A METHOD FOR THE IDENTIFICATION AND QUANTITATION OF VOLATILE NITROSAMINES

JAMES HIGGINS WOLFRAM

Follow this and additional works at: https://scholars.unh.edu/dissertation

Recommended Citation
https://scholars.unh.edu/dissertation/1087

This Dissertation is brought to you for free and open access by the Student Scholarship at University of New Hampshire Scholars' Repository. It has been accepted for inclusion in Doctoral Dissertations by an authorized administrator of University of New Hampshire Scholars' Repository. For more information, please contact nicole.hentz@unh.edu.
INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106
WOLFRAM, James Higgins, 1943-  
A METHOD FOR THE IDENTIFICATION AND QUANTITATION  
OF VOLATILE NITROSOAMINES.  

University of New Hampshire, Ph.D., 1975  
Chemistry, biological  

Xerox University Microfilms, Ann Arbor, Michigan 48106
A METHOD FOR THE IDENTIFICATION AND QUANTITATION
OF VOLATILE NITROSAMINES

by

JAMES H. WOLFRAM
B.S., Ohio State University, 1965

A THESIS

Submitted to the University of New Hampshire
In Partial Fulfillment of
The Requirements for the Degree of

Doctor of Philosophy
Graduate School
Department of Biochemistry
May, 1975
This thesis has been examined and approved.

Edward J. Herbst
Thesis director, Edward J. Herbst, Professor of Biochemistry

George O. Eates, Associate Professor of Plant Science

Robert E. Lyle, Professor of Chemistry

James A. Stewart, Associate Professor of Biochemistry

Miyoshi Ikawa, Professor of Biochemistry

March 17, 1975
Date
ACKNOWLEDGEMENTS

The author expresses gratitude to Dr. Edward J. Herbst for his patience and guidance during the course of this research. The author appreciates the excellent technical assistance and advice of Mr. John Wakefield and Mr. Frank Hoose. The author also appreciates the valuable discussion concerning this work offered by Dr. Frederick Liberatore.

The author acknowledges the award of a Graduate Research Assistantship supported by a grant from USDA, CSRS grant #116-15-09, "Carcinogenic Nitrosamines and Precursors in the Biological Environment"
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>METHODS AND MATERIALS</td>
<td>28</td>
</tr>
<tr>
<td>A. Photolysis of Nitrosamines</td>
<td>28</td>
</tr>
<tr>
<td>B. Nitroso-Group Determination</td>
<td>28</td>
</tr>
<tr>
<td>C. Amine Determination</td>
<td>29</td>
</tr>
<tr>
<td>D. Acid Denitrosation of Nitrosamines</td>
<td>29</td>
</tr>
<tr>
<td>E. Dansylation Procedure</td>
<td>30</td>
</tr>
<tr>
<td>F. Derivatization with NBD-C1</td>
<td>31</td>
</tr>
<tr>
<td>G. TLC, Fluorometric and Quantitation Procedures</td>
<td>32</td>
</tr>
<tr>
<td>H. Seafood Sample Preparation</td>
<td>32</td>
</tr>
<tr>
<td>I. Determination of Dimethylamine (DMA) in Seafood</td>
<td>35</td>
</tr>
<tr>
<td>RESULTS AND DISCUSSION</td>
<td>37</td>
</tr>
<tr>
<td>A. Separation of Dansylated Secondary Aliphatic Amines</td>
<td>37</td>
</tr>
<tr>
<td>B. Photochemical Denitrosation of Nitrosamines</td>
<td>41</td>
</tr>
<tr>
<td>C. Recovery of Amine from Photochemical Denitrosation</td>
<td>44</td>
</tr>
<tr>
<td>D. Acid Denitrosation of Nitrosamines</td>
<td>49</td>
</tr>
<tr>
<td>E. Derivatization with NBD-C1</td>
<td>55</td>
</tr>
<tr>
<td>F. Seafood Survey</td>
<td>66</td>
</tr>
<tr>
<td>G. Survey of Seafood for Nitrosamines</td>
<td>73</td>
</tr>
<tr>
<td>H. Dimethylamine in Seafood</td>
<td>79</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>83</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>84</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Solvent Systems Tested for the Separation of Dansyl-ated Secondary Amines on Silica Gel Plates................. 40
2. Rf Values for Dansyl-Amino and NBD-Amino Derivatives.... 42
3. Denitrosation and the Loss of Nitrite vs. Time of UV-Treatment................................................................. 43
4. Effect of Na$_2$CO$_3$ Concentration on the Recovery of the Nitroso-Group.......................................................... 45
5. Denitrosation of N-Nitrosamines by Photochemical Splitting: Comparison with the Procedure of Fan and Tannenbaum.......................................................... 48
6. UV-Denitrosation of Nitrosopiperidine as Determined by Recovery of Piperidine................................................. 50
7. Acid-Denitrosation of Nitrosamines as Determined by Recovery of Nitrite.......................................................... 52
8. Initial Results of Acid-Denitrosation of Nitrosamines................................................................. 53
9. Effect of pH on the Derivatization of Amines with NBD-Cl................................................................. 57
10. Effect of Volume of MIBK and the Amount of NBD-Cl on The Fluorescence Yield of Secondary Amine Derivatives.... 58
11. Acid-Denitrosation of Nitrosamines in Methylene Chloride and Derivatization of the Amine with NBD-Cl................................................................. 67
12. Recoveries of Volatile Nitrosamines at Various Stages of the Procedure.......................................................... 72
LIST OF TABLES (cont'd)

13. Recoveries of N-Nitrosamines from Fresh or Frozen Clams .................................................... 74
14. Survey of nitrosamines in Frozen and Processed Seafoods .................................................................................................................... 75
15. Effect of Frozen Storage on Nitrosamine Production........ 78
16. Recovery of Secondary Amines After Steam Distillation .................................................................................................................. 81
17. DMA Content of Seafood Products.................... 82
LIST OF FIGURES

1. Structural Formulae of Seven Volatile N-Nitrosamines.......................... 39

2. Linear Calibration Curves of Absorbance vs. Concentration for Photo-Denitrosation of Nitrosamines............................................................... 47

3. Linearity of Fluorescence with Concentration of Three Cyclic Secondary Amine-NBD Derivatives................................. 60

4. Linearity of Fluorescence with Concentration of Four Secondary Amine-NBD Derivatives............................... 62

5. Chromatographic Separation by TLC of the Mixture of NBD-Amino Derivatives................................. 65

6. A Tracing from a TLC Plate Scan.................................................... 77
ABSTRACT

A METHOD FOR THE IDENTIFICATION AND QUANTITATION
OF VOLATILE NITROSAMINES

by

JAMES H. WOLFRAM

Research on an improved method for the detection of N-nitrosamines was initiated because of the increased concern about the occurrence of these carcinogenic compounds in foods. The procedure that was developed is applicable to the identification and quantitation of seven volatile N-nitrosamines at a sensitivity of approximately 10 ppb. The method is based on the denitrosation of the nitrosamine to yield the parent secondary amine. The amine is reacted with a fluorogenic reagent producing a highly fluorescent derivative.

The denitrosation of the nitrosamines was initially attempted by irradiating acidified aqueous samples with short-wave UV light. The compounds underwent photodecomposition as was determined by the release of the nitroso-group which was detected as a highly colored diazo compound and quantitated colorimetrically. However, on analyzing the irradiated samples of dimethyl-, diethyl-, dipropyl-, and dibutyl-nitrosamines quantities of amine products could not be detected.

Denitrosation was achieved by using a modification of the acid denitrosation technique, first reported by Eisenbrand, in which viii
the nitroso-group is removed from the nitrosamine in a medium of glacial acetic acid and hydrobromic acid yielding the secondary amine entity. In the modified procedure the nitrosamines were denitrosated in a medium of anhydrous methylene chloride containing 3% hydrobromic acid at room temperature. The reaction was terminated by the addition of aqueous acid and the mixture was taken to dryness. The residue containing the amine salts was dissolved and reacted with NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol. The resulting derivatives are highly fluorescent compounds. Aliquots of the sample containing the fluorescent derivatives were placed on a silica gel thin-layer chromatography plate and separated by ascending chromatography. Quantitation was achieved by direct scanning with a fluorometer. The peak heights of the unknowns were compared to standards developed on the same plate. The sensitivity allowed detection of 1-2 ng of dimethylamine which is equivalent to approximately 5 ppb of dimethyl-nitrosamine.

This method was applied in a survey of seafoods with emphasis on shellfish species. The extraction procedure consisted of homogenizing the sample followed by steam distillation. The distillate was partitioned against aqueous acid to remove amine contaminants. The sample was concentrated and, for additional purification, was applied to an alumina column, eluted with methylene chloride and further concentrated to a small volume.

The concentrated sample was divided into two equal fractions, only one of which was denitrosated, and both of which were taken to dryness and derivatized. The fluorometric scans of both denitrosated ("split") and "unsplit" samples were recorded and compared
thus allowing correction for background amine contaminants. Recoveries of volatile N-nitrosamines ranged from 50-80 percent except for nitrosopyrrolidine which was recovered to the extent of 25-40 percent.

N-dimethylnitrosamine was found in several processed seafood samples and in several samples of frozen clams and mussels which had been stored for an extended period. Although stringent comparisons of this method with gas-liquid chromatography-mass spectrometry have not been completed, it appears that the fluorescence-scanning procedure will provide an inexpensive and simplified alternative to these standard but complex procedures for the analysis of N-nitrosamines.
INTRODUCTION

Historical Background

N-nitroso and C-nitroso compounds have the nitroso group attached either to a nitrogen atom or to a carbon atom respectively. The chemical reactivity of these two groups of organic compounds differ considerably and is adequately covered by Feuer (1) in two volumes. Biologically many of the N-nitrosamines are carcinogenic, mutagenic and teratogenic, whereas among the C-nitroso compounds, only a few compounds, e.g., p-nitroso-N, N-dimethylaniline (2) have been tested and have shown weak carcinogenesis in laboratory animals.

Although Barnes and Magee (3) are credited as the first to report the carcinogenicity of dimethylnitrosamine (DMN), two earlier reports by Freund (4) in 1937 and Hamilton and Hardy (5) in 1949 described poisonings involving DMN as the possible causative agent.

Barnes and Magee's report (3) pertained to an industrial accident where two of three workers who were using DMN as a solvent became very ill. Both workers showed severe hepatic impairment. Magee and Barnes (6) followed up their report with laboratory studies performed on animals and the results of the tests indicated that DMN caused liver tumors in nearly all of the animals that received the compound.

The Army Chemical Center in Maryland in 1955 (7) reported symptoms of humans exposed to DMN vapors. Diagnostic tests showed liver damage and abnormally high body temperatures in the exposed patients. Jacobson and collaborators (6) within that laboratory
conducted animal tolerances to different levels of DMN vapors. Rats, mice and dogs were tested, with dogs responding to the effects of the vapor at lower concentrations than mice or rats. Two of three dogs died within two days after being exposed to the lowest concentration tested (16 ppm or 48 mg/M³ for four hours).

By 1967 other workers had further tested not only DMN but other nitroso compounds. Two comprehensive reports were published in that year: Magee and Barnes' review (8) covers the chemistry of the nitroso compounds, pathological effects (both acute and chronic aspects), and metabolism and biochemical effects of the nitrosamines. During that same year Druckery et al. (9) published an extensive review on 65 nitrosamines and nitrosamides which had been tested in various laboratory animals. To date, over 100 nitroso compounds have been tested with 80 percent of them demonstrating carcinogenicity in animals.

Momentum for the early research on these compounds from approximately 1954-1967 was stimulated by the potential human toxicity associated with industrial contact. Many toxicity studies on the chemicals' effect on animal physiology were conducted; the toxic chemical in all these investigations was administered by the experimenter and the incidence of tumor production or increase/decrease in activities of various biological functions were measured.

In Norway, during 1960-1962 several outbreaks of liver toxicity in fur-bearing animals occurred (10) and similar disorders among sheep were reported by Koppang in 1964 (11). Koppang (12) demonstrated the toxic element responsible for the liver injury which caused many fatalities had originated in the herring meal fed these animals.
Ender et al. (13) isolated and identified the toxic compound as DMN. Therefore, it was established that a toxic substance was produced in processing the fish meal by the interaction of naturally occurring amines and a preservative, nitrite. Since nitrite is also used as a preservative in processed foods for humans the food chain of not only animals but also of man became implicated. Attention was initially focused on the possible occurrence of these compounds in foodstuffs for human and animal consumption.

Several serious obstacles were encountered. Procedures for the detection of these compounds in foodstuffs were found to be quite inadequate in specificity and sensitivity. Furthermore, the question arose as to which compounds in addition to DMN should be included in the survey. This dilemma although not yet resolved is currently receiving considerable attention.

**Properties of Nitrosamines**

To the chemist, N-nitrosamines have been important compounds for several reasons: high chemical reactivity of the nitroso-group, important intermediates in reactions, compounds for investigating P-π* conjugation, and n-π* and π-π* transitions. In 1863, Geuther (14) first reported the reaction of diethylamine-hydrochloride with sodium nitrite in acidic solution to yield N-diethylnitrosamine (DEN). A.L. Fridman et al. (15) recently published a review covering the chemical literature of nitrosamines.

**Physical Properties**

The smaller N-nitroso dialkylamines are yellowish, oily liquids with a density near 1.0 and boiling points in the range of 150° - 220°C at 760 mm pressure. The dissociation energy of the
nitrogen-nitrogen bond is comparatively low in comparison with that of other organic bonds. The dissociation energy of the nitrogen-nitrogen bond obtained by thermochemical analysis was 32 Kcal mole\(^{-1}\). Kinetic data placed its strength at 43 Kcal mole\(^{-1}\). Recently the energy of dissociation was recalculated to be 55.2 Kcal mole\(^{-1}\). The following energies of dissociation illustrate the "weakness" of this bond in comparison to others expressed in Kcal mole\(^{-1}\): N-N, 32-55; N=N, 61; N=N, 225; C-N, 66; C-C, 80; C=C, 145; C=O, 198 (16).

