AN ABSTRACT OF THE THESIS OF

Timothy McIntyre for the degree of Master of Science

in Food Science and Technology presented on December 15, 1986

Title: Food as a Source of Nitrosatable Amines

Abstract Approved:

R.A. Scanlan

Beer, nonfat dry milk, fried bacon and microwave baked fish were treated under simulated gastric and extreme nitrosation conditions to estimate their capacity for generation of endogenously formed N-nitrosamines. The level of nitrosamines in beer increased from 0.1 ppb N-nitrosodimethylamine to 0.4 ppb N-nitrosodimethylamine ($p < 0.05$) under simulated gastric nitrosation. The levels in the other food products were not increased under the same simulated gastric conditions ($p < 0.05$). Extreme nitrosation illustrated that food has a large nitrosatable amine content. Beer produced elevated levels of N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosopyrrolidine and N-nitrosomorpholine. Nonfat dry milk produced elevated levels of N-nitrosodimethylamine, N-nitrosopiperidine, N-nitrosopyrrolidine and N-nitrosomorpholine. Fish produced elevated levels of N-nitrosodimethylamine and N-nitrosopyrrolidine. Overall, certain food products provide exposure to ample amounts of nitrosatable amines, but it appears that these food products do not produce a significantly increased risk from endogenous N-nitrosamine exposure.

FOOD AS A SOURCE OF NITROSATABLE AMINES

by

Timothy McIntyre

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the
degree of
Master of Science

Completed December 15, 1986

Commencement June 1987

APPROVED:

Professor of Food Science and Technology in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented December 15, 1986

Typed by the author Timothy McIntyre

ACKNOWLEDGMENTS

The author wishes to thank Dr. Richard A. Scanlan, his major advisor, for providing this opportunity and for the direction of this research. Sincere appreciation also goes to Dr. Leonard M. Libbey for his assistance in obtaining mass spectral data and in editing this thesis and to Mr. James F. Barbour for his assistance with the Thermal Energy Analyzer.

This investigation was supported in part by grant No. CA25002, awarded by the National Cancer Institute, Dept. of Health and Human Services and by grant No. 82-CRSR-2-2034, awarded by the U.S. Department of Agriculture. The Thermal Energy Analyzer is on loan from the National Cancer Institute, DHHS under contract No. N01-CP-85610.

TABLE OF CONTENTS

Page

I. INTRODUCTION 1

II. LITERATURE REVIEW 4

A. Background on Nitrosamines 4
1: Toxicity 4
2: Epidemiology 5
3: The Nitrosation Reaction 6
4: Catalysis and Inhibition 8
B. Routes of Exposure 11
1: Exogenous 11
2: Endogenous 13

III. EXPERIMENTAL 24

A. Materials 24
B. Methods 24
1: Simulated Gastric Nitrosation 24
a: Beer 24
b: Nonfat Dry Milk 25
c: Bacon 25
d: Baked Fish 26
2: Extreme Nitrosation 26
a: Beer 26
b: Nonfat Dry Milk or Baked Fish 27
3: Extraction 27
a: Beer 27
b: Nonfat Dry Milk 28
c: Bacon or Baked Fish 29
4: Quantitation 30

IV. RESULTS 32
A. Simulated Gastric Nitrosation 32
B. Extreme Nitrosation 34

V. DISCUSSION 61
A. Simulated Gastric Nitrosation 61
B. Extreme Nitrosation 67

VI. SUMMARY 71

VII. BIBLIOGRAPHY 72

VIII. APPENDIX 81

LIST OF FIGURES

Figure		Page
I.	Gas Chromatography Trace of Beer After Simulated Gastric Nitrosation	36
II.	Gas Chromatography Trace of NDM After Simulated Gastric Nitrosation	39
III.	Gas Chromatography Trace of Bacon After Simulated Gastric Nitrosation	41
IV.	Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NMDA from extreme nitrosated beer and (Bottom) standard NDMA solution.	45
V.	Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NDEA from extreme nitrosated beer and (Bottom) standard NDEA solution.	47
VI.	Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NPYR from extreme nitrosated beer and (Bottom) standard NPYR solution.	49
VII.	Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NMOR from extreme nitrosated beer and (Bottom) standard NMOR solution.	51
VIII.	Gas Chromatography Trace of Beer After Extreme Nitrosation	54
IX.	Gas Chromatography Trace of NDM After Extreme Nitrosation	57

LIST OF TABLES

			Page
Table	I.	Foods reported to contain N-Nitrosamines.	10
Table	II.	Foods tested under *in-vitro* conditions for N-Nitrosamine formation	21
Table	III.	Simulated Gastric Nitrosation: Domestic Beer Survey.	35
Table	IV.	Simulated Gastric Nitrosation: Non-fat Dry Milk Survey	38
Table	V.	Simulated Gastric Nitrosation: Fried Bacon Survey.	38
Table	VI.	Simulated Gastric Nitrosation of Fish	43
Table	VII.	Extreme Nitrosation: Domestic Beer Survey.	53
Table	VIII.	Extreme Nitrosation: Non-fat Dry Milk Survey . . .	56
Table	IX.	Extreme Nitrosation: Fish Survey	59

FOOD AS A SOURCE OF NITROSATABLE AMINES

I. INTRODUCTION

Over the past 15 years considerable attention has been given to the relationship between diet and cancer. Current research suggests that the diet possesses some role in human cancer, but the effects of specific dietary components are still mostly unknown. Clearly, more research is necessary to elucidate the effects of these dietary components. One class of carcinogens known to be a component in foods, the N-nitrosamines, have received a great deal of research in the attempt to analyze this relationship.

N-Nitrosamines can be divided into two groups; volatile and non-volatile. Much less is known about the latter group due to analytical difficulties. Volatile N-nitrosamines on the other hand, have been found in several foods (Scanlan, 1983). As a result of these findings, attempts have been made to determine sources of nitrosamine exposure, the amount of total nitrosamine exposure and methods of reducing this exposure from food. It stands to reason that further study of nitrosamine exposure and its relation to the overall burden to the human body can aid in determining the role of diet in cancer.

There are two routes of exposure to nitrosamines, exogenous and endogenous. Exogenous exposure is exposure to nitrosamines formed

Abbreviations: NDEA, N-Nitrosodiethylamine; NDMA, N-Nitrosodimethylamine; NDPA, N-Nitrosodipropylamine; NMOR, N-Nitrosomorpholine; NPIP, N-Nitrosopiperidine; NPRO, N-Nitrosoproline; NPYR, N-Nitrosopyrrolidine; NTHZ, N-Nitrosothiazolidine; NTPRO, N-Nitrosothiazolidine-4-carboxylic acid (Nitrosothioproline) and NMTPRO, N-Nitrosomethylthiazolidine-4-carboxylic acid (Nitrosomethylthioproline).

outside the body, while endogenous exposure is exposure to nitrosamines formed within the body. Exogenous exposure has been well documented and decreasing trends of exposure are evident. Particular examples are beer (Mangino and Scanlan, 1982) and fried bacon (Havery and Fazio, 1985). Endogenous exposure, however, has more recently come under intense investigation.

The concept of endogenous nitrosation has been considered for over 20 years. Some of the early evidence supporting this type of nitrosation was the induction of tumors in laboratory animals after the feeding of both nitrite and a secondary amine (Sander and Burkle, 1969, Lijinsky, et al., 1972 and Greenblatt, et al., 1971). One of the first articles directly reporting endogenous formation of N-nitrosamines in humans was provided by Sander and Seif (1969). The authors reported measuring N-nitrosodiphenylamine in humans after feeding nitrate and diphenylamine. Other factors supporting endogenous nitrosation in humans are the widespread distribution of reactive amines in food, the acidic pH of the stomach, the presence of nitrite in the saliva, the presence of nitrosation catalysts in the gastric system and certain unusual stomach conditions that are thought to place some humans at an increased risk to endogenously formed nitrosamines. A number of recent reports positively indicate that endogenous nitrosation does occur in humans, but the extent of this type of nitrosation still needs to be determined (Ohshima and Bartsch, 1981, Wagner and Tannenbaum, 1985 and Tannenbaum, 1986).

The relevance of nitrosatable amines to endogenous N-nitrosamine formation is clear. The relevance of the same amines to cancer has

yet to be determined. The International Agency for Research on Cancer (IARC) has therefore urged further studies on endogenous nitrosation of amines which occur in foods. Previous work in this area has utilized realistic levels of reactants under *in vitro* conditions. The results concerning endogenous nitrosamine formation from those studies were mainly negative (Walter, et al., 1976, Groenen, et al., 1982 and Sen, et al., 1985). The fact that unusual circumstances might lead to increased levels of nitrite in the body exemplifies the need for experiments submitting the nitrosatable amines in foods to these higher levels.

The objective of this study was to explore the possibility of endogenous N-nitrosamine formation through the nitrosation of foods under simulated gastric and extreme nitrosation conditions.

II. LITERATURE REVIEW

Nitrosamines are a diverse class of carcinogens. Concern over the presence of carcinogens in food was voiced due to the nature of the disease and resulted in the addition of the so-called Delaney Clause to the Food Additive Amendment of 1958. This legislation was concerned with the addition of carcinogenic materials to foods. In order to assess exposure to nitrosamines already present in foods, one must possess an understanding of nitrosation reactions. This review will discuss nitrosation reactions with emphasis being placed on the formation of N-nitrosamines in food and their possible formation endogenously from precursor amines in food.

A. Background on Nitrosamines

This background section will cover toxicity, epidemiology and the nitrosation reaction in general.

1. Toxicity: N-Nitroso compounds illustrate multiple toxic effects, with perhaps the most important of these being their carcinogenicity. N-Nitroso compounds (N-nitrosamines, N-nitrosamides and others) have been extensively tested for their carcinogenic activity starting with NDMA by Magee and Barnes in 1956. At the present time, over 300 N-nitroso compounds have now been tested for carcinogenicity by many researchers (Scanlan and Reyes, 1985). Approximately 90% of the compounds tested have been shown to be carcinogens in animals. Additionally, N-nitroso compounds have been shown to be carcinogenic in 40 species of animals and no species is

known to be resistant to these compounds (Bartsch and Montesano, 1984). The N-nitroso compounds have proven to be organ specific, and many require activation to an intermediate alkylating species. Their specific site of action will reflect the structure of the N-nitroso compound, dose of compound and length of exposure. At this time no direct evidence has implicated N-nitroso compounds as carcinogens in humans, even though the indirect evidence is overwhelmingly positive.

2. Epidemiology: Epidemiological studies to date have been inconclusive in determining the role of N-nitroso compounds in human cancer (Preussmann, 1984). Possible reasons for this are the low levels of exposure (ppb), presence of other carcinogens in the environment and an uncertainty in how to measure total human exposure to nitrosamines (Miller, et al., 1984). A typical epidemiological procedure for the diet/cancer hypothesis is to attempt to correlate nitrate and nitrite content of water, or the consumption of a certain food, with the incidence of a certain cancer. This procedure has been followed since an increased level of nitrate or nitrite in the body has been implicated as the most important nitrosating source in the nitrosamine portion of the diet/cancer hypothesis. There seems to be a slightly positive correlation between exogenous exposure to N-nitrosamines and cancer in humans (Miller, et al., 1984). When endogenous nitrosation is correlated with the incidence of cancer the results are even less conclusive. Recent work on tobacco products, snuff in particular, may lead towards a more positive correlation of exogenous nitrosamine exposure, since the only known carcinogens typically present are the tobacco specific N-nitrosamines (Hoffmann,

et al., 1984). Finally, new epidemiological models, termed the molecular epidemiological approach, include laboratory data in the analysis, and perhaps can better estimate total nitrosamine exposure (Choi, 1985).

3. The Nitrosation Reaction: The structure of nitrosamines depends primarily on the precursor amines. The amines can vary from simple aliphatic amines, to amino acids and complex ring structures. The common structural characteristic among the N-nitrosamines is the N-N=O moiety. This characteristic moiety is responsible for the detection and quantification of nitrosamines by the Thermal Energy Analyzer (TEA).

The formation of nitrosamines have been studied both chemically and kinetically. The reaction occurs between secondary and/or tertiary amines and a nitrosating species. Primary amines form unstable structures which can breakdown into N_2 and various deaminated products. The reaction rate usually proceeds according to the equation:

$$\text{rate} = k[\text{amine}][\text{nitrite}]^2$$

The rate is pH dependent, and the optimum range for nitrosation of most secondary amines is in the pH range of 2-4 (Mirvish, 1975). Since the unprotonated form of the secondary amine participates in the reaction, an inverse relationship occurs between amine basicity and reactivity. This results in a pattern of amines with decreasing basicity (lower pK_a) having an increased rate of nitrosation (Scanlan, 1983).

The second important nitrosation reactant is the nitrosating species. Different forms exist including nitrous anhydride (N_2O_3), dinitrogen tetroxide (N_2O_4), nitrosyl halides and even other nitroso compounds. Perhaps the most common nitrosating agent, especially in food, is N_2O_3. It is formed from two molecules of nitrous acid, HNO_2 (Mirvish, 1975). HNO_2 is formed from nitrite under acidic conditions and does not normally react directly with an amine. Nitrite is very reactive at low pH, higher temperatures and elevated concentrations. Under these conditions, it will persist in solution only a short time. Other nitrosating species derived from HNO_2 are the nitrosyl halides. These will be discussed in more detail under catalysis.

Nitrogen oxides are a nitrosating species that can be formed naturally by combustion and also from chemical or microbial reduction of nitrate and nitrite salts (Committee on Nitrite and Alternative Curing Agents in Food, 1981). Nitrogen oxides do not require acidic conditions to react with amines, and in fact, they have been shown to react under alkaline conditions (Challis, et al., 1978). The nitrosating species formed under these conditions are N_2O_3 and N_2O_4. These species can exist in gases and solvents, and are especially important nitrosating agents in drying processes using combustion gases (Scanlan, 1983). Although existing as the same empirical species as those mentioned for acidic conditions, they tend to react faster under gaseous conditions.

A final nitrosation reaction that could be of significance, particularly with foods, is transnitrosation. This is a reaction in which a nitroso compound transfers the nitroso moiety to another amine precursor (Singer, et al., 1978). It can be important since non-carcinogenic nitroso compounds, such as NPRO, can transnitrosate to lead to formation of carcinogens. The reaction proceeds in dilute acid, and can be catalyzed by thiocyanate or iodide (Singer, et al., 1978). The catalysis is similar to that of acid catalyzed nitrosation (Singer, et al., 1980). Experimental evidence for transnitrosation in food, however, is sparse.

4. Catalysis and Inhibition: This subject has been covered in detail elsewhere (Archer, 1984 and Mirvish, 1975). What will be discussed here is a summary of information important to endogenous nitrosation. Compounds that can increase or catalyze nitrosation tend to be anionic or nucleophilic in nature. Of significance to man would be ions such as thiocyanate, iodide and chloride since they are naturally found in the body. These ions form nitrosyl derivatives in the presence of nitrous acid, for example nitrosyl chloride is NOCl. Their order of activity is: $I^- > SCN^- > Br^- > Cl^-$ (Ridd, 1961).

The thiocyanate ion is important endogenously since it is known to be present in saliva, and smokers tend to possess higher levels. The nitrosyl derivatives in general catalyze reaction rates according to the equation:

$$\text{rate} = k[\text{amine}][\text{nitrite}][X^-].$$

Note that the dependency on nitrite is no longer squared. The thiocyanate nitrosyl reactant will be favored over N_2O_3 nitrosation when the pH is less than 3.0, and also when nitrite concentration is very low (Fan and Tannenbaum, 1973). Both of these situations are common occurrences in the human stomach.

Inhibition of nitrosation in food, or the human body, is most typically accomplished by binding or reducing the nitrosating species to an inactive form. The most effective scavengers in foods are ascorbic acid and α-tocopherol (Scanlan, 1983).

Ascorbic acid and α-tocopherol are both naturally occurring vitamins, and their addition or presence in food is commonplace. Their actions are similar in that they reduce the nitrosating species to nitric oxide (NO), a non-reactive species. Their use can cover both aqueous (ascorbate) and lipid (tocopherol) systems.

The effective use of ascorbic acid and α-tocopherol in food and animal studies for blocking nitrosation has already been documented. For example, addition of ascorbate to cured meats high in nitrite has inhibited NDMA formation (Fiddler, et al., 1973). Cardesa, et al., (1974) also found that gavaging rats with dimethylamine and nitrite plus ascorbate formed less NDMA that a gavage without ascorbate. At the present time, bacon is required to be cured with ascorbate while use of α-tocopherol is optional in the United States.

Other compounds have also been found to inhibit nitrosamine formation. For example, sulfur dioxide (SO_2) is used during the drying of malt (O'Brien, et al., 1980). 1,4- and 1,2-

Table I. Foods Reported to Contain N-Nitrosamines

Food Product	N-Nitrosamine
Fried Bacon	NDMA, NDEA, NPYR, NPIP, NTHZ
Nonfat Dry Milk	NDMA, NPIP, NPYR
Fish	NDMA, NPYR
Beer (domestic and imported)	NDMA, NDEA
Cheese	NDMA
Dried Buttermilk	NDMA
Other Cured Meats	NDMA, NTHZ, NPYR, NPIP, NDEA
Soy Protein Foods	NDMA, NMOR
Scotch Whisky	NDMA
Edible Oils	NMOR

From: Havery and Fazio, 1985; Scanlan, 1983 and Committee on Nitrite and Alternative Curing Agents in Food, 1981.

Dihydroxyphenols have been shown to inhibit nitrosamine formation by a mechanism similar to that shown for ascorbic acid (Pignatelli, et al., 1980). The same authors point out, however, that 1,3-dihydroxyphenols possess a catalytic effect. Tannic and gallic acids are multi-hydroxy phenolic compounds found in teas and fruits. These acids have been shown to destroy nitrite (Bogovski, et al., 1972). These compounds are important since through natural occurrence, or by the addition of these naturally occurring compounds, nitrosamine formation could be inhibited. They may also be of importance during processing or handling of food products, and perhaps more importantly, after eating when endogenous formation of N-nitrosamines could occur.

B. Routes of Exposure

1. Exogenous Exposure: For some time food has been known to contain N-nitrosamines. A non-exhaustive list of food products reported to contain N-nitrosamines has been compiled in table I. These products obviously contain certain levels of nitrosatable amines, and their ingestion could lead to further nitrosamine exposure via endogenous nitrosation. Not mentioned in the table, but of importance to certain foods, is the case of baby bottle rubber nipples.

Rubber nipples were recently found to contain N-nitrosamines (Ireland, et al., 1980). The level of nitrosamines found was shown to increase after heating the nipples. This was due to the presence of both the precursor amines and the nitrosating species in the rubber (Havery and Fazio, 1985). This was significant since the

authors also illustrated N-nitrosamine migration into the contents of the bottle, especially when the nipples and the contents were heated together. The rubber industry has since worked on reducing the total volatile N-nitrosamine level from the rubber material. Sen, et al., (1985), has reported the levels to be decreasing and that various liquid infant juices, such as orange and apple juices, inhibited the formation of N-nitrosamines from migrated reactants under *in vitro* conditions.

Other sources of N-nitrosamine exposure do exist. The most notable is from tobacco products. The Committee on Nitrite and Alternative Curing Agents in Food (1981) estimates average daily intake of N-nitrosamines from tobacco products to be about 17 times higher than the highest single food source. Cosmetics (Fan, et al., 1977), pesticides (Bontoyan, et al., 1979), drugs (Eisenbrand, et al., 1979), water (Fiddler, et al., 1977) and paper products (Hotchkiss and Vecchio, 1983) have also been found to contain N-nitrosamines. These non-food sources are important since they could introduce the N-nitrosamines and also the precursor amines into food or the body. A good example is the presence of NMOR in margarines. Sen and Baddoo (1986) have recently reported that certain packaging materials made of wax paper are responsible for NMOR migrating into the margarine.

