

**THE CONTRIBUTION OF CHEMISTRY  
TO FOOD SUPPLIES**

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I. MORTON, D. N. RHODES

## TRACE NITROSAMINE ANALYSIS

Aaron E. Wasserman

Eastern Regional Research Center\*  
Philadelphia, Pennsylvania 19118

### ABSTRACT

N-nitroso compounds have been found in trace amounts in some samples of food treated with sodium nitrite. Since nitrosamines are carcinogenic they constitute a potential public health hazard, depending on the concentration present and the amount needed for pharmacological activity. The latter is still unknown. The determination of nitrosamines in ug/kg concentrations requires analytical procedures that are sensitive and accurate; until recently there has been no accord on methodology. Standardization of the procedures is being investigated currently by IUPAC and IARC.

The analytical procedure entails several problem areas. The nature of the food products analyzed and the chemical characteristics of the nitrosamines are so varied that an adequate separation procedure capable of satisfactory performance under all conditions is not currently available. A number of methods in use are described. Contaminating substances arising from the food matrix or reagents used must be removed prior to quantitation and identification by thin layer or gas chromatography and colorimetric techniques. Interference occurs in all methods of analysis so confirmation of the identity of the nitrosamine is essential to prevent false positive identifications. The present method of choice is confirmation of molecular structure by mass spectrometry, but other methods may be satisfactory in the absence of a mass spectrometer.

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### INTRODUCTION

Prior to the development of canning by Appert about 170 years ago, man's food could be classed as perishable, and what would be considered today as food additives were used liberally to preserve food products beyond their natural spoilage period, or to disguise the aroma and flavor if the former efforts were unsuccessful. Curing or "corning" with salt and sodium nitrate, and/or nitrite in recent years, has been used to preserve meat and fish products.

In 1956 Magee and Barnes (1) reported the carcinogenic activity of N-nitrosodimethylamine, one of a class of compounds called nitrosamines. Considered as industrial chemicals, these compounds were studied for a number of years purely on the basis of their cancer-inducing activity. In 1964, however, a nitrosamine was identified in an ingestible product and demonstrated to be the causal agent of undesirable liver conditions in animals (2). In less than a decade nitrosamines have become important subjects of investigation in the food industry, particularly the cured food segment. In view of their role as a potential public health hazard, it is necessary to be able to determine the concentration of these compounds in foods. Particularly in the past few years, there has been intensive pressure to develop sensitive analytical procedures. Information about the present state of knowledge of the relationship between nitrosamines and the compounds that react to form them may be of interest in understanding some of the analytical problems.

Nitrosamines are formed by the chemical reaction between nitrite and secondary amines. In food the source of nitrite is either the salt itself or its precursor, nitrate. Vegetables may have high levels of nitrate, depending on conditions of fertilization, climate, genetics and other factors (3). Reduction of nitrate to nitrite occurs as the result of the action of enzymes in the tissue or from adventitious bacteria under appropriate conditions of storage and temperature. Water also may contain high levels of nitrate which, when used in processing foods, can serve as a reservoir for nitrite formation by the action of contaminating bacteria. The major source of nitrite, however, is the nitrite and nitrate salts that are added directly to a number of foods, either as curing agents for meat or as an anti-bacterial preservative for meat, fish and cheese. The use of nitrite and nitrate under such conditions is often controlled by Governmental regulations and a number of countries are either forbidding or restricting use of these salts at the present time.

The second component of the reaction, the amine, originates in the food tissue itself, although in some instances amines can be introduced with other ingredients during processing. Primary amines, such as most amino acids, react with nitrite and are deaminated without formation of nitrosamines. Secondary amines, however, react with nitrite yielding the N-nitrosamine. N-nitrosodimethylamine (DMNA) has been the most frequently identified nitroso compound in food to date. The source of the dimethylamine, however, cannot be identified with assurance in every product in which it is found. This amine is probably present in the greatest concentration in salt water fish as a result of enzymic degradation of trimethylamine, a natural constituent. A number of other physiological amines - secondary, tertiary and quaternary - have yielded DMNA in model systems, as shown in Table 1. Creatine degrades to N-nitrososarcosine, which breaks down further to DMNA (4). Fiddler, et al. (5) demonstrated the formation of DMNA from choline, betaine, carnitine and a number of related tertiary amines. Lecithin recently was reported to yield DMNA and traces of N-nitrosodiethylamine and N-nitrosodipropylamine when nitrosated in a model system (6).

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The only other major nitrosamine identified in food to date is nitrosopyrrolidine (NOPyr) which occurs in approximately 90% of all cured bacon after frying for consumption (7-9). While the precursor for NOPyr is still unknown, it is postulated that the amino acid proline is nitrosated and decarboxylated in some fashion to yield the nitrosamine (10).

Other amines, or amine precursors, that have been nitrosated in model systems, or are potentially nitrosatable, exist in foods. Arginine, citrulline and hydroxyproline have been nitrosated in the test tube but the nitroso compounds have not been found in food at this time. Putrescine, cadaverine, spermine, spermidine and other polyamines are potentially nitrosatable or are precursors for nitrosamines. Many plants and vegetables are the source of large concentrations of betaine-type compounds (11) that may be able to react with nitrite.

Morpholine is a nitrogenous substance normally not found in food, but it has been used extensively as a rust inhibitor for boilers and may appear as a contaminant in food exposed to steam. Morpholine nitrosates readily and is a potent carcinogen (12, 13), therefore it is a nitroso compound that should be considered in the analysis of foods for this class of compounds.

Research on nitrosamines has centered principally around the volatile compounds that are separated from the food matrix with relative ease. It is conceivable, therefore, that there may be other nitrosamines present in food, particularly non-volatile compounds whose detection is difficult because of inadequate analytical procedures.

Ender and Ceh (2), and Ender (14) were the first to identify a nitrosamine in an edible product, reporting substantial amounts of DMNA in cooked fish meal made from herring preserved with nitrite. Since their paper in 1964 nitrosamines have been reported in a number of foods used for human consumption, including fish (15,16), meat (17-19), cheese (8, 20, 21), flour (22-25), mushroom (26), alcoholic beverages (27) and a solanaceous fruit (28), as shown in Table 2. Unfortunately, however, the identifications in most of these reports are now open to question. Analytical procedures have become more sophisticated in recent years and we have become aware of the presence of interfering contaminants, improper analytical methodology and interpretive errors that could result in false positive responses. Many authors today refer, uncritically, to these earlier reports in their reviews of the literature, perpetuating the impression that nitrosamines are more prevalent, and are present in a greater variety of foods, than is actually the case. Responsible reporting of analytical data is extremely important, particularly for substances in such a sensitive area.

