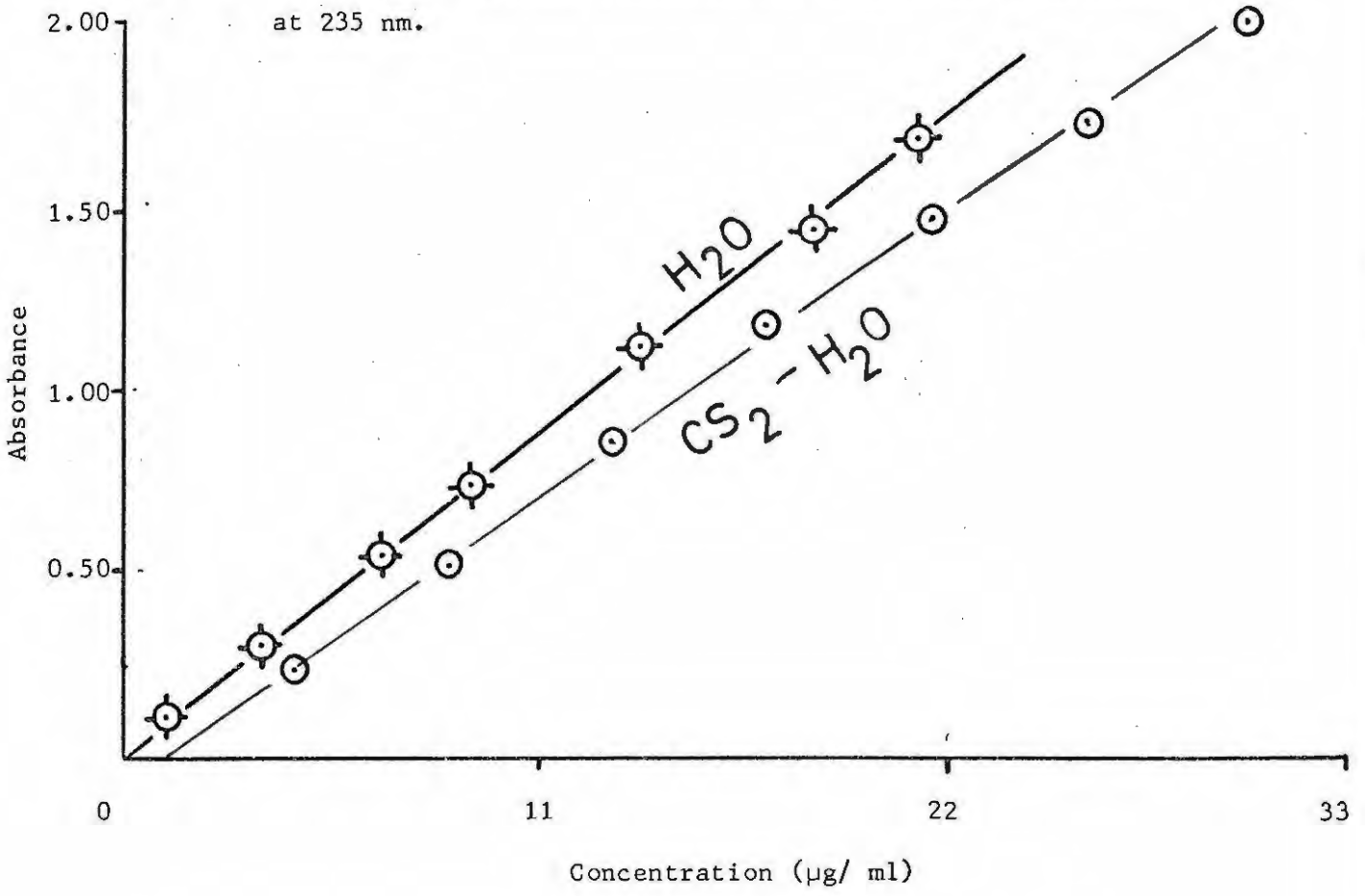
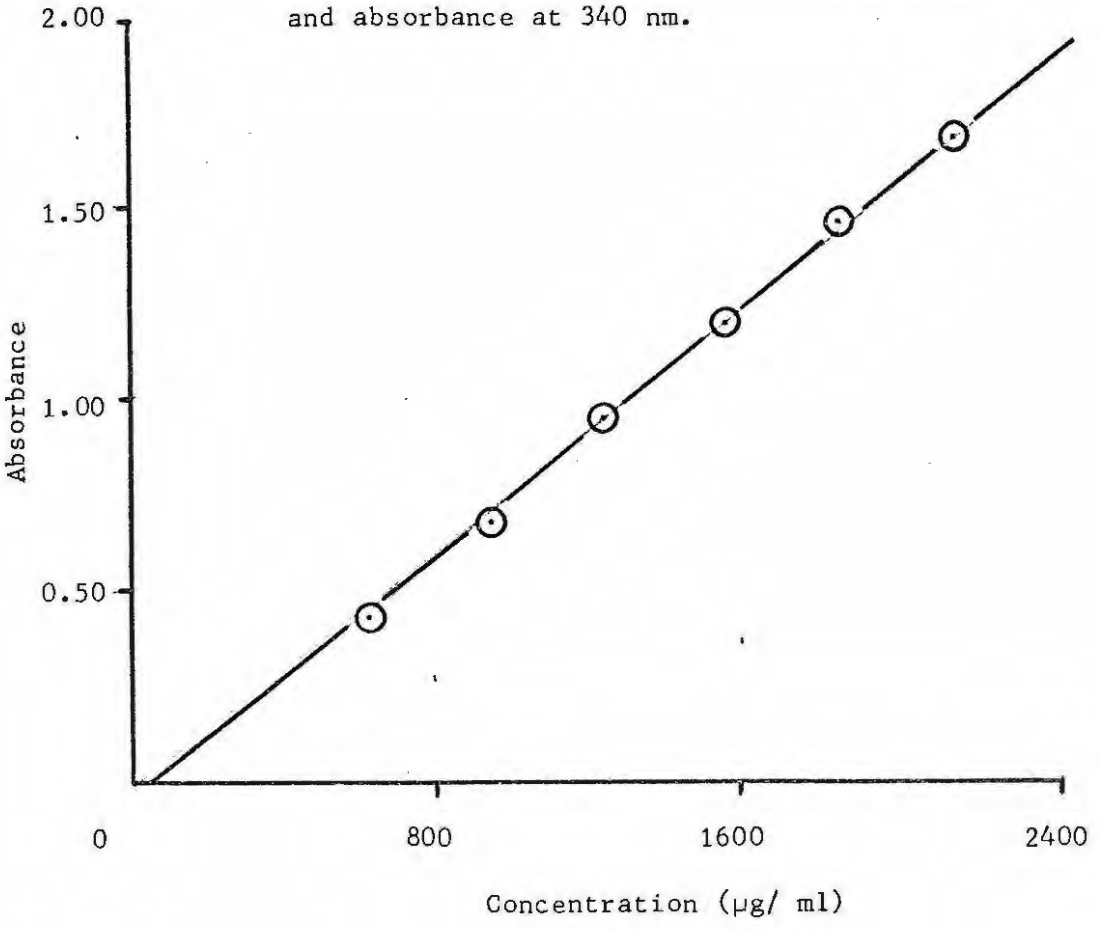


Fig. 4. Relationship between concentration of DENA and absorbance at 340 nm.