The nitroso group is bonded to the nitrogen atom of the amine with the general structure of \(R_1 R_2 N-N=O\). (In addition to the designation as N-nitrosamines these compounds are also referred to as nitrosamines or N-nitroso compounds).

The variation in the structure of the "R" groups can give a wide range of physical properties and chemical reactivities to these compounds. Nitrosamines can exist in a monomeric or dimeric form, form hydrogen bonded complexes and even become protonated under some conditions. These forms can also alter physical properties, e.g., absorption spectra. Feuer (1) discussed and reviewed the IR studies performed on nitrosamines and describes the differences in the spectra of monomeric and dimeric forms in a variety of solvents. NMR investigations show the presence of cis-trans isomers for many of the compounds, e.g., Karabatsos (17); Looney (18).

Two major absorption bands appear in the ultraviolet spectra of nitrosamines. The intense band at 235 nm is due to \(\pi\rightarrow\pi^*\) transition and has a molar extinction coefficient of approximately 10,000. The lower intensity absorption maximum is near 350 nm and has been assigned to the \(n-\pi^*\) transition. The molar extinction coefficient
is approximately 100. The protonated species of nitrosamines do not absorb in the 350 nm range. Pensabene et al. (19) recently published spectra, boiling points and gas chromatographic characteristics of 25 nitrosamines.

**Chemical Properties**

The discussion of the chemical reactions of these compounds will focus on the \((N-N=O)\) entity of the molecule, unless stated otherwise. Reduction, oxidation, pyrolysis, photolysis and rearrangement will be discussed.

The principal reaction products these compounds yield upon reduction are secondary amines by elimination of the nitroso group (denitrosation) or unsymmetrical hydrazines through reduction of the nitroso group. Although the choice of products can be enhanced by the conditions and reducing agents chosen, both products are likely to be produced to some degree.

Synthesis of hydrazine products has been accomplished by the use of: acetic acid and zinc dust (20), lithium aluminium hydride (21), catalytic hydrogenation in the presence of palladium (22) and electrochemical reduction (23).

Secondary amines are formed when the nitroso compounds are treated with zinc or tin dust in hydrochloric acid solution (24). Hydrogen chloride is a more efficient denitrosating compound than hydrochloric acid and methods have been proposed for the synthesis of pure secondary amines (25). These methods involve the denitrosation of nitrosamines with hydrogen chloride in organic solvents, e.g., toluene or ether.
According to Fan and Tannenbaum (26) nitrosamines are stable under mild acidic conditions even at elevated temperatures like those needed for distillation. Eisenbrand et al. (27) was able to distill many of the dialkyl and alicyclic nitroso-compounds quantitatively at atmospheric pressure from solutions of 0.2 N acids. However, under anhydrous conditions in the presence of hydrogen bromide Eisenbrand (28) was able to quantitatively denitrosate many of the nitrosamines. A secondary amine and nitrosyl bromide could be recovered as the products. Lunt et al. (29) accomplished denitrosation of several nitrosamines in an anhydrous solution of methylene chloride containing 25% thionyl chloride. In this case only the nitrosyl species was measured. The effects of temperature, time and concentration of hydrogen bromide were studied by Eisenbrand (28). Although quantitative denitrosation was obtained over a wide range of temperature, time and concentration of hydrogen bromide, the presence of small amounts of water greatly reduced denitrosation.

Oxidation of the nitroso-species results in addition of oxygen to form \( N - N \). The resulting compounds are named nitramines. Emmons (30) prepared nitramines from nitrosamines in greater than 90% yield using a mixture of trifluoroacetic acid and hydrogen peroxide.

Richatson and Stevens (31) investigated the thermal degradation products of nitrosamines. The reaction products thermally derived supported the theory that two pathways were involved. Richatson further determined that substituents on the amino nitrogen of the nitrosamines governed the pathway taken. The products observed were those resulting from the combination of the radical fragments produced under these conditions. The basic difference between the pathways is the
elimination of nitrogen gas by one route. In the second pathway both fragment components retain a nitrogen atom and thus form nitrogen-containing products. Temperatures used in these pyrolysis studies ranged from 190° - 240°C.

Photolysis of the chemical bond between the two nitrogen atoms has been investigated several times since the work of Bramford (32). Bramford observed that DMN and DEN in the vaporous state in the presence of UV light yielded a mixture of secondary amines and other compounds formed from free radical combination. Since this work, others have investigated the photolysis of nitrosamines and examined the products more closely. Different theories have been proposed on the mechanism of photodissociation. Variable conditions under which the irradiation of the nitroso compounds was carried out may account for the differing ideas. Bramford suggested a dialkylamino radical as an intermediate prior to the formation of the secondary amine. Burgess and Lavanish (33) found no photo decomposition of dibutynitrosamine (DBN) in hydrocarbon solutions at wavelengths above 300 nm. However, upon addition of a proton donor, photolysis took place and an amidoxime was recovered in 50% yield. No photolysis occurred when the nitrosamines were in 9.0 M H_2SO_4. They also observed that in the presence of electron transfer agents that the radical dissociates and results in the quantitative conversion to secondary amines. The proposal was made that the photochemically-generated radical intramolecularly abstracts a hydrogen. Chow (34) confirmed the stability of N-nitrosamines in hydrocarbon solutions (and also in water) to UV light. Addition of dilute acid (less than 0.3 N HCl) quickly eliminated the absorbance of the nitrosamine in the 330 nm range. The iso-
lated products resulting from photodecomposition of DBN were as follows: butyraldehyde (9%), N-butyl-butyramid-oxime (60%) and di-
butylamine (21%).

In 1967 Chow (35) reported that the primary photolytic products were (NOH) and the corresponding alkylidene imine which underwent further reactions. Evidence was presented indicating that any secondary amine formed did not result from direct hydrolysis of the parent nitrosamine.

Evidence presented by these investigators (33, 35) and others (15) show that nitrosamines do photo-decompose in short and long wave ultraviolet light when in dilute aqueous solutions of either acid or base. The products generated are often complex in structure. However, at wavelengths over 300 nm nitrosamines in the gaseous state, neat or aprotic solvents do not denitrosate readily. In concentrated acidic solutions (approximately 4 M H₂SO₄ or greater) these compounds are not photolabile.

Aromatic secondary nitrosamines exhibit reversible rearrangement under acidic environments. The nitroso-group rearranges inter-
molecularly to the para position. This reaction is termed the Fischer-Hepp rearrangement. If the para position is occupied the nitroso-group can orient in the ortho position although yields of this product are generally reduced (36).

**Synthesis**

Nitrosation and nitrosating agents have been studied by Ridd (37). Nitrosating agents are related to nitrous acid having the structure NOX where "X" can represent an -OALK, -NO₂, -NO₃ halide
ion or -OH_2^+ group. However, one of the most active nitrosating agents is the nitrosonium cation (NO^+) and is often used in laboratory synthesis. The nitrosating agents attack the lone pair of electrons on the nitrogen atom of the unprotonated amine. Therefore, highly basic amines, e.g., aliphatic secondary amines, are favorably nitrosated in weakly acidic solutions. Dinitrogen trioxide or nitrosyl-halides are the best nitrosating agents in this case. Amines with low electron density on the nitrogen atom, e.g., aniline, can be nitrosated in highly acidic solutions with the nitrosonium ion.

Laboratory synthesis of aliphatic and aromatic nitrosamines in an acidic medium is presented by Fieser and Fieser (38). Temperature and pH of the solution are important criteria in achieving optimum yields. Fridman et al. (15) in their review of nitrosamines have covered other routes of synthesis.

A novel synthesis was demonstrated by Keefer et al. (39). Formation of nitrosamines from secondary amines and nitrite occurred in alkaline solution when formaldehyde was used as a catalyst. At room temperature only 1% of theoretical yield was produced while under reflux conditions nitrosopyrrolidine was formed at a yield of 46%. Nitrosation did not occur above pH 7.5 if formaldehyde was not added. A mechanism was proposed illustrating the direct collapse of an adduct with the regeneration of formaldehyde and production of the nitrosamine.

Almost one hundred years of controversy prevailed since Guether claimed in 1864 (40) that he had produced a nitrosamine from the reaction of a tertiary amine and nitrous acid. In 1959 Smith and Pars (41) presented convincing proof of the synthesis of nitrosamines from a tertiary amine and nitrous acid. In a study of the mechanism
they concluded that it involves an attack on the nucleophilic nitrogen atom of the amine by the nitrosating agent resulting in formation of the nitrosammonium ion. This species undergoes hydrolysis yielding a secondary amine and a ketone or aldehyde. Another molecule of the nitrosating agent then reacts with the secondary amine and forms the nitrosamine.

Controversy has arisen in the mechanism proposed for the production of nitrosamines from tertiary amines. Malins et al. (42) reported that under certain conditions DMN was formed from trimethylamine (TMA) and not from dimethylamine (DMA). This led Scanlan et al. (43) to compare production of DMN from DMA and TMA, by varying the ratio of these amines to nitrite the production of DMN varied.

Equal ratios of amine to nitrite yielded eight times the amount of DMN formed from DMA vs. TMA. At a 30:1 ratio almost equal amounts of DMN was produced from either amine. Increasing the amine to nitrite ratios above this point resulted in more DMN being formed from TMA than DMA. The other conditions were pH 6.4, temperature 100°C and reaction time of 2.5 hours. These results indicated some other mechanism must be operating other than degradation of tertiary amines to secondary amines.

Smith and Pars (41) degraded amine oxides in acid or with sulfur dioxide and achieved secondary amines as products which could be nitrosated.

Fiddler et al. (44) achieved nitrosation of naturally occurring quaternary ammonium compounds and tertiary amines, e.g., carnitine, neurine, trimethylamine, etc. The yields varied with the individual compounds but approached 50% of the yield as compared to
the yield obtained from nitrosating DMA. Their reaction conditions were pH 5.6 and 78°C for four hours.

**Methods for Assay of Nitrosamines in Biological Samples**

The low concentrations found in biological samples, volatility of the nitrosamines and the diversity and complexity of biological samples being examined and the lack of knowledge of the basic properties of these compounds, e.g., stability, reactivity, etc. have posed many problems to the analyst. The rate of growth in this field has also produced a wide variety of methods for qualitative and quantitative analysis of biological samples for these compounds.

Wasserman (45) and DuPlessis (46) have reviewed the analytical procedures. The major problem consists of concentrating the nitrosamines being extracted while simultaneously eliminating the contaminants without loss of the nitrosamines. Five major steps are involved in the procedure for the analysis of these compounds; sample preparation, extraction, purification, identification and quantitation.

**Sample Preparation**

Solids and semisolids are minced, macerated, or homogenized for short periods of time (1-10 minutes). Salting out with K₂CO₃ and pH adjustment are often done at this step. Mixing in the presence of an organic solvent, e.g., methylene chloride is preferred by Sen et al. (47). Emulsions often occur in this step and various additives, e.g., NaCl and Na₂SO₄, are utilized. If a digestive reflux is applied homogenation is done in an alkaline, aqueous-alcoholic media (48).

Aqueous samples are prepared for extraction with addition of salting out agents and pH adjustments. Gases, e.g., smoke, are con-
densed or trapped and prepared as the aqueous samples (49).

**Extraction**

One of two methods is usually employed to achieve separation of the nitrosamine from the matrix; solvent partitioning or distillation. Many procedures use both methods: one for the initial separation followed by the second as an initial "clean up" step. Methylene chloride has found favor as the solvent used in partitioning. This solvent not only removes the nitrosamines efficiently, but in addition, it has a low boiling point and is immiscible with water. Methylene chloride has been utilized in Soxhlet extraction (50), liquid-liquid extraction (51, 52) or extraction via separatory funnel (53).

Many analysts have applied various distillation techniques in removing nitrosamines from the homogenate. Steam distillation at reduced or normal atmospheric pressures is commonly applied to the aqueous alkaline homogenate. The pH is adjusted above neutrality to prevent artifactual production of nitrosamines if residual nitrite is present. However, Sen et al. (54) reported the *in situ* formation of nitroso compounds in 3 N NaOH while Fazio et al. (48) could not detect any formation of nitroso-compounds using the same procedure. Anti-foam agents are commonly added prior to distillation. Telling (55) finds that distillation at reduced pressure aids in reducing the foam production.

**Purification**

The "clean up" portion of the procedure prior to separation of the nitrosamines has received the most attention since contaminants have caused problems in the identification of the nitroso-compounds.
With the existence of multiple methods of detection, contaminants interfering in one method may not affect another procedure. A combination of two or more of the following techniques have been utilized in removing interfering compounds: ion exchange (56) and adsorption resins (55), partitioning between an aqueous fraction and an organic solvent (57), distillations from media of different pH (27) and preparative TLC (50). Almost all of the procedures incorporate a concentration and a drying step. The best results have been obtained by concentrating the organic solvent in an apparatus called a Kuderna-Danish. Water contamination results in losses during concentration and inhibits some methods of detection (58). Therefore, most samples after being partitioned in the organic phase are percolated through a bed of anhydrous sodium sulphate.

These purification steps have often been very tedious and time consuming, requiring scrupulously clean glassware, solvents of ultra high purity and work arrangements to achieve maximum safety for the researchers. Many laboratories have altered their procedures from time to time to achieve better recoveries on different biological samples and to save time. Techniques which have not found wide acceptance in analysis of biological samples for volatile nitrosamines are: adsorption on charcoal (59), fractional distillation (60), preparative TLC (50), freeze drying (61), and separation on Sephadex (62).