One of the earliest controversies over the presence of nitrosamines in the food supply involved an interesting exchange between Thewlis (24,25) and Marquardt and Hedler (22,23). The latter authors reported extracting N-nitrosodiethylamine (DENA) from German flour and, subsequently, from flour supplied by Thewlis when treated by their procedure. Thewlis was unable to find the nitrosamine in English

and Canadian flour. Analytical procedures included thin layer chromatography and spraying with the palladium chloride-diphenylamine reagent of Preussmann et al. (29) to separate and visualize the nitrosamine. Thewlis (25) later indicated that phenols, and possibly other compounds, gave interfering spots on the plates. Other authors, Krolller (30) and Mohler and Mayerhofer (31), also were unable to find DENA in flour and reported interfering artifacts.

Devik (32) reported nitrosamine formation arising from the Maillard reaction in heated mixtures of carbohydrates and amino acids. This has serious implications for the food industry, and for people in general, because so many food processing procedures, in the home as well as commercially, involve a heating step in which the browning reaction can occur. However Devik used polarography to identify and quantitate the apparent nitrosamines. A number of investigators (33-35) subsequently demonstrated, by gas chromatography-mass spectrometry (GC-MS) that pyrazines formed in the Maillard reaction, give polarographic responses that may be interpreted as nitrosamines. Chemical components of alcoholic beverages also have reduction potentials resembling those of nitrosamines, but GC-MS confirmational investigations detected the differences (36-38). The gravity of such incorrect identification is indicated in one study in which it was suggested that the supposed nitrosamine in the alcoholic beverage could be the causal agent of esophageal cancer (27).

A variety of substances have been reported to give false positive values in a number of analytical procedures. Unsaturated hydrocarbons, dicarboxylic acids and compounds with alpha-unsaturated carbonyl groups react with the diphenylamine-palladium chloride colorimetric reagent of Preussmann et al. (29). Sen et al. (39) reported that the presence of amines and yellow pigments from fish and cheese interfered with nitrosamine identification by thin layer chromatography (TLC). Linoleic acid, alpha-tocopherol, xanthophyll and carotene also yield spots that may be mistaken for nitrosamines (30). Propionic acid from cheese produces a spot with the Preussmann reagent at the same Rf value as nitrosodiethylamine (40). Solvents may also contain trace impurities that, on concentration, interfere with nitrosamine identification. Furfural, which occurs in a variety of food products (bread, spirits, dried milk powder, honey, flour, some meat products) is difficult to separate from N-nitrosodiethylamine by TLC (41) and gives a polarographic response similar to DMNA (36). The difficulties encountered in GC analysis will be discussed in the appropriate section.

#### ANALYTICAL PROCEDURES FOR ANALYSIS OF NITROSAMINES

With the advent, in recent years, of specialized instrumentation and more sensitive techniques the concentrations at which nitrosamines can be quantitated have become smaller and smaller. It is generally accepted that an analytical procedure should be capable of measuring nitrosamines in the order of 10 ug/kg and there are reports of quantitative analyses at the level of 1 ug/kg (8). The sensitivity of detection of nitrosamines by some methods is picograms of material (42).

The determination even of pure substances at such levels imposes rigid requirements on the procedure; when the analysis involves a food, further complications are added. The preparation and analysis of a sample requires several steps: 1) separation of the nitrosamine from the food matrix; 2) clean-up, to remove interfering substances; 3) separation of nitrosamine; 4) quantitation; 5) confirmation. A number of procedures have been reported for the analysis of nitrosamines, but, unfortunately, some were developed with pure solutions in model systems only. The analytical difficulties can be appreciated only by the use of a food base, and preferably the food to be analyzed, for determining recovery yields, sensitivity and specificity of the procedure. A review of the literature of the past five or six years reveals the variations used to improve the analysis for nitrosamines (43). Since it is not possible to discuss all of the methods, selected examples will be used to illustrate the various stages. Details of the procedures can be obtained from the original papers.

#### Separation of nitrosamine from food matrix

In order to analyze nitrosamines it is necessary to remove them from the food under investigation. Volatile nitrosamines can be separated by distillation procedures: atmospheric distillation, as well as vacuum and steam distillation, has been employed by various investigators. Telling et al. (44) claim steam is faster than vacuum distillation, but the recovery of nitrosamine is lower. Distillation may be carried out on a slurry or on an homogenate of the food material. In the Laboratory of the U. S. Food and Drug Administration (FDA), as well as in our Laboratory, meat products are digested first for 5-6 hrs with methanolic KOH to disintegrate the matrix and liberate any available nitrosamine that may be present (45,46). Alkaline conditions are generally established prior to distillation in order to prevent further nitrosamine formation during heating with residual nitrite present.

Extraction, or solvent partition, procedures have been used to remove nitrosamines from the food under investigation; dichloromethane is the solvent of choice. Distillation of the extract generally follows.

A combined distillation-extraction procedure utilizing the Likens-Nickerson apparatus has been described by Essigmann and Issenberg (47).

#### Clean-up procedures

Analytical studies with pure nitrosamines pose few problems with interfering contaminants, but isolates from natural products are invariably accompanied by other tissue components. Interference may range from retardation of travel of nitrosamines in TLC or column chromatographic procedures to confusion with the nitrosamine, yielding spurious quantitative or qualitative information. Acid treatment of extracts, either as a wash with HCl or in the form of a column, will remove many of the basic contaminants.

Since most contaminants have not been identified, clean-up procedures are usually empirical. The most commonly used treatments include passage through columns containing silica gel, celite or alumina. Adsorption of nitrosamines on activated carbon has also been reported as a clean-up technique (48).

The solvents used in attempting to clean-up nitrosamine-containing samples may add contaminants. Commercial methanol, ethanol or ether may contain substances that increase the background and lead to difficulty in the identification of nitrosamines. Dichloromethane contains components that interfere with analysis of DMNA by GC under the conditions used in our Laboratory. Even distillation did not purify the solvent sufficiently in some cases to permit the normal concentration of extracts without interference. We have resorted to purchasing lots of glass-distilled dichloromethane and testing each lot for its content of interfering material.