When discussing nitrosamine exposure it is important to state the level of exposure, since it is a major factor in carcinogenesis. Most of the products discussed here will produce an average daily exposure to humans of less than 1 μg (Committee on Nitrite and Alternative

Curing Agents in Food, 1981). It is important to view these estimates as average daily exposures, since certain populations may have more or less than the amount indicated (Scanlan, 1983). It is this low level of human exposure to N-nitrosamines that contributes to the controversy of whether a nitrosamine hazard exists from food sources.

2. Endogenous Exposure: After the hypothesis of endogenous N-nitrosamine formation was first put forth, feeding trials and other studies were incorporated into laboratory animal testing. With some of the results reflecting endogenous nitrosamine formation (Sander and Burkle, 1969 and Lijinsky, et al., 1972), researchers sought methods of extending these tests to the human body. The researchers wanted to determine where nitrosation occurred, develop techniques to measure nitrosation, identify populations particularly susceptible to endogenous nitrosation, and devise methods of preventing further nitrosamine exposure. One of the first areas studied concerned the sites of nitrosation and the conditions promoting nitrosation at these sites.

At first, nitrosation was thought to occur in three main areas of the body. These were the stomach, large intestine and areas of microbial infection (Mirvish, 1975). Of major concern to this study is the stomach, therefore a brief discussion of its physiology is included. The other two areas of nitrosation involve mainly the microbial action of nitrate reduction or enzyme catalyzed nitrosamine formation. As will be mentioned later, another endogenous nitrosation source has been identified.

Gastric physiology can affect possible nitrosation reactions. The physiology determines pH, enzyme action and other secretions. Possibly the most important secretion from the stomach lining in terms of nitrosation comes from the parietal cells.

Parietal cells secrete hydrochloric acid for digestion. The pH of normal gastric juice is about 1-2 (Lane and Bailey, 1973 and Malagelada, et al. 1977). This pH is known to vary after ingesting a meal, and then return to initial levels within two to three hours, as was shown by Malagelada, et al. (1977). This is of importance to food nitrosation, since the optimum for dimethylamine nitrosation in gastric juice has been reported to be at a pH of 2.5 (Lane and Bailey, 1973), and the range of optimum nitrosation for most amines could be obtained during this pH increase.

Other secretions from the stomach lining include pepsinogen, mucous, hormones and salts. Pepsinogen is converted to pepsin, a proteolytic enzyme, by acid and by autocatalytic cleavage. Pepsin activity cleaves proteins mainly at tyrosine, tryptophan and phenylalanine residues and to a lesser extent at leucine, glutamic acid and glutamine residues (Lehninger, 1982). Pepsin may thus play a minor role in determining which amines or amino acids are available for nitrosation.

Of additional importance is the possibility of dimethylamine secretion into the stomach. Dogs and ferrets have been reported to efficiently transport dimethylamine from the blood into the stomach (Zeisel, et al., 1986). This is significant since these animals have stomachs very similar to humans. Although this transport activity

has not yet been illustrated in humans, it does bring to light the importance of the secretory action of the stomach.

Saliva is secreted into the oral cavity and enters the stomach via the esophagus. The main contribution of saliva to possible nitrosation reactions in the stomach involves some of the anions discussed previously. It can provide a fairly continuous supply of the important anionic constituents nitrate, nitrite and thiocyanate.

Nitrate is a ubiquitous ion found in soil and many foods. It was found to range from a non-detectable level up to 3800 ppm in vegetables (Spiegelhalder, et al., 1976). The nitrate content of saliva is due mainly to the diet, and has a direct effect on the nitrite content in the saliva.

Nitrite is not transported into the saliva like nitrate, but is produced by nitrate reduction in the oral cavity, and such a reduction is most likely brought about by bacteria (Tannenbaum, et al., 1974). The nitrite content of saliva is dependant on the quantity of the precursor nitrate, concentration of the nitrate source and the specific oral microflora (Tannenbaum, et al., 1976). The salivary nitrite concentration can usually be found in the range of 0.1 to 200 ppm. It has been reported that the average increase in the level of salivary nitrite was 20 ppm per 100 mg nitrate ingested (Spiegelhalder, et al., 1976). The nitrite level in the normal fasting stomach can be similarly affected. The level has been shown to range from less than 0.1 ppm to 1.4 ppm (Mueller, et al., 1986 and Ruddell, et al., 1976). White (1975) further supports saliva as the main source of nitrite by suggesting that over two-thirds of the

nitrite entering the stomach originates from saliva, and less than one-third from cured meats.

Another important anion found in saliva is thiocyanate. It has already been pointed out that thiocyanate catalyzes nitrosation reactions. Ruddell, et al., (1976) found average thiocyanate levels in gastric contents to be about 90 ppm in a group of both smokers and non-smokers. Other studies show average levels of thiocyanate from smokers and non-smokers to be about 55 ppm, of which the smokers separately averaged about 90 ppm (Walters, et al., 1976).

The conditions stated above appear to make the stomach the most likely site of endogenous nitrosation. Wagner, et al. (1985), however, in determining endogenous formation in the stomach were not able to account for all of the nitrosamines that were excreted in the urine. It was suggested that another site of nitrosation in addition to the stomach was contributing to nitrosamine formation.

Other mechanisms for nitrosation are known to involve bacterial action, enzyme catalyzed reactions, and more recently, macrophages. Bacteria are known to reduce nitrate to nitrite, especially in saliva. This action can continue in the stomach until the pH inactivates the bacteria. It has been suggested that certain bacteria can also enzymatically catalyze nitrosation (Hawksworth and Hill, 1971 and Mills and Alexander, 1976). Macrophages have been shown to be capable of forming N-nitrosamines under physiological conditions previously thought to inhibit nitrosamine formation (Miwa, et al., 1986).

Macrophages are the end products in the mononuclear phagocyte system that is derived from bone marrow. Macrophages exist in various organs and tissues, and play a role in the immune response by actively killing bacteria, fungi and tumor cells (Douglas, 1980). The macrophages are a known source of cell mediated nitrate/nitrite biosynthesis. The macrophages have also formed N-nitrosamines in the cell culture if properly stimulated.

The above mentioned sites and mechanisms are strongly suggestive of endogenous nitrosation in humans. Further research on endogenous nitrosation has attempted to measure nitrosamines in the body, or estimate their formation under *in vitro* conditions. Initially, biological fluids were measured for nitrosamine content. Human blood, feces and urine were found to contain nitrosamines (Fine, et al., 1977, Wang, et al., 1978 and Lakritz, et al., 1980). The analytical methods employed were open to artifact formation, so the studies cannot be considered as conclusive evidence that endogenous formation occurred (Eisenbrand, et al., 1981). In 1981, a new method for demonstrating endogenous nitrosation was developed using the amino acid proline. This method, referred to as the NPRO test, did not have the difficulties mentioned above.

The NPRO test consisted of feeding a nitrate source and proline, and then measuring the NPRO content excreted in the urine (Oshima and Bartsch, 1981). The basis for this technique is that nitrate is transported to the saliva and reduced to nitrite. Proline is consumed a short time after the nitrate and reacts with the nitrite in the stomach. The resulting NPRO is largely unmetabolized and is

non-carcinogenic in rats and other species, (Mirvish, et al., 1980). The reaction was of no harm to the human subjects and was strictly an endogenous process. Further use of this technique found two levels of NPRO excreted. Subjects fed a diet low in nitrate excreted a basal level of NPRO. When fed the nitrate source and proline, the NPRO excretion was significantly increased (Wagner and Tannenbaum, 1985). The source of the basal NPRO excretion has not been identified. Tannenbaum (1986) suggested that the basal level came from either cell-mediated synthesis or nitrogen oxide mediated synthesis. Results using this technique indicated that most humans excrete NPRO in their urine, and that the NPRO came from endogenous nitrosation (Tannenbaum, 1986).

Assuming that a majority of the endogenous nitrosation of dietary constituents occurs in the stomach, the NPRO test can be used to determine whether or not inhibitors or catalysts present in the diet have any effect on endogenous nitrosation. In the NPRO study by Wagner, et al. (1985), ascorbic acid and α-tocopherol were found to reduce endogenous NPRO formation. In the same study, the authors used ^{15}N-labeling to show NPRO excretion without dietary precursors. Ascorbic acid and α-tocopherol had no effect on this basal level of NPRO, thus positively indicating a non-gastric source of endogenous nitrosamine exposure. Other naturally occurring compounds that are known to be nitrosation inhibitors have been tested using the NPRO technique.

Stich, et al. (1984) showed that various polyphenolics inhibited NPRO formation from dietary sources. These authors found a 50-80% reduction in excreted NPRO after consumption of tea and coffee, which contain some of these polyphenolics. Chinese wild plum juice contains a newly discovered nitrite scavenger (Normington, et al., 1986). This action from the nitrite scavenger was overshadowed by high ascorbic acid levels, but still serves to point out that certain food products may contain unknown naturally occurring nitrosation inhibitors.

Finally, the NPRO test has been used to evaluate the extent of *in vivo* nitrosation from dietary constituents. Wagner and Tannenbaum (1985) could not find an increase above basal NPRO excretion after feeding a cured meat and a nitrate source. This suggests no additional N-nitrosamine exposure from the consumption of cured meats, which were previously thought to increase the exposure risk due to the addition of nitrite during the meat curing process.

A major problem in measuring most endogenously formed nitrosamines is they are metabolized at very high rates (Bartsch and Montesano, 1984). It has been shown that NDMA metabolism in the rat can be partially blocked with ethanol. This allows excretion of NDMA in the urine (Swann, et al., 1984). Using this "ethanol effect", Spiegelhalder and Preussmann (1985) fed a readily nitrosatable drug (aminopyrine), a nitrate source and ethanol. They reported a recovery of about 1-2% of the NDMA that could theoretically be formed. Further use of the "ethanol effect" does not appear justified in light of the low yields and conflicting reports on its

reliability.

Other N-nitrosamines have also been detected in the urine. Tsuda, et al. (1986) have directly measured the nitrosamines NTPRO and NMTPRO. These authors showed a 2-fold increase for these two nitrosamines due to cigarette smoking. Other methods used have been indirect, and rely on measuring a metabolic product of the N-nitrosamine. Such is the case with NMOR. Morrison and Hecht (1984) were able to quantify N-nitroso-(2-hydroxyethyl)glycine, a major urinary metabolite in rats. The possibility of using these compounds as markers of endogenous nitrosation is still being investigated.

Intentional nitrosation of dietary constituents has been another valuable method used to estimate endogenous nitrosamine formation. These nitrosations follow one of two lines of attack; simulated gastric nitrosation and extreme nitrosation. A relevant question one may ask is whether *in vitro* simulations accurately represent what actually occurs endogenously. The answer to this question is debatable, so the results of these simulations must take this question into consideration.

Simulated gastric conditions have been established for studying endogenous nitrosation (Groenen, et al., 1980, Walters, et al., 1976, Gillatt, et al., 1985 and Sen, et al., 1985). The experimental conditions have usually consisted of varying amounts of nitrite, pepsin, amine source, inhibitors or accelerators, pH and digestion time. The above variables are considered either to be factors influencing nitrosation in the stomach or play a role in nitrosation

Table II. Foods Tested Under *in vitro* Conditions for N-Nitrosamine Formation

Beer	Bread
Cereal products	Cheese
Chicken (fried)	Coffee (brewed)
Cola	Cured Meats
Eggs	Fish
Fruits and Vegetables	Ketchup
Mayonnaise	Mustard
Peanuts	Pork
Potatoes	Soya Sauce
Spices	Steak
Tea (brewed)	Water
Yoghurt	

From: Groenen, et al., 1980 and Walters, et al. 1976.

as discussed above. Table II lists foods previously examined under various *in vitro* conditions. Results of these tests indicated that very few of the food products showed nitrosamine formation, and the foods that did form N-nitrosamines did so at very low levels (Groenen, et al., 1982). Gillatt, et al. (1985), however, reported that several drugs were nitrosated under simulated gastric conditions.

In another approach to study *in vitro* nitrosation, very high levels of nitrite have been added to food with variable results. For example, Walters, et al. (1974) found elevated levels of NPIP from nitrosated milk, while Walker, et al. (1979), reported no large increases in the levels of N-nitrosamines after nitrosating beer and other alcoholic beverages. This indicates that some food products do contain nitrosatable amines and can produce N-nitrosamines in the presence of nitrosating agents.

Further studies of the diet/cancer hypothesis might involve use of the above *in vitro* and *in vivo* techniques in regions known to have a high incidence of cancer. Conditions of high cancer risk could also be identified from environmental factors and the body's condition. These include: achlorhydria, gastrectomies by Bilroth techniques, and regions of high nitrate/nitrite levels in drinking water. The first two conditions can lead to an increased pH of stomach contents, and this increase allows abnormally high bacterial levels to exist. Some of these bacteria might reduce nitrate to nitrite, and this could lead to increased levels of gastrointestinal cancers from N-nitrosamines (Eisenbrand, et al., 1984). Certain high

nitrate areas, such as Colombia, are known to have a higher incidence of cancer, and investigations are being carried out to see if the probable cause is the diet. The levels of nitrate, nitrite and thiocyanate in the gastric system of Colombian natives indicate a strong possibility of endogenous nitrosamine formation (Tannenbaum, et al., 1979). The selection of these abnormal situations for study may provide opportunities not seen elsewhere.

To conclude, exogenous N-nitrosamine exposure seems to be largely understood and is well documented. As new sources of exposure are determined, little time lapses before corrective action is evident. In contrast, endogenous exposure is just now coming under intense investigation. The extent of its' contribution to total N-nitrosamine exposure, and techniques for possible control are not yet fully understood. New techniques being developed for N-nitrosamine analysis and studies of high risk populations are directed at solving these problems. Further research in this area will identify which source of N-nitrosamine exposure, endogenous or exogenous, is predominant and contributes the most insight into the diet/cancer hypothesis.

III. EXPERIMENTAL

A. Materials

Beer was purchased in retail stores in Oregon, Illinois and North Carolina. Non-fat dry milk (NDM), bacon and fish were purchased in retail stores in Oregon. The following chemicals were of analytical grade and obtained from the J.T. Baker Chemical Co.; sodium nitrite, ammonium sulfamate, sodium hydroxide, anhydrous sodium sulfate, sodium chloride, isopentane, sulfuric acid, hydrochloric acid and glacial acetic acid. Methylene chloride was obtained from Burdick and Jackson, Inc.; porcine pepsin (3200 units/mg) and sodium thiocyanate from Sigma Chemicals Co.; heavy white mineral oil from Mallinckrodt, Inc. and finally, food chemical grade Celite was obtained from Manville Products Corp. N-Nitrosamine standards were purchased from the Aldrich Chemical Co., Inc.

B. Methods

1. Simulated Gastric Nitrosation: a. *Beer.* All beer samples were stored at 4°C until used. The beer was degassed according to the American Society of Brewing Chemists (ASBC) standard method by equilibrating to room temperature overnight, then swirling or pouring back and forth in one liter flasks until gas evolution was severely reduced.

Simulated gastric juice was prepared by mixing the following in a 150 ml beaker: 10 ml of 0.19 M NaCl (2.82 g/250 ml), 5 ml of 11.1 mM NaSCN (0.225 g/250 ml), 5 ml of pepsin (0.25 g/5 ml) made fresh

daily, and 5 ml of one of the two $NaNO_2$ solutions 5.80 mM (40 mg/100 ml) or 0.14 mM (1 mg/100 ml). The simulated chyme was prepared by adding 25 ± 0.2 g beer to the simulated gastric juice and adjusting the pH to 3.0 with 1 M HCl. The chyme was thoroughly mixed, then separated equally into two Teflon-lined screw cap test tubes (20 x 150 mm). There were two test tubes for each individual beer sample and these were later combined into one flask. One of the tubes was spiked with 1 ml of the internal standard, NDPA (0.1 μg/ml), and both tubes were then sealed. The simulated digestion was initiated by placing the tubes in a covered, constant temperature waterbath at 37 ± 1°C. The digestion was stopped after two hours by first placing the tubes in ice water for several minutes and then adding 5 ml of 88 mM ammonium sulfamate (1 g/100 ml, brought to about pH 1 with sulfuric acid) to quench excess nitrite.

b. *Nonfat Dry Milk.* The simulated gastric juice was prepared as given above for beer. The simulated chyme was produced by adding 25 ± 0.2 g non-fat dry milk (NDM) to the simulated gastric juice in a Teflon-lined screw cap Erlenmeyer flask (125 ml). The pH was adjusted to 3.0 with 2.5 M HCl. There was one flask for each NDM sample, and the chyme was digested in the same manner as the beer.

c. *Bacon.* Raw bacon was fried in an electric skillet (Model 61B-1, Sunbeam Corp.) two minutes per side. The skillet temperature controller was adjusted to the bacon setting of 340. The bacon was stored at -12°C until used. Whole cooked rashers were ground with a mortar and pestle for 30 to 60 seconds or until broken into small particles. The bacon (25 ± 0.2 g) was made into a chyme and then

digested in the same manner as NDM.

d. *Fish.* 450 ± 25 g raw fish was wrapped in Saran Wrap and baked in a microwave oven (Tappan, model PN 560T762P01) on the "High" setting for 15 minutes to a temperature of approximately xxx °C. The cooked fish was then placed in plastic bags, sealed and stored at -12°C until used. Thawed fish was manually ground into small fragments and 25 ± 0.2 g of fish was made into a chyme and digested in the same manner as the NDM.

2. Extreme Nitrosation: a. *Beer.* The beer was degassed as in the simulated gastric nitrosation procedure. The reaction mixture was made by mixing 30 ± 0.2 g beer and 10 ml of 0.58 M $NaNO_2$ (4 g/100 ml) in a 150 ml beaker and adjusting the pH to 3.25 with glacial acetic acid. The reaction mixture was then divided equally into two Teflon-lined screw cap test tubes. Each tube contains one sample, so each pair of tubes represents duplicates of the same sample. One milliliter of the internal standard, NDPA (1.5 μg/ml), was added to each tube. The tubes were sealed and incubated at 100°C for 1 hour. After incubation, the tubes were cooled in ice water for 10 to 15 minutes. The tubes were opened carefully to release any remaining pressure and poured into 600 ml beakers. Excess nitrite was quenched by first adding 5 ml of 1.54 M ammonium sulfamate (17.5 g/100 ml, brought to pH 1 with sulfuric acid) to the empty test tube. The sulfamate was then swirled in the tube for 15 to 30 seconds and then poured into the beaker containing the beer. Finally, the beer was swirled and allowed to stand at least 5 minutes.

b. *NDM or Baked Fish.* The reaction mixture was produced by adding 15 ± 0.2 g NDM or fish, 25 ml distilled water and 5 ml of 0.58 M $NaNO_2$ into a Teflon-lined screw cap Erlenmeyer flask (125 ml). The contents were mixed thoroughly, and the pH was adjusted to 3.25 with 2.5 M HCl. The flasks were spiked with 1 ml of the internal standard, NDPA (1.5 μg/ml) and sealed. The flasks were then incubated for 1 hour at 100°C. After incubation, the flasks were cooled and quenched in the same manner as for beer.

