" A STUDY OF THE PROPERTIES AND METHODS OF ANALYSIS OF HIGH MOLECULAR WEIGHT N-NITROSAMINES "

by

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SUMMARY

Various high molecular weight dialkylnitrosamines were prepared including, for the first time, methyl-n-octadecylnitrosamine and di-n-dodecylnitrosamine. The infrared, ultraviolet and mass spectra of a selection of these compounds were recorded and studied. Gel permeation chromatography was used for the isolation of individual nitrosamines in standard nitrosamine mixtures, while ion-exchange chromatography effected complete clean-up of amine-contaminated nitrosamine solutions. Thin-layer and gas-liquid chromatographic methods were developed for the detection, separation and analysis of nanogram quantities of these lipophilic nitrosamines. In addition the above chromatographic systems were used for the analysis of distillates of spiked wheat flour samples. High recoveries of dicyclopentylnitrosamine, di-n-heptylnitrosamine and di-n-octylnitrosamine, from the spiked wheat flour samples, were achieved using a specially developed freeze-drying/vacuum distillation technique, the distillates obtained being relatively free from major contaminants.

1. INTRODUCTION

In a general review of the chromatography of alkylating agents Fishbein and Falk [1] (1969) briefly outlined chromatographic methods for the analysis of nitrosamines, while in 1971 Wasserman [2] and Walters [3] both reviewed general analytical procedures. Du Plessis [4] (1972) reviewed many aspects of nitrosamine analysis in a very comprehensive and broad based review.

The following is a critical and comparative study of recent isolation and chromatographic techniques in N-nitrosamine analysis.

1.1 Isolation of N-nitrosamines

Eisenbrand [5] extracted samples (1 kg) of flour in a Soxhlet apparatus and the extract was evaporated to about 50 ml in a Kuderna-Danish apparatus. The syrupy residue was transfered to a steam distillation apparatus, an equal volume of sodium hydroxide solution (0,4M) added and the mixture distilled. The distillate was then redistilled from 0,1M sulphuric acid and this second distillate, after saturation with sodium chloride, was extracted twice with an equal volume of dichloromethane. After drying over sodium sulphate, the dichloromethane extract was concentrated to 2-3 ml in a Kuderna-Danish evaporator; the residue was further concentrated to 0,5 ml with a gentle stream of nitrogen. The extract was further 'cleaned-up' by a preparative thin-layer chromatography (TLC) method (see TLC section). Denitrosation of any nitrosamine present was effected with 3% hydrogen bromide and the heptafluorobutyryl chloride derivatives (HFB-Cl) of the resulting amines were formed and quantitatively determined by gas-liquid chromatography (GLC). Even after this multi-step extraction and clean-up procedure, GLC traces of the developed TLC extract zones (corresponding to the approximate R_f-values of the authentic individual nitrosamines) showed the presence of many strong contaminating peaks

which would make positive identification and accurate estimation of the nitrosamines very uncertain. No GLC flour 'blank' trace was given for background comparison. Nevertheless, the use of an additional clean-up system was suggested which looks promising for the removal of low and high molecular weight contaminating amines from food-stuff extracts. This system consists of a 30 X 5 mm ion exchange column packed with a cellulose matrix ion exchanger (given as SE-cellulose). The author claims that amines adsorbed, in amounts up to 100 μg, on the above column system could be quantitatively eluted with 5 ml of 4M hydrochloric acid in 50% aqueous methanol.

Sen [6] (1971), who used an ion exchange resin Rexyl 101 and polyamide composite column for clean-up of low molecular weight nitrosamine extracts, stated that the above system was not suitable for analysing N-nitrosopyrrolidine (N-Pyrr) and other high molecular weight dialkylnitrosamines owing to the poor recoveries of such compounds, and remarked that Telling [7] also experienced the same problem.

Walters et al [8] (1970) used an adsorptive method to extract nitrosamines from aqueous solution. On shaking for two hours with activated carbon (2g/100 ml) N-nitrosamines were removed, virtually quantitatively, from 10 ppm aqueous solutions with the exception of dimethylnitrosamine (DMN) and N-nitrosomethyl-2-hydroxyethylamine, for which the efficiencies of adsorption were 75% and 70% respectively. Desorbing the nitrosamines with boiling methanol (20 ml/g dry carbon) resulted in reasonably good extraction of the lower molecular weight nitrosamines, but very poor desorption of the more lipophilic nitrosamines (for example zero for di-n-pentylnitrosamine).

Eisenbrand et al [9] performed atmospheric and reduced pressure distillations of 16 different N-nitrosamines from acid, alkaline and neutral media (0,1M tartaric acid, 0,2M sodium hydroxide and water

respectively). The temperatures of the heating baths used for the atmospheric and reduced pressure (14 mm) distillations were 160-170°, and 110-120° respectively. Nitrosamine recoveries of 90-100% from the various media were claimed except in the cases of di-n-octyl and methyl-2-hydroxyethyl nitrosamines, which gave average recoveries of 39% and 24% respectively when distilled under reduced pressure. However, atmospheric pressure steam distillation of di-n-octylnitrosamine (DON) gave yields better than 90%. The authors also reported that for all the nitrosamines studied no difference in yield was observed when 0,2M phosphoric or 0,1M sulphuric acids were used in place of tartaric acid. A Zeiss DMR 21 recording spectrophotometer was used for estimating recoveries.

No attempt was made, in the above work, to separate any of the nitrosamines from organic or biological material, but it was suggested that post steam distillation lipid removal could be achieved by a procedure such as acetonitrile/n-heptane partition [10]. The authors stated that large amounts of inherent lipids could reduce the recoveries of the higher lipophilic nitrosamines.

Crosby et al [11] extracted N-nitrosamines from various foodstuffs by a steam distillation method. The minced sample (250 g) was mixed with sodium chloride (54 g) and steam distilled. To the distillate (350 ml) was added 3M sulphuric acid (6 ml) and anhydrous sodium sulphate (42 g). This solution was again steam distilled and to the final distillate (250 ml) was added solid potassium hydroxide (0,2 g) and sodium chloride (50 g). The solution was extracted with dichloromethane (3 X 50 ml) and the combined extracts evaporated to 0,25 ml in a Kuderna-Danish evaporator.

The nitrosamine content of the extracts was estimated by GLC-mass spectrometry (see Isolation section) and levels of between 1 and 9 μg/kg DMN, diethylnitrosamine (DEN) and N-Pyrr were found in the meat and

fish products, and a maximum of 4 μg/kg of DMN and DEN in some cheese samples. In one fried bacon product the exceptionally high N-Pyrr level of 16-40 μg/ kg was recorded. The above steam distillation method when applied to recovery trials (10 μg/ kg level) resulted in variable nitrosamine recoveries which normally fell within the range 70-90%. However the overall recoveries of N-Pyrr were exceptionally low and generally below 30%. The authors stated that no correction factor was applied to the concentration ranges quoted; thus the nitrosamine levels given, particularly in the case of N-Pyrr, must be considered only very approximate.

In contrast with the results above, Du Plessis and Nunn [12] found that the results obtained on steam distilling food samples, followed by extraction into dichloromethane, were inconsistent; the best recovery was 58% for DEN. Sen et al [13] abandoned the use of steam distillation for food-stuff extraction after it was found that steam distillation of food-stuffs, in the presence of nitrite and secondary amines, produced nitrosamine artefacts.

Bryce et al [14] found vacuum distillation preferable to steam distillation for meat product extraction. The authors studied a large number of dialkylnitrosamines, from carbon numbers 2 to 10, and two heterocyclic nitrosamines, N-nitrosopyrrolidine and N-nitrosopiperidine. Recoveries of nitrosamine, at the 10 μg level, from a 250 g sample of ham ranged from 88% for diethylnitrosamine to 31% for di-n-pentylnitrosamine, when steam distillation was employed, and 93% to 31% respectively for vacuum distillation.

Telling [15], using the same vacuum distillation method referred to above, produced further initial clean-up on a neutral alumina column and then oxidised the nitrosamines, in the purified extract, to the corresponding nitramines with peroxytrifluoroacetic acid (PTFA). This

nitrosamine solution was given a final clean-up on a magnesium oxide/alumina column, the eluting solvent being 1% v/v diethyl ether in n-pentane. After final concentration of the eluate to 2 ml in a Kuderna-Danish evaporator detection was achieved by use of a gas chromatograph fitted with an electron capture detector (ECD) (see GLC section). The efficiency of conversion of N-nitrosamines, at the 50 μg level, to the corresponding nitramines varied between 86% for DMN to 25% for N-Pyrr, the percentage conversion being reasonably consistent for all the dialkylnitrosamines chosen. Dibutyl nitrosamine was the highest molecular weight compound investigated.

Foreman et al [16] used a steam distillation method as described by Heath and Jarvis [17] in which DMN and DEN at the 1 ppm level were recovered to 95% and 97% respectively from spiked corned beef. A GLC method of detection, which is applicable to aqueous samples, is described (see GLC section).

Eisenbrand et al [18] describe a possible method for the extraction and clean-up of spiked wheat flour samples. The method is based on solvent extraction of commercial wheat flour with dichloromethane in a Soxhlet apparatus. The solution was evaporated in a rotary evaporator and the residue partitioned in n-heptane/acetonitrile [10]. The acetonitrile phase was evaporated together with 100 g of neutral alumina, after which the latter was packed into a column and eluted with 50% aqueous methanol. Attempts to separate and identify N-nitrosamines in the above flour concentrate, by gel chromatography/UV spectrophotometry, failed because UV absorbing contaminants in the wheat flour made the gel separation monitoring impossible, and no alternate procedure was adopted to determine the efficiency of the clean-up method.

Sen et al [19] extracted various food-stuffs with ether, this being followed by steam distillation of the extract. In each case a food

sample of 100 g yielded a final extract of about 0,5 ml. This method afforded DEN recoveries of 65% and 90% for spiked flour samples containing 10 μg and 400 μg of the nitrosamines respectively (see TLC section).

A method for the estimation of nitrosamine in alcoholic beverages has been described by Williams et al [20]. Spiked aqueous ethanol solutions and a potable spirit of 70° proof were studied. Two standard aqueous ethanol (35%) samples, one containing 1 ppm each of DMN and DEN and the other 2 ppm, were prepared. Acetic and palmitic acid were added in suitable amounts to simulate the total acidity and pH of commercial spirits. The solutions were distilled in vacuum almost to exhaustion and distillates collected in an ice cooled receiver and two solid CO_2-acetone traps in series. The trap contents were combined, neutralized with saturated potassium carbonate solution, salted, diluted with an equal volume of water and extracted with dichloromethane. The extracts were dried over sodium sulphate followed by calcium sulphate, and final concentration to 0,5 ml was achieved by distillation through fractionating columns packed with stainless steel mesh. The authentic spirit distillates were collected in two fractions, the first up to 31° and the second 31-32°. GLC examinations (peak height comparison) of the final extracts before and after distillation and extraction revealed recoveries for the two nitrosamines of only 20% and 30% respectively. Apparently the added organic acids had no effect on their recovery levels. The GLC study revealed that the higher boiling fraction (after n-pentane extraction clean-up) gave the cleanest trace but still contained strong contaminating peaks which would interfere with the identification and estimation of the two nitrosamines present. The GLC-MS investigation gave no indication of possible inherent nitrosamine in the potable spirit.

Sen and Dalphe [21] also developed a method for nitrosamine isolation from alcoholic beverages. The recoveries of the spiked nitrosamines DMN, DEN and di-n-propylnitrosamine (DPN) were very inconsistent (DMN 20-100%, DPN 50-100%). The variance does not appear to have been due solely to alcohol content but varied considerably within a beverage type. Although the recovery estimations were based on semiquantitative TLC intensity comparisons of extract and authentic standard samples, the following method [21] would appear to result in rather higher nitrosamine recovery than the method of Williams et al [20]. Aliquots of the beverage (100 ml) were distilled after addition of sodium hydroxide (12 g) and 50 ml distillate were collected. The distillate (after addition of potassium carbonate) was extracted with dichloromethane and the extracts washed, first with glycine-hydrochloric acid buffer, and finally with potassium carbonate solution. The dried dichloromethane extracts were finally evaporated down to about 0,4 ml using a micro-snyder distillation apparatus. It was recommended that spirits of high alcololic content should be initially diluted with water (3 times dilution) and 4 times the normal amount of sodium hydroxide. It was further suggested that a proportionately greater volume of distillate should be collected.

Rhoades and Johnson [22] isolated DMN from cigarette tar and obtained positive identification of the compound by high resolution mass spectrometry. Basically the method consisted of collection of the condensate of cigarettes by impingement [23] in small weighing bottles. The combined condensates were slurried with sodium hydroxide and transferred to a 'stripping flask' (a container with provision for a sweep gas to enter at the bottom and exit at the top). The stripping flask exit tube extended, via $\frac{1}{16}$" O.D. Teflon tubing, to a collection vessel immersed in ice water containing a small volume of 5M hydrochloric acid. The nitrogen 'sweep gas' was passed through the system until a

predetermined volume of sample was obtained. The sample in the receiver was shaken with n-hexane, the n-hexane fraction was discarded, and the aqueous phase placed in a clean stripping flask and the process repeated. Collection was continued until only a few drops remained in the stripping flask. This aqueous condensate was saturated with sodium chloride, extracted with dichloromethane, the extract evaporated to 0,5 ml and detection achieved by means of a Coulson electrolytic conductivity detector (CECD) operated in the pyrolytic mode. The method was unfortunately not quantitized to enable accurate estimation of possible nitrosamine content in the extracts. Thus an estimation of the losses of nitrosamine during the stripping and clean-up processes could not be made; losses of DMN and DEN must have been quite high due to their high vapour pressures.

1.2 Thin-layer chromatography (TLC)

Because of the speed, convenience and moderate-to-high sensitivity of the TLC method it has persisted as one of the prime modes of nitrosamine detection. Despite its rejection by some workers on the grounds of insensitivity and unspecificity it has been shown, with recent developments, to be a steadily improving detection system and probably the best general food extract monitoring system available.

Sen and Dalphe [21] described three methods by which extracts of alcoholic beverages, including rum, whiskey, sherry, wine and beer, could be analysed for nitrosamine content by TLC. The thin layers (0,25-0,3mm) were prepared with MN-silica gel G-HR. The mobile phase was n-hexane-diethyl ether-dichloromethane (4:3:2) and the spray reagents were Griess [24,25] NEDSA [21,26] (1:1 solution of 1% w/v sulphanilic acid in 30% v/v acetic acid and 0,1% w/v of N-1-naphthylethylenediamine hydrochloride) and ninhydrin solution (0,3% in ethanol). They reported that a double spray technique [19] using Griess or NEDSA reagent and

ninhydrin solution is specific for N-nitrosamines. Two of the methods described were directed to the detection of the free nitrosamines while the third was used for the detection of the nitramines (the nitrosamines were oxidised to the appropriate nitramines with trifluoroperacetic acid [13, 27]). They claimed the methods to be sensitive down to 0,025 ppm. In the alcoholic beverages studied no identifiable volatile nitrosamines were detected. However, the need was expressed for sensitive methods for detecting non-volatile nitrosamines which might occur in undistilled alcoholic drinks.

The methods employed in the above work are meritorious in general content in that they appear to offer a successful solution to the difficult problem of nitrosamine detection in the presence of many contaminating and interfering species However, the authors suggested that the whole of the final cleaned up extract volume of 0,3 ml be applied to the plate as a single spot, the diameter of which should be 8-10 mm. If this were the case reproducible semiquantitative TLC would be almost impossible since spots of the above dimensions would, on development, result in relatively poor detection and resolution of the nitrosamines.

Eisenbrand and Preussmann [26] developed a method in which stoichiometric production of nitrite as nitrosyl bromides was possible through the action of hydrobromic acid (in glacial acetic acid) or N-nitrosamines. The NO^+ species formed was reacted with sulphanilic acid followed by coupling with N-1-naphthylethylenediamine. The resulting chromophore was estimated colorimetrically. Virtually quantitative release of nitrosyl bromide was obtained from seventeen simple nitrosamines (dialkyl, heterocyclic and aromatic) but a lower percentage conversion was obtained for N-methyl-N-vinylnitrosamine (92,8%). No percentage NO^+ conversion estimates were given for the higher dialkyl nitrosamines (i.e. carbon number greater than 10), They found that hydrolysis was seriously impaired by the presence of more that 5% water.

Eisenbrand [5] used a preparative TLC clean-up procedure for flour extracts in which the extract (in dichloromethane) was applied as a streak on a preparative silica gel PF 254 TLC plate and authentic nitrosamines (DMN, DEN and diamylnitrosamine (DAN)) were co-chromatographed on one side of the plate. The plate was developed in the dark at 4° with n-pentane:dichloromethane:diethyl ether (5:2:2), and the layer then divided into three zones according to the R_f-value of the reference compound; each zone was eluted with glacial acetic acid. Each eluate was treated with an equal volume of 3% hydrogen bromide in glacial acetic acid to denitrosate the nitrosamines. Heptafluorobutyryl chloride derivatives were then formed and quantitatively estimated according to the method of Walle and Ehrsson [28]. They estimated that this clean-up procedure resulted in losses less than or equal to 10%. A 5% SE-30/AW-DMCS chromosorb W GLC column and electron capture detector (ECD) were used to estimate the recoveries of the above nitrosamines, and the degree of contamination of their fractions. The mean recoveries for DMN, DEN and DAN were respectively 69%, 82% and 62%. However, all three preparative TLC fractions showed quite heavy contamination from other compounds present and no 'blank' flour extract traces were recorded for comparison. The author suggested the use of an ion exchanger based on a cellulose matrix (SE cellulose) for further clean-up of the fractions by the adsorption of amines (both low molecular weight and higher lipophilic amines). However, no indication was given of its success or otherwise with spiked flour extracts.

Serfontein and Hurter [29] (1966) employed a method which they claimed was particularly useful for the identification and estimation of nitrosamines in complex organic mixtures and had been successfully applied to the analysis of N-nitrosamines in cigarette smoke. The procedure involved reduction of the nitrosamines by lithium aluminium

hydride followed by reaction with 4-nitro-azobenzene-4'-carboxylic acid chloride to produce the hydrazides, according to the method of Neurath and Doerk [30]. The hydrazide derivatives were separated by TLC on silica gel G using 60-80° petroleum ether/chloroform (5:9) as developing solvent. The hydrazides, being coloured, eliminated the need for a spray reagent. No lower detection limit was determined but they claimed that 2,5 µg of the derivative was easily detectable. However, because of the undoubted presence of other nitrogeneous compounds in tobacco, and the high possibility of artefact formation in the burning tobacco, one cannot conclude that positive 'hydrazide' spots confirm the presence of nitrosamine.

Sen et al [19] extracted various food-stuffs with diethyl ether, and steam distilled the extract. The food-stuffs concerned included cheese, fish, canned meats, flour; the weight extracted in each case was 100 g. The final volume of extract after evaporation was 0,5 ml, and 100-200 µl aliquots were spotted onto conventionally prepared silica gel G-HR TLC plates. Various nitrosamine standards were also run on similar plates using three different mixtures of n-hexane:diethyl ether: dichloromethane as eluting solvents. The R_f-values for fourteen nitrosamines were recorded. They found that, using the above conditions a detection of 0,05-0,15 ppm of nitrosamine could be achieved. Difficulties were encountered when TLC and GLC methods were applied to the analysis of flour extracts, prepared according to the method of Marquardt and Hedler [31], due to interfering co-extractants; also recoveries were low (viz, DEN was less than 30%). However, when using their own method of extract preparation as referred to above, the authors obtained DEN recoveries of 65 and 90% from spiked flour samples containing 10 µg and 400 µg of the nitrosamine respectively. They claimed that the diphenylamine/palladium chloride [32,33] spray reagent

gave many false positive spots for nitrosamines. The other spray reagents used (Griess [24,25] and ninhydrin reagents) did not give these false colour reactions, and it was concluded that the latter reagents were more specific for N-nitrosamines.

The danger of assuming the presence of nitrosamines, both natural and as artefacts in food-stuffs, from polarographic and simple chromatographic evidence has been well illustrated by a number of workers; for instance, Scanlan and Libbey [34] challenged the findings of Devik[35], who reported the formation of N-nitrosamines from heat induced reactions between D-glucose and several L-amino acids. The production of a number of the simpler dialkylnitrosamines was implicated by polarographic TLC and GLC techniques. In the work of Scanlan and Libbey D-glucose and L-alanine were adsorbed on potato starch in a slurry at pH 8,3 and heated at 100° for 20 hr. The products were vacuum distilled, as described by Devik [35] and the dichloromethane extracts of the aqueous distillates were analysed by tandem GLC-mass spectrometry. No evidence for the presence of the carcinogenic lower molecular weight nitrosamines was found. Their results agreed with those of Heyns and Koch [36] who indicated that Devik probably misidentified non-enzymatic browning products, such as pyrazines and acetylpyrrole as N-nitrosamines.

Sen et al [37] reported the presence of DMN and N-Pyrr in bacon The bacon was purchased from local stores (Ottowa, Canada) and analysed raw or after frying in an electric pan. The N-Pyrr was detected by the TLC method already referred to in this review. Samples (0,1 to 0,3 ml) of the final extract were spotted on silica gel TLC plates, and an acetic acid-ninhydrin reagent [21] combination was used for the final detection. N-Pyrr featured as an orange fluorescent spot when the heated plate was examined inside a chromoview chamber (Ultra-violet Products Inc., San Gabriel, California). The authors claimed that the above

technique is very sensitive and specific, 50 ng of authentic N-Pyrr being clearly visible. The N-Pyrr in the extracts were semiquantitatively estimated by visual comparison of intensities of the unknown with those of authentic standards. The recoveries of added N-Pyrr (at the 0,01 ppm level) varied between 60-80%. The nitrosamine was extracted and purified by an earlier method of Sen [6]. A table of bacon analyses is given in which eight fried bacons were claimed to contain from 0,002 to 0,03 ppm DMN and 0,004 to 0,02 ppm N-Pyrr. It was further stated that none of the raw bacon samples contained any detectable amount of N-Pyrr and that only one contained DMN (0,03 ppm). The authors concluded that N-Pyrr may be formed during frying. Confirmation of nitrosamine identity was made by means of GLC-mass spectrometry (GLC-MS).