The degree of activation of alumina is also a critical feature in the clean-up procedure. We have found variations in the recovery of N-nitrosopyrrolidine and the amount of interfering material from alkaline-treated fried bacon which were attributable to the alumina clean-up procedure.

Solvent partition procedures for the separation of nitrosamines from the food matrix generally yield preparations with fewer contaminants, making the clean-up step less of a problem. Alkaline digestion of the food, while releasing all of the nitrosamine available for analysis, also degrades other food components into compounds that create difficulties in the isolation of the nitrosamine.

#### Quantitation

There is no standard method of analysis for nitrosamines at present, and the investigator uses a system modified to suit his needs. It is difficult, therefore, to evaluate data in the literature. To alleviate this situation, the International Agency for Research on Cancer, with the invitation and encouragement of IUPAC, is conducting an international collaborative assay. The results will be reported by IARC on completion of the tests. In general, however, sufficient information is available in the literature on the quantitative analysis of nitrosamines to permit a discussion of the techniques at this time.

#### Gas chromatography (GC)

The use of gas chromatography for separation and quantitation is probably the most popular procedure for the analysis of volatile nitrosamines. The sensitivity of the system permits quantitation of nanogram concentrations of material. The flame ionization detector, however, is not sufficiently selective to differentiate between nitrosamines and other components extracted from the food. Clean-up procedures and special detectors are necessary to achieve the desired degree of responsiveness.

Fig 1a Alkali Flame Ionization  
Detector

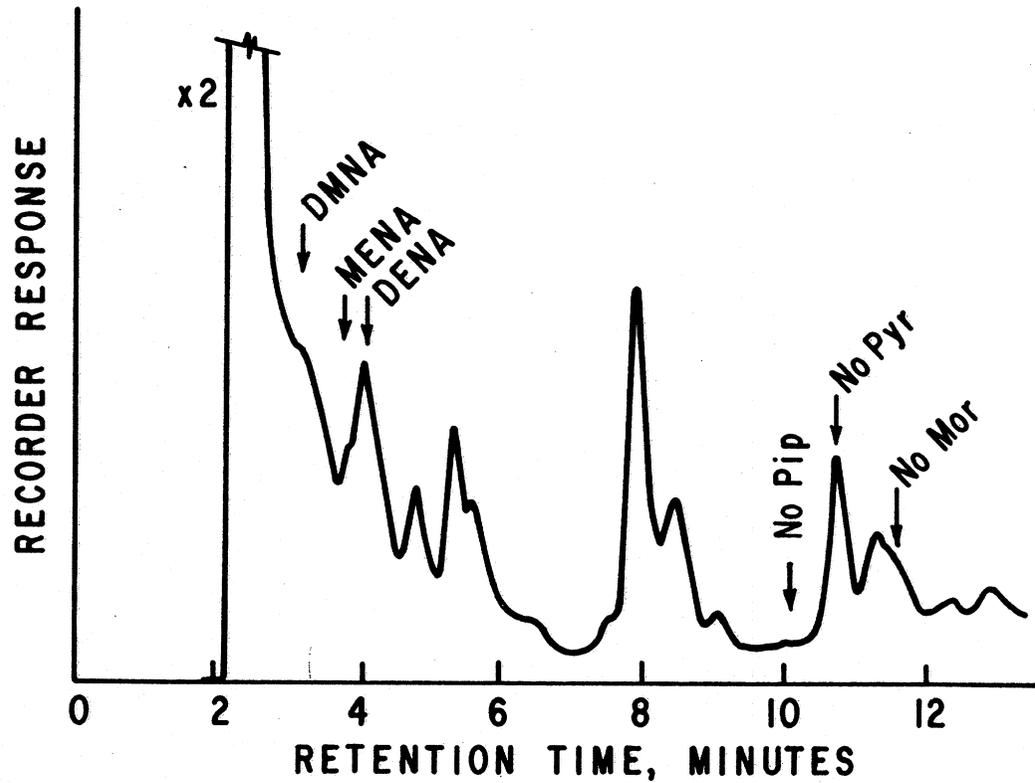
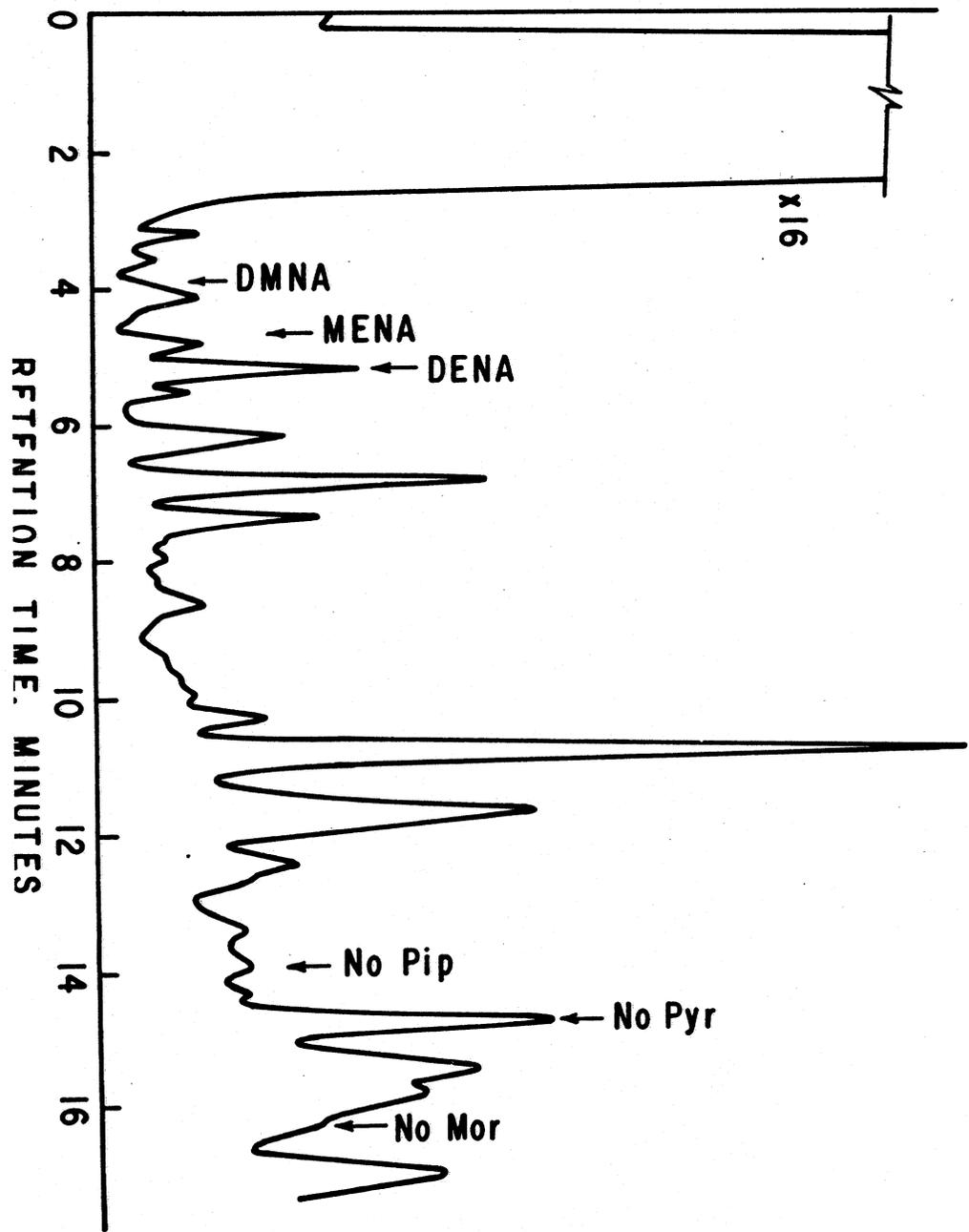