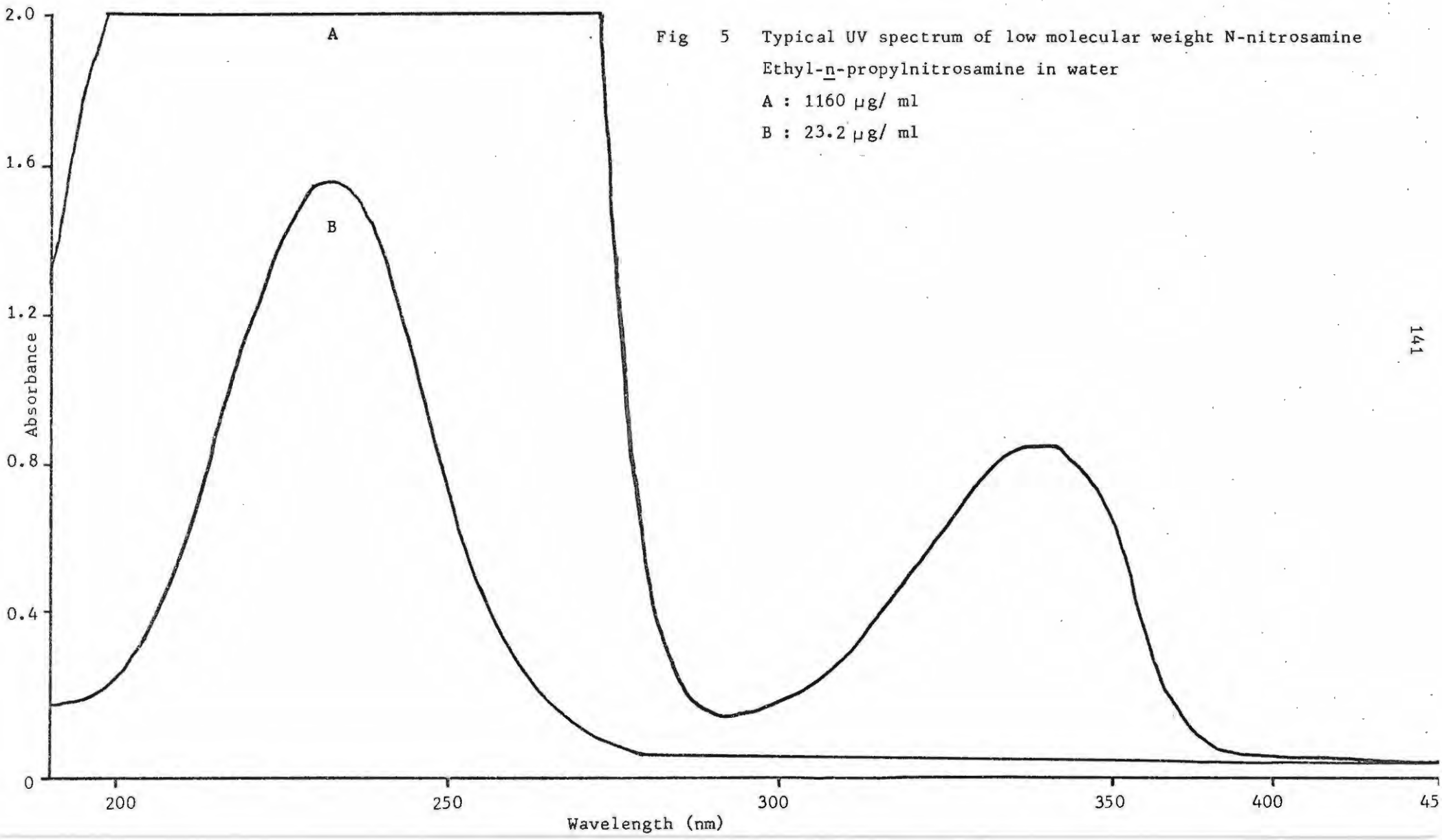


Fig 5 Typical UV spectrum of low molecular weight N-nitrosamine

Ethyl-n-propylnitrosamine in water

A : 1160 µg/ ml

B : 23.2 µg/ ml

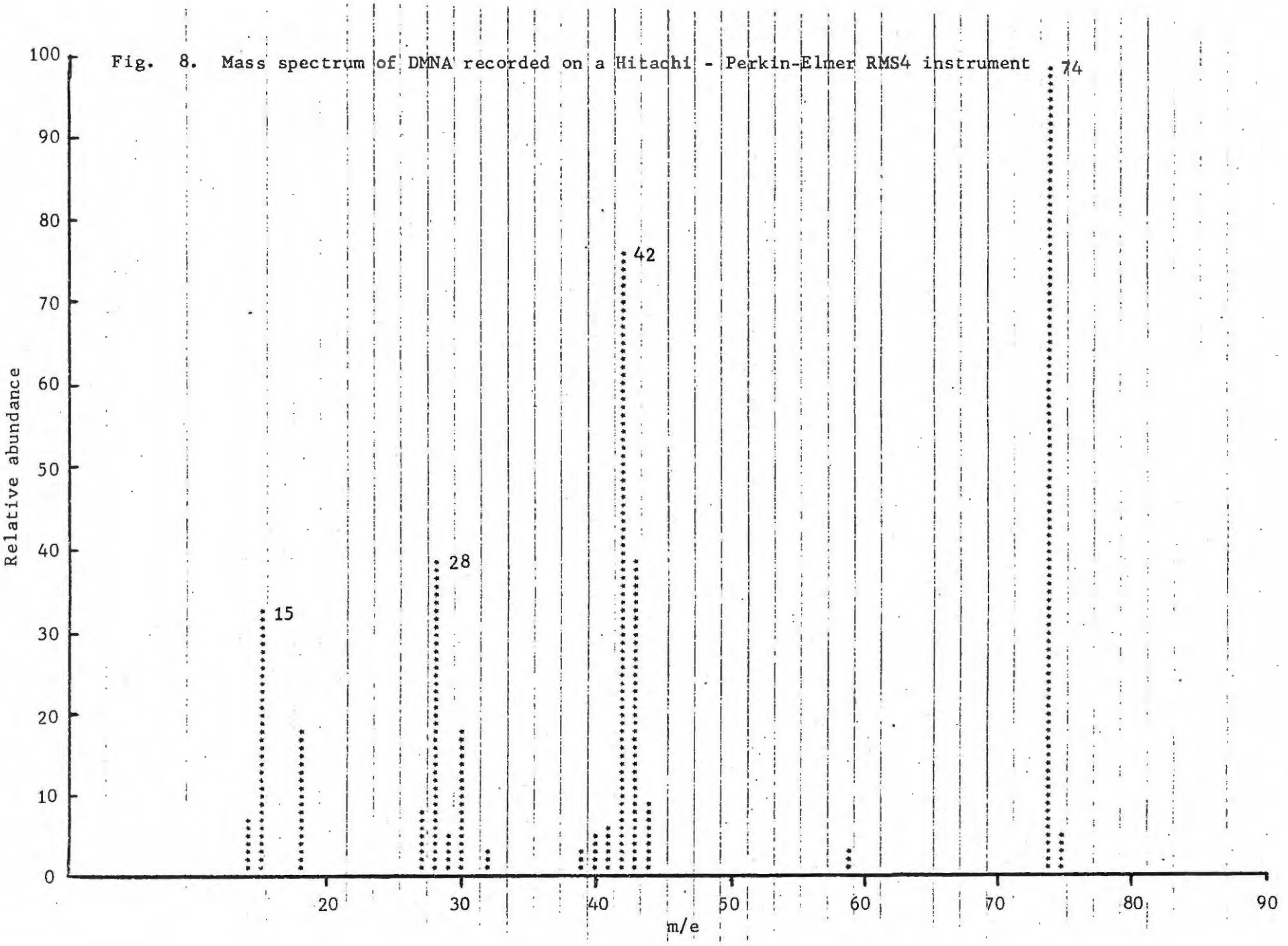


Fig. 8. Mass spectrum of DMNA recorded on a Hitachi - Perkin-Elmer RMS4 instrument

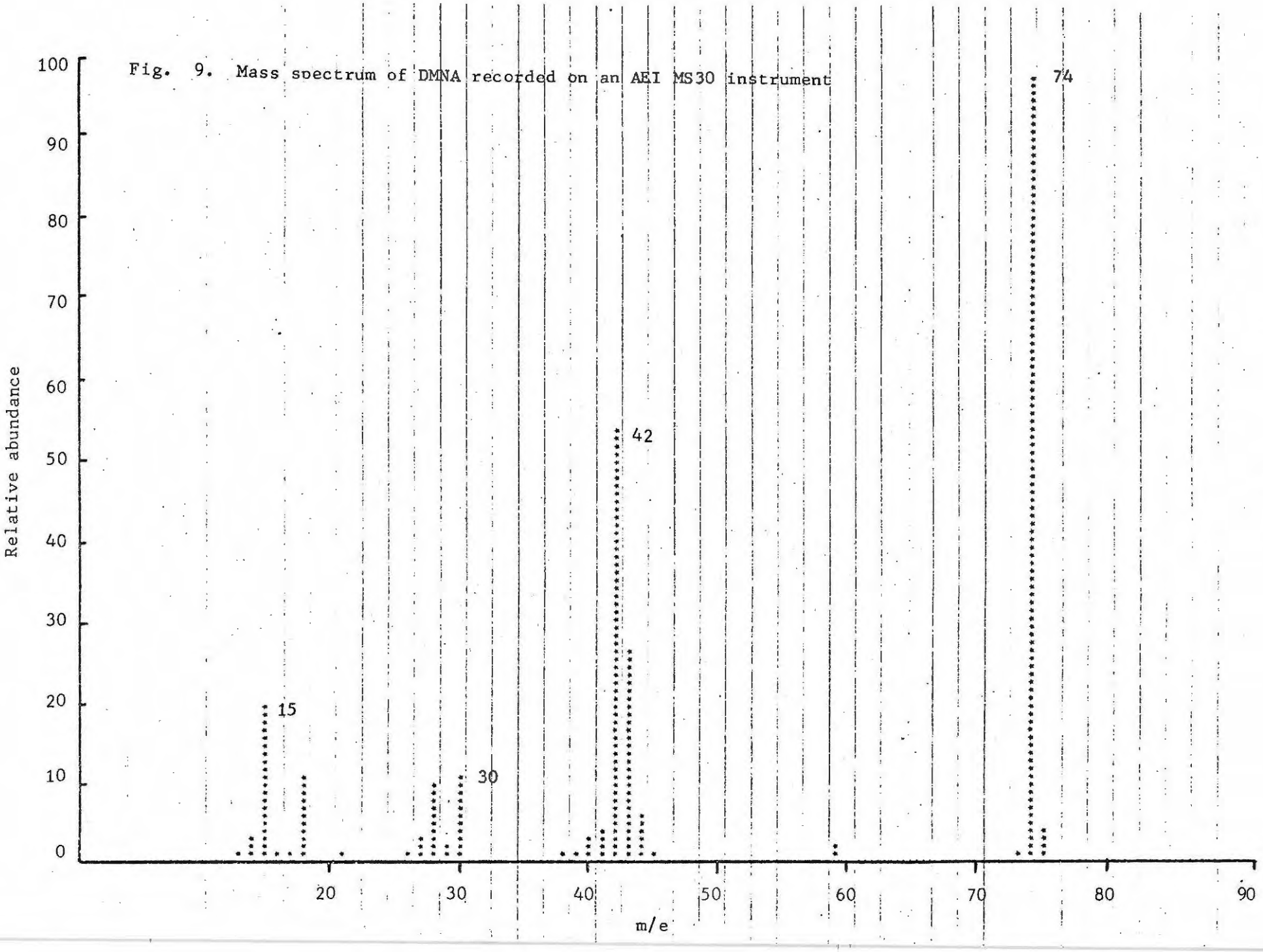


Fig. 9. Mass spectrum of DMNA recorded on an AEI MS30 instrument

Fig. 10. Mass spectrum of DENA recorded on a Hitachi - Perkin-Elmer RMS4 instrument

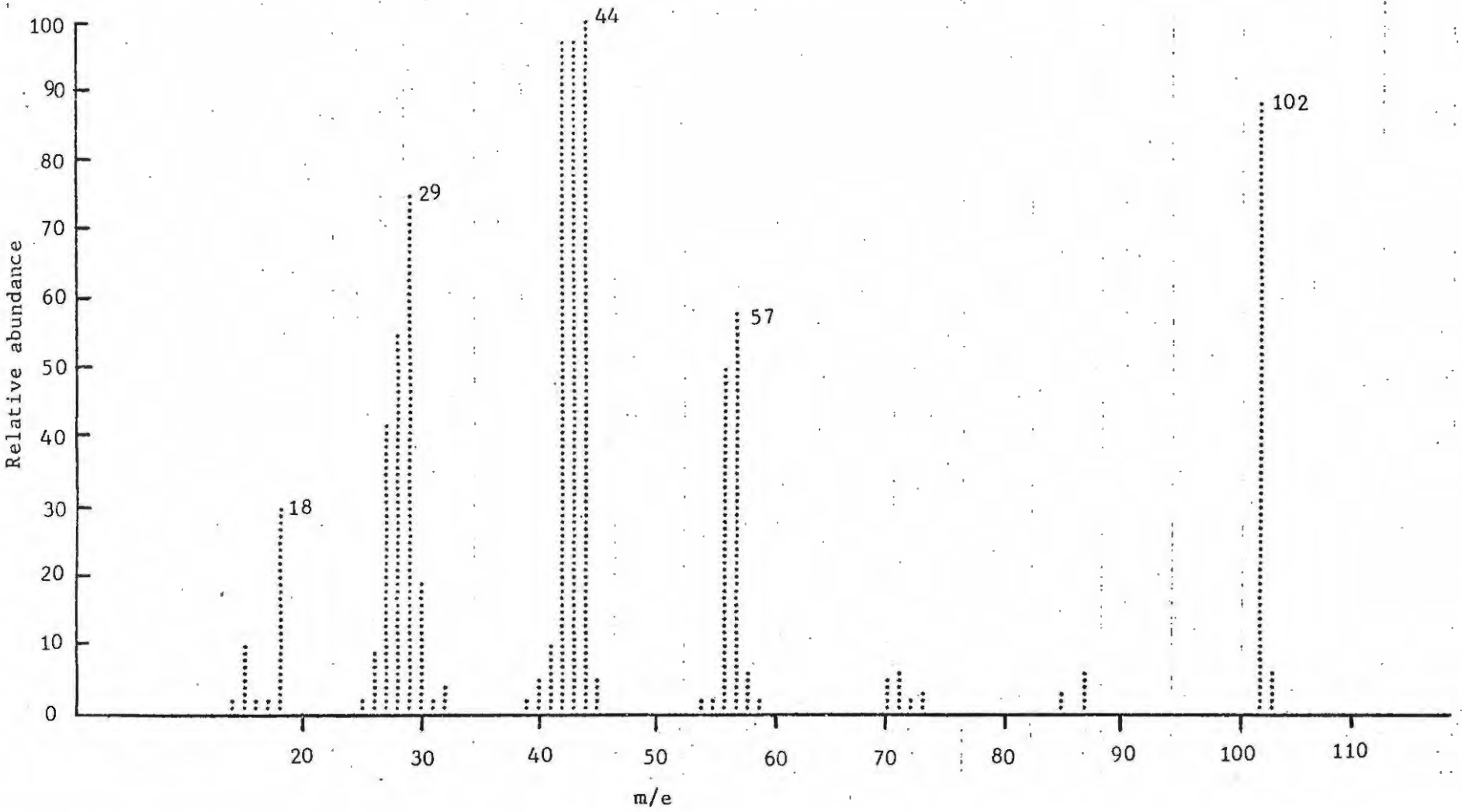


Fig. 11. Mass spectrum of DENA recorded on an AEI MS30 instrument

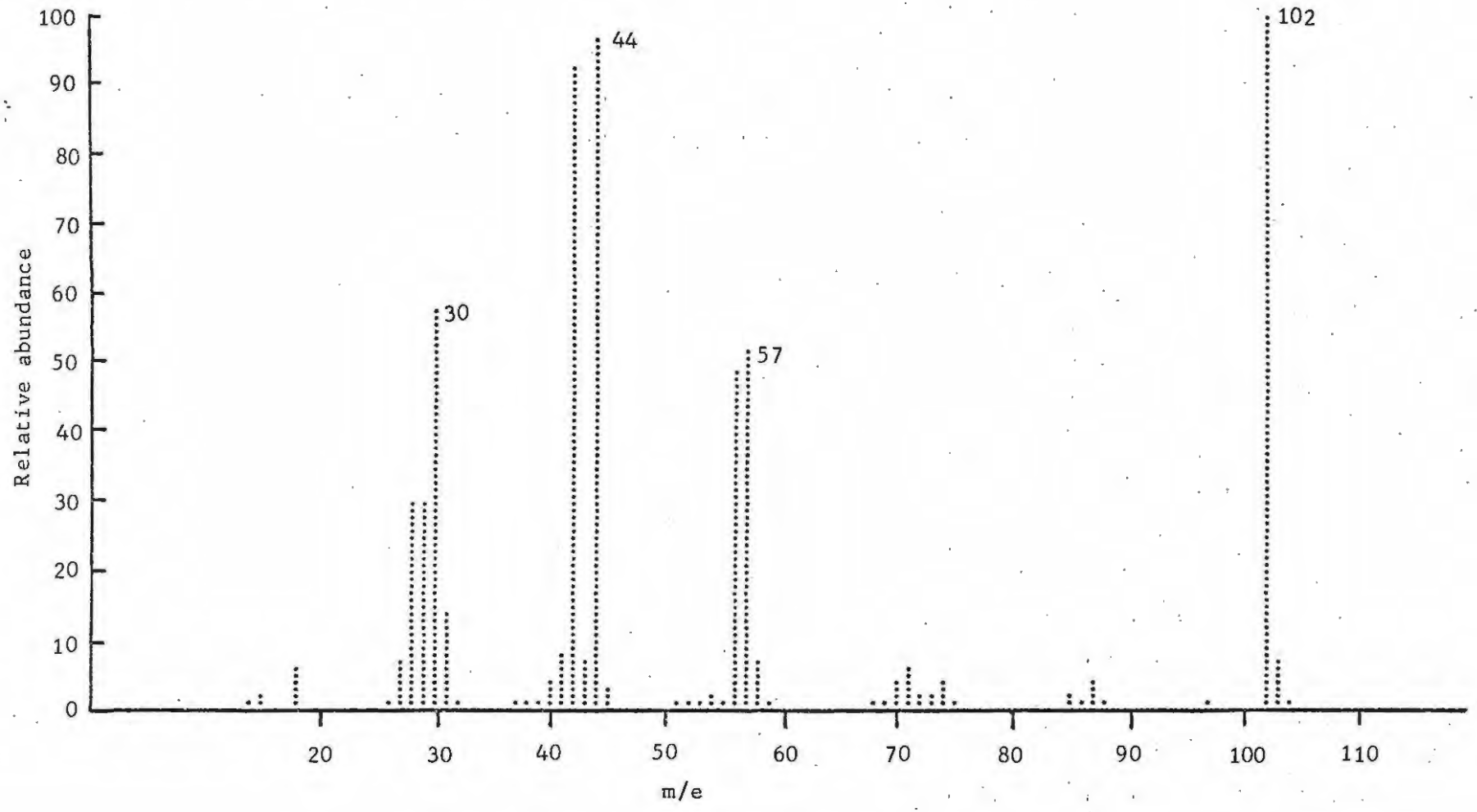


Fig. 12. Mass spectrum of MPNA recorded on a Hitachi - Perkin-Elmer RMS4 instrument