1.3 Gas-liquid chromatography (GLC)

GLC and GLC-MS methods of nitrosamine estimation and identification have become much more important, almost obligatory, in recent years. The sophistication of extraction and clean-up methods, the specificity of GLC detectors such as the Coulson electrolytic conductivity detector (CECD), the alkali flame ionisation detector and the even greater progress in the sensitivity and computer based technology of high resolution double beam mass spectrometers, are all contributory factors in this trend. Workers in the field of nitrosamine analysis, now have the means of unequivocal determination of specific nitrosamines, which should result in a more rigid analytical standardisation for future food-stuff monitoring.

Palframan et al [38] evaluated the Coulson electrolytic conductivity detector (CECD) and the alkali flame ionisation detector (AFID) in N-nitrosamine analysis. Under the conditions and concentration range chosen (0-200 ng), the AFID detection appeared to give the greater

peak height to concentration ratio, but the authors found that both detectors had a limit of about 100 pg, calculated as nitrogen. The linear concentration versus peak height relationship for the AFID, falls off above a concentration of 60 ng nitrogen (DEN) and that for the CECD above 100 ng nitrogen. The maximum column loading for the AFID is about 20 µl when using a special vent valve system (VALCO). The capacity of the gas chromatographic column would of course govern the limit of sample volume injected. A chromatogram was given of a 50 µl injection of a dilute aqueous nitrosamine solution using the CECD detector; this indicated that the latter detector may be more suitable for dilute aqueous samples. Investigation of the relative selectivity of both detectors showed that the CECD was nitrogen specific in the reductive mode when an 'in line' strontium hydroxide scrubber was used. The use of this detector in the pyrolytic mode as described by Rhodes and Johnson [39] (see later) and Issenberg and Tannenbaum [40] was not advocated since the nitrosamine/amine specificity was gained only at the expense of sensitivity and was found unsatisfactory for this work. The AFID operating parameters were adjusted for a maximum sensitivity/selectivity compromise but it was found that large quantities of contaminating hydrocarbons in extracts produced considerable and troublesome negative peaks. However, standard nitrosamine samples, contaminated with hydrocarbons and an alcohol, produced a comparable degree of nitrosamine sensitivity and selectivity with both detectors. By comparison, a standard flame ionisation detector (FID) was fully sensitive to all components present. It was found that, on examination of various food-stuffs for low levels of nitrosamine, both AFID and CECD gave satisfactory results. However, considering the overall requirements of sensitivity, reproducibility and noise level in respect of food-stuff trace analysis, it was concluded that the CECD may be more

convenient and reliable.

It is of interest to note that the authors found FFAP a suitable column liquid phase, when either the AFID or CECD was used, even though this phase contains nitrogen. Generally these nitrogen detectors were largely insensitive to increased column bleed on temperature programming.

Rhoades and Johnson [39] made use of the CECD, in the pyrolysis mode to eliminate GLC detection of all but amine type compounds in spiked cigarette tar extracts. Even the presence of large amounts of other nitrogen compounds did not appear to interfere to any really noticeable extent with nitrosamine detection. These findings should, however, be compared with those of Palframan et al [38] in assessing the overall suitability of the CECD used in the above mode, for a specific analytical requirement. The GLC column, packed with Carbowax 1540 on 80-100 mesh chrom Q was temperature programmed from 70-150^{o}C at 5^{o}C/min, argon being used as carrier gas. The carrier gas was saturated with water vapour at room temperature to reduce DMN tailing. The authors compared the above method with that of injecting the spiked tar extract onto the same GLC column, but employing the CECD in the reductive mode. The DMN, DEN and di-n-butylnitrosamine (DBN) peaks were completely masked by other nitrogeneous species in the latter detection mode but clearly defined, at the same concentration, when the pyrolytic mode was employed. The authors also stated that the standard hydrogen FID would be useless for the GLC examination of the neutral fraction of cigarette tar. It was considered that the limit of sensitivity on the above system would allow analysis of 1 ng of low molecular weight nitrosamine/ cigarette.

Issenberg and Tannenbaum [40] compared the relative sensitivities of various nitrosamines to the CECD operated in the

pyrolytic mode and reported that the detector response was dependent on nitrosamine structure citing a number of comparative examples to corroborate the claim. Palframan et al [38] suggested that, in the above mode of operation, incomplete fragmentation of the molecule occurs. This would appear to be the case since Issenberg and Tannenbaum [40], and Rhoades and Johnson [39] independently report the CECD (in pyrolytic mode) response to N-Pyrr to be one eighth and one tenth, respectively, that of DMN. This can be compared with the data of Palframan et al. who report a response of N-Pyrr, to the CECD (in the reductive mode), of 73% that of DMN.

Rhoades and Johnson [22] examined the cleaned up extract (see Isolation section) of 20 non-spiked cigarettes by GLC. A 40 µl portion of this extract was injected on a gas chromatograph fitted with a Carbowax 1540 column and Coulson electrolytic conductivity detector (operated in the pyrolytic mode) and a peak showed up which was thought to be DMN. The total remaining extract (0,46 ml) was evaporated to about 40 µl and, although large losses presumably occurred, this total residue when injected on the same GLC system revealed two peaks, one which had a retention coincident with that of DMN and the other with that of methylethylnitrosamine (MEN). These coincident retentions also occurred on a Porapak Q column. Thus preparative GLC was carried out by injection of a sample, obtained from the extract of 600 cigarettes, on a 10% Carbowax column at 65°C and subsequent collection on Porapak Q at room temperature. This was continued until the entire extract of 1,0 ml had been thus processed. On applying gas flow and controlled heating to the Porapak Q column the nitrosamine 'band', determined from preliminary elution of pure DMN, was collected in 0,2 ml dichloromethane at dry ice/ acetone

temperature. It was found necessary to repeat the GLC separation to obtain a satisfactorily clean sample for mass spectrometry. High resolution mass spectrometry positively identified the major component as DMN.

Reineccius and Coulter [41] studied the isolation of volatile nitrosamines from non fat dried milk by a vacuum distillation method of isolation, followed by conversion of the nitrosamines to nitramines, and detection of the latter by gas chromatography using an electron capture detector (ECD). The vacuum distillate was extracted with dichloromethane, and the extract concentrated to about 0,5 ml under a stream of nitrogen. The nitrosamines in the concentrate were oxidised to the nitramine derivitives according to the method of Sen [42]. The solution thus obtained was neutralised and extracted with dichloromethane. The extract was dried over magnesium sulphate and concentrated to 5 ml under a nitrogen stream. Final concentration to about 0,2 ml was effected after addition of 1 ml n-hexane. Gas chromatographic analysis was effected on a Hewlet-Packard model 5756 gas chromatograph, equipped with a ^{63}Ni ECD. The 1,83m x 3,2 mm stainless steel column was packed with 6% Rheoflex 400 on AW-DMCS Chromosorb W (60-80 mesh), and maintained at 175^{o}C for the nitramine separation (dimethyl, diethyl dipropyl and dibutyl nitramines). The authors claimed that the ECD has a sensitivity of 100-1000 times that of a FID. Unfortunately no comparative ECD/FID GLC traces were shown to enable estimation of the improved elimination of interfering co-extractants. The authors noted that distillation under reduced pressure, instead of atmospheric pressure also helped in the diminution of interfering substances. A normal isolation/detection level of 50 ng/g was reported but it was claimed that tentative detection of 10 ng/g could be made. No volatile nitrosamines were found in freshly prepared or stored (30 day) dry milk

which had been dried by indirect steam heat or direct gas firing, although a substance was detected which co-chromatographed with dimethylnitramine, and which could not be identified by mass spectrometry because of heavy background contamination. Chromatography on another column (3% OV-1) showed a considerable difference in retention times between the compounds, and thus the presence of DMN, at the detection level studied, was eliminated. The authors thought that this interfering compound may have been a furfural or pyrazine type compound, and a reference was made to work by Havre and Ender [43], who demonstrated that heat induced artefacts such as 2,5-dimethyl-pyrazine may be misidentified as nitrosamine, if one does not use sufficiently specific confirmatory methods (see also work by Scanlan and Libbey [34], Heynes and Koch [36] and Devik [35] referred to in the TLC section).

Saxby [44] developed a precolumn GC method of differentiating between N-nitrosamines and pyrazines by selectively adsorbing the latter on a precolumn containing copper thiocyanate. Since pyrazines release nitrite under photolysis [45], they could be mistaken for nitrosamines when TLC methods are used. The author made use of the complexing ability of pyrazines with copper thiocyanate, the products of which are essentially non-volatile [46] and of the type $(CuSCN)_4(C_4H_4N_2)_3$. To test this differentiation a mixture of four pyrazines, commonly found in roasted food-stuffs [47], and two dialkyl nitrosamines (methylethyl nitrosamine and di-n-propylnitrosamine) was analysed by GLC. On a column of 10% DEGA/silanised Chromosorb W packed in 2 m x 3 mm o.d. Teflon tubing, the temperature being programmed from 50°-180°C at 5°/ min, with a nitrogen flow of 35 ml/min. By omitting the precolumn, methylethyl nitrosamine and 2,5-dimethylpyrazine were shown to exhibit similar retention times

and the other pyrazines showed up strongly alongside the N-nitrosamines. However when the precolumn (Teflon 10 cm x 3 mm o.d. copper thiocyanate and silanized Chromosorb W 10% w/w) was employed in series to the polyester column only the nitrosamines were detectable. Although no quantitative details were supplied it would appear, from the GLC traces, that little or no loss in nitrosamine response occurred after selective removal of the pyrazines.

Crosby et al [11] estimated steam-volatile N-nitrosamines in various food stuffs by GLC-mass spectrometry. The distillates (see Isolation section) obtained by doubly steam distilling the food-stuffs were extracted with dichloromethane and this extract was evaporated to 0,25 ml in a Kuderna-Danish evaporator. This concentrate was examined on two different gas chromatographic systems;

1. A Varian 1200 gas chromatograph fitted with a CECD detector and a 5,5 m x 3 mm i.d. stainless steel column packed with 15% FFAP on 80-100 mesh Chromosorb W.
2. A Varian 1740 gas chromatograph fitted with a modified flame thermionic detector (rubidium sulphate tip) and a 5,5 m x 3 mm i.d. stainless steel column packed with 15% Carbowax 20M on 80-100 mesh Chromosorb W.

For confirmation of nitrosamine identity, an AEI MS 902 high resolution mass spectrometer was used in conjuction with a Pye (model R) gas chromatograph fitted with a 5,5 m FFAP column. The effluent stream was split in a single stage membrane separator and the technique was essentially that of Gough and Webb [48].

The foods studied, (mainly cured and processed meats and fish as well as a range of cheeses), were those likely to contain possible precursors for nitrosamine formation. A minimum detection level of around 1 μg/Kg DMN, DEN and N-Pyrr was claimed and in one fried bacon

product the nitrosopyrrolidine content was estimated at 16-40 μg/Kg. This value was obtained by MS molecular ion intensity measurements. It is unlikely that this method would be suitable or even feasible for N-nitrosamine estimation at the above levels due to the very small molecular ion intensities produced with these compounds. It also follows that, since the authors did not apply correction factors (see Isolation section) to the concentration ranges quoted, this method cannot be considered a sufficiently accurate method of estimation.

A general precolumn method of clean-up for spiked N-nitrosamine algal suspensions has been described by Mosier and Andre [49]. The precolumn consisted of a 8 cm x 4,23 mm i.d. FEP Teflon tube packed with 20-30 mesh Ascarite and was placed in the injection port. The precolumn, fitted to a Tracor MT-220 GC equipped with FID and a 2 m x 4 mm i.d. glass column packed with 50-60 mesh Chromosorb 103, was used to examine spiked aqueous algal suspensions (the algal cultures contained approximately 3 x 10^7 cells/ml suspended in Knops solution at pH 7,2) containing 10-500 ng/ml DMN, DEN, DPN and DBN. The column temperature was maintained at 200°C for quantitative analysis of the mixed nitrosamines, and the carrier gas flow rate was 120 ml/min. The Ascarite precolumn clean-up of the algal suspension, was examined by GLC, and the only real interference resulted from the large volume of water (which came off the column first). The authors reported that this direct analysis of aqueous algal suspensions allows greater sensitivity than one involving solvent extraction before GLC. A detection level of 10 ng/ml was claimed (c.f. Hawksworth and Hill [50], Sen et al [51] and Rhoades and Johnson [39]). The plot of DMN peak height (FID response) versus sample concentration, over the range 10-100 ng/ml was shown to be linear. The same system was used for the quantitative estimation of DPN and DBN, using a carrier gas flow of

140 ml/min and column temperature of 210°, but no details of the quantitative work or estimation of recoveries of these nitrosamines were given. The above system is claimed to be 100 times more sensitive than methods of N-nitrosamine analysis previously reported.

1.4 Liquid and gel permeation chromatography

Although thin-layer chromatography has been a very important tool in N-nitrosamine analysis, separation by liquid chromatography (LC) of individual nitrosamines on conventional adsorbents and eluting solvents, has been largely unsuccessful. This is especially evident in the case of the highly lipophilic nitrosamines. High speed, high efficiency liquid chromatography packings for use in high pressure column systems (particularly liquid-liquid phases) may prove to be effective separation media, but as yet, no reports of nitrosamine separations on these systems have been published. Gel permeation chromatography (GPC) on Sephadex LH-20 has been the most successful liquid phase column chromatographic method for general nitrosamine separation.

Sen and Dalphe [21] used alumina column chromatography to clean-up spiked extracts of alcoholic beverages containing DMN, DEN and DPN. The column packing was Woelm basic alumina (activity grade 1.) and the initial eluting solvent was n-pentane. The distilled, extracted and concentrated (see isolation section) extract was passed through the freshly prepared column of approximate dimensions 30 mm x 10 mm. After all the extract had passed through, the column was successively washed with n-pentane containing increasing proportions of dichloromethane (up to 20%). Finally the 'cleaned-up' nitrosamines were eluted from the column with dichloromethane at a rate of 2-3 ml/min. Since the use of LC, in this case, was to effect clean-up and elution

of a mixed nitrosamine band on which subsequent TLC analysis was performed, no attempt was made to apply the method to the isolation of individual nitrosamines.

Eisenbrand et al [18] described the separation of various nitrosamines on the gel Sephadex LH-20 using methanol:water 50:50 as eluting solvent. This solvent was apparently suitable for even the highly lipophilic nitrosamines such as di-n-hexylnitrosamine. The gel was allowed to swell for 12 hr in the solvent and was then packed into a 100 x 1 cm column. The sample (0,5 ml), containing between 10,5 and 74 μg (depending on molecular weight) of individual nitrosamines, was eluted at 4,3 ml/hr and fractions of 2-3 ml each we were collected. The fractions were monitored by UV spectrometry for nitrosamine content and the time taken to elute all nitrosamines was 55,8 hr. They claimed that the six symmetrical nitrosamines studied, dimethylnitrosamine up to di-n-hexylnitrosamine, could be clearly separated but that DMN was eluted first and di-n-hexylnitrosamine last. The authors concluded that the separation on LH-20 is not a gel filtration process but an absorptive one and that the nitrosamine affinity for the gel matrix increases with lipophilic character. An attempt was made to resolve three unsymmetrical compounds — methyl-ethyl-nitrosamine, methyl-n-butyl-nitrosamine and methyl-n-pentyl nitrosamine, by the same process. The time taken for complete elution of these compounds was approximately 28 hr and , although methylethyl nitrosamine was clearly separable from the other two compounds, the resolution between methyl-n-butyl nitrosamine and methyl-n-pentyl nitrosamine was relatively poor.

2. DISCUSSION

2.1 Introduction

There are few reports on the chemistry, isolation and analysis of high molecular weight dialkylnitrosamines. In particular little work appears to have been done on chromatographic methods of analysis. In view of the now well-established carcinogenic activity of a wide range of N-nitroso compounds in certain test animals and of the widespread occurrence of amines [52-57] in great variety, especially those of moderately high molecular weight, which can, under suitable conditions [58-64], give rise to nitrosamines, it is of some considerable importance that methods for the isolation and analysis of all these compounds are available. Up to now the possible carcinogenic activity of the highly lipophilic nitrosamines has not received much attention. The question, whether these nitrosamines might exist in the environment, particularly in foodstuffs, or whether these materials could be formed *in vivo* from the separate consumption of nitrite or nitrate and certain amines or peptides, has yet to be investigated. However, the possibility exists that these compounds may give rise to cancers, and may be specific for the lower intestinal tract [65] due to their highly lipophilic properties.

Both low and high molecular weight nitrosamines may be employed as anti-oxidants in fuels, lubricating oils and rubbers [66] and there are prospects of employing these compounds as stabilizers for aromatic halogeno-compounds, fungicides, insecticides and as intermediates in the manufacture of medicinal preparations [66,67]. Di-*n*-dodecylamine and other high molecular weight amines (primary, secondary and tertiary) in emulsions with water and bitumen have been employed as water proofing agents for masonry and road pavements [68]. In

recent years high molecular weight dialkylamines and their salts have been used in connection with solvent extraction studies [69-72].

The nitrosamines used in the following work, dicyclopentylnitrosamine (DCPN), di-n-heptylnitrosamine (DHN), di-n-octylnitrosamine (DON), methyl-n-octadecylnitrosamine (MODN) and di-n-dodecylnitrosamine (DDDN) were synthesised and their infrared, ultraviolet and mass spectra recorded and studied. Methods of isolation and analysis were developed to enable unequivocal identification of individual members of this class of lipophilic compounds.

2.11 Synthesis of the nitrosamines

The only nitrosamines which presented any difficulty in their syntheses were DDDN and MODN. In particular nitrosation of di-n-dodecylamine was attempted by a variety of methods before a sufficiently efficient method was developed. The amines, as obtained commercially, were very impure and repeated recrystallisation and subsequent conversion to the respective hydrochlorides with further recrystallisation, was necessary to produce products pure enough for nitrosamine synthesis.

Difficulties in attempted nitrosation methods arose because of the very high lipophilic character of the above amines and, although preparation of DDDN and MODN from the parent amine was achieved by reaction with hydrochloric acid and sodium nitrite as in the syntheses of the lower molecular weight dialkylnitrosamines, it proved necessary to dissolve the amines in a large excess of ethanol. Unfortunately, addition of small quantities of water to the ethanol solvent, to help dissolve the added sodium nitrite, often precipitated the amine or hydrochloride. A careful balance, therefore, had to be maintained between dissolution of the amine and that of the nitrite.

The preparation of the nitrosamines via the recrystallised

hydrochlorides (see sections 3.22 and 3.23) produced very pure products and yields of 81 and 77% were obtained for MODN and DDDN respectively. In addition to the above solvent composition requirements it was found necessary to keep the temperature of the solution at about 70°C and to add the additional hydrochloric acid and excess sodium nitrite as described.

Temperature is in fact an important factor in the nitrosation reaction. In an acidic medium it is desirable to carry out the process at a low temperature to avoid denitrosation, which is acid catalysed. If the acidity in the reaction zone is low, as is the case in the synthesis of DDDN or MODN, nitrosation is greatly facilitated by temperature elevation [73,74]. It may be of interest to note that, in the synthesis of nitrosamines, many investigators treated secondary amine hydrochlorides with an excess of sodium or potassium nitrite at room or higher temperature without additional acidification [75-81].

2.12 The ultraviolet and infrared spectra of trans-Dichlorobisdi-n-dodecylnitrosaminepalladium(II)

The above complex was prepared by heating di-n-dodecylnitrosamine with finely powdered anhydrous palladium (II) chloride (see section 3.3). UV and IR spectrophotometry was performed using the instruments and apparatus described in sections 3.13 and 3.14.

The UV spectra in ethanol and 2,2,4-trimethylpentane were recorded at ten min intervals over two hour periods and it was found that an approximately 3×10^{-4} M solution of the complex (see section 3.3) in 95% ethanol gave an initial absorbance of 0,88 (λ_{N-N}) which increased to 1,30 after 2 hr. Concomitant with this absorbance increase there was a λ_{max} decrease from 237 to 235 nm. In iso-octane solution (approx. 3×10^{-4} M) the reverse trend was exhibited, a 0,31 optical density units decrease being observed over a similar time

period. In addition there was a very distinct change in peak shape as a 'shoulder' at about 210 nm gradually disappeared producing eventually the symmetrical 'nitrosamine' peak at 236 nm. Little or no change in wavelength was noted as the above transition occurred.

The KBr disc spectrum of the complex revealed a new strong absorbance doublet at 1621 cm^{-1}, 1633 cm^{-1}. No absorbance in the solid disc spectrum of pure DDDN was evident in this region. Other strong absorbances occurred at 1485 (probably $\nu_{N=O}$ [82]), 1468 (same value for the CH_3/CH_2 vibrational mode as in the spectrum of pure DDDN, see table 3), 1393, 1172 (probably ν_{N-N}) and 721 cm^{-1}. In chloroform solution the spectrum changed greatly from the above KBr disc spectrum. The absorbance doublet had disappeared and the probable N = O frequency had shifted to 1490 cm^{-1}. A medium intensity broad band made an appearance in the 1200 - 1220 cm^{-1} region and the previously assigned strong intensity N — N frequency had also disappeared. A much more comprehensive solvent study will be required to account for the observed behaviour of this complex in solution. Work on the structure and properties of low molecular weight nitrosamine/$PdCl_2$ complexes has been undertaken by Schmidpeter[83] and Brown and Coates [82]. The latter authors conclude that, in these complexes co-ordination is from the oxygen atoms, since co-ordination from the tertiary nitrogen would result in an increased N — O bond order and no strong band in the 1550-1600 cm^{-1} region was observed in the IR spectra of their complexes.

2.13 Infrared spectra of high molecular weight N-nitrosamines

Attempts to assign the N = O and N — N frequencies for the lower dialkylnitrosamines have been made by Haszeldine *et al* [84,85] and Tarte [86]. Some of the assigned values are, however, somewhat removed from the more accurate data of recent workers. The conclusion that

dimerisation was extensive in these compounds led the above authors to assign dimer stretching frequencies. Later work by Looney et al [87] supported the dimerisation concept, the structure fig. 1 having been suggested.

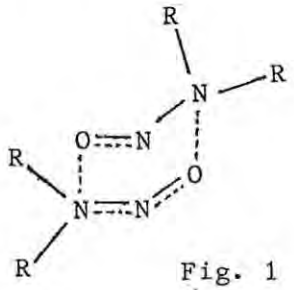


Fig. 1

However, Williams et al [88] rejected the dimer theory on the grounds of the insensitivity of certain previously assigned dimer bands towards solvent change. The work of Levin et al [89] represents a very detailed infrared and Raman spectral study of DMN, DMN-d_6 and DMN-[^{15}N nitroso]. Their assignments for DMN and conclusions regarding dimerisation largely agree with those of Williams et al [88].