Figure 1 - Comparison of gas chromatographic profiles of a bacon sample following alcoholic digestion, extraction, distillation and purification. a. Alkali Flame Ionization Detector; b. Coulson Electrometric Conductivity Detector. Arrows indicate retention times of the following: N-Nitroso-dimethylamine (DMNA), -diethylamine (DENA), -methylethylamine (MENA), -pyrrolidine (NO<sub>Pyr</sub>), -piperidine (NO<sub>Pip</sub>), -morpholine (NO<sub>Mor</sub>).

# RECORDER RESPONSE

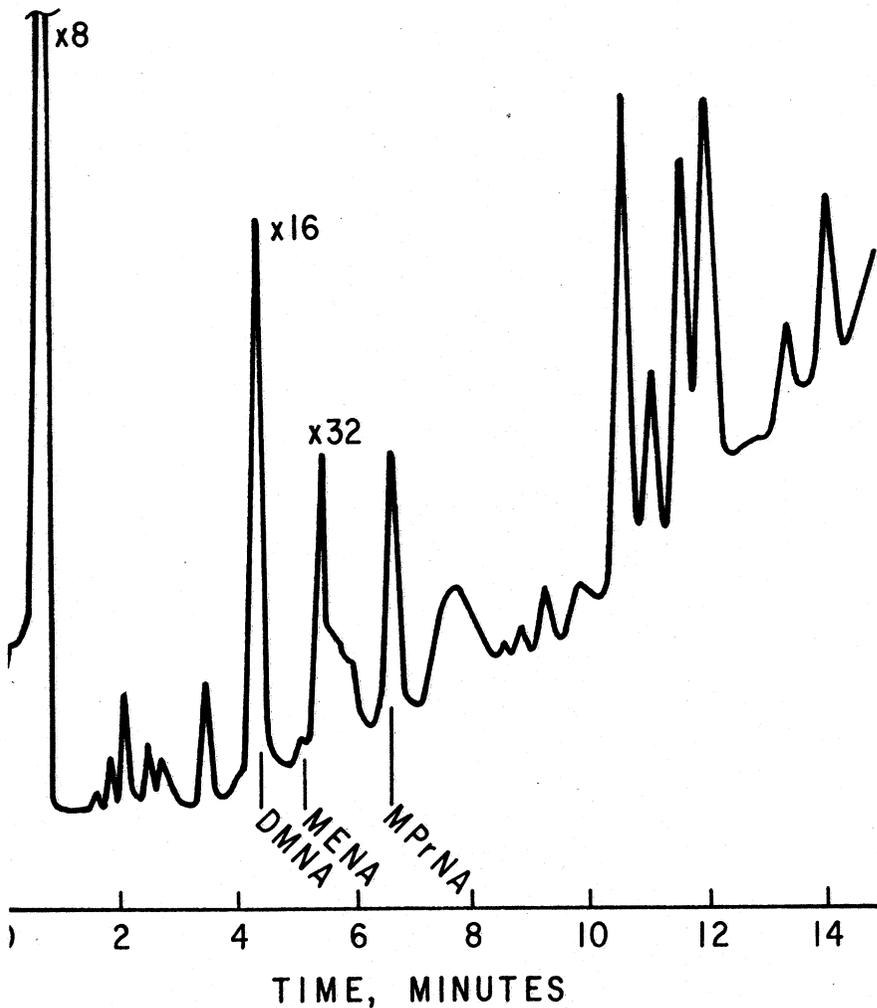


(a) The Alkali Flame Ionization Detector (AFID) is a flame ionization detector modified by the presence of an alkali salt in the flame. Ionization of the salt selectively depresses, in some yet-unknown manner, the response to hydrocarbons but not to nitrogen, phosphorus and compounds containing several other elements. Use of the AFID for nitrosamines was first described by Howard (45). A potassium chloride coil is used in the procedures of the FDA and the Department of Agriculture whereas the Laboratory of the Government Chemist reports using a rubidium sulfate pellet (49) with success. Operational characteristics of the detector depend on the detector geometry, nature of the carrier gas, hydrogen flow, ratio of air to hydrogen flow, and other undefinable factors (50-53). Detector response is reduced by chlorinated hydrocarbons, compared to other solvents such as water, hexane, ether, pentane, etc. Concentrations of nitrosamines in the order of a few tenths of a nanogram can be quantitated.