3. Extraction: a. *Beer.* The procedure utilized was an Association of Official Analytical Chemists (AOAC) adopted method with minor changes. Chromatography columns (28 x 450 mm) were prepared by placing a glass wool plug in the bottom of the tube and then placing a layer of sodium sulfate over it, and finally covering the sodium sulfate with a layer of Celite. The Celite was prepared by heating at 700°C for 6 to 15 hours, followed by air cooling. Thirty grams of Celite was added to the quenched beer and thoroughly mixed. The Celite-beer mixture was poured into the column with the aid of a plastic funnel and tamping rod; each test tube was placed on a single column. The test tubes from the reaction mixtures were rinsed twice with 20 ml of methylene chloride (DCM) and the beaker used to mix the Celite and beer was rinsed once with 50 ml of DCM. All of the DCM rinsings were placed on the column. The funnel and tamping rod were then removed from the column. The flow was adjusted to 1-2 ml per minute into a 125 or 250 ml separatory funnel and the columns were allowed to run dry. The simulated gastric nitrosation samples had two columns combined per sample, while the extreme

nitrosation samples had only one column per sample. Keeping this in mind, the two columns for simulated gastric nitrosation samples were run at the same time, and as close to the same flow rate as possible. The DCM eluate was extracted with 40 ml of 2.5 M NaOH. At this point, the eluate of the two columns from the simulated gastric nitrosation samples were combined into the same flask. The combination was accomplished when the DCM layers were passed through a bed of anhydrous sodium sulfate and collected in a 250 ml Kuderna-Danish boiling flask fitted with a 10 ml concentrator tube. The bed of sodium sulfate was washed twice with 20 ml of DCM. Two silicon carbide boiling chips were added, and a macro Snyder column was attached. The DCM was concentrated to about 4 ml in a 58°C water bath. When samples were held overnight, they were held at 4 ml and stored at -12°C. Before quantitation, the samples were further concentrated to 1 ml under a stream of nitrogen while attached to a micro Snyder column.

b. *Nonfat Dry Milk*. The procedure utilized was an AOAC adopted method with minor changes. Simulated gastric and extreme nitrosation samples were handled the same. Quenched milk samples were poured into a 600 ml beaker with 40 g Celite and mixed thoroughly. The Celite-milk mixture was packed into the chromatographic columns as described above for beer. Special care was taken not to pack the columns too tightly. The reaction mixture flasks were rinsed twice with 30 ml of DCM and the beaker was rinsed once with 70 ml of DCM. All rinsings were placed on the column. The columns were eluted and concentrated in the same manner as for the beer, except that the NaOH

extraction was omitted.

c. *Fried Bacon or Fish.* The quenched reaction mixtures were transferred to a one liter round bottom flask containing a thermometer well. Ammonium sulfamate (1-2 g) was then added to the flask. The reaction mixture flask was rinsed twice with 20 ml of 1 N sulfuric acid and once with 20 ml of DCM. All rinsings were added to the one liter flask. Fifty ml of mineral oil were then added to the flask to make a slurry. The flask was then attached to a double-trap vacuum distillation apparatus. The traps rest in Dewar flasks filled with liquid nitrogen. The distillation was carried out at 10 microns vacuum and the Variac was set at 45 volts before starting the distillation. The distillation was stopped when the thermometer reached 105-110°C. The two traps were removed and allowed to warm at room temperature for 10-15 minutes. The frozen contents of the traps were melted under warm tap water and then transferred to a 250 ml separatory funnel. The large trap was rinsed four times with 20 ml of 1 N sulfuric acid, and the small trap was rinsed three times with 10 ml of 1 N sulfuric acid. All rinsings were added to the separatory funnel. The sulfuric acid solutions were next extracted three times with 35 ml of DCM. The DCM was then collected and extracted once with 40 ml of 2.5 M NaOH. The DCM extracts were dried over a bed of anhydrous sodium sulfate in a coarse fritted glass funnel. Concentration was carried out as described above.

4. Quantitation: The analysis was carried out by gas chromatography coupled to a Thermal Energy Analyzer (GC-TEA). The GC was a Varian model 3700 and it was coupled to a model 502 TEA. Five microliter aliquots were injected onto one of the two columns employed. Column A was a 2 mm I.D. x 3 meter nickel column packed with 10% Carbowax 20M on Chromosorb WAW (80/100 mesh). Column B was a 2 mm I.D. x 3 meter nickel column packed with 10% Carbowax 20M on Chromosorb GAW-HMDS (60/120 mesh). Helium was the carrier gas for both columns and the column flow was 18 ml/min. GC conditions were as follows: Column A was operated at 160°C isothermally, and Column B was held at 165°C for 10 minutes, then programmed at 6°C/minute to 190°C and held for 15 minutes. The TEA conditions were a vacuum of 1.2 mm Hg, pyrolyzer at 500°C, and the TEA cold trap was immersed in a liquid nitrogen-isopentane slush.

Standard curves were prepared for each nitrosamine by plotting the ratio of peak heights for the nitrosamine to the internal standard versus concentration of nitrosamine added as described by Marinelli, et al. (1981). The standard curves were examined by linear regression. The ratios obtained for the samples were then compared to the standard curve to determine the concentration of N-nitrosamine present in the sample.

To confirm the presence of several of the N-nitrosamines identified, mass spectral data was obtained. Samples were extracted from the food matrix as described above. The extracts were cleaned up on an alumina column by eluting in methanol. The N-nitrosamine fractions were trapped from an OV 275 column and stored in a nickel

loop (trap) at -12°C. The mass spectra were obtained using an Aerograph 1400 GC interfaced to a Finnigan 1015C quadrupole MS system. The GC conditions were as follows: a 60 m x 0.75 mm glass capillary column with a 1 μm layer of bonded Supelcowax 10, a helium flow rate of 10 ml/minute and the column temperature was 80°C for 5 minutes and then programmed at 4°C/minute to 180° and held. The MS operating conditions were as follows: electron energy of 70 eV, ionizing current of 450 μA, manifold temperature of 100°C, and the mass range scanned was 15-200 m/z. GC-MS data was acquired and processed using a Riber 400 data system.

IV. RESULTS

Some terms and rationale used throughout the results and discussion need to be clarified before proceeding. The use of the term "background level" will refer to the level of nitrosamines in food available in retail stores. The rapid column screening methods recently adopted by the Association of Official Analytical Chemists (AOAC) and the American Society of Brewing Chemists (ASBC) were employed to decrease the time of analysis. For bacon, the column method did not provide sufficiently clean DCM extracts for the analysis of nitrosamines by the TEA, so a vacuum distillation procedure was substituted. The choice of two different gas chromatographic (GC) columns was based on previous experience in our laboratory. Column A yields quick separation of NDMA, NDEA and NDPA, but was not suitable for NPIP and NPYR. Therefore, column A was used for quantifying beer samples where NPIP or NPYR were not found. Column B retained NDMA and NDPA longer, but separated NPIP from NPYR. Column B was used for analysis of all the extreme nitrosation and simulated gastric nitrosation samples that might contain NPIP or NPYR. Finally, nitrite concentrations in ppm, referred to in simulated gastric nitrosations, were calculated as $NaNO_2$ in the final reaction volume unless otherwise stated.

A. Simulated Gastric Nitrosation

Commercially available beer was purchased from three different regions of the United States. Samples of each brand were from the

same package of 6 or 12 containers. NDMA was the only nitrosamine consistently found in beer and usually at levels of less than 1 ppb. NDEA was found sporadically in beer and at trace levels. The range for background NDMA in beer was none detected to 0.8 ppb, with a mean of 0.1 ppb. The detection limit for this procedure was 0.1 ppb. Recoveries of N-nitrosamines for the beer samples were above 85% in nine out of ten analyses. No analyses were accepted with a recovery below 70%. Calculations were performed using a standard addition curve described by Marinelli, et al., (1981). The standard curves for all of the simulated gastric nitrosation analyses can be found in the appendix. The curves were accepted only if the regression coefficients were greater than or equal to 0.997.

The beer samples nitrosated under simulated gastric conditions at 1 and 40 ppm $NaNO_2$ showed small increases for NDMA. The results are reported in Table III. Figure I is a typical GC trace from the simulated gastric nitrosated beer. For all three purchase areas, the means of the nitrosated samples were significantly greater than their background levels as determined by a Student t-test ($p < 0.05$).

Nonfat dry milk (NDM), fried bacon and baked fish were also nitrosated under simulated gastric conditions. NDMA was the only nitrosamine found in the background of NDM, and it ranged from 0.3 to 1.2 ppb with a mean of 0.7 ppb. Table IV illustrates the food surveyed, and Figure II is a typical GC trace from a NDM sample. Very small increases in N-nitrosamine levels were evident when the NDM samples were nitrosated under simulated gastric conditions. The means of the nitrosated samples, however, were not significantly

different ($p < 0.05$) from the background levels.

Three volatile N-nitrosamines were found in the background of fried bacon. These were NDMA, NDEA and NPYR. The quantities of each background nitrosamine varied widely. No statistical difference was found for any of the nitrosamines between the background and the simulated gastric nitrosation samples. The bacon survey is summarized in Table V, and Figure III is a typical GC trace from nitrosated bacon.

The background fish samples were not found to contain any significant level (> 0.1 ppb) of NDMA. Upon simulated gastric nitrosation, slight increases were evident, although they were not statistically significant. Table VI summarizes the survey data for fish.

All values in the tables represent the mean of duplicate analyses except for the fried bacon and fish. Calculations were performed on standard addition curves prepared using NDM, bacon and fish as components for the stock solutions. Detection limits were 0.1 ppb for NDM, 0.2 ppb for bacon and 0.1 ppb for fish. Identification of background N-nitrosamines and N-nitrosamines in simulated gastric nitrosation samples was by GC-TEA retention times.

B. Extreme Nitrosation

The potential for beer to form N-nitrosamines was further investigated by exposing beer to extreme nitrosation conditions. The beer samples were found to yield several positive responses on the TEA. Four major N-nitrosamines were formed repeatedly in beer. They

Table III. Simulated Gastric Nitrosation: Domestic Beer Survey

Sample		NDMA (ppb)		
		Background	1 ppm $NaNO_2$	40 ppm $NaNO_2$
West Coast:	1	ND	--	0.2
	2	ND	--	0.3
	3	ND	--	0.2
	4	ND	--	0.3
	5	ND	--	0.2
	6	ND	--	0.3
	7	ND	--	0.2
	8	ND	--	0.2
	9	ND	0.3	0.2
	10	ND	0.4	0.2
	11	0.1	--	0.2
	12	0.1	--	0.2
	13	0.1	--	0.2
	14	0.1	--	0.3
	15	0.1	--	0.2
	16	0.1	--	0.2
	17	0.2	--	0.2
	18	0.2	--	0.3
	19	0.2	--	0.2
	20	0.2	--	0.3
	21	0.2	--	0.4
	22	0.2	--	0.3
	23	0.2	0.6	0.5
	24	0.3	0.2	1.2
	25	0.4	0.4	1.1
Mean	x	0.1	0.4 A	0.3 A
Midwest:	1	0.1	0.5	0.4
	2	0.2	0.6	0.6
	3	0.3	--	0.5
	4	0.5	0.6	0.7
Mean	x	0.3	0.6 A	0.6 A
East Coast:	1	0.2	--	0.6
	2	0.3	--	0.6
	3	0.4	--	0.6
	4	0.4	--	0.9
	5	0.4	0.8	0.8
	6	0.8	0.9	0.8
Mean	x	0.4	0.8 A	0.7 A

ND, None Detected; --, No Analysis performed
A, Significantly different from background ($p < 0.05$)

Figure I. Gas Chromatography Trace of Beer After Simulated Gastric Nitrosation. Column A, Attenuation 1.

Figure I.

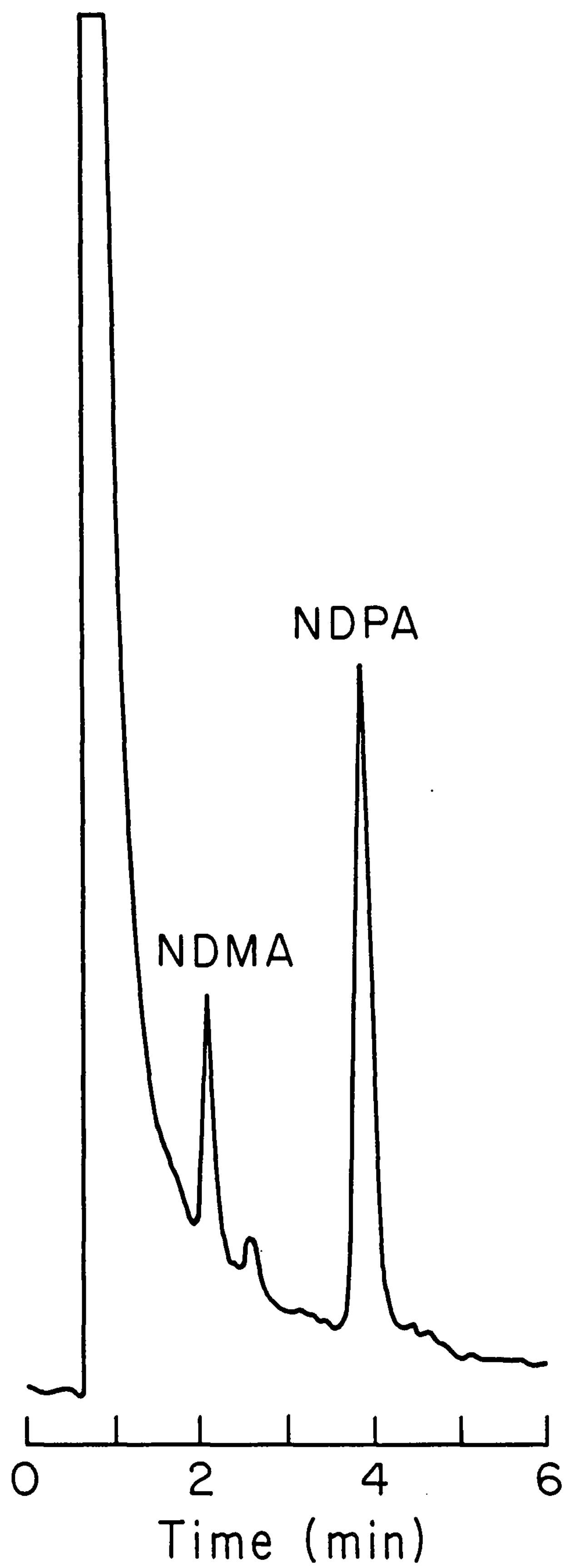


Table IV. Simulated Gastric Nitrosation: Non-fat Dry Milk Survey

Sample		NDMA (ppb)		
		Background	1 ppm $NaNO_2$	40 ppm $NaNO_2$
	1	0.3	0.4	0.6
	2	0.4	0.6	0.5
	3	0.7	0.7	1.1
	4	0.8	0.7	1.1
	5	0.9	0.7	1.0
	6	1.2	0.7	1.4
Mean	x	0.7	0.6	0.9

Table V. Simulated Gastric Nitrosation: Fried Bacon Survey*

N-Nitrosamine	Sample	Background	1 ppm $NaNO_2$	40 ppm $NaNO_2$
NDMA	1	ND	5.0	1.9
	2	1.8	6.6	2.2
	3	4.5	1.5	1.7
	4	5.5	7.7	5.2
	5	8.7	7.9	5.5
Mean	x	4.1	5.7	3.3
NDEA	1	ND	4.3	0.9
	2	ND	5.2	ND
	3	ND	ND	ND
	4	4.1	7.4	4.1
	5	6.6	6.4	TR
Mean	x	2.1	4.7	1.0
NPYR	1	8.9	6.0	9.3
	2	23.2	8.8	12.8
	3	32.8	24.9	25.7
	4	5.5	3.7	5.4
	5	14.6	24.8	43.4
Mean	x	17.0	13.6	19.3

*, All nitrosamine values reported are in ppb.
ND, None Detected
TR, Trace: < 0.1 ppb

Figure II. Gas Chromatography Trace of NDM After Simulated Gastric Nitrosation. Column B, Attenuation 1.

Figure II.

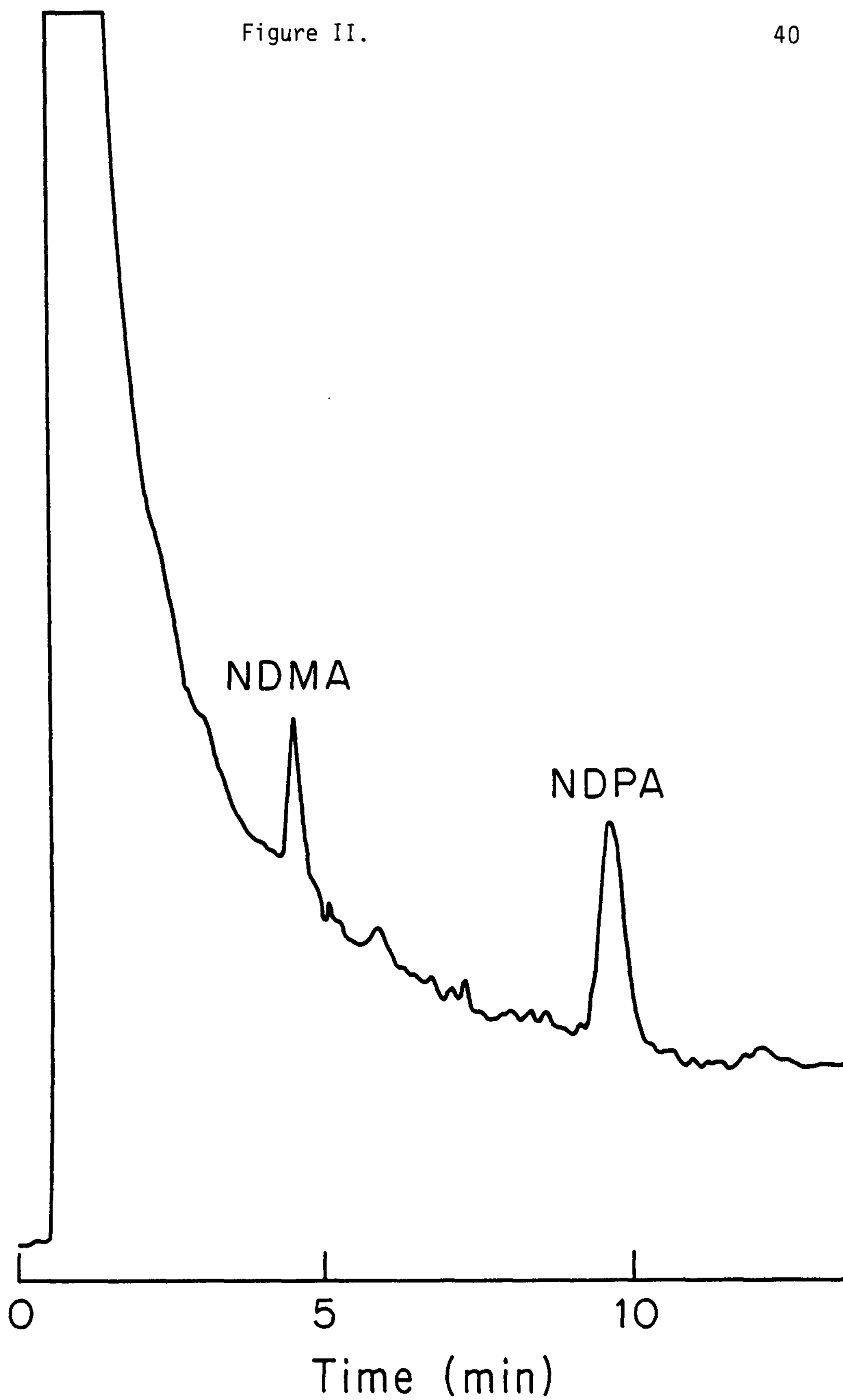


Figure III. Gas Chromatography Trace of Bacon After Simulated Gastric Nitrosation. Column B, Attenuation 1.

Figure III.