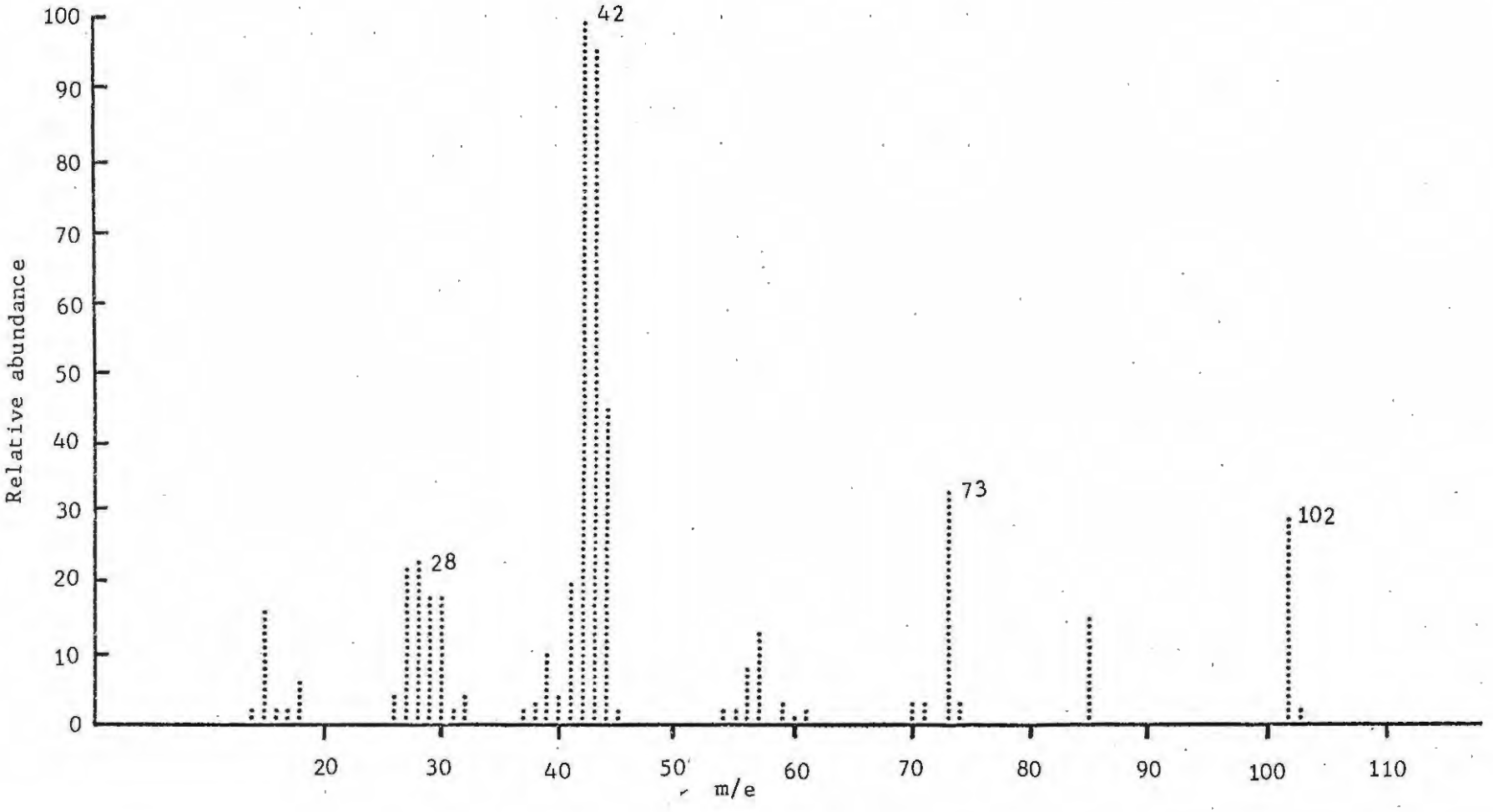


Fig. 13. Mass spectrum of MPNA recorded on an AEI MS30 instrument

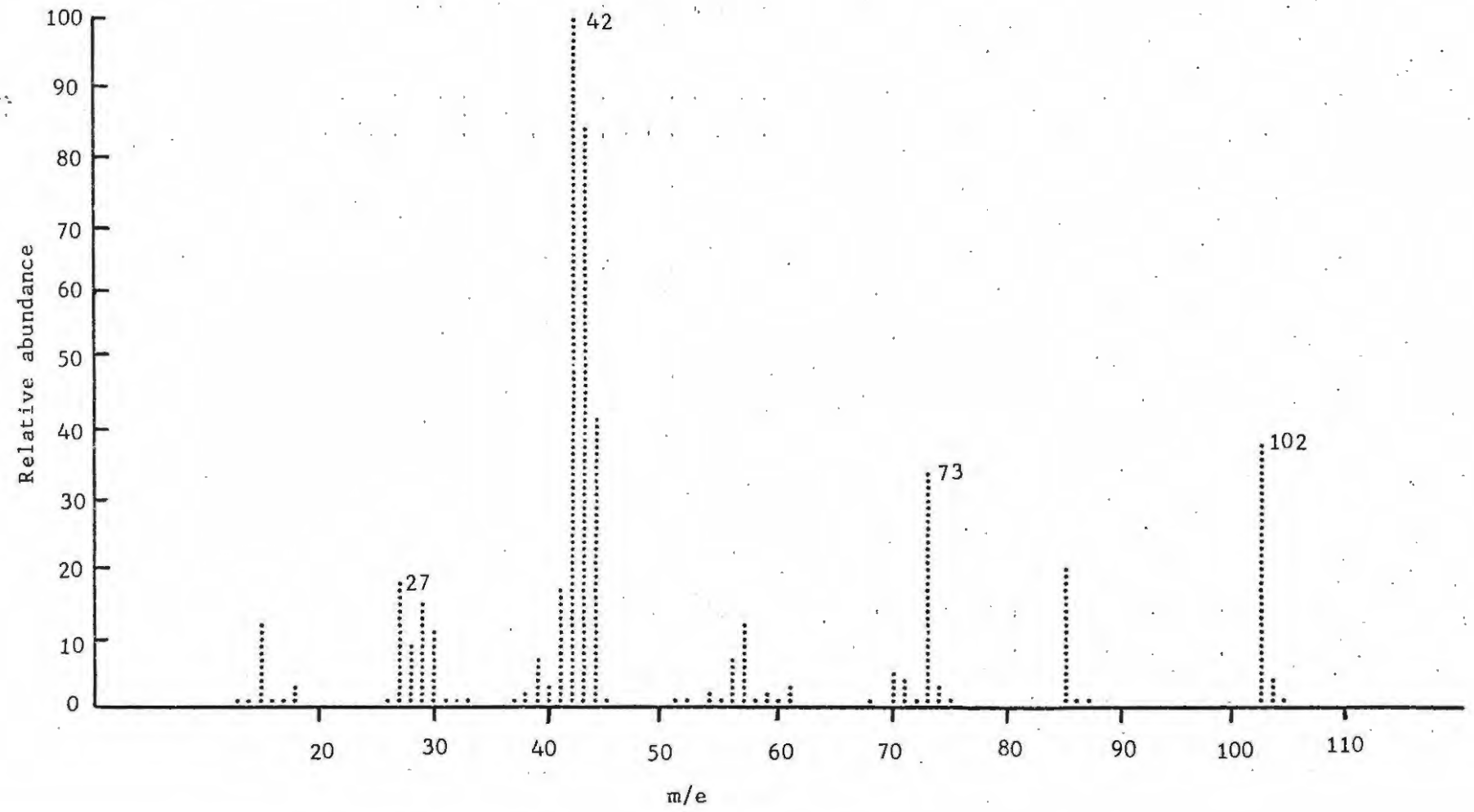


Fig. 14. Mass spectrum of EPNA recorded on a Hitachi - Perkin-Elmer RMS4 instrument