**Identification and Quantitation**

Most of the initial work relied on TLC or polarography end points as a means of detection and quantitation. TLC found wide acceptance because of its speed and simplicity (46). DuPlessis and
Nunn (46) reported the Rf value of 25 nitrosamines using a solvent system composed of various ratios of n-hexane/ether/methylene chloride. Supporting systems of silica gel, Kieselguhr or aluminum oxide have been utilized. Several spray reagents producing a visible spot on contact became the basis of identification. However, none were specific for nitrosamines. Ninhydrin (63), Griess (64) and diphenylamine palladium chloride (65), the common spray reagents, produced visible spots with degradation products produced by UV light. Several workers used a combination of these sprays to avoid interference by contaminants (60). At best these methods are today used as a qualitative means of identification and estimation of nitrosamines.

Walters et al. (59) and others (66) have detected nitrosamines using the half-wave potential of these compounds in a polarographic technique. DuPlessis (46) published the half-wave potential of 19 nitroso compounds in five media. This method, although modified by several workers (59, 66), is subject to interference from many contaminants, e.g., aldehydes and pyrazines.

Colorimetric determinations have been developed. Several techniques trap the nitroso-group which when coupled chemically produces a highly colored stable diazo complex that is quantitated spectrophotometrically. Fan and Tannenbaum (67) automated this system. Although the sensitivity is high, no individual identification can be achieved.

Denitrosation and reduction of nitrosamines coupled with derivative formation of the parent amine or unsymmetrical hydrazine have been accomplished. Ender (68) and Neurath (69) have determined nitrosamines using derivatives of hydrazine. Eisenbrand (70) denitro-
sated nitrosamines and formed the heptafluorobutyryl chloride derivatives of the resulting secondary amines. Identification and quantitation (71, 72) was achieved by using gas liquid chromatography (GLC) a technique that has been widely applied as a means of separating, identifying and quantitating nitrosamines. However, the sole use of GLC even with multiple liquid phases on different column supporting material is insufficient for absolute identification. With the advancement in technology, GLC coupled to a mass spectrometer allows simultaneous quantitation, identification and confirmation. The GLC-mass spectrometry method is now recognized as the most reliable detection method and all other methods should be confirmed by this means.

The main areas of improvement with the GLC equipment has been in suppression of background noise and interfering peaks accomplished through specific detectors. Three types of detectors are now available and the principles of operation of these detectors have been reviewed (73). The detectors are: the Coulson electrolytic conductivity detector, alkali flame ionization detector and the electron capture detector. Recently, Dooley (74) reported the first contamination of the mass spectrometer system of a compound with the identical mass peak as that of DMN.

The sensitivity of most of these methods is in, or approaches, the low microgram per kilogram (ppb) range. Several new separation and detection systems, e.g., Fine's thermal energy analyzer (TEA) (75), ESR end point detection, and high pressure liquid chromatography (76) coupled with a sensitive end point detector appear to have potential as a means of identification along with mass spectrometry confirmation.
Occurrence of Nitrosamines

Exposure of human beings to nitrosamines utilized in industry and the presence of nitrosamines in foods are serious, real and potential problems. Naturally occurring nitrosamines, production of nitrosamines through processing, and formation of nitrosamines in vivo from precursors may contribute to the contamination of the biological environment.

Naturally occurring nitrosamines are rare. There have been tentative reports of a nitrosamine, N-nitroso-4-methylamino benzaldehyde, found in a species of mushroom (77). DuPlessis (78) identified nitrosamines in a fruit from the species Solanum incanum grown and used in South Africa. The etiological aspects of cancer in this region supports the concept of a naturally occurring carcinogen. Only a few reported findings of nitrosamines in raw and unprocessed fish and meat have been made. In vivo formation will be discussed in another section.

Production of nitrosamines resulting from processing is presently under vigorous investigation. The precursors are present and the chemical conditions are often favorable for nitrosation. Sen (79) has compiled a list of the reported findings of nitrosamines through 1972. This list also includes the product from which the compound was isolated, quantity present, identification of the compound and the laboratory doing the analysis. This summary covers fish products and meals, meat and meat products, and cheese. Except for fish meals and samples of salt-dried fish which contained DMN in the ppm range, the examples cited were of foods containing less than 80 ppb and most below 20 ppb of DMN or other nitrosamines.
More recently Sen and coworkers (80) reported on the presence of volatile nitrosamines in 197 cured meat products. Another survey on approximately 100 meat products was published in 1974 (81). This list included seventeen samples having nitrosopyrrolidine (NPyr) at levels of 13-105 ppb; 29 samples had 2-35 ppb of DMN, and DEN was found in 9 samples at 2-35 ppb. Sixty-two of the 100 samples contained no trace of the compounds. Confirmation by mass spectrometry was performed on some of the positive cases.

Fazio (82, 83) has reported and confirmed the presence of DMN in smoked nitrate/nitrite treated fish and from some meat products. Fazio (82) found NPyr in eight out of eight samples of cooked bacon but none in raw bacon. The fat drippings were analyzed separately and were found to contain even higher levels.

The USDA meat laboratory has also reported positive findings of DMN and NPyr in some meat products (84) and fried bacon (85).

Fong and Chan (86) purchased marine salt cured fish directly from Hong Kong markets and confirmed the presence of 50-300 ppb of DMN.

Sen and Dalpe (60) found no trace of volatile nitrosamines in the alcoholic beverages tested. Vegetables have also been assayed with negative results (87, 88). Recently Heisler et al. (88) searched for nitrosamines in spinach and beet samples which had been "abused". All samples gave negative tests for six volatile nitrosamines even though levels of nitrite reached 1000 ppm in one sample and many samples contained nitrate over 1500 ppm.

Milk and milk products have not shown any positive presence for these compounds as tested by Reineccius and Coulter (89).
Nitrosamines have been discovered in tobacco and tobacco smoke condensates. A non-volatile nitrosamine, N'-nitroso nornicotine, a proven carcinogen in mice, was found in unburned tobacco at the level of 2000-88000 ppb by Hoffman et al. (90). This compound occurred in cigarettes, cigars and chewing tobaccos. McCormick et al. (91) and other investigators (92) have found several volatile nitrosamines in tobacco smoke condensates. Tobaccos grown in high nitrogen soils yield higher levels of nitrosamines in their smoke condensates which reach a level of 180 ng/cigarette. However, the condensate trapping procedure may cause artifactual formation of nitrosamines.

Occurrence of Precursors of Nitrosamines, Secondary Amines and Other Amino Compounds

Although amino compounds are ubiquitous in living systems, free secondary amines are much less prevalent and, if present, occur only in trace amounts. Extensive studies on levels and identification of free amines have not been completed with the exception of surveys of di- and tri-methylamines and trimethylamine oxide (93) in various marine fish. Gadoid fishes, e.g., hake, cod, cusk, etc., have been studied by Castell et al. (94) and Miller et al. (95). Effects of storage in ice on levels of DMA and TMA in frozen fillets indicates large amounts of these compounds can be generated reaching levels of 400 ppm. Trimethylamine oxide content in tuna, which is thought to be the precursor to DMA, in fish was studied by Yamagata et al. (96) and Dyer et al. (97)

Several researchers, Ito et al. (98) Kawamura et al. (99)
determined the occurrence of secondary amines in commercial foods. A comparative survey on levels of amines in raw foods and cooked or processed foods was performed. Variation in roasting or cooking times and the effect on levels of amines was also established. Miller et al. (100) determined methylamines in fish protein concentrate and found 5-10 ppm of TMA and 25-150 ppm of DMA. Waste water from industrial processes was studied for the presence of dimethylamine (101) and amine levels reaching 158 mg of DMA per liter were found.

Many amino compounds can yield secondary amines and then generate volatile nitrosamines. For example, polyamines and diamines have been found in many organisms (102). These compounds under conditions of heating can produce pyrrolidine and piperidine, cyclic aliphatic secondary amines (103). Spinelli et al. (104) identified and quantitated several of these polyamines and diamines along with other amines in processed and unprocessed pork. Levels were not significantly altered in processing and ranged from 0.12 mg to 9.1 mg/100 g of tissue.

Plants do not contain high levels of free aliphatic amines. However, many species have high levels of heterocyclic nitrogen compounds called alkaloids. Many of these alkaloids have piperidine, morpholine or pyrrolidine nuclei within their chemical structures (105, 106). However, embryos of cereal crops according to Moruzzi and Caldarera (107) contain polyamines.

Nitrate, Nitrites and Other Oxides of Nitrogen

Nitrates, nitrite and other oxides of nitrogen constitute the second potential reactant required for synthesis of nitrosamines.
Although the occurrence of these compounds is universal, the manipulation, to some extent, of their levels can more easily be controlled by man than levels of amino compounds.

Various nitrogen oxides are produced upon combustion of fossil fuels. Levels of nitric and nitrous oxide, the largest nitrogen products of combustion, are 1 ppm and 0.5 ppm/M³ respectively (108), and can reach much higher levels in the atmosphere over industrial centers under various environmental conditions, e.g., smog (109).

Nitrate, the highest oxidation state of nitrogen, is very stable and is the form that accumulates in the environment. Nitrate per se, is non-toxic to man and is not a chemical reactant in the formation of nitrosamines. However, reduction to nitrite, either prior or after injection, can produce acute toxic symptoms and holds the potential for the formation of nitrosamines. Many surveys indicate only low levels of nitrite exist, but the contrary is often the case for nitrate. The water supply, specific vegetables, and a variety of processed foods are the major sources of our nitrate intake. Within the plant kingdom nitrate is the main form from which a nitrogen supply is derived. Many plants quickly reduce nitrate into a form which is then incorporated into complex biological molecules. Nevertheless, some plants can accumulate high levels of nitrate in various tissues. Nitrate levels in fruit are usually low while leaves, stems, and tubers of plants, e.g., spinach, radish, lettuce, beet, etc. contain higher levels. Forage crops used as animal feeds can accumulate nitrate. Factors contributing to nitrate content of plants are: genetic, environmental and stage of maturity. Surveys (110, 111) on
vegetables and vegetable products, e.g., baby food; indicate levels can reach over 1000 ppm and are still fit for human consumption (88). Animal and human poisonings attributed to high nitrate intake have been reported and reviewed by Fassett (112).

Water is another medium which can be contaminated with high levels of nitrate, particularly shallow wells, commonly found in rural areas (113). Entrance of nitrate in water can be gained because of its mobility in soil and solubility in water. Sources of this ion are: natural deposits, agricultural application, nitrogen fixation by plants and microorganisms, human and animal excretions.

Nitrate levels can undergo rapid and short term changes which makes monitoring difficult. Health officials have set limits on the maximum amount of nitrate allowed in potable water supplies at 45 ppm of the nitrate ion (114). The World Health Organization recommends a daily intake level for adults not to exceed 5 mg/kg of nitrate and 0.4 mg/kg of nitrite (115).

Nitrate and nitrite and their curing properties as an additive in processing of meat and fish products and in some countries in cheese production (not U.S.) has recently been reviewed (116). Their role in these processes is still obscure. Reports (117) indicate nitrate alone is not bacteriostatic and its role as a reserve for nitrite production may also be questioned. However, by regulation 3.5 oz of sodium or potassium nitrate per 100 lbs of meat for dry cure and 2.75 oz/100 lbs of chopped meat or meat by-products are allowed (118).

Nitrite, the agent directly responsible for acute toxicity resulting from reduction of high nitrate levels ingested, reacts with
the oxygen carrying blood protein, hemoglobin. The iron in this complex is oxidized forming methemoglobin. Thus the hemoglobin complex no longer can function as an oxygen carrier and if extensive levels of methemoglobin occur toxic symptoms will appear in the organism.

Since 1925, nitrite has been allowed as an additive in the curing of meats. The action of nitrite is fourfold in this process; producing coloration, cured flavor, stability and antibacterial activity. With the increased use of refrigeration, the need for preservation of meats has decreased. New agents which might contribute to the flavor and coloration, shelf-life and stability of these processed meat products are being reviewed (119, 120). Other studies are underway to ascertain if lower levels of these additives will produce effective bacteriostatic action as might be indicated.

**In Vitro Formation of Nitrosamines**

Druckery et al. (121) first made the suggestion that conditions in the human stomach might be conducive to the formation of nitrosamines in the presence of both nitrite and secondary amines. Sanders (122) was the first to demonstrate formation of nitrosamines in gastric juice upon addition of the precursors. Ridd (37) has extensively studied the chemistry of nitrosation, deamination and diazo reactions. The act of nitrosation appears to be the first step in the reaction of amino groups with a nitrosating agent. The ultimate reaction products are determined in the chemistry of the nitrosated intermediate and conditions employed (123).

Mirvish (124), using tritium labeled DMA, investigated the kinetics of nitrosation for this amine. The reaction rate depends on
three factors; pH, concentration of the amine, and the square of the nitrous acid concentration. The optimum pH for DMA nitrosation was 3.4.

Based on this type of data, many investigations have been performed on various systems, e.g., physiological, food systems and synthetic chemical media. Most experimental protocols have used high concentrations of one or both precursors.

Ender and Ceh (125) studied the formation of DMN over a wide range of conditions in an attempt to determine the most favorable conditions for nitrosation in food products.

Gastric juice from many mammalian stomachs has been utilized as the medium for the nitrosation of amines. Sen et al. (56) removed gastric solutions from rabbits, rats, cats, dogs and humans to which was added diethylamine and sodium nitrite. Larger concentrations of DEN were detected at a pH of 1-2 than at pH 4-5. Recently Lane et al. (126) applied a sensitive detection method for nitrosamines to in vitro experiments in gastric juice. Formation of DMN occurred with levels of precursors comparable to concentrations expected to exist physiologically. The optimum pH was 2.5.

Huxel et al. (127), using elevated temperatures, formed NPyr from various pyrrolidine nuclei-containing compounds after Bills (128) reported that N-nitrosoproline, proline, and other straight chain diamines could also be potential precursors for NPyr. Lijinsky et al. (129) not only found that high yields of nitroso-derivatives were formed from various amino acid compounds under conditions similar to those existing in the mammalian stomach, but decarboxylation could take place forming potent carcinogenic dialkylnitrosamines.
Archer et al. (130) worked with similar compounds and obtained similar results.