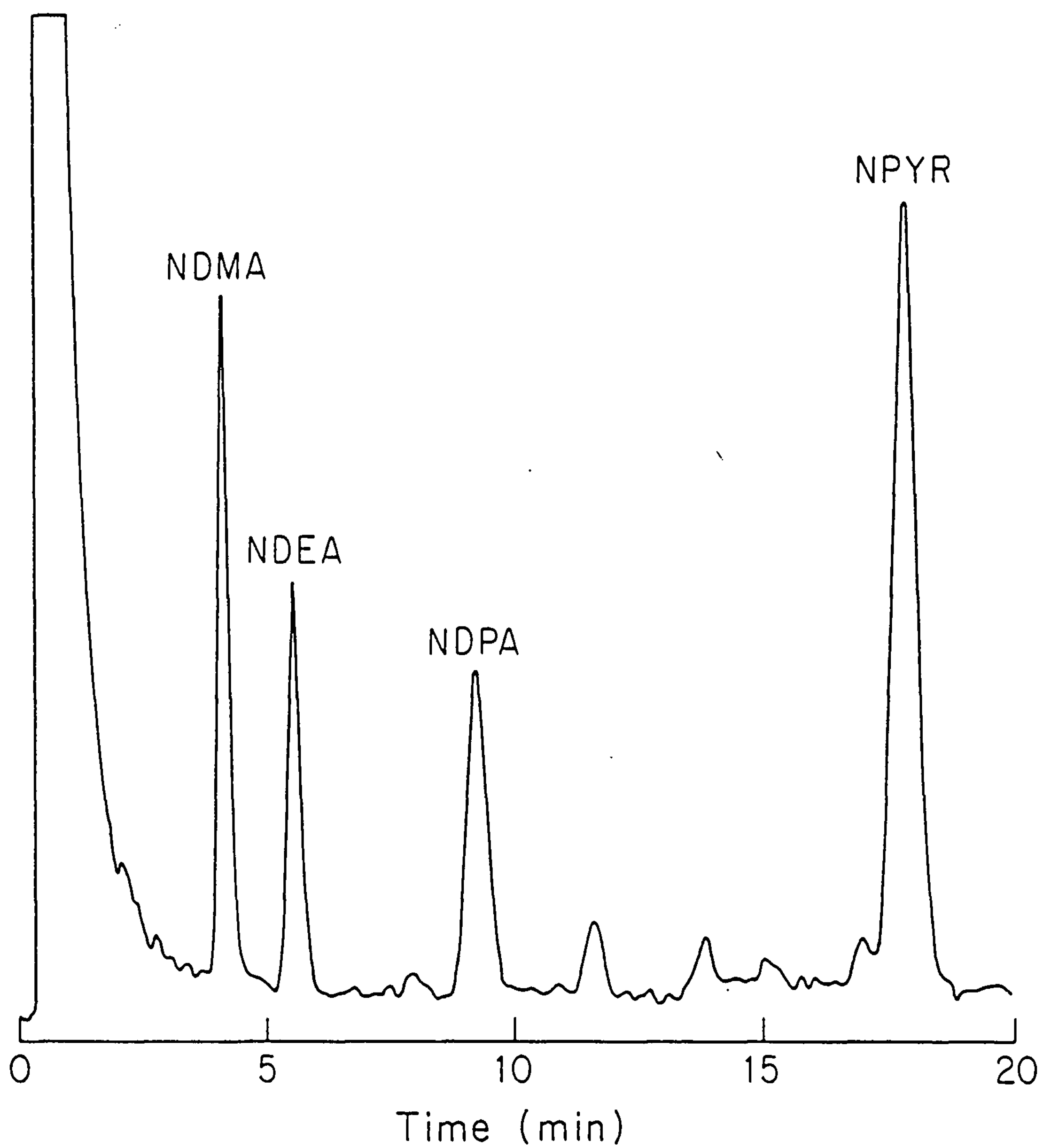


Table VI. Simulated Gastric Nitrosation of Fish

	NDMA (ppb)		
Sample	Background	1 ppm $NaNO_2$	40 ppm $NaNO_2$
Salmon	ND	TR	0.1
Tuna	TR	TR	TR
Halibut	ND	TR	TR
Cod	ND	TR	0.2
Rockfish	ND	ND	TR
Mean	ND	TR	TR

ND, None Detected
TR, Trace: < 0.1 ppb

were trapped and identified by gas chromatography-mass spectrometry (GC-MS) as NDMA, NDEA, NPYR and NMOR. Figures IV-VII compare the mass spectra of the N-nitrosamine standards with the N-nitrosamines extracted from beer. It was observed that the spectra of the nitrosated beer N-nitrosamines resembled the standard N-nitrosamine spectra very closely. NDMA levels were found to range from 496 to 2.41×10^3 ppb. The other N-nitrosamines occurred at lower levels and NMOR was only found in 9 out of 36 samples. The extreme nitrosation beer survey is summarized in Table VII, and a typical GC trace can be found in Figure VIII.

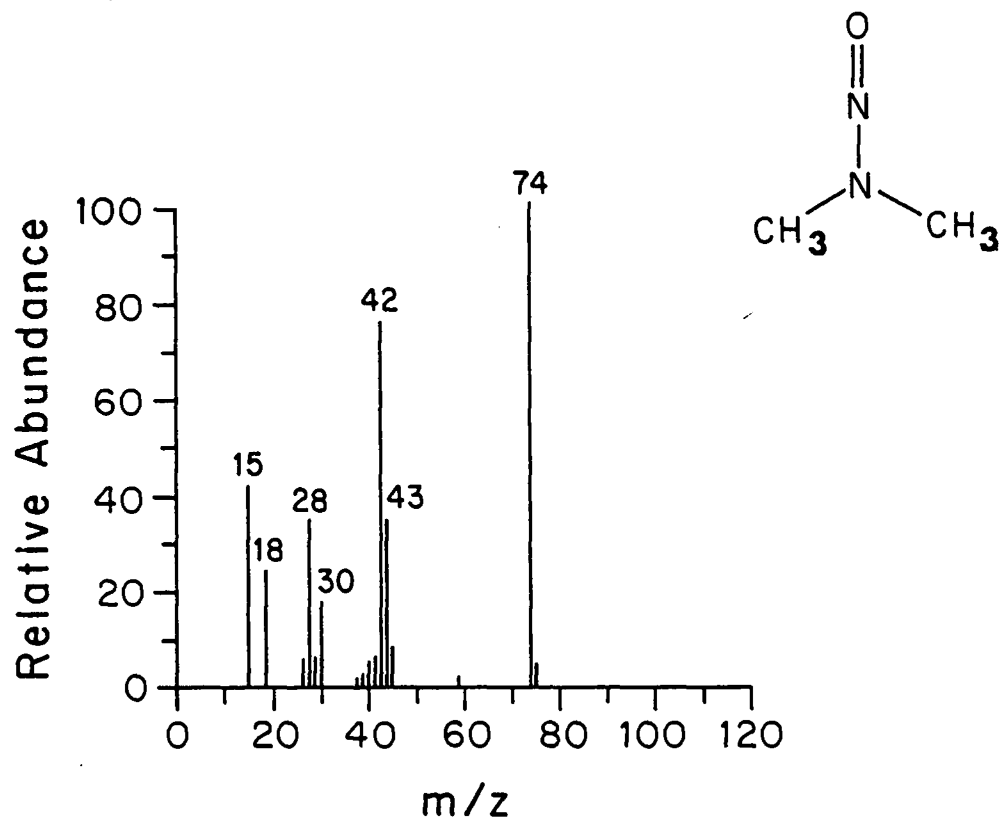
As with beer, many TEA positive peaks were produced when NDM and fish were subjected to extreme nitrosation. Again, four major peaks were present in most samples of NDM. These peaks were identified by GC-TEA retention times as NDMA, NPIP, NPYR and NMOR. They were not confirmed by GC-MS. NDMA levels ranged from 57 to 193 ppb, with a mean of 142 ppb. The levels for the other N-nitrosamines were all below 100 ppb. The NDM survey is summarized in Table VIII, and a typical GC trace is found in Figure IX.

The extreme nitrosation fish samples consistently yielded two peaks. These peaks were identified by retention times as NDMA and NPYR. They were not confirmed by GC-MS. NDMA levels ranged from 1.23×10^3 to 1.89×10^4 ppb with a mean of 1.09×10^4 ppb. NPYR occurred at levels much lower than the levels of NDMA. The fish survey data is reported in table IX.

Calculations for beer, NDM and fish were performed by the standard addition curves previously mentioned, except that new curves

Figure IV. Mass spectra of (Top) the peak with a GC-TEA retention time corresponding to NDMA from extemely nitrosated beer and (Bottom) standard NDMA solution

Figure IV.



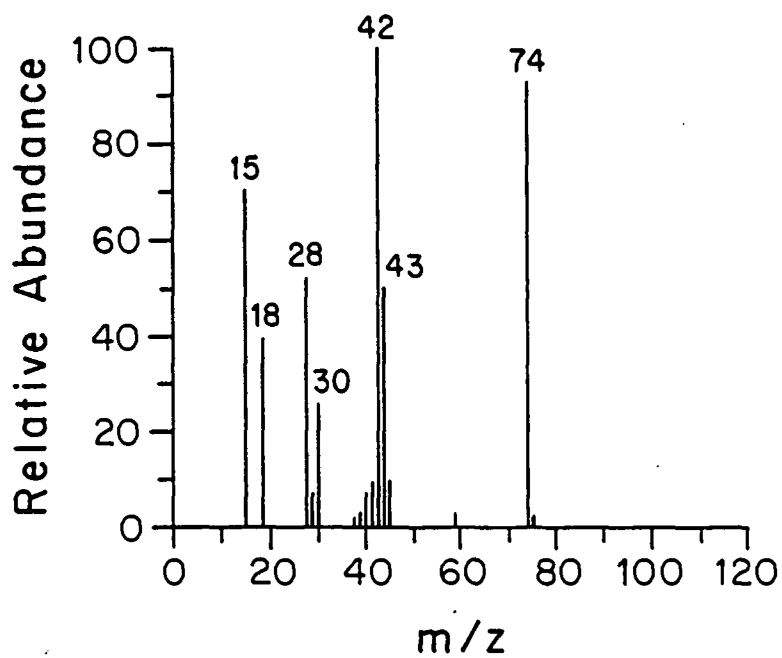
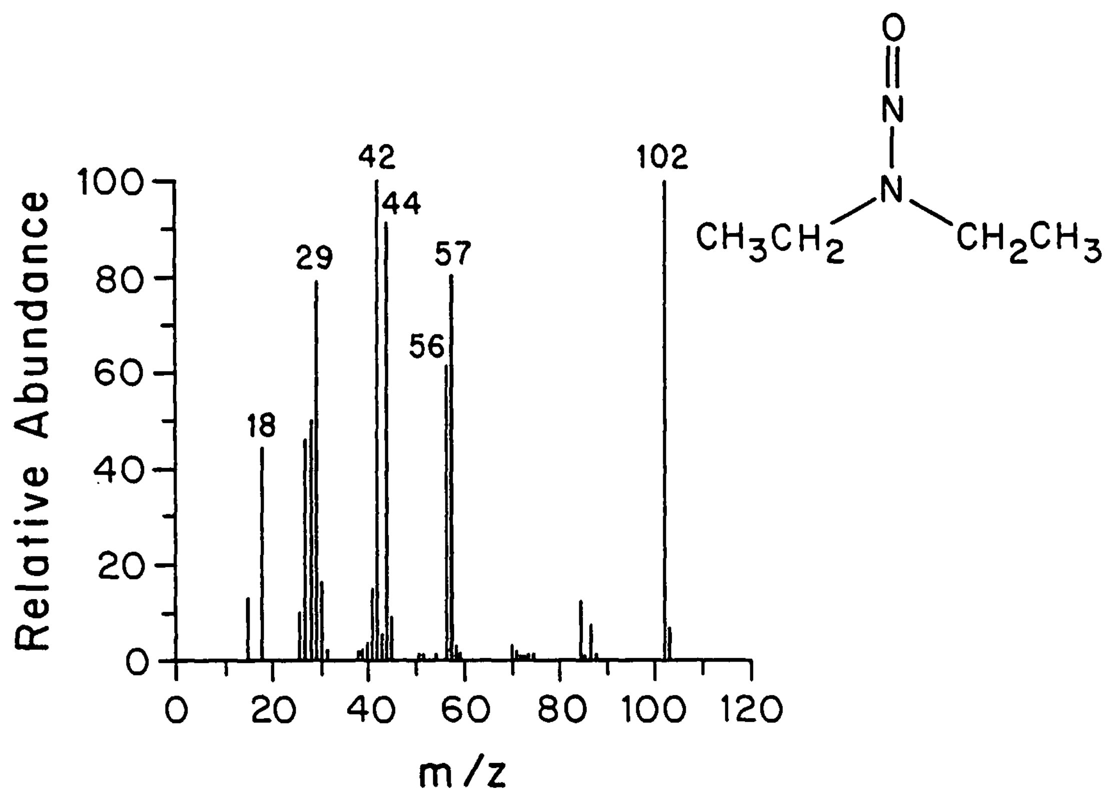


Figure V. Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NDEA from extremely nitrosated beer and (Bottom) standard NDEA solution

Figure V.



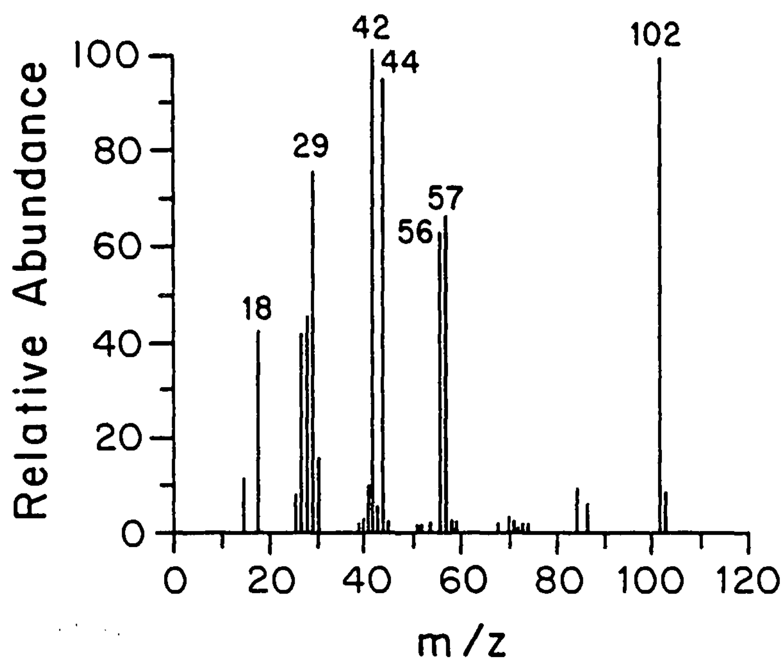


Figure VI. Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NPYR from extremely nitrosated beer and (Bottom) standard NPYR solution

Figure VI.

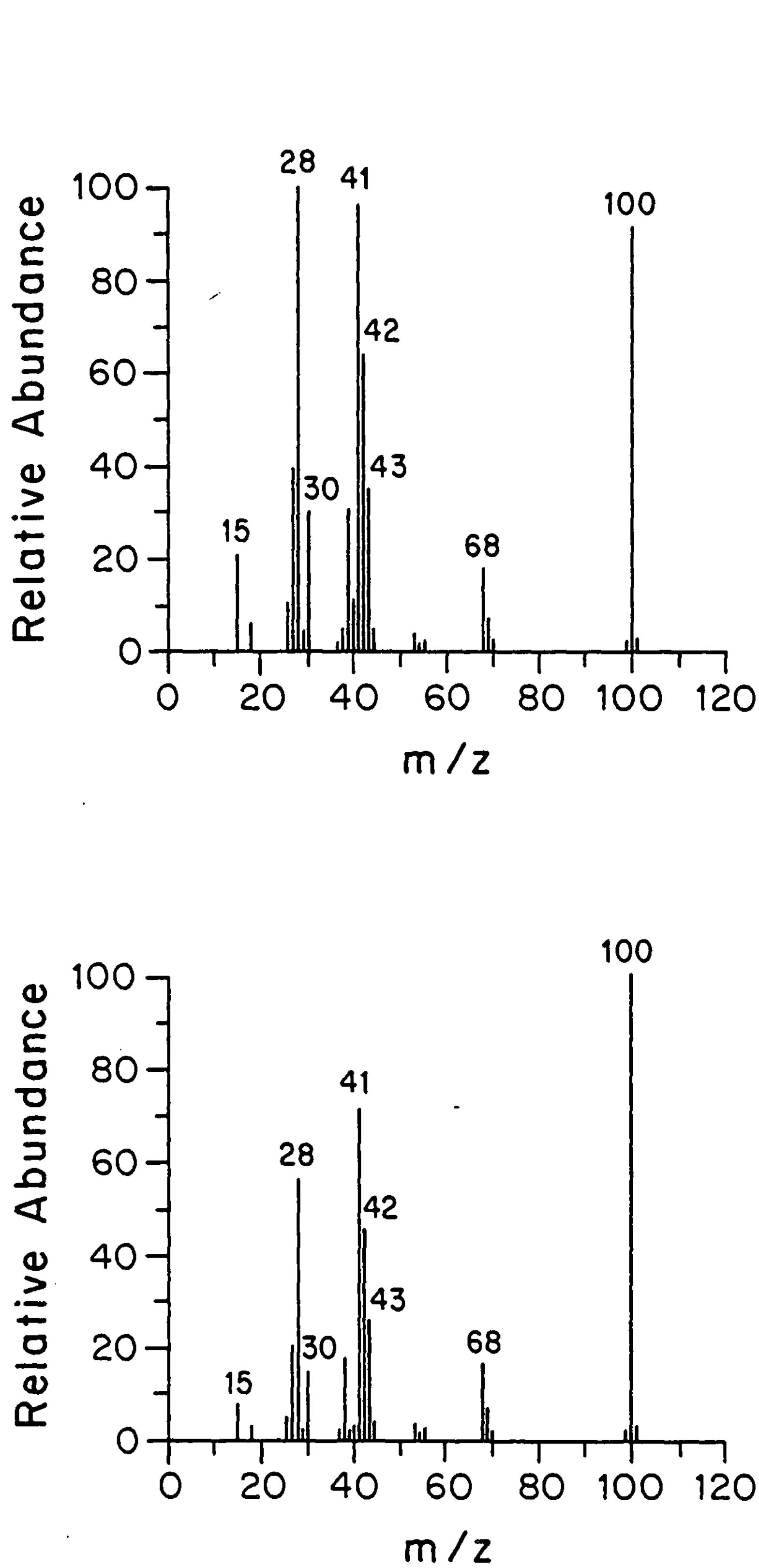
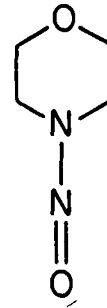


Figure VII. Mass Spectra of (Top) the peak with a GC-TEA retention time corresponding to NMOR from extremely nitrosated beer and (Bottom) standard NMOR solution

Figure VII.



Relative Abundance

100 80 60 40 20 0

28 30 42 56 86 116

0 20 40 60 80 100 120

m/z

Relative Abundance

100 80 60 40 20 0

28 30 42 56 86 116

0 20 40 60 80 100 120

m/z

Table VII. Extreme Nitrosation: Domestic Beer Survey

Sample		N-Nitrosamine (ppb)			
		NDMA x 10^3	NDEA	NPYR	NMOR
West Coast:	1	1.04	31	259	ND
	2	0.96	30	279	ND
	3	1.29	81	312	ND
	4	1.16	36	153	ND
	5	1.10	39	248	ND
	6	0.89	80	243	51
	7	1.05	35	330	ND
	8	0.67	30	195	24
	9	0.89	38	246	ND
	10	1.32	52	269	ND
	11	1.10	71	222	ND
	12	1.01	49	185	ND
	13	0.93	107	218	ND
	14	1.22	121	203	ND
	15	1.02	49	232	ND
	16	0.98	59	265	ND
	17	0.89	52	198	ND
	18	0.75	38	177	ND
	19	0.63	45	220	ND
	20	1.30	144	230	ND
	21	1.14	48	256	ND
	22	1.08	46	237	111
	23	0.76	67	153	24
	24	0.87	43	229	24
	25	0.88	40	219	82
Mean	x	1.00	57	231	13
Midwest:	1	2.41	33	1.09×10^3	ND
	2	1.06	40	242	29
	3	1.04	28	245	38
	4	1.18	30	302	ND
Mean	x	1.42	33	470	17
East Coast:	1	1.56	51	297	ND
	2	1.11	56	306	ND
	3	0.50	53	401	ND
	4	1.28	67	249	68
	5	1.32	62	249	ND
	6	0.74	56	295	ND
Mean	x	1.08	57	299	11

ND, None Detected

Figure VIII. Gas Chromatography Trace of Beer After Extreme Nitrosation. Column B, Attenuation 8.