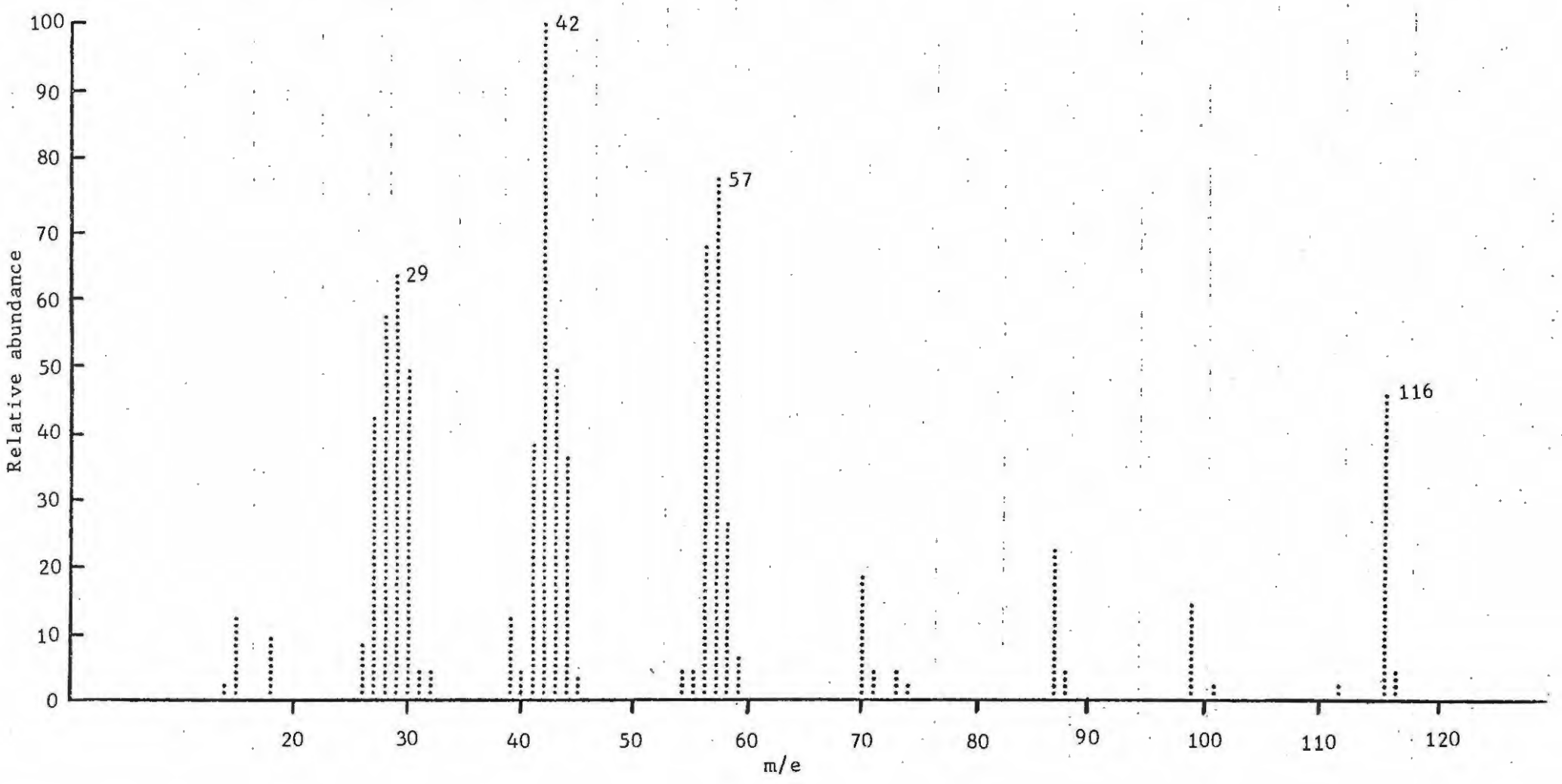


Fig. 15. Mass spectrum of EPNA recorded on an AEI MS30 instrument

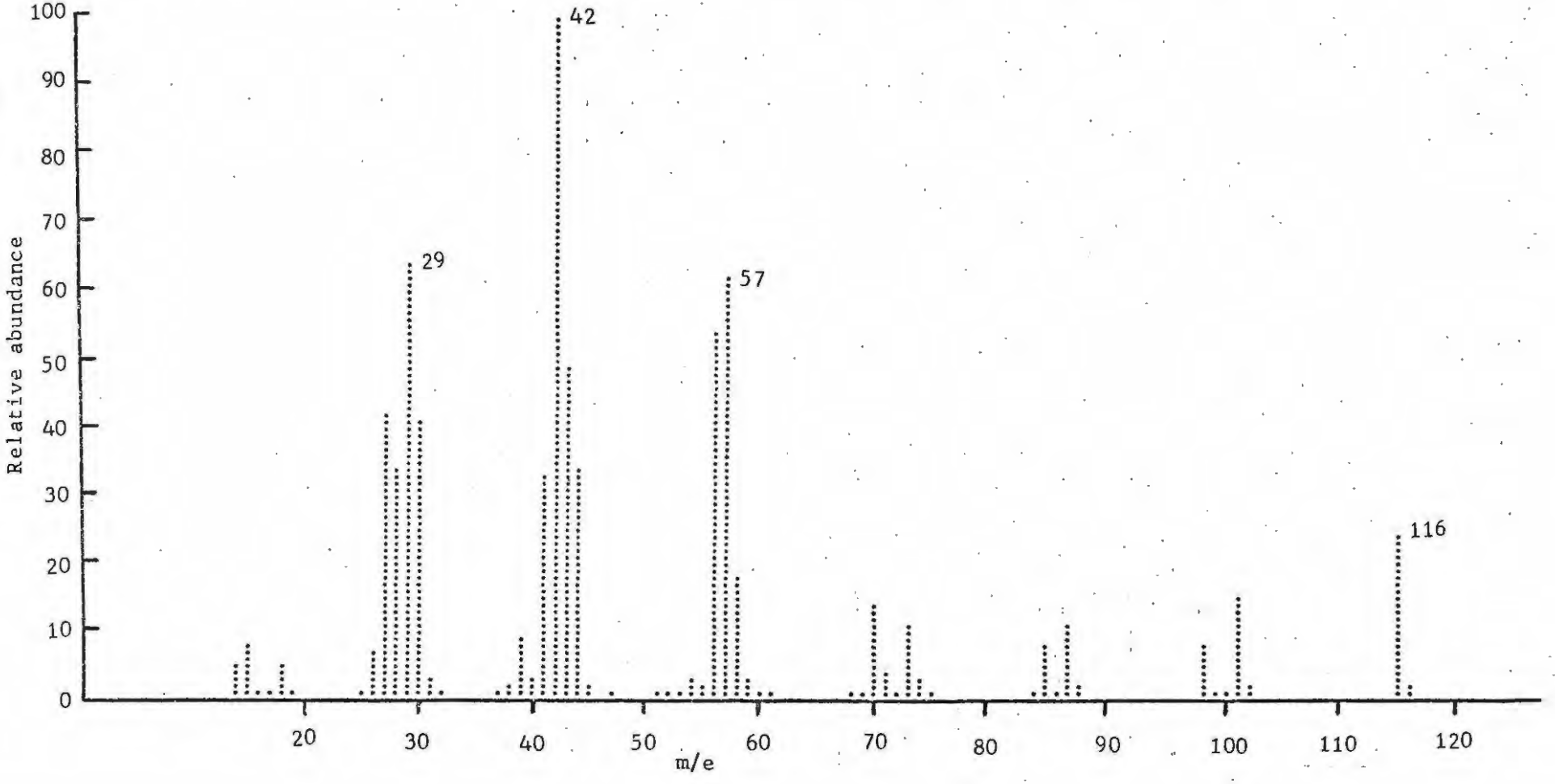


Fig. 16. Mass spectrum of DPNA recorded on a Hitachi - Perkin-Elmer RMS4 instrument

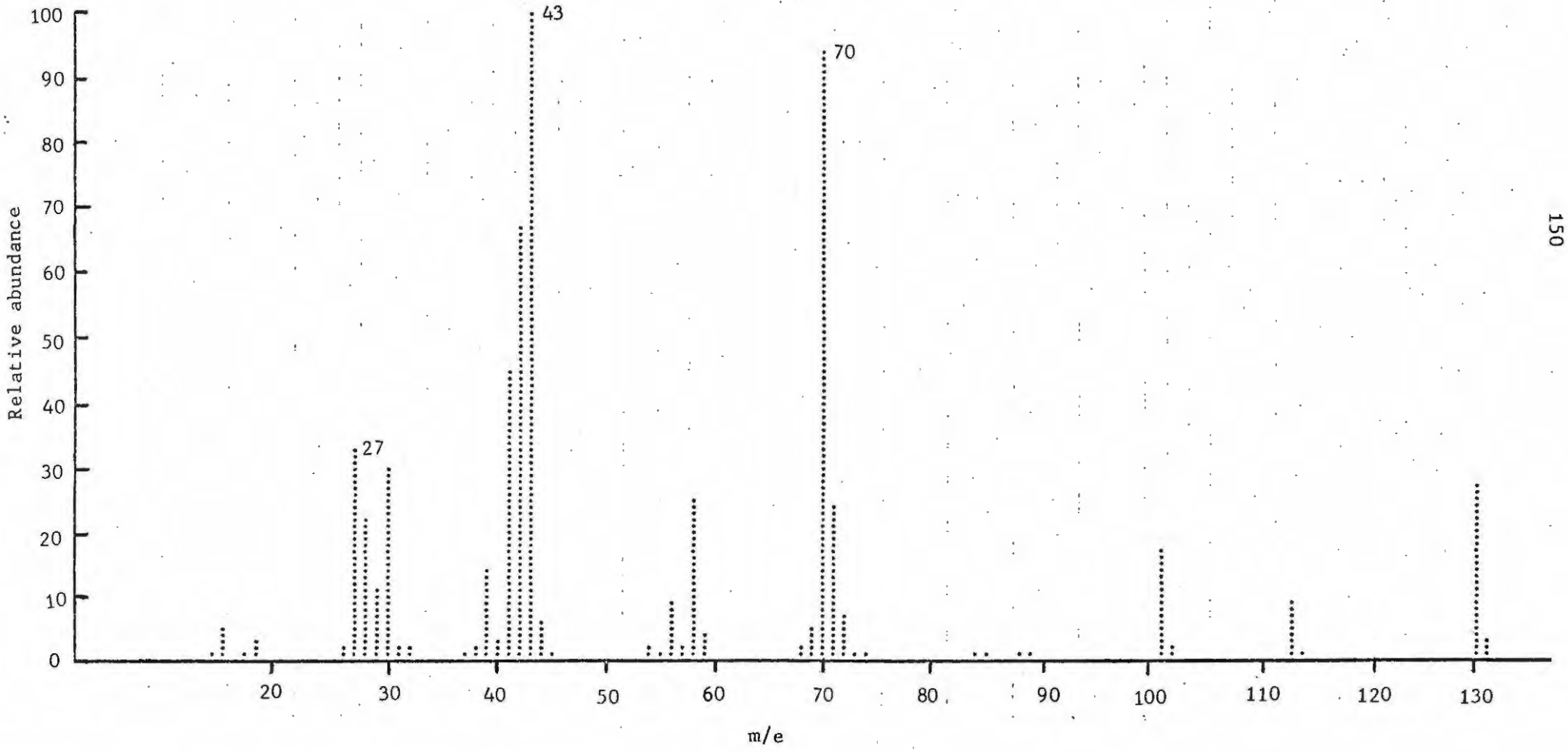


Fig. 17. Mass spectrum of DPNA recorded on an AEI MS30 instrument

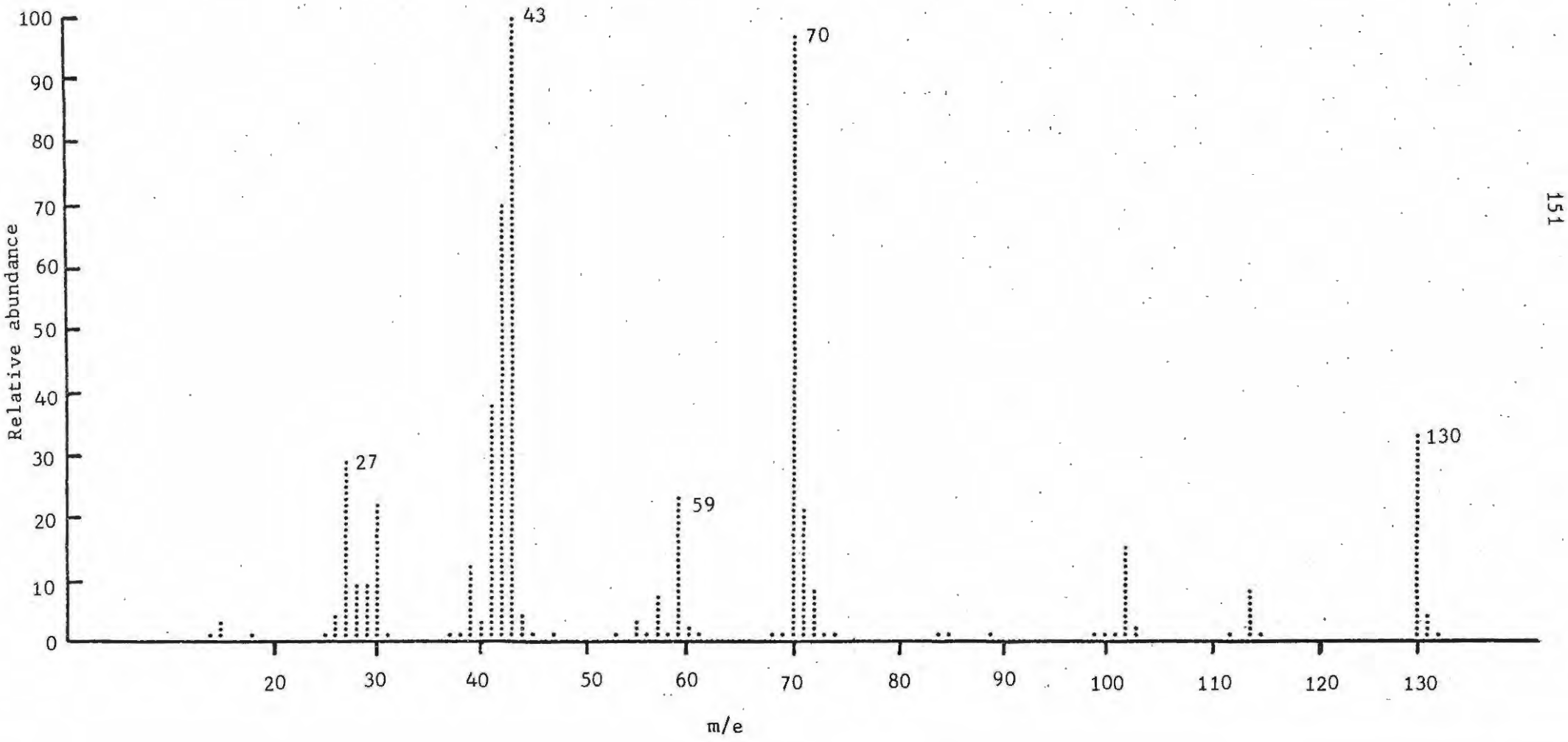


Fig. 18. Mass spectrum of DBNA recorded on an AEI MS30 instrument

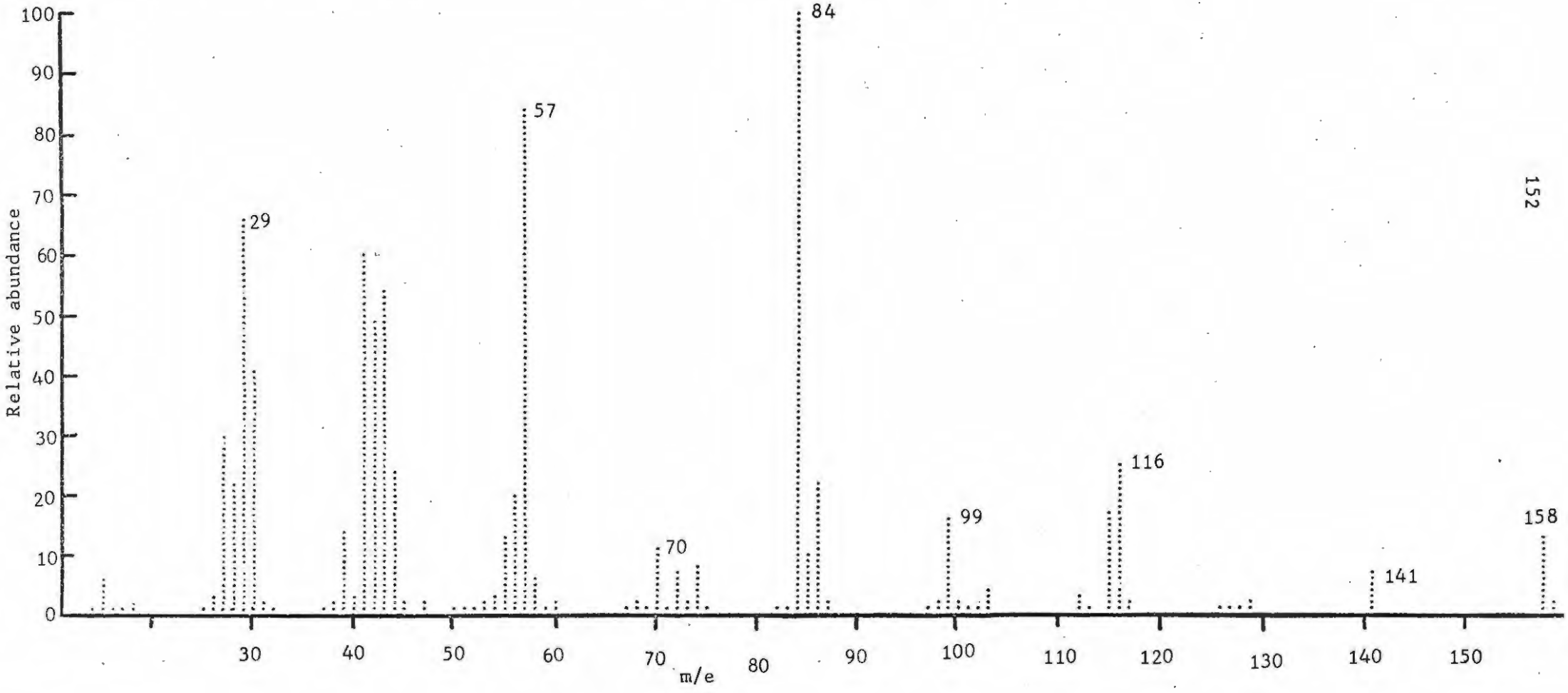


Fig. 19. Mass spectrum of NPIP recorded on a Hitachi - Perkin-Elmer RMS4 instrument