The viscosities of DMN, DEN and DPN in binary mixture with either water or propionic acid show large increases above the values of ideal mixing [90] and no such increase is observed in the absence of a proton donor, as with mixtures of DMN-dioxan, DMN-propionic anhydride or DMN-benzene. Extensive solvent extraction work on aqueous DMN solutions with dichloromethane [4] (conc. range 7-16 ppm) did not result in a constant proportion of the nitrosamine being progressively extracted from the same aqueous DMN sample. The amount extracted increases with increasing dilution. DEN and DPN also do not appear to behave ideally, but with these nitrosamines the amount extracted decreases with increasing dilution. It would appear that no satisfactory explanation can be given for the above observations apart from assuming the existance, in solution, of some type of nitrosamine-solvent (H-bonding) or nitrosamine-nitrosamine dipole complex.

The assigned N = O stretching frequencies of DHN, DON and DDDN decrease (see tables 1 - 3) from non-polar to polar solvents while the N — N stretching frequencies undergo progressively increasing shifts. The absorption intensities of the N — N frequencies decreased whilst those of the N = O frequencies increased when proceeding from dichloromethane to carbon tetrachloride. Chloroform (hydrogen bonding solvent), in each case, produced the greatest N = O and N — N frequency shifts. However, in all the solvents listed in the tables the N — N frequency increased with increasing molecular weight indicating not only the expected trend of increased double bond contribution to the N — N frequency stretch in polar solvents as shown in fig. 2, but also that this increasing double bond character appeared to be progressive with increasing dialkyl chain length.

Fig. 2

The above N — N frequency trend could be due to two possible factors:- (a) A mechanical coupling between the C — C frequencies of the alkyl groups and the ν_{N-N} of the nitrosamine group. An interesting trend, noted during this solvent study, would, at first sight, seem to corroborate this suggestion. As can be seen from table 1 a broad band appears in DHN at 1033 cm^{-1} alongside the ν_{N-N} stretch. This band increases rapidly in frequency and intensity with increasing dialkyl chain length and in the case of DDDN exhibits itself as a very strong band with the most non-polar solvents (see table 3). With decreasing solvent polarity, especially with respect to DDDN, this band (1086 cm^{-1} for DDDN KBr disc) intensity increases with decreasing ν_{N-N} intensity. This diminishing N — N/increasing 'shoulder' trend appears to begin

in the C_7 chain length region since in the case of DHN the change was only just noticeable. If this effect were due to Fermi resonance coupling with the N — N bond it would be expected that this 'shoulder' absorbance would also decrease in intensity from polar to non-polar solvents since the frequency difference between them, in these solvents, changed by a comparatively small amount.

It is interesting to note that in assigning the N — N stretching frequency of N-nitrosopyrrolidine in CCl_4 (1153 cm^{-1}), Williams et al[88] accounted for its raised value by suggesting that this was the result of interaction with ring C — C frequencies; however the same argument may be expected to apply to N-nitrosopiperidine. This would, therefore, appear to be only a secondary contribution due to the much lower N — N frequency (1087 cm^{-1}) of the latter nitrosamine.

(b) A progressive decrease in abundance of a nitrosamine-solvent or nitrosamine dipole-dipole complex as in fig. 3.

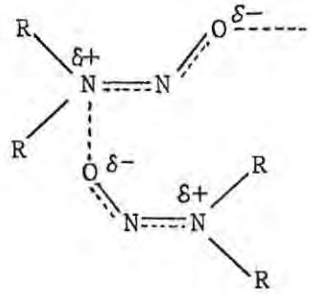


Fig. 3

The increasing dialkyl chain length would cause a distinct and progressive weakening of the dipole-dipole interaction, due to increased shielding of the $\delta+$ on the amino nitrogen, thereby permitting greater freedom of the species to exist as a resonance hybrid as shown in fig. 2. This would in part explain the raised N — N frequencies of isopropylnitrosamine and sec.-butylnitrosamine [88], a major contribution of which, however, may be attributed to the +I (inductive) effects of the alkyl groups (cf. ν_{C-O} for primary, secondary and

tertiary alcolols [91]).

However, if such a dipole-dipole complex was possible and decreased in abundance with increasing steric shielding, one would expect that in non-polar solvents the N = O frequency would increase as ν_{N-N} decreases. This in fact does happen to a slight degree with DHN ($\nu_{N=O}$, 1462 cm^{-1} in cyclyhexane) but does not occur with DON and DDDN, the limiting value of $\nu_{N=O}$ for the latter compounds being 1460 cm^{-1}. Even though the $\Delta\nu_{N-N}$ total shift [ν_{N-N} (in CH_2Cl_2) − ν_{N-N} (in CCL_4)] is maintained from DHN to DDDN the concomitant $\Delta\nu_{N=O}$ value decreased steadily.

2.14 Ultraviolet spectra of N-nitrosamines

The spectra of N-nitrosamines are characterised by a relatively intense band around 235 nm due to the $\pi \rightarrow \pi^*$ transition and a low absorption maximum around 360 nm which exhibits fine structure in non-polar solvents due to the $n \rightarrow \pi^*$ transition. On changing from non-polar to polar solvents this band exhibits a marked blue-shift [92].

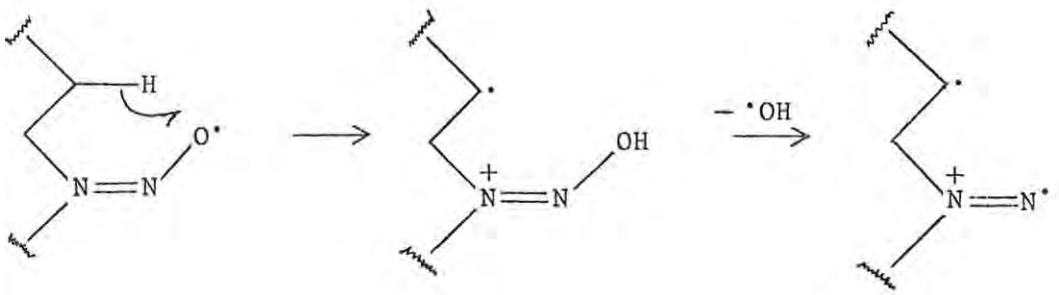
The UV spectra of DCPN, DHN, DON and DDDN were recorded and of these DHN and DDDN were chosen for the construction of calibration curves (figs. 4 and 5). Ethanol and iso-octane (2,2,4-trimethylpentane were the solvents chosen. The $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ absorbance wavelengths and respective extinction coefficients are given in table 4. Corresponding values [84, 93] for the absorbances of diethyl, di-n-propyl and di-n-pentyl-nitrosamines are given for comparison. The $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ absorption intensities increased and decreased respectively with decreasing solvent polarity. In ethanol solution about 5 and 8 ppm of DHN and DDDN respectively could be estimated (see fig. 4). Dimethylnitrosamine and diethylnitrosamine can be estimated at the 2 ppm level [4] in aqueous solution.

2.15 Mass spectrometry of high molecular weight N-nitrosamines

The mass spectra of three high molecular weight N-nitroso compounds were studied, viz. dicyclopentylnitrosamine, di-n-heptylnitrosamine and di-n-dodecylnitrosamine. Several workers [4, 94-96] have reported the mass spectra of a number of the low molecular weight dialkyl N-nitroso compounds. In addition several N-nitrosoazacyclic compounds [96, 97] have been studied but no aliphatic dicyclo-N-nitrosamines were included.

One of the most notable features of the mass spectra of the high molecular weight N-nitrosamines is the rapidly diminishing molecular ion abundance as the molecular weight increases. In the case of DDDN the molecular ion is only just discernible from background intensities and in figs. 9 and 10, this ion is shown at five times the actual observed intensity, all other relative abundances remaining the same. Table 5 gives a comparison of the percentage abundance of some of the more common m/e ions exhibited in the mass spectra of the low and high molecular weight N-nitrosamines.

The M-17 ion [4, 94, 96, 98] occurred for the three high molecular weight N-nitrosamines but was only just noticeable for DCPN. This is probably due to the rigidity of the dicyclo structure initially preventing the six membered Mc Lafferty type rearrangement [99] which has been proposed [97] for this fragment loss (i.e. transfer of a H-atom β to the amine nitrogen, see scheme 1).



Scheme 1

The m/e peaks which occur in all the dialkylnitrosamines (except DPN) is probably due to loss of $^{\cdot}NO$ [97] and the m/e 30 peak is always of medium abundance. The increasing abundance trend of the M - 30 peak occurs concomitant with an increasing M - 17 abundance going from DBN to DDDN. ApSimon and Cooney [97] believe that the ability to lose $^{\cdot}NO$ indicates the relative strengths of the N — N bonds; a large M - NO peak indicating a weaker N — N bond and a small M - NO peak a stronger one. According to these authors the M - 17 peaks should also be indicative of the N — NO bond strength. Their investigations of the mass spectra of various N-nitrosoazacyclic compounds showed that, when the M - NO peak was small the M - OH peak was large, and vice versa. This prediction of N — N bond strength is not easily correlated with the trends in the mass spectra of the dialkylnitrosamines, since the largest M - 17 peak occurs in DDDN (38%) and both the M - 17 and M - 30 ion abundances increase with increasing molecular weight.

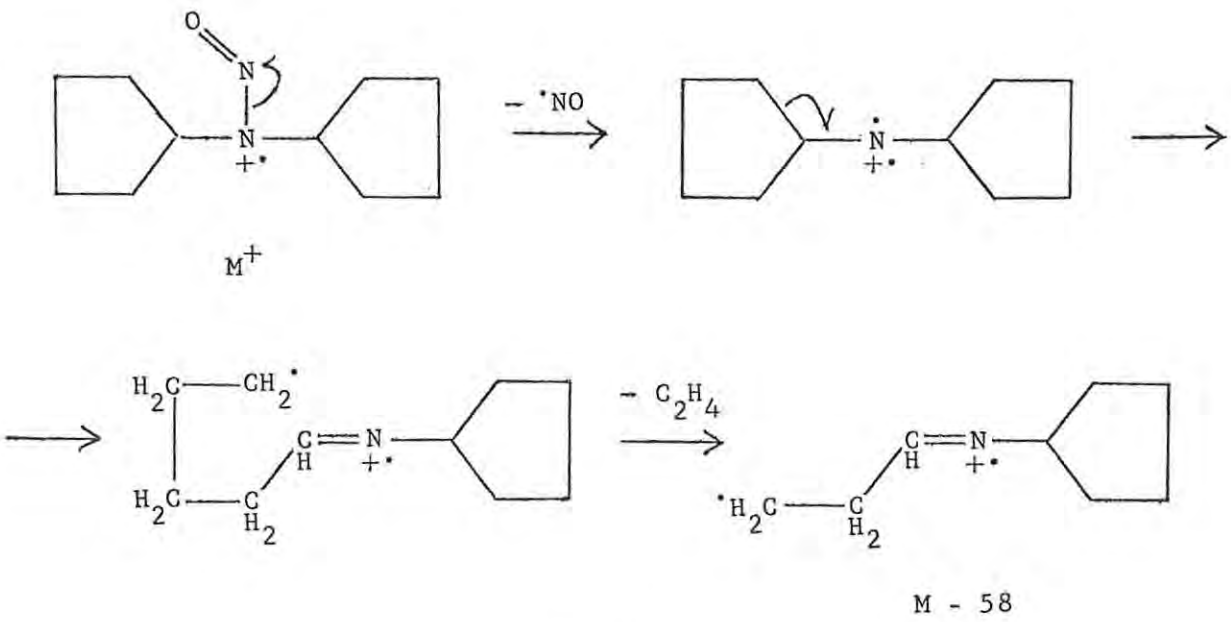
An interesting feature of the spectra of DON and DDDN was the regular mass 14 cleavage pattern (see figs. 9 and 10). In the case of DDDN this appeared to begin, with low species abundance, at m/e 322 (1%) and increased steadily to reach a maximum at m/e 196 (57%). However, this increasing relative abundance trend was interrupted at m/e 210 (2%) and the dominant peak in this region was m/e 211 (33%) i.e. m/e 196 + 15. This unexpected odd mass number species was probably due to a CH_3 abstraction prior to probable formation of the charge stabilized species $C_{12}H_{25}\overset{+}{N} \equiv CH$ (m/e, 196). After m/e 196 the intensity of the CH_2 fragmentation dropped greatly but again began an upward trend from m/e 182 (3%) to 56 (46%). This fragmentation trend resembles that of a long chain hydrocarbon [100] with peak

groups 14 mass units apart and increasing in intensity with decreasing fragment weight. The CH_2 clevage pattern of DON followed the same CH_2 cleavage trend as that of DDDN. It appeared to begin at m/e 238 and continued regularly with increasing relative abundance to m/e 140. Here also the same discontinuity in intensity increase occurred at 15 mass units before the appropriate charge stabilized species (m/e 140, 76%) probably $C_8H_{17}\overset{+}{N} \equiv CH$. After m/e 140 the trend was the same as for DDDN with the peaks at 14 mass units apart increasing again from m/e 126 (6%) to 42 (57%), probably $CH_2 = \overset{+}{N} = CH_2$ [101]. By analogy with accurate mass measurements of DPN and DBN [96], and noting the abundance (98%) of this latter species in the mass spectra of DMN, the most likely composition is C_2H_4N.

It is opportune at this point to give the probable compositions for the most prominent peaks in the individual mass spectra of the three nitrosamines studied.

(a) Dicyclopentylnitrosamine (fig. 8)

This mass spectrum differs greatly from the from the mass spectra of the two straight chain compounds studied. The most intense peak occurred at m/e 41, probably $C_3H_5^+$ [101] and other prominent peaks appeared at m/e 28 (76%), 54 (36%), 56 (38%), 69 (76%), 84 (60%) and 124 (48%). Comparison of accurate mass measurements of DBN and DPN [96] suggests compositions $C_3H_6N + C_4H_8$ and $C_5H_{10}N$ for m/e 56 and 84 respectively. The other species m/e 28, 54 69 and 124 may possibly have the compositions $CH_2N + C_2H_4$, $C_3H_4N(C_2H_4CN)$, C_5H_9(cyclopentyl ring or $\cdot(CH_2)_3\overset{+}{N} \equiv CH$) and $C_8H_{14}N$. To account for the latter fragment (48%) a possible mechanism is given in scheme 2.



Scheme 2

(b) Di-n-octylnitrosamine (fig. 9)

The molecular ion is shown, on the bar graph, at five times the observed intensity (1,2%). By comparing accurate mass measurements of DBN and DPN [96] the base peak at m/e 43 may be assigned the complex composition $C_2H_5N + C_3H_7$. Similarly, the prominent peaks at m/e 41 (95%), 44 (64%), 57 (89%) may be assigned compositions C_3H_5, C_2H_6N and $C_4H_9 + C_3H_7N$ respectively. Other prominent peaks occurred at m/e 71 (see DDDN). The M - 17 peak at m/e 253 is more abundant (27%) than the corresponding peak in any of the lower molecular weight nitrosamines (see table 5). The significance of the mass 14 peak groups has already been discussed.

(c) Di-n-dodecylnitrosamine (fig. 10)

As was the case with DON, a dominant feature of this nitrosamine was the mass 14 cleavage pattern. The m/e 43 peak was again (see b) the base peak and the M^+ relative abundance was very small (approx. 0,6%),

shown in fig. 10 at five times the observed intensity. The peak at M - 17, (m/e 365, 38%) had the greatest M - 17 relative abundance of the three high molecular weight nitrosamines studied and this species is referred to in the earlier discussion. Other prominent peaks occurred at m/e 41 (68%), 44 (46%), 55 (51%), 57 (71%) and 71 (33%). By comparison of accurate mass measurements of DBN and DPN [96] these peaks, with the exceptions of m/e 55 and 71, may be assigned the compositions C_3H_5, C_2H_6N and C_3H_7N respectively. In the absence of accurate mass measurement data the compositions of m/e 55 and 71 are uncertain.

2.16 Thin-layer chromatography of high molecular weight N-nitrosamines

Thin-layer chromatography (TLC), although rejected by some workers in the field of nitrosamine analysis, on the grounds of insensitivity and unspecificity, was found to be a most effective and sensitive method for the separation and detection of the high molecular weight dialkyl nitrosamines.

Remarkably low detection levels (see section 3,54) were achieved by use of a special reversed phase system and a very effective spot applicator (see section 3.54 and fig. 11). The very low limits of visualization are especially noteworthy when one considers the decreasing proportion of active group (— NNO) in the molecule as the alkyl chain length increases. Using the developing solvent and reversed phase system described the development time was approximately 1 hr for a 75 mm solvent run distance and the R_f-values for DCPN, DHN, DON and DDDN were 0,69 0,36 0,25 and 0,0 respectively. Many TLC runs were made to confirm reproducibility of R_f-values and detection levels. The low limit of visualization, per spot, of the high molecular

weight nitrosamines should be compared with the detection levels of N-nitroso compounds on thin-layers, reported by previous workers [4, 19, 32, 102]. Walters et al [103] reported that the sensitivity of detection of non-volatile N-nitrosamines, using the reagents of Preussmann et al [32, 33] was seldom less than 0,5 mg (sic). Du Plessis [4] found that the mobile phases of Preussmann et al [32, 33] did not adequately separate all the nitrosamines the author investigated, (dimethylnitrosamine, diethylnitrosamine, di-n-propylnitrosamine, nitrosopyrrolidine and diphenylnitrosamine). A carbon tetrachloride : dichloromethane :: 6 : 4 mobile phase was used with greater success.

Preussmann et al [33] placed considerable reliance on analysis by TLC using sprays A and B (see section 3.54) and UV irradiation as visualizing agents. The authors consider that if both tests are positive the presence of N-nitroso compounds can be accepted. While this may be true for many cases it is preferable to regard these methods as very useful guides to the possible presence of N-nitroso compounds. The detection of the secondary amine formed after UV-splitting is also possible by spraying the plate with a ninhydrin reagent [104] when, normally, blue spots are obtained.

It should be noted that when the thin-layers, as described in section 3.54, were baked in an oven at 110°C, prior to use, for periods greater than about two hours, both R_f reproducibility and spot resolution deteriorated slightly. The optimum 'activation' time was found to be between 20 to 30 min. As can be seen from sections 3.54 and 3.55 the high molecular weight nitrosamines exhibited great stability in the aqueous acetic acid developing solvent. This contrasts with the caution expressed by Sen et al [19] concerning

the use of a benzene - acetic acid - water TLC mobile phase.

The combination of the above reversed phase TLC method with GLC (see sections 3.54, 3.57 and 3.58) provided a very sensitive and specific measure of high molecular weight nitrosamine analysis.

2.17 Separation of conformational isomers of methyl-n-octadecyl-nitrosamine (MODN)

Although MODN was repeatedly recrystallised the melting point range remained constant at 46-51,5°C. GLC (see section 3.23) revealed a single peak in the chromatogram which was not resolvable on reduction of the column over temperature or carrier gas flow rate. Elemental analysis of the compound indicated a high degree of purity (see section 3.23). When paper chromatography of the nitrosamine was performed on the reversed phase system described in section 3.56, two spots were evident at R_f-values 0,3 and 0,4 which were approximately equal in intensity. It is unlikely that these spots were caused by acid catalysed decomposition products since similarly high molecular weight nitrosamines have been shown to be very stable in concentrated aqueous acetic acid (see section 3.55). No equilibrium studies on fractionally crystallised MODN was attempted and no NMR investigation was undertaken.

Previous reports of isolable conformational isomers of nitrosamines involved only alkyl-aryl and diaryl-N-nitroso compounds (105, 106, 107).

2.18 Liquid-liquid column chromatography (LLC) of ethyl-n-propyl-nitrosamine (EPN) and di-n-propylnitrosamine (DPN)

Although low molecular weight dialkylnitrosamines were not considered for extensive study, various liquid chromatographic

systems (see sections 3.41 and 3.42) were investigated to attempt the efficient separation of the above compounds which differ by one methylene group. Apparently the only previously reported method for low molecular weight dialkylnitrosamine separation by liquid column chromatography was that of Eisenbrand et al [18] who used a gel chromatographic system (see section 1.4). However, these authors found that only partial separation was achieved between dialkylnitrosamine homologues differing by one methylene group (i.e. methylbutylnitrosamine and methylpentylnitrosamine), the time taken to elute the partially separated nitrosamines being 28 hr.

In contrast, the LLC system described in section 3.41 afforded almost complete resolution of EPN and DPN in a total elution time of 43 min (see fig 12). The order of elution was DPN first followed by EPN.

The GLC (see section 3.41) traces of the nitrosamine positive fractions contained only the nitrosamine peaks (retention times for EPN and DPN, 10,2 min and 13,0 min respectively) and no contaminating peaks were recorded at any stage during GLC fraction monitoring.

2.19 Gel permeation chromatography (GPC) of high molecular weight N-nitrosamines

GPC of dicyclopentylnitrosamine (DCPN), di-n-heptylnitrosamine (DHN), di-n-octylnitrosamine (DON) and di-n-dodecylnitrosamine (DDDN) was performed on Sephadex LH-20 using isopropanol - water (17:3) as eluting solvent (see section 3.59). The only previous report of the separation of N-nitrosamines on Sephadex LH-20 is that of Eisenbrand et al [18] (see below).

Good resolution was obtained (see fig. 13) between DDDN (I), the DON/DHN (II/III) band and DCPN (IV), the order of elution being

DDDN first and DCPN last. The isopropanol - water eluting solvent was found to give the best results although the use of methanol - water and ethanol - water solvents (both 17:3) also gave reasonable separations under the same conditions. Little or no loss of nitrosamines occurred from adsorption on the column. The separation is probably based on a simple gel filtration process.

Eisenbrand et al [18] (see section 1.4) separated various dialkyl nitrosamines (dimethylnitrosamine up to di-n-hexylnitrosamine) on Sephadex LH-20 using methanol water (1:1) as eluting solvent and reported that dimethylnitrosamine was eluted first, followed by the higher homologues in the order of increasing molecular weight. They concluded that the separation was not based on a gel filtration process and that the affinity of a given nitrosamine for the Sephadex matrix increases with lipophilic character. However, no reports were made concerning the behaviour of these nitrosamines on a similar gel column using increased proportions of organic solvent in the eluting solvent mixture.