Figure VIII.

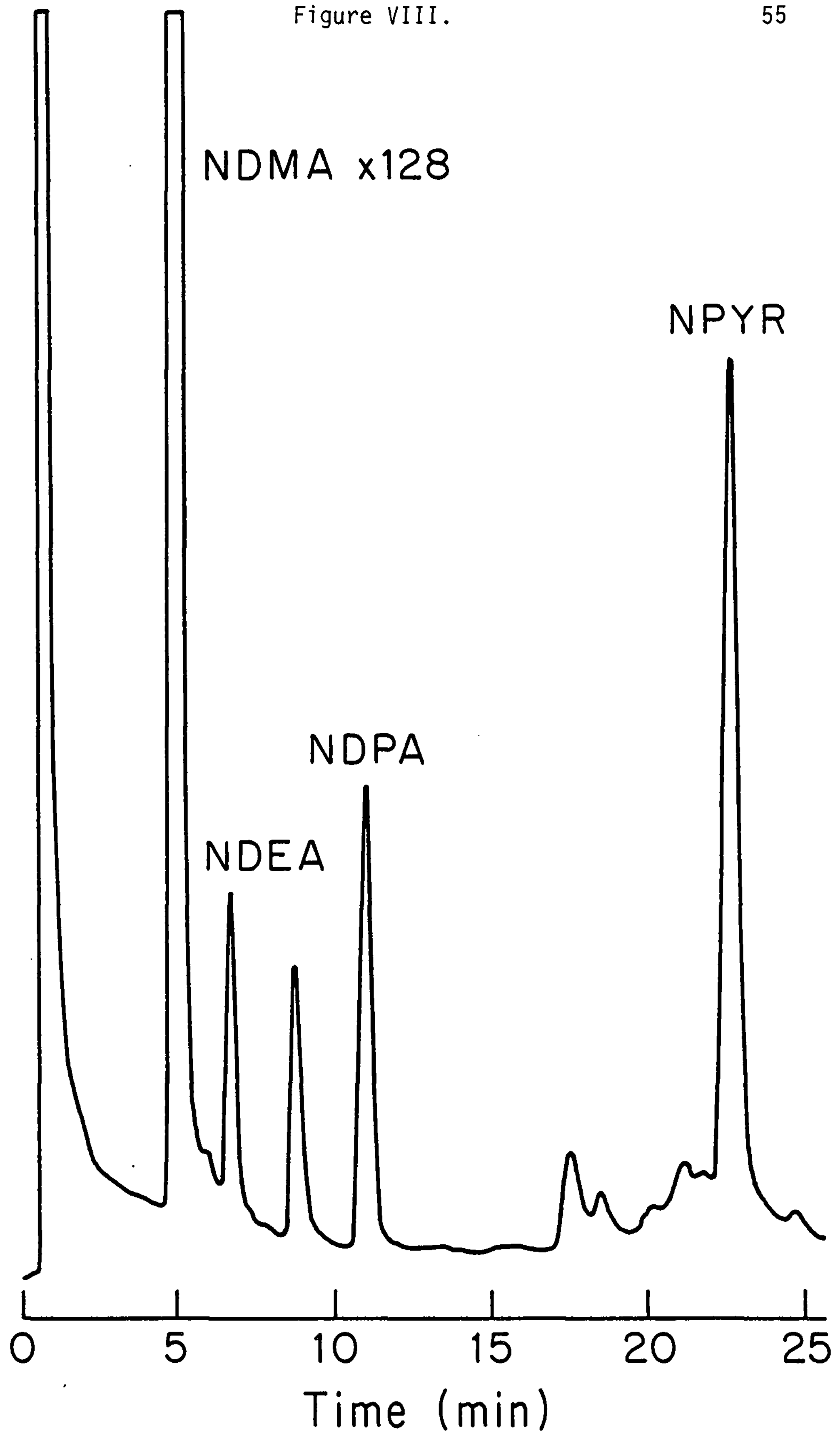


Table VIII. Extreme Nitrosation: Non-fat Dry Milk Survey

		N-Nitrosamine (ppb)			
Sample		NDMA	NPIP	NPYR	NMOR
	1	130	40	28	27
	2	122	54	29	10
	3	165	99	44	14
	4	183	62	40	13
	5	193	35	32	78
	6	57	38	30	7
Mean	x	142	55	34	25

Figure IX. Gas Chromatography Trace of NDM After Extreme Nitrosation. Column B, Attenuation 8.

Figure IX.

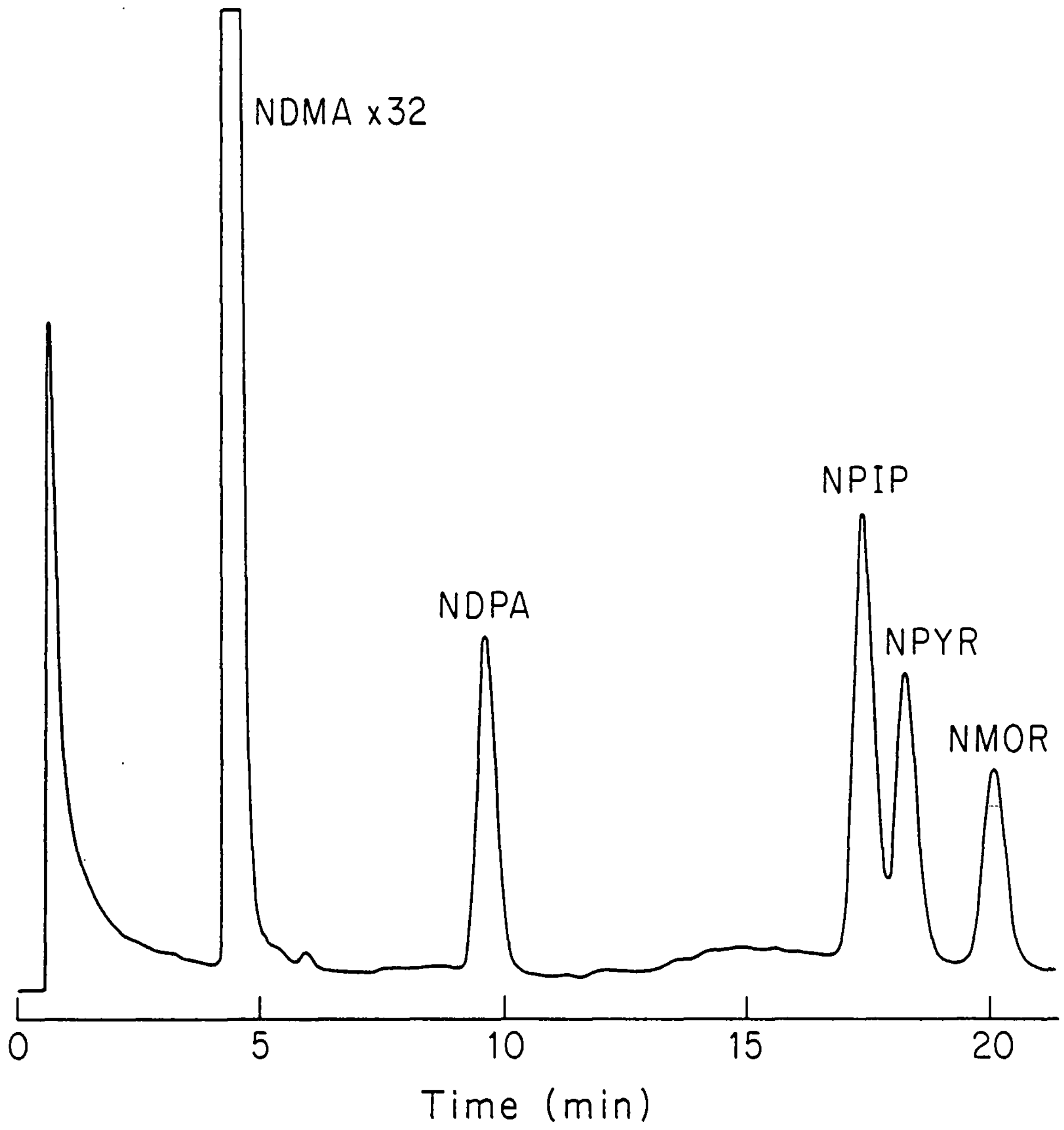


Table IX. Extreme Nitrosation: Fish Survey

Sample	N-Nitrosamine (ppb)	
	NDMA x 10^4	NPYR
Salmon	0.23	9
Tuna	0.12	ND
Halibut	1.87	2
Cod	1.89	55
Rockfish	1.34	2
Mean	1.09	14

ND, None Detected

were prepared for the increased levels of N-nitrosamines. The curves can be found in the appendix, and were only accepted if the regression coefficient was greater than or equal to 0.997.

Extreme nitrosation of fried bacon resulted in many analytical problems. Due to these problems no results are presented. However, it can be stated that fried bacon appeared to yield a large number of TEA positive responses indicating a large nitrosatable amine content.

V. DISCUSSION

A. Simulated Gastric Nitrosation of food

The choice of which food to study was an important one. The most logical choice would be food known to contain N-nitrosamines. Such food products would already illustrate some level of nitrosatable amine, and for *in vitro* studies, the food chosen should represent the more likely suspects for endogenous nitrosation. Since previous work in our laboratory has involved malt and beer, the major emphasis of this study was on beer.

The food products selected were beer, fried bacon, nonfat dry milk (NDM) and fish. Beer was sampled across the United States to identify differences in nitrosating capacity due to different barley varieties or malting practices. Bacon has probably been the most thoroughly investigated of all the foods known to contain N-nitrosamines. NDM and fish have been known to contain trace levels of NDMA for some time. Another factor in selecting several of these food products, was the well developed analytical procedures available for volatile nitrosamines. The column extraction procedures and GC-TEA system make working with a large number of samples less time consuming. With the appropriate food products identified, the next step was to establish simulated gastric conditions.

This was a critical step, since one must make the conditions as realistic as possible, and yet still obtain data on the reaction(s) of interest. This can be accomplished by identifying the major variables involved, and establishing the desired conditions and

levels of reactants. The basic variables of a simulated gastric system are pH, enzyme concentration and activity, and digestion time (Miller and Schricker, 1982). Additional variables of importance to endogenous nitrosation are the ions that can participate in the nitrosation reaction. These ions include nitrite, thiocyanate and chloride.

The two sets of conditions used in this study to simulate gastric nitrosation differed only in nitrite concentration. The selection of 40 ppm $NaNO_2$ (27 ppm NO_2^- or 0.587 mM NO_2^-) was not altogether arbitrary. Nitrite concentration in the gastric juice of healthy, fasting humans is typically less than 1 ppm. However, within 30 to 120 minutes after ingesting a meal rich in nitrate, the salivary nitrite concentration can increase to several hundred ppm (Tannenbaum, et al., 1976). This indicates an increased level of salivary nitrite in the stomach. The nitrite level has been shown not to regain its basal level for about 24 hours after ingestion of nitrate. Considering that certain individuals with gastric disorders have been shown to have higher levels of nitrite in their stomach, it seemed reasonable to also select a significantly higher level of nitrite in which to incubate the foods than exists in normal fasting gastric juice. To compliment the results at 40 ppm, several of the samples were tested at a lower level (1 ppm) which more realistically simulates healthy, fasting gastric conditions.

The concentrations for the two other ions, thiocyanate and chloride, were selected with the same line of reasoning; to promote nitrosation. Normal concentrations of thiocyanate in gastric juice

range from 50 to 90 ppm. Smokers, in general, are found to have levels in the upper part of this range (Ruddell, et al., 1976). Ninety ppm NaSCN (65 ppm SCN^- or 1.12 mM SCN^-) was selected to be on the high side, while still approximating realistic values. Chloride is a normal, abundant constituent of gastric juice and can catalyze nitrosation under certain conditions. The catalytic role of Cl^- may not be of great importance, since the pH of the simulated gastric conditions was 3.0, and the catalytic effects of Cl^- are observed at pH values below 3.0 (Hildrum, et al., 1975).

The basic variables mentioned earlier are described below. The pH of the incubation was selected as 3.0. A constantly changing pH environment occurs during the consumption of a meal. The pH has been illustrated to increase from the normal of 1-2 up to about 5 (Malagelada, et al., 1977). To create a system that could adjust the pH throughout simulated digestion would lead to many complexities. It was decided, therefore, to select a single pH in the optimum range for nitrosation. The pH of 3.0 seems logical since it is close to the optimum for nitrosation of dimethylamine in gastric juice.

Pepsin is the principal proteolytic enzyme in the stomach. Previous *in vitro* studies using simulated gastric conditions used from 6 to 60 mg pepsin per gram of contents (Groenen, et al., 1980 and Miller and Schricker, 1982). The latter authors point out that different enzyme concentrations would produce different rates of digestion, but the important concept was that precise duplication of the concentration was necessary to compare one food to another. The pepsin obtained was the highest activity available (3200 units/mg) so

a lower concentration of 5 mg/g was selected.

To remain comparable to the times used in previous studies, two hours were allowed for the simulated digestion. Malagelada, et al. (1977) also reported a return to the basal gastric volume after three hours post prandial in humans.

The last variable to consider was food dilution, since the food consumed is diluted by the gastric secretions. Although human fasting gastric juice volumes are normally 50 to 75 ml, this may change drastically due to various stimuli. A moderate quantity of food would be about 250-300 g, and this resulted in gastric volumes of about 500 ml (Scheunig and Ziebarth, 1976). The dilution chosen was therefore 1:2 for food to chyme. The last step in the study was to compare the results.

Food may contain background levels of N-nitrosamines, and for comparisons it is important to measure these levels. The beer survey reported here found the N-nitrosamine background to be almost identical to a survey reported by Mangino and Scanlan (1982). The authors reported a range of ND to 0.7 ppb NDMA with a mean of 0.2 ppb. In the same report, the authors indicated that in 1979 NDMA had ranged up to 14 ppb, with a mean of 5.9 ppb. This trend towards lower levels of N-nitrosamines in beer continues according to the present survey.

NDMA in non-fat dry milk was found in previous surveys to range from a non-detectable level to 4.5 ppb, with a mean of less than 1 ppb (Havery, et al.,1982, Sen, et al., 1980 and Libbey, et al., 1980). The present results are comparable to those previously

published.

The major volatile nitrosamine in fried bacon is NPYR. It has been found to range from a trace amount to 45 ppb (Scanlan, 1983). Background levels found in the present survey were in a comparable range. Other volatile nitrosamines in fried bacon in the present study, NDMA and NDEA, were found at lower levels, yet still consistent with previous work. The frying temperature used in the present study possibly accounts for these relatively lower levels, as Gough, et al. (1976) found that over three-fourths of the NDMA formed in bacon was distributed in the frying vapor depending on the temperature.

Fish has previously been found to contain low background levels of NDMA (none detected to 10 ppb). The amount formed will depend on the species of fish, method of preservation and cooking conditions (Smith, 1980, Matsui, et al., 1980 and Sen, et al., 1985). The results of the present study indicate NDMA levels at the extreme lower end of the range mentioned above. This was attributed partly to the species of fish, and partly to the method of cooking. The precursor amine levels will vary widely from species to species, and only two samples were of the Gadoid species which is known to be high in dimethylamine. The use of microwave cooking in the present study does not produce nitrogen oxides that could further nitrosate the amine precursors, so the method of cooking used in this study did not contribute to the background level of N-nitrosamines.

Under both sets of simulated gastric conditions established, beer was found to yield slightly, yet significantly increased amounts of NDMA. Groenen, et al. (1980) reported no increase upon simulated gastric nitrosation of beer. Although the present findings do indicate an increase in NDMA, the small differences found might be expected from experimental variation.

No significant nitrosation differences were observed between beer samples obtained from different regions of the United States, although the sampling was quite limited. This suggests that the capability to form N-nitrosamines endogenously from beer amines tends to be uniform from different regions.

The non-fat dry milk, bacon and fish were not found to be significantly increased in N-nitrosamine content by the simulated gastric conditions. The milk and fish consistently showed a pattern of slight increases at the 40 ppm nitrite treatment. The small number of samples used may have prevented this from becoming statistically significant in the case of the dry milk. The fried bacon data gave larger variations, and showed no pattern of increased nitrosation under simulated gastric conditions. Overall, the results for the latter three products are similar to previously published results for *in vitro* food nitrosation.

Some difficulty was experienced in transferring the bacon from container to container due to the large amount of lipid present. Dichloromethane was used as a solvent wash and reduced the variation, but did not completely eliminate it. The analyses were accepted only if the recovery was above 70%. Recoveries for most samples were

above 80%. Several of the N-nitrosamine recoveries for bacon were in the 70 to 80% range, and this may be an indication of the problem stemming from lipid residues. The cooking conditions for bacon have also been shown to affect the quantity of N-nitrosamines formed (Hotchkiss and Vecchio, 1985), which may also account for some of the variability observed for the bacon samples.

B. Extreme Nitrosation of foods

Extreme nitrosation conditions were used in this study for three reasons. First of all, the results from the extreme nitrosation experiments established whether large amounts of nitrosatable amines existed in the food. Secondly, these experiments allow the identification of the major N-nitrosamines that could possibly be formed in a particular food. Finally, extreme nitrosation determines an upper range or "theoretical maximum" for N-nitrosamine formation that can be used for comparison purposes. These conditions are recognized as not likely to be found in food, or in the human body, but they do provide some insight into the theoretical maximum capacity of a food to form N-nitrosamines.

The conditions for extreme nitrosation were intended to produce a large quantity of N-nitrosamines from the food amines. The nitrite level was selected to be in large excess. The pH optimum for nitrosation of dimethylamine, the major precursor of NDMA in beer, is 3.4 in aqueous solution (Mirvish, 1975). Since acetate ions have been reported to form nitrosyl compounds which are effective nitrosating agents (Masui, 1974), the pH of the beer was adjusted to

3.25 with glacial acetic acid. The reaction mixture was not buffered, but the pH was measured after incubation of several samples, and the pH was found to vary from 3.4 to 3.7. This is not out of the optimum nitrosation range for formation of N-nitrosamines. The pH of NDM and fish was adjusted with hydrochloric acid, since an almost ten-fold increase in acetic acid would be required to lower the pH to 3.25.

Beer produced relatively large amounts of N-nitrosamines under extreme nitrosation, as compared to the amount formed under simulated gastric nitrosation. This conflicts with the results for nitrosated beer reported by Walker, et al. (1979). These authors reported little or no increase in the levels of N-nitrosamines after extreme nitrosation. Their incubation was carried out at 37°C,while the temperature used in the present study was 100°C; which may account for some of the difference. The great disparity between the results reported here and those of Walker, et al. (1979) still remains somewhat puzzling.

It was curious to observe that one midwestern beer sample formed about twice as much NDMA as the other samples under conditions of extreme nitrosation. This beer was a bock beer that was produced differently from most domestic beer, and thus may contain a higher level of NDMA precursors. This beer did not produce an exceptionally large level of NDMA in the simulated gastric survey, and therefore is not likely to be an additional risk for consumers.