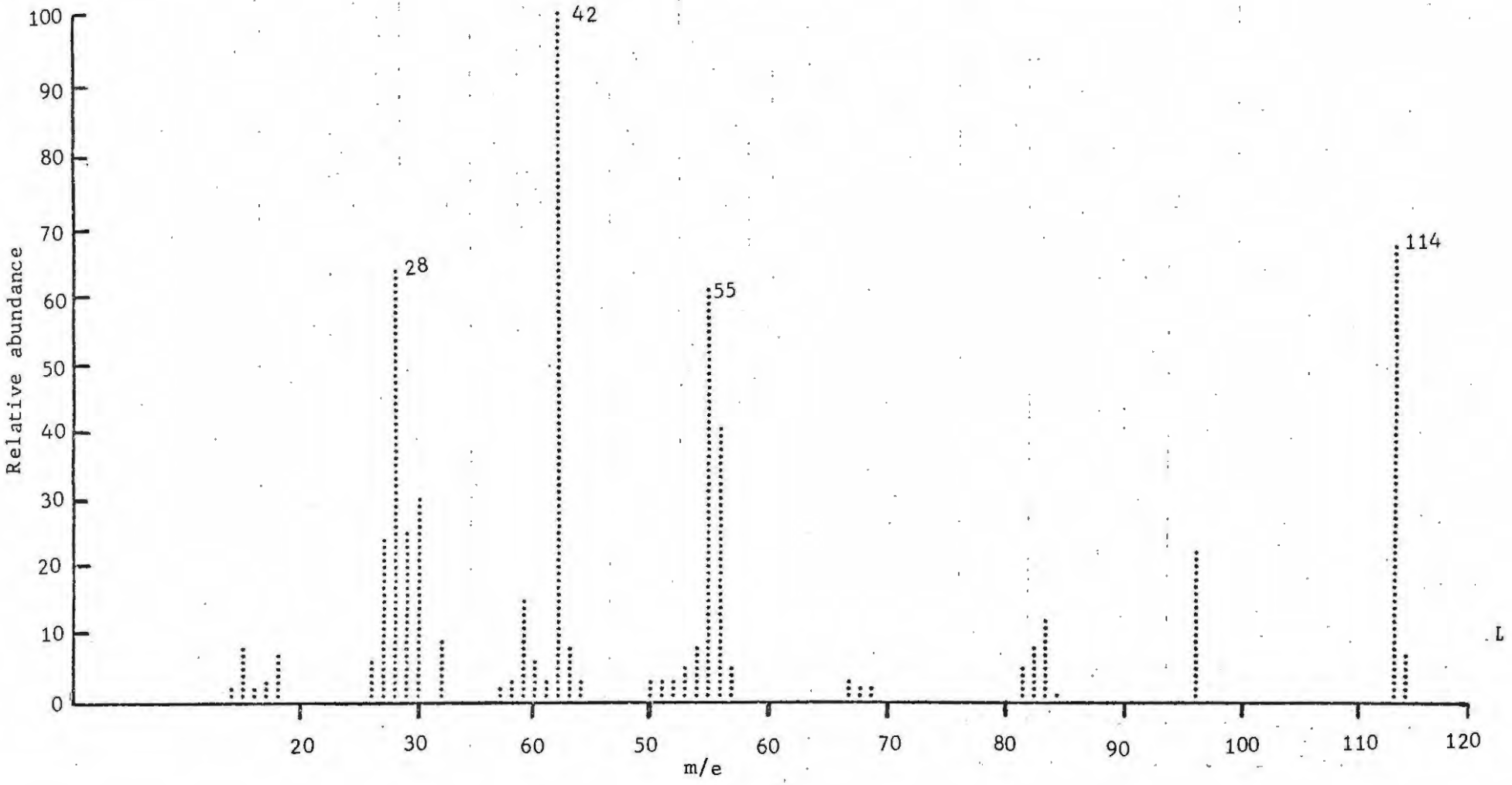
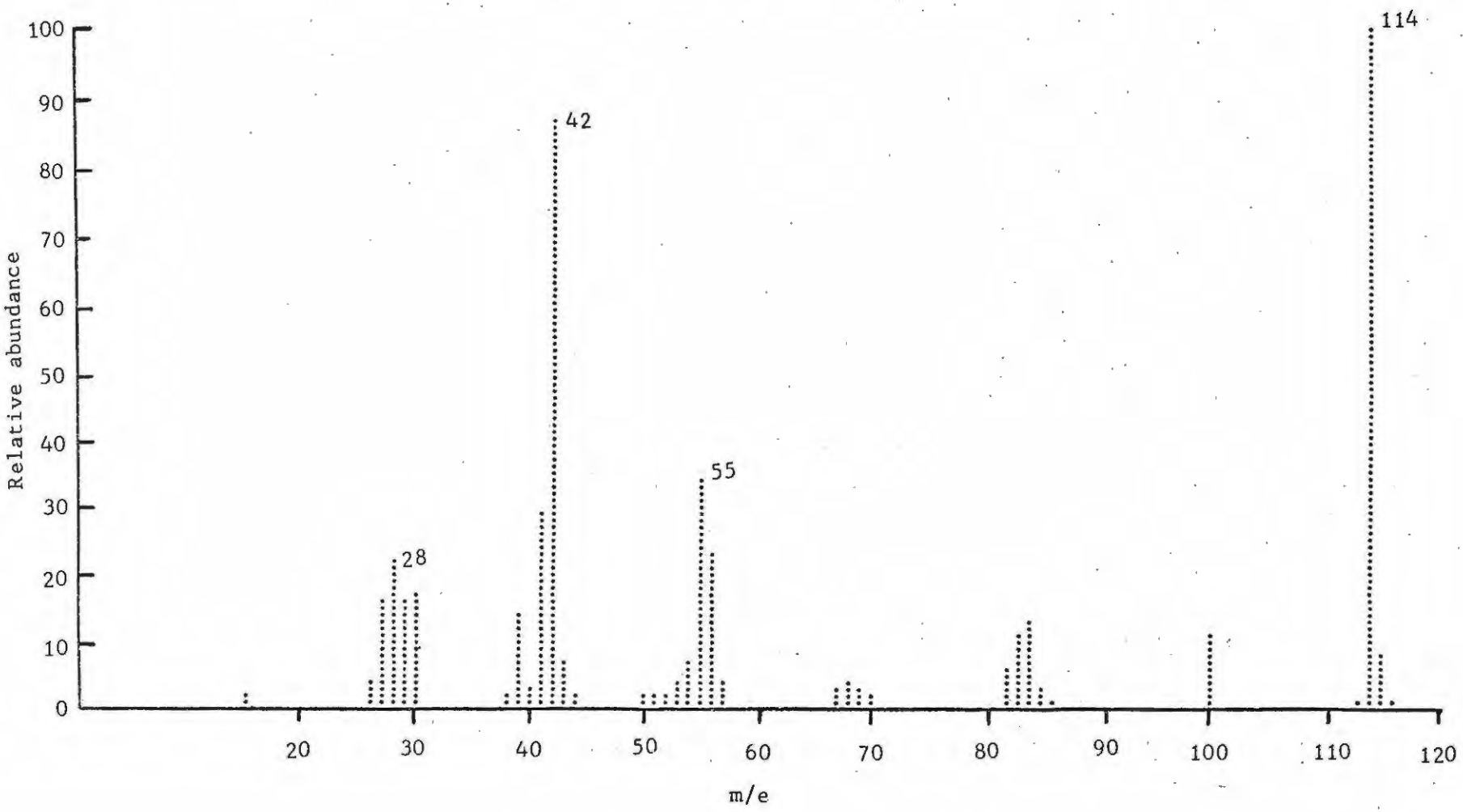