(b) The Coulson Electrolytic Conductivity Detector (CECD) was first described for the analysis of nitrosamines by Rhoades and Johnson (54), and Essigmann and Issenberg (47) have presented data utilizing this technique. The effluent from the gas chromatograph is led through a furnace at temperatures on the order of 700 to 800°C. Ammonia, produced from nitrogen-containing compounds is dissolved in deionized water and passed through a microcell in which changes in electrical conductivity are measured. The less volatile cyclic nitrosamines, such as nitrosopyrrolidine, give significantly lower detector response in the pyrolytic mode, possibly due to incomplete reduction. A nickel catalyst may be used in the furnace and the sample mixed with hydrogen after leaving the gas chromatograph in order to achieve complete reduction.

A paper has appeared recently in which Palframan, et al. (49) described and evaluated the operations of the AFI and CEC detectors. In their opinion, based on the criteria that a detector should respond to nitrogen-containing material without interference from other molecules, should be highly sensitive and reproducible and should be free from high noise level and baseline fluctuations, the CECD is more convenient and reliable than the AFID. Their evaluations, using the reductive mode of operation, were reported principally on the basis of standard solutions of nitrosamines. They note however, that peaks not corresponding to nitrosamines are present in food samples, particularly cooked foods or those stored for extended periods.

Experiences in our Laboratory confirm the difficulties encountered on analyzing food samples. Figure 1a, 1b are a comparison of a typical profile of bacon preparations obtained with the AFI and CEC detectors. There is sufficient background material at the retention times of the six nitrosamines followed in our Laboratory to create difficulties in both quantitation and identification. Although the background is less when the sample was analyzed with the CECD, technical difficulties with the CECD make the AFID the detector of choice in our Laboratory. The alkaline digestion of protein products liberates more nitrogenous material, as previously indicated, that would contribute to the background of a sample analyzed by CECD.



(c) The Electron Capture Detector (ECD) has been used to determine the presence of nitrosamines after oxidation to nitramines by peroxytrifluoroacetic acid (Sen (42), Althorpe, et al. (55)). Detection of picogram quantities of nitrosamines were reported with this sensitive detector. Telling presented greater details of this procedure recently (56).

Nitrosamines also may be detected by ECD following reduction or cleavage to the corresponding amines with subsequent derivatization with heptafluorobutanoyl chloride. Alliston et al. (57) have developed a procedure for electrochemical reduction of nitrosamines in alkaline solution which they claim to be sensitive to 1 ug nitrosamine/kg.

### 2-Colorimetric

Although gas chromatographic procedures are highly suitable for the separation and quantitation of nitrosamines, studies are still being carried out by other procedures. The nitrosamines are amenable to separation and semi-quantitation by thin layer chromatography. A comprehensive review of this field has been published (Fishbein and Falk (58)). The nitrosamines are separated by a variety of solvents, then visualized by spraying with sulfanilic acid and  $\alpha$ -naphthylamine (or naphthyl-1-ethylenediamine) after irradiation by UV light, or by spraying with palladium chloride and diphenylamine prior to UV irradiation. As a check on the identity of the compound, both types of sprays could be used or ninhydrin may be used in conjunction with the sulfanilic acid: $\alpha$ -naphthylamine treatment (Sen (39)). Comparison of spot size and color intensity with standards of known concentrations will furnish an approximation of the nitrosamine concentration in the sample.

A colorimetric procedure that was of interest in determining total nitrosamines, described by Eisenbrand and Preussmann (59), utilized the formation of nitrosyl bromide following treatment of the nitrosamines with HBr in glacial acetic acid. The liberated  $\text{NO}^+$  - diazotized sulfanilic acid and the product was coupled with naphthyl-1-ethylenediamine. A logical extension of this procedure permits identification of the amines formed, and thus identification of the parent nitrosamines (Eisenbrand (60)). Derivatization with 1-dimethylaminonaphthalene-5-sulfonyl (DANSYL) chloride results in fluorescent amine derivatives that can be separated on thin layer plates, scraped off and quantitated by spectrophotofluorimetry. Alternatively, the amines can be derivatized with heptafluorobutyryl chloride and analyzed gas chromatographically using the EC detector. A number of laboratories are working with some form of this procedure but too few data have been reported to permit evaluation of the results.

### Confirmation

In spite of the apparent selectivity of the GC detectors described above, contaminants or artifacts have been observed in gas chromatographic determinations of nitrosamines. Retention values alone on a single column are insufficient for identification purposes. Reineccius and Coulter (61) reported a contaminant that co-chromatographed with, and behaved like, dimethylnitrosamine on three types of columns and required some ingenuity to separate. We have also observed peaks that coincided with known nitrosamines (Fig.2) (62) and similar reports have been published by others (31, 36, 37, 44).

In view of the serious public health implications of inaccurately reporting the presence of nitrosamines in foods, it is essential that scrupulous attention be paid to the identification of these compounds. At the present time it appears that retention times in gas chromatography,  $R_f$  values and color reactions in thin layer chromatography, and possibly the other techniques mentioned above, may be insufficient for positive identification of nitrosamines. It has been stated that confirmation of the molecular structure obtained from the mass spectral fragmentation pattern of the compound is the only positive identification of a nitrosamine known today. With an interfaced GC-mass spectrometer, material separated in the gas chromatograph is introduced directly into the mass spectrometer, permitting a peak to be analyzed several times very rapidly.

Telling et al. (44) proposed using the MS as a specific detector for  $\text{NO}^+$  which is present in the fragmentation pattern of all nitrosamines and compounds containing NO or  $\text{NO}_2$  groups. An MS resolution of 15,000 in the peak matching mode was sufficient to separate  $\text{NO}^+$  with an  $m/e$  29.99799 from the relatively few compounds with almost the same mass. Low resolution mass spectrometry, which permitted visualization of the entire fragmentation pattern was used to confirm compounds giving positive responses. The  $\text{NO}^+$  ion alone may not be satisfactory for identifying or confirming, a nitrosamine; so very little is present it may be difficult to detect at low concentrations of nitrosamines. It is more satisfactory to monitor the molecular ion of the particular nitrosamine under study (63).