The non-fat dry milk survey indicates there are significant quantities of nitrosatable amines in milk. Walters, et al. (1974) reported that large amounts of NPIP and NPYR were formed in milk under extreme nitrosation conditions. The values in the present study were comparable, except that NDMA was the N-nitrosamine found in the largest amount. The amount of N-nitrosamines produced from NDM were about a factor of ten lower than from beer under extreme nitrosation conditions. This might explain why the NDM did not increase in NDMA content under simulated gastric nitrosation.

The data from fish samples showed a similar pattern of yielding much larger quantities of NDMA from extreme nitrosation conditions when compared to simulated gastric conditions. NDMA was the major N-nitrosamine expected, since cod and rockfish are members of the Gadoid species, which contain high levels of dimethylamine and trimethylamine oxide.

The extreme nitrosation treatment of bacon resulted in a TEA response that masked all the peaks of interest when the Celite column method was used. A sulfamic acid, vacuum distillation procedure also produced a similar, yet weaker masking. A sodium hydroxide vacuum distillation method showed promise, but analytical problems still persisted. It was thus decided not to pursue the extreme nitrosation of bacon at this time.

A general pattern for the foods studied can be observed from a comparison of results from simulated gastric and extreme nitrosation. The pattern indicates a nitrosative blocking action by food constituents under gastric nitrosation conditions. For example,

the polyphenolics that give beer some of its body and flavor, may scavenge some of the relatively limited amount of nitrite present under simulated gastric conditions. Certain phenolics have indeed been shown to inhibit nitrosation by the scavenging of nitrite (Pignatelli, et al., 1980 and Stich, et al., 1984). Reconstituted, dehydrated milk was shown to partially inhibit NMOR formation when morpholine and nitrite were added (Fan and Tannenbaum, 1973). The authors, however, could not attribute nitrite scavenging to the protein in milk.

The N-nitrosamines identified in the surveys reported here illustrate only a partial array of the nitrosatable amines present in food. This is by no means a complete list of N-nitrosamines that can form. It is important to realize that the precursor amines for nonvolatile nitrosamines are also likely to be present. The detection of many of these nonvolatile N-nitrosamines is difficult, since the GC-TEA requires volatile components. A HPLC-TEA interface has been developed, but has met with only limited success due to interferences from water and other problems. Further advances into nonvolatile N-nitrosamine analysis have been slow. Human exposure to this type of N-nitrosamine has been less well elucidated and clearly deserves intensive investigation.

VI. SUMMARY

It was very apparent that some human foods possess nitrosatable amines which can lead to the formation of carcinogenic N-nitrosamines. However, given the experimental limitations of simulated gastric nitrosation, it does not appear that amines from the foods studied are nitrosated in large amounts under gastric conditions. From the results reported here, there is some indication of a nitrosative blocking action by food constituents. The long term health effects of endogenous nitrosation have yet to be established, so any further conclusions on the potential cancer risk can only be speculative at this time. Further work is needed to elucidate the total human exposure to N-nitrosamines from both endogenous and exogenous exposure routes. Since it is very likely that nonvolatile N-nitrosamines are also formed, research towards including exposure to this class of N-nitrosamines is badly needed.

VII. BIBLIOGRAPHY

Archer, M.C. (1984) Catalysis and inhibition of N-nitrosation reactions. In: N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. Eds: I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long and H. Bartsch; International Agency for Research on Cancer; Publication #57; Lyon, France; p.263-274.

Bartsch, H. and Montesano, R. (1984) Relevance of nitrosamines to human cancer. Carcinogenesis, 5(11):1381-1393.

Bogovski, P., Castegnaro, M., Pignatelli, B. and Walker, E.A. (1972) The inhibiting effect of tannins on the formation of nitrosamines. In: N-Nitroso Compounds: Analysis and Formation. Eds: P. Bogovski, R. Preussmann and E.A. Walker; International Agency for Research on Cancer; Publication #3; Lyon, France; p. 127-129.

Bontoyan, W.R., Law, M.W. and Wright Jr., D.P. (1979) Nitrosamines in agricultural and home-use pesticides. Journal of Agricultural and Food Chemistry, 27(3):631-635.

Cardesa, A., Mirvish, S.S., Haven, G.T. and Shubik, P. (1974) Inhibitory effect of ascorbic acid on the acute toxicity of dimethylamine plus nitrite in the rat. Proceedings of the Society of Experimental Biological Medicine, 145:124-128.

Challis, B.C., Edwards, A., Hunma, R.R., Kyrtopoulos, S.A. and Outram, J.R. (1978) Rapid formation of N-nitrosamines from nitrogen dioxides under neutral and alkaline conditions. In: Environmental Aspects of N-Nitroso Compounds. Eds: E.A. Walker, L. Griciute, M. Castegnaro and R.E. Lyle; International Agency for Research on Cancer; Publication #19; Lyon, France; p. 127-142.

Choi, B.C.K., (1985) N-Nitroso compounds and human cancer: A molecular epidemiological approach. American Journal of Epidemiology, 121(5):737-743.

Committee on Nitrite and Alternative Curing Agents in Foods, (1981) The Health Effects of Nitrate, Nitrite and N-Nitroso Compounds. National Academy Press, Washington, D.C.

Douglas, S.D., (1980) Cells involved in immune responses. In: Basic and Clinical Immunology. Eds: H.H. Fudenberg, D.P. Stites, J.L. Caldwell and J.V. Wells; Lange Medical Publication; Los Altos, CA; p.108.

Eisenbrand, G., Spiegelhalder, B., Kann, J., Klein, R. and Preussmann, R. (1979) Carcinogenic N-nitrosodimethylamine as a contamination in drugs containing 4-dimethylamine-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (amidopyrine, aminophenazone). Arzneimittel-Forsch, 29:867.

Eisenbrand, G., Spiegelhalder, B. and Preussmann, R. (1981) Analysis of human biological specimens for nitrosamine contents. In: Gastrointestinal Cancer: Endogenous Factors. Eds: W.R. Bruce, P. Correa, M. Lipkin and S.R. Tannenbaum; Cold Spring Harbor Press, NY; Banbury Report 7; p. 275-283.

Eisenbrand, G., Adam, B., Peter, M., Malfertheiner, P. and Schlap, P. (1984) Formation of nitrite in gastric juice of patients with various gastric disorders after ingestion of a standard dose of nitrate-A possible risk factor in gastric carcinogenesis. In: N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. Eds: I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long and H. Bartsch; International Agency for Research on Cancer; Publication #57; Lyon, France, p. 965-970.

Fan, T.Y. and Tannenbaum, S.R. (1973) Factors influencing the rate of formation of nitrosomorpholine from morpholine and nitrite: Acceleration by thiocyanate and other anions. Journal of Agricultural and Food Chemistry, 21(2):237-240.

Fan, T.Y. and Tannenbaum, S.R. (1973) Natural inhibitors of nitrosation reactions: The concept of available nitrite. Journal of Food Science, 38:1067-1069.

Fan, T.Y., Goff, U., Song, L., Fine, D.H., Arsenault, G.P. and Biemann, K. (1977) N-nitrosodiethanolamine in cosmetics, lotions and shampoos. Food and Cosmetics Toxicology, 15:423-430.

Fiddler, W., Pensabene, J.W., Piotrowski, E.G., Doerr, R.C. and Wasserman, A.E. (1973) Use of sodium ascorbate or erythorbate to inhibit formation of N-nitrosodimethylamine in frankfurters. Journal of Food Science, 38:1084.

Fiddler, W., Pensabene, J.W., Doerr, R.C. and Dooley, C.J. (1977) The presence of dimethyl- and diethyl-nitrosamines in deionized water. Food and Cosmetics Toxicology, 15:441-443.

Fine, D.H., Ross, R., Rounbehler, D.P., Silvergleid, A. and Song, L. (1977) Formation *in vivo* of volatile N-nitrosamines in man after ingestion of cooked bacon and spinach. Nature, 265:753-755.

Gillatt, P.N., Palmer, R.C., Smith, P.L.R., Walters, C.L. and Reed, P.I. (1985) Susceptibilities of drugs to nitrosation under simulated gastric conditions. Food and Cosmetics Toxicology, 23(9):849-855.

Gough, T.A., Goodhead, K. and Walter, C.L. (1976) Distribution of some volatile nitrosamines in cooked bacon. Journal of the Science of Food and Agriculture, 27:181-185.

Greenblatt, M., Mirvish, S.S. and So, B.T. (1971) Nitrosamine studies: Induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice. Journal of the National Cancer Institute, 46:1029-1034.

Groenen, P.J., De Cock-Bethbeder, M.W., Bouwman, J. and Dhont, J.H. (1980) Formation of N-nitrosamines and N-nitrosamino acids from food products and nitrite under simulated gastric conditions. In: N-Nitroso Compounds: Analysis, Formation and Occurrence. Eds: E.A. Walker, L. Griciute, M. Castegnaro and M. Borzsonyi; International Agency for Research on Cancer; Publication #31; Lyon, France; p. 215-227.

Groenen, P.J., Luten, J.B., Dhont, J.H., De Cock-Bethbeder, M.W., Prins, L.A. and Vreeken, J.W. (1982) Formation of volatile N-nitrosamines from food products, especially fish, under simulated gastric conditions. In: N-Nitroso Compounds: Occurrence and Biological Effects. Eds: H. Bartsch, M. Castegnaro, I.K. O'Neill and M. Okada; International Agency for Research on Cancer; Publication # 41; Lyon, France; p. 99-112.

Havery, D.C., Hotchkiss, J.H. and Fazio, T.F. (1982) Rapid determination of volatile nitrosamines in nonfat dry milk. Journal of Dairy Science, 65:182-185.

Havery, D.C. and Fazio, T.F. (1985) Human exposure to nitrosamines from food. Food Technology, 39(1):80-83.

Hawksworth, G.M. and Hill, H.J. (1971) Bacteria and the N-nitrosation of secondary amines. British Journal of Cancer, 25:520-526.

Hildrum, K.I., Williams, J.L. and Scanlan, R.A. (1975) Effect of sodium chloride concentration on the nitrosation of proline at different pH levels. Journal of Agriculture and Food Chemistry, 23(3):439-442.

Hoffman, D., Brunneman, K.D., Adams, J.D. and Hecht, S.S. (1984) Formation and analysis of N-nitrosamines in tobacco products and their endogenous formation in consumers. In: N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. Eds: I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long and H. Bartsch; International Agency for Research on Cancer; Publication #57; Lyon, France; p. 743-762.

Hotchkiss, J.H. and Vecchio, A.J. (1983) Analysis of direct contact paper and paperboard food packaging for N-Nitrosomorpholine and morpholine. Journal of Food Science, 48:240-242.

Hotchkiss, J.H. and Vecchio, A.J. (1985) N-nitrosamines in fried-out bacon fat and its use as a cooking oil. Food Technology, 39(1):67-73.

Ireland, C.B., Hytrek, F.R. and Lasoski, B.A. (1980) Aqueous extraction of N-nitrosamines from elastomers. American Industrial Hygeine Association Journal, 41:895.

Lakritz, L., Simenhoff, M.L., Dunn, S.R. and Fiddler, W. (1980) N-nitrosdimethylamine in human blood. Food and Cosmetics Toxicology, 18:77-79.

Lane, R.P. and Bailey, M.E. (1973) The effect of pH on dimethylnitrosamine formation in human gastric juice. Food and Cosmetics Toxicology, 11:851-854.

Lehninger, A. (1982) Principles of Biochemistry. Worth Publishers, Inc., New York, NY; p. 688.

Libbey, L.M., Scanlan, R.A. and Barbour, J.F. (1980) N-nitrosodimethylamine in dried dairy products. Food and Cosmetics Toxicology, 18:459-461.

Lijinsky, W., Conrad, E. and Van de Bogart, R. (1972) Formation of carcinogenic nitrosamines by interaction of drugs with nitrite. In: N-Nitroso Compounds: Analysis and Formation. Eds: P. Bogovski, R. Preussmann and E.A. Walker; International Agency for Research on Cancer; Publication #3; Lyon, France; p.130-

Magee, P.N. and Barnes, J.M. (1956) The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. British Journal of Cancer, 10:114-122.

Malagelada, J., Longstreth, G.F., Deering, T.B., Summerskill, W.H.J. and Go, V.L.W. (1977) Gastric secretion and emptying after ordinary meals in duodenal ulcer. Gastroenterology, 73:989-994.

Mangino, M.M. and Scanlan, R.A. (1982) Formation of N-nitrosodimethylamine in direct-fired dried malt. American Society of Brewing Chemists Journal, 40(2):55-57.

Marinelli, L., Bell, G., Clark, T., Clarke, B., Fazio, T., Feit, M., Griffith, A., Herwig, W., Koski, P., Kuroiwa, Y., Marks, E., Moll, M., O'Brien, T., Scanlan, R., Thomas, G., Wessa, S. and Likens, S. (1981) Nitrosamines in malt and beer. American Society of Brewing Chemists Journal, 39(3):99-106.

Masui, M., Nakahara, H., Ohmori, H. and Sayo, H. (1974) Kinetic studies on the formation of dimethylnitrosamine. Chemical and Pharmacological Bulletin, 22:1846-1849.

Matsui, M., Ohshima, H. and Kawabata, T. (1980) Increase in the nitrosamine content of several fish products upon broiling. Bulletin of the Japanese Society of Scientific Fisheries, 46(5):587-590.

Miller, A.B., Choi, B.C.K., Howe, G.R., Burch, J.D. and Sherman, G.J. (1984) Epidemiological assessment of risk to humans from exposure to nitrosamines. In: N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. Eds: I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long and H. Bartsch; International Agency for Research on Cancer; Publication #57; Lyon, France; p. 929-936.

Miller, D.D. and Schricker, B.R. (1982) *In vitro* estimation of food iron availability. In: Nutritional Bioavailability of Iron. Ed: Constance Kies; American Chemical Society; Symposium series #203; Washington, D.C.; p. 11-26.

Mills, A.L. and Alexander, M. (1976) N-nitrosamine formation by cultures of several microorganisms. Applied and Environmental Microbiology, 31(6):892-895.

Mirvish, S.S. (1975) Formation of N-nitroso compounds: Chemistry, kinetics and *in vivo* occurance. Toxicology and Applied Pharmacology, 31:325-351.

Mirvish, S.S., Bulay, O., Runge, R.G. and Patil, K. (1980) Study of carcinogenicity of large doses of dimethylnitramine, N-nitroso-L-proline and sodium nitrite administered in drinking water to rats. Journal of the National Cancer Institute, 64(6):1435-1442.

Miwa, M., Steuhr, D.J., Marletta, M.A., Wishnok, J.S. and Tannenbaum, S.R. (1986) N-nitrosamine formation by macrophages. In: Proceedings of IX International Symposium on N-Nitroso Compounds; Vienna, Austria. International Agency for Research on Cancer; Lyon, France.

Morrison, J.B. and Hecht, S.S. (1984) A sensitive new method for the detection of N-nitrosomorpholine formation *in vivo*. In: N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer. Eds: I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long and H. Bartsch; International Agency for Research on Cancer; Publication #57; Lyon, France; p. 185-192.

Mueller, R.L., Hagel, H.J., Wild, H., Ruppin, H. and Domschke, W. (1986) Nitrate and nitrite in normal gastric juice. Oncology, 43:50-56.

Normington, K.W., Baker, I., Molina, M., Wishnok, J.S., Tannenbaum, S.R., and Puju, S. (1986) Characterization of a nitrite scavenger, 3-hydroxy-2-pyranone, from Chinese wild plum juice. Journal of Agricultural and Food Chemistry, 34:215-217.

O'Brien, T.J., Lukes, B.K. and Scanlan, R.A. (1980) Control of N-nitrosodimethylamine in malt through the use of liquid/gaseous sulfur dioxide. MBAA Technical Quarterly, 17(4):196-200.

Ohshima, H. and Bartsch, H. (1981) Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in urine. Cancer Research, 41:3658-3662.

Pignatelli, B., Friesen, M. and Walker, E.A. (1980) The role of phenols in catalysis of nitrosamine formation. In: N-Nitroso Compounds: Analysis, Formation and Occurrence. Eds: E.A. Walker, L. Griciute, M. Castegnaro and M. Borzsonyi; International Agency for Research on Cancer; Publication #31; Lyon, France; p. 95-109.

Preussmann, R. (1984) Carcinogenic N-nitroso compounds and their environmental significance. Naturwissenschaften, 71:25-30.

Ridd, J.H. (1961) Nitrosation, diazotization and deamination. Quarterly Reviews of the Chemical Society (London), 15:418-441.

Ruddell, W.S.J., Bone, E.S., Hill, M.J., Blendis, L.M. and Walters, C.L. (1976) Gastric juice nitrite: A risk factor for cancer in the hypochlorhydric stomach. Lancet, November 13, p. 1037-1039.

Sander, J. and Burkle, G. (1969) Induction of malignant tumors in rats by simultaneous feeding of nitrite and secondary amines. Z. Krebsforsch, 73:54-66.

Sander, J. and Seif, F. (1969) Bakterielle reduktion von nitrat im magen des menschen als ursache einer nitrosamin-bildung. Arzneimittel-Forsch., 19:1091-1093.

Scanlan, R.A. (1983) Formation and occurrence of nitrosamines in food. Cancer Research, 43(May):2435s-2440s.

Scanlan, R.A. and Reyes, F.G. (1985) An update on analytical techniques for N-nitrosamines. Food Technology, 39(1):95-99.

Scheunig, G. and Ziebarth, D. (1976) Formation of nitrosamines by interaction of some drugs with nitrite in human gastric juice. In: Environmental N-Nitroso Compounds-Analysis and Formation. Eds: E.A. Walker, P. Bogovski and L. Griciute; International Agency for Research on Cancer; Publication #14; Lyon, France; p. 269-277.

Sen, N.P., Seaman, S. and McPherson, M. (1980) Further studies on the occurrence of volatile and non-volatile nitrosamines in foods. In: N-Nitroso Compounds: Analysis, Formation and Occurrence. Eds: E.A. Walker, L. Griciute, M. Castegnaro and M. Borzsonyi; International Agency for Research on Cancer; Publication #31; Lyon, France; p. 457-463.

Sen, N.P., Seaman, S. and Bickis, M. (1982) Gas-liquid chromatographic-Thermal Energy Analyzer determination of N-nitrosodimethylamine in beer: Collaborative study. Journal of Association of Official Analytical Chemists; 65(3):720-729.

Sen, N.P. Seaman, S. and Karpinsky, K. (1984) Determination of N-nitrosodimethylamine in nonfat dry milk: Collaborative study. Journal of Association of Official Analytical Chemists; 67(2):232-236.

Sen, N.P., Tessier, L., Kushwaha, S.C. and Clarkson, S.G. (1985) Nitrosamines in baby bottle nipples and pacifiers: Occurance, migration and effect of infant formulas and fruit juices on *in vitro* formation of nitrosamines under simulated gastric conditions. Journal of Agricultural and Food Chemistry, 33:428-433.

Sen, N.P. and Baddoo, P.A. (1986) Origin of N-nitrosomorpholine contamination in margarine. Journal of Food Science, 51(1):216-217 & 236.

Singer, S.S., Lijinsky, W. and Singer, G.M. (1978) Transnitrosation: An important aspect of the chemistry of aliphatic nitrosamines. In: Environmental Aspects of N-Nitroso Compounds. Eds: E.A. Walker, M. Castegnaro, L. Griciute and R.E. Lyle; International Agency for Research on Cancer; Publication #19; Lyon, France; P.175-181.

Singer, S.S., Singer, G.M. and Cole, B.B. (1980) Alicyclic nitrosamines and nitrosamino acids as transnitrosating agents. Journal of Organic Chemistry, 45:4931-4935.

Smith, T.A. (1980) The amine content of foods. Food Chemistry, vol. 6, 169-200.