Fig. 20. Mass specrtum of NPIP recorded on an AEI MS30 instrument



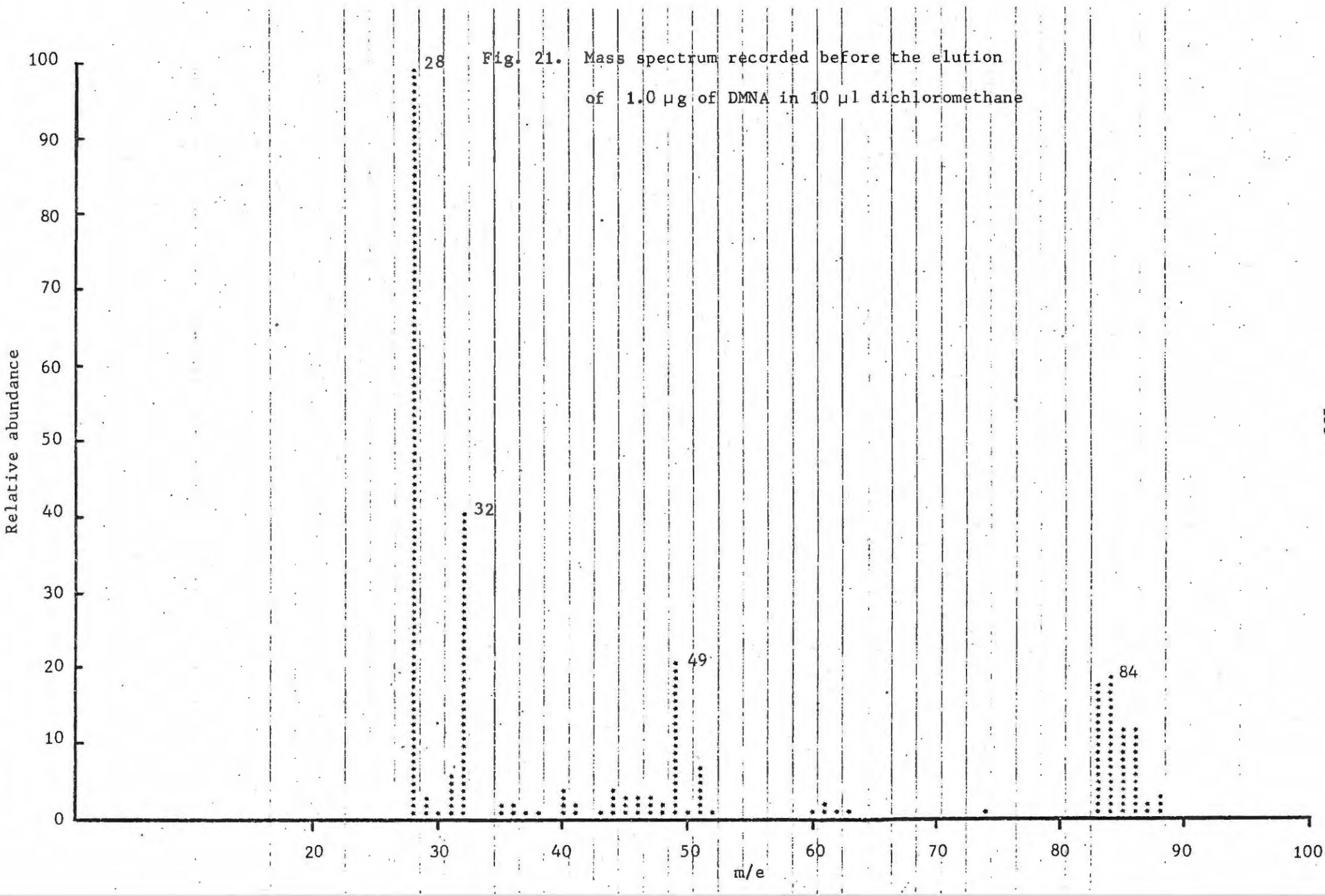
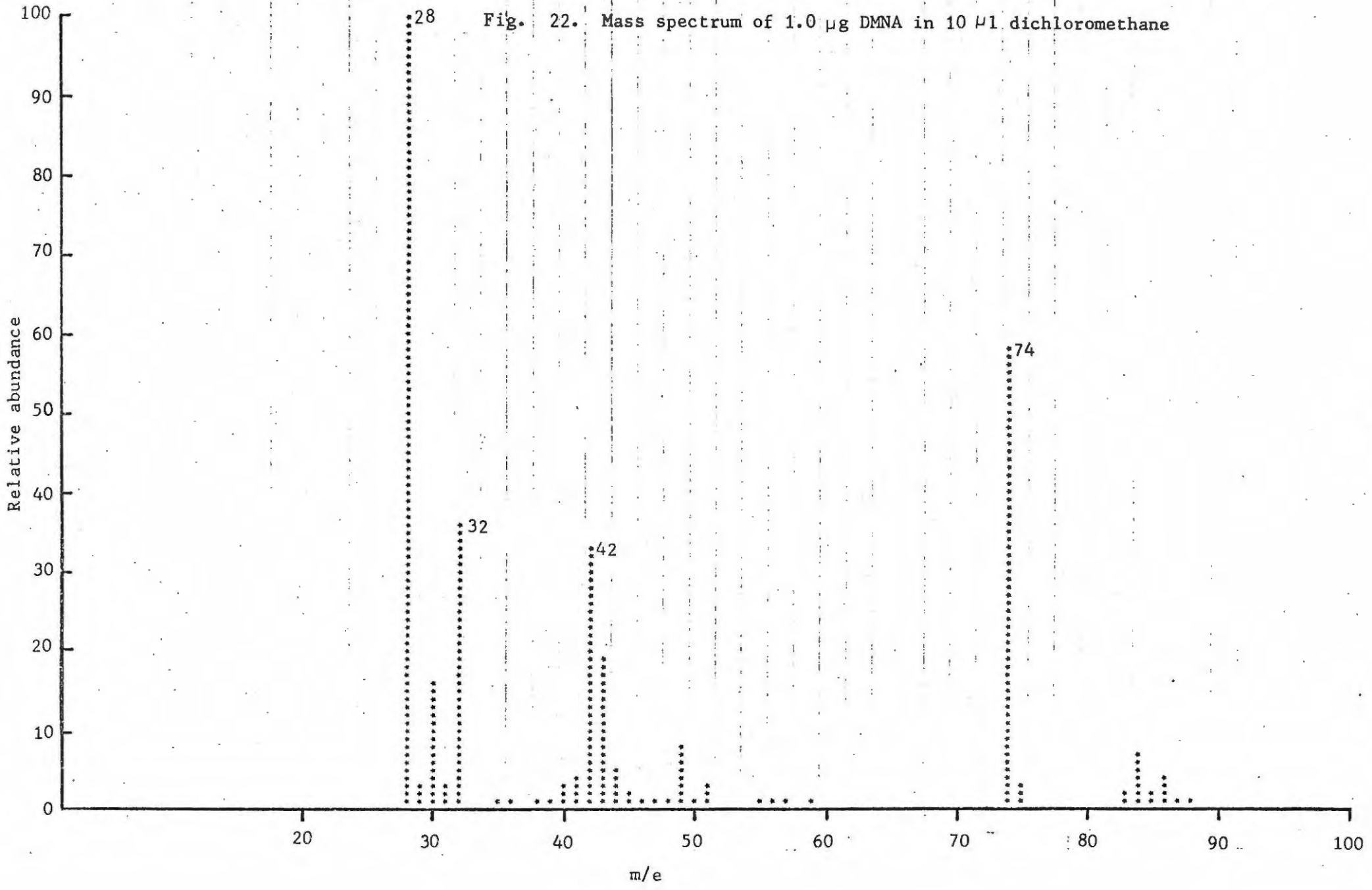


Fig. 21. Mass spectrum recorded before the elution of 1.0 µg of DMNA in 10 µl dichloromethane

Fig. 22. Mass spectrum of 1.0 µg DMNA in 10 µl dichloromethane



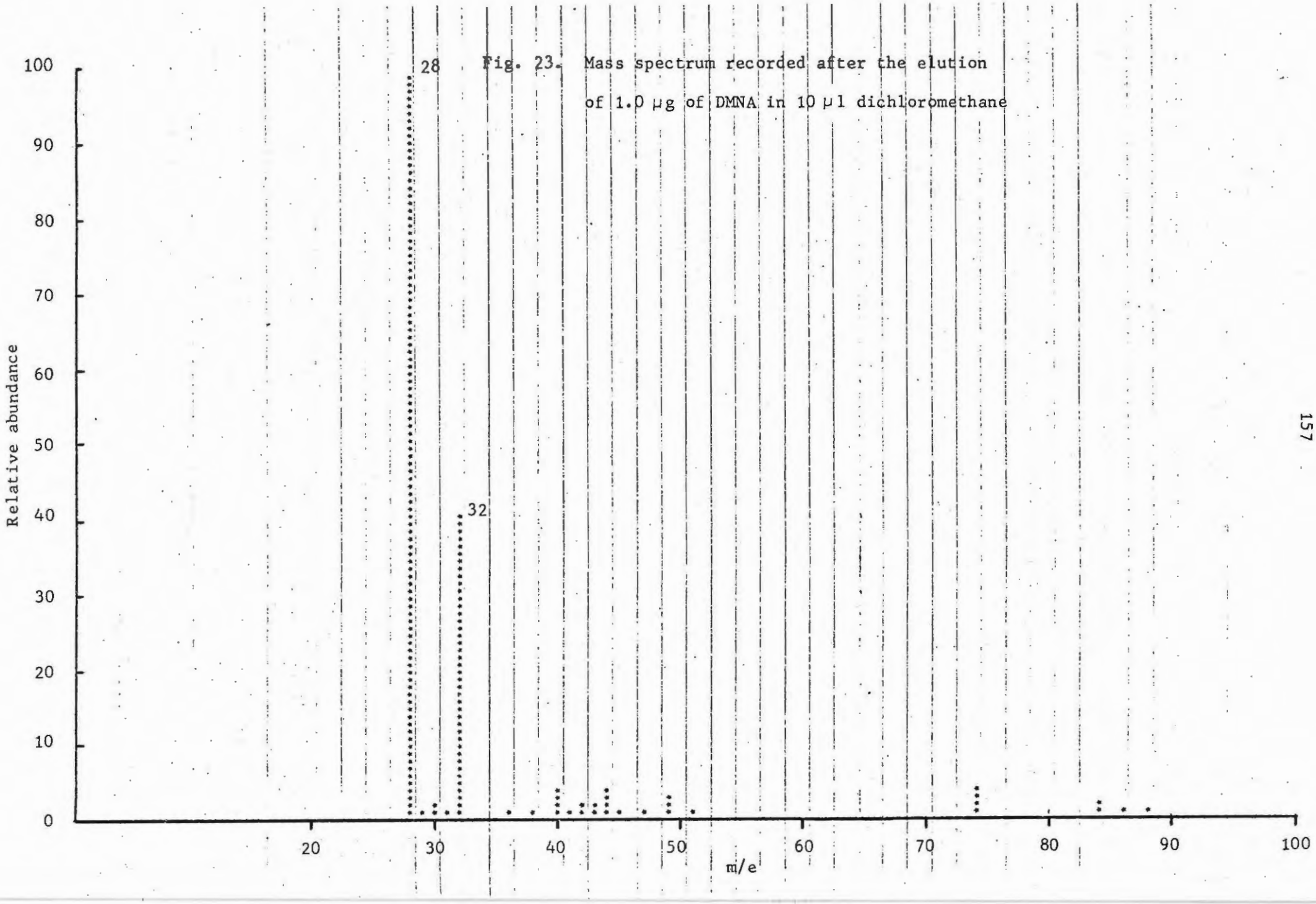
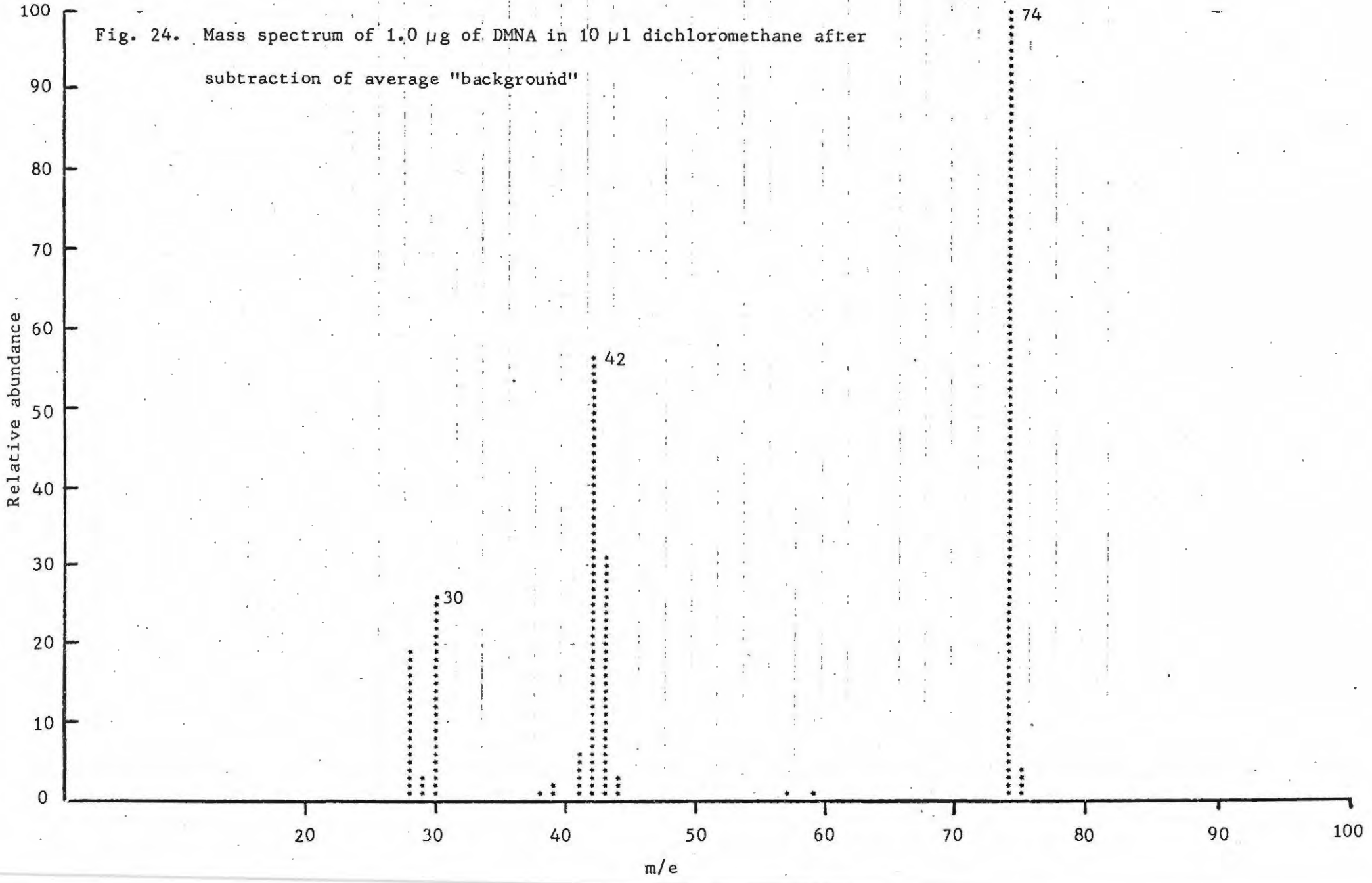


Fig. 23. Mass spectrum recorded after the elution of 1.0 µg of DMNA in 10 µl dichloromethane

Fig. 24. Mass spectrum of 1.0 µg of DMNA in 10 µl dichloromethane after subtraction of average "background"