Although the GC-MS system is acclaimed to be the most authoritative method at this time for confirming the identity of nitrosamines, several instances of potential interferences have been reported. Hydroxyacetone and DMNA elute from the GC at almost the same time under the conditions of chromatography used by Telling (44). In the presence of large amounts of hydroxyacetone, traces of DMNA were difficult to confirm by low resolution MS because the molecular weights and fragmentation patterns of these two compounds are similar. These authors recommended monitoring the molecular ion of a nitrosamine because "if a positive result is found it need not be confirmed by recording a low resolution mass spectrum as it is exceedingly unlikely that any other compound of the same retention time as the nitrosamine will contain a fragment ion in its mass spectrum which corresponds to the composition of the nitrosamine molecular ion." In our Laboratory, however, we have found that a silicon-containing compound with a GC retention time very close to that of DMNA formed the trimethylsilyl ion in the mass spectrometer with an  $m/e$  similar to that of DMNA (64). The  $m/e$  of the molecular ion for DMNA is 74.0480 while the  $m/e$  of the  $^{13}\text{C}$  isotope of the trimethylsilyl ion is 74.0502 and the  $m/e$  of the  $^{29}\text{Si}$  isotope is 74.0469. The range is on the order of 3 parts in 74000 which is too small to distinguish at a resolution of 1:12,000. The difficulty was overcome by observing the characteristic patterns on low resolution MS. The trimethylsilyl ion interference has also been noted by Gough (65) in the Laboratory of the Government Chemist. To overcome this problem it may be necessary to: 1) use GC column packings that separate the interfering compounds from the nitrosamines; 2) monitor additional peaks in the

MS to identify the compounds more precisely; 3) check all positive high resolution confirmation by low resolution mass spectrometry to observe the total fragmentation pattern.

While it is highly desirable that all nitrosamines found in foods should be confirmed by MS, not all laboratories have this capability. It is essential, therefore, that all positive reports should be based on as much evidence as possible, using two or three unrelated procedures to identify the nitrosamine in question. While this may require a considerable effort, particularly in surveys, every precaution should be taken to prevent false positive identifications.

In recent years, through the use of techniques as described above, nitrosamines have been identified in a number of food products, and confirmed by MS. These are indicated in Table 3. Several factors can be seen from this compilation. DMNA and NOPyr are the major, if not the only, nitrosamines identified to date. It is difficult to know whether these are, in fact, the only nitroso compounds formed in natural food products or whether others have not been reported because of inadequate analysis. The number of potential nitrosamines is large but only a very few are being investigated on a routine basis. It must also be remembered, as indicated previously, the major analytical emphasis has been on the few volatile nitrosamines; the non-volatile compounds still remain to be identified.

Another factor of interest appears from the reports of positive findings: the concentration of nitrosamines found in foods is very low. In contrast with earlier reports of nitrosamine concentrations in the mg/kg range, the largest amount of nitrosamine, confirmed, was approximately 100 ug/kg. For the most part, nitrosamine levels are on the order of 10-30 ug/kg, and a few investigators have reported less than 5 ug/kg, where they can confirm the identification. With improvement in methodology and accuracy in evaluation of results the determination of amount of known nitrosamines in food products becomes less of a problem, but more and more food items are found to contain low levels of these compounds.

Analytical capabilities are expanding and the isolation, identification, quantitation, and confirmation of nitroso compounds at the ng/kg level may be possible soon.

Table 1

Some Physiological Amines Nitrosatable in Model Systems

Dimethylamine	Carnitine	Sarcosine
Trimethylamine	Lecithin	Creatine
Choline	Spermine	Proline
Acetylcholine	Spermidine	Hydroxyproline
Putrescine	Arginine	Pyrrolidine
Ureine	Citrulline	Piperidine

Table 2

Some Unconfirmed Reports of Nitrosamines in Foods

<u>No. of Samples</u>		<u>No. Positive ug/kg</u>		<u>Ref.</u>
8	Cheese	1 (120)	DMNA*	20
30	Flour (heated)	22	DENA	23
2	Cheese	2		
1	Milk	1		
12	Fish	12 (0.5-4.0)	DMNA	
7	Cured meat	7 (0.6-6.5)		66
11	Mushrooms	11 (0.4-3.0)		
8	Alcoholic drinks	8 (2000)	DMNA	27
12	Apples, raw	(0-1.3)	DMNA	
11	Milk, raw	(1.3-2.7)		67
	Cantonese salt-dried fish-various varieties	600-9000 1200-21000	DMNA DENA	68

\*DMNA - N-Nitrosodimethylamine; DENA - N-Nitrosodiethylamine

Table 3

Nitrosamines in Food Confirmed by Mass Spectrometry

<u>No. of mples</u>	<u>Type</u>	<u>No. Positive (<math>\mu\text{g}/\text{kg}</math>)*</u>		<u>Ref.</u>
59	Bacon, corned beef, sausage, salami, etc.	4 (10-80)	DMNA**	18
24	Bacon - various types	11 (1-4)	DMNA;	
35	Fish	15 (1-9)	NOPyr	8
12	Cheese	6 (1-4)		
6	Salami	1 (1-4)		
25	Salmon, sable, shad	21 (4-26)	DMNA	16
7	Fish meal	6 (120-450)	DMNA	15
16	Bacon	1 (30)	DMNA; NOPyr	7
40	Frankfurters	3 (11-84)	DMNA	19
44	Alcoholic drink	0 (100-900 by GC alone)		37
8	Bacon	8 (10-108)	NOPyr	
31	Drippings Canadian bacon, ham	(45-207) 0		9
51	20 cold cuts, 7 sausage, 4 baby food, 5 canned meat, 9 bacon, ham, pork products, 6 misc. beef products	1 (5)	DMNA	17