Spiegelhalder, B., Eisenbrand, G. and Preussmann, R. (1976) Influence of dietary nitrate on nitrite content of human saliva: Possible relevance to *in vivo* formation of N-nitroso compounds. Food and Cosmetics Toxicology, 14:545-548.

Spiegelhalder, B. and Preussmann, R. (1985) *In vivo* nitrosation of amidopyrine in humans: Use of 'ethanol effect' for biological monitoring of nitrosodimethylamine in urine. Carcinogenesis, 6(4):545-548.

Stich, H.F., Dunn, B.P., Pignatelli, B., Ohshima, H. and Bartsch, H. (1984) Dietary phenolics and betel nut extracts as modifiers of N-nitrosation in rat and man. In: N-Nitroso Compounds Occurrence, Biological Effects and Relevance to Human Cancer. Eds: I.K. O'Neill, R.C. Von Borstel, C.T. Miller, J. Long and H. Bartsch; International Agency for Research on Cancer; Publication #57; Lyon, France; p. 213-222.

Swann, P.F., Coe, A.M. and Mace, R. (1984) Ethanol and dimethylnitrosamine and diethylnitrosamine metabolism and disposition in the rat. Possible relevance to the influence of ethanol on human cancer disease. Carcinogenesis 5(10):1337-1343.

Tannenbaum, S.R., Sinskey, A.J., Weisman, M. and Bishop, W. (1974) Nitrite in human saliva. Its possible relation to nitrosamine formation. Journal of the National Cancer Institute, 53:79-84.

Tannenbaum, S.R., Weisman, M. and Fett, D. (1976) Effect of nitrate intake on nitrite formation in human saliva. Food and Cosmetics Toxicology, 14:549-552.

Tannenbaum, S.R. (1986) Endogenous formation of N-nitroso compounds: A current perspective. In: Proceedings of IX International Symposium on N-Nitroso Compounds; Vienna, Austria. International Agency for Research on Cancer; Lyon, France.

Tsuda, M., Nagai, A., Suzuki, H., Hayashi, T., Ikeda, M., Kuratsune, M., Sato, S. and Sugimura, T. (1986) Effect of cigarette smoking and dietary factors on the amounts of N-nitrosothioproline and N-nitroso-2-methylthioproline in human urine. In: Proceedings of IX International Symposium on N-Nitroso Compounds; Vienna, Austria. International Agency for Research on Cancer; Lyon, France.

Wagner, D.A., Shuker, D.E.G., Bilmazes, C., Obiedzinski, M., Baker, I., Young, V.R. and Tannenbaum, S.R. (1985) Effects of vitamin C and E on endogenous synthesis of N-nitrosamino acids in humans: Precursor-product studies with ^{15}N-nitrate. Cancer Research, 45(December):6519-6522.

Wagner, D.A. and Tannenbaum, S.R. (1985) *In vivo* formation of N-nitroso compounds. Food Technology, 39(1):89-90.

Walters, C.L., Newton, B.E., Parke, D.V. and Walker, R. (1974) The precursors of N-nitroso compounds in foods. In: N-Nitroso Compounds in the Environment. Eds: P. Bogovski and E.A. Walker; International Agency for Research on Cancer; Publication #9; Lyon, France; p.223-228.

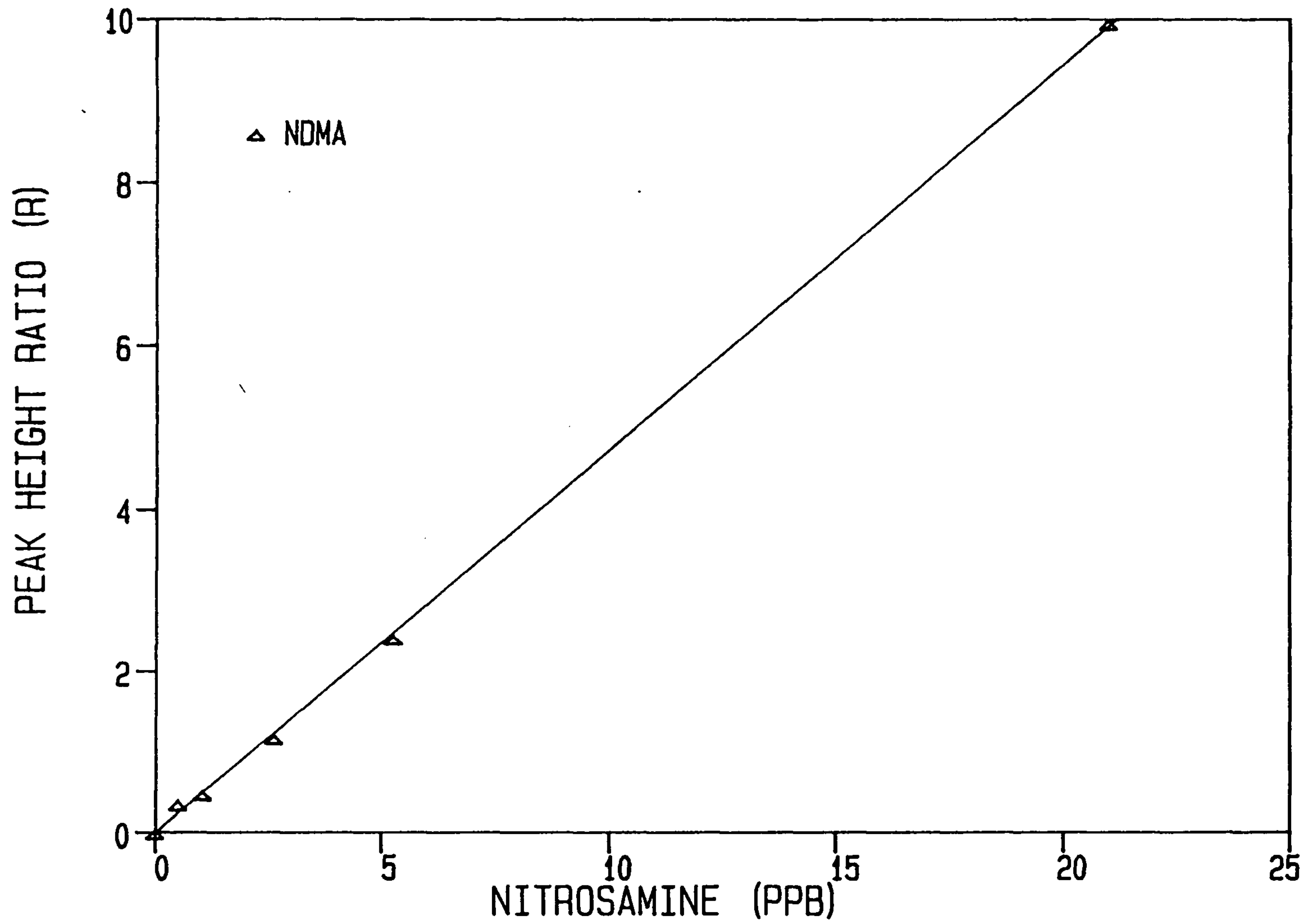
Walters, C.L., Dyke, C.C., Saxby, M.J. and Walker, R. (1976) Nitrosation of food amines under stomach conditions. In: Environmental N-Nitroso Compounds-Analysis and Formation. Eds: E.A. Walker, P. Bogovski and L. Griciute; International Agency for Research on Cancer; Publication #14; Lyon, France; p. 181-193.

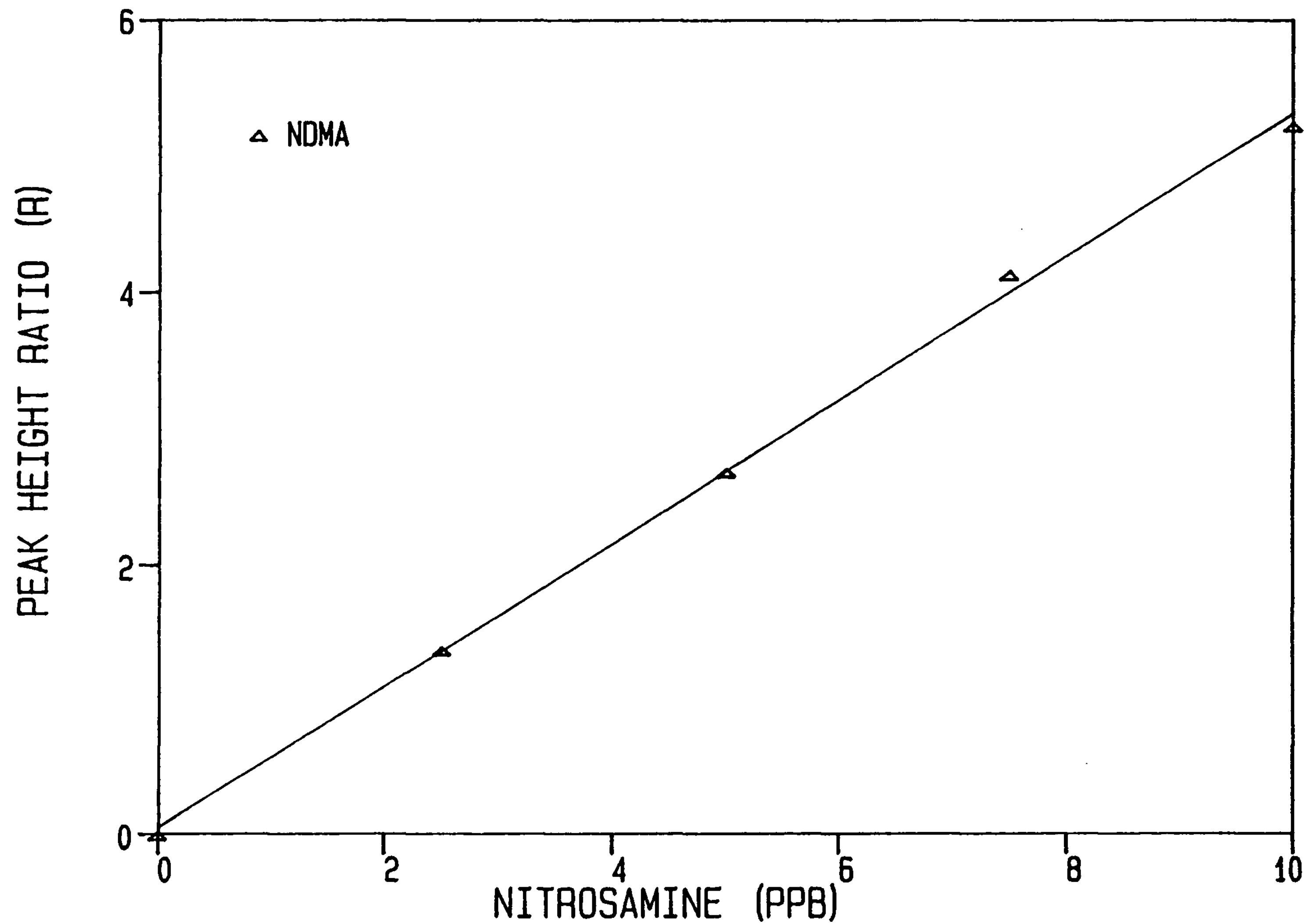
Wang, T., Kakizoe, T., Dion, P., Furrer, R., Varghese, A.J. and Bruce, W.R. (1978) Volatile nitrosamines in normal human faeces. Nature, 276:280-281.

White, J.W. (1975) Relative significance of dietary sources of nitrate and nitrite. Journal of Agricultural and Food Chemistry, 23(5):886-891.

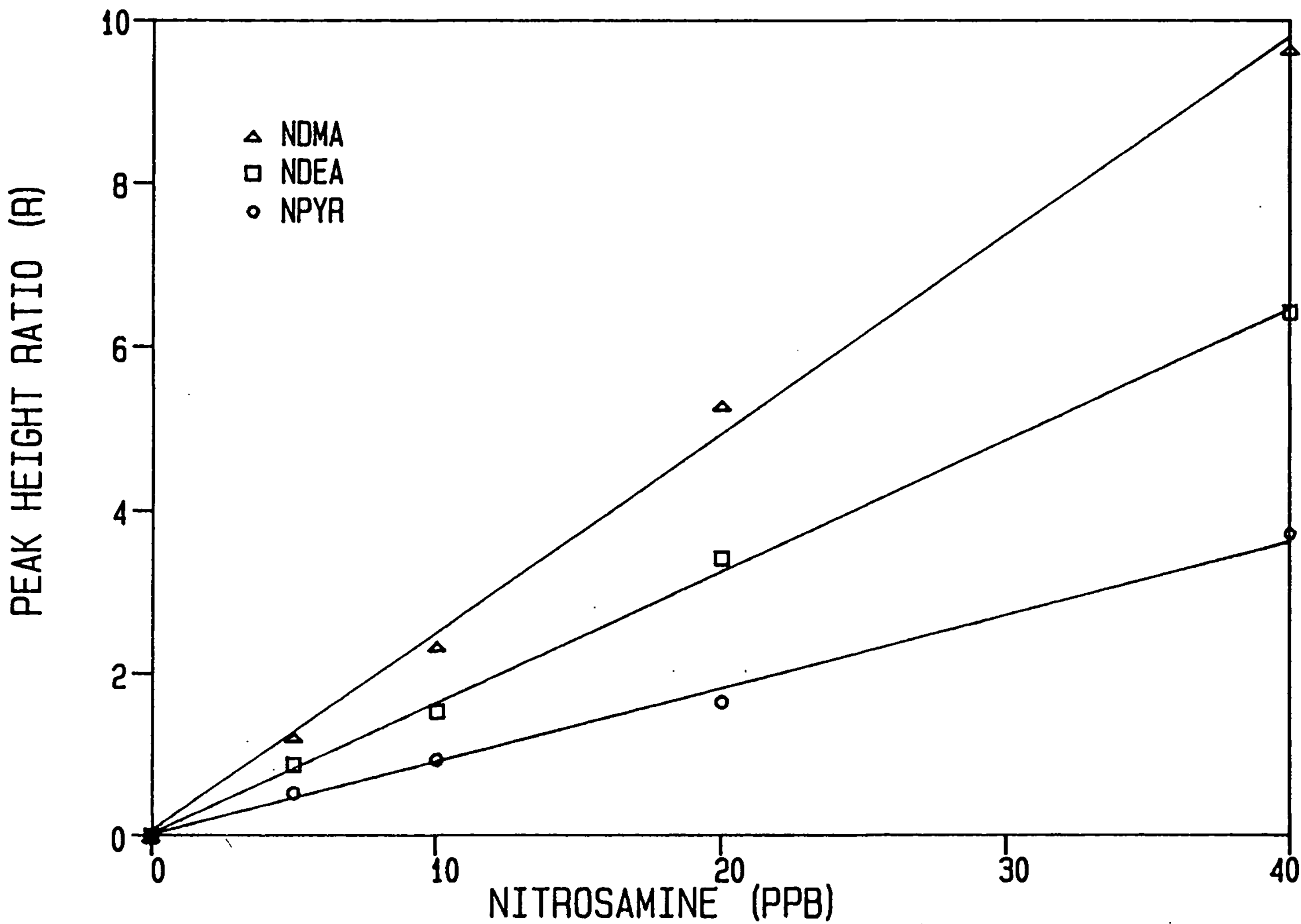
Zeisel, S.H., DaCosta, K., Edrise, B.M. and Fox, J.G. (1986) Transport of dimethylamine, a precursor of nitrosodimethylamine, into stomach of ferret and dog. Carcinogenesis, 7(5):775-778.

VIII. APPENDIX

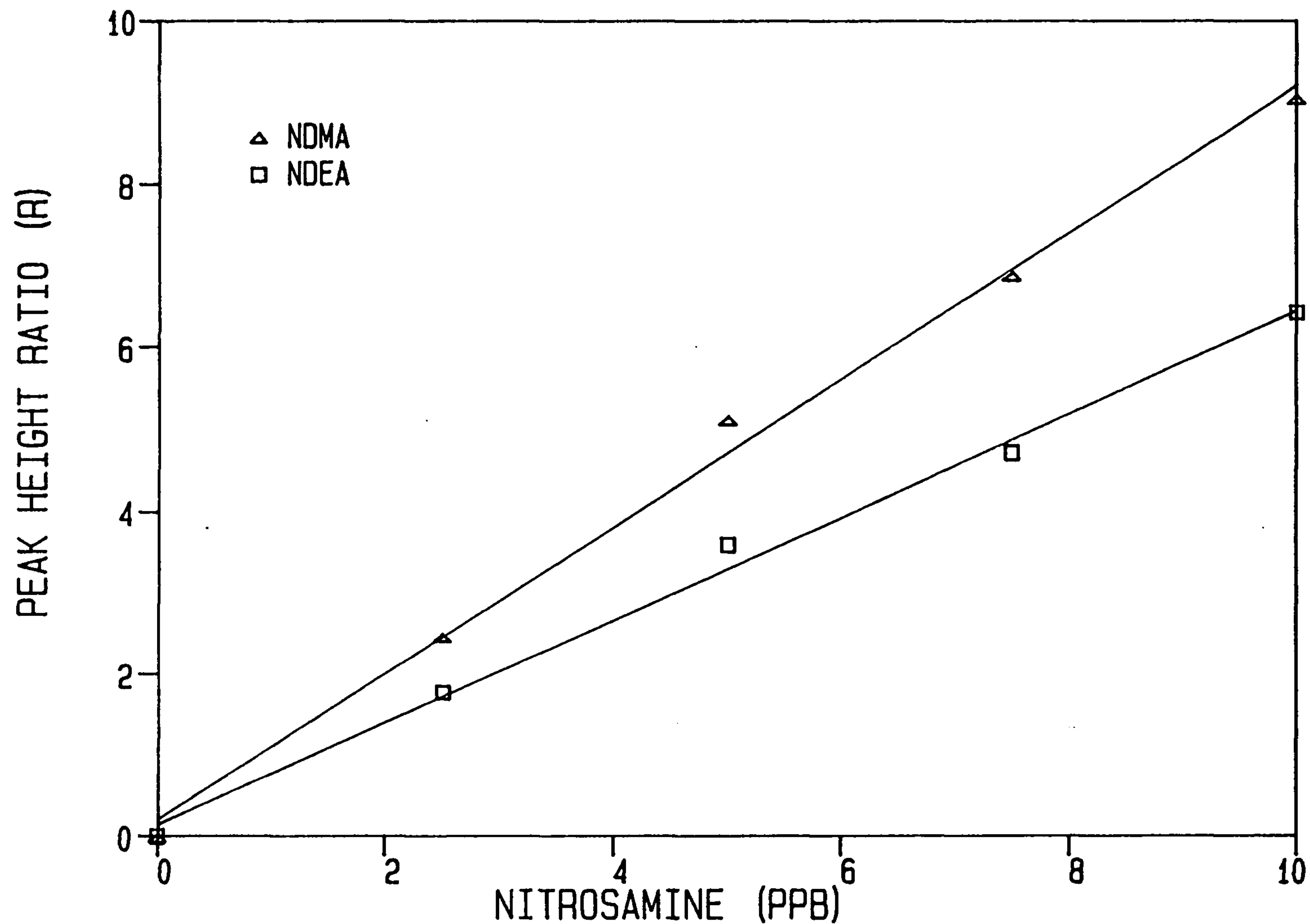

STANDARD CURVE-BEER SIMULATED GASTRIC NITROSATION
NDMA
PEAK HEIGHT RATIO (R)
NITROSAMINE (PPB)
0
2
4
6
8
10
0
5
10
15
20
25

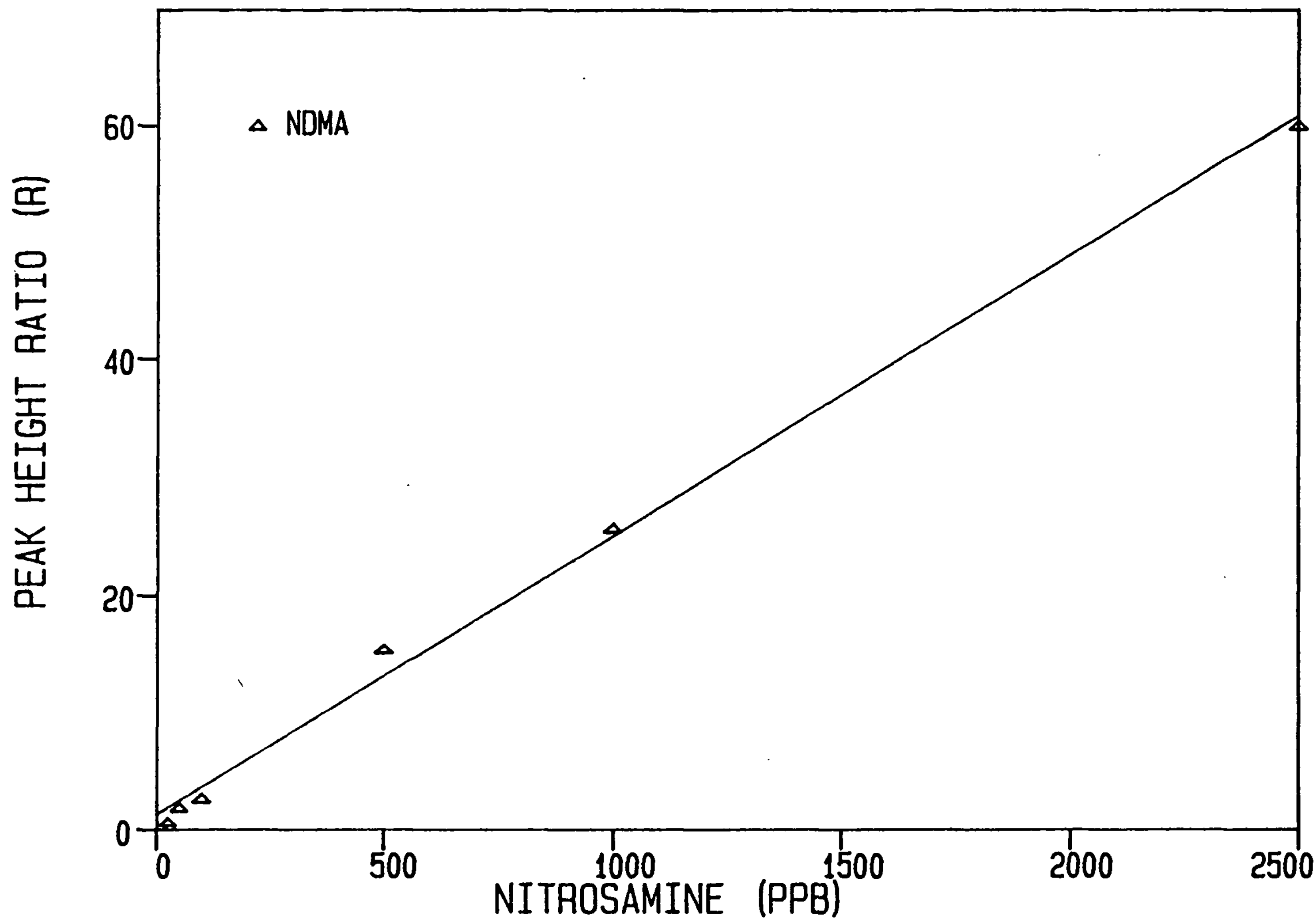

STANDARD CURVE-NDM SIMULATED GASTRIC NITROSATION
NDMA
PEAK HEIGHT RATIO (R)
NITROSAMINE (PPB)
0
2
4
6
8
10

STANDARD CURVE-BACON SIMULATED GASTRIC NITROSATION

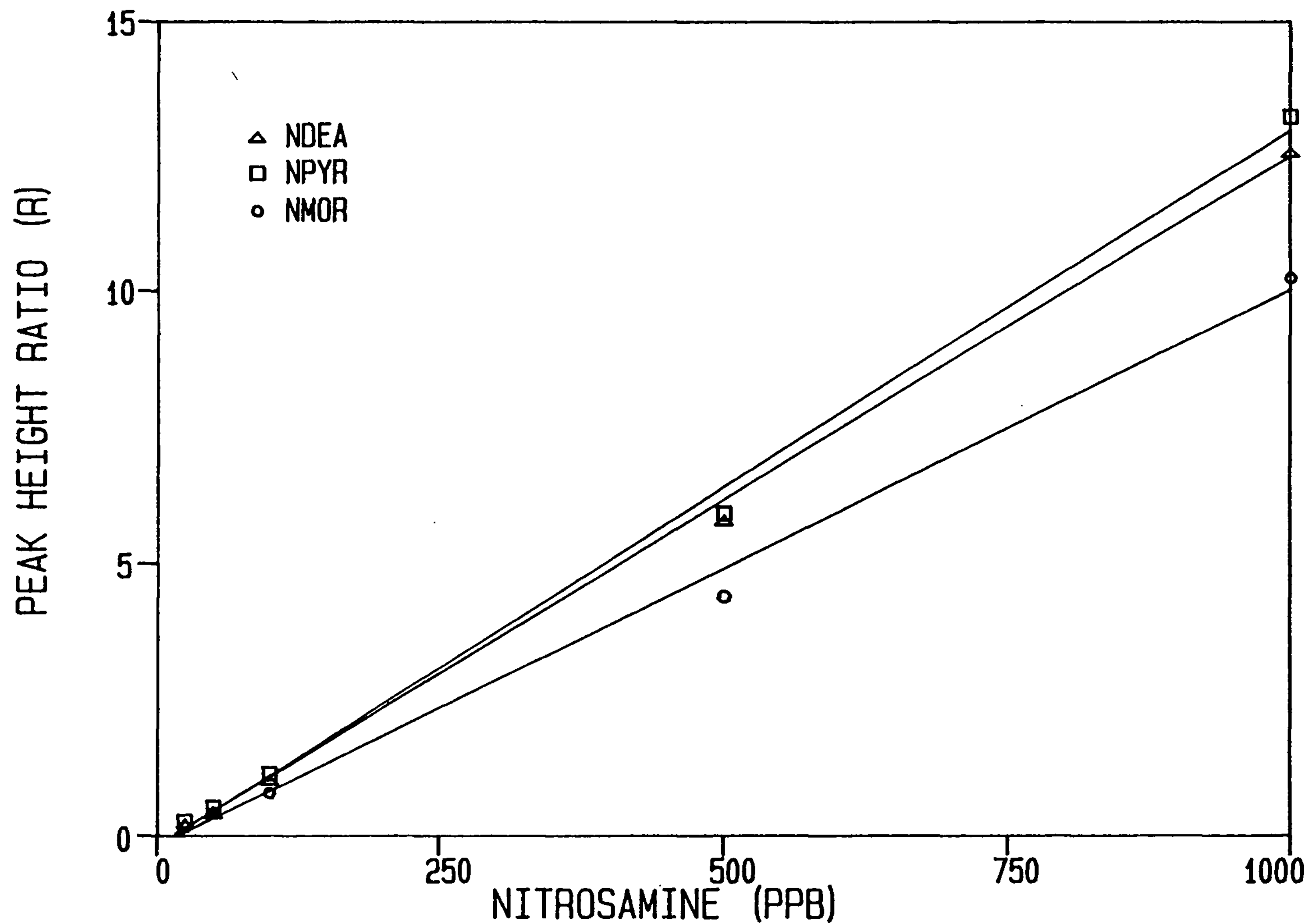


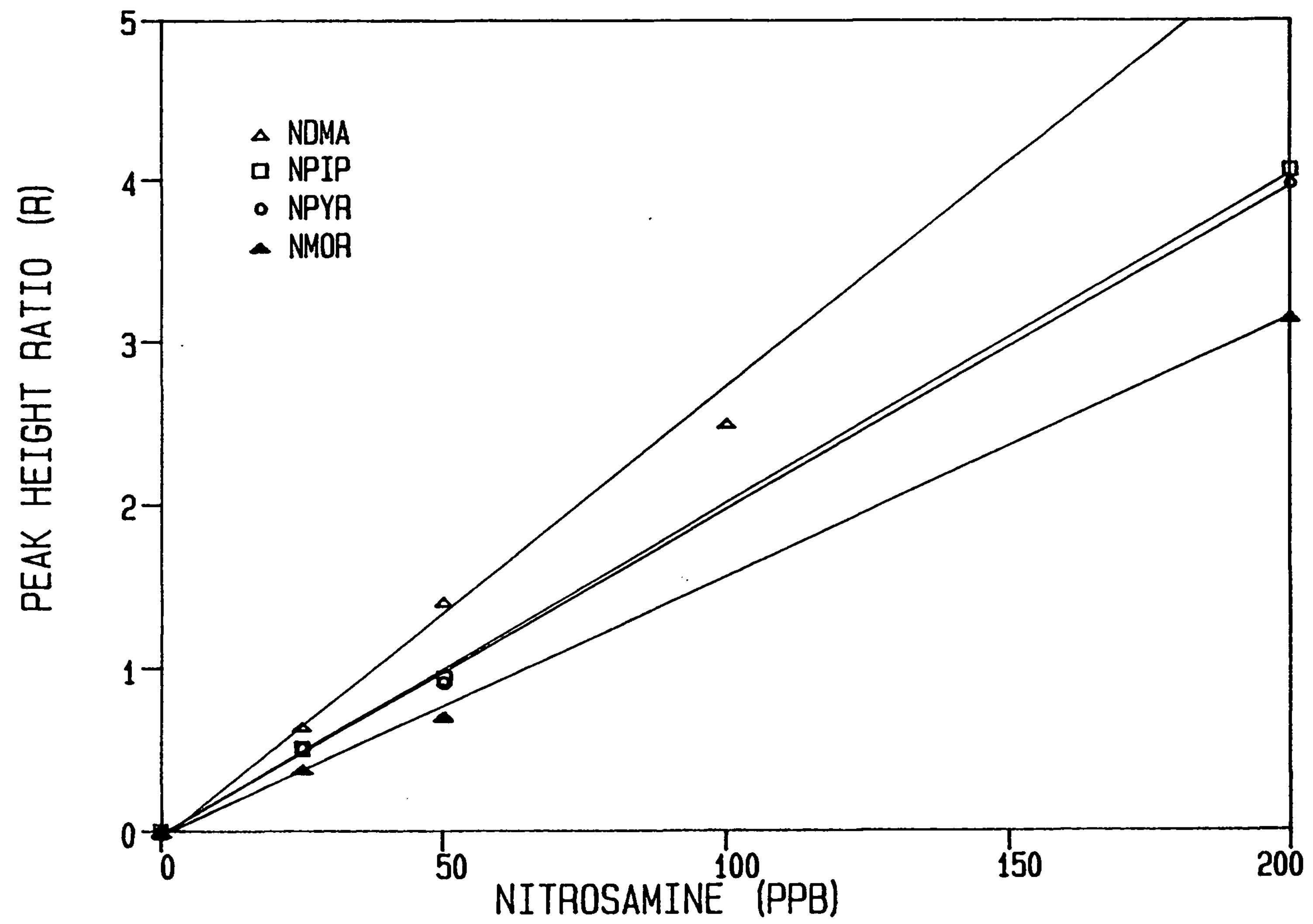
STANDARD CURVE-FISH SIMULATED GASTRIC NITROSATION



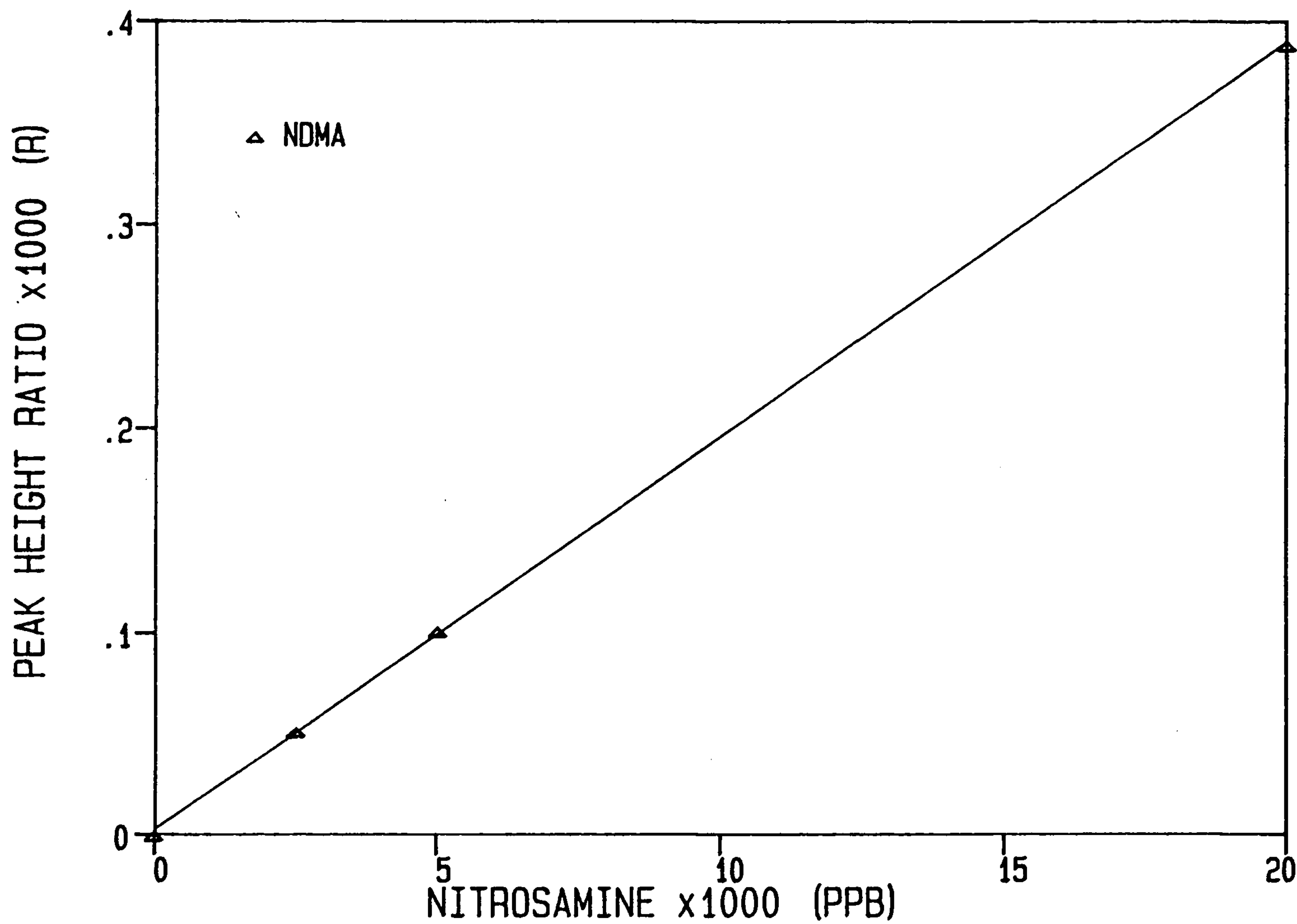

STANDARD CURVE: BEER EXTREME NITROSATION
NDMA
PEAK HEIGHT RATIO (R)
0
20
40
60
0
500
1000
1500
2000
2500
NITROSAMINE (PPB)

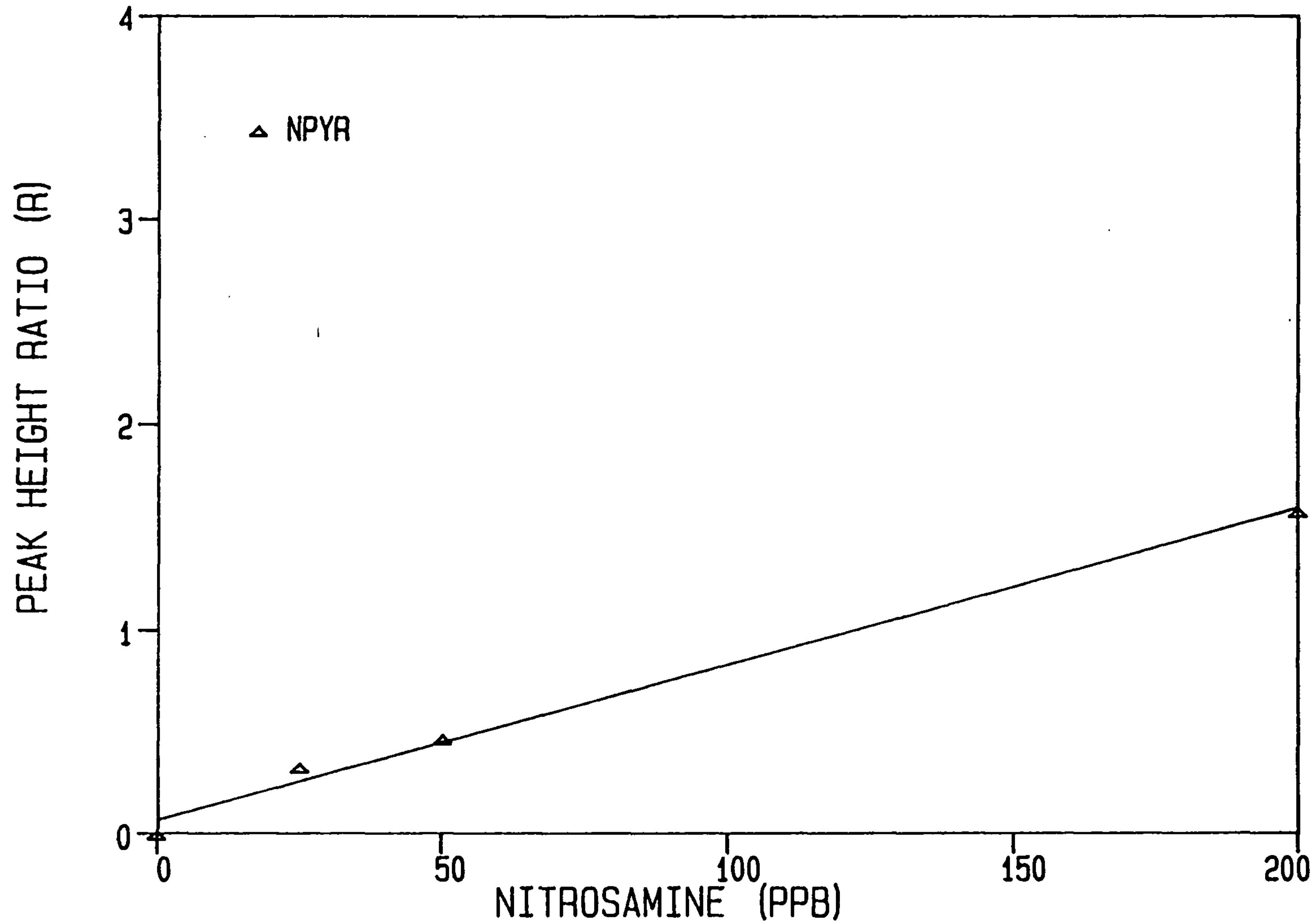
STANDARD CURVE: BEER EXTREME NITROSATION




STANDARD CURVE: NDM EXTREME NITROSATION
PEAK HEIGHT RATIO (R)
NITROSAMINE (PPB)
NDMA
NPIP
NPYR
NMOR
0
1
2
3
4
5
0
50
100
150
200

STANDARD CURVE: FISH EXTREME NITROSATION




STANDARD CURVE: FISH EXTREME NITROSATION
NPYR
PEAK HEIGHT RATIO (R)
0
1
2
3
4
0
50
100
150
200
NITROSAMINE (PPB)