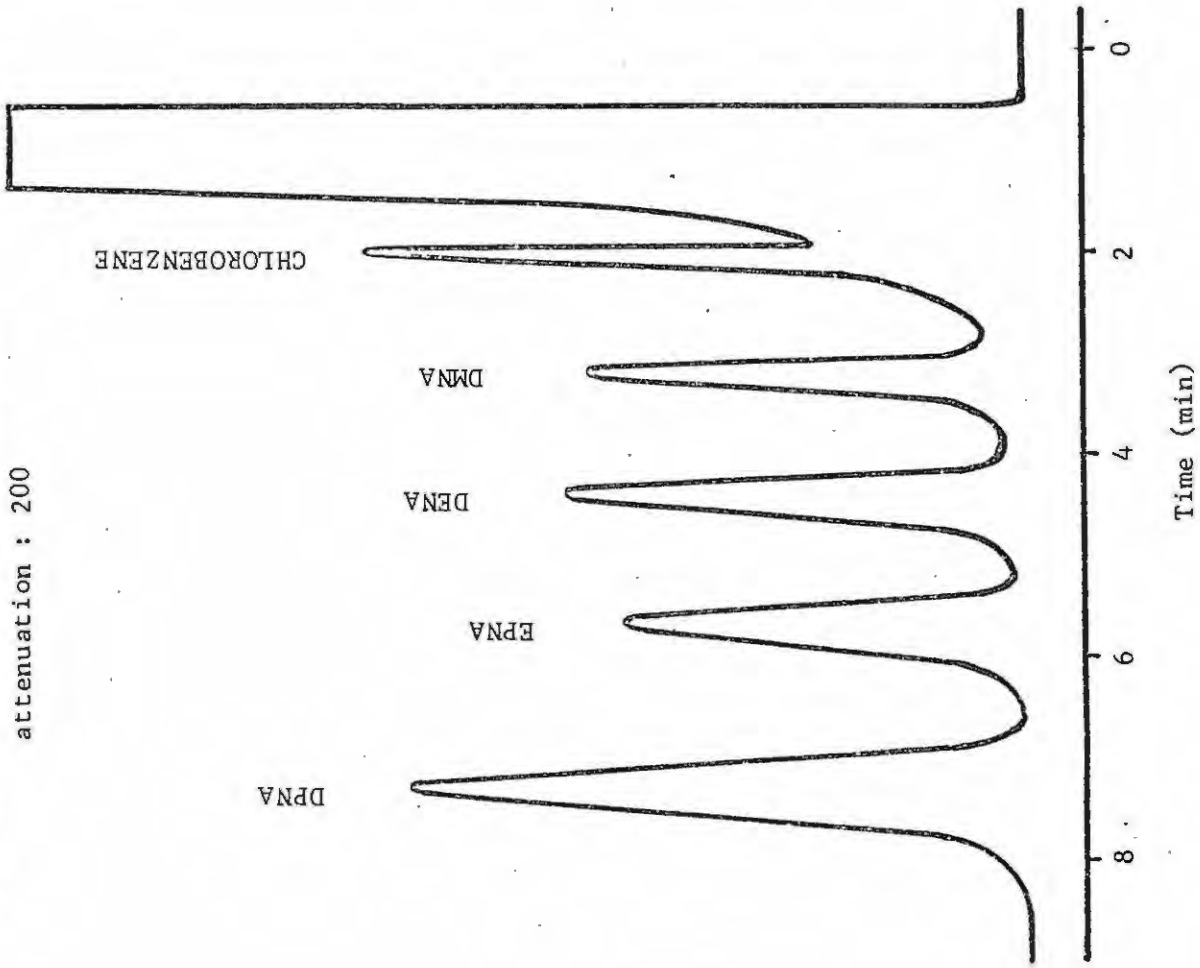


Fig. 25. A typical gas chromatogram
attenuation : 200

Fig. 26. Calibration curves for nitrosamines
attenuation : 200

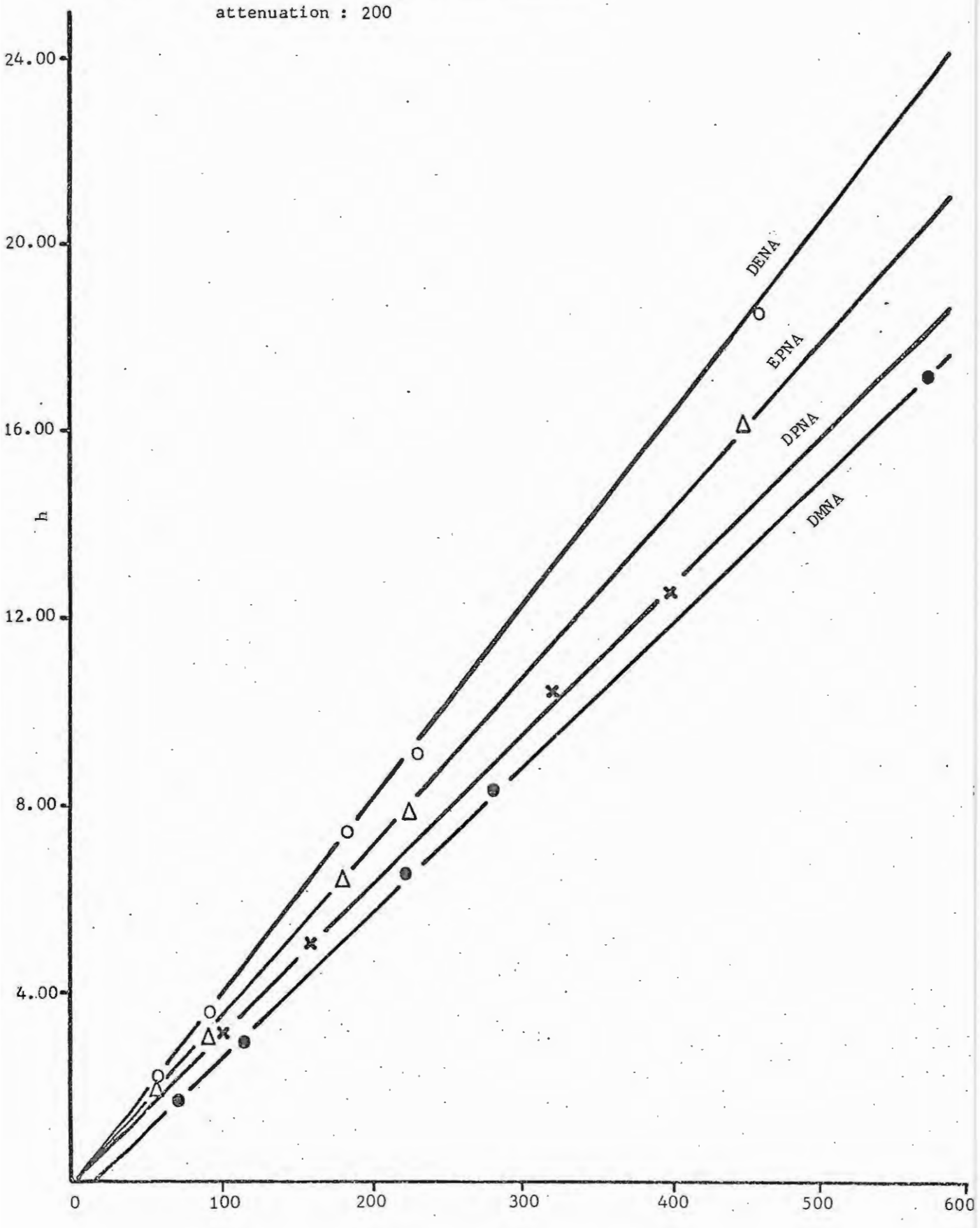


Fig. 27. Calibration curves for nitrosamines
attenuation : 20

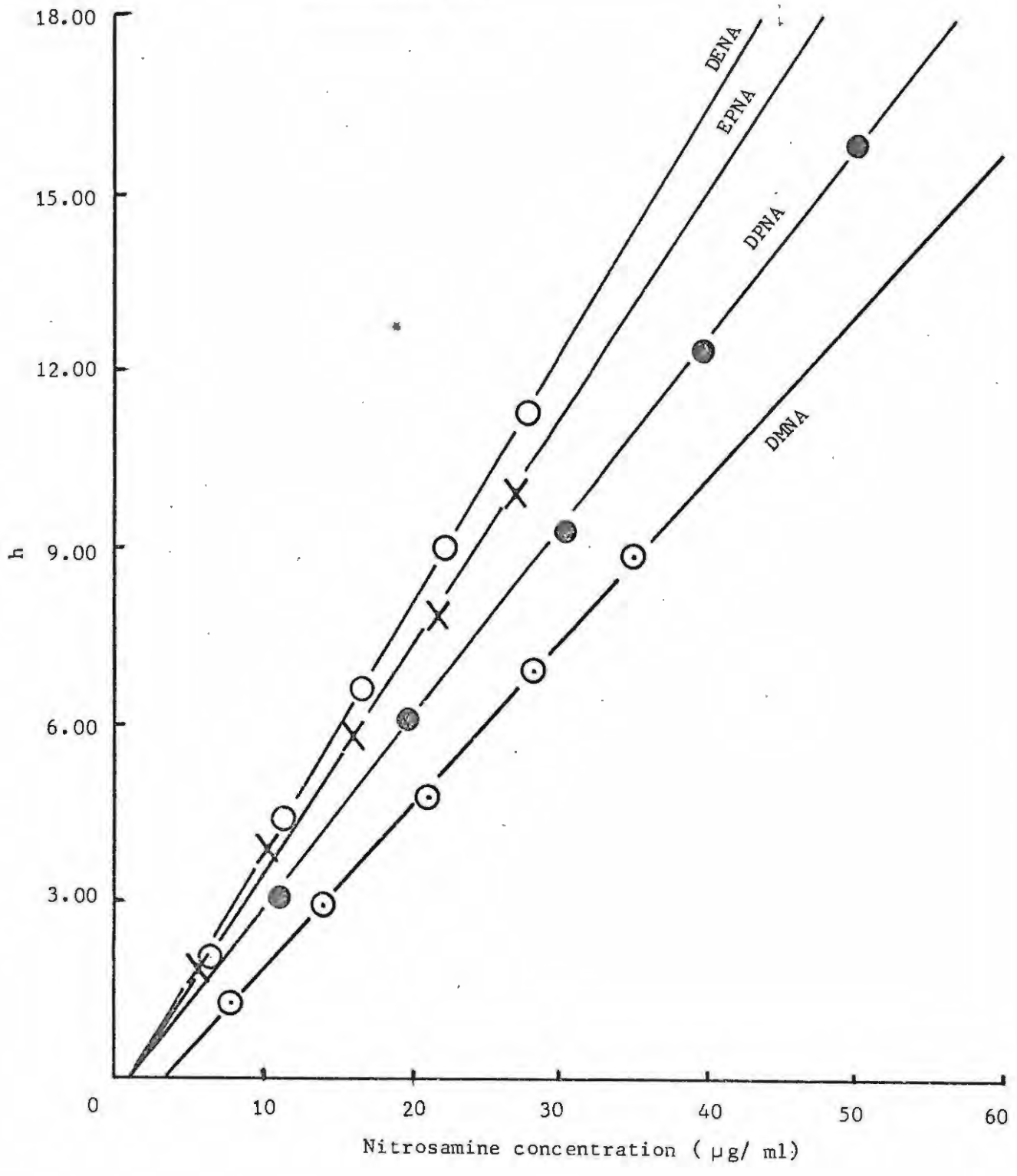


Fig. 28. Relationship between volume of extractant and recovery of DMNA

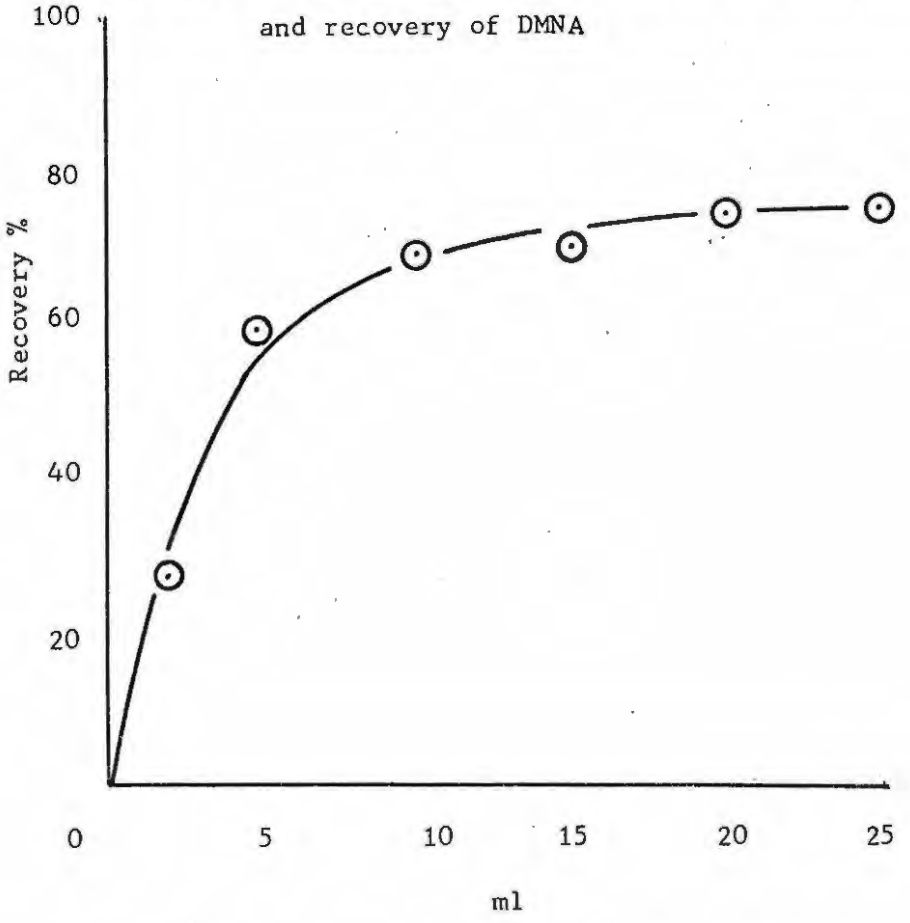


Fig. 29. Relationship between number of extractions and recovery of DMNA, DENA, EPNA and DPNA