The USDA and FDA laboratories in this country and Sen's group in Canada have been investigating conditions of nitrosamine formation in food processing. Both groups have studied the effect of NaNO₂, used as an additive in processing, in the production of volatile nitroso compounds within various matrices, e.g., bacon (131), curing mixture (132), fish (54) and meat products (133). Although the applied conditions varied, formation of DMN, NPIP and NPyr have been demonstrated without addition of the amine precursor. Gray and Dugan (134) investigated parameters in a low moisture system on the synthesis of nitrosamine.

Commonly employed agricultural chemicals, e.g., insecticides and fungicides have been reacted with nitrite in mild acidic conditions and formation of dialkylnitrosamines has resulted (135). Many of these agri-chemicals are tertiary amino compounds, e.g., alkylureas and alkylcarbamic acids.

Some medicinal compounds contain secondary and tertiary amino-groups. Lijinsky et al. (136) found that addition of nitrite to six drugs produced measurable yields of nitrosamines of 0.1% to 73% of the expected theoretical yield. The analgesic drug, aminopyrene, consistently produced yields of 31 to 73% after incubation for 3-4 hours at 37°C under varying conditions of pH and nitrite concentration. The antibiotic oxytetracycline, was also a precursor in the synthesis of nitrosamines; DMN was the product in each instance of nitrosation of the precursor drug.

Another aspect of in vitro synthesis of nitrosamines has been
demonstrated in bacterial cultures. The data thus far obtained indicates both nonenzymic (137) and enzymic (138) processes may result in the formation of nitroso-compounds.

Investigations on the effect of catalyst and inhibitors of nitrosation are being examined in vitro. Halide ions and thiocyanate enhance nitrosation at rather low concentrations. Thiocyanate ion, a normal constituent of human saliva, is found in elevated amounts in saliva of smokers when compared to non-smokers (139). Normal levels are 0.1 to 0.5 mM and levels found in smoker's saliva are raised 5 to 10 times the normal level.

Ridd et al. (37) have shown the order of enhancement resulting in the increased rate of nitrosation of halide ions is; I⁻, Br⁻, Cl⁻. Boyland (139) describes the activity of thiocyanate to be in the order of the iodide ion as a catalyst in the nitrosation reaction. Fan and Tannenbaum (140) explored factors influencing formation of nitrosomorpholine including acceleration by anions. Keefer (39) recently reported that formaldehyde acted directly to increase nitrosation at neutral and alkaline pH values.

Although inhibition by primary amino groups offers the greatest potential in preventing nitrosation of secondary and tertiary amines only recently has this been investigated since the work of Taylor and Price in 1929 (141). Lane et al. (126) using synthetic gastric juice as a media observed that glycine competed for nitrite therefore lowering the production of DMN.

Ascorbic acid, sodium ascorbate and sodium erthyrobate have been shown to inhibit nitrosation. Dahn (142) studied the reaction of nitrous acid and ascorbic acid and determined the products to be
nitric oxide and dehydroascorbic acid. Mirvisch et al. (143) and Kamm et al. (144) have demonstrated in vitro inhibition using ascorbate as an additive in nitrosation conditions.

In Vivo Formation of Nitrosamines

Evidence is accumulating suggesting that nitrosamines are a major class of human carcinogens. The correlation of in vivo tumor production resulting from the incorporation of secondary amines and sodium nitrite in feeding experiments is good in comparison with the actual administration of the nitroso-compound itself. Sanders and Schweinsberg (145) also report, "the results of the in vivo experiments agree very well with those obtained in vitro, i.e., tumors were induced by compounds that were easily nitrosated in vitro and were not induced by those amines that were not nitrosated in vitro."

Studies of certain locales of Africa, Asia and South America indicate environmental agents may be responsible for the high incidence of specific types of cancer in these areas. Nitrosamines have been implicated especially in Transkei section of South Africa and particularly with the Bantu people (146).

Sanders et al. (147) performed experiments of intragastric feeding of thirty one humans with sodium nitrate and diphenylamine. Nitrosodiphenylamine was formed. Numerous experiments have shown in vivo formation of nitrosamines in animals. No threshold limits have been established for humans.

Objectives Achieved in Thesis Research

Reported in this work is the development of an indirect method for the detection of N-nitrosamines. The method permits the
identification and quantitation of the nitrosamines through the derivatization of the amine products into highly fluorescent compounds. This method of analysis was applied to a survey of seafood for the presence of volatile N-nitrosamines.
METHODS AND MATERIALS

Photolysis of Nitrosamines

An ultraviolet light irradiating chamber was built out of 1/4 inch plywood with outside dimensions of 10" high x 21" long x 11" wide. Two sylvania G15T8 short wave 18" lamps were installed in the top of the box and controlled by a two position toggle switch. This chamber also functioned as a detoxifying area for unused aqueous nitrosamine solutions.

Nitroso-Group Determination

Nitrosamine samples were pipetted into 20 ml glass beakers followed by the addition of 0.5 ml of 0.5% Na₂CO₃. The total volume was adjusted to 1.0 ml with distilled water. The samples were placed in a pan containing crushed ice in the U.V. chamber. The beakers were aligned so that they were directly below the light source and the samples were irradiated for 15 minutes. Quantitation of the denitrosation process was accomplished by the formation of a colored diazo compound resulting from the addition of the Griess-Ilosvay reagent. This reagent was freshly prepared from a 1:1 mixture of 1% sulfanilic acid in 30% acetic acid, and 0.1% 1-naphthylamine in 30% acetic acid. 3.0 ml of the Griess-Ilosvay reagent was added to each sample and the total volume adjusted to 5.0 ml with distilled water. After 15 minutes of development in the dark at room temperature, the samples were read at 525 nm in a Bausch and Lomb Spectronic 20 colorimeter and quantitated by reference to a standard curve prepared with known concentrations of NaN₃O₂.
Amine Determination

The photolysis procedure was the same as previously described. However, the pH of the sample was maintained below neutrality by the addition of 0.01 N HCl or pH 5.0 sodium acetate buffer. The total volume was 0.5 ml or less. An aliquot representing one-half of the sample was withdrawn, after irradiation had taken place, and reacted with the fluorescence reagent, 5-dimethylaminonaphthalene-1-sulfonyl chloride, (dansyl-Gl from Pierce Chemical Company). The fluorescence procedure as described by Seiler and Wiechman (148) and as modified by Dion and Herbst (149) was followed.

Acid Denitrosation of Nitrosamines

The sample was mixed and aliquots of equal size were pipetted into a Buchler Evapo-mix tube (150mm x 18mm). One tube containing one-half of the sample will be referred to as the "unsplit" fraction while the other is the "split" or denitrosated fraction. The "unsplit" fraction received 1.0 ml of 0.1 N HCl. The "split" sample received an aliquot of 30-32% hydrobromic acid in glacial acetic acid (Eastman Kodak Co., to give a final concentration of 3% hydrobromic acid). Both tubes were mixed, stoppered and allowed to stand for 30 minutes at room temperature. The denitrosation reaction was terminated by the addition of 1.0 ml of 0.1 N HCl followed by vigorous mixing. During this mixing process a color change from deep orange to light yellow occurred. The "split" and "unsplit" samples were taken to dryness on a Buchler Evapo-mix under conditions of continuous shaking, reduced pressure at 60°C. After the addition of 1.0 ml of 0.1 N HCl to the residue in each tube, the samples were again evaporated to
dryness. The samples were taken up in two aliquots of 0.5 ml of 0.1 N HCl (Vortex mixing) and stored in capped 100 x 13 mm test tubes in the refrigerator.

**Dansylation Procedure**

The amine salts derived from nitrosamines or standard stock solutions of amines were dissolved in 1.0 ml of 0.1 N HCl and pipetted into 150 mm x 13 mm screw-capped test tubes followed by the addition of 2.0 ml of spectral grade acetone containing 1.0 mg of dansyl-Cl. Enough NaHCO₃ was added to saturate the system and each tube was thoroughly mixed. The reaction took place overnight at room temperature in the dark with constant shaking. To remove excess reagent 0.1 ml of 100 mg/ml of proline was added and the reaction continued for 30 additional minutes. Acetone was removed under reduced pressure and the amine derivatives were extracted into 1.0 ml of benzene. Extensive mixing (Vortex mixer) was required to insure complete extraction into benzene followed by a short low speed centrifugation to break the emulsion. The tubes were placed in ice and the benzene layer was pipetted into a small capped test tube and stored in the freezer.

TLC plates were prepared in our laboratory by applying a slurry of silica gel G (Merck A.G., Darmstadt, Germany) on clean glass plates (20 x 20 cm) with a Brinkmann-Desaga variable thickness applicator (Brinkmann Instruments) set at 250 μ. The silica gel slurry was prepared by vigorously shaking 30 g of silica gel G with 60 ml of distilled water in a 500 ml Erlenmeyer flask. The plate was activated for 1 hour in an oven at 110°C prior to spotting. Microliter
quantities of samples were applied to the plates with a Hamilton Micro syringe equipped with a Chaney adapter. Ascending chromatography was performed in the dark using an ethylacetate:cyclohexane (1:4 V/v) solvent system. After chromatography, the plate was sprayed with triethanolamine:isopropanol (1:4 V/v) and dried at room temperature \textit{in vacuo} for one hour in the dark.

Quantitation of the amine derivatives was accomplished by direct scanning with a Turner model 111 fluorometer (G.K. Turner Assoc.) equipped with a TLC scanner and a Heath Kit IR-18, 10 inch recorder. The UV light source was a long-wave lamp (Turner Catalog #110-850). A Turner Primary Filter #7-37 permitted transmission of the wavelength of 365 nm. The secondary filter, Turner #2A-12, allowed emission of wavelengths longer than 512 nm.

Quantitation was achieved by measuring the peak area, i.e., peak height times the width at one-half peak height, and referring to a standard curve of peak area vs. concentration derived from amine standards chromatographed on the same plate.

\textbf{Derivatization with NBD-C1}

One milliliter of solution containing 6.0 mg of NBD-C1 (Regis Chemical Co.) in redistilled methyl isobutyl ketone (MIBK) was added to each sample and to an amine standard mixture in 1.0 ml of 0.1 N HCl. To this two layer system, sufficient anhydrous Na$_2$CO$_3$ was introduced to saturate the aqueous layer (bottom layer). The tubes were shaken and placed in a hot water bath at 80°C for one hour. After cooling to room temperature, the tubes were placed in the freezer until the bottom layer was frozen. The amber organic layer, contain-
ing the amine derivatives, was removed with a disposable pipette and transferred to a 100 x 13 mm capped tube which was stored in the freezer.

**TLC, Fluorometric and Quantitation Procedures**

Prepared silica gel 60 plates, 20 x 20 cm with a layer thickness of 250 \( \mu \) (E. Merck Laboratories) were activated at 100°C for one hour. Aliquots of the amine derivatives were applied with a 10 \( \mu \)l Hamilton syringe equipped with a Chaney adaptor. The plate was developed in a solvent system of cyclohexane:diethyl ether:ethylacetate:chloroform:glacial acetic acid (50:30:30:2:1) at 12°C. When the solvent front had traveled to 16 cm or more up the plate, the plate was removed, air dried and held in vacuo at room temperature for a minimum of 30 minutes. Fluorometric analysis was accomplished with a Turner Model 111 Fluorometer equipped with a TLC plate scanning accessory. The fluorometer was fitted with a 436 nm light source (G.K. Turner Catalog #110-853 Blue Lamp) in a T-5 envelop. The primary filter was a combination of the 47B and 2A and the secondary filter was a 2A-12 allowing transmission of wavelengths longer than 512 nm. The tracings from a scan were recorded on a Coleman Model 165, 10 inch Recorder. Quantitation was computed by comparing peak height measurements in mm of unknowns to those of standards chromatographed on the same plate.

**Seafood Sample Preparation**

A 100 g sample of fresh, frozen or processed seafood, e.g., clams, oysters, kippers, etc. was placed in the screw top jar of a Waring blender. Ten grams of potassium carbonate and 7-8 grams of
potassium hydroxide were added followed by 200-250 ml of methylene chloride. The sample was homogenized for several short periods (10-30 sec) with several minutes between each homogenization. This procedure was followed until a uniform homogenate was obtained. The fresh and frozen seafood samples would often form highly viscous emulsions. To reduce emulsification, these samples were homogenized as previously mentioned but no methylene chloride was added until sufficient homogenization was achieved. Then 200-250 ml of the organic solvent was added and after several short periods of homogenizing the samples were allowed to stand approximately five minutes prior to filtering through glass wool. The filtrate was collected in a two liter round bottom flask and the blender jar was rinsed with 50-75 ml of methylene chloride and this was also filtered. The filter cake was discarded. To the yellowish filtrate 150 ml of distilled water was added along with Micro-Porous Boiling Chips (Todd Scientific Co.), which had previously been boiled in CH₂Cl₂ and Antifoam A (Dow Corning). A Snyder column (Kontes #503000) was inserted into the flask, the contents were swirled, and the organic solvent was evaporated in a hot water bath in the hood. After all the methylene chloride was removed, the apparatus was cooled to room temperature and 10-20 ml of distilled water was added to the top of the Snyder column and allowed to percolate into the flask. The cooled aqueous fraction was added to a 1 liter, 3 necked steam pot of a steam distillation system. The round bottom flask was rinsed with distilled water and more Antifoam A was added to the steam pot. The Graham condenser was cooled with tap water. 200-250 ml of distillate was collected and stored in the refrigerator or partitioned immediately.
Partitioning was performed in 500 ml Squibb separatory funnels fitted with teflon stopcocks. The distillate was poured into a separatory funnel followed by the addition of 10 ml of 6 N HCl. The distillation flask was rinsed with 70 ml of CH$_2$Cl$_2$ and this was added to the separatory funnel and shaken 3 minutes. The layers were allowed to separate (3-5 minutes) and the organic layer (bottom) was drained into another separatory funnel. This extraction procedure was repeated two times without further addition of the 6 N HCl. Each time, the organic fractions were combined and the aqueous distillate was discarded. To the combined organic fraction 50 ml of 1.0N HCl was added and after shaking, the CH$_2$Cl$_2$ layer was drained into a third separatory funnel containing 50 ml of 2.0N NaOH. The aqueous acid layer (50 ml of 1 N HCl) was extracted twice more with 25-50 ml aliquots of CH$_2$Cl$_2$. These organic fractions were also combined into the third separatory funnel. Then a 1-2 minute shake was done followed by a 15 minute standing period. If a colloidal layer formed, NaCl was added and after shaking briefly a clean separation of the two layers was usually accomplished. The organic layer was percolated through a bed of anhydrous Na$_2$SO$_4$ (pre-wetted with 25 ml of methylene chloride) supported in a glass-fritted funnel. The methylene chloride was collected in a 500 ml Kuderna-Danish flask fitted with a concentrating tube (Ace Glass Co. #6707-12, 10 ml capacity). Several prewashed boiling chips were added and a Snyder column was inserted. The sample was concentrated to 2-4 ml on a hot water bath and after cooling the Snyder column was rinsed with a small aliquot of hexane. The sample was either stored in a freezer or applied directly to an alumina column.