\*Range of nitrosamine concentration in positive samples

\*DMNA-N-Nitrosodimethylamine; NOPyr - N - Nitrosopyrrolidine

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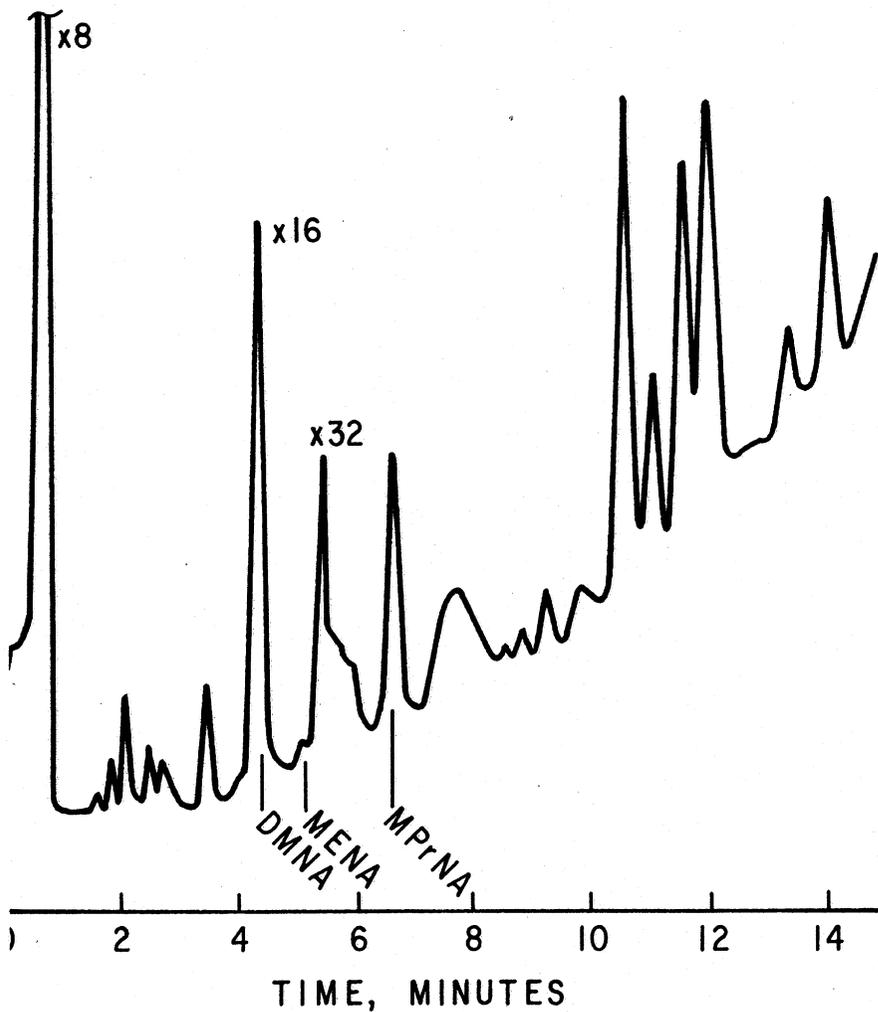


Figure 2 - Gas chromatograph of the extract of a bacterial culture medium. Arrows indicate retention times for the following nitrosamines: N-Nitroso-dimethylamine (DMNA), -methylethylamine (MENA), -methylpropylamine (MPrNA).

(c) The Electron Capture Detector (ECD) has been used to determine the presence of nitrosamines after oxidation to nitramines by peroxytrifluoroacetic acid (Sen (42), Althorpe, et al. (55)). Detection of picogram quantities of nitrosamines were reported with this sensitive detector. Telling presented greater details of this procedure recently (56).

Nitrosamines also may be detected by ECD following reduction or cleavage to the corresponding amines with subsequent derivatization with heptafluorobutanoyl chloride. Alliston et al. (57) have developed a procedure for electrochemical reduction of nitrosamines in alkaline solution which they claim to be sensitive to 1 ug nitrosamine/kg.

### 2-Colorimetric

Although gas chromatographic procedures are highly suitable for the separation and quantitation of nitrosamines, studies are still being carried out by other procedures. The nitrosamines are amenable to separation and semi-quantitation by thin layer chromatography. A comprehensive review of this field has been published (Fishbein and Falk (58)). The nitrosamines are separated by a variety of solvents, then visualized by spraying with sulfanilic acid and  $\alpha$ -naphthylamine (or naphthyl-1-ethylenediamine) after irradiation by UV light, or by spraying with palladium chloride and diphenylamine prior to UV irradiation. As a check on the identity of the compound, both types of sprays could be used or ninhydrin may be used in conjunction with the sulfanilic acid: $\alpha$ -naphthylamine treatment (Sen (39)). Comparison of spot size and color intensity with standards of known concentrations will furnish an approximation of the nitrosamine concentration in the sample.

A colorimetric procedure that was of interest in determining total nitrosamines, described by Eisenbrand and Preussmann (59), utilized the formation of nitrosyl bromide following treatment of the nitrosamines with HBr in glacial acetic acid. The liberated  $\text{NO}^+$  - diazotized sulfanilic acid and the product was coupled with naphthyl-1-ethylenediamine. A logical extension of this procedure permits identification of the amines formed, and thus identification of the parent nitrosamines (Eisenbrand (60)). Derivatization with 1-dimethylaminonaphthalene-5-sulfonyl (DANSYL) chloride results in fluorescent amine derivatives that can be separated on thin layer plates, scraped off and quantitated by spectrophotofluorimetry. Alternatively, the amines can be derivatized with heptafluorobutyryl chloride and analyzed gas chromatographically using the EC detector. A number of laboratories are working with some form of this procedure but too few data have been reported to permit evaluation of the results.

### Confirmation

In spite of the apparent selectivity of the GC detectors described above, contaminants or artifacts have been observed in gas chromatographic determinations of nitrosamines. Retention values alone on a single column are insufficient for identification purposes. Reineccius and Coulter (61) reported a contaminant that co-chromatographed with, and behaved like, dimethylnitrosamine on three types of columns and required some ingenuity to separate. We have also observed peaks that coincided with known nitrosamines (Fig.2) (62) and similar reports have been published by others (31, 36, 37, 44).

In view of the serious public health implications of inaccurately reporting the presence of nitrosamines in foods, it is essential that scrupulous attention be paid to the identification of these compounds. At the present time it appears that retention times in gas chromatography,  $R_f$  values and color reactions in thin layer chromatography, and possibly the other techniques mentioned above, may be insufficient for positive identification of nitrosamines. It has been stated that confirmation of the molecular structure obtained from the mass spectral fragmentation pattern of the compound is the only positive identification of a nitrosamine known today. With an interfaced GC-mass spectrometer, material separated in the gas chromatograph is introduced directly into the mass spectrometer, permitting a peak to be analyzed several times very rapidly.