With respect to the above work on the high molecular weight dialkylnitrosamines, both UV and GLC methods of fraction monitoring were employed; hence, unequivocal identification of a particular nitrosamine in a given fraction could be made. It seems likely from this work, that complete resolution of the diheptyl- and dioctyl-nitrosamines could be achieved on a longer or a recycling Sephadex LH-20 column system. In any event, pure samples of each nitrosamine were reproducibly obtained from mixed samples applied to the above short column. From an estimation of the GLC detection limit, under the conditions described in section 3.59, the minimum concentrations of individual nitrosamines in a mixture which could usefully be monitored by GLC (1 µl injections) are approximately 6, 9, 18 and 34 µg/ml of

DCPN, DHN, DON and DDDN respectively.

2.20 Clean-up of amine contaminated N-nitrosamine samples by ion-exchange chromatography

It is generally accepted today that a detection level, for carcinogenic nitrosamines, of 5-10 µg/kg is a necessary requirement of nitrosamine analytical methods (see sections 1.1 - 1.4). Because of this proposed low level of detection it is of utmost importance that macro amounts of interfering contaminants in foodstuff extracts should be removed prior to analysis for nitrosamines. Many of the frequently encountered contaminants are of a basic nature and a number of methods [18, 19, 104, 108-110] have been proposed for their removal from nitrosamine containing extracts. However due to the strong lipophilic properties and relatively low volatility of high molecular weight dialkylnitrosamines, most of the earlier clean-up methods (especially those involving polystyrene type ion-exchangers [6, 7, 110] resulted in high losses of these nitrosamines. The results below indicate that the strong cation exchanger SE-Sephadex is probably the ideal medium for clean-up of base contaminated nitrosamine solutions.

The procedure adopted in the use of the SE-Sephadex ion-exchanger is given in section 3.6. The estimation of nitrosamine recovery was based only on GLC peak height measurements (mean height from five 0,5 µl GLC injections). Reasonably accurate estimations were thus obtained by comparing the above measurements with the peak heights obtained from injections (0,5 µl) of the pure nitrosamine standard (see section 3.6 and chromatogram 2). The traces obtained for the amine and amine/nitrosamine mixtures are given in chromatograms 1 and 3 respectively. Complete selective retention of the amines on the ion-exchange column was achieved (see chromatograms 3 and 4) and the

mean recoveries of the nitrosamines from three successive nitrosamine/amine mixture samples (each 0,5 ml) were 82,2 91,5 and 95,6 for DCPN, DHN and DON respectively. The results show that almost quantitative elution of the latter two nitrosamines is possible under the described conditions and that, as expected, the affinity of the nitrosamines for the swollen gel matrix decreases with increasing dialkyl chain length.

The passage of approximately 100 ml of the 90% aqueous ethanol solvent (i.e. the volume of solvent used for three successive nitrosamine/amine runs) through the ion-exchange column, did cause some shrinkage in gel volume. However, the gel volume was quickly regenerated (less than 10 min) by addition of 1:1 ethanol/water to the column.

Because of the success of the above clean-up process the following system is proposed, which may be sufficient to remove all basic and acidic contaminating materials from nitrosamine containing samples and, at the same time, allow very high nitrosamine recoveries.

The system should consist of a composite ion-exchange column containing a short column packing (5 x 2 cm) of SE-Sephadex separated from a similar bed volume of QAE-Sephadex (strong anion exchanger) by a glass wool plug or silica disc of suitable porosity. The gel conditioning and the elution procedure should be carried out as described in section 3.6 except that the order of base and acid washing should be reversed for the anion exchanger.

By employing a gradient aqueous ethanol eluting system (20→90% ethanol) clean-up of both low and high molecular weight N-nitrosamines should be possible in a single eluting run.

2.21 Isolation of high molecular weight N-nitrosamines from wheat flour

Apart from the difficulties of the 'clean up' of extracts prior to the analysis of nitrosamines there are added difficulties associated with the analysis of high molecular weight nitrosamines, viz. their decreased volatility and increased lipophilic character as compared with the lower molecular weight homologues, thus increasing the difficulty of selective isolation from biological materials.

Because of the strongly lipid absorbing properties of flour, this foodstuff was chosen as the substrate from which lipophilic nitrosamine extraction may yield the poorest results.

The full experimental procedure for isolation of the above nitrosamines is given in section 3.7. The nitrosamines were distilled (see fig. 14) from the flour samples under controlled conditions and the distillate, which collected in a trap cooled in liquid air, was extracted with dichloromethane. The latter solution was washed with acid and base, dried and evaporated to a small volume, and finally analysed by both TLC and quantitative GLC (see section 3.54 and 3.58). As can be seen in table 6 (section 2.21) the average recoveries of DCPN and DHN were almost quantitative, 93,7 and 93,2% respectively, while that of DON was 75,7%. These values are the means of three consecutive spiked flour extractions. Even allowing for the great care taken in 'working-up' and concentrating the extract, the recovery levels were surprisingly consistent. The above extraction procedure would appear to be the most successful nitrosamine-from-flour extraction method to date. The above results should be compared with those of Sen *et al* [19] and Eisenbrand [5]. The former authors extracted 100 g portions of wheat flour with diethyl ether in a Soxhlet apparatus and reported recoveries of 65 and 90% for, respectively, 10 and 400 μg of

diethylnitrosamine added to the flour. They did not, however, attempt the extraction of higher molecular weight nitrosamines. When the authors used the flour extraction method of Marquardt and Hedler [31] they were unable to get a satisfactory recovery (less than 30%) for diethylnitrosamine.

Eisenbrand [5] extracted flour spiked with dimethylnitrosamine, diethylnitrosamine and diamylnitrosamine with dichloromethane. The extract, after 'work-up' was separated by preparative TLC The zones on the plates were extracted, reacted with heptafluorobutyryl chloride and then analysed by GLC. He reported recoveries of 69, 82 and 62% respectively of the above nitrosamines. However, the GLC traces in his paper show many high intensity contaminant peaks rendering quantitative estimation uncertain.

2.22 Analysis of N-nitrosamines in spiked wheat flour extracts

The gas chromatograms (see chromatogram 5) of spiked flour extracts (see section 3.7) were relatively free from interfering peaks, considering the GLC amplifier attenuation (32), with the exception of a prominent peak at retention time 11,6 min. Chromatogram 6 is the result of a blank flour extraction done under exactly the same conditions. The results of three consecutive spiked flour extractions are given in table 6.

Table 6

Nitrosamine	Amount Added	Recoveries			Mean recovery
	µg	µg			%
		1	2	3	
DCPN	66,8	62,3	63,7	61,9	93,7
DHN	134,0	124,3	126,9	123,5	93,2
DON	248,4	185,8	190,4	187,6	75,7

Quantitative GLC extimation was carried out using calibration tables and curves (see section 3.58 and fig. 15) and each recovery level was estimated from the average of five 0,6 µl injections of the individual extract solutions.

On thin-layer chromatography (see sections 3.12 and 3.54) of the extracts using spray reagent A followed by UV irradiation, spots were observed at R_f 0,25 0,36 0,57 and 0,69. (R_f-values for DCPN, DHN and DON are 0,25 0,36 and 0,69 respectively). The component at R_f 0,57 , thought to be the same as the unknown at retention time 11,6 min in the gas chromatogram, gave a more intense purple colour with spray reagent A than any of the known nitrosamines present. However, when a newly developed plate was first irradiated with UV for 40 min and then sprayed with reagents B or C this spot was absent and only the N-nitroso compounds appeared, showing that the unknown substance was not an N-nitroso compound. Extracts from various wheat flour samples, when washed as described in section 3.7 behaved normally on the prepared thin-layers the nitrosamine spots being consistently well defined and detection levels remaining constant. Co-extracted flour contaminants did not interfere with the separation, definition or limit of visualisation of the nitrosamines (see section 3.54). No trace of a purple zone was observed at or near the thin-layer solvent fronts of any of the spiked or blank flour extract runs, thus elimimating the possibility of the presence of lower molecular weight nitrosamines in the flour samples at the levels of detection studied, under normal conditions.

3 EXPERIMENTAL

3.1 GENERAL

3.11 Gas liquid chromatography was performed on a Perkin-Elmer model 900A gas chromatograph equipped with standard dual hydrogen FIDs and a Hitachi QPD_{54} recorder. Nitrogen was used as carrier gas and the following column systems and conditions were used:-

(a) Glass column (1,92 m x 4,5 mm I.D.) packed with 15% HI-EFF-4BP on Gas Chrom P 80-100 mesh. Respective oven, injector and manifold temperatures were 150°, 240° and 300°C while the nitrogen flow rate was 16 ml/min. This column system was used only for monitoring the fractions obtained from liquid chromatography of the low molecular weight di-n-propylnitrosamine and ethyl-n-propylnitrosamine.

(b) Glass column (1 m x 4,1 mm) packed with 1% silicone SE-30 on AW-DMCS chromosorb G 80-100 mesh. Respective oven, injector and manifold temperatures were 170-220°C (depending on resolution required and molecular weights of compounds investigated), 250°and 320°C. The carrier gas (nitrogen) flow rate was 15 ml/min.

(c) Glass column (2,4 m x 4,4 mm) packed with 5% FFAP on AW-DMCS chromosorb G 80-100 mesh. Respective oven, injector and manifold temperatures were 80-150°, 250°and 320°C while carrier gas (nitrogen) flow rate was varied between 18-30 ml/min.

3.12 Thin-layer chromatography was performed on a reversed phase system (see section 3.54) and the samples were applied on the prepared plates by means of the applicator shown in fig. 11 The legend for the apparatus is as follows:-

A. steel bolt and clamped positioning nuts

B. syringe plunger head flattened and polished to minimise friction

with the bolt

C. stainless steel spring for supporting syringe plunger

D. 50 or 100 µl syringe

E. 9 x 4 cm plates coated with 5% silicone SE-30 on TLC grade silica gel G

F. adjustable platform

G. heavy wooden block support to which retort stand was screwed

Lead weights were also placed on the stand base to ensure complete stability of the unit.

The mode of operation consisted of drawing up the requisite amount of sample into the syringe with the platform in a lowered position and the bolt screwed out of contact with the syringe plunger head. The syringe plunger was then simply located in the desired position by a positioning spring. A prepared plate was placed on the adjustable platform and the platform raised until the orthogonally tipped syringe needle just made contact with the TLC plate. The top bolt was then screwed down until contact was made with the syringe plunger head. Thereafter the rate of application was easily controlled by a gradual downward screwing of the bolt.

3.13 Infrared spectra were recorded on a Beckmann IR-8 instrument equipped with a reference beam attenuator (R11C no. AT-02). The sample chamber was purged with dry nitrogen before recording any spectra. Normal and micro KBr/sample discs were made in 16 mm and 5 mm dies respectively. Solution spectra were measured in teflon stoppered, KBr window, solution cells (RIIC no. F-05).

3.14 Ultraviolet spectra were recorded on a Unicam SP.800B instrument. Sample solutions and reference solvents were contained in matched UV grade silica cells (1 cm) equipped with teflon stoppers.

3.15 Mass spectrometry was performed on an AEI MS30 instrument, equipped with a solid probe inlet and a heated glass inlet system. The instrument was fitted to a Pye series 104 gas chromatograph by means of a membrane separator and spectra were recorded (AEI UV series 10-430S recorder) at an ionising voltage of 70eV.

3.16 Freeze drying and vacuum distillation of flow samples was carried out using the apparatus illustrated in fig. 14 and the legend is as follows:-

A. extractant in a 500 ml flask

B. thermostat controlled bath

C. supporting springs

D. silanised glass wool plug

E. glass indentations

F. trap

G. liquid air

H. to high vacuum pump

The shaded areas are well-fitting Quickfit joints.

3.2 PREPARATION OF N-NITROSAMINES

3.21 N-nitrosopyrrolidine, dicyclopentylnitrosamine, di-n-heptylnitrosamine and di-n-octylnitrosamine (b.p. 63°/0,3 mm, 102°/0,3 mm, 128°/0,3 mm and 200°C/13 mm Hg respectively) were prepared from the respective amines essentially according to the method of Pensabene et al [111] using hydrochloric acid and sodium nitrite (both analytical grade). The only departure from this method was that the latter two amines were dissolved in 5 times their volume of ethanol-water (9:1) solvent. The resulting nitrosamines, after ether extraction, were washed sequentially with M sulphuric acid, 10% sodium carbonate

solution and then water. The N-nitrosopyrrolidine, dicyclopentylnitrosamine and di-n-heptylnitrosamine were purified by vacuum distillation and the di-n-octylnitrosamine by recrystallisation from ethanol at a low temperature according to the method of Williams et al [88]. All the nitrosamines were free of contaminants as shown by gas chromatography (column packing, 1% silicone SE-30 on AW-DMCS chromosorb G).

3.22 Di-n-dodecylnitrosamine

Di-n-dodecylamine was converted to the hydrochloride, which was purified by repeated crystallisation from ethanol, m.p. 204-206°C. To the hydrochloride (1,2 g) in ethanol (50 ml) at 30°C was added Analar sodium nitrite (0,25 g) with vigorous stirring, the temperature being then raised to 70°C and water (5,0 ml)added to completely dissolve the sodium nitrite. After 1 hr of heating, GLC (using the same column system as above at a temperature of 220°C and nitrogen flow rate of 15 ml/min) revealed the presence of a trace of amine but the complete absence of nitrosamine. Concentrated Analar hydrochloric acid (1,0 ml) was added, followed by sodium nitrite (1,0 g) and heating continued for a further hour, when both amine and nitrosamine (1:7 GLC peak heights) were present. After a further 2 hr, more sodium nitrite (1,5 g) and water (3,0 ml) were added and heating was continued at 70°C for another 3 hr, when GLC revealed peaks at retention times 9,15 11,0 and 26,5 min in the ratio 3:6:148 the latter being that of the nitrosamine, which separated as a yellowish oil and solidified on cooling. It was filtered off, washed with aqueous ethanol (1:1) and recrystallized from ethanol (95%) yielding the colourless nitrosamine (0,75 g) m.p. 45-46°C. (Found: C 75,57; H 13,23; N 7,24% calc. for $C_{24}H_{50}N_2O$: C 75,33; H 13,17; N 7,32%). The product (10 mg) gave a positive Liebermann's

nitroso-test on heating with phenol (0,2 g) and concentrated sulphuric acid for 4 min.

3.23 Methyl-n-octadecylnitrosamine

Methyl-n-octadecylamine was converted to the hydrochloride, which was purified by repeated crystallisation, m.p. 162-164°C. Essentially the same method of conversion to the nitrosamine was adopted as in the case of di-n-dodecylamine hydrochloride (1,2 g recrystallised hydrochloride being used). Stirring and heating at 70°C was continued for a total of 6½ hr during which time Analar sodium nitrite (1,1 g) and Analar concentrated hydrochloric acid (0,5 ml) were added. After this period GLC analysis revealed a very pure product with almost complete freedom from amine and other contaminants. The cream coloured solid nitrosamine formed on cooling was filtered, washed well with 50% aqueous ethanol and recrystallised three times from 90% ethanol yielding the pure product (0,95 g) m.p. 46-51,5°C. (Found: C 73,13; H 13,07; N 8,85% calc. for $C_{19}H_{40}N_2O$: C 73,02; H 12,90; N 8,96%). As with didodecylnitrosamine Liebermann's nitroso-test was positive but over a shorter time period (less than 2 min). The GLC analysis of the recrystallised nitrosamine on the same column system as above (column temperature 200°C and nitrogen flow rate 15 ml/min) revealed a single peak at retention time of 26,3 min.

3.24 Reaction between di-n-octadecylamine and nitrosyltetrafluoroborate ($NOBF_4$)

The amine was repeatedly recrystallised from 95% ethanol to yield colourless feathery crystals, m.p. 69-70°C. The preparation of the corresponding nitrosamine was attempted according to the method of Olah et al [112]. To a solution of the above amine (1,0 g) in anhydrous diethyl ether (30 ml) was added $NOBF_4$ (0,4 g) gradually, with vigorous stirring by a magnetic stirrer. The solution temperature was maintained

at approximately 25°C during stirring (1½ hr). Ethanol (about 10 ml) was then added, and the cream coloured suspension filtered off. The cream precipitate was recrystallised from ethanol to yield the off-white product, the melting point of which was indistinct (170-200°C). This material was rather insoluble in non-polar solvents and very insoluble in water.

3.25 Di-n-dodecylnitrosamine (using nitrosyl tetrafluoroborate)

The above procedure of Olah et al [112] was used to prepare di-n-dodecylnitrosamine. The purified secondary amine (1,8 g) was dissolved in anhydrous diethyl ether (20 ml) and $NOBF_4$ (0,27 g 0,5 mole) was added gradually as the solution (cooled on an ice bath) was vigorously stirred on a magnetic stirrer. When addition was complete stirring was continued for a further 25 min. The white precipitate which formed, as in the di-n-octadecylamine/$NOBF_4$ reaction, had a high melting point and was filtered off. The resulting ethereal solution was evaporated down to yield a yellowish precipitate which was taken up in n- pentane (30 ml). This n-pentane solution was evaporated down to half its original volume, some residual precipitate of the high melting point complex filtered off, and the remaining solution evaporated down to yield a pale yellow product. This was recrystallised from 95% ethanol to yield the nitrosamine (m.p. 45°C). The overall yield was poor (12,8%).

3.3 trans-Dichlorobisdi-n-dodecylnitrosaminepalladium (II)

This complex was prepared by heating di-n-dodecylnitrosamine (0,4 g) at 65°C with finely powdered anhydrous palladium (II) chloride (0,1 g) for 15 min. Absolute ethanol (5 ml) was then added and the solution heated under mild reflux for 1½ hr. After this time only a

trace of $PdCl_2$ remained unreacted. The solution was filtered and the filtrate cooled in ice to produce a very pale green crystalline precipitate which was filtered off in a micro-Buchner and dried in a vacuum desiccator over magnesium perchlorate to yield crystals of m.p. 79-80°C.

The UV spectra of the complex in 2,2,4-trimethyl pentane and absolute ethanol were recorded at regular intervals over the same time period (2 hr) and the IR spectra of the solid (KBr disc) and its solution in chloroform were also recorded.

3.4 LIQUID COLUMN CHROMATOGRAPHY

3.41 Liquid-liquid column chromatography (LLC) of ethylpropylnitrosamine (EPN)* and di-n-propylnitrosamine (DPN)*

The column chromatography of a standard mixture of EPN and DPN (in n-hexane) was performed on a 60 cm x 4,0 mm I.D. column packing of Carbowax 4000 on Porasil S (80-100 mesh), a GLC grade packing obtainable from Waters Associates Inc. To minimise progressive pooling or elution of the stationary phase some Carbowax was dissolved in the reservoir of eluting solvent (n-hexane). The standard solution contained 568 µg/ml EPN and 631 µg/ml DPN and a volume of 0,2 ml was applied to the column. Under a 100 ml n-hexane solvent head the elution rate was 0,4 ml/min and a total of 34 x 0,5 ml fractions were collected on an ISCO model 270 fraction collector. The fractions were monitored by GLC on a Perkin-Elmer model 900A gas chromatograph equipped with a standard hydrogen FID and a 1,92 m x 4,5 mm I.D. glass column packed with 15% HI-EFF.4BP on Gas chrom P 80-100 mesh (column oven temperature 150°C, carrier gas flow 16 ml/min and amplification attenuation 32). A very good separation of the above nitrosamines was obtained on this

*obtained from Dr. L.S. du Plessis, Rhodes University

system (see fig.12).

3.42 Other liquid chromatographic systems used for the possible separation of EPN and DPN

The glass columns had internal diameters varying between 2,5 and 6 mm and column packing lengths varied between 35 and 65 cm. Using a polar to non-polar range of solvents (n-hexane to ethanol/water) the packings which were found largely unsuccessful for this separation were as follows:- silica gel (column quality), Porasil E, Porasil F, Porapak Q-S, deactivated Porasil (BX), Corasil I (all these packings were obtained from Waters Associates Inc.), Acidic, basic and neutral alumina (Woelm) and the gel Sephadex LH-20. The same fraction collecting and GLC monitoring systems as in 3.41 were used.

3.5 DETECTION AND SEPARATION OF HIGH MOLECULAR WEIGHT N-NITROSAMINES

3.51 Infrared spectra of di-n-heptylnitrosamine (DHN), di-n-octylnitrosamine (DON) and di-n-dodecylnitrosamine (DDDN)

These were recorded on a Beckmann IR8 spectrophotometer using KBr cells with 0,1 mm lead spacers. The N=O frequencies were readily distinguishable from the CH_3/CH_2 vibrations as these former and latter bands are well resolved in long chain dialkyl compounds. The accurate assignment and estimation of the N=O frequency was often facilitated by adding a small amount of the respective parent amine to the reference beam solvent cell, when the common CH_3/CH_2 modes were diminished in the spectrum relative to the N=O stretching frequency which resulted in greater resolution.

Approximately 0,2 M solutions of each nitrosamine were used, in all solvents, thus eliminating complications which may have arisen from change in concentration.

3.52 Ultraviolet spectrophotometry

Standard solutions were prepared in volumetric flasks by diluting the pure weighed nitrosamines, dicyclopentylnitrosamine (DCPN), DHN DON and DDDN with the appropriate solvent. The solvents used for normal qualitative analyses and solvent effect determinations were, ethanol, chloroform, dichloromethane, n-heptane and 2,2,4-trimethylpentane (iso-octane), all of which were spectroscopically pure except dichloromethane, which was specially distilled and dried to remove UV absorbing contaminants. Calibration curves were constructed for the high intensity absorption at ca. 235 nm (solvent, ethanol), and the low intensity absorption at ca. 365 nm (solvent, iso-octane), the nitrosamines chosen being DHN and DDDN. The latter absorption at 365 nm was the most intense component absorption of the low intensity band, due to the $n - \pi^*$ transition, which shows fine structure in the above non-polar solvent. The respective concentrations for DHN and DDDN used in the construction of the high intensity maxima calibration curves were 11,6 - 46,5 and 27,4 - 68,7 µg/ml and those for the low intensity maxima 439,1 - 1090,9 and 388,6 - 1164,7 µg/ml (see figs. 4, 5).

3.53 Mass spectrometry

The mass spectra of three high molecular weight N-nitrosamines, DCPN, DON and DDDN were recorded using an AEI MS30 mass spectrometer linked to a Pye Series 104 gas chromatograph. The nitrosamines were repeatedly distilled and recrystallised respectively until GLC pure before being introduced into the instrument via a solid probe inlet (sample and inlet temperature approximately 150°C). Mass spectra were recorded (AEI UV Series 10-430S recorder) at an ionising voltage of 70 eV. The spectra were normalised by a computer program written in Fortran IV and were displayed in the form of computed bar graphs.