The alumina column was prepared as follows. A small glass wool
pad was placed at the bottom of a water jacketed column (1.5 x 24 cm) which was filled with hexane (B.P. 66-69°C). Three grams of 3% deactivated alumina (Woelm Basic, Brockman Activity 1) was slowly added. On top of the alumina bed a 1 cm layer of anhydrous Na₂SO₄ was added. Approximately 50 ml of hexane was passed through the column before addition of the sample (deactivation was accomplished by adding 3.0 ml of distilled water slowly to 100 g of oven dried alumina. The alumina was then thoroughly mixed by rotating slowly on a Buchi Rotary Evaporator for 4 hours). The alumina column was refilled with hexane. The sample was added to the hexane and a flow rate of 60-100 drops per minute was maintained. As the column head reached the Na₂SO₄ layer, 77 ml of a wash solution (10% CH₂Cl₂ in hexane, v/v) was added to a reservoir and passed through the column and discarded. The nitrosamines were eluted by passing 75 ml of glass-redistilled methylene chloride (B.P. 39.5-40.5°C) through the column. This fraction was collected in a glass stoppered flask for storage or added to a Kuderna-Danish flask (250 ml reservoir) fitted with a concentrating tube (Kontes Glass Co. Catalog #570050, 4 ml capacity) and concentrated to approximately 6 ml. After cooling to room temperature, a micro Snyder column was attached to the concentrating tube and the sample was further concentrated to 1-2 ml. This concentrated sample was either stored in the freezer in a ground glass stoppered tube or denitrosated immediately as previously described.

**Determination of Dimethylamine (DMA) in Seafood**

A 20% homogenate of seafood was prepared with cold 10% perchloric acid (PCA) in a Waring blender. The samples were held on
ice for two hours and were centrifuged in an International PR-2 refrigerated centrifuge. 100 ml of the supernatant was poured into the steam pot and the Graham condenser was "charged" with 5-10 ml of 6 N HCl. 30-50 ml of 1N HCl was placed in the 1 liter collecting flask. The condenser exit was placed beneath the aqueous acid layer. 20 grams of NaOH was added to the steam pot and approximately 200 ml of distillate was collected. The distillate was taken to dryness on a Buchi Rotary Evaporator. The precipitate containing amine salts was dissolved in 25 ml of 0.1 N HCl and kept in a refrigerator until either NBD-Cl or dansyl-Cl derivatization was performed as described in this section.
RESULTS AND DISCUSSION

Separation of Dansylated Secondary Aliphatic Amines

In Figure 1 the structures of the N-nitrosamines for which a method of detection is proposed are presented. Initially the parent secondary amines of these N-nitrosamines were derivatized to form the highly fluorescent dansyl-amino compounds by the procedure developed in this laboratory (149). Separation and quantitation of these compounds had not previously been accomplished by one dimensional TLC. Seiler and Weichman (150) while working with perchloric acid extracts of brain tissue detected and separated several of these small secondary aliphatic amines from many other biogenic amines and amino acids as dansyl derivatives by two dimensional paper chromatography and high-voltage electrophoresis. Several solvent systems that Seiler and Weichman utilized were tried and found ineffective in separating these secondary amine derivatives and, therefore a number of other solvents were tested (see Table 1). Although none of these solvent systems provided the separation of all six secondary amines, (DMA, DEA, DPA, DBA, PYR and PIP) in one dimensional chromatography, the solvent system composed of ethyl acetate and cyclohexane appeared promising and was further tested. The separation of the four straight chain aliphatic amines was achieved by a 1:4 v/v ratio of the ethyl acetate and cyclohexane system. Other adsorbents besides silica gel used in TLC were also tried, i.e., polyamide and kieselguhr plates. Nevertheless, the separation of the dansyl-derivatives of pyrrolidine and piperidine from the other compounds could not be achieved. Eisenbrand (28) also
Figure 1. Structural Formulae of Seven Volatile N-Nitrosamines
\[
\begin{align*}
&\text{CH}_3\text{C}=\text{N-N}=\text{O} \\
&\text{CH}_3\text{C}\longrightarrow\text{N-N}=\text{O} \\
&\text{N-NITROSODIMETHYLAMINE (DMN)} \\
&\text{CH}_3\text{CH}_2\text{N-N}=\text{O} \\
&\text{CH}_3\text{CH}_2\text{N-N}=\text{O} \\
&\text{N-NITROSODIETHYLAMINE (DEN)} \\
&\text{CH}_3\text{CH}_2\text{CH}_2\text{N-N}=\text{O} \\
&\text{CH}_3\text{CH}_2\text{CH}_2\text{N-N}=\text{O} \\
&\text{N-NITROSODIPROPYLAMINE (DIPN)} \\
&\text{CH}_3\text{(CH}_2\text{)}_3\text{N-N}=\text{O} \\
&\text{CH}_3\text{(CH}_2\text{)}_3\text{N-N}=\text{O} \\
&\text{N-NITROSODIBUTYLAMINE (DBN)} \\
\end{align*}
\]
TABLE 1. Solvent Systems Tested for the Separation of Dansylated Secondary Amines on Silica Gel Plates

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Degree of Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol-ethylacetate-cyclohexane</td>
<td>−</td>
</tr>
<tr>
<td>(20:20:60)</td>
<td></td>
</tr>
<tr>
<td>Methanol-ethylacetate-cyclohexane-NH$_4$OH</td>
<td>+</td>
</tr>
<tr>
<td>(10:20:65:5)</td>
<td></td>
</tr>
<tr>
<td>Isopropanol-ethylacetate-cyclohexane-NH$_4$OH</td>
<td>−</td>
</tr>
<tr>
<td>(35:45:20)</td>
<td></td>
</tr>
<tr>
<td>Methanol-chloroform-glacial acetic acid</td>
<td>−</td>
</tr>
<tr>
<td>(20:75:5)</td>
<td></td>
</tr>
<tr>
<td>Hexane-ethylacetate-cyclohexane-pet. ether-methylene chloride (20:20:30:10:10)</td>
<td>+</td>
</tr>
<tr>
<td>Ethylacetate-cyclohexane (40:60)</td>
<td>++</td>
</tr>
<tr>
<td>Butylacetate-heptane (50:50)</td>
<td>+</td>
</tr>
<tr>
<td>Benzene-triethylamine (90:15)</td>
<td>−</td>
</tr>
<tr>
<td>Benzene-triethylamine-cyclohexane (10:10:80)</td>
<td>+</td>
</tr>
<tr>
<td>Toluene-dioxane (50:50)</td>
<td>−</td>
</tr>
<tr>
<td>Toluene-dioxane-hexane-pet. ether (33:33:16:16)</td>
<td>−</td>
</tr>
<tr>
<td>Chloroform-isobutanol-hexane-pet. ether (25:25:25)</td>
<td>−</td>
</tr>
<tr>
<td>Benzene-acetone (98:2)</td>
<td>−</td>
</tr>
<tr>
<td>Butylacetate-cyclohexane (20:80)</td>
<td>++</td>
</tr>
</tbody>
</table>

− No separation, at solvent front, or at origin
+ Some separation of at least one spot
++ Several spots separated
reported three solvent systems which separated the four aliphatic straight chain amino-dansyl derivatives but he was unable to separate the dansyl derivatives of pyrrolidine and piperidine from the other compounds. The Rf values of the dansyl-amino compounds are listed in Table 2.

**Photochemical Denitrosation of Nitrosamines**

Determination of nitrosamines via photochemical denitrosation and the quantitative recovery of the nitroso-group was performed by Daiber and Preussman (151). Since they reported 95-100% denitrosation, it was reasoned that the second product of photochemical splitting would be the parent secondary amine that could be recovered in high yield. Due to the differences between my photo-splitting system and the Daiber and Preussman system, i.e., UV light source, etc., the recovery of the nitroso-group was determined. A time study was done to determine the maximum denitrosation achieved by the photochemical reaction (see Table 3). A maximum release and recovery of the nitroso-group occurred in 15 minutes with a decrease in recovery occurring by 30 minutes. Daiber and Preussman (151) also achieved a maximum denitrosation in 15 minutes. They reported, however, a decrease in recovery of the nitroso-group as the concentration of the nitrosamine was increased due to the formation of nitrate which they were able to detect. In my time study the standard nitrite solution did not show an appreciable loss, as indicated by the O.D. values, even after 60 minutes of the UV treatment (Table 3). However, Daiber and Preussman did not report the effect of UV on a standard nitrite solution. These investigators indicated that the concentration of sodium carbonate was
TABLE 2. Rf Values for Dansyl-Amino and NBD-Amino Derivatives.

The solvent system for the dansyl derivatives was ethylacetate and cyclohexane (1:4 v/v). The NBD-derivatives were chromatographed in a solvent of cyclohexane, diethyl ether, ethylacetate, chloroform, glacial acetic acid (50:30:20:2:1 v/v) at 12°C. Chromatography of both derivatives was done in the dark on silica gel G plates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dansyl-Derivatives</th>
<th>NBD-Derivatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>0.13</td>
<td>0.16</td>
</tr>
<tr>
<td>Methylamine</td>
<td>0.26</td>
<td>0.23</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>0.40</td>
<td>0.11</td>
</tr>
<tr>
<td>Morpholine</td>
<td>0.21</td>
<td>0.11</td>
</tr>
<tr>
<td>Pyrrolidine</td>
<td>0.41</td>
<td>0.20</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>0.64</td>
<td>0.29</td>
</tr>
<tr>
<td>Piperidine</td>
<td>0.62</td>
<td>0.35</td>
</tr>
<tr>
<td>Dans-OH</td>
<td>0.67</td>
<td>--</td>
</tr>
<tr>
<td>Dipropylamine</td>
<td>0.74</td>
<td>0.53</td>
</tr>
<tr>
<td>Dibutylamine</td>
<td>0.83</td>
<td>0.67</td>
</tr>
</tbody>
</table>
TABLE 3. Denitrosation and the Loss of Nitrite vs. Time of UV Treatment

(A) Each sample contained 6 x 10^{-5} m moles of DMN and 0.5 ml of 0.5% Na₂CO₃. The volume was adjusted to 1.0 ml by the addition of water. The samples in 20 ml beakers were placed in a pan containing ice and exposed to the UV source. After the UV treatment, the samples were analyzed as described in the Materials and Methods section.

(B) Each sample contained 3 ug of NaNO₂ and the total volume was adjusted to 1.0 ml and analyzed as described above.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Time of UV Treatment in Minutes</th>
<th>0</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D. at 525 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.0</td>
<td>0.19</td>
<td>0.45</td>
<td>0.39</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.0</td>
<td>0.12</td>
<td>0.49</td>
<td>0.37</td>
<td>--</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.41</td>
<td>0.41</td>
<td>0.41</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
</tbody>
</table>
critical in the assay. Therefore, the effect of the sodium carbonate concentration on the recovery of nitroso-group from nitrosopiperidine was determined (Table 4). A maximum photochemical splitting was attained with 0.5 ml of 0.5% Na₂CO₃ and this amount was incorporated into the procedure.

The linear calibration curves derived from the nitrite standards and from the recovery of the nitroso-group, as nitrite, are presented in Figure 2. The denitrosation and recovery of the nitroso-group from these nitrosamines is not 100% as is evident from the difference in the slopes of curves of the compounds compared to that of the nitrite standard curve. Although Daiber and Preussman (151) reported nearly quantitative denitrosation as determined by the recovery of the nitroso-group it is impossible to account for the differences observed in the recovery of the nitroso-group in the two studies due to the differences in the two systems. Our system employed a different UV source, a lower temperature (samples were cooled on ice), and the distance from the light source was greater. However, our results are comparable to those obtained by Fan and Tannenbaum (67). Their system utilized a UV source similar to our system and a comparison of denitrosation of several nitroso-compounds achieved in our system and theirs is presented in Table 5.

**Recovery of Amine in Photochemical Denitrosation**

According to Burgess and Lavanish (33), UV irradiation of an aqueous solution of nitrosamine in the presence of a proton donor, i.e., dilute hydrochloric acid, quickly denitrosated the compound but products other than the parent secondary amine frequently occurred.
TABLE 4. Effect of Na$_2$CO$_3$ Concentration on the Recovery of the Nitroso-Group

Each sample contained 0.3 ml of $2 \times 10^{-4}$ M NPIP, the indicated volume of a Na$_2$CO$_3$ solution and water to give a total volume of 1.0 ml. The samples were cooled on ice during the 15 minutes UV treatment. The analysis for nitrite is described in the Materials and Methods Section.