Telling et al. (44) proposed using the MS as a specific detector for  $\text{NO}^+$  which is present in the fragmentation pattern of all nitrosamines and compounds containing NO or  $\text{NO}_2$  groups. An MS resolution of 15,000 in the peak matching mode was sufficient to separate  $\text{NO}^+$  with an  $m/e$  29.99799 from the relatively few compounds with almost the same mass. Low resolution mass spectrometry, which permitted visualization of the entire fragmentation pattern was used to confirm compounds giving positive responses. The  $\text{NO}^+$  ion alone may not be satisfactory for identifying or confirming, a nitrosamine; so very little is present it may be difficult to detect at low concentrations of nitrosamines. It is more satisfactory to monitor the molecular ion of the particular nitrosamine under study (63).

Although the GC-MS system is acclaimed to be the most authoritative method at this time for confirming the identity of nitrosamines, several instances of potential interferences have been reported. Hydroxyacetone and DMNA elute from the GC at almost the same time under the conditions of chromatography used by Telling (44). In the presence of large amounts of hydroxyacetone, traces of DMNA were difficult to confirm by low resolution MS because the molecular weights and fragmentation patterns of these two compounds are similar. These authors recommended monitoring the molecular ion of a nitrosamine because "if a positive result is found it need not be confirmed by recording a low resolution mass spectrum as it is exceedingly unlikely that any other compound of the same retention time as the nitrosamine will contain a fragment ion in its mass spectrum which corresponds to the composition of the nitrosamine molecular ion." In our Laboratory, however, we have found that a silicon-containing compound with a GC retention time very close to that of DMNA formed the trimethylsilyl ion in the mass spectrometer with an  $m/e$  similar to that of DMNA (64). The  $m/e$  of the molecular ion for DMNA is 74.0480 while the  $m/e$  of the  $^{13}\text{C}$  isotope of the trimethylsilyl ion is 74.0502 and the  $m/e$  of the  $^{29}\text{Si}$  isotope is 74.0469. The range is on the order of 3 parts in 74000 which is too small to distinguish at a resolution of 1:12,000. The difficulty was overcome by observing the characteristic patterns on low resolution MS. The trimethylsilyl ion interference has also been noted by Gough (65) in the Laboratory of the Government Chemist. To overcome this problem it may be necessary to: 1) use GC column packings that separate the interfering compounds from the nitrosamines; 2) monitor additional peaks in the

MS to identify the compounds more precisely; 3) check all positive high resolution confirmation by low resolution mass spectrometry to observe the total fragmentation pattern.

While it is highly desirable that all nitrosamines found in foods should be confirmed by MS, not all laboratories have this capability. It is essential, therefore, that all positive reports should be based on as much evidence as possible, using two or three unrelated procedures to identify the nitrosamine in question. While this may require a considerable effort, particularly in surveys, every precaution should be taken to prevent false positive identifications.

In recent years, through the use of techniques as described above, nitrosamines have been identified in a number of food products, and confirmed by MS. These are indicated in Table 3. Several factors can be seen from this compilation. DMNA and NOPyr are the major, if not the only, nitrosamines identified to date. It is difficult to know whether these are, in fact, the only nitroso compounds formed in natural food products or whether others have not been reported because of inadequate analysis. The number of potential nitrosamines is large but only a very few are being investigated on a routine basis. It must also be remembered, as indicated previously, the major analytical emphasis has been on the few volatile nitrosamines; the non-volatile compounds still remain to be identified.

Another factor of interest appears from the reports of positive findings: the concentration of nitrosamines found in foods is very low. In contrast with earlier reports of nitrosamine concentrations in the mg/kg range, the largest amount of nitrosamine, confirmed, was approximately 100 ug/kg. For the most part, nitrosamine levels are on the order of 10-30 ug/kg, and a few investigators have reported less than 5 ug/kg, where they can confirm the identification. With improvement in methodology and accuracy in evaluation of results the determination of amount of known nitrosamines in food products becomes less of a problem, but more and more food items are found to contain low levels of these compounds.

Analytical capabilities are expanding and the isolation, identification, quantitation, and confirmation of nitroso compounds at the ng/kg level may be possible soon.

Table 1

Some Physiological Amines Nitrosatable in Model Systems

Dimethylamine	Carnitine	Sarcosine
Trimethylamine	Lecithin	Creatine
Choline	Spermine	Proline
Acetylcholine	Spermidine	Hydroxyproline
Putrescine	Arginine	Pyrrolidine
Ureine	Citrulline	Piperidine

Table 2

Some Unconfirmed Reports of Nitrosamines in Foods

<u>No. of Samples</u>		<u>No. Positive ug/kg</u>		<u>Ref.</u>
8	Cheese	1 (120)	DMNA*	20
30	Flour (heated)	22	DENA	23
2	Cheese	2		
1	Milk	1		
12	Fish	12 (0.5-4.0)	DMNA	
7	Cured meat	7 (0.6-6.5)		66
11	Mushrooms	11 (0.4-3.0)		
8	Alcoholic drinks	8 (2000)	DMNA	27
12	Apples, raw	(0-1.3)	DMNA	
11	Milk, raw	(1.3-2.7)		67
	Cantonese salt-dried fish-various varieties	600-9000 1200-21000	DMNA DENA	68

\*DMNA - N-Nitrosodimethylamine; DENA - N-Nitrosodiethylamine

Table 3

Nitrosamines in Food Confirmed by Mass Spectrometry

<u>No. of samples</u>	<u>Type</u>	<u>No. Positive</u> <u>(µg/kg)*</u>		<u>Ref.</u>
59	Bacon, corned beef, sausage, salami, etc.	4 (10-80)	DMNA**	18
24	Bacon - various types	11 (1-4)	DMNA;	
35	Fish	15 (1-9)	NOPyr	8
12	Cheese	6 (1-4)		
6	Salami	1 (1-4)		
25	Salmon, sable, shad	21 (4-26)	DMNA	16
7	Fish meal	6 (120-450)	DMNA	15
16	Bacon	1 (30)	DMNA; NOPyr	7
40	Frankfurters	3 (11-84)	DMNA	19
44	Alcoholic drink	0 (100-900 by GC alone)		37
8	Bacon	8 (10-108)	NOPyr	
31	Drippings Canadian bacon, ham	(45-207) 0		9
51	20 cold cuts, 7 sausage, 4 baby food, 5 canned meat, 9 bacon, ham, pork products, 6 misc. beef products	1 (5)	DMNA	17

\*Range of nitrosamine concentration in positive samples

\*DMNA-N-Nitrosodimethylamine; NOPyr - N - Nitrosopyrrolidine

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