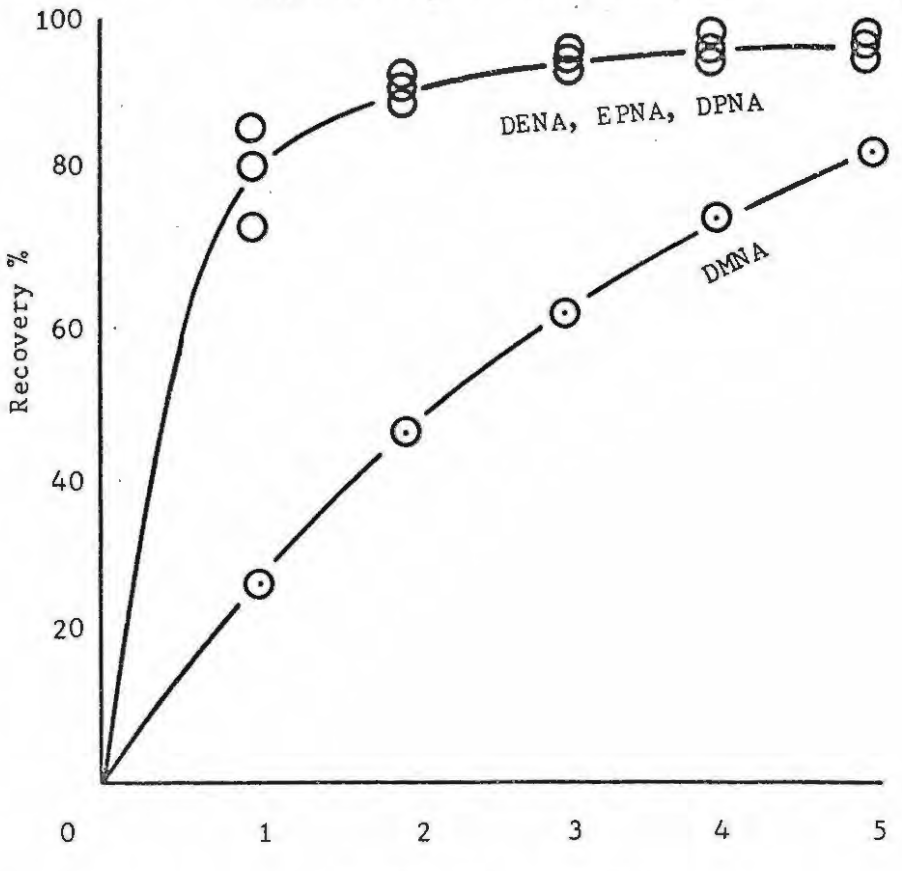


Fig. 30. NMR spectrum of DMNA

A : pure DMNA

B : isolated from Solanum incanum

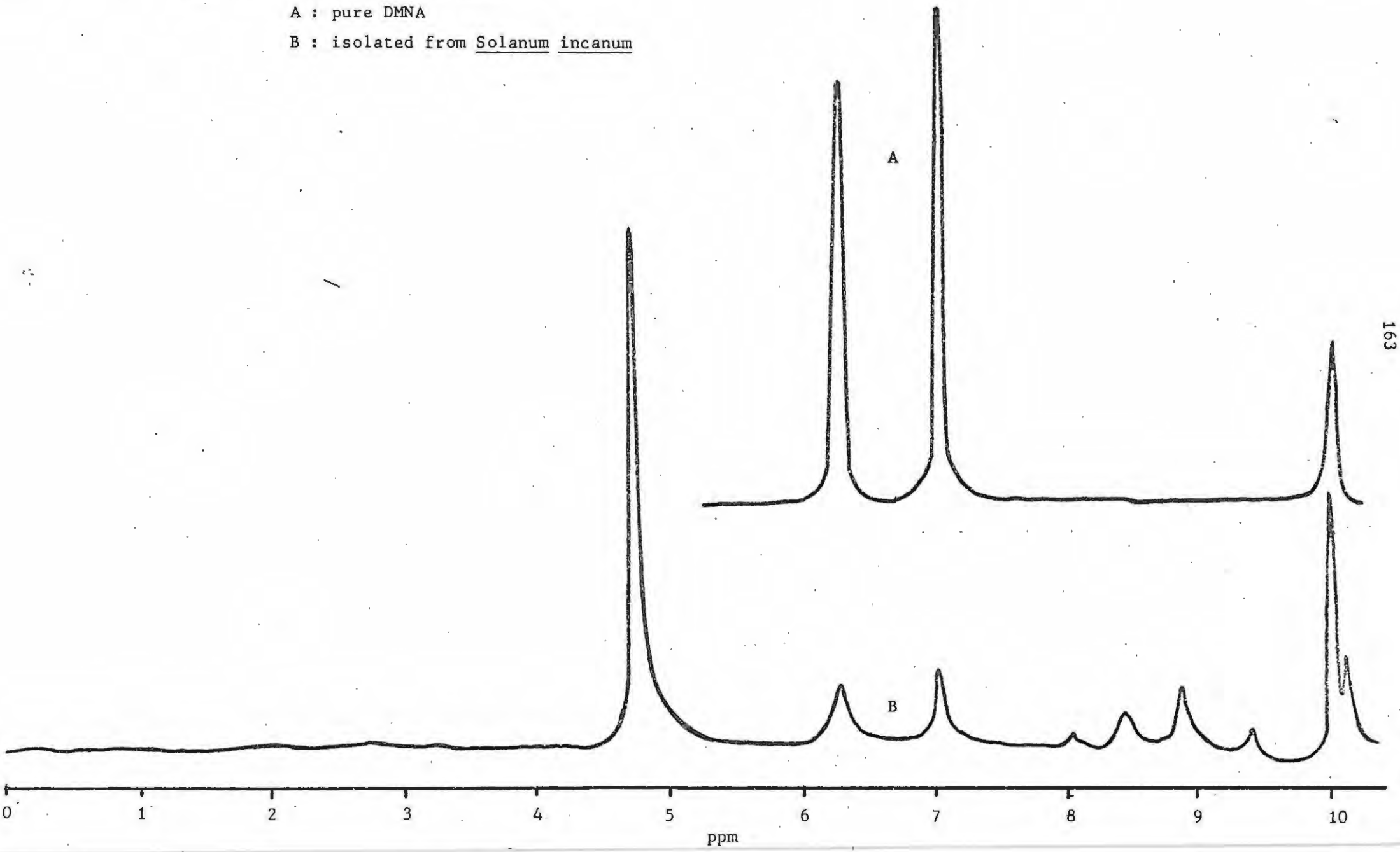


Fig. 31. Infrared spectrum of dimethylnitrosamine fraction isolated from Solanum incanum

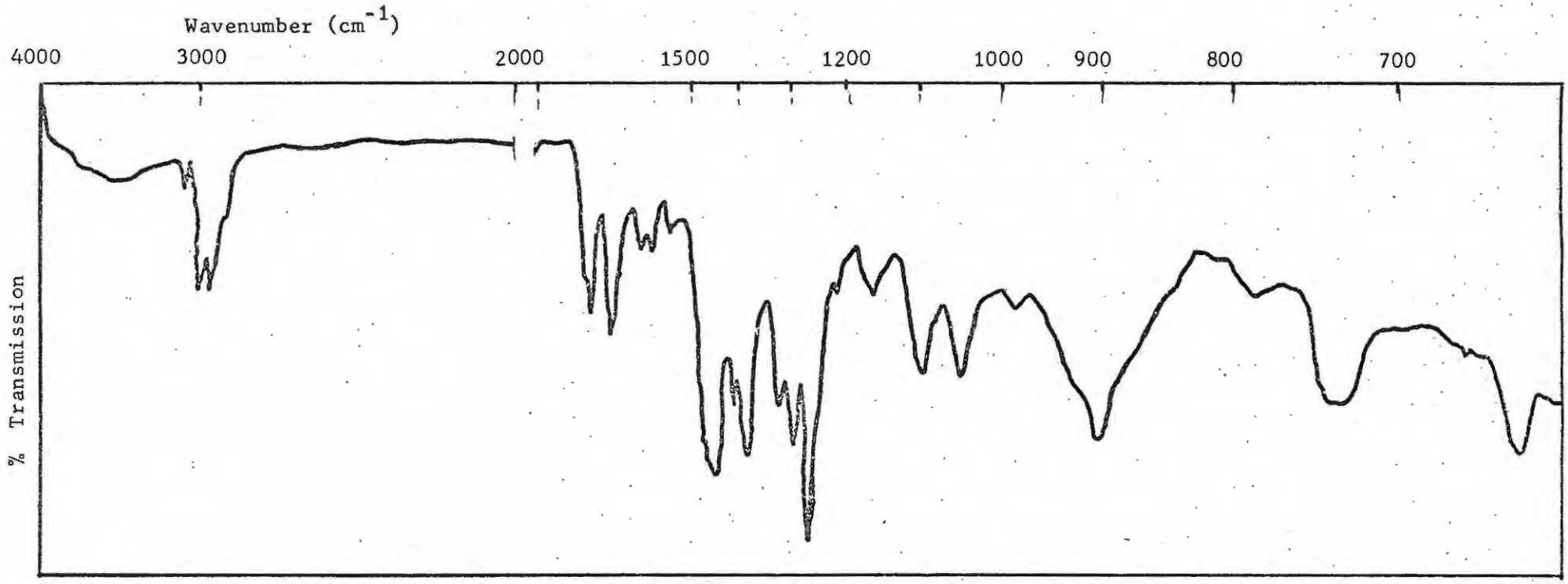
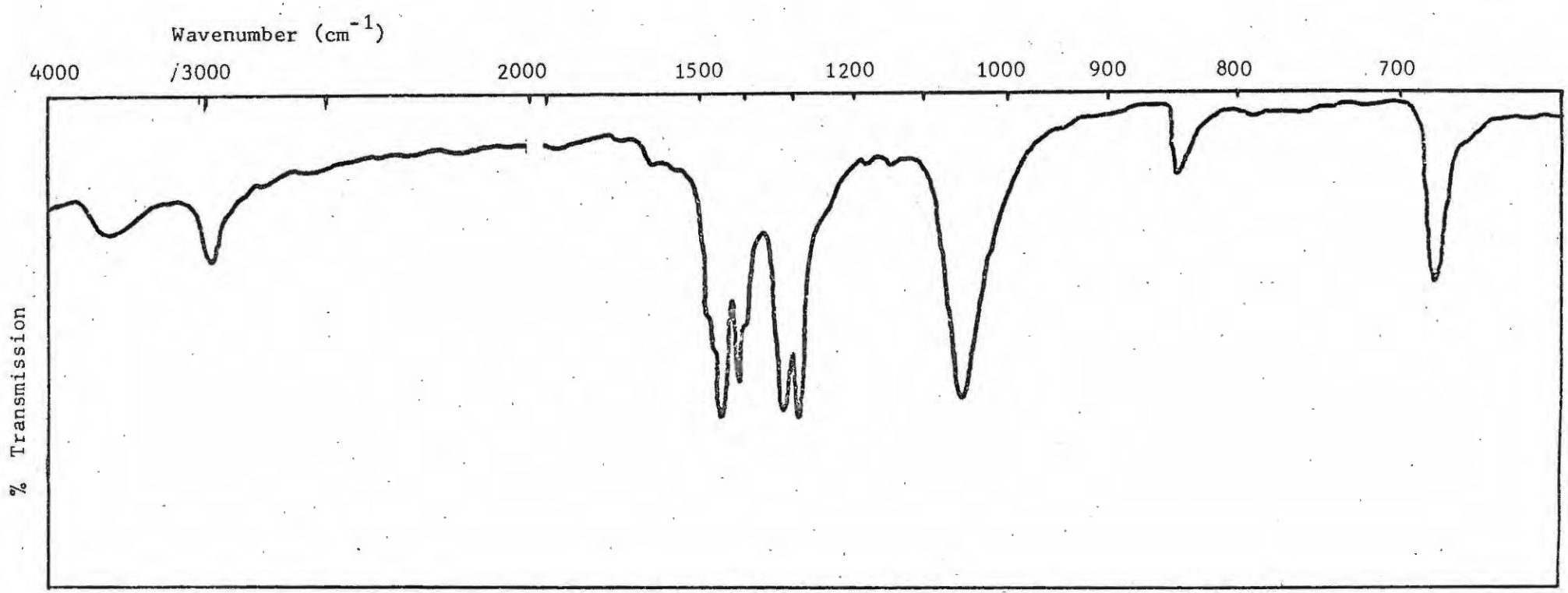


Fig. 32. Infrared spectrum of pure dimethylnitrosamine



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