<table>
<thead>
<tr>
<th>Concentration of Na$_2$CO$_3$</th>
<th>Volume of Na$_2$CO$_3$</th>
<th>A$_{525}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>ml</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.1</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.39</td>
</tr>
<tr>
<td>1.0</td>
<td>0.3</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.36</td>
</tr>
<tr>
<td>2.0</td>
<td>0.1</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.26</td>
</tr>
<tr>
<td>5.0</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td>10.0</td>
<td>0.1</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Figure 2. Linear Calibration Curves of Absorbance vs. Concentration for Photo-Denitrosation of Nitrosamines.

Aliquots of sample from standard stock solutions of 1 x $10^{-4}$ M were transferred to 20 ml beakers and treated as described in the Materials and Methods section. After completion of the UV treatment 1.5 ml of a 1:1 mixture of the developing solution was added. The samples were read at 525 nm after 15 minutes of development. The curve labelled MAN represents the photo-denitrosation of N-methyl-N-aniline nitrosamine and the standard nitrite curve is labeled, $\text{NO}_2^-$. 
TABLE 5. Denitrosation of N-Nitrosamines by Photochemical Splitting: Comparison with the Procedure of Fan and Tannenbaum (67).

The Fan and Tannenbaum system, like ours, used two Model G 15T8 short-wave lamps (General Electric Co.). Their sample size was approximately 0.25 ml and the UV treatment was for 30 minutes in contrast to 15 min. by our procedure. The distance from the sample to the UV light source was variable in their system averaging 5.0 cm compared to 15 cm in our system.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Present System</th>
<th>Fan &amp; Tannenbaum</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPIP</td>
<td>53 (8)</td>
<td>NPYR 55</td>
</tr>
<tr>
<td>DEN</td>
<td>47 (4)</td>
<td>DEN 60</td>
</tr>
<tr>
<td>DPN</td>
<td>45 (4)</td>
<td>DMN 66</td>
</tr>
<tr>
<td>MAN</td>
<td>25 (3)</td>
<td>-</td>
</tr>
</tbody>
</table>

*MAN = N-Methyl-N-Aniline Nitrosamine

( ) = # of samples analyzed
They reported that the presence of electron transfer agents such as hydroquinone to the system led to quantitative conversion of the nitrosamines to secondary amines.

Initially small aliquots of nitrosamines were irradiated with short wave UV light in a dilute hydrochloric acid solution (0.001 N). Under these conditions nitrosopiperidine was denitrosated and piperidine was recovered in 65-80% yield (Table 6). The dialkyl nitrosamines, however, did not yield more than 10% recovery of the theoretical amount of the secondary amine under the same conditions. A number of additives to the reaction mixture were tried, i.e., methanol, hydroquinone and zinc dust, and the UV treatment was extended to 4 hours. Nevertheless, no increase in the conversion to secondary amines was observed. Chow (35) concluded after thoroughly studying the photochemical reactions of N-nitrosamines that the primary product formed is an oxime. He further showed that irradiation of dibutyl nitrosamine in aqueous methanol or in aqueous acid solution yielded 17 and 21% of dibutylamine respectively.

Acid Denitrosation of Nitrosamines

Dialkyl nitrosamines are relatively stable in aqueous solution at low pH values even at elevated temperatures (35). Fan and Tannenbaum (26) stated that the half-life of DMN and NPYR is 150 days at a pH of 2.2 at 110°C. The nitroso-group, however, has been used as a protective group in peptide synthesis and removed in a solution of dioxane saturated with hydrogen chloride gas (152). Eisenbrand (58) using an anhydrous mixture of glacial acetic acid and HBr developed a system for denitrosating both alkyl and aryl nitrosamines. The acid
TABLE 6. UV Denitrosation of Nitrosopiperidine as Determined by Recovery of Piperidine

Each sample of NPIP was irradiated for 15 minutes in a solution of 0.001 N HCL (pH 3.0) or 0.2 M sodium acetate buffer (pH 5.0). After denitrosation, an aliquot was withdrawn and dansylated.

<table>
<thead>
<tr>
<th>NPIP</th>
<th>pH</th>
<th>Amine Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>3</td>
<td>75%</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>82%</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>81%</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>65%</td>
</tr>
</tbody>
</table>
denitrosation was quantitative as determined by the amount of \( \text{NO}^+ \) species released and recovered as a highly colored product after diazotization. The results obtained at room temperature and at \( 50^\circ\text{C} \) are compared with the splitting reported by Eisenbrand at \( 50^\circ\text{C} \) in Table 7. In our system, better denitrosation and recovery was obtained at room temperature. A time study was also done at a lower temperature \( (10^\circ\text{O}-15^\circ\text{C}) \). The results of the test showed that after 30 minutes a maximum of 90% denitrosation had taken place but by 80 minutes only 50% of the nitrite could be recovered.

When the reaction volume was reduced from 10 ml to 2.0 ml with a corresponding reduction of the amount of sample used by a factor of 10, i.e., 50 \( \mu \text{g} \) to 5 \( \mu \text{g} \), 89% denitrosation and recovery of nitrite from DMN was achieved.

Eisenbrand (28) identified the parent secondary amine product after acid denitrosation of nitrosamines. The amine entity was determined by forming derivatives with dansyl-Cl, 4-nitroazobenzene-4'-carboxylic acid chloride (NABS-Cl) or heptafluorobutyl chloride (HFB-Cl). Although no quantitative determinations were performed with the dansyl derivatives, GLC quantitation of the HFB-derivatives allowed detection of picomole quantities.

Initially the results (Table 8) indicated a great variability in the denitrosation and/or the production of the secondary amine. Nevertheless, when the secondary amines were added directly to the reaction mixture, quantitative recovery was accomplished. After all of the parameters had been thoroughly checked, i.e., redistillation of glacial acetic acid, freshly prepared glacial acetic acid solution containing HBr, standardization by titration, etc., the sporadic re-
TABLE 7. Acid Denitrosation of Nitrosamines as Determined by Recovery of Nitrite

1.0 ml of sample containing 25 or 50 μg of a nitrosamine was placed in test tubes followed by the addition of 9.0 ml of glacial acetic acid containing 0.6 ml of 30-32% HBr. The samples were held at room temperature for 15 minutes or were incubated in a 50°C water bath for 3-5 minutes. 1.0 - 3.0 ml aliquots were withdrawn and added to 5.0 ml of Griess-Ilosvay reagent, adjusted to 10 ml with water and developed in the dark at room temperature for 15-30 min. The samples were read at 525 nm. In the third column are the values Eisenbrand obtained by acid denitrosation.

<table>
<thead>
<tr>
<th>present Study</th>
<th>50°C</th>
<th>Eisenbrand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm. Temp.</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>DMN</td>
<td>100⁻¹</td>
<td>89⁻¹</td>
</tr>
<tr>
<td>DBN</td>
<td>89⁺⁻⁵</td>
<td>76⁺⁻³</td>
</tr>
<tr>
<td>NPIP</td>
<td>109⁺⁻⁶</td>
<td>90⁺⁻⁶</td>
</tr>
</tbody>
</table>
TABLE 8. Initial Results of Acid Denitrosation of Nitrosamines

Nitrosamines were denitrosated with 1.5% HBr at room temperature for 15 minutes in a 1.0 ml of glacial acetic acid. The samples were taken to dryness in a Buchler Evapo-Mix at 80-90°C under reduced pressure. The amine salts were dansylated according to the procedure of Dion and Herbst (149).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample Size</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ug</td>
<td>%</td>
</tr>
<tr>
<td>DMN</td>
<td>37.0</td>
<td>88</td>
</tr>
<tr>
<td>DMN</td>
<td>37.0</td>
<td>85, 85, 89</td>
</tr>
<tr>
<td>DMN</td>
<td>7.4 - 74.0</td>
<td>82, 88, 92, 96, 80</td>
</tr>
<tr>
<td>DMN</td>
<td>3.7 - 37.0</td>
<td>61, 77, 74, 79</td>
</tr>
<tr>
<td>DEN</td>
<td>5.0</td>
<td>50, 73, 69, 69</td>
</tr>
<tr>
<td>DEN</td>
<td>10.0</td>
<td>29, 71, 81</td>
</tr>
<tr>
<td>DEN</td>
<td>5.0</td>
<td>86, 81</td>
</tr>
<tr>
<td>DBN</td>
<td>5.0</td>
<td>55, 61, 55, 58</td>
</tr>
<tr>
<td>DBN</td>
<td>10.0</td>
<td>100, 91, 118</td>
</tr>
<tr>
<td>DBN</td>
<td>5.0</td>
<td>95, 100</td>
</tr>
<tr>
<td>DBN</td>
<td>5.0</td>
<td>98, 98</td>
</tr>
<tr>
<td>NPYR</td>
<td>5.0</td>
<td>95, 105, 80, 71</td>
</tr>
<tr>
<td>NPIP</td>
<td>11.4, 22.8</td>
<td>75, 87, 90, 89, 97</td>
</tr>
</tbody>
</table>
sults still persisted. Variations in the fluorescent derivatization procedure were also tested and as a result a modified procedure was developed. The new procedure, described in the Materials and Methods section eliminated many of the variations that had been observed.

Eisenbrand had developed the acid denitrosation of nitrosamines on standard nitrosamine solutions. However, this method had to be applied to methylene chloride extracts of biological materials containing nitrosamines. Initially an aliquot of acetic acid was added to the concentrated methylene chloride extract and, after the methylene chloride had been removed under reduced pressure, an aliquot of HBr was added. After 15 minutes the sample was taken to dryness. This technique did not result in good recoveries. A modified method of denitrosation was developed. The acid denitrosation was performed by the direct addition of HBr to the methylene chloride solution of the nitrosamines. Quantitative results were achieved by adding an aliquot of 30% HBr in glacial acetic acid to give a final concentration of 3% HBr followed by 30 minutes incubation at room temperature. In addition to this modification it was discovered that it was essential to terminate the reaction by the addition of an aliquot of aqueous acid, i.e., 0.1 N HCl prior to taking the reaction mixture to dryness.

This procedure prevented any loss of nitrosamines while removing the methylene chloride since the addition of the aqueous acid prevented side reactions which occur during the concentration and removal of the anhydrous medium. Another advantage was achieved with this procedure in that milder conditions and less time was required in taking a sample to dryness. Removal of the acetic acid and HBr required a temperature of 80-90°C and reduced pressure for 15-30
minutes while the methylene chloride and HBr solution could be removed at 60°C in a few minutes at reduced pressure. While this work was in progress, Lunt et al. (29) reported the denitrosation of several nonvolatile nitrosamines in a methylene chloride solution containing 25% thionyl chloride with approximately 90% efficiency. The nitrosyl chloride released was trapped in an aqueous solution and reacted with the Bratton-Marshall reagent to form a colored diazo compound. This procedure did not allow identification of the parent nitrosamine but could be applied for the estimation of total nitrosamine compounds.

**Derivatization with NBD-Cl**

Although the denitrosation of nitrosamines was achieved in the methylene chloride medium there were still severe limitations due to the lack of sufficient separation of the dansyl-amino derivatives from each other and the interference by two persistent contaminants. The two recurring contaminants were dansyl-OH and dansyl-DMA. Dansyl-OH, a fluorescent hydrolytic by-product of dansyl-Cl was apparently generated during the reaction. The DMA background fluorescence was found to originate from the degradation of the dimethylamino group of the reagent. Although this background varied from time to time it severely hampered the quantitation of small quantities of DMA. Therefore, another fluorogenic reagent was sought. NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazone, first used by Ghosh and Whitehouse (153) in fluorescence labeling of amino acids, was selected. Lawrence and Frei (154) reported the successful application of this reagent for the determination of methylamine and dimethylamine decomposition products of carbamate pesticides. The intense fluorescence of the NBD-
derivatives permitted the detection of 1 ng or less of DMA and methyl-amine. Fager et al. (155) separated amino acid-NBD derivatives and found the sensitivity of detection to vary for different amino acids. The lowest sensitivity was reported for glutamic and asparatic acid derivatives (2 nmoles). Proline and hydroxyproline derivatives were detected at the highest sensitivity of 0.02 nmoles while the other amino acid derivatives were detected at sensitivities between these limits.

The method employing NBD-Cl and the separation of the secondary amine derivatives was developed in this laboratory as described in the Materials and Methods section. In order to maximize the fluorescence yield, several parameters were checked. The fluorescence yield was not greatly affected between the pH range of 9-11 (Table 9). In acidic solutions of 0.4N PCA or 0.1NHCl the reaction did not take place as was evident from the absence of fluorescent products. The procedure of Lawrence and Frei (154) utilized a temperature of 80°C for the derivatization reaction. Since several of the free secondary amines have very low boiling points, a study was done on the effect of the volume of MIBK and the concentration of the reagent used (Table 10). Six mg of the reagent in 1.0 ml of redistilled MIBK was selected and to prevent any contaminants from being carried over from the aqueous fraction, the reaction mixture was frozen prior to removal of the organic layer containing the NBD-amino derivatives. The excess reagent did not interfere because unreacted NBD-Cl is not fluorescent. Figures 3 and 4 illustrate the linear standard curves obtained with the NBD-derivatives of the secondary amines. The working concentration range was 0.1-0.8 nmoles of secondary amines derivatives although
TABLE 9. Effect of pH on the Derivatization of Amines with NBD–Cl

Aliquots of an amine standard solution containing DMA, PYR, and PIP in 0.1 M HCl were lyophilized to dryness. 1.0 ml of an aqueous alkaline solution was added to achieve variable pH followed by 1.0 ml of MIBK containing 6 mg of NBD–Cl.