However, for convenience the bar graphs were reproduced in the form shown in figs. 6-10 by using a Hewlett-Packard 9800 series model 10 calculator linked to the calculator plotter 9800 series model 62A.

3.54 Thin-layer chromatography (TLC)

TLC was performed on a reversed phase system consisting of silica gel G impregnated with 6% silicone SE-30, using glacial acetic acid/water 70:30 as developing solvent. Glass plates (9 x 4 cm) were coated by dipping a pair, back to back, into a slurry of the above coated silica gel G in chloroform (100 g/250 ml $CHCl_3$). Prepared plates were dried in an oven at 110°C for 20 minutes. Plates prepared in this way gave consistently reproducible R_f-values. Normally volumes of sample solution (in dichloromethane) of between 2 and 25 µl were analysed by TLC. Application of the sample was made by the simple but effective applicator shown in fig. 11. Not only did this device enable one to accurately measure the volume being applied, but it enabled application of relatively large volumes of solvent within the very small spot diameter of 1 to 1,5 mm. This latter fact alone produced a significant lowering of the detection level. The end of the syringe needles (Hamilton) were carefully filed down to produce orthogonal tips. After application of the sample, the platform was easily lowered without the needle disturbing the plate in any way.

The spray reagents used were;

A. 1 part 0,1% $PdCl_2$ in 0,2% NaCl solution plus five parts 1,5% diphenylamine in 95% ethanol [32,33].

B. Griess reagent [24,25], a 1:1 mixture of 1,0% w/v sulphanilic acid in 30% acetic acid and 0,1% α-naphthylamine in 30% acetic acid.

C. A 1:1 mixture of 1% w/v sulphanilic acid in 30% acetic acid and 0,1% w/v N-1-naphthylethylenediamine hydrochloride in 30% acetic acid [21]. The latter solution was stored at 0-5°C and the reagents

were mixed just before use.

Excellent TLC separations were obtained on the above reversed phase system in a development time of 1 hour. DCPN, DHN, DON and DDDN appeared at R_f 0,69 0,36 0,25 and 0,0 respectively when the 70% acetic acid solvent was used (90% acetic acid was required to elute DDDN as a well defined spot). The lowest levels of detection per TLC spot with spray A (followed by UV irradiation) were, DCPN 80 ng; DHN 150 ng; DON 170 ng and DDDN 140 ng. The low level of the latter resulted from its zero R_f-value. UV irradiation of at least 20 min was usually required to achieve maximum intensity of the purple spots with spray A. Spraying with reagents B and C was preceded by UV irradiation (normally 30 min) but rapid background colouration with the latter sprays inevitably resulted in higher detection levels than for spray A. It may be of interest to note that UV irradiation of the developed plates alone visualized DCPN as a yellow spot which turned brownish after spraying with reagents B or C and further UV irradiation (maximum spot intensity achieved only after about 30 min irradiation). The other nitrosamines showed up as reddish spots and the respective minimum detection levels, using the latter spray reagents, were 120 ng, 300 ng, 300 ng, and 250 ng for DCPN, DHN, DON and DDDN respectively.

3.55 Estimation of the stability of the nitrosamines in aqueous acetic acid (70%)

The stability of the high molecular weight nitrosamines in the above solvent system was checked by UV spectrometry and TLC. A solution (0,1 ml) containing 0,476 mg/ml DCPN, 0,868 mg/ml DHN and 1,10 mg/ml DON in UV grade chloroform, and diluted to 3 ml with 70% aqueous acetic acid displayed no alteration in optical density at 252 nm (i.e. λmax N—N in the acetic acid solvent) after 12 hr. In addition TLC

examination of the solution after this period gave the expected R_f-values for each of the three nitrosamines, with no tailing of spots, confirming that no degradation had occurred.

3.56 Paper chromatography

Whatman no.1 strips (55 x 5 cm) were coated with silicone SE-30 by drawing the papers slowly through a solution containing 4% SE-30 in n n-hexane. The solvent system used for the separation of DHN, DON and DDDN was acetic acid/water 70:30 and the papers were eluted by the descending method. Very good separations of the above nitrosamines were achieved on this system giving R_f-values 0,0 0,3 and 0,57 for DDDN, DON and DHN respectively. Spray reagent A followed by UV irradiation were the visualizing agents and spot dimentions after 17 cm solvent elution were 3,5 x 3,5 mm, 11,0 x 4 mm and 11,0 x 5,5 mm for DDDN, DON and DHN respectively.

To gain a measure of the sensitivity of this paper chromatographic system 2,0 µl, 4,0 µl and 6,0 µl of a solution containing 476 µg/ml, 868 µg/ml and 1100 µg/ml of DCPN, DHN and DDDN respectively were spotted. The paper, on completion of the run, was sprayed twice with spray reagent A at 2 min intervals. After UV irradiation for 30 min the 6,0 µl spotting was only just detectable for all three nitrosamines.

The paper chromatography of methyloctadecylnitrosamine (MODN) was performed on the same system as above but using acetic acid/water 4:1 as eluting solvent. A 5% solution of MODN in absolute ethanol was spotted onto the paper (spot diameter 4 mm) and the sample eluted as before. After 4½ hr the solvent front had travelled 22,0 cm and on treatment with spray A and UV irradiation two spots were evident at R_f-values 0,3 and 0,4 (unfortunately resolution was not complete

and some overlap occurred).

3.57 Gas-liquid chromatography (GLC)

Gas chromatography of the nitrosamines was carried out using the three column systems referred to in 3.1. On column (b) (column, injector port and manifold temperatures, 170° 250° and 320°C respectively) the retention times for DCPN, DHN and DON were 3,54 8,86 and 19,00 min ± 1% respectively while MODN at a column temperature of 200°C had a retention time of 26,4 min and that of DDDN, at a column temperature of 220°C was 26,5 min.

3.58 Quantitative GLC

Quantitative analysis of DCPN, DHN and DON was performed on column (b) under the above operating conditions and carrier gas flow rate of 15 ml/min. The internal standard used was 2,2'-dinitrodiphenyl (Rt 15,1 min) which was also used in the construction of calibration curves (see fig. 15). The latter were plotted from data obtained from the chromatography of standard solutions (prepared in grade 'A' volumetric flasks) each of which contained 75,8 µg/ml of the above internal standard. The standard solutions contained 20,4 to 66,8 µg/ml of DCPN, 40,2 to 134,0 µg/ml of DHN and 74,52 to 248,4 µg/ml of DON. The operating attenuation was 32 and each point on the calibration curves was an average result of five 0,6 µl injections. The ratios of the nitrosamine to internal standard peak heights were plotted against nitrosamine concentration. Correlation coefficients were 0,999 0,998 and 0,999 for DCPN, DHN and DON respectively. Improved accuracy and convenience were achieved by computing the peak height ratio versus concentration values to obtain, in tabular form, exact values for a large number of these correlations.

3.59 Gel permeation chromatography (GPC) of DCPN, DHN, DON and DDDN

The GPC of the above nitrosamines was performed using Sephadex LH-20 as column packing and isopropanol/water 17:3 as eluting solvent. The gel was allowed to equilibriate in the above solvent for 12 hr prior to packing into a 12 mm I.D. glass column. The glass column used was fitted with a 2 mm solid glass tap and the column base was plugged with silanized glass wool. The column packing dimensions were 62 cm x 12 mm and elution was performed under normal gravity conditions, the solvent being fed onto the column via a 100 ml Quickfit separating funnel placed on top of the column. A standard solution of the above nitrosamines was made up in 95% ethanol and contained 115 µg/ml, 174 µg/ml, 280 µg/ml and 224 µg/ml of the above nitrosamines respectively and 0,5 ml injections of this solution were made onto the column. Elution fractions were collected on an ISCO Model 270 fraction collector at the rate of 1/12 min and the solvent elution rate was maintained at 0,1 ml/min. The total volume of eluate collected was 50 ml, the first nitrosamine-positive fraction being determined by UV spectrophotometry (λ_{max} N–N, 235 nm in ethanol). Thereafter fractions were monitored on a Perkin-Elmer Model 900 gas chromatograph fitted with a FID and a 1m x 4 mm I.D. glass column, packed with 1% Silicone SE-30 on AW-DMCS chromosorb G (80-100 mesh).

The GLC column temperature was maintained at 175°C for the estimation of the first three nitrosamines and was increased to 230°for estimation of the highest molecular weight compound, the nitrogen carrier gas flow rate remaining constant at 17 ml/min.

Graphs of nitrosamine peak height versus gel column elution volume were then constructed (see fig.13).

The peak designation for fig.13 is as follows:-

I Di-n-dodecylamine

II Di-n-octylnitrosamine

III D-n-heptylnitrosamine

IV Dicyclopentylnitrosamine

3.6 CLEAN-UP OF AN AMINE CONTAMINATED NITROSAMINE MIXTURE ON A CELLULOSE BASED CATION EXCHANGER

When a small quantity of the strong cation exchanger SE-sephadex (about 2 g) was added to water (100 ml) a very swollen product was obtained. This was left in contact with distilled water for 12 hr and was then transferred to a 10 cm I.D. Buchner funnel where it was successively washed (under mild suction) with 0,5 M sodium hydroxide (50 ml), water (50 ml), 0,5 M hydrochloric acid (50 ml) and finally thoroughly with distilled water. The product was lightly drained and then transfered to a flask containing 90% ethanol in which it was left to equilibriate for 10 hr. Enough of the activated exchanger was transferred to a short glass column, plugged at the base with silanized glass wool, to produce a packing of dimensions 5 x 2 cm.

A solution of N-nitrosamines (in 95% ethanol) containing DCPN (163 μg/ml), DHN (140 μg/ml) and DON (258 μg/ml) was mixed with an exactly equal volume of an amine solution containing di-n-heptylamine (742 μg/ml) and di-n-octylamine (972 μg/ml). The above mixture (0,5 ml) was injected onto the column and was eluted through the ion exchanger with a total of 35 ml 90% ethanol. The eluate was collected in approximately 2,5 ml fractions on an ISCO model 270 fraction collector and the flow rate from the column was maintained at 2,1 ml/min, a total of 14 fractions being collected. The total eluate was evaporated down to 0,5 ml in a pear-shaped flask equipped with a Vigreaux column and the sample examined by GLC (column, 1% SE-30 on AW-DMCS Chromosorb G, oven temperature 175°C, carrier gas (nitrogen) flow 16 ml/min,

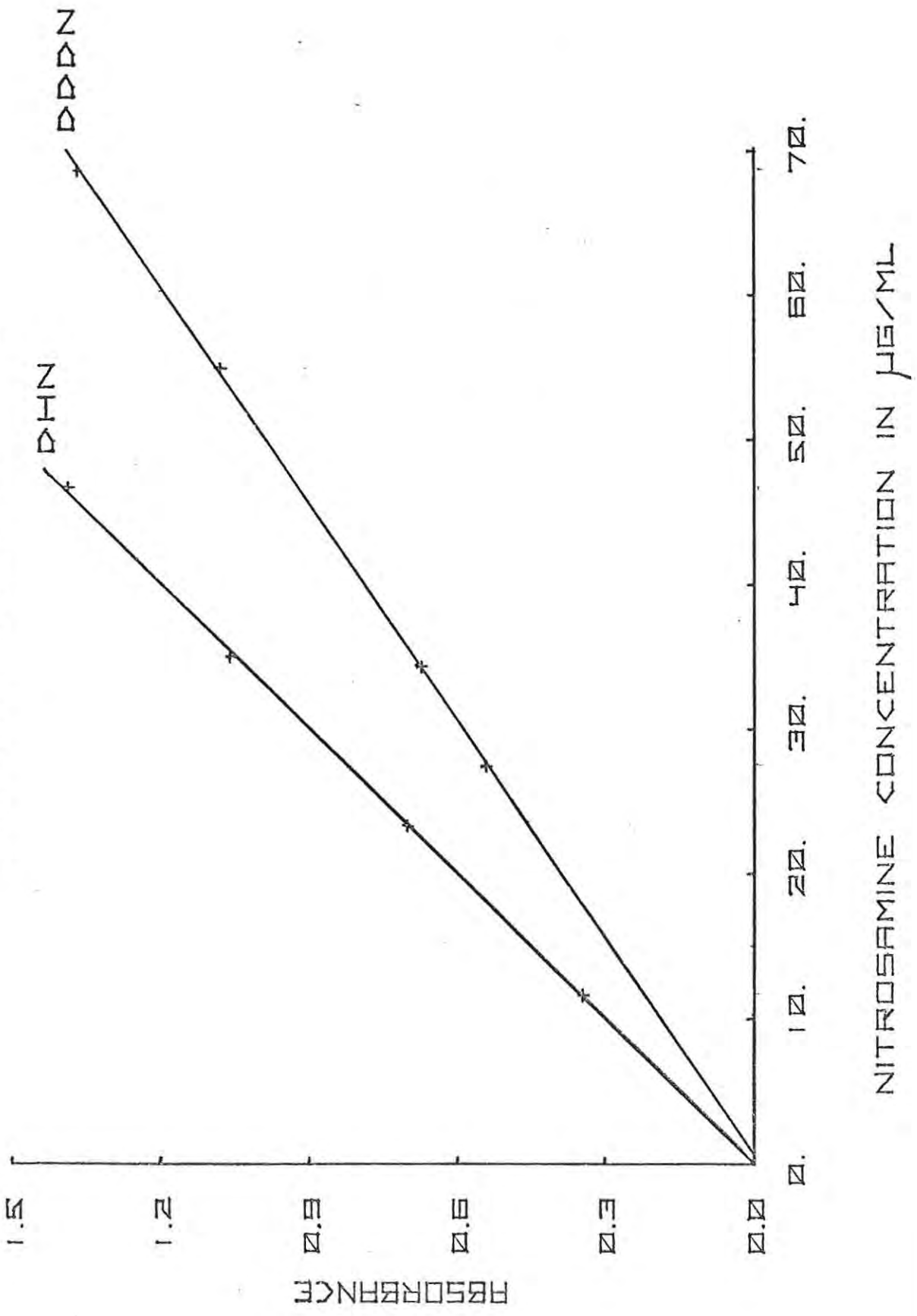
attenuation 64). Samples (0,5 µl) of the pure amine mixture (x 2 dilution), pure N-nitrosamine mixture (x 2 dilution) and the N-nitrosamine/amine 1:1 mixture were also injected onto the above column system to obtain comparative GLC traces (chromatograms 1, 2 and 3 respectively).

3.7 VACUUM DISTILLATION OF DCPN, DHN AND DON FROM FLOUR SAMPLES

To flour (50 g) was added a dichloromethane solution (1,0 ml) containing 66,8 µg/ml DCPN, 134,0 µg/ml DHN and 248,4 µg/ml DON. Addition of dichloromethane (35 ml) produced a thick slurry which was allowed to stand for 1 hr to allow adsorption of the nitrosamines onto the flour. The flask (500 ml) containing the slurry was cooled in liquid air, and then connected to the freeze drying apparatus shown in fig. 15. After freeze drying for 2 hr (0,01 to 0,05 mm), the sample flask was immersed in a thermostated water bath at 72°C and distillation (vacuum degassing), initially under the same reduced pressure as above, continued for a further 16 hr. After this period the vacuum was still good (about 0,1 - 0,15 mm). The distillate, after thawing, was washed with 0,5 M sulphuric acid (2 x 25 ml), followed by M sodium hydroxide (2 x 25 ml) and finally with water (2 x 25 ml). The dichloromethane solution was dried (anhydrous sodium sulphate) and evaporated to about 0,25 ml in a flask attached to a Vigreaux column. To this was added a solution (1,0 ml) of the internal standard (see section 3.58), 2,2'-dinitrodiphenyl (151,6 µg/ml) and the volume made up to 2,0 ml in a grade A volumetric flask with dichloromethane. The mixture was examined by both gas and thin-layer chromatography.

APPENDIX I - Figures

Fig. 4 NITROSAMINE CONC. VS. ABSORBANCE AT 235 nm



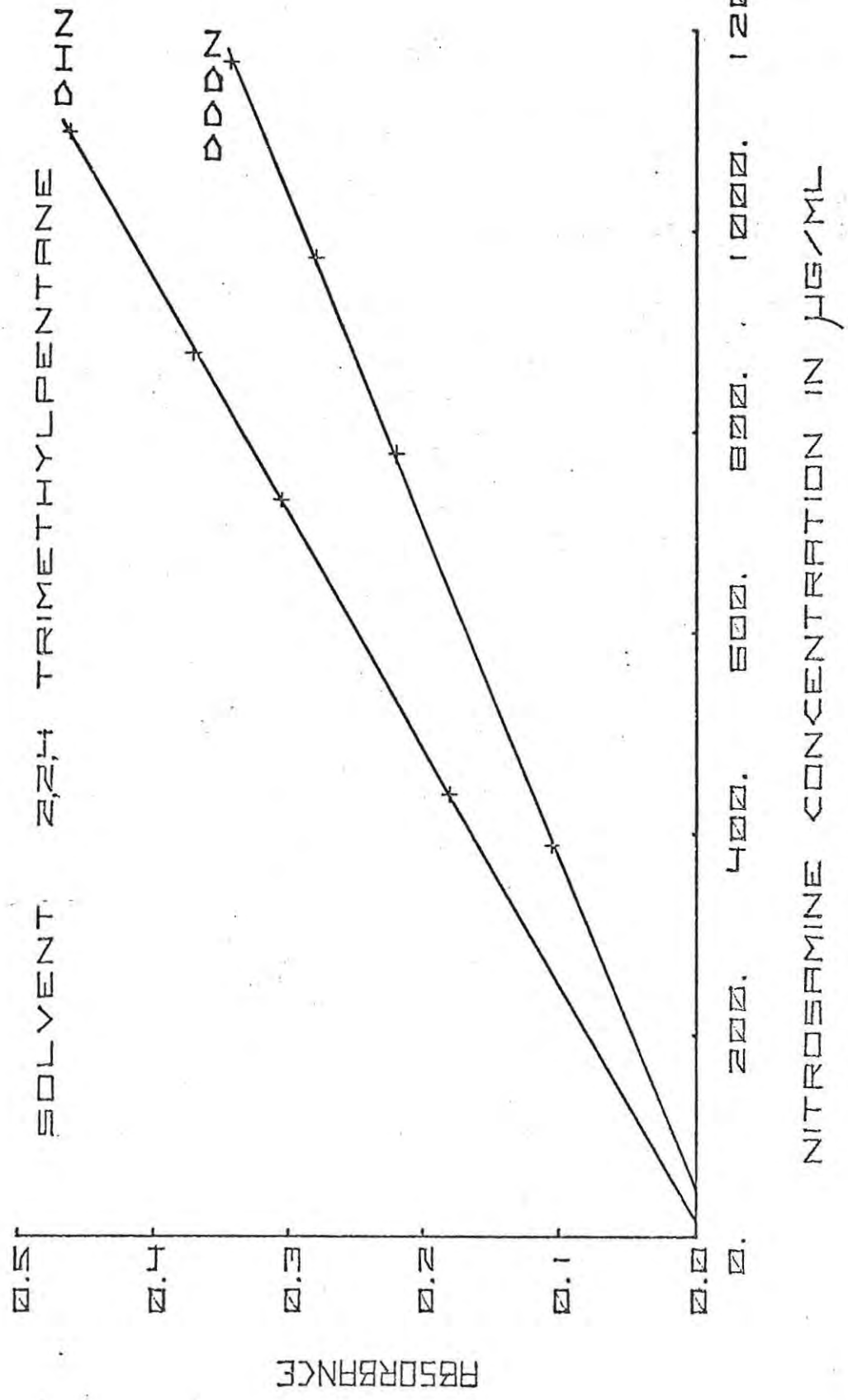

NITROSAMINE CONC. VS. ABSORBANCE AT
365nm FOR. DHN AND 365nm FOR DDDN.
SOLVENT 2,2,4 TRIMETHYLPENTANE
DHN
DDDN
ABSORBANCE
0.0
0.1
0.2
0.3
0.4
0.5
0.
200.
400.
600.
800.
1000.
1200.
NITROSAMINE CONCENTRATION IN µG/ML

Fig. 5

Fig. 6 Mass spectrum of DPN recorded on an AEI MS30 instrument

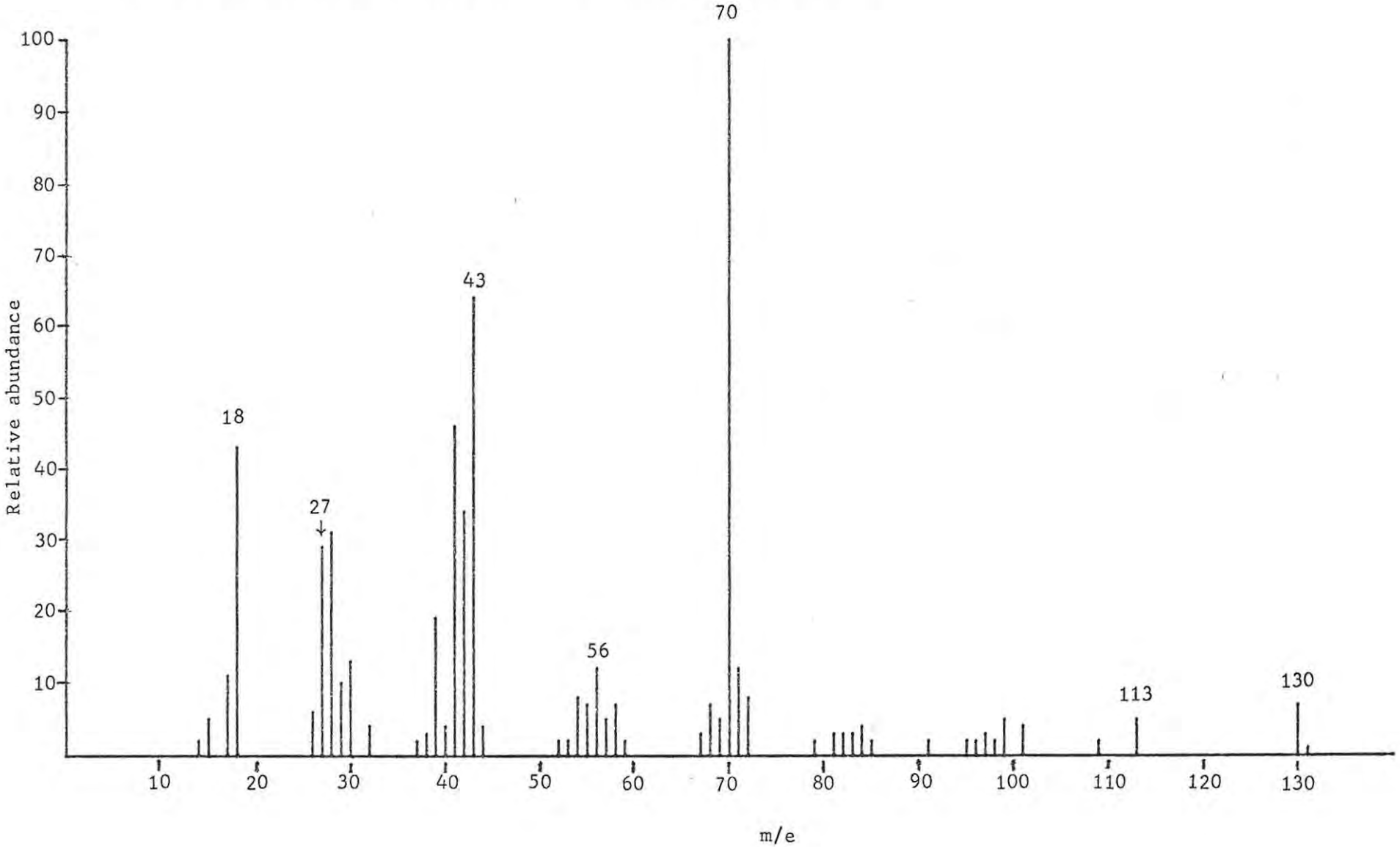


Fig. 7 Mass spectrum of DBN recorded on an AEI MS30 instrument

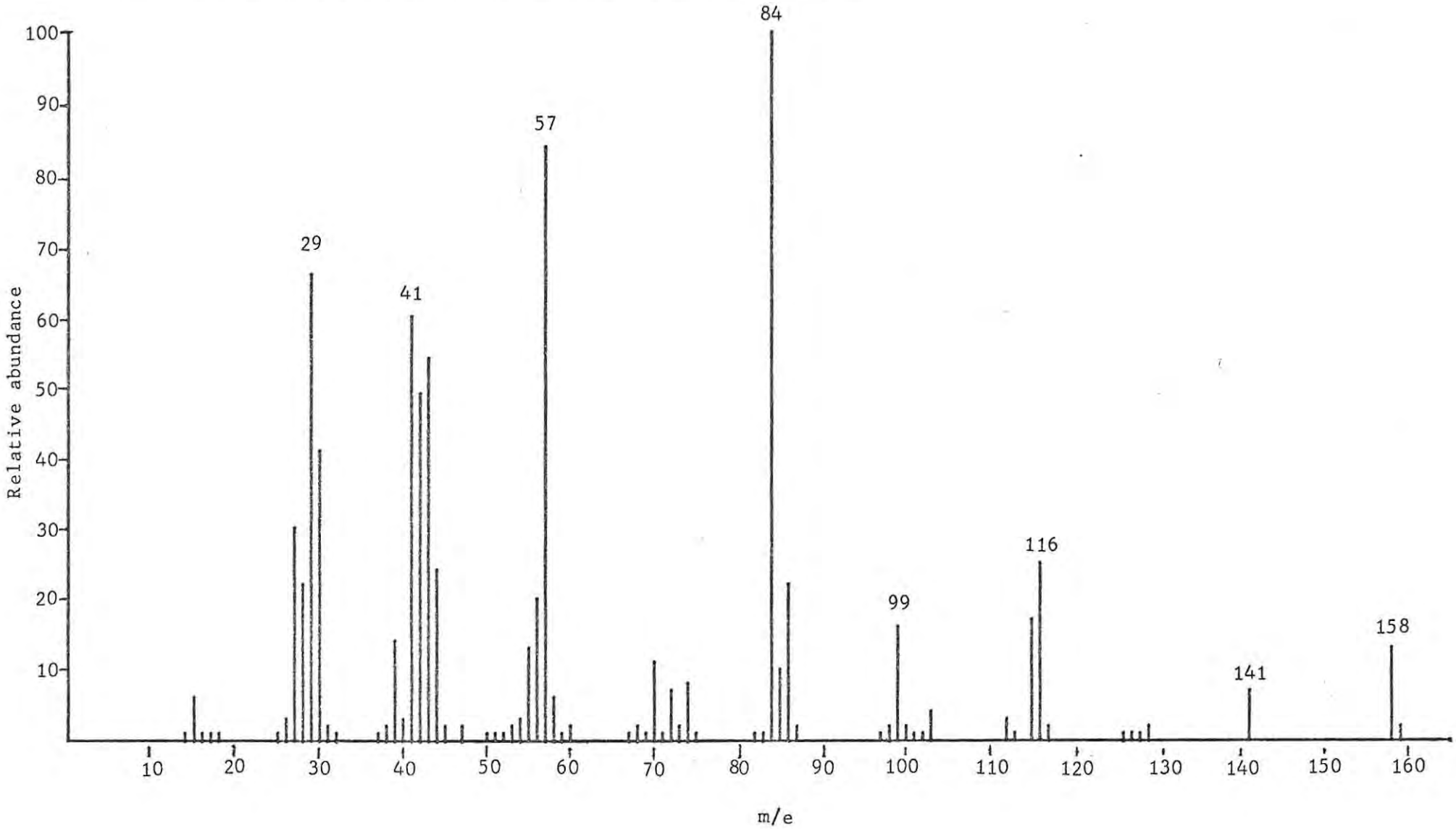


Fig. 8 Mass spectrum of DCPN recorded on an AEI MS30 instrument

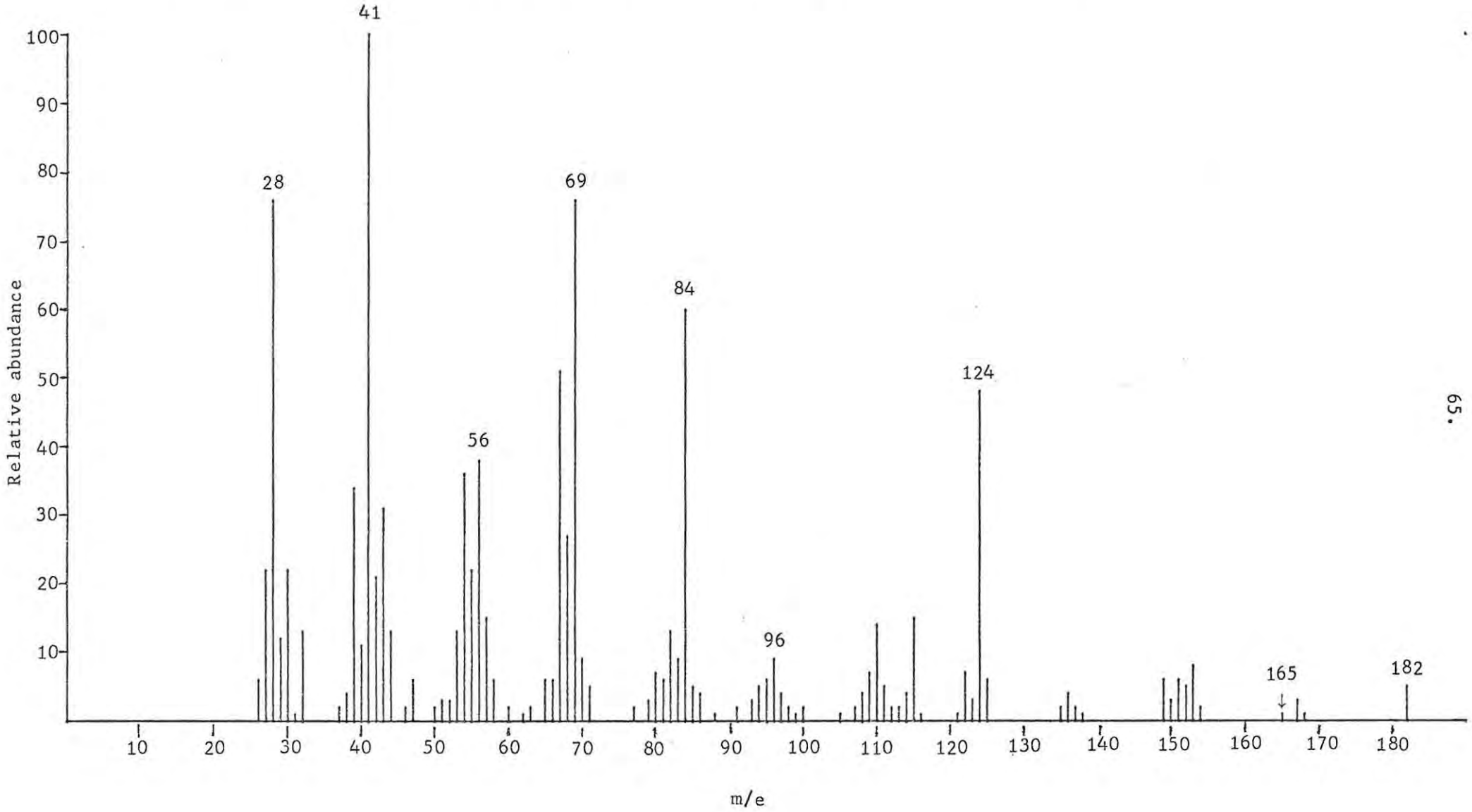


Fig. 9 Mass spectrum of DON recorded on an AEI MS30 instrument (Molecular ion shown 5 times observed intensity)

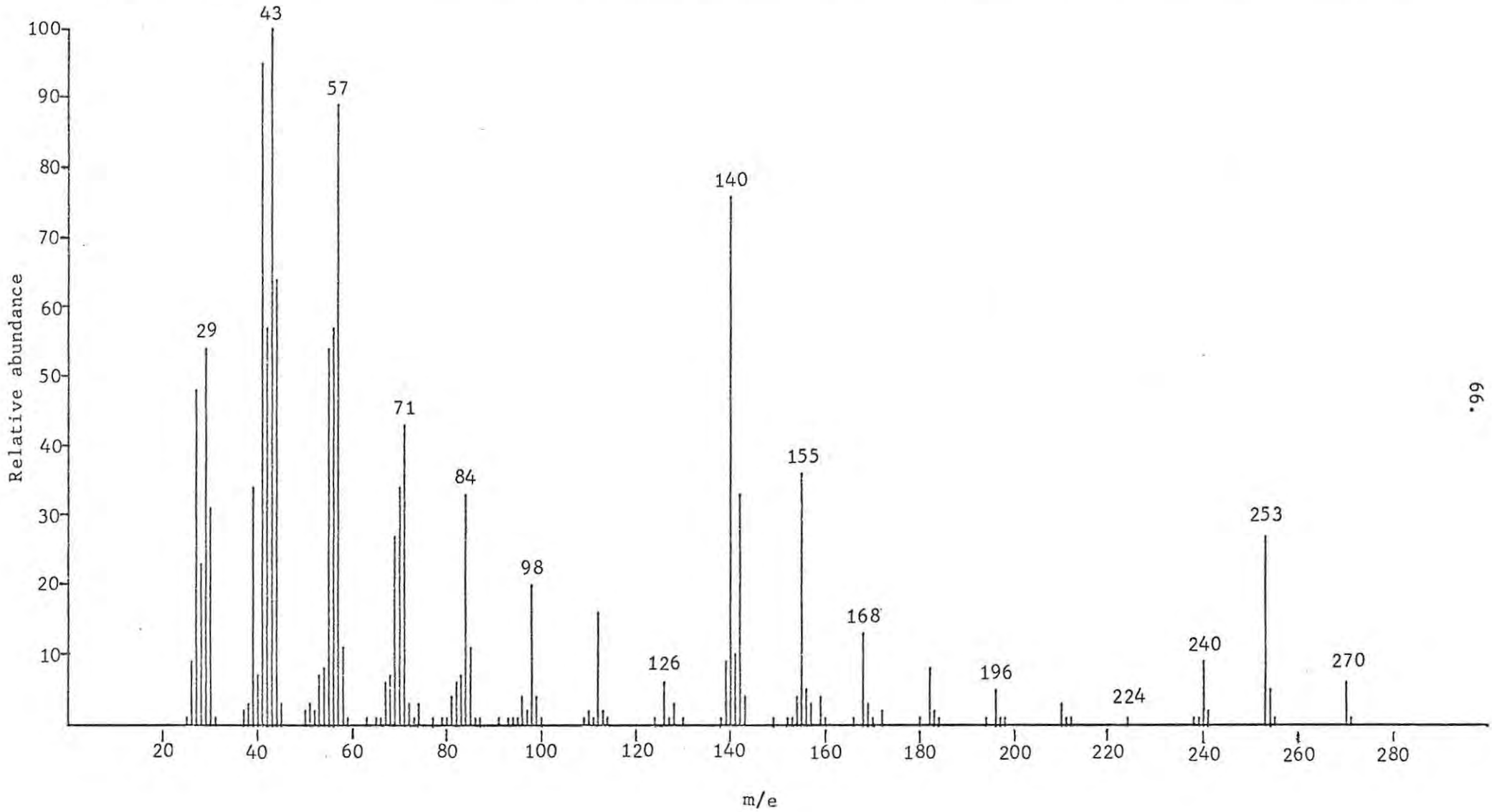


Fig. 10 Mass spectrum of DDDN recorded on an AEI MS30 instrument (Molecular ion shown 5 times observed intensity)

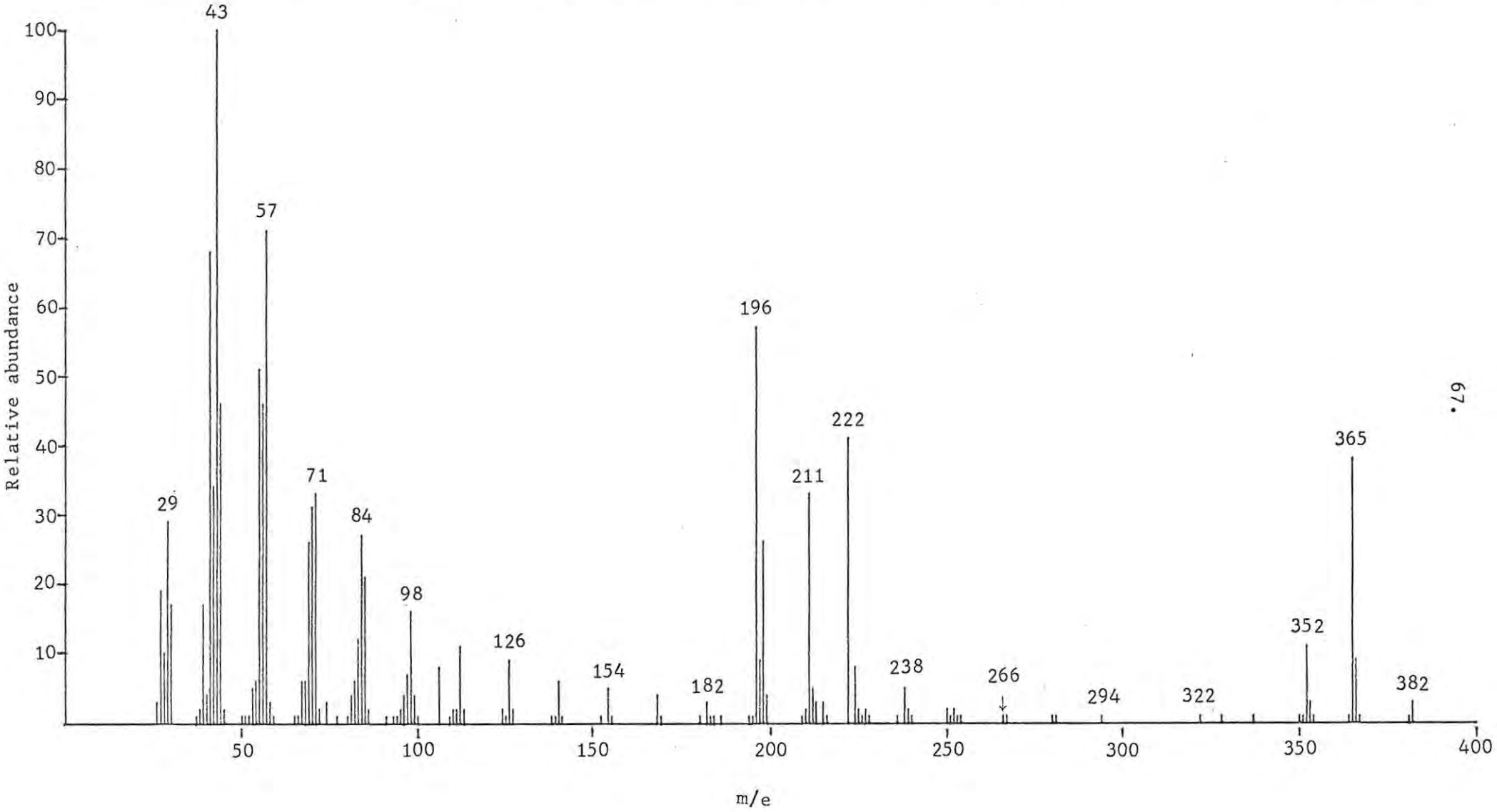
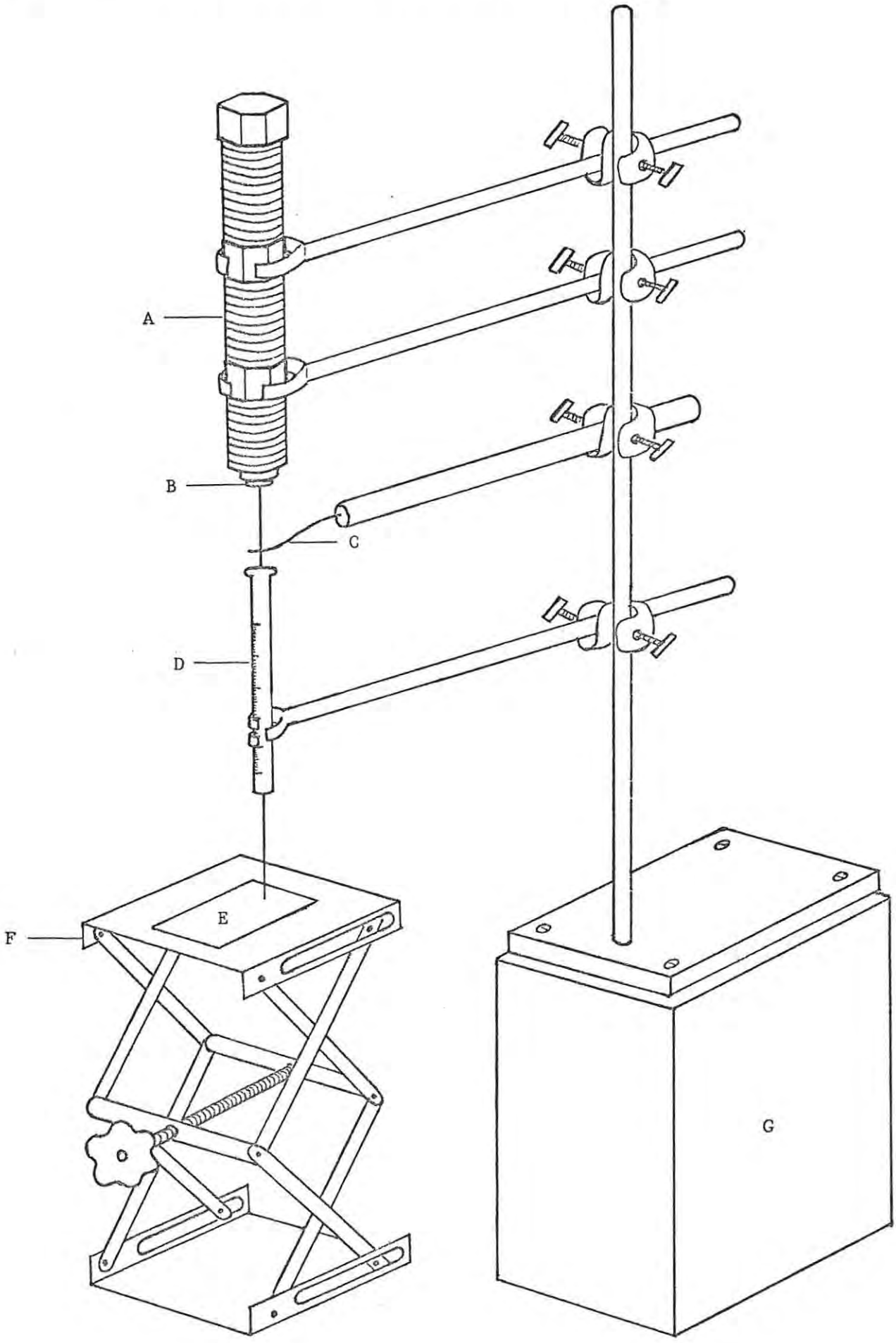


Fig. 11 Applicator apparatus for thin-layer chromatography.



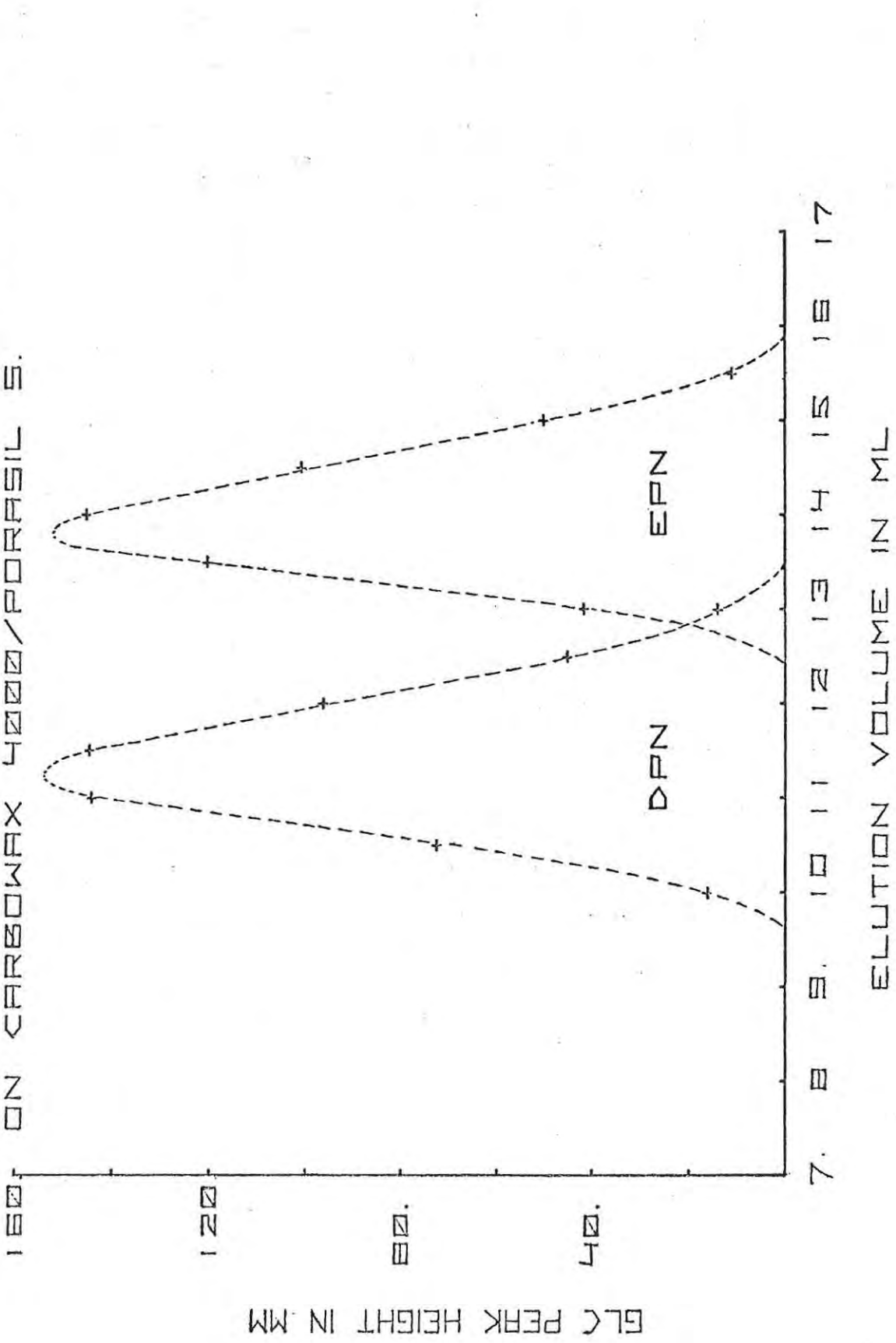

LIQUID COL. CHROMATOGRAPHY OF. EPN + DPN
ON CARBOWAX 40000/PORASIL 5.
GLC PEAK HEIGHT IN MM
160
120
80.
40.
7. 8 9. 10 11 12 13 14 15 16 17
ELUTION VOLUME IN ML
EPN
DPN

Fig. 12

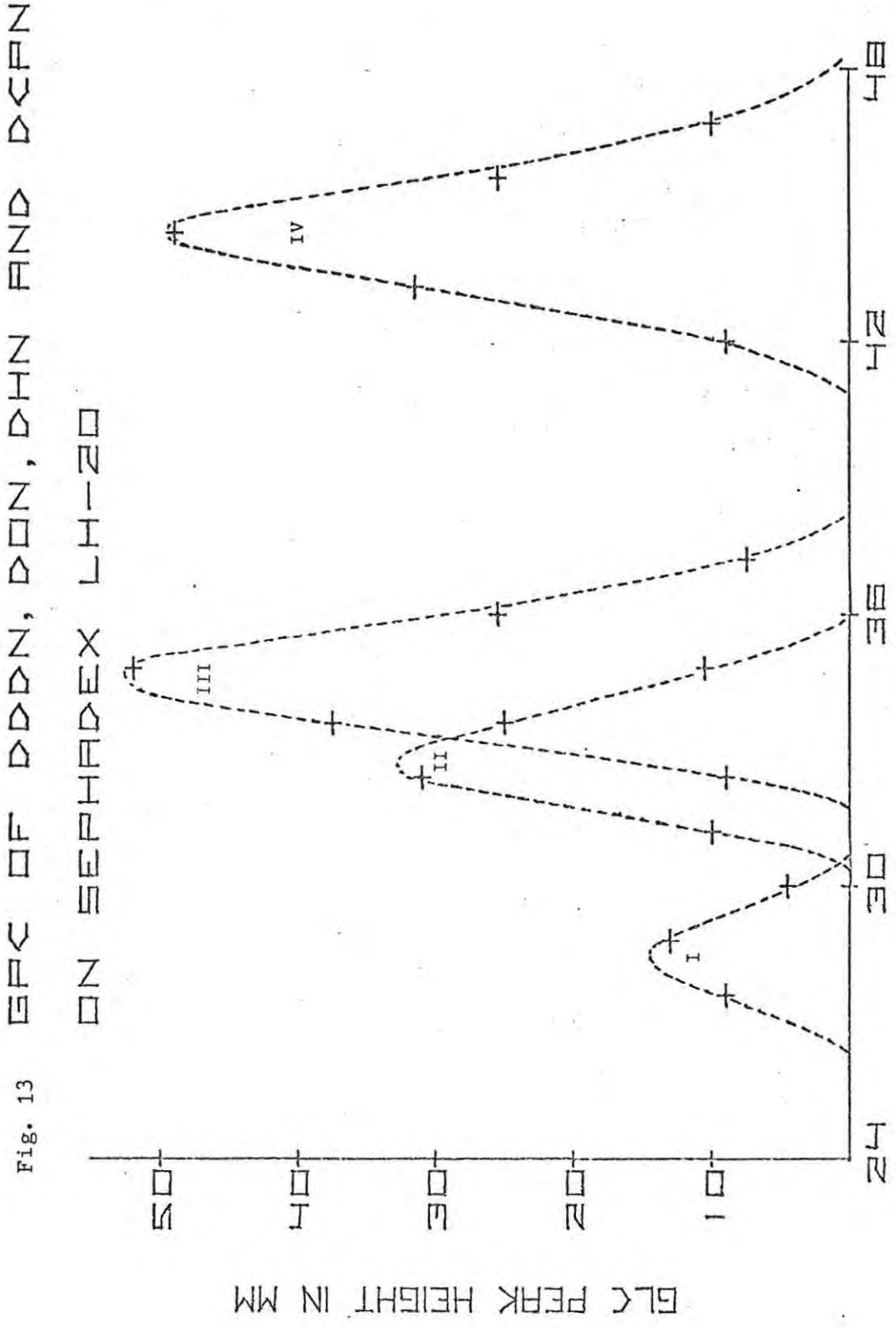
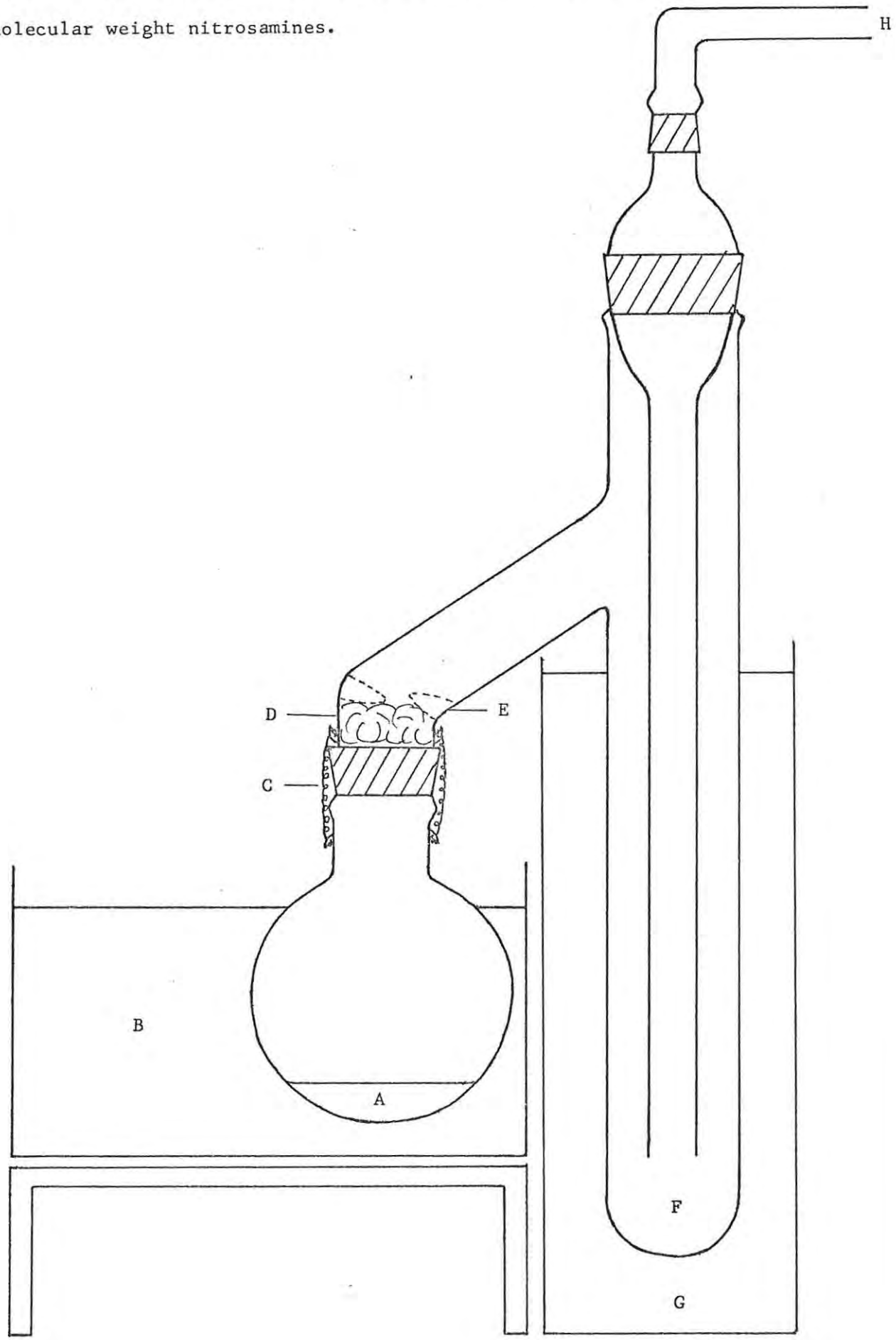


Fig. 13 GPC OF DADN, DGN, DHN AND DCPN ON SEPHADEX LH-20

Fig. 14 Vacuum distillation apparatus for isolation of high molecular weight nitrosamines.



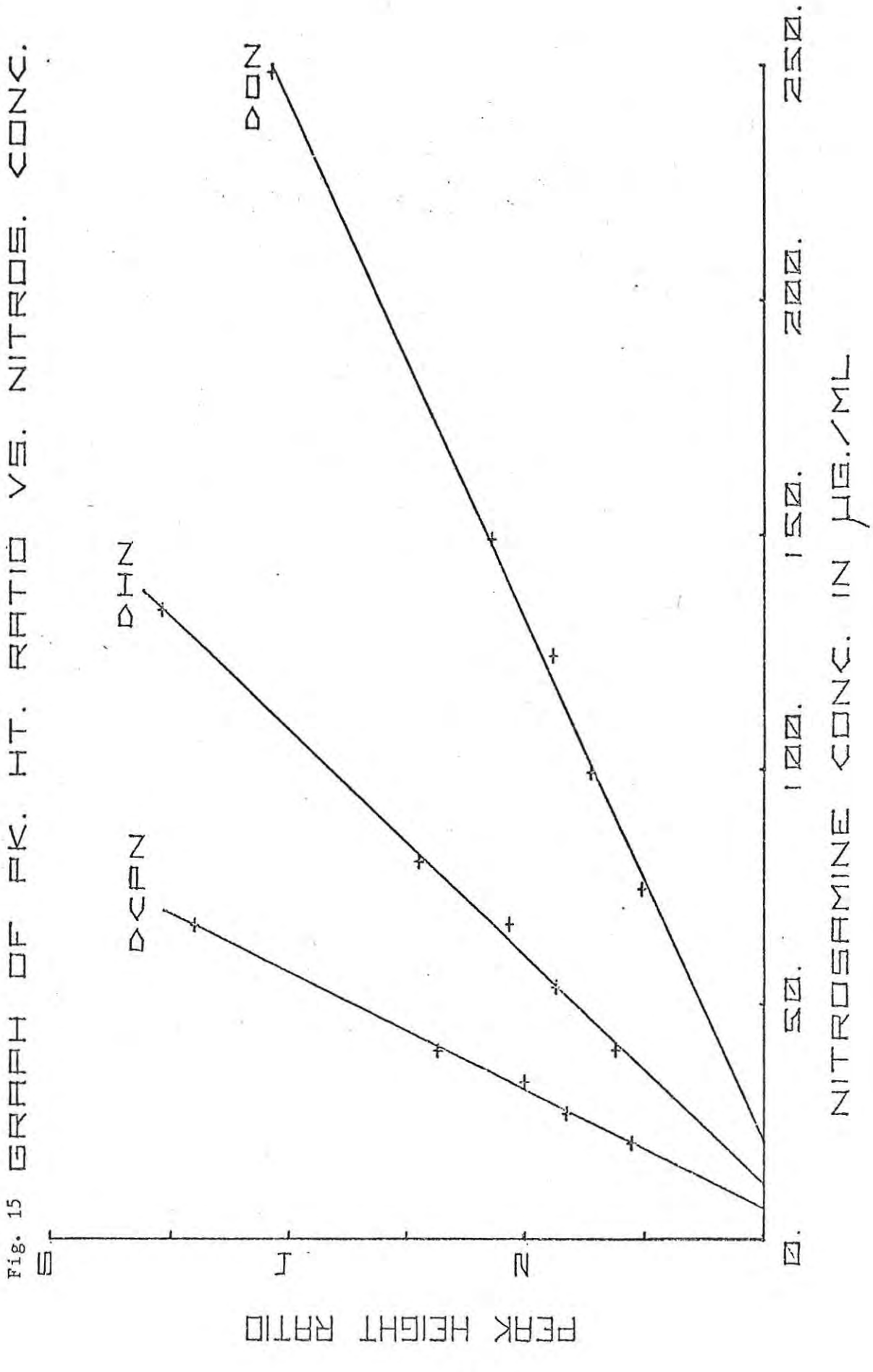

GRAPH OF PK. HT. RATIO VS. NITROS. CONC.
PEAK HEIGHT RATIO
NITROSAMINE CONC. IN µG./ML
0.
50.
100.
150.
200.
250.
DNZ
DHN
DCPN

Fig. 15

APPENDIX II - Tables

Table 1

Infrared frequencies of DHN (range 2000 – 625 cm^{-1})

Solvent			N=O str.									N–N str.	C–N symm. str.		
	S	S	S	M	W-M	MS	S	W-M	W-M	W-M	M	S	M + bd	W + bd	MS
Liquid film	1468	1464	1457	1438	1425	1381	1361	1316	1274	1233	1158	1091	1033	918	724
	MS	S	S	MS	MS	MS	S	W-M	W-M		M	S	W		
Dichloro-methane	1468	1464	1454	1436	1418	1380	1361	1315	1263	–	1157	1096	1033	–	–
	S	S	S	M	S	M	S	W-M	W-M	W-M	W-M	S	W		
Chloroform	1468	1465	1453	1436	1421	1377	1362	1315	1272	1232	1157	1097	1028	–	–
				M	M	M	S	W	W	W + bd	W-M	S	M + bd	W + bd	M + bd
Carbon disulphide	–	–	–	1438	1416	1377	1358	1315	1273	1230	1153	1086	1030	914	722
	MS	S	S	S	W-M		S	W-M	W	W	W-M	S	M	W + bd	M + bd
Carbon tetrachloride	1469	1465	1461	1440	1422	–	1359	1319	1277	1234	1156	1086	1024	922	723
	W-M	S	S	S	M		S	W-M	W	W + bd	W-M	S	M + bd	W + bd	M + bd
Cyclohexane	1469	1465	1462	1440	1418	–	1359	1319	1274	1233	1153	1082	1023	919	722
					M		S	M	VW	VW	W-M	S	M + bd	W + bd	M + bd
Iso-octane	–	–	–	–	1418	–	1357	1319	1264	1228	1155	1082	1023	919	623
		W			M		S	M	W	W	W-M	S	M + bd	W + bd	M + bd
n-heptane	–	1465	–	–	1418	–	1357	1319	1273	1228	1154	1082	1025	919	718
					M		S	M	VW		W-M	S	M + bd		
n-pentane	–	–	–	–	1418	–	1356	1316	1263	–	1154	1082	1025	–	–

S, M, W, V, bd and sh denote strong, medium, weak, very, broad and sharp respectively.

Table 2

Solvent	Infrared frequencies of DON (range 2000 – 625 cm^{-1})														
			N=O str.									N–N str.	C–N symm.str.		
	MS	S	S	M	W-M	MS	S	W	W	W-M	W-M	S	M + bd	VW + bd	M
Liquid film	1468	1465	1457	1438	1425	1381	1361	1320	1264	1224	1154	1093	1048	955	722
	S	S	S	MS	M	MS	S	W	W	VW	M	S	M + bd		
Dichloro methane	1469	1465	1456	1439	1423	1380	1361	1319	1258	1228	1154	1098	1044	–	–
	S	S	S	MS	M	MS	S	W	W	VW	W-M	S	M + bd	W + bd	
Chloroform	1469	1465	1455	1439	1423	1378	1362	1319	1266	1230	1157	1099	1045	953	–
					M	M	S	W	W	VW	W-M	S	M	W + bd	M + bd
Carbon disulphide	–	–	–	–	1419	1377	1359	1317	1268	1228	1154	1090	1044	956	723
	S	S	S	M	W-M	M	S	W	W	VW	W-M	S	M	W + bd	
Carbon tetrachloride	1469	1465	1460	1439	1423	1377	1360	1319	1266	1230	1155	1090	1044	954	–
					W-M			W		VW	W-M	S	M-S		W-M
Iso-octane	–	–	–	–	1423	–	–	1317	–	1228	1153	1088	1040	–	723
		W			W-M			W		VW	W-M	S	MS		W-M
n-heptane	–	1466	–	–	1422	–	–	1317	–	1228	1153	1088	1042	–	722
				M			S	W	W	VW	W-M	S	MS	W + bd	W-M
n-pentane	–	–	–	1434	–	–	1355	1312	1260	1221	1154	1088	1042	946	720

S, M, W, V, bd and sh denote strong, medium, very, broad and sharp respectively.

Table 3

Solvent	Infrared frequencies of DDDN (range 2000 – 625 cm^{-1})														
			N=O str.									N–N str.	C–N symm. str.		
		S-Sh	S	W-M	M	MS	S	M	M	W		S	S	W	S
KBr disc	–	1468	1458	1434	1419	1381	1350	1321	1252	1193	–	1099	1086	939	720
Dichloro-methane	S 1469	S 1465	S 1457	MS 1436	M 1421	MS 1380	S 1362	M 1315	W 1260	W 1207	W 1151	S 1105	S 1080	–	–
Chloroform	S 1469	S 1465	S 1456	MS 1436	M 1420	M 1378	S 1361	W-M 1311	–	W 1211	W 1149	S 1106	S 1080	W 943	–
Carbon tetrachloride	S 1469	S 1465	S 1460	M 1438	W 1421	M 1377	S 1361	M + bd 1310	–	–	W 1149	MS 1093	S 1067	–	–
Iso-octane	–	–	–	–	W 1419	–	–	M + bd 1313	–	–	VW 1146	MS 1093	VS 1064	–	S 722
n-heptane	–	–	–	–	W 1419	–	–	M + bd 1313	–	–	VW 1146	MS 1093	VS 1064	–	W 721
n-pentane	–	–	–	–	M 1413	–	S 1356	M + bd 1313	–	–	VW 1145	MS 1092	VS 1062	–	W 722

S, M, W, V, bd and sh denote strong, medium, very, broad and sharp respectively.

Table 4

Electronic absorption spectral data on nitrosamines

Compound	Solvent	max, nm[a]			
Diethylnitrosamine	Light petroleum	233	358	366	378
		(6500)	(82)	(105)	(90)
	Ethanol	233		350	
		(7400)		(90)	
Di-n-propylnitrosamine	Light petroleum	235		366	
		(6100)		(110)	
	Ethanol	233		350	
		(7000)		(90)	
Di-n-pentylnitrosamine	Light petroleum	236	356	366	378
		(6300)	(85)	(105)	(90)
	Ethanol	235		350	
		(7400)		(93)	
Di-n-heptylnitrosamine	Iso-octane	236	355	366	379
		(6100)	(80)	(99)	(82)
	Ethanol	253		349	
		(7260)		(82)	
Di-n-dodecylnitrosamine	Iso-octane	237	356	365	379
		(6330)	(95)	(110)	(98)
	Ethanol	235		348	
		(7570		(86)	

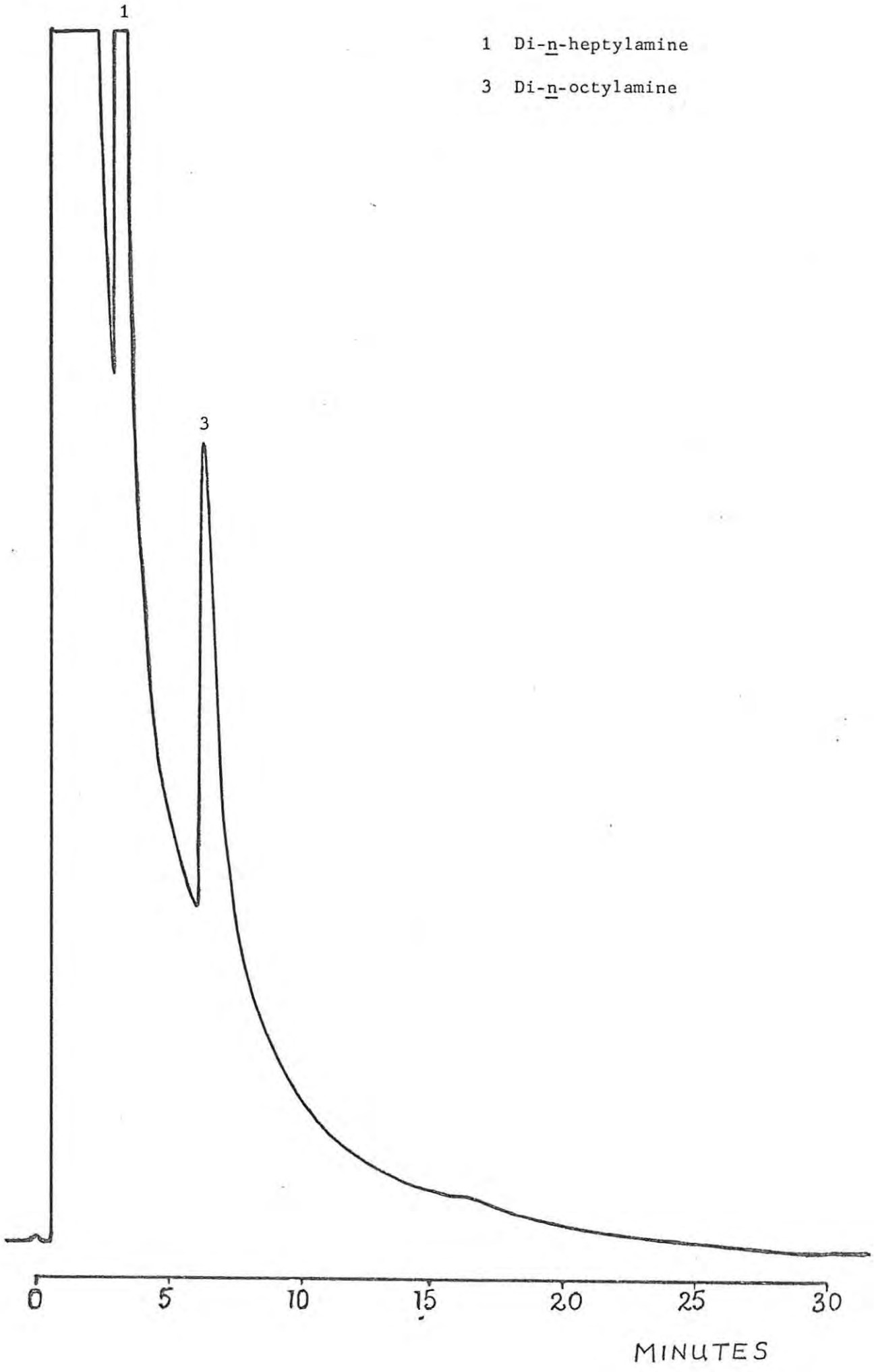
[a] The numbers in parentheses are the extinction coefficients of the absorption bands.

Table 5

m/e				27	30	41	42	43	57	71	84
Probable Compositions / Nitrosamine	M^+	M-17	M-NO	HCN	$^{\cdot}NO$	C_2H_3N, C_3H_5	C_3H_6, C_2H_4N	C_3H_7, C_2H_5N	C_3H_7N, C_4H_9	C_4H_9N, C_5H_{11}	$C_5H_{10}N$
	%										
DEN (97)	58	–	7	47	42	12	100	6	42	–	–
DPN (4)	7	5	–	29	13	46	34	64	5	12	4
DBN (4)	13	7	1	30	41	60	49	54	84	1	100
DCPN	5	1	5	22	22	100	21	31	15	5	60
DON	1,2	27	9	48	31	95	57	100	89	43	33
DDDN	0,6	38	11	19	17	68	34	100	71	33	27

APPENDIX III - Chromatograms

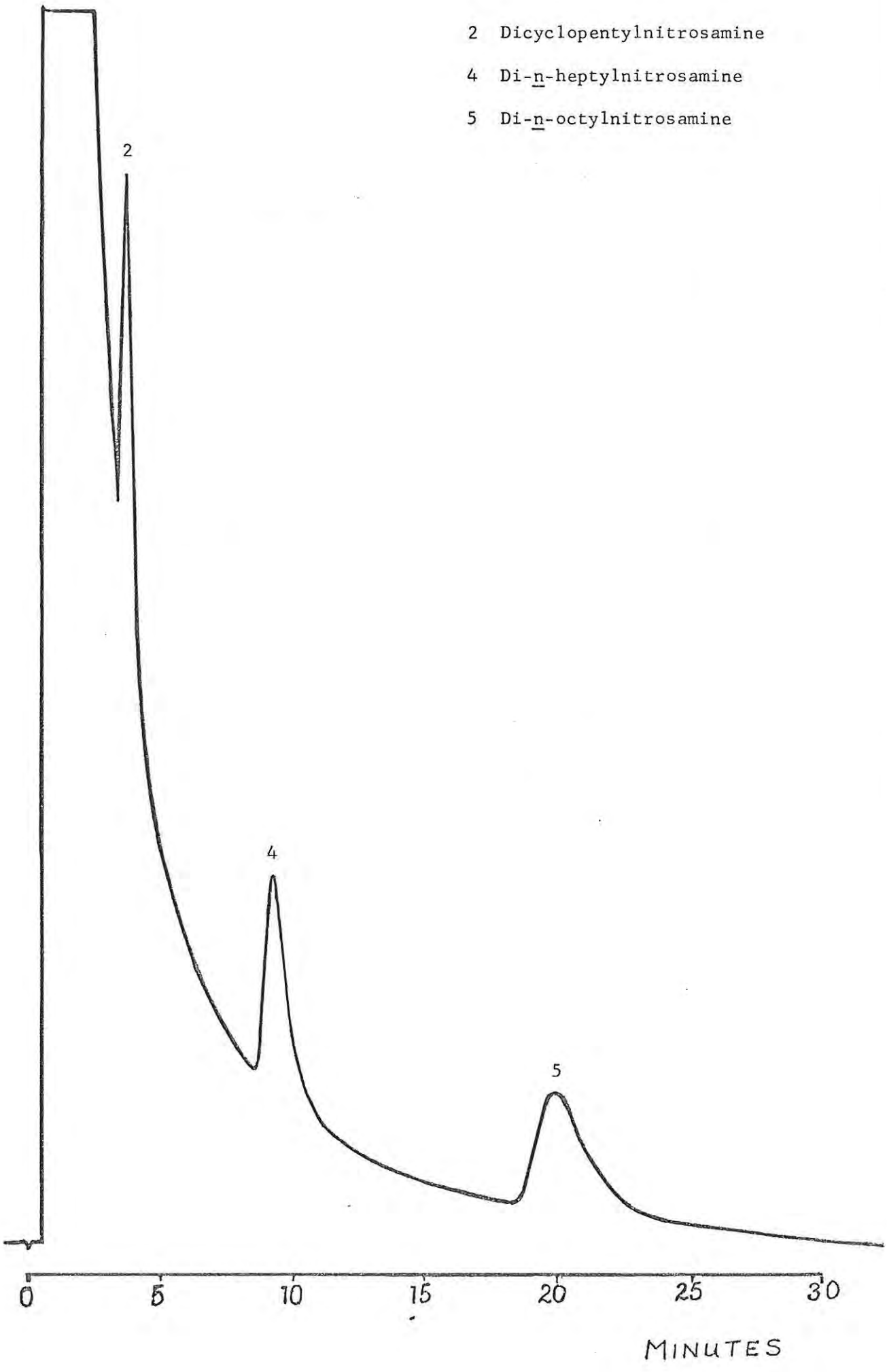
Chromatogram 1.



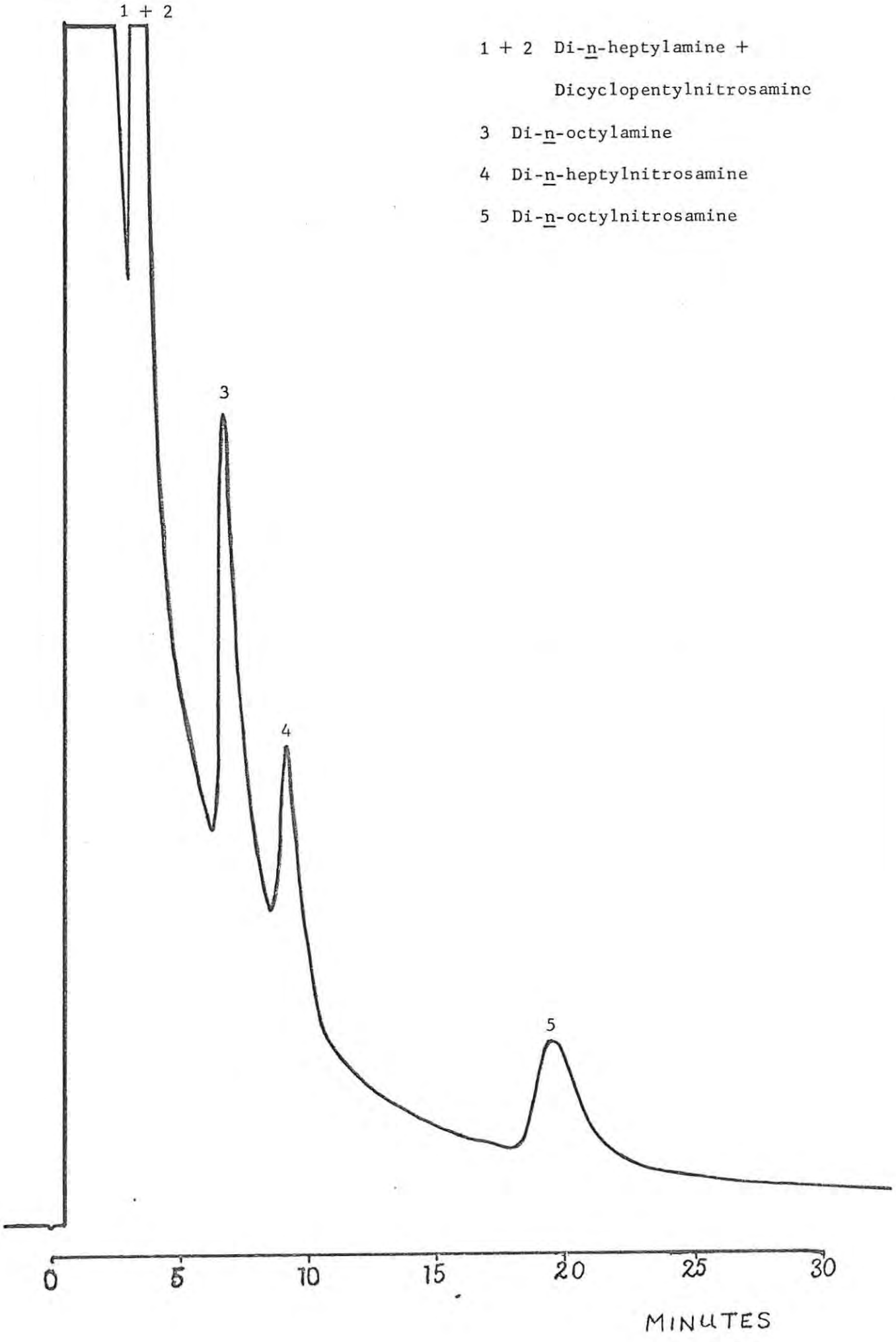
1 Di-n-heptylamine

3 Di-n-octylamine

Chromatogram 2



Chromatogram 3



1 + 2 Di-n-heptylamine + Dicyclopentylnitrosamine

3 Di-n-octylamine

4 Di-n-heptylnitrosamine

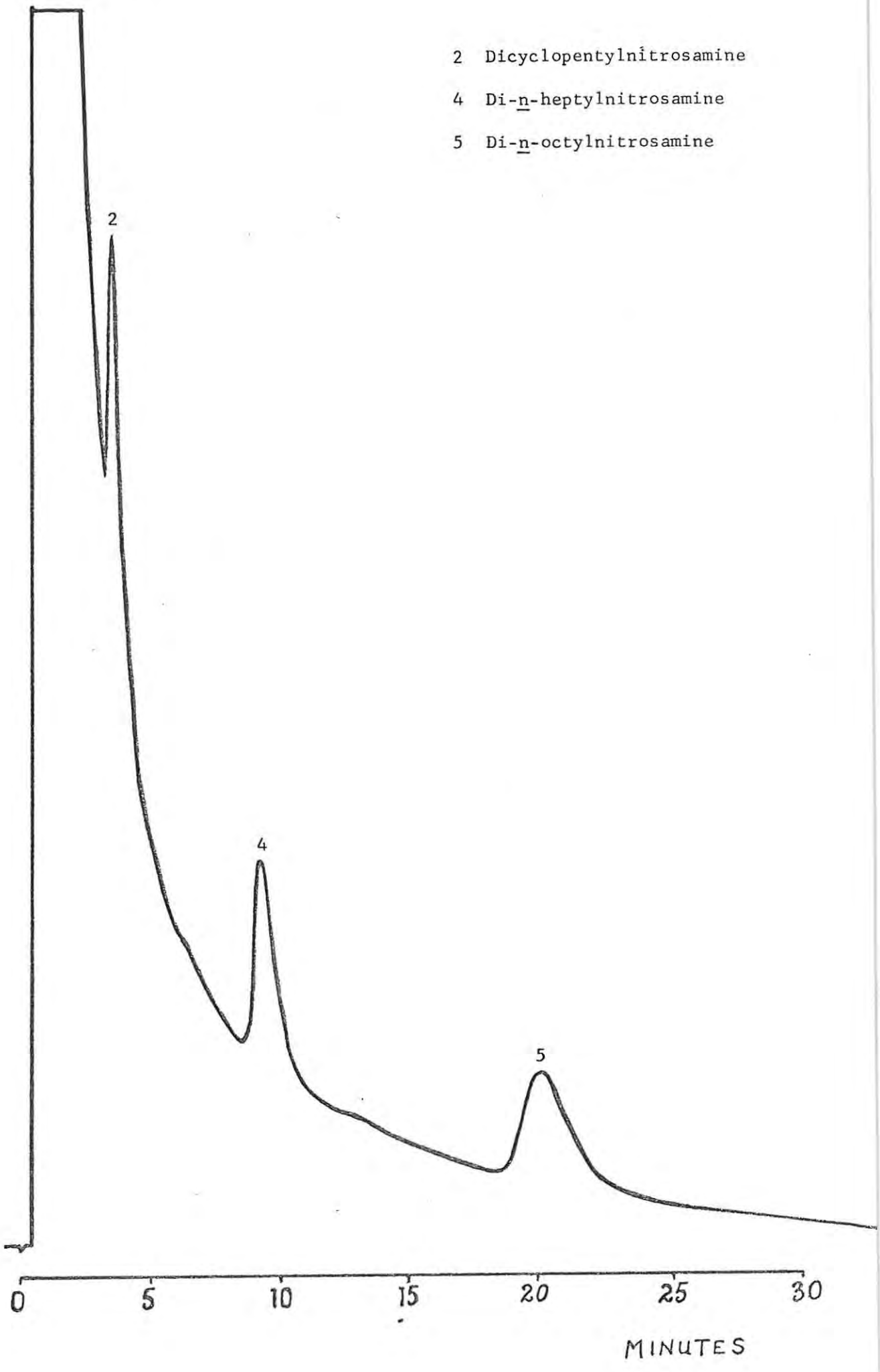
5 Di-n-octylnitrosamine

Chromatogram 4

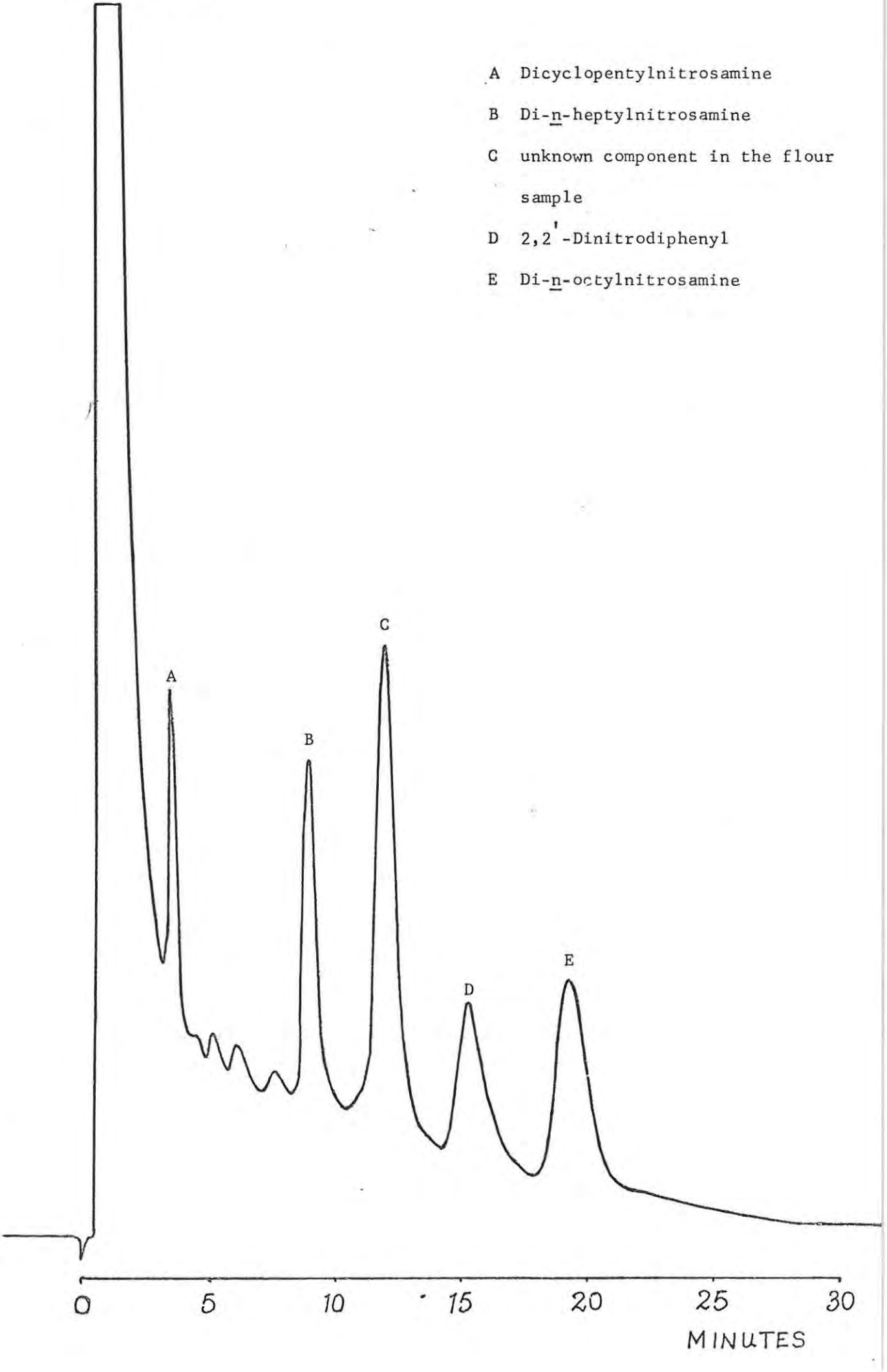
2 Dicyclopentylnitrosamine

4 Di-n-heptylnitrosamine

5 Di-n-octylnitrosamine

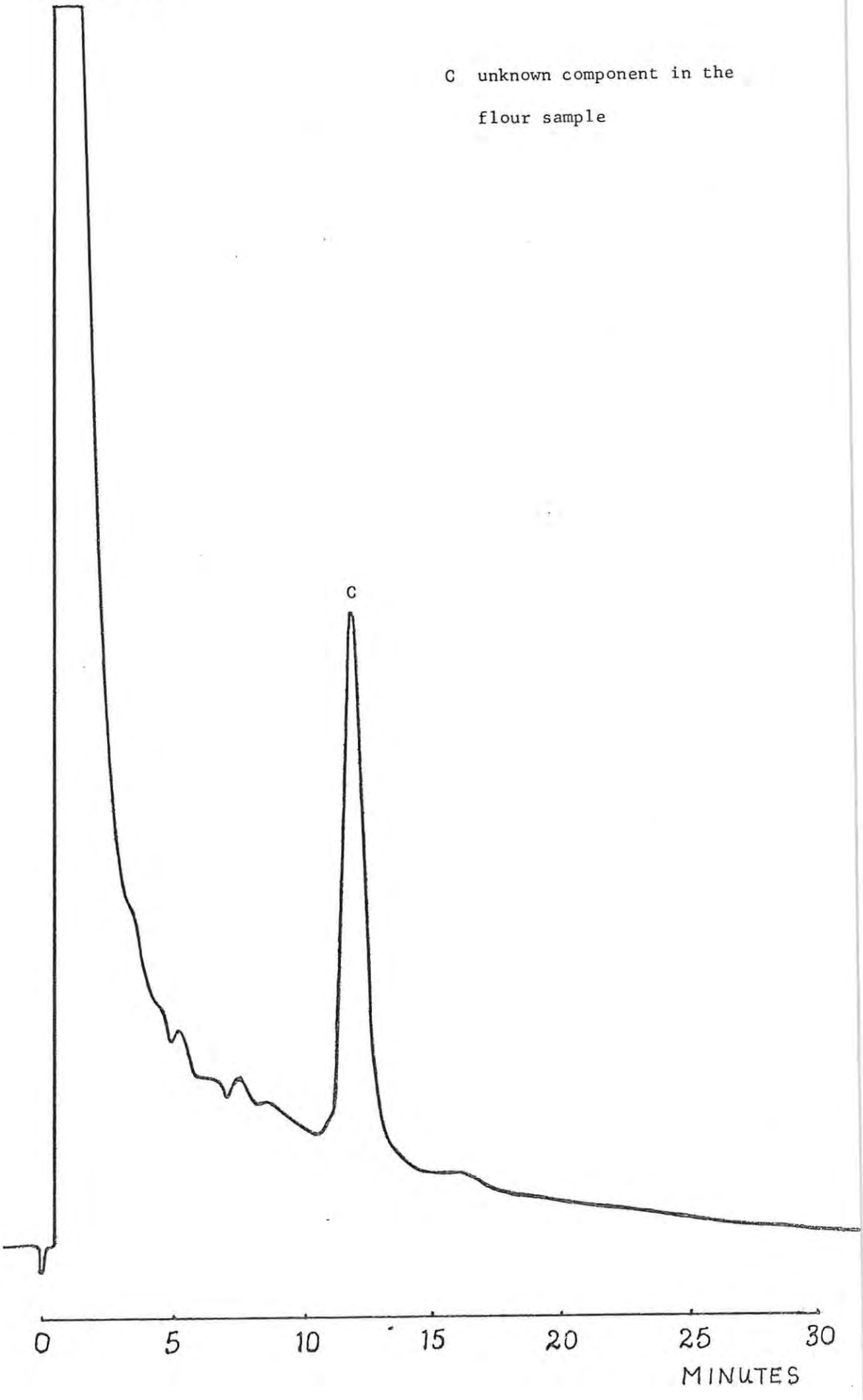


Chromatogram 5



Chromatogram 6

C unknown component in the flour sample



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