<table>
<thead>
<tr>
<th>*pH</th>
<th>DMA</th>
<th>PYR</th>
<th>PIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1</td>
<td>87, 85</td>
<td>108, 105</td>
<td>54, 51</td>
</tr>
<tr>
<td>9.1</td>
<td>121, 117</td>
<td>159, 152</td>
<td>63, 61</td>
</tr>
<tr>
<td>9.7</td>
<td>153, 148</td>
<td>193, 189</td>
<td>68, 67</td>
</tr>
<tr>
<td>10.2</td>
<td>166, 155</td>
<td>202, 191</td>
<td>70, 63</td>
</tr>
<tr>
<td>11.1</td>
<td>160, 152</td>
<td>210, 201</td>
<td>67, 63</td>
</tr>
</tbody>
</table>

* Ratios of 1.0 M NaHCO₃ and 2.0 M Na₂CO₃ were used to obtain the pH values. pH of 1.0 M NaHCO₃ was 8.1 while 2.0 M Na₂CO₃ was 11.1.
TABLE 10. Effect of Volume of MIBK and the Amount of NBD-C1 on the Fluorescence Yield of Secondary Amine Derivatives.

To 1.0 ml of \(1 \times 10^{-4}\) M amine standard solution containing four amines, varying amounts of NBD-C1 and MIBK were added and solution was incubated for 1 hr. at 80°C. The MIBK layer was removed either after freezing of the aqueous layer or without freezing. Aliquots were spotted and peak heights reported.

<table>
<thead>
<tr>
<th>NBD-C1</th>
<th>MIBK</th>
<th>F/NF*</th>
<th>DMA</th>
<th>DBA</th>
<th>PYR</th>
<th>PIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg</td>
<td>ml</td>
<td>Peak Height in mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>F</td>
<td>72</td>
<td>197</td>
<td>88</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>F</td>
<td>71</td>
<td>196</td>
<td>89</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>0.5</td>
<td>F</td>
<td>96</td>
<td>160</td>
<td>124</td>
<td>38</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>F</td>
<td>74</td>
<td>192</td>
<td>94</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>F</td>
<td>94</td>
<td>220</td>
<td>120</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>F</td>
<td>103</td>
<td>191</td>
<td>136</td>
<td>39</td>
</tr>
<tr>
<td>12</td>
<td>1.0</td>
<td>F</td>
<td>101</td>
<td>166</td>
<td>133</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>NF</td>
<td>74</td>
<td>182</td>
<td>95</td>
<td>29</td>
</tr>
<tr>
<td>12</td>
<td>1.0</td>
<td>NF</td>
<td>101</td>
<td>158</td>
<td>128</td>
<td>36</td>
</tr>
<tr>
<td>**12</td>
<td>1.0</td>
<td>F</td>
<td>0</td>
<td>4</td>
<td>13</td>
<td>0</td>
</tr>
</tbody>
</table>

* (F) Aqueous layer frozen; (NF) Aqueous layer not frozen.

** Reagent blank (no nitrosamines and no amines)
Figure 3. Linearity of Fluorescence with Concentration of Three Cyclic Secondary NBD-Amino Derivatives.
The graph shows the relationship between peak heights and nanomoles for three different compounds: Morph (MORPH), Pyr (PYR), and Pip (PIP). The x-axis represents nanomoles, ranging from 0 to 0.6, and the y-axis represents peak heights, ranging from 0 to 180. The graph includes linear trends for each compound, indicating a direct proportionality between peak heights and nanomoles.
Figure 4. Linearity of Fluorescence with Concentration of Four Secondary NBD-Amino Derivatives.
linearity extended for an order of magnitude greater. The lower limit of sensitivity was 1-2 ng of the NBD-derivative of DMA. This agrees well with the sensitivity reported by Lawrence and Frei (154) for the same derivative. This amount of DMA is equivalent to 3.5 ng of DMN and allows the detection of 5-10 ppb of DMN in a food sample. The lowest sensitivity was experienced in the detection of pyrrolidine due to a contaminant in the reaction mixture which migrates at the same rate as the pyrrolidine derivative. Recently, this background contamination was almost totally eliminated by carrying out the reaction at a lower temperature (55°C). In order to obtain linear standard curves at this temperature frequent shaking of the reaction mixture was essential.

The TLC procedure is described in the Materials and Methods section. By this procedure the NBD-amino derivatives derived from six nitrosamines are well resolved. Figure 5 is a typical fluorescence scan illustrating separation of the six NBD-amino compounds achieved by this method. The NBD-derivative of morpholine co-chromatographs with DMA but a solvent system consisting of chloroform and tetrahydrofuran (98:2 v/v) separates these two compounds. The Rf values for DMA and morpholine are 0.54 and 0.33 respectively in this solvent. While this work was in progress a procedure was published using NBD-C1 for the detection of secondary amines and their separation by TLC (156). The sensitivity of the method allowed the detection of 15 ng of DMA as the lower limit.

The reproducibility and quantitation of the acid denitrrosation technique was improved in our system as a result of denitrrosating in a medium of methylene chloride. The results illustrating
Figure 5. Chromatographic Separation by TLC of a Standard Mixture of NBD-Amino Derivatives.

The derivatization and chromatography of this mixture was done according to the procedure described in the Materials and Methods section. Each peak represents 0.3 nmoles of the secondary amine derivative.
the recovery of fluorescent NBD-amino derivatives are shown in Table 11.

**Seafood Survey**

Several laboratories have identified dimethylnitrosamine in processed seafood or seafood products. For example, the initial reports of a nitrosamine-induced hepatotoxicity in sheep and cattle were traced to the feeding of a nitrite-processed herring meal (10, 11, 12). Sen and co-workers (47) reported the possible presence of DMN in frozen and pickled herring although no quantitation was reported. These investigators also detected nitrosamines in various fish products cooked with sodium nitrite but nitrosamines were not detected in samples cooked in the absence of nitrite (54). More recently Sen et al. (157) reported and confirmed by mass spectrometry the presence of 120-450 ppb of DMN in fish meal. This study was undertaken as a result of a report indicating a large number of mink deaths traced to the feeding of a dried fish meal. Although the processing of this fish meal did not include the addition of nitrite or nitrate as a preservative, according to the manufacturer, nitrate was detected in some samples (13-22 ppm) while no nitrite was found. Samples of the mink feed contained DMN; one sample had 400 ppb while the other had 180 ppb and only one out of seven samples of the processed fish meal did not contain DMN. Koppang (158) has shown that mink are very susceptible to the effects of dimethylnitrosamine and prolonged feeding of 0.05 mg of DMN/kg will produce liver tumors.

Howard et al. (48) of the FDA laboratories reported trace amounts of DMN in nitrite-treated smoked chub. The GLC method they
TABLE 11. Acid Denitrosation of Nitrosamines in Methylene Chloride and Derivatization of the Amine Product with NBD-C1

1.0 ml of sample containing $0.25 \times 10^{-4}$ mmoles of a nitrosamine in methylene chloride was placed in a Evapo-Mix tube followed by the addition of 0.1 ml of 30% HBr in G acetic acid. This mixture was allowed to stand at room temperature for 30 minutes. The reaction was stopped by the addition of 1.0 ml of 0.1 N HCl and the sample was prepared for analysis as described in the Material and Methods section.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Denitrosation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp. A</td>
</tr>
<tr>
<td>DMN</td>
<td>100</td>
</tr>
<tr>
<td>DEN</td>
<td>95</td>
</tr>
<tr>
<td>DPN</td>
<td>98</td>
</tr>
<tr>
<td>DBN</td>
<td>104</td>
</tr>
<tr>
<td>NPYR</td>
<td>95</td>
</tr>
<tr>
<td>NPIP</td>
<td>105</td>
</tr>
<tr>
<td>NMORPH</td>
<td>100</td>
</tr>
</tbody>
</table>
employed could detect nitrosamines in the 1-5 ppb range. This group later published a survey encompassing several species of fish and DMN was found in raw sable as well as in smoked and smoked nitrate-treated samples (53). No DMN was found in raw samples of shad or salmon but in smoked nitrate-treated salmon and shad levels of 10-17 ppb were reported. These workers also stated that the skins were removed prior to analysis which may have reduced the recovery of nitrosamines formed during the smoking process.

Fong and Chan (86) found DMN in almost every sample of salt fish purchased from the local markets in Hong Kong. White herring, yellow croaker, anchovies and croaker contained 10-300 ppb DMN and one spoiled sample was found to contain 1000 ppb of DMN. The presence of DMN in many of these samples was confirmed by GLC-mass spectrometry. These workers also did nitrite and nitrate determinations on the samples finding 0-4 ppm of nitrite and 6-40 ppm of nitrate. They further studied the crude salt mixture used in curing and found 17-40 ppm of nitrate and not more than 1 ppm of nitrite. Nitrate reducing bacteria were also found in these fish samples. In another report (159) these investigators showed that the production of DMN did not increase in a sterilized sample whereas an inoculated broth sample with $10^9$ per ml of *Staphylococcus aureus* and a non-inoculated sample accumulated 500 ppb and 400 ppb respectively after 5 days.

Although many fish products have been examined for nitrosamines, very little work, if any, has been done on shellfish, fresh or processed. Therefore the research effort was concentrated in this area. The indirect method of determination of nitrosamines, i.e., acid denitrosation, followed by NBD-derivatization of the recovered
amine salts, was adapted to seafood extracts.

Most previous methods of detection for nitrosamines have been found to be non-specific for nitrosamines and, even after extensive purification, interference from contaminants cannot be avoided. False positive results have been reported, e.g., in the application of polarography to the analysis of nitrosamine (160). Even though the nitrosamines have a specific half-wave potential, various aldehydes also exhibit a similar half-wave potential. Another commonly used detection method is that of GLC. GLC procedures which have been developed are not specific for the separation and detection of nitrosamines and even with the incorporation of multiple columns, false positive peaks occur frequently. Special detectors for the GLC-system have been developed which provide selectivity for nitrogen-containing compounds. The alkali flame ionization detector (AFID), by discharging alkali metal ions, i.e., potassium or rubidium, into the flame is able to enhance the response to nitrogen-containing compounds. This enhancement factor for nitrogen containing compounds can be 10,000 times that for hydrocarbons. The other detector often used for the analysis of compounds containing nitrogen atoms is the Coulson electrolytic conductivity detector (CECD). Using this detector the GLC column effluent is diverted, mixed with hydrogen, passed through a furnace containing a nickel catalyst whereby all nitrogen is reduced to ammonia. Ammonia is then dissolved in deionized water and, as the solution is passed through a micro-cell, the conductivity is monitored. However, interference from contaminants is not completely prevented with either of these detectors. Therefore,
the GLC method has been coupled to a mass spectrometer which enables the inspection of each peak of a specific retention time to be further examined. This system appears to be the most reliable and specific presently in use. The system, however, is very complex, delicate and expensive. Due to the probability of the occurrence of nitrosamines in small quantities, generated either naturally or through processing of foods, alternate methods with high sensitivity, reliability, less complex instrumentation and reduced work-up time would be beneficial in the survey of the environment for these potent carcinogens.

The method described in this thesis employs the acid cleavage first reported by Eisenbrand (28) on pure chemical samples and as modified allows specific and sensitive detection of volatile nitrosamines. We have applied this method to biological extracts. The detection instruments are less complex and much less expensive than the GLC-mass spectrometer system. The work up time per sample is still extensive, requiring a full day.

One advantage of this indirect detection system is that each sample is divided into two fractions prior to denitrosation; one-half of the sample acts as a control or background indicator and is not acid-cleaved but is reacted with the fluorogenic reagent. Therefore, any amine background will be detected in this fraction and can be subtracted from the other fraction which is denitrosated.

This procedure does not completely eliminate the possibility of the generation of amine compounds from contaminants as a result of the HBr-denitrosation process. Johnson and Walters (161) and Eisenbrand (58) have found no serious generation of nitrite from other organic compounds containing a nitro or nitroso-group as a
result of the HBr treatment. However, instead of trying to generate amines from numerous chemical compounds randomly selected from the shelf, I applied this method to numerous fresh shellfish extracts. Although some of the extraction methods have been modified or changed during this work no trend can be seen in the production of amines in the denitrosated fraction compared to the "unsplit" fraction. Approximately 20-40 fresh samples of mussels and clams have been tested without detection of nitrosamines.

An experiment was done to check for artefactual production of DMN as a result of steam distillation in the presence of nitrite and secondary amines as mentioned by Sen et al. (54). In this experiment a clam filtrate was steam distilled. After cooling to room temperature, 250 ppm of DMA and TMA were added followed by the addition of 200 ppm of NaNO₂. The system was closed and the sample was steam distilled a second time. No increase in the DMA area could be seen after work up and denitrosation had been performed.

Various extraction procedures have been tried, i.e., methanolic-KOH digestion and purification steps employed by Howard et al. (48). The present method as described in the Materials and Methods has provided the best recoveries of nitrosamines and adaptability to the seafood products examined. Table 12 shows the recoveries at different stages of purification. Deactivated alumina was found to be superior to silica gel for the removal of contaminants and several organic solvents have been compared in the washing of the alumina column. No difference could be detected in the recovery of nitrosamines or the removal of added amines to the column when either n-hexane or mixed hexanes were used as a wash.
<table>
<thead>
<tr>
<th>Procedural Steps</th>
<th>Compound and Percent Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>STM distillation, HCl partition and Kuderna-Danish conc.</td>
<td>DEN (60), DBN (60-80)</td>
</tr>
<tr>
<td>Basic liquid-liquid extraction and concentration</td>
<td>NPIP (55)</td>
</tr>
<tr>
<td>Acid liquid-liquid extraction and concentration</td>
<td>NPIP (55), DMN (57)</td>
</tr>
<tr>
<td>Silica gel column and concentration</td>
<td>NPYR (40), DBN (61)</td>
</tr>
<tr>
<td>*STM distillation</td>
<td>DEN (85)</td>
</tr>
<tr>
<td>*Alumina column</td>
<td>DEN (94-95)</td>
</tr>
</tbody>
</table>

* Determined by spectrophotometric analysis at 230 nm.
Data on recovery of nitrosamines from fresh or frozen clams are given in Table 13. These recoveries are comparable to those reported by other laboratories. Recoveries of nitrosopyrrolidinide were consistently lower than the recoveries of other N-nitrosamines. This was found to be a result of losses at the steam distillation step. This loss is believed to be due to degradation of the compound although the breakdown products have not been identified. Distillation under reduced pressure at a lower temperature might improve the recovery of this compound as indicated by the results obtained by Telling et al. (162). Nitrosomorpholine was not tested for recovery and only denitrosation data has been accumulated for this compound.

**Survey of Seafood for Nitrosamines**

Processed seafood products were purchased from local grocery stores and consequently the originating area of the seafood and the procedure of processing are unknown. The brand name will not be identified due to the lack of confirmation by another method and the limited survey of each seafood type. In Table 14 the results obtained on the seafood products are summarized. Figure 6 displays a scan of an "unsplit" or "split" fraction of clam that contains no DMN. To insure that quantitative denitrosation was taking place in biological samples an aliquot of nitrosopiperidinide that served as an internal standard was added to the "split" fraction just prior to the addition of HBr.

Table 15 summarizes the results of a time study performed on frozen clams. A large batch of shucked clams were purchased from a local supplier and packaged in 200 g lots which were stored in a -20°C freezer. At intervals, a batch was withdrawn, thawed and
TABLE 13. Recoveries of N-Nitrosamines from Fresh or Frozen Clams.

The nitrosamine "Spike" was added to the filtrate after homogenation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DMN</th>
<th>DEN</th>
<th>DPN</th>
<th>DBN</th>
<th>NPIP</th>
<th>NPYR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Fresh)</td>
<td>--</td>
<td>77</td>
<td>--</td>
<td>--</td>
<td>65</td>
<td>--</td>
</tr>
<tr>
<td>2 (Fresh)</td>
<td>62</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>73</td>
<td>--</td>
</tr>
<tr>
<td>3 (Fresh)</td>
<td>--</td>
<td>80</td>
<td>84</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>4 (Frozen)</td>
<td>66</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>54</td>
<td>--</td>
</tr>
<tr>
<td>5 (Frozen)</td>
<td>57</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>50</td>
<td>--</td>
</tr>
<tr>
<td>6 (Fresh)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>72</td>
<td>--</td>
<td>32</td>
</tr>
<tr>
<td>7 (Fresh)</td>
<td>82</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>58</td>
<td>--</td>
</tr>
<tr>
<td>8 (Frozen)</td>
<td>--</td>
<td>80</td>
<td>82</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>9 (Fresh)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>66</td>
<td>75</td>
<td>--</td>
</tr>
<tr>
<td>10 (Fresh)</td>
<td>--</td>
<td>--</td>
<td>57</td>
<td>63</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>11 (Fresh)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>25</td>
</tr>
<tr>
<td>12 (Frozen)</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>DMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen shrimp</td>
<td>N.D. (3)</td>
</tr>
<tr>
<td>Canned shrimp</td>
<td>N.D. (1)</td>
</tr>
<tr>
<td>Smoked canned kipper fillets</td>
<td>N.D. (3)</td>
</tr>
<tr>
<td></td>
<td>10, 10, 6 *</td>
</tr>
<tr>
<td></td>
<td>93, 137</td>
</tr>
<tr>
<td>Anchovies canned with and</td>
<td>N.D. (4)</td>
</tr>
<tr>
<td>without capers</td>
<td>93</td>
</tr>
<tr>
<td>Smoked canned clams</td>
<td>N.D. (2)</td>
</tr>
<tr>
<td></td>
<td>87, 140</td>
</tr>
<tr>
<td></td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>21</td>
</tr>
<tr>
<td>Fresh oysters</td>
<td>N.D. (2)</td>
</tr>
<tr>
<td>Smoked, canned oysters</td>
<td>N.D. (2)</td>
</tr>
<tr>
<td></td>
<td>98, 102</td>
</tr>
<tr>
<td>Frozen mussels</td>
<td>N.D. (6)</td>
</tr>
<tr>
<td></td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>75</td>
</tr>
<tr>
<td>Smoked, canned mussels</td>
<td>N.D. (2)</td>
</tr>
<tr>
<td></td>
<td>155, 185</td>
</tr>
</tbody>
</table>

N.D. Not detected

( ) Numbers in parenthesis represent the number of samples processed

* Entrees with numbers separated by a comma represent samples assayed separately but originating from the same batch.
Figure 6. Tracings from TLC Plate Scans

(A) Standard mixture of NBD-amino derivatives from a standard stock solution. Each peak represents 0.2 nmoles of NBD-amino derivative.

(B) Unsplit sample of clam extract which has been taken through the entire extraction and derivatization procedure described in the Methods and Materials section.

(C) Reagent blank containing 1.0 ml of 0.1 N HCl and derivatized according to the procedure described in the Materials and Methods Section.
TABLE 15. Effect of Frozen Storage on Nitrosamine Production.

<table>
<thead>
<tr>
<th>Length of Storage</th>
<th>DMN</th>
<th>Recovery of Spike</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weeks</td>
<td>ppb</td>
<td>%</td>
</tr>
<tr>
<td>0 (Fresh)</td>
<td>N.D.</td>
<td>DMN (62)</td>
</tr>
<tr>
<td>0 (Fresh)</td>
<td>N.D.</td>
<td>DMN (73)</td>
</tr>
<tr>
<td>1</td>
<td>N.D.</td>
<td>DEN (77), NPIP (65)</td>
</tr>
<tr>
<td>1.5</td>
<td>N.D.</td>
<td>NPIP (54)</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>DPN (56), DBN (62)</td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>126</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

N.D. None detected.
analyzed for nitrosamines. The results indicate that the formation of DMN may occur in the clams after a period of frozen storage. Storage for eight weeks and longer appeared to result in DMN formation in 5 of 6 samples whereas, during the first 8 weeks no DMN was found. The mechanism of nitrosation during cold storage occurred is unknown although there was most likely an increase in DMA content.

Confirmation of the presence of DMN in these samples has not been obtainable by mass spectrometry. Several samples were sent to laboratories with GLC-mass spectrometry systems but background contaminants that interfered with the interpretation of mass spectra precluded the unequivocal confirmation of DMN.

**Dimethylamine in Seafood**

Many marine fish, i.e., herring, cod, dogfish, hake, skate, haddock, etc., contain large quantities of trimethylamine oxide (TMAO). Quantities of TMAO in these fish range from 50-250 mg of nitrogen per 100 g of wet weight (94). Invertebrates, i.e., scallop, lobster and squid contain similar quantities of TMAO. But oyster, clam and mussel contain less than 1 mg of TMAO nitrogen per 100 g of wet weight.

Storage of these marine fish accelerates production of trimethylamine (TMA) and DMA as a result of bacterial breakdown of TMAO. The concentration of TMA and DMA increases with storage time and can reach levels over 50 mg/100 g of tissue for each compound (94). Castell et al. (163) report that more DMA than TMA is produced in frozen storage and the reverse is true during storage on ice. Miller et al. (95) reported the occurrence of over 400 ppm of DMA in hake frozen for prolonged periods.
The extraction of DMA from seafood products was performed as described in the Materials and Methods section. The acid extract was placed in the steam distillation flask, KOH was added and the alkaline solution was steam-distilled. The recovery of secondary amines from solutions of pure compounds and from a diluted clam extract is summarized in Table 16.

A survey of the DMA content of seafood products (Table 17) was performed by the procedure described in the Materials and Methods section. No attempt was made to follow the increase in DMA with the length of storage. However, more DMA was present in frozen clams than in fresh clams. The highest concentration of DMA was found in canned anchovies (46-64 ppm) and smoked canned herring fillets (70-240 ppm). Although no data could be found in the literature on the DMA content of anchovies and herring, Castell et al. (94) reports levels reaching approximately 60 ppm of DMA in fish stored on ice and 100 ppm of DMA in frozen fish. No quantitation data on the DMA content of shellfish has been reported although DMA has been detected in these species.
TABLE 16. Recovery of Secondary Amines After Steam Distillation

An aliquot of amine standard solution containing secondary amines in 0.1 N HCl was added to 65 ml of distilled water. 150 ml of distillate was collected and the recoveries are shown in column A. Column B illustrates the recoveries from a diluted previously distilled clam extract. The distillates from both trials were taken to dryness, redissolved in a small volume and an aliquot was derivatized with NBD.

<table>
<thead>
<tr>
<th>Compound</th>
<th>A (%)</th>
<th>B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>DEA</td>
<td>84</td>
<td>--</td>
</tr>
<tr>
<td>DBH</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td>PIP</td>
<td>68</td>
<td>81</td>
</tr>
<tr>
<td>PYR</td>
<td>79</td>
<td>65</td>
</tr>
</tbody>
</table>
TABLE 17. DMA Content of Seafood Products

<table>
<thead>
<tr>
<th>Sample</th>
<th>DMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clams (Fresh)</td>
<td>4 - 16 *</td>
</tr>
<tr>
<td>(Frozen)</td>
<td>27 - 32</td>
</tr>
<tr>
<td>(Smoked)</td>
<td>4 - 20</td>
</tr>
<tr>
<td>Mussels (Fresh)</td>
<td>22</td>
</tr>
<tr>
<td>(Smoked)</td>
<td>16</td>
</tr>
<tr>
<td>Oysters (Fresh)</td>
<td>5</td>
</tr>
<tr>
<td>(Smoked)</td>
<td>27</td>
</tr>
<tr>
<td>Herring (Smoked)</td>
<td>70 - 240</td>
</tr>
<tr>
<td>Anchovies</td>
<td>46 - 64</td>
</tr>
</tbody>
</table>

* The high and low values of the results on separate samples are indicated by values separated by a hyphen.
SUMMARY

A method for the detection and quantitation of nitrosamines has been developed. The nitrosamines are denitrosated in an anhydrous medium of methylene chloride to which hydrobromic acid is added. The resulting amine salts are reacted with NBD-Cl to yield highly fluorescent derivatives. The fluorescent derivatives are separated by TLC and quantitated by direct scanning of the TLC plates. The sensitivity is of the order of 1-5 ng of fluorogenic compound allowing a detection of 5 ppb of DMN.

The method has been applied to a survey of fresh, frozen and processed seafood for N-nitrosamines. Although many samples showed no evidence of nitrosamines, several samples contained DMN. Other volatile nitrosamines, for which this method is applicable, were absent.

Several samples of processed seafoods contained DMN and it is possible that the nitrosamine was formed during the processing stage. However, the presence of DMN in frozen clams and mussels is difficult to explain by the current concept of the formation of nitrosamines in the presence of secondary or tertiary amines and nitrite in an acid medium.

Several recent reports suggest that nitrosamines may be formed under other conditions. Formaldehyde has been shown to catalyze the nitrosation reaction at neutral and alkaline pH (39). The relative ease of formation of nitrosamines in low moisture systems has been demonstrated in cold traps used in the collection of smoke condensates. Thus the formation of nitrosamines in natural foods during storage or processing is possible and further studies are required.
BIBLIOGRAPHY


10. Koppang, N., Severe Liver Disease in Fur-Bearing Animals in


20. Fischer, E., Ueber die Hydrazinverbindungen der Fettreihe, Ber., 8, 1587 (1879).


58. Eisenbrand, G. and R. Preussmann. Eine neue Methode zur Kolor-
90


68. Ender, F. and L. Ceh, Alkylierend Wirkende Vergindungen, Second Conf. on Tobacco Research, Freiburg, Germany, pp. 83 (1967).
77. Herrmann, H., P-Methylnitrosamino-benzaldehyde, a Metabolic Product of Clitocybe suaveolens, Naturwissenschaften 47, 162 (1960).
78. DuPlessis, L.S., J.R. Nunn and W.A. Roach, Carcinogen in Transk-


80. Panalaks, T., J.R. Iyengar and N.P. Sen, Nitrate, Nitrite and
Dimethylnitrosamine in Cured Meat Products, J.A.O.A.C. 56,
621 (1973).

81. Panalaks, T., J.R. Iyengar, B.A. Donaldson, W.F. Miles and N.P.
Sen, Further Survey of Cured Meat Products for Volatile N-

82. Fazio, T., H. White, L.R. Dusold, and J.W. Howard, Nitroso-

83. Fazio, T., R.H. White and J.W. Howard, Analysis of Nitrite and/
or Nitrate Processed Meats for N-Nitroso Dimethylamine, J.A.
O.A.C. 54, 1157 (1971).

84. Wasserman, A.E., W. Fiddler, R.C. Doerr, S.F. Osman, C.J. Dooley,
Dimethylnitrosamine in Frankfurters, Fd. Cosmet. Toxicol. 10,
681 (1972).

85. Pensabene, J.W., W. Fiddler, R.A. Gates, J.C. Fagan and A.E. Wass-
erman, Effect of Frying and Other Cooking Conditions on Nitroso-

86. Fong, Y.Y. and W.C. Chan, Dimethylnitrosamine in Chinese Marine
Salt Fish, Fd. Cosmet. Toxicol. 11, 841 (1973).

into the Possible Presence of Nitrosamines in Nitrite-Bearing

Schwartz, Changes in Nitrate and Nitrite Content and Search


97. Dyer, W.J., F.E. Dyer and J.M. Snow, Amines in Fish Muscle:


107. Moruzzi, G. and C.M. Caldarera, Occurrence of Polyamines in the


115. Cyanosis of Infants Produced by High Nitrate Concentration in Rural Wells, WHO 13, 19 (1949).


117. McLean, R.A., H.D. Lilly and J.A. Alford, Effects of Meat-Curing Salts and Temperature on Production of Staphylococcal Enteroc-

118. Code of Federal Regulations, Title 21, Sec. 121.1064 and Sec. 121.1230, Revised 1971.


125. Ender, F. and L. Ceh, Conditions and Chemical Reaction Mechanisms by which Nitrosamines may be Formed in Biological Products with Reference to Their Possible Occurrence in Food Products, Z. Lebensmittel-Untersuch und Forsch. 145, 133 (